การศึกษาทางเภสัชจลนศาสตร์ของสารสกัดมาตรฐานบัวบกอีซีเอ 233 ในหนูแรท

นายทศพล อนุกูลวิทยา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชศาสตร์ชีวภาพ ภาควิชาชีวเคมีและจุลชีววิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

PHARMACOKINETIC STUDY OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 IN RATS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biopharmaceutical Sciences
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2016

Thesis Title	PHARMACOKINETIC STUDY OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 IN RATS
By	Mr. Tosapol Anukunwithaya
Field of Study	Biopharmaceutical Sciences
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ทศพล อนุกูลวิทยา : การศึกษาทางเภสัชจลนศาสตร์ของสารสกัดมาตรฐานบัวบกอีซีเอ 233 ในหนูแรท (PHARMACOKINETIC STUDY OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 IN RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภก. ดร. พิสิฐ เขมาวุฒฒ์, 81 หน้า.

สารสกัดมาตรฐานบัวบกอีซีเอ 233 ประกอบด้วยสารสำคัญในกลุ่มไตรเทอร์พีนอยด์ไกลโคไซด์ ได้แก่ มาดีแคสโซไซด์ (Madecassoside) 53.1% และเอเชียติโคไซด์ (Asiaticoside) 32.3% ซึ่งสารสกัดนี้มีฤทธิ์ทางเภสัชวิทยาที่ดีต่อระบบประสาทส่วนกลาง ทั้งยังมีความเป็นพิษต่ำจากการศึกษาถึงความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรัง ในการศึกษานี้จึงทำการศึกษาทางเภสัชจลนศาสตร์ของสารสกัดมาตรฐานบัวบกอีซีเอ 233 ในหนูแรท สารสกัดดังกล่าวได้ถูกบริหารโดยการฉีดเข้าทางหลอดเลือดดำและการป้อนทางปากให้หนูแรทด้วยขนาด 50 ถึง 200 มิลลิกรัมต่อกิโลกรัม จากนั้นเก็บตัวอย่างเลือด เนื้อเยื่อ ปัสสาวะ และอุจจาระภายหลังได้รับสารตั้งแต่ 0 ถึง 48 ชั่วโมงเพื่อวัดระดับของสารสำคัญและเมแทบอไลต์เป้าหมาย ได้แก่ มาดีแคสซิค แอซิด (Madecasssic acid) และเอเชียติค แอซิด (Asiatic acid) โดยใช้เทคนิค Liquid chromatography-tandem mass spectrometry จากการศึกษาพบว่า สัตว์ทดลองมีความทนต่อสารสกัดอีซีเอ 233 ได้ดีในทุกขนาดยาที่ทดสอบ สามารถตรวจพบสารมาดีแคสโซไซด์และเอเชียติโคไซด์ที่ระดับความเข้มข้นสูงสุดในพลาสมา ณ เวลา 5 ถึง 15 นาทีภายหลังจากการบริหารสารสกัดอีซีเอ 233 โดยการป้อนทางปาก ซึ่งมีค่าชีวประสิทธิผลต่ำกว่า 1% อย่างไรก็ตามพบว่าสารสำคัญทั้งสองชนิดนั้นสามารถแพร่กระจายไปยังสมอง กระเพาะอาหาร และผิวหนังได้ภายในเวลา 1 ชั่วโมงและคงอยู่ในอวัยวะดังกล่าวอย่างน้อยเป็นเวลา 4 ชั่วโมงภายหลังจากสัตว์ทดลองได้รับสารสกัด ในส่วนมาดีแคสซิค แอซิดและเอเชียติคแอซิดนั้นพบได้ในระดับน้อยมากทั้งในพลาสมาและเนื้อเยื่อต่างๆ แต่สารทั้งสองตัวเป็นเมแทบอไลต์หลักที่ถูกขับออกและสามารถพบได้ที่อุจจาระของสัตว์ทดลองในปริมาณสูง ผลการทดลองทางเภสัชจลนศาสตร์นี้จะเป็นประโยชน์ในการเลือกใช้ขนาดยาที่เหมาะสมสำหรับการศึกษาสารสกัดอีซีเอ 233 ต่อไปในอนาคต การศึกษานี้ยังเป็นการค้นพบครั้งแรกที่แสดงถึงการแพร่กระจายของสารมาดีแคสโซไซด์และเอเชียติโคไซด์ไปที่อวัยวะเป้าหมายจากการให้ยาทางปาก

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชศาสตร์ชีวภาพ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5576451833 : MAJOR BIOPHARMACEUTICAL SCIENCES
KEYWORDS: CENTELLA ASIATICA / APIACEAE / ECA 233 / MADECASSOSIDE / ASIATICOSIDE / PHARMACOKINETICS

TOSAPOL ANUKUNWITHAYA: PHARMACOKINETIC STUDY OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 IN RATS. ADVISOR: ASST. PROF. PHISIT KHEMAWOOT, Ph.D., 81 pp.

The standardized extract of *Centella asiatica* ECa 233 is consist of triterpenoid glycosides, which are madecassoside (53.1%) and asiaticoside (32.3%). This extract has been found to exhibit various positive pharmacological effects on the central nervous system, and have a good safety profile in both acute and sub-chronic toxicity studies. The present study aimed to explore the disposition kinetics of ECa 233 in rats. The extract was intravenously or orally administered at doses from 50 to 200 mg/kg. Plasma, tissues, urine, and feces were collected at time points from zero to 48 h after dosing. The levels of madecassoside and asiaticoside, as well as their postulated triterpenic metabolites – madecassic acid and asiatic acid – in biological samples, were simultaneously measured by liquid chromatography-tandem mass spectrometry. The results showed that all animals had good tolerability for ECa 233. Whereas madecassic and asiatic acids were found in negligible amounts after pharmacokinetic assessment, madecassoside and asiaticoside demonstrated rather similar absorption and tissue distribution profiles. They were rapidly absorbed, reaching maximum levels within 5-15 minutes after oral administration, but they had poor oral bioavailability, less than 1%. Both triterpenoids were extensively distributed in the brain, stomach, and skin within 1 h and remained there for at least 4 h after dosing. A negligible amount of madecassic acid and asiatic acid were found in plasma and tissues, despite these two metabolites were extensively detected in feces after dosing. The pharmacokinetic results obtained could provide some guidance for appropriate dosing regimen of ECa 233 in future studies. This study also provided the first evidence demonstrating the distribution of madecassoside and asiaticoside to their target tissues from oral administration.

Department:	Biochemistry and Microbiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Biopharmaceutical Sciences	
Academic Year:	2016	

ACKNOWLEDGEMENTS

To complete this Ph.D. thesis, I would like to express my earnest gratitude to my advisor, Assistant Professor Dr. Phisit Khemawoot, for his patience, motivation, and immense knowledge, especially in preclinical pharmacokinetics which fulfilled my research of interest. His guidance and continuous support helped me in all the time of research and writing of this thesis.

Besides my advisor, I also would like to commend all of my thesis committee and my external examiner, Associate Professor Dr. Mayuree Tantisira, for their insightful comments, suggestions, and encouragements which persuaded me to widen my research from various perspectives.

This thesis research was financially supported by the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University (RES560530205-HR). A Ph.D. scholarship from the Graduate School of Chulalongkorn University to commemorate the 72nd anniversary of His Majesty King Bhumibala Aduladeja, and an overseas research experience scholarship for graduate student are gratefully acknowledged.

Finally, I would like to kindly thank my fellow labmates, all friends and staffs in the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for everything in past 5 years. Last but not least, I must express my profound gratitude to my parents, my siblings, and my fiancée for encouraging me spiritually throughout my Ph.D. study and daily life in general. This accomplishment would not have been possible without them. Thank you so much.

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
ASA	asiatic acid
ASS	asiaticoside
AST	aspartate aminotransferase
dL	deciliter
DMSO	dimethylsulfoxide
ECa 233	standardized extract of *Centella asiatica*
g	gram
h	hour
IS	internal standard
IV	intravenous administration
L	liter
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MDA	madecassic acid
MDS	madecassoside
mg	milligram
min	minute
mL	milliliter
ng	nanogram
NSS	normal saline solution
PO	oral administration
QC	quality control
XlogP	partition coefficient
µg	microgram
µL	microliter

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Centella asiatica (L.) Urban, also known as Asiatic pennywort, gotu kola or bua-bok, belongs to the family Apiaceae. It is commonly found in tropical areas of Southeast Asia, China, India, South Africa, South America and Eastern Europe [1-3]. Traditionally, this plant has been increasingly used to treat various conditions [4-11], so it is recognized as an interesting target for phytopharmaceutical product development. There are several commercial standardized extracts of *C. asiatica*. However, most of them are enriched in triterpenic acids, whereas the indigenous plant contains mainly triterpenoid glycosides, not triterpenic acids which exist naturally in very low amounts [12, 13]. To develop a standardized extract of *C. asiatica* as an alternative medicine in which the natural proportion of triterpenoid glycosides is maintained, ECa 233 was established in the Faculty of Pharmaceutical Sciences, Chulalongkorn University, and has subsequently been extensively investigated for its pharmacological and toxicological profiles. ECa 233 is a white to off-white extracted powder of *C. asiatica* containing triterpenoid glycosides, not less than 80%, and a ratio of madecassoside to asiaticoside of 1.5 ± 0.5 : 1 [14, 15].

When topically applied, ECa 233 has exhibited wound healing activity on second-degree burns in rats [16]. An oral paste containing 0.05% ECa 233 showed efficacy in reducing pain, ulcer size and erythema in minor recurrent aphthous ulceration [17]. Furthermore, oral ECa 233 at 100 mg/kg demonstrated significant anxiolytic effects in mouse models of acute and chronic stress [15]. Interestingly, oral ECa 233 at 10 and 30 mg/kg improved learning and memory deficits induced by intracerebroventricular injection of amyloid peptide [18] or by transient occlusion of bilateral common carotid arteries in mouse models [19]. A study of the neuritogenic effect on human neuroblastoma cells suggested that ECa 233 at a concentration of 1-

100 µg/mL promoted neurite outgrowth by up-regulating the levels of activated ERK1/2 and Akt [20]. Moreover, up to 10 g/kg of ECa 233 demonstrated a good safety profile with no evidence of lethality in acute toxicity tests in mice. Sub-chronic toxicity tests of oral ECa 233 at 10-1000 mg/kg in rodent models revealed modest toxicity [21] and no significant alterations in major drug-metabolizing enzymes [22].

The fact that ECa 233 has demonstrated favorable pharmacological and very good safety profiles warranted further development of the extract for human use, but its pharmacokinetic data have not yet been explored. Previous pharmacokinetic studies of pure madecassoside, administered orally as a single compound in rats, demonstrated a time to maximum plasma concentration and half-life of 0.90 ± 0.14 h and 3.47 ± 0.68 h, respectively [23], whereas the complete pharmacokinetics of asiaticoside have been unexplored. We therefore conducted experiments in rats to determine the pharmacokinetic profiles, including the oral absorption, tissue distribution, metabolism and excretion, of both madecassoside and asiaticoside in ECa 233 using LC-MS/MS.

1.2 Objectives

1.2.1 To determine the pharmacokinetic profiles of ECa 233 in rats.

1.2.2 To investigate tissue distribution patterns of active components and metabolites of ECa 233 in rats.

1.2.3 To identify the metabolites and metabolic pathway of ECa 233 in rats.

1.3 Hypothesis

ECa 233, standardized extract from *Centella asiatica* has a suitable pharmacokinetic profiles in rats, and the active compounds can distribute into pharmacological related organs.

1.4 Expected benefits from the study

1.4.1 Pharmacokinetic data of ECa 233 in rats which will be used and required for investigational new drug filing of ECa 233.

1.4.2 Promising results are useful information for further pharmacological studies of ECa 233.

CHAPTER II

LITERATURE REVIEW

2.1 *Centella asiatica*

General name: Asiatic pennywort, Gotu kola, Bua-bok

Scientific name: *Centella asiatica* (L.) Urban

Family: Apiaceae

Figure 1 *Centella asiatica* plant.

Centella asiatica is a clonal perennial creeper growing in swampy areas. It is an odorless plant with kidney-shaped green leaves, white or light purple-to-pink flowers and it bears small oval fruit. It can be found in tropical and subtropical areas, including Southeast Asia, China, India, Pakistan, Sri Lanka, Madagascar, South Africa, South America and Eastern Europe [1-3].

Centella asiatica has been widely used in traditional and herbal medicine for treating various illness both in preclinical and clinical stages for many pharmacological indications, such as wound healing effect [4, 5], memory enhancement [6], gastric ulcer healing [7, 11] venous ulcers treatment [8], anti-inflammatory [9], anti-tumor, anti-anxiety, antifungal, and antibacterial properties [10].

2.2 Chemical compositions of *Centella asiatica*

There are various chemical compositions contained in *C. asiatica* [1, 10].

2.2.1 Glycosides

- asiaticoside A
- asiaticoside B
- asiaticoside
- madecassoside
- centelloside
- brahmoside

2.2.2 Triterpene acids

- asiatic acid
- madecassic acid
- terminolic acid
- centic acid
- centellic acid
- centolic acid
- indocentolic acid
- isobrahmic acid
- betulic acid
- brahmic acid
- madasiatic acid

2.2.3 Volatile and fatty oil

- glyceride of palmitic acid
- stearic acid
- lignoceric acid
- oleic acid
- linoleic acid
- linolenic acid

2.2.4 Alkaloid

- hydrocotylin

2.2.5 Flavonoids

- 3-glucosylquercetin
- 3-glucosylkaempferol
- 7-glucosylkaempferol

2.2.6 Others

- mesoinositol
- oligosaccharide centellose
- kaempferol
- quercetin
- stigmasterol
- sitosterol
- campesterol
- polyacetylenes
- carotenoids
- vitamin B
- vitamin C

There are also several commercial standardized extracts of *C. asiatica*. However, most of them are enriched in triterpenic acids, whereas the indigenous plant contains mainly triterpenoid glycosides, not triterpenic acids which exist naturally in very low amounts [12, 13].

2.3 ECa 233 and its pharmacological activities

ECa 233 was the standardized extract of *Centella asiatica*, prepared and developed by the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The extract was characterized as white to off-white powder containing triterpenoid glycosides not less than 80% and the ratio of madecassoside and asiaticoside was 1.5 ± 0.5 : 1 [14, 24].

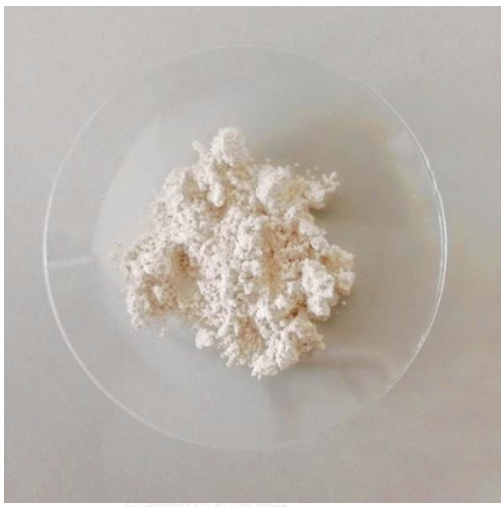


Figure 2 Physical appearance of ECa 233.

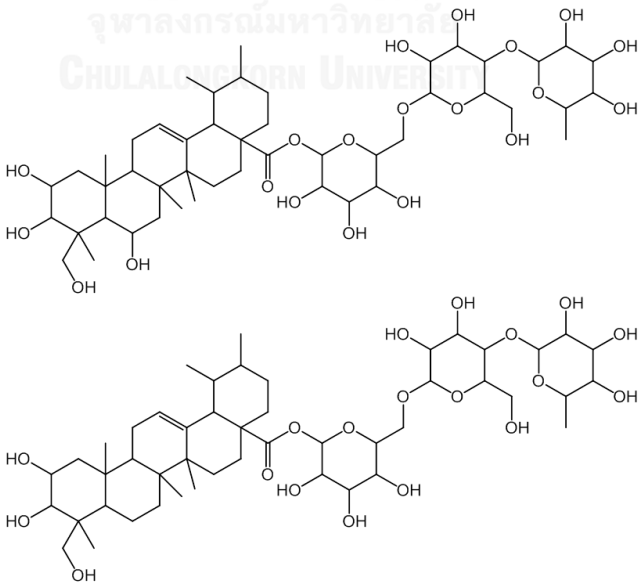


Figure 3 Chemical structures of madecassoside (top) and asiaticoside (below).

