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## Original article

## Identification of Icaritin Metabolites in Rats by LC-MS/MS

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## ABSTRACT

**Objective** Icaritin is the main aglycone and also active intestinal metabolite of prenylflavonoids from the Chinese materia medica *Epimedii Herba*. Modern pharmacological studies have demonstrated that icaritin has a wide range of biological activities. However, its metabolites and biotransformation pathways have not yet been comprehensively investigated. The present study aims to identify icaritin metabolites in rats by using a sensitive and effective LC-MS/MS method. **Methods** The plasma and urine samples of rats were collected before (blank) and after oral administration of icaritin, and subjected to liquid-liquid extraction with ethyl acetate. The full-scan LC-MS chromatograms of the plasma and urine samples were compared with those of blank samples to detect the possible metabolites, which were later detected by their product ion spectra. **Results** A total of 23 metabolites were identified, and conjugated icaritins produced by glucuronidation, glycosylation, and sulfation were its major metabolites. Minor demethylation, hydrogenation, and oxidation metabolites were also found. **Conclusion** Phase II metabolism is the main metabolic pathway of icaritin.

## Key words

*Epimedium* L.; icaritin; LC-MS/MS; metabolites

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## 1. Introduction

*Epimedii Herba*, the dried aerial part of the species in the genus *Epimedium* L. (Berberidaceae), has a long history for the medicinal purpose and is believed “to nourish the kidney and reinforce the *Yang*” in traditional Chinese medicine (Li et al, 2011a; Ma et al, 2011). A number of pharmacological studies demonstrated that this herb had a wide range of

biological activities, such as improving bone health and cardiovascular function, modulating immunological function, regulating hormone levels, antitumor, anti-oxidation, anti-aging, and hepatoprotection (Chang et al, 2012; Ma et al, 2011; Jiang et al, 2015; Wang et al, 2013). To date, more than 200 compounds have been identified from various species of *Epimedium*. Icaritin, a prenylflavonoid, is one of the main and important active components from this herb.

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Icaritin (Figure 1) is the aglycone and also the active intestinal metabolite of icariin (Chang et al, 2012) with a broad spectrum of pharmacological activities, such as promoting anabolic and antiresorptive effects on osteoporotic bone (Peng et al, 2013), enhancing osteoblastic differentiation, facilitating matrix calcification (Huang et al, 2007a), anti-inflammation (Lai et al, 2013), antileukemia (Zhu et al, 2011), inhibiting human endometrial cancer cells (Tong et al, 2011) and breast cancer cells (Huang et al, 2007b), and stimulating neuronal and cardiomyocyte differentiations from mouse embryonic stem cells (Wang et al, 2009; Wo et al, 2008). Several studies have been published for the metabolite profiles of the components from the plants of genus *Epimedium*, which mainly focused on the metabolism of icariin and other icaritin glycosides in rats (Chen et al, 2009; Qian et al, 2012; Zhao et al, 2010) and zebrafishes (Li et al, 2011b). It was recognized that glycoside hydrolysis and further glucuronidation in the gut lumen were the major metabolism processes of these prenylflavonoids. While, up to now, there is no any publication directly involving icaritin metabolites except for a study using zebrafishes.

Our previous study revealed that icaritin could be rapidly absorbed into the body and converted to its conjugated metabolites in rats after oral administration (Chang et al, 2012). However, it is still unclear where and how this conjugation occurs so far. Therefore, the present study aims to identify the metabolites of icaritin after oral administration to rats by LC-MS/MS techniques.

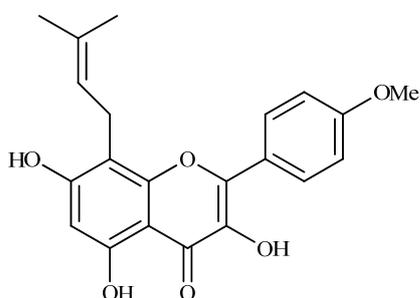


Figure 1 Chemical structure of icaritin

## 2. Materials and methods

### 2.1 Chemicals and reagents

Icaritin (purity of 98.0%) was purchased from Shanghai Ronghe Pharmaceutical Science Co., Ltd. (China). Methanol and acetonitrile of HPLC-grade were obtained from Fisher Co., Ltd. (USA). Formic acid and other reagents were all of analytical grade and purchased from Beijing Chemical Reagent Company (China). Milli-Q (USA) water was used throughout the study.

