Tropical Journal of Pharmaceutical Research December 2017; 16 (12): 2985-2990

**ISSN:** 1596-5996 (print); 1596-9827 (electronic)



Available online at http://www.tjpr.org

**http://dx.doi.org/10.4314/tjpr.v16i12.24**

## Original Research Article

# Characterization and identification of *in vitro* metabolites of (-)-epicatechin using ultra-high performance liquid chromatography-mass spectrometry

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*Sent for review: 4 June 2017* *Revised accepted: 27 November 2017*

## Abstract

***Purpose:*** *To characterize and identify metabolites of (-)-epicatechin in microsomal fraction of rat hepatocytes (MFRHs).*
***Methods:*** *A single incubation of (-)-epicatechin (1 mL, 50 µg/mL) in MFRH (0.5 mg/mL) was used for the generation of metabolites. Thereafter, the sample was subjected to protein precipitation prior to analysis with ultra-high performance liquid chromatography coupled to linear ion-trap orbitrap mass spectrometry (UHPLC-LTQ-Orbitap MS).*
***Results:*** *Nine metabolites of (-)-epicatechin were characterized on the basis of high resolution mass measurement, MS spectra and literature data. Based on their structures, the major metabolic routes of (-)-epicatechin in MFRHs were identified as hydroxylation, dihydroxylation and glycosylation.*
***Conclusion:*** *This is the first report on metabolites of (-)-epicatechin in MFRHs, and it is helpful in gaining deeper insight into the metabolism of (-)-epicatechin in vivo. The results will also provide guidance in research on the pharmacokinetics of new drugs.*

***Keywords:*** *(-)-Epicatechin, Metabolites, Hydroxylation, Dihydroxylation, Glycosylation, Rat liver microsomes, Pharmacokinetic studies*



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## INTRODUCTION

(-)-Epicatechin and its isomer (+)-catechin, which belong to the flavan-3-ol family, are ubiquitously distributed in plants [1]. They have been used in many fields, such as food and medical industries due to their anti-oxidative [2], anti-microbial [3,4], and cardio-protective properties [5]. Research on the metabolism of drugs is a very important step in discovery of new drugs. It is also vital for drug development, pharmacokinetics, and clinical pharmaceutics [6]. However, not much is known about the metabolites of (-)-epicatechin [7]. On the other hand, more than 40 metabolites of the isomer of (-)-epicatechin, (+)-catechin have been isolated and characterized through *in vitro* and *in vivo* studies [8-11].

The technique of liquid chromatography coupled with mass spectrometry (LC-MS) is a useful tool for studying the metabolites of drugs in *vitro or in vivo* [12]. However, ultra–high performance liquid

chromatography (UHPLC) can provide a higher and faster separation, and less solvent consumption than LC-MS [13], while high-resolution mass spectrometry (HRMS) can provide elemental composition by accurate mass measurement. Therefore, UHPLC-HRMS has been widely used for the characterization of drug metabolites among several different LC/MS platforms [14-15].

The aim of this investigation was to isolate and characterize (-)-epicatechin metabolites from MFRHs.

## EXPERIMENTAL

### Reagents

Authentic (-)-epicatechin standard (purity ＞98.0 %) was product of Chengdu Biopurify Phytochemicals Co, Ltd (Sichuan, China). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Fisher, Fair Lawn, USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was supplied by Zhong Sheng Rui Tai Biotech (Beijing, China), while a Milli-Q system (Millipore, MA, USA) was used for preparing distilled water. Rat liver microsomes were product of BD Biosciences (Bedford, MA, USA). Magnesium chloride and Tris-HCL buffer used in this experiment (pH 7.4) were of analytical grade.

