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Identification of CYP isoforms involved in the metabolism of thymol and carvacrol in human liver microsomes (HLMs)

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Carvacrol and thymol are phenolic compounds with similar structures isolated from many aromatic plants, and have been demonstrated to exert multiple pharmacological effects. The metabolic and pharmacokinetic behaviour of thymol and carvacrol has received much attention. Carvacrol and thymol have been demonstrated to undergo phase I metabolism such as hydroxylation reaction. However, drug-metabolizing enzymes involved in this process remain unclear. Given that cytochrome P450 s (CYPs) are involved in most phase I metabolism, the aim of the present study was to investigate the role of CYPs in the metabolism of thymol and carvacrol. After incubation with human liver microsomes (HLMs) in the presence of NADPH, a new metabolite and two metabolites were detected for thymol and carvacrol, respectively. A combination of chemical inhibition studies and assays with recombinant CYP isoforms demonstrated that CYP2A6 was the predominant drug-metabolizing enzyme involved in the metabolism of thymol and carvacrol. All these results remind the researchers that special attention should be paid on pharmacokinetic and clinical outcomes when thymol or carvacrol was co-administrated with other compounds mainly undergoing CYP2A6-mediated metabolism.

1. Introduction

Thymol and carvacrol (Fig. 1) are two phenolic compounds with similar structures found in numerous aromatic plants, and have been reported to exhibit multiple biochemical and pharmacological activities including antibacterial, antifungal and antioxidative effects (Veldhuizen et al. 2006; Chami et al. 2004; Nafisi et al. 2004).

The metabolism of thymol and carvacrol has been drawing much attention. Williams (1959) reported that thymol was extensively metabolized through glucuronidation and sulphation reaction, and the small amounts underwent ring oxidation to form thymoquinol (2,5-dihydroxy-p-cymene) in rabbits, dogs and men. The experiment carried out by Austgulen et al. (1987) showed that thymol and carvacrol could undergo hydroxylation and glucuronidation reactions. To date, investigation of thymol and carvacrol metabolism mainly focuses on the elucidation of the metabolites. However, the involved drug-metabolizing enzymes remain unclear.

Cytochrome P450 s (CYPs), a superfamily of heam-containing isoenzymes mainly located in hepatocytes, are the enzymes most commonly involved in the phase I metabolism of xenobiotics (Guengerich 2006). Generally, CYP enzymes bind two atoms of oxygen and result in the formation of a water molecule together with the generation of a polar metabolite. The reactions catalyzed by CYPs contain hydroxylation, dealkylation and even reduction reaction (Fang et al. 2011a).

Given the fact that CYPs play a key role in phase I metabolism and thymol/carvacrol has been demonstrated to undergo phase I metabolism, the aim of the present study was to investigate the role of various CYP isoforms in the phase I metabolism of thymol and carvacrol.

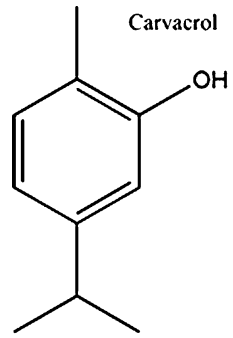
2. Investigation and results

2.1. Detection of thymol and carvacrol metabolites in HLM

After incubation of thymol with HLMs and NADPH-generating system, a new peak was eluted at 14.5 min (M) for thymol (Fig. 2A). This new peak was not detected in the negative controls without NADPH, without substrate, and without microsomes. For the metabolism of carvacrol, two new peaks were eluted at 14.7 min (M-1) and 15.3 min (M-2) (Fig. 2B). These two new peaks were not detected in the negative controls without NADPH, without substrate, and without microsomes.

