

Acetylcholinesterase inhibitory-active components of *Rhodiola rosea* L.

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Abstract

An activity-directed fractionation and purification process was used to identify the acetylcholinesterase inhibitory-active components of *Rhodiola rosea* L. (RR). Dried rhizome of RR was extracted with boiled ethanol. After removal of tannins, the extract was separated into chloroform, ethyl acetate, *n*-butanol and water fractions. Among these, chloroform and *n*-butanol fractions showed stronger activity by bioassay for anti-cholinesterase activity than did ethyl acetate and water fractions. The chloroform fraction was then subjected to separation and purification using silica gel column chromatography and Sephadex LH-20 chromatography. One compound, showing strong anti-cholinesterase activity, was identified by spectral methods (NMR, UV and MS) and by comparison with authentic samples. It proved to be hydroquinone.

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Keywords: *Rhodiola rosea* L.; Acetylcholinesterase; Anti-cholinesterase activity; Hydroquinone

1. Introduction

Acetylcholinesterase (AChE) inhibitors have been widely used in the treatment of Alzheimer's disease (AD), a common neurodegenerative disease that affects the elderly population, the primary symptom is loss of memory. Currently, the only effective treatment for AD has been aimed at the cholinergic system, using anti-cholinesterase compounds.

The rhizome of *Rhodiola rosea* L. (Crassulaceae) (Abbreviated as RR) has been used in the Orient as a tonic and antiaging agent since ancient times. Clinically, RR is used to increase physical endurance and longevity and to treat fatigue, depression, anemia and nervous system disorders. The medicinal effect of RR in the treatment of AD is probably mediated by the acetylcholinesterase inhibitory capability of this plant (Wu, Yao, Gao, & Wang, 2003). However, the specific compounds responsible for the anti-cholinesterase activity of RR remain unknown.

This study was designed to identify the anti-acetylcholinesterase constituents in *Rhodiola rosea* L. to elucidate their chemical structures, and to help our further understanding of the anti-acetylcholinesterase activity of RR.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (ATCI), AChE, from human erythrocytes, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and tacrine were purchased from Sigma. All of the solvents used for the isolation and purification were of analytical grade.

2.2. Plant materials

Plant material was the dried rhizome of *Rhodiola rosea* L., purchased from Nanjing Medicinal Materials Company, Jiangsu Province, China. This medicinal plant was authenticated by Professor Minjian Qin (College of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, PR China).

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2.3. Extraction and isolation procedures

The plant material (200 g) was powdered with a blender and extracted in boiled ethanol (1 l) for 1 h. The extract was filtered through a filter paper and extracted once again under the same conditions with fresh solvent. Filtered extracts were mixed and evaporated to dryness (46.4 g) under reduced pressure by a rotary evaporator. Extract (40 g) was dissolved in 400 ml of deionized water. After centrifugation (3000 rpm, 5 min), the supernatant (360 ml) was precipitated with gelatine (1% water solution, 100 ml) and centrifuged at 3000 rpm for 10 min to remove all the tannins. The supernatant (420 ml) was reprecipitated with ethanol (1680 ml) and centrifuged at 3000 rpm for a further 10 min to remove the remnant gelatine. The supernatant (2081 ml) was evaporated to dryness (7.6 g) under reduced pressure by a rotary evaporator and fractionated by partition between H₂O (100 ml) and chloroform (CHCl₃) (2 × 50 ml), ethyl acetate (EtOAc) (2 × 50 ml) and *n*-butanol (BuOH) (2 × 50 ml) to give 3.0 g, 0.4 g, 1.7 g and 2.1 g of dried masses by rotary evaporation under reduced pressure, respectively. The dry chloroform extract (0.4 g) was then subjected to column chromatography (CC) on silica gel (200 × 25 mm), eluted with a solvent mixture of chloroform/ethyl acetate (1:1, 200 ml), and collected into 100 fractions (A). Fractions A36–A59, showing strong AChE inhibitory effect were combined and subjected to a Sephadex LH-20 column (350 × 10 mm), eluted with methanol/water (1:1, 200 ml), and collected into 100 fractions (B). Fractions B29–B39 were combined and compound 1 (21.4 mg) was obtained. The compound was redissolved in a small volume of ethanol. A 500 µl aliquot of sample was injected into an AKTA™ purifier HPLC system (Amersham Biosciences Co., Sweden). The HPLC analysis was carried out on a reverse phase polystyrene/divinyl column (100 × 6.4 mm). Solvent A (water) and solvent B (acetonitrile) were used as mobile phases. The elution was allowed to run for 4 min with 100% A, and then solvent B increased from 0% to 100%, while solvent A decreased

