SAFETY PROFILE OF SUBACUTE EXPOSURE TO *CURCUMA COMOSA* ETHANOLIC EXTRACT IN FEMALE RATS

Wanida Sukketsiri[1, 4], Laddawal Phivthong-ngam[2], Chaiyo Chaichantipyuth[1], Nuansri Niwattisaiwong[1, 5], Supatra Srichairat[3, 5], Somsong Lawanprasert[1, 5, *]

[1] Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, 10330, Thailand
[2] Faculty of Medicine, Srinakharinwirot University, Bangkok, 10110, Thailand
[3] Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, 10330, Thailand
[4] Present address: Faculty of Science, Prince of Songkla University, Songkhla, 90110, Thailand
[5] Present address: School of Pharmacy, Eastern Asia University, Pathumthani, 12110, Thailand

ABSTRACT:

Background: Rhizomes of *Curcuma comosa* Roxb. (Zingiberaceae) have been widely used traditionally to alleviate abnormal painful uterine symptoms. This study aims to investigate subacute toxicological effects of a *C. comosa* ethanolic extract (CCE) in female rats. Effects of the extract on the activities of hepatic cytochrome P450 (CYP) including CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A were also investigated.

Methods: Forty female Wistar rats were randomly divided into 4 groups of 10 rats each. The extract was given orally to female rats at doses of 0, 100, 250 and 500 mg/kg/day for 30 days. At the end of the treatment, blood samples were collected by heart puncture and were measured for hematology and blood chemistry parameters. Microsomes were prepared from livers for enzyme assays.

Results: CCE at all dosages used in this study had no effects on the parameters of hematology and blood chemistry. Only serum alkaline phosphatase and potassium levels were significantly increased in the animals receiving the extract at 500 mg/kg/day. In addition, CCE did not change hepatic total CYP content and the activities of CYP1A2, CYP2E1 and CYP3A. However the activities of CYP1A1 and CYP2B1/2B2 were significantly increased following administration of CCE at dosages of 250 and 500 mg/kg/day.

Conclusions: Results from this study indicated that CCE does not cause serious toxicities to many important rat organs/systems. In addition, this study provides information regarding the possibility of herb-drug interactions and the increased risks from any bioactivation reactions of the drugs or compounds that are metabolized or bioactivated *via* CYP1A1 and CYP2B1/2B2.

Keywords: *Curcuma comosa* Roxb.; Blood chemistry; Cytochrome P450; Hematology; Subacute toxicity

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INTRODUCTION

Herbal medicines are now being used more frequently as alternatives to or as supplements with other medicines. Also, many herbal plants are being used as food or dietary supplements. Herbal medicine may have beneficial effects for humans; however long-term use can possess some toxic effects. *Curcuma comosa* Roxb., in Thai called Waan Chak Mod Look, is a plant in the family Zingiberaceae. *C. comosa* has been shown to contain many interesting chemical constituents such as diarylheptanoids and acetophenones [1, 2]. Thai traditional medical practitioners have used its rhizome for the relief of postpartum uterine pain and for reducing any inflammation of the uterus. Many studies have revealed pharmacological effects of *C. comosa* that are related to estrogenic-like

* *Correspondence to: Somsong Lawanprasert*
E-mail: lsomsong@chula.ac.th

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activities [3, 4], hypolipidemic effect [5, 6], bone sparing effect [7, 8], anti-inflammatory activity [9, 10] and anti-oxidative effects [11, 12]. These observed pharmacological properties are due to the presence of active chemicals in *C. comosa*. Diarylheptanoid, (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol, a novel phytoestrogen isolated from *C. comosa* [1] has several pharmacological properties including estrogenic-like activity [3, 4] and anti-inflammatory effects [13].

