Development of Simultaneous Analysis Method for Multi-Compounds Content of New Shilajit Using HPLC-UV and the Cognitive Enhancing Effect: Mongolian Shilajit

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Abstract
Shilajit has a longstanding use as an anti-aging and memory enhancing drug. It is known to have excellent anti-bacterial effects and is believed to be effective for cognitive enhancement, but is difficult to standardize because of the lack of quality control standards. This study, for the first time, proposes a quality control standard using a simultaneous analytical method for the drug’s multi-compound content using high-performance liquid chromatography-ultraviolet detection (HPLC-UV) as an aid for the internationalization of Mongolian Shilajit. Phenolic compounds 1-6 were isolated from Mongolian Shilajit extract using bioassay-guided isolation, and the isolated compounds were evaluated for cognitive-related anti-Alzheimer’s disease (AD) activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), and advanced glycation end-product (AGE) formation assays. The isolated compounds showed good effects for each activity. In addition, the isolated compounds were successfully quantified using a validated quantitative HPLC analysis method. As a result, the isolated compounds were suggested as standard marker compounds for Mongolian Shilajit. Also, we proved that the original material of Mongolian Shilajit is a lichen named Xanthoparmelia somloensis (Gyel.) Hale using HPLC-UV, ultra-high-performance liquid chromatography-electrospray ionization/hybrid linear trap-quadruple-orbitrap-high-resolution mass spectrometry (UHPLC-ESI/LTQ-HRMS).

Keywords
Alzheimer’s disease, HPLC-UV, Mongolian Shilajit, phenolics, simultaneous analysis, Xanthoparmelia somloensis (Gyel.) Hale

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Shilajit is a natural multi-complex material. For centuries, it has been obtained as a product mixed with rock minerals through the process of accumulation and decomposition of materials such as plants, fungi, and lichens. Therefore, its components vary depending on various conditions such as the mineral content of the rocks, surrounding plants, humidity, and bacteria. In general, Shilajit contains hippuric acid and benzoic acid. Because the raw material of Shilajit contains lower plants, such as lichen, moss, and liverwort, as well as animal urine and feces, these compounds do not vary.

Shilajit has been reported to have anti-inflammatory, antioxidant, memory enhancement, anti-Alzheimer’s disease (AD), and anti-diabetes activities. Nevertheless, little is known about its constituents and biological activities, which limits its international use. First, quality control standards are difficult because the standard marker compound of Shilajit has not been established. Second, there is a lack of scientific research on Shilajit. Thirdly, the focus is on research of Indian Shilajit. Shilajit is found in mountainous areas, such as the Himalayas, Altai, and the Urals, hence the chemical properties of the drug differ from mountain to mountain. Mongolian Shilajit is known for its anti-bacterial effects that are attributable to its rich benzoic acid content.

Various plants, mosses, lichens, and liverworts are associated with Shilajit. We have collected the lichen, Xanthoparmelia somloensis (Gyel.) Hale.
(Gyel.) Hale around Mongolian Shilajit. Through high-performance liquid chromatography-ultraviolet detection (HPLC-UV) and ultra-high-performance liquid chromatography-electrospray ionization/hybrid linear trap-quadrupole-orbitrap-high-resolution mass spectrometry (UHPLC-ESI/LTQ-HRMS), the relationship between Mongolian Shilajit and *X. somloensis* (Gyel.) Hale was established.

For the first time, this study proposes the standardization of quality of Mongolian Shilajit through isolation of standard marker compounds from Mongolian Shilajit extract, simultaneous analysis through HPLC-UV, validation, cognitive-related anti-Alzheimer’s disease (AD) assay, and identification of origin. The purpose of this study was to establish a quality control standard for Mongolian Shilajit.

**Results and Discussion**

**Identification of Compounds 1-6 Isolated From Mongolian Shilajit**

Compounds 1-6 were identified as syringaldehyde (1), benzoic acid (2), phthalic acid (3), hippuric acid (4), urolithin B (5), and (+)-usnic acid (6) using $^1$H and $^{13}$C-NMR spectroscopy, UHPLC-ESI/LTQ-Orbitrap-HRMS and by comparison with results of previous studies. After identification of compounds 1-6, HPLC-UV analysis was performed to determine the major compounds of Mongolian Shilajit extract (Figure 1). However, compounds 5 and 6 were not detected at any of the UV wavelengths.

