Research on the Neuro-protective Compounds in *Terminalia chebula* Retz Extracts in-vivo by UPLC–QTOF-MS

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We have developed a strategy to analyze the components absorbed in the plasma and brain tissue of rats after intragastric administration of *Terminalia chebula* Retz extracts by ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC–QTOF-MS). Nine components (gallic acid, methyl gallate, ferulic acid, ethyl gallate, brevifolin carboxylic acid, ellagic acid, galloflavin, arjugenin, and arjunic acid) and four metabolites were identified in plasma, and five components (ethyl gallate, brevifolin carboxylic acid, ellagic acid, arjugenin, and arjunic acid) were identified in the rat brain based on their fragmentation behaviors. The components present in the plasma were associated with the antioxidant activity of *T. chebula* Retz, and the components absorbed in the brain were associated with its neuro-protective effects. This approach allowed us to rapidly determine the active components of *T. chebula* Retz and develop a method for its quality control. This analysis method showed good resolution and high sensitivity, and is a potentially powerful tool for the determination of effective components of natural products.

Keywords: Extracts of *Terminalia chebula* Retz, absorbed components in plasma and in brain, UPLC–QTOF-MS, effective components

Introduction

The Tibetan medicinal plant *Terminalia chebula* Retz, which belongs to the Combretaceae family, is commonly known as haritaki or chebulic myrobalans, and is indigenous to South China, India, Thailand, Burma, and other Southeast Asian countries. The dried ripe fruit of *T. chebula* Retz or *T. chebula* Retz var. *tomentella* Kurt is the part of the plant used for medicinal purposes [1]. In Tibet and Mongolia, *T. chebula* Retz is regarded as the "king of medicines" [2]. It has a bitter and sour flavor and has astringent properties. It is commonly used to treat nervous disorders, stroke neuropathy, colic pain, sore throat, and diarrhea. *T. chebula* Retz has been reported to exhibit a variety of biological activities including neuroprotective, antioxidant, cardioprotective, antibacterial, antitumor, and antidiabetic [3, 4]. Phytochemical studies have reported that *T. chebula* Retz contains various chemical components including tannins, triterpenoids, flavonoids, and others [5]. Some reports indicate that the main active components of *T. chebula* Retz are derived from tannins and triterpenoids [6, 7]. Various tannins have been demonstrated to show medicinal properties; for example, ellagic acid, galloyl glucose, and gallate have significant neuroprotective, antioxidant, and cardioprotective properties [8–12]. Gallic acid has demonstrated antioxidant and hepatoprotective effects on several mammalian tissues and cells [13, 14]. Triterpenoids also exhibit many pharmacological properties including antioxidant, antidiabetic [7], cardioprotective, and nephrotoxicity prevention effects [15–17]. The absorption and distribution characteristics of *T. chebula* Retz in the body are still unclear, and further research in this area will facilitate determination of its active ingredients and mode of action. Previous studies have reported various methods for analyzing tannins and triterpenoids, such as nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS), lipid raft stationary phase chromatography (LRSC), and capillary electrophoresis (CE) [18–22]. However, there has been no relevant report of qualitative assay of the absorbed components in plasma and brain tissue after intragastric administration of *T. chebula* Retz extracts to rats. The complex matrix and the large number of chemical components of *T. chebula* Retz make the determination of its active components a significant challenge. Additionally, there has been no comprehensive investigation of the active components of *T. chebula* Retz. It is known that, generally, only components of Chinese medicine that are absorbed into the blood or into the brain can produce any biological effect [23, 24]. Thus, in order to rapidly determine the active components of *T. chebula* Retz, we developed a strategy to determine the components absorbed in plasma and in brain after intragastric administration of *T. chebula* Retz extracts to rats using ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC–QTOF-MS). This analysis method has the advantages of high sensitivity, good resolution, and high accuracy. The main active components of *T. chebula* Retz were determined using this method, which provides the foundation for measurement of individual components as part of quality control and facilitates the further development of this medicine.

