Effect of Ethyl-*p*-Methoxy Cinnamate from *Kaempferia galanga* on Cytochrome P450 Enzymes Expression in Mouse Hepatocytes

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ABSTRACT

Kaempferia galanga, a plant in the Zingiberaceae family, has been widely used as a spice, food flavoring and folk medicine. Its rhizome contains ethyl-p-methoxycinnamate (EPMC) as a major constituent. This study aimed to investigate the effect of EPMC on cytochrome P450 expression in mouse primary hepatocytes. EPMC was isolated from the dichloromethane extract of dried rhizome of *K. galanga* by column chromatographic method. For cytotoxicity test, EPMC at a concentration of 2.42 mM is relatively non-toxic to primary cultured hepatocytes. EPMC at this concentration induced CYP1A1 and CYP1A2 mRNA expressions in the mouse hepatocytes. However, EPMC at this concentration did not show significant induction of CYP2B9 and CYP3A11 mRNA expressions. Moreover, EPMC can modulate the inductive expression of CYP1A1 mRNA by specific inducers, B[a]A and β-NF. In which the synergistic effects were observed at 72 and 48 h for B[a]A and β-NF treatments, respectively. Therefore, the consumption of *K. galanga* may be potentially caused herbal-drug or herbal-chemical interactions.

Keywords: *Keampferia galanga*, ethyl-*p*-methoxycinnamte, cytochrome P450, hepatocyte, mRNA.

1. INTRODUCTION

Currently, there are increasing in the utilization of the complementary and alternative medicine. In contrast to chemical conventional medicines, herbs have sometimes been claimed to be non-toxic, because of their natural origin and long-term use as folk medicine. However, problems may arise due to intrinsic toxicity, adulteration, substitution, contamination, misidentification, and herbal-drug interaction [1]. Interactions between herbs and medications can be caused by either pharmacodynamic or pharmacokinetic, and

most of current evidence of pharmacokinetic drug interactions involves cytochrome P450 (CYP) metabolizing enzymes. A variety of herbal components are capable of interacting with CYP enzyme system in several ways including induction or inhibition of CYP mRNA expression. In general, it was indicated that most CYPs are modulated via receptor-mediated mechanisms leading to gene transcription [2, 3]. CYPs are involved in a variety of xenobiotic substances metabolism [4]. In humans, among abundant three CYPs isoforms, CYP1A1, CYP2, and CYP3A families, CYP3A4 subtype seems to be the most important due to its participation in the metabolism of more than 50 % of conventional drugs [5]. While CYP1A1, CYP1A2 and CYP2B are important in metabolism of wide variety of xenobiotic substances such as therapeutic drug, (pro)carcinogen and toxicant [6]. Therefore, changes of CYP mRNA expression may affect the detoxication and excretion of drugs and toxicants.

Kaempferia galanga L. ("Proh hom" in Thai), belonging to the Zingerberaceae family, has been widely used as a spice, food flavoring, and folk medicine in South India and Southeast Asia such as Malaysia, Indonesia, and Thailand [7]. The biological activities of this plant extract have been reported such as antibacterial and antifungal activities [8, 9] and anti-ulcer activity in various experimental model [10]. Ethyl-*p*-methoxycinnamate, a major constituent found in the essential oil from *K. galanga* rhizomes, has been reported to have activities against *Candida albicans*, and Mycobacterium tuberculosis [7]. Moreover, it has also reported to have strong anti-carcinogenic potential in *in-vitro* studies [11]. Herbal-drug interaction causes alterations in the way of a natural medicine affects a metabolic pathway of a herbal medicine and the ability of other therapeutic agents by producing synergistic or additive actions or antagonizing activities on

P450 enzymes. Taking herbal or natural products may represent a potential risk to patients taking conventional medication. This study aimed to investigate the effect of EPMC isolated from *K. galanga* on cytochrome P450, especially on CYP1A1, CYP1A2, CYP2B9 and CYP3A11 mRNA expressions in mouse hepatocytes.

