การศึกษาเปรียบเทียบเภสัชจลนศาสตร์ของสารเอเชียติโคไซด์และมาดีแคสโซไซด์ในรูปสารบริสุทธิ์
และในรูปของผสมในสารสกัดมาตรฐานบัวบกอีซีเอ 233 ในหนูแรท

นางสาวพัชราภรณ์ เฮงจำรัส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยาและพิษวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

COMPARATIVE PHARMACOKINETIC STUDY OF ASIATICOSIDE AND MADECASSOSIDE AS THE PURE COMPOUNDS AND MIXTURE IN THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA*, ECA 233 IN RATS

Miss Patcharaporn Hengjumrut

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology and Toxicology
Department of Pharmacology and Physiology
Faculty of Pharmaceutical Sciences
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Academic Year 2016

Thesis Title	COMPARATIVE PHARMACOKINETIC STUDY OF ASIATICOSIDE AND MADECASSOSIDE AS THE PURE COMPOUNDS AND MIXTURE IN THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA*, ECA 233 IN RATS
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พัชราภรณ์ เฮงจำรัส : การศึกษาเปรียบเทียบเภสัชจลนศาสตร์ของสารเอเชียติโคไซด์และมาดีแคสโซไซด์ในรูปสารบริสุทธิ์และในรูปของผสมในสารสกัดมาตรฐานบัวบกอีซีเอ 233 ในหนูแรท (COMPARATIVE PHARMACOKINETIC STUDY OF ASIATICOSIDE AND MADECASSOSIDE AS THE PURE COMPOUNDS AND MIXTURE IN THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA*, ECA 233 IN RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภก. ดร.พิสิฐ เขมาวุฒฒ์, 70 หน้า.

สารสกัดมาตรฐานบัวบกอีซีเอ 233 ประกอบด้วยสารสำคัญในกลุ่มไตรเทอร์พีนอยด์ไกลโคไซด์ไม่น้อยกว่า 80% โดยมีสารมาดีแคสโซไซด์ (Madecassoside) และเอเชียติโคไซด์ (Asiaticoside) ในอัตราส่วนคงที่คือ 1.5 (± 0.5): 1 ในการศึกษานี้ทำการศึกษาเปรียบเทียบเภสัชจลนศาสตร์ของสารมาดีแคสโซไซด์และเอเชียติโคไซด์ เมื่อบริหารโดยการฉีดเข้าทางหลอดเลือดดำและการป้อนทางปากในรูปของผสมในสารสกัดมาตรฐานบัวบกอีซีเอ 233 หรือในรูปสารบริสุทธิ์ซึ่งมีปริมาณสารสำคัญสมมูลกัน จากนั้นเก็บตัวอย่างเลือด เนื้อเยื่อ ปัสสาวะ และอุจจาระภายหลังได้รับสาร เพื่อวัดระดับสารสำคัญในร่างกาย โดยใช้เทคนิค Liquid chromatography-tandem mass spectrometry จากการศึกษาพบว่าเมื่อให้สารในรูปของสารสกัดมาตรฐานบัวบกอีซีเอ 233 ระดับมาดีแคสโซไซด์และเอเชียติโคไซด์ในเลือดมีค่าเพิ่มสูงขึ้นเมื่อเปรียบเทียบกับการให้ในรูปสารเดี่ยว ค่าครึ่งชีวิตของสารทั้งสองชนิดและค่าเฉลี่ยของเวลาคงอยู่ของสารเอเชียติโคไซด์ในร่างกายยาวนานขึ้นเมื่อให้ในรูปสารสกัดมาตรฐานบัวบกอีซีเอ 233 โดยการป้อนทางปาก (12.79 vs 7.20 ชั่วโมง) นอกจากนั้น พบการเปลี่ยนแปลงแบบผันกลับได้ระหว่างมาดีแคสโซไซด์และเอเชียติโคไซด์ในร่างกาย ซึ่งเป็นผลให้ระดับสารทั้งสองในพลาสมาเพิ่มสูงขึ้นและสามารถคงอยู่ได้ยาวนานขึ้นเมื่อให้สารในรูปของสารสกัดมาตรฐานบัวบกอีซีเอ 233 จากการศึกษาการกระจายตัวในเนื้อเยื่อ พบว่า สารทั้งสองชนิดสามารถแพร่กระจายไปยังอวัยวะต่างๆได้ดี เอเชียติโคไซด์และมาดีแคสโซไซด์มีการเปลี่ยนแปลงอย่างรวดเร็วภายหลังการให้สารโดยฉีดเข้าทางหลอดเลือดดำ พบว่าประมาณ 80-90% ของขนาดสารที่ให้ถูกขับออกผ่านทางอุจจาระพบในรูปมาดีแคสซิค แอซิด (Madecasssic acid) และเอเชียติค แอซิด (Asiatic acid)

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยาและพิษวิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5776120233 : MAJOR PHARMACOLOGY AND TOXICOLOGY
KEYWORDS: ECA 233 / MADECASSOSIDE / ASIATICOSIDE / CENTELLA ASIATICA

PATCHARAPORN HENGJUMRUT: COMPARATIVE PHARMACOKINETIC STUDY OF ASIATICOSIDE AND MADECASSOSIDE AS THE PURE COMPOUNDS AND MIXTURE IN THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA*, ECA 233 IN RATS. ADVISOR: ASST. PROF. PHISIT KHEMAWOOT, Ph.D., 70 pp.

ECa 233, the standardized extract of *Centella asiatica,* contains not less than 80% triterpenoid glycosides, in a madecassoside:asiaticoside ratio of 1.5 (± 0.5): 1. In this study, we performed a pharmacokinetic comparison of madecassoside and asiaticoside following intravenous and oral administration of ECa 233, or an equivalent dose of the individual compounds, in rats. Blood, tissues, urine and feces were collected after dosing for the determination of drug and metabolite levels using liquid chromatography-tandem mass spectrometry. The results revealed that plasma levels of madecassoside, and to a lesser extent asiaticoside, were higher after administration of ECa 233 than the corresponding values for the pure compounds. The elimination half-life of madecassoside and asiaticoside were significantly prolonged after administration of ECa 233 ($p < 0.05$). The mean resident time of asiaticoside appeared to be prolonged following the oral administration of ECa 233 compared with following administration of the pure asiaticoside (12.79 vs 7.20 h). Interestingly, there was a bidirectional interconversion between asiaticoside and madecassoside consistent with the increased exposure of madecassoside and asiaticoside in ECa 233. In tissue distribution studies, asiaticoside and madecassoside appeared to be widely distributed in several organs. Both madecassoside and asiaticoside were metabolized extensively; following intravenous administration of either compound, approximately 80-90% of the dose was recovered as madecasssic acid and asiatic acid in the feces.

Department:	Pharmacology and Physiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Pharmacology and Toxicology	
Academic Year:	2016	

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
ASS	asiaticoside
AST	aspartate aminotranferase
dL	deciliter
DMSO	dimethylsulfoxide
ESI	electrospray ionization
g	gram
h	hour
HPLC	high-performance liquid chromatography
IV	intravenous
kg	kilogram
LC/MS/MS	liquid chromatography tamdem mass spectrometry
LLOQ	lower limit of quantitation
MDS	madecassoside
mg	milligram
min	minute
mL	milliliter
ng	nanogram
QC	quality control
µg	microgram

CHAPTER I

INTRODUCTION

Background and rationale

Centella asiatica (L.) urban, a tropical plant in the Apiaceae family, has been considered a traditional herb for hundreds of years. Its predominant pharmacological effects are thought to include wound healing, memory improvement, and anti-inflammatory, anticancer, antidepressant and antimicrobial activities [1-3]. The most prominent active compounds of *C. asiatica* are the triterpenoid glycosides, madecassoside and asiaticoside. These two triterpenoid glycosides are recognized as biomarker components and recorded for wide varieties of pharmacological activities [4, 5]. To avoid variation in the compositions of bioactive constituents, the researchers from the Faculty of Pharmaceutical Sciences, Chulalongkorn University have developed a standardized extract of *C. asiatica* known as ECa 233 [6]. ECa 233 contains not less than 80% triterpenoid glycosides and the ratio of madecassoside and asiaticoside is constant at 1.5 ($\pm$ 0.5): 1. The extract has been shown to have wound healing [6], neuritogenic [7] and anxiolytic effects in mice after administration of doses ranging from 10-300 mg/kg [8]. ECa 233 has also been found to enhance learning and memory in rats [9]. Toxicity studies of ECa 233 in rats and mice showed that the standardized extract could be orally administered up to 10 g/kg without acute toxicity [10].

In earlier studies, the distribution kinetics and metabolism of asiaticoside and madecassoside were investigated in rats [11, 12]. After intravenous administration of 40 mg/kg asiaticoside, the result showed maximum plasma concentrations and $AUC_{0-\infty}$ at 3,347 ± 786 ng/mL and 81,904 ± 57,112 ng.h/mL, respectively. Meanwhile, oral administration of 100 mg/kg madecassoside exhibited maximum plasma levels and $AUC_{0-\infty}$ at 303.75 ± 28.53 ng/mL and 1,458.74 ± 202.99 ng.h/mL, respectively. The elimination half-life of madecassoside was 3.47 h. The major metabolic pathways of madecassoside and asiaticoside have been proposed to be madecassic acid and asiatic

acid resulted from cleavage of a sugar moiety [13, 14]. Furthermore, the pharmacokinetics of madecassoside and asiaticoside after administration of water-soluble titrated extract of *C. asiatica*, TECA (41.6% madecassoside, 46.3% asiaticoside, 3.0% madecassic acid, and 1.7% asiatic acid) has been reported. The result demonstrated that the absorption of madecassoside and asiaticoside were very low with oral bioavailability less than 6.0%. Oral administration of 20 mg/kg TECA could reach maximum concentration of madecassoside within 0.5 h after dosing. After intravenous administration 20 mg/kg TECA, terminal half-life of madecassoside and asiaticoside were estimated to be 0.23 and 0.16 h, respectively [15].

In recent years, there have been several reports of interactions among components present in extracts, resulting in pharmacokinetic and pharmacodynamic alterations in major bioactive compounds [16-22]. Interestingly, the *C. asiatica* extract showed superiority in healing gastric ulcers [21] and anxiolytic effect [8, 22] compared to when asiaticoside was administered as a pure compound. It is possible that the multiple-components present in this extract demonstrated synergistic therapeutic actions [23]. However, it was unclear whether this results from pharmacokinetic or pharmacodynamic interactions.

In light of the above, the present study was designed to compare the pharmacokinetic behaviors of asiaticoside and madecassoside after administration to male Wistar rats as either pure compounds or in the standardized extract of *C.asiatica,* ECa 233. There is possibility that existence of two compounds in one standardized extract might have the synergistic effect due to pharmacokinetic interactions. Enhancements of oral bioavailability, increment of exposure or bidirectional interconversion need to be elucidated. In addition, measurement of proposed active metabolite, madecassic acid and asiatic acid should be explored in all biological samples by highly specificity and sensitivity method, LC-MS/MS. It is expected that the results obtained from this study will provide valuable information for further drug development research involving *C. asiatica* and ECa 233.

Objectives

To compare the pharmacokinetic parameters and bioavailability, distribution and excretion between asiaticoside and madecassoside when administered as a pure compound and their respective counterparts existing in standardized extract of *Centella asiatica,* ECa 233.

Hypothesis

1. Combination of madecassoside and asiaticoside in ECa 233 has synergistic effect on pharmacokinetic profiles compared with madecassoside and asiaticoside alone.
2. Pharmacokinetic interactions between madecassaside and asiaticoside in ECa 233 have an effect on pharmacokinetic parameter, bioavailability, tissue distribution and excretion of madecassoside and asiaticoside.

Expected benefits from the study

The results from this research including an evaluation of pharmacokinetics parameters, oral bioavailability, tissue distribution and excretion of asiaticoside and madecassoside when administered to the rats as a pure compound or ECa 233. These information can be used to supports their pharmacological studies, toxicological properties and further development of ECa 233 as phytopharmaceutical product of Thailand.

CHAPTER II

LITERATURE REVIEW

Centella Asiatica

C. asiatica (L.) Urban (Figure 1), a tropical plant in the Apiaceae family, which grows abundantly in the warmer regions including Southeast Asia, China, India, Australia and Africa. The other common names of *C. asiatica* are Gotu kola, Indian Pennywort or Asiatic Pennywort (English), and Bua-bok in Thai [3].

This plant is creeping, aromatic herb with up to 2 m slender and tender reddish prostate stolon. Leaves are thin and soft, 1-3 arising from each node, long and 1.5-5 cm wide, orbicular, reniform. Flowers occurring in July-September are in fascicled umbels, white or light violet color, covered by bracts and 3-6 flowers are arranged in an umbel. Fruits are small, compressed, 8 mm long, mericarps are curved, rounded at the top, broad and 7-9 ridged [1, 2].

