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Chemical Composition and Acetylcholinesterase Inhibitory Activity of Essential Oils from *Piper* Species

Cai-Peng Xiang,[†,‡] Jia-Xin Han,[†,‡] Xing-Cong Li,[§] Yun-Hui Li,[∥] Yi Zhang,[∥] Lin Chen,[†] Yan Qu,[⊥] Chao-Yun Hao,[#] Hai-Zhou Li,[†] Chong-Ren Yang,[†,∥,⊥] San-Jun Zhao,[*,¶] and Min Xu[*,†]

[†]Center for Pharmaceutical Sciences, Faculty of Life Science and Technology, Kunming University of Science and Technology, Chenggong Campus, Kunming 650500, P. R. China

[¶]School of Life Sciences, Yunnan Normal University, Kunming 650500, P. R. China

[§]National Center for Natural Products Research, School of Pharmacy, University of Mississippi, Mississippi 38677, United States

[∥]Yunnan Engineering Research Center of Ethnopharmacy, Yunnan Provincial Academy of Science and Technology, Kunming 650101, P. R. China

[#]Chinese Academy of Tropical Agricultural Sciences, Hainan 100049, P. R. China

[⊥]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China

Supporting Information

ABSTRACT: The essential oils (EOs) derived from aromatic plants such as *Piper* species are considered to play a role in alleviating neuronal ailments that are associated with inhibition of acetylcholinesterase (AChE). The chemical compositions of 23 EOs prepared from 16 *Piper* spp. were analyzed by both gas chromatography with a flame ionization detector (GC-FID) and gas chromatography−mass spectrometry (GC-MS). A total of 76 compounds were identified in the EOs from the leaves and stems of 19 samples, while 30 compounds were detected in the EOs from the fruits of four samples. Sesquiterpenes and phenylpropanoids were found to be rich in these EOs, of which asaricin, caryophyllene, caryophyllene oxide, isospathulenol, (+)-spathulenol, and β-bisabolene are the major constituents. The EOs from the leaves and stems of *Piper austrosinense*, *P. puberulum*, *P. flaviflorum*, *P. betle*, and *P. hispidimervium* showed strong AChE inhibitory activity with IC_{50} values in the range of 1.51 to 13.9 mg/mL. A thin-layer chromatography (TLC) bioautography assay was employed to identify active compound(s) in the most active EO from *P. hispidimervium*. The active compound was isolated and identified as asaricin, which gave an IC_{50} value of 0.44 ± 0.02 mg/mL against AChE, comparable to galantamine with an IC_{50} 0.15 ± 0.01 mg/mL.

KEYWORDS: *essential oil, Piper, P. hispidimervium, asaricin, acetylcholinesterase inhibitory activity*

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disorder with grave concerns in the elderly population. About 44 million people worldwide are affected by AD or related dementia.[1] The disease is characterized by deposition of amyloid-β (Aβ) plaques and neurofibrillary tangles in the brain, accompanied by synaptic dysfunction and neurodegeneration.[2] This not only seriously impacts the health and quality of life of the patient but also brings considerable burden to the family as well as society due to the required long-term care and management.[3] It has been demonstrated that the neuropsychological impairments of AD are attributed, at least partially, to cholinergic disturbance.[4] Rivastigmine and galanthamine derived from natural products are the commonly prescribed cholinergic enhancers as acetylcholinesterase (AChE) inhibitors.[4]

Aromatic plants have been used to alleviate and cure neuronal ailments for many centuries by different cultures around the world.[5] The EOs and volatile compounds in such plants might be potential drugs for AD therapies.[5] Many species of the *Piper* plants are widely used as dietary spices in cuisine worldwide due to their delicious and unique taste. Some are used as functional foods due to their health-promoting effects.[6] For example, the fruit of *Piper nigrum*, namely, peppers, is one of the most important spicy crops in the world, and has gained the reputation of the "king of the species".[7] The leaf of *P. betle* is chewed by the local people in a wrapped package along with the areca nut.[8] The root or rhizome of *P. methysticum* is known as kava piper and is used as a beverage to relieve pain and reduce stress by the local people living in the South Pacific tropical islands.[9] Extensive phytochemical investigations on the *Piper* plants have been conducted since the 1960s. EOs are one of the major chemical constituents of *Piper* spp., of which monoterpenes, sesquiterpenes, and phenylpropanoids are predominant compounds (Supporting Information Table 1 and Figure 6). However, the yields and chemical compositions of the EOs have been found to vary significantly in the samples depending on species and collection conditions. The *Piper* EOs