from 100% to 0% for 60 min, linearly. The compound was detected at 280 nm, and the peak fraction (P1 in Fig. 1) was collected and then freeze-dried, so 19.7 mg of purified compound 1 was obtained.

2.4. Bioassay for anti-cholinesterase activity

Inhibition of AChE was assessed by the colorimetric method of Ellman, Courtney, Andres, and Featherstone (1961). ATCI (0.3 mM) was used as substrate for the assay of AChE. The mixture, including substrate (1 ml), sodium phosphate buffer (0.1 mM, pH 7.4), 1 ml and 0.1 ml of plant extract, was incubated at 37 °C for 5 min, 0.1 ml of enzyme was added and the mixture was incubated at 37 °C for a further 8 min. The reaction was terminated by adding 3% SDS (1 ml), then 0.1 ml of 0.2% DTNB was added to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The colour was measured spectrophotometrically at 440 nm. Tacrine was used as a positive control. False-positive reactions were eliminated by the method of Rhee, van Rijn, and Verpoorte (2003) with a few modifications. The mixture, including substrates (1 ml), sodium phosphate buffer (1 ml) and enzyme (0.1 ml), was incubated at 37 °C for 5 min. Plant extract (0.1 ml) was added and the mixture was incubated at 37 °C for another 8 min. The following process was carried out in accordance with the above described method.

2.5. UV-visible spectrophotometric analysis

Sample (1 mg) was dissolved in 10 ml of ethanol. The sample solution was scanned from 200 to 700 nm, using a Multiskan Spectrum Microplate Spectrophotometer (Thermo, Finland) and the spectra were recorded.

2.6. Molecular weight estimation

A MS system (GCT TOF MS, Micromass, UK) was used to determine the molecular weight. Electron

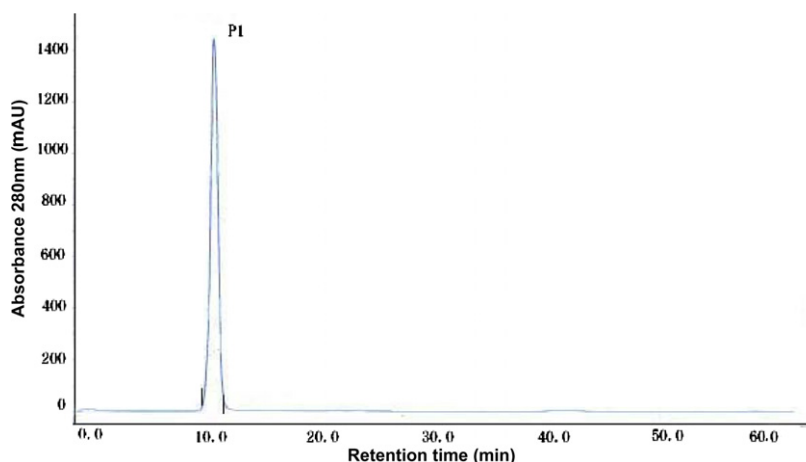


Fig. 1. Elution profiles of the chloroform fraction of RR separated by reverse phase HPLC.

bombardment ionization energy was 70 eV; ion source temperature was 230 °C. The scan range of m/z was 40–800.

