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Multiple Oral Dosing Pharmacokinetics of Standardized Extract of *Centella asiatica* ECa 233 and Its Inductive Effect on Efflux Transporters in Rats

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ABSTRACT

ECa 233 is a standardized extract of *Centella asiatica*, characterized as a white powder containing triterpenoid glycosides not less than 80 % with a ratio of madecassoside to asiaticoside of 1.5 ± 0.5:1. Although pharmacological and toxicological profiles of ECa 233 have been successively reported, the pharmacokinetic data needed for further therapeutic development are not fully elucidated. This study aimed to investigate the pharmacokinetics of multiple oral dosing of ECa 233 at 100 mg/kg/day for 7 days in rats. Plasma, tissues, urine, and feces were collected from 0 to 24 h after dosing on days 1 and 7. The concentrations of asiaticoside, madecassoside, asiatic acid, and madecassic acid were simultaneously analyzed by liquid chromatography-tandem mass spectrometry. No significant change was observed in physical and blood biochemical parameters of the animals treated with ECa 233 for 7 days. The maximum plasma concentration and area under the curve at day 7 of madecassoside and asiaticoside decreased by 70–80 % from day 1. However, both triterpenoid glycosides were extensively distributed and accumulated, resulting in significantly higher concentrations at pharmacologically relevant organs. Madecasssic acid and asiatic acid are major metabolites mainly found in and excreted via feces. Moreover, multiple dosing of ECa 233 increased mRNA expression of *Abcb1a* and *Abcc2* in the small intestine by approximately 2- to 3-fold. This is the first study to identify an inductive effect of a standardized extract of *C. asiatica* after multiple oral dosing in rats. Potential drug-herb interactions when ECa 233 is coadministered with *Abcb1a* and *Abcc2* substrates calls for further investigations.

Abbreviations

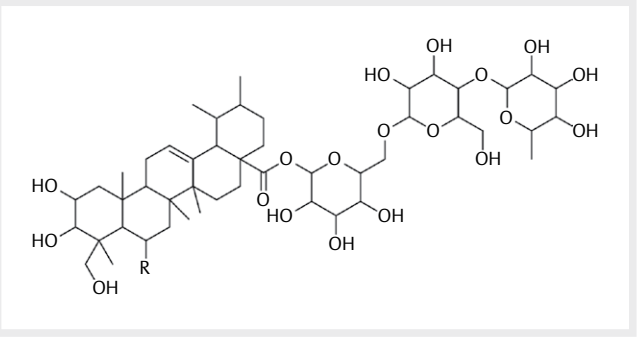
ALT	alanine aminotransferase
AST	aspartate aminotransferase
$AUC_{(0\text{-}t)}$	area under the curve from time 0 to the observed time
C_{max}	maximum plasma concentration
ECa 233	standardized extract of *Centella asiatica*
IS	internal standard
MRP2	multidrug-resistant protein 2
NSS	normal saline solution
PGP	p-glycoprotein
qPCR	real-time polymerase chain reaction
T_{max}	time to reach maximum plasma concentration
XlogP	partition coefficient

Introduction

Centella asiatica (L.) Urb. (Asiatic pennywort, Gotu kola, or Bua-bok) belongs to the Apiaceae family and is commonly found in tropical areas of Southeast Asia, India, South Africa, South America, and Eastern Europe [1–3]. The plant has been used as traditional medicine for a long time and, in line with this, supporting pharmacological effects have been well documented in a number of preclinical studies, suggesting the potential for further development of *C. asiatica* into a phytopharmaceutical product [4–11]. Considerable fluctuations in the chemical composition of the plant from different origins have resulted in inconsistencies of bioactive constituents of the plant extract, which poses a major problem for its application as a natural medicine. To overcome such a problem, we prepared ECa 233 which is a standardized extract of *C. asiatica* [12] defined as a white to off-white powder containing triterpenoid glycosides not less than 80 % with a ratio of madecassoside to asiaticoside of 1.5 ± 0.5:1 (▶ **Fig. 1**) [13, 14].

