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Potent AChE and BChE inhibitors isolated from seeds of *Peganum harmala* Linn by a bioassay-guided fractionation

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ABSTRACT

Ethnopharmacological relevance: Seeds of *Peganum harmala* Linn are traditionally used as folk medical herb in Uighur medicine in China to treat disorders of hemiplegia and amnesia. Previously studies have proved that dominating alkaloids in *P. harmala* show significant inhibitory activities on the cholinesterase.

Aim of the study: The aim of the present study is to isolate trace ingredients from seeds of *P. harmala* and evaluate its inhibitory activities on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

Materials and methods: For sake of screening effective cholinesterase inhibitors, trace compounds were isolated from seeds of *P. harmala* through a bioassay-guided fractionation and their structures were determined via detailed spectral analysis. The inhibitory activities on AChE and BChE were assessed using an improved Ellman method by UPLC-ESI-MS/MS to determine the common final product choline.

Results: The activity-guided fractionation led to the isolation of two new alkaloids 2-aldehyde-tetrahydroharmine (**10**), 2-carboxyl-3,4-dihydroquinazoline (**19**), one syringin structure analog 1-O-*β*-*D*-xylopyranose sinapyl alcohol (**22**), and along with 19 known compounds. Compounds acetylnorharmine (**6**), harmic acid methy ester (**7**), harmine *N*-oxide (**13**), 6-methoxyindoline (**14**), syringin (**21**) were first found from genus *Peganum* and compounds 3-hydroxylated harmine (**4**), 1-hydroxy-7-methoxy-*β*-carboline (**5**) were new natural products. The results showed that the 2-aldehyde-tetrahydroharmine (**10**) has a potential inbibitive effect on both AChE and BChE with IC_{50} values of 12.35 ± 0.24 and 5.51 ± 0.33 μM, respectively. Deoxyvasicine (**15**) and vasicine (**16**) showed the strongest BChE inhibitory activity with IC_{50} values of 0.04 ± 0.01 and 0.1 ± 0.01 μM. The analysis of the structure-activity relationship indicated that the saturation of pyridine ring and the presence of substitution at indole ring, C-1, C-3, C-7 and N-2, for β-carbolines, were essential for effective inhibition of both AChE and BChE and the five-membered ring between C-2 and N-3 as well as the substituent groups sited at C-4 and C-9, for quinazolines, were important to both the AChE/BChE-inhibitory activity.

Conclusions: Bioassay-guided fractionation has led to the isolation of AChE and BChE inhibitors from the seeds of *P. harmala*. These results are in agreement with the traditional uses of the seeds of *P. harmala*.

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease and has become an urgent public health problem in most areas of the world (Grutzendler and Morris, 2001). Since the introduction of the first cholinesterase inhibitor (ChEI) in 1997, most clinicians and probably most patients would consider the cholinergic drugs, donepezil, galantamine and rivastigmine, to be the first line pharmacotherapy for moderate AD (Birks, 2006). Recent studies indicated that the main cause of the loss of cognitive functions in AD patients is a continuous decline of the cholinergic neurotransmission in cortical and other regions of the human brain (Schuster et al., 2010). The drugs above can block the acetylcholinesterase (AChE) and inhibit the breakdown of acetylcholine (ACh) accordingly. The selective inhibitors on butyrylcholinesterase (BChE) could elevate extracellular levels of ACh in the brain and improve cognitive performance

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in rodents without the classic adverse side effects induced by the selective or nonselective inhibitors on AChE, such as nausea and vomiting (Hartmann et al., 2007).

Ever since two AChE inhibitors derived from natural products (galantamine and rivastigmine) currently being licensed to alleviate cognitive symptoms in dementia, extensive research has been directed towards the identification of other AChE inhibitors, with the majority of these arising from the plant kingdom (Williams et al., 2011). While structurally diverse, these strongest AChE inhibitors are primarily alkaloids (Houghton et al., 2006; Williams et al., 2011). *Peganum harmala* is a perennial herb growing in Africa, the Middle East, India, South America, Mexico, southern America and China (Kartal et al., 2003; Cheng et al., 2010). In China, *P. harmala* has been used as a folk medicine since antiquity among the Uighur, Kazakh, and Mongolia for the treatment of cold, asthma, malaria, rheumatism, lumbago, hemiplegia, forgetfulness, and some skin diseases (Liu et al., 2013; Cheng et al., 2010). Previous reports have indicated that *P. harmala* show a potential therapeutic effect on AD, due to the cholinesterase inhibitory activities of harmine, harmaline, harmalol, harmol, and vasicine presented in plant (Rook et al., 2010; Zheng et al., 2009; Zhao et al., 2013; Frost et al., 2011; Liu et al., 2014). It was determined that the total alkaloids content (mainly harmine and harmaline) in dry seeds of *P. harmala* could accumulated up to 4.3% and 5.6% (w/w) (Herraiza et al., 2010; Wang et al., 2002).

Many trace ingredients isolated from plants such as paclitaxel may have strong biological activities (Rowinsky and Donehower, 1995). A few studies have reported that some abundant alkaloids from *P. harmala* have potent inhibitory activities on AChE and BChE (Zhao et al., 2013; Zheng et al., 2009; Liu et al., 2014). More interesting result is that some selective BChE inhibitors, such as vasicine, deoxyvasicine, deoxyvasicinone, and vasicinone, have been found from *P. harmala* (Zhao et al., 2013; Liu et al., 2014), which prompted us to find other trace alkaloids as potent AChE/BChE inhibitors from *P. harmala*. The present study performed a bioactive guided isolation of the trace ingredients from *P. harmala* and determined their inhibitory activities against AChE and BChE, using a UPLC-ESI-MS/MS method established by our team earlier (Liu et al., 2014). It would provide some guidance for the design and synthesis or semi-synthesis of potential inhibitors on AChE or BChE.

