Acetylcholinesterase Inhibitive Activity-Guided Isolation of Two New Alkaloids from Seeds of *Peganum Nigellastrum* Bunge by an *In Vitro* TLC- Bioautographic Assay

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Acetylcholinesterase inhibitors (AChEIs) currently form the basis of the newest drugs available for the treatment of Alzheimer’s disease. For the aim of screening effective AChEIs, the methanol extracts of the seeds of genus *Peganum* were found to show significant inhibitory activity of acetylcholinesterase enzyme (AChE) using an *in vitro* TLC-bioautographic assay. In further studies to seed of *P. nigellastrum* Bunge, activity-guided fractionation led to the isolation of two new alkaloids nigellastrine I (9) and nigellastrine II (10), and along with eight known alkaloids, vasicinone (1), vasicine (2), harmine (3), deoxyvasicinone (4), deoxyvasicine (5), harmane (6), harmol (7), harman (8), in which harmol and harman were first isolated from species *P. nigellastrum* Bunge. As active constituents, all compounds showed good inhibitory activities against AChE. The results of *in vitro* semi-quality TLC-bioautographic assay showed that harmine, harmaline and harmol displayed a similar AChE inhibitive activities comparing to galanthamine. These results indicated that these alkaloids in *P. nigellastrum* Bunge could be a potent class of AChEIs.

**Key words:** *Peganum nigellastrum* Bunge, Acetylcholinesterase, TLC-bioautographic assay, Alzheimer’s disease, Alkaloid, Nigellastrine I, Nigellastrine II

INTRODUCTION

Alzheimer’s disease (AD) is a progressive, neurodegenerative disease, which primarily affects the elderly population, and is estimated to account for 50-60 percent of dementia cases in those over 65 years of age (Francis et al., 1999). AD is a highly prevalent neurodegenerative disease, characterized initially by selective loss of cholinergic neurons in the basal forebrain (Whitehouse et al., 1982), followed by cognitive and behavioral impairments that progressively disrupt activities of daily living, leading to institutionalization and eventually death (Blennow et al., 2006). Cholinergic neurotransmission dysfunction in the brain contributes to the salient cognitive decline in AD. The loss of cholinergic cells, particularly in the basal forebrain, is accompanied by loss of the neurotransmitter, acetylcholine. Acetylcholinesterase inhibitors (AChEIs) currently form the basis of the newest drugs available for the management of this disease. AChEIs are general chemical classes, such as physostigmine, galanthamine, tacrine and heptylphysostigmine, and have been tested for the symptomatic treatment of AD (Becker et al., 1988; Fulton and Benfield, 1996). However, the non-selectivity of these drugs, and their limited efficacy, poor bioavailability, adverse cholinergic side effects in the periphery, narrow therapeutic ranges and hepatotoxicity are some of the severe limitations to their therapeutic success (Bores et al., 1996; Forette et al., 1999). It is necessary for other studies on the AChEIs
derived from medicinal plants or from designing and development of synthesis.

Genus *Peganum* (*Zygophyllaceae*), including six species and one variety, is perennial plant native of the central Asia region but now grows wild and discontinuously in Africa, the middle east, India, south America, Mexico, and southern USA. It was reported that three species, *P. harmala* Linn, *P. multisectum* (Maxim) Bobr, and *P. nigellastrum* Bge, grow wild in northwest China (Ma and Wang, 1998; Ma and Li, 1996; Wang et al., 2002). In China, the entire plant and seeds of *P. harmala* Linn, listed in the UyguR Drug Standard of Ministry of Public Health (Pharmacopoeia Commission, 1998), have been used as traditional folk medicinal substances in the Xinjiang Uygur autonomous region and in the Mongolian autonomous region. It has been used to treat diseases such as cough, asthma, rheumatoid arthritis and swelling pain, etc. Previous studies on the plants from the genus *Peganum* have reported the isolation of various alkaloids and its biological properties such as psychopharmacological and behavioral effects in the brain (Airaksinen and Kari, 1981; Pfau and Skog, 2004), cytotoxicity and anti-tumour activities (Lutes et al., 2001), tremoregenesis (Lutes et al., 1988), cardiovascular actions (Aarons et al., 1992), antimicrobial activity (Ahmad et al., 1992), anti-parasitic activity (Di Giorgio et al., 2004), and strong reversible inhibition of monoamine oxidase (MAO) (Kim et al., 1997; Schwarz et al., 2003).

