PHARMACOKINETIC AND METABOLOMIC STUDIES OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* (ECa 233) CAPSULE IN THAI HEALTHY VOLUNTEERS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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การศึกษาเภสัชจลนศาสตร์และเมแทบอโลมิกส์ ของแคปซูลสารสกัดมาตรฐานบัวบก (อีซีเอ 233) ในอาสาสมัครไทยสุขภาพดี

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สารสกัดมาตรฐานบัวบก อีซีเอ 233 ปัจจุบันได้มีการพัฒนาต่อยอดเป็นเภสัชภัณฑ์จากพืชสมุนไพรที่มีเป้าหมายเพื่อการนำมาใช้ประโยชน์ในมนุษย์ จึงนำมาสู่การนำมาใช้ศึกษาข้อมูลในทางคลินิกของงานวิจัยนี้ โดยมีวัตถุประสงค์ข้อแรกเพื่อ ศึกษากระบวนการเปลี่ยนแปลงทางเภสัชจลนศาสตร์ของอาสาสมัครสุขภาพดีภายหลังได้รับแคปซูลสารสกัดมาตรฐานบัวบก อีซีเอ 233 และวัตถุประสงค์ข้อที่สองเพื่อ ศึกษาข้อมูลทางเมแทบอโลมิกส์ และติดตามการเปลี่ยนแปลงแบบแผนของเมแทบอไลต์ที่สำคัญในร่างกายเมื่อรับประทานแคปซูล อีซีเอ 233 โดยในการศึกษาเภสัชจลนศาสตร์นั้น เป็นการศึกษาทางคลินิกระยะที่ 1 ในอาสาสมัครไทยสุขภาพดี จำนวน 12 คน ที่ได้รับแคปซูลสารสกัดมาตรฐานบัวบก อีซีเอ 233 ในขนาด 250 และ 500 มิลลิกรัม และอาสาสมัครอีก 12 คน ได้รับแคปซูลที่มีการปรับปรุงการละลายทั้งสองขนาด โดยทั้งสองกลุ่มมีการศึกษาเปรียบเทียบการรับประทานแบบครั้งเดียว และแบบรับประทานต่อเนื่องกันเป็นระยะเวลา 7 วัน จากการศึกษาพบว่า สารสำคัญซึ่งเป็นองค์ประกอบหลักของสารสกัดมาตรฐานบัวบก อันได้แก่ มาเดคาสโซไซด์ (madecassoside) และ เอเชียติโคไซด์ (asiaticoside) ถูกดูดซึมเข้าสู่กระแสเลือดได้ค่อนข้างน้อย อีกทั้งยังมีส่วนหนึ่งที่ถูกขับออกทางปัสสาวะในรูปของสารเดิมนี้ อย่างไรก็ตามสารทั้งสองจะถูกเปลี่ยนแปลงให้อยู่ในรูปเมแทบอไลต์ ได้แก่ กรดมาเดคาสสิก (madecassic acid) และกรดเอเชียติก (asiatic acid) จากนั้นสารเมแทบอไลต์ทั้งสองจะอาศัยกระบวนการขับออกจากร่างกายโดยผ่านทางอุจจาระเป็นหลัก นอกจากนี้จากผลการศึกษายังพบว่า เมื่อได้รับสารสกัดมาตรฐานบัวบก อีซีเอ 233 ในขนาดที่สูงขึ้น ส่งผลให้ระดับของสารสำคัญโดยเฉพาะสารออกฤทธิ์ในรูปเมแทบอไลต์ทั้งสองนี้สูงขึ้นในกระแสเลือดด้วย อีกทั้งยังพบการสะสมของกรดเอเชียติก (asiatic acid) อย่างมีนัยสำคัญเมื่อได้รับสารสกัดดังกล่าวต่อเนื่องกัน 7 วัน ทั้งนี้เมื่อพิจารณาร่วมกับผลการศึกษาทางเมแทบอโลมิกส์พบว่า สารสกัดอีซีเอ 233 มีผลต่อการเปลี่ยนแปลงรูปแบบของเมแทบอโลมในร่างกาย ซึ่งจะพบการเปลี่ยนแปลงระดับ แอลโฮโมเซอรีน (L-homoserine) ซิทรูลีน (citrulline) โอซัคซินิลแอลโฮโมเซอรีน (O-succinyl L-homoserine) โฮโมคาร์โนซีน (homocarnosine) และ โคลีน (choline) โดยเฉพาะอย่างยิ่งพบการเพิ่มขึ้นของโคลีนซึ่งเป็นเมแทบอไลต์ที่มีความสำคัญและจำเป็นต่อกระบวนการเรียนรู้และการจดจำ จึงนำไปสู่ข้อมูลสนับสนุนการดำเนินการศึกษาเพื่อใช้ประโยชน์จากสารสกัดมาตรฐานบัวบก อีซีเอ 233 ในผู้ที่มีภาวะบกพร่องทางการเรียนรู้และการจดจำ และสามารถนำตัวแทนเมแทบอไลต์เหล่านี้มาใช้ติดตามการเปลี่ยนแปลงทางเมแทบอโลมิกส์ภายหลังได้รับสารสกัดมาตรฐานบัวบก ในการศึกษาทางคลินิกระยะที่สองต่อไป

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Phanit Songvut : PHARMACOKINETIC AND METABOLOMIC STUDIES OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* (ECa 233) CAPSULE IN THAI HEALTHY VOLUNTEERS. Advisor: Asst. Prof. ROSSARIN TANSAWAT, Ph.D. Co-advisor: Assoc. Prof. PHISIT KHEMAWOOT, Ph.D.,Asst. Prof. PAJAREE CHARIYAVILASKUL, M.D.

A well-characterized standardized extract of *Centella asiatica* (ECa 233) being developed as a phytopharmaceutical product for human use was explored in this clinical study. The first objective of this research was to understand changes in disposition kinetics after oral administration of both the original-formula capsule and a newly-modified enhanced-dissolution capsule of ECa 233. Secondly, this study aimed to investigate alteration of human metabolomes following administration of the modified capsule of ECa 233, using an NMR-based metabolomics approach. For pharmacokinetics, the study involved a phase I clinical trial in twelve healthy Thai volunteers, who each received 250 and 500 mg of the original ECa 233 capsule and of the modified ECa 233 capsule, first as a single dose and then a week later as once-daily doses for 7 consecutive days. The results demonstrated that two major parent compounds, madecassoside (MDS) and asiaticoside (ASS), were rarely absorbed but rather underwent extensive biotransformation with minimal renal excretion. Those unabsorbed parent compounds were substituted with two mainly-active metabolites, madecassic acid (MDA) and asiatic acid (ASA). These metabolites were likely excreted through hepatobiliary system by feces elimination. Interestingly, increasing the dose of ECa 233 resulted in significantly greater plasma levels of these active metabolites, with accumulation of asiatic acid after multiple oral administration. Considering metabolomics, this accumulation behavior could affect human metabolome, partly through an alteration between pre- and post-dose of endogenous metabolites detected in plasma. The changes in five relevant metabolites were thoroughly considered to identify candidate biomarkers; these were L-homoserine, citrulline, O-succinyl L-homoserine, homocarnosine, and choline. In particular, ECa 233 was associated with a significant increase in levels of choline, an endogenous metabolite reported to have benefits for learning and memory. This finding suggests that ECa 233 may adjust human metabolic profiles and in this way play a role in fulfilling endogenous metabolites, which might be useful in mitigating cognitive impairment in phase II clinical study.

Field of Study: Pharmacology and Toxicology Student's Signature .....

Academic Year: 2020 Advisor's Signature .....

Co-advisor's Signature .....

Co-advisor's Signature .....

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RESEARCH ORIENTATION

This compilation thesis was written as a thesis by publication, comprising two published articles assembled according to the Chulalongkorn University's regulations. The thesis outline includes 5 chapters as follows:

Chapter I: background and rationale, which clarifies the main research questions of the thesis and explains connections with these two articles

Chapter II: literature review, a comprehensive overview of relevant information from other research documents

Chapter III: published article I, which is written according to the Planta Medica journal's guideline

Songvut, P., Chariyavilaskul, P., Tantisira, M. H. & Khemawoot, P. Safety and pharmacokinetics of standardized extract of *Centella asiatica* (ECa 233) capsules in healthy Thai volunteers: a phase 1 clinical study. Planta Med. 85, 483–490 (2019).

Chapter IV: published article II, which is written according to the Scientific Reports journal's guideline

Songvut, P., Chariyavilaskul, P., Khemawoot, P. Tansawat R. Pharmacokinetics and metabolomics investigation of an orally modified formula of standardized *Centella asiatica* extract in healthy volunteers. Sci Rep 11, 6850 (2021).

Chapter V: summary, a conclusion that integrates all pieces of work in this thesis and coherently presents key findings of both publications. This chapter also includes a discussion of the study's limitations and suggestions for future avenues of inquiry building on this research.

CHAPTER I

BACKGROUND AND RATIONALE

Centella asiatica (L.), belonging to the family Apiaceae, was developed as a standardized extract of *C. asiatica* and named 'ECa 233' [1]. The constituents of ECa 233 were characterized and the results demonstrated that it contains major contents of triterpenoid glycosides not less than 80% in composition, with a proportion of madecassoside (MDS) and asiaticoside (ASS) at 1.5 ± 0.5:1 [2]. This extract has been continuously investigated for safety and for its pharmacological activities. The evidences indicated that ECa 233 possesses neuroprotective effects by reducing learning deficits in preclinical (*in-vivo)* studies [3,4]. These findings increased interest in the efficacy of *C. asiatica* in terms of improving cognitive impairment, including enhancing learning and memory and mitigating Alzheimer's disease [5]. A recent consolidated report on safety evaluation of ECa 233 has shown the tolerability of this extract in preclinical toxicology studies in rats [6].

For pharmacokinetic studies, the possible kinetics was first determined in a rat model [7]. Then, a study with beagle dogs was carried out in order to observe the interspecies differences. Later, a study on safety and clinical pharmacokinetics were undertaken in healthy volunteers by carrying out oral administration of ECa 233 capsules in dosages of 250 and 500 mg of ECa 233, which is the first part of this thesis [8]. The objectives of the first study were, therefore, to investigate safety profiles of ECa 233 and to determine pharmacokinetics of the four major bioactive compounds: madecassoside, asiaticoside, and their metabolites (madecassic acid and asiatic acid). The study found that ECa 233 were rarely absorbed and had limited dissolution, leading to a restriction of its oral bioavailability [8]. Hence, a modified formula for an ECa 233 capsule was developed with enhanced dissolution and used in the second part of this research. Furthermore, when considering the results from the first part, most pharmacokinetic parameters were illustrated; however, excretion

data was still limited, since neither elimination half-life nor clearance of ECa 233 were discussed. The additional objectives in the second part of this thesis, therefore, were to investigate the completed clinical pharmacokinetics profiles, including the excretion pathways, and to provide all possible-determined pharmacokinetic parameters after the volunteers had received the modified formula of ECa 233 [9].

By taking the approach to develop ECa 233 as an alternative herbal medicine, metabolomic profiling was also investigated in the second part of this research, since metabolome can directly reflect changes in the physiological state after oral administration of specific compounds. For these reasons, the objective of this metabolomics study was to understand the metabolic consequences of taking ECa 233 [9]. This investigation of human metabolome was carried out by using metabolites-driven development of biomarkers. Detectable candidate metabolites with observable alterations in their levels in plasma were investigated through their comparing profiles before and after participants received the modified ECa 233 capsule. In addition, possible association between these changes in levels of the candidate metabolites and the metabolite-related biomarkers of neurodegenerative disease were also thoroughly considered. All in all, findings in the two published articles in this thesis strongly support further investigation of ECa 233 in phase II clinical trial in mild cognitive impairment patients.

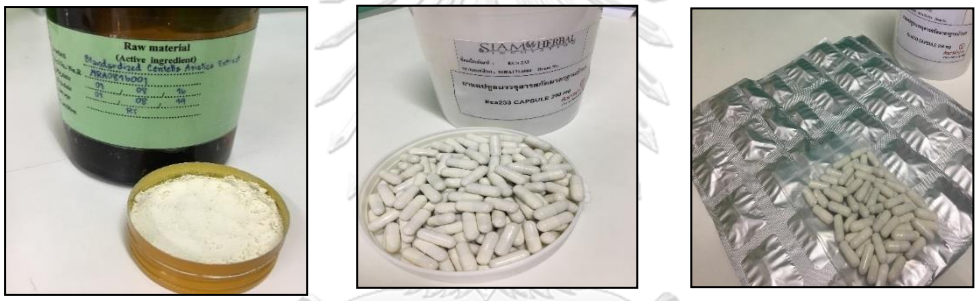
CHAPTER II

REVIEW LITERATURE

Table 1 Scientific Classification

Classification [10]	**Name**
Family	Apiaceae or Umbelliferae
Genus	Centella
Species	*Centella asiatica*
In Thailand, this plant is known as Bua-Bok. In India, it is commonly known as 'Indian Pennywort' or Gotu Kola.	

Figure 1 Standardized extract (ECa 233)



ECa 233 powder | ECa 233 Capsules | ECa 233 capsule in Alu-Foil Package

In vitro pharmacological studies of *Centella asiatica* extracts

C. asiatica extract contains more than one bioactive component; however, asiatic acid (ASA), a triterpenic acid found in ethanolic extract, is one that is most examined for its potential neuroprotective effects. The study [11] illustrated that ASA could exhibit 80% preventing amyloid-induced neurotoxicity and shows protective effects on free radical injury by reducing H_2O_2-induced cell death and lowering intracellular free radical concentration. Another *in vitro* study found that ASA promoted neurite elongation [5]. In addition, the results from a structure-activity relationship study suggested that triterpenic structure of ASA could be responsible for its biological activities related to neuroprotective effects [11].

Considering the other related pathways, phosphorylated form of CREB or pCREB, the cyclic AMP response element binding protein, also demonstrated an important role in learning and memory formation [12]. An evidence showed a reduction in the level of phosphorylated CREB in cases of Alzheimer's Disease in both human and animal models [13]. Examination of molecular mechanism of ECa 233 on CREB was therefore conducted. The results showed that *C. asiatica* extract containing ASA could elevate the level of phosphorylated CREB and might enhance the arborization of neurons leading to improvement of cognitive functions [14].

In vitro pharmacological studies of standardized extract (ECa 233)

In vitro studies have been carried out to understand the mechanisms that ECa 233 extract reduces cognitive impairment and improves neurodegenerative diseases. One potential mechanism is through neurite outgrowth in which ERK1/2 and Akt signaling pathways are the underlying drivers of its neuritogenic effects in human IMR-32 neuroblastoma cell line [15]. I*n vitro* studies found several possible molecular mechanisms through which *C. asiatica* extract, especially in terms of ECa 233 may have impact neurological disorders. Accordingly, *in vivo* studies of ECa 233 in animal models are still needed to consider its potential benefits on enhancing learning and memory.

In vivo efficacy studies of _Centella asiatica_ extracts

The neuropharmacological effects of *C. asiatica* extract have been investigated in *in vivo* studies, generating findings that are in line with the results of *in vitro* studies. The effects of *C. asiatica* aqueous extract in animal study, the results showed correlation between the extract and cognitive enhancement by improving learning and memory [16]. Furthermore, oral administration of *C. asiatica* ethanolic extract was linked with accelerated repair of damaged neurons and nerve

regeneration in the peripheral nervous system [5]. According to the data from these *in vitro* studies, ASA was considered to be the most potential active component of *C. asiatica* extract; therefore, preclinical research studies in the therapeutic use of ASA were also conducted. Male Sprague-Dawley rats received drinking water containing ASA demonstrated more rapid functional recovery and increased axonal regeneration [5]. Furthermore, the results in the mouse model illustrated that ASA could provide neuroprotective effects and improve neurological functions [17].

***In vivo* efficacy studies of standardized extract (ECa 233)**

To investigate the effects of ECa 233 on cognitive impairment in preclinical studies, a preliminary study was conducted in an animal model. Mice were pretreated with ECa 233 at doses of 10 mg/kg twice a day for 7 days, and then they received β-amyloid peptide by intracerebroventricular injection to induce Alzheimer-like cognitive deficits. Finally, learning and memory improving on spatial memory was evaluated by using Morris water maze test. The results suggested that pretreatment with ECa 233 was effective in ameliorating the cognitive deficits induced by β-amyloid peptide [4].

Preclinical toxicity studies of ECa 233

Table 2 Acute, sub-chronic, and chronic toxicity studies

Type of study	Species	Number/group	Unit dose	Route of administration, Dose interval, Duration of dosing	Duration of post-exposure follow-up
Acute toxicity	ICR mice	10 mice/group	10 g/kg	Oral, once	Observed twice daily for 14 days
Sub-chronic toxicity	Wistar rats	12 rats/group	10, 100, 1,000 mg/kg/day	Oral, once a day for 90 consecutive days	Observed every week for 90 days
Chronic toxicity	Wistar rats	28 rats/group	10, 100, 1,000 mg/kg/day	Oral, once a day for 180 consecutive days	180 days

Acute toxicity studies of ECa 233

The oral acute toxicity study found that single doses of 10 g/kg of ECa 233 were safe in mice, since no acute toxic effects were observed in the group receiving the highest dose and no lethality or pathological lesion was observed as well, the good safety profiles of single oral administration of ECa 233 was then demonstrated. Therefore, the 50% lethal dose (LD_{50}) in mice should be more than 10 g/kg [6].

Sub-chronic toxicity studies of ECa 233

In order to study the sub-chronic toxicity of ECa 233, Wistar rats were given 10, 100 and 1,000 mg/kg/day of ECa 233 by oral administration for 90 days. The results showed that the extract did not affect body weight, food consumption, or any aspect of the animals' health. Nonetheless, female rats receiving 1,000 mg/kg/day of ECa 233 showed a significant increase in white blood cells (WBC), but this increase could not be considered as leukocytosis. In addition, the blood chemistry evaluated in ECa 233-treated rats was not significantly different in

comparison with their sex-corresponding control groups, except for elevated sodium levels in male rats that received the highest dose. This change was still within normal sodium ranges, however, and these doses of ECa 233 did not cause any significant microscopic changes to vital organs in a dose-dependent manner. To summarize, no significant sub-chronic toxicity was observed in rats receiving 10-1,000 mg/kg of ECa 233 [6].

Chronic toxicity studies of ECa 233

The results of a chronic toxicity study of ECa 233 in rats revealed that orally given ECa 233 in doses of 10, 100 and 1,000 mg/kg/day for 180 consecutive days did not cause any effects on hematological or urine clinical chemistry parameters. Moreover, no gross pathological lesions were found for any of the doses. Although there was a change in the serum electrolytes in rats treated with 100 and 1,000 mg/kg/day, this alteration was still in the normal range of previously reported serum electrolytes. Therefore, this study could identify the NOAEL as more than 1,000 mg/kg/day, and the LOAEL (Lowest Observed Adverse Effect Level) was expected to be over 1,000 mg/kg/day (unpublished data).

Preclinical pharmacokinetics of ECa 233

Previous study demonstrated the pharmacokinetics of ECa 233 at 50 up to 200 mg/kg by oral or intravenous administration. The results showed that all animals had a good tolerability and none of them showed any observed changes or abnormalities throughout the experiments. A significant increase in aspartate aminotransferase (AST) after oral dosing at 50 mg/kg and a decrease in alanine aminotransferase (ALT) after oral dosing at 200 mg/kg of ECa 233 were noted; however, these changes were within the normal physiological ranges for Wistar rats [7].

Absorption

Madecassoside and asiaticoside were rapidly, but not completely, absorbed from the gastrointestinal tract. The maximum plasma concentration (C_{max}) and time to reach maximum concentration (T_{max}) of madecassoside were observed within 5–15 minutes after oral administration. The poor oral bioavailability of madecassoside and asiaticoside could be explained by their large molecular sizes and the presence of a sugar moiety in their structures. Madecassoside and asiaticoside have low lipid solubility, which could restrict their ability to cross the intestinal membrane [7]. In addition, madecassoside is a substrate for efflux transporters P-glycoprotein (P-gp) and multidrug-resistant protein 2 (MRP2), which might efflux the absorbed drug back into the gastrointestinal tract [18]. These effects provide a reasonable explanation for the poor absorption and low bioavailability of ECa 233.

Distribution

The first pharmacokinetic evidence of tissue distribution in an animal study demonstrated the presence of the parent compounds (madecassoside and asiaticoside) in target organs, especially in brain tissue. In addition, both parent

compounds reached the brain within 1 h after dosing and remained in those tissues until 4 h after dosing [7]. Another study showed accumulation of the mainly compounds of ECa 233 in hippocampus, the critical brain region for spatial memory formation, after receiving ECa 233 for 30 days [unpublished data].

Metabolism

Based on studies in rats receiving ECa 233, madecassic acid and asiatic acid have been suggested to be active metabolites formed by acid hydrolysis or esterase hydrolytic cleavage of the sugar moiety of their parent, triterpenoid glycoside [7]. After oral administration, ECa 233 was metabolized by hydrolase isozymes in intestinal bacteria [19]. Madecassoside and asiaticoside might be hydrolyzed by microflora and its enzymes in gastrointestinal tract.

Effects of ECa 233 on hepatic CYPs were investigated both *in vivo* and *in vitro*. Results from the *in vivo* study indicated that ECa 233 did not cause any changes in total CYP contents or the activities of CYP1A1, 1A2, 2B1/2B2, and 2E1 and 3A. Likewise, no effects of the extract on CYP1A1, 2E1 and 3A were observed in the *in vitro* study using rat liver microsomes [20].

Elimination

After an initial downward trend in the plasma concentration-time curves, the time period 4 h to 8 h showed an abrupt increasing of plasma concentrations possibly due to enterohepatic recirculation. This effect produced a significant prolongation of the terminal elimination half-life of madecassoside, which is similar to that of asiaticoside. The findings implied that madecassoside and asiaticoside might return to the blood circulation after elimination, probably by a pathway of enterohepatic circulation [21]. In order to test whether the hepatobiliary transporters

were involved in madecassoside elimination, the specific inhibitors of P-gp and MRP2 were used in a transporter experiment. The results demonstrated that both inhibitors significantly inhibited the excretion of madecassoside as a parent drug from bile, and that P-gp or MRP2 might be the main contributors to hepatobiliary elimination of madecassoside [18].

Comparative pharmacokinetics of ECa 233 and its pure compounds

A comparative pharmacokinetic study after intravenous administration of madecassoside, asiaticoside and ECa 233 found that plasma levels of madecassoside and asiaticoside were increased in the treated group of ECa 233. For pharmacokinetic parameters, the AUC of madecassoside and asiaticoside was significantly higher than pure compound administration. In addition, the elimination half-life of asiaticoside was significantly prolonged from 4.09±1.61 hours to 8.73 ± 2.25 hours [21]. Considering the plasma level of madecassoside after oral administration of ECa 233, C_{max} and AUC were at higher levels when compared to data in the group receiving pure compounds. The elimination half-life of madecassoside was also significantly prolonged from 4.33 ± 0.74 hours to 7.68 ± 1.96 hours after ECa 233 was administered [21].

This study found a bidirectional interconversion between asiaticoside and madecassoside. This phenomenon was observed in plasma at all sampling time points after oral and intravenous administration of individual compounds. The interconversion pharmacokinetic parameters were calculated to determine the rate and extent of conversion **(Figure 2)** [21].

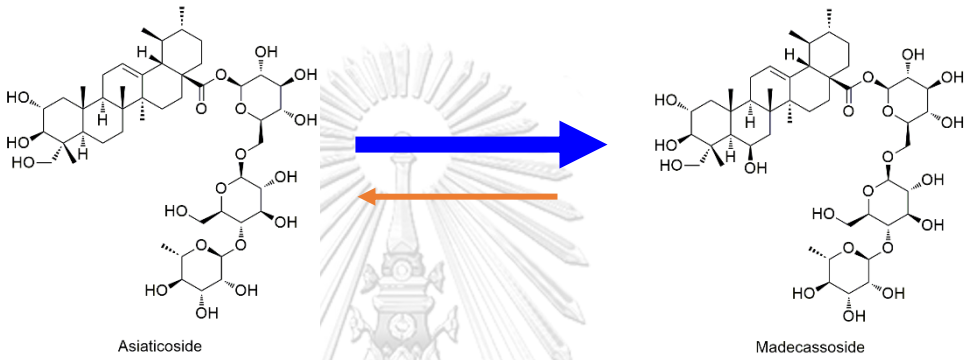


Figure 2 Interconversion between asiaticoside and madecassoside.

Metabolomics Reviews

Metabolomics is defined as the systematic biology study of metabolite profiles or products of endogenous metabolism in biological samples [22]. Metabolomics has been applied in the investigation of neurological pathologies including cognitive impairments and memory deficits, which lack effective biomarkers [23]. Going further, application of metabolomics strategies for diagnosis of the disease is now an attractive avenue to explore since the metabolome may directly reflect the specific physiological and pathological state of patients [24]. Several studies point to the use of advanced metabolomics in drug discovery and development in order to gain understanding through the effect of drug related human metabolites on specific biomarkers of the disease.

Considering the limitations of AD diagnosis, currently available hallmarks for AD are measurements of proteins in the CSF, which closely reflects the extracellular composition in the brain and is likely the most effective clinical biomarker at present. The limitation of this diagnostic method, however, is that taking CSF from the spinal cord likely invasive procedure, making it unsuitable for routine use, particularly in elderly individuals [25]. For these reasons, the recently investigated biomarkers have been identified in plasma by using metabolites-driven development of biomarkers. The identification of associated metabolites in plasma samples could be more widely applicable in AD diagnosis, as collection of these samples is far less invasive compared to CSF diagnosis.