ECa 233 has demonstrated various pharmacological effects such as the acceleration of second degree burn wound healing in Wistar rats [15], as well as a reduction in pain, ulcer size, and erythema score of minor recurrent aphthous ulcerations in human subjects [16]. In the central nervous system, the anxiolytic effects of 100 mg/kg ECa 233, given orally, have been shown in mouse models of acute and chronic stress [14]. ECa 233 was also found to ameliorate learning and memory deficits induced by intracerebroventricular injection of amyloid peptide in a mouse model [17]. The extract also has a neuritogenic effect on human neuroblastoma cells via the upregulation of activated ERK1/2 and Akt [18]. An oral dose up to 10 g/kg has modest toxicity in both acute and subchronic tests in rodents [19]. Taken together, ECa 233 may be a good phytomedicine candidate for the treatment of some neurological disorders. However, the pharmacokinetic data needed for appropriate dosing are rather scant.

Recently, a single-dose pharmacokinetic study of orally given ECa 233 was conducted in rats [20]. It was found that, after a single oral doses of ECa 233 at 50, 100, and 200 mg/kg, madecassoside and asiaticoside were rapidly absorbed, reaching their respective C_{max} (madecassoside 1 654 ± 884, 5 664 ± 3 947, and 9 020 ± 5 744 µg/L; asiaticoside 318 ± 192, 1 283 ± 1 089, and 4 028 ± 3 157 µg/L) within 5–15 min. Both compounds were distributed into the brain and other tissues and were eliminated mainly as madecassic acid and asiatic acid via feces [21]. The present study



▶ **Fig. 1** Structures of two triterpenoid glycosides, madecassoside (R = OH) and asiaticoside (R = H), which are the major components of ECa 233.

▶ **Table 1** Physical and biochemical profiles of rats pre- and post-treatment with oral dosing of ECa 233 (100 mg/kg/day) for 7 consecutive days.

Biochemical parameters	Pretreatment at day 1	Post-treatment at day 7
Physical appearance	Normal	Normal
Creatinine (mg/dL)	0.21 ± 0.03	0.22 ± 0.04
Aspartate transaminase (U/L)	61.56 ± 11.45	74.11 ± 35.94
Alanine transaminase (U/L)	18.67 ± 7.53	22.00 ± 7.27
Data are shown as the mean ± S.D. (n = 9). * P < 0.05 pretreatment on day 1 vs. post-treatment on day 7		

further examines the single and multiple dosing pharmacokinetic behavior of ECa 233 at its effective dose for the treatment of chronic neurological conditions (100 mg/kg daily) in Wistar rats. The tissue distribution of the major components of ECa 233, namely madecassoside and asiaticoside, into pharmacologically related organs was also conducted to determine their steady state levels after multiple oral dosing for 7 days. Determination of triterpenoid glycosides and triterpenic acid metabolites was performed by LC-MS/MS. In addition, the mRNA expression of efflux transporters in the liver and small intestine was determined by qPCR in order to access their effects on the pharmacokinetic behaviors of madecassoside and asiaticoside after multiple oral dosing of ECa 233. The data obtained from this study will help with determining the appropriate dose of ECa 233 for long-term use and highlights possible drug-herb interactions of ECa 233.