### 2.2 Animals

Male Sprague-Dawley rats ( $n = 5$ ,  $200 \pm 20$  g) were

supplied by Vital River Experimental Animal Co., Ltd. (Beijing, China). The animal experiments were approved by Animal Ethics Committee at Institute of Medicinal Plant Development of Chinese Academy of Medical Sciences. The rats were housed under standard conditions of temperature, humidity, and light with free access to a standard rodent diet and water before the experiment. The day before the experiment, the rats were subjected to a light surgery, and a polyethylene catheter (0.50 mm ID, 1.00 mm OD, Portex Ltd., England) was cannulated into the right jugular vein under anesthesia condition by an ip dose of 10% chloral hydrate at 3.50 mL/kg. After surgery, the rats were placed individually in metabolism cages to recover for at least 24 h. The rats were fasted overnight with free access to water before dosing.

### 2.3 Sample collection

Icaritin dosing solution was freshly prepared by dissolving in water containing 0.2% DMSO and orally given to five rats at 20 mg/kg. Blood samples (0.5 mL) were collected into heparinized tubes through the catheter before dosing and at 0.5, 1, and 2 h post dosing, respectively. At the same time, urine samples were also collected into urine reservoir, which contained 2 mL of 0.1% HCl to prevent possible degradation of metabolites, over 12 h before and post dosing. The collected blood samples were immediately centrifuged for 5 min for plasma separation at 3000  $g$  and 4 °C. The collected plasma or urine samples from five rats before dosing were pooled together and mixed to obtain blank plasma or blank urine sample, and all plasma at different time points or urine samples post dosing were also separately mixed to obtain dosing plasma or dosing urine sample. These samples were stored at -20 °C until assay.

### 2.4 Sample preparation

An aliquot (1 mL) of plasma or urine sample was spiked with 0.3 or 0.5 mL HCl (1 mol/L) for adjusting the pH value at 4–5, and then extracted with 5 mL of ethyl acetate by vortex mixing for 10 min. After centrifugation, the ethyl acetate layer was transferred into a glass tube and concentrated to dryness by a gentle steam of nitrogen at 25 °C. The residue was reconstituted in 100  $\mu$ L of 80% methanol, and 20  $\mu$ L of the resulting sample was injected into an LC-MS/MS system for metabolite identification.

### 2.5 Chromatographic and mass spectrometric conditions

The LC-MS/MS system consisted of an Agilent 1200 HPLC system (USA) and an Applied Biosystem 3200 Q-Trap Mass Detector (USA) equipped with an electrospray ionization (ESI) interface. The separation was performed using a Thermo BDS Hypersil C<sub>18</sub> column (100 mm  $\times$  4.6 mm, 2.4  $\mu$ m, USA) maintained at 40 °C. The mobile phase consisted of acetonitrile (A) and water (B), both containing

0.1% formic acid, and ran in the following linear gradient programs at 0.4 mL/min, 20%–70% A (0–8 min), 70%–90% A (8–9 min), 90% A (9–20 min), 90%–20% A (20–21 min), and 20% A (21–25 min). The total run time was 25 min. The Turbo Ion Spray interface was operated in positive mode at 5500 V. The mass conditions were set as the followings: curtain gas at  $1.38 \times 10^5$  Pa, ion source temperature at 500 °C, collision gas at high, ion source of Gas 1 and Gas 2 both at  $4.14 \times 10^5$  Pa. Full-scan mass spectra at a mass range of  $m/z$  100–800 were acquired and the corresponding LC-MS chromatograms were recorded. The extracted LC-MS chromatograms of the molecular ion ( $[M + H]^+$ ) of icaritin and its metabolites were used for finding the potential metabolites, by comparison of chromatographic differences of the plasma or urine samples before and after dosing. The mass spectra were analyzed using the Analyst 1.4.2 software packages (USA).

### 3. Results

A total of 23 metabolites (M1–M23) were found and identified, and their extracted mass chromatograms were illustrated in Figure 2. The metabolites were then confirmed by their product ions through LC-MS/MS experiments in the positive ESI mode. The major fragment ions of identified metabolites were presented in Table 1.

