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Effects of *Eulophia macrobulbon* Extract and Its Main Active Compound, 1-(4´-hydroxy-benzyl)-4,8-dimethoxyphenanthrene-2,7-diol on Hepatic Cytochrome P450 Activities in Rats

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Abstract

The *Eulophia macrobulbon* (EM) ethanolic extract and its active component, 1-(4´-hydroxy-benzyl)-4,8-dimethoxyphenanthrene-2,7-diol, have shown potential therapeutic benefits in the treatment of erectile dysfunction and cardiovascular diseases. However, their safety in relation to herb-drug interactions remains unclear. This study aimed to evaluate the potential for herb-drug interactions mediated by cytochrome P450 (CYP) modulation. CYP1A, CYP2B, CYP2C, and CYP3A activities were assessed in primary rat hepatocytes exposed to EM extract or phenanthrene, and in rat liver microsomes following 21-day oral administration. *In vitro*, EM extract decreased CYP2C activity and increased CYP1A and CYP3A activities, whereas phenanthrene showed no significant effects. *In vivo,* EM extract inhibited CYP2C and CYP3A activities at low doses (15 mg/kg) and induced CYP1A activity at higher doses (450 mg/kg), with no significant changes at 1,000 mg/kg. Phenanthrene decreased CYP1A activity at 750 µg/kg. Overall, CYP modulation was approximately 2-fold compared with the control. These findings indicate that EM extract can modulate key CYP enzymes and may pose a risk of herb-drug interactions, particularly through inhibition of CYP2C and CYP3A at low *in vivo* doses. The discrepancies between *in vitro* and *in vivo* results suggest that metabolites may contribute to CYP modulation.

Keywords: *Eulophia macrobulbon*, Cytochrome P450, Liver microsomes, Herb-drug interaction, Primary hepatocytes, Natural product safety

Introduction

The concomitant use of herbal medicines and conventional pharmacotherapy is widespread [1,2]. While this practice offers several advantages, it also presents the risk of herb-drug interactions [3,4]. Such interactions may alter drug pharmacokinetics and/or pharmacodynamics, particularly in drugs with a narrow therapeutic index (e.g., digoxin, warfarin, cyclosporine A, and rifampicin). The inhibition and/or induction of cytochrome P450 (CYP) enzymes, which play a central role in drug metabolism, are major mechanisms underlying pharmacokinetic interactions. CYPs are heme-containing monooxygenases involved in the metabolism of a wide range of chemically diverse compounds, including drugs. Although CYPs are present in most tissues, the liver expresses the highest levels of CYP isoforms [5,6]. To date, more than 30 human CYP isoenzymes have been identified. Among these,

CYP1A2, CYP3A4, CYP2C9 and CYP2D6 are the major subfamilies responsible for drug metabolism [7,8]. Therefore, the pharmacological evaluation of new herbal preparations should include assessment of their potential to cause drug interactions via these CYP enzymes. However, natural compounds, including polyphenolic and lipophilic phytochemicals, have been reported to modulate major drug-metabolizing enzymes, particularly cytochrome P450 isoforms such as CYP1A2, CYP2C9, and CYP3A4 [9-11]. Clinically, these enzymes are responsible for the metabolism of a large proportion of clinically used drugs. Modulation of CYP3A4, the most abundant hepatic isoform, may significantly alter the pharmacokinetics of drugs, including PAH-specific therapies such as phosphodiesterase-5 inhibitors (sildenafil and tadalafil), endothelin receptor antagonists (ambrisentan and bosentan), and calcium channel blockers (e.g., nifedipine) [12], while CYP2C9 is involved in the metabolism of drugs such as warfarin and non-steroidal anti-inflammatory drugs [13,14]. In addition, CYP1A2 participates in the metabolism of xenobiotics and endogenous compounds. Therefore, inhibition or induction of these CYP isoforms by natural products may result in clinically relevant herb-drug interactions [9,10]. Herbal extracts, including polyphenolic and lipophilic phytochemicals, may modulate CYP enzymes through both direct enzyme interaction and transcriptional regulation [9,10,15-17]. Therefore, EM extract and its phenanthrene constituent may influence hepatic CYP-mediated drug metabolism.

*Eulophia macrobulbon (*EM) (E.C.Parish & Rchb.f.) Hook.f (Syn; *Crytopera macrobulbon, Graphorkis macrobulbon*, etc.) is a member of the Orchidaceae family and is widely distributed in Thailand, Cambodia, Laos, Vietnam, and Myanmar. It has been used in Thai traditional medicine to treat gangrene and insect bites, as well as to relieve pain and fatigue [18,19]. Recent studies have reported that the ethanolic extract of EM tubers possesses antioxidant, anti-inflammatory, and anticarcinogenic activities [19]. In addition, our previous studies [20,21] demonstrated that EM extract exerts both endothelium-dependent and endothelium-independent vasorelaxant effects in pulmonary and systemic vascular beds. Furthermore, our *in vivo* studies in rats have shown that EM extract exerts hypotensive effects [20], and reduces the severity of pulmonary hypertension [21]. Improved erectile function has also been reported [22,23]. These therapeutic effects have been linked, at least in part, to the presence of 1-(4′-hydroxy-benzyl)-4,8-dimethoxyphenanthrene-2,7-diol [24], a potent phosphodiesterase-5 inhibitor [25]. This phenanthrene derivative has been identified as a phosphodiesterase-5 (PDE5) inhibitor, which enhances cyclic guanosine monophosphate (cGMP) signaling and promotes smooth muscle relaxation. PDE5 inhibition is a well-established therapeutic mechanism in conditions such as erectile dysfunction and pulmonary hypertension [26,27]. Moreover, toxicological safety assessment of the ethanolic extract of EM roots, in rodents, revealed no significant adverse effect on mortality, biochemical or hematological parameters, or macroscopic organ morphology following acute or chronic oral administration [28]. These findings support the potential of this extract and its active constituent as candidates for further studies in humans.