2. Materials and methods

2.1. General experimental procedures

UV spectra were determined using a TU-1901 spectrophotometer (Purkinje General Instrument Co., Ltd., Beijing, China). Scanning IR spectroscopy was performed on a Thermo Nicolet 380 FT-IR spectrometer (Thermo Electron Corp., San Jose, Calif., USA) using KBr pellets. Optical rotations were measured on Autopol VI (Rudolph Research Analytical). 1 and 2-D NMR spectra were recorded at Bruker AV-400 instrument at 400 (^{1}H) and 100 MHz (^{13}C) in MeOD or DMSO soln., with TMSi as an internal standard. ESI-MS were taken at Bruker Daltonics, Inc. APEXIII 7.0 T FTMS and HR-EI-MS were taken at Finnigan LC QDECA mass spectrometers. Analytical and preparative TLC was performed on silica gel plates ($HSGF_{254}$, Yantai Jiangyou Guijiao Kaifa Co., Ltd., PR China). The spots were visualized by exposure to UV radiation under 254 nm and 365 nm. Column chromatography (CC) was performed on silica gel (SiO_2; 200–300 mesh; Qingdao Haiyang, Co., Ltd., PR China), Sephadex LH-20 (Amersham Biosciences, GE Health Care) and MCI gel CHP-20P (Mitsubishi Chemical Corporation). High performance liquid chromatography (HPLC) was performed using an Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA) coupled with an analytical column (Diamonsil C18, 5 μm, i.d.4.6 × 250 mm).

AChE from *Electrophorus electricus*, BChE from equine serum, acetylcholine (Ach) chloride, butyrocholine (BCh) chloride, choline (Ch) chloride, chlormequat (internal standard, IS), galantamine and harmane were purchased from Sigma-Aldrich (MO, USA). HPLC-grade methanol was obtained from Fisher Co. (NJ, USA). Formic acid was obtained from Tedia Inc. (OH, USA). Water was produced with a Milli-Q Academic System (Millipore, Billerica, MA). Other reagents were of analytical grade.

2.2. Plant material

The seeds of *P. harmala* were collected in wild in Urumuqi, Xinjiang Uyghur Autonomous Region, China, in September 2009. The plant material was authenticated by professor Chang-hong Wang and the voucher specimen (No. PH09-1) was deposited at the Herbarium of the Shanghai R&D Center for Standardization of Traditional Chinese Medicine, Shanghai, China.

2.3. Extraction and isolation

The dried seeds of *P. harmala* (400 kg) were extracted three times with 85% EtOH (3200 L) at 85 °C after immersion overnight. The combined extracts were concentrated under vacuum to afford a viscous residue (114 kg, total ethanol extract, TEE). The concentrated extracts (TEE) were first defatted by using petroleum ether to remove liposoluble constituents and fatty oil (17 kg, petroleum ether extract, PEE). The residual extracts (97 kg, total alkaloid extract, TAE) were dissolved in 4% HCl (80 L) and centrifuged to remove non-alkaloid extracts (54.6 kg, NAE). Then the filtrate was adjusted to pH 11 by using NaOH solution and stand overnight to precipitate the crude alkaloids (21.4 kg, abundant alkaloid extract, AAE), which mainly contained compounds **3** and **8**. The rest alkali solutions (70 L) were extracted with CH_2Cl_2 (3 × 70 L) and led to 800 g concentrated extracts (trace alkaloid extract, TrAE). Then the extract (TrAE) was subjected to silica gel (5 kg) column and gradient eluted with mixture of AcOEt–MeOH (100:0, 50:1, 20:1, 10:1, 0:100, v/v). The next series of effluents were concentrated under vacuum to obtain Fr. A to Fr.G. Compounds **18** (5 g) and **16** (80 g) were found as precipitate and filtered out from Fr. D and Fr. F, respectively.

Fr. D (21 g) was dissolved in 30% MeOH and the precipitate was filtrated and discarded. The filtrate (300 mL) was fractionated by MCI gel with the elution of MeOH–H_2O (3:7, 4:6, 6:4, 7:3, 9:1, and 10:0, v/v) to obtain 9 fractions. Fr.D.4 (0.8 g) gave compound **22** (10 mg) after purifying by ODS column chromatography under isocratic elution with acetonitrile–H_2O (8: 92). Fr.D.5 (1.2 g) gave compound **20** (66.8 mg) after purifying by preparative HPLC under isocratic elution with MeOH–H_2O (4.5:5.5). Fr.D.8 (0.8 g) and Fr.D.9 (0.6 g) were subjected to Sephadex LH-20 (MeOH) to afford compound **5** (16.6 mg) and compound **12** (10 mg).

Fr.E (40 g) was subjected to silica gel (800 g) column chromatography with the elution of AcOEt–MeOH (100:0, 40:1, 15:1, 10:1.5, 0:100, v/v) and 12 fractions were collected. Fr.E.4 (4 g) was further separated by Sephadex LH-20 (250 g), eluted with MeOH to give three fractions. Fr.E.4.1 was then purified by preparative HPLC with the eluent of MeOH–H_2O (1:1) to give compound **7** (12 mg). Compound **10** (7.5 mg) was obtained from Fr.E.11 (7 g) by MCI gel eluted with MeOH–H_2O (3:7, 4:6, 6:4, 7:3, 1:9, v/v). In addition, compound **21** (23 mg) was separated from Fr. E.9 by MCI gel eluted with MeOH–H_2O (4:6).

Fr.G (60 g) was fractionated by silica gel (1.5 kg) column chromatography by applying mixture of CH_2CL_2–MeOH (20:1, 15:1, 10:1, 1:1, 0:100, v/v) as eluent and 7 fractions were collected. Fr.G.1 (2 g) was fractionated by silica gel (60 g) column chromatography with the

eluent of petroleum ether: EtOAc (15:1, 1:1, 0:1) to afford six fractions. Fr.G.1.2 was then subjected to Sephadex LH-20 (MeOH) to obtain compound **6** (15 mg). Compound **14** (49.1 mg) was obtained from Fr. G.2 (3.4 g) by silica gel column chromatography with the elution of AcOEt. Fr.G.5 was subjected to MCI gel (500 g) with the MeOH–H_2O (3:7, 4:6, 7:3, 9:1, v/v) as eluent to give 18 fractions. Compound **19** (40 mg, white crystal) was crystallized out from Fr.G.5.4 after being stood overnight at room temperature. Fr.G.5.12 was further purified by preparative TLC with the developing solvent system of AcOEt–MeOH–ammonia (10:1:0.2) and yielded compound **13** (32.9 mg). Fr.G.6 (8 g) was subjected to silica gel (200 g), employed with AcOEt–MeOH–ammonia (10:1:0, 10:1:0.5) as developing solvent system to afford nine sub-fractions. Fr.G.6.8 yielded compound **4** (44.8 mg) after concentration and being extracted with 30% MeOH. Fr.G.6.9 was loaded on MCI gel to afford compounds **15** (2 g) and **17** (13.7 mg) eluted with MeOH–H_2O (3:7, 8:2, respectively).

