Effects of the standard extract of *Centella asiatica* (ECa233) on rat hepatic cytochrome P450

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Abstract:

*Centella asiatica* (L) Urban (Umbelliferae) has been used traditionally in various diseases. The aim of this study was to examine subchronic effects of the standard extract of *C. asiatica* (ECa233) on hepatic xenobiotic/drug metabolizing enzymes, particularly cytochrome P450 (CYP) such as CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A in rats. 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 1,000 mg/kg/day whereas rats in the control group were given distilled water for 90 consecutive days. At the end of treatment, rats were sacrificed and liver microsomes were prepared for enzyme activity assay. The results showed that ECa233 did not affect total CYP content as well as the activities of CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A in both male and female rats. Inhibitory effect of ECa233 was examined *in vitro*, using rat liver microsomes. ECa233 caused a concentration-dependent decrease of the activity of CYP2B1/2B2 (BRD: IC₅₀ = 523 mg/ml, PROD: IC₅₀ = 563 mg/ml) and very slightly effect on CYP1A2 (MROD: IC₅₀ > 1,000 mg/ml). ECa233 had no inhibitory effect on the activities of CYP1A1, CYP2E1 and CYP 3A at concentrations up to 2,000 mg/ml. The inhibitory effect of ECa233 on CYP2B1/2B2 indicated a possibly beneficial effect of the compound regarding the protection of chemical-induced carcinogenesis but the concern regarding the possibilities of drug-drug interaction with the medicines metabolized by these CYPs. Effects of ECa233 on the activities of other CYP isoforms and human liver CYP should be further investigated.

Keywords: Cytochrome P450; Drug interaction; ECa233, Standard extract of *C. asiatica*
Introduction

Centella asiatica (L.) Urban (Umbelliferae) has been used traditionally in various diseases. The active compounds isolated from C. asiatica are the triterpene acids and their glycosides, including asiatic acid, madecassic acid, asiaticoside and madecassoside [1]. Asiaticoside which is the most abundant triterpene glycoside is effective in wound healing [2]. Additionally, the extracts of this plant and its active compounds possessed several pharmacological effects, such as anti-oxidant effect [3], cardioprotective effect [4], learning and memory improvement [5], decreasing lipid peroxidation in brain [6], anti-cancer activity [7]. Thus, this plant was subjected to extensive studies aiming to be developed as an alternative for several pathological symptoms.

Tantisira M.H. and collaborates at the Faculty of Pharmaceutical Sciences, Chulalongkorn University found that the standard extract of C. asiatica (ECa233), characterized with well-defined ratio of the active ingredients, attenuated learning and memory deficit induced by an intracerebroventricular injection of β-amyloid peptide in mice [8]. Toxicity study and other pharmacological aspects are still required before performing clinical study in human. In addition to the safety information of this extract regarding subchronic/chronic toxicity, the study on the effect of the extract on cytochrome P450 (CYP) would be important in term of the possibility of drug-drug interaction when the extract is co-administered with medicines that are metabolized by CYPs.

During the research and development process of a new compound, drug-drug interaction study is one of the essential processes in pre-clinical and clinical studies particularly effect of the compound on CYP enzymes. CYP is an enzyme system in phase I metabolism, that plays an important role in detoxification or in the other hand, metabolic activation of xenobiotics so as to be further detoxified by the conjugative reactions of phase II metabolism then readily excreted from the body. Thus, inhibition and/or induction of CYP enzymes by any compounds may lead to the change of plasma level of drugs that are metabolized by the particular CYP isoforms resulting in an increase of side effects/drug toxicity or in the other hand decrease of drug efficacy. Therefore, an attempt to develop ECa233 for use clinically, safety study regarding drug-drug interaction should be clarified.

In addition to the drug-drug interaction issue, effect of ECa233 on CYP would address whether this extract affects human susceptibility to exogenous mutagens/pro-carcinogen. This is due to the fact that many CYP isoforms play a role in xenobiotic bioactivations. As a result, this study investigated effects of ECa233 on CYP isoforms that play key role in drug metabolism and the bioactivation of mutagens and/or carcinogens. Such CYP isoforms included CYP1A1, 1A2, 2B1/2B2, 2E1 and 3A. The study was performed in vivo in rats and in vitro using rat liver microsomes.