Experimental

Apparatus, materials, and animals

Apparatus. The chromatographic separation and mass spectrometry analysis were performed using UPLC–QTOF-MS

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(UPLC: Shimadzu, Japan; QTOF-MS: Triple TOF5600+, Allen-Bradley, USA). Sample concentration was carried out using a QGC-24 T-type Termovap sample concentrator (Shanghai Spring Island Company, China). Centrifugation of samples was performed on a D-37520 Osterode-type high-speed tabletop refrigerated centrifuge (Thermo Electron Corporation, USA). *T. chebula* Retz extracts were concentrated using a V-850-type rotary evaporator (Buchi Labortechnik AG).

Materials. Standards of gallic acid (batch number: M-017-150129), methyl gallate (batch number: M-014-150730), ethyl gallate (batch number: M-015-150730), and ellagic acid (batch number: R-004141216) were purchased from Chengdu Herbpurify Co., Ltd. Standards of brevifolin carboxylic acid (batch number: T06J7Z15823), arjugenin (batch number: PM0529SA14), and arjunic acid (batch number: P29F7F10218) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. The dried ripe fruits of *T. chebula* Retz were purchased from a local pharmacy in He Bei Province, China. The samples were identified as Burma *T. chebula* Retz by Dr. Xiao-mei Fu, Associate Professor in Jiang Xi University of Traditional Chinese Medicine. The voucher specimen of the herb was preserved in pharmaceutical analysis department and retained as a reference sample. Heparin sodium (lot 425C 0211) and phosphate-buffered saline (PBS) were purchased from the Wuhan Boster Biological Technology Co. Ltd., China. Acetonitrile (ACS, HPLC grade), alcohol, formic acid and ethyl acetate were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd., China. Purified water was obtained from a Milli-Q ultra-pure water system (Millipore, Billerica, USA). All other chemicals and solvents were of analytical grade.

Animals. Male SD (Sprague-Dawley) rats (n = 24, 230 ± 20 g; age, 5–6 weeks) were supplied by Hunan SJA Laboratory Animal Co. Ltd., Hunan, China (License Number: SCXK2015-0004). They were allowed at least 1 week to adapt to their environment before being used for experiments. The animals were housed in 6 cages, 4 animals per cage, under a normal 12 h/12 h light/dark schedule with the lights on at 07:00 a.m. They were kept at room temperature (23 ± 2 °C) with a relative humidity of 55 ± 5% and given standard food and water. This animal experiment was approved by the Animal Ethics Committee of Jiangxi University of Traditional Chinese Medicine.

Preparation of *T. chebula* Retz extracts. About 100 g of *T. chebula* Retz powder was weighed and then ultrasonically extracted for 60 min in 40% alcohol (the ratio of 40% alcohol–*T. chebula* Retz powder was 40:1). The extract samples were evaporated by rotary evaporation under vacuum, and the residue was then vacuum-dried. The dried powder of the extracts (5.0 g) was dissolved in 100 mL water and then extracted with 100 mL ethyl acetate three times. The obtained ethyl acetate extracts were combined and evaporated to dryness (for intragastric administration).

Preparation of test solution. About 0.5 g of the above dried ethyl acetate extract was dissolved and diluted to a volume of 50 mL with acetonitrile, and then filtered by a 0.22 μm membrane filter before analysis by UPLC–QTOF-MS. As standards, 1 mg gallic acid, 1 mg methyl gallate, 1 mg ethyl gallate, 1 mg ellagic acid, 1 mg brevifolin carboxylic acid, 1 mg arjugenin, and 1 mg arjunic acid were separately dissolved and diluted to a volume of 25 mL with methanol, and then filtered by a 0.22 μm membrane filter before analysis by UPLC–QTOF-MS.

Collection of plasma and brain tissue samples. Twenty-four SD rats were randomly divided into the drug group (n = 12) and the control blank group (n = 12) and were fasted for 12 h before lavage administration, but had free access to water. Extracts of *T. chebula* Retz were administered to rats in the drug group by infusion into the stomach (equivalent to 35 g/kg of crude drugs). Rats in the control group received the same volume of normal saline. Blood samples were drawn from the orbit of six randomly selected rats at 5, 15, 25, 35, 45, and 55 min after treatment, and the blood was transferred to centrifuge tubes containing heparin sodium. The blood samples were incubated at room temperature for 15 min, and then, the plasma was collected by centrifugation (3000 r/min, 4 °C, 10 min). Next, the chest cavities of the remaining 6 rats were opened under ether anesthesia 1 h after drug treatment. PBS was flushed through from the left ventricular to right auricle until the outflow was clear liquid. Then, after decapitation, the brain tissues were removed, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until used.