Although there are several studies reporting pharmacological effects of *C. comosa*, the safety data of this plant is limited. A subchronic toxicity study of the ethanolic extract of *C. comosa* is available [14], while the safety data regarding herb-drug interaction are shown by the effect of *C. comosa* hexane and ethanolic extracts at the dosages of 250 and 500 mg/kg/day on phase I and phase II hepatic drug metabolizing enzymes in male rats [15, 16]. To elucidate more safety information of *C. comosa* in females who are the main target population due to the estrogenic application of this plant, this study aims to investigate the safety profile of *C. comosa* ethanolic extract (CCE) in female rats. Subacute toxic effects of CCE were assessed via effect of the extract on blood chemistry and hematology parameters. Herb-drug interaction potential of CCE was evaluated via the effect of the extract on the activities of hepatic cytochrome P450 (CYPs) including CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A.

MATERIALS AND METHODS

Preparation of CCE

C. comosa rhizomes were acquired from a traditional drug store in Bangkok. Identification was made by Associate Professor Chaiyo Chaichantipyuth, Ph.D., Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The rhizomes were washed, dried, and cut into pieces before drying at 60 °C for 12 hour then ground into a fine powder. The powder was extracted with 95% ethanol for 24 hours. The ethanolic fraction was dried under vacuum using a rotary evaporator. The extraction yield of the CCE was 14.5 % w/w. The CCE extract was analyzed by HPLC. Identification of the 1,7-diphenyl-4,6-heptadiene-3-ol was carried out by comparing the retention times to that of the standard compound.

Experimental animals

Forty female Wistar rats with body weights of between 180-250 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornprathom, Thailand. All animals were maintained under standard environmental conditions (12 h light-dark cycle, 25±2°C temperature, 50% humidity). Rats were allowed free access to food and drinking water. The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Approval no.13/2005). Forty rats were randomly divided into 4 groups as follow: rats in group I (control group) were orally given corn oil at 1 mL/kg/day, rats in group II-IV were orally given CCE at dosages of 100, 250 and 500 mg/kg/day, respectively for 30 days. At the end of the treatment, animals were fasted for 12 hours before being anesthetized. Blood samples were collected by heart puncture for analysis of hematology and blood chemistry parameters. Livers were used for microsomal preparation and analysis of CYP activities.

Preparation of microsomes and determination of hepatic microsomal total CYP contents and CYP activities

Microsomal suspensions were prepared according to the method described by Lake [17]. Liver microsomal protein concentrations were determined according to the method of Lowry et al. [18]. Hepatic microsomal total CYP contents were determined spectrophotometrically according to the method of Omura and Sato [19]. Rate of hepatic microsomal alkoxyresorufin *O*-dealkylation was determined according to the method of Burke and Mayer [20] and Lubet et al. [21]. Benzyloxyresorufin (BR) and Pentoxyresorufin (PR) were used as selective substrates for CYP2B1/CYP2B2. Ethoxyresorufin (ER) and Methoxyresorufin (MR) were used as selective substrates for CYP1A1 and CYP1A2, respectively. Catalytic activity of CYP2E1 was determined based on the rate of hepatic microsomal aniline *4*-hydroxylation, according to the method of Schenkman et al. [22]. Aniline hydrochloride was used as the selective substrate in this reaction. Rate of hepatic microsomal erythromycin *N*-demethylation was determined using the method of Nash et al. [23] and Friedli [24]. Erythromycin stearate was used as the selective substrate for CYP3A.