Fr.G.7 (10 g) was applied to MCI gel using MeOH–H_2O (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 9:1) to afford eleven fractions. Fr.G.7.7 was then subjected to Sephadex LH-20 and eluted with MeOH to yield 2 fractions. Fr.G.7.7.2 was then loaded on a reverse-phase C18-ODS column with the eluent of MeOH–H_2O (15: 85) to yield compound **11** (36.4 mg).

2.3.1. Compound 10

Amorphous white powder: α_t^{20}: −41.3°(C 0.17 MeOH), UVλ_{max}^{MeOH}nm (log ε): 226 (4.49); IRν_{max}^{KBr}cm^{-1}: 3277, 2967, 2874, 2828, 1655, 1644, 1630, 1428, 1216, 1249, 1195, 1154, 1036, 805; HR-EI-MS *m/z* $[M+H]^+$ 245.1318, calcd. 245.1290, $C_{14}H_{16}N_2O_2$; ^{1}H and ^{13}C NMR shown in Table 1.

2.3.2. Compound 19

Colorless acicular crystal: UVλ_{max}^{MeOH}nm (log ε): 220 (4.12); IRν_{max}^{KBr}cm^{-1}: 3535, 3159, 1650, 1579, 1501, 1623, 1395, 1369, 1294, 1223, 757; HR-EI-MS *m/z* $[M+H]^+$ 177.0677, calcd. 177.0664, $C_9H_8N_2O_2$; ^{1}H and ^{13}C NMR are shown in Table 1.

2.3.3. Compound 22

Amorphous white powder: UVλ_{max}^{MeOH} nm (log ε): 221 (4.43); IRν_{max}^{KBr}cm^{-1}: 3419, 2917, 2613, 1589, 1508, 1467, 1421, 1338, 1245, 1130, 1085, 1025, 960, 632. HR-ESI-MS *m/z* $[M++Na]^+$ 365.1221, calcd. 365.1207, $C_{16}H_{22}O_8$; ^{1}H and ^{13}C NMR are shown in Table 1.

2.4. Acidic hydrolysis of compounds 22

A soln. of **22** (1–2 mg) in 3 M aq. CF_3COOH (200 μL) and 80 mg/mL aq. borane-4-methylmorpholine (50 μL) was heated for 5 min at 80 °C. After cooling, 50 μL of aq. borane-4-methylmorpholine (80 mg/mL) was added to the mixture and heated for 60 min at 120 °C in oil bath. The mixture was cooled and added with 100 μL aq. borane-4-methylmorpholine (80 mg/mL) and then concentrated until anhydrous. Acetic anhydride/anh. (100 μL) and CF_3COOH (100 μL) were added to the hydrolyzate and the mixture was allowed to react for 20 min at 50 °C. After addition of 1 mL of $CHCl_3$, the mixture was washed three times with saturated Na_2CO_3 solution and H_2O, successively. The organic phase was dried (Na_2SO_4) and subjected to GC/MS (Thermo TR-5 MS, 60 m × 0.25 mm × 2.5 mm); carrier gas He, flow rate 1 mL/min; oven temp.: 180–220°C (4°C/min), 220 °C for 2 min, 220–270°C (1°C/min), and 270°C for 1 min. By comparison with the retention times of authentic samples, the configuration (*D*) of the sugar moieties was determined.

2.5. Biological activities

The AChE and BChE inhibitory activities of fractions and isolated compounds were assessed using a UPLC-ESI-MS/MS method previously established by our lab. (Liu et al., 2014), employing galantamine as reference compounds.

All the tested extracts were dissolved in DMSO to obtain 10 mg/mL solutions, and the test compounds were dissolved in 0.2% DMSO to obtain 10 mM or 5 mM solutions. All the stock solutions were diluted to a series of concentrations with 20 mM sodium phosphate buffer solution (pH 7.6) before the experiment.

Table 1
^{13}C and ^{1}H NMR data of compounds **10**, **19**, and **22.**

Position	Compound **10**		Compound **19**		Compound **22**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	45.8	5.42 (1H, q, 6.60)			135.2	
1a			132.1			
2			154.0		154.4	
3	42.4	3.51 (1H, m) 3.92(1H, m)			104.9	6.76 (1H, s)
4	23.3	2.75 (2H, overlapped)	42.1	4.71 (2H, s)	134.9	
4a			118.1			
5	119.4	7.26 (1H, d, 8.6)	126.8	7.16 (1H, overlapped)	104.9	6.76 (1H, s)
6	109.7	6.66 (1H, dd, 8.6, 2.16)	127.0	7.16 (1H, overlapped)	154.4	
7	157.6		128.8	7.24 (1H, t, 7.13)	131.3	6.57 (2H, d, 15.84)
8	95.8	6.84 (1H, d, 1.76)	117.8	7.36 (1H, d, 7.88)	129.9	6.34 (1H, dt, 15.84, 5.56)
9			154.8		63.5	4.24 (2H, d, 5.52)
10	133.6				56.7	3.86 (3H, s)
11	107.3				56.7	3.86 (3H, s)
12	122.4					
13	138.8					
14	19.5	1.49 (3H, d, 6.68)				
15	163.9	8.17 (1H, s)				
16	56.0	3.89 (3H, s)				
1'					104.9	5.01 (1H, d, 6.24)
2'					74.3	3.58 (1H, dd, 7.68, 6.44)
3'					76.0	3.48 (1H, overlapped)
4'					71.0	3.61 (1H, overlapped)
5'					66.3	3.99 (1H, dd, 11.72, 4.6)
						3.21 (1H, dd, 11.72, 8.28)