2.2. Kinetic study

Under the experimental conditions used, the metabolism of thymol in HLMs obeyed typical Michaelis-Menten kinetics, as evidenced by Eadie-Hofstee plots (Fig. 3A). The kinetic parameters (apparent V_{max} and K_m) were calculated to be 0.58 ± 0.02



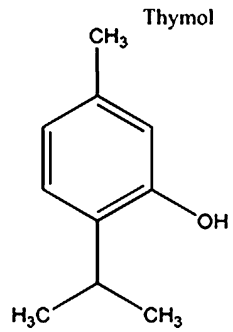
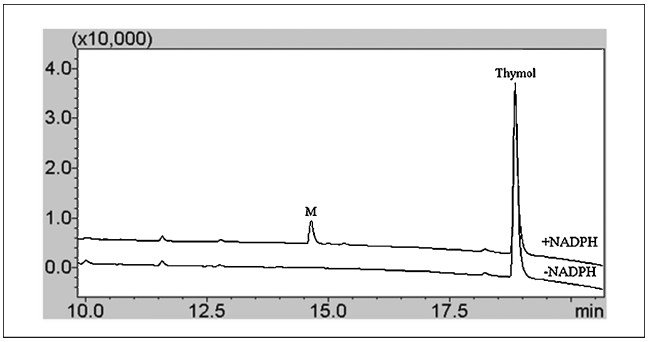
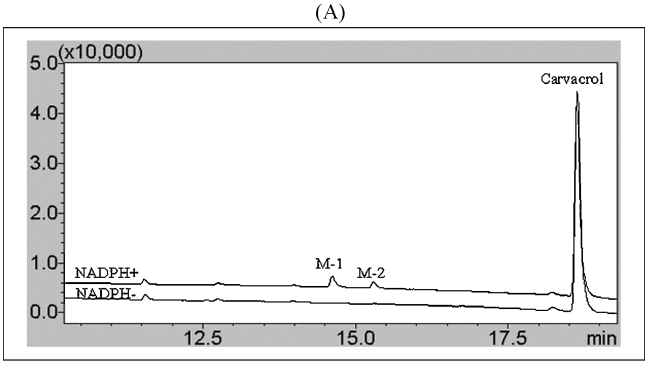


Fig. 1: Structures of thymol and carvacrol

nmol/min/mg pro and 19.8 ± 2.2 μM. The metabolism of carvacrol in HLMs obeyed the typical Michaelis-Menten kinetics, as evidenced by Eadie-Hofstee plots (Fig. 3B and Fig. 3C). The kinetic parameters (K_m and V_{max}) were 9.8 μM and 0.78 nmol/min/mg pro for M-1, and 9.3 μM and 0.37 nmol/min/mg pro for M-2.

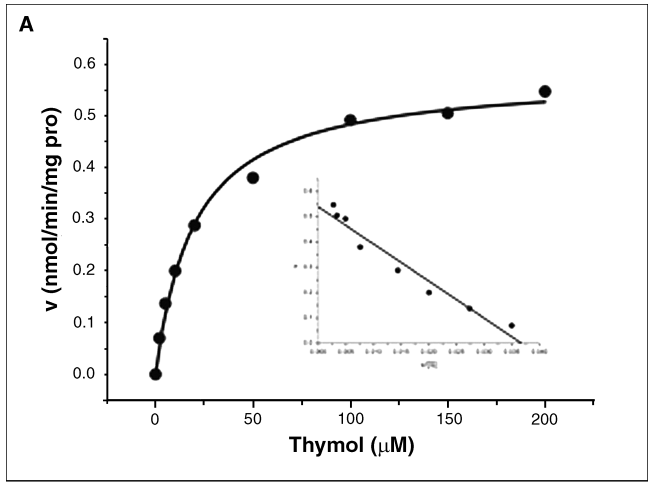


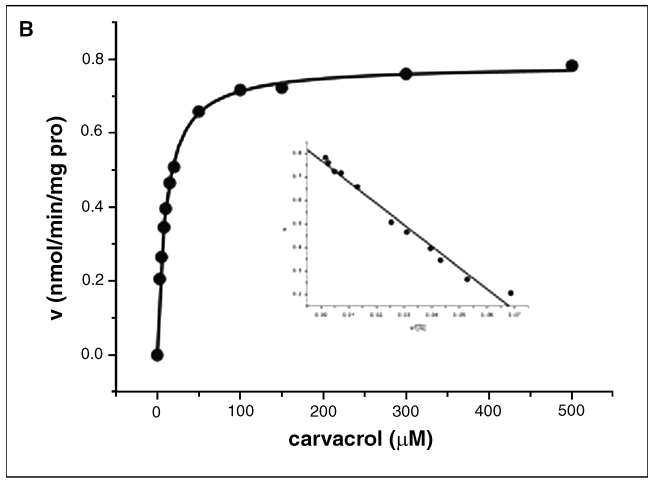
(A)