Statistical analysis

All the numerical data were presented as

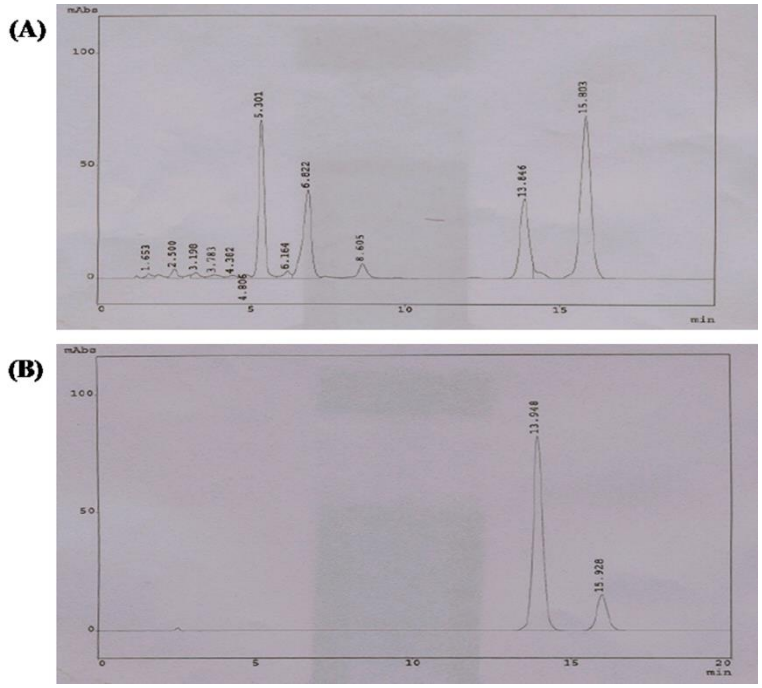

Figure 1 HPLC chromatogram of CCE. Twenty microlitres of solution of CCE in methanol were injected into the HPLC. The chromatogram of CCE (A) and the chromatogram of the reference standard, 1,7 diphenyl-4,6-heptadiene-3-ol (B) are shown with the presence of 1,7 diphenyl-4,6-heptadiene-3-ol at the retention time of 13.9 min.

Table 1 Effect of CCE on body weight, liver weight, % relative liver weight, food and water consumption

	Treatment group			
	Control	**CCE 100 mg/kg/day**	**CCE 250 mg/kg/day**	**CCE 500 mg/kg/day**
Initial body weight (g)	233.01±6.38	234.31±3.73	234.93±6.36	232.54±4.09
Final body weight (g)	237.68±4.18	233.78±4.01	234.79±5.66	234.20±6.21
Food intake (g/day)	13.53±1.14	10.36±1.21	11.03±0.88	10.34±1.04
Water intake (mL/day)	31.23±2.12	28.76±1.96	23.11±3.20	34.18±6.98
Liver weight (g)	7.50±0.36	8.59±0.52	10.67±0.73[#]	11.58±0.54[#]
% relative liver weight (g/100 g of body weight)	3.15±0.12	3.67±0.19	4.52±0.24[#]	4.95±0.24[#]

Data shown are mean ± SEM (n=10)
[#]$p<0.05$ as compared to the control group

mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) and Tukey's test were used for statistical comparison at a significance level of $p < 0.05$.

RESULTS

Chemical identification test

CCE was identified by HPLC on the basis of its UV-spectra and retention time in comparison with the reference standard, 1,7 diphenyl-4,6-heptadiene-3-ol (Figure 1). The total 1,7 diphenyl-4,6-heptadiene-3-ol in CCE used in the study was 23.6% w/w.

Effect of CCE on body weight, liver weight, % relative liver weight, food and water consumption

CCE at the doses of 100, 250 and 500 mg/kg/day given orally to female rats for 30 days did not cause any effects on body weight, food and water intake. Significant increases of liver weight and % relative liver weight were observed in rats given CCE at dosages of 250 and 500 mg/kg/day as compared to those of the control group (Table 1). All rats were alive at the end of the experiment and exhibited no apparent signs of toxicity.

Table 2 Effects of CCE on hematology

Hematology	Control	CCE 100 mg/kg/day	CCE 250 mg/kg/day	CCE 500 mg/kg/day
Hematocrit (%)	45.2±0.96	44.72±1.62	41.57±3.70	43.33±0.67
Hemoglobin (g/dl)	14.51±0.12	14.06±0.52	13.31±1.16	13.85±0.39
RBC count (10^6 cell/cumm)	7.64±0.12	7.85±0.30	7.35±0.64	7.52±0.07
MCV (fL)	59.23±1.52	53.3±1.74	56.1±0.68	56.93±1.38
MCH (pg)	19.1±0.29	17.9±0.22	18.09±0.13	18.15±0.44
MCHC (g/dl)	32.2±0.59	31.39±0.62	32.3±0.44	31.92±0.39
RBC morphology	Normal	Normal	Normal	Normal
Platelet count (10^3 cell/cumm)	830.3±39.24	935.12±58.96	915.29±85.80	940.83±42.58
WBC count (cell/cumm)	1179.2±282.94	1131.75±143.28	976.29±134.74	1718.5±589.23
PMN (%)	21.5±1.98	31.12±8.1	22.29±3.32	22.33±3.86
Lymphocyte (%)	74.1±2.07	65.62±8.34	74.43±3.40	71.17±3.75
Monocyte (%)	3.2±0.47	2.5±0.38	2.71±0.36	3.67±0.8
Eosinophil (%)	1.1±0.35	0.75±0.41	0.57±0.43	0.5±0.22