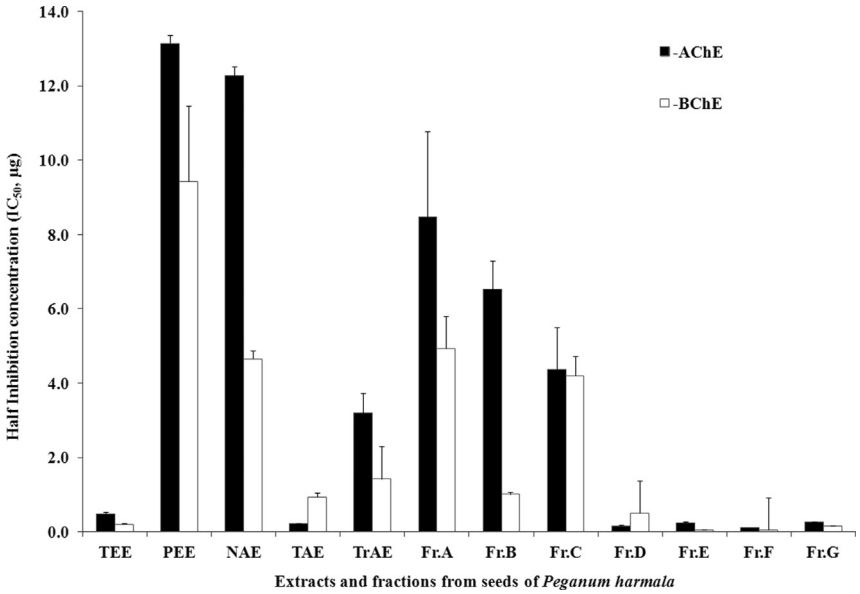

Fig. 1. The AChE/BChE inhibitory activities (the IC_{50} values are expressed as μg/mL) of the extract fractions from seeds of *Peganum harmala* (TEE: total ethanol extracts; PEE: petroleum ether extract; NAE: non-alkaloid extract; TAE: total alkaloids extract, TrAE: trace alkaloid extract. Fr.A–Fr.G: the fractions separated from TrAE).

For the AChE and BChE assay, 10 μL of the solutions of test extracts or compounds, 40 μL enzyme preparation (with final concentrations: 0.0035 unit/mL for AChE, or 0.008 unit/mL for BChE,) were mixed and pre-incubated in ice bath for 15 min. To the mixture, 50 μL substrate solutions (final concentrations 1 mg/mL for acetylcholine chloride, or 1.5 mg/mL for butyrocholine chloride) was added and incubated for 30 min at room temperature, the reaction was terminated by the addition of 300 μL acetonitrile (0°C and added 300 ng/mL chlormequat as internal standard) immediately, and the supernatant was used for analysis.

For controls, the test solutions were replaced by the corresponding volume of phosphate buffer solutions (pH 7.6) or galantamine solution in concentrations corresponding to their IC_{50} values (50% of inhibition). For blanks, the test solutions and the enzyme preparation were all replaced by the corresponding volume of phosphate buffer solutions (pH 7.6).

The separation was performed on a Waters-ACQUITY^{TM}UPLC system (Waters Corp., Milford, MA, USA) using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm) maintained at 40 °C. The mobile phase consisted of A (0.1% formic acid) and B (methanol) at a flow rate of 0.3 mL/min, using an isocratic elution with 98% A and 2% B. The injection volume was 5 μL using a partial loop with needle overfill mode (Liu et al., 2014).

The assays were conducted in triplicate, and all tabulated results were expressed as means ± ± SD, and the IC_{50} values were calculated from concentration-response curves by a nonlinear regression analysis using Prism software (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Isolation and characterization of compounds

Two new alkaloids (**10**, **19**) and one new syringin structure analog (**22**) were isolated from *P. harmala*, along with 18 known alkaloids (**1**–**9**, **11**–**18**, **20**) and syringin (**21**). Besides, compounds **6**, **7**, **13**, **14**, **21** were first found from *P. harmala* and compound **4**, **5** were new natural products. Their structures were elucidated on the basis of spectroscopy data using ^{1}H and ^{13}C NMR, 2D NMR (HMQC, HMBC, ^{1}H–^{1}H COZY, ROESY), as well as HR-ESI-MS.

3.1.1. Activity-guided isolation of compounds

The total ethanol extracts (TEE) of the dried seeds of *P. harmala* were first treated with petroleum ether in order to remove the oil (PEE). In order to remove the non-alkaloids ingredients (NAE), the skimmed residue was first dissolved in acid to dissolve alkaloid components and filtrated. Then the most abundant alkaloids (AAE) such as harmaline and harmine were precipitated from the acid solution by alkalization. Some trace alkaloid ingredients would be retained in the alkaline solution section (Liu et al., 2013). The trace alkaloid extract (TrAE) was obtained from the alkalization solution by dichloromethane extraction. The extracts obtained above were assayed on AChE/BChE using a UPLC-ESI-MS/MS method and calculated the IC_{50} values to evaluate their inhibitory activity (Fig. 1). A high inhibitory activity (AChE IC_{50}=0.481 μg, BChE IC_{50}=0.205 μg) of the total ethanol extracts (TEE) was observed. The total alkaloid extract (TAE) and the trace alkaloid extract (TrAE) which all were yielded from the total ethanol extracts (TAE) also exhibited a strong inhibitory activities against AChE with IC_{50} values of 0.218 μg and 3.194 μg, BChE with IC_{50} values of 0.935 μg and 1.424 μg, respectively. While the petroleum ether extract (PEE) and the non-alkaloid extract (NAE) showed much weaker inhibitory activity on both AchE and BChE.

The total alkaloids extract (TAE), which mainly containing harmine and harmaline, have been proved potential inhibitory activity on AChE and BChE (Zhao et al., 2013). The trace alkaloid extract (TrAE), which has been removed off most harmine and harmaline and enriched a mass of other trace alkaloids according to principle of dissolution, became as the target for further separation of active compounds with potential inhibitory activity on AChE and BchE. The trace alkaloid extract (TrAE) were subjected to silica gel column and eluted with AcOEt–MeOH to give 7 fractions. The concentrated extracts of every fractions were tested on AChE/BChE inhibitory activity. From the results shown in Fig. 1, the fractions A–C did not show a desired inhibitory activity on AChE and BChE, while the fractions D–G showed potential inhibitory activities (AChE IC_{50}=0.151, 0.236, 0.103, 0.264 μg, BChE IC_{50}=0.508, 0.052, 0.056, 0.159 μg, respectively). After multiple separation processes by normal and reverse column chromatogram and preparative HPLC of fractions D–G, total 22 compounds containing 20 alkaloids were obtained (Fig. 2), and their structures were elucidated on the basis of spectra data using ^{1}H and ^{13}C NMR, 2D (HMQC, HMBC, $^{1}H-^{1}H$ COSY, ROESY) NMR.