Preparation of plasma and brain tissue samples. To a 0.5 mL aliquot of the plasma sample in a 5 mL Eppendorf tube, 1.5 mL acetonitrile was added and vortex-mixed for 1 min to release the analytes and precipitate the plasma protein. The supernatant was separated after centrifugation at 3000 r/min at 4 °C for 10 min and then dried using a Termovap sample concentrator. The residue was redissolved in 1 mL acetonitrile, vortexed for 1 min to mix, and centrifuged for 10 min at 14,000 r/min and 4 °C, and the supernatant was removed for analysis. Separately, brain tissues were thawed on ice. Once thawed, ice-cold saline (2 mL saline/100 mg sample) was added and the sample was homogenized using a tissue homogenizer for 2 min in an ice bath. Aliquots of 1 mL homogenate was suctioned, vortex-mixed with 5 mL acetonitrile for 2 min, and centrifuged at 3000 r/min at 4 °C for 10 min. The upper organic layer was transferred to a separate tube and evaporated to dryness under nitrogen stream, and the resulting residue was dissolved with 700 μL acetonitrile and vortexed for 1 min. The mixture was centrifuged for 10 min at 14,000 r/min and 4 °C, and the supernatant was filtered and stored at −80 °C for UPLC–MS/MS analysis. The control plasma and brain tissue samples were prepared by the same procedure.

LC–MS conditions. Separation and detection of the components were performed on a Shimadzu UPLC system (Shimadzu, Japan) coupled with an AB5600 Series MS–MS instrument (Allen-Bradley, USA). A Zorbax RRHD Eclipse Plus C18 (2.1 × 100 mm, 1.8 μm, Agilent, USA) column was used for chromatographic separation. For gradient elution with the mobile phase, the following solutions were prepared: solvent A, 0.1% formic acid solution in water; and solvent B, acetonitrile. The gradient was programmed as follows: 0 min, 5% B; 5 min, 15% B; 15 min, 30% B; 25 min, 100% B; 30 min, 100% B; and, finally, 35 min, 5% B. The flow rate was 0.3 mL/min. The column temperature was 30 °C, and the injection volume was 10 μL.

The mass spectrometric full-scan data were acquired in the negative ion mode from 100 to 2000 Da with a 0.9 s scan time. The electrospray ionization (ESI) conditions were: gas 1, nitrogen (60 psi); gas 2, nitrogen (60 psi); curtain gas (35 psi); ion spray voltage, −4500 V; collision energy, −40 eV; fragmentor, −100 V; and ion source temperature, 500 °C.

Results and Discussion

Identification of the components absorbed in plasma and brain. The absorbed components in the rat plasma and brain after intragastric administration of *T. chebula* Retz extracts were detected and analyzed by QTOF-MS in the negative full-scan mode. The total ion chromatograms of the absorbed components in the plasma obtained from treated animals (a) and plasma obtained from untreated animals (b) are shown in Figure 1, where the molecular ion peaks are labeled “1 to 16.” The chromatographic and spectrometric data of the total ion

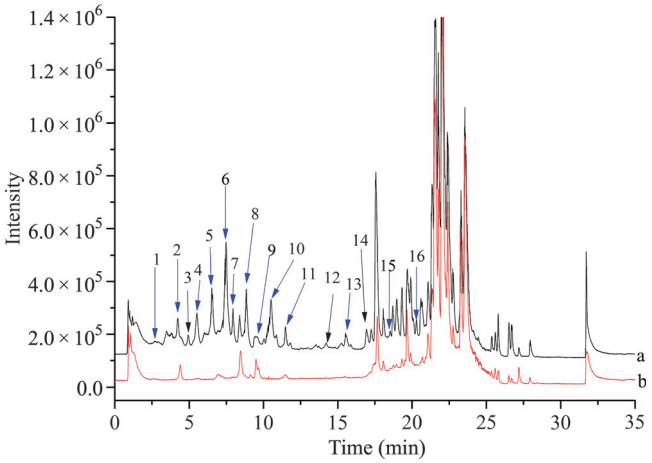

Figure 1. The total ion chromatogram of the absorbed components in plasma (a) and blank plasma (b), and the molecular ion peaks (Nos. 1–16)