2. MATERIALS AND METHODS
2.1 Chemicals
Materials for culturing hepatocytes were purchased from Gibco® Invitrogen Cell Culture (Carlsbad, CA) Bio Whittaker™ Cambrex Bio-Sciences (Walkerville, MD), and Wako Pure Chemical (Osaka, Japan). D-glucose, 2-mecaptoethanol, sodium acetate, phenol, guadinium thiocyanate, isopropanol, Waymouth's medium and Lactate dehydrogenase cytotoxic (LDH) test kit were purchased from Wako Pure Chemical (Osaka, Japan). Percoll was from GE Healthcare Bio-Science AB (Uppsala, Sweden). β-naphthoflavone (β-NF) and benz(a) anthrazene (B[a]A) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The TaqMan® Gold RT-PCR kit and TaqMan® Gene Expression Assays were products of Applied Biosystem (Branchburg, NJ, USA). SYBR® Green *Premix Ex Taq*™ (Perfect Real Time) was supplied by TaKaRa Biomedicals Inc. (Shiga, Japan). All other chemicals are high purity grade from commercial suppliers. The standard EPMC was kindly provided by Associate Professor Dr. Chavi Yenjai, Faculty of Science, Khon Kaen University, Thailand.

2.2 Plant Materials
The rhizomes of *K. galanga* were collected from Khon Kaen province, Thailand. The dried rhizome powder (1kg) was macerated with dichloromethane (2.5 liters) for 7 days, and then filtered through a Whatman No.1

filter paper. Then the solvent was evaporated by a rotary evaporator (Eyela, SB-1,000, Japan). The obtained dichloromethane extract of *K. galangal* (DEK) was applied to siliga gel column chromatography (70-230 mesh) and eluted with 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 contained EPMC were pooled and dried for further used.

2.3 Identification of EPMC

The identification of isolated EPMC was examined by Fourier Transform Infrared spectrophotometer (FT-IR spectrum-one spectrometer, Perkin-Elmer, USA). Chromatographic purity was determined by HPLC. (Agilent 1,100 series and UV-VWD, Japan.), using an isocratic solvent, acetronitrile: methanol: 20mM NaH_2PO_4 (30:40:30 v/v/v) at a flow rate of 1 ml/min, with a Thermo Hypersyl-Keystone ODS HYPERSYL; 5μm, 4.6×250 mm (Agilent, Germany) and guard column, μBondpack 10 μm C18(Water, U.S.A.) for the separation. The UV detection was at 270 nm. Identification of spectral data and chromatographic purity was compared to the standard EPMC.

2.4 Preparation of Primary Hepatocyte Culture

Primary hepatocyte culture was performed by method as previously described [12]. The liver of a ddY male mouse (Sankyo Laboratories, Shizuoka, Japan) at 8 weeks of age was perfused with 0.01% collagenase-containing Hanks' solution, and viable hepatocytes were isolated by means of Percoll isodensity centrifugation. The cells were dispersed in Waymouth MB 752/1 medium containing bovine serum albumin (2 g/L), insulin (0.5 mg/L), transferin (0.5 mg/L), and selenium

(0.5 μg/L), and seeded at density of 5×10^5 cell/1.5 ml/35mm collagen coated dishes. The culture dishes were maintained at 37°C in a CO_2 humidified incubator. The medium was renewed 3 h after plating and then 24 h after the treatment was performed as indicated.

