Differences in xanthotoxin metabolites in seven mammalian liver microsomes

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Abstract. Xanthotoxin, abundantly occurring in fruits, vegetables, grapefruit juice and oils, is widely used in medicine for the treatment of psoriasis and vitiligo. Xanthotoxin possesses the ability to inhibit mechanism-based cytochrome P450 (CYP450)-mediated activities in rats and mice. Furthermore, it time-dependently obstructs a number of CYP450-mediated functions in humans. CYP450 enzymes are most abundant in the liver and induce metabolic activation of numerous xenobiotic compounds. The present study aimed to identify the similarities and differences in xanthotoxin metabolism in liver microsomes of 7 mammalian species, including human liver microsomes (HLM), Rhesus monkey liver microsomes (RMLM), Cynomolgus monkey liver microsomes (CMLM), Sprague Dawley rat liver microsomes (RLM), mouse liver microsomes (MLM), Dunkin Hartley guinea pig liver microsomes (PLM) and Beagle dog liver microsomes (DLM). Ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometric analysis was used to determine the metabolites. A total of 3 metabolites were detected in RMLM, CMLM and RLM. Furthermore, two metabolites were observed in MLM, HLM, PLM and DLM. By analyzing the type and quantity of metabolites, the metabolism of xanthotoxin in MLM was indicated to be most similar to that in HLM. The metabolic transformations of xanthotoxin in the liver microsomes of the 7 species were analyzed in further detail. On the whole, the results of the present study provide a deeper understanding of the metabolic patterns of xanthotoxin in liver microsomes of different species, which may prove to be advantageous regarding the metabolic mechanisms of action of xanthotoxin. Further insight into drug metabolism with respect to different species will also aid in the selection of appropriate animal models for further research.

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Introduction

Furocoumarins are biologically potent organic compounds occurring in various traditional herbal medicines and foods, particularly those belonging to the Umbelliferae and Rutaceae families (1,2). Xanthotoxin (8-methoxypsoralen) is an organic substance prevalent in various edible plants. It may be used in combination with long-wavelength ultraviolet (UV) light as a treatment for psoriasis, vitiligo and T-cell lymphoma (3). It is a photosensitive substance mostly used in clinical psoralen UV A (PUVA) therapy for vitiligo, psoriasis, as well as other skin conditions. PUVA is a UV light (320-360 nm) therapy for skin diseases, using the sensitizing effects of the drug psoralen. Previous studies have reported the modulation of various cytokines and the decontamination of platelet concentrates by methoxypsoralen and long-wavelength UV radiation (4,5).

Cytochrome P450 (CYP450) enzymes, which are abundant in the liver, are responsible for promoting the metabolic stimulation of a sizable number of xenobiotic compounds. However, under certain conditions, CYP450 catalysis is able to bring about reactive species that bind to and inhibit CYP450, and the complex attaches to other cellular proteins which may lead to cell death (6). Xanthotoxin is a substance with a recognized ability to suppress rat and mouse CYP450-mediated activities (7-11) and is known as a time-dependent inhibitor of various human CYP450-mediated activities (12). Certain studies have suggested that xanthotoxin is able to function as an efficient mechanism-based inhibitor of CYP2A6 activities, which discriminatorily incapacitates this enzyme in human liver microsomes (HLM) when utilized at lower concentrations and for limited exposure durations (13,14).

In addition, since animal microsomes are routinely employed for determining metabolic patterns in humans, it is imperative to identify inter-species differences between humans and animals with regard to metabolic activities mediated by CYP450 (15-17). Therefore, the present study sought to identify the similarities and differences in xanthotoxin metabolism in liver microsomes of 7 mammalian species,

including HLM, Rhesus monkey liver microsomes (RMLM), cynomolgus monkey liver microsomes (CMLM), Sprague Dawley rat liver microsomes (RLM), mouse liver microsomes (MLM), Dunkin Hartley guinea pig liver microsomes (PLM) and Beagle dog liver microsomes (DLM). To date, at least to the best of our knowledge, no studies are available on the differences in xanthotoxin metabolites in humans and other mammalian liver microsomes. The present study therefore aimed to provide insight into metabolic functions with regard to xanthotoxin and to identify specific differences among species. The present results may aid future investigations on xanthotoxin, which may prove to be beneficial to humans in the future.

