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Chemical Components and Anticholinesterase Activity of *Piper ornatum* N.E.Br Volatile Oil

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This study reports volatile oil components and anticholinesterase inhibitory activity of *Piper ornatum* N.E.Br (Piperaceae) essential oil collected from Malaysia. The essential oil was obtained by hydrodistillation and fully analysed by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Twenty-seven chemical components were identified representing 79.6% of the total oil. The essential oil contains caryophyllene oxide (31.5%), spathulenol (5.9%), aromadendrene (4.9%), and β-caryophyllene epoxide (4.5%). Anticholinesterase activity was evaluated using Ellman method. The essential oil showed significant inhibitory activity against acetylcholinesterase (I%: 70.2%) and butyrylcholinesterase (I%: 75.8%) assays.

Key words: *Piper ornatum*; essential oil; acetylcholinesterase; caryophyllene oxide

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Alzheimer disease (AD) is a neurodegenerative disease associated with the loss of cholinergic neurons in parts of the brain and deposition of β-amyloid in the form of neurofibrillary tangles and amyloid plaques [1,2]. There is no therapeutic approach that can delay AD progression and all available treatments only provide symptomatic relief. There is two main classes of drugs available for AD; cholinesterase inhibitors, such as donepezil, galantamine, and rivastigmine, and the glutamate antagonist memantine [3]. Two cholinesterases; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), participate in cholinergic neurotransmission by hydrolysing acetylcholine (AChl) in the central and peripheral nervous system. AChE is responsible for the degradation of AChl in the synaptic cleft of cholinergic synapses and neuromuscular junctions into choline and acetate. It has a crucial role in regulating many functions such as learning, memory, cerebral blood flow control, and cortical organization of movement. On the other hand, BChE has a higher activity in liver, heart, intestine, kidney, and lung. Both enzymes share 65% amino acids' sequence homology and show similar molecular forms and active sites [4]. Thus, restoring the level of acetylcholine may be proposed in the treatment and management of AD.

Essential oils are complex mixtures containing mostly two or three dominant components. Major components often determine the essential oil main biological properties while other minor oil components may possess a variety of biological effects. Therefore, examination of the chemical composition of essential oils is important for better understanding of their biological activities, broaden their applications and for understanding any possible side effects [5].

The genus *Piper* (Piperaceae) comprises more than 700 species of worldwide distribution. Species in this genus have high commercial and medicinal importance. Extensive phytochemical investigations of *Piper* species from different parts of the world led to isolation of several physiologically active compounds including alkaloids, flavones, dihydrochalcones, kawapyrones, lignans, neolignans, propenylphenols, and terpenoids [6-11]. *Piper ornatum*, locally known in Indonesia as Celebes Pepper, is a sprawling shrub with wiry stems that spread across the ground and climb into low vegetation. It can grow to about 15 ft. (4.5 m) tall. Its heart-shaped leaves are nearly as wide as they are long 10 cm. The upper leaf surfaces are a mottled pattern of green, pink and silver while the undersides are flushed purple-red [12]. There has been no information of this plant in traditional or folk medicine practice.

Following our previous studies [13-16], the present work aimed to analyse the chemical composition and the anticholinesterase inhibitory activity of *P. ornatum* essential oils. The chemical profile, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) inhibitory activity have been investigated.

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MATERIALS AND METHODS

Plant Material

The leaves of *P. ornatum* were collected from Muar, Johor (August 2021) and were identified by Dr. Shamsul Khamis at Universiti Kebangsaan Malaysia. The voucher specimen (NP05/08/22) was deposited at FSM Herbarium, UPSI.

Isolation of Essential Oil

The fresh leaves of *P. ornatum* (250 g) were subjected to hydrodistillation for 5 h in Clevenger-type apparatus. The obtained essential oil was dried over anhydrous magnesium sulphate and stored at 4–6°C. The oil yield (w/w) was 0.35% based on the fresh weight basis.

