

Comparison of the Pharmacokinetic Profiles of a Standardized Extract of *Centella asiatica* and A Mixture of Madecassoside and Asiaticoside in Rats



Authors

Phisit Khemawoot^{1,2}, Patcharaporn Hengjumrut¹, Tosapol Anukunwithaya¹, Leng Chee Chang³, Supakit Wongwiwatthananut³, Mayuree H. Tantisira⁴

Affiliations

- 1 Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
- 2 Preclinical Pharmacokinetics and Interspecies Scaling for Drug Development Research Unit, Chulalongkorn University, Bangkok, Thailand
- 3 Daniel K. Inouye College of Pharmacy, University of Hawai'i, Hilo, HI, USA
- 4 Faculty of Pharmaceutical Sciences, Burapha University, Chonburi, Thailand

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Correspondence

Phisit Khemawoot, PhD

Department of Pharmacology and Physiology

Faculty of Pharmaceutical Sciences

Chulalongkorn University, Phyathai Road

Bangkok 10330

Thailand

Tel.: +66/2/218 8343, Fax: +66/2/218 8340

phisit.k@chula.ac.th

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ABSTRACT

Centella asiatica is a tropical plant commonly used as an herbal remedy in traditional medicines in many countries. In an attempt to establish an herbal extract with well-defined characteristics, a standardized extract of *C. asiatica*, ECa 233, was developed. This extract contains at least 80% triterpenoid glycosides with the major constituents madecassoside and asiaticoside at a ratio of 1.5 ± 0.5:1. In the present study, comparative pharmacokinetics of ECa 233 with its mixture of madecassoside and asiaticoside were conducted in rats. Following intravenous or oral administration of the test compounds, blood, tissues, urine, and feces were collected for the determination of madecassoside, asiaticoside, and their metabolite levels using liquid chromatography tandem mass spectrometry. Plasma levels of madecassoside and asiaticoside in the ECa 233-treated group were found to be higher than their respective counterparts in the mixture. Madecassoside and asiaticoside in both test formulae appeared to be widely distributed in several organs, and more than 50% of the administered doses were recovered as madecassic acid and asiatic acid in the feces within 24 to 48 h. The results clearly demonstrated the pharmacokinetic advantage of a standardized extract of *C. asiatica*, ECa 233, compared with a mixture of madecassoside and asiaticoside at an equivalent amount. Other minor constituents that naturally exist in ECa 233 appeared to positively modulate the pharmacokinetics of its major constituents, resulting in relatively better pharmacokinetic profiles than those from a mixture of pure compounds.

Abbreviations

ALT alanine aminotransferase
AST aspartate aminotransferase

AUC_{0–24} area under the curve from time zero to 24 h
AUC_{0–inf} area under the curve from time zero to infinity
C_{max} maximal plasma concentration

Cl_{app}	apparent clearance
ECa 233	standardized extract of <i>Centella asiatica</i>
IS	internal standard
IV	intravenous
Kp	tissue to plasma ratio
MRT	mean resident time
NSS	normal saline solution
PO	per oral
$T_{1/2}$	elimination half-life
T_{max}	time to reach maximal plasma concentration
V_{app}	apparent volume of distribution
XlogP	partition coefficient

Introduction

Traditional medicine serves as an alternative choice for disease prevention and treatment for people in developing countries. *Centella asiatica* (L.) Urb., a tropical herb belonging to the Apiaceae family, is commonly used in alternative and traditional medicines in Asia [1–3]. *C. asiatica* from different sources showed significant variations in the quality and quantity of its bioactive compounds, leading to unpredictable pharmacological activities. Using a strictly controlled method, we developed a standardized extract of *C. asiatica*, ECa 233 [4, 5], which was defined as a white to off-white extracted powder of *C. asiatica* that contained triterpenoid glycosides, namely, madecassoside and asiaticoside, in an amount of at least 80% and the ratio between them was maintained at $1.5 \pm 0.5:1$ (► Fig. 1). ECa 233 exhibited pharmacological profiles that corresponded to the traditional use of *C. asiatica* in humans; that is, wound healing properties, neuroprotective properties, and memory-enhancing effects [6–9]. In parallel with the favorable pharmacological profiles, ECa 233 demonstrated minimal toxicity in both acute and subchronic toxicity tests in rodents [10].

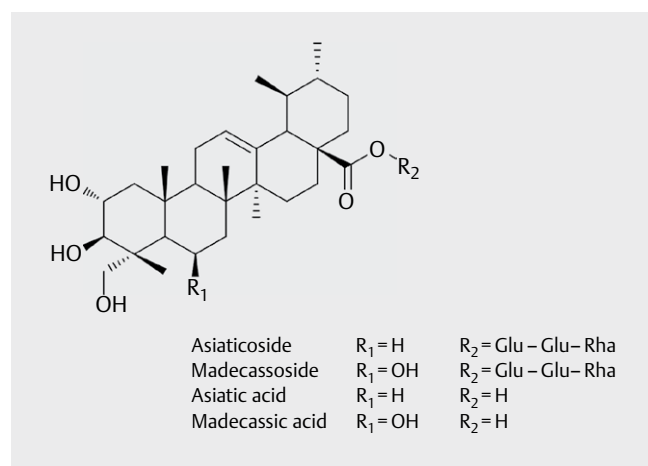
Recently, our laboratory reported pharmacokinetic profiles following single and multiple oral dosings of ECa 233 in rodents [11, 12]. This standardized extract showed dose linearity within a pharmacologically effective dose range of 50–200 mg/kg PO in male Wistar rats. Madecassoside and asiaticoside, in a clear solu-