Materials and methods

Materials and testing agents. Xanthotoxin was obtained from The Chinese National Institute for the Control of Pharmaceutical and Biological Products. NADPH, KH_2PO_4-K_2HPO_4 buffer and DMSO were purchased from Sigma-Aldrich (Merck KGaA). Human, Rhesus and Cynomolgus monkey, Sprague Dawley rat, mouse, Dunkin Hartley guinea pig and Beagle dog microsomes were obtained from the Research Institute for Liver Diseases Shanghai, Co., Ltd. Liver microsomes were stored at -80˚C. High-performance liquid chromatography (HPLC) was used to separate, identify and quantify all mixture components.

Ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometric analysis (UPLC/Q-TOF MS). The UPLC/Q-TOF MS procedure was performed using an Agilent 6520 Q-TOF LC/MS system and MassHunter Workstation (Agilent Technologies Inc.). An Extend-C18 column (1.8 μm, 2.1x50 mm) was used. The elution gradient consisted of mobile phase A (0.1% aqueous formic acid) and B (100% methanol). The UPLC gradient curriculum employed was thus motile phase A, which was maintained at 90% for a duration of 3 min, and thence a linearly set up gradient lowered moving phase A out of 90% to become 10% at the end of 14 min and maintained at 10% through a time of 3 min. This was finally increased to 90% A to equilibrate the column. The column temperature was maintained at 30˚C together with the flow rate kept at 0.2 ml/min. Acquisition of mass was performed in the duo of positive ion mode [electron spray ionization $(ESI)^+$] and negative ion mode (ESI^-) from a mass-to-charge ratio (m/z) of 80 towards 1,000 Da, making use of a source temperature of 100˚C, a desolvation temperature of 350˚C and a dissolution gas flow standing at 600 l/h.

Metabolism of xanthotoxin in liver microsomes. Xanthotoxin (final concentration, 50 μM) was dissolved in DMSO (final concentration <0.5%) and diluted (PBS; 0.1 m, pH 7.4). For each of the 7 species individually, the liver microsomes (50 μl; final concentration, 2 mg/ml) were mixed with standard solution (50 μl) and pre-incubated for 5 min at 37˚C. The above reaction mixture was added to NADPH solution (100 μl; final concentration, 1 mM), which was diluted in PBS (0.1 M; pH 7.4). A total of 400 μl ice-cold methanol was utilized to terminate the reaction at the end of the 120-min incubation at a temperature of 37˚C. Following centrifugation at 10,000 x g for a total duration of 10 min at 4˚C, the supernatant was filtered through a 0.22-μm filter and was collected for analysis by UPLC/Q-TOF MS. A control reaction was set up without xanthotoxin. An aliquot of 5 μl of the solution was analyzed by UPLC/Q-TOF MS and HPLC/MS/MS.

Statistical analysis. The content of each metabolite was compared according to the peak area of the metabolite. Values are expressed as the mean ± standard deviation of three samples. One-way ANOVA with the post hoc Student-Newman-Keuls test was performed to confirm inter-group differences with SPSS 11.5 software (SPSS Inc.). $P<0.05$ was considered to indicate statistical significance.

Results

Q-TOF MS analysis of xanthotoxin metabolites. Xanthotoxin had a retention time (Rt) of 14.0595 min and in ESI^+ mode, the protonated molecule $[M+H]^+$ exhibited a peak at m/z 217.0956. For xanthotoxin, the noteworthy fragment ions at m/z 202.0657, 189.0898, 174.0662, 161.0958 and 146.0708 in the collision energy 25 eV mass spectrum were generated by means of the loss of -CH_3 and small neutral molecules, e.g. CO. Q-TOF mass spectra and xanthotoxin metabolite and fragment information are provided in Fig. 1.