chromatograms were compared with authentic standards or data from the literature, as shown in (a) and (b). A total of 9 prototypes (Nos. 1, 2, 4, 6, 8, 9, 10, 15, and 16) and 4 metabolites (Nos. 5, 7, 11, and 13) were identified or tentatively characterized in the plasma samples, and 3 components (Nos. 3, 12, and 14) were not identified. The total ion chromatogram of the absorbed components in the brain samples from treated animals (a) and the brain samples from untreated animals (b) is shown in Figure 2, where the molecular ion peaks are labeled "6, 8, 9, 15, and 16." By comparison of the total ion chromatograms shown in (a) and (b), a total of five prototype constituents were identified in the brain tissue by comparison of their LC–MS characteristics to literature data. The mass fragmentation patterns of the 13 components are shown in Figures 3–5. The molecular formula for each fragment was generated with the help of PeakView data processing software. Table 1 presents the results including retention time, extraction mass, molecular formulae, and other characteristics of the fragments.

Mass fragmentation behavior of the 13 components. Compound 1 was characterized as gallic acid. The $[M-H]^-$ precursor ion at *m/z* 169.0192 gave one prominent fragment ion at *m/z* 125.0302 $[M-H-CO_2]^-$, and subsequent fragmentation patterns showed ions at *m/z* 124.0223 and 107.0212, a fragmentation pathway consistent with the pure standard. The presence of methyl gallate in fruits of *T. chebula* Retz was reported previously, and together with the mass spectrum of

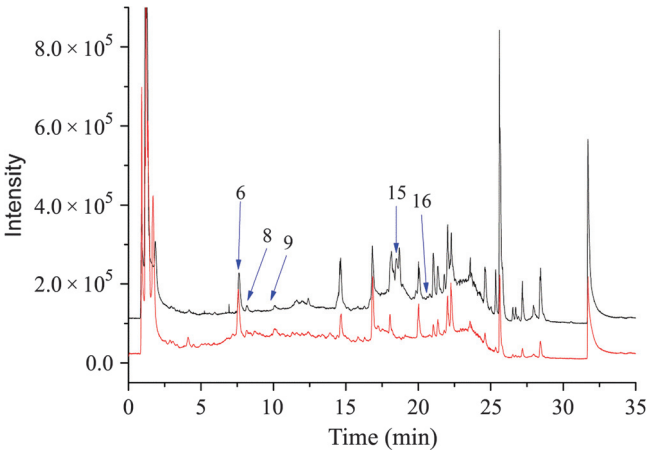

Figure 2. The total ion chromatogram of the absorbed components in brain (up/black chromatogram) and blank brain tissue (low/red chromatogram), and the molecular ion peaks (Nos. 6, 8, 9, 15, and 16)