Analysis of Essential Oil

Gas chromatography-flame ionisation detection (GC-FID) analysis was performed on a Hewlett Packard 6890 series II A gas chromatograph equipped with HP-5 column (30 m× 0.25 mm× 0.25 µm film thickness). Helium was used as a carrier gas at a flow rate of 0.7 mL/min. Injector and detector temperatures were set at 250 and 280°C, respectively. The oven temperature was kept at 50°C, then gradually increased to 280°C at 5°C/min rate and finally held isothermally for 15 min. Diluted samples (1.0 µL, 1/100 v/v in diethyl ether) were injected manually (split ratio 50:1). Injection was repeated three times and peak area percentages were reported as means ±SD of the triplicates. Peak area percentages were calculated from flame ionisation detection (FID) using GC HP Chemstation software (Agilent Technologies).

Gas chromatography-mass spectrometry (GC-MS) chromatograms were recorded using a gas chromatograph Hewlett Packard Model 5890A and a Hewlett Packard Model 5989A mass spectrometer. The GC was equipped with HP-5 column. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was 250°C. The oven temperature was programmed from 50°C (5 min hold) to 250°C at 10°C/min and finally held isothermally for 15 min. For GC-MS detection, an electron ionization system, with ionization energy of 70 eV was used. A scan rate of 0.5 s (cycle time: 0.2 s) was applied, covering a mass range from 50 to 400 amu.

Identification of Components

The chemical components were identified by co-injection with standards (major components) and their comparison with reported retention indices and mass spectra found in Adams, NIST08 and FFNSC2 libraries [17]. Semi-quantification of essential oil components was made by peak area normalization considering the same response factor for all volatile components.

Anticholinesterase Inhibitory Activity

The acetylcholinesterase/butyrylcholinesterase (AChE/BChE) inhibitory activity of the essential oil was measured using a slightly adapted spectrophotometric method reported elsewhere [18, 19]. Electric eel AChE and horse serum BChE (Sigma-Aldrich, St. Louis, MO, USA) were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction and (5,5′-dithiobis-[2-nitrobenzoic]) DTNB acid (Sigma-Aldrich, St. Louis, MO, USA) was used for the measurement of the anticholinesterase activity. Briefly, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of essential oil and 20 µL of AChE/BChE (0.22 U/mL) solution were added into a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated by adding 10 µL of acetylthiocholine iodide/butyrylthiocholine chloride. Hydrolysis of acetylthiocholine iodide/butyryl-thiocholine chloride was monitored by the formation of the yellow 5-thio2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, Winooski, VT, USA). The percentage of inhibition (I%) of AChE/BChE was determined by comparing the reaction rates of the relative to the blank sample (EtOH in phosphate buffer pH 8) using the formula: $I\% = [E – S / E] \times 100$; where E is the activity of enzyme without the test sample and S is the activity of the enzyme with the test sample. Analyses were expressed as means ± SD of the triplicates and galantamine (Sigma-Aldrich, St. Louis, MO, USA) at the same concentration as essential oil was used as a positive control.

Statistical Analysis

Data obtained from essential oil analysis and their anticholinesterase inhibitory activity were expressed as mean values. The statistical analyses were carried out by employing one way ANOVA ($p<0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

RESULTS AND DISCUSSION

The essential oil was obtained at a 0.35% yield. Identified components of the essential oil are listed in Table 1, according to their Kovats indices (KI) on an HP-5 column. The GC-FID and GC-MS analysis (Figure 1) of the essential oil revealed the presence of 27 chemical components constituting about 79.6%. Oxygenated sesquiterpenes were the most dominant components found in the essential oil which include 9 components, accounting for 49.5% of the total composition. In addition, sesquiterpene hydrocarbons comprise 18 components, accounting for 30.1% of the total composition. The essential oil is rich in caryophyllene oxide (31.5%), spathulenol (5.9%), aromadendrene (4.9%), and β-caryophyllene epoxide

(4.5%). Meanwhile, minor components; exceeding 2%, were β-elemene (3.8%), viridiflorol (3.7%), α-amorphene (2.7%), α-copaene (2.1%), β-bisabolene (2.1%), and *cis*-calamenene (2.1%).