To investigate the correlation between blood and CSF samples, candidate metabolomes of AD patients were identified and the data from both samples were integrated. The results showed that nearly 60% of the metabolic pathways altered in patients are affected in both CSF and plasma samples [23]. This finding might be

explained by the confluence of biomolecules, which are generally transported between blood and CSF; therefore, the alteration of metabolites in blood could possibly reflect the CSF changing and imply the CNS working. For these reasons, applying blood sample in metabolomics to explore the relevance of changing endogenous metabolites may not only provide the specific candidate biomarkers for drug related disease, but also facilitate the development of new therapeutic strategies.

Overall objectives

To investigate pharmacokinetics and metabolomics of standardized extract of *Centella asiatica* (ECa 233) capsule in Thai healthy volunteers after single and multiple oral administration.

Specific objectives

1. To investigate the clinical pharmacokinetic profiles of standardized extract of *Centella asiatica,* ECa 233 both in parent compounds and their metabolites, including pharmacokinetic parameters and dose accumulation, after oral administration of ECa 233 capsules.
2. To identify the alteration of endogenous human metabolomic profiling following administration of ECa 233 capsules and propose the candidate endogenous metabolites that could support further studies in phase II clinical trial.

Expected benefits

5.1) This study will present the clinical pharmacokinetic profiles after oral administration of ECa 233 capsules, reported as first in human evidence, including its pharmacokinetic parameters and the dose accumulation of its bioactive components.

5.2) This study will discover the candidate endogenous human metabolites that change in plasma after administration of the ECa 233 capsule. These candidates will also be compared with the metabolite biomarkers of Alzheimer's patients, potentially supporting the further investigation of ECa 233 in clinical applications through a phase II study.

Scope of the research

This study mainly focuses on pharmacokinetics and metabolomics in healthy volunteers after receiving standardized extract of *Centella asiatica*, ECa 233 capsules: A phase I clinical study.

CHAPTER III

PUBLISHED ARTICLE I

Title: Safety and Pharmacokinetics of Standardized Extract of *Centella asiatica* (ECa 233) Capsules in Healthy Thai Volunteers: A Phase 1 Clinical Study

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Safety and pharmacokinetics of standardized extract of *Centella asiatica* (ECa 233) capsules in healthy Thai volunteers: A phase 1 clinical study.

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Abstract

The aim of this study was to investigate the safety and pharmacokinetic profiles of a newly developed, standardized extract of *Centella asiatica* (ECa 233) capsule in healthy Thai volunteers. This study was designed as an open-labeled, two-sequence dosage, single- and repeated-dose study investigated under fasting conditions. Plasma concentrations of the parent compounds and their relative acid metabolites were measured and pharmacokinetic parameters were calculated using non-compartmental analysis. Tolerability was assessed based on physical examinations, monitoring of vital signs, clinical laboratory tests, and any observed adverse events. A key finding of this study was that the pharmacokinetics of ECa 233 in healthy volunteers did not correspond with its pharmacokinetics in animal studies. As indicated in human pharmacokinetic parameters, the mean maximum plasma concentration (Cmax) and mean area under the curve ($AUC_{(0-t)}$) of the parent compounds (madecassoside and asiaticoside) were very low, while their respective metabolites (madecassic acid and asiatic acid) demonstrated higher values. Based on the pharmacokinetic results observed in the dose comparison, accumulation of active metabolites after repeated dose is highly suggestive. In addition, the asiatic acid profile showed two-fold increase in Cmax and $AUC_{(0-t)}$ after increasing dose from 250 to 500 mg of ECa 233. Lastly, the safety and tolerability evaluation illustrated that single and multiple doses in both 250 and 500 mg oral administration of ECa 233 were well tolerated, and none of the volunteers discontinued their participation due to adverse effects during the study.

Keywords

Centella asiatica, Apiaceae, pharmacokinetics, madecassic acid, asiatic acid, triterpenoid glycosides

Abbreviations

AEs, Adverse events

ALT, Alanine aminotransferase

ASA, Asiatic acid

ASS, Asiaticoside

AST, Aspartate aminotransferase

$AUC_{(0-t)}$, Area under the curve from time zero to the last sampling time

BMI, Body mass index

CL/F, Apparent total clearance of the test substance removed from plasma after oral administration

Cmax, Maximum plasma concentration

CV, Coefficient of variation

ECa 233, Standardized extract of *Centella asiatica*

HED, Human equivalent dose

IS, Internal standard

LLOQ, Lower limit of quantification

MDA, Madecassic acid

MDS, Madecassoside

MRP2, Multidrug resistance-associated protein 2

MRSD, Maximum recommended starting dose

NOAEL, No observed adverse effect level

P-gp, P-glycoprotein

p.o. , Per os or oral administration

RE, Relative error

Tmax, Time to reach maximum plasma concentration

Vd/F, Apparent volume of distribution after non-intravenous administration

Introduction

Cognitive impairment has been described as a neurological disorder that usually causes deficits in specific brain functions, particularly a decline in learning memory and a loss of thinking ability (1). These cognitive deficits mostly result from progressive neurodegeneration, leading to an increase in the risk of developing Alzheimer's disease or other forms of dementia (2). As the accumulation of beta-amyloid is considered a hallmark of Alzheimer's disease, approaches dealing with amyloid have been proposed to slow disease progression (3). Additionally, the role of traditional medicine substances is also being investigated. *Centella asiatica* (Linn.), or Bua-Bok in Thai, is a member of the plant family Apiaceae that has been used for several medicinal purposes (4). Based on several preclinical pharmacology studies, there is increased interest in the efficacy of *C. asiatica* in terms of improving cognitive impairment, including enhancing learning memory (5,6) and its role in mitigating Alzheimer's disease (7).

Researchers from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand have developed a standardized extract of *C. asiatica* named 'ECa 233' (8). The major contents of ECa 233 are triterpenoid glycosides, with more than 80% with a constant proportion of madecassoside (MDS) and asiaticoside (ASS) at 1.5 ± 0.5:1 (9). *In-vivo* pharmacological investigations of ECa 233 showed a reduction in learning deficit and memory in mice (10,11). An acute toxicological study showed that the median lethal dose of ECa 233 was more than 10 g/kg after oral administration in mice (12). Moreover, the toxicity test in rats at doses of 10, 100 and 1,000 mg/kg/day of ECa 233 for 180 days revealed no significant differences between the treatment and control groups (unpublished data). These led to an estimation of the no-observed-adverse-effect level (NOAEL) of more than 1,000 mg/kg/day after oral administration in rats.

A pharmacokinetic study of ECa 233 in rats demonstrated that the parent compounds, MDS and ASS, were rapidly, but not completely, absorbed in the gastrointestinal tract. The maximum plasma concentration (Cmax) was detected within 5–15 min after oral administration. However, these parent compounds had poor oral bioavailability, whereas the tissue distribution was unrestricted in rats. The predominant existence of both parent compounds was shown in many target organs, particularly brain tissue, possibly contributing to the cognitive-enhancing effect of ECa 233 (13). Both MDS and ASS are metabolized by intestinal microflora via a hydrolytic cleavage, resulting in active metabolites: madecassic acid (MDA) and asiatic acid (ASA), respectively (14). These compounds are then finally excreted in the urine and feces (13,15). All rats in these pharmacokinetic studies showed normal physical appearances and normal liver and kidney functions, both pre-dose and post-dose (24 h) of ECa 233.

Although preclinical studies reported benefits of ECa 233, some knowledge gaps in human safety data and human pharmacokinetic profiles still remain, especially in terms of the pharmacokinetics of the four major bioactive compounds (MDS, ASS, MDA, and ASA; **Fig. 3**). The objectives of this study were, therefore, to investigate the pharmacokinetic and safety profiles of ECa 233 oral administration to healthy individuals. To justify the dose selection of ECa 233 in this study, a conversion from the relevant safety and efficacy doses in animals was completed. To calculate the first dose in humans, guideline for estimating maximum recommended starting dose (MRSD) in initial clinical trials was applied (16). Considering the safety profile of ECa 233, the animal NOAEL (> 1,000 mg/kg/day, p.o.) was determined based on results of toxicity studies in rats and it was then converted to the human equivalent dose, HED. This calculated HED was further converted to the MRSD;

however, the pharmacologically active dose in animals (100 mg/kg, p.o.) was also taken into consideration for determining the first dose in humans (5–10 mg/kg, p.o.).

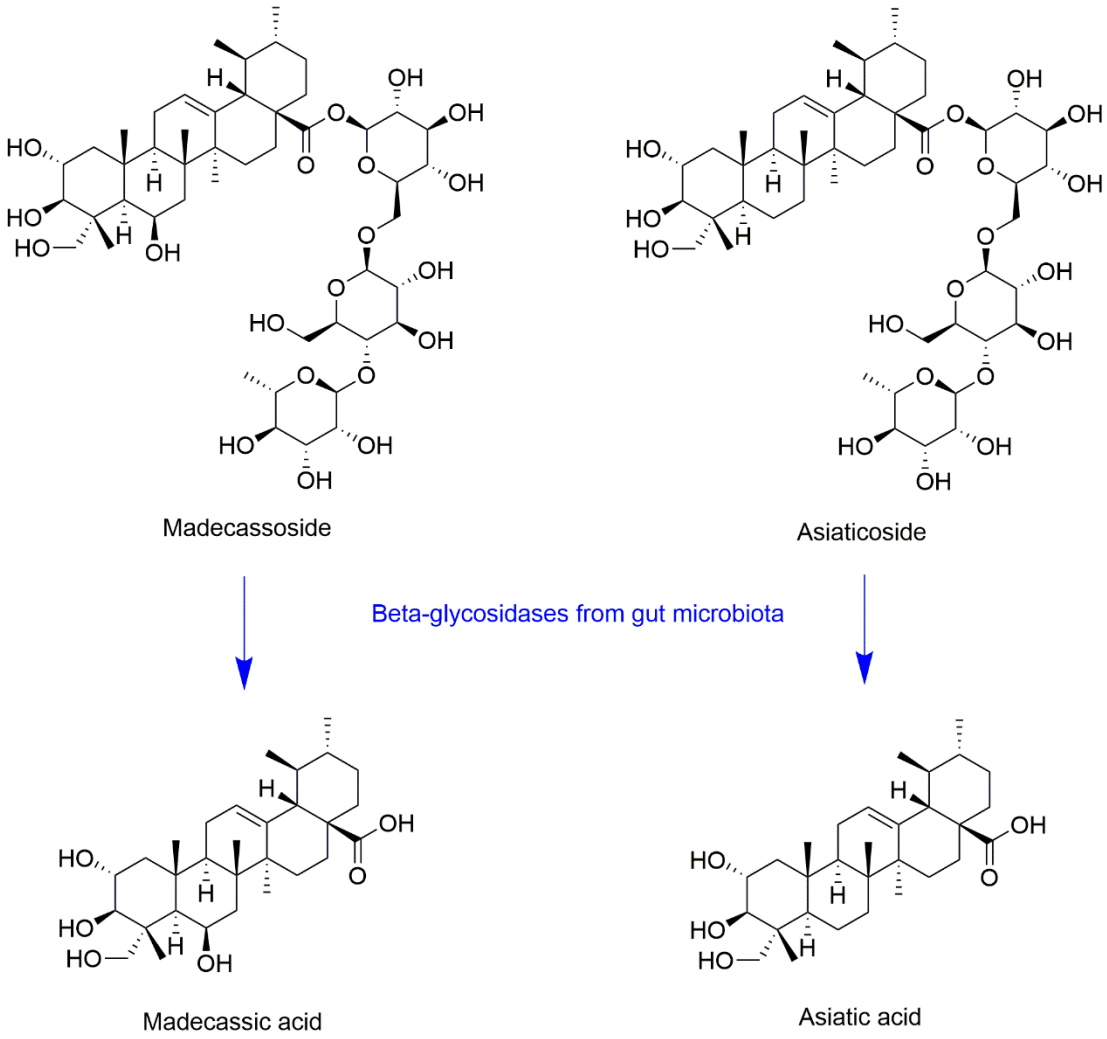

Figure 3 Chemical structures of madecassoside, asiaticoside, madecassic acid, and asiatic acid.

Results and Discussion

Twelve Thai healthy volunteers were enrolled in this study. Baseline characteristics of the subjects are shown in **Table 3**. One subject did not complete the first period of the study due to abnormal blood pressure prior to the drug administration and one subject withdrew their consent prior to the second study period. As such, there were 11 and 10 subjects in the first and second period of the study, respectively. Clinical laboratory screening, including hematology, electrolytes, kidney and liver functions, was completed after oral administration of multiple-dose ECa 233 for 7 days **(Table 3)**. All participants showed normal range of clinical laboratory analysis throughout the first period of the study (250 mg) and then they were permitted to receive a higher dose (500 mg) in the second study period. This dose escalation of ECa 233 (250 to 500 mg) was well tolerated in subjects whether it was administered as single or multiple doses.

Table 3 Demographic and baseline characteristic data of participants administered 250 and 500 mg ECa 233 capsules.

Demographic data		Baseline	ECa 233, 250 mg	ECa 233, 500 mg
Gender, % (n)	Female		36% (n=4)	40% (n=4)
	Male		64% (n=7)	60% (n=6)
Age at enrollment[b] (year)			31.3 ± 8.79	30.55 ± 8.71
Body mass index[a,b] (kg/m^2)			22.15 ± 1.94	21.93 ± 1.98
Systolic blood pressure (mmHg)		118 ± 10	112 ± 9	115 ± 13
Diastolic blood pressure (mmHg)		66 ± 8	65 ± 12	65 ± 12
Body temperature (ºC)		36.7 ± 0.3	36.5 ± 0.2	36.6 ± 0.2
Clinical laboratory screening [b]				
White blood cell (x10^3/µL)		7.37 ± 1.67	6.76 ± 1.42	6.16 ± 1.42
Red blood cell (x10^6/µL)		5.31 ± 0.36	5.15 ± 0.40	4.99 ± 0.40
Hemoglobin (g/dL)		13.8 ± 1.3	13.3 ± 1.2	13.0 ± 1.3
Platelet (x10^3/µL)		270 ± 40	277 ± 66	274 ± 58
Fasting blood glucose (mg/dL)		87.36 ± 6.36	85.09 ± 5.92	83.50 ± 5.84
Blood urea nitrogen (mg/dL)		12 ± 4	13 ± 4	14 ± 4
Creatinine (mg/dL)		0.81 ± 0.15	0.85 ± 0.19	0.86 ± 0.14
Albumin (g/dL)		4.33 ± 0.25	4.26 ± 0.24	4.26 ± 0.23
Total bilirubin (mg/dL)		0.72 ± 0.35	0.59 ± 0.23	0.62 ± 0.30
SGOT, AST (U/L)		21 ± 7	20 ± 6	20 ± 8
SGPT, ALT (U/L)		25 ± 17	22 ± 11	22 ± 12
Alkaline phosphatase (U/L)		64 ± 21	58 ± 17	60 ± 18
Total cholesterol (mg/dL)		179 ± 25	189 ± 25	178 ± 27
Triglycerides (mg/dL)		92 ± 47	78 ± 32	72 ± 17
HDL cholesterol (mg/dL)		57 ± 8	53 ± 7	54 ± 8
LDL cholesterol (mg/dL, calculated)		103 ± 24	120 ± 24	110 ± 26
Sodium (mmol/L)		139 ± 1	139 ± 1	142 ± 1
Chloride (mmol/L)		105 ± 2	106 ± 3	107 ± 2
Potassium (mmol/L)		4.2 ± 0.2	4.3 ± 0.3	4.1 ± 0.2
Calcium (mg/dL)		9.5 ± 0.3	9.5 ± 0.3	9.2 ± 0.3
Magnesium (mg/dL)		2.4 ± 0.2	2.5 ± 0.1	2.4 ± 0.2
Phosphorus (mg/dL)		3.5 ± 0.5	3.6 ± 0.5	3.8 ± 0.5

[a]BMI was defined as the body mass divided by the square of the body height.

[b]Data expressed as mean ± SD, (n = 10-11).

Decimal numbers were reported according to laboratory standard of Chula Clinical Research Laboratory, Chula Clinical Research Center, Faculty of Medicine, Chulalongkorn University.

The plasma concentration-time profiles of the four major bioactive components of ECa 233 after single and multiple dose oral administration are shown in **Fig. 4**. The plasma concentrations of the metabolites (MDA and ASA) were much higher than the concentrations of their respective parent compounds (MDS and ASS) in both the single and multiple dose study, significantly increasing with multiple dosing. These data support the previous hypothesis that the two major parent compounds are biotransformed in the human body to their respective metabolites (14,17). Human pharmacokinetic findings were not consistent with results in rats, where parent compounds were observed in rat plasma and tissue compartments (13,15). This discrepancy between human and animal data suggests that there may be a species difference in the biotransformation of the major components of ECa 233, which could be associated with microflora and its enzyme. One study indicated that β-glycosidase from human intestinal bacteria can hydrolyze glycosides, whereas animal β-glycosidase does not hydrolyze these glycosides (18). According to the current understanding of the biotransformation of ECa 233, MDS and ASS are not easily absorbed from the gastrointestinal tract since they are glycosides, which contain large hydrophilic sugars. They are also resistant to gastric acid or any digestive enzymes (18), so they are mostly retained in the GI tract. Thus, these glycosides can pass through the upper intestinal tract then continue to exist in the lower tract, where numerous anaerobes are present as resident microbiota. Consequently, these unabsorbed glycosides (MDS and ASS) are hydrolyzed to aglycones (MDA and ASA) by enzymes produced by intestinal bacteria; this is probably Eubacterium spp., which can produce β-glycosidase, an enzyme responsible for hydrolyzing glycosides (14,18).

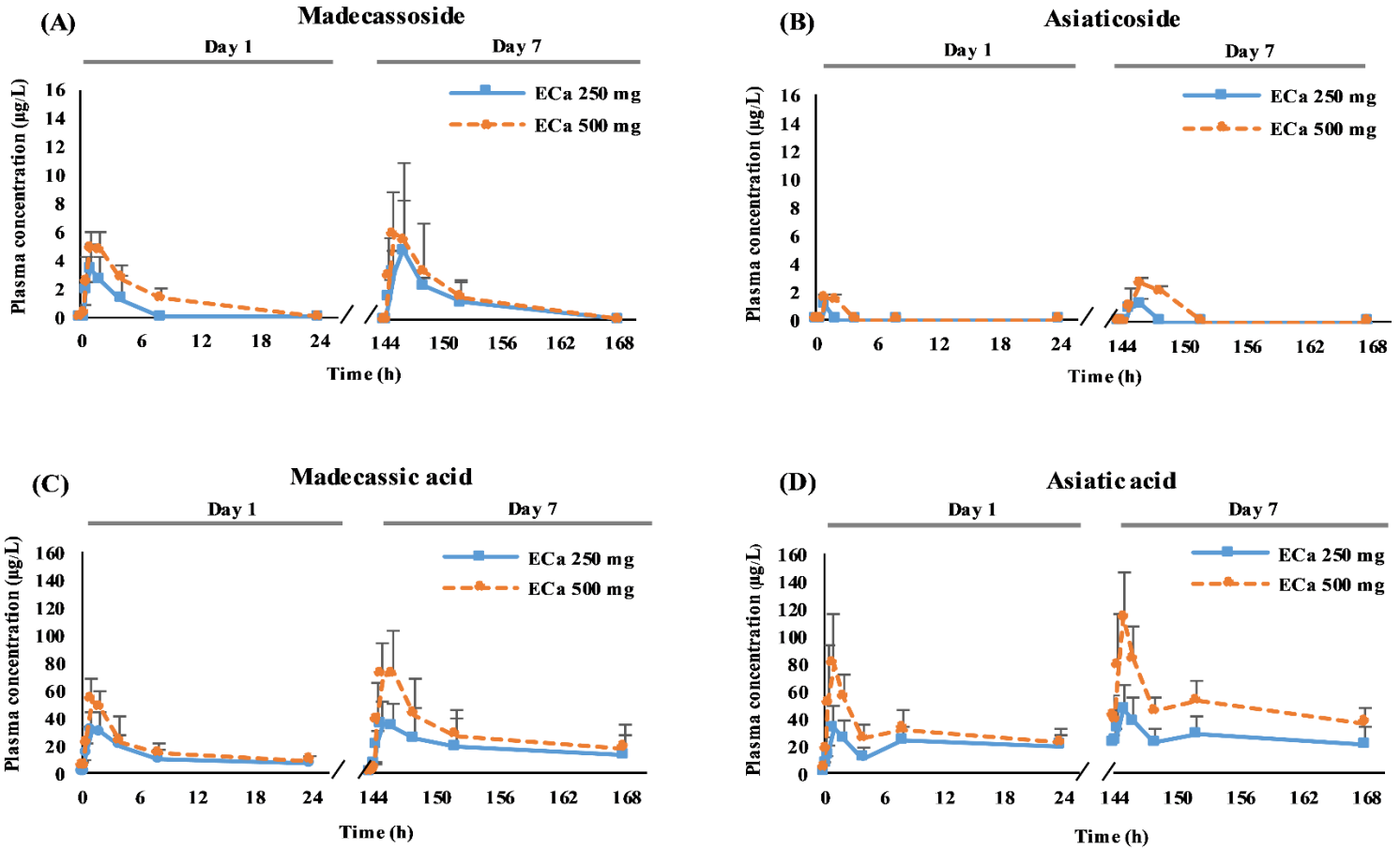

Figure 4 Plasma concentration versus time profiles of (A) madecassoside, (B) asiaticoside, (C) madecassic acid, and (D) asiatic acid after single or multiple dose oral administration of 250 mg or 500 mg ECa 233 capsules. Data are presented as means ± SD (n = 10-11).

Table 4 Pharmacokinetic parameters of madecassoside and asiaticoside (A), madecassic acid and asiatic acid (B) after oral administration of ECa 233 capsules, comparing single and multiple doses.

(A)

	Parent Compounds (Triterpenoid glycosides)							
	Madecassoside				Asiaticoside			
	Day 1		Day7		Day 1		Day7	
Pharmacokinetic parameters	250 mg (n =11)	500 mg (n =10)	250 mg (n =11)	500 mg (n =10)	250 mg (n =11)	500 mg (n =10)	250 mg (n =11)	500 mg (n =10)
Cmax[a] (µg/L)	3.55 ± 1.79	5.67 ± 0.62	5.75 ± 3.17	5.23 ± 1.84	1.043 ± 0.14	1.50 ± 0.18	5.93 ± 21.20	2.71 ± 1.08
Tmax[b] (h)	1 [2]	1 [1]	2 [2]	1 [0]	1 [0]	1 [1]	2 [0]	2 [0]
AUC[a] (µg·h/L)	12.43 ± 6.70	30.12 ± 7.12	22.1 ± 11.68	37.29 ± 16.52	1.0 ± 0.23	1.70 ± 0.53	10.8 ± 10.18	11.40 ± 9.67
Vd/F[c] (L/kg)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cl/F[c] (L/h/kg)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Half-life[c] (h)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Notes: [a] data are expressed as mean ± SD; [b]data is expressed as median [IQR].

[c] data were not reported since most values of plasma concentration were below LLOQ.

Abbreviations: Cmax, maximum plasma concentration; Tmax, time to reach Cmax; AUC, area under the plasma concentration–time curve from time zero to the last measurable concentration; Vd/F, the apparent volume of distribution; Cl/F, the apparent clearance.

(B)

	Active metabolites (Triterpenic acid)							
	Madecassic acid				Asiatic acid			
	Day 1		Day7		Day 1		Day7	
Pharmacokinetic parameters	250 mg (n =11)	500 mg (n =10)	250 mg (n =11)	500 mg (n =10)	250 mg (n =11)	500 mg (n =10)	250 mg (n =11)	500 mg (n =10)
Cmax[a] (µg/L)	40.92 ± 25.78	52.14 ± 18.67	42.71 ± 21.06	80.79 ± 27.76	38.02 ± 12.21	84.08 ± 33.91*	51.28 ± 17.91	116.62 ± 32.26*
Tmax[b] (h)	2 [1]	1.5 [1]	1 [1]	1.5 [1]	1 [7]	1 [0]	1 [1]	1 [0]
AUC[a] (µg·h/L)	348.44 ± 362.47	357.20 ± 116.65	412.66 ± 375.88	681.05 ± 413.17	434.13 ± 195.72	724.75 ± 259.98*	624.97 ± 277.14	1202.29 ± 293.37*
Vd/F[a] (L/kg)	32.45 ± 17.43	50.80 ± 58.02	28.43 ± 26.30	57.20 ± 42.12	35.89 ± 28.08	35.29 ± 9.68	24.45 ± 11.65	23.51 ± 9.24
Cl/F[a] (L/h/kg)	5.98 ± 5.41	4.55 ± 2.86	4.06 ± 3.13	3.68 ± 3.25	1.05 ± 1.58	0.92 ± 0.41	0.55 ± 0.52	0.52 ± 0.29
Half-life[a] (h)	15.11 ± 2.42	35.28 ± 36.22	20.94 ± 9.21	27.27 ± 17.82	31.57 ± 14.97	38.52 ± 29.51	29.19 ± 17.46	38.17 ± 18.33

Notes: [a]data are expressed as mean ± SD; [b]data is expressed as median [IQR]; * $p < 0.05$ for significant differences.