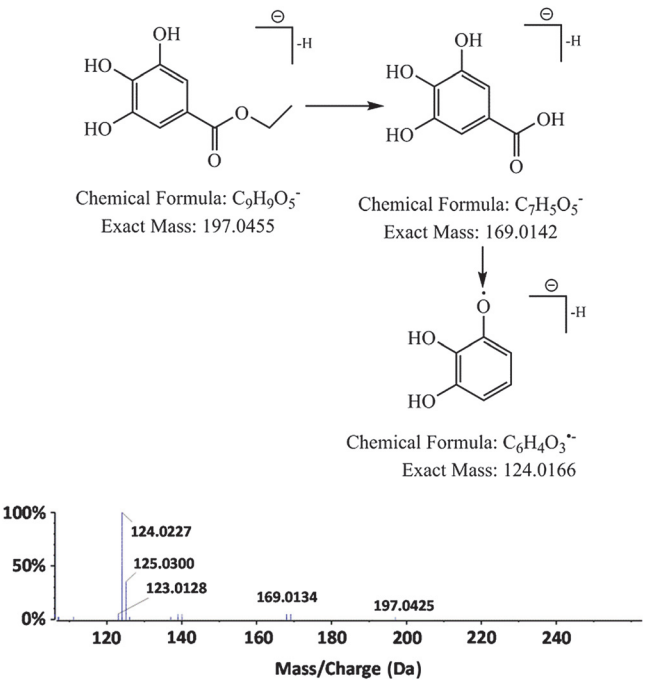

Figure 3. The mass fragmentation patterns of ethyl gallate (No. 6) absorbed in plasma and brain

pure standard, compound 2 was identified as methyl gallate. The $[M-H]^-$ at *m/z* 193.0516 of compound 4 produced MS/MS daughter ions at *m/z* 178.0519 and 134.0628, which indicated the sequential loss of a methyl moiety and carboxyl moiety. From this information, we concluded that compound 4 was ferulic acid. Compound 6 corresponded to ethyl gallate, the structure and fragmentation pathway of which are in complete agreement with that of the ethyl gallate standard sample (Figure 3) [25]. For compound 8, the $[M-H]^-$ at *m/z* 291.0057 produced MS/MS daughter ion at *m/z* 211.0560, which indicated a neutral loss of $2H_2O$ and CO_2. Moreover, $[M-H]^-$ was reduced to a fragment of *m/z* 196.0361 on the loss of *m/z* 95. From this information, we concluded that compound 8 was brevifolin carboxylic acid; this assignment was supported by comparison of its MS/MS spectra with that of the pure standard. Ellagic acid was widely reported to be the key constituent of *T. chebula* Retz. Compound 9 displayed a MS/MS spectra that matched that of the pure standard of ellagic acid. It had $[M-H]^-$ at *m/z* 300.9848, which gave rise to three fragments of *m/z* 283.9837, 257.0011, and 245.0010 with the loss of $^{\cdot}OH$, CO_2, and 2CO, respectively. Compound 10 was tentatively characterized as galloflavin by comparison of its chromatographic and spectrometric data. Triterpenoids are also found in *T. chebula* Retz and are mainly pentacyclic triterpene compounds such as oleanane-type compounds. Compounds 15 and 16 showed similar mass fragmentation regularity with similar product ions. After comparison with pure standards, the two compounds were identified as arjugenin (Figure 4) [26] and arjunic acid. Additionally, 4 metabolites were identified in the plasma in rats after oral administration, compounds 5 (Figure 5), 7, 11, and 13 of the metabolic pathways of methylation and glycosylation [27].

Mass fragmentation regularity of the 9 prototype constituents absorbed in plasma and brain. As shown in Figures 1 and 2, there are two types of components absorbed in the plasma and brain, tannins and triterpenoids. Typical mass fragmentation patterns and similarities were observed.

The components of tannins were easily fragmented into a fragment of *m/z* [M-18] on neutral loss of H_2O due to the adjacent hydroxyl group [28]. Gallic acid and its esters (such as

