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Original Article

Effects of *Kaempferia galanga* L. and ethyl-*p*-methoxycinnamate (EPMC) on hepatic microsomal cytochrome P450s enzyme activities in mice

Wanna Sirisangtragul[1], and Bungorn Sripanidkulchai [1,2]*

[1] *Faculty of Pharmaceutical Science,*

[2] *Center for Research and Development of Herbal Health Products, Khon Kaen University, Mueang, Khon Kaen, 40002 Thailand.*

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Abstract

Kaempferia galanga L. (*K. galanga* L.), belonging to the Zingiberaceae family, has been widely used in Thai traditional medicine for treatment of hypertension, asthma, rheumatism, indigestion, cold and headache and relief abdominal pain. This study aimed to investigate the effect of dichloromethane extract of *K. galanga* L. (DEK) and its major component ethyl-*p*-methoxycinnamate (EPMC) on hepatic microsomal cytochrome P450s enzyme activities in mice. After oral administration with DEK (100 mg/kg which is equivalent to EPMC 80 mg/kg), EPMC (120 and 160 mg/kg) for 28 consecutive days, the microsomal P450 content decreased. The CYP1A1 and CYP2B activities significantly increased, whereas CYP2E1 activity was significant inhibited only at the highest concentration of EPMC (160 mg/kg). None of the treatments did affect CYP1A2 and CYP3A4 activity when compared to the control group. The results indicated that *K. galanga* L. and its active compound, EPMC may participate in herbal-drug interaction and may also increase risk of toxicity and chemical carcinogenesis from drugs and compounds that are metabolized via CYP1A1, CYP2B and CYP2E1.

Keywords: *Kaempferia galanga* L., ethyl-*p*-methoxycinnamate, cytochrome P450, CYP, microsome, herbal-drug interaction

1. Introduction

Kaempferia galanga L. (*K. galanga* L.) (Proh Hom in Thai name) is a plant belonging to the Zingiberaceae family. This plant has been found in South India and Southeast Asia such as Malaysia, Indonesia, and Thailand. As folk medicine, the rhizome of *K. galanga* L. is employed for antibacterial, treatment of hypertension, asthma, rheumatism, indigestion, cold and headache, relief abdominal pain and toothache (Mustafa *et al*., 1996; Kanjanapothi *et al*., 2004; Ridtitid *et al*., 2008). The volatile oil obtained from water distillation of this plant had activity against gram-positive and gram-negative bacteria, and fungi (Tewtrakul *et al*., 2005). The plant methylene chloride extract possessed amebicidal activity (Chu *et al*., 1998). Aroonrerk and Kamkaen (2009) reported the anti-inflammatory effect by inhibition of IL-6 production by this plant. Moreover, other pharmacological actions such as smooth muscle relaxant and vasorelaxant effects were also reported (Mustafa *et al*., 1996; Othman *et al*., 2006). According to the high content of essential oil in the dried rhizome, phytochemical studies revealed several constituents, including the major component; ethyl-*p*-methoxycinnamate (EPMC), ethyl cinnamate, *p*-methoxystyrene, borneol, camphene, carene, *p*-methoxycinnamic acid, kaempferal and 3-carene-5-one, carvone, methylcinnamate and eucalyptol (Kiuchi *et al*., 1987; Tewtrakul *et al*., 2005). The ethyl-*p*-methoxycinnamate obtained from methanolic extract of *K. galanga* L. was highly cytotoxic to Hela cells (Kosuge *et al*., 1985). Moreover, EPMC showed relatively strong anti-carcinogenic potential in *in-vitro* study (Xue and Chen, 2002) and it also has activi-

* Corresponding author.
Email address: bungorn@kku.ac.th

ties against *Candida albicans*, and *Mycobacterium tuberculosis* (Yenjai *et al*., 2003).

Cytochrome P450 (CYP) plays a critical role in biotransformation of a wide variety of xenobiotic substances such as therapeutic drug, carcinogen and toxicant (Omiecinski *et al*., 1999). In human, three CYP families, CYP1, CYP2, and CYP3 are the most abundant of hepatic microsome CYPs and they are active in the metabolism of several xenobiotics (Zhou *et al*., 2003). In CYP1 family, both CYP1A1 and CYP1A2 subfamilies are involved in the metabolism of many planar aromatic drugs, and are highly active in oxidation of carcinogens (Yan and Caldwell, 2001; Guengerich, 1995). The CYP2 family contains several subfamilies including CYP2A, CYP2B, CYP2C, CYP2D and CYP2E. Among them, the expression of CYP2B6 is rather low compared with other human P450s, but it is the most important CYPs, regarding to the number of metabolic pathways of drugs and xenobiotics catalysis. It has been shown to be responsible for metabolizing over 30% of market drugs. (Zanger and Erichelbaum, 2000; Yan and Caldwell, 2001). Their role has been strongly implicated in the metabolism of chemotherapeutic drugs such as cyclophosphamide (Parkinson, 1996).