The metabolites M1 and M2 yielded the same  $[M + H]^+$  of  $m/z$  355 and product ion of  $m/z$  299, which were all 14 Da

less than that of icaritin ( $m/z$  369). Therefore, M1 and M2 were identified as two demethylcaritins. This observation is similar to previous report by Qian et al (2012), in which three demethylcaritins had been found. Even though the compound icaritin has only one position at 4' C-O of the ring B for the demethylation, the exact isomer and formation mechanism were not clear and reserve to further study. The metabolite M3 showed  $[M + H]^+$  of  $m/z$  371, which was 2 Da more than that of icaritin, produced by demethylation, and followed by hydroxylation. M3 showed a fragment ion at  $m/z$  355 ( $[M + H]^+ - 16$ , loss of an OH unit), indicating the possibility of the formation of C-OH metabolite. M4 showed  $[M + H]^+$  ion of  $m/z$  385, which was 16 Da more than that of icaritin and yielded the product ions at  $m/z$  369 ( $[M + H]^+ - 16$ , loss of an OH unit) and  $m/z$  313 ( $[M + H]^+ - 16 - 56$ , loss of a OH and a prenyl units). This indicates that M4 might be the icaritin oxidate. The oxidation position should be at the terminal methyl of the isopentene group at the position 8 of the ring A (Qian et al, 2012). The  $[M + H]^+$  ion of M5 shown at  $m/z$  387 was 18 Da, more than that of icaritin and might gain one  $H_2O$  via an addition reaction to form wushanicaritin (Li et al, 2012). M6 showed  $[M + H]^+$  ion of  $m/z$  401, which was 32 Da more than that of icaritin. M6 produced the fragment ions, including  $m/z$  387 ( $[M + H]^+ - 14$ , loss of a methyl group), 371 ( $[M + H]^+ - 14 - 16$ , loss of a methyl group and an OH unit) and 357 ( $[M + H]^+ - 14 \times 2 - 16$  loss of two methyl groups and one OH unit). These results indicated that the metabolite M6 might be icaritin di-oxidate.

