some limitations to the existing treatment with AChEIs, which are accompanied with varying degrees of toxicity and side effects. Thus, developing an efficient and low-toxicity AChEIs from traditional Chinese medicine has become a main research direction.

Pathogenesis of AD is complex, and current studies are focusing on three aspects which include oxygen-free radicals, inflammatory cytokines, and central neuronal apoptosis.[4,5] Yinhuang, an oral liquid, is a Chinese medicine preparation collected in 2015 from the edition of "Chinese Pharmacopoeia." Yinhuang oral liquid consists of flos lonicerae extract and scutellaria extract. Modern pharmacological studies have shown that organic acids in *Lonicera japonica* Thunb and flavonoids in *Scutellaria baicalensis* Georgi play an important role as antioxidants and anti-inflammatory agents.[6] Moreover, aqueous extract of *S. baicalensis* Georgi could inhibit inflammation by cholinergic anti-inflammatory pathway which promotes the release of Ach.[7-10] N-butanol extract of *L. japonica* Thunb has a significant effect on acetylcholinesterase inhibition and antioxidant activity at 1 mg/mL,[11] suggesting that both *S. baicalensis* Georgi and *L. japonica* Thunb contain AChEIs.

To rapidly screen AChEIs from Yinhuang oral liquid, ultrafiltration–liquid chromatography–electrospray ionization tandem mass spectrometry (UF-LC-ESI-MS/MS) was used. Briefly, small-molecule ligands with potential activity were mixed with acetylcholinesterases receptors to obtain ligand–receptor complexes and small unbounded molecules. By removing small unbound molecules with UF membranes, small-molecule ligands were released from the compounds by organic solvents, and active small molecules were isolated and identified by LC-MS. The whole screening process was carried out in a liquid phase system, which is more suitable for the biological environment of interaction between small molecules of traditional Chinese medicine and enzymes. Furthermore, this technique is sensitive, rapid, and suitable for high-throughput screening which has been widely used in screening target enzyme combined with drug ligand.

Hence, in this study, considering acetylcholinesterase as a drug target, Yinhuang oral liquid was chosen, which is composed of two Chinese herbal medicines (*S. baicalensis* Georgi and *L. japonica* Thunb). These are rich in flavonoids, phenolic acids, and AChEIs which were then screened and measured. For the first time, active compounds were extracted from Yinhuang oral liquid by affinity UF and then were isolated and identified by LC-MS.

MATERIALS AND METHODS

Instruments and reagents

Agilent 1100 series LC-MSD instrument (America Agilent Co.), including the Agilent SL-type multistage ion trap mass spectrometer, a low-pressure quaternary pump, a diode array detector (DAD), an autosampler, a column oven, and ChemStation; a hundred thousandth electronic balance of type BP211D (Beijing Sartorius balance Ltd.); automatic microplate reader (Bio-Rad); XL90 ultracentrifuge (Beckman); and rotary temperature oscillator (SuZhou Pei Ying Experimental Equipment Co., Ltd.) were the instruments used.

Yinhuang oral liquid (batch number: 160301) was provided by JiLin AoDong Medicine Industry Group CO., Ltd; chlorogenic acid, cryptochlorogenic acid, baicalin, wogonoside, baicelein, wogonin, caffeic acid, and huperzine-A were purchased from Institute of Chinese Pharmaceutical and Biological Products, China; acetylcholinesterase (derived from yeast) was purchased from Fluka; YM-10 UF membrane was purchased from Millipore Corporation; 96-well plates (Corning, USA); high-performance liquid chromatography-(HPLC) acetonitrile, methanol, and formic acid (Fisher, USA); water was from Wahaha; and other reagents were of analytical grade.

Sample Preparation

Preprocess of solid-phase extraction for Yinhuang oral liquid

Reverse-phase column C_{18} SPE (laboratory made) was activated by 10 mL ethanol and balanced with 10 mL water. 10 mL Yinhuang oral liquid described above was added into the column, and impurities were eluted with 10 mL water. Then, eluent was collected followed by 10 mL of 50% ethanol elution. Finally, the 1.0 mL ethanol eluent was diluted to 10 mL with 50% ethanol and filtered through 0.22 μm UF and used for analysis with UF-LC-ESI-MS/MS and *in vitro* experiment of enzyme activity.