Based on these considerations, we hypothesized that EM extract and its main phenanthrene constituent may modulate hepatic CYP activities. To evaluate the potential for herb-drug interactions, this study investigated the effects of EM extract and its main constituent, 1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (phenanthrene), on CYP-dependent activities using primary rat hepatocytes (*in vitro*) and liver microsomes following sub-chronic treatment in rats (*in vivo*).

Materials and methods

Chemicals

Dulbecco's modified eagle medium (DMEM), William's E culture medium, insulin, fetal calf serum (FCS) and penicillin G/streptomycin were purchased from Invitrogen Corporation (France). Bovine serum albumin (BSA), β-naphthoflavone (BNF), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT), phenobarbital (PB), HEPES, KCl, Tris-HCl, sucrose, nicotinamide-adenine dinucleotide phosphate (NADPH), resorufin, ethoxyresorufin and collagenase type IV were obtained from Sigma-Aldrich (France). The highest purity laboratory chemicals were used, purchased from a commercial supplier. P450-Glo™

CYP3A and CYP2C assay kits were obtained from Promega (Charbonnières-les-bains, France).

Preparation of the extract

EM fresh tubers were collected from the area of Prachinburi province, Thailand. The voucher specimen (No. 002716) was identified by Associate Professor Dr. Anupan Kongbungkerd of the Department of Biology in the Faculty of Sciences, Naresuan University, and kept at the Faculty. As previously described [25], after the tubers were cut into small pieces, dried in a circulating oven (55 °C), ground into fine powder (2 kg), they were macerated 2 times with 14 L of 95% ethanol for 3 days, then filtered and evaporated by vacuum to yield 15.78% (w/w) of extract. This EM extract contained the phenanthrene compound; 1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (0.52% w/w), which was a major constituent of the extract and used as a bioactive marker [25]. This constituent was isolated from EM extract, purified and analysed by NMR and HPLC-UV/Vis system as described by Temkitthawon *et al*. [25]. The EM extract and the phenanthrene were stored at -20 °C until used.

***In vitro* studies**

Isolation, culture, and treatment of rat hepatocytes

All procedures were approved by the local ethics committee for animal experimentation of Franche-Comté University (Besançon, France; protocol No. 2015/001-CD/12PR; approved on 29/03/2015). Male Sprague-Dawley (SD) rats, aged 7 - 8 weeks and weighing 250 - 300 g, were used for hepatocyte isolation. All experimental procedures were conducted in accordance with institutional guidelines for animal care and use. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg; Ceva Santé Animale, France). The hepatocytes were isolated and cultured as previously described by Cornu, Rougier [29]. Briefly, livers were perfused in situ with a calcium-free washing buffer followed by a collagenase digestion buffer at 37 °C. The liver was excised, and released hepatocytes were filtered, washed, and purified by Percoll density gradient centrifugation. Cell viability was assessed using trypan blue exclusion. Viable hepatocytes were seeded in William's E medium supplemented with 10% fetal calf serum (FCS), 4 µg/mL insulin, 10 µM hydrocortisone, 10 U/mL penicillin, and 10 µg/mL streptomycin. After attachment, cultures were maintained in serum-free William's E medium before treatment. Hepatocytes were exposed to different concentrations of EM extract or phenanthrene for 24, 48, and 72 h, with daily renewal of treatment. EM extract and phenanthrene were dissolved in DMSO to prepare stock solutions and diluted in culture medium immediately before use. The final concentration of DMSO was maintained at 0.1% (v/v) in all treatment and control groups.

Cell viability assay

To determine cell viability, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 0.5 mg/mL for 3 h at 37 °C. After incubation, the medium was removed, and the formazan crystals were solubilized in DMSO. Absorbance was then measured at 570 nm.

Cytochrome P450 (CYP)-dependent activities in hepatocyte cultures

Rat hepatocytes were treated for 24, 48, and 72 h, with EM extract or phenanthrene at non-cytotoxic concentrations, based on the MTT assay. β-naphthoflavone (BNF, 50 µM), and phenobarbital (PB, 1 mM for CYP2B and 2 mM for CYP2C and CYP3A assays) were used as reference CYP inducers to validate the responsiveness of the rat hepatocyte model [30,31]. Reference CYP inducers were selected to assess CYP modulation under time-dependent exposure conditions and to evaluate overall enzyme regulation. At each time point, the CYP1A and CYP2B activities were determined by measuring ethoxyresorufin-O-deethylase (EROD) and benzyloxyresorufin O-dealkylase (BROD) activities, respectively, according to previous studies [29-31]. Briefly, the cells were exposed for 1 h to a reaction buffer consisting of a culture medium containing salicylamide (1 M) and a specific substrate (ethoxyresorufin or benzyloxyresorufin, 10 mM). Fluorescence was measured at excitation 530 nm and emission 580 nm. The results were expressed as pmol resorufin formed/min/mg protein. CYP2C and 3A activities were measured using the P450-Glo™ CYP2C9 and CYP3A4 assay kits, respectively, following the manufacturer's protocol [32,33]. Briefly, the cells were exposed for 1 h (CYP3A) or 3 h (CYP2C) at 37 °C to a specific luciferin. After incubation with the luciferin detection reagent at room temperature for 20