For the CYP 3A family, CYP 3A4 is the predominant P450 enzyme that is responsible for approximately 50% of P450-mediated metabolism of drugs in therapeutic use today, implying that this enzyme is important with respect to the action, duration and disposition of drugs and their metabolites (Yan and Caldwell, 2001).

Induction and/or inhibition of CYPs by herbal medicines is the important current evidence for herbal-drug interaction. The interaction can cause activation or inactivation of therapeutic agents (Delgoda and Westlake, 2004). Thus, it is important to identify the potential of herbal-drug interaction in order to prevent adverse effect outcome in patients who take drugs in combination with herbal supplement. Moreover, changes in some cytochrome P450 activity are involved in chemical carcinogenesis such as CYP1A1, CYP1A2 and CYP2E1 (Mckinnon and Miners, 2000; Carpenter and Raucy, 2000). Induction of these CYPs by herbs may increase the risk of carcinogenesis. On the other hand, inhibition of CYPs activity may represent anti-carcinogenic effect of herb. Since *K. galanga* L. has been traditionally used as a remedy herb, it may be involved in bioactivation and/or detoxication of carcinogen. Thus the effects of *K. galanga* L. and its major constituent, EPMC, on cytochrome P450 isoforms including CYP1A1, CYP1A2, CYP2B, CYP2E1 and CYP3A4 were examined in this study.

2. Materials and Methods

2.1 Chemicals

Pregnenolone 16α-carbonitrile (PCN), potassium chloride, tris(hydroxymethyl)-aminomethane, reduced nicotinamide adenine dinucleotide phosphate (NADPH), ethoxyresorufin (ER), methoxyresorufin (MR), penthoxyresorufin (PR), standard resorufin, *p*-nitrophenol, 4-nitrocatechol (4NC), testosterone, 6-b-hydroxytestosterone, and dimethylsulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-methylchloranthene (3-MC) was supplied by Eastman (Rocchester, USA). Phenobarbital (PB) was a product of Avantis (USA). Pentobarbital was provided by Abbott S.p.a, Campoverde, Lt (Italy). Potassium chloride, glycerol, acetronitrile, ethanol, methanol and trifluoroacetic acid were supplied by BDH (England). Silica gel for column chromatography and silica gel, GF 254 for thin layer chromatography (TLC) were purchased from Merck (Germany). All other chemicals were analytical grade obtained from the local distributor. The standard EPMC was kindly provided by Assoc. Prof. Dr Chavi Yenjai, Faculty of Science, Khon Kaen University, Thailand.

2.2 Plant material

The dried rhizome powder of *K. galanga* L. was obtained from the local drug store in Khon Kaen province, Thailand (Identified by Assoc. Prof. Dr Bungorn Sripanidkulchai). A voucher specimen was kept in our laboratory (HHP-07K).

2.3 Extraction of *Kaempferia galanga* L. and purification of the major compound, EPMC

The dried rhizome powder of *K. galanga* L. was macerated with dichloromethane (1 kg/ 2.5 liters) for 7 days. After filtration through a Whatman No.1 filter paper, the solvent was evaporated in a rotary evaporator (Eyela, SB-1000, Japan). The brown jelly-like dichloromethane extract of *K. galanga* L. (DEK) was kept at 4°C until used. To obtain the major compound, ethyl-p-methoxycinnamate (EPMC), the DEK was applied to siliga gel column chromatography (70-230 mesh) and eluted with a gradient of dichloromethane-hexane (70:30 to 95:5). The eluted fractions were collected and examined by TLC (silica gel, GF 254) with dichloromethane-hexane (95:5) as a developing solvent. The fractions containing EPMC were pooled and dried. The purity of EPMC was checked by using HPLC method as follows; HPLC (Agilent 1100 series and UV-VWD, Agilent Japan), using an isocratic solvent, acetronitrile: methanol: 20 mM NaH_2PO_4 (30:40:30 v/v/v) at a flow rate of 1 ml/min, with a ThermoHypersyl-Keystone ODS HYPERSYL; 5 µm, 4.6x250 mm (Agilent, Germany) and guard column, µBondpack 10 µm C18 (Water, U.S.A.). The UV detection was at 270 nm. DEK gave a similar major peak which contained 80 mg% of EPMC (Figure 1).