The metabolization of xanthotoxin comprises the hydroxylation of the phenyl ring, hydrogenation and hydrolysis of the lactone ring (18,19). Oxidation of the furan ring produces a furanoepoxide or γ-ketoenal intermediate. Furanoepoxide then forms a dihydrodiol, while γ-ketoenal is converted to hydroxy coumaryl acetic acid. The structures and formation of metabolites of xanthotoxin are presented in detail in Fig. 2. M1-3 are metabolizations of xanthotoxin detected in RMLM, CMLM and RLM, MLM, HLM, PLM and DLM. The metabolites M1 (Fig. 2A) included protonated molecules $[M+H]^+$ in the vicinity of m/z 235.1831. Meticulous mass analysis revealed the chemical formula of $C_{12}H_{10}O_5$, propounding the hydroxylation and hydrogenation of xanthotoxin. The steep energy mass spectra of M1 revealed a major product ion with m/z 176.1473 and the major fragment ion was observed at m/z 162.1224. The indicated remnant ions suggested that the alteration took place at the site C5 of xanthotoxin.

The metabolites M2 (Fig. 2B) included the molecule $[M+H]^+$ at m/z 251.1780 along with $[M-H]^-$ at m/z 249.1275. The fragment ions suggested that xanthotoxin is transformed into an epoxide intermediate that reacts to become a dihydrodiol (M2). This result is in agreement with that of an earlier study (20). The deprivation of H_2O and the ensuing deficiency of CO leads to the formation of the compound with m/z 205 and corroborates the structure of M2.

The metabolite M3 (Fig. 2C), characterized by $[M-H]^-$ with m/z 251.1041, is in accordance with a hydroxylation on the furan ring, and is also an oxidation product of the dihydrodiol (M2). The fragments include ions at m/z 193.1379, 221.1317 and 165.0960.

Identification of the types and amount of metabolites in liver microsomes from various species. To identify probable metabolic avenues of xanthotoxin, the metabolites obtained from 7 liver microsome incubations were initially analyzed by

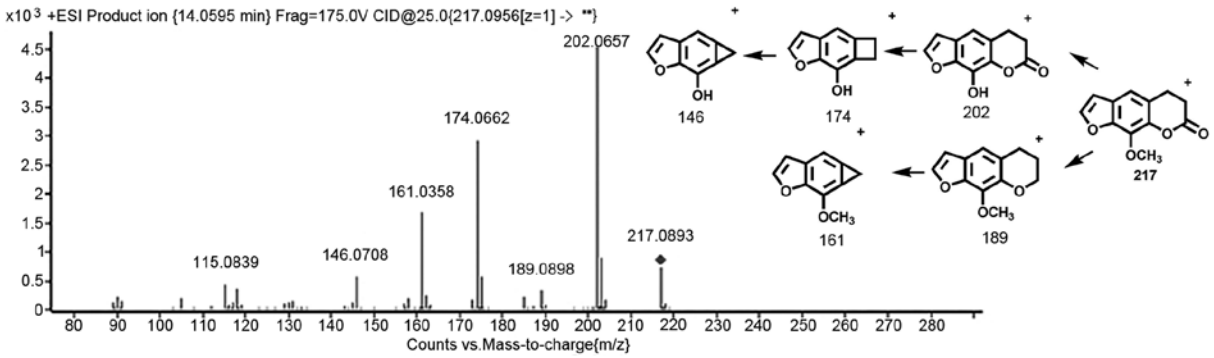

Figure 1. Quadrupole Frag information of xanthotoxin. Secondary mass spectrometry was performed to obtain protonated ion information. Frag, time-of-flight mass spectrum and fragment; CID, collision-induced dissociation.