ECa 233 has already been indicated for some pharmacological effects. When topically applied, ECa 233 has exhibited wound healing activity on second-degree burns in male Wistar rats on day 7 post-burning [16]. An oral paste containing 0.05% ECa 233 showed efficacy in reducing pain, ulcer size and erythema of minor recurrent aphthous ulceration completely within 10 days [17].

Furthermore, oral ECa 233 at 100 mg/kg demonstrated significant anxiolytic effects in mouse models of acute and chronic stress [15]. Interestingly, oral ECa 233 at 10 and 30 mg/kg improved learning and memory deficits induced by intracerebroventricular injection of amyloid peptide [18] or by transient occlusion of bilateral common carotid arteries in mouse models [19]. A study of the neuritogenic effect on human neuroblastoma cells suggested that ECa 233 at a concentration of 1-100 µg/mL promoted neurite outgrowth by up-regulating the levels of activated ERK1/2 and Akt [20].

2.4 Toxicity studies of ECa 233

2.4.1 Acute toxicity

ECa 233 was tested in 10 male and 10 female mice by oral administration of a single dose of 10 g/kg. It was found that the given high dose of ECa 233 did not produce any toxic signs, mortality and gross pathological lesions within 14 days of the study [21].

2.4.2 Sub-chronic toxicity

ECa 233 was also studied for sub-chronic toxicity using 4 groups of Wistar rats by oral administration; three experimental groups treated with ECa 233 solution at doses of 10, 100, and 1,000 mg/kg/day for 90 days comparing to control group given distilled water. The result showed that all given doses of the extract did not affect body weight, food consumption and animal health. At the dose of 1,000 mg/kg/day, female rats had significantly higher white blood cell counts and male rats had significantly higher sodium level than the control group ($p<0.05$); however, the levels of the incidences were still within normal range and it did not affect any internal organ pathology confirmed by histopathological test [21].

2.5 Pharmacokinetic studies of *Centella asiatica*

2.5.1 Absorption

The studies in various animal models found that single dose of madecassoside, asiaticoside, madecassic acid or asiatic acid showed a bioavailability between 30 and 50% [25] and the peak plasma levels were reached at 0.9-4.0 h after intramuscular injection or oral administration [12, 23, 26, 27].

Moreover, the pharmacokinetic study in human was investigated in 12 healthy volunteers by single oral administration of total triterpenic fraction (TTF) of *Centella asiatica* (the product of 60% asiatic and madecassic acids together with 40% asiaticoside) at doses of either 30 or 60 mg. The results demonstrated the maximum plasma levels of asiatic acid at 4.5 ± 0.4 and 4.2 ± 0.3 h after dosing, and 2.2 ± 0.3 and 3.4 ± 0.7 h of elimination half-lives, for doses of 30 and 60 mg respectively. Comparing to chronic treatment for 7 days with either 30 mg or 60 mg of TTF twice daily, it was observed that peak plasma concentrations, AUC_{0-24}, and half-life were significantly higher than after single dose administration [13].

2.5.2 Metabolism

A study in 12 healthy volunteers found that asiaticoside was converted to asiatic acid *in vivo* by hydrolytic cleavage of the sugar moiety [28].

Another metabolism study of radiolabeled asiatic and madecassic acids in rats, it was reported that 45% of the both acids were metabolized to glucuronide (31.5-36.0%) and sulfate (9.0-13.5%) metabolites, later secreted into bile [29].

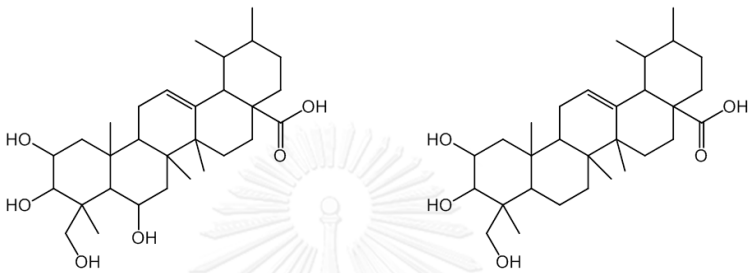


Figure 4 Chemical structures of madecassic acid (left) and asiatic acid (right).

The study in subchronic toxicity of ECa 233 on hepatic drug metabolizing enzymes, cytochrome P450 (CYP), was examined in rats by oral administration at doses of 10, 100 and 1,000 mg/kg/day for 90 days. The results showed that ECa 233 did not significantly affect either total CYP content or the activities of CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A in both male and female rats [22].

Furthermore, ECa 233 was also investigated for its inhibitory effects on major human cytochrome P450 (CYP) using *in vitro* recombinant human CYPs. The results exhibited that ECa 233 can inhibit CYP3A4, CYP2C19 and CYP2B6; meanwhile, it did not affect CYP1A2, CYP2C9, CYP2D6 and CYP2E1. This observation will be useful for further consideration of drug-drug interaction in using ECa 233 [30].

2.5.3 Excretion

Madecassol, the extract that was composed of 29-30% asiatic acid, 29-30% madecassic acid, 1% madasiatic acid and 40% asiaticoside, was totally excreted in feces within 24-76 h after ingestion, injection, or application, with a minimal amount metabolites through the kidneys [12]. Whereas, the elimination of asiatic and madecassic acids in rats were almost entirely eliminated from rats in feces within 4-6 days (81.0-89.6% in first 48 h after dosing), with little percentage found in urine after monitoring by radiolabeled technique [29].

From the up-to-date review of all pharmacokinetic studies in *Centella asiatica* (Table 1–3), there were some research groups which already reported the pharmacokinetic parameters of the plant extracts or pure active compounds, such as madecassoside and asiaticoside, using various animal models, routes of administration and methods of detection [23, 26, 27, 31, 32]. This can be summarized that:

- For extravascular administration, peak plasma levels were reached between 0.90-2.70 h after dosing and elimination half-lives were between 3.5-5.0 h.
- For intravenous injection, elimination half-lives were between 15-33 min.

Table 1 Previous pharmacokinetic study of madecassoside.

Compounds	**Madecassoside**
Research group	Han, *et al*. [23]
Country	China
Journal	Biomed. Chromatogr.
Year	2012
Subject species	6 Male Sprague-Dawley rats
Weight range	220 ± 30 g
Dose	100 mg/kg
Route of administration	PO, Oral administration
Analytical system	LC-MS
Blood uptake	Orbital veins
T_{max} (h)	0.90 ± 0.14
C_{max} (ng/mL)	303 ± 28
K_e (1/h)	0.21 ± 0.04
$T_{1/2}$ (h)	3.47 ± 0.68
AUC_{0-t} (ng h/mL)	1,368 ± 90 (t = 24 h)
$AUC_{0-\infty}$(ng h/mL)	1,458 ± 202

Table 2 Previous pharmacokinetic studies of asiaticoside.

Compounds	**Asiaticoside**		
Research group	Baek, *et al*. [31]	Liu, *et al*. [32]	
Country	South Korea	China	
Journal	J. Chromatogr. B	Chromatographia	
Year	1999	2010	
Subject species	Male Sprague-Dawley rats	8 Sprague-Dawley rats	
Weight range	200-220 g	230 ± 20 g	
Dose	10 mg/kg	40 mg/kg	200 mg/kg
Route of administration	IV, Intravenous administration	IV, Single caudal vein administration	IG, Single intragastric administration
Analytical system	HPLC-UV	LC-MS/MS	LC-MS/MS
Blood uptake	-	-	-
T_{max} (h)	-	0.08 (5 min)	*The amount of drug absorbed leads to very low concentrations in the circulation, lower than a few ng/mL*
C_{max} (ng/mL)	-	3,347 ± 786	
K_e (1/h)	-	-	
$T_{1/2}$ (h)	0.26 ± 0.10	0.39 ± 0.16 (beta)	
AUC_{0-t} (ng h/mL)	25,530 ± 4,560 (t = 1 h)	81,443 ± 57,156 (t = 2 h)	
$AUC_{0-\infty}$(ng h/mL)	-	81,904 ± 57,112	

Table 3 Previous pharmacokinetic studies of asiatic acid.

Compounds	**Asiatic acid**	
Research group	Nair, *et al*. [26]	Zheng, *et al*. [27]
Country	India	China
Journal	Rapid Commun. Mass Spectrom.	J. Chromatogr. B
Year	2012	2009
Subject species	30 Female Albino Wistar rats	5 Male Beagle dogs
Weight range	253.8 ± 12.9 g	12 ± 1.5 kg
Dose	CAE equivalent to 3 mg asiatic acid	CAE containing 540mg total glucosides (72% asiaticoside and 6% madecassoside)
Route of administration	Oral administration	Oral administration
Analytical system	LC-MS/MS	HPLC-UV
Blood uptake	Retro-orbital plexus	Limb veins
T_{max} (h)	1.33 ± 0.26	2.70 ± 0.45
C_{max} (ng/mL)	34.28 ± 4.90	740 ± 130
K_e (1/h)	0.16 ± 0.04	-
$T_{1/2}$ (h)	4.69 ± 0.40	4.29 ± 0.70
AUC_{0-t} (ng h/mL)	73.54 ± 0.57 (t = 48 h)	3,740 ± 420 (t = 24 h)
$AUC_{0-\infty}$(ng h/mL)	85.55 ± 11.01	3,820 ± 440

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Animal models

Male Wistar rats aged 8 weeks old were obtained from the National Laboratory Animal Center, Mahidol University. The animals were housed for 3 months in a controlled environment with free access to food and water. Adult rats were used in pharmacokinetic experiments after their body weights exceeded 400 g. The rats were placed in metabolic cages 12 h before the experiment and kept in the cages until 48 h post-administration. These rodents were anesthetized with 5% isoflurane by a chamber induction method during intravenous injection and blood collection. The animal experiments were conducted according to a protocol approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (approval number 13-33-007 and approval date: March 4, 2013).

3.1.2 Test compound

ECa 233 was kindly supplied by Siam Herbal Innovation Co., Ltd., as a white powder containing 53.1% madecassoside and 32.3% asiaticoside.

3.1.3 Chemicals and Reagents

- Madecassoside (Sigma-Aldrich, Corp., USA)
- Asiaticoside (Sigma-Aldrich, Corp., USA)
- Madecassic acid (Wako Pure Chemical Industries, Ltd., Japan)
- Asiatic acid (Sigma-Aldrich, Corp., USA)

- Glycyrrhetinic acid (Wako Pure Chemical Industries, Ltd., Japan)
- Dimethyl sulfoxide (Sigma-Aldrich, Corp., USA)
- β-Glucuronidase enzyme from *E. coli* (Sigma-Aldrich, Corp., USA)
- Potassium phosphate monobasic (Sigma-Aldrich, Corp., USA)
- Potassium phosphate dibasic (Sigma-Aldrich, Corp., USA)
- Isoflurane, Terrell (MINRAD, Inc., USA)
- Heparin (LEO Pharma A/S, Denmark)
- 0.9% Normal saline solution (General Hospital Products Public, Co., Ltd., Thailand)
- Methanol, HPLC grade (Honeywell Burdick & Jackson International, Inc., USA)
- Water, HPLC grade (Honeywell Burdick & Jackson International, Inc., USA)
- Formic acid (Merck KGaA, Corp., Germany)

3.1.4 Equipment

- QTRAP 6500 LC-MS/MS system (AB Sciex, Pte. Ltd., USA)
- HPLC C18 column, model Synergi Fusion-RP (Phenomenex, Inc., USA)
- Guard C18 column, model SecurityGuard Fusion-RP (Phenomenex, Inc., USA)
- Solid phase extraction column (Phenomenex, Inc., USA)
- Metabolic cage (Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand)
- Microliter centrifuge, model MIKRO 120 (Andreas Hettich, GmbH & Co. KG, Germany)
- Tabletop centrifuge, model EBA 20 (Andreas Hettich, GmbH & Co. KG, Germany)

- Micropipette (Labnet International, Inc., USA)
- Homogenizer, model Yellowline DI 18 Basic (IKA-Werke GmbH & Co. KG, Germany)
- Homogenizer (Tissue tearor), model WT-130 (Success Technic, Malaysia)
- Vortex mixer, model VX-200 (Labnet International, Inc., USA)
- Dry Bath Incubator, model EL-02 Dual Block (Major Science Co., Ltd., USA)
- Analytical balance, model AG135 (Mettler-Toledo International, Inc., Switzerland)
- Analytical balance, model UMT2 (Mettler-Toledo International, Inc., Switzerland)
- pH meter, model S220 SevenCompact (Mettler-Toledo International, Inc., Switzerland)
- Chest freezer, -20°C (Singer, Sdn Bhd, Malaysia)
- Insulin syringe 1 mL (0.4x12 mm.) (NIPRO, Corp. Ltd., Thailand)
- Stopwatch (Canon, Co., Ltd., China)
- Gavage needle 13G, 3 inches, ball diameter
- Surgical instruments
- Computer set

3.2 Methods

3.2.1 Test compound preparation

ECa 233 was freshly prepared at the concentration of 50 mg/mL (20% DMSO in NSS) for administration to the animals at 50, 100 and 200 mg/kg body weight, with following procedures,

1) ECa 233 was weighed out using analytical balance, Metler Toledo AG.
2) The compound was dissolved with 100% DMSO to the target volume (20% of final volume).
3) NSS was filled to complete 100% final volume and mixed to obtain clear solution of ECa 233 solution of 50 mg/mL (20% DMSO in NSS).
4) The solution was filtered using a 0.2-micron membrane filter by a sterile technique.

3.2.2 Pharmacokinetic study of ECa 233 in rat model

The pharmacokinetic study in rats was conducted for 4 periods with 2 weeks interval as shown in the table below,

Table 4 Dosages of administration.

Period	Control group (n=2)	Experimental group (n=4)
1	20% DMSO in NSS PO	ECa 233 50 mg/kg PO (20% DMSO in NSS)
2	20% DMSO in NSS PO	ECa 233 100 mg/kg PO (20% DMSO in NSS)
3	20% DMSO in NSS PO	ECa 233 200 mg/kg PO (20% DMSO in NSS)
4	20% DMSO in NSS IV	ECa 233 50 mg/kg IV (20% DMSO in NSS)

Period 1-3: Oral gavage administration

Method

1) All rats were placed in the metabolic cages for 12 h before the pharmacokinetic experiment.
2) On the experiment, rats in the experimental group were administered the test compounds at a dose of 50, 100 and 200 mg/kg body weight.
3) Rats in the control group were administered by oral gavage at various volume of 20% DMSO in NSS in accordance with ECa 233 doses.
4) All rats were anesthetized with isoflurane during oral administration and sample collection.
5) Blood: 400 µL of blood was collected via lateral tail vein at 0, 0.083 (5 min), 0.25 (15 min), 0.5 (30 min), 1, 2, 4, 8, 24 h after dosing. Blood samples were then centrifuged at 1,500*×g* for 10 min to acquire approximately 200 µL of plasma which was equally aliquoted into 2 tubes (one for pharmacokinetic study and another for stock). The plasma samples were kept at -20°C until analysis.
6) Urine: the samples were separately collected for 2 periods (0-24 and 24-48 h) after dosing from metabolic cages. Urine samples volume were recorded and then centrifuged at 3,000*×g* for 10 min. The 200 µL of urine supernatant was equally aliquoted into 2 tubes (one for pharmacokinetic study and another for stock), and the rest was used in metabolite identification. All urine samples were kept at -20°C until analysis.
7) Feces: the samples were separately collected for 2 periods (0-24 and 24-48 h) after dosing from metabolic cages. Feces samples were weighed and added methanol up to 10 mL in 15 mL tube. The samples were then kept at -20°C until analysis.

Period 4: Intravenous bolus administration

Method

1) All rats were placed in the metabolic cages for 12 h before the pharmacokinetic experiment.
2) On the experiment, rats in the experimental group were administered the test compounds at a dose of 50 mg/kg body weight.
3) Rats in the control group were administered by intravenous bolus in accordance with ECa 233 dose.
4) All rats were anesthetized with isoflurane during intravenous administration and sample collection.
5) Blood: 400 µL of blood was collected via lateral tail vein at 0, 0.083 (5 min), 0.25 (15 min), 0.5 (30 min), 1, 2, 4, 8, 24 h after dosing. Blood samples were then centrifuged at 1,500*xg* for 10 min to acquire approximately 200 µL of plasma which was equally aliquoted into 2 tubes (one for pharmacokinetic study and another for stock). The plasma samples were kept at -20°C until analysis.
6) Urine: the samples were separately collected for 2 periods (0-24 and 24-48 h) after dosing from metabolic cages. Urine samples volume were recorded and then centrifuged at 3,000*xg* for 10 min. The 200 µL of urine supernatant was equally aliquoted into 2 tubes (one for pharmacokinetic study and another for stock), and the rest was used in metabolite identification. All urine samples were kept at -20°C until analysis.
7) Feces: the samples were separately collected for 2 periods (0-24 and 24-48 h) after dosing from metabolic cages. Feces samples were weighed and added methanol up to 10 mL in 15 mL tube. The samples were then kept at -20°C until analysis.