Materials and Methods

Chemicals

The following chemicals were obtained from Sigma Chemical Co. (USA): Acetylacetone, 4-aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin, cupric sulfate, ethoxyresorufin (ER), Folin and Ciocalteu’s phenol reagent, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), methoxyresorufin (MR), nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR) and resorufin. All other chemicals were analytical grade. Erythromycin stearate was kindly provided by Siam Pharmaceutical Co. Ltd., Thailand.

The standard extract of C. asiatica (ECa233)

ECa233 was prepared by Assoc. Prof. Ekarin Saifah and collaborates at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The extract was characterized as white to off-white powder containing triterpenoid glycosides not less than 80% which consisted of 43% of madecassoside and 39% of asiaticoside.

Experimental animals

Forty adult male and forty adult female Wistar rats of body weight between 250-300 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 seven days prior to the experiment. All animals were maintained at 25 °C on a 12-hour alternate light-dark cycle in controlled humidified room and allowed freely access to food (C.P. Company) and drinking water. The protocol of animal housing and treatment used in this study was approved by the Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Approval no. 07-33-003).

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/day whereas rats in the sex-corresponding control group were given distilled water for 90 consecutive days. At the end of treatment, rats were sacrificed and liver microsomes were prepared for enzyme activity assay.

**Preparation of microsomes**

At the end of the treatment, animals were fasted for 12 hours before anesthetized with diethyl ether by inhalation. Livers were immediately excised and perfused with ice-cold 0.9% sodium chloride. Rat liver microsomes were prepared according to the method described by Lake [9] with some modifications. Briefly, rat livers were quickly perfused in situ with ice-cold 0.9% w/v sodium chloride until the entire organ became pale. The livers were removed from the bodies and rinsed with ice-cold 0.9% w/v sodium chloride, and blotted dry with gauzes. The whole livers were weighed, cut into pieces, and homogenized with 3 times of its weight by phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 10,000 g for 30 minutes at 4 °C, using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei, and mitochondria. The supernatants were transferred to centrifuge at 100,000 g for 60 minutes at 4 °C, using refrigerated ultracentrifuge. The pellets (microsomal subfractions) were resuspended with 5 ml of 0.1 M phosphate buffer, pH 7.4 containing 20% v/v glycerol. The microsomal suspensions were aliquoted, kept in microtubes, and stored at -80 °C until the time of enzyme activity assays. Liver microsomal protein concentrations were determined according to the method of Lowry et al. [10].

**Enzyme assays**

Hepatic microsomal total CYP contents were determined spectrophotometrically according to the method of Omura and Sato [11]. Rate of hepatic microsomal alkoxyresorufin O-dealkylation was determined according to the method of Burke and Mayer [12] and Lubet et al. [13]. BR and PR were used as selective substrates of CYP 2B1/2B2. ER and MR were used as selective substrates of CYP 1A1 and CYP1A2, respectively. The catalytic activity of CYP2E1 was determined based on the rate of hepatic microsomal aniline 4-hydroxylation, according to the method of Schenkman et al. [14]. Aniline hydrochloride was used as a selective substrate in this reaction. Rate of hepatic microsomal erythromycin N-demethylation was determined using the method of Nash et al. [15] and Friedli [16]. Erythromycin stearate was used as a selective substrate of CYP3A.