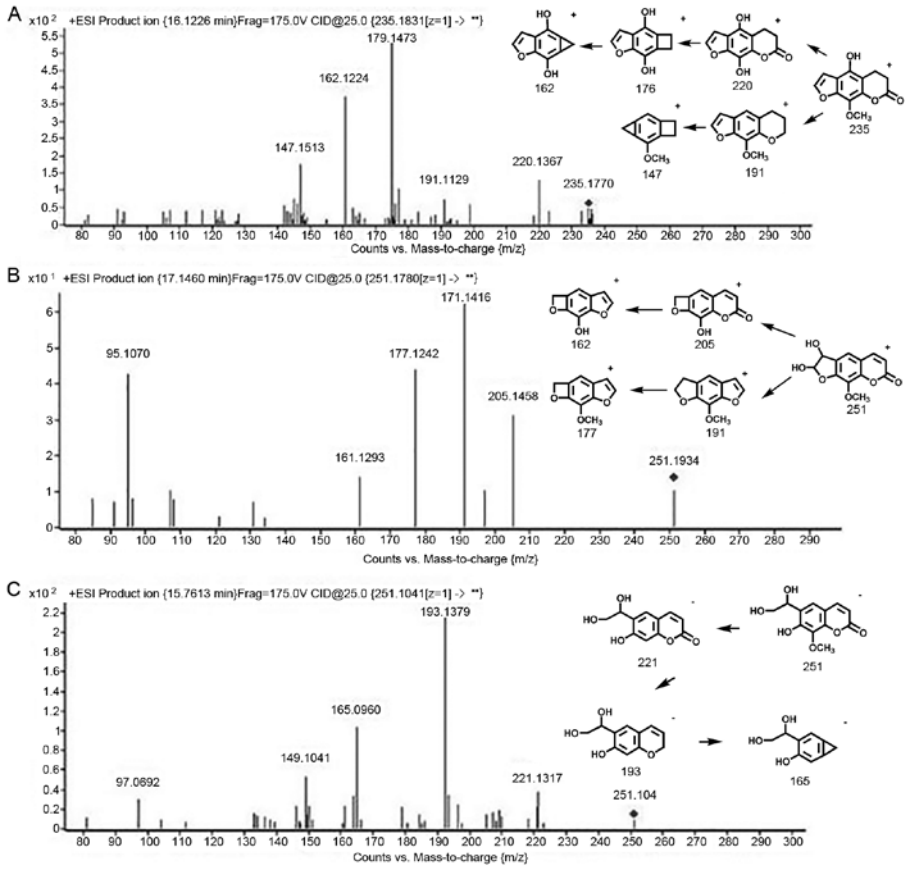

Figure 2. Quadrupole Frag spectra of xanthotoxin metabolites. (A) M1; (B) M2; (C) M3. Structural analysis of three metabolites in combination with the original nuclear structural formula. Frag, time-of-flight mass spectrum and fragment; CID, collision-induced dissociation; M1-3, metabolizations of xanthotoxin detected in RMLM, CMLM and RLM, MLM, HLM, PLM and DLM.

UPLC/Q-TOF MS. A total of 3 metabolites were detected in RMLM, CMLM and RLM, two metabolites were observed in MLM, HLM, PLM and DLM. The array of the metabolites may be contradistinguished by MS and Rt.

As presented in Fig. 3, regarding the amount of metabolites, the metabolites M2 and M3 in MLM were closest to those in HLM.

Table I displays the chemical formulas, authentic masses and Rts of these metabolites of xanthotoxin in the liver microsomes of the 7 species. A total of 3 metabolites of xanthotoxin were detected in RMLM, CMLM and RLM. A duo of metabolites were identified in DLM, PLM, HLM and MLM. The peaks of ion current chromatograms of the metabolite with m/z 250 in ES^+ and ES^- modes for the liver microsomes from all species.