min, the luminescence was measured. The results were expressed as relative luminescent units (RLU)/min/mg protein. Protein concentration was determined by the bicinchoninic acid assay kit (Sigma) and using the bovine serum albumin (BSA) as a standard.

In vivo studies

Experimental design

All procedures were approved by the local ethics committee for animal experimentation of Franche-Comté University (Besançon, France, protocol No. 2015/001-CD/5PR, date 04/03/2015). Male Sprague-Dawley (SD) rats aged 6 - 7 weeks and weighing 160 - 200 g were randomly assigned to treatment groups (n = 5 - 6 per group). Rats were administered the EM extract (15, 450, and 1,000 mg/kg/day) or phenanthrene at 75, 250, and 750 µg/kg/day by oral gavage, for 21 consecutive days, at a volume of 1 mL/kg body weight. Rats from the control group were administered the vehicle (propylene glycol, PG) by the same route. The EM extract doses were chosen based on its positive effect on pulmonary hypertension in a rat model [21], while the doses of phenanthrene were extrapolated from the EC_{50} of its vasorelaxant effect on isolated arteries *ex vivo* [20]. Rats were weighed daily and sacrificed 24 h after the last administration. Different organs (liver, spleen, lungs, kidneys, and heart) were immediately removed and weighed. Liver pieces were immediately frozen in liquid nitrogen and stored at –80 °C until the microsome preparation.

Rat liver microsome preparation

Rat liver microsomes were prepared in-house using a standardized protocol. Briefly, rat liver samples were homogenised in 4 volumes of cold homogenisation buffer (50 mM Tris–HCl, pH 7.4, 150 mM KCl, 2 mM EDTA). The homogenates were centrifuged for 20 min (at 9,000×g, 4 °C), and the collected supernatant fractions were centrifuged twice for 1 h (at 100,000 g, 4 °C). The final microsomal pellets were resuspended in 0.25 M sucrose buffer [34,35] and stored at –80 °C until use. To ensure consistency across samples, all liver tissues were processed simultaneously for microsome preparation using identical experimental conditions. Protein concentrations of microsomal preparations were determined prior to CYP activity assays, and equal amounts of protein were used across all samples to ensure comparability between groups.

Cytochrome P450 (CYP)-dependent activities in rat liver microsomes

Microsomal EROD (for CYP1A) and BROD (for CYP2B) activities were measured as previously described [29,34,36]. The enzyme activities were expressed as pmol resorufin formed/min/mg microsomal protein. CYP3A and CYP2C activities were measured using the P450-Glo™ CYP3A4 and CYP2C9 assay kits, respectively, according to the manufacturer's instructions. Protein content was determined as described above.

Statistics

All *in vitro* and *in vivo* data are expressed as means ± standard error of the mean (SEM). For chemical testing, statistical analysis was performed when fold changes were < 1 or ≥ 2, in accordance with OECD guidelines [37]. Statistical analyses were performed using GraphPad Prism software (version 5.03). For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc comparison test was used. For time-course data (e.g., body weight changes over time), two-way ANOVA with treatment and time as factors followed by Tukey's post hoc test was applied. For time-course or concentration-dependent experiments, comparisons were performed within each timepoint to the corresponding control group. A p-value < 0.05 was considered statistically significant.

Results

Cytotoxicity of EM extract and phenanthrene in cultured hepatocytes

EM extract induced a time- and concentration-dependent decrease in cell viability (**Figure 1**). The EM extract is a crude mixture of multiple constituents; therefore, its concentration is expressed as mass concentration (µg/mL). In contrast, phenanthrene is an isolated pure compound with a defined molecular weight, and its concentration is expressed as molar concentration (µM). A statistically significant reduction greater than 80% was observed at 1,000 µg/mL (24 h), 500 µg/mL (48 h) and 400 µg/mL (72 h). IC_{50} values for EM extract were 1.73 ± 0.25 mg/mL (24 h), 1.01 ± 0.15 mg/mL (48 h) and 0.53 ± 0.07 mg/mL (72 h). Similar effects were observed with phenanthrene, which induced a reduction in cell viability greater than 80% at 50 µM (24 h), 25 µM (48 h) and 20 µM (72 h). IC_{50}

values for phenanthrene were 74.7 ± 8.32 µM (24 h), 36.4 ± 7.22 µM (48 h) and 22.5 ± 1.73 µM (72 h).