tion of ECa 233, rapidly reached the maximum concentration in plasma within 1 h following oral dosing and were prominently detected in most pharmacologically relevant organs (e. g., skin and brain). Negligible amounts of the proposed active metabolites, madecassic and asiatic acids, were detected in rat plasma, and, unexpectedly, we found a bidirectional interconversion between madecassoside and asiaticoside [13]. Both triterpenoid glycosides were mainly excreted via the hepatobiliary system, and were extensively biotransformed into their respective triterpenic acids, madecassic and asiatic acids, likely by normal flora in the gastrointestinal tract [14, 15]. Interestingly, in relation to the pharmacokinetics of its respective single compounds, an increased exposure of the two triterpenoid glycosides present in ECa 233 was observed. Questions on whether other minor constituents in ECa 233 may underlie increased exposure to its major bioactive constituents, madecassoside and asiaticoside, therefore, emerged and required clarification. To obtain more insight into the results previously observed, we compared the pharmacokinetic behavior of ECa 233 compared it with that exhibited by a mixture of madecassoside and asiaticoside at equivalent amounts. The pharmacologically active dose of ECa 233 at 100 mg/kg PO, containing 51% madecassoside and 38% asiaticoside, was administered to male Wistar rats. Accordingly, a mixture of 51 mg/kg madecassoside and 38 mg/kg asiaticoside was prepared for the comparative pharmacokinetic study. In addition, a study of the pharmacokinetics following intravenous administration of ECa 233 at 10 mg/kg and a mixture of madecassoside and asiaticoside at an equivalent amount was also conducted. In addition to madecassoside and asiaticoside, other minor constituents are also present in ECa 233 and their impact on the major constituents remains elusive. Information obtained from this study could illustrate the significance of interactions among the different chemical constituents that are present in ECa 233.

Results

All rats had a normal physical appearance both before and after receiving ECa 233 or the mixture of madecassoside and asiaticoside. There were no significant changes in the creatinine level, reflecting normal kidney function after dosing for all test groups at 24 h. Similarly, stable AST and ALT levels were observed after dosing in all of the test groups. There were no significant alterations in liver markers for any of the animals tested, either via IV or PO routes of administration (► Table 1).

Significant differences in the plasma concentration-time profiles between madecassoside and asiaticoside present in ECa 233 and their respective counterparts at equivalent doses in the mixture groups were demonstrated (► Fig. 2, 3). After IV dosing, rats in the ECa 233 group exhibited significantly higher plasma concentrations of madecassoside and asiaticoside compared with their respective counterparts in the mixture group. Similarly, oral administration of ECa 233 could prolong exposure of both madecassoside and asiaticoside compared to the results observed with the mixtures. Determination of pharmacokinetic parameters from the plasma concentration-time curves by non-compartmental analysis is presented in ► Table 2, 3. In comparison with the pharmacokinetic profiles of the mixture of madecassoside and asiaticoside, an



► Fig. 1 Chemical structures of triterpenes from *C. asiatica* (Glu: glucose, Rha: rhamnose).

► **Table 1** Physical appearance and biochemical profiles at time zero and 24 h after dosing.

Parameters	ECa 233 10 mg/kg IV		Mixture (MD + AS) IV		ECa 233 100 mg/kg PO		Mixture (MD + AS) PO	
	Pre-dose	Post-dose	Pre-dose	Post-dose	Pre-dose	Post-dose	Pre-dose	Post-dose
Physical appearance	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Creatinine (mg/dl)	0.23±0.06	0.20±0.00	0.23±0.04	0.23±0.06	0.20±0.00	0.23±0.02	0.22±0.04	0.20±0.00
AST (U/L)	71.50±19.36	68.25±13.48	73.83±9.80	58.00±2.96	48.75±3.30	55.00±21.22	57.83±7.00	61.17±15.34
ALT (U/L)	21.50±12.24	21.75±8.88	25.00±6.60	20.17±6.84	14.75±12.94	37.75±30.22	20.17±5.20	13.50±9.42

