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Effects of the standardized extract of *Centella asiatica* ECa233 on hepatic Phase II drug-metabolizing enzymes in rats

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ABSTRACT

Introduction: The standardized extract of Centella asiatica ECa233 (ECa233) was shown to possess neuroprotective and wound healing effects. **Purpose of the Study:** The purpose of this study was to investigate effect of ECa233 on the activities of Phase II drug-metabolizing enzymes including uridine 5'-diphosphoglucuronosyltransferase (UDPGT), sulfotransferase (SULT), glutathione S-transferase (GST), and NAD(P)H quinone oxidoreductase (NQOR) in rats. Method: ECa233 was orally given to both male and female Wistar rats at doses of 10, 100, and 1000 mg/kg/d for 90 days. **Result and Conclusion:** The results showed that all regimens of ECa233 did not affect the activities of UDPGT in both male and female rats. Furthermore, in line with our previous study in male rats, it was found that neither the activities of GST nor NQOR in female rats was modulated by ECa233. However, the activity of SULT, which had been found to significantly decrease in male rats, was unaltered in female rats. The obtained results provide the proof of evidence of safety of ECa233 on Phase II drug-metabolizing enzymes in animals, then interpretation of these data from animals to human remains to be further investigated.

INTRODUCTION

Centella asiatica (Linn.) Urban, a member of Apiaceae family, has long been consumed as food, beverage, and traditional medicines. It contains triterpenoids, principally asiaticoside, madecassoside, asiatic acid, and madecassic acid [1]. In agreement with its traditional use as medicinal herb, experimental studies revealed several pharmacological effects of *C. asiatica* extracts. Recently, reports on wound healing activity [2,3], protective effect on gastric ulcer formation [4,5], learning- and memory-enhancing effect [6-9], antioxidant activity [10-12], antitumor

activity [13,14], and immunological activity [15-17] of *C. asiatica* or its constituents were markedly increased.

The standardized extract of *C. asiatica* ECa233 (ECa233) has been developed with well-defined ratio of the active ingredients (madecassoside and asiaticoside). It possesses several striking pharmacological activities with positively safety profile. The neuroprotective effect of orally given ECa233 was demonstrated in animal models of memory impairment induced by transient global ischemia or intracerebroventricular injection of β-amyloid peptide in mice [18,19]. Anxiolytic effect of ECa233 was also observed in animals subjected to acute or chronic stress [20]. Furthermore, neurite outgrowth-promoting activity of ECa233 was demonstrated in human IMR-32 neuroblastoma cell line [21]. Topical application of ECa233 was found to accelerate wound healing processes of burn wounds in rats [22]. Preliminary clinical trial demonstrated that ECa233 was effective in reducing pain, ulcer size, and erythema of minor recurrent aphthous ulceration [23]. Other pharmacological activities of the extract and the pharmacokinetic studies [24] of its active constituents are currently ongoing.

Regarding the safety concern, ECa233 at the oral dose up to 10 g/kg produced no acute toxic effects. No significant subchronic toxicity was observed in rats receiving 10-1000 mg/kg/d of the extract [25]. Safety information regarding potential drug-herb interaction of ECa233 is assessed both *in vitro* [26] and *in vivo* in male and female rats [27] through investigating effect of the extract on hepatic cytochrome P450, the important Phase I enzymes which are commonly associated with drug interaction. Phase II drug-metabolizing enzymes, for example, uridine 5'-diphospho-glucuronosyltransferase (UDPGT), sulfotransferase (SULT), glutathione *S*-transferase (GST), etc., are also involved in drug interaction. These Phase II enzymes are primarily responsible for detoxification of the reactive metabolites of Phase I reactions or the parent toxic compounds. Likewise, NAD(P)H quinone oxidoreductase (NQOR), a flavoprotein enzyme, also plays an important role in detoxification of various quinone metabolites. Modulation of these enzymes through either induction or inhibition of the enzymes can affect metabolism of drugs that are detoxified by the particular enzyme. To investigate herb-drug interaction potential of ECa233, effects of the extract on some Phase II enzymes have been investigated in male rats [28]. Since sex variation is also existed in Phase II enzymes [29,30], in this study, we aimed to investigate the subchronic effects of ECa233 on hepatic Phase II enzymes in female rats as well as other Phase II enzyme that had not yet been reported in male rats [28].