Standard preparation

Seven reference standards were accurately weighed and dissolved in 50% ethanol. All of them, which were chlorogenic acid (110753–201412), cryptochlorogenic acid (130490–201404), baicalin (110715–201318), wogonoside (112002-20150), baicalein (111595–201306), wogonin (111514–201304), caffeic acid (110885–201402), and huperzine (#SHBC7961V), were then diluted to appropriate concentration ranges for the establishment of calibration curves. All stock and working standard solutions were stored in brown bottles at 4°C until used for analysis and *in vitro* experiment of enzyme activity.

Acetylcholinesterase activity test

The improved Ellman method was used for the test,[12,13] in which 140 μL phosphate-buffered saline (PBS) buffer, 20 μL sample solution, and 15 μL of 0.5 U/mL enzyme were mixed and added into a 96-well plate and placed at 4°C for 20 min. After that, 10 μL 2 mM DTNB and 10 μL 15 mM ATCI were added into the probes. Reaction took place at 37°C for 20 min. Finally, the absorbance at 405 nm was measured. 15 μL enzyme solution was replaced by 15 μL PBS buffer for control measurement, and the 20 μL sample solution was replaced by 20 μL PBS buffer as well.

Inhibitory rate of enzyme = A (control – complete inhibition)–(A sample – A sample control)/A (control – complete inhibition) ×100%.

In the above equation, "A" control was the absorbance value for fully reacted enzyme and substrate without sample; "A sample" was the absorbance value for fully reacted enzyme and substrate with sample; and "A sample control" was the absorbance value for the sample and substrate without enzyme. Three isocratic concentrations were set in the sample group to detect acetylcholinesterase inhibition rate of the sample.

Affinity ultrafiltration screening

According to the methods in previous studies,[14-16] 200 μL sample solution was added into a 24-well plate together with 100 μL acetylcholinesterase enzyme solution with the concentration of 10U/mL. After adding 200 μL PBS buffer, sample was incubated at 37°C for 30 min. The incubated solution was transferred to an UF centrifuge tube (YM-10) for centrifugation at 12,000 r/min for 20 min. 500 μL PBS buffer was added to wash unbound small-molecule compounds by centrifuging at 12,000 r/min for 20 min, three times. Then, 500 μL methanol–water (90:10, V/V) was added to the filter and placed at room temperature for 10 min to release the binding ligand by centrifuging at 12,000 r/min for 15 min, three times. After combining filtrate and drying under reduced pressure, the sample was eluted with 1 mL methanol/water (50:50, v/v) for LC-MS/MS analysis. Control group with inactivated enzyme was processed in the same way.

Chromatography and mass spectrum conditions

Chromatography conditions

HPLC separation was carried out with a Agilent 1100 series system, equipped with a G1315B DAD detector at 320 nm (Agilent Technologies Co. Ltd., USA) and a Agilent Eclipse Plus C_{18} column (250 × 4.6 mm, 5 μm, Agilent

Technologies Co. Ltd.). The column temperature was controlled at 30 °C and the sample volume was 1.0 μL. The flow rate was 1.0 ml/min and the eluting gradient was as follows: methanol (a) and 0.1% of formic acid (b) [Table 1].

Mass spectrum condition

ESI which acquires negative ions; scanning scope: *m/z* 50–1200; target molecule quantitation: 350; dryer temperature: 350°C; dryer flow: 9.0 L/min; nebulizer pressure: 0.24MPa (35.0psi); and capillary voltage: 4kv.

Simultaneous determination of seven AChEIs in Yinhuang Oral Liquid.

Chromatographic conditions

The HPLC conditions of the determination coincide with those used for HPLC analysis in LC-MS.

Liquid chromatography method validation

The HPLC analysis method used in this study has been validated through several tests. Linearity was evaluated by the value of R^2 (correlation coefficient) in the calibration curve of serial concentrations. Sensitivities were determined by the value of limit of detection (LOD) and limit of quantification (LOQ). They were calculated with the corresponding standard solution on the basis of signal-to-noise ratio (S/N) of 3 and 10, respectively. The stability of the analysis method was assessed by the measurements of the intra- and interday variability. To evaluate the accuracy, the recovery test was performed, spiking each standard compound to the sample solution.