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Table 1. Yields of the Essential Oils from *Piper* Species

no.	Latin name	parts	collectors	collecting locations	collecting time	yield (v/w, %)
YP-1	*P. austrosinense* Y. Q. Tseng	leaves and stems	Fang Xiao-Ming	Guangzhou	July 2014	0.12
YP-2	*P. sarmentosum* Roxb.	leaves and stems	Fang Xiao-Ming	Guangzhou	July 2014	0.19
YP-3	*P. hancei* Maxim.	leaves and stems	Fang Xiao-Ming	Guangzhou	July 2014	0.18
YP-4	*P. wallichii* DC.	leaves and stems	Hao Yun-Chao	Hainan	Jan., 2014	0.09
YP-5	*P. laetispicum* C. DC.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.61
YP-6	*P. austrosinense* Y. Q. Tseng	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.15
YP-7	*P. bavinum* C. DC.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.03
YP-8	*P. puberulum* (Benth.) Maxim.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.43
YP-9	*P. hancei* Maxim.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.40
YP-10	*P. betle* L.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.49
YP-11	*P. senporeiense* Yamam in Contrib Fl. Kainan.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.40
YP-12	*P. aduncum* L.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.39
YP-13	*P. hainanense* Hemsl.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.10
YP-14	*P. thomsonii* (C. DC.) Hook. f.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.04
YP-15	*P. boehmeriaefolium* (Miq.) C. DC.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.34
YP-16	*P. flaviflorum* C. DC.	leaves and stems	Hao Yun-Chao	Hainan	July 2014	0.37
YP-17	*P. nigrum* L.	leaves and stems	Liu Jin-Ping	Hainan	July 2014	0.07
YP-18	*P. betle* L.	leaves and stems	Liu Jin-Ping	Hainan	May 2012	0.07
YP-19	*P. hispidimervium* C. DC.	leaves and stems	Xu Bing-Qiang	Guangzhou	May 2012	0.29
YP-20	*P. nigrum* L.	black piper (fruits)	Yang Chong-Ren	Yunnan	June 2016	1.28
YP-21	*P. nigrum* L.	red piper (fruits)	Yang Chong-Ren	Cambodia	June 2016	1.43
YP-22	*P. nigrum* L.	black piper (fruits)	Yang Chong-Ren	Cambodia	June 2016	1.34
YP-23	*P. nigrum* L.	white piper (fruits)	Yang Chong-Ren	Cambodia	June 2016	1.23

have been used in pharmaceutical and nutraceutical industries[10] due to their antifungal,[11] antibacterial,[12] amebicidal,[13] larvicidal,[14] antioxidant,[15] antinociceptive, and anti-inflammatory activities.[16] In the present work, we prepared 23 EOs from 16 *Piper* species by the direct distillation extraction method (DDE). We analyzed their chemical compositions by GC-MS and tested their AChE inhibitory activity. The active compound in the EO of *P. hispidimervium* was detected by a thin-layer chromatography (TLC) bioautography assay, and subsequent isolation and structural characterization led to the identification of asaricin, which showed strong AChE inhibitory activity.

MATERIALS AND METHODS

Chemicals. Acetylthiocholine iodide (ATCI) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Adamas Reagent Co., Ltd.. Fast Blue B salt, 1-naphthyl acetate, and acetylcholinesterase (from electric eel (EC 3:1.1.7), Sigma C 2888; 1000 U) were purchased from Sigma. Galanthamine was purchased from Shanghai Mindray Chemical Technology Co., Ltd., purity >98% by HPLC. Deionized water was used in the bioassay.

General Procedures. TLC was carried on precoated 0.2−0.25 mm thick silica gel GF254 plates (Qingdao Haiyang Chemical Co., Qingdao, China). Spots were detected by spraying with 10% H_2SO_4−EtOH reagent followed by heating. 1D NMR spectra were recorded in $CDCl_3$ with Bruker AM-800 spectrometers operating at 800 MHz for ^1H, and at 200 for ^{13}C, respectively. Coupling constants were expressed in hertz, and chemical shifts were given on a ppm scale with tetramethylsilane as internal standard. ESIMS were measured with Agilent 1260-6530-Q-TOF. The acetylcholinesterase inhibitory assay was performed in 96-well microplates, and absorbances were measured by using an Emax precision microplate reader.

Plant Materials. The leaves and stems of *Piper* spp. (YP1−19) were collected from Hainan and Guangzhou provinces, People's Republic of China, and identified by Dr. Chao-Yun Hao at Chinese Academy of Tropical Agricultural Sciences, People's Republic of China. Black pepper (YP20) was collected from Lvchun county, Yunnan province, People's Republic of China, and was identified by Dr. Yuan-Xue Lu at Kunming Institute of Botany, Chinese Academy of Science. Black, white, and red peppers (the fruits of *P. nigrum*, YP21−23) were purchased from Cambodia and identified by Prof. Chong-Ren Yang at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Science. Voucher specimens were deposited at the Center for Drug Discovery & Technology Development of Yunnan Traditional Medicine, Kunming, Yunnan, China. Detailed information on all the aforementioned samples is shown in Table 1.

Extraction of EOs. A Dean−Stark apparatus and a thermostatic heating magnetic stirrer (Gongyi Yuhua Instrument Co., Ltd.) were used for extraction of EOs. To a round-bottom flask was added the dried ground *Piper* material (20.0 g) and distilled water (500 mL). After standing alone overnight the mixture was refluxed for 6 h in the essential oil extractor (keeping microboiling). The refluxing liquid was collected and extracted 3 times with ether, then, dried over anhydrous sodium sulfate, and evaporated to dryness. The dried EO was dissolved in constant volume with *n*-hexane in a 5 mL volumetric flask.

