SUPPLEMENTARY MATERIAL

Anticholinesterases and antioxidant alkamides from *Piper nigrum* fruits

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The anticholinesterase and antioxidant effects of different extracts of *Piper nigrum* were evaluated. Twenty-one known alkamides were isolated from active ethyl acetate extract and investigated for their cholinesterase inhibitory and antioxidant effects. Among them, piperine (**2**), piperettine (**5**) and piperettyline (**20**) exhibited dual inhibition against AChE and BChE, and feruperine (**18**) was the most potent selective inhibitor of BChE. Molecular docking simulation was performed to get insight into the binding interactions of the ligands and enzymes. In addition, *N*-trans-feruloyltyramine (**3**) contributed to the strongest DPPH radical scavenging activity. The self-induced A β aggregation inhibition of **2**, **5**, and **18** was further evaluated. Results indicated that some alkamides could be multifunctional lead candidates for Alzheimer's disease therapy.

Keywords: *Piper nigrum*; alkamides; anticholinesterase; antioxidant; A β_{1-42} aggregation inhibition

Experimental

General experimental procedures

Column chromatography was performed on silica gel (Qingdao Marine Chemical, Inc.) and Sephadex LH-20 (Amersham Biosciences). Electric eel AChE (Type-VI-S, EC 3.1.1.7), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8), 5, 5'-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide and galantamine were purchased from Sigma Aldrich (St. Louis, MO, USA). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent and thioflavin T were obtained from Changzheng Co. Ltd. (Chengdu, China). A β ₁₋₄₂ samples were purchased from GL Biochem. (Shanghai, China). All other chemicals and solvents used were of analytical grade.

UV spectra were taken in Rayleigh UV-2100 UV spectrophotometer. NMR spectra were recorded on Bruker Avance II-400 (400MHz for ¹H and 100MHz for ¹³C) spectrometer using CDCl₃ as solvent. HRESIMS spectra were taken in quadrupole time of flight (Q-TOF) premier spectrometer coupled with an ESI source (Micromass Co., Ltd.).

Plant material

Black pepper was purchased from a local herbal medicine store (Chengdu, Sichuan Province, China) in July, 2011. The plant was identified by Yanfang Li. A voucher specimen (D201101) was deposited at the Department of Pharmaceutics & Bioengineering, Sichuan University.

Extraction, fractionation and isolation

The dried seed of Black pepper (1.0 kg) were powdered and extracted three times with 95% ethanol (6 L, 3 days each) at room temperature. After filtration and evaporation under reduced pressure, the combined ethanol extract (yield, w/w: 115 g) was suspended in H₂O (1.8 L) and then successively partitioned with petroleum ether (PE, 3 × 1 L), EtOAc (3 × 1 L), and *n*-butanol (*n*-BuOH, 3 × 1 L) to afford PE, EtOAc, and *n*-BuOH extracts. Each extract was evaporated to dryness under reduced pressure to yield "PE ex." (yield, w/w: 32g), "EtOAc ex." (yield, w/w: 42.3 g), "*n*-BuOH ex." (yield, w/w: 17 g), and "H₂O ex." (yield, w/w: 18 g), respectively.

The EtOAc extract (42.3 g) was subjected to CC over silica gel (62 × 270 mm, 200–300 mesh, 250 g) using mixtures of PE and acetone with increasing polarity (v:v = 20: 1, 15: 1, 10:1, 8: 1, 5: 1, 2: 1, 1: 1, 1: 2, 1: 5, 0: 1, 3 L each) to give ten fractions (A-J) according to TLC patterns (detection at 254 and 365 nm).

Twenty-one compounds (**1-21**) were isolated from the EtOAc extract by repeated silica gel CC (PE:EA=20:1 to 5:1), Sephadex LH-20 CC (CH₂Cl₂: MeOH = 1:1), and pTLC (PE:EA=2:1, 1:1). Structure elucidation of all isolates was carried out using spectroscopic techniques: mass spectrometry (ESI-TOF-MS), ¹H and ¹³C NMR.

