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Chemical Profiling and Biological Activities of *Alphonsea elliptica* (Annonaceae) Essential Oil

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The chemical composition of the essential oil from the leaves of *Alphonsea elliptica* (Annonaceae) growing in Malaysia was investigated for the first time. The essential oil was obtained by hydrodistillation and fully characterized by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). A total of 45 components (98.4%) were successfully identified in the essential oil, which were characterized by high proportions of β-caryophyllene (20.2%), β-elemene (8.6%), α-humulene (8.5%), germacrene D (7.2%), and bicyclogermacrene (6.0%). The cytotoxicity of the essential oil was evaluated using an MTT assay. The essential oil exhibited cytotoxicity against three cancer cell lines, which are HepG2, MCF7, and A549, with the IC_{50} values 75.2, 65.8, and 68.5 µg/mL, respectively. The tested essential oil showed moderate *in vitro* lipoxygenase activity with an IC_{50} value 64.8 µg/mL. The present study highlights the potential of using essential oil as an alternative for developing chemopreventive or cosmetic agents for the pharmaceutical industry.

Keywords: *Alphonsea elliptica*; Annonaceae; essential oil; cytotoxicity; lipoxygenase; β-caryophyllene

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Annonaceae family is a part of the Angiosperma flowering plants, which was described as one of the largest in the family. They comprise approximately 128 genera and around 3,000 species [1]. *Alphonsea* is a genus that is labeled as part of the Annonaceae family. *Alphonsea* were commonly discovered in Hainan and South Yunnan, China, while in the Asian regions, they can be discovered around Cambodia, Sri Lanka, Papua New Guinea, India, Thailand, Myanmar, Laos, Vietnam, Malaysia, and Indonesia [2]. Phytochemical investigation of *Alphonsea* species resulted in the isolation of alkaloids [3], phenolics [4], and flavonoids [5]. Their extracts and phytochemicals have been reported for anticancer, antioxidant, antifungal, anti-inflammatory, and anti-trypanosomal activities [6].

Alphonsea elliptica is a tree which can grow up to 26 m tall. It is mainly distributed in Sumatra, Peninsular Malaysia, Borneo, and Thailand. In Peninsular Malaysia, it is locally known as *terbak* or *mempisang* and is found primarily in terrestrial (primary rainforest, freshwater swamp forest) and tropical forests [7]. It produces timber used for light construction, such as wooden packing crates and tool handles. Likewise, numerous *Alphonsea* species have served as a vital source of herbal supplements for centuries based on their wide range of medicinal properties [6]. The leaves and fruits decoction of *A. elliptica* are used indigenously for postpartum swelling, inflamed joints, and rheumatism in Southeast Asia countries, including Myanmar, Thailand, and Laos [8]. Besides, the bark and flower extracts reduce fever and diarrhea and stimulate menstrual flow in Vietnam and Indonesia [9]. Moreover, the stems are used to construct kayaks, boats, carts, and agricultural implements in Sarawak, Malaysia [10].

Essential oils, which serve as secondary metabolites, involve complex mixtures of natural compounds and versatile organic structures [11]. They are an involute cumulation of terpenic compounds, especially monoterpenes, sesquiterpenes, alcohols, aldehydes, ethers, esters, ketones, and phenols that are mainly responsible for aroma and odor. The oils are mainly responsible for the fragrance in spices and condiments and are used as a repellent agent in insecticides and herbicides [12]. Essential oils from aromatic and medicinal plants have been known to

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possess biological activity since antiquity, most notably antibacterial, antifungal, and antioxidant properties [13]. Researchers worldwide produce medicines from the essential oils of natural products like plants. Natural products have played a pivotal role in cancer chemotherapy and chemoprevention for over half a century and established anticancer drugs, e.g., camptothecin, doxorubicin, paclitaxel, vinblastine, and vincristine [14]. Hence, discovering new anticancer hits derived from natural sources is a feasible strategy and is in dire need.

As a continuation to our systematic study on pharmacologically active volatiles from Malaysian plants, this study evaluates the chemical compositions of the essential oils derived from the leaves of *A. elliptica*. To the best of our knowledge, this is the first report on the essential oil of this species and its cytotoxicity and lipoxygenase inhibitory activities.

EXPERIMENTAL

Plant Material

The leaves of *A. elliptica* were collected from Fraser Hill, Pahang (3.7119° N, 101.7366° E) (January 2022) and identified by Shamsul Khamis, a botanist from Universiti Kebangsaan Malaysia. The voucher specimen (SB-61-48) was deposited at UKM Herbarium.