Data are presented as the mean±S.D. (n =4). AS: asiaticoside, MD: madecassoside

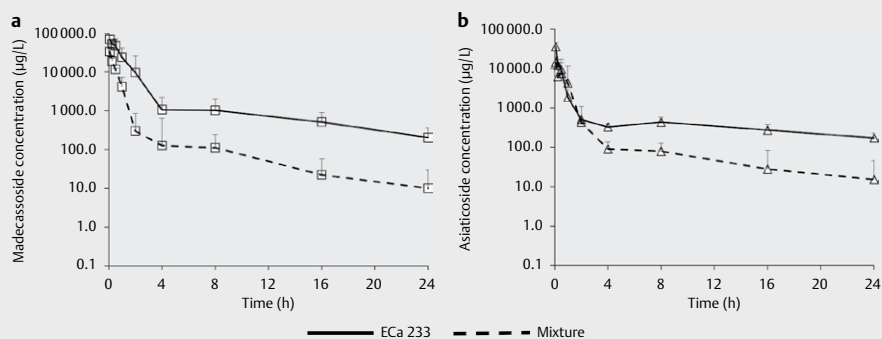
increase of approximately 400 % of the madecassoside area under the curve (AUC) and an increase of 50 % of the asiaticoside AUC were detected following IV dosing of ECa 233. Similarly, a significant increase of both the madecassoside and asiaticoside AUCs were also observed following oral dosing with ECa 233. The MRT of both madecassoside and asiaticoside following dosing with ECa 233 showed a tendency to prolong in comparison to these components in the mixture.

The tissue to plasma values (Kp) of madecassoside and asiaticoside following IV dosing of ECa 233 or the mixture at an equivalent dose are presented in ► **Fig. 4**. Madecassoside and asiaticoside could be concentrated in and distributed to several organs, including the skin and brain, which were target sites of pharmacological actions. The accumulation of madecassoside and asiaticoside in the spleen was observed from 1 to 4 h after dosing with ECa 233 or the mixture. Interestingly, the Kp values of both madecassoside and asiaticoside increased significantly from 1 to 4 h following IV dosing of both test formulae. ECa 233 showed higher Kp values of both madecassoside and asiaticoside in some organs at 1 and 2 h compared with the mixture.

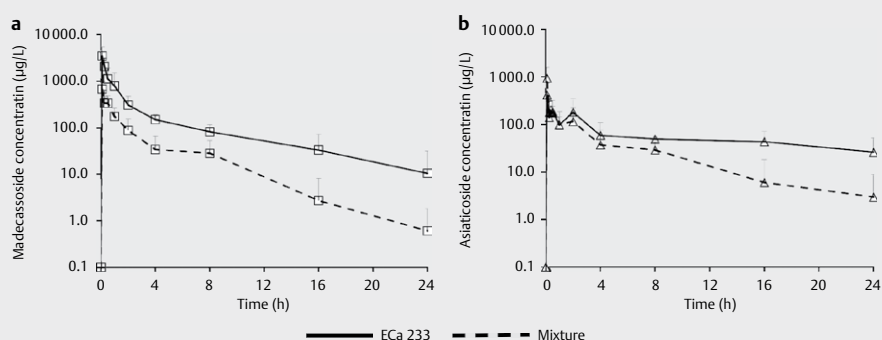
Negligible amounts of unchanged madecassoside and asiaticoside were found in excreta following the IV administration of all test formulae (► **Table 4**). After dosing for 24-48 h, it was likely that most of the administered madecassoside and asiaticoside were extensively biotransformed into madecassic and asiatic acids, before being excreted in feces. There were no significant differences in the concentrations of madecassic and asiatic acids in feces between the ECa 233 and mixture groups.

The compounds of the ECa 233 extract were analyzed using HPLC ESI-QTOF-MS/MS and two standards (madecassoside and asiaticoside). The first major peak of the mass chromatogram was detected at 7.0 min with a molecular weight of 997.4995 daltons (Da). This molecular weight was deduced to be a sodium adduct $[M + Na]^+$ of madecassoside. The second major peak detected at 7.95 min exhibited a molecular weight of 981.5034 Da (► **Fig. 5**). This molecular weight was deduced to be a sodium-adduct $[M + Na]^+$ of asiaticoside. These two major peaks were confirmed using standards that had the same retention time eluted with the same solvent systems and molecular weight. MS/MS fragmentation data provided further evidence for the presence of madecassoside and asiaticoside in the ECa 233 extract. In addition, a minor peak at 7.38 min with a molecular weight of 981.5047 Da was detected. This compound was considered to likely be a sodium adduct $[M + Na]^+$ of centellasaponins, which had an exact molecular weight corresponding to 958.51 Da plus sodium 22.99 Da. However, the specific type of centellasaponins (A, C, D) remained undetermined due to the lack of commercially available centellasaponins. Overall, by matching retention times, mass spectra of sodium ion adducted molecular ions, and fragmentation patterns of madecassoside and asiaticoside, it was evident that the minor components of ECa 233 extracts contain a centellasaponin.

Additionally, two small peaks of minor components at 13.0 and 15.0 min were found. These signals were confirmed to be madecassic and asiatic acids, consistent with their retention times and molecular weights compared with analytical standards. These triterpenic acids accounted for less than 1.0 % of ECa 233 using quantitative analysis with a triple quadrupole LC-MS/MS system.



► **Fig. 2** Plasma concentration-time profiles of madecassoside (a) and asiaticoside (b) following intravenous administration of ECa 233 (10 mg/kg) and a mixture of madecassoside and asiaticoside at an equivalent dose. Data are presented as the mean \pm S.D. (n = 4); * $p < 0.05$ for ECa 233 vs. mixture.



