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CONTENT

TDF/3TC/EFV Regimen-Related Renal and Neuropsychiatric Toxicity in Vietnam HIV-Infected Patients

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Abstract

Introduction: ARV regimen constituting of tenofovir, lamivudine and efavirenz (TDF/3TC/EFV) has been selected as basic regimen for HIV treatment in Vietnam. Despite the increasing use of this regimen, available data about EFV-related neuropsychiatric toxicity and TDF-related renal dysfunction is still limited. **Objectives:** To determine incidence of neuropsychiatric and renal toxicity and to identify possible related risk factors. **Materials and Methods:** A prospective study was conducted at ten clinics in Vietnam, including adult HIV-infected patients initiated with TDF/3TC/EFV regimen. Neuropsychiatric toxicity was recorded by interviewing patients while renal toxicity was defined as a decrease of creatinine clearance (CrCl) by over 25% from the baseline level. Associating risk factors were identified using multivariate analyses. **Results:** There were 838 patients enrolled with a median of 10.4 months monitoring period. 324 (38.66%) experienced at least one neuropsychiatric adverse events, mainly in the first month of treatment (94.5%). Most patients only experienced mild ADR (76.9%). TDF-related renal dysfunction was identified in 78 (14.47%) patients. Multivariate analysis showed that EFV-related CNS toxicity was associated with higher age (HR=1.218; 95%CI 1.076-1.363), lower weight (HR=0.822; 95%CI 0.686-0.959), higher hemoglobin (HR=1.111; 95%CI 1.055-1.168) and TDF-related renal toxicity was associated with lower hemoglobin (HR=0.846; 95%CI 0.745-0.948), higher age (HR=1.363; 95%CI 1.062-1.672) and higher baseline CrCl (HR=1.177; 95%CI 1.140-1.214). **Conclusion:** The EFV-related CNS toxicity was common yet mild while the incidence of TDF-associated renal toxicity was relatively low. Some identified risk factors may be useful for clinical management.

Keywords: ART; TDF; EFV; adverse drug reactions.

1. INTRODUCTION

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) recommendedwidely in combination with two other nucleoside reverse transcriptase inhibitors (NRTI) in highlyactive antiretroviral therapy(HAART) [1], [2]. Research has shown that efavirenz is related to neuropsychiatric adverse reactions such as dizziness, vertigo and sleep disorders [3], [4]. Tenofovir disoproxil fumarate (TDF) is a nucleoside reverse-transcriptase inhibitor (NRTI). Currently, TDF is widely recommended as a first choice in HIV treatment guidelines [5]. There are a number of reports on renal toxicity appearing in HIV patients treated with TDF[6], [7].

Since 2015, the Vietnam Ministry of Health has recommended the combination of TDF/3TC/EFV as first line regimen for naïve HIV-infected patients and it is expected that the use of TDF and EFV will increase in the near future in Vietnam. Although Vietnamese patients are likely to have lower body weight than Whites and African Americans, a limited number of studies reported the EFV-associated neuropsychiatric adverse effects and TDF-associated renal dysfunction data in Vietnam populations [8], [9]. This present study was conducted to determine incidence of neuropsychiatric and renal toxicity and to identify possible risk factors associated with these adverse effects.

2. METHODS

2.1. Study design

We conducted a prospective cohort study including HIV-infected patients in 10 clinics in 7 cities in Vietnam. Inclusion criteria were as follows: (1) age ≥18 years old, (2) antiretroviral naivety, (3) initiation of TDF/3TC/EFV regimen between March 16, 2015 and July 15, 2016 and (4) non-pregnant during monitoring period. Moreover, exclusion criteria were defined for each group as follow: (for EFV cohort) (1) did not have at least one follow-up visit at the HIV clinic after EFV initiation; (for TDF cohort) (1) did not have information on baseline weight and serum creatinine and (2) did not have at least one follow-up serum creatinine results after TDF initiation.

Basic demographic information and baseline laboratory parameters were considered as potential risk factors and recorded closest to and prior the initiation of HAART within 90 days from the medical records. These included (where possible): demographic variables (weight, sex and age); CD4 cell count (cell/mm^3); haemoglobin, alanine aminotransferase (ALT), serum creatinine (µmol/L); and clinical stage (based on WHO Guidelines) [5].

Adverse effects on neuropsychiatric system and renal were monitored and reported by trained healthcare professionals. EFV-related neuropsychiatric disorders were determined by physicians by interviewing patients on each visit. The severity of neuropsychiatric adverse events was classified by the Division of AIDS grading tables [10]. To assess the renal toxicity of TDF, follow-up parameters including body weight and serum creatinine were collected every 6 months after TDF initiation based on recommendation by Vietnam Ministry of Health. Creatinine clearance (CrCl) was calculated using the Cockcroft-Gault formula. Study endpoint definition, which was renal function decline, was defined by a 25% decline in CrCl from the baseline level.

2.2. Statistical analysis

Mean (standard deviation, SD), median (interquartile range, IQR) and frequencies (%) were used to describe patients' characteristics. Censored cases represented those who died, dropped out, switched to non-EFV or non-TDF based regimen or were referred to other clinics before the end of follow-up period at 15 July 2016. The impact of basic demographics and baseline

laboratory data was estimated by multivariate analyses which was performed by using Bayesian Information Criterion (BIC) to find the most appropriate model. For the purpose of handling with a large number of missing values, the PMM method was used to create values replacing missing values of continuous variates. All statistical analyses were performed by using RStudio.

3. RESULTS

3.1. Baseline characteristics

From March 16, 2015 to July 15, 2016, a total of 838 HIV-infected patients were included in the study. All of 838 patients was eligible for inclusion in the analysis for neuropsychiatric toxicity (EFV cohort). Due to lack of baseline or follow up serum creatinine data, 299 patients were excluded from renal function monitoring cohort (TDF Cohort). Thus, 539 patients were included in the analysis for renal toxicity. Baseline characteristics and laboratory investigations were relatively similar between the two cohorts. These data were described in Table 1.

Table 1. *Baseline demographics and laboratory investigationsof EFV and TDF cohort*

		EFV cohort	**TDF cohort**
Number of patients		838 (100.0)	539 (100.0)
Age	Median (IQR)	33 (29, 38)	34 (29, 39)
Gender	Male	561 (66.9)	365 (67.7)
	Female	277 (33.1)	174 (32.3)
Weight	Median (IQR)	53 (48, 59)	53 (48, 59)
ALT	Median (IQR)	30 (20-51) N=804	30 (20-50) N=519
CD4 (cells/mm^3)	Median (IQR)	318 (122-478) N=801	292 (113-455) N=523
Hb (g/L)	Median (IQR)	131 (115-143) N=817	132 (116-144) N=532
Crcl	Median (IQR)	86.0 (72.5-101.0)	86 (72-102)
Clinical stage	1	523 (62.4)	330 (61.2)
	2	109 (13.0)	78 (14.5)
	3	91 (10.9)	58 (10.8)

	4	115 (13.7)	73 (13.5)
Follow-up time, months	Median (IQR)	10.4 (7.9-12.8)	11.1 (8.7-13.0)

3.2. EFV-associated neuropsychiatric toxicity

Median (IQR) duration of receiving EFV was 10.4 (7.9-12.8) months. The percentage of patients suffering at least one psychiatric disorder was 38.7% (324 patients). Among these patients, 94.5% experienced adverse events in the first month of treatment, median (IQR) time to EFV-associated neuropsychiatric adverse events was 7.0 (1.0-10.0) days.

The most common manifestations of neuropsychiatric toxicity were dizziness, headache and fatigue, which were observed in at least 163 patients (50.3%), while nausea and hot flush occurred in at least 82 patients (25.3%). The number of patients experiencing mild and moderate adverse events were 249 (76.9%) and 72 (22.3%), respectively. Only 3 patients had severe symptoms.

By multiple logistic regression, higher age (per 10 years, HR=1.218; 95%CI, 1.076-1.363), lower weight (per 10kg increment, HR=0.822; 95%CI, 0.686-0.959), and higher hemoglobin at baseline (per 1g/dL increment, HR =1.111; 95%CI, 1.055-1.168) were statistically associated with EFV-related CNS toxicity.

3.3. TDF-associated renal toxicity

Median (IQR) duration of receiving TDF was 11.1 (8.7-13.0) months. There were 78 (14.5%) patients with renal dysfunction defined as a 25% decrease in CrCl from the baseline. Among these patients, median (IQR) time to a 25% decrease in CrCl was 6.2 (5.1-7.7) months, the earliest occurrence was in the first month of TDF initiation, and the latest occurrence was in the 16^{th} month after TDF initiation. Among those experiencing TDF-related renal dysfunction, creatinine level in 60 (76.9%) remained normal, 18 patients (22.8%) had serum creatinine concentration exceeding normal limits, wherein 15 cases increased up to 1.5 times and 3 cases increased up to 3 times the normal upper limit.

By multiple logistic regression, lower haemoglobin at baseline (per 1g/dL increment, HR=0.846; 95%CI, 0.745 – 0.948), higher age (per 10 years increment; HR=1.363; 95%CI, 1.062 – 1.672) and higher baseline CrCl (per 10ml/min increment, HR=1.177; 95%CI, 1.140-1.214) were statistically associated with a 25% decrease in CrCl.

4. DISCUSSIONS

To our knowledge, the present study was one of the first multicentre studies regarding EFV-related neuropsychiatric disorders and TDF-associated renal dysfunction among Vietnamese HIV-infected patients.

The percentage of patients experiencing neuropsychiatric adverse events related to EFV was 38.7% (324 patients). This incidence rate was lower than the results in other studies, which reported that the prevalence rates fluctuated from 40% to 70% [2], [3]. This difference may be due to different study designs, including sample size, patient selection criteria, definition of adverse events on the neuropsychiatric system and the method of investigation and assessment [2]. The majority of reactions were mild, transient, and easily confused with clinical symptoms, particularly on IDU patients. As a result, they might be ignored and not recorded in the medical record.

The most common neuropsychiatric adverse effects were dizziness, headache and fatigue while some severe adverse effects including depression and suicidal intention were reported rarely. Most of the adverse effects were mild or moderate (99.1%). Only 3 cases were severe (0.9%). This result was similar to the data in some reviews and medical literature [1], [11], [12].

In our study, the ADR in the neuropsychiatric system mainly appear within the first month of initiation (94.5%). This lag period was also reported in other studies, wherein the most severe of adverse effects were recorded within the first 2–4 weeks [2].
Results from multivariate analysis in this study suggested that lower weight, higher age, or higher hemoglobin at baseline were the factors significantly associated with EFV toxicity on neuropsychiatric system. Lower body weight may lead to high EFV plasma concentration and increased risk of toxicity. The association between plasma concentration of EFV and neuropsychiatric disorder was well documented but does not always occur [13]. In addition, it was recommended by WHO that physicians should consider to reduce the dose of EFV to 400mg/day to reduce CNS adverse events when the symptoms occurred. This finding put an emphasis on monitoring for neuropsychiatric disorder for an appropriate and effective consultancy and treatment for lighter patients, which are common among Asian populations. The reason why older patients and those who had high haemoglobin level at baseline are associated with higher risk of neuropsychiatric disorder remains unclear to us. Future study is needed to explore this association.

We found that 14.5% of the patients developed a TDF-associated renal function decline defined by a 25% decrease in CrCl from the baseline. This proportion seemed to be higher than other studies in Asian population with different definitions of nephrotoxicity [8][14] and lower than those having similar definitions of adverse events [15]. In addition to the difference in definitions of nephrotoxicity, this can be explained by difference in the formula used to estimate CrCl, patients' characteristic and duration of observation [16]. Factors associated with renal dysfunction were higher baseline CrCl, lower haemoglobin level at baseline and higher age. The mechanism of the association between high baseline renal function (or CrCl) and TDF-related renal dysfunction was not clearly known. However, this finding was also observed in other cohort studies on Asian populations [15][17]. It can be explained by the predefined criteria detecting nephrotoxicity in this study estimated by a 25% decrease in CrCl from baseline. Therefore, patients with higher baseline CrCl are more sensitive to suffer a decrease in renal function [18]. Old age was also suggested as a risk for TDF-induced renal dysfunction, which is similar to previous data [17][8].

The study had several important limitations. Firstly, there was noticeable amount of missing data, especially information of concurrent nephrotoxic drugs and comorbidities, which were potential risk factors for neuropsychiatric or renal toxicity. However, this was predicted in view of the routine of examining and recording patient data in HIV care practice, especially in resource-limited settings in Vietnam. Secondly, renal function was estimated at 6-months interval, additionally, some patients did not follow the recommended schedules of serum creatinine test. Thus, this might not represent the exact incidence and time-to-onset of the nephrotoxicity. Thirdly, other TDF associated nephrotoxicity, such as Fanconi syndrome were not observed because other laboratory tests including urine analysis and serum electrolyte levels were not collected.

5. CONCLUSIONS

Our study shows that proportions of EFV-associated neuropsychiatric toxicity was common yet mild while the incidence of TDF-associated renal toxicity was relatively low among Vietnam HIV-infected patients. Careful consulting and close monitoring is essential for early detection and appropriate management for both toxicities, especially among high risk patients. Further research is needed to confirm the impact of age or haemoglobin level on neuropsychiatric disorder and renal dysfunction.

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Common Technique Errors in Patients When Using Insulin Pen

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Abstract

Introduction: Technique of using insulin pen plays an important role in treatment of diabetes. Improper use may not only reduce therapeutic effect of the drug but also cause adverse drug reactions such as hypoglycemia, itch, pain at injection site and lipodystrophy. Thus pharmacist's counselling practice should be focused to improve patients' outcome. **Objectives:** Evaluate patients' practical skills of using insulin pens in the outpatient department of a national hospital. **Methods:** This was a prospective observational study involving 203 patients who performed injection using their prescribed insulin pen on a model, simulating the steps how they use their pens at home. Steps were observed and recorded including missing and incorrect steps. Patients who have more than one pen were evaluated one time on their more sophisticated and less common one. The steps of using pens are also classified as low-important, important and the most important steps. **Results:** The study used seven types of injection pens divided into four main categories by injection technique. In total, error rates in the operation of important steps such as "safety test" and "holding the injection button" were 43.8% and 26.6% respectively. The most important step, "choosing dose", had the error percentage of 7.4%. In general, 62.6% of patients had poor technique, 9.9% had optimal technique and 1.5% did not know how to use the pen. Errors in low-important steps occurred in around 67% patients for "removing inside cap" and "removing and discarding needle". **Conclusion:** Not many patients were wrong at the most important step but still a low percentage of patients can operate perfectly. More effort should be made to improve patients' technique in using insulin pens.

Keywords: insulin pen, injection technique, error

1. INTRODUCTION

Vietnam, like other emerging countries, has a high prevalence of diabetes. Insulin-based regimens play key roles in achieving glycaemic control for diabetic patients including type 2 diabetes mellitus patients, who will need insulin because of the progressive nature of the disorder.Nowadays, thanks to technological advances, insulin pens were introduced and gradually replaced vials and syringes because they are more accurate, less painful, and easier to use. However, errors in the administration technique remain an issue. Improper use may not only reduce therapeutic effect of the drug but also cause adverse drug reactions such as hypoglycaemia, itch, pain at injection site and lipodystrophy (1)

The National Hospital of Endocrinology is the leading specialty hospital for endocrine and metabolic diseases in Vietnam, where more than 70% of outpatients are diabetic patients and

more than half of them are treated with insulin. In this hospital, more pens are used for patients. Therefore, evaluating the practice of insulin use in outpatients is of great importance in improving the quality of drug use and the effectiveness of treatment for diabetes in the hospital.

2. MATERIALS AND METHODS

This study was conducted in the outpatient department of National Institute of Endocrinology of Vietnam. It included type 1 and type 2 diabetes mellitus patients who were using insulin pens during their follow-up visits from September 2016 to January 2017. Pregnant patients were excluded. There were 203 patients who met the criteria and agreed to participate in the study.

Patients were asked to perform injection using their prescribed insulin pens on a model, simulating the steps how they use their pens at home. Patients who have more than one pen are evaluated one time on their more sophisticated and less common one. Steps were observed and recorded by a researcher and a clinical pharmacist independently and in parallel based on their checklists of steps with clear technical description, which had been built upon each product manufacturer's instruction of use. Only data of the patients who had the same evaluations was used. After evaluation, the patient was counselled about the knowledge and practice of using insulin pens through a visual model and product introduction brochure. Other related information was extracted from their outpatient records.

The steps of using pens are classified as low-important, important and the most important steps based on the consent of physicians in the hospital. The most important step is "choosing dose", whereas the important steps are "safety test" in which patients have to select 2 units, point the tip of the pen up, tap gently on the pen to remove air bubbles, press and hold until a drop of insulin is seen at the tip of the needle; and the step of "holding the injection button" after pressing the injection button. The other steps are low-important. Patients' levels of pen operation are as follow: "No technique" if they do not have any correct steps, "poor technique" if they are wrong in at least one of the most important and important steps, "adequate technique" if none of the most important and important steps is wrong, and "optimal technique" is without any incorrect steps.

3. RESULTS

3.1 Patient characteristics

The analysed dataset comprised a total number of 199 individuals with T2DM and 4 with T1DM. Median age was 64 years with a range of 12-88 years. Of 203 study participants, 105 (51.7%) were male whereas 98 (48.3%) were female. Median time of treatment with insulin with range was 3 (0-16) years and median time of treatment with insulin pens was 1 year (range 0-10). There were seven types of insulin pens which were used for the patients and several patients had more than one pen (Table 1).

Table 1. *Demographic characteristics of patients*

Characteristics	**Median, Interquartile (Min-Max)**
Gender*	
Male	105 (51.7%)

Female	98 (48.3%)
Age (years old)	64.0, 12 – 88, (57 – 69)
Type*	
DM Type 1	4 (2.0%)
DM type 2	199 (98.0%)
Years treated with insulin	3, 2-6, (0-16)
Years treated with insulin pens	1,1-3, (0-10)
Insulin pens*	
NovoMix	80 (39.4%)
Lantus	54 (26.6%)
Humanlog Mix	38 (18.7%)
NovoRapid	29 (14.3%)
Insulatard	28 (13.8%)
Apidra	26 (12.8%)
Levemir	3 (1.5%)

** The values are expressed as frequency (percentage)*

3.2 Technique errors when using insulin pens

In this study, correct insulin injection techniques were analysed with regard to parameters such as shaking/mixing, removing inside cap of the needle, safety test, choosing dose, holding injection button, removing and discarding needle. In general, there were less errors in the most important step of "choosing dose" than other steps. However, there were still 7.4% of patients who failed the most important step. Error rates in the operation of important steps such as "safety test" and "holding the injection button" were 43.8% and 26.6% respectively (**Table 2**).

Table 2. *The proportion of patients having errors in different steps of insulin injection*

Steps of injection	**Group 1 Pts used Humalog Mix (Eli Lilly)**	**Group 2 Pts used NovoRapid/ Levemir (Novo Nordisk)**	**Group 3 Pts used Insulatard/ NovoMix (Novo Nordisk)**	**Group 4 Pts used Apidra/ Lantus (Sanofi Aventis)**	**Total**
	N=35	**N=32**	**N=71**	**N=65**	**N=203**
	Number of patients (%)				
Shaking/mixing	15 (42.9)	-	43 (60.6)	-	-
Removing inside cap	24 (68.6)	23 (71.9)	53 (74.6)	38 (58.8)	138 (68.0)
Safety test *****	**15 (42.9)**	**15 (46.9)**	**30 (42.3)**	**29 (44.6)**	**89 (43.8)**
Choosing dose ******	**4 (11.4)**	**3 (9.4)**	**3 (4.2)**	**5 (7.7)**	**15 (7.4)**
Holding the injection button *****	**9 (25.7)**	**9 (28.1)**	**12 (16.9)**	**24 (36.9)**	**54 (26.6)**

Steps of injection	Group 1 Pts used Humalog Mix (Eli Lilly) N=35	Group 2 Pts used NovoRapid/ Levemir (Novo Nordisk) N=32	Group 3 Pts used Insulatard/ NovoMix (Novo Nordisk) N=71	Group 4 Pts used Apidra/ Lantus (Sanofi Aventis) N=65	Total N=203
	Number of patients (%)				
Removing and discarding needle	28 (80.0)	23 (71.9)	52 (73.2)	33 (50.8)	136 (67.0)

* *important step.*

** *the most important step.*

- *the step is not required.*

Patients were also classified in 4 categories of technique: No technique, poor technique, adequate and optimal technique (**Table 3**). Only under 10% of tested patients showed optimal technique. The majority of patients (62.6%) had low technique when they were asked to do the injection with their prescribed pens and a model. There were still some patients who even did not know how to use the pens (No technique: 1.5%).

Table 3. *Levels of patients' injection techniques*

Levels of injection techniques	Group 1 Pts used Humalog Mix (Eli Lilly) N=35	Group 2 Pts used NovoRapid/ Levemir (Novo Nordisk) N=32	Group 3 Pts used Insulatard/ NovoMix (Novo Nordisk) N=71	Group 4 Pts used Apidra/ Lantus (Sanofi Aventis) N=65	Total N=203
	Number of patients (%)				
No technique	3 (8,6)	0	0	0	3 (1,5)
Poor technique	17 (48,6)	19 (59,4)	48 (23,6)	43 (21,2)	127 (62,6)
Adequate technique	13 (37,1)	6 (3,0)	19 (9,4)	15 (7,4)	53 (26,1)
Optimal technique	2 (5,7)	7 (3,4)	4 (2,0)	7 (3,4)	20 (9,9)

4. DISCUSSIONS

In this study, patients' injection techniques were directly evaluated by observing and recording steps of operation. This can help to reflect more accurately the practice of patients at home. "Choosing dose" which is considered the most important step by the hospital's doctors has the lowest rate of mistake (7.5%). As it is supposed to be very crucial, this could have been emphasized more to patients which may have led to less error.

The most common steps of error were "removing inside cap" and "removing and discarding needle" with similar rates of 68% and 67% respectively. These two steps are closely related, almost all patients who kept the inner cap had the intention of reusing it after injection; they would close the needle with the inner cap consequently. These patients therefore did not remove and discard needles but reuse them for the subsequent injections. These steps are not considered important ones but when patients do not follow them, there are a series of risks. Firstly, patients may suffer from hurting their hands or bleeding when they put the inner cap on the needle. Secondly, at the injection sites, they may have pain, bruising and bleeding because when the needles are reused several times, their silicone lubricants are lost, and they may be bent or chipped, especially small ones (2, 3). The third risk is reduced accuracy of subsequent doses, and increased risk of infection and pinch points due to residual insulin in the needle or air may come through to insulin pen (2-4). Finally, we see that patients had the habit of reusing needles. Although these are not important steps but it is very easy to be in error. Thus when training patients, medical staffs should observe patients to follow the right instruction to avoid the potential risks.

In the 2 important steps, "safety test" was performed incorrectly more often (43.8%) than "Holding the injection button" (26.6%)."Safety test", or in other words removal of air bubbles prior to injection, helps to test the flow of insulin as the bubbles reduce or stop the insulin infusion rate (2-5). If this is not done, patients are at risk for a broken needle or inadequate insulin dose resulting in hyperglycaemia. In a Lebanon study, this rate was even higher, 60% (6). In the rest of the important steps, patients usually did not hold the pen at injection site for a sufficient time or did not hold the injection button until they took out the pen. Some studies in Europe show patients did not hold for a sufficient duration, whereas Chinese patients tend to hold for more than 10 seconds. In this study, patients using different pens showed different rates of error in this step. Every pen has its own duration of holding the injection button, for example SolaStar requires 10 seconds of holding, Plexpen and KwikPen require 6 and 5 seconds respectively. This may be confusing to patients especially those who have more than one pen, however it is important to remember to keep the injection button pressed down completely until the needle is removed from the skin, as this helps to inject the full dose of insulin without leaking or missing.

Some insulin in the form of a suspension should be shake or mixed well before injection but patients still forgot this step (40% in group 1 and 60% in group 3) which may lead to too high or too low blood glucose levels.

5. CONCLUSION

Our study adds much information to our knowledge about patient injection practices. Not many patients are wrong at the most important step but still a low percentage of patients can operate perfectly. More effort should be made to improve patients' technique in using insulin pens.

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Attitudes Towards E-Learning Among Community Pharmacists in Hanoi, Vietnam: An Exploratory Study

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Abstract

Introduction: E-learning is now emerging as the advance paradigm for continuing pharmacy education and it has been widely utilized for continuous professional development (CPD) in most countries. However, e-learning has still been a new teaching method and has not been widely used in pharmacy education in Vietnam. **Objectives:** The aim of this study was to identify the attitudes of Vietnamese pharmacists towards e-learning for CPD and the factors influencing the e-learning adoption of participants. **Methods:** A cross-sectional survey of 111 community pharmacists was conducted from January to April 2017 at three districts in Hanoi, Vietnam. A self-administered questionnaire was used to measure pharmacist's access to the internet, internet facilities and attitudes toward e-learning. The dependent variable of study was the participants' intention of using e-learning in continuing education. Multivariable logistic regression model was performed to identify factors significantly associated with the participants' acceptance of e-learning courses. **Results:** 61.26% of respondents used high speed internet. The percentage of participants using smart phone to connect to the internet accounted for 91.89%. In general, the participating pharmacists had a positive attitude toward e-learning for continuous professional development (CPD). Access to internet at work significantly decreased the likelihood of participants taking e-learning in continuing education (OR= 0.22, 95%CI=0.06-0.75). Accordingly, pharmacists having sufficient skills of using internet devices (OR=2.52, 95%CI=1.44-4.41) and internet usage for study (OR=4.67, 95%CI=1.82-11.98) were more likely to adopt e-learning in continuing education. **Conclusion:** The study found some variables affected the behavioral intentions related to web-based e-learning systems. The e-learning courses should be designed as simple and flexible courses so that pharmacists with different levels of skill can participate in at anytime.

Keywords: E-learning, pharmacy, attitude, continuing education, continuous professional development

1. INTRODUCTION

The pharmacy profession has been changing continuously and pharmacists are increasingly involved in patient monitoring and consultation with other healthcare professionals as partners. Therefore, in more and more countries, continuing professional training is a requirement for pharmacists, as is for many healthcare professions, to keep their license valid (1). The new Vietnamese pharmacy law (No 105/2016/QH13) also obligated pharmacists to take part in CE courses. Continuing education (CE) plays an important role in maintaining and updating pharmaceutical skills and knowledge (1). CE courses for pharmacists, provided by professional associations, pharmacy boards, universities, teaching hospitals, and pharmaceutical companies, vary widely in their scope and breadth of content (2).

E-learning is a method of teaching and learning using electronic media (3). E-learning is also called web-based, online learning, distributed learning, computer-assisted instruction and internet-based learning (4). E-learning is now emerging as the advance paradigm for continuing pharmacy education and it has been widely utilized for CPD in most countries (5). Numerous studies on medical and pharmaceutical staff have been conducted in the past decade to assess the efficiency of e-learning in their current and continuing education. The results have been varied but there exists a consensus on the benefits of this alternative medium of learning: high accessibility, flexibility, time and cost/investment benefits (5). Some studies conducted on the efficiency of e-learning have shown that well-designed courses resulted in similar or more knowledge gaining as on-site learning (6, 7). In addition, several courses have shown evidence of significant self-reported practice change (5).

Lately, e-learning has started to make way into developing countries and is believed to have enormous potential for governments struggling to meet a growing demand for education while facing an escalating shortage of teachers (8). E-learning is also an attractive solution to educate large numbers of geographically diverse populations. Nevertheless, e-learning has still been a new teaching method and it has not been widely used in pharmacy education in Vietnam. Therefore, determining the factors associated with pharmacists' intention of using e-learning courses for CPD is vital in order to improve the number of participants in continuing pharmacy education. Besides, it is necessary to assess conditions to implement e-learning courses in CE. Challenges mentioned were network connectivity, bandwidth availability and learner computer skills (9, 10). Therefore, the goal of the study is explore the attitudes towards e-learning for CE and the factors influencing the e-learning adoption of participants. We hypothesized that pharmacists who had better internet facilities or more positive attitude towards e-learning would be more likely to intend using e-learning in CE. Findings from the study may inform future interventions to improve e-learning system in CE in the country.

2. MATERIALS AND METHODS

A cross sectional survey was conducted from November to March 2017 in three districts in Hanoi, Vietnam. A validated self-administered questionnaire was distributed among retailers at pharmacies. Drug retailers were approached and recruited through a convenient sampling technique. Participants will be selected according to the following criteria: retailers had graduated from a pharmacy university or college and agreed to be interviewed.

Based on validated instruments about attitudes and internet facilities used in previous studies, we devised 25 items in to criteria to assess retailers' access to internet facilities, attitudes and e-learning adoption. These items were translated from English into Vietnamese. The translation was an iterative process with principles of translation/back translation. The questionnaire was piloted to assure the clarity of each item. 30 pharmacists participated in the pilot survey.

The study outcome was the participants' intention of using e-learning in continuing education. Accordingly, the respondents were classified as "no e-learning adoption" if they have not intended to use e-learning in CE and "e-learning adoption" if they have intended to use e-learning in CE by the time of the survey.

Internet related items encompassed basic internet information about connection speed, where, how long to connect to the internet, what purpose to connect to the internet and which devices

to connect to the internet. Attitude related items assessed perceived usefulness, e-learning self-efficacy and technology factor. Each item was measured with a five-point Likert scale ranging from strongly disagree to strongly agree. In addition, personal characteristics (i.e, age and gender), professional factors (i.e, length of career and professional qualification) and experience with e-learning were also collected.

Descriptive statistics were conducted to compare the respondents' characteristics by e-learning adoption status (No e-learning adoption vs. e-learning adoption). Frequencies and distributions of variables related to internet facilities and attitude towards e-learning were also described. Student t-test and Mann-Whitney test were used for normally and non-normally distributed continuous variables, respectively. Chi-square statistics were used for categorical variables. Logistic regression was performed to identify factors significantly associated with intention of using e-learning in CE. Models were built based on the strategy recommended by Hosmer & Lemeshow (11). Accordingly, any variable whose univariate test had a p-value <0.25 was a candidate for the multivariable model. We then applied a stepwise backward model approach based on the log-likelihood ratio test including variables with a p-value<0.1. Well-documented predictors (i.e internet usage for study) were kept in the final models regardless of statistical significance. Collinearity was checked by variance inflation factors. All potential interactions were examined. We assessed model calibration using Hosmer-Lemeshow goodness-of-fit test (11). All tests of hypotheses were two-tailed with a significance level of α less than 0.05. Statistical analyses were performed with STATA version 12.0.

3. RESULTS

3.1 Characteristics of the respondents

Table 1. *Sociodemographic characteristics of respondents*

	No e-learning adopter	**E-learning adopter**	**Total**	**P value**
N (%)	67 (60.36)	44 (39.64)	111 (100.00)	
Age, mean (SD)	29.54 (6.309)	27.68 (6.459)	28.81 (6.402)	0.0735
Gender, n (%)				
Female	58 (86.57)	42 (95.45)	100 (90.09)	0.125
Male	9 (13.43)	2 (4.55)	11 (9.91)	
Years of practice, mean (SD)	6.49 (5.915)	4.73 (4.801)	5.78 (5.538)	0.0861
Professional qualification, n (%)				
Graduated from a college	12 (17.91)	2 (4.55)	14 (12.61)	0.116
Graduated from a junior college	25 (37.31)	19 (43.18)	44 (39.64)	
Had associate degrees in	30 (44.78)	23 (52.27)	53 (47.75)	

pharmacy				
E-learning experience, n (%)	9 (13.64)	12 (27.27)	21 (19.09)	0.075

A total of 137 pharmacists participated in the survey. The response rate was 68.5%. Twenty-six subjects were excluded due to missing responses on the outcome variable, resulting in 111 participants included in data analysis. 39.64% respondents had intention of using e-learning in CE. The proportion of pharmacists graduating from a university, pharmacists graduating from a college and people having associate degrees in pharmacy in the sample were 12.61%, 39.64% and 47.75%, respectively. The majority was female (90.09%), had a mean age of 28.808 years old (SD=6.402), and had an average practice of 5.78 years (SD=5.538). E-learning adoption group (vs no e-learning adoption group) was more likely to be younger, had shorter practice time but more e-learning experience (Table 1).

3.2 Internet facilities and purpose

Table 2. *Internet facilities and purpose*

	No e-learning adopter	**E-learning adopter**	**Total**	**p-value**
Frequent internet connection, n (%)	57 (91.94)	43 (100.00)	100 (95.24)	0.077
Access to the internet, n (%)				
At home	43 (64.18)	35 (79.55)	78 (70.27)	0.083
At work	58 (86.57)	33 (75.00)	91 (81.98)	0.121
High speed internet, n (%)	38 (56.72)	30 (68.18)	68 (61.26)	0.225
Device connected to the internet, n (%)				
Smart phone	61 (91.04)	41 (93,18)	102 (91.89)	1.000
Desktop or Laptop	27 (40.30)	20 (45.45)	47 (42.34)	0.591
Tablet	11 (16.42)	5 (11.36)	16 (14.41)	0.585
Average internet usage time per day (hours), mean (SD)	5 (2.799)	4.83 (3.01)	4.93 (2.87)	0.611
The purpose of internet usage, n (%)				
Business	44 (67.69)	36 (81.82)	80 (73.39)	0.124
Study	24 (36.92)	32 (72.73)	56 (51.38)	**<0.01**
Entertainment	55 (84.62)	35 (79.55)	90 (82.57)	0.608

Internet facilities and purpose were shown in Table 2. The percentage of access to the internet at home and at work of participants in the survey were 70.27% and 81.98%, respectively. The most popular devices connected to the internet was smartphone (91.89%), followed by the percentage of using computer (desktop or laptop) to connect to the internet (42.34%). The average hour of using internet per day was 4.93 (SD=2.87). The purpose of using internet was multiform. 82.57% participants used the internet for entertainment compared with 73.39% for business and 51.38% for study. The percentage of the internet usage for study was the lowest and had significant difference between two groups ($p<0.01$). 72.73 % respondents of e-learning adoption group used internet for study compared with 36.92% respondents of no e-learning adoption group.

Table 3. *Attitude of participants about e-learning*

	No e-learning adopter, mean (SD)	E-learning adopter, mean (SD)	Total	p
I have experience to use handled device (such as laptops, tablets and smartphones…)	3.16 (1.039)	3.68 (0.909)	3.369 (1.017)	0.003
I have experience to use internet	3.12 (1.038)	3.57 (0.899)	3.30 (1.005)	0.008
I have the necessary skills for using an e-learning system	2.97 (0.887)	3.64 (0.865)	3.23 (0.934)	<0,01
Using the e-learning enhanced my effectiveness in learning	3.34 (0.827)	3.75 (0.781)	3.50 (0.829)	0.001
E-learning could make it easier to study course content	3.30 (0.871)	3.79 (0.765)	3.49 (0.862)	0.0005
E-learning helps to save time	3.34 (0.897)	3.73 (0.758)	3.49 (0.862)	0.009
E-leaning helps to save cost	3.36 (0.899)	3.73 (0.817)	3.50 (0.883)	0.007
I have the flexibility of learning with regard to place	3.52 (0.746)	3.77 (0.773)	3.62 (0.763)	0.020
I have the flexibility in choice of learning time.	3.51 (0.823)	3.77 (0.711)	3.61 (0.788)	0.022
E-learning give me self-confidence	3.36 (0.899)	3.70 (0.795)	3.49 (0.873)	0.013
E-leaning provides up to date content and information	3.19 (0.802)	3.45 (0.875)	3.29 (0.838)	0.040
I have the necessary devices and internet facilities to use e-learning system	3.28 (0.794)	3.55 (0.951)	3.39 (0.865)	0.026

Table 3 shows the attitude about e-learning of two groups. We found significant differences in all e-learning attitudes between two groups. The attitude means of e-learning adoption group was higher than the attitude means of no e-learning adoption group. Some benefits of e-learning such as flexibility of learning with regard to time and place had the highest mean at 3.62 (SD=0.763) and 3.61(SD=0.788), respectively. Meanwhile, the mean of having the necessary skills for using an e-learning system was the lowest at 3.23 (SD=0.934).

Table 4. *Associated factors of choosing e-learning among study participants*

	Univariate regression		Multivariate regression	
	Unadjusted OR (95% CI)	P	Adjusted OR (95% CI)	p
Age	0,95 (0.89-1.02)	0.153		
Gender				
Male	1			
Female	3.25 (0.67-15.86)	0.144		
Year of practice	0.94 (0.86-1.02)	0.123		
Professional qualification				

Graduated from a college	1.00			
Graduated from a Junior college	4.56 (0.91-22.85)	0.065		
Had associate degrees in pharmacy	4.6 (0.94-22.61)	0.060		
e-learning experience	0.42 (0.16-1.11)	0.079		
Access to the internet:				
At home	2.17 (0.89-5.27)	0.087		
At work	0.47 (0.17-1.24)	0.126	0.22 (0.06-0.75)	**0015**
Highspeed internet	1.63 (0.74-3.63)	0.227		
Internet usage for				
Business	2.15 (0.85-5.42)	0.106		
Study	4.56 (1.98-10.48)	<0,01	4.67 (1.82-11.98)	**0.001**
I have experience to use handled device (laptops, tablets, smartphones…)	1.76 (1.14-2.71)	0.011		
I have experience to use internet	1.63 (1.07-2.49)	0.024		
I have the necessary skills for using an e-learning system	2.46 (1.48-4.08)	<0,01	2.52 (1.44-4.41)	**0.001**
Using the e-learning enhanced my effectiveness in learning	2.04 (1.15-3.65)	0.015		
E-learning could make it easier to study course content	2.34 (1.29-4.23)	0.005		
E-learning helps to save time	1.82 (1.07-3.09)	0.026		
I have the flexibility of learning with regard to place	1.59 (0.92-2.78)	0.096		
E-leaning helps to save cost	1.72 (1.03-2.85)	0.036		
I have the flexibility in choice of learning time.	1.59 (0.93-2.74)	0.088		
E-learning give me self-confidence	1.69 (1.01-2.85)	0.047		

Results from the multivariate logistic regression are presented in Table 4. After adjusting for other variables, those found to be significantly associated with intention of using e-learning in CE were access to the internet at work, the purpose of internet for study and the necessary skills for using an e-learning system. Accordingly, access to the internet at work significantly decreased the likelihood of e-learning adoption in CE (OR= 0.22, 95%CI=0.06-0.75). However, the respondents who using the internet for study were more likely to adopt e-learning in CE (OR=4.67, 95%CI=1.82-11.98). Besides, other advantage for intention of using the internet were having the necessary skills for using an e-learning system (OR=2.52, 95%CI=1.44-4.41).

4. DISCUSSION

The study sets out to assess whether the internet facilities among Vietnamese community pharmacists can meet the requirement of e-learning courses and the e-learning courses are accepted by pharmacists. To our knowledge, the current study is one of the first to address internet facilities and associated factors on e-learning adoption in CE among Vietnamese community pharmacies.

This research collected basic information about internet conditions. This is valuable information because network connectivity and bandwidth availability are the key obstacles to the effective delivery of online e-learning (9, 10). 95.25% participants used the internet frequently and 61.26% respondents connected to the high speed internet. Therefore, there was advantage of implementing e-learning in CE in Vietnam. Learners can now select a distance learning environment to meet their needs. The percentage of internet usage at pharmacy was higher than that at Bachmai hospital (56%) (12). When the living standards were raised, the percentage of Vietnamese people having connected devices improved. By June 30, 2017, according to Internet World Stats, the number of internet users in Vietnam had been 64 million people. Vietnam is in the top 20 countries with highest number of internet users (13). Most retailers had access to the internet at both home and workplace (70.27% and 81.98%) respectively. Therefore, the participants could use internet and take part in e-learning anywhere.

Some pharmacy universities or colleges in Health Human Resources Sector Development Program were educated to design e-learning courses on Modular Object Oriented Dynamic Learning Environment system. In order to benefit from e-learning, learners should have a personal computer (PC) and an internet connection or other network connection. However, 91.2% participants in this study used smart phone to connect the internet, while the figure for using PC was 42.34%. These results were the same as the results of a previous study among health workers. The percentage of connecting internet by smartphone was the highest (over 75%) (12). Accordingly, we should design online pharmacy courses in CE on mobile (m-learning). Currently, many well-known online learning sites at Vietnam such as www.coursera.org designed both e-learning and m-learning. They have designed more software applications on mobile phones to increase the number of students using online learning.

Only 19.09% respondents had e-learning experience. This percentage was the same as results of some previous studies in Vietnam and Malaysia, while the figure for other developing countries was higher (12, 14, 15). Hence, e-learning is still a new teaching method and it has not been widely used in pharmacy education in Vietnam. The effectiveness of e-learning in pharmacy education was examined by Sandra et al. The study confirmed that e-learning in pharmacy education is effective at increasing knowledge immediately after training. Additionally, in comparisons, e-learning was as effective as traditional learning and superior to no training (16).

In the atmosphere of constant changes, updates and new findings in pharmaceutical sciences, nobody doubts the need to keep knowledge up to date in order to stay professionally active and reliable. More and more countries implement CE as obligatory lifelong learning programs. The role of e-learning in CE has become crucial in recent years. It is a convenient way of learning, which can take place anytime and anywhere. This study has shown that some benefits of e-learning such as flexibility of learning with regard to time and place had the highest mean (3.62 (SD=0.763) and 3.61 (SD=0.788), respectively). We found that pharmacists recognized that this learning model had certain strengths such as flexibility in time and space, consistent with some previous studies (15, 17). This flexibility is particularly attractive to professionally active pharmacists who often have no time to travel and attend conventional courses in big academic centers. E-learning helps to save cost (3.50 (SD=0.883)) since it reduces e-cost for participants (i.e, travel and accommodation) as well as for providers (i.e., renting the venue and printing materials). However, this means that learners need to take more responsibility for their own learning.

In our study, respondents having the necessary skills for using and e-learning system were more likely to adopt e-learning in CE (OR=2.52, 95%CI=1.44-4.41). This is consistent with previous researches which found that perceived skillfulness at using e-learning influenced attitudes toward the use of e-learning (18, 19). The lack of technological or internet skills was the biggest challenge, therefore, learning courses must be designed with multiple-level to fit pharmacists with diverse levels of skills. Designers should plan their courses for easy access, navigation, and participation, to increase the positive experience of first time users and to increase repeat participation. Additionally, learning should be enjoyable and simple. Therefore, course designers must consider several issues regarding their designs: simplicity/intuitiveness, visual/esthetic appeal, level of complexity, and degree of interactivity and engagement. Efforts to motivate potential participants to take advantage of online CE should be considered beyond uncomplicated design. Besides, training necessary e-learning skills for pharmacists should be considered to encourage the participation of e-learning courses in continuing pharmacy education.

Participants using internet for learning were more likely to use it in CE. Apparently, because of their experience these people were in an advance stage and adopted e-learning system easier. Still, others felt that access to the internet at work pose challenge on e-learning adoption (OR= 0.22, 95%CI=0.06-0.75). Working time seem to not be the appropriate time for learning, therefore, flexible courses should be implied to benefit learners.

Several limitations should also be considered. First, due to the nature of a cross-sectional design, we cannot establish temporal or causal relationships between independent variables and e-learning adoption. Second, the generalization of our study results is limited by the convenience sampling strategy. Therefore, caution should be taken in generalizing findings from our study to other pharmacist populations in Vietnam. Third, the sample size was small. Fourth, data gathered using Likert scales are subject to biases caused by participants who may agree to a given survey statement, simply out of the desire to give a favorable response; alternately, we suggest that some participants profess to being neutral on some issues because they wish to avoid extreme response. We believe the latter may be the case for several statements with high percentage of neutral responses. Besides, most previous studies used technology acceptance model theory to evaluate the intention of using e-learning in CE. However, we could not use this theory in this study due to insufficient databases and e-learning course shortage for community pharmacists. We suggest that further studies be conducted.

5. CONCLUSION

This is the first study to explore the attitude of pharmacists towards e-learning in Vietnam. The results showed that most pharmacists had a positive attitude toward e-learning, implying that it may be used as a new in-service educational method for pharmacists. Besides, this study found that variables, including "*where have access to the internet", "purpose to connect the internet*" and "*necessary skills for using the e-learning system*". Providers should design e-learning as simple and flexible courses so that pharmacists with different levels of skill can participate anytime. Besides, training necessary e-learning skills for pharmacists should be considered to encourage the participation of e-learning courses in continuing pharmacy education.

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Applying the 2015 Updated Beers Criteria to Assess Potentially Inappropriate Medications in Elderly Patients at Discharge: A Geriatric Hospital-Based Study

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Abstract

Introduction: Inappropriate prescribing is an important risk factor of adverse drug reactions and hospitalizations in elderly people, resulting in increased health care burden. However, there is a paucity of studies in understanding the prescribing pattern in elderly patients in Vietnam. For this purpose, the 2015 updated Beers criteria, developed and validated by American Geriatrics Society, was used to assess the Potentially Inappropriate Medication in elderly patients in this study. **Objectives:** To describe the pattern of Potentially Inappropriate Medication (PIM) in elderly patients at discharge in a Vietnamese Central geriatric hospital, and to determine the relationship between the prevalence of PIMs and the rate of ADE occurred in patients during a 45-day-period after discharge. **Methods:** A prospective observational study was conducted from 1st November 2016 to 15th April 2017 in a geriatric hospital in Hanoi, involving 206 discharge patients aged ≥65 years. Patient information including sociodemographic, clinical data and medication prescription at discharge were obtained from medical records. The discharge prescription of each patient was assessed to detect Potentially Inappropriate Medication (PIM) according to the 2015 updated Beers criteria. Patients were monitored for 45 days after discharge for ADEs and readmission events by telephone interview. **Results:** According to the 2015 Beers criteria, at least one PIM was identified in 12.6% of the study population (n=206). 18 types of PIMs were detected, with the two most common PIMs being antipsychotics (6.8%) and digoxin (2.4%). 100 ADEs (44.3% of patients) and 27 readmission events (15.5%) were recorded. The risk of having ADE in the group of patients with at least 1 PIM was significantly higher than the non-PIM group (OR:3.643; 95% CI:1.425 – 9.315; p= 0.005). **Conclusion:** The prevalence of PIMs in discharge prescriptions of elderly patients is relatively high in Vietnam. Our study also demonstrates the positive association between PIM and ADE in elderly patients. This finding would support more active intervention to minimize PIM at discharge in elderly patients in Vietnam.

Keywords: Potentially Inappropriate Prescribing, elderly, Beers criteria, discharge

1. INTRODUCTION

Population aging is an increasing worldwide phenomenon that influences the healthcare systems of many countries. Due to age-related physiological changes, the high prevalence of polypharmacy and multiple comorbid conditions, elderly patients naturally are at higher risk of adverse drug reactions, drug-drug and drug-diseases interactions.

Potentially Inappropriate Medications (PIM) are defined as the medications in which risk of treatment potentially outweighs the benefit, particularly when safer or more effective alternative therapy is available for the same condition [1]. The prescribing of PIMs is an important risk factor of adverse drug reactions and hospitalizations in elderly people, resulting in increased health care burdens [2][3][4]. Explicit criteria for PIM have been developed in many countries, including the United States, Canada, France, Ireland, Australia and Norway [5-7]. Using these criteria, many studies confirmed the high prevalence of PIM in elderly patients in all healthcare settings (including primary care, secondary care and long-term care), and also showed significant correlation between the prescribing of PIM and the incidence of adverse drug events (ADE) [8][9].

Up to now, there is a paucity of studies in understanding the prescribing pattern in discharged elderly patients in Vietnam. For this purpose, the 2015 updated Beers criteria, developed and updated by American Geriatrics Society, was used to assess the Potentially Inappropriate Medication in elderly patient in this study.
The objectives of this study is to describe the pattern of Potentially Inappropriate Medication (PIM) in elderly patients at discharge in a Vietnamese Central geriatric hospital, and to explore the relationship between the prevalence of PIM and the rate of ADE occurred in patients during a 45-day-period after discharge.

2. METHODS

2.1. Study design

A prospective observational study was conducted from 1st November 2016 to 15th April 2017 in a public geriatric hospital in Hanoi.

2.2. Inclusion criteria

Patients were included in the study if they matched these criteria: (1) aged ≥65 years; (2) discharged from a ward of internal medicine of hospital; and (3) having discharge prescription. Exclusion criteria included: (1) Discharge due to transfer to another hospital; (2) Discharge due to death.

2.3. Data collection

Patients' information including sociodemographic (age, gender), clinical data (principle and secondary diagnoses) and medication prescription (full data on name, dosage and duration of drug treatment) at discharge were obtained from medical records. The complement information from interview included the history of diseases, history of medication, history of falls and other functional data.

Patients were monitored for 45 days after discharge for ADEs and readmission events by telephone interview. In the first week after discharge, telephone interview was conducted twice, followed by one phone call per week thereafter.

2.4. Assessment

The discharge prescription of each patient was assessed to detect Potentially Inappropriate Medication (PIM) according to the 2015 updated Beers criteria. The Charlson Comorbidity Index (CCI) was used to determine the patient health status and comorbidities. The Independent level in Activities of Daily Living (ADL) was assessed for each patient. To assess cognitive function and the risk of bleeding, the Mini Mental State Examination (MMSE) and HAS-BLED score were used respectively.

The possible symptoms of ADE were documented and judged by the researcher. To identify the ADE-induced drugs, the National Formula of Vietnam and Summary Product Characteristics labels were used.

2.5. Data analysis

All collected data were collated using Microsoft Excel 2015 and analyzed by SPSS Version 22.0. To assess the factors related to ADE, univariate analysis using the chi-square test was applied. Statistical significance level was set at $p = 0.05$.

3. RESULTS

3.1 Characteristics of the study population

The main characteristics of the study population are described in Table 1. A total of 206 elderly patients (40.8% male, mean age 78.7 years) met the selection criteria. The mean number of medication per discharge prescription was 4.1 ± 1.6 (range 1 - 8). Patients were mainly discharged from Cardiovascular ward (26.2%), Endocrine & Metabolism ward (21.8%) and General Internal medicine ward (21.4%). 57.3 % of patients had CCI score in range of 3 to 4. The history of falls was recorded in 35 (17.0%) patients, while 13 (6.3%) patients were assessed to have impaired cognitive status.

Table 1. *Characteristics of the study population (N=206)*

Characteristics		Number of patients (%)
Age (years) (Mean ± SD)*		78.7 ± 7.6
Gender Male		84 (40.8)
Number of disease per patient (Mean ± SD)*		3.0 ± 1.1
Charlson Co-morbidity Index (CCI)	2	63 (30.6)
	3-4	118 (57.3)
	≥ 5	25 (12.2)
Activities for Daily Living (ADL)	Independent	93 (45.1)
	Dependent ≥ 1 ADL	113 (54.9)
Cognitive function	Normal	183 (88.3)
	Impaired	13 (6.3)
	Unevaluated	10 (4.9)
History of fall		35 (17.0)
Most common diseases	Hypertension	136 (66.0)
	Stroke	77 (37.4)
	Diabetes	40 (19.4)
	Pneumonia	27 (13.1)
	Osteoporosis	24 (11.7)

**SD: Standard Deviation*

The proportion of patients exposed to polypharmacy (5 or more medications) was 36.4%. The total number of drugs prescribed was 781 (related to 106 active ingredients). Of these, vitamin was the most prescribed drug (9.1%), followed by AT1 receptor blockers and ACE inhibitors (8.8%). (Table 2)

Table 2. *Characteristics of discharge prescribing*

Characteristics		Number of prescription
Number of medications per prescription (N =206)	Mean ± SD*	4.1 ± 1.6
	Min - Max	1 – 8
	< 5	131 (48.1)
	≥ 5	75 (36.4)
Top 5 drug groups prescribing (N= 781)	Vitamin	71 (9.1)
	ACEI/AT1 blockers**	69 (8.8)
	Anticoagulation	61 (7.8)
	Antidiabetics	59 (7.6)
	Lipid modifying	49 (6.3)

** SD: Standard Deviation*

*** ACEI/AT1 blockers: Angiotensin converting enzyme inhibitors/Angiotensin II receptor blockers*

3.2 Potentially Inappropriate Medication determined by the 2015 Updated Beers criteria

According to 2015 Beers criteria, one or more medications defined as PIMs had been prescribed in 12.9% of discharge patients in the study. Most of these patients had one PIM (22/26 patients), and only 1 patient was prescribed with 3 PIMs. Table 3 lists 12 types of PIMs with a total of 31 PIMs in the study population. The most common PIM was the use of antipsychotics (6.8%), followed by the use of digoxin (2.4%), long acting benzodiazepine and amitriptyline (1%).

Table 3. *The distribution of PIM according to 2015 Beers criteria*

Characteristics		**Number of patient**	**Percentage (%)**
Number of PIM per patient (n = 206)	0 PIM	180	87.4
	1 PIM	22	10.7
	2 PIM	3	1.5
	3 PIM	1	0.5
Type of PIMs			
Independent of Diagnosis Medication	Antipsychotics (first and second generation)	14	6.8
	Digoxin > 0,125mg/day	5	2.4
	Long acting benzodiazepine (diazepam)	2	1.0
	Amitriptyline	2	1.0
	Metoclopramide	1	0.5
	Trihexyphenidyl	1	0.5

Dependent of Diagnosis	Antipsychotics: Sulpiride – history of fall Risperidone – history of fall Antiepileptics: Pregabaline – History of fall	1 1 1	0.5 0.5 0.5
	Quetiapine – dementia	1	0.5
	Quetiapine – delirium	1	0.5
	Meloxicam in patients who have Clcr* <30 ml/min	1	0.5
Total		**31**	

** Clcr: Clearance creatinine*

3.3 Characteristics of ADEs

Table 4 shows the number of ADEs and re-admission events that had occurred. There was a total of 100 ADEs and 27 re-admission events recognized in 77 patients (44.3% of 174 follow-up patients). Every one of these patients had at least an ADE, with one patient had 4 ADEs. There were 5 ADE-inducing medications (including long acting benzodiazepine, trihexyphenidyl, pregabaline, meloxicam and digoxin) that have been defined as PIM according to Beers criteria. One re-admission event was suspected to be caused by digoxin.

Table 4. The number of ADE occurred (N = 174)

Number of ADE	**Number of patients (%)**	**Percentage (%)**
- 0 ADE	97	55.7
- 1 ADE	59	33.9
- 2 ADE	14	8.0
- 3 ADE		

- 4 ADE	3 1	1.7 0.6
- Readmission event	27 (15.5)	15.5

3.4 Univariate analysis of factors associated with ADEs

To explore for factors associated with ADEs, the occurrence of ADEs and several independent variables were analysed: patient characteristics (age, gender), number of medication per patient, CCI score, number of diseases per patient, and number of PIMs. A positive association was found between the number of medications and the number of PIM and ADEs (Table 5).

Table 5. Univariate analysis of factors related to ADE

Independent factors	**No. of patient having ADE**	**OR (95%CI)**	**p**
Number of medications			
< 5 drugs (n=112)	43 (38.4)	1.949 1.039 – 3.654	0.036
≥ 5 drugs (n=62)	34 (54.8)		
Number of PIMs			
0 (n = 150)	60 (40.0)	3.643 1.425 – 9.315	0.005
≥ 1 (n = 24)	17 (70.8)		

4. DISCUSSION

4.1 Characteristics of PIMs

In our study, the percentage of PIM prescription was 12.6% according to Beer criteria. To compare with other studies, many factors should be considered including the differences in the study setting, the healthcare system and the tool of assessment. The time of discharge represents a high-risk time especially for elderly patients, as new drugs are usually prescribed [10]. However, there is few studies conducted in this time up to now. A recent cross-sectional study in Brazil show that the prevalence of PIM in elderly patients at discharge was 13.9% [11], and this figure in another study in Spain [12] was 22.9 %. These findings of our study showed that, like other countries, PIM might be commonly prescribed for elderly patients at discharge in Vietnam.

The most frequent PIM in our study concerned the use of antipsychotics (14/31 of all PIMs identified), digoxin (5/31) and benzodiazepine (2/31). While there is difference about the order of top common PIMs, these PIMs are also reported as the most common PIMs identified in many previous studies.

4.2 Characteristic of ADEs

The present study found that the prevalence of ADEs and readmission events occurred after discharge in elderly patients was relatively high. ADE occur more often in patients with polypharmacy and patients prescribed PIMs. These findings were consistent to many previous studies[13], [14][15, 16].

The present study has several limitations. The study only identified PIMs of discharge prescriptions in a small sample size from one hospital. The result may not be totally representative across differing elderly patient populations in other settings. The second limitation is the short duration of monitoring after discharge that may result in under-estimating some long-term PIM-related ADEs. Nevertheless, this study at least provides more evidence that the practice of PIM is a concerned phenomenon in Vietnamese healthcare setting. Re-evaluation of drug treatment and removal of PIMs in discharge prescription of elderly patients should be considered more by physicians and pharmacist.

5. CONCLUSION

The prevalence of PIMs in discharge prescriptions of elderly patients is relatively high in Vietnam. Our study also demonstrates the positive association between PIM and ADE in elderly patients. This finding would support more active intervention to minimize PIM at discharge in elderly patients in Vietnam.

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Vietnamese pharmacists' perceptions of Facilitators and Barriers to Continuing Education: An exploratory study

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Abstract

Introduction: Contemporary pharmaceutical care requires pharmacists to maintain and improve knowledge, skills and performance. Continuing Education (CE) is a lifelong learning approach to enhance professional competencies. However, in Vietnam, little is known about pharmacists' perceptions of facilitators and barriers to Continuing Education. **Objectives:** The purpose of this study was to identify facilitators of and barriers to pharmacists' participation in Continuing Education. **Materials and methods**: A cross-sectional study design using questionnaires was conducted in some districts of Hanoi. Self-administrated questionnaires were distributed to 200 community pharmacists. The difference between participants in CE and non-participants in CE in relation to mean motivational and attitudinal barrier scores was identified using Mann-Whitney test. Chi-squared test or Fisher exact test was used to identify association between categorical variables. **Results:** The response rate was 68.00%. Approximately two-third participants (61.03%) had not attended any CE programs. The findings from the study reveal that the major motivating factors identified by the participants were keeping up to date with the information, meeting job requirements and meeting/ interacting/ exchanging ideas with others (the mean scores were 3.82, 3.64 and 3.74, respectively). Pharmacists who participated in past CE had a significantly higher attitude score of funding support (p= 0.024). Major obstacles included poor timing, lack of funding and family constraints (the mean scores were 3.48, 3.34 and 3.33, respectively). **Conclusions:** Respondents of the survey expressed a great interest toward CE activities and reported the participation motivations and constraints as well. To address factors identified as facilitators and barriers, CE providers should focus more on pharmacists' time constraints and pharmacists' motivations to learn (keeping up to date with the information, meeting job requirements).

Keywords: continuing education, continuing professional development, facilitators, barriers, pharmacy

1. INTRODUCTION

During the last decades, the role of the community pharmacist has changed from medication dispenser to counselor and provider of pharmaceutical care. Contemporary pharmaceutical care requires sustained pharmacist competency through improvement of knowledge, skills, and performance. Many pharmacists seek to accomplish this by participating in continuing education (CE). In Vietnam, a pharmacy practitioner must have the certificate of completion of training program and refresher program in pharmacy within 3 years from the issuance date of the pharmacy practice certificate or the issuance date of the latest certificate of completion of training program and refresher program in pharmacy.

Although CE programs are available nationwide, published research with regard to pharmacists' preferences for CE programs is lacking. To our knowledge, in Vietnam, no prior studies have addressed pharmacists' feedback on the adequacy of currently available CE programs.

Additionally, meeting pharmacists' needs and matching their preferences is an important prerequisite for effective CE programs. Pharmacists are more likely to better grasp information when presented topics meet their preferences. Therefore, the need to explore pharmacists' perceptions and attitudes and identifying their educational preferences is of great value. Furthermore, with technology advancement, continuing education has become very accessible where someone can attend live lectures from their home, or even listen and watch from their phones. Such research can guide the development and implementations of future CE programs that more effectively support the educational requirements.
The aim of this study was to get answers on the following research questions:

1. Which facilitators and barriers influence pharmacists' participation in CE courses?
2. Do pharmacists differ in opinion on these issues according to whether they take up CE courses or not?
3. What are the preferences for course topics and teaching methods?

2. MATERIALS AND METHODS

A questionnaire was developed by the investigators based on the available literature and expert opinions in four main sections (demographics, motivation to participation, barriers, and preferences for content). Five likert items were designed to assess the pharmacists' perceptions of facilitators and barriers to CE (5 points unipolar scaled, one to five). The survey was introduced and launched through distribution to 200 pharmacies which were privately owned in 3 districts in Hanoi. The data were collected over 6 months from November 2016 to April 2017. A pilot study was performed on 10 pharmacists who evaluated the questionnaire to assess if the questions were clear, easy to understand, in a logical order, and totally representative of the questionnaire objective.

All data analyses were conducted using Stata standard version release 12.0. Categorical data are presented as percentages of frequency. Participants were categorized into groups based on their participation in CE. The difference between participants in CE and non-participants in CE in relation to mean motivational and attitudinal barrier scores was identified using Mann-Whitney test. Chi-squared test or Fisher exact test was used to identify association between categorical variables and p value ≤ 0.05 was considered as significant.

3. RESULTS

3.1 Socio-demographics characteristics of the respondents

Table 1 shows the differences in the demographic characteristics between two groups (participated and non-participated in CE). The average pharmacist age was 28.36 years (SD = 6.128). Most of the respondents were female (90.4%). The average of years of experience since graduation from a pharmacy degree program was 5.3 years. Regarding professional qualification, most of them are pharmacists graduating from colleges and vocational schools (42.7% and 45.6% respectively). There was a statistically significant difference in the number of years of experience between both groups.

Table 1. *Socio-demographics characteristics of respondents*

	CE		**Total**	**P**
	Participated	Non-participated		
Age, mean (SD)	30.04±7.123	27.35±5,245	28.36± 6,128	0,063
Gender, n (%)				
Male	3 (5,7)	10 (12.1)	13 (9.6)	0.250
Female	50 (94.3)	73 (87.9)	123 (90.4)	
Professional qualification, n (%)				
Pharmacists graduating from universities	8 (15.1)	7 (8.4)	15 (11.0)	0.475
Pharmacists graduating from colleges	22 (41.5)	36 (43.4)	58 (42.7)	
Pharmacists graduating from vocational schools	23 (43.4)	40 (48.2)	63 (46.3)	
Experience years, mean (SD)	6,89 (6.319)	4,35 (4.118)	5.34 (5.213)	**0.036**

3.2 Pharmacists' perceptions of facilitators to CE

Motivation to participation was shown in Table 2. The motivational factors for updating knowledge, meeting job requirements and improving the quality and efficiency of work are more highly valued than the remaining factors (average score from 3.6 to 3.8). The facilitator "get support from the drugstore owners" among the participated group had a higher mean score ($p<0.05$) than the other group.

Table 2. *Pharmacists' perceptions of facilitators to CE*

Facilitators	Participated mean (SD)	Non participated mean (SD)	Total mean (SD)	P
Content helps to update knowledge	3.89 (0.847)	3.78 (0.682)	3.82 (0.749)	0.126
Content meets job requirements	3.642 (0.901)	3.638 (0.691)	3.640 (0.776)	0.334
Improve the quality and efficiency of work	3.792±0.817	3.711±0.672	3.74±0.730	0.159
Sufficient resources (time, money)	3.36±0.942	3.48±0.902	3.43±0.917	0.674
Get support from the drugstore owner	3.60±0.768	3.31±0.840	3.42±0.822	***0.024***
Meet with colleagues	3.736±0.858	3.73±0.717	3.735±0.772	0.456

3.3. Pharmacists' perceptions of barriers to CE

The perceptions of barriers of both groups is shown in Table 3. Time factor is the main obstacle to continuous training with an average of 3.5 (SD = 0.9). This is followed by cost factors and family issues (average score of 3.3). Elements of course content, distance learning, or difficult accessibility had the lowest average scores (3.0-3.2).

Table 3. *Pharmacists' perceptions of barriers to CE*

Barriers	Participated mean (SD)	Non participated mean (SD)	Total mean (SD)	P
Lack of time	3.36±1.076	3.55±0.830	3.48±0.935	0.368
Lack of funds	3.25±1.072	3.39±0.882	3.34±0.960	0.586
Family issues	3.21±1.026	3.41±0.856	3.33±0.927	0.379
Course content is not interesting	2.906±0.946	3.20±0.793	3.09±0.865	0.083
Course content is too intensive	3.0±0.919	3.20±0.761	3.13±0.829	0.313
Geographical distance	3.05±1.027	3.25±0.778	3.18±0.885	0.325
Difficult accessibility	2.98±1.047	3.27±0.831	3.16±0.929	0.117

3.4. Preferences for CE course topics and teaching methods

Pharmacists were also asked about their preference of CE topics and teaching methods. The results are shown in Table 4.

Table 4. *Preferences for CE course topics and teaching methods*

		n	Percentage (%)
Course topics	Medication counselling	79	70.5
	Pharmacology	59	52.7
	Communication skills in GPP	57	51.0
	Computer skills in pharmacy management	46	41.1
	Pharmacy related legislation	37	33.0
Teaching methods	Online learning	44	39.6
	Traditional learning	20	18.0
	Distance learning (through textbooks)	10	9.0
	Blended learning	43	38.8

Topics related to medication counselling was of highest interest to 70.5%, closely followed by pharmacology topics, communication skills in GPP (52.7% and 51.0%, respectively). Online learning was preferred by 39.6% of the respondents while blended learning was preferred by 38.8% of the respondents.

4. DISCUSSION

This study investigated pharmacists' perceptions regarding their CE needs (course topics, teaching methods) as well as factors (facilitators, barriers) that impact their selection of CE programs. The facilitator "get support from the drugstore owners" among participated group had a higher mean score ($p<0.05$) than other group. When 530 Scottish pharmacists were surveyed to express their views and attitudes towards CE, they too were motivated by workplace challenges and peer interaction, but also felt they lacked support (56%) and time (76%) to achieve their CE goals. Therefore, in order to increase the number of participants in short-term courses, we need to assure that drug owners can support their employees. In addition, other motivational factors such as "update knowledge", "improve the quality of work performance", or "meet with colleagues" are also the main factors with average scores from 3.6-3.8. This result was quite similar to the results from other studies in the United States, Flanders.

Time factor is the biggest obstacle for drug retailers to attend continuous training courses (the highest average is 3.5). "Lack of time" was also mentioned among pharmacists in Scotland (76%). Topics related to medication counselling was of highest interest (70.5%). Other skill required for training is pharmacology (52.7%). The results of this study are similar to some previous studies conducted in Quatar, Egypt and Galicia. Two kinds of training methods that pharmacists choose to participate in are online training (39.6%) and combined online and traditional training (38.8%). This result is similar to the results of the study among the health

workers in Bach Mai Hospital, the high proportion of combined forms (76%) or in Egypt and Galicia.

5. CONCLUSIONS

The primary purpose of this study was to identify facilitators and barriers among pharmacists to participate in pharmacy CE programs as well as perceptions regarding CE needs. The top-ranked facilitators to continuing education are “keeping up to date with the information” and “meeting job requirements”. The top-ranked barriers to continuous learning is related to the time of pharmacists. The results of this study might be used to design more engaging programs and provide CE to pharmacists in a way that is more convenient and interesting for them.

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QSAR studies of chromone derivatives as cyclooxygenase-2 inhibitors

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Abstract

Introduction: In our recent study, our chromones which were varied substituents at position 2, 3, 5, 6,7 and 8 exhibited very potent in COX-2 inhibition. Subsequently, their structure should be scrutinized rationally for developing higher potent COX-2 inhibitors. **Objectives:** The goal of our study was to develop an ideal QSAR model for extracting necessary chromone features that required for understanding and designing more active COX-2 inhibitors. **Materials and methods:** Our chromones were drawn and generated 2D features using padel and 3D features (comparative molecular field analysis (comfa) and comparative molecular similarity indices (comsia)) using SYBYL-X2.0. Then the data set were splitting into training-test set using sorting activity, hierarchical and k-means clustering methods. The 2D descriptors selected by stepwise and genetic algorithm (GA) were used to develop multilinear regression (MLR) models while partial least square used to develop 3D QSAR models. The models should be evaluated by correlation coefficient (R^2 >0.6), leave-one-out cross validation (Q^2_{LOO}>0.6), and external validation (r^2_{pred}>0.5). **Results:** Our chromones were clustered into eight training-test sets from three clustering methods and their 2D and 3D QSAR models provided R^2>0.7, Q^2_{LOO} = 0.334-0.865, and r^2_{pred} = -9.720-0.546. Most satisfactory 2D model was developed from the training-test set from hierarchical method (structure) and descriptors selected by GA (R^2= 0.853, Q^2_{LOO} = 0.797, r^2_{pred} = 0.532). The model revealed that solvation, lipophilic, electronic and neighborhood symmetry were necessary features of chromones for COX-2 inhibition. While most acceptable comfa (R^2 = 0.918, Q^2_{LOO} = 0.443, r^2_{pred} = 0.547) and comsia (R^2 = 0.831, Q^2_{LOO} = 0.465, r^2_{pred} = 0.470) were generated from the training-test sets from k-means (activity) and hierarchical methods (structure), respectively. According comfa and comsia contour plots, it revealed that a less steric and positive group needed to replace at R_2 while more steric and negative group required to substitute at R_3. Moreover, R_6 and R_7 substituted with hydroxy groups exhibited higher potency than those non-substituents. **Conclusion:** Although those generated models were low robustness and predictivity, useful information of chromone derivatives for designing more potent COX-2 inhibitors were revealed.

Keywords: QSAR, CoMFA, CoMSIA, chromone, cyclooxygenase-2

1. INTRODUCTION

Cyclooxygenases (COXs) are endogenous enzymes which play the key roles in prostaglandins biosynthesis and inflammatory pathway (1). The most interesting COX isoforms are COX-1 whose functions involve the maintenance of physiological homeostasis and COX-2 which induces the increase of inflammation process (2). Nonsteroidal anti-inflammatory drugs (NSAIDs) competitively inhibit COXs and relieve inflammation and pain. Conventional NSAIDs, i.e., aspirin, ibuprofen and naproxen have some side effects such as nephrotoxicity and gastric ulcers. It was found that the binding affinity of conventional NSAIDs with COX-1 is greater than COX-2 (3-5). To reduce these adverse effects, new inhibitors with more selective COX-2 inhibitory activity have been thoroughly studied. Consequently, numerous selective COX-2 inhibitors have been marketed such as celecoxib, valdecoxib and etoricoxib. Unfortunately, selective COX-2 inhibitors are associated with serious cardiovascular adverse effects (6-8). Therefore, new and higher selective COX-2 inhibitors must be developed.

Quantitative structure-activity relationship (QSAR) is useful to study the relationship between physicochemical parameters of the interested molecules and their biological activity. An ideal QSAR model is generated from careful steps, i.e., data set selection, descriptor generation and selection, developing model and validation, to produce high accuracy model for predicting new hypothetical molecules (9).

In our recent study, a series of chromone derivatives have been evaluated for potential COX-2 inhibitors and it was found that these compounds showed potent inhibitory activity. In this study, two dimensional (2D) QSAR and 3D QSARs, i.e., comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) were performed. Three training-test set selection methods (sorting activity, hierarchical, and κ-means clustering), and 2 descriptor-selection methods (stepwise and genetic algorithm) were used to evaluate the influences of data and descriptors selection methods in 2D QSAR study using multiple linear regression (MLR) analysis. The training-test set obtained from those 3 data selection methods were used to develop CoMFA and CoMSIA models. The developed QSAR models will provide

important information about necessary features of the chromone compounds for higher COX-2 inhibitory activity and for further designs of more potent inhibitors.

2. MATERIALS AND METHODS

The data set consists of 42 chromone derivatives whose inhibitory activity have been evaluated using COX-2 (human) inhibitor screening assay kit (Cayman Chemical). The percentage inhibition collected from our previous study (15) are presented in Table 1. Compounds **17**, **22**, **25**, **42**, **45** and **49** were outliers and excluded from the studies, therefore only 36 compounds were used as data set.

The 2D structures of the chromone derivatives were drawn with SYBYL-X 2.0 software and the molecular descriptors were calculated using PaDEL software (16). The descriptors with very low variance and correlation between descriptors higher than 0.9 were omitted.

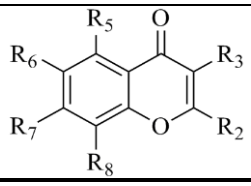
In this study, training set (80% of the data set) and test set (20% of the data set) were selected using sorting activity, hierarchical clustering and κ-means clustering methods. In sorting activity technique, the data set was arranged according to their COX-2 inhibitory activity in ascending manner. The member of test set was assigned for every 5 compounds ascendingly and the rest of the compounds were assigned as training set. The Hierarchical clustering method analyzed similarities of the data and the result was shown in 2D diagrams so called dendrograms. Thirty-six chromone derivatives were clustered hierarchically using statistical package for the social sciences (SPSS) program based on their biological activity and 3D structure properties (i.e., CoMFA descriptors representing electrostatic and steric properties of the molecules) individually, and based on both biological activity and 3D properties. The members of the test sets which provided the best model were selected from those clusters and the rest were assigned as the training sets. In κ-means clustering, a data set was clustered into a certain number of clusters. The number of clusters of the data set was analyzed randomly using the same parameters as in hierarchical clustering until an optimal number of cluster was reached. The members of training and test sets were assigned in the same manner as hierarchical clustering method.

The descriptors selection methods, stepwise (S) and genetic algorithm (GA) were used to develop the MLR models using DTC LAB software (17). Based on the selected descriptors by S and GA, MLR analysis were performed on the training sets and evaluated on the test sets. For evaluation of the QSAR models, internal and external validation were performed. The qualities of the models were justified by the correlation coefficients of the training set (R^2 >0.6), leave-one-out cross validation (Q^2_{LOO} > 0.6), external validation (R^2_{pred} > 0.5), standard deviation of error of prediction (SDEP), standard deviation of error of estimate (SDEE), and fitness score (*F*-value) (5).

CoMFA and CoMSIA studies were performed using Sybyl-X 2.0. Because there is no X-ray crystallographic data of the studied chromones, compound **48** which exhibited the highest COX-2 inhibitory activity (IC_{50} = 3.3 μM) was used as the template molecule in fit atom alignment. CoMFA descriptors of all aligned compounds in the data set were generated under default setting. For both steric and electrostatic interactions, the energy cutoff value was set at 30 kcal/mole. CoMSIA descriptors were generated using steric, electrostatic, hydrophobic, hydrogen bond donor and hydrogen bond acceptor properties. The attenuation factor was set at 0.3.

CoMFA and CoMSIA descriptors were analyzed statistically using partial least squares (PLS) analysis. Each PLS model was cross-validated using leave-one-out procedure, and column filtering was set at 2.0 kcal/mole. The result of optimal principle component was used for non-cross-validated PLS analysis. The quality of the 3D QSAR models was evaluated using statistical measurements. The acceptable qualities of models should show R^2> 0.6, Q^2_{LOO} > 0.6, and R^2_{pred} > 0.5. The results of best CoMFA and CoMSIA models were visualized as 3D contour plots and their correlation between actual (experimental) and predicted (calculated) biological activities were investigated (data not shown).

Table 1. *Structures and COX-2 inhibitory activity of chromone derivatives*



No.	R_2	R_3	R_5	R_6	R_7	R_8	% of COX-2 Inhibition ±SE (at 30 µM)	IC_{50} (µM)
1	Phenyl	H	H	H	H	OH	45.06±1.80	-
3	Benzyl	H	H	H	OH	OH	49.01±4.04	-
4	Phenyl	H	H	H	OH	OH	53.18±3.96	-
5	CH3	H	H	H	OH	H	60.90±1.65	-
11	3'-(CF_3)-Phenyl	H	H	H	OH	H	74.69±0.53	-
12	4'-(F)-Phenyl	H	H	H	OH	H	74.59±2.34	-
13	3', 5'-(diNO_2)-Phenyl	H	H	H	OH	H	49.55±2.12	-
14	3'-(Cl)-Phenyl	H	H	H	OH	H	64.82±1.81	-
15	3', 4'-(diCl)-Phenyl	H	H	H	OH	H	60.96±1.87	-
16	4'-(t-butyl)-Phenyl	H	H	H	OH	H	56.07±0.61	-
17	3'-(CF_3)-Phenyl	H	OH	H	OH	H	25.55±4.26	-
18	4'-(F)-Phenyl	H	OH	H	OH	H	51.36±4.26	-
19	3', 4'-(diF)-Phenyl	H	OH	H	OH	H	50.66±4.26	-
20	4'-(t-butyl)-Phenyl	H	OH	H	OH	H	54.70±3.51	-
22	3', 5'-(diNO_2)-Phenyl	H	OH	H	OH	H	75.14±1.73	-
23	3'-(Cl)-Phenyl	H	OH	H	OH	H	61.23±4.07	-
24	3', 4'-(diCl)-Phenyl	H	OH	H	OH	H	45.75±2.14	-
25	4'-(OCH_3)-Phenyl	H	OH	H	OH	H	82.92±2.15	-
26	3'-(OCH_3)-Phenyl	H	OH	H	OH	H	47.78±1.15	-
27	3'-(OCH_3)-Phenyl	H	H	OH	H	H	55.91±2.54	-
28	3'-(Cl)-Phenyl	H	H	OH	H	H	61.56±1.57	-
29	4'-(F)-Phenyl	H	H	OH	H	H	85.98±0.68	-
31	4'-(t-butyl)-Phenyl	H	H	OH	H	H	89.59±0.40	-
32	3'-(CF_3)-Phenyl	3'-(CF_3)-Benzoyl	H	H	OH	OH	76.82±0.84	-
33	3'-(Cl)-Phenyl	3'-(Cl)-Benzoyl	H	H	OH	OH	55.64±1.82	-
34	3'-(OCH_3)-Phenyl	3'-(OCH_3)-Benzoyl	H	H	OH	OH	89.14±1.35	-
35	4'-(F)-Phenyl	4'-(F)- Benzoyl	H	H	OH	OH	93.50±0.81	7.46
36	4'-(NO_2)-Phenyl	4'-(NO_2)- Benzoyl	H	H	OH	OH	44.53±5.82	-
37	4'-(OCH_3)-Phenyl	4'-(OCH_3)- Benzoyl	H	H	OH	OH	56.48±1.35	-
38	3', 4'-(diF)-Phenyl	3', 4'-(diF)- Benzoyl	H	H	OH	H	92.75±1.88	-
39	3'-(CF_3)-Phenyl	3'-(CF_3)- Benzoyl	H	H	OH	H	95.47±0.75	7.36
40	3'-(Cl)-Phenyl	3'-(Cl)- Benzoyl	H	H	OH	H	74.96±0.87	-
41	3'-(OCH_3)-Phenyl	3'-(OCH_3)- Benzoyl	H	H	OH	H	83.00±0.67	6.86
42	4'-(F)-Phenyl	4'-(F)- Benzoyl	H	H	OH	H	54.37±2.29	-
43	4'-(NO_2)-Phenyl	4'-(NO_2)- Benzoyl	H	H	OH	H	59.20±0.40	-
44	4'-(OCH_3)-Phenyl	4'-(OCH_3)- Benzoyl	H	H	OH	H	70.35±0.76	-
45	4'-(t-butyl)-Phenyl	4'-(t-butyl)- Benzoyl	H	H	OH	H	52.73±1.26	-
46	3'-(OCH_3)-Phenyl	3'-(OCH_3)- Benzoyl	OH	H	OH	H	52.80±1.05	-
47	4'-(NO_2)-Phenyl	4'-(NO_2)- Benzoyl	OH	H	OH	H	45.95±2.16	-
48	4'-(t-butyl)-Phenyl	4'-(t-butyl)- Benzoyl	H	OH	H	H	93.52±3.16	3.30
49	3'-(OCH_3)-Phenyl	H	H	OH	OH	H	97.51±0.28	12.97
50	3'-(OCH_3)-Phenyl	3'-(OCH_3)- Benzoyl	H	OH	OH	H	82.70±0.63	-

*SE = Standard error

3. RESULTS AND DISCUSSIONS

For 2D QSAR study, the MLR equations derived from 3 training-test sets selection methods, i.e., sorting activity, hierarchical and k-means clustering, and the 2D descriptors from S and GA descriptor selection methods provided high correlation (R^2>0.7), good robustness (Q^2_{LOO}>0.4) but low predictivity (R^2_{pred}<0.5). The best model was generated from training-test set selected by hierarchical clustering method, based on 3D structure properties, and from 2D descriptors selected by GA. The best QSAR equation was shown in the following equation:

%COX-2 Inhibition = -251.621(±62.83) - 110.623(±33.67) VE1_Dzp + 53.448(±7.25) MLFER_BH + 38.232(±7.46) minssO + 7.642(±2.73) AATSC8s + 61.604(±30.93) SIC2 + 1.004(±0.32) minsF

Number of compounds in training set = 29, R^2 = 0.853, SDEE = 7.267, *F* = 21.242, Q^2_{LOO} = 0.797, SDEP = 7.438, R^2_{pred} = 0.532

where VE1_Dzp = coefficient sum of the last eigenvector from Barysz matrix weighted by polarizabilities (18); MLFER_BH = overall or summation solute hydrogen bond basicity (19); minssO = minimum atom-type electrotopological state (E-state) for oxygen atom (20); AATSC8s = average centered Broto-Moreau autocorrelation lag 8 weighted by intrinsic-state (21); SIC2 = structural information content index (neighborhood symmetry of second order) (22); minsF = minimum atom-type E-state for fluorine atom (20).

The best model exhibited high correlation (R^2 = 0.853) between biological activity and the 2D descriptors, i.e., VE1_Dzp, MLFER_BH, minssO, AATSC8s, SIC2 and minsF. The low standard error of estimate (SDEE = 7.267) indicated the accuracy of the model. In leave-one-out cross-validation procedure, good cross-validated R^2 (Q^2_{LOO} = 0.797) and low standard error of prediction (SDEP = 7.438) demonstrated good internal predictivity of the model. Moreover, in external validation, the acceptable external validated (R^2_{pred} = 0.532) showed the predictive power of the model. The 6 descriptors used in the study were investigated for multi-colinearity and they illustrated low internal correlation (correlation coefficient < 0.65 and variation inflation factors < 5). According to the obtained MLR equation, increasing the hydrogen bond basicity (MLFER_BH), atom-type electrotopological state (minssO and minsF), autocorrelation

descriptor deriving structure properties of a molecule (AATSC8s) and second order neighborhood symmetry (SIC2) enhanced COX-2 inhibitory activity while increasing the polarization property (VE1_Dzp) decreased the activity.

In 3D QSAR study, the statistical results of CoMFA and CoMSIA using different sorting methods are summarized in Table 2. All CoMFA models exhibited high correlation ($R^2 > 0.89$), acceptable robustness (Q^2_{LOO} = 0.443-0.524), and low predictivity ($R^2_{pred} < 0.6$). The best CoMFA model was generated from the training-test set from κ-means clustering method based on activity with $R^2 = 0.918$, $Q^2_{LOO} = 0.443$ and $R^2_{pred} = 0.547$. The contributions of the steric and electrostatic fields were 0.509 and 0.491, respectively. The QSAR results produced by CoMFA and CoMSIA models are usually represented as 3D coefficient contour maps (Figure 1) and these contour maps are useful for exploring SARs. The molecular structure of compound **48** was displayed inside the field as the reference structure. In steric contour map, the green areas represent the regions of favorable steric substituents, and yellow areas represent regions of disfavorable steric substituents. As shown in Figure 1A, the steric group was preferred at C-3. This steric contour map was in good correlation with the experimental activity that most of the compounds bearing substituent at C-3, i.e., compounds **34**, **35**, **38-41**, **48** and **50** exhibited COX-2 inhibitory activity higher than 74% inhibition.

The electrostatic contour map shown in Figure 1B indicated that electronegative substituent (the red contours) should be located around C-6 of ring A, C-2'' of ring D, and C-4' of ring B. The electropositive substituent (the blue contours) should be positioned between C-7 and C-8 of ring A, and between C-2' and C-3' of ring B.

As shown in Table 2, the statistical qualities of CoMSIA ($R^2 > 0.8$, Q^2_{LOO} = 0.334-0.473, $R^2_{pred} <$ 0.6) of the models gave similar results compared to those of CoMFA. The best CoMSIA model was developed from the training-test set from hierarchical sorting based on 3D structure properties with $R^2 = 0.831$, $Q^2_{LOO} = 0.465$ and $R^2_{pred} = 0.470$. The relative contributions of steric, electrostatic, and H-bond acceptor fields were 0.125, 0.535, and 0.340, respectively. The steric and electrostatic contour maps (Figures 1C and 1D) were corresponding to those of

CoMFA. The H-bond acceptor fields gave more contribution (34.0 %) to the QSAR equation than the steric fields. The CoMSIA hydrogen bond acceptor contour map (Figure 1E), magenta and red contours showed regions where H-bond acceptor group favored and disfavored, respectively. These H-bond acceptor contours indicated that H-bond acceptor group should be located around benzoyl group at C-3 (ring C) and at C-6 of ring A. Figure 2 summarizes the structural features of the chromone compounds which are important for enhancing the COX-2 inhibitory activity.

Table 2. *The statistical results of 3D QSAR models*

3D QSAR model	Selection Method in training-test set	Based on	R^2	SDEE	PC	Q^2_{LOO}	SDEP	R^2_{pred}	F-value	Electrostatic	Steric	Hydrophobic	H-bond acceptor	H-bond donor
CoMFA														
	Sorting Activity	Activity	0.944	4.446	6	0.471	13.599	0.273	61.246	0.462	0.538			
	Hierarchical	Activity	0.893	5.950	5	0.486	13.034	0.513	38.364	0.476	0.524			
		3D Structure	0.909	5.728	6	0.462	13.889	0.471	36.435	0.491	0.509			
		Activity&3D Structure	0.924	5.066	5	0.482	13.250	0.340	55.798	0.483	0.517			
	κ-Means	Activity	0.918	5.344	6	0.443	13.909	0.547	40.958	0.491	0.509			
		3D Structure	0.903	5.787	5	0.524	12.843	0.402	42.994	0.503	0.497			
		Activity&3D Structure	0.952	3.997	6	0.482	13.071	0.383	72.089	0.511	0.489			
CoMSIA														
	Sorting Activity	Activity	0.840	7.029	3	0.473	12.744	0.206	43.608	1.000				
	Hierarchical	Activity	0.800	7.800	3	0.469	12.712	0.461	33.333	1.000				
		3D Structure	0.831	7.447	4	0.465	13.259	0.470	29.587	0.535	0.125		0.340	
		Activity&3D Structure	0.832	7.375	4	0.431	13.549	0.414	29.619	0.536	0.112		0.352	
	κ-Means	Activity	0.764	8.338	2	0.415	13.120	0.170	42.003	0.521	0.147			0.332
		3D Structure	0.805	7.880	3	0.407	13.754	0.407	34.449	1.000				
		Activity&3D Structure	0.818	7.278	3	0.334	13.903	0.553	37.350	1.000				

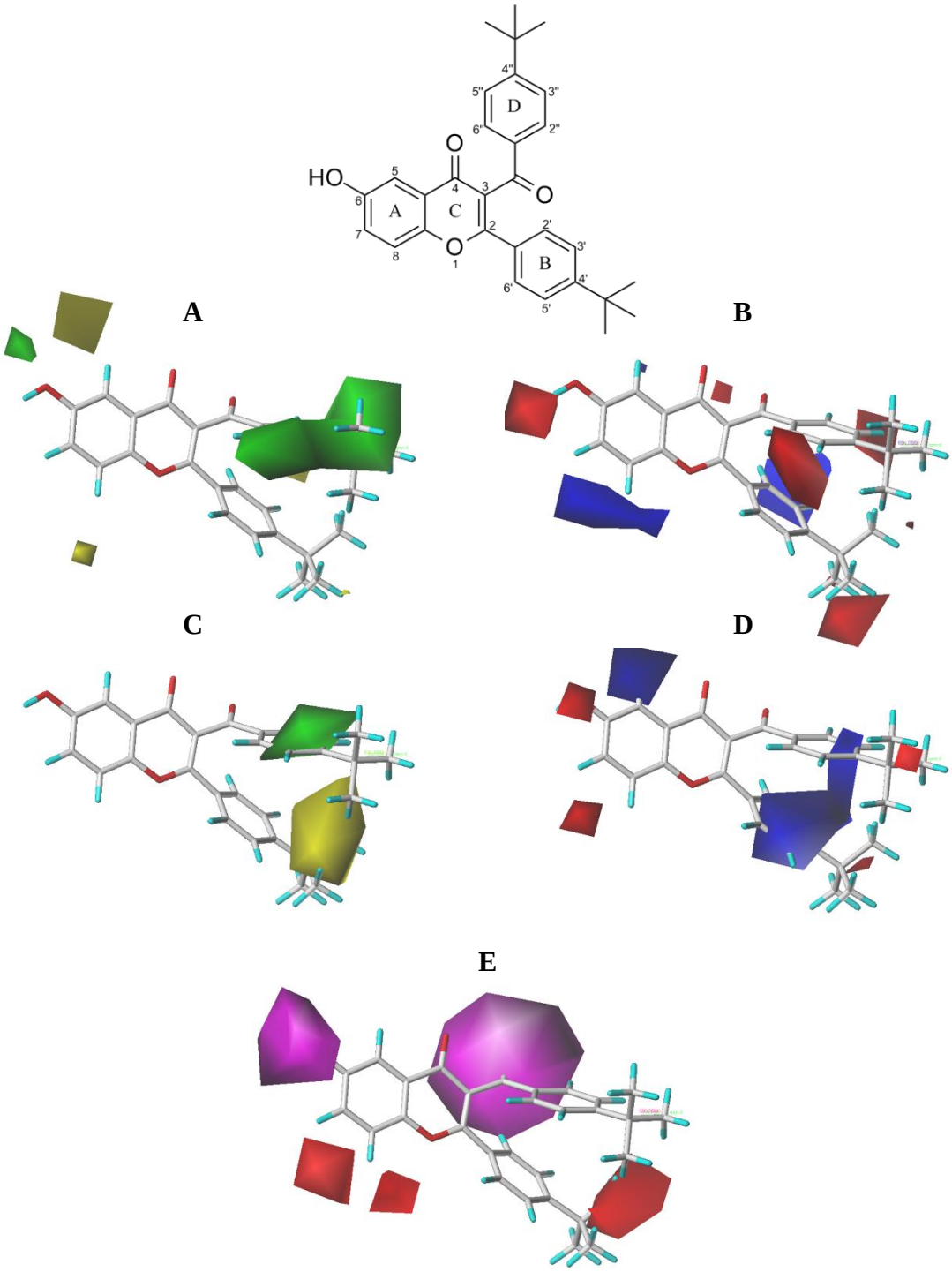


Figure 1. *CoMFA steric contour map (A) and electrostatic contour map (B). The green contour refers to sterically favored region; the yellow contours indicate disfavored areas. The blue contour indicates region where electropositive substituent is favored and red contour refers to region where electronegative substituent is favored. CoMSIA steric contour map (C),*

electrostatic contour map (D). CoMSIA H-bond acceptor contour map (E), magenta and red contours indicate regions where hydrogen bond acceptor group favored and disfavored, respectively.

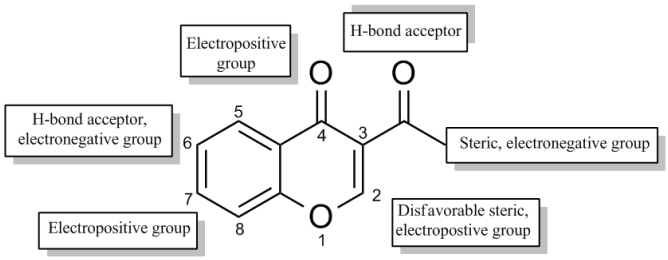


Figure 2. *Summary of the structural features important for increasing COX-2 inhibitory activity*

4. CONCLUSION

In this study, the 2D QSAR and 3D-QSAR have been applied to a set of novel chromone series which have been evaluated for their COX-2 inhibitory activity. The best QSAR model using MLR analysis was obtained from training-test set selected by hierarchical clustering method based on 3D structure properties and from 2D descriptors selected by GA. This model exhibited high correlation, R^2 = 0.853, good cross-validated R_2 (Q^2_{LOO} = 0.797), and acceptable external validated (R^2_{pred} = 0.532). The obtained model indicated that the COX-2 inhibitory activity of the studied compounds mainly depended on electronic properties and hydrogen bonding property of the molecules. The 3D QSAR, CoMFA and CoMSIA gave the statistical parameters which showed that the established CoMFA and CoMSIA models were reliable. The best CoMFA model showed R^2 = 0.918, Q^2_{LOO} = 0.443 and R^2_{pred} = 0.547 and the best CoMSIA model provided R^2 = 0.831, Q^2_{LOO} = 0.465 and R^2_{pred} = 0.470. The CoMFA and CoMSIA contour maps provided the relationships between structural features and inhibitory activity. Consequently, this study also gave meaningful structural insights into possible modifications of chromone derivatives which could improve the COX-2 inhibitory activity.

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Application of Mean Centering of Ratio Spectra Based Spectrophotometric Method for Quantitative Determination of Acetaminophen and Chlorzoxazole

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Abstract

The aim of this study was to develop a mean centering of ratio spectra based spectrophotometric method for determination of acetaminophen and chlozoxazole in combination. Two sets of standard solutions, calibration set and test set were prepared. Calibration set composed of 12 synthetic mixtures with vary concentrations of acetaminophen (1-10 µg/mL) and chlorzoxazole (1-10 µg/mL). The concentrations of calibration set were selected by mean of central composite design. The absorbance data of calibration set samples were collected and used to construct the mean centering of ratio spectra models for the two drugs. The MCR models were constructed with Microsoft® Excel. The resulted models were tested by determination of the test set samples which were not part of the models building. The plots of the second ratio spectra (y-axis) and concentrations (x-axis) show the maximum amplitude at 246 nm for acetaminophen and 287 nm for chlorzoxazole. The good linear relationship was proved with the correlation coefficient (r) values higher than 0.99 for acetaminophen and chlorzoxazole. The accuracy of the models was tested by determination the test set samples. Accuracy results, expressed in term of % recovery between the determination values and the true values were 98.3% ± 2.3% (mean ± % RSD, n = 7) and 99.5% ± 6.3% (mean ± % RSD, n = 7) for acetaminophen and chlorzoxazole, respectively. The results were implied to the accuracy, precision and reliable of the invented method.

Keyword: Acetaminophen, chlorzoxazole, mean centering of ratio spectra

1. INTRODUCTION

Acetaminophen (paracetamol, ACP) is widely used in normal and deep pain and chlorzoxazone (5-chloro-2- hydroxybenzoxazole, CZX) is a muscle relaxant which interacts to central nervous system. Combination of ACP and CZX has been commercial available in tablets dosage form. Determination of these drugs in tablet, according to the United States Pharmacopoeia (USP) 40 and other studies, were performed by HPLC method. Analysis of ACP and CZX in combinations could not be achieved by direct UV spectrophotometer without chromatographic separation since the overlapping of their UV spectra. Application of mean centering of ratio spectra (MCR) based spectrophotometric approach may overcome this limitation.

2. MATERIALS AND METHODS

UV spectrophotometry was carried out by a double-beam UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a pair of 1 cm quartz cells. The spectral bandwidth was 1 nm over the wavelengths range 200-400 nm with medium speed scanning. A 50% methanol was used as a blank. Two sets of standard solutions, calibration set and test set were prepared. Calibration set composed of 12 synthetic mixtures with vary concentrations of ACP (1-10 µg/mL) and CZX (1-10 µg/mL). The concentrations of calibration set were selected by mean of central composite design (CCD). The absorbance data of calibration set samples were collected and used to construct the MCR models for ACP and CZX. The MCR models were constructed with Microsoft® Excel. The resulted models were tested by determination of the test set samples which were not part in models building. The composition of calibration set and test set are showed in Table 1. The principle of MCR, described by Afkhami and Bahram, was used to overcome the spectra overlapping of ACP and CZX in UV region. Mathematical explanation of MCR method was described as the following equations.

$$A_m = a_{ACP}\, bc_{ACP} + a_{CZX}\, bc_{CZX} \dots\dots\dots\dots\dots\dots (1)$$

$$B = \frac{A_m}{a_{CZX}} = \frac{a_{ACP}\, c_{ACP}}{a_{CZX}} + c_{CZX} \dots\dots\dots\dots\dots\dots . (2)$$

$$MC(B) = MC\left[\frac{a_{ACP}\, c_{ACP}}{a_{CZX}}\right] + 0 \dots\dots\dots\dots\dots\dots . . (3)$$

Where "A_m" is the absorbance at a certain wavelength, "a" is absorptivity coefficient, "b" is path length and equal 1 for a 1 cm quartz cells and "c" is concentration.

The plot of mean centering of the second ratio spectra (y-axis) and wavelength (x-axis) will show the maximum amplitude at a certain wavelength. Eventually, the calibration curve of each drug was plotted between the amplitude at such wavelength (y-axis) and concentration (x-axis).

3. RESULTS AND DISCUSSION

The plots of the second ratio spectra (y-axis) and wavelength (x-axis) show the maximum amplitude at 246 nm for ACP and 287 nm for CZX (Figure 1). The good linear relationship were proved with the correlation coefficient (r) values higher than 0.99 for ACP and CZX (Figure 2). The accuracy of the models was tested by determination the test set samples. Accuracy results, expressed in term of % recovery between the determination values and the true values were 98.3% ± 2.3% (mean ± % RSD, n = 7) and 99.5% ± 6.3% (mean ± % RSD, n = 7) for ACP and CZX, respectively. The determination results of test set sample were showed in Table 2.

4. CONCLUSION

The developed MCR method had ability to determine ACP and CZX by using UV-absorbance data. The method was simple, accurate and precise. The other advantages of this method could be listed including only spectra data were required, data analysis could be performed by Microsoft® Excel that was widely used, high samples throughput and less time consuming.

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Table 1. *Composition of calibration set (C) and test set (T) samples*

Sample	Concentration (ug/mL)	
	ACP	CZX
C1	0.00	8.00
C2	19.20	8.00
C3	9.60	0.00
C4	9.60	16.00
C5	2.80	2.34
C6	2.80	13.60
C7	16.40	13.60
C8	16.40	2.34
C9	9.60	8.00
C10	9.60	8.00
C11	9.60	8.00
C12	9.60	8.00
T1	12.00	10.00
T2	9.60	8.00
T3	6.00	5.00
T4	14.00	7.00
T5	3.00	16.00
T6	5.00	14.00
T7	8.00	12.00

Table 2. *Determination results of the test set samples*

ACP			CZX		
Added (µg/mL)	Found (µg/mL)	%Recovery	Added (µg/mL)	Found (µg/mL)	%Recovery
12.00	12.29	97.63	10.00	9.49	94.86
9.60	9.62	99.84	8.00	8.83	110.37
6.00	6.02	99.59	5.00	5.01	100.17
14.00	13.91	100.66	7.00	6.40	91.38
3.00	3.18	94.44	16.00	15.70	98.10
5.00	5.19	96.37	14.00	13.61	97.19
8.00	8.06	99.23	12.00	12.50	104.16
Mean		98.25	Mean		99.46
SD		2.22	SD		6.26
% RSD		2.26	% RSD		6.29

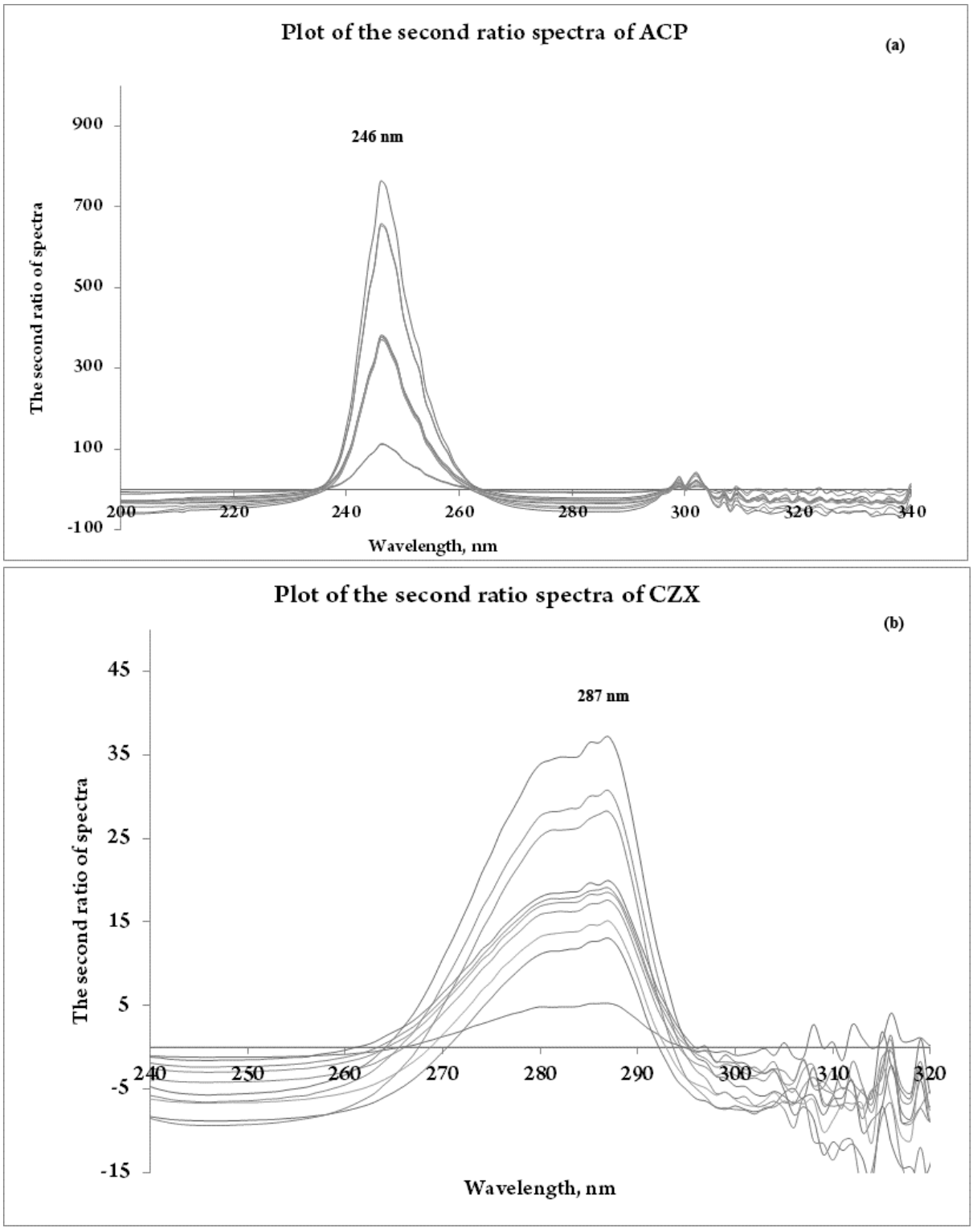

Figure 1. *The plots of the second ratio spectra (y-axis) and wavelength (x-axis) of (a) ACP and (b) CZX*

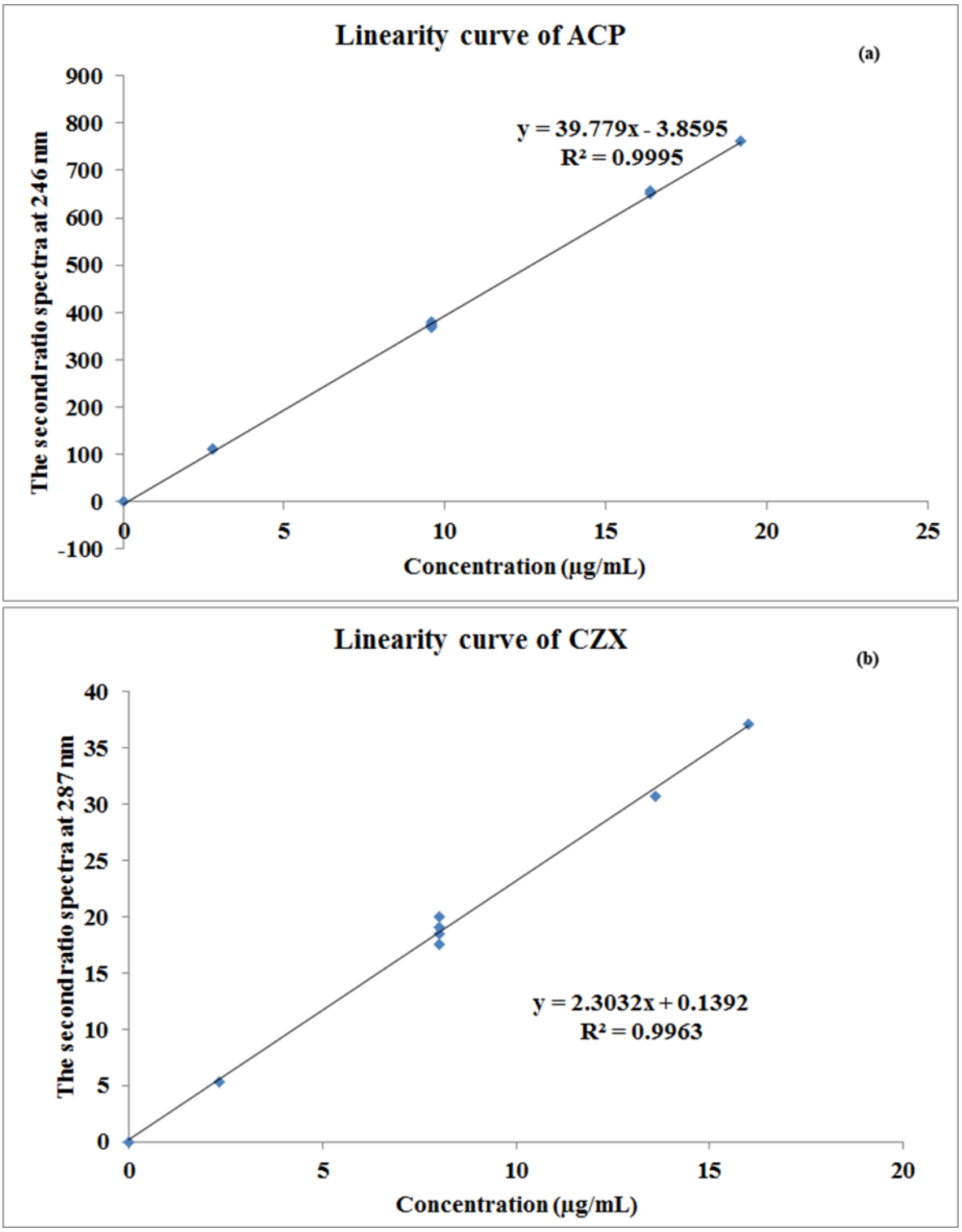

Figure 2. *Linearity curves of (a) ACP and (b) CZX*

Synthesis of 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate, and ethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate as Fenofibrate Impurities

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Abstract

Introduction: Fenofibrate impurity B (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid), fenofibrate impurity D (methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoate), and fenofibrate impurity E (ethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoate) are three of the related compounds of fenofibrate. These impurities arise from the synthesis and/or the degradation of fenofibrate during storage. The presence of these impurities may affect the treatment efficacy and safety of fenofibrate. BP 2017, and USP 39 require conducting the impurities test in fenofibrate pharmaceutical substances as well as corresponding finished products. Additionally, three mentioned impurities reference standards are costly with limited accessibility. **Objectives:** The aim of this study was to synthesize fenofibrate impurities B, D and E. **Materials and Methods:** Fenofibrate impurity B (fenofibric acid) was synthesized by the alkaline hydrolysis of fenofibrate under reflux in 2 hours. The reaction mixture was then acidified and the precipitated solid product was filtered. Fenofibrate impurity D and E was synthesized by acid-catalyzed esterification between fenofibric acid (impurity B) and methanol/ethanol, respectively. The reaction mixture was neutralized and excess alcohol was evaporated under reduced pressure. By adding cold water, the product was precipitated and then filtered. Then, these crude synthesized products were furthermore purified by precipitation and recrystallization. The structure of synthesized compounds was elucidated by their UV, MS, and NMR data. Finally, the purity of these impurities was determined by HPLC based on peak area normalization method. **Results and Discussion:** Three impurities B, D and E were synthesized with 74.5%, 84.4% and 61.5% yield, respectively. By using HPLC-PDA, their chromatographic purity was determined as over 99%. **Conclusion**: Fenofibrate impurities B, D and E were successfully synthesized by the proposed methods, which are simple and cost effective. These impurities could be used as reference standards for impurity testing in pharmaceuticals.

Keywords: Fenofibrate, fenofibrate impurity B, fenofibric acid, fenofibrate impurity D, fenofibrate impurity E.

1. INTRODUCTION

Fenofibrate is used to treat high cholesterol and high triglyceride levels. Fenofibric acid, the active metabolite of fenofibrate, reduces cholesterol and triglycerides in the blood by the activation of peroxisome proliferator activated receptor α (PPARα). Impurity removal is a critical and important task in pharmaceutical process research, where the final product meets stringent purity requirements. The presence of impurities in an active pharmaceutical ingredient (API) can have a significant impact on the quality and safety of the drug product. These impurities are required in pure form to understand the impurity profile and development of an accurate analytical method during the research and development phase.

Fenofibrate impurities B (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid), D (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate) and E (ethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate) are three related compounds of fenofibrate, arising from the synthesis of fenofibrate and/or during drug storage. The presence of these impurities may influence the treatment efficacy and safety of fenofibrate. BP 2017[1] and USP 39[2] require conducting the impurity test in fenofibrate pharmaceutical substance as well as corresponding finished products. Additionally, three mentioned impurity reference standards are costly with limited accessibility. There have been several published studies on chemical synthesis of fenofibrate impurities so far[3,4]. Herein, we report the impurity profile for fenofibrate, including detailed experimental procedures and characterization of these synthesized impurities.

2. MATERIALS AND METHODS

2.1 Chemicals, solvents and standards

Fenofibrate (100.11% purity) was supplied by Jiangsu Nhwa/China. Sodium hydroxide, ethanol and sulfuric acid were of analytical grade. Other employed reagents and solvents were of commercial grade. TLC has performed on Kieselgel 60 F_{254} silica-coated aluminum plates (Merck) and visualized by UV light ($\lambda = 254$ nm).

2.2 Equipment

The melting points were determined in an open capillary using a Sanyo GallenKamp melting point instrument. The IR spectrum was obtained on a Shimadzu IRAffinity-1S instrument (powder for solids). The chromatogram was recorded from HPLC Waters Alliance, e2695 XE series. Mass spectrum was recorded with an Agilent 1200 series. NMR spectrum was recorded on Bruker Advanced II 500 MHz (^{1}H-NMR and ^{13}C-NMR).