Gas Chromatography (GC-FID) Analysis of EOs. The EOs were filtered by a 0.45 μm filter membrane and subjected to GC spectroscopy for analysis using an Agilent Technologies 7890B GC apparatus equipped with a FID detector and DB-17 MS capillary column (30 m × 0.320 mm; film thickness, 0.25 μm). A sample of

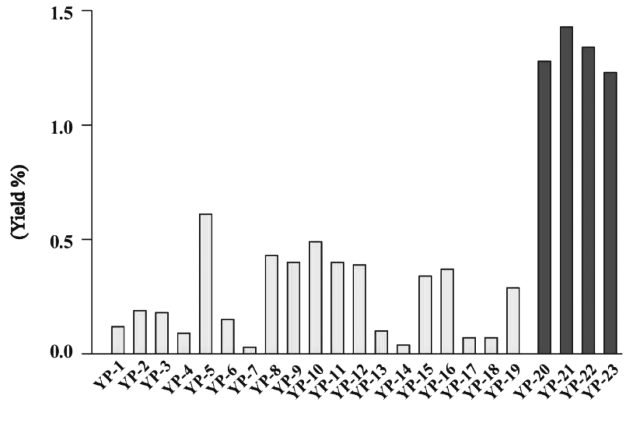

Figure 1. Yields of *Piper* species EOs.

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Table 2. Composition of the EOs of from the Leaf and Stem of *Piper* Species

	composition[a] (%)																		
compds	YP-1	YP-2	YP-3	YP-19	YP-4	YP-5	YP-6	YP-7	YP-8	YP-9	YP-10	YP-11	YP-12	YP-13	YP-14	YP-15	YP-16	YP-17	YP-18	ID[b]
1-menthol															1.84			1.61	0.94	MS
(*E*,*Z*)-1,3-cyclododecadiene						2.01														MS
linalool																	0.87			MS
(+)-spathulenol	6.28			0.57	18.8	2.48	2.02	8.04		7.78	8.00	7.72	8.32	3.76			4.59	6.22		MS
t-cadinol	6.78						2.62													MS
δ-cadinol	23.9						8.14	2.49			9.32	3.52				1.83			1.50	MS
a-cadinol	0.62							2.71			5.62	4.38			2.23			1.53		MS
elemol			0.98	0.64	1.57					2.22							0.77			MS
hinesol					1.68															MS
viridiflorol	2.60					0.98	1.42													MS
γ-eudesmol					1.54															MS
isospathulenol					3.12															MS
α-eudesmol				2.04	5.82					6.94							2.47			MS
β- eudesmol				0.36	6.01			1.67		6.84							0.87			MS
nerolidol										1.94										MS
guaiac alcohol										2.01							4.64			MS
eudesm-7(11)-en-4-ol										3.19										MS
t-muurolol											2.67	1.97			1.79		0.99			MS
caryophyllenol II																			3.06	MS
bulnesol																	3.57			MS
α-bisabolol																17.6			1.17	MS
(−)-glogulol						3.78		1.95												MS
δ-cadinene	1.68		0.87	1.80		1.26	8.13			2.02	3.15	4.66				3.81	1.87	1.17	2.91	MS
γ-cadinene							2.34													MS
caryophyllene oxide	5.78	1.40	1.13	1.68				7.78		1.99	2.71	2.14	1.65	1.81	21.6		1.45	6.00	8.78	MS
cadina-1,4-diene																				MS
isocaryophillene		2.43																		MS
β-caryophyllene		1.94	1.76	4.95		8.79	2.15					1.23	1.68				5.46	13.8		MS
α-caryophyllene				0.60		9.62				2.66								1.53		MS
germacrene d						5.82	2.08						5.76							MS
β-elemene						4.62							3.34							MS
α-gurjunene						4.69														MS
β-selinene						23.8				7.99			2.23					1.37		MS
α-selinene						19.1				7.14						2.81		1.33		MS
β-bisabolene																1.48				MS
γ-muurolene							4.27				1.72	1.90							1.41	MS
α-muurolene							1.41													MS
bicyclogermacrene													3.87				2.15			MS
cis-*δ*-bisabolene																1.10				MS
α-copaene																	2.33			MS
12-oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-																			2.91	MS