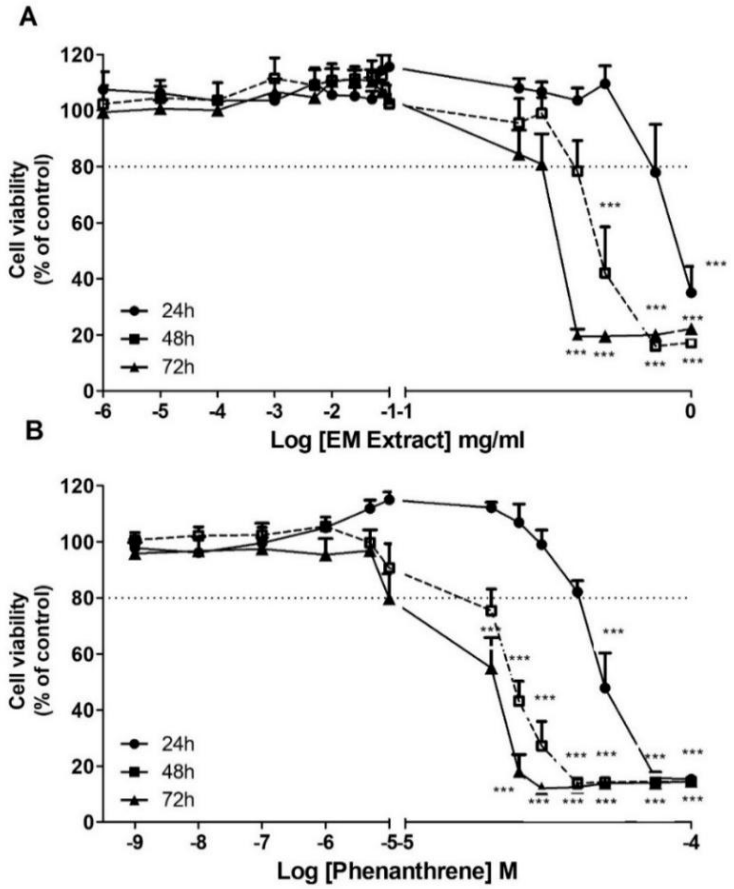


Figure 1 Effect of EM extract and phenanthrene on cell viability in primary rat hepatocyte cultures. Cell viability was evaluated in rat hepatocyte cultures after 24 h (●), 48 h (□), and 72 h (▲) of treatment with EM extract (A), phenanthrene (B), and expressed as a percentage of control (DMSO 0.1%). Values are expressed as means ± SEM of triplicate of 3 - 5 independent cultures. ***$p < 0.001$ *vs* control at each time point (one-way ANOVA with Tukey's post hoc test).

Effects of EM extract and phenanthrene on CYP activities in isolated hepatocytes

CYP1A, CYP2B, CYP2C and CYP3A activities were measured in rat hepatocytes after 24, 48 and 72 h of exposure to EM extract, phenanthrene or reference inducers (**Figures 2** and **3**). Non-cytotoxic concentrations of EM extract (0.01 to 300 µg/mL) and phenanthrene (0.01 to 10 µM) were selected based on the results shown in **Figure 1**. BNF was used as a reference inducer for the CYP1A activity (**Figure 2(A)**) and PB was used for CYP2B, CYP2C and CYP3A activities (**Figures 2(B) - 2(D)**). As expected, BNF significantly induced CYP1A activity (4.9- and 8-fold increases at 48 and 72 h), while PB significantly increased CYP2B (8.2- and 5.9-fold), CYP2C (4.7- and 4.9-fold) and CYP3A (6- and 19.4-fold) activities at 48 and 72 h. EM extract inhibited CYP2C at 300 µg/mL, at all-time points (**Figure 2(C)**). By contrast, EM extract increased CYP1A activity (at 50, 100 and 300 µg/mL after 48 - 72 h, **Figure 2(A)**) and CYP3A activity (at 300 µg/mL after 72 h of treatment, **Figure 2(D)**). In contrast to EM extract, exposure to the phenanthrene did not significantly affect CYP activities, whatever the time of exposure (**Figures 3(A) - 3(D)**).