Results

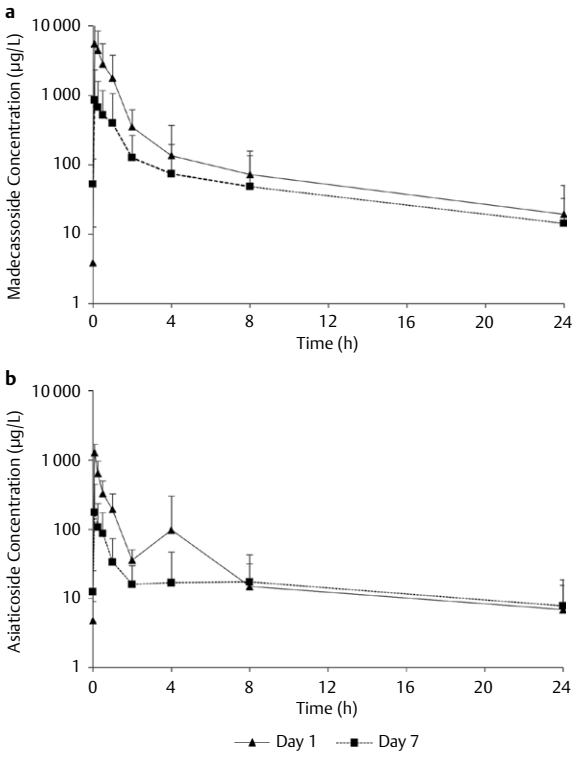
Rats receiving 100 mg/kg/day of oral ECa 233 for 7 days showed no significant physical changes or abnormalities. AST and ALT levels at post-treatment (24 h on day 7) did not change significantly from those of pretreatment (0 h on day 1), indicating marginal effects of ECa 233 on liver functions. Creatinine levels, reflecting kidney status, showed no significant changes after multiple oral dosing for 7 days. All biochemical parameters were within the normal ranges of healthy Wistar rats (▶ **Table 1**).

Plasma concentration-time profiles of madecassoside and asiaticoside, the major components of ECa 233, were constructed on

days 1 and 7 (▶ **Fig. 2**), and the pharmacokinetic parameters were determined (▶ **Table 2**). Multiple oral doses of ECa 233 (100 mg/kg) for one week resulted in a significant decrease in the C_{max} and $AUC_{(0-24)}$ of madecassoside and asiaticoside compared with the single oral dose on day 1 ($p < 0.05$). C_{max} decreased by 83.4 and 83.6 % and $AUC_{(0-24)}$ decreased by 78.2 and 72.2 % for madecassoside and asiaticoside, respectively. T_{max} at day 7 was slightly delayed, but did not differ significantly from that observed on day 1.

Madecassoside and asiaticoside showed good tissue distribution after oral administration of 100 mg/kg ECa 233 for 7 consecutive days (▶ **Table 3**). Both compounds reached the brain, skin, spleen, liver, kidney, and stomach in similar distribution patterns with the $AUC_{(0-4)}$ up to 44.5 µg · h/g of tissue. Interestingly, the $AUC_{(0-4)}$ of all tissues, except the stomach, after ECa 233 dosing on day 7 was significantly higher than that after single oral dosing (day 1) in which madecassoside and asiaticoside in the brain were increased by approximately 4- and 6-fold, respectively. Excretion of triterpenoid glycosides was observed within 24 h after dosing on days 1 and 7 (▶ **Table 4**). Madecassoside and asiaticoside were detected in negligible amounts in the urine. Madecassic acid and asiatic acid, the expected triterpenic acid metabolites of madecassoside and asiaticoside, which were found at negligible levels in plasma, were detected mainly in the feces and had higher percent recoveries than the parent compounds on days 1 and 7.

The mRNA expression levels of *Abcb1a*, *Abcb1b*, *Abcc2*, and *Abcg2* in the liver and small intestine were quantified (▶ **Fig. 3**). There were no notable changes in any of the observed efflux transporter mRNAs in the liver of most tested groups. The mRNA expression of *Abcb1a* and *Abcc2* was significantly upregulated in the small intestine. There was about a 2-fold increase in *Abcb1a* expression in the duodenum and jejunum, and a 2- to 4-fold increase of *Abcc2* mRNA expression was clearly observed throughout the small intestine of the animals receiving multiple doses of ECa 233 for 7 days.



▶ **Fig. 2** Plasma concentration-time profiles of madecassoside **a** and asiaticoside **b** following oral administration of ECa 233 (100 mg/kg/day) for 7 consecutive days. Data are shown as the mean ± S.D. (n = 9).