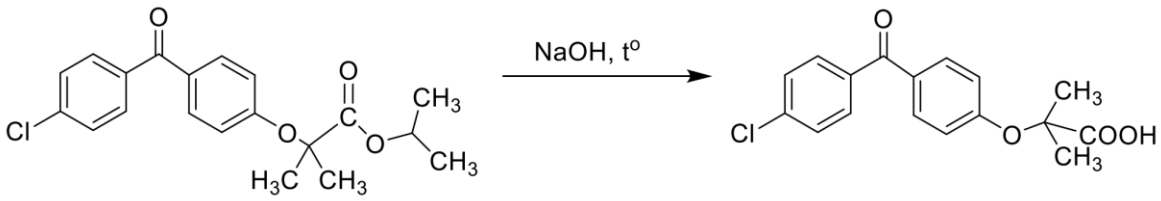
2.3 Methods

Fenofibrate impurity B (fenofibric acid) was synthesized by the alkaline hydrolysis of fenofibrate under reflux in 2 hours. The reaction mixture was then acidified and the precipitated solid product was filtered. Fenofibrate impurity D and E was synthesized by acid-catalyzed esterification between fenofibric acid (impurity B) and methanol/ethanol, respectively. The reaction mixture was neutralized and excess alcohol was evaporated under reduced pressure. By adding cold water, the product was precipitated and then filtered. Then, these crude synthesized products were furthermore purified by precipitation and recrystallization. The structure of synthesized compounds was elucidated by their UV, MS, and NMR data. Finally, the purity of these impurities was determined by HPLC on the basis of peak area normalization method.

3. RESULTS AND DISCUSSION

3.1 Preparation of impurity B

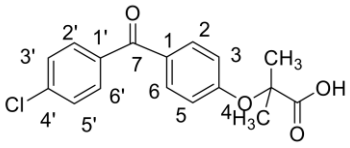
Fenofibrate (3.0 g, 0.0083 mole) was dissolved in ethanol (30 mL) in a round-bottomed flask, then sodium hydroxide (0.4 g, 0.009 mole) in water (2 mL) was added. The reaction mixture was then stirred under reflux at 84^{o}C for 2 hours, cooled to room temperature, concentrated under reduced pressure to remove excess ethanol and finally added to cold water (50 mL). The mixture was filtered through cellulose membrane. The filtrate was acidified with a solution of 10% hydrochloric acid to pH < 3. The precipitated solid product was filtered, then was washed with cold water until the filtrate was neutralized. The crude product was recrystallized in a 50% ethanol solution and dried in an air oven at 40^{o}C to constant weight (1.97 g, 74.5% yield).



Fenofibrate Fenofibric acid (impurity B)

Figure 1. *Synthesis of fenofibrate impurity B from fenofibrate*

The IR spectrum of the product (cm^{-1}) showed characteristic absorption peaks of functional groups, including C-H aromatic (1595.13), C=O ketone (1645.28), and C=O acid (1705.07). The positive ESI-MS of the product revealed a $[M+Na]^+$ at m/z = 341.05 that indicated the relative molecular mass similar to impurity B ($C_{17}H_{15}ClO_4$, M=318.8). The structure of product was elucidated by its ^{1}H-NMR and ^{13}C-NMR data: **^{1}H-NMR [(500 MHz, MeOD), δ_H (ppm)]:** 7.748 (*dt*; 4H; *J* = 9; 2.5; H_2, H_6, $H_{2'}$, $H_{6'}$), 7.547 (*dt*; 2H; *J* = 9; 2; H_3, H_5), 6.984 (*dt*; 2H; *J* = 9; 2; $H_{3'}$, $H_{5'}$), 1.680 (*s*; 6H; $2CH_3$). **^{13}C-NMR [(125 MHz, MeOD), δ_C (ppm)]:** 196.11 (C_7), 176.91 (COOH), 161.46 (C_4), 139.55 ($C_{4'}$), 137.86 ($C_{1'}$), 133.03 ($C_{2'}$, $C_{6'}$), 133.35 (C_2, C_6), 131.42 (C_1), 129.68 ($C_{3'}$, $C_{5'}$), 118.75 (C_3, C_5), 80.54 (qC), 25.79 ($2CH_3$). This product was confirmed as 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid (impurity B).



Impurity B (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid)

3.2 Preparation of impurity D and impurity E

Impurity D and impurity E were synthesized from impurity B.

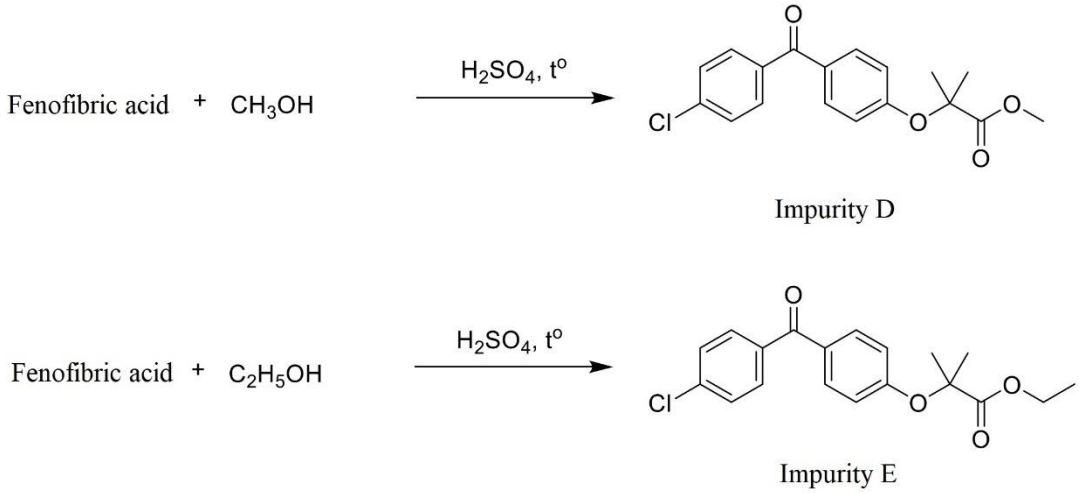


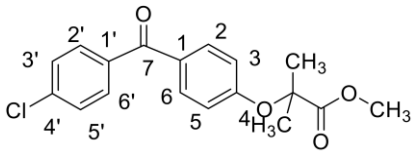
Figure 2. *Synthesis of fenofibrate impurities D and E from impurity B*

Fenofibric acid (impurity B) (1 g, 0.0031 mole) was dissolved in 20 ml of alcohol (methanol for impurity D and ethanol for impurity E), the solution was added concentrated sulfuric acid (1 mL) and stirred at 80^{o}C for 4 hours. After that, the reaction mixture was allowed to cool, the excessive acid was neutralized by a solution of concentrated sodium bicarbonate. Then 50 mL of water was added, filtered under reduced pressure to get a white solid product. The crude product was dissolved in ethyl acetate (20 mL) and extracted with saturated sodium bicarbonate solution (2 × 25 mL). The ethyl acetate layer was washed with 2 × 25 ml purified water, dried over anhydrous sodium sulfate, filtered, evaporated to dryness under vacuum, and dried in an air oven at 40^{o}C to constant weight to get white colored solid product D (0.88 g, 84.4% yield) or E (0.67 g, 61.5% yield).

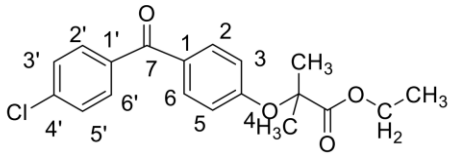
The IR spectrum of the product D (cm^{-1}) showed characteristic absorption peaks of functional groups, including C-H aromatic (1595.13), C=O ketone (1651.07), and C=O ester (1751.36). The positive ESI-MS of the product revealed an $[M+H]^+$ at m/z = 333.18 that indicated the relative molecular mass similar to impurity D ($C_{18}H_{17}ClO_4$, M=332.8). The structure of the product was elucidated by its ^{1}H-NMR and ^{13}C-NMR data: **^{1}H-NMR [(500 MHz, MeOD), δ_H (ppm)]:** 7.725 (*dt*; 2H; *J* = 9; 2; $H_{2'}$, $H_{6'}$), 7.695 (*dt*; 2H; *J* = 8.5; 2; H_2, H_6), 7.507 (*dt*; 2H; *J* = 8.5; 2; H_3, H_5), 6.899 (*dt*; 2H; *J* = 9; 2; $H_{3'}$, $H_{5'}$), 3.767 (*s*; 3H; CH_3 ether), 1.661 (*s*; 6H; $2CH_3$). **^{13}C-NMR [(125**

MHz, MeOD), δ_C (ppm)]: 195.82 (C_7), 175.39 (COOH), 161.18 (C_4), 139.49 ($C_{4'}$), 137.72 ($C_{1'}$), 133.09 ($C_{2'}$, $C_{6'}$), 132.32 (C_2, C_6), 131.51 (C_1), 129.65 ($C_{3'}$, $C_{5'}$), 118.60 (C_3, C_5), 80.62 (qC), 53.13 (CH_3 ether), 25.76 ($2CH_3$). This product was confirmed as methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoate (impurity D).

The IR spectrum of the product E (cm^{-1}) showed characteristic absorption peaks of functional groups, including C-H aromatic (1593.20), C=O ketone (1654.92), and C=O acid (1743.65). The positive ESI-MS of the product revealed an $[M+Na]^+$ at m/z = 369.09 that indicated the relative molecular mass similar to impurity E ($C_{19}H_{19}ClO_4$, M=346.8). The structure of the product was elucidated by its ^{1}H-NMR and ^{13}C-NMR data: **^{1}H-NMR [(500 MHz, MeOD), δ_H (ppm)]:** 7.734 (*dt*; 2H; *J* = 9; 2; $H_{2'}$, $H_{6'}$), 7.709 (*dt*; 2H; *J* = 8.5; 2; H_2, H_6), 7.521 (*dt*; 2H; *J* = 8.5; 2; H_3, H_5), 6.917 (*dt*; 2H; *J* = 9; 2; $H_{3'}$, $H_{5'}$), 4.238 (*q*; 2H; CH_2 ether), 1.668 (*s*; 6H; $2CH_3$), 1.234 (*t*; 3H; CH_3 ether) . **^{13}C-NMR [(125 MHz, MeOD), δ_C (ppm)]:** 195.89 (C_7), 174.89 (COOH), 161.27 (C_4), 139.51 ($C_{4'}$), 137.76 ($C_{1'}$), 133.05 ($C_{2'}$, $C_{6'}$), 132.32 (C_2, C_6), 131.47 (C_1), 129.67 ($C_{3'}$, $C_{5'}$), 118.60 (C_3, C_5), 80.65 (qC), 62.80 (CH_2 ether), 25.77 ($2CH_3$), 14.32 (CH_3 ether). This product was confirmed as ethyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoate (impurity E).



Impurity D (methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoate)



Impurity E (ethyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoate)

3.3 Purity assessment of impurity B, impurity D, and impurity E by HPLC

The chromatographic separation was performed with an isocratic elution. A Phenomenex Gemini NX column (250 × 4.6 mm; 5 µm) was used for purity determination. The mobile phase contained acetonitrile and acetic acid (pH 2.5) (70:30). The analysis was performed at a flow rate of 1.0 ml/min, and an injection volume of 10 µl. The effluents were monitored at 286 nm. The purity of impurities B, D and E was determined as over 99% on the basis peak area normalization method (excluding peaks from mobile phase, acetonitrile as blank sample, and signals below 3 times of noise.

3. CONCLUSIONS

Fenofibrate impurities B, D and E were successfully synthesized by the proposed method, which is simple and cost effective. These impurities conform fully to establish reference standards.

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A New Practical Method for Extraction of Nuciferine From Lotus (*Nelumbo Nucifera* Gaertn) Leaves

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Abstract

Introduction: Nuciferine, a major aporphine alkaloid from leaves of *Nelumbo nucifera* Gaertn (sacred lotus), is a substance of great interest because of its important pharmacological activities, particularly amelioration of hyperlipidemia, stimulation of insulin secretion, antimicrobial and anti-HIV activities. The previous methods for extraction of nuciferine have been performed in laboratory scale using ethanol as extraction solvent, and the purification has carried out by column or thin-layer chromatography, which are not suitable for scaling up and further industrial purposes. **Objectives:** The aim of our study was to optimise a method for extraction of nuciferine (with purity > 95%) in scale of 2.5kg of dried lotus leaves/batch without using chromatography. **Materials and methods:** Extraction of nuciferine from lotus leaves was carried out by direct-immersion in high-temperature mode in kerosene. Nuciferine was purified by recrystallization from ethanol 96^o. The quality of the product was evaluated by TLC, melting point, ESI-MS, IR, ^{1}H-NMR spectroscopies and HPLC analysis. R**esults:** Extraction parameters: batch size: 2.5 kg dried lotus leaves, solvent: kerosene in ratio solvent:leaves as 8:1 (L/kg), temperature 90-100^{o}C, extraction time 2 h, three times per batch. Purification: isolation of the total alkaloid by the general methods, then crystallization of the total alkaloid from ethanol (2 times) to obtain 6.22g of nuciferine as white crystal with mp. 164.9-165.2^{o}C. TLC: R_f 0.40 [*n*-hexane-acetone-NH_4OH 25% (3:1:0.1)]. ESI-MS, *m/z:* 296,0 ($[M+H]^+$). IR (KBr), ν (cm^{-1}): 3010; 2958; 1591, 1497; 1243; 1104. ^{1}H-NMR (500 MHz, DMSO-d_6), δ (ppm): 8.16 (1H, d, *J*=7,5Hz, H-11); 7.27 (2H, m, H-8, H-9); 7,22 (1H, m, H-10); 6,77 (1H, s, H-3); 3,80 (3H, s, H-1'); 3,57 (3H, s, H-2'); 3.13 (1H, dd, J_1=3.5Hz, J_2=14.0Hz, H-7β); 2.96 (2H, m, H-5); 2.82 (1H, dd, J_1=4.0Hz, J_2=14.0Hz, H-6a); 2.63 (1H, d, *J*=15.5Hz, H-7α); 2.34 (2H, m, H-4); 2.42 (3H, s, H-3'). Purity: 95.64% by HPLC (Column: Supelco C_{18}. Mobile phase: acetonitrile-Et_3N 0.1% (70:30, v/v). Detector: PDA with λ=280nm. Flow rate: 1.0 mL/min. Injection volume: 20 µL). **Conclusion:** Kerosene was employed for high-temperature extraction of alkaloids from lotus leaves. Nuciferine was successfully separated from alkaloids

by crystallization using ethanol, without chromatography. From 2.50 kg dried leaves, 6.22 g of nuciferine were obtained in a batch with a purity of 95.64%.

Keyword: Extraction, lotus, nelumbo, nuciferine, kerosene

1. INTRODUCTION

Nuciferine ((*R*)-1,2-dimethoxyaporphine, see **Fig. 1**) is one of the major aporphine alkaloids of the leaves of *Nelumbo nucifera* Gaertn (also known as Indian lotus, sacred lotus, bean of India, or simply lotus - a plant in the family Nelumbonaceae). Recent phytomedicine studies have shown that nuciferine possesses remarkable pharmacological effects, including smooth muscle relaxation, stimulation of insulin secretion, vasodilation, antioxidant, anti-obesity, hypolipidemic, anti-hypotensive, anti-arrhythmic, anti-atherosclerotic, antimicrobial and anti-HIV activities [1-4]. Nuciferine was recorded as the major constituent of lotus leaf in the 2005 Chinese Pharmacopoeia [2]. To obtain pure nuciferine, some conventional separation methods, such as using organic solvents to extract and column chromatography including silica gel, polyamide column, thin-layer chromatography (TLC), Sephadex LH-20 chromatography and high-performance liquid chromatography (HPLC), or high-speed counter-current chromatography (HSCCC) have been used to isolate and purify aporphine alkaloids from leaves of N. nucifera [1,4,5]. However, these extracts and purification methods are time-consuming, requiring multiple steps, in which column chromatography requires a long time and a large volume of organic solvent, worse still the sample are adsorbed onto the stationary phase irreversibly, and difficult to scale-up. Consequently, to further study the biological activity of nuciferine, an efficient method for the preparative separation is necessary. The main goal of the current protocol was to optimise a method for extraction of nuciferine (with purity > 95%) in scale of 2.5kg of dried lotus leaves/batch without using chromatography.

2. MATERIALS AND METHODS

Lotus leaves (*Nelumbo nucifera* Gaertn, Vietnam), standard nuciferine (purity of 100.11%, Vietnamese national institute of drug quality control). Sulfuric acid, sodium carbonate (chemically pure, China). Acetonitrile and triethylamine (for HPLC, Merck). Kerosene, slaked lime and ethanol 96% (industrial, Vietnam).

Extraction of nuciferine from lotus leaves was carried out by direct-immersion in high-temperature mode in kerosene. Nuciferine was purified by recrystallization from ethanol 96^{o}. The quality of the product was evaluated by TLC, melting point, ESI-MS, IR, ^{1}H-NMR spectroscopies and HPLC analysis.

3. RESULTS (AND DISCUSSION)

3.1 Extraction, separation and purification of nuciferine

Lotus leaves were dried at 50-60^{o}C, ground to size about 5 mmm. Mix 2.5 kg of powdered herbs with the suspension obtaining from 250 g of slaked lime and 3.5 L of water. Then incubate the mixture for 24 hours. Transfer the alkalized poweder mass into the 12-bottle extraction system, then 20.4 L of kerosene. After maintaining the extraction system at 90-100^{o}C during 2 hours, collect the extract. Add new solvent to extract for the 2nd and 3rd times by the similar procedure.

Transfer the 1st extraction solution into 2.5 L of 0.5 % H_2SO_4 solution. Stir it well for 30 minutes. Then allow the mixture to separate phases, collect the acidic layer (recycle the kerosene - organic solvent layer for the next extraction). Transfer the 2nd extract into this acidic phase, stir it well, separate phase and collect acidic layer (pH < 2). The 3rd extraction solution is used as new solvent for the next extraction. Add gradually saturated Na_2CO_3 solution in the aqueous phase to pH ~ 10, allow to stand for 30 minutes to precipitate completely. The alkaloid precipitate was filtered out, washed with distilled water to neutral pH and dried at 60^{o}C. 12.84 g of total alkaloids was obtained.

Dissolve the precipitate alkaloids in about 1.0 L of 96 % ethanol, add 2.0 g of activated carbon. Allow to reflux the mixture for 15 minutes, then filter off. The filtrate was allowed to crystallize at room temperature during 48 hours. The product (crude nuciferine) was filtered out, washed with 96 % ethanol. After recrystallization from 96 % ethanol, 6.22 g of nuciferine was obtained.

3.2 Evaluation of quality of nuciferine

The product is a white or pale yellowish-green crystalline powder (see **Fig. 1**) with **mp.** 164.9-165.2^{o}C.

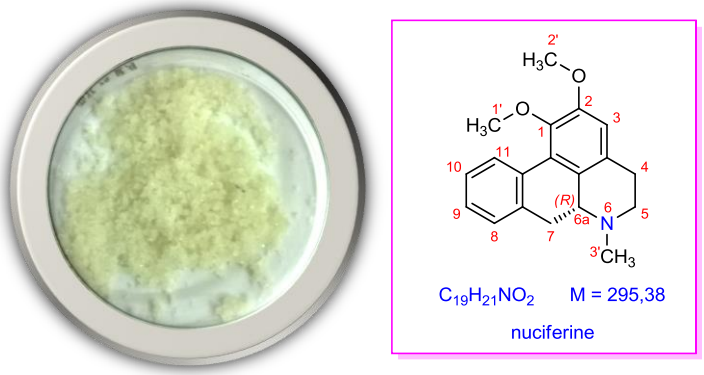

Figure 1. Nuciferine from lotus leaves (crystals and chemical structure)

TLC: R_f 0.40 [*n*-hexane - acetone - ammonia solution 25% (3 : 1 : 0.1)]; 0.24 [chloroform - ethyl acetate (5 : 1)].

ESI-MS (MeOH), *m/z:* 296,0 $[M+H]^+$ ($C_{19}H_{21}NO_2$, molecular weight : 295.38).

IR (KBr), ν max (cm^{-1}): 3010; 2958, 2838; 1591, 1497, 1452; 1243 (C-O); 1104 (C-N).

^{1}H-NMR (500 MHz, DMSO-d_6), δ (ppm): 8.16 (1H, d, *J*=7,5 Hz, H-11); 7.27 (2H, m, H-8, H-9); 7,22 (1H, m, H-10); 6,77 (1H, s, H-3); 3,80 (3H, s, H-1'); 3,57 (3H, s, H-2'); 3.13 (1H, dd, J_1=3.5 Hz, J_2=14.0 Hz, H-7β); 2.96 (2H, m, H-5); 2.82 (1H, dd, J_1=4.0 Hz, J_2=14.0 Hz, H-6a); 2.63 (1H, d, *J*=15.5 Hz, H-7α); 2.34 (2H, m, H-4); 2.42 (3H, s, H-3').

HPLC parameters: Column: Supelco C18 (150 mm × 4.6 mm, 5 µm). Mobile phase: acetonitrile - 0.1% solution of Et_3N in water (70:30, v/v). Detector: PDA with λ=280 nm. Flow rate: 1.0 mL/min. Injection volume: 20 µL. Concentration of the standard nuciferine is 100 µg/mL in 96% ethanol. Retention time of the nuciferine samples were detemined about 25.877 (see **Fig. 2**). Content of isolated nuciferine was 95.64%.

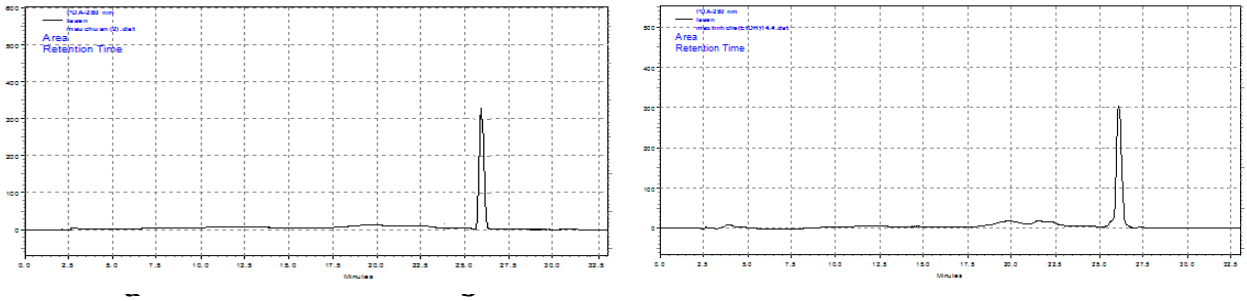

Figure 2. HPLC chromatograms of standard (**a**) and isolated nuciferine (**b**)

4. DISCUSSIONS

Kerosene is a mixture of liquid hydrocarbons (C8-C15) with boiling point from 150 to 275^{o}C, and has some shortcomings, such as toxic and flammable. However, by the application of this solvent as extraction solvent, the process of isolation / purification of nuciferine has significantly simplified: less stage, less time and no need to chromatography. Also, within that process, solvent can be recovered to reuse after extraction, and soaking method requires simple equipment, which brings many advantages for the expansion of the production scale (the pilot and the industrial). In this study, after shaking 1st and 2nd extraction solutions with sulfuric acid and collecting the aqueous phase, the oil phase is recycled directly for the next extractions. The recovered kerosene is in average of 14 L / batch (about 70% of the original solvent).

5. CONCLUSIONS

In current study, kerosene was employed for high-temperature extraction of alkaloids from *Nelumbo nucifera*'s leaf. The optimum conditions of this extraction process were following: batch size: 2.5 kg, solvent: kerosene in ratio solvent:leaves as 8:1 (L/kg), temperature 90-100^{o}C, extraction time 2 h, three times per batch. Nuciferine was successfully separated from alkaloids by crystallization using ethanol, without chromatography. From 2.50 kg/batch dried leaves, 6.22 g of nuciferine were obtained in a batch with a purity of 95.64% (HPLC).

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Synthesis and Purity Determination of *N*-butyryl-*N*-{[2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine as Related Valsartan Impurity

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Abstract

Introduction: Valsartan impurity B (*N*-butyryl-*N*-{[2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine), arising from synthesis, may influence the treatment efficacy and safety of valsartan. The EP 8.0, BP 2016 and USP 40 require conducting the impurity test in valsartan pharmaceutical substance as well as corresponding finished products. However, the mentioned impurity reference standard is costly with limited accessibility and there have been no published local studies on chemical synthesis of valsartan related compound B so far. This was our motivation to study on synthesis of impurity B from valsartan to serve for establishment of reference standard. **Objectives:** The aim of this study was to synthesize valsartan impurity B and determine its purity by HPLC method in order to establish the reference standard. **Materials and Methods:** Firstly, (*S*)-3-methyl-2-((2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl methyl)-amino)-butyric acid (DOP) was synthesized by refluxing valsartan in hydrochloric acid in the dark. Then butyryl chloride and mixture of pyridine and acetonitrile were added to DOP suspension in acetonitrile at -5^{o}C. After that, methanol and water were added to the mixture at room temperature. The resultant mixture was adjusted to pH 7.5 by sodium carbonate, evaporated, added into by chloroform, and adjusted to pH 2 by hydrochloric acid. The chloroform extract was separated and evaporated to mucus form. Methanol was added to make clear solution, then water was added to form suspension. Finally, the suspension was evaporated at 50°C in vacua until the white solid was formed. The purity of the synthesized product was preliminarily determined by TLC, and then the structure of synthesized compound was elucidated by IR, MS and NMR data. Finally, the purity of valsartan impurity B was determined by HPLC with photodiode array

detector on the basis of peak area normalization method. **Results:** *N*-butyryl-*N*-{[2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine (valsartan impurity B) was synthesized by acylating DOP with butyryl chloride. The purity was more than 98% and yield approximately 70.24%. Additionally, a HPLC method for assay of impurity B was developed and validated according to International Conference on Harmonization guidelines. The method showed selectivity, wide linearity range, and high precision. **Conclusion:** Valsartan impurity B was successfully synthesized by the proposed method, which is simple and cost-effective. The impurity conforms fully to establish reference standard for impurity testing in pharmaceuticals.

Keywords: Valsartan impurity B, *N*-butyryl-*N*-{[2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine.

1. INTRODUCTION

Valsartan (L-Valine, *N*-(1-oxopentyl)-*N*-[[2'-(1*H*-tetrazole-5-yl)[1,1'-biphenyl]-4-yl] methyl]) was invented by Novartis, launched under trade name Diovan. The drug is a potent, orally active and highly selective antagonist of the angiotensin II AT1-receptor, that is widely employed for treatment of all grades of hypertension[1]. Valsartan (VAL) has recently acquired official status in the most relevant compendia, including EP 9.0[2], BP 2017[3] and USP 40[4]. Therefore, the monograph valsartan describes two liquid chromatography determinations for limiting a total of three related compounds. One of them is *N*-butyryl-*N*-{[2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine (valsartan impurity B, VALB). This impurity arises from synthesis, may influence the treatment efficacy and safety of VAL, and must be controlled in pharmaceutical substance as well as corresponding finished products. However, the mentioned impurity reference standard is costly with limited accessibility and there have been no published local studies on chemical synthesis of VALB so far. This was our motivation to study on synthesis of impurity B from VAL to serve for establishment of reference standard.

2. MATERIALS AND METHODS

2.1 Chemicals, solvents, and standards

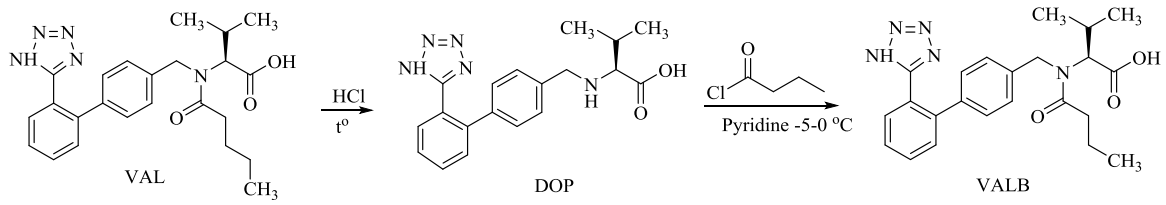
Valsartan (99.76% purity) was supplied by Wohler laboratories Pvt. Ltd, India. Butyryl chloride was provided from Sigma-Aldrich, Germany. Acetonitrile was of liquid chromatography grade, and the other solvents and chemicals were of analytical grade. TLC was performed on silicagel 60 F_{254} plates (Merck) and visualized by UV light ($\lambda = 254$ nm).

2.2 Equipment

NMR spectra were recorded on a Bruker Advance III 500 MHz with TMS as an internal standard. The IR spectra were recorded in the solid state as KBr dispersion using FT-IR Alpha–T, Bruker. The mass spectra were recorded on MS Waters Xevo TQD. Purity of synthesized products was determined with HPLC system from Hitachi.

2.3 Methods

Firstly, (*S*)-3-methyl-2-((2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl methyl)-amino)-butyric acid (DOP) was synthesized by refluxing valsartan in hydrochloric acid in the dark. Then butyryl chloride and mixture of pyridine and acetonitrile were added to DOP suspension in acetonitrile at -5^{o}C (scheme 1). After that, methanol and water were added to the mixture at room temperature. The resultant mixture was adjusted to pH 7.5 by sodium carbonate, evaporated, added into by chloroform, and adjusted to pH 2 by hydrochloric acid. The chloroform extract was separated and evaporated to mucus form. Methanol was added to make clear solution, then water was added to form suspension. Finally, the suspension was evaporated at 50°C in vacua until the white solid was formed. The purity of the synthesized product was preliminarily determined by TLC, and then the structure of synthesized compound was elucidated by IR, MS and NMR data. Finally, the purity of valsartan impurity B was determined by HPLC with photodiode array detector on the basis of peak area normalization method.



Scheme 1. *Synthesis of valsartan impurity B*

3. RESULTS AND DISCUSSION

3.1. Preparation of (S)-3-methyl-2-((2'-(1H-tetrazole-5-yl)-biphenyl-4-yl methyl)-amino)-butyric acid (DOP)

Valsartan solution in 3M HCl (2 g/150 mL) was stirred, refluxed constantly in the dark until the clear solution, cooled at room temperature to form crystal. The resultant mixture was filtered to remove solvent, washed with distilled water (10 mL × 3 times), and dried at 60^{o}C to obtain the solid product (1.35 g, 83.74% yield). Thin layer chromatography with three different polar solvent systems showed that the synthesized product had a single spot.

The IR spectrum of the product (KBr, ν) showed characteristic absorption peaks of functional groups, including 1718 (C=O (acid)), 1560 (N-N), 1476 (C-OH), and 1397 (C-O) cm^{-1}.

The positive ESI-MS of the product revealed a $[M+H]^+$ at m/z = 352.1 that indicated the relative molecular mass similar to DOP ($C_{19}H_{21}N_5O_2$, MW = 351.4 Daltons).

The structure of product was elucidated by its ^{1}H-NMR and ^{13}C-NMR data: **^{1}H-NMR [(500 MHz, DMSO), δ_H (ppm)]:** 0.95 and 1.04 (2 x *d*, 2 x 3H, *J* = 7.0, H-8 and H-9), 2.40–2.45 (*m*, 1H, H-7), 3.70 (*d*, 1H, *J* = 3.0, H-6), 4.13 (*d*, 1H, *J* = 13.5, H-11), 4.17 (*d*, 1H, *J* = 13.0, H-11), 7.17 (*d*, 2H, *J* = 8.0, H-13 and H-17), 7.59 (*m*, 1H, H-21), 7.52 (*dd*, 2H, *J* = 4.5 and 2.0, H-14 and H-16), 7.55 (*d*, 1H, *J* = 1.0, H-19), 7.69 (*t*, 1H, *J* = 7.5, H-20) and 7.71 (*d*, 1H, *J* = 1.5, H-22); **^{13}C-NMR [(125 MHz, DMSO), δ_C (ppm)]:** 16.9 and 19.6 (C-8 and C-9), 28.1 (C-7), 49.4 (C-11), 64.0 (C-6), 128.0 (C-23), 129.0 (2C, C-13 and C-17), 130.4 (2C, C-14 and C-16), 130.5 (C-21), 130.6 (C-19), 130.7 (C-22), 131.0 (C-20), 140.1 (C-12), 141.0 (C-18), and 168.9 (C-10). This product was confirmed as 2(*S*)-3-methyl-2-((2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl methyl)-amino)-butyric acid (DOP).

3.2. Preparation of N-butyryl-N-{[2'-(1H-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine (valsartan impurity B, VALB) from DOP

Suspension of DOP (1.12 g; 3.2 mmole) in acetonitrile (20 mL) was freezed to -5°C, and butyryl chloride (1 mL; 9.6 mmole) was added into, followed by slow addition of pyridine (1.0 ml; 12.8 mmole) diluted with acetonitrile (10 mL). The reaction mixture was added with methanol (3 mL), water (10 mL) at room temperature, stirred for 1 hour, adjusted to pH 7.5 by 10% sodium carbonate solution, evaporated at 50°C in vacuo to remove organic solvent. The concentrated aqueous solution was added by chloroform (40 mL), adjusted to pH 2 at 0-5°C by 1M HCl. The chloroform extract was separated, evaporated to mucus form, added by methanol to obtain clear solution and by water to form suspension. Finally, the suspension was evaporated at 50°C in vacuo until the formed white solid, and dried at 60^{o}C to obtain the white crystals (0.95 g, 70.24% yield). Thin layer chromatography with three different polar solvent systems showed that this synthesized product had a single spot.

The IR spectrum of the product (KBr, ν) showed characteristic absorption peaks of functional groups, including 1732 (C=O (acid)), 1604 (C=O (amid)), 1514 (N-N), 1470 (C-OH), 1410 (C-O) cm^{-1}.

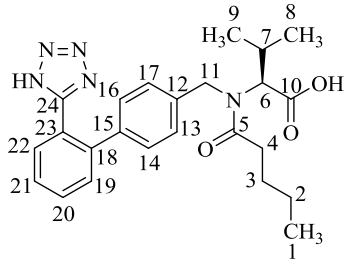
The positive ESI-MS of the product revealed a $[M+H]^+$ and a $[M+Na]^+$ at m/z = 422.1 and 444.1, respectively, that indicated the relative molecular mass similar to VALB ($C_{23}H_{27}N_5O_3$, M=421.5).

Based on the spectral data in table 1, the product was confirmed as *N*-butyryl-*N*-{[2'-(1*H*-tetrazole-5-yl)-biphenyl-4-yl]methyl}-L-valine (valsartan impurity B).

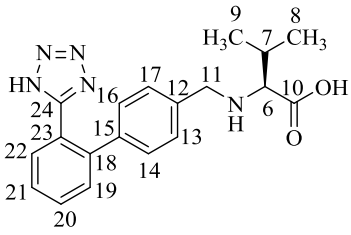
Table 1. *Comparison of ^{1}H-NMR and ^{13}C-NMR chemical shifts (in ppm) of valsartan impurity B and valsartan in DMSO-d6 solutions*

Number	Major Conformer						Minor Conformer					
	δ_C			δ_H			δ_C			δ_H		
	VALB	VAL	VAL[5]	VALB	VAL	VAL[5]	VALB	VAL	VAL[5]	VALB	VAL	VAL[5]
5	173.4	173.5	173.4				173.4	173.5	173.4			
10	171.9	171.9	171.8				171.6	171.6	171.5			
24	155.1	No signal	155				155.1	No signal	155.0			
18	141.2	141.2	141.1				141.3	141.2	141.3			
12	137.7	137.1	137.7				137.1	137.1	137.1			
15	137.8	137.8	137.7				138.2	138.2	138.2			
22	131.0	131.0	130.9	7.68 *m*	7.68 *m*	7.68	131.0	131.0	130.9	7.68 *m*	7.68 *m*	7.68
20	130.6	130.6	130.5	7.63 *m*	7.64 *m*	7.63	130.6	130.6	130.5	7.63 *m*	7.64 *m*	7.63
19	130.5	130.5	130.5	7.54 *d* (8.0 Hz)	7.55 *d* (6.0 Hz)	7.53	130.5	130.5	130.5	7.52 *d* (8.0 Hz)	7.52 *d* (6.5 Hz)	7.53

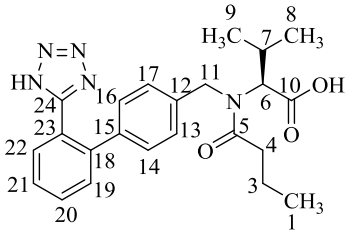
16	128.8	128.8	128.7	7.06 *d* (8.0 Hz)	7.06 *d* (8.5 Hz)	7.06	128.3	128.3	128.2	6.97 *d* (8.0 Hz)	6.97 *d* (8.0 Hz)	6.97
14	128.8	128.8	128.7	7.06 *d* (8.0 Hz)	7.06 *d* (8.5 Hz)	7.06	128.3	128.3	128.2	6.97 *d* (8.0 Hz)	6.97 *d* (8.0 Hz)	6.97
21	127.7	127.7	127.6	7.57 *d* (7.5 Hz)	7.57 *d* (7.5 Hz)	7.57	127.6	127.5	127.6	7.56 *m*	7.57 *d* (7.5 Hz)	7.57
17	126.3	126.3	126.2	7.20 *d* (8.0 Hz)	7.20 *d* (8.0 Hz)	7.20	126.9	126.9	126.9	7.10 *d* (8.0 Hz)	7.09 *d* (8.0 Hz)	7.09
13	126.3	126.3	126.2	7.20 *d* (8.0 Hz)	7.20 *d* (8.0 Hz)	7.20	126.9	126.9	126.9	7.10 *d* (8.0 Hz)	7.09 *d* (8.0 Hz)	7.09
23	123.6	No signal	123.5				123.5	No signal	123.5			
6	62.9	62.9	62.9	4.44 *m*	4.43 *m*	4.45	65.7	65.7	65.7	4.08 *d* (10.5 Hz)	4.08 *d* (10.5 Hz)	4.08
11	48.7	48.7	48.7	4.62 *s*	4.62 *s*	4.62	45.5	45.5	45.5	4.49 *d* (16.0 Hz)	4.49 *d* (17.0 Hz)	4.47
4	34.6	32.4	32.4	2.00 *m*; 2.23 *d* (7.0 Hz)	2.03 *m*; 2.23 *d* (8.0 Hz)	2.04; 2.20	34.8	32.4	32.4	2.44 *m*	2.45 *m*	2.46
7	27.5	27.5	27.5	2.18 *m*	2.19 *m*	2.21	27.6	27.5	27.5	2.13 *m*	2.13 *m*	2.13
3	18.1	26.8	26.7	1.51 *m*; 1.43 *m*	1.30 *m*; 1.40 *m*	1.37; 1.39	18.3	27.0	26.9	1.58 *dd* (14.5; 7.5 Hz)	1.54 *m*	1.54
2		21.6	21.6		1.15 *m*	1.15		21.8	21.7			1.31
9	20.1	20.1	20.0	0.93 *m*	0.93 *m*	0.93	19.4	19.4	19.3	0.93 *m*	0.93 *m*	0.93
8	18.8	18.8	18.7	0.76 *t* (7.5 Hz)	0.76 *t* (7.5 Hz)	0.75	18.5	18.5	18.4	0.70 *d* (6.5 Hz)	0.70 *d* (6.5 Hz)	0.70
1	13.6	13.7	13.6	0.76 *t* (7.5 Hz)	0.76 *t* (7.5 Hz)	0.75	13.7	13.8	13.6	0.89 *m*	0.88 *t* (7.0 Hz)	0.88



Valsartan



DOP



Valsartan impurity B

3.3. Purity assessment of impurity B by HPLC

The chromatographic separation was performed with a gradient elution. A Thermo Acclaim™ Polar Advantage C16 (250 × 4.6 mm; 5 µm) was used for purity determination. The mobile phase contained solvent A (water adjusted to pH 2.5 by orthophosphoric acid) and solvent B (acetonitrile). The gradient program (minute/%B) was set as 0/73, 5/73, 6/50, 45/50, 46/73 and 60/73. The analysis was performed at a flow rate of 1.0 mL/min., and an injection volume of 10 µL. The effluents were monitored at 225 nm. VALB solution was prepared at 450 ppm, using solvent A and solvent B in the ratio of 1:1 as solvent mixture.

The proposed HPLC-DAD method was validated accroding to ICH guidelines[6] concerning system suitability test, selectivity, linearity and precision. The validated results were shown in table 2, 3 and figure 1.

Table 2. *Results of system suitability test*

Parameter	Retention time (min)	Peak area (µAU x s)	Asymmetry factor
Average	14.62	53551231	0.97
RSD	0.02%	0.53%	0.99%

RSD value of all chromatographic parameters were below 2% and the asymmetry factor of VALB is in the range of 0.8 - 1.5.

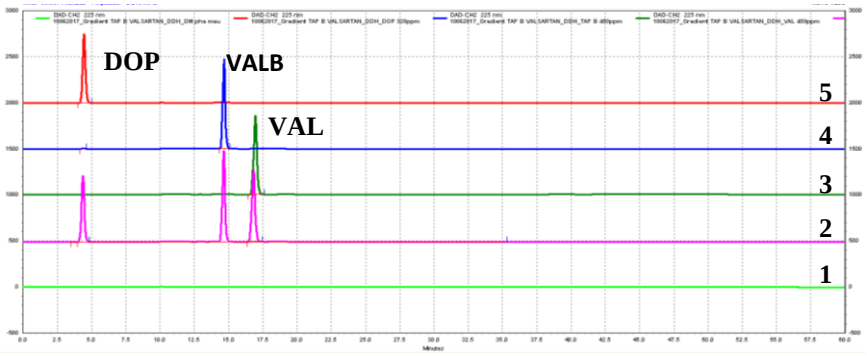


Figure 1. *Chromatograms of selectivity tests.*

(1) Blank sample; (2) Mixture of DOP, VALB and VAL; (3) VAL; (4) VALB; (5) DOP

The selectivity results showed that DOP, VALB and VAL appeared at various retention time. The blank sample chromatogram did not appear peaks having retention time equal to one of DOP, VALB and VAL. Critical separations in chromatography were demonstrated with resolution factor being above 1.5. Peak purity test was used to show that the analyte chromatographic peak was not attributable to more than one component. As a consequence, the analytical procedure showed the selectivity.

Table 3. *Results of linearity and precision*

Regression equation	$\hat{y}$ = 116433.97x
Linearity range (µg/ml)	112.5 – 675
Correlation coefficient (R)	0.9997
Precision (RSD, n=6)	0.02%
Purity of VALB*	98.89%

**Be determined from the precision test.*

Statistical results showed that this procedure had a wide range with high correlation coefficient value and acheived the precision requirements. The purity of VALB was more than 98% based on the basis peak area normalization method (excluding peaks from blank sample, and signals below 3 times of noise).

4. CONCLUSIONS

Valsartan impurity B was successfully synthesized by the proposed method which is simple and cost-effective. The impurity conforms fully to establish reference standard for impurity testing in pharmaceuticals.

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Synthesis of Novel Curcumin-valine Conjugate Using Ethylene Group as Linker

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Abstract

Introduction: Curcumin is a major active polyphenol of *Curcuma Longa L.* (turmeric) which has been used for thousands of years in traditional medicine. The evidences of the modern medicine have shown that, curcumin is safe, provides many health benefits and exhibits non-toxicity even at high doses (up to 12 g/day). However, extremely low oral bioavailability (approximately 1%) is the main limitation to use curcumin as a therapeutic agent. Therefore, numerous formulation techniques have been undertaken over the past decades to increase its solubility, stability, and consequently, bioavailability. However, many questions and challenges still exist. In this work, the chemical amino acid conjugation approach was performed to improve the solubility of curcumin in water. **Objectives:** The aim was to use ethylene group as linker to obtain a water-soluble curcumin-valine conjugate. **Materials and methods:** Curcumin, L-valine, 2-bromoethanol, 4-dimethyl-aminopyridine (DMAP), *N,N'*-dicyclohexylcarbodiimide (DCC), potassium carbonate, di-*tert*-butyl dicarbonate, sodium hydroxide, anhydrous sodium sulfate and common solvents are suitable for synthesis. The synthesis was performed by 4 steps from curcumin and L-valine: *O*-alkylation of curcumin with 2-bromoethanol to obtain bis-*O*-(2-hydroxyethyl)-curcumin (**1**), protection of L-valine by *t*-butyloxycarbonyl group, conjugation of **1** and protected L-valine using DCC and DMAP, deprotection with methanolic HCl to obtain final product (**LHD-62017**). The product was purified by silica gel column chromatography (EtOAc : 96% EtOH = 4 : 1, *v/v*). Its structure was analyzed by spectral methods, including ESI-MS and ^{1}H-NMR. **Results: LHD-62017** was obtained with total yield of 8.75% as brownish yellow powder. $\mathbf{R_f}$ = 0.30 (EtOAc : 96% EtOH = 2 : 1). **ESI-MS**, *m/z:* 655.2 $[M+H]^+$, 677.2 $[M+Na]^+$. **^{1}H-NMR** (500 MHz, DMSO-d_6), δ (ppm): 8.33 (1H, s, H-8), 6.94-7.19 (10H, m, H-1, H-2, H-6, H-7, H-2', H-2'', H-5', H-5'', H-6', H-6''), 6.66 (1H, s, H-4), 5.47 (4H, s, H-13', H-13''), 4.21 (8H, t, *J*=4.5 Hz, H-7', H-7'', H-8', H-8''), 3.81 (6H, s, H-14', H-14''), 3.80 (2H, d, *J*=5.0 Hz, H-9', H-9''), 1.82-1.86 (2H, m, H-10', H-10''), 0.82 (12H, d, *J*= 6.5 Hz, H-11', H-11'', H-12', H-12''). **Conclusion:** The novel water-soluble curcumin-valine conjugate by ethylene linker was successfully synthesized with total yield of 8.75% from curcumin and L-valine.

Keywords: Conjugate, curcumin, synthesis, turmeric, L-valine

1. INTRODUCTION

Curcumin (diferuloylmethane) is the major active polyphenol of *Curcuma Longa* L. (turmeric) which has been used for thousands of years in traditional medicine for treating various diseases in many countries of Asia where it is grown for commercial use [1,2]. The continually reported evidences of the modern medicine have shown that curcumin possesses remarkable pharmacological effects, including anti-tumor, anti-inflammatory, anti-oxidation, anti-amyloid, anti-fungal and anti-bacterial activities [3,4]. Furthermore, it has been clinically confirmed that, curcumin is safe, non-toxic even at high doses (up to 12 g/day), exhibits good tolerability and provides many health benefits for human. However, extremely low oral bioavailability (approximately 1%) is the main limitation to use curcumin as a therapeutic agent [5]. This is related to its poor absorption, instability in physiological fluids, rapid metabolism and rapid systemic elimination from body [5]. Two different approaches were undertaken to resolve this issue. The first involved the formulation techniques, namely, combination with adjuvants (piperine), phospholipid complexes, β-cyclodextrin complexation, solid dispersion systems, microemulsions, water-based nano emulsions, polymeric micelles, liposomes, and nanoparticles which have been extensively investigated over past decades [1,6,7]. The second approach involved the chemical modification of curcumin. In this connection, prodrug design is a widely known molecular modification strategy that aims to optimize the physicochemical and pharmacological properties of drugs, such as improving their solubility, pharmacokinetic features and decreasing their toxicity. In the last 10 years, several prodrugs were successfully developed by this tool [8]. Amino acids, especially L-valine, are the water-soluble promoieties widely used to form ester and amide prodrugs, for example, valaciclovir, valganciclovir and valbenazine [8-11]. Furthermore, some chemical groups, such as glycols (e.g., polyethylene glycol and ethylene glycol) were also applied as a spacer linking the carrier to the parent drug [8]. Thus, the main goal of current work was to develop a new ethylene-glycol-linked valine prodrug (conjugate) of curcumin with increased solubility in water.

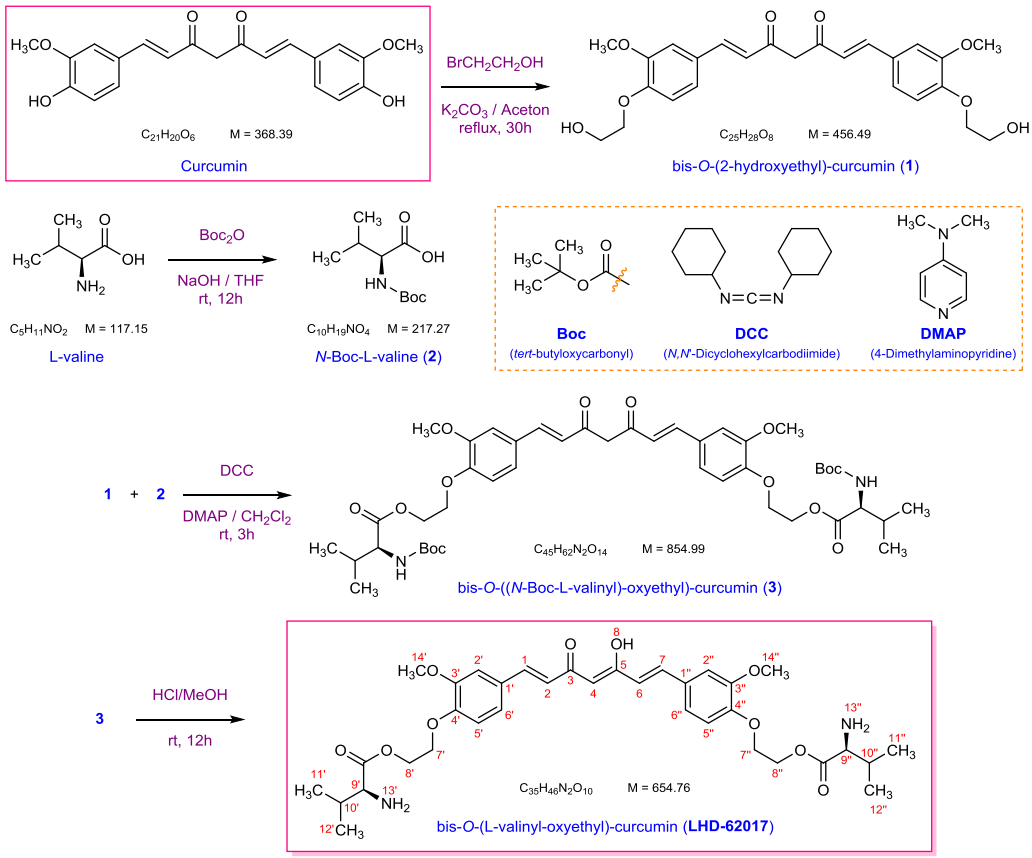
2. MATERIALS AND METHODS

2.1. Materials

Curcumin was extracted and separated from rhizome of *Curcuma longa* L. (provided by Bac Ninh pharmaceutical JSC, Vietnam). All chemicals used for synthesis were in AR (analytical reagent) grade: L-valine, 2-bromoethanol, 4-dimethyl-aminopyridine (DMAP), *N, N'*-dicyclohexylcarbodiimide (DCC), potassium carbonate, di-*tert*-butyl dicarbonate (Boc_2O), sodium hydroxide, sodium hydrocarbonate, 37% hydrochloric acid, anhydrous sodium sulfate, acetic acid (AcOH); acetone, dicloromethane (DCM), diethyl ether, ethanol (EtOH), 96% EtOH, ethyl acetate (EtOAc), methanol (MeOH) and tetrahydrofuran (THF).

2.2. Methods

The synthesis process was performed by 4 steps from curcumin and L-valine as shown in Scheme 1, namely, *O*-alkylation of curcumin with 2-bromoethanol to obtain bis-*O*-(2-hydroxyethyl)-curcumin (**1**), protection of L-valine by *t*-butyloxycarbonyl group to prepare *N*-Boc-L-valine (**2**), conjugation of **1** and **2** using DCC and DMAP to synthesize **3**, followed by deprotection with methanolic HCl to obtain final curcumin-valine conjugate (**LHD-62017**).



Scheme 1. *Synthesis of **LHD-62017** from curcumin and L-valine*

The reactions were monitored by thin layer chromatography (TLC) using precoated silica gel 60 F254 plates (Merck) and suitable mobile phase solvent system. Spots on TLC were detected under UV light (254, 366 nm) or by spraying with ninhydrin reagent followed by heating. The product was purified by precipitation or column chromatography using Merck silica gel 60.

Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. The structure was analyzed by spectral methods, including mass-spectrometry (MS) and proton nuclear magnetic resonance (^{1}H-NMR). The mass spectrum was obtained in an electrospray ionization (ESI) mode using a LC-MS/MS Agilent 1290/6460 mass spectrometer. ^{1}H-NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer.

3. RESULTS

3.1. Synthesis of bis-O-(2-hydroxyethyl)-curcumin (1) (construction of ethylene group as linker)

A mixture consisting of curcumin (1.00 g, 2.7 mmol), anhydrous potassium carbonate (1.69 g, 12.2 mmol) and anhydrous acetone (80 mL) was stirred in a 250 mL two-necked flask for 30 minutes at room temperature. Afterwards, 2-bromoethanol (1.9 mL, 26.8 mmol) was added to the

flask. The reaction mixture was stirred well under reflux for 30 hours (reaction monitoring was carried out by TLC with mobile phase: DCM : MeOH = 9.0 : 1.0, *v/v*). The reaction mass was cooled, filtered off to remove potassium carbonate. Acetone was evaporated from the filtrate under vacuum. The residue was washed with distilled water, subsequently dissolved in 15 ml of hot (~60 °C) ethanol in a flask, cooled to room temperature. 7 mL of diethyl ether was slowly added. The mixture was allowed to completely precipitate at 0 °C. The precipitate was filtered off, washed with diethyl ether and dried at 60-70 °C to give 0.76 g of **1** (61.0% of yield) with mp. 176.4-178.0 °C, R_f = 0.63 (DCM : MeOH = 9.0 : 1.0), 0.21 (*n*-hexane : EtOAc = 3.0 : 7.0) [12,13].

3.2. Synthesis of N-Boc-L-valine (2) *(protection of L-valine)*
A mixture consisting of L-valine (1.76 g, 15.0 mmol), THF (30 mL), water (15 mL) and 1 M NaOH (20 mL) was stirred and cooled in an ice bath. Afterwards, di-*tert*-butylpyrocarbonate (3.60 g, 16.5 mmol) was added and the reaction mixture. was further stirred for 12 h at room temperature (reaction monitoring was carried out by TLC with mobile phase: *n*-butanol : AcOH : H_2O = 9.0 : 2.0 : 2.5, *v/v*). The reaction mass was acidified with 1M HCl to pH ~ 1 and extracted with ethyl acetate (3 x 75 mL). The organic phase was washed with water (3 × 50 mL), dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to give **2** (2.75 g, yield 82.40%) as a gummy white mass with R_f = 0.72 (*n*-butanol : AcOH : H_2O = 9.0 : 2.0 : 2.5) [9].

3.3. ***Synthesis of bis-O-((N-Boc-L-valinyl)-oxyethyl)-curcumin (3)*** *(conjugation of 1 with 2)*
A mixture consisting of 4-dimethylaminopyridine (0.20 g, 1.6 mmol), **1** (1.00 g, 2.2 mmol), **2** (2.10 g, 4.5 mmol), dichloromethane (16 mL) was stirred and cooled in an ice bath for 30 minutes. Afterwards, dicyclohexylcarbodiimide (5.0 g, 25.0 mmol) was added. Stir the mixture for 3 hours at room temperature (reaction monitoring was carried out by TLC with mobile phase: DCM : MeOH = 9.0 : 1.0, *v/v*). The reaction mass was filtered off to remove dicyclohexylurea (DCU) evaporated under reduced pressure to dryness. The residue was dissolved in 50 mL of ethyl acetate and washed with 10% solution of sodium carbonate (3 × 20 mL), then with distilled water. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to give **3** (1.25 g, yield 66.70%) as dark yellow mass with R_f = 0.80 (DCM : MeOH = 9.0 : 1.0).

3.4. Synthesis of bis-O-(L-valinyl-oxyethyl)-curcumin (LHD-62017) *(deprotection)*
0.5 g (0.6 mmol) of **3** was dissolved in 30.0 mL of methanol and 20 mL of 1.3 M methanolic HCl. The reaction was stirred for 12 hours at room temperature (reaction monitoring was carried out by TLC with mobile phase: EtOAc : 96% EtOH = 2 : 1, *v/v*). The reaction mass was evaporated, dissolved in 30 mL of water and washed with dichloromethane (3 × 30 mL). Aqueous phase was neutralized by saturated solution of sodium hydrocarbonate to pH = 9, subsequently extracted with ethyl acetate (3 x 30 mL). Organic phase was washed with distilled water, dried over anhydrous sodium sulfate, filtered off and evaporated to dryness. Purification by silica gel column chromatography with mobile phase : EtOAc : 96% EtOH = 4 : 1, *v/v* gave **LHD-62017** (0.10 g, yield 26.11%) as brownish yellow powder. $\mathbf{R_f}$ = 0.30 (EtOAc : 96% EtOH = 2 : 1). **ESI-MS**, *m/z:* 655.2 $[M+H]^+$, 677.2 $[M+Na]^+$. **^{1}H-NMR** (500 MHz, DMSO-d_6), δ (ppm): 8.33 (1H, s, H-8), 6.94-7.19 (10H, m, H-1, H-2, H-6, H-7, H-2', H-2'', H-5', H-5'', H-6', H-6''), 6.66 (1H, s, H-4), 5.47 (4H, s, H-13', H-13''), 4.21 (8H, t, *J*=4.5 Hz, H-7', H-7'', H-8', H-8''), 3.81 (6H, s, H-14', H-14''), 3.80 (2H, d, *J*=5.0 Hz, H-9', H-9''), 1.82-1.86 (2H, m, H-10',

H-10''), 0.82 (12H, d, *J*= 6.5 Hz, H-11', H-11'', H-12', H-12'').

4. DISCUSSIONS

The synthesis of bis-*O*-(2-hydroxyethyl)-curcumin (**1**) was described by C. Changtam in the previous study with yield of 32-35% [12,13]. Herein the hydroxyethylation was carried out with 2-bromoethanol, and the purification has undertaken by column chromatography. In current study, we used molar ratio of 2-bromoethanol : curcumin = 10 : 1 to reduce by-product mono-*O*-(2-hydroxyethyl)-curcumin, and have successfully purified **1** by precipitation without column chromatography. The synthesis yield was 61.0% higher than that of Changtam's work. Melting point and retention factor data of the product were consistent with the reported values [12,13].

Protection of valine was perform by the classical technique in organic synthesis using *tert*-butyloxycarbonyl (Boc) as a protecting group. We used the conditions completely equaled to the reported data [9], and have obtained similar results.

In fact, the TLC monitoring has shown that conjugation reaction of 1 with 2 gave a mixture of the mono- and bis-*O*-((*N*-Boc-L-valinyl)-oxyethyl)-curcumin which were difficult to separate. Thus, we carried out steps 3 and 4 as *one-pot* procedure to obtain final product **LHD-62017.** The deprotection yield was low (26.11%) because the acidic condition of methanolic HCl hydrolyzed not only Boc groups but also ester bonds of ethylene spacer and valine fragment.

5. CONCLUSIONS

In current study, we have preliminarily synthesized the novel curcumin-valine conjugate (**LHD-62017**) by ethylene linker from curcumin and L-valine with total yield of 8.75%. The novel molecule is more water-soluble than original curcumin. The comparison of their biological activities will be further researched.

ACKNOWLEDGEMENT

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Study on Botany and Genetic Diversity of *Clinacanthus nutans* (Burm. f.) Lindau in Vietnam

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Abstract

The genetic diversity of 17 *Clinacanthus nutans* samples collected from different provinces of Vietnam were conducted by using Inter-Simple Sequence Repeat (ISSR) marker - the method has been widely used to assess genetic diversity of plants due to its effectiveness and reliability. ISSR analysis was performed with 10 primers to evaluate genetic diversity among samples (*Clinacanthus nutans* (Burm. f.) Lindau) grown in Vietnam. A dendogram of genetic diversity was officially constructed using UPGMA method. The result showed that *Clinacanthus nutans* could be placed into four groups which have differences in morphology and genotype; *Clinacanthus nutans* has the low genetic diversity among samples despite of their differences in geographic conditions. The result of the chloroplast barcode marker (rbcL) showed that *Clinacanthus nutans* grown in Vietnam could genetically be treated as groups.

Keywords: *Clinacanthus nutans*, ISSR, rbcL, botanical characteristic, genetic diversity

1. INTRODUCTION

Clinacanthus nutans Burm.f.) Lindau (*Bim bip* in Vietnamese) is one of the medical important species from Acanthaceae within tropical and sub-tropical Asian countries, including Malaysia, Thailand, Indonesia, China and Vietnam to treat skin lesions and inflammations caused by virus (Fong *et al.*, 2014; Alam *et al.*, 2016). It was collected in the wild and even cultivated to meet consumer demand. Morphological investigation revealed the differences among samples. However, the genetic diversity of the plant grown in Vietnam was still little known. The use of two different species: *Clinacanthus nutans* (Burm. f.) Lindau and *Clinacanthus siamensis* Bremek, in traditional medicine due to their similar morphology (Alam *et al.*, 2016) are commonly happen in practice.

Numerous attempts approaching to this issue based on morphological characters were made. It still has numbers of disadvantages. In 2009, DNA barcoding for plants were officially established via the Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL), and two plastid genes, matK and rbcL, were completely discovered and recommended as the standard plant DNA barcodes for plant identification (Natasha *et al.*, 2012; Combik *et al*.,

2015). Molecular markers have been broadly used as a standard method to study genetic variability among closely related taxa (Weising *et al*., 1995). Inter-simple sequence repeat (ISSR), microsatellite marker, was presented as an efficient molecular marker based on quick PCR amplification of polymorphic DNA fragments (Zietkiewicz *et al.*, 1994). ISSR technique has successfully identified the genetic diversity of crops including mango (Rocha *et al.* 2011), durian (Vanijajiva, 2012), sweet potato (Moulin *et al.*, 2012) and *C. nutans* (Fong *et al*. 2014 and Ismail *et al.* 2016).

2. MATERIALS AND METHODS

2.1. Plant materials

Fresh leaf samples of 17 *Bim bip* samples collected in provinces of Vietnam were presented in Table 1.

Table 1. *The list of 17 collected samples in Vietnam*

Code	Sample names	Locations (District, Province)
1	CN2201	Tuyen Quang, Tuyen Quang
2	CN2202	Tuyen Quang, Tuyen Quang
3	CN60	Xuan Loc, Dong Nai
4	CN50	Ho Chi Minh city
5	CN71	Mo Cay, Ben Tre
6	CN8401	Chau Thanh, Tra Vinh
7	CN8402	Tra Vinh city, Tra Vinh
8	CN64	Binh Tan, Vinh Long
9	CN6501	Can Tho city, Can Tho
10	CN6502	Con Au, Can Tho
11	CN95	Phung Hiep, Hau Giang
12	CN6701	Long Xuyen city, An Giang
13	CN6702	Nui Cam, An Giang
14	CN68	Hon Dat, Kien Giang
15	CN83	Ke Sach, Soc Trang
16	CN6901	Ca Mau city, ***
17	CN6902	U Minh, Ca Mau

2.2. Genomic DNA isolation

CTAB method (Doyle and Doyle, 1990) was applied for DNA extraction, and all accessions were stored at -20^{o}C for further use. The DNA quantity and quality were measured via Eppendorf BioPhotometer D.30 spectrometer **(**Eppendorf, German) before ISSR analysis and sequencing.

2.3. ISSR-PCR analysis

Each PCR reaction contains 20 µl mixture including 2X Master mix (Taq DNA Polymerase, dNTPs, $MgCl_2$ and Reaction buffers from NEXpro, Korean), Ultrapure water and ISSR primers.

ISSR profiles (Table 2) and PCR reactions were performed via GeneAmp PCR System 2700 (Applied Biosystems – USA). There were two melting steps at 95 C^0, the first step was started for 5 min and 20 sec. for the second one, followed by 35 cycles. Annealing step was setup based on Tm of each primer (Table 2), such as 48 ^{0}C and 52 ^{0}C for ISSR05 and ISSR10, respectively. The primer extension step was performed at 72 ^{0}C for 30 sec. and 5 min as the final step. All ISSR-PCR products were separated by 8% polyacrylamide gel electrophoresis, and the gels were stained with GelRed, Ethidium bromide substitution, eventually. To determine the size of each DNA band, 1 kb plus DNA ladder was used as the molecular weight marker (M).

Table 2. *The ISSR profiles using in the experiment*

ISSR Primers	Sequence (5' – 3')	Tm (^{0}C)	Reference
ISSR01	CACACACACACAAG	40	Nirmaladevi *et al*. (2016)
ISSR05	CTCTCTCTCTCTCTTG	48	Naganur *et al*. (2017)
ISSR06	GAGAGAGAGAGAGAGAC	48	
ISSR08	GAGAGAGAGAGAGAGAT	50	
ISSR10	ACACACACACACACACG	52	
ISSR11	CACCACCACGC	38	Nirmaladevi *et al*. (2016)
ISSR14	AGCAGCAGCAGCGT	55	Naganur *et al*. (2017)
ISSR15	TCCTCCTCCTCCTCC	42	Latif *et al*. (2013)
ISSR17	GTGTGTGTGTGTCC	55	
ISSR18	AGGTCCAGCAGCAGCAGCAG	51	

2.4. Data analysis

PIC (Polymorphism information content) of ISSR markers as the mean of the PIC of each allele (Kempf *et al*., 2016). ISSRs are the dominant markers (Botstein *et al.*, 1980) so the formula for dominant markers from **(**Roldan Ruiz *et al.*, 2000) was used to calculate the PIC value as: $PIC_i = 2f_i(1-f_i)$, where PIC_i is the polymorphism information content of allele i, f_i is the frequency of the amplified fragments and $1-f_i$ is the frequency of non-amplified fragments. A dendrogram among 17 accessions was constructed using the hierarchical cluster analysis based on the unweighed pair-group method algorithm (UPGMA) by STATISTICA ver 5.5 (StatSoft, 2000). The molecular weights were measured by GelAnalyzer sofware (Istvan, 2010). One-way analysis of variance (ANOVA) were used to examine the relationship between leaf length and width using IBM SPSS Statistics 22. The data set includes data on leaf length, width and length to width ratio. There were two stages of data analysis process. The first stage included a descriptive analysis to describe the distribution of the data. The second were used one-way analysis of variance to measure the data. Four unidentified individuals contained CN6702, CN6701, CN6501 and CN50 were selected for the chain termination method of DNA sequencing, also known as the Sanger's method (Sanger *et al*., 1977). The results of sequencing were checked visually using BioEdit Sequence Alignment Editor v.7.2.5 (Hall, 1999), and additionally

searched on The BOLD Identification System (IDS) for identification (Ratnasingham *et al*., 2007).

3. RESULTS AND DISCUSSIONS

3.1. DNA profiles

DNA fragments of 17 accessions were revealed on agarose gel 1% (Figure 1), and as the result of DNA quantification (Table 3). High quality DNA template with the ratio between 1.71 and 2.15 under $OD_{260/280}$ by Nanodrop Spectrophotometer indicating that CTAB was a suitable method to extract high quality DNA from samples. The highest yield absorbed from experiments was 21,165 ng (Sample 12), and the lowest was 6,453 ng (Sample 17). All DNA samples were efficient to perform the PCR step.

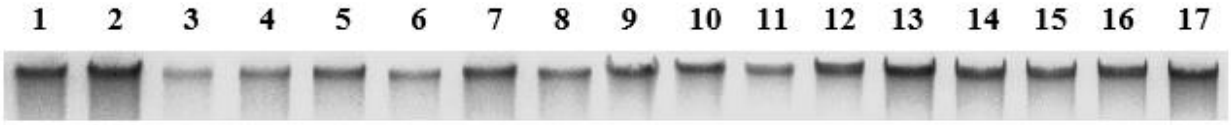

Figure 1. *DNA bands of 17 accessions on agarose gel 1%*

Table 3. *The quality and quantity of total DNA isolated from 17 protocols*

Code	Concentration (ng/µl)	$OD_{260/280}$	Productivity (ng/100mg)
1	383.8	1.87	11,514
2	427.2	1.87	12,816
3	324.1	1.79	9,723
4	576.5	1.80	17,295
5	695.7	1.92	20,871
6	639.2	1.88	19,176
7	497.1	1.96	14,913
8	264.1	2.15	7,923
9	332.6	1.71	9,978
10	264.3	1.96	7,929
11	459.7	1.75	13,791
12	705.5	1.86	21,165
13	357.4	1.83	10,722
14	447.4	1.87	13,422
15	545.8	1.92	16,374
16	598.2	1.96	17,946
17	215.1	1.81	6,453

3.2 The assessment of genetic diversity among 17 accessions in this study

The results of ISSR analysis were shown in Table 5 indicating that there were 106 scorable bands in total within 94 bands were polymorphisms, and sharing an average of 9.40 scorable bands per primer. Three primers scored 100% of polymorphism were ISSR05, ISSR14 and ISSR15 in which the average proportion of polymorphic band was good, 85.90% per primer. Following to Botstein *et al*. (1980), PIC value was confirmed based on variation between 0.0 and 0.5, and above 0.5 were effective and informative markers. In this study, PIC_{Av} values absorbed from ISSR analysis mostly ranged from 0.18 (ISSR11) to 0.32 (ISSR15), and ISSR15 marker showed the highest value of PIC_{max} (0.50).

Table 5. *Genetic diversity parameters estimated by using 10 ISSR primers*

Primer	TB	PB	PPB (%)	PIC_{Av}	PIC_{min}	PIC_{max}	Size (bp)
ISSR01	15	13	86.67	0.27	0.00	0.50	600 – 6,000
ISSR05	3	3	100	0.26	0.21	0.29	1,000 – 3,000
ISSR06	11	10	90.91	0.25	0.00	0.50	1,500 – 10,000
ISSR08	15	14	86.67	0.28	0.00	0.48	500 – 10,000
ISSR10	12	10	84.62	0.24	0.00	0.46	1,000 – 4,000
ISSR11	6	3	66.67	0.18	0.00	0.48	1,500 – 10,000
ISSR14	18	18	100	0.31	0.11	0.50	1,000 – 10,000
ISSR15	13	13	100	0.32	0.11	0.50	900 – 10,000
ISSR17	6	5	85.71	0.27	0.00	0.50	1,300 – 10,000
ISSR18	7	5	71.42	0.27	0.00	0.48	700 – 10,000
Total	106	94					
Average	10.60	9.40	85.90	0.27			
SD	±4.88	±5.19	±15.59	±0.04			

Note: (TB) Total bands, (PB) Polymorphic bands, (PPB) Percentage of polymorphic bands, (PIC) Polymorphic information content, (PIC_{Av}) PIC average, (PIC_{min}) PIC minimum, (PIC_{max}) PIC maximum, (SD) Standard deviation

3.3 ISSR profile of 17 accessions

ISSR06 could be used as a marker in order to identify CN95 by the specific polymorphic fragments at 3837 bp (Figure 2).

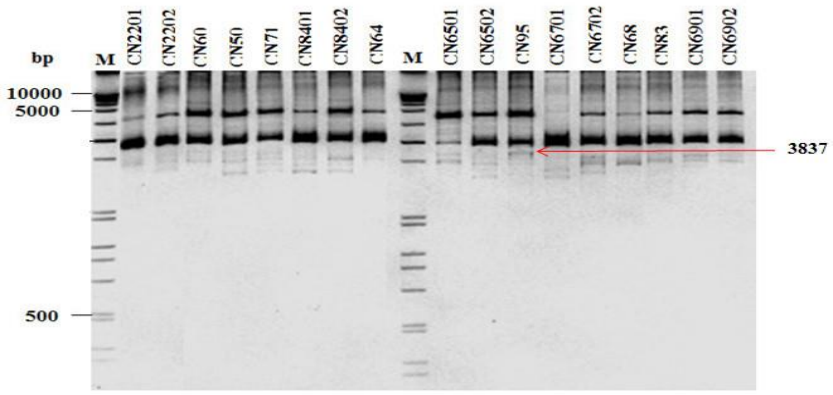

Figure 2. *ISSR profile from 17 accessions using ISSR06 primer (M:1 kb plus molecular DNA ladder - Invitrogen, USA)*

3.4 The results of DNA analysis of 4 unidentified individuals using rbcL marker

An amplified fragment of 546 bp was scored by 4 unidentified samples (CN6702, CN6701, CN6501, CN50) using rbcL marker. According to the result (Table 6), three accessions (CN6702, CN6701, CN6501) presented the highest similarity index of percentage (99.83%) after using BLAST method compared to numerous identified species. CN50 showed correct plant identification of 100% of *Clinacanthus nutans* (Burm.f.) Lindau. Interestingly, point (substitution) mutations occurred in three samples (CN6702, CN6701, CN6501) in which one base (Adenine) located at position of 266 of original nucleotide sequence was exchanged to Guanine (CN6702), while both CN6501 and 6701 shared similar replacement at position of 397 (Figure 3). In the case, these three samples were considered to be a subgroup of *Clinacanthus nutans* population in Vietnam, thereby, numerous analysis of evaluation were performed in order to demonstrate the genetic diversity of selected accessions.

Table 6. *Basic results of DNA analysis of 4 samples*

Sample	BLAST method on The BOLD Identification System			Reference
	Species	GenBank Accession	Similarity (%)	
CN6702	*Clinacanthus nutans* L.	GQ436501	99.83	Chen *et al*. (2009)
CN6701	*Clinacanthus nutans* L.	GQ436501	99.83	
CN6501	*Clinacanthus nutans* L.	GQ436501	99.83	
CN50	*Clinacanthus nutans* L.	GQ436501	100	

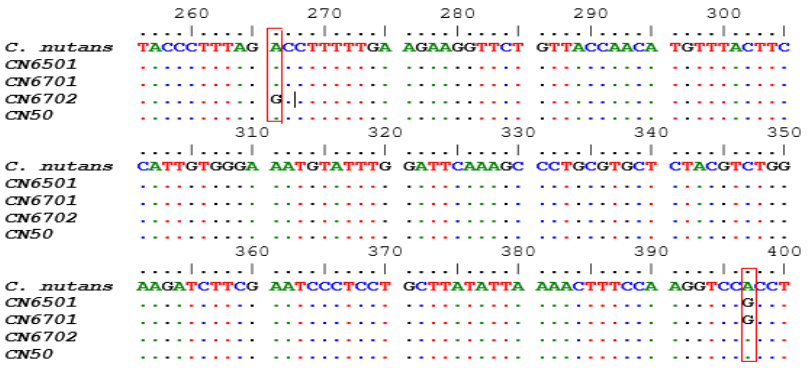

Figure 3. *Result of point mutations of 4 individuals compared to orignal DNA sequence*

3.5 Relationship among accessions

Figure4.*Morphology of four representative samples*

After analyzing several databases of leaf length, leaf width and L:W ratio carefully (Table 7), the results of one way ANOVA with Duncan analysis were summerized (Table 8). A bar chart of leaf length/width (L:W) ratio (Figure 4) was designed to provide a closer demonstration of the relationship of 17 accessions based on (Table 8) within a significant effect ($p<0.001$).

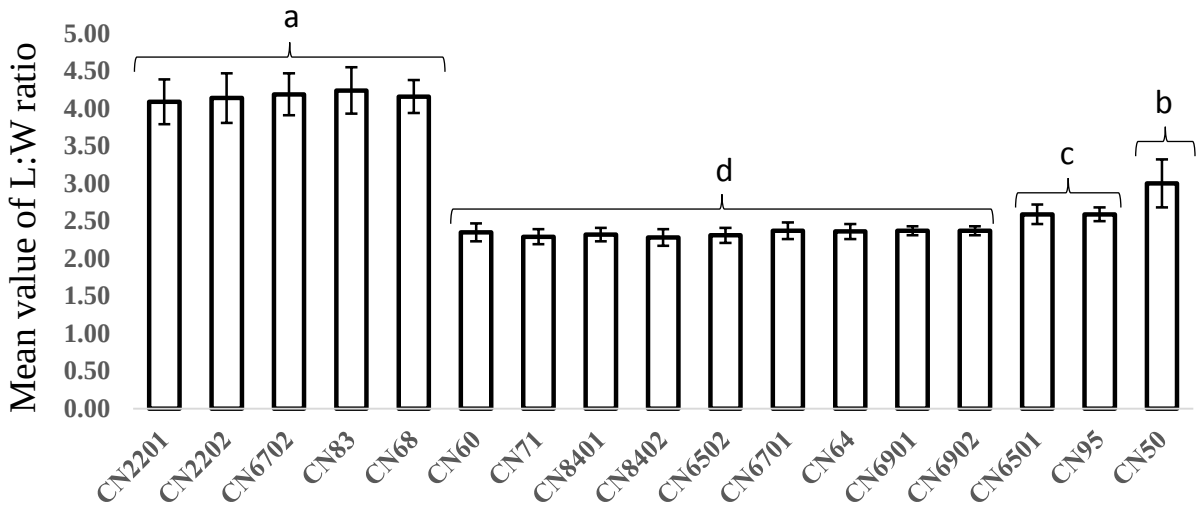

Figure 5. *Bar chart of mean value of leaf length/width measuring by 17 accessions within a significant effect (p<0.001); (a,b,c,d: groups of individuals).*

Additionally, a dendrogram in Figure 5 showed the relationship analysis of 17 accessions orginated from Vietnam, by grouping based on the band pattern. The coefficients of relationships (Figure 5) ranged from 0.01 to 0.70, consisted of four clusters with the average genetic distance of approximately 0.301.

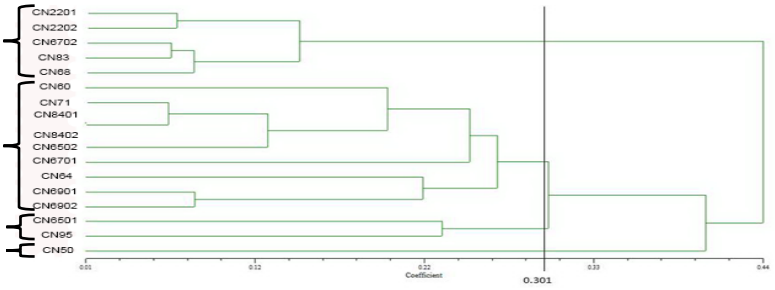

Figure 6. *The dendrogram illustrated the genetic similarity and relationship of 17 a obtained from ISSR markers based on UPGMA cluster analysis using STATISTICA ver. 5.5.*

Interestingly, the classification (Figure 4) was completely matched with the dendrogram based on genetic similarity (Figure 5). By the way, 17 accessions were officially divided into four main cluster. The first cluster contained 5 accessions (CN2201, CN2202, CN6702, CN83, CN68). The second cluster included 9 accessions (CN60, CN71, CN8401, CN8402, CN6502, CN6701,

CN64, CN6901, CN6902). The third cluster consisted of two accessions (CN6501 and CN95). The fourth cluster had only one accession (CN50). With p-value less than 0.001, this conclusion was perfectly acceptable. On the other side, it could be concluded that the *Bim bip* (*Clinacanthus nutans* Burm.f.) Lindau population in Vietnam was able to initially classify based on phenotypic expression rather than genotypic evaluation.

4. CONCLUSION

The result of this study shows that 17 samples of *Clinacanthus nutans* collected in Vietnam cuold be classified into four groups which have differences in morphology and genotype. This study shows that the genetic diversity of *Clinacanthus nutans* is low and may affect its long-tem survival and evolution if current methods of vegetative propagation from the same parent plant continue. Therefore, understanding and obtaining information on the levels and patterns of genetic diversity are essential for assisting the design of effective species convervation strategies. Studies on genetic diversity are necessary to discover novel factors that can contribute to increased heterogeneity and are valuable for preserving biodiversity. The result in species discrimination by determination of gene sequence shows that *Clinacanthus nutans* exists in Vietnam and there are some lower taxa of *Clinacanthus nutans*. Therefore, there is diversity in population of *Clinacanthus spp* in Vietnam. To our knowledge, this study is the first to report on the botany, the genetic diversity and species discrimination by determination of gene sequence of *Clinacanthus nutans* population in Vietnam. This study provides fundamental knowledge on existing cultivars and may assist in future practices and breeding programs to increase biodiversity and favourable traits.

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Comparison of Phytochemicals, Microscopicals and TLC Profiles of Thanaka Bark and Wood

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Abstract

Thanaka (*Naringi crenulata* (Roxb.) Nicolson Family Rutaceae), its stem bark has been traditionally used as natural cosmetics. Different Thanaka extracts have also been proven by both *in vitro* and *in vivo* studies for having antioxidant, anti-inflammatory, antityrosinase, anti-aging and antimicrobial activities. Nowadays, there is an increasing demand in cosmetics industry and due to the higher yield of wood than bark, the wood may be used instead of bark or both parts are used. However, the commercial Thanaka in the market are usually in the form of powder which cannot be identified. The aim of the study was to find out the phytochemicals, microscopical characters, and Thin Layer Chromatography (TLC) fingerprints of Thanaka bark and wood. For both Thanaka bark and wood, phytochemical screening tests for active constituents of methanol extract were initially carried out. Microscopical characters of powder materials were studied. Two developing solvent systems, hexane-ethyl acetate (7 : 3) and dichloromethane-acetonitrile-formic acid (5 : 5 : 0.1), were used for development of TLC profiles. The results showed that the combination of the microscopical characters and TLC fingerprints of both solvent systems can be used to distinguish Thanaka bark from wood and thus this combination can be utilized for quality control purpose of commercially available Thanaka products.

Keywords: Thanaka, *Naringi crenulata*, phytochemicals, microscopical characters, TLC fingerprint

1. INTRODUCTION

Thanaka, *N. crenulata* (Synonyms *Hesperethusa crenulata* (Roxb.) M. Roem. and *Limonia crenulata* Roxb.), is a tropical plant species grown in Indian subcontinent and South East Asia[1, 2]. It is a deciduous spinuous small tree and generally distributed in the dry regions. Leaves compound, unipinnate, imparipinnate, petiole and rachis jointed, petiole narrowly winged and rachis broadly winged as if the leaflets. Leaflets 2-3 pairs, notched at the top, crenulate or

crenuate margin, opposite, sessile, oblong, obtuse. On the surface of the leaflets, there are conspicuous gland-dotted. Flowers umbelliform, racemose. Calyx 4, petal 4, stamen 8, ovary 4 with one ovule per locule. Ovary supported on a disk stipitiform. The fruits globose, around 6 mm in diameter, rind black, texture smooth, firmness membraneous[1].

Thanaka is used in Myanmar and in India. In India, Thanaka is commonly known as Magavilvam in *Tamil*, Beli in *Hindi*, and Kattunarakan in *Malayalam* [3].In Indian folk medicines, leaf can cure digestive disorder and it is also a remedy for epilepsy, fruit decoction is used as antidote to insect poison, root extract is used to induce vomiting and to treat dysentery and colic disorders, and bark juice is applied externally to relief sprain [4, 5] .In Myanmar, although Thanaka is used as traditional medicine, it is commonly used and widely known for other purpose as natural skin care and cosmetics. The bark of Thanaka is ground to a pale yellow paste with water and applied on the skin for many purposes such as photo protection, acne treatment and prevention, skin lightening, skin cooling, cosmetic purpose as make up, wrinkle reduction, pruritus relief, scar reduction, and odor prevention [6].
Thanaka leaf extracts showed *in vivo* anti-inflammatory activity, antimicrobial activity, *in vitro* anti-oxidant activity [7, 8]. Thanaka bark showed varied in vitro antioxidant, antityrosinase, anti-inflammatory, and anti-aging activity based on the polarity of extraction solvents [7-10]. Water extract of Thanaka wood also shows *in vitro* antioxidant activity [11].

Only the outer bark of the stem of Thanaka is used traditionally for cosmetic purpose and for prevention and treatment of some dermatological related problems [6]. The inner wood that has high fibre contents and difficult to grind and thus the wood is not used. Nowadays, due to the increasing demand of Thanaka in cosmetics industry and the higher yield of wood than bark, the wood may be used instead of bark or both parts are used together. Moreover, the commercially available Thanaka is usually in powder form so it cannot be identified the contents with the naked eyes. Therefore, the microscopical characters of powder material samples and the phytochemical constituents and phytochemical profiles of methanol extract of both Thanaka bark and wood were studied.

2. MATERIALS AND METHODS

2.1. Plant materials

Both authentic samples and commercial samples of Thanaka were studied. The authentic samples were collected from Ayadaw, Sagaing Region, Myanmar (including fertile specimens, sterile specimens and wood collections). The plant was checked through by local harvesters first and then identified and characterized by comparing morphological and microscopical characters with herbarium specimens at the Forest Herbarium, Bangkok, Thailand (BKF). The commercial

Thanaka samples were bought from the local market, Mandalay, Myanmar and identified. The bark and wood of Thanaka stems were prepared separately. The samples were cut into small pieces, dried in the hot air oven at 60°C for 1 week, and then ground to get the powdered plant materials.

2.2. Preparation of Plant extracts

The dried powdered plant materials were macerated with methanol for 24 hours and filtered. The filtrates were evaporated to dryness by using rotary evaporator to get the crude methanol extract. These extracts were used for carrying out phytochemical screening and TLC fingerprints.

2.3. Phytochemical screening of crude methanol extracts

Crude methanol extracts were tested for the presence of active principles including alkaloids, flavonoids, tannins, coumarins, triterpenoids, steroids, lactones, and deoxy sugars according to the following procedures [12-16].

2.3.1 Test for Alkaloids

Crude methanol extracts are re-extracted with 5% HCl and filtered. The filtrate was used as the test solution.

Mayer's Test: Test solution was treated with Mayer's reagent. Formation of a cream coloured precipitate indicates the presence of alkaloids.

Wagner's Test: Test solution was treated with Wagner's reagent. Formation of reddish-brown precipitate indicates the presence of alkaloids.

Dragendroff's Test: Test solution was treated with Dragendroff's reagent. Formation of reddish-brown precipitate indicates the presence of alkaloids.

Hager's Test: Test solution was treated with Hager's reagent (saturated picric acid solution). Formation of yellow coloured precipitate indicates the presence of alkaloids.

2.3.2 Test for Flavonoids

Test solution was prepared by re-dissolving the extracts in 95% ethanol.

Shinoda's test: Small pieces of magnesium ribbons were added into the test solution followed by adding concentrated hydrochloric acid dropwise. A pink or red colour indicates the presence of flavonoids.

Sodium hydroxide test: A few drops of 10% NaOH solution was added into the test solution, the changing of solution into yellow color indicates the presence of flavonoids. This yellow color will turn to colorless when adding dilute hydrochloric acid.

2.3.3 Test for Tannins

Test solution was prepared by reconstituting the extracts with 95% ethanol.

Gelatin test: 1% gelatin solution containing sodium chloride was added into the test solution. Formation of white precipitate indicates the presence of tannins.

Ferric chloride test: A few drops of diluted solution of $FeCl_3$ was added into the test solution, production of a blue or greenish-black color that changes to olive green as more ferric chloride was added indicates the presence of tannins.

Lead acetate solution test: Test solution when treated with few drops of lead acetate (10%) solution would result in the formation of yellow precipitate.

2.3.4 Test for steroids and triterpenoids

Crude methanol extracts was re-extracted with chloroform and filtered. The filtrate was used as the test solution.

Lieberman-Burchard's test: Equal volume of acetic anhydride was added into the test solution and gently mixed. After that 1 ml of concentrated sulfuric acid was gently then added into the tube. The appearance of a brownish-red ring at the contact zone of the two liquids layers and a greenish color in the separation layer indicates the presence of sterols and triterpenes .

Salkowski's test: A few drops of concentrated sulfuric acid was added into the test solution, shaken, and then allowed to stand. The appearance of golden yellow color at the lower phase indicates the presence of triterpenes and the formation of reddish-brown colour at the inter phase indicates the presence of steroidal ring.

2.3.5 Test for coumarins

Test solution was prepared by reconstituting the extracts in 95% ethanol and put into the Erlenmeyer flasks. A sodium hydroxide paper was prepared by rapidly immersion of a piece of filter paper into 1M sodium hydroxide solution and then air-dried in a fukme hood. The sodium hydroxide paper is placed on the month of the Erlenmeyer flasks which were further warm on the water bath. After 5 minutes, the sodium hydroxide paper was examined under UV 366nm. The intense green fluorescence indicates the presence of coumarins.

2.3.6 Test for lactone ring (Kedde's test)

Test solution was prepared by re-extracting with chloroform. Kedde's reagent and sodium hydroxide solution were added. The presence of bluish violet or pink violet indicates the presence of compounds containing lactone ring.

2.3.7 Test for deoxy sugar (Keller-Killiani's test)

Test solution was prepared by re-extracting with chloroform. 10% Ferric chloride in glacial acetic acid was added. Concentrated sulphuric acid was then added dropwise. The presence of decoxy sugar can be notified with the presence of reddish-brown color ring between two layers which gradually becomes blue.

2.4. Microscopical characters studies

The fine powdered plant samples were firstly cleared with chloral hydrate solution and then mounted with glycerine water and staining reagents such as aniline sulphate, iodine water, and sudan Ⅲ for microscopic studies.

2.5. Thin Layer Chromatography (TLC) fingerprint analysis of Thanaka bark and wood

Methanol extracts of Thanaka bark and wood were reconstituted with methanol to get 1mg/30 ul concentration. These reconstituted methanol extracts were spotted on a precoated silica gel 60 GF_{254} TLC aluminum plates. The TLC plates were developed in two solvent systems, which were hexane : ethyl acetate (7:3 v/v) and dichloromethane : acetonitrile : formic acid (5: 5: 0.1 v/v/v). The developing distance was 65 mm. After being removed from the developing chamber, the TLC plates were air dried in a fume hood and then examined under UV light at wavelengths of 254 and 366 nm and under white light after spraying with anisaldehyde and sulphuric acid visualizing reagent. The 0.2% DPPH solution was prepared and used as spraying reagent for TLC plate to screen for the antioxidant activity of the separated compounds. The image of TLC fingerprints of Thanaka extracts were recorded.

3. RESULTS AND DISCUSSION

3.1 Taxonomic identification

The plant samples (THlaing001) from Ayadaw, Sagaing Region, Myanmar were identified as *Naringi crenulata* (Roxb.) Nicolson Family Rutaceae base on taxonomic key and by comparison with herbarium specimens at the Forest Herbarium, Bangkok, Thailand (BKF). The voucher specimens were deposited at the Pharmaceutical Botany, Mahidol University Herbarium (PBM), Faculty of Pharmacy, Mahidol University, Thailand.

3.2 Phytochemical screening

From the preliminary phytochemical screening, both methanolic extracts of Thanaka bark and wood contained alkaloids, flavonoids, tannins, steroid and triterpenoid, coumarin, lactone ring, and deoxy sugars as shown in Table 1. Since both bark and wood extracts were precipitated by all four reagents used for testing alkaloids thus alkaloids were present in both.

The green to blue-green coloration with ferric chloride indicate the presence of phenolic hydroxy groups. Moreover, both bark and wood were positive to Shinoda's test and sodium hydroxide test, thus flavonoids were present in both. The white precipitate formed from gelatin test was the good indicator of the presence of astringent principles, tannins in the extracts. The distinct rings were seen in the contact zone of two from Lieberman-Burchard's test and from the Salkowski's test, it is noticeable that the lower liquid layer was the yellow color. This means that the extracts had not only steroids but also triterpenoid compounds. By testing with alkali solution, sodium hydroxide, in UV 366 nm, intense light blue fluorescence was seen in both bark and wood, thus it was possible that umbelliferone, 7-hydroxycoumarins were present. The positive reaction to Keller-Killiani's test means that the extracts had deoxy sugar. It is probable that many different kinds of glycosides were present in these methanol extracts.

3.3 Microscopical characteristics of powder materials

From powder materials of authentic Thanaka bark, plenty of cork cells, sclereids, oily materials, fibres with crystals insides, calcium oxalate crystals, and a few of starch granules were found (Figure 1.). In Thanaka wood samples, plenty of medullary rays, bordered pitted xylem vessels, xylem fibres with a few of oily materials, calcium oxalate crystals, and starch granules were found (Figure 2.). It is cleared that cork cells and sclereids are the anatomical components of bark, whereas medullary rays, xylem vessels, and xylem fibres are the components of wood. However, even though oily materials and calcium oxalate crystals were found out in both bark and wood, the ratio of their contents varied very differently. The amount of oil globules and calcium oxalate crystals in the wood were lower in proportion than the bark. Since the traditional use of Thanaka is the bark, base on the microscopical characteristics, any adulterant of bark with wood can easily be identified.

3.4 Thin layer chromatographic analysis

The district TLC fingerprints of Thanaka bark and wood can be observesed by TLC developing in both solvent systems of hexane-ethyl acetate (7: 3 v/v) and dichloromethane-acetonitrile-formic acid (5: 5: 0.1 v/v/v) (Figure 3.) which are suitable for separation of non-polar and polar components, respectively.

By TLC developing with hexane-ethyl acetate (7: 3 v/v) solvent system (Figure 3; 1A-C), the quenching bands under UV 254nm at R_f value of around 0.45 and 0.75 were only noticed in the bark extracts (lanes 1-4) which were not seen in wood extracts (lanes 5-8). Moreover, bark extracts also present the intense fluorescent bands under 366nm at R_f value of 0.47. These bands can act as the markers for non-polar compounds of bark extracts. Whereas, for the wood, it is

cleared that at the R_f value of 0.7, there were blue bands after spraying with anisaldehyde and sulphuric acid visualizing agents.

By TLC developing with dichloromethane-acetonitrile-formic acid (5: 5: 0.1) solvent system (Figure 3; 2A-C), all the bark extracts (lanes 1-4) had common quenching bands under UV 254nm at R_f of 0.35 whereas all wood extracts had quenching bands under UV 254 nm which is a little bit higher than that in bark, at R_f of 0.44. Moreover, in the plate both UV 366 nm and white light after derivatizing with anisaldehyde and sulphuric acid visualizing agent, it is noticable that at the R_f value less than 0.3, there were many bands in wood extracts (lanes 1-4) but not in the bark. Therefore, these bands could be the markers for wood extracts and the presence of these bands can easily identified out Thanaka wood if adulteration in Thanaka bark. By spraying the developed TLC plate with 0.2% DPPH solution, it can be seen that bands in both bark extracts and wood extracts present DPPH free radicals scavenging activity. The compounds in bark showed stronger inhibition than compounds in wood.

4. CONCLUSION

Since authentication of medicinal plant materials is the first and foremost step for the research purpose and commercially use. The differences in microscopic characters of both bark and wood and TLC fingerprints of bark and wood extracts in both developing solvent systems described could be used as markers for identification of bark and wood. Therefore, the combination of microscopic anatomical characters and phytochemical markers of TLC fingerprint results can be a very cost effective and remarkably straightforward procedure for identification and quality control of commercially available Thanaka samples.

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Legends for figures

Figure 1. *Microscopic identification of Thanaka bark (400×). A, cork cells in surface view stained with Sudan III solution; B, sclereids; C, starch granules stained with iodine solution; D, oily materials stained with Sudan III solution; E, fibres with prism of calcium oxalate crystals inside stained with aniline sulphate; F, prism of calcium oxalate crystals.*

Figure 2. *Microscopic identification of Thanaka wood (400×). A, medullary rays stained with aniline sulphate; B, bordered pitted xylem vessels stained with aniline sulphate; C, starch granule stained with iodine solution; D, brownish yellow oily materials; E, xylem fiber; F, prisms of calcium oxalate crystals.*

Figure 3. *TLC chromatogram of methanol extracts of Thanaka bark and wood. Lane 1, authentic Thanaka bark sample; lanes 2-4, commercial Thanaka bark samples; lane 5, authentic Thanaka wood sample; lanes 6-8, commercial Thanaka bark samples. Solvent system: 1, hexane-ethyl acetate (7: 3 v/v); 2, dichloromethane-acetonitrile-formic acid (5 : 5 : 0.1) Detection : A, UV 254 nm; B, UV 366 nm; C, white light after spraying with anisaldehyde and sulphuric acid visualizing reagent.*

Figure 4. *TLC chromatogram of methanol extracts of Thanaka bark and wood. Lane 1, authentic Thanaka bark sample; lane 2, authentic Thanaka wood sample. Solvent system, dichloromethane-acetonitrile-formic acid (5 : 5 : 0.1) Detection: A, UV 254 nm; B, UV 366 nm; C, white light after spraying with 0.2% DPPH solution.*

Legend for table

Table 1. *Phytochemical screening of methanolic extracts of Thanaka bark and wood*

Figure 1

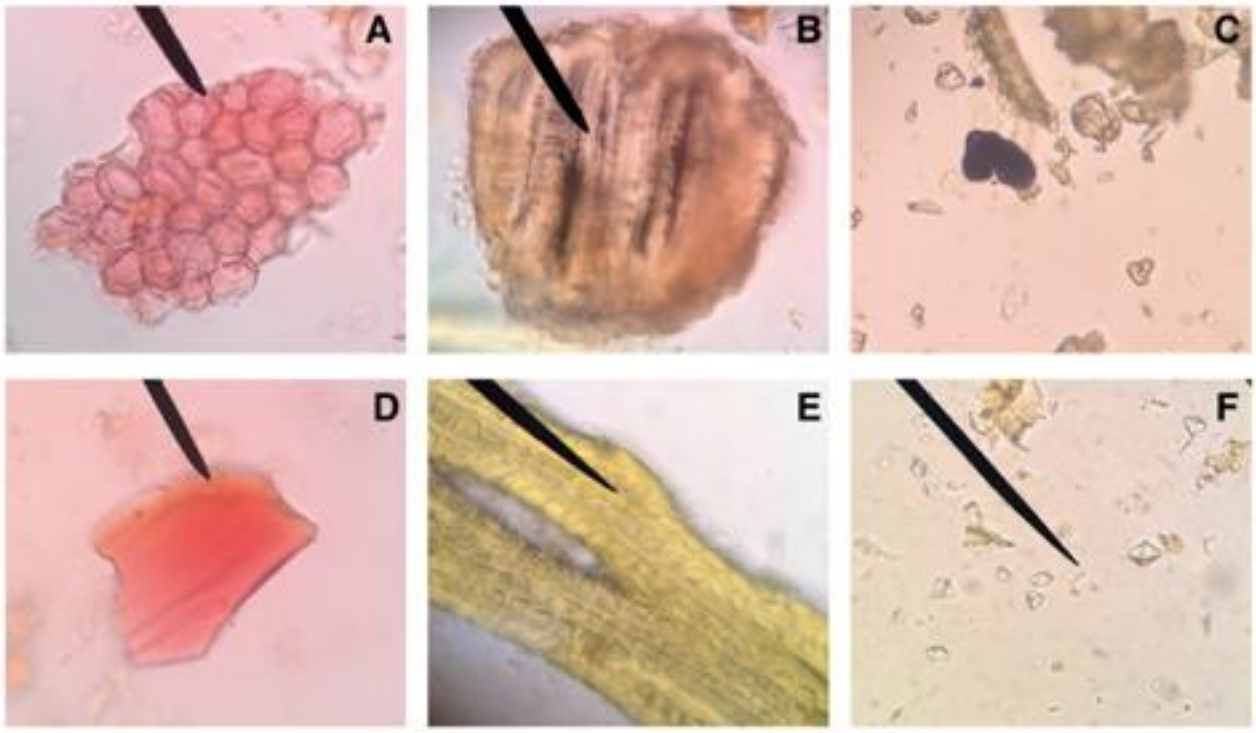

Figure 2

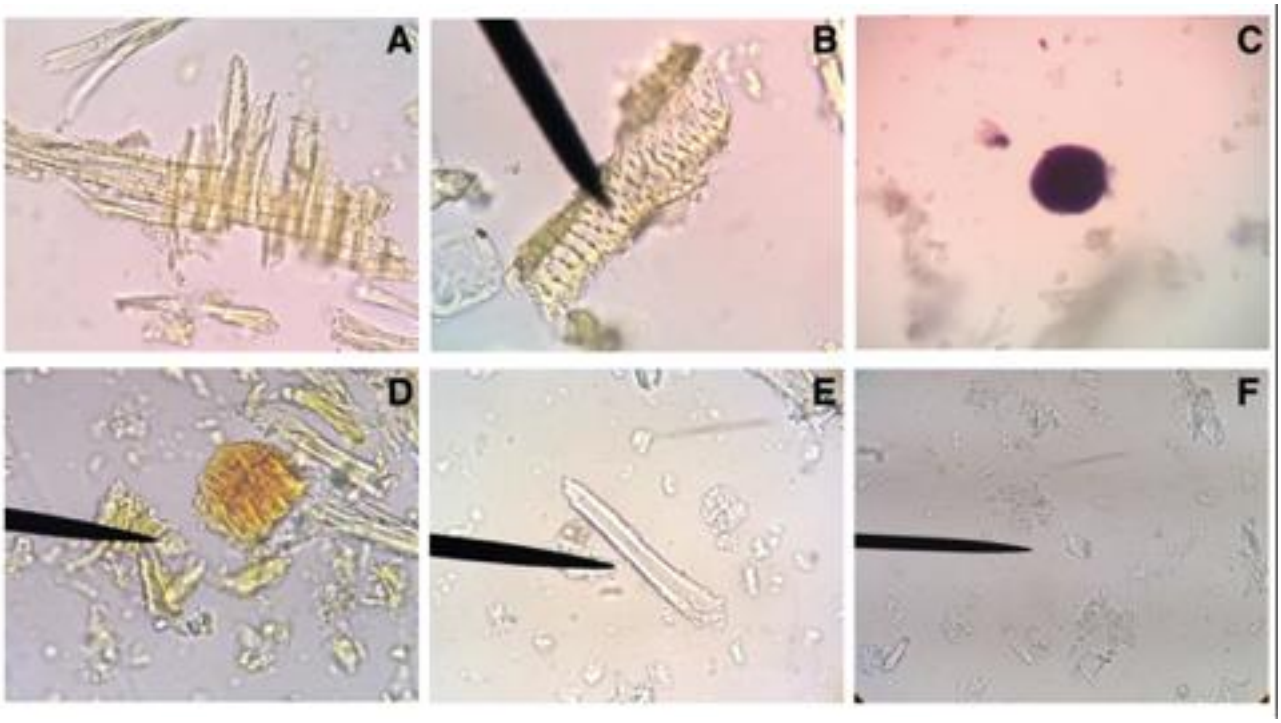

Figure 3

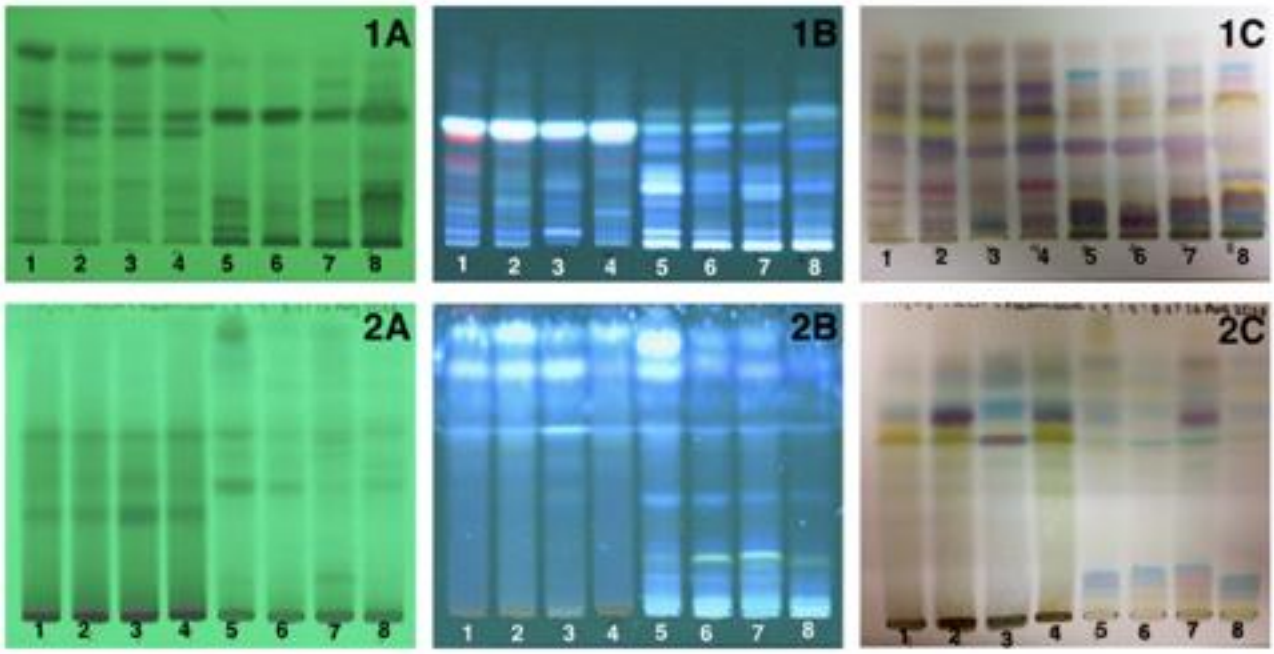

Figure 4

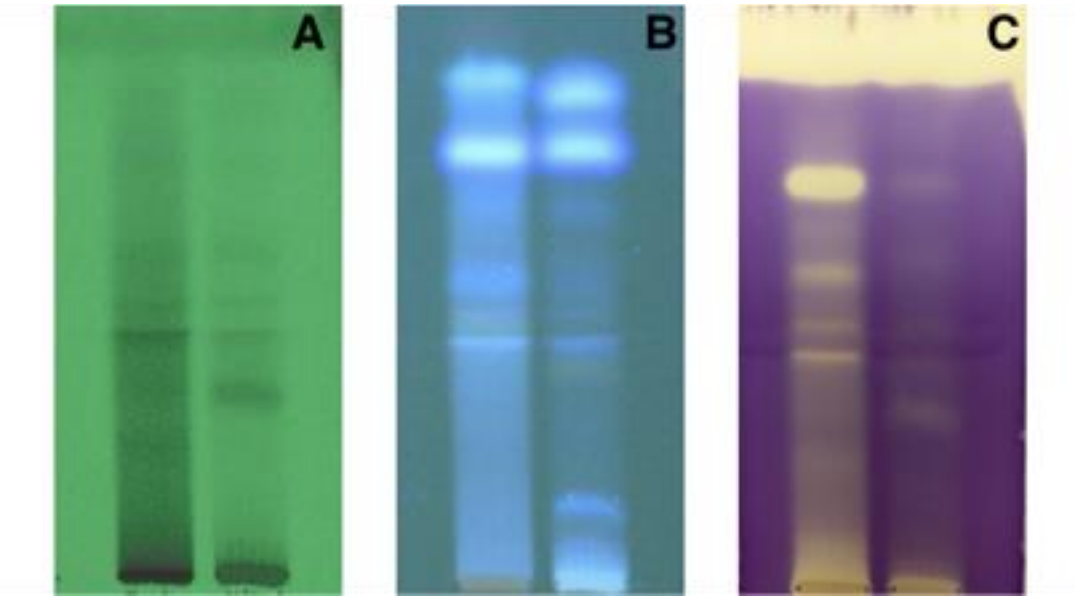

Table 1

Phytochemical tests	Bark	Wood
Alkaloid		
Mayer's test	+	+
Wagner's test	+	+
Dragendroff's test	+	+
Hager's test	+	+
Flavonoid		
Shinoda's test	+	+
Sodium hydroxide test	+	+
Tannin		
Gelatin test	+	+
Ferric chloride test	+	+

Lead acetate solution test	+	+
Steroid and triterpenoid		
Lieberman-Burchard's test	+	+
Salkowski's test	+	+
Coumarin	+	+
Lactone ring	+	+
Deoxy sugar	+	+

Simultaneous and Direct Determination of Glycine, Cysteine and Ammonium Glycyrrhizinate by Hydrophilic Interaction Liquid Chromatography

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Abstract

Introduction: Hydrophilic Interaction Liquid Chromatography (HILIC) is a chromatographic technique characterized using a polar stationary phase and a relatively nonpolar mobile phase containing a small amount of water. It is therefore exploited for separation of polar and hydrophilic compounds. **Objectives:** In this study, HILIC was used to simultaneously determine glycine, cysteine and ammonium glycyrrhizinate in a hepatic formula. **Materials and methods:** Various analytical conditions were screened i.e. sample treatment, stationary phase, mobile phase (phosphate buffer : acetonitrile ratio, pH and concentration of buffer), temperature, and detection wavelength. The method was validated according to AOAC 2016 in terms of system suitability, selectivity, linearity, repeatability and accuracy. It was then applied for determination of glycine, cysteine and ammonium glycyrrhizinate in an intravenous formulation. **Results:** Samples were diluted 1:10 with water and 5-µl aliquots were then chromatographically analyzed using an Agilent Zorbax silica column (250 × 4.6 mm, 5 µm) maintained at 30^{o}C. The mobile phase consisted of a mixture of acetonitrile : phosphate buffer 3 mM, pH 3.25 (75 : 25 v/v). The detector wavelengths were set at 200 nm for glycine and cysteine, and 254 nm for ammonium glycyrrhizinate. The linearity ranges were of 54.3-162.8 µg/ml; 987.8-2963.4 µg/ml and 133.2-399.6 µg/ml for cysteine, glycine and ammonium glycyrrhizinate, respectively. Total analysis time lasted around 13 minutes without having to use sample pretreatment. The method proved to be selective, repeatable (RSD < 2%), and accurate (percentage recovery 98.5-101.7%). It was successfully applied to assay glycine, cysteine and ammonium glycyrrhizinate (95.4-99.4 % of the content stated on the label). **Conclusion:** A reliable and simple HILIC method was developed to directly and simultaneously analyze cysteine, glycine and ammonium glycyrrhizinate in an intravenous formulation. It could significantly improve analytical throughput of glycine and cysteine (high polarity and low ultra-violet absorbance compounds), which usually requires derivatization for HPLC analysis.

Keywords: glycine, cysteine, ammonium glycyrrhizinate, hydrophilic interaction liquid chromatography, HILIC, HPLC

1. INTRODUCTION

Amino acids play an essential role in the body as building blocks of proteins and intermediates in metabolism. Because humans can only produce 10 of the 20 amino acids, the others must be supplied in food and pharmaceuticals to treat debilitating symptoms. They may be also integrated

with other active ingredients for other purposes [1,2]. For instance, formulation containing glycine (GLY), cysteine (CYS) and ammonium glycyrrihizinate (AG) (Figure 1) has been used to reduce adverse effects of escalating aldosterone and enhance detox reactions in the liver [3].

In practice, it is difficult to directly analyze GLY and CYS by RP-HPLC due to their high polarity and weak UV absorption. These amino acids were derivatized before or after chromatogaphic analysis with fluorescence or ultraviolet detection [4]. The sample treatment in a derivatization step is usually complicated and time-consuming.

Hidrophillic interaction liquid chromatography (HILIC) is a chromatographic technique based on the integration of a polar stationary phase and a polar mobile phase containing a small amount of water. Separation mechanism in HILIC is believed to be adsorption and/or partition due to intermolecular forces (e.g. electrostatic interactions, hydrogen bonding, dipole-dipole interactions and weak hydrophobic interaction). Analytes are partitioned between the mobile phase and the water layer covering the stationary phase i.e. polar compounds show higher retention and elute later from the column. Therefore, this technique could be used to retain and separate polar and hydrophilic compounds such as amino acids. The aim of this study was to develop an HILIC method for direct and simultaneous determination of CYS, GLY and AG in an intravenous formulation.