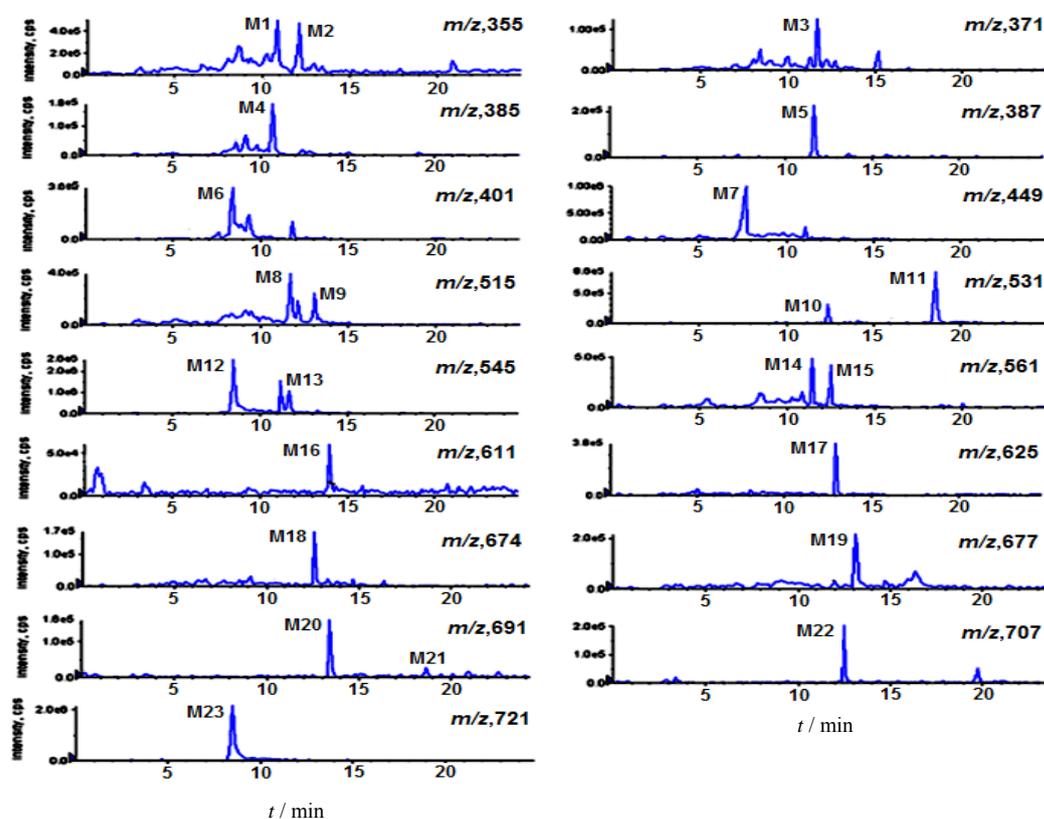


Figure 2 Extracted metabolite ion chromatograms of rat plasma and urine after oral administration of icaritin

**Table 1** Icaritin metabolites in plasma and urine of rats after oral administration

No.	RT / min	Metabolic pathway	[M + H] <sup>+</sup> / <i>m/z</i>	Major fragment ions / <i>m/z</i>	Detection
M0	15.80	parent (icaritin)	369	313 ([M + H] <sup>+</sup> -prenyl)	plasma, urine
M1	10.85	demethylation	355	299 ([M + H] <sup>+</sup> -prenyl)	plasma, urine
M2	12.13	demethylation	355	299 ([M + H] <sup>+</sup> -prenyl)	plasma
M3	11.68	demethylation hydrogenation	371	355 ([M + H] <sup>+</sup> -OH)	plasma, urine
M4	11.73	oxidation	385	369 ([M + H] <sup>+</sup> -OH) 313 ([M + H] <sup>+</sup> -OH-prenyl)	urine
M5	11.08	hydration	387	369 ([M + H] <sup>+</sup> -H <sub>2</sub> O)	plasma, urine
M6	8.38	di-oxidation	401	387 ([M + H] <sup>+</sup> -Me), 371 ([M + H] <sup>+</sup> -Me-OH) 357 ([M + H] <sup>+</sup> -2Me-OH)	urine
M7	7.69	sulfation	449	369 ([M + H] <sup>+</sup> -SO <sub>3</sub> )	urine
M8	11.68	rhamnosidation	515	369 ([M + H] <sup>+</sup> -Rha)	plasma, urine
M9	13.08	rhamnosidation	515	369 ([M + H] <sup>+</sup> -Rha)	plasma, urine
M10	12.38	glucosylation	531	369 ([M + H] <sup>+</sup> -Glc)	urine
M11	18.56	glucosylation	531	369 ([M + H] <sup>+</sup> -Glc)	plasma, urine
M12	8.52	glucuronidation	545	369 ([M + H] <sup>+</sup> -Gln)	plasma, urine
M13	11.22	glucuronidation	545	369 ([M + H] <sup>+</sup> -Gln)	urine
M14	11.44	glucuro-oxidation	561	369 ([M + H] <sup>+</sup> -Gln-O)	urine
M15	12.49	glucuro-oxidation	561	369 ([M + H] <sup>+</sup> -Gln-O)	plasma, urine
M16	14.01	sulfation glucosylation	611	531 ([M + H] <sup>+</sup> -SO <sub>3</sub> ), 369 ([M + H] <sup>+</sup> -SO <sub>3</sub> -Glc)	plasma, urine
M17	12.96	sulfation glucuronidation	625	545 ([M + H] <sup>+</sup> -SO <sub>3</sub> ), 369 ([M + H] <sup>+</sup> -SO <sub>3</sub> -Gln)	plasma, urine
M18	12.61	glutathionylation	674	369 ([M + H] <sup>+</sup> -GSH)	urine
M19	13.12	glucosylation rhamnosidation	677	515 ([M + H] <sup>+</sup> -Glc), 369 ([M + H] <sup>+</sup> -Glc-Rha)	plasma, urine
M20	13.43	glucuronidation rhamnosidation	691	545 ([M + H] <sup>+</sup> -Rha), 369 ([M + H] <sup>+</sup> -Gln-Rha)	plasma
M21	18.69	glucuronidation rhamnosidation	691	545 ([M + H] <sup>+</sup> -Rha), 369 ([M + H] <sup>+</sup> -Gln-Rha)	plasma, urine
M22	12.50	glucosylation glucuronidation	707	545 ([M + H] <sup>+</sup> -Glc), 369 ([M + H] <sup>+</sup> -Glc-Gln)	plasma, urine
M23	8.50	di-glucuronidation	721	545 ([M + H] <sup>+</sup> -Gln), 369 ([M + H] <sup>+</sup> -Gln-Gln)	plasma, urine

