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Research Article

Anticholinesterase and Antityrosinase Activities of Ten *Piper* Species from Malaysia

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Abstract

Purpose: The aim of this study was to investigate acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and antityrosinase activities of extracts from ten *Piper* species namely; *P. caninum*, *P. lanatum*, *P. abbreviatum*, *P. aborescens*, *P. porphyrophyllum*, *P. erecticaule*, *P. ribesioides*, *P. miniatum*, *P. stylosum*, and *P. majusculum*.

Methods: Anticholinesterase and antityrosinase activities were evaluated against *in vitro* Ellman spectroscopy method and mushroom tyrosinase, respectively.

Results: The EtOAc extract of *P. erecticaule* showed the highest AChE and BChE inhibitory with 22.9% and 70.9% inhibition, respectively. In antityrosinase activity, all extracts of *P. porphyrophyllum* showed the highest inhibitory effects against mushroom tyrosinase, compared to standard, kojic acid.

Conclusion: This study showed that *P. erecticaule* and *P. porphyrophyllum* have potential AChE/BChE and tyrosinase inhibition activities. The respective extracts can be explored further for the development of novel lead as AChE/BChE and tyrosinase inhibitors in therapeutic management of Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common form of dementia, characterized by a decline in memory and cognitive function.[1] Acetylcholine (ACh) is a neurotransmitter which plays an important role in the pathology of AD. Decreement of ACh due to inhibition by acetylcholinesterase (AChE) will cause AD.[2] Therefore, AChE and BChE inhibitors have become the remarkable alternatives in treatment of AD. The history of drug discovery showed that plants are highly rich sources in the search for new active compounds and they have become a challenge to modern pharmaceutical industry. The plants have been used in treatment of memory dysfunction in some folk medicines since centuries. Therefore, the present study was undertaken to evaluate the anticholinesterase potential of a number of *Piper* species to discover new candidates for anticholinesterase inhibitors. Tyrosinase, a multifunctional copper-containing oxygenase which is widely distributed in nature catalyzes the hydroxylation of a monophenol and the conversion of *O*-diphenols to the corresponding *O*-quinones.[3] As the key of melanin biosynthesis, it is responsible for melanisation in animals and browning in plants and fungi. Recently, it has been discovered that various dermatological disorders, such as age spots and freckles, were also caused by excessive level of epidermal pigmentation. Furthermore, tyrosinase inhibitors are also imperative in cosmetic applications for skin whitening effects.[4] Since plants are a rich source of bioactive chemicals, and mostly free of harmful side effects, there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as cuminaldehyde, oxyresveratrol, kaempferol, quercetin and gallic acid derivatives have been isolated from various plants.[5] Moreover, kojic acid which is fungal metabolites known as one of the most popular tyrosinase inhibitors has been widely used as a skin whitening and antibrowning agent.[6]

The genus *Piper* has over 1000 species distributed worldwide and it is the most representative of the family Piperaceae. There was an estimated total of 1200 species of *Piper* distributed in the pantropical and neotropical regions of the world and over 400 species were recorded from the Malaysian region alone.[7] The uses of *Piper* species from Peninsular Malaysia were documented which obviously included the cultivated *P. nigrum*, the primary sources of spices worldwide. *Piper* species have often been used in folk medicine to treat several conditions and they have shown several biological activities such as anti-inflammatory, antihypertensive, antimicrobial, antioxidant and antifungal activities.[8-12] Herein, we report the AChE, BChE, and antityrosinase activity of the *n*-hexane, ethyl acetate and methanol leaves extracts of ten *Piper* species from Piperaceae family. To the best of our knowledge, this is the first report describing these activities on the species.

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Materials and Methods

Plant materials

Ten *Piper* species were collected at different location in Malaysia as listed in Table 1. Sample was identified by Mrs. Mohizar Mohamad and Dr. Shamsul Khamis, while the voucher specimens were deposited at the Natural Products Research & Development Centre (NPRDC), UiTM Sarawak and Institute of Bioscience, UPM Serdang.

