ARTICLE

Metabolism, pharmacokinetics, and hepatic disposition of xanthenes and saponins on *Zhimu* treatments for exploratively interpreting the discrepancy between the herbal safety and timosaponin A3-induced hepatotoxicity

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Timosaponin A3, a saponin in *Zhimu*, elicited hepatotoxicity via oxidative stress. However, the clinical medication of *Zhimu* has been historically regarded as safe, probably associated with the antioxidants it contains. However, the related information on the in vivo levels of timosaponin A3 and antioxidants remained unclear on *Zhimu* treatments. Therefore, a combination of the in vitro metabolism, including microbiota-mediated and liver-mediated metabolism, and in vivo pharmacokinetics and hepatic disposition, was conducted for three xanthenes (neomangiferin, mangiferin, and norathyriol) and three saponins (timosaponin B2, timosaponin B3, and timosaponin A3) on *Zhimu* treatments. Consequently, following oral administration of *Zhimu* decoction to rats, those saponins and xanthenes were all observed in the plasma with severe liver first-pass effect, where mangiferin was of the maximum exposure. Despite the ignorable content in the herb, timosaponin A3 elicited sizable hepatic exposure as the microbiota-mediated metabolite of saponins in *Zhimu*. The similar phenomenon also occurred to norathyriol, the microbiota-mediated metabolite of xanthenes. However, the major prototypes in *Zhimu* were of limited hepatic exposure. We deduced the hepatic collection of norathyriol, maximum circulating levels of mangiferin, and timosaponin B2 and mangiferin interaction may directly or indirectly contribute to the whole anti-oxidation of *Zhimu*, and then resisted the timosaponin A3-induced hepatotoxicity. Thus, our study exploratively interpreted the discrepancy between herbal safety and timosaponin A3-induced hepatotoxicity. However, given the considerable levels and slow eliminated rate of timosaponin A3 in the liver, more attention should be paid to the safety on the continuous clinical medication of *Zhimu* in the future.

Keywords: *Zhimu*; timosaponin A3; mangiferin; pharmacokinetics; metabolism; hepatotoxicity; antioxidant

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INTRODUCTION

With the increasing consumption of herb as medicine or dietary supplements around the world, the safety of herb, especially for the herbal hepatotoxicity, has become a major public concern [1]. According to the estimation by the US Drug-Induced Liver Injury Network, herbal consumption-induced liver injury accounted for 16% of all cases reported [2]. More importantly, this proportion increased from 7% in 2004–2005 to 20% in 2013–2014 [2]. Nowadays, there has been a hot and sharp controversy on the relationship between the liver cancer and aristolochic acids (AA), a metabolism-based toxic ingredient widely distributed in the genera *Aristolochia* and *Asarum*, which triggered by the report that 78% of hepatocellular carcinomas samples showed distinctive mutational signature of AA among 98 patients in Taiwan, China [3]. Once observing the toxic ingredients in the herb, or the toxic metabolites in vivo, it is timely indispensable and essential to clarify the exposure of those toxic substances in vivo, especially paying more attention to the organs associated with toxicity. It is worth noting, however, that the herbs containing toxic ingredients

or metabolites do not equal to their unsafe usage in clinic. Given the inherent nature of herb containing various ingredients, the possible drug–drug interaction (DDI) at pharmacokinetic or pharmacodynamic levels probably cause the toxicity enhancement or alleviation on herb treatments [4].

Zhimu (*Rhizoma anemarrhenae*), botanically from the dried rhizome of *Anemarrhena asphodeloides* Bge, has been one of the most frequently used herb medicines in China for centuries with various activities, such as antipyretic, anti-inflammatory, anti-oxidation, anti-depressant, and anti-diabetes [5–9]. According to the isolated compounds and pharmacological screenings, the active ingredients in *Zhimu* were mainly xanthenes, represented by neomangiferin and mangiferin (Fig. 1a), and saponins, represented by timosaponin B2 and timosaponin B3 (Fig. 1b), with lots of activities [9]. Nowadays, the anti-diabetic activities of mangiferin, as well as the anti-depressant and anti-dementia effect of timosaponin B2, attracted extensive attention [10–12]. Our team has been focused on those active compounds in *Zhimu* for many years, and a great body of investigations have been

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Fig. 1 Chemical structures of three xanthones (**a**) and three saponins (**b**) in *Zhimu*

performed, including the metabolic patterns of mangiferin [13, 14], timosaponin B2 [15, 16], timosaponin B3 [17] and timosaponin A3 [18], the pharmacokinetic profiles of mangiferin [19, 20], the hepatobiliary disposition of timosaponin B2 [21], and the DDI between timosaponin B2 and mangiferin [20]. Those provided much scientific data for understanding the effective material base of *Zhimu*.

Although there were no related reports regarding the toxicity during the historically clinical medication of *Zhimu* [9], we previously discovered obvious timosaponin A3-induced hepatotoxicity in rats, a steroid saponin isolated from *Zhimu*, following oral administration of timosaponin A3 for consecutive 14 days (100 mg/kg per day) [22]. We also confirmed this hepatotoxicity was highly associated with timosaponin A3-induced oxidative stress, and the presence of antioxidants can powerfully fight against the timosaponin A3-induced hepatotoxicity in rat hepatocytes [22]. As published, xanthones were good scavengers for various oxidizing species, and the antioxidant capacity of xanthones was profoundly correlated with the number and position of hydroxyl and catechol groups [23]. Correspondingly, we also previously confirmed mangiferin, a xanthone with strong antioxidant ability in *Zhimu* [24, 25], can powerfully attenuate the oxidative stress induced by timosaponin A3 in hepatocytes [22]. Nevertheless, such an in vitro results were far less than comprehensively interpreting the safe clinical medication of *Zhimu* for centuries, particularly in the absence of the in vivo levels of antioxidants and timosaponin A3 on *Zhimu* treatments. Moreover, according to our previous study, timosaponin A3 was detected as a major metabolite after oral administration of timosaponin B2 or timosaponin B3 in rats [15, 17], two saponins with the high content in *Zhimu*, suggesting a high probability of substantial biodistribution of timosaponin A3 on *Zhimu* treatments. Therefore, in light of the complicated transformation among saponins, and the confrontation relationship on the hepatotoxicity between timosaponin A3 and xanthones, it was imperative to make it clear for the in vivo dynamic disposition of those xanthones and saponins, especially for the hepatic

disposition. However, up to date, in spite of several studies only concerning the circulating levels of those ingredients on *Zhimu* treatments [26–28], the major issues regarding the safety of *Zhimu*, such as the liver distribution of timosaponin A3, the formation pathway of timosaponin A3, and the exposure relationship between timosaponin A3 and the prototypes or metabolites with antioxidant activity, and so on, remained largely unclear.