MATERIAL AND METHODS

Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA): Adenosine 3′-phosphate-5′-phosphosulfate (PAPS), bovine serum albumin, cupric sulfate, 1-chloro-2,4-dinitrobenzene (CDNB), DCPIP, dicumarol, Folin and Ciocalteu's phenol reagent, glutathione (GSH)-reduced form, nicotinamide adenine dinucleotide (NADH)-reduced form, *p*-nitrophenol sulfate, and UDP-glucuronic acid (UDPGA). *p*-nitrophenol was purchased from Merck, Darmstadt, Germany. 2-Naphthol was purchased from Aldrich, St. Louis, MO, USA. Other chemicals were analytical grade.

The Standardized Extract of *C. asiatica* ECa233

ECa233 was prepared by Associate Professor Ekarin Saifah and collaborates at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The extract is a white-powdered extract of *C. asiatica* containing 85% of total triterpenoid glycosides that comprises 53% of madecassoside and 32% of asiaticoside. The chromatogram of madecassoside and asiaticoside in ECa233 is shown in Wanasuntronwong *et al*. [20].

Experimental Animals

Forty adult male and forty adult female Wistar rats of body weight between 250 and 300 g were obtained from the Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed three per cage at the Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health and acclimatized for at least 7 days before the experiment. All animals were maintained at 25°C on a 12-h alternate light-dark cycle in controlled humidified room and allowed freely access to food (C.P. Company, Thailand) and drinking water. The protocol of animal housing and treatment used in this study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Approval no. 08-33-009).

Both male and female Wistar rats were randomly divided into 4 groups of 10 rats each. Rats in the treatment groups were given orally with ECa233 at 10, 100, and 1000 mg/kg/d, whereas rats in the control group were given distilled water for 90 days. At the end of treatment, rats were sacrificed and liver microsomes as well as cytosols were prepared for enzyme activity assays.

Preparation of Microsomes and Cytosols and Enzyme Activity Assays

At the end of the treatment, animals were fasted for 12 h before anesthetized with diethyl ether. Livers were excised and perfused with ice-cold 0.9% sodium chloride. Rat liver microsomes and cytosols were prepared according to the method described by Lake [31]. Liver microsomal and cytosolic protein concentrations were determined according to the method of Lowry *et al*. [32]. UDPGT activity in microsomes was measured using *p*-nitrophenol as a substrate and UDPGA as a coenzyme according to the method of Bock *et al*. [33]. SULT activity in cytosols was determined using 2-naphthol as a substrate as well as paps and *p*-nitrophenol sulfate as coenzymes according to the method of Frame *et al*. [34]. GST activity in cytosols was measured using 1-chloro-2, 4-dinitrobenzene as a substrate and the reduced form of GSH as a coenzyme according to the method of Habig *et al*. [35]. NQOR activity in cytosols was determined using 2, 6-dichlorophenol-indophenol as a substrate and NADH as a coenzyme according to the method of Lind *et al*. [36]. Methods for determination of UDPGT, SULT, GST, and NQOR activities were verified by performing the reactions with various concentrations of the microsomal or cytosolic protein and measuring the absorbance of the corresponding final products. Linearity of the method was demonstrated by the coefficient of determination (R^2).

Statistics

All quantitative data were presented as mean ± standard error of the mean. A one-way analysis of variance (ANOVA) and Student-Newman-Keuls test were used for statistical comparison at a significant level of $P < 0.05$.

RESULTS

Linearity of the methods for determination of UDPGT, SULT, GST, and NQOR activities was demonstrated with the R^2 of 0.9907, 0.9958, 0.9917, and 0.9947, respectively (Figure 1).

ECa233 orally given to male or female rats had no significant effect on the activity of UDPGT. As shown in Figure 2a, the activity of UDPGT of male rats receiving ECa233 at the doses of 10, 100, and 1000 mg/kg for 90 days was not statistically different from that of their control. Similar results were observed in female rats (Figure 2b).