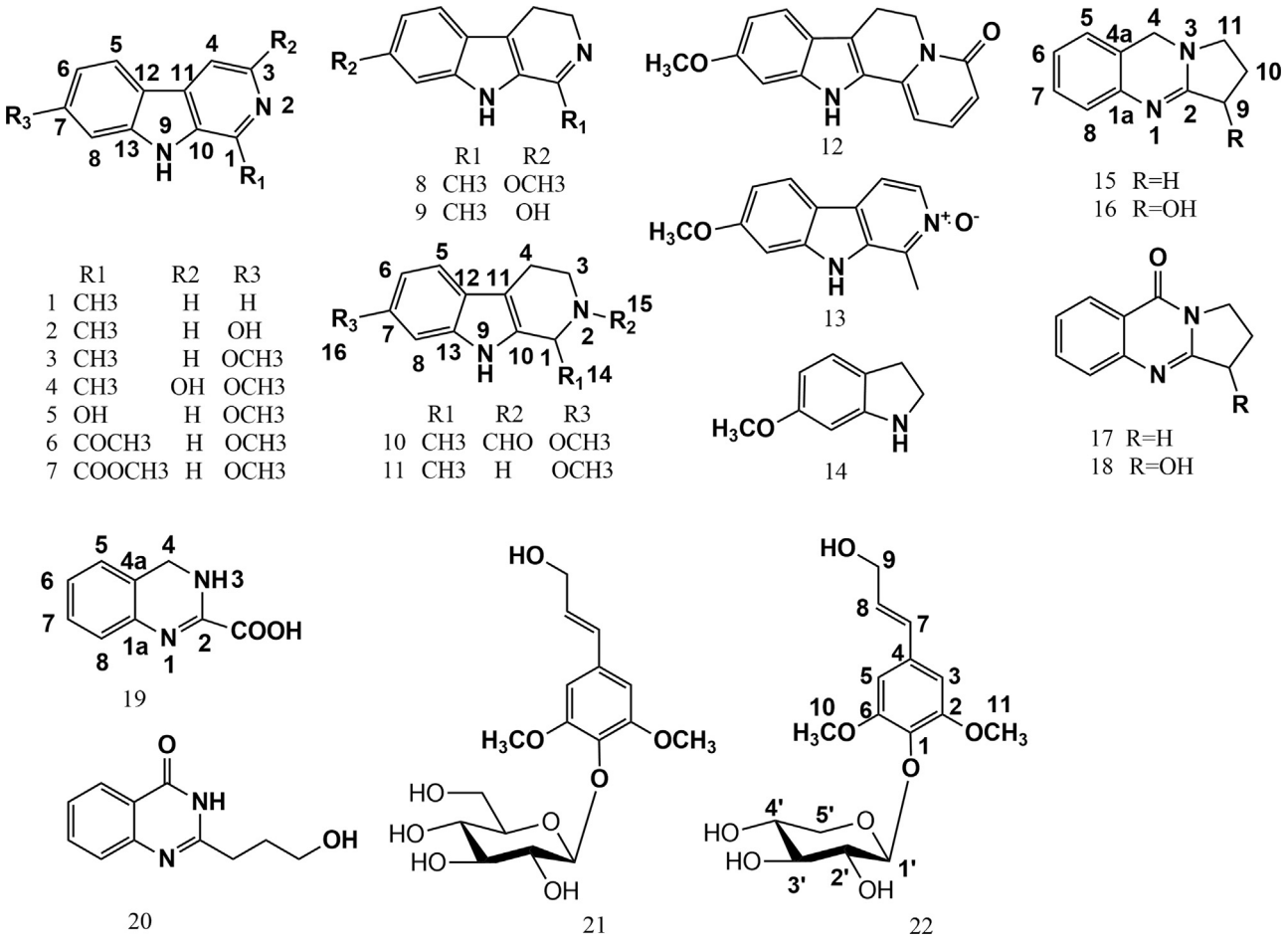

Fig. 2. Structures of compounds separated and identified from seeds of *P. harmala*.

3.1.2. Characterization of the three new compounds

2-Aldehyde-tetrahydroharmine (**10**) was obtained as a white amorphous powder, with the molecular formula as $C_{14}H_{16}N_2O_2$ determined by HR-ESI-MS (*m/z* $[M+H]^+$ 245.1318, calcd. 245.1290). UV absorption at 226 and 268 nm suggested the presence of an indole ring, and the IR spectrum indicated the presence of NH (3277 cm^{-1}), carbonyl (1655 cm^{-1}), and benzene ring (1630 cm^{-1}) groups. The ^1H NMR signals (Table 1) of an ABX coupling benzene ring [δ_H 6.84 (1H, d, J=1.9 Hz, H-8), 6.66 (1H, dd, J=8.6, 1.9 Hz, H-6) and 7.26 (1H, d, J=8.6 Hz, H-5)], together with five quaternary carbons at δ_C 157.6 (C-7), 133.6 (C-10), 107.3 (C-11), 122.4 (C-12) and 138.8 (C-13) confirmed a 2,3,6-trisubstituted indole ring structure. HMBC correlations of H-15 (1H, δ_H 8.17, s) with C-1 (δ_C 45.8) and C-3 (δ_C 42.4) suggested their connection through a nitrogen atom. $^1H-^1H$ COZY correlations (Fig. 3) from H-4 (δ_H 2.75) to H-3 (δ_H 3.51 and 3.92) in combination with HMBCs of H-1(δ_H 5.42) with C-10 (δ_C 133.6), and H-4 (δ_H 2.75) with C-11 (δ_C 107.3) demonstrated that compound **10** had a 1,2,3,4-tetrahydro-β-carboline skeleton. In addition, the HMBCs of H-14 (δ_H 1.49) with C-1 (δ_C 45.8) and C-10 (δ_C 133.6), and H-OMe (δ_H 3.89) with C-7 (δ_C 157.6) indicated the methyl and methoxyl was connected with C-1 and C-7, respectively. The absolute configuration of compound **10** was established as 1S by X-ray crystallographic analysis (Fig. 4). Accordingly, the structure of compound **10** was determined as shown in Fig. 3, and named as (1S) 2-aldehyde-tetrahydroharmine.