**Identifying the Origin of Mongolian Shilajit**

To standardize Mongolian Shilajit, we first identified the substance from which it originated. Mongolian Shilajit extract, *X. somloensis* extract, and standard mixtures were analyzed by HPLC-UV. Peaks of compounds 1, 2, and 4 were identified in both the Mongolian Shilajit extract and *X. somloensis* extract. UHPLC-ESI/LTQ-Orbitrap-HRMS analysis of both extracts showed that compounds 1, 2, and 4 were present in both. As a result, *X. somloensis* was found to be the original material of Mongolian Shilajit.

**DPPH Free Radical-Scavenging Activity of Mongolian Shilajit Extract and Fractions**

The accumulation of active oxygen induces oxidative stress, resulting in cell damage, which could lead to several chronic

![HPLC chromatograms of Mongolian Shilajit extract (1) and standard mixtures (2) (A) compound 4 (hippuric acid); (B) compound 1 (syringaldehyde); (C) compound 3 (phthalic acid); (D) compound 2 (benzoic acid).](image-url)
Compounds scavenging activity, whereas the Mongolian Shilajit extract and therefore, the potential anti-oxidant activity of the test samples was determined through analysis of DPPH activity, which can be used as ROS inhibitors whereas compounds 1 and 6 showed mild activity. These results suggest that these bioactive compounds can be used as ROS inhibitors whereas compounds 1 and 4 showed only mild activity (Table 1).

### Inhibitory Activity of the Extract, Fractions, and Compounds 1-6 of Mongolian Shilajit on AChE, BChE, and BACE1 Activity and AGE Formation

AD has several causes, including excessive degradation of the neurotransmitters ACh and BCh, resulting in decreased levels as a result of increased levels of the enzymes AChE and BChE, which ultimately lead to the development of neurodegenerative diseases, including AD, whereas the anti-oxidant defense systems of the body protect against these chronic diseases. Therefore, the potential anti-oxidant activity of the test samples was determined through analysis of DPPH activity, which is widely used to assay free radical-scavenging activity. The extract, fractions, and compounds 1-6 of Mongolian Shilajit were tested for DPPH free radical-scavenging activity; the results are summarized in Table 1. The FSC50 values of the positive control in inhibiting AChE, BChE, and BACE1 activity and AGE formation were similar to previously reported values. The Shilajit extract and n-BuOH fraction inhibited AChE activity (IC50 422.83 ± 6.11 and 240.28 ± 8.58 µg/mL, respectively), whereas the n-BuOH fraction and extract showed greater inhibition of BChE activity (IC50 55.85 ± 1.01, 59.36 ± 0.76 µg/mL, respectively) than the other fractions. The CH2Cl2 and water fractions exhibited mild activity (IC50 197.52 ± 6.42, 110.44 ± 2.89 µg/mL, respectively). The extract and n-BuOH and water fractions showed greater inhibition of BACE1 activity (IC50 54.83 ± 11.41, 56.64 ± 1.54, and 158.70 ± 14.97 µg/mL, respectively) than the other fractions. Finally, the n-BuOH fraction (IC50 141.77 ± 9.38 µg/mL) most potently inhibited AGE formation, followed by the water and EtOAc fraction (IC50 209.69 ± 10.12 and 214.42 ± 7.10 µg/mL). The n-BuOH fraction showed good inhibitory efficacy against the activity of AChE, BChE, and BACE1 activity and AGE formation.