2.5 Cytotoxicity Assessment

Cytotoxicity of EPMC on primary hepatocytes was assessed using lactate dehydrogenase (LDH) leakage assay kit under the instruction of the supplier (Wako, Japan). Primary hepatocytes were treated with indicated concentration of EPMC (0.12-24.24 mM). At the end of incubation, 400 μl of medium was harvested for enzyme leakage determination at 540 nm using a microtiter plate reader (Nalge Nunc International, ImmunoMini NJ-2,300). The percentage of cell injury rate was calculated by comparing to the standard curve of hepatocytes that were treated with 0.3% of tween 80 in Waymouth's medium

2.6 Induction of P450s in Primary Hepatocytes

Primary hepatocyte cultures as described above were changed to the new medium and then treatment with EPMC or simultaneously treatment with EPMC plus specific CYP1A inducers, which are B[*a*]A or β-NF were performed immediately. At the end of incubation time, the cell was harvested for RNA isolation.

2.7 Isolation of RNA

Hepatic total RNA was isolated as described by Chomczynski and Sacchi [13] according to the guadinium thiocyanate/phenol method. Briefly, 0.45 ml of solution D (containing 74.2% guadinium thiocyanate, 0.8% N-laurylsarkosine sodium and 0.04 M citrate buffer, pH 7.0) containing 0.72% of 2-mecaptoethanol was used to isolate intact

RNA. Each sample was collected and put into a sample tube containing 55 μl of 2 M sodium acetate, pH 4.0 and 0.5 ml phenol solution. Then 150 μl of chloroform was added into the sample tube and vigorously shaken. After centrifugation at 20,000 g, 4°C for 10 min, the chloroform layer was transferred to eppendorff tube containing 0.65 ml of isopropanol, mixed and centrifuged again at 20,000 g, 4°C for 10 min. Pellet was collected and suspended in 0.35 ml of solution D and 0.35 ml isopropanol, and then centrifuged at 20,000 g, 4°C for 10 min for RNA harvesting. RNA was washed by gentle mixed with 80% ethanol, and centrifuged at 20,000 g, 4°C for 5 min, and then the supernatant was discarded. RNA in the pellet was dried at room temperature before reconstitution in 100 μl of 0.5% of sodium dodecyl sulfate, and kept at -80°C until used.

2.8 Real-time reverse transcriptase – polymerase chain reaction (RT-PCR)

The mouse CYP1A1, CYP1A2, CYP2B9, CYP3A11 and house keeping gene; GAPDH mRNAs were quantified by real-time RT-PCR as described [14]. Hepatic total RNA was reverse-transcribed and cDNA was synthesized under the condition recommended by the supplier of the TaqMan® Gold RT-PCR kit (Applied Biosystem, Branchburg, NJ) using a specific TaqMan® Gene Expression Assay (Inventoried) for *Cyp1a1* (assay ID, Mm00487218_m1), *CYP1a2* (assay ID, Mm00487224_m1), *CYP2b9* (assay ID, Mm00657910_m1), *CYP3a11* (assay ID, Mm00731567_m1), and the SYBER® Premix Ex Taq™ (Perfect Real Time) for GAPDH in which the forward, and reverse primers were 5′-TCC ACT CAC GGC AAA TTC AAC G-3′ and 5 -TAG ACT CCA CGA CAT ACT CAG C-3′. The PCR condition was as follows: denaturation at 95°C for 15s, annealing and extension at 60°C for 1 min. Real-time PCR

was performed using the ABI Prism® 7,000 Sequence Detection System (Applied Biosystems, Brunchburg, NJ) with ABI Prism® 7000 SDS software.

The amplified products of CYP1A1, CYP1A2, CYP2B9 and CYP3A11 were detected directly by monitoring the fluorescence of the reporter dye (FAM), by which the increment in fluorescence signal was detected if the target sequence was complementary to the probe and amplified by the PCR. Whereas the amplified PCR products of GAPDH was monitored by measuring the increase in SYBR® Green that was bound to double-strand DNA amplified by PCR. The standard curves of *Cyps* and GAPDH were generated by the linear regression of a plot of threshold cycle (Ct) value versus the log of the amount of total RNA of control mice added to the reaction. The relative expression level of *Cyps* mRNA was normalized by that of GADPH mRNA.

2.9 Statistical analysis

All data were expressed as mean ± S.D. The analysis of variance (ANOVA) or Student's *t-test* was performed. P-values less than 0.05 were considered as statistical significance.

