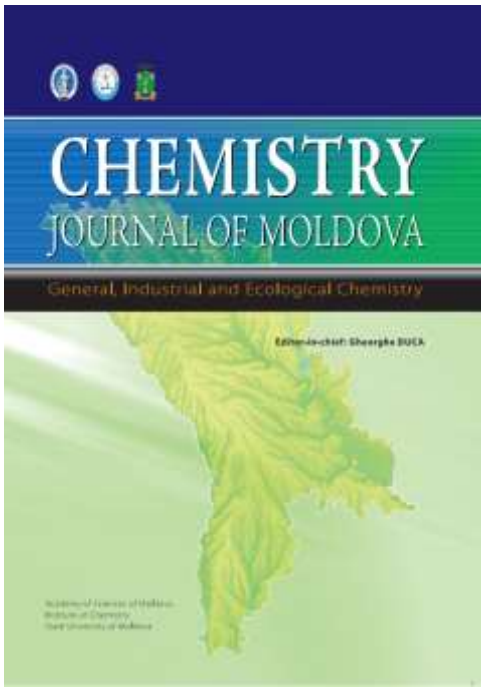

CHEMISTRY JOURNAL OF MOLDOVA.
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Accepted version posted online: 8 September 2021

To cite this article: W.M.N.H.W. Salleh, M.H. Nadri, S. Khamis. Chemical Composition and Lipoxygenase Inhibitory Activity of the Essential Oil of *Alstonia Angustiloba*. *Chemistry Journal of Moldova, 2021,* DOI: dx.doi.org/10.19261/cjm.2021.848

CHEMISTRY JOURNAL OF MOLDOVA. General, Industrial and Ecological Chemistry
ISSN (p) 1857-1727 ISSN (e) 2345-1688
http://cjm.asm.md
http://dx.doi.org/10.19261/cjm.2021.848

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Abstract. This study was aimed to investigate the chemical compositions and lipoxygenase inhibitory activity of the essential oil extracted from *Alstonia angustiloba* growing in Malaysia. The essential oils were obtained by hydrodistillation and fully characterized by gas chromatography and gas chromatography-mass spectrometry methods. Analysis of the *A. angustiloba* essential oil resulted in the identification of twenty-five chemical components, which constitute 90.8% of the total oil. The most abundant components of *A. angustiloba* oil were linalool (21.2%), 1,8-cineole (16.8%), *α*-terpineol (9.5%), terpinen-4-ol (8.5%), *β*-caryophyllene (6.2%), and caryophyllene oxide (5.2%). The essential oil displayed moderate activity towards lipoxygenase inhibitory activity with an IC_{50} value of 45.8 μg/mL.

Keywords: essential oil, hydrodistillation, lipoxygenase, *Alstonia angustiloba*, Apocynaceae.

Received: 29 June 2021/ Revised final: 21 August 2021/ Accepted: 24 August 2021

Introduction

Alstonia is a genus of evergreen trees and shrubs, of the family *Apocynaceae* and consists of about 40-60 species. It is distributed in deciduous, evergreen forests and is widespread in tropical and subtropical Africa, Central America, Southeast Asia, Polynesia, and Australia, with most species in the Malaysian region [1]. The bark, the latex from the bark of *Alstonia*, is are widely used in traditional medicine throughout Southeast Asia, being employed in the treatment of liver and intestinal troubles, heart diseases, asthma, various skin diseases, fever, vulnerary, and emmenagogue [2]. *Alstonia angustiloba* is locally known as *pulai* or *jelutong* in Malaysia; it is native to Thailand, Peninsular Malaysia, Singapore, Sumatra, and Java, and is commonly found in a variety of habitats from sea level to 200 meters (700 ft) altitude [3]. Phytochemical studies of this genus have resulted in the isolation of indole alkaloids [4], triterpenes [5], flavonoids [6], caffeoylquinic acids [7] and found to possess various biological properties such as antioxidant, antidiabetic [8], antiplasmodial [9], antimalarial [10], antibacterial, antibiofilm [11], and antiproliferative [12] activities. Essential oils are one of the promising candidates amongst natural compounds for the development of safe therapeutic agents. There are few literature reports on the chemical components of the essential oils of this genus, mainly on *A. scholaris* [13]. Meanwhile, several studies have been reported on the essential oils of *A. angustiloba* from various locations [14-16].

The goal of the study was to evaluate the chemical compositions and lipoxygenase inhibitory activity of the essential oils of *A. angustiloba* for further exploring the potential nutraceutical and pharmaceutical applications.

Experimental

Materials

Analytical grade methanol, ethanol, diethyl ether and dimethylsulfoxide (DMSO), HPLC grade chloroform, and anhydrous magnesium sulphate were purchased from Merck (Darmstadt, Germany). The lipoxygenase inhibitor screening assay kit (Item No. 760700) was purchased from Cayman Chemical (USA).