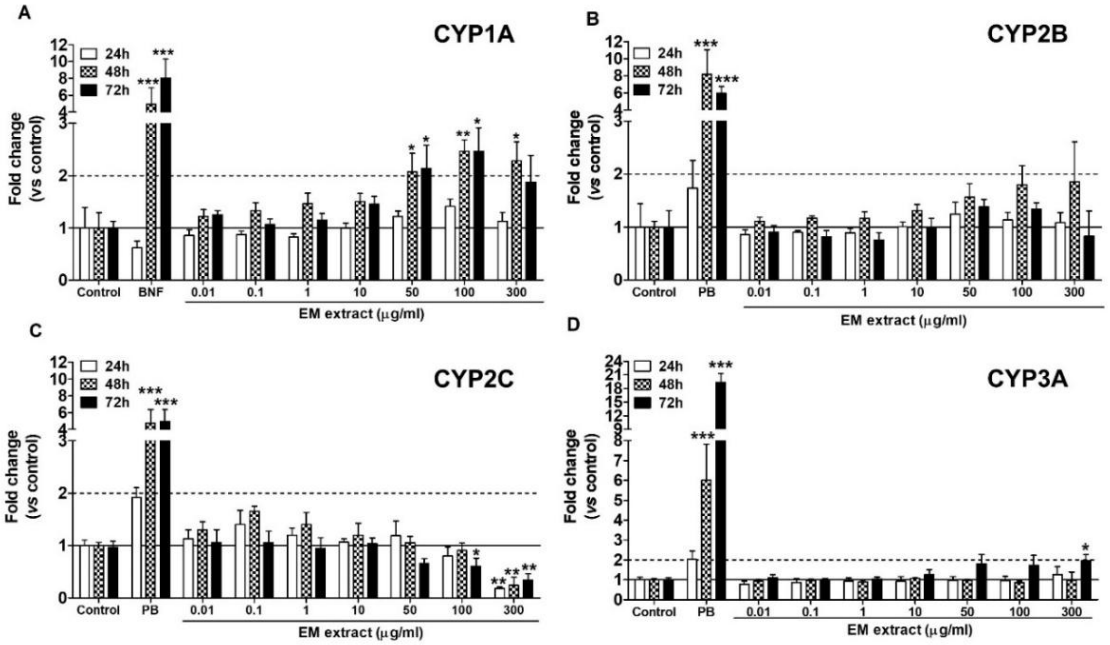


Figure 2 Effects of EM extract on CYP activities in isolated rat hepatocytes. Rat hepatocytes were incubated with EM extract for 24h (□), 48h (■) and 72h (■), with DMSO (0.1%) as the control. CYP1A (**A**), CYP2B (**B**), CYP2C (**C**) and CYP3A (**D**) activities were measured. β-Naphthoflavone (BNP, 50 μM) and phenobarbital (PB, 1 mM for CYP2B and 2 mM for CYP2C and CYP3A) were used as reference inducers. CYP activities were expressed as fold change relative to the control (DMSO 0.1%), which was set at 1. Values are presented as means ± SEM from triplicate from 3 - 5 independent experiments. *$p <$ 0.05, ** $p < 0.01$, ***$p < 0.001$ *vs* the control at each time point (one-way ANOVA with Tukey's post hoc test).

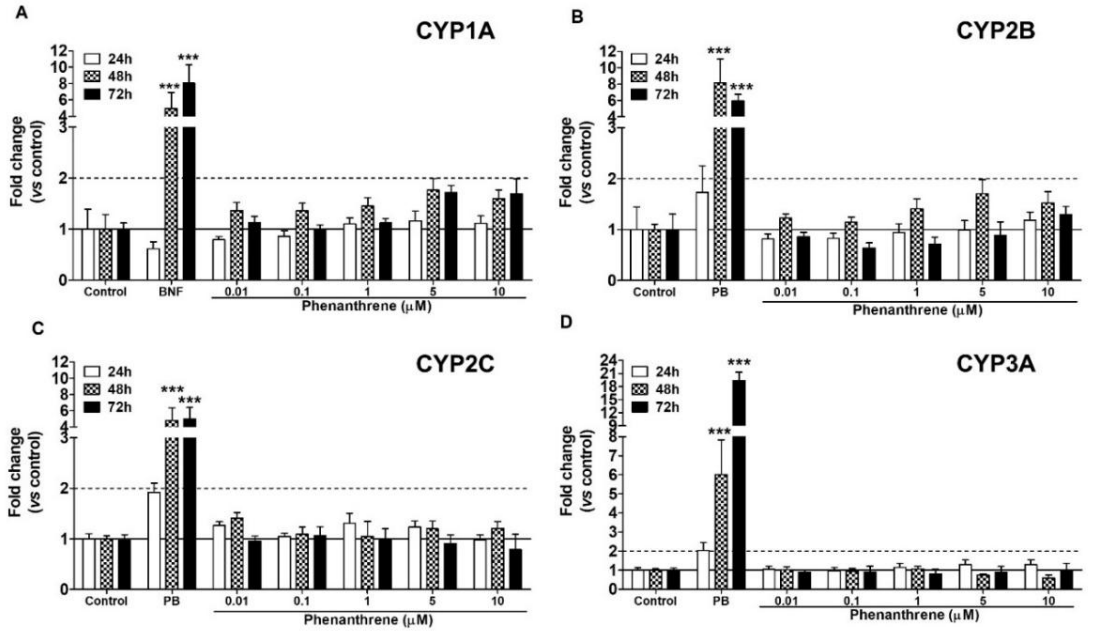


Figure 3 Effects of phenanthrene on CYP activities in isolated rat hepatocytes. Rat hepatocytes were incubated with EM extract for 24h (□), 48h (■) and 72h (■), with DMSO (0.1%) as the control. CYP1A (**A**), CYP2B (**B**), CYP2C (**C**) and CYP3A (**D**) activities were measured. β-Naphthoflavone (BNP, 50 μM) and phenobarbital (PB, 1 mM for CYP2B and 2 mM for CYP2C and CYP3A) were used as reference inducers. CYP activities were expressed as fold change relative to the control (DMSO 0.1%), which was set at 1. Values are presented as means ± SEM from triplicate from 3 - 5 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* the control at each time point (one-way ANOVA with Tukey's post hoc test).

Effects of EM extract and phenanthrene after *in vivo* treatment

After 21-days of oral treatment with EM extract, no changes in body weight were observed at any dose (**Figure 4(A)**). In contrast, treatment with phenanthrene significantly reduced body weight gain compared with the vehicle group at all tested doses (75, 250, and 750 μg/kg) (**Figure 4(B)**). No change in organ-to-body weight ratios (liver, spleen, lungs, kidneys, and heart) were observed following treatment with either EM extract or phenanthrene (**Figures 4(C) - 4(G)**).