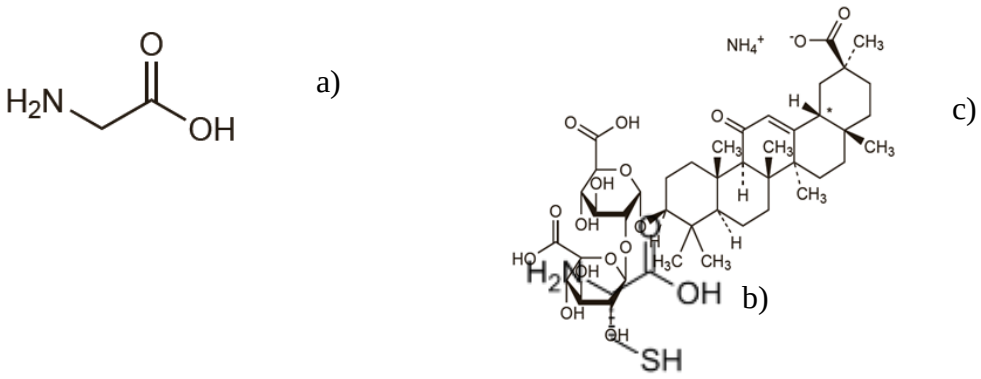

Figure 1. *Chemical structure of a) glycine, b) cysteine and c) ammonium glycyrrhizinate*

2. MATERIALS AND METHODS

2.2. Materials and equipment

Amiphargen IV injection was produced by Taiwan Biotech Co., Ltd. This product contains 53 mg AG, 400 mg GLY, 20 mg L-cysteine HCl, 20 mg sodium sulfite, 12 mg monoethanolamine

and water enough to constitute 20 ml solution. Standards [glycine (98.7%) and L-cysteine hydrochloride (98.5%) from Kyowa Hakko Bio Co., Ltd. and ammonium glycirrhizinate (99.7%) from Shaanxi Fujie Pharmaceutical Co., Ltd.] were used as purchased. Sodium sulfite and monoethanolamine were of excipient grade (Fisher). Acetonitrile and potassium dihydrogen phosphate (Merck, Germany) and phosphoric acid (Scharlau, Spain) were of HPLC grade.

Aqueous solution of sodium sulfite 1 mg/ml (A) was used to prepare CYS, GLY and AG standard solutions as well as monoethanolamine 5% (v/v). A 10-ml placebo solution consisted of 1 ml of monoethanolamine 5% and 1 ml of solution A. 10-ml standard mixtures were prepared from 1 ml of placebo solution and 1 ml of standard solution. Samples were diluted 10 times prior to analysis. All solutions were freshly prepared and light-protected on the day of analysis.

Chromatographic analysis was carried out using an Agilent 1100 HPLC system coupled with Diode Array Detector and Chemstation software.

2.3.Methods

Standard solutions were prepared using different solvents (water, phosphate buffer and a mixture of acetonitrile and phosphate buffer) and chromatographically analyzed using an Apollo column (150 × 4.6 mm; 5 μm) and a mixture of acetonitrile : phosphate buffer 3 mM, pH 3.5 (75: 25) as the mobile phase. The dilution solvent was chosen with reference to peak tailing factor and stability of the analytes under study.

Chromatographic conditions were selected by screening of mobile and stationary phases. The mobile phase consisted of a mixture of acetonitrile and potassium dihydrogen phosphate buffer (1.5-3 mM, pH 2.5-3.5). Two mixture ratios of acetonitrile : phosphate buffer were investigated i.e. 75:25 and 72:28 (v/v). Bare silica stationary phases were from Agilent (250 × 4.6 mm; 5 μm) and Apollo (150 × 4.6 mm; 5 μm). Column temperature was set at 26 or 30^{o}C to increase the repeatability of the assay. The retention time of AG and total analysis time were taken into consideration.

The developed method was validated according to AOAC guideline (2016) [4] in terms of system suitability, selectivity, linearity, repeatability and accuracy. It was then applied to analyze CYS, GLY and AG in Amiphargen IV injection samples.

3. RESULTS AND DISCUSSION

3.2.Dilution solvent selection

Figure 2 displays the chromatograms recorded at 254 nm of standard mixtures in 3 different dilution solvents.

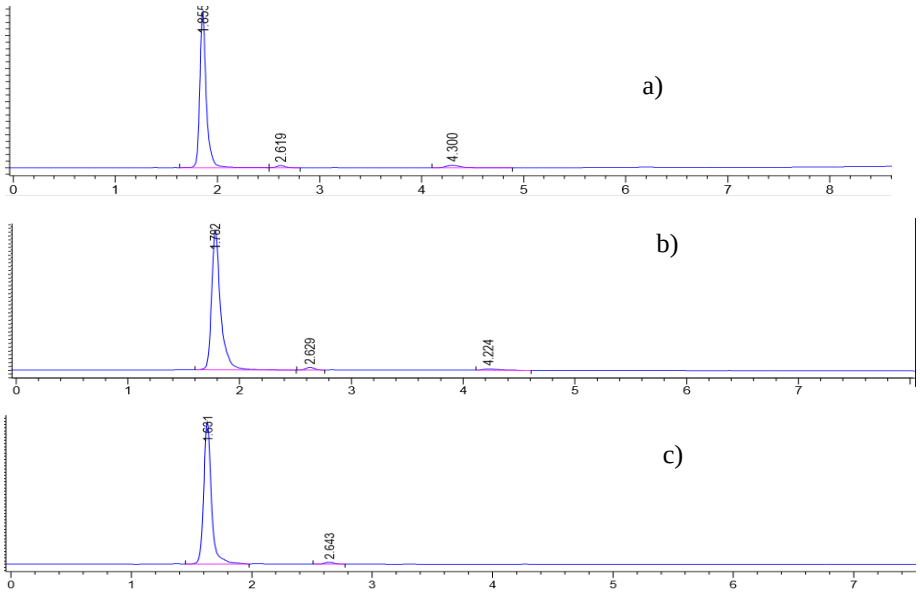

Figure 2. *Chromatograms of standard mixtures in a) phosphate buffer, b) phosphate buffer and acetonitrile mixture and c) water*

It is clearly shown that an additional peak was seen at about 4.2 - 4.3 minutes with the use of phosphate buffer and solvent mixture of phosphate buffer and acetonitrile. This peak was probably due to the decay of CYS as it also appeared in the chromatogram of CYS when these two solvents used (data not shown). Therefore, water was chosen as the dilution solvent for further chromatographic analysis.

3.2. Chromatographic conditions

Table 1 presents the retention time of the analytes when being chromatographed with a mixture of acetonitrile : phosphate buffer 3 mM, pH 3.5 (75: 25, v/v).

Table 1. *Retention times of AG, CYS and GLY*

Flow rate (ml/min)	Retention time (min)					
	AG		CYS		GLY	
	Apollo	Agilent	Apollo	Agilent	Apollo	Agilent
1.0	1.886	2.389	4.314	5.854	5.881	7.634
0.8	2.385	3.120	5.423	7.993	7.778	10.664

The Apollo column (150 mm) was selected at first because this shorter column decreased analysis time and it was for the sake of acetonitrile saving. However, AG had poor retention on

this column (retention time less than 2.5 minutes) leading to its unreasonable retention time repeatability. As a result, the Agilent column (250 mm) was chosen. The column temperature was set at 30^oC to have a better repeatability of retention time. This temperature is in compliance with sample storage condition.

Increasing acetonitrile content in the mobile phase improved the retention time of the analytes. Mixture ratio 72:28 of acetonitrile : phosphate buffer (2.5 mM, pH 3) cut down on the acetonitrile consumption, however, it shortened the retention time of AG (less than 2 minutes). Reducing acetonitrile ratio (75:25) resulted in better retention of AG. Increasing acetonitrile would lead to the stronger retention of GLY (the last eluted analyte), lengthening the analysis time and raise the acetonitrile consumption. Therefore, the mixture of acetonitrile : phosphate buffer (75: 25, v/v) was chosen for further investigation.

AG showed maximum absorption at λ_{max} 254 nm, while CYS and GLY did not have a specific λ_{max} due to the lack of chromophores in their molecules. Because the mobile phase has a low UV cut-off wavelength, the elution of CYS and GLY was monitored at 200 nm and AG at 254 nm.

Chromatographic data show that at phosphate buffer pH < 3, serious peak tailing was observed with GLY. All analytes gave a good peak shape when using the mobile phase containing phosphate buffer 3 mM, pH 3. The total analysis time was less than 13 minutes when the flow rate fixed at 0.8 ml/min.

3.2. Method validation

Repeated injections of standard mixtures (n = 6) showed good RSD values for retention time (< 1.7%) and peak area of AG, CYS and GLY (< 0.6%) indicating the suitability of the chromatographic system under study. The chromatograms of the placebo, AG, CYS and GLY standards, and their corresponding mixture and sample clearly showed no significant matrix effect (Figure 3).

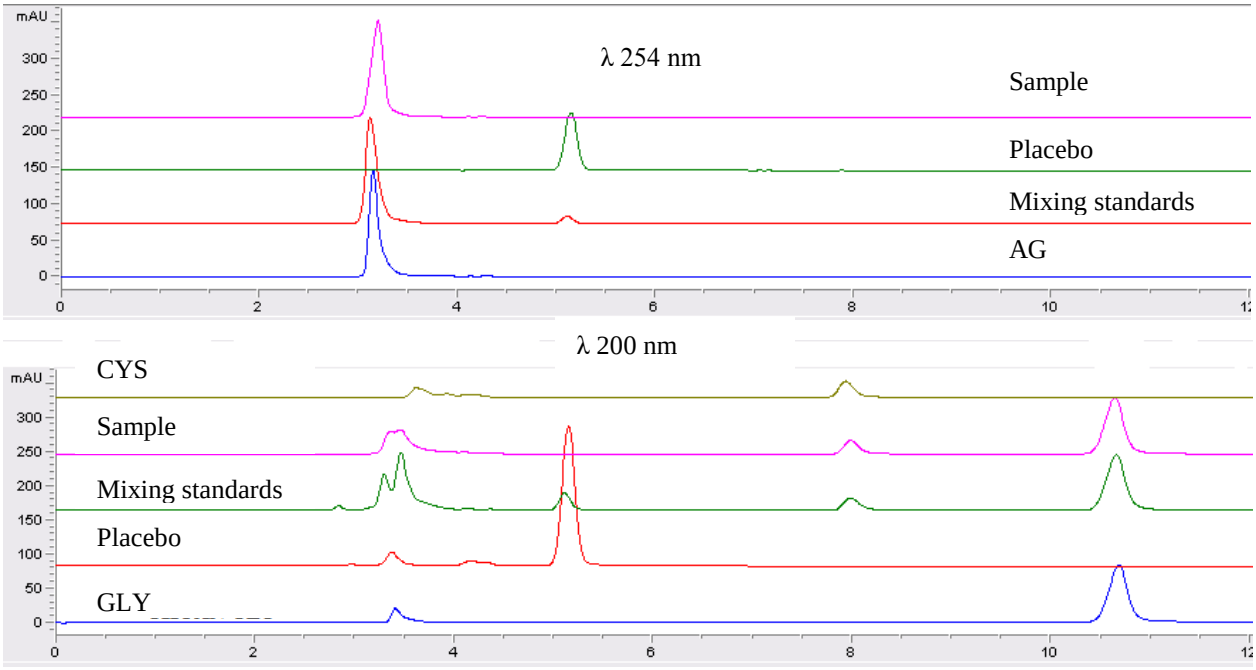

Figure 3. *Chromatogram at 254 nm and 200 nm*

Linearity ranges were recorded for AG (133.1 – 399.6 µg/ml), CYS (54.3 – 162.8 µg/ml) and GLY (987.8 – 2963.4 µg/ml) with correlation coefficients > 0.997. The repeatability of the method was accessed by analyzing six different samples (RSD values were < 1.8% for AG, CYS and GLY). The standard addition procedure was used for accuracy determination. The percentage recovery of AG, CYS and GLY was from 99.2 – 100.9% with RSD < 1.4%.

3.3. Application

The proposed method was applied to determine the content of AG, CYS and GLY in Amiphargen IV injection samples. This formulation was found to have 95.5, 98.6 and 98.0% of the AG, CYS and GLY content stated in the label, respectively.

4. CONCLUSIONS

A reliable and simple HILIC method was developed to directly and simultaneously analyze cysteine, glycine and ammonium glycyrrhizinate in an intravenous formulation. It could significantly improve analytical throughput of glycine and cysteine (high polarity and low ultra-violet absorbance compounds), which usually requires derivatization for HPLC analysis.

ACKNOWLEDGEMENT

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In silico screening for *Andrographis paniculate* Pharmalogical Activity of Diterpenlactone (*Andrographispaniculata* (burm.f.) Nees)

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Abstract

Hyperglycemia is a condition in which an excessive amount of glucose circulates in blood plasma. Cancer is uncontrolled growth of abnormal cells in the body. They are two serious diseases on which extensive research is being done all over the world. Structure-based approach offers a computer-aided drug design to identify the potential leads which can be developed into a drug. *Andrographispaniculata* has been known as a potential drug, especially diterpenlactone compounds. Institute of Drug Quality Control HCMC extracted four diterpenlactone compounds but the biological essays of these compounds have not been done. This study was conducted to identify the potential compounds targeting on hypoglycemia and anti cancer effects for their *in vitro* and *in vivo* testing.

Keyword: diterpenlactone, hypoglycemia, anti cancer, anti HIV, *in silico* screening

1. INTRODUCTION

1.1 Hyperglycemia

The ATP sensitive K^+ channel (KATP channel) activity plays a cruial role in glucose simulated insulin secretion by coupling □-cells metabolism to calcium entry 5.

Insulin receptor: insulin initates its cellular responses by binding to its cellular receptor, a transmembrane, multisubunit glycoprotein that contains insulin-stimulated tyrosine kinase activity 6.

Phosporylase kinase plays an important role in stimulating glycogen breakdown into free glucose by phosphorylating glycogen phosphorylase and stabilizing its active conformation. This activity is particularly important in liver and muscle cells, while muscle cells generally break down glycogen to power their immediate activity, liver cells are responsible for maintaining glucose concerntration in the bloodstream 7.

Pyruvate dehydrogrenasekinase-the primary function of pyruvate kinase is to catalyze the transphosphorylation from PEP (phospohenolpyruvate) to ADP as the last step of glycolysis to generate ATP 9.

Glycogen phosphorylase inhibitors (GPI) have been shown to be more potent inreducing hepatic glucose output especially when glucose exists in high concentration. Thus, as blood glucose concentration diminishes, compound potency is attenuated, and the patient is better protected from periods of hypoglycemia.

PPAR-□□□(peroxisome proliferator-activated receptor)-recent evidence suggests premises of the peroxisome proliferator-activated receptor (PPAR) ligands in the combat against type 2 diabetes and metabolic syndrome including obesity and insulin resistance. PPAR-□ is highly expressed in fat to facilitate glucose and lipid uptake, stimulate glucose oxidation, decrease free fatty acid level and ameliorate insulin resistance.

DPP4 (dipeptidyl peptidase 4) Recently recognized class of oral hypoglycemic, dipeptidyl peptidase (DPP4) inhibitors could block the dipeptidyl peptidase 4 (DPP4) enzymes. DPP4 is an intrinsic membrane glycoprotein and a serine exopeptidase that playsamajor role in glucose metabolism and responsible for the degradation of incretins such as GLP-1, therefore providing a useful treatment to diabetes mellitus type 28.

□□glucosidase and □□amylase inhibitors are used to control high blood sugar problems. □-amylase is related to the breakdown of long chain carbohydrates and □-glucosidase is associated with breakdown of disaccharide into monosaccharide 7.

1.2 Cancer

Epidermal growth factor receptor (EGFR) belongs to receptor tyrosine kinase family, and overexpression of EGFR is associated with poor prognosis and progression of manly human cancers, including oral cancer3.

Protein kinase BCR-Abl - the first anticancer agent specifically targeted to a protein kinase was Imanitib, which acts as an inhibitor of the oncogenic kinase BCR-Abl and is active in the chronic myelogenous leukemia1.

cAMP dependent protein kinase-cAMP/PKA signaling pathway is altered in different cancers, and may be exploited for cancer diagnosis and/or therapy[12].

1.3.HIV

HIV protease (HIV-1 PR) inhibitors has drastically decreased the mortality and morbidity associated with AIDS4.

HIV reverse transcriptase-HIV-1 RT inhibitors can be classified into two cateogories: nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) 2.

2. OBJECTIVE

This study was conducted to identify the potential compounds targeting on hypoglycemia and anti cancer effects for their *in vitro* and *in vivo* testing.

3. MATERIALS AND METHODS

3.1. Target identification

Hyperglycemia against: Proteins selected for molecular docking

Table 1. The targets protein for hyperglycemia against

Protein mụctiêu	**PDB id**	**Độphângiải (Å)**	**In complex-with**
ATP sensitive K^+ channel	2WLK	2,8	Spermine
Insulin receptor	1IR3	2,3	ATP analog
Phosphorylase kinase	2Y7J	2,5	Sunitinib
Pyruvate kinase	4MP2	1,75	(5-bromo-2,4-dihydroxy-phenyl)(1,3-dihydro-2H-isoindol-2-yl)methanon
Glycogen phosphorylase	3DDS	1,8	Anthrnilimid
PPAR-γ	3DZY	3,1	Rosiglitazon
DPP4	2QT9	2,1	(2S,3S)-3-Amino-4-[(3S)-3-floropyrrolidin-1-yl]-N,N-dimethyl-4-oxo-2-(trans-4 [1,2,4]triazolo-[1,5-A]pyridyl-5-yl-cyclohexyl)butanamid
α-glucosidase	2QMJ	1,9	Acarbose
α-amylase	4GQR	1,2	Myricetin

3.2 Anti cancer

The targets were selected the 3 Dimensional strutures of the target proteins Epidemal growth factor receptor complexed with erlotinib (**Figure 5**), human cAMP dependent protein kinase (**Figure 6**) and human Ab1-kinase in complex with nilotinib (**Figure 7**) were downloaded form Protein Data Bank (PDB).

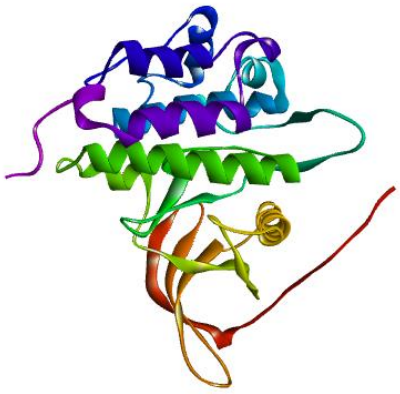

Figure 5. *The crystal structure of the target EGFR (PDB id: 1M17)*

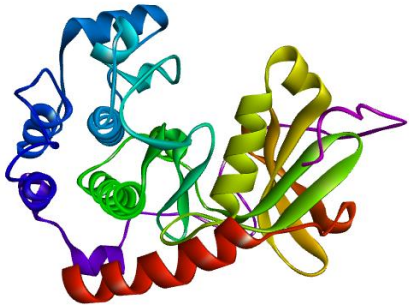

Figure 6. *The crystal structure of the target cAMP dependent on protein kinase (PDB id: 3OVV)*

Figure 7. *The crystal structure of the target Ab1-kinase (PDB id: 3CS9)*

3.3. Anti HIV

The targets were selected the 3 Dimensionalstrutures of the target proteins HIV protease (**Figure 8**) and HIV reverse transcriptase (**Figure 9**) were downloaded form Protein Data Bank (PDB).

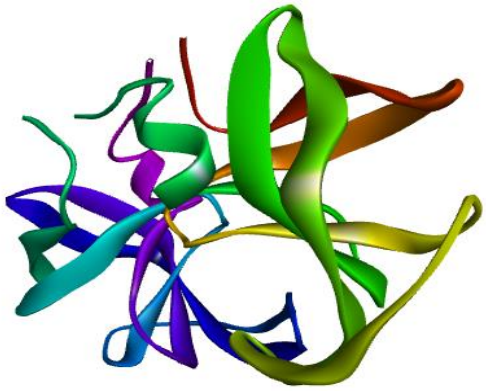

Figure 8. *The crystal structure of the target HIV protease (PDB id: 1HVR)*

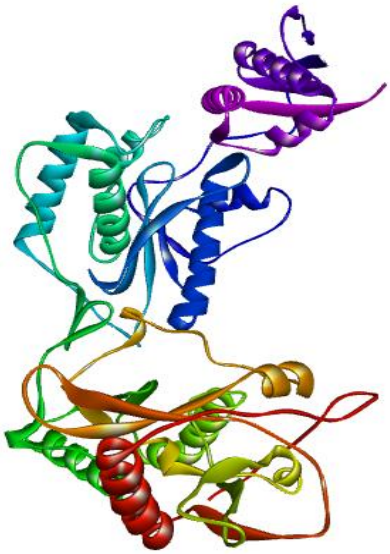

Figure 9. *The crystal structure of the target HIV reverse transcriptase (PDB id: 1VRT)*

3.4 Preparation of compounds

The structure of four deterpenlacton compounds andrographilide, neoandrographolide, dehydroandrographolide and andrograpanin were downloaded from Pubchem in SDF format and converted to PDB format by using Open BABEL tools (http://openbabel.org/wiki/Category:Installation). These ligands were energy minimized and considered for docking.

3.5 Molecular docking

Using AutoDock Vina Tools for docking studies and BIOVIA Discovery Studio Tools for interactions analysis. Docking studies were performed by five steps including: protein preparing

(adding hydrogen for proteins), ligand preparing (energy minimizing for ligands), binding site definition, molecular docking and docking results analysis.

4. RESULTS

4.1 Hyperglycemia against

Redocking on nine targets for hypoglycemia effect proteins relation.

Table 2. *Redocking results for hypoglycemia effect proteins relation*

Proteins	**PDB id**	**Redocking score (kcal.mol^{-1})**	**Resolution (Å)**	**RMSD (Å)**
Kênh K^+nhạycảm ATP	2WLK	-6,5	2,8	4,2396
Insulin receptor	1IR3	-7,3	2,3	3,0098
Phosphorylase kinase	2Y7J	-8,0	2,5	9,9532
Pyruvat dehydrogenase kinase	4MP2	-8,0	1,75	6,6101
Glycogen phosphorylase	3DDS	**-9,5**	**1,8**	**1,4408**
PPAR-γ	3DZY	-8,4	3,1	9,8250
DPP4	2QT9	-8,4	2,1	10,6295
α-glucosidase	2QMJ	-7,8	1,9	5,8869
α-amylase	4GQR	-8,1	1,2	2,4614

As the results (
Table 2), 3DDS shows the best compatible with software. So, glycogen phosphorylase (PDB id: 3DDS) is chosen as the target protein for hypoglycemia effect. Docking four diterpenlacton compounds into 3DDS protein. Neoandrographolideshows the best potential on decreasing glycemia (-8,2 kcal.mol^{-1}) compare to andrographolide (-7,4 kcal.mol^{-1}), dehydroandrographolide (-7,2 kcal.mol^{-1}) and andrograpanin (-6,8 kcal.mol^{-1}). Neoadrographolide situates in the binding site and forms interactions with Tyr75, Arg242, Asp306, Arg309 and Arg310 (**Figure 10**).

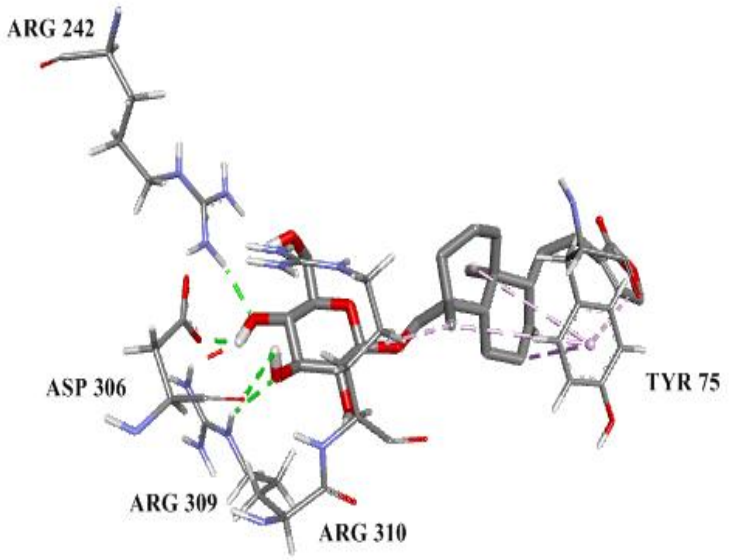

Figure 10. *Interaction between neoandrographolide and 3DDS*

Summarizing, the most potential compound for hypoglycemia effect is neoanrographolide, which should be continued*in vitro* and *in vivo* researches.

4.2 Anti Cancer

Redocking on three targets for anti cancer proteins relation.

Table 3. *Redocking results for anti cancer proteins relation*

Protein	PDB id	Redocking (kcal.mol^{-1})	Resolution (Å)	RMSD (Å)
EGFR	1M17	-7,3	2,6	6,1690
Receptor PKA/cAMP	3OVV	-10,5	1,58	9,9882
Receptor tyrosine kinase Bcr-Ab1	3CS9	-10,6	2,2	8,6776

As the results (**Table 3**), 3CS9 shows the best compatible with software. Therefore, choosing Tyrosine kinase Bcr-Abl (PDB id: 3CS9) as the target protein for cancer against.

Docking simulations of four compounds andrographilide, neoandrographolide, dehydroandrographolide and andrograpanin with 3CS9 target protein. Dehydroandrographolide shows the best potential on cancer against (-9,3 kcal.mol^{-1}) and interactions with Leu248, Tyr253, Val256, Ala269, Lys271, Val299, Phe317, Gly321 and Phe382 (**Figure 11**).

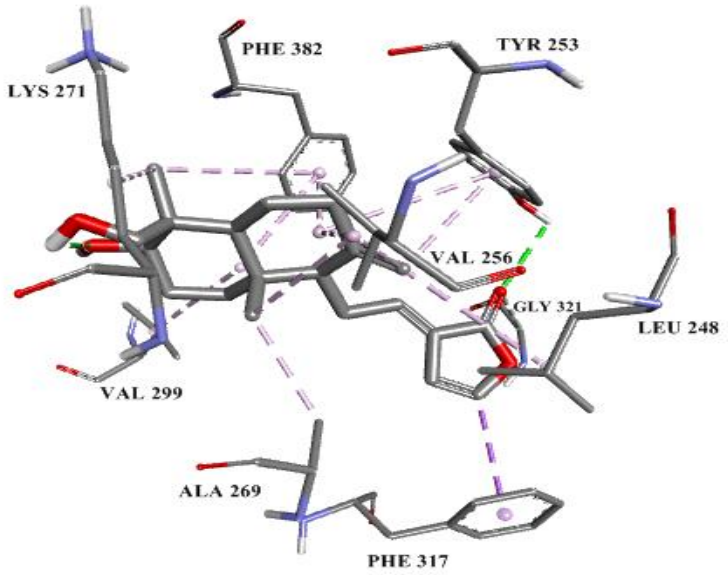

Figure 11. *Interaction between dehydroandrographolide and 3CS9*

As the results, the most potential compound for anti cancer effect is dehydroandrographolide which should be continued*in vitro* and *in vivo* researches.

4.3 Anti HIV

Redocking on three targets for anti cancer proteins relation.

Table 4. *Redocking results for anti HIV proteins relation.*

Protein mụctiêu	PDB id	Redocking (kcal.mol^{-1})	Resolution (Å)	RMSD (Å)
HIV protease	1HVR	-14	1,8	0,5397
HIV reverse transcriptase	1VRT	-10,5	2,2	0,3669

As the results (**Table 4**), 1HVR shows the best compatible with software. Therefore, choosing HIV protease (PDB id: 1HVR) as the target protein for cancer against.

Docking simulations of four compounds andrographilide, neoandrographolide, dehydroandrographolide and andrograpanininto 1HVR. The results showsneoandrographolide is the most potential on HIV against (-9,4 kcal.mol^{-1}). Binding site between 1HVR and neoandrographolide are surrounded by GlyA27, AlaA28, AspA29, AspA30 and IleA50 as well as AspB29, AspB30 and IleB47 (**Figure 12**).

Summary, the most potential compound for anti HIV effect is neoandrographolide which should be continued *in vitro* and *in vivo* researches.

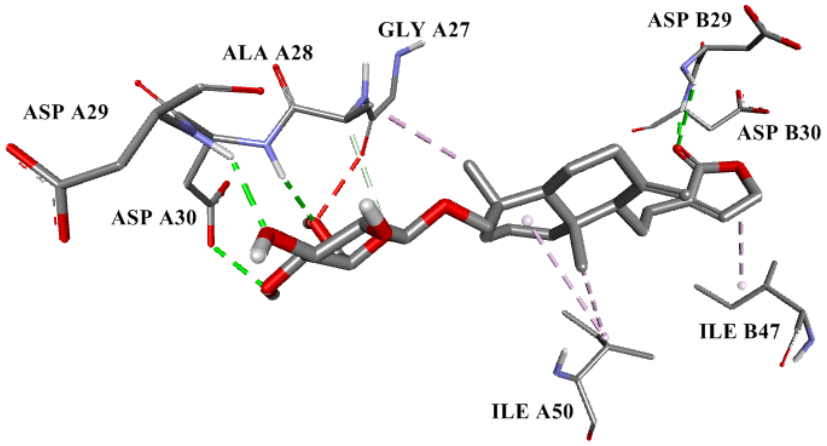

Figure 12.*Interaction between neoandrographolide and 1HVR*

CONCLUSION

As the results, continuing *in vitro* and *in vivo* researches on diterpenlactone compounds gained from *in silico* study. Including, the most potential on reducing blood sugar and anti HIV is neoandrographolide and the best potential on anti cancer is dehydroandrographolide.

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Identification of potential inhibitors for Ebola virus

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Keyword: Ebola virus, docking, VP24, VP30, VP 40, VP 35, potential inhibitor

1. INTRODUCTION

Ebola virus belongs to the genus *Ebolavirus*, family *Filoviridae*, order *Mononegavirales*, which causes the serious hemorrhagic fever in human and other mammals, and leads to the lethal for thousands of people in the world, especially the outbreak in 2014. Moreover, recently, there is any drug available approved by the Drug Administration to against Ebola virus infection.

2. OBJECTIVES

The aim of our study was to discover potential compounds to inhibit Ebola virus.

3. MATERIALS AND METHODS

Initially, the target proteins VP24, VP30, VP35, VP40 which are involved in the pathogenesis were identified and ligands were taken from some drugs which are in clinical testings or from other anti-viruses potential compounds. The blind docking study was carried out using AutoDock Vina. As the results of the study, the interactions between each target and ligand were analyzed. Based on the binding affinity (docking score), the drug lead compounds were identified. Moreover, the potential binding sites on the proteins where ligands usually bind were predicted by blind docking and Define Receptor Cavities tool of BIOVIA Discovery Studio 4.5.

4. RESULTS AND DISCUSSION

The target proteins identified were VP24, VP30, VP35, VP40. Most of the potential binding sites predicted by blind docking and by BIOVIA are similar. The docking result of the trial drugs suit to experiment. In the group of other potential compounds, there were some ligands which had abilities to well bind with Ebola proteins such as Silybin (-9.5 $kcal.mol^{-1}$), Harringtonine (-8.0 $kcal.mol^{-1}$), and Homoharringtonine (-8.3 $kcal.mol^{-1}$). Among these ligands, after choosing through Lipinski 5 rules, Silybin is the only suitable one.

5. CONCLUSION

This study provided helpful information to considerably assist in drug discovery of antiviral agents for Ebola virus.

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Development of UPLC-MS/MS Method for Simultaneous Determination Carbamate Pesticides Residue in Vegetables and Fruits and Pilot Survey in Real Samples

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Abstract

Introduction: The evaluation of pesticide residues in food is nowadays a priority objective to ensure food safety and to protect consumers against possible health risks. As the residues of pesticides are frequently found in fruits and vegetables, they may easily become a worrying risk for consumer's health. To obtain a practical, rapid and sensitive method for determining pesticide residues in food complex matrices, several sample preparation methods were developed. However, some of these have faced difficulty because of the limit of preparation and detection method. **Objectives:** The aim of this study was to develop and validate method for simultaneous determination of methomyl, aldicarb, carbaryl, carbofuran and thiram in vegetables and fruits using Acquity UPLC H-Class tandem Xevo TQD mass spectrometer and QuEChERS for sample preparation and its application to quantitative study of these popular carbamates in fruits and vegetables samples that were collected in three different wholesale markets in Can Tho city, Vietnam. **Materials and methods:** 120 samples of many kinds of vegetables and of fruits were collected and homogenized finely. The experiments were carried out using blank samples spiked standard solution of five carbamate pesticides. Firstly, the sample was spiked EDTA, and then according to extracted steps in QuEChERS protocol. The final extract was analyzed by UPLC-MS/MS system using positive and negative mode, separated on a hydro-C_{18} reversed phase column, and eluted with methanol and water. **Results:** The optimized condition for separation of five carbamate pesticides were obtained using methanol and water with gradient mode, kinetex phenyl-hexyl column (50mm × 2.1mm i.d ×1.7µm), flow rate 0.2 ml/min. Validation result showed that the method was suitable for UPLC-MS/MS system, selectivity, specificity, linearity (R^2> 0.995), with low limit of detection (0.05- 0.3ppb), precision (RSD < 11%) and mean recovery of accuracy in the range of 90.57-10.85%. The validated method was used in a real samples survey. The results revealed that over 20% of vegetables and fruits samples were positive detected and there was one sample contained concentration of carbamate above the Vietnam Ministry of Health's MRLs. **Conclusion:** A sensitive and rapid LC-MS/MS method for the simultaneous quantification of five popular carbamate pesticides residue in vegetables and fruits was successfully developed and validated. Practical applicability of the assay was shown by determination of vegetables and fruits samples contain carbamate content in higher than MRLs.

Keywords: Carbamates, thiram, UPLC-MS/MS, QuEChERS, EDTA

1. INTRODUCTION

Carbamates, a class of highly effective commercial pesticides. However, the increasing use of carbamate pesticides poses a risk to aquatic systems and is a potential hazard for the human's lives [1]. Thiram is a dithiocarbamate fungicide used as a seed protectant and in foliar treatments on fruits and vegetables to control a number of fungal diseases, as well as to protect harvested crops from deterioration in storage or transport. It is also extremely susceptible to environmental condition especially ambient temperature, pH and enzymatic activities [2]. Hence, although there are many approaches published to determine carbamate pesticides residue in agriculture products, few researches detecting both carbamate and thiram in over the world. In Vietnam, to the best our knowledge LC-MS/MS method has never been applied for this simultaneous quantification. This was our motivation to develop and validate the first LC-MS/MS assay for the simultaneous these carbamates and increased the extraction efficiency of thiram by adding EDTA in extraction process.

2. MATERIALS AND METHODS

2.1. Reagents and Standard

Reference standards of all the test pesticides were purchased from the Sigma–Aldrich (USA). Acetonitrile, methanol, water, formic acid was purchased from Merck with MS grade. All solvents or chemicals for sample preparation used for analysis were of analytical grade from Merck. Primary secondary amine (PSA) and graphitized carbon black (GCB) were obtained from Agilent (USA). Anhydrous magnesium sulfate and sodium acetate were all ACS grade and obtained from Beijing Chemical Works (Beijing, China). Anhydrous magnesium sulfate (MgSO4) was heated in bulk to 500°C for >5 h to remove phthalates and residual water.

2.2. Instrument

The Acquity UPLC H–Class system includes a quaternary solvent manager, sample manager-flow through needle, column heater module coupled with a triple quadrupole mass spectrometer, Xevo TQD (Waters Corporation, Milford, USA). The Masslynx 4.1 software version was used to control operation and data processing. Chromatographic separation was performed on UPLC Kinetex Phenyl-Hexyl column (50 mm × 2.1 mm, 1.7 µm) analytical column (Phenomenex Inc., CA, USA) maintained at room temperature.

2.3. Methods

The MS parameters consist of capillary, cone voltage, desolvation temperature and gas flow, collision energy, dwell time, etc. were optimized by directly infusion of methomyl (MTM), aldicarb (ADC), carbaryl (CBR), carbofuran (CBF), thiram (THI) and internal standard (triphenyl diphosphate: TPP) solutions prepared in the reasonable solvents at 500 ng/mL with negative and positive ESI source and MRM, SIR to obtain the highest signal of product ion mass. A reverse phase chromatographic technique using acetonitrile, methanol, water or added pH-modifier as mobile phase. Factors influence to separation were investigated including stationary phase, mobile phase, pH, flow rate, column temperature and conditions for sample treatment were also investigated.

3. RESULT AND DISCUSSION

3.1 Method development

3.1.1 Optimization of MS parameters and chromatographic conditions

During the optimization of the mass spectrometric parameters, strong and stable signals of analysis of 5 carbamates and TPP (IS) were noted. The results indicate that ESI ionizer was operated in positive and MRM mode using the transition of *m/z* 162.99 →88.01, *m/z* 212.97 → 88.91, *m/z* 225.15 →123.10, *m/z* 201.10 →127.08, *m/z* 240.99 →88.06, *m/z* 326.99 →76.95 to quantify MTM, ADC, CBF, CBR, THI and TPP (IS), respectively. The ionizer and MS parameters were optimized as shown in Table 1.

Table 1. *Optimum ESI-MS/MS conditions*

Compound (MW)	Retention time (min)	$[M+H]^+$	Capillary voltage (kV)	Cone voltage (V)	Quant-ion CE[a] (V)	Ident-ion CE[a] (V)
Methomyl	2.79	162.99	3.5	14	88.01 10	106.07 10
Aldicarb	3.12	212.70		28	88.91 12	116.00 16
Carbofuran	3.23	222.10		30	165.21 12	123.10 12
Carbaryl	3.27	202.10		18	145.09 12	127.08 30
Thiram	3.49	240.99		18	88.06 14	120.03 12
Triphenyl Phosphate	3.9	326.99		52	76.95 40	152.13 32

[a] *: Collision energy (CE)*

Important parameters such as pH of the mobile phase, concentration and type of the buffer solution, percentage and type of the organic modifier (acetonitrile, methanol, water), stationary phase and flow rate were attempted for better sensitivity, chromatographic separation and peak shape of MTM, ADC, CBR, CBF, THI and TPP. The best separation and peak symmetries of six compounds was found to be significantly affected by using UPLC Kinetex Phenyl-Hexyl column (50 mm × 2.1 mm, 1.7µm) and utilizing mixing of methanol: water using a gradient elution is better than mixing of acetonitrile: water, acetonitrile: acid formic 0.1% at different pH or ammonium acetate buffer as well. In fact, by adding acid formic 0.1% has improved ionization efficiency and increased slightly signal of ADC, CBR, CBF and TPP. However, peak MTM and THI were unstable, badly peak shape and accelerated degradation of THI. Yet, several trials were performed of buffer concentration, flow rate (0.2 mL/min), temperature column (room temperature), injection volume (10 µL). They either had no impact or only modified separation time, without sharply improving peak resolution. Totally, optimum MS and chromatographic conditions were shown in Fig 1.

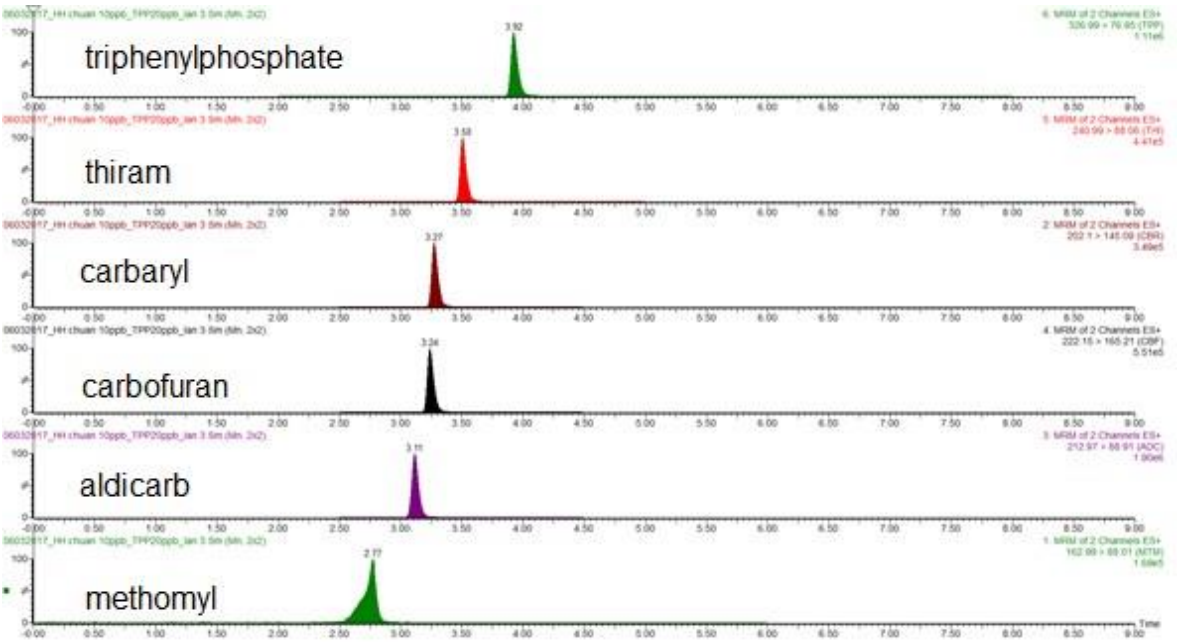

Fig 1. *Chromatogram of blank samples spiked mixture of standards at optimum MS and chromatographic condition*

3.2. Sample preparation

Homogenized sample (3g) was weighed into a 15 mL Teflon centrifuge tube and added EDTA, TPP, acetonitrile containing 1% acetic acid to reach 3mL and then the tube was vigorously shaken by hand for 1 min. The next step, anhydrous CH_3COONa (0.3 g) and anhydrous MgSO4 (1.2 g) were added, secondly shaking was immediately performed within 1 min manual shaking and 1 min mechanical shaking in vortex mixer. The sample was centrifuged at 4000 rcf in 3 min. An aliquot of 2 mL of the upper layer was placed in another 15mLTeflon centrifuge tube containing 50 mg of PSA, 300 mg of MgSO4 and 15 mg of GCB. It was also vigorously shaken by hand in 1 min, following with the vortex mixer in 1 min. Finally, 1mL of supernatant layer was diluted with 1 mL of water and then filtered through 0.22µm filter to carry out the LC-MS/MS analysis. In this experiment, the QuEChERS method was modified by adding EDTA to reduce the analytical cost and time, during 15 min for the whole extraction process without compromising extraction efficiency. The concentration of EDTA was investigated to gain the good purification and recovery for THI, which is extremely susceptible to enzymes combined with metal ions copper, zinc etc. [3].

The result showed in Figure 2 indicates that the recovery of THI had improved while the recoveries of other substances tended to decrease if the concentration of EDTA went over 0.01N. Consequently, 0.01N is the appropriate concentration of EDTA added to the first extraction stage.

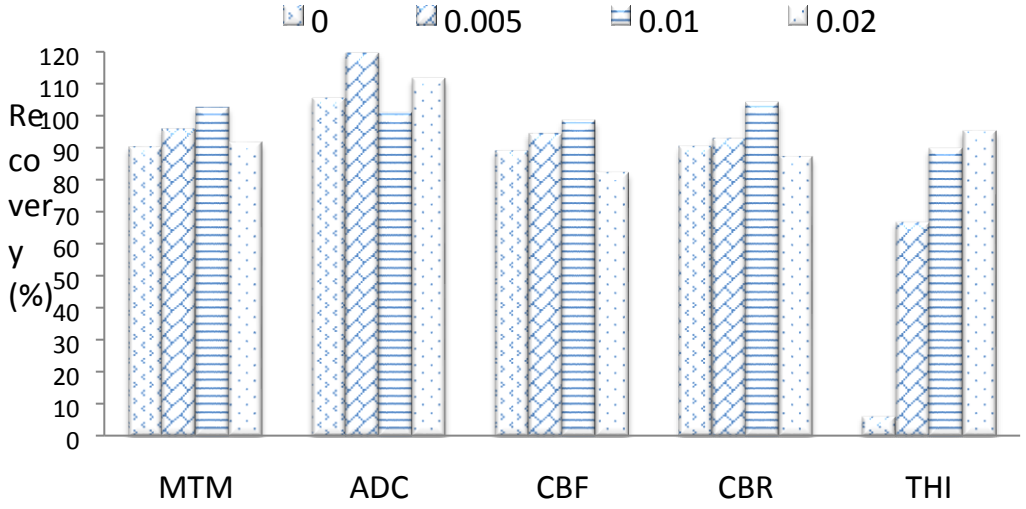

Fig 2. *The recoveries of all analytes of different concentration of added EDTA in QuEChERS extraction process.*

3.3. Method validation

3.3.1. System suitability

To evaluate the system suitability of the methods, six consecutive injections of standard mixture spiked in blank sample were conducted. Table 2 listed statistical data of retention time, peak area, and ratio of analyte peak area and IS peak area.

Table 2. *System suitability test at LQC (n=6)*

Analytes		Retention time	Peak area	$A_{analyte}/A_{IS}$
MTM	Average	2.797	11093.197	0.233
	RSD (%)	0.292	0.362	0.465
ADC	Average	3.122	29816.040	0.626
	RSD (%)	0.131	0.576	0.598
CBF	Average	3.235	12404.808	0.261
	RSD (%)	0.259	0.863	0.715

CBF	Average	3.273	3834.687	0.080
	RSD	0.249	1.766	1.603
THI	Average	3.495	3784.962	0.080
	RSD (%)	0.239	0.949	0.903
TPP	Average	3.900	47601.298	-
	RSD (%)	0.418	0.221	-

The RSD values of all pesticides were lower 2%, which indicated that this system is adaptable to intended analysis.

3.3.2. Specificity and selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained from standard MTM, ADC, CBR, CBF, THI and TPP spiked blank sample with those obtained from the blank samples. As a result, there was no significant interference observed at the retention time of the analytes and IS assessed by calculating % interference derived from the processed blank sample against the mean peak area of the samples. The results of specificity are shown in Figure 3 and 4.

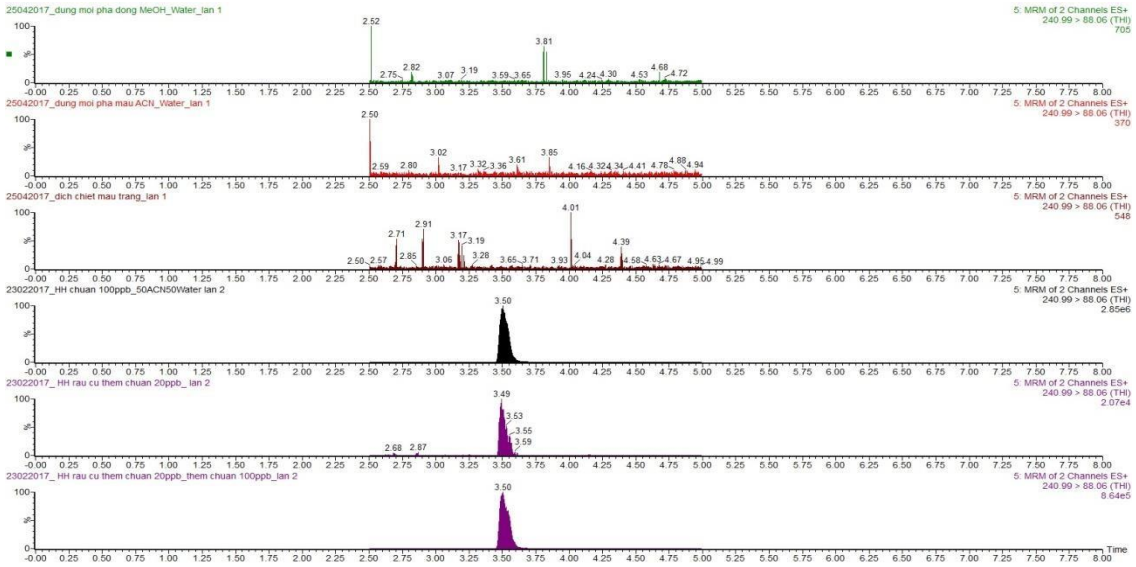

Fig 3. *Chromatogram of blank and blank sample spiked standards of THI*

Moreover, MS parameters were optimized with the objective obtaining two ion pairs for identification and quantification each target compound. The results were summarized in Table 3.

Table 3. *The ratio of identification and quantification ion of analytes.*

Compounds	Identification ion (MW)	Quantification ion (MW)	Ion ratio (%)		Difference (%)
			Sample	Standard	
Methomyl	106.07	88.01	80.7	83.9	3.2
Aldicarb	116.00	88.91	24.4	22.9	1.5
Carbofuran	123.10	165.21	22.9	22.7	0.2
Carbaryl	127.08	145.09	33	37.8	4.8
Thiram	88.06	120.03	59.3	56.5	2.8

3.3.3 Linearity, LOD, LOQ

The calibration curve was shown to be linear for the tested concentration range of MTM, ADC, CBR, CBF, THI. The mean correlation coefficient of the weighted ($1/X^2$ i.e, $1/[\text{concentration}]^2$) calibration curve generated in the validation was always > 0.995 and between the peak area ratios and the concentration of five analytes in the range 0.5-200 ng/mL. The experiments were performed using a standard seven-point series of each carbamate. LOD and LOQ were calculated according to the AOAC guideline for validation of analytical procedure based on standard deviation of the response and the slope of the calibration curve. Calculations of six replicate experimental injections LODs of 0.10, 0.3, 0.1, 0.2, 0.05 ng/mL and determined LOQs of 0.5, 0.5,0.4, 0.6, 0.6 ng/mL for MTM, ADC, CBR, CBF, THI, respectively.

3.3.4 Accuracy and precision

The accuracy and precision of the proposed method were determined by intra-day and inter-day replicate analyses of human gastric juice spiked at three concentration level standards covering the linear ranges. The accuracy is evaluated by the recovery.

The results for precision and accuracy are performed in table 4.

Table 4. *Validated parameters for the developed analytical method.*

Parameters		Range values (of all pesticides)
Coefficient of determination		0.996-0.998
Linearity range (ng/mL)		0.5-200
LOD (ng/mL)		0.05-0.3
LOQ (ng/mL)		0.4-0.6
Precision (intra-day) (n=6) (%RSD)	LQC*	3.1-6.1
	MQC	3.7-7.5
	HQC	3.6-5.9
Precision (inter-day) (n=3)(%RSD)	LQC	4.6-7.4
	MQC	5.1-7.0
	HQC	6.3-5.3
Accuracy (%recovery)	LQC	78.1-102.7
	MQC	80.2-99.9
	HQC	86.4-98.4

(*) LQC, MQC, HQC are low, medium and high-quality control sample.

3.4. Method application to real samples

The proposed method was applied to the analysis of 120 samples containing 40 varieties of fruits and vegetables collected in 3 wholesale markets. As a consequence, five carbamates in 27 positive samples were found with detected average rate is 22.5% (27/120). MTM and CBR were most frequently detected. Notably, one tested sample showed the concentration of MTM higher than the criterion from Vietnam Ministry of Health's MRLs. No pesticides were observed in the other 93 samples.

4. CONCLUSIONS

A simple, selective, sensitivity, accurate and reproducible LC-MS/MS method was developed and validated successfully for simultaneous determination MTM, ADC, CBR, CBF, THI. Practical applicability of the assay was shown by quality analyzing 120 samples
containing 40 varieties of fruits and vegetables. This method can be useful for monitoring quality of residue frequently using carbamates in food safety with great precision and accuracy.

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Development of Direct Infusion Mass Spectrometry Method for Rapid and Simultaneous Determination of Eleven Corticosteroids Adulterated in Herbal Medicines Used in Arthritis Treatment

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Abstract

Introduction: Recently, herbal medicines are widely used all over the world to treat rheumatoid arthritis because they are perceived as non-toxic products in comparison with synthetic medicines. To accelerate fast and effective treatment, synthetic drugs, such as corticosteroids, may be intentionally mixed with the herbal medicines. However, the adulteration of herbal medicines with synthetic steroids is prohibited. Due to the extremely complex matrices, it is difficult to develop a method that can rapidly identify multiple corticosteroids with low solvent consumption, short run time, cost reduction, simple sample preparation and extreme sensitivity. Among several analytical approaches, direct infusion technique in mass spectrometry is a very suitable one. **Objectives:** The purpose of this study was to apply direct infusion mass spectrometry for rapidly simultaneous identification of eleven corticosteroids (prednisolone, prednisone, methylprednisolone, prednisolone acetate, hydrocortisone acetate, dexamethasone, dexamethasone acetate, betamethasone, triamcinolone acetonide, fluocinolone acetonide, and clobetasol propionate) adulterated in herbal medicines used in arthritis treatment. **Materials and methods:** The blank sample was formed by combining seventeen kinds of pulverized herbal. The sample preparation process was established by studying the blank sample added standards. The mass spectrometry (MS) conditions including the solvent composition, the additive formic acid and MS settings were investigated under both positive and negative electrospray ionization mode. The typical precursor ion and fragments of each corticosteroid were recorded by MS full scan, MS/MS scan and daughters (product ion) mode. The existence of corticosteroid was recognized by comparing the spectrums between standards with samples. **Results and Discussion:** The suitable direct infusion MS/MS conditions for determination of eleven corticosteroids were obtained with the mixture of methanol-water containing 0.1% formic acid (70:30), 3 kV capillary voltage, 15 V cone voltage, desolvation temperature of 900^{o}C, and gas flow of 400 L/h. The method was validated and shown high selectivity, and very good limit of detection (0.1-0.3 ppm) and was applied for analyzing forty-five herbal medicines products. The results revealed that 40% of the ones were detected adulterated corticosteroids. **Conclusion:** A sensitive and rapid direct infusion MS method for simultaneous identification of eleven corticosteroids adulterated in herbal drugs used in arthritis treatment was successfully developed and validated. Practical applicability of the validated method was proved by detecting corticosteroids adulterated in real herbal samples.

Keywords: Corticosteroids; direct infusion mass spectrometry; herbal medicines

1. INTRODUCTION

Recently, the arthritis and rheumatic diseases have become more and more popular in our modern society. The treatments could be based on traditional foundation as well as up-to-date techniques. The trend of applying traditional medicines is highly noticeable because of their proven medical effects and safety. However, many synthetics drugs, particularly corticosteroids, adulterated in herbal medicines is an intense matter in the market. When administering these products, patients may suffer from host side effects and toxicity without any warning. Because of the complex matrices of herbal medicine and the low content of corticosteroids adulteration, determination these adulterant is difficult but essential. Several methods are reported in the literature for the estimation of corticosteroids by TLC, HPLC and LC-MS [2], [5]. All the methods mentioned are studied on different matrices, groups of adulterants. Moreover, because the presence of adulterant is prohibited, the present work aims to develop a selective and rapid corticosteroid identification method. An attempt is made to apply the mass spectrometry technique associated with low solvent consumption, short run time, cost reduction, simple sample preparation and extreme sensitivity. Based on the general structure of glucocorticoid and the fragments elucidation, directly infusion technique of mass spectrometry is an ideal method adapting those requirements.

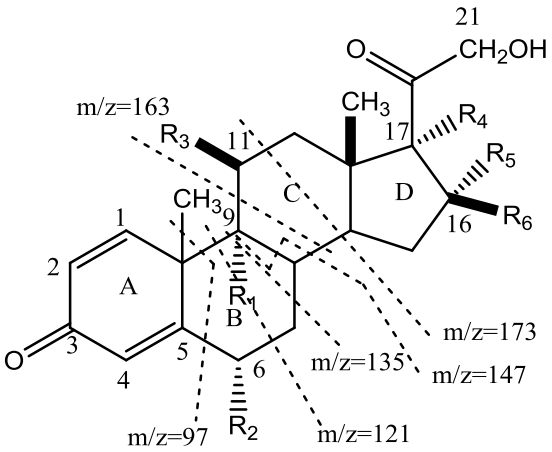

Figure 1. *General structure of glucocorticoids*

2. MATERIALS AND METHODS

2.1 Chemicals, solvents, and standards

Reference standards of Glucocorticoids (Betamethasone, dexamethasone acetate, methylprednisolone, prednisolone, prednisone, dexamethasone, fluocinolone acetonide, triamcinolone acetonide, clobetasol propionate, hydrocortisol acetate, prednisolone acetate) were supplied by Institute of Drug Quality Control Ho Chi Minh City and National Institute of Drug Quality Control. All solvents used in spectrometry operation (water, acetonitrile, methanol, formic acid) were of MS grade, and purchased from Merck. All solvents or chemicals for sample preparation (ethyl acetate, n-hexan, chloroform) used for analysis were of analytical grade from Merck.

2.2 Equipment

Mass spectrum was recorded with Xevo TQD (Waters Corporation, Milford, USA) triple quadrupoles mass spectrometer. The Masslynx 4.1 software version was used to control operation and data processing mass spectroscopy.

2.3 Methods

The MS parameters consist of capillary, cone voltage, desolvation temperature and gas flow, collision energy, dwell time, etc. were optimized by directly infusion of Glucocorticoids solutions prepared in the reasonable solvents at 500ng/mL with negative, positive ESI source and MS^1 and MS^2 full-scan, daughter mode to obtain the highest signal of product ion mass. The blank sample was formed by combining seventeen kinds of pulverized herbal. The sample preparation process was established by studying the blank sample added standards. The mass spectrometry (MS) conditions including the solvent composition, the additive formic acid and MS settings were investigated under both positive and negative electrospray ionization mode. The typical precursor ion and fragments of each corticosteroid were recorded by MS full scan, MS/MS scan and daughters (product ion) mode. The existence of corticosteroid was recognized by comparing the spectrums between standards with samples.

3. RESULTS AND DISCUSSION

3.1. Optimizing mass spectrometry conditions

During the optimization of the mass spectrometric parameters, strong and stable signals of analysis of 11 glucocorticoids were noted. The result was shown in Tables 1 and 2.

Table 1. *The optimized mass spectrometry condition*

Parameter	Value
Solvent	Methanol – water containing 0.1% formic acid (70:30)
Capillary voltage	3kV
Cone voltage	15V
Collision energy	12V, 18V
Desolvation gas temperature	900^{o}C
Desolvation flow rate	400 L/h
Flow rate	30µl/min

Table 2. *The fragments for identification*

Glucocorticoid	Precursor ion (ESI^+)	Fragments	Collision energy	Precursor ion (ESI^-) ($[M+H-HCOOH]^-$)
BMX	393 ($[M+H]^+$)	*373*, *355*, *337*, 319, *311*, *309*, 279, 237, *215*, 185,*153*	12V	437
CLP	467 ($[M+H]^+$)	*447*, *373*, *355*, *337*, *319*, 291, 279, 263	12V	511
DXA	435 ($[M+H]^+$)	415, *397*, *379*, *355*, *337*, 309, *319*, 291, 237, 227	12V	479
DXM	393 ($[M+H]^+$)	*373*, *355*, *337*, *319*, *309*, 291, 279, 253, 237	12V	437
FLA	435 ($[M+H-H_2O]^+$)	*412*, *403*, *389*, *371*, *321*, 279, 223	18V	497
HCA	405 ($[M+H]^+$)	387, *369*, *339*, *321*, 303, *293*, 279, 253	18V	449
MPR	375 ($[M+H]^+$)	*339*, *321*, 303, 293, *279*, 253, *185*, 161	18V	419
PRA	403 ($[M+H]^+$)	385, *367*, *349*, *325*, *307*,	12V	447

		289, 279, 265, 237, 223		
PRL	361 ($[M+H]^+$)	*343*, *325*, *307*, 289, 279, 237, 223, 171	18V	405
PRS	359 ($[M+H]^+$)	*341*, *323*, *313*, *305*, 295, 267, 237, 183, 171	12V	403
TCA	435 ($[M+H]^+$)	415, *397*, *379*, *357*, *339*, 321, 311, 293, 279, 253, 225	12V	479

The solvent was chosen according to the dissoluble property of glucocorticoids in study, the ionization type, the ability of ionization. The solvent was checked with methanol, methanol: water, acetonitrile, acetonitrile: water. The practical result showed that the ionization increased with the existence of water. However, there was no significant difference between the proportions of methanol and water in the solvent. Moreover, the increase of water proportion in the solvent leaded to the increase in desolvation gas temperature, which is limited at the rate of no more than 1000^{o}C. As a result, the mixture of methanol: water at the proportion of 7:3 (v/v) was selected as the optimized solvent. The capillary and cone voltage was established for recording all spectrum of eleven glucocorticoids with the least interferences from the matrix. The collision energy could be privately chosen for each glucocorticoid. This process aims to determine quickly, so there was no need to have the specific rate of collision energy for each one. Therefore, two range collision energy of 12V and 18V was selected. The main conditions are shown in the Table 1.

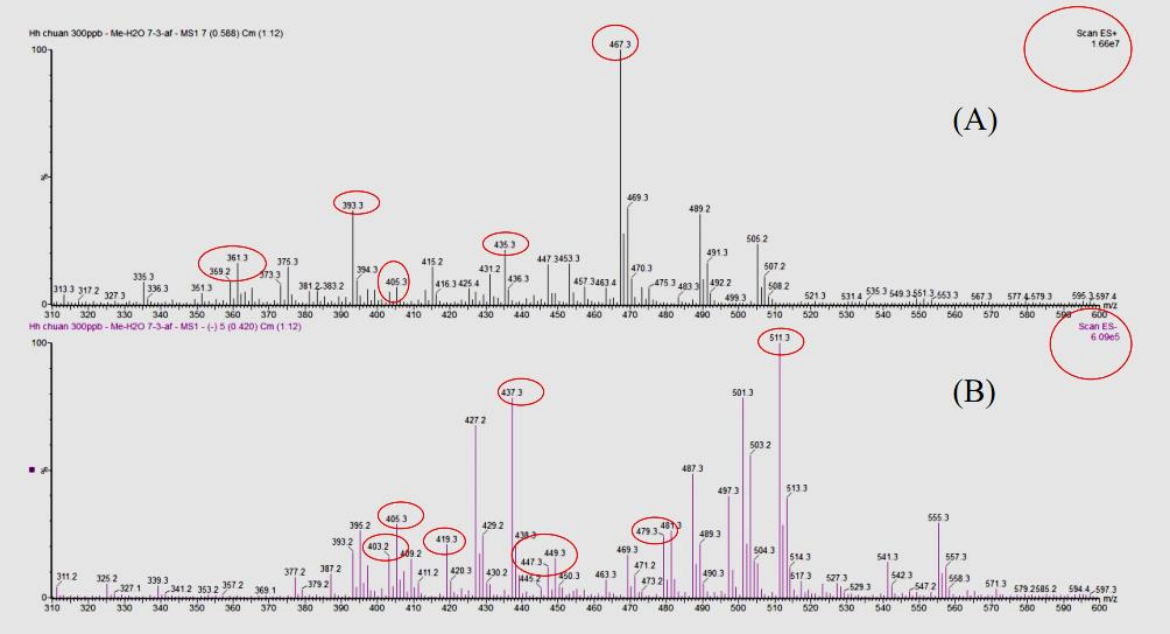

Figure 2. *The spectrum of spiked sample at the optimized conditions*

A small difference in mass spectra can be a hint to distinguish between dexamethasone and betamethasone and has been showed in figure 5. The elucidation of betamethasone can be: precursor ion m/z (393) as $[M+H]^+$, m/z 375 due to loss of water, m/z 373 as loss of hydrochloric, m/z 355, 337 and 319 as loss of water. The other ion recorded was m/z 279 ($[C_{20}H_{22}O + H]^+$) as the loss of HF, the loss of two water from C11- and C17-, the loss of 1,2-ethanedial (OHCCHO) from C17-, m/z 237 ($[C_{17}H_{16}O + H]^+$) as loss of propene ($CH_3CH=CH_2$) from D ring, and other carbocations with m/z 171 ($[C_{12}H_{11}O]^+$), m/z 147 ($[C_{10}H_{11}O]^+$) and m/z 121 ($[C_8H_9O]^+$). The other substances are complied with the similar elucidation. This elucidation was suitable with [1]. Table 2 illustrates the fragments used to compare and confirm the presence of glucocorticoids in the sample.

The identification of one glucocorticoid in a sample was confirmed by the similar of the spectrum of sample and standard. According to USP 38, an identification will be accepted with at least three similar structural ions (including one ion represents for the molecular mass among them). Thus, a sample was scanned through two modes MS and MS/MS mode. The figure 3 and figure 4 shows an example of the existence of dexamethasone in a sample. There is an ion representing for molecular mass at MS mode and more than two structural ions at MS/MS mode.

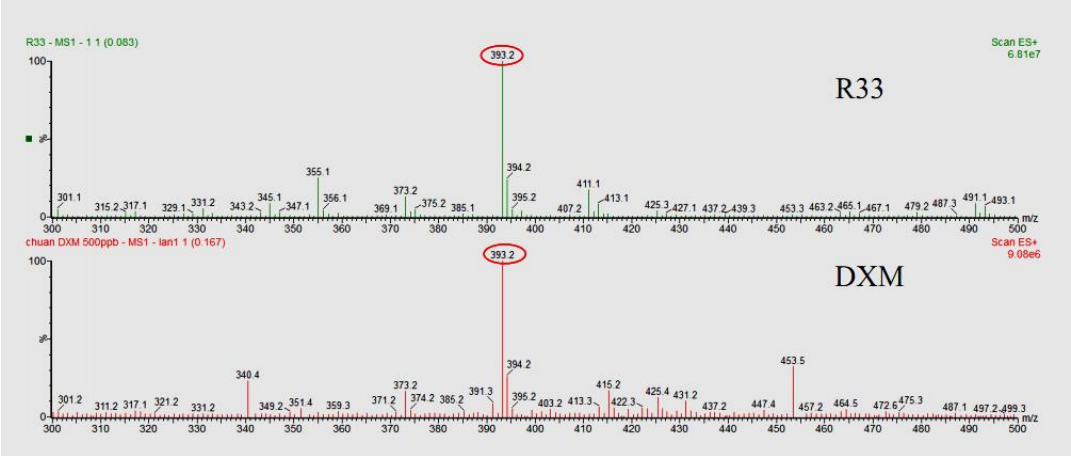

Figure 3. *The identification of dexamethasone in the sample R33 at MS mode*

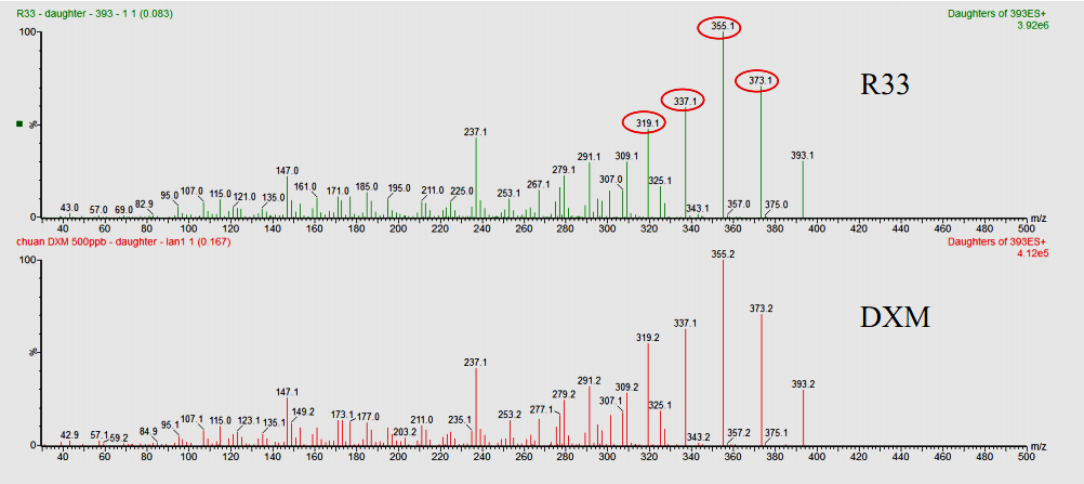

Figure 4. *The identification of dexamethasone in the sample R33 at MS/MS mode*

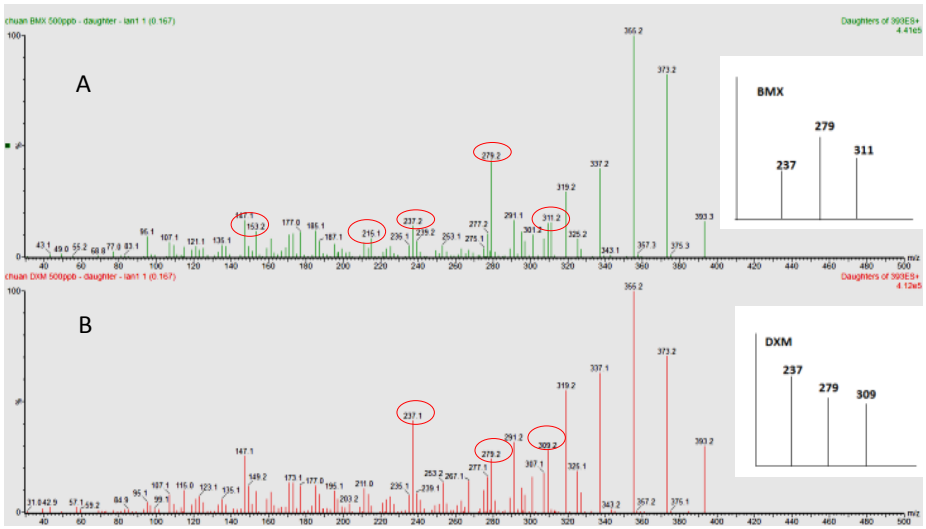

Figure 5. *The difference between betamethasone (A) and dexamethasone (B)*

3.2. Optimization of sample preparation

The sample preparation was studied on spiked samples which were the blank sample adding the mixture of glucocorticoid standards. The blank sample was created by mixing seventeen kinds of herb usually used for treating arthritis, dividing into three popular forms: tablet, honey pill, and liquid type. The spiked samples contained blank sample and the mixture of glucocorticoids whose concentration was 300 ppb for each one.

The sample that was collected on the market was classified into two main type of preparations: as solid type (including tablets, capsules, honey pills, medications) and liquid type (including oral solution, syrup). For the sample preparation of capsules, the husks were removed; tablets were pulverized; honey pills were split in small pieces. At first, 1.5 mg of powders or pieces or 15 ml solution was extracted with 15 ml *n*-hexane for two times. The *n*-hexane extraction was removed. The remaining was extracted with 30 ml mixture of methanol - water (3:7, v/v). The extraction then was extracted with 30 ml ethyl acetate. The ethyl acetate extraction was vaporized and then dissolved in the solvent proposed in table 1 and filtered through 0.22 µm filter membrane. Reference from the two research [3][4] combining with a change in the amount has resulted in an effect and economical preparation process. A comparison between using ethyl acetate and chloroform was also studied. Both can extract the certain glucocorticoids. However, the intensity of the same amount on the spectrum of chloroform extraction was lower than ethyl acetate extraction.

3.3. Selectivity and limit of detection (LODs)

The selectivity of the method was proved by acquiring the solvent, the blank matrix sample (created by mixing 17 different herbs), the mixture of standard (at the concentration of 100 ppb for each glucocorticoid), the spiked sample (at the concentration of 300 ppb for each glucocorticoid in the blank matrix), the spiked sample at the higher rate (at the concentration of 500 ppb for each glucocorticoid in the blank matrix). The selectivity was validated. The limit of detection of each glucocorticoid was obtained by acquiring the spiked sample solvents of each glucocorticoid with different dilutions. The limit was set up at the concentration that the rate of signal to noise was 3. The expected LOD should be lower than a dose one time. The result was summarized in Table 3.

Table 3. *LOD of glucocorticoids in spiked samples*

Glucocorticoid	LOD (ppb)		
	Solid	**Honey pill**	**Liquid**
Betamethasone	200	50	100
Clobethasol propionate	200	10	25
Dexamethasone acetate	300	100	100
Dexamethasone	200	50	100
Fluocinolone acetonide	300	100	100
Hydrocortisol acetate	300	200	150
Methyl prednisolone	300	100	100
Prednisolone acetate	300	200	150
Prednisolone	300	100	100
Prednisone	300	100	100
Triamcinolone acetonide	300	100	100

The limit of detection of each glucocorticoid in matrices of seventeen herbs is shown in table 3. All LODs are under the dosage in one time of each glucocorticoid. The spectrum of standard can be recorded and saved to use for a period. Thus, to compare with other methods existed, the process was not required using standard for each sample, so this process is suitable for applying in a large amount of market samples.

3.3. Survey on the herbal medicine market

The result of the survey was 19/45 samples positive with glucocorticoid, accounted for over 40%. In addition, because of comparison of spectrums between spectrum of standard in library spectrum and spectrum of samples, it was clearly that the products also adulterated paracetamol.

4. CONCLUSIONS

The developed direct infusion method is simple, selective and sensitive for identification of glucocorticoids. The proposed method is completely validated and applied on numerous samples on the market.

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Development and Validation of LC-MS/MS Method for Simultaneous Determination of Valsartan and Nifedipine in Human Plasma

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Abstract

Introduction: Combination of valsartan 80 mg/day and nifedipine 30 mg/day can help to control better high blood pressure. However, there is a few analytical method reported for simultaneous determination of these drugs in human plasma that can be useful for routine therapeutic drug monitoring and pharmacokinetic studies. **Objectives:** The aim of this study was to develop and validate ultra performance liquid chromatopraphy-tandem mass spectrometry method to quantify plasma levels of both valsartan and nifedipine. **Materials and methods:** A method for detection and quantification of vasartan and nifedipine in human plasma was been developed using Acquity LC H–Class system and Xevo TQD mass spectrometer equipped with an electrospray ionization source. Valsartan, nifedipine and losartan as internal standard (IS) in human plasma were extracted by protein precipitation with acetonitrile, then seperated on a C_{18} reversed phase column, and finally eluted with acetonitrile mixed with 0.015% formic acid solution. The assay was validated in compliance with US FDA and EMA guidelines. **Results:** Valsartan and nefedipine were extracted from human plasma using acetonitrile with ratio of 1:3, and separated on a C18 column (250 mm × 4 mm; 5 µm). The mobile phase was a mixture of acetonitrile and 0.015% formic acid (65:35, v/v) at flow rate of 1 mL/min. Electrospray ionnization source was operated in positive mode and multiple reaction monitoring (MRM) mode, using the transition of *m/z* 436.15 → 235.05, *m/z* 349.03 → 315.03 and *m/z* 423.16 → 207.04 to quantify valsartan, nifedipine and IS, respectively. The linearity was obtained over the concentration range of 20-6000 ng/mL and of 0.25-75 ng/mL with the lower limits of quantitation of 20 ng/mL and 0.25 ng/mL for valsartan and nifedipine, respectively. The intra and inter-day precision and accuracy, stability and recoveries of all the analytes were in the acceptable range. **Conclusion:** A highly sensitive, specific, reproducible and rapid LC-MS/MS method for simultaneous determination of valsartan and nifedipine in human plasma was successfully developed and validated. This method can be useful for pharmacokinetic studies and monitoring these therapeutic drugs with desired precision and accuracy.

Keywords: Valsartan; nifedipine, human plasma, LC–MS/MS

1. INTRODUCTION

Valsartan (VAL) and nifedipine (NIF) are widely used for hypertension treatment. VAL is an angiotensin II receptor antagonist while NIF is a dihydropyridine calcium channel blocker. The combination of those drug with 80 mg dose for VAL and 30 mg dose for NIF has been proved to have a good effect on controlling blood pressure[1-2]. Therefore, a sensitive and specific analytical method is needed for simultaneous determination of two drugs in human plasma for routine therapeutic drug monitoring and pharmacokinetic studies.

There have been several published studies on application of modern analytical methods for single determination of each drug, such as capillary electrophoresis (CE)[3], gas chromatography (GC)[4], high-performance liquid chromatography (HPLC) in combination with ultraviolet (UV)[5-6], or even mass tandem mass spectrometry (MS/MS)[7-8] detectors. Herein, a highly sensitive, specific, reproducible, and rapid LC-MS/MS method for determination of VAL and NIF in human plasma, was reported.

2. MATERIALS AND METHODS

2.1. Standards, chemicals and solvents

Reference standard of valsartan was purchased from Sigma-Aldrich. Nifedipine, and losartan (LOR) were provided by Institute of Drug Quality Control at Ho Chi Minh City, Vietnam. Other chemicals and organic solvents such as acetonitrile, methanol, formic acid etc. were of liquid chromatography grade, and purchased from Merck.

2.2. Equipment

The chromatograms and mass spectra were recorded from ACQUITY LC H-CLASS SYSTEM with Xevo TQD (Waters).

2.3. Methods

2.3.1. Preparation of standards and samples

VAL, NIF and LOR as internal standard (IS) were individually weighed and dissolved in methanol for the concentration of 1000 µg/mL. The stock standard solution was diluted with mixture of acetonitrile and 0.015% formic acid (65:35, v/v) to obtain the standard solutions with the concentration in a range of 20-6000 ng/mL, and of 0.25-70 ng/mL for VAL and NIF, respectively.

2.3.2. LC-MS/MS conditions

The MS/MS conditions were optimized by direct infusion of VAL, NIF and LOR standard solutions with concentration of 500 ng/mL each. Electrospray ionnization source was operated in either negative or positive mode by multiple reaction monitoring (MRM) mode. The parameters

such as desolvation temperature, capillary and cone voltage, gas flow, collision energy, and product ion mass were optimized to obtain the highest signal of VAL, NIF, and IS. For the LC conditions, the main parameters were optimized such as stationary phase, composition of mobile phase, flow rate, column temperature, volume of injection to achieve the high signals and reasonable run time.

2.3.3. Sample treatment

A protein precipitation method was applied to remove protein from plasma sample. Several organic solvents in acidic pH medium were investigated to increase sensitivity and specification as well as to achieve a consistent recovery.

2.3.4. Method validation

The method followed FDA's 2011 and EMA's 2011 guideline with main criteria namely system suitability, selectively, recovery, linearity, lower limit of quantification (LLOQ), precision and accuracy, stability and matrix effect[9-10].

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS conditions

By using Auto-tune function, the optimized MS/MS conditions were showed in Table 1.

Table 1. *The optimized MS/MS conditions for analysis of VAL, NIF, and LOR*

Parameters	**VAL**	**NIF**	**LOR**	**Parameters**	**VAL**	**NIF**	**LOR**
Ion mode		ES+		Parent ion (Da)	436	347	423
Capillary (kV)		1.0		Collision energy	20	8	20
Cone voltage (V)	40	20	24	Product ion	235	315	207
Desolvation temperature (^{o}C)		500		Dwell time (second)		0.052	
Desolvation gas flow (L/Hr)		1000					

As the results of the optimization, the three analytes were separated on a reversed phase column C_{18} Acclaim^{TM} 120 (250 × 4.6 mm; 5 µm). The mobile phase was a mixture of acetonitrile and 0.015% formic acid (65:35, v/v) at flow rate of 1 mL/min. The column temperature was set at room temperature and the injection volume was 10 µL. A mixture of tetrahydrofuran, methanol, and 10% acetic acid (50:40:10, v/v/v) was used to wash the column after every 10 injections.

3.2. Sample treatment

Several experiments using methanol or acetonitrile with formic acid to precipitate protein showed that acetonitrile (ACN) in combination with 0.045% formic acid (AF) was selected because of high and consistent recovery at three levels in plasma. Figure 1 shows process of plasma sample treatment.

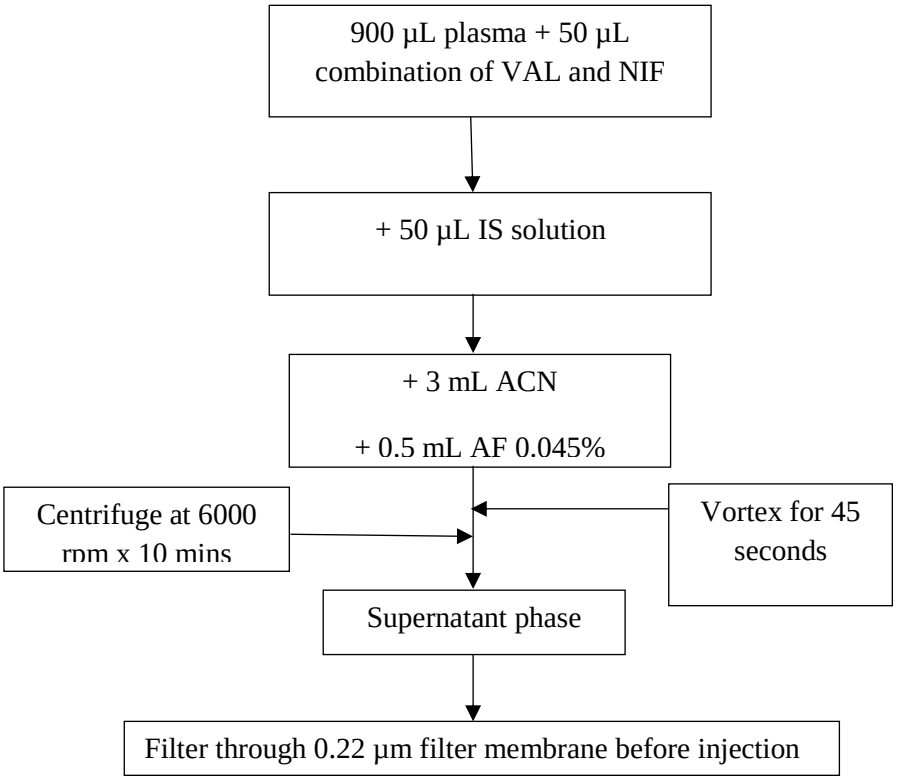

Figure 1. *Process of plasma sample treatment*

3.3. Method validation

3.3.1. System suitability

To evaluate the system suitability of the methods, six consecutive injections of low quality control (LQC) samples and high-quality control (HQC) samples were performed. Table 2 and Table 3 list statistical data of retention time, peak area, and ratio of analyte peak area and IS peak area.

Table 2. *System suitability test at LQC (n=6)*

	Statistical data	t_R (min.)	S	S/S_{IS}
VAL (100 ppb)	Average	4.73	46372	1.42
	RSD	0.09%	1.48%	1.27%
NIF (0.25 ppb)	Average	5.25	5912	0.18
	RSD	0.12%	2.03%	2.16%
LOR	Average	3.80	32634	
	RSD	0.14%	0.68%	

Table 3. *System suitability test at HQC (n=6)*

	Statistical data	t_R (min.)	S	S/S_{IS}
VAL (2500 ppb)	Average	4.74	1472986	44.63
	RSD	0.09%	0.88%	1.54%
NIF (60 ppb)	Average	5.265	437752	13.27
	RSD	0.10%	1.42%	2.27%
LOR	Average	3.803	33006	
	RSD	0.14%	1.13%	

RSD values of S/S_{IS}, peak area, and retention time were below 3%.

3.3.2. Selectivity

Selectivity was determined by comparing the chromatograms of six different batches of blank human plasma with those of plasma samples at LLOQ concentration. As shown in figure 2, no signal of three analytes was observed at the retention time of IS, valsartan, and nifedipine in figure 3 (3.80, 4.76, and 5.31 minutes, respectively).

3.3.3. Accuracy and precision

The intra- and inter-day accuracy of VAL and NIF at three levels of QC samples were within the range of 89.0% to 94.5% and 98.5% to 102.2%, respectively. The intra- and inter-day precision with RSD values were in a range of 2.41% - 4.30% for VAL and of 3.56% - 5.41% for NIF. Those criteria met the FDA guidance (not exceeding 15%).

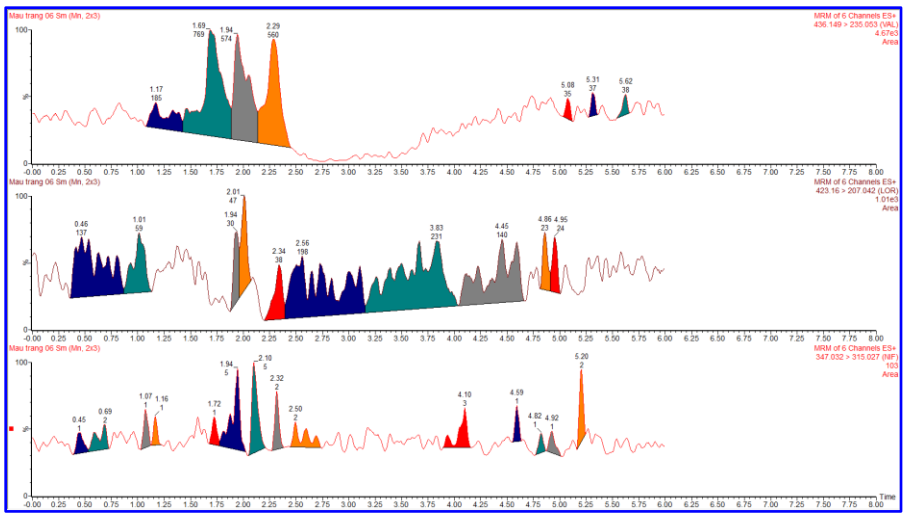

Figure 2. *Chromatograms of blank plasma sample*

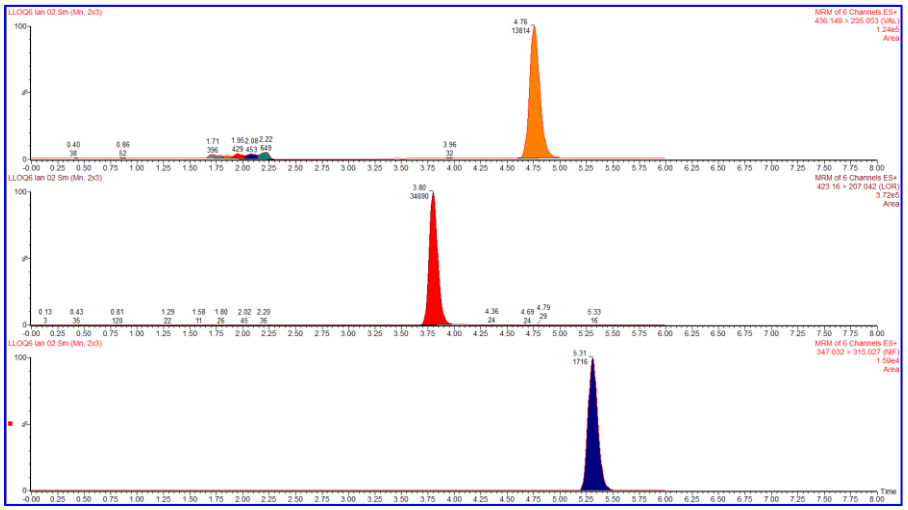

Figure 3. *Chromatogram of plasma sample at LLOQ concentration*

3.3.4. Recovery

The recovery values at three concentration levels were 95.18%-102.22%, 99.48%-105.35%, and 100.47%-104.80% for VAL, NIF and LOR, respectively. The average values of RSD were 3.62%, 2.90%, and 3.12% for VAL, NIF, and LOR, respectively. The results showed consistency and reproducibility recovery for all analytes and IS.

Table 4. *Recovery and accuracy – precision of VAL, NIF, and LOR in plasma*

Analyte	Analyte Conc. (ng/mL)	Extraction Recovery		Accuracy - Precision			
				Intra-day (n=5)		*Inter-day (n=15)*	
		Mean Recovery	*RSD%*	*Mean Recovery*	*RSD%*	*Mean Recovery*	*RSD%*
VAL	100	102.22	2.72%	89.2%	2.18	89.0%	2.41
	1500	95.18	1.14%	89.3%	2.61	93.5%	4.30
	2500	97.73	7.48%	94.7%	1.72	94.5%	3.04
NIF	0.25	101.75	1.60%	100.5%	5.88	98.5%	5.41
	40	99.48	4.57%	97.6%	3.46	99.5%	3.56
	60	105.35	5.70%	106.3%	2.27	102.2%	4.21
LOR (IS)	LQC[1]	104.80	8.13%	-	-	-	-
	MQC[1]	102.32	2.70%	-	-	-	-
	HQC[1]	100.47	3.06%	-	-	-	-

[1]*: Recoveries of internal standard in 3 level of VAL and NIF samples*

3.3.5. Linearity and lower limit of quantitation (LLOQ)

Eight-point calibration curves of the VAL and NIF for a specified range were set up. The curves were obtained by plotting the peak area ratio of the analytes to IS against the corresponding concentration of the analytes in the spiked plasma. The parameters of slope, intercept and correlation coefficient (R) were calculated. An achievement of R value greater than 0.995 indicated the good level of linearity. The lower LOQ of the method was 20 ppb, and 0.25 ppb for VAL and NIF, respectively, that met the requirements of precision (RSD < 20%) and accuracy (recovery ranging from 80 to 120%). The LLOQ value of VAL was equal to 1/82 C_{max} with a dose of 80 mg and the LLOQ value of NIF was equal to 1/80 C_{max} with a dose of 30 mg.

Table 5. *Linearity of VAL and NIF in plasma*

Analyte	Calibration curve	R
VAL	y = 0.0191324x – 0.00623828	0.9982
NIF	y = 0.218333x – 0.00161203	0.9998

3.3.6. Stability

The stability of VAL and NIF in stock solutions and in plasma was investigated. At -20^{o}C in freezer and at room temperature, the stock solutions were stable within 20 days and 6 hours, respectively. They indicated that the analytes were stable after three cycles of freeze and thaw, for 6 hours at room temperature, for 24 hours in auto sampler at 20^{o}C, and for 30 days at -20^{o}C.

3.3.7. Matrix effect

Table 6 summarizes the matrix factor (MF) value of VAL, NIF, and IS at low and high concentration levels.

Table 6. *The matrix effect on response of VAL, NIF, and LOR*

Plasma	**MF_{VAL}**		**MF_{NIF}**		**MF_{IS}**		**MF_{VAL}/ MF_{IS}**		**MF_{NIF}/ MF_{IS}**	
batch	**LQC**	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**
1	0.97	0.87	0.98	1.00	1.00	0.97	0.96	0.90	0.98	1.03
2	0.94	0.99	0.98	0.99	0.98	1.01	0.95	0.98	0.99	0.98
3	1.06	1.04	1.01	0.98	0.96	1.04	1.10	0.99	1.05	0.94
4	1.14	1.05	1.04	0.99	0.94	1.06	1.22	1.00	1.11	0.93
5	1.13	1.06	1.03	0.99	0.94	1.07	1.21	0.99	1.09	0.93
6	1.14	1.08	1.01	1.08	0.91	1.06	1.25	1.02	1.12	1.02
Average	1.05	1.02	1.01	1.00	0.96	1.04	1.10	0.98	1.06	0.97
RSD%	8.62	7.66	2.49	3.84	3.58	3.76	11.82	4.25	5.71	4.73

The RSD values of the ratio of MF_{VAL}/ MF_{IS} and MF_{NIF}/ MF_{IS} at low and high concentration levels were in a range of 11.82%-4.25%, and of 5.71%-4.73%, respectively. The results indicated that the matrix effect for valsartan and nifedipine was removed.

4. CONCLUSIONS

A highly sensitive, specific, reproducible and rapid LC-MS/MS method for simultaneous determination of valsartan and nifedipine in human plasma was successfully developed and validated. From the results of all the validation parameters, this method can be useful for pharmacokinetic studies and monitoring these therapeutic drugs with desired precision and accuracy.

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Isolation of Triterpenoids from the Stems of *Clinacanthus Nutan* (Burm. F.) Lindau

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Abstract

Friedelin, morolic acid acetate, betulinic acid and (3β)-3-hydroxyolean-12-en-28-oic acid were isolated from the dichloromethane fractions. The dichloromethane extract was obtained by liquid-liquid distribution from 96% ethanol extract of stem barks of Clinacanthus nutans. Main components were isolated and purified by using conventional column chromatography. Their structures were elucidated by 1D-NMR, 2D-NMR and MS spectroscopic data and by comparison of these data with those of the literatures published. The known lupane-type triterpene betulinic acid was isolated for the first time from *Clinacanthus nutans* (Burm. f.) Lindau. It has many biological effects interested such as anti-HIV, anti-inflammation, anti-bacteria, anti-malaria and anti-cancer.

Keywords: *Clinacanthus nutans*, friedelin, morolic acid acetate, betulinic acid, (3β)-3-hydroxyolean-12-en-28-oic acid

1. INTRODUCTION

Clinacanthus nutans (Burm. f.) Lindau, known as Sabah Snake Grass, belongs to the family Acanthaceae, is one of famous medicinal plants in Asian folklore medicine [1] or "belalai gajah" in Malaysia, has been interested in the recent years due to its high medicinal values. *C. nutans* has been reported to have antiviral, anti-oxidant, anticancer and anti-inflammatory activity [2]. *Clinacanthus nutans* is also used for the treatment of herpes simplex infection, herpes zoster infection, diabetes mellitus, skin rash, dysuria and cancer [4]. Several bioactive constituents such as *β*-sitosterol, stigmasterol, *C*-glycosyl flavones, orientin, vitexin, sulfur containing glycosides and glycoglycerolipids have been isolated and identified from the leaves of *this plant* [5]. Because of the reasons mentioned above, this report was carried out to make clear the chemical constituents of this plant.

2. OBJECTIVES

The objective of the paper was to isolate and elucidate the structures of some triterpenoids from the stems *Clinacanthus nutans* (Burm. f.) Lindau, Acanthaceae.

3. MATERIALS AND METHODS

3.1. Plant material

Clinacanthus nutans (Burm. f.) Lindau was collected in the Nui Cam, Chau Doc, Viet nam in December, 2015 and determined by gene sequence. It was numbered Bb1215 and deposited at the Department of Pharmacognosy, University of Medicine and Pharmacy, Ho Chi Minh City.

3.2. Experimental methods

The air-dried and powdered stems barks of *Clinacanthus nutans* was extracted with 96% ethanol. By liquid-liquid extraction with dichloromethane and ethyl acetate, the fractional dichloromethane and ethyl acetate extracts were obtained. These extracts were isolated purified using column chromatography. The chemical structures of the isolated compounds were elucidated with MS, 1D-2D-NMR technique and by comparison with those in literatures.

EIMS spectrum was obtained using a Autospec primer, ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer and TMS was used as an internal standard. Column chromatography (CC) was performed on silica gel (240-430 mesh, Merck), TLC: silicagel F254 (Merck).

4. RESULTS AND DISCUSSIONS

4.1. Extraction and isolation

The stems were chopped to small pieces and dried in the shade. The dried stems were powdered and percolated with 90 L of 96% ethanol. The crude extract was obtained by evaporation of the solvent in vacuum (860 g).

The ethanol extract (300 g) was distributed with dichloromethane (6 L) and ethyl acetate (4 L) to give dichloromethane extract (56.68 g) and ethyl acetate extract (15.18 g).

The dichloromethane extract (56.68 g) was chromatographed on silica gel with dichloromethane-methanol as the eluent in gradient mode to give 34 fractions (CN1-CN34). The fraction CN3, CN12 and CN16 was rechromatographed over silica gel and eluted with n-hexan and gradient ethyl acetate to yield four compounds. From the fraction CN3, the compound CN3-1 (8 mg) and CN3-6 (6 mg) were obtained. The compound CN12-2 (40 mg) was obtained from the fraction CN12 and the compound CN16-2 (6 mg) from the fraction CN16.These compounds were subjected to physical and spectral studies for characterization.

4.2. Determination of the structures

Four triterpenoids were isolated from the dichloromethane fraction of *Clinacanthus nutans*, and were identified as friedelin, morolic acid acetate, acid betulinic and (3β)-3-hydroxyolean-12-en-28-oic acid. The known compounds were identified by comparison of their physical and spectrocopic data with those reported in the literatures. The structures of the new compounds were elucidated using their spectroscopic data 1D, 2D-NMR.

CN3-1. colorless needles, **ESI-MS** *m/z*: 427.2 $[M+H]^+$; **^{1}H-NMR** ($CDCl_3$, 500 MHz) δ_H: 1.67 (1H, *m*, H-1b), 1.96 (1H, *m*, H-1a), 2.29 (1H, *m*, H-2b), 2.38 (1H, *qd*, *J*=7.0; 2.0 Hz, H-2a), 2.24 (1H, *d*, *J*=7.0 Hz, H-4), 1.26 (1H, *m*, H-6b), 1.75 (1H, *dd*, *J*=12.5; 2.5 Hz, H-6a), 1.39 (1H, *m*, H-7b), 1.47 (1H; *m*, H-7a), 1.35 (1H, *m*, H-8), 1.48 (1H, *m*, H-10), 1.23 (1H, *m*, H-11b), 1.38 (1H, *m*, H-11a), 1.32 (2H, *m*, H-12), 1.28 (1H, *m*, H-15b), 1.50 (1H, *m*, H-15a), 1.55 (2H, *m*, H-16), 1.51 (1H, *m*, H-18), 1.20 (1H, *m*, H-19b), 1.27 (1H, *m*, H-19a), 1.41 (1H, *m*, H-21b), 1.45 (1H, *m*, H-21a), 0.93 (1H, *m*, H-22b), 1.48 (1H, *m*, H-22a), 0.88 (3H, *s*, Me-23), 0.72 (3H, *s*, Me-24), 0.87 (3H, *s*, Me-25), 1.01 (3H, *s*, Me-26), 1.05 (3H, *s*, Me-27), 1.18 (3H, *s*, Me-28), 1.00 (3H, *s*, Me-29), 0.95 (3H, *s*, Me-30); **^{13}C-NMR** ($CDCl_3$, 125 MHz) δ_C: 22.2 (C-1), 41.5 (C-2), 213.2 (C=O, C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.5 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.3 (C-19), 28.1 (C-20), 32.8 (C-21), 39.2 (C-22), 6.8 (C-23), 14.6 (C-24), 17.9 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28), 31.7 (C-29), 35.0 (C-30). The spectral data are the same as in [3] reference.

CN3-1 was identified as friedelin (Figure 1). Its EI-MS molecular ion peak at *m/z* 426 corresponded to the molecular formula of $C_{30}H_{50}O$. One secondary and seven tertiary methyls of the Friedelane skeleton were observed in the 1H-NMR spectrum as a singlet at δ 0.88 (*J* = 6.0

Hz, Me-23), δ 0.72 (Me-24), 0.87 (Me-25), 1.01 (Me-26), 1.05 (Me-27), 1.18 (Me-28), 1.00 (Me-29), 0.95 (Me-30), respectively. The most downfield carbon signal at δ 213.2 represented the 3-keto group of friedelin.

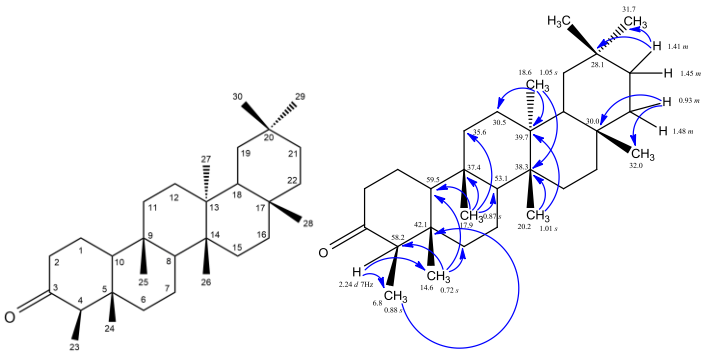

Figure 1. *Structure of friedelin*

CN3-6. colorless needles; **ESI-MS** *m/z*: 497.3 $[M-H]^-$; **^{1}H-NMR** ($CDCl_3$, 500 MHz) δ_H: 1.74 (1H, *d*, *J*=13.0 Hz, H-1), 1.04 (1H, *m*, H-1), 1.63 (2H, *m*, H-3), 4.48 (1H, *dd*, *J*=11.0 Hz, 6.0 Hz H- 4), 0.80 (1H, *d*, *J*= 11.5 Hz, H-4), 1.49 (1H, *s*, H-6), 1.35 (1H, *m*, H-6), 1.44(1H, *m*, H-7), 1.33(1H, *m*, H-7), 1.28(1H, *s*), 1.55(1H, *m*), 1.26 (1H, *s*), 1.25(1H, *s*), 1.63(1H, *m*), 2.21 (1H, *d*, *J*=11.0 Hz, H-13), 1.21 (1H, *d*, *J*=13.0 Hz, H-15), 1.63 (1H, *m*, H-15), 1.63 (1H, *m*, H-16), 2.16 (1H, *d*, *J*=13.5 Hz, H-16), 5.18 (1H, *s*, H-19), 1.38 (1H, *m*, H-21), 1.42 (1H, *s*, H-21), 1.46 (1H, *s*, H-22), 1.99 (1H, *m*, H-22), 0.85 (3H, *s*, Me-23), 0.84 (3H, *s*, Me-24), 0.89 (3H, *s*, Me-25), 0.99 (3H, *s*, Me-26), 0.77 (3H, *s*, Me-27), 1.00 (3H, *s*, Me-29), 0.98 (3H, *s*, Me-30), 2.05 (3H, *s*, Me-32); **^{13}C-NMR** ($CDCl_3$, 125 MHz) δ_C: 38.6 (C-1), 23.7 (C-2), 80.9 (C-3), 37.8 (C-4), 55.6 (C-5), 18.1 (C-6), 34.5 (C-7), 40.7 (C-8), 51.1 (C-9), 37.2 (C-10), 20.9 (C-11), 26.0 (C-12), 41.4 (C-13), 42.6 (C-14), 29.4 (C-15), 33.4 (C-16), 48.0 (C-17), 136.7 (C-18), 133.3 (C-19), 32.1 (C-20), 33.5 (C-21), 33.5 (C-22), 27.9 (C-23), 16.5 (C-24), 16.7 (C-25), 16.0 (C-26), 14.9 (C-27), 180.9 (C-28), 30.4 (C-29), 29.1 (C-30), 171.1 (C-31), 21.3 (C-32). The spectral data are the same as in [6] reference.

CN3-6 was identified as Morolic acid acetat (Figure 2). Its EI-MS molecular ion peak at *m/z* 498 corresponded to the molecular formula of $C_{32}H_{50}O_4$.

In ^{13}C-NMR of CN3-6 there are 32 carbon resonance signals: 9 C_{IV}, 5 C_{III}, 10 C_{II} and 8 C_I, C_{III} δ_C 133.3 ppm (C-19) double bond with C_{IV} δ_C 136.7 (C-18) ppm, C_{IV} δ_C 180.9 ppm (C-28) is –COOH group, C_{IV} δ_C 171.1 ppm (C-31) is -$COOCH_3$.

In ^{1}H NMR spectrum of CN3-6 displays signals for seven tertiary methyl groups at δ_H 0.85 (3H, *s*, Me-23), 0.84 (3H, *s*, Me-24), 0.89 (3H, *s*, Me-25), 0.99 (3H, *s*, Me-26), 0.77 (3H, *s*, Me-27), 1.00 (3H, *s*, Me-29), 0.98 (3H, *s*, Me-30), signals for one tertiary methyl at δ_H 2.05 (3H, *s*, Me-23) mounted to –COO group. The above data and comparison of ^{1}H- NMR et ^{13}C- NMR data with those of the known morolic acid acetat [6] suggests that the compound is morolic acid acetat.

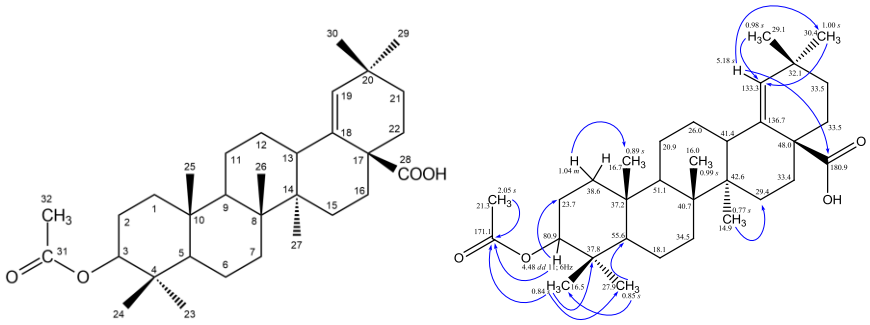

Figure 2. *Structure of morolic acid acetat*

CN12-2. colorless needles; **ESI-MS** *m/z*: 457.3 $[M+H]^+$, 439.2 $[M+H-H_2O]^+$, 455.2 $[M-H]^-$ **^{1}H-NMR** ($CDCl_3$ + CD_3OD, 500 MHz) δ_H: 0.89 (1H, *m*, H-1), 1.68 (1H, *m*, H-1), 1.57 (2H, *m*, H-2), 3.17 (1H, *t*, *J*=8.0 Hz, H-3), 0.68 (1H, *d*, *J*=9.5 Hz, H-5), 1.40 (1H, *m*, H-6), 1.51 (1H, *m*, H-6), 1.40 (2H, *m*, H-7), 1.27 (1H, *m*, H-9), 1.40 (1H, *m*, H-11), 1.27 (1H, *m*, H-11), 1.03 (1H, *m*, H-12), 1.68 (1H, *m*, H-12), 2.25 (1H, *m*, H-13), 1.40 (1H, *m*, H-15), 1.95 (1H, *m*, H-15), 1.40 (1H, *m*, H-16), 2.25 (1H, *m*, H-16), 3.01 (1H, *m*, H-18), 1.57 (1H, *m*, H-19), 1.51 (1H, *m*, H-21), 1.19 (1H, *m*, H-21), 1.40 (1H, *m*, H-22), 1.95 (1H, *m*, H-22), 0.95 (3H, *s*, Me-23), 0.75 (3H, *s*, Me-24), 0.83 (3H, *s*, Me-25), 0.96 (3H, *s*, Me-26), 0.98 (3H, *s*, Me-27), 4.60 (1H, *s*, H-29), 4.73 (1H, *s*, H-29), 1.69 (3H, *s*, Me-30); **^{13}C-NMR** ($CDCl_3$+CD_3OD, 125 MHz) δ_C: 39.0 (C-1), 27.2 (C-2), 79.0 (C-3), 55.6 (C-5), 18.5 (C-6), 34.6 (C-7), 40.9 (C-8), 50.8 (C-9), 37.4 (C-10), 21.1 (C-11), 25.8 (C-12), 38.6 (C-13), 42.7 (C-14), 30.8 (C-15), 32.5 (C-16), 56.5 (C-17), 47.2 (C-18), 49.5 (C-19), 151.0 (C-20), 30.0 (C-21), 37.4 (C-22), 28.1 (C-23), 15.5 (C-24), 16.1 (C-25), 16.2

(C-26), 14.8 (C-27), 179.3 (C-28), 109.6 (C-29), 19.4 (C-30). The spectral data are the same as in [7] reference.

CN12-2 was identified as betulinic acid (Figure 3). Its EI-MS molecular ion peak at *m/z* 456 corresponded to the molecular formula of $C_{30}H_{48}O_3$.

In ^{13}C-NMR of CN12-2 there are 30 carbon resonance signals: 6 C_{IV}, 8 C_{III}, 10 C_{II}, 6 C_{I}. C_{IV} δ_C 179.3 ppm (C-28) is COOH group, C_{IV} δ_C 151.0 ppm (C-20) is double bond with C_{II} δ_C 109.6 (C-29). C_{III} δ_C 79.0 ppm (C-3) mounted to hydroxyl.

In ^{1}H-NMR spectrum at δ_H 0.95 (H-23); 0.75 (H-24); 0.83 (H-25); 0.96 (H-27); 1.69 (H-30) and two singles at 4.60 and 4.73 (H-29a and H-29b). In view of biogenetic considerations, one of the hydroxyl functions was placed at C-3, the *β* orientation of which was supported by a triplet at δ_H 3.17 ppm (*J*=8,0 Hz, H-3). The above data and comparison of ^{1}H- NMR and ^{13}C- NMR data with those of the known betulinic acid [7] suggests that the compound is betulinic acid.

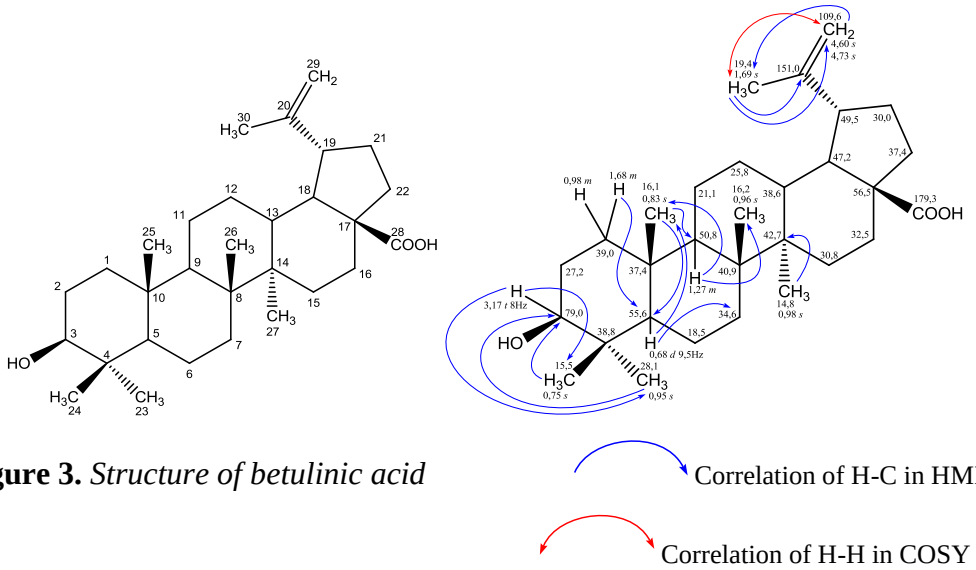

Figure 3. *Structure of betulinic acid*

CN16-2. solid white powder, **ESI-MS** *m/z*: 457.4 $[M+H]^+$, 439.2 $[M+H-H_2O]^+$. **^{1}H-NMR** ($CDCl_3$, 500 MHz) δ_H: 1.65 (2H, *s*, H-1), 1.60 (2H, *s*, H-2), 3.21 (1H, *dd*, *J*=4.0; 11.5 Hz, H-3), 0.72 (1H, *s*, H-5), 1.57 (2H, *m*, H-6), 1.42 (2H, *m*, H-7), 1.56 (1H, *m*, H-9), 1.88 (2H, *m*, H-11), 5.28 (1H, *t*, *J*=3.5 Hz, H-12), 1.60 (2H, *m*, H-15), 1.98 (2H, *m*, H-16), 2.82 (1H, *dd*, *J*=4.5; 14.0

Hz, H-18), 1.16 (1H, *s*, H-19), 1.62(1H, *s*, H-19), 1.22 (1H, *m*, H-21), 1.34 (1H, *m*, H-21), 1.25 (2H, *m*, H-22), 0.94 (3H, *s*, H-23), 0.77 (3H, *s*, H-24), 0.93 (3H, *s*, H-25), 0.74 (3H, *s*, H-26), 1.13 (3H, *s*, H-27), 0.90 (3H, *s*, H-29), 0.93 (3H, *s*, H-30); **^{13}C-NMR** ($CDCl_3$, 125 MHz) δ_C: 38.4 (C-1), 27.2 (C-2), 79.0 (C-3), 37.1 (C-4), 55.2 (C-5), 18.3 (C-6), 32.4 (C-7), 39.3 (C-8), 47.6 (C-9), 38.7 (C-10), 24.9 (C-4'), 22.7 (C-2'), 14.1 (C-1'). The spectral data are the same as in [8] reference.