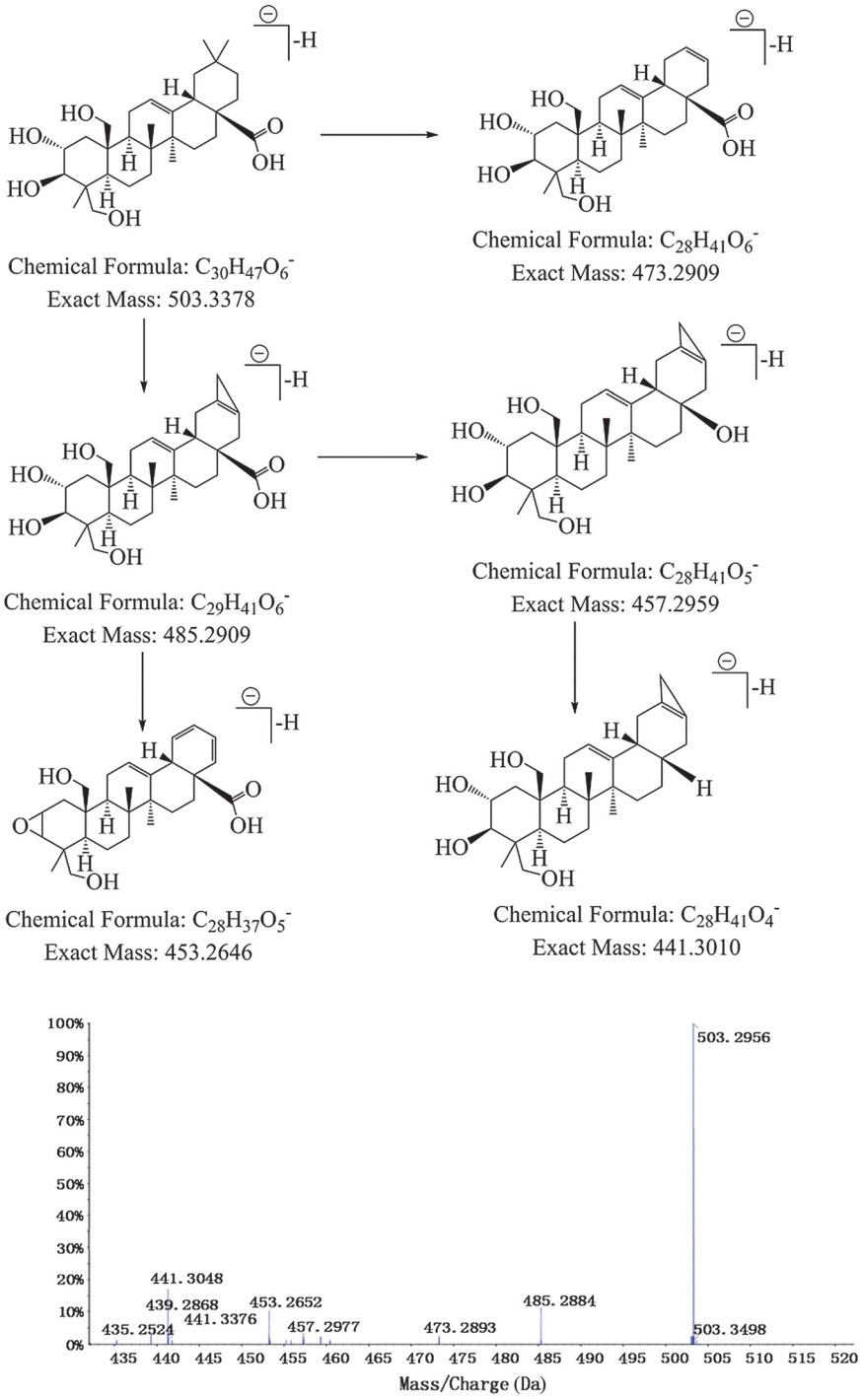

Figure 4. The mass fragmentation patterns of arjugenin (No. 15) absorbed in plasma and brain

methyl gallate and ethyl gallate) easily fragmented into two fragments of *m*/*z* 124.0179 and *m*/*z* 168.0074 with the loss of the ester group (COOR) and the alkyl group (R), respectively. The components containing ionic groups such as the carboxyl or ester moiety were likely to lose either carboxyl group, CO or CO_2, to give products such as brevifolin carboxylic acid and ellagic acid.

Triterpenoids such as arjugenin and arjunic acid have two methyl branches on the six-membered ring and easily gave a fragment of *m*/*z* [M-30] with the loss of $2CH_3$, as well as a fragment of *m*/*z* [M-45] with the loss of COOH.