Data shown are mean ± SEM (n=10)

Table 3 Effects of CCE on blood chemistry parameters

Clinical blood chemistry	Control	CCE 100 mg/kg/day	CCE 250 mg/kg/day	CCE 500 mg/kg/day
AST (U/L)	187.78±15.38	186.62±21.03	194.0±20.03	209.0±16.88
ALT (U/L)	46.11±2.46	53.0±7.66	54.29±7.08	58.71±6.83
ALP (U/L)	67.11±6.26	92.62±8.15	88.71±11.31	156.29±29.67*
Total bilirubin (mg/dl)	0.1±0	0.1±0	0.13±0.02	0.21±0.06
Direct bilirubin (mg/dl)	0.02±0.01	0.0±0.0	0.06±0.03	0.13±0.06
Total protein (g/dl)	6.9±0.1	7.0±0.1	7.23±0.11	6.81±0.26
Albumin (g/dl)	3.73±0.1	3.9±0.09	4.2±0.06	3.57±0.26
Globulin (g/dl)	3.17±0.07	3.1±0.1	3.03±0.08	3.24±0.07
BUN (mg/dl)	22.9±1.23	30.5±4.83	31.43±2.62	30.37±4.33
SCr (mg/dl)	0.67±0.03	0.8±0.08	0.83±0.06	0.78±0.05
Glucose (mg/dl)	92.0±6.83	88.87±4.34	87.71±7.86	82.5±3.37
Total cholesterol (mg/dl)	55.9±3.96	51.0±2.28	52.0±2.24	66.57±8.41
TG (mg/dl)	58.4±4.08	65.12±3.01	71.0±8.32	67.37±10.63
HDL-C (mg/dl)	50.33±4.17	38.75±4.94	40.14±6.39	52.5±5.35
LDL-C (mg/dl)	2.44±0.44	2.12±0.29	2.57±0.3	4.0±0.60
Sodium (mEq/L)	148.44±1.57	154.37±3.78	150.29±2.71	150.29±4.09
Potassium (mEq/L)	4.37±0.14	4.57±0.27	4.64±0.16	5.40±0.28*
Calcium (mg/dl)	10.1±0.12	10.32±0.19	10.06±0.20	10.39±0.16
Chlorine (mEq/L)	110.44±0.96	112.87±3.00	107.71±2.67	109.86±2.94

Data shown are mean ± SEM (n=10)

*p<0.01 as compared to the control group

Effects of CCE on the parameters of hematology and blood chemistry

Subacute exposure (30 days) of CCE at doses of 100, 250 and 500 mg/kg/day in female rats did not cause abnormal changes on most of the hematology and blood chemistry parameters (Table 2). Serum alkaline phosphatase (ALP) and potassium levels were significantly increased in rats receiving 500 mg/kg/day of CCE as compared to the control group (Table 3).

Effects of CCE on hepatic microsomal total CYP contents and CYP activities

CCE of all 3 experimental doses did not cause significant changes of the total CYP contents as well as the activities of CYP1A2, 2E1 and 3A (Table 4). CYP1A1 activity which is represented by the rate of ethoxyresorufin *O*-dealkylation (EROD) was significantly increased by administration of CCE at the dosages of 250 and 500 mg/kg/day (Figure 2A). Similarly, rates of both benzyloxy- and

Table 4 Effects of CCE on hepatic microsomal total CYP contents and CYP activities