A review of existing literature on essential oils of the genus *Piper* revealed that caryophyllene oxide was shown to be the principal sesquiterpene component of the essential oils of *P. caldense* (13.9%) [20], *P. curtistilum* (28.6%) [21], *P. miniatum* (20.3%) [22], *P. aleyreanum* (11.5%) [23], *P. chaba* (12.2%) [24], and *P. peltatum* (22.9%) [25]. Chemical differences in the essential oil composition of plant species concerning their geographical origins and harvesting season have been reported showing that the chemical and biological diversity of aromatic and medicinal plants depend on factors such as cultivation area, climatic conditions, vegetation phase, and genetic modifications. In fact, these factors influence the plant's biosynthetic pathways and consequently, the relative proportion of the main characteristic components [26].

Table 1. Chemical components identified from *P. ornatum* essential oil

No	Components	KI[a]	KI[b]	Percentage (%)	Identifications[c]
1	β-Elemene	1332	1335	3.8	RI, MS, Std
2	α-Copaene	1375	1374	2.1	RI, MS
3	β-Bourbonene	1390	1387	0.9	RI, MS
4	β-Gurjunene	1430	1431	0.5	RI, MS
5	Aromadendrene	1440	1439	**4.9**	RI, MS, Std
6	α-Humulene	1452	1452	0.5	RI, MS
7	β-Caryophyllene	1475	1473	1.5	RI, MS
8	γ-Muurole	1478	1478	1.3	RI, MS
9	α-Amorphene	1482	1483	2.7	RI, MS
10	Germacrene D	1485	1484	1.0	RI, MS
11	α-Curcumene	1487	1487	1.2	RI, MS
12	β-Selinene	1488	1489	1.5	RI, MS
13	Zingiberene	1492	1493	1.8	RI, MS
14	α-Selinene	1498	1498	0.8	RI, MS
15	α-Muurolene	1501	1500	0.8	RI, MS
16	β-Bisabolene	1505	1505	2.1	RI, MS
17	*cis*-Calamenene	1528	1528	2.1	RI, MS
18	α-Calacorene	1545	1544	0.6	RI, MS
19	Spathulenol	1575	1577	**5.9**	RI, MS, Std
20	Caryophyllene oxide	1582	1582	**31.5**	RI, MS, Std
21	*epi*-Globulol	1590	1590	0.7	RI, MS
22	Viridiflorol	1592	1592	3.7	RI, MS
23	Ledol	1600	1602	0.6	RI, MS
24	β-Caryophyllene epoxide	1620	1623	**4.5**	RI, MS
25	Isoaromadendrene epoxide	1638	1639	0.3	RI, MS
26	t-Muurolol	1640	1640	1.1	RI, MS
27	α-Cadinol	1652	1652	1.2	RI, MS
Group components					
	Sesquiterpene hydrocarbons			**30.1**	
	Oxygenated sesquiterpenes			**49.5**	
Total identified				**79.6**	

[a]Linear retention index experimentally determined using homologous series of C_6-C_{30} alkanes

[b]Linear retention index taken from Adams, Wiley and literatures

RI, based on comparison of calculated RI with those reported in Adams; MS, based on comparison with Wiley databases; Std, based on comparison with standard compounds