### Incubation of microsomes

Metabolic transformation was carried out *in vitro* in a final volume of 1 mL by incubating (-)-epicatechin with RLMs in a shaking water bath at 37 $^{o}$C. (-)-Epicatechin (50 µg/mL) was pre-incubated for 5 min in 0.1 mol/L Tris-HCl buffer (pH 7.4) containing 5mM $MgCl_2$ and 0.5 g/mL MFRHs. Next, NADPH (1 mM) was added to initiate the reaction. The reaction was allowed to proceed for I h, and then stopped by introduction of ice-cold acetonitrile (1 mL) to the reaction mixture. The mixture was vortexed and clarified by centrifugation at 4 °C for 10 min at 15,000 rpm. The metabolites were identified by injection of 5 µL of the supernatant into UHPLC-LTQ-Orbitrap MS. A solution prepared in a similar manner but lacking (-)-epicatechin served as blank. All analyses were done in triplicate.

### UHPLC-LTQ-Orbitrap analysis conditions

All UHPLC-LTQ-Orbitrap analyses were performed with LTQ/Orbitrap XL hybrid mass spectrometer (Thermo Electron, Germany) equipped with An Accela UHPLC system (Thermo Fisher Scientific) comprising an auto sampler, a de-gasser component and a quaternary pump, via an electrospray ionization source (ESI) (Thermo Electron, Bremen, Germany). Column chromatography was carried out at room temperature using an Acquity™ UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) with a mobile phase of water (solvent A) and acetonitrile (solvent B) through gradient elution (0.2mL per/min) as follows: 0.2 mL/min: 0 – 2 min, maintained at 5 % B; 2 – 3 min, increased from 5 to 10 % B; 3 – 15 min, increased from 10 to 15 % B; 15 – 17 min, increased from 15 to 80 % B; 17 – 23 min, maintained at 80 % B; 23 – 24 min, decreased from 80 to 5 % B; 24 – 28 min, maintained at 5 % B.

Negative ion mode was used in the MS/MS operations, and mass analysis was carried out at a resolution of 30,000 in the range of 100 to 800 *m/z*. The capillary voltage, source voltage, capillary temperature, tube lens sheath gas flow and auxiliary gas flow rate were 35 V, 3.0 kV, 350 °C, 110 V, 30 and 10 arbitrary units, respectively.

### Data processing

Data acquisition and processing were performed with Thermo X caliber 2.1 workstation (Thermo Fisher Scientific), and the results were compared with that from blank MFRH samples obtained under identical conditions.

## RESULTS

### Metabolic routes of (-)-epicatechin

In order to identify the metabolites of (-)-epicatechin, the first step of this work was to study the $MS^2$ fragmentation pattern of (-)-epicatechin. The parent ion $[M-H]^-$ was at *m/z* 289.0704 (-0.9 ppm, $C_{15}H_{13}O_6$) and the $MS^2$ spectrum yielded fragment ions at m/z 245.0811 (1.1 ppm, $C_{14}H_{13}O_4$), *m/z* 205.0499 (1.8 ppm, $C_{11}H_9O_4$) and *m/z* 179.0346 (4.0 ppm, $C_9H_7O_4$) by loss of $CO_2$ moiety (44 Da), $C_4H_4O_2$ (84 Da), and $C_6H_6O_2$ (110 Da) (Figure 1). These product ions aided the identification of the metabolites of (-)-epicatechin.

### Identified metabolites

The results from high resolution extracted ion chromatography (HREIC) are shown in Figure 2. Nine metabolites of (-)-epicatechin were tentatively characterized on the bases of high resolution mass measurements, MS spectra, and literature data in negative ion mode. The UPLC-MS data are summarized in Table 1.

Metabolite M0 was confirmed as (-)-epicatechin through comparison of the retention time, high resolution mass measurements, and $MS^2$ spectra with authentic references.