Therefore, aimed at clarifying the metabolic patterns, the in vitro tools, rat primary hepatocytes and enterobacteria, were employed for evaluating the liver-mediated and microbiota-mediated metabolism of xanthones and saponins in *Zhimu*, respectively. On the basis of those results, after oral administration of *Zhimu* decoction in rats, the determination of the prototypes and metabolites with the skeleton structure of saponin and xanthone was systemically conducted in the plasma before and after hepatic disposition, namely portal vein plasma and systemic plasma, as well as liver. Serving as the technical foundation, a sensitive and accurate HPLC–MS/MS method was developed and validated for the simultaneous determination of three xanthones, neomangiferin, mangiferin, and norathyriol, and three saponins, timosaponin B2, timosaponin B3, and timosaponin A3, in the plasma and liver. Hence, via the systemic evaluation on the pharmacokinetics, metabolism, and hepatic disposition of ingredients in *Zhimu*, this study was aimed at potentially understanding the discrepancy between the historically clinical safety of *Zhimu* and timosaponin A3-induced hepatotoxicity.

MATERIALS AND METHODS

Materials

Zhimu was purchased from Anhui Shuzhong traditional Chinese Medicine Co., Ltd (Shanghai, China), and were identified as dried rhizome of *Anemarrhena asphodeloides* Bge by Dr. Zhixiong Li. Norathyriol (purity >98%) was isolated from rat urine post-mangiferin dosing in our laboratory [13]. Calycosin-7-O- β -D-glucopyranoside (C7G), mangiferin, neomangiferin, timosaponin B2, timosaponin B3, timosaponin A3, and tolbutamide (internal

standard, IS) were bought from the National Institute for Control of Pharmaceutical and Biological Products Co., Ltd (Beijing, China). Acetonitrile and formic acid of HPLC grade were obtained from Fisher Scientific Co., Ltd (Tustin, CA, USA). All other analytical-grade reagents were provided by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) or Sigma-Aldrich Co., Ltd (St. Louis, MO, USA). Triple-deionized water was prepared using a Milli-Q System (Millipore, Billerica, MA, USA). Anaerobic bags were bought from AnaeroPouch™-Anaero, Mitsubishi Gas Chemical Co., Ltd (Tokyo, Japan), and anaerobic culture solution was purchased from Qingdao Hope Biotechnology Co., Ltd (Qingdao, China).

Instrumentation

The determination of three xanthenes and three saponins in different biological samples was performed by an Agilent 6460 triple quadrupole mass spectrometer with Dual Agilent Jet Stream Electrospray Ionization (Agilent Technologies, Palo Alto, CA, USA), combining with an Agilent 1260 Series Liquid Chromatography System (Agilent Technologies, Palo Alto, CA, USA). The MS parameters were optimized as follows: gas temperature, 350 °C; gas flow rate, 12 L/min; nebulizer, 45 psi; capillary, 3500 V; nozzle voltage, 1000 V; sheath gas temperature, 400 °C; sheath gas flow rate, 8 L/min. All quantifications of compounds were monitored in the negative multiple reaction monitoring (MRM) mode, and the relevant optimized parameters were shown in Table 1. The chromatographic separation was carried out using an ACE Super C18 (100 mm × 2.1 mm, 3 μm, Advanced Chromatography Technologies Limited, Aberdeen, Scotland) at the column temperature of 40 °C. A binary gradient consisting of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was employed as follows: 0–3 min, 92% A; 3.5–4 min, 88%–60% A; 5.5–6 min, 60%–55% A; 6.6–7 min, 55%–5% A; 11–11.01 min, 5%–92% A; 11.01–13.5 min, 92% A. The flow rate remained at 0.35 mL/min at the time range of 0–6.6 min, and it kept at 0.45 mL/min at the time range of 7–13.5 min. The system operation and data analysis were performed using Masshunter Workstation software (Agilent Technologies, Palo Alto, CA, USA).

Preparation of the *Zhimu* decoction

After soaking for 30 min, the *Zhimu* pieces (560 g) were extracted by direct heating with 5.6 L of water for 2 h, and after filtration, the residue was extracted by direct heating with 4.48 L of water for an additional two hours. After filtration, the combined supernatant was concentrated to 800 mL (0.7 g/mL) for analysis and oral administration to rats.

In vitro: microbiota-mediated metabolism

Anaerobic culture was performed as our previous literature described [14, 19, 29]. In brief, fresh intestinal contents obtained from Wistar rats were homogenized with saline solution (0.9%) in the ratio of 1 g: 5 mL, and then filtrated with gauze immediately. *Zhimu* decoction and the above filtrate contacting enterobacteria

were added to above intestinal flora anaerobic cultural solution to generate the concentration of *Zhimu* at 0.7 mg/mL, and then cultured in the anaerobic incubation bags at 37 °C ($n = 3$). C7G (20 μg/mL) was used as a positive control. After incubation for 0, 8, and 24 h, the incubated solution (200 μL) was terminated by adding ice-cold methanol (600 μL) immediately. The enterobacteria samples were diluted with methanol by a factor of 10, and then extracted by acetonitrile containing 3% acetic acid precipitation and prepared for analysis by HPLC–MS/MS.

In vitro: liver-mediated metabolism

The fresh hepatocytes were isolated from male Wistar rats by a previously described two-step perfusion [21, 30]. Briefly, the hepatocytes were incubated in six-well plates at a density of 0.5×10^6 per well followed by immediate addition of *Zhimu* decoction (0.7 mg/mL), and then incubated in an orbital shaker at 37 °C for 3 h ($n = 3$). Testosterone (10 μmol/L) was used as positive control. Cell suspensions (200 μL) were obtained at 0, 1.5, and 3 h, and then ice-cold methanol (600 μL) was immediately added to terminate the potential reactions. The hepatocyte samples were lysed after three successive freezes (–80 °C)–thaw (37 °C) cycles, diluted with methanol by a factor of 10, extracted by acetonitrile containing 3% acetic acid precipitation, and prepared for analysis by HPLC–MS/MS.