Figure 1 *C. asiatica (L.)* Urban

Chemical constituents

C. asiatica contains several chemical compositions. These chemical constituents can be classified as following [1-5] :

1. **Triterpenes**:
    - 1.1 Asiaticoside
    - 1.2 Madecassoside
    - 1.3 Centelloside
    - 1.4 Asiatic acid
    - 1.5 Madecassic acid
    - 1.6 Centellic acid
    - 1.7 Terminolic acid
    - 1.8 Indocentoic acid
2. **Flavonoids glycosides**:
    - 2.1 Quercetin-3-glucoside
    - 2.2 Kaempferol-3-glucoside
    - 2.3 Kaempferol-7-glucoside
3. **Alkaloids**:
    - 3.1 hydrocotylin
4. **Free amino acids**:
    - 4.1 Alanine and serine (major components)
    - 4.2 Aminobutyrate
    - 4.3 Aspartate
    - 4.4 Glutamate
    - 4.5 Histidine
    - 4.6 Lysine
    - 4.7 Threonine

5. **Volatile and fatty oils**:

5.1 Glyceride of palmitic acid

5.2 Stearic acid

5.3 Oleic acid

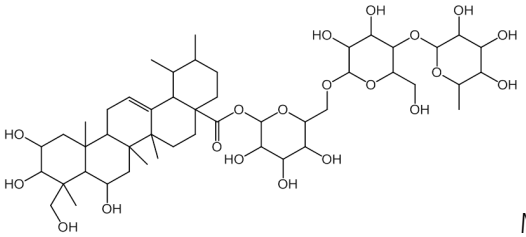
5.4 Linoleic acid

5.5 Linolenic acid

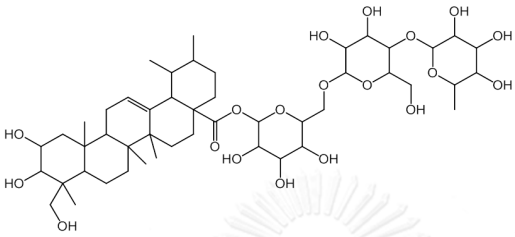
6. Others components: mesoinositol, oligosaccharide, stigmasterol, sitosterol, campesterol, polyacetylenes, carotenoids, vitamin B and vitamin C

The most prominent active compounds of *C. asiatica* are the triterpenoid glycosides, madecassoside and asiaticoside. These two triterpenoid glycosides are recognized as biomarker components and recorded for wide varieties of pharmacological activities [4, 5].

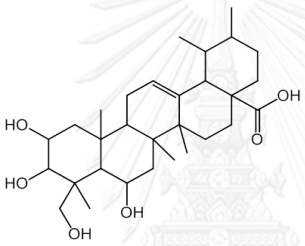
Asiaticoside and madecassoside, pentacyclic triterpenoid saponins from *C. asiatica* are secondary metabolites. The triterpenoid synthesis depend on environmental conditions via isoprenoid pathway. The process involves conjugation of aglycone (hydrophobic structure) with hydrophilic sugar molecule to improve solubility [4]. Moreover, the sugar moiety may be play an important role in pharmacokinetic parameter such as absorption, distribution and bioavailability possible to change the efficacy of aglycone molecules [24] .



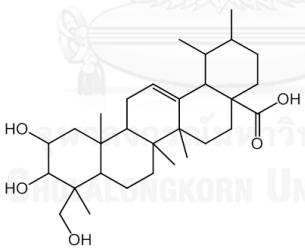
Madecassoside



Asiaticoside



Madecassic acid



Asiatic acid

Figure 2 Chemical structures of major triterpenes in *C. asiatica*.

Medicinal uses and pharmacological effects

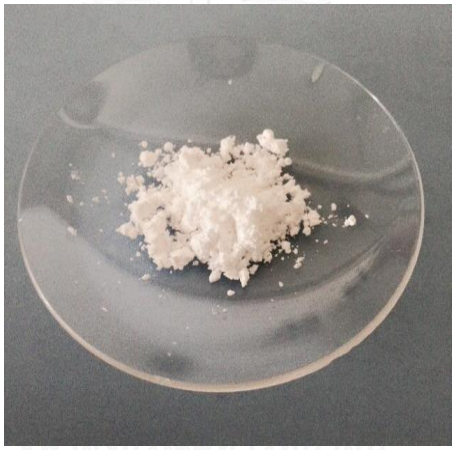
C. asiatica is considered to be a traditional medicinal herb for hundreds of years. This plant has been used for various medicinal proposes including [1-4] :

- Wound healing agent and protective agent for gastric ulcer
- Memory enhancer
- Antioxidant
- Anti-inflammatory and analgesic
- Antidepressant and anxiolytic
- Tranquilizing and sedative
- Antimicrobial such as antibacterial, antifungal, antiviral, antiprotozoa
- Anticancer and immunomodulating activities
- Antileprotic and antitubercular activities
- Neuroprotective agent
- Hepatoprotective agent
- Antispasmodic
- Anticonvulsant
- Anti-hyperglycemic agent

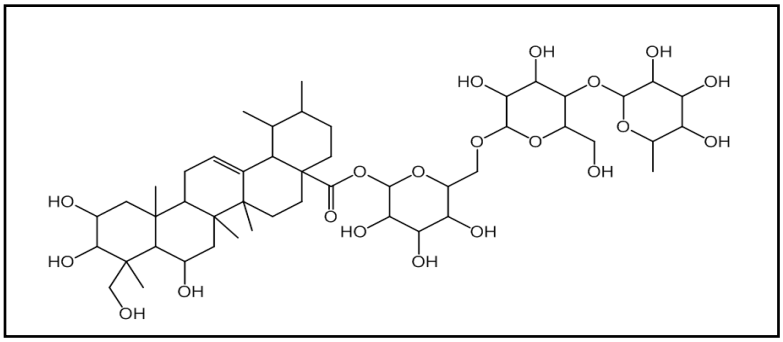
Madecassoside

Madecassoside is a triterpenoids glycoside in *C. asiatica* and the main active ingredient in ECa 233, characterized as white powder with molecular weight 975.12 g/mol and chemical formula is $C_{48}H_{78}O_{20}$.

Many studies indicated that madecassoside possessed various pharmacological effects including anti-inflammatory in rheumatoid, antioxidant effect, increasing collagen synthesis, increasing angiogenesis, preventing scars, wounds healing effect and neuroprotective effect [25-27].



(a)



(b)

Figure 3 Physical appearance (a) and chemical structure of madecassoside (b)

Pharmacological activities

Anti-rheumatoid arthritis effect

Anti-rheumatoid arthritis effect of madecassoside has been investigated in mice. Madecassoside 10, 20 and 40 mg/kg. orally administered for twenty consecutive days. In treated groups, the severity of joint swelling and erythema has reduced with decrease of inflammatory cells [25].

Neuroprotective agent

In field of drug discovery, madecassoside has been a drug candidate for neuroprotective agent. In ischemic-reperfusion injury rat, madecassoside significantly reduced brain damage through decreasing malondialdehyde, nitric oxide and pro-inflammatory cytokine levels [26]. Moreover, the neuroprotective effect of madecassoside has been studied in early stage of Parkinson disease induced by MPTP in rats. Treatment with madecassoside could prevent Parkinsonian sign, improve locomotor dysfunction and protect dopaminergic neuron by antagonizing the effect of MPTP [27].

Anti-inflammatory effect

Anti-inflammatory effect of madecassoside has been investigated in collagen – induced arthritis mice. Madecassoside 3, 10 and 30 mg/kg intragastric administered from days 21 to 42 after immunization. The results demonstrated that joint tissue pathological damage and clinical arthritis score were decreased in madecassoside treated group. Anti-inflammatory effect of madecassoside can be attributed to inhibition of inflammatory mediators such as TNF-α and IL-6 levels [28].

Preventive scar formation

The effect of madecassoside on the formation of keloid scars have been studied in keloid-derived fibroblasts. The cells treated with madecassoside showed decrease F-actin filaments and diminished p-cofilin. The result demonstrated that madecassoside inhibits the migration of keloid-derived fibroblast and could be used in prevention of scar and keloids [29].

Pharmacokinetic studies of madecassoside

The pharmacokinetics of madecassoside was conducted in male Sprague-Dawley rat by oral administration of madecassoside at single dose of 100 mg/kg. Madecassoside exhibited maximum plasma levels after 0.90 ± 0.14 h at 303.75 ± 28.53 ng/mL, half-life approximately 3.47 h, AUC_{0-t} and $AUC_{0-\infty}$ were 1,368.37 ± 90.71 and 1,458.74 ± 202.99 ng.h/mL respectively [12].

According to the study on disposition and metabolism, madecassoside was quickly distributed to heart, liver, spleen, lungs, and kidney of rats after 100 mg/kg orally administered. The levels of madecassoside in kidney and liver were higher than other organs. For elimination study, rats were given a single oral administration of madecassoside after bile duct cannulation. Madecassoside was predominantly excreted in metabolite form and mainly excreted in feces. Madecassoside exhibited in bile as parent form within 12 h was 7.16%. Feces excretion was 24.68% within 54 h while about 0.25% madecassoside was found in urine at 0-72 h. These author proposed the metabolic pathways of madecassoside that sugar moiety of madecassoside was stepwise hydrolysed to obtain M1, M2, M3 or madecassic acid. In addition, coadministration of madecassoside and digoxin (P-glycoprotein inhibitor) or madecassoside and probenecid (MRP2 inhibitor) reduced the excretion of madecassoside in bile, indicating that P-glycoprotein and MRP2 contributed to bile elimination of madecassoside [14].

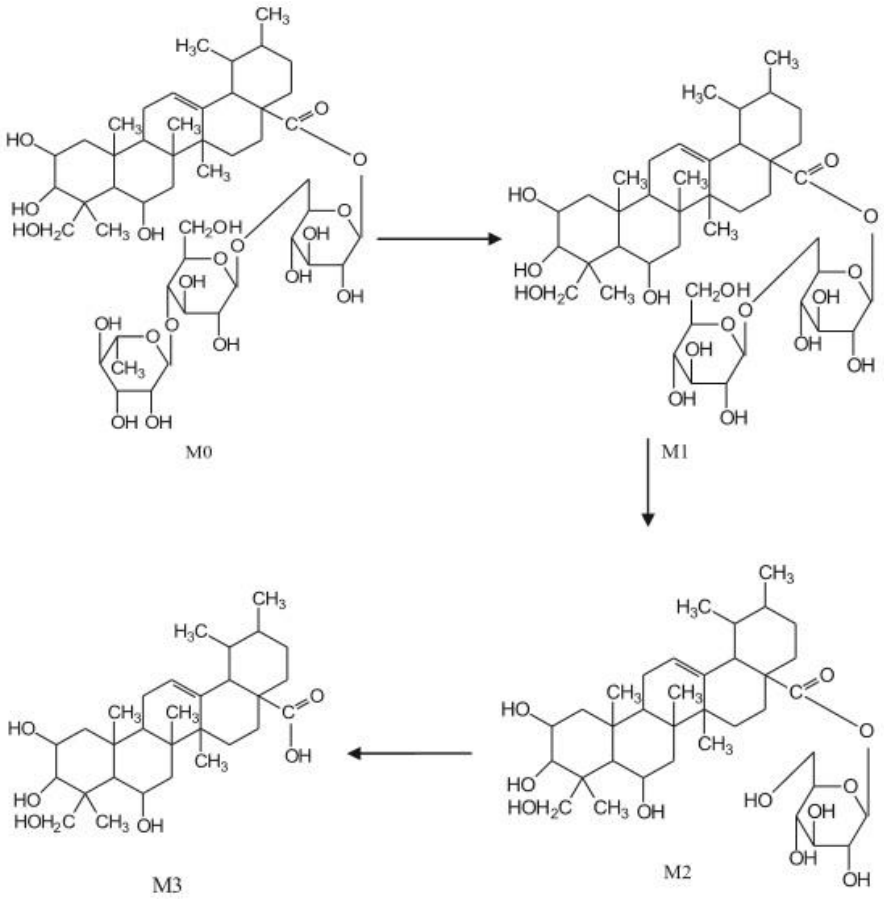
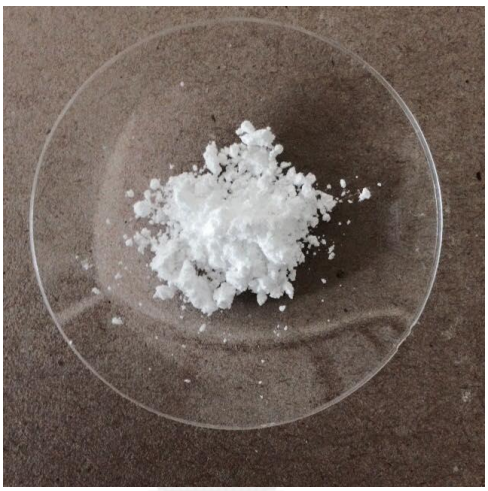