Despite a number of studies on genus *Peganum*, few have regarded the active principals and AChE inhibitory activity. In continuation of our on-going study to search the bioactive constituents of seeds of genus *Peganum* by a rapid bioautographic assay on TLC plates developed for the screening of AChE inhibition by plant extracts (Marston et al., 2002), it was found that the alkaloids fraction showed potential inhibitory effects on the AChE activity. Herein, the isolation and structure elucidation of two new alkaloids (9, 10) and 8 known alkaloids vasicinone (1), vasicine (2), harmine (3), deoxyvasicinone (4), deoxyvasicine (5), harmaline (6), harmol (7), harman (8) from seeds of *P. nigellastrum* Bunge, as well as the AChE inhibitory of the isolated compounds are reported.

**MATERIALS AND METHODS**

**Materials**

The seeds of genus *Peganum, P. harmala* Linn, *P. multisectum* (Maxim) Bobr, and *P. nigellastrum* Bunge, and a probable *P. variety* were collected in wild in Xinjiang, Ningxi, Gansu, inner Mongolia and Shaanxi provinces, China, in september 2006. All of these plant materials were authenticated by professor Chang-hong Wang and the voucher specimens were deposited at the herbarium of the Shanghai R&D center for standardization of traditional chinese medicine, Shanghai, China.

Acetylcholinesterase from electric eel (EC3.1.1.7) was purchased from Sigma (St. Louis, MO; product no. C3389). Bovine serum albumin, fast blue B salt, Tris, and 1-naphthyl acetate were obtained from Sigma. Galanthamine was obtained from Shanghai R&D centre for standardization of Chinese medicines. All other chemical solvents used for isolation were of analytical grade. Column chromatography (CC) and preparative-TLC were carried out using precoated silica gel 60 F 254 (0.5 mm, obtained from Qingdao Ocean Chemical Co.). C 18-ODS (Merck) and Sephadex LH-20 (Amersham Biosciences, GE Health Care) were used. NMR Spectra: at 500 MHz for 1H and at 125 MHz for 13C on a Bruker AV-500 spectrometer. ESI-MS and HR-ESI-MS: LCQ Deca XP plus (Thermo Finnigan) and Finigan MAT95 spectrometers, respectively.

**Samples test and fraction screening on AChE inhibitory activity**

**Samples preparation and TLC analysis:** In each case, 0.5 g seeds of *P. harmala* Linn, *P. multisectum* (Maxim) Bobr, and *P. nigellastrum* Bunge, and a probable *P. variety* were extracted overnight with 90% methanol (15 mL) before ultrasound extraction 20 min, and filtration, respectively.

In order to establish active fraction by the TLC bioautographic assay, 10 and 1 µL of the four stock solutions in methanol were applied onto the TLC plate A and plate B respectively, and migrated with ethyl acetate-methanol-ammonia (10:1.5:0.5) in duplicates. The developed plate A was firstly inspected under ultraviolet light (366 nm) and then was colored by spraying Dragendorff’s reagent (potassium heptaiodobismuthate solution) and plate B was introduced a bioautographic assay (Marston et al., 2002), respectively.

**TLC bioautographic assay:** The TLC bioautographic assay was carried out as described previously (Marston et al., 2002) by some modification. AChE (1000 U) was dissolved in 150 mL of 0.05 M Tris-hydrochloric acid buffer at pH 7.8; bovine serum albumin (150 mg) was added to the solution in order to stabilize the enzyme during the bioassay. The stock solution was kept at 4°C. TLC plates were eluted with methanol in order to wash them, and were thoroughly
dried just before use. After applying samples solution onto plate and migration of the TLC plate with ethyl acetate-methanol-ammonia (10:1:5:0.5) (or direct deposition of samples), the TLC plate was dried with a hair dryer for complete removal of solvent. The plate was then drenched with enzyme stock solution and thoroughly dried again. For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing a little water; by this means, water did not come directly into contact with the plate but the atmosphere was kept humid. The cover was placed on the tank and incubation was performed at 37°C for 20 min. The enzyme had satisfactory stability under these conditions. For detection of the enzyme, solutions of 1-naphthyl acetate (250 mg) in ethanol (100 mL) and of Fast Blue B salt (400 mg) in distilled water (160 mL) were prepared immediately before use (in order to prevent decomposition). After incubation of the TLC plate, 10 mL of the naphthyl acetate solution and 40 mL of the Fast Blue B salt solution were mixed and sprayed onto the plate to give a purple coloration after 1-2 min.