Extraction of Essential Oil

The fresh leaves (300 g) were subjected to hydrodistillation in a Clevenger-type apparatus for 4 hours. The essential oil obtained was dried over anhydrous magnesium sulfate and stored at 4-6°C. The oil yield (w/w) was 0.19% based on a fresh weight basis.

Analysis of Essential Oil

Gas chromatography (GC-FID) analysis was performed on an Agilent Technologies 7890B equipped with a DB-5 capillary column (30 m long, 0.25 µm thickness, and 0.25 mm inner diameter). Helium was used as a carrier gas at a 0.7 mL/min flow rate. Injector and detector temperatures 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 triplicate. The peak area percentage was calculated using the GC HP Chemstation software (Agilent Technologies) [15]. Gas chromatography-mass spectrometry (GC-MS) chromatograms were recorded using an Agilent Technologies 7890A/5975C MSD equipped with HP-5MS fused silica capillary column (30 m long, 0.25 µm thickness and 0.25 mm inner diameter). Helium was used as the carrier gas at a 1 mL/min flow rate. 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 and electron, an 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 50-400 amu [15].

Identification of Essential Oil Components

For identification of essential oil components, co-injections with the standards (β-caryophyllene, β-elemene, α-humulene, germacrene D, and bicyclogermacrene) were used, together with the correspondence of their retention indices and mass spectra as reported in Adams [16], NIST 08 and FFNSC2 libraries. Semi-quantification of essential oil components was made by peak area normalization, considering the same response factor for all volatile components.

Cytotoxicity

The cytotoxicity of the essential oil against HepG2, MCF-7, and A549 cell lines was assessed using the MTT assay [17,18]. Cells were plated in a 96-well microplate at a density of 5×10^4 cells per well in a 200 µL mixture. An initial stock solution of the essential oil was prepared in dimethyl sulfoxide (DMSO). Subsequently, the essential oil samples were created by diluting the stock solution to achieve a range of concentrations from 1 to 100 µg/mL. These samples, along with doxorubicin used as a positive control (ranging from 0.05 to 1.56 µg/mL), were added to the 96-well microplate. The cells were then incubated at 37°C for 48 hours in a 5% CO_2 environment. Subsequently, 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) was added to the wells and further incubated at 37°C for 4 h. After that the formazan dye was dissolved with 100 µL of dimethyl sulphoxide. Absorbance was recorded at 540/720 nm using a Spark multimode reader (Tecan). Each experiment was conducted in triplicate.

Equation 1:

$$\text{Cell viability (\%)} = [1 - OD_{sample}/OD_{conc}] \times 100$$

where; OD_{sample} and OD_{conc} stand for the optical densities of the samples and the control, respectively. Data obtained from the cytotoxicity assay were expressed as mean values. 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. A probit analysis was carried out to determine the IC_{50} values using the SPSS version 11.0 software.

Lipoxygenase Inhibitory Activity

The LOX inhibition was largely based on the previous technique [19,20], in which 5 µL of essential oil was buffered with 1.74 mL borate (0.2 M, pH 9.2) and added with 5 µL (50,000 U/mL) of 5-LOX enzyme. The reaction began upon adding 250 µL linoleic acid