CN16-2 was identified as (3*β*)-3-hydroxyolean-12-en-28 oic acid (Figure 4). Its **EI-MS** molecular ion peak at *m/z* 456 corresponded to the molecular formula of $C_{30}H_{48}O_3$.

In ^{13}C-NMR of CN16-2 there are 30 carbon resonance signals: 8 C_{IV}, 5 C_{III}, 10 C_{II}, 7 $C_{I.}$ C_{IV} δ_C 181.8 ppm (C-28) is COOH group. One C_{III} δ_C 79.0 ppm is carbon mounted to OH group, one C_{III} δ_C 122.7 ppm (C-12) double bond with C_{IV} δ_C 143.6 (C-13) ppm. The chemical shifts of C-l2 and C-l3 of olean-l2-enes are δ_C 122.0 and 145.0 ppm, respectively. From the above data and comparison of ^{1}H- NMR and ^{13}C- NMR data with those of the known (3*β*)-3-hydroxyolean-12-en-28 oic acid [8], suggests that the compound is (3*β*)-3-hydroxyolean-12-en-28 oic acid.

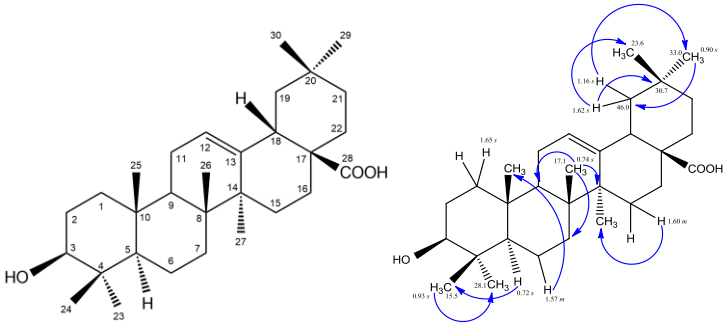

Figure 4. *Structure of (3β)-3-hydroxyolean-12-en-28 oic acid*

5. CONCLUSIONS

Four compounds: friedelin, morolic acid acetat, betulinic acid and (3*β*)-3-hydroxyolean-12-en-28 oic acid from the stems of *Clinacanthus nutans* were reported for the first time from this plant.

To our knowledge, betulinic acid is a new lupan tritepenoid isolated from stems of *Clinacanthus nutans*. There are many valuable effects that have been studied on betulinic acid such as the suppression of human immunodeficiency virus (HIV), antibacterial, parasitic resistance, anti-inflammatory and anti-cancer activity. This compound can be also used as a reference standard for qualification and standardization of herbal products containing the stems of *Clinacanthus nutans*.

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Bio-Screening for the Reduction Uric Acid Level in Blood of Some Southern Vietnamese Medicinal Plants

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Abstract

Introduction: Gout is a chronic disease which increases rapidly in devoloping countries such as Vietnam. Gout is caused by elevated levels of uric acid, the final metabolite of purine. Options for acute treatment of Gout include nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine and steroids that lead to many side effects on stomach or kidney. Thus, using natural products for supportive treatment of Gout is considered the optimal solution to help mitigate the side effects of synthetic drugs.**Objectives:** The aims of this study were to investigate the *in vitro* xanthine oxidase inhibitory activity and the *in vivo* effect of reducing uric acid level of the extracts prepared from different parts of plants which were collected in Southern Vietnam known for supporting treatment of Gout. **Materials:** Material samples of 21 plants of seven families as Acanthaceae, Amaranthacase, Caprifoliaceae, Flacourtiaceae, Gnetaceae, Lamiaceae and Moraceae were collected in provinces of Southern Vietnam in August, 2015. By selecting plants which have great inhibition, the inhibitory activity of extracts against xanthine oxidase from bovine milk was determined using modified assay procedure. The most active fractions were chosen based on the *in vitro* results of the lowest serum uric acid levels in a model of hyperuricemia in mice pretreated with oxonate. **Results:** Fifty-five samples of the used parts of 21 selected plants were extracted to give 165 extracts. Among the 165 extracts tested, the result showed that the most active one was the extract from sterm of *Gnetum latifolium* collected in Kien Giang province (IC_{50} = 190.54 μg/ml). Fractionation and determining the IC_{50} of fractions from this extract result in the most inhibitory effect one was chloroform fraction (IC_{50} = 150.37 μg/ml), in comparison with the ethyl acetate (IC_{50} = 186.20 μg/ml), and the water fraction (IC_{50} = 216.67 μg/ml). The chloroform fraction of *G. latifolium* stem wood significantly reduced uric acid levels in blood of hyperuricemic mice in a dose-dependent manner (150, 100 and 50 mg/kg). **Conclusion:** This research could be used not only for finding and learning about the medicinal plants resource that are active in the treatment of Gout, but also promoted the investigation of active constituents and using them for development of natural-based medicines from this medicinal plant.

Keywords: Xanthine oxidase inhibitory activity, Potassium oxonate induced hyperuricemia mice, *Gnetum latifolium* Blume *var. latifolium*

1. INTRODUCTION

Gout is the most common form of inflammatory arthritis caused by the elevated levels of serum uric acid that leads to monosodium urate crystal formation in and around the joints. The prevalence and incidence of gout are increasing in many parts of the world over the past 50 years with the contribution of many risk factors such as hyperuricemia, genetics, nutrition, and medications.[1] Nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine and steroids are frequently indicated for acute treatment of gout but with many side effects on stomach or kidney. Thus, using natural products for supportive treatment of gout is considered the optimal solution to help mitigate the side effects of synthetic drugs. The aims of this study were investigation the *in vitro* xanthine oxidase inhibitory activity and the *in vivo* uric acid reducing effect of the extracts prepared from different plant parts which were known for supporting treatment of Gout collected in Southern Vietnam.

2. MATERIALS AND METHOD

2.1. Plant materials

55 plant samples from 21 species of seven families (Table 1) i.e. Acanthaceae, Amaranthacase, Caprifoliaceae, Flacourtiaceae, Gnetaceae, Lamiaceae, and Moraceae were collected in provinces of Southern Vietnam in August, 2015. The plant materials were authenticated base on the reference literatures,[6,8] separated into different used parts, dried and grinded to powders.

2.2. Experimental animal

Male *Swiss albino* mice with the mean body weight of 20-30 g were acquired from the Institute of Vaccines and Medical Biologicals (Nha Trang, Vietnam) and allowed one week to adapt to their environment before used for experiments. Standard food and water were given *ad libitum* during the period of study.

2.3. Chemicals

Xanthine oxidase (X1875), xanthine (X4002), allopurinol (A8003), potassium oxonate (156124), monopotassium phosphat, dipotassium phosphat, and uric acid assay kit (MAK077) were purchased from Sigma-Aldrich (Missouri, USA). Other chemicals and reagents were of high quality and obtained from commercial sources.

Table 1. *List of plant materials used for xanthine oxidase inhibition activity assay*

Latin name	Family	Used parts	Location
Artocarpus altilis (Park.) Fosb.	Moraceae	Leaves, stem, root	Ho Chi Minh C.
Artocarpus heterophyllus Lamk.	Moraceae	Leaves, stem, root	Ho Chi Minh C.
Artocarpus integer (Thunb.) Merr.	Moraceae	Leaves, stem	Ho Chi Minh C.
Clinacanthus nutans	Acanthaceae	Leaves, stem, root	Ho Chi Minh C.
Ficus auriculata Lour.	Moraceae	Leaves, stem, root	Binh Duong
Ficus benjamina L.	Moraceae	Leaves, stem, root, rhizome	Ho Chi Minh C.
Ficus elastica Roxb. ex Horn.	Moraceae	Leaves, stem, root, rhizome	Ho Chi Minh C.
Ficus heterophylla L. f.	Moraceae	Leaves, stem, root	Binh Phuoc
Ficus hirta Vahl	Moraceae	Leaves, stem, stem root	Binh Phuoc
Ficus hispida L. f.	Moraceae	Leaves, stem, root	Binh Phuoc
Ficus microcarpa L. f.	Moraceae	Leaves, rhizome	Dong Thap
Ficus pumila L.	Moraceae	Leaves, stem	Ho Chi Minh C.
Ficus racemosa L.	Moraceae	Leaves, stem, root	Ho Chi Minh C.
Ficus religiosa L.	Moraceae	Leaves, stem, root	Ho Chi Minh C.
Flacourtia rukam Zoll. Et Mor.	Flacourtiaceae	Leaves, stem, root	An Giang
Gnetum montanum Markgr.	Gnetaceae	Stem	Kien Giang
Gnetum latifolium Blume *var. latifolium*	Gnetaceae	Stem	Kien Giang
Gomphrena celosioides Mart	Amaranthacase	Flower, leaves, stem	Ho Chi Minh C.
Lonicera japonica Thunb.	Caprifoliaceae	Flower	Ho Chi Minh C.
Morus alba L.	Moraceae	Leaves, stem, root	Lam Dong
Perilla frutescens (Burm.f.) Lindau	Lamiaceae	Leaves, stem	Ho Chi Minh C.

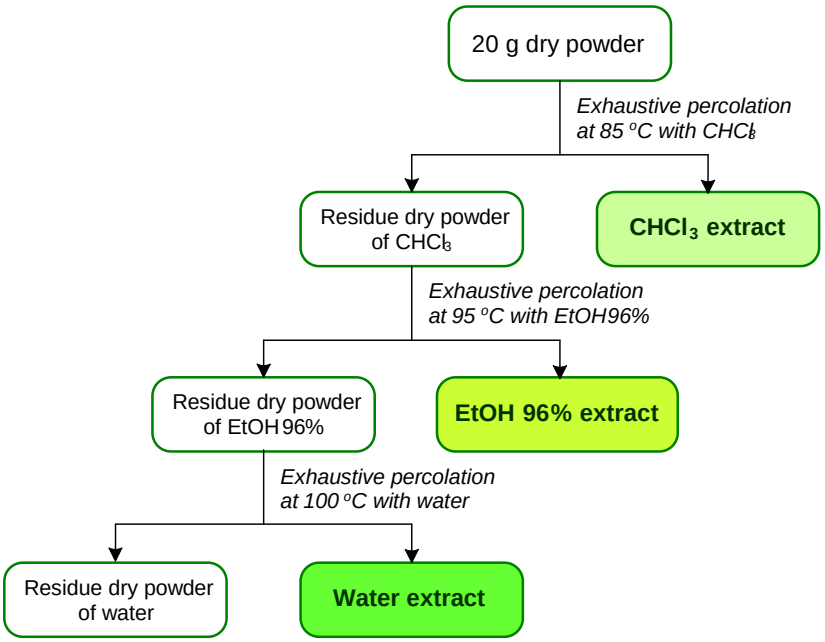


Figure 1. *Scheme of sample preparation for xanthine oxidase inhibitory activity assay*

2.4. Assay for xanthine oxidase inhibitory activity

Dry powder (20 g) of each samples was extracted sequentially with chloroform, ethanol 96% and water (Fig. 1) to give the respective crude extracts for *in vitro* screening assay. The inhibition activities of extracts against bovine milk's xanthine oxidase with xanthine as the substrate was measured spectrophotometrically based on the procedure reported by Lin et al. (2009)[2] with modifications. The assay mixture included 50 µl of test solution, 60 µl of phosphate buffer (70 mM, pH 7.5), and 30 µl of xanthine oxidase (0.01 U/ml, prepared prior to use). After pre-incubation at 25^{o}C for 15 minutes, the reaction was initiated by adding 60 µl of xanthine (150 µM). The final assay mixture was incubated at 25^{o}C for 30 minutes. After that, the optical density wers measured by ELISA EPOCH Microplate Spectrophotometer from BioTek (Winooski, USA) at 290 nm. A blank, control and test control mixtures were prepared in the same way without certain component as shown in table 2. The crude extracts were dissolved initially in DMSO and followed by dilution with the buffer. Xanthine oxidase inhibitory activity was expressed as the percentage inhibition of xanthine oxidase in the above assay system, calculated as:

$$\% \text{ Inhibiton} = \left(1 - \frac{Abs\ test - Abs\ test\ control}{Abs\ control - Abs\ blank}\right) \times 100$$

Table 2. *Experimental design of components for bank, control, test control and test*

	Sample	Xanthine oxidase	Xanthine	Phosphate buffer
Blank			+	+
Control		+	+	+
Test control	+		+	+
Test	+	+	+	+

2.5. Assay for the lowering of uric acid on potassium oxonate induced hyperuricemia mice

The effect on serum uric acid levels in a model of hyperuricemia in mice pretreated with oxonate was determined by modification of the procedure reported by Sheu et al. (2016).[7] The experimental animals were divided into 7 groups based on the basis of their body weight (8 mice per group). Different dose of test samples were orally administered every two days during the 14 days of the experiment. On days 0, 7, and 14, experimental hyperuricemia was induced in *Swiss albino* mice by intraperitoneally injection of the uricase inhibitor potassium oxonate (250

mg/kg). Uric acid in tail vein blood was measured 2 hours after PO injection using Uric acid Assay Kit of Sigma-Aldrich. Allopurinol was used as a positive control at the dose of 10 mg/kg.

Table 3. *Experimental design of animal groups*

Groups	Intraperitoneally injection	Orally treatment
Vehicle control	0.9% Saline	Distilled water
Solvent control		Tween 80 – Distilled water (1:9, v/v)
Negative control	Potassium oxonate (250 mg/kg)	Distilled water
Positive control		Allopurinol (10 mg/kg)
Test 1		Test sample (150 mg/kg)
Test 2		Test sample (100 mg/kg)
Test 3		Test sample (50 mg/kg)

2.6. Statistical analysis

All data were expressed as the mean ± standard deviation. The IC_{50} values was estimated from the % inhibition versus concentration plot using a linear regression algorithm by Sigma Plot 13.0. Statistical analyses were performed using the Student's t-test method for two-group comparison by SPSS 20. P values of less than 0.05 were considered statistically significant.

Table 4. *The results of assay for xanthine oxidase inhibition activity*

Plants	Used parts	% Xanthine oxidase inhibition											
		1 mg/ml			0.5 mg/ml			0.25 mg/ml			0.125 mg/ml		
		C	E	W	C	E	W	C	E	W	C	E	W
A. altilis	Leaves	–	28.04±0.002	28.68±0.004	36.57±0.007	22.40±0.017	27.41±0.017	4.81±0.041	26.62±0.039	28.22±0.028	–	6.51±0.014	5.14±0.032
	Stem	17.05±0.058	14.10±0.173	23.41±0.033	4.22±0.029	17.86±0.024	24.79±0.028	6.16±0.023	22.27±0.010	25.84±0.014	–	4.17±0.025	7.63±0.011
	Root	10.49±0.026	17.62±0.047	20.85±0.014	7.28±0.045	19.28±0.009	47.52±0.028	7.76±0.011	28.79±0.024	31.50±0.018	4.31±0.023	7.28±0.019	13.79±0.028
A. heterophyllus	Leaves	–	18.80±0.029	27.50±0.008	–	20.22±0.040	7.98±0.021	9.86±0.029	32.28±0.002	33.64±0.029	–	14.08±0.033	12.66±0.008
	Stem	–	15.13±0.033	22.91±0.005	–	4.20±0.058	30.73±0.046	7.65±0.011	28.37±0.023	30.43±0.042	–	13.73±0.022	14.78±0.019
	Root	–	17.68±0.018	16.15±0.012	77.88±0.036	7.28±0.024	9.53±0.026	44.87±0.033	12.77±0.014	21.07±0.031	10.67±0.020	11.54±0.001	9.84±0.022
A. integer	Leaves	3.39±0.018	35.17±0.034	31.50±0.046	–	3.80±0.013	13.86±0.057	7.04±0.014	12.57±0.016	17.77±0.009	3.21±0.019	0.37±0.026	9.68±0.010
	Stem	–	31.56±0.024	20.74±0.023	–	10.30±0.026	10.97±0.008	–	11.74±0.009	14.84±0.005	0.85±0.036	5.60±0.010	7.48±0.002
C. nutans	Leaves	47.10±0.027	45.81±0.034	38.56±0.005	17.09±0.009	22.17±0.031	20.35±0.025	24.36±0.032	25.78±0.014	41.52±0.012	9.25±0.016	14.10±0.009	25.48±0.072
	Stem	22.00±0.034	26.14±0.007	23.55±0.017	8.99±0.033	33.32±0.017	16.98±0.026	39.28±0.042	31.84±0.018	31.22±0.022	16.52±0.046	18.57±0.049	17.55±0.059
	Root	28.38±0.158	28.45±0.030	27.98±0.018	–	20.31±0.020	16.28±0.013	21.39±0.030	36.84±0.016	39.38±0.029	10.50±0.008	16.07±0.057	16.18±0.005
F. auriculata	Leaves	–	24.21±0.035	19.51±0.027	–	–	10.28±0.025	6.37±0.012	16.56±0.015	20.86±0.038	6.63±0.010	7.54±0.039	14.23±0.036
	Stem	–	17.03±0.007	18.51±0.023	–	5.49±0.032	12.18±0.012	5.02±0.013	22.83±0.008	18.55±0.034	8.38±0.024	23.04±0.036	11.31±0.036
	Root	1.65±0.011	28.87±0.015	26.91±0.033	–	–	5.49±0.016	4.66±0.023	14.96±0.021	15.60±0.023	4.06±0.026	–	9.70±0.036
F. benjamina	Leaves	–	22.14±0.009	17.01±0.026	–	7.91±0.042	8.87±0.011	3.48±0.029	10.73±0.010	13.29±0.042	6.45±0.007	24.86±0.021	13.31±0.025
	Stem	–	36.97±0.013	21.52±0.008	–	13.36±0.037	12.72±0.023	7.33±0.009	22.68±0.031	20.32±0.021	5.81±0.032	14.58±0.016	14.13±0.019
	Root	–		19.38±0.020	–	0.94±0.010	8.48±0.009	2.16±0.006	10.73±0.012	16.26±0.005	–	7.16±0.015	22.10±0.033
	Rhizome	–	14.77±0.008	7.54±0.004	–	5.81±0.045	15.56±0.030	2.74±0.009	17.76±0.026	15.07±0.031	–	15.96±0.041	20.03±0.009
F. elastica	Leaves	31.90±0.070	28.20±0.001	17.32±0.009	–	–	3.74±0.041	28.92±0.027	20.93±0.029	25.55±0.026	4.42±0.004	4.73±0.033	7.01±0.022
	Stem	–	13.82±0.024	22.27±0.014	–	–	2.50±0.014	16.42±0.022	21.11±0.011	25.93±0.026	2.91±0.006	5.67±0.018	8.43±0.017
	Root	6.85±0.021	35.43±0.013	54.98±0.032	–	–	3.92±0.018	19.49±0.016	18.90±0.009	24.30±0.046	2.17±0.013	–	7.49±0.027
	Rhizome	–	23.80±0.006	20.32±0.008	–	6.66±0.022	10.55±0.019	23.17±0.016	28.71±0.025	18.94±0.013	10.86±0.005	5.71±0.017	7.07±0.009
F. heterophylla	Leaves	–	17.44±0.004	20.09±0.048	–	15.71±0.020	12.93±0.037	4.62±0.025	15.90±0.013	14.53±0.012	2.29±0.026	15.02±0.021	11.71±0.019
	Stem	–	9.23±0.017	14.83±0.014	–	10.62±0.026	13.02±0.020	–	14.23±0.016	24.86±0.017	2.05±0.011	15.79±0.025	7.82±0.016
	Root	–	18.29±0.073	17.78±0.028	–	19.30±0.017	11.97±0.013	14.19±0.021	26.69±0.018	15.07±0.029	6.71±0.025	10.19±0.025	19.09±0.041
F. hirta	Leaves	–	29.38±0.031	19.20±0.023	–	13.03±0.033	12.24±0.028	–	23.43±0.002	23.74±0.017	–	5.87±0.026	11.30±0.026
	Stem	–	33.35±0.047	22.25±0.005	–	24.04±0.043	13.86±0.042	12.98±0.020	11.96±0.026	19.31±0.007	0.99±0.013	3.26±0.009	5.45±0.025
	Stem root	15.55±0.028	28.18±0.015	16.66±0.020	0.68±0.012	22.79±0.028	15.94±0.059	0.79±0.025	14.80±0.013	14.67±0.021	–	8.56±0.019	2.12±0.003
F. hispida	Leaves	34.31±0.022	8.65±0.025		–	10.09±0.028	19.71±0.014	–	12.06±0.008	10.38±0.005	–	4.75±0.010	5.15±0.006
	Stem	–	39.26±0.014	20.25±0.032	–	16.55±0.008	24.87±0.016	–	20.28±0.018	9.59±0.012	–	6.90±0.020	18.41±0.028
	Root	–	16.55±0.012	22.07±0.004	–	4.86±0.024	22.05±0.024	3.92±0.009	13.25±0.020	19.44±0.015	4.12±0.018	7.38±0.024	4.88±0.009
F. microcarpa	Leaves	31.82±0.074	54.38±0.003	58.68±0.005	–	–	11.91±0.006	5.12±0.023	18.46±0.011	17.51±0.050	–	–	0.68±0.030
	Rhizome	–	28.93±0.020	16.65±0.013	–	–	1.32±0.022	3.97±0.006	11.40±0.012	12.02±0.033	–	–	3.68±0.017

F. pumila	Leaves	–	44.48±0.015	29.24±0.020	–	10.06±0.012	10.21±0.031	–	8.36±0.024	16.27±0.026	–	5.95±0.020	8.31±0.027
	Stem	2.45±0.026	22.51±0.022	22.78±0.032	–	–	10.50±0.022	9.81±0.019	12.64±0.018	16.80±0.015	–	–	2.12±0.015
F. racemosa	Leaves	8.60±0.009	21.41±0.046	20.60±0.005	1.70±0.020	1.68±0.011	1.94±0.024	9.97±0.007	17.27±0.003	17.02±0.018	–	4.67±0.007	7.48±0.032
	Stem	6.31±0.027	10.17±0.015	29.17±0.020	–	–	–	9.20±0.003	21.87±0.006	17.33±0.005	1.04±0.031	12.79±0.038	7.74±0.037
	Root	–	17.00±0.015	18.83±0.024	–	–	5.49±0.015	4.87±0.019	16.74±0.014	18.02±0.029	1.74±0.002	12.41±0.023	10.61±0.045
F. religiosa	Leaves	0.60±0.035	18.06±0.042	8.16±0.024	–	4.01±0.022	5.27±0.006	–	12.41±0.011	16.76±0.021		9.00±0.017	8.60±0.013
	Stem	–	31.62±0.014	19.10±0.004	–	5.60±0.011	6.75±0.018	–	13.32±0.023	15.33±0.013	1.72±0.037	12.55±0.021	26.46±0.029
	Root	–	9.70±0.002	21.41±0.029	–	–	5.71±0.018	1.43±0.014	17.66±0.002	17.24±0.004	–	8.80±0.013	23.99±0.074
F. rukam	Leaves	–	100.00±0.009	102.93±0.006	15.45±0.041	87.34±0.038	87.96±0.016	5.13±0.055	35.83±0.029	32.84±0.032	–	28.53±0.027	29.98±0.025
	Stem	98.58±0.005	99.22±0.005	94.19±0.016	48.01±0.011	68.14±0.030	45.97±0.065	24.01±0.019	33.18±0.017	28.72±0.031	18.06±0.011	25.27±0.032	22.58±0.037
	Root	73.05±0.008	85.82±0.009	91.91±0.022	19.86±0.025	39.93±0.029	59.27±0.034	17.01±0.048	35.51±0.020	41.22±0.031	14.75±0.027	20.36±0.032	20.54±0.042
G. montanum	Stem	90.66±0.012	96.71±0.004	101.18±0.011	36.01±0.019	99.62±0.010	78.28±0.028	29.68±0.024	80.05±0.014	27.03±0.022	25.41±0.145	38.47±0.021	19.04±0.033
G. latifolium	Stem	95.43±0.001	**96.45±0.002**	–	67.72±0.025	**93.89±0.007**	83.35±0.018	37.89±0.029	**82.09±0.020**	51.52±0.030	25.15±0.043	**43.12±0.049**	28.21±0.045
G. celosioides	Flower	31.42±0.035	55.36±0.006	27.25±0.012	12.56±0.009	20.86±0.021	13.37±0.018	20.28±0.019	19.28±0.034	23.39±0.014	9.41±0.023	6.53±0.006	9.16±0.028
	Leaves	15.90±0.057	37.46±0.035	21.47±0.007		18.09±0.001	12.35±0.009	25.04±0.004	27.63±0.018	21.55±0.046	7.11±0.030	10.07±0.015	8.34±0.015
	Stem	26.02±0.007	18.99±0.011	26.79±0.005	4.86±0.015	12.52±0.006	10.55±0.015	19.11±0.013	12.98±0.010	21.10±0.026	3.42±0.004	3.56±0.022	8.01±0.015
L. japonica	Flower	30.65±0.016	62.37±0.003	58.47±0.015	19.72±0.059	28.88±0.033	25.94±0.031	17.42±0.028	21.22±0.009	22.10±0.040	4.52±0.049	7.09±0.007	11.87±0.039
M. alba	Leaves	8.03±0.027	22.56±0.043	23.46±0.008	–	4.30±0.030	7.32±0.015	0.37±0.034	23.44±0.012	22.40±0.008	–	3.75±0.018	4.98±0.012
	Stem	2.51±0.019	49.50±0.014	16.71±0.037	–	9.86±0.035	6.62±0.006	6.44±0.011	18.28±0.020	20.40±0.008	–	12.77±0.008	17.00±0.019
	Root	42.34±0.011	88.36±0.001	26.97±0.020	29.61±0.027	53.58±0.101	8.75±0.014	–	8.67±0.025	17.84±0.0009	–	–	14.31±0.057
P. frutescens	Leaves	–	42.27±0.010	93.51±0.014	–	25.06±0.017	35.59±0.018	24.40±0.041	30.40±0.029	28.09±0.019	10.03±0.031	16.05±0.013	15.84±0.015
	Stem	49.01±0.007	26.03±0.007	29.00±0.034	17.91±0.031	23.02±0.017	15.18±0.011	25.29±0.038	29.76±0.023	25.86±0.032	15.86±0.008	13.74±0.017	11.96±0.007

3. RESULTS AND DISCUSSION

3.1. Assay for xanthine oxidase inhibition activity

The xanthine oxidase inhibitory activity of 165 extracts from 55 different used parts of 21 plants were tested at 4 concentrations ranging from 0.125-1 mg/ml. The results of this bio-screening assay were shown in Table 4. Among 165 experimental of xanthine oxidase inhibitors, the ethanol extract of *Gnetum latifolium* stem wood showed the strongest xanthine oxidase inhibitory activity, with the IC_{50} is 190.54 µg/ml.

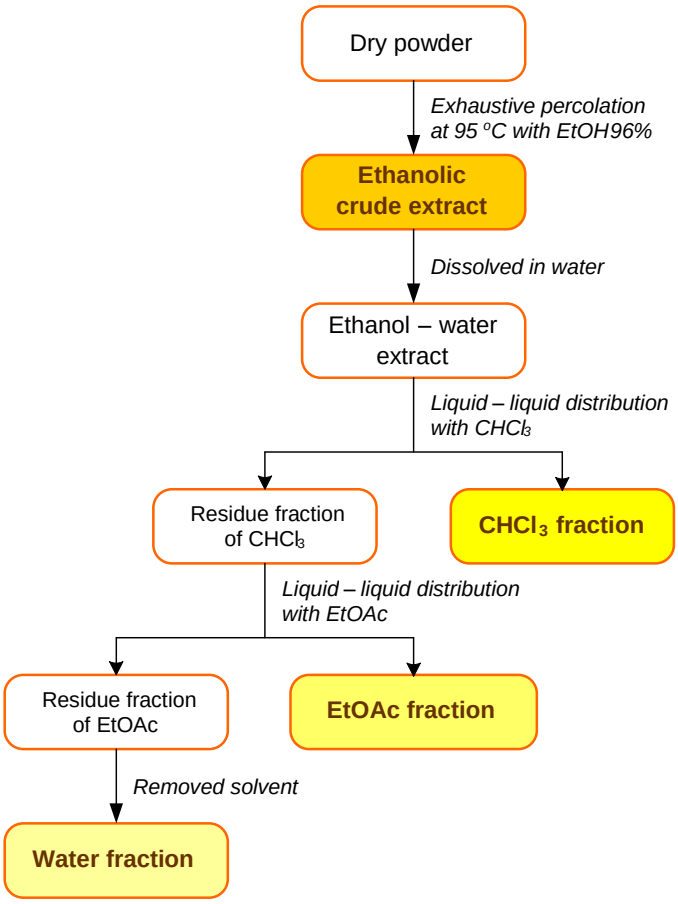
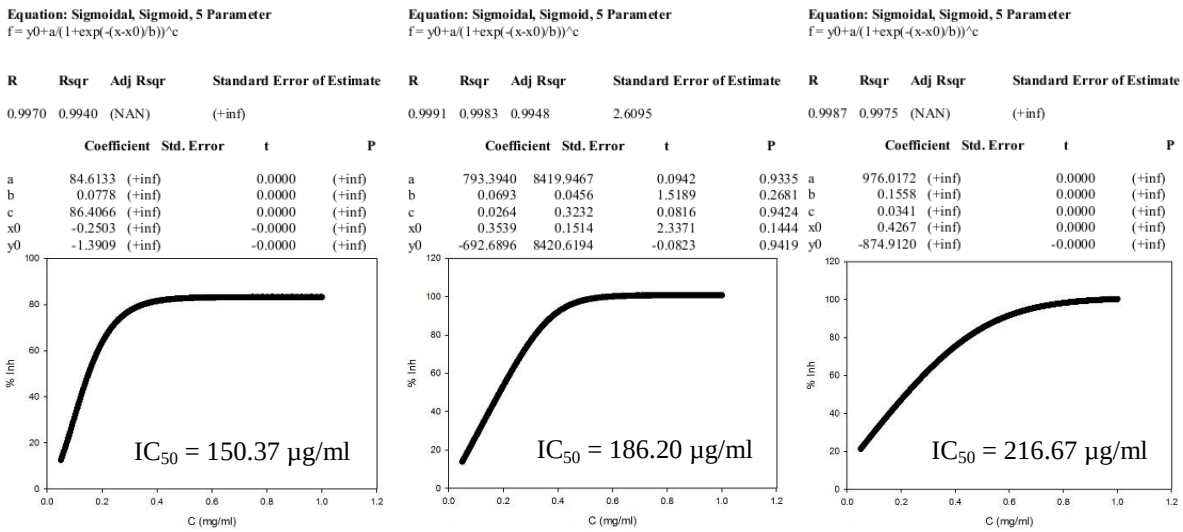


Figure 2. *Scheme for fractionation of the ethanol extract from G. latifolium stem wood*

The ethanol crude extract of *G. latifolium* stem wood was sequentially partitioned with chloroform, ethyl acetate, and water to yield respective fractions. The IC_{50} of xanthine oxidase inhibitory activity of these 3 fractions were determined (Fig. 3). The result revealed that chloroform fraction was the most active one (IC_{50} of 150.37 µg/ml). This fraction was chosen for further study on serum uric acid levels in a model of hyperuricemia in mice pretreated with oxonate.



Chloroform fraction *Ethyl acetat fraction* *Water fraction*

Figure 3. *Xanthine oxidase IC_{50} values of 3 fractions from the ethanol extract of G. latifolium stem wood*

3.2. Assay on the uric acid lowering effect on potassium oxonate induced hyperuricemia mice

The chloroform fraction of *G. latifolium* stem wood significantly reduced serum uric acid levels of hyperuricemic mice in a dose-dependent manner (150, 100 & 50 mg/kg). The highest concentration,150 mg/kg, of *G. latifolium* chloroform fraction showed the strongest effect while compared with 2 others concentrations. After 14 experimental days, this concentration also increased the effect of reducing serum uric acid level by time. To the best of our knowledge, this is the first scientific report to prove the rationale use of *G. latifolium* for anti-hyperuricemia.

Table 5. *The average serum uric acid levels of experimental groups at day 0, 7 and 14*
($^{@}$: $p < 0,01$ compared with average serum uric acid levels of vehicle control group
**: $p < 0,01$ compared with average serum uric acid levels of negative control group*
$^{\#}$: $p < 0$, compared with average serum uric acid levels of the same group at day 0)

Groups	Average serum uric acid levels (mg/dL)		
	Day 0	Day 7	Day 14
Vehicle control	1,780 ± 0,019	1,608 ± 0,176	1,685 ± 0,052
Solvent control	1,901 ± 0,104	1,710 ± 0,061	1,734 ± 0,059
Negative control	3,576 ± 0,009$^{@}$	3,455 ± 0,165$^{@}$	3,588 ± 0,021$^{@}$
Positive control	1,609 ± 0,003*	1,615 ± 0,008*	1,577 ± 0,206*
Test 1 (150 mg/kg)	1,779 ± 0,015*	1,546 ± 0,041*$^{\#}$	1,512 ± 0,105*$^{\#}$
Test 2 (100 mg/kg)	1,976 ± 0,045*	1,796 ± 0,110*$^{\#}$	1,853 ± 0,093*$^{\#}$
Test 3 (50 mg/kg)	2,088 ± 0,022*	2,068 ± 0,111*	2,062 ± 0,052*

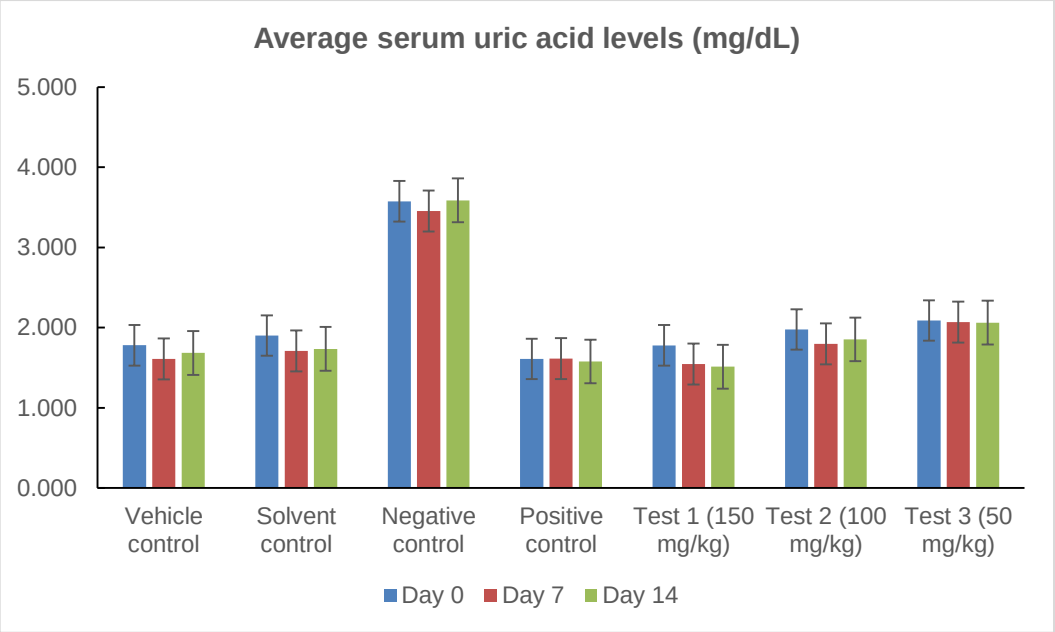


Figure 4. *The average serum uric acid levels of experimental groups at day 0, 7 and 14*

4. CONCLUSION

55 plant sample of the used parts of 21 selected plants were extracted to give 165 chloroform, ethanol and water extracts. Among these 165 extracts, the most active one was the one from sterm wood of *Gnetum latifolium* collected in Kien Giang Province (IC_{50} = 190.54 µg/ml). Fractionation of this extract by liquid-liquid extraction result in the most inhibitory effect one was chloroform fraction (IC_{50} of 150.37 µg/ml), in comparison with the ethyl acetat (IC_{50} of 186.2 µg/ml), and the water fraction (IC_{50} of 216.67 µg/ml). The chloroform fraction of *G. latifolium* stem wood significantly reduced uric acid levels in blood of hyperuricemic mice in a dose-dependent manner (150, 100, and 50 mg/kg). This result could be use not only for further study on the effect of this plant in the treatment of Gout, but also promoted the investigation of active constituents and using them for development of natural-based medicines from this medicinal plant.

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Optimization of The Synthesis of Methoxycarbonyl-*β*-Cyclodextrin as Chiral Selector

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Abstract

Introduction: The chiral separation of racemic drugs has been becoming a necessary stage in pharmaceutical industry as well as in clinical therapeutics. Thus, the new trend in chiral separation research is developing new chiral separation techniques or discovering new chiral selectors. *β*- cyclodextrin (*β*-CD) and its derivatives have been used widely for the separation of enantiomers by high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE). In this study, we have prepared and optimized the sythetic process of methoxycarbonyl-*β*-cyclodextrin, a derivative of *β*-CD, which has been studied recently and has not been introduced in the market. The researches of this derivative are still too little and there have been no published local studies on chemical synthesis of methoxycarbonyl- *β*-cyclodextrin so far. **Objectives:** The aim of this study was to optimize the synthesis of methoxycarbonyl-*β*-cyclodextrin from *β*-cyclodextrin. **Materials and methods:** Methoxycarbonyl-*β*-cyclodextrin was synthesized by nucleophilic substitution reaction of *β*-cyclodextrin, dimethyl carbonate (DMC) in *N*, *N*-dimethylformamide (DMF) with alkaline condition (potassium carbonate). The crude product was purified by precipitation with acetone and diethyl ether. Influences of reaction temperature, reaction duration and reactant ratio on the substitution degree and the yield of CM-*β*-CD were discussed. The synthetic process was optimized by Modde 5.0 software using Box-Behnken model to achieve the highest yield and the degree of substitution (DS). The structure of the synthesized compound was elucidated by IR, MS, and NMR data. **Results:** Methoxycarbonyl-*β*-cyclodextrin was synthesized from *β*-cyclodextrin under optimized conditions (reacting for 15 h at 83 ℃ and the molar ratios of DMC to β-CD 38,5:1) with optimized yield and DS approximately 45,57% and 1,3; respectively. **Conclusion:** We have successfully developed the practically optimized procedure for synthesis of methoxycarbonyl-*β*-cyclodextrin as the chiral selector. This study provides a theoretical and experimental basis for further scale-up research. In the future, we are going to developing the application of this selector for separation of enantiomer drugs by capillary electrophoresis.

Keywords: Methoxycarbonyl-*β*-cyclodextrin, *β*-cyclodextrin derivatives, chiral selector

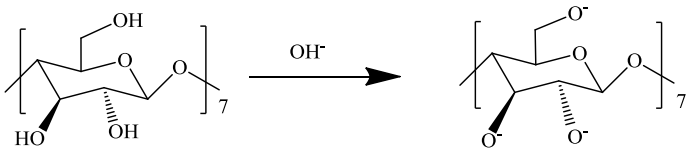
1. INTRODUCTION

The chiral separation of racemic drugs has been becoming a necessary stage in pharmaceutical industry as well as in clinical therapeutics. Thus, the new trend in chiral separation research is developing new chiral separation techniques or discovering new chiral selectors. *β*-cyclodextrin (*β*-CD) and its derivatives have been widely used for the separation of enantiomers by high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE)[1-6]. *β*-CD is a natural torus-shaped cyclic oligosaccharide consisting of seven *α*-D-glucose units connected through *α*-(1.4)-glycosidic linkages with the hydrophobic intracavity and the hydrophilic extracavity. The special structure makes *β*-CD have analytical applications[2,4-5]. However, *β*-CD's application is limited because of its low solubility, so that modifying *β*-CD to improve its solubility has been a trend[7]. In this study, we reported an optimization of the synthetic process of methoxycarbonyl-*β*-cyclodextrin, a derivative of *β*-CD, which has been studied recently and has not been introduced in the market. There have been several studies of this derivative and none of them was developed in Viet Nam.

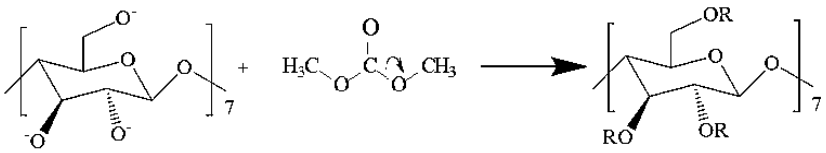
2. MATERIALS AND METHODS

β-CD and dimethyl carbonate (DMC) were purchased from Himedia and Sigma-Aldrich, respectively. Potassium carbonate, dimethyl carbonate (DMC), *N*, *N*-dimethylformamide (DMF), and other solvents were of analytical grade. Thin layer chromatography (TLC) was performed on Kieselgel 60 F_{254} silica-coated aluminum plates (Merck).

Methoxycarbonyl-*β*-cyclodextrin (MC-*β*-CD) was synthesized by nucleophilic substitution reaction of *β*-cyclodextrin, DMC in DMF with alkaline condition (potassium carbonate)[8-11].



Scheme 1: *Alkalizing alcoholic hydroxyl group of β-CD*



R= -$COOCH_3$ or –H

Scheme 2: *Synthesis of MC-β-CD from β-CD and DMC*

1 gram (0.88 mmole) of *β*-CD was alkalize by 1.5 gram of potassium carbonate in 20 ml DMF at 80^{o}C in 20 minutes, then 3.5 ml (39.6 mmole) of DMC was added, the reaction mixture was stirred thoroughly and adjusted to the temperature of 90^{o}C. The end point of reaction was monitored by TLC with the solvent mixture consisting of isopropanol-water-

ethyl acetate (6:3:1). The resulting mixture was filtered, then distilled off the organic solvent by using evaporator to obtain the yellow-brown solid. Adding 20 ml acetone and collecting the solid by filtration, the crude product (spM) was purified three times by 10 ml diethyl ether. The product was dried at 50^{o}C under vacuum. The structure of synthesized product was elucidated by IR, MS, and NMR data. Influences of reaction temperature, reaction duration and reactant ratio on the substitution degree and the yield of MC-*β*-CD were discussed. The synthetic process was optimized by Modde 5.0 software using Box-Behnken model to achieve the highest yield and the degree of substitution (DS).

3. RESULTS AND DISCUSSION

3.1. Synthesis of MC-β-CD from β-CD

0.29 gram of spM was synthesized from 1 gram of *β*-CD. SpM is in a form of white powder, soluble in water and insoluble in ether, and acetone. Melting point of spM is 250-260^{o}C and $[\alpha]_D^{20}$ = 160° (1% solution in water).

The spM was checked the purity by using TLC with two different polar solvent systems which were isopropanol-water-ethyl acetate (6:3:1) (R_f of β-CD and spM were 0.34 and 0.77, respectively), and dichloromethane-methanol (2:1) (R_f of *β*-CD and spM were 0.18 and 0.64, respectively).

The IR spectrum of spM (KBr, cm^{-1}) showed characteristic absorption peaks of functional groups, including -OH (3383 cm^{-1}), -CH_3 (2929 cm^{-1}), C=O (1753 cm^{-1}), and C-O-C (1050 cm^{-1}). The MS (positive ESI) of spM revealed an $[M+K]^+$ at m/z = 1463 that indicated the relative molecular mass similar to MC-*β*-CD $(C_6H_{10}O_5)_7(COOCH_3)_5$, and the MS (negative ESI) of spM revealed an $[M-H]^-$ at m/z = 1191 that indicated the relative molecular mass similar to MC-*β*-CD $(C_6H_{10}O_5)_7(COOCH_3)_1$. ^{1}H-NMR data were showed in table 1. As characterized by the data of IR, MS and NMR spectroscopy, spM was assigned to be MC-*β*-CD with DS approximately 1.33.

Table 1. *^{1}H-NMR (D_2O, 500 MHz) of spM*

Chemical shift δ (ppm)	Number of proton	Peaks	Position of peaks
5.12	7	*s*	H/C_1
3.63 – 4.13	46	*m*	H/C_2 – H/C_6 H/C_c

The MC-*β*-CD product, wherein the number of substituent groups per mole cyclodextrin is n methoxycarbonyl groups, "n" represents the average degree of substitution of methoxycarbonyl groups (DS). The proton number of H/C_2 – H/C_6 is 42, so the H_a number = 46 – 42 = 4. Therefore, the DS = 4/3 = 1.33.

3.2. Effect of reaction conditions on yield

3.2.1 Reaction duration

Figure 1 shows the effect of reaction time on yield. Synthetic yield was increased when reaction time increased from 14 to 16 hours and decreased when reaction time was increased from 10 to 16 hours. A value, which was near the maximum, was obtained approximately from 14 to 16 hours.

3.2.2 Reaction temperature

The influence of reaction temperature on synthetic yield was performed within a range of 70-100°C. Results were shown in Figure 2. The yield was increased when temperature increased from 70 to 80°C and decreased when temperature was increased from 80 to 100°C. The reaction temperature which gave the best yield, was 80^{o}C.

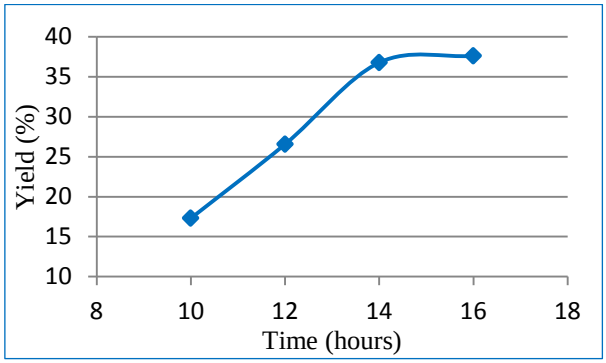


Figure 1. *Effect of reaction time on synthetic yield. Synthetic conditions: ratio of DMC and β-CD = 45, and 90°C*

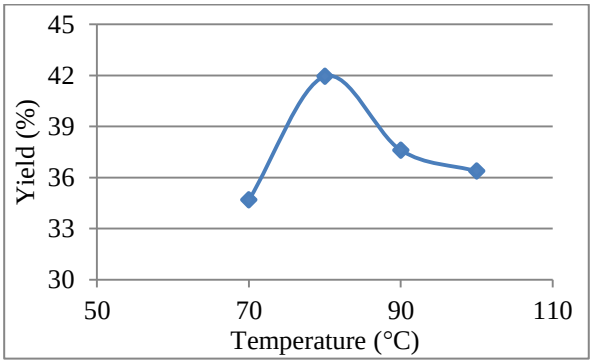


Figure 2. *Temperature effect on synthetic yield. Synthetic conditions: ratio of DMC and β-CD = 45, and 16 hours*

Ratio of DMC and β-CD

From Figure 3, it can be seen that the synthetic yield was increased when the ratio of DMC and *β*-CD was from 25 to 35, and it was decreased when the ratio of DMC and *β*-CD was increased from 35 to 55.

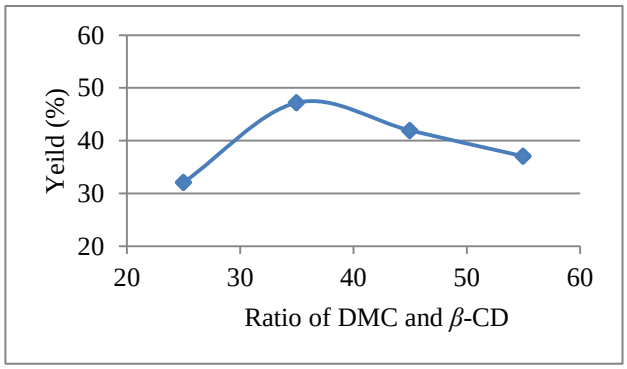


Figure 3. *Effect of ratio between DMC and β-CD on synthetic yield. Synthetic conditions: 16 hours, and 80°C*

3.3. Optimization of synthetic process by Modde 5.0 software using Box-Behnken model

Several preliminary experiments on synthesis of MC-*β*-CD showed that there are three factors impacting the reaction yield, including reaction duration (12-16 hours), reaction temperature (70-90°C) and ratio of DMC and *β*-CD (25-45 mole/mole) to achieve the best yield. Modde 5.0 software was applied for design experiments and calculating results of them (figure 4 and table 2), the optimized synthetic conditions were found as follows: 15 hours, 83^{o}C, and ratio of 38.5. In the conditions, the predicted yield was 45.57%.

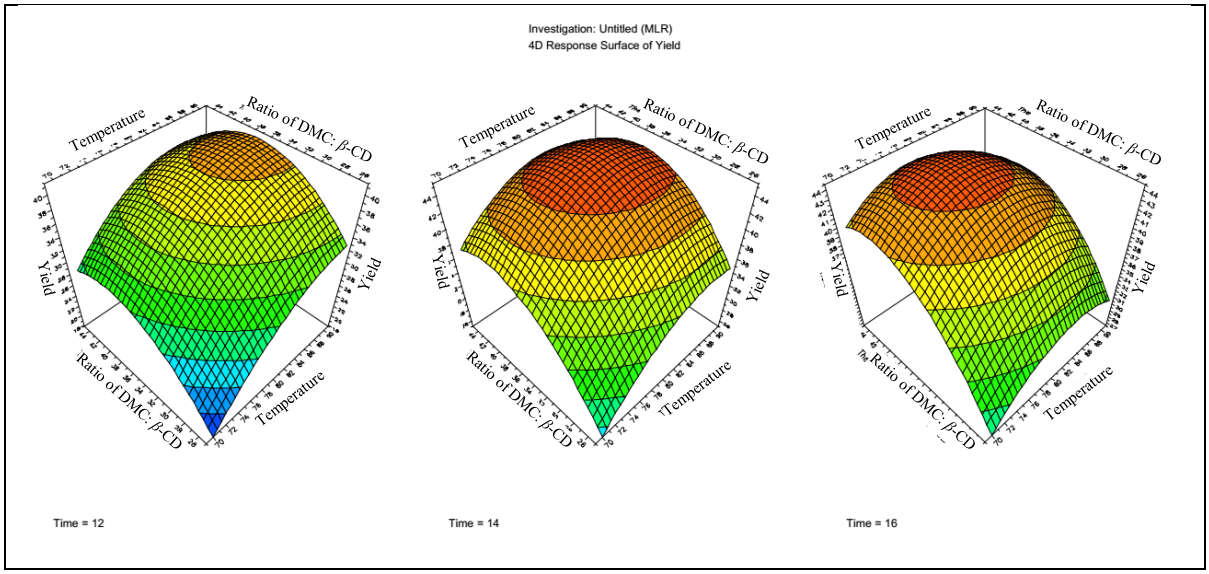


Figure 4. *The relation between 3 experimental variables and synthetic yield*

Table 2. *Effect of reaction duration (X1), reaction temperature (X2) and ratio of DMC and β-CD (X3) on synthetic yield*

Yield	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int(±)**
Constant	43.9133	0.49067	3.30132e-009	1.26131
X1	2.37375	0.300473	0.000522809	0.772391
X2	3.5825	0.300473	7.31489e-005	0.772391
X3	4.27625	0.300473	3.08539e-005	0.772391
X1*X1	-2.99542	0.442284	0.00106678	1.13693
X2*X2	-3.72292	0.442284	0.000388028	1.13693
X3*X3	-5.66541	0.442284	5.16062e-005	1.13693
X1*X2	-2.735	0.424932	0.00134547	1.09233
X1*X3	0.767498	0.424932	0.130716	1.09233
X2*X3	-1.5	0.424932	0.0167402	1.09233
N = 15	Q2 =	0.891	Cond. no. =	4.2385
DF = 5	R2 =	0.993	Y-miss =	0
	R2 Adj. =	0.980	RSD =	0.8499
			Conf. lev. =	0.95

The optimal conditions were experimentally repeated for three times. The actual yields (45.59%) was equivalent to predicted one.

4. CONCLUSIONS

The synthesis of methoxycarbonyl-*β*-cyclodextrin was successfully optimized with the yield and DS approximately 46% and 1.33, respectively. This chiral selector could be used to separate the enantiomer drugs by capillary electrophoresis.

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Analysis of Tramadol Enantiomers by Capillary Zone Electrophoresis With *Beta*-Cyclodextrin Derivatives as Chiral Selective Agents

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Abstract

Introduction: Chirality has become a key parameter in the development of pharmaceuticals. CE has been shown to be a powerful tool for chiral analysis. Beta-cyclodextrin (*β*-CD) and its derivatives become well established for the chiral discrimination of enantiomers by means of CE. **Objectives:** The aim of our study was to apply capillary zone electrophoresis (CZE) using *β*-CD derivatives as the chiral selectors for separation of tramadol enantiomer drugs. Moreover, we would like to develop and apply CE, a rather new technique, in quality control of chiral drugs in Vietnam. **Materials and methods:** The separation of the tramadol enantiomers was studied varying the experimental electrophoretic conditions. The pH value and concentrations of background electrolyte (BGE), the nature of chiral selectors and their concentrations were optimized by Modde 5.0 software using Box-Behnken model to achieve the best resolution. Then, the optimized method was validated in system suitability, selectivity, linearity and range, limit of detection, precision, and accuracy. **Results:** The optimized electrophoretic conditions were obtained using a 50 mM tris-phosphate buffer with pH 3 containing 0.5% carboxymethyl-*β*-cyclodextrin; fused-silica capillary (56/50 cm × 50 μm); 25^{o}C; 25 kV; 50 mbar * 5 s; 225 nm. Validation results showed that the method was suitable for the CE system, selective, linear (R > 0.995), precise (RSD < 2% for inter-day variations), and accurate (mean recoveries in the range of 98.0-106.0% of the expected values). **Conclusion:** The CZE approach for analysis of tramadol enantiomers was successfully developed using carboxymethyl-*β*-CD. The analytical procedure was proven to be selective, low limit of detection, precise and accurate. This procedure can be applied to analyse tramadol enantiomers in raw material or in pharmaceutical product.

Keywords: Tramadol enantiomers, capillary zone electrophoresis, carboxymethyl-β-cyclodextrin.

1. INTRODUCTION

Chirality has become a key parameter in the development of pharmaceuticals. Many drugs used as therapeutic agents, are chiral compounds which may have very different pharmacological properties and lower side effects compared to racemic mixture. The separation of enantiomeric mixtures is one of challenges in quality control of enantiomeric drugs. CE has been shown to be a powerful tool for chiral analysis. *Beta*-cyclodextrin (*β*-CD), and its derivatives become well established for the chiral discrimination of enantiomers by means of CE, etc.[1-6]. Recently, the requirement for quality control of chiral drugs is increasingly while the number of commercial chiral selectors is limited and sold with high price. In Vietnam, some of *β*-CD derivatives were successfully synthesized by our group. With the desire to prove the effect of these chiral selectors, this study was carried out to application of chiral selectors for analysis of tramadol enantiomers by capillary zone electrophoresis. Moreover, we would like to develop and apply CE, a rather new technique, in quality control of chiral drugs in Vietnam.

2. MATERIALS AND METHODS

2.1. Chemicals, solvents and drugs

All chemicals and organic solvents used in this work were of analytical grade. Tramadol was supplied from Institute of Drug Quality Control at Ho Chi Minh City, Vietnam. *β*-CD was purchased from Sigma. Carboxymethyl-*β*-CD (CM-*β*-CD), hydroxypropyl-*β*-CD (HP-*β*-CD) and methyl-*β*-CD (Me-*β*-CD) were synthesized and standardized by Department of Analytical Chemistry and Drug Quality Control, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam. The studied drugs were provided by local pharmaceutical manufacturers.

2.2. Preparation of standards and samples

Tramadol hydrochloride stock standard solution (1000 µg/ml) was prepared by dissolving 100 mg of *tramadol hydrochloride* in methanol to obtain 100 ml of solution, diluted daily with deionized water, added ephedrine hydrochloride stock standard solution 1000 µg/ml to appropriate concentration 50 µg/ml as internal standard, and then filtered through a 0.22 µm membrane filter.

Tramadol hydrochloride sample solution was prepared by dissolving an amount of drug powder that is equivalent to 100 mg of *tramadol hydrochloride*, in methanol to obtain 1000 µg/ml solution, diluted daily with deionized water, added ephedrine hydrochloride stock standard solution 1000 µg/ml to appropriate concentration 50 µg/ml as internal standard, and then filtered through a 0.22 µm membrane filter.

2.3. CE system and conditions

An Agilent-CE 7100 equipped with DAD and a temperature control system was used. The CE instrument was controlled by commercial software supplied from Agilent. All separations were carried out at 25^{o}C in a fused silica capillary having 50 µm i.d., 56 cm total length, and 50 cm effective length with on-line UV detection. The analytical solutions were injected by pressure applying 50 mbar × 5 sec. The applied voltage was 25 kV. The UV signals were recorded at 225 nm. The background electrolyte (BGE) used in this study was tris-phosphate or phosphate buffers. The appropriate amount of chiral selector was dissolved in BGE to obtain concentration of 0.3-1.0 %. This running buffer was filtered through a 0.22 µm membrane filter prior to use. The new capillary was conditioned by flushing with 1 M NaOH for 30 min, followed by water for 30 min. At the beginning of each working day, the capillary was rinsed with 0.1 M NaOH for 15 min, water for 15 min, and the background electrolyte for 10 min. Between injections, the capillary was rinsed for 5 min with water, and conditioned for 5 min with BGE to maintain proper reproducibility.

The separation of the tramadol enantiomers was studied varying the experimental electrophoretic conditions. The pH value and concentrations of background electrolyte, the nature of chiral selectors, and their concentrations were optimized by Modde 5.0 software using Box-Behnken model to achieve the best resolution. Then, the optimized method was validated according to ICH guideline[7].

3. RESULTS AND DISCUSSION

3.1. Effect of electrophoretic conditions on enantioresolution (R_s)

3.1.1 Background electrolyte

Buffer and buffer pH are important parameters in chiral CE separation because they affect resolution and migration time. The enantiomer separation was investigated with tris-phosphate buffer and phosphate buffer.

pH: Because the pH affects the resolution and the migration time, the pH dependence of the chiral resolution was investigated in the pH range from 2.0 to 4.0. Enantiomer resolution was increased when pH was increased from 2 to 3 and decreased when pH was increased from 3 to 4.

Electrolyte concentration: A tris-phosphate concentration in the range of 20-60 mM were found to be optimal. Higher buffer concentrations reduced EOF and increased the electric current and the Joule heat.

CD types and their concentrations: Chiral CE separations are mainly carried out by adding an appropriate chiral selector to the background electrolyte which can modify selectively effective mobility of two enantiomers. *β*-CD, Me-*β*-CD, HP-*β*-CD and CM-*β*-CD were used as chiral additives for enantiomeric resolution of tramadol. The resolution values obtained in this study were greater than 1.5 for CM-*β*-CD whereas other derivatives

showed incomplete separation. The influence of concentration (0.3-0.9%) of CM-*β*-CD on enantioresolution was investigated further.

3.2. Optimization of CE conditions by Modde 5.0 software using Box-Behnken model

Several preliminary experiments on CE separation of enantiomeric tramadol showed that there are three factors impacting the enantiomeric resolution, including pH (2.5-3.5), BEG concentration (40 mM-60 mM), and CM-*β*-CD concentration (0.3%-0.7%) to achieve the best resolution. Modde 5.0 software was applied for design experiments and calculating results of them (Table 1), the optimized electrophoretic conditions were found, as follows: 50 mM tris-phosphate buffer pH 3 containing 0.5% CM-*β*-CD, capillary (56 cm × 50 μm i.d., 50 cm), 225 nm, 25^{o}C, 25 kV, injection 5 s at 50 mbar. With the conditions, the predicted resolution was 2.80.

Table 1. *Optimizing result output by Modde 5.0 software*

Resolution	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int (±)**
Constant	2.78667	0.033183	4.53728e-009	0.0852997
pH	-0.13	0.0203203	0.00138287	0.0522352
BGE	-0.0524999	0.0203203	0.049216	0.0522352
CM-*β*-CD	0.0625	0.0203203	0.0276031	0.0522352
pH*pH	-0.492083	0.0299107	1.51427e-005	0.0768881
BGE*BGE	-0.307083	0.0299107	0.000150659	0.0768881
CM-*β*-CD*CM-*β*-CD	-0.742083	0.0299107	1.98448e-006	0.0768881
pH*BGE	0.0124997	0.0287373	0.681721	0.0738717
pH*CM-*β*-CD	0.00749999	0.0287373	0.804513	0.0738717
BGE*CM-*β*-CD	0.0125	0.0287373	0.681714	0.0738717
N = 15	Q2 =	0.968	Cond. no. =	4.2385
DF = 5	R2 =	0.995	Y-miss =	0
	R2 Adj. =	0.985	RSD =	0.0575
			Conf. lev. =	0.95

The optimal conditions were experimentally repeated for six times. The actual resolution value 2.73 was equivalent to predicted one.

3.3. Method validation

To evaluate the stability of the methods, six consecutive injections were performed. Table 2 lists the mean values of resolution, CorrArea, and migration time (t_M). The values demonstrated a good reproducibility of the method.

Table 2. *Mean values of electrophoretic parameters and their relative standard deviation values calculated from 6 consecutive injections*

Electrophoretic parameter	Statistical data (n=6)	Tramadol enantiomer 1	Tramadol enantiomer 2
Ratio CorrArea*	Mean	1.073	1.063
	RSD (%)	1.65	1.99
Ratio t_M *	Mean	1.47	1.53
	RSD (%)	0.40	0,74
Resolution	Mean	2.73	

**CorrArea (Correlated Area) is defined as ratio of peak area and migration time.*
Ratio CorrArea is defined as ratio of CorrArea of tramadol hydrochloride's peak and CorrArea of ephedrine hydrochloride's peak.
Ratio t_M is defined as ratio of t_M of tramadol hydrochloride's peak and t_M of ephedrine hydrochloride's peak.

According to ICH guideline, the suitable electrophoretic optimazed conditions was validated on selectivity, linearity, LOD, precision and accuracy.

3.3.1 Selectivity

The selectivity of the method was demonstrated by the baseline separation of the enantiomers in the electropherograms. No significant electrophoretic interference in blank sample was found. The peak purity check with photodiode array detector was used to confirm the spectra consistency of the obtained peaks and the results indicated that no interference was noted for tramadol drug. All of the experiments evidently indicated the selectivity of the developed CZE methods (Figure 1).

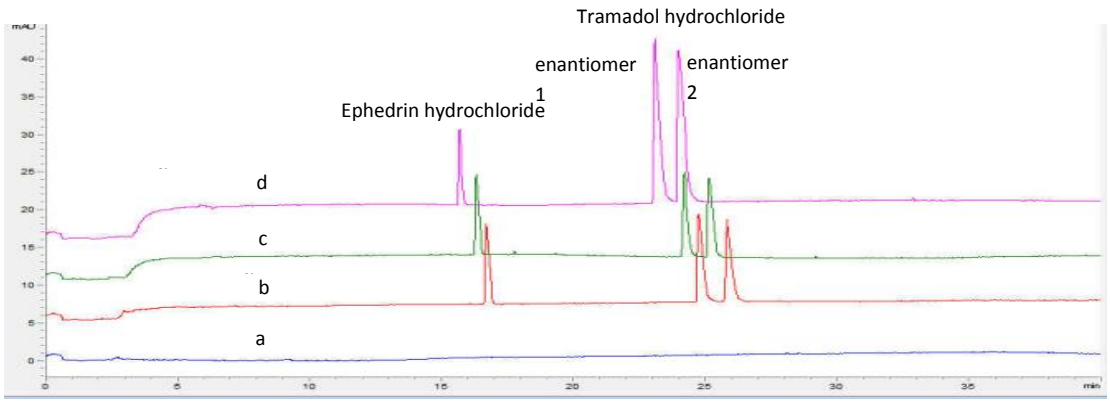

Figure 1. *Electropherograms for the chiral separation of tramadol hydrochloride at the optimized electrophoretic conditions. (a): BGE, (b) racemic sample, (c): racemic tramadol hydrochloride standard, (d): racemic sample spiked tramadol hydrochloride standard.*

3.3.2 Linearity, LOD, precision and accuracy

The linearity, LOD, precision and accuracy were summarized in table 3. The linearity range for both enantiomers was 2.5-125 μg mL^{-1}. Intra-day relative standard deviation (RSD; *n*=6) was below 3%. Recovery (n=9) was in the range of 94-106% of the expected values.

Table 3. *Validation parameters of analytical procedure*

Validation characteristics	Tramadol enantiomer 1	Tramadol enantiomer 2
Linearity range (µg/ml)	2.5 – 125 (R^2 = 0.999)	2.5 – 125 (R^2 = 0.999)
Precision (n = 6)	RSD = 0.70%	RSD = 0.89%
Accuracy (n = 9, $p < 0.05$)	99.6 - 106.0%	98.6 – 106.0%

4. CONCLUSION

The CZE approach for quality control of tramadol was successfully developed using CM-*β*-CD as a chiral selector. The analytical procedure was proven to be selective, a wide range with high correlation coefficient value, precise and accurate. This procedure can be applied to analyse tramadol enantiomers in raw material or in corresponding pharmaceutical products.

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The Effects of "Ha Mo Mau" Granule on Experimental Blood Hyperlipidemia Profile

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Abstract

The study was conducted to assess the effect of "Ha mo mau"- a blood lipid lowering granulate. The granulate was made from 6 herbal medicines: *Pericarpium Citri Reticulatae perenne, Rhizoma Pinelliae, Poria, Radix Glycyrrhizae, Radix Achyranthes bidentatae* and *Rhizoma Alocaciae odorae.* Two experimental models were applied for exogenous and endogenous hyperlipidemia evaluation by using cholic acid and propylthiouracil (PTU) with white rats and poloxamer 407 (P-407) with white mice. On the exogenous hyperlipidemia model, with the dose of 3.15g/kg/day and 9.45 g/kg/day "Ha mo mau" granulate made the TC, TG, and LDL-C levels decreasing in white rats. On the endogenous hyperlipidemia model, with the dose of 5.4 g/kg/day and 16.2 g/kg/day, "Ha mo mau" granulate made TC, TG, and LDL-C levels reducing in white mice. It was observed that "Ha mo mau" granule was effective in regulating dyslipidemia in both models of exogenous and endogenous hyperlipidemia.

Keywords: "Ha mo mau" granule, regulating dyslipidemia, endogenous hyperlipidemia, exogenous hyperlipidemia

1. INTRODUCTION

High blood cholesterol is a major cause of atherosclerosis by gradually narrowing the arteries that supply blood to the heart and other organs of the body [1]. The incidence of atherosclerosis is increasing, especially in developed and developing countries, so the treatment of dyslipidemia is one of the primary and urgent goals for national health protection in every single country [2]. Many western medicines classes have been widely used to treat dyslipidemia such as statin, ezetimibe - inhibitor of cholesterol absorption, bile acid sequestrants, nicotinic acid, and fibrates [3]. In addition to the development of modern medicine, traditional medicine has been being asserted to make significant contributions to the health care of the community. Lipid dysfunction (dyslipidemia) has been described in several damp-phlegm causing diseases [4]. "Nhi tran thang" is a Vietnamese traditional remedy, which was useful for dry dampness and expelphlegm

commonly chosen for the treatment of dyslipidemia. “Ha mo mau” granule is made on the basis of this remedy, adding *Radix Achyranthes bidentatae* and *Rhizoma Alocaciae odorae* to increase the performance of the remedy. For the purpose to provide the basic scientific information for clinical research, this study was conducted with the objectives:

1. To assess the effect of regulating the dyslipidemia on the exogenous hyperlipidemia model of cholesterol reduction in experimental animals.
2. To assess the effect of regulating the dyslipidemia on the endogenous hyperlipidemia model of cholesterol reduction in experimental animals

2. MATERIALS AND METHODS

2.1. Research Subjects

2.1.1. Research Materials and Chemicals

‘Ha mo mau’ granule (made from 6 herbal medicines: *Pericarpium Citri Reticulatae perenne* (0.64 g), *Rhizoma pinelliae* (0.64 g), *Poria* (0.80 g), *Radix Glycyrrhizae* (0.27 g), *Radix Achyranthes bidentatae* (0.80 g) (satisfying to Vietnamese pharmacopoeia IV) and *Rhizoma Alocaciae odorae* (0.64 g) (satisfying to basic standards) on the experimental exogenous and endogenous hyperlipidemia model. Dosage for adult: 1 bag/time × 3 times daily.

Cholesterol (Acros-Belgium), Acid Cholic (Sigma-Aldrich), Propylthiouracil tablet 50 mg (Rieserstat, Pharma Chemicals GmbH, Germany), Poloxamer 407 (Sigma-Aldrich), Atorvastatin tablet 10mg (Lipistad STADA, Vietnam).

Biochemical testing machine: BTS-350 (Spain).

Kit quantifying substances in the blood: Total cholesterol, Triglycerid, HDL-Cholesterol, LDL-Cholesterol made by DIALAB GmbH (Austria)

2.1.2. Animals

Swiss white mice, both gender, healthy, 25 ± 2 g, provided by the National Institute of Hygiene and Epidemiology, Vietnam.

Wistar white rats both gender healthy, weight 150 - 180 grams, provided by Military Medical University, Vietnam.

Animals are fed with adequate food provided by the National Institute of Hygiene and Epidemiology, Vietnam.

Standard laboratory conditions and drinking water at the Department of Experimental Studies-Institute of Military Traditional Medicine, Vietnam.

2.2. Research Methods

2.2.1. To assess the effect of dyslipidemia regulation on the exogenous hyperlipidemia model of cholesterol reduction

The exogenous model was performed by method of Nassiri *et al.* (2009) [5], adjusted for cholic and propylthiouracil content (PTU) based on the results of Thanh N.P (2011) [7]. Rats were feed by mixture of cholesterol-containing oil (every 1ml of cholesterol-oil mixture contains: 0.1 g cholesterol, 0.01 g of cholic acid, 0.005 g of propylthiouracil, 1ml of peanut oil) at a dose of 10 ml/kg/day (l ml/100 g), for 4 weeks continuously (28 days) to cause dyslipidemia.

Randomly divided to 5 lots, each of 10 rats, receiving within 4 weeks the oral doses as follows:

Lot 1 (Biological controls): daily fed with 1 ml/100 g body weight (b.w.) distilled water and repeated after 2 hours.

Lot 2 (Controls with disease): daily fed with 1 ml/100 g (b.w.) mixture of cholesterol oil, followed by 1ml/100g (b.w.) distilled water after 2 hours.

Lot 3 (Positive controls, Atorvastatin): daily fed with 1 ml/100 g (b.w.) mixture of cholesterol oil, followed by 1ml (10mg Atorvastatin)/kg (b.w.) after 2 hours.

Lot 4 ("Ha mo mau" granule, equivalent clinical dose): daily fed with 1 ml/100 g (b.w.) mixture of cholesterol oil, followed by 1ml (3.15 g "Ha mo mau")/kg (b.w.) after 2 hours.

Lot 5 ("Ha mo mau" granule, triple dose): daily fed with 1 ml/100 g (b.w.) mixture of cholesterol oil, followed by 1 ml (9.45 g "Ha mo mau")/kg (b.w.) after 2 hours.

At 2 weeks and 4 weeks before and after study, the experimental rats were fasted overnight (still drink water), canthus rat blood was taken for examination of total cholesterol (TC), TG, HDL-C, LDL-C.

2.2.2. To assess the effect of dyslipidemia regulation on the endogenous hyperlipidemia model of cholesterol reduction

Use and modify the endogenous hyperlipidemia model with P-407 according to Millar et al. [6]. P-407 solution (2%) was prepared by mixing 0.4g of P-407 in 20ml of 0.9% NaCl, freezing overnight. The mice were randomly divided into 5 lots of 10 mice each. Different lots were fed/injected according to the following program:

Lot 1 (Biological controls): daily fed with 0.2ml/10g b.w. distilled water, in the 7^{th} day, intraperitoneally injected with 0.1ml 0.9% NaCl/10g (b.w).

Lot 2 (Controls with disease): daily fed with 0.2ml/10g (b.w.) distilled water, in the 7th day, intraperitoneally injected with 0.1ml 2% P-407/10g b.w (200mg/kg) (b.w.).

Lot 3 (Positive controls, Atorvastatin): daily fed with 0.2ml 50% Atorvastatin solution/10 g (b.w.) (10 mg/kg), in the 7th day, intraperitoneally injected with 0.1 ml 2% P-407/10 g (200 mg/kg) (b.w.).

Lot 4 ("Ha mo mau" granule, equivalent clinical dose): daily fed with 0.2 ml extract of "ha mo mau" granule solution/10 g (b.w.) (4.2 g/kg), in the 7th day, intraperitoneally injected with 0.1ml 2% P-407/10g (200mg/kg) (b.w.).

Lot 5 ("Ha mo mau" granule, triple dose): daily fed with 0.2 ml extract of "Ha mo mau" granule solution/10g (b.w.) (12.6g/kg), in the 7th day, intraperitoneally injected with 0.1 ml 2% P-407/10g (200mg/kg) (b.w.).

After injected P-407, the mice were completely starved without drinking water. After 24 hours, artery blood was taken for quantitative testing of TC, TG, LDL-C, HDL-C.

2.3. Data processing

The collected data were processed by t-test student biomedical method and Avant-après method. Data is expressed as: $\overline{X} \pm SD$.The difference was statistically significant when $p < 0.05$.

3. RESULTS

3.1. To assess the effect of regulating the dyslipidemia on the exogenous hyperlipidemia model.

Table 1. *The body weight of experiment rats on exogenous model (n=10)*

Lot	***Body weight* ($\overline{X} \pm SD$, gram)**				
	Before	***After 1 week***	***After 2 weeks***	***After 3 weeks***	***After 4 weeks***
Lot 1: Biological controls	154.3 ± 3.9	175.7 ± 4.9	196.7 ± 5.5	214.3 ± 5.5	224.4 ± 6.1
Lot 2: controls with disease	155.1± 4.3	174.6 ± 4.8	193.7 ± 5.7	207.8 ± 8.8	220.7 ± 6.0
Lot 3: Atorvastatin (10mg/kg)	157.7 ± 5.3	175.8 ± 5.7	197.5 ± 6.9	212.8 ± 5.0	222.4 ± 4.3
Lot 4: HMM (3.15g/kg)	156.8 ± 7.2	176.8 ± 4.8	195.1 ± 5.4	211.8 ± 7.0	223.6 ± 5.7
Lot 5: HMM (9.45g/kg)	157.4 ± 5.8	175.4 ± 6.4	195.4 ± 7.9	213.5 ± 8.7	222.3 ± 6.2

The results on the Table 1 showed that, after 1 week, 2 weeks, 3 weeks and 4 weeks of the study, the body weight of rats in all lots tended to increase. Lots of disease controls, body weight of rats tended to gain weight more slowly than biological ones, but there was no

statistical difference between the biological, disease and medication lots at the same time of observation ($p > 0.05$).

Table 2. Blood lipid indicator before study on exogenous model (n=10)

Lot	Indicator ($\overline{X} \pm SD$, mmol/l)			
	TC	**TG**	**HDL - C**	**LDL - C**
Lot 1: Biological controls	2.49 ± 0.10	0.51 ± 0.09	0.62 ± 0.08	1.64 ± 0.12
Lot 2: controls with disease	2.51 ± 0.33	0.52 ± 0.11	0.60 ± 0.08	1.67 ± 0.34
Lot 3: Atorvastatin	2.52 ± 0.24	0.51 ± 0.07	0.63 ± 0.13	1.66 ± 0.28
Lot 4: HMM (3.15g/kg)	2.50 ± 0.20	0.53 ± 0.07	0.59 ± 0.07	1.67 ± 0.22
Lot 5: HMM (9.45g/kg)	2.51 ± 0.23	0.52 ± 0.05	0.58 ± 0.08	1.69 ± 0.27

The results on the Table 2 showed that, before the study, there was no statistical difference of the observation indicators between lots ($p > 0.05$), it means that, all the indicators of each lot are similar to each other.

Table 3. *Exogenous hyperlipidemia modeling*

Indicators	After 2 weeks		After 4 weeks	
	Biological controls	***Controls with disease***	***Biological controls***	***Controls with disease***
Cholesterol	2.48 ± 0.17	4.68 ± 0.37***	2.49 ± 0.16	4.97 ± 0.49***
Triglicerid	0.52 ± 0.07	0.59 ± 0.03**	0.55 ± 0.13	0.67 ± 0.10*
HDL - C	0.61 ± 0.09	1.65 ± 0.21***	0.60 ± 0.07	1.67 ± 0.18***
LDL - C	1.64 ± 0.19	2.76 ± 0.48***	1.65 ± 0.17	3.00 ± 0.39***

(Note: *: $p<0.05$;**: $p<0.01$; ***: $p<0.001$)

The results (Table 3) showed that, after exogenous dyslipidemia modeling, mice were suffering from dyslipidemia. The lipid indicators in the disease control group increased in comparison to the biological controls. The indicators TC, LDL-C, HDL-C were increased ($p <0.001$); TG index was significantly increased ($p <0.01$).

Table 4.*Blood lipid indicators at 2 weeks of the study in exogenous model (n = 10)*

Lot	Indicator ($\overline{X} \pm SD$, mmol/l)			
	TC	**TG**	**HDL - C**	**LDL - C**
Lot 2: controls with disease	4.68 ± 0.37	0.59 ± 0.03	1.65 ± 0.21	2.76 ± 0.48
Lot 3: Atorvastatin % compared to lot 2	3.44 ± 0.41*** (↓26.5%)	0.53 ± 0.08* (↓10.1%)	1.55 ± 0.18 (↓6.0%)	1.65 ± 0.44*** (↓40.2%)
Lot 4: "ha mo mau" (3.15g/kg) % compared to lot 2	3.74 ± 0.31*** (↓20.0%)	0.54 ± 0.07* (↓8.5%)	1.56±0.13 (↓5.4%)	1.94 ± 0.34*** (↓29.7%)
Lot 5: HMM (9,45g/kg) % compared to lot 2	3.71 ± 0.37*** (↓20.7%)	0.53 ± 0.07* (↓10.1%)	1.59 ± 0.19 (↓3.6%)	1.88 ± 0.48*** (↓31.9%)

(Note: : *: p<0.05;**: p<0.01; ***: p<0.001)

After 2 weeks of study (Table 4):

Atorvastatin at the dose of 10mg/kg: Effective in regulating dyslipidemia on exogenous hyperlipidemia model: decrease TC (↓26.5%), LDL-C (↓40.2%), decrease TG (↓10.1%) compared to the disease controls (p <0.05).

"Ha mo mau" granule at the dose of 3.15g/kg/day: reduced TC (↓20.0%), LDL-C (↓29.7%), decreased TG (↓8.5 %) compared to the disease lot (p <0.05). The effect was comparable to that of Atorvastatin taking controls (p> 0.05).

"Ha mo mau" granule at dose of 9.45g/kg/day: reduced TC (↓ 20.7%), LDL-C (↓ 31.9%), reduced TG (↓ 10.1) %) compared to the disease lot (p <0.05). The effect was comparable to that of Atorvastatin taking controls (p> 0.05).

Table 5. *Blood lipid concentration at 4 weeks of study (n = 10)*

Lot	Indicators ($\overline{X} \pm SD$, mmol/l)			
	TC	**TG**	**HDL - C**	**LDL - C**
Lot 2: controls with disease	4.97 ± 0.49	0.67 ± 0.10	1.67 ± 0.18	3.00 ± 0.39
Lot 3: Atorvastatin % compared to lot 2	3.49 ± 0.41*** (↓29.8%)	0.56 ± 0.06** (↓16.4%)	1.57 ± 0.17 (↓6.0%)	1.67 ± 0.42*** (↓44.3%)
Lot 4: "Ha mo mau" (3.15g/kg) % compared to lot 2	3.75 ± 0.31*** (↓24.5%)	0.58 ± 0.08* (↓13.4%)	1.58 ± 0.18 (↓5.4%)	1.91±0.40*** (↓36.3%)
Lot 5: "Ha mo mau" (9.45g/kg) (% compared to lot 2)	3.72 ± 0.38*** (↓25.1%)	0.57 ± 0.09* (↓15.0%)	1.63 ± 0.13 (↓2.4%)	1.83 ± 0.42*** (↓39.0%)

(Note: *: p<0.05; **: p<0.01; ***: p<0.001)

The results on the Table 5 showed that after 2 weeks of study:

Atorvastatin at dose of 10mg/kg: Effective in reducing TC (↓29.8%), LDL-C (↓44.3%), reducing TG (↓16.4%) compared to the disease controls ($p<0.05$).
"Ha mo mau" granule at dose of 3.15g/kg/day: effective in reducing TC (↓24.6%), LDL-C (↓36.3%), reducing TG (↓13.4%) compared to the disease controls ($p<0.05$). The effect is comparable to that of Atorvastatin taking controls ($p>0.05$).
"Ha mo mau" granule at dose of 9.45g/kg/day: effective in reducing TC (↓25.1%), LDL-C (↓39.0%), reducing TG (↓15.0%) compared to the disease controls ($p<0.05$). The effect was comparable to that of Atorvastatin taking controls ($p>0.05$).

3.2. To assess the effect of regulating the dyslipidemia of "Ha mo mau" granule on the exogenous hyperlipidemia model

Table 6. *Model of dyslipidemia by P-407*

Blood lipid indicator	Biological controls (1)	Disease controls (2)	P_{2-1}
Cholesterol	2.80 ± 0.27	6.71 ± 0.81	< 0.001
Triglicerid	0.65 ± 0.16	7.54 ± 1.59	< 0.001
HDL-C	1.03 ± 0.39	2.26 ± 0.13	< 0.001
LDL-C	0.63 ± 0.22	1.90 ± 0.45	< 0.001

The results (Table 6) showed that, after modeling dyslipidemia by P-407, the total cholesterol, triglyceride, HDL-C, and LDL-C levels in mice blood statistical significantly increased over the biological controls ($p <0.001$).

Table 7: Impact of "Ha mo mau" on lipid concentration in endogenous hyperlipidemia model

Lot	TC	TG	HDL - C	LDL - C
Lot 2: (controls with disease)	6.71 ± 0.81	7.54 ± 1.59	2.26 ± 0.13	1.90 ± 0.45
Lot 3: Atorvastatin (% compared to lot 2)	4.94 ± 0.37*** (↓26.37%)	5.85 ± 0.46** (↓22.41%)	2.41 ± 0.55 (↓6.63%)	1.21 ± 0.20*** (↓36.32%)
Lot 4: "Ha mo mau" (5.4g/kg) (% compared to lot 2)	5.45 ± 0.83** (↓18.78%)	6.10 ± 0.21* (↓19.00%)	2.33 ± 0.31 (↓3.09%)	1.35 ± 0.10** (↓28.95%)
Lot 5: "Ha mo mau" (16.2g/kg) (% compared to lot 2)	5.42 ± 0.63*** (↓19.22%)	6.09 ± 0.35* (↓19.23%)	2.36 ± 0.13 (↓4.42%)	1.33 ± 0.14** (↓30.00%)

(Note: *: $p<0.05$;**: $p<0.01$; ***: $p<0.001$)

The results on the Table 7 showed that, at the doses of 5.4 g/kg and 16.2 g/kg, "Ha mo mau" granule effectively regulated dyslipidemia in the endogenous dyslipidemia model. At the dose of 5.4 g/kg, LDL-C level decreased in rat blood (18.78%, 28.95%), 19.00% reduction in triglyceride compared to the control group ($p <0.05$), similar to Atorvastatin at dose of 10 mg/kg ($p> 0.05$).

“Ha mo mau” granule at dose of 16.2 g/kg could reduce TC level by 19.22%, reduced TG by 19.23%, reduced LDL-C by 30.00% compared to disease controls ($p<0.05$), equivalent to Atorvastatin at dose of 10 mg/kg ($p> 0.05$).

4. DISCUSSION

4.1. Effect of dyslipidemia regulating by “Ha mo mau” granule on the exogenous hyperlipidemia model

To study medication for dyslipidemia treatment, it is required first of all to create a model of hyperlipidemia. Many animals have been used and many pharmacological models have been successfully studied and applied on experimental animals. The type of models may be: exogenous hypercholesterolemia or lipoproteinemia (inputting cholesterol and fat by digestive tract), endogenous hypercholesterolemia (increased cholesterol synthesis) or a combination of both.

We selected the exogenous hyperlipidemia model according to the method of Nassiri et al. (2009) [5], adjusting the content of cholic and propylthiouracilic acids (PTU) in line with the research by Thanh N.P. (2011) [7] to cause hyperlipidemia. Results showed that after 2 weeks and 4 weeks of the study, the indicators TC, TG, HDL - C and LDL - C in modeling lots increased compared to the biological controls, the changes were statistically significant with $p <0.05$.

“Ha mo mau” granule was effective in regulating dyslipidemia on exogenous hyperdyslipidemia model, demonstrated by reduced TC, TG and LDL-C levels.
- Low dose (3.15g/kg): decreased TC (19.3%), LDL - C (29.7%) compared to the control group ($p <0.001$) %), decreased TG (8.5%) compared to the control group ($p <0.05$) after 2 weeks of study; reduced TC (24.6%), LDL-C (36.3%) compared to the control group ($p<0.001$), reduced TG (15%) compared to the disease controls ($p <0.05$) after 4 weeks of the study.

- At the dose of 9.45 g/kg, “Ha mo mau” granule could decrease TC (20.7%), LDL - C (31.9%) compared to the control group ($p <0.001$) %) decrease TG (10,1%) compared to the control group ($p <0.05$) after 2 weeks of study; reduce TC (25,1%), LDL – C (39%) compared to the control group ($p<0.001$), reduce TG (15%) compared to the disease controls ($p <0.05$) after 4 weeks of the study.
- “Ha mo mau” granule at both experimental doses did not alter the HDL-C indicator after modeling ($p> 0.05$).
- The effect of dyslipidemia regulating by two remedies of “Ha mo mau” granule were similar and equivalent to Atorvastatin 10 mg/kg ($p>0.05$).
- According to Thanh N.P. (2011): the effect of dyslipidemia regulating proposed by Monacholes on exogenous hyperlipidemia model after 2 weeks of the study was not reported, but after 4 weeks it was reported to be effective in reducing TC at dose of 2g/kg

by 27.6%, and 21.5% at 4g/kg; LDL-C level was reduced at 2g/kg by 34.8% and 26.9% at 4g/kg [7].
- According to Huong D.Q. (2016): the effect of dyslipidemia regulating using Lipidan at 0.7 g/kg and 1.4 g/kg on exogenous hyperlipidemia model helped to reduce TC by 3.1% and 25.3% after 2 weeks and TC by 31.2% and 23.0% after 4 weeks of study. TG level tended to decrease with no difference [8].
- According to Thuy T.T. (2016), the effect of the effect of dyslipidemia regulating using "Dai An" liquid jelly at dose of 4.8 g/kg and 9.6 g/kg on exogenous hyperlipidemia model reduced TC, LDL-C levels compared to the statistically significant sample size (modeling lot). TG tended to decrease with no difference [9].

4.2. Effect of dyslipidemia regulating of "Ha mo mau" granule on the endogenous hyperlipidemia model

We selected the endogenous hyperlipidemia model using p-407 according to the method of Millar et al. [6]. Intraperitoneal injection of p-407 caused marked dyslipidemia in white mice. The difference was statistically significant with $p<0.001$. This model was developed by Thanh N.P. [7], Huong D.Q. [8] and Thuy T.T. [9], all showed similar results suggesting that endogenous hyperlipidemia according to Millar et al. was proved stable.

"Ha mo mau" granule is effective in regulating dyslipidemia on endogenous hyperdyslipidemia model, using Poloxamer-407 on white mice demonstrated by the following results:

At the dose of 5.4 g/kg, it reduced the concentration of: TC by 18.78% ($p<0.01$), TG by 19% ($p<0.05$), LDL - C by 28.95% ($p<0.001$) in mice blood compared to the disease controls but did not alter the HDL-C indicator with $p> 0.05$.

At the dose of 16.2 g/kg, it reduced concentration of: TC by 19.22% ($p<0.001$), TG by 19.23% ($p<0.05$), LDL - C by 30% ($p<0.01$) in mice blood compared to the disease controls but did not alter the HDL-C indicator with $p> 0.05$.

According to Thanh N.P. (2011), the effect of dyslipidemia regulation proposed by Monacholes at dose of 30g/kg on endogenous hyperlipidemia model was reported to be effective in reducing TC, HDL-C and LDL-C levels in comparison to the control group with $p < 0.05$, did not alter TG with $p> 0.05$ [7].

According to Huong D.Q. (2016): the effect of dyslipidemia regulating using oral Lipidan at 6.0 g/kg and 12.0 g/kg in 7 days helped to reduce TC significantly by 14.1% and 25.6%. At dose of 12.0 g/kg also reduced TG level significantly by 26.8% compared to the sample size (modeling lot) [8].

According to Thuy T.T. (2016), the effect of the effect of dyslipidemia regulating using "Dai An" liquid jelly at dose of 9.6g/kg and 19.2g/kg on endogenous hyperlipidemia model reduced TC levels statistically-significantly compared to the sample size [9].

“Ha mo mau” granule was prepared from “Nhi tran thang” remedy, adding *Radix Achyranthis bidentatae* and *Alocasia odora. Rhizoma Pinelliae* plays the role of monarch drug, aiming for dry dampness and expelphlegm, *Pericarpium Citri Reticulatae perenne*. is the minister drug, that aims to regulation “qi”, dry dampness. *Poria* is an adjuvant, useful in strengthening spleen and stomach, excreting dampness. *Radix Glycyrrhizae* is the guide drug, useful to regulate medicines, tonify and replenish spleen, strengthening the spleen, dissipating sputum. The two added adjuvants: *Alocasia odora* for enhancing dry dampness and expelphlegm; *Radix achyranthis bidentatae* for active blood as the liquid phlegm is characterized by locating in system of acupuncture spots, making this system obstructed, difficult blood circulation and the dyslipidemia in the modern medicine is closely related to liquid phlegm from point of view of the traditional medicine. In addition, *Typhonium divaricatum* contains β-sitosterol, while *Ganoderma* contains ergosterol, *Alocasia odora* contains phytosterol-all are substances that reduce blood cholesterol. Nhu D.T. (1991) demonstrated that *Radix achyranthis bidentatae* is useful for blood cholesterol lowering. *Pericarpium Citri Reticulatae perenne* is also beneficial for bile, contributing to cholesterol lowering. Therefore, the “Ha mo mau” granule proves the significant effect in regulating dyslipidemia on both exogenous and endogenous dyslipidemia models.

5. CONCLUSION

“Ha mo mau” granule was proved useful for regulating dyslipidemia on exogenous dyslipidemia model at dose of 3.15 g/kg/day and 9.45 g/kg/day, equivalent to Atorvastatin 10 mg/kg.

“Ha mo mau” granule was proved useful for regulating dyslipidemia on endogenous dyslipidemia model at dose of 5.4 g/kg/day and 16.2 g/kg/day, equivalent to Atorvastatin 10 mg/kg.

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Preparation and Characterization of Niosome Encapsulated Curcumin

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Abstract

Niosomes encapsulated curcumin were prepared by ethanol injection method with continuous ethanol evaporation using non-ionic surfactant span 80, cholesterol and curcumin. Curcumin-niosomes were purified from unencapsulated curcumin and impurities by size exclusion chromatography. Field emission scanning electron microscopy (FE-SEM) demonstrated that curcumin-niosomes had relatively uniform, spherical shapes with estimated diameters of 50 nm. Dynamic light scattering (DLS) analysis indicated that the size of particles hardly depended on the solvent evaporation time. Niosomal encapsulation efficiency of curcumin (EE), which was determined by fluorospectrometer, incredibly increased after 2-hour ethanol evaporation. It reached the highest value of 98% after 5 hours of ethanol evaporation for the mixture of span80, cholesterol, curcumin in a molar ratio of 45: 30: 1.

Keywords: Niosomes, curcumin, nanocarier, encapsulation.

4. INTRODUCTION

Niosomes (non-ionic surfactant vesicles) are one of the promising drug nanocarriers since they can supply high encapsulation efficiency and control release of both hydrophilic and hydrophobic drugs. Niosomes have been used for encapsulation of separate drugs including antibiotics [1], hormones [2], genes [3] etc. as well as for encapsulation of two drugs (doxorubicin hydrochloride and curcumin) at the same time for enhancing the

anticancer effect [4]. Curcumin is a hydrophobic polyphenolic natural compound found in *Curcuma longa*. Although curcumin has various therapeutic relevancies (for instance anticancer, anti-inflammatory, antioxidant and antimicrobial activities) its poor solubility in water and degradability in alkaline conditions severely limits its bioavailability. Curcumin has been recently encapsulated in niosomes by diverse methods including solvent evaporation [5], thin film hydration [4], reserve phase evaporation [6] from different nonionic surfactants with various ratios. However, the obtained niosomal encapsulation efficiency was not very high. The aim of our work was to prepare niosomes by a simple method with highest curcumin encapsulation efficiency.

5. MATERIALS AND METHODS

2.1. Materials

Cholesterol was obtained from MP Biomedicals North America (USA). Curcumin was purchased from Biobasic (Canada). Absolute ethanol was obtained from VWR Prolabo Chemicals (USA). Deionzed water with resistivity 18Ω was used for all the experiments. Size exclusion chromatography column ilustra^{TM} NAP-25 was purchased from GE Healthcare Life Sciences (USA).

5.2. Methods

2.2.1. Preparation of curcumin- niosomes

Curcumin-niosomes were prepared by ethanol injection method with continuous solvent evaporation. Briefly, 10 ml preheated (65°C) ethanol solution of span 80, cholesterol (in a molar ratio of 3:2) and various concentrations of curcumin were slowly injected through a needle (27Gx3 $^{1/2}$) into 100 ml preheated (65°C) distilled water in 3 minutes under magnetic stirring. Continue stirring at 25°C for further appropriate time (1h, 2h, 4h, 5h, 6h, 24h) for ethanol evaporation and curcumin-niosome formation. Resulted curcumin-niosomes were purified from unencapsulated curcumin and impurities by size exclusion chromatography using ilustra^{TM} NAP-25 (GE Healthcare Life Sciences).

2.2.2. Characterization of curcumin- niosomes

Morphology and size distribution of curcumin-niosomes were analyzed by field emission scanning electron microscopy (FE-SEM) on a Hitachi S-4800 system and dynamic light scattering (DLS) on a Horiba LB-550, respectively.

2.2.3 Determination of niosomal encapsulation efficiency of curcumin

Niosomal encapsulation efficiency of curcumin (EE) was determined by NanoDrop 3300 Fluorospectrometer (Thermo Scientific) using curcumin fluorescence at the wavelength of 525 nm. Fluoroscent calibration curve of free curcumin was made with solutions of curcumin in ethanol. The EE was calculated by the following equation:

$$EE(\%) = \frac{C}{C_0}.100$$

where C_0 is initial concentration of curcumin, C is concentration of encapsulated curcumin after destruction of purified curcumin-niosomes by absolute ethanol.

3. RESULTS AND DISCUSSION

3.1. Morphology and size distribution of curcumin-niosomes

FE-SEM image illustrated that both curcumin- and blank niosomes had relatively uniform, spherical shapes with estimated diameters of 50 nm (Figure 1). On the other hand, dynamic light scattering analysis (DLS) showed that curcumin-niosome particles had narrow size distribution with the average diameter of approximately 150 nm, which was much larger than those indicated by the FE-SEM (Figure 2.a). It was supposed that the particle size shown by the DLS corresponded to the cluster of curcumin-niosomes. Therefore it is necessary to investigate the condition to obtain monodisperse niosomes. When ethanol evaporation varied from 1 hour to 24 hours, the average particle size changed insignificantly from 141 nm to 161 nm, indicating hard dependence of the particle size on the solvent evaporation time (Figure 2.b).

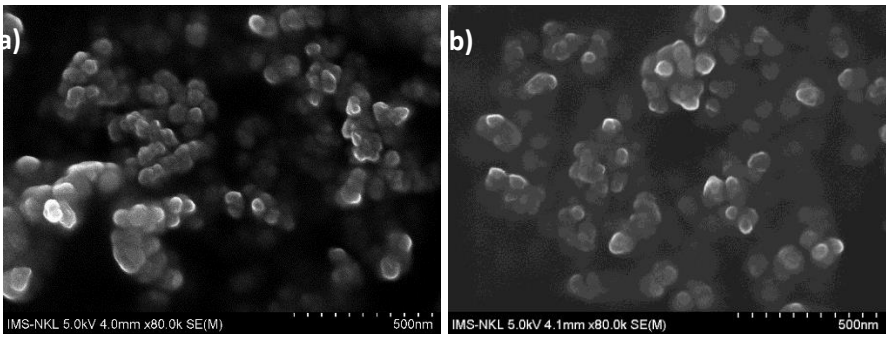

Figure 1. *FESEM images of blank niosomes (a) and curcumin-niosomes (b)*

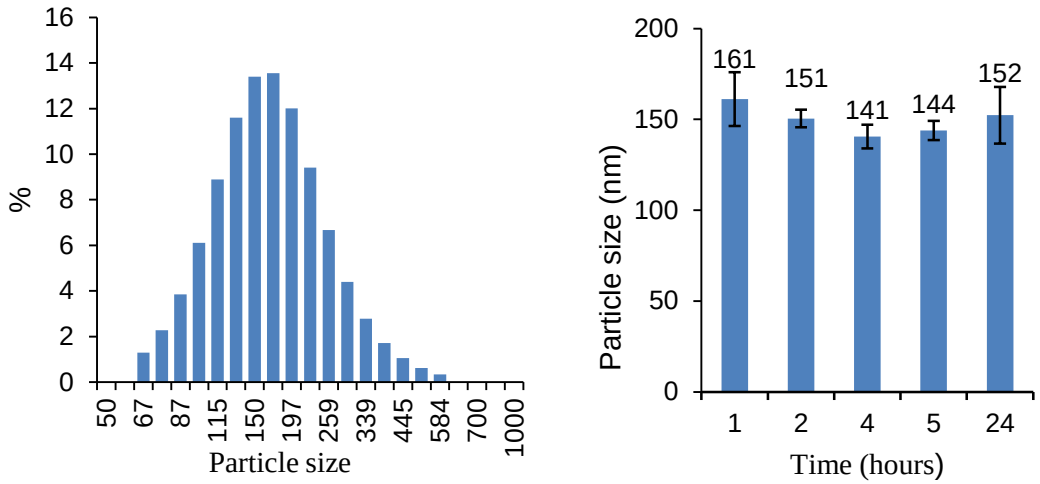

Figure 2. a. *Size distribution of curcumin-niosomes (ethanol evaporation time: 5h, curcumin concentration: 0.05 mg/ml)* ***b****. Dependence of curcumin-niosome particle size on ethanol evaporation time (curcumin concentration: 0.05 mg/ml).*

3.2 Fluorescence of niosomal encapsulated curcumin

Blank niosomes made of span80 and cholesterol had no fluorescence, whereas curcumin exhibited good fluorescent emission efficiency with maximum fluorescence peak at a wavelength of 525 nm. Therefore, fluorescence spectra were utilized for determination of niosomal encapsulation efficiency of curcumin. The fluorescence spectra of curcumin in ethanol with various concentrations 0.00625, 0.025, 0.05, 0.0625 and 0.125 (mg/ml) are showed in figure 3.a. A fluorescent calibration curve of free curcumin in ethanol was built and used for determination of curcumin concentration (Figure 3.b)

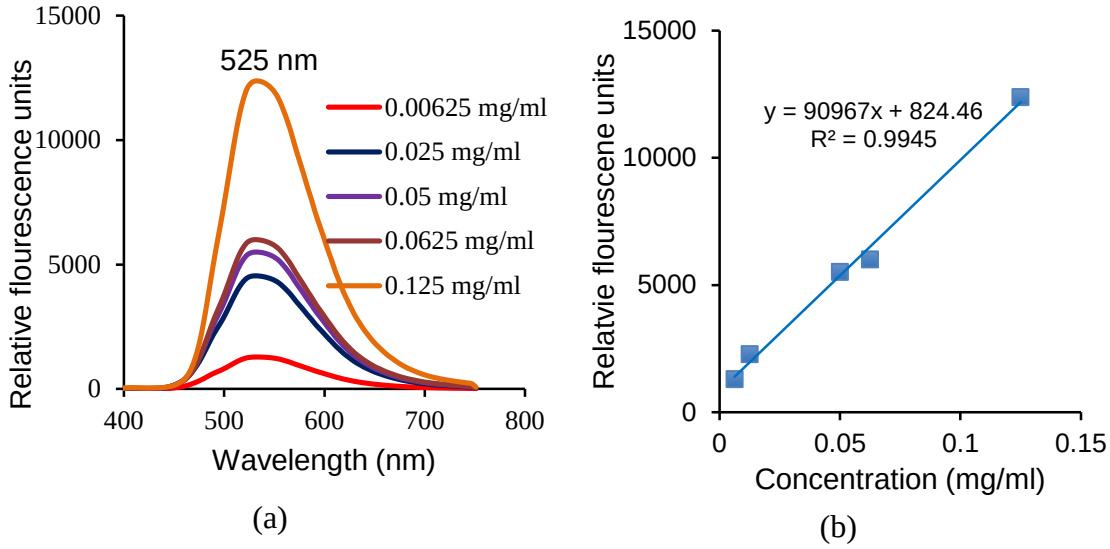

Figure 3. a. *Fluorescence spectra of curcumin in ethanol with various concentrations 0.00625, 0.025, 0.05, 0.0625 and 0.125 (mg/ml).* ***b.*** *Fluorescent calibration curve of free curcumin in ethanol.*

After encapsulation of curcumin in niosomes the fluorescent intensity of curcumin increased approximately two times. In addition, the fluorescent peak of curcumin shifted from 525 nm to 495 nm (Figure 4). These changes of fluorescent characteristics may be due to the hydrophobic interaction between curcumin and span-80, cholesterol that indicated the successful loading of curcumin into niosomes.

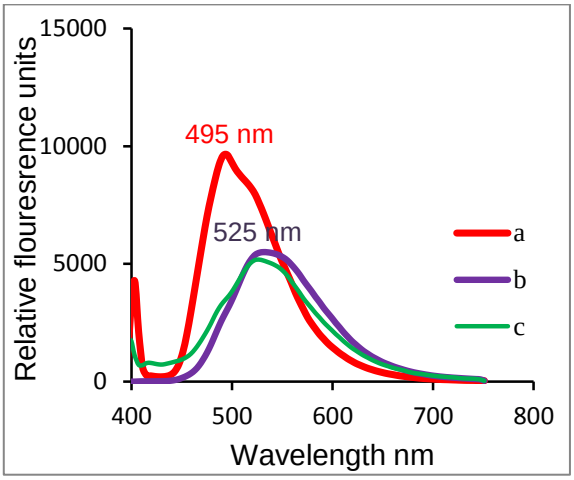

Figure 4. *Fluorescence spectra of niosomal encapsulated curcumin (a), free curcumin in ethanol (b) and niosomal encapsulated curcumin after destruction of niosomes by absolute ethanol (c) (curcumin concentration: 0.05 mg/ml).*

3.3. Determination of niosomal encapsulation efficiency of curcumin

Two experimental parameters were explored for curcumin-niosome formation: (1) ethanol evaporation time and (2) initial concentration of curcumin (the molar ratio of span 80 and cholesterol was kept constant). Niosomal encapsulation efficiency (EE) and mass of encapsulated curcumin were investigated for different ethanol evaporation times and initial concentrations of curcumin using fluorospectrometry.

The EE and mass of encapsulated curcumin at a particular initial concentration of curcumin (0.05 mg/ml) for different intervals of time are shown in figure 5.a. Since EE increased sharply after two hours of ethanol evaporation (89%) and rose up to the maximum value (98%) after five hours of ethanol evaporation, these results suggested that two hours was the necessary time for niosome formation, but five hours was the time for reaching the highest EE. This value is greater than the encapsulation efficiency of curcumin in niosomes made from span 80, poloxamer 188 [5] and from span 60 and cholesterol [6].

The EE and mass of encapsulated curcumin of different initial curcumin concentrations kept for 5 hours of ethanol evaporation time are plotted in figure 5.b. Mass of encapsulated curcumin rose from 1 mg to approximately 5 mg when initial concentration of curcumin increased from 0.01 mg/ml to 0.05 mg/ml (figure 5. b). In this case the molar ratio of

span80: cholesterol: curcumin was 45: 30: 1. Thus, in the present study, the highest encapsulation efficiency (98%) and mass of encapsulated curcumin (5mg) were achieved with an initial curcumin concentration of 0.05 mg/ml and ethanol evaporation time of 5 hours.

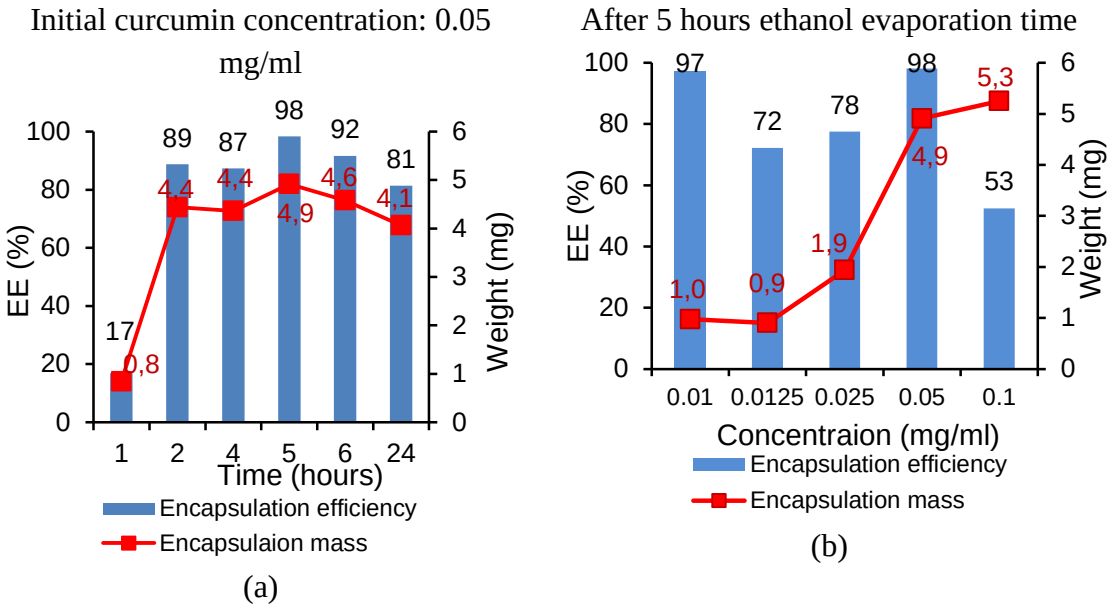

Figure 5. *Influence of the ethanol evaporation time (a) and the initial concentration of curcumin (b) on the niosomal encapsulation efficiency of curcumin and the mass of niosomal encapsulated curcumin.*

6. CONCLUSIONS

In this study, a simple method preparation of niosomes from non-ionic surfactant span 80 and cholesterol in ethanol for curcumin encapsulation was proposed. The highest encapsulation efficiency (98%) and mass of encapsulated curcumin (5mg) were achieved with an initial curcumin concentration of 0.05 mg/ml and ethanol evaporation time of 5 hours. With high niosomal encapsulation efficiency this system could improve therapeutic effect of curcumin.

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Investigation on Physical Stability and Microstructure Characteristics of Fatty Alcohol – Tween 60 – Water Ternary System

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Abstract

Introduction: Fatty alcohol – surfactant – water ternary systems, models to represent the continuous phases of multiple phase oil-in-water emulsions have been successfully developed in literature. **Objectives:** The aim of our study was to investigate fatty alcohol – Tween – water ternary systems as potential dermatological vehicles with respect to their macro- and microscopic, rheological, and thermal characteristics. **Materials and methods:** In this study, fatty alcohol (16g) – Tween 60 (4g) – water (180g) ternary systems were prepared by adding molten mixture of fatty alcohol and Tween 60 to hot water and homogenizing with the use of Silverson. These systems were then characterized by macroscopic and macroscopic observation, rheological test (continuous and viscoelastic), thermal analysis (differential scanning calorimetry). **Results:** The consistency of Tween 60 ternary systems was higher when prepared with Tween 60 batch containing greater amount polyoxyethylenesorbitan/isosorbide monoesters. The dominant role of swollen lamellar α-crystalline gel phase on the microstructure and stability of ternary systems containing various C16-C18 fatty alcohol combinations was confirmed. The gel phase remaining stable on storage was mainly responsible for the stability of semisolid oil-in-water emulsion and the deterioration from semisolid to milky systems was observed when this gel phase converted into non-swollen β,γ crystals. **Conclusion:** The influence of excipient composition and functionality on the microstructure and drug release characteristics of fatty alcohol – Tween 60 – water ternary system was successfully investigated. Our results suggest that cetostearyl alcohol – Tween 60 – water ternary system could be a good candidate to deliver pharmaceutical and cosmetic agent to the skin.

Keywords: polysorbate, fatty alcohol, ternary system, semisolid oil-in-water emulsion, thermal analysis, rheology, microscopy

1. INTRODUCTION

In theory, the o/w type creams stabilizied with mixed emulsifier have at least four phases: (i) crystalline/hydrophilic gel phase composed of bilayers of surfactant and fatty amphiphile that inserts water molecules to form interlamellar water; (ii) free-bulk water layer in equilibrium with the interlamellarly fixed water; (iii) lipophilic gel phase composed of the excess of the fatty amphiphile; and (iv) dispersed oil phased mainly immobilized mechanically from the lipophilic gel phase (reviewed by Eccleston).[1]

The physical stability and microstructure of these systems may affect their *in vitro* drug release profile. In practice, they are commonly characterized by different techniques such as rheology, differential scanning calorimetry (DSC), microscopy.....
The objective of this study was to investigate the influence of excipient batch variation on microstructure and consistency of Tween 60-fatty alcohol-water ternary systems, which were used as representative models of continuous phases of the corresponding semisolid o/w emulsions.

2. MATERIALS AND METHODS

2.1. Materials

Fatty alcohols were purchased from Sigma, UK. A batch of Cetostearyl alcohol BP (CSA) was from J.M.Loveridge plc (UK). 3M Health Care limited, UK provided two batches of Tween 60 and one batch of cetyl alcohol (C16). Freshly distilled water was used throughout.

2.2. Preparation of ternary systems

In this study, fatty alcohol (16g) – Tween 60 (4g) – water (180g) ternary systems were prepared by adding molten mixture of fatty alcohol and Tween 60 to hot water and homogenizing with the use of Silverson SL2T (Silverson machines, Chesham, UK).