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Table 2. continued

compds	composition[a] (%)																			ID[b]
	YP-1	YP-2	YP-3	YP-19	YP-4	YP-5	YP-6	YP-7	YP-8	YP-9	YP-10	YP-11	YP-12	YP-13	YP-14	YP-15	YP-16	YP-17	YP-18	
tetramethyl-[1*R*-(1*R*,3*E*,7*E*,11*R*)]-																				
1,6-dimethyl-4-(1-methylethyl)naphthalene	4.92														7.35			3.26		MS
3,4-dimethyl-3-cyclohexen-1-carboxaldehyde	2.18																			MS
6,10,14-trimethyl-2-pentadecanone	2.42		0.76												1.39			1.13	1.57	MS
1-propanone, 1-(1,3-benzodioxol-5-yl)-					8.58		12.5	7.83	23.4	12.2	5.75	21.1	7.14	5.64						MS
2,6-di-*tert*-butylbenzoquinone	1.27						1.27													MS
tetradecanoic acid	1.64																			MS
hexadecanoic acid	3.47	8.20	2.45												1.92			1.96	0.75	MS
nonanoic acid															2.10					MS
decanoic acid															3.71					MS
benzenepropanoic acid		2.00	0.83																	MS
1-octadecanol															1.94				1.06	MS
3,7,11-trimethyl-1,6,10-dodecatriene-3-ol		1.14	0.98	3.33						1.98						10.4	48.4	1.72		MS
trans-2-methoxy-4-propenylphenol		1.05				0.98					10.3	1.65	1.72	1.79					44.8	MS
methyleugenol		1.06	1.35	1.21					0.90				3.79	0.92					0.89	MS
4-allyl-2-methoxyphenol							1.12		0.73		1.21	1.53	29.1	4.32						MS
cis-methyleugenol			1.06																	MS
trans-methyl-isoeugenol									2.35											MS
2,4,6-trimethyl-benzenepropanol								26.9	0.77											MS
asaricin		54.5	65.9	64.6			4.38		2.59											MS
methyl salicylate			0.68		0.73										9.19					MS
benzyl benzoate													1.96							MS
methyl *trans*-cinnamate																40.74				MS
elemicin		3.24	4.08	3.01			1.65		2.64											MS
cis-isoelemicin			3.49	0.57	4.90		6.16	3.00	10.1	1.57	3.20	4.38		1.49						MS
cis-asarone		0.80		1.10	2.34		4.68	2.39	11.5	2.24	3.35	5.09		1.48						MS
asarone		2.69	1.71	5.03	1.63		3.20		28.5	1.78	1.76	3.62								MS
apiol							4.42	2.93	2.68											MS
trans-phytol					1.27		0.65		0.43	0.59	4.11	0.74	1.30							MS
phytol	4.85	2.91	1.20		7.38				1.55	3.06					2.65			1.73	1.92	MS
2-benzothiazolone		1.40	1.00																	
6-ethoxyquinaldine							1.40				10.28									MS
N-TFA-4-hydroxy-benzeneethanamine								2.81												MS
1*H*-imidazole,1-[(2,4,6-trimethylphenyl)methyl]-															11.0					MS

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Table 2. continued

compds	composition[a] (%)																	ID[b]
	YP-1	YP-2	YP-3	YP-19	YP-4	YP-5	YP-6	YP-7	YP-8	YP-9	YP-10	YP-11	YP-12	YP-13	YP-14	YP-15	YP-16	YP-17	YP-18	
2-propenamide, 3-phenyl-*N*-2-propenyl-																			8.53	MS
diphenylamine	2.09	1.05	0.89		1.72			2.27							3.02				2.06	MS
monoterpenoids						2.01									1.84		0.87	1.61	0.94	
sesqueterpenoids.	52.6	5.77	4.73	12.6	38.5	85.0	34.6	24.6		52.7	33.2	27.5	26.8	5.56	32.9	28.6	31.2	36.2	21.7	
aldehyde	2.18																			
ketones	3.70		0.76		8.58		13.8	7.83	23.4	12.2	5.75	21.1	7.14	5.64	1.39			1.13	1.57	
fatty acid	5.11	10.20	3.28												7.72			1.96	0.75	
alcohols		1.14	0.98	3.33											1.94	10.4	48.4	1.72	1.06	
phenylpropanoids		56.6	68.3	65.8		0.98	5.50	26.9	7.33		1.21	3.18	34.6	7.02					45.6	
esters			0.68		0.73								1.96		9.18	40.7				
ethers		6.73	9.28	9.71	8.86		20.1	8.32	55.5	5.59	8.31	13.1		2.96						
phytols	4.85	2.91	1.20		8.6		0.65		1.98	3.65	4.11	0.74	1.30		2.65			1.73	1.90	
nitrogenous compounds	2.09	2.44	1.89		1.72		1.40	5.08			10.3				14.0				10.6	
total	70.5	85.8	91.1	91.5	67.1	88.0	76.0	72.8	88.2	74.2	62.8	65.6	71.9	21.2	71.6	79.3	81.3	44.3	84.2	

[a]Percent calculated from MS data.. [b]Identification method: MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and Mass Finder libraries and comparison with literature data.

0.8 μL of essential oil was injected manually, and the GC split ratio used was 10:1. Helium was the carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were both set at 250 °C. The injection port was operated in a splitless mode, and the oven temperature was programmed as follows: 90 °C for 2 min, raised to 140 °C at a rate of 5 °C/min, held isothermal for 2 min, then from 140 to 200 °C for 5 min, at a rate of 1 °C/min, and finally raised to 260 °C at 4 °C/min for 1 min.

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis of EOs. The EOs were filtered by a 0.45 μm filter membrane and subjected to gas chromatography−mass spectrometry (GC-MS) for analysis using a DB-17 MS capillary column (30 m × 0.320 mm; film thickness, 0.25 μm). A sample of 0.8 μL of EOs was injected manually, and the GC split ratio used was 10:1. Helium was the carrier gas at a flow rate of 1 mL/min. The mass-selective detector was operated in an electron-impact ionization (EI) mode with a mass scan range from m/z 50 to 550 at 70 eV. Injector and MS transfer line temperatures were both set at 250 °C. The oven temperature was programmed as in the GC-FID analysis.

