Isolation and identification of highly active anticholinesterase ingredients from fermented soybean products

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ABSTRACT
Inhibitory activities of ethanol extracts from seven fermented soybean products, including douchi, sufu, stinky tofu, to acetylcholinesterase were determined, and crude extract from Yongchuan douchi, which showed the strongest inhibitory effect, was further extracted systematically. Highly active anticholinesterase ingredients were mainly found in petroleum ether extracts; after multistep column chromatography, three compounds were purified finally with high-speed countercurrent chromatography. By their spectral data of MS, 1H-NMR, and 13C-NMR, three compounds were identified as genistein, daidzein, and kaempferol, whose further research could lay foundation for research and development of new drugs and foods against Alzheimer’s disease.

1. Introduction
Alzheimer’s disease (AD), one of the most common types of dementia in the elderly (Adewusi, Moodley, & Steenkamp, 2011), is characterized by amnesia, disorientation, cognitive dysfunction, behavioral disorders, and personality changes (Yoon, Ngo, & Kim, 2009) and has become the third leading cause of death, after cardiovascular disease and cancer, in developed countries (Ma & Gang, 2008). Acetylcholine (ACh), whose decrease in the hippocampus and cortex of the brain is the most prominent biochemical change of AD patients (Orhan et al., 2007), is a neurotransmitter assisting the process of intercellular communication and plays an indispensable role in the excitatory neurotransmission (Aidoo & Ward, 2006; Garhyan, Mahecha-Botero, & Elnashaie, 2006). It is synthesized by choline acetyltransferase (ChAT) and hydrolyzed by acetylcholinesterase (AChE). Low activity of ChAT or high activity of AChE unbalances the cholinergic system and that is predisposition of AD (Garhyan et al., 2006; Rahim et al., 2016). Consequently, inhibition to AChE is regarded as a promising remedy against AD (Lenigk et al., 2000; Orhan & Üstün, 2011).

Currently, AChE inhibitors, which can inhibit AChE from hydrolyzing ACh to augment ACh levels in the brain, are extensively used to alleviate the symptoms of AD patients (Cho, Kim, Ahn, & Je, 2011; Zhang et al., 2007). However, several AChE inhibitors licensed by the US Food and Drug Administration recently, including tacrine, donepezil, rivastigmine, and galanthamine (Ma & Gang, 2008), are reported to have insufficient activity and some side effects, such as hepatotoxicity and gastrointestinal disturbances (Racchi, Mazzucchelli, Porrello, Lanni, & Govoni, 2004; Rahim et al., 2016; Sancheti, Sancheti, Um, & Seo, 2010). Therefore, search for AChE inhibitors from natural products, especially diets, which are more secure, has been a current research priority (Aremu, Amoo, Ndhlala, Finnie, & Staden, 2011; Moyo, Ndhlala, Finnie, & Staden, 2010; Sacan & Yanardag, 2010; Stasiuk, Bartosiewicz, & Kozubek, 2008). Douchi, sufu, and stinky tofu are used traditionally as food condiment and pharmaceutical since ancient times, and today douchi is still added to some traditional Chinese medicines (Chao, Tomii, Watanabe, & Tsai, 2008; Han, Cao, Rombouts, & Nout, 2004; Moy, Lu, & Chou, 2012; Tsai, Kung, Chang, Lee, & Wei, 2007; Wang et al., 2007). So far, there are many published studies on the angiotensin-I-converting enzyme inhibitory activity (Zhang, Tatsumi, Ding, & Li, 2006), anti-α-glucosidase activity (Chen, Cheng, Yamaki, & Li, 2007), antioxidant activity (Wang et al., 2008) of douchi extracts, antioxidative and antihypertensive effects, anti-...
AChE activity of sufu extracts (Chen, Quan, Cheng, Sun, & Li, 2012) but little information about AChE inhibitory activity of douchi extracts.