(B)

Fig. 2: Representative HPLC-UV profile of thymol and carvacrol metabolism. (A) Thymol (100 uM) was incubated with human liver microsomes (HLMs) (0.5 mg/ml) with (upper) and without (lower) NADPH-generating system. (B) Carvacrol (100 uM) was incubated with human liver microsomes (HLMs) (0.5 mg/ml) with (upper) and without (lower) NADPH-generating system





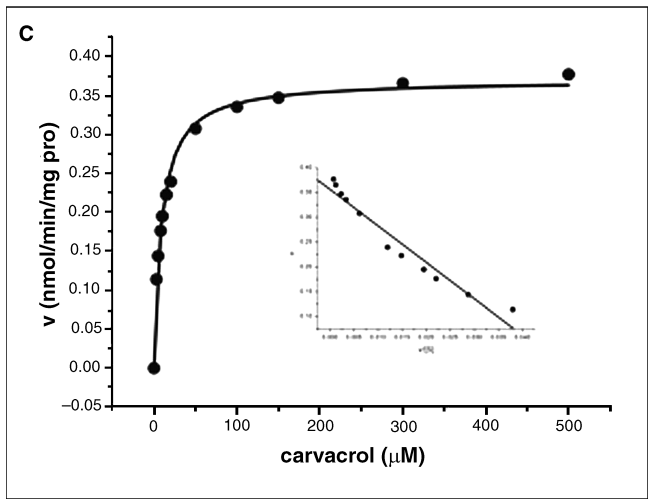


Fig. 3: Enzyme kinetics of thymol and carvacrol metabolites formation. (A) The kinetics of thymol metabolism. Thymol (1–200 uM) was incubated with pooled HLMs (0.3 mg/ml) at 37°C for 30 min. (B) The kinetics of carvacrol M-1 metabolite formation. Carvacrol (1–500 uM) was incubated with pooled HLMs (0.3 mg/ml) at 37°C for 30 min. (C) The kinetics of carvacrol M-2 metabolite formation. Carvacrol (1–500 uM) was incubated with pooled HLMs (0.3 mg/ml) at 37°C for 30 min

2.3. Chemical inhibition study

The effect of various chemical inhibitors on the metabolism of thymol was investigated in pooled HLMs (Fig. 4A). ABT, the broad CYP inhibitor, strongly inhibited the formation of thymol metabolite, suggesting that CYPs were the major drug-metabolizing enzymes involved in the metabolism of thymol. Among the selective inhibitors of nine CYP iso-