Table 1. List of *Piper* species and their traditional uses

Species	Collection localities/herbarium number/dates	Traditional uses
P. caninum	Bau, Sarawak/UiTMKS-3003/July 2010	Chewing, hoarseness, flavour, throatache, antiseptic
P. lanatum	Lundu, Sarawak/UiTMKS-03/July 2010	malaria, toothache, rheumatism, deworming, fever, influenza, ulcer
P. abbreviatum	Lundu, Sarawak/UiTMKS-01/March 2012	splenomegaly, stimulant, carminative, coughs and colds, flatulence
P. aborescens	Bau, Sarawak/UiTMKS-3001/July 2010	rheumatism, antiplatelet aggregation, cytotoxic
P. porphyrophyllum	Bau, Sarawak/UiTMKS-3002/July 2010	leprosy, stomach-aches, skin diseases, postpartum treatment, bone pain
P. erecticaule	Lundu, Sarawak/UiTMKS-02/March 2012	No reports
P. ribesioides	K.Berang, Terengganu/SK1962-11/June 2011	asthma, diarrhea, abdominal pain, flavor, alleviate chest congestion, treat urticaria
P. miniatum	Bangi, Selangor/HTBP-1286/July 2006	spice, food flavor, food natural preservative, antibacterial
P. stylosum	K. Berang, Terengganu/SK1963-11/June 2011	vegetables, seasoning, poultice/decoction, confinement
P. majusculum	Padang, Indonesia/EM02-1205/January 2008	No reports

Plant extraction

The dried and powdered leaves of the above-mentioned *Piper* species, 50 g for each was extracted firstly with *n*-hexane (*n*-Hex) (2×200 mL), filtered and the solvent was removed under vacuum using a rotary evaporator (Eyela, Germany). The remaining samples were then extracted again with ethyl acetate (EtOAc) and methanol (MeOH), consecutively followed by filteration and concentration. Finally the *n*-Hex, EtOAc and MeOH extracts were obtained and kept in freeze.

AChE and BChE activity

AChE and BChE inhibitory activities were measured by slightly modifying the spectrophotometric method developed by Ellman.[13] Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic) acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the anticholinesterase activity. Briefly, in this method, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of extracts and 20 µL of AChE/BChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µL of acetylthiocholine iodide/butyrylthiocholine chloride. Hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition (I%) of AChE/BChE was determined by comparison of the rate of reaction of each sample relative to blank sample (ethanol in phosphate buffer pH = 8) using the following formula:

$$\mathrm{I}\,(\%) = \frac{\mathrm{E} - \mathrm{S}}{\mathrm{E}} \times 100$$

where E is the activity of enzyme without the test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galantamine was used as the standard.

Antityrosinase activity

Tyrosinase inhibition assay (EC1.14.18.1) was performed according to previous study with slight modifications.[14] Briefly, all the crudes and kojic acid (Sigma, St. Louis, MO, USA) were dissolved in DMSO prepared as 0.1 mg/mL. The reaction was carried out using 96-well microplate and ELISA microplate reader (VersaMax Molecular Devices, USA) was used to measure the absorbance at 475 nm. The extract (40 µL each) was dissolved in DMSO with 80 µL of phosphate buffer (pH 6.8), 40 µL of tyrosinase enzyme and 40 µL of L-Dopa (Sigma, St. Louis, MO, USA) were placed in each well. Each sample was accompanied by a blank that had all the components except for L-Dopa. Kojic acid was used as the standard inhibitor. The percentage of tyrosinae inhibition was calculated as follows:

$$\mathrm{I}\,(\%) = \frac{A_{\mathrm{control}} - A_{\mathrm{sample}}}{A_{\mathrm{control}}} \times 100$$

where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the extracts or standard. Analyses were run in triplicate and the result was

expressed as average values with standard error mean (SEM).

Statistical analysis

The assays were conducted in triplicate and all tabulated results were expressed as means ±SEM and compared using Student's t-test. A $p<0.05$ was considered significant.