In vivo study

Male Wistar rats (220 ± 20 g) were provided by Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). They were acclimated for seven days before use. The animals were housed under a controlled temperature of 22 ± 2 °C, relative humidity of $50\% \pm 10\%$, and 12 h light/dark cycle in the air-conditioned animal quarters. The rats were given ad libitum access to standard diet and water. The experiments were carried out in accordance with the guidelines by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica, China Academic Science (Shanghai, China).

Fifty rats were fasted overnight with the free access to water before use. After oral administration of *Zhimu* decoction (7 g/kg, 0.7 g/mL), the rats were anaesthetized with urethane (1.4 g/kg) at 0.167, 0.5, 1, 2, 4, 6, 8, 11, 24, 48 h post-dose ($n = 5$ for each time-point). Hepatic portal vein blood (2 mL) was collected firstly by syringe, and the systemic blood was obtained via drawing plasma from the abdominal aorta (6–8 mL). Finally, the right liver lobe was excised by tweezers. The blood samples were centrifuged at $9500 \times g$ for 5 min to obtain the supernatants; the liver samples were washed with saline for three times; all the above samples were stored at –80 °C until analysis.

Sample preparation

Standard stock solutions of neomangiferin, mangiferin, norathyriol, timosaponin B2, timosaponin B3, and timosaponin A3 were separately prepared in acetonitrile at 500 μg/mL. The stock solutions were serially diluted with acetonitrile to prepare the working solutions. The calibration standards were prepared by spiking the working solutions to the biological samples to yield the final concentrations at 3, 10, 25, 50, 100, 250, 500, and 1000 ng/mL for each analyte, along with tolbutamide (IS) at 5 ng/mL. The low, middle, and high concentration levels of quality control (QC) samples containing each analyte (8, 80, and 800 ng/mL) and IS (5 ng/mL) were prepared in the same manner.

The liver samples (1 g) were homogenized with three volumes (3 mL) of saline. The liver homogenates (100 μL) or plasma samples (100 μL) were precipitated through protein precipitation using acetonitrile containing 3% acetic acid (300 μL). After mixed for 5 min and centrifuged at $13,680 \times g$ for 10 min, the supernatants (250 μL) were separated and evaporated to dryness under vacuum at 40 °C. The residues were reconstituted with solvents (100 μL), a combination of water, acetonitrile and acetic acid in the ratio of 92:8:0.1. After vortexed for 5 min and centrifuged at $13,680 \times g$ for

Table 1. The optimized parameters of six ingredients in *Zhimu* and IS in the negative MRM mode of HPLC–QQQ–MS/MS

Analytes	Transition (m/z)	Fragmentor voltage (V)	Collision energy (V)
Neomangiferin	582.9 → 330.9	209	39
Mangiferin	420.9 → 330.9	149	22
Norathyriol	258.9 → 150.8	138	42
Timosaponin B2	919.2 → 756.9	285	49
Timosaponin B3	901.3 → 739.1	310	46
Timosaponin A3	739.1 → 577.2	255	38
Tolbutamide (IS)	269 → 170	100	32

10 min, the supernatant (70 μ L) was obtained and prepared for the analysis by HPLC–MS/MS.

Method validation

The full validations, including of selectivity, linearity, accuracy, precision, matrix effect, extraction recovery, and stability, were performed in the plasma and liver matrix.

Selectivity was assessed by analyzing six different sources of blank biological samples, blank samples spiked with analytes and post-dose biological samples from rats to determine potential interferences for the analytes and IS. Standard calibration curves were generated by plotting the peak area ratios of the analytes to the IS versus the concentrations of the analytes using a $1/C$ - or $1/C^2$ -weighted linear least-squares regression model. The concentrations of ingredients in the biological samples were all calculated based on the regression parameters derived from the standard calibration curves prepared on the same day. The requirements for the lowest limit of quantification (LLOQ) were the lowest concentration with a

signal-to-noise ratio >10 , an accuracy within $100\% \pm 20\%$, and a precision (relative standard deviation, RSD) $<20\%$.

The accuracy and precision of the method were evaluated by analyzing QC samples ($n = 5$) at three levels on one day (intra-day) and three successive days (inter-day). The stability was assessed by analyzing QC samples ($n = 5$) at three levels under different conditions, including after storage at an ambient temperature for 4 h (short-term stability), after storage at -80°C for 2 weeks (long-term stability), after three successive freeze-thaw cycles (free-thaw stability) and after storage at room temperature for 12 h (post-preparative stability). Variation in the precision (RSD) and accuracy should be no more than 15% for the precision and stability validation.

Extraction recovery was evaluated by comparing the mean peak areas of the analytes and IS after the above-mentioned routine processing with those of the post-extraction spiked samples at the same concentration. The matrix effect was determined by comparing the mean peak areas of the analytes and IS of the post-extraction spiked samples with those of the standard

Fig. 2 Representative MRM chromatograms of six ingredients in *Zhimu* and IS in the blank plasma, blank plasma spiked with the analytes (3 ng/mL, LLOQ) and IS (5 ng/mL), and plasma samples after oral administration of *Zhimu* decoction (7 g/kg) at 2 h

Table 2. Precision and accuracy of determination of six ingredients of *Zhimu* in the plasma (inter-day, $n = 5$; intra-day, $n = 5 \times 3$)

Compound	Spiked (ng/mL)	Inter-day			Intra-day		
		Measured (ng/mL)	Accuracy (%)	Precision (RSD%)	Measured (ng/mL)	Accuracy (%)	Precision (RSD%)
Neomangiferin	8	8.2	102.5	2.9	7.8	97.4	4
	80	85.9	107.4	6.7	90.2	112.7	2
	800	792.9	99.1	5.3	839.9	105	5.2
Mangiferin	8	8.3	103.8	3.8	7.8	97	2.4
	80	85.5	106.9	5	83.5	104.4	4.8
	800	810.1	101.3	4.4	806.4	100.8	4.4
Norathyriol	8	7.4	92.5	4.9	7.9	98.4	4.9
	80	84.1	105.1	5.9	70.4	88	9.5
	800	827.1	103.4	10.1	765.5	95.7	7.4
Timosaponin B2	8	8.1	101.3	7.1	7.9	99	5
	80	76.7	95.9	7.9	80.7	100.9	11.4
	800	807.3	100.9	4.5	789.4	98.7	7.5
Timosaponin B3	8	7.7	96.3	4.9	7.8	97.6	5.7
	80	77.4	96.8	6.2	79.1	98.9	4.2
	800	803.9	100.5	9.3	780.4	97.6	10
Timosaponin A3	8	8	100	6.9	8.2	101.9	3.2
	80	83.1	103.9	2.2	77.1	96.4	7.5
	800	820.5	102.6	9.1	840.5	105.1	7.6