Determination of pharmacodynamic activities of *T. chebula* Retz extracts. Our previous research showed that *T. chebula* extract HZ4 has neuro-protective effects. It can decrease infarct volume, improve sport ability, and promote the rehabilitation of model animals [29–31]. In animals and humans, only drugs that cross the blood–brain barrier can protect the central nervous system [32]. Thus, the components absorbed in brain are the components that can present neuro-protective effects. Due to the specificity of the blood–brain barrier, only molecules with low molecular weight and higher lipophilicity can easily enter the membrane [33]. The five components that were identified in the brain tissue include ethyl gallate, brevifolin carboxylic acid, ellagic acid, arjugenin, and arjunic acid, all of which have these physical characteristics and can cross the blood–brain barrier into the brain. Thus, the five components that were absorbed in the brain may confer the neuro-protective effects of *T. chebula* Retz.

T. chebula Retz has been reported to show various medicinal effects as described above. *T. chebula* Retz can increase the contents of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) in mouse blood serum, decrease the contents

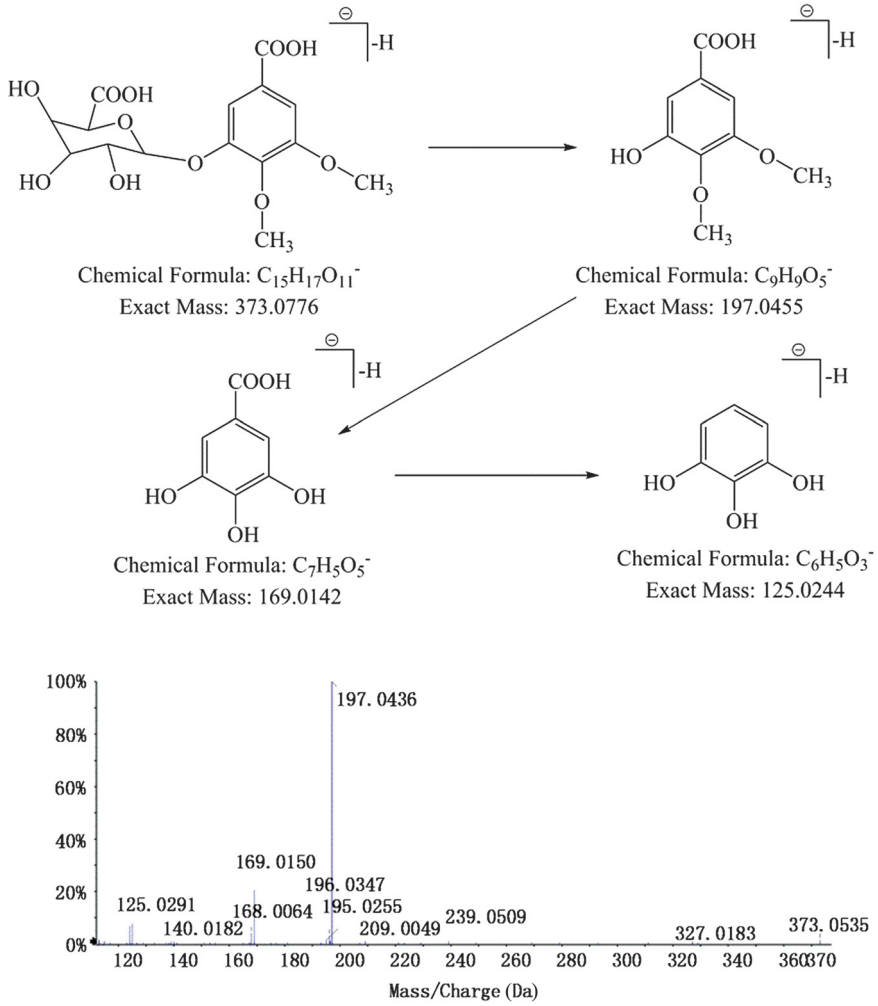

Figure 5. The mass fragmentation patterns of methylation of gallic acid glucuronide conjugate product (No. 5)

of monoamine oxidase (MAO) and malonaldehyde (MDA) in mouse blood serum and liver, and decrease levels of lipofuscin (LF) in rat liver [34]. It has also been shown to confer significant cardiac protection in isoproterenol-induced myocardial necrosis [15]. Gallic acid, methyl gallate, ferulic acid, ethyl gallate, brevifolin carboxylic acid, ellagic acid, galloflavin, arjugenin, and arjunic acid were detected in plasma and have antioxidant, hepatoprotective, cardiotonic, and