Results and Discussion

AChE, BChE, and tyrosinase inhibitory activities of the plant extracts are summarized in Table 2. Most of the extracts possessed some AChE and BChE inhibitory activities at 1 mg/mL concentration. The EtOAc extract of *P. erecticaule* displayed potent inhibitory activity on AChE (22.9%), compared to the standard which exhibited 95.9% inhibitions, followed by the MeOH and *n*-Hex extracts which gave 21.9% and 20.8% inhibition, respectively. As to BChE, all extracts of *P. erecticaule* (*n*-Hex 52.7%; EtOAc 70.9%; MeOH 50.4%) showed the most potent inhibitory activity as well as *P. ribesioides* (67.3%), *P. miniatum* (48.1%), and *P. abbreviatum* (42.5%). The remaining extracts had activity below 50%. All of the extracts had lower activity against AChE, but higher activity against BChE. This may suggest that these extracts might be interacting with the enzymes in different mechanisms. In the light of these findings, we can conclude that most of the plant extracts screened herein showed inhibitory activity against both of the enzymes in dose-dependent manner and they could be considered for further studies in the treatment of AD.

Table 2. Anticholinesterase and antityrosinase activities of extracts of ten *Piper* species

Species/Extracts	Inhibitory activity against tyrosinase (%±SEM)			(A) Inhibitory activity against AChE (%±SEM) (B) Inhibitory activity against BChE (%±SEM)		
-	n-Hex	EtOAc	MeOH	n-Hex	EtOAc	MeOH
P. caninum	NA	NA	NA	12.6 ± 0.2 32.4 ± 0.3	11.2 ± 0.2 36.3 ± 0.4	1.8 ± 0.2 NA
P. lanatum	7.2 ± 0.2	9.7 ± 0.2	NA	13.5 ± 0.1 33.1 ± 0.2	1.8 ± 0.3 NA	1.5 ± 0.2 13.1 ± 0.1
P. abbreviatum	NA	NA	14.7 ± 0.1	12.9 ± 0.2 42.5 ± 0.4	12.4 ± 0.2 35.6 ± 0.4	1.9 ± 0.3 4.4 ± 0.3
P. aborescens	80.1 ± 0.4	84.1 ± 0.4	94.9 ± 0.3	4.0 ± 0.3 35.1 ± 0.4	1.4 ± 0.3 15.2 ± 0.2	3.0 ± 0.3 16.8 ± 0.2
P. porphyrophyllum	91.2 ± 0.2	91.5 ± 0.5	98.3 ± 0.4	8.4 ± 0.2 NA	NA NA	1.6 ± 0.3 NA
P. erecticaule	NA	NA	7.9 ± 0.1	20.8 ± 0.2 52.7 ± 0.4	22.9 ± 0.3 70.9 ± 0.4	21.9 ± 0.1 50.4 ± 0.2
P. ribesioides	NA	15.3 ± 0.1	NA	3.8 ± 0.2 27.3 ± 0.3	11.9 ± 0.2 67.3 ± 0.4	1.8 ± 0.3 NA
P. miniatum	NA	5.7 ± 0.1	NA	12.9 ± 0.2 32.7 ± 0.4	13.4 ± 0.3 48.1 ± 0.3	2.4 ± 0.2 NA
P. stylosum	NA	NA	NA	2.1 ± 0.3 NA	2.2 ± 0.2 1.8 ± 0.2	1.8 ± 0.3 6.8 ± 0.2
P. majusculum	NA	NA	NA	NA 12.3 ± 0.2	NA NA	NA 11.9± 0.2
Kojic acid		81.8 ± 0.5			-	
Galantamine		-			(A) 95.9 ± 0.2 (B) 88.7 ± 0.2	

Data represent mean ±SEM of three independent experiments; NA-not active.

