CHAPTER V

DISCUSSION AND CONCLUSION

1. Inhibitory effects of ECa 233 on human CYP enzymes: *in vitro* study

This study primarily investigated inhibitory effects of the standardized extract of *C. asiatica* (ECa 233) on the major human drug-metabolizing CYPs, namely CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 by using recombinant human CYP in the *in vitro* study. This would partly provide preliminary information of ECa 233 in terms of drug-drug interaction potential when the extract was co-administered with other currently used medicines. No inhibitory effects on any CYPs if demonstrated, would rule out the potential of drug-drug interaction caused by that particular CYP inhibition. In the other hand, if positive result was exhibited and the inhibition was significant, the need for further *in vivo* drug-interaction study was recommended. In addition, if ECa 233 inhibited any CYPs investigated in this study, which were the isoforms that bioactivated many environmental toxicants, would provide preliminary information that ECa 233 may possess advantageous property to decrease risk of human from bioactivated toxicants.

Result from this study showed that ECa 233 demonstrated a concentration-related inhibitory effects on the activities of CYP2B6, CYP2C19 and CYP3A4 but not or very small effects on CYP1A2, CYP2C9 and CYP2D6 at the range concentrations of ECa 233 in the reaction mixture up to 1,000 μg/ml. Only CYP2E1, the study was performed using the concentrations of ECa 233 limited up to 250 μg/ml due to the limited solubility of ECa 233 in DMSO which could not exceed 0.1% in the reaction mixture that suggested by the supplier's protocol, the inhibitory effect of ECa 233 on CYP2E1 was not observed.

No inhibitory effect of ECa 233 on CYP1A2, CYP2C9, CYP2D6 and CYP2E1 ruled out the potential effect of the extract to interact with various currently used medicines that were metabolized by these CYP isoforms (see Table 3). These results were in some parts, consistent to the results previously reported by Kulthong (2007). In the study of Kulthong (2007), ECa 233 did not demonstrate any inhibitory effects on CYP1A2 and CYP2E1 in rats both *in vivo* and *in vitro* study using rat liver microsomes. Because *in vitro* metabolism data of CYP1A2, CYP2C9, CYP2D6 and

CYP2E1 were studied using human (not animal) recombinant CYPs, the only *in vitro* data were sufficient and no further *in vivo* studies were need (U.S. FDA, online).

Even though the inhibitory effects of ECa 233 on CYP2B6, CYP2C19 and CYP3A4 were found in this study, the effect seemed to be small for CYP2B6 and CYP2C19 if approximately compared to the known inhibitors. Table 14 showed the IC_{50} of ECa 233 on CYP2B6, CYP2C19 and CYP3A4 compared to the IC_{50} (with the same unit of μg/ml) of the known inhibitors. Inhibitory effect of ECa 233 was greater on CYP3A4 than on CYP2C19 and CYP2B6 as shown by the lower IC_{50}. Because CYP3A4 plays a major role in metabolism of many currently used medicines, effect of ECa 233 on this CYP isoform, despite not prominent, was most concern. Marketed drugs that are metabolized by CYP3A4, CYP2C19 and CYP2B6 were shown in Table 3. The preliminary information of inhibitory effect of ECa 233 on CYP3A4, CYP2C19 and CYP2B6 found in this study suggested that effect of ECa 233 on these CYP isoforms needed to be further investigated clinically *in vivo*.

Table 14 IC_{50} of ECa 233 on CYP2B6, CYP2C19 and CYP3A4 compared to the IC_{50} of the known inhibitors

CYP isoforms	IC_{50} of ECa 233 (μg/ml)	IC_{50} of known inhibitor (μg/ml)
CYP2B6	871.14	0.084 (Ticopidine; Turpeinen, 2005)
CYP2C19	365.18	0.029 (Miconazole; Mark and Larson, online)
CYP3A4	210.98	0.069 (Ketoconazole; Mark and Larson, online) 0.032 (Ketoconazole; Krippendorff, 2006)

Inhibitory effect of ECa 233 on CYP3A4, CYP2C19 and CYP2B6 were advantageous for ECa 233 in the aspect of a protection (or decrease risk) of human toxicity from xenobiotic bioactivation. Several xenobiotics that were metabolic bioactivated by these CYP isoforms were shown in Table 4.