Metabolites M3, M6, M8 and M9 eluted at 7.59, 12.53, 14.53, and 16.87 min, respectively, and possessed un-protonated molecular ion $[M-H]^-$ at *m/z* 305.0652 (-1.3 ppm, $C_{15}H_{13}O_7$), *m/z* 305.0655 (0 ppm, $C_{15}H_{13}O_7$), *m/z* 305.0654 (-0.7 ppm, $C_{15}H_{13}O_7$), and *m/z* 305.0651 (-1.7 ppm, $C_{15}H_{13}O_7$). These ions were by 16 Da higher than that of (-)-epicatechin, implying that they were derived from (-)-epicatechin by hydroxylation. The diagnostic product ions at *m/z* 179.0342 (1.8 ppm, $C_9H_7O_4$), *m/z* 179.0337 (-1.0 ppm, $C_9H_7O_4$), *m/z* 179.0342 (1.8 ppm, $C_9H_7O_4$), and *m/z* 179.0342 (1.8 ppm, $C_9H_7O_4$) were observed in their $MS^2$ spectra, which confirmed that they were metabolites of (-)-epicatechin. Therefore, they were tentatively characterized as hydroxylated products of (-)-epicatechin.

The compounds M1 and M2 were eluted at 6.54 and 7.38 min with the same deprotonated molecules at *m/z* 331.0806 (-1.9 ppm, $C_{17}H_{15}O_7$), which was a +36 Da (2O)-shift from that of (-)-epicatechin. The MS2 spectra of M1 and M2 showed the major fragment ions at *m/z* 313, *m/z* 287, and *m/z* 269, due to loss of $H_2O$, $CO_2$, and $H_2O+ CO_2$, respectively from the parent drug which had similar fragmentation pattern with (-)-epicatechin. Based on previous analyses, they were identified as dihydroxylated products of (-)-epicatechin.