Table 3. Extraction recovery and matrix effect of six ingredients in *Zhimu* and IS in the plasma ($n = 6$)

Compound	Mean extraction recovery (%)			Matrix effect (%)		
	Low	Medium	High	Low	Medium	High
Neomangiferin	70.1 ± 1.2	70.6 ± 1.7	76.3 ± 1.5	110.3 ± 5.3	105 ± 1.1	111.1 ± 4.5
Mangiferin	56.9 ± 5.2	53.9 ± 2.8	60.1 ± 2	110.2 ± 6.6	101.2 ± 2.4	105.1 ± 3.5
Norathyriol	50 ± 3.3	47.9 ± 1.5	51.7 ± 1.1	111.3 ± 4.3	108.5 ± 2	101.4 ± 4.2
Timosaponin B2	43.2 ± 1.2	47.8 ± 1.6	47.7 ± 0.6	91.6 ± 3.9	93.9 ± 3.2	89.5 ± 3.2
Timosaponin B3	58.7 ± 3.4	58.2 ± 0.7	63.1 ± 1.2	85.1 ± 2.9	85.4 ± 2.8	89.6 ± 3.3
Timosaponin A3	43 ± 3	42.7 ± 1.6	43.9 ± 0.6	86.1 ± 5.8	90.3 ± 2	87.2 ± 3
Tolbutamide (IS)		51 ± 2.3			112.7 ± 2.9	

compound samples directly dissolved by solvents at the same concentration.

Data analysis

A non-compartmental analysis utilizing WinNonlin software (Pharsight 6.2, NC, USA) with sparse algorithm was applied for the PK parameters calculation. An unpaired Student's *t*-test was used to assess the significance of the in vitro results.

Liver extraction ratio (ER) indicated the fraction of hepatic clearance and first-pass effect, and it was calculated as follows:

$$ER = (AUC_{por} - AUC_{sys}) / AUC_{por} \quad (1)$$

Where AUC_{por} and AUC_{sys} correspond to the areas under the concentration-time curves of compounds in the portal vein plasma and systemic plasma, respectively.

AUC_D was used for evaluating the utilized efficacy per unit in the herb, and it was calculated as follows:

$$AUC_D = AUC / Dose. \quad (2)$$

Where AUC represents the area under the concentration-time curves of compounds in vivo, and dose represents the relevant oral dosage of compounds in rats on the herb treatments.

RESULTS

Method validation

The full validation, including of selectivity, linearity, accuracy, precision, matrix effect, extraction recovery, and stability, was performed for the simultaneous determination of three xanthenes and three saponins in the plasma and liver matrix by HPLC-MS/MS.

The xanthenes of neomangiferin, mangiferin, and norathyriol were detected at 5.58, 5.91, and 6.80 min, respectively, and the saponins of timosaponin B2, timosaponin B3 and timosaponin A3 were observed at 6.16, 6.87, and 8.68 min, respectively, along with tolbutamide (IS) at 8.27 min (Fig. 2). Neither endogenous nor exogenous interferences were found at the retention time of those analytes in the biological samples.

The calibration curve of analytes exhibited an excellent linearity with a correlation coefficient (r^2) higher than 0.99, over the

Table 4. Results of stability analysis for six ingredients in *Zhimu* in rat plasma ($n = 5$)

Compound	Spiked (ng/mL)	Short-term stability			Long-term stability		
		Measured (ng/mL)	Accuracy (%)	Precision (RSD%)	Measured (ng/mL)	Accuracy (%)	Precision (RSD%)
Neomangiferin	8	8.4	105	6.8	7.5	93.8	6.6
	80	83.7	104.6	5.2	83	103.8	5.2
	800	823.5	102.9	6.2	830.4	103.8	6.2
Mangiferin	8	8.4	105	8.2	7.4	92.5	8
	80	83.7	104.6	5.8	84.5	105.6	5.8
	800	853.1	106.6	1.6	833.6	104.2	1.6
Norathyriol	8	7.8	97.5	9.1	7.6	95	10.3
	80	82.1	102.6	6	80.7	100.9	6
	800	797.6	99.7	9.1	825.4	103.2	9.1
Timosaponin B2	8	8.4	105	6.7	7.8	97.5	6.6
	80	82.5	103.1	4.9	84.3	105.4	4.9
	800	811.6	101.5	6.9	817.5	102.2	6.9
Timosaponin B3	8	8.2	102.5	6.1	7.1	88.8	5.9
	80	82.9	103.6	2.9	83.5	104.4	2.9
	800	771.4	96.4	6.5	809.8	101.2	6.5
Timosaponin A3	8	7.1	88.8	9.8	7.4	92.5	11.3
	80	82.7	103.4	5.8	78.9	98.6	5.8
	800	837.6	104.7	8.3	857.9	107.2	8.3

Compound	Spiked (ng/mL)	Freeze-thaw stability			Post-preparative stability		
		Measured (ng/mL)	Accuracy (%)	Precision (RSD%)	Measured (ng/mL)	Accuracy (%)	Precision (RSD%)
Neomangiferin	8	7.3	91.3	6.4	8.9	111.3	4.4
	80	80.6	100.8	5.2	88.5	110.6	5
	800	806.4	100.8	6.2	871	108.9	6.1
Mangiferin	8	7.2	90	7.8	8.9	111.3	5.8
	80	82	102.5	5.8	88.5	110.6	5.6
	800	809.6	101.2	1.6	902.3	112.8	1.5
Norathyriol	8	7.4	92.5	10.1	8.3	103.8	7.8
	80	78.3	97.9	6	86.9	108.6	5.7
	800	801.5	100.2	9	843.6	105.5	9.1
Timosaponin B2	8	7.6	95	6.3	8.8	110	4.3
	80	81.9	102.4	4.8	87.3	109.1	4.6
	800	793.9	99.2	6.8	858.4	107.3	6.8
Timosaponin B3	8	7	87.5	5.6	8.7	108.8	3.7
	80	81	101.3	2.8	87.6	109.5	2.6
	800	786.5	98.3	6.4	815.9	102	6.4
Timosaponin A3	8	7.2	90	12.3	7.5	93.8	9.8
	80	76.6	95.8	5.7	87.5	109.4	5.5
	800	833.2	104.2	8.3	885.9	110.7	8.3

concentration ranged from 3–1000 ng/mL in the plasma samples. The regression equations were as follows: $Y = 0.042415 \times -0.000492$ ($r^2 = 0.9931$) for neomangiferin, $Y = 0.077848 \times -0.020584$ ($r^2 = 0.9902$) for mangiferin, $Y = 0.021752 \times -0.003810$ ($r^2 = 0.9921$) for norathyriol, $Y = 0.006466 \times +0.001473$ ($r^2 = 0.9916$) for timosaponin B2, $Y = 0.042993 \times -0.004487$ ($r^2 = 0.9913$) for timosaponin B3, and $Y = 0.004918 \times +0.002913$ ($r^2 = 0.9929$) for timosaponin A3.