The inhibitory effect of ECa 233 on CYP2B1/2B2 was also found in the *in vitro* study using the catalytic reaction of pentoxyresorufin and benzyloxyresorufin *O*-dealkylation with rat liver microsomes (Kulthong, 2007). In this study, IC_{50} of ECa 233 on CYP2B1/2B2 were 563 μg/ml and 523 μg/ml as using PROD and BROD

reactions, respectively. CYP2B1/2B2 in rats was demonstrated to be closely analogous to CYP2B6 in human (Soucek and Gut, 1992). However, the inhibitory effect of ECa 233 on CYP3A4 was not found in the study of Kulthong (2007). This was not surprised because the sensitivity of the screening kit used in this study was far higher than the assay of erythromycin *N*-demethylation *in vitro* which may not be sufficiently sensitive to detect the small inhibition.

In this study, recombinant human CYP enzymes were used in the *in vitro* inhibition study. Commercial screening kits comprised microsomes containing cDNA expressed human CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. CYP isozyme-specific substrates were used as following: BOMCC were used as substrates for CYP2B6, CYP2C9 and CYP3A4 whereas EOMCC were used as substrates for CYP1A2, CYP2C19, CYP2D6 and CYP2E1. The Vivid® substrates (BOMCC and EOMCC) were metabolized by the specific CYP450 enzyme into products that were highly fluorescent according to the supplier's protocol. Before using the protocol that suggested by the supplier for determination the inhibitory effect of ECa 233 on human CYPs, the assays of every CYP were verified by determination of IC_{50} of a particular known inhibitor for each CYP isoform. α-Naphthoflavone, sulfaphenazole, imipramine and ketoconazole were used as inhibitors for CYP1A2, CYP2C9, CYP2E1 and CYP3A4, respectively whereas miconazole was used as inhibitor for CYP2B6, CYP2C19 and CYP2D6 (Mark and Larson, online; Marks et al., 2002). Table 15 showed the IC_{50} of each known inhibitor in this study compared to the IC_{50} reported by other studies. IC_{50} of most known inhibitors were similar to IC_{50} reported by other studies, except IC_{50} of sulfaphenazole and miconazole on CYP2C9 and CYP2C19, respectively.

In conclusion, inhibitory effects of ECa 233 on the major human CYPs, including CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 using recombinant human CYP were investigated. The results showed that ECa 233 inhibited CYP3A4, CYP2C19 and CYP2B6 with an IC_{50} of 210.98 μg/ml, 365.18 μg/ml and 871.14 μg/ml, respectively. ECa 233 did not/or very slightly affected CYP1A2, CYP2D6 and CYP2C9. Inhibitory effect of ECa 233 on CYP2E1 was not observed at concentration up to 250 μg/ml. These findings provided preliminary information that ECa 233 possessed a potential effect to interact with other medicines that were metabolized by CYP3A4, CYP2C19 and CYP2B6. Whether or not this effect was clinically significant needed to be further studied. The mechanistic

inhibitory effects of ECa 233 on CYP3A4, CYP2C19 and CYP2B6 were needed to clarified.

Table 15 IC_{50} of each known inhibitor in this study compared to the IC_{50} reported by other studies

CYP isoforms	Inhibitor	IC_{50} (95% Confidence limits), (μM) this study	IC_{50} (μM) from other studies*
CYP1A2	α-Naphthoflavone	0.060 (0.019-0.152)	0.029
CYP2B6	Miconazole	0.805 (0.515-1.211)	-
CYP2C9	Sulfaphenazole	1.076 (0.735-1.747)	0.310
CYP2C19	Miconazole	0.068 (0.027-0.163)	0.069
CYP2D6	Miconazole	1.991 (1.709-2.313)	0.430
CYP2E1	Imipramine	345.930 (272.027-444.996)	380.000
CYP3A4	Ketoconazole	0.107 (0.075-0.145)	0.130

(*) IC_{50} from other studies of all CYP according to the data by Mark and Larson, online and Marks et al., (2002).

II. Effects of ECa 233 on phase II drug metabolizing enzymes

This study also investigated effects of ECa 233 on phase II drug metabolizing enzymes such as UDPGT, SULT, GST and NQOR. Modulation of phase II enzymes, unlike of phase I enzymes, did not mainly contribute to the metabolic-based drug-drug interaction. However, changes of phase II enzymes, in some circumstance, causes drug-drug interaction. Induction of phase II enzymes, which mostly detoxified xenobiotics and/or reactive metabolites, explained the protection of animal/human from toxicity of xenobiotics/their reactive metabolites.