Acetylcholinesterase Inhibition Assay. The acetylcholinesterase (AChE) inhibitory activity was tested by a modified Ellman's method.[17] PBS buffer solution was prepared by dissolving 3.121 g of $NaH_2PO_4{\cdot}2H_2O$ in 100 mL of distilled H_2O to obtain 0.2 mol/L NaH_2PO_4 solution. We dissolved 71.64 g of $Na_2HPO_4{\cdot}12H_2O$ in 1000 mL of distilled H_2O to get 0.2 mol/L Na_2HPO_4 solution. We added 26.5 mL of 0.2 mol/L NaH_2PO_4 and 473.5 mL of 0.2 mol/L Na_2HPO_4 in 1000 mL of distilled H_2O to obtain 0.1 mol/L PBS buffer solution of pH 8.0. Then, we added 19.5 mL of 0.2 mol/L NaH_2PO_4 and 30.5 of mL 0.2 mol/L Na_2HPO_4 in 100 mL of distilled H_2O to get 0.1 mol/L PBS buffer solution of pH 7.0. AChE solution was prepared by dissolving acetylcholinesterase in PBS buffer solution of pH 8.0 to get 1000 U/mL AChE solution. The solution was diluted into 4 U/mL and kept at −40 °C. The solution was diluted into 0.4 U/mL when tested. ATCI solution (0.6 mmol/L) was prepared by dissolving 8.8 mg of ATCI in 50 mL of distilled H_2O. The solution is always made immediately before use and kept at 4 °C. DTNB solution (0.6 mmol/L) was prepared by dissolving 11.9 mg of DTNB and 0.75 g of $NaHCO_3$ in a 50 mL PBS buffer of pH 7.0 solution. The solution is always made immediately before use and kept in a dark place. The EOs were dissolved in MeOH. The experiment was carried out on 96-well cell culture plates, on which it included these groups: a blank control, a blank control, a blank, a test, and a test control. Each group processed 3 wells. In the test group, 40 μL of PBS buffer pH 8.0, 10 μL of essential oil, 10 μL of 0.4 U/mL AChE, and 20 μL of 0.6 mmol/L DTNB were placed in 96-well microplates and incubated for 10 min at 37 °C. Then 20 μL of 0.6 mmol/L ATCI was added into the reaction mixture and incubated for 30 min. Finally, 50 μL of anhydrous ethanol was added into the 96-well microplates to stop reaction. In the test control group, 10 μL of buffer pH 8.0 was used instead of 10 μL of 0.4 U/mL AChE. In the blank group, 10 μL of MeOH was used to replace 10 μL of test EO. In the blank control group, 10 μL of MeOH and 10 μL of buffer pH 8.0 were used to replace 10 μL of test EO and 10 μL of 0.4 U/mL AChE, respectively. Galanthamine was used as a positive control. AChE activity was evaluated by measuring the absorbance at 412 nm using a microplate absorbance reader (Tecan-Infinite 200 PRO, Austria), and the percentage inhibition was calculated according to the following formula:

$$\begin{aligned}&\text{inhibition activity (\%)}\\&= \{[(\text{blank group} - \text{blank control group})\\&\quad - (\text{test group} - \text{test control group})]/[\text{blank group}\\&\quad - \text{blank control group}]\} \times 100\%\end{aligned}$$

Statistical Analysis. All experiments were carried out in triplicate. Data were expressed as means ± standard deviation (SD). The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression using Excel 2007 for Windows (Microsoft Office Software, 2007, USA).

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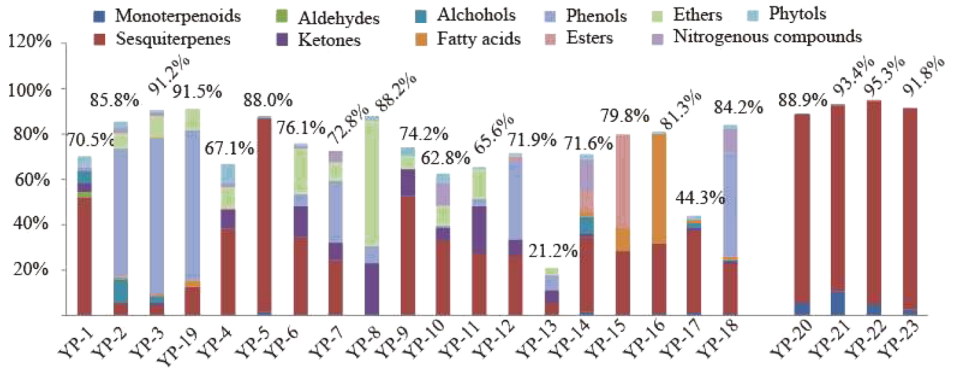

Figure 2. Chemical constituents of EOs of *Piper* species.

TLC-Bioautography Assay. Acetylcholinesterase was dissolved in 150 mL of 0.05 M Tris-HCl 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. The experiment was done by a modified Marston's method.[18] The plant-derived alkaloid galanthamine was used as the positive control. TLC is using petroleum ether/acetone (3:1, v/v).