3.2.3 Blood chemistry

Plasma samples from pharmacokinetic study at 0 and 24 h after dosing were sent to Professional Laboratory Management Corp Co., Ltd., for biochemical parameters evaluation in order to determine effects of ECa 233 or its vehicle (20% DMSO in NSS) on physiological conditions. The major organs of interests were kidney and liver which involved in drug metabolism and excretion, and biochemical markers those were needed to determine are:

- Creatinine
- Aspartate aminotransferase (AST)
- Alanine aminotransferase (ALT)

A Cobas 6000 automated analyzer (Roche Diagnostics, Ltd.) was used in an enzymatic method (chemiluminescence) for creatinine quantitation and a kinetic method (according to the International Federation of Clinical Chemistry and Laboratory Medicine recommendations) for AST and ALT quantification.

The acquired results were reported as mean ± standard deviation (S.D.) and statistical analyzed by student's t-test (significant level = 0.05) using SPSS software, version 17 (SPSS Inc., USA) to determine significant differences of biochemical parameters between pretreatment and posttreatment for 24 h. The results were also compared with the reference values of healthy male Wistar rats in Table 5.

Table 5 Reference ranges of blood chemistry in male Wistar rats.

Test (Units)	**Reference Range** [33]
Creatinine (mg/dL)	0.2-0.8
AST (U/L)	45.7-80.8
ALT (U/L)	17.5-30.2

3.2.4 Tissue Distribution of ECa 233

Rats were divided into 3 groups; all rats treated with ECa 233 were used to collect tissues at the time of 1, 2 and 4 h, and determined for the ECa 233 level.

Method

1) Rats were administered ECa 233 by intravenous injection via lateral tail vein at a dose of 50 mg/kg body weight. Then, they all were euthanized at the time of 1, 2 and 4 h after dosing and designated tissues (brain, liver, kidney, spleen, stomach, and skin) were collected.
2) 400 µL of blood was collected via lateral tail vein at time 0 h and the designated time. Blood samples were then centrifuged at 1,500*xg* for 10 min to acquire approximately 200 µL of plasma which was equally aliquoted into 2 tubes (one for tissue distribution study and another for stock). The plasma samples were kept at -20°C until analysis.
3) All rats were anesthetized with isoflurane during intravenous administration and before euthanized.
4) Tissue samples were washed in NSS to remove blood from the tissues. The tissue samples were kept at -20°C until analysis.

3.2.5 Sample preparation for determination by LC-MS/MS

All samples were prepared by protein precipitation using methanol as precipitating agent before analyzed by LC-MS/MS.

Plasma

1) Plasma samples were thawed at room temperature and 50 µL of each samples was pipetted to 1.5 mL tube.
2) 200 µL of methanol containing internal standard was added and mixed by vortex mixer for 10 min.
3) The samples were centrifuged at 5,000*xg* for 10 min.
4) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

Urine

1) Urine samples were thawed at room temperature and 50 µL of each samples was pipetted to 1.5 mL tube.
2) 200 µL of methanol containing internal standard was added and vortex mixed for 10 min.
3) The samples were centrifuged at 5,000*xg* for 10 min.
4) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

Feces

1) Feces samples which previously added methanol were thawed at room temperature and homogenized in the ice bath for 5 min.
2) The homogenized samples were vortex mixed for 10 min and then centrifuged at 2,500*xg* for 10 min.
3) 50 µL of primary supernatants was transferred to 1.5 mL tube, and 200 µL of methanol containing internal standard was added and vortex mixed for 10 min.
4) The samples were centrifuged at 5,000*xg* for 10 min.

5) 150 µL of secondary supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

Tissues

1) Tissue samples were thawed at room temperature and weighed out for 50 mg in 1.5 mL tube.
2) 200 µL of methanol containing internal standard was added, and the samples were homogenized in the ice bath for 5 min.
3) The samples were mixed for 10 min and the centrifuged at 5,000*xg* for 10 min.
4) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

For some biological samples for which the concentrations were higher than the linearity range of quantification, blank matrices were used to lower the concentration prior to processing for protein precipitation.

3.2.6 Sample preparation for determination of glucuronide metabolites by LC-MS/MS

In this experiment, β-glucuronidase enzyme from *E. coli* in 75 mM phosphate buffer pH 6.8 was used in enzymatic hydrolysis of ECa 233 glucuronide metabolites. Using curcumin glucuronide as a positive control, the condition for the reaction was first needed to study by varying enzyme units (100 and 500 units) and incubation time at 37°C (15, 30, 60 and 90 min) to acquire the optimal conditions used for all samples. Consequently, the hydrolyzed samples were then prepared by protein precipitation using methanol as precipitating agent before analyzed by LC-MS/MS.

Plasma

1) Plasma samples from pharmacokinetic study were thawed at room temperature and 50 µL of each samples was pipetted to 1.5 mL tube.
2) Added 50 µL of β-glucuronidase solution and the mixture was incubated at 37°C, with the optimized conditions.
3) 400 µL of methanol containing internal standard was added and mixed by vortex mixer for 10 min.
4) The samples were centrifuged at 5,000×*g* for 10 min.
5) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

Urine

1) Urine samples from pharmacokinetic study were thawed at room temperature and 50 µL of each samples was pipetted to 1.5 mL tube and diluted by 950 µL of 75 mM phosphate buffer pH 6.8.
2) 50 µL of the 10X diluted samples was transferred into new 1.5 mL tube.
3) 50 µL of β-glucuronidase in phosphate buffer pH 6.8 were added, and the mixture was incubated at 37°C with the optimized conditions.
4) 400 µL of methanol containing internal standard was added and vortex mixed for 10 min.

5) The sample tubes were centrifuged at 5,000*xg* for 10 min.
6) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

Feces

1) 50 µL of primary supernatant of feces samples from pharmacokinetic study (prepared in previous section) was transferred to 1.5 mL tube.
2) 50 µL of β-glucuronidase in 75 mM phosphate buffer pH 6.8 were added, and the mixture was incubated at 37°C with the optimized conditions.
3) 400 µL of methanol containing internal standard was added and vortex mixed for 10 min.
4) The sample tubes were centrifuged at 5,000*xg* for 10 min.
5) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

Tissues

1) Tissue samples from tissue distribution study were thawed at room temperature and weighed out for 50 mg in 1.5 mL tube.
2) 100 µL of 75 mM phosphate buffer pH 6.8 was added, and the samples were homogenized in the ice bath for 5 min.
3) 50 µL of tissue homogenates were added with 50 µL of β-glucuronidase solution, and the mixture was incubated at 37°C with the optimized conditions.
4) 400 µL of methanol containing internal standard was added and vortex mixed for 10 min.
5) The sample tubes were centrifuged at 5,000*xg* for 10 min.
6) 150 µL of supernatants were collected and 10 µL were analyzed by LC-MS/MS with optimized conditions and parameters.

3.2.7 LC-MS/MS analysis

Standard chemical tuning

1) Madecassoside, Asiaticoside, Madecassic acid, Asiatic acid and Glycyrrhetinic acid (as internal standard) were dissolved in methanol at concentration of 1, 10, 100 ng/mL. Each chemical solution was injected into mass analyzer to determine appropriate condition for MS measurement.
2) The following parameters of each compound were optimized for appropriate values,
    - Declustering potential (DP)
    - Entrance potential (EP)
    - Collision energy (CE)
    - Collision exit potential (CXP)
3) Mixer of chemical solutions was analyzed with LC-MS/MS to determine proper LC conditions and exact retention time.

Calibration curve construction

The calibration curves were individually constructed for each matrices, plasma, urine, feces, and various tissues by using blank samples.

1) Standard mixtures of chemicals were mixed with blank matrices.
2) The samples were centrifuged and supernatant were collected.
3) Samples were analyzed by using LC-MS/MS with optimized conditions.
4) The ratio of drug/internal standard chromatogram area was calculated and standard curve of each compounds was constructed in order to determine correlation of chromatogram area and concentrations.
5) From the linear regression ($R^2 > 0.99$) in the calibration curve, the standard equations were used in next step.

Method validation and quality control

Refer to Guidance for Industry: Bioanalytical Method Validation of US Food and Drug Administration [34, 35], partial method validation was developed to verify the quality of ECa 233 measurement which was composed of:

- Lower limit of quantification (LLOQ), defined as the lowest detectable concentration of analyte with a signal-to-noise ratio greater than 5.
- Linearity, defined as the range of analyte concentration that can be fitted with the calibration curve with $R^2 > 0.99$.
- Accuracy, determined by comparing the measured concentration to the actual concentration of quality control (QC) samples at low, medium and high concentrations. Accuracy were reported as % bias which should be within 15% of the actual value. The % bias was calculated by (measured value - true value) ÷ true value × 100.
- Precision, determined concurrently with accuracy by analyzing QC samples for intra-day (5 replicates within a day) and inter-day (once a day for 5 consecutive days). Precision were reported as % RSD which should not exceed 15%. The % RSD was calculated by (SD ÷ mean) × 100.
- Recovery, calculated by comparing the peak-ratio of the prepared sample to that of the standard solution containing the same concentration. The % recovery would be indicated the quality of sample preparation.

Table 6 Quality of sample preparation determined by % recovery.

% Recovery	**Interpretation** [36]
> 85%	Sample preparing process was qualified.
< 80% but > 60%	Sample preparing process was needed improvement.
< 60%	Sample preparing process was not acceptable.

3.2.8 Data analysis

Pharmacokinetic parameters determination (PK analysis)

1) From the calculated plasma concentrations of madecassoside and asiaticoside at various time-points, the plasma concentrations were plotted against time to construct plasma concentration-time profile of ECa 233.
2) PK solutions 2.0 software (Summit Research Services, CO, USA) with non-compartmental analysis was used to calculate the pharmacokinetic parameters:
   - Area under the curve from time zero to 24 h after dosing (AUC_{0-24})
   - Area under the curve from time zero to infinity ($AUC_{0-\infty}$)
   - Maximum plasma drug concentration (C_{max})
   - Time to reach C_{max} (T_{max})
   - Volume of distribution (V_d)
   - Mean residence time (MRT)
   - Elimination half-life ($T_{1/2}$)
   - Total Clearance (CL)
3) Oral bioavailability was calculated by $(AUC_{po} \div AUC_{iv}) \times 100$

Tissue distribution analysis

The tissue-to-plasma concentration ratios of madecassoside and asiaticoside were calculated as the tissue level divided by the plasma level at the same sampling time points. The tissue AUC_{0-4} were also calculated by trapezoidal rule.

%Recovery of ECa 233

The percentage recoveries of madecassoside, asiaticoside, madecassic acid and asiatic acid were determined from the total amount of the drug found in urine or feces divided by the dose based on molar unit.

All pharmacokinetic data were reported in mean ± standard deviation (S.D.) and statistical analyzed by student's t-test (significant level = 0.05) using SPSS software, version 17 (SPSS Inc., USA) to determine significant differences.

CHAPTER IV

RESULTS

4.1 Validated LC-MS/MS analysis

LC-MS/MS was performed using an Eksigent ekspert UPLC 100 liquid chromatograph equipped with a QTRAP 6500 mass spectrometer and controlled by Analyst software, version 1.6 (AB Sciex, Pte. Ltd.). The stationary phase was a Synergi Fusion-RP C18 column (Phenomenex, Inc.) with a 40 ℃ oven temperature. Methanol and 0.2% formic acid in water (pH 2.5) were used as the mobile phase in a gradient elution pattern at a flow rate of 0.5 mL/min, as follows: 10% methanol for 0.5 min, increased to 90% methanol at 1.5 min and maintained for 2 min, then decreased to 10% methanol at 4.0 min and maintained for 4.5 min as the run time (Figure 5). Standards of madecassoside, asiaticoside, madecassic acid, asiatic acid and glycyrrhetinic acid were dissolved in methanol for tuning in the negative ion mode $[M-H]^-$ to obtain optimal MS conditions. The parameters for electrospray ionization were as follows: curtain gas, 25.0 units; collision gas, medium; ion spray voltage, -4,500 V; temperature, 500 ℃; ion source gas 1, 40.0 units; and ion source gas 2, 50.0 units.

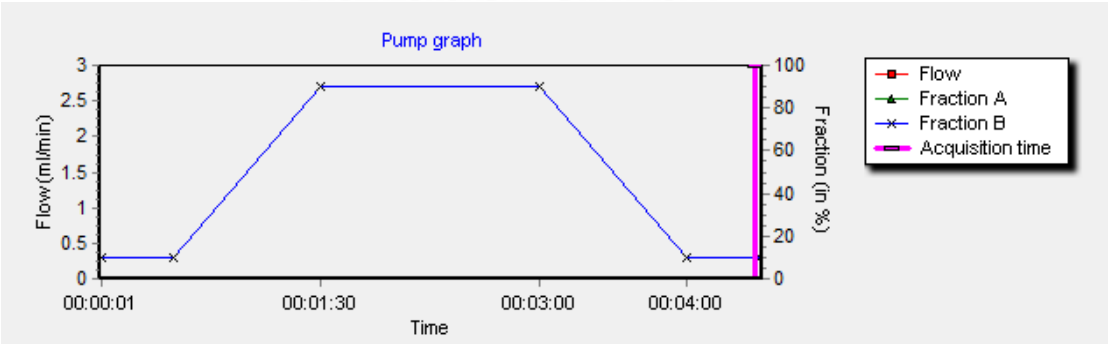
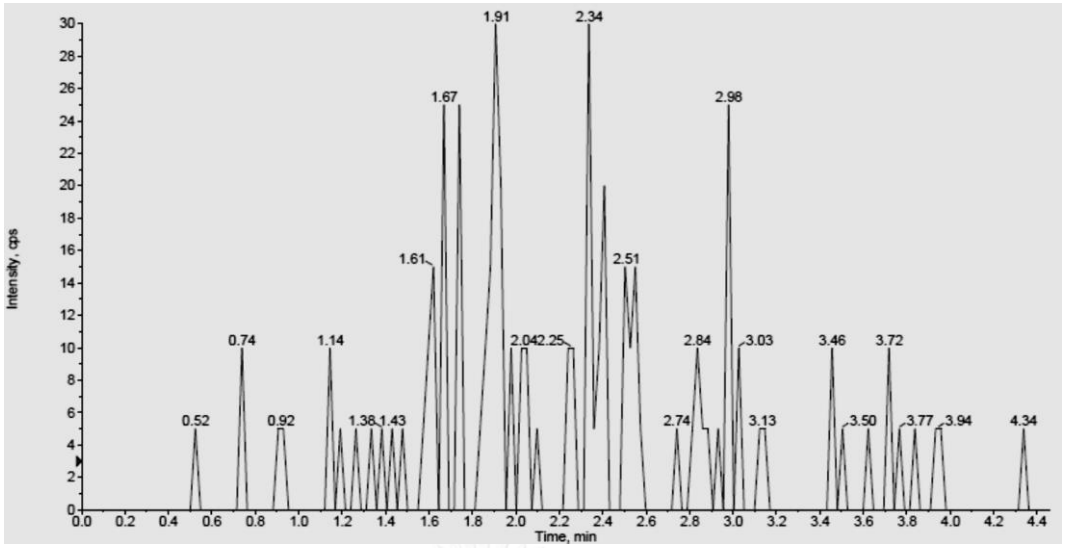


Figure 5 Mobile phase in a gradient elution pattern.