3. RESULTS
3.1 Identification of EPMC

The dichloromethane extract from dried rhizome of *K. galanga* gave a dark brown oily solution with yield of 3.4%. By using silica gel column chromatography, EPMC fractions were pooled and dried to give a white pale crystal of EPMC at yield of 10.88 %. The obtained EPMC was identified by using FT-IR, which gave the same pattern as standard EPMC (Figure 1). HPLC chromatogram of the purified EPMC demonstrated a single peak with retention time of 12.32 min as observed of the standard EPMC (Figure 2).
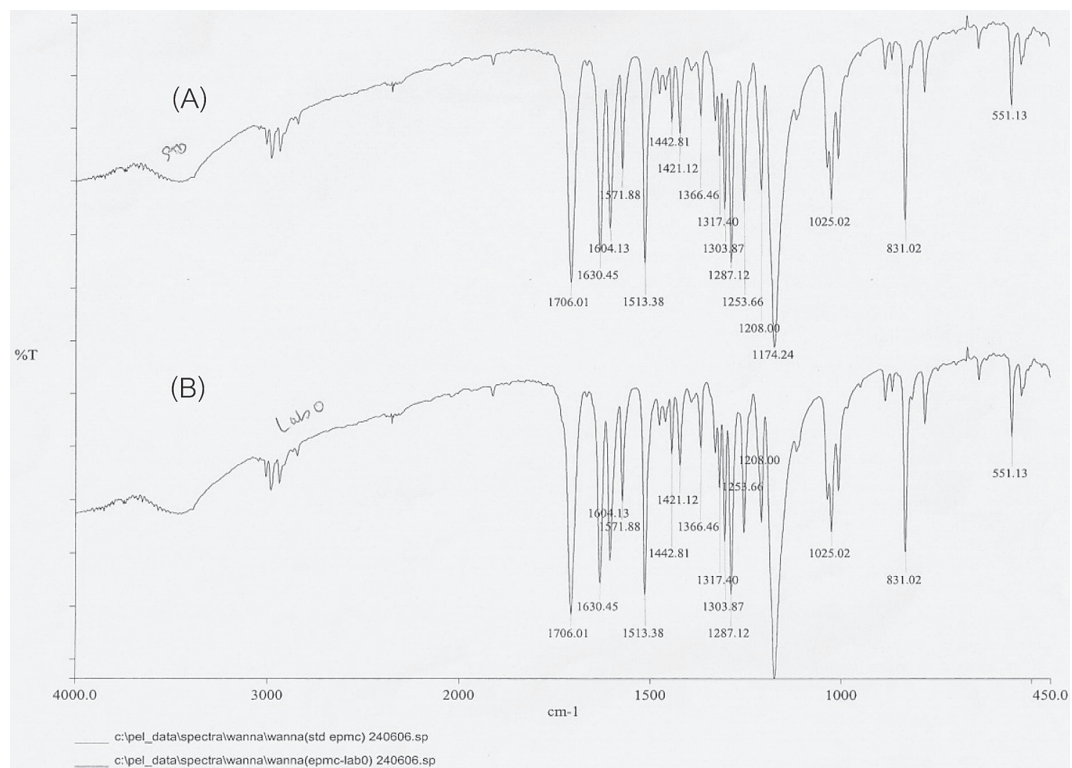
Figure 1. FT-IR pattern of standard EPMC (A) and purified EPMC obtained from column chromatography(B).
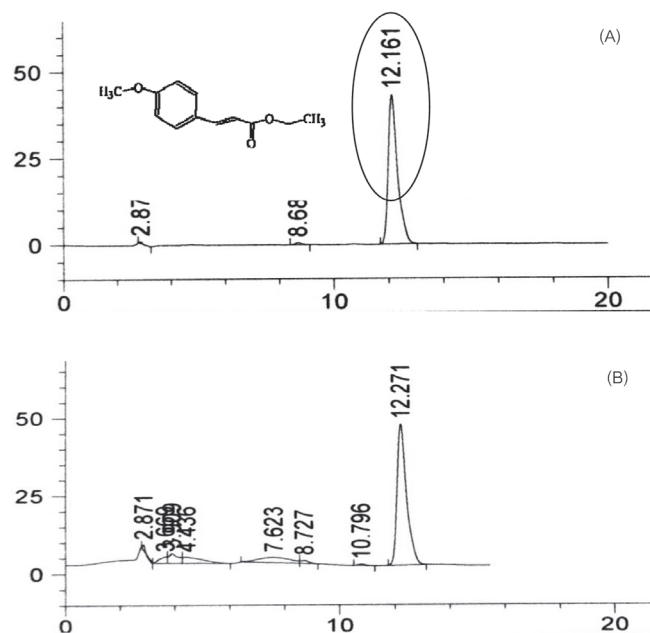