► **Fig. 3** Plasma concentration-time profiles of madecassoside (a) and asiaticoside (b) following oral administration of ECa 233 (100 mg/kg) and a mixture of madecassoside and asiaticoside at an equivalent dose. Data are presented as the mean \pm S.D. (n = 4); * $p < 0.05$ for ECa 233 vs. mixture.

► **Table 2** Pharmacokinetic parameters of madecassoside and asiaticoside after intravenous administration of ECa 233 10 mg/kg and a mixture of madecassoside and asiaticoside at equivalent doses.

Pharmacokinetic parameters	Madecassoside		Asiaticoside	
	ECa 233 10 mg/kg	Mixture MD + AS	ECa 233 10 mg/kg	Mixture MD + AS
AUC ₀₋₂₄ (µg.h/L)	86571.91 \pm 40136.51	22034.21 \pm 7637.14 *	20139.85 \pm 7173.22	14707.54 \pm 18920.30 *
AUC _{0-inf} (µg.h/L)	88907.03 \pm 41437.76	22362.07 \pm 7655.40 *	22558.42 \pm 7997.22	15174.02 \pm 18848.86 *
V _{app} (L/kg)	0.55 \pm 0.13	2.27 \pm 0.97 *	1.99 \pm 0.72	4.45 \pm 3.26
MRT (h)	3.00 \pm 1.42	1.19 \pm 1.07 *	7.29 \pm 5.20	3.41 \pm 4.33
Elimination half-life (h)	6.21 \pm 1.47	6.14 \pm 2.58	7.80 \pm 3.08	5.59 \pm 2.48
CL _{app} (L/hr/kg)	0.07 \pm 0.02	0.25 \pm 0.07	0.19 \pm 0.08	0.52 \pm 0.29

Data are presented as the mean \pm S.D. (n = 4); * $p < 0.05$ for ECa 233 vs. mixture. AS: asiaticoside, MD: madecassoside

Discussion

Unlike other commercially available extracts of *C. asiatica*, ECa 233 is a white to off-white natural extract of *C. asiatica*, with known and standardized consistent amounts of its bioactive markers [4]. In agreement with traditional uses, ECa 233 exerts numerous pharmacological activities with minimal toxicity [6–9, 16]. Some pharmacokinetic studies of ECa 233 at its effective dose range have been conducted in recent years [12]. In the present study, comparative pharmacokinetics between ECa 233 and a mixture of madecassoside and asiaticoside at equivalent amounts were conducted in

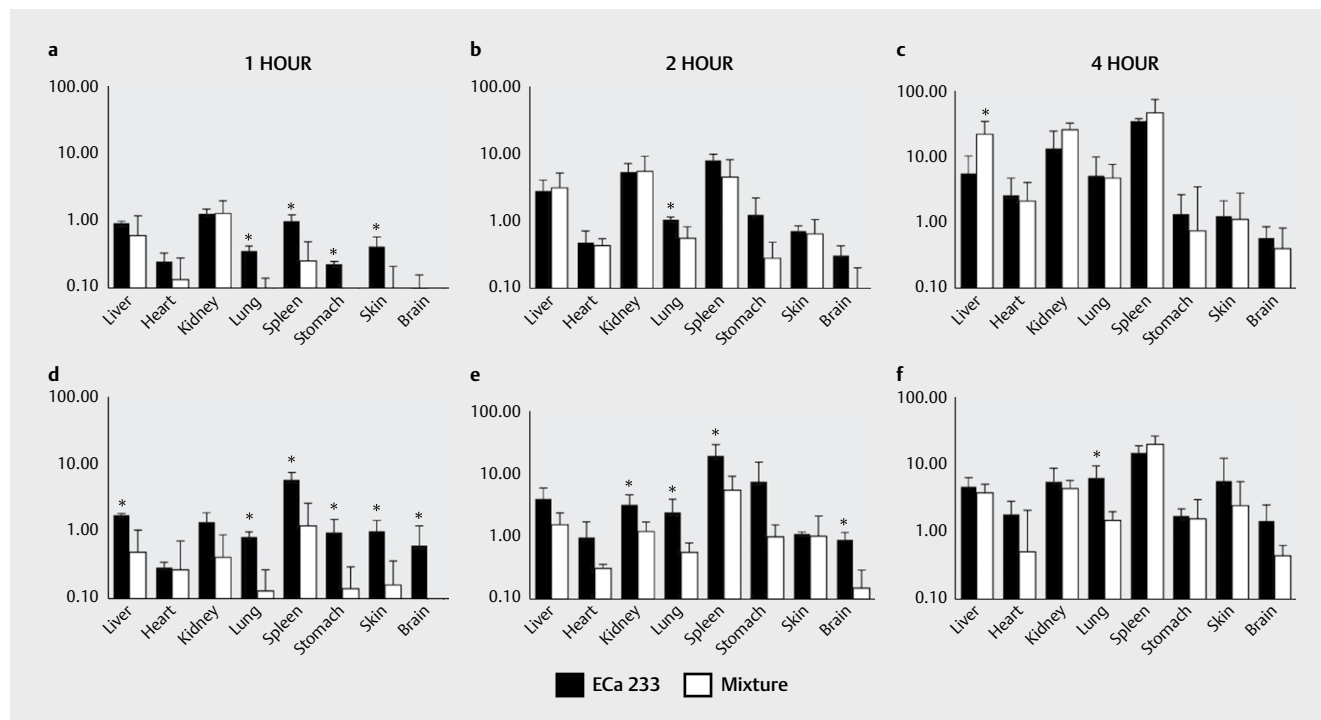
male Wistar rats. All animals showed good tolerability following IV or PO dosing of the test formulae. No significant changes were observed in physical appearance or kidney and liver markers, which indicates a good safety profile of all test formulae. The results of the present study corresponded well with previous pharmacokinetic studies and toxicity tests of ECa 233 [10].