2-Carboxyl-3,4-dihydroquinazoline (**19**) was isolated as a colorless acicular crystal with the molecular formula established as $C_9H_8N_2O_2$ by HR-ESI-MS (*m/z* 177.0677, $[M+H]^+$, calcd. 177.0664). The IR spectrum indicated the presence of OH (3160 cm^{-1}), carbonyl (1651 cm^{-1}) groups. The ^1H NMR signals (Table 1) of a 1,2-disubstituted benzene ring [δ_H 7.16 (1H, m, overlapped, H-5), 7.16 (1H, m, overlapped, H-6), 7.24 (1H, t, J=7.13 Hz, H-7) and 7.36 (1H, d, J=7.88 Hz, H-8)], together with the characteristic carbon signals at δ_C 154.0 (C-2), 42.1 (C-4), 118.1 (C-4a) and 132.1 (C-1a) implied a 2-substituted-3,4-dihydroquinazoline ring structure. Furthermore, $^1H-^1H$ COZY correlations (Fig. 3) from δ_H 7.16 (H-5) to δ_H 7.36 (H-8) and HMBCs (Fig. 3) of H-4 (δ_H 4.71) with C-2 (δ_C 154.0), C-5 (δ_C 126.8), C-4a (δ_C 118.1), and C-1a (δ_C 132.1) confirmed the above skeleton. Subtracting the structure of 3,4-dihydroquinazoline ring from molecular formula calculated by HR-ESI-MS led to a carboxyl, and the diamagnetic shift (C-9,δ_C 154.8) suggested its attachment to C-2 (δ_C 153.9). Based on the above evidences, the structure of **19** was as shown in Fig. 3, and named as 2-carboxyl-3,4-dihydroquinazoline.

1-O-β-D-xylopyranose sinapyl alcohol (compound **22**) was obtained as a white amorphous powder, with the molecular formula $C_{16}H_{22}O_8$ determined by the HR-ESI-MS (*m/z* 365.1221, $[M+Na]^+$, calcd. 365.1207). The ^1H NMR signals (Table 1) of δ_H 6.76 (2H, s, H-3,5) and two methoxy group signals (6H, δ_H 3.86, s, H-10,11) of magnetic equivalence demonstrated the presence of a symmetrical quadri-substituted benzene ring stucture. In addition, a trans-disubstituted alkenyl [δ_H 6.57 (2H, d, J=15.84 Hz), 6.34 (1H, dt, J=15.84, 5.56 Hz)] was also observed. $^1H-^1H$ COZY data from H-9 (δ_H 4.24) to H-8 (δ_H 6.34) and HMBC of H-7 (δ_H 6.57) with C-4 (δ_C 134.98) indicated the presence of a sinapyl alcohol structure. The NMR characteristics were very similar to those of syringin (**21**). However, ^1H and ^{13}C NMR data revealed a β-xylopyranose moiety with anomeric proton at δ_H 5.01 (1H, d, J=6.24, H-1′). HMBC data (Fig. 2) from H-1' (δ_H 5.01) to C-1 (δ_C 135.25) demonstrated that the β-xylopyranose moiety was

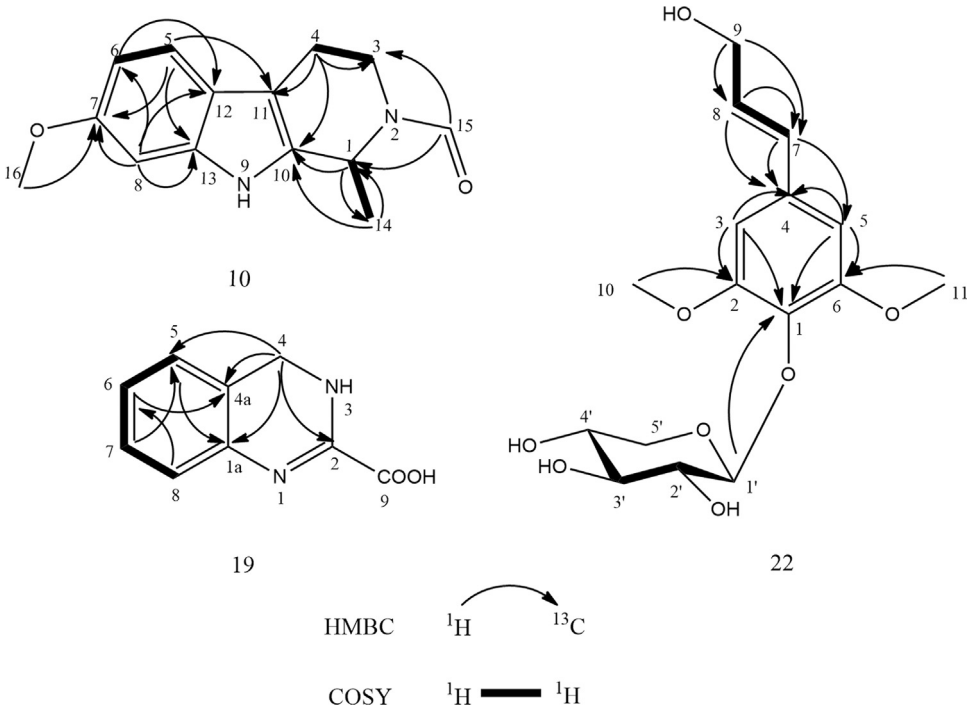

Fig. 3. The ^{1}H–^{1}H COSY and selected HMBC of compounds **10**, **19**, **22**.