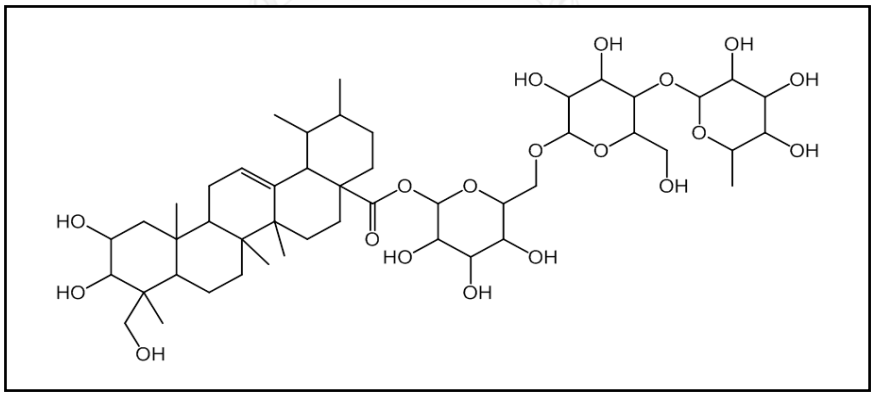
Figure 4 The proposed metabolic pathways of madecassoside in rat. [14]

Asiaticoside

Asiaticoside, a major pentacyclic triterpenoid glycoside component of *C. asiatica* and a main component in ECa 233, is characterized as white powder with molecular weight 959.12 g/mol and chemical formula $C_{48}H_{78}O_{19}$



(a)



(b)

Figure 5 Physical appearance (a) and chemical structure of asiaticoside (b)

Pharmacological activities

Wound healing effect

In vitro study on normal human skin cell, asiaticoside showed a promising wound healing activity and skin regeneration. This study demonstrated that asiaticoside increased the migration rate, improved attachment of skin cells and promoted human dermal fibroblast proliferation. In addition, asiaticoside increased the expression of matrix-metalloproteinase-1 to induce cell adhesiveness in skin repairing process [30].

The wound healing activity of asiaticoside has been studied in normal skin and delayed-type wound of guinea pig. The result demonstrated that topical application of 0.2% asiaticoside solution produced 57% increased in tensile strength of skin, 56% increase in hydroxyproline, increased content of collagen. These study also investigated the effect of asiaticoside in delayed-type wound healing by using streptozotocin diabetic rats. The result showed that topical application of 0.4% asiaticoside solution increased tensile strength, collagen content and epithelisation thereby facilitating the healing. However, asiaticoside was active by oral dosing at 1 mg/kg [31].

Antidepressant effect

In animal model, antidepressant-like action of asiaticoside was investigated in unpredictable chronic mild stress model in mice using clomipramine as positive control. The results showed that 10 mg/kg asiaticoside was equally effective as 50 mg/kg clomipramine [32].

Anxiolytic-like effect

Anxiolytic-like effect of asiaticoside was investigated using a number of experimental paradigms of anxiety in male Swiss mice with positive anxiolytic control, diazepam. The results indicated that 10 mg/kg asiaticoside exhibited anxiolytic-like effect in the elevated plus-maze, light/dark box and hole-board tests same as 0.3 mg/kg diazepam [33].

Anti-inflammatory effect

Asiaticoside has been shown to possess anti-oxidant and anti-inflammatory activities. One study has showed that the survival of septic lung cancer mice and lung pathological damage was significantly increased in 40 mg/kg asiaticoside treated animals for 24 h. Asiaticoside could inhibit mitogen-activated protein kinase (MAPKS) and nuclear factor-KB (NF-KB) which decreased the levels of TNF-α, IL-6 and the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in lung tissue [34].

Pharmacokinetic and toxicity studies of asiaticoside

The pharmacokinetic of asiaticoside was investigated in male Sprague-Dawley rat by intravenous administration of asiaticoside at single dose of 10 mg/kg. Plasma levels of asiaticoside were analyzed by HPLC system. The result showed area under the curve and elimination half life ($T_{1/2}$) of 25.53±4.56 μg.h/mL and 0.26 ± 0.10 h, respectively. The excretion of asiaticoside in bile at 24 h post-dosing was 81.4±1.6% [35].

The metabolic pathway of asiaticoside was proposed in previous pharmacokinetic study of total triterpenic fraction of *Centella asiatica* (asiatic acid and madecassic acids 60% and asiaticoside 40%) in healthy volunteers that asiaticoside could be converted to asiatic acid *in vivo* [13].

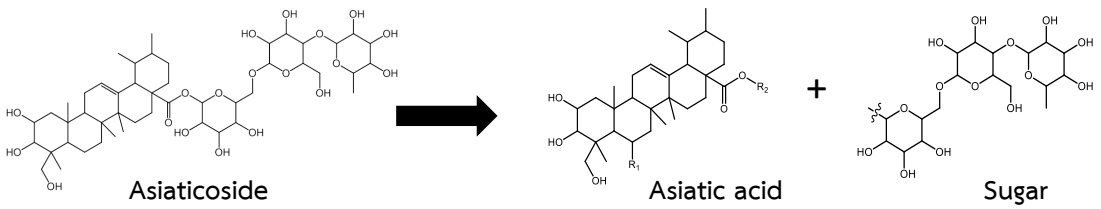


Figure 6 The proposed metabolic pathways of asiaticoside.

In another metabolism study, the effect of asiaticoside and madecassoside on human cytochrome P450 enzyme were studied by median inhibitory concentration (IC_{50}) measurement. It was reported that asiaticoside and madecassoside had no effect on CYP1A2, CYP2C9, CYP2D6 and CYP2E1 but exhibited non-competitive inhibitor of CYP2C19 and CYP3A4 [36].

Toxicity studies of asiaticoside [37]

Asiaticoside was tested for acute toxicity in Swiss albino rats by oral administration at doses of 300-5,000 mg/kg. The animals were observed after 1 h and after 14 days of administration for mortality and morbidity. Only animals treated group with 4,000-5,000 mg/kg of asiaticoside shown decreased body weight. It was also reported that the weighs of kidney decreased and the weights of liver weight increased significantly. They found the median lethal dose (LD_{50}) was greater than 2,000 mg/kg.

The subchronic toxicity of asiaticoside was examined in rats by repeated dose of 200, 500 and 1,000 mg/kg for 90 days. No sign of toxicity were observed at all given dose. The result revealed that at dose 1,000 mg/kg, the animals had significant decreased body weight. The statistically significant elevated hepatic enzyme and increase in the weight of liver were observed. The histopathological observation of liver sections showed sign of inflammation in liver with non-significant change in architecture of the cell.

ECa 233

ECa 233 is the standardized extract of *C. asiatica*, prepared and developed from a group of researchers from the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The extract characterized as white to off-white powder containing triterpenoid glycosides not less than 80% and the ratio between madecassoside and asiaticoside within 1.5 ± 0.5 : 1 [6, 8].

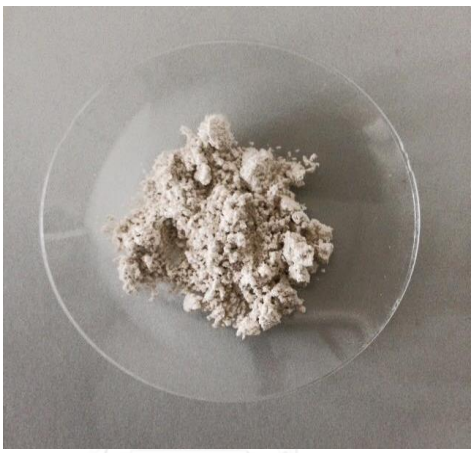


Figure 7 Physical appearance of ECa 233.

The pharmacological studies of ECa 233 were observed on second degree burn in male Wistar rats. Formulation of 0.05% ECa 233 gel was directly applied to wound and evaluated the wound healing including appearance, rate of healing and cutaneous blood flow. Rate of wound healing in rats treated group with 0.05% ECa 233 gel was higher than control group and also found that the cutaneous blood flow was increased in day 7 post-treatment [6].

The preliminary study of ECa 233 on cognitive deficits induced by intracerebro-ventricular injection β-amyloid peptide in male ICR mice revealed that pretreatment with 10 mg/kg of ECa 233 oral administered twice daily for 7 days before the injection of β-amyloid significantly improved deficit in learning and memory [9]. Moreover, ECa 233 has been shown some interesting pharmacological effects, such as anxiolytic

effects in stressed mice model [8], neuritogenic effect on human neuroblastoma cells by upregulating the level of ERK1/2 and Akt [7].

ECa 233 was also investigated for the effects on major human cytochrome P450 using *in vitro* recombinant human CYPs. The results showed that ECa 233 did not affect CYP1A2, CYP2C9, CYP2D6 and CYP2E1 but inhibited CYP3A4, CYP2C19 and CYP2B6 [38].

Toxicity studies of ECa 233 [10]

ECa 233 was tested for acute toxicity in both male and female mice by oral administration at single dose up to 10 g/kg. No sign of toxicity and gross pathological lesions were observed within 14 days of experiment.

Sub-chronic toxicity of ECa 233 have been investigated in Wistar rats by repeated dose of 10, 100, and 1,000 mg/kg/day for 90 days. No sign of toxicities such as body weight, food consumption and animal health were observed at all given dose. At the dose of 1,000 mg/kg of ECa 233, female rats had statistically significant higher white blood cell counts and male rats had higher sodium level, however, it was within normal range.

Table 1 Previous pharmacokinetic studies of madecassoside and asiaticoside

Compounds	Madecassoside	Madecassoside	Asiaticoside	Asiaticoside	
First author	Han WJ	Ting W	Baek M	Liu SJ	
Subject species	Male Sprague-Dawley rats	Female Wistar rats	Male Sprague-Dawley rats	Sprague-Dawley rats	
Weight range	220 ± 30 g	160 – 180 g	200-220 g	230 ± 20 g	
Dose	100 mg/kg	30 mg/kg	10 mg/kg	40 mg/kg	200 mg/kg
Route of administration	Oral administration	Oral administration	IV bolus	IV, Single caudal vein administration	IG, Single intragastric administration
Detection system	LC-ESI-MS	LC-ESI-MS	HPLC-UV	LC-MS-MS	
Blood uptake	Orbital veins	Orbital veins	-	-	-
T_{max} (h)	0.90 ± 0.14	1.32 ± 0.87	-	0.08 ± 0.00	Not detected under analytical system
C_{max} (ng/mL)	303.75 ± 28.53	27.14 ± 9.70	-	3,347.88 ± 786.89	
K_e (1/h)	0.21 ± 0.04	0.14 ± 0.01	-	-	
$T_{1/2}$ (h)	3.47 ± 0.68	5.01 ± 0.30	0.26 ± 0.10	0.39 ± 0.16 (beta)	
AUC_{0-t} (ng.h/mL)	1,368.37 ± 90.71	130.56 ± 17.30	25,530 ± 4,560	81,443.67 ± 57,156.81	
$AUC_{0-\infty}$ (ng.h/mL)	1,458.74 ± 202.99	243.11 ± 83.69	-	81,904.99 ± 57,112.45	

Table 2 Previous pharmacokinetic studies of *Centella asiatica*

Compounds	Titrated extract of Centella asiatica (TECA) TECA contains 41.6% madecassoside, 46.3% asiaticoside [15]			
First author	Jeong DC			
Subject species	Male Sprague-Dawley rats			
Weight range	210-250 g			
Dose	20 mg/kg		20 mg/kg	
Route of administration	IV administration		Oral administration	
Detection system	HPLC-UV			
	Asiaticoside	Madecassoside	Asiaticoside	Madecassoside
T_{max} (h)	-	-	Not detected under analytical system	0.33 ± 0.05
C_{max} (ng/mL)	-	-		0.793 ± 0.174
K_e (1/h)	-	-		-
$T_{1/2}$ (h)	0.16 ± 0.05	0.23 ± 0.06		0.10 ± 0.03
CL (L/h/kg)	2.237 ± 0.813	0.317 ± 0.129		-
AUC_{0-t} (ng.h/mL)	4.11 ± 2.41	26.98 ± 10.35		1.52 ± 0.372
Vd (L/kg)	0.524 ± 0.214	0.103 ± 0.031		-

Comparative pharmacokinetic studies

In recent years, the comparative pharmacokinetic between extract and pure compound has been extensively reported. The interaction between main active compound and other components in the extract affected pharmacokinetic behavior. One study investigated the comparative pharmacokinetic of osthole, a coumarin compound from dried root and rhizome of *Libanotis buchtormensis.* The result showed different plasma concentration-time profile of osthole after oral administration of pure osthole and *Libanotis buchtormensis* supercritical extract (LBSE) and suggested that non-osthole component of LBSE has pharmacokinetic interaction with osthole resulting in decrease of absorption level [39]. Another study investigated pharmacokinetic of gastrodin, the main active ingredient in *Gastrodia elata*. The pharmacokinetic of gastrodin following administred parishin, which converse to gastrodin *in vivo* and *Gastrodia elata* extract showed significantly prolonged elimination half-life and higher mean residence time values than pure gastrodin administered [40].