Table 1. Chemical components identified from *A. elliptica* essential oil

No	Components	KI[a]	KI[b]	Percentage (%)	Identification[c]
1	α-Pinene	932	930	1.0	RI, MS, Std
2	Camphene	946	944	1.1	RI, MS
3	α-Terpinene	1014	1016	1.2	RI, MS
4	Limonene	1033	1032	1.5	RI, MS
5	(Z)-β-Ocimene	1044	1045	0.5	RI, MS
6	γ-Terpinene	1055	1056	0.4	RI, MS
7	α-Terpinolene	1086	1085	0.6	RI, MS
8	Linalool	1092	1090	2.0	RI, MS
9	Borneol	1165	1165	1.2	RI, MS
10	Terpinen-4-ol	1175	1172	0.5	RI, MS
11	α-Terpineol	1186	1185	1.5	RI, MS, Std
12	δ-Elemene	1335	1335	0.4	RI, MS
13	α-Cubebene	1345	1345	0.5	RI, MS
14	α-Ylangene	1372	1373	0.1	RI, MS
15	α-Copaene	1374	1374	0.5	RI, MS
16	β-Bourbonene	1387	1388	0.5	RI, MS
17	β-Cubebene	1388	1387	0.6	RI, MS
18	**β-Elemene**	1390	1389	**8.6**	RI, MS, Std
19	Longifolene	1407	1407	0.2	RI, MS
20	α-Gurjunene	1409	1410	0.6	RI, MS
21	**β-Caryophyllene**	1415	1417	**20.2**	RI, MS, Std
22	β-Gurjunene	1431	1431	0.6	RI, MS
23	γ-Elemene	1435	1434	2.0	RI, MS
24	**α-Humulene**	1435	1436	**8.5**	RI, MS, Std
25	Aromadendrene	1439	1439	1.2	RI, MS
26	(*E*)-β-Farnesene	1455	1454	2.5	RI, MS
27	*allo*-Aromadendrene	1458	1458	1.0	RI, MS
28	Germacrene B	1460	1559	2.0	RI, MS
29	γ-Gurjunene	1475	1475	0.5	RI, MS
30	γ-Muurolene	1476	1478	0.2	RI, MS
31	α-Amorphene	1482	1483	2.2	RI, MS
32	**Germacrene D**	1484	1484	**7.2**	RI, MS, Std
33	β-Selinene	1485	1489	2.0	RI, MS
34	δ-Selinene	1490	1492	2.2	RI, MS
35	α-Selinene	1495	1498	2.1	RI, MS
36	**Bicyclogermacrene**	1500	1500	**6.0**	RI, MS, Std
37	α-Muurolene	1502	1501	2.2	RI, MS
38	δ-Cadinene	1520	1522	2.8	RI, MS
39	α-Cadinene	1538	1537	0.2	RI, MS
40	α-Calacorene	1544	1545	0.4	RI, MS
41	Spathulenol	1576	1577	2.0	RI, MS, Std
42	Viridiflorol	1595	1595	2.2	RI, MS
43	τ-Muurolol	1645	1644	1.8	RI, MS
44	β-Eudesmol	1648	1649	1.5	RI, MS
45	α-Cadinol	1650	1652	1.4	RI, MS
Group components					
	Monoterpene hydrocarbons			**6.3**	
	Oxygenated monoterpenes			**5.2**	
	Sesquiterpene hydrocarbons			**78.0**	
	Oxygenated sesquiterpenes			**8.9**	
Total identified				**98.4**	

[a]Linear retention index experimentally determined using a 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

(5 mg linoleic acid mixed with 15 µL ethanol and 15 mL borate in brisk shaking). The absorbance at 234 nm was assayed for 5 min in a UV-visible spectrophotometer (Genesys 10Se, Thermo Scientific, USA). Dimethyl sulfoxide (5 µL) served as a negative control, while quercetin (Sigma-Aldrich, St. Louis, MO, USA), a positive control, was prepared in the same strength as the essential oil. The percentage inhibition (I%), which is equivalent to the concentration of drug required for 50% inhibition (IC_{50}) in µg/mL, was computed by the equation below:

Equation 2:

$$\text{Inhibition (\%)} = [A_{\text{initial activity}} - (A_{\text{inhibitor}} / A_{\text{initial activity}})] \times 100$$

where; $A_{\text{initial activity}}$ is the absorbance of the control, and $A_{\text{inhibitor}}$ is the absorbance of the test sample. Averaging the absorbance values of triplicate and quercetin yielded the LOX inhibitory activity.

RESULTS AND DISCUSSION

The identified components with their percentages are listed in the order of their elution from the HP-5 column in Table 1. The GC-FID and GC-MS analysis of the essential oil revealed the presence of 45 components with the constitution of 98.4%. The essential oil was characterized by sesquiterpene hydrocarbons which constitute 78.0% of the total composition. Meanwhile, oxygenated sesquiterpenes, monoterpene hydrocarbons, and oxygenated monoterpenes were present in substantial amounts accounting for 8.9%, 6.3%, and 5.2% of the total composition, respectively. The most prominent components were β-caryophyllene (20.2%), β-elemene (8.6%), α-humulene (8.5%), germacrene D (7.2%), and bicyclogermacrene (6.0%). Other components identified in noteworthy levels (>2.0%) were δ-cadinene (2.8%), (*E*)-β-farnesene (2.5%), viridiflorol (2.2%), α-amorphene (2.2%), δ-selinene (2.2%), α-muurolene (2.2%), α-selinene (2.1%), linalool (2.0%), γ-elemene (2.0%), germacrene B (2.0%), β-selinene (2.0%), and spathulenol (2.0%).

