Supplemental material

Supplementation with Tribulus terrestris extract exhibits protective effects on MCAO rats via modulating inflammation-related metabolic and signaling pathways

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1 HPLC analysis of GSTTF

1.1 HPLC analysis

The separation of the GSTTF (1mg/mL) sample was conducted using an Agilent 1200 series LC system equipped with an Agilent ZorBax SB-C18 column (2.7 μm, 4.6 × 250 mm) at 25 °C with an injected volume of 10 μL. The mobile phase consisted of acetonitrile (phase A) and water (phase B). It was carried out under the following gradient procedure: 0-5 min, 15-19%B; 5-30 min, 19-23%B; 30-50 min, 23-23.5%B; 50-55 min, 23.5-29%B; 55-70 min, 29-37%B; 70-90 min, 37-73%B; 90~100 min, 73-80%B. The chromatogram was monitored by an ELSD.

1.2 Results of HPLC analysis

The HPLC chromatogram of GSTTF was shown in **Figure S1**. By comparing the retention times with reference standards, we identified nine peaks as 26-O-β-D-glucopyranosyl-(25S)-5α-furostan-12-one-2α,3β,22α,26-tetraol-3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**peak 1**), 26-O-β-D-glucopyranosyl-(25S)-5α-furostan-12-one-3β,22α,26-triol-3-O-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**peak 2**), 26-O-β-D-glucopyranosyl-(25R)-5α-furostan-12-one-3β,22α,26-triol-3-O-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**peak 3**), 26-O-β-D-glucopyranosyl-(25S)-5α-furostan-20(22)-en-2α,3β,26-triol-3-O-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**peak 4**), 26-O-β-D-glucopyranosyl-(25R)-5α-furostan-20(22)-en-12-one-2α,3β,26-triol-3-O-β-D-glucopyranosyl(1→4)-β-D-galactopy-ranoside (**peak 5**), 26-O-β-D-glucopyranosyl-(25R)-5α-furostan-20(22)-en-12-one-3β,26-diol-3-O-β-D-galactopyranosyl-(1→2)-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**peak 6**), 26-O-β-D-glucopyranosyl-(25S)-5α-furostan-20(22)-en-12-one-3β,26-diol-3-O-β-D-galactopyranosyl-(1→2)-β-D-glucopyranosyl(1→4)-β-D-galac-topyranoside **(peak 7)**, 26-O-β-D-glucopyranosyl-(25S)-5α-furostan-20(22)-en-12-one-2*α*,3β,26-triol-3-O-β-D-glucopyranosyl-(1→2)-o-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside **(peak 8)**, and 26-O-β-D-glucopyranosyl-(25S)-5α-furostan-20(22)-en-12-one-2*α*,3β,26-triol-3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside **(peak 9)**.

2 Animal and treatments

2.1 MCAO model establishment

The MCAO surgery was performed following the previous report with modifications [1]. Briefly, after rats were anesthetized, a 1 cm long midline skin incision was made on

the neck; then, the muscle on either side of the trachea was separated to expose the common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA). Then a silicone-coated suture was inserted from the left ECA into the lumen of the ICA to occlude the origin of the MCA. The rats in the sham-operated group were subjected to an identical procedure without ligation. All animals were maintained at 25-28 °C during the surgery.

2.2 Infarct volume measurement

The brains were removed and sliced into 2 mm thick coronal sections and stained with 2,3,5-triphenyltetrazolium chloride (TTC, 2% TTC in phosphate-buffered saline). All coronal slices were digitalized, and the area of cerebral damage was analyzed using Image-J software (National Institutes of Health, Bethesda, MD, USA).

2.3 Evaluation of neurological defects

Neurological functional deficiency scores were following Longa's five-point scale[1]: zero points: no neurobehavioral dysfunction; one point: failure to flex the contralateral front limb completely; two points: circling counter-clockwise; three points: turning around to the affected side seriously; four points: cannot walk spontaneously. The higher the score, the more serious the impairment of animal behavior.

3 LC-MS-based metabolomics analysis

3.1 Brain tissue extraction

Sample preparation was performed according to our previous report[2]. An 80 mg brain tissue sample was homogenized with 1.5 mL prechilled methanol/water (v/v 1:1), then the obtained mixture was centrifuged at 15000 g for 15 min at 4 °C. The supernatant aqueous extracts were transferred into a centrifuge tube and were lyophilized with a freeze dryer. The retained pellet was extracted with a 1.6 mL prechilled dichloromethane/methanol (v/v 3:1) solution. After homogenization, the sample was centrifuged at 15000 g for 15 min at 4 °C, and the supernatant organic extracts were transferred into a centrifuge tube and were lyophilized with a freeze dryer. The aqueous and organic extracts were reconstituted in 120 μL of methanol/water (v/v 1:1), respectively, and centrifuged at 15000 g for 10 min at 4 °C. An aliquot of 5 μL supernatant was used for LC-MS analysis. The pooled aqueous and organic quality control (QC) sample was prepared by mixing an equal mass of each sample and prepared by the above procedures to perform method validation.