2.4 Experimental animals

ICR male mice at 8 weeks of age were obtained from the National Laboratory Animal Centre, Salaya Mahidol University, Nakorn Pathom, Thailand. The animals were maintained at 25±3°C with 12 h of dark-light cycle, and provided pellet diet with *ad libitum* water. All experiments

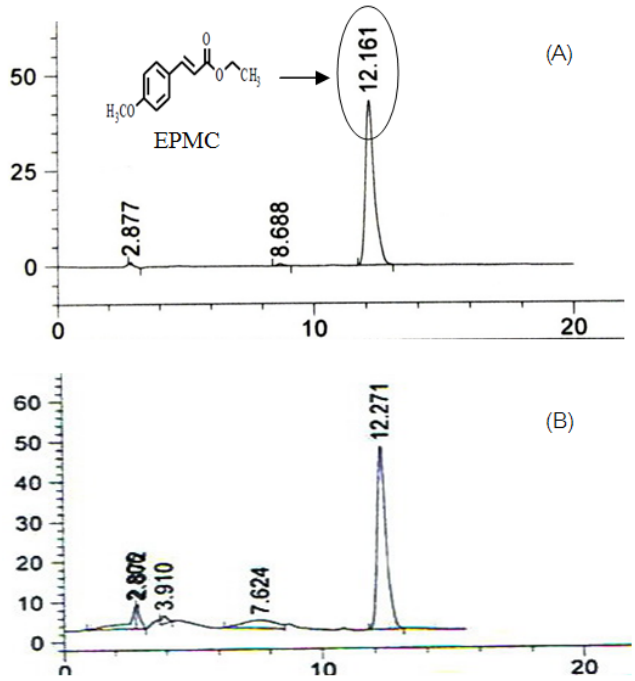

Figure 1. HPLC Chromatograms of standard EPMC (A) and DEK which contained 80 mg% of EPMC (B) using mobile phase containing acetronitrile: methanol: 20mM NaH_2PO_4 (30:40:30) and detection at 270 nm.

were conducted under the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publication No.80-23).

Mice were randomly divided into 8 groups (N= 5) and treated as follows: Group 1 (negative control group) was orally administered with olive oil at 100 µl daily. Groups 2-5 were treated with specific positive inducer of each CYP (Jarukamjorn *et al*., 2006), Group 2 (positive control for CYP1A1 and CYP1A2 induction) was subcutaneously administered with a typical CYP inducer, 3-methylchloranthene (3-MC) at a daily dose of 100 mg/kg for 5 days, Group 3 (positive control for CYP2B induction) was intraperitoneally injected with phenobarbital (PB) at a daily dose of 100 mg/kg for 5 days. Group 4 (positive control for CYP3A4 induction) was subcutaneously administered with pregnenolone 16α-carbonitrile (PCN) at a daily dose of 50 mg/kg for 5 days and Group 5 (positive control for CYP2E1 induction) was orally administered with 10% ethanol (EtOH) in drinking water for 14 days (Forkert *et al*., 1991). Group 6 (DEK-treated groups) was orally administered with DEK at a daily dose of 100 mg/kg (equivalent to 80 mg/kg of EPMC) for 4 weeks. Groups 7-8 (EPMC-treated groups) were orally administered with EPMC at a daily dose of 120 and 160 mg/kg for 4 weeks, respectively. The animals were observed for behavioral changes over the experimental period. At 24 h after the last of treatment, mice were sacrificed by decapitation. After perfusion with 1.15% ice-cold KCl until the liver became pale yellow, it was quickly removed and immediately frozen in liquid nitrogen and stored at -80°C for microsomal preparation.

2.5 Preparation of hepatic microsome

Livers were thawed and minced, and then homogenized in 3 volumes of ice-cold 1.15% (W/V) KCl in 0.1 M potassium phosphate buffer pH 7.4 by a motor-driven Teflon pestle in a glass homogenizing vessel placed in an ice bath. The crude homogenate was centrifuged at 9,000 g, 4°C for 20 minutes. The free lipid layer of supernatant was then centrifuged at 100,000 g, 4°C for 1 hr. The microsomal fraction was obtained by suspension of the sediment with 0.1 M of potassium phosphate pH 7.4 containing 20% glycerol (v/v), 1 mM EDTA and 0.1 mM dithiotrietol. The microsomal protein concentration was determined under the instruction of protein assay (BioRad laboratory) with bovine serum albumin as a standard. The total amount of protein in the final volume was 10-20 mg/ml. Aliquots of the microsomal fraction were kept at -80°C until further analysis.