Discussion

At present, multitudinous herbal medicines are used worldwide. Various Chinese traditional medicines and plants have been approved for nutritional and medical purposes (21,22). Traditional Chinese Medicine has stood the test of time for thousands of years and has a long history of usage. Rhizoma ginseng and Radix *Angelicae dahuricae* are all medicinal

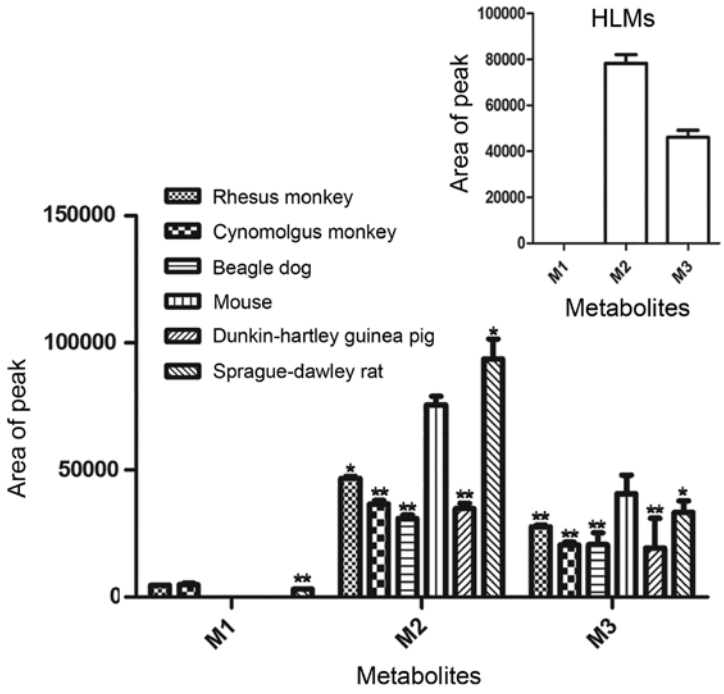

Figure 3. Content of xanthotoxin metabolites in liver microsomes of 7 species (n=3). The metabolism of xanthotoxin in mouse liver microsomes was most similar to that in human liver microsomes. *P<0.05, **P<0.01 compared with the HLM group. HLM, human liver microsomes.

plants belonging to the plant family of Umbelliferae, which are widely used in Traditional Chinese Medicine. Xanthotoxin is a medicinal component of furocoumarins derived from Umbelliferae plants. It is widely used in Traditional Chinese Medicine, including the Chinese patent drug Xiaoyan Xuanshi ointment. Xanthotoxin is widely used in clinical practice for conditions including psoriasis and vitiligo (4,5). Clinical studies have reported on side effects of xanthotoxin (23-25). The present study provided information on the metabolic behavior and routes of xanthotoxin in liver microsomes of 7 species for the first time, to the best of our knowledge. This is useful for investigating the metabolic reactions of xanthotoxin. It provides an important basis for further research and developments. The present study elucidated the biotransformation/metabolism of xanthotoxin, which may have far-reaching and important effects; this knowledge may contribute to the rational and safe use of traditional Chinese and western medicine in the future.

It has been indicated that certain dietary supplements, including grapefruit and hop tea, influence CYP activity and may affect the pharmacokinetics of drugs (26). The effects of grapefruit juice and its inhibitory effects against CYP3A4 have been investigated. Studies have revealed that components of grapefruit juice inhibit CYP3A4 and alter the absorption and metabolism of midazolam (27), vinblastine (28) and digoxin (29), *in vitro* as well as *in vivo*. Furanocoumarin derivatives in grapefruit have been suggested to exert an inhibitory effect on CYPs (30). Previous studies have suggested that xanthotoxin is an influential mechanism-based inhibitor of CYP2A6 activity and immobilizes this enzyme in HLM when applied at low concentrations for short exposure times (31). Another study investigated the reactive metabolites of xanthotoxin as the epoxide intermediate, demonstrating that CYP450 enzymes are responsible for the metabolic activity of xanthotoxin (20). These studies have provided pertinent knowledge for the prudent usage of xanthotoxin. However, extensive data on the metabolites of xanthotoxin, particularly the analogy of metabolic traits amidst liver microsomes from divergent mammalian species, are currently limited, at least to the best of our knowledge. The scope of the present study was geared towards obtaining definitive knowledge regarding the comparison of metabolites and metabolism in liver microsomes among mammalian species.