RESULTS AND DISCUSSION

Acetylcholinesterase inhibitory activity of Yinhuang oral liquid

Common clinical drug huperzine was used as a positive control drug, and AChE inhibitory activity of Yinhuang oral liquid was measured according to the previous mentioned method. The inhibitory rate was used to evaluate the sample activity. When the concentration of huperzine was 1.0 μg/mL, the inhibitory rate of acetylcholinesterase was up to 49.64%. At the same concentration, inhibitory rate of acetylcholinesterase in Yinhuang oral liquid was 49.14%. Detailed results are shown in Table 2. The results showed that Yinhuang oral liquid had a strong inhibitory effect on acetylcholinesterase, and there was a need to carry out affinity UF experiment to screen more active AChEI.

Analysis of affinity ultrafiltration–liquid chromatography–electrospray ionization tandem mass spectrometry of Yinhuang oral liquid

Experiments of enzyme activity showed that Yinhuang oral liquid had a strong inhibitory activity for AChE. A UF-LC-ESI-MS/MS method was used to screen potential AChEIs. As shown in Figure 1a, after the specific binding to AChE, seven constituents were observed in the LC chromatogram. These compounds specifically bind to AChE. The results proved that they may be the AChEIs. The LC chromatogram of Yinhuang oral liquid sample was displayed in Figure 1b, in which about 20 compounds are in it. However, two-thirds of them did not bind to AChE specifically. The LC chromatogram of control assay is displayed in Figure 1c, which showed that there was no false-positive peak.

To identify the potential AChEIs obtained from Yinhuang oral liquid, seven compounds were analyzed using HPLC-DAD-ESI-MS/MS. In ESI-MS experiment, molecular mass of the components can be obtained. Moreover, in HPLC-DAD experiment, the UV absorption characteristics are obtained. Comparing MS results with the chlorogenic acid, cryptochlorogenic acid, caffeic acid, baicalin, baicalein, wogonoside and wogonin, standard, and those in the literature,[17-20] we have deduced seven structures of potential AChEIs in the HPLC peaks. Since negative ionization ESI mode was used, all of the *m/z* data are $[M-H]^-$. The retention times and mass spectra of products exactly matched with the corresponding standard compounds, which are shown in Table 3 and Figures 2 and 3.

Table 1: List of gradient elution conditions for mobile phase

T	*A*%	*B*%
0	25	75
10	27	73
12	45	55
22	55	45
55	55	45

Table 2: Inhibition activity of Yinhuang oral liquid and acetylcholinesterase inhibitors against acetylcholinesterase

Sample	Concentration (μg/mL)	Inhibition (%)
Chlorogenic acid	25	30.89±6.01
	12.5	26.03±4.74
	6.25	10.37±0.42
Cryptochlorogenic acid	25	16.02±3.03
	12.5	17.35±2.97
	6.25	9.79±1.67
Caffeic acid	25	33.77±4.28
	12.5	20.62±3.45
	6.25	16.87±2.73
Baicalin	25	41.54±7.65
	12.5	39.31±2.29
	6.25	20.66±3.05
Baicalein	25	12.1±1.14
	12.5	9.96±1.03
	6.25	2.48±0.46
Wogonoside	25	26.22±1.24
	12.5	13.25±1.43
	6.25	9.48±1.31
Wogonin	25	22.81±1.27
	12.5	17.58±3.01
	6.25	5.92±0.71
Yinhuang oral liquid	25	49.14±6.21
	12.5	30.29±4.42
	6.25	19.48±1.35
Huperzine	10.0	92.32±4.11
	1.0	49.64±4.54
	0.1	10.41±1.68

Data are represented in mean±SD; *n*=3. SD: Standard deviation

Evaluation of acetylcholinesterase inhibitors inhibition activity of the inhibitors was identified in Yinhuang oral liquid

To validate the screened inhibitors from Yinhuang oral liquid, the AChE inhibitory activities of related compounds were estimated using *in vitro* AChE inhibition assays based on microplate reader. The results suggested that each screened monomer compound had inhibitory activity for AChE. However, they had different degrees of reduction compared with Yinhuang oral liquid; this was due to the fact that inhibitory activity of acetylcholinesterase of Yinhuang oral liquid functioned with many ingredients synergistically. The results of the experiment are shown in Table 2.