As shown in the plasma concentration-time profiles, all rats in the ECa 233 group demonstrated significantly higher levels of madecassoside in absorption, distribution, and elimination phases compared with those of the mixture group. Similar response pro-

► **Table 3** Pharmacokinetic parameters of madecassoside and asiaticoside after oral administration of ECa 233 100 mg/kg and a mixture of madecassoside and asiaticoside at equivalent doses.

Pharmacokinetic parameters	Madecassoside		Asiaticoside	
	ECa 233 100 mg/kg	Mixture MD + AS	ECa 233 100 mg/kg	Mixture MD + AS
C_{max} ($\mu\text{g/L}$)	3474.63 \pm 1969.04	618.99 \pm 307.64 *	509.05 \pm 213.46	697.95 \pm 445.78
T_{max} (h)	0.08 \pm 0.00	0.19 \pm 0.20	0.19 \pm 0.21	0.08 \pm 0.00
AUC_{0-24} ($\mu\text{g}\cdot\text{h/L}$)	3,572.14 \pm 1,001.28	747.37 \pm 270.94 *	1409.40 \pm 652.63	749.35 \pm 331.33 *
AUC_{0-inf} ($\mu\text{g}\cdot\text{h/L}$)	4241.76 \pm 1005.12	1131.52 \pm 840.01 *	1787.54 \pm 737.60	888.50 \pm 438.71 *
MRT (h)	8.52 \pm 3.21	7.21 \pm 6.77	12.72 \pm 4.04	6.48 \pm 4.17
Elimination half-life (h)	7.80 \pm 2.24	6.77 \pm 4.99	7.13 \pm 1.75	5.07 \pm 2.01

Data are presented as the mean \pm S.D. (n = 4); *p < 0.05 for ECa 233 vs. mixture. AS: asiaticoside, MD: madecassoside



► **Fig. 4** Tissue to plasma ratio of madecassoside (a, b, c) and asiaticoside (d, e, f) in internal organs at 1, 2, and 4 h after intravenous administration of ECa 233 (10 mg/kg) and a mixture of madecassoside and asiaticoside at an equivalent dose. Data are presented as the mean \pm S.D. (n = 4).

files were also exhibited by asiaticoside in the ECa 233-treated group where prolonged plasma levels, especially during the elimination phase, were observed. These results were similar to those reported by Hengjumrut et al. [13] in which longer exposure of madecassoside and asiaticoside was observed following administration of ECa 233 compared with the single compounds administered separately. Oral bioavailability of both madecassoside and asiaticoside was increased approximately 2- to 4-fold following administration in the form of ECa 233, suggesting that other minor components present in ECa 233 might play a substantial role as a bioenhancer of madecassoside and asiaticoside [17–19]. Therefore, increases of madecassoside and asiaticoside levels following dosing with ECa 233 were observed from the absorption through the elimination phase.

Determination of the minor components in ECa 233 was conducted using high-resolution LC-MS/MS analysis. It appears likely that ECa 233 contains a certain amount of centellasaponin(s) and

is known to contain a small amount of triterpenic acids. In general, appropriate concentrations of minor components to act as bioenhancers should constitute 10% of the major components. Therefore, it is possible that centellasaponin(s) could act as bioenhancers of the two triterpenoid glycosides in ECa 233. Centellasaponins have structures similar to madecassoside and asiaticoside, which consist of pentacyclic triterpenes and glucose-glucose-rhamnose. These saponins in ECa 233 might have competitive or inhibitory activities toward efflux transporters of madecassoside and asiaticoside. Therefore, improvement of the pharmacokinetic profiles of the two major bioactive components in ECa 233 was observed from the absorption, distribution, and elimination phases. The MRTs of both madecassoside and asiaticoside were improved by intravenous or oral administration of ECa 233 compared with the mixture of pure madecassoside and asiaticoside. Further studies of the bioenhancer activity of centellasaponins could be developed if pure chemicals become commercially available in the future.

► **Table 4** Percent recovery of madecassoside and asiaticoside via urine and feces after administration of ECa 233 and a mixture of madecassoside and asiaticoside at an equivalent dose.