Abbreviations: Cmax, maximum plasma concentration; Tmax, time to reach Cmax; AUC, area under the plasma concentration–time curve from time zero to the last measurable concentration; Vd/F, the apparent volume of distribution; Cl/F, the apparent clearance.

The pharmacokinetic parameters of bioactive compounds from ECa 233 are listed in **Table 4A** and **4B**. The plasma levels of MDS and ASS reached Tmax within 1-2 h of oral dosing, but Cmax and AUC were very low. This could be explained by the large molecular size and sugar moiety in the chemical structures of triterpenoid glycosides, which could lead to a low lipid solubility, restricting their ability to pass through human intestinal membranes (13). Actually, the parent compounds of ECa 233 seems to be mostly biotransformed into active metabolites (MDA and ASA) prior to absorption (19), which become predominant compounds in the plasma concentration-time curve. As expected, the upward trend of these metabolites appeared adjacent to the downward trend of their parent compounds. The metabolites reached Tmax within 3 h after ingestion of ECa 233. Noticeably, Cmax and AUC of ASA obtained during a period of 500 mg oral administration of ECa 233 were approximately two-fold greater than those parameters obtained during a period of 250 mg oral administration of ECa 233. Furthermore, the accumulation of ASA is indicated by the manner in which Cmax and AUC showed statistically significant differences between single and multiple doses (day 1 vs. day 7, $p < 0.05$). This accumulation could possibly account by ASA which may strongly inhibit P-gp expression and may have increased the systemic exposure of ASA (20). Interestingly, plasma concentration-time curves of ASA are much higher when compared to the concentrations of other compounds presented in healthy volunteers, implying that ASA seems to be the most therapeutically active metabolite in humans. As mentioned in the *in vitro* studies, some evidences indicated that ASA could exhibit 80% preventing on amyloid-induced neurotoxicity and show the strongest protective effects on free radical injury by reducing H_2O_2-induced cell death and lowering intracellular free radical concentration. In addition, the results from a structure-activity relationship study suggested that a triterpenic structure of ASA could be responsible for its biological activities on neuroprotective effects (21). The preclinical

research in therapeutic potential of ASA indicated that ASA could improve neurological function in a mouse model and provide neuroprotective effects (22). It is considered that ASA showed the physicochemical properties, an un-ionized highly lipophilic molecule (log P = 5.7) with 488.709 g/mol molecular weight and nine cumulative hydrogen bonds in chemical structure, which could lead to blood-brain barrier (BBB) permeability related to BBB restoration and neural tissue survival (23).

Taken together, the comparative steady-state bioavailability of ASS and ASA (equivalent doses) was evaluated in healthy volunteers. The result was reported that AUC0-12h values following dosing with ASS or ASA were not significantly different (P = 0.90). Thus, the bioavailability of ASS is comparable to ASA when taken orally equivalent dose. Considering ASA administered directly, it provided a favorable Cmax and a slightly rapid Tmax; however, direct receiving ASS appeared to prolong the terminal elimination half-life of ASA. Taking into consideration that mitigating cognitive impairment requires a long-lasting stimulation of brain functions at well-tolerated doses, ECa 233 containing ASS seems to be a reasonable candidate for Alzheimer treatment. The therapeutic effect of ASS might be mediated through its conversion into ASA in human plasma levels and prolonged the therapeutic exposures compared with ASA direct administration (14).

For safety and tolerability, ECa 233 was safe and well tolerated as no serious adverse events were reported. Mild to moderate adverse events were reported in five subjects; four adverse events were categorized as unrelated to the medication, while six events were considered ECa 233-related (four probably and two unlikely). Adverse events were moderate drowsiness (n = 3), mild stomach irritation (n = 1), moderate frequent urination (n = 1), and moderate headache (n = 1). All adverse events started after dosing and remained only in the period of the single-dose study.

The participants, however, could tolerate the repeated doses even at the high dosage regimen **(Table 5).** Moreover, results showed that there was consistent safety data in humans and animals, since there were no significant changes in screening parameters or any physical appearances throughout the study.

This is the first report for a clinical pharmacokinetic study of ECa 233 in healthy volunteers where active metabolites were mainly observed. The daily doses of 250 mg and 500 mg are safe and suitable for clinical applications, whether taken in a single or multiple dosage regimen. Results support further use of ECa 233 capsules in phase II clinical studies.

Table 5 Safety and tolerability after single and multiple dose oral administration of ECa 233 capsules in different dosage regimens.

Symptoms	ECa 233, 250 mg (n=11)		ECa 233, 500 mg (n=10)		Relation to study drug
	Single n (%)	Multiple n (%)	Single n (%)	Multiple n (%)	
Drowsiness	1 (9%), moderate	0	2 (20%), moderate	0	2-probable, 1-unlikely
Gastrointestinal Disorder					
Nausea	0	0	0	0	-
Vomiting	0	0	0	0	-
Burning stomach	1 (9%), mild	0	0	0	probable
Abdominal discomfort	0	0	0	0	-
Flatulence	0	0	0	0	-
Increased urinary frequency	1 (9%), moderate	0	0	0	probable
Others					
Runny nose	1 (9%), moderate	0	0	0	unrelated
Cough	1 (9%), moderate	0	0	0	unrelated
Fever	1 (9%), moderate	0	0	0	unrelated
Headache	1 (9%), moderate	0	0	0	unlikely
Thirsty	1 (9%), moderate	0	0	0	unrelated

Materials and Methods

Chemicals

Standardized extract of *Centella asiatica*, ECa 233, as a raw material was provided by Siam Herbal Innovation Co., Ltd. (Lot number MRA 0816001). The newly developed ECa 233 capsules were formulated by Pharma Neuva Co., Ltd. (101.8% label amount of total triterpene derivatives, Lot number K17EC003002/1) in accordance with an approval quality standard of Good Manufacturing Practice. Analytical standards of asiaticoside (purity > 98.5%) and asiatic acid (purity > 97.0%) were purchased from Sigma-Aldrich Corp.; madecassoside (purity > 96.7%) and madecassic acid (purity > 97.5%) were purchased from Chromadex Corp. The glycyrrhizin (purity > 90.0%) and glycyrrhetinic acid (purity > 98.0%) used as the internal standard (IS) were obtained from Wako Pure Chemical Industries, Ltd. The HPLC grade methanol was obtained from Merck, Ltd.

Subjects

Twelve healthy Thai volunteers were enrolled. They were aged 18-50 years old, with a body mass index of 18-25 kg/m^2. All subjects were healthy based on their medical history, physical examination and laboratory screening, including hematology and chemistry. Subjects with a history of excessive smoking (> 10 cigarettes/day) and a history of alcoholic or moderate drinking (> 3 drinks/day) were excluded. Pregnant or breast-feeding females were not allowed to be enrolled in the study. Sample size in this study was determined according to the general guideline for phase 1 clinical pharmacokinetic studies (24).

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and Thai Clinical

Trials Registry (Number: TCTR20171107002, approval date: November 6, 2017). The clinical part was conducted at Chula Pharmacokinetic Center, Chula Clinical Research Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All clinical procedures were performed in compliance with the International Conference on Harmonization - Good Clinical Practice and the Declaration of Helsinki. All subjects provided written informed consent prior to the start of the study.

Study design

This was an open-labeled, single and multiple dosing safety and pharmacokinetic study in healthy Thai volunteers under fasting conditions. The study was divided into two periods **(Fig. 5)** with sequentially different doses: 250 and 500 mg of ECa 233. Two weeks prior to the first period of study and until the study was completed, all participants were advised to abstain from any concomitant medication or other herbal medicines, especially treatment that induces or inhibits drug metabolizing enzymes and medication related to drug transporters, P-glycoprotein and MRP2 (17). For the first period of the single dose study, after overnight fasting, one capsule of ECa 233 (250 mg) was administered orally to all subjects with 240 mL of water. Venous blood samples (9 mL) were collected for pharmacokinetic study at pre-dose and 0.25, 0.50, 1, 2, 4, 8 and 24 h post-dose via a forearm vein catheter. A standard meal contained 650-850 kilocalories (carbohydrate: fat: protein, 60%:30%:10%) was provided at 4 h after ECa 233 administration. In order to continue the multiple dosing of ECa 233, one 250 mg capsule was administered orally to subjects every morning (before breakfast) for the following seven consecutive days. Then, pharmacokinetic blood samples were collected at the same times as described for the single dose section. After a three-week washout, the second period of the study was conducted in the same group of subjects with a 500

mg dose of ECa 233, using the same pharmacokinetic blood sampling time points used for both single and multiple dosing.

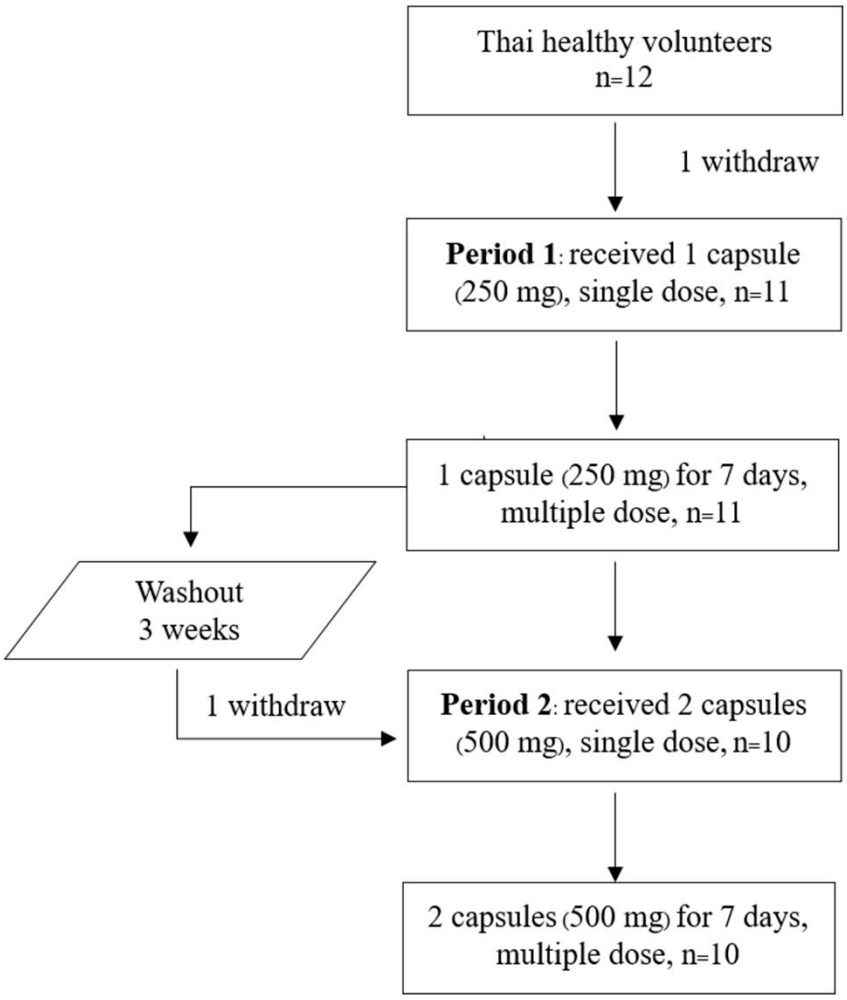

Figure 5 Flow diagram illustrating the study design of the two-sequence dosage, single and multiple dosing, safety and pharmacokinetics study in healthy Thai volunteers.

Safety and tolerability assessments

Safety and tolerability were evaluated throughout the study by vital sign monitoring, physical examination, adverse events (AEs) monitoring and clinical laboratory testing. Adverse events were self-reported in the subjects' diaries; a clinical investigator also defined these AEs according to the possible relationship to ECa 233 (i.e. unrelated, unlikely, probable, possible, or definitely) and graded the severity (i.e. mild, moderate, severe, or life-threatening). Clinical laboratory tests were done at screening and post-dose at the end of each study period. Blood samples were centrifuged at 4 °C and 1,500 x *g* for 10 min and plasma samples were kept at -70 °C until further analysis.

Bioanalysis of ECa 233 bioactive compounds in plasma

The bioanalytical part of this study was performed at the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. All major components of human plasma were determined according to a modification of a previously published method (13) and were fully validated according to the International Conference on Harmonization (ICH) Guidelines "Validation of Analytical Procedures" (25). This validated method met all acceptance criteria, as shown in the supporting information **(Table 6** and **7)**. The method had good specificity since there were no other impurity interferences observed at the retention times for target compounds and IS. The calibration curves displayed good linearity (R2 > 0.995) over the plasma concentration range of 1-800 µg/L for parent compounds (madecassoside and asiaticoside) and 5-400 µg/L for acid metabolites (madecassic acid and asiatic acid). The lower limits of quantification (LLOQ) were 1.00 µg/L and 5.00 µg/L for parent and metabolite compounds, respectively.

For the sample preparation process, extractions of the major compounds in human plasma were conducted by protein precipitation in the presence of two internal standards. Briefly, 50 µL of plasma samples were extracted with 200 µL of methanol containing two internal standards (glycyrrhizin and glycyrrhetinic acid) to correct the analysis of the parent compounds (triterpenoid glycosides) and their metabolites (triterpenic acids), respectively. After vortex mixing for approximately 10 min, the mixtures were then centrifuged at 12,000 x *g* and 4 °C for 10 min. The supernatants were subsequently filtered through a 0.2 - µM PVDF membrane (Chrome Tech, Inc.) prior to the LC-MS/MS analysis. The LC-MS/MS system was run on LCMS-8060 (Shimadzu Corp.) equipped with a binary pump, an autosampler and a triple quadrupole mass spectrometer with an electrospray ionization source. Sample analysis was run using a mobile phase consisting of 100% methanol and 0.2% formic acid in water with a gradient system (0.0–0.5 min 10% methanol, 0.5–3.0 min 90% methanol, 3.0–5.0 min 10% methanol) at a flow rate of 0.5 mL/min and 10 µL injection volume. Chromatographic separation was achieved on C18 reversed phase column, model Phenomenex Synergi Fusion-RP equipped with Guard C18 column (Phenomenex, Inc.). The column oven was maintained at 40 °C throughout the analytical procedure. The mass spectrometric system was operated using negative electrospray ionization and in multiple reaction monitoring mode. The mass-to-charge ratio of madecassoside, asiaticoside, madecassic acid, asiatic acid, glycyrrhizin, and glycyrrhetinic acid was 973.40/503.30, 957.40/469.20, 503.25/437.15, 487.30/409.45, 821.25/350.90, and 469.35/409.40, respectively (Supporting Information, **Fig. 6** and **7**). All the operations, acquisition and analysis of data were controlled by LabSolution software, version 5.86 (Shimadzu Corp.).

Pharmacokinetic analysis

Pharmacokinetic parameters were determined by non-compartmental analysis using PK solutions software, version 2.0 (Summit Research Services). The maximum concentration of target compounds (Cmax) and the time to reach maximum concentration (Tmax) were taken directly from the individual plasma concentration versus time profile. The area under the curve from time zero to the last sampling time (AUC0-t) was estimated using the linear trapezoidal rule and extrapolated to time infinity (if possible) by the equation AUC (0-∞) = AUC(0-t) + (Clast/kel), where Clast is the concentration at the last quantifiable sampling time and kel is the elimination rate constant, determined by a log-linear least square regression of the terminal phase. The apparent total clearance of the test substance removed from plasma after oral administration (Cl/F) was estimated based on the equation, Cl/F = Dose/AUC, where F is absolute oral bioavailability. The apparent volume of distribution after non-intravenous administration (Vd/F) was estimated as Vd/F = Dose/(AUC x kel).

Statistical analysis

Statistical analysis was performed using SPSS statistical software (Version 22.0). Continuous data were expressed as means ± standard deviation (SD). Data were tested for a normal distribution using the Shapiro–Wilk test, as well as the histograms of distribution test.

Differences in pharmacokinetic parameters between the two related groups (single dose on day 1 and multiple doses on day 7) were compared using a paired Student's t-test or Wilcoxon signed-rank test, where appropriate. Differences in pharmacokinetic parameters between two-dosage regimens (250 and 500 mg ECa

233) were evaluated using a Student's t-test or a Mann–Whitney U test. All differences were considered statistically significant at p-values < 0.05.

Supporting information

Method validation for LC-MS/MS analysis of ECa 233 and its chromatograms with mass spectrums are available as supporting information.

Acknowledgments

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Conflict of interest

All authors declare no conflict of interest.

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SUPPORTING INFORMATION

Safety and pharmacokinetics of standardized extract of *Centella asiatica* (ECa 233) capsules in healthy Thai volunteers: A phase 1 clinical study.

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Table 6 Intra- and inter-day precision and accuracy of madecassoside, asiaticoside, madecassic acid, asiatic acid in human plasma.

Concentration (µg/L)		Madecassoside			Asiaticoside		
		Concentration found (µg/L)	(%) RE	(%) CV	Concentration found (µg/L)	(%) RE	(%) CV
Intra-day							
High	**800**	774.24 ± 17.45	-3.22	2.25	815.81 ± 12.99	1.98	1.59
Medium	**100**	109.74 ± 3.63	9.74	3.31	106.57 ± 3.79	6.57	3.56
Low	**10**	10.33 ± 0.85	3.27	8.19	10.73 ± 0.77	7.28	7.18
LLOQ	**1**	0.93 ± 0.09	-6.78	9.50	1.12 ± 0.15	11.68	13.54
Inter-day							
High	**800**	774.06 ± 15.83	-3.24	2.05	817.21 ± 12.49	2.15	1.53
Medium	**100**	108.83 ± 3.76	8.83	3.45	106.49 ± 5.06	6.49	4.75
Low	**10**	10.61 ± 0.83	6.05	7.86	10.71 ± 0.93	7.09	8.69
LLOQ	**1**	0.95 ± 0.10	-4.72	10.36	1.05 ± 0.14	5.3	13.18
Concentration (µg/L)		**Madecassic acid**			**Asiatic acid**		
		Concentration found (µg/L)	(%) RE	(%) CV	Concentration found (µg/L)	(%) RE	(%) CV
Intra-day							
High	**400**	387.67 ± 22.07	-3.08	5.69	421.57 ± 19.39	5.39	4.60
Medium	**100**	103.48 ± 2.36	3.48	2.28	106.05 ± 2.59	6.05	2.44
Low	**25**	26.13 ± 0.92	4.52	3.50	26.83 ± 1.00	7.33	3.74
LLOQ	**5**	5.45 ± 0.58	9.09	10.57	5.55 ± 0.56	10.91	10.07
Inter-day							
High	**400**	394.79 ± 28.38	-1.30	7.19	413.60 ± 25.07	3.40	6.06
Medium	**100**	103.44 ± 4.15	3.44	4.02	105.17 ± 2.61	5.17	2.48
Low	**25**	25.98 ± 1.51	3.94	5.83	26.02 ± 1.40	4.10	5.39
LLOQ	**5**	5.45 ± 0.65	9.08	11.96	5.35 ± 0.56	7.08	10.52

Data expressed as mean ± SD, (n=6)

Table 7 Stability of madecassoside, asiaticoside, madecassic acid, and asiatic acid in human plasma under different storage conditions (n=6).

	Storage conditions	Concentration (μg/L)	Concentration found (μg/L, mean±SD)	SD	%RE	%CV
Madecassoside	10 h at room temperature	800	805.80	9.57	0.73	1.19
		100	102.73	6.70	2.72	6.52
		10	9.77	1.09	-2.28	11.18
	3 freeze-thaw cycles	800	795.51	16.43	-0.56	2.07
		100	103.06	6.21	3.06	6.02
		10	10.59	0.75	5.89	7.13
	3 months at -20 °C	800	807.69	14.75	0.96	1.83
		100	104.74	3.73	4.74	3.56
		10	10.47	0.95	4.71	9.10
	24 h at autosampler	800	802.93	14.78	0.37	1.84
		100	106.46	6.60	6.46	6.20
		10	10.21	1.30	2.13	12.69
Asiaticoside	10 h at room temperature	800	806.55	12.25	0.82	1.52
		100	97.13	5.49	-2.87	5.65
		10	10.21	1.11	2.12	10.85
	3 freeze-thaw cycles	800	802.51	11.83	0.31	1.47
		100	102.12	3.41	2.12	3.34
		10	10.16	0.85	1.56	8.33
	3 months at -20 °C	800	798.97	23.40	-0.13	2.93
		100	97.53	6.16	-2.47	6.31
		10	9.78	0.82	-2.24	8.37
	24 h at autosampler	800	805.48	12.68	0.68	1.57
		100	100.74	6.62	0.74	6.57
		10	10.23	0.92	2.25	8.96
Madecassic acid	10 h at room temperature	400	404.09	9.41	1.02	2.33
		100	96.57	6.45	-3.43	6.68
		25	25.11	2.07	0.44	8.26
	3 freeze-thaw cycles	400	402.15	11.04	0.54	2.74
		100	104.72	5.47	4.72	5.22
		25	25.34	1.34	1.36	5.28
	3 months at -20 °C	400	400.94	10.16	0.23	2.53
		100	100.90	7.63	0.90	7.56
		25	26.17	1.58	4.67	6.02
	24 h at autosampler	400	409.92	11.97	2.48	2.92
		100	103.70	6.85	3.80	6.61
		25	25.21	1.92	0.85	7.62
Asiatic acid	10 h at room temperature	400	405.92	6.36	1.48	1.57
		100	103.35	5.84	3.35	5.65
		25	25.28	1.80	1.13	7.10
	3 freeze-thaw cycles	400	405.06	8.52	1.26	2.10
		100	100.11	5.65	0.12	5.65
		25	25.46	1.37	1.82	5.37
	3 months at -20 °C	400	397.02	10.16	-0.75	2.56
		100	105.23	7.43	5.23	7.06
		25	25.55	1.80	2.19	7.03
	24 h at autosampler	400	404.88	11.66	1.22	2.88
		100	105.23	6.19	5.23	5.88
		25	25.05	1.44	0.19	5.75

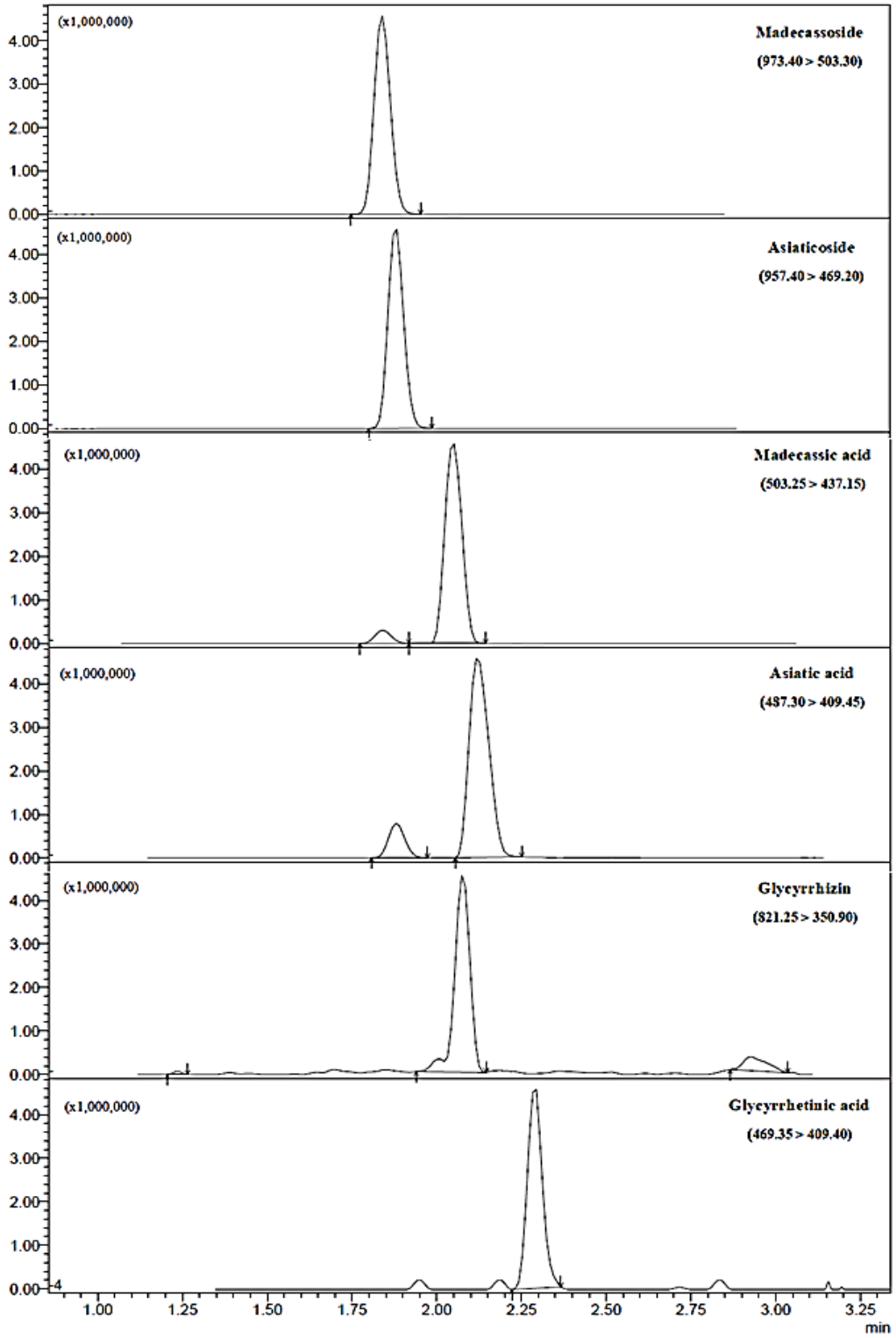

Figure 6 chromatograms of madecassoside, asiaticoside, madecassic acid, asiatic acid, and internal standards.