2.6 Assessment of total P450 content and CYPs activities

The total P450 content was determined as described by Omura and Sato (1964). CYP1A1, CYP1A2, and CYP2B activities were determined as described by Sakuma *et al.* (1999), and Jarukamjorn *et al* (1999) using alkoxyresorufin *O*-dealkylation (AROD) activities assay. Ethoxyresorufin *O*-dealkylase (EROD), methoxyresorufin *O*-dealkylase (MROD), and penthoxyresorufin *O*-dealkylase (PROD) activities were performed for CYP1A1, CYP1A2, and CYP2B enzymes, respectively. CYP2E1 activity was determined by measurement of *p*-nitrophenol hydroxylase activity. The end product, 4-nitrocatechol was separated using reverse phase-HPLC and monitoring at wavelength of 345 nm (Elbrabry *et al.*, 2006, Mischin e*t al*., 1996). The CYP3A4 activity was examined using testosterone as a selective marker. Testosterone hydroxylation and its metabolite, 6β-hydroxytestosterone, were determined with reverse phase-HPLC and monitoring at wavelength of 254 nm (Baltes *et al.*, 1998). For each determination, the number of samples is specified in each figure.

2.7 Statistical analysis

All data were expressed as mean ± S.E. Statistical analysis was performed by the analysis of variance (ANOVA) followed by Student's *t-test*, which was set at *P*-value lower than 0.05.

3. Results

The effects of oral administration of dichloromethane extract of *K. galanga* L. (DEK) (100 mg/kg, which is equivalent to 80 mg/kg of EPMC) and ethyl-p-methoxycinnamate (EPMC) (120 and 160 mg/kg) on body and organ weights were observed. There was no animal death during the treat-

ment period in any studied group. Neither DEK nor any dose of EPMC caused any detectable changes in behavior activities. There were no changes in body, liver or kidney weights (data not shown). The internal organs of all animals showed no signs of abnormality in appearance to the naked eye. These results indicated that oral administration of DEK (100 mg/kg) and EPMC (120, 160 mg/kg) did not produce toxicity.

The treatments with DEK (100 mg/kg) and EPMC (120 and 160 mg/kg) for 28 days had significantly reduced total microsomal P450 content. In contrast, the administrations of PB and PCN, which are specific CYP2B and CYP3A4 inducers, markedly increased total microsomal P450 content up to 3.2- and 2.3-fold, respectively. Whereas the treatment of 3-MC, a specific CYP1A1 and CYP1A2 inducer, and ethanol, a specific CYP2E1 inducer, did not affect total microsomal P450 content (Figure 2A).

Using ethoxyresorufin O-dealkylation (EROD as an activity-probe for selective measurement of CYP1A1 iso-