As shown in Figure 3, ECa233 had no significant effect on the activities of SULT, GST, and NQOR in female rats. Lack of significant effect of ECa233 on GST and NQOR was similar to our results previously reported in male rats. However, the decreased activity of SULT demonstrated in male rats and was not noted in female rats in the present study.

DISCUSSION AND CONCLUSION

This study investigated effects of ECa233 on Phase II drug-metabolizing enzymes such as UDPGT, SULT, GST, and NQOR in rats. Modulation of Phase II enzymes, unlike of Phase I enzymes, does not mainly contribute to the metabolic-based drug-drug interaction. However, changes of Phase II enzymes, in some circumstances, cause drug-drug interaction. In addition, induction of Phase II enzymes, which mostly detoxify xenobiotics and/or reactive metabolites, explains the protection of animal/human from toxicity of xenobiotics/their reactive metabolites .

Results from this study demonstrated that all regimens of ECa233 given to female rats did not significantly affect the activities of UDPGT, SULT, GST, and NQOR as compared to their respective control groups. Lack of effects of ECa233 on UDPGT, SULT, GST, and NQOR activities indicated that these extracts had no additional advantage in terms of potential decrease risks of toxicity, antimutagenetic and/or anticarcinogenic effect against many drugs, and/or xenobiotics that are detoxified by these enzymes. UDPGT plays major role in detoxification of both endogenous compounds, such as bilirubin and steroid hormones, as well as exogenous compounds, including various drugs and xenobiotics either in the form of parent compounds or their metabolites from Phase I metabolism. Example of drugs/xenobiotics or their metabolites that are detoxified by UDPGT is morphine, acetaminophen, etc. [37]. SULT plays an important role in the detoxification of many xenobiotics such as acetaminophen, hydroxyl-tamoxifen, and phenol [37]. Several major classes of GST play an important role in detoxification of electrophilic drugs and toxic metabolites from Phase I metabolism. Example of drugs/xenobiotics or their reactive metabolites that are detoxified by GST is acetaminophen, various epoxide metabolites of aromatic hydrocarbon xenobiotics. NQOR, a flavoprotein, also plays an important role in detoxification of various quinone metabolites. Drugs/xenobiotics or their reactive metabolites that are detoxified by NQOR are menadione, benzo(*a*)pyrene-3,6-quinone, 1,4-naphthoquinone, etc. [36].

In contrast to our findings in female rats in the present study, activity of SULT in male rats treated with ECa233 at all doses was significantly decreased in comparison to their male control group [28]. Decrease of SULT activities in male rats indicated the possibility of either increase or decrease risks of

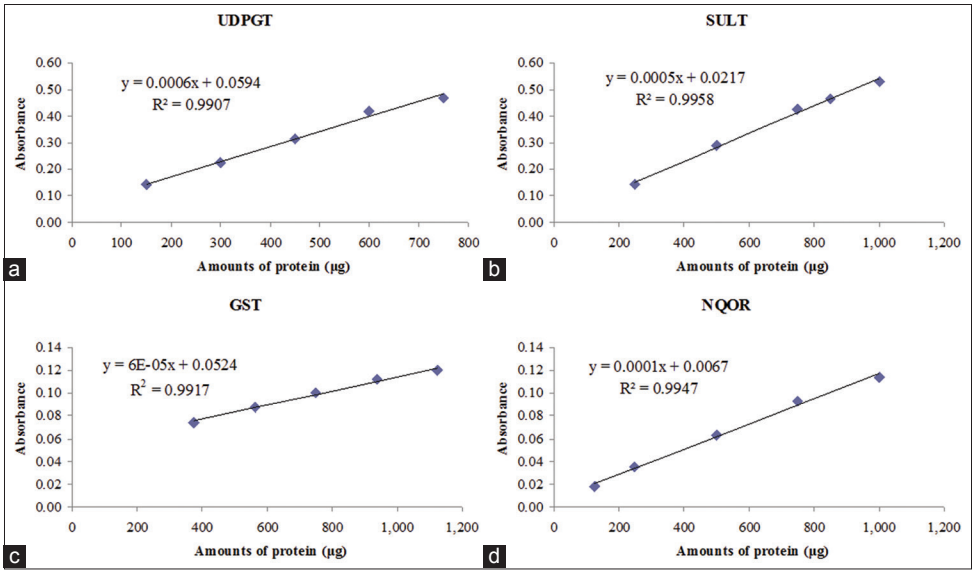

Figure 1: Linearity of the method for determination the activities of (a) uridine 5'-diphospho-glucuronosyltransferase, (b) sulfotransferase, (c) glutathione *S*-transferase, and (d) NAD(P)H quinone oxidoreductase. The line was acquired by plotting the amounts of microsomal or cytosolic protein against the corresponding spectrophotometrically absorbance of the final products. Each point represented the mean of duplicated reactions