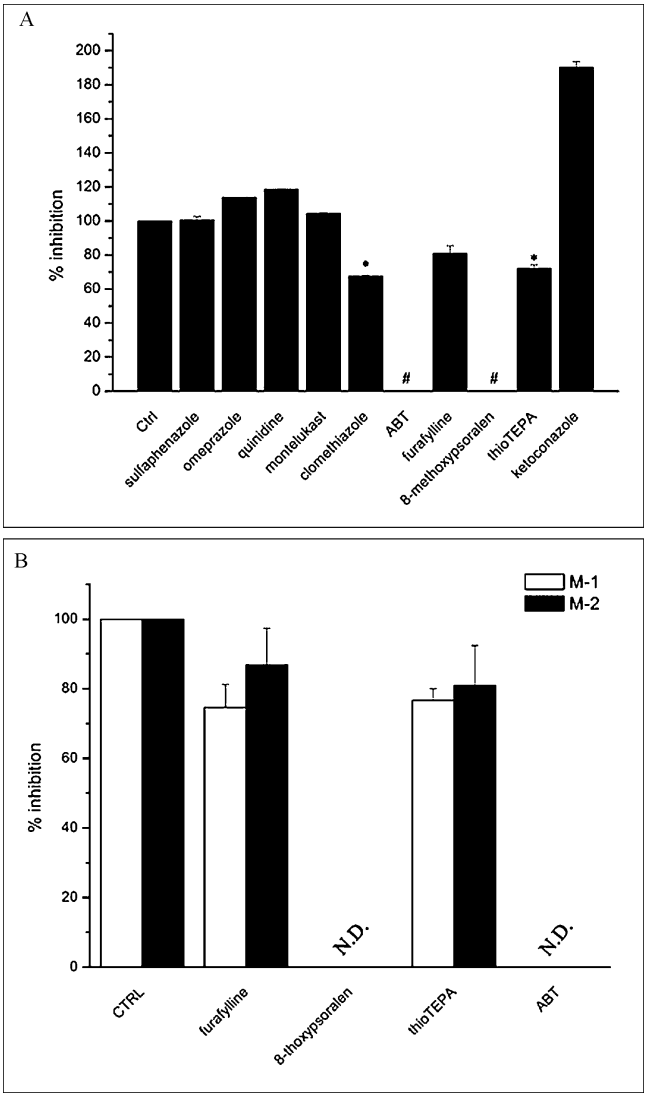


Fig. 4: The inhibition of thymol (A) and carvacrol (B) metabolism by CYP selective inhibitors. Data was given as mean ± standard evidation (S.D.). Statistical differences were considered significant at the $*p < 0.05$, $\#p < 0.01$ level. All incubations were carried out in duplicate

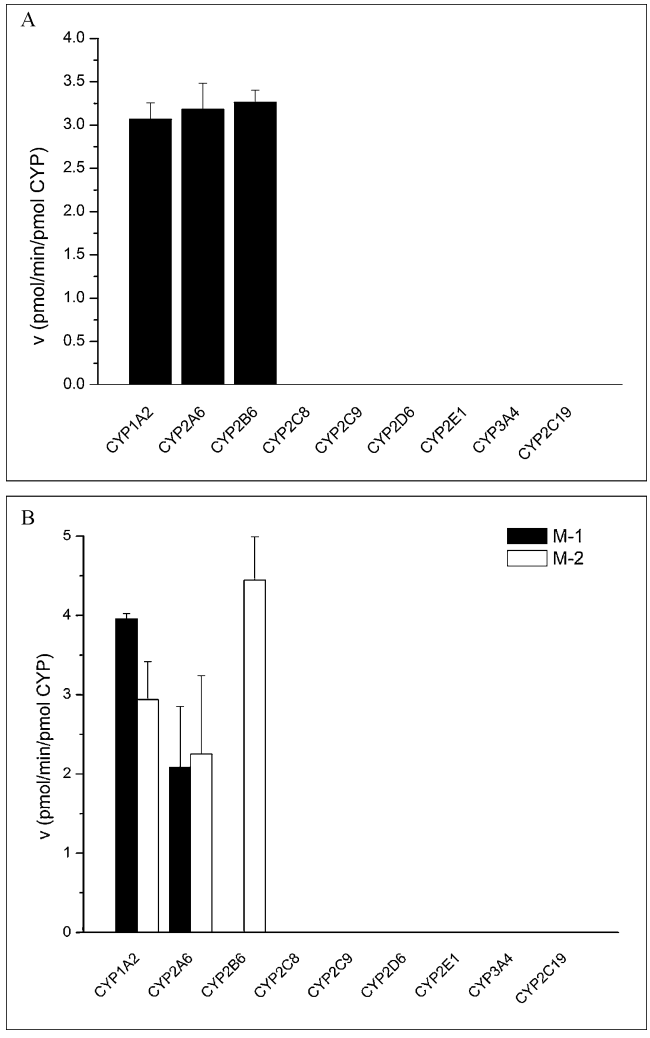


Fig. 5: Formation of metabolites from thymol (A) and carvacrol (B) by cDNA-expressed CYP isoforms. All incubations were carried out in duplicate. Data were given as mean ± standard evidation (S.D.)

forms, 8-methoxypsolaren (the selective inhibitor of CYP2A6) almost completely inhibited the formation of thymol metabolite. As shown in Fig. 4B, ABT, the broad CYP inhibitor, strongly inhibited the formation of carvacrol metabolites (M-1, M-2), suggesting that CYPs were the major drug-metabolizing enzymes involved in the metabolism of carvacrol. 8-methoxypsolaren (the selective inhibitor of CYP2A6) almost completely inhibited the formation of M-1 and M-2.