In addition, many studies have reported the effect of non-active compound in herbal extract on P-glycoprotein transporter, the permeability glycoprotein at intestinal epithelial cells which pumps xenobiotic back into intestinal lumen resulting in decrease of drug absorption. One study has evaluated the pharmacokinetic of **α**-mangostin in C57BL/6 mice when administered as a pure **α**-mangostin or standardized mangosteen fruit extract (equivalent amount of **α**- mangostin). The results showed that when **α**-mangostin was administered with additional xanthone in mangosteen fruit extract, the activity of P-glycoprotein was decreased resulting in increasing **α**-mangostin absoption [20]. In relating to previous report on comparative pharmacokinetic of tectorigenin (the metabolite of tectoridin) after oral administration of *Iris tectorum* Maxim extract and pure tectoridin, the absorption of tectorigenin was

higher in extract group resulting with increase of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} and also prolonged $T_{1/2}$ value. The researcher suggested that the coexisting ingredients in *Iris tectorum* Maxim extract could promote the absorption of tectoridin by inhibition of P-glycoprotein and MRP2 protein [18].

However, the pharmacological effects of asiaticoside as a pure compound and as extract have been comparatively studied. For healing effects on gastric ulcer, the *C. asiatica* extract showed superiority in healing gastric ulcers. *C. asiatica* water extract induced higher level of basic fibroblast growth factor expression than that of asiaticoside alone. This phenomenon could result in increasing angiogenesis activity and promoting cell proliferation [21]. In addition, the study on anxiolytic effect revealed that both *C. asiatica* extract and asiaticoside had the anxiolytic activity in rodent but asiaticoside had no effect on locomotor activity and sedative effect [22].

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

The animal experiments were conducted under the protocol approved by the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Protocol number 15-33-002, approved April 22, 2015). Male Wistar rats, 8 weeks old, were purchased from the National Laboratory Animal Centre, Mahidol University, Thailand. All rats were housed in an environmentally controlled temperature of 25 ± 2 °C, relative humidity 50-60% with a 12 h light/dark cycle, and free access to food and water. The rats were fasted overnight in metabolic cages with free access to water before experiments.

3.1.2 Chemicals

ECa 233 was kindly supplied by Siam Herbal Innovation Co., Ltd. (Thailand). The percent labeled amount of madecassoside and asiaticoside in ECa 233 was quantitatively determined by LC-MS/MS analysis. The total triterpenoid glycoside content of ECa 233 was 89%; represented by 51% madecassoside and 38% asiaticoside.

Analytical standards of chemical use in LC-MS/MS analysis were purchased from following companies,

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

Pharmaceutical produce used in our experiments were purchased from following companies,

- Isoflurane, Terrell™ (MINRAD, Inc., USA)
- Heparin (LEO Pharma A/S, Denmark)
- 0.9% Normal saline solution (General Hospital Products Public, Co. Ltd., Thailand)

HPLC grade of the solvent used in this study were purchased from following companies,

- Dimethyl sulfoxide (Sigma-Aldrich, Corp., USA)
- Methanol, HPLC grade (Honeywell Burdick & Jackson International, Inc., USA)
- Water, HPLC grade (Honeywell Burdick & Jackson International, Inc., USA)
- Formic acid (Merck KGaA, Corp., Germany)
- Acetonitrile (Merck KGaA, Corp., Germany)
- Ethyl acetate (Merck KGaA, Corp., Germany)

3.1.3 Instruments

- QTRAP® 6500 LC/MS/MS system (AB Sciex, Pte. Ltd., USA)
- UPLC column, Synergi™ Fusion-RP (Phenomenex, Inc., USA)
- Guard column, SecurityGuard™ Fusion-RP (Phenomenex, Inc., USA)
- Metabolic cage, 3701M081 (Tecniplast, S.p.a., Italy)
- Vortex mixer, model VX-200 (Labnet International, Inc., USA)
- Gavage needle 13G, 3 inches, (Biolascothai Co., Ltd., Thailand)
- Insulin syringe 1 ml, 0.4x12 mm. (Nipro, Corp. Ltd., Thailand)
- Micropipette (Labnet International, Inc., USA)
- Homogenizer (Glas-Col®, LLC, USA)
- Tissue homogenizer (Tissue Terror model WT-130 with Shaft set 5, Success, Malaysia)

- Dual-range analytical balance, AG135 (Mettler-Toledo International, Inc., Switzerland)
- Microliter Centrifuge, model MIKRO 120 (Andreas Hettich, GmbH & Co.KG, Germany)
- Tabletop Centrifuge, model EBA 20 (Andreas Hettich, GmbH & Co.KG, Germany)
- Chest freezer,- 20°C (Singer, Sdn Bhd, Malaysia)
- Stopwatch, CT-20 (Canon, Co., Ltd., China)

3.2 Methods

3.2.1 Test compound preparation

All test compounds were freshly prepared in 50% dimethylsulfoxide in normal saline solution before the animal experiments and the final concentrations are shown in the table below:

Table 3 The concentration of test compounds for pharmacokinetic studies.

Chemical	Oral administration	Intravenous administration
ECa 233	100 mg/ml	20 mg/ml
Madacassoside	51 mg/ml	10.2 mg/ml
Asiaticoside	38 mg/ml	7.6 mg/ml

1. The test compounds were weighed out using analytical balance.
2. The test compounds were dissolved with DMSO to target volume.
3. The test compound solution were added with 0.9% normal saline to complete 100% final volume.
4. The solution of test compounds were mixed until obtaining clear solution.

3.2.2 Pharmacokinetic study

3.2.2.1 Route of administration

Thirty rats were randomly divided into six groups. The sample size was minimized to five rats per group based on our pilot study. In each experiment, all rats were fasted overnight in the metabolic cages for 24 h before the pharmacokinetic experiment. A single dose of the test compound was administered to rats by intravenous administration via the lateral tail vein or by oral gavage. All rats were anesthetized by inhalation of isoflurane prior to administration and sample collection in order to reduce pain and injury to animals. The dose of administration was calculated based on the percent labeled amount of ECa 233 as shown in the table below:

Table 4 Dose of administration

Group	Chemical	Dose	Route
1	ECa 233	100 mg/kg	Oral
2	Madecassoside	51 mg/kg	administration
3	Asiaticoside	38 mg/kg	
4	ECa 233	10 mg/kg	Intravenous
5	Madecassoside	5.1 mg/kg	administration
6	Asiaticoside	3.8 mg/kg	

3.2.2.2 Sample collection

1. **Plasma**

Blood samples (approximately 300 µL) were collected through lateral tail veins into preheparinized tubes prior to dosing and at designed time, 0.08, 0.25, 0.5, 1, 2, 4, 8, 16, 24 h post-dosing under light anesthesia with isoflurane. The blood samples were centrifuged at 1,500 x g for 10 min to collect plasma and stored at -20 °C until analysis.

2. **Urine sample**

Urine samples were collected at two intervals; 0-24 h and 24-48 h from metabolic cages after intravenous dosing. The volume of urine were recorded, and all samples were stored at -20 °C until analysis.

3. **Feces sample**

Feces samples were collected at two intervals; 0-24 h and 24-48 h from metabolic cages after intravenous dosing. The weight of feces were recorded, and all samples were stored at -20 °C until analysis.

3.2.3 Tissue distribution study

1. Rats were divided into 3 groups. Each group contains 4 rats.
2. A single dose of 10 mg/kg ECa 233, 3.8 mg/kg asiaticoside or 5.1 mg/kg madecassoside was intravenously administered via the lateral tail vein.
3. At 1, 2 and 4 h after administration, blood samples approximately 500 µL were collected from lateral tail vein for analysis of drug levels in plasma. The blood samples were centrifuged at 1,500 x g for 10 min to collect plasma and stored at -20 °C until analysis.
4. Animals were then euthanized and tissue samples, including liver, kidney, heart, lung, stomach, spleen, brain and skin, were removed and immediately rinsed with 0.9% normal saline solution.
5. All tissue samples were stored at -20 °C until analysis.

3.2.4 Sample preparation for LC-MS/MS analysis

To determine the concentrations of madecassoside and asiaticoside and its metabolites in all biological samples, protein precipitation methods were used for sample preparation prior to LC-MS/MS analysis.

1. **Plasma**

1.1 50 µL of plasma samples were added to 200 µL of methanol containing 10 ng of internal standard in 1.5 mL tube.

1.2 The mixtures were centrifuged at 5,000 × g for 10 min.

1.3 150 µL of supernatants were collected and analyzed by LC-MS/MS.

2. **Urine**

2.1 50 µL of each urine samples were diluted with 450 µL of methanol containing 20 of internal standard in 1.5 mL tube.

2.2 The mixtures were centrifuged at 5,000 × g for 10 min.

2.3 150 µL of supernatants were collected and analyzed by LC-MS/MS.

3. Feces

3.1 Feces samples were added methanol up to 10 mL.

3.2 The samples were homogenized and centrifuged at 1,500 × g for 10 min to collect 500 µL of primary supernatant.

3.3 50 µL of primary supernatant were diluted with 450 µL of methanol containing 20 ng of internal standard.

3.4 The mixtures were centrifuged at 5,000 × g for 10 min.

3.5 150 µL of supernatants were collected and analyzed by LC-MS/MS.

4. Tissues

4.1 The tissue samples 50 mg were weighed out in 2 mL tube.

4.2 The sample were added 450 µL of methanol containing 20 ng of internal standard and homogenized in the ice bath for 10 min.

4.3 The mixtures were centrifuged at 5,000 × g for 10 min.

4.4 150 µL of supernatants were collected and analyzed by LC-MS/MS.

3.2.5 Blood chemistry test

Plasma sample approximately 500 mL from pharmacokinetic study were collected at pre-dosing and 24 h post-dosing via lateral tail vein. The blood samples were centrifuged at 1,500 x g for 10 min to collect plasma sample. The plasma sample were measured for level of creatinine, AST, and ALT by enzymatic method and kinetic method (IFCC without pyridoxal phosphate activation) using Modular P800 (Roche diagnostics, Ltd., Thailand) to evaluate the effect of test compound and vehicle on kidney and liver function.

The level of creatinine, AST and ALT between pre-dosing and 24 h post-dosing were reported as mean ± standard deviation and statistical analyzed by non-parametric test (significant level = 0.05) to determine significant differences of biochemical parameters.

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

Parameter	Reference Range	Unit
Creatinine	0.39-0.74	mg/dL
AST	64-247	U/L
ALT	22-64	U/L

3.2.6 LC-MS/MS analysis

3.2.6.1 LC-MS/MS system

The LC-MS/MS analyses were performed on an Eksigent ekspert UHPLC 100 liquid chromatograph (Eksigent, Canada) equipped with QTRAP 6500 mass spectrometer (AB Sciex, Pte. Ltd., USA). The analysis was operated on a Synergi Fusion-RP C18 reverse phase column (Phenomenex, Inc., USA). Gradient elution with 100% methanol and 0.2% formic acid in water (pH 2.5) was used for separation. Elution occurred at a flow rate of 0.5 mL/min, with 10% methanol for 0.5 min, increasing to 90% methanol at 1.5 min for 2 min, then decreasing to 10% methanol at 4.0 min until 4.5 min. The instrument was operated in negative ionisation mode. The detector was set to monitor mass to charge ratios of 973.4/503.5 for madecassoside, 957.7/469.1 for asiaticoside, 503.5/219.2 for madecassic acid, 487.3/379.2 for asiatic acid, and 469.3/409.2 for glycyrrhetinic acid. The retention times were 1.79 min for madecassoside, 1.82 min for asiaticoside, 1.93 min for madecassic acid, 1.99 min for asiatic acid and 2.12 min for glycyrrhetinic acid.

3.2.6.2 Method validation and quality control

Method validation was developed to verify the quality of asiaticoside and madecassoside measurement which is composed of:

1. Lower limit of quantification (LLOQ), defined as the lowest detectable concentration of analyte with a signal-to-noise ratio greater than 5.
2. Linearity, defined as the range of analyze concentration that can be fitted with the calibration curve with $R^2 > 0.99$.

3. Accuracy, determined by comparing the measured concentration to the actual concentration of quality control (QC) samples at low, medium and high concentrations.
4. 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).
5. Recovery, calculated by comparing the peak-area of the prepared sample to that of the standard solution containing the same concentration.