**In vitro study**

To investigate the inhibitory effect of ECa233 on CYP1A1, CYP1A2 in vitro, various concentrations of ECa233 were prepared and added concomitantly with ER and MR, respectively, in the incubation mixture of ethoxyresorufin O-dealkylation and methoxyresorufin O-dealkylation, respectively. The final concentrations of ECa233 in the reaction mixture were 0, 100, 250, 500, 1,000 and 2,000 µg/ml and the β-naphthoflavone-induced rat liver microsomes were used in the reaction. Likewise, to investigate the inhibitory effect of ECa233 on CYP2B1/2B2 in vitro, various concentrations of ECa233 were prepared and added concomitantly with BR or PR in the incubation mixture of benzyloxyresorufin O-dealkylation or pentoxyresorufin O-dealkylation, respectively. The final concentrations of ECa233 in the reaction mixture were 0, 100, 250, 500, 1,000 and 2,000 µg/ml and the phenobarbital-induced rat liver microsomes were used in the reaction. To investigate the inhibitory effect of ECa233 on CYP2E1 in vitro, various concentrations of ECa233 were added concomitantly with aniline hydrochloride in the incubation mixture of aniline hydrochloride.
4-hydroxylation. The final concentrations of ECa233 in the reaction mixture were 0, 100, 250, 500, 1,000 and 2,000 µg/ml and the normal rat liver microsomes were used in the reaction. To investigate the inhibitory effect of ECa233 on CYP3A \textit{in vitro}, various concentrations of ECa233 were added concomitantly with erythromycin stearate in the incubation mixture of erythromycin N-demethylation. The final concentrations of ECa233 in the reaction mixture were 0, 100, 250, 500, 1,000 and 2,000 µg/ml and the normal rat liver microsomes were used in the reaction. Percent inhibition of CYP activities were calculated from comparing the rate of ethoxyresorufin O-dealkylation (EROD), methoxyresorufin O-dealkylation (MROD), benzyloxyresorufin O-dealkylation (BROD), pentoxyresorufin O-dealkylation (PROD), aniline 4-hydroxylation or erythromycin N-demethylation at various concentrations of ECa233 with the corresponding rate of the reactions without ECa233.

**Statistics**

All quantitative data were presented as mean ± standard error of the mean (SEM). An one-way analysis of variance (ANOVA) and Student-Newman-Keuls test were used for statistical comparison at a significant level of $p < 0.05$. For calculation of IC$_{50}$, percent of enzyme inhibition was transformed to probit unit. The linear regression method was used to fit a curve between probit unit and log dose. The IC$_{50}$ was calculated from the linear regression equation.

**Results**

In the \textit{in vivo} study, ECa233 given to rats at the doses of 10, 100 and 1,000 mg/kg/day for 90 days did not affect total CYP content as well as the activities of CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A in both male (Figure 1) and female rats (Figure 2).

In the \textit{in vitro} study using rat liver microsomes, the results showed that ECa233 possessed an inhibitory effect on CYP2B1/2B2 in a concentration-dependent manner with a median inhibitory concentration (IC$_{50}$) of 523 µg/ml as determined by BROD reaction and 563 µg/ml as determined by PROD reaction (Figure 3).

Very slightly decrease or no effect of the extract was shown on the activity of CYP1A2 resulting in a high IC$_{50}$ of more than 1,000 µg/ml (Figure 4). Likewise, no inhibitory effects of ECa233 were shown on the activities of CYP1A1, CYP2E1 and CYP3A (Table 1).

**Discussion**

Results from this study showed that ECa233 did not modulate CYP1A1, 1A2, 2B1/2B2, 2E1 and 3A \textit{in vivo} in rats of both male and female. No inductive and inhibitory effects of the extract in the \textit{in vivo} study indicated an advantage of the extract in terms of drug-drug interactions when the extract was administered concomitantly with other currently used medicines. The examples of drugs that are metabolized by CYP1A1 are R-warfarin, amiodarone, etc.; by CYP1A2 are acetaminophen, amitriptyline, theophylline, etc.; by CYP2B1 & 2B2 are phenobarbital, cyclophosphamide, etc.; by CYP 2E1 are acetaminophen, chlorzoxazone, etc., and by CYP3A are erythromycin, terfenadine, omeprazole, etc. [17, 18].