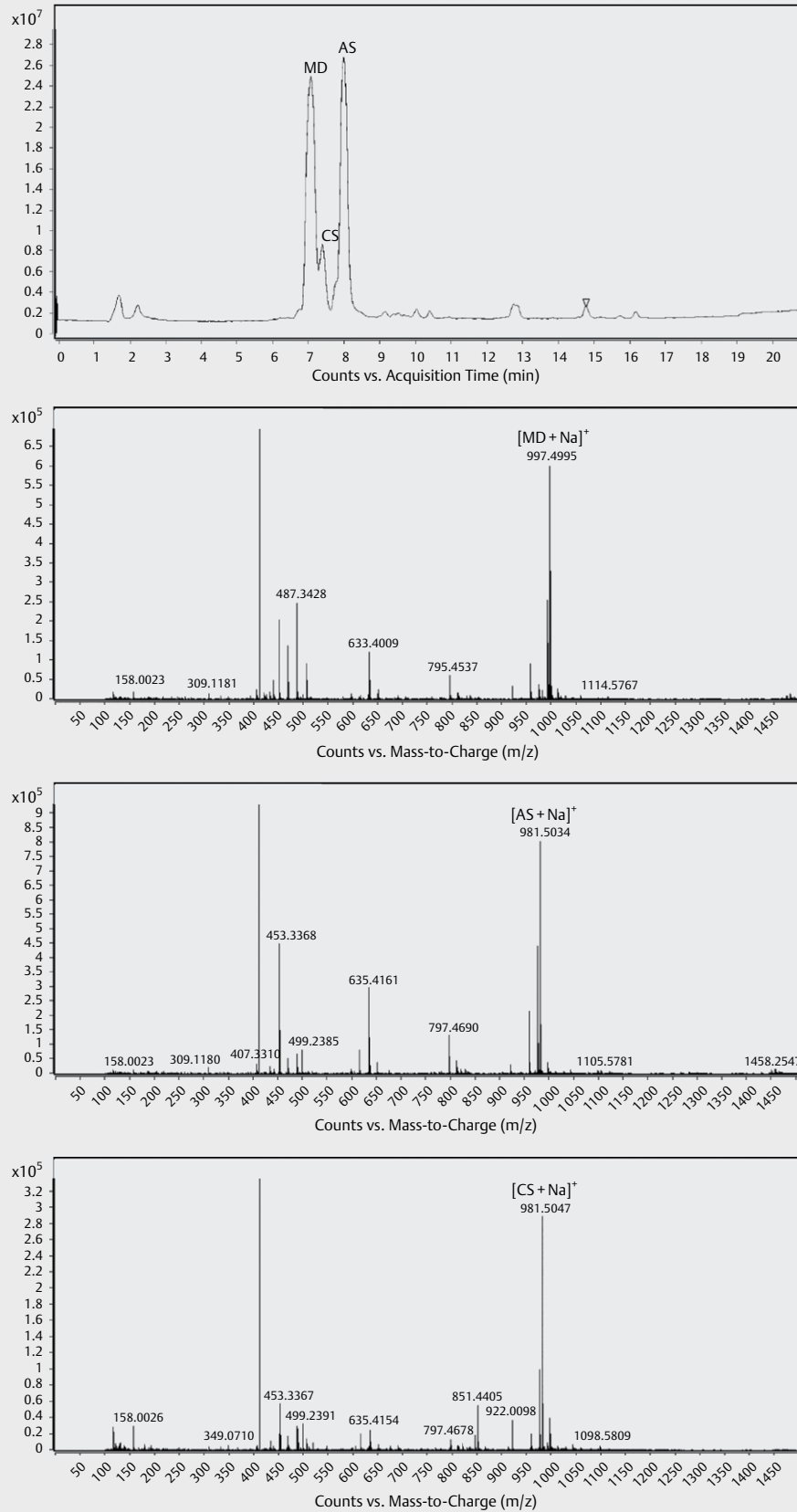
Percent recovery	Intravenous administration				Oral administration				
	ECa 233 10 mg/kg		Mixture (MD + AS)		ECa 233 100 mg/kg		Mixture (MD + AS)		
	0–24 h	24–48 h	0–24 h	24–48 h	0–24 h	24–48 h	0–24 h	24–48 h	
Urine	Madecassoside	1.95 ± 1.62	0.07 ± 0.04	1.73 ± 2.08	0.08 ± 0.02	0.02 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Asiaticoside	0.28 ± 0.24	0.02 ± 0.04	0.23 ± 0.36	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Feces	Madecassoside	0.04 ± 0.02	0.04 ± 0.04	0.08 ± 0.04	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.02	0.01 ± 0.00	0.01 ± 0.00
	Asiaticoside	0.05 ± 0.02	0.05 ± 0.04	0.13 ± 0.06	0.04 ± 0.02	0.06 ± 0.04	0.02 ± 0.00	0.04 ± 0.02	0.03 ± 0.02
	Madecassic acid	25.44 ± 5.02	9.80 ± 7.42	22.19 ± 6.58	10.45 ± 3.68	8.84 ± 3.20	9.11 ± 5.16	6.45 ± 2.42	6.03 ± 1.98
	Asiatic acid	32.45 ± 7.20	17.99 ± 13.44	35.56 ± 2.94	25.76 ± 2.60	11.19 ± 6.98	7.68 ± 2.62	15.76 ± 4.76	20.15 ± 7.40