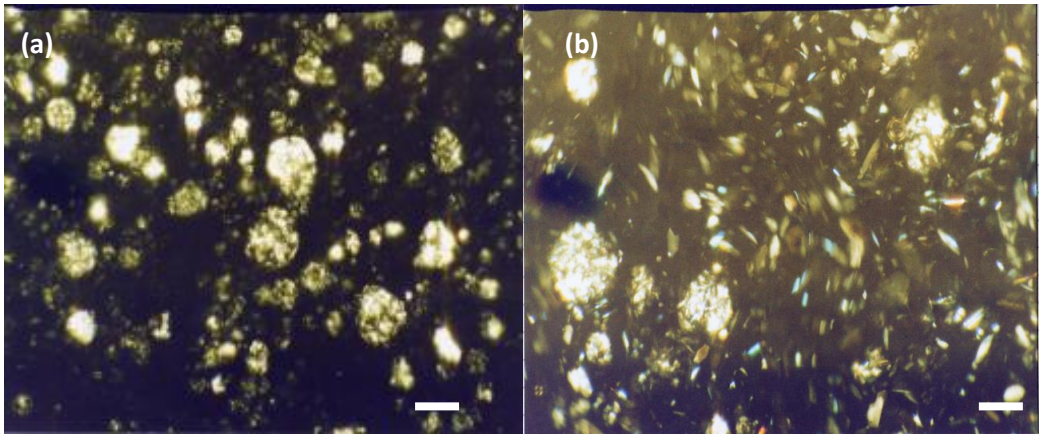
2.3. Characterization of ternary systems

The ternary systems were examined visually and microscopically using polarized and normal light with Polyvar system (Reichart – Jung, Austria). Rheological tests were performed at 25^{o}C using a STRESSTECH Rheometer, RheoLogica Instruments AB, Sweden with 4^{o}, 4cm cone and plate. Flow curves (shear rate vs. shear stress) were recorded when the samples were exposed to increasing and decreasing shear rate sweep (0.3-100s^{-1}) in 10 minutes (5 minutes up; 5 minutes down). Samples were tested under oscillation frequency sweep mode (i.e. 0.01-10Hz) at 1Pa. DSC experiments of hermetically sealed pans containing ca. 10 mg between 15-80°C were performed with 3 cycles: heating, cooling and re-heating at the rate of 10°C/min using a TC 15 (Mettler Toledo, Switzerland).

3. RESULTS

All CSA ternary systems with both batches of Tween 60 were shiny white semisolids immediately after preparation. Little change was observed after 4-month storage although ternary systems were shinier. In these ternary systems, microscopy revealed spherical structures, which were Maltese crosses in polarized light (Figure 1 a). In addition, large anisotropic "clumps" were visible. Some of which were masses of Maltese crosses, others were less well defined. In contrast, ternary systems prepared with pure C16 alcohol were very mobile on the day of preparation. Their semisolid structure formed extensively during the first week storage. On further ageing, they gradually thinned whilst becoming pearlescent. In these ternary systems, some crystallization appeared progressively as they aged (Figure 1 b).
Figure 2 shows typical DSC diagrams of Tween 60 ternary systems after 6-7 month storage. In 6-month old CSA system, there was only a broad asymmetrical endothermic peak (starting around 50°C and peaking at ~ 60°C, first run). The second heating cycle on the same sample after a cooling process showed a different spectrum i.e. a very board peak divided into doublet at ~ 56°C. The repetition of DSC tests on the same sample left

undisturbed in the pan after one more month kept at 25°C was also performed. The first run of this sample aged for 7 months gave a broad endotherm starting around 50°C peaking at ~ 61°C with a shoulder at the low temperature side. This was similar to the first run of 6-month sample. The second runs after 6 and 7 month-storage of this sample were nearly identical. On the contrary, two endothermic peaks were observed in 6-month old C16 systems i.e. a low temperature stating at ~ 32°C and peaking at ~ 37°C and a high temperature peak resolved as a doublet ranging 58-66°C.



Figures 1. *Typical photomicrographs of (a) CSA systems and (b) C16 systems. Bar: 50 μm.*

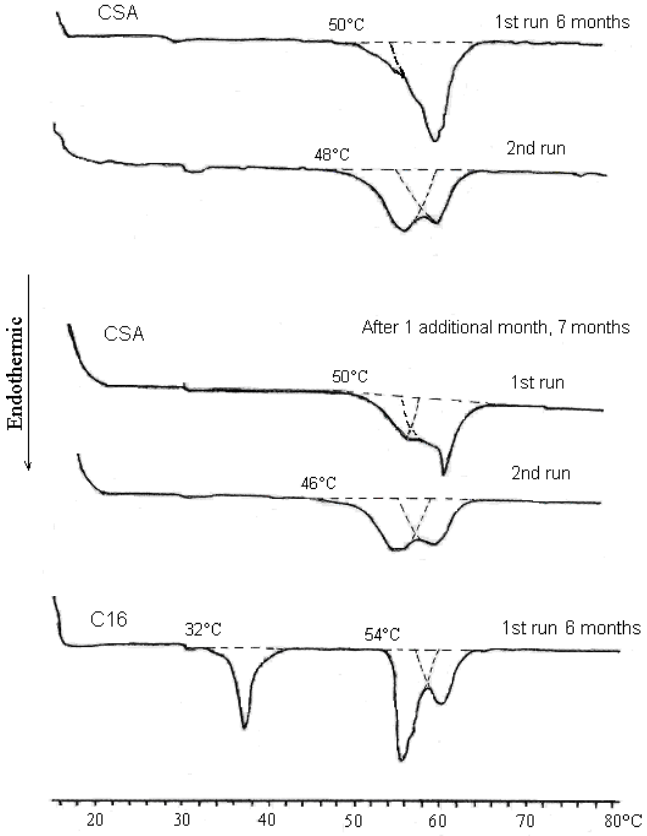


Figure 2. *Typical DSC diagrams of CSA and C16-Tween 60 ternary systems after 6-7 months. 1st run: heating; 2nd run: heating after cooling.*

All the formulations were shear thinning throughout. Flow curves were in the form of anti-clockwise hysteresis loops (data not shown). The loops show that some structure was present in all systems on the day of preparation and their semisolid structure formed extensively during the first week storage as evidenced by the movement of flow curves towards greater values along the shear stress axis. The variation of apparent viscosity η_{app} of each ternary system, derived at the apex of each loop ($100s^{-1}$), with storage time is plotted in Figure 3.

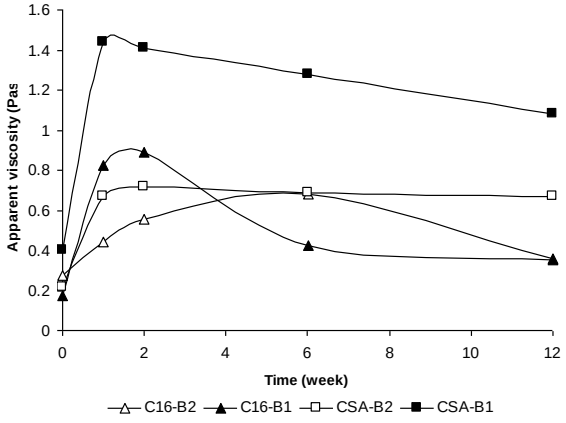
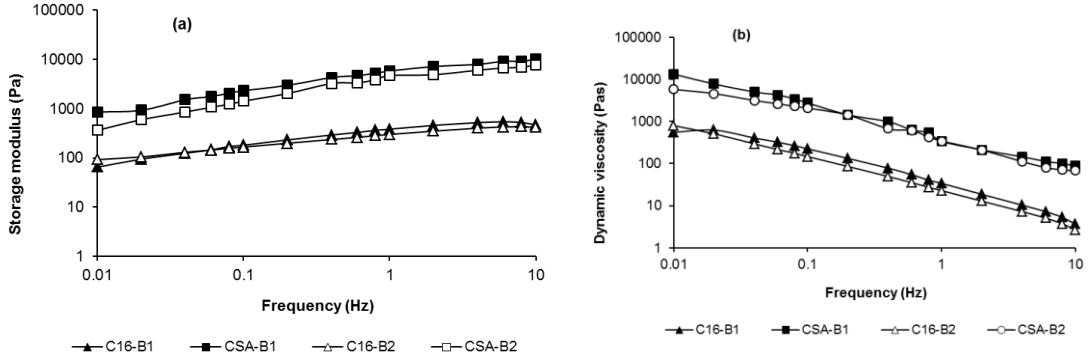


Figure 3. *Variation of apparent viscosity of CSA and C16-Tween 60-water ternary systems vs. storage time. B1 and B2 represent 2 batches of Tween 60 used.*

Figures 4 a, b shows G' (storage modulus) and η'(dynamic viscosity) of all ternary systems 12 weeks after preparation. As frequency increased G' increased and η' fell almost monotonically; the latter through several logarithmic scales. Clearly, CSA systems had significantly higher G', η' values than C16 systems independently of the Tween 60 batch used.



Figures 4. *Comparison of (a) G' and (b) η' of 12-week-old CSA and C16-Tween 60-water ternary systems. B1 and B2 represent 2 batches of Tween 60 used.*

7. DISCUSSION

In CSA ternary systems, the existence of the gel network microscopically viewed as maltese crosses (Figure 1 a), was thermally evidenced by a broad endothermic peak with a

low temperature shoulder representing the melting of hydrated fatty alcohol crystals and the main peak representing the swollen α-lamellar gel phase transition (Figure 2). A different spectrum was observed when the same sample was cooled and then re-heated, for there was a prominent doublet indicating that the ternary system did not fully form in the absence of mixing. The first part of the doublet is due to the hydrated fatty alcohol crystals and the second part due to the swollen α-lamellar gel phase.[2] The repetition of DSC test on the same sample after one more month kept at 25°C confirmed the gradual reformation of the microstructure of lamellar gel phase on storage but not to the same extent as it had been.

Basically, the increases in consistency of all the CSA ternary systems during the first week storage (Figure 3) can be explained by the gel network theory developed by Eccleston and Barry (reviewed by Eccleston).[1] Ternary systems prepared with CSA batches thickened slowly on storage because of additional hydration of polyoxyethylene chains at 25oC. On further ageing, slight reductions in consistency occurred.

In a series of studies on the aged samples of the CSA – POE (20) glycerol monostearate (PGM20) – water system, de Vringer and co-workers found the decrease in thickness of the lamellae indicated by X-ray diffraction data and the repulsion of water from the lamellae ascertained by the measurements of spin lattice relaxation rates of water.[2-5] According to de Vringer and co-workers, the explanation was related to changes in the distribution of surfactant molecules among lipophilic bilayers.[4] Immediately after preparation, the gel network of CSA – Tween 60 – water systems can exist in two possible extreme situations with reference to the distribution of Tween 60 among the lipophilic bilayers: (i) "inhomogeneous" (Tween 60 molecules are grouped to form clusters) and (ii) "homogeneous" (Tween 60 molecules are distributed more randomly throughout the system). Actually, a mixture of intermediate situation can be thought of. On storage, they propose that changes from inhomogeneous to homogeneous situations happen as a result of slow lateral diffusion of the hydrocarbon tails of Tween 60 molecules in the gel network. In the homogeneous situation, the possibility of polyoxyethylene chain folding to a certain extent is more likely to give the reduction in bilayer structure whereas very unlikely in inhomogeneous situation. In other words, the steric hindrance in the direction normal to the lipophilic sheets that counteracts the attractive Van der Waals forces (which are responsible for the decrease of the thickness of the lamellae) is higher in inhomogeneous situation.

In this study, our aged samples of CSA – Tween 60 – water systems got thinner after 2-week storage as shown by rheological data. This might be due to the expulsion of physically trapped water within the lamellae and is in a good agreement with de Vringer and co-workers' observations. When considering these rheological results in relation to LC-MS and NMR analysis data of Tween 60 batch variation[6], some important remarks can be reached. The fact that Tween 60 batch 1 system was thicker than Tween 60 batch 2 system (as illustrated in both continuous shear and viscoelastic tests) independent of which CSA batches were used (Figure 6) appears to correlate with higher ratio of POE sorbitan stearate/POE sorbitan palmitate in batch 1 (i.e. batch 1: batch 2 ratio of 1.5 : 0.8).

Similar observations were obtained when CSA was replaced by pure C16 homologue to formulate C16 – B1/B2 ternary systems. Generally, all C16 ternary systems showed increase in consistency over approximately the first 2 weeks, followed by a more prominent breakdown of the structure with storage time (from week 2 to week 12) than CSA- ternary systems. The structural breakdown of these C16 systems was explained by the transformation of swollen α-lamellar gel phase into non-swollen β,γ crystals on

storage.[7,8] The existence of β,γ crystals at 25°C storage was evidenced by additional low temperature β,γ to α-crystal transition in our DSC experiments (Figure 2) and microscopic observation under polarized light (Figure 1b). Interestingly, rheological data always show that CSA ternary systems with all batches of Tween 60 were thicker than corresponding C16 ternary systems on storage (Figures 3 and 4).

8. CONCLUSION

CSA ternary systems were thicker than all C16 ternary systems independently of Tween 60 batches used. All the ternary systems showed the same trend on storage. An initial increase in consistency over the first 2 weeks of storage, followed by slight changes in consistency (CSA systems) due to the re-allocation of Tween 60 in the gel network; or significant breakdown of structure (pure C16 systems) due to the transformation of swollen α lamellar gel phase into β,γ crystals on 25°C storage. The consistency of Tween 60 ternary systems is expectedly higher when prepared with Tween 60 batch containing a greater amount of POE sorbitan/isosorbide monoesters and (ii) sorbitans, POE-diester substances are non-constructional gel network elements.

ACKNOWLEDGEMENT

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Formulation of Floating Tablets as Captopril Delivery System Using Direct Compression Method

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Abstract

Captopril short elimination half-life (2-3 hours) and good stability at pH 1,2 with its major absorption in the upper part of Gastro Intestinal Tract (GIT) was the reason of this drug formulated into sustained release floating tablets. This research was conducted in order to obtain several formulations and evaluate the physical characteristics, floating lag time and dissolution rate of the floating tablets. The captopril floating tablets were produced by direct compression technique using combination of polymers (HPMC, ethyl cellulose and CMC-Na in different concentrations). The gel forming agent, gas generating agent and another polymer were used to control their floating capability and drug dissolution rate profile. The tablet floating capability was evaluated by determining lag time and floating duration. All formulas remained buoyant for more than 12 hours. The drug release evaluated for 12 hours using USP paddle-type II dissolution apparatus in a 900ml 0,1N HCl as dissolution medium. The dissolution efficiency of control tablets (Formula 1) was 90,98%. There was a significance difference compared to dissolution efficiency value of Formula 2 (2% CMC-Na), Formula 3 (4% CMC-Na), Formula 4 (6% CMC-Na), Formula 5 (10% Ethyl cellulose), Formula 6 (20% Ethyl cellulose), Formula 7 (30% Ethyl cellulose) as 86,35%, 83,88%, 80,35%, 81.25%, 75.81%, and 78.16%, respectively. The result of this research indicates that tablet formulation with different in composition of the polymers (CMC-Na and Ethyl cellulose) affects the floating capability and dissolution rate of the tablets, which is important in consider floating tablet as captopril sustained release delivery system.

Keywords: captopril, HPMC, CMC-Na, ethyl cellulose, floating tablet, sustained release

1. INTRODUCTION

The conventional tablets commonly used today have its limitations. The limitations of conventional tablets are on fluctuations in drug plasma concentrations (Brahmankar & Jaiswal, 2009). Controlled release tablets made to answer that limitations, because of the use of controlled release tablets can avoid fluctuations of drug levels in plasma. Controlled release tablets also used to improve the patient with chronic diseases compliance in taking the drug (Kumar, 2012). The use of controlled release tablet still has limitations, where the

controlled release tablet that has been designed could not survive in the absorption place during the drug release from the matrix (Prinderre *et al.,* 2011).
The way to make controlled release dosage forms can survive in the gastrointestinal tract is by using the *Gastro Retentive Drug Delivery System* (GRDDS) (Chikhalikar & Wakade, 2012). Drugs that require *GRDDS* work locally in the gut, mostly absorbed in the gastric, insoluble in an alkaline pH, and drugs that unstable at alkaline pH (Mishra & Gupta, 2012). One example of a drug that is absorbed in the stomach and degraded at an alkaline pH is captopril (Sweetman 2011; Vijayasankar *et al.,* 2011).

Floating drug delivery system is GRDDS dosage form that has smaller density than gastric fluid, so that it can float on the top of the gastric juices and is not influenced by the gastric emptying time (Narang, 2011). Materials such as HPMC in floating system are used as gelling materials and swelling agents. Swollen HPMC then locks the air inside and shows floating effect of the systems (Mishra & Gupta, 2012). In this experiment, ethyl cellulose and CMC-Na as polymers can protect the penetration of medium, lowering the floating lag time, and improve floating duration (Dhole *et al.,* 2011; Chowdary *et al.,* 2013).

The floating ability and dissolution profiles will be tested in this research based on percent dissolved, dissolution efficiency and floating duration of 7 kinds of formulas with different ethyl cellulose and CMC Na composition in each formula. It is expected that the formula can provide buoyancy and good dissolution profiles at pH 1, 2 for 12 hours.

2. MATERIALS AND METHODS

2.1 Equipments

Erweka AR 402 tablet compression machine, Monsato hardness tester, Pharmex Y cone mixer, Hanson Research SR-8 dissolution apparatus, Sartorius BP10 analytical balance, Erweka friability tester, Shimadzu spektrofotometer.

2.2 Material

Captopril p.g (Huahai), HPMC K100M p.g (Colorcon), ethyl cellulose p.g (Colorcon), spray dried lactose p.g (Foremost), $NaHCO_3$ (Inner Mongolia Ihjuchen), citric acid (Anugrah Buana), magnesium stearate p.g (Bratachem), HCl p.a (Sigma).

2.3 Method

2.3.1 Tablets Formulation

The process of producing the tablet was done by direct compression method. The powder mixture was made by mixing of captopril, HPMC K100M, ethyl cellulose, CMC Na, SDL, NaHCO3 and citric acid. Mixed mixture was screened by siever. Magnesium stearate then added and mixed to form the 240mg weight tablets.

Table 1 *Floating Tablet Formula*

Materials	Weight (mg)						
	F1 (control)	F2	F3	F4	F5	F6	F7
Captopril	25	25	25	25	25	25	25
HPMC K100M	48	48	48	48	48	48	48
CMC- Na	-	4,8	9,6	14,4	-	-	-
Etil Selulosa	-	-	-	-	24	48	72
Mg. Stearat	1,2	1,2	1,2	1,2	1,2	1,2	1,2
SDL (Spray Dried Lactose)	ad 240	ad 240	ad 240	ad 240	ad 240	ad 240	ad 240

2.3.2 Powder Mixture Evaluation

The moisture content of the powder is tested by weighing it for 5 grams and checked it in the moisture content balance. Drying will take place until it showed no change of material weight. Good moisture content value was 1-4%. Compressibility also tested with a number of material in the tap density volumenometer. The density of material before and after tapping process was determined and calculated to get the compressibility value. Powder good compressibility value was <20% (US Pharmacopoeial Convention ., 2017).

2.4 Tablet Evaluation

2.4.1 Physical Characteristics of Tablets

Tablet weight variation is measured by weighing one by one of 10 tablets with analytical balance. Weight variation data is quite good, if the acceptance value is not more than 15% (US Pharmacopoeial Convention ., 2017). Tablet hardness was measured with 10 tablets prepared. The tablet to be tested is placed on Monsanto hardness tester. The tool base rotated until the tablet breaks and the scale was read that indicates the numbers of tablet hardness. In the tablet hardness testing all tablets should be crushed by the load 4,0 to 8,0 kg (Lieberman *et al.*, 1990).

Tablet friability was measured from 20 tablets prepared, then weighed and included in the test apparatus. After rotated, the tablet is then removed and then weighed again. It said to be good if the weight loss is not more than 1% (Lieberman *et al.*, 1990).

2.4.2 Content Uniformity of Tablets

Preparation of captopril standart solution performed by weighing 50,0 mg captopril, then dissolved in 0,01N HCl until 50,0 ml. The standard solution then prepared into several working standart Maximum wavelength were observed between 200-300 nm using a UV spectrophotometer. Standard curve obtained from observations of absorption value of the standard solution at each concentration of captopril was observed at maximum wavelength. (Vijayasankar *et al.*, 2011; Mehta *et al.*, 2011).

Captopril content uniformity in tabletis measured by weighted 10 tablets are taken randomly and ground into powder. Powder weighed equal with 25 mg captopril and diluted to 100,0ml. The solution was filtered using Whatmann filter paper (0,45 µm). Dilution was observed using a UV spectrophotometer at maximum wavelength (Vijayasankar *et al.*,

2011) (Mehta *et al.*, 2011).

2.4.3 Floating Lag Time and Floating Duration

Floating lag time and floating duration time test was done with Measure Glass equipment, heater, thermometer and stop watch. 200ml 0,01N HCl solution putted into a measuring cup 250 ml in temperature of 37^{o}C, then the tablet was inserted. Floating lag time was observed. The time required for the tablet to rise up to the surface and begin to float on the medium surface is recorded as floating lag time, while the time for the tablet remains constant on the dissolution media surface is recorded as floating duration time. Floating duration time was expected to be not less than 12 hours to be able to ensure the drug is in the stomach within 12 hours (Vijasankar *et al.*, 2011).

2.4.4 Dissolution Test

The dissolution media used were 900ml 0,01N HCl solution (pH 1.2) and using a type II dissolution apparatus 50rpm. Temperature and time for dissolution test were 37 ± 0.5 ^{0} C for 12 hours. Sampling was done at 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660, 720 minutes. Measurements performed with a UV spectrophotometer at the maximum wavelength (US Pharmacopoeial Convention., 2017) (Vijayasankar et al., 2011) (Mehta et *al.*, 2011). The drug release profile of captopril is accomplished by making the captopril released curve (% Q) per time. Dissolution profile curves calculated from AUC (Area Under the Curve) to determine the efficiency of dissolution. The results obtained were floating lag time, floating duration, percent of dissolution efficiency (%ED), percent dissolved (%Q) then analyzed using statistical methods to determine whether there were significant differences between the groups were tested.

3. RESULTS AND DISCUSSION

Materials were mixed into homogenous mixture. Before compressed into tablet, the powder mixture was tested first. The mixture showed good result in compressibility and moisture content, then it continued with compression using direct compression method.

Table 2. *Characteristics of powder mixture*

Formula	Compressibility (%)	Moisture content (%)
F1	12.00%	1.96%
F2	13,16%	2,04%
F3	12,82%	1,98%
F4	13,16%	2,08%
F5	14.29%	1.98%
F6	13.64%	1.72%
F7	14.63%	1.50%

Table 3. *Physical Characteristics of Tablets*

Evaluation	F1 (control)	F2	F3	F4	F5	F6	F7
Weight variation (mg)	244,54 ± 1,18	240,56 ± 0,59	243,80 ± 0,77	244,66 ± 0,91	243,21 ± 1,05	242,27 ± 0,94	240,87 ± 0,76
Tablet Hardness (kg)	7,00 ± 0,41	5,50 ± 0,53	5,20 ± 0,26	5,15 ± 0,24	5,05 ± 0,28	4,50 ± 0,33	4,20 ± 0,24
Tablet Friability (%)	0,16 ± 0,06	0,18 ± 0,08	0,18 ± 0,10	0,18 ± 0,06	0,12 ± 0,07	0,16 ± 0,05	0,09 ± 0,06
Floating Lag time (sec)	16,33 ± 0,57	16,67 ± 0,58	17,00 ± 1,00	17,33 ± 0,58	19,67 ± 1,00	24,67 ± 1,15	32,33 ± 2,52
Uniformity of content (%)	105,30 ± 0,67	105,01 ± 0,10	104,35 ± 0,46	104,16 ± 0,52	104,64 ± 0,72	109,55 ± 0,96	103,33 ± 0,47

Based on evaluation results, shown that all of formula had good physical characteristics. The results determined that tablets weight variation, hardness and friability of all formula met the requirements. Floating duration results showed the entire formula could float for more than 12 hours. Floating lag time of each formula was different, according to the amount of polymer. Addition of CMC-Na was not very influences the floating lag time, but it became statistically influences when ethyl cellulose added into components of formula, provided longer floating lag time. This floating resistance could be caused by the ethyl cellulose properties as a hydrophobic polymer that inhibits penetration of the medium into the tablet, so it takes longer time for HPMC K100M to expand and form a system that can make the tablet floated.

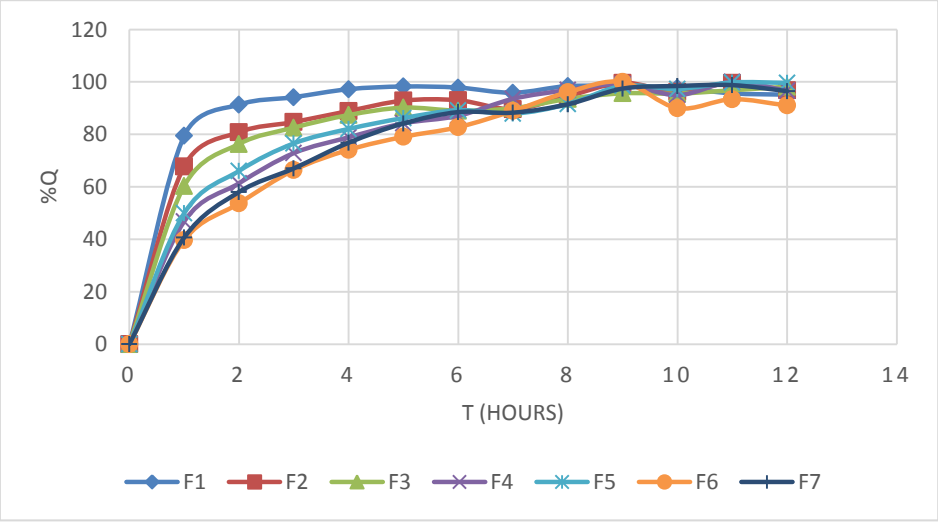

Figure 1. *Dissolution rate of Captopril*

The dissolution test results showed the release resistance on the addition of CMC-Na and ethyl cellulose in the formula. This results showed that the addition of CMC-Na and ethyl cellulose in the formula could decrease the dissolution efficiency, where the decrease in %ED indicates a decrease in the dissolution rate, but on increasing the ethyl cellulose concentration from 10% to 30% did not indicate a significant %ED change.

Table 4. *Dissolution Efficiency Results*

Formula	Dissolution Efficiency (%)
F1	90,98 ± 0,47
F2	86,35 ± 2,48
F3	83,88 ± 6,82
F4	80,35 ± 1,32
F5	81,25 ± 3,50
F6	75,81 ± 0,98
F7	78,16 ± 0,72

4. CONCLUSIONS

The ethyl cellulose and CMC-Na use as polymer showed the influence on controlled release floating tablet result. The addition of ethyl cellulose and CMC-Na reduced the dissolution rate, and showed the sustained release profile of captopril. Addition of ethyl cellulose and CMC-Na affected the floating lag time, significantly made it slower based on higher concentration of the polymer. The differences could be influenced by improvement of viscosity surrounds the tablets. Amount of ethyl cellulose and CMC-Na must be considered, also the possibility on using both of polymer as combination to get faster floating lag time, longer floating duration and sustained release effect on captopril.

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Application of Starch Nanocrystals in Pulsatile Release Pellets

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Abstract

Pulsatile release system is a time or site controlled drug delivery system which released the drug rapidly after the predetermined lag time. Ethylcellulose is frequently used as rupturable coating polymer in the rupturable type pulsatile drug delivery system, but its high sensitivity of coating level on lag time causes to make less robust system. This study was aimed to investigate cassava starch nanocrystals as an ethylcellulose film modifier to fabricate more robust system for pulsatile delivery. The rupturable pulsatile release pellets consisted of (i) a drug (theophylline) layered core pellets; (ii) a swelling layer (croscarmellose sodium) and, (iii) a rupturable layer of ethylcellulose with cassava starch nanocrystals were developed. The effect of amount of cassava starch nanocrystals in rupturable layer, coating levels of swellable and rupturable layers on lag time and drug release were investigated. The results indicated that lag time of the pulsatile pellets was increased with increasing levels of swellable and rupturable coatings, whereas increasing amount of nanocrystals in rupturable coating subjected to decrease lag time. Increasing level of swellable layer and amount of starch nanocrystals in rupturable layer led to rapid drug release after lag time while higher level of rupturable layer caused a slower drug release. In conclusion, rapid drug release after the lag time were obtained from the pulsatile release pellet with ethylcellulose and cassava starch nanocrystals as rupturable layer, indicating the potential to produce the desired pulsatile release for various drugs which address the chronopharmacological needs of certain diseases.

Keywords: Pellets, Pulsatile release, Starch nanocrystals, Drug delivery system

1. INTRODUCTION

The main focus of pulsatile drug delivery is to release drugs on a specific preprogrammed pattern, i.e. at appropriate time and/or at appropriate site of action (1). Drug release under appropriate time is primarily controlled by the system, while the other is governed by the biological environment in the gastrointestinal tract e.g., pH, enzymes. Pulsatile delivery system offers advantages over the conventional delivery system such as, minimization of side effects, decreased incidence and/or intensity of undesirable side effects, reduced dose and frequency improving patient compliance, protected the drugs which show extensive first pass metabolism, protection of mucosa from irritating drugs and obtained the special pharmacokinetic features according to circadian rhythm of body (2). Ethylcellulose is a

frequently using film forming polymer in rupturable type pulsatile drug delivery system, because of its water insolubility, adjustable water permeability and mechanical properties (3). Nowadays, polymer blends are more common than pure ethylcellulose as they offer major advantages, including: (i) adjusting the desired drug release patterns (ii) improving film formation and storage stability (iii) developing site specific drug delivery within the gastrointestinal tract (4). Moreover, since the coating level of ethylcellulose is high sensitive on lag time, the modifications with other materials such as hydrophobic particulate material, magnesium stearate, (1, 5) are able to develop a less sensitive and more robust coating system. Therefore, it was decided to develop a successive rupturable pulsatile release pellets using a polymer blend of ethylcellulose and cassava starch nanocrystals as polymeric rupturable layer.

2. MATERIALS AND METHODS

2.1 Materials

Starch nanocrystal powder was provided from National Nanotechnology Center, National Science and Technology Development Agency (NSTDA), Thailand). Sugar sphere 1000-1190 □m, (IPS, Italy), theophylline (A.C.S., China), ethylcellulose NF standard 10 Premium (Dow Chemical, USA), hydroxypropyl methylcellulose (Methocel® E15LV) (Dow Chemical, USA), Povidone K90 (BASF, Germany), croscarmellose sodium (Nichirin Chemical Industries Ltd., Japan), dibutyl sebacate NF (Morflex, Greensboro, NC, USA), magnesium stearate (Peter Greven Nederland C.V., Venlo, Netherlands) were used as received. All of other materials are of pharmaceutical grade.

2.2 Preparation of pulsatile release pellets

2.2.1 Drugs layering on sugar pellets

Theophylline (15% w/v) was dispersed in ethanol/water (60:40 w/w) mixture containing HPMC (6% w/v) and the dispersion was stirred until clear dispersion was obtained. Then this dispersion was layered on the pre-warmed sugar pellets in a fluidized bed bottom spray coater equipped with a Wurster insert (GPC 1.1, Glat, Germany) until 20% (w/w) weight gain was achieved.

2.2.2 Coating for swellable layer

A swelling agent, croscarmellose sodium (ADS) (20% w/v) was layered onto the drug layer using Povidone® K90 (6% w/v) as a binder. Povidone® K90 was dissolved in ethanol 96% (v/v) and the mixture was stirred until clear solution was obtained. ADS was dispersed in the binder solution and agitated for 30 minutes to obtain a homogeneous dispersion, prior to coating. The coating suspension was layered on pre-warmed pellets with drug layer to obtain the coating levels of 40% (w/w) in a fluidized bed bottom spray coater equipped with a Wurster insert. The conditions used are shown in Table 1.

2.2.3 Coating for rupturing layer

The coated pellets with drug and swellable layers were subsequently coated with ethanolic ethylcellulose solution, using dibutyl sebacate as a plasticizer, in a fluidized bed bottom spray coater equipped with a Wurster insert. The coating solution was prepared by dissolving the polymer, ethylcellulose (6% w/v), in 96% (v/v) ethanol. The polymer suspension was stirred overnight until a clear solution was obtained. A plasticizer (10% w/w based on the weight of polymer) was added in to the polymer solution and the solution was further stirred to obtain a homogeneous solution for at least 30 minutes before coating. In the application of starch nanocrystals, it was incorporated as an additive in the ethylcellulose rupturing layer. The ratio of ethylcellulose polymer to starch nanocrystals 90:10, 80:20, and 75:25 (based on non-plasticized polymer dry mass) were investigated.

Table 1. *Operations for preparing pulsatile pellets in a fluidized bed bottom spray coater equipped with a Wurster inserts.*

	Operating conditions		
	Drug layer	Swellable layer	Rupturable layer
Batch size (g)	1000	1000	1000
Inlet air temperature (°C)	48-50	35-37	35-37
Product temperature (°C)	38-40	30-32	30-32
Air flow (m^3/h)	75-80	70-75	70-75
Atomizing air pressure (bar)	2.5-2.7	2.3-2.5	2.3-2.5
Spray rate (g/ min)	9-12	15-18	15-18
Spray nozzle diameter (mm)	1.2	1.2	1.2
Final drying temperature (°C)	55-60	40-45	40-45
Final drying time (min)	15-20	15-20	15-20

2.2.4 Evaluation of coated pellets with rupturable layer

2.2.4.1 Morphology

Surface morphology and cross section of the coated pellets were photographed using scanning electron microscope. To obtain cross-sectional view, a coated pellet were placed on a glass slide and cut through from the top center with a razor blade. The samples were coated with gold prior to microscopic examination.

2.2.4.2 Drug release study

A sample of pellets equivalent to 25 mg theophylline was accurately weighed and processed in a USP paddle apparatus (900 ml 0.1 N HCl or pH 7.4 phosphate buffer USP, 37±0.5 °C, 50 rpm, n=3). An aliquot of 10 ml was withdrawn at predetermined time points and analyzed by UV spectrophotometrically at a wavelength of 269 nm.

2.2.4.3 Lag time

Lag time was determined by extrapolation of the upward part of release profile to the time axis.

3. RESULTS AND DISCUSSION

The rupturable pulsatile pellets were prepared in a fluidized bed bottom spray coater equipped with a Wurster insert by the solution and/or suspension layering technique. Inert sugar spheres were used as starting core and three coating layers were subsequently deposited on it. First, innermost layer was a drug layer and swellable layer was a second layer. Outer layer was a rupturable layer where ethylcellulose with starch nanocrystal was investigated. The design of pulsatile release pellets is shown in Figure 1.

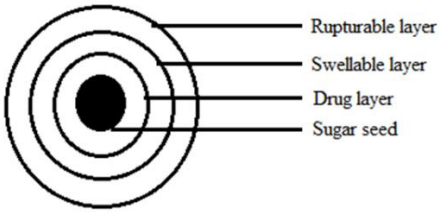

Figure 1. *Design of rupturable pulsatile release pellets.*

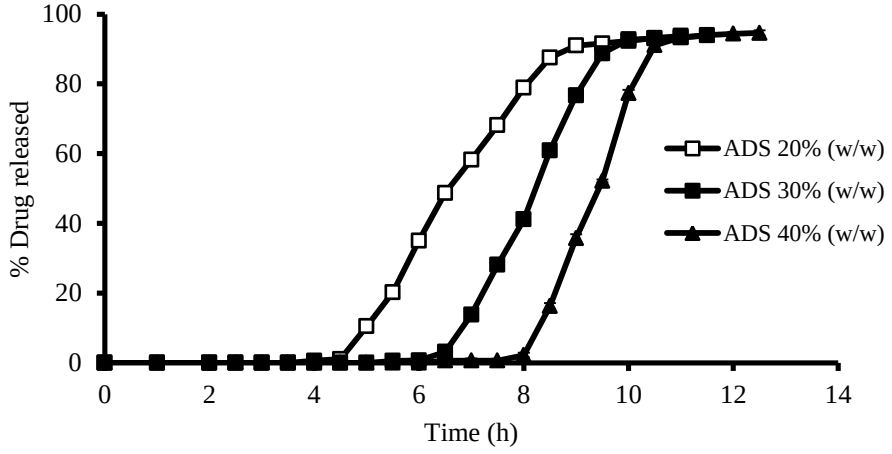

Figure 2. *Effect of swellable coating (ADS) level on drug release of pulsatile pellets with rupturable coating (EC) 5.0% (w/w) level in 0.1 N HCl.*

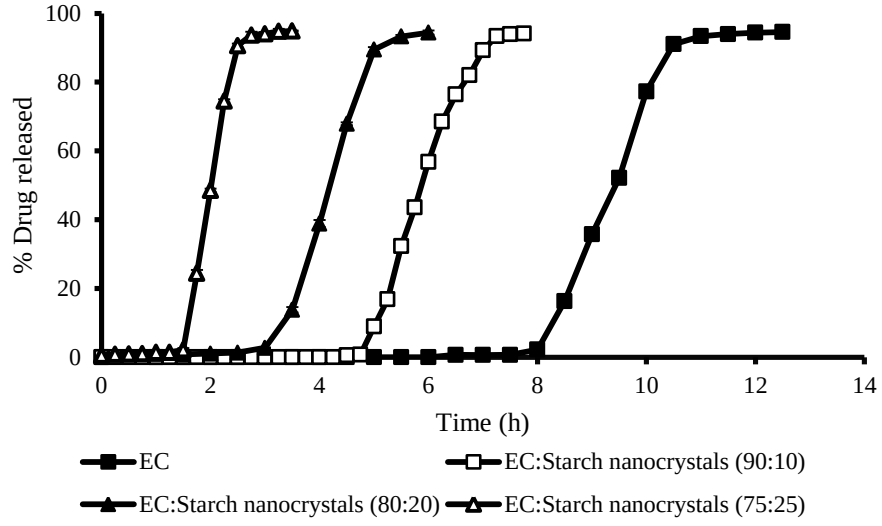

Figure 3. *Effect of amount of starch nanocrystals in rupturable coating on drug release of pulsatile pellets with 40% (w/w) swellable coating with 5.0% (w/w) rupturable coating in 0.1 N HCl.*

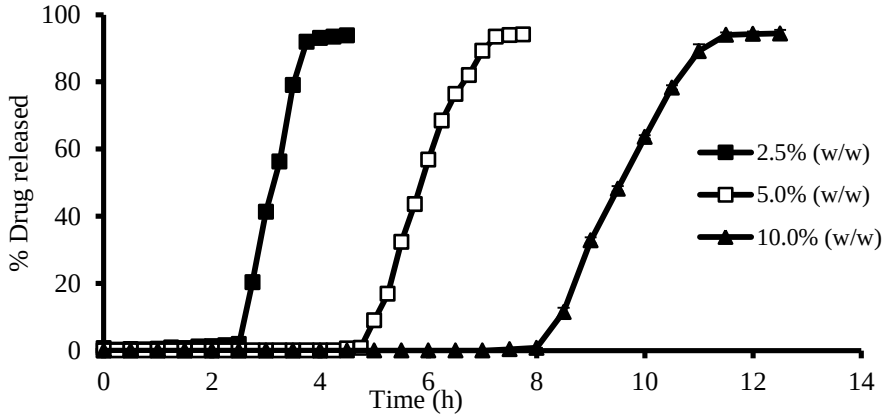

Figure 4. *Effect of rupturable coating EC: Starch nanocrystals (90:10) levels on drug release of pulsatile pellets with ADS 40% (w/w) swellable coating levels in 0.1 N HCl.*

3.1 Effect of swellable coating level on lag time and drug release

Effect of swellable coating level on lag time and drug release of the pulsatile release pellets were investigated. Increasing level of swelling agent (ADS) increased lag time and seemed to improve pulsatile release of the pellets (Figure 2). This finding was in agreement with a study of Sungthongjeen et. al (1). It could be attributed to the hardness of the pellets according to higher level of swelling layers (1). When ADS exposed to the aqueous medium, it was swelled and retarded the further water penetration in to the inner part of swelling layer and hence time was prolonged to obtain the complete swelling force. It was clearly shown that increase level of swellable layer subjected to increase the drug release

rate because of the higher swelling force thus resulted in completely rupturing and thereafter rapid drug release was obtained.

3.2 Effect of amount of cassava starch nanocrystals in rupturable layer

Effect of amount of starch nanocrystals in rupturable coating on drug release of pulsatile pellets with 40% (w/w) swellable coating ADS and rupturable coating levels 5.0% (w/w) in 0.1 N HCl is shown in Figure 3. Increased amount of starch nanocrystals in rupturable membrane further enhanced the drug release rate facilitating to ensure typical type of pulsatile release pattern. This could be attributed to the porous structure of the starch nanocrystals in rupturable layer, created by shape of starch nanocrystals. Starch nanocrystals formed three dimensional networks in the film matrix of rupturable membrane increasing the porosity and water penetration (6). Increased amount of starch nanocrystals caused to fabricate more disorganized, porous film, encouraging water penetration through the rupturable membrane.

3.3 Effect of coating level of rupturable layer

Effect of rupturable coating levels of ethylcellulose and starch nanocrystals 90:10 (w/w) on drug release of pulsatile pellets coated with different swellable coating levels in 0.1 N HCl is exhibited in Figures 4. The release profile had a typical pulsatile type at low level of rupturable coatings as, 2.5%, 5.0% (w/w). At higher coating level, 10.0% (w/w) the drug release was slow after lag time (Figure 4). Additionally, premature drug release was not observed during the lag time period hence, it confirmed that accidental crack formation did not occurred at all investigated rupturable coating levels. This might be explained due to shielded effect of drug layer by two continuous polymer layers, swellable and rupturable, respectively (5).

4. CONCLUSION

The rupturable pulsatile release pellets consisted of a drug (theophylline) layered core pellets; a swelling layer (croscarmellose sodium) and, a rupturable layer of ethylcellulose with cassava starch nanocrystals were successfully prepared. In conclusion, rapid drug release after the lag time were obtained from the pulsatile release pellet with ethylcellulose and cassava starch nanocrystals as rupturable layer, indicating the potential to use starch nanocrystals as a modifier to produce the desired pulsatile release.

ACKNOWLEDGEMENT

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Formulation of Controlled Porosity Osmotic Pump Tablets Containing Venlafaxine Hydrochloride

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Abstract

Venlafaxine hydrochloride is an antidepressant agents, it has a short biological half-life (5 h) thereby requiring twice a day dosing to maintain adequate plasma levels of drug. Controlled porosity osmotic pump (CPOP) were introduced, the CPOP consists of (a) an osmotic core that contains the active ingredient and along with an osmogent and (b) a semi-permeable membrane coating that contains a water-soluble pore-forming agent, instead of the delivery orifice created by laser drill. The aim of this study was to formulate CPOP tablets containing venlafaxine hydrochloride. In this study, the core tablets consist of microcrystalline cellulose pH 101 and osmogents (sodium chloride, mannitol, lactose) were prepared by wet granulation method. The core tablets were coated semipermeable membrane of cellulose acetate containing polyethylene glycol 400 as a pore former and plasticizer, Tween 80 as a surfactant. The coating operation was performed using a pan coating machine. The formulation variables affecting on drug release *in vitro,* coating weight, concentration of pore former and surfactant were investigated using D-optimal design. The optimal tablet formulation has been proposed. An invention product, efexor® XR extended release capsules was used as reference for study *in vitro*. It was found that drug release rate was inversely proportional to coating weight and directly proportional to the concentration of pore former and surfactant in membrane. The drug release profile from the optimized formulation was similar to that from reference drug. CPOP tablets of venlafaxine hydrochloride were successfully prepared, the drug release from the tablets was extended for 24 hours. The CPOP tablets were prepared simply by coating the core tablets with the pore forming agent which is likely to be most cost-effective than laser drilling.

Keywords: controlled porosity osmotic pump, venlafaxine hydrochloride, semipermeable membrane, cellulose acetate.

1. INTRODUCTION

Venlafaxine hydrochloride, (R/S)-1-[2-(dimethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexanol hydrochloride, is an antidepressant unrelated to tricyclic, tetracyclic, or other available antidepressant agents. It has also been used for treatment of generalized anxiety disorder and social anxiety disorder. Hydrochloride salt of venlafaxine is having high aqueous solubility (572 mg/ml) and short biological half-life (5 h), thereby requiring twice a day dosing to maintain adequate plasma levels of drug, which often leads to patient non-compliance [6], [4]. So, it is a suitable candidate for the development of one a day formulation.

Controlled porosity osmotic pump (CPOP) were introduced, the CPOP consists of (a) an osmotic core that contains the drug and along with an osmogent and (b) a semi-permeable

membrane coating that contains a water-soluble pore-forming agent, instead of the delivery orifice created by laser drill [1]. Upon contact with the aqueous fluids, the pore forming agent was dissolved resulting an in situ formation of a microporous membrane that is substantially to both water and dissolved solutes. It was reported that the rate of drug release from these systems depends on the thickness of the semi-permeable membrane, level of the pore forming agent in membrane, the drug solubility in the core tablet, and the osmotic pressure difference across membrane [2].

In the current work, the ingredients of core tablets were selected suitably. For developing coating membrane formulation: D-optimal design in Modde 8.0 software was used to design of experiments. FormRules v2.0 software was used to evaluate the effects of input variables on output variables and optimized by INForm 3.1 software. An innovation drug, efoxor XR extended release capsules was a reference for *in vitro* dissolution study.

2. MATERIALS AND METHODS

Materials

Venlafaxine chloride was bought from Cipla Ltd. Lactose, talc, polyvinyl pyrrolidon K30, Tween 80, ethanol; aceton, sodium chloride, polyethylene glycol 400, mannitol, magnesium stearate, Avicel PH 101, Hydroxy propyl methyl cellulose K4M (HPMC K4M) were gifted by Department of pharmaceutics, Hanoi University of Pharmacy. Cellulose acetate (with 40% acetyl) was bought from Daejung Chemicals & Metals CO., LTD.

Methods

2.1. Drug-excipients compatibility study FTIR

The use of FTIR technique allows pointing out the implication of the different function groups of drug and excipients by analyzing the significant changes in the shape and position of the absorbance bands. The individual samples such as pure drug (venlafaxine hydrochloride), the mixture of drug and excipients were ground and mixed thoroughly with potassium bromide and compressed into disc. The mixture was kept in the sample holder and scanned from 2000 to 400 cm^{-1} in FTIR spectrophotometer. The peak characteristics of all samples were obtained.

2.2. Preparation of core tablets

The formula of core tablets is listed in **table 1**. The core tablets were prepared by wet granulation technique. Talc and magnesium stearate were passed through the 80 mesh sieve. The drug and other excipients were passed through the 60 mesh sieve. PVP K30 was dissolved in ethanol to obtain concentration of 10 % (w/v). The drug and other excipients except talc and magnesium stearate were blended homogeneously in a mortar. The mixture was moistened with PVP K30 solution, and granulated through 18 mesh sieve. The granulation was dried at 60^0C for 15 minutes. The dried granulation was passed through 20 mesh screen to break the lumps and to get uniform size of granules, the granulation was dried continuously at 60^0C till the moisture content of granules reached 2-3%. The granules were blended with lubricants (talc and magnesium stearate). The homogeneous blend was the compressed into tablets using 10 mm diameter, concave punches. The compression force was adjusted to give tablets with approximately 6-8 $kg.cm^2$ hardness.

2.3. Coating solution

Required quantity of cellulose acetate was accurately weighted and dissolved in a beaker containing acetone using mechanical stirrer. Required quantity of PEG 400 and Tween 80

were dissolved in ethanol in other beaker and was added to mixture of cellulose acetate with stirring for 2 hours.

2.4. Coating of tablets

Core tablets were placed in a coating pan with 30 g of core tablets. The coating pan was rotated at 20 rpm and heated air was passed through the tablets. Coating process was started when temperature pan reaches to 40±5^{0}C. The coating solution was sprayed at the rate 3 ml/min. Coating was continued until desired coating weight was obtained. The coated tablets were dried at 40^{0}C for 3 hours in vacuum oven.

2.5. In-vitro drug release

Apparatus: USP I (basket type)
Medium: Water, 0,1N HCl pH 1,2; acetate buffer pH 4,5 and phosphate buffer pH 6.8.
Volume of medium: 900 ml
Sample volume: 10 ml
Replacement volume: 10 ml
The collected samples were analyzed by UV-spectrophotometer method at 235 nm using the medium as blank. The percentage drug release (%CDR) was calculated.

3. RESULTS

3.1. Compatibility study

Figure 1, the images scanned by FT-IR method for the drug and mixture of dug and difference excipients (lactose, talc, polyvinyl pyrrolidon K30, Tween 80, sodium chloride, mannitol, magnesium stearate, Avicel PH 101, Hydroxy propyl methyl cellulose K4M) show that there is no significant change in the peaks of drug-excipient mixtures in comparison to pure drug. It means that there is no incompatibility of excipients with the drug.

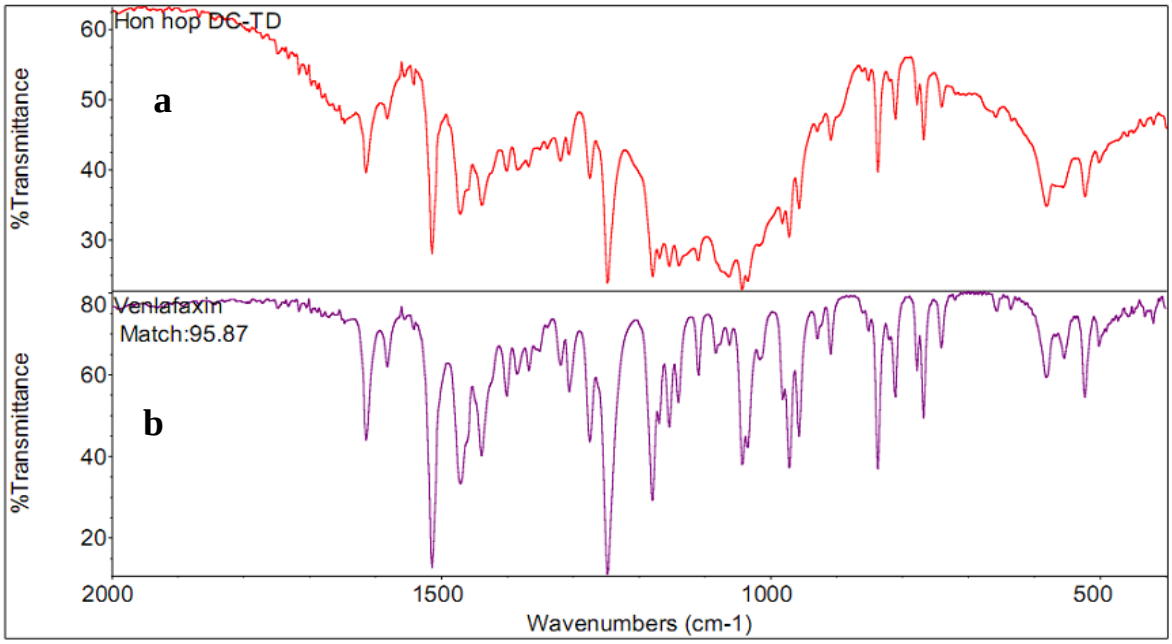


Figure 1. FTIR spectrum of mixture of the drug excipients (a), pure drug (b)

3.2. Development of the core tablet formulation

To develop formulation of core tablets, the formulas as in **table 1** were prepared and coated with coating membrane formulation containing CA 4%, PEG 400 20% and coating weight was 3%, 5%, 7%. The results of dissolution studies shown that the coating membrane did not remain intact till the end of dissolution study and ruptured after a short period for all levels of coating weight of formulas N1-N6. The coating membrane of tablets of formulas N7, N8 remains intact for 24 hours during dissolution testing. The formulas (N1-N6) containing HPLC K4M from 50 mg to 10 mg in core tablets, HPMC K4M is a swellable polymer increase hydrostatic pressure inside the pump [3] due to their swelling nature that could lead to rupture of the system. An amount 5 mg of HPMC K4M incorporated in core tablets of formula N7 is small, so that it's hydrostatic pressure inside the pump could be not high enough to break the membrane. However, the drug dissolution profile from formulas N7 and N8 with 5% of coating weight was not difference significantly as showed in **figure 2**, so formula N8 was used for further investigations.

Table 1. *Formulations of core tablets*

Ingredients	N1	N2	N3	N4	N5	N6	N7	N8
Venlafaxine.HCl (mg)	86.1	86.1	86.1	86.1	86.1	86.1	86.1	86.1
HPMC K4M (mg)	**50**	**30**	**30**	**15**	**15**	**10**	**5**	**0**
NaCl (mg)	10	10	10	10	10	10	10	10
Manitol (mg)	50	50	50	50	50	50	50	50
Lactose (mg)	75	75	75	75	75	75	75	75
Avicel PH101 (mg)	75	75	**20**	75	**20**	75	75	75
Magnesium stearate (mg)	2	2	2	2	2	2	2	2
Talc (mg)	2	2	2	2	2	2	2	2
PVP K30 (mg)	10	10	10	10	10	10	10	10

3.3. Development of coating membrane formulation

3.3.1Preliminary studies

To select the range of input variables (formulation ingredients and coating weight of the coating membrane) that influences on response variables (percentage of released venlafaxine), preliminary studies were carried out. The tablets from formula N8 was coated with different membrane formulas and coating weight as listed in **table 2**.

Table 2. *Membrane formulas with difference ingredients and coating weight*

Ingredients / Formulas	PEG 400	Tween 80	Coating weight
M1	20%	-	3%
M2	20%	-	5%
M3	20%	-	7%
M4	25%	-	5%

M5	10%	-	3%
M6	10%	5%	3%
M7	10%	3%	3%

Coating weight: The core tablets was coated with coating weight 3% (M1), 5% (M2) and 7% (M3). The results of dissolution studies shown that the drug release rate was decreased with increasing in coating weight and lag time increases with increasing in coating weight, **figure 3**. The percentage of drug dissolution from tablets with coating weight 7% (M3) is so low compared to the given requirement as shown in table 3, base on dissolution data of the reference drug. So the range of coating weight from 3% to 5 % was used for experimental design.

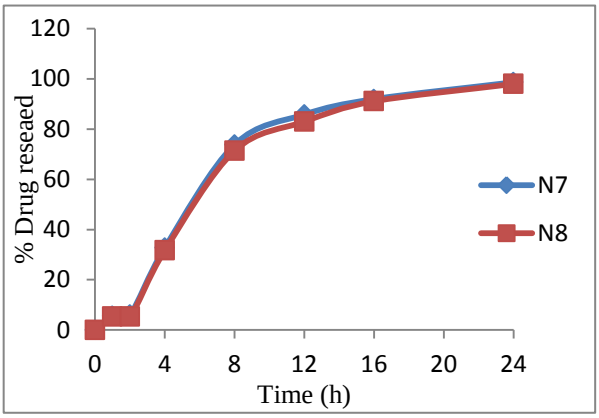


Figure 2. The dissolution profiles from formula containing 5 mg of HPMC K4M (N7) and without HPMC K4M (N8)

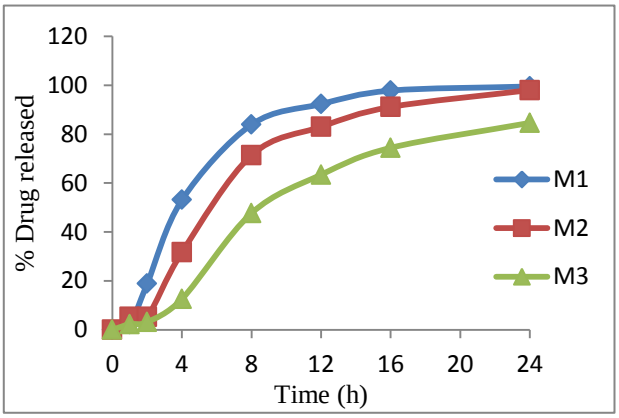


Figure 3. *Dissolution profiles of CPOP tablets with difference coating weight, M1 (3%), M2 (5%), M3 (7%)*

Coating membrane ingredients: The pore forming agents as PEG 400 was used individual or combination with Tween 80 in coating membrane formulations as shown in **table 2** to investigate their influences on the drug release rate. The results of dissolution studies showed that the drug release rate increases as ratio of PEG 400 in membrane increased as shown in **figure 4** (M1, M5). The drug release rate from formula with PEG 400 25% is high at 12 h compared to the given requirement. So, the ratio of PEG 400 in range of 10% to 20% was used for experimental design.

For formulas containing PEG 400 combination with Tween 80, ratio of Tween 80 effects clearly on the drug release rate at early stage (2 h, 4 h) but it did not effect significantly at lately hours as shown in **Figure 4** (M5, M6, M7). This effect of Tween 80 decrease lag-time that was usually observed for osmotic tablets, and it was suitable for developing the coating membrane formulation. The drug release rate from formula containing 5% of Tween 80 was high compared to the given requirement. So, the ratio of Tween 80 in the membrane is kept in range of 0% - 3% to experimental design of the coating membrane.

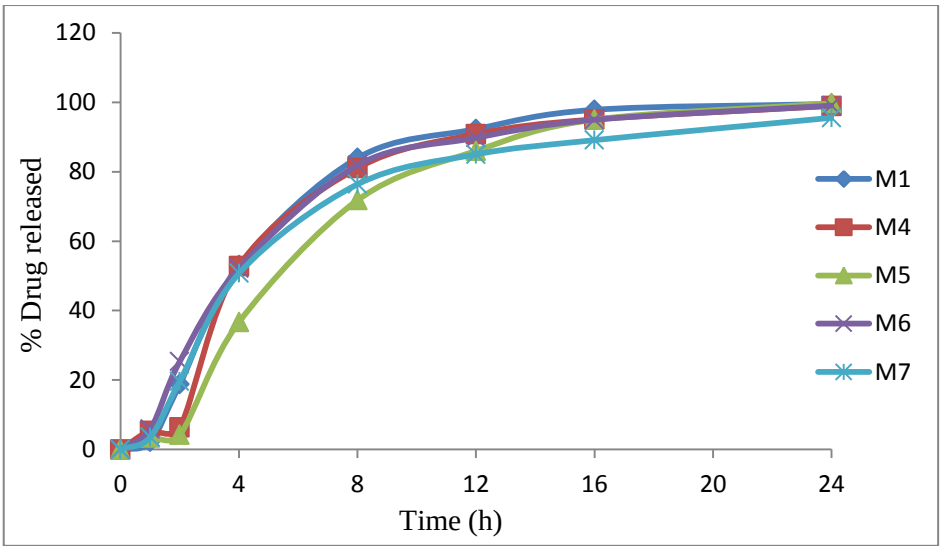


Figure 4. *Effect of PEG 400, PEG 400 combination with tween 80 on the drug release rate in preliminary study.*

3.3.2 Experimental design

D-optimal design in modde 8.0 software was used to design experimental formulas for the coating membrane and evaluation of the effects of the independent variables on dependent variables that is dissolution percentages as showed in **table 3**.

Table 3. *Independent and dependent variables*

Independent variables	**Levels**	
	Low	Height
X1= amount of PEG 400 (%)	10	20
X2= amount of Tween 80 (%)	0	3
X3=coating weight (%)	3	5
Dependent variables	Requirements	
Y_{2h}= Percent of drug dissolved at 2 h	$10 \leq Y_{2h} \leq 30\%$	
Y_{4h}= Percent of drug dissolved at 4 h	$33 \leq Y_{4h} \leq 53\ \%$	
Y_{8h}= Percent of drug dissolved at 8 h	$58 \leq Y_{8h} \leq 78\ \%$	
Y_{12h}= Percent of drug dissolved at 12 h	$68 \leq Y_{12h} \leq 88\%$	
Y_{24h}= Percent of drug dissolved at 24 h	$Y_{24h} \geq 90\ \%$	

Table 4. *Formulas and dissolution data of venlafaxine from CPOP tablets (n=6)*

Formulations	PEG 400 (%)	Tween 80 (%)	Coating weight (%)	Y_{2h}	Y_{4h}	Y_{8h}	Y_{12h}	Y_{24h}
CT1	20	0	3	18.8 ± 1.9	53.1 ± 2.0	83.9 ± 1.4	92.3 ± 2.3	99.5 ± 1.9
CT2	10	3	3	19.6 ± 0.5	50.7 ± 1.4	76.4 ± 1.0	85.0 ± 0.8	95.5 ± 0.7
CT3	20	3	3	39.3 ± 0.8	75.6 ± 1.7	86.6 ± 2.2	95.7 ± 2.4	100.8 ± 2.7
CT4	10	0	5	4.7 ± 1.8	15.5 ± 4.0	58.8 ± 3.1	73.1 ± 1.1	90.9 ± 1.9

CT5	20	0	5	5.3 ± 0.6	31.7 ± 4.4	71.4 ± 4.9	83.0 ± 3.6	98.0 ± 1.3
CT6	10	3	5	4.2 ± 0.6	30.0 ± 1.9	61.2 ± 0.4	77.2 ± 0.3	93.8 ± 1.2
CT7	20	3	5	5.8 ± 0.1	41.5 ± 4.8	72.6 ± 4.4	85.0 ± 5.1	95.3 ± 1.1
CT8	10	0	3.7	10.4 ± 2.7	42.8 ± 0.9	75.8 ± 1.4	84.2 ± 1.6	94.4 ± 1.4
CT9	10	1	3	11.5 3.4	47.0 ± 2.4	76.6 2.4	84.5 ± 1.5	93.7 ± 1.5
CT10	13.3	0	3	22.1 ± 1.2	51.2 ± 1.8	75.7 ± 2.9	86.6 ± 0.9	99.0 ± 1.1
CT11	16.7	0	3	18.2 ± 2.4	52.3 ± 2.3	79.7 ± 1.8	88.3 ± 2.2	98.8 ± 1.0
CT12	20	1.5	4	7.9 ± 0.5	49.1 ± 0.9	79.8 ± 0.1	87.7 ± 0.8	97.5 ± 0.4
CT13	15	3	4	17.8 ± 2.8	58.6 ± 1.8	83.0 ± 2.4	91.2 ± 2.9	100.2 ± 1.4
CT14	15	1.5	5	4.7 ± 1.5	37.3 ± 3.2	73.2 ± 0.7	85.6 ± 0.3	97.7 ± 0.5
CT15	15	1.5	4	9.8 ± 2.1	40.5 ± 0.9	72.2 ± 1.0	83.7 ± 2.3	96.0 ± 1.1
CT16	15	1.5	4	11.7 ± 3.4	42.1 ± 1.9	74.3 ± 1.3	87.6 ± 2.4	98.1 ± 1.2
CT17	15	1.5	4	8.7 ± 2.9	38.6 ± 0.9	75.8 ± 0.8	84.2 ± 2.2	94.4 ± 2.0

Effects of independent variables on dependent variables:

Base on the results of dissolution of 17 experimental formulations in **table 4**, analyzing ANOVA statistical data and evaluating the effect of the input variables on out put variables was analyzed by FormRules v2.0 software. R^2-values and effect of the input variables on out put variables shown in **table 5** and **table 6**.

Table 5. *R^2-values*

Parameter	Y_2 (2 h)	Y_4 (4 h)	Y_8 (8 h)	Y_{12} (12 h)	Y_{24} (24 h)
R^2	97,8	98,8	98,1	97,6	93,5

Table 6. *The effects of independent variables of dependent variables*

Parameters	Y_2 (2 h) (%)	Y_4 (4 h) (%)	Y_8 (8 h) (%)	Y_{12} (12 h) (%)	Y_{24} (24 h) (%)
X1	+	+	+	+	+
X2	+	+	+	+	-
X3	+	+	+	+	+

Where: + Effect, - Not effect

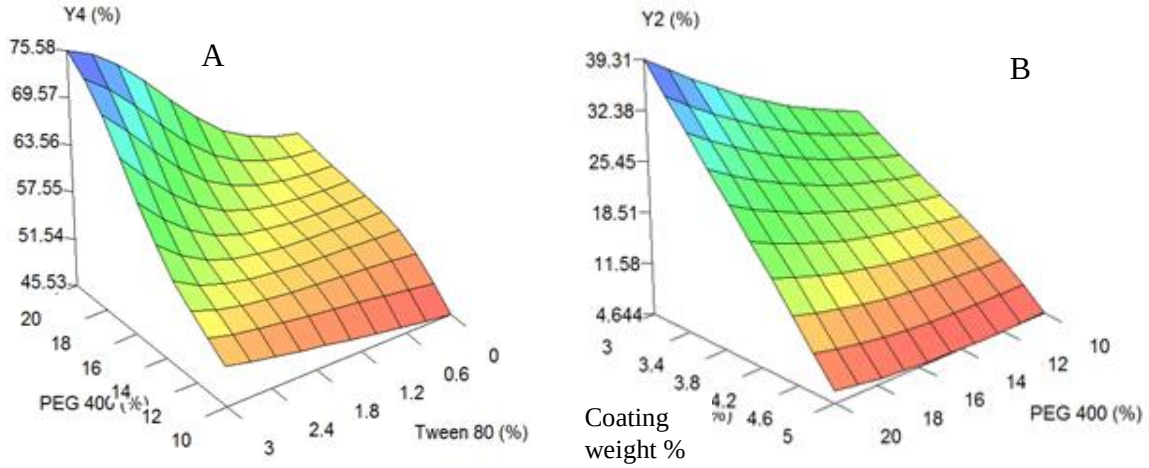


Figure 5. *Response surface plot (A) showing effects of PEG 400 and Tween 80 on drug release at 4h, and (B) showing effects of PEG 400 and coating weight on drug release at 2h.*

3.3.3 Optimization of coating membrane

Base on the data from the experimental results in **table 4** and requirement of dissolution for dependent variables (Y_2, Y_4, Y_8, Y_{12}, Y_{24}) as presented in **table 3**, INForm 3.1 software was used for optimizing the coating membrane. The optimized parameters of coating membrane were presented in **table 7**.

Table 7. *The optimized coating membrane formulation*

Parameters	Ratio
PEG 400	12,2%
Tween 80	1,2%
Coating weight	3,8%

3.3.4 Optimal tablet formulation

The CPOP tablets containing a core tablets of N8 with optimized coating membrane as showed in **table 7** was proposed as the optimal CPOP tablets. Dissolution profile of venlafaxine from the optimal CPOP tablets was similar to that from invention tablets (exfexor) as shown in **figure 6**, and f2-value was 64.2.

Figure 6. Dissolution profile of reference (efexor XR) and optimal CPOP tablets in water, (n=12)

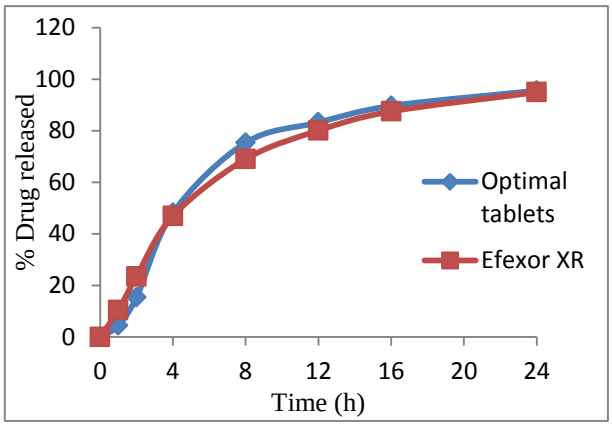
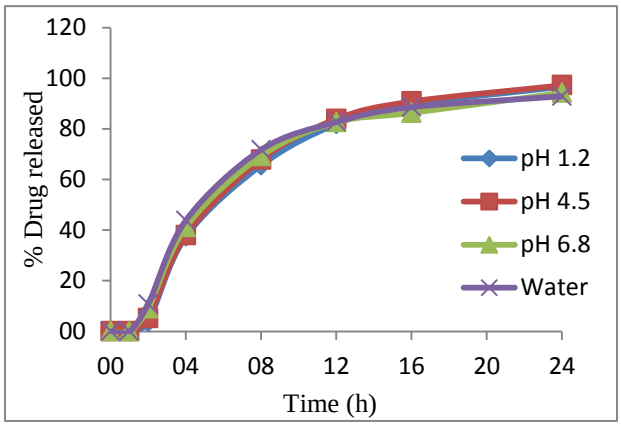


Figure 7. *Effect of pH of dissolution on the drug release rate, (n=6)*



3.4. Effect of pH on the drug release rate

The dissolution profile of the drug from optimal CPOP tablets in water and different pH media was shown in **figure 7**. The drug release rate in different media was almost similar. The pH of dissolution media has not significant effect on the drug release. So, the drug release from the osmotic pump tablet was independent from pH.

3.5. SEM micrographs

Figure 8 compares SEM micrographs of the optimized coating membrane before and after the dissolution studies. Before contact with the dissolution medium, SEM micrographs revealed that the membrane has a rough surface and no pores were observed (Figure 8a). After 24 hours of dissolution, SEM micrographs revealed pores formed, possibly, due to the dissolution of PEG 400, a pore-forming agent, upon contact of the tablets with dissolution medium (Figure 8b). Base on observation it could be suggested that release of the drug from CPOP tablets passes through the following steps: dissolution of fore forming agents upon contact with the dissolution medium, penetration of the tablet by the dissolution medium through the formed pores, dissolution the drug particles within the tablet and release of the drug through the pores.

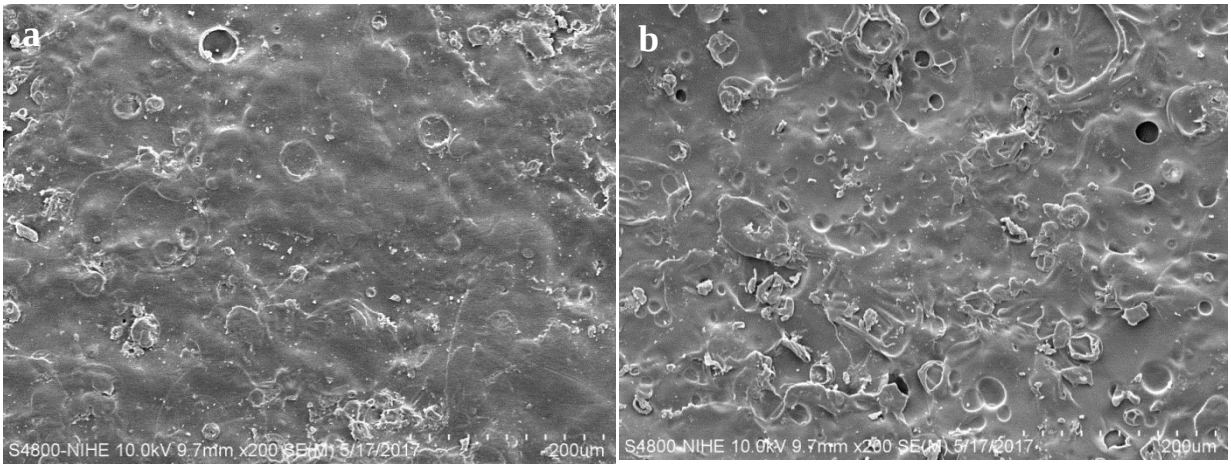


Figure 8. *SEM micrographs of optimized coating membrane, **(a)** taken before dissolution study and **(b)** taken after dissolution study for 24 h*

4. DISCUSSION

The results in **table 5** and **table 6** shown that, the R^2-value at all time points was higher than 80, so there was good regression between input variables (coating membrane formulation and coating weight) and out put variable (percentage of the drug release). All input variables (X1= PEG 400, X2 = Tween, X3 =Coating weight) affect the out put variables at all time points except a time point 24 h, Tween does not affect on.

Base on analyzing response surface as in the **figure 5 (B),** the drug release rate decreased as coating weight increases, the effects of PEG 400 on the drug release rate from coating weight 3% was higher than that at 5% **figure 5 (A)**. In a previous study, *Shokri et al.* [7] proved that thickening the semipermeable membrane can decrease the rate of water penetration through the membrane resulting in a decrease in the drug release rate. It can be

seen from equation (1), that release rate from osmotic system is inversely proportional to membrane thickness.

$$dM/dt = (A/h)*k*\Pi*C \qquad (1)$$

Where, *dM/dt* is drug delivery rate, *A* an *h* are the membrane area and thickness, respectively. *C* is the soluble fraction of the drug, *Π* is the osmotic pressure of the system and *k* is the equation constant [7].

PEG 400 had a direct effect on the drug release rate, the higher the PEG 400 concentration, the faster the drug release rate, specially at the time after 4 h. Similar results were reported by *Ahmed Abd-Elbary et al [1]*. This behavior could be related to the hydrophilic nature of PEG 400. After coming into contact with the aqueous environment, the higher PEG 400 levels would leach out the membrane easily, resulting in more porous structure and faster drug release rate.

The results of dissolution test in table 4 and figure 4 showed that the drug release rate from formulas incorporated Tween 80 was faster at early hours. In previous study [7], an initial lag-time of 1 h is necessary to moisten the device and penetration of water into the core. This time may be reduced by the addition of a surface-active agent to the coating material. Surfactants are useful when added to wall forming material also. They produce an integral composite that is useful for making the wall of device operative [5]. The surfactant act by regulating the surface energy of material to improve their blending into the composite and maintain its integrity in environment of use during the dug release period [5].

5. CONCLUSION

Extended release tablets of venlafaxine HCl were developed based on CPOP technology, the drug release from the tablets was extended for 24 hours. The drug release rate was inversely proportional to coating weight of membrane and directly proportional to the concentration of pore former. The surfactant in membrane improves the drug release rate at early time. The drug release profile from the optimized formulation was similar to that from reference drug (efexor XR). The CPOP tablets were prepared simply by coating the core tablets with the a pore forming agent which is likely to be most cost-effective than laser drilling.

ACKOWLEDGEMENT

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Development and Evaluation of *In Situ* Gel of Chlorhexidine Gluconate

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Abstract

Periodontal diseases affect gingival in a reversible or irreversible way. Chlorhexidine gluconate (chx) – the gold standard of anti-plaque agent – found to be beneficial for patients. However, chx conventional topical form present some disadvantages either rapid washout or low penetration in the gingival pocket. This study aimed at development and evaluation of a chx *in situ gel* to overcome the shortcoming of conventional formulations. *In situ* gels containing chx were prepared by using cold method. The effect of each excipient on the properties of formulation was investigated in order to choose appropriate concentration. The *in situ* gel was evaluated for appearance, gelation temperature (GT), spreadability, pH, drug content and *in vitro* release. This formulation was put into a preliminary stability study. As the result, each excipient affects the *in situ* gel properties by different rules. Benzalkonium chloride and PEG 400 make increased GT with increasing the ratio. Meanwhile, poloxamer 407, HPMC, glycerin and propylene glycol reduce GT when increasing the ratio. The final formulation containing 0.5% chx; 16.25% poloxamer 407; 0.2% HPMC E15; 1% PEG 400 and 0.2% benzalkonium chloride was selected. This clear gel has, GT around 31.7 °C, a spreadibility value comparable to the reference gel, pH 6.0, chx dosage at 98.71% and gel released *in vitro* 93.70% of chx in 5 hours which fitted by a Korsmeyer – Peppas kinetic. The *in situ* gel was found stable under long term storage condition for 4 weeks. The *in situ* gel of chx developed exhibited potential in treatment of periodontal disease.

Keywords: *In situ* gel, chlorhexidine, periodontal, gelation temperature, poloxamer

1. INTRODUCTION

Periodontitis is a group of oral diseases affecting many people around the world. It is defined as an inflammation and progressive destruction of the tooth – supporting structures (gums, periodontal ligaments, alveolar bone, and dental cementum)[1], that if left untreated, can lead to tooth loss[2, 3]. The disease is often silent, meaning symptoms may not appear until an advanced stage of the disease. In the advanced phase of the disease, the supporting collagen of the periodontium is disintegrated, alveolar bone begins to resorb, gingival epithelium migrates along the tooth surface forming a "periodontal pocket"[4] and this is irreversible. These treatment aims to remove dental plaque and plaque-retentive factors. Mechanical therapy such as scaling and root planing has difficulty in contacting with pathogenic bacteria in the deep periodontal pockets, periodontal tissues or dentinal tubules. Among the chemical therapy, the use of an antiseptic is shown to be more advantageous than an antimicrobial agent, being effective while avoiding of development of bacterial resistance and other side-effects.

Over the past, chlorhexidine (chx) was used as an antimicrobial agent. In chemotherapy of periodontitis, chx is the gold standard of antiplaque and antigingivitis agent[5]. It is a component of various preparations such as mouth rinsing, gels, varnishes, PerioChip,

film,… However, conventional topical form of chx present some disadvantages either rapid washout (mouthwash solution or spray) or low penetration in the gingival pocket (gel).

The development of *in situ* gel for controlled drug delivery systems has received considerable attention over the past few years. In particular, it shows some promise in periodontal therapy[2, 4, 6]. *In situ gel* is polymeric system, which is liquid aqueous solutions before administration, but undergoes sol – gel transition under physiological conditions. Gelation occurs because of different factors like pH, temperature, ionic, solvent and ultra violet irradiation[7, 8].

The present work is based on the temperature dependent system and polymer which was used in formulation is Poloxamer or Pluronic. Poloxamers are the series of commercially available non-ionic triblock copolymers of poly (oxyethylene) and poly (oxypropylene). They are available in various grades differing in molecular weights and the PEO/PPO ration. Aqueous solution of poloxamer 407 has ability to change from a low viscosity solution to a gel.
This study aimed at development and evaluation of a chx *in situ* gel to overcome the shortcoming of conventional formulations for treatment of periodontal diseases.

2. MATERIALS AND METHODS

Chx gluconat was purchased from Evonik Company (Germany). Poloxamer 407 was obtained from BASF (Germany). Hydroxyl propyl methyl cellulose (HPMC E15) were supplied from Colorcon (Singapore). All other chemicals and solvents were analytical grade and from Bach Khoa chemical Company (Viet Nam).). Reference gel (gel X) from France.

2.1 Preformulation studies

2.1.1 Selection of polymers

In order to select the appropriate polymer and its ratio, different gel with poloxamer or a combination of poloxamer and HPMC E15 was investigated in **Table 1**. The periodontal *in situ* gel was prepared by cold method. Formulations were evaluated for appearance and GT. Each experience was performed in triplicate.

Table 1. *Formulation of in situ gel for selection of polymer*

	Concentration (% w/w)							
	Chx	**Poloxamer**	**HPMC**	**Benzalkonium chloride**	**PEG 400**	**PG**	**Glycerol**	**Ditilled water**
F1	0.5	16						q.s 100
F2	0.5	16		0.02				q.s 100
F3	0.5	15		0.02				q.s 100
F4	0.5	15.5		0.02				q.s 100
F5	0.5	16		0.02				q.s 100
F6	0.5	16.25		0.02				q.s 100
F7	0.5	16.5		0.02				q.s 100
F8	0.5	17		0.02				q.s 100
F9	0.5	17.5		0.02				q.s 100
F10	0.5	18		0.02				q.s 100

F11	0.5	16	0.1					q.s 100
F12	0.5	16	0.5					q.s 100

2.1.2. Effects of types and concentrations of excipients on GT

In order to evaluate the effect of type and concentration of excipients on GT of an *in situ* gel, different formulations were prepared as shown in **Table 2**. The *in situ* gel was prepared by cold method and the gelling temperature of was determined. Each formulation was evaluated for gelling temperature in order to select optimum concentration of poloxamer and other excipients for a reversible *in situ* gel.

Table 2. *Formulation of in situ gel for investigation of the effect of excipients on GT*

	Concentration (% w/w)							
	Chx	**Poloxamer**	**HPMC**	**Benzalkonium chloride**	**PEG 400**	**PG**	**Glycerin**	**Ditilled water**
F13	0.5	16		0.01				q.s 100
F14	0.5	16			1			q.s 100
F15	0.5	16			2.5			q.s 100
F16	0.5	16			5			q.s 100
F17	0.5	16			7.5			q.s 100
F18	0.5	16			10			q.s 100
F19	0.5	16				1		q.s 100
F20	0.5	16				2.5		q.s 100
F21	0.5	16				5		q.s 100
F22	0.5	16				7.5		q.s 100
F23	0.5	16				10		q.s 100
F24	0.5	16					1	q.s 100
F25	0.5	16					2.5	q.s 100
F26	0.5	16					5	q.s 100
F27	0.5	16					7.5	q.s 100
F28	0.5	16					10	q.s 100

2.2 Formulation of in situ gel

The *in situ* gels of chx gluconate were prepared using cold method. The required amount of Poloxamer was dispersed in distilled water with continuous stirring for 1 hour at room temperature. Then, the beaker was stored at 4ºC for around 12 hours until the entire polymer was completely dissolved. Accurately weighed quantity of HPMC was dispersed slowly in another beaker with continuous stirring in water until a uniform and clear solution was obtained. HPMC solution was added to Poloxamer solution with continuous stirring. Accurately weighed quantity of chx was added to above mixture with continuous stirring until thoroughly mixed. Benzalkonium chloride solution was added as preservative in all solutions. Other excipients were added above mixture with continuous stirring until thoroughly mixed. Weight of the gel was adjusted using distilled water, with continuous

stirring until a uniform and clear solution was obtained stirred and stored in ambient temperature for around 12 hours.

Table 3. *Formulation of in situ gel*

Ingredients	Concentration (% w/w)				
	F29	**F30**	**F31**	**F32**	**F33**
Chlorhexidine gluconate	0.5	0.5	0.5	0.5	0.5
Poloxamer 407	16	16	16	16.25	16.25
HPMC E15	0.3	0.4	0.5	0.2	0.3
PEG 400	1	1	1	1	1
Benzalkonium chloride	0.02	0.02	0.02	0.02	0.02
Ditilled water	q.s 100	q.s 100	q.s 100	q.s 100	q.s 100

2.3 Characterization of in situ gel

2.3.1 Appearance

The clarity of formulated solutions was determined by visual inspection under black and white background, with the shaking and overturning action[9].

2.3.2 Gelation temperature (GT)

5g of the sample and a magnetic bar were put into a transparent vial that was placed in a low temperature water bath (15 °C). A thermometer with accuracy of 0.5 °C was immersed in the water bath. The solution was heated at the rate of 1 °C/min with the continuous stirring at a constant speed. The temperature was determined as GT, at which the magnetic bar stopped moving completely due to gelation. Each sample was measured at least in triplicate[7, 10].

2.3.3 Spreadability

Two glass slides (15 cm × 15 cm) were used in this test. The studied formulation was heated at 37 °C to completely gelation. Accurately weighed quantity of X or gel under study (1 g) was placed on the first slide. The gel was then sandwiched between this slide and another glass slide. The standardized 0.5 kg weight was placed on the top of two slides. After 1 minute, the spreadability of the gel formulation was determined, by measuring diameter *d* of gel (mm). Each sample was measured at least in triplicate. The spreadability was calculated by using the formula: $S = d^2 * \pi / 4$ (mm^2).

2.3.4 pH

pH is checked by using a calibrated digital pH meter. The formulation temperature was maintained at 20°C.

2.3.5 Drug content

The drug content was determined by taking accurately 0.5 g of gel in 100 ml volumetric flask. Around 20 ml of distilled water was added to the volumetric flask and shaken well until the gel was totally dispersed to give a uniform and clear solution. Final volume was adjusted to the mark with distilled water. The drug concentrations were determined by measuring the absorbance at 254 nm using UV Visible spectrophotometer. Each sample was measured at least in triplicate.

2.3.6 In vitro drug release studies

In vitro release studies were performed using non-membrane model. Accurately weighed quantity of each formulation (100 mg) was placed on an eppendorf. The eppendorf was put in a horizontally shaker and thermostatically controlled water bath. The temperature of the medium and shaking rate were maintained at 37°C ± 0.5 °C and 80 rpm, respectively. The eppendorf was then filled with 1 ml phosphate buffer pH 6.8. The total volume of the receptor solution (1 ml) were withdrawn at intervals (5, 10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 minutes) and replaced with the fresh medium previously heated to 37±1°C. The withdrawn samples are diluted in a volumetric flask with distilled water to specific volume and analyze by UV spectrophotometer at 254 nm. The drug content is calculated using the equation generated from standard calibration curve then the % cumulative drug release (%CDR) is calculated. The data obtained is further subjected to curve fitting for drug release data. Each sample was measured at least in six times[2, 3].
Drug release kinetics was studied by curve fitting method to different kinetic models of zero order, first order, Higuchi models or Korsmeyer-Peppas equation[11]:

2.3.7 Preliminary stability study

The preliminary stability studies were carried out by storing the samples at ambient temperature (under 30°C) for 1 month. The sample is analyzed at every week for appearance, pH, drug content and gelation temperature.

3. RESULTS AND DISCUSSION

3.1 Preformulation studies

3.1.1 Selection of polymers

In this study, poloxamer 407 was used as a thermosensitive *in situ* gel forming polymer. Beside, some different polymers such as HPMC E15 were used the combination of polymers to improve the gelling properties. The formulation which has just poloxamer 407 form thermoreversible gel at at least 16% as shown in **Table 4.** The combination of poloxamer and 0.1% HPMC E15 gelled at 31.17 ± 0.29 °C.

3.1.2 Effects of types and concentrations of excipients on GT

The most important characterization of an thermoreversible *in situ* gel is GT. So, any excipient which was used in this work should consider its effects on this criterion. The GT result in **Table 4** show Benzalkonium chloride and PEG 400 make increased GT with increasing the ratio. In detail, F2 with 0.02 % Benzalkonium chloride has the highest GT (32.83 °C) in F1, F2, F13. The GT of F14 to F15 was found to increase with increasing ratio of PEG 400.
Meanwhile, poloxamer 407, HPMC, glycerine and propylene glycol reduce GT when increasing the ratio. The GT of F5-F6 was found to be within the range of 30°C-37°C. This was nearer to the body temperature. If GT is higher than the body temperature, it will remain liquid at the application site and if it is lower than the environment temperature, it causes difficulty in storage and administering. The concentrate of Poloxamer in F5 and F6 are 16% and 16.25% in turn. So, 16-16.25% w/v concentration of Poloxamer 407 was used for further studies. The table indicate increasing the concentration of HPMC from 0.1% to 0.5% (F11- F12) also decreases the GT of *in situ* gel.

Table 4. *GT of formulation of in situ gel*

Formulation	GT (°C)	Formulation	GT (°C)
F1	32.50 ± 0.50	F15	34.33 ± 0.58
F2	32.83 ± 0.29	F16	-
F3	-	F17	-
F4	-	F18	-
F5	32.67 ± 0.58	F19	31.00 ± 0.00
F6	30.33 ± 0.58	F20	30.33 ± 0.58
F7	29.17 ± 0.29	F21	30.00 ± 0.00
F8	27.33 ± 0.58	F22	28.67 ± 0.58
F9	27.17 ± 0.29	F23	26.33 ± 0.58
F10	26.50 ± 0.50	F24	29.33 ± 0.58
F11	31.17 ± 0.29	F25	29.00 ± 0.00
F12	29.33 ± 0.29	F26	28.67 ± 0.58
F13	31.33 ± 0.58	F27	26.67 ± 1.15
F14	33.33 ± 0.58	F28	25.67 ± 0.58

- No gelation

3.2 Characterization of in situ gel

3.2.1 Appearance

The formulations (F29–F33) were prepared by using various concentrations of Poloxamer along with HPMC in different ratios. All the formulations prepared were clear without any turbidity and suspended particles or impurities.

3.2.2 Gelation temperature (GT)

The Gelation temperature of F29-F33 was found to be within the range of 30°C-37°C. Among that, F32 was nearest to the body temperature.

Table 5. *Characterization of in situ gel*

	Appearance	GT (°C)	Spreadability (mm^2)	pH	Drug content (%)
F29	Clear	31.00 ± 0.00	3883 ± 64	6.155	98.69 ± 1.07
F30	Clear	30.33 ± 0.58	3489 ± 60	5.989	98.37 ± 0.98
F31	Clear	29.17 ± 0.29	3215 ± 0	6.168	98.24 ± 0.37
F32	Clear	31.17 ± 0.29	3420 ± 104	6.000	98.87 ± 0.64
F33	Clear	29.50 ± 0.50	3249 ± 58	6.037	98.56 ± 0.55
X			3419 ± 0		

3.2.3 Spreadability

Spreadability of formulation decrease as concentration of polymer increases. The spreadability of F32 was found to be the same as the reference gel.

3.2.4 pH

The pH of F29-F33 was found to be average of 6.0 which is near the gingival crevicular fluid pH. This indicates formulations can be used without any irritation in the oral cavity.

3.2.5 Drug content

Drug content was one of a significant requirement for any type of dosage form. Amount of the drug present in the formulation should not deviate beyond certain specified limits from

the labeled amount. All formulations were found to having drug content in the range of 98.24-98.87%, representing homogenous drug distribution throughout *in situ* gel.

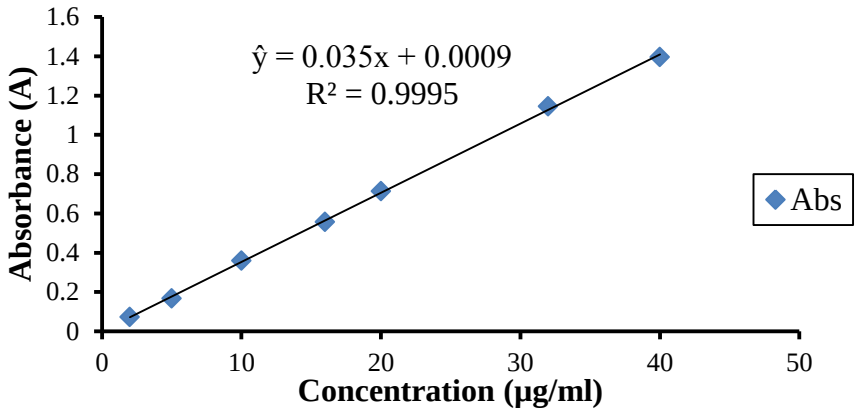

Fig. 1. *Standard calibration curve of chx by UV spectrophotometer at 254 nm*

3.2.6 In vitro drug release studies

Release profiles of the drug from the prepared chx *in situ* gel were exhibited in **Fig. 2**. It could be seen that the release of drug from these *in situ* gels was characterized by an initial phase of high release (initial 5 min) and as the gelation proceeded, the remaining drug was released at a slower rate (second phase) and reached the plateau after 5 hours.

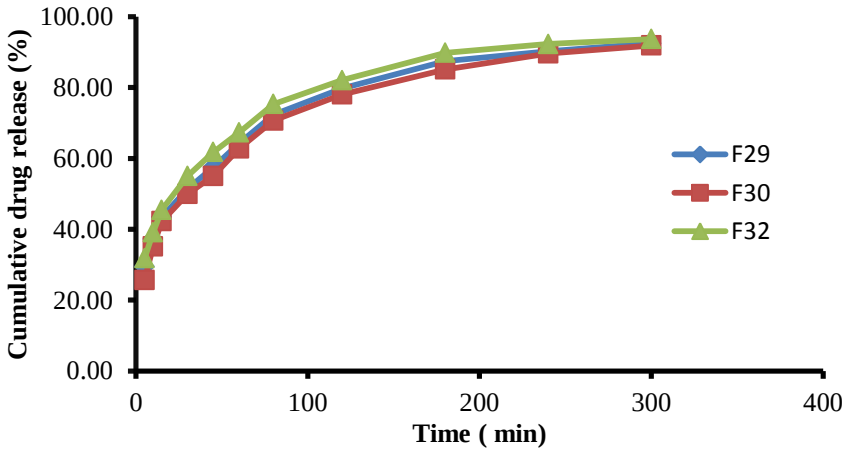

Fig. 2. *In vitro drug release profile of chx from formulation F29, F30, F32*

In vitro release kinetics of chx from *in situ* gel during the studied period was best fitted to Korsmeyer-Peppas equation with the r^2 value of 0.989. Therefore, the release of chx from this formulation was the log cumulative percentage of drug release versus log time.

Table 6. *Correlation coefficient (R^2 value) of chx release from in situ gels by different kinetic models obtained from curve fitting method*

Formulation	Zero order	First order	Higuchi	Korsmeyer-Peppas
F32	0.803	0.965	0.941	0.989

3.2.7 Preliminary stability study

From the result it was concluded that no significant change was observed in the formulation. So the *in situ* gel was stable under long term storage condition for 4 weeks.

Table 7. *Result of preliminary stability study for optimized formulation*

Time (week)	Appearance	pH	GT (°C)	Drug content (%)
0	Clear	5.893	31.17 ± 0.29	98.87 ± 0.64
1	Clear	5.884	31.17 ± 0.29	98.81 ± 0.72
2	Clear	5.895	31.33 ± 0.29	98.49 ± 0.41
3	Clear	5.889	31.50 ± 0.50	98.70 ± 0.20
4	Clear	5.921	31.50 ± 0.50	98.61 ± 0.35

4. CONCLUSIONS

Chx – a broad-spectrum antiseptic used for antiplaque and antigingivitis activity, was successfully formulated in thermoresponsive *in situ* gel using 16.25% Poloxamer 407 as the gelling agent and 0.2% HPMC E15 as a copolymer. The formulation was liquid in storage conditions and transferred to the gel form upon physiologic conditions (37ºC). The *in situ* gel afforded sustained drug delivery over an 5 hour period. The developed formulation could be a premise solution for treatment of periodontal diseases.

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Purification of Acarbose from *Actinoplanes* Sp. Mutant Strains and Evaluation of the Hypoglycaemic Effect in Mice

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Abstract

Acarbose is an alpha-glucosidase inhibitor, which reduces the postprandial glucose level in human blood by delaying the digestion of carbohydrates in the human small intestine, and therefore widely used to treat non-insulin-dependent diabetes mellitus. Acarbose can be obtained by fermentation of *Actinoplanes* sp. mutant strains. Isolating acarbose from the fermentation broth, therefore, is an essential process to prepare highly pure acarbose which is suitable for pharmaceutical use. Our study aims to isolate and purify acarbose from *Actinoplanes* sp. KCTC 9161 – L14 mutant strains and evaluate the hypoglycemic effects of purified acarbose in mice. Acarbose from the fermentation of *Actinoplanes* sp. KCTC 9161 – L14 mutant strains was absorbed by activated charcoal. The adsorbed material was eluted with ethanol solution at different concentrations from 0% to 20%. The presence and purity of acarbose in the eluted solutions were determined using TLC and HPLC. An oral glucose tolerance test, a fasting blood glucose test and HbA1c measurement wereconducted to evaluate the hypoglycemic effect of purified acarbose in mice. Our results showed that the purified acarbose is 98% pure, reaching the concentration of arcabose in the eluted solution at 191.5 g/L, indicating that active charcoal column chromatography can be used to purify acarbose from fermentation broth of *Actinoplanes* sp. mutant strains. The purified acarbose treatment alonesignificantly lowered postprandial blood glucose levels. Obtained purified acarbose plus insulin treatmentstabilized the fasting blood glucose and HbA1c levels in mice. There were no significant differences in posprandial hypoglycemic effect and the effect on fasting plasma glucose and HbA1c between groupstreated with purified acarbose and Glucobay (an anti-diabetic product from Bayer) alone or in the combination with insulin therapy. In conclusion, acarbose was isolated and purified from the fermentation of *Actinoplanes* sp. KCTC 9161 – L14 mutant strains and the purified acarbose can be used as be a pharmaceutical raw material.

***Keywords*:**Acarbose, active charcoal, Actinoplanes sp., α-glucosidase inhibitor, purification.

1.INTRODUCTION

Foods containing carbohydrates such as rice, white flour, generally provide a large percentage of the total daily caloric intake. The rate of carbohydrate digestion and the subsequent absorption are key factors affecting the postprandial hyperglycemia, which

have been proved to be linked with microvascular and macrovascular complications in type 2 diabetics [1].

Acarbose, a pseudo-tetrasaccharide, can blunt postprandial hyperglycaemia by competitively inhibiting α-glucosidase, which is responsible for breaking down carbohydrates to smaller sugar particles like glucose, therefore delaying the digestion and absorption of carbohydrates in the human small intestine. Acarbose has been demonstrated to provide euglycaemia and reduce cardiovascular events. Additionally, acarbose has minimal risk for hypoglycaemia and, when titrated slowly, is generally well tolerated. These advantages make acarbose receive preferred status even when compared to other oral glucose lowering drugs and widely used for the treatment of diabetes.

So far the commercial production of acarboseisvia microbial fermentation, exclusively with strains from the genera of *Actinoplanes*. Isolating and purifying acarbose from the fermentation broth, therefore, are essential processes to prepare highly pure acarbose which is suitable for pharmaceutical use. The strains selection and fermentation optimization for acarbose production from *Actinoplanes* sp. have been conducted in our previous studies [2,3]. In this researchaims to isolate and purify acarbose from *Actinoplanes* sp. KCTC 9161 – L14 mutant strains and evaluate the hypoglycemic effects of purified acarbose in mice.

2.MATERIALS AND METHODS

2.1. Microorganism

Actinoplanes sp. KCTC 9161 – L14 mutant strain was supplied by the Department of Enzyme Biotechnology, Institute of Biotechnology, Vietnam Academy of Science and Technology

2.2. Chemicals

Acarbose (Sigma Chemical Co-USA), p-nitrophenyl-α-D-glucopyranoside, α-glucosidase, peptone (Biobasic INC, Canada), activated charcoal, amberlite XAD 1600T, amberlite IRA67 (Sigma Chemical Co-USA), N-butanol, ethanol, methanol, ethyl acetat, acid fomic, $CaCl_2$, $CaCO_3$, KCl, $FeSO_4.7H_2O$, K_2HPO_4, $K_2HPO_4.3H_2O$, $MgSO_4$(AR Co-China), streptozocin (Sigma), insulin (Lantus), acarbose (Glucobay -Bayer), diabetic test strips (Accu-chek active – Roche Diagnostics), HbA1C assay kit (Trinity Biotech), soluble starch (Sigma).

2.3. Equipments

Silica gel TLC plates (Merck), chromatography column (4x50 cm) (Korea), blood glucose meter (Accu-chek active – Roche Diagnostics), HbA1c analyzer (Trinity Biotech).

2.3. Methods

2.3.1. Purification of acarbose from the fermentation of Actinoplanes sp. mutant strains.

After the 144 hours of fermentation, fermentation broth was centrifuged at 12000rpm, 4°C for 20 minutes. The supernatant was cleaned by ethanol (1 sample: 4 ethanol (v/v)) and concentrated for 10-20 times in *vacuo*. The concentrated supernatant was adsorbed by activated carbon in the condition of pH 2-3, slightly stirring for 1 hour and then keeping at 4° C for 12 hours. The mixture was washed with 200 ml water 3 to 5 times and was was stuffed into a chromatography column. The column was eluted with a gradient elution of

ethanol from 0% to 20% . The flow-through contained acarbose whose presence was checked by using TLC (Merck silica gel 60 F254, 0.25 mm thick with a solvent system 94% A and 6% B (A contain ethyl acetate: methanol =1:1 and B contain H_2O: acid formic 5:2), then by the color burst slightly acid (10% H_2SO_4in ethanol) at 121^oC in 15 minutes. The acarbose containing fractions were collected, concentrated (90%) and were purified by activated carbon once again,followingthe process described above, except that the elution solutions were5% and 15%. EtOH. The acarbose containing fractions were collected, concentrated (90%) and applied to amberlite XAD 1600T cation-exchange column which was then eluted with water/acetone mixture (9/1-v/v). The eluted fractions were appliedto amberlite IRA67 anion-exchange column which was afterwards eluted with water. The acarbose containing fractions were evaporated for the purity analysis (by HPLC method) and for the molecular structure determination (ESI-MS, ^{13}C NMR, ^{1}H NMR methods).

2.3.2. Hypoglycemic effect evaluation

Animals and treatment:

Six week-old male Swiss mice (*Mus musculus*) were housed in groups of 10-15 mice with a 12-/12-hour light (10 p.m.)/dark (10 a.m.) cycle and free access to water and food at least 7 days before the experiments. On day one, mice were intra-peritoneally injected with a single dose of 150mg/kg Streptozocin (STZ). On day four, fasting blood glucose test (FGT) was performed and only mice with FGT equal to or higher than 200 mg/dL (11mmol/L) were selected for the next experiments. The animal experimental procedure was approved by the local animal ethical committee of theHanoi University of Pharmacy.

Oral glucose tolerance test (OGTT):

Diabetic mice were divided into 4 groups: nondiabetic mice (ND), diabetic mice (D), diabetic mice treated with purified acarbose (oral routh, 50mg/kg/day) (AcaVN) or Glucobay (oral route, 50mg/kg/day) (Glucobay). The OGTT (2g/kg of soluble starch) was performed in 6 h-fasted mice after 1 week of treatment. Blood from the tip of the tail vein was sampled at 30 minutes before and at 0, 30 min, 60, 90, 120 min after oral starch soluble administration for plasma glucose analysis, using a glucose meter (Roche Diagnostic). The area under the curve (AUC) between 0 and 60 minutes was calculated for each group of mice.

Fasting blood glucose test (FGT) and HbA1c measurement:

Diabetic mice were divided into 5 groups: nondiabetic mice (ND), diabetic mice (D), mice treated with Insulin (subcutaneous route, 1 UI/kg/day),mice treated with Insulin (1 UI/kg/day) plus purified acarbose (50mg/kg/day) (AcaVN+Ins)or plus Glucobay (oral route, 50mg/kg/day) (Glucobay+Ins).Fasting blood glucose and HbA1c levels were determined in 6 h-fasted mice after 4 weeks of treatment. Blood glucose concentrations were monitored from the tip of the tail vein with a glucose meter (Roche Diagnostic). Blood HbA1c levels weremeasured using boronate affinity HPLC (Trinity Biotech)[4].

3. RESULTS AND DISCUSSION

3.1. Purification of acarbose

We firstly used activated charcoal for the purification of acarbose from the fermentation broth. After two passages through activated charcoal column, purified acarbose was detected in 13 fractions, from fraction 11 to 23 (Figure 1)

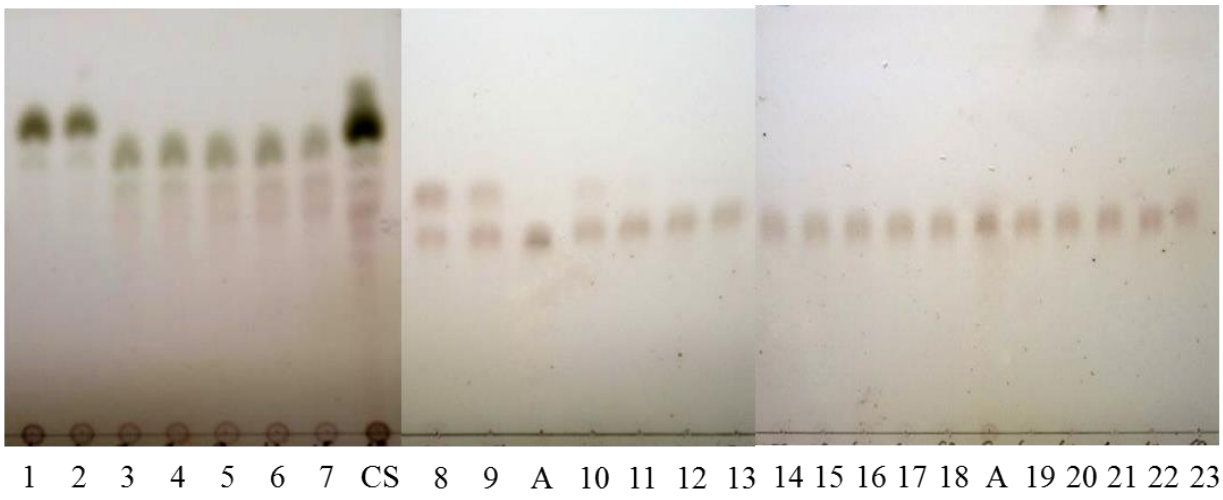

Figure 1. ***TLC profiles of eluted fractions from activated charcoal column:*** *1-23: eluted fractions; CS: concentrated supernatant; A: Acarbose (Sigma).*

Activated charcoal can be used for the purification of fructooligosaccharides, monosaccharides, and disaccharides [5]. As a pseudo-tetrasaccharide, acarbose can be also purified from the microbial fermentation broth. The adsorption behavior of each sugar depends on the micropores diameter distribution of the pore channel and the strength of the interaction between sugars and activated charcoal correlates to molecular weight and size of the adsorbed molecules. While small molecules pass through the channel, large ones are kept inside the matrix. Therefore, in the case of sugar, activated charcoal absorbs more oligosaccharides than smaller saccharides (e.g. glucose or maltose). In addition, the major portion of the carbon surface of the charcoal is hydrophobic, therefore, oligosaccharides which contain more – CH group than smaller saccharides are better adsorbed by active charcoal. The step of activated charcoal column, however, provides acarbose having a content of only 72% by weight. (Figure 2).

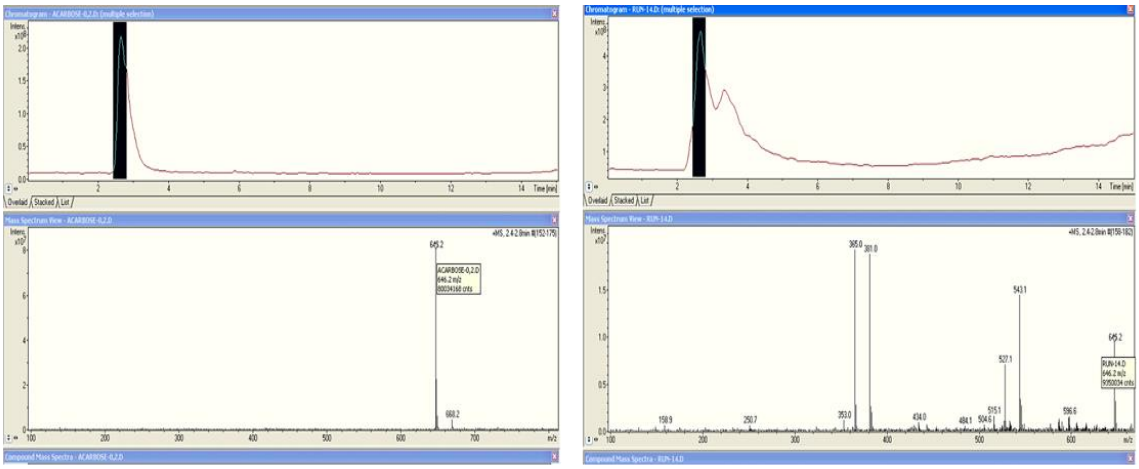

Figure 2. *HPLC profiles of acarbose purchased from Sigma (A) and the acarbose purified from fermentation broth using activated charcoal column (B)*

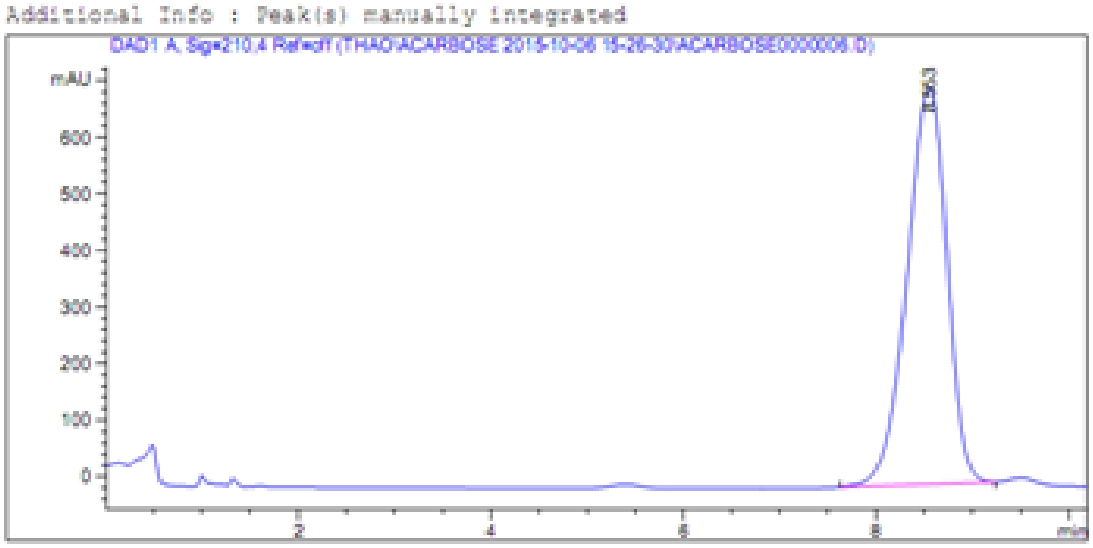

Figure 3. *HPLC profiles of the final purified acarbose*

In order to improve the purity of acarbose, acarbose-containing solutionfrom activated charcoal column were concentrated (90%) and applied to amberlite XAD 1600T cation-exchange column before to amberlite IRA67 anion-exchange column. The results showed that the obtained acarbose is 98% pure (by HPLC method), reaching the concentration of arcabose in the eluted solution at 191.5 g/L, at a yield of more than 40% (Figure 3).Finally, the molecular structure of purified product was determined by ESI-MS, ^{13}C NMR, ^{1}H NMR**.** Dataindicated that molecular formula of purified product was $C_{25}H_{43}NO_{18}$ and its structure of acarbose (Figure 4)indicated that the chemical formula of purified product was $C_{25}H_{43}NO_{18}$and confirmed its molecular structure as acarbose (Figure 4).From our results, acarbose obtained by our method is highly pure (98%) and can be used as pharmaceutical raw material.

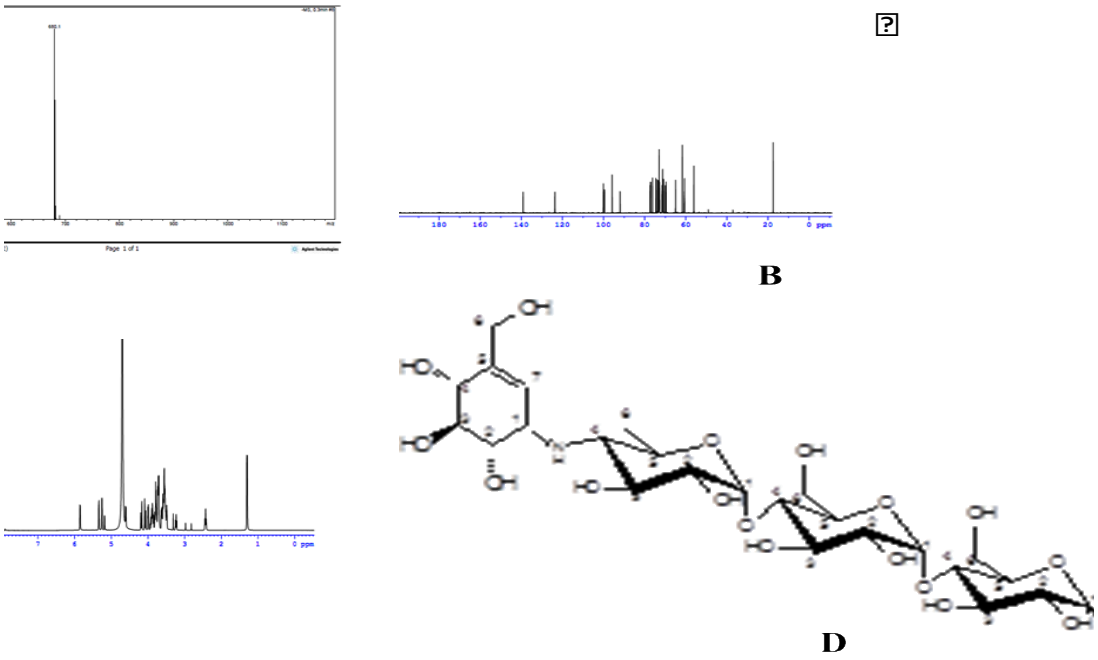

Figure 4. *ESI-MS (A), 1H NMR (B), 13C NMR (C) profiles and molecular structure of the final purified product (D).*

3.2. Hypoglycemic effect evaluation of purified acarbose

3.2.1. Effect of purified acarbose on postprandial blood glucose levels in mice

The therapeutic activity of acarbose is to provide euglycaemia, partially by blunting postprandial spikes of glucose. To evaluate the effect of purified acarbose on postprandial blood glucose levels, an OGTT was conducted in diabetic mice after 1 week of treatment of acarbose. The results showed that both acarbose groups significantly reduced postprandial spikes of glucose in diabetic mice at each tested time and there was no significant difference in the effect between two groups (Figure 5).