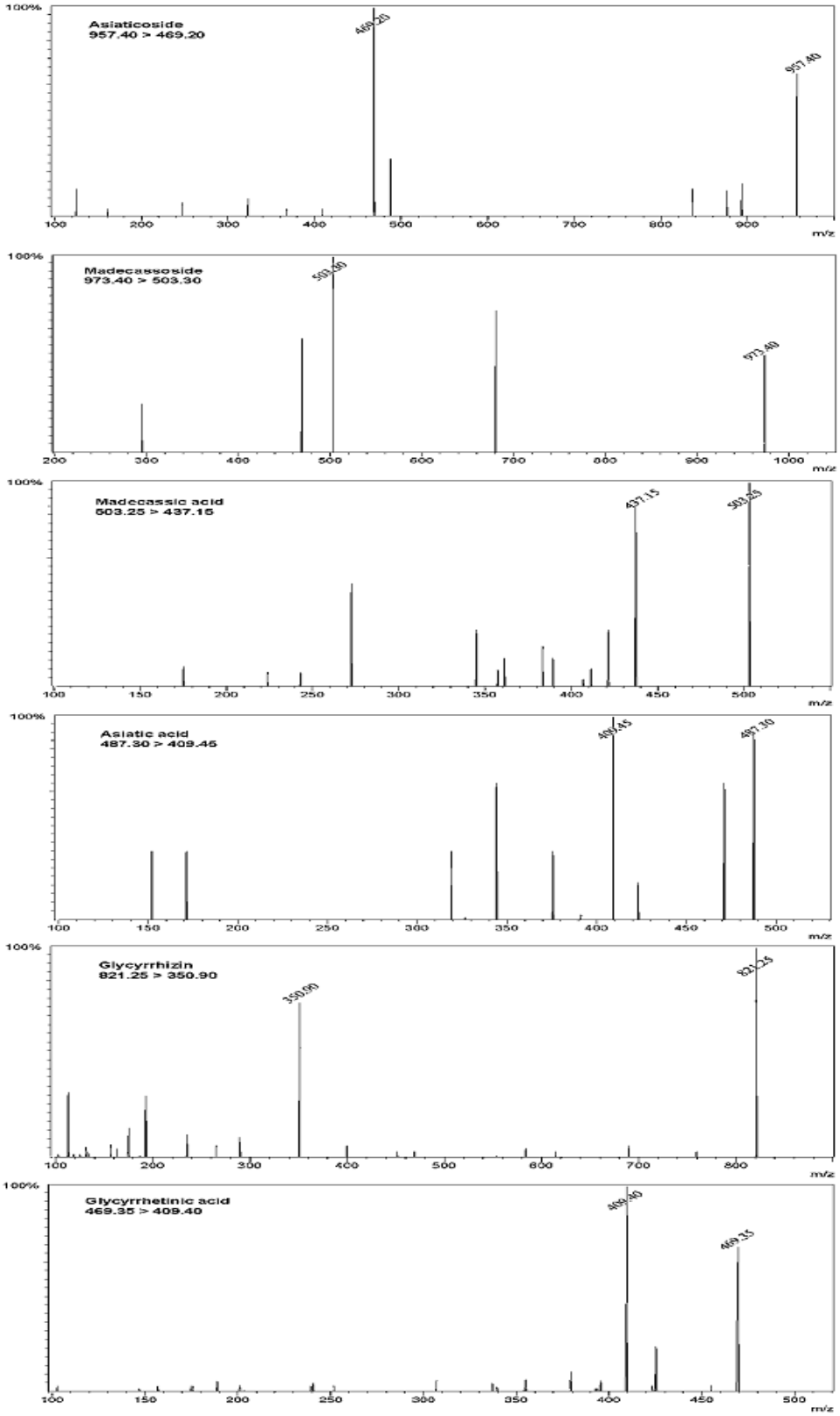

Figure 7 Mass Spectrum of madecassoside, asiaticoside, madecassic acid, asiatic acid, and internal standards.

CHAPTER IV

PUBLISHED ARTICLE II

Title: Clinical pharmacokinetics and metabolomics investigation of a modified formula of standardized *Centella asiatica* extract orally administered in healthy volunteers

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Clinical pharmacokinetics and metabolomics investigation of a modified formula of standardized *Centella asiatica* extract orally administered in healthy volunteers

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Abstract

The formula of a standardized extract of *Centella asiatica* (ECa 233) was modified to improve its dissolution, with implications for pharmacokinetics and metabolomics profiles. This study aimed to understand the resultant changes in disposition kinetics of ECa 233 and alterations to human metabolome after oral administration. This study was a two-sequence of dosages (250 and 500 mg), with an open-label phase I clinical trial. The modified formula was administered in single and multiple doses to twelve healthy Thai volunteers. The major parent compounds, madecassoside and asiaticoside, were rarely absorbed, instead undergoing biotransformation into active metabolites, madecassic acid and asiatic acid with possibility to be eliminated via fecal route. Increasing dose of ECa 233 resulted in significantly greater plasma levels of those active metabolites, with accumulation of asiatic acid after multiple oral administration for seven days. Examining the impacts of accumulation behavior on metabolomics, the study traced changes in levels pre- and post-dose of five relevant human metabolites to identify candidate biomarkers. Administration of ECa 233 was found to be significantly associated with an increase of choline, an endogenous metabolite with documented benefits for learning and memory. Therefore, ECa 233 may be useful in mitigating cognitive impairment, through its role in modulating human metabolites.

Keywords

pharmacokinetics; metabolomics; *Centella asiatica*; madecassoside; asiaticoside; madecassic acid; asiatic acid

Abbreviations

AChEI, Acetylcholinesterase inhibitor or cholinesterase inhibitor

AEs, Adverse events

ASA, Asiatic acid

ASS, Asiaticoside

AUC, Area under plasma concentration-time curve

BMI, Body mass index

CL/F, Apparent total clearance of the test substance removed from plasma after oral administration

Cmax, Maximum plasma concentration

CPMG, Carr-Purcell-Meiboom-Gill pulse

ECa 233, Standardized extract of *Centella asiatica*

ESI, Electrospray ionization

F, Absolute oral bioavailability

HMDB, Human metabolome database

HPLC, High performance liquid chromatography

IQR, Interquartile range

IS, Internal standard

LC-MS/MS, Liquid chromatography tandem mass spectrometry

LLOQ, Lower limit of quantification

MDA, Madecassic acid

MDS, Madecassoside

MRM, Multiple reaction monitoring

NMR, Nuclear magnetic resonance spectroscopy

NOAEL, No observed adverse effect level

OPLS-DA, Orthogonal-signal correction-projection to latent structure-discriminant analysis

PCA, Principal component analysis

P-gp, P-glycoprotein

p.o. (Per os), Oral administration

SD, Standard deviation

STOCSY, Statistical total correction spectroscopy

Tmax, Time to reach maximum plasma concentration

TSP, Trimethylsilylpropanoic acid

$t_{1/2}$, Elimination half-life

Vd/F, Apparent volume of distribution after non-intravenous administration

Introduction

Centella asiatica (Linn.), commonly known as Asiatic pennywort or Gotu kola, belonging to the family Apiaceae, has been widely used in traditional medicine and possesses well-documented neuroprotective effects[1]. A standardized extract of *C. asiatica* was fully researched and developed as ECa 233 and was then characterized to contain more than 81% of triterpenoid glycosides [2]. Recent reports have shown the tolerability of ECa 233 in preclinical toxicology studies with NOAEL ≥ 1,000 mg/kg/day in rats [3]. Pretreatment with 10 mg/kg of ECa 233 in mice was effective in mitigating the Alzheimer-like cognitive deficits induced by β-amyloid peptide [4,5]. Hence, ECa 233 has a potential to become a phytomedicine candidate for human use in terms of improving patients' cognitive function.

Pharmacokinetics profiles of ECa 233 were first determined in a rat model[6]. Later, 250- and 500-mg capsules of ECa 233 were reported to be well tolerated in a phase 1 study in healthy subjects [7]. The parent compounds of ECa 233, which are MDS and ASS, were extensively biotransformed into active metabolites, MDA and ASA, respectively [7]. Most of the pharmacokinetic parameters of ECa 233's parent compounds and active metabolites were illustrated; however, data about excretion and metabolomics are still limited [7]. To date, information in regard to the metabolome may directly reflect changes in the physiological state after oral administration of a specific drug and has been applied in the process of identifying biomarkers in metabolic disorder-involved diseases [8]. Understanding the associations between changes in the levels of candidate endogenous metabolites and the metabolite-related biomarkers of neurodegenerative disease [9] is the way to support the next step in the use of ECa 233 in clinical practice.

A previous study found that ECa 233 had limited dissolution of formulation, leading to a restriction of its oral bioavailability [7]. Hence, the modified formula to improve oral bioavailability of ECa 233 was developed and was used in this clinical study. This research aimed to investigate the clinical pharmacokinetics profile and excretion pathway including metabolomic profiles of the modified-formula ECa 233 capsules in healthy Thai volunteers.

Materials and Methods

Chemicals

The standardized *C. asiatica* extract is a white to off-white powder that contains the triterpenoid glycosides MDS (53.1%) and ASS (32.3%) as determined by the LC-MS/MS method. For this clinical study the extract was provided by Siam Herbal Innovation Co., Ltd (Thailand) (Lot number MRA 0816001). The modified formula of ECa 233 capsules (Lot number 18EC003005) was manufactured by Pharma Nueva Co., Ltd. (Thailand) under Good Manufacturing Practice quality standards. The appropriate dosage of *C. asiatica* extract used in this clinical study was determined by calculating the first dose in humans (FIH dose) [10]. The estimated maximum recommended starting dose (MRSD) was converted from the human equivalent dose (HED)[7] based on the results of safety and pharmacological studies of ECa 233.

For LC-MS/MS analysis, methanol (HPLC grade) was obtained from Merck (Germany). The internal standards of glycyrrhizin (purity > 90%) and glycyrrhetinic acid (purity > 98%), and the reference standard of ASS (purity > 98.5%) were purchased from Sigma-Aldrich (Corp. USA). MDS (purity > 96.7%) was purchased from Chromadex, (Inc. USA). MDA and ASA, determined to be >97.5% and 97.0% purity, respectively, were purchased from Wako Pure Chemical Industries, (Ltd. Japan).

For NMR analysis, deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories, (Inc. USA). Sodium hydrogen phosphate (Na_2HPO_4) for phosphate buffer preparation, sodium azide (NaN_3) and trimethylsilyl propionic acid sodium salt (TSP) were purchased from Sigma-Aldrich, (Corp. USA).

Ethical Statement and Informed Consent

This study protocol was approved by the Institutional Review Board of Chula Clinical Research Center, Chulalongkorn University (IRB number: 479/61, COA number 850/2018, date of approval: 06/09/2018) and was registered in the Thai Clinical Trials Registry, a trial registration data set required by World Health Organization, (TCTR identification number: TCTR20180922001, date of first registration: 20/09/2018). The clinical study was conducted at Chula Pharmacokinetic Center, Maha Chakri Sirindhorn, Clinical Research Center, Faculty of Medicine, Chulalongkorn University, under the International Conference on Harmonization - Good Clinical Practice, and in accordance with the Declaration of Helsinki. All participants provided written informed consent for their participation prior to the start of the study.

Eligibility Criteria for Participants

Twelve Thai healthy volunteers aged between 18 and 50 years were enrolled and recruited from March to April 2018. Inclusion criteria were BMI between 18.0 and 25.0 kg/m^2, with normal medical histories, physical examinations, vital signs, and clinical laboratory tests. Participants were excluded if they smoked more than 10 cigarettes/day, had a history of alcoholism or of moderate drinking (more than 3 drinks/day), were pregnant or breastfeeding, or planned to become pregnant during the study period. The number of subjects was defined according to the general guidelines for clinical pharmacokinetic studies, a concise guide used in clinical

research[11]. Sample size determination was calculated for a 95% level of confidence according to the standard normal distribution ($Z_{1-\alpha/2}$ = 1.96), and a tolerated margin of error at 15% of the mean (γ = 0.15) [12].

Study Design and Sample Collection

This was a single-center, with an open-label, two-sequence of dosages (250 and 500 mg), two-periods of study (single and multiple dose) conducted in fasting condition **(Figure 8)**. In study period 1, all participants fasted overnight for at least 10 hours before drug administration. The 250 mg of ECa 233 with 240 mL of drinking water were administered orally. Blood samples (3 mL) were collected from each individual at pre-dose (0 hour) and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, and 72 hours post dose. All blood samples were centrifuged at 4 °C, 3,200 x *g* for 10 minutes. Urine samples were collected at 0 hour (the day prior to dosing) and 0-4, 4-8, 8-12, and 12-24 hours after dosing. Feces were also collected from one of the volunteers for detecting the possible presence of any eliminated compounds. Feces samples were collected each time that a volunteer defecated on day 1-3, 4-6 and 7-9 during the study period. These samples were immediately fixed with methanol and prepared in following to our previously described method of protein precipitation. After a 3-week washout period, all participants continued period 2 of the study, which they received a 500-mg dose of ECa 233, and sample collection procedures remained consistent with period 1 **(Figure 9)**. Plasma, urine and fecal samples were then stored at -80 °C until analysis.

For multiple oral dosing, participants continued to take 250 mg or 500 mg of ECa 233 every morning for 7 consecutive days. Then they had another set of blood and urine samples collected similar to the single dose study. The primary outcome

was determination of pharmacokinetic parameters, including the profiles of the metabolites, and the secondary outcome was consideration of the safety profiles of ECa 233 after period 2 (end of the study).

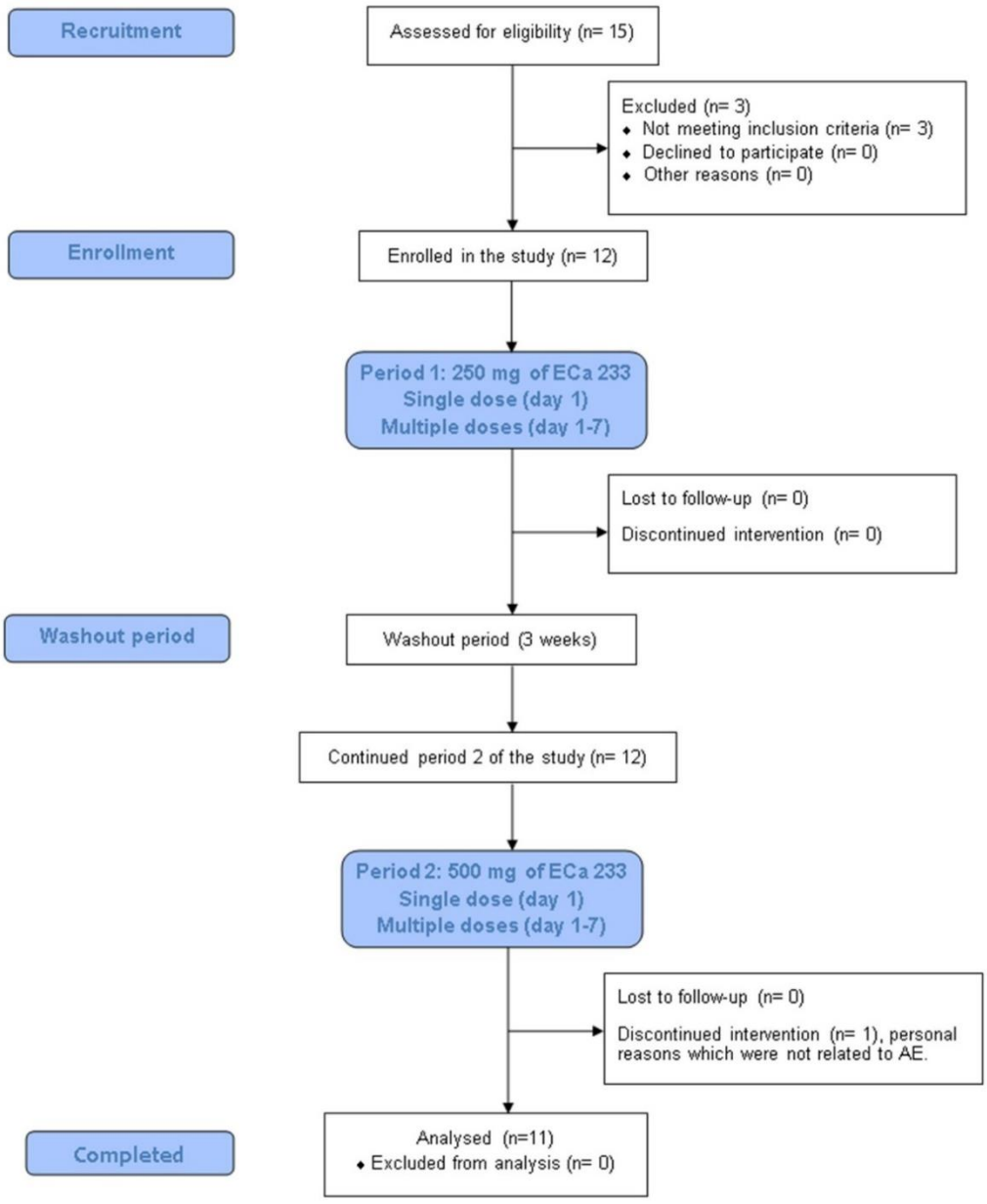

Figure 8 Flow diagram illustrates study design of the clinical trial.

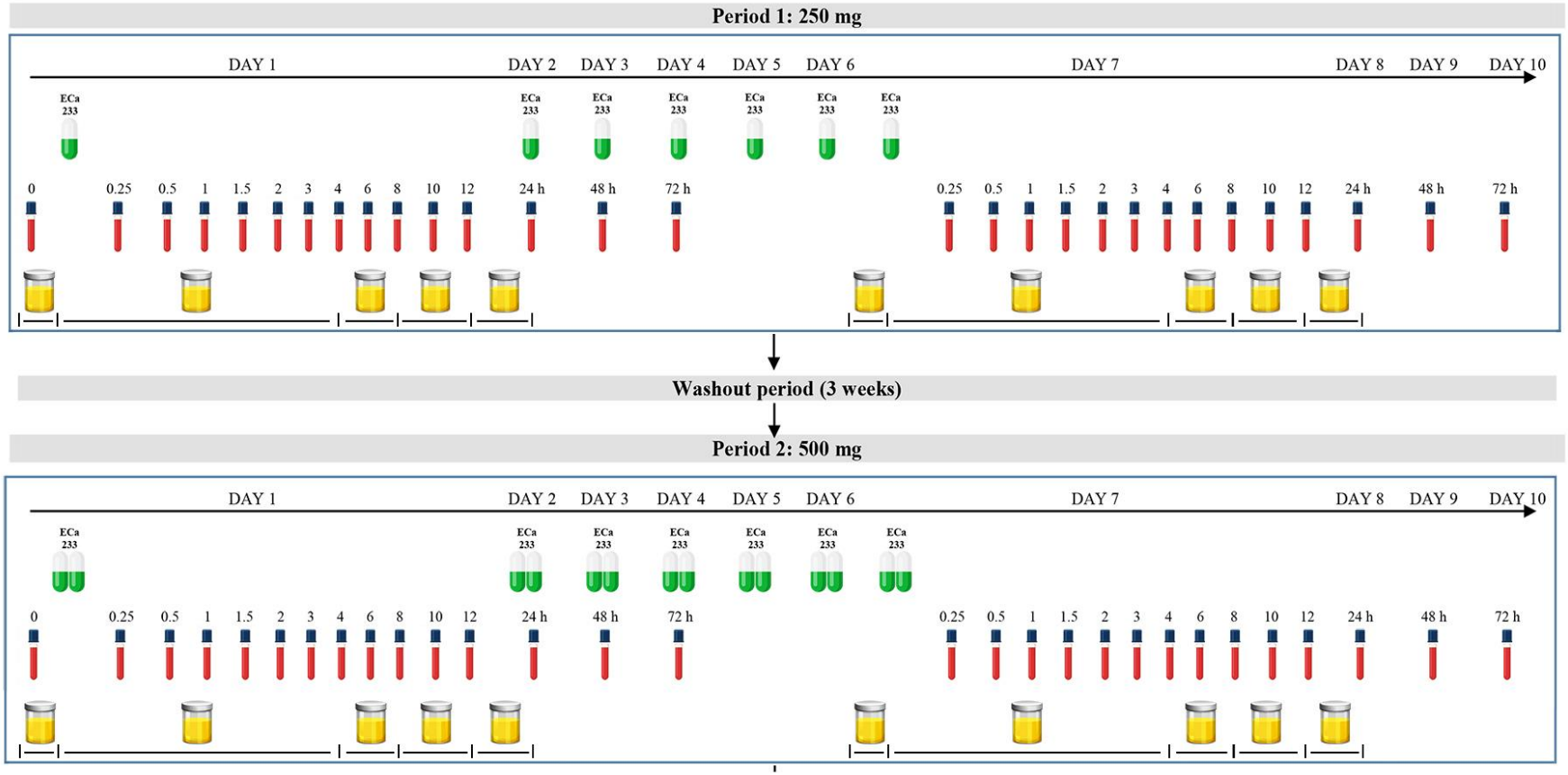

Figure 9 Timeline for oral administration, blood and urine collection.

Sample Preparation and Pharmacokinetic Analysis

Sample preparation was performed by protein precipitation with two internal standards (glycerrhizin and glycerrhetinic acid) to correct the quantitative analysis of parent compounds and of their metabolites. Briefly, 200 µL of methanol was added followed by vortex mixing with 50 µL of each sample for 10 minutes and then centrifuged at 12,000 x *g*, 4 °C, 10 minutes. Quantitative determination of the parent compounds and their active metabolites in plasma and urine were carried out by using liquid chromatography tandem mass spectrometry (LC-MS/MS): LCMS-8060 (Shimadzu Corp. Japan) according to a previously validated and published bioanalytical method[7]. Chromatographic separation of the extracted sample was then performed on a Phenomenex Synergi 4u Fusion-RP (80A, 4 microns, size 50 x 2.0 mm) C18 column (column temperature = 40°C) and eluted by two mobile phases: 0.2% formic acid in water (solvent A) and 100% methanol (solvent B), following gradients 0-0.5 minutes 10% B, 0.5-3 minutes 90% B, and 3-5 minutes 10% B, at a constant flow rate of 0.5 mL/min with 10 µL of injection volume. The MS/MS system was operated using an ESI source in negative mode with MRM. For m/z transition; MDS, ASS, MDA, ASA, glycyrrhizin, and glycyrrhetinic acid showed a precursor ion and product ion at 973.50/503.30, 957.55/469.00, 503.20/437.20, 487.60/409.25, 821.25/350.95, and 469.45/409.40, respectively (**Supplementary Figures 15, 16** and **17**).

Sample Preparation and Metabolomics Analysis

Plasma samples at predose, 0.5, 1, and 2 hours were used in the metabolomics part of the study. In brief, 400 µL of plasma were mixed with 400 µL of 0.142 M phosphate buffer in D_2O solution (pH 7.4) containing 3 mM sodium azide (NaN_3) used as a bacteriostatic reagent, and 0.08% trimethylsilylpropanoic acid (TSP)

used as a chemical shift reference. The mixer was vortexed, sonicated at 25 °C for 15 minutes and then centrifuged at 13,000 x *g*, 4 °C for 10 minutes. Lastly, 600 µL of supernatant was transferred into a 5-mm diameter NMR tube for analysis.

All ^{1}H NMR spectra of plasma samples were acquired at 600.13 MHz on a 600 MHz NMR spectroscopy (Bruker Avance III, Bruker Biospin, Germany) and were determined using a modified version of a previously published method [13]. The samples were measured at a temperature of 300 K using the presaturation sequence (64 scans). ^{1}H NMR spectra were recorded by a standard one-dimensional (1-D) with the suppression of water signal, CPMG pulse. The spectra region was set to a range from -1 to 10 ppm and the parameters were as follows: 64 K data points; relaxation delay of 2 s and mixing time (tm) of 100 ms.

Data Processing and Statistical Analysis of Pharmacokinetics

Non-compartmental analysis was applied to determine pharmacokinetic parameters and performed by PK solutions software (version 2.0). $AUC_{(0-t)}$ was calculated using the linear trapezoidal rule. t½ was calculated by $t_{(1/2)} = \ln 2/k_{el}$. The apparent clearance (Cl/F) after oral administration was calculated as Cl/F = Dose/AUC, where F is the absolute oral bioavailability. The apparent volume of distribution (Vd/F) was estimated using Vd/F = Dose/(AUC x k_{el}). The Cmax and Tmax were determined directly from the observed concentration-time curve.