Both of the intra-day and inter-day precision (RSD) of six analytes at three levels were no more than 11.4%, and the corresponding accuracy ranged from 88% to 112.7% in the plasma samples (Table 2).

The mean extraction recovery of each analyst was similar at low, middle and high levels in the plasma samples, and those were

69.4%–71.2% for neomangiferin, 53.1%–61.2% for mangiferin, 45%–54.6% for norathyriol, 41.4%–46% for timosaponin B2, 56.5%–59.4% for timosaponin B3, and 35.1%–41.7% for timosaponin A3, along with $51\% \pm 2.3\%$ for IS respectively (Table 3). All of the matrix effect of analysts were at the range of 85.1%–110.1% at low, middle and high levels, and this was $112.7\% \pm 2.9\%$ for IS, suggesting neither ion suppression nor enhancement occurred in the plasma samples.

The analysts in the plasma samples remained stable after storage for 4 h at ambient temperature, -80°C for 2 weeks, 12 h post-preparative and three freeze-thaw cycles, evidenced by the accuracy of six analysts within the range of 87.5%–112.8%, and the precision below 12.3% at each level (Table 4).

Fig. 3 Evaluation the microbiota-mediated metabolism of xanthenes and saponins in *Zhimu* decoction in the enterobacteria samples after incubation of *Zhimu* decoction at 0, 8, and 24 h, C7G used as a positive control (a). Evaluation of liver-mediated metabolism of xanthenes and saponins in the rat primary hepatocytes after incubation of *Zhimu* decoction 0, 1.5, and 3 h, testosterone used as a positive control (b). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control

As with the plasma matrix, full validations were investigated and verified in the liver samples. Consequently, these results met the demands for the accurate determination of six ingredients in the liver matrix (seen in the supplementary materials). Those results confirmed the favorable availability for the simultaneous determination of three xanthenes and three saponins in the plasma and liver matrix by HPLC–MS/MS.

Determination of xanthenes and saponins in the *Zhimu* decoction Among the ingredients, timosaponin B2 was of the highest content at the concentration of 18,150 $\mu\text{g}/\text{mL}$ in the *Zhimu* decoction (0.7 g/mL), followed by mangiferin, timosaponin B3, and neomangiferin at 6100, 4790, and 840 $\mu\text{g}/\text{mL}$, respectively. However, the content of timosaponin A3 remained extremely low at 97 $\mu\text{g}/\text{mL}$, only accounting for 0.5% of that of timosaponin B2, along with an undetected amount of norathyriol in the *Zhimu* decoction. When rats taking the *Zhimu* decoction at the oral dosage of 7 g/kg, it was equivalent to timosaponin B2, mangiferin, timosaponin B3, and neomangiferin at the oral dosage of 181.5, 61.0, 47.9, and 8.4 mg/kg, respectively, when the oral dosage of timosaponin A3 and norathyriol can be negligible with <1 mg/kg on *Zhimu* treatments.

In vitro study

After incubation for 8 h, the positive control (C7G, 20 $\mu\text{g}/\text{mL}$) was completely metabolized in the enterobacteria samples, indicating of the excellent applicability for evaluating microbiota-mediated metabolism (Fig. 3a). Regarding xanthenes, after incubation of *Zhimu* decoction, neomangiferin were all metabolized, and the levels of mangiferin elicited a bit increased trend ($P > 0.05$), and those decreased with difference ($P < 0.001$) until incubation for 24 h (Fig. 3a). This was due to neomangiferin was metabolized into mangiferin firstly by lose of one glucose unit, and then mangiferin was converted into norathyriol after elimination of the other glucose group. Correspondingly, norathyriol, the aglycone of neomangiferin and mangiferin, was generated after incubation of

Zhimu decoction for 8 h, and an apparently increased concentration happened by hundreds of times during the incubated time range of 8–24 h (Fig. 3a). As for saponins, similar to xanthenes, after incubation of *Zhimu* decoction for 8 h, timosaponin B2 and timosaponin B3 were all metabolized, corresponding to the dramatically increased concentration of timosaponin A3 by 218-fold during the incubated time for 8 h, although with slight decrease by 8.8% during the time range of 8–24 h (Fig. 3a).

The positive control (10 $\mu\text{mol}/\text{L}$ testosterone) was all metabolized in the primary hepatocytes within 1.5 h, thus confirming the excellent metabolic activity of those cells (Fig. 3b). However, there is no obvious change on the concentration of all the prototypes, involved of neomangiferin, mangiferin, timosaponin B2, and timosaponin B3, before and after *Zhimu* decoction incubation ($P > 0.05$), indicating of the limited metabolic activity for those ingredients in *Zhimu* in the liver (Fig. 3b).

Briefly, xanthenes, including of neomangiferin, mangiferin, and saponins, involved of timosaponin B2 and timosaponin B3, could be respectfully transformed into norathyriol and timosaponin A3 via the microbiota-mediated metabolism, but those were limited to the liver-mediated metabolism.

In vivo study

The established and validated HPLC–MS/MS method was successfully applied to the determination of xanthenes and saponins in the portal vein plasma, liver, and systemic plasma, following oral administration of *Zhimu* decoction at the dosage of 7 g/kg (Figs. 4, 5; Table 5).