Postprandial hyperglycaemia is known to be linked with microvascular and macrovascular complications in type 2 diabetics [1]. Moreover, glycaemic excursions coupled with elevated free fatty acids have a pro-oxidant effect on β cells of the pancreas, leading to β-cell exhaustion, which can precipitate overt clinical diabetes [6]. Hence, the effect of acarbose to blunt postprandial hyperglycaemia may be useful for reducing cardiovascular disease risk and possibly aids in preventing type 2 diabetes or delay its onset.

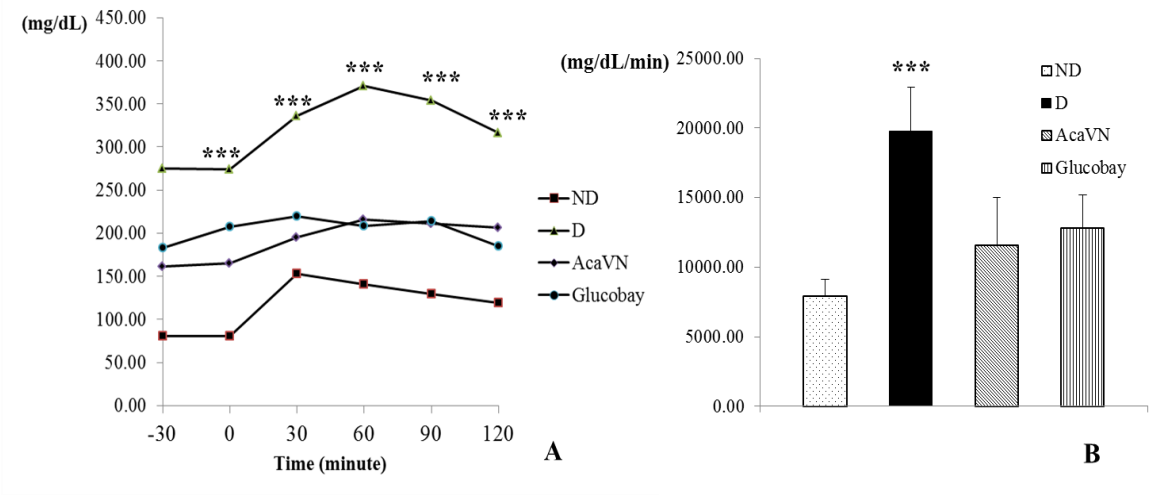

Figure 5. *Acarbose significantly reduced postprandial spikes of glucose in diabetic mice*

A) Glycemic profiles (mg/dL) of nondiabetic mice (ND), diabetic mice (D), diabetic mice treated with purified acarbose (AcaVN) or with Glucobay (Glucobay) for one week and

B) The area under the curve for glucose (AUC); Data are presented as mean ± S.E.M. *** statistically different between groups when $p<0.001$, as analyzed Statistical significance of differences was calculated by one-way analysis of variance (ANOVA) followed by Tukey test, ***$p<0.001$.

3.2.2. Effect of purified acarbose on fasting blood glucose and HbA1c levels

In order to evaluate the anti-diabetic effect of adding purified acarbose in diabetic mice already on insulin therapy we compared the effects of acarbose (Glucobay or purified acabose) plus insulin therapy with insulin therapy aloneon fasting blood glucose and HbA1c levels. After four-week treatment, both therapies significantly reduce fasting blood glucose level in diabetic mice ($p<0.001$). However a significant decrease in HbA1c level was observed only in acarbose plus insulin groups (6,44% in AcVNn+Ins group and 6,75% in Glucobay+Ins group) but not in insulin therapy group. These results indicated that acarbose significantly reduced HbA1c level but did not affect fasting glycemia in diabetic mice.There was no significant difference in fasting blood glucose and HbA1c levels between two acarbose groups (Figure 6).

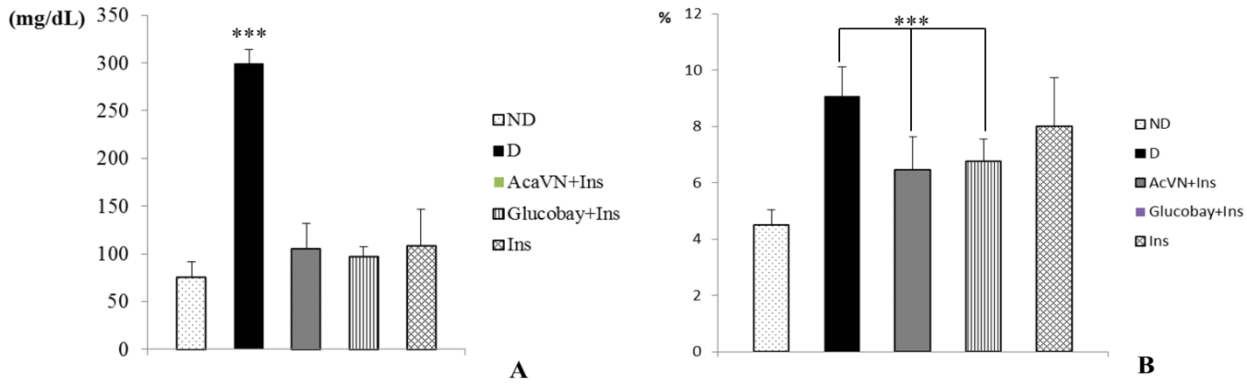

Figure 6. *Acarbose significantly reduced HbA1c in diabetic mice*

A) Fasting glycemia (mg/dL) of nondiabetic mice (ND), diabetic mice (D), diabetic mice treated with the Insulin plus the purified acarbose (AcaVN+Ins) or plus Glucobay (Glucobay+Ins) for four weeks.
B) % HbA1c; Data are presented as mean ± S.E.M. Statistical significance of differences was calculated by one-way analysis of variance (ANOVA) followed by Tukey test, ***p<0.001.

HighHbA1clevels are shown to be strongly associated with an increased risk of cardiovascular disease (CVD) in people with and without diabetes [7]. Our results showed that purified acarbose plus insulin therapy significantly reduced HbA1c level in diabetic mice to the level of 6,44% while insulin therapy alone group still showed high HbA1c level (8%) which was not significantly lower than that in normal group. It is unknown about how Acarbose induces a reduction in HbA1c level. Unlikethe glycaemic effect of acarbose on postprandial glucose, which varies with dose, acarbose decreases HbA1c level in a dose-independent manner. No matter how it is induced, the effect of acarbose on HbA1c level indicates its potential benefitin reducing risk microvascular complications in heart, kidney and eyes in patients with type-2 diabetes [8].

4. CONCLUSIONS

In conclusion, we isolated and purified acarbose from the fermentation of *Actinoplanes* sp. mutant strains. Our process can be used for preparing highly pure acarbose (98%) as medicine for the treatment of diabetes.

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Evaluation of Essential Fatty Acids, Vitamin E and EPA Content in *Nanochodopsisocculata*Microalgae Powders and Its Bioactivities

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Abstract

Introduction: Microalgae are important sources of unsaturated fatty acids that the body cannot synthesize itself, as well as non-substituted amino acids. Most notable are eicosapentaenoic fatty acids (EPA) (20: 5n-3, EPA). EPA is a well establish substance that plays an important role in enhancing the resistance and prevention of diseases for humans and animals due to its antioxidant and anti-inflammatory activities.

Objectives: The aim of our study was to detect essential fatty acids, vintamin E and EPA content in *Nanochodopsisocculata* microalgae cultured in closed bioreactor systems with different conditions in spring and summer. Its antioxidant and acute toxicity activities were also determined.

Materials and method: *Nanochodopsisocculata* microalgae was cultured in closed bioreactor systems in August and September (summer) with average temperature 32^0C; pH 7,8 - 8,0; light 15900 lux and in March and April (spring) with average temperature $23,2^0$C; pH 7,7 - 8,4; light 11800 lux. The culture biomass were collected and dried to powder.The microalgae powders were analyzed the content of fatty acid components include EPA and vitamin E by gas chromatography.The acute toxicity effect was tested in white mice and the antioxidant activities was determined with DPPH by optical measurement method.

Results: Results showed that the content of unsaturated fatty acids including omega 3, omega 6, pentadecanoic acid, palmitoleic acid, palmitic acid, oleic aids, stearic acid, vitamin E and EPA were quite high on both tested samples. The microalgae showed strong antioxidant effect. No acute toxic effect were observed. In addition, No mice died after treatment with a maximum dose of microalgae (40 mg/kg).

Conclusion: *Nanochodopsisocculata* microalgae grown in sealed bioreactors can be used as a source of organic essential fatty acids, vitamins E and EPA for pharmaceuticals and natural products.

Keywords:*Nanochodopsisocculata*, fatty acids, EPA, antioxidant

1. INTRODUCTION

The microalgae generally contain an abundance of nutrients that necessary for the body. In addition to basic nutrients such as protein, lipid, and carbondydrate, these are high density constituents of the total dry weight of algae (protein 6-52%, lipids 7-23%, carbohydrates 5-23%), algae also contains many types of unsaturated fatty acids in the omega 3 group (n-3HUFA), especially the eicosahexaenoic group (22: 5n-3, EPA), arachidonic acid (20: 4n-6, AA), docosapentaenoic acid (22: 6n- 3, DHA) and docopentaenoic (DPA C22: 5n-6)[2]
Microalgae are important sources of unsaturated fatty acids that the body can not synthesize itself, as well as non-substituted amino acids. Of particular importance are linoleic acid, gamma-linolenic (GLA), arachidonic ... that help prevent heart disease, atherosclerosis, and obesity.

N. oculata contains a wide variety of polyunsaturated fatty acids (PUFAs). Most notable are the eicosapentaenoic fatty acids EPA (20: 5n-3, EPA), which makes up about 24.5 - 40% total fatty acids. EPA is a proven substance that plays an important role in enhancing the resistance and prevention of diseases for humans and animals. EPA helps the body fight against asthenia, prevents fatty liver disease, cardiovascular disease, atherosclerosis, and reduces inflammation[5,8],reducing blood clots[7]. EPA is an important ingredient in many foods and medicines that support brain development in children and protection against declining memories in older people. And in particular, according to many authors, such as [1,9], EPA is an anti-tumor and is considered as a supportive element of cancer prevention and treatment.

N. oculata contains higher vitamin E (α-tocopherol) than *E. gracilis, Dunaliellasalina*and*Tetraselmissuecica,* which are used as vitamin E sources [10].Tocopherols are fat-soluble mixtures that have the potential for antioxidant activity [3]. In the body, α-tocopherol helps prevent pathological conditions caused by light to the eyes and skin [4], preventing degenerative disorders, cardiovascular diseases and cancer[6]. Because animal cells cannot synthesize these substances themselves[3], they must be obtained from the sources of photomicrographs.

With all of the above advantages, *Nannochloropsisoculata* is recommended as a healthy nutritional supplement for humans.
The aim of our study was to detect essential fatty acids, vitamin E and EPA content in *Nanochodopsisocculata* microalgae cultured in closed bioreactor systems with different conditions in spring and summer. Its antioxidant and acute toxicity activities were also determined.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1.Nanochodopsisocculatamicroalgaepowder

Nanochodopsisocculata microalgae was cultured in closed bioreactor systems in August and September (summer) with average temperature 32^0C; pH 7,8 - 8,0; light 15900 luxnamed PBR1 and in March and April (spring) with average temperature $23,2^0$C; pH 7,7 - 8,4; light 11800 luxnamed PBR4. The culture biomass were collected and dried to powder.

2.1.2. Animals

Swiss mice, male and female, both healthy, weighing 18-22 g, are provided by the National Institute of Hygiene and Epidemiology. Animals were kept stable under laboratory conditions for at least 5 days prior to the study, fed with standard foods, free-drink.

2.2. Methods

2.2.1. Determination of total lipid content in algae powder

- Determination of total lipid content in algae by ether according to Soxhlet extraction method . After evaporating the ether extracion, weigh the remaining fat and calculate the lipid content in 100g of sample.
- Determination of fatty acids component by gas chromatography. Fatty acids in foods were analyzed according to the "Standard Methods of Analysis for Hygiene Chemists", Pharmaceutical Society of Japan Edition (1995). The lipid obtained from the total lipid determination procedure is used to determine the fatty acid composition. The analysis process is summarized as follows:

Gas chromatography system is GC-17A of Shimadzu (Japan), with flame ionization detector (FID). The Supelco Wax-10 chromatography column (30m x 0.25mm x 0.25μm, Supelco, US). The conditions are as follows:

- Program temperature: 50^0C(2 mins) $\rightarrow$ increase to 120^0C (20/mins) $\rightarrow$ 240^0C (40/mins) and maintain for 35 minutes. Total analysis time is 65 minutes / sample.
- Temperature detector: 260^0C; Pump port temperature: 250^0C
- N2 make-up pressure:: 70kPa; Pressure H_2 = 60 kPa; Compressed air pressure = 50kPa
- Sample pump volume: 1 μl; Split rate 1:10

2.2.2. Quantifying vitamin E, beta-carotene

Beta-carotene and vitamin E in the sample is extracted with petroleum ether andare quantified by high performance liquid chromatography (HPLC) method.The β-carotene and vitamin E standard used in this study was purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). Other reagents were of analytical or HPLC grade.

The concentration of vitamin E and beta-carotenewas determined by HPLC (Hewlett Packard HP1100, FLD). HPLC analysis was performed using C18 (5 μ, 4.6 mm × 250 mm) at 30^0C. The used mobile phase was methanol/hexane/0.1 M ammonium acetate in H2O (500:25:25, by vol.) and the flow rate was 1 ml/min. Total runtime for each standard and sample was 40 minutes. The injection volume was 20 μL. Detection was performed

using a fluorescence detector at excitation 295 nm and emission 330. The results were calculated by the following formula:

χ ppm = Vs/Ws x As/ Astd x VIstd/ VIs x Cstd

Where:

Vs = Volume of sample

Ws = Weight of sample

As = Area of sample

Astd = Area of standard

VIstd = Volume of standard injected

Vis = Volume of sample injected

Cstd = Concentration of standard

2.2.3. Acute toxicity

Perform acute toxicity tests with classical model and PRB4 powder was used for this test. Micesdon't feed for 4 hours before taking the test, normal drinking water. After 4 hours, the mice were divided into 6 batchs (8 mice per each batch), using the test composition. The volume of the test composition administered to mice was 0.2 ml / 10g.

- Reference batchs: taking solvent used for mixing (NaCMC 0,5%)
- Sample batchs: taking algae powder with the highest dose possible for oral administration (40 mg / kg in 0.5% NaCMC)

Mice are fed back after 2 hours, normal drinking water. Continuous follow-up within the first 4 hours and 7 days after the test.

Tracking Targets:

- General condition of the mice: natural activity, posture, color (nose, ears, tail), feathers, faeces, urine
- Mortality rate within 72 hours.
- When mice died, surgery to observe the organs of the organs. If necessary, add microscope to determine the cause.

2.2.4. Antioxidant Activity Test

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) and L-(+)-Ascorbic acid were obtained from Sigma-Aldrich ChemieGmbH,Germany; others chemicals were obtained from Merck Chemicals, Germany.The percentage of antioxidant activity (AO%) of samples was assessed by DPPH free radical assay. 0.5 ml of the sample at the investigated concentrations were reacted with 0.5 ml of DPPH 0.8 mM mixed in MeOH. The mixture is left to stand at room temperature for 30 minutes. Optical measurement at wavelength λ = 515 nm. Ascorbic acid is used as positive control andPRB4 powder was used as sample for this test. The inhibition ratio AO(%) was obtained from the following equation: AO (%) = (Ac – As)/ Ac x 100 with Ac is absorbance of control and As is absorbance of sample. Each measurement was repeated 3 times and do the average.

3. RESULTS AND DISCUSSION

3.1. Lipid content and composition

Table 1. *Lipid composition and other ingredients in Nanochrodopsis occulata*

Compositions	Unit	PBR1	PBR4
Lipid	g/100g	14.5	22.33
Omega 3	g/100g	5.577	10.497
Omega 6	g/100g	1.376	1.450
Pentadecanoic acid	g/100g	0.049	0.623
Palmitoleic acid	g/100g	4.167	3.543
Palmitic acid	g/100g	6.542	2.400
Oleic aid	g/100g	0.895	0.511
Stearic acid	g/100g	0.235	0.125
Vitamin E	mg/100g	57.85	58.60
EPA	g/100g	2.787	2.892
Carotenoid	mg/100g	180.67	178.44

3.2. Antioxidant activity

Table 2. *In vitro antioxidant activity of algae in experimental DPPH.*

Concentration µg/ml	OD Average	AO%
Reference (Vitamin C 50µg/ml)	0.775	
2000	0.277	64.23
1500	0.370	52.23
1000	0.477	38.41
500	0.605	21.89
250	0.686	11.43
100	0.721	6.97
50	0.767	0.97

In the DPPH test, DPPH is a purple free radical because of unmatched N electrons, but after reacting with the oxygen atom of the radical extinguisher it is reduced to purple. The antioxidant activity of the algal powder was shown by DPPH color reduction, which led to a decrease in the absorption at 515 nm wavelength. Table 2 shows that the antioxidant activity of PRB4 algae powder was the highest at 2000 µg / ml in the DPPH test and the antioxidant activity at the concentration of 50 µg/mlsimilar to vitamin C of 50 µg/ml. This result showed that PRB4 algae powder has strong antioxidant activity.

3.3.Acute toxicity results:

The results of acute toxicity experimental are shown in tables 3.1 and 3.2

Table 3.1. *Number of dead mice within 72 hours*

Batch	**Samples / dosages**	**n**	**Use volume / 10g mouse**	**Number of dead mice within 72 hours**
Reference	-	8	0,2ml/per times	0
Sample	Algae powder 40 mg / kg	8	0,2 ml /per times	0

Table 3.2. *Describe the condition of mice in batches in 7 days*

Batchs	**Samples / dosages**	The first 4 hours	72 hours	7 days
Reference	-	Normal activity, normal urine, good reflexes with stimulation	Normal activity, normal urine, smooth feather, well reflected with stimulation	Normal activity and eating, normal urine, smooth feathers
Sample	Algae powder 40 mg / kg	Normal activity, mild diarrhea, normal urine, reflex well with stimulation	Normal activity, normal urine, smooth feather, well reflected with stimulation	Normal activity and eating, normal urine, smooth feathers

The results showed that the PBR4 algae powder did not show any toxicity when tested with the highest possible feed dose (40 mg / kg).

5. CONCLUSIONS

The content of unsaturated fatty acids including omega 3, omega 6, pentadecanoic acid, palmitoleic acid, palmitic acid, oleic aids, stearic acid, vitamin E and EPA were quite high on *Nanochodopsisocculata* microalgae was cultured in closed bioreactor systems. The microalgae showed a strong antioxidant effect and safety because acute toxic effect were observed. In addition, no mice died after treatment with a maximum dose of microalgae (40 mg/kg). Thus *Nanochodopsisocculata* microalgae grown in sealed bioreactors can be used as a source of organic essential fatty acids, vitamins E and EPA for pharmaceuticals and natural products.

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Determination of Pb, Cd and Cr Contamination on Sea Water, *Tegillarcanodifera* and *Pernaviridis* from The Kenjeran Beach, Surabaya with Inductively Coupled Plasma Spectrometry

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AbstraResearch has been done to measure Pb, Cd, and Cr on *Tegillarca nodifera*and *Perna viridis* and sea water from The Kenjeran Beach Surabaya during September until November 2016. *Tegillarca nodifera*is a species of ark clam known as the blood cockle or blood clam, while *Pernaviridis*is an economically important mussel, known as green mussel. They are found in the coastal water and catch by fisherman of Kenjeran Beach Surabaya. It is one of the favored foods by people to consume. Furthermore they can be used as bioindicator of environment pollution, which is biomonitor to indicate pollution caused by heavy metals, organochlorides and petroleum products due to its filter feeder capability, and settling on a substrat. The sampling is using unrestricted random sampling technique. The shellfish are destructed by wet destruction and analized with Inductively Coupled Plasma Spectrometry Fison ARL 3410+. The results show that sea water contains Pb 9,6806 - 10,0278 ppm, Cd 0,1422 - 0,1522 ppm, Cr 0,7470 - 0,8675 ppm; the *Tegillarca nodifera* contains Pb 4,2870-4,6574 ppm and Cd 0,2083 - 0,2515 ppm; and the *Pernaviridis* contains Pb 4,4259 - 5,2593 ppm and Cr 0,4659 - 0,7470 ppm. The Pb, Cd and Cr content exceed the limit that is defined in Ministerial Decree of Environment,Republic of Indonesia No. 51 of 2004, on seawater quality standards, that is Pb in the amount of 0,008 mg/L, Cd in 0,001 mg/L, and Cr in 0,005 mg/L.

Keywords: Pollution, Pb, Cd, Cr, *Tegillarca nodifera*, *Pernaviridis*, sea water, Kenjeran

1. INTRODUCTION

Recently development of Indonesia growing rapidly, especially in big cities as Surabaya. This is followed by the increasing number of industries. Industrialization has an impact which is positive or negative. One of the impacts of industrialization is environment pollution from the waste water.

The increasing number of industries will lead to an increas in the amount of waste, i.e solid waste, waste water and emission. There is another type of waste called heavy metals that are categorized as hazardous waste. On 2005, Ririn *et al.*,reported that biota from Kenjeran Beach contains Cd, Cr, Cu dan Pb at high level. Biota that were studiedare*Anadara antiquota*, *Corbula faba*, *Meretrix meretrix*. As bioindicator, they can be used as reference that other biota (especially those consumed by humans) also exposed to heavy metals, so it will endanger human health.[1] Heavy metals from seawater and marine sediments are

known to be accumulated by many species of marine invertebrates such as oysters, mussels, clams and shells.[2]The body burdens of trace metals in most bivalves have been used to identify and map areas with exceedingly high levels of trace metals and organic pollutants, hence they can be used as biomonitors for aquatic environment.[3-5]

The presence of heavy metals does not only degrade the water quality, but it will cause accumulation in the sedimentand bioaccumulation occurs in the biota that live in these water.[6] The accumulation process of heavy metals in bivalves bodycalled bioaccumulation or atau biomagnification. This process is related to the characteristics of the bivalves, that is *sessile* (immobile) and a biota that is *depositefeeder*. One type of shellfishis blood cockle or blood clam.[6]Shellfish can be used as a good indicator in monitoring an environmental pollution caused by its sedentary nature in a particular habitat. If within the shells contained high levels of metal and exceed the normal limit that has been determined can be used as an indicator of the occurrence of pollution in the environment.[7]

Blood clam (*Tegillarca nodifera*)dangreen mussel (*Perna viridis*)arethe most frequent seawater obtained by fishermen at Kenjeran Beach Surabaya. Blood clam (*Tegillarca nodifera*)dangreen mussel (*Perna viridis*)are a mineral-rich, vitamin and low-fat marine products that are highly favored by people to be processed into different types of food. Therefore, many restaurants serve blood clams and green mussels as their menu. The high public interest on the consumption of blood clams and the existence of previous research as bioindicator and the discovery of heavy metals in small biota in Kenjeran Beach Surabaya, then in this study selected Blood Shells (*Tegillarcanodifera*) and green mussel (*Pernaviridis*), as well as seawater around the habitat of Blood Clams and green mussel as samples to see how far Pb, Cd and Cr pollution at Kenjeran Beach Surabaya. Blood clams have demonstrated to be a good bio-indicator for Pb, Cd and possibly Se while green mussels may be used for Cr.[8]In this study, the sample was taken from Kenjeran Beach Surabaya because along the river that flows towards the Kenjeran Beach Surabaya there are many industries which discard their waste into the river and taken in 3 periods because heavy metal accumulated are not only depending on the quality but also seasonal factor.[9] Those industrial waste will flow towards Kenjeran Beach Surabaya.heavy metal content of Pb, Cd, and Cr will be compared with prevailing regulations, namely Ministerial Decree of Environment regulation Republic of Indonesia no. 51 of 2004, on seawater quality standards, that is Pb in the amount of 0.008 mg / L, Cd in 0.001 mg / L, and Cr in 0.005 mg / L.

2. MATERIALS AND METHODS

2.1 Material

Sample of Blood Clam(*Tegillarca nodifera)*, Green Mussel (*Perna viridis*)and sea waterfrom Pantai Kenjeran Surabaya that were taken on 22nd September, 23rd October, and20 November 2016, Standard Solution Pb (Pro Analyze, Merck), Standard Solution Cd (Pro Analyze, Merck), HNO_3 (Pro Analyze, Merck), HNO_3 50%, HNO_3 2%, H_2O_2 30%, Whatmanno.41 filter paper, demineralized water (Qualitative Laboratory of Faculty of Pharmacy University of Surabaya), Argon Gas (welding grade, Samator gas).

2.2 Instrument

Inductively Coupled Plasma Spectrometry (ICPS) ARL-3410+ Fisons, Laboratory glassware, Hot plate, Magnetic stirrer, Thermometer, Oven, Analytical Scales (Ohaus Pioneer type).

2.3 Sampling

The sample of Blood clam(*Tegillarca nodifera)*, Green mussel (*Perna viridis*) and sea water from Kenjeran Beach Surabaya weretaken in 3 period, on 22nd september, 23rd Oktober and 20 November 2016. The sampling used the technique of unrestricted random samples because the samples were drawn directly from the population. The population is not pre-divided over subsamples.[10]

2.4 Working Standard

2.4.1 Pb

Standard raw solution 1000 ppm of Pb metal element is made standard solution between 10 ppm. Then made a working standard solution of Pb with concentration of 0.02 ppm; 0.5 ppm; 1 ppm; 2 ppm; 8 ppm. Then observed the intensity using ICPS and made a curve of the relationship between concentration and intensity.

2.4.2 Cd

Standard raw solution 1000 ppm of Cd metal element is made standard solution between 5 ppm. Then made a working standard solution of Cd with concentration of 0.05 ppm; 0.3 ppm; 0.5 ppm; 0.8 ppm; 1 ppm. Then observed the intensity using ICPS and made a curve of the relationship between concentration and intensity.

2.4.3 Cr

Standard raw solution 1000 ppm of Cr metal element is made standard solution between 10 ppm. Then made a working standard solution of Cd with concentration of 0.3 ppm; 1.0 ppm; 4.0 ppm; 6.0 ppm; 10.0 ppm. Then observed the intensity using ICPS and made a curve of the relationship between concentration and intensity.

2.5 Qualitative Analysis

Qualitative tests were performed by comparing the working standard intensity of 5.0 or 10.0 ppm of each metal with samples at each selected wavelength.

2.6 Quantitative Analysis

Analysis of Pb, Cd, and Cr Metals on Blood Clams and Green Mussels using Wet Destruction

Each sample of blood clams and green mussels was accurately weighed approximately 3 g and put into a 100 ml beaker glass, and note the weighing result was then added 10 ml of 50% HNO_3, then covered with a glass, heat up to 95 ° C, and let the solution be heated for 15 minutes. The solution was allowed to cool, added 5 ml of concentrated HNO_3 and covered with a glass, heated for 30 min at 95 ° C. The solution is then evaporated to less than 20 ml remaining. The solution was allowed to cool, added 2 ml of demineralized water and 3 ml of 30% H_2O_2, covered with a glass and warmed to 45 ° C until the bubbles were reduced or subside. The solution is allowed to cool and then filtered with Whatman filter paper No. 41 to remove residual particles. The solution was diluted to 25.0 ml with 2% HNO_3 in a measuring flask and homogenized.[10-11] The result of sample preparation was then analyzed by using ICPS Fisons ARL 3410+ to find out the metal content of Pb, Cd and Cr in the sample. Performed as much as 3x for each capture period.

2.7 Analysis of Metals Pb, Cd, and Cr on Sea Water Samples

Seawater samples obtained from Blood clams (*Tegillarcanodifera*) and Green mussels(*Pernaviridis*) were measured 50.0 ml and then inserted in 100 ml beaker glass. Added 10 ml 50% HNO_3, then covered with a glass and heated for 15 minutes at 95ºC. The solution was allowed to cool, added 5 ml of concentrated HNO_3 and covered with a glass, heated for 30 min at 95ºC, then evaporated to the remaining 5 ml. The solution was diluted to 50.0 ml with 2% HNO_3 in a measuring flask and homogenized, filtered with whatman filter paper No. 41, then the first 10 ml was thrown into a glass beaker.[11-12] analyzed it by using ICPS to know the metal content of Pb, Cd, and Cr in the sample.

3. RESULT AND DISCUSSION

Table 1. *The result of qualitative analysis*

Sample	Pb 283,306 nm	Cd228,802 nm	Cr283,536 nm
Tegillarca nodifera	+	-	
Perna viridis	+		+

Table 2. *Result of Intensity of Standard Solution PbandCd*

Pb (ppm)	Intesity		Cd (ppm)	Intesity		Cr (ppm)	Intensity
0	0,034		0	0,041		0	0.02
0,05	0,037		0,02	0,042		0.3	0.024
0,3	0,066		0,5	0,046		1	0.031
0,5	0,084		1	0,049		4	0.053
0,8	0,113		2	0,056		6	0.07
1	0,134		8	0,099		10	0.104

The regression equation and r value for metal Pb, Cd, Cr is obtained y = 0.0418 + 0.0072x and 0.9998; y = 0,0338 + 0,1002x and 0,9995; y = 0,0083x + 0,0208 and 0,9993 respectively.

The content of Pb, Cd, Cr in seawater samples was calculated using the regression equation of standard metal curves Pb, Cd, Cr. Each metal was observed using ICPS ARL Fisons 3410+ at wavelength 283,306 nm (for Pb), 228,802 nm (for Cd), and 283,536 nm (for Cr). The result of Pb, Cd, and Cr content on sea water sample can be seen in table 3.

Table 3. *Results of Cd and Pb Metals Level on Sea Water Samples*

Sampling	Cd (ppm)	Pb (ppm)	Cr (ppm)
I (22ndseptember 2016)	**0,1422**	**9,6806**	**0,7470**
II (23rdoktober 2016)	**0,1472**	**9,7500**	**0,8072**
III (20 november 2016)	**0,1522**	**10,0278**	**0,8675**

Table 4. *Results of Cd and Pb Metals Level on Blood Clam Samples*

Sampling	Cd (ppm)	Pb (ppm)
I (22ndseptember 2016)	**0,2083**	**4,2870**
II (23rdoktober 2016)	**0,2149**	**4,3796**
III (20 november 2016)	**0,2515**	**4,6574**

From table 4 above, on the September 22nd, 2016, the average Pb content = 4.2870 ppm was obtained = 20.73 mg / kg dry weight. On the October 23rd, 2016, the average Pb content = 4, 3796 ppm was obtained = 21,51 mg / kg weight. On the November 20, 2016, the average Pbcontent = 4.6574ppm = 22.97 mg / kg dry weight.

on the September 22nd, 2016, the averageCd content was found 0.2083 ppm = 1.01 mg / kg dry weight. On the October 23rd, 2016, the average Cd content = 0.2149 ppm was obtained = 1.06 mg / kg dry weight. On the November 20, 2016, the average Cd content = 0.2515 ppm was obtained = 1.24 mg / kg dry weight.

Table 5. *Results of Cr and Pb Metals Level on Green Mussel Samples*

Sampling	Cr (ppm)	Pb (ppm)
I (22 september 2016)	**0,4659**	**4,8426**
II (23 oktober 2016)	**0,5864**	**4,4259**
III (20 november 2016)	**0,7470**	**5,2593**

From table 5 above, on the September 22nd, 2016, the average Pb content = 4,8426 ppm was obtained = 26,32 mg / kg dry weight. On the October 23rd, 2016, the average Pb content = 4, 4259 ppm = 23,38 mg / kg weight. On the November 20, 2016, the average Pb content = 5,2593 ppm= 24.13 mg / kg dry weight. on the September 22nd, 2016, the average Cr content was found = 0.4658 ppm = 2,53 mg / kg dry weight. On the October 23rd, 2016, the average Cr content = 0.5864 ppm was obtained = 3,16 mg / kg dry weight. On the November 20, 2016, the average Cr content = 0.7470 ppm was obtained = 4,07 mg / kg dry weight.

In this study sea water samples and shellfish were collected at Kenjeran Beach Surabaya because it is an estuary for river in the city of Surabaya, where along the river flow there are many industries that allegedly produce Pb, Cd, Cr waste and dispose of waste into the river

Pb, Cd, Cr was selected because these metals is categorized as non-essential heavy metals or toxic. Pb may inhibit Hb synthesis, cause brain and kidney damage and may also cause ECG abnormalities in children. Cd can cause proteinuria, swelling of the lungs, the fragility of the bone, At a certain concentration Cd can kill sperm cells in men, impotence, cause decrease testosterone levelsin blood. Carcinogenic Chromium is usually caused by Cr hexavalent Cr^{6+}, which is corrosive and insoluble in water. Cr^{6+} can also induce necrosis of the kidneys. Cr6 + ions in the body's metabolism will inhibit or inhibit the action of benzopiren hydroxylase enzyme causing a change in the ability of cell growth, so the cells grow irregularly and this is better known as cancer.[13]

Wet destruction method was used to examine Pb and Cd content analysis because the temperature used is relatively low, the equipment is simpler, the oxidation process is faster, and the time required is relatively faster. Wet destruction is carried out by addition of HNO_3 to break the bond of complex compounds between organic and metal (organometallic) materials and also by the addition of H_2O_2 as an oxidizing agent and can reduce the carbon content of the destruction results so that the solution becomes clearer. After the destruction process ends, the resulting solution of the destruction is cooled then the solution is filtered using Whatrmann no. 41 to filter out the remaining particles. The filtered solution was then subjected to a measuring flask until the boundary mark was then homogenized and observed for intensity using ICPS ARL 3410+.

This study uses ICPS ARL 3410+ because this tool can analyze multielement, and sensitive to small sample concentration. The wavelength selected for Pb observation is 283,306 nm,for Cd observation is 228,802 nm, and for Cr observation is 228,802 nm. The wavelength selection is based on the highest sensitivity and no interference with other metals.

Previous to the quantitative test, qualitative tests were performed on blood clams. The result of qualitative test shows that there is a peak at wavelength 283,306 nm. So, on the blood clams from the Kenjeran beach Surabaya containing Pb metal. In the qualitative test of wavelength 228,802 nm there is no peak, but it is not signify there is no content of Cd in blood clam samples, there is the possibility of blood clams containing Cd metal although the level is relatively small.The heavy metal Cr on the sample looks a close peak at a wavelength of 283.536 nm

In this study also conducted examination of heavy metal content in seawater of Kenjeran Beach Surabaya. This is intended to determine the ratio of heavy metal content in the seawater to the level of metal in blood clams. The seawater is taken from the environment around the blood clamsand green mussel habitat. From the research, the content of Pb in seawater is as follows: at the first sampling (22nd September 2016) is 9,68056 ppm, the second sampling (23rd October 2016) is 9.75 ppm, on the third sampling (20November 2016) is 10,0278 ppm. Thus, during the period of September-November 2016 the level of Pb in sea water was 9.68056 ppm- 10,0278 ppm. The Cd content in seawater is as follows: the first sampling (22nd September 2016) is 0.1422 ppm, the second sampling (23rd October 2016) is 0.1472 ppm, on the third sampling (20November 2016) is 0.1522 ppm. Thus, during the period September-November 2016 the Cd content in sea water was 0.1422 ppm - 0.1522 ppm.The Cr content in seawater is as follows: the first sampling (22nd September 2016) is 0.7470 ppm, the second sampling (23rd October 2016) is 0.8072 ppm, on the third sampling (20 November 2016) is 0.8675 ppm. Thus, during the period September-November 2016 the Cr content in sea water was 0.7470 - 0.8675 ppm. Results during the period September-November 2016 the level of Pb and Cd in blood clams were4.28704 - 4,65741 ppmand 0.20825 - 0.2515 ppm, respectively. the level of Pb and Cr in green mussel were4.4259 - 5,2593 ppm and 0.4659 - 0.7470 ppm, respectively.These levels exceed the limits established by Decree of the Minister of EnvironmentRepublikof Indonesia No. 51 year 2004 on Wastewater Quality Standard is for Pb of 0.008 mg/L, for Cd of 0.001 mg/L, and for Cr of 0,005 mg/L.[15]
From the results of the study also found that the level of Pb in the blood clam (*Tegillarcanodifera*) has increased from first sampling to second sampling and third sampling. Where at the third sampling the content of Pb greater than at the second sampling and the level of Pb and Cd at second sampling also bigger than at first sampling. From the research result also found that the concentration of Cd in the blood clam (*Tegillarcanodifera*) has increased from first sampling to second sampling and third sampling. Where at the third sampling the content of Cd is bigger than at the second sampling and the content of Cd at second sampling is also bigger than at the first sampling. The increase of Pb and Cd content at each sampling is due to the "bioaccumulative" nature in the body of living creature including shells. Another thing that is also the cause of increased levels of Pb and Cd in the body of the blood clam (*Tegillarcanodifera*) is due to an increase in levels of Pb and Cd in the environment around the shells in this case is an increase in levels of Pb and Cd metal in sea water and sediments at Kenjeran Beach Surabaya.[16]

Cd concentrations found in blood clams, 0.2083 - 0.2515 ppm, greater than those found in seawater 0.1422 ppm - 0.1522 ppm, show blood clams can accumulate heavy metals Cd, so that blood clams can be used as bioindicators and biomonitoring of heavy metal pollution Cd on territorial waters.[8] Accumulation can also be caused by blood clams are less able to metabolize heavy metals Cd, thus accumulating in the blood clams tissue.[17]

In the same period, Pb concentrations in blood clams and green mussel were found 4.2870 - 4.6574 and 4.4259 - 5.2593, respectively, smaller than those found in sea water of 9.6806 ppm - 10,0278 ppm. This may be because Pb levels in the blood clams and green mussel have exceeded the limits of the ability of them to metabolize heavy metals Pb. Previous research has suggested that blood clams can be used as biomonitoring of Pb heavy metals because they can accumulate the heavy metals.[8-9,17]Pb concentration in green mussel 4.4259 - 5.2593 ppm, almost the same as that found by blood clams of 4.2870 - 4.6574 ppm. Indicating green mussel can be used as a biomonitor that is as good as blood clams.Cr concentration on green mussel 0.4659 - 0.7470 ppm, smaller than that found in sea water of 0.7470 - 0.8675 ppm. Indicates if the green mussel is poorly used as a biomonitor of Cr heavy metal contamination in the waters, in contrast to previous research.[8]

5. CONCLUSION

Blood clams and green mussel that were taken at Kenjeran Beach Surabaya during the period September-November 2016 contain metal Pb, Cd, dan Cr. The level of Pb, Cd, and Cr in seawater from September to November 2016 were 9,6806 - 10,0278 ppm; 0.1422 - 0.1522 ppm; 0.7470 - 0.8675 ppm, respectively.Pband Cd content in blood clams amounted to 4.28704 - 4,65741 ppm and 0.20825 - 0.2515 ppm, respectively.Pband Cr content in green mussel amounted to 4.4259 - 5,2593 ppm and 0.4659 - 0.7470 ppm, respectively.These levels exceed the limits established by Decree of the Minister of Environment Republik of Indonesia No. 51 year 2004 on Wastewater Quality Standard is for Pb of 0.008 mg/L, for Cd of 0.001 mg/L, and for Cr of 0,005 mg/L. Based on the research conducted, it is recommended to do further research on heavy metal content in blood clams over a longer time range and research the concentration of heavy metals in blood clams based on differences in shell size and seasonal differences. Further research needs to be done regularly so that intensive monitoring of heavy metals in Kenjeran Beach Surabaya can be done. Blood clams can be used as biomonitoring and bioindicator which is good for heavy metal Cd pollution in the waters. Blood clams and green mussel can be used equally well for biomonitoring and Pb metal pollution bioindicators in the waters. Green mussel is less well used as biomonitoring and bioindicator of heavy metal pollution Cr.

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Developmentand Validation of a High-Performance Liquid Chromatography Method To Determine Plasma Methotrexate Concentrations

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ABSTRACT

Introduction: Methotrexate (MTX) is one of the most widely used chemotherapy agentsand immunosupressantsto treat cancer diseases in children and adult patients. However, MTX induced many side effects on hepatic, renal and nervous system. **Objectives:** The aim of this study is to develop and validate a high performance liquid chromatography (HPLC) method to determine plasma MTX concentrations in order to contribute to optimize the treatment efficacy and reduce the adverse effects of MTX. **Methods:** MTX and Para-aminoaetophenone (PAAP) as an internal standard were extracted from plasma samples with methanol. Sample purity was performed using Osis HLB 1cc cartridges. 10 µL of sample were injected onto Lichrocart Supersil 125-4 column C18 maintained at 40 °C on a Waters 2695 XE equipped with a PDA detector set at 303 nm. The mobile phase consisted of a mixture of phosphate buffer (pH 6.0) and methanol (80:20, v:v) set at a flow rate of 1 ml/min. The validation criteria were evaluated according to FDA guidelines 2001. **Results:** The total HPLC run time was 10min for isocratic MTX and PAAP separation, and then a gradient mode was used for 5min to clean the column. The retention times for MTX and PAAP were 2.3 and 5.2 min, respectively. MTX linearity was in the range from 0.5-25 mg/L. Mean intra-day and inter-day imprecision levels (coefficient of variation, CV%) for MTX were in the range from 3.42 – 8.12%. The lower limit of quantification was 0.5 mg/L. MTX extraction effects were above 77%. Interference was not found in the assay. **Conclusion:** We described a robust, rapid and simple method suitable for the determination of MTX concentrations in plasma. This analytical method could be used to quantify plasma MTX concentrations in patients under MTX therapy.

Keywords: methotrexate, high performance liquid chromatography, plasma

1. INTRODUCTION

Methotrexate (MTX) is one of the most widely used chemotherapy agents and immunosuppressants to treat cancer, autoimmune diseases in children and adult patients, ectopic pregnancy[1,2,3]. However, MTX induced many side effects on hepatic, renal and nervous system and severe skin rashes[4,5]. People on long-term treatment should be regularly monitored for side effects, especially in those with insufficient kidney function[6-9]. Pharmacokinetic monitoring of the MTX in the patients under MTX therapy may optimize the treatment efficacy and reduce the adverse effects of MTX[10]. Determination of MTX levels in plasma contributes to adjust MTX dosing. The aim of this study is to develop and validate a HPLC method to quantify the plasma MTX concentrations.

2. MATERIALS AND METHODS

MTX and p-aminoacetophenone (PAAP) were obtained from Sigma-Aldrich. HPLC-grade methanol (MeOH) was purchased from Merck and JT Baker, respectively. Potassium dihydrogenphosphatewas obtained from Merck.

The MTX calibrator and quality control (QC) samples were prepared separately by adding 100 µL of PAAP10mg/L as internal standard (IS) into 400 µL of plasma fortified appropriate amounts of MPA to achieve calibrators and QC concentrations of 0.5, 1, 5, 15, 25 mg/L and 0.5, 10, 20mg/L, respectively. For samples obtained from patient under MTX therapy, only 100 µL of 10mg/L PAAP were adding to 400 µL of plasma. Subsequently, proteins in samples were precipitated after 400 µL of 4% H_3PO_4 addition and mixing for 30s. After centrifuging at 10000rpm for 10 min at RT and then transferring 800 µL of supernatants into OASIS HLB cartridge 1ml (Waters) previously activated with 1 ml of pure MeOH and 1 ml of HPLC water, impurities were removed by subsequently adding 1 ml of 5% MeOH and 1 ml of 2% HCOOH. Then MTX extraction was performed by adding 1mL of pure MeOH.The solution eluted from cartridge was collected into a 1.5-mL eppendorf and evaporated to dryness at 50 °C. The residues were reconstituted with 200 µL of 0.01MNaOH (vortexed for 1 min). After centrifuging at 10000 rpmfor 5 min, 10 µL of supernatants were injected into the HPLC system.The analytical column was LichrocartSupersil C18 column (125 x 4 mm, 5 µm) maintained at 40 °C. The tray temperature in the autosampler was kept at 4 °C. The isocratic mobile phase consisted of a mixture of 0.01MKH_2PO_4 (pH 6.0) and MeOH (80:20, v:v). The total HPLC run time was 10 min for isocratic MTX and PAAP separation, and then a gradient mode was used for 5 min to clean the column before the next run.

The assay was fully validated according to the U.S. Food and Drug Administration (FDA) guidelines [11]. Statistics were performed using Microsoft Excelsoftware 2010.

The linearity of the method has been assessed over the 5 calibrators in replicate of 3 in 3 runs. The lower limit of quantification (LLOQ) is considered being the lowest concentration, with an acceptable accuracy and imprecision (≤ 20%), and a signal-to-noise ratio > 10:1. The limit of detection (LOD) was calculated as the smallest detectable peak above baseline noise (signal-to-noise ratio > 3:1). Inter and intra-assay precision and

accuracy were assessed on QC samples at 3 concentrations (0.5, 10, 20 mg/L) in replicate of 5 in 3 runs. Recovery of plasma MTX was determined by comparing the results from extracted samples in replicate of 5 at 0.5, 10, 20 mg/L with standard MTX in 0.01M NaOH.

Method specificity and selectivity were assessed by analysing blank and fortified plasma extracts from different healthy volunteers to differentiate the MTX and IS from endogenous components in the matrix.The stability of MPA in plasma was investigated in triplicate at 0.5, 10, 20 mg/L after 12h at RT by comparing freshly prepared samples. Additionally, the stability of MPA and IS in samples processed and stored in the autosampler (4 °C) was determined after 24h.

3. RESULTS

*Based on HPLC conditions, diode array*HPLC*detector*(PDA) showed the maximum absorbance of MTX at 303 nm. The retention times for MTX and IS were 2.3 min and 5.2 min, respectively. No interfering peak was detected at retention times of interests (See Fig 1).

The coefficient of determination (R^2) for MTX calibration curve was > 0.99 with a mean value of 0.9979 ± 0.001. LLOQ was found to be 0.5 mg/L. The CV% of inter-day and intra-day accuracy was < 10% at the low, medium, and high concentrations and < 20% at the LLOQ.

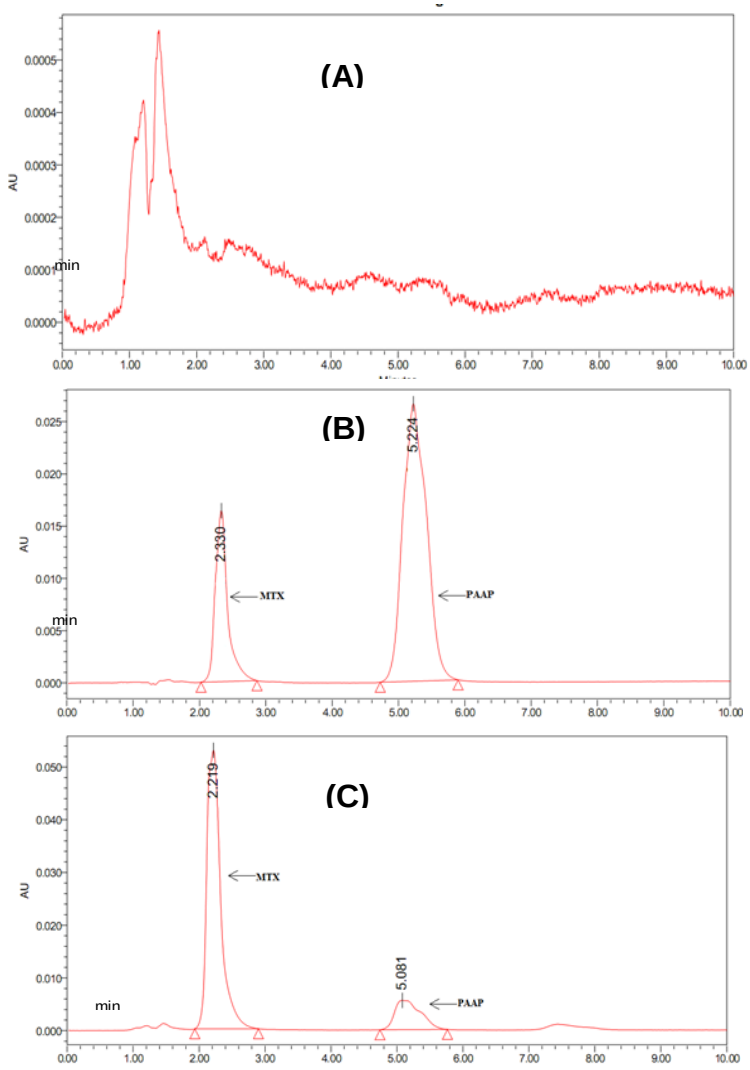

Figure 1. *Typical chromatogram of extracts from (A) blank plasma sample (placebo), (B) standard mixture of MTX (10mg/L) and PAAP (5 mg/L), and (C) plasma reconstituted with mixture of MTX (5mg/L) and PAAP (5 mg/L)*

MTX recovery at 0.5, 10 and 20 mg/L displayed a good reproducibility, with a mean value above77% (range: 70.08–81.98%). At the 3 concentrations mentioned above, mean inter-assay (3 days) and intra-assay imprecision (CV%) and accuracy ranged from 2,93 to 8,1% and 100,04 to 106,65%, and from 4,5 to 7,44% and 101,05 to 105,02%, respectively (See table 1)

.

Table 1. *Intra-day and inter-day accuracy and imprecision, and recovery for MTX at three levels of Quality Control (QC) Samples*

QC concentration (mg/L)	Intra-day (n=3) Accuracy/imprecision (%)	Inter-day (n=3) Accuracy/imprecision (%)	Recovery (n=3) (%)
0.5	98.84/3.42	92.4/7.50	70.08

10	98.57/6.69	99.2/6.00	81.98
20	89.07/8.12	98.3/6.05	80.19

Stability

No significant difference in MTXlevels was found between theinitial plasma samples and samplesafter 12h at RT, and extracts maintained at 4°C into the autosampler for 24 hrs.

4. DISCUSSION AND CONCLUSIONS

The previous studies demonstrated that renal dysfunction causes delayed MTX elimination and unsuccessful rescue by leucovorin. MTX-induced acute nephrotoxicity is thought to be due to the precipitation of MTX or its insoluble metabolites in the renal tubules or to a direct toxic effect of MTX on the tubules[6,7,11,12].

Therapeutic drug monitoring of MTX may contribute to prevent early occurrence of adverse reactions.To date, several quantitative methods have been developed to measure MPA plasma concentrations such as HPLC) equipped with either ultraviolet (UV) or PDA (Photodiode array) or mass-spectrometric (MS) detectors, or immunoassays such as ELISA (enzyme-linkedimmunosorbent assay)[11-18].

The method most commonly used for routine MTX measurement in clinical laboratories is fluorescence polarization immunoassay (FPIA). In addition to immunoassays, capillary zone electrophoresis and HPLC methods have been described for measuring MTX and its metabolites in biological fluids [12].

The choice of the method may depend on different factors such as the equipment available in the laboratory, the experience of the laboratory technicians and the number of assays to run. In the present study, we described a simple, robust, fast, specific and sensitive method suitable for the determination of MTX concentrations in plasma, with a LLOQ level of 0.5 mg/L. This method could be applied in routine to monitor patients under MTX therapy.

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Optimization of Extracellular Lipase Production from *Geotrichum* Geo 26.3 Using Response Surface Method-Central Composite Design (Rms-Ccd)

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Abstract

The production of some specific microbial lipases (EC 3.1.1.3) has aroused considerable interest because of their great potential for industrial applications as well as for medical applications. Among these microbial lipases, fungal lipases are the most attractive one due to their remarkable advantages such as easy extraction, substrate specificity and stability under varied chemical and physical conditions. The aim of our study was to optimize the production of extracellular lipases from *Geotrichum*Geo 26.3, an isolated native marine fungi using response surface method-central composite design (RMS-CCD).Several cultural parameters were investigated to determine the three most significant factors influencing the high yield of extracellular lipase production. The interactive effects of these three factors were then studied using RMS-CCD. Each factor was studied at five different levels (-α, -1, 0, +1, +α) in the CCD of 20 experiments. The relationship among the factors was expressed mathematically in the form of a polynomial model, as shown in Eq. (1).

$$Y = bo + b1A + b2B + b3C + b11A2 + b22B2 + b33C2 + b12AB + b23BC + b13AC \quad (1)$$

where, Y was the predicted response (lipase production in terms of lipase activity); A,B,C were the three input variables; b1,b 2, b3 were the linear coefficients, b11 b22, b33 were the quadratic coefficients and b12 b23, b13 were the interactive coefficients of each factors couple. Data were analyzed by Design expert 10.0.0® software (Stat-Ease Inc. USA). Concentration of yeast extract, concentration of soybean oil and concentration of ammonium nitrate were shown to be three most significant factors influencing the high yield of extracellular lipase production from *Geotrichum* Geo 26.3. The optimum conditions for extracellular lipase production from *Geotrichum* Geo 26.3 were: 0.5% yeast extract, 0.5% soybean oil, 0.31% ammonium nitrate, 0.1% soybean meal, after 72h of culture. At this optimum condition, lipase activity obtained was 56,456 IU / ml which was 4,5 folds increase as compared to the non-optimized environmental factors in the basal medium.In concluding, the production of extracellular lipase production from *Geotrichum* Geo 26.3 was successfully optimized using response surface method-central composite design (RMS-CCD).

Keywords: *Geotrichumcandidum* Geo26.3, lipase, optimization, response surface method (RMS), central composite design (CCD)

1. INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3), a class of hydrolase which catalyze the hydrolysis of triglycerides, have recently received considerable attention due to their great potential in a wide array of industrial applications, especially in pharmaceuticals. Lipase-catalyzed reaction has been used in the resolution process to obtain the desired optical isomer of many active pharmaceutical ingredients such as, ibuprofen(1), diltiazem(2), fluoxetine (3)…etc. In addition, lipases from some microbial species are effective for the enrichment or the concentration of polyunsaturated fatty acids (PUFA)in oils via selective hydrolysis(4). Recently, lipase has been proved to be an efficient tool in the synthesis of antiviral prodrugs from racemic to increase their bioavailability (5).

Microorganisms have long been known as potential producers of lipase. Among these microbial lipases, fungal lipases are the most attractive one due to their remarkable advantages such as easy extraction, substrate specificity and stability under varied chemical and physical conditions. *Geotrichumcandidum* (*G.candidum*), in particular, is one of the most commercially important lipase-producing fungi. It is because of not only the high enzyme production capacity but also the remarkable properties of the produced enzyme, such as fatty acid specificity, linkage specificity, stereochemical specificity and the safety (6). In Vietnam, the Institute of Biotechnology has successfully isolated *G.candidum* Geo26.3 strain from marine samples. Initial screening studies have shown that this strainmight become a promising producer of extracellular lipase. The aim of our study was to optimize the production of extracellular lipases from *Geotrichum Geo* 26.3 using response surface method-central composite design (RMS-CCD).

2. MATERIALS AND METHODS

2.1. Microorganism

The *G.candidum* Geo26.3 was supplied by the Department of Enzyme Biotechnology at the Institute of Biotechnology, Vietnam Academy of Science and Technology

2.2. Chemicals

Yeast extract (Bio Basic Canada Inc), ammonium nitrate (Xilong-China), peptone (Bio Basic Canada Inc), Glucose (Bio Basic Canada Inc), soybean powder (Vietnam), Flour (Vietnam), soybean oil (Vietnam).

2.3. Methods

2.3.1. Experimental design

Effects of several factors, such as carbon and nitrogen sources, fermentation time, ammonium nitrate concentration, soybean oil concentration and yeast extract concentrations, on the extracellular lipase production of *Geotrichumcandidum* Geo26.3 wereevaluatedby lipase activity determination in the supernatant (obtained by centrifuging the fermentation broth at 4000 rpm for 20 min. at 4^oC). Three most significant factors were then selected to study of their interactive effects using response surface method-central composite design.

2.3.2. Fermentation process

A single colony of *Geotrichumcandidum* Geo26.3 on SDA agar plate wastransferredto SD medium and incubated at 30^oC for 24h with shaking(200 rpm). 4% inoculum was then added to fermentation medium and incubatedat 30^oC with shaking(200 rpm).

2.3.3. Lipase activity assays

Lipase activity was determined by volumetric method, which based on the titrimetric determination of the free fatty acids released from triacylglycerols by lipase catalyzed hydrolysis. A lipid-water emulsion (2% v/v soybean oil, 1% w/v gum arabic) was used as substrate. The NaOH10mM was continuous titrated by pH-stat titrator (Metrohm 718 STAT Titrino).

One unit of lipase activity was defined as the amount of enzyme that liberates 1 µmol of fatty acid per minute under assay conditions(7).

2.3.4. Response surface method (RSM) and central composite design (CCD)

Each of the three most extracellular lipase production influencing factors was studied at five different levels (-α, -1, 0, +1, +α) in the CCD of 20 experiments. The relationship among the factors was expressed mathematically in the form of a polynomial model, as shown in Eq. (1).

$$Y = bo + b1A + b2B + b3C + b11A2 + b22B2 + b33C2 + b12AB + b23BC + b13AC \quad (1)$$

where, Y was the predicted response (lipase production in terms of lipase activity); A,B,C were the three input variables; b1,b 2, b3 were the linear coefficients, b11 b22, b33 were the quadratic coefficients and b12 b23, b13 were the interactive coefficients of each factors couple. Data were analyzed by Design expert 10.0.0® software (Stat-Ease Inc. USA) (8).

3. RESULTS AND DISCUSSION

*3.1. Effects of cultural factors on extra cellular lipase production of Geotrichumcandidum*Geo26.3

Different culture medium formulations and conditions were used to evaluated the effects of carbon and nitrogen sources, fermentation time, yeast extract concentrations, soybean oil concentration and ammonium nitrate concentration on the extracellular lipase production of *Geotrichumcandidum* Geo26.3 (Figure 1)

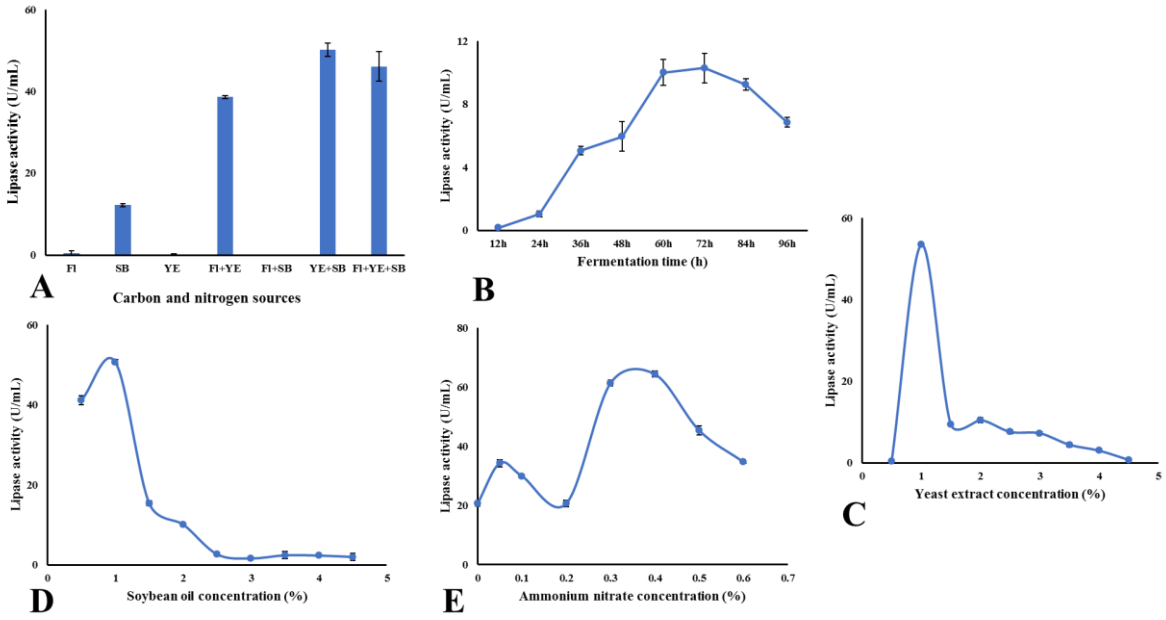

Figure 1. *Effect of carbon and nitrogen sources (A), fermentation time (B), yeast extract concentration (C), soybean oil concentration (D), ammonium nitrate concentration (E) on*

lipase production ofGeotrichumcandidum Geo26.3. (Fl: Flour, SB: Soybean, YE: Yeast extract)

The results showed that all of the examined factors affected thelipase production of *G.candidum* Geo26.3. However, the three most influential factors were yeast extract concentration, soybean oil concentration and ammonium nitrate concentration. Therefore, they were chosen for the next RSM – CCD experiments.

*3.2. Optimization for the extra cellular lipase production of Geotrichumcandidum*Geo26.3

The three chosen factors were optimized using RSM – CCD and Design expert® 10.0 software. The range of interest and code level for each factor were presented in Table 1.

Table 1.*Value of studied factors in RSM – CCD*

Variables	Unit	Symbol	Range of interest	Code level				
				-α	-1	0	+1	+α
Yeast extract concentration	% (w/v)	A	0,03 – 3,97	0,03	0,5	2,0	3,5	3,97
Soybean oil concentration	% (v/v)	B	0,8 – 2,82	0,18	0,5	1,5	2,5	2,82
Ammonium nitrate concentration	% (w/v)	C	0,04 – 0,56	0,04	0,1	0,3	0,5	0,56

20 experiments were then designed and conducted. The observed results were presented in Table 2.

Table 2.*Experiment design and observed results*

Experiment	Factors			Observed criteria
	Yeast extract concentration	Soybean oil concentration	Ammonium nitrate concentration	Lipase activity (IU/ml)
	A	B	C	
1	0	+α	0	46,19 ± 0,512
2	0	0	0	50,02 ± 0,101
3	0	0	+ α	41,74 ± 0,405
4	1	-1	1	50,66 ± 0,101
5	0	0	0	53,76 ± 0,456
6	0	0	- α	31,67 ± 0,163
7	-1	1	1	36,89 ± 0,358
8	-1	1	-1	22,22 ± 0,111
9	0	0	0	55,14 ± 0,219
10	1	-1	-1	60,74 ± 0,211
11	- α	0	0	39,98 ± 0,318
12	1	1	1	45,43 ± 0,405
13	0	0	0	57,24 ± 0,488
14	0	- α	0	68,72 ± 0,101

15	+α	0	0	60,89 ± 0,608
16	-1	-1	-1	47.99 ± 0,203
17	0	0	0	49,78 ± 0,152
18	-1	-1	1	50,42 ± 0,152
19	0	0	0	53,87 ± 0,101
20	1	1	-1	37,78 ± 0,152

After performing ANOVA, lipase production yield was estimated via regression equation in the following model:

$Y = 53,58 + 5,64A – 8,47B + 2,44C – 2,44AC + 3,75BC – 9.64C^2$

In which Y was lipase activity (IU/mL). A,B,C were the three input variables : yeast extract concentration, soybean oil concentration and ammonium nitrate concentration, respectively. Regression coefficient (R^2) was identified as 0.9342, which means 93.42 % of experiment data was compatible with the value predicted via the model. $R^2 > 0.75$ means the model was also compatible with experiments. Predicted R^2 value (0.8376) was on accord with modified R^2 (0.9039) with deviation of $0.0663 < 0.2$. Signal: noise ratio was $21.787 > 4$, which means the signals were sufficient. The C.V (7.03%) was low, which means that the plot was on a more uniform location and the data is more precise.

The three- dimensional response surface diagram presented the interaction between each couple of factors (Figure 2).

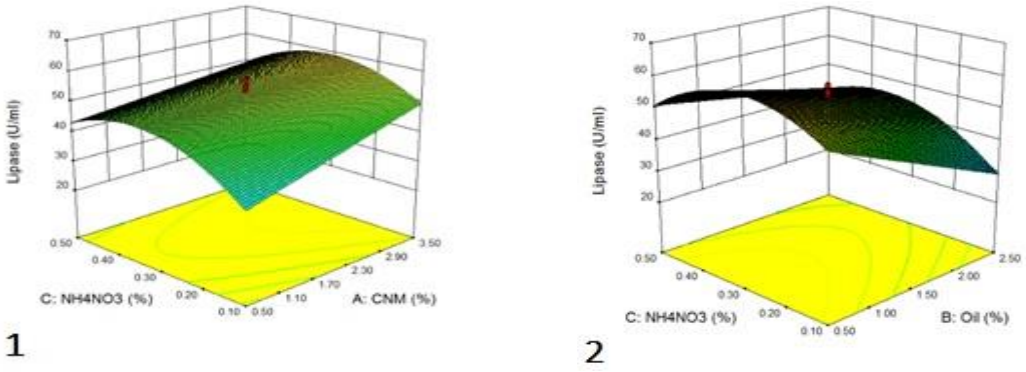

Figure 2. *Three-dimensional Lipase activity response surface by yeast extract concentration and ammonium nitrate concentration (1) and by soybean oil concentration and ammonium nitrate concentration (2) (CNM: Yeast-extract)*

From the diagram it was possible to identify the optimalof each ones for the maximum value of response function. Considering to the economic issue (the cost of the ingredient) several solutions were supposed. The lipase activity and the desirability of these solutions were presented in Table 3.The optimal solution were chosen based on two criteria: high lipase activity and high desirability.

Table 3.*Lipase activity and the desirability of supposed optimal solutions*

Number	Variable	Goal	Solution	Lipase activity (IU/mL)	Desirablility
1	Yeast extract concentration	Minimum	0,5%	56,5	0,858
	Soybean oil concetration	In range	0,5%		
	Ammonium nitrate concentration	In range	0,31%		
2	Yeast extract concentration	Minimum	0,50%	56,456	0,903
	Soybean oil concetration	Minimum	0,50%		
	Ammonium nitrate concentration	In range	0,31%		
3	Yeast extract concentration	Minimum	0,5%	50,55	0,807
	Soybean oil concetration	In range	0,5%		
	Ammonium nitrate concentration	Minimum	0,16%		
4	Yeast extract concentration	Minimum	0,5%	50,918	0,851
	Soybean oil concetration	Minimum	0,5%		
	Ammonium nitrate concentration	Minimum	0,16%		

The results showed that the solution number 2 is the optimal one. The lipase activity increased by 4.5 folds while yeast extract concentration and soybean oil concentration decreased by 2 folds.when compared to the non-optimized conditions (lipase activity is 12 IU/mL with 96h fermentation in culture containing: 1% yeast extract; 1% soybean oil; 0.2% ammonium nitrate).

4. CONCLUSIONS

The optimal fermentation conditions for the biosynthesis of extracellular lipase from *G.candidum* Geo26.3 strain were successfully determined using RSM-CCD, which were as follows: 72h fermentation in medium containing 0.5% (w/v) yeast extract; 0.5% (v/v) soybean oil; 0.31% (w/v) ammonium nitrate and 1% (w/v) soybean. Under this condition, lipase activity obtained was 56,456 IU / ml which was 4,5folds increase as compared to the non-optimized environmental factors in the basal medium. These results open the prospect of lipase production in large volume to serve the pharmaceutical industry.

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Enhanced Cellular Uptake and Reduced Toxicity of Doxorubicin-Loaded Stealth Liposomes

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Abstract

Stealth liposomes form an important subset of liposomes, demonstrating prolonged circulation half-life and improved safety *in vivo*. The aim of this work is to compare the cellular uptake efficacy of our doxorubicin-loaded stealth liposomes with Lipo-Dox (a commercial available doxorubicin loaded stealth liposomes) in breast cancer cells (MCF-7 and MCF-7/MDR cellines). Then, the toxicity reduction of the nanosystems in comparison with free doxorubicin was also determined. The intracellular uptake of doxorubicin from the stealth liposomal formulations,commercial available stealth liposome and free doxorubicin were evaluated *in vitro* with breast cancer cells (MCF-7 and MCF-7/MDR cellines) using laser scanning confocal microscopy (LSCM) and flow cytometer (FACS).Liposomes were also administered intravenously to rabbits in doses at 1.5 mg/kg once weekly for 3 weeks, in a reference along with free doxorubicin. The toxicity reduction was examined by biochemical and histopathological tests.The results of LSCM observations and flow cytometry analysis indicated that doxorubicin-loaded stealth liposomes mediated an efficient cellular uptake in both cellines. In addition, doxorubicin-loaded stealth liposomes promoted and enhanced their subsequent uptake by multidrug resistance cells (MCF-7/MDR cellines), whereas free doxorubicin enter cells with difficulty. Furthermore, the biochemical and histopathological test results from *in vivo* studies in rabbits have shown that free doxorubicin damaged the liver cells, toxic to the heart and reduces peripheral blood cells. Our nano formulation significantly reduced systemic side effects, including cardiotoxicity of doxorubicin. This study demonstrates that our nano formulation provide an effective delivery of drug to sensitize cells to circumvent MDR and to enhance the therapeutic index of the chemotherapy.

Keywords: Cancer therapy; Doxorubicin hydrochloride; Multidrug resistance; Stealth liposomes

1. INTRODUCTION

Anthracyclines are known as one of the most widely used chemotherapeutic agents for treatment
of a broad range of cancers such as hematologic and solid tumors [1]. Doxorubicin (Dox) is a conventional anthracycline isolated from Streptomyces peucetius caesiusin [2]. However, its efficacy remains unsatisfactory due to two major factors: multidrug resistance (MDR) and toxicity. Although Dox is considered as a potent antineoplastic agent, its clinical application is still limited because of its associated side effects. In addition to bone marrow depression, cardiotoxicity is a very important limiting factor for utilization of Dox [3,4]. The chronic dilated cardiomyopathy is the most common and the most serious type

of Dox cardiotoxicity and may result in congestive heart failure. Many different animal models of Dox induced cardiomyopathy have been developed particularly to test various substances for their potential cardioprotective properties [5-7]. The chronic cardiotoxic effect of Dox was detected particularly in rabbits [8] and many experimental studies proved the suitability and reproducibility of the rabbit model of Dox-induced cardiomyopathy [9-10].
The use of liposomes as drug carriers to achieve controlled, targeted drug release has emerged as an attractive option for drugs with narrow therapeutic indices. Liposomes of appropriate sizes are capable of accumulating within solid tumors by a phenomenon known as the enhanced permeation and retention (EPR) effect [11], thereby increasing therapeutic efficacy and decreasing systemic toxicity of anticancer drugs. Therefore, liposomes provide an excellent mode of drug delivery by ameliorating the toxicity ofencapsulated drugs. However, conventional liposomes are rapidly removed by the reticuloendothelial system from the circulation or may lose their encapsulated drug too fast [12].

To overcome this limitation, by covalently attaching polyethylene glycol (PEG) to the lipid bilayers, smaller and more rigid liposomes are produced. PEG forms a protective layer over the liposome surface and PEGylated liposomal has long circulation time and provides slow release of an encapsulated drug [13]. Therefore, PEG coated liposomes (stealth liposomes) can reduce the uptake by the cells of the RES and have a longer circulation time, consequently, results in an increased accumulation in tumors [14].Doxil®, Caelyx®, Lipo-Dox are marketed pegylated (polyethylene glycolcoated) liposome-encapsulated (PLD) form of Dox which approved in US, Europe, Taiwan for treatment of advanced breast cancer, ovarian cancer as well as in Kaposi's sarcoma in AIDS patients [15].

Therefore in this study, we compared the cellular uptake efficiency of our doxorubicin-loaded stealth liposomes with Lipo-Dox (a commercial available doxorubicin loaded stealth liposomes) in breast cancer cells (MCF7 and MCF7/MDR cellines). Then, we checked the toxicity reduction of the nanosystems in comparison with free doxorubicin. This experimental study was aimed to compare the chronic toxic effects of free Dox and doxorubicin loaded stealth liposomes using an identical study design in rabbits.

2. MATERIALS AND METHODS

2.1 Materials

Doxorubicin hydrochloride (Dox), hydrogenated soybean phosphatidylcholine (HSPC) (Lipoid), (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol) -2000] (ammonium salt) (DSPE-(PEG)2000-(NH2)), Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), cholesterol, chloroform, sodium hydroxide, ammonium sulfate, potassium dihydrogen phosphate, disodium hydrogen phosphate, phosphoric acid, triton X 100 (octyl phenol ethoxylate).All other reagents and solvents used to meet requirements for pharmaceutical and analytical grade. Reference drugs: Solution Dox Ebewe for injection 25 ml vials, 2 mg/ml (Ebewe Pharma, Austria) and Lipo-Dox Injections, 20mg/10ml (TTY Bio pharm, Taiwan).

2.2.Methods

2.2.1. Preparation of the doxorubicin-loaded stealth liposomes (Dox stealth liposome)

Liposomes were prepared by thin-film hydration. HSPC, cholesterol and DSPE-PEG2000 (3:1:1 w/w) were dissolve in chloroform and dried usinga rotary evaporator under vacuum (R-210, Büchi, Switzerland) at 50^{o}C for 12 h to form the dry lipidic film on the flask wall. The thin lipid film was hydrated by adding a buffer citrate solution pH 4.0 at 60^{o}C for 2h.

The liposome suspension was then sequential extruded through mm pore sized 0.2 μm and 0.1 μm polycarbonate membrane filters (Whatman, UK) using a High Pressure Homogenizers EmulsiFlex (Avestin-Canada) with nitrogen gases. Then, the external buffer environment of liposome was changed with buffer Hespes pH 7.5 using tangential flow filtration. Diluted Dox solutions were incubated with blank liposomes at appropriate drug-to-lipid (D/L) molar ratios at 65°C for 2 hours with constant shaking with 80 rpm for 30 min, temperature maintaining at 50 ± 2^{o}C.Mixtures were allowed to cool to room temperature (25°C) before removal of unencapsulated drugs using Sephadex G-50 columns (equilibrated with 5% glucose solution). The product was filtered through membrane filter 0.2 μm and then packed into 10 ml glass closed with rubber and aluminum cap, keep in a refrigerator from 8-10 ^{o}C.

2.2.2. Liposome size, distribution and Zeta potential

Using the method of dynamic light scattering (DLS) with instrument Zetasizer ZS90 (Malvern Instruments Ltd, Malvern, UK). The suspension of liposomes was diluted 200 times with deionized water.

2.2.3. Quantification of doxorubicin

Using a HPLC method. *Mobil phase:*Dissolved 1 g of sodium lauryl sulfate in 1000 mL mixture of water-acetonitril-methanol-phosphoric acid (400:450: 150:2), adjusted to pH 3.6 ± 0.1 by solution sodium hydroxyde 2 N. *Detector:* UV –VIS, 254 nm. *Flow rate*: 1.2 ml/ min *Injection volume*: 20 μL

2.2.4. Encapsulationefficiency

1 mL of Dox stealth liposome suspension was added into dialysis bag and hangs the bag in an Erlenmeyer flask containing 100 ml of phosphate buffer pH 4.0 systems at temperature 8-10 ^{o}C for 12 hours. The solution was measured optical density at 233 nm wavelength.The percentage encapsulation efficiency of the drug was calculated by:

Encapsulation efficiency (%) = (Dox amounts in the filtered liposomes (mg))/ (Total Dox amounts in unfiltered liposomes (mg)) ×100

2.2.5. Confocal microscopy studies

MCF7 and MCF7-MDR cells were seeded at a density of 3×10^{5}cells per well in six-well plates and incubated at 37°C overnight to allow cell attachment. The medium was replaced with cell culture medium containing Dox-loaded stealth liposomes, lipo-Dox and free Dox (these preparations have the same concentration of 10 μg/mL), respectively.Then, the cells were incubated at 37°C for 6 hours. After 6 hours, the medium was then removed, and cells were washed three times with ice-cold PBS followed by fixing with 4% paraformaldehyde in PBS in 30 minutes. After fixing, the cells were washed twice with PBS and stained with PBS containing 0.1% Hoechst 33258 at 37 ^{o}C for 10 minutes and then washed twice with PBS. The fluorescent images of the cells were analyzed using a LSM 780 confocal microscope (Zeiss, Germany).

2.2.6. Flow cytometry analysis

MCF7 and MCF7/MDR cells were seeded at a density of 3×10^{5}cells per well in six-well plates and incubated at 37°C overnight to allow cell attachment. The medium was replaced with cell culture medium containing Dox stealth liposomes, lipo-Dox and free Dox (these preparations have the same concentration of 10 μg/mL), respectively. Then, the cells were incubated at 37°C for 6 hours and washed three times with PBS solution. The cells were then harvested by trypsinization and centrifuged at 2000 rpm for 5 minutes, resuspended with 0.25% paraformaldehyde solution in PBS, and examined by flow cytometry using an

FACScan instrument (Becton Dickinson, San Jose, CA, USA). Cell-associated DOX was excited with an argon laser (488 nm), and fluorescence was detected at 570 nm.

2.2.7. Determination of biochemical, haematological parameters and histological changes
Female and male rabbits of average weight of 2.4 kg at the beginning of the experiment were used. All animal experiments were performed in accordance with the guidelines of Vietnam Military Medical University. Animals were divided into three groups: control group (n=10) and free Doxgroup (n=10)and Doxstealth liposomes group. The substances were administered intravenously once weekly for 3 weeks: both free Dox and Dox stealth liposomes group were given in a Dox dose of 1.5 mg/kg per week, control rabbits received saline (1 mL/kg). All manipulations were performed under the ketamine anesthesia (50 mg /kg, i.m.). Biochemical and haematological parameters were evaluated from the blood sampled at 4 time points: T_0 (before injection); T_1 (1 week after injection); T_2 (2 weeks after injection) and T_4 (4 weeks after injection). Standard biochemical parameters were determined in serum using a Architech CI 16200 (Abbott, USA). Haematological parameters were measured by means of a CellDyn 1700 (Abbott, USA).Then the rabbits were sacrificed by an overdose of pentobarbitone, an autopsy was performed and the heart, liver and kidney were examined histologically. An autopsyand histological examination was also performed in animals that had died prematurely.

2.3 Statistical analysis
All data are shown as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine significance among groups. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Physicochemical characterization of liposomal formulations
We have prepared three batches of Dox stealth Liposomes at scale 100 vial/batch (1000ml/batch) and analyzed some properties of the obtained Dox-loaded stealth Liposomes in comparison with that of the commercial available stealth liposomes (lipo-Dox). The results were shown in Table 1. All drug-loaded liposomes exhibited mean particle diameters smaller than 90 nm, good polydispersity (PDI<0.2) and negative zeta potentials (Table 1). The encapsulation efficiency of Dox in lipo-Dox and Dox-loaded stealth liposomes were almost similar, about 95% (w/w).

Table 1. *The size, PDI, Zeta potential and encapsulation efficiency (EE) (%) of different liposomes.*

formulations	Size (nm)	PDI	Zeta potential (mV)	EF (%)
Lipo Dox	80.6±0.5	0.09±0.02	-89.3±0.39	95.31±1.62
Dox stealth Liposomes	85.2±0.7	0.08±0.05	-33.9±0.05	92.78±1.96

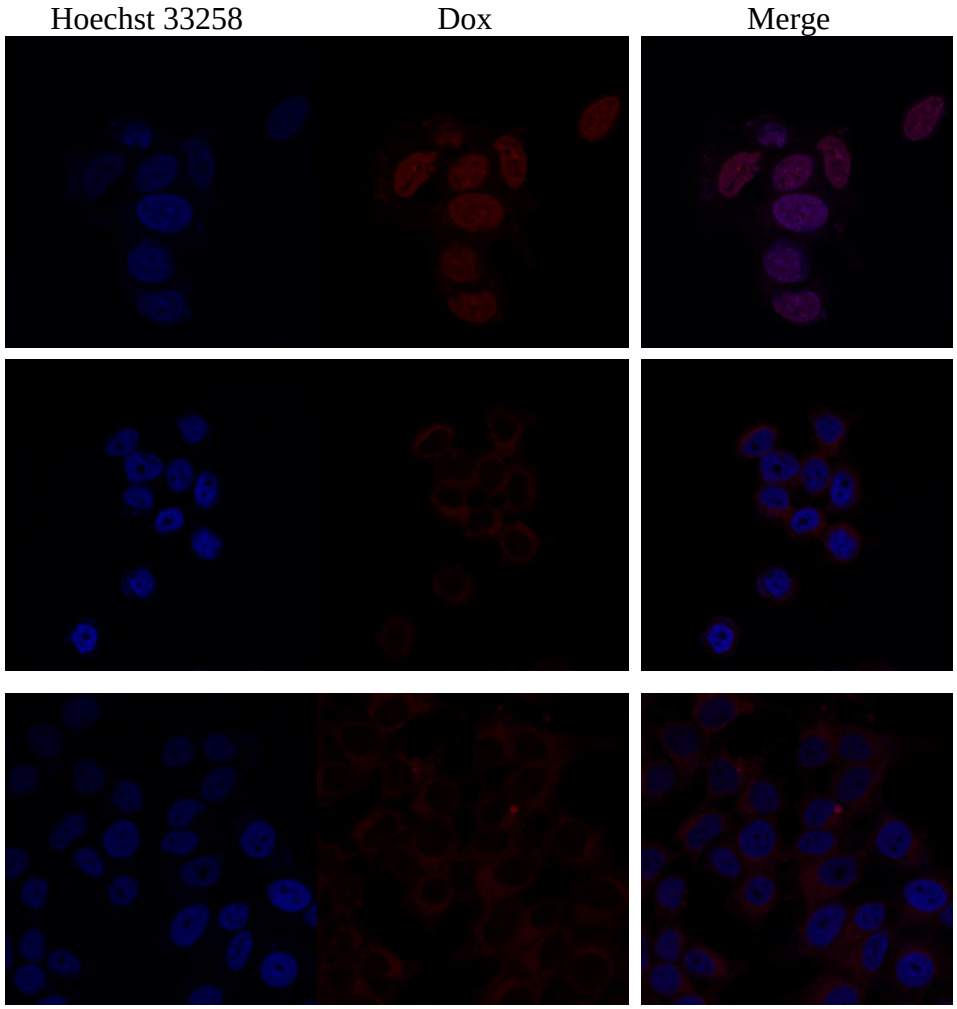
Data represent the mean±SD (n=3).

There are no significant differences in some parameters such as size, PDI, Zeta potential patterns of Lipo-Dox and Dox stealth Liposomes. The size of particles plays important role due to their interaction with the biological environment. When particles are loaded by intravenous administration, their ability to pass or leave the vascular capillaries effectively is dependent on the size [16]. Referring to Table 1, Dox stealth Liposomes has a size of 85.2 ± 0.7 nm. That means our liposomes with small particle size (< 200 nm) could increase the accumulation of drug in the tumor by augmented permeability and retention effect. The polydispersity index (PDI) value is a measure of the heterogeneity of particle sizes in a compound. Liposomes with PDI value between 0.1 and 0.25 display more uniformity and physical stability. In case of PDI value more than 0.5 indicates the poor uniformity of mixture [17]. Our PDI values of liposomes are 0.08±0.05, are confirmed as uniformity and homogeneity. The zeta potential (ZP) is a key indicator of the stability of colloidal dispersions. It presents the repulsive forces between the particles. Particles having a ZP of less than −25 mV or more than +25 mV are usually considered stable. Our Dox stealth liposome preparations have zeta potential values -33.9 ± 0.05 mV (Table 1), are considered stable. As showed in Table 1, our liposomes contained very high entrapment efficiency, 92.78±1.96 %. These confirmed that our liposome preparations have meet requirements of a liposome product.

3.2 Cellular uptake using confocal microscopy and flow cytometry

Figure 1 shows the confocal microscopic images of the MCF7 and MCF7/MDR cell lines after incubation with free Dox, Lipo-Dox and Dox stealth liposome. As shown in Figure 1A, the visible red fluorescence of Dox was mainly distributed in the nuclear compartment in the free Dox treatment group.Contrary, in MCF7/MDR cells, free Dox was not effectively uptaken due to the P-gp expressed on the plasma membrane (Figure 1B). On the other hand, the cellular uptake of lipo-Dox and Dox stealth liposomes exhibited no significant differences in both cell lines (Figure 1A and 1B), indicating that effective cellular uptake achieved by our drug-loaded liposomes (Dox stealth liposome) was similar to that of commercial available stealth liposomes (Lipo-Dox). MCF7/MDR cells treated with lipo-Dox and Dox stealth liposome displayed intensive and punctate Dox fluorescence in both nuclear and intracytoplasmic vesicles.Interestingly, bothstealth liposomes promoted and enhanced their subsequent uptake by multidrug resistance cells (MCF7/MDR cellines), whereas free doxorubicin enter cells with difficulty (Figure 1B).This suggesting that the drug must be encapsulated or complexed to the liposomes to overcome MDR and did not impact the cellular uptake efficiency in MCF7/MDR cells(Figure 1B).

A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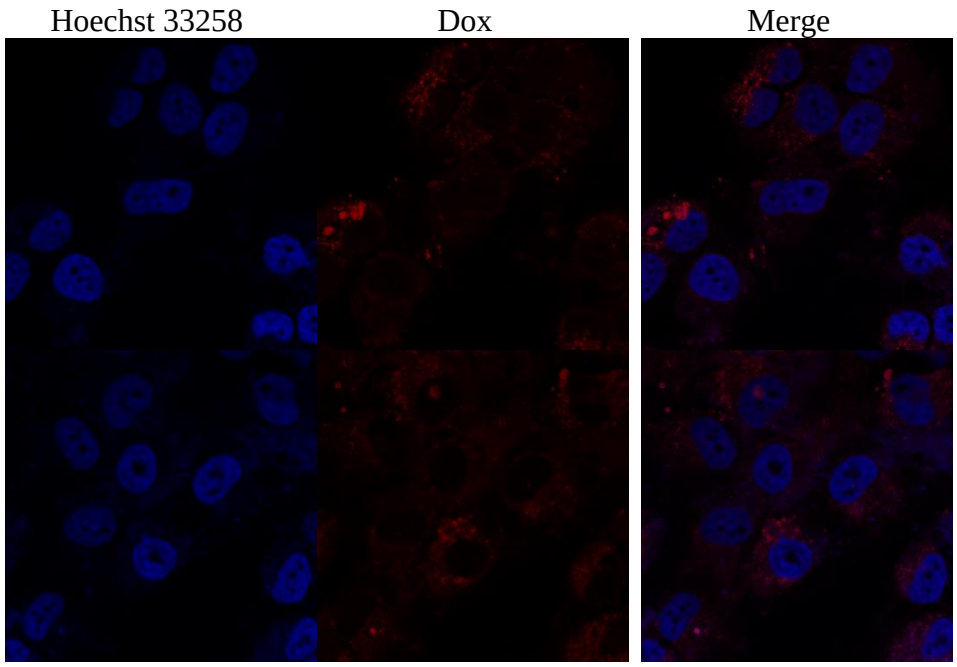
Dox stealth
liposome
MCF7 cell line

B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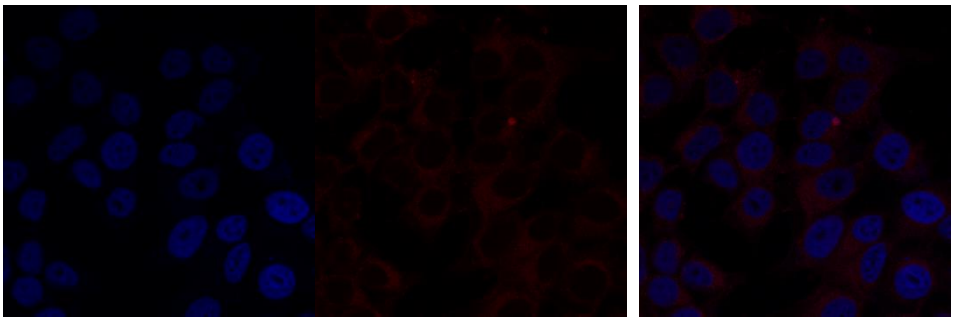

Figure 1. *The confocal microscopy images of MCF7 cells (A) and MCF/MDR cells (B) incubated with free Dox,Lipo-Dox and Dox stealth liposome.Cells were treated with Hoechst 33258 for nucleus staining. Red: DOX fluorescence, Blue: Fluorescence of the nucleus. A. Comparison of cellular uptake of free Dox, Lipo-Dox and Dox stealth liposome for the MCF7 cell line; B. Comparison of cellular uptake of free Dox, Lipo-Dox and Dox stealth liposome for the MCF7/MDR cell line.*

Next, low cytometry was used to quantify the total Dox uptake by MCF7 cells and MCF/MDR cells for different Dox formulations. Two cell lines were incubated with free Dox, Lipo-Dox and Dox stealth liposome. The results showed that, in both cell linescellular Dox level showed no significant difference between Lipo-Dox and Dox stealth liposomes (The data not shown). These flow cytometry results confirmed the finding from the confocal microscopy observations that our Dox stealth liposomes could effectively uptaketo cancer cells and overcome MDR. These results indicate that the new nano-formulation possess similar efficacy in cellular uptake in comparison with Lipo-Dox.

3.3 Toxic effects

In our study, Dox were administered to rabbits in the same dosing-schedule to compare their chronic toxic effects. The doses of Dox administered to animals were comparable with those used in patients.

Biochemical parameters

The doxorubicin-induced nephropathy in rabbits was also described in other papers [18]. However, no significant difference in serum levels of urea and creatinin was observed in animals
exposed to nano-formulations of Dox and free Dox ($P > 0.05$) at different time points (Table 2). Thus, in our study, Dox has not influenced kidney function during the study period.

Table 2*: The effects of various Dox formulations treatment on serum levels of urea and creatinin*

Time points of blood draw	Urea (μmol/l)		Creatinin (μmol/l)	
	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)
T_1	6,58 ± 0,57	6,67 ± 0,35	80,0 ± 16,1	82,1 ± 13,6
T_2	6,95 ± 1,2	7,06 ± 1,09	89,2 ± 23,2	96,4 ± 11,3
T_3	6,37 ± 1,21	6,84 ± 0,83	86,7 ± 23,8	96,8 ± 14,3

T_4	6,96 ± 0,31	6,50 ± 0,50	91,4 ± 30,1	87 ± 16,1
p	p_{2-1}> 0,05 p_{3-1}> 0,05 p_{4-1}> 0,05	p_{2-1}> 0,05 p_{3-1}> 0,05 p_{4-1}> 0,05	p_{2-1}> 0,05 p_{3-1}> 0,05 p_{4-1}> 0,05	p_{2-1}> 0,05 p_{3-1}> 0,05 p_{4-1}> 0,05
	p_{I4-II4} > 0,05		p_{I4-II4} > 0,05	

Notes: 4 time points: T1 (before injection); T2 (1 week after injection); T3 (2 weeks after injection) and T4 (4 weeks after injection)

As shown in Table 3, liver injury markers including AST, ALT and GGT has been observed. The AST and ALT levels in both groups significantly increased just after a week of injection. Most of these changes were more expressed in free Dox than in Dox stealth liposome group. The activity of GGT in Dox stealth liposome groups was not changed at all, whereas in the free Dox administration increased significantly after four weeks of injection (Table 3).

The liver appeared relatively resistant to Dox toxicity unlike heart, probably due to higher activities of antioxidant enzyme systems and lower accumulation of Dox in comparison with heart [19]. Nevertheless, the hepatotoxic effect of doxorubicin in rabbits was reported in one study [20].Surprisingly, in our study, significantdistinct changes in thebiochemical parameters characteristic for hepatotoxicitywere present in our free Doxorubicin group ofanimals (Table 3).These results contributed further confirmation of the previous studies [20]. However, the results in Table 3 shows that Dox stealth liposome seem to slowdown this process compared to free Dox on hepatocytes.

Table 3: *The effects of various Dox formulations treatment on serum levels of AST, ALT, GGT.*

Time points of blood draw	AST (U/l)		ALT (U/l)		GGT (U/l)	
	Dox stealth liposome (I) (X_{TB} ± SD)	Free Dox (II) (X_{TB} ± SD)	Dox stealth liposome (I) (X_{TB} ± SD)	Free Dox (II) (X_{TB} ± SD)	Dox stealth liposome (I) (X_{TB} ± SD)	Free Dox (II) (X_{TB} ± SD)
T_1	15,3 ± 6,6	17,8 ± 6,2	30,7 ± 7,5	36,1 ± 6,8	7,5 ± 1,5	8,4 ± 1,6
T_2	26,4 ± 10,8	24,8 ± 9,8	33,3 ± 10,7	56,4 ± 9,3	6,7 ± 1,2	8,1 ± 2,3
T_3	26,0 ± 5,2	23,5 ± 7,2	43,6 ± 13,3	63,8 ± 17,6	7,0 ± 2,0	9,0 ± 4,1
T_4	38 ± 11,2	49,0 ± 22,1	69,7 ± 18,8	91,8 ± 11,1	8,0 ± 2,0	12,6 ± 4,6
p	p_{2-1}< 0,05 p_{3-1}< 0,05 p_{4-1}< 0,05	p_{2-1}< 0,05 p_{3-1}< 0,05 p_{4-1}< 0,01	p_{2-1}< 0,05 p_{3-1}< 0,05 p_{4-1}< 0,01	p_{2-1}< 0,05 p_{3-1}< 0,01 p_{4-1}< 0,01	p_{2-1}> 0,05 p_{3-1}> 0,05 p_{4-1}> 0,05	p_{2-1}> 0,05 p_{3-1}> 0,05 p_{4-1}< 0,05
	p_{I4-II4} < 0,05		p_{I4-II4} < 0,05		p_{I4-II4} < 0,05	

Notes: 4 time points: T1 (before injection); T2 (1 week after injection); T3 (2 weeks after injection) and T4 (4 weeks after injection)

Total CK activity in both groups were not changed significantly at the all the time points of sampling. CK-MB is a golden standard indicator of myocardial damage or death [21]. Marked increase of serum CK-MB was observed in free Dox-injected rabbits compared with nano-formulation group after 4 weeks of injections ($P < 0.05$). In addition to CK-MB, the release of LDH which is another biochemical index of myocardial injury [22]. LDH levels in both groups increased significantly after 4 weeks of injection and there was no difference between the two groups (Table 4). These indicated that encapsulation in

liposomes might reduce the cardiac cell injury of Dox.These findings were partly consistent to results of other authors, who stated that administrationof free Dox in doses 250±340mg/m2 (22.7-31 mg/kg) caused severe myocardial damage in the rabbits [23-24].

Table 4: *The effects of various Dox formulations treatment on serum levels of CK, CK-MB and LDH*

Time points of blood draw	CK (U/l)		CK-MB (U/l)		LDH (U/l)	
	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)
T_1	394± 90,7	420,2 ± 120	239,3± 64,2	222,1±57,9	57,8 ± 8,3	68,3 ± 18,6
T_2	387 ± 146	346,3± 120	241,2 ± 90,4	201,9 ± 34	54,6 ± 19,4	64,3 ± 7,2
T_3	341,5± 85,5	416,4±157	202,2 ± 27,4	229,6±92	53,6 ± 16,2	56,7 ± 22,1
T_4	432,3 ± 157	474,2 ± 221	209,2 ± 61,8	301,6±165	98,6 ± 43,2	103 ± 18,8
p	$p_{2-1} > 0,05$ $p_{3-1} > 0,05$ $p_{4-1} > 0,05$	$p_{2-1} > 0,05$ $p_{3-1} > 0,05$ $p_{4-1} > 0,05$	$p_{2-1} > 0,05$ $p_{3-1} > 0,05$ $p_{4-1} > 0,05$	$p_{2-1} > 0,05$ $p_{3-1} > 0,05$ $p_{4-1} < 0,05$	$p_{2-1} > 0,05$ $p_{3-1} > 0,05$ $p_{4-1} < 0,05$	$p_{2-1} > 0,05$ $p_{3-1} > 0,05$ $p_{4-1} < 0,05$
	$p_{I4-II4} > 0,05$		$p_{I4-II4} < 0,05$		$p_{I4-II4} > 0,05$	

Notes: 4 time points: T1 (before injection); T2 (1 week after injection); T3 (2 weeks after injection) and T4 (4 weeks after injection)

3.4 Haematological parameters

Decrease of erythrocytes, haemoglobin and haematocrit was not so conspicuous in the rabbits treated with Dox stealth liposome, whereas the decrease in these parametes was statistically significant even in comparison with the free Dox group. Free Dox administration led to significant decrease of erythrocytes, haemoglobin and haematocrit just 2 weeks after injection (Table 5). In the Dox stealth liposome group, the number of erythrocytes, haemoglobin and haematocrit after the first 2 weeks of injection did not change significantly, butsignificant decrease was present after 4 weeks of injection.Decrease levels of erythrocytes and haemoglobin were lower in the Dox stealth liposome group than that in the free Dox group (Table 5).These findings might indicate the bone marrow damage when treated with free Dox, as indicated by a significant decrease in erythrocytes count, haemoglobin and haematocrit (Table 5). Surprisingly, using Dox stealth liposome group could significantly improve thisdamage.

From the results shows Table 6, both free Dox and Dox stealth liposome administration led to significant decrease of leucocytes, neutrophils, lymphocytes after the first injection. These parameters also significantly reduced after the second injection but tended to recover after two weeks of discontinuation. Dox stealth liposome groups had a slower decline (after the second injection) and lower leucocytes counts compared with the free doxorubicin group.

Table 5. *The effects of various Dox formulations treatment on serum levels of erythrocytes, haemoglobin and haematocrit*

Time points of blood draw	erythrocytes (T/l)		haemoglobin (g/l)		haematocrit (l/l)	
	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)
T_1	5,76 ± 0,55	5,64 ± 0,68	115,6 ± 11	115,5 ± 13	0,36 ± 0,03	0,35 ± 0,04
T_2	5,65 ± 0,76	5,70 ± 0,85	112,9 ±15,9	116,7±17,2	0,34 ± 0,05	0,35 ± 0,05
T_3	5,24 ± 1,05	4,92 ± 0,85	102,0 ± 19,9	81,3 ± 31,2	0,31 ± 0,06	0,29 ± 0,04
T_4	4,25 ± 0,79	3,51 ± 0,67	85,7 ± 13,6	73,5 ± 11,4	0,25 ± 0,03	0,22 ± 0,02
P	$p_{2-1}> 0,05$ $p_{3-1}> 0,05$ $p_{4-1}<0,05$	$p_{2-1}> 0,05$ $p_{3-1}< 0,05$ $p_{4-1}< 0,01$	$p_{2-1}> 0,05$ $p_{3-1}> 0,05$ $p_{4-1}< 0,05$	$p_{2-1}> 0,05$ $p_{3-1}< 0,05$ $p_{4-1}< 0,01$	$p_{2-1}> 0,05$ $p_{3-1}> 0,05$ $p_{4-1}< 0,05$	$p_{2-1}> 0,05$ $p_{3-1}< 0,05$ $p_{4-1}< 0,01$
	$p_{I4-II4} < 0,05$		$p_{I4-II4} < 0,05$		$p_{I4-II4} > 0,05$	

Notes: 4 time points: T1 (before injection); T2 (1 week after injection); T3 (2 weeks after injection) and T4 (4 weeks after injection)

Incidence of thrombocytopenia and leukopenia was reported in most of anthracycline studies [25-26]. The results finding in this study were consistent with these studies and proved that our liposome product could improve this toxicity.

Table 6. *The effects of various Dox formulations treatment on serum levels of leucocytes, neutrophils, lymphocytes*

Time points of blood draw	leucocytes(G/l)		neutrophils (G/l)		lymphocytes (G/l)	
	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)	Dox stealth liposome (I) ($X_{TB} \pm SD$)	Free Dox (II) ($X_{TB} \pm SD$)
T_1	5,84 ± 1,18	5,53 ± 0,87	3,95 ± 0,89	3,47 ± 0,65	1,58 ± 0,66	1,79 ± 0,76
T_2	5,80 ± 1,9	5,01 ± 1,9	3,41 ± 1,73	2,96 ± 0,6	1,85 ± 1,1	1,13 ± 0,76
T_3	3,28 ± 1,55	2,42 ± 0,88	1,81 ± 1,20	1,35 ± 0,8	0,91 ± 0,4	0,28 ± 0,15
T_4	3,62 ± 2,6	3,73 ± 0,51	2,15 ± 1,1	2,97 ± 0,67	1,15 ± 0,6	0,60 ± 0,2
P	$p_{2-1}> 0,05$ $p_{3-1}< 0,01$ $p_{4-1}<0,05$	$p_{2-1}< 0,05$ $p_{3-1}< 0,01$ $p_{4-1}< 0,05$	$p_{2-1}> 0,05$ $p_{3-1}< 0,01$ $p_{4-1}< 0,01$	$p_{2-1}> 0,05$ $p_{3-1}< 0,01$ $p_{4-1}< 0,05$	$p_{2-1}> 0,05$ $p_{3-1}< 0,05$ $p_{4-1}< 0,05$	$p_{2-1}> 0,05$ $p_{3-1}< 0,01$ $p_{4-1}< 0,01$
	$p_{I4-II4} > 0,05$		$p_{I4-II4} < 0,05$		$p_{I4-II4} < 0,05$	

Notes: 4 time points: T1 (before injection); T2 (1 week after injection); T3 (2 weeks after injection) and T4 (4 weeks after injection)

3.5 Histological evaluation

Last, we checked the morphological changes in rabbit liver and heart treatedwith free Dox and Dox stealth liposomes. The histopathology results of samples taken from the liver did not reveal distinct pathological changes when treated with Dox stealth liposomes, whereas in the free Dox group, pitting degenerated central vein, congestive sinus vessels, and lymphocytic invasive lobes appeared.Moreover, there was toxic injury of the heart with the appearance of degenerated myocardial cells appeared for the free Dox administration, but almost no change with the samples treated with Dox stealth liposomes (The data not shown). Thus, in our study, these histological findings closely correlated with the significant alterations in some of biochemical parameters found in the above results (Table 2,3,4).

4. CONCLUSIONS

The assessment performed in this study has indicated that our nano formulation (Dox stealth liposome) and commercial available stealth liposome (Lipo-Dox) are similar in terms of cellular uptake efficiency. Dox stealth liposome group could reduce significantly toxicity for heart, liver andbone marrow.This study demonstrated that stealth liposome-associated doxorubicin provide an effective delivery of drug to sensitize cells to circumvent MDR and to enhance the therapeutic index of the chemotherapy.

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In silico Study of The Pharmacologic Properties of Bioactive Compounds Isolated from The Fruits of Three Species of *Cleistanthus* genus (Euphorbiaceae)

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Abstract:

Several Cleistantoxin analogues, the major active component of *Cleistanthus* genus, were characterized in this work by theoretical methods. The molecular modeling approaches were explored to screen the molecular targets to which Cleistantoxin and other lignans can interact to produce the cytotoxic effects. Molecular docking simulations revealed that these compounds tightly bound to DNA topoisomerase IIB and induced apoptosis *via* extrinsic pathway. Subsequently, the predicted physicochemical properties are in agreement with the solubility and cLogP values, the penetration across the cell membrane, and absorption values, as well as with a possible apoptosis-activated mechanism of cytotoxic action. Finally *in silico* ADMET (Absorption, Metabolism, Distribution, Excretion and Toxicity) profiling revealed that all compounds fulfilled Lipinski's drug-like rules, while showing quite favorable pharmacokinetic and low toxicological profiles.

Keywords: Chem-bioinformatics, cleistantoxin, aryltetralin lignans, *Cleistanthus* genus, anti-cancer drug.

1. INTRODUCTION

The discovery of anti-cancer drug from natural resources is becoming a main direction of pharmaceutical research all over the world [1]. Hartwell, in his review of plants used against cancer, lists more than 3000 plan species that have long been used for the treatment of different cancer types. Over 60% of currently utilized anti-cancer agents are derived in

one way or another from natural sources, including plants, marine organisms and micro-organisms [2]

Recently, our screening program in Vietnam indicated that the ethyl acetate extracts of the fruits of 3 species of *Cleistanthus* genus *(C. indochinensis* Merr. ex Croiz; *C. tonkinensis* Jabl; *C. eberhardtii* Gagn Euphorbiaceae) showed strong cytotoxicity against KB cells (>90% inhibition at 1 μg/mL) [3, 4]. Cleistantoxin and some aryltetralin lignans were found from those species (Figure 1), in which Cleistantoxin, the major active component, had significant cytotoxic against MCF-7 (IC_{50}=0.036 μM), MCF-7R (IC_{50}=0.014 μM), and HT29 (IC_{50}=0.035 μM) cancer cell lines [3]. However, the therapeutic mechanism related to their potential bioactivity remains unclear. It is of great interest to determine the possible receptor of these natural products as well as the mechanism of their cytotoxicity effect.

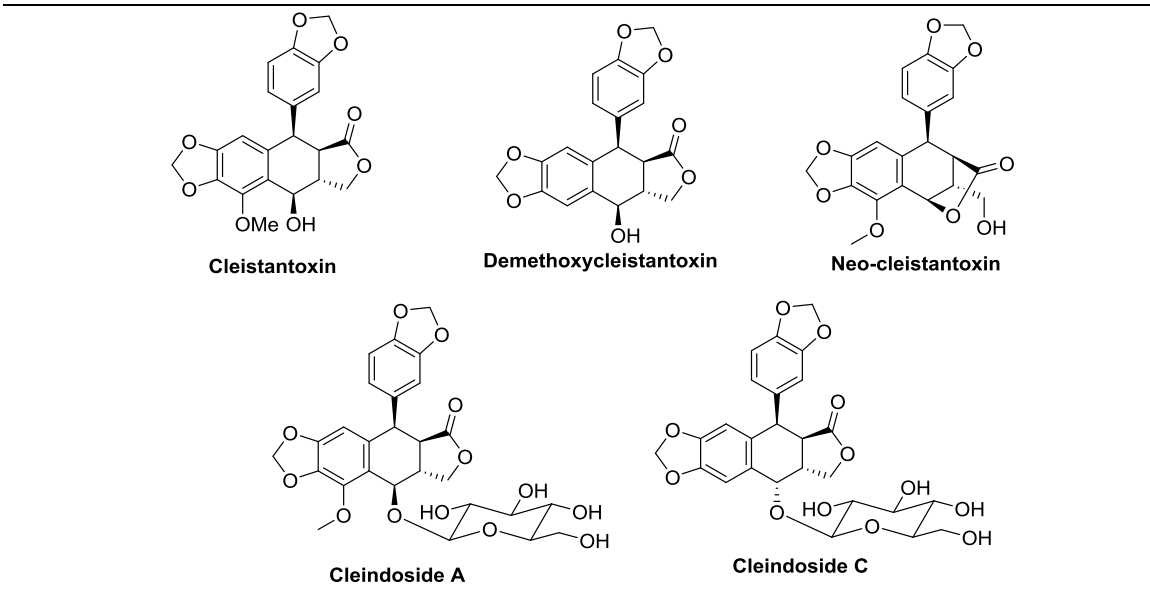


Figure 1. *Cleistantoxin and other aryltetralin lignans extracted from several Cleistanthus genus in Vietnam.*

In this context, computational chemistry methods (also known as *in silico* approaches) have widely been applied in both virtual screening of new bioactive agents and the study of their possible mechanisms of action [5]. Recently, Natasenamat *et al*. have revised numerous up-to-date computational approaches used for the research and development of new natural-occurring drugs [6]. Among the many approaches mentioned, docking studies provide models of the possible interactions of protein residues with ligands, and can also furnish potential explanations for the therapeutic action on a protein receptor [6]. Throughout docking assays, binding affinity, hydrogen bonds, and bonding energy values can be estimated as the main criteria for the elucidation of ligand-receptor interaction mechanisms and the possible therapeutic action of drugs under study. Binding poses with the lowest binding free energy, the number and energy of hydrogen bonds and bonding energy are important to select the "optimum conformation" of the ligand in the binding site of the receptor protein [7]. On the other hand, the chemoinformatics approaches allow characterizing certain physicochemical properties of studied molecules that define the successfulness of novel chemical agents in the drug discovery process, such as ability of the molecules to transport through cell membrane, the aqueous solubility, or metabolism processes [5, 7].

Taking into account the aforementioned issues, this study aims at exploring the molecular modeling approaches to screen the molecular targets to which Cleistantoxin and other lignans can interact to produce the cytotoxic effects. In addition we investigated the physicochemical and ADMET (Absorption, Metabolism, Distribution, Excretion and Toxicity) properties of these compounds.

2. MATERIALS AND METHODS

2.1 Docking Study

The compounds were firstly subjected to a similarity searching using Pubchem database (https://pubchem.ncbi.nlm.nih.gov/), using Tanimoto index threshold of 0.85 as the criteria of structural similarity. After identifying the lead templates, we performed precise docking simulation using Glide SP to study the binding modes of the compounds against the template-bound targets.

The crystal structure of the target protein was selected and prepared using the Protein Preparation Wizard in Maestro v.9.2 (Schrödinger, LLC, NY, USA).

For ligand preparation, the 3D structure of the compounds was generated with softwares

from OpenEye Scientific Software Inc (https://www.eyesopen.com/). First, a library of conformers of molecular fragments was created with the makefraglib program, setting the force field to mmff94s, which is the exact reproduction of the 94s variant of the Merck Molecular force Field (MMFF94s). Then, the model builder in OMEGA version 2.3.2 was used to generate a maximum of 10 conformations per compound in the dataset keeping the same force field.

During the docking process, the ligand was not rigid and allowed to torsion in the first blind docking processes. The grid box was 15 Å x 15 Å x 15 Å, encompassing the ligand binding cavity of the protein. Only a maximum of five surfaces for docking were fixed for the typical docking process. The binding modes were clustered using the root-mean-square deviation among the cartesian coordinates of the ligand atoms. The docking results were ranked according to the binding free energy and the root mean standard deviation RMSD < 2 Å).

The binding free energy (ΔG_{bind}) was subsequently calculated using the coupled parameters of Prime tool from Schrodinger suite. Local optimization refinement within 5 Å of the docked ligand was performed. During this process, the side chain conformations of the selected protein residues were predicted and minimized and subsequently minimized along with the docked ligand. The results were further energy-minimized using a Monte Carlo sampling algorithm in 6000 steps.

2.2 Pharmacological and Metabolic Properties

Absorption and metabolic properties of the studied compounds were calculated by using the admetSAR server [8], which predict about 50 ADMET endpoints using a chemoinformatics-based toolbox, called ADMET-Simulator, which integrates high quality and predictive QSAR models. The proposed human metabolism for the target compounds was made using MetaPrint2D-Reaction software, a prediction set of xenobiotic metabolism by means of data-mining and statistical analysis of known metabolic transformations reported in scientific literature [9].