CYP activity	Control group	CCE 100 mg/kg/day	CCE 250 mg/kg/day	CCE 500 mg/kg/day
Total CYP contents (nmole/mg protein)	0.39±0.03	0.38±0.03	0.37±0.02	0.44±0.03
CYP1A2 activity (pmole/mg protein/min)	8.30±0.88	7.90±1.08	9.40±2.09	7.44±1.18
CYP2E1 activity (pmole/mg protein/min)	0.04±0.01	0.04±0.01	0.04±0.01	0.03±0.01
CYP3A activity (nmole/mg protein/min)	0.99±0.07	0.91±0.07	0.79±0.07	0.82±0.09

Data shown are mean ± SEM (n=10)

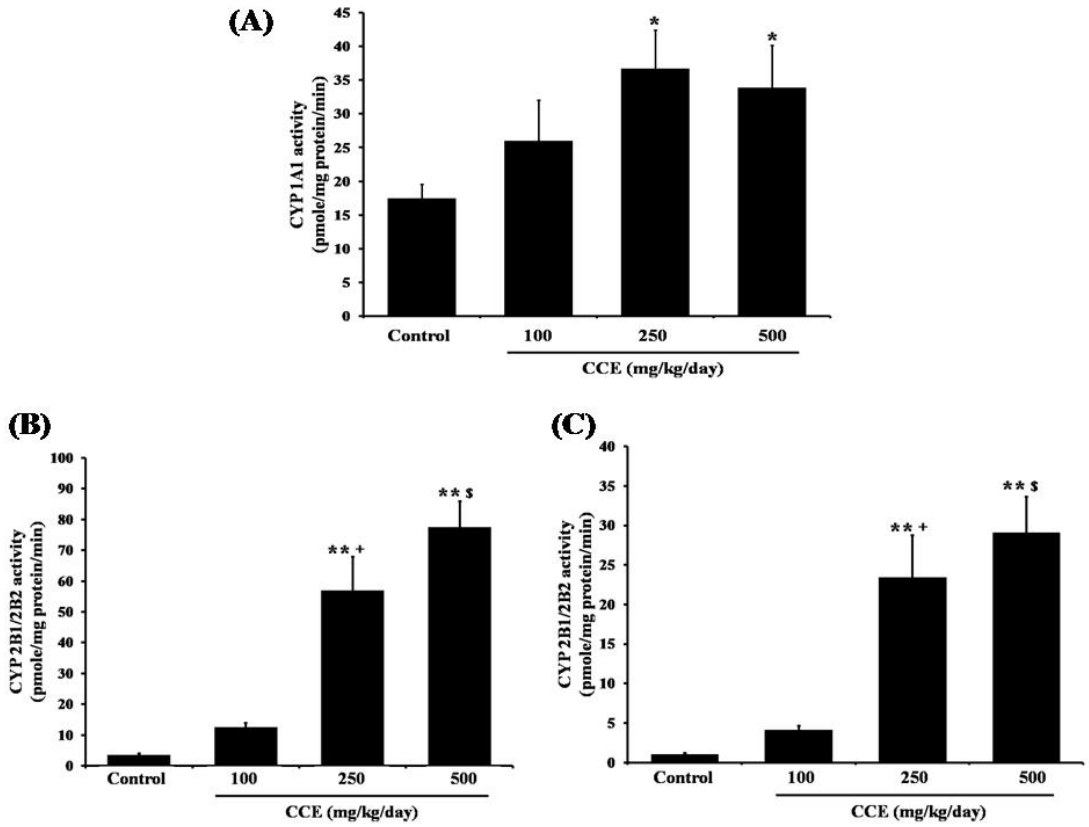

Figure 2 Effects of CCE on hepatic CYP1A1 and CYP 2B1/2B2 activities. Rats administered orally with 1 mL/kg/day of corn oil (control group), or 100, 250 and 500 mg/kg/day of CCE, respectively for 30 days. Liver microsomes were assayed for EROD (A), BROD (B) and PROD (C) activities. The individual bar graph is mean of the activity showing with the error bar of SEM (n=10). *p<0.01 as compared to the control group, **p<0.001 as compared to the control group, ^{+}p<0.001 CCE 250 mg/kg/day vs CCE 100 mg/kg/day, $^{\$}p$<0.001 CCE 500 mg/kg/day vs CCE 100 mg/kg/day

pentoxyresorufin *O*-dealkylation (BROD and PROD, respectively), which represented the activities of CYP2B1/2B2, were significantly increased by CCE administration at the dosages of 250 and 500 mg/kg/day and the increase seemed to be dose-related (Figure 2B and 2C).