PK before and after hepatic disposition

Following oral administration of *Zhimu* decoction, three xanthenes and three saponins were all accurately determined in the portal vein plasma and systemic plasma, and the compounds were of higher levels in the former (Fig. 4a). Among the xanthenes, mangiferin was of the greatest exposure both in the portal vein plasma and systemic plasma, where the AUCs of mangiferin were

Fig. 4 Mean concentration-time curves of the xanthenes and saponins in the portal vein plasma and systemic plasma (a) and liver (b), after oral administration of *Zhimu* decoction at 7 g/kg in rats (n = 5)

at $12,434.4 \pm 1106.1$ ng/mL/h, and 8008.9 ± 1148.7 ng/mL/h, respectively, corresponding to its ER at 35.6%. Although no detection in the *Zhimu* decoction, there were substantial exposure of norathyriol in the portal vein plasma with the AUC at 4658.6 ± 874 ng/mL/h, and after undergoing serious hepatic clearance with the ER at 84.9%, the AUC of norathyriol was at 705.2 ± 133.1 ng/mL/h in the systemic plasma. Neomangiferin was of the lowest levels with the AUC only at 154 ± 13.9 and 72.3 ± 9.5 ng/mL/h in the portal vein plasma and systemic plasma, along with its ER at 53%. Likewise, the order of C_{max} from high to low was mangiferin, norathyriol, and neomangiferin both in the portal vein plasma and systemic plasma. There was an observable multiple-peak phenomenon for mangiferin and neomangiferin, but not for norathyriol in the time-concentration curves. The first T_{max} s of neomangiferin and mangiferin happened at the time range of 0.5–2 h, and the second ones occurred at 6 h both in the portal vein plasma and systemic plasma. The T_{max} s of norathyriol happened at 8 and 11 h in the portal vein plasma and systemic plasma, respectively, probably associated with the slow metabolized rate of xanthenes on the effect of enterobacteria (Fig. 3a). Except of neomangiferin with $T_{1/2}$ at about 2 h, those for mangiferin and norathyriol were at the time range of 6.4–10 h both in the portal vein plasma and systemic plasma.

Similarly, the levels of saponine were obviously higher in the portal vein plasma than those in the systemic plasma, including timosaponin B2 (3580.6 ± 373.5 vs. 1163.7 ± 197.4 ng/mL/h), timosaponin B3 (2730.2 ± 357.3 vs. 808.7 ± 109.1 ng/mL/h),

timosaponin A3 (1656.7 ± 121.1 vs. 650.5 ± 45.2 ng/mL/h). Those corresponded to the severe liver first-pass effect of those saponins, whose ERs were at the range of 60.7%–70.4%. Timosaponin B2 was of the highest AUC in both the portal vein plasma and systemic plasma. The C_{max} of timosaponin B3 was a bit higher than that of timosaponin B2 (875.1 ± 187 ng/mL vs. 635.7 ± 155.5 ng/mL) in the portal vein plasma, and those were very similar in the systemic plasma (200.7 ± 85.1 vs. 220.1 ± 43.7 ng/mL). Timosaponin A3 was of the lowest C_{max} and AUC in both the portal vein plasma and systemic plasma. There was a significant multi-peak phenomenon in the time-concentration curves of timosaponin B2 and timosaponin B3, while that remained obscure to timosaponin A3 in both portal vein plasma and systemic plasma. The T_{max} s of those saponins all appeared at 6 h. The $T_{1/2}$ of timosaponin B3 was at 7.9 h, a bit smaller than those of timosaponin B2 and timosaponin A3 at 9.5 and 10.3 h, respectively.

After normalizing the AUC by the oral dosage, the AUC_D of mangiferin was the maximum, followed by timosaponin B3, timosaponin B2, and neomangiferin in both the portal vein plasma and systemic plasma. This reflected that among the ingredients in *Zhimu*, mangiferin exerted the highest utilized efficacy in the plasma both before and after hepatic clearance following oral administration.

Liver distribution

Only the metabolites- norathyriol and timosaponin A3, but none of any prototypes, were observed in the liver. The levels of

Fig. 5 Comparison on the AUC_s of the xanthenes and saponins in the portal vein plasma, liver and systemic plasma after oral administration of *Zhimu* decoction at 7 g/kg, along with the content in the herb

timosaponin A3 were as high as $71,386 \pm 10,285.1$ ng/g/h, about 40.7-fold of that of norathyriol at 1754.4 ± 288.8 ng/g/h (Fig. 4b). Similarly, the C_{max} of timosaponin A3 was 39.1-time of that of norathyriol. The T_{max} s of norathyriol and timosaponin A3 occurred at 11 and 6 h, respectively, probably correlated with the slow metabolized rate for xanthenes and saponins on the effect of enterobacteria (Fig. 3a). The $T_{1/2}$ s of timosaponin A3 and norathyriol were a little long at 12.1 and 10.6 h, respectively.

Given their negligible content in the herb, as well as the microbiota-mediated metabolism of xanthone and saponin prototypes can generate norathyriol and timosaponin A3 (Fig. 3a), the substantial hepatic exposure of timosaponin A3 and norathyriol was mainly attributed to the microbiota-mediated metabolism of prototypes, rather than direct absorption from the herb. This was also consistent with the non-ignorable levels of norathyriol and timosaponin A3 in the portal vein plasma, by which they were transported to the liver after microbiota-mediated metabolism and gut absorption.

DISCUSSION

Owing to the inherent nature containing multiple ingredients, the herbal medicine exerts the therapeutic efficacy or toxicity as a whole, probably involved of synergistic, additive or antagonistic effect via the complicated DDIs [31]. Hence, it is well acknowledged that there may be grand disparity on the efficacy or safety between the herb and pure compound treatments. Serving as the material base for comprehensively understanding the herbal efficacy or safety, the primary work is to make it clear of the in vivo dynamic disposition of main prototypes and metabolites in a holistic way, particularly with the observation of the toxic substances. Hence, via the in vitro and in vivo combination, the metabolism, pharmacokinetics and hepatic disposition of xanthenes and saponins was comprehensively clarified on *Zhimu* treatments in this study.

Given the giant variation of the chemical structures among the analytes in *Zhimu* (Fig. 1), much efforts were made for the optimization for the simultaneous determination of three xanthenes and three saponins in *Zhimu*, including of verification of matrix inference between the positive and negative mode, comparison of sensitivity between organic solvent precipitation and liquid–liquid extraction, determination of precipitator (acetonitrile/methanol), the choice on the acidity of acetonitrile (1%–10% of formic/acetic acid) and confirmation of appropriate internal standard (IS). Finally, acetonitrile containing 3% acetic acid precipitation, negative MRM mode, and tolbutamide as internal standard were employed, considering the comprehensive benefits of high sensibility, less matrix inference and perfect peak shapes.