3. RESULTS AND DISCUSSION

3.1 Docking study

Cleistantoxin showed structural similarity to podophyllotoxin, while Cleidoside A and C, Demethoxycleistantoxin and Neo-cleistantoxin are similar to etopoide and teniposide with Tanimoto index > 0.90. To our best knowledge, podophyllotoxin is a potent inhibitor of microtubule assembly which can interact with numerous targets involved into the cytotoxicity pathway (Figure 2) [10, 11]. Table 1 summarized possible biological targets for podophyllotoxin based molecules.

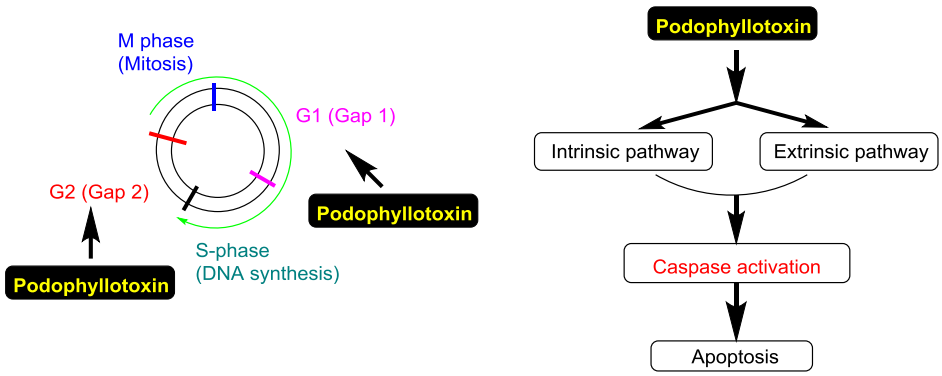
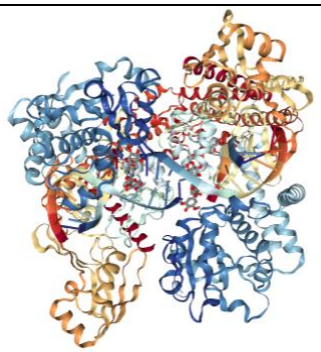
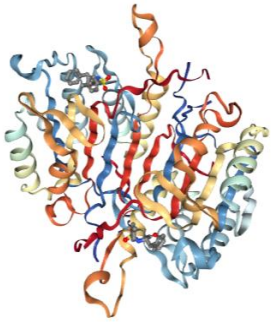
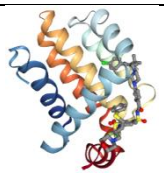
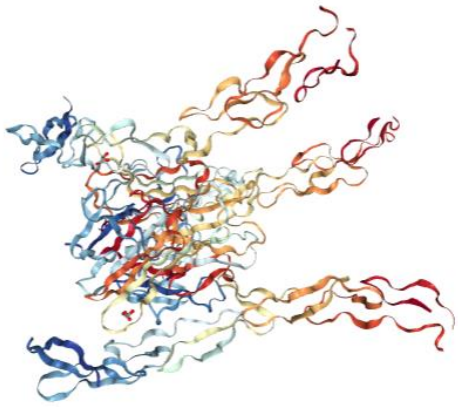
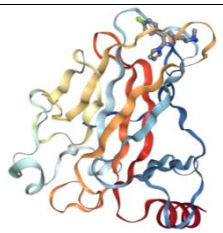


Figure 2. *Graphical representation of mechanism of action of podophyllotoxin*

Table 1. *Some possible targets of podophyllotoxin and studied molecules*

No	Protein	PDB code	3D structure
1	DNA topoisomerase IIB	3QX3 [12]	
2	Caspase 3	2XYG [13]	

3	Bcl-2	4LVT [14]	
4	Cd95l	4MSV [15]	
5	p53	3ZME [16]	

Molecular docking simulations revealed that these compounds tightly bound to DNA topoisomerase IIB (PDB ID: 3qx3) [12]. Main interactions involve hydrogen bonds between cyclolignan moieties and nitrogen-containing nucleobases of DNA units, such as DC8, AD12, and DG13, and numerous stacking interactions with hydrophobic residues, including Asp479, Arg503, and Met782. According to intrinsic pathways of apoptosis, we considered the activation of procaspase 3 and 7 as critical in the first step. The docking results on caspase 3, however, showed no interaction between Cleistantoxin and the protein. In addition, Cleistantoxin could not bind towards the active site of activate B cell lymphoma Bcl-2, which regulates the proapopototic BAX by BCLXL through constant retrotranslocation of BAX from the mitochondrial outer membrane (MOM) to the cytosol of malignant cells [14]. Therefore we rejected the hypothesis of apoptosis mechanism *via* tumor suppressor p53-independent route. The next step involved in docking Cleistantoxin and other analogues into the binding site of extrinsic pathway targets.

The extrinsic pathway was analyzed by interaction of the target molecules with the membrane receptors such as CD95L (4MSV) [15]. The molecules Cleistantoxin, Neo-cleistantoxin and Cleindoside A and C have good docking results with these proteins, showing the best interaction with Cleistantoxin (score = -98.245 kcal/mol), followed by

Cleindoside A (score = -90.112 kcal/mol) and a lesser interaction with Neo-cleistantoxin (score = -70.981 kcal/mol). The docking results on tumor suppressor p53 protein were also good. The activation of these receptors makes that the caspases 8 and 10 were activated. In addition, these enzymes can activate the caspases 7 and 3, or stimulate the conversion of BID in t-BID, promoting an interaction of t-BID with BAX or BAK and consequently producing mitochondrial stress.

Based on our analysis, we proposed that those studied compounds can interact with the FasL (CD95L) receptor, so these molecules can inhibit the interaction among the caspase 8 and BID and suppress the extrinsic apoptotic pathway. Since it does not have a molecular docking conformation with PARP-1, the prospective pathway inside of the cell (considering the big predicted membrane permeability) is the activation of the proteins t-Bid and BAK (considering the moderate affinity in comparison with the other target molecules) [16].

3.2 Pharmacological and Metabolic Properties

Complementary and interesting results, considering the potential use of the studied molecules as drug, are the prediction of its absorption, metabolic and excretion properties of the studied molecules in humans [8].

The behavior in different absorption, metabolism and excretion models were predicted using admetSAR methodology and the results are given in Table 2 and 3 [2].

Table 2. *Absorption, distribution and elimination profiling of Cleistantoxin and other compounds*

Compounds	BBB[a]	HIA[b]	Caco-2 Perm.[b]	Pgp subs.	Pgp inhibitor	Renal Organic Cation Transporter
Cleistantoxin	High (0.64)	High (0.99)	High (0.71)	NS (0.51)	NI (0.65)	NI (0.80)
Demethoxycleistantoxin	High (0.84)	High (0.99)	Low (0.53)	NS (0.84)	NI (0.79)	NI (0.82)
Neo-cleistantoxin	High (0.71)	High (0.98)	High (0.74)	NS (0.51)	I (0.87)	NI (0.75)
Cleindoside A	Low (0.94)	High (0.51)	Low (0.77)	S (0.68)	NI (0.79)	NI (0.86)
Cleindoside C	Low (0.94)	High (0.51)	Low (0.77)	S (0.68)	NI (0.69)	NI (0.86)

[a]Blood-Brain Barrier distribution; [b]Human Intestinal Absorption; [c]intestinal Caco-2 cell permeability; S = substrate, I = inhibitor, NI = non-inhibitor NS = non-substrate, number in parenthesis is prediction probability.

As can be observed in Table 2, all the compounds showed good intestinal absorption profiles. A model considered the "gold standard" for the drug permeability is the human colon adenocarcinoma (Caco-2) monolayer cell culture which is widely used in drug discovery and has been recommended by the Food and Drug Administration (FDA) [17], in this sense the prediction for the target molecules show higher permeability for cleistantoxin and neo-cleistantoxin compared to other analogues. Regarding the P-glycoproteins, this molecule is one of the mayor ABD transporters, distributed in several tissues, and involved in the clearance of xenotoxins against steep concentration gradients, at the expense of ATP hydrolysis [18]. In this sense, three molecules Cleistantoxin, Demethoxycleistantoxin, and Neo-cleistantoxin showed possibilities of efflux metabolism escape.

Another interesting result was to predict the metabolism of the studied compounds using admetSAR and MetaPrint2D-React methodology. The behavior as substrate or inhibitor of the target molecules in the most important isoforms of the cytochrome P450 participants in the metabolism of therapeutic drugs was evaluated [19]. During the biotransformation of the drugs, the molecules are broken down and/or converted into more soluble molecules, which play important roles in the pharmacokinetic and therapeutic action of drug molecules. According to the metabolism prediction, Cleindoside A and C were determined to be potent CYP3A4 substrates. On the other side, Demethoxycleistantoxin showed desirable first-pass metabolism behavior, as it can inhibit numerous cytochrome isoforms (CYP1A, CYP2C and CYP3A). Given this advantage, this compound could significantly enhance the bioavailability of co-administrated compounds.

At last, toxicological and physicochemical profiles of cleindosides were evaluated using admetSAR server. The toxicity risk predictors showed that the compounds have low risk of undesirable effects, such as: mutagenicity, tumorgenicity, irritant and reproductive effects. In addition, the drug scores (DS), which are the combination of drug likeness, cLogP, log S, molecular weight and toxicity risks was calculated for all the compounds. The results suggested that our analyzed compounds are suitable for drug use.

Table 3. *Prediction of metabolism*

Metabolism Model	Cleistantoxin	Demethoxycleistantoxin	Neo-cleistantoxin	Cleindoside A	Cleindoside C
CYP450 2C9 Substrate	NS (0.80)	NS (0.84)	NS (0.74)	NS (0.80)	NS (0.80)
CYP450 2D6 Substrate	NS (0.89)	NS (0.89)	NS (0.86)	NS (0.88)	NS (0.88)
CYP450 3A4 Substrate	NS (0.59)	NS (0.67)	NS (0.59)	S (0.58)	S (0.58)
CYP450 1A2 Inhibitor	NI (0.62)	I (0.61)	NI (0.71)	NI (0.93)	NI (0.93)
CYP450 2C9	I (0.93)	I (0.69)	I (0.81)	NI (0.81)	NI (0.82)

Inhibitor					
CYP450 2D6 Inhibitor	NI (0.65)	NI (0.76)	NI (0.54)	NI (0.94)	NI (0.93)
CYP450 2C19 Inhibitor	I (0.91)	I (0.62)	I (0.82)	NI (0.76)	NI (0.70)
CYP450 3A4 Inhibitor	I (0.79)	I (0.57)	I (0.82)	NI (0.63)	NI (0.61)

S = substrate, I = inhibitor, NI = non-inhibitor NS = non-substrate, number in parenthesis is prediction probability.

4. CONCLUSION

Our results suggest that bioactive compounds extracted from the fruits of the *Cleistanthus* genus may potentially inhibit DNA topoisomerase IIB and induced tumor cell apoptosis via p53-dependent extrinsic pathway. These substances also have ADMET characteristics suitable for future oral anti-cancer drug development.

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Prevalence of OXA-type ESBLs Producing *Escherichia coli* in Thailand

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Abstract

Extended spectrum β-lactamases (ESBLs) producing Gram-negative bacteria become a health threat worldwide. ESBLs are resistant to many penicillin and cephalosporins. The OXA-type ESBLs are mostly detected in *Pseudomonas* spp. and *Acinetobacter*spp. but there is less reported in *E. coli*. The aim of this study was to determine the prevalence of OXA-type ESBLs producing *E. coli* in Thailand.Total of 350 *E. coli* isolates were collected from tertiary care hospitals located in Thailand during 2014 to 2015. ESBLs producing *E. coli* were screened by disc diffusion method by cephalosporins and confirmed using combination disc diffusion method. Antimicrobial susceptibility of ESBLs producing *E. coli* was determined by broth dilution. The *bla*OXA gene was detected by PCR method andwas identified by DNA sequencing.ESBLs producing *E. coli* were found 285 out of 350 isolates. Interestingly, the *bla*OXA gene could be detected only one isolate. Further study was identified it as OXA-10.In addition, OXA-10isolaterevealed strong resistance to imipenem. This study demonstrated that the *bla*OXA type ESBLs producing *E. coli* did exist in Thailand. Although the *bla*OXA genes were mostly reported in *Acinetobacter*spp. and *Pseudomonas* spp., this study showed the presence of OXA-type ESBLs producing *E. coli*. The OXA-10 type was known to have narrow spectrum β-lactamase activity. This enzyme has been associated with infection of Gram-negative bacteria in the last two decades restricting therapeutic options which was posed a serious threat for health care-associated infections.

Keywords: ESBLs, *E. coli*, OXA, cephalosprins, beta-lactamases.

1. INTRODUCTION

Strains of *E. coli* have been reported worldwide.*E. coli*is the most common cause of UTIs, accounting for about 85% of community-acquired and 50% of hospital-acquired infections (1). Resistance of *E.coli* to broad-spectrumcephalosporins may be mediated by ESBLs which areenzymesthat have ability to hydrolyzed penicillin and 3rdgeneration cephalosporins. These enzymes are encoded by different gene located in chromosome or plasmid. ESBLs have several types including TEM, SHV, CTX-M, OXA, VEB, GES and PER. Most of them are belonging to molecular class A except OXA-type. OXA-type ESBLsare classified in the molecular class D(2).The *bla*OXA gene was first detected in the 1980s isolated from *Pseudomonas* spp. (3) The OXA-type confers resistance to ampicillin and cephalothin. They are characterized by their high hydrolytic activities against oxacillin and cloxacillin (4). Infections from ESBLs producing strainscan result in high rates of mortality and morbidity, and consequently high economic costs are associated with treatment (5).The OXA-type ESBLs are mostly detected in *Pseudomonas* spp. and *Acinetobacter* spp. but there is less reported in *E. coli*. Therefore, this study was aimed to determine the prevalence of OXA-type ESBLs producing *E. coli* in Thailand.

2. MATERIALS AND METHODS

2.1 E.coli isolation

Total of 350 *E. coli* isolates were collected from tertiary care hospitals located in Thailand from 2014 to 2015. *E. coli* strains were confirmed by MALDI-TOF Mass Spectrometry and biochemical tests.

This study was approved by Mahidol University Institutional Review Board (MU-IRB) [Approval No. MU-IRB 2014/019.0705].

2.2 Combination disc diffusion method

Strains of *E. coli*were grown in Mueller-Hinton broth (MHB) at 37^{o}C, for 18 h. *E. coli* suspension were adjusted turbidity at0.5 McFarland (approximately 10^{8} CFU/ml). Then, bacteria cells were cultivated on Mueller-Hinton agar (MHA). Discs of ceftazidime (30 µg), ceftazidime/clavulanate (30/10 µg), cefotaxime (30 µg) and cefotaxime/clavulanate (30/10 µg) were placed on MHA at distance 30 mm center to center. Finally, the plate was then incubated at 37^{o}C for 18 h. Interpretation criteria were followed the CLSI2016 guideline (6). The test result is positive if the inhibition zone diameter is 5 mm or larger with clavulanate than without. The strain of *K. pneumoniae* ATCC700603

(carrying*bla*SHV-18 gene) was used for a positive control and *E. coli* ATCC25922 was used for a negative control in this study.

2.3 Antimicrobial susceptibility

ESBLs producing *E. coli* weredetermined MIC values by broth micro-dilution with 4 selected antibiotics including ceftazidime, imipenem, meropenem and piperacillin/tazobactam. Strains of ESBLs producing *E. coli*were grown in MHB. Then,*E. coli* isolates were adjusted turbidity at0.5 McFarland(approximately 10^8 CFU/ml)and diluted to adjust the cell to 10^6 CFU/ml before added into 96-well plates containing antibiotics in triplicates. Concentrations of antibiotics were performed based on the CLSI 2016 guideline. Finally, the plates were incubated at 37^{o}C for 18 h. The results were evaluated by observed the MIC values of the minimum concentration of the drugs that gives no visible bacterial growth.

2.4 Detection of the OXA gene

DNA templates were preparedby the commercial kit (Favorgen, Taiwan). ESBLs producing *E. coli*were determined OXA-type ESBLs by PCR method. Two sets of primers were used in this study including *bla*OXA-2 group (OXA-2 group F 5' ATG GCA ATC CGA ATC TTC GC 3' and OXA-2 group R 5' GCA CGA TTG CCT CCC TCT T 3') and *bla*OXA-10 group (OXA-10 group F 5' ATG AAA ACA TTT GCC GCATAT G 3' and OXA-10 groupR 5' TTA GCC ACC AAT GAT GCC CT 3').PCR reactions including 1Xbuffer, 1.5 mM of $MgCl_2$, 400 µM of dNTPs, 0.5 µM of forward and reverse primers each, 1U Taq and 2 ng/µl of DNA template. PCR conditions were performed with pre-heat at 96^{o}C for 3 minute, followed by 30 cycles of DNA denaturation at 96^{o}C for 30 sec, primer annealing at 60^{o}C for 30 sec, extension at 72^{o}C for 30 sec and final extension at 72^{o}C for 10 minute. PCR products were analyzed by agarose gel electrophoresis. Finally, PCR product was analyzed for DNA sequence bases.

3. RESULTS

The CLSL2016guideline was recommended to ESBLs confirmation by combination disc diffusion method**.** To interpret the result, the inhibition zone diameter of cephalosporin combined with clavulanate disc and inhibition zone diameter of cephalosporin disc alone were compared**.** The test is positive if the inhibition zone diameter is ≥5 mm larger with

clavulanate than without **(**Fig 1**).** The result showed that about 285 **(**81**.**42**%)** out of 350 *E.* *coli* isolates were ESBLs producing strains**.**

For antimicrobial susceptibility, four antibiotics including ceftazidime, imipenem, meropenem and piperacillin/tazobactam were used in micro-dilution method**.** The result showed high resistance to ceftazidime at 80**% (**228/285 isolates**)** followed by piperacillin/tazobactam at 5.61% (16/285 isolates) and imipenem at 0.70% (2/285 isolates)**.** Molecular detectionrevealedthat the *bla*OXA gene could be detected only in one isolate from 285 ESBLs producing *E. coli***.** This strain was collected from North-eastern Thailand**.** Further study was identified it as the OXA-10**.** In addition, this strain was highly resistant to ceftazidime and cefotaxime**.** Moreover, OXA-10 producing strain demonstrated strong resistance to imipenem**.**

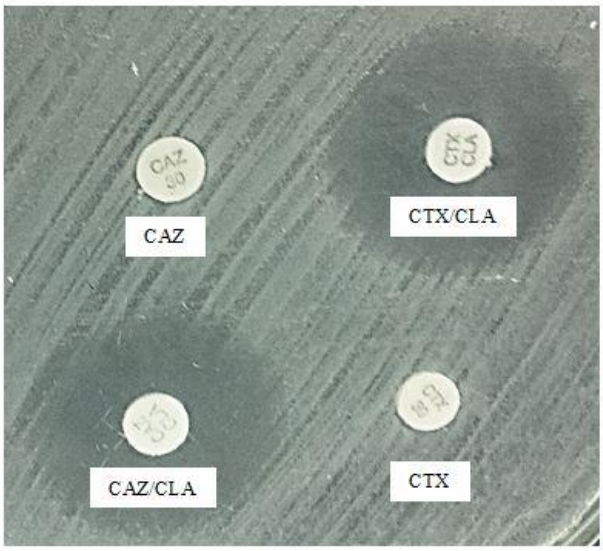

Figure 1. *Confirmation of ESBL production by combination disc diffusion method.*

4. DISCUSSIONS

Our finding based on the evaluation of clinical isolates of *E. coli* obtained from tertiary care hospitals in Thailand. The result from this studyshowed that ESBLs producing *E. coli*possessedhigh level resistance to cefotaxime and ceftazidime. Although, carbapenem and β-lactamase inhibitor were used to treat infection from ESBLs producing bacteria interestingly, some were found some of these strains resistance to imipenem (carbapenem) and piperacillin/tazobactam (penicillin/β-lactamase inhibitor). All isolates of ESBLs

producing *E. coli* susceptible to meropenem.The study of Kiratisin P. in 2008 reported molecular characterization and epidemiology of ESBLs producing *E. coli* and *K. pneumoniae*. The result showed ESBLs producing *E. coli*was predominantly CTX-M type and did not reportthe present of OXA-type ESBLs. In addition, the study of Katvoravutthichai C. found OXA-10 at 15.25%. OXA-10 was reported in *Pseudomonas aeruginosa* isolated from a tertiary-level hospital in Bangkok, Thailand but not in *E. coli* (7).Our findings arethe first detection of OXA-typeESBLs from *E. coli* and reported the OXA-10 producing strain.

5. CONCLUSIONS

This study demonstrated that*bla*OXA type ESBLs producing *E. coli* do exist in Thailand. Although the *bla*OXA genes were mostly reported in *Acinetobacter*spp. and *Pseudomonas* spp., this study showed the presence of *bla*OXA gene in *E. coli* in Thailand. It is interesting that the OXA-10 type has been known to have narrow spectrum β-lactamase activity although variants of this enzyme family have expanded-spectrum activity. This enzyme has been associated with Gram-negative bacterial infections in the last two decades restricting therapeutic options which were posed a serious threat for health care associated infection.

In summary, this study indicated the presence of OXA-type producing in *E. coli*. From all isolates of ESBLs producing *E. coli*, *bla*OXA gene only one strain. Further study will be determined other types of ESBLs.

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Development and Validation of LC-MS/MS Method for Simultaneous Determination of Metformin Hydrochloride and Sitagliptin In Human Plasma

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Abstract

Introduction: Combination in the clinic of metformin hydrochloride 500 mg and sitagliptin 50 mg when monotherapy does not improve glycaemic control. Measurement of systemic concentrations of two drugs is thus of interest both pre-clinically and clinically for therapeutic drug monitoring of diabetic patients to prevent toxicity and ensure adherence to prescribed medications. However, there is a few analytical methods reported for simultaneous determination of these drugs in human plasma that can be useful for routine therapeutic drug monitoring studies. **Objectives:** The purpose of this study was to develop and METidate method for simultaneous quantitative analysis of metformin hydrochloride and sitagliptin in human plasma using liquid chromatography coupled with triple quadrupoles mass spectrometry technique. **Materials and methods:**A method for detection and quantification of metformin and sitagliptin in human plasma was been developed using Acquity H–Class system and Xevo TQD mass spectrometer equipped with an electrospray ionization source. Metformin hydrochloride (MET), sitagliptin (SGT) and phenformin (PF) as internal standard in human plasma were extracted by protein precipitation with methanol, then separated on a C18 reversed phase column, and finally eluted with methanol mixed with 0.1% formic acid solution. The assay was validated in compliance with US-FDA guidelines. **Results:** Metformin and sitagliptin were extracted from human plasma using methanol with 1:5 proportion and separated on a Gemini C18 column (250 mm x 4 mm; 5 µm) with mixture of methanol – water containing 0.1% formic acid (45:55) as eluent. ESI interface was operated in positive mode under MRM conditions, using the transition of *m/z* 130.15 → 71.07, *m/z* 408.02 → 235.11 and *m/z* 206.04 →105.06 to quantify metformin, sitagliptin and phenformin, respectively. The

method has shown high selectivity, good reproducibility, as intra-day and inter-day within 5.23% and linearity of 0.995, LLOQ were 5ppb (metformin) and 1ppb (sitagliptin) and mean recovery of accuracy in the range of 90.59- 104.52%. **Conclusion:** A reliable, simple and robust LC-MS/MS method for the simultaneous quantification of metformin and sitagliptin in human plasma was successfully developed and METidated. Practical applicability of the assay for the bioequivalence study metformin and sitagliptin after oral administration of a fixed dose of the combination.

Keywords: metformin hydrochloride; sitagliptin, human plasma, triple quadrupoles mass spectrometry.

1. INTRODUCTION

Metformin hydrochlorideand sitagliptin are two well known hypoglycemic drugs.The combination of those drug with 500 mg dose for metforminhydrochloride (MET) and 50 mg dose forsitagliptin(SGT) has been proved to have a very good effect on controlling glycaemic. Therefore, a sensitive and specific analytical method is needed for simultaneous determination of two drugs in human plasma for routine therapeutic drug monitoring and pharmacokinetic studies.There have been several published studies on application of modern analytical methods for single determination of each drug, such asHPLC with ultraviolet detector[1,2], capillary zone electrophoresis[3], and especiallyLC–MS/MS[4,5] were reported for the quantitation of either metformin or sitagliptin.This was our motivation to develop and validate a highly sensitive, specific, reproducible, and rapid LC – MS/MS method for simultaneous determination metformin and sitagliptin in human plasma.

2. MATERIALS AND METHODS

2.1.Standards, chemicals and solvents

Reference standard of sitagliptin phosphateand was purchased from Glenmark Generics Ltd., India. Metformin hydrochloride were provided by Institute of Drug Quality Control at Ho Chi Minh City, Vietnam. Phenformin hydrochlorid (internal standard) was purchased from Sigma-Aldrich, USA. Other chemicals and organic solvents such as acetonitrile, methanol, formic acid, etc. were of liquid chromatography grade, and purchased from Merck. Human plasma containing K_2EDTA anticoagulant waspurchased from Blood transfusion hematology hospital in Can Tho city, Vietnam.

2.2.Equipment

The chromatograms and mass spectra were recorded from ACQUITY LC H-CLASS SYSTEM with Xevo TQD (Waters).

2.3. Methods

2.3.1 Preparation of stock and working dilution and spiking solutions

The calibration standards (CS) and quality control samples (QC) were prepared by spiking blank plasma with respective working solutions. CS were made at concentration ranges of 5-3200 ng/mL for metformin hydrochloride and 1-799 ng/mL for sitagliptin. Quality controls were prepared at 5 ng/mL (low limit of quality control, LLOQC), 15 ng/ml (LQC), 1600 ng/mL (MQC) and 2400 ng/mL (HQC) for metformin; 1 ng/mL (LLOQC), 3 ng/mL (LQC), 400 ng/mL (MQC) and 600 ng/mL (HQC) for sitagliptin. Spiked plasma samples were performed in centrifuge tubes and stored at -20ºC until use.

2.3.2. Sample treatment

A protein precipitation method was applied to remove protein from plasma sample. Several organic solvents in acidic pH medium were investigated to increase sensitivity and specification as well as to achieve a consistent recovery.

2.3.3.Liquid chromatography and mass spectrometric condition

The MS/MS conditions were optimized by direct infusion of MET, SGT and PF standard solutions with concentration of 500 ng/mL each. Electrospray ionnization source was operated in either negative or positive mode by multiple reaction monitoring (MRM) mode. The parameters such as desolvation temperature, capillary and cone voltage, gas

flow, collision energy, and product ion mass were optimized to obtain the highest signal of MET, SGT and PF. For the LC conditions, the main parameters were optimized such as stationary phase, composition of mobile phase, flow rate, column temperature, volume of injection to achieve the high signals and reasonable run time.

2.3.4. Method validation

The method followed US-FDA[6] and EMA[7] 2011 guideline with main criteria namely system suitability, selectively, recovery, linearity, lower limit of quantification (LLOQ), precision and accuracy, stability and matrix effect.

3. RESULTSAND DISCUSSION

3.1. LC-MS/MS conditions

By using Auto-tune function, the optimized MS/MS conditions were showed in table 1.

Table 1. *The optimized MS/MS conditions for analysis of MET, SGT and PF*

Parameters	Analytes MET, SGT, PF	Parameters	MET	SGT	PF
Ion mode	ES+	Parent ion (Da)	130.1	407.9	206.0
Capillary (kV)	3.0	Collision energy	20	18	18
Cone voltage (V)	40	Product ion	71.1	235.1	105.1
Desolvation temperature (^oC)	500	Dwell time (second)		0.02	
Desolvation gas flow (L/Hr)	1000				

It can be seen that important parameters such as pH of the mobile phase, concentrate and type of the buffer solution, percentage and type of the organic modifier (acetonitrile, methanol, water), stationary phase were attempted for better sensitivity, chromatographic separation and peak shape of MET, SGT, PF.The best separation and peak symmetries of three compounds was found to be significantly affected by using Phenomenex Gemini RP C_{18} (150mm x 4.6mm i.d, 5 µm) and utilizing mixing of that methanol: 0.1% aqueous formic acid (45:55, v/v) is better than mixing of acetonitrile: water, methanol: water, acetonitrile: acid formic 0,1% or ammonium acetate buffer.As a result,the latter has improved signal of MET, SGTand PF.However, SGT peak was unstable, not good peak shape and higher baseline in MRM mode. Yet, several trials were conducted of buffer concentration, flow rate, temperature column, which either had no impact or only modified

separation time, without sharply improving peak resolution. Totally, optimum MS and chromatographic conditions was shown in Fig 1.

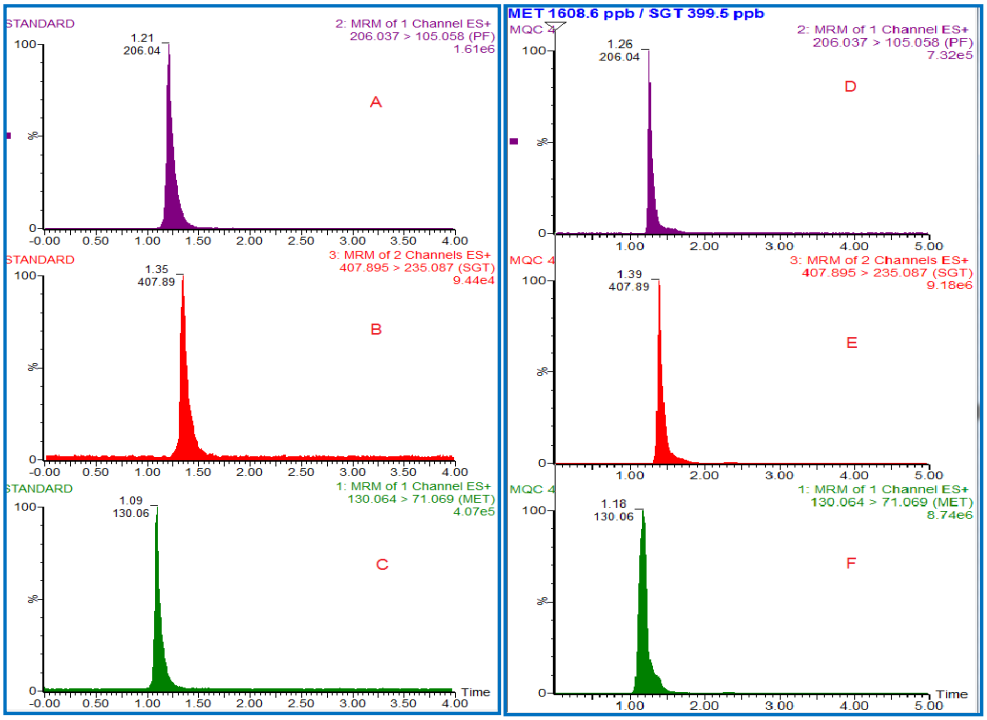

Fig.1 *Chromatogram standards mixture of MET, SGT and PF (A, B, C) and MQC (D, E, F) samples at optimum MS and chromatographic condition.*

3.2. Sample treatment

Several experiments using methanol or acetonitrile with formic acid to precipitate protein showed that acetonitrile (ACN) in combination with 0.045% formic acid (AF) was selected because of high and consistent recovery at three levels in plasma. Figure 1 showed processofplasmasampletreatment.

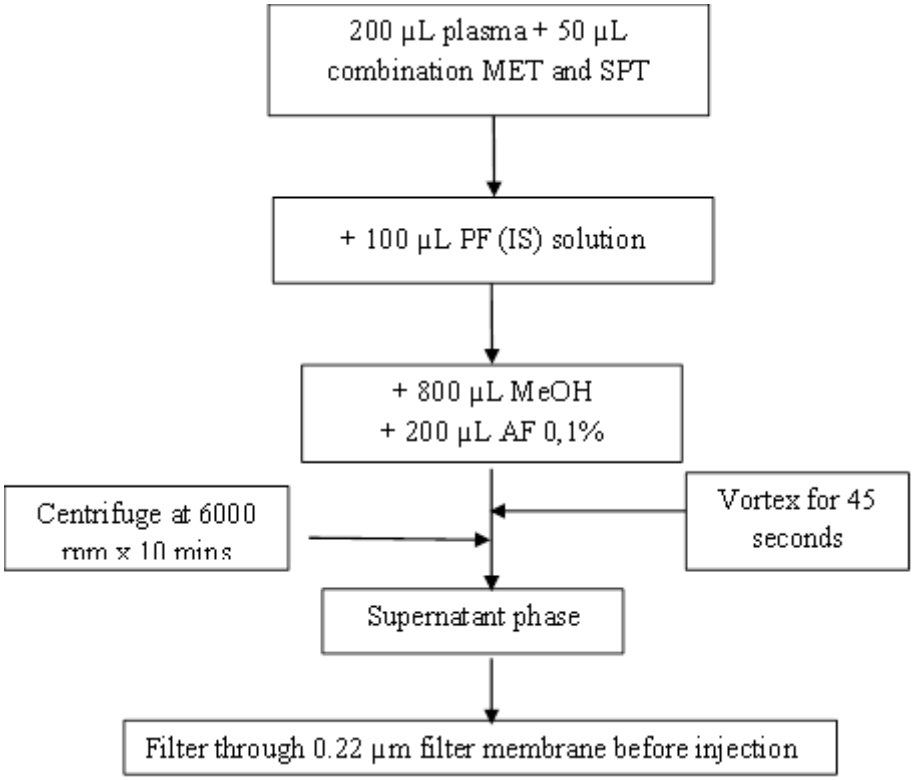

Figure 2. *Process of plasma sample treatment*

3.3. Method validation

3.3.1. System suitability

To evaluate the system suitability of the methods, six consecutive injections of low quality control (LQC) samples and high quality control (HQC) samples were performed. Table 2 listed statistical data of retention time, peak area, and ratio of analyte peak area and IS peak area.

Table 2. *System suitability test at LQC (n=6)*

MET		**Retention time**		**Peak area**		**MET/IS peak area**
		MET	PF	MET	PF	MET/PF
LQC	SD	0	0	11747.94	1328.50	0.65
	RSD (%)	0	0	0.97	2.70	2.65
Standard	SD	0	0	5843.49	1439.94	0.06
	RSD (%)	0	0	0.54	1.10	0.78
SGT		**Retention time**		**Peak area**		**SGT/IS peak area**
		SGT	PF	SGT	PF	SGT/PF
LQC	SD	0	0	6118.97	1328.5	0.462
	RSD (%)	0	0	0.74	2.70	2.73
Standard	SD	0	0	8131.97	1439.94	0.06
	RSD (%)	0	0	0.78	1.10	0.79

RSD values of S/S_{IS}, peak area, and retention time were below 3%.

3.3.2.Specificity and selectivity

Selectivity was determined by comparing the chromatograms of six different batches of blank human plasma with those of plasma samples at LLOQ concentration. There is no signal of three analytes was observed at the retention time of MET, SGT and PF (1.20, 1.29, and 1.42 minutes, respectively).The results were shown in table 3 and Fig 3.

Table 3. *Result of Analyte/IS peak ratio (%) of blank plasma, spiked plasma sample (n=6)*

	Blank sample			**Spiked plasma sample LLOQ**			**Analyte/IS peak ratio (%)**		
	MET	**SGT**	**PF**	**MET**	**SGT**	**PF**	**MET**	**SGT**	**PF**
Mean respond	86.18	73.20	5.83	7876.83	2562.70	100353.23	1.09	2.83	0.01
						Criteria	≤ 20	≤ 20	≤ 5
						Result	Passed	Passed	Passed

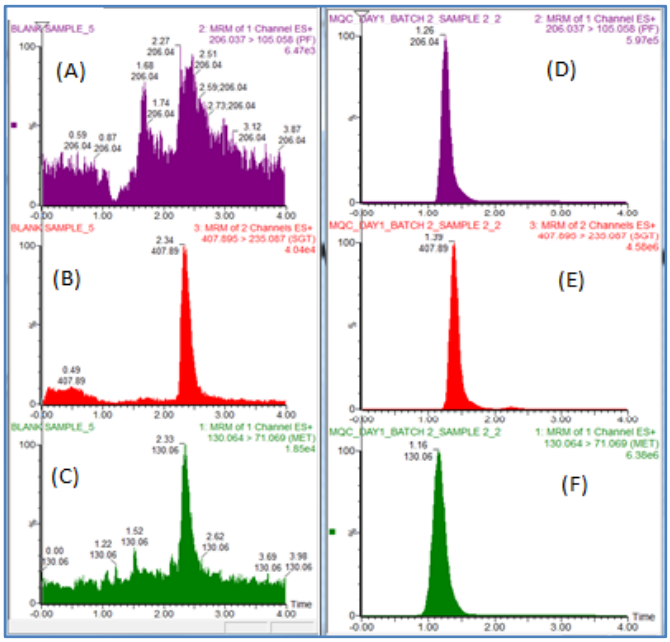

Fig. 3 *It shows the typical chromatograms of blank plasma, spiked plasma sample at LLOQ with metformin (C, F), sitagliptin (B, E) and IS (A,D).*

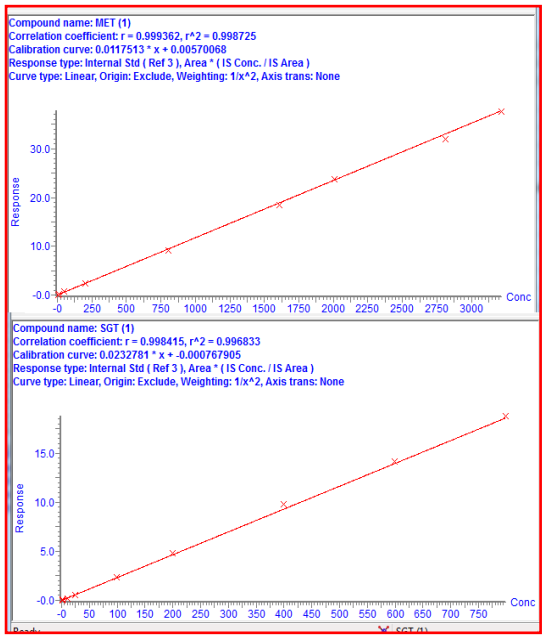

Fig.4 *Calibration curves of metformin and sitagliptin was calculated and built automatically by Masslynx 4.1*

3.3.3.Extraction recovery and accuracy, precision

The extraction recovery values at three concentration levels were 90.92% - 101.59%, 86.96% - 89.49%, and 100.47% - 105.32% for MET, SGT, and PF (IS)respectively. The average values of RSD were 6.90%, 1.52%, and 3.01% for MET, SGT, and PF,respectively.It showed consistency and reproducibility recovery for all analytes and IS.The results of were summarized in table 4.

Table 4.*Extraction recovery and accuracy – precision of MET, SGT, and PF in plasma*

Analyte	Analyte Conc. (ng/mL)	Extraction Recovery		Accuracy - Precision			
				Intra-day (n=5)		*Inter-day (n=15)*	
		Mean Recovery	*RSD%*	*Mean Recovery*	*RSD%*	*Mean Recovery*	*RSD%*
MET	15	90.92	2.72	92.20%	4.18	90.90%	4.37
	1600	95.18	1.14	95.33%	3.78	95.52%	5.23
	2400	101.59	7.48	99.60%	4.37	97.73%	4.76
SGT	3	86.96	1.60	101.80%	5.08	100.80%	5.76
	400	89.49	4.57	99.97.6%	3.97	101.56%	5.09
	600	87.48	5.70	104.52%	5.71	102.80%	6.40
PF (IS)	LQC[1]	103.80	7.13	-	-	-	-
	MQC[1]	105.32	5.50	-	-	-	-
	HQC[1]	100.47	3.16	-	-	-	-

[1]*: Recoveries of internal standard in 3 level of MET and SGT samples*

3.3.4.Linearity and lower limit of quantitation (LLOQ)

Night-point calibration curves of the MET and SGT for a specified range were performed. The curves (y = 0.0117513X + 0.0570068 for MET and y = 0.232781X – 0.000767905 for SGT) were obtained by plotting the peak area ratio of the analytes to IS against the corresponding concentration of the analytes in the spiked plasma. The parameters of slope, intercept and correlation coefficient (R) were calculated. An achievement of r^2 value greater than 0.995 indicated the good level of linearity. The lower LOQ of the method was 5 ppb, and 1 ppb for MET and SGT, respectively, that met the requirements of precision (RSD < 20%) and accuracy (recovery ranging from 80 to 120%). The result of linearity and lower limit of quantitation were revealed in Fig 4 and table 4.

Table 5. *Result of LLOQ determination of MET and SGT (n=6)*

Analyte	Blank sample	QC sample			S/N
	Mean Area	Mean Area	Observed conc. (ng/ml)	RSD (%) precision	RSD (%) accuracy	
MET	77.09	7924.13	5.033	6.113	99.998	>10
SGT	277.02	2562.70	1.000	6.325	99.967	>10
IS	5.83	84587.70	-	-	-	>10

3.3.5. Matrix effect

Experiments for evaluation matrix factor (MF) value of MET, SGT, and PF at low and high concentration levels were carried out of six different plasma batches.The RSD values of the ratio of MF_{MET}/ MF_{IS} and MF_{SGT}/ MF_{IS} at low and high concentration levels were in a range of 12.34% and 5.29%, 10.43% and 3.98%, respectively. The results indicated that the matrix effect for MET and SGT was removed.

3.3.6. Stability

The stability of MET and SGT in stock solutions and in plasma was investigated at – 20^{o}C in freezer and at room temperature, the stock solutions were stable within 20 days and 6 hours, respectively. It indicated that the analytes were stable after three cycles of freeze and thaw, for 6 hours at room temperature, for 24 hours in auto sampler at 20^{o}C, and for 30 days at -20^{o}C.Different stability experiment in plasma was shown in table 5.

Table 6.*Stability of metformin in plasma under different storage conditions*

Stability check	Samples (n=5)	Initial conc. (ng/mL)	Observed conc. (ng/mL)	%RSD	%Stability
Bench top for (6 h)	LQC	15.60	14.88	3.31	95.39
	HQC	2247.38	2185.38	3.12	97.25
Auto Sampler Stability for 24 hrs.	LQC	15.25	15.06	2.86	98.78
	HQC	2192.24	2218.29	1.85	101.19
Freeze–thaw at -20^{o}C (three cycles)	LQC	15.25	15.40	2.53	100.99
	HQC	2192.24	2160.27	2.10	98.54

4. CONCLUSIONS

A highly sensitive, specific, reproducible and rapid LC-MS/MS methodfor the simultaneous quantification ofmetformin hydrochloride and sitagliptin in human plasma was successfully developed and validated.From the results of all the validation parameters, this method can be useful for bioequivalence studies and monitoring these therapeutic drugs with great precision and accuracy.

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Development of LC-MS/MS Method for Simultaneous Determination of Pepsin, Lipase, Alpha- Amylase in Human Gastric Juice

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Abstract

Introduction: Pepsin, lipase, and alpha-amylase are crucial digestive enzymes. Analysis of human gastric juice biomarker enzymes is important for understanding oral health and disease pathogenesis. However, simultaneous determination of these enzymes by spectrophotometric methods is difficult due to low repeatability. To the best of our knowledge LC-MS/MS method has never been applied for this simultaneous quantification so far. **Objectives:** The aim of this study was development and validation of method for simultaneous determination of pepsin, lipase, and alpha-amylase in human gastric juice using AcquityLC H-Class tandem Xevo TQD mass spectrometer and its application to determine concentration of these bio-makers in gastric juice samples of patientsat CTUMP hospital. **Materials and methods:** 130 samples of human gastric juice were supplied from CTUMP hospital, including 40 samples of *Helicobacter pylori* infectiouspatients. Firstly, the human gastric juice was precipitated protein by acetonitrile, then pepsin, lipase, and alpha- amylase in supernatant layer were hydrolyzed into tyrosine, butyric acid, and maltose by Tyr-Ala, tributyrin, and starch substrates, respectively. Finally, the hydrolytic products were determined by the validated LC-MS/MS, using positive and negative mode, separated on a hydro-C18 reversed phase column, and eluted with acetonitrile and water.

Results: The suitable LC- MS/MS conditions for separation of tyrosine, butyric acid and maltose were obtained with Synergi Hydro RP 80A column (250 x 4.6mm; 4µm) and mixture of acetonitrile and water (58:42) as eluent. ESI ionizer was operated in positive and MRM mode using the transition of *m/z* 181.19 →90.9, *m/z* 342.10 → 203.10, *m/z* 151.16 →92.73 to quantify tyrosine, maltose and IS (paracetamol), respectively and in negative with selected ion recording mode using *m/z* 87 for butyric acid. The method was validatedand shown high selectivity, good reproducibility (RSD < 15%) and linearity (R > 0.995), low limit of detection (0.1- 100 ppb) and mean recovery of accuracy in the range of90.57- 112.26%. The assay was applied for analyzing gastric juice sample of patients. The results revealed that all samples of *Helicobacter pylori* infectious patients had pepsin and lipase concentration higher than one in samples without *Helicobacter pylori* infection. **Conclusion:** A sensitive and rapid LC-MS/MS method for the

simultaneous quantification of pepsin, lipase, and alpha- amylase in human gastric juice was successfully developed and validated. Practical applicability of the assay was shown by analyzing 130 human juice samples from patients with and without *Helicobacter pylori* infection.

Keywords: Pepsin; Lipase; Alpha-amylase; Human gastric juice, triple quadrupoles.

1.INTRODUCTION

The change of enzymatic reaction of one or a few enzymes in body is a health sign which is worth caring. Specially, this change plays a key role in diagnosing and monitoring some diseases. The typical case among them is the unstableness of enzyme activity in gastritis caused by Helicobacter pylori. One of the criteria used for evaluating the efficacy of treatment is to monitor enzyme activity of pepsin, lipase and amylase in the gastric fluid. The evaluation requires methods offering high specificity, sensitivity, precision and accuracyto obtain the most reliable results.

A number of methods have been developed to individually estimate these biomarker enzymesconcentration, including spectrophotometricin the UV range for determination of the pepsin activity in human gastric juice[1],colorimeter for lipase activity in complex system [2], [3].All of spectrophotometry methods offer a lower specificity and reproducibility. Recently, there has been growing interest in exploring qualitative and quantitative analysis by using LC-QTOF technique with high sensitivity and reliability for identification proteins in human salivary[4]. A survey of the literature reveals that there is no reported method for the simultaneous determination of pepsin,lipase,α-amylase in human gastric juice by LC-MS/MS. This was our motivation to develop and validate the first LC-MS/MSassay for the simultaneousthese biological compounds.

2. MATERIALS AND METHODS

2.1 Chemicals, solvents, and standards: standard compounds (purity ≥ 98.5%) of tyrosine (TYR), butyric (BTR), maltose (MAL), phenylalanine, dipeptide tyrosine-alanine, tributyrin, soluble starch and pepsin, lipase, α – amylase were supplied by Sigma. Paracetamol(PARA)was fromThe Institute of Drug Quality Control – Ho Chi Minh City. Acetonitrile, methanol, water,formic acidwaspurchased from Merck with MS grade.All solvents or chemicals for sample preparation used for analysis were of analytical gradefrom Merck.

Human gastric juice samples from patients with and without *Helicobacter pylori* infection were kindly supplied by MD. Nguyen Phan Hai Sam, CTUMP hospital.

2.2 Instruments: The Acquity H–Class system includes a quaternary solvent manager, sample manager-flow through needle, column heater module coupled with a triple quadrupolemass spectrometer, Xevo TQD (Waters Corporation, Milford, USA). The Masslynx 4.1 software version was used to control operation and data processing. Chromatographic separation was performed on Synergi Hydro RP 80A (250 x 4.6mm; 4μm) analytical column (Phenomenex Inc., CA, USA) maintained at room temperature.

2.3 Method: The MS parameters consist of capillary, cone voltage, desolvation temperature and gas flow, collision energy, dwell time, etc. were optimized by directly infusion of tyrosine, butyric acid, maltose and internal standard standard solutions prepared in the reasonablesolventsat 500ng/mL withnegative and positive ESI sourceand MRM, SIR, daughter mode to obtain the highest signal of product ion mass.A reverse phase chromatographic techniqueusing ACN, MeOH, water or added pH-modifier as mobile phase. Factors influence to separation were investigatedincluding stationary phase, mobile phase, pH, flow rate,column temperature andconditions for hydrolysis reaction of substrates by pepsin,lipase, amylase were also investigated.

The assay was validated according to the US FDA[6] at system suitability, selectivity, sensitivity, linearity, precision, accuracy and applied to analyze human gastric juice samples. For quantification, the peak area ratios of the target ions to those of the IS were compared with weighted (1/ concentration^2) least square calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

3. RESULTS AND DISCUSSION

3.1. Method development

3.1.1 Optimization of MS parameters and chromatographic conditions

During the optimization of the mass spectrometric parameters, strong and stable signals of analysis of analytes and IS were noted. The results indicate that ESI ionizer was operated in positive and MRM mode using the transition of *m/z* 181.19 →90.96, *m/z* 342.10 → 203.10, *m/z* 151.16 →92.73 to quantify tyrosine, maltose and IS (paracetamol), respectively and in negative with selected ion recording mode using *m/z* 87.10 for butyric acid. Furthermore, ACE is suitable for being internal standard because it is not available in human gastric juice sample and has better signal response than to phenylalanine. The source and compound parameters were optimized as follow to table 1.

Table 1. *Optimization of MS parameters.*

Capillary voltage (kV)				3
Desolvation gas flow (L/min)	4	1000	500	1000
Desolvation temperature (^{o}C)				500
Parameters	TYR	MAL	PARA	BTR
Positive/negative	ES^{+}	ES^{+}	ES^{+}	ES^{+}
Modes of scanning and acquiring	MR	MRM	MRM	SIR
Cone voltage (V)	25	50	30	30
Collision energy (V)	25	22	22	-
Dwell time (s)	0,039	0,039	0,039	0,039

It is well known that important parameters such as pH of the mobile phase, concentrate and type of the buffer solution, percentage and type of the organic modifier (acetonitrile, methanol, water), different columns (Synergi C12 Max RP and C18 Hydro RP 80A)and flow rates (0.2-0.8 mL/min) were attemptedfor better sensitivity,chromatographic separation and peak shape of TYR, MAL, BTR, ACE. The best separation and peak symmetriesof four compounds was found to be significantly affected by using C18 Hydro RP and utilizing mixing of acetonitrile: water (58:42) isbetter than mixing of methanol: water, acetonitrile: acid formic 0.1% at gradient pH or ammonium acetate buffer as well. In fact, by adding acid formic 0,1% has improved only slightly signal of TYR, MAL and ACE. However, peak BTR was unstable, badly peak shape and higher baseline in MRM mode. This can be explained that formic acid competed with BTR acid. Yet, several trials were performed of buffer concentration, flow rate (0.6 mL/min), temperature column (room temperature). They either had no impact or only modified separation time, without sharply improving peak resolution. Totally, optimum MS and chromatographic conditions was shown in Fig 1.

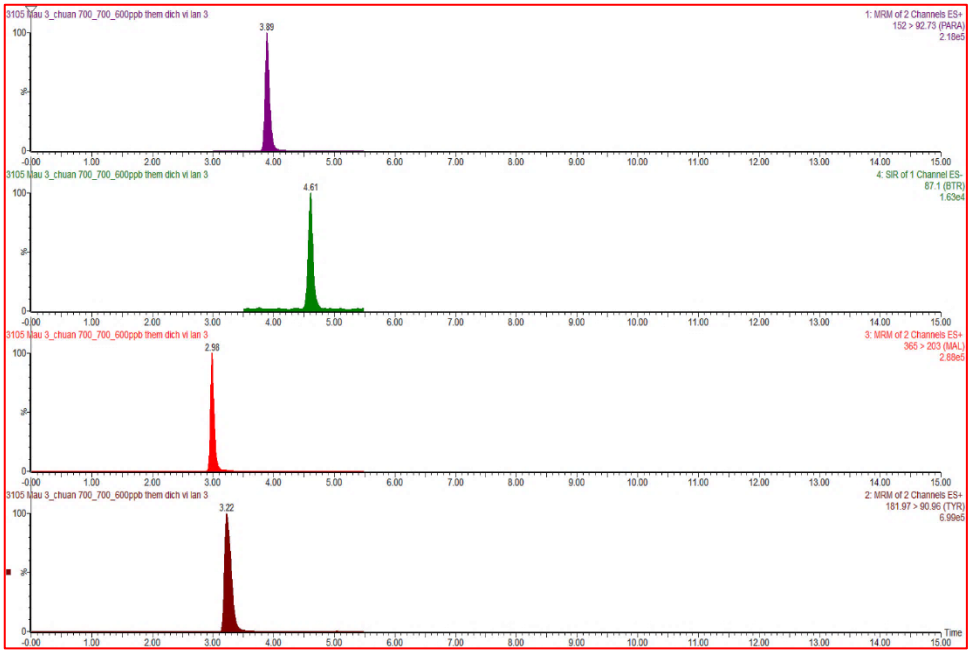

Fig 1. Chromatogram of TYR, BTR, MAL and PARA standards at optimum condition

3.1.2 Sample preparation

Based on experiments of reaction time of pepsin, lipase and α-amylase for hydrolysis was chosen 20, 60, 60 minutes with substrate Tyr – Ala 2mg/ml in solution of formic acid at pH 2.3, tributyrin (TBR) 2 µL/mL and soluble starch 2 mg/ml in buffer of acid formic: trietylamine at pH 6.8, respectively. The temperature for all reaction was adjusted at 37 ^{o}C.Procedure for human gastric juice sample preparation was showed in **Fig 2**.

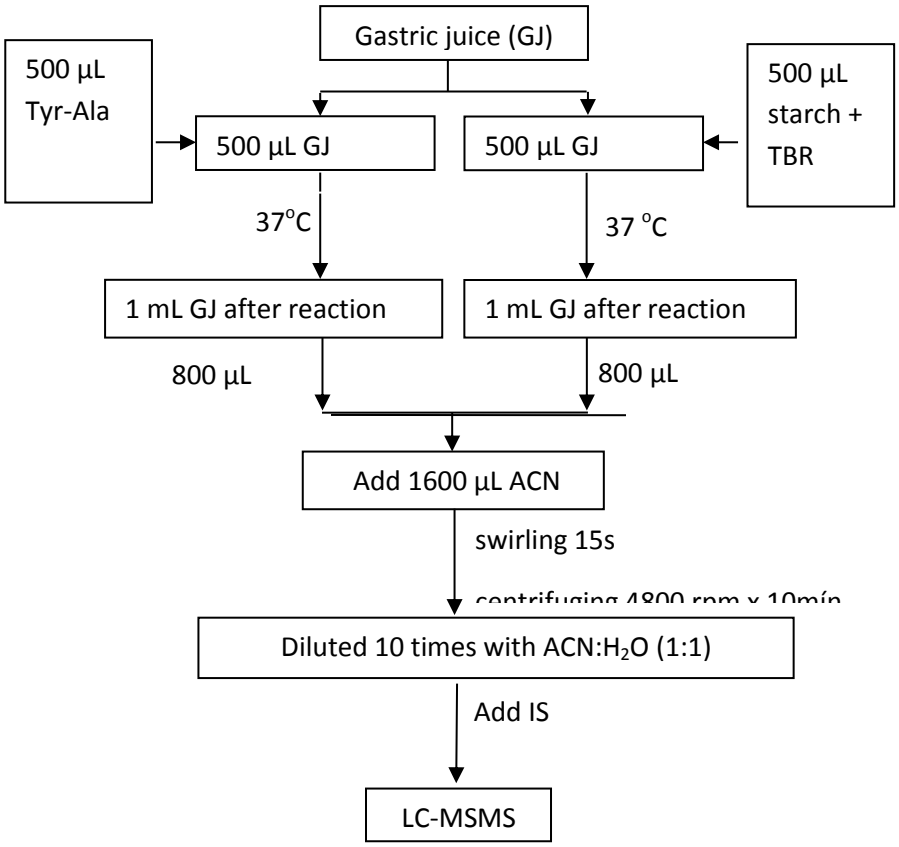

Fig 2. *Procedure of human gastric juice sample preparation*

3.2. Method validation

3.2.1 System suitability

Six consecutive injections were performed. Table 2lists RSD% values of peak area (S_{peak}), retention time (t_R) and rate of peak area and IS area (S_{peak}/S_{IS}). The values demonstrate a good reproducibility of the method.

Table 2. *Relative standard deviation values calculated from 6 consecutive injections (n=6)*

Analyte		t_R	S_{peak}	S_{peak}/S_{IS}
TYR	TB	3,21	44494,004	2,369
	RSD%	0,144	1,333	1,145
BTR	TB	4,59	661,744	0,035
	RSD%	0,116	1,661	2,159
MAL	TB	2,99	25925,573	1,374
	RSD%	0,155	1,850	1,812
PARA	TB	3,90	18788	
	RSD%	0,133	1,579	

3.2.2 Specificity

The selectivity of the method was evaluated by comparing the chromatograms obtained from TYR, MAL, BTR samples and the PARA with those obtained from the blank samplesandassuredby scanning and filtering of precursor ions and product fragments (MS^1 and MS^2 full-scan, SIR, MRM, daughter mode) of TYR, MAL, BTR and PARA with othersubstances under the experimental conditions.The results of specificity are shown infigure 3 and 4.

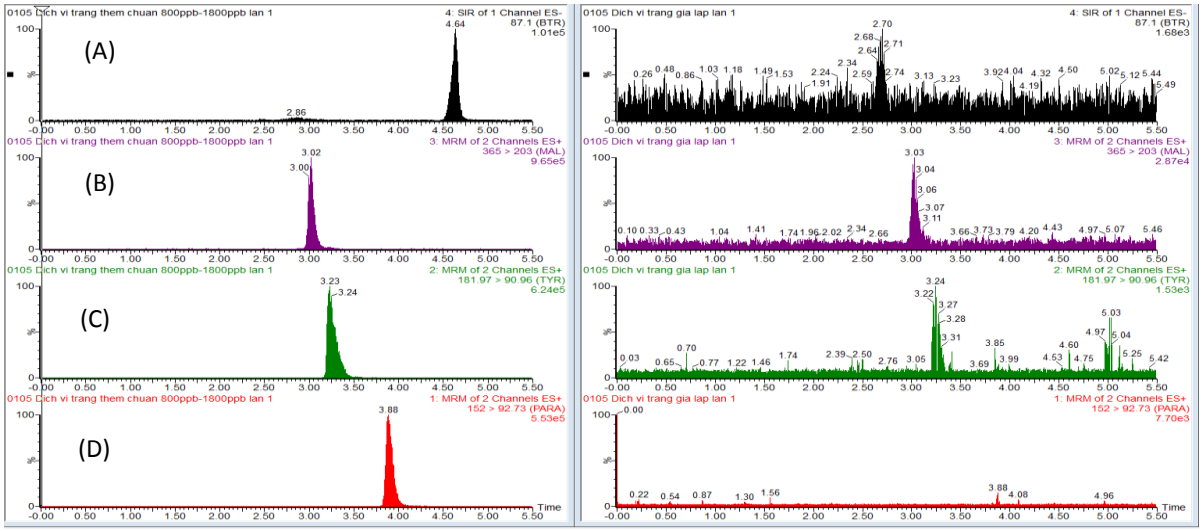

Fig 3. *Chromatogram of blank and blank spiked standards of butyric (BTR), maltose (B), TYR (C) andparacetamol (D).*

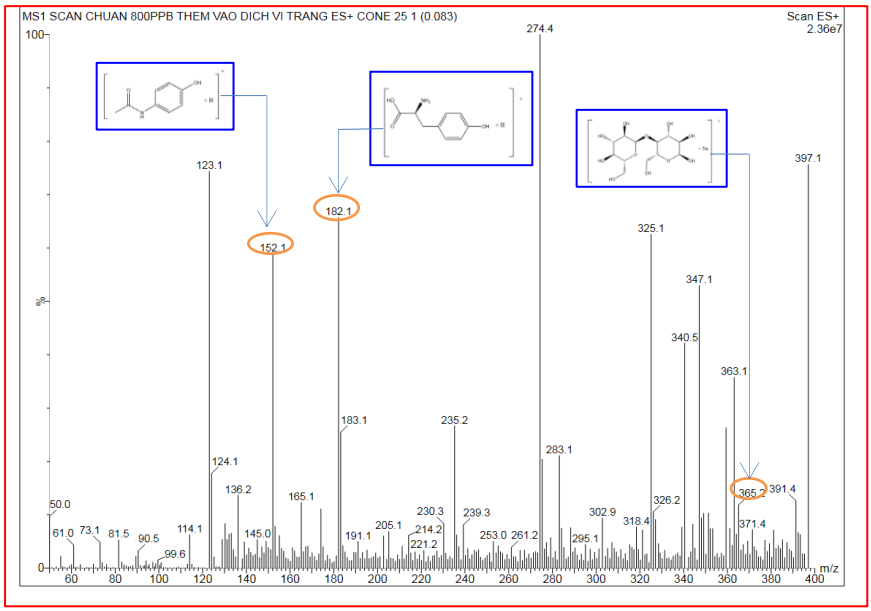

Fig 4. *MS^1full-scan (ES^+) of blank human gastric juice spiked mixture of standards TYR, MAL and BTR.*

Moreover, MS parameters were optimized with the objective obtaining two ion pairs for identification and quantification each target compound [7]. The results were shown in Table 3.

Table 3. *The confirmatory and quantifiable ion ratios of the TYR and MAL*

Analytes	Identified ion	Quantified ion	Ion ratio (%)		Tolerance (%)
			Sample	Standard	
TYR	122,93	90,96	64,6	64,3	0,47
MAL	185,0	203,10	27,0	21,9	23,29

Tolerance of ion TYR and MAL are 0.47% and 23.29% respectively and less than criteria (<20% for TYR and <25% for MAL).

3.3 Linearity, LOD, LOQ

The calibration curve was shown to be linear for the tested concentration range of TYR, MAL, BTR. The mean correlation coefficient of the weighted ($1/X^2$i.e, $1/[\text{concentration}]^2$) calibration curve generated in the validation was always > 0.995 and between the peak area ratios and the concentration of three analytes in the range 1-3000 ng/mL. The experiments were performed using a standard ten-point series of each biological compound.LOD and LOQ were calculated according to the ICH guideline for validation of analytical procedure based on standard deviation of the response and the slope of the calibration curve. Calculations of six replicate experimental injections LODs of 0.10, 0.13, 50 ng/mL and determined LODs of 5, 25, 100 ng/mL for TYR, MAL, BTR, respectively.

3.4 Accuracy and precision

The accuracy and precision of the proposed method were determined by intra-day and inter-day replicate analyses of human gastric juice spiked at three concentration level standards covering the linear ranges. Theaccuracy is evaluated by the recovery.The results for precision and accuracy are summarized in table 5.

Table 4. *Precision and accuracy of the quality control samples of TYR, MAL, BTR*

Analyte	QC sample	Accuracy		Precision	
		% intra –day	% inter-day	% intra –day	% inter-day
TYR	LQC	94.53	96.42	14.19	15.05
	MQC	98.31	94.54	14.64	14.05
	HQC	98.60	99.89	13.27	14.34
MAL	LQC	100.03	102.18	12.23	14.07
	MQC	110.85	112.26	14.34	13.99
	HQC	102.17	106.13	13.26	12.15
BTR	LQC	99.63	97.46	13.78	13.24
	MQC	90.57	93.40	11.65	11.97
	HQC	94.33	97.54	14.82	12.79

The intermediate-precision and accuracy of TYR,MAL, BTR ranged from 11 to 14.82% and from 90.57 to 112.26%, respectively. The results were found to be acceptable, which endorse practical applicability of the method to analyze many human gastric juice samples.

3.3. Method application determine pepsin, lipase, α-amylase in human gastric juicesamples from patients with and without *Helicobacter pylori* infection. * (not sure)

130 human gastric juice samples were divided into case group (*Helicobacter pylori* infectious group) and control group (without *Helicobacter pylori* infectious group)and were analyzed to determined activity of pepsin, lipase and amylase. The results were shown in table 6.

Table 5. *Concentration (ppm) and activity (U/ml) of enzyme in base gastric juice of cases and controls groups*

Enzymes	Cases (n=40) $\overline{X}$ ±SD	Controls (n=90) $\overline{X}$ ±SD	P
Conc. Pepsin	18,65 ± 2,38	13,2 ± 2,4	
Conc. Lipase	14,47 ± 8,08	6,76 ± 2,89	>0,05
Conc. Amylase	18,66 ± 1,12	41,6 ± 1,91	
Activity Pepsin	4,66 ± 5,95	3,31 ± 6,0	
Activity Lipase	4,34 ± 2,42	2,03 ± 8,67	>0,05
Activity Amylase	0,56 ± 3,36	1,24 ± 5,74	

The average concentration and activity of pepsin and lipase enzymes in the cases group tend to be higher than those in the controls group, while for amylase enzyme concentrations and activity in the controls group are higher.

5. CONCLUSIONS

In this study, a simple, selective, sensitivity, accurate and reproducible LC-MS/MS method was developed and validated for simultaneous determination pepsin, lipase and amylase in human gastric juice samples. The method shows good performance with respect to all the validation parameters tested. Practical applicability of the assay was shown by successfully analyzing more than 130 different gastric juice samples. As a result, activity of pepsin and lipase show an upward tendency at cases. On the contrary, activity of amylase is a downward tendency at controls.

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Simultaneous Quantitative Determination of Metoprolol and Amlodipine in Human Plasma by LC-MS/MS

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ABSTRACT

Introduction: Metoprolol (MET) is the prototype of cardio selective β-adrenoceptor antagonist. Amlodipine (AMP) is the calcium channel blocker, pharmacokinetically, the most distinct dihydropyridines. A fixed combination of metoprolol 50 mg and amlodipine 5 mg as tablet formulation is used in treatment of angina pectoris, cardiac arrhythmia and hypertension. However, there are a few analytical method reported for simultaneous determination of these drugs in human plasma that can be useful for routine therapeutic drug monitoring and pharmacokinetic studies. **Objectives:** The aim of this study was development and validation of method for simultaneous determination of MET and AMP in human plasma using Shimadzu LCMS-8040 system. **Materials and Methods:** Blank plasma samples were spiked with mixed standard solutions including amlodipine besylate, metoprolol succinate, and hydrochlorothiazide as internal standard (IS). Amlodipine and metoprolol were extracted by mixture of diethyl ether and dichloromethane, the extract was dried under nitrogen at 40^{o}C and the dryness was finally dissolved in mixture of methanol and water. A method for quantification of MET and AMP in human plasma was developed using Shimadzu LCMS-8040 system equipped with an electrospray ionization source. The assay was validated in compliance with US FDA and EMA guidelines. **Results and Discussion:** MET and AMP were separated on a C18 column (100 x 3 mm; 5 µm) with mixture of methanol and 0.4% formic acid (80:20) as mobile phase at 40^{o}C column temperature, flow rate of 0.3 ml/min. Electrospray ionnization source was operated in positive mode and multiple reaction monitoring (MRM) mode, using the transition of *m/z* 268.15 →

116.00, *m/z* 409.10 → 238.00, and *m/z* 296.00 → 126.00 to quantify MET, AMP and IS, respectively. The linearity was obtained over the concentration range of 0.3-100 ng/ml (R^2 = 0.9939), 0.045-15 ng/ml (R^2 = 0.9977) with the lower limits of quantitation of 0.3 ng/ml and 0.045 ng/ml for MET and AMP, respectively. The intra and inter-day precision and accuracy, stability, recoveries and sample carry over of all the analytes were in the acceptable range. **Conclusion:** A rapid, sensitive, and specific LC-MS/MS method was successfully developed and validated for simultaneous quantitative determination of metoprolol succinate and amlodipine besylate in human plasma. This method can be applied to bioequivalence study of fixed dose combination of MET and AMP formulation product.

Keywords: metorpolol, amlodipine, LC-MS/MS, human plasma.

1. INTRODUCTION

Metoprolol (MET) is the prototype of cardio selective β-adrenoceptor antagonist. Amlodipine (AMP) is the calcium channel blocker, pharmacokinetically, the most distinct dihydropyridines. In current hypertension treatment guidelines, combination therapy may be favored by the fact that multiple factors contribute to hypertension, and achieving control of blood pressure with a single agent acting through one particular mechanism may not be possible. The advantages of combinations include greater blood pressure decrease and response rates than monotherapy; fewer side effects with small doses of two drugs than with large doses of one agent; and improved adherence to treatment. Therefore, a sensitive and specific analytical method is needed for simultaneous determination of two drugs in human plasma for routine therapeutic drug monitoring and pharmacokinetic studies.

There have been several published studies on application of modern analytical methods for single determination of each drug, such as high-performance liquid chromatography (HPLC) in combination with UV, RP-HPLC and especially LC-MS/MS[1-3] detectors. Herein, a highly sensitive, specific, reproducible, and rapid LC-MS/MS method for determination of MET and AMP in human plasma, was reported.

2. MATERIALS AND METHODS

2.1. Standards, chemicals and solvents

AMP (100,43% purity) (Batch No. QT145 090516), MET (99.80% purity) (Batch No. HOL334) and HCTZ (99,55% purity) (Batch No. 0114308.01) as internal standard (IS) were supplied by Institute of Drug Quality Control at Ho Chi Minh city, USP and nation institute of Drug Quality Control at Ha Noi city, respectively. Other chemicals and organic solvents such

as methanol, diethylether, dicloromethan and acid formic was also purchased from J.T.Baker, Fisher chemical and Prolabo, respectively.

2.2. Equipment

The chromatograms and mass spectra were recorded from Shimadzu LCMS-8040 system (equipped with a LC-30AD pump, a SIL-30AC autosampler, LCMS-8040 mass detector and Lab Solution analyst data processing software).

2.3. Methods

2.3.1 Chromatographic conditions

Chromatographic separation was achieved on a C18 column (100 x 3mm, 5µm). Mobile phase used for separation of the analytes was methanol:formic acid 0.4% (8:2, v/v). The flow rate was set at 0.3 ml/min. The injection volume was 2,5µl. The column temperature was 40^{o}C. HCTZ was used as an internal standard. Electrospray ionization (ESI) with multiple reaction monitoring (MRM) was used to acquire the mass spectra of the compounds.

Nebulizing gas flow: 3 l/min

Drying gas flow: 15 l/min

Temperature DL: 250^{o}C

Temperature heat block: 400^{o}C

Voltage 4500V

		m/z	Q1 (V)	CE	Q3 (V)
Amlodipine	MRM (+)	409,10 → 238,00	-30	-10	-24
Metoprolol	MRM (+)	268,15 → 116,00	-28	-11	-11
Hydroclorothiazide	MRM (-)	296,00 → 126,00	9	31	23

2.3.2 Preparation for standard curve samples

Original standard solution of standard amlodipine (AS1) in methanol was at the exact concentration about 150 µg/ml. Dilute these solution in plasma to obtain standard curve samples of amlodipine at exact concentration about 0,045; 0,15; 0,75; 1,5; 3; 4,5; 7,5; 9; 12; 15 ng/ml, respectively.

Original standard solution of standard metoprolol (AS2) in methanol was at the exact concentration about 100 µg/ml. Dilute these solution in plasma to obtain standard curve samples of metoprolol at exact concentration about 0,3; 1; 5; 10; 20; 30; 50; 60; 80; 100 ng/ml respectively.

2.3.4 Preparation for verification samples

Original standard solution of standard amlodipine (AS1) in methanol at the exact concentration about 150 µg/ml. Dilute these solution in plasma to obtain verification samples at exact concentration of AS about 0,045; 0,15; 0,75; 12; 15ng/ml respectively.

Original standard solution of standard metoprolol (AS2) in methanol at the exact concentration about 100 µg/ml. Dilute these solution in plasma to obtain verification samples at exact concentration of AS about 0,3; 1; 50; 80; 100ng/ml, respectively.

2.3.5 Process to handle samples

Standard curve samples and verification samples are handled before injection as follow: take exactly 1 ml of plasma which consist of AMP and MET into a tube, add 50 µl of solution of internal standard of HCTZ at concentration of 2000nf/ml in methanol, swirl it for 10 seconds, extract with mixture of diethylether and dichloromethan (6:2) (2 x 3ml), swirl for a minute, shake at 300 rmp for 5 minutes then centrifuge at 4000 rmp for 5 minutes. Collect the upper layers of extraction solutions, evaporate by nitrogen gas at 40^{O}C then dissolve in 500µl mixture of water and methanol (20:80), swirl for a minute, sonicate for 5 minute, centrifuge at 4000 rmp for 5 minutes at 0^{O}C, filter through 0.22 µm membrane.

2.3.6 Method validation

The method followed FDA's 2011 and EMA'2 2011 guideline with main criteria namely system suitability, specificity, lower quatitative limit, linear range, efficiency of extraction, intra-day and inter-day accuracy; precision, cross-contamination, effect of sample matrix and stability[4-5].

3. RESULTS AND DISCUSSION

3.1 System suitability

Table 1. *System suitability parameters*

	AS1 (amlodipine)		AS2 (metoprolol)		IS (HCTZ)		Rt Ratio AS/IS		Peak area ratio AS/IS	
Sample	R_t	Peak area	R_t	Peak area	R_t	Peak area	AS1/ IS	AS2/ IS	AS1/ IS	AS2/ IS
Mean	1,26	301796	1,24	398621	1,55	34819	0,815	0,801	8,678	11,465
CV%	0,17	1,65	0,10	2,3	0,18	3,83	0,25	0,23	4,08	4,92
Standard				CV ≤ 5%						
Results				Good						

3.2 Specificity and selectivity

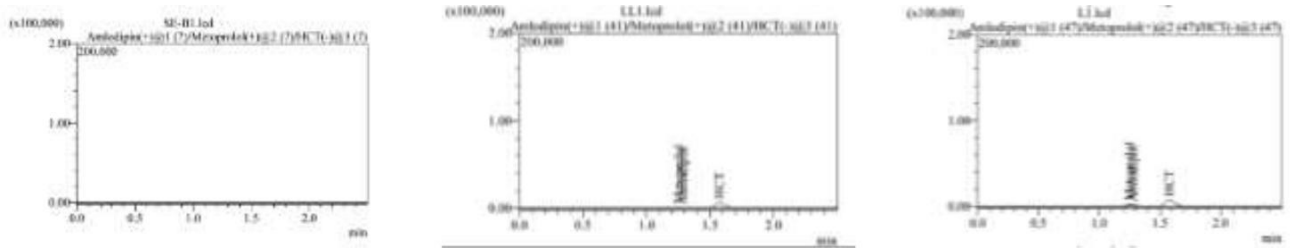
On chromatography, LLOQ has a peak of AMP, MET and HCTZ (1.27, 1.24, 1.55 minute, respectively). The ratio of retention time of AMP to HCTZ is 0.82 and that of MET to HCTZ is 0.8. The blank sample of plasma has no peak at the position of AMP, MET and HCTZ.

***Table* 2.** *Selectivity data*

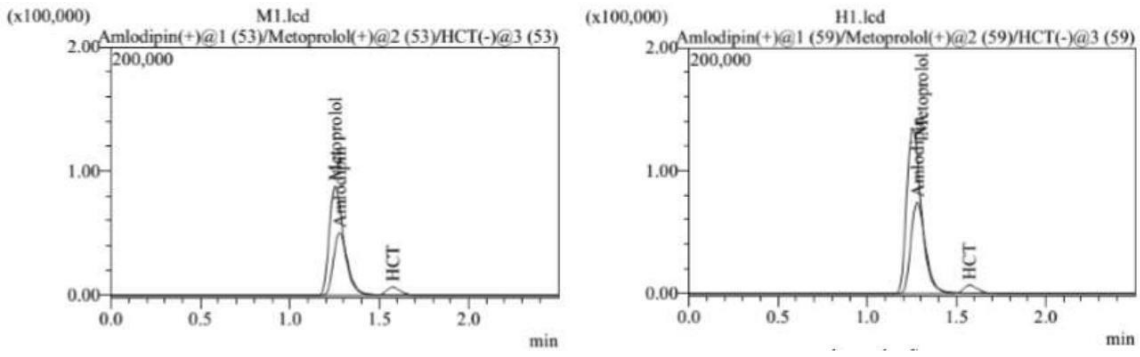
Sample	Peak area						Noise In%			S/N	
	Blank sample			LLOQ sample							
	AS1 (1)	AS2 (2)	IS (3)	AS1 (4)	AS2 (5)	IS (6)	AS1 = (1)*100 /(4)	AS2 = (2)*100 /(5)	IS = (3)*100 /(6)	AS1	AS2
Mean	0,00	0,00	0,00	1110	1627	26743	0,00	0,00	0,00	9,15	7,98
Standard							In ≤ 20%		In ≤ 5%	≥ 5	
Comment							Good		Good	Good	

3.3 Lower quantitative limit

At the concentration of AMP 0.45ng/ml and MET 0.3ng/ml, the results after analysising give that the S/N ratio is over 5 and the precision and accuracy are achieved as requirement. Therefore, this concentration is accepted as the lower quantitative limit of the method (LLOQ).



Chromatogram of blank plasma *Chromatogram of LLOQ samples samples* *Chromatogram of LQC*



Chromatogram of MQC samples Chromatogram of HQC samples

Figure 1. *Chromatograms of blank plasma, LLOQ, LQC, MQC, HQC sample*

3.4 Standard curve and linear range

Evaluate the correlation between the concentration of AS in plasma and the ratio of peak area measured in the survey concentration.

***Table* 3.** *The correlation between the concentration of AS in plasma and the ratio of peak area*

Concentration Sample (ng/ml)		Peak area			Ratio		Concentration found (ng/ml)		Accuracy (%)	
AS1	AS2	AS1	AS2	IS	AS1/IS	AS2/IS	AS1	AS2	AS1	AS2
At least 75% of the calibration standards must fulfil:										
Standard	LLOQ: 80-120%									
LQC, MQC, HQC: 85-115%										
Comment	Good									

Table 4. *The regression equation of AMP, MET*

Drug	Regression equation	Correlation coefficient R^2
AMP	$\hat{y}$ = 1,1742x + 0,0019	0,9977
MET	$\hat{y}$ = 0,2553x + 0,1140	0,9939

3.5 Extraction efficiency

Table 5. *Extraction efficiency of AMP, MET và HCTZ (n = 6)*

Sample	In plasma			In solvent			Extraction (H%)		efficiency
	AS1	AS2	IS	AS1	AS2	IS	AS1	AS2	IS
Mean LQC (%)	5686	7630	33866	6083	8938	35571	93,47	85,36	95,21
CV (%)							2,11	5,72	2,00
Mean MQC (%)	277368	415855	34208	300827	426304	35360	92,20	97,55	96,74
CV (%)							9,32	2,20	3,91
Mean HQC (%)	385643	543160	27616	537708	642661	32787	71,72	84,52	84,23
CV (%)							9,57	1,47	4,29
Standard	CV ≤ 15%								
Comment	Good								

Intra-day and inter-day precision and accuracy

The result of intra- and inter-day precision and accuracy is shown in table 6.

Table 6. *Intra-day precision and accuracy (n=6)*

Analyte	Amlodipine				Metoprolol			
QC sample (ng/ml)	LLOQ (0,045)	LQC (0,15)	MQC (7,502)	HQC (12,004)	LLOQ (0,299)	LQC (0,997)	MQC (49,866)	HQC (79,786)
Mean	0,047	0,147	7,004	12,056	0,269	0,905	46,689	75,906
CV (%)	6,96	6,808	5,71	8,92	11,73	5,849	6,01	7,95
Accuracy %	105,15	98,01	93,36	100,43	90,02	90,7	93,63	95,14
Accuracy Standard	LLOQ: 80% - 120%; LQC, MQC, HQC: 85% - 115%							
Precsion Standard	LLOQ: CV ≤ 20%; LQC, MQC, HQC: CV ≤ 15%							
Comment	Good							

The result of inter-day precision and accuracy is shown in table 7.

Table 7. *Inter-day precision and accuracy for amlodipine and metoprolol (n = 6)*

Analyte		Amlodipine				Metoprolol			
QC sample (ng/ml)		LLOQ (0,045)	LQC (0,15)	MQC (7,502)	HQC (12,004)	LLOQ (0,299)	LQC (0,997)	MQC (49,866)	HQC (79,786)
Day 1	Mean (SD)	0,047	0,147	7,004	12,056	0,269	0,905	46,689	75,906
	CV (%)	6,96	6,808	5,71	8,92	11,73	5,849	6,01	7,95
	Accuracy %	105,15	98,01	93,36	100,43	90,02	90,7	93,63	95,14
Day 2	Mean (SD)	0,044	0,147	7,577	11,991	0,329	0,894	51,316	78,739

	CV (%)	4,23	5,05	8,76	0,89	5,77	4,22	7,59	7,16
	Accuracy %	97,09	97,67	100,99	99,89	110,04	89,62	102,91	98,69
	Mean (SD)	0,047	0,155	7,806	11,526	0,31	1,048	56,312	84,784
Day 3	CV (%)	8,04	8,18	4,49	8,65	6,64	7,69	1,94	6,54
	Accuracy %	103,35	103,12	104,05	96,02	103,78	105,05	112,92	106,26
MEAN		0,044	0,147	7,577	11,991	0,303	0,949	51,439	79,810
CV (%)		6,41	6,68	6,32	6,15	8,05	5,92	5,18	7,22
Accuracy (%)		101,86	99,6	99,47	98,78	101,28	95,12	103,15	100,03
Accuracy Standard		LLOQ: 80% - 120%; LQC, MQC, HQC: 85% - 115%							
Precsion Standard		LLOQ: CV ≤ 20%; LQC, MQC, HQC: CV ≤ 15%							
Comment		Good							

Cross-contamination

Table 8. *Cross-contamination (n = 6)*

Sample	LLOQ		ULOQ			Blank-CR			CR (%)		
	AS1	AS2	AS1	AS2	IS	AS1	AS2	IS	AS1	AS2	IS
Mean	1692	2077	447482	600001	24295	0	0	0	0	0	0
Standard									≤ 20%		≤ 5%
Comment									Good		Good

Stability of original standard solution

Table 9. *Stability of AMP and MET in original solution at MQC concentration (AMP 7,502ng/ml và MET 49,866ng/ml) (n = 6)*

Sample	Short term		Long term			
	After 6 hours		80 Day		110 Day	
	AS1	AS2	AS1	AS2	AS1	AS2
Mean	100,03	98,99	92,46	96,61	99,24	97,40
CV (%)	1,91	2,40	1,16	3,76	2,98	1,26
Bias (%)	0,03	-1,01	-7,54	-3,39	-0,76	-2,60
Standard	Bias ≤ ± 10%					
Comment	Good					

Stability of original internal standard solution

Table 10. *Stability of HCTZ in original solution at 100µg/ml (n = 6)*

Sample	Short term	Long term	
	After 6 hours	80 Day	110 Day
Mean	102,58	90,19	90,21
CV (%)	4,11	4,46	2,16
Bias (%)	2,58	-9,81	-9,79
Standard	Bias ≤ ± 10%		
Comment	Good		

3.6 Stability of active ingredients at LQC concentration

Table 11. *Stability of active ingredients at LQC concentration (AMP 0,150ng/ml and MET 0,997ng/ml) (n = 6)*

Sample	Short term				Long term			
	Freeze-thaw		After 6 hours		80 Day		110 Day	
	AS1	AS2	AS1	AS2	AS1	AS2	AS1	AS2
Mean (%)	98,14	108,27	104,70	99,47	99,60	86,91	104,78	103,10
CV (%)	7,78	3,00	3,65	6,27	5,38	2,01	6,87	8,08
Bias (%)	-1,86	8,27	4,70	-0.53	-0,40	-13,09	4,78	3,10
Standard	Bias ≤ ± 15%							
Comment	Good							

Table 12. *Stability of active ingredients at HQC concentration (AMP 12,004ng/ml và MET 79,786ng/ml) (n = 6)*

Sample	Short term				Long term			
	Freeze-thaw		After 6 hours		80 Day		110 Day	
	AS1	AS2	AS1	AS2	AS1	AS2	AS1	AS2
Mean (%)	97,40	89,72	102,28	108,52	110,35	104,29	94,51	96,72
CV (%)	1,94	3,72	3,82	2,27	4,99	7,02	5,36	5,57
Bias (%)	-2,60	-10,28	2,28	8,52	10,35	4,29	-5,49	-3,28
Standard	Bias ≤ ± 15%							
Comment	Good							

Table 13. *Stability of active ingredients in autosampler at 10 ^{o}C (n = 6)*

Sample	AMP		MET		IS (100µg/ml)
	LQC (0,150ng/ml)	HQC (12,004ng/ml)	LQC (0,997ng/ml)	HQC (79,786ng/ml)	
Mean (%)	109,44	107,86	101,85	108,00	96,73
CV (%)	2,50	7,50	5,67	6,37	1,90
Bias (%)	9,44	7,86	1,85	8,00	-3,27
Standard	Bias ≤ ± 15%				
Comment	Good				

4. CONCLUSION

A rapid, sensitive, and specific LC-MS/MS method was successfully developed and validated for simultaneous quantitative determination of metoprolol succinate and

amlodipine besylate in human plasma. This method can be applied to bioequivalence study of fixed dose combination of MET and AMP formulation product.

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Evaluation on the Potential Effect of A Patient Decision Aid to Improve Patient Adherence in the UK: A Case Study in Venous Thromboembolism Patients

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Abstract

Introduction: Medicines have always been the most common therapeutic intervention in healthcare. However, many studies showed that patients often fail to follow treatment recommendations, both intentionally and unintentionally. In the UK, in order to increase patient adherence, The National Institute for Health and Care Excellence published the NG5 guideline to encourage shared decision making between patients and doctors. The Royal United Hospital NHS Foundation Trust (RUH) is planning to implement the use of Patient Decision Aid (PDA) in consultation for deep vein thrombosis (DVT) patients. The researchers, in collaboration with the chief pharmacist and consultants at RUH developed an initial PDA, which was used throughout this research. **Aim:** The aim of our study was to conduct initial testing on the usability, comprehensibility and feasibility of the adopted PDA from the perspectives of both clinicians and patients, as well as to evaluate its potential effect on improving patient adherence. **Materials and methods:** The research was designed as a cross-sectional study and conducted using qualitative methodology. The research included semi-structured interviews with patients and health care professionals of the RUH, 2 focus group with students and staffs of the University of Bath. Using thematic analysis, codes were generated from transcribed data freely and were not bound to any pre-existing coding frame or grounded theory. Those initial codes were then developed and grouped into final sub-themes and themes. **Results:** The majority of participants found the PDA to be better than the current RUH medicine information leaflet and believed that PDA would benefit future DVT consultation and improve their understanding and adherence. The PDA met most of patient' and HCP's expectations and received many positive comments. Participants also suggested some changes and future developments that could be adapted into the final PDA version. **Conclusion**: The PDA would be beneficial for both patients and HCPs in future DVT consultation, as well as be effective in improving patient adherence. However, it is arguable whether the need for a PDA in this field is crucial and patients might get more benefit from an updated medicine information leaflet.

Keywords: Patient decision aid, Ppatient adherence, share decision making, venous thromboembolism, deep vein thrombosis.

1. INTRODUCTION

Medicines have always been the most common therapeutic intervention in healthcare. Significant resources have been invested in the discovery and development of safe and effective drugs, so it is expected that patients will take their medicines at the right dose and at the right time to achieve the maximum effectiveness. However, many studies showed that patients often fail to follow treatment recommendations, both intentionally and unintentionally. It is estimated that for patients with long-term conditions, 30 to 50% of prescribed medicines are taken in the wrong way. 55% of non-adherence cases were described as unintentional, in which patients do not realise they are taking their medicines incorrectly, or forget to take their medication. In the UK, in order to increase patient adherence, The National Institute for Health and Care Excellence published the NG5 guideline to encourage shared decision making between patients and doctors. A range of different tools and approaches have been developed to assist the process, namely patient decision aids (PDAs), patient-held and electronic records, group sessions, individual education and health coaching. This study will focus on PDAs, which were designed to encourage active engagement, support patient knowledge about their condition and treatment options, and help patients think through what is important to them. Based on a careful review of the evidence, a PDA contains all treatment options available to patients and the outcome of those options (including benefits, harms, and uncertainties). The Royal United Hospital NHS Foundation Trust (RUH) is planning to implement the use of PDA in consultation for deep vein thrombosis (DVT) patients.At the moment, a PDA support DVT consultation has not been developed. It is expected that the use of PDA would increase patient adherence and lead to improvement of patient's satisfaction and their treatment outcome. The researchers, in collaboration with the chief pharmacist and consultants at RUH developed an initial PDA, which was used throughout this research.

2. METHOD

2.1. Settings

The Royal United Hospitals NHS Foundation Trust (RUH), in Bath is considering implementing PDAs into routine practice for DVT consultation but at present, the HCPs there do not have experience in using them. Furthermore, there has been some doubts expressed by HCPs, in view that patients might not want to be involved in making decisions, due to the lack of capacity or ability that might lead to 'bad' decisions, or the time constraints in consultations. The research team, in accordance with the RUH, will conduct a study exploring whether PDAs would be beneficial in supporting DVT consultations between the patient and the HCPs. The new PDA will be developed according to local and national guidance by the consultant haematologist and the chief pharmacist.

2.2 Design

The design of this study has been informed by the recommendations by the International Patient Decision Aid Standards (IPDAS) collaboration on a systematic development process for PDAs[31]. The development of PDAs followed published guidelines and evidence on the treatment options. Any feedback from HCP and patient will be listened and considered for further modification. The PDA's content was prepared by clinicians (consultant haematologist and chief pharmacist) at the RUH and had been sent to the research team to be reformatted.

The study was designed as a cross-sectional study, as the aim of the study is to evaluate the PDA before any improvement can be made. The design of choice fits the purpose of the study while remaining inexpensive and less time consuming to conduct and suits the ability of the researcher to commit follow-up.

2.3. Data collection

The study requires an in-depth understanding and exploration of patients', HCPs', as well as layperson views about the usability, comprehensibility and practicability of the PDA. Therefore, qualitative methodology was chosen as the method of choice due to the focus on capturing expressive information from participants, namely beliefs, feelings, values, and underlying motivation[33]. This methodology allowed the generation of detailed information and enabled researchers to have an insight into patient's view to understand their underlying opinions, feelings of using the PDA. In the other hand, the findings would not be applied to general populations with the same degree of certainty compared to quantitative analyses[34].

The qualitative data will be gathered through semi-structured interviews with patients and HCPs while focus groups are the method of choice for collecting layperson views on the PDA. Semi-structured interviews was chosen for HCPs and patients as it allows the collection of more detailed and in-depth data, fostering the generation of rich description, which in turn opened up more chances to probe and explore participants views, both in clinical area and design of the PDA. Compared to focus groups, interviews help to remove the potential negative effect of thepower dynamic between different HCPs, namely consultant, staff grade doctor and nurse. While for the general population, who were not required to have any specific knowledge or interest in DVT or PE, focus group allowed researchers to gather a broader range of information with minimum time and money compared to semi-structured interviews that are more time intensive.

For recruitment, the Chief Pharmacist generated a list of 52 potential patient participants from a list of discharged patients. An invitation letter, a study information sheet and an express of interest form were posted to those patients. Invitation letters and postage stamps were prepared by researchers and sent by RUH staff to ensure patient anonymous. Eight patients express their interest in taking part by post, and one patient called the PI to say sorry that she could not join the study (response rate equal 17.3%). Researchers contacted the participants to organise a convenient time to carry out the interview, but one patient cancelled the interview and one was not able to meet the researcher in time for the project. In the end, 6 patient semi-structured interview was conducted.

12 HCPs were invited to take part through both email invitation and direct letter deposit into their pigeonhole, accompanied by the information sheet and details on how to express an interest in participating in the study. Seven HCPs expressed (response rate equal 58.3%) their interest but researchers could not contact two of them. 5 HCPs semi-structured interview was conducted.

The students and staffs of the focus group were recruited by an emailed invitation letter sent by Director of Study, while the research team sent an emailed invitation letter to staffs, which included the email address of the researcher to whom they should reply to if they are interested in the study.

2.4 Population

Participants for the semi-structured interviews that formed the basis of the alpha test will include newly or recently diagnosed VTE patients that meet the required inclusion criteria, and the healthcare professionals who had experience working with DVT patients.

2.5 Data analysis

Data collected was analysed using thematic analysis, which was the method of choice due to its flexibility and the ease to access.

3. RESULTS & DISCUSSION

In general, participants found the concept of PDA to be very good. Patients reported that they found no disadvantages of a PDA and thought that PDA would be harmless. HCPs shared the positive view of the PDA. They thought that a well-designed PDA would be helpful and effective.

On another hand, many participants stated that while PDA is helpful for those who were engaged, it might not suit everyone. Some patients might not interest in information and would like the HCPs to make the decision for them.

3.1 Benefits of PDA

Participants reported many benefits of using a PDA. Many suggested that it can be used as something to take away and read it quietly at their own time. Patients thought that PDA would be a useful reference point for them and their family and caused they found the amount of verbal information in a short consultation is a lot for them to remember. A patient stated that PDA can save her from going back to doctor to ask for information all the time. One HCP added that patients liked written information and PDA can ensure that patients are fully informed, as the amount of verbal information given out might vary amongst staffs

Patients reported that PDA prompted them to think more about their treatment options and can trigger further questions.PDA is believed to improve patient satisfaction, empowering patients and give patients a feeling of being in control.

HCPs also stated that they think PDA could boot consultation process. PDA can provide more information to patients during diagnosis and treatment process

According to participants, PDA is a source of accurate information, which can improve patient's understanding of treatment options and side effects, which could result in an increasing of patient's adherence.

3.2 Disadvantages

Both patients and HCPs were concerned that PDA might provide too much information which could lead to patient confusion and worryness. They were afraid that some patients might get stress from having to make the decision themselves. Another patient added that the use of PDA might delay treatment decisions, which should be made quickly. The student FG argued that whether PDA could be bias and guiding patient toward cheaper medications. An

HCPs suggested difficulties of implementing of PDA in practice, stated that it would be difficult to ensure that patients understand all the information and implications in order to make a truly informed decision.

Participants reported many factors that may affect decision-making process. Many mentioned the 'Doctor knows best' belief that patient would accept what the Doctor say and do not want to have any discussion. One HCP shared that he thought making treatment decision was Doctor's job and HCPs should make the decision themselves to save patient from stress.

4. CONCLUSION

The finding showed that the majority of patients and HCPs believe that the PDA is better than the current leaflet and will benefit future DVT consultation. However, certain places require improvement before it could be implemented in routine practice.

Both patients and HCPs expressed concerned with the amount and variability of verbal information in consultation and thought of PDA as a reference or an accurate source of information, which is not the main desired benefit of PDA. Many believed that patient would accept what the Doctor say and do not want to have any discussion, which saved them from the stress. The need for a PDA in this field might not be crucial and the RUH should spend resources on revised the current medicine information leaflet so it can meet patient's demands. In any cases, all feedback and recommendations collected would be beneficial for the future development of PDA or medicine information leaflet.

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Population Pharmacokinetics of Imipenem in Burn Patients

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Abstract

Introduction: Burn is complex injury with high risk of hospital resistant organism infection.The use of broad spectrum antibiotics such as imipenemis common. Nevertheless, substantial change in physiopathology including augmented renal clearance(ARC) observed in severe burn patients results in high pharmacokineticvariability.Toxicity or sub-therapeutics may occur. **Objectives:** This study aimed to estimate PKparameters of imipenem and those potential covariates. **Methods:** Burn patients with body surface area injured >20% andimipenem indicationwere recruited. Two set of plasma samples (30 min post-dose and 1-2 hours before next dose)were obtainedat imipeneminitiation and before the end of imipenem use. ARC was defined if 8h-urinary creatinine clearance (8hClcr) was above 130 mL.min-1173 m2. PK samplewas quantified by validated HPLC method. Population pharmacokinetic analysis were performedusingMonolix2016R1. **Results:** A total of47 sets with 94 plasma samples were collected from 24 patients. Of which 18 sets were obtainedat ARC time. One compartmental model with proportional error fitted the data best. The inclusion of inter-individual (IIV) and inter-occasion variation (IOV) improved the goodness of fit of the model. Population volume of distribution was 33.5 L with IIV and IOV of 18.2 % and 15.6%, respectively. Population clearance and the respective IIV and IOV were 18.8L.h-1, 27.0 % and 28.1 %. Age and ARC showed to besignificant covariates ($p<0.001$). Targeted PK/PD attainment appeared to be affectedas a consequent. **Conclusions:** Imipenem pharmacokinetics had significant IIV and IOV on burn patient and the ARC may influence the targeted PK/PD attainment.

Keywords:imipenem, burn, population pharmacokinetics, inter occasion variation, augmented renal clearance.

1. INTRODUCTION

Burn is a complicated injury with high mortality and cause serious consequence to patients. The pathophysiology of burn is represented by inflamatory response after injury related to increased microvascular permeability, eodema or infection and lead to severe conditions like sepsis or progresivemultiorgan dysfunction syndrome or death [1]. In burn patient, a severe complication is acute renal failure with poor pronosis. In constrast, patients may experience augmented renal clearance (ARC) at several occasions, especially in the early phase of burn[2]. The variation in renal function may have unfavorable effect to the treatment if drugs have large urinary elimination[3]. The pharmacokinetic characteristic of many drugs including antibiotics are changed in which two fundamental parameters including volume of distribution (Vd) and Clearance (Cl) are directly affected by renal function variation and the eodemacondition[4-6]. As a result, patient may experience risk of antibiotics over exposure or subtherapeutic and higher dose for empirical regimen were recommended[7-9].

Carbapenemswith time-dependent activity are backbone antibiotics for the treatment of hospital infectionswhichcommonly occur in burn patients. Unfortunately, microorganisms are gradually resistant to these reserved antibiotics. Under high resistant pressure environment like intensive care unit, the requirement of cautious use of these antibiotics deserved awareness. Antibiotics should be usedin a manner not only to cure serious infection but also to minimize the risk of resistant emergence. Therefore, the application of pharmacokinetic/pharmacodynamicappeared to be a rational approach[5-6]. In order to improve the probability targeted attainment for typical microorganisms in burn patients, increasing dose is the common recommendation [7]. Nevertheless, the high variation of pharmacokinetic during the treatment with high inter-occasion variability of PK parameters was observed for meropenem and therefore the fixed empirical dose should be questioned[9]. Indeed, a real-time therapeutic drug monitoring on the dose of carbapenems in critically ill burn patient was proposed[10].

The use of carbapenems including imipenem National Institute of Burns of Vietnam follow empirical approach where the pharmacokinetic characteristic on this special population had not been well understood. To evaluate the appropriateness of current practice, this study aimed to estimate the population pharmacokinetics parameters and the potential covariates influencing pharmacokinetics properties of imipenem on burn patients.

2. METHODS

2.1 Patients

From December 2016 to March 2017, burn patients recruited in Intensive Care Unit, National Burns Institute of Vietnam were received routine care for their injury. Eligible patients were adults (≥18 years old) patients hospitalized within 72 hours after injury with injured body surface area of more than 20% and imipenem indication. Patients with renal failure or any other serious conditions before the injury were excluded. The study procedure were reviewed

and approved by local ethical committee and informed consents were obtained from patients or their caregivers.

2.2 Sample collections

Eligible patients receiving two hours intermittent imipenem infusionwith the dose of 0.5 or 1.0 g and the interval of 6 or 8 hour relying on decision of physicians. A set of two plasma samples of 30 minutes post dose and one or two hours before next dose were obtained at least 12 hours after imipenem commencement to ensure that steady state condition were achieved.

Second set of two plasma samples were collectedbefore the end of imipenem course and an additional third set of plasma samples were considered if acute kidney injury occurred. An eight-hour- urinary creatinine clearance (8h-Clcr) was measuredin parallel with each occasions ofplasma sampling. Augmented renal clearance (ARC) was identified if 8h-Clcr> 130mLmin^{-1} 1.73 m^{-2}. For remaining days, patientswere daily monitored according to routine practice in which estimated glomerular filtration rate (eGFR) were calculated using Cockroft and Gault equation.

For each plasma sample, three milliliter of venous blood were collected into heparinized vacutainer. After centrifuging at 1800g, one milliliter of plasma was obtained and was mixed immediately with one milliliter of 3-(N-morpholino) propanesulfonic acid (MOPS) 0.5M. The sample were stored at -40^{0}C for no more than 7 days before analysis.

2.3 Sample analysis

Plasma samples stabilized with MOPS were analyzed using a validated high performance liquid chromatography (HPLC) method. In short, 400 µL of plasma was mixed with 100 µLmeropenem1mg/mL as internal standard and subsequently precipitated by 500 µL of acetonitrile. After centrifuging at 3900g in 10 minutes, the supernatant was evaporated under nitrogen stream and the residual were dissolved in 200 µL MOPS 0.5M. An injection volume of 50 µL was operated byAgilent 1200 chromatography system consist of columm XDB-C8 (4.6 x 150 mm, 5µm); mobile phase of phosphat buffer 0.1M pH 7.4: methanol (60:40); eluent rate of 0.5mL/minute for 19 minutes with UV detector monitoring at 298nm. The method showed acurate and precise (bias:-2.6%, 5% and -3.5%; precision:5.91%; 4.73% and 6.31% at concentrations of 0.5, 20 and 60 µg/mL, respectively) with lower limit of quantification of 0.5µg/mL. The linearity range were 0.5 to 80 µg/mL and the stability was proved at -40^{0}C for one week.

2.4 Population Pharmacokinetic

Population pharmacokinetic modeling was performedusing non-linear mixed effect model approached with the help of Monolix2016R1. Assumptions of one or two compartmentfor structural modelwith inter-individual and/or inter-occasion variability (IIV and IOV) of pharmacokinetic parameters weretested. The model selection were firstly performedwith basic pharmacokinetic model in which no covariates were added. The Bayesian information criterion (BIC) was used to test the significant improvement in description of data by the model as the number of observations in this study is limited. BIC reduction by more than 2 was considered to be significant improvement.The covariate models were subsequently tested using the best-fitted basic pharmacokinetic model in which covariates of consideration were age, gender, weight, burn area and 8h-Clcr. Likelihood ratio test was applied and the -2 log-likelihood reduction threshold of 3.84 ($p<0.05$) were considered to defined significant covariate. Individual pharmacokinetic parameters estimated from the last model were used to calculated targeted *f*T>MIC values.

3. RESULTS

A total number of 24 patients with burned surface areas of 50.8 ± 17.3 (%) hospitalized within median of 4.5 (IQR: 3-9.5) hours after injury. Patient had mean age of 38.9±17.5 yearsin which 15 (62.5%) were male. Most of patients have preserved renal function with eGFR of 85.9 ± 29.4 mL min^{-1} 1.73 m^{-2}. Patients were received intensive care during first days of admission to stabilized the injury condition and imipenem treatment were commenced after 5 (IQR: 3.3 – 7.0) days. Most of patient received 2 hours intermittent infusion dosing at 1g three to four times per day and the duration of imipenem treatment course lasted after 7 (IQR: 6-10) days. A total 47 pharmacokinetics sampling occasions were attained in which ARC was observed in 18 (38,3%) occasions of 13 (54.2%) patients. (Table 1).

Table1. *Characteristics of patients (n=24), imipenem usage and sampling.*

Parameters	**n (%)**
Gender (Male)	15 (62.5)
Age (yearrs)(#)	38.9 (17.5)
SOFA score($)	5 (4 - 6)
APACHE II score($)	14 (11 – 18)
eGFR (ml/phút/1,73m^2)(#)	85.9 (29.4)
Burned surface area (m^2) (#)	50.8 (17.3)
Time of hospitalization since injury (hrs)($)	4.5 (3 - 9.5)
Time of imipenem initiation (days)($)	5 (3.3– 7.0)
Imipenem dosage (n=47)	
1g q.i.d.	38 (80.9)
1g t.i.d.	7 (14.9)
0,5g q.i.d.	1 (2.1)
0,5g t.i.d.	1 (2.1)

Duration of Imipenem courses (days) [$]	7.5 (6 – 10)
Patients with ARC (N=24)	13 (54.2)
Occations with ARC(N = 47)	18 (38.3)
Sampling occation(s) per patient (*)	
1	6
2	30
3	3
4	8

[$] mean (interquartile range); [#] mean (standard deviation); (*) Patient may have two courses of imipenem, the second sampling occasion may not be available due to antibiotic switching, patient transfer or death.

In population pharmacokinetics modeling, log-normal distribution was assumed for pharmacokinetic parameters and the respective variation components. One compartmental model appeared to fit better as BIC were significantly lower. The subsequent incorporation of IIV and then IOV components to Vd and Clshowed significantly improvement in comparison with zero model (BIC of 512 and 505 vs. 615) and that defined the basic model. In basic model, IIV of Vd and Cl were 18.2% and 27% while theIOVwere 15.6% and 28.1%, respectively.

Among selected covariates, 8h-Clcr and age had significant impact(likelihood ratio test, $p<0.001$). In addition, these two covariates wereremained in multivariate model since withdrawing any of those resulted in significant increase of -2 log-likelihood value (466 and 463 vs. 446). For better interpretation, ARC occurrence was used instead of 8h-Clcr and it appeared to have similar effect on the model. Age showed significant inverse relationships with both PK parameters in which 10 year older was accounted for 13 percent reduction in Cl and Vd. ARC had no impact on Vd while Cl at ARC occasions were 1.5 folds higher than those without ARC (Table 2).

Table 2. *Population pharmacokinetics parameters of imipenem on burn patients*

	Estimations (95% CI)	
Basic pharmacokinetic model		
Volume of distribution (L)	33.5 (28.2-38.8)	
Inter-individual variability (CV%)	18.2	
Inter-occasion variability (CV%)	15.6	
Clearance (L/h)	18.8 (15.9-21.7)	
Inter-individual variability (CV%)	27.0	
Inter-occasion variability (CV%)	28.1	
Residual variability (CV%)	27.2	
Covariate model		**p**
Volume of distribution (L)		
Non-ARC, Age = 38.9	32.6 (26.7-38.5)	
ARC	33.6 (26.5-40.7)	0.83
Age (10 years) (*)	0.874 (0.802-0.952)	0.002
Clearance (L/h)		
Non-ARC, Age = 38.9	16.4 (14.24-18.56)	
ARC	24.9 (20.6-29.2)	<0.001
Age (10 years) (*)	0.872 (0.816-0.932)	<0.001

Age was centralized by mean value of 38.9; (*) present relative reduction of parameters;

As patients with ARC condition had higher imipenem clearance, the probability of targeted PK/PD attainment also was affected. The PTA for the target of 40% *f*T>MIC and 70% *f*T>MIC was presented in Figure.

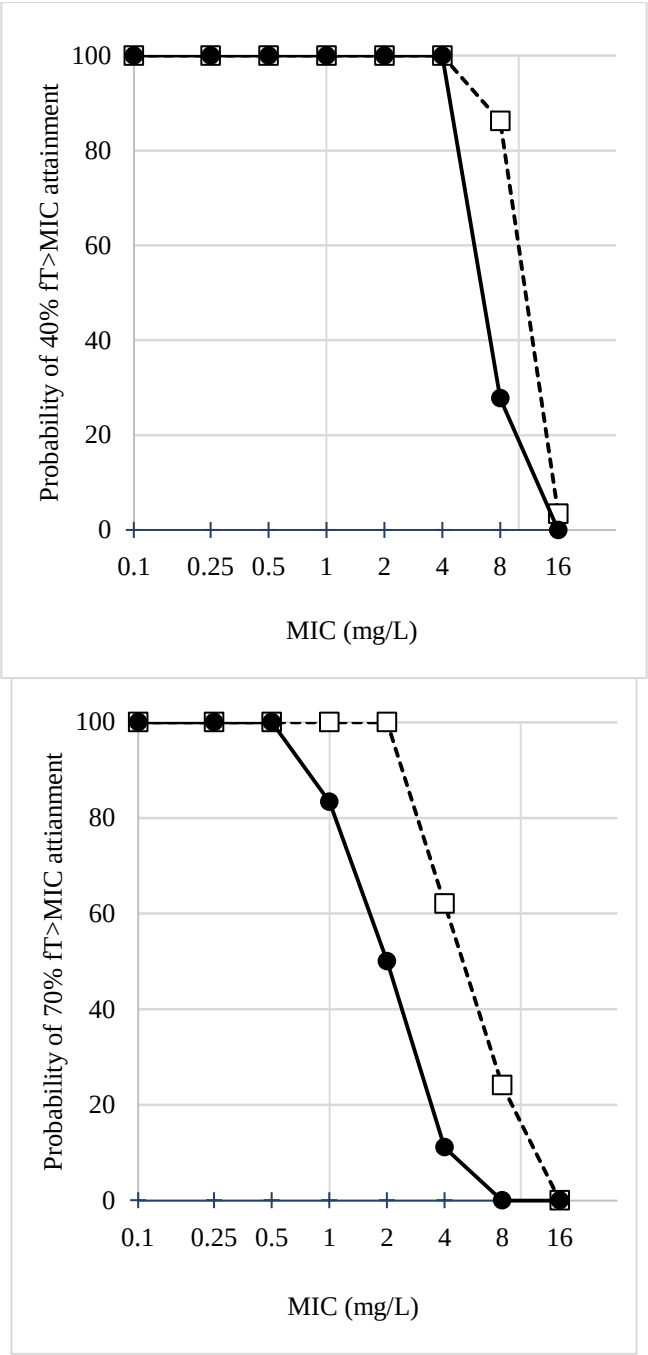

Figure 1. *The probability of target attainment (PTA) at 40% (left) and 70% fT>MIC (right) of imipenem on ARC (solid line, closed circle) and non-ARC (dash line, open square) burn patient*

4. DISCUSSION

This study showed that imipenem pharmacokinetics varied substantiallynot only between burn patients but also between occasions during the treatment. In addition, the age of patient and the development of ARC were significant covariates predicting pharmacokinetic alteration.

The population PK parameters including Vd and Cl estimated in this study are inline with previous finding [11]which was higher than those in healthy subjects. As limited sampling scheadule, the one compartment model appeared to better present PK data. The high IIV in Cl and Vd observed in this study suggested that empirical imipenem dose in approved label may not fit all patients and the risk of sub-therapeutic could be aware. Belzeberg et al. could not predict the pharmacokinetic of imipenemin critical ill patient with preserved renal function due to the high variability of PKparameters. Efforts were put on exploring covariates that could explain the high IIVof burn patients and only creatinine clearance appeared to have significant impact [11-12]. In our study, age showed significant prediction in which older patient may have lower Vd and Cl. Nevertheless,combiningeffect on half-life may be neutralized because this parameter depended on both Vd and Cl but in opposite direction. The drug exposure therefore was of minor alteration.