2.4. Assay with human recombinant CYP isoforms

Nine recombinant CYP isoforms were employed to identify the CYP isoforms involved in the metabolism of thymol and carvacrol. The results (Fig. 5A) showed that CYP1A2, CYP2A6 and CYP2B6 could catalyze the formation of thymol metabolite. The levels of involvement of other CYP isoforms in the metabolism of thymol were negligible. For the metabolism of carvacrol, the results (Fig. 5B) showed that CYP1A2, and CYP2A6 could catalyze the formation of M-1, and CYP1A2, CYP2A6 and CYP2B6 could catalyze the formation of M-2. The levels of involvement of other CYP isoforms in the metabolism of carvacrol were negligible.

3. Discussion

Studies on the metabolism of a new chemical entity (NCE) have an important role for determining an optimal window between the drug safety parameters and its therapeutic potential (Lin and Lu 1997; Fang et al. 2011b). Therefore, identification of the drug-metabolizing enzymes involved in metabolism of thymol and carvacrol is very important. The present experimental data show that CYP2A6 is the major drug-metabolizing enzyme involved in the thymol and carvacrol metabolism. This conclusion was drawn from the following observations: 1) For thymol and carvacrol metabolites formation in HLMs, monophasic Eadie-Hofstee plot was obtained, indicating the participation of one primary drug-metabolizing enzyme. 2) CYP1A2, CYP2A6 and CYP2B6 could catalyze the formation of thymol metabolite. For carvacrol, CYP1A2, and CYP2A6 could catalyze the formation of M-1, and CYP1A2, CYP2A6 and CYP2B6 could catalyze the formation of M-2. 3) ABT, the broad CYP inhibitor, almost completely inhibited the formation of metabolites of thymol and carvacrol, which indicated that CYPs are the major enzymes involved in the thymol and carvacrol metabolites formation. Among the selective inhibitors of CYP isoforms, the specific CYP2A6 inhibitor (8-methoxypsoralen) significantly inhibited the thymol and carvacrol metabolites formation.

CYP2A6 is mainly expressed in human liver and also found in other tissues, such as nasal epithelium, trachea, lung and esophagus (Koskela et al. 1999; Godoy et al. 2002). High

inter-individual variability was found in hepatic CYP2A6 expression (30-fold) (Yun et al. 1991). A variation in expression of CYP2A6 has also been found in lung (50-fold) (Zhang et al. 2007) and esophagus (41-fold) (Godoy et al. 2002). Additionally, CYP2A6 alleles might significantly influence the function of this CYP isoforms. For example, *CYP2A6*4* presents a gene deletion, which accounts for majority of poor metabolizer individuals (Oscarson et al. 1999). *CYP2A6*20* allele, resulting in a truncated protein without activity, was found in African-Americans with a frequency of 0.016, but not among European-Americans, Japanese and Koreans (Nakajima et al. 2006; Fukami et al. 2005). The different response to the pharmacokinetic and clinical outcomes within people after administration of thymol and carvacrol can be forecasted due to high interindividual viability of CYP2A6. Additionally, when thymol and carvacrol were co-administered with the compounds mainly undergoing CYP2A6-mediated metabolism, potential interactions should be paid more attention.

In conclusion, CYP2A6 was demonstrated to be the major drug-metabolizing enzyme involved in the metabolism of thymol and carvacrol, which is helpful for a deeper understanding of metabolic and pharmacokinetic behaviours of thymol and carvacrol.

4. Experimental

4.1. Chemicals and reagents

Thymol (purity, 99%) and carvacrol (purity, 99%) were purchased from Aladdin Corp. (Shanghai, China). Quinidine, 1-aminobenzotriazole (ABT), D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, sulfaphenazole, clomethiazole, furafylline, 8-methoxypsoralen, and omeprazole were obtained from Sigma-Aldrich (St Louis, MO, USA). Ketoconazole (KTZ) was purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA). Triethylenethiophosphoramide (thioTEPA) was purchased from Acros Organics (Geel, Belgium). Montelukast was purchased from Beijing Aleznova Pharmaceutical (Beijing, China). All other reagents were of high-performance liquid chromatography (HPLC) grade or the highest purity commercially available.

cDNA-expressed recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 derived from baculovirus-infected insect cells co-expressing NADPH-P450 reductase were obtained from BD Gentest (Woburn, MA USA). cDNA-expressed CYP2C8 and CYP2C19 in Escherichia coli co-expressing NADPH-P450 reductase were purchased from New England Biolabs (Ipswich, MA).