The result of the \textit{in vivo} study in this study was consistent to the result reported earlier by Phongjit [19]. In the study of Phongjit [19], ethanolic extract of \textit{C. asiatica} was given to male rats at doses of 250 and 1,000 mg/kg/day for 30 days. No effects (either induction or inhibition) of the ethanolic extract of \textit{C. asiatica} were found on CYP1A1, 1A2, 2B1/2B2, 2E1 at both dosages of the extract. Even though the \textit{in vivo} study can be used to determine both the inductive effect and the inhibitory effect of any compounds, limitation of the detection is existed for the inhibitory study. Normally, the inhibitory effect is detected by the \textit{in vivo} study if the inhibition is an irreversible type. In contrast, if the inhibition is a reversible type especially inhibition via competitively binding at the substrate binding site, the \textit{in vitro} study is a more appropriate model than the \textit{in vivo} study. Thus, \textit{in vitro} study was also performed in this study even though the inhibition was not found in the \textit{in vivo} study. In the \textit{in vitro} study, CYP2B1/2B2 was shown to be inhibited by ECa233 with an IC$_{50}$ of 523 µg/ml (as determined by BROD assay) and 563 µg/ml (as determined by PROD assay). Rat CYP2B1/2B2 and human CYP2B6 share approximately 80% nucleotide
sequence identity [20]. A potent CYP2B6 inhibitor, ticlopidine demonstrated an inhibitory effect clinically [21], while in an in vitro study this compound possessed an inhibitory effect on this CYP isoform with an IC$_{50}$ of 0.084 µg/ml [22]. The slightly inhibitory effect of ECa233 found in this study may or may not be clinically significant, the issue of which needed to be clarified. Likewise, the inhibitory effect of this extract on CYP1A2 with an IC$_{50}$ more than 1,000 µg/ml (2,895 µg/ml) was rather small in comparison to furafylline, a potent inhibitor on CYP 1A2. Effect of furafylline on CYP1A2 was shown to be clinically significant corresponding with an in vitro study showing an inhibitory effect on this CYP with the IC$_{50}$ of 0.72 µg/ml [23].

**Figure 1** Effect of ECa233 on hepatic total CYP contents, CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A activities in male rats (mean ± SEM; n=10)
Figure 2 Effect of Eca233 on hepatic total CYP contents, CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A activities in female rats (mean ± SEM, n=10)
Figure 3 Effects of ECa233 on CYP2B1/2B2 in an in vitro study. Eca233 was co-incubated simultaneously with BR (A) and PR (B) under condition described in the Materials and Methods. IC$_{50}$ values for BROD and PROD were 523 and 563 µg/ml respectively. Data shown were mean ± SD; n=4.

Figure 4 Effects of ECa233 on CYP1A2 in an in vitro study. Eca233 was co-incubated simultaneously with MR under condition described in the Materials and Methods. IC$_{50}$ value was > 1,000 µg/ml. Data shown were mean ± SD; n=4.
Table 1 Effect of ECa233 on CYP1A1, CYP2E1 and 3A in the in vitro study. Various concentrations of ECa233 were co-incubated simultaneously with ER (for CYP1A1), Aniline hydrochloride (for CYP2E1) or Erythromycin stearate (for CYP3A) under condition described in the Materials and Methods. Data shown were mean ± SD; n=4.

<table>
<thead>
<tr>
<th>Concentrations of the ECa233 in the reaction mixture (µg/ml)</th>
<th>CYP activities</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1A1 (pmol/mg protein/min)</td>
</tr>
<tr>
<td>0</td>
<td>685.67 ± 80.10</td>
</tr>
<tr>
<td>100</td>
<td>748.36 ± 58.12</td>
</tr>
<tr>
<td>250</td>
<td>750.27 ± 45.08</td>
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<tr>
<td>500</td>
<td>794.49 ± 41.23</td>
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<tr>
<td>1,000</td>
<td>835.88 ± 38.06</td>
</tr>
<tr>
<td>2,000</td>
<td>866.55 ± 33.84</td>
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An inhibitory effect of ECa233 on CYP2B1/2B2 found in an in vitro study in which the extract was simultaneously added into the reaction with BR or PR, a selective substrate of CYP2B1/2B2. However, no inhibitory effect was noted in the in vivo study. This finding suggested that some constituents in the extract could probably be a substrate of these CYP isoforms. Thus, adding together with the selective substrate during performing the reaction showed a decreased rate of the selective substrate oxidation. In addition, inhibitory effect that was shown in vitro but not shown in vivo would rather indicate that the inhibition was reversible. Thus, the inhibitory effect could not be detected in vivo due to the recovery of the enzyme. Further study is needed to be clarified regarding types of inhibition on these CYPs isoforms of ECa233.