2.7. NMR spectroscopy

^{13}C NMR spectra were recorded with a Bruker AV-300 Instrument at 303 K using 5 mm tubes. Sample (10 mg) was dissolved in 0.5 ml of MeOH- d_6 . ^{13}C chemical shifts were expressed in parts per million (ppm) relative to tetramethyl silane (TMS) as an internal standard.

^1H NMR spectra were recorded with a Bruker AV-500 Instrument at 303 K using 5 mm tubes. Sample (10 mg) was dissolved in 0.5 ml of DMSO- D_6 or D_2O . ^1H chemical shifts were expressed in parts per million (ppm) and when the solvent was DMSO- D_6 , tetramethyl silane (TMS) was employed as an internal standard.

3. Results

3.1. AChE inhibitory activity of four different fractions of RR

The dry chloroform, ethyl acetate, *n*-butanol and water fractions were redissolved in deionized water to 10 mg/ml. At this concentration, they showed inhibitory effects with various intensities. The results are shown in Table 1. Among them, the chloroform and *n*-butanol fraction showed stronger inhibitory effects on AChE compared to ethyl acetate and water fraction. Tacrine was used as a standard AChE inhibitor in this study. It showed that the concentration that inhibited 50% of AChE activity (IC_{50}) was 8.63×10^{-5} mg/ml. At the concentration of 10 mg/ml, tacrine had completely inhibited the activity of the enzyme.

3.2. UV–visible spectrophotometric analysis

The UV–visible spectrophotometric analysis showed that compound 1 had maximum absorbance around 285 nm. This result was used to direct the isolation and purification of compound 1. The detectable wavelength of the purification equipment was at 260 nm and 280 nm. 280 nm was utilized, because compound 1 had noticeable absorbance at 280 nm.

Table 1

Comparison of AChE inhibitory activity of chloroform, ethyl acetate, *n*-butanol and water fraction at 10 mg/ml

Fractions	Inhibition (%)
Chloroform fraction	85.8 ± 0.39
Ethyl acetate fraction	38.6 ± 4.20
<i>n</i> -Butanol fraction	83.2 ± 1.43
Water fraction	4.04 ± 1.82

The dry fractions of RR were redissolved in deionized water to 10 mg/ml. AChE inhibitory activities of these were determined according to the method of Ellman et al. (1961). Measurements were carried out in triplicate. Means and standard deviations are indicated.

3.3. Identification of compound 1

Compound 1 was identified as hydroquinone (Fig. 2), by UV, MS and NMR analyses, based on the following characteristics.

Hydroquinone: acicular crystals; UV_{\max} at 285 nm; $[M-1]^-$ peak at m/z 110; ^1H NMR (500 MHz, D_2O) δ 6.78 (2H, s), 4.70 (4H, s); ^1H NMR (500 MHz, DMSO- D_6) δ 8.57 (2H, s), 6.55 (4H, s); ^{13}C NMR (300 MHz, DMSO- D_6) δ 149.9 (s, C-1, 4), 115.8 (s, C-2, 3, 5, 6) [identical with the Nist database and SDDBS database].

3.4. AChE inhibitory activity of HQ

In order to search for ingredients with AChE inhibitory activity, the chloroform fraction of RR revealing the strongest activity, was used for further purification. As shown in Table 2, HQ which was isolated from the chloroform fraction of RR, showed strong inhibitory effect in a dose-dependent manner (with IC_{50} is 9.81×10^{-3} mg/ml). Moreover, as shown in Table 3, the observed inhibition was due to a true enzyme inhibition, not the inhibition of the reaction between thiocholine and DTNB.

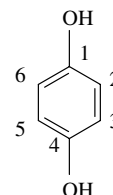


Fig. 2. Structure of hydroquinone.

Table 2

AChE inhibitory activity of HQ

Concentration of HQ (mg/ml)	Inhibition (%)
5×10^{-2}	96.4 ± 0
10^{-2}	32.8 ± 3.97
5×10^{-3}	15.8 ± 8.34
10^{-3}	4.85 ± 2.35

AChE inhibitory activities were determined according to the method of Ellman et al. (1961). Measurements were carried out in triplicate. Means and standard deviations are indicated.