DISCUSSION

Results from this study showed that all three doses of CCE did not affect body weight, food and water consumptions. In contrast, an increase of liver weight or % relative liver weight was observed in rats receiving the extract at 250 and 500 mg/kg/day as compared to the control group. The explanation for this increment might be find out if histopathological of the liver was explored whether it is due to an accumulation of fat in the liver or other causes. An induction of hepatic drug metabolism enzymes [25], an increased blood flow to the liver and an inflammation of the liver associated with the accumulation of fat in the liver [26], etc., can be a contribution cause of this increase of liver weight. Our results also revealed that CCE given orally at 100, 250 and 500 mg/kg/day did not cause any toxic effects to the hematopoietic system and many important organs such as liver, kidneys as well as

most serum electrolytes, carbohydrate and lipid metabolism. Only serum ALP and potassium levels were significantly increased in rats receiving the 500 mg/kg/day dosage of CCE as compared to those of the control group. The significantly higher potassium level (mean ± SEM of 5.40 ± 0.28 mEq/L) was within the normal range (5.40-7.00 mEq/L) [27]. However, the significantly higher of ALP (mean ± SEM of 156.29 ± 29.67 U/L) in rats receiving CCE at 500 mg/kg/day was higher than the normal range (56.80-128.00 U/L) [27]. The subacute toxicity data was quite comparable to a previous study done by Chivapat, et al. [14], on the investigation of the subchronic toxicity of *C. comosa* ethanolic extract by giving oral administration of 100, 200, 400 and 800 mg/kg/day doses to male and female rats for 90 days and found that male rats treated with 800 mg/kg/day and female rats receiving 400 and 800 mg/kg/day doses demonstrated a significant increase of ALP level. However, they also reported that the significantly higher ALP level was within the normal ranges. Normally, an increase of serum ALP indicates an injury of bile duct epithelium which can lead to liver cholestasis [28]. Therefore, these two findings of an increase of serum ALP by CCE indicated that hepatic biliary function may be affected if this extract is administered repeatedly at high dose.

In this study, *C. comosa* was extracted with 95% ethanol, the fraction which was shown to possess many pharmacological effects. Moreover, ethanolic extract was closely similar to the way of using this plant traditionally. Before using this extract in the experiment, the extract was identified via a chemical identification. While, the CCE was determined for 1, 7 diphenyl-4,6-heptadiene-3-ol which was the active ingredient found in the extract using HPLC. The lowest dose (of 100 mg/kg/day) shown to decrease plasma triglyceride level and increased liver triglyceride content [6], whereas the dosage of 250 mg/kg/day was shown to possess uterotrophic effect [29]. The highest dose (of 500 mg/kg/day) shown to possess uterotrophic effect and estrogenic-like action [6, 29, 30] and also possess antihypercholesterolemic effect [6] as well as increase bile flow rate [5]. Effects of *C.comosa* extracts on phase I (CYP enzymes) and phase II enzymes were also assessed in male rats at the dosages of 250 and 500 mg/kg/day [15, 16]. In this study, we treated rats for 30 days, the duration which covered many cycles of rat estrous cycle (about 6-7 estrous cycles). This is because rats are normally become puberty at 50±10 days of age and the length of estrous cycle was 4-5 days [27]. Body weights of rats used during the experiment in this study were between 180-250 g (approximately 8 weeks) when the animals were already had estrous cycle. Outbreed rats in the same lot of animals were used by sampling to each group (control group and treated groups) which were maintained in the same condition such as environmental handling and housing during the experiment. These conditions are designed to reduce bias due to estrous cycles of the rats as well as the environmental conditions.