In this study, inhibitory activities of ethanol extracts from seven fermented soybean products, including douchi, sufu, and stinky tofu, to acetylcholinesterase were investigated, highly active anticholinesterase ingredients were isolated and purified from the sample that showed the strongest inhibitory effect, and identified by their spectral data.

2. Materials and methods

2.1. Chemicals and adsorbents

Magnesium chloride, sodium dihydrogen phosphate, sodium dihydrogen phosphate, n-hexane, methanol, ethanol, petroleum ether, dichloromethane, ethyl acetate, and n-butanol were analytical reagents purchased from Sinopharm Chemical Reagent Co., Ltd. Acetylcholinesterase (AChE), acetylthiocholine iodide, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), galantamine, sodium dodecyl sulfate (SDS), and Sephadex LH-20 were purchased from Sigma-Aldrich, Co. Column chromatography silica gel was purchased from Qingdao Haiyang Chemical Co., Ltd.

2.2. Fermented soybean products and pretreatment

Seven samples, including Yongchuan douchi, Yangjiang douchi, Buerjia red oil sufu, Buerjia white sufu, Wangzhihe red oil sufu, Wangzhihe white sufu, and Wangzhihe stinky tofu, were bought from Trust-Mart supermarket in Shanghai. The oil and pepper on the samples’ surface were removed by petroleum ether. After vacuum freeze-drying, the samples were sieved with 60-mesh sieve and preserved sealed.

2.3. Sample screening

Each dried sample (10 g) was added into conical flask (250 mL) and extracted with 120 mL ethanol (80%, v/v) under reflux at 70°C for 1 h. Extract was vacuum filtered while hot, filtrate was centrifuged at 4000 r/min for 20 min for obtaining supernatant, which was condensed into extractum applying a rotary evaporator. Extractum was dissolved by ethanol (10%, v/v) into solution (1.0 mg/mL), which was prepared for the determination of inhibition ratio to AChE.

2.4. Determination of inhibition ratio to AchE

Inhibition ratio to AChE was determined by slightly modifying the spectrophotometric method of Ellman, Courtney, Jr, and Featherstone (1961). The principle of the method was based on two reactions as illustrated in Figure 1. With the catalysis of AChE, the substrate acetylthiocholine was hydrolyzed into thiocholine, which reacted with 5,5'-dithio-bis-(2-nitrobenzoate) ion to produce the yellow 5-thio-2-nitrobenzoate anion, whose production could be measured at 412 nm by the spectrophotometer.

About 2900 μL of phosphate buffer solution, 20 μL of magnesium chloride (0.75 M), 20 μL of AChE (0.75 U/mL), and 100 μL of extractum solution were mixed and preheated for 10 min at 37°C. With the addition of 50 μL of DTNB (15 mM) and 50 μL of acetylthiocholine iodide (15 mM), the reaction was initiated and maintained at 37°C water bath for 5 h, then 1000 μL of SDS (4%, w/w) was added to terminate the reaction, and absorbance A1 of solution t5 was determined rapidly. About 20 μL of Phosphate buffer solution was substituted for 20 μL of AChE; absorbance A2 of solution was determined after the reaction’s termination. Ethanol (10%, v/v) and phosphate buffer solution were substituted for extractum solution successively, and absorbances A3 and A0 were determined. All the reactions were performed in triplicate. The inhibition rate was calculated by the following formula.

\[ \text{Inhibition ratio} = \frac{\left| A_3 - (A_1 - A_2) \right|}{A_0} \times 100\% \]

Galantamine was used as the positive control.

2.5. High-speed countercurrent chromatography (HSCCC) separation

The solvent system dichloromethane–methanol–water (4:3:2, v/v/v) was thoroughly equilibrated in a separatory funnel, and upper phase (stationary phase) and lower phase (mobile phase) were separated before use.