UPLC/Q-TOF MS revealed that the metabolic behavior of xanthotoxin comprises diverse chemical modifications to form a multitude of metabolites that may have various biological functions. Fig. 4 illustrates the suggested metabolic routes of xanthotoxin in the liver microsomes of the 7 species.

Previous studies focusing on the metabolism of furanocoumarin compounds have disclosed that the transformations include hydroxylation of the phenyl ring (32), hydrogenation of unsaturated lactone ring and hydrolyzation of the lactonic ring (33). However, the production of furanoepoxide/γ-ketoenal via oxidation of the furan ring is the fundamental and ultimately interesting metabolic behavior of the furanocoumarins. The metabolic transformation of furanocoumarins by CYP450 is presented in Fig. 4.

In the present study on the liver microsomes of 7 species, dihydrodiol or hydroxy coumaryl acetic acid was indicated to be derived from the furanoepoxide or γ-ketoenal intermediate, respectively. These results suggest that in diverse species, the furan ring is one utmost integral site of metabolic transformation for furanocoumarin compounds. The present investigation on xanthotoxin may provide a basis for metabolic research with inclusively furan-containing compounds. It may provide important information for further study of drug-drug interactions and food-drug interactions.

In conclusion, in the present study, xanthotoxin and NADPH were incubated in the liver microsomes of 7 mammalian

Table I. Identification of xanthotoxin metabolites in liver microsomes.

		Rt (min)						
Metabolites	Molecular formula	HLM	RMLM	CMLM	RLM	MLM	PLM	DLM
M1	$C_{12}H_{10}O_5$	-	16.1036	16.0826	16.1295	-	-	-
M2	$C_{12}H_{10}O_6$	17.1753	17.2637	17.1670	17.1411	17.2245	17.2146	17.1331
M3	$C_{12}H_{12}O_6$	15.7499	15.8695	15.7310	15.6837	15.6819	15.7781	15.8149
Number	-	2	3	3	3	2	2	2

Rt, retention time; HLM, human liver microsomes; RMLM, Rhesus monkey liver microsomes; CMLM, Cynomolgus monkey liver microsomes; RLM, Sprague Dawley rat liver microsomes; MLM, mouse liver microsomes; PLM, Dunkin Hartley guinea pig liver microsomes; DLM, Beagle dog liver microsomes.