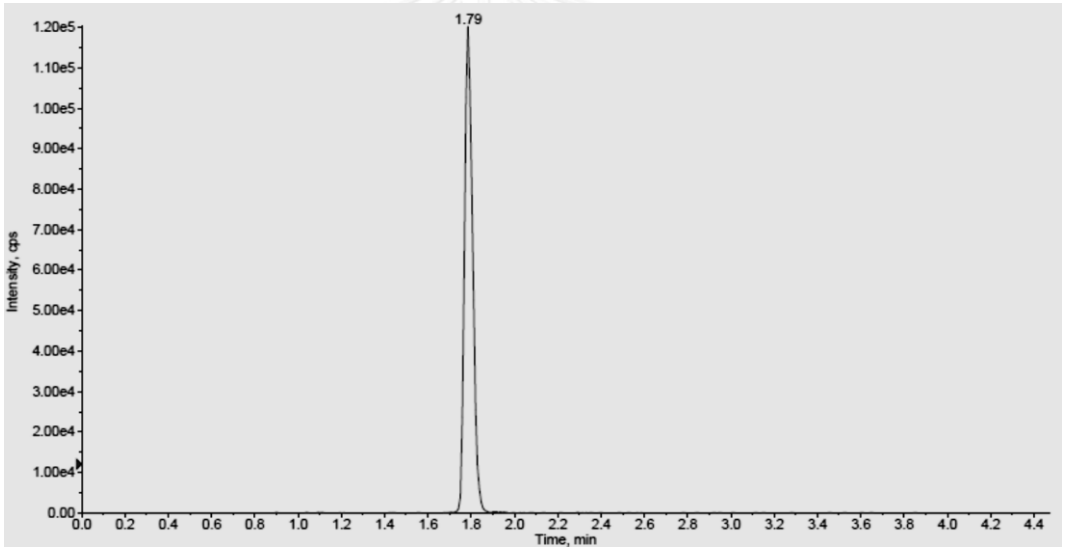
The MS conditions for the standard chemicals were tuned to establish the best conditions for the LC-MS/MS experiments (Table 7). Using the optimized LC-MS/MS conditions, the retention times of madecassoside, asiaticoside, madecassic acid, asiatic acid and glycyrrhetinic acid were 1.79, 1.82, 1.93, 1.99 and 2.12 min, respectively (Figure 6). Calibration curves showed good linearity with correlation coefficients of $R^2 > 0.99$ ranging at 0.1-400 μg/L for madecassoside and asiaticoside, and at 0.5-300 μg/L for madecassic acid and asiatic acid. Standard equations were used to calculate the amounts of active components in biological samples. The lower limits of quantification were 0.1 μg/L for madecassoside and asiaticoside, and were 0.5 μg/L for madecassic acid and asiatic acid. The percentage recovery was over the range of 78-93% for all of the compounds. The intra-day and inter-day precision and accuracy were within ± 10% (Table 8) which were not exceed 15% recommended by 'Guidance for Industry: Bioanalytical Method Validation of US Food and Drug Administration [34]'.

Table 7 Mass spectrometry parameters of standard chemicals used for measurements in biological samples.

MS Parameters	Madecassoside	Asiaticoside	Madecassic acid	Asiatic acid	Glycyrrhetinic acid
Parent ion (m/z)	973.4	957.7	503.5	487.3	469.3
Daughter ion (m/z)	503.5	469.1	219.2	379.2	409.2
Declustering potential (Volt)	-118.21	-60.70	-124.58	-23.85	-226.20
Entrance potential (Volt)	-7.68	-13.99	-5.62	-11.10	-7.70
Collision energy (Volt)	-69.86	-36.29	-31.10	-58.00	-59.36
Collision exit potential (Volt)	-15.50	-45.16	-31.80	-30.78	-15.01

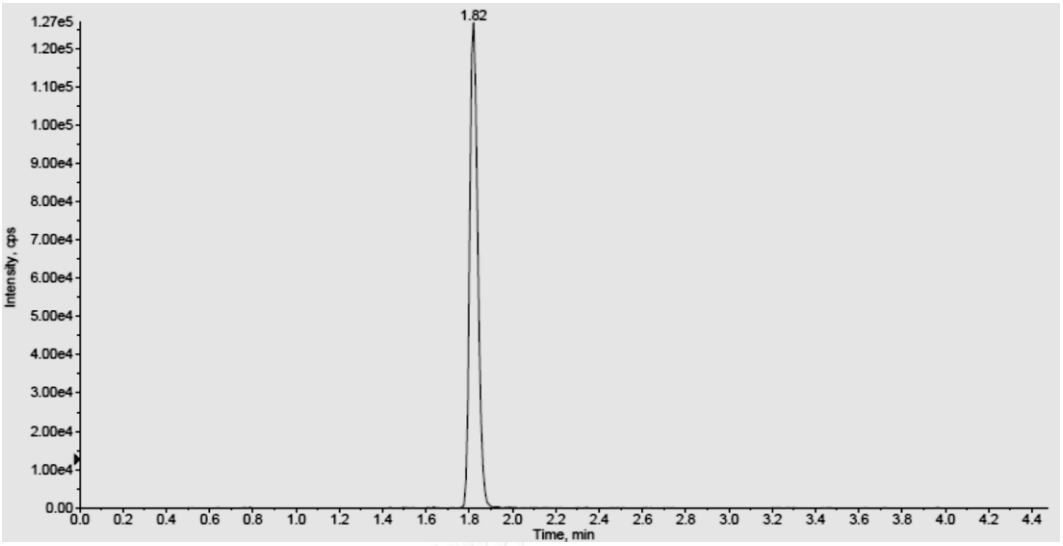


(a) Blank plasma

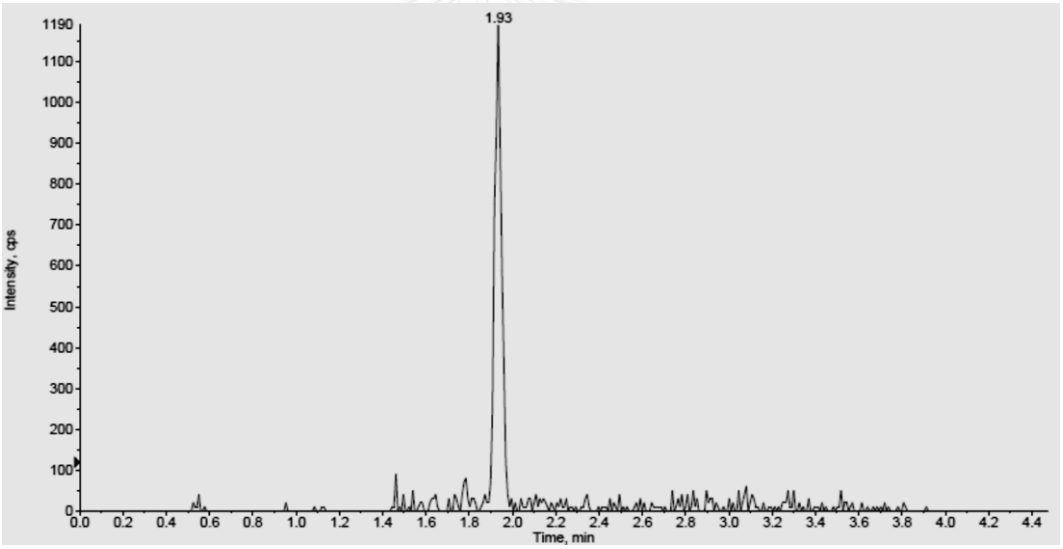


(b) 100 µg/L of madecassoside spiked into plasma

Figure 6 LC-MS/MS chromatograms for ECa 233.

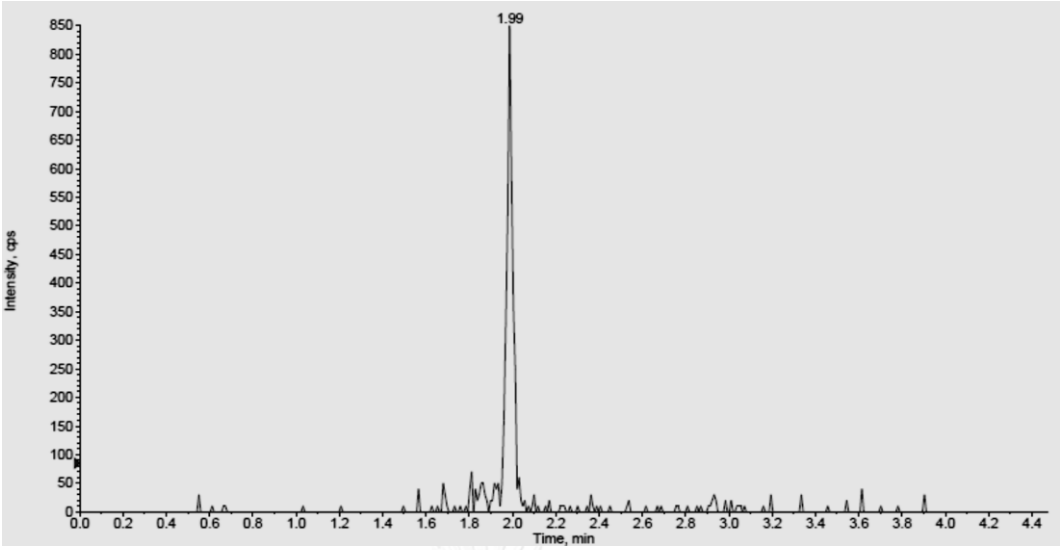


(c) 100 µg/L of asiaticoside spiked into plasma

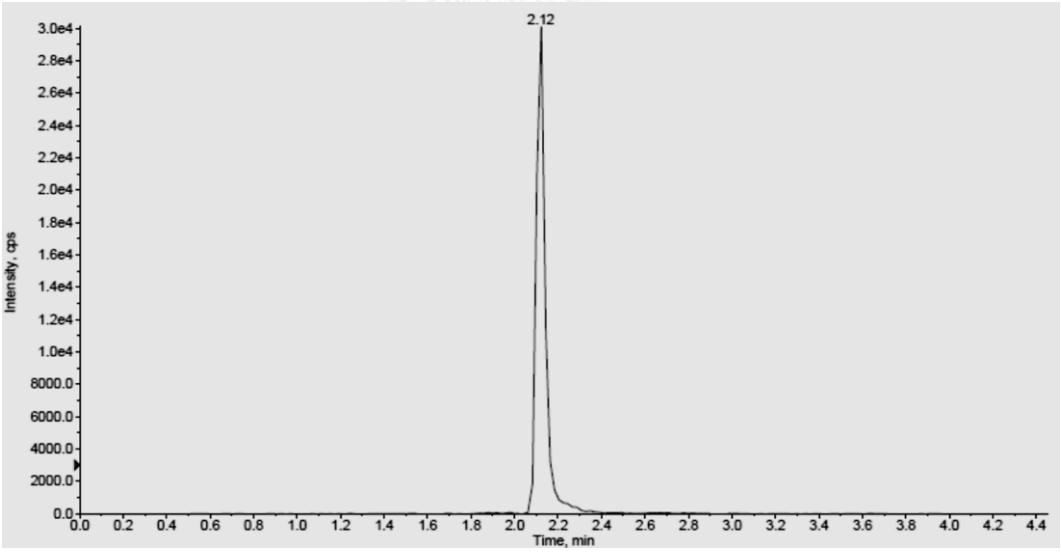


(d) 100 µg/L of madecassic acid spiked into plasma

Figure 6 LC-MS/MS chromatograms for ECa 233. (cont.)



(e) 100 µg/L of asiatic acid spiked into plasma



(f) 10 µg/L of glycyrrhetinic acid (IS) spiked into plasma

Figure 6 LC-MS/MS chromatograms for ECa 233. (cont.)

Table 8 The intra- and inter-day precision, accuracy, and recovery method validation of compounds using LC-MS/MS.

Compounds	Intra-day			Inter-day		
	Precision (% RSD)	Accuracy (%)	Recovery (%)	Precision (% RSD)	Accuracy (%)	Recovery (%)
Madecassoside						
- Low concentration	9.20	101.60	88.93	7.77	95.15	91.55
- Medium concentration	1.43	103.08	84.02	9.81	107.45	82.07
- High concentration	1.00	97.38	80.41	8.08	99.82	78.27
Asiaticoside						
- Low concentration	8.40	108.05	93.28	6.19	95.90	88.36
- Medium concentration	8.96	101.18	92.70	8.31	100.52	78.09
- High concentration	4.04	92.13	87.54	6.42	97.13	80.12
Madecassic acid						
- Low concentration	9.40	98.10	78.31	6.63	104.78	79.97
- Medium concentration	4.24	94.28	89.70	7.75	96.00	78.11
- High concentration	3.12	99.50	84.68	6.31	100.02	79.74
Asiatic acid						
- Low concentration	6.11	93.10	87.97	4.88	106.87	89.18
- Medium concentration	7.47	106.67	82.54	4.78	109.40	90.53
- High concentration	5.60	96.58	79.52	9.43	96.88	77.61

4.2 Animal tolerability

ECa 233 was well tolerated. None of the rats in the control and experimental groups showed any significant changes or abnormalities throughout the experiments. Furthermore, no significant alteration of biochemical parameters reflecting kidney or liver function was noted at 24 h after administration of ECa 233 at almost all of the doses tested. Although a statistically significant increase in AST was noted after an oral dose of ECa 233 of 50 mg/kg, and a statistically significant decrease in ALT was found after an oral dose of ECa 233 of 200 mg/kg (Table 9), these changes were not clinically significant. The observed biochemical parameters were also within the normal physiological ranges of Wistar rats from 'Exotic Companion Medicine Handbook for Veterinarians [33]' (Table 5).

Table 9 Physical and biochemical profiles of rats in the control and experiment groups at 0 and 24 h after ECa 233 dosing.

Biochemical parameters		Experimental groups				
		Control	50 mg/kg IV	50 mg/kg PO	100 mg/kg PO	200 mg/kg PO
Physical appearance	Pre-treatment	Normal	Normal	Normal	Normal	Normal
	Post-treatment	Normal	Normal	Normal	Normal	Normal
Creatinine (mg/dL)	Pre-treatment	0.31 ± 0.04	0.25 ± 0.06	0.23 ± 0.05	0.25 ± 0.06	0.30 ± 0.08
	Post-treatment	0.33 ± 0.05	0.33 ± 0.05	0.28 ± 0.05	0.33 ± 0.05	0.28 ± 0.05
AST (U/L)	Pre-treatment	67.75 ± 3.28	63.75 ± 9.74	72.25 ± 2.22	63.75 ± 9.74	65.50 ± 4.36
	Post-treatment	72.50 ± 8.93	75.75 ± 4.86	89.75 ± 7.72 *	75.75 ± 4.86	73.25 ± 9.00
ALT (U/L)	Pre-treatment	21.50 ± 5.43	20.75 ± 4.99	17.75 ± 2.06	20.75 ± 4.99	17.50 ± 2.08
	Post-treatment	19.00 ± 4.15	20.50 ± 4.12	17.25 ± 1.50	20.50 ± 4.12	14.50 ± 3.32 *

Data are shown as the mean ± S.D. (n = 4); * $p < 0.05$ pre-treatment vs. post-treatment.

4.3 Plasma concentration-time profiles

Madecassoside and asiaticoside were rapidly, but not completely, absorbed from the gastrointestinal tract, given a bioavailability of less than 1%. The maximum plasma concentrations (C_{max}) of madecassoside (1,654 ± 884, 5,664 ± 3,947 and 9,020 ± 5,744 μg/L) and asiaticoside (318 ± 192, 1,283 ± 1,089 and 4,028 ± 3,157 μg/L) were observed within 5-15 min after the provision of oral doses of ECa 233 of 50, 100 and 200 mg/kg, respectively. As shown in Figure 7, the concentration-time curves of orally administered ECa 233 at all doses were rather similar, albeit of smaller magnitudes, compared to those exhibited in Figure 8 by intravenously administered ECa 233 (50 mg/kg), in which the maximal plasma concentrations of madecassoside (approx. 6,000 mg/L) and asiaticoside (approx. 1,500 mg/L) gradually decreased to 200-500 μg/L 8 h after dosing. The area under the curve from time 0 to 24 h ($AUC_{(0-24)}$) of madecassoside (2,712 ± 1883, 7,778 ± 4,672 and 8,071 ± 3,285 μg·h/L) and asiaticoside (796 ± 910, 2,831 ± 2,843 and 3,390 ± 3,325 μg·h/L) were found after oral administration of ECa 233 at doses of 50, 100 and 200 mg/kg, respectively. The compounds had volumes of distribution of 0.32-0.88 L/kg and clearances of 0.04-0.10 L/h/kg. The elimination half-life was approximately 5.4-8.0 h for madecassoside and 6.3-8.8 h for asiaticoside (Table 10 and 11).

Madecassic acid and asiatic acid, the proposed metabolites, were not found in plasma at any time points after oral administration of all given doses. On the other hand, small amounts of madecassic acid (7 mg/L) and asiatic acid (10 mg/L) were exclusively found in plasma only at 5 min after intravenous injection of ECa 233. The plasma concentration-time profiles of these two triterpenic acids could not constructed and their pharmacokinetic parameters could not calculated.