Isolation of Asaricin from the EO of YP19. The active compound in the EO of YP19 (10 mg) was purified by preparative TLC using petroleum ether/acetone (3:1, v/v). The compound had an R_f value of 0.87 and was identified as arasicin (>98%, 3 mg) by the NMR and MS shown below. ^{1}H NMR (800 MHz, $CDCl_3$): δ 6.65 (1H, s, H-2), 6.52 (1H, s, H-5), 5.91−5.93 (1H, ddt, J = 16.8, 10.2, 6.6 Hz, H-2′), 5.89 (2H, s, H-3a), 5.02 (1H, dd, J = 10.2, 1.7, H-3′a), 5.00 (1H, dd, J = 16.8, 1.7, H-3′b), 3.76 (3H, s, −OCH_3), 3.29 (2H, d, J = 6.6 Hz, H-1′). ^{13}C NMR (200 MHz, $CDCl_3$): δ 152.1 (C-6), 146.3 (C-3), 140.9 (C-4), 137.2 (C-2′), 120.1 (C-1), 115.2 (C-3′), 109.7 (C-2), 100.9 (C-3a), 94.9 (C-5), 56.6 (−OMe), 34.0 (C-1′). ESIMS: m/z 215 $[M + Na]^+$ $C_{11}H_{12}O_3Na$.

RESULTS AND DISCUSSION

The Yields of the *Piper* EOs. The yields of 23 EOs are shown in Figure 1 and Table 1. The results indicated that the yields of the EOs in the *Piper* samples were different due to the plant species, location, and collection dates. For example, the yield of *P. hancei* growing in Guanzhou province was 0.18%, approximately two times less than that from Hainan province (0.40%). The fruit of *P. nigram* (red pepper, black pepper, and white pepper) produced EOs about 20 times higher than its leaf and stem. Among them, the yield of the red pepper from Cambodia is the highest one (1.43%)

The Chemical Compositions of the EOs from the Leaves and Stems of *Piper* Samples. Table 2 and Figure 2 show the chemical compositions of 19 *Piper* EOs from 16 different *Piper* species collected in China. Among them, the chemical constituents of the EOs from seven *Piper* species have been reported, which are *P. sarmentosum* (YP-2), *P. hancei* (YP-3 and -9), *P. betle* (YP-10 and -18), *P. aduncum* (YP-12), *P. boehmeriaefolium* (YP-15), *P. flaviflorum* (YP-16), and *P. nigrum* (YP-17) (Supporting Information Figure 7 and Table 1). Herein, 76 volatile compounds were identified from the 19 EOs, including 39 sesquiterpenes, three monoterpenes, one aldehyde, three ketones, five fatty acids, seven phenylpropanoids, two alcohols, three esters, five ethers, six nitrogenous compounds, and two phytols.

Except for *P. puberulum* (YP-8), the other 18 EOs contained sesquiterpenes. Among them, the total contents of the sesquiterpenes in YP-1 (*P. austrosinense*, 52.6%), YP-5 (*P. laetispicum*, 85.0%), YP-9 (*P. hancei*, 52.7%), and *P. nigrum* (YP-17, 36.2%) are higher. The high amount of sesquiterpenes was checked previously in the EOs from leaf and stem of *P. nigrum*.[19] The samples containing high contents of phenylpropanoids include YP-2 (*P. sarmentosum*, 56.6%), YP-3 (*P. hancei*, 68.3%), YP-18 (*P. betle*, 45.6%), and YP-19 (*P. hispidimervium*, 65.8%). The aforementioned data supported that EOs of *P. sarmentosum*[20] and *P. hancei*[21] have been characterized mainly by aromatic compounds and devoid of monoterpenes. However, the previous publication suggested that the major constituents of EO of *P. betle* are primarily sesquiterpenes.[22] EO of YP-12 (*P. aduncum*) is made up predominantly by phenylpropanoids (34.6%) and sesquiterpenes (26.8%). It was previously reported that EO of *P. aduncum* contained high content of monoterpenes[23,24] except for a high percentage of phenylpropanoids[25,26] and sesquiterpenes.[23,27,28] YP-15 (*P. boehmeriaefolium*) and YP-16 (*P. flaviflorum*) were found to have the highest esters (40.7%) and alcohols (48.4%), respectively. However, the previous publication suggested that *P. flaviflorum*[29] was made up predominantly by sesquiterpenes and aromatic compounds.[30]

Furthermore, our results showed that some bioactive volatile constituents are present in some *Piper* EOs in high contents. For example, asaricin was detected in YP-2 (54.5%, *P. sarmentosum*), YP-3 (65.9%, *P. hancei*), and YP-19 (64.6%, *P. hispidimervium*). This compound was previously reported to show larvicidal[31] and antifungal[32] activities. 1-(1,3-Benzodioxol-5-yl)-1-propanone with antifungal properties[33] existed in YP-6 (12.6%, *P. austrosinense*), YP-8 (23.4%, *P. puberulum*), YP-9 (12.2%, *P. hancei*), and YP-11 (21.1%, *P. senporeiense*).

Compared with previous reports, the chemical constituents of EOs of *Piper* species were increased and diversified. The variation in EO constituents may depend on the light conditions and is related to phenotypic and genetic factors. The aforementioned data provided chemical confirmation to the EOs from some leaves and stems of *Piper* spp. being good candidates to develop as good agents for pharmaceutical, nutraceutical, and food industries.

