Research Article

Acetylcholinesterase Inhibitory Activities of Flavonoids from the Leaves of *Ginkgo biloba* against Brown Planthopper

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Ginkgo biloba is a traditional Chinese medicinal plant which has potent insecticidal activity against brown planthopper. The MeOH extract was tested in the acetylcholinesterase (AChE) inhibitory assay with IC_{50} values of 252.1 $\mu\text{g/mL}$. Two ginkgolides and thirteen flavonoids were isolated from the leaves of *Ginkgo biloba*. Their structures were established on the basis of spectroscopic data interpretation. It revealed that the 13 isolated flavonoids were found to inhibit AChE with IC_{50} values ranging from 57.8 to 133.1 $\mu\text{g/mL}$ in the inhibitory assay. AChE was inhibited dose dependently by all tested flavonoids, and compound **6** displayed the highest inhibitory effect against AChE with IC_{50} values of 57.8 $\mu\text{g/mL}$.

1. Introduction

Ginkgo biloba (Ginkgoaceae) is the oldest living tree, with a long history of use in traditional Chinese medicine [1]. The extract of dried green leaves which was extracted with an acetone/water mixture has been standardized to contain 6% terpene trilactones (ginkgolides and bilobalide) and 24% flavonoids [2]. Those flavonoids are almost flavonol-O-glycosides, a combination of aglycones kaempferol, quercetin, and isorhamnetin, with glucose or rhamnose or both linking to different positions of the flavonol moiety [3]. Moreover, brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) is a major rice pest in many countries of Asia. And its infestation is initiated every year by a windborne from the tropics and subtropics. Acetylcholinesterase (AChE) is a substrate-specific enzyme that degrades the neurotransmitter acetylcholine in the nerve synapse [4]. As reported, linarin was isolated from *Buddleja davidii*, with a detection limit of 10 ng in the bioautographic TLC assay, which is at the same level as the known active compound galantamine [5].

In this study, we describe the isolation and structures as well as acetylcholinesterase inhibitory activity of thirteen

flavonoids together with two ginkgolides from the leaves of *Ginkgo biloba* (Figures 1 and 2).

2. Experimental

2.1. Chemicals. Chlorpyrifos was purchased from Sigma Chemicals Co. (St. Louis, Mo, USA). Acetylcholine iodide (ACHI) was purchased from Fluka Chemical Co. (Milwaukee, WI, USA), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was purchased from Biological Engineering Co. (Huzhou, Zhejiang, China). And other reagents used were of the highest available quality and obtained from China National Medicines Co., Ltd. (Beijing, China).

2.2. Plant Material. The plant materials, leaves of *Ginkgo biloba*, used in this study were purchased from Fujian Medicine Co., Ltd, Fujian Province, People's Republic of China, in April 2011, and identified by Professor Ke-Cuo He, College of Plant Protection, Fujian Agriculture and Forestry University. A voucher specimen (no. 110406) was deposited in the College of Plant Protection, Fujian Agriculture and Forestry University.

FIGURE 1: Structure of flavonoids.

2.3. Extraction and Isolation. The air-dried leaves of *Ginkgo biloba* (5.0 kg) were powdered and extracted exhaustively by maceration with MeOH at room temperature. The extract solution was concentrated under diminished pressure to afford a residue, which was partitioned between ethyl acetate (5×1 L) and water (5×1 L) to give 25 g and 34 g of extracts from these two layers, respectively. The ethyl acetate extract was subjected to column chromatography over silica gel and eluted with a gradient CHCl_3 -MeOH (100:1-2:1) to afford eight fractions. Fr. 3 (3.2 g) was submitted to RP-18 column chromatography and eluted with 60–100% MeOH to afford three fractions. Afterwards, SFr. 2 (862 mg) was analyzed by normal phase semipreparative HPLC with CHCl_3 -acetone (10:1) to yield 14 (139.8 mg) and 15 (221.0 mg). Fr. 5 (2.8 g) as mentioned above, was subjected to step-gradient silica gel column chromatography with a solvent consisting of CHCl_3 -MeOH to afford five fractions. And then, SFr. 3 (365 mg) was subjected to column chromatography over RP-18 and eluted with a gradient of 40–90% MeOH. Further, it was purified by RP-18 semi-preparation HPLC and eluted with 85% MeOH to yield 1 (12.7 mg) and 3 (29.6 mg). The water extract was subjected to step-gradient silica gel column chromatography with a solvent consisting of CHCl_3 -MeOH (20:1-1:1) to afford seven fractions. And Fr. 1 (303 mg) was purified over RP-18 with 80% MeOH to yield 2 (22.4 mg) and 6 (24.5 mg). Fr. 3 (303 mg) was purified over RP-18 with 75% MeOH to yield 4 (42.8 mg) and 5 (104.1 mg). Fr. 4 (2.6 g) was subjected

FIGURE 2: Structure of ginkgolides.

to step-gradient silica gel column chromatography with a solvent consisting of CHCl_3 -MeOH to afford 8 (23.1 mg), 11 (18.4 mg), and five fractions. SFr. 2 (356 mg) was purified by RP-18 semi-preparation HPLC and eluted with 85% MeOH to yield 9 (11.9 mg) and 12 (24.5 mg). Dealing with SFr. 4 (216 mg), it yielded 7 (16.9 mg) by RP-18 semi-preparation HPLC. SFr. 5 (216 mg) was purified by Sephadex LH-20 to yield 10 (15.3 mg) and 13 (17.8 mg) with MeOH.

2.4. Insect. The brown planthopper, used in this study was first collected from the experimental field of Fujian Agriculture and Forestry University, Fuzhou, Fujian China. Then, it was reared on rice seedling in laboratory, at $25 \pm 1^\circ\text{C}$, 16 L/8 D.

