Acetylcholinesterase Inhibitor From Tabernaemontana pandacaqui Flowers

Anan Athipornchai1,2, Pattapon Ketpoo1, and Rungnapha Saeeng1,2

Abstract
The inhibition of acetylcholinesterase (AChE) is still considered a strategy for the treatment of Alzheimer’s disease. The aim of this study was the search for potential drugs from natural sources which can inhibit AChE. The methanol extract of fresh flowers of Tabernaemontana pandacaqui was partitioned with n-hexane and ethyl acetate. All extracts were evaluated for AChE inhibitory activity. The ethyl acetate fraction, which showed the strongest AChE inhibitory activity, was fractionated using various chromatographic techniques, leading to the isolation of 6 compounds (1-6), which were identified mainly by spectroscopic techniques; this is the first report of these compounds from T. pandacaqui flowers. Astragalin (6) was the major active constituent. The structure-AChE inhibitory activity relationship of 6 and its derivatives was studied. The results suggest that T. pandacaqui flowers and its flavonoid compounds could be potentially used for the treatment of Alzheimer’s disease.

Keywords
Tabernaemontana pandacaqui, acetylcholinesterase inhibitory activity, Alzheimer’s disease, astragalin, flavonoids

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spectroscopic and physical data with the literature values, their structures were identified as 7α-hydroxyindoleninevoacangine (1),11,12 β-sitosterol (2),13,14 p-anisic acid (3),15,16 ursolic acid (4),13,14,17 sitosterol-3-O-β-D-glucopyranoside (5),13,18 and astragalin (6).14,19 The chemical constituents from T. pandacaqui flowers are reported for the first time.

A number of flavonoid constituents have been reported to be AChE inhibitors, such as quercitrin, tiliroside, 3-methoxyquercetin, and quercetin from Agrimonia pilosa,20 and catechin and epigallocatechin gallate from Camellia sinensis.21 Therefore, a major constituent, astragalin (6) and its derivatives, including kaempferol (7), quercetin (8), myricetin (9), and apigenin (10), was also evaluated for their AChE inhibitory activity using the modified Ellman method to study the structure-activity relationship.

A major flavonoid glucoside constituent, kaemferol-3-O-β-D-glucopyranosyl or astragalin (6), isolated from the ethyl acetate fraction, was evaluated for AChE inhibitory activity and shown to have good activity producing 41.3% ± 0.5% inhibition at 100 µg/mL (Table 1). As this active constituent might play an important role in the treatment of Alzheimer’s disease, it was of great interest to study the structure-AChE activity relationship of flavonoid 6 and its derivatives. All flavonoids showed good inhibitory activity on AChE at 100 µg/mL (Table 1).

Based on the structure-activity relationship analysis, it was found that the effect of a free hydroxyl group at position 3 in the flavonoid moiety on AChE was observed. The isolated 3-O-β-D-glucopyranosyl flavonoid 6 was 1.3-fold more active than the aglycone 7 (32.8% ± 0.3%). With the absence of a hydroxyl group at position 3, apigenin (10, 46.5% ± 0.8%) was 1.1- and 1.4-fold more active than compounds 6 and 7, respectively. The results indicated that the absence of a hydroxyl group at position 3 on the 5,7,4′-trihydroxyflavonoids moiety caused an increase in AChE properties. In order to see the influence of the number of hydroxyl groups on ring B of the flavonoid moiety for AChE inhibitory activity, 3′,4′,5′-trihydroxyflavonoid 9 was found to be 1.7- and 2.1-fold more active.

![Figure 1. Anti-acetylcholinesterase activity of methanol extract and other fractions.](image)

Table 1. Acetylcholinesterase Inhibitory Activity of Astragalin and Its Derivatives.

| Conc. (µg/mL) | 6      | 7      | 8      | 9      | 10     | Galanthamineb |
|--------------|--------|--------|--------|--------|--------|---------------|
| 1.56         | 7.4 ± 0.3 | 0.8 ± 0.3 | 1.2 ± 0.1 | 3.2 ± 0.3 | 1.7 ± 0.2 | 44.7 ± 0.3   |
| 3.13         | 11.1 ± 0.3 | 3.5 ± 0.5 | 3.9 ± 0.1 | 6.9 ± 0.4 | 4.4 ± 0.3 | 58.7 ± 0.3   |
| 6.25         | 15.4 ± 0.1 | 6.8 ± 0.4 | 5.4 ± 0.4 | 9.4 ± 0.3 | 7.6 ± 0.2 | 74.6 ± 0.1   |
| 12.50        | 21.0 ± 0.5 | 11.4 ± 0.2 | 8.5 ± 0.2 | 14.3 ± 0.6 | 10.0 ± 0.3 | 84.3 ± 0.4   |
| 25.00        | 25.8 ± 0.4 | 16.8 ± 0.3 | 15.9 ± 0.1 | 26.1 ± 0.1 | 17.7 ± 0.5 | 93.3 ± 0.2   |
| 50.00        | 32.5 ± 0.3 | 25.2 ± 0.4 | 26.6 ± 0.1 | 47.2 ± 0.2 | 31.1 ± 0.1 | 97.7 ± 0.4   |
| 100.00       | 41.3 ± 0.5C | 32.8 ± 0.3D | 41.3 ± 0.4C | 68.6 ± 0.4A | 46.5 ± 0.8B | 99.8 ± 0.3   |

Data followed by different letters indicate statistically significant differences (P < 0.05).

All values are mean ± SD based on 3 replicates.