Table 3

False-positive effect of HQ in the colorimetric method of Ellman et al. (1961)

Concentration of HQ (mg/ml)	Inhibition (%)
5×10^{-2}	4.56 ± 0.67
10^{-2}	5.77 ± 6.29
5×10^{-3}	1.61 ± 3.75
10^{-3}	1.02 ± 4.03

Measurements were carried out in triplicate. Means and standard deviations are indicated.

4. Discussion and conclusion

This AChE inhibitory activity-directed fractionation and isolation experiment confirmed the report (Wu et al., 2003) that AChE inhibitory-active components are present in the chloroform fraction of RR.

Alzheimer's disease (AD) is the most common form of dementia among the elderly. In AD patients, there is a decreased level of acetylcholine in the brain areas related to memory and learning (Lahiri, Farlow, Greig, & Sambamurti, 2002). Based on the cholinergic hypothesis that memory impairments in patients suffering from AD result from a defect in the cholinergic system, an important approach to treat this disease is to enhance the acetylcholine level in the brain by inhibition of the enzyme AChE (Shetty & Woodhouse, 1999). AChE inhibitors have been widely used in the treatment of AD. Currently, the only effective treatment for AD has been aimed at the cholinergic system, using anti-cholinesterase compounds.

In this experiment, the AChE inhibitory-active components in the chloroform, ethyl acetate and *n*-butanol fractions were determined. Among them, the chloroform and *n*-butanol fractions showed stronger inhibitory effects on AChE than did the ethyl acetate and water fractions. Interestingly, in our former experiment, the ethyl acetate and *n*-butanol fractions showed higher inhibitory activity than did the chloroform and water fractions. The reason is probably that tannins were not removed before fractionation, which non-specifically precipitated protein, including AChE, and made fractions show higher activity than they really had. Another condition that may lead to this phenomenon is that the sample only inhibits the chemical reaction between thiocholine and DTNB, but not the enzymatic reaction. This is called a false-positive effect. In order to screen for AChE inhibitors from natural products, the colorimetric method of Ellman et al. (1961) has been developed. This method, however, is known to give many of these false-positive effects, but techniques to distinguish these false-positive effects from true enzyme inhibition do not appear to have been described. In the present work, the method of Rhee et al. (2003), with a few modifications, to determine false-positive effects was developed. HQ was tested for possible false-positive effect in the assay system by adding sample, followed by the enzyme (AChE) and the substrate which had been mixed previously to produce thiocholine, the product of the enzyme reaction. Thus it could be established whether the inhibition effect was on the enzymatic reaction or on the chemical reaction between thiocholine and DTNB.

Through further work related to the isolation and purification of the active constituents of the chloroform fraction, a yield of 20 mg of pure compound 1 was obtained and it was determined to be hydroquinone (HQ). Its structure, as shown in Fig. 2, was determined by NMR, MS, UV and confirmed by the literature. This purified compound

demonstrated AChE inhibitory activity with an IC_{50} of 9.81×10^{-3} mg/ml, confirming the result obtained in the initial fractionation process. HQ occurs naturally in plants or plant-derived products (Deisinger, Hill, & English, 1996). Exposure of humans occurs mostly through the diet, as HQ is a natural antioxidant found in many foods. Since safety is the first priority for oral care products, numerous toxicity studies have been conducted (DeCaprio, 1999). These results suggest that low-level exposure to HQ may even be beneficial to health.

In summary, the activity-directed fractionation and identification study has resulted in the identification of one compound from the rhizome of *Rhodiola rosea* L. It showed strong anti-acetylcholinesterase activity. This not only helped our further understanding of the anti-acetylcholinesterase activity of RR, but also had an implication in the food industry for use as a natural AChE inhibitor and supplement against AD. The fact that the *n*-butanol fraction displays AChE inhibitory activity suggests that there may be active components in this fraction. Further work related to the isolation of the active constituents of the *n*-butanol fraction is in progress.

Acknowledgements

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