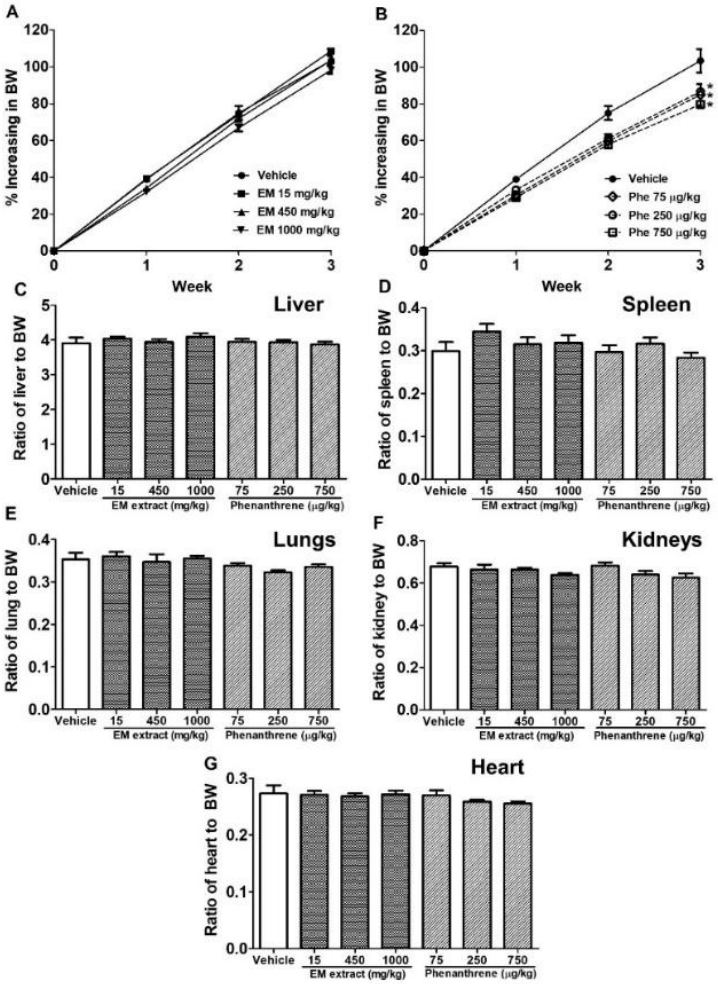


Figure 4 Effects of EM extract or phenanthrene on body and organ weights following sub-chronic oral administration. Rats were treated daily with EM extract, phenanthrene, or vehicle (propylene glycol, PG) by oral gavage for 21 days. Body weight (BW) gain was expressed weekly as a percentage relative to BW at week 0 for EM extract (**A**) or phenanthrene (**B**). At day 21, organs (liver **C**, spleen **D**, lungs **E**, kidneys **F** and heart **G**) were collected and weighed. Organ weights were expressed as organ-to-body weight ratios at the end of treatment. Values are presented as means ± SEM (n = 5 - 6 rats). *$p < 0.05$ *vs* vehicle.

To assess the effects of EM extract and phenanthrene on CYP-dependent activities, liver microsomes were isolated at the end of the treatment period. Treatment with EM extract decreased both CYP2C (**Figure 5(C)**) and CYP3A activities (**Figure 5(D)**), this effect was observed only at the low dose of 15 mg/kg. When administered at the intermediate dose (450 mg/kg), EM extract increased CYP1A activity (**Figure 5(A)**). At the highest dose (1,000 mg/kg), EM extract did not significantly affect CYP activities as compared to the vehicle (**Figure 5**). In contrast, phenanthrene did not affect CYP2B, CYP2C, or CYP3A activities (**Figures 5(B) - 5(D)**), but significantly decreased CYP1A activity at the highest dose of 750 μg/kg (**Figure 5(A)**).