The Chemical Compositions of the EOs from the Fruits of *Piper* Samples. Table 3 shows the chemical compositions of the EOs from four pepper samples (the fruits of *P. nigrum*). Eighteen compounds, including eight monoterpenes (MS), nine sesquiterpenes (SS), and one ester (ES), were identified in the EOs of black pepper collected in Yunnan. Twenty-five (15 MS, 9 SS, and 1 ES), 22 (10 MS, 11 SS, and 1 ES), and 15 (4 MS, 10 SS, and 1 ES) chemical compositions were identified in the EOs of the black, red, and white peppers from Cambodia, respectively. The aforementioned results (Figure 2) showed that the chemical compositions of the EOs of different pepper were similar including only three classes of

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Table 3. Composition of the EOs of from the Fruit of *P. nigrum* (Pepper)

compds	composition[a] (%) YP-20	YP-21	YP-22	YP-23	ID[b]
3-carene	0.67	1.37	0.20	0.28	MS
D-limonene	0.61	0.77	0.23	0.30	MS
β-pinene		0.23	0.03		MS
β-myrcene		0.10			MS
α-phellandrene	0.78	1.24			MS
p-cymene	0.07	0.19	0.06		MS
γ-terpinene		0.01			MS
α-terpinolene [1-methyl-4-(1-methylethylidene)cyclohexene]		0.03			MS
1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-cyclohexane	1.84	4.27	3.07	1.52	MS
linalool		0.06	0.07		MS
levomenthol		0.02	0.03		MS
α-terpineol	0.04	0.02			MS
(1S,3S,6R)-(−)-4-carene		0.18	0.03		MS
α-guaiene	0.95	2.16	1.31		MS
heptane, 2,2,4,6,6-pentamethyl	0.67		0.23	0.70	MS
(1S)-2,6,6-trimethylbicyclo[3.1.1] hept-2-ene		0.10			MS
copaene	0.71		1.37	4.62	MS
caryophyllene	42.0	55.2	51.8	58.9	MS
humulene	3.96	6.29	5.45	3.86	MS
guaia-9,11-diene				2.14	MS
β-bisabolene	2.10	3.25	3.48	0.61	MS
caryophyllene oxide	13.5	5.09	4.69	1.74	MS
isospathulenol	4.86	1.36	3.40	7.56	MS
4-ethenyl-4-methy-3-(1-methylethenyl)-1-(methylethyl)-, (3R-*trans*)-cyclohexene		3.44	3.12		MS
pogostol	1.03	1.21	1.68		MS
decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4aα,7α,8aβ)]	5.10		7.97	1.50	MS
1,2,4a,5,8,8a-hexahyro-4,7-dimethyl-1-(1-methylethyl)- , [1S-(1α,4aβ,8aα)]				5.59	MS
decahydro-4a-methylene-7-(1-methylethylidene)-, (4aR-*trans*)		5.60	6.20	2.21	MS
10,10-dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5β-ol	9.98	1.23	1.01		MS
methyl salicylate		0.02	0.03	0.20	MS
					MS
monoterpenoids	5.62	10.7	5.26	2.80	MS
sesquiterpenes	83.3	82.7	90.0	88.7	MS
ester		0.02	0.03	0.20	MS
total	88.9	93.4	95.3	91.8	MS

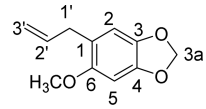
[a]Percent calculated from MS data.. [b]Identification method: MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and Mass Finder libraries and comparison with literature data.

compounds. Monoterpenes had lower contents (less than 10%) than those of sesquiterpenes, while they showed more plentiful structural diversity than sesquiterpenes. The total contents of sesquiterpenes were more than 80%, especially black pepper from Cambodia with 90.0% contents of sesquiterpenes. Among them, β-caryophyllene was the major sesquiterpene with more than 50% contents from Cambodia and 42% contents from Yunnan, China. β-Caryophyllene is approved by United States Food and Drug Administration and European agencies as food additive, taste enhancer, and flavoring agent and termed as a phytocannabinoid. It has been reported that β-caryophyllene

Table 4. Acetylcholinesterase Inhibitory Activity of the *Piper* EOs[a]

	inhibition (%)	IC_{50} (mg/mL)
YP-1	147 ± 9.2	NT
YP-3	89.0 ± 3.5	NT
YP-4	93.0 ± 4.6	NT
YP-5	130 ± 3.5	NT
YP-6	102 ± 3.0	12.4 ± 0.13
YP-8	148 ± 9.6	4.47 ± 0.37
YP-10	124 ± 3.5	NT
YP-11	97.7 ± 4.6	NT
YP-13	103 ± 3.5	NT
YP-14	NA	NA
YP-15	94.9 ± 4.5	NT
YP-16	96.1 ± 3.7	13.9 ± 1.85
YP-17	128 ± 4.5	NT
YP-18	108 ± 1.9	14.0 ± 0.01
YP-19	95.4 ± 4.6	1.51 ± 0.05
asaricin	NT	0.44 ± 0.02
galanthamine	104 ± 2.9	0.15 ± 0.01

[a]Concentration 100 mg/mL. Data are expressed as mean ± SD (n = 3). NT: not tested. NA: not active.