3.2 LC-MS condition

The brain tissue extract was analyzed by a Vanquish Duo UHPLC coupled with a Q-

Orbitrap mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific, San Jose, CA, USA). The aqueous extract was separated on an ACQUITY HSS T3 column (2.1 mm×100 mm, 1.8 μm) with a column temperature of 50 °C and an injection volume of 5 μL. The mobile phases are 0.1 % formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). The gradient, at 0.4 mL/min, was 0.1%B (0-2 min); 0.1-25%B (2-6 min); 25-80%B (6-10 min); 80-90%B (10-12 min); 90-99.9%B (12-21 min); 99.9%B (21-23 min); 99.9-0.1%B (23-30 min), 0.1%B (30-35 min).

For the organic extract, the chromatographic separation was conducted on an Hypersil GOLD ™ column (2.1 mm × 50 mm, 1.9 μm) at 50 °C, with an injection volume of 5 μL. The mobile phase consisted of 0.1% aqueous formic acid (phase A) and methanol containing 0.1% formic acid (phase B) under the following gradient program: 40%B (0-1 min) 40-70%B (1-6 min); 70%B (6-10 min); 70-80%B (10-15 min); 80%B (15-17 min); 80-91%B (17-18 min); 91-99.9%B (18-29 min); 99.9%B (29-32 min); 75-40%B (33-34 min) 40%B (34-35 min) with a flow rate of 0.4 mL/min.

The mass spectrometer was operated in both positive and negative ion modes, and the profile data was recorded in the range of *m/z* 100-1500. The full scan acquisition was performed at the resolution of 35000, while the tandem MS information was acquired under ddMS2 (TOP 10) mode with a resolution of 17500, and the ramped normalized collision energy of 25-45. The key parameters of the ionization source were set as follows: capillary voltage of 3.5 kV in positive ion mode or -3.2 kV in negative ion mode, sheath gas flow 50 arb, auxiliary gas flow 15 arb, sweep gas flow 2 arb, capillary temperature 300 °C. Before sample analysis, the mass spectrometer was calibrated using the vendor-provided Pierce™ calibration solution (Thermo Fisher Scientific, San Jose CA, USA). All samples were maintained at 4 °C through the analysis.