First, the multilayer coiled column was entirely filled with the stationary phase. The mobile phase was then pumped into the head end of the column at the flow rate of 2.0 mL/min while the apparatus was rotated at 900 r/min. After hydrodynamic equilibrium, which was indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected into the injection valve. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm, and the chromatogram was recorded, and peak fractions were collected manually.

Figure 1. Reactions about determination of inhibition ratio to acetylcholine.

Figura 1. Reacciones de la determinación de la proporción inhibitoria de la acetilcolina.
according to the chromatographic profile and prepared for structure identification.

### 2.6. Chemical structure identification

Chemical structure identification was completed in Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

The structure of compounds separated from sample had been identified by MS, $^1$H-NMR, and $^{13}$C-NMR.

### 3. Results and discussion

#### 3.1. Inhibition ratio of fermented soybean products to AchE

As illustrated in Figure 2, samples were varied in inhibition ratio to AchE, because douchi, sufu, and stinky tofu were naturally fermented soybean products, whose qualities were influenced by technological processes, production seasons, and produce districts. Since ethanol extracts from Yongchuan douchi, whose inhibition ratio reached 52.36%, showed the strongest inhibitory effect, further isolation and purification of acetylcholinesterase inhibitor was focused on ethanol extracts from Yongchuan douchi. Galantamine was used as a positive control (standard drug) in this article (inhibition ratio 96.04%). Galantamine showed a stronger AChE inhibitory activity than fermented soybean products in this work.

#### 3.2. Systematical extraction of Yongchuan douchi crude extract

As shown in Figure 3, extractum of freeze-dried Yongchuan douchi was prepared following the steps in the sample screening. Then extractum was dispersed in a little distilled water and extracted three times with petroleum ether, dichloromethane, ethyl acetate, and $n$-butanol respectively and successively. Extractive solutions were combined and back-extracted with the same volume of water; organic solvents were recycled from organic phase to obtain petroleum ether extracts (80.111 g), dichloromethane extracts...
(40.134 g), ethyl acetate extracts (9.136 g), and n-butanol extracts (17.345 g). After diluted to 1.0 mg/mL by ethanol (80%, v/v), organic solvents extracts were prepared for the determination of inhibition ratio to AChE, respectively.

### 3.3. Organic solvents extracts’ inhibition ratio to AchE

As shown in Table 1, petroleum ether extracts exhibited the highest inhibition to AChE, and dichloromethane extracts’ and ethyl acetate extracts’ inhibitions to AChE were relatively high, while n-butanol extracts’ inhibition was low. According to the theory that substances with similarity in polarity have better solubility, the active ingredients, which were isolated and purified from petroleum ether extracts, dichloromethane extracts, and ethyl acetate extracts by column chromatography, were no polar or weakly polar substances. For with the highest inhibition activity to AChE and the heaviest weight, petroleum ether extracts were selected for further investigation.

| Extracts                | Mass (g) | Inhibition ratio to AChE (%) |
|-------------------------|----------|-----------------------------|
| Petroleum ether extracts| 88.111   | 75.52                       |
| Dichloromethane extracts| 40.134   | 61.10                       |
| Ethyl acetate extracts  | 9.930    | 70.84                       |
| n-butanol extracts      | 17.400   | 26.73                       |

### 3.4. Gradient elution

Petroleum ether extracts (80.111 g) were dissolved with 30% ethanol and then filtrated, and filtrate’s inhibition ratio to AChE was 86.31%. HZ-818 macroporous resin was immersed with the filtrate (50 mg/mL) for 3 h and then filtrated, and the remaining solution’s inhibition ratio to AChE was 24.64%. Saturated HZ-818 macroporous resin was desorbed successively by 60%, 80%, 100% ethanol with the flow rate of 1.0 mL/min, and each 100 mL eluent’s ratio to AChE was measured. As shown in Figure 4, highly active anticholinesterase ingredients were mainly found in the eluent of 60% and 80% ethanol. After rotary evaporation and vacuum freeze-drying, the eluents of 60% and 80% ethanol were dried and yellow powders were obtained as Pr. 1 (14.331 g) and Pr. 2 (38.106 g), respectively.