For statistical analysis in the pharmacokinetics study, all data were expressed as mean ± SD except for Tmax, which was expressed as median (IQR). The descriptive data were used to describe demographic characteristics and to summarize the

continuous variables. Data processing was performed using STATA software (version 10.0; StataCorp, USA) and displayed in the graphical charts using GraphPad Prism 8.
A paired t-test or Wilcoxson Signed Rank Test was used to compare pharmacokinetics parameters between single and multiple doses. To determine statistically significant differences of the pharmacokinetics parameters of two dosages between 250- and 500-mg, Student's t-test or Mann-Whitney U test was used where appropriate. A p-value of less than 0.05 was considered statistically significant.

Data Pre-processing, Multivariate Analysis, and Metabolite Identification for Metabolomics

All plasma spectra were pre-processed using TopSpin 3.1 software (Bruker, Germany) including baseline correction and TSP calibration at 0.00 ppm. The NMR spectra were subsequently transferred into MATLAB software (MathWorks, R2014a, USA). The spectral regions containing TSP peak (δ^1H -1 to 0.66 ppm) and water peak δ^1H 4.599 to 5.053 ppm) were omitted. The spectral alignment was then applied to adjust the position for all remaining peaks, and those aligned NMR spectra were imported into SIMCA 14.0 (Umetrics Umea, Sweden). In order to provide a general overview on clustering, data were initially analyzed by an unsupervised principal component analysis (PCA) with unit variance (UV) scaling. In considering the possible influences of ECa 233, a clear separation of suspicious metabolome changes between pre- (T0) and post-dose (T 0.5, 1, 2 h, on day 7) was further analyzed by OPLS-DA in MATLAB (R2014a) using the in-house developed scripts at Imperial College London. The OPLS-DA model was evaluated according to two parameters: R^2X explained the fitness and Q^2Y represented the predictive capability of the model. The permutation test was assessed by using 1,000 times for the validation of all models involved in this study. The coefficient loadings plot of OPLS-DA models was

used to indicate the significantly different metabolites as compared between pre-dose and post-dose. The permutation p-values were used to illustrate the level of differences that were considered as statistically significant at p-values less than 0.05.

For metabolite identification, plasma endogenous metabolites were determined using Chenomx NMR Suite 8.2 (Chenomx Inc., Canada), and were further justified in statistical total correction spectroscopy (STOCSY) using MATLAB software. In addition, the human metabolome database (HMDB) as well as the previously published assignments and in-house chemical shift databases were also used to support the metabolite identification in this study.

Results of pharmacokinetics and metabolomics

Participants baseline clinical characteristics

All subject demographics and characteristics are summarized in **Table 8**. The age limit for study volunteers was 18-50 years; people in this age range were eligible to participate. The age of enrolled participants, however, was within a smaller range, from 21 to 38 years. One subject withdrew consent during the second period of the study due to personal reasons, which were neither ECa 233-related problems nor intolerable side effects. Therefore, there were eleven subjects that completed the study and remained for the per-protocol analysis.

Table 8 Subject Demographic and baseline characteristics of the study volunteers.

Demographic data		Baseline	ECa 233, 250 mg	ECa 233, 500 mg
Gender, % (n)	Female		50% (n=6)	45.5% (n=5)
	Male		50% (n=6)	54.5% (n=6)
Age[a] (year)			26 [9.5]	26.5 [9.25]
Body mass index[b] (kg/m^2)		21.49 ± 1.66	21.37 ± 1.70	20.95 ± 1.58
Systolic blood pressure[b] (mmHg)		110 ± 10	117 ± 7	115 ± 11
Diastolic blood pressure[b] (mmHg)		65 ± 11	64 ± 6	59 ± 5
Body temperature (°C)		36.6 ± 0.4	36.3 ± 0.6	37.0 ± 0.1
Clinical laboratory screening [b]				
White blood cell ($x10^3/\mu L$)		6.58 ± 0.89	6.11 ± 1.29	6.74 ± 1.91
Red blood cell ($x10^6/\mu L$)		5.05 ± 0.31	4.84 ± 0.29	4.83 ± 0.42
Hemoglobin (g/dL)		13.6 ± 1.9	13.0 ± 1.7	13.0 ± 2.1
Platelet ($x10^3/\mu L$)		307 ± 69	299 ± 77	317 ± 85
Fasting blood glucose (mg/dL)		84.58 ± 3.99	87.25 ± 6.28	89.55 ± 6.42
Blood urea nitrogen (mg/dL)		13 ± 3	12 ± 4	11 ± 4
Creatinine (mg/dL)		0.72 ± 0.19	0.73 ± 0.20	0.79 ± 0.21
Albumin (g/dL)		4.48 ± 0.26	4.58 ± 0.23	4.45 ± 0.27
Total bilirubin (mg/dL)		0.70 ± 0.42	0.68 ± 0.37	0.60 ± 0.30
AST (U/L)		18 ± 5	23 ± 15	19 ± 5
ALT (U/L)		18 ± 9	19 ± 9	20 ± 14
Alkaline phosphatase (U/L)		52 ± 9	52 ± 9	52 ± 9
Total cholesterol (mg/dL)		173 ± 25	174 ± 21	172 ± 25
Triglycerides (mg/dL)		76 ± 46	77 ± 34	77 ± 48
HDL cholesterol (mg/dL)		55 ± 8	57 ± 8	56 ± 8
LDL cholesterol (mg/dL, calculated)		104 ± 22	101 ± 20	100 ± 24
Sodium (mmol/L)		137 ± 1	137 ± 1	138 ± 1
Chloride (mmol/L)		105 ± 2	107 ± 2	105 ± 2
Potassium (mmol/L)		3.9 ± 0.3	3.9 ± 0.3	4.2 ± 0.3
Calcium (mg/dL)		9.5 ± 0.4	9.7 ± 0.3	9.5 ± 0.4
Magnesium (mg/dL)		2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.2
Phosphorus (mg/dL)		3.5 ± 0.4	3.9 ± 0.5	3.9 ± 0.4

[a] data is expressed as median [IQR]; [b]data are expressed as mean ± SD, (n = 11-12).
Decimal numbers were reported according to laboratory standard of Maha Chakri Sirindhorn Clinical Research Center, Faculty of Medicine, Chulalongkorn University.
Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Pharmacokinetics

Pharmacokinetic profiles of the modified formula ECa 233

Pharmacokinetic profiles after oral administration of modified formula ECa 233 capsules are represented in plasma concentration-time curves of the parent compounds (MDS or ASS) and metabolites (MDA or ASA) **(Figures 10a - 10d).** Cmax of MDS or ASS was observed within 1 hour after dosing, followed by the appearance of MDA or ASA that reached their Cmax within 1-2 hours. A notably higher Cmax with a greater AUC was observed in both parent compounds and metabolite profiles of the modified formula of ECa 233 when compared to its previous formulation.

In considering a dose comparison between the 250 mg and 500 mg, the Cmax of MDA and ASA was found to increase significantly; however, no significant changes were observed in Tmax and $t_{1/2}$ **(Table 9).** Likewise, the apparent volume of distribution (Vd/F) and the apparent clearance (Cl/F) for MDA and ASA did not show significant differences when compared between the two dosage regimens.

Multiple oral dosing of the modified formula ECa 233 capsule resulted in greater plasma concentrations of ASA as compared to the single oral dosing. The AUC and Cmax of ASA on day 7 were significantly higher than those on day 1 ($p = 0.001$ and 0.007, respectively), indicating that there was an accumulation of active metabolites after the repeated doses. The accumulation ratio of ASA was approximately 1.891.

Table 9 Clinical pharmacokinetic parameters of parent compounds (MDS and ASS) and their metabolites (MDA and ASA) following oral administration of the modified ECa 233 capsules in healthy Thai volunteers.

	Single dose oral administration, Day 1 ($T_{0\text{-}24\ h}$)							
	250 mg (n=12)				500 mg (n=11)			
	Parent compounds		Metabolites		Parent compounds		Metabolites	
Parameters[#]	MDS	ASS	MDA	ASA	MDS	ASS	MDA	ASA
C_{max}[a] (µg/L)	9.57 ± 3.34	5.18 ± 3.43	66.09 ± 17.09	79.76 ± 19.28	12.73 ± 2.73*	9.81 ± 3.53*	72.26 ± 16.33	102.32 ± 20.76
T_{max}[b] (h)	1 [0.5]	1.25 [0.625]	1.5 [0.5]	1.5 [0]	1 [0.5]	1 [3.5]	1.5 [0.25]	1.5 [0.5]
AUC[a] (µg·h/L)	75.38 ± 26.60	25.20 ± 29.15	410.35 ± 100.24	647.73 ± 117.41	141.16 ± 41.15*	176.59 ± 177.06*	476.02 ± 116.59	733.09 ± 149.90
	Multiple dose oral administration, Day 7 ($T_{0\text{-}72\ h}$)							
	250 mg (n=12)				500 mg (n=11)			
	Parent compounds		Metabolites		Parent compounds		Metabolites	
parameters	MDS	ASS	MDA	ASA	MDS	ASS	MDA	ASA
C_{max}[a] (µg/L)	15.34 ± 6.96	10.93 ± 4.60	61.38 ± 20.47	78.23 ± 26.42	23.00 ± 4.99	12.77 ± 2.87	99.78 ± 27.57*	170.35 ± 53.79*
T_{max}[b] (h)	1 [0.125]	1 [0.125]	2 [0.625]	1.5 [0.5]	1 [0.5]	1 [0.5]	1.5 [0.75]	1.5 [0]
AUC[a] (µg·h/L)	162.27 ± 140.81	114.98 ± 54.12	437.49 ± 174.96	1013.55 ± 221.14	176.24 ± 72.68	106.82 ± 34.59	516.43 ± 156.64	1386.50 ± 332.86*
Vd/F[a] (L/kg)	371.84 ± 386.32	155.55 ± 489.17	37.12 ± 28.53	10.53 ± 12.55	453.64 ± 376.55	458.14 ± 462.33	57.89 ± 24.35	23.39 ± 23.03
Cl/F[a] (L/h/kg)	27.99 ± 23.88	17.09 ± 13.52	3.29 ± 1.70	0.74 ± 0.25	31.33 ± 16.15	30.48 ± 15.50	5.09 ± 1.64	1.11 ± 0.49
$t_{1/2}$[a] (h)	8.06 ± 5.62	10.37 ± 8.84	8.25 ± 5.85	9.86 ± 9.77	9.97 ± 9.39	9.35 ± 8.18	8.71 ± 5.46	14.13 ± 12.79

Notes: adata are expressed as mean ± SD; bdata is expressed as median [IQR]; * $p < 0.05$ for significant differences.
Abbreviations: Cmax, maximum plasma concentration; Tmax, time to reach maximum concentration; AUC, area under the plasma concentration–time curve from time zero to the last measurable concentration; Vd/F, the apparent volume of distribution; Cl/F, the apparent clearance; t1/2, elimination half-life.
The desired pharmacokinetic parameters of Vd, Cl and t1/2 of both the parent compounds during single oral administration (day 1, 24 hours) were not included in this table. These parameters are omitted for the parent compounds because the slope in their elimination phase did not end at 24 hours post-dosing. Therefore, these parameters were calculated only for the metabolites.

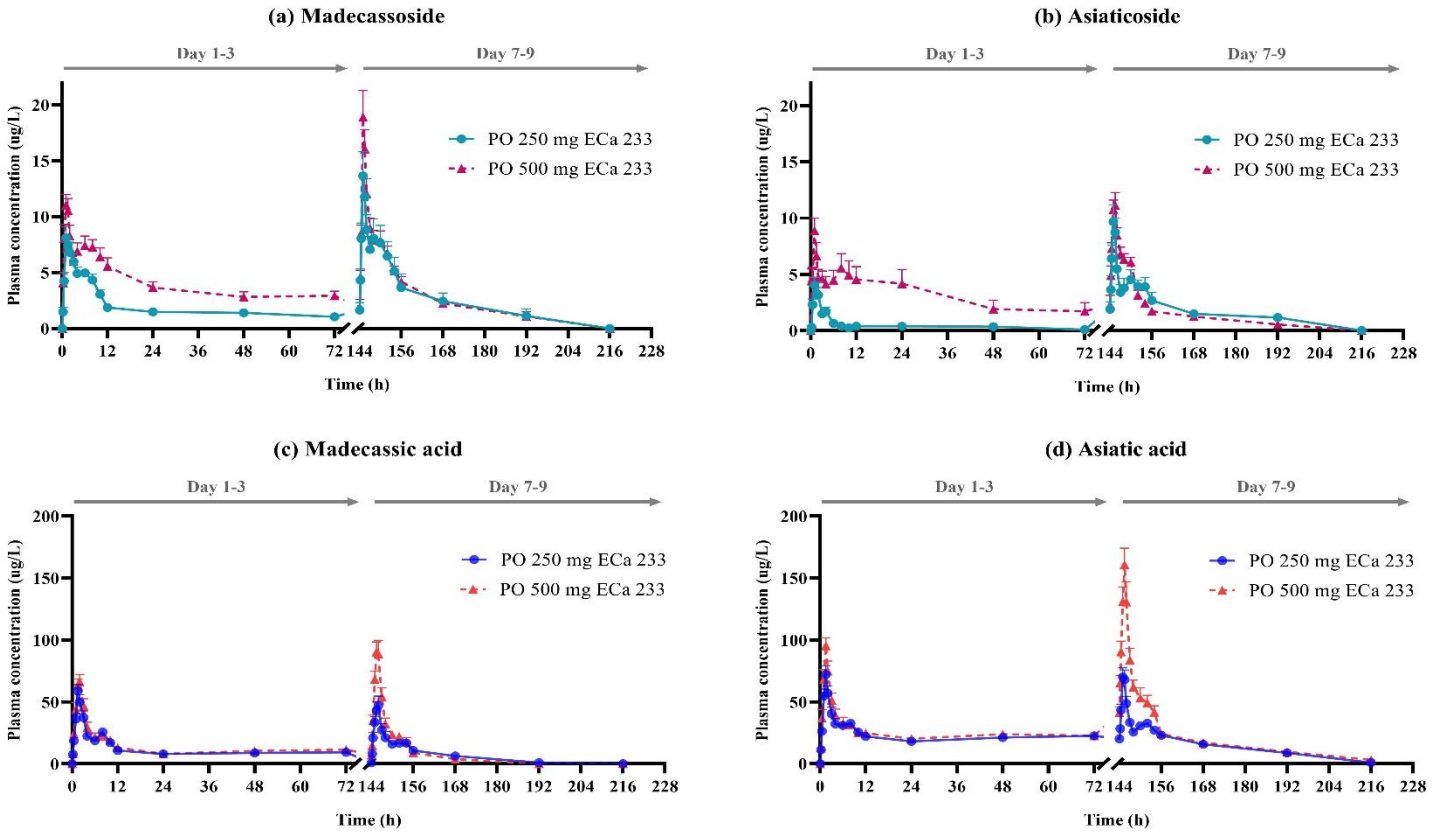

Figure 10 Mean plasma concentration-time profiles of the modified dissolution ECa 233; (a) madecassoside, (b) asiaticoside, (c) madecassic acid, and (d) asiatic acid, after single or multiple oral administration of 250 or 500 mg doses in healthy Thai volunteers. Data are presented as means ± SD (n = 11-12).

The unchanged forms of MDS and ASS in plasma were primarily eliminated into the urine from the first 4 hours until 24 hours after dosing **(Figure 11)**. The highest cumulative contents of MDS and ASS were within 8 hours, where only a small amount of the unchanged compounds was eliminated through the kidneys during 12-24 hours. The active metabolites (MDA and ASA), however, did not follow the same pattern observed for their respective parent compounds, as evidenced by the absence of the metabolites in the urine. Fecal samples collected from one of the volunteers also corroborated this assumption, as MDA and ASA were both found in the feces.

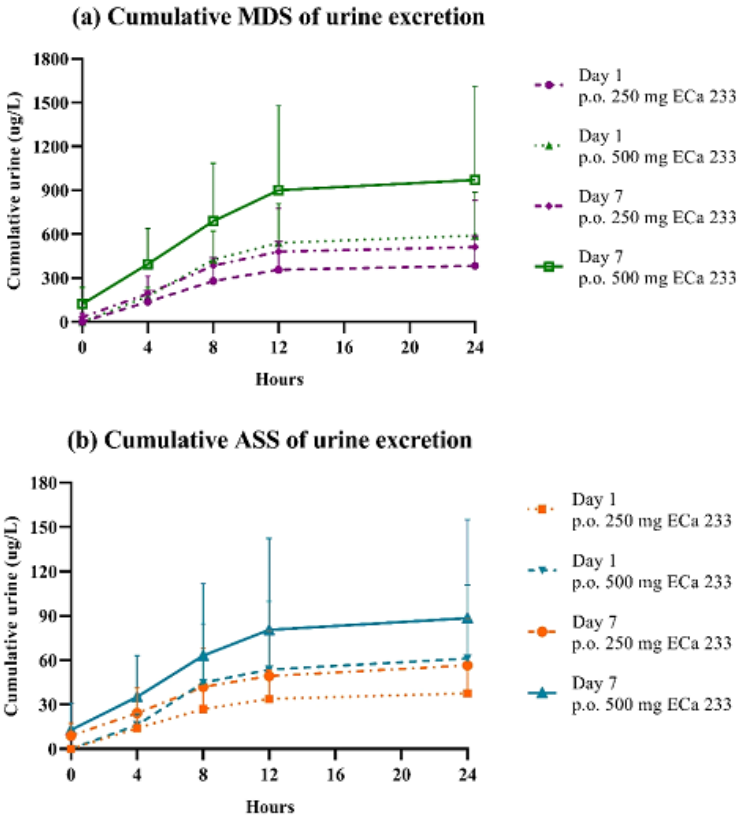

Figure 11 Cumulative urine excretion of (a) madecassoside and (b) asiaticoside after single or multiple oral administration of 250 or 500 mg doses in healthy Thai volunteers. Data are presented as means ± SD (n = 11-12).

Metabolomics

Human metabolomic profiling

Following oral administration of a once-daily dose of 250 mg or 500 mg of modified-formula ECa 233 for 7 consecutive days, metabolomic profiling detected a total of 31 endogenous metabolites from 1D ^{1}H-NMR spectra. Twenty-nine metabolites in plasma were identified according to the Chenomx NMR and human metabolome database (HMDB; **Supplementary Figure 18**). Clustering between pre- and post-dose receiving the repeated 500 mg of modified-formula was clearly separated based on the OPLS-DA score plot, indicating the discrimination of metabolites observed among those two clusters **(Figure 12)**.

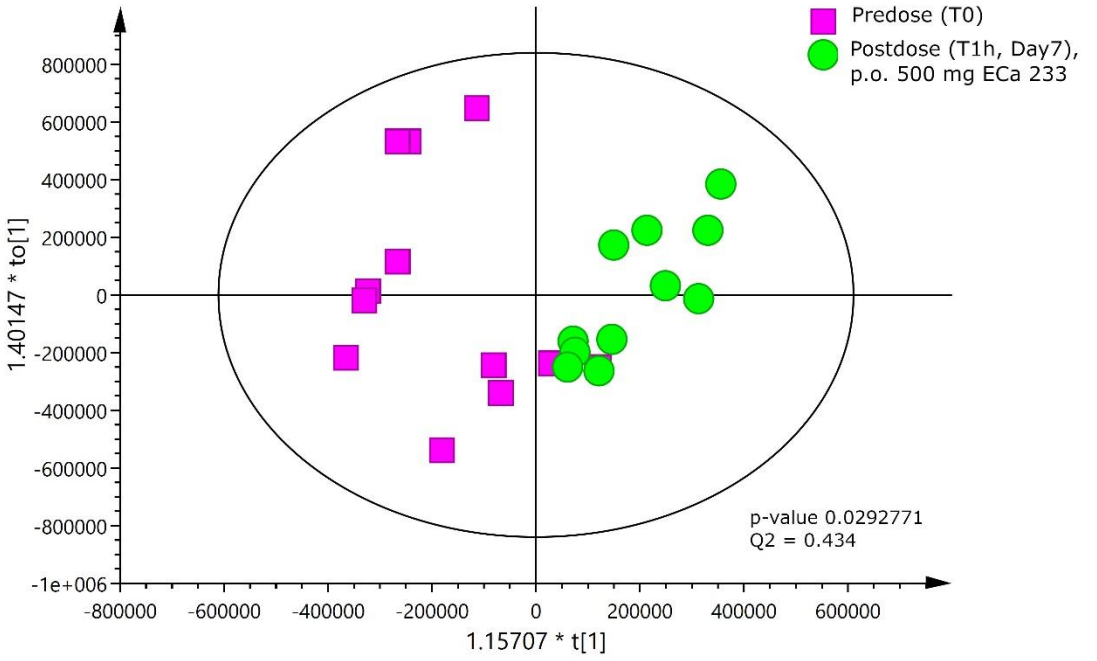

Figure 12 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose, for repeated administration of 500 mg of ECa 233

The results on OPLS-DA score plot suggested that a two-dose treatment (250 and 500 mg in multiple dose) had the same patterns of metabolome changes. The permutation p-value of the model validity was only significant for the group of 500-mg. Therefore, these findings may indicate a dose-dependent relationship in metabolomics. The OPLS-DA coefficient loadings plot was further used to investigate in the group of 500 mg repeated doses. The correlation of variables with classification of OPLS-DA models is shown in **Figure 13**. Based on the results in the loadings plot, 5 potential metabolites **(Table 10)** were indicated as possible biomarkers for the metabolomics alterations related effects when taking repeated doses of 500 mg modified-formula ECa 233.

Table 10 Metabolic changes in human plasma and chemical shift of 1H NMR profiles (predose-T0 vs postdose-T1h comparison). The validity of the models was evaluated using permutation p-values with R2X and Q2Y values.

Metabolite	**Chemical Shift**	**OPLS-DA Models Predose (T_0) vs MH (T_{1h})** R^2X = 93.63% Q^2Y = 0.4861 Permutation *p = 0.004*
O-Succinyl-L-homoserine	1.85 (m); 2.06 (m); 2.47 (d); 2.52 (m); 3.65 (m); 4.24 (dd)	-0.7492
Homocarnosine	3.17 (dd); 3.21 (t); 4.48 (m); 7.01 (s); 7.90 (s)	-0.7315
Choline	3.21 (s); 3.52 (m); 4.07 (m)	-0.7114
Homoserine	2.03 (m); 2.16 (m); 3.79 (m); 3.86 (dd)	+0.7645
Citrulline	1.58(m); 1.88(m); 3.15(t); 3.76(t)	-0.6722

Notes: (+) indicates metabolite that was higher in pre-dose group, whereas (-) indicates higher metabolites at post-dose (T_{1h}) after multiple oral dosing of the modified ECa 233 at 500 mg.
permutation p value obtained from n = 1000 permutation tests.
Abbreviations: s, singlet; d, doublet; dd; double of doubles; t, triplet; m, multiplet

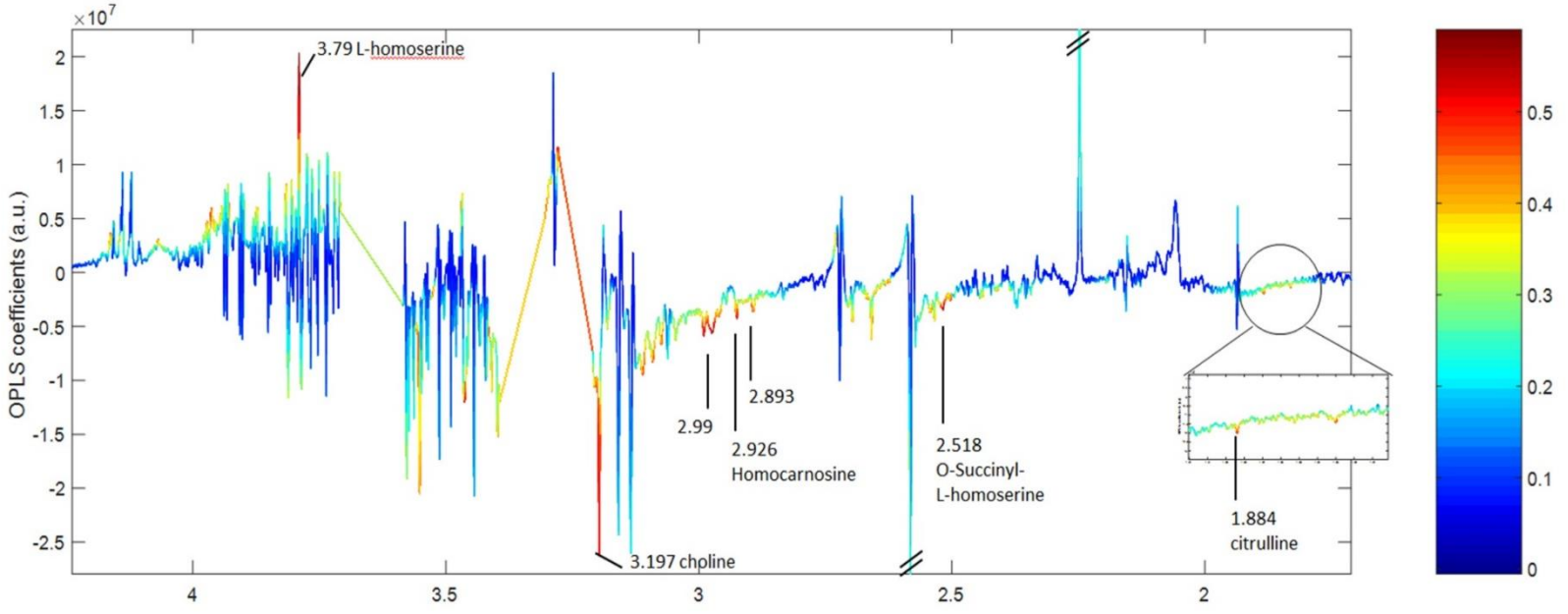

Figure 13 OPLS-DA coefficient loadings plot of different metabolites that were altered in the pairwise comparison (predose, T0 vs postdose, T1h). Resonances pointing upwards reflect metabolites that were greater in the pre-dose group relative to the post-dose group, whereas resonances pointing downwards indicate lower concentrations of metabolites in the pre-dose group relative to the post-dose group.