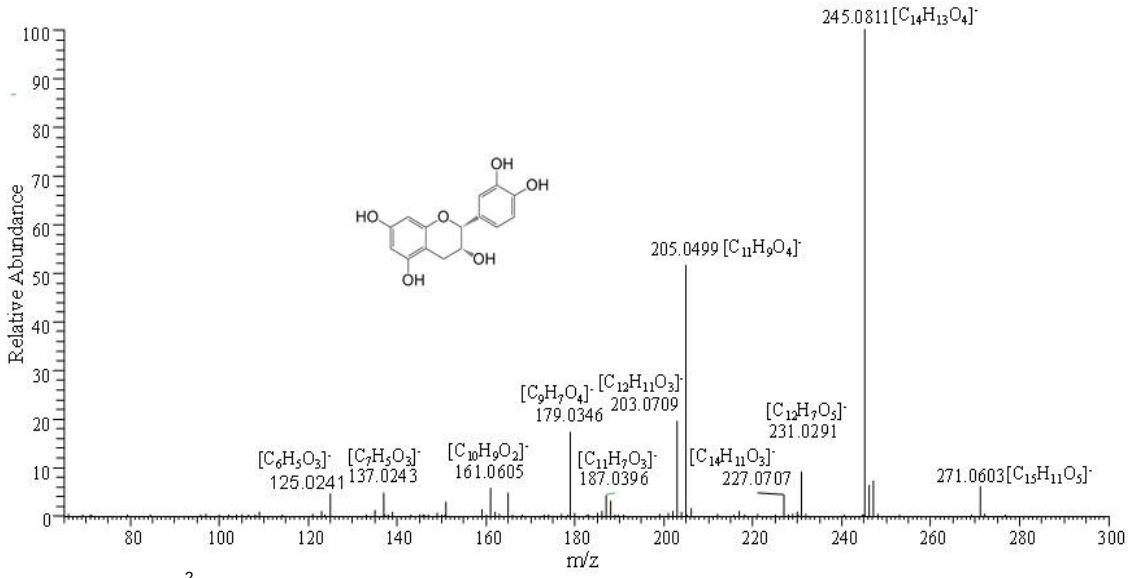


**Figure 1:** $MS^2$ spectrum of (-)-epicatechin

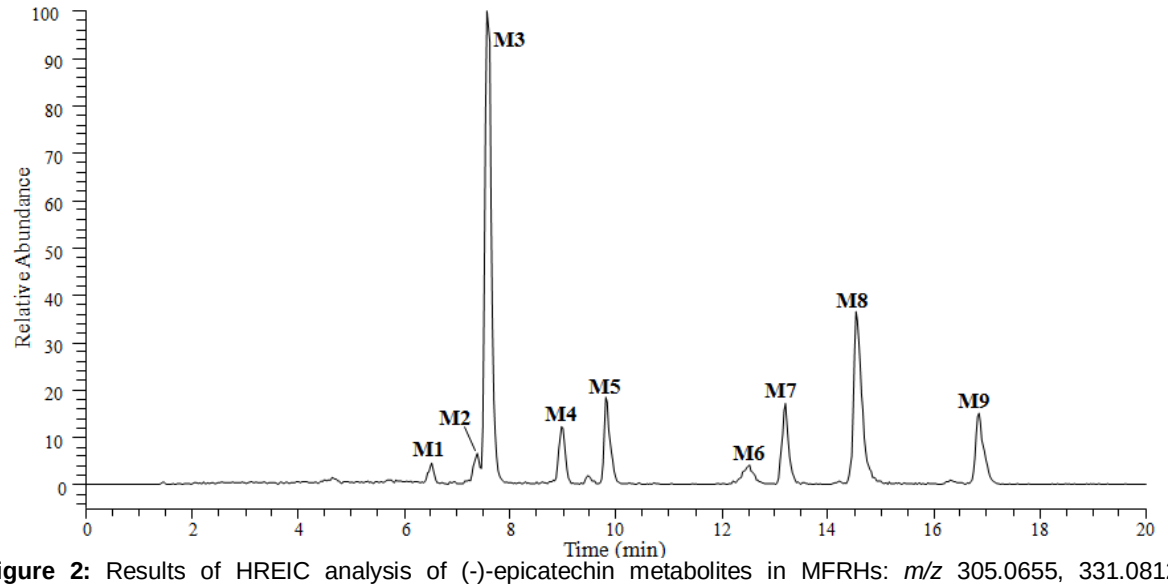


**Figure 2:** Results of HREIC analysis of (-)-epicatechin metabolites in MFRHs: *m/z* 305.0655, 331.0812, 451.1235

**Table 1:** Fragment ions from (-)-epicatechin metabolism in MFRHs identified by UHPLC-LTQ-Orbitrap MS

| Peak | $t_R$ | Theoretical mass *m/z* | Experimental mass *m/z* | Error (ppm) | Formula $[M-H]^-$ | MS/MS fragment | Transformation |
|---|---|---|---|---|---|---|---|
| 1 | 6.54 | 331.0812 | 331.0806 | -1.9 | $C_{17}H_{15}O_7$ | $MS^2$ [331]: 313.0700 (100), 287.0910 (49), 269.0794 (32), 205.0495 (22) | Dihydroxylation |
| 2 | 7.38 | 331.0812 | 331.0806 | -1.9 | $C_{17}H_{15}O_7$ | $MS^2$ [331]: 313.0699 (100), 287.0909 (49), 269.0798 (38), 205.0498 (16) | Dihydroxylation |
| 3 | 7.59 | 305.0655 | 305.0652 | -1.3 | $C_{15}H_{13}O_7$ | $MS^2$ [305]: 179.0342 (100), 221.0444 (76), 219.0651 (74) | Hydroxylation |
| 4 | 8.97 | 451.1235 | 451.1230 | -1.2 | $C_{21}H_{23}O_{11}$ | $MS^2$ [451]: 313.0700 (100), 355.0803 (63), 289.0701 (8) | Glycosylation |
| 5 | 9.82 | 451.1235 | 451.1229 | -1.4 | $C_{21}H_{23}O_{11}$ | $MS^2$ [451]: 313.0911 (100), 289.0701 (66), 245.0805 (37) | Glycosylation |
| 0 | 12.29 | 289.0707 | 289.0704 | -0.9 | $C_{15}H_{13}O_6$ | $MS^2$ [209]: 245.0500 (100), 205.0810 (40), 203.0709 (19),179.0345 (15), 231.0291 (9), 271.0603 (6) | (-)-epicatechin |
| 6 | 12.53 | 305.0655 | 305.0655 | 0.0 | $C_{15}H_{13}O_7$ | $MS^2$ [305]: 179.0337 (100), 219.0653 (75), 221.0446 (70) | Hydroxylation |
| 7 | 13.20 | 451.1235 | 451.1228 | -1.6 | $C_{21}H_{23}O_{11}$ | $MS^2$ [451]: 289.0703 (100), 245.0806 (58) | Glycosylation |
| 8 | 14.53 | 305.0655 | 305.0654 | -0.7 | $C_{15}H_{13}O_7$ | $MS^2$ [305]: 179.0342 (100), 219.0652 (76), 221.0445 (72) | Hydroxylation |
| 9 | 16.87 | 305.0655 | 305.0651 | -1.7 | $C_{15}H_{13}O_7$ | $MS^2$ [305]: 179.0342 (100), 219.0650 (79), 221.0443 (74) | Hydroxylation |