**Extraction and isolation**

The dried seed of *Peganum nigellastrum* Bunge (350 g) was extracted three times with 90% ethanol under reflux after immersion overnight, and the combined extract was concentrated under vacuum to afford a viscous residue (114 g). The residue was treated three times with 1.05 L of 2% HCl and filtrated. Then the acidic solution was basified with concentrated ammonia (10:1:5:0.5), to afford compound 5 (7 mg). Compound 6 (2000 mg) was obtained from fraction E by crystallization with a chloroform-methanol (1:1) solvent system. Fraction F was subjected to a silica gel column, eluted with chloroform-methanol (10:1 and 5:1), afforded three subfractions (F1~F3). Compound 7 (13 mg) was obtained from subfraction F1. Fraction K was chromatographed on a reverse-phase C18-ODS column with water-methanol gradient system to yield four subfractions (K1~K4). Subfraction K2 (30% methanol) was subjected to a preparative-TLC, eluted with chloroform-methanol-ammonia (10:2:5:0.5), to afford compound 8 (19 mg). Fraction L was subjected to further chromatography on C18-ODS column with water-methanol gradient system to yield five subfractions (L1~L5). Compound 9 (3 mg) and compound 10 (3 mg) were obtained from subfraction L2 (30% methanol), using Sephadex LH-20 with chloroform-methanol (1:1) elution and preparative-TLC with ethyl acetate-methanol-ammonia (10:3:0.5).

**Semiquantitative assay of acetylcholinesterase activity**

Reference substance of galanthamine and the ten isolated compounds 1-10, purity were more than 98% determined by HPLC, were accurately weighed and dissolved in methanol and diluted to make serial concentration solutions of 1.0, 0.1, 0.01, 0.001, and 0.0001 mol/L, respectively. 1 µL of the above serial concentration solutions were applied onto the TLC plate. The AChE activity assay was carried out using an *in vitro* semiquantitative method as described under TLC bioautographic assay previously.

**RESULTS AND DISCUSSION**

It can be seen from Fig. 1A, there were some similar green to blue fluorescence spots from seed extracts of *P. harmala* Linn, *P. multisectum* (Maxim) Bobr, and *P. nigellastrum* Bunge, and a probable *P*. variety. These fluorescence spots were indicated from Fig. 1B to be alkaloids because of giving an orange-red color with Dragendorff’s reagent on TLC plate. Accordingly, white spots in the TLC-bioautographic assay (Fig. 1C) indicated that these alkaloids spots represented biochemical ingredients with AChE inhibitory activity of genus *Peganum*.

In order to isolate the compounds responsible for inhibition of the activity of AChE, a activity-guided fractionation strategy was performed throughout the separation procedure (see extraction and isolation described above). Repeated column chromatography of the ethanol fraction from the seeds of *Peganum*...
nigellastrum Bunge led to the isolation of ten compounds (1-10) (Fig. 2), including two new compounds, nigellastrine I (compound 9) and nigellastrine II (compound 10), and as well as eight known compounds 1-8. The structures of the known compounds 1-8 were confirmed as vasicinone (1), vasicine (2), harmine (3), deoxyvasicinone (4), deoxyvasicine (5), harmaline (6), harmol (7), harman (8), by comparison of their physical and spectral data with the published data (Duan et al., 1998a, 1998b). Among them, harmol and harman were first isolated from *P. nigellastrum* Bunge.

Compound 9, obtained as pale yellow powder, showed a positive response with Dragendorff’s reagent. The HR-ESI-MS signal at m/z 383.1501 gave rise to the empirical formula C_{23}H_{19}N_{4}O_{2} (M^+; calc. 383.1508). The 1H-NMR spectrum exhibits signals for nine aromatic protons [H.23 (1H, 5-H), 8.20 (1H, 4’-H), 8.16 (1H, 6’-H), 7.23 (1H, 7-H), 7.19 (1H, 8-H), 7.13 (1H, 6-H), 7.03 (1H, 8’-H), 6.96 (1H, 9-H), 6.87 (1H, 7’-H)], one methyl groups [3.09 (3H, 2’C-CH_{3})], one methylene groups [2.49 (1H, 10-H)], 2.08 (1H, 10-H), and two methine groups [5.09 (1H, 9-H), 3.74 (1H, 11-H)]. The 13C-NMR spectrum exhibits signals for 23 C atoms [17.4 (C_{2’}-CH_{3}), 30.7 (10-C), 55.9 (11-C), 72.6 (9-C), 109.4 (9’-C), 114.4 (7’-C), 115.2 (8’-C), 115.9 (5’-C), 118.0 (6’α-C), 122.3 (2-C), 127.0 (6’-C), 128.5 (6-C), 128.7 (7-C, 8-C), 130.6 (5-C), 131.7 (5’α-C), 132.7 (2’α-C), 135.0 (9’α-C), 136.3 (2’-C), 139.7 (4’-C), 145.5 (5’α-C), 162.2 (8α-C), 165.1(C=O)].