4.2. Preparation of human liver microsomes

Human liver samples were obtained from Dalian Medical University (Dalian, Liaoning province, China) with the approval of the local ethics committee at the university. A panel of human liver microsomes (HLMs) was prepared from twelve liver samples obtained from male and female patients by differential ultracentrifugation as described previously (Fang et al. 2010; Sun et al. 2010; Qu et al. 2011; Dong et al. 2012). Microsomal protein concentrations were determined by the Lowry method with bovine serum albumin as standard (Lowry et al. 1951).

4.3. Incubation system

The incubation mixture, with a total volume of 200 μl, contained 100 mM potassium phosphate buffer (pH 7.4), NADPH generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase and 4 mM $MgCl_2$), human liver microsomes (0.5 mg/ml) and thymol/carvacrol (100 μM). The reaction was initiated by adding the NADPH-generating system after 3-min pre-incubation at 37 °C. After incubation for 10 min in a shaking water bath, the reaction was terminated by the addition of methanol (100 μl). The mixture was kept on ice until it was centrifuged at 20000× g for 10 min at 4 °C. Aliquots of supernatants were transferred for HPLC analysis. Control incubations without NADPH or without substrate or without microsomes were included to ensure that metabolites formation were microsomes and NADPH dependent. All incubations were carried out in duplicate.

4.4. LC/UV method

Chromatography was performed with a Shimadzu (Kyoto, Japan) HPLC system, which was equipped with a SCL-10A system controller, two LC-10AT pumps, a SIL-10A auto injector and a SPD-$10A_{vp}$ UV detector. A C_{18} column (4.6 mm × 200 mm, 5 μm, Kromasil) was used to separate thymol and its metabolites. The mobile phase consisted of CH_3CN (A) and H_2O containing 0.5 % (v/v) formic acid (B). For thymol, the following gradient conditions were used: 0–4 min, 95–85% B; 4–18 min, 85–5% B; 18–25 min, 5% B; 25–33 min, 95% B. For carvacrol, the mobile phase consisted of CH3OH (A) and H_2O containing 0.5 % (v/v) formic acid (B) (A:B = 55:45, v:v). The flow rate of the mobile phase was set at 1 ml/min. The injection volume was 50 μl and the scan wavelength was set at 277 nm.

4.5. Chemical inhibition study

The chemical inhibition study was performed as previously reported (Fang et al. 2011a; Huang et al. 2010). Briefly, chemical inhibition studies were carried out by adding different human CYP inhibitors to the incubation mixture of thymol (20 μM) or carvacrol (10 μM) before the addition of the NADPH-generating system. The inhibitors utilized were as follows: furafylline (10 μM) for CYP1A2, 8-methoxypsoralen (2.5 μM) for CYP2A6, thioTEPA (50 μM) for CYP2B6 (Rae et al. 2002), montelukast (5 μM) for CYP2C8 (Walsky et al. 2005), sulfaphenazole (10 μM) for CYP2C9, omeprazole (20 μM) for CYP2C19, quinidine (10 μM) for CYP2D6, clomethiazole (50 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A4, and ABT (500 μM) for broad CYPs (Emoto et al. 2005). Inhibiton by furafylline, 8-methoxypsoralen, thioTEPA and ABT were examined by adding thymol/carvacrol after pre-incubation with NADPH-generating system at 37 °C for 20 min.

4.6. Assay with recombinant CYPs

The incubation for each CYP isoform was carried out as described for the microsomal study. Thymol (20 μM) or carvacrol (10 μM) was incubated with each of the recombinant CYPs (10–20 nM) at 37 °C for 60 min and plausible metabolites were monitored.

4.7. Kinetic study

To evaluate kinetic parameters, thymol (1–200 μM) or carvacrol (1–500 μM) was incubated with pooled human liver microsomes (0.3 mg/ml) for 30 min. Preliminary experiments were performed to make sure that the formation of metabolites was in the linear range of both reaction time and the concentration of microsomes. The apparent V_{max} and K_m values were calculated from nonlinear regression analysis of experimental data according to the Michaelis-Menten equation. Due to the absence of authentic standards for metabolites of thymol and carvacrol, quantification of the metabolites in the incubation mixtures was achieved using a standard curve for thymol or carvacrol.

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