Simultaneous determination of seven acetylcholinesterase inhibitors in Yinhuang oral liquid

Liquid chromatography method validation

The calibration curves of seven compounds showed good linearity ($r2 > 0.999$) within the test range. LOD and LOQ under the

Table 3: High-performance liquid chromatographic–diode array detector–electrospray ionization tandem mass spectrometry data of acetylcholinesterase inhibitors of the Yinhuang oral liquid in the negative ion mode

Number	Retention time (min)	UV absorption characteristics λmax (nm)	Mass data (m/z) $[M-H]^-$	MS/MS characteristic ions (m/z)	Compound identified
1	7.9	240,325	353	191	Chlorogenic acid
2	8.7	240,325	353	173,135	Cryptochlorogenic acid
3	11.6	240,325	179	135	Caffeic acid
4	22.0	278,316	445	269,175	Baicalin
5	26.4	274,350	459	283,175	Baicalein
6	33.7	275,325	269	251	Wogonoside
7	41.6	268,320	283	268	Wogonin

UV: Ultraviolet; MS: Mass spectrometry

Table 4: Content of acetylcholinesterase inhibitors in Yinhuang oral liquid

Analyte	Regression equation	Linear range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)	Recovery (%)	RSD (%)		Content (mg/ml)
							Intraday	Interday	
Wogonoside	$Y=4.56\times10^4X-69.14$	1.79–71.6	0.9991	0.179	1.79	105.6	0.8	3.0	6.19±0.19
Caffeic acid	$y=2.289\times10^4X+35.11$	0.56–108	0.9995	0.056	0.56	98.7	1.7	2.5	0.29±0.01
Baicalin	$y=1.613\times10^4X+24.203$	45–480	0.9992	0.23	4.5	108.5	1.2	3.0	23.97±0.72
Chlorogenic acid	$y=3.247\times10^4X+2.212$	25–348	0.9997	0.25	2.5	92.7	2.2	2.8	21.04±0.63
Baicalein	$Y=5.56\times10^4X-95.12$	0.35–105	0.9995	0.035	0.35	96.8	2.4	2.7	0.57±0.02
Wogonin	$Y=7.85\times10^4X-67.9$	0.27–54	0.9996	0.054	0.27	99.5	2.9	2.8	0.61±0.02
Cryptochlorogenic acid	$y=1.735\times10^4X+48.62$	0.67–134	0.9995	0.134	0.67	92.3	2.7	2.6	0.37±0.01

Data are represented in mean±SD; *n*=3. LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; SD: Standard deviation