Timosaponin A3 has arisen attention because of the high potentials on anticancer, anti-dementia and antiplatelet aggradation [32–34]. However, we previously discovered timosaponin A3

(100 mg/kg per day) significantly increased the levels of alanine aminotransferase (ALT) and total bile acid (TBA), the typical pathological indicators of cholestasis, after continuous oral administration of timosaponin A3 for 14 days [22]. Rather than directly inhibiting the bile acid transporters, timosaponin A3 disrupted the polarization of the bile acid transporter system by generating reactive oxygen species (ROS) [22]. Excessive ROS can oxidize the proteins, DNA, lipid and other macromolecules, resulting in destroying the cellular physiological structure and causing hepatotoxicity [35]. In the presented study, the content of timosaponin A3 was too low in the *Zhimu* decoction to be taken into consideration, which only accounted for less than 0.5% of that of timosaponin B2 in the herb decoction. However, given the in vitro results that timosaponin B2 and timosaponin B3 were metabolized entirely into timosaponin A3 through deglycosylation in the enterobacteria samples (Fig. 3a), timosaponin A3 may exist as metabolites in vivo. In fact, following oral administration of *Zhimu* decoction at the dosage of 7 g/kg, there was substantial exposure of timosaponin A3 in the portal vein plasma, corresponding to the in vitro results that the generation of timosaponin A3 contributed to the microbiota-, but not liver-, mediated metabolism of other saponins in *Zhimu* (Fig. 3). After transported by the portal vein plasma, timosaponin A3 elicited the C_{max} as high as 5085.3 ± 1581.5 ng/g (about 7 μ M) in the liver (Fig. 4). It should be alert that timosaponin A3 even at 5 μ M can obviously induce oxidative stress and downregulate bile acid transporters in the hepatocytes [22]. In addition to the microbiota-regulated conversion from other saponins in *Zhimu*, the substantial hepatic exposure of timosaponin A3 was also attributed to its high membrane permeability resulting from its high hydrophobicity ($\log P$ at 2.978 ± 0.884), and low bile excretion with the bile excretion index (BEI) only at 12.8% (data not shown). After the hepatic clearance, just 39.3% of timosaponin A3 from the portal vein plasma got into the circulating system. The serious liver first-pass effect of timosaponin A3 (ER at 60.7%) corresponded to the sizable levels in the liver, but not with the limited hepatic metabolism of timosaponin A3, according to the incubated results in the primary hepatocytes (Figure S2). Notably, timosaponin A3 also elicited slow absorption and elimination rate in the liver, where the $T_{1/2}$ and T_{max} of timosaponin A3 were at 12.1 and 6 h, respectively (Table 5). This indicated that there was very likely to be the high hepatic accumulation of timosaponin A3 after continuous oral administration of *Zhimu*.

Interestingly, in contrast to the substantial exposure of timosaponin A3, there were no detectable levels of timosaponin B2 and timosaponin B3, the saponins with high amounts in *Zhimu*, in the liver (Fig. 5). On the one hand, this was associated with the microbiota-mediated metabolism of timosaponin B2 and timosaponin B3 (Fig. 3a). On the other hand, given the chemical structures containing one more glucose molecule (Fig. 1), timosaponin B2 and timosaponin B3 exerted poorer membrane

Table 5. The PK parameters of the xanthones and saponins in the portal vein plasma, liver and systemic plasma after oral administration of *Zhimu* decoction at 7 g/kg in rats ($n = 5$)

PK parameters	Neomangiferin		Mangiferin		Norathyriol	
	Portal vein plasma	Systemic plasma	Portal vein plasma	Systemic plasma	Portal vein plasma	Systemic plasma
$T_{1/2}$ (h)	2	2.3	6.4	7.6	6.7	10
T_{max} (h)	1	0.5	2	6	8	11
$C_{max} \pm SE$ (ng/mL)	58.9 ± 11	26.3 ± 2.6	1503.6 ± 167	1105.2 ± 336.8	408.1 ± 108	57.6 ± 21.2
$AUC \pm SE$ (ng/mL/h)	154 ± 13.9	72.3 ± 9.5	$12,434.4 \pm 1106.1$	8008.9 ± 1148.7	4658.6 ± 874	705.2 ± 133.1
AUC_D (g/L/h)	18.3	8.6	203.8	131.3	—	—
ER	53%		35.6%		84.9%	

PK parameters	Timosaponin B2		Timosaponin B3		Timosaponin A3	
	Portal vein plasma	Systemic plasma	Portal vein plasma	Systemic plasma	Portal vein plasma	Systemic plasma
$T_{1/2}$ (h)	9.5	7	7.9	8.2	10.3	9.2
T_{max} (h)	6	6	6	6	6	6
$C_{max} \pm SE$ (ng/mL)	635.7 ± 155.5	200.7 ± 85.1	875.1 ± 187	220.1 ± 43.7	125 ± 33.9	54.6 ± 6
$AUC \pm SE$ (ng mL/h)	3580.6 ± 373.5	1163.7 ± 197.4	2730.2 ± 357.3	808.7 ± 109.1	1656.7 ± 121.1	650.5 ± 45.2
AUC_D (g/L/h)	19.7	6.4	57	16.9	—	—
ER	67.6%		70.4%		60.7%	

PK parameters	Norathyriol	Timosaponin A3
	Liver	Liver
$T_{1/2}$ (h)	10.6	12.1
T_{max} (h)	11	6
$C_{max} \pm SE$ (ng/g)	130.1 ± 32.6	5085.3 ± 1581.5
$AUC \pm SE$ (ng/g/h)	1754.4 ± 288.8	$71,386 \pm 10,285.1$

permeability than timosaponin A3, resulting in the limited passive diffusion in the liver. In spite of the limited hepatic accumulation (Fig. 5) and metabolism (Fig. 3b), timosaponin B2 and timosaponin B3 underwent severe liver first-pass effect, whose ER was at 67.6% and 70.4%, respectively. Those were probably associated with their bile excretion, based on our previous conclusion that timosaponin B2 was the substrate of breast cancer resistance protein (Bcrp) and multidrug resistance-associated protein 2 (Mrp2), the efflux transporters responsible for the bile excretion, in line with the BEI of timosaponin B2 at 44% [21], although those for other ingredients awaited further study. Given the limited hepatic exposure and no reports regarding the anti-oxidation, timosaponin B2, and timosaponin B3 were likely to have insignificant role in the whole hepatoprotective activity on *Zhimu* treatments.