3.2.7 Data analysis

1. Pharmacokinetic parameters of asiaticoside and madecassoside are calculated by non-compartmental analysis using PK solution 2.0™ (Summit Research Service, USA). The following pharmacokinetic parameters were reported as follow,

- Maximum plasma drug concentration (C_{max})
- Time to reach maximum plasma drug concentration (T_{max})
- Area under the curve from time zero to the last sampling point (AUC_{0-t})
- Area under the curve from time zero to infinity ($AUC_{0-\infty}$)
- Apparent volume of distribution (V_{app})
- Mean residence time (MRT)
- Elimination half-life ($T_{1/2}$)
- Apparent clearance (CL_{app})
- Absolute oral bioavailability, calculated as

$$(AUC_{po}/DOSE_{po})/(AUC_{iv}/DOSE_{iv})$$

2. For tissue distribution analysis, the tissue to plasma concentration ratio (K_p) was calculated as the drug level in tissue divided by the drug level in plasma at the same time point

3. Percent recovery of drugs and metabolites in urine and feces at all time periods was determined from drug or metabolite recoveries in the excreta divided by drug doses based on molar unit.

4. The interconversion between madecassoside and asiaticoside was determined using previously described methods (Ebling et al., 1986; Prueksaritanont et al., 2005). The proposed metabolic scheme of asiaticoside and madecasosside interconversion was presented in Figure 8

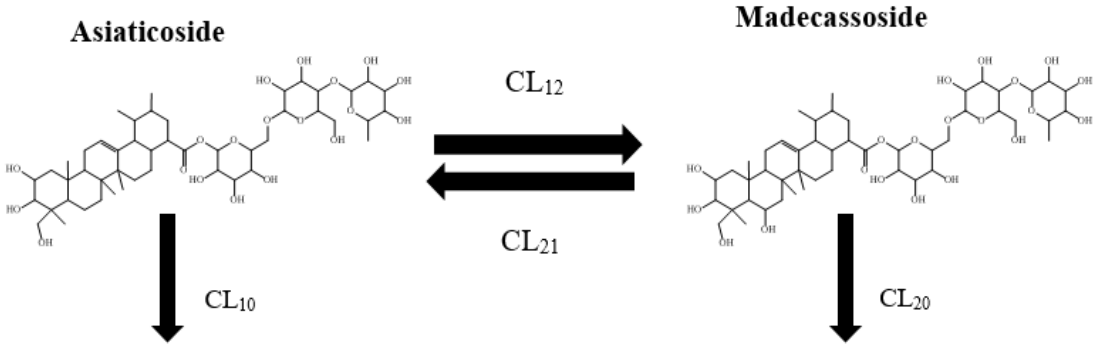


Figure 8 The proposed metabolic scheme of asiaticoside and madecassoside interconversion.

The four fundamental clearances, namely irreversible elimination clearance of asiaticoside (CL_{10}) and madecassoside (CL_{20}), and the interconversion clearance of asiaticoside to madecassoside (CL_{12}) and madecassoside to asiaticoside (CL_{21}) were calculated following the equations:

$$CL_{10} = \frac{[(\text{Dose}^{ASS} \times AUC^{MDS}_{MDS}) - (\text{Dose}^{MDS} \times AUC^{ASS}_{MDS})]}{[(AUC^{ASS}_{ASS} \times AUC^{MDS}_{MDS}) - (AUC^{ASS}_{MDS} \times AUC^{MDS}_{ASS})]}$$

$$CL_{20} = \frac{[(\text{Dose}^{MDS} \times AUC^{ASS}_{ASS}) - (\text{Dose}^{ASS} \times AUC^{MDS}_{ASS})]}{[(AUC^{ASS}_{ASS} \times AUC^{MDS}_{MDS}) - (AUC^{ASS}_{MDS} \times AUC^{MDS}_{ASS})]}$$

$$CL_{12} = \frac{\text{Dose}^{MDS} \times AUC^{ASS}_{MDS}}{[(AUC^{ASS}_{ASS} \times AUC^{MDS}_{MDS}) - (AUC^{ASS}_{MDS} \times AUC^{MDS}_{ASS})]}$$

$$CL_{21} = \frac{\text{Dose}^{ASS} \times AUC^{MDS}_{ASS}}{[(AUC^{ASS}_{ASS} \times AUC^{MDS}_{MDS}) - (AUC^{ASS}_{MDS} \times AUC^{MDS}_{ASS})]}$$

Percent conversion between madecassoside and asiaticoside in plasma after administration was calculated by $AUC_{metabolite}/AUC_{substrate}$ to determine the extent of conversion.

All pharmacokinetic data of madecassoside and asiaticoside were expressed as mean ± standard deviation (SD.). Comparison of data between standardized extract and pure compounds was performed by non-parametric test. P-value < 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS ver. 16 (SPSS Inc, USA).

CHAPTER IV

RESULTS

4.1 Animal tolerability

The animals exhibited a good tolerability to all of the test compounds. No signs of toxicity or physiological changes were observed in rats after treatment with any of the test compounds. The blood levels of aspartate transaminase (AST), alanine transaminase (ALT) pre-dose and 24 h post-dose did not indicate liver damage. Although a slight increase in the AST levels was observed 24 h after oral administration of 100 mg/kg ECa 233 and intravenous administration of 3.8 mg/kg asiaticoside, these values were still within normal ranges for healthy Wistar rats. Blood creatinine levels did not indicate kidney toxicity after administration of any of the test compounds.

Table 6 Physical appearance and biochemical profiles of rats at time zero and 24 h after intravenous dosing.

Parameters	ECa 233 10 mg/kg IV		Madecassoside 5.1 mg/kg IV		Asiaticoside 3.8 mg/kg IV	
	Pre-dose	Post-dose	Pre-dose	Post-dose	Pre-dose	Post-dose
Physical appearance	Normal	Normal	Normal	Normal	Normal	Normal
Creatinine (mg/dL)	0.23 ± 0.10	0.20 ± 0.00	0.22 ± 0.09	0.23 ± 0.11	0.20 ± 0.00	0.20 ± 0.00
AST (U/L)	67.17 ± 36.66	63.17 ± 29.01	74.33 ± 50.53	67.17 ± 22.19	65.00 ± 22.34	98.33 ± 72.89
ALT (U/L)	23.00 ± 23.88	21.83 ± 17.52	44.50 ± 69.24	27.33 ± 12.96	22.17 ± 22.45	40.67 ± 40.14

Data are presented as mean ± SD. (n = 5)

AST aspartate transaminase; ALT alanine transaminase

Table 7 Physical appearance and biochemical profiles of rats at time zero and 24 h after oral dosing.

Parameters	ECa 233 100 mg/kg PO		Madecassoside 51 mg/kg PO		Asiaticoside 38 mg/kg PO	
	Pre-dose	Pre-dose	Pre-dose	Post-dose	Pre-dose	Post-dose
Physical appearance	Normal	Normal	Normal	Normal	Normal	Normal
Creatinine (mg/dL)	0.22 ± 0.09	0.23 ± 0.11	0.22 ± 0.09	0.22 ± 0.09	0.20 ± 0.00	0.22 ± 0.09
AST (U/L)	48.83 ± 8.03	83.33 ± 50.75	51.50 ± 13.29	67.17 ± 21.74	53.50 ± 42.77	72.17 ± 34.61
ALT (U/L)	15.83 ± 11.65	31.00 ± 25.64	19.17 ± 19.71	28.83 ± 23.37	16.83 ± 10.83	11.00 ± 9.61

Data are presented as mean ± SD. (n = 5)

AST aspartate transaminase; ALT alanine transaminase

4.2 Plasma concentration-time profiles and oral bioavailability

The mean plasma concentration-time profiles of madecassoside and asiaticoside after administration of intravenous doses of ECa 233, madecassoside and asiaticoside are shown in Figure 9. After intravenous administration of pure madecassoside, the plasma concentration reached a maximum of approximately 40,000 μg/L, which then declined to 100 μg/L 24 h after dosing. The elimination half-life of madecassoside was 4.99 ± 2.29 h. After intravenous administration of pure asiaticoside, the plasma concentration was also detected in rat plasma up to 24 h. The elimination half-life of asiaticoside was 4.09 ± 1.61 h. Compared with the intravenous administration of 10 mg/kg ECa 233, the pharmacokinetic characteristics of madecassoside and asiaticoside were similar to that of the pure compounds. However, the plasma levels of madecassoside and asiaticoside were remarkably increased in the ECa 233 treated group as compared to the groups treated with the individual compounds. The pharmacokinetic parameters, including AUC_{0-24} and $AUC_{0-\infty}$ of madecassoside, were significantly higher than pure compound administration ($p< 0.05$). In addition, the elimination half-life of asiaticoside was significantly prolonged from 4.09 ± 1.61 h to 8.73 ± 2.25 h after administration of ECa 233.

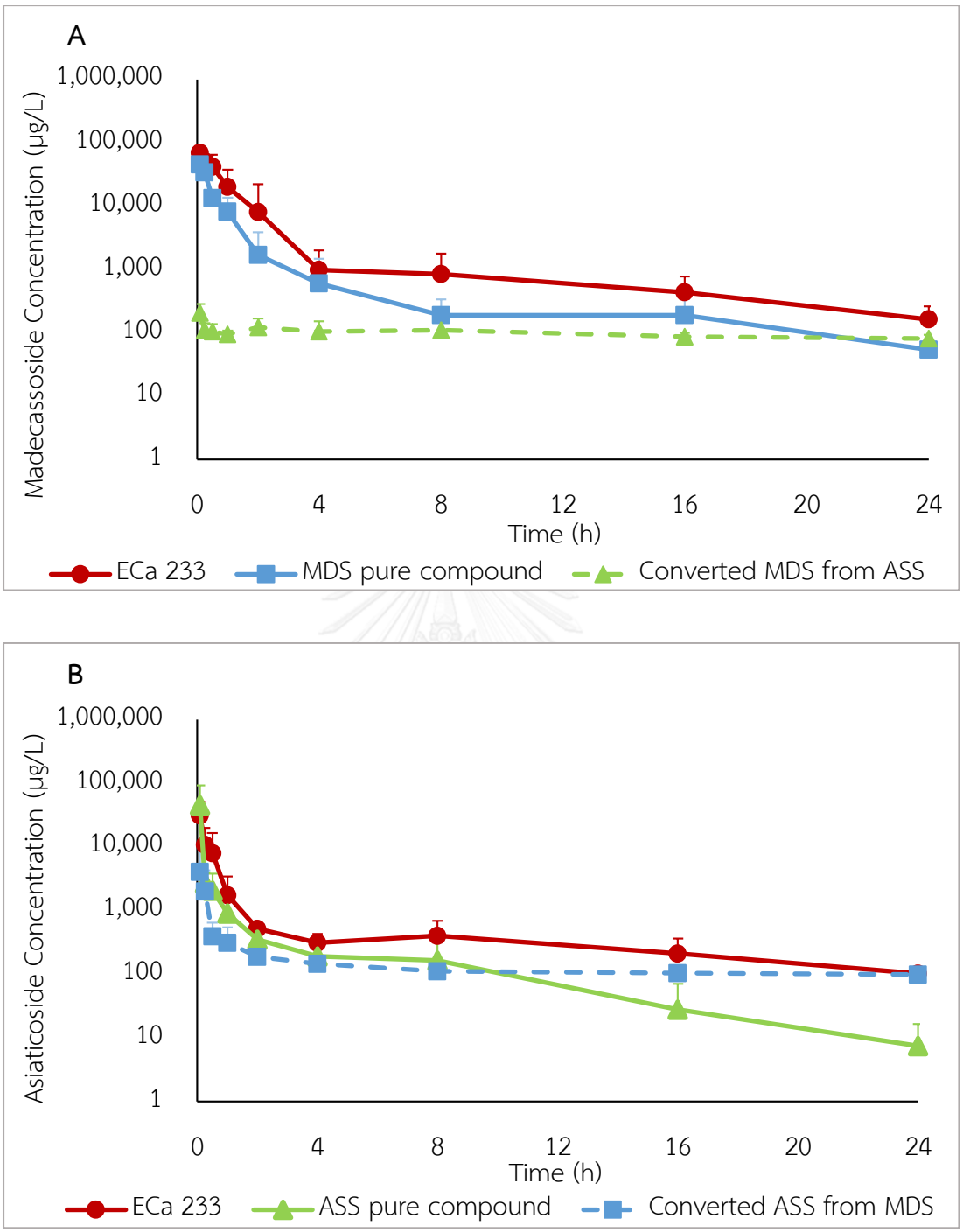


Figure 9 Plasma concentration-time profiles of madecassoside (A) and asiaticoside (B) following the intravenous administration of ECa 233, madecassoside and asiaticoside.

Table 8 Pharmacokinetic parameters of madecassoside and asiaticoside after intravenous administration of 10 mg/kg ECa 233 and the equivalent doses of madecassoside (5.1 mg/kg) and asiaticoside (3.8 mg/kg).