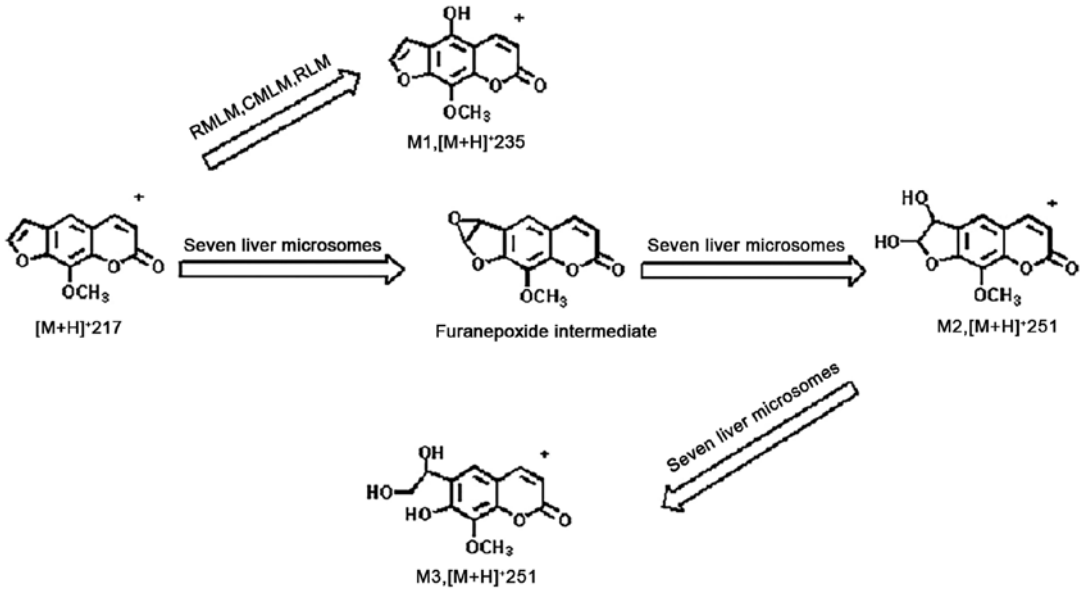

Figure 4. Proposed pathways of metabolism of xanthotoxin in seven liver microsomes: M2 and M3 are metabolized by the production of active intermediates, while M1 is not. RMLM, Rhesus monkey liver microsomes; CMLM, Cynomolgus monkey liver microsomes; RLM, Sprague Dawley rat liver microsomes. M1-3, metabolizations of xanthotoxin detected in RMLM, CMLM and RLM, MLM, HLM, PLM and DLM.

species, including human, Rhesus monkey, Cynomolgus monkey, Sprague Dawley rat, mouse, Dunkin Hartley guinea pig and Beagle dog. Identification of three metabolites of xanthotoxin was achieved. The metabolites of xanthotoxin include a dihydrodiol and hydroxy coumaryl acetic acid from furanoepoxide and γ-ketoenal intermediates, respectively, originating from the oxidation of the furan ring, hydroxylation of the phenyl ring, and hydrogenation and hydrolysis of the unsaturated lactone ester, as well as the lactonic ring. Umbelliferae medicinal plants, including Rhizoma ginseng and *Angelica dahurica*, have been widely used in Traditional Chinese Medicine. Furocoumarins, including psoralen and isopsoralen, are widely used in the clinic. Previous studies by our group have indicated that their metabolites do produce certain serious drug interactions and toxicity through biotransformation and other processes (34,35). Relevant documents include studies by Yang *et al* (18,19). Xanthotoxin is also derived from a medicinal plant of the Umbelliferae, and hence, it is speculated that there may be a similar association between its metabolites. Further studies by our group will aim to expand on the association between the formation of these metabolites and their toxicity in these species.

Due to inter-species differences, different animal liver microsomes exhibit differences in their metabolites of xanthotoxin. Only two metabolites were detected in HLM. The present study mainly focused on the metabolic behavior of xanthotoxin in the liver microsomes of 7 different mammalian species. The activity of CYP450 enzymes is reflected indirectly by the metabolism of substrate. Regarding the types of metabolites, the metabolites of xanthotoxin in MLM, PLM and DLM were the same as those in HLM; regarding the amount of metabolites, the metabolites M2 and M3 of MLM were closest to those in HLM. Analysis of the type and quantity of metabolites indicated that the metabolism of xanthotoxin in MLM was most similar to that in HLM. The results of the present study may provide further knowledge on the metabolic functions of xanthotoxin in liver microsomes of different species and may prove to be useful for future study of the metabolic mechanisms

of action of xanthotoxin. Identification of differences among species regarding drug metabolic processes will aid in the selection of appropriate animal models for future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL performed the liver microsome incubation experiments, analyzed the data and wrote the manuscript. DZ and LZ were responsible for the UPLC/Q-TOF MS experiments and analysis of the data. XH, LW, AY and JAM designed the experiments, analyzed the data and modified of the manuscript. XH received funding for the project, conceived the study and performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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