Determination of ChE inhibition activity

Evaluation of anti-ChE activity by samples was measured by a microplate assay based on Ellman's method (Ellman et al. 1961) with modification. In brief, 25 µL of 15 mM ATCI or butyrylthiocholine iodide in Millipore water, 125 µL of 3 mM DTNB in buffer C (50 mM Tris-HCl, pH 8.0; 0.1 M NaCl; 0.02 M MgCl₂·6H₂O), and 65 µL of buffer B (50 mM Tris-HCl, pH 8.0; 0.1% bovine serum albumin) were sequentially added to wells of 96-well plates. Subsequently, 10 µL of each test sample dissolved in methanol or galanthamine (as positive control) was added to the respective wells. Changes in the absorbance were measured eight times at 405 nm every 45 s with a Model 680 Microplate Reader (Bio-Rad Co., USA) to assess the spontaneous hydrolysis of the substrate. In the second stage, 25 µL of 0.226 U/mL AChE or BChE in buffer B was added to wells to initiate the reaction, and the plates were immediately shaken at medium speed for 15 s before the absorbance measurements were repeated under the same conditions as previously described. The reaction rate was calculated by Microplate Manager software version 5.2. Given the spontaneous hydrolysis of the substrate, the real enzyme rate was corrected by subtracting the reaction rate before the enzyme was added. Blanks were set up by adding 10 µL of methanol instead of the 10 µL sample solutions. All assays were performed in triplicate. The inhibitory activity against ChE (%) was calculated using the following equation:

$$\text{Inhibition rate (\%)} = (1 - V_{\text{sample}} / V_{\text{blank}}) \times 100\%$$

Where V_{sample} is the reaction rate of the test sample and V_{blank} is that of the blank.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Based on the scavenging activity of the stable DPPH free radical, the antioxidant activity of the isolated compounds was determined via the assay described by Blois (1958) with slight modification. In brief, 2 mL of the test samples with varying concentrations was added to the DPPH solution (2 mL, 1.5 mM) and dissolved in ethanol for a reaction mixture with a final volume of 4 mL. The DPPH solution was freshly prepared before the assay. After each reaction mixture was incubated for 30 min at room

temperature in the dark, the change in the absorption of the reaction mixtures was read at 517 nm. The standard antioxidant ascorbic acid was used as a positive control. A mixture of 2 mL of DPPH and 2 mL of ethanol was prepared as the blank. The errors caused by the absorbance of sample itself were notably corrected by subtracting the absorbance readings of the sample from the corresponding readings obtained in the presence of DPPH. The DPPH free radical scavenging activity (I%) was calculated as follows:

$$I\% = [1 - (A_{\text{mixture}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100\%$$

Where A_{mixture} is the absorbance of the reaction mixture, and A_{sample} is that of the sample in the absence of DPPH.

$A\beta_{1-42}$ aggregation inhibition activity assay

$A\beta_{1-42}$ samples were dissolved in DMSO to prepare a 1 mM stock solution, which was aliquoted into smaller samples and frozen at $-20\text{ }^{\circ}\text{C}$. Before use, the $A\beta_{1-42}$ stock solution was incubated in an ultrasonic bath for 1 min and diluted with a 10 mM phosphate buffer (pH 7.4) containing 10 mM NaCl. The experiments were performed by incubating the peptide at $37\text{ }^{\circ}\text{C}$ for 48 h (final $A\beta$ concentration of $50\text{ }\mu\text{M}$) with and without the test compounds at 50, 100, or $200\text{ }\mu\text{g/mL}$. The thioflavin T fluorescence method described by Shan et al. (2011) was used with slight modification to quantify the resulting $A\beta_{1-42}$ aggregation inhibition. After incubation, $80\text{ }\mu\text{L}$ of each above mentioned test solution was diluted to a final volume of $600\text{ }\mu\text{L}$ with a phosphate buffer containing $10\text{ }\mu\text{M}$ thioflavin T. Finally, the fluorescence intensity was measured after subtracting the background fluorescence of the $10\text{ }\mu\text{M}$ thioflavin T solution ($\lambda_{\text{exc}} = 446\text{ nm}$; $\lambda_{\text{em}} = 490\text{ nm}$; Cary Eclipse fluorometer; Varian, USA). Curcumin was used as the positive control. The percentage of inhibition on aggregation attributed to the presence of the plant extract was calculated with the following equation: $(1 - IF_i / IF_c) \times 100\%$, where IF_i and IF_c are the fluorescence intensities obtained for $A\beta_{1-42}$ in the presence and absence of the inhibitors, respectively.