The findings demonstrated the significant differences in levels of metabolites that varied in the pairwise comparison (predose T0 vs postdose T1h), **(Figure 13)**. In contrast, focusing on the metabolites at post-dose (T1h, day 7), these results showed that relative plasma concentrations of L-homoserine significantly decreased while levels of citrulline, O-succinyl L-homoserine, homocarnosine, and choline were significantly increased after receiving modified formula ECa 233 in comparison to pre-dose levels at T_0.

Metabolomics shifts

A shift in human endogenous metabolite profiles as a result of administered modified formula ECa 233 was observed between a single low dose (250 mg dose taken once) and multiple high dose (500 mg dose taken once daily for 7 days), **Figure 14.** The PCA trajectory scores plot between single low dose and multiple high doses at different time points (0, 0.5, 1, 2 and 4 hours) revealed remarkable metabolic alterations. At pre-dose, the first time point demonstrates similar metabolic baselines of both groups suggesting that human endogenous metabolites at the beginning of the study are similar in both groups **(Figure 14)**. After receiving the modified formula ECa 233, distinct metabolic shifts were observed during 4 hours at different magnitudes **(Figure 14).**

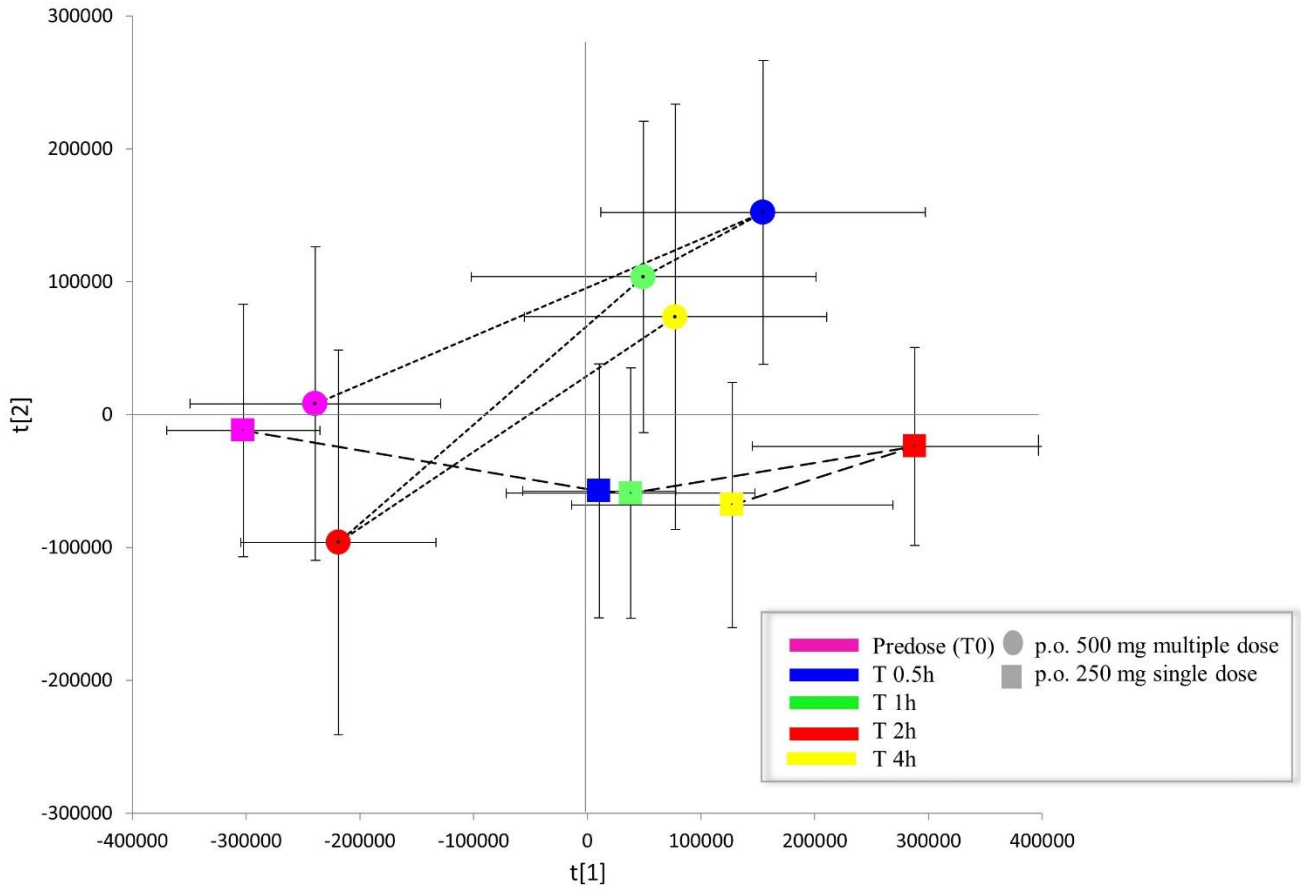

Figure 14 PCA trajectory scores plot of plasma data sets obtained from single low dose and multiple high dose; error bars are shown by standard deviation of the mean.

Safety and tolerability

The modified formula ECa 233 was well-tolerated throughout the study **(Table 11).** No serious adverse events were reported. The most commonly reported AEs were associated with the gastrointestinal system; mild abdominal discomfort was occasionally (n = 2/12) found during the 250-mg multiple oral dosing, and moderate flatulence (n = 1/11) with constipation (n = 1/11) were rarely reported in the group that received 500 mg doses. One participant reported serious drowsiness after receiving the multiple high doses of ECa 233; this AE was also included in the previous report after oral administration with high doses of *C. asiatica* [14].

Table 11 Summary of safety and adverse events of the modified-formula ECa 233 after single and multiple oral administration in different dosage regimens.

Adverse events	250 mg (n=12)		500 mg (n=11)		Relation to medication
	Single n/12 (%)	**Multiple** n/12 (%)	**Single** n/11 (%)	**Multiple** n/11 (%)	
Drowsiness	3/12 (25%) Mild-moderate	3/12 (25%) Mild-severe	3/11 (27.27%), mild-severe	1/11 (9.09%) severe	unlikely
Gastrointestinal Disorder					
Nausea	0	1/12 (8.33%) mild	0	0	possible
Vomiting	0	0	0	0	-
Burning stomach	0	0	0	0	-
Abdominal discomfort	0	2/12 (16.67%) mild	0	0	probable
Flatulence	0	1/12 (8.33%) mild	1/11 (9.09%) moderate	1/11 (9.09%) moderate	probable
Increased urinary frequency	1/2 (8.33%) mild	1/12 (8.33%) moderate	0	0	1-possible 1-probable
Others					
Headache	1/12 (8.33%) moderate	0	0	0	unlikely
Constipation	0	0	1/11 (9.09%) mild	1/11 (9.09%) mild-moderate	probable

Discussion

This study found that daily doses of the modified formula ECa 233 was safe and appropriate for clinical uses. This newly developed ECa 233 capsule causes changes not only in disposition kinetics but also in endogenous human metabolomics profiling.

In comparison with the previous formulation [7], AUC and Cmax of MDS and ASS exhibited the improved absorption kinetics of this modified-formula, which indicates an increased solubility of the parent compounds. This can be explained by the formula's enhanced dissolution from the addition of a small amount of solubilizer, an excipient that increases the bioavailability of pharmaceutical ingredients. The higher dissolution of this formula could lead to the increased MDS and ASS absorption and result in the improved ECa 233 oral bioavailability. Interestingly, a significantly greater systemic exposure was clearly seen in their respective metabolites (MDA and ASA). These results reflect a biotransformation of the parent compounds, the unabsorbed triterpenoid glycosides that can be completely hydrolyzed to become their respective metabolites (triterpenic acids). This process is carried out by an enzyme produced by intestinal bacteria with the role of hydrolyzing glycosides[15]. In addition, the previous study reported the possible hydrolysis mechanism of ASS. This mechanism involves hydrolysis of sugar moiety of the glycoside (ASS) under acidic conditions, converting ASS into aglycone (ASA) [16], which becomes the active component of *C. asiatica* extract that has pharmacological effects[17]. The modified formula ECa 233 had greater accumulation of active metabolites as seen in the increased AUC and Cmax of ASA ($p<0.05$), corresponding to the increased amount of ECa 233 in the administered dose. This accumulation supports the role of ASA in inhibition of P-glycoprotein (P-gp) [18], an efflux membrane

transporter that limits cellular uptake of xenobiotics. As a consequence of inhibiting P-gp expression, ASA (a substrate of P-gp) increases, then accumulates in the systemic circulation[19]. Other studies have also found an accumulation of ASA after oral administration of *C. asiatica* extract [20].

In regard to the excretion behaviors, the study findings fill a gap in the knowledge around ECa 233. MDS and ASS are rarely absorbed but rather undergo extensive biotransformation with minimal renal excretion. Their active metabolites, MDA and ASA, which are highly lipophilic molecules, were likely eliminated through the hepatobiliary system and then mainly excreted by the fecal route. The results suggest that both major parent compounds were excreted mainly as metabolites via the feces. This is supported by the findings of other research in elimination pathways of *C. asiatica* extracts, which indicates that the excretion (0-72 hours) of MDS was 0.25% in urine and 24.68% in feces [21]. Another study showed that the extract was excreted in feces over 24-76 hours, while only a small amount of extract was eliminated via the kidneys [20].

The accumulation behavior of the modified formula ECa 233 affected human metabolomic profiles, partly through an alteration of endogenous metabolites detected in plasma between pre- and post-dose. The changes in 5 relevant human metabolites were thoroughly considered to identify the candidate biomarkers; including L-homoserine, citrulline, O-succinyl L-homoserine, homocarnosine, and choline. In particular, increasing doses of ECa 233 to a 500-mg multiple oral dosing had a significant effect on increasing levels of choline, an endogenous metabolite reported to have benefits for learning and memory. It has been reported that higher concentrations of choline are believed to slow the progression of cognitive impairment[9]. The previous studies have suggested that a possible mechanism by

which *C. asiatica* enhances cognitive function is through inhibition of acetylcholinesterase activity (AChEI). The studies also demonstrated this extract's protective effect against ß-amyloid formation. The results indicated that ECa 233, an extract from of *C. asiatica*, might be considered as an AChEI alternative and responsible for increasing level of choline. Choline is a precursor of acetylcholine, a neurotransmitter that serves the neural activities in the learning and memory system. Furthermore, the reduction of choline activity in cholinergic neurons is also involved with cognitive impairment as detected in Alzheimer's disease[22,23]. This finding in healthy volunteers suggests that the recommended dose of ECa 233 should not be less than 500 mg/day in future studies investigating metabolite changes or exploring metabolic pathways related to cognitive deficits in patients.

Metabolite shifts were observed over 4 hours after administration of multiple doses of ECa 233 **(Figure 14)**. The results indicated that changes of metabolomic profiling could be obviously detected over 1-2 hours post-dose. This interval time is also considered as Tmax in the pharmacokinetic results (Tmax = 1-1.5 hour, **Table 9**). These findings could imply that the relationship between drug disposition kinetics and the changes in human endogenous metabolites are affected by time after administration of high oral doses of ECa 233. Lastly, at 4 hours later, the metabolic features had returned to nearly the starting point or closely to the initial metabolites again **(Figure 14)**, presumably because of the decreased concentration of the parent compounds and their active metabolites in plasma **(Figure 10)**. This pattern at end points conformed with the termination phase of ECa 233 as reflected in its excretion.

Conclusion

This study presents the first reported evidence on the elimination kinetics of standardized extract of *C. asiatica* (ECa 233), indicating that the unchanged forms (MDS and ASS) are excreted partly through renal elimination. Fecal excretion is assumed to be the major pathway for their active metabolites (MDA and ASA). The enhanced-formula ECa 233 indicated higher absorption, which significantly increased its bioactive metabolites in healthy volunteers. This study reveals that administering a daily dose of at least 500 mg of ECa 233 can alter human metabolic profiling. Five human endogenous metabolites (L-homoserine, citrulline, O-succinyl L-homoserine, homocarnosine, and choline) should be further investigated as candidate biomarkers in a phase II clinical trial.

Supporting information

Chromatograms of LC-MS/MS and NMR analysis are available in supplementary information.

Acknowledgements

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Professor Daren Cornforth for proofreading. Lastly, we are grateful to all participants who kindly participated in this study.

Conflict of interest

All authors declare no conflict of interest.

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SUPPLEMENTARY DATA

Clinical pharmacokinetics and metabolomics investigation of a modified formula of standardized *Centella asiatica* extract orally administered in healthy volunteers

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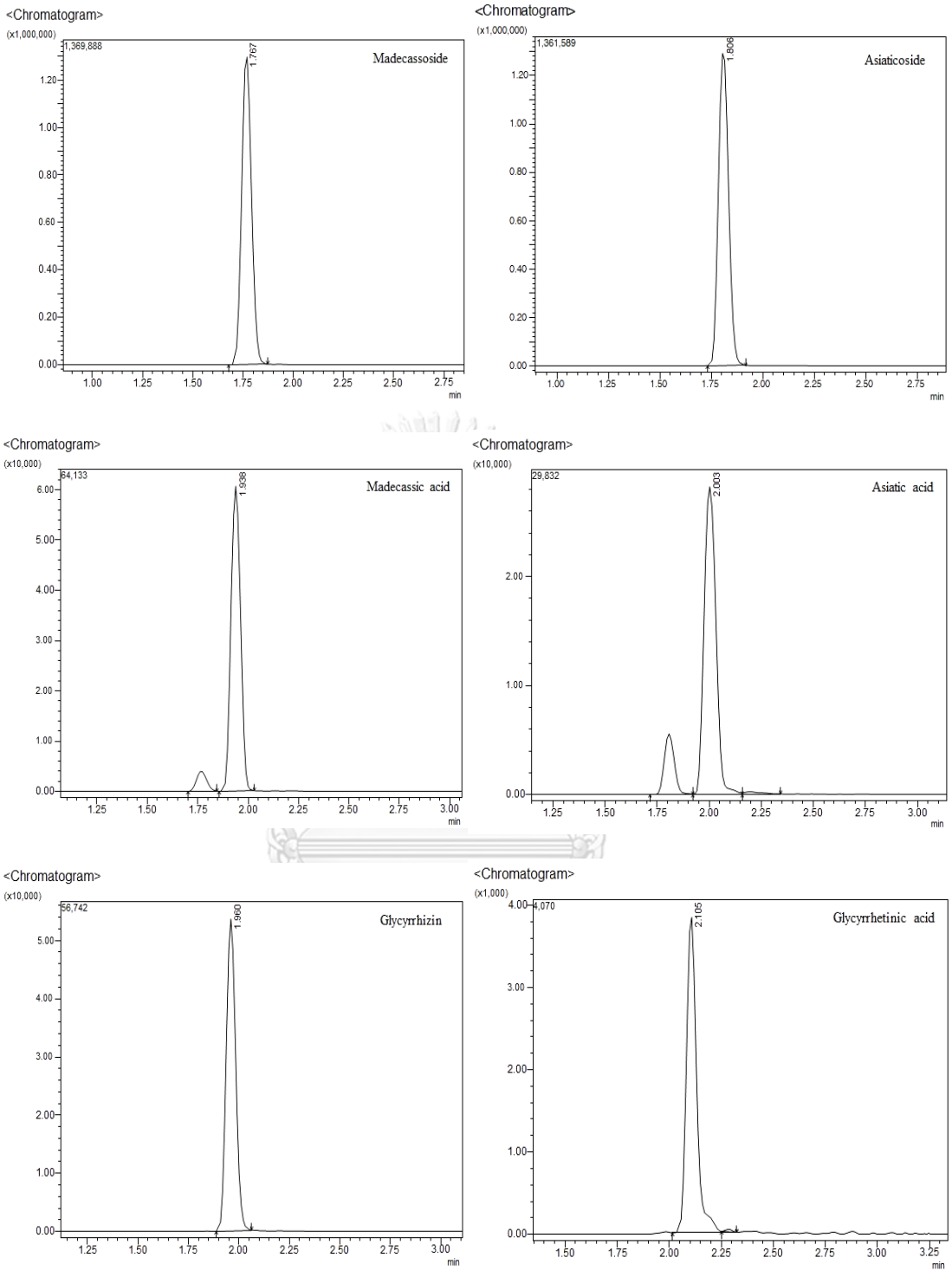

Figure 15 Chromatogram and retention time of madecassoside, asiaticoside, madecassic acid, asiatic acid, glycyrrhizin, and glycyrrhetinic acid.

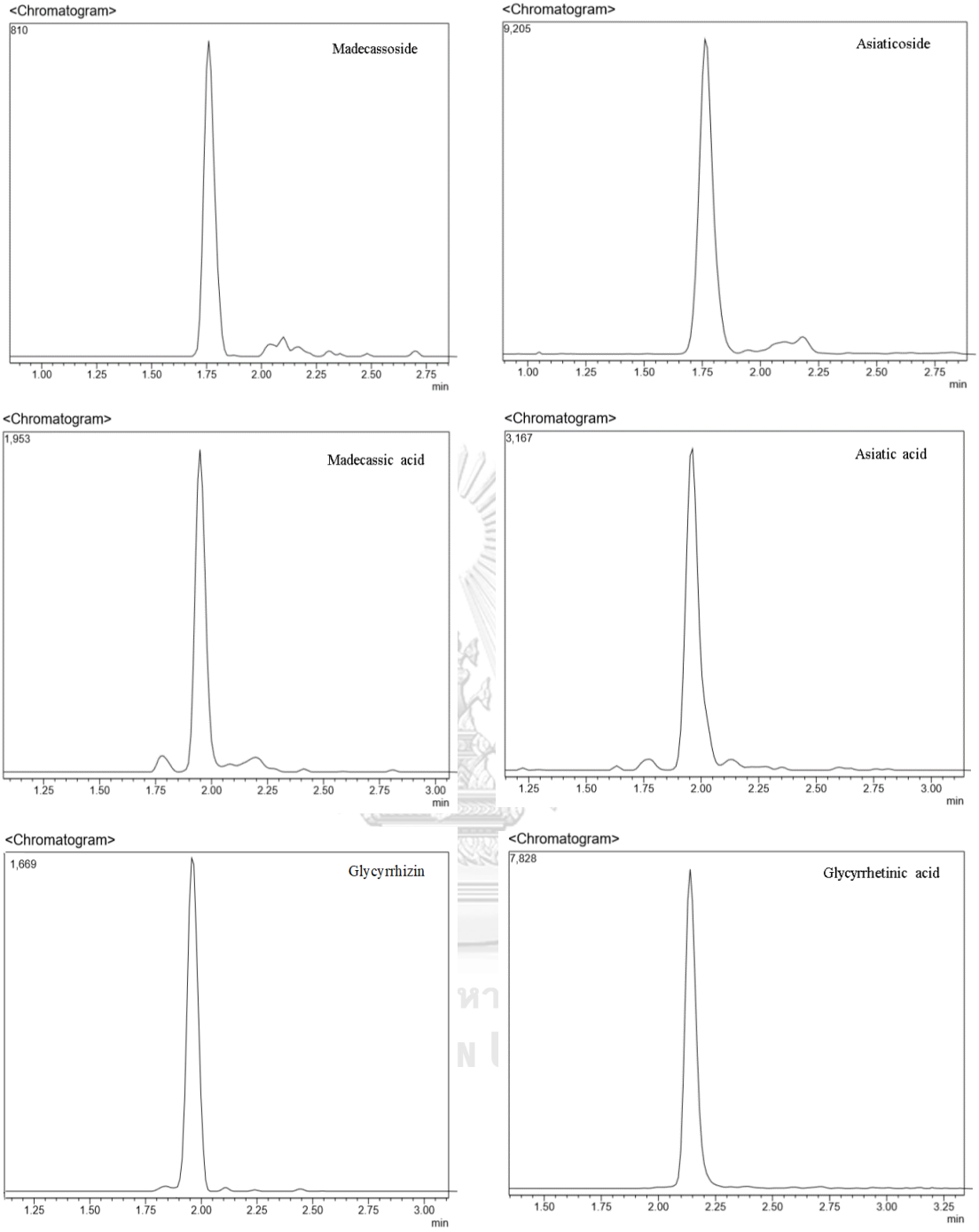

Figure 16 Chromatogram and retention time in plasma.

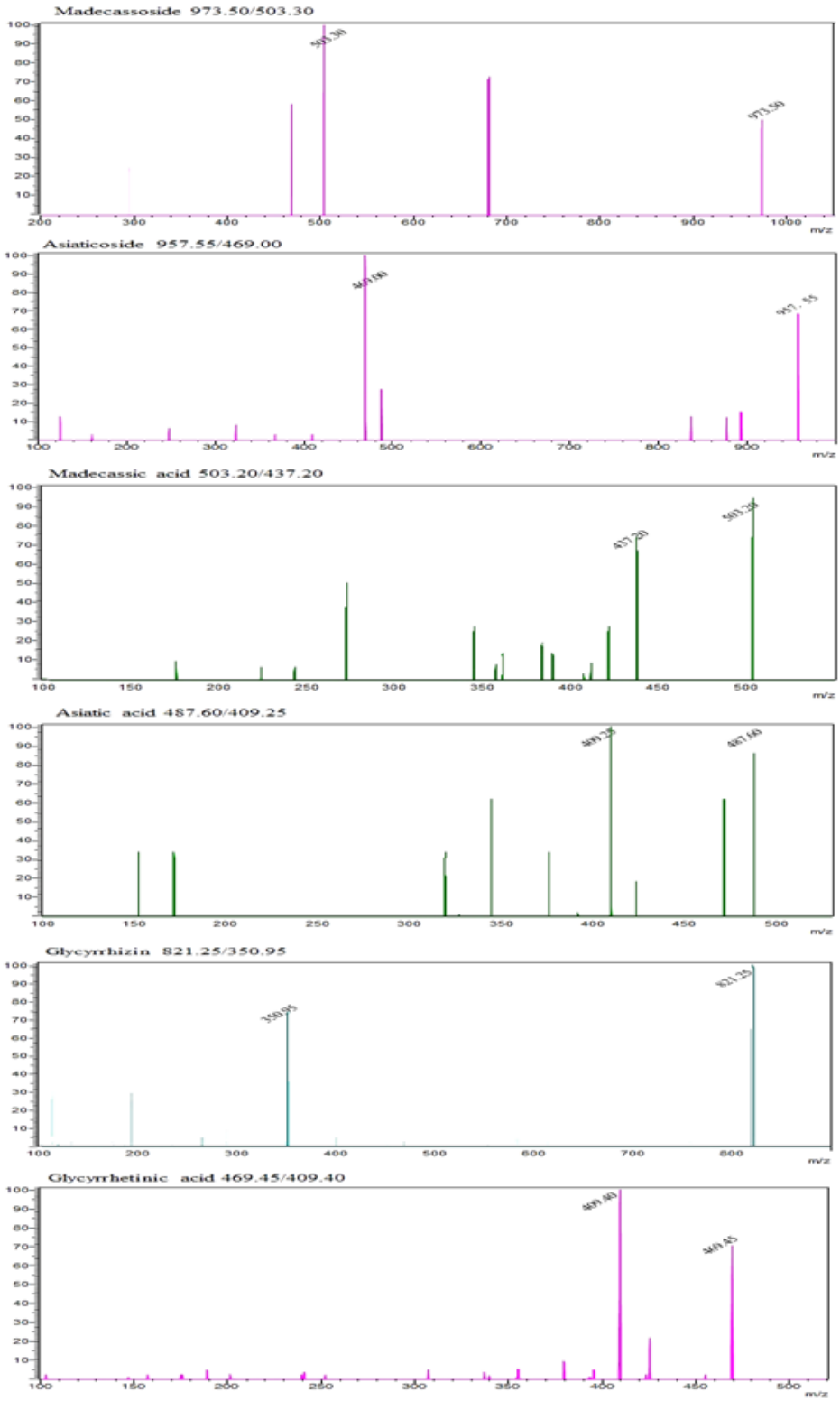

Figure 17 Mass spectrum of madecassoside, asiaticoside, madecassic acid, Asiatic acid, glycyrrhizin, and glycyrrhetinic acid