Since most of the AChE inhibitors are known to contain nitrogen, the higher activity of these extracts may be due to their rich alkaloidal content. Piperine, isolated from *P. nigrum* has been reported to improve memory impairment and neurodegeneration in the hippocampus of animal models with AD.[15] *P. capense* contains amide alkaloids: piperine and 4,5-dihydropiperine which have previously been shown to have AChE activity.[16] *P. methysticum* has been reported to possess local anaesthetic, sedating, anticonvulsive, musclerelaxant and sleep-stimulating effects which is due to the presence of kavopyrones.[17] The ethanol extract of *P. sarmentosum* had shown as the strongest AChE inhibitory activity using Ellman's method with new technique of Flow Injection Analysis (FIA).[18] *P. betel* extract had shown potential of AChE and BChE inhibition in TLC bioautography and 96 well microtiter plate assays. It was reported that hydroxychavicol and chlorogenic acid are responsible for the bioactivity of *P. betel*.[19] Some of *Piper* species have been studied and showed significant activity on their AChE/BChE activity such as *P. guineense*, *P. hymenophyllum*, *P. interruptum*, and *P. longum*.[17,20-22]

Evaluation of the antityrosinase activity of the extracts was subjected to tyrosinase inhibition assay with L-DOPA as the substrate. The tyrosinase inhibitory activity was compared with kojic acid as the positive control. Results showed that extracts of *P. aborescens* and *P. porphyrophyllum* exhibited potent inhibition on mushroom tyrosinase with percentage inhibition ranged from 80.1-98.3% at concentration of 0.1 mg/mL, compared with the

reference standard inhibitor, kojic acid (81.8±0.5%). The MeOH extract from *P. porphyrophyllum* gave the highest inhibition which was 98.3±0.3% followed by *P. aborescens* with 94.9±0.3% inhibition. The MeOH extract with polar constituents showed higher tyrosinase inhibition effect compared to other solvent extracts and may have some bioactive materials. The inhibition of tyrosinase ability might be due to the hydrogen bonding formation of the hydroxyl groups of the phenolic compounds of the extracts with the active site of the enzyme.[23]

Flavonoids were the major phytochemicals isolated from *P. porphyrophyllum*.[24] The present of flavonoids seems to justify the high tyrosinase inhibition, which flavonoids is well known to show good inhibition of tyrosinase activity.[25] Previous studies on *P. aborescens* had showed the present of cyclobutanoid amides and pyridine alkaloids.[26-28] These compounds might be contributed in the tyrosinase activity. Nevertheless, the other *Piper* species (*P. lanatum*, *P. abbreviatum*, *P. erecticaule*, *P. ribesioides*, *P. miniatum*) showed less inhibition at the tested concentrations. The decrease in inhibition might be presumably related to their secondary metabolittes and also related to different inhibitory mechanism. The tyrosinase activity has been previously assessed on the extracts, chemical constituents and essential oils of *Piper* species which are *P. nigrum*, *P. longum*, *P. betle*' and *P. vuscumentosa*.[29-34] In addition, piperlonguminine from *P. longum* was discovered to inhibit melanin production in melanoma B16 cells stimulated with α-melanocyte stimulating hormone (α-MSH), 3-isobutyl-1-methylxanthine or protoporphyrin IX, where the compound exhibited stronger depigmenting efficacy than kojic acid.[35] Besides, 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin), a dihydrochalcone from *P. elongatum* had showed the strongest tyrosinase activity among the test compounds, and had almost the same activity of kojic acid.[36]

Conclusion

We have herein screened the extracts of ten *Piper* species of Malaysia origin for enzyme (AChE, BChE, and tyrosinase) inhibitory potentials by three different methods, due to their traditional utilization for memory improvement. In this current study, the extracts of *P. aborescens* and *P. porphyrophyllum* showed strong AChE and BChE inhibition, which led us to conclude that memory-enhancing property of these species may result from AChE/BChE inhibition. To the best of our knowledge, this is the first report on anticholinesterase and antityrosinase inhibition of the above mentioned ten *Piper* species.

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Conflict of Interest

There is no conflict of interest to be reported.

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