Discussion

ECa 233 is a standardized extract of *C. asiatica* that consistently contains designated levels of madecassoside and asiaticoside [12]. The pharmacological effects of ECa 233 in decreasing anxiety [14] and ameliorating learning and memory deficits indicate the therapeutic potential of this extract [17, 18]. Development of ECa 233 as an alternative medicine for the treatment of some central nervous system disorders requires pharmacokinetic data following long-term administration. In this study, the multiple-dose pharmacokinetics of 100 mg/kg given orally for 7 days were examined in adult male Wistar rats. No animals showed significant changes in physical appearance or biochemical parameters, indicating normal kidney and liver function. These results are consistent with the good safety profiles of ECa 233 in previous acute and subchronic toxicity studies [19].

The pharmacokinetic parameters of madecassoside and asiaticoside were determined from the plasma concentration-time profiles on days 1 and 7 using non-compartmental analysis. The significant 80 % decreases in C_{max} and $AUC_{(0-24)}$ of madecassoside and asiaticoside after 7 consecutive days of ECa 233 treatment indicate the possibility that the extract might have an inductive effect on efflux transporters or on drug metabolizing enzymes, as previously demonstrated by other herbal products [22, 23]. Madecassoside has been found to be a substrate for efflux transporters such as PGP and MRP2 [24]. Accordingly, our qPCR results did demonstrate in-

▶ **Table 2** Pharmacokinetic parameters of madecassoside and asiaticoside after oral dosing of ECa 233 (100 mg/kg/day) on days 1 and 7.

Pharmacokinetic parameters	Madecassoside		Asiaticoside	
	Day 1	Day 7	Day 1	Day 7
C_{max} (µg/L)	5 713.00 ± 5 069.38	948.03 ± 1 449.37 *	1 281.60 ± 407.48	209.62 ± 250.74 *
T_{max} (h)	0.17 ± 0.14	1.30 ± 2.72	0.08 ± 0.00	1.50 ± 3.19
$AUC_{(0-24)}$ (µg · h/L)	7 361.88 ± 4 195.27	1 604.40 ± 2 066.20 *	1 104.98 ± 713.29	306.78 ± 234.37 *
Normalized $AUC_{(0-24)}$ (µg · h · kg/L · mg)	138.64 ± 79.01	30.21 ± 38.91 *	34.21 ± 22.08	9.50 ± 7.26 *

Data are shown as the mean ± S.D. (n = 9). * $P < 0.05$ pharmacokinetic parameters on day 1 vs. day 7

▶ **Table 3** Area under the curve of madecassoside and asiaticoside in internal organs from time 0–4 h after oral dosing of ECa 233 (100 mg/kg/day) on days 1 and 7.

Compounds	Organs	$AUC_{(0-4)}$ (ng · h/g of tissue)	
		Day 1	Day 7
Madecassoside	Skin	613 ± 97	2 019 ± 947 *
	Spleen	206 ± 97	4 048 ± 4 104 *
	Brain	84 ± 32	309 ± 194 *
	Stomach	43 538 ± 11 383	44 496 ± 25 460
	Kidney	1 602 ± 2 110	21 814 ± 18 301 *
	Liver	1 524 ± 2 191	6 597 ± 3 967 *
Asiaticoside	Skin	116 ± 16	1 410 ± 970 *
	Spleen	35 ± 28	2 847 ± 2 796 *
	Brain	20 ± 3	120 ± 97 *
	Stomach	22 372 ± 4 599	35 313 ± 21 714
	Kidney	305 ± 419	18 105 ± 15 629 *
	Liver	343 ± 529	4 119 ± 2 828 *

Data are shown as the mean ± S.D. (n = 6). * $P < 0.05$ tissue $AUC_{(0-4)}$ on day 1 vs. day 7

▶ **Table 4** Percent recovery of triterpenoid glycosides and triterpenic acid metabolites for 0–24 h after oral dosing of ECa 233 (100 mg/kg/day) on days 1 and 7.