Compounds 1-6 were tested for AChE, BChE, and BACE1 activity and AGE formation, and the results are summarized in Table 2. Compound 4 showed the greatest inhibitory effects against AChE and BChE activity. Compound 4 has an N-acylglycine unit and N-acylglycine can play a potential role in the nervous system. Compound 5 exhibited good efficacy against BChE and BACE1 and inhibited significant AGE formation, in that order. Compound 5 is a dibenzo-α-pyron. This is known to be able to inhibit β-amyloid aggregation. In addition, compound 1 showed inhibitory effects on BACE1 and inhibited significant AGE formation, whereas compound 3 showed greater inhibitory effects against AGE formation than the other compounds. Compounds 1, 3, and 5 showed better inhibition of AGE formation than the positive control AG hydrochloride. Compound 6 showed greater inhibition of BACE1 activity than the other compounds. Compound 6 is a dibenzofuran. Benzoferan is the basic structure of galantamine, an acetylcholinesterase inhibitor. Recently, benzoferan has been shown to have the ability to suppress BACE1, in addition to acetylcholinesterase inhibition, whereas the effect of compound 2 was mild. These results suggest that these bioactive compounds have the potential as inhibitors for the treatment of AD.

### Validation of HPLC Analysis

Compounds 1-4 are benzenoids, so they are detected at similar wavelengths by HPLC-UV. Compounds 5 and 6 were not detected at all by HPLC-UV. Thus, we have selected compounds 1-4 as the marker compounds of Mongolian Shilajit.
Specificity
Optimal ultraviolet wavelength screening was conducted using HPLC-PDA between 210 and 400 nm, and a wavelength of 232 nm was selected in the PDA spectrum. Using the developed HPLC analytical method, the 4 major compounds of Mongolian Shilajit extract were separated without interference.

Linearity
The linearity of the 4 compounds was measured at five final concentrations within 2.5 to 50 µg/mL. In all calibration curves, the correlation coefficient ($r^2$) of compounds 1-4 was 0.99 or higher. The results are shown in Figure 2.

Limit of Detection (LOD)/Limit of Quantification (LOQ)
The LOD values of compounds 1, 2, 3, and 4 ranged from 0.27 to 0.75 µg/mL, and LOQ values of compounds 1, 2, 3, and 4 ranged from 0.82 to 2.27 µg/mL (Table 3).

Intra—Inter Day Precision & Trueness
The precision and trueness of analysis were evaluated by 4 standard mixture solutions. Intra-day precision measurement was conducted in a day and repeated on 3 days. The intra-day precision of compounds 1, 2, 3, and 4 ranged from 0.81% to 2.09%, and the inter-day precision from 1.01% to 1.99%. The trueness of intra-day ranged from 100.1% to 109.5%, and inter-day trueness from 100.6% to 110.5% (Table 4).

Quantitative HPLC Analysis of Isolated Compounds
The isolated compounds 1-6 showed an effect in each active experiment. However, only compounds 1-4 were detected simultaneously by HPLC-UV. Thus, in this study, we developed a standard extraction method that established quality control standards based on 1-4. The 4 major compounds were extracted using different solvent systems and extraction times. The optimal extraction solvent system for these 4 compounds was 50% methanol, which further showed an optimal extraction time range of 30-120 min. The results showed that an extraction time of 120 minutes was superior to the others (30, 60, and 90 minutes). The contents of the bioactive compounds 1-4 were 180, 10, 10, and 60 µg/g, respectively. The content analysis using HPLC-UV compares to previously reported values. These findings suggest that the developed HPLC analytical method for these 4 compounds was successfully conducted and quality control standards criteria were established for Mongolian Shilajit.