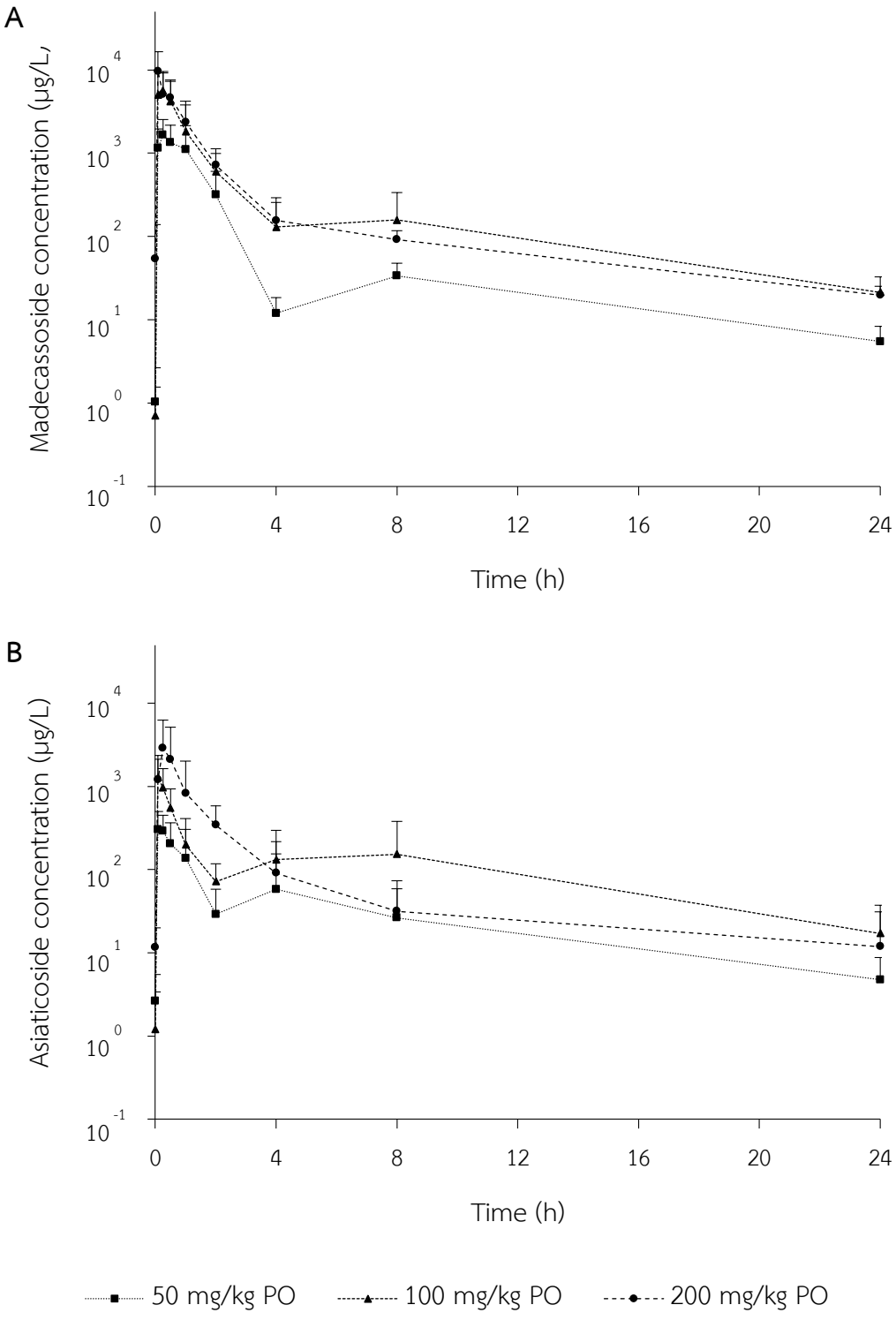


Figure 7 Plasma concentration-time profiles of madecassoside **(A)** and asiaticoside **(B)** after oral dosing of ECa 233. The results are shown as the mean ± S.D. (n = 4).

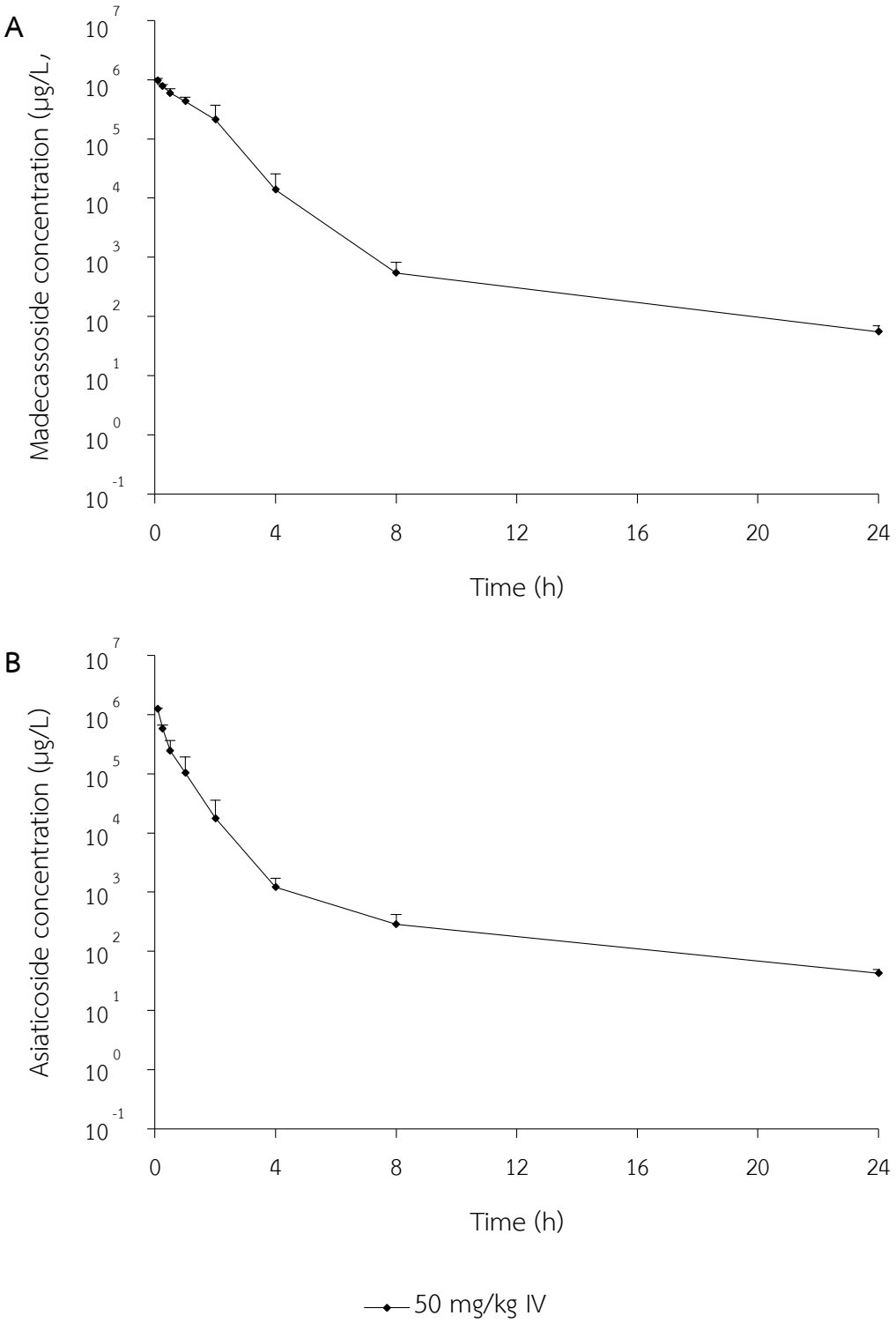


Figure 8 Plasma concentration-time profiles of madecassoside **(A)** and asiaticoside **(B)** after intravenous dosing of ECa 233. The results are shown as the mean ± S.D. (n = 4)

Table 10 Pharmacokinetic parameters of madecassoside, a main constituent of ECa 233.

Pharmacokinetic parameters	ECa 233			
	50 mg/kg IV	50 mg/kg PO	100 mg/kg PO	200 mg/kg PO
C_{max} (µg/L)	5,910,697 ± 4,489,761	1,654 ± 884	5,664 ± 3,947	9,020 ± 5,744
T_{max} (h)	NA	0.25 ± 0.00	0.25 ± 0.00	0.08 ± 0.00
$AUC_{(0-24)}$ (µg·h/L)	1,436,900 ± 562,001	2,712 ± 1,883	7,778 ± 4,672	8,071 ± 3,285
$AUC_{(0-\infty)}$ (µg·h/L)	1,437,323 ± 562,035	2,767 ± 1,868	8,025 ± 4,502	8,280 ± 3,224
Vd (L/kg)	0.32 ± 0.21	265 ± 220	290 ± 396	291 ± 132
MRT (h)	0.91 ± 0.13	3.43 ± 1.29	5.86 ± 5.08	3.60 ± 1.88
Elimination half-life (h)	5.40 ± 1.63	6.51 ± 1.97	8.05 ± 4.91	7.28 ± 0.97
CL (L/h/kg)	0.04 ± 0.01	26.17 ± 19.54	18.28 ± 14.88	27.63 ± 12.20
Bioavailability (%)	100.00	0.19	NA	NA

Data are shown as the mean ± S.D. (n = 4). NA: not applicable.

Table 11 Pharmacokinetic parameters of asiaticoside, a main constituent of ECa 233.

Pharmacokinetic parameters	ECa 233			
	50 mg/kg IV	50 mg/kg PO	100 mg/kg PO	200 mg/kg PO
C_{max} (µg/L)	1,466,774 ± 82,830	318 ± 192	1,283 ± 1,089	4,028 ± 3,157
T_{max} (h)	NA	0.19 ± 0.10	0.08 ± 0.00	0.14 ± 0.10
$AUC_{(0-24)}$ (µg·h/L)	543,530 ± 156,158	796 ± 910	2,831 ± 2,843	3,390 ± 3,325
$AUC_{(0-\infty)}$ (µg·h/L)	543,921 ± 156,217	858 ± 919	2,974 ± 2,952	3,605 ± 3,703
Vd (L/kg)	0.88 ± 0.36	2,085 ± 2,144	1,871 ± 3,297	1,055 ± 610
MRT (h)	0.49 ± 0.13	7.21 ± 6.42	6.95 ± 3.86	3.07 ± 2.28
Elimination half-life (h)	6.30 ± 1.51	8.29 ± 4.49	7.53 ± 6.06	8.43 ± 3.37
CL (L/h/kg)	0.10 ± 0.03	200 ± 251	101 ± 129	105 ± 75
Bioavailability (%)	100.00	0.16	NA	NA

Data are shown as the mean ± S.D. (n = 4). NA: not applicable.

4.4 Tissue distribution

Madecassoside and asiaticoside in ECa 233, administered orally, were rapidly distributed to the skin, spleen, stomach, kidneys, liver and brain, with an $AUC_{(0-4)}$ of 73.64 ± 19.59 and 20.52 ± 2.95 ng·h/g of brain tissue, respectively. Whereas the highest tissue $AUC_{(0-4)}$ of madecassoside and asiaticoside from intravenously administered ECa 233 (50 mg/kg) was found in the kidneys, their corresponding values in orally administered ECa 233 (100 mg/kg) were found in the stomach (Table 12). All of the tissue-to-plasma concentration ratios of these two triterpenoids were stepwise increased to their respectively highest level at 4 h after intravenous dosing (Figure 9). In contrast, the tissue-to-plasma concentration ratios of madecassoside and asiaticoside at 1, 2 and 4 h after provision of an oral dose of ECa 233 were found to be rather consistent or even slightly decreased at 4 h (Figure 10).

The expected triterpenic acid metabolites, madecassic acid and asiatic acid, were not found in any observed tissues.

Table 12 Area under the curve of madecassoside and asiaticoside in internal organs from time 0 to 4 h after intravenous injection of 50 mg/kg or oral administration of 100 mg/kg ECa 233.

Compounds	Organs	$AUC_{(0-4)}$ (ng·h/g of tissue)	
		50 mg/kg IV	100 mg/kg PO
Madecassoside	Skin	32,795 ± 457	667.22 ± 121.06
	Spleen	24,963 ± 679	219.65 ± 132.44
	Brain	11,608 ± 398	73.64 ± 19.59
	Stomach	10,299 ± 671	38,593 ± 14,716
	Kidney	164,319 ± 30,999	248.51 ± 20.71
	Liver	50,685 ± 6,393	155.27 ± 54.14
Asiaticoside	Skin	8,432 ± 448	114.50 ± 12.07
	Spleen	23,885 ± 2,237	53.49 ± 30.39
	Brain	8,465 ± 588	20.52 ± 2.95
	Stomach	6,954 ± 246	19,292 ± 3,908
	Kidney	88,154 ± 20,979	37.00 ± 3.68
	Liver	33,381 ± 3,060	19.99 ± 7.76

Data are shown as the mean ± S.D. (n = 3).

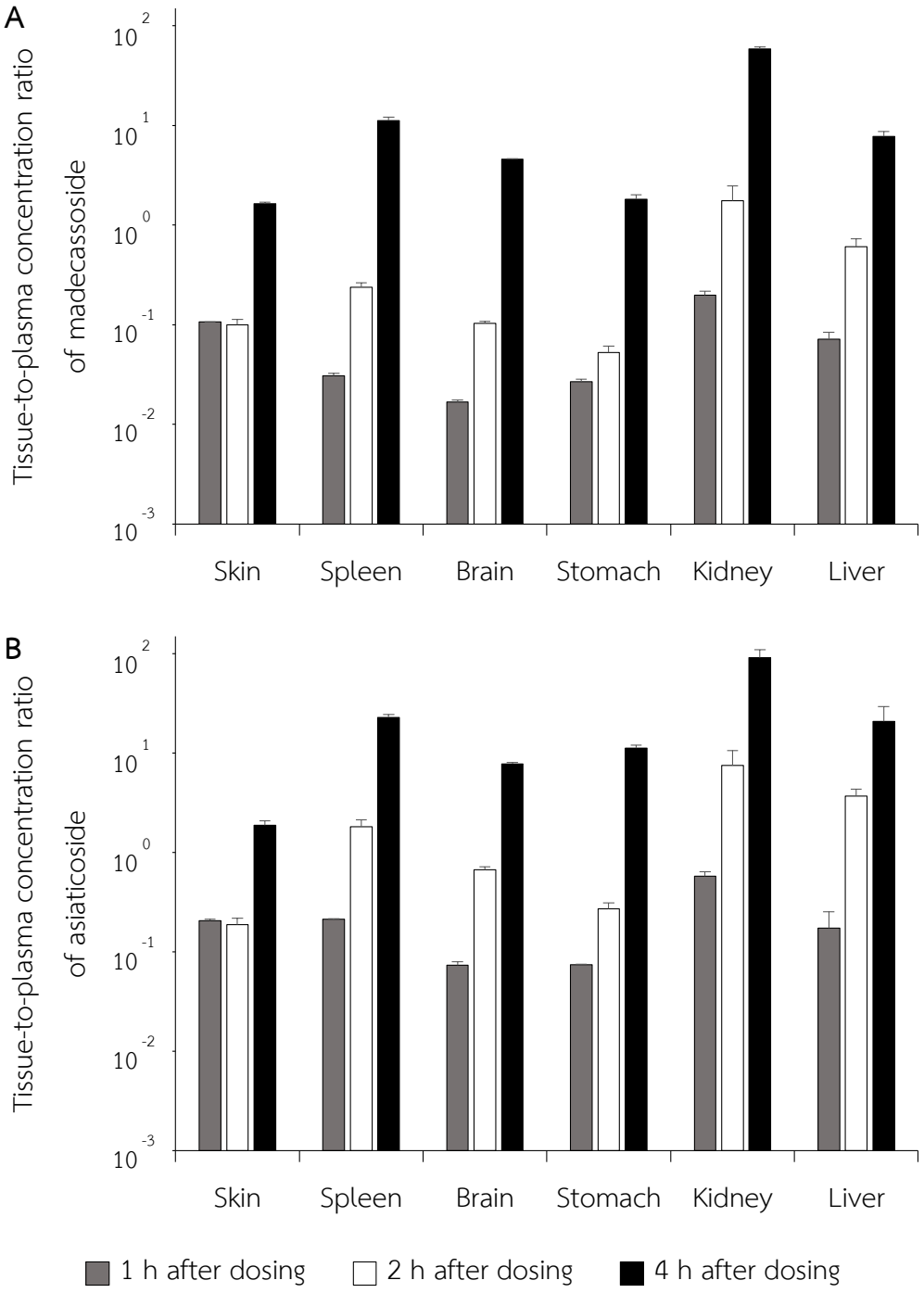


Figure 9 Tissue-to-plasma concentration ratio of madecassoside **(A)** and asiaticoside **(B)** in internal organs after intravenous injection of 50 mg/kg ECa 233. The results are shown as the mean ± S.D. (n = 3).