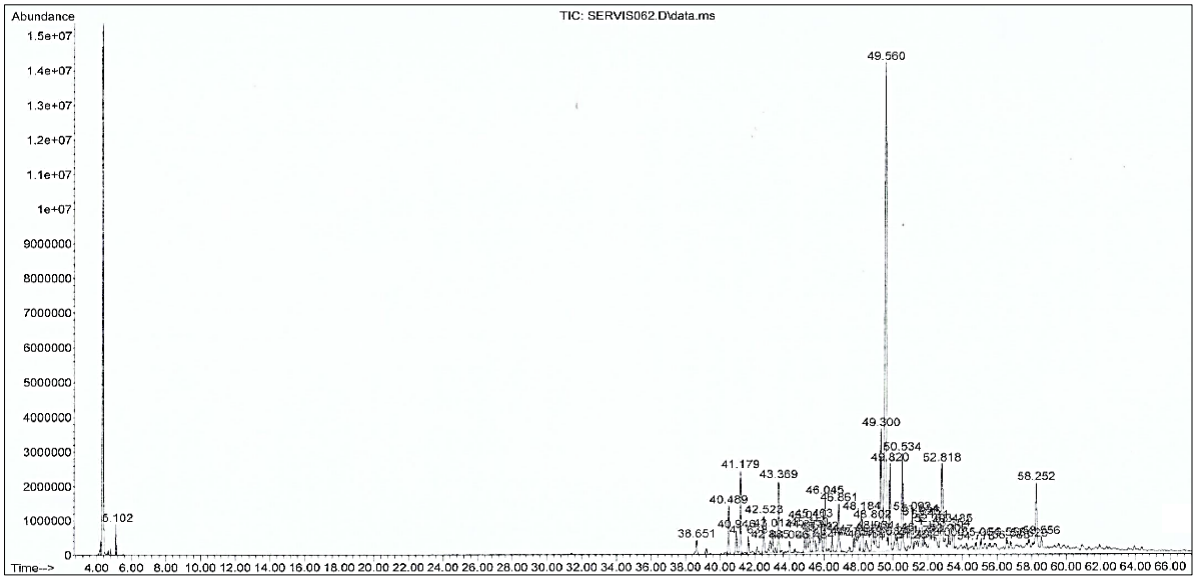

Figure 1. Chromatogram of *P. ornatum* essential oil

Anticholinesterase inhibitory activity (I%) was tested against AChE and butyrylcholinesterase BChE enzymes where it was compared with that of galantamine, as a standard drug in the treatment of Alzheimer's disease. AChE is the key enzyme that catalyzes the breakdown of some choline ester compounds that are functioning as neurotransmitters. The most important among them is acetylcholine, which is found in all autonomic ganglia and different synapses in the central nervous system. Reversible inhibitors of acetylcholine have been used for the treatment of some neurodegenerative disorders, especially Alzheimer's disease [27]. BChE is another major form of cholinesterases, and unlike AChE, it is also present in liver and plasma. Reportedly, the increase of the BChE levels could promote the transformation of benign plaques to malignant plaques which could lead eventually to neurodegeneration [28]. In this study, the essential oil indicated significant AChE (I%: 70.2%) and BChE (I%: 75.8%) inhibitory activity at 1000 mg/mL concentration, compared to galantamine which gave 95.9% (AChE) and 88.7% (BChE) inhibition, at the same concentration. In previous reports, AChE/BChE inhibition was explained by the presence of β-caryophyllene and caryophyllene oxide which have shown cholinesterase activity. This study shows that the high content of these components obtained in the essential oil may contribute, at least in part, to the activity ascribed to the plant [29].

The structural variation of the active anti-cholinesterase terpenoids cause inconveniences for the estimation of the potency of structure-activity relations. One feature related to AChE and BChE inhibitory activities is the opening of the epoxide ring. Sesquiterpene hydrocarbons comprise an epoxide ring that might contribute to their cholinesterase inhibitory activity [30]. In this investigation, caryophyllene oxide were principal component of the leaves oil from *P. ornatum*, and displayed AChE and BChE inhibitory activities.

Caryophyllene oxide contain epoxide ring. According to the results obtained, it can be suggested that epoxide ring on the caryophyllene oxide was turned into a diol group. It may result by the molecular interactions of the caryophyllene oxide with enzyme active sides. Hydrogen bonding interactions of the hydroxyl groups on the compounds with the active sides of AChE and BChE is an increasing factor for the enzyme-inhibitory complex. The component exist in two isomers such as *cis* or *trans*. This situation may also influence the bioactivity potency positively.

CONCLUSION

This work constitutes the first study to report the essential oil composition and anticholiesterase inhibitory activity of *P. ornatum* obtained from Malaysia. A study on the essential oil of *P. ornatum* revealed the existence of sesquiterpenes as the major class of components, dominated by caryophyllene oxide. Further studies are needed to investigate the safety of *P. ornatum* essential oil to be used as a therapeutic against neurodegenerative diseases.

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