### 3.5. Multistep column chromatography

Pr. 2, which had more weight and higher anticholinesterase activity, was dissolved with methylene chloride–methanol (30:1, v/v) and then filtrated and separated with silica gel column chromatography. Every 10 min, 100 mL eluent was collected and prepared for the determination of inhibition ratio to AChE; the results are shown in Figure 5.

Eluents that had high anticholinesterase activity were merged and concentrated. After vacuum freeze-drying, Pr. 2.1 (10.120 g) was obtained.

Pr. 2.1 was dissolved with methylene chloride–methanol (60:1, v/v) and then filtrated and separated with silica gel column chromatography. Every 5 min, 10 mL eluent was collected and prepared for the determination of inhibition ratio to AChE, and the result is shown in Figure 6.

Eluents that had highly anticholinesterase activity were merged and concentrated, and Pr. 2.1.1 (3.077 g) was obtained after vacuum freeze-drying.

Pr. 2.1.1 was dissolved with 5 mL methanol and then filtrated and separated with Sephadex LH-20 column chromatography; the elution curve was shown in Figure 7. After reiterative purification and vacuum freeze-drying, Pr. 2.1.1.1 (1.112 g) was obtained and prepared for separation by HSCCC. The HSCCC of Pr. 2.1.1.1 is shown in Figure 8.

### 3.6. Structure identification of Compound 1

Compound 1, whose melting point was 297–298°C, was white acicular crystal. Its spectral data is as follows:

- EI-MS m/z: 271 [M + H]+, 270 [M+]2, 269 [M-H]+, 241 [M-H-CO]1, 213 [M-H-CO-CO]1, 153[A1 + H]+, 152 + , 124 [A1-CO]2, 118 [B1]2.
H-NMR (DMSO-d$_6$) $\delta$: 6.21 (1 H, d, $J = 2.1$ Hz, H-6), 6.37 (1 H, d, $J = 2.1$ Hz, H-8), 6.81 (2 H, dd, $J = 8.7$, 2.1 Hz, H-3ʹ,5ʹ), 7.36 (2 H, dd, $J = 8.7$, 2.1 Hz, H-2ʹ,6ʹ), 8.31 (1 H, s, H-2), 9.158 (1 H, s, OH-4ʹ), 10.87 (1 H, s, OH-7), 12.95 (1 H, s, OH-5).

13C-NMR (DMSO-d$_6$) $\delta$: 94.0 (C-8), 99.3 (C-6), 104.8 (C-10), 115.4 (C-3ʹ, 5ʹ), 121.5 (C-3), 122.6(C-1ʹ), 130.5(C-2ʹ, 6ʹ), 154.2 (C-2), 157.7 (C-9), 157.9 (C-4ʹ), 162.3 (C-5), 164.6 (C-7), 180.5 (C-4).

The spectral data of Compound 1 was consistent with that of genistein reported by Kinjo et al. (1987). And the melting point of mixture of Compound 1 and genistein was still 297–298°C, so Compound 1 was genistein and showed the highest enzyme inhibition activity against AchE with an IC$_{50}$ value of 17.48 µg/mL. (The IC$_{50}$ values were calculated from inhibition curves: inhibition percentage vs. percent of inhibition.)

3.7. Structure identification of Compound 2

Compound 2, whose melting point was 320–321°C, was colorless acicular crystal. Its spectral data is as follows:

EI-MS m/z: 255 [M + H]$^+$, 254 [M]$^+$, 253 [M-H]$^-$, 236 [M-H$_2$O]$^+$, 225 [M-H-CO]$^+$, 197[M-H-CO-CO]$^+$, 137 [A$_1$ + H]$^+$, 118 [B$_3$]$^+$, 108 [A$_1$-CO]$^-$.