Conclusions
This study conducted standard marker compound isolation from Mongolian Shilajit extract, cognitive-related AD activity assay,
development of a simultaneous analysis method for multi-compounds content using HPLC-UV, and origin identification to establish the standard quality control criteria for Mongolian Shilajit. The Mongolian Shilajit extract and fractions were evaluated for anti-oxidant and anti-AD activity. Among them, the n-BuOH fraction showed the greater anti-oxidant and anti-AD activity. Syringaldehyde, benzoic acid, phthalic acid, hippuric acid, urolithin B, and (+)-usnic acid were identified in the extracts. Hippuric acid (4) showed good inhibition of AChE and BChE activity, whereas syringaldehyde (1) inhibited BACE1 activity and AGE formation. Moreover, syringaldehyde (1), phthalic acid (3), and urolithin B (5) showed significant inhibitory effects on AGE formation. Benzoic acid (2) showed mild inhibitory activity against BACE1, whereas urolithin B (5) and (+)-usnic acid (6) exhibited DPPH free radical-scavenging activity and inhibition of BACE1. The isolated compounds 1-6 showed activity in the various AD tests. This indicated that Mongolian Shilajit extract was effective for the treatment of AD. In addition, we established an HPLC method for the simultaneous analysis of compounds 1-4 as quality control standards of Mongolian Shilajit. These results suggest the isolated compounds as standard marker compounds of Mongolian Shilajit. Furthermore, we have identified through HPLC and UHPLC-ESI/LTQ-Orbitrap-HRMS that X. somboensis is the original material of Mongolian Shilajit. Overall, the results present quality control standards for Mongolian Shilajit.

Materials and Methods

Plant and Raw Materials
X. somboensis and Mongolian Shilajit samples (Figure 3) were both collected from the Khovd aimag - Xoba aimarin,

Table 3. Calibration Curves and Linear Range of Compounds 1-4.

| Compound | Calibration equation\(^a\) | Correlation Coefficient (\(r^2\))\(^b\) | Linear Range (μg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|----------|----------------------------|--------------------------------|----------------------|-------------|-------------|
| 1        | \(y = 5077.3 x + 5071.8\)  | 0.9994                        | 2.5-50               | 0.38        | 1.15        |
| 2        | \(y = 3027.4 x - 1774.9\)  | 0.9997                        | 2.5-50               | 0.75        | 2.27        |
| 3        | \(y = 1839.8 x + 2075.3\)  | 0.9998                        | 2.5-50               | 0.48        | 1.45        |
| 4        | \(y = 1252.9 x + 1145.9\)  | 0.9996                        | 2.5-50               | 0.27        | 0.82        |

\(^a\) \(Y = \text{peak area, } X = \text{concentration of standard (μg/mL)}\).
\(^b\) \(r^2 = \text{correlation coefficient for five final concentrations in the calibration curve}\).
Mongolia in April 2020. They were authenticated by Professor Wan Kyunn Whang, and Professor Enkhjargal Enkhtaivan of the Mongolian Academy of Sciences Institute of General and Experimental Biology in Mongolia.

**Instruments and Reagents**

Ethanol (EtOH), methanol (MeOH), n-hexane, dichloromethane (CH₂Cl₂), ethyl acetate (EtOAc), n-butanol (n-BuOH), and distilled water were used as solvents for extraction, fractionation, and open column chromatography. The open column chromatography was conducted using Sephadex LH-20 (25, 100 µm; Pharmacia, Stockholm, Sweden), MCI CHP 20P gel (Supelco, St. Louis, MO, USA), and octadecylsilane (ODS) gel (400, 500 mesh; Waters, Milford, MA, USA) columns. Dimethyl sulfoxide-d₆ (DMSO-d₆), MeOH-d₄ (CD₃OD), and chloroform-d (CDCl₃; Sigma Aldrich Co., St. Louis, MO, USA) were used as solvents for nuclear magnetic resonance (NMR) imaging. The proton (¹H)- and carbon (¹³C)-NMR spectra were recorded at 600 and 150 MHz, respectively, using a JEOL ECZ600R spectrometer (JEOL, Tokyo, Japan). Chemical shifts are presented as parts per million (ppm) on the δ scale, and coupling constants (J) are shown in Hertz. Electron ionization-mass spectrometry (EI-MS) was performed using UHPLC-ESI/LTQ-HRMS with an Ultimate 3000 rapid separation LC system (Thermo, Darmstadt, Germany). HPLC was conducted using Empower Pro 2.0 software, a Waters 2695 system pump with a Waters 2489 photodiode array detector (Waters, Milford, MA, USA), and a Nouryon Kromasil C₁₈ separation column (4.6 × 250 mm, 5 µm, Bohus, Sweden). HPLC-grade solvents, such as acetonitrile, distilled water, acetic acid, and formic acid were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The DPPH free radical-scavenging activity and anti-AD inhibitory assays were performed using an Infinite® F200 PRO spectrophotometer (Männedorf, Zürich, Switzerland). Other solvents and reagents, including DMSO, L-ascorbic acid, DPPH, sodium phosphate buffer, sodium azide, electric-eel AChE, horse serum BChE, acetylthiocholine iodide, butyrylthiocholine chloride, 5,5-dithiobis