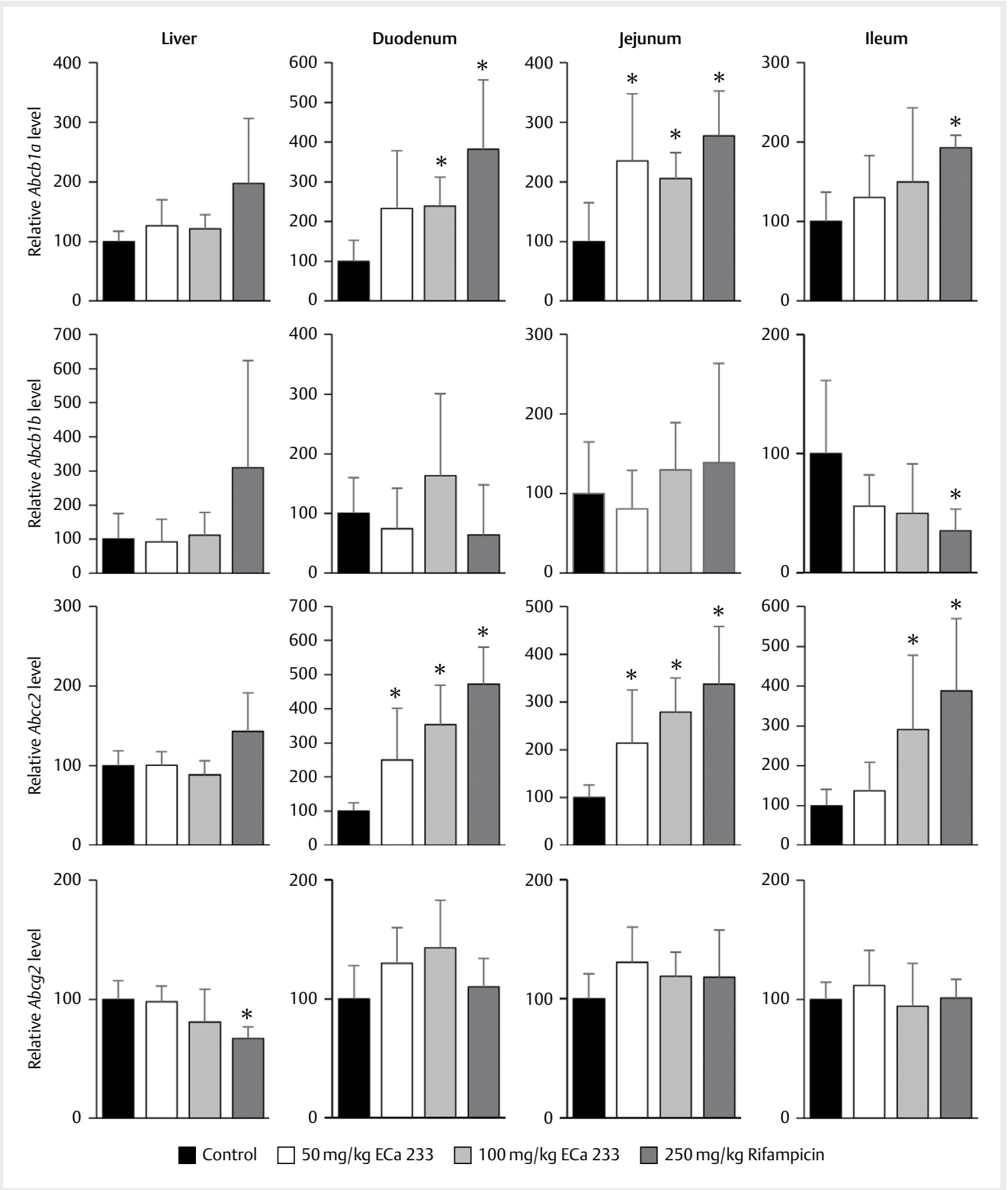
Percent recovery	Day 1			Day 7		
	Urine	Feces	Total	Urine	Feces	Total
Madecassoside	0.01 ± 0.01	9.57 ± 20.14	9.58 ± 20.13	0.01 ± 0.02	0.09 ± 0.18	0.11 ± 0.18
Asiaticoside	0.00 ± 0.00	2.61 ± 4.70	2.61 ± 4.70	0.00 ± 0.00	0.10 ± 0.20	0.10 ± 0.20
Madecassic acid	0.00 ± 0.00	17.43 ± 8.50	17.43 ± 8.50	0.00 ± 0.00	21.34 ± 12.93	21.34 ± 12.93
Asiatic acid	0.00 ± 0.00	10.95 ± 5.23	10.95 ± 5.23	0.00 ± 0.00	5.40 ± 2.92	5.40 ± 2.92 *