RT: retention time; [M + H]<sup>+</sup>: positively charged molecular ion; Me: methyl unit; Glc: glucosyl unit; Rha: rhamnose unit; Gln: glucuronyl unit

M7, with a relatively high intensity, showed a molecular ion [M + H]<sup>+</sup> of *m/z* 449 and yielded fragment ion of *m/z* 369 ([M + H]<sup>+</sup>-80, loss of SO<sub>3</sub>), suggesting that the metabolite M7 should be icaritin sulfate. M16 showed [M + H]<sup>+</sup> ion at *m/z* 611, which was 162 Da more than M7, and yielded fragment ions of *m/z* 531 ([M + H]<sup>+</sup>-80, loss of SO<sub>3</sub>) and *m/z* 369 ([M + H]<sup>+</sup>-80-162, loss of SO<sub>3</sub> and a glucosyl unit). This indicated that the metabolite M16 might be icaritin glucoside sulfate. M17 with [M + H]<sup>+</sup> ion of *m/z* 625 was 176 Da more than M7 and 256 Da more than icaritin,

indicating that the metabolite M17 might be icaritin glucuronide sulfate.

The molecular ions [M + H]<sup>+</sup> of the two metabolites M8 and M9 were both at *m/z* 515, which was 146 Da (a rhamnose unit) more than that of icaritin. This suggested that M8 and M9 might be icaritin rhamnosides, with a rhamnose moiety at different positions. The two metabolites M10 and M11 were the highest abundance [M + H]<sup>+</sup> ions both at *m/z* 531, which were 162 Da more than that of icaritin. This indicated that these ions might be the

monoglucoside of icaritin, with a glucosyl group at different positions. The two metabolites M14 and M15 showed the  $[M + H]^+$  ions both at  $m/z$  561, which were 192 Da more than that of icaritin. This indicated that they were the metabolites of icaritin glucuro-oxidation. The metabolite M18 with  $[M + H]^+$  of  $m/z$  674, which was 305 Da more than that of icaritin, was detected in urine but not in plasma and the result of icaritin glutathionylation.

The metabolite M19 with  $[M + H]^+$  ion of  $m/z$  677 yielded the fragment ions of  $m/z$  559 ( $[M + H]^+ - 146$ , loss of a rhamnose unit) and  $m/z$  515 ( $[M + H]^+ - 162$ , loss of a glucosyl unit). This suggests that the metabolite should be icaritin glucoside rhamnoside. The metabolites M20 and M21 with  $[M + H]^+$  ions both of  $m/z$  691 yielded fragment ions of  $m/z$  545 ( $[M + H]^+ - 146$ , loss of a rhamnose unit) and  $m/z$  515 ( $[M + H]^+ - 176$ , loss of a glucuronyl unit). This indicated that the metabolites should be icaritin glucuronide rhamnosides.

The metabolite M22 showed a  $[M + H]^+$  at  $m/z$  707, which yielded the fragment ions of  $m/z$  545 ( $[M + H]^+ - 162$ , loss of a glucosyl unit) and  $m/z$  369 ( $[M + H]^+ - 162 - 176$ , loss of a glucosyl and a glucuronyl unit). This indicated that the metabolite might be icaritin glucuronide glucoside.

The two metabolites M12 and M13, with  $[M + H]^+$  ions both of  $m/z$  545, were 176 Da (one glucuronyl unit) more than that of icaritin, indicating that they should be icaritin monoglucuronides. The metabolite M23 exhibited the highest abundance at  $m/z$  721  $[M + H]^+$  that was 352 Da (two glucuronyl units) more than that of icaritin, indicating that the metabolite might be icaritin diglucuronide.