In this study, ECa 233 was orally administered to thirty male and thirty female albino Wistar rats at the doses of 10, 100 and 1,000 mg/kg/day for 90 days. Ten male and ten female rats which were the control groups, were given water in the same manner as the treatment group. The dose of 10 mg/kg/day of ECa 233 was found to possess learning and memory enhancing effects in rats (unpublished data). This study was performed in both male and female rats and the duration of ECa 233 administation was 90 days, because the experiment was designed according to the

protocol suggested for subchronic toxicity testing by WHO guideline (WHO, 2001). Although design of the experiment was not mainly intended for the metabolism study, the information regarding the effects of ECa 233 on phase II drug-metabolizing enzymes in both sexes of rats and long-term use was more or less benefit in terms of drug-drug interaction and the possibility to decrease/increase risks to xenobiotic-induced toxicities, mutagenicities and/or carcinogenicities.

The results from this study demonstrated that all dosage regimens of ECa 233 did not significantly affect the activities of UDPGT, GST and NQOR as compared to the control groups in both male and female rats. No effects of ECa 233 on UDPGT, GST and NQOR activities indicated that no advantageous effects of these extracts in term of a potential decrease of toxicity, antimutagenetic and/or anticarcinogenic effect against many drugs and/or xenobiotics that were 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 form of parent compounds or their metabolites from phase I metabolism. Example of drugs/xenobiotics or their metabolites that are detoxified by UDPGT are morphine, cyproheptadine, *p*-nitrophenol and acetaminophen etc., (Zhang et al., 2007). Several major classes of GST play an important role in detoxification of electrophillic drugs and xenobiotics that are toxic metabolites from phase I metabolism. However, in some cases can actually bioactivate compound, for example, 1, 2-dibromoethane and sevoflurane (Zhang et al., 2007). Example of drugs/xenobiotics or their reactive metabolites that are detoxified by GST are anticancer agents (i.e. busulfan) and inhalation anesthetics (i.e. halothane and enflurane) etc. (Zhang et al., 2007). NQOR, a flavoprotein also play an important role in detoxification. Drugs/xenobiotics or their reactive metabolites that are detoxified by NQOR are menadione, benzo(*a*)pyrene-3,6-quinone, and 1,4-naphtholquinone etc. (Ernster, 1990).

Activity of SULT was significantly decreased in male rats treated with ECa 233 at all doses as compared to the control group, whereas the activity of this enzyme did not change in female rats at all dose of ECa 233 administration. Decrease of SULT activities in male rats indicated that the possibility of either increase or decrease risk of drugs and/or xenobiotics -induced toxicities, mutagenicities and/or carcinogenicities depending upon the fact that SULT plays role in the detoxification or bioactivation of those xenobiotics. Examples of drugs/xenobiotics that are

detoxified by SULT such as acetaminophen, hydroxyl-tamoxifen, phenol (Zhang et al., 2007), *p*-nitrophenol, 2-naphthol (Gibson and Skett, 2001). And the 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 (Moon et al., 2006). In this study, the inhibition of SULT was different between in male rats and female rats. This finding was probably due to the sex-variation of this enzyme. Sex difference of SULT activities have been previously reported in rats (Iwasaki et al., 1994; Surh et al., 1991).

To investigate inhibitory effects of ECa 233 on phase II drug metabolizing enzymes, selective substrates of individual enzymes were used. *p*-Nitrophenol, which was the substrate of UDPGT1A4 or UDPGT1A8/9 isoform (Zhang et al., 2007) was used to determine UDPGT activity. 2-Naphthol, which was the substrate of SULT1As isoform (Gibson and Skett, 2001) was used to determine SULT activity. 1-Chloro-2,4-dinitrobenzene (CDNB), which was the substrate of GST alpha, GST mu, GST pi and GST omega classes (Coleman, 2005; Zhang et al., 2007) was used to determine GST activity. 6-Dichlorophenol-indophenol (DCPIP), which was the substrate of NQOR (Ernster, 1960) 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 coefficient of determination (R^2) between the amount of microsomal protein/cytosolic protein and the absorbance of final product solution.

In conclusion, effects of ECa 233 on phase II drug metabolizing enzymes such as UDPGT, SULT, GST and NQOR were investigated. All doses (10, 100 and 1,000 mg/kg/day) of ECa 233 were orally administered to both male and female rats for 90 days. The results showed that all dosage regimens of ECa 233 did not show significant effects on the activities of UDPGT, GST and NQOR as compared to the control group in both male and female rats. The activity of SULT was significantly decreased in male rats treated with ECa 233 at all doses as compared to the control group, whereas the activity of this enzyme was not changed in female rats. This study provided the information of effects of ECa 233 on phase II drug-metabolizing enzymes in animals, thus interpretation of these data from animal to human should be further clarified. In addition, effects of ECa 233 on other phase II drug-metabolizing enzymes should be further determined.