Molecular docking

Molecular docking was performed using Autodock 4.0 along with AutoDockTools (ADT) (Tan et al. 2014). The structure of **5** and **18** were constructed by using ChemBio3D Ultra 12.0 and energy minimization was performed. Crystal structures of AChE from *Torpedo californica* (PDB ID: 1EVE) and BChE from *Homo sapiens* (PDB ID: 2WIJ) were obtained from Protein Data Bank, respectively. Both proteins were prepared by using ADT to remove water molecules, add hydrogens and assign Gasteiger

partial charges. A grid box of $44 \times 52 \times 50$ points for AChE and BChE was defined at the center of active site gorge. Ten independent dockings were carried out for each docking experiment. The lowest docked energy of each conformation in the most populated cluster was selected.

C log P calculation of alkamides and statistical analysis

The C log P values of all isolates from *P. nigrum* were estimated by ChemDraw Ultra 12.0 to evaluate the possibility of simple blood-brain barrier penetration.

All bioassays were measured in triplicate. The IC₅₀ values (the concentration providing 50% ChE or DPPH inhibition, in µg/mL) was calculated by the four-parameter logistic equation analysis of the GraphPad Prism software (version 5.0 for Windows). Data were expressed as the mean $\pm$ standard error (SEM). Differences between the sample groups and the reference compounds were assessed by one-way ANOVA. Dunnett's tests for multiple comparisons were used as post hoc tests, with $p < 0.05$ considered as significant.

References

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Table S1 Cholinesterase inhibitory and antioxidant activities of extracts and pure compounds of *Piper nigrum*^a.

Samples	% of inhibition at 100 µg/mL		IC ₅₀ Values (µg/mL)		Selectivity for BChE ^c	DPPH radical scavenging activity		CLogP
	AChE ^b	BChE	AChE	BChE		200 µg/mL	EC ₅₀ (µg/mL)	
95% EtOH	58.11 ± 3.10	86.12 ± 1.25 ^b	74.06 ± 7.74 ^b	17.80 ± 0.84	4.16	42.51 ± 2.02 ^b	259.93 ± 20.31 ^b	
PE	48.69 ± 1.43	80.74 ± 2.45 ^b	108.97 ± 7.58 ^b	26.26 ± 1.17 ^b	4.15	7.63 ± 0.12 ^b	>400	
EtOAc	61.50 ± 1.69	90.60 ± 0.92	71.64 ± 2.90 ^b	15.39 ± 1.12	4.65	62.81 ± 2.27 ^b	129.33 ± 10.99 ^b	
<i>n</i> -BuOH	38.65 ± 1.01	50.79 ± 1.03 ^b	139.87 ± 2.30 ^b	92.88 ± 6.45 ^b	1.51	75.24 ± 0.46 ^b	101.63 ± 1.87 ^b	
Water	5.64 ± 1.38	6.95 ± 2.55 ^b	>500	>500	/	42.48 ± 1.11 ^b	222.50 ± 9.14 ^b	
1	55.75 ± 1.42	71.35 ± 0.04 ^b	69.88 ± 1.11 ^b	36.19 ± 1.22 ^b	1.93	12.92 ± 1.11 ^b	NT	2.75
2	59.77 ± 1.24	76.88 ± 0.67 ^b	63.16 ± 1.09 ^b	25.11 ± 0.21 ^b	2.52	10.82 ± 1.60 ^b	NT	3.31
3	12.70 ± 1.07	33.07 ± 1.04 ^b	NT	NT	/	86.92 ± 0.50	11.82 ± 1.58	2.42
4	13.33 ± 1.37	7.24 ± 1.83 ^b	NT	NT	/	66.06 ± 5.24 ^b	108.62 ± 14.44 ^b	2.27
5	61.29 ± 2.46 ^d	59.46 ± 0.57 ^{b,d}	10.57 ± 1.23	14.89 ± 0.51	0.71	24.14 ± 3.49 ^b	NT	3.92
6	47.29 ± 0.92	47.96 ± 1.78 ^b	119.23 ± 2.56 ^b	107.66 ± 8.24 ^b	1.11	12.53 ± 2.06 ^b	NT	5.76
7	57.79 ± 0.47	29.10 ± 3.57 ^b	74.37 ± 3.08 ^b	>200	<0.37	13.53 ± 2.81 ^b	NT	6.82
8	19.93 ± 0.56	35.55 ± 2.00 ^b	NT	NT	/	13.61 ± 2.39 ^b	NT	4.15
9	26.55 ± 3.27	50.79 ± 2.21 ^b	NT	NT	/	10.82 ± 1.46 ^b	NT	5.21
10	7.62 ± 0.69	4.09 ± 0.86 ^b	NT	NT	/	NT ^g	NT	6.84
11	10.99 ± 0.82	34.60 ± 2.23 ^b	NT	NT	/	13.23 ± 2.54 ^b	NT	7.90
12	45.78 ± 1.27	56.63 ± 2.06 ^b	112.40 ± 0.80 ^b	74.90 ± 9.83 ^b	1.50	15.48 ± 3.35 ^b	NT	4.76
13	58.24 ± 1.77	58.13 ± 1.55 ^b	49.01 ± 2.96 ^b	53.20 ± 5.10 ^b	0.92	13.04 ± 2.54 ^b	NT	3.71
14	52.33 ± 2.34	65.97 ± 4.42 ^b	84.35 ± 6.75 ^b	21.43 ± 2.17	3.94	25.87 ± 6.25 ^b	NT	5.28
15	24.08 ± 1.09	47.98 ± 1.00 ^b	NT	NT	/	22.28 ± 5.79 ^b	NT	7.40
16	13.82 ± 0.61	32.53 ± 1.64 ^b	NT	NT	/	19.30 ± 1.58 ^b	NT	7.88
17	26.54 ± 2.20	44.75 ± 2.41 ^b	NT	NT	/	13.22 ± 1.39 ^b	NT	6.34
18	26.45 ± 0.57	85.76 ± 1.52 ^b	>200	12.88 ± 0.35	>15.53	42.84 ± 4.55 ^b	NT	2.53
19	27.58 ± 2.95	47.88 ± 2.25 ^b	NT	NT	/	21.85 ± 2.62 ^b	NT	4.30
20	61.51 ± 1.97 ^d	57.67 ± 1.07 ^{b,d}	11.80 ± 0.34	15.51 ± 0.29	0.76	29.68 ± 0.56 ^b	NT	3.36