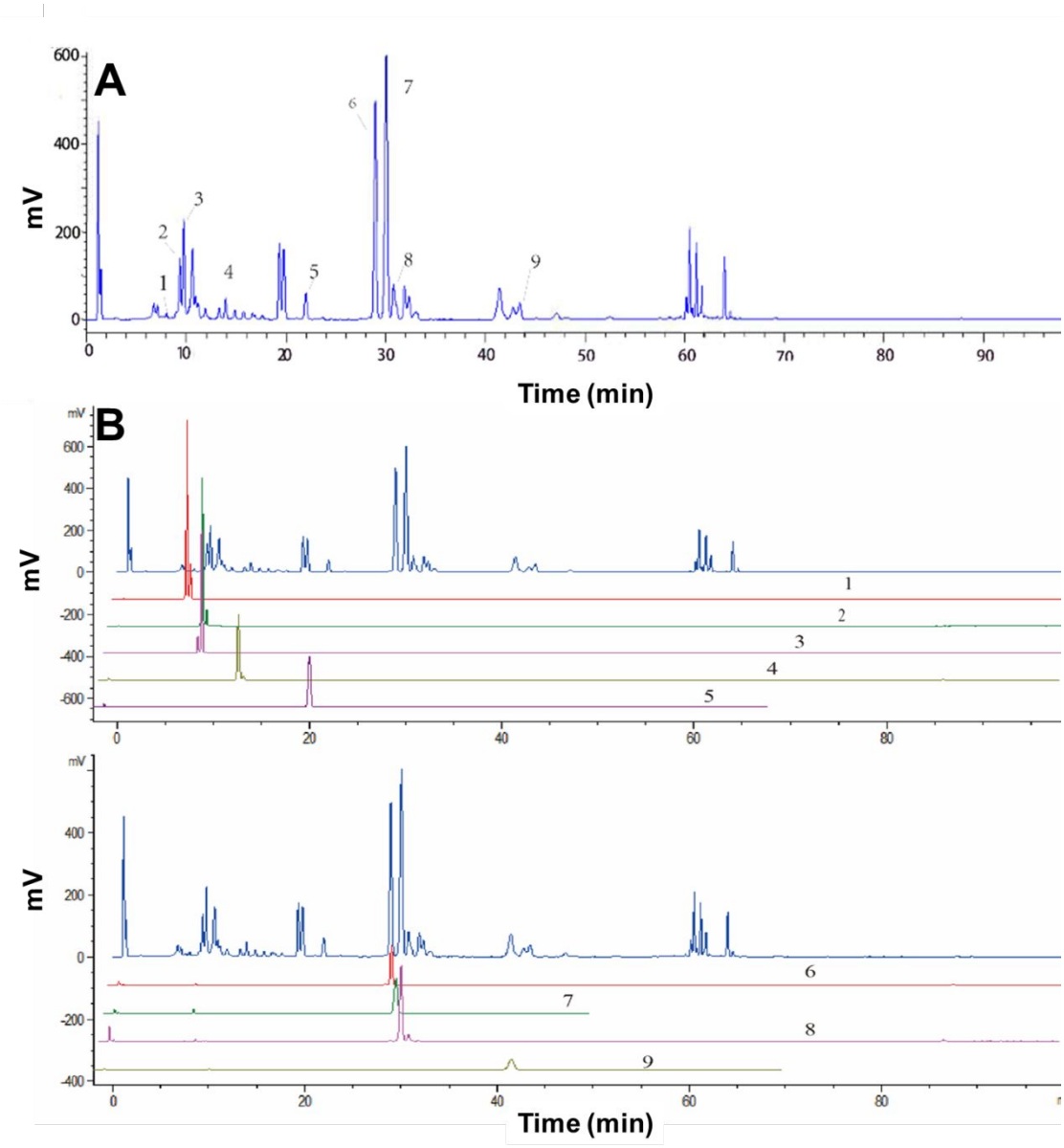

Figure S1 HPLC chromatograms of GSTTF (A), and the chromatograms of 9 standard references (B).

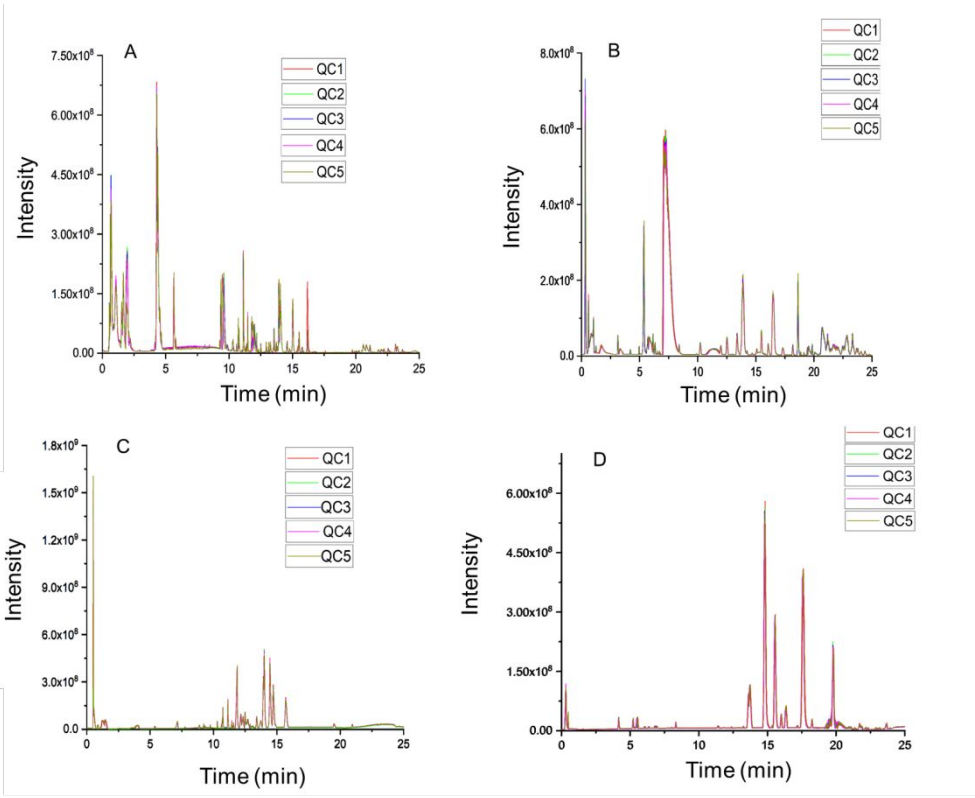

Figure S2 The base peak chromatograms of QC sample from aqueous (A, ESI+, and C, ESI-) and organic extracts (B, ESI+, and D, ESI-).