Data are shown as the mean ± S.D. (n = 9). * $P < 0.05$ total percent recovery on day 1 vs. day 7

creasing mRNA levels of *Abcb1a* and *Abcc2*, which are responsible for PGP and MRP2 expression. These two transporters could be upregulated in the small intestine, especially the duodenum and jejunum where most drug absorption takes place. Together with our previous findings, ECa 233 neither altered the total CYP content nor the CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1, and CYP3A activities after 90 days of treatment [25]. It is suggestive that upregulation of the efflux transporters, PGP and MRP2, might underlie the decreases in the C_{max} and $AUC_{(0-24)}$ of madecassoside and possibly asiaticoside as well. On day 1 of oral dosing with ECa 233, the T_{max} of madecassoside and asiaticoside was found to be 0.08–0.17 h and tended to be delayed after multiple dosing of ECa 233. A small second peak 4 h after dosing could possibly be accounted for by the enterohepatic circulation as proposed for pure madecassoside in a linked-rat model [24].

The tissue distribution study showed that madecassoside and asiaticoside reached various organs such as the skin, stomach, and especially the brain in which ECa 233 apparently exerts its pharmacological effects. High amounts of both triterpenoid glycosides in brain tissue is key evidence to confirm that madecassoside and asiaticoside are the bioactive compounds responsible for the pharmacological effects of ECa 233, as previously stated in a single dosing pharmacokinetic study [20]. In the present study, both triterpenoid glycosides appeared to be rapidly absorbed from the intestine into the circulatory system and extensively distributed into the internal organs after multiple oral dosing. Accumulation of these two glycosides in the target organs was apparent and resulted in a significantly higher tissue $AUC_{(0-4)}$ of madecassoside and asiaticoside on day 7, while their respective plasma concentrations were decreasing. Hence, our study suggests that it is best to determine the tissue levels of the active constituents of the herbal extract in concurrent with their respective plasma concentration-time profiles in multiple dosing pharmacokinetic studies.

Madecassic acid and asiatic acid were initially suggested to be active triterpenic metabolites [26] formed *in vivo* by esterase hydrolytic cleavage or acid hydrolysis of the sugar moiety from the parent triterpenoid glycosides [27] in a stepwise process [24]. The levels of these metabolites were minimal in plasma and tissue samples, but they were abundantly found in feces. The calculated percent recoveries within 24 h post-treatment on days 1 and 7 were consistent with our previous studies showing that ECa 233 was primarily excreted via feces and mainly as madecassic acid and asiatic acid [20, 21]. This result could imply that the parent triterpenoid glycosides underwent a hydrolytic reaction to remove the sugar moiety and were then metabolized into the acid metabolites in the gastrointestinal tract. A relatively larger amount of triterpenic acid metabolites, in comparison with their respective parent triterpenoid glycosides, were found in the feces on days 1 and 7. A small amount of triterpenic acid metabolites was excreted via the urinary system, consistent with their lipophilic properties (madecassic acid:



▶ **Fig. 3** Relative mRNA expression level (% of control) of *Abcb1a*, *Abcb1b*, *Abcc2*, and *Abcg2* in the liver and small intestine after multiple oral dosing for 7 consecutive days. Data are shown as the mean ± S.D. (n = 6). * $P < 0.05$ vs. control group.

