Nat. Volatiles & Essent. Oils, 2020; 7(4): 8-13
DOI: 10.37929/nveo.770303

NVEO
Natural Volatiles & Essential Oils

RESEARCH ARTICLE

Chemical composition and acetylcholinesterase inhibition of the essential oil of *Cyathocalyx pruniferus* (Maingay ex Hook.f. & Thomson) J. Sinclair

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Submitted: 16.07.2020; Accepted: 24.11.2020

Abstract
The chemical composition and acetylcholinesterase inhibitory activity of the essential oil from the leaf of *Cyathocalyx pruniferus* growing in Pahang, Malaysia was investigated for the first time. The essential oil was obtained by hydrodistillation and fully characterized by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). A total of thirty chemical components (95.5%) were successfully identified in the essential oil which was characterized by high proportions of α-pinene (25.4%), germacrene D (20.2%), β-caryophyllene (10.8%), and δ-cadinene (6.4%). Acetylcholinesterase inhibitory activity was evaluated against the Ellman method, and the essential oil showed good inhibition against acetylcholinesterase (Percentage inhibition: 75.5%) assay.

Keywords: Essential oil, *Cyathocalyx pruniferus*, Annonaceae, α-pinene, germacrene D, acetylcholinesterase

Introduction

The genus *Cyathocalyx* belonging to the Annonaceae family consists of approximately 36 species of monopodial trees. It is widely distributed in primary and secondary tropical lowland forests of Southeast Asia, with a center of diversity in western Malaysia (Peninsular Malaysia, Sumatra, and Borneo) (Wang and Saunders, 2006). *Cyathocalyx pruniferus* (Maingay ex Hook.f. & Thomson) J. Sinclair. is locally known as *Antoi beludu* in Malaysia (Burkill, 1966). International Plant Names Index reported that the plant is a synonym of *Drepananthus pruniferus* Maingay ex Hook.f. & Thomson (IPNI, 2020). Literature reviews indicated that only a small number of species in the genus *Cyathocalyx* have been investigated for its chemical compounds and biological activities. Previous phytochemical investigation on *Cyathocalyx* species led to the isolation of alkaloids and diterpenoids (Wijeratne et al., 1995a, 1995b). However, there is little information about the chemical composition and the biological properties of the essential oil of the genus *Cyathocalyx.* Previous study on the essential oil of *Cyathocalyx* have been reported on *Cyathocalyx zeylanicus* collected from India (Hisham et al., 2012). In continuation of our systematic studies on pharmacologically active volatiles from Malaysian plants (Salleh et al.,2014, 2015a, 2015b, 2016a, 2016b), we describe in this paper an evaluation of the chemical composition and acetylcholinesterase inhibitory activity of the essential oil from the leaf of *C. pruniferus*.

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Materials and Methods

Plant material

Sample of *Cyathocalyx pruniferus* was collected from Gambang, Pahang in September 2019, and identified by Dr. Shamsul Khamis from Universiti Kebangsaan Malaysia (UKM). The voucher specimen (SK242/19) was deposited at UKMB Herbarium, Faculty of Science and Technology UKM.

Extraction of essential oil

The fresh leaf (300 g) was subjected to hydrodistillation in Clevenger-type apparatus for 4 hours. The essential oil obtained was dried over anhydrous magnesium sulphate and stored at 4-6°C.

Gas chromatography (GC) analysis

GC analysis were performed on an Agilent Technologies 7890B and an Agilent 7890B FID equipped with DB-5 column. Helium was used as a carrier gas at a flow rate of 0.7 mL/min. Injector and detector temperature were set at 250 and 280 °C, respectively. The oven temperature was kept at 50 °C, then gradually raised to 280 °C at 5 °C/min and finally held isothermally for 15 min. Diluted samples (1/100 in diethyl ether, v/v) of 1.0 µL were injected manually (split ratio 50:1). The injection was repeated three times and the peak area percentages were reported as means ± SD of triplicates. Calculation of peak area percentage was carried out by using the GC HP Chemstation software (Agilent Technologies).

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS chromatograms were recorded using Agilent Technologies 7890A and Agilent 5975 GC MSD equipped. The GC was equipped with HP-5MS column. Helium was used as carrier gas at a flow rate of 1 mL/min. 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 40–400 amu.

Identification of chemical components

For identification of essential oil components, co-injection with the standards (major components) were used, together with correspondence of retention indices and mass spectra with respect to those occurring in Adams, NIST 08 and FFNSC2 libraries (Adams, 2007). Semi-quantification of essential oil components was made by peak area normalization considering the same response factor for all volatile components. Percentages values were the mean of three chromatographic analyses.

Acetylcholinesterase inhibitory activity

AChE inhibitory activity of the essential oil was measured by slightly modifying the spectrophotometric method developed by Salleh et al. (2014b). Electric eel AChE was used, while acetylthiocholine iodide was employed as substrates of the reaction. DTNB acid was used for the measurement of the acetylcholinesterase activity. Briefly, in this method, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of essential oil and 20 µL of AChE 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. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalysed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of

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inhibition (%I) of AChE was determined by comparison of rates of reaction of samples relative to blank sample (EtOH in phosphate buffer pH 8) using the formula: %I = [E - S / E] × 100; where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galantamine was used as a reference.

Statistical analysis

Data obtained from essential oil analysis and bioactivity 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 isolated by hydrodistillation from the fresh leaf of *C. pruniferus* was analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The yield of the essential oil obtained was 0.13% (w/w). The percentage composition of the essential oil, retention time and retention indices of the components are shown in Table 1.