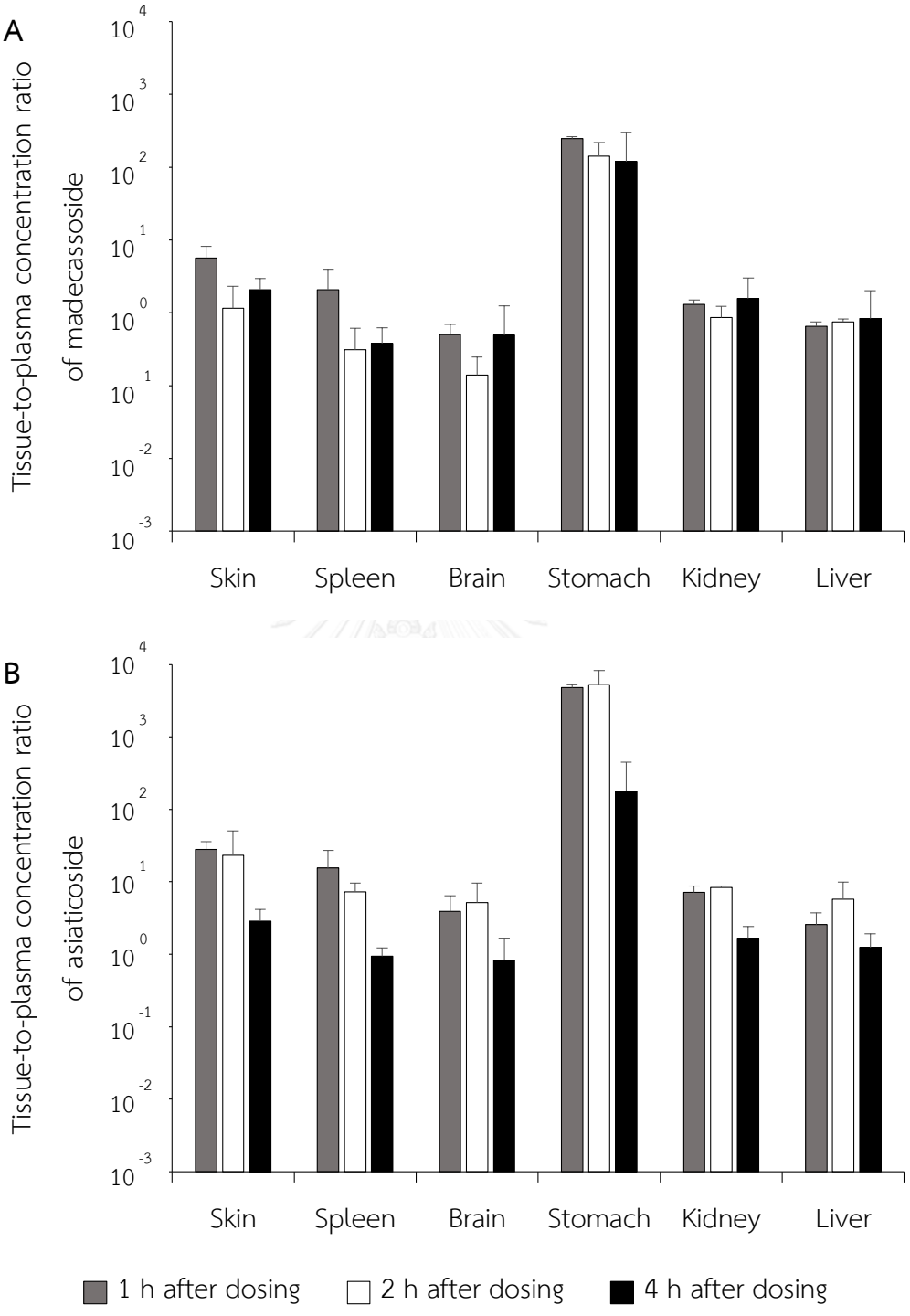


Figure 10 Tissue-to-plasma concentration ratios of madecassoside **(A)** and asiaticoside **(B)** in internal organs after oral administration of 100 mg/kg ECa 233. The results are shown as the mean ± S.D. (n = 3).

4.5 Metabolism

The triterpenic acid metabolites, madecassic acid and asiatic acid (Figure 4), were detected at mass-to-charge ratios of 503/219 and 487/379, respectively. These metabolites were confirmed by the fragmentation patterns of standard chemicals and were later quantified in all of the biological samples by LC-MS/MS (Table 6). These triterpenic acids were barely detected in any plasma or tissue samples, except that low concentrations of madecassic acid and asiatic acid were detected in plasma 5 min only after intravenous administration of ECa 233. However, madecassic and asiatic acid were found in feces (0-48 h) after the administration of ECa 233 by intravenous and oral routes.

For glucuronide conjugate determination, the best condition validated by curcumin glucuronide was using 500 units of β-glucuronidase from *E. coli* and 30 min for incubation time at 37 °C prior to processing for protein precipitation. However, we could not determine the concentrations of any glucuronide metabolites in any biological samples after hydrolytic processes with β-glucuronidase, excluding glucuronidation as a metabolic pathway of madecassoside and asiaticoside in ECa 233.

4.6 Excretion

After provision of an intravenous dose of ECa 233 of 50 mg/kg, almost all of the madecassoside was excreted via urine, whereas asiaticoside was similarly excreted in both urine and feces in unchanged forms. Their respective metabolites, madecassic acid and asiatic acid, were detected in feces with percentage recoveries of 6.97 ± 6.51% and 16.41 ± 3.75%, respectively. However, when ECa 233 was administered orally, madecassoside seemed to be mainly eliminated in feces as madecassic acid (25.92 ± 16.24%) and as relatively small amounts of madecassoside (approx. 10%). Renal clearance played a minor role in the elimination of madecassoside in orally provided ECa 233. Similarly, asiaticoside in all doses of orally provided ECa 233 was excreted in feces as asiatic acid (20.84 ± 12.44%), with approximately 5-20% as asiaticoside (Table 13).

Table 13 Percent recovery calculated from elimination of the main constituents and major metabolites of ECa 233 during 0-24 h and 24-48 h after dosing.

Percent recovery		50 mg/kg IV		50 mg/kg PO		100 mg/kg PO		200 mg/kg PO	
		0-24 h	24-48 h	0-24 h	24-48 h	0-24 h	24-48 h	0-24 h	24-48 h
Madecassoside	Urine	50.46 ± 6.60	0.63 ± 0.25	Below LLOD	Below LLOD	0.37 ± 0.45	Below LLOD	0.66 ± 0.41	Below LLOD
	Feces	0.11 ± 0.06	0.06 ± 0.04	10.88 ± 21.45	0.09 ± 0.03	0.77 ± 1.04	Below LLOD	6.60 ± 11.83	0.10 ± 0.07
Asiaticoside	Urine	14.75 ± 7.50	0.28 ± 0.37	Below LLOD	Below LLOD	0.18 ± 0.23	Below LLOD	0.25 ± 0.29	Below LLOD
	Feces	14.91 ± 7.40	0.36 ± 0.40	23.04 ± 45.83	0.10 ± 0.05	5.16 ± 8.07	0.16 ± 0.23	17.61 ± 28.33	0.28 ± 0.25
Madecassic acid	Urine	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD
	Feces	4.87 ± 4.85	2.11 ± 1.71	15.59 ± 11.06	26.60 ± 6.19	12.01 ± 8.50	7.48 ± 5.29	5.89 ± 7.29	10.20 ± 4.08
Asiatic acid	Urine	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD	Below LLOD
	Feces	8.52 ± 1.54	7.89 ± 2.92	13.87 ± 5.16	21.16 ± 7.54	9.83 ± 5.22	5.88 ± 3.51	4.65 ± 4.35	7.12 ± 2.86

Data are shown as the mean ± S.D. (n = 4). Below LLOD: below limit of detection.

CHAPTER V

DISCUSSION

Unlike other standardized extracts of *Centella asiatica*, ECa 233 was a natural extract of *C. asiatica* that was prepared by a specially controlled process to contain at least 80% madecassoside and asiaticoside, the natural major phytochemical constituents of this plant [24]. Orally administered ECa 233 demonstrated positive neurological activities in various animal models of anxiety [15] and memory deficits [18, 20]. In agreement with *C. asiatica* being widely consumed as a vegetable, ECa 233 had a very good safety profile in both acute and sub-chronic toxicity in rodents [21]. To study the bioavailability of ECa 233, a clear solution of ECa 233 was prepared using 20% DMSO/NSS. The safety of intravenously and orally administered ECa 233 dissolved in this vehicle was noted in the present study. None of the experimental animals showed any physiologically relevant alterations in their general appearance or biochemical parameters related to either renal or liver function after the administration of ECa 233, indicating the tolerability of the test compound and the vehicle used.

The plasma concentration-time profiles of madecassoside and asiaticoside were analyzed using non-compartmental analysis to obtain pharmacokinetic parameters. C_{max} was achieved within 5-15 min after oral dosing, which was more rapid than the previously reported value of 0.90 ± 0.14 h after dosing with pure madecassoside [23]. The relationships among the three oral doses of ECa 233 and their respective C_{max} values were linear ($R^2 > 0.99$ for madecassoside and $R^2 > 0.94$ for asiaticoside). A small second peak was observed 4 or 8 h after oral dosing and could possibly indicate enterohepatic circulation, as proposed in a disposition study of pure madecassoside in a linked-rat model [37].

The apparent oral bioavailability of both parent compounds was less than 1%, which could be explained by their large molecular sizes and the presence of a sugar moiety in their structures. The fact that madecassoside and asiaticoside have low lipid solubility could restrict their ability to cross the intestinal membrane. In addition, the high volume of distribution of triterpenoid glycosides after orally given ECa 233 which might infer to short residence time in blood circulation could lessen the plasma AUC_{po} values which used in the calculation of the oral bioavailability (($AUC_{po} \div AUC_{iv}$) × 100). On the other hand of *in vivo* explanation, madecassoside was also a substrate for efflux transporters, p-glycoprotein and multidrug-resistant protein 2, which might efflux the absorbed drug back into the gastrointestinal tract [37]. The low oral bioavailability is common phenomenon of the active compounds from natural product, and need further structural modification [38, 39]. Bisphosphonates are well-known drugs which also showed oral bioavailability lower than 1%, but they are commonly used to treat or prevent osteoporosis in a wide varieties of patients [40].

In the tissue distribution study, ECa 233 showed rapid tissue penetration after intravenous injection. Asiaticoside showed more rapid distribution than madecassoside, with a sharper decrease in plasma levels in the distribution phase of the plasma concentration-time profile and a comparatively greater tissue-to-plasma concentration ratio than that exhibited by madecassoside, which correlated well with its higher volume of distribution (0.88 vs 0.32 L/kg). Both parent compounds, either by intravenous or by oral administration, reached various target organs, such as the skin, the stomach and especially the brain, within one hour after dosing and resided in those tissues until the fourth hour of observation. This study provided the first pharmacokinetic evidence demonstrating the presence of madecassoside and asiaticoside, but not madecassic acid or asiatic acid, from ECa 233 in brain tissue. This observation strongly supported our previous findings in animal models of anxiety and memory deficits which showed that these two triterpenoid glycosides, but not their triterpenic acid metabolites, were the bioactive molecules responsible for the effects observed [41].

Madecassic acid and asiatic acid have primarily been suggested to be active metabolites formed by acid hydrolysis or esterase hydrolytic cleavage of the sugar moiety of their parent triterpenoid glycoside [28] in a proposed stepwise process [37]. Such hydrolysis could possibly explain the detection of small amounts of madecassic acid (7 mg/L) and asiatic acid (10 mg/L) exclusively in plasma at 5 min after intravenous injection of ECa 233. As these metabolites accounted for approximately 1% of their respective parent compounds from ECa 233 in plasma, and because the levels of madecassic and asiatic acids in other biological samples collected were below the detection limit, it was suggested that the two triterpenoid glycosides barely underwent the hydrolytic pathway. Considering that 36.0% asiatic acid glucuronide and 31.5% madecassic acid glucuronide were found by another investigator in bile after oral administration of a radiolabeled mixture of asiatic acid, madecassic acid, and asiaticoside using a Desaga radiochromatogram scanner [29], it was not surprising that glucuronide metabolites were not detected in our biological samples, in which the amounts of triterpenic acids were negligible. Our findings indicated that neither plasma hydrolysis nor glucuronide conjugation was a major metabolic pathway of madecassoside or asiaticoside in ECa 233.

Intravenously administered ECa 233 was excreted mainly as unchanged parent glycosides through pathways consistent with the partition coefficients (XlogP) of these compounds. Madecassoside preferably resides in plasma (XlogP -1.2) and was excreted renally, whereas asiaticoside (XlogP 0.1) was detected in both urine and feces at a ratio of 1:1. The primary excretion route after oral administration of ECa 233 occurred via feces within 48 h. In agreement with previous reports [12, 29], madecassic acid and asiatic acid, rather than madecassoside and asiaticoside, were found in significant amounts in feces, suggesting hydrolytic cleavage of the sugar moiety by intestinal esterase or gut microflora, converting triterpenoids, from orally administered ECa 233 or excreted bile, into triterpenic acids that were excreted via feces. A discrepancy in the route of excretion of intravenously or orally administered ECa 233 might reflect

different activities of plasma and intestinal esterases [42], which could play important roles in the metabolic and excretory pathways of the triterpenoid glycosides, the major components of ECa 233.

CHAPTER VI

CONCLUSION

ECa 233 is the standardized extract of *Centella asiatica* containing triterpenoid glycosides not less than 80%, and a ratio of madecassoside to asiaticoside is maintained at 1.5 ± 0.5 : 1. The extract has been tested for interesting pharmacological indications and toxicity studies. This research have been conducted to acquire an essential preclinical pharmacokinetic information in Wistar rats which is needed for further steps of phytopharmaceutical product development.

The single oral dosing pharmacokinetics of ECa 233 could be summarized as shown in purposed metabolic pathway of this extract (Figure 11). Madecassoside and asiaticoside from ECa 233 were rapidly absorbed with low oral bioavailabity of less than 1%; however, the absorbed triterpenoid glycosides could distribute to target internal organs such as brain and skin since 1 h and reside there until 4 h after dosing, correlated with their high volume of distribution. This pharmacokinetic study of ECa 233 is the first evidence to identify madecassoside and asiaticoside as bioactive compounds. Approximately 50% of oral ECa 233 might be metabolized into triterpenic acids, madecassic acid and asiatic acid, via hydrolysis by intestinal esterase or gut microflora and excreted into feces, whereas approximately 20% was also excreted into feces as unchanged forms.

The information obtained from this study is beneficial for further pharmacokinetic evaluation of ECa 233 in repeated dose studies and serves as a guidance for pharmacokinetic study in dogs, monkeys and healthy volunteers leading to an appropriate selection of dosage regimen for clinical evaluation of this standardized extract. Some results also suggested for additional molecular studies in tissue penetration, blood-brain-barrier permeability, and efflux transporters induction.