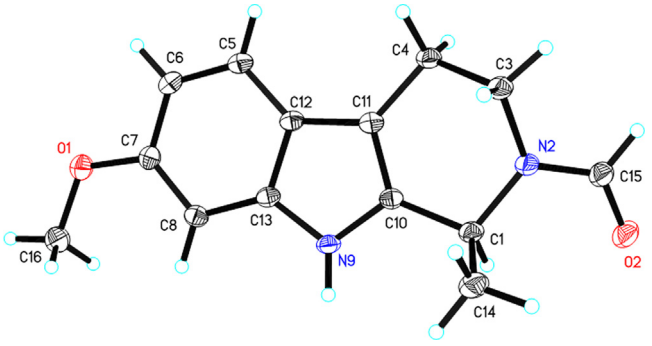

Fig. 4. The X-ray crystallographic structure of compound **10**.

attached to C-1 of the sinapyl alcohol aglycone. Acidic hydrolysis of **22** gave D-xylose as the sugar moiety. Based on the above evidences, compound **22** was determined to be 1-O-β-D-xylopyranose sinapyl alcohol.

In addition, 12 known β-caboline alkaloids of harmane (**1**) (Kusurkar and Goswami, 2004), harmol (**2**) (Duan et al., 1998a), harmine (**3**) (Duan et al., 1998a,b), 3-hydroxylated harmine (**4**) (Zhao et al., 2012), 1-hydroxy-7-methoxy-β-carboline (**5**) (Ghazala, 1980), acetylnorharmine (**6**) (Hashimoto and Kawanishi, 1976), harmic acid methy ester (**7**) (Hashimoto and Kawanishi, 1975), harmaline (**8**) (Duan et al., 1998a), harmalol (**9**), tetrahydroharmine (**11**), harmalanine (**12**) (Salimuzzaman et al., 1988), harmine *N*-oxide (**13**) (Hashimoto and Kawanishi, 1975), one known indole alkaloid 6-methoxyindoline (**14**), five known quinazoline alkaloids of deoxyvasicine (**15**) (Duan et al., 1998a), vasicine (**16**) (Duan et al., 1998a,b), dexyvasicinone (**17**) (Duan et al., 1998b), vasicinone (**18**) (Duan et al., 1998a,b), pegamine (**20**) (Ma et al., 1999), and one other type compound of syringin (**21**) (Gao et al., 2012) were isolated. Their structures were identified by comparison of their spectroscopic data with those reported in the literatures or the authentic compounds.

3.2. Cholinesterase inhibition evaluations

The IC_{50} values of inhibitive activity against AChE and BChE of the 22 compounds were evaluated and shown in Table 2.

It was observed that compounds of harmane (**1**), harmol (**2**), harmalol (**9**), deoxyvasicine (**15**) and vasicine (**16**) were able to strongly inhibit AChE and BChE activity with IC_{50} values of 3.64, 1.90, 3.45, 2.37, 3.38 μM for AChE and IC_{50} values of 1.04, 0.35, 0.66, 0.04, 0.10 μM for BChE, respectively. The BChE IC_{50} values displayed by deoxyvasicine (**15**) and vasicine (**16**) were lower than that of galantamine at the same test conditions. Moreover, harmine (**3**) and harmaline (**8**) were selective AChE inhibitors with the IC_{50} values 1.21 μM and 1.95 μM, respectively. Besides, 2-aldehyde-tetrahydroharmine (**10**), 3-hydroxylated harmine (**4**) and tetrahydroharmine (**11**) also showed weak inbibitive effect on both AChE and BChE.

β-carboline is a tricyclic structure of indole ring fused to a pyridine ring with the nitrogen 2-sited. From the AChE and BChE inhibitory potencies of β-carboline alkaloids (compound **1–13**) are shown in Table 2, it could be found that the compounds **1**, **2** and **3** were more active for AChE, which demonstrated that the presence of methoxyl or hydroxide at C-7 of the β-carbolines led to an increase in potency for AChE. Furthermore, compounds **3** and **8** have a more effective inhibitory activity against AChE than compounds **2** and **9**, respectively. It suggested that methoxyl (as an electron-withdrawing group sited on C-7) would make the β-carboline skeleton more inhibitory potency on AChE than a hydroxy.

A further observation was that the compound **2** and **9** gave the AChE selectivity index (S.I., defined as IC_{50} BChE/IC_{50} AChE affinity ratio) of 0.18 and 0.19, respectively. Both of them displayed more potent inhibition on BChE than the compounds which has a methoxy sited on C-7, such as compounds **3**, **5**,**8**, etc. It implied that the hydroxy at C-7 would make the β-carboline skeleton have a higher inhibition on BChE.

Simple indoles with substitution of methoxy, carboxy or hydroxy at the benzene ring showed a low percent of inhibitory activity on AChE. Adding a side chain at the pyrrole ring, such as serotonin, β-carbolines and quinolines (the bioisostere of indole),

Table 2
The half Inhibition concentration (IC_{50}) and selectivity index (S.I.) of the compounds investigated against AChE and BChE.

Compounds		IC_{50} (μM ± SD)		S.I.[a]
No.	Name	AChE	BChE	
1	Galantamine	0.05 ± 0.00	0.25 ± 0.00	5.28
2	Harmane	3.64 ± 0.19	1.04 ± 0.05	0.29
3	Harmol	1.90 ± 0.02	0.35 ± 0.03	0.18
4	Harmine	1.21 ± 0.04	2.79 ± 0.27	2.30
5	3-hydroxylated harmine	23.61 ± 1.30	3.25 ± 0.06	0.14
6	1-hydroxy-7-methoxy-β-carboline	7.19 ± 0.47	5.15 ± 0.23	0.72
7	Acetylnorharmine	4.04 ± 0.07	26.5 ± 2.82	6.56
8	Harmic acid methy ester	14.1 ± 0.74	21.48 ± 0.87	1.52
9	Harmaline	1.95 ± 0.08	5.38 ± 0.64	2.76
10	Harmalol	3.45 ± 0.08	0.66 ± 0.09	0.19
11	(1S)2-aldehyde-tetrahydroharmine	12.35 ± 0.24	5.51 ± 0.33	0.45
12	Tetrahydroharmine	22.95 ± 0.56	10.41 ± 0.63	0.45
13	Harmalanine	16.95 ± 0.56	3.24 ± 0.29	0.19
14	Harmine *N*-oxide	> 1000	> 1000	/
15	6-methoxyindoline	67.85 ± 8.60	21.37 ± 1.41	/
16	Deoxyvasicine	2.37 ± 0.40	0.04 ± 0.01	0.02
17	Vasicine	3.38 ± 0.03	0.10 ± 0.00	0.03
18	Dexyvasicinone	35.16 ± 1.34	17.82 ± 0.93	0.51
19	Vasicinone	76.60 ± 8.46	10.20 ± 2.08	0.13
20	2-carboxyl-3,4-dihydroquinazoline	344.80 ± 40.82	> 1000	/
21	Pegamine	155.10 ± 7.04	105.70 ± 13.50	0.68
22	Syringin	31.47 ± 2.25	2.77 ± 0.12	0.09
23	1-O-β-D-xylopyranose sinapyl alcohol	> 1000	> 1000	/

IC_{50} values were determined by regression analyses and expressed as the means ± SD of three replicate determinations.
/: no S.I. values.