2.5. Determination of IC_{50} of Test Compounds to AChE. One hundred 3rd instar larvae were homogenized in a glass homogenizer with 2 mL of 0.02 mol/L phosphate buffer (PH 7.0, containing 0.1% Triton X-100) using the method of Park and Choi [6], and the homogenizer was washed with 1 mL of phosphate buffer twice which was adapted for use in a microplate reader. The homogenate was centrifuged at 6000 r/min, 4°C for 30 min. the supernatant was used as the source of AChE. All compounds and chlorpyrifos were dissolved in acetone before diluting into the solution of AChE, and five concentrations of test compounds and chlorpyrifos solution were made by 2-fold dilution using acetone. 5 μL of test solution of each concentration was mixed with 95 μL of the solution of AChE, and the mixture was placed in wells of 96-well microtiter plate (Nunc, 300 μL volume per well) for 1 hour. Then, the 100 μL DTNB (0.3 mmol/L) and 100 μL ATCHI (1.5 mmol/L) were added successively. The residual activity of AChE was measured at 405 nm on the microplate reader [7]. A control reaction was carried out using phosphate buffer instead of test compounds and was considered as 100% activity. Inhibition, in %, was calculated in the following way:

$$I(\%) = 100 - \left(\frac{A_{\text{compound}}}{A_{\text{control}}} \right) \times 100, \quad (1)$$

where A_{compound} is the absorbance of the test compounds containing reaction and A_{control} is the absorbance of the reaction control. IC_{50} was obtained by plotting the inhibition percentage against using concentrations.

3. Results and Discussion

With the aim of researching active compounds by measuring acetylcholinesterase-inhibiting activity from the leaves of *Ginkgo biloba*, the MeOH extract and compounds **1–15** were tested in the acetylcholinesterase inhibitory assay. The MeOH extract proved to be active with $IC_{50} = 252.1 \mu\text{g/mL}$. In order to substantiate the result and find out which compound inhibited the enzyme, the extract was subjected to column chromatography over silica gel, RP-18, Sephadex LH-20, and semipreparation HPLC to yield the active compounds **1–13**.

3.1. Structural Elucidation of Compounds 1–15. Thirteen flavonoids, namely, kaempferol (**1**) [8], quercetin (**2**) [9], ermanin (**3**) [10], quercetin-3-*O*- α -L-rhamnopyranoside (**4**) [11], quercetin-3-*O*- α -D-glucopyranoside (**5**), quercetin-3-*O*- β -D-glucopyranoside (**6**) [12], quercetin-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranoside (**7**) [13], quercetin-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (**8**) [14], acacetin-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (**9**), quercetin-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranoside (**10**) [15], kaempferol-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (**11**) [16], taxifolin (**12**), and genistein (**13**) [17]; two

TABLE 1: AChE inhibitory activity of compounds **1–15**.

Compound	IC_{50} ($\mu\text{g/mL}$) ^a
1	100.5
2	95.8
3	88.5
4	110.9
5	79.4
6	57.8
7	77.5
8	73.1
9	97.8
10	112.6
11	101.7
12	133.1
13	103.8
14	— ^b
15	—
Chlorpyrifos	12.4

^aAll compounds were examined in a set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss. ^bNo inhibitory activity.

ginkgolides, namely, ginkgolide B (**14**) and ginkgolide C (**15**) [18] were obtained from the leaves of *Ginkgo biloba*. The structure determination of compounds **1–15** was established using NMR spectral method, and their spectral data were compared to previous literature values.

3.2. AChE Inhibitory Activity. The AChE inhibitory activity of the evaluated extract of *Ginkgo biloba* at different concentrations (31.25, 62.5, 125, 250, and 500 $\mu\text{g/mL}$) showed that the extract has moderate inhibitory activity with the $IC_{50} = 252.1 \mu\text{g/mL}$ (Table 1). Ginkgolides B and C were found to be inactive in the AChE inhibitory test, while their effect target seemed to be GABA receptor [19].

Flavonoids have previously been reported to be multitargeted agents in combating Alzheimer's disease (AD) by enhancing acetylcholine levels. Among the 13 isolated flavonoids, they were found to inhibit AChE with IC_{50} values ranging from 57.8 to 133.1 $\mu\text{g/mL}$ in the inhibitory assay. AChE was inhibited dose dependently by all tested flavonoids. As shown in Table 1, compound **6** exerted the most promising activity with IC_{50} values of 57.8 $\mu\text{g/mL}$ following by the inhibitory property of compound **8** that exhibited an IC_{50} value of 73.1 $\mu\text{g/mL}$ as compared with a positive control, chlorpyrifos ($IC_{50} = 12.4 \mu\text{g/mL}$). Compounds **4**, **10**, and **12** showed weak inhibitory activity, with IC_{50} values of 110.9, 112.6, and 133.1 $\mu\text{g/mL}$, respectively.

4. Conclusion

In conclusion, we isolated two ginkgolides and thirteen flavonoids from the leaves of *Ginkgo biloba*. The AChE inhibitory activity of test compounds revealed that flavonoids were found to inhibit AChE with IC_{50} values ranging from 57.8 to 133.1 $\mu\text{g/mL}$, and that ginkgolides were inactive. These

inhibitors enhance the signal transmission in nerve synapses by prolonging the effect of acetylcholine, which is harmful to brown planthopper. The isolation and identification of AChE inhibitors of these compounds may be of interest to clarify the physiological role of this enzyme and to provide novel pesticide.

Conflict of Interests

There is no conflict of interests among all authors.

Acknowledgments

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