Samples of *A. angustiloba* were collected from Gambang, Pahang (N 3° 42’ 59.99” E 103° 05’ 60.00”) in October 2019. The voucher specimens (SK43/18) have been identified by

Dr. Shamsul Khamis and deposited at Universiti Kebangsaan Malaysia Herbarium.

Methods

Isolation procedure of essential oils

The fresh leaves of *A. angustiloba* (220 g) were weighed and then subjected to hydrodistillation using a Clevenger-type apparatus for 4 hours. The selected runtime allowed obtaining optimum yield without drastically affecting the oil components. Afterwards, the obtained oil was dried using anhydrous magnesium sulphate, weighed, and stored in dry amber vials at 4°C until analysis. The average yield of oil was calculated as percentage weight by weight (% w/w) of the plant material.

GC-FID analysis

Gas chromatography with flame ionization detection (GC-FID) analysis was performed on an Agilent Technologies 7890B and an Agilent 7890B FID spectrometer (U.S.A.) equipped with HP-5 column (30 m × 0.25 mm × 0.25 μm). At a flow rate of 0.7 mL/min, helium was used as a carrier gas. Injector and detector temperatures were set at 250 and 280°C, respectively. The oven temperature was maintained at 50°C, then slowly increased to 280°C at 5°C/min and lastly detained 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 percent was reported as means ±SD of triplicates.

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) chromatograms were recorded using Agilent Technologies 7890A and Agilent 5975 GC MSD spectrometer (U.S.A.) equipped with HP-5MS column (30 m × 0.25 mm × 0.25 μm). Helium was used as 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 [17]. For GC-MS detection, an electron ionization system, with an 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 m/z 50-400 amu. In order to determine the chemical components of essential oil, standards (major components) need to be injected, together with correspondence of retention indices. The data of mass spectra were compared with those occurring in Wiley, NIST08, and FFNSC2 libraries [18]. Each peak was considered the same response factor for all components for semi-quantification of essential oil components. Quantification was done by the external standard method using calibration curves generated by running GC analysis of representative authentic compounds.

Lipoxygenase inhibitory activity

The lipoxygenase inhibitor screening assay kit was used in this study. The reagents were prepared according to the previous study and followed the standard protocol [19]. Stock solutions of essential oil were prepared to obtain concentrations of 125-7.8 μg/mL in the respective wells. The prepared solutions were then placed onto 96 well plates where the cells were distributed as blanks 1A-2A-1D (triplicate), positive control 1B-2B (duplicate), and 100% initial activity wells 1C-2C-2D (triplicate). The remaining wells were designated for inhibitor (essential oil) solutions in duplicate. The addition of the reagents was done according to the standard protocol, according to which, 100 μL of assay buffer was added to the blank wells and 90 μL of lipoxygenase enzyme (5-LOX) and 10 μL of assay buffer were added to positive control wells. For the 100% initial activity wells, 90 μL of lipoxygenase enzyme and 10 μL of solvent (DMSO) were added. The inhibitor (essential oil) wells were charged with 90 μL of 5-LOX and 10 μL of respective stock (essential oil) solution. The reaction was initiated by adding 10 μL of the substrate to all wells. The plate was then shaken for 5 min on an orbital shaker. Ultimately, 100 μL of the obtained chromogen solution was added to each well to stop the enzyme catalysis. The plate was incubated for 30 min and was read at 500 nm. Quercetin was used as a standard. The percentage inhibition (I%) of the tested sample was calculated using the Eq.(1).

$$I\% = [\, A_{in.activ.} - A_{inhibitor} \,/\, A_{in.activ.} \,] \times 100 \quad (1)$$

where, $A_{in.activ.}$ is the absorbance of 100% initial activity wells without sample;

$A_{inhibitor}$ is the absorbance of sample/reference.

The sample concentration that gave 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. The outcomes of the analyses are expressed in terms of means ±SD of triplicates.

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

Chemical composition of A. angustiloba essential oil

Hydrodistillation of the fresh leaf of *A. angustiloba* yielded (w/w) 0.15% of essential oil. The list of chemical components identified in the essential oils is shown in Table 1. The analysis of *A. angustiloba* essential oil revealed the presence of 25 components with a percentage of 90.8%. The oil was characterized by a high concentration of oxygenated monoterpenes (64.2%). The oil composition was demonstrated by its richness in linalool (21.2%), 1,8-cineole (16.8%), *α*-terpineol (9.5%), terpinene-4-ol (8.5%), *β*-caryophyllene (6.2%), and caryophyllene oxide (5.2%). Other components identified in noteworthy levels (> 2.0%) were bornyl acetate (3.2%), spathulenol (3.2%), germacrene D (2.5%), *α*-terpinene (2.1%), and globulol (2.1%). In comparison to the previous studies, linalool has also been reported as the major component in various *A. scholaris* essential oils such as from Vietnam [14], Bangladesh [15], and India [16], which constituted 35.7%, 8.8%, and 28.14%, respectively. To the best of the authors' knowledge, this is the initial study that investigated the essential oil composition of *Alstonia angustiloba* collected from Malaysia.