Data are presented as the mean ± S.D. (n = 4). AS: asiaticoside, MD: madecassoside

The Kp of both madecassoside and asiaticoside increased over time from 1 to 4 h following IV dosing of both test formulae. This result correlated well with our previous finding that continuous dosing of 100 mg/kg PO of ECa 233 for 7 days could significantly increase tissue levels of both triterpenoid glycosides [11]. Madecassoside and asiaticoside are large hydrophilic molecules with molecular weights of more than 900 Da. These large triterpenoid molecules might have limited channels to enter tissue compartments compared with small lipophilic molecules. In general, small lipophilic molecules enter tissues by simple diffusion, depending on the concentration gradient of the diffusing molecules [20]. It is likely that the distribution patterns of both triterpenoid glycosides are time dependent rather than concentration dependent. Madecassoside and asiaticoside were able to reach the brain and skin, two major targeted organs of pharmacological activities. The Kp values in these organs increased from 1 to 4 h following IV dosing of both test formulae, suggesting the potential for ECa 233 to be used as a neuroprotective substance for brain injuries.

In the present study, madecassic and asiatic acids, which have previously been proposed to be the active metabolites [15], were not detected either in plasma or tissue compartments. Therefore, it is likely that administered madecassoside and asiaticoside were absorbed and distributed as unchanged triterpenoid glycosides, not triterpenic acids. Subsequently, the triterpenoid glycosides in the systemic circulation were excreted into the gut lumen via the hepatobiliary system [21]. From there, the unchanged madecassoside and asiaticoside were further biotransformed by the gut flora into triterpenic acids before being excreted in feces [14, 15]. We found abundant amounts of the two triterpenic acid metabolites, madecassic and asiatic acids, in feces; the percent of recovery was greater than 50% of the administered doses of triterpenoid glycosides. These two triterpenic acids had very high lipophilic properties, with XlogP ranging from 4.4 to 5.7. Therefore, excretion of these lipophilic molecules should occur via the biliary system rather than the urinary system. P-glycoprotein and multidrug resistance-associated protein 2 are two major transporters responsible for the excretion of madecassoside and asiaticoside into the biliary system [21]. Further information on the hepatobiliary transport and biotransformation of the two triterpenoid glycosides in the gastrointestinal tract will be essential for the development of phytopharmaceutical products from *C. asiatica*.

Overall, ECa 233, the standardized extract of *C. asiatica* containing mainly madecassoside and asiaticoside, demonstrated superior pharmacokinetic profiles compared with the mixture of madecassoside and asiaticoside at an amount equivalent to their counterparts presented in ECa 233. Higher oral bioavailability and more rapid tissue distribution of triterpenoid glycosides were observed in rats treated with ECa 233. There were no significant differences between the metabolism and excretion profiles of the two triterpenoid glycosides in ECa 233 and the mixture. Pharmacokinetic information obtained from this study clearly demonstrated prolonged exposure of the two major bioactive substances, madecassoside and asiaticoside, of the standardized extract of *C. asiatica* ECa233 compared with a mixture of the two pure compounds. The significant role of the minor constituents on the pharmacokinetic profiles of the major constituents calls for precaution in the interpretation and extrapolation of the data of different extracts.



► **Fig. 5** MS chromatogram and spectra from high-resolution LC-MS/MS identifying major and minor components of ECa 233.

Materials and Methods

Chemicals

ECa 233, madecassoside, and asiaticoside were provided by Siam Herbal Innovation Co., Ltd. The standardized extract (batch number MRA1214004) contained 51 % madecassoside and 38 % asiaticoside, and was analyzed by LC-MS/MS. Analytical standards of asiaticoside (>98.5 %), madecassoside (>95.0 %), and asiatic acid (>95.0 %) were purchased from Sigma-Aldrich Corp. Madecassic acid (>98.9 %) and glycyrrhetic acid (>99.0 %) used for LC-MS/MS experiments were purchased from Wako Pure Chemical Industries.

Animals and treatments

Male Wistar rats were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were housed under 12-h light-dark cycles, 24 ± 2 °C, 50 ± 10 % humidity, and ad libitum access to food and water. Rats aged 16–20 weeks that weighed more than 400 g were used in the pharmacokinetic experiments; they were placed into metabolic cages and fasted overnight with free access to water. The animal protocols were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (approval number 15-33-002, approval date April 22, 2015).