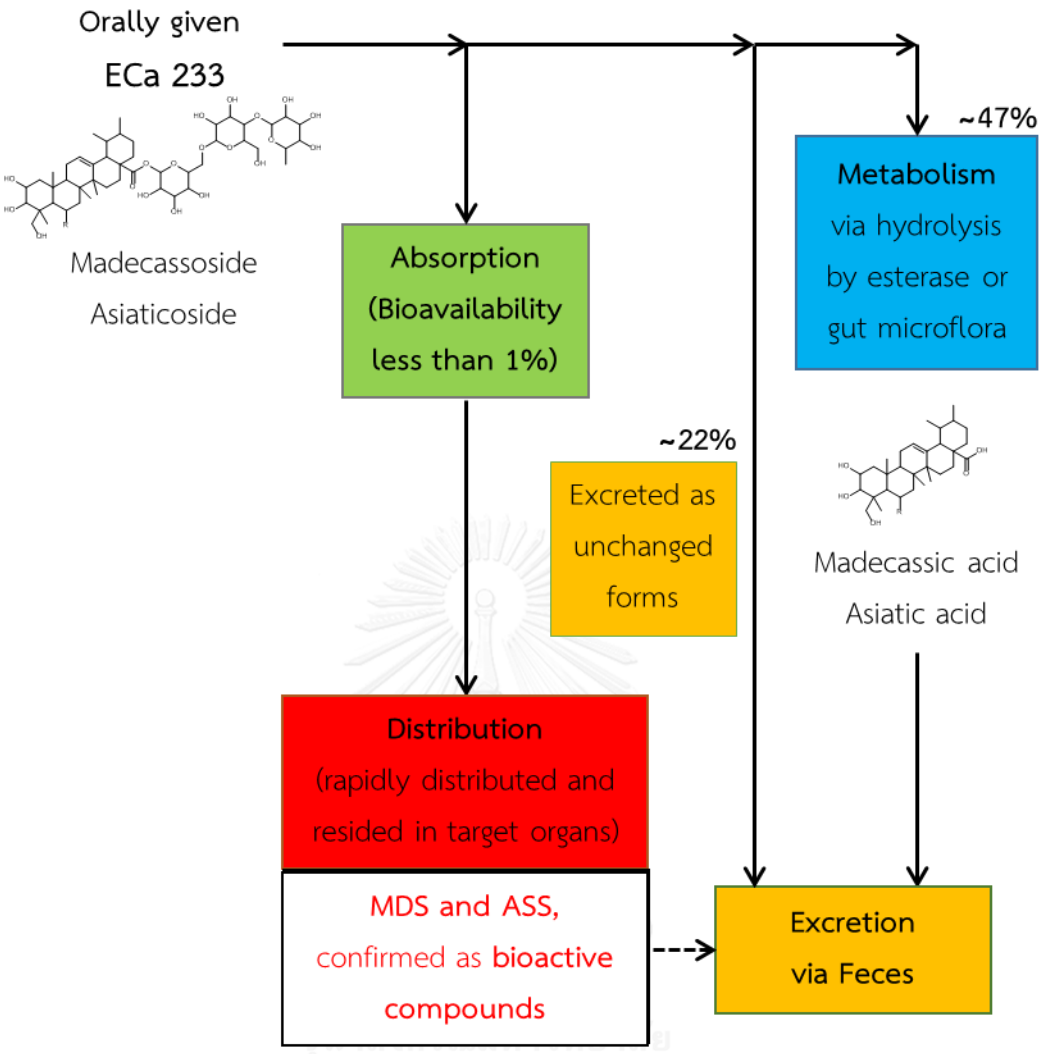


Figure 11 Purposed metabolic pathway of ECa 233.

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APPENDIX

APPENDIX A

Animal use protocol approval

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 13-33-007	**Approval No. 13-33-007**

Protocol Title
Pharmacokinetics study of ECa233 in rodents

Principal Investigator
PHISIT KHEMAWOOT, Ph.D.

Certification of Institutional Animal Care and Use Committee (IACUC)
This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
March 4, 2013	March 4, 2015

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title **NARUEPORN SUTANTHAVIBUL, Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

APPENDIX B

Examples of LC-MS/MS chromatograms from biological samples

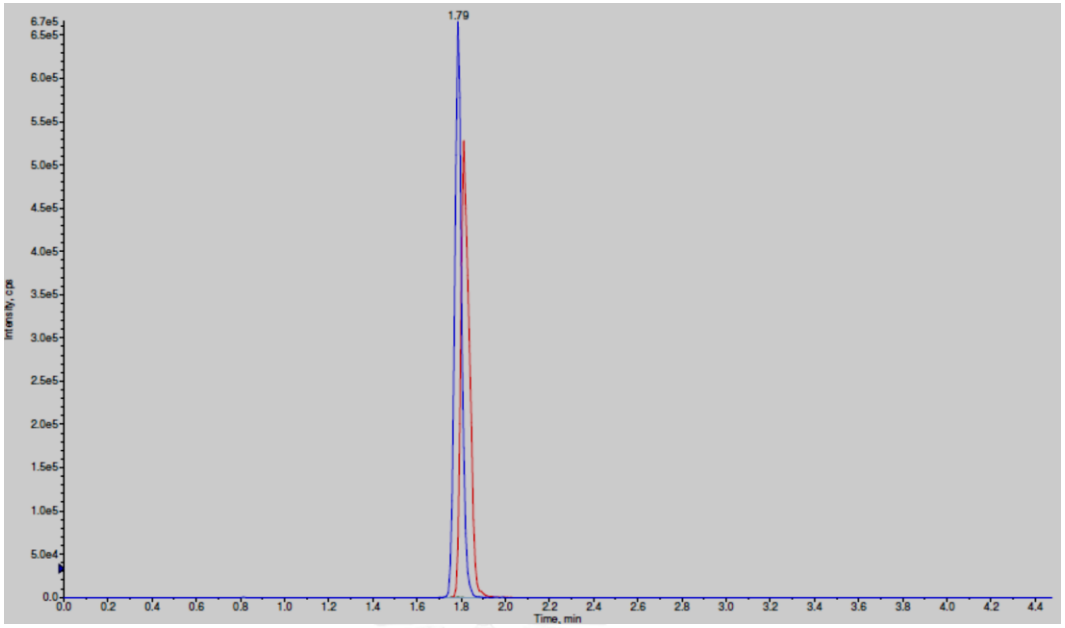


Figure 1B Example of LC-MS/MS chromatogram from plasma sample at 2 h after single intravenous dosing of 50 mg/kg ECa 233.

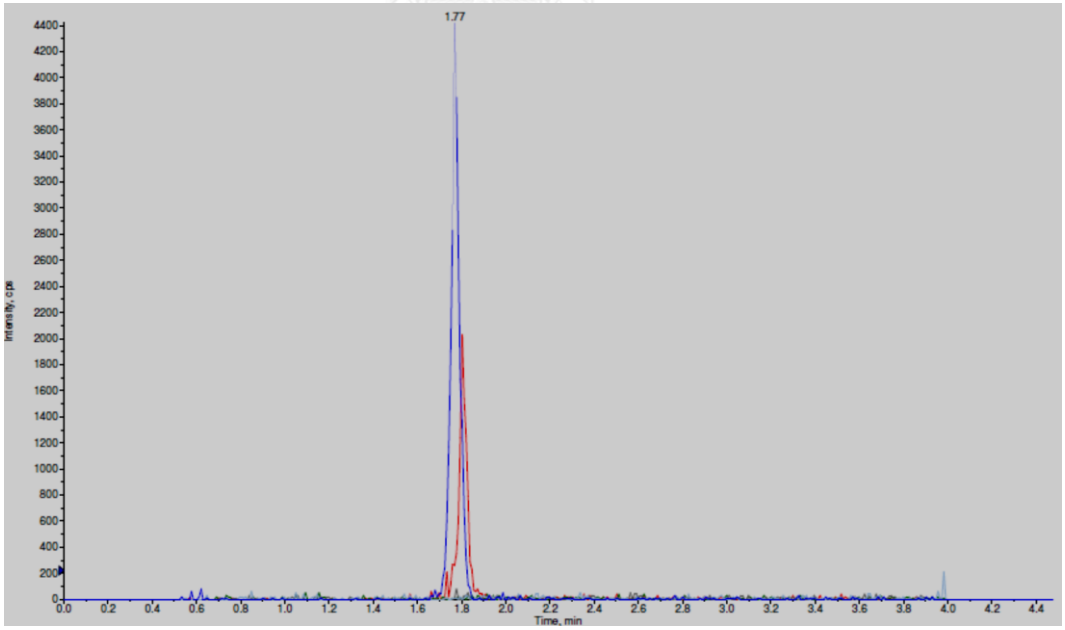


Figure 2B Example of LC-MS/MS chromatogram from skin sample at 1 h after single oral dosing of 100 mg/kg ECa 233.

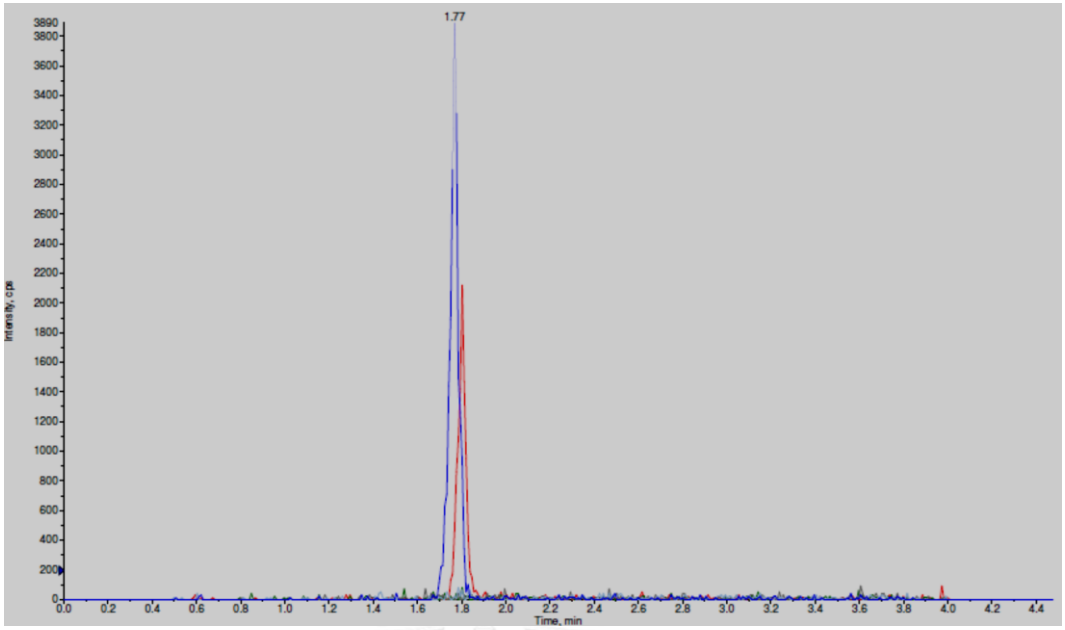


Figure 3B Example of LC-MS/MS chromatogram from spleen sample at 1 h after single oral dosing of 100 mg/kg ECa 233.

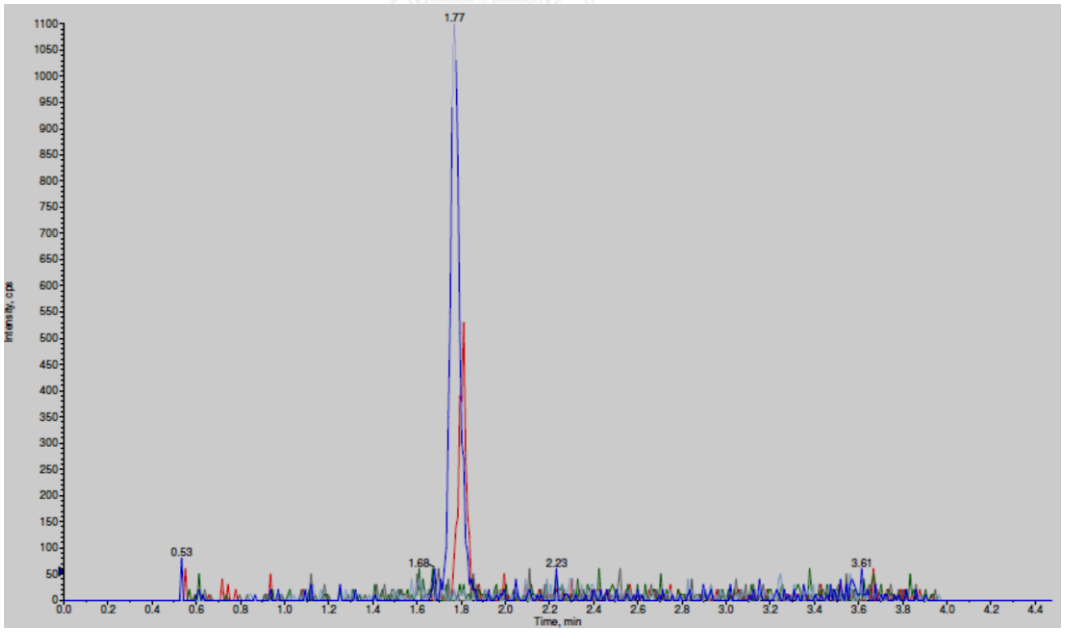


Figure 4B Example of LC-MS/MS chromatogram from brain sample at 1 h after single oral dosing of 100 mg/kg ECa 233.

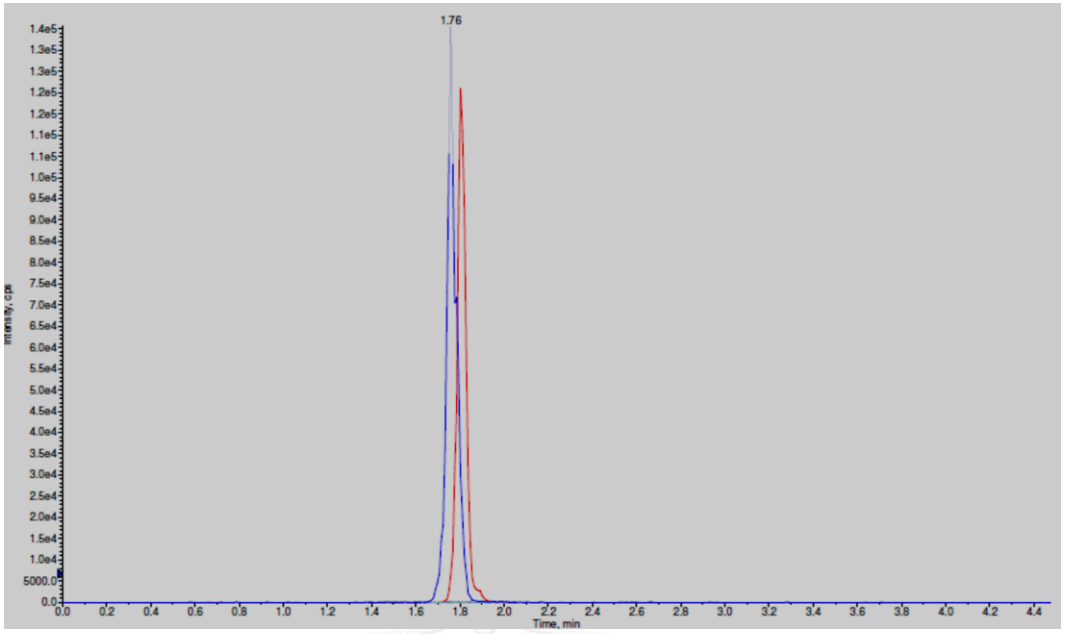


Figure 5B Example of LC-MS/MS chromatogram from stomach sample at 1 h after single oral dosing of 100 mg/kg ECa 233.

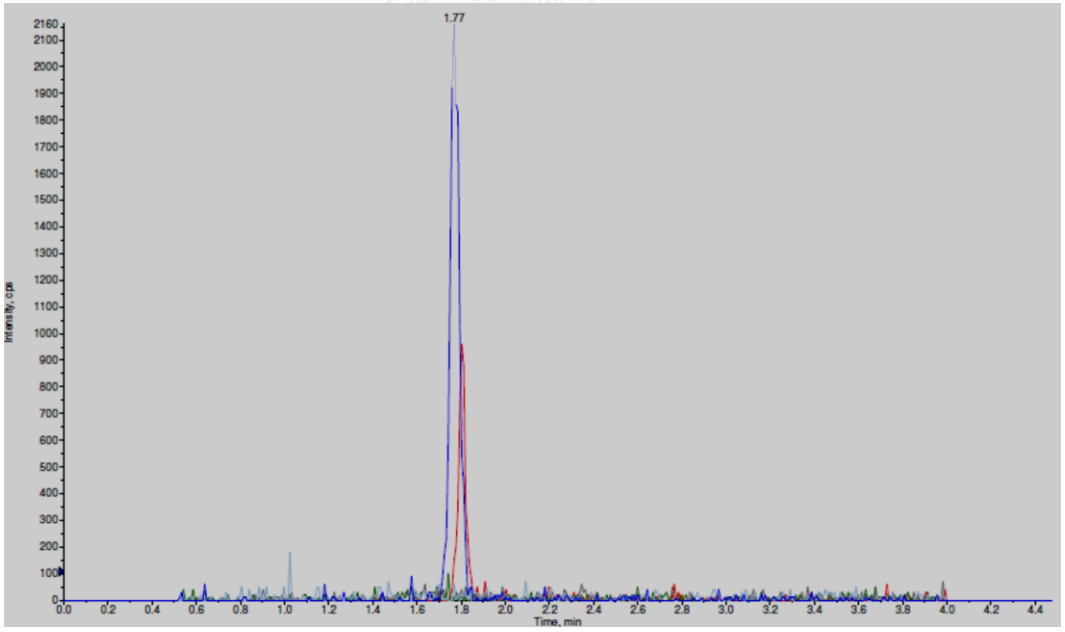


Figure 6B Example of LC-MS/MS chromatogram from kidney sample at 1 h after single oral dosing of 100 mg/kg ECa 233.