Mangiferin, a well-established naturally antioxidant, can neutralize various reactive species and regulate the expression and activity of key detoxification enzymes, involved of superoxide, catalase, and glutathione-S-transferase [36]. Compared to the frequently used antioxidants, such as vitamin C, vitamin E and β -carotene, mangiferin showed preferable or comparative antioxidative capacity in vivo [25]. In our previous study, mangiferin at 25 μ M can completely restore the ROS, ATP and LDH induced by timosaponin A3 to the normal levels in hepatocytes, and such an astonishing effect even can not be achieved on *N*-acetyl-L-cysteine treatments [22]. To our surprise, although with the highest exposure in the portal vein plasma, there were limited exposure of mangiferin in the liver (Fig. 5). This was in accord with our previous study that there were limited exposure of mangiferin in the liver, which was highly associated to the poor membrane permeability of mangiferin, following oral intake of mangiferin

even at the high dosage of 200 mg/kg in animals [19]. However, some researchers have documented that mangiferin can efficiently resist the oxidative stress-mediated liver dysfunction and exerted the hepatoprotective activity with the oral dosage of mangiferin no more than 100 mg/kg in animals [25, 37, 38]. Thus, based on the discrepancy of significant efficacy and limited exposure in liver, it was reasonably deduced that there might be some specific sites, but not liver, where mangiferin took action as antioxidants and indirectly caused the liver protection on mangiferin treatments. Analogously, in views of the maximum exposure in the circulating system (Fig. 5), mangiferin was very likely to be carried to the specific organs where it took actions of anti-oxidation and fighting against timosaponin A3-induced hepatotoxicity indirectly on *Zhimu* treatments. Besides, according to our previous study, compared to the pure compound treatments, the AUC of mangiferin was increased by 11.5-fold in the plasma on the *Zhimu* decoction treatments, because of drug–drug interaction (DDI) of timosaponin B2 and mangiferin at pharmacokinetic levels [20]. This also potentially explained why mangiferin elicited the maximum AUC and AUC_D in the plasma on herb treatments, although the bioavailability of mangiferin was only at 1.2% [39]. Given the C7-OH position occupied by a glucose molecule, neomangiferin should have relatively poor oxidation resistance and make an ignorable contribution to the whole antioxidant ability of *Zhimu* with its low exposure in vivo (Fig. 5).

Interestingly, in contrast to no observation of xanthone prototypes, norathyriol, the aglycone of mangiferin, showed certain hepatic accumulation (Fig. 5) on *Zhimu* treatments. This was in contrast to no observation of norathyriol on mangiferin treatments alone [19], probably due to the timosaponin B2 and mangiferin interaction leading to the increased exposure of

norathyriol [20], as well as the generation of norathyriol from neomangiferin except of mangiferin (Fig. 3a), on *Zhimu* treatments. Similar to that of timosaponin A3, the exposure of norathyriol in vivo was attributed to the microbiota-, but not liver-, mediated metabolism of xanthenes in *Zhimu* (Fig. 3). In agreement with generation of norathyriol via microbiota-mediate metabolism, there was also non-ignorable levels of norathyriol in the portal vein plasma, where the AUC of norathyriol took up of 37.4% of those of mangiferin. Nevertheless, the levels of norathyriol only accounted for 8.8% of those of mangiferin in the systemic plasma. Correspondingly, the ER of norathyriol was as high as 84.9%, and this was correlated with the substantial hepatic accumulation (Fig. 5) and obvious hepatic metabolism of norathyriol (Figure S2). Moreover, via the comparison on their $T_{\max}$ s and $T_{1/2}$ s, norathyriol elicited similar absorption and elimination rate to timosaponin A3 in the liver. Those implied the comparable probability of hepatic accumulation of timosaponin A3 also happened to norathyriol on repeated *Zhimu* treatments. As reported, norathyriol was of similar antioxidative capacity to mangiferin with the IC_{50} both at $9\ \mu\text{M}$ [37]. This was also in line with the conclusion that B-ring, 6-OH and 7-OH on the chemical structure of xanthenes made the most significant contribution to the antioxidative ability, according to the theoretical bond dissociation enthalpy (BDE) analysis [40]. Thus, norathyriol, as a powerful antioxidant in the liver, was likely to have a direct role in fighting against timosaponin A3-induced hepatotoxicity. Given the levels of norathyriol only accounted up of 2.5% of those of timosaponin A3 in the liver, more studies are still in demand on the quantitative relationship of timosaponin A3 and norathyriol on balancing hepatic status between oxidation and anti-oxidation in the future. Besides, compared to the mangiferin treatments, the DDI between mangiferin and timosaponin B2 also cause the increased exposure of norathyriol by 5.9-folds in the plasma on the *Zhimu* treatments [20]. Thus, such a DDI should also contribute indirectly to the whole anti-oxidation of *Zhimu* via improving the levels of mangiferin and norathyriol in vivo.

In spite of limited content in the herb, timosaponin A3, who can induce the hepatotoxicity via oxidative stress, elicited substantial hepatic exposure as the microbiota-mediated metabolite of other saponins in *Zhimu*, following oral administration of *Zhimu* in rats. To sum up, the discrepancy of the safe clinical medication of *Zhimu* and timosaponin A3-induced hepatotoxicity can be exploratively interpreted by the presence of antioxidants in vivo. Norathyriol, the microbiota-mediated metabolites of xanthenes with specific hepatic exposure, should have a direct role in fighting against the oxidative stress induced by timosaponin A3 in the liver. Mangiferin, a xanthone with the maximum exposure in the plasma, may take the action of as antioxidants in some specific sites and exerted the hepatoprotective activity indirectly. The DDI of timosaponin B2 and mangiferin at pharmacokinetic levels could also make significant contribution indirectly to the overall anti-oxidation of *Zhimu* by increasing the in vivo levels of mangiferin and norathyriol. More scientific evaluation is still urgently required to explore the quantitative relations in vivo between anti-oxidation generated by xanthenes and oxidative stress induced by timosaponin A3. Notably, given the sizable levels and slow elimination rate of timosaponin A3 in the liver, more attention should be paid to the safety on the continuous clinical medication of *Zhimu* in the future.

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AUTHOR CONTRIBUTIONS

Y.X., Z.X., X.-t.T. and C.-g.H. were responsible for the research design; Y.X., Z.X., P.H. and M.-c.C. conducted the experiments; Z.-l.S. and Y.-r.X. contributed new reagents or analytical tools; Y.X., X.-t.T. and C.-g.H. performed data analysis and wrote the manuscript.

ADDITIONAL INFORMATION

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