Pharmacokinetic experiments

The rats were divided into four groups ($n = 4$): ECa 233 10 mg/kg IV, ECa 233 100 mg/kg PO, a mixture of madecassoside and asiaticoside (5.1 and 3.8 mg/kg, respectively) IV, and a mixture of madecassoside and asiaticoside (51 and 38 mg/kg, respectively) PO. All formulations were freshly prepared as a clear solution in 20 % DMSO/NSS before administration via the lateral tail vein or oral gavage. The rats were anesthetized with isoflurane during drug administration and blood or organ collections. We collected 300 μ L of blood via the lateral tail vein at 0, 5, 15, and 30 min, and 1, 2, 4, 8, 16, and 24 h after dosing. Blood samples were collected from these rats and centrifuged at $1500 \times g$ for 15 min to collect 150 μ L of plasma. Plasma samples at 0 and 24 h were used to determine creatinine, AST, and ALT levels. Tissue samples were collected at 1, 2, and 4 h after IV dosing, and washed with cold saline solution. Rat excreta, urine, and feces were collected separately from the metabolic cages 24–48 h after dosing to determine the excretion routes of drugs and metabolites. All biological samples were stored at -80 °C until analysis.

Sample preparation

Methanol was used as a precipitating agent in the protein precipitation method. Fifty microliters of plasma or urine samples were thawed at room temperature and vigorously mixed with 200 μ L of methanol containing 10 ng of glycyrrhetic acid as the IS. The mixture was centrifuged at $3000 \times g$ for 15 min, and the supernatants (10 μ L) were analyzed by LC-MS/MS. Fifty milligrams of feces or tissues were mixed with 200 μ L of methanol that contained 10 ng of IS, homogenized in an ice bath, and processed in the same manner as the liquid samples. In the case that the drug or metabolite levels exceeded the linearity of the calibration curves, blank matrices were used to dilute the samples prior to protein precipitation.

LC-MS/MS analysis

LC-MS/MS analysis was conducted under conditions and methods previously described in a pharmacokinetic study of ECa 233 [12]. In brief, an LC-MS/MS system was conducted with an Eksigent UPLC 100 and a QTRAP 6500 mass spectrometer and Analyst software version 1.6 (AB Sciex Pte., Ltd.). The stationary phase was a Synergi Fusion-RP C18 column (Phenomenex, Inc.) with a 40 °C oven temperature. The retention times of madecassoside, asiaticoside, madecassic acid, asiatic acid, and glycyrrhetic acid were 1.79, 1.82, 1.93, 1.99, and 2.12 min, respectively. The standard curves of the triterpenoid glycosides and triterpenic acids showed good linearity, with $R^2 > 0.99$ for 0.5–300 μ g/L. The lower limit of detection was 0.1–0.5 μ g/L, and intra- and inter-day precision and accuracy were within ± 10 %. The calculated percent recoveries were more than 70 % for all analytes.

Minor components identification

In this study, the identification of the minor components of ECa 233 was conducted with high-resolution LC-MS/MS analysis. LC-MS/MS analysis was performed on an Agilent 6530 QTOF mass spectrometer with an Agilent HPLC 1260 binary pump. A Kinetex Phenomenex C₁₈ column (3.5 μ m, 2.1 \times 150 mm) was used as the stationary phase. The mobile phase was run with a gradient of 0.1 % formic acid in water and acetonitrile (20–80 %) at a flow rate of 0.2 mL/min. The mass spectrometer was equipped with an Agilent Jet Stream ESI source. LC-MS/MS was performed in the positive ionization mode over a mass range of m/z 100–1700 at a scan rate of 3 spectra/second. ECa 233 (10 μ L) at a concentration of 0.1 mg/mL was injected into the LC system, with the column oven maintained at 25 °C. The elution gradient of the mobile phase started with 20 % acetonitrile at time zero and increased to 80 % acetonitrile after 20 min, was maintained at 80 % acetonitrile for 3 min, decreased to 20 % acetonitrile over 5 min, and was equilibrated with 20 % acetonitrile for 5 min. Analysis of the eluates was conducted in the positive ion mode of ionization, and employed a QTOF mass spectrometer (QTOF 6530, Agilent Technologies). Qualitative analysis of mass spectra was performed with MassHunter software B.07.00 (Agilent Technologies).

Data analysis

The pharmacokinetic parameters of madecassoside and asiaticoside were calculated by non-compartmental analysis using PK solution software (Summit Research Service). The C_{max} and T_{max} were directly observed from real experimental data. The AUC_{0-24} , AUC_{0-inf} , V_{app} , $T_{1/2}$, Cl_{app} , and MRT were reported. The K_p values were calculated from the drug concentrations in the tissue divided by the drug concentration in the plasma at the same time point. The percent of recovery of the drugs and metabolites in the excreta was calculated as the total drugs or metabolites found in the excreta divided by the administered dose based on molarity. All parameters were reported as the mean \pm standard deviation. Statistical differences in pharmacokinetic parameters between the two experimental groups were analyzed using a nonparametric test ($p < 0.05$). SPSS statistical analysis (version 16) was used for all data analyses (SPSS, Inc.).

Supporting information

A representative LC-MS/MS chromatogram from a plasma sample and MS/MS fragments of major and minor components of ECa 233 are available as Supporting Information.

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Conflict of Interest

All authors declare no conflicts of interest.

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