#### 4. Discussion

In the present study LC-MS/MS techniques were used to identify icaritin metabolites in rat body. Both negative and positive modes for ESI full scan of icaritin were tested to establish the most sensitive and effective mass conditions for the detection of the icaritin metabolites. In the negative mode condition, icaritin could produce a lot of product ions but no major product ions appeared. These ions might be helpful for identifying the structures of metabolites, but their intensities were not high enough to find the metabolites from the complex matrix samples. However, in the positive ion mode conditions, fewer product ions with relatively higher sensitivities were produced (Figure 3). This was helpful to find out the peaks of metabolites from the extracted mass chromatograms. Therefore, the positive ionization was chosen for the mass detection in the present study. To identify the metabolites as many as possible, the mixed plasma at 0.5, 1, and 2 h and urine over 12 h from five rats were used for monitoring their full-scan LC-MS chromatograms that were compared with those of blank samples.

Icaritin and some of its metabolites were weak acidic due to their phenolic hydroxyl groups. To obtain satisfactory extraction efficiency of these compounds from plasma and urine, 1 mol/L HCL was spiked to the samples for adjusting their pH value at 4–5.

A previous study demonstrated that icaritin, a glucoside of icaritin, was biotransformed its metabolites in rats by phase I and phase II metabolic pathway, including hydrolysis

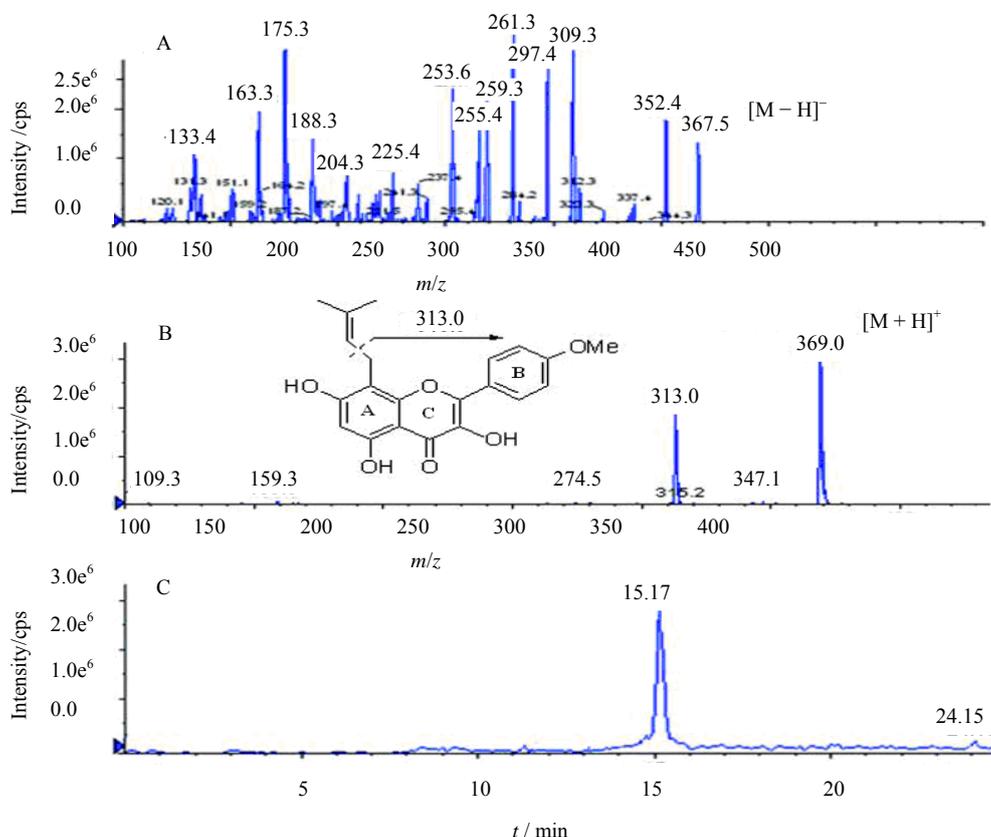


Figure 3 MS ion chromatogram (C) of icaritin and its negative (A) and positive (B) MS

(deglucosidation), demethylation, dehydrogenation, and glucuronidation (Qian et al, 2012). Icaritin was the metabolite of icariin in the body, by the hydrolysis of glucosidic bond, therefore, it might have the similar biotransformation process with that of icariin. In our study, conjugated metabolites by glucuronidation, glycosylation and sulfation (Table 1) were found the major metabolites of icaritin. In addition, a small amount of icaritin oxidates, demethylcaritin, and its isomers were also detected, suggesting that phase II metabolism might be the main metabolic pathway of icaritin. The enzymes involved in the synthesis of these phase II metabolites might be glucuronosyltransferase and sulfotransferase, which have been found in the small intestine (Chen et al, 2003; Mullen et al, 2007).