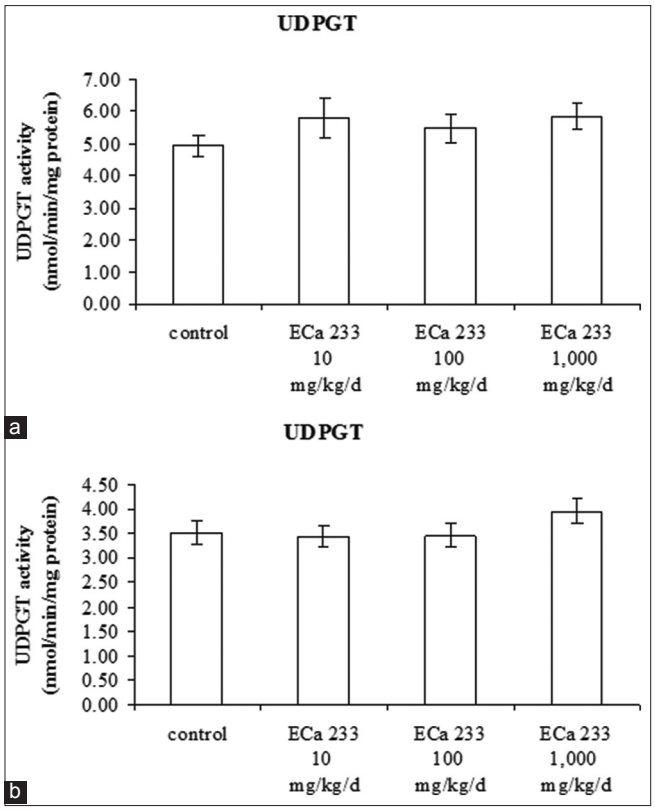

Figure 2: Effects of ECa233 on hepatic microsomal uridine 5'-diphospho-glucuronosyltransferase activity in (a) male and (b) female rats. Rats were treated orally with water (1 ml/kg/d) in the control group, ECa233 (10, 100 and 1000 mg/kg/d) for 90 days. Data were presented as mean ± standard error of the mean of 10 rats/group. For statistical analysis, one-way ANOVA and Student-Newman-Keuls test were used, $P < 0.05$ was considered statistically significant