[a] S.I. is the AChE selectivity index defined as IC_{50} BChE/IC_{50} AChE affinity ratio.

improved the inhibitory activity on AChE significantly (Khorana et al., 2012). While, the presence of pyridine ring substituted at indole ring seemed to be important to inhibitory activities on AChE/BChE, concluding from the lower activity of compound **14** (AChE IC_{50}=67.850 μM, BChE IC_{50}=21.37 μM) and comparing to that of β-carbolines (compounds **1**–**13**).

Compounds **3**, **8** and **11** showed a progressively lower AChE/BChE-inhibitory activity. Moreover, compound **2** showed more AChE/BChE-inhibitory than compound **9**. All of above results suggested that the nonsaturation of pyridine ring was very essential for both the cholinesterase inhibition. Further observation on the results, the compound **8** had a higher S.I. value of 2.76 than the compound **11** (S.I., 0.45). It implied that the saturated bond of C1–N2 would make the compound to have a BChE-selectivity inhibitory activity. The compound **2** had a similar S.I. value with compound **9** and also a similar S.I. value was found between the compound **3** and **8**. It indicated that the double bond between C3 and C4 would not contribute to the change of cholinesterase inhibition selectivity.

When comparing with compound **11**, the compound **10** with an aldehyde group substituted at tetrahydropyridine nitrogen and the compound **12** with an unsaturated ring between C-1 and N-2 displayed the increased IC_{50} values on both AChE and BChE. It suggested that the electron withdrawing group sited on N-2 would make the β-carboline more active on cholinesterase inhibition. In addition, the similar S.I. between compounds **11** and **10** implied that the aldehyde group substitute on N-2 made the compound **10** show corresponding BChE-selective inhibition as compound **11**. Moreover, the compound **12** had a lower S.I. value than compound **11**, which meant that the unsaturated ring between C-1 and N-2 would observably improve the selective inhibition on BChE of compound **11**.

When comparing the IC_{50} values of the compounds **3**, **6**, **5** and **7** to each other, it could be found that the methyl cited on C-1 of the β-carboline mother nucleus would improve both the AChE and BChE inhibitory activity. Besides, the highest S.I. value 2.303 of compound **3** implied that the methyl on C1 would make the β-carbolines showing selective AChE inhibition.

When the S.I. values of compounds **3** and **4** were compared, it was observed that the hydroxyl sited on C-3 would cut down obviously the inhibitory activity on AChE but slightly on the BChE.

In consideration of other natural and synthetic compounds in the literature (Torres et al., 2012), quaternalization of the pyridine nitrogen of the β-carboline nucleus leads to an increase in potency for both cholinesterase inhibitions. However, compound **13**, which is quaternalized in the pyridine nitrogen, shows non-inhibitory activity in present test with the IC_{50} values above 1000 μM on both the AChE and BChE.

For quinazoline alkaloids obtained in *P. harmala*, the structure-function relationship seemed simple. Compared to the compounds **15** and **17**, the compounds **19** and **20** had no five-membered ring between N-3 and C-2. Both of them exhibit weak inhibitory activities on either AChE or BChE. It suggested that the five-membered ring between C-2 and N-3 was important to both the AChE/BChE-inhibitory activities.

It was observed that compounds **15** and **16** have a much more potential inhibitory activity than compounds **17** and **18** on both AChE and BChE. Therefore, the non-substitution on C-4 of the quinazolines is important to the cholinesterase inhibitory activities.

Compared with the compound **16**, the compound **15** has a higher inhibitory activity on both AChE and BChE. Furthermore, the compound **17** is more active than compound **18** on both AChE and BChE. Thus, the hydroxy sited on C-9 might decrease the AChE/BChE inhibitory activity of the quinazolines.

In the process of drug development, toxicity and safety of drug is one of the important factors that should be considered first. The acute toxicity of the seeds of the genus *Peganum* growing in China, including *Peganum harmala*, *Peganum multisectum*, *Peganum nigellastrum*, and *Peganum variety*, have been determined previously (Zhao, 2011). The results indicated that the strength of the toxicity is in order of *P. nigellastrum* > > *P. variety* > > *P. multisectum* > > *P. harmala*, and the toxicity potency is proportional to

the harmaline content in the extract of plants. According to previous reports, the half lethal dose (LD_{50}) of harmine (417.45 mg/kg) is about 2 times larger than that of harmaline (144.07 mg/kg) (Massoud et al., 2002; Wang, 2002). In order to evaluate the potential for these new compounds becoming drug candidates, it is very important to further understand their toxicity. This will be conducted in a follow-up study.

4. Conclusion

For sake of screening effective cholinesterase inhibitors, a bioactive guided isolation of the trace ingredients from *P. harmala* led to two new alkaloids 2-aldehyde-tetrahydroharmine (**10**) and 2-carboxyl-3,4-dihydroquinazoline (**19**), one new syringin structure analog 1-O-*β*-D-xylopyranose sinapyl alcohol (**22**), as well as 19 known compounds (**1–9**, **11**–**18**, **20**–**21**, **23**) were obtained. Among them, the new compound 2-aldehyde-tetrahydroharmine (**10**) has a potential inbibitive effect on both AChE and BChE, and two known compounds deoxyvasicine (**15**) and vasicine (**16**) show the strongest BChE inhibitory activity. The analysis of the structure-activity relationship indicates that, for β-carbolines, the saturation of pyridine ring and the presence of substitution at C-1, C-3, C-7 and N-2 of indole ring, are very essential for inhibition both AChE and BChE, and for quinazolines, the five-membered ring between C-2 and N-3 as well as the substituent groups sited at C-4 and C-9 are important to both the AChE/BChE-inhibitory activity.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.03.070.

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