Standard drug.
than the dihydroxy 8 and monohydroxy 7, respectively. From these results, it was found that the number of hydroxyl groups on ring B of the flavonoid moiety is important for high AChE inhibitory activity. The structure-activity relationship of the isolated flavonoid and its derivatives is shown in Figure 2.

The inhibition of AChE is still considered an important strategy for the treatment of Alzheimer's disease. The aim of this study was to search for new drugs from natural sources. Tabernaemontana pandacaqui flowers were found to contain constituents which have AChE inhibitory activity. In particular, the flavonoid glucoside astragalin (6), which is the major active constituent, showed good inhibitory activity on AChE. Tabernaemontana pandacaqui flowers and its flavonoid compounds should be further explored in the possible treatment of Alzheimer's disease.

**Experimental**

**General Experimental Procedures**

All the organic solvents and chemicals used in this study were of analytical grade. ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer operating at 400 and 100 MHz, respectively. Unless indicated otherwise, column chromatography was carried out using Merck silica gel 60 (finer than 0.063 mm) and Pharmacia Sephadex LH-20. For Thin layer chromatography (TLC), Merck precoated silica gel 60 F254 plates were used. Reversed phase column chromatography was performed on Merck silica gel 60 RP-18. Compounds on TLC were detected under UV light and spraying with anisaldehyde-H₂SO₄ reagent followed by heating.

**Plant Material**

The flowers of *T. pandacaqui* (Apocynaceae) were purchased from a local market in Saensuk district, Chonburi province, Thailand during the month of May 2014. A voucher specimen has been deposited at the Faculty of Science, Burapha University.

**Preparation of the Extract and Fractionation**

The fresh powdered flowers of *T. pandacaqui* (2.0 kg) were immersed in methanol at room temperature and the extracts filtered. The solvent was then removed under reduced pressure at 50°C to give the dry methanol extract (150 g), which was dissolved in methanol-water (4:1) and extracted successively with equal volumes of *n*-hexane and ethyl acetate. Each fraction was then concentrated under reduced pressure at 50°C to yield the hexane, ethyl acetate, and residual fractions. The methanol extract and its fractions were freeze-dried and then refrigerated until further use.

**Isolation of Active Compound From Tabernaemontana pandacaqui Flowers**

The ethyl acetate fraction of TPF(M) (7.02 g) was subjected to silica gel 60 (Merck) QCC using a gradient system of *n*-hexane-ethyl acetate and ethyl acetate-methanol to give 7 fractions (A1-A7). Fraction A2 (309.1 mg) was purified by column chromatography using a gradient system of *n*-hexane-ethyl acetate to give 1 (1.6 mg) and 2 (1.2 mg). Purification of fraction A3 (4.45 g) by repeated column chromatography using a gradient system of *n*-hexane-dichloromethane and recrystallization from methanol yielded 3 (3.4 mg) and 4 (7.8 mg). Methanol was added to fraction A5 (1.25 g) to give insoluble and soluble subfractions (B1 and B2). Subfraction B1 was identified as compound 5 (7.1 mg). Purification of soluble subfraction B2 by Sephadex LH-20 column chromatography using isocratic conditions of methanol gave 8 subfractions (C1-C8). Subfraction C6 was also purified by silica gel 60 column chromatography using isocratic conditions of 1.0% methanol in ethyl acetate to yield 10.6 mg of compound 6. The structures of all isolated compounds were elucidated on the basis of spectroscopic analysis and also by comparison of the ¹H NMR and ¹³C NMR spectral data with those reported in the literature. Compounds 1 to 6 were identified as 7α-hydroxyindole-3-carboxylic acid, 2-α-sitosterol, 3-α-anisic acid, 4-ursolic acid, 5-sitosterol-3-O-β-D-glucopyranoside, and astragalin, respectively.
Acetylcholinesterase Inhibitory Activity

Acetylcholinesterase inhibition was determined spectrophotometrically using acetylthiocholine iodide (ATCI) as substrate, by modifying the method of Ellman. Briefly, in a 96-well plate, 150 µL of 10 mM phosphate buffer (pH 8.0), 20 µL of a solution of AChE (4.0 U/mL in 10 mM phosphate buffer, pH 8.0), and 10 µL of the test compound solution dissolved in dimethyl sulfoxide (DMSO) were mixed and incubated at room temperature for 15 minutes. The reaction was started by adding 20 µL of either a solution of 5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB in 10 mM phosphate buffer (pH 8.0), containing 0.1% bovine serum albumin and 5 mM ATCI in 10 mM phosphate buffer, pH 8.0 (3:1). The hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate 8.0 (3:1). The hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as the product of the reaction with DTNB and thiocholines, catalyzed by enzymes at the wavelength of 405 nm using an EPOCH-2 microplate reader spectrophotometer and the absorbance was measured after 5 minutes of incubation at room temperature. Galanthamine was used as a reference standard. The percentage of inhibition was calculated by comparing the rate of enzymatic hydrolysis of ATCI for the sample to that of the blank (DMSO in buffer), which was calculated by the following equation:

\[
\text{inhibition} \% = \frac{\text{B} - \text{C}}{\text{B}} \times 100
\]

where A is the activity of the enzyme without the inhibitor; B is the control of A without the inhibitor and enzyme; and C and D are the activities of the inhibitors with and without AChE, respectively. All experiments were carried out in triplicate.

Statistical Analysis

All analyses in this study were performed in 3 replicates. Minitab software version 18 was used for statistical analyses. Tukey’s tests were used to determine the variations between the means. Differences at 5% (\(P < 0.05\)) level were considered as significant.

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ORCID ID

Anan Athipornratchi https://orcid.org/0000-0002-3565-0789

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