H-NMR (DMSO-d$_6$) $\delta$: 6.80 (2 H, dd, $J = 8.7$, 2.1 Hz, H-3ʹ,5ʹ), 6.85 (1 H, d, $J = 2.1$ Hz, H-8), 6.93 (1 H, dd, $J = 8.7$, 2.1 Hz, H-6), 7.38 (2 H, dd, $J = 8.7$, 2.1 Hz, H-2ʹ, 6ʹ), 7.95 (1 H, d, $J = 8.7$ Hz, H-5), 8.28 (1 H, s, H-2), 9.52 (1 H, s, OH-4ʹ), 10.78 (1 H, s, OH-7).

13C-NMR (DMSO-d$_6$) $\delta$: 102.1 (C-8), 114.9 (C-3ʹ, 5ʹ), 115.1 (C-6), 116.6 (C-10), 122.5 (C-3),123.5 (C-1ʹ), 127.3 (C-5), 130.1 (C-2ʹ, 6ʹ), 152.8 (C-2), 157.2 (C-9), 157.4 (C-4ʹ), 162.5 (C-7), 174.7 (C-4).

The spectral data of Compound 2 is consistent with that of daidzein reported by Kinjo et al. (1987). And the melting point of mixture of Compound 2 and daidzein was still 320–321°C, so Compound 2 was daidzein and the inhibition activity against AchE with an IC$_{50}$ value of 17.29 µg/mL.

3.8. Structure identification of Compound 3

Compound 3, whose melting point was 269–270°C, was yellow powder. Its spectral data is as follows:

EI-MS m/z: 287 [M + H]$^+$, 286[M+], 285 [M-H]$^-$, 257 [M-H$_2$O]$^+$, 229 [M-H-CO]$^+$, 197[M-H-CO-CO]$^+$, 137 [A$_1$ + H]$^+$, 118 [B$_3$]$^+$, 108 [A$_1$-CO]$^-$.

H-NMR (DMSO-d$_6$) $\delta$: 6.18 (1 H, d, $J = 2.0$ Hz, H-6), 6.43 (1 H, d, $J = 2.0$ Hz, H-8), 6.81 (2 H, dd, $J = 8.7$, 11.6 Hz, H-3ʹ, 5ʹ), 7.36 (2 H, dd, $J = 8.7$, 2.1 Hz, H-2ʹ, 6ʹ), 8.31 (1 H, s, H-2), 9.158 (1 H, s, OH-4ʹ), 10.87 (1 H, s, OH-7), 12.95 (1 H, s, OH-5).

13C-NMR (DMSO-d$_6$) $\delta$: 94.0 (C-8), 99.3 (C-6), 104.8 (C-10), 115.4 (C-3ʹ, 5ʹ), 121.5 (C-3), 122.6(C-1ʹ), 130.5(C-2ʹ, 6ʹ), 154.2 (C-2), 157.7 (C-9), 157.9 (C-4ʹ), 162.3 (C-5), 164.6 (C-7), 180.5 (C-4).

The spectral data of Compound 3 was consistent with that of kaempferol reported by Markham, Ternai, Stanley, Geiger, and Mabry (1978). And the melting point of mixture of Compound 3 and kaempferol was still 269–321°C, so Compound 3 was kaempferol and the inhibition activity against AchE with an IC$_{50}$ value of 17.29 µg/mL.
4. Conclusion

In conclusion, inhibitory activities of ethanol extracts from seven fermented soybean products, including douchi, sufu, and stinky tofu, to acetylcholinesterase were studied, and crude extract from Yongchuan douchi, which showed the strongest inhibitory effect, was further extracted systematically. And highly active anticholinesterase ingredients were mainly found in petroleum ether extracts; after multistep column chromatography, three compounds were purified finally with HSCCC. By their spectral data of MS, 1H-NMR, and 13C-NMR, three compounds were identified as genistein, daidzein, and kaempferol, which should be recommended for further research on the relationship between their structure and high anticholinesterase activity.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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