XlogP 4.4, and asiatic acid: XlogP 5.7). Therefore, both of compounds were excreted mainly by the hepatobiliary system, rather than the urinary system.

In conclusion, a significant accumulation of madecassoside and asiaticoside in the tissue compartments was apparent despite an 80 % decrease in their respective plasma concentrations at day 7. This decrease in plasma triterpenoid glycosides could be accounted for by the inductive effect of ECa 233 on intestinal *Abcb1a* and *Abcc2* mRNA expression. The information obtained in this study suggests that no dosage adjustment is required for long-term use of ECa 233, but calls attention to the possibility of drug-herb interactions when ECa 233 is coadministered long term with conventional PGP and MRP2 substrate drugs.

Materials and Methods

Chemicals

ECa 233 was kindly supplied by Siam Herbal Innovation Co., Ltd. (batch number MRA1214004) as a white powder containing 53.1 % madecassoside and 32.3 % asiaticoside. Analytical standards of asiaticoside (> 98.5 %), madecassoside (> 95.0 %), and asiatic acid (> 95.0 %) were purchased from Sigma-Aldrich Corp. Rifampicin (> 90.0 %), madecassic acid (> 98.9 %), and glycyrrhetinic acid (> 99.0 %) were obtained from Wako Pure Chemical Industries.

Animals

Male Wistar rats aged 12 weeks old were obtained from the National Laboratory Animal Center, Mahidol University. They were housed with free access to food *ad libitum* under a controlled temperature and 12-h light-dark cycle prior to multiple dosing experiments. Rats with a weight of 400 to 600 g were placed in metabolic cages and fasted for 12 h with free access to water before a daily dosing of ECa 233, rifampicin, or vehicle (20 % DMSO/NSS). The animal experiments were conducted according to a protocol approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (approval number 13-33-007 and approval date: March 4, 2013).

Pharmacokinetic experiments

Rats received a daily oral administration of freshly prepared ECa 233 (50 mg/mL in 20 % DMSO/NSS) at a dose of 100 mg/kg for 7 days. Blood (400 µL) was collected via the lateral tail vein into preheparinized tubes on days 1 and 7 at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h after dosing. Blood samples were centrifuged at 1 500 × *g* for 10 min to acquire approximately 200 µL of plasma. Urine and feces were collected separately from metabolic cages on days 1 and 7 for 0–24 h after dosing. Urine volume and feces weight were recorded for the calculation of percent recovery. On day 7, some rats were euthanized at 1, 2 and 4 h after oral dosing to collect the brain, liver, kidney, spleen, stomach, and skin. Tissue samples were washed in normal saline to remove blood. All rats were anesthetized with isoflurane to reduce pain and injury during drug administration and sample collection. All biological samples were kept at − 20 °C until analysis. Plasma samples at 0 h on day 1 and 24 h on day 7 were also used for evaluation of creatinine, AST, and ALT levels to examine the effects of ECa 233 on kidney and liver functions.

Sample preparation

Methanol was used as a precipitating agent to prepare all biological samples by protein precipitation. Plasma and urine samples were thawed at room temperature, and 50 µL of each sample were mixed with 200 µL of methanol containing 10 ng of glycyrrhetinic acid (IS). The mixture was centrifuged at 5 000 × *g* for 10 min, and 10 µL of supernatants were analyzed by LC-MS/MS using optimized conditions and parameters. Approximately 50 mg of feces or tissue were combined with 200 µL of methanol containing 10 ng of IS, homogenized in an ice bath, and then processed in the same manner as the plasma and urine samples. Blank matrices were used to dilute some samples before protein precipitation if the concentration of analytes exceeded the linearity range of detection in LC-MS/MS.