In comparison with the previous report, β-caryophyllene has also been reported as the major component in other *Alphonsea* species. The large amount of β-caryophyllene identified in the oil of *A. elliptica* was consistent with the data obtained for the leaf oil of *A. tonkinensis* [21] and *A. monogyma* [22] previously analyzed from Vietnam. In addition, bicyclogermacrene, β-caryophyllene, and α-humulene present in *A. elliptica* were also present in the leaves oil of *A. philastreana* and *A. gaudichaudiana* [23]. According to chemotaxonomic analysis, the essential oils of Alphonsea plants from Asia can be classified as belonging to the group with a high content of sesquiterpene hydrocarbons. Conclusively, the qualitative and quantitative variations in the essential oils derived from the leaves of *A. elliptica* compared to other prior studies may be attributed to various factors, such as genetic variations, environmental conditions, harvesting time, drying period, or extraction temperature [24-26]. To the authors' knowledge, this is the first study that investigated the essential oil composition of *A. elliptica* collected from Malaysia.

The essential oil was subjected to cytotoxic examination using an MTT assay. The essential oil showed activity against three cancer cell lines HepG2, MCF7, and A549 with the respective IC_{50} values of 75.2, 65.8, and 68.5 µg/mL, as compared with those of the positive control doxorubicin (IC_{50} 0.76 µg/mL for HepG2, IC_{50} 0.20 µg/mL for MCF7, and IC_{50} 0.95 µg/mL for A549). At the highest concentration of 100 µg/mL, the essential oil responds with 82.5% inhibition at least. The antiproliferative effect of β-caryophyllene on several cancer cell lines was previously reported [27]. They found that treatment with β-caryophyllene obtained from the essential oils of *Aquilaria crassna* stem bark led to strong growth inhibition in two colon cancer cell lines, HCT-116 and HT-29, as well as in pancreatic cancer cells, PANC-1. In contrast, the other tested cancer cell lines demonstrated moderate susceptibility to β-caryophyllene. Interestingly, the compound also showed that human skin fibroblasts were resistant to *Commiphora gileadensis* stem extracts, in which β-caryophyllene was a major compound [28]. The present result suggests that the occurrence of this compound as the major component (20.2%) could be associated with the cytotoxic activity detected in the essential oil. However, a complete mechanistic study is required to understand the mode of cell death and the proposed molecular targets involved in the observed anticancer potential.

Arachidonate 5-lipoxygenase is the key enzyme in leukotriene biosynthesis and catalyzes the initial steps in converting arachidonic acid to biologically active leukotrienes [29]. Leukotrienes are considered potent mediators of inflammatory and allergic reactions. Regarding their pro-inflammatory properties, inhibiting the 5-lipoxygenase pathway is considered interesting in treating a variety of inflammatory diseases [30]. Besides 5-lipoxygenase inhibitors, drugs able to block the 5-lipoxygenase and the cyclooxygenase metabolic pathway are also of therapeutic value [31]. The LOX inhibitory effect of essential oil extracted from the bark was modest, with an inhibition of 64.8%, compared to that of quercetin, with an inhibition of 92.5%. Sesquiterpene hydrocarbons of essential oils extracted from various plant species (e.g., *Syzygium aromaticum*, *Cannabis sativa*, *Rosmarinus officinalis*, and *Tagetes minuta*) were generally reported to show anti-inflammatory effects [32,33]. Hence, it is plausible that the high amount of β-caryophyllene, β-elemene, α-humulene, germacrene D, and bicyclogermacrene in the essential oil of *A. elliptica* might at least partially contribute to the anti-inflammatory potential. This study provides valuable and useful information and indications for further exploring the genus Annonaceae's potential nutraceutical and pharmaceutical applications. The next phase of our research will involve evaluating the essential

oil's in vivo effects, aiming to harness the ecological significance of this species. This step is pivotal for optimizing its potential in scientific and ecological contexts, ensuring a comprehensive understanding of its applications.

CONCLUSION

The medicinal plant of *Alphonsea* has exhibited various biological and pharmacological activities. To date, many traditional medicines have used this medicinal ingredient. The sesquiterpenes have many biological activities, particularly inhibition activities against cancer cells. Thus, this study has identified the main components of the sesquiterpene group present in the medicinal plant *A. elliptica* and evaluated the inhibition activity against cