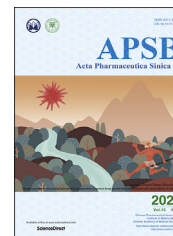




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SHORT COMMUNICATION

A systematic strategy for screening therapeutic constituents of *Schisandra chinensis* (Turcz.) Baill infiltrated blood–brain barrier oriented in lesions using ethanol and water extracts: a novel perspective for exploring chemical material basis of herb medicines



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Pharmacodynamics;
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Components into nidi;
Effective material basis;
Traditional Chinese
medicine

Abstract *Schisandra chinensis*, a widely used Chinese herbal medicine, was considered as central nervous system (CNS) drug for years. Both ethanol extracts (EES) and water extracts (WES) of it were applied clinically. Unfortunately, the difference of their efficacy and even effective material foundation of *S. chinensis* remains obscure. In this study, to explore the active constituents of *S. chinensis*, we compared pharmacodynamics and chemical profiles *in vitro/in vivo* of EES/WES for the first time using multiple chemical analysis, pharmacological and data processing approaches. It was proved that there was no significant difference in the anti-depressive effects between WES and EES. However, the contents of most components *in vitro* and in plasma were higher in EES than those in WES, which was unconvincing for their similar efficacy. Therefore, we further explored components of *S. chinensis* targeted onto brain and the results showed that 5 lignans were identified with definite absorptivity respectively both in EES and WES caused by the limitation of blood–brain barrier. Moreover, bioinformatic analysis predicted their anti-depressive action. Above all, the systematic strategy screened 5 brain-targeted effective

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substances of *S. chinensis* and it was suggested that exploring the components into nidi would promote the studies on herbs effective material basis.

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1. Introduction

Schisandra chinensis (Turcz.) Baill, a Magnoliaceae family traditional Chinese medicine (TCM), was abundant in orient with related records traced back before Christ^{1,2}. Based on extensive studies done by researchers, complex and various chemical ingredients have been identified^{3–5}, abounding in triterpenoids, lignans, polysaccharides, organic acids, and essential oils. Recently, *S. chinensis* has attracted increasing attention for its high nutritional and officinal values^{6,7}. In previous reports, *S. chinensis* has been proved to be effective in alleviating diverse central nervous system (CNS) diseases and our past research demonstrated that *S. chinensis* has satisfying anti-depression action^{8–11}. Over the years, ethanol extracts (EES) and water extracts (WES) have been widely applied clinically, whereas WES was considered as a traditional administration for humans for thousands of years. However, the difference of the pharmacodynamics of these refinements has always been neglected and even the active constituents of *S. chinensis* has been indistinct for now.

Interestingly, our study suggested that there was no significant difference in the anti-depressive effects between WES and EES though contents of most components in EES were higher than those in WES *in vitro* and in plasma samples. According to published literatures, blood–brain barrier (BBB) possesses high selectivity for foreign materials, limiting the delivery of CNS drugs to reach the site of action in brain^{12,13}. Thus, we speculated that as a natural anatomical gateway, BBB may adjust the type and content of *S. chinensis* components into the nidi to further display pharmacodynamic.

In present research, we explored the selection of BBB on the constituents of *S. chinensis* by comparing the therapeutic mechanism and chemical contents *in vitro/in vivo* of EES/WES, to ascertain its brain-targeted effective substances. The exploration strategy was shown as Fig. 1. In brief, we proved the similar anti-depression action of WES and EES on chronic unpredictable mild stress (CUMS) depressive rats by measuring the levels of neurotransmitters and cytokines. Then, after contrast research on the chemical profiles of EES and WES *in vitro/in vivo*, only 5 lignans, schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N, could be identified in the brain lesions with similar contents in both EES and WES, which might be selected and leveled by BBB independent on their contents *in vitro* or in plasma. It was indicated that the 5 brain-oriented components might contribute to the similar effect of WES and EES greatly. Furthermore, bioinformatics was employed to predict the pharmacology of these 5 potential active ingredients, suggesting their potential anti-depression effects on modulating neurotransmitters and inflammatory pathways respectively. This research suggested that ingredients pointed at lesions were vital for the

pharmacodynamics of TCM and the established integrated strategy on nidi-directed ingredients was significant for the development of studies on herbs effective material basis.

2. Materials and methods

2.1. Materials and reagents

The fruits of *S. chinensis* were purchased from the TCM dispensary—Tongrentang (Shenyang, China) grown in Ji'an, Jilin, China. Fluoxetine, bifendatum, dopamine (DA), norepinephrine (NE), serotonin (5-HT) were all purchased from Sigma (St. Louis, MO, USA). The reference substances of schisandrol A, gomisin D, gomisin J, schisandrol B, gomisin O, epigomisin O, schisandrin A, schisandrin B and gomisin N were obtained from Yuanye of Biological Engineering (Shanghai, China). The commercial enzyme-linked immunosorbent assay (ELISA) kits of corticosterone (CORT), interleukin-1b (IL-1 β), cyclooxygenase-2 (COX-2), tumor necrosis factor α (TNF- α) and tyrosine protein kinase (TPK) were purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

2.2. Animals

Male Sprague–Dawley rats weighing between 200–250 g were used in present study. Standards for Animal Experimentation of Shenyang Pharmaceutical University were used in all experiments, which was in line with the rules of the Animal Ethics Committee of the institution (Shenyang, China). Before the experiments, rats were housed in groups at indoor temperature with 40%–60% humidity for seven days. Food and water were available *ad libitum*. Rats were divided into control, CUMS, WES, EES and positive groups.

2.2.1. CUMS procedure

CUMS protocol used in this study was derived from a model established by Willner^{14,15}. Rats were suffered from various stressors for 28 days. The process of CUMS in the first 14 days was shown as Supporting Information Table S1 and was repeated in the next 14 days. The experiment process was shown as Supporting Information Fig. S1. The rats in CUMS, WES, EES and positive groups were placed in a different room from the rats in control group, and they were bear two or three stressors every single day. In additional, all stressors were inflicted randomly.

2.2.2. Behavioral tests

Forced-swim test (FST), locomotor activity test and novelty suppressed feeding (NSF) test were conducted during the animals'

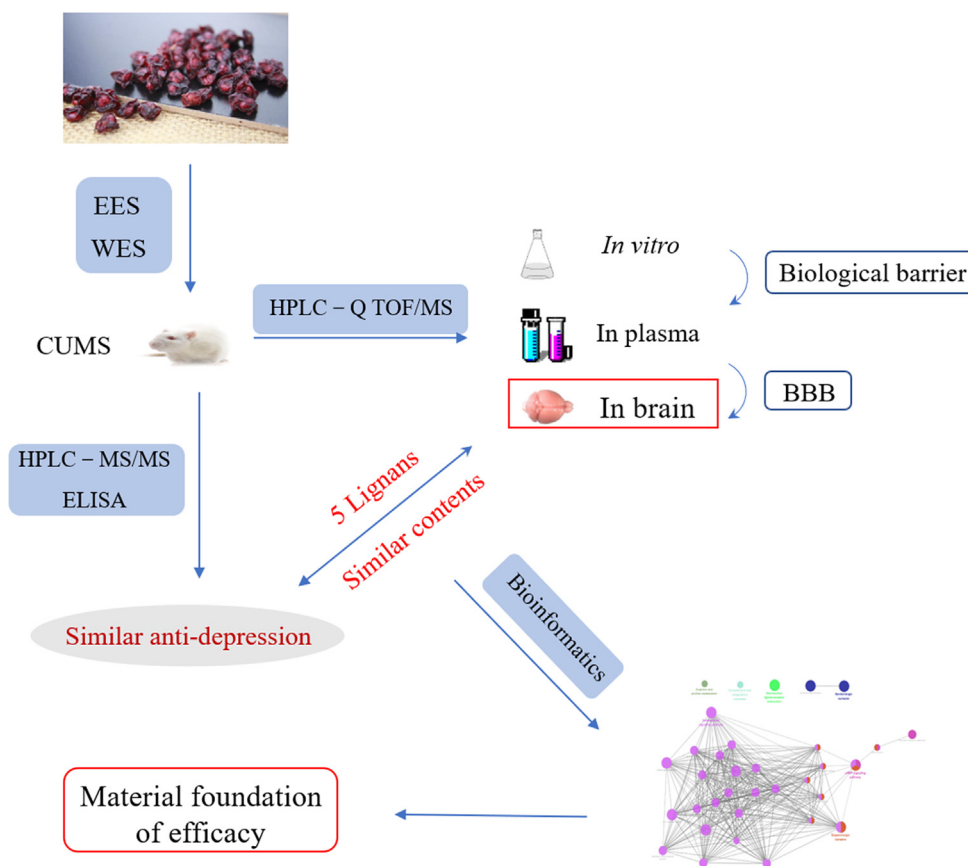


Figure 1 The research strategy for finding the material basis for efficacy of *Schisandra chinensis* by comparing the chemical profiles and pharmacodynamics of EES and WES.

light cycle. Rats were gavage ahead of time and given 1 h to habituate to the room before behavioral tests.

2.2.2.1. FST test. It was carried out as designed before. Rats were coerced to swim separately in a metallic box (18 cm in diameter \times 40 cm in height) full with water (approximately 30 cm deep at 25 °C). The observation time on the rats was 6 min and the total immobile time in last 4 min was recorded, which was defined as short of activities in spite of needful movements avoiding their heads into water.

2.2.2.2. Locomotor activity test. Spontaneous locomotor activity of the experimental rats was proceeded in an open filed analysis system (Jiliang Bio-Apparatus Co. Ltd., Shanghai, China) to exclude the possibility that the change of the immobility time in the FST was resulted by the locomotor activity. The analysis system was placed in a dark and quiet room. The total path of spontaneous locomotive was observed for 5 min. The equipment was cleaned using 75% ethanol after each test.

2.2.2.3. NSF test. Rats were abstained from food for 24 h and placed in a corner of a top-opening square box individually. The box covered with 2 cm padding and 12 food pellets placed in the center. The feeding latency period to eat (up to 5 min) was

recorded. Eating was defined as chewing or biting, not merely sniffing or toying with the food.

2.3. Quantitative analysis of neurotransmitters using HPLC-MS/MS

2.3.1. Working standard solutions and sample solutions

We prepared the standard stock solutions (1.0 g/mL) of NE, DA, 5-HT and internal standard isoprenaline stock solution in methanol respectively (stored at 4 °C). In addition, we prepared the mixed brain tissue standard solution with different concentrations as follow: DA of 1.00, 2.00, 10.00, 20.00, 100.00 and 200.00 μ g/mL; NE, 5-HT of 5.00, 10.00, 50.00, 100.00, 500.00 and 1000.00 μ g/mL. Besides, the quality control (QC) samples were prepared ditto. The working solution of the internal standard (IS) at 10 μ g/mL was prepared.

Twenty-four hours after all the behavior tests, all the experimental rats were killed and brain samples were stored at -80 °C. The brain samples were homogenized in a 10-fold volume of methanol and centrifuged at 4 °C, 12,000 rpm (TGL-16, Cence Xiangyi, Hunan, China) for 10 min. 600 μ L supernatant was transferred to a 1.5 mL centrifugal tube, then added 60 μ L IS solution and 60 μ L methanol into it and the mixture was vortexed for 45 s. Next, the solution was blown with air at 35 °C to dry.

After that, the residue was dissolved in 100 μ L methanol. Then, the solution was vortexed for 5 min and centrifuged at 4 $^{\circ}$ C, 12,000 rpm (TGL-16) for 10 min. Finally, 3 μ L supernatants was used for the HPLC–MS/MS analysis.

2.3.2. Conditions of HPLC–MS/MS

In this study, an XR LC-20AD Prominence™ HPLC system (Shimadzu, Kyoto, Japan) combined with a QTRAP™ 4000 MS/MS system (AB Sciex, Redwood City, CA, USA) was used. The chromatographic separation was proceeded on a Phenomenex OOD-4496-EO (2.6 μ m, 100 mm \times 4.6 mm) column and the column temperature was set at 30 $^{\circ}$ C. The mobile phase was water–0.1% formic (A) and acetonitrile (B) at 0.4 mL/min. The gradient elution program was performed as follows (*t* in min): 10% B maintained for 0.5 min, increased to 20% at 1.0 min, increased to 90% at 3.0 min, and then decreased to 10% and held for 2.0 min for equilibration. The MS was operated in positive ion multiple reaction monitoring mode. Supporting Information Table S2 showed all optimized parameters. The other parameters were listed as follows: ion spray voltage of 4500 V, curtain gas of 20 psi; source temperature of 500 $^{\circ}$ C; ion source gas 1 and gas 2 of 50 psi.

We abide by the U.S. Food and Drug Administration Guidance for Industry Bioanalytical Method Validation and European Medicines Agency Guidelines Principle in this study. The inspection items included calibration curve, selectivity, accuracy and precision, lower limit of quantification (LLOQ), matrix effect and stability. Samples were prepared according to “sample preparation”. On every validation day, a series stock solution was added to brain samples to prepare the QC and calibration standards samples. The concentrations of neurotransmitters were calculated by calibration curves.

2.4. Qualitative and semi-quantitative analysis of contents of *S. chinensis* using HPLC–Q-TOF/MS

2.4.1. Preparation of samples

Samples were smashed into powder and sieved with 60 mesh. The medicine powder was extracted by reflux in 75% ethanol or water for 1 h for 3 times. The extracts were collected and being filtered. Then, under vacuum, the filtrate was concentrated. The residues were then dissolved in water at concentration of 0.9 g/mL as the intragastric solutions (WES or EES). The dosage used for rats was according with human dosage listed on the Chinese Pharmacopoeia. To ensure the parallelity of the detected samples *in vivo* and *in vitro*, the method for preparation of test solution of WES and EES *in vitro* was same as the intragastric solutions.

One hour after the last administration, we collected plasma samples from suborbital vein and transferred into heparinized tubes. The plasma samples were immediately centrifuged at 4000 rpm (TGL-16) for 10 min and then stored at -80° C. Protein precipitation was adopted to extract the plasma samples in this study. 1 mL supernatant of plasma was evaporated to dryness under a gentle stream of nitrogen at 35 $^{\circ}$ C. Then, the residue was reconstituted with 100 μ L solution of bifendatatum (1 mg/mL) and vortex-mixed for 3 min, and sonicated for 5 min followed by centrifuged at 12,000 rpm (TGL-16) for 5 min. Finally, 5 μ L supernatants was injected into the HPLC–Q-TOF/MS system for analysis.

The rats were weighed and anesthetic using chloral hydrate. Then, we opened the rats' abdominal cavity and freed the heart tissue from their chest. Opened the constant-current pump to

pump 200 mL of warm normal saline from the heart to clear the blood. Rapid whitening of liver, eyes and claws is an effective observation index for blood expulsion. At last, rat brain tissues were collected and stored at -80° C. Five-fold methanol was added to the brain samples, and the mixtures were homogenized on the ice and then centrifugated at 4 $^{\circ}$ C, 12,000 rpm (TGL-16) for 10 min. The following procedures were the same as that for the plasma samples.

2.4.2. Conditions of HPLC–Q-TOF/MS

The constituents of *S. chinensis in vivo/in vitro* were analyzed on the Agilent 1260 HPLC system (Santa Clara, CA, USA) interfaced with a Triple TOF 5600⁺ (AB Sciex, Redwood City, CA, USA) outfitted with a DuoSpray™ ion source (Sciex). The Phenomenex OOD-4496-EO (2.6 μ m, 100 mm \times 4.6 mm) column was used for the separation at a flow rate of 0.8 mL/min in LC–MS grade water (A) and acetonitrile (B). The gradient elution program was as follows: 10% held for 5.0 min; increased to 40% at 10 min; increased to 57% at 75 min; increased to 90% at 85 min and held for 5 min followed by 10% for 5.0 min for equilibration. The scan acquisition for the analysis was over the range of *m/z* 100–2000 in positive ion mode. The other parameters were listed as follows: ion spray voltage of 5500 V; declustering potential (DP) of 80 V; the turbo spray temperature of 550 $^{\circ}$ C; curtain gas of 30 psi; ion source gas 1 and gas 2 of 50 psi. Nitrogen was kept as nebulizer and auxiliary gas. SciexPeakView® (version 2.2) and Master View™ (version 1.1) software were used for the further data process.

2.5. Statistical analyses

All values were showed as means \pm SD in the study. GraphPad Prism (GraphPad software, San Diego, CA, USA) and SPSS (SPSS Inc., Armonk, NY, USA) were used for the data process.

3. Results

3.1. Exploration of the anti-depressant effects of EES and WES

3.1.1. Improvement of the behavioral deficits induced by CUMS

After 4-week CUMS process and EES/WES/fluoxetine (10 mg/kg) treatment, rats were assessed on their behaviors and the level of corticosterone in plasma to evaluate the success of depression model. All treatments showed no significant alterations to the total path in spontaneous locomotive activity test, which suggesting that the locomotor activity has few impacts on the alteration of immobility time in FST. Moreover, the process of CUMS successfully induced depression on rats according to depressive symptoms showed in behaviors (immobility time and latency time remarkably prolonged, Fig. 2A and C, $n = 6$, $P < 0.01$), and increased level of corticosterone in rats' plasma samples compared with the control group (Fig. 2D, $n = 6$, $P < 0.01$). However, they were consistently restored after pharmacological treatments (Fig. 2A, C and D, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$), and have no significant difference between WES and EES groups (Fig. 2).

3.1.2. Effects of WES and EES on neurotransmitters and inflammation markers

In this study, an HPLC–MS/MS method was employed to detect 5-HT, NE and DA in rat brain samples. The retention

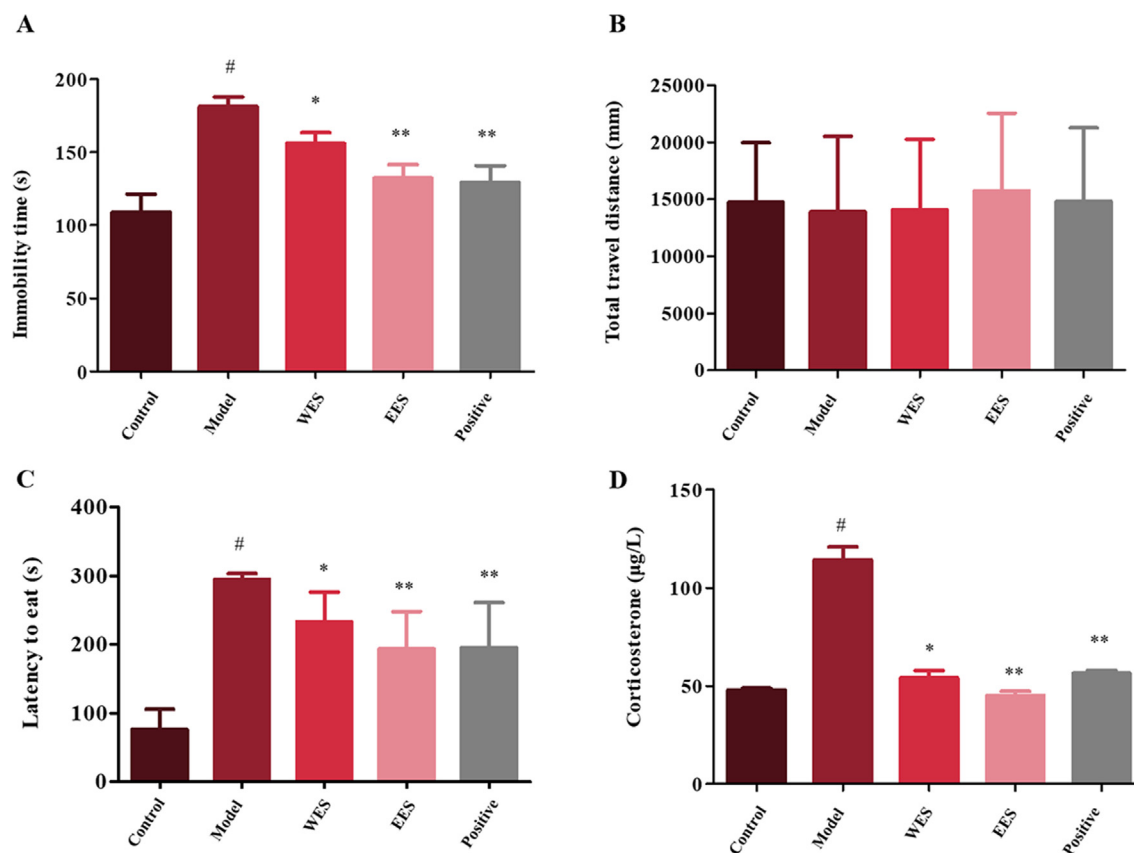


Figure 2 (A) The immobility time in FST of groups. (B) The travel paths in the locomotor activity test. (C) The latency time to feed in NSF test. (D) The level of corticosterone in plasma samples. Values shown are means \pm SD, $n=6$; [#] $P < 0.01$ compared with control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with model group.

time of analytes or IS was without interference based on the chromatography (Supporting Information Fig. S2). The developed method was validated with respect to the lower limit of quantification, calibration curve, accuracy and precision, matrix effect and stability (Supporting Information Tables S3–7). It was suggested that the HPLC–MS/MS method was well validated to quantify the target analytes in this study. Besides, inflammatory enzyme COX-2, TNF- α , TPK and IL-1 β were measured in brain samples using ELISA assays. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), was selected as the positive drug.

In CUMS group, obvious reduction of the expression of neurotransmitters and significant elevation of the levels of inflammatory markers were observed as shown in Table 1 and Fig. 3. However, the samples from both EES and WES groups showed significantly reverse tendency in both two tests based on the results of t test, and there was no obvious deference between them.

3.2. Analysis of chemical profiles *in vivo* and *in vitro* using HPLC–Q-TOF/MS

3.2.1. Identification of constituents in WES and EES

An efficient and selective HPLC–Q-TOF/MS method was employed to identify and semi-qualify the ingredients of WES and EES *in vitro/in vivo*. The absolute value of errors was defined lower than 3. Total 45 constituents including 33 lignans, 10 triterpenes and 2 organic acids were identified in EES, while 36 components (the first 36 ingredients in Table 2, including 30 lignans, 4 triterpenes and 2 organics) of them were also found in WES, all their structures were shown in Supporting Information Fig. S3. Moreover, 15 lignans were found in the plasma samples and 5 of them (schisandrol A, gomisin J, schisandrin A, gomisin N and schisandrin B) were found in the brain samples both in EES and WES. All components of EES and WES *in vitro/in vivo* were listed in Table 2 and total compound chromatogram (TCC) for ingredients *in vivo* shown as Fig. 4. Nine lignans were confirmed

Table 1 The concentrations of 5-HT, DA, NE in samples WES and EES.

Analyte	Control group (ng/mg)	CUMS group (ng/mg)	WES (ng/mg)	EES (ng/mg)	Fluoxetine group (ng/mg)
5-HT	19.9 \pm 4.94	4.34 \pm 1.89 ^{##}	8.04 \pm 2.14 [*]	15.20 \pm 3.77 ^{**}	13.9 \pm 2.26 ^{**}
DA	5.49 \pm 2.29	2.40 \pm 1.22 [#]	3.54 \pm 1.28	6.60 \pm 2.04 [*]	5.51 \pm 3.13
NE	6.93 \pm 1.08	2.41 \pm 0.97 [#]	5.30 \pm 1.60 [*]	7.82 \pm 1.16 ^{**}	5.50 \pm 1.38 [*]

For statistical significance ^{**} $P < 0.01$, ^{*} $P < 0.05$ compared with CUMS group, ^{##} $P < 0.01$, [#] $P < 0.05$, compared with control group.

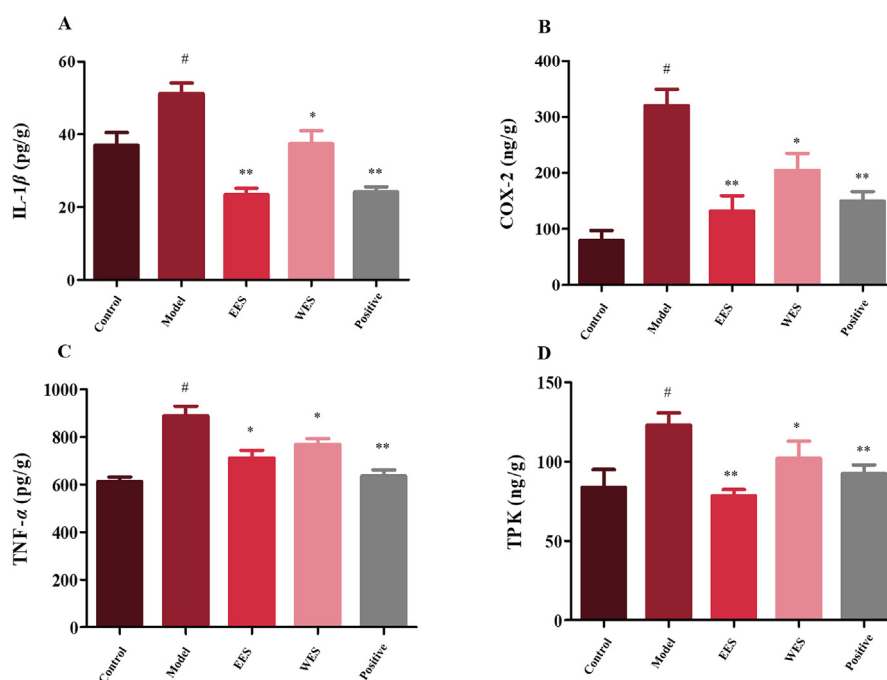


Figure 3 Effects of EES and WES on inflammation markers. Values shown are means \pm SD; [#] $P < 0.01$ compared with control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with CUMS.

with standard substance among them (Supporting Information Fig. S4).

3.2.2. Semi-quantitation of constituents in EES and WES

In present study, bifendate was taken as the IS for semi-quantitative analysis. The contents of all lignans except for 9 included benzoylgomisin Q, angeloylgomisin Q, schisantherin D, schisantherin E, schisanhenol, schisanwilsonin D, schindilactone D, schisphenlignan A and schisanlactone E were decreased significantly in WES but those of organic acids and triterpenes were similar in EES and WES *in vitro* (Fig. 5). In addition, in the plasma samples, contents of 10 lignans, schisanwilsonin D, schisandrol A, gomisin O, epigomisin O, wilsonilignan C, gomisin G, schisandrin A, gomisin N, schisandrin B and schisandrin C, in EES were obviously higher than those in WES, whereas others were basically equal in both groups. However, schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N were figured out not only in the brain samples of EES but in WES, and there was no significant difference between their contents (Fig. 6), which might be caused by the selectivity of BBB and they might be the direct acting components for CNS disease of *S. chinensis*. Bioinformatics was adopted following to predict the pharmacodynamics of these 5 lignans.

3.2.3. Targets prediction of contents in brain by bioinformatic analysis

The prognostic gene targets of these constituents were analyzed by Swiss Target Prediction (<http://www.swisstargetprediction.ch/>). Furthermore, pathway enrichment analysis was proceeded on Cytoscape using Cluego. The target pathway network of the 5 potential active chemical compositions was shown as Fig. 7.

The main functional groups *in vivo* modulated by the 5 lignans, schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N, were shown as Fig. 8. The predicted pathways were mainly concentrated on dopaminergic synapse, serotonergic

synapse, cAMP signaling pathway and sphingolipid signaling pathway, which were largely involved in neurogenic diseases plus the inflammation, and they all have relevant to the etiology of depression. This result showed their potential antidepressant effects and indicated that they were the strongest candidates for the effective constituents of *S. chinensis*.

4. Discussion

4.1. Comparing the anti-depressive effects of EES and WES

In this study, we compared the antidepressant effects of WES and EES first. The results of behavioral tests and the level of CORT were indicated the success of CUMS model and the similar activity of anti-depression of EES/WES preliminarily. Previous researches have been demonstrated that neurotransmitters and inflammation were all involved in the etiology of depression^{16–18}. Therefore, we detected neurotransmitters 5-HT, DA, NE and cytokines IL-1 β , Cox-2, TPK, TNF- α in brain to further compare the pharmacodynamics of these two groups at the molecular level. It was proved that EES showed a little bit stronger effect with no significant difference ($P > 0.05$) on depression than WES in reversing the levels of analyzed endogenous substances. Thereof, it was speculated that resemblance effective material basic might be responsible for the similar effects of EES and WES. For exploring the active substances of *S. chinensis*, we following proceeded comparative studies on the chemical ingredients *in vitro/in vivo* of EES and WES.

4.2. Chemical profiles of EES and WES *in vitro/in vivo*

It has been figured out 45 constituents in EES, whereas 36 of them were identified both in EES and WES. The 9 components peculiar to EES covered 6 triterpenes and 3 lignans with low-polarity and

Table 2 The components in WES and EES *in vitro/in vivo*.

Peak No.	Identification	Error Δ (ppm)	Retention time (min)	[M+H] ⁺ / [M+Na] ⁺ (m/z)	Fragment ions (m/z)
1	Citric/isocitric acid	2.2	2.02	193.0278 [M+H] ⁺	216.1779, 148.1526, 129.0188, 111.0081, 87.0087
2	1,5-Dimethyl citrate	0.7	5.05	221.0583 [M+H] ⁺	185.0400, 157.0493, 129.0185, 111.0082, 87.0088
3	Schindilactone D	2	15.05	561.2341 [M+H] ⁺	501.2136, 423.1792, 401.1073, 320.6077, 242.2852
4	Schisanwilsonin B	0.0	15.06	501.2046 [M+H] ⁺	483.1986, 455.2089, 437.1956, 365.1784, 317.1752
5	PRXFPPAVWQDTHT- UHFFFAOYSA-N	0.7	16.82	419.1992 [M+H] ⁺	426.1799, 401.1980, 395.1494, 369.1716, 337.1453
6	Schisanwilsonin D ^a	0.6	18.39	503.2203 [M+H] ⁺	525.2078, 425.1567, 354.1457, 339.1205, 266.0913
7	Schisandrol A ^b	0.4	24.49	433.2148 [M+H] ⁺	400.1885, 384.1930, 346.1413, 331.1184, 300.0996
8	Schisantherin E	0.8	27.35	539.2203 [M+H] ⁺	561.2099, 439.1719, 417.1922, 357.1347, 342.1487
9	Gomisin D ^a	0.9	27.79	531.2152 [M+H] ⁺	485.2177, 467.2040, 401.1594, 326.1139, 353.1371
10	Rubrisandrin A	1.5	29.14	389.1886 [M+H] ⁺	389.1977, 357.1684, 325.1451, 288.0977, 227.0713
11	Gomisin J ^b	1.5	29.44	389.1886 [M+H] ⁺	357.1708, 326.1457, 319.1162, 287.0908, 255.0646
12	Gomisin O ^a	0.8	30.02	417.1835 [M+H] ⁺	399.1813, 369.1630, 337.1419, 316.0960, 277.1228
13	Epigomisin O ^a	0.8	30.33	417.1835 [M+H] ⁺	399.1813, 369.1630, 337.1419, 316.0960, 277.1228
14	Wilsonilignan C ^a	0.8	31.12	417.1835 [M+H] ⁺	399.1813, 368.1681, 355.1648, 314.1040, 299.0895
15	Schisphenlignan A	1.7	31.59	523.189 [M+H] ⁺	423.1425, 383.1530, 352.0834, 341.1024, 326.1151
16	Schisandrol B ^a	0.8	31.90	417.1835 [M+H] ⁺	399.1804, 369.1700, 330.1100, 229.0921, 263.1079
17	Angeloygomisin H/Tigloylgomisin H	1.0	40.69	501.2412 [M+H] ⁺	483.2374, 451.2159, 401.1963, 370.1779, 337.1443
18	Benzoylgomisin H	-0.4	43.94	523.2254 [M+H] ⁺	545.2141, 515.1654, 503.1874, 455.1392, 355.1554
19	Benzoylgomisin Q	-0.6	44.56	553.2359 [M+H] ⁺	453.1896, 387.1882, 356.1620, 343.1177, 301.1071
20	Angeloylgomisin Q/Tigloylgomisin Q	-0.5	46.07	531.2516 [M+H] ⁺	553.2389, 453.1875, 431.2050, 371.1477, 341.1371
21	Gomisin C ^a	0.2	50.60	537.2046 [M+H] ⁺	559.1936, 437.7814, 415.1754, 341.1380, 299.0911
22	Benzoylgomisin P ^a	0.2	50.62	537.2046 [M+H] ⁺	437.1569, 415.1754, 371.1482, 340.1303, 310.1189
23	Schisanhenol	0.0	62.02	403.2042 [M+H] ⁺	371.1845, 340.1667, 301.1063, 271.0948, 270.0885
24	Schisantherin D	-2.9	64.26	521.1733 [M+H] ⁺	421.1237, 399.1439, 355.1172, 325.1084, 295.0084
25	gomisinE	-0.5	69.17	515.2203 [M+H] ⁺	469.2176, 438.1765, 423.1801, 385.1647, 355.1543
26	Schisantherin B	-0.5	69.20	515.2203 [M+H] ⁺	469.2207, 421.1801, 385.1635, 355.1531, 302.0772
27	Gomisin F	-0.5	69.25	515.2203 [M+H] ⁺	469.2176, 438.2017, 385.1638, 355.1537, 315.0859
28	Schisantherin C	-0.5	70.17	515.2203 [M+H] ⁺	469.2222, 438.1801, 385.1635, 355.1531, 316.0940
29	Gomisin G ^a	0.0	71.18	537.2046 [M+H] ⁺	491.2034, 475.2089, 407.1452, 316.0940, 302.0772
30	Schisandrin A ^b	-0.9	77.81	417.2199 [M+H] ⁺	417.2251, 402.2034, 386.2088, 316.1291, 301.1059
31	Gomisin N ^b	-0.5	80.27	401.1886 [M+H] ⁺	386.1718, 370.1772, 300.4007, 285.0755, 242.0929
32	Schisandrin B ^b	-0.4	80.36	401.1886 [M+H] ⁺	386.1718, 370.1772, 300.4007, 285.0755, 242.0929
33	BenzoylisogomisinO	-0.9	84.84	521.2097 [M+H] ⁺	543.1984, 421.1605, 399.1779, 369.1692, 315.0846
34	Schisandrin C ^a	-0.1	85.09	385.1573 [M+H] ⁺	385.1636, 355.1532, 338.1132, 315.0857, 299.0876
35	Schisanlactone E	-0.5	85.60	469.3242 [M+H] ⁺	451.3176, 433.3098, 423.3237, 337.2525, 311.2370
36	Mexicanolide	-0.8	86.61	469.2148 [M+H] ⁺	491.2019, 476.0341, 435.3217, 387.1814, 339.1210
37	Lancifodilactone C	0.9	14.64	545.2386 [M+H] ⁺	527.2292, 485.2170, 467.2073, 439.2131, 403.1909
38	Schisanlactone B	1.5	15.52	501.2119 [M+H] ⁺	487.2031, 425.1936, 345.1683, 379.1521, 288.0826
39	Schisanwilsonin A	2.8	16.94	523.1914 [M+Na] ⁺	469.1657, 425.1433, 353.0876, 337.1054, 311.0901
40	Schindilactone A	0.7	20.30	543.2152 [M+H] ⁺	563.2048, 503.1967, 463.1752, 437.2334, 407.1871
41	Gomisin S	2.9	30.27	419.2077 [M+H] ⁺	402.2005, 369.1720, 337.1448, 323.1274, 301.1042
42	Pregomisin	-0.3	38.36	413.1934 [M+Na] ⁺	391.1702, 359.1829, 351.1555, 327.1581, 299.1555
43	Kadsuphilactone B	0.7	51.14	505.2924 [M+H] ⁺	483.3090, 464.2474, 438.6657, 425.1936, 345.1663
44	Propindilactone G	-0.2	69.49	514.2566 [M+H] ⁺	479.4076, 400.1871, 384.1931, 369.1698, 338.1511
45	Schisanlactone D	-2.5	91.21	453.3325 [M+H] ⁺	435.3240, 313.2222, 270.1898, 245.1885, 111.1018

^aIdentified in plasma samples.^bIdentified in both plasma and brain samples.

low-contents, which might be resulted in their inexistence in WES. Furthermore, as shown in Fig. 5, the results of semi-quantification illuminated that the contents of 33 ingredients (27 lignans and 6 triterpenes) were higher in EES. From the above, not only the compound types but also the contents of most components in EES were more than WES. Considering their similar anti-depressive activity, it was suggested that the highly concentrated ingredients in EES *in vitro* might not be absorbed *in vivo* in quantity and some of them even couldn't reach the nidus on account of the selectivity of diverse biological barriers. The

exploration of chemical profiles of EES and WES *in vivo* was conducted next.

The components in plasma are the potential direct acting ingredients of TCM^{19–21}. In this study, 15 lignans have been identified in the plasma samples of both WES and EES group. Among them, contents of 10 lignans were significantly higher in EES than WES with varied absorptivity independent upon their contents *in vitro*, while others were nearly equal, as shown in Table 3. It was indicated the selectivity of organisms on ingredients of *S. chinensis* and there might be saturation points for

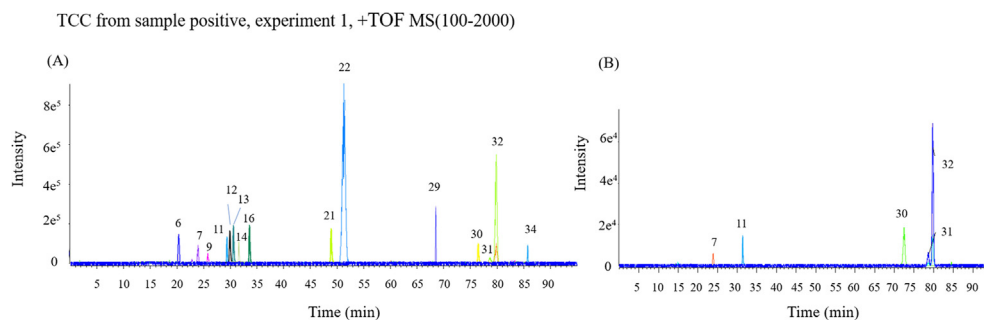


Figure 4 TCC of (A) plasma and (B) brain samples in positive ion mode.

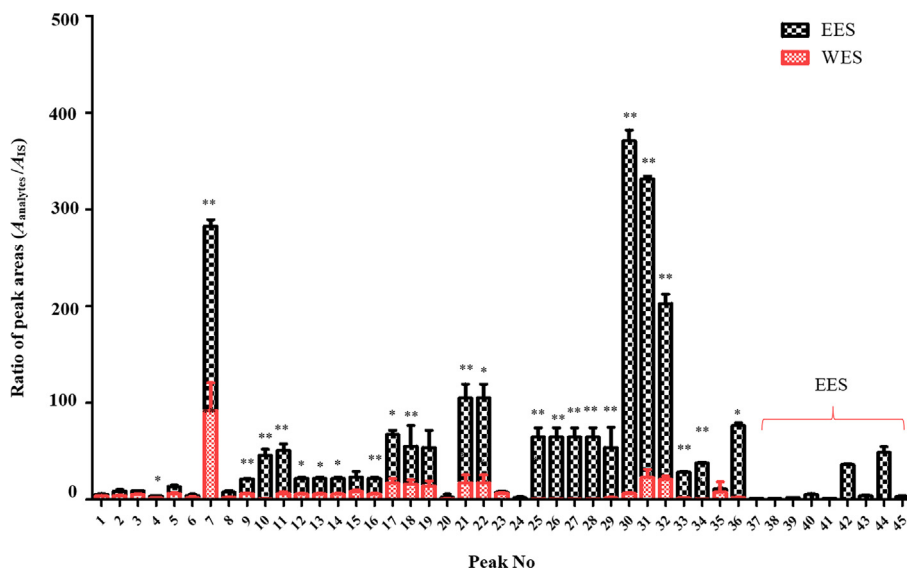


Figure 5 Peak areas of lignans corrected by Bifendate *in vitro*. For statistical significance, * $P < 0.05$, ** $P < 0.01$.

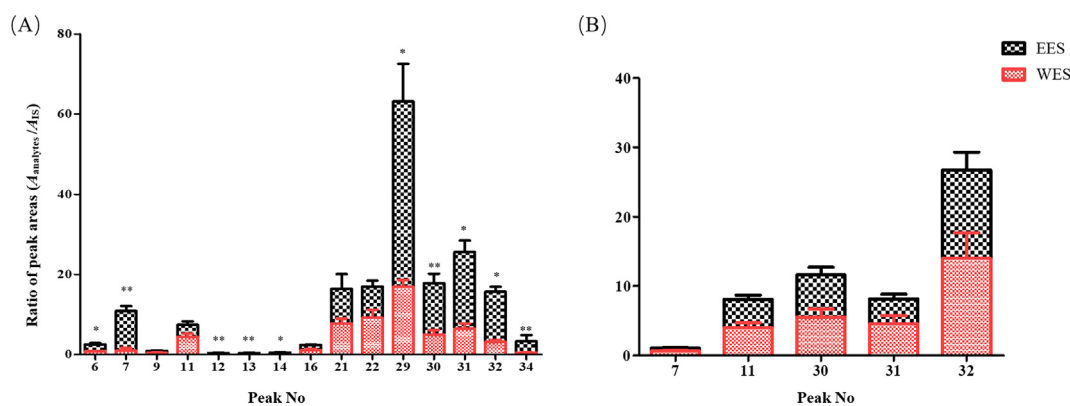


Figure 6 Peak areas of lignans corrected by Bifendate *in vivo*. (A) Contents in plasma samples. (B) Contents in brain samples. For statistical significance, * $P < 0.05$, ** $P < 0.01$.

the absorption of part of components. Besides, the complicated physiological process, including metabolism, tissue distribution and such, for each constituents according to their characteristic physicochemical property based on the theory of TCM serum pharmaco-chemistry might also be an important factor resulted in their diverse absorptivity²². For the stability of dibenzocyclooctene lignans' parent nucleus, the metabolic pathways of the lignans were mainly focused on their side chains, included

hydroxylation, demethylation, phosphorylation, dehydration and even the mutual transformation between prototypes. The proposed metabolic pathways of transform process between schisandrols A/B and schisandrins A/B were shown as the example of lignans metabolism in Supporting Information Fig. S5. However, the specific metabolic mechanism (involved with the metabolic rate, distribution in bodies and so on) of these bioactive components needs further investigation. Although these results might lead to

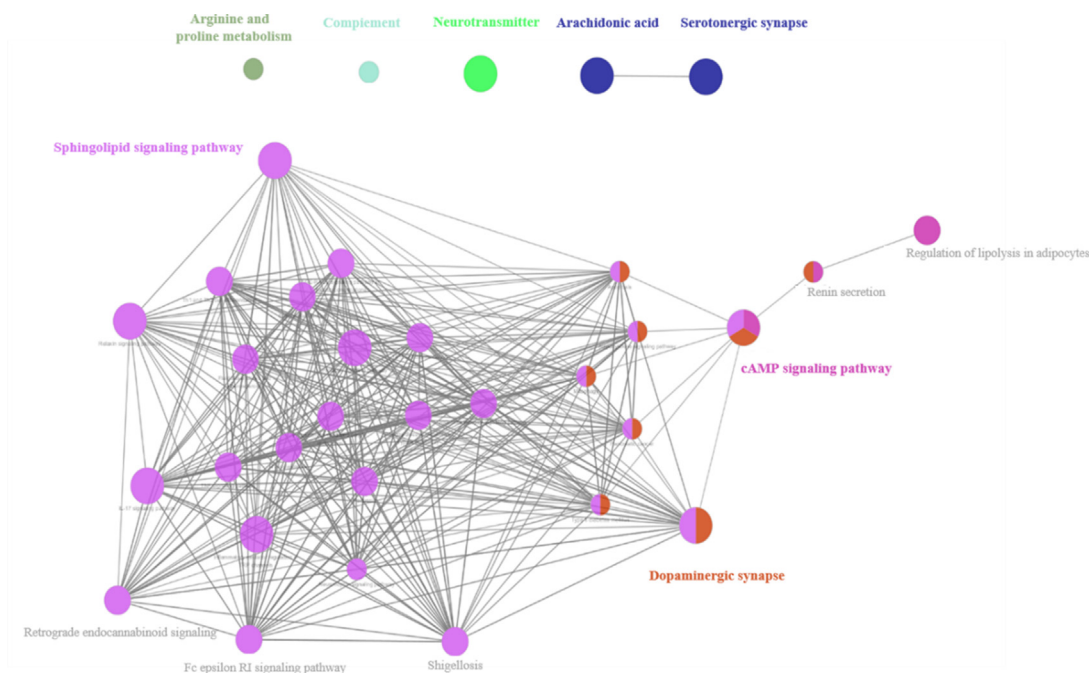


Figure 7 Pathways of all active chemical compositions based on bioinformation analysis.

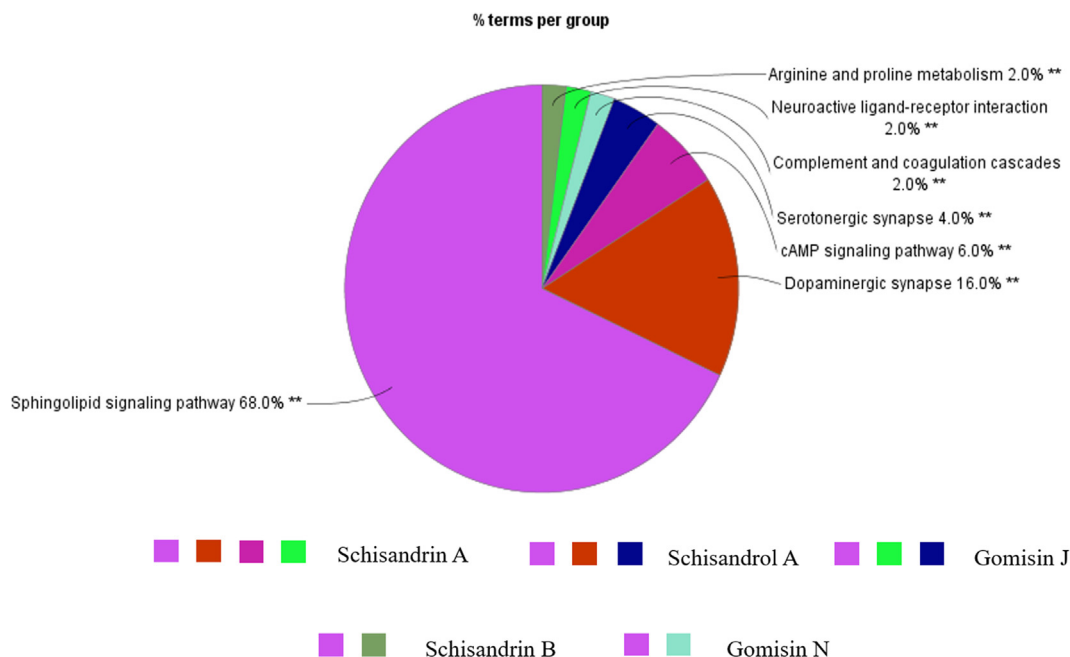


Figure 8 The enrichment degree of target pathways on function groups. Corresponding functional groups related to schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N respected with relevant color, respectively.

preferable anti-depressant effect of EES, they were far fetched explaining the similar efficacy of these two groups. It was worthy to comparatively investigate the constituents, permeated BBB absorbed in the position of depression—brain, of WES and EES since they might contribute to the effects of *S. chinensis* greatly. To avoid the influence of the constituents in numerous blood capillary in the brain tissue, the *in vivo* perfusion method was used to wash away the blood in rats in this study.

BBB, a high selectivity biological barrier structure, was composed of microvascular endothelial cells connected by tight

junctions of the cerebral capillary endothelium and plenty of transporters, prevented substances in circulations from CNS. In present research, only 5 lignans (schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N), infiltrated BBB were figured out in rat brains with almost equivalent contents both in EES and WES, while those were all higher in plasma samples of EES (Table 3). It was demonstrated that the species absorbed in brain were definite and even their absorptive amount were leveled severally by BBB. Moreover, it was also indicated the limitation of BBB on medicines mainly according to their characteristic instead

Table 3 The ratio of $A_{\text{analytes}}/A_{\text{IS}}$ of EES and WES *in vitro/in vivo*.

Lignan	<i>In vitro</i> ($A_{\text{analytes}}/A_{\text{IS}}$)		Ratio (E/W)	Plasma ($A_{\text{analytes}}/A_{\text{IS}}$)		Ratio (E/W)	Brain ($A_{\text{analytes}}/A_{\text{IS}}$)		Ratio (E/W)
	WES	EES		WES	EES		WES	EES	
6	1.84±0.58	2.19±0.27	1.19	0.84±0.01	1.76±0.54	2.08*	—	—	—
7	92.07±18.61	190.60±6.71	2.07**	1.02±0.38	9.88±2.13	9.67**	0.64±0.30	0.41±0.36	0.64
9	6.13±0.59	15.32±0.37	2.50**	0.41±0.35	0.54±0.20	1.32	—	—	—
11	6.17±0.26	44.75±6.62	7.25**	4.42±1.37	3.02±1.54	0.68	3.98±2.30	4.10±1.30	1.03
12	5.79±0.59	16.72±1.68	2.89*	0.02±0.01	0.41±0.12	20.50**	—	—	—
13	5.69±0.67	16.07±1.48	2.83*	0.02±0.01	0.38±0.18	19.06**	—	—	—
14	6.03±0.81	17.17±2.06	2.85*	0.12±0.05	0.52±0.20	4.33*	—	—	—
16	5.53±0.61	17.07±1.62	3.09**	1.13±0.53	1.29±0.28	1.14	—	—	—
21	17.53±7.12	87.62±14.15	5.00**	7.79±2.68	8.62±1.33	1.11	—	—	—
22	20.03±7.48	85.12±11.97	4.25*	9.19±4.56	7.82±3.31	0.85	—	—	—
29	1.71±0.79	52.19±20.88	30.58**	16.92±3.48	46.23±18.76	2.73*	—	—	—
30	6.45±0.75	364.49±10.98	56.52**	5.04±0.84	13.06±2.97	2.60**	5.53±3.74	6.07±3.54	1.10
31	22.73±8.63	308.96±2.78	13.59**	6.47±2.28	19.13±4.94	2.96*	4.54±3.77	3.62±2.11	0.80
32	20.79±3.48	181.85±9.68	8.75**	3.19±1.09	12.47±2.87	3.91**	13.99±5.78	12.74±3.13	0.91
34	0.56±0.12	37.11±0.96	66.22**	0.48±0.23	2.83±2.73	11.63**	—	—	—

For statistical significance, * $P < 0.05$, ** $P < 0.01$. —Not applicable.

of their contents in blood or *in vitro*, which was of great significant for exploring the effective ingredients of neuroprotective TCM. Above all, these 5 lignans might be the chemical material foundation for similar effects of EES and WES. In order to ascertain the active substances of *S. chinensis*, the pharmacodynamics of potential active ingredients need to be explicit in further.

4.3. Ascertaining the active substances of *S. chinensis*

The bioinformatics analysis predicted the anti-depression effects of 5 lignans absorbed in brain on modulating the neurotransmitter and inflammation pathways. Besides, according to previous researches, CNS disease lead to the elevation of mitochondrial membrane potential-9 (MMP-9) and the degradation of tight junction proteins, which were responsible for the BBB destruction. Under the pathological state, antidepressants like fluoxetine and reboxetine could perfusion in the pathological brain to attenuate the damage of BBB²³. Schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N are the liposoluble constituents and their molecular weight are close to fluoxetine, which was supported that their mechanisms of permeating BBB were similar with fluoxetine. In addition, neuroinflammation was caused by impaired BBB through releasing an excess of inflammatory factors^{24–26}. Moreover, cytokines could in turn modulate MMP-9 activation and subsequent BBB disruption²⁷. It has been demonstrated that schisandrol A, schisandrin A, schisandrin B, gomisin J and gomisin N all have anti-inflammatory and neuroprotective effects on LPS-induced inflammation in microglia *in vitro* mainly by inhibiting the expression of nuclear translocation of nuclear factor- κ B (NF- κ B) and diverse inflammatory markers as well as the mitogen-activated protein kinases (MAPK) signaling pathway, and then relieving the destruction of BBB^{28–32}. Furthermore, accompanied by impaired BBB, the redox balance as well as mitochondrial function are disrupted, which is resulted in the inhibition of antioxidant enzymes expression simultaneously^{33,34}. As reported, schisandrin A and schisandrin B manifested anti-oxidative effects on regulating the levels of diverse enzymes^{35,36}. Besides, TLR4, belonging to Toll-like receptors (TLRs), is widely expressed in the central nervous system and plays an important role in inflammatory responses³⁷. After interaction of TLR4 with ligands, the NF- κ B was stimulated

by MyD88-dependent pathways, which also caused damage of BBB³⁸ (Supporting Information Fig. S6). Schisandrin B could downregulate the expression of proteins TLR4 and MyD88, also the levels of NF- κ B based on previous investigations³⁹. Moreover, schisandrol A, schisandrin A, schisandrin B, gomisin J and gomisin N could modulate the levels of neurotransmitters in CNS disorder rats^{38,40}. Given the pharmacodynamic studies as above and the similar antidepressant effects of WES and EES concluded in this study, it was indicated that schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N were the pharmacodynamic substances for CNS diseases of *S. chinensis*.

In conclusion, the study demonstrated that the absorptivity of TCM constituents *in vivo* did not depend on their contents *in vitro* entirely because of the high selectivity of organisms. Furthermore, the biological barriers in bodies could limit not only the species but also the quantity of components absorbed *in vivo*, and the constituents targeted on lesions were crucial for the effects of TCM.

5. Conclusions

The current study has demonstrated that BBB could level the quantity of schisandrol A, gomisin J, schisandrin A, schisandrin B and gomisin N respectively in brain samples in both EES and WES, which might be resulted in their similar antidepressant effects. Based on bioinformatics analysis and lots of previous researches, it has been suggested that the 5 lignans were the strong candidates for active substances of *S. chinensis*. Furthermore, the research proved the selectivity of bodied on TCM ingredients and provided a novel perspective for exploring the material foundation of TCM efficacy by considering the limitation of diverse biological barriers in organisms. It was suggested that the chemical constituents directed into locations of lesion were vital for TCM effects, which was expected to further advance the development of the study on effective substance basis of TCM.

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Author contributions

Yiwen Zhang and Kaishun Bi conceived and designed the experiments; Xinyan Lv, Jiameng Qu, Xin Zhang, Mingyang Zhang, Qian Zhang and Hao Gao performed the experiments; Ran Liu, Huarong Xu, Yiwen Zhang and Qing Li analyzed the data; Yiwen Zhang wrote the paper.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at <https://doi.org/10.1016/j.apsb.2019.10.008>.

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