Pharmacokinetic parameters	Madecassoside			Asiaticoside		
	ECa 233 10 mg/kg	MDS 5.1 mg/kg	Converted MDS from ASS	ECa 233 10 mg/kg	ASS 3.8 mg/kg	Converted ASS from MDS
AUC_{0-24} (μg.h/L)	75,049.13 ± 40,979.46	32,362.13 ± 11,146.09*	2,546.37 ± 519.43	17,578.04 ± 9,199.41	11,383.71 ± 6,437.39	3,965.17 ± 1079.00
$AUC_{0-\infty}$ (μg.h/L)	77,154.66 ± 42,227.64	33,301.72 ± 11,073.39*	7,086.60 ± 2,399.28	20,182.08 ± 9,608.92	12,020.89 ± 6,237.93	7,836.75 ± 814.04
V_{app} (L/kg)	0.73 ± 0.38	1.28 ± 0.81	-	2.83 ± 0.60	2.30 ± 1.52	-
MRT (h)	2.61 ± 1.20	2.92 ± 1.73	-	7.37 ± 4.73	3.64 ± 2.28	-
Elimination half-life (h)	6.45 ± 1.38	4.99 ± 2.29	-	8.73 ± 2.25	4.09 ± 1.61*	-
CL_{app} (L/h/kg)	0.08 ± 0.03	0.17 ± 0.08*	-	0.24 ± 0.14	0.62 ± 0.24	-
% Conversion	-	-	58.95	-	-	23.53

Data are presented as the mean ± SD. (n = 5)

AUC_{0-24} area under concentration-time curve from time 0-24 h; $AUC_{0-\infty}$ area under concentration-time curve from time 0-∞; V_{app} apparent volume of distribution; MRT mean resident time; CL_{app} apparent clearance; MDS madecassoside; ASS asiaticoside

* $p < 0.05$ for ECa 233 vs. pure compound, madecassoside and asiaticoside.

The mean plasma concentration-time profiles of madecassoside and asiaticoside after administration oral doses of ECa 233, madecassoside and asiaticoside are shown in Figure 10. The main pharmacokinetic parameters are summarized in Table 9. Both pure compounds were rapidly absorbed from the gastrointestinal tract and could be detected in plasma in 5 min after dosing. The plasma levels of madecassoside and asiaticoside reached maximum concentrations of 1,227.47 ± 1,283.74 μg/L and 658.17 ± 597.56 μg/L, respectively, within 1 h. However, absolute bioavailabilities of madecassoside and asiaticoside were very low, with values of 0.67% and 1.86%, respectively. Compared to the administration of the pure compound, the plasma level of madecassoside from ECa 233 was increased, resulting in higher C_{max} and AUC values than pure compound. The elimination half-life of madecassoside was also significantly prolonged from 4.33 ± 0.74 h to 7.68 ± 1.96 h after ECa 233 administration ($p < 0.05$). In addition, the pharmacokinetic profile of asiaticoside after ECa 233 administration showed significantly prolonged elimination half-life and higher mean residence time values than pure asiaticoside ($p < 0.05$).

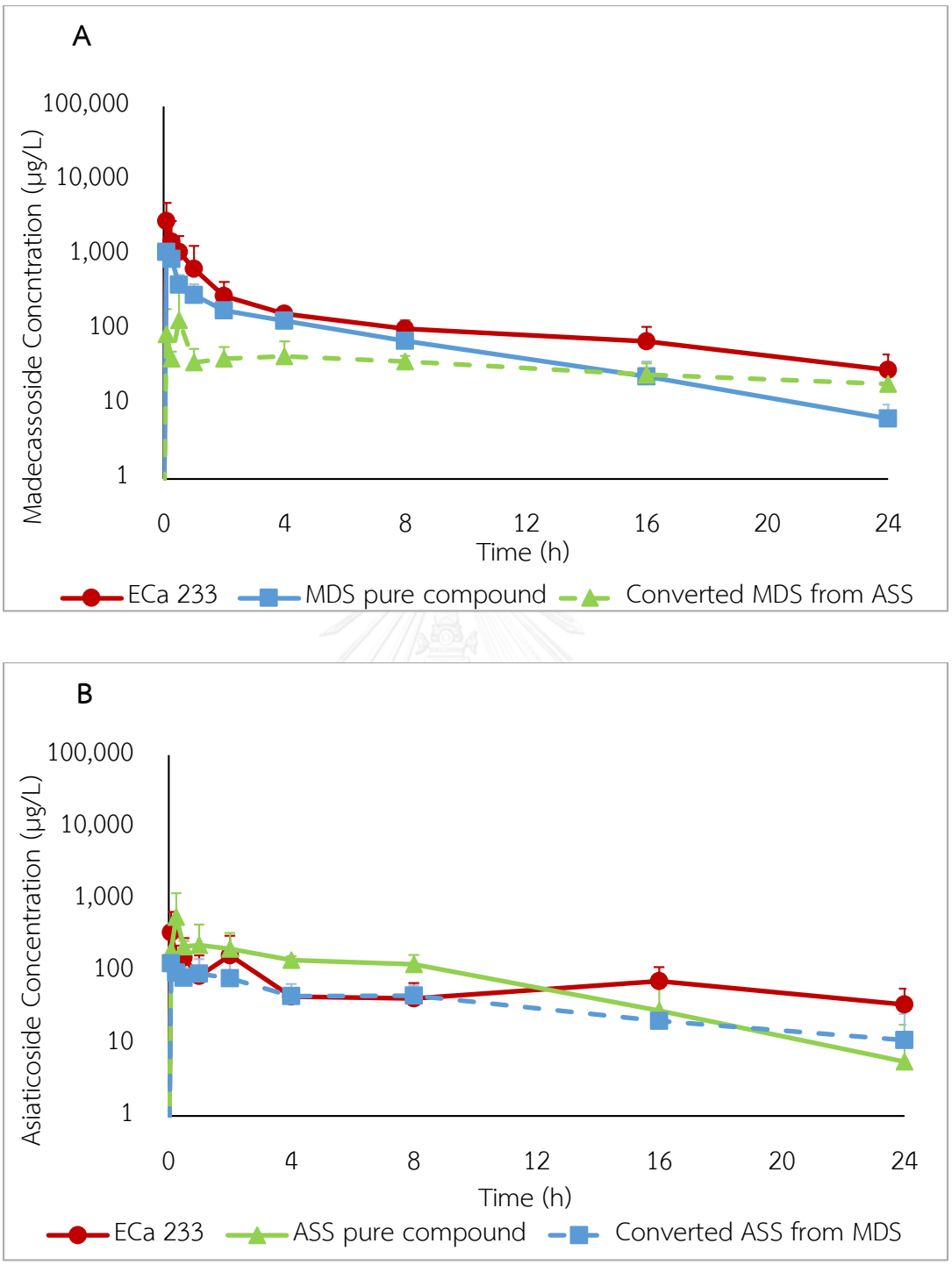


Figure 10 Plasma concentration-time profiles of madecassoside (A) and asiaticoside (B) following the oral administration of ECa 233, madecassoside and asiaticoside.

Table 9 Pharmacokinetic parameters of madecassoside and asiaticoside after oral administration of 100 mg/kg ECa 233 and the equivalent doses of madecassoside (51 mg/kg) and asiaticoside (38 mg/kg).

Pharmacokinetic parameters	Madecassoside			Asiaticoside		
	ECa 233 100 mg/kg	MDS 51 mg/kg	Converted MDS from ASS	ECa 233 100 mg/kg	ASS 38 mg/kg	Converted ASS from MDS
C_{max} (µg/L)	3,013.27 ± 1,993.01	1,227.47 ± 1,283.74	190.38 ± 172.04	442.74 ± 236.97	658.17 ± 597.56	157.06 ± 50.25
T_{max} (h)	0.17 ± 0.19	0.22 ± 0.07	0.33 ± 0.23	0.20 ± 0.18	0.62 ± 0.79	0.50 ± 0.84
AUC_{0-24} (µg.h/L)	3,419.48 ± 1,531.67	2,047.13 ± 963.57	826.11 ± 173.63	1,217.45 ± 714.94	1,937.19 ± 464.40	1,146.91 ± 267.57
$AUC_{0-\infty}$ (µg.h/L)	4,210.00 ± 1,698.85	2,241.44 ± 1,104.43*	1,886.76 ± 1,156.16	1,588.19 ± 834.85	2,240.27 ± 389.78	1,538.78 ± 607.52
MRT (h)	8.50 ± 1.88	5.69 ± 1.84	-	12.79 ± 3.20	7.20 ± 2.09*	-
Elimination half-life (h)	7.68 ± 1.96	4.33 ± 0.74*	-	8.40 ± 1.88	4.20 ± 0.91*	-
% Bioavailability	1.26	0.67	-	1.32	1.86	-
% Conversion	-	-	84.22	-	-	68.65

Data are presented as the mean ± SD. (n = 5)

C_{max} maximum concentration; T_{max} time to reach maximum concentration; AUC_{0-24} area under concentration-time curve from time 0-24 h; $AUC_{0-\infty}$, area under concentration–time curve from time $_{0-\infty}$; MRT mean resident time; MDS madecassoside; ASS asiaticoside

* $p < 0.05$ for ECa 233 vs. pure compound, madecassoside and asiaticoside

As shown in Figure 9 and 10, plasma concentration-time profiles demonstrated the *in vivo* interconversion between madecassoside and asiaticoside. This phenomenon was observed in plasma at all sampling time points after oral and intravenous dosing of individual compounds. The interconversion pharmacokinetic parameters were calculated to determine the rate and extent of conversion. The irreversible elimination clearances of asiaticoside (CL_{10}) and madecassoside (CL_{20}) were 0.25 and 0.09 L/hr/kg, respectively. The formation clearance of madecassoside from asiaticoside (CL_{12} = 0.11 L/hr/kg) was approximately 2 fold greater than the reverse reaction (CL_{21} = 0.07 L/hr/kg). Percent conversion of asiaticoside to madecassoside after intravenous and oral administration were higher than reverse reaction with 58.95% vs 23.53 % and 84.22 % vs 68.65 %, respectively.

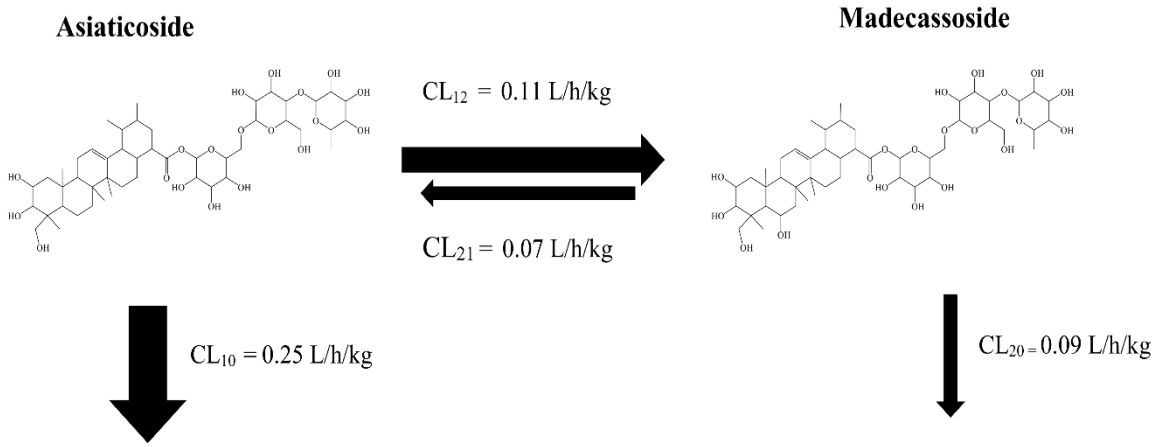
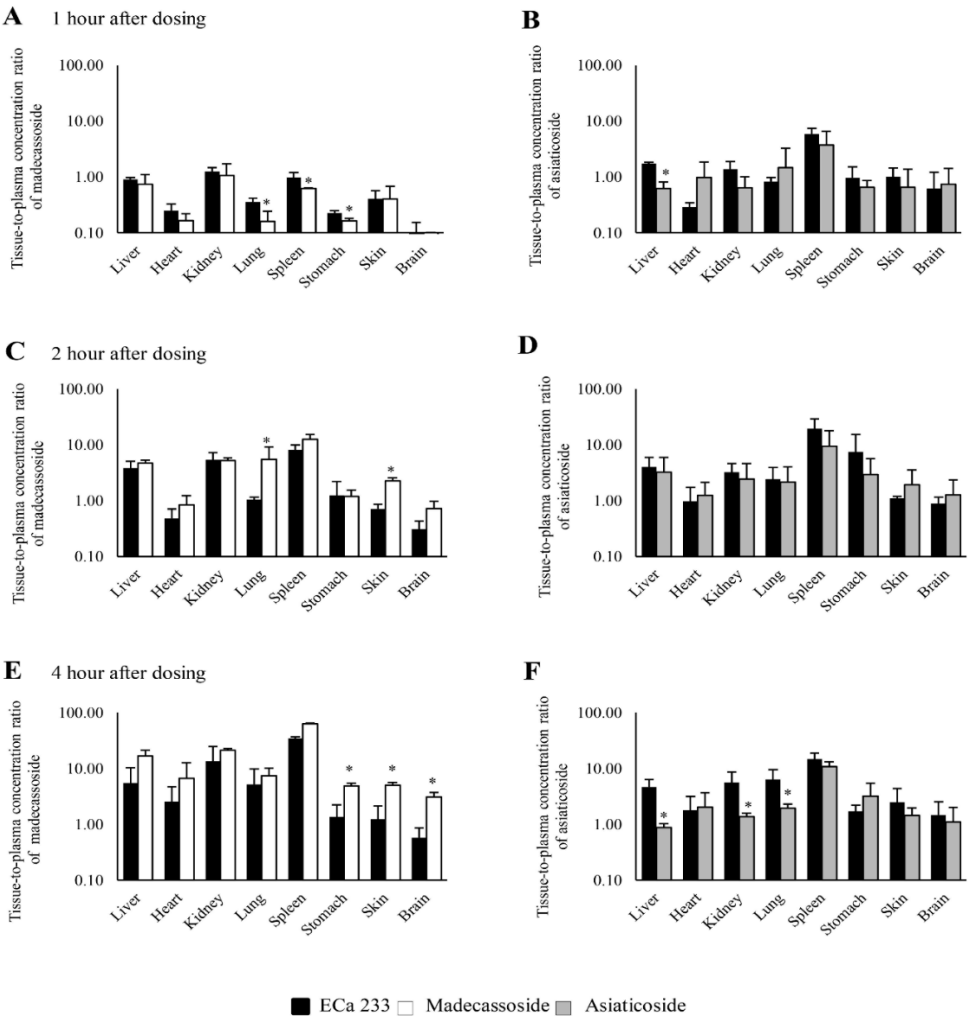