Compounds M4, M5, and M7 appeared after 8.97, 9.82 and 13.20 min with un-protonated $[M-H]^-$ at *m/z* 451.1230 (-1.2 ppm, $C_{21}H_{23}O_{11}$), *m/z* 451.1229 (-1.4 ppm, $C_{21}H_{23}O_{11}$), and *m/z* 451.1228 (-1.6 ppm, $C_{21}H_{23}O_{11}$), 162 Da ($C_6H_{10}O_5$) higher when compared with (-)-epicatechin. Fragments at *m/z* 289.0701 (-2.0 ppm, $C_{15}H_{13}O_6$), *m/z* 289.0701 (-2.0 ppm, $C_{15}H_{13}O_6$), and *m/z* 289.0703 (-1.3 ppm, $C_{15}H_{13}O_6$) by loss glucosyl ($C_6H_{10}O_5$, 121 Da) moiety relative to the precursor ion at m/z 451 in their $MS^2$ spectra indicated that a glucosyl moiety was present. Thus, they were presumed to be glucosyl products of (-)-epicatechin.

## DISCUSSION

The results obtained from preliminary trials with various mobile phase systems in this study showed that good chromatographic peak shapes could be achieved by using mobile system without formic acid, while much lower column pressure was afforded by the inclusion of acetonitrile in the mobile phase. Therefore, the mixture of acetonitrile and water was chosen as the mobile phase solvent system in this study. A gradient elution pattern was adopted.

(-)-Epicatechin is characterized by the presence of many hydroxyl groups on the flavanol skeleton, which make it suitable for detection by ESI in negative mode [15]. To the best of our knowledge, many studies have so far focused mainly on the metabolites of (+)-catechin, while not much has been done on the elucidation of the metabolites of (-)-epicatechin. For example, 40 and 58 metabolites of (+)-catechin were found in *in vitro* and *in vivo* investigations, respectively [8-11]. Among these, 7 metabolites were identified in MFRHs [16]. It is worth mentioning that (-)-epicatechin has a metabolic pathway similar to that of (+)-catechin in MFRHs. Therefore, it is scientifically reasonable to assume that the *in vivo* metabolisms of (-)-epicatechin and (+)-catechin will follow a similar route. This will be helpful in the identification of metabolic products of (-)-epicatechin *in vivo*.

## CONCLUSION

The *in vitro* metabolites of (-)-epicatechin have been successfully identified based on UHPLC-LTQ-Orbitrap analysis. Based on the 9 metabolites identified and their MS data, the major metabolic pathways of (-)-epicatechin in RLMs are hydroxylation, dihydroxylation and glycosylation. This is the first report on the metabolites of (-)-epicatechin in RLMs and is considered a useful guide for understanding its metabolism *in vivo.* In addition, the methodology used in this study offers a new perspective for studying the metabolism and pharmacokinetics of new drugs.

## DECLARATIONS

### *Acknowledgement*

The authors gratefully acknowledge Dr di Jia for supporting this project.

### *Conflict of interest*

The authors declare that no conflict of interest is associated with this study.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiao Ling Yin planned and designed the research; Rui Jun Cai and Jing Liu performed the experiments, Ruijun Cai wrote the manuscript, while Da Xu Qin and Gui Zhen Zhao analyzed the data.

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