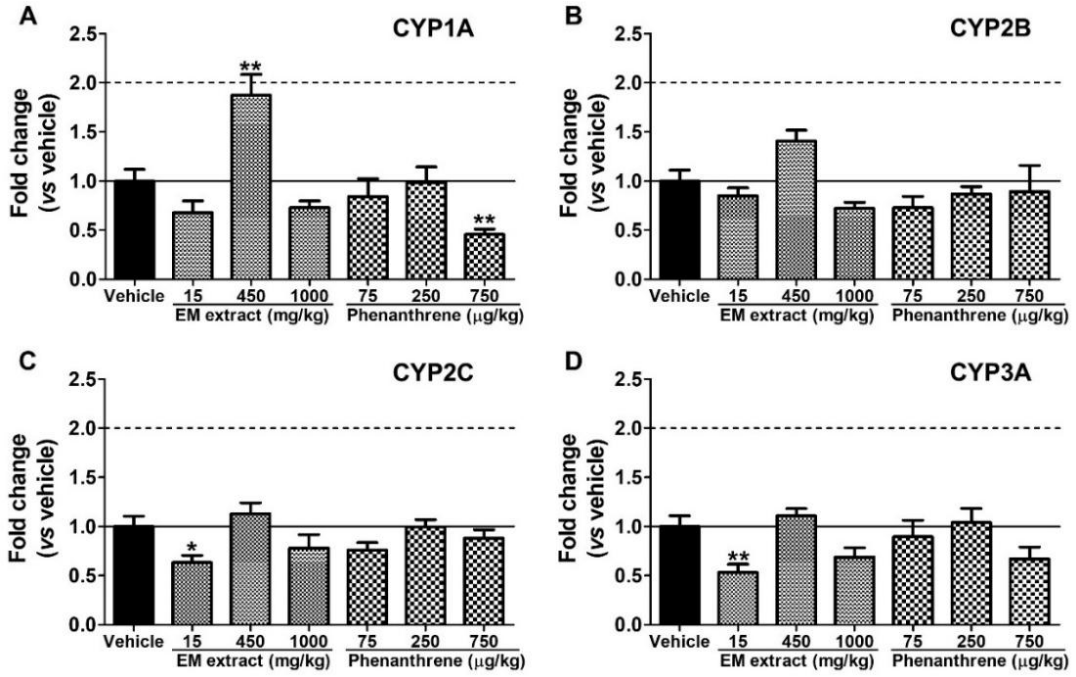


Figure 5 Effects of EM extract and phenanthrene on CYP activities in rat liver microsomes after a sub-chronic oral treatment. Rats received EM extract, phenanthrene, or vehicle (PG) by oral gavage for 21 days. At the end of the treatment, liver microsomes were prepared to measure CYP1A (**A**), CYP2B (**B**), CYP2C (**C**) and CYP3A activities (**D**). Data are expressed as fold change relative to the vehicle (PG) group, which was set at 1. Values are presented as means ± SEM (n = 5 - 6 rats). *$p < 0.05$, **$p < 0.01$ *vs* vehicle (one-way ANOVA with Tukey's post hoc test).

Discussion

Although recent studies have reported promising therapeutic applications for the EM extract and its main phenanthrene 1-(4′-hydroxy-benzyl)-4,8-dimethoxyphenanthrene-2,7-diol in PAH, arterial hypertension [20,21] and erectile dysfunction [22,23], information regarding their potential for herb-drug interactions remains limited. Recent studies show that phenanthrene-enriched extract of EM exhibit enhanced PDE5A1 inhibitory activity, suggesting increased pharmacological potency and emphasizing the need to assess their safety and interaction potential [38]. In the present study, EM extract modulated several CYP isoforms, inducing CYP1A activity *in vitro* and at selected *in vivo* doses, inhibited CYP2C activity, and exhibited divergent effects on CYP3A activity between *in vitro* and *in vivo* models.

Before investigating the effect of the EM extract and phenanthrene on CYP activities in primary rat hepatocyte, we first evaluated their potential impact on cell viability. Our data demonstrated that both EM extract and phenanthrene induced concentration- and time-dependent cytotoxicity. These findings are consistent with previous reports showing cytotoxic effects of crude extracts from other *Eulophia* species (*Eulophia petersii* Rchb. F. and *Eulophia nuda*), in various human cancer cell lines [39,40]. Similarly, the main phenanthrene of EM extract, has been reported to exert cytotoxic effects in various cancer cell lines [19]. These findings are consistent with reports of cytotoxic phenanthrene derivatives from *Eulophia nuda* [41]. In the present study, the IC_{50} value for EM extract was 530 µg/mL after 72 h of treatment. In previous pharmacological studies investigating its vascular effects, the half-maximal effective concentration (EC_{50}) to induce a vasorelaxation was approximately 50 µg/mL in resistance arteries and 172 µg/mL in pulmonary arteries [20,21], corresponding to IC_{50}:EC_{50} ratios between 3 and 11. These findings suggest a relative low cytotoxicity of EM extract at pharmacologically relevant concentrations. In contrast, the pure phenanthrene exhibited the cytotoxicity in hepatocytes, with and IC_{50} of 22 µM after 72 h of treatment. Previous reported EC_{50} values for its vasorelaxant effects were approximately 8 µM in

mesenteric arteries and 34 μM in pulmonary arteries [20,21], corresponding to IC_{50}:EC ratios of 2.7 and 0.7, respectively. Even though definitive conclusions cannot be drawn from these *in vitro* observations, the data suggest the need for further investigation into the potential hepatotoxicity of this compound when used for PAH treatment.

The effect of the EM extract and its main phenanthrene on CYPs activities were first explored in rat hepatocyte cultures at non-cytotoxic concentrations. Since EM extract altered CYP activities *in vitro*, we further examined whether similar effects were observed following sub-chronic (21-days) oral treatment in rats. CYP1A, CYP2B, CYP2C, and CYP3A were selected due to their major roles in the metabolism of many drugs including drugs with narrow therapeutic index [8]. CYP1A was also included because it plays an important role in the metabolism of environmental chemicals and pollutants [42]. *In vitro*, EM extract induced CYP1A and CYP3A activities and inhibited CYP2C activity. Importantly, these effects were observed only at the highest concentrations tested (between 50 to 300 μg/mL), and the variations of CYP activities measured were low (approximately 2-3-fold relative to control). These changes were modest compared with strong induction observed with the reference inducers BNF and PB. Although CYP activity assays provide functional insight into enzyme modulation, they do not allow discrimination between direct enzyme induction or inhibition, altered protein expression, or transcriptional regulation mediated by nuclear receptors [9,10]. Therefore, the observed changes in CYP activity should be interpreted as net functional outcomes rather than specific mechanistic effects. Future studies incorporating specific CYP inhibitor controls may help further distinguish direct enzyme inhibition from other regulatory mechanisms.