Figure 11 Metabolic scheme and interconversion pharmacokinetic parameters between asiaticoside and madecassoside after intravenous administration of asiaticoside and madecassoside as pure compounds

4.3 Tissue distribution

The tissue to plasma concentration ratios of madecassoside and asiaticoside after intravenous administration of 10 mg/kg ECa 233, and equivalent doses of madecassoside and asiaticoside, are provided in Figure 10. The concentration of madecassoside and asiaticoside in observed organ were reported in Table 10 and 11. Madecassoside and asiaticoside were rapidly distributed to internal organs within 1 hour after dosing. Both compounds were mainly distributed to liver, kidney, heart, spleen and lung, and to pharmacologically related organs including stomach, skin and brain. The highest tissue to plasma ratios of both madecassoside and asiaticoside were observed in the spleen. In asiaticoside treated group, it was likely that administration of asiaticoside in form of ECa 233 had higher tissue to plasma ratio compared with pure compound.



*P< 0.05 for ECa 233 vs. pure compound, madecassoside or asiaticoside

Figure 12 Tissue to plasma concentration ratio of madecassoside (A, C, E) and asiaticoside (B, D, F) in internal organs at 1, 2, and 4 hour after intravenous administration of 10 mg/kg ECa 233, 5.1 mg/kg of madecassoside and 3.8 mg/kg asiaticoside.

Table 10 The concentration of madecassoside in internal organs after intravenous administration of 10 mg/kg ECa 233 and the 5.1 mg/kg of madecassoside.

Tissue samples	Madecassoside Concentrations (ng/g tissue)					
	1 hour		2 hour		4 hour	
	ECa 233	MDS	ECa 233	MDS	ECa 233	MDS
Liver	520.67 ± 28.95	311.12 ± 64.13	322.21 ± 25.19	166.97 ± 1.03	148.44 ± 3.59	180.48 ± 30.13
Heart	158.63 ± 27.84	82.33 ± 13.46	37.96 ± 8.44	24.62 ± 2.14	28.88 ± 30.64	74.20 ± 37.02
Kidney	859.15 ± 98.54	547.22 ± 169.04	375.96 ± 50.28	215.65 ± 12.49	207.26 ± 26.53	224.35 ± 9.14
Lung	229.59 ± 17.07	78.07 ± 19.53	90.00 ± 9.89	86.15 ± 0.37	207.41 ± 37.68	76.36 ± 11.15
Spleen	589.77 ± 103.48	315.36 ± 7.25	676.39 ± 26.55	401.99 ± 62.92	388.98 ± 38.35	665.87 ± 66.83
Stomach	144.39 ± 8.55	80.02 ± 4.33	43.43 ± 1.03	45.87 ± 6.87	49.86 ± 8.56	50.19 ± 8.71
Skin	263.90 ± 48.41	199.40 ± 67.40	62.68 ± 13.04	91.49 ± 3.96	28.20 ± 1.35	45.80 ± 2.70
Brain	52.96 ± 21.47	40.10 ± 4.48	23.50 ± 4.59	26.61 ± 4.07	9.50 ± 0.10	29.48 ± 3.57

Data are presented as mean ± SD. (N=4)

Table 11 The concentration of asiaticoside in internal organs after intravenous administration of 10 mg/kg ECa 233 and 3.8 mg/kg of asiaticoside.

Tissue samples	Asiaticoside Concentrations (ng/g tissue)					
	1 hour		2 hour		4 hour	
	ECa 233	ASS	ECa 233	ASS	ECa 233	ASS
Liver	76.62 ± 2.33	42.81 ± 7.32	61.83 ± 3.88	75.13 ± 18.98	62.76 ± 15.95	14.32 ± 1.66
Heart	10.17 ± 1.26	49.12 ± 14.04	11.98 ± 4.07	64.66 ± 28.66	17.50 ± 7.66	37.52 ± 15.65
Kidney	59.86 ± 11.92	39.01 ± 3.13	50.42 ± 3.54	43.46 ± 7.01	77.05 ± 26.64	24.41 ± 2.33
Lung	34.55 ± 3.65	62.11 ± 28.08	33.68 ± 3.01	63.28 ± 11.84	87.33 ± 28.14	36.10 ± 3.64
Spleen	267.61 ± 36.57	221.28 ± 30.38	305.84 ± 4.15	183.16 ± 22.68	192.54 ± 13.51	212.80 ± 19.55
Stomach	41.22 ± 12.54	59.14 ± 18.10	85.50 ± 40.55	53.32 ± 9.39	20.58 ± 4.61	62.78 ± 23.71
Skin	43.01 ± 10.12	33.42 ± 13.08	9.49 ± 0.15	38.64 ± 4.40	81.38 ± 51.35	26.12 ± 5.83
Brain	11.83 ± 2.65	30.29 ± 15.73	8.02 ± 2.16	23.46 ± 3.79	18.17 ± 8.62	19.08 ± 9.28

Data are presented as mean ± SD. (N=4)

4.4 Metabolism and excretion

Less than 2% of unchanged madecassoside and asiaticoside were excreted in urine and feces within 48 h after dosing, suggesting that madecassoside and asiaticoside underwent extensive metabolism in rats. Negligible amount of proposed active metabolites, madecassic acid and asiatic acid were found in plasma only 5 min after intravenous dosing of individual compound or the standardized extract. These triterpenic acid metabolites were observed in fecal excretion with 80-90% recovery after dosing of either madecassoside and asiaticoside or the standardized extract ECa 233 (Table 12).

Table 12 Percent recovery of madecassoside and asiaticoside via urine and feces after intravenous administration of ECa 233, madecassoside and asiaticoside.

Percent recovery		Intravenous administration					
		10 mg/kg ECa 233		5.1 mg/kg Madecassoside		3.8 mg/kg Asiaticoside	
		0-24 h	24-48 h	0-24 h	24-48 h	0-24 h	24-48 h
Urines	Madacassoside	0.92 ± 0.88	0.06 ± 0.03	1.13 ± 1.26	0.25 ± 0.20	0.02 ± 0.04	0.00 ± 0.01
	Asiaticoside	0.21 ± 0.28	0.02 ± 0.03	0.37 ± 0.71	0.00 ± 0.00	0.05 ± 0.06	0.01 ± 0.00
Feces	Madacassoside	0.04 ± 0.02	0.04 ± 0.04	0.17 ± 0.11	0.08 ± 0.02	0.02 ± 0.01	0.03 ± 0.01
	Asiaticoside	0.05 ± 0.03	0.05 ± 0.03	0.19 ± 0.14	0.08 ± 0.04	0.10 ± 0.04	0.09 ± 0.05
	Madacassic acid	25.44 ± 5.01	9.80 ± 2.42	24.70 ± 2.96	22.76 ± 2.95	25.48 ± 13.71	7.96 ± 1.65
	Asiatic acid	32.45 ± 7.21	17.99 ± 13.44	22.06 ± 11.67	24.34 ± 21.95	41.62 ± 5.32	21.69 ± 9.52

Data are presented as the mean ± SD. (n = 5)

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

ECa 233, the standardized extract of *Centella asiatica*, has been reported to possess pharmacological effects including wound healing [41 ,6] anxiolytic [8] and neuroprotective effects [7, 9]. The standardized extract contains the pentacyclic triterpenoid glycosides madecassoside (51%) and asiaticoside (38%). The chemical structures of madecassoside and asiaticoside are very similar, and are differentiated by the hydroxyl group at C6. To examine pharmacokinetic changes of the two triterpenoid glycosides in the standardized extract, the comparative pharmacokinetics of madecassoside and asiaticoside following administration as pure compounds and those presented in ECa 233 in male Wistar rats were investigated. In our experiment, all animal showed normal physical appearances. The biochemical profiles, including AST, ALT and creatinine levels, of the rats at pre-dose and 24 h post-dose, were within normal ranges. The animals could tolerate the test compound after intravenous and oral administration.

In our pharmacokinetic experiments, pure asiaticoside and madecassoside could be detected in plasma in 5 min and reached maximum plasma concentrations within 1 h after oral administration. The value of T_{max} of madacassoside and asiaticoside following oral administration were 0.22 and 0.62 h, respectively. Similarly, Jeong *et al*., 2014 reported T_{max} of madecassoside at 0.33 h after oral dosing of TECA 20 mg/kg solution in rats [15]. The test compounds used in our study was prepared in 50% DMSO in normal saline solution to obtain a clear solution for both intravenous and oral administration. This formulation was readily absorbed, resulting in higher C_{max} and shorter T_{max} compared to that for other formulations in different studies. Han *et al*., 2012 also mentioned that madecassoside could reach T_{max} within 1 h after single oral

dosing of 100 mg/kg madecassoside in rats [12]. These evidences might imply that the triterpenoid glycosides were rapidly but not completely absorbed from gastrointestinal tract. The percent bioavailability of madecassoside and asiaticoside was less than 2%, which is consistent with a previous study from Liu *et al.*, 2010. After a single intragastric administration of 200 mg/kg asiaticoside, the authors could not detect asiaticoside in rat plasma. The authors suggested that the circulating concentration of asiaticoside was lower than the detection limit of their assay at 38 µg/L [11]. Meanwhile, our LC-MS/MS system had better sensitivity, with a detection limit at 0.1-0.5 µg/L, therefore we were able to detect both triterpenoid glycosides in biological samples during 24-48 h after dosing. Leng *et al*., 2013 also reported that madecassoside is a substrate for efflux transporters, p-glycoprotein and multidrug-resistant protein-2, which may decrease the absorption of madecassoside [14]. The low bioavailability of both triterpenoid glycosides might be accounted by incomplete absorption and/or excretion of absorbed triterpenoid glycosides by transporters in the gastrointestinal tract. The $AUC_{0-\infty}$ of madecassoside from ECa 233 was about 2 fold higher than pure madecassoside. The absolute bioavailability of madecassoside was remarkably improved from 0.67 % to 1.26 % after administration of ECa 233. The low oral bioavailability is common phenomenon of the active compounds from natural products [42, 43]. However, the plasma levels of both triterpenoid glycosides were still be detected with significant amount at 24 h after single oral dose of ECa 233. Interestingly, we could detect both triterpenoid glycosides in most internal organs with Kp approximately 1-100 fold at 1-4 h postdose of ECa 233. Accumulation of the two triterpenoids in specific sites of pharmacologic actions needs to be determined after multiple oral dosing in animal models. This information will be useful to clarify that lead compounds with poor oral bioavailability could be developed as therapeutic agent. The adequate levels of active compounds at the sites of actions seem to be a critical factor in this circumstance.

Intravenous administration of pure madecassoside showed an apparent volume of distribution of 1.28 L/kg, meanwhile intravenous asiaticoside exerted a Vd of 2.30 L/kg. This parameter correlated with the superior lipophilicity of asiaticoside, as

estimated by the partition coefficients; XlogP 0.1 vs -1.2 for asiaticoside and madecassoside, respectively. This implies that asiaticoside may distribute in lipophilic tissues and internal organs better than madecassoside. This finding was consistent with the results from our tissue distribution study. Asiaticoside showed a higher tissue to plasma concentration ratio in most internal organs 1-2 h after intravenous dosing, compared with madecassoside. We detected madecassoside and asiaticoside in many tissues including liver, kidney, spleen, lung, heart, skin, stomach and brain after intravenous dosing of the individual compounds or ECa 233. This result correlated with a previous study of madecasoside deposition from Leng *et al*., 2013. They reported that oral dosing of 100 mg/kg madecassoside could distribute into various organs in rats [14]. In contrast to pharmacokinetic study of TECA in which madecassocide was not detected in the brain [15], our result clearly demonstrated the presence of madecassoside and asiaticoside from ECa 233 in the brain as well as in other tissues including stomach and skin where ECa 233 has been reported to exert its pharmacological effects [6-8, 41]. These might reflect limitation of the detection method used in previous study. The levels of madecassoside and asiaticoside in brain at 4 h after intravenous dosage of ECa 10 233 mg/kg in rat were within the range of ~10 20 ng/g tissue which certainly can be detected by LC-MS/MS system (detection range from 100-0.1 ng/g tissue) used in the present study but probably not by HPLC with UV detection used in previous study of Jeong *et al*., 2015.