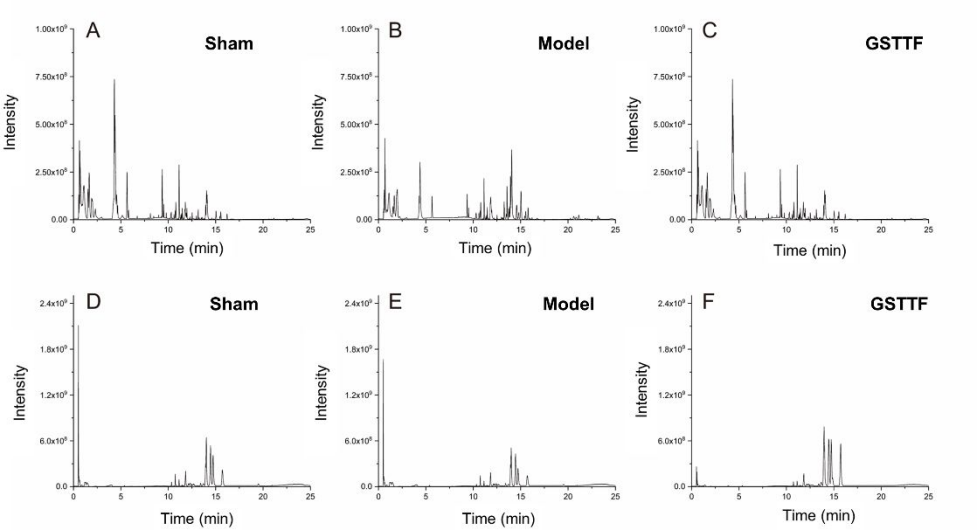

Figure S3 The representative BPC of aqueous brain tissue extract in three groups acquired in positive ion mode (A-C) and negative ion mode (D-F).

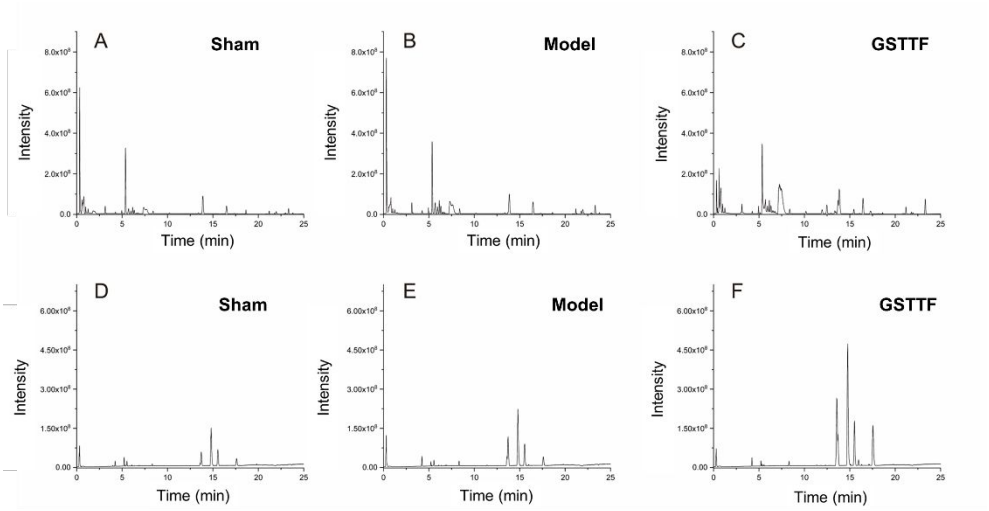

Figure S4 The representative BPC of organic brain tissue extract in three groups acquired in positive ion mode (A-C) and negative ion mode (D-F).

References

1. Longa, E.Z.; Weinstein, P.R.; Carlson, S.; Cummins, R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* **1989**, *20*, 84-91.
2. Wang, Y.; Guo, W.; Xie, S.; Liu, Y.; Xu, D.; Chen, G.; Xu, Y. Multi-omics analysis of brain tissue metabolome and proteome reveals the protective effect of gross saponins of Tribulus terrestris L. fruit against ischemic stroke in rat. *J. Ethnopharmacol.* **2021**, *278*, 114280.