![Mongolian shilajit, X. Somloensis (Gyel.) Hale.](image-url)
[2-nitrobenzoic acid] (DTNB), bovine serum albumin (BSA), glucose, fructose, berberine chloride, and aminoguanidine (AG) hydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BACE1 FRET assay kits (β-Secretase) were purchased from PanVera Co. (Madison, WI, USA).

**Extraction, Fractionation, and Isolation of Mongolian Shilajit**

Dried and powdered Mongolian Shilajit (1.0 kg) was extracted with MeOH (5 L × 3) at 28 °C. The solution was filtered, and the dry extract (340.5 g) was obtained after the solvent was removed in vacuo. This was suspended in water and sequentially partitioned with n-hexane, CH₂Cl₂, EtOAc, and n-BuOH (each, 5 L × 3 times), and the yield of the various solvent fractions was obtained (5.20 g, 12.24 g, 9.76 g and 145.74 g, and that of the water fraction was 142.52 g). Among the fractions, the n-BuOH fraction showed the strongest inhibitory effect in the 4 AD inhibitory assays conducted; therefore, we further analyzed this fraction using open column chromatography. The n-BuOH fraction (145.74 g) was also subjected to MCI gel column chromatography with step gradient elution with 5% to 80% MeOH, and 5 sub-fractions were obtained. Sub-fraction 1 (10.1 g) was separated using ODS column chromatography with 5% MeOH, and sub-fractions 1-1 (3.2 g) to 1-2 (5.1 g) were obtained. Sub-fraction 1-1 was separated using Sephadex LH-20 column chromatography with 20% MeOH to obtain compound 1 (37.2 mg). Recrystallization of sub-fraction 1-2 in 50% EtOH led to the isolation of compound 2 (120.1 mg). Sub-fraction 2-2 (5.2 g) was obtained from sub-fraction 2 (11.7 g) using ODS column chromatography with a 10% MeOH solvent system. Sub-fraction 2-2-1 (3.4 g) was separated from sub-fraction 2-2 using MCI gel column chromatography with 10% MeOH. Sub-fraction 2-2-1 was recrystallized in 10% EtOH to obtain compound 3 (29.1 mg). Sub-fraction 3 (18.4 g) was obtained from sub-fraction 3-1 to 3-4 using MCI gel column chromatography using a 10% MeOH solvent system. Among the fractions, sub-fraction 3-4 (3.6 g) was separated from sub-fraction 3-4-2 (1.7 g) using Sephadex LH-20 with 20% MeOH. Sub-fraction 3-4-2 was subjected to MCI gel column chromatography with 10% MeOH to obtain compound 4. Compound 4 (30.1 mg) was recrystallized after using Sephadex LH-20 from 10% EtOH. Sub-fraction 5 (16.9 g) was separated to obtain sub-fraction 5-2 (7.2 g) using a Sephadex LH-20 column with 5% MeOH, and further separation using Sephadex LH-20 with 5% MeOH yielded sub-fraction 5-2-3 (3.1 g) and 5-2-4 (1.4 g). Sub-fraction 5-2-3 was subjected to Sephadex LH-20 with 10% EtOH to obtain compound 5 (4.6 mg). Sub-fraction 5-2-4 was separated using Sephadex LH-20 with 10% EtOH to yield compound 6 (7.1 mg).