Table 1. Chemical composition of *C. pruniferus* essential oil

No.	Components	KI[a]	KI[b]	Percentage (%)
1	α-Pinene	930	0932	25.4 ± 0.3
2	Camphene	945	946	0.4 ± 0.1
3	Sabinene	970	0969	0.4 ± 0.1
4	β-Pinene	972	0974	1.1 ± 0.1
5	α-Terpinene	1015	1014	0.4 ± 0.2
6	Limonene	1025	1024	0.3 ± 0.1
7	β-Elemene	1336	1335	0.5 ± 0.1
8	α-Cubebene	1346	1345	0.9 ± 0.1
9	α-Copaene	1376	1374	1.1 ± 0.1
10	β-Caryophyllene	1415	1417	10.8 ± 0.3
11	Aromadendrene	1440	1439	1.3 ± 0.1
12	α-Humulene	1450	1452	0.3 ± 0.2
13	Alloaromadendrene	1460	1458	0.5 ± 0.2
14	α-Amorphene	1485	1483	1.1 ± 0.1
15	Germacrene D	1486	1484	20.2 ± 0.2
16	β-Selinene	1490	1489	0.3 ± 0.2
17	α-Muurolene	1505	1500	0.5 ± 0.2
18	β-Bisabolene	1502	1505	0.8 ± 0.1
19	(*E,E*)-α-Farenesene	1505	1505	2.6 ± 0.2
20	γ-Cadinene	1510	1513	0.5 ± 0.1
21	δ-Cadinene	1520	1522	6.4 ± 0.1
22	Elemol	1550	1548	0.7 ± 0.1
23	Germacrene B	1560	1559	2.4 ± 0.3
24	(*E*)-Nerolidol	1560	1561	1.2 ± 0.1

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25	Spathulenol	1575	1577	2.8 ± 0.2
26	Caryophyllene oxide	1580	1582	2.9 ± 0.2
27	Globulol	1590	1590	3.0 ± 0.2
28	Guaiol	1600	1600	2.9 ± 0.2
29	t-Muurolol	1645	1644	1.3 ± 0.1
30	α-Cadinol	1650	1652	2.7 ± 0.2
	Monoterpene hydrocarbons			28.0 ± 0.1
	Sesquiterpene hydrocarbons			50.2 ± 0.2
	Oxygenated sesquiterpenes			17.4 ± 0.2
	Total (%)			95.53 ± 0.2

[a]Linear retention index, experimentally determined using homologous series of C6-C30 alkanes; [b]Linear retention index taken from Adams (2007) or NIST 08 (2008) and literature; [c]Relative percentage values are means of three determinations ± SD

The chemical components identified from the essential oil were thirty, forming 95.5% of the total oil composition. The essential oil consisted mainly of sesquiterpenes hydrocarbon (50.2%), monoterpenes hydrocarbon (28.0%), and oxygenated sesquiterpenes (17.4%). The most abundant components of the essential oil were α-pinene (25.4%), germacrene D (20.2%), β-caryophyllene (10.8%), and δ-cadinene (6.4%). The other minor components detected in the essential oil in more than 2% were globulol (3.0%), caryophyllene oxide (2.9%), guaiol (2.9%), spathulenol (2.8%), α-cadinol (2.7%). (*E*, *E*)-α-farnesene (2.6%), and germacrene B (2.4%). In comparison to the previous study (Hisham et al., 2012), analyses of the leaf oil of *Cyathocalyx zeylanicus* has successfully identified thirty-two compounds, comprising 98.0% of total oil. The essential oil was reported to show high amounts of β-caryophyllene (21.6%), α-pinene (20.4%) and (*E*)-β-ocimene (11.8%). 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 (Salleh et al., 2016b).

Acetylcholinesterase inhibitory activity (I%) was tested against acetylcholinesterase (AChE) enzyme. It was compared with that of galantamine, as a standard drug against Alzheimer's disease. The essential oil indicated good AChE (I%: 75.5%) inhibitory activity at 1,000 mg/mL concentration, compared to galantamine which gave 85.6% inhibition. In previous reports, AChE inhibition can be explained by the high content of α-pinene and β-pinene have anticholinesterase activity (Picollo et al., 2008). This study shows that α-pinene as the major component in this oil hence may contribute to the AChE inhibition.

α-Pinene is a bicyclic monoterpene widely found in nature, acting as an insect-repellent agent in plant defence (Huang et al., 2013). A variety of interesting pharmacological properties have been attributed to α-pinene, including anti-inflammatory, bronchodilator, hypoglycemic, sedative, antioxidant, and broad-spectrum antibiotic activities (Mercier et al., 2009; Violante et al., 2012; Da Silva et al., 2012). Previous studies have reported the presence of α-pinene in the essential oils obtained from Annonaceae family, such as *Polyalthia korintii* (leaf oil: 43.2%) (Sherin et al., 2018), *Guatteria costaricensis* (leaf oil: 36.3%) (Palazzo et al., 2009), and *Xylopia langsdorffiana* (fruit oil: 34.6%) (Moura et al., 2016). Meanwhile, germacrene D was also present as the predominant component in the oil. This component is one of the most common plant volatiles considered to be a biogenetic precursor of many sesquiterpenes such as cadinane, muurolane, and

amorphane derivatives (Fujita, 1990). This metabolite is involved in plant-insect interaction acting as a pheromone on receptor neurons (Stranden et al., 2002). Germacrene D was also shown as an important deterrent and insecticidal agent against different parasites such as mosquitos, aphids, and ticks (Bruce et al., 2005). Previously, germacrene D was found abundant in *Cardiopetalum calophyllum* (flower oil: 37.03%) (Xavier et al., 2016), *Uvaria rufa* (stem oil: 38.4%) (Thang et al., 2014) and *Fissistigma pallens* (leaf oil: 30.2%) (Hoferl et al., 2013), another member of the Annonaceae family.

ACKNOWLEDGMENT

The author would like to thank Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris (UPSI) for research facilities.

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