Table 1. QTOF-MS data of fragments of the components absorbed in the plasma and brain tissue of rats after intragastric administration of *Terminalia chebula* Retz extracts

No.	$[M–H]^+$					Product ion(s) (*m*/*z*)	Name
	Retention time (min)	Extraction mass (Da)	Found at mass (Da)	Error (ppm)	Formula		
1	2.92	169.01425	169.01499	4.4	$C_7H_6O_5$	125.0302, 124.0223, 107.0212	Gallic acid
2	4.53	183.0299	183.0303	2.2	$C_8H_8O_5$	168.0074, 124.0179	Methyl gallate
3	4.94	249.0979	249.0981	0.7	$C_{10}H_{18}O_7$	212.0026, 197.8081, 162.8397	–
4*	5.96	193.0506	193.0510	2.2	$C_{10}H_{10}O_4$	178.0519, 134.0623	Ferulic acid
5	6.54	373.0776	373.0769	−1.9	$C_{15}H_{18}O_{11}$	197.0436, 169.0150, 125.0291	Methylation of gallic acid glucuronide conjugate product
6	7.48	197.0455	197.04603	2.4	$C_9H_{10}O_5$	169.0134, 124.0227	Ethyl gallate
7	7.95	283.0823	283.0823	−0.1	$C_{13}H_{16}O_7$	283.0710, 107.0572	p-Cresol glucuronide
8	8.85	291.0146	291.01767	3.4	$C_{13}H_8O_8$	211.0560, 196.0361, 166.9996, 123.0141	Brevifolin carboxylic acid
9	9.51	300.9989	300.9989	0.1	$C_{14}H_6O_8$	283.9837, 257.0011, 245.0010	Ellagic acid
10*	10.512	276.9990	277.0022	11.5	$C_{12}H_6O_8$	277.0027, 198.0497, 197.0467	Galloflavin
11	11.81	401.1089	401.1078	−2.9	$C_{17}H_{22}O_{11}$	225.0704, 169.0143, 124.0207	3-*O*-methylpropyl gallate glucuronide conjugate product
12	14.21	305.0303	305.0329	8.6	$C_{14}H_{10}O_8$	225.0768, 197.8029, 186.0567	–
13	15.54	397.1140	397.1125	−3.8	$C_{18}H_{22}O_{10}$	221.0753, 177.0922, 149.0979	Monobutyl phthalate acyl-D-glucuronide
14	16.937	543.2811	543.2834	4.3	$C_{27}H_{44}O_{11}$	498.2856, 365.2315, 305.1597	–
15	18.69	503.33781	503.33502	2.5	$C_{30}H_{48}O_6$	485.2884, 473.2893, 457.2977 453.2652, 441.3048	Arjugenin
16	20.81	487.3429	487.33983	3.3	$C_{30}H_{48}O_5$	469.2873, 457.2869, 441.2956 437.2639, 425.3039	Arjunic acid

Note: "*" indicates that compound was tentatively identified from *Terminalia chebula* Retz; "–" indicates that compound was not identified.

nephrotoxicity preventive activities [6–17, 35, 36]. Thus, the 9 components identified in the rat plasma are likely the main components responsible for these beneficial medicinal effects of *T. chebula* Retz.

Conclusions

In this work, the components absorbed in the plasma and brain of rats after intragastric administration of *T. chebula* Retz extracts were systematically investigated. The 9 components that were absorbed in plasma and 5 components absorbed in the brain tissue were rapidly identified by UPLC–QTOF-MS. Based on the results of this work and related literature reports, we can confirm that the antioxidant, hepatoprotective, cardiotonic, and antidiabetic activities of *T. chebula* Retz are related to the components absorbed in plasma, and the neuroprotective effects are related to the components that are absorbed in the brain. This experiment allowed rapid determination of the active components of *T. chebula* Retz and guided the establishment of a method for its quality control. The results of this study demonstrate a rapid and highly efficient method for the determination of the effective components of Chinese herbal medicines.

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