**UHPLC-ESI/LTQ-Orbitrap-HRMS Conditions**

The molecular weights of compounds 1-6 were determined using UHPLC-ESI/LTQ-Orbitrap-HRMS. One mg of isolated compounds 1-6 in 1 ml of MeOH was filtered through a 0.45 µm syringe filter. This was diluted to 0.25 to 5 µg/mL and used for UHPLC-ESI/LTQ-Orbitrap-HRMS analysis for mass determination. A Hypersil GOLD column (C₁₈, 2.1 × 50 mm, 1.9 µm) was used for the analysis. The mobile phase was run on a gradient schedule with solvent A (water, 0.1% formic acid, v/v) and solvent B (acetonitrile, 0.1% formic acid, v/v). Solvent B was increased from 10% to 50% for 18 minutes and then from 50% to 100% for 5 minutes, maintained for 3 minutes, decreased from 100% to 10%, and maintained for 10 minutes. The flow rate of the mobile phase was 0.3 mL/min, the injection volume was 10 µL, the column temperature was maintained at 30 °C, and UV detection was not used. The optimal UHPLC-ESI/LTQ-Orbitrap-HRMS analysis conditions were as follows: heater and capillary temperatures, 300°C and 360 °C, respectively, auxiliary and sheath flow rate, 10 and 45 L/h, respectively; S-lens RF level, 50.0 V; spray capillary voltage, 3.0 kV; full MS AGC target, 3e⁶; and full MS resolution, 35,000.

**HPLC-UV Analysis**

**Sample preparation.** One mg of powdered Mongolian Shilajit and X. somloensis were dissolved in 50% MeOH (1 ml) and filtered through a 0.45 µm syringe filter. For simultaneous content analysis, samples were sonicated with MeOH and EtOH at different concentrations (30%, 50%, 70%, and 100%) and extraction times (30, 60, 90, and 120 minutes). All samples were filtered through a 0.45 µm syringe filter.

**Preparation of standard solution.** One mg of isolated compounds 1-4 was dissolved in 1 ml of MeOH and prepared as a standard solution of 1000 µg/mL. This was diluted to 2.5-750 µg/mL and used for HPLC-UV analysis to establish and validate the analysis conditions.

**Analytical conditions.** For the analysis of compounds 1-4 and other samples, the mobile phases A and B consisted of water containing 1% acetic acid and acetonitrile, respectively, and were run on the following gradient schedule. Mobile phase B was maintained for 20 minutes, increased from 25% to 40% for 6 minutes, and maintained for 9 minutes. The flow rate of the mobile phase was 0.7 mL/min, and the injection volume was 10 µL. The column temperature was maintained at 28 °C, whereas the detector was set at a UV absorbance wavelength of 232 nm.

**Validation.** To verify the reproducibility and trueness of the HPLC-UV method, specificity, linearity, limit of detection (LOD) and limit of quantitation (LOQ), precision, and trueness were evaluated through the recovery.

**Specificity.** Optimal ultraviolet wavelength screening was conducted using HPLC-PDA under UV 210-400 nm.

**Linearity.** The stock standard solution (compounds 1, 2, 3, and 4) was manufactured with 5 levels of concentrations of
standard mixtures (0.25, 1000 µg/mL). The calibration curves of the 4 standards were calculated using linear least-squares regression.

Limit of detection and limit of quantitation (LOD, LOQ). These was determined as the quantifiable concentration of the compounds having a signal-to-noise ratio of ≥3.3 and ≥10, respectively.

Precision and trueness. Standard mixtures (40, 60, and 100 µg/mL) were used for intra and inter day precision analysis. Precision (coefficient of variation [C.V%]) were calculated using the linear range of the standard curves of the 3 different concentrations (40, 60, and 100 µg/mL) within 1 day or 3 consecutive days. Intra-inter day trueness was marked as the observed concentration value relative to the true concentration. The intra-inter day precisions was marked as the relative standard deviation (RSD).