LC-MS/MS analysis

LC-MS/MS analysis was carried out using methods previously mentioned in a pharmacokinetic study of a single oral dosing of ECa 233 [20]. In brief, an LC-MS/MS system comprising of an Eksigent Ekspert UPLC 100 liquid chromatograph equipped with a QTRAP 6500 mass spectrometer controlled by Analyst software version 1.6 (AB Sciex Pte. Ltd.) was used for analytical procedures. The stationary phase was a Synergi 4 µm Fusion-RP C18 column 50 × 2 mm (Phenomenex, Inc.) with a 40 °C oven temperature. With the optimized LC-MS/MS system, the retention times of madecassoside, asiaticoside, madecassic acid, asiatic acid, and glycyrrhetinic acid were 1.79, 1.82, 1.93, 1.99 and 2.12 min, respectively. The calculated percent recoveries were 78–93 % for all analytes. Calibration curves of triterpenoid glycosides and triterpenic acids showed good linearity with $R^2 > 0.99$ for 0.5–300 µg/L. The lower limit of detection was 0.1–0.5 µg/L, and intraday and inter-day precision and accuracy were within ± 10 %.

Quantitative real-time PCR

Rats were divided into four groups and orally treated for 7 days as vehicle (negative control), 50 mg/kg ECa 233 (low dose), 100 mg/kg ECa 233 (high dose), and 250 mg/kg rifampicin (positive control). Subsequently, they were euthanized to collect liver and intestinal mucosal cells from the duodenum, jejunum, and ileum parts of the small intestine. Total RNA was then isolated from tissue homogenates using a Gen Elute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Corp.) according to the company instructions. The cDNA was consequently prepared by reverse transcription of each RNA sample with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed on an Mx3000P Real-Time QPCR System (Agilent Technologies Inc.). The following primers were used for amplification of rat *Gapdh*: forward 5'-CTG TGG TCA TGA GCC CCT CC-3' and reverse 5'-CGC TGG TGC TGA GTA TGT CG-3', *Abcb1a*: forward 5'-TGA ACT GTG ACC ATG CGA GAT GTT AAA TA-3' and reverse 5'-GTC TCT GAA GAC TCT AAA ATG GAC TAA ATG-3', *Abcb1b*: forward 5'-CCA GGA GAG AAG ACT TAG TTC G-3' and reverse 5'-GGC AAA CAC TGG TTG TAT GCA C-3', *Abcc2*: forward 5'-TTC ACG GGC ACA TCA CCA-3' and reverse 5'-ATT CGG ACC CAA ACA GGA TG-3', *Abcg2*: forward 5'-GTT TGG ACT

CAA GCA CAG CA-3' and reverse 5'-TGA GTT TCC CAG AAG CCA GT-3', respectively. The qPCR conditions were one cycle at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, and a last cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s.

Data analysis

Biochemical parameters for the liver and kidney were analyzed by Student's t-test ($p < 0.05$) to determine significant differences between pretreatment (0 h on day 1) and post-treatment (24 h on day 7). Pharmacokinetic parameters of madecassoside and asiaticoside, including C_{max}, T_{max}, $AUC_{(0-24)}$, and normalized $AUC_{(0-24)}$, and tissue $AUC_{(0-4)}$ were reported. The calculated concentrations of madecassoside, asiaticoside, madecassic acid, and asiatic acid in urine and feces were used to determine percent recoveries using the total drug found in urine and feces divided by the administered dose based on a molar basis. Statistical differences in pharmacokinetic parameters, tissue $AUC_{(0-4)}$, and percent recoveries between day 1 and day 7 were analyzed using Student's t-test ($p < 0.05$). The expression level of each mRNA was normalized by the expression of *Gapdh* as an internal control, and reported as % of the control group. Student's t-test ($p < 0.05$) was also used to evaluate the statistical differences between the ECa 233 treated groups and the control group.

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Conflict of Interest

All authors declare no conflicts of interest.

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