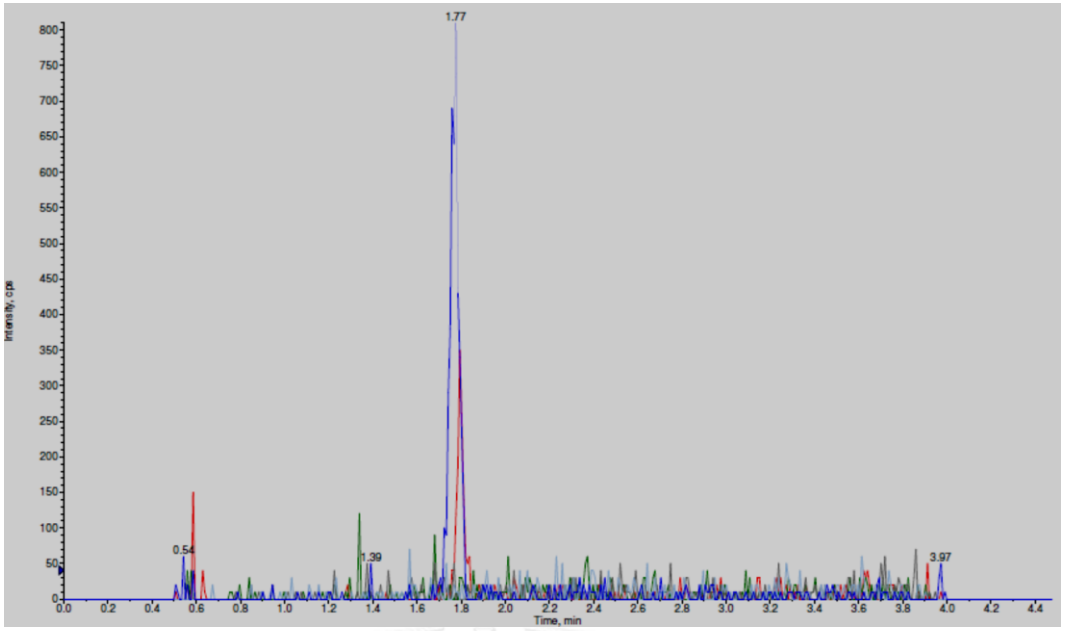


Figure 7B Example of LC-MS/MS chromatogram from liver sample at 1 h after single oral dosing of 100 mg/kg ECa 233.

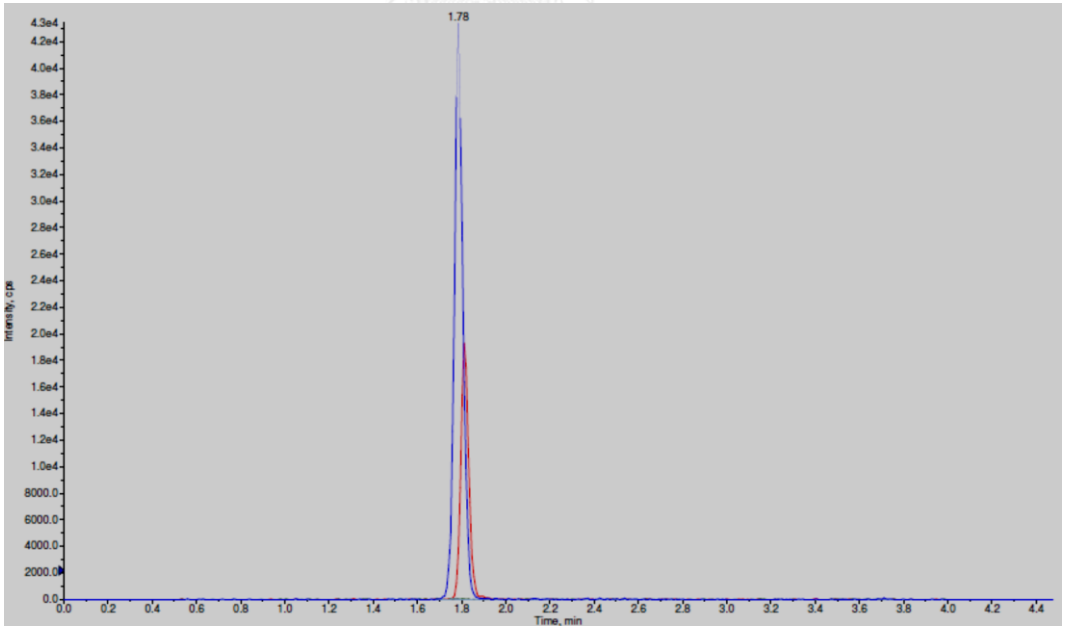


Figure 8B Example of LC-MS/MS chromatogram from urine sample during 0-24 h after single intravenous dosing of 50 mg/kg ECa 233.

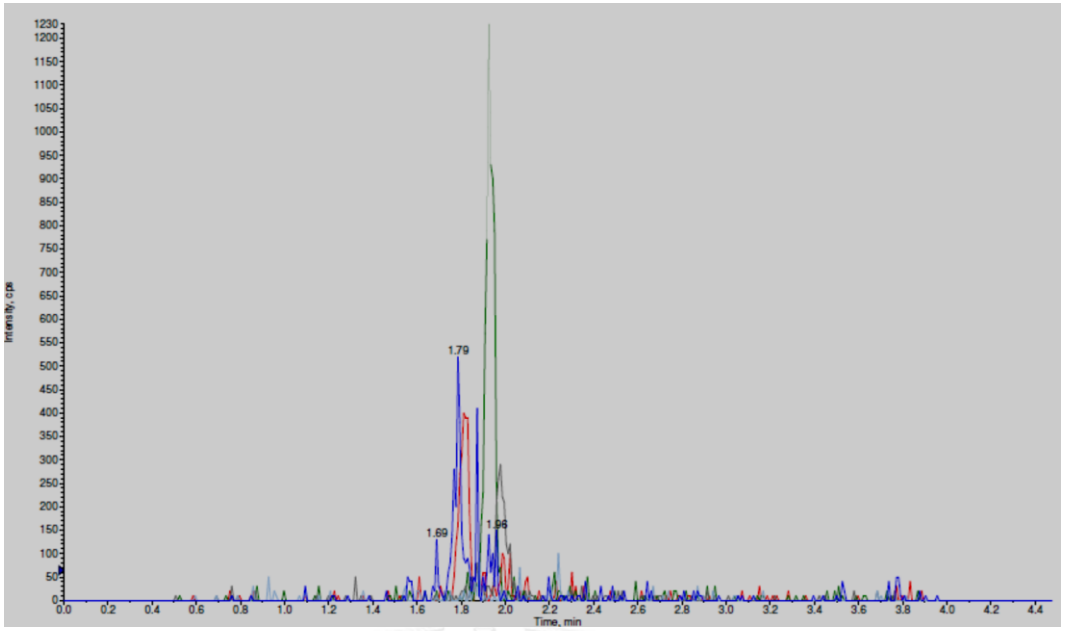


Figure 9B Example of LC-MS/MS chromatogram from feces sample during 24-48 h after single oral dosing of 100 mg/kg ECa 233.

Notes for figure 1B – 9B

1) Retention times in LC-MS/MS chromatograms represented each compounds:
   - Madecassoside at 1.79 min
   - Asiaticoside at 1.82 min
   - Madecassic acid at 1.93 min
   - Asiatic acid at 1.99 min
2) The shift of retention time could be occurred between batches of analysis; however, a retention time shift between ± 0.02 min was acceptable.

APPENDIX C

Tissue concentration

Table 1C Tissue concentration of madecassoside and asiaticoside in internal organs from time 0 to 4 h after intravenous injection of 50 mg/kg ECa 233.

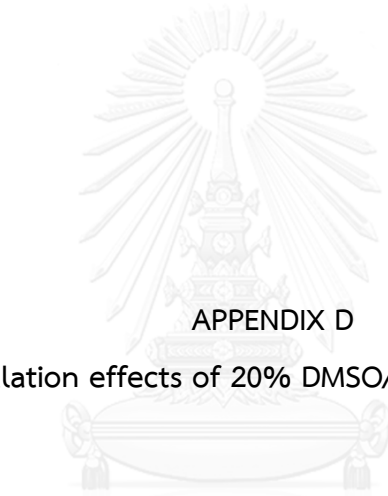
Compounds	Organs	Tissue Concentration (ng/g of tissue)		
		1 h after dosing	2 h after dosing	4 h after dosing
Madecassoside	Skin	27,256 ± 174	3,099 ± 395	891 ± 24
	Spleen	7,867 ± 475	7,357 ± 800	6,060 ± 453
	Brain	4,304 ± 180	3,212 ± 155	2,486 ± 24
	Stomach	6,864 ± 408	1,636 ± 252	981 ± 105
	Kidney	50,578 ± 4,962	54,692 ± 22,227	31,702 ± 1,332
	Liver	18,409 ± 3,152	18,724 ± 3,906	4,191 ± 518
Asiaticoside	Skin	6,813 ± 244	765 ± 119	471 ± 52
	Spleen	7,014 ± 144	7,414 ± 1,267	5,749 ± 390
	Brain	2,430 ± 205	2,717 ± 209	1,960 ± 74
	Stomach	2,470 ± 9	1,100 ± 171	2,834 ± 209
	Kidney	19,092 ± 1,976	30,700 ± 12,199	23,011 ± 4,698
	Liver	5,713 ± 2,705	14,945 ± 2,718	5,251 ± 2,154

Data are shown as the mean ± S.D. (n = 3).

Table 2C Tissue concentration of madecassoside and asiaticoside in internal organs from time 0 to 4 h after oral administration of 100 mg/kg ECa 233.

Compounds	Organs	Tissue Concentration (ng/g of tissue)		
		1 h after dosing	2 h after dosing	4 h after dosing
Madecassoside	Skin	486.45 ± 216.34	70.50 ± 70.11	75.02 ± 32.31
	Spleen	177.61 ± 163.10	18.88 ± 18.31	13.72 ± 8.74
	Brain	43.23 ± 16.54	8.43 ± 6.62	17.76 ± 27.01
	Stomach	21,260 ± 1,534	8,665 ± 4,566	4,336 ± 6,558
	Kidney	113.05 ± 15.28	52.38 ± 22.33	56.89 ± 51.65
	Liver	56.18 ± 8.85	45.84 ± 4.44	30.33 ± 41.92
Asiaticoside	Skin	72.79 ± 19.37	17.08 ± 20.18	16.09 ± 7.24
	Spleen	40.22 ± 29.81	5.34 ± 1.74	5.25 ± 1.52
	Brain	10.13 ± 6.57	3.83 ± 3.29	4.65 ± 4.69
	Stomach	12,459 ± 1,312	3,895 ± 2,232	992 ± 1,520
	Kidney	18.52 ± 4.07	6.14 ± 0.28	9.27 ± 4.23
	Liver	6.66 ± 3.01	4.27 ± 3.05	6.92 ± 3.81

Data are shown as the mean ± S.D. (n = 3).



APPENDIX D

Formulation effects of 20% DMSO/NSS vs 0.5% CMC

The formulation used in this research was 20% DMSO/NSS as we needed to obtain a clear solution of 50 mg/mL ECa 233 for intravenous dosing; however, the previous pharmacodynamic experiments of ECa 233 were formulated in 0.5% carboxymethyl cellulose (CMC). To clarify the formulation effects on pharmacokinetics of ECa 233, some preliminary results from 'Food and Formulation Effects on Pharmacokinetics of ECa 233' study were provided to demonstrate the similarity of AUC and tissue distribution of madecassoside and asiaticoside in ECa 233 using 0.5% CMC and 20% DMSO/NSS as vehicle.

Table 1D Pharmacokinetic parameters of orally 100 mg/kg ECa 233 with different formulations in rats.

Compounds	Pharmacokinetic Parameters	Formulation for preparing ECa 233	
		0.5% CMC	20% DMSO/NSS
Madecassoside	$AUC_{(0-24)}$ (μg·h/L)	8,250 ± 3,305	8,630 ± 2,529
Asiaticoside	$AUC_{(0-24)}$ (μg·h/L)	2,938 ± 1,177	2,683 ± 786

Data are shown as the mean ± S.D. (n = 3).

Table 2D Tissue concentration at 1 h after orally 100 mg/kg ECa 233 dosing with different formulations.

Compounds	Tissue concentration (ng/g of tissue)	Formulation for preparing ECa 233	
		0.5% CMC	20% DMSO/NSS
Madecassoside	Brain	47.62 ± 15.92	49.85 ± 18.84
Asiaticoside	Brain	22.81 ± 7.63	24.92 ± 9.42

Data are shown as the mean ± S.D. (n = 3).

APPENDIX E

Optimization of glucuronidase assay

Using curcumin glucuronide as a positive control, the condition for the reaction was first needed to study by varying enzyme units (100 and 500 units) and incubation time at 37°C (15, 30, 60 and 90 min) to acquire the optimal conditions used for all samples.

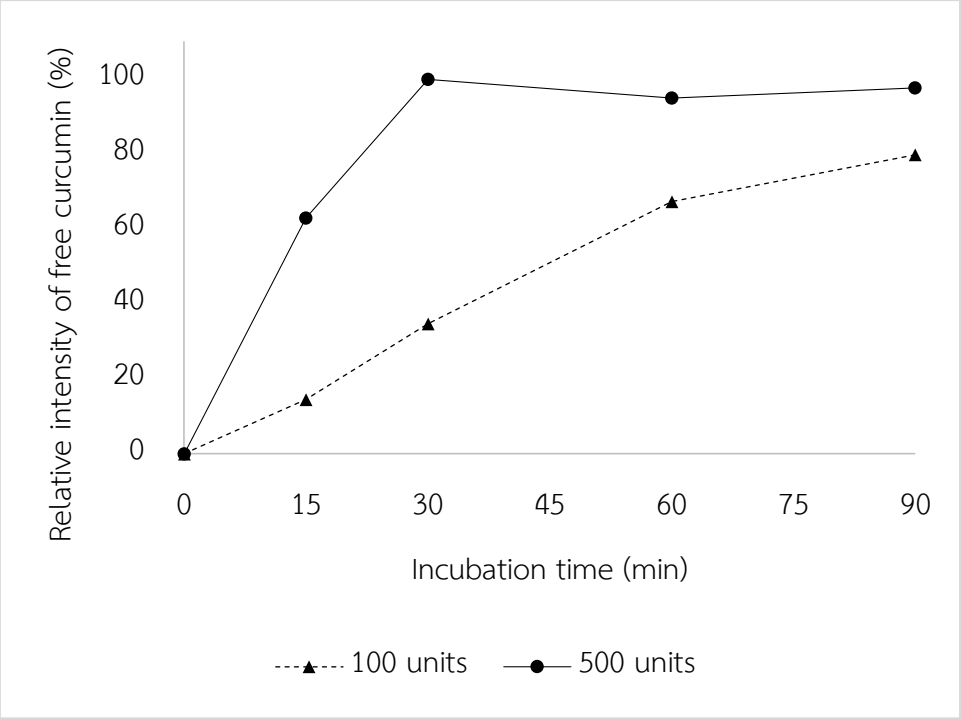


Figure 1E Relative intensity of free curcumin after incubating the same curcumin glucuronide sample with various conditions of β-glucuronidase.

For glucuronide conjugate determination, the best condition validated by curcumin glucuronide was using 500 units of β-glucuronidase from *E. coli* and 30 min for incubation time at 37 °C prior to processing for protein precipitation.

VITA

Mr. Tosapol Anukunwithaya was born on September 7, 1987, in Bangkok, Thailand. He finished from Mahidol Wittayanusorn School, the first science high school of Thailand in 2005. After that, he got a scholarship for undergraduate study in the Young Scientist and Technologist Program (YSTP) from the National Science and Technology Development Agency (NSTDA) to attend a Bachelor of Science degree program at the Department of Chemistry, Faculty of Science, Mahidol University. In 2009, he joined the Medical Molecular Biology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC) as a research assistant. His responsibility was on the Project of Antimalarial Drug Development in Protein-Ligand Engineering and Molecular Biology Laboratory. He has continued his doctoral study in the Ph.D. Program of Biopharmaceutical Sciences at the Faculty of Pharmaceutical Sciences, Chulalongkorn University since 2012.