Anti-inflammatory activity

In this present study, the anti-inflammatory activity was assessed by using lipoxygenase inhibitory activity. The essential oil demonstrated moderate activity (IC_{50} value 45.8 µg/mL), when compared to quercetin (IC_{50} value of 10.5 µg/mL). The high quantities of oxygenated monoterpenes detected in the essential oil may contribute, at least in part, to the anti-inflammatory activity ascribed to the plant.

Table 1

Chemical components identified in essential oil of *A. angustiloba* from Malaysia.

No.	*Components*	*KI*[a]	*KI*[b]	*Percentage (%)*	*Identifications*[c]
1	Camphene	946	945	0.5	RI, MS
2	*β*-Pinene	974	974	1.2	RI, MS
3	*α*-Terpinene	1015	1014	2.1	RI, MS
4	1,8-Cineole	1026	1025	16.8	RI, MS, Std
5	Linalool	1095	1092	21.2	RI, MS, Std
6	Terpinene-4 ol	1174	1175	8.5	RI, MS
7	*α*-Terpineol	1186	1185	9.5	RI, MS
8	Bornyl acetate	1287	1286	3.2	RI, MS
9	*δ*-Elemene	1335	1335	1.5	RI, MS
10	*α*-Copaene	1375	1374	0.8	RI, MS
11	*β*-Cubebene	1387	1387	0.2	RI, MS
12	*α*-Bergamotene	1410	1411	0.2	RI, MS
13	*β*-Caryophyllene	1415	1417	6.2	RI, MS, Std
14	Aromadendrene	1440	1439	0.2	RI, MS
15	*α*-Humulene	1450	1452	0.5	RI, MS
16	*α*-Amorphene	1480	1483	0.9	RI, MS
17	Germacrene D	1485	1484	2.5	RI, MS
18	*α*-Muurolene	1502	1500	1.5	RI, MS
19	*δ*-Cadinene	1520	1522	1.2	RI, MS
20	Spathulenol	1575	1577	3.2	RI, MS
21	Caryophyllene oxide	1582	1582	5.2	RI, MS, Std
22	Globulol	1590	1590	2.1	RI, MS
23	Guaiol	1602	1600	0.2	RI, MS
24	*t*-Muurolol	1642	1644	0.2	RI, MS
25	*α*-Cadinol	1650	1652	1.2	RI, MS
	Monoterpene hydrocarbons			3.8	
	Oxygenated monoterpenes			64.2	
	Sesquiterpene hydrocarbons			15.7	
	Oxygenated sesquiterpenes			12.1	
	Total identified (%)			90.8	

RI: based on comparison of calculated RI with those reported in Adams;
MS: based on comparison with Wiley, Adams, FFNSC2, and NIST08 MS databases;
Std: based on comparison with standard compounds;
[a]*Linear retention index experimentally determined using homologous series of C_6-C_{30} alkanes;*
[b]*Linear retention index taken from Adams, Wiley or NIST08 and literature;*

[c]*Quantification was done by the external standard method using calibration curves generated by running GC analysis of representative authentic compounds.*

In addition, linalool, a major component present in basil, bergamot, and bitter orange essential oils were also found to possess anti-inflammatory activities as demonstrated in carrageenan-induced edema in rats [20]. Generally, inflammation involves the formation of both prostaglandins and leukotrienes as mediators followed by the liberation of neutrophils and the production of reactive oxygen species (ROS). In mammals, lipoxygenases catalyze reactions on arachidonic acid that generate metabolites important to the mediation of inflammatory responses. Because of their involvement in lipid oxidation and inflammation, lipoxygenases have been implicated in the development of inflammatory vascular diseases such as atherosclerosis and diabetes [21]. Nevertheless, the outcomes of *in vitro* anti-inflammatory activity reported in this study demand verification with *in vivo* assays before considering the use of those essential oils in human care. This study also provides valuable and useful information and indications for further exploring the potential nutraceutical and pharmaceutical applications of the genus *Alstonia.*

Conclusions

The GC-FID and GC-MS analysis of the essential oil allowed us to identify oxygenated monoterpenes as the major group components with the presence of linalool, 1,8-cineole, *α*-terpineol, terpinene-4-ol, *β*-caryophyllene, and caryophyllene oxide as the most abundant components.

According to lipoxygenase inhibitory activity evaluation, the essential oil revealed moderate anti-inflammatory activity with IC_{50} value of 45.8 µg/mL. The species might be a source of natural products for further investigation into the development of new anti-inflammatory agents, which could be used as natural additives in the food, cosmetic and pharmaceutical industries. Thus, further phytochemical and biological studies should be carried out to identify their active constituents and toxicities.

Acknowledgments

The authors would like to thank the Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, Malaysia for providing research facilities.

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