Bioactivity assay. Anti-oxidant activity

DPPH Free Radical-Scavenging Activity

DPPH free radical-scavenging activity was measured using methods described in previous studies, with modifications. All test samples were dissolved in 10% DMSO at 5 different concentrations (10, 500 µg/mL for the extract and fractions and 100-1000 µM for the compounds). The assay mixture consisted of 180 µL 0.1 mM DPPH in EtOH and 20 µL of the test samples. The mixture was shaken for 10 s using a vortex mixer and incubated at 37 °C for 20 minutes. The reaction was performed in a 96-well plate, and the activity was measured using a UV-Visible (VIS) spectrophotometer at 517 nm with L-ascorbic acid as a positive control. The inhibitory activity was calculated using the following formula: (Ac − As/Ac) ×100, where Ac and As represent the change in absorbance of the control and sample, respectively. The half-maximal inhibitory concentration (IC₅₀) values of triplicate measurements of the samples and L-ascorbic acid as a control, were calculated and expressed as the mean ± standard deviation (SD).

Anti-AD Activity

Cholinesterase (ChE) inhibitory activity. The cholinesterase (ChE) inhibitory activity was measured as previously reported, with modifications, using AChE and BChE. All test samples were dissolved in 10% DMSO at 5 different final concentrations (10, 800 µg/mL for the extract and fractions and 10-500 µM for the compounds). The assay mixture consisted of 20 µL of the test samples, 140 µL of 100 mM sodium phosphate buffer at pH 8.0, and 20 µL of 0.36 U/mL of either AChE or BChE. The mixture was incubated in a 96-well plate at room temperature for 15 minutes; then, 10 µL each of 0.5 mM DTNB and either ACh or BCh was added, followed by 15 minutes incubation. Subsequently, the mixture was analyzed at 412 nm using a spectrophotometer. Berberine, a typical ChE inhibitor, was used as a positive control. Inhibitory activity was calculated using the following formula: (Ac − As/Ac) ×100, where Ac and As are the absorbance for the control and sample, respectively, after 15 minutes. The half-maximal inhibitory concentration (IC₅₀) values of triplicate measurements of the samples and berberine, as the control, were calculated and expressed as the mean ± SD.

BACE1 inhibitory activity. BACE1 activity was measured using a kit according to the manufacturer’s recommended protocol. All test samples were dissolved in 50 mM sodium acetate (pH 4.5) at 5 different final concentrations (25, 250 µg/mL for the extract and fractions and 25-250 µM for the compounds). The assay mixture containing 10 µL of the test samples and 10 µL 1.0 U/mL BACE1 substrate (75 µM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate) was shaken for 5 s in a vortex mixer. To start the reaction, 10 µL BACE1 enzyme was added to the assay mixture in a blank 384-well plate, followed by incubation for 60 minutes at room temperature; then 10 µL BACE1 stop solution (50 mM sodium acetate at pH 4.5) was added. The fluorescence was subsequently measured using a spectrofluorometer at excitation and emission wavelengths of 545 and 585 nm, respectively, with quercetin as a positive control. The inhibitory activity was calculated using the following formula: (Ac − As/Ac) ×100, where Ac and As represent the change in fluorescence of the control and sample, respectively, after 60 minutes. The IC₅₀ values of triplicate measurements of the samples and quercetin were calculated as the mean ± SD.

Inhibitory activity against AGE formation. Inhibition of AGE formation was measured as described in previous studies, with modifications. All test samples were dissolved in 10% DMSO to 5 different final concentrations (10, 500 µg/mL for the extract and fractions and 10-100 µM for the compounds). The assay mixture contained the following constituents: BSA (10 mg/mL), substrate as 0.4 M fructose and glucose, and 50 mM phosphate buffer (pH 7.4) with 0.02% sodium azide. The assay mixture was incubated in a black 96-well plate at 60 °C for 48 hours, and the fluorescence was measured using a spectrofluorometer at excitation and emission wavelengths of 350 and 450 nm, respectively. AG hydrochloride was used as a positive control. The inhibitory activity was calculated using the following formula: (Ac − As/Ac) ×100, where Ac and As represent the fluorescence of the control and sample, respectively. The IC₅₀ values of triplicate measurements of the samples and AG hydrochloride were calculated and expressed as mean ± SD.

Statistical Analysis

All assays were performed in triplicate, and the data, which are presented as the mean ± SD, were analyzed using one-way
analysis of variance. Differences in the results were considered statistically significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

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Declaration of Conflicting Interests

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