Asaricin

Figure 3. Structure of asaricin.

has agonist action on the cannabinoid 2 (CB2) receptor[34] and peroxisome proliferated activator receptor (PPAR) isoforms.[35] Additionally, β-caryophyllene was an antagonist of homomeric nicotinic acetylcholine receptors (α7-nAChRs).[35] Moreover, it also alters the gene expression or signaling pathways or works through direct interaction to modulate numerous molecular targets.[35] So β-caryophyllene showed polypharmacological properties such as cardioprotective,[36] neuroprotective,[37] and immune-modulative effects.[35] In addition, the contents of caryophyllene oxide were higher than those of the other compositions, especially in the black pepper from Yunnan with 13.5%, which might be the typical chemical composition of EOs in the black pepper from Yunnan. Caryophyllene oxide has been reported to show anticancer,[38] acaricidal,[39] analgesic,[40] anti-inflammatory,[40] and antifungal activities.[41] The previous results suggested that high concentrations of α-thujene (1.07%), β-pinene (6.04%), 3-carene (10.75%), limonene (9.22%), and caryophyllene (34.23%)[7] were observed in the EO of white pepper, while β-caryophyllene (29.9%), piperine (39.0−63.9%), limonene (35.06%), and germacrene (11.01%) were the major components in the EO of black pepper.[42] Our results of chemical investigations were slightly different from the previously reported work on black and white pepper. The differences in components may be due to geographical factors, post crop processing, and different nutritional status of the plants.[43]

The Comparison of Chemical Compositions of the EOs from the *Piper* Samples. Compared with the leaves and stems of *Piper* spp., the chemical compositions of the EOs from the fruits of *Piper* spp. were totally different. Moreover, it is noted that the structural diversity of EOs from fruits was less abundant than that from the leaves and stems. The chemical compositions of leaves and stems of *Piper* showed marked

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structural diversities which included 11 classes of compounds, while only three classes of compounds were found in the fruits of *Piper*. Though the chemical compositions from leaves and stems of *Piper* were totally different from those of the fruits, the sesquiterpenes were the main volatile compounds in leaves and stems of *Piper* spp., as well as in fruits of *Piper* spp. Among them, *β*-caryophyllene (sesquiterpene) was the major volatile compound in the fruits [YP-20 (42.0%), YP-21 (55.2%), YP-22 (51.7%), and YP-23 (58.9%)], while it was low in leaves and stems of *Piper* spp. Although monoterpenes were detected in both fruits and leaves and stems, the diversity of monoterpenes in leaves and stems was less than that in fruits.

Acetylcholinesterase Inhibitory Activities of the EOs. The acetylcholinesterase (AChE) inhibitory bioassay was utilized to identify cholinergic compounds for the potential treatment of Alzheimer's disease (AD). Fifteen *Piper* EOs (YP-1, YP-3–YP-6, YP-8, YP-10, YP-11, and YP-13–YP-19) were tested for AChE inhibitory activities by a modified Ellman's method. The results indicated that most of the EOs showed strong activities at the concentration of 100 mg/mL except for YP-14 (Table 4). The EOs of YP-6, YP-8, YP-16, YP-18, and YP-19 have significant inhibitory activities with IC_{50} values of 12.4 ± 0.13, 4.47 ± 0.37, 13.9 ± 1.85, 14.0 ± 0.01, and 1.51 ± 0.05 mg/mL, respectively. However, the IC_{50} values of other EOs were not tested due to the limited amounts. A TLC-bioautography assay was employed to guide the isolation of active compound(s) in YP-19 (see Supporting Information Figure 1). Asaricin (Figure 3) was isolated and identified to be the active compound with an IC_{50} value of 0.44 ± 0.02 mg/mL. The results indicated that EOs from the *Piper* species are an important resource for exploring AChE inhibitors for AD therapy.

In summary, the differences for the chemical compositions of the *Piper* EOs have been demonstrated in the present study. Sesquiterpenes and phenylpropanoids were found to be rich in these EOs, of which caryophyllene and asaricin are the major constituents. Caryophyllene had an extremely high content in the four EOs from the *Piper* fruits (>40%). The three EOs from the stems and leaves of *P. sarmentosum* (YP-2), *P. hancei* (YP-3), and *P. hispidimervium* (YP-19) were made up predominantly by asaricin (>60%). The examination of the *Piper* EOs against AChE for the first time has led to the identification of asaricin, which showed potent inhibitory activity. This study suggests that the *Piper* EOs may be a good natural product source for compounds with therapeutic potential for AD or other CNS diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b01350.

TLC bioautography, ^{1}H and ^{13}C NMR, MS, GC-FID, and GC-MS, and GC spectra, and short review on chemical compositions of EOs of *Piper* (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Phone: +86-0871-65920570. Fax: +86-0871-65920570. E-mail: xumin8121@hotmail.com.

*Phone: +86-0871-65920570. Fax: +86-0871-65920570. E-mail: sanjunzhao@hotmail.com.

ORCID

Min Xu: 0000-0003-0318-9040

Author Contributions

‡C.-P.X. and J.-X.H. contributed equally to this work. M.X. and S.-J.Z. designed research; C.-P.X. and J.-X.H. performed experiments; M.X., S.-J.Z., and X.-C.L. analyzed data and wrote the paper. All authors revised and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GC-FID, gas chromatography with a flame ionization detector; GC-MS, gas chromatography–mass spectrometry; AChE, acetylcholinesterase; AD, Alzheimer's disease; TLC, thin-layer chromatography; EI, electron-impact ionization; DDE, distillation extraction method; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ATCI, acetylthiocholine iodide

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