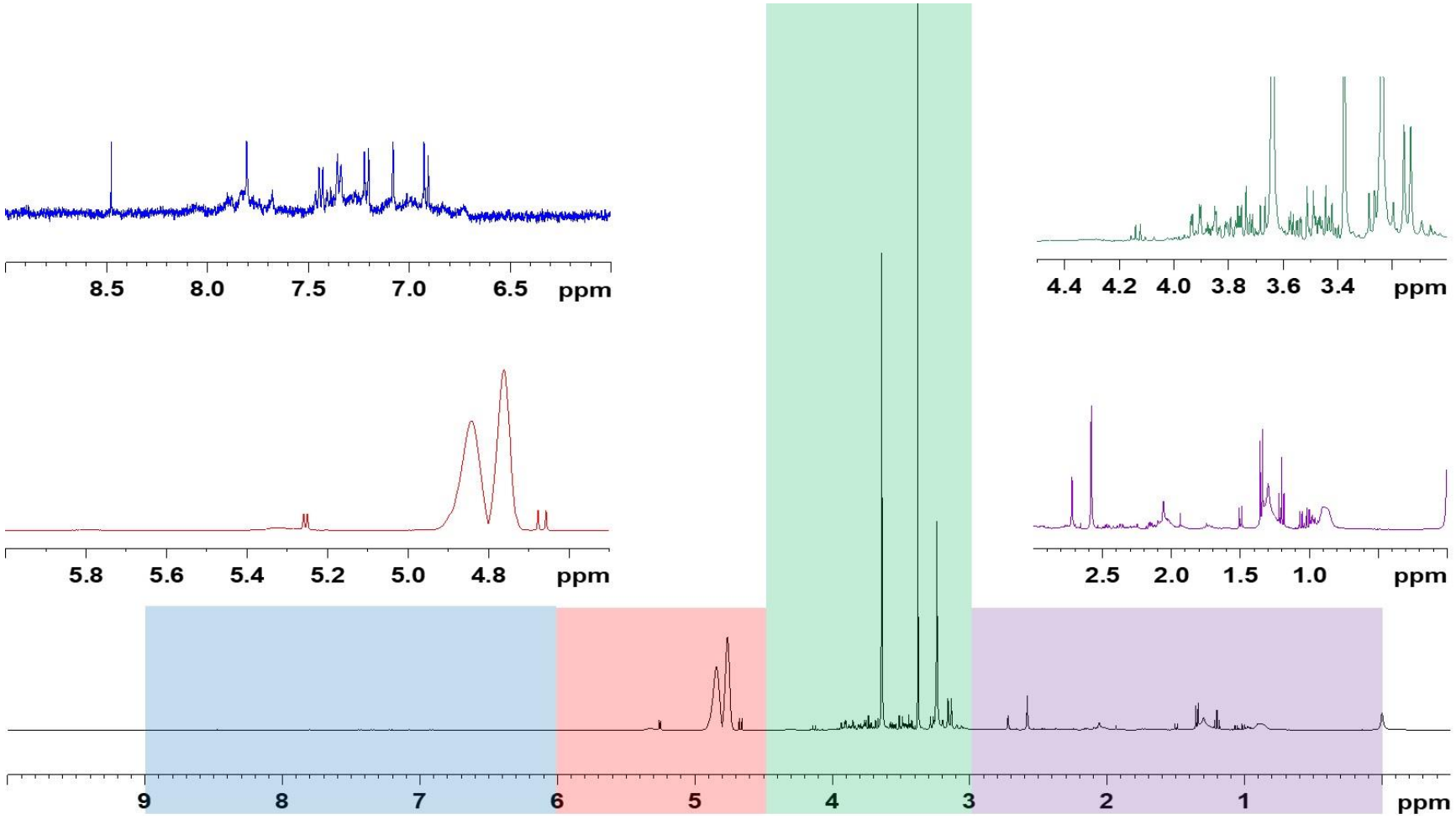

Figure 18 NMR chromatogram

CHAPTER V

Conclusion of this thesis

To summarize key findings of both research studies in the thesis, a standardized extract of ECa 233 was found to be potentially useful in alternative medicine as it demonstrates a good tolerability, whether it was administered as single or multiple doses in healthy volunteers. The modified formulation of ECa 233 was developed by adding surfactant to increase solubility of the poorly water-soluble substances, and this improved formulation was found to expand pharmacokinetics and to result in greater systemic exposure of ECa 233's active metabolites (MDA and ASA). For pharmacokinetics profiles, parent compounds were extensively metabolized into their respective metabolites found in blood circulation. In terms of excretion, this is the first evidence of human elimination kinetics of ECa 233, indicating that the unchanged forms (MDS and ASS) are excreted partly through renal elimination, with fecal excretion assumed as the major route for their active metabolites. For metabolomics, significant changes in human endogenous metabolites were detected in levels of L-homoserine, citrulline, O-succinyl L-homoserine, homocarnosine, and choline from the blood samples of participants after receiving the modified ECa 233 capsule. Increasing doses to a 500-mg multiple oral dosing was associated with a significant increase in levels of choline, an endogenous metabolite with benefits for learning and memory.

Limitations of this study

The pharmacokinetics and safety profile results from the phase I clinical trial support further investigation of this standardized extract of *C. asiatica* (ECa 233) in a phase II clinical study. The results suggest that the modified ECa 233 capsule possesses enhanced pharmacokinetics due to its improved dissolution. Oral administration of the modified capsule may affect the plasma metabolome through alteration of endogenous metabolite levels between pre- and post-dose. This study indicates that a daily dose of at least 500 mg of the modified ECa 233 can balance the levels of necessary plasma metabolites.

This study possesses some limitations. It does not include any potential effects on the human gut microbiome of ECa 233's metabolism. Also, data on the elimination of ECa 233 via the hepatobiliary system or fecal route was collected from only one research subject. Another limitation is that the NMR-metabolomics results do not account for lipid species identification, due to restrictions of the NMR technique. Therefore, further studies should consider effects of using phytopharmaceuticals, specifically this standardized *C. asiatica* (ECa 233) extract, on human lipid alterations.

Suggestions for future research

Future work on pharmacokinetics should focus on the following aspects: (1) understanding the effects of enzymes responsible for hydrolyzing glycosides, a possible metabolism pathway of this standardized extract (ECa 233); and (2) confirming the major route of eliminating ECa 233's parent compounds and their metabolites with use of a larger sample size. This research suggests fecal elimination as the major route of elimination based on data from one study participant; in this individual, MDS and ASS were excreted mainly as MDA and ASA via the feces.

Regarding further studies on metabolomics, human lipidome alteration that can regulate the neuronal cell function should be explored. Taken together with the effects of lipidome alteration on neuronal cell functions, an imbalance in levels of plasma lipid metabolites also indicates the progression of neurodegenerative diseases [26]. For these reasons, future work should focus on changes of endogenous lipid profiles, including lipid biomolecules. In addition, network analysis on changed lipidome should subsequently be provided, based on understanding of phospholipid metabolism with possible alteration in cognitive impairment.

Based on the findings of significant changes in human endogenous metabolites reported in this study, the next step should be a clinical trial phase II to confirm these metabolic changes including measurement of the metabolites;

(1) L-homoserine, a reactive variant of the amino acid serine (HMDB database);

(2) citrulline which participates in a number of enzymatic reaction (HMDB database);

(3) O-succinyl L-homoserine, a member of the class of L-alpha-amino acids found in a number of food items (FOODB);

(4) homocarnosine, a normal human metabolite which is the brain-specific dipeptide of gamma-aminobutyric acid (GABA) and histidine (HMDB database);

(5) choline, an important precursor of acetylcholine (HMDB database).

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APPENDIX

Adverse Event Reporting

Adverse Events

Adverse event (AE) is defined as any untoward medical occurrence in a clinical investigation where subjects are administered a pharmaceutical product which does not necessarily have a causal relationship with this treatment. AE can, therefore, be any unfavorable and unintended sign, (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product [28].

All AEs regardless of treatment group or suspected causal relationship to the investigational product(s) will be mainly reported as per clinical research department SOPs. For examples; Clinical investigator will evaluate all AEs as to:

- Level of severity (refer to Topic "Severity Assessment") [29]:
  - Mild;
  - Moderate:
  - Severe;
  - Life-threatening
  - Lethal;
- Outcome: The outcome occurred after any AE at any dose that:
  - resolved without sequelae, or
  - resolved with sequelae, or
  - ongoing (recovering or not yet recover), no further follow-up required, or
  - resulted in death, or
  - others e.g. loss of follow up
- Duration: Record, at least, the start and stop dates of the adverse experience. If less than 1 day, indicate the appropriate length of time and units.
- Action taken

- Relationship to the test drug: Use the following scale of criteria [30];
  - **Certainly** related to test drug: guidance
    - Event or laboratory test abnormality, with plausible time relationship to drug intake
    - Cannot be explained by disease or other drugs
    - Response to withdraw plausible (pharmacologically, pathologically)
    - Event definitive pharmacologically or phenomenologically (i.e. an objective and specific medical disorder or a recognized pharmacological phenomenon)
    - Rechallenge satisfactory, if necessary
  - **Probably/likely** related to test drug: guidance
    - Event or laboratory test abnormality, with reasonable time relationship to drug intake
    - Unlikely to be attributed to disease or other drugs
    - Response to withdrawal clinically reasonable
    - Rechallenge not required
  - **Possibly** related to test drug: guidance
    - Event or laboratory test abnormality, with reasonable time relationship to drug intake
    - Could also be explained by disease or other drugs
    - Information on drug withdrawal may be lacking or unclear
  - **Unlikely** related to test drug: guidance
    - Event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable (but not impossible)
    - Disease or other drugs provide plausible explanations
  - **Unclassified/conditional** related to test drug: guidance
    - Event or laboratory test abnormality

- More data for proper assessment needed, or
- Additional data under examination

- **Unrelated** to test drug: guidance
  - The subject did not receive the test drug or
  - Temporal sequence of the AE onset relative to administration of the test drug is not reasonable or
  - There is another obvious cause of the AE

For all AEs, the Clinical Investigator must pursue and obtain adequate information adequate both to determine the outcome of the AE and to assess whether it meets the criteria for classification as an SAE requiring immediate notification to Sponsor or not. For all AEs, sufficient information should be obtained by the investigator to determine the causality of the AE. The clinical Investigator is required to assess causality. Follow-up by the investigator may be required until the event or its sequelae resolved or stabilized at a level acceptable to the investigator.

All adverse events, medical events or laboratory abnormalities will be reported in the final report in part of clinical study report.

Common Terminology Criteria for Adverse Events (CTCAE) v5.0

Publish Date: November 27, 2017

Introduction
The NCI Common Terminology Criteria for Adverse Events is a descriptive terminology which can be utilized for Adverse Event (AE) reporting. A grading (severity) scale is provided for each AE term.

SOC
System Organ Class (SOC), the highest level of the MedDRA[1] hierarchy, is identified by anatomical or physiological system, etiology, or purpose (e.g., SOC Investigations for laboratory test results). CTCAE terms are grouped by MedDRA Primary SOCs. Within each SOC, AEs are listed and accompanied by descriptions of severity (Grade).

CTCAE Terms
An Adverse Event (AE) is any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medical treatment or procedure that may or may not be considered related to the medical treatment or procedure. An AE is a term that is a unique representation of a specific event used for medical documentation and scientific analyses. Each CTCAE v4.0 term is a MedDRA LLT (Lowest Level Term).

Grades
Grade refers to the severity of the AE. The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each AE based on this general guideline:

Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
Grade 2 Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL*.
Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL**.
Grade 4 Life-threatening consequences; urgent intervention indicated.
Grade 5 Death related to AE.

A Semi-colon indicates 'or' within the description of the grade.

A single dash (-) indicates a Grade is not available. Not all Grades are appropriate for all AEs. Therefore, some AEs are listed with fewer than five options for Grade selection.

Grade 5
Grade 5 (Death) is not appropriate for some AEs and therefore is not an option.

Definitions
A brief Definition is provided to clarify the meaning of each AE term. A single dash (-) indicates a Definition is not available.

Navigational Notes
A Navigational Note is used to assist the reporter in choosing a correct AE. It may list other AEs that should be considered in addition to or in place of the AE in question. A single dash (-) indicates a Navigational Note has not been defined for the AE term.

Activities of Daily Living (ADL)
*Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
**Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

[1] CTCAE v5.0 incorporates certain elements of the MedDRA terminology. For further details on MedDRA refer to the MedDRA MSSO Web site (https://www.meddra.org/).

Additional tables and figures

Table 12 List of metabolites in plasma

No.	metabolites	Chemical shift
1	leucine	1.01 (d); 1.07 (d); 1.71 (m); 3.72 (m)
2	valine	0.99 (d); 1.07 (d); 2.30 (m); 3.70 (d)
3	isoleucine	0.96 (t); 0.99 (d); 1.01 (d); 1.96 (m); 3.70 (d)
4	lactate	4.14 (q); 1.34 (d)
5	alanine	3.80 (q); 1.50 (d)
6	acetate	1.93 (s)
7	glutamate	3.70 (q); 2.46 (m); 2.16 (m)
8	methylamine	2.58 (s)
9	dimethylamine	2.50 (s)
10	choline	4.07 (m), 3.55 (m), 3.19 (s)
11	glucose	5.25 (d); 4.63 (d); 3.90 (dd); 3.82 (m); 3.72(m); 3.57 (m); 3.39 (m); 3.22 (dd)
12	myo-inositol	4.06 (t); 3.71 (t); 3.5 (dd); 3.26 (t)
13	3-Hydroxyisobutyrate	3.50 (m); 2.46 (m); 1.11 (d)
14	serine	3.95(m); 3.83 (dd)
15	propionate	2.17 (q); 1.03 (t)
16	glycine	3.54 (s)
17	pyruvate	7.65(s); 2.46(s)
18	dopamine	6.90 (d); 6.84 (d); 6.74 (dd); 3.22 (t); 2.86 (t)
19	histidine	7.90 (d); 7.09 (d); 3.98 (dd); 3.23 (dd); 3.16 (dd)
20	tyrosine	7.17 (m); 6.87 (m); 3.92 (dd); 3.17 (dd); 3.02 (dd)
21	octopamine	7.17 (m); 6.76 (m); 4.66 (dd); 3.44 (dd); 3.22(dd); 1.90 (s)
22	urocanate	7.85 (s); 7.37 (s); 7.27 (d); 6.36 (d)
23	3-hydroxykynurenine	7.42 (d or dd); 7.04 (d or dd); 6.73 (t); 4.11 (t); 3.74 (d)

24	1-methylhistidine	7.67 (s); 7.00(s); 3.95 (dd); 3.68 (s); 3.16 (dd); 3.06 (dd)
25	O-Succinyl-L-homoserine	1.85 (m); 2.06 (m); 2.47 (d); 2.52 (m); 3.65 (m); 4.24 (dd)
26	Homocarnosine	3.17 (dd); 3.21 (t); 4.48 (m); 7.01 (s); 7.90 (s)
27	Homoserine	2.03 (m); 2.16 (m); 3.79 (m); 3.86 (dd)
28	Citrulline	1.58(m); 1.88(m); 3.15(t); 3.76(t)
29	Serine	3.85 (dd); 3.95 (dd); 4.00 (dd)

Following oral administration of a once-daily dose of 250 mg or 500 mg of modified-formula ECa 233 for 7 consecutive days, metabolomic profiling detected a total of 31 endogenous metabolites from 1D ^{1}H-NMR spectra. Twenty-nine metabolites in plasma **(Table 12)** were identified according to the Chenomx NMR and human metabolome database.

Table 13 Summary of metabolic changes in plasma 1H NMR profiles

No.	Comparison	Group	Q^2	models	Figure
1	SL vs MH	Single low dose (250 mg) vs Multiple high dose (500 mg)	0.590	OPLS-DA	**Figure 19**
2	SLT01 vs MHT03	Pre-dose (T_0) vs Post-dose (500 mg, $T_{0.5h}$)	0.550	OPLS-DA	**Figure 20**
3	SLT01 vs MHT04	Pre-dose (T_0) vs Post-dose (500 mg, T_{1h})	0.508	OPLS-DA	**Figure 21**
4	SLT01 vs MHT06	Pre-dose (T_0) vs Post-dose (500 mg, T_{2h})	0.515	OPLS-DA	**Figure 22**
5	SLT01 vs MHT08	Pre-dose (T_0) vs Post-dose (500 mg, T_{4h})	0.498	OPLS-DA	**Figure 23**
6	SLT01 vs SLT03	Pre-dose (T_0) vs Post-dose (250 mg, $T_{0.5h}$)	0.453	OPLS-DA	**Figure 24**
7	SLT01 vs SLT04	Pre-dose (T_0) vs Post-dose (250 mg, T_{1h})	0.434	OPLS-DA	**Figure 25**
8	SLT01 vs SLT06	Pre-dose (T_0) vs Post-dose (250 mg, T_{2h})	0.503	OPLS-DA	**Figure 26**
9	SLT01 vs SLT08	Pre-dose (T_0) vs Post-dose (250 mg, T_{4h})	0.445	OPLS-DA	**Figure 27**

Summary of score plot and loading plot in OPLS-DA model

O-PLS-DA models were constructed in order to visualise the metabolic profiles indicating the differences in human endogenous metabolites. Pairwise comparison including single low dose (250 mg) vs multiple high doses (500 mg) and predose (T0) vs. postdose (T0.5, 1, 2, and 4 hour) were carried out using O-PLS-DA in order to extract the detail of metabolic alterations after oral administration of ECa 233.

Single low dose (250 mg) vs Multiple high dose (500 mg)

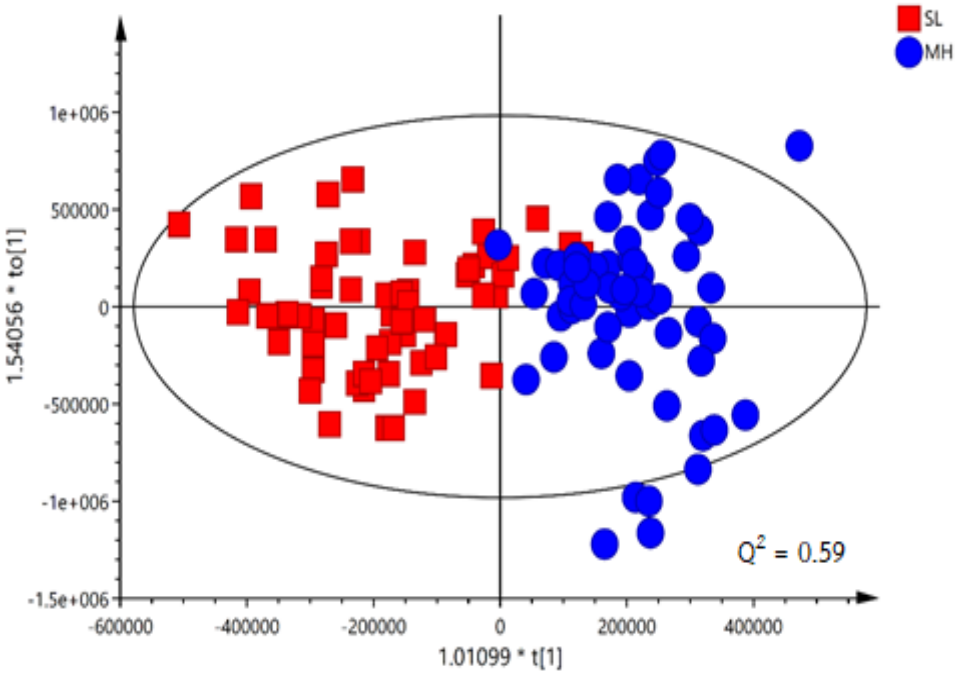

Figure 19 OPLS-DA score plot of human endogenous metabolites between the two clusters, repeated administration of 250 mg and 500 mg of ECa 233

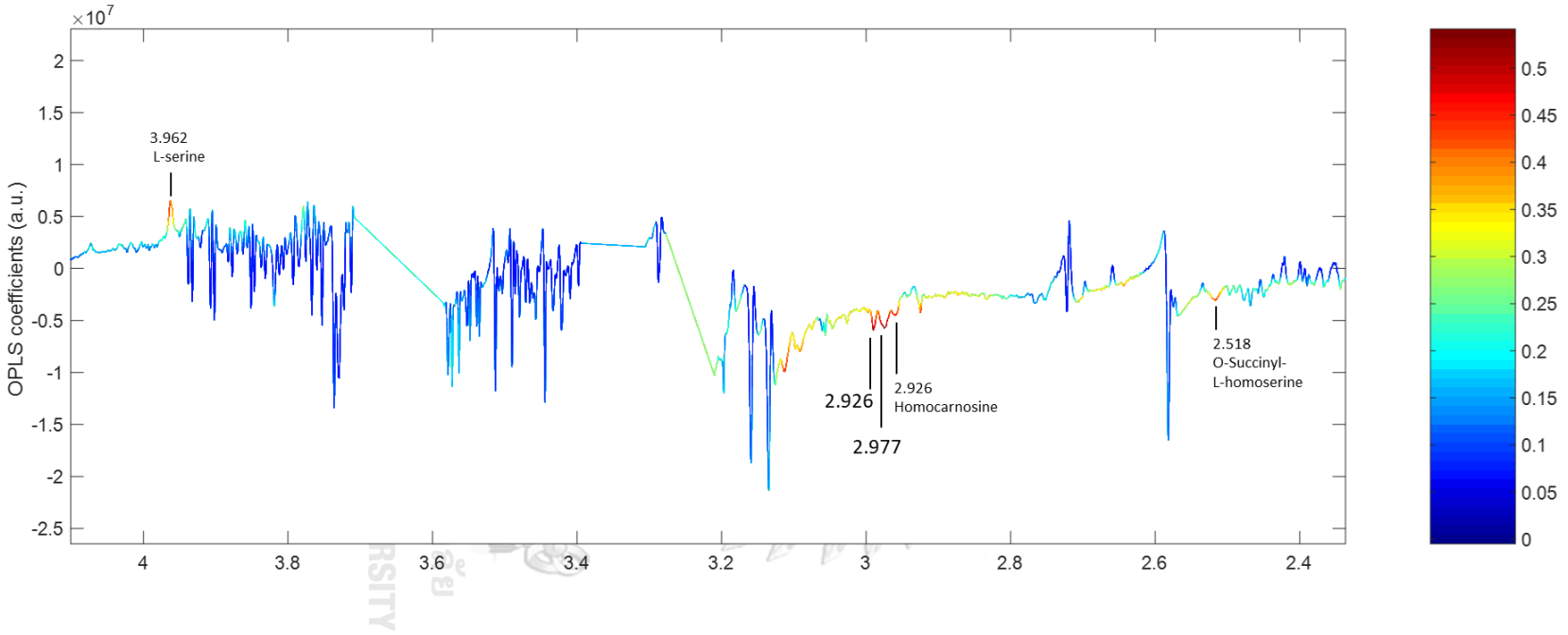

Figure 20 OPLS-DA coefficient loadings plot of different metabolites that were altered in the pairwise comparison (single low dose, 250 mg vs. multiple high doses, 500 mg).

Table 14 Metabolic changes in human plasma and chemical shift of ^{1}H NMR profiles (single low dose vs multiple high dose comparison). The validity of the models was evaluated using permutation p-values with R2X and Q2Y values.

		O-PLS-DA Models
Metabolite	**Chemical Shift**	SL vs. MH R^2X =60.01% Q^2Y = 0.342 p-value =0.002
O-Succinyl L-homoserine	1.85 (m); 2.06 (m); 2.47 (d); 2.52 (m); 3.65 (m); 4.24 (dd)	-0.6228
Homocarnosine	3.17 (dd); 3.21 (t); 4.48 (m); 7.01 (s); 7.90 (s)	-0.6522
L-Serine	3.85 (dd); 3.95 (dd); 4.00 (dd)	0.6189

Notes: (+) indicates metabolite that was higher in single low dose group, whereas (-) indicates higher metabolites in multiple high dose group after multiple oral dosing of the modified ECa 233
permutation p value obtained from n = 1000 permutation tests.
Abbreviations: s, singlet; d, doublet; dd; double of doubles; t, triplet, m, multiplet

Clustering between single low dose (250 mg) and multiple high doses (500 mg) of modified-formula was clearly separated based on the OPLS-DA score plot, indicating the discrimination of metabolites observed among those two clusters **(Figure 19).**

Figure 20 showed a pairwise comparison of O-PLS-DA coefficient loadings plot between single low dose (250 mg) and multiple high doses (500 mg). Resonances pointing upwards reflect metabolites that were greater in the single low dose group relative to the multiple high doses group, whereas resonances pointing downwards indicate lower concentrations of metabolites in the single low dose group relative to the multiple high doses.

This finding indicated that the alteration of endogenous metabolites showed the significant differences between two doses. The results show that relative plasma concentrations of L-serine significantly increased while levels of O-succinyl L-homoserine and homocarnosine were significantly decreased in a group of 250 mg. The significantly altered features, which were related to dosage of ECa 233, are summarised in **Table 14**. These findings could imply that administration of higher dosage (500 mg) of ECa 233 was found to be significantly associated with an increase level O-succinyl L-homoserine and homocarnosine.