Different metabolites may display different pharmacokinetic profiles and excretion routes due to their different chemical structures, which would be involved with different metabolite enzymes and transportation rates. In the detection of metabolites, M2 and M20 were found only in plasma but not in urine, indicating that the two metabolites might not be mainly excreted from the kidney. In contrast, the metabolites M6, M7, M10, M13, M14, and M18 were found only in urine but not in plasma, indicating that these metabolites might be rapidly excreted into urine after their formation.

LC-MS/MS technique is the most versatile tool for the qualitative and quantitative analysis of herb constituents and drug metabolites, and is an integral part of pharmaceutical researches due to its superb sensitivity and selectivity (Chen et al, 2012; Han et al, 2013; Zhang et al, 2009). The metabolites of a compound may be vitally related to its pharmacological activities. Although the accurate structures of icaritin metabolites were not identified in the present study, the results are helpful to understand the metabolic pathway of icaritin and further investigate its pharmacological mechanism.

## 5. Conclusion

In the present study, the metabolites of icaritin in rats are studied for the first time using LC-MS/MS techniques. The conjugated icaritin converted by glucuronidation, glycosylation and sulfation are the major metabolites, accompanying with minor demethylation, hydrogenation, and oxidation metabolites. Phase II metabolism is the main metabolic pathway of icaritin.

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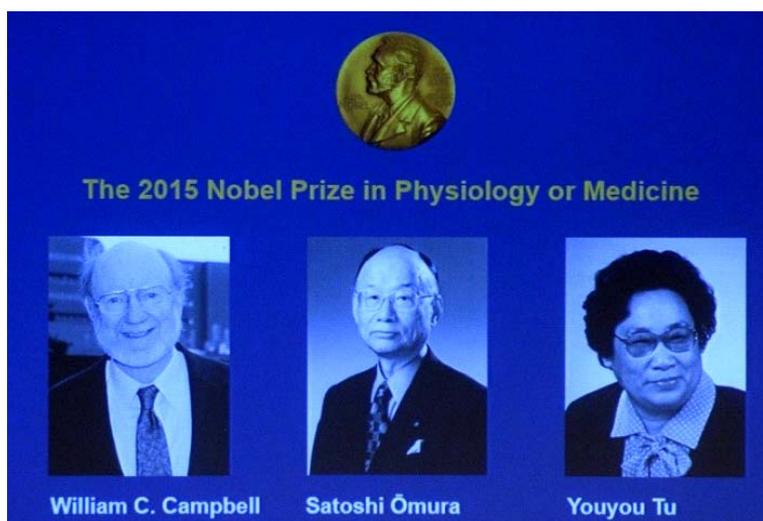
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## Special News

# China's You-you Tu was awarded Nobel Medicine Prize in Physiology or Medicine

**2015 Nobel Prize for Physiology or Medicine was jointly awarded to William C. Campbell and Satoshi Ōmura for their work on infections caused by roundworm, and to You-you Tu for her work on malaria.**



You-you Tu is the first Chinese Nobel laureate in physiology or medicine and the first citizen of the People's Republic of China to receive the Nobel Prize in natural sciences. You-you Tu, who was born in 1930, had an unusual background. She studied pharmacy in college, and then spent a few years on training in traditional Chinese medicine. Nearly 50 years ago. Tu is a Chinese medical scientist, pharmaceutical chemist, pharmacist, and educator. Tu began working on a classified Communist military project using clues from ancient Chinese medicine in search of new cures for malaria. Tu shared the Nobel Prize for Medicine in recognition of her work, which has led to one of the world's final and most potent defenses against a tropical disease that kills over half a million people each year. Tu's discovery of a cutting-edge drug developed from an ancient Chinese folk remedy was hardly known beyond the country's borders. She is best known for discovering artemisinin (also known as *qinghaosu*) and dihydroartemisinin, used to treat malaria, which saved millions of lives. Her discovery of artemisinin and its treatment of malaria are regarded as a significant breakthrough of tropical medicine in the 20th century and health improvement for people of tropical developing countries in South Asia, Africa, and South America.