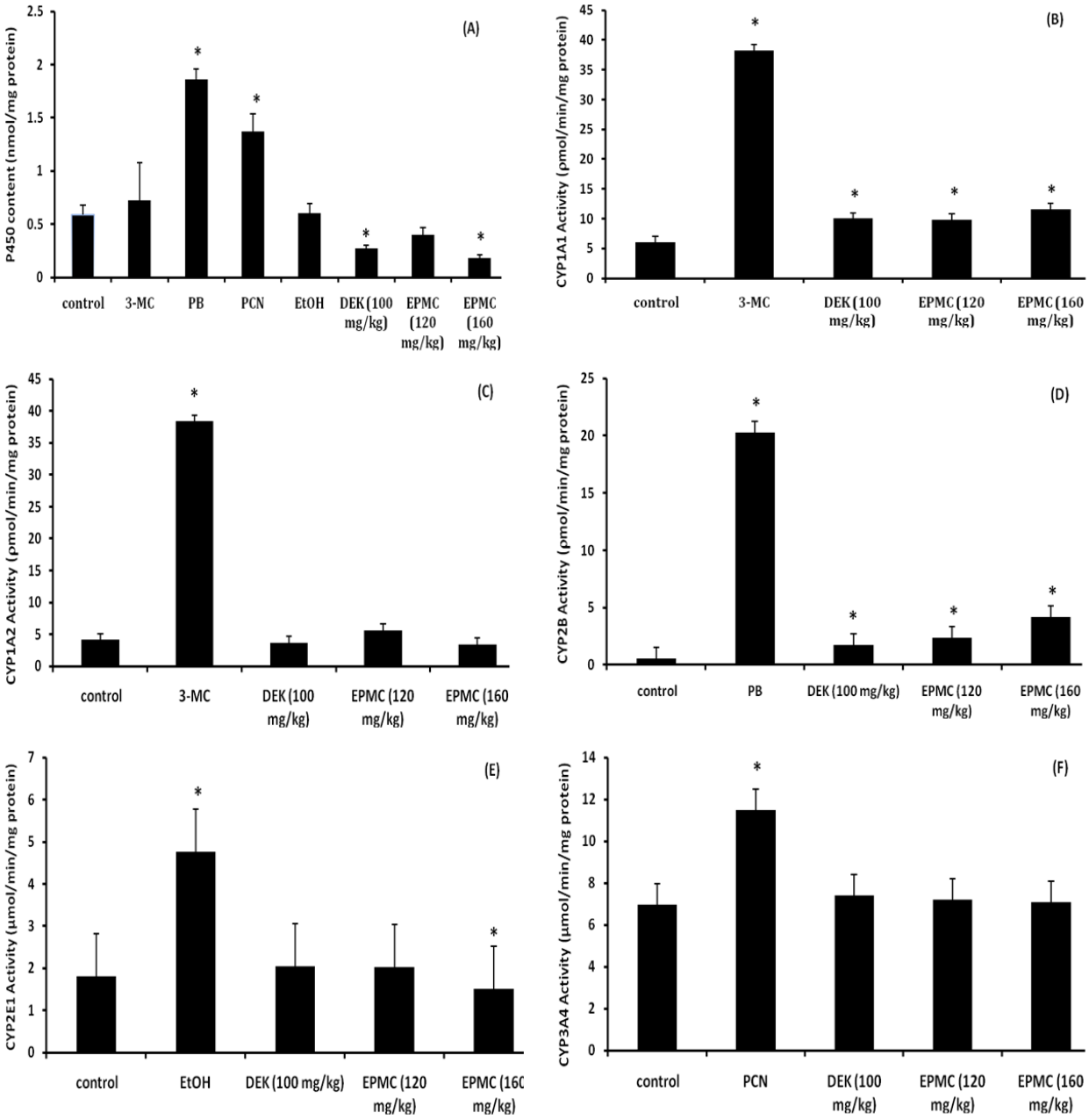

Figure 2. Effects of dichloromethane extract of *K. galanga* L. (DEK) and EPMC on total P450s content (A) and hepatic cytochrome P450, CYP1A1 (B), CYP1A2 (C), CYP2B (D), CYP2E1 (E) and CYP3A4 (F) activities in mice. Values are expressed as mean ± SD (n = 5). Columns represent control, 3MC: 3-methylchloranthrene (100mg/kg), PB: Phenobarbital (100mg/kg), PCN: Pregnenolone 16α-carbonitrile (50mg/kg), EtOH: Ethanol (10%), DEK (100 mg/kg) and EPMC (120 and 160 mg/kg).
* Significant differences when compared to control group at $P<0.05$.

forms, both DEK and EPMC significantly increased CYP1A1 activities in mice (Figure 2B). In contrast, the methoxyresorufin *O*-dealkylation (MROD) that represented CYP1A2 activities of the DEK and EPMC treated groups did not change (Figure 2C). For positive control groups, a specific inducer, 3-MC, administration markedly increased CYP1A1 and CYP1A2 activities up to 6.3- and 9.3- fold, respectively. Both DEK and EPMC significantly increased the penthoxyresorufin *O*-dealkylase (PROD) reaction, that was a specific probe for CYP2B. The CYP2B activities were significantly elevated to 3.18-, 4.22- and 7.66- fold in DEK (100 mg/kg) and EPMC (120 and 160 mg/kg) treated groups (Figure 2D). For CYP2E1 activities, DEK (100 mg/kg) and EPMC (120 mg/kg) did not cause significant difference of the *p*-nitrophenol hydroxylase activity (Figure 2E). However, when the concentration of EPMC was increased to 160 mg/kg, CYP2E1 activity reduced, whereas the CYP3A4 activities of all treated groups did not change (Figure 2F). Positive control groups, PB, EtOH, and PCN significantly elevated CYP2B, CYP2E1 and CYP3A4 activities to 38-, 2.6-, and 1.6-fold, respectively.