In vivo, EM extract did not affect body weight gain, or organ to body weight ratios after sub-chronic treatment, which is consistent with previous reports showing no significant toxicity following acute or long-term administration [28]. Under our experimental conditions, EM extract induced some changes in CYP activities that were dependent on the administered dose. Previous studies in rats have identified doses of 450 and 1,000 mg/kg as effective for treating PAH in the monocrotaline model [21], and doses of 15, 150 and 450 mg/kg for reversing erectile dysfunction [23]. At 15 mg/kg, EM extract inhibited CYP3A and CYP2C activities, whereas at 450 mg/kg it increased CYP1A activities. This discrepancy may be explained by differences in experimental conditions [43], including oral absorption, first-pass metabolism, and the formation of active metabolites *in vivo*, whereas *in vitro* hepatocyte models are directly exposed to the parent compounds. Moreover, the observed pattern of CYP modulation, with inhibition at low doses and induction at higher doses, suggests a potential non-linear pharmacological response, possibly involving both direct enzyme interaction and nuclear receptor-mediated transcriptional regulation [9,44]. The results suggest that phenanthrene is unlikely to be the sole contributor to the CYP-modulating effects observed with the EM extract. Indeed, unlike the EM extract, phenanthrene did not affect CYP activities *in vitro*. *In vivo*, phenanthrene only moderately decreased CYP1A activity at the highest administered dose, whereas EM extract exerted the opposite effect. A limitation of the present study relates to the use of isolated phenanthrene compound. Due to its limited availability, it was not feasible to perform *in vivo* efficacy studies in established disease models such as pulmonary arterial hypertension (PAH) or erectile dysfunction (ED). As a result, the dose selection for phenanthrene in the *in vivo* study was based on previously reported *ex vivo* vasorelaxation data rather than on *in vivo* efficacy or pharmacokinetic parameters. Therefore, the relationship between administrated dose, systemic exposure, and pharmacological efficacy remains unclear. This limitation may partly explain the lack of pronounced CYP modulation despite observable physiological effects, such as reduced body weight gain. Further studies are required to determine the pharmacokinetic profile and optimal *in vivo* efficacy dose of phenanthrene. Notably, phenanthrene treatment resulted in reduced body weight gain. Even though we did not detect differences in organ weights, this finding warrants further toxicological investigation of this phenanthrene. Recent formulation studies have improved the delivery of EM extracts, potentially increasing bioavailability and exposure to active compounds [45].

Overall, the present findings provide important preliminary insight into the effects of *Eulophia macrobulbon* extract and its phenanthrene constituent on CYP-mediated drug metabolism using integrated *in*

vitro and *in vivo* approaches. The findings also highlight the potential relevance of multi-component herbal extracts in modulating CYP-related herb-drug interaction risks. Nevertheless, some limitations should be considered when interpreting the results. First, CYP modulation was assessed using activity-based assays, which reflect overall changes in enzyme activity but do not distinguish between direct enzyme inhibition, altered expression, or nuclear receptor-mediated regulation. Further mechanistic studies (e.g., RT-qPCR, Western blot, or receptor assays) would help clarify the underlying mechanisms. While rat models are widely used in preclinical CYP studies because of their well-characterized hepatic metabolism, reproducibility, and partially conserved CYP regulatory pathways involved in enzyme induction and drug metabolism, they remain suitable for evaluating *in vivo* enzyme regulation and herb-drug interaction potential. Nevertheless, species differences between rat and human CYP isoforms should still be considered when extrapolating the findings to human drug metabolism. Therefore, further studies using human-derived systems would help improve translational relevance. In addition, the lack of pharmacokinetic and *in vivo* efficacy data for phenanthrene limits interpretation of dose-exposure-response relationships. Collectively, these findings provide a useful foundation for future mechanistic and translational studies investigating the herb-drug interaction potential of *Eulophia macrobulbon* and related phenanthrene-containing herbal preparations.

Conclusions

The ethanolic extract of *Eulophia macrobulbon* and its major phenanthrene constituent, 1-(4′-hydroxy-benzyl)-4,8-dimethoxyphenanthrene-2,7-diol, were shown to modulate hepatic CYP activities and may therefore contribute to potential herb-drug interactions in future clinical applications. Although the observed changes were generally modest, inhibition of CYP2C and CYP3A activities by the EM extract was detected at low *in vivo* doses. The discrepancies between *in vitro* and *in vivo* findings suggest that additional metabolites, systemic exposure, first-pass metabolism, and interactions among multiple constituents within the extract may contribute to CYP modulation. Overall, these findings provide preliminary insight into the CYP-modulating effects and safety considerations of *Eulophia macrobulbon* and its bioactive constituents.

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Declaration of Generative AI in Scientific Writing

The authors declare that no generative AI tools or AI-assisted technologies were used in the writing, reviewing, or grammar checking of this research manuscript. All content was solely developed, written, and edited by the authors.

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