Figure 2. HPLC chromatograms of standard ethyl-p-methoxycinnamate (A) and EPMC obtained from column chromatographic purification (B).

3.2 Cytotoxicity of EPMC on Mouse Hepatocytes

To examine the cytotoxicicty of EPMC, mouse hepatocytes were treated with indicated doses of EPMC for 24 h. Low doses of EPMC (0.12 – 2.42 mM) slightly increased the LDH release, but the toxicity was dramatically observed at a concentration of 24.24 mM of EPMC (Figure 3).
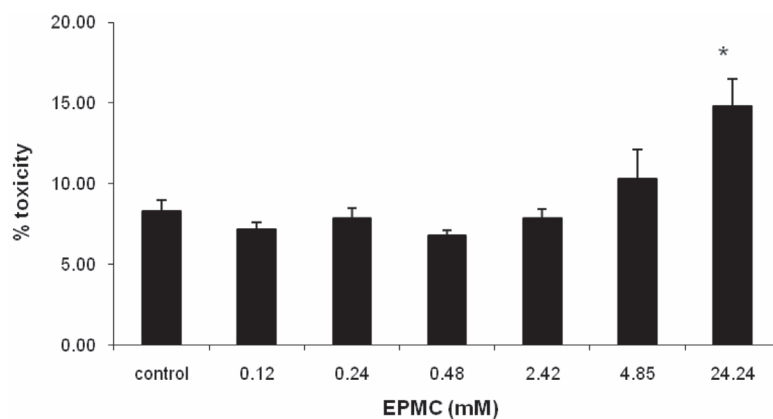

Figure 3. Effect of EPMC on LDH leakage in mouse hepatocytes. Data were presented as mean ± S.D (n=3). *Significant differences when compared to control group at $P<0.05$.
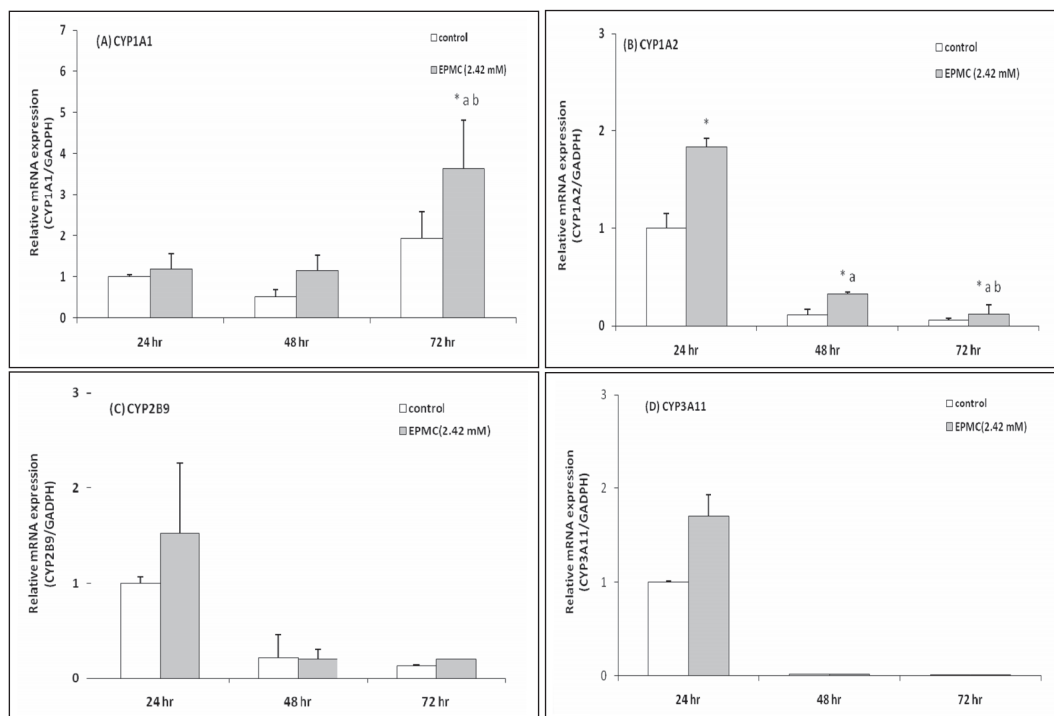