Pre- and Post-dose Comparison

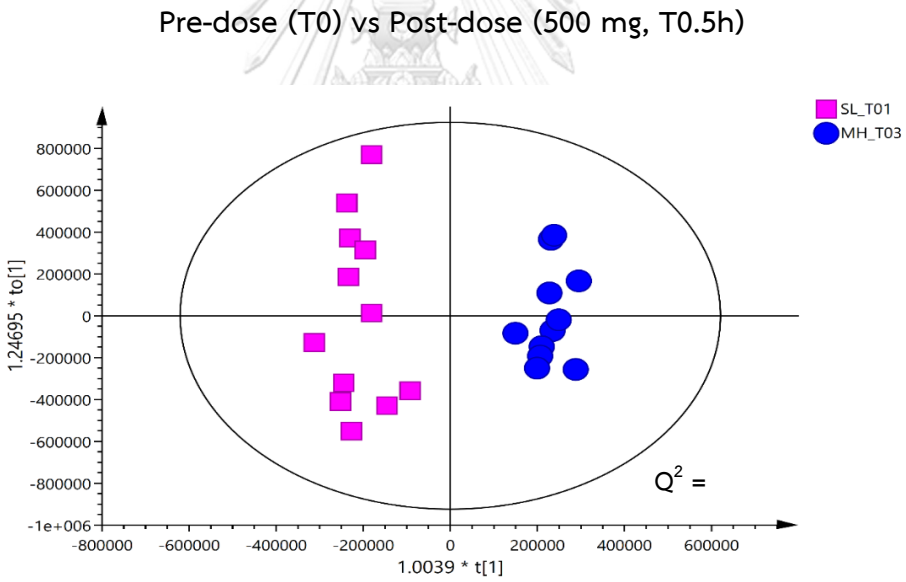

Figure 21 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T0.5h), for repeated administration of 500 mg of ECa 233

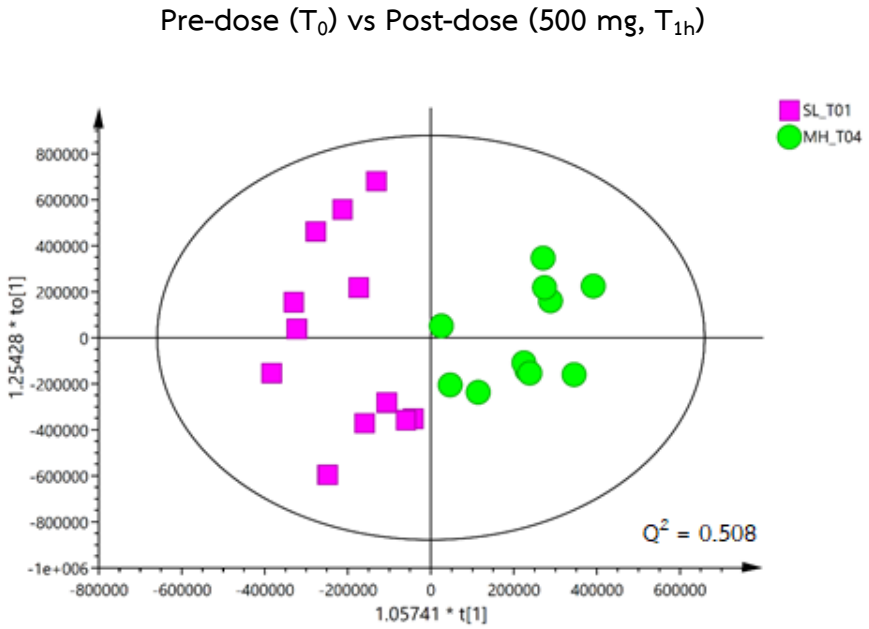

Figure 22 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T1h), for repeated administration of 500 mg of ECa 233

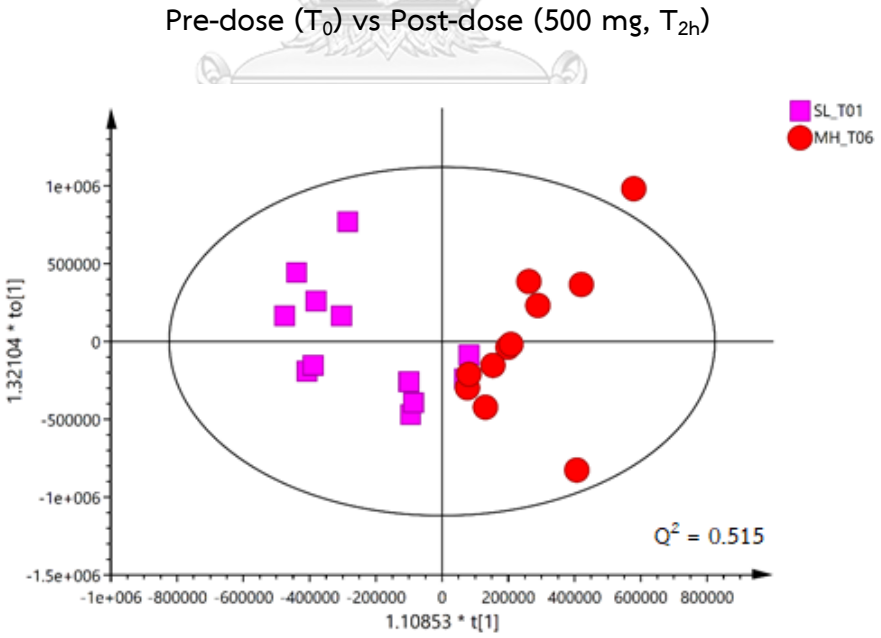

Figure 23 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T2h), for repeated administration of 500 mg of ECa 233

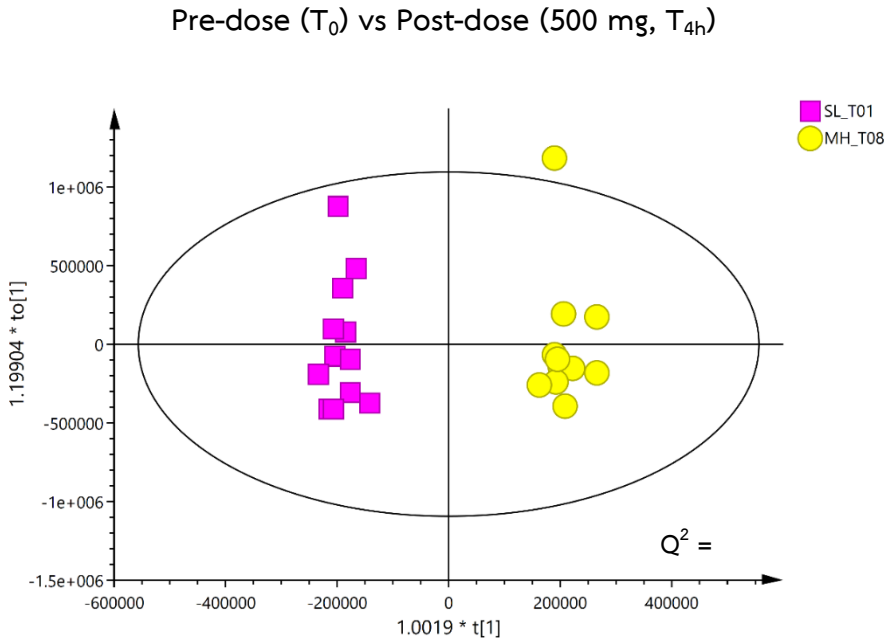

Figure 24 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T4h), for repeated administration of 500 mg of ECa 233

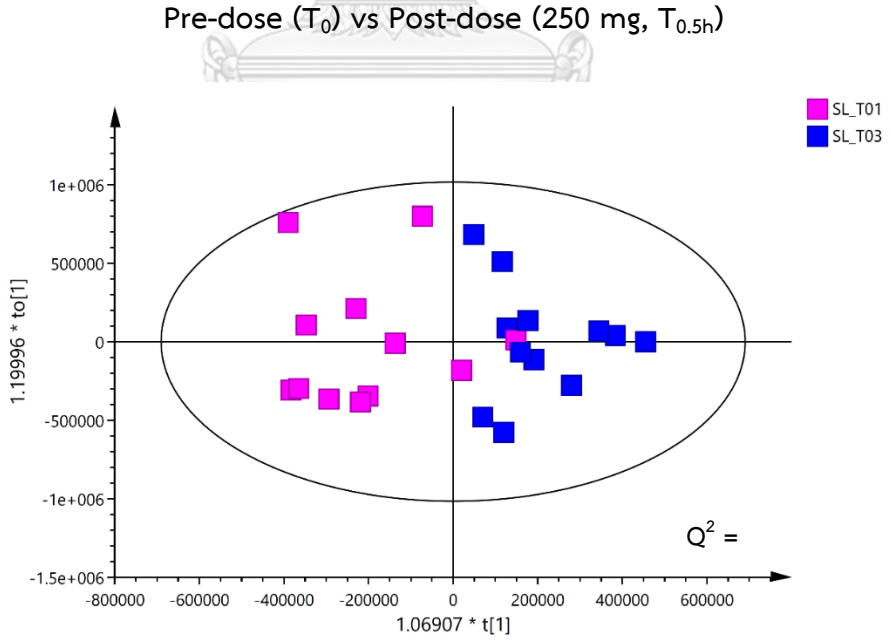

Figure 25 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T0.5h), for repeated administration of 250 mg of ECa 233

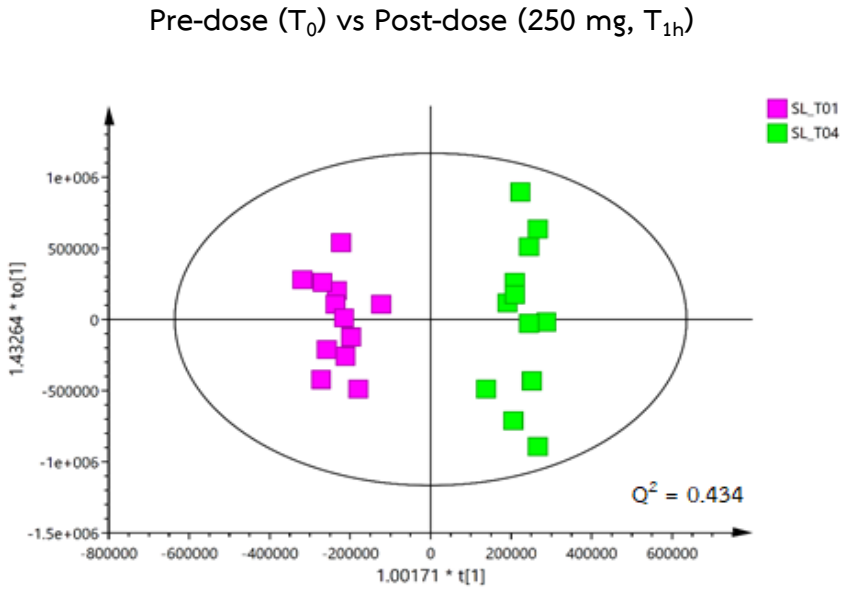

Figure 26 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T1h), for repeated administration of 250 mg of ECa 233

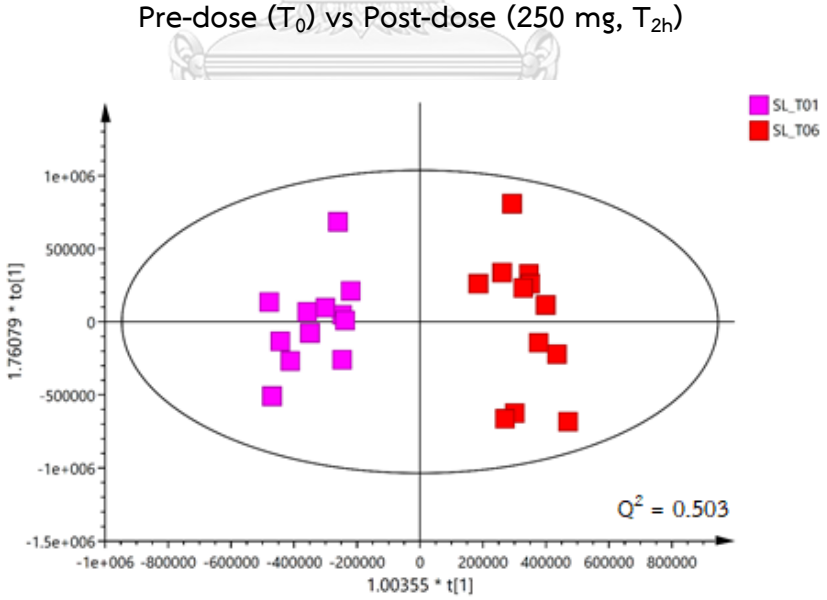

Figure 27 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T2h), for repeated administration of 250 mg of ECa 233

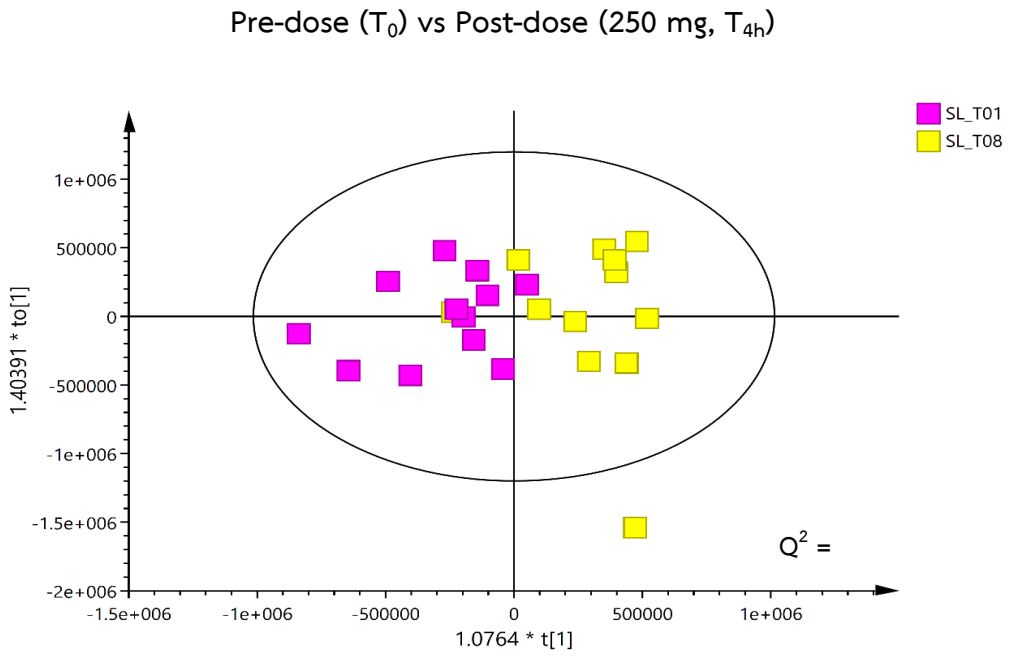

Figure 28 OPLS-DA score plot of human endogenous metabolites between the two clusters, pre- and post-dose (T4h), for repeated administration of 250 mg of ECa 233

Plasma metabolic profiles of predose were compared with those of postdose **(Figure 21-28)** and the results showed that distinct metabolic difference was observed at postdose, 0.5, 1, 2, and 4-hour after multiple oral administration of 250 and 500 mg ECa 233. Although these O-PLS-DA models comparing metabolome between pre- and post-dose demonstrated possibly valid statistical parameters **(Table 13),** there was no significant discriminatory metabolite obtained at T 0.5, 2, and 4 hours after Benjamini-Hochberg FDR correction. Therefore, this research thesis presented the OPLS-DA score plot of human endogenous metabolites between the two clusters only for the repeated administration of 500 mg at T 1 hour in the second publication (**Figure 12, Chapter IV**).

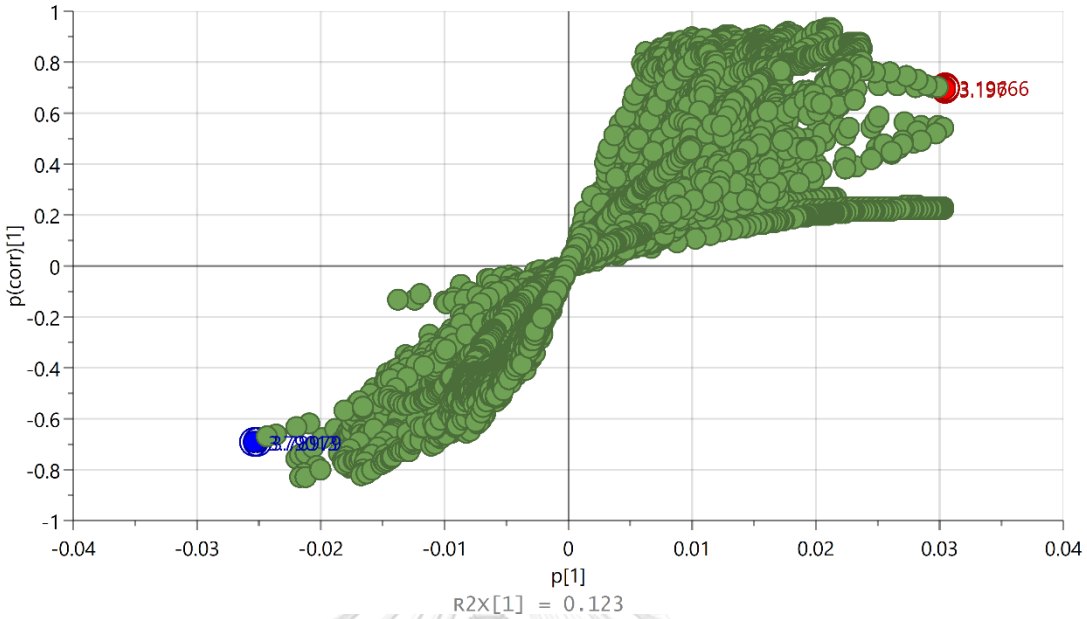

Figure 29 S-line plots of the model between pre- and post-dose (T1h) after oral administration of 500 mg ECa 233, showed the correlated features to either increased or decreased relative abundance of the metabolites.

O-PLS regression analysis of the significantly altered metabolites detected in loading plot (**Figure 13**) between predose (T0) vs postdose (T1h), corresponded to S-line plots showing the correlated features to either increased or decreased relative abundance of human metabolites including citrulline, O-succinyl L-homoserine, homocarnosine, L-homoserine and choline.

Dissolution investigation

The standardized *C. asiatica* (ECa 233) extract mainly consisted of madecassoside and asiaticosode (>80% of triterpenoids); however, these compounds were characterized as poorly soluble in water. To improve oral bioavailability of these compounds, the modified ECa 233 capsule was prepared by adding sodium lauryl sulphate (SLS) as one of the surfactant in the formulation ingredients.

The contents of mainly active ingredients of ECa 233 were determined to be triterpene derivatives following the United States Pharmacopeia (USP). The results showed that the newly-developed ECa 233 possessed higher solubility, with a higher dissolution percentage. The content of triterpene derivatives could reach 80% of dissolution within 45 minutes in an environment with an acidic pH (0.1 M HCl), which simulated the acidic conditions of human stomach.

Considering the effect of SLS in terms of surfactant enhanced dissolution was described through a process of micelle formation [27]. Micelles facilitated an improved dissolution in gastrointestinal tract to achieve the desired plasma concentration of bioactive compounds, which were further determined in pharmacokinetics profiles.

Table 15 Dissolution profiles of modified ECa 233

% Dissolution		Peak area of triterpene derivative						
		Capsule 1	Capsule 2	Capsule 3	Capsule 4	Capsule 5	Capsule 6	Average
standard		327.834	326.514	327.595	327.418	323.916	-	326.655
45 min	Asiaticoside	333.342	291.832	306.208	294.149	335.277	303.658	310.744
	Madecassoside	141.318	125.073	129.031	123.919	139.294	127.282	130.986
	Asiaticoside B	217.640	190.468	199.185	191.548	216.968	196.453	202.044
% Dissolution		97%	85%	89%	85%	97%	88%	89%
Max		97%						
Min		85%						
60 min	Asiaticoside	347.554	324.584	329.591	325.936	346.110	337.303	335.180
	Madecassoside	143.560	135.126	135.966	134.324	141.711	138.838	138.254
	Asiaticoside B	225.624	211.407	214.299	211.982	224.620	218.431	217.727
% Dissolution		100%	94%	95%	94%	100%	97%	97%
Max		100%						
Min		94%						

Institutional Review Board and Certificate of approval

COA No. 911/2017

IRB No. 446/60

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University

1873 Rama 4 Road, Patumwan, Bangkok 10330, Thailand, Tel 662-256-4493

Certificate of Approval

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the following study in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title : Safety and pharmacokinetics study of standardized extract of Centella asiatica (ECa233) capsule in Thai healthy volunteers

Study Code : -

Principal Investigator : Assist.Prof. Pajaree Chariyavilaskul, M.D., MSc., Ph.D.

Affiliation of PI : Department of Pharmacology,
Faculty of Medicine, Chulalongkorn University.

Review Method : Full board

Continuing Report : Every 3 months.

Document Reviewed :

1. STUDY PROTOCOL Version 2.0, Dated 10-10-2017
2. Protocol Synopsis Version 2.0, Dated 10-10-2017
3. Information sheet for research participant Version 2.0, Dated 10-10-2017
4. Informed consent for participating volunteers Version 2.0, Dated 10-10-2017
5. Invitations for volunteers to participate in research projects Version 1.0, dated 20-05-17

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;
2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for the record;
3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;
4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;
5. If the study cannot be finished within the expire date of the approval certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.
6. If the research project is completed, the researcher must be form the Faculty of Medicine, Chulalongkorn University.

* A list of the Institutional Review Board members (names and positions) present at the meeting of Institutional Review Board on the date of approval of this study has been attached. All approved documents will be forwarded to the principal investigator.

6. CASE REPORT FORM Version 1.0, dated 20-05-17
7. Curriculum Vitae and GCP Training
   - Assist.Prof. Pajaree Chariyavilaskul, M.D., MSc., Ph.D.
   - Miss Phanit Songvut
   - Phisit Khemawoot
   - Mayuree H. Tantisira

Signature .....
(Emeritus Professor Tada Sueblinvong MD)
Chairperson
The Institutional Review Board

Signature .....
(Napakkawat Buathong, PhD.)
Member and Assistant Secretary, Acting Secretary
The Institutional Review Board

Date of Approval : October 17, 2017
Approval Expire Date : October 16, 2018

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;
2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for the record;
3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;
4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;
5. If the study cannot be finished within the expire date of the approval certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.
6. If the research project is completed, the researcher must be form the Faculty of Medicine, Chulalongkorn University.

* A list of the Institutional Review Board members (names and positions) present at the meeting of Institutional Review Board on the date of approval of this study has been attached. All approved documents will be forwarded to the principal investigator.

COA No. 850/2018

IRB No. 479/61

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University

1873 Rama IV Road, Patumwan, Bangkok 10330, Thailand, Tel 662-256-4493

Certificate of Approval

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the following study in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title : Safety and pharmacokinetics study of a modified formulation of standardized extract of Centella asiatica (ECa233) capsule in Thai healthy volunteers.

Study Code : -

Principal Investigator : Assist.Prof. Pajaree Chariyavilaskul, MD., MSc, PhD.

Affiliation of PI : Department of Pharmacology,
Faculty of Medicine, Chulalongkorn University.

Review Method : Full board

Continuing Report : At least once annually or submit the final report if finished.

Document Reviewed :

1. STUDY PROTOCOL Version 2.0, Dated 31-08-2018
2. Protocol Synopsis Version 1.0, Dated 11-07-2018
3. Information sheet for research participant Version 2.0, Dated 31-08-2018
4. Informed consent for participating volunteers Version 2.0, Dated 31-08-2018

Approval granted is subject to the following conditions: (see back of this Certificate)

5. CASE REPORT FORM Version 1.0, dated 11-07-2018
6. Invitations for volunteers to participate in research projects Version 1.0, dated 11-07-2018
7. Curriculum Vitae and GCP Training
   - Assist.Prof. Pajaree Chariyavilaskul, MD., MSc, PhD.
   - Miss Phanit Songvut
   - Assist.Prof. Phisit Khemawoot, Ph.D.
   - Assoc.Prof. Mayuree H. Tantisira, Ph.D.

Signature .....
(Associate Professor Unnop Jaisamrarn MD, MHS)
Vice-Chairman, Acting Chairman
The Institutional Review Board

Signature .....
(Assistant Professor Prapapan Rajatapiti MD, PhD)
Member and Secretary
The Institutional Review Board

Date of Approval : September 6, 2018
Approval Expire Date : September 5, 2019

Approval granted is subject to the following conditions: (see back of this Certificate)

VITA

NAME	PHANIT SONGVUT
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PUBLICATION	Songvut, P., Chariyavilaskul, P., Khemawoot, P. et al. Pharmacokinetics and metabolomics investigation of an orally modified formula of standardized Centella asiatica extract in healthy volunteers. Sci Rep 11, 6850 (2021). Songvut P, Chariyavilaskul P, Tantisira M.H. and Khemawoot P. Safety and Pharmacokinetics of Standardized Extract of Centella asiatica (ECa 233) Capsules in Healthy Thai Volunteers: A Phase 1 Clinical Study. Planta medica 2019; 85(06), 483-490. Boondam Y, Songvut P, Tantisira M.H, Tapechum S, Tilokskulchai K. and Pakaprot N. Inverted U-shaped response of a standardized extract of Centella asiatica (ECa 233) on memory enhancement. Scientific reports 2019; 9(1), 8404. Junsaeng D, Anukunwithaya T, Songvut P, Sritularak B, Likhitwitayawuid K, Khemawoot P. Comparative

pharmacokinetics of oxyresveratrol alone and in combination with piperine as a bioenhancer in rats. BMC complementary and alternative medicine. 2019; 19(1):235.

Pholphana N, Panomvana D, Rangkadilok N, Suriyo T, Puranajoti P, Songvut P, et al. Andrographis paniculata: Dissolution investigation and pharmacokinetic studies of four major active diterpenoids after multiple oral dose administration in healthy Thai volunteers. J Ethnopharmacol 2016;194:513-521.

AWARD RECEIVED

Received 1st place "the excellent poster presentation award", Pharmacological and Therapeutic Society of Thailand Meeting