4. Discussion

Regarding the high content of volatile oil in the rhizome, the non-polar dichloromethane extract (DEK) and the pharmacologically active compound, EPMC, were used for the investigation of the potential of *K. galanga* L. for herbal-drug interaction. The results in the present study showed that both DEK and EPMC significantly affected P450 content and the hepatic microsomal CYP enzyme activities in mice.

DEK at 100 mg/kg and EPMC up to 160 mg/mg did not affect the body or organ weights of mice at 28 consecutive days of treatment. These results indicate the safety of the selected doses of *K. galanga* L. used in this study. Our results confirm the previous report on the safety exposures of ethanolic *K. galanga* L. extract in rat (Kanjanapothi *et al*., 2004). For CYP activities, CYP1A1 and CYP2B were significant increased in the DEK-treated group; likewise in the EPMC-treated group. These findings suggest that EPMC may contribute at least in part to the induction of CYP1A1 and CYP2B by *K. galanga* L. However, the effect of these extracts was lower than that of 3-MC and phenobarbital, which are specific inducers of CYP1A1 and CYP2B enzyme, respectively. These results are in agreement with the findings of previous studies that herbal remedy can interact with P450 activity, such as *Gingko biloba* (*G. biloba*) extract that markedly inducing CYP2B activity (Umegaki *et al*., 2002). The induction of CYP enzyme isoform which is responsible for the metabolism of a drug can reduce the drugs expected therapeutic capacity due to depletion of its plasma concentration. For example, *G. biloba* extract reduced the hypnotic potency of phenobarbital in rats, with the reduction of phenobarbital C_{max} and $AUC_{0\text{-}24}$ (Kubota *et al*., 2004). Baicalin, a flavone glucuronide extracted from *Radix scutellariae* induced the CYP2B6-catalyzed hydroxylation of bupropion in healthy male volunteers (Fan *et al*., 2009). Our result reveals the effect of DEK and EPMC on CYP1A1 and CYP2B activity. These findings may suggest a risk of interactions between *K. galanga* L. with conventional drugs. Induction of CYP1A1 and CYP2B activity can decrease drug plasma concentrations to sub-therapeutic levels leading to loss of pharmacological effect. Therefore, co-administration of *K. galanga* L. with drugs that metabolize via these CYP isoforms such as caffeine, phenacetin, warfarin, phenobarbital, cyclophosphamide and efavirence should be avoided. In addition, repeat exposure of this herbal remedy may raise the risk of chemical carcinogenesis from metabolic activation process via these CYP such as benzo(a) pyrene, 4-4(bis)methylenechloroaniline, or alfatoxin B_1.

However, inconsistencies between P450 content and CYPs activities were observed. A significant decrease of total hepatic P450 content in the present study reflects the changes of P450 content and these may not represent alteration of individual CYP isoform content. Moreover, the changes in each CYP activity suggest the specific induction and inhibition of DEK and EPMC treatment since CYP1A1 and CYP2B6 had rather low expression when compared to other human P450s (Zanger and Erichelbaum, 2000; Yan and Caldwell, 2001). The induction of these two CYPs by DEK and EPMC may not much contribute to the total P450s content. Furthermore, whether or not change in the other CYPs activities participate in the reduction of P450 content needs to be further investigated.

The findings that DEK or EPMC did not have modulatory effect on CYP3A4 and CYP1A2 activities in this study may suggest certain safety of this plant on herbal-drug interaction and human risk assessment on chemical carcinogenesis as more than 50% of conventional drugs are metabolized via these CYP isoforms. For example, theophylline, imipramine, propranolol are metabolized by CYP1A2 and cyclosporine, erythromycin, simvastatin are metabolized by CYP3A4 (Anzenbacher and Anzenbacherrova, 2001). Additionally, slight reduction of CYP2E1 activity may suggest slight anti-carcinogenic activity of EPMC. Regarding CYP2E1 is involved in metabolic activation of many low molecular weight toxins and carcinogen such as carbon tetrachloride, chloroform, methylchloride, vinylchloride (Anzenbacher and Anzenbacherrova, 2001). Inhibition of this enzyme activity may decrease risk of chemical-induced toxicity, mutagenesis, and carcinogenesis.

5. Conclusion

In conclusion, the results demonstrate induction of CYP1A1 and CYP2B by DEK and EPMC in mice, which indicate the possible role of *K. galanga* L. in herbal-drug interaction. However, to indicate a clinical significance, the potential interaction between DEK or EPMC and CYP1A1 or CYP2B substrates needs to be further investigated.

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