21	47.90 ± 2.13	55.18 ± 0.61 ^b	104.21 ± 3.43 ^b	59.77 ± 1.37 ^b	1.74	8.67 ± 2.89 ^b	NT	4.20
Galanthamine ^c	98.89 ± 0.44	94.60 ± 0.83	0.52 ± 0.01	4.27 ± 0.25	0.12			
ascorbic acid ^f						96.61 ± 0.05	4.09 ± 0.10	

^a Data presented as Mean ± Standard error mean (SEM).

^b p<0.05, being regarded as significant compared to control.

^c Selectivity for BChE is defined as IC₅₀(AChE)/ IC₅₀(BChE).

^d Sample was tested at 20 µg/mL due to turbidity at high concentration in assay.

^e Standard drug for AD.

^f Standard antioxidant.

^g Amount of the compound **10** was not adequate to test.

NT, not tested.

Table S2 Binding interactions data for compounds **5** and **18** docked into active site gorge of *TcAChE* and *hBChE*.

Ligand	Enzyme	Binding energy (kcal)	Residue	Type of interaction	Distance(Å)	Ligand interacting moiety
5	<i>TcAChE</i>	-9.83	Ser 200	Hydrogen	2.06	O at MDP ring
			His 440	Hydrogen	2.20	O at MDP ring
			Phe 288	Hydrogen	1.88	O at C-1
	<i>hBChE</i>	-8.76	Ser 198	Hydrogen	1.71	O at C-1
			His 438	Hydrogen	1.98	O at C-1
			Tyr 332	Hydrophobic	/	Aromatic ring
18	<i>hBChE</i>	-8.66	Ser 198	Hydrogen	1.92	O at C-1
			His 438	Hydrogen	2.13	O at C-1
			Tyr 128	Hydrogen	1.75	OH at aromatic ring
20	<i>TcAChE</i>	-9.75	Ser 200	Hydrogen	1.86	O at MDP ring
			His 440	Hydrogen	2.14	O at MDP ring
			Phe 288	Hydrogen	1.76	O at C-1
	<i>hBChE</i>	-8.92	Trp 82	Hydrogen	2.72	O at MDP ring

Figure S1. Cholinesterase inhibitory activity of five different extracts of *Piper nigrum*.

Figure S2. $A\beta_{1-42}$ aggregation inhibition activity of three compounds (**2**, **5**, **18**) from *Piper nigrum*. The concentrations of compounds were 50, 100 and 200 $\mu\text{g/mL}$.

Figure S3. (A-E) Binding orientation and interaction of **5** and **18** with protein residue at the active site of AChE and BChE. A and B: docking between **5** and *Tc*AChE and *h*BChE, respectively; C: docking between **18** and *h*BChE. D and E: docking between **20** and *Tc*AChE and *h*BChE