Figure 4. Effect of EPMC on CYP1A1 (A), CYP1A2 (B), CYP2B9(C), and CYP3A11(D) mRNA expressions in monolayer cultured hepatocytes. Data are expressed as relative mRNA expressions (CYPs target gene/GAPDH). Each column represents the maen ± S.D. (n=3). *[a, b] Significant differences when compared to control at the same duration of treatment, EPMC at 24 hr of treatment and EPMC at 48 hr of treatment, respectively.

3.3 Effect of EPMC on P450 mRNA Expression

To evaluate the effect of EPMC on P450 enzyme, the expressions of P450 mRNAs were examined in monolayer-cultured hepatocytes at indicated times. As compared to the control group, EPMC at a non-toxic dose (2.42 mM) increased CYP1A1 mRNA expression, but the significant difference was observed only at 72 h of treatment. The CYP1A2 mRNA expression was significantly increased at 24 h of treatment, and declined at 48 and 72 h. In contrast, CYP2B9 and CYP3A11 mRNA expressions, at 24 h of EPMC treatment were slightly increased, but without statistical significance. However, when the incubation time was increased to 48 and 72 h, both CYP mRNA expressions were

decreased to normal level (Figure 4).

3.4 Modulatory Effect of EPMC on CYP1A1 mRNA Expression under Specific Induction

The combination effect of EPMC and specific CYP1A1 inducer on CYP1A1 mRNA expression was investigated. EPMC at a non-toxic concentration (2.42 mM) markedly affected CYP1A1 mRNA expression. At 48 h of treatment, EPMC significantly antagonized the inductive effect of B[a]A, but synergized the inductive effect of β-NF on CYP1A1 mRNA expression (Figure 5A). In contrast, at 72 h of treatment, EPMC synergized the inductive effect of B[a]A, but did not change the inductive effect of β-NF (Figure 5B). However, there were no significant differences
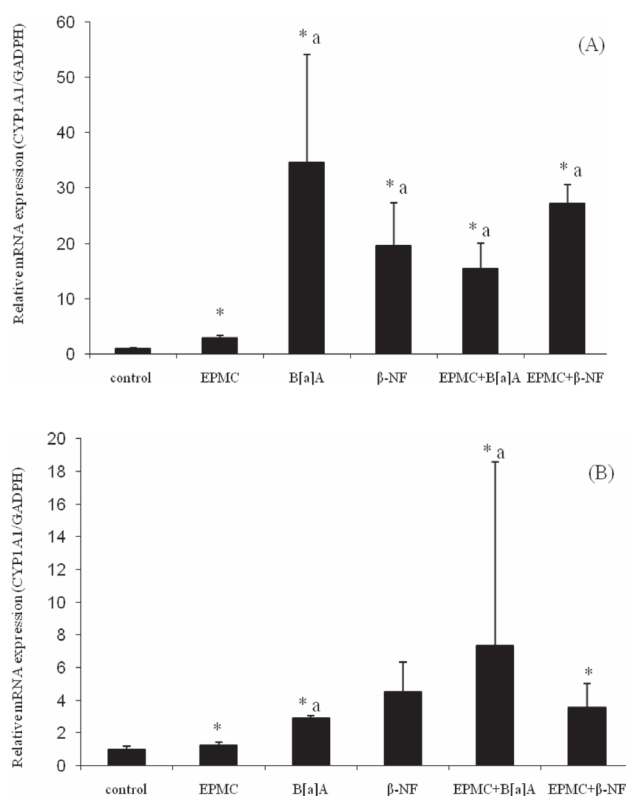