Compound 10, obtained as a pale yellow powder, has a molecular formula of C_{23}H_{21}N_{4}O_{2} based on its positive-ion HR-ESI-MS (m/z 385.1662 [C_{23}H_{21}N_{4}O_{2}^+; calc. 385.1665], and gave an orange-red color with Dragendorff’s reagent on a TLC plate. The 1H-NMR spectrum exhibits signals for eight aromatic protons [H.28 (1H, 5-H), 8.20 (1H, 6’-H), 7.20 (1H, 7-H), 7.12 (1H, 8-H), 7.02 (1H, 6-H), 7.00 (1H, 8’-H), 6.90 (1H, 7’-H), 6.86 (1H, 9’-H)], one methyl groups [3.09 (3H, 2’C-CH_{3})], two methylene groups [3.33 (2H, 4’-H), 2.52 (1H, 10-H), 1.91 (1H, 10-H), and three methine groups [5.18 (1H, 9-H), 3.65 (1H, 11-H), 3.21 (1H, 5’-H)]. The 13C-NMR spectrum exhibits signals for 23 C atoms [17.1 (C_{2’}-CH_{3}), 30.9 (10-C), 31.6 (5’-C), 56.0 (11-C), 57.8 (4’-C), 73.2 (9-C), 108.9 (9’-C), 114.7 (7’-C), 115.3 (8’-C), 117.8 (6’α-C), 122.3 (2-C), 127.2 (6’-C), 128.2 (6-}

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**Fig. 1.** Fraction detection and screening on AChE inhibitory activity of *P. harmala* Linn (1), *P. multisectum* (Maxim.) Bobr (2), *P. varity* (3), and *P. nigellastrum* Bunge (4) by different chromogenic methods: (A) inspected under UV 366, (B) Dragendorff’ reagent, (C) Bioautograph (Samples’ concentration in the plate C is one-tenth of that in plate A and B)

**Fig. 2.** Structures of compounds 1-10 isolated from seeds of *P. nigellastrum* Bunge. vasicinone 1, vasicine 2, harmine 3, deoxyvasicinone 4, deoxyvasicine 5, harmaline 6, harmol 7, harman 8, nigellastrine I 9, nigellastrine II 10
Activity-Guided Isolation of *P. Nigellastrum* Bunge

The structures of compound 9 and compound 10 (Fig. 2.) were primarily verified by comparison of 1H-NMR and 13C-NMR data with that of authentic compounds of vasicinone, harmine, and harmaline (Table I), and were denominated as nigellastrine I and nigellastrine II.

In order to establish detection limits for the bioautographic assay, above-mentioned ten compounds and galanthamine (a known inhibitor of AChE) were applied at varying concentrations onto the TLC plate, and the concentration that produced the spot with the least observable whiteness was noted. From Fig. 3, it could be seen that galanthamine, harmine, harmaline and harmol inhibited the AChE activity down to 0.01 µg, while the least amount of vasicine and harman required for activity on AChE inhibition was 0.1 µg.

The alkaloids of vasicinone, deoxyvasicinone, deoxyvasicine, nigellastrine I and nigellastrine II, also had shown weak activity on AChE inhibition (1 µg). In considered of the AChE inhibitory activity and construction of investigated compounds, β-carboline alkaloids such as harmine, harmaline, harmol and harman displayed a similar AChE inhibition comparing to

### Table I. Comparison of 1H NMR and 13C NMR data of nigellastrine I and nigellastrine II with that of authentic compounds of vasicinone, harmine, and harmaline

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<sup>a</sup> Come from the published data (Duan et al., 1998a, 1998b)
galanthamine, in comparison to quinoline alkaloids (vasicinone, deoxyvasicinone, deoxyvasicine). The new compounds of nigellastrine I and nigellastrine II, constructed of quinoline and carboline alkaloids, also displayed a weak activity on AChE inhibition.

In conclusion, by activity-guided isolation on AChE inhibition, ten alkaloids including two new compounds were achieved from *P. nigellastrum* Bunge. The results indicated that alkaloids form *P. nigellastrum* Bunge shown potential inhibitive activity on AChE.

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