With the sampling schedule at different occasion, it enabled us to estimate the high IOV in both Vd and Cl. The IOV of about 20% in this study may partly explain the high PK variation of imipenemobserved in published result.It should be of note that this type of variation reflected the change of pathophysiological characteristic of patientsduring the treatment. Without monitoring drug level, afixedempirical dosemay result in sub-therapeutic or toxicity depending on patient's condition. Therefore, thereal-time therapeutic drug monitoring was proposed for this special situationand it was proved to have impact on altering empirical dosing of imipenem[10]. Nevertheless, real-time (TDM) was not simple practice for limited resources facilities. The alternative approach may come from our finding that the ARC emergence was the significant covariate and showed a high correlation with the estimated Cl. ARC were commonly observed in severe injured population [2] at the prevalence of about 50%. Closely monitoring this condition as a surrogate marker for the change in drug clearance could help to adjust the dose in time. With current empirical dose, the PTA for 40% *f*T>MIC may not be sufficient in ARC patient at MIC 8mg/L. The PTA curves of ARC and non-ARC patientswere futher split with the target of 70% *f*T>MIC and PTA of ARC patient were only 50% at MIC 2mg/L, the common threshold for defining susceptibility.

This study has several limitations. Small sample size and limited PK sampling schedule may lower the precision of estimated parameters and prevent the extrapolation in to larger population.

5. CONCLUSIONS

Pharmacokinetic of imipenem in burn patients characterized by a high inter-individual and inter-occasion variation which may undermine the empirical drug use. Close monitoring of renal function may help in dose adjustment during treatment to ensure the treatment efficacy.

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Economic Evaluation of Exemestane for Patients with Postmenopausal Hormone-Receptor-Positive Advanced Breast Cancer: A Systematic Review

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Abstract

Althoughexemestane has been used as an effective hormonal therapy for advanced breast cancer (ABC) to prolong progression, it is not listed in the National List of Essential Medicines (NLEM). In Thailand, economic evaluation evidence has played a crucial role to inform Thai policymakers. However, there has beenno systematic reviewof economic evaluation studies on exemestane for the treatment of ABC patients. **Objectives**: This study aimed to systematically review the methodology and results of economic evaluation studies on exemestane for the treatment of postmenopausal-hormone-receptor-positive ABC patients. **Methods**: A literature search was conducted using PubMed, Scopus, and Center for Reviews and Dissemination databases from January 2000 to March 2016. The studies that comparedexemestane to other treatments in these patientsand published in English were included. Costinganalysis and reviews were excluded. **Results**: A total of124 relevant publications were retrieved. Five publications which were conducted in Australia, Europe, Canada and the United States were included. Three used Markov model, one used hazard-driven model, and the other did not provide details on the modeling. Cycle lengths were used as monthly and six weeks. The time horizons were 3, 5, 10 years, 120 weeks and 1,000 days. All publications used direct medical cost and one included cost of adverse event. The discount rates were 3%, 3.5%, and 5%. One-way and probabilistic sensitivity analyses were employed in three and two studies, respectively. The outcomes were reported as quality adjusted life years (QALYs), quality adjusted progression free years (QAPFYs), and life years (LYs) gained. All studies concluded that exemestane was cost-effective for these patients.There were a limited number of economic evaluation studies of exemestane for the treatment of ABC. Moreover, no cost-effectiveness or cost-utility analysis of exemestane study has been conducted in Asian countries including Thailand.

Keywords: Exemestane, Advanced breast cancer, Economic evaluation, Systematic review

1. INTRODUCTION

Breast cancer is a serious most common cancer in females all over the world. The worldwide incidence and mortality of breast cancer were available in the GLOBOCAN series of the international agency for research on cancer. The data were derived from population-based cancer registries form many countries in 2012. The GLOBOCAN reported that breast cancer

was the second most common cancer in the world and most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012, approximately 25% of all new cancer cases. Breast cancer is ranked as the fifth cause of death from overall cancer, around 522,000 deaths. According to the National Cancer Institute Thailand, 943 new breast cancer were reported, approximately 24% of all new cancer cases[1]. Approximately one-fifth of these patients had five year survival depending on risk factor of the patients[2]. The risk factors affecting recurrence and treatment outcomes is estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (HER2)[3]. The costs of this disease are very high in terms of both human suffering and healthcare consumption[1].

Hormonal therapy,chemotherapy, and targeted therapy have been the backbone treatment for the advanced breast cancerpatients[4]. The aromatase inhibitors (AI), anastrozole, letrozole and exemestane, was recommended as the first line hormonal therapy for postmenopause hormone receptor positive recurrent and metastasis breast cancer due to response rate (RR) and time to progression (TTP) compared withtamoxifen[2]. The mechanism of action of steroidal AI and non-steroidal AI (NSAI) is different and not cross resistant[5]. In the case of treatment failure from any sub-group AI, the treatment can be switched to another sub-group. The sequence using hormone therapy such as tamoxifen and fulvestrant might be considered as the second and third line therapy after treatment failure. The use of chemotherapycan be delayed[2, 5]. Moreover, exemestane, a steroidal aromatase inhibitor, strongly inhibits the enzyme aromatase that converts androgen to estrogen by irreversible binding to the enzyme which makes itunable to work permanently.

According to the protocol for the treatment of breast cancer patients with recurrent or metastatic breast cancer, exemestanehas not been included in the National List of Essential Medicines (NLEM) in Thailand. Only tamoxifen, letrozole, and megestrol are included in NLEM. The Subcommittees of the NLEM has requested the economic evaluation study ofexemestane for the treatment loco-regional recurrent or metastasis or advanced breast cancer (ABC) for post-menopause and hormone receptor positive patients for making decision whether exemestane should be added into the NLEM in Thailand. Therefore, the purpose of the study was to systematically review the methodology and results of economic evaluation studies on exemestane for the treatment of postmenopausal-hormone-receptor-positive advanced breast cancer (ABC) patients.

2. MATERIALS AND METHODS

2.1 Literature search

A systematic review was conducted by searching the economic evaluation studies on exemestane for the treatment of ABC patients from PubMed, Scopus and CRD from January 2000 to March 2016. The search termswere “exemestane”[Title/abstract],“advance*or metas* and breast cancer*”[Title/abstract], “economic* evaluation* or cost effective* or cost utility”

2.2 Selection criteria

The inclusion criteriawere applied to select the economic evaluation studiesfocusing on exemestane, published in English. Target population was postmenopausal, hormone receptor positive, locoreginal recurrent or metastatic stage breast cancer. The guidelines, reviews and costing analysis studies were excluded.

2.3 Quality assessment

The quality of relevant publications was assessed by two independent authors in accordance with the Guidelines for Health Technology Assessment in Thailand focusing on three areas i.e., data sources, result reporting and analysis methodology[6]. The data extraction sheetwas performed in Microsoft Excelincludingpublication year, country of the study, populations, comparator, perspective, modeling method, cycle length timehorizon, discounting rate,costs, outcome, and sensitivity analysis.

Table 1. ***Key topics in data extraction***

1. Populations
2. Comparators
3. Modeling
4. Perspective
5. Cycle length
6. Time horizon
7. Cost component
8. Outcome
9. Discounting rate
10. Sensitivity analysis

3. RESULTS AND DISCUSSIONS

A total of 124 relevant publications were retrieved fromthe systematic search. Nine duplication publications were found by Endnote program. One hundred fifteen titles and abstracts were reviewed. After reviewing, 108 publications were excluded because 88 studies were not economic evaluation and 20 studies focused on early stage or adjuvant setting of breast cancer. Seven studies with full text were retrieved, and two studies i.e., costing analysis and review of cost effectiveness analysis were excluded. Finally five publications i.e.,Xie et al., 2015[7], Diaby et al., 2014[8], Verma&Rocchi, 2003[9], Lindgren, Jonsson, Redaelli, &Radice, 2002[10] and Hillner&Radice, 2001[11]were included.

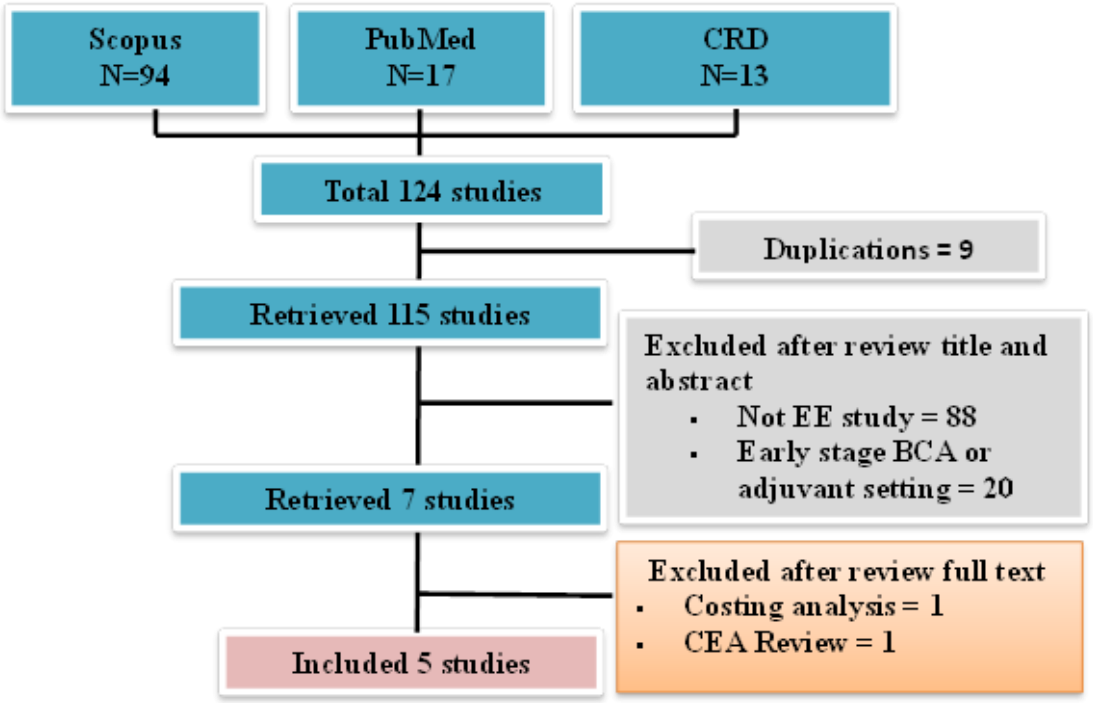

Figure 1. *Flowchart of study selection*

There are three cost effectiveness studies in theUS, Australia and Europe (Belgium, France Germany, Italy, Netherlands, Spain, and UK) and Canada conducted and published during2001-2003. Then during 2014-2015, two cost effectiveness studies in the US were published because the treatment guidelines recommended to useeverolimus in combination with exemestane.

Four publications used payer perspective, while only one applied a societal perspective. Target populations of all five studies werepostmenopausal hormone positive advanced breast cancer after failure from tamoxifen(3 studies) and those after failure from letrozole or anastrozole(2 studies). Markovmodel (3 studies), hazard driven model (2 studies) and modeling method (1 study) were used. Monthly (1 study) and 6 week(1 study) cycle lengths

were used, whereas three studiesdid not state. The reason for short cycle length was thatthe advanced breast cancer couldbe progressed in a short time period. The time horizon of 3,5, or 10 years wasused (3 studies), and time horizon periodsof 120 weeks and 1000 days waere used in the cost effectiveness studiesconducted alongside with other clinical trials. Five publications used direct medical cost as medicine and treatment cost, only one publication addedadverse event cost. The adverse event cost was not included in most publications because the side effectsof exemestanewereheadache and hot flushwhich were common and did not require costly treatment. Discount rates of 3%, 3.5% and 5% were used. Five publications conducted one way sensitivity analysis and two publications conducted probabilistic sensitivity analysis. The outcome was reported in quality adjusted life years (OALY) and life years gained (LYG). Five studies suggested that exemestane would be cost effective.

Studies	Xie et al., 2015	Diaby et al., 2014	Verma & Rocchi, 2003	Lindgren, Jonsson, Redaelli, & Radice, 2002	Hillner & Radice, 2001
YEAR	2015	2014	2003	2002	2001
Country	US	US	Canada	Europe and Australia	US
Perspective	Payer	Payer	Payer	Payer	Societal
Population	Postmenopausal, HR+,advanced Breast cancers				
	After failured of letrozole, anastrozole	After failured of letrozole, anastrozole	After failured of tamoxifen	After failured of tamoxifen	After failured of tamoxifen
	HER2+ and -	HER2-	HER2 N/A	HER2 N/A	HER2 N/A
Treatment	Everolimus+Exemestane	Everolimus+exemestane	Exemestane	Exemestane	Exemestane
Comparator	Exemestane Fulvestrant Tamoxifen	Exemestane	Anaztrozole Letrozole Megestrol	Megestrol	Megestrol
Modeling	Markov model	Markov model	Markov model	Hazard driven model	Modeling
Cycle length	monthly	6 weeks	N/A	N/A	N/A
Time horizon	10 years	120weeks (Equal to final progression free analysis of BOLERO-2 trial)	5 years	3 years	1,000 days (2.75 years)
Direct medical cost	Drug, treatment, AE	Drug, treatment	Drug, treatment	Drug, treatment	Direct medical cost
Discount rate	3%	3.5%	5%	3%	3%
Sensitivity analysis	1-ways SAs, Multivariate PSA	1-ways SAs, Multivariate PSA	1-ways SAs	1-ways SAs	1-ways SAs
Outcome	QALYs	QAPFYs	Life year gain	Life year gain	Life year gain
Result	EVE+EXE was associated with 1.99 QALY and total direct cost was $258,648over 10 years Incremental cost per QALY of EXE+EVE compared with EXE was $139,740 FUL was $157,749 TAM was 115,624 at the willingness to pay $130,000/QALY or above EXE+EVE might be considered a cost effective treatment	EVE+EXE had incremental benefit of 0.22 QAPFY compared to EXE and incremental cost of $60,574. ICER of $265,498.5/ QAPFY	EXE and anastrozole both cost Canadian $9000 per life year gained, and letrozole saved Canadian $300 annually, with no life year gain.	EVE+EXE was associated with 1.99 QALY and total direct cost was $258,648 over 10 years Incremental cost per QALY of EXE+EVE compared with EXE was $139,740 FUL was $157,749 TAM was 115,624 at the willingness to pay $130,000/QALY or above EXE+EVE might be considered a cost effective treatment	EVE+EXE had incremental benefit of 0.22 QAPFY compared to EXE and incremental cost of $60,574. ICER of $265,498.5/ QAPFY

Figure 2. *Methodological characteristics on economicevalucuation on exemestane for treatment of postmenopausal HR+ve advanced breast cancer.*

4. CONCLUSIONS

There were very few economic evaluation studies of exemestane in advanced breast cancer. Some of the previous studies did not explain in details onthe model such as transition stage and cycle length. None of these studies was conducted in Asia and Thailand. Therefore, it is important that cost-effectiveness analysis of exemestane in Thailand should be performed in order to help policy makers making policy decision whetherexemestane should be added into the NLEM.

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Ceftriaxone Usage in Neonates in a Hospital of Obstetrics and Gynecology

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Abstract

Introduction: Bacterial infection is a major cause of morbidity and mortality in neonates, especially those were born prematurely. Narrow-spectrum antibiotics are considered for several infection conditions or prophylaxis, i.e. gentamicin with penicillin or ampicillin are specific for group B Streptococcus or *E. coli* - the most frequently identified organisms. Empirical therapy should not start with a broad spectrum antibiotic such as the third generation cephalosporin or carbapenem because of the high risk of antibiotic resistance. Among third generation cephalosporins, cefotaxime is preferred to ceftriaxone in patients with hyperbilirubinemia or with intravenous calcium. However, a study in 2015 in the neonatal intensive care center of the research hospital revealed that ceftriaxone was the most commonly used antibiotic. **Objectives:** The study described characteristics of the neonates given ceftriaxoneand the explored pattern of ceftriaxone usage in the investigated institute. **Methods:** Medical records with ceftriaxone prescription were stratified randomly selected from discharge list in 2015. Information of patient demographic characteristics, neonatal infection features, microbiological culture results, and antibiotic regimens was collected into an established data collection form. **Results:** A total of 200 medical records were included. Preterm neonates accounted for 60.5% of those,15% was very low birth weight. According to British national formulary for children (BNFC) all patients had at least onecontraindication to ceftriaxone. The contraindications included corrected gestational age < 41 weeks (98.5%), jaundice (39.5%), hyperbilirubinemia (24.5%) and hypoalbuminemia (51.0%). About quarter (51/200) had a definite diagnosis of bacterial infection while the remaining patients (143/149) had risk of infection only. All patients were prescribed ceftriaxone for initial empirical treatment of infection (25.5%), prophylaxis (67.5%) and unknown reasons (7%). Combination between ceftriaxone and amikacin was the most common initial regimen for either treatment (74.5%) or prophylaxis (57.0%). All patients were prescribed ceftriaxone at a dose of 100mg/kg once daily intravenous injection which might not be appropriate for patient without meningitis. **Conclusion**: Physicians should reconsider ceftriaxone in initial regimen for the treatment and prophylaxis of neonatal infections, especially in patients with gestational age < 41 weeks, jaundice, hyperbilirubinaemia, hypoalbuminemia. Ceftriaxone should be given at lower doses than current practice by intravenous infusion. Intravenous injection is not preferred for research population.

Keywords: ceftriaxone, neonate, neonatal infection

1. INTRODUCTION

Bacterial infection is a significant cause of morbidity and mortality in newborn babies. In addition, risk factors for infection in neonates are common, most of which are related to the care given to pregnant women and newborn babies. In neonates with definite or suspected bacterial infections, as well as one have risk factors for infection, the use of antibiotics for the treatment or prevention of neonatal infection is unlikely to be delayed[3], [6], [11].

The most frequently identified organisms in neonatal infection are group B Streptococcus or *E. coli* so the preferred treatment regimen is ampicillin (or penicillin) in combination with gentamicin or amikacin[2]. In case of septicemia ormeningitis, the choice may be ampicillin plus gentamicin of cefotaxime[4], [7]. The first recommended antibiotic prophylaxis for early onset septicemia in neonates is combination between ampicillin (or penicillin, amoxicillin) and an aminoglycoside[3], [6], [11]. Empirical therapy should not start with a broad spectrum antibiotic such as the third generation cephalosporin or carbapenem because of the high risk of antibiotic resistance. Among third generation cephalosporins, cefotaxime is more preferred to ceftriaxone in patients with hyperbilirubinemia or with intravenous calcium[7].

In Vietnam, there is no study on the usage of antibiotics forthe treatment and preventionof neonatal infections. In a small trial in the research hospital, we found that among neonates receiving antibiotics, more than 80% of patients were prescribed ceftriaxone. Therefore, this study was conducted with the aim of describing the characteristics of the neonates given ceftriaxone and the characteristics of the indication, dosage, and administration of ceftriaxone on them.

2. MATERIALS AND METHODS

Medical records with ceftriaxone prescription were stratified randomly selected from discharge list in 2015. Information of patient demographic characteristics, neonatal infection features, microbiological culture results, and antibiotic regimens was collected into an established data collection form. Research outcomes included: (1) Percentage of patients according gestational age at birth and birth weight,characteristics related to ceftriaxone contraindications in newborn babies,definite and suspected infections, risk factors for infection and microbiological tests; (2) Percentage of indications of ceftriaxoneand antibiotic regimens with ceftriaxone; (3) Percentage of administration route, average dosage and median duration of use of ceftriaxone.Data was analyzed with Stata 13.0 program.

3. RESULTS

3.1. Profile of patients

200 medical records were included to be investigated. The majority of patients were preterm (60.5%) andaverage gestational age was 35.4 ± 3.5 weeks. Mean birth weight was 2314 ± 795.5 grams, 15.0% of newborns had very low weight (weight at birth <1,500 grams). Before administration and throughout the course of ceftriaxone, we investigated whether or not the features of the contraindication of ceftriaxone are mentioned in literatures, including corrected gestational age less than 41 weeks, jaundice, hypoalbuminemia or hyperbilirubinemia. The results were shown in Table 1.

Table 1.*Characteristics associated with contraindications to ceftriaxone*

Characteristics	Number of patients	Percentage
Corrected gestational age less than 41 weeks	197	98.5
- Full-term neonates	76	38.0
- Premature neonates	121	60.5

(gestational age at birth< 37 weeks)		
Jaundice	79	39.5
Hyperbilirubinemia(> 10mg/dL)	49	24.5
- Dangerous hyperbilirubinemia (>15mg/dL)	13	6.5
Hypoalbuminaemia(<36 g/l)	102	51.0

Furthermore, all patients had at least one characteristic and 69.0% had two or more simultaneous characteristics.About quarter of patients (51/200) had a definite or suspected diagnosis of bacterial infection whilethe remaining patients (143/149) had risk of infection only (see Table 2).

Table 2.*Risk factors for neonatal infection in neonates without infection diagnosis*

Risk factors	**Number of patients**	**Percentage (N=149)**
Risk factors from mother		
Suspected of confirmed infection of amniotic sac and membranes	29	19.5
Suspected or confirmed rupture of membranes for more than 18 hours in a preterm birth	19	12.8
Prelabour rupture of membranes	14	9.4
Maternal infection in labour	13	8.7
Parenteral antibiotic treatment given to the woman for confirmed or suspected invasive bacterial infection at any time during labour, or in the 24-hour periods before and after the birth [This does not refer to intrapartum antibiotic prophylaxis]	3	2.0
Risk factors from baby		
Premature	94	63.1
Mechanical ventilation	92	61.7
Feeding tube sonde	69	46.3
Parenteral nutrition	66	44.3
Catheter	57	38.3
Very low birth weigh	26	17.5

In terms of microbiological tests, 96 patients (48.0%) had microbiological tests. All samples were bloodspecimen, however, only 4 tests were positive.

3.2. Ceftriaxone regimens

25.5% of patientswere prescribed with ceftriaxonefor treatment, 67.5% for prophylaxisand7.0% for unknown purposes.Total regimens with ceftriaxone were 298 and all patients were prescribed with ceftriaxone for initial empirical therapy.

Table 3. *Ceftriaxone regimens*

Regimes	Initialregimens n (%) (N=200)	Alternative regimens n(%) (N=98)	Total n(%)(N=298)
Ceftriaxone only	**69 (34.5%)**	**37 (37.8%)**	**106 (35.6%)**
Ceftriaxone + an other antibiotic	**122 (61.0%)**	**32 (32.6%)**	**155 (51.7%)**
Ceftriaxone + amikacin	122 (61.0%)	10 (10.2 %)	132 (44.3%)
Ceftriaxone + imipenem/cilastatin	-	20 (20.4%)	20 (6.7%)
Ceftriaxone + metronidazole	-	2 (2.0%)	3 (0.7%)
Ceftriaxone + 2 other antibiotics	**9 (4.5%)**	**29 (29.6%)**	**38 (12.7%)**
Ceftriaxone +amikacin+ imipenem/cilastatin	8 (4.0%)	24 (24.5%)	32 (10.7%)
Ceftriaxone + amikacin+ metronidazole	1 (0.5%)	3 (3.1%)	4 (1.3%)
Ceftriaxone +imipenem/ cilastatin + colistin	-	2 (2.0%)	2 (0.7%)
Total	**200 (100%)**	**98 (100%)**	**298 (100%)**

Table 4. *Initial antibiotic regimens with ceftriaxone*

Regimens	Early neonatal infection n (%)(N=21)	Suspected early neonatal infection n (%)(N=30)	Prophylaxis for neonatal infection n (%)(N=135)
Ceftriaxoneonly	2 (9.5%)	6 (20.0%)	54 (40.0%)
Ceftriaxone + amikacin	16 (76.0%)	22 (73.3%)	77 (57.0%)
Ceftriaxone + amikacin + imipenem/cilastatin	3 (14.3%)	2 (6.7%)	3 (2.2%)
Ceftriaxone + amikacin + metronidazole	-	-	1 (0.7%)

3.3. Dosage and administration of ceftriaxone

All patients received intravenous injection ceftriaxone at a dose of 100 mg/kg once daily (calculated on birth weight). The median duration of ceftriaxone was 5 dayswhich accounted for two third of length ofthe hospital stay (median 7.5 days).

4. DISCUSSIONS

4.1 Characteristics associated with contraindications to ceftriaxone

In British National Formulary for Children (BNFC), ceftriaxone is contraindicated in neonates with corrected gestational age less than 41 weeks, or jaundice, hyperbilirubinaemia, hypoalbuminaemia[9]. Meanwhile according to the summary of product characteristics (SPC) of Rocephin (ceftriaxone sodium), ceftriaxone is contraindicated in premature neonates with

corrected gestational age less than 41 weeks, full term neonates with hyperbilirubinaemia, jaundice, or who are hypoalbuminaemia[5]. In Vietnamese National Formulary, ceftriaxone is contraindicated in neonates with hyperbilirubinaemia [1].Therefore, in this study, according to BNFC all patients had at least one contraindicationto ceftriaxone use, half of patients, if followedSPC of Rocephin,had contraindications to the antibiotic andquarter of them had contraindications to ceftriaxone in accordance with Vietnamese National Formulary. Neonates with these characteristics will have high risk of bilirubin encephalopathy if they were prescribed with ceftriaxone. Normally bilirubin is bound to albumin in plasma. Ceftriaxone can displace bilirubin fromserum albumin, which results in increased free bilirubin level in the blood whichcan penetrates in to nervous system and develop bilirubin encephalopathy. Using ceftriaxone in neonates with hypoalbuminaemia increases the risk of hyperbilirubinaemia. The relationship between hyperbilirubinemia and neurotoxicity is most significant in preterm and low birth weight infants [8].

4.2 Ceftriaxone in initial regimens

In the current study, more than a quarter of neonates were given ceftriaxone for treatment and nearly three quartersreceived it for prevention ofneonatal infections.All patients in this study were treated with ceftriaxonein initial regimens. In the current study combination ofceftriaxone and amikacin was the most common initial regimens prescribed for the treatment of early neonatal infection (76.0%), suspected early neonatal infection (73.3%) or prophylaxis (57.0%).Previous literature suggested thatinitial experimental antibiotic therapy for treatment and prophylaxis of early neonatal infection is ampicillin (or penicillin) in combination with gentamicin [4]; for late onset neonatal infection, flucloxacillin and gentamicin combination becomes the preferred regimen[12]. In case of meningitis, the initial experimental therapy includes cefotaxime with or without ampicillin [10], however, no meningitis case was reported in this study. It can be seen that β-lactame with aminoglycoside is reasonable according to recommendations, but ceftriaxone is not recommended in these patients. The third-generation cephalosporin antibiotic combined with an aminoglycoside is recommended for the treatment of suspected or identified infections by Gram (-) bacteria[11]. Cefotaxime is superior third-generation cephalosporin to ceftriaxone for patients with hyperbilirubinemia [7].

4.3 Dosage and administration of ceftriaxone

All patients in the study were given ceftriaxone at dose of 100 mg/kg once daily. This dose is higher than recommendation by BNFC and VietnameseNational Formularywhich standard dose of ceftriaxone in the newborns is 20-50mg/kg/ day andmaximum dose of 100mg/kg/day is usually recommended for severe infections such as meningitis[1], [9].However, there was no meningitis cases found among the patients in our study. In neonates, ceftriaxone is recommended for administration by deep intramuscular injection or intravenous infusion over 60 minutes with doses of 50 mg/kg or over [1], [9]. Intravenous infusion over 60 minutes reduces the risk of hyperbilirubinemia in neonates[5]. All patients were given ceftriaxone intravenously which is not recommended in neonates.

5. CONCLUSIONS

Physicians should reconsider the use of ceftriaxone in initial regimen for the treatment and prophylaxis of neonatal infections, especially in patients with gestational age < 41 weeks, jaundice, hyperbilirubinaemia, hypoalbuminemia. Ceftriaxone should be given at lower doses than the current practice by intravenous infusion. Intravenous injection is not preferred for neonates

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The Use of Herbal Medicine Among Cancer Patients at Department of Chemotherapy, University Medical Center, Ho Chi Minh City

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Abstract

Introduction: Cancer is increasingly a global health issue. A large proportion of cancer patients are estimated to use herbal medicines, but data on type of herbal medicines, the benefits from taking them are poorly understood. **Objectives:** This study aims to examine the prevalence and patterns of herbal medicine usage by cancer patients. Clinical evidence of these herbal medicines is also investigated. **Materials and methods:** This study used a cross-sectional survey on outpatients at the Department of Chemotherapy - Medical University Medical Center, Ho Chi Minh City from 1st May 2016 to 31st May 2016. The Pubmed database was searched to review clinical evidence of these herbal medicines. **Results:** Of 243 patients participated in the study, 54.7% were male. Of the patients surveyed, 25.5% (n = 62) of patients took herbal medicines. Of total 35 recorded herbal medicines, the most common was papaya leaf (n = 20; 32.3%), lingzhi (n = 15; 24.2%), turmeric (n = 10; 16.1%), lemongrass and fucoidan (n = 9; 14.5%). Only 4.8% cancer patients got information about herbal medicine from medical practitioners. Patients did not inform their doctor about the self-medication uses. The Pubmed database yielded 3778 studies, with 76 papers (2%) fitting the criteria for reviewed. 34 out of 35 herbs had low clinical evidence, and especially four herbs had no clinical evidence. **Conclusion:** A substantial number of people with cancer are likely to be taking herbal medicine. However, most patients got information from unreliable information resources. The majority of herbal medicine had low clinical evidenceand limited references from the medical practitioner shows the urgent need for professionals advising in cancer patient for herbal medicine.

Keywords: cancer patient, herbal medicine, review, level of evidence

1. INTRODUCTION

Cancer is a leading cause of death, affecting millions of people around the globe.For reducing or eradicating cancers, surgery, chemotherapy and radiation therapy have been applied as conventional treatments. Survival of cancer patients has improved over the years resulting from improvement in conventional therapies and early presentation. However, herbal medicines are widely used as an adjunctive treatment in cancer patients, but the prevalence, patterns, reasons, and benefits from taking herbal medicine arepoorly understood.So far, very few studies have described the use of herbal medicine in developing countries. To our knowledge, no previously published study has investigated the used herbal medicine pattern ofcancer patients in Vietnam, where the use of traditional herbs and remedies is well known and relatively common. Thus, the aims of this study were to determine the prevalence, pattern, reason for using herbal medicine, the perceived effectiveness as well as sources of information and scientific evidence of these medicines.

2. MATERIALS AND METHODS

Thiscross-sectional survey study was conducted at the Department of Chemotherapy- Medical University Medical Center, Ho Chi Minh City, Vietnam. The study was approved by the EthicalCommittee of the hospital (1503-DHYD-HDDD).

2.1. Study Subjects. All outpatientcancer patients attending chemotherapy at the Department of Chemotherapy - Medical University Medical Center, Ho Chi Minh City, Vietnam from 1st May 2016 to 31st May 2016 were recruited into the study. The inclusion criteria were adult above18-year old with diagnosis of cancer and agreedto participate in the study.

2.2. Data collection. Prior to the interview, the patients were informed about the purpose of thestudy and ensuredthat all personal information would remain confidential and be used for research purposes only. Physicians who were in any way involved in the treatment of each patient were not present during the administration of the questionnaire.

2.3. The Questionnaire. The questionnaire was developed based on previous studies byMolassiotis et al and Dameryet al[1], [2]. To evaluate the applicability of questionnaire, a pilot study was conducted on10 randomly selected patients. The results from the pilot study were not included in the final analysis of the data. The majority of the questions had pre-prepared answers asking patients on demographic details; disease-related characteristics; information about use of herbal medicine; purpose of the use of herbal medicine, source of information; and outcomes.

2.4. Review of the literature. In order to investigate the levels of clinical evidence of the taken herbal medicine, published papers were collected and reviewed. The search was based on various databases (PubMed, Google scholar). The scientific and English common names of the identified herbs were used to carry out a Medical Subject Headings (MeSH) search. The search included the following terms: neoplasm, tumor, neoplasia, cancer, efficacy, effectiveness, effect, treatment, prevention, antitumor, anticancer.

2.5. Levels of evidence. Levels of evidence of the finding literature were ranked from level I to level V, adapted and modified from those used by the NHMRC [3], which categorizes studies based on study design in accordance with their general capacity to minimize or eliminate bias (Table 1).

2.6. Statistical Analysis. All analyses were performed using SPSS software version 20.0. The demographic characteristic datawere calculated by descriptive statistics. Cancer patients participated in the study were classified as either herbal medicine users or non-users according to whether or not they used herbal medicine. Categorical data weredescribed with frequency and percentage and compared byusing chi-square.

3. RESULTS

There were 243 cancer patients participatedin thestudy.Sixty two (25.5%) of the total participantsreported having used at least one herbal medicine since their diagnosisof cancer. Table 2 shows the demographic characteristics ofherbal users and non-herbal users. There were no significantdifferences in the proportion of herbal users by age, gender, education level, annual income, cancertype, or cancer staging ($p > 0.05$).

3.1. The reasons for using herbal medicine

When they were asked to provide a primary reason for the particular herbal medicine use, themost commonly reported reason was to improve physical health or stimulate the immune system (n=46, 74.2%). Other reported reasons are shown in Table 3.

When the nonusers were asked why they did not use herbal medicine, 89.0% answered that they were happy with the conventional treatment received and 69.6% said they never thought of herbal medicine, as shown in Table 4.

3.2. Sources of information about herbal medicine.

The main sources of recommendations for herbal products use in the user group were from friends (58.1%),mass media (53.2%);followedbythe family members (16.1%),other patients (9.7%)andmedical staff (3.4%).

3.3. Levels the evidence

Following the information on herbal used from the survey, we have searched and reviewed a total of 3778 studies, of which 76 met the described criteria for reviewing the clinical evidence (Table 1). Most of theherbal medicines used had low evidence, one was graded level I (lingzhi), four had level II, two had level III-2 and two were leveled IV (Table 5).

4. DISCUSSION

To our knowledge, this is the first study of the use of herbal medicine bypatients with a variety of cancers in Vietnam. The use of herbal medicine by cancerpatients is very common and varies widely among populations.

Friends and media constitute the greatest source ofinformation on herbal use. In spite of the fact that massive channels on TV, radio and internet nowadays, its content withrespect to traditional medicine is hardly regulated, that potentiallyinfluences patient behavior and preference.

In our surveyed population, the most frequently used(>10% of users) was papaya (*Carica papaya*), followed by lingzhi (*Ganodermalucidum*), turmeric (*Curcuma longa*), lemon grass(*Cymbopogoncitratus*) and fucoidan.The systematic review finding of scientific evidence of papaya was from acase report of47-year-old female patient withstomach cancer that had metastasized to the pancreas. In the report, it was stated that the patient drank approximately 750 ml of the papaya leaf extract every day for a total of 180-days, which cause the pancreatic metastasis of the stomach cancer to be disappeared [4]. Out of 5 RCTs included in this systematic review, four studies showed that patients in the *G. lucidum* group had relatively improved quality of life in comparison to controls. One study recorded minimal side effects, including nausea and insomnia. No significant hematological or hematological toxicity was reported [5]. Lingzhihad highest evidence in our study (Level I). Although 31 out of 35 herbs had scientific evidences, just five were graded to be atlevel I to II, with most of reporteduse of herbal medicines had low or no evidence.This finding highlights the importance of performing pharmacological tests and clinical trials to establish for the presence of side effects, toxicity and efficacy for these herbal medicines.

Our study is limited by the rather low number of participants, non-probability sampling method used, and the fact that it was performed in a single institution.

5. CONCLUSION

Herbal medicine use is common among cancer patients on treatmentwith chemotherapy. However, most patients receivedinformation related to herbal use from unreliable resources. The majority of herbal medicines used by the patients in this study had low clinical evidencewithlimited consultation from theirdoctor. We recommend that physicians caring for cancer patients should be aware of the use herbal medicine by theirpatients.

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Table1. *Levels of evidence*

Levels of evidence	Evidence
I	Evidence obtained from a systematic review of all relevant RCT.
II	Evidence obtained from an RCT.
III-1	Evidence obtained from a pseudo-RCT.
III-2	Evidence obtained from case control/cohort studies or a meta-analysis/systematic review of case control/cohort studies
IV	Evidence obtained from case series.
V	Evidence obtained from animal studies.

Table 2. *Patient characteristics and herbal medicine use*

Variables	N	User (%)	Nonuser (%)	P value
Total	243	62 (25.5)	181 (74.5)	
Age (years)				0.841
<50	50	13 (26.0)	37 (74.0)	
50-59	68	18 (26.5)	50 (73.5)	
60-69	89	24 (27.0)	65 (73.0)	
≥ 70	36	7 (19.4)	29 (80.6)	
Gender				0.984

Male	133	34 (25.6)	99 (74.4)	
Female	110	28 (25.5)	82 (74.5)	
Educational level				0.255
Illiterate	8	0 (0.0)	8 (100.0)	
Primary	57	12 (21.1)	45 (78.9)	
Secondary	134	36 (26.9)	98 (73.1)	
Technician, college	9	4 (44.4)	5 (55.6)	
University	35	10 (28.6)	25 (71.4)	
Annual income (in million VND)				0.078
< 5	121	27 (22.3)	94 (77.7)	
5 - 10	85	24 (28.2)	61 (71.8)	
10 - 18	31	9 (29.0)	22 (71.0)	
18 - 32	4	0 (0.0)	4 (100.0)	
≥ 32	2	2 (100.0)	0 (0.0)	
Cancer type				0.053
Colorectal cancer	102	23 (22.5)	79 (77.5)	
Stomach cancer	34	4 (11.8)	30 (88.2)	
Lung cancer	31	16 (51.6)	15 (48.4)	
Hematological cancers	7	1 (14.3)	6 (85.7)	
Gynecological cancers	9	2 (22.2)	7 (77.8)	
Breast cancer	13	6 (46.2)	7 (53.8)	
Genitourinary cancers	4	1 (25.0)	3 (75.0)	
Prostate cancer	12	1 (8.3)	11 (91.7)	
Pancreatic cancer	9	3 (33.3)	6 (66.7)	
Periampullary cancer	3	0 (0.0)	3 (100.0)	
Liver cancer	7	1 (14.0)	6 (86.0)	
Thymus cancer	1	0 (0.0)	1 (100.0)	
Duodenal cancer	1	0 (0.0)	1 (100.0)	
Esophageal cancer	8	4 (50.0)	4 (50.0)	
Head and neck cancer	1	0 (0.0)	1 (100.0)	
Bone cancer	1	0 (0.0)	1 (100.0)	
Cancer stage				0.074
Stage 1	0	0 (0.0)	0 (0.0)	
Stage 2	9	0 (0.0)	9 (100.0)	
Stage 3	234	62 (26.5)	172 (73.5)	
Type of therapy				0.931
Chemotherapy	46	13 (28.3)	33 (71.7)	
Chemotherapy+ surgery	187	47 (25.1)	140 (74.9)	
Chemotherapy+ radiation	4	1 (25.0)	3 (75.0)	
Chemotherapy+ surgery + radiation	6	1 (16.7)	5 (83.3)	

Table 1.*Stated reasons for using herbal medicine*

Reason	n	(%)
Improving physical health	46	74.2
Stimulating the immune system	46	74.2
Adjuvant therapy	44	71.0
Reducing side effects of drug and chemotherapy	42	67.7
Controlling the development of cancer	36	58.1
Protecting against recurrent symptoms	36	58.1

Curing cancer	34	54.8
Achieving a longer life-span	30	48.4
Seeking a new treatment for cancer	27	43.5

Table 4. Stated reasons for not using herbal medicine

Reasons	n	%
They were happy with the conventional treatment	161	89.0
They never thought of herbal medicine	126	69.6
They did not believe in herbal medicine	83	45.9
Lacking of information about herbal medicine	32	17.7
Inability to pay for herbal medicine	1	0.6
They were interested in herbal medicine but they had not decided to use any as yet	37	20.4

Table 5. *Frequencies of use, number of studies and evidence level for specific herbal medicines*

No	Common name (Latin name)	Number of users (%)	Number and type of studies	Evidence level
1	Lingzhi (*Ganodermalucidum*)	15 (24.2)	1 systematic review of 5 RCTs	I
2	Turmeric(*Curcuma longa*)	10 (16.1)	2 RCTs	II
3	Garlic (*Allium sativum*)	1 (1.6)	2 RCTs	II
4	Ginger (*Zingiberofficinale*)	1 (1.6)	3 RCTs	II
5	Green tea (*Camellia sinensis*)	1 (1.6)	3 RCTs	II
6	Carrot (*Daucuscarota*)	2 (3.2)	1 meta-analysis of 2 cohort and 8 case-control studies	III-2
7	Walnut*(Juglansregia*)	1 (1.6)	1 case-control study	III-2
8	*Scutellariabarbata*	2 (3.2)	2 case series report	IV
9	Papaya (*Carica papaya*)	20 (32.3)	1 case report	IV
10	Chaga mushroom (*Inonotus obliquus*)	1 (1.6)	5 *in vivo* studies	V
11	Common plantain (*Plantago major*)	1 (1.6)	2 *in vivo* studies	V
12	Moringa (*Moringaoleifera*)	1 (1.6)	2 *in vivo* studies	V
13	Cordyceps mushroom (*Ophiocordycepssinensis*)	3 (4.8)	1 *in vivo* study	V
14	Lemon grass (*Cymbopogoncitratus*)	9 (14.5)	1 *in vivo* study	V
15	Fucoidan	9 (14.5)	10 *in vivo* studies	V
16	Aloe (*Aloe vera*)	4 (6.5)	6 *in vivo* studies	V
17	Honey	4 (6.5)	5 *in vivo* studies	V
18	Pseudoginseng (*Panaxpseudoginseng*)	2 (3.2)	3 *in vivo* studies	V
19	Blue scorpion (*RhopalurusJunceus*)	2 (3.2)	1 *in vivo* study	V
20	Black garlic	1 (1.6)	1 *in vivo* study	V

21	Chamber bitter *(Phyllanthusurinaria*)	1 (1.6)	2 *in vivo* studies	V
22	Graviola (*Annona muricata*)	1 (1.6)	2 *in vivo* studies	V
23	*Hedyotisdiffusa*	3 (4.8)	4 *in vivo* studies	V

Healthcare Costs Associated with Diabetic Mellitus among Vietnam Social Insurance Enrollees in Thaibinh Province

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Abstract

Introduction: Diabetes has been becoming one of the most common chronic illnesses in Vietnam. Increased healthcare costs draw the attention of third-party payer seeking strategies to use the fund more effectively. However, there are limited researches on calculating the healthcare cost for treating diabetic patients in Vietnam. **Objectives:** This study aimed to identify and estimate the healthcare costs of Vietnam Social Insurance program for treating diabetic patients in Thaibinh Province. **Materials and Methods**: A retrospective cohort study was conducted with 17,244 diabetic patients under Health Insurance Program coverage in the first quarter of 2015 in Thaibinh province including inpatient and outpatient data. Study cohort subjects were selected as having diabetes according to disease codes. Indirect costs of diabetes were not estimated. **Results:** Among 17,244 patients, there had been 418 times of hospitalization and 35,170 visits to outpatient clinics. Healthcare costs of treating diabetes reached 16.4 billion VND, accounted for 9.97% total reimbursed expenditures of insurance agency. Over 50% of diabetes expenditures were paid for medicines, followed by laboratory testing. Average costs of outpatient visits increased from 5,716 VND (SD= 3,787.34VND) in commune health stations to 607,043 VND (SD= 486,610.48VND) in provincial hospitals ($p<0.05$). When diabetic patients had to hospitalize, a suddenly rise in healthcare costs could be seen. On average, the duration staying in hospital was 11 days and the cost reached over 3,000,000 VND. **Conclusion:** The findings provided the estimation of diabetes healthcare cost from a payer perspective in Vietnam and groundwork to policy makers for reasonable budget allocation.

Keywords:diabetes, healthcare costs, insurance, Vietnam

5. INTRODUCTION

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. The International Diabetes Federation estimated that there are now 415 million adults aged 20-79 with diabetes worldwide, including 193 million who are undiagnosed (1).The global prevalence of diabetes among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014. Especially, the prevalence has been rising more rapidly in middle- and low-income countries(2).Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation.In 2015, an estimated 1.6 million deaths were directly caused by diabetes. Another 2.2 million deaths were attributable to high blood glucose in 2012(3), (4).

Diabetes is a burden for social and economic development because of the prevalence and serious consequences of the disease for late detection and treatment. In 1997, the world spent $ 1.030 billion for diabetes treatment, primarily for the treatment of diabetes, the average cost for each patient with diabetes over 30 years was $ 47,420. Based on the cost estimates of a recent systematic review, the world spent more than $ 827 billion annually on direct costs(5). The IDF estimates that global diabetes expenditure worldwide tripled in the period 2003 to 2013 (1).

In Vietnam, the prevalence of diabetes is growing at alarming rates and has almost doubled within the past 10 years. Currently, it's estimated that there were over 3 million Vietnamese people had diabetes. In addition, the number of people with a pre-diabetic condition is three times higher than those with diabetes.

Severe complications, such as feet ulcers, gangrene and resulting amputations, cardiovascular diseases, blindness and kidney failures are common in diabetic patients. These complications are the main causes of death and disability for people with diabetes. It's estimated that about 53,458 deaths were attributed to diabetes in 2015 in Viet Nam.

The treatment costs, along with travel costs to hospitals as well as the loss of productivity due to illness and prolonged stays in hospitals can debilitate a whole family and drain funds for basic subsistence. According to International Diabetes Federation, the diabetes related expenditures in Vietnam are on average 162.7 USD per patient per year in 2015. This is more than the average monthly salary of 150 USD in Vietnam(1).

Increased healthcare costs draw the attention of third-party payer seeking strategies to use the fund more effectively. However, there are limited researches on calculating the healthcare cost for treating diabetic patients in Vietnam.
Thaibinh is a coastal eastern province in the Red River Delta region of northern Vietnam which subdivided into 8 district-level sub-divisions. The province had a population of 1.78 million people. The database of diabetes patients in the province was complete with a large number of participants.

This study aimed to identify and estimate the healthcare costs of Vietnam Social Insurance programme for treating diabetic patients in Thaibinh Province.

6. MATERIALS AND METHODS

2.1. Study designs and data collection

Participants of the study was formed using the database of the National Health Insurance Fund. This database covering about 82,19% of Vietnamese population. Study participants were selected according these inclusion criteria: diagnosed diabetes mellitus (ICD-10 diagnosis codes E10; E11; E13 and E14). A total of 17,244 patients with diabetes mellitus were selected from the National Health Insurance Fund database. All the patients with these inclusion criteria attending primary healthcare centers had the same probability to be recruited in this study.

The data on resources utilized and costs between January 1, 2015 and March 31, 2015, was obtained from the same National Health Insurance Fund database. The database included information about these patients' data: treatments, ambulatory and hospital inpatient care costs, as well socio-demographic and disease variables (age, gender, type of diabetes). All costs are given in 2015 prices and presented in Vietnamese currency Dong (VND), the costs are expressed in USD (in the study year the rate of exchange was approximately 1 USD = 21,890 VND).

2.2. Methods of evaluation direct healthcare costs

The analysis of direct healthcare costs of patients with diabetes mellitus included these variables: consultations of doctor, laboratory tests, diagnostic imaging, medicine and medical supplies, surgical procedures, hospitalization. Direct cost on patients with diabetes mellitus healthcare was calculated per person in 2015.

2.3. Statistical analysis

A statistical data analysis was performed with Statistical Package for the Social Sciences (SPSS) 17.0 software packages. For descriptive statistical analysis means, standard deviation (SD), and frequencies were calculated. The statistical significance of differences identified in mean costs at different levels of healthcare facilities was evaluated using Kruskal–Wallistest. The difference between the results was considered statistically significant, if $P < 0.05$.

7. RESULTS

Table1. *Distribution of admissions in different kinds of health facilities*

	Provincial hospitals	District hospitals	Commune health stations	Total
OUTPATIENT				
Number of facilities	3	21	154	178
Number of admission (%)	1680 (4.8)	32127 (91.3)	1363 (3.9)	35,170 (100)
Frequency (patient/hospital/month)	286.67	509.95	2.95	197.58
INPATIENT				
Number of facilities	1	14	0	15
Number of admission (%)	272 (65.07)	146 (30.14)	0 (0)	418 (100)
Frequency (patient/hospital/month)	90.67	3.48	0	9.29

Healthcare system of Thaibinh province had 178 facilities taking part in treatment and taking-care of diabetes patients. In the firrst quarter of 2015, the National health Insurance database recorded 35,170 examinations and 418 hospitalizations related to diabetes in the province. Although Thaibinh had a wide network of commune health stations, the proportion of patients chose the facilities was very low (3.9%). District hospitals were the most favourite choice of diabetes patients. However, when the situation became worse and they had to hospitalize, provincial hospitals seemed to be prefered with 65.07% of inpatients.On average, patients had 2.05 (range 1–8) general practitioner consultations within 3 months.

Table 2. *Characteristics of study participants*

	Inpatient (N=371) No (%)	Outpatient (N=17,160) No (%)
Female	137 (36.9)	8757 (51.0)
Age (SD)	**63.69 (11.82)**	**62.88 (11.22)**
Age group (year)		
<20	1 (0.3)	33 (0.2)
20-39	9 (2.4)	403 (2.3)
40-59	107 (28.8)	5669 (33.0)
≥60	254 (68.5)	11055 (64.4)
Diabetes type		
I	18 (4.85)	671 (3,91)
II	350 (94.34)	14,994 (87.38)
Other specified diabetes	3 (0.81)	1495 (8.7)

The all of socio-demographic and disease-related characteristics are shown in table 2. A total number of 17,244 patients with diabetes mellitus participated in this study in which there were

287 subjects examined both inpatient and outpatient. 51.0% of outpatient were women while the proportion in inpatient group was 36.9%. The mean age of outpatient was 62.88 years (SD=11.22), which was a little lower than inpatient group (63.69 years- SD=11.82).

It was identified that the most frequent type of diabetes wastype 2 (94.34% in inpatient group and 87.38% in outpatient group).

Mean direct healthcare costs per admission ofoutpatient accounted to 430,986.45VND (USD 19.69). Average costs of outpatient visits increased from 5,716 VND (USD 0.26) in commune health stations to 607,043 VND (USD 27.73) in provincial hospitals ($p<0.05$). Despite of the availability and lower cost of treatment at commune health stations, the most popular choice of patients was district facilities with the average cost of 439,822 VND (SD= 172,663.4).

Table 3. *Mean cost per admission of diabetes patient in different level of healthcare facilities*

		Provincial hospitals	District hospitals	Commune health stations	Total
Outpatient					
N (%)		1680 (4.78)	32127 (91.35)	1363 (3.88)	35170 (100)
Sum	VND	1,019,833,414	14,130,167,656	7,792,210	15,157,793,280
	USD	46,589	645,508	356	692,453
Meancost per admission	VND	607,043.70	439,822.19	5,716.96	430,986.45
	USD	27.73	20.09	0.26	19.69
SD	VND	486,610.48	172,663.40	3,787.34	217,023.82
	USD	22.23	7.89	0.17	9.91
p (Kruskal–Wallis test)		p<0.001	p<0.001	p<0.001	
Inpatient					
N (%)		272	145	0	417
Sum	VND	1,091,901,854	174,055,579		1,265,957,433
	USD	49,881.31	7,951.37		57,832.68
Treatment duration	Day (SD)	12.8 (5.6)	7.8(3.7)		
Mean cost per admission	VND	4,014,345.05	1,200,383.31		3,035,869.14
	USD	183.39	54.84		138.69
SD	VND	2,458,009.70	579,022.47		2,419,140.68
	USD	112.29	26.45		110.51
p (Mann- Whitney test)		p<0.001			

Hospitalization mean cost was VND 3,035,869.14 (USD 138.69) in 2015(Table 3). On average, patient had been treated in district hospitals for 7.8 days (range from 1 to 22 days) while treatment duration in provincial hospitals was 12.8 days (range 1-42 days). The longer duration as well as higher level technique in provincial hospitals cost an average cost of VND4,014,345.05 which was 3 times higher than district hospitals.

Mean cost per person increased statistically significantly with level of healthcare facilities ($P < 0.001$ and $P < 0.001$).

Table 4. ***Direct medical costs of diabetes outpatient***

	N	Sum		Mean		SD	
		VND	USD	VND	USD	VND	USD
Medicine	32258	7,869,341,611	359,494.82	243,950.08	11.14	151,617.90	6.93
Laboratory tests	31283	6,426,267,682	293,570.93	205,423.64	9.38	65,344.94	2.99
Diagnostic imaging	11008	407,957,518	18,636.71	37,060.09	1.69	40,683.86	1.86
Consultation fee	35142	346,162,521	15,813.73	9,850.39	0.45	2,095.39	0.10
Surgical procedure	497	98,865,144	4,516.45	198,923.83	9.09	482,287.87	22.03
Medical supplies	284	9,198,800	420.23	32,390.14	1.48	9,306.32	0.43
Total	35170	15,157,793,279	692,452.87	430,986.45	19.69	217,023.82	9.91

Healthcare costs of treating diabetes reached 16,4 billion VND, accounted for 9.97% total reimbursed expenditures of insurance agency. The majority of the expenditure belonged to outpatient (92.07%).51.92% of diabetes expenditures were paid for medicines, followed by laboratory testing which accounted for 42.39%.

8. DISCUSSION

Our study results were different with the cost of diabetes studies in other nation. American Diabetes Association study data (2012) showed that the largest components of medical expenditures are hospital inpatient care (43% of the total medical cost), prescription medications to treat the complications of diabetes (18%), antidiabetic agents and diabetes supplies (12%) (6). These results also confirm Schmitt-Koopmann et al., their accounted that total direct costs consisted of 53% hospitalization and 30% medication costs (7). However, the cost of inpatient in our study accounted for only 7,7% of diabetes expenditure. The largest part of direct costs in diabetes mellitus healthcare composed medicine and laboratory tests expenditures. The reason for this difference was the consultation fee and hospitalization fee in Vietnam seemed to be much lower than developed countries.

Despite of the fact that Vietnam had a very good net of commune health stations (CHS), for example Thaibinh had 154 CHSs, the proportion of diabetes patients coming to the facilities was quite low. This was a understandable situation because the stations lacked of specialist phycians and laboratory for test. Besides, medicine allowed to use in commune health stations were limited. Insurance agency just accepted to reimburse for metformin, glibenclamid, gliclazid, glimepirid but insulin or other antidiabetes medicine (8).

5. **CONCLUSIONS**

The findings provided the estimation of diabetes healthcare cost from a payer perspective in Vietnam. Finding out the capacity of different levels of healthcare facilities and healthcare cost provided groundwork to policy makers for reasonable budget allocation.

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Adverse Events During Treatment of Multidrug-Resistant Tuberculosis: The First Cohort Event Monitoring in Vietnam

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Abstract

Introduction: The safety data during multidrug-resistant tuberculosis (MDR-TB) treatment have varied for not only Vietnamese patients but also patients in other areas of the world. ***Objectives:*** This study was conducted to determine the incidence of adverse events (AEs) that occurred during MDR-TB treatment in Vietnam and to assess risk factors associated with adverse events. ***Methods:*** AEs were collected from 659 MDR-TB patients enrolled from April to December 2014 through a cohort event monitoring (CEM) program. Patients were monitored with a follow-up of approximately 20 months. Adverse events were determined by clinical criteria and laboratory tests. Cox proportional hazard regression models were used to explore factors associated with the reported adverse events. ***Results:*** The cohort enrolled 659 patients in which 81.3% experienced at least one AE during treatment. Of those with AEs, 18.3% required adjustment of MDR-TB regimen. The most common AEs including arthralgia, hepatotoxicity and hyperuricemia were observed in 34.7%, 32.2% and 29.3% of patients, respectively. Multivariate regression analysis indicated that the independent predictors for hepatotoxicity were baseline levels of alanin amino transferase (HR 1.023; 95%CI 1.008-1.037) and alcoholic status (HR 4.255; 95%CI 1.239-14.616) while pyrazinamide daily dose (HR 1.025; 95%CI 1.002-1.048) and alcoholic status (HR 2.016; 95%CI 1.084-3.751) were associated with the elevation of serum uric acid. ***Conclusions:*** Adverse events were common during MDR-TB treatment in Vietnam including serious ones that required proper interventions. Predictors for hepatotoxicity and hyperuricemia observed in this study underlined the importance of patient history investigation, baseline physical and laboratory examination and close monitoring.

Keywords: MDR-TB; cohort event monitoring; adverse event; Vietnam

1. INTRODUCTION

The complicated epidemiological situation of drug-resistant TB in Vietnam as well as in other countries has been a global concern and become a great challenge for our efforts to control TB [1]. TB treatment requires long term chemotherapy with a combination of many antibiotics simultaneously, so problems related to drug safety, especially severe adverse events, can cause a great impact on treatment adherence, thus leading to drug resistance and difficulty in monitoring TB. As a result, ensuring safe and rational drug use has been considered as one of the most important objectives of MDR-TB treatment. Activities that monitor, detect, evaluate and prevent adverse drug reactions related to drugs against TB hold important roles in the enforcement of treatment efficiency, saving costs, preventing drug resistance and contributing in patients life quality [2]. To date, the rates of yearly spontaneous reports received by the National DI & ADR Center related to MDR-TB drugs are rather low and does not reflect the safety of MDR-TB treatment in Vietnam [3]. Thus, it is not possible to detect problems related to drug safety and to provide data for recommendations on regimen change. Consequently, the implementation of programs enforcing the collection of ADR reports the evaluation of ADR from MDR-TB drugs is becoming more urgent.

This study may help determine the incidence of adverse events (AEs) that occurred during MDR-TB treatment in Vietnam. We also aim to assess risk factors associated with the occurrence of the most reported adverse events.

2. METHODS

2.1 Study design

We conducted an observational, prospective study based on a Cohort Event Monitoring (CEM) program.

2.2 Setting and study population

Nine TB treatment centers in Vietnam were chosen as sentinel sites of the study. The targeted population of this study was adult (≥ 16 year-old) patients starting MDR-TB treatment at chosen sentinel sites and enrolled between April 2014 to December 2014. Patients taking part in other studies (e.g. the STREAM trial) were excluded.

2.3 Treatment protocol and follow-up

Patients treated in sentinel sites received MDR-TB therapy based on drug susceptibility test (DST) results and their treatment history. The standardized regimens (IVa and IVb) consisted of six drugs: kanamycin (or capreomycin), levofloxacin, prothionamide, cycloserine (or p-aminosalicylic acid PAS), pyrazinamide and ethambutol. The only difference between IVa and IVb regimens was the injectable drugs. A standardized regimen would be modified based on DST results and history of allergy.

Patients were treated for MDR-TB for up to 24 months. At each clinic visit, the patient was examined for treatment response and undesirable effects. Patients were assessed by clinical and sub-clinical manifestations at baseline (before starting MDR-TB treatment), during routine follow-up visits (once a month) and at any time that AEs occurred/were reported. Table 1 shows the definitions of important adverse drug reactions from scientific medical literature regarding all of MDR-TB drugs in the standardized regimens.

2.4 Data collection and analysis

Information was filled into paper collection forms, afterwards transfered to Microsoft® Access 2010 then to SPSS® Statistics 22. For descriptive statistics, nominal and ordinal variables were presented as percentages, continuous variables with normal distribution were represented as mean ± SD (standard deviation). Cox multivariate regression analysis was conducted using stepwise backward method to look for independent factors associated with AEs due to MDR-TB therapy.

2.5 Ethics

As this was a study of routinely collected monitoring data and did not affect therapeutic practice, informed consent from the patients was not obtained.

Table 1. *Definitions of adverse events of MDR-TB standardized regimens*[4]

Adverse event	Definition
Hepatoxicity	
Identified	Presence of jaundice, conjunctival discolouration, nausea, vomiting, loss of appetite, urine abnormal, abdominal pain, pruritus and elevated AST or ALT level > 3 ULN*, or AST or ALT level > 5 ULN without symptoms or diagnosed with hepatotoxicity by physician.
Suspected	Presence of one or some symptoms but no findings to confirm the diagnosis.
Psychiatric disorders	Presence of one or more of the followings: paranoid reaction, delusion, abnormal behaviour, bad mood lasts over 2 weeks, insomnia, distraction, suicide attempt or other psychiatric symptoms, unless the cause was known such as TB in the central nervous system, cerebrovascular accident, alcoholism.
Arthralgia	Presence of joint pain, unless the cause was known, e.g. musculoskeletal tuberculosis, rheumatoid arthritis…
Hypersensitivity reactions	Presence of one of the followings: pruritus, rash, photosensitivity or other hypersensitivity reactions including anaphylaxis, unless the cause was known, e.g. food allergy, hepatitis…
Renal toxicity	Presence of oliguria, oedema, at least one elevated serum level of creatinine, urea after starting MDR-TB treatment, creatinine clearance < 50 ml/min or diagnosed by physician.
Vision disorders	Vision abnormal or decrease eyesight after starting MDR-TB treatment or difficult to distinguish colour with no other symptoms or diagnosed by physician.
Hearing and vestibular disorders	Deaf or hearing loss after starting MDR-TB treatment or diagnosed by physician or confirmed by audiometry; symptoms consistent with vestibular disorders such as vertigo and/or loss of balance.
Hypothyroidism	
Identified	Presence of fatigue, tiredness, depression, constipation, arthralgia, excessive menstrual bleeding, distraction, loss of appetite, weight gain, dry skin, dry hair, and elevated TSH level > 5 uU/l, T3 level < 1 nmol/l, T4 level < 64 nmol/l.
Suspected	

	Presence of one or some symptoms but no findings to confirm the diagnosis
Hypokalemia	At least one serum potassium value ≤ 3,5 mmol/l.
Hyperuricemia	Serum uric acid level > 420 umol/l (70 mg/dl) in men and > 360 umol/l (60 mg/dl) in women or diagnosed by physician.
Blood disorders	Anemia (hemoglobin < 12 g/dL in men and < 13 g/dL in women) or leucopenia (< 3000 x 10^9/l) or thrombocytopenia (< 100 x 10^9/l) or diagnosed with blood disorders by physician.

**ULN: Upper Limit of Normal*

3. RESULTS

3.1 Patient characteristics

Properties and characteristics of the cohort are presented in Table 2. Between April 2014 and December 2014, 659 patients were enrolled in the study. Of these, 631 (95.8%) patients were treated with regimen IVa, 22 (3.3%) and 6 (0.9%) patients received regimen IVb and individualized therapy, respectively. The median duration of MDR-TB therapy was 19.2 months (Interquartile range [IQR] 17.5 - 20.2).

Table 2. *Characteristics of patients treated with MDR-TB therapy (n = 659)*

Characteristics	**n (%)**
Male sex	517 (78.5)
Age (year). mean ± SD	42.4 ± 13.8
Weight (kg). mean ± SD	48.3 ± 9.3
Baseline conditions	
Diabetes mellitus	104 (15.8)
HIV co-infection	57 (8.7)
Hepatic disease	33 (5.0)
Renal insufficiency	5 (0.8)
Alcoholism	16 (2.4)
History of allergy	13 (2.0)
Number of months on treatment. median [IQR]	19.2 (17.5 - 20.2)
Treatment outcome	
Cure/completion	512 (77.7)
Transfer-out	17 (2.6)
Default	61 (9.3)
Failure	20 (3.0)
Death	49 (7.4)

3.2 Adverse events

Overall, at least one type of AE was experienced by 536 (81.3%) of 659 MDR-TB patients. Table 3 demonstrates the frequency and duration of occurence of each type of adverse event in this cohort. The most common types of AE were arthralgia (34.7%) and hepatotoxicity (32.2%), respectively. Out of those patients experienced AEs, 18.3% of patients required a significant change in MDR-TB chemotherapy due to adverse events: dose reduction (5.2%), temporary discontinuation (10.1%), and drug substitution (3.0%).

Table 3. *Frequency of significant adverse events observed during MDR-TB treatment*

Type of AE	Patients experienced AE (n = 659) n (%)	Type of AE	Patients experienced AE (n = 659) n (%)
Arthralgia	229 (34.7)	Vision disorders	69 (10.5)
Hepatotoxicity	212 (32.2)	Hypokalemia	60 (9.1)
Nausea, vomiting	210 (31.9)	Peripheral neuropathy	52 (7.9)
Hyperuricemia	193 (29.3)	Abdominal pain	47 (7.1)
Anorexia	188 (28.5)	Hyperglycaemia	42 (6.4)
Dizziness	151 (22.9)	Hematologic disorders	23 (3.5)
Headache	127 (19.3)	Diarrhea	20 (3.0)
Dermatologic disorders	119 (18.1)	Hypothyroidism	15 (2.3)
Gastritis	116 (17.6)	Convulsions	10 (1.5)
Octotoxicity	100 (15.2)	Anaphylaxis	4 (0.6)
Psychiatric disorders	94 (14.3)	Vision disorders	69 (10.5)
Nephrotoxicity	85 (12.9)	Hypokalemia	60 (9.1)

3.3 Risk factors

Multivariate regression model was used to assess risk factors on the most reported adverse events including hepatotoxicity, arthralgia and hyperuricemia (Table 4). While male gender ($p = 0.019$), alcoholic status ($p = 0.011$) and baseline AST ($p = <0.001$) were found to be associated with suspected hepatotoxicity, independent factors associated with the development of identified hepatotoxicity were baseline ALT ($p = 0.002$) and alcoholism ($p = 0.021$). Pyrazinamide daily dose ($p = 0.034$) and alcoholic status ($p = 0.027$) were associated with the elevation of serum uric acid. No factors in relation with the occurrence of arthralgia were found to be statistically significant.

Table 4. *Multivariable analysis of factors associated with adverse events*

Adverse events	Variable	Cases[1] n (%)	Controls[2] n (%)	p value	HR	(95% CI)
Suspected hepatotoxicity	*Sex*					
	Male	155 (85.6)	362 (75.7)	**0.019**	1	
	Female	26 (14.4)	116 (24.3)		0.593	(0.384-0.916)
	Alcoholism					
	No	173 (95.6)	470 (98.3)	**0.011**	1	
	Yes	8 (4.4)	8 (1.7)		2.512	(1.231-5.129)
	Baseline AST (U/L)			**<0.001**	1.005	(1.002-1.007)
Identified hepatotoxicity	*Age (year)*			0.074	1.026	(0.998 -1.056)
	Alcoholism					
	No	28 (90.3)	615 (97.9)	**0.021**	1	
	Yes	3 (9.7)	13 (2.1)		4.255	(1.239-14.616)
	Baseline AST (U/L)			0.083	0.983	(0.963-1.002)
	Baseline ALT (U/L)			**0.002**	1.023	(1.008-1.037)
Hyperuricemia	*Alcoholism*					
	No	180 (93.3)	463 (99.4)	**0.027**	1	
	Yes	13 (6.7)	3 (0.6)		2.016	(1.084-3.751)
	Diabetes mellitus					
	No	178 (92.2)	377 (80.9)	0.057	1	
	Yes	15 (7.8)	89 (19.1)		0.563	(0.311-1.017)
	Pyrazinamid daily dose (mg/kg per day)			**0.034**	1.025	(1.002-1.048)

[1]experienced AE; [2]not experienced AE; HR: hazard ratio.

5. DISCUSSION

This study was not only the first cohort event monitoring but also the largest study to date of MDR-TB treatment in Vietnam. It was based on an active surveillance system with designed forms, had a large sample size, and provided valuable information about adverse events in Vietnamese population.

Initially, we found that 81.3% of patients developed at least 1 type of AEs after MDR-TB treatment. The frequency of adverse events in our study was rather higher than that in previous studies. A study in Russia of 244 MDR-TB patients showed that 73.3% patients had experienced at least one adverse event [5]. The rate of patients experienced AEs in an observational cohort study on drug resistant TB in Pakistan was 72.3% [6]. A cross-sectional study on the treatment of MDR-TB patients in Vietnam observed 143 (50.7%) patients with at least one adverse event [7]. There were several factors that may have contributed to this result. Firstly, unlike restropective studies with high probability of missing data, this study method allowed us to collect and record information more properly. Secondly, patients enrolled in the study were monitored regularly during treatment by trained healthcare workers following a complete procedure, thus increasing the ability of detecting adverse events. In addition, a list of definitions was compiled with detailed descriptions of expected adverse reactions of MDR-TB medication to optimize AE detection. Therefore, the results of this study could reflect the incidence of adverse reactions following standardized MDR-TB therapy in Vietnam.

The severity of AEs varied from mild rash or nausea to the life-threatening anaphylaxis. The most common adverse events were arthralgia (34.7%), hepatotoxicity (32.2%), nausea/vomiting (31.9%), hyperuricemia (29.3%) and anorexia (28.5%). Arthralgia and gastrointestinal disorders were consistent with other published studies as the most reported. In Nathanson et al., the most observed adverse events were nausea/vomiting (32.8%), diarrhea (21.1%) and arthralgia (16.4%)[8]. In Hoa et al., the most common undesirable reactions of MDR-TB drugs were arthralgia (35.8%), followed by gastrointestinal disturbance (14.2%) [7]. Other clinically significant adverse events were also observed such as ototoxicity (15.2%), psychiatric disorders (14.3%), nephrotoxicity (12.9%), vision disorders (10.5%) and hypokalemia (9.1%).

Both of arthralgia and hyperuricemia were common in our study and probably related to each other. Hyperuricemia, in fact, may potentially lead to severe gouty arthritis if it is not under control, and therefore, should not be underestimated. Although many studies have showed arthralgia as one of the most reported undesirable effects during MDR-TB treatment, hyperuricemia seems to be not significant enough to be noticed.

In a systematic review on adverse events of MDR-TB drugs, we found that out of 69 studies, there were 35 studies had observed arthralgia/joint pain but hyperuricemia was presented in only 2 studies [9]. Besides, the rates of hyperuricemia in those studies were quite low: 2.8% [10] and 12.6% [11]. The results of multivariable analysis also indicated that alcoholism and pyrazinamide daily dose could affect on serum uric acid level, suggesting that this drug should be prescribed according to body weight.

In this study, hepatic adverse events were reported in 212 (32.2%) of patients. However, only 31 (4.7%) patients were classified as identified hepatotoxicity. A study on hepatic events during MDR-TB treatment in Russia, in which 91/658 (16.5%) patients experienced hepatoxicity, showed that elevated transaminases (ALT, AST) at baseline were associated factors of hepatotoxicity [12]. The results of our study confirmed that baseline levels of ALT was one of the independent predictors for identified hepatotoxicity, which can be explained because an increase in ALT serum levels, compared to AST, is more specific for liver damage [13].

One of the limitations of our study was the lack of consistency in detecting and reporting AEs among nine sentinel sites, which was caused by their differences in human and technical resources. Moreover, some types of AE requiring specific measurements (e.g. audiometry) might be underestimated. In spite of these limitations, the results are encouraging and we believe that our study has provided important information regarding the side effects of second-line anti-TB drugs in Vietnam. The methodology of this study could be applied to other studies especially for new drugs, or standardized yet high-cost regimens.

5. CONCLUSIONS

Adverse events were encountered in most patients during MDR-TB treatment in Vietnam and may result in treatment change. The findings in this study demonstrate that adverse events can be detected in a timely and effective way through baseline examination and routine monitoring. On the basis to understand the significance of undesirable effects in MDR-TB treatment, further investigation is suggested to emphasizing the occurrence of adverse events in different phases of MDR-TB treatment, and corresponding relationships with other risk factors.

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The Vietnamese Version of the Coronary Artery Disease Education Questionnaire – Short Version (CADE-Q SV): Translation and Cross-cultural Adaptation

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Abstract

Introduction: Coronary artery disease, including acute coronary syndrome, is one of the leading causes of mortality worldwide. Insufficient knowledge about the disease may increase the mortality rate among patients with coronary artery disease. There has been no validated Vietnamese questionnaire to assess patient's knowledge about coronary artery disease. **Objectives:** The objectives of this study are to translate and cross-culturally adapt the Coronary Artery Disease Education Questionnaire – Short Version (CADE-Q SV) into Vietnamese. **Methods:** The 5-stage guideline by Beaton et al. (2000 & 2007) was strictly followed during the process of translation and adaptation. First, two independent translations into Vietnamese were produced. Second, these two translations were synthesized. Third, two translators blinded with the outcome measurements independently created two back-translations into English. Fourth, nine experts reached consensus on all items of the Vietnamese version of CADE-Q SV. Fifth, the pilot study was conducted on 35 Vietnamese patients with acute coronary syndrome to explore the clarity of the Vietnamese version of the questionnaire. After that, the Vietnamese version of CADE-Q SV was created and reached a good equivalence with the original questionnaire in all four criteria including semantic, idiomatic, experiential and conceptual equivalence (the total mean score was 0.99 for each criterion). **Results:** The patients participated in the pilot study also evaluated that our instrument was easy to understand. This result was demonstrated by the high clarity score of 20-item (9.85 ± 0.78) and no item has been rated lower than 9. **Conclusion:** In conclusion, the Vietnamese version of CADE-Q SV can be considered as a clear instrument. However, psychometric properties of the questionnaire should be evaluated prior to broader dissemination.

Keywords: coronary artery disease, questionnaire, patient's knowledge, translation and adaptation.

1. INTRODUCTION

Coronary artery disease (CAD) is one of the leading causes of mortality worldwide.[1] According to a report from American Heart Association (AHA), CAD is responsible for 1 of every 7 deaths and there are approximately 16.5 million patients with CAD in the United States.[2] The mortality rate among patients with CAD may increase due to the lack of knowledge about disease. In addition, good knowledge can motivate patient to change lifestyle such as physical activities, diet, weight management, attainment of lipid level goals, and medication adherence.[1,3] Unfortunately, many studies have indicated that the patient's knowledge about CAD still remains at low level. Ammouri (2016) reported that 60.5% of patients had inadequate knowledge regarding risk factors of CAD.[4] Among 3,522 patients involved in a study of Dracup (2008), more than 60% of participants have low knowledge level.[5] Evaluating the patient's knowledge can help develop public health programs as well as the education plans to improve knowledge, reduce modified risk factors, and enhance the medication adherence.[6]

In Vietnam, there has been no Vietnamese questionnaire to assess patient's knowledge about coronary artery disease. In 2016, Ghisi developed and psychometrically validated the coronary artery disease education questionnaire - short version (CADE-Q SV) in English. CADE-Q SV was designed as a 20-item questionnaire.

The respondents were asked to select one answer out of three choices including "yes", "no" and "do not know". If patients give a correct answer, they will get 1 point. This questionnaire was considered as a short, quick and appropriate tool to apply in clinical and research.[7] However, using a questionnaire in another country, culture and/or language requires the translation and cross-cultural adaptation to reach equivalence between the original source and target language.[8] The goal of this study was to translate and cross-culturally adapt the Coronary Artery Disease Education Questionnaire – Short Version (CADE-Q SV) into Vietnamese.

2. MATERIAL AND METHODS

2.1. Study population

Patients were recruited from the Heart Institute of Ho Chi Minh City, Vietnam between February 21st 2017 and March 12th 2017. Eligible patients had discharge diagnosis of acute coronary syndrome including unstable angina (UA), non ST segment elevation myocardial infarction (NSTEMI) and ST segment elevation myocardial infarction (STEMI). Patients were excluded if they were unable to communicate in Vietnamese or

were in serious health condition which would prevent them from answering the questionnaire.

2.2. Methods

The process of translation and cross-cultural adaptation includes five stages described by Beaton et al.[8, 9]

Stage 1: Translation

The CADE-Q SV questionnaire was translated from English into Vietnamese. Two independent translators who are fluent in English and speak Vietnamese as their mother tongue involved in this stage. One had clinical background and knowledge about research concepts while the other was not informed about the objectives of the research as well as had no clinical or medical background. They produced the T1 and T2 translations and a written report for each version.

Stage 2: Synthesis

Two translations were synthesized by another person who had experiences in methodology. The result of stage 2 was developing one common translation called T-12.

Stage 3: Back translation

Two translators blinded with the original questionnaire and outcome measurements independently created two back-translations of T-12 version into English. These two back translations were used to compare with the original CADE-Q SV in the next stage.

Stage 4: Expert committee

The Expert Committee consisted of nine professionals: a methodologist, two clinicians, all translators in previous stages, and the author of original questionnaire. They reviewed all CADE-Q SV versions including the original and translated versions, questionnaire instructions and scoring documentation.

Step 4.1: the equivalence between the original and the translated version was assessed on four criteria: semantic equivalence, idiomatic equivalence, experiential equivalence and conceptual equivalence. For each item, the expert could choose 1 (equivalence) or 0 (non-equivalence). Based on the average score of items, the experts suggested some changes for T-12 version. This step aimed to improve the equivalence between the original and the translated version.

Step 4.2: the equivalence between the original and the modified T-12 version was determined with the same criteria and score range in step 4.1.

Stage 4 resulted in the pre-final version which was used for the pilot testing in the next stage.

Stage 5: The pilot study

Patients from the target population were surveyed using the pre-final CADE-Q SV version. The ideal sample size was about 30 to 40 people.[8] The purpose of this stage was to explore the clarity of the pre-final version. Therefore, patients were asked to rate each statement on a Likert scale ranging from 0 (unclear) to 10 (very clear). If the mean score

of some statements were less than 9, the expert would review these items to make some necessary modifications. In addition, the characteristics of patients including age, gender, discharge diagnosis, comorbidities and educational level were collected through medical reports and interviews. Data were entered and analyzed using SPSS 20.0 software package.

3. RESULTS AND DISCUSSION

3.1 Characteristics of the study population

There were 35 patients participating in the pilot study. The demographic characteristics of the subjects were reported in Table 1.

Table 1. *The demographic characteristics of participants*

Demographic characteristics		**Values**	
		Number	**Percentage (%)**
Gender	Male	22	62.90
	Female	13	37.10
Diagnosis	UA	21	60.00
	NSTEMI	8	22.90
	STEMI	6	17.10
Comorbidities	Hypertension	21	60.00
	Diabetes	10	28.60
	Dyslipidemia	6	17.10
Smoking history	Yes	14	40.00
	No	21	60,00
Educational level	Elementary school	5	14.30
	High school	25	71.43
	College/University /Post graduate	5	14.30

The average age was 59.97 ± 7.20 years which was similar to the data reported in previous studies conducted in Vietnam, such as the research of Nguyen (2015) (60.36 years) and Quach (2015) (60.40 years).[10,11] The male participants accounted for over 60.0%. This percentage was in line with the results reported in the study of Ghisi (2016) (69.7%). This also fits the epidemiology of patients with acute coronary syndrome reported by AHA (the proportion of male patients was much higher than their female counterparts). In term of educational level, more than 85% patients graduated from high school or higher level. In our study, the percentage of people having the high educational level including colleges, universities and postgraduate was lower than that reported in the

article of Ghisi (2016) (14.3% compared to 71.3%).[7] This difference could be explained by the low socio-economic conditions in Vietnam. The prominent comorbidity was hypertension with 60.0% while diabetes and dyslipidemia represented for 28.60% and 17.10% respectively. There were about 40.0% patients having the smoking history.

3.2 Assessment of the equivalence by the expert committee:

The results of the equivalence evaluation in step 4.1 was presented in Table 2.

Table 2. *Assessment of the equivalence of the T-12 version of CADE-Q SV*

Items	Semantic equivalence	Idiomatic equivalence	Experiential equivalence	Conceptual equivalence
A1*	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
A2	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
A3	0.89 (8/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
A4	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
1	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
2	0.78 (7/9)	1.00 (9/9)	1.00 (9/9)	0.78 (7/9)
3	0.89 (8/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
4	0.67 (6/9)	0.67 (6/9)	0.67 (6/9)	0.56 (5/9)
5	0.67 (6/9)	0.78 (7/9)	0.78 (7/9)	0.67 (6/9)
6	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
7	0.78 (7/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
8	0.89 (8/9)	0.89 (8/9)	0.89 (8/9)	0.89 (8/9)
9	0.89 (8/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
10	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
11	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
12	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
13	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
14	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
15	0.67 (6/9)	0.89 (8/9)	0.89 (8/9)	0.89 (8/9)
16	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
17	0.78 (7/9)	0.89 (8/9)	0.89 (8/9)	0.78 (7/9)
18	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
19	0.78 (7/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
20	0.89 (8/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)

**A1, A2, A3, A4 belong to the questionnaire instructions.*

Among 24 statements, 12 items met the absolute equivalence in all of four criteria (semantic equivalence, idiomatic equivalence, experiential equivalence, and conceptual equivalence). The remaining statements, which had the mean score lower than 1.00, were reviewed and modified to improve the equivalence. These modified items were presented in Table 3.

Table 3. *The modifications of the questionnaire items*

Statements	Original version	T-12 translation	Modified items
2.	Lifestyle changes like healthy eating *can lower your chances of developing* heart disease.	Thay đổi lối sống như ăn uống lành mạnh *giúp giảm nguy cơ mắc bệnh tim.*	Thay đổi lối sống như ăn uống lành mạnh có thể *giúp giảm nguy cơ tiến triển bệnh tim*
4.	Resistance training (lifting weights or *using elastic bands)* can strengthen your muscles and help lower your blood sugar	Tập thể dục *(ví dụ như* tập với tạ tay hay với *dây thun đàn hồi)* giúp cơ bắp khỏe mạnh và *làm* giảm lượng đường trong máu	Tập thể dục *(như* tập với tạ tay hay với *dây đàn hồi*) giúp cơ bắp khỏe mạnh và giảm lượng đường trong máu
5.	Eating more meat and milk products is *a good way* to add more fibre to your diet.	Để bổ sung chất xơ vào khẩu phần ăn, nên ăn nhiều thịt và các sản phẩm từ sữa	*Một cách tốt* để bổ sung chất xơ vào khẩu phần ăn *là* ăn nhiều thịt và các sản phẩm từ sữa.
8.	A warm-up before exercising raises your heart rate and lowers your chance of getting angina	Khởi động trước khi tập thể dục giúp tăng nhịp tim và *làm* giảm nguy cơ lên cơn đau thắt ngực	Khởi động trước khi tập thể dục giúp tăng nhịp tim *từ từ* và giảm nguy cơ lên cơn đau thắt ngực
15.	Sleep apnea (pauses in breathing during sleep) can increase your chance of *having another heart attack.*	Chứng ngưng thở khi ngủ có thể làm tăng nguy cơ lên cơn đau tim *ở những người mắc bệnh tim*	Chứng ngưng thở khi ngủ có thể làm tăng nguy cơ lên cơn đau tim *ở những người đã từng bị đau tim*

After making some necessary changes to the T-12 version, the expert evaluated the equivalence between the original and the modified T-12 version. The results of this were illustrated in Table 4.

Table 4. *Assessment of the equivalence of the pre-final version of CADE-Q SV*

Items	Semantic equivalence	Idiomatic equivalence	Experiential equivalence	Conceptual equivalence
A1	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
A2	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
A3	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
A4	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
1	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
2	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
3	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
4	0.78 (7/9)	0.78 (7/9)	0.78 (7/9)	0.78 (7/9)
5	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
6	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
7	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
8	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
9	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
10	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
11	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
12	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
13	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
14	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
15	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
16	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
17	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
18	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
19	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
20	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)	1.00 (9/9)
Mean	0.99	0.99	0.99	0.99

All the statements achieved higher equivalence for each criterion, compared to the result in step 4.1. The mean score was 0.99 for all four criteria. These results indicated that the pre-final version was equivalent to the original CADE-Q SV.

3.3 Assessment of clarity in the pilot study:

The evaluation of clarity was obtained from calculating the rating scores of 35 patients. The data was displayed in Table 5.

Table 5. *Assessment of clarity*

Item	Score (Mean ± SD)
A1	10.00 ± 0.00
A2	10.00 ± 0.00
A3	10.00 ± 0.00
A4	10.00 ± 0.00
1	10.00 ± 0.00
2	9.89 ± 0.68
3	9.74 ± 0.95
4	9.69 ± 1.11
5	9.94 ± 0.34
6	9.86 ± 0.85
7	9.60 ± 1.17
8	10.00 ± 0.00
9	9.83 ± 0.71
10	9.91 ± 0.51
11	10.00 ± 0.00
12	10.00 ± 0.00
13	10.00 ± 0.00
14	10.00 ± 0.00
15	10.00 ± 0.00
16	9.46 ± 1.27
17	9.91 ± 0.51
18	9.29 ± 2.05
19	9.94 ± 0.34
20	9.94 ± 0.34
Mean score of 20-item	9.85 ± 0.78

All of 4 items belong to the instructions got the maximum score (10/10) which demonstrated that patients could understand how to answer the CADE-Q SV. The average score of 20-item was 9.85 ± 0.78 with no item had the rating lower than 9. Therefore, no statement had to be considered for modification. This score was similar to the rating reported in the original article. The mean clarity of the original CADE-Q SV was 9.10 ± 1.12 in the pilot study with 20 participants.[7] This results indicated that the Vietnamese version of CADE-Q SV is easy to understand and could be used for the validation process.

4. CONCLUSIONS

In conclusion, the CADE-Q SV was translated from English into Vietnamese. The translated version achieved the high equivalence to the original version. The Vietnamese questionnaire can be considered as a clear instrument. However, psychometric properties of the questionnaire should be evaluated prior to broader dissemination.

5. ACKNOWLEDGEMENT

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Unit Cost of Healthcare Services for Provincial Hospital in Central Highlands of Vietnam

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Abstract

Introduction: In Vietnam, the major of medicalservices' costs are covered by government insurance programmes, however, there is still a controversial issue about the different health care services costs in different hospitals. Therefore, Vietnamese healthcare system need information on the unit cost of medical services. **Objective:** The objective of this study was to identify unit costs of medical services at General hospital in Lam Dong Province during the fiscal year 2015 in Vietnam. **Methods:** This study was a retrospective cost analysis, which standard costing approach was applied for to calculate unit cost of medical services. All cost centers in the institute were identified and classified into two categories: temporary cost centres (TCCs) and absorbing cost centres (ACCs). Unit cost of each medical service was estimated by cost-to-charge ratio method. **Results:** It was found that total costs of General Hospital in Lam Dong Province in the fiscal year 2015 were 4,560,891 USD. Cost-to-charge ratios of the remaining medical services were from 0.36 – 164.68. **Conclusion:** The result of this study can help hospital administrators understand their cost structures and run their facilities more efficiently. The study is also looked forward to providing the unit costs to the governmental data, contribute to complete the Vietnam standard cost list and be helpful for health economicevaluation.

1. INTRODUCTION

In developed countries, unit costs are critical to establishing repayment rates, meanwhile, in the developing nations, unit costs are important to determine the reimbursement rate [1]. However, a study by the World Health Organization (WHO) is in the project WHO-CHOICE has been conducted and the results showed that in the majority of countries, especially in low-income countries and the average income nations, very few detailed researchers have been carried out on the economics of hospitals [2, 3]. In recent years, the needs of a community in using medical services has been growing up in both developed and developing countries, which requires the hospital managers provide these services at an acceptable level of quality and at the least possible cost.

In the past decade in Vietnam, an example of a developing country, increasing the demand for using health care services had encouraged the population to use healthcare insurance in order

to decrease the healthcare expenditures. Nevertheless, the cost of medical services is still a controversial issue because of the difference of health care service costs among different hospitals. Therefore, Vietnamese health care system needs information on the actual cost of medical services by conducting cost analysis in various type of hospitals in Vietnam, which helps to calculate healthcare service costs more accurate, fuller and support in the development of national costing data. This study aimed to describe unit costs of medical services in Lam Dong General Hospital in order to contribute to healthcare data in Vietnam in standardization unit cost of hospital services and support the health economic evaluation.

2. MATERIALS AND METHOD

2.1. Study Design

This retrospective descriptive study was carried out at provincial Hospital in the Central Highlands region of Vietnam. An economic analysis of hospital medical services was approached by using the ratio of cost to charge (RCC) method from a hospital perspective. All costs were estimated based on resources consumed in the fiscal year 2015.

2.2. Geographic location

Lam Dong General Hospitalis a governmental hospital which serves both urban and rural population in the highland area of Central Southern Vietnam. General Hospital is located in Lam Dong Province. Lam Dong General Hospital was chosen for this study based on their willingness to cooperate and the accessibility of hospital data.

2.3. Data collection and management

The period of time spent on collecting the required data for the research is defined as time horizon during which costs and outputs are to be considered. Ideally, unit cost analysis of the whole year is practiced so that to avoid the effect caused by seasonal variation in the number of patients which might ultimately affect cost per unit of the fixed cost. Annual data were collected in the fiscal year 2015. The main sources of data were the hospitals' activity and accounting reports.

2.4. Costing methodology

The accounting and economics fields offer various methodologies for measuring and valuing resources for the costing of health services. In this study, the unit cost of health care services have been calculated using the standard costing method. The standard costing method is composed of six steps [4]: 1) Study design and planning; 2) Organization analysis and cost center classification; 3) Direct cost of cost centers determination; 4) Indirect cost determination; 5) Full cost determination; 6) Unit cost of hospital services calculation. (**Figure 1**).

2.5. Data analysis and presentation

The real exchange rate in 2015 was used in the calculation of all costs in US dollars (USD). According to the report of Vietnam State Bank in 2015, 1 USD was equivalent 22,547 VND [5]. The total cost of the hospital is presented together with the percentage of the cost component.

The unit cost of services in Lam Dong General Hospital was calculated by dividing cost to charge in 2015. Costing and the use of Microsoft Excel 2013 can be applied to the development of a costing template for unit cost analysis of health care services.

3. RESULTS

3.1. Organization analysis and cost center classification

The organization structures of the study hospital were based on the function as supporting departments or patient service producing departments. They were classified as Temporary cost centers - TCCs or Absorbing cost centers – ACCs, respectively.

3.2. Direct cost

Table 1 displays the figures of total direct costs obtained by multiple types of the cost center.
Table 1. The component of total direct costs and their contribution (USD, 2015), [(cost,%)]

3.3. Indirect cost determination

Direct costs of TCCs are allocated according to several methods, the most accurate of which is the simultaneous equation method. Appropriate allocation criteria of each TCCs were selected.

3.4 Full cost determination of ACCs without drugs.

After allocating the direct cost of TCCs to cost of ACCs, the full cost of absorbing cost centers was calculated. As the summation needed to calculate the full cost of ACCs is already presented in **Table 2**, it is simple to see that the total value of ACCs full cost (4,560,891 USD) was made of two factors, one of which is the total direct cost with its figure taking 78.72% (3,590,168 USD) of the total ACCs full cost and the other one is the indirect cost with the percentage of 21.28 % (970,723 USD)
Table 2. Full cost of each ACCs (USD,2015), [(cost,%)]

3.5. Unit cost of hospital services

Table 3 displays the RCC of some departments in hospital and **Table 4** the unit cost of basic services for study hospital. The results reveal that average RCC was 11.72 (range from 0.36 to 164.68) which means the cost for practicing the service is higher than the current price of the service itself.
Table 3. Ratio cost of to charge (RCC) in absorbing cost centers, 2015
Table 4. RCC and unit costs of some medical services in Lam Dong General Hospital (USD, 2015)

4. DISCUSSION

4.1. Total direct costs of cost centers

In what may be the first comprehensive study on the economics of hospital, this study has presented the total direct costs and unit costs of medical services. In terms of cost center

groups, the total direct costs of ACCs which is 3.590.168 USD (78.72%) is greater than TCCs 970.723 USD (21.28%) by 2015 in General Hospital in Lam Dong Province. In terms of total direct costs, Material costs accounted for the largest proportion and Capital cost made up the lowest proportion, which is 2,237,945 USD (49.07 percent) and 746.195 USD (16.36 percent), respectively.

4.2. Unit cost of health care services

For unit cost calculation, cost allocation was used in transferring full costs of departments to the services provided. There are several units cost allocation methods. Cost-to-charge ratio approach was employed in this study. According to RCC methodology, this is the less complicated method in practice since it is easy to calculate and allocate indirect or overhead cost; less time-consuming and less costly to implement; less extensive primary data collection requirements. This is also useful in the analysis of the rate of return, which is important in terms of financial or business management.

5. CONCLUSIONS

The results of this simulation analysis not only provided an empirical basis for health policymakers in Vietnam to assess and negotiate over different provider payment reform options, make decisions that are more likely to advance health system objectives but also highlight the importance of costing exercises as a hospital planning and management tool. Ours was a preliminary study on the cost of services in province hospitals in Vietnam. Further studies on this issue, with larger sample size and more sophisticated design, should be done as soon as possible to provide clearer and more detailed data about the levels and determinants of hospital costs in Vietnam.

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Figure 1. Six steps of the standard costing method

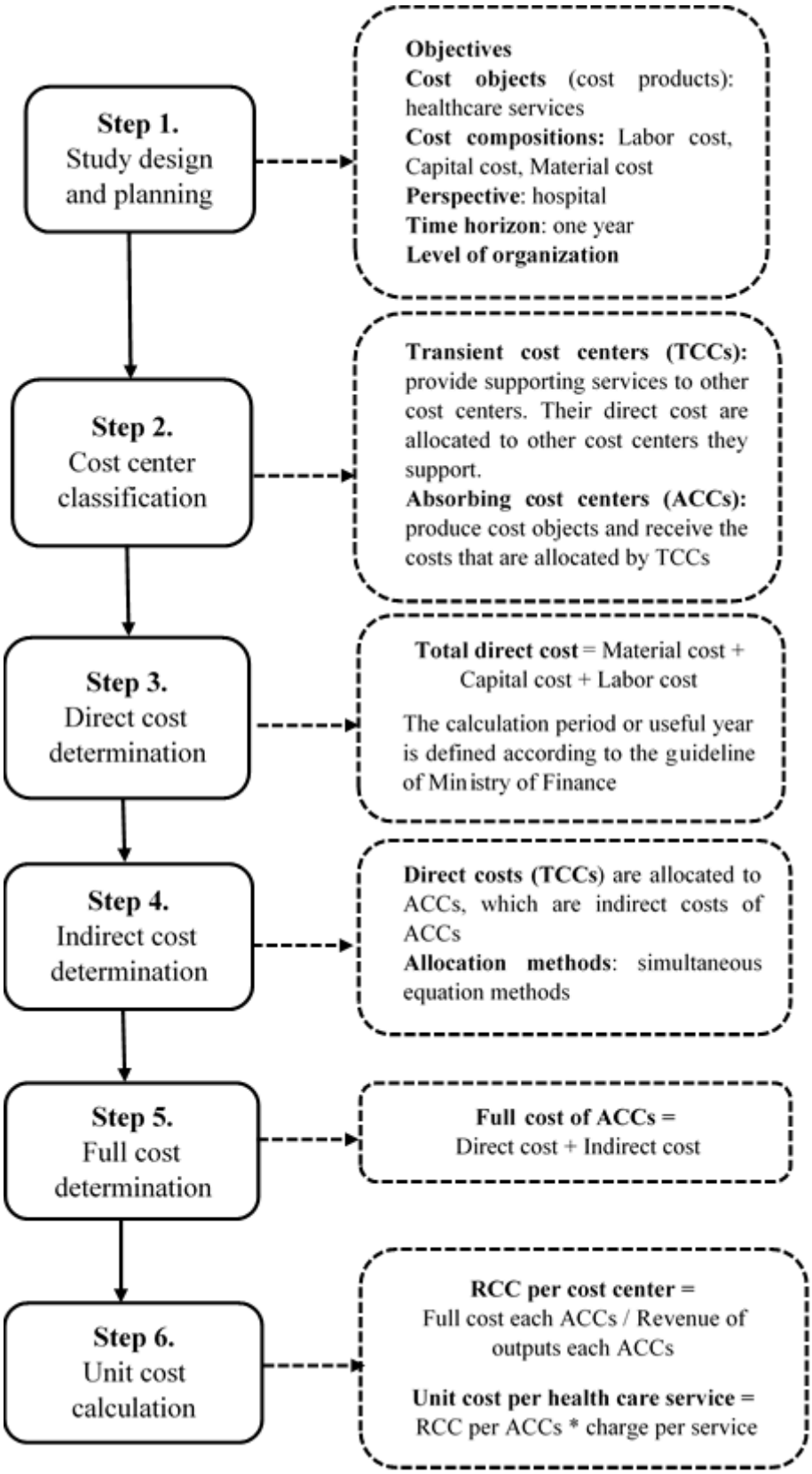

Table 1. *The component of total direct costs and their contribution (USD, 2015), [(cost,%)].*

Cost centers	Labor cost	Capital cost	Material cost	Direct cost
TCCs	277,191 (28.56)	2,212,755 (22.76)	472,617 (48.68)	970,723
ACCs	1,299,560 (36.20)	525,279 (14.63)	1,765,328 (49.17)	3,590,168
Total	**1,576,752 (34.57)**	**746,195 (16.36)**	**2,237,945 (49.07)**	**4,560,891**

Abbreviations: TCCs, Temporary cost centers; ACCs, Absorbing cost centers.

Table 2. *Full cost of each ACCs (USD,2015), [(cost,%)].*

Departments	Cost centers	Direct cost	Indirect cost	Total cost
Pharmacy	A1	73,376.22(89.12)	89,561.61(10.88)	823,326.83
Laboratory	A2	192,411.82(84.69)	34,779.66(15.31)	227,191.47
Imaging and Functional Exploration	A3	363,585.73(85.07)	63,786.42(14.93)	427,372.15
Examination – Emergency	A4	260,703.30(76.90)	78,330.27(23.10)	339,033.57
Hemodialysis	A5	93,878.04(68.16)	43,858.34(31.84)	137,736.38
ICU - Antitoxic	A6	228,527.89(77.89)	64,888.58(22.11)	293,416.47
Ophthalmological	A7	66,354.29(77.27)	19,522.92(22.73)	85,877.22
ENT	A8	63,998.03(75.76)	20,473.27(24.24)	84,471.30
Dental	A9	84,262.61(77.51)	24,443.82(22.49)	108,706.43
Maternity Unit	A10	273,972.40(72.66)	103,091.28(27.34)	377,063.68
Pediatrics	A11	163,230.75(70.39)	68,649.13(29.61)	231,879.88
Infectious Disease - Dermatology	A12	88,037.00(77.55)	25,483.53(22.45)	113,520.53
Surgery	A13	193,929.83(79.27)	50,723.41(20.73)	244,653.24
General Surgery	A14	152,512.53(75.61)	49,194.94(24.39)	201,707.47
Traumato Surgery	A15	155,568.00(73.73)	55,424.22(26.27)	210,992.22
Internal Medicine A	A16	99,681.39(67.51)	47,975.38(32.49)	147,656.77
Internal Medicine B	A17	100,980.51(72.81)	37,718.94(27.19)	138,699.45

Internal Medicine II	A18	51,527.26(69.54)	22,573.43(30.46)	74,100.69
Traditional medicine	A19	23,785.27(61.71)	14,757.43(38.L29)	38,542.70
Rehabilitation and Physiotherapy	A20	31,327.86(80.93)	7,383.99(19.07)	38,711.85
Tuberculosis	A21	61,991.62(71.35)	24,896.45(28,65)	86,888.06
Anapathology	A22	18,367.49(78.40)	5,060.55(21.60)	23,428.04
Interventional Cardiology	A23	8,056.67(62.37)	4,861.42(37.63)	12,918.09
Nuclear medicine and Tumour	A24	79,712.48(85.72)	13,284.41(14.28)	92,996.89
Total		**3,590,167.99(78.72)**	**970,723.39(21.28)**	**4,560,891.38**

Abbreviations: ICU, Intensive care unit; ENT, Ear, nose and throat .

Table 3. *Ratio cost of to charge (RCC) in absorbing cost centers, 2015.*

Cost center	Total cost	Revenue	RCC	Cost center	Total cost	Revenue	RCC
A2	823,326.83	628.419	0,36	**A14**	244,653.24	334.833	0,60
A3	227,191.47	708.030	0,60	**A15**	201,707.47	337.515	0,63
A4	427,372.15	208.642	1,62	**A16**	210,992.22	92.475	1,60
A5	339,033.57	5.741	23,99	**A17**	147,656.77	163.789	0,85
A6	137,736.38	622.650	0,47	**A18**	138,699.45	22.391	3,31
A7	293,416.47	55.315	1,55	**A19**	74,100.69	23.422	1,65
A8	85,877.22	88.408	0,96	**A20**	38,542.70	5.187	7,46
A9	84,471.30	41.252	2,64	**A21**	38,711.85	73.080	1,19
A10	108,706.43	500.923	0,75	**A22**	86,888.06	-	-
A11	377,063.68	172.661	1,34	**A23**	23,428.04	78	164,68
A12	231,879.88	41.158	2,76	**A24**	12,918.09	15.380	6,05
A13	113,520.53	5.491	44,56				

Table 4. RCC and unit costs of some medical services in Lam Dong General Hospital (USD, 2015).

Departments	Services	Charge	RCC	Unit cost
Laboratory	Triglyceride	3.99	0.36	1.45
	Troponin I	6.43	0.36	2.34
	CEA	3.10	0.36	1.12
	Glucose	1.15	0,36	0.42
Examination - Emergency	Cut suture	0.53	1.63	0.87
	Cut the boil, small abscess	0.53	1.63	0.87
	Out-patient visit	0.53	1.63	0.87
Ophthalmological	Tomonetry	15.52	1.60	24.87
	Trabeculectomy	17.74	1.60	28.42
	Measure Javal	0.53	1.60	0.85
General Surgery	Endoscopy nose	2.88	0.60	1.73
	Endoscopy ENT	6.65	0.60	3.99
	Cut a part of a small intestine	96.51	0.60	57.91
Traditional medicine	Galvanopuncture	1.11	1.65	1.83
	Speech/ activity therapy	0.67	1.65	1.11
	Infrared	0.80	1.65	1.32
Rehabilitation and Physiotherapy	Massage by vibrator	0.44	7.46	3.28
	Respiratory physiotherapy	0.44	7.46	3.28
	Massage by hand (60 minutes)	1.33	7.46	9.92

Abbreviations: RCC, Ratio of cost to charge; ICU, Intensive care unit; TSH, Thyreostimulin Hormon; CEA, Carcinoma Embryonic Antigen; EEG, Electroencephalogram; ENT, Ear, nose and throat.

Survey on the Use and Sensitivity of Bacteria to Antibiotics on Upper Respiratory Infection in Elderly Patients at Trung Vuong Hospital

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ABSTRACT

Introduction: The uncontrolled used of antibiotics, especially the broad spectrum may cause bacterial resistance to the antibiotic. The consequences of bacterial resistance are prolonged hospitalization, increased risk of mortality, increased costs and bacterial resistance outbreaks. **Objectives:** To evaluate the frequency of prescriptions antibiotics and the sensitivity of bacteria to β – lactam antibiotics group on elderly patients with acute exacerbation of chronic bronchitis, COPD and multiple infections of pneumonia at Trung Vuong hospital. **Method:** This study was conducted as cross-sectional observation of inpatient records on elderly patients with diagnosis of upper respiratory infection who were prescribed with β-lactam antibiotic group and Fluoroquinolone from January to October 2016. **Results**: There were 118 patients included in the study. The average age of the patients were 76.84 ± 9.36 years with age range between 60 and 99. The majority of the patients aged between 70-80 (35.59%) and two third of them are male (60%). Average length of hospital stay was 15.25 ± 10.66 days (range between 3 to 63 days). Half of the patients had chronic bronchitis exacerbations (52.54%), while the rest had multiple pulmonary infections (30.51%) and acute exacerbations of COPD (16.95%). Antibiotics are mainly used as monotherapy (53%). Antibiotics Map Results showed that 50% of the patients had the β - lactam resistant bacteria. Of these, 71% had bacterial resistant to Fluoroquinolone. Selection on antibiotics showed nothing specific with uneven distribution trend with wide use of broad spectrum antibiotics. Majority of the patients were prescribed with the antibiotic between 7 and 14 days (57%). The current study did not find any undesirable effects on antibiotics used. **Conclusion:** The current study found that β - lactam antibiotics were more commonly used for exacerbation of respiratory tract infection (81.81%) than Fluoroquinolone (18.19%). Selecting empiric antibiotics against antibiotic results can be done based on the following findings: β - lactam antibiotics had 50% resistant, 40% sensitivity and 10% with intermediate sensitivity. While 71.4% of the quinolone case was found to be resistant with only 28.57% had bacterial sensitivity.

Keywords: antibiotics, elderly patient, Ceftriaxone, Imipenem + Cilastatin

1. INTRODUCTION

The advent of antibiotics is a great contribution to the world's medicine. The invention and application of antibiotic treatment has evolved constantly with many new antibiotics were developed. However, it is very unfortunate that there is also an increasing trend of antibiotic-resistance due to antibiotics being overuse or non-selectively used or used appropriately.

The uncontrolled used of antibiotics especially the broad spectrum may cause bacterial resistance to the antibiotic. The consequences of bacterial resistance are prolonged hospitalization, increased risk of mortality, increased costs and bacterial resistance outbreaks. In the Trung Vuong Hospital, the treatment costs for antibiotics were high which encounter about 25% of treatment expenditure of the hospital. In the in-patient setting, elderly patients were found to commonly contract exacerbation of chronic bronchitis, acute exacerbations of COPD and lung infection which require the use of antibiotics. β-lactam antibiotics and Fluoroquinolone was found to be the most indicated antibiotics for the in-patients hospital populations. Due to that, the current study aims to evaluate the use and bacterial sensitivity pattern of β – lactam antibiotics and Fluoroquinolone among elderly in-patient patients at Trung Vuong hospital with acute exacerbation of chronic bronchitis, COPD and pneumonia.

2. METHODS

This study was conducted as cross-sectional observational study of elderly in-patient patients at Trung Vuong Hospital from January to Oktober 2016. Patients were included in the study if they were over 60 years old, prescribed with β-lactam antibiotic or fluoroquinolone for exacerbations of respiratory tract infections during the study duration. Patients who used β-lactam or fluoroquinolone for other indication such as for pre- or post-surgical prophylaxis were excluded from the study. The appropriate use of β – lactam or fluoroquinolone on the studied population were evaluated based the American Thoracic Society 2005 clinical practice guidelines [(4)] and 2013 Cho Ray Hospital's protocol of COPD treatment. The antibiotic's dosage was accessed according to the National Pharmacopoeia Vietnam (2010) and drug prescribing in renal failure (2011).

3. RESULTS

A total of 118 patients' records were included in the current study. The summary of the patients' demographic and characteristic is presented in Table 1. The average age of the patients were 76.84 ± 9.36 years with age range between 60 and 99. The majority of the patients aged between 70 and 80 (35.59%) and are male (60%). Half of the patients had chronic bronchitis exacerbations (52.54%), while the rest had multiple pulmonary infections (30.51%) and acute exacerbations of COPD (16.95%). Antibiotics were mainly used as monotherapy (53%). Antibiotics Map Results showed that 50% of the patients had the β -lactam resistant bacteria. Of these, 71% had bacterial resistant to Fluoroquinolone.

Table 1. *Characteristics of the patients (n=118)*

	Characteristics	n	%
Age (years)	60 – 70	32	27.12
	70 – 80	42	35.59
	80 - 90	38	32.2
	90 -100	6	4.24
Gender	Male	71	60.17
	Female	47	39.83
Diagnosis	Exacerbation chronic bronchitis	62	52.52
	Lung multiple infection	36	30.51
	Exacerbation of COPD	20	16.95
Antibiotic administration	Single agent	63	53.39
	Combination of two antibiotics	59	42.37
	Triple antibiotics	5	4.42
Results of bacterial sensitivity pattern	**β - lactam:**		
	Sensitive	34	39.53
	Resistant	50	58.14
	Intermediate	2	2.23
	Fluoroquinolone:	10	27.78
	Sensitive		
	Resistant	25	69.44
	Intermediate	1	2.78
Length of hospital stay	< 7 days	21	17.80
	7 -14 days	52	43.22
	14 -21 days	22	18.64
	>21 days	24	20.34

Commonly used antibiotic combination by the prescribers in the current study is summarized in Table 2. Selection on antibiotics showed nothing specific with uneven distribution trend with wide use of broad spectrum antibiotics.

Table 2: *Frequency of use of antibiotics*

Antibiotics group	Common combination	n=143	%
β – lactam		117	81.81
	Cefoperazon+ Sulbactam	24	16.78
	Ampicillin + Sulbactam	18	12.59
	Piperacillin +Tazobactam	10	6.99
	Imipenem + Cilastatin	12	8.39
	Ceftriaxone	12	8.39
	Cefepim	11	7.69
	Amoxicillin + clavulanic acid	11	7.69
	Cefuroxim	5	3.49
	Ceftazidime	4	2.79

	Meropenem	3	2.10
	Cefoperazon	3	2.10
	Ticarcillin + Clavulanat	1	0.70
Fluoroquinolone		38	18.19
	Levofloxacin	33	23.08
	Ciprofoxacin	5	3.49

A total of 32 patients (27.12%) had a duration of antibiotics of <7days. The majority were prescribed with antibiotic between 7 and 14 days (n= 79, 57%). The rest were prescribed with the antibiotic between 14 and 21 days (n = 18, 15.25%). The current study did not find any undesirable effects on antibiotics used. Based on the antibiotic map results, there were 50% of the β-lactam antibiotics had bacteria resistant to antibiotics. Only 40% of the empirical therapy was found to be bacterial sensitive while 10% of the patients had intermediate sensitivity to β-lactam antibiotics. For quinolone group, 71.4% of the cases included in the current study were resistance to the antibiotic with only 28.57% were sensitive to it (see Table 4).

Table 4: *Empirical antibiotic treatment*

Empirical antibiotic treatment	n	Sensitivity (n,%)	Intermediate (n,%)	Resistance (n,%)
β -lactam	**22**	**9 (40.1)**	3 (9.9)	**11 (50**)
• Cefoperazon+ Sulbactam	8	3 (37.5)	1 (12.5)	4(50)
• Piperacillin +Tazobactam	1	1 (100)	0	0
• Imipenem+ Cilastatin	2	1(50)	0	1 (50)
• Ceftriaxon	5	2 (40)	1(20)	2(40)
• Cefepim	1	0	0	1(100)
• Amoxicillin + clavulanic acid	1	0	0	1 (100)
• Meropenem	3	1 (33.3)	0	2 (66.7)
Fluoroquinolone	**13**	**3 (28.57**)	0	**10((71.4**)
• Levofloxacin	11	2 (18.18)	0	9 (81.8)
• Ciprofoxacin	2	1 (50)	0	1 (10)

4. DISCUSSION

Through the survey, 118 patients were included the study. The majority of the patients aged between 70-80 years old which may require special attention when using antibiotic, for example, as they may have compromised organ function e.g. liver or renal impairment. There were more male patients in this study which is consistent with the inclusion criteria of the studied population of exacerbation of COPD, which is more associated to male smokers.

In the current study, the antibiotics were commonly used as monotherapy (53%), followed by a combination of two antibiotics (42%) and three antibiotics (5%). Coordination of antibiotic use may increases bactericidal action thus reduce resistance.

There was no particular pattern on selection of antibiotics used. [2,4] In order, Levofloxacin> Cefoperazon + Sulbactam> Cefepim> Ceftriaxone > Imipenem + Cilastatin were used more than the others. The used of broad spectrum antibiotic in the current study could be due to the treatment were provided as empirical. However, injustice use of broad-spectrum antibiotics often increases the risk of antibiotic-resistant bacteria. De-escalation of therapy should be done appropriately following culture and sensitivity result.

5. CONCLUSION

The current study found that β- lactam antibiotics were more commonly used for exacerbation of respiratory tract infection (81.81%) than Fluoroquinolone (18.19%). Selecting empiric antibiotics can be done following the reported sensitivity results which show that 50% of β - lactam antibiotics were resistance to the bacteria, only 40% of the cases were found to be sensitive and 10% had intermediate sensitivity. For quinolone group, 71.4% of the cases, the organism were found to be resistance with only 28.57% were sensitive to the antibiotic.

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