To investigate effects of CCE on hepatic CYPs, selective substrate of the individual CYP was used and rate of selective substrate oxidation was determined to represent the corresponding CYP activity in hepatic microsomes of rats treated with the extract. The ER, MR and PR&BR have been proved to be selective substrates of CYP 1A1 [20], CYP 1A2 [31] and CYP 2B1&2B2 [21, 31], respectively. Aniline *4*-hydroxylation was shown to represent CYP 2E1 activity [22] while erythromycin *N*-demethylation was classically used for determining CYP 3A activity [23, 24]. Results from this study showed that these three dosages of CCE did not changes hepatic microsomal total CYPs contents as well as the activities of CYP1A2, CYP2E1 and CYP3A in female rats whereas increases of CYP1A1 and CYP2B1/2B2 activities were shown in the groups of 250 and 500 mg/kg/day doses. Effects of CCE on the activities of these CYPs in female rats found in this study are consistent to the results found in male rats [15] regardless of the sex-difference that may influence the expression of drug metabolizing enzymes in the liver [32]. No effects of CCE on CYP1A2, CYP2E1 and CYP3A would be an advantageous characteristic of this extract in term of herb-drug interactions as well as risks to xenobiotic-induced toxicities, mutagenesis, and/or carcinogenesis. Meanwhile, no effects of this extract on these CYPs excluded the possibilities of herb-drug interactions if CCE is consumed concomitantly with any medicines that are metabolized by these CYPs. Examples of such therapeutic drugs that are metabolized by CYP 1A2 are paracetamol, theophylline; paracetamol and chlorzoxazone for CYP 2E1; and clarithromycin, carbamazepine, erythromycin, for CYP 3A, [33]. No induction effects on these isoform of CYPs ruled out the potential increased risks of the extract on xenobiotic-induced toxicities, mutagenesis, and/or

carcinogenesis. Examples of xenobiotics which are bioactivated by the individual CYPs isoform are as following: 2-aminofluorene and aflatoxin B1 are activated by CYP 1A2; *N,N'*-nitrosodimethyllamine and acetaminophen are activated by CYP 2E1; aflatoxin B1 and benzo(a)pyrene are activated by CYP 3A [34].

Results from this study showed an increase activity of CYP1A1 and CYP2B1/2B2 by CCE administration at the dosages of 250 and 500 mg/kg/day. Induction effects of this extract on CYP1A1 and CYP2B1/2B2 indicated the possibilities of herb-drug interaction if CCE is consumed concomitantly with other medicines that are metabolized by these CYP isoforms. Examples of therapeutic drugs that are metabolized by CYP1A1 is warfarin and phenobarbital and cyclophosphamide for CYP 2B1/2B2 [35]. Induction effects of this extract on these isoforms of CYPs indicated potential increased risks of the extract on xenobiotic-induced toxicities, mutagenesis, and/or carcinogenesis. Examples of xenobiotics bioactivated by CYP1A1 and CYP 2B1/2B2 are benzo(a)pyrene and aflatoxin B1 [34]. Actually, CYP2B1/2B2 is not presented in human. Comparing the similarity of cDNA and sequences of the protein enzyme, rat CYP 2B1 is closely analogous to human CYP 2B6 [34, 36] which is expressed at very low level in human liver, approximately 0.2 % of total CYP [37]. CYP 2B6 is reported to play a role in the bioactivation reactions of 6-aminochrysene [38] and the metabolisms of bupropion, cyclophosphamide, efavirenz [37]. Thus, induction of CYP2B1/2B2 by CCE administration in rat may affect the metabolism or bioactivation of xenobiotics that are metabolized or bioactivated by CYP2B6 in human. Further study is suggested to explore the effect of this extract on other CYP isoforms that had not been assessed in this study.

CONCLUSION

Subacute exposure to CCE did not cause any serious toxicity on many important organs/systems such as the liver, kidney, hematopoietic, electrolytes, as well as the carbohydrate and lipid metabolism. CCE did not affect hepatic microsomal total CYP contents and the activities of CYP1A2, CYP2E1 and CYP3A but significantly increased CYP1A1 and CYP2B1/2B2 activities in female rats. This information provides the possibility of herb-drug interactions and the increased risks from any bioactivation reactions of the drugs or compounds that are bioactivated *via* CYP1A1 and CYP2B1/2B2.

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