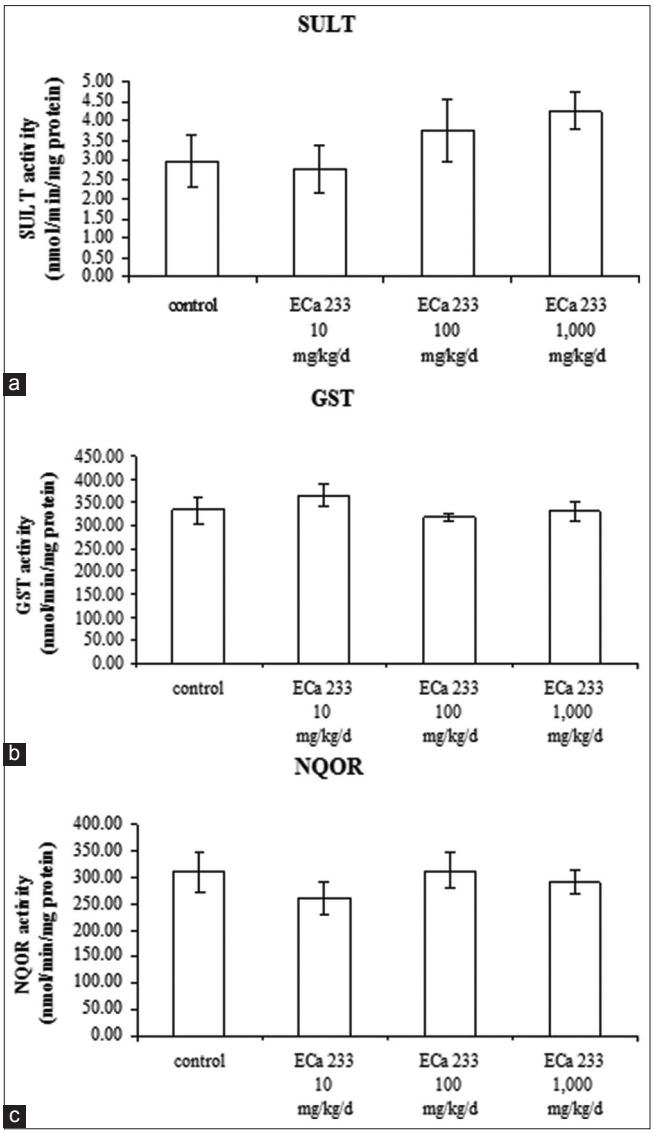

Figure 3: Effects of ECa233 on the activities of hepatic cytosolic (a) sulfotransferase, (b) glutathione *S*-transferase, and (c) NAD(P)H quinone oxidoreductase in female rats. Rats were treated orally with water (1 ml/kg/d) in the control group, ECa233 (10, 100 and 1000 mg/kg/d) for 90 days. Data were presented as mean ± standard error of the mean of 10 rats/group. For statistical analysis, one-way ANOVA and Student-Newman-Keuls test were used, $P < 0.05$ was considered statistically significant

drugs - and/or xenobiotics-induced toxicities, mutagenicities, and/or carcinogenicities depending on the fact that SULT plays a role in the detoxification or bioactivation of those xenobiotics. Examples of xenobiotics that are bioactivated by SULT are hydroxymethyl polycyclic aromatic hydrocarbons, N-hydroxyarylamines, since their sulfate ester are electrophiles that covalently bind to nucleic acids and other macromolecules [38]. In this study, the inhibition of SULT was not found in the female rats. The discrepancy observed was probably due to the sex variation of this enzyme in male and female rats as previously reported [39,40].

To investigate inhibitory effects of ECa233 on Phase II drug-metabolizing enzymes, selective substrates of the individual enzymes were used. *p*-nitrophenol, which was the substrate of UDPGT1A4 or UDPGT1A8/9 isoform [37], was used to determine UDPGT activity. 2-Naphthol, which was the substrate of SULT1As isoform [29], was used to determine SULT activity. CDNB, which was the substrate of GST alpha, GST mu, GST pi, and GST omega classes [30,37], was used to determine GST activity. DCPIP, which was the substrate of NQOR [41], was used to determine NQOR activity. Before using all protocols for determination, the activities of UDPGT, SULT, GST, and NQOR, verifications of the methods were performed. Linearity of the methods was shown by the acceptable coefficient of determination (R^2) between the amount of microsomal protein/cytosolic protein and the absorbance of the final product solution.

In this study, ECa233 was orally given to both male and female rats for 90 days, according to the protocol suggested for subchronic toxicity testing [42]. Therefore, **t**he information providing effects of ECa233 on Phase II drug-metabolizing enzymes in both sexes of rats was beneficial in terms of safety in long-term use as well as lack of potential to cause drug-herb interaction. In addition, the possibility of the extract to decrease/increase risks to xenobiotic-induced toxicities, mutagenicities, and/or carcinogenicities in association with decreasing activity of SULT in male rats should be concerned.

In conclusion, effects of ECa233 on Phase II drug-metabolizing enzymes such as UDPGT, SULT, GST, and NQOR were investigated. ECa233 at doses of 10, 100, and 1000 mg/kg/d were orally administered to both male and female rats for 90 days. The results showed that all regimens of ECa233 did not show significant effects on the activities of UDPGT in both male and female rats. ECa233 did not modulate the activities of SULT, GST, and NQOR in female rats. This study provided the information of effects of ECa233 on Phase II drug-metabolizing enzymes in animals; however, interpretation of these data from animal to human should be further clarified.

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