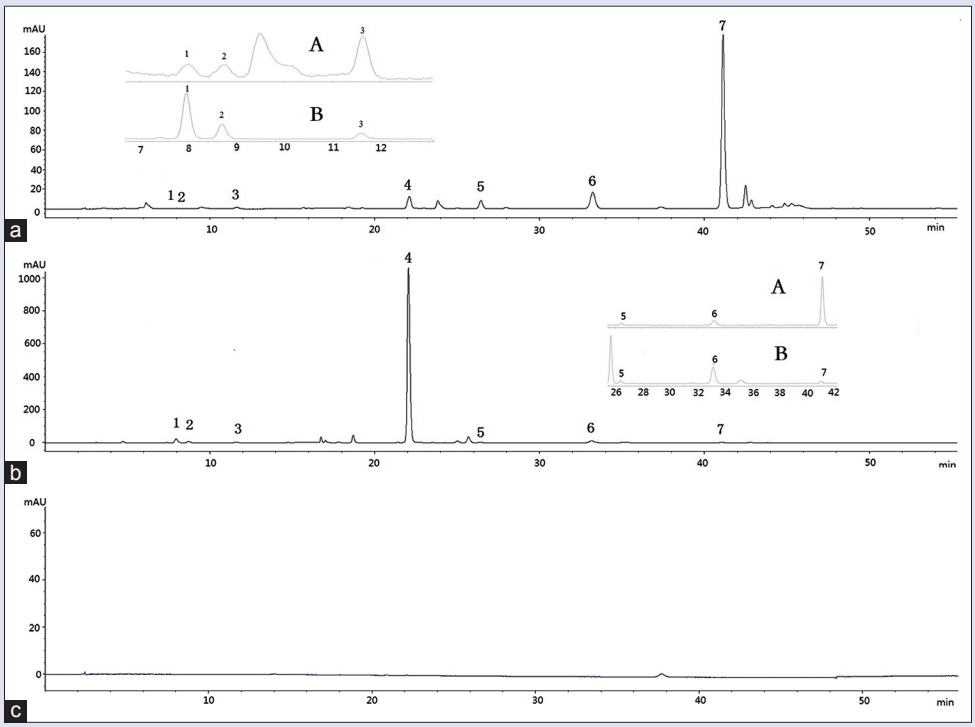

Figure 1: High-performance liquid chromatography-chromatogram of Yinhuang oral liquid being performed ultrafiltration (a), not being performed ultrafiltration(b) and the chromatogram of control assay (c). The two insets enlarged high-performance liquid chromatography chromatogram in two time periods, which are from 7 to 12 min and from 26 to 42 min

chromatographic conditions were calculated, respectively. The regression equation, linear ranges, LOD, LOQ, and content of seven compounds in Yinhuang oral liquid are presented in Table 4.

Repeatability, recovery, and stability

The results of repeatability showed that the intraday and interday RSD values of seven compounds were <3% [Table 4], which indicated that the method had good reproducibility.

The mean recovery was 100% ±5%, and the RSD was <3%[Table 4]. The results of the recovery test demonstrated that the method was accurate.

Stability results showed that all solutions were stable under the room temperature condition within 3 days [R <3%, Table 4].

Quantitative determination of seven acetylcholinesterase inhibitors

To better explain the active components of Yinhuang oral liquid, seven AChEIs, chlorogenic acid, cryptochlorogenic acid, baicalin, wogonoside,

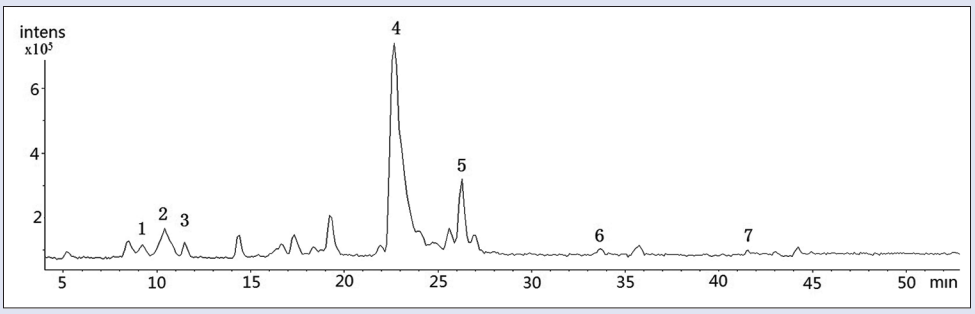

Figure 2: The total ion chromatogram in negative ion mode of Yinhuang oral liquid

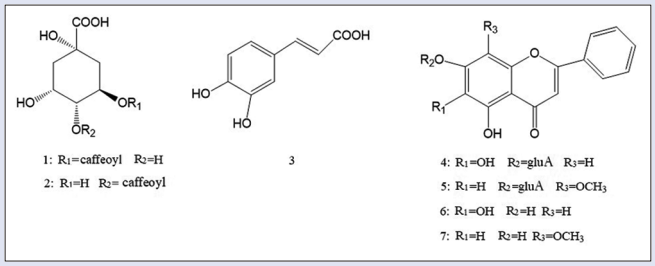

Figure 3: Structures of seven identified acetylcholinesterase inhibitors of Yinhuang oral liquid

baicalein, wogonin, caffeic acid, and huperzine were determined simultaneously. Each sample was determined in triplicate. The HPLC chromatograms of the Yinhuang oral liquid sample are shown in Figure 1a. Peaks in the chromatograms were identified by comparing the retention times and online UV spectra with those of the standards. Table 4 summarizes the content of the seven AChEIs in Yinhuang oral liquid.

CONCLUSION

The *in vitro* experiment proved that Yinhuang oral liquid had AChE inhibitory activity. This study successfully screened seven compounds which had potential inhibition against acetylcholinesterase through UF-LC-ESI-MS/MS technique, but even the strongest activity baicalin had a low inhibitory rate of AChE compared with Yinhuang oral liquid. In conclusion, there are synergistic components in Yinhuang oral liquid, and further studies are required to explore the role and mechanism of active substances that have a synergistic effect in the treatment and prevention of AD.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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