After administration of the pure compounds, the elimination half-life of madecassoside and asiaticoside were approximately 4 h, which is similar to that reported in previous studies [12]. In the ECa 233 treated group, the elimination half-life of madecassoside and asiaticoside were prolonged, and the mean residence time of asiaticoside was significantly higher than the pure compound ($p<0.05$). To our knowledge, this is the first report of the bidirectional interconversion of madecassoside and asiaticoside *in vivo* when administered individually. The results demonstrated that percent conversion of asiaticoside to madecassoside after administration was higher than opposite direction. This phenomenon occurred after oral administration more than intravenous administration. The clearance of asiaticoside (CL_{10}) was about three

times faster than that of madecassoside (CL_{20}) and more rapid than the interconversion clearances (CL_{12}, CL_{21}) resulting in higher levels of madecassoside in plasma after administration of individual compound or ECa 233. CL_{12}, which reflects the formation of madecassoside from asiaticoside, was slightly higher than the reverse reaction (CL_{21}). As summarized in Figure 10, the result implies that the bidirectional interconversion favored the formation of madecassoside due to an oxidation reaction. Based on these observations, it is possible that this phenomenon might increase the counterpart level and prolong the elimination half-life of both madecassoside and asiaticoside after ECa 233 administration. The mechanism on increment of madecassoside and asiaticoside exposure still unclear and need to further investigated. However, the result from our study revealed that the Cl_{app} of madecassoside and asiaticoside from ECa 233 were decreased about 2 fold compared with pure compound. It possible that pharmacokinetic interactions between madecassoside and asiaticoside, which have similar chemical structure in ECa 233, may have an effect on metabolism and excretion pathway of madecassoside and asiaticoside and seem to be a critical factor in an increment exposure of madecassoside and asiaticoside in the standardized extract. Han *et al*., 2012 also reported that single oral dosing of 100 mg/kg madecassoside in rats demonstrated elimination half-life at 3.47 h [12]. Meanwhile, based on the sampling time of 0-4 h the HPLC-UV of orally given 20 mg/kg TECA was found to be 0.10 h by Jeong *et al*., 2014 [15]. In comparison to the present experiment in which sampling time from 0-24 h was used and determination of plasma concentration was carried out by high precision instrument, LC-MS/MS, it is suggestive that 0.33 was a distribution half-life rather than an elimination half-life.

In general, glycosides are hydrolyzed by intestinal flora to the corresponding aglycone [44]. According to previous reports, madecassoside and asiaticoside undergo hydrolysis of the sugar molecule to release madecassic acid and asiatic acid *in vivo* [12, 45] which subsequently being excreted in feces through glucuronide conjugation and sulfation of triterpenic acid have been proposed as possible metabolic pathways [46], we could not detect the glucuronide of triterpenic acid in urine or feces after oral dosing of the pure compounds or ECa 233. In our study, negligible amounts of

unchanged madecassoside and asiaticoside were detected in urine and feces within 48 h after dosing. The excretion of madecassoside and asiaticoside following administration of ECa 233 and pure compounds occurred mostly in the form of triterpenic acid metabolites, madecassic acid and asiatic acid, approximately 80-90% of the dose. We found negligible amounts of these triterpenic acid metabolites in plasma samples, but high levels of these metabolites were detected in fecal excretions. We also found that madecassoside, asiaticoside, madecassic acid and asiatic acid could be detected in fecal excretions after the individual administrations of madecasoside or asiaticoside. It is possible that madecassoside and asiaticoside could be interconverted and the sugar molecule hydrolyzed to obtain madecassic acid and asiatic acid. Formation of aglycone was likely to occur in the gastrointestinal tract rather than systemically.

5.2 Conclusion

In summary, our findings suggested that the pharmacokinetic behavior of madecassoside and asiaticoside could be improved when administered as ECa 233, a standardized extract of *Centella asiatica*. Interconversion between the two triterpenoid glycosides could prolong the levels of madecassoside in plasma and asiaticoside in most tissues after administration of ECa 233. This interconversion might have influential impact on pharmacodynamics activities of both triterpenoid glycosides when administered as the standardized extract. Further study to identify the molecular mechanisms of the bidirectional interconversion and biochemical reactions between madecassoside and asiaticoside should be investigated for future drug development of ECa 233.

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APPENDIX

APPENDIX A

Animal care and use protocol

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 15-33-002	**Approval No. 15-33-002**

Protocol Title
COMPARATIVE PHARMACOKINETIC OF ECA233 AND ITS PURE COMPOUND, ASIATICOSIDE AND MADECASSOSIDE IN RATS

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**
April 22, 2015	April 22, 2018

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

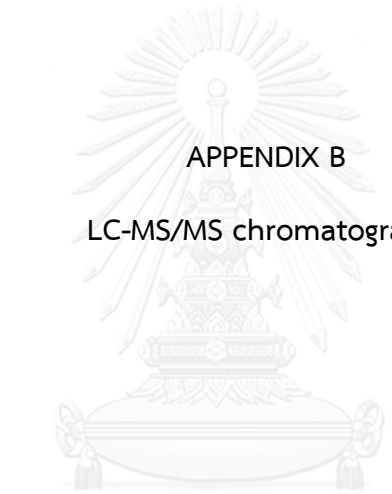
Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title **PORNCHAI ROJSITTHISAK, Ph.D.** **Associate Dean (Research and Academic Service)**

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.

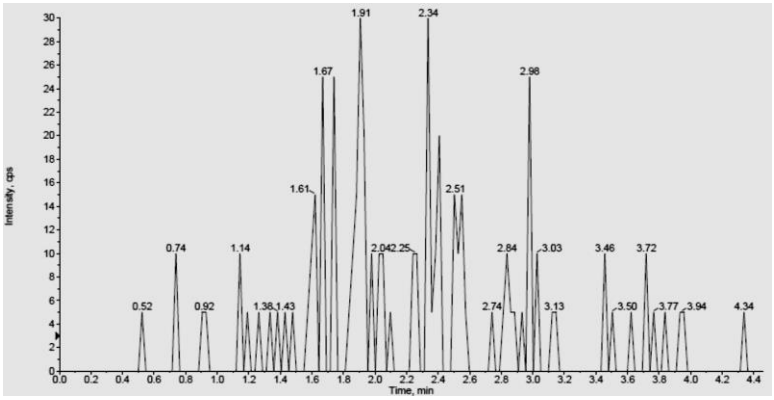
APPENDIX B

LC-MS/MS chromatograms

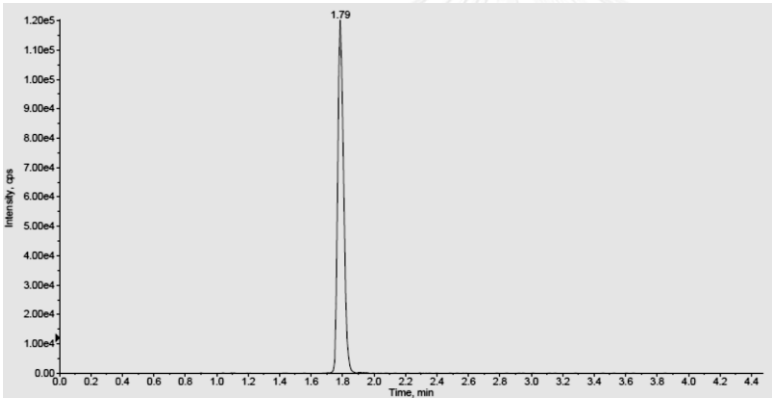


LC-MS/MS chromatograms for ECa 233.

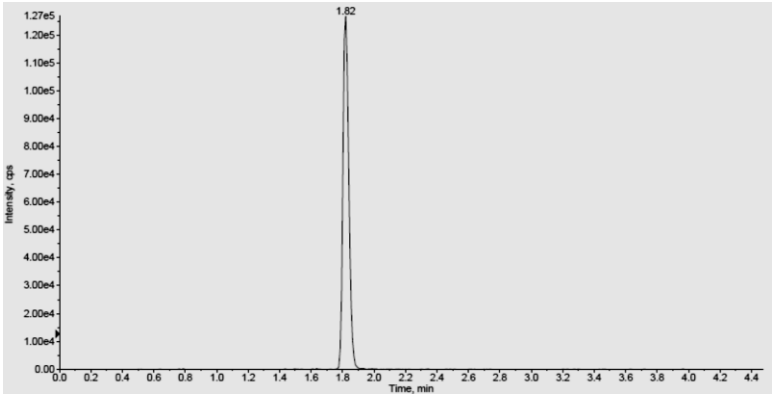
(a) Blank plasma



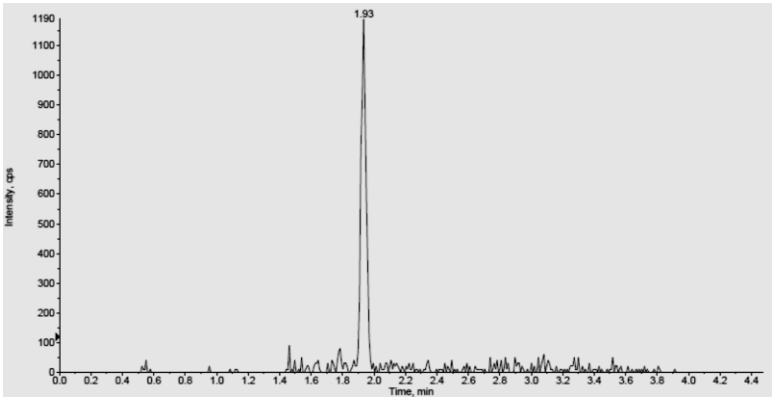
(b) 100 µg/L of madecassoside spiked into plasma



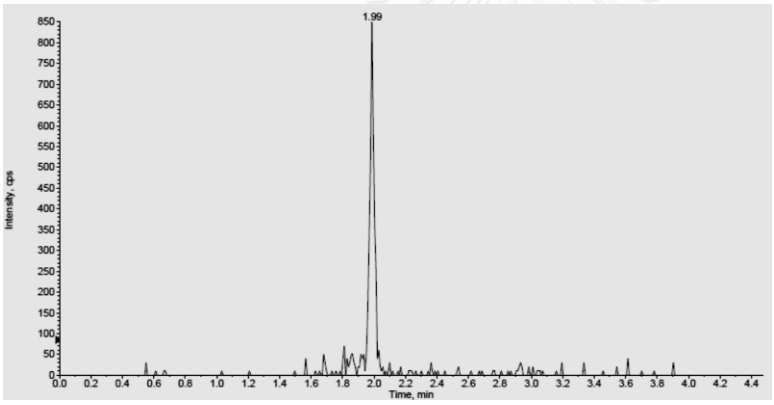
(C) 100 µg/L of asiaticoside spiked into plasma



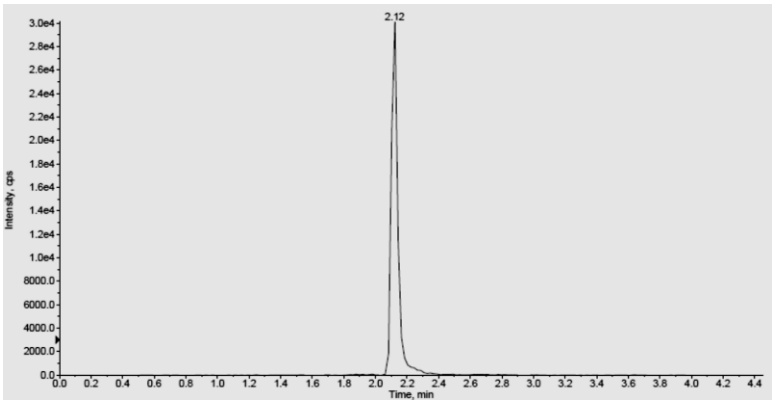
(d) 100 µg/L of madecassic acid spiked into plasma



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



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



APPENDIX C

LC-MS/MS conditions and validation of analytical system

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

The intra- and inter-day precision, accuracy and recovery 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

VITA

Miss Patcharaporn Hengjumrut was born on November 12, 1989 in Samutsakhon, Thailand. She graduated from Silpakorn University, Nakornpathom, Thailand in 2013 with a Bachelor of Pharmacy.