With regards to the drug-drug interaction issue, no inductive effects of ECa233 was observed on CYP1A1, 1A2, 2B1/2B2, 2E1, 3A which normally bioactivate many procarcinogen, medicines and other environmental xenobiotics, offer an advantage of this extract in term of chemical bioactivation. Safety in term of no increase risk to toxicity, mutagenesis and/or carcinogenesis from other xenobiotics that are metabolic activated by these CYP isoforms is offered following long term use of the extract. Procarcinogens that are bioactivated by CYP 1A1 include environmental polycyclic aromatic hydrocarbon (PAHs), such as benzo(a)pyrene, 7, 12-dimethylbenz(a)anthracene, 6-nitrochrysene, etc.; by CYP1A2 include 2-acetylaminofluorene, 2-aminofluorene, aflatoxin B1, 2-aminoantrachene, 2-naphthylamine, etc.; by CYP2B1/2B2 include aflatoxin B1, benzo(a)pyrene, 3-methylcholanthrene, etc.; by CYP2E1 include acrylonitrile, benzene, carbon tetrachloride, chloroform, trichloroethylene, etc., and by CYP3A include aflatoxin B1, aflatoxin G1, benzo(a)pyrene, 6-aminochrysene, etc. [24].

In this study, Wistar rats of both sexes, male and female were used and the duration of treatment was 90 days. This was due to the fact that this study actually designed for subchronicity study of the ECa233. Thus, the experiment was designed according to the protocol suggested by WHO guideline [25]. In addition to the subchronicity toxicity data, the data on long term use of the extract obtained from the same group of experimental animals were also beneficial in term of safety regarding drug-drug interaction and the possibility to increase/decrease risk to xenobiotic exposure. Doses of ECa233 used in this study were 10, 100 and 1,000 mg/kg/day. The lowest dose (10 mg/kg/day) was the dose that was found to possess the beneficial effect of learning and memory enhancer in rodent [8].

To investigate effects of ECa233 on hepatic CYP activities, selective substrate of the individual CYP were used. Rate of the selective substrate oxidation was considered to represent the corresponding CYP activity in hepatic microsome of rats treated with the extract. ER, MR and BP&PR have been proved to be selective substrate of CYP1A1, 1A2, 2B1/2B2, respectively.
and 3A were shown in the vivo. Likewise, no effects of the extract on CYP1A1, 2E1 activities of CYP1A1, 1A2, 2B1/2B2, and 2E1 and 3A in not cause any changes on total CYP contents and the same manner. The results showed that ECa233 did correspond sex-control group given distilled water in vivo. In an vitro study, both male and female rats were given orally with ECa233 at the doses of 10, 100 and 1,000 mg/kg/day for 90 days, compared to the corresponding sex-control group given distilled water in the same manner. The results showed that ECa233 did not cause any changes on total CYP contents and the activities of CYP1A1, 1A2, 2B1/2B2, and 2E1 and 3A in vivo. Likewise, no effects of the extract on CYP1A1, 2E1 and 3A were shown in the in vitro study using rat liver microsomes. Decrease of CYP2B1/2B2 and very slight effect on CYP1A2 activities by ECa233 were shown in the in vitro study and in vivo study with the IC50 of 523 µg/ml (BROD), 563 µg/ml (PROD) for CYP2B1/2B2 and IC50 of more than 1,000 µg/ml (2895 µg/ml) for CYP1A2. Further study regarding types of inhibition on CYP2B1/2B2 as well as effects of the extract on other human CYP isoforms should be performed.

Conclusion

In conclusion, subchronic effects of ECa233 on hepatic CYPs were investigated both in vivo and in vitro. In an in vivo study, both male and female rats were given orally with ECa233 at the doses of 10, 100 and 1,000 mg/kg/day for 90 days, compared to the corresponding sex-control group given distilled water in the same manner. The results showed that ECa233 did not cause any changes on total CYP contents and the activities of CYP1A1, 1A2, 2B1/2B2, and 2E1 and 3A in vivo. Likewise, no effects of the extract on CYP1A1, 2E1 and 3A were shown in the in vitro study using rat liver microsomes. Decrease of CYP2B1/2B2 and very slight effect on CYP1A2 activities by ECa233 were shown in the in vitro study and in vivo study with the IC50 of 523 µg/ml (BROD), 563 µg/ml (PROD) for CYP2B1/2B2 and IC50 of more than 1,000 µg/ml (2895 µg/ml) for CYP1A2. Further study regarding types of inhibition on CYP2B1/2B2 as well as effects of the extract on other human CYP isoforms should be performed.

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References