Figure 5. Combination effect of EPMC and typical CYP1A1 inducers, B[a]A and β-NF on the expression of CYP1A1 mRNA in monolayer-culture hepatocytes at 48 h (A) and 72 h (B) of treatment. Data were expressed as maen ± S.D. (n=3). [*a] shows significant differences to control and EPMC at $P<0.05$, respectively.

when compared between B[a]A and EPMC+ B[a]A or β-NF and EPMC+ β-NF treated groups. This finding may reflect the interaction of EPMC with these two inducers.

4. DISCUSSION

The present study revealed that EPMC, a major compound from the rhizome of *K. galanga*, affected CYP1A1 and CYP1A2 mRNA expressions in mouse primary hepatocytes. EPMC – induced CYP1A1 mRNA expression was clearly observed only at 72 h treatment, whereas EPMC – induced CYP1A2 was found at 24, 48 and 72 h treatments. It is suggested that EPMC is a weak inducer of CYP1A subfamily. These results are rather different from the previous report of the strong induction of andrographolide on CYP1A subfamily [15]. *CYP1A1* and *CYP1A2* were P450 genes identified as responsive to xenobiotic exposure. CYP1A1 is generally more sensitive to be induced than CYP1A2, and the induction at predominantly transcriptional level is mediated by *aryl hydrocarbon* receptor (AhR) [16]. It is concurrent with our results that EPMC induced CYP1A1 mRNA expression rather than induced CYP1A2 mRNA expression. Moreover, EPMC did not affect CYP2B9 and CYP3A11 mRNA expressions. This reflects the plant differentiated effect on CYP mRNA expressions. The combination of EPMC with, B[a]A or β–NF, specific inducers of CYP1A1 mRNA expression indicated that there are synergistic effects of EPMC to B[a]A and β–NF, but with time-dependence manner. However, the influences of EPMC on the enhancement of B[a]A or β–NF regulates the expression of CYP1A1 mRNA are needed to be further investigated.

Since the expression of CYP1A1 mRNA markedly influenced the activation of human-related chemical carcinogens [17-20]. CYP1 is responsible for activating a number of chemicals carcinogen that we are exposed to daily life via diet and environment such as polycyclic aromatic hydrocarbon, aromatic amines and heterocyclic amines. According CYP1A1/2 can metabolize a range of substrates, therefore the induction of these enzymes by one substrate may increase the metabolism of other chemical and clinical drug, and such interaction can be clinically significant [21, 22]. We showed here that EPMC significantly induced CYP1A mRNA expression and synergized the inductive effect of B[a]A and β–NF on CYP1A subfamily. Our finding suggests certain precaution on the utilization of *K. galanga* in traditional medicine therapy. However, the possible mechanism of the herbal-drug or herbal-chemical interactions is needed to be further investigated. In conclusion, EPMC, a major component of *K. galanga* rhizome, is a weak inducer of CYP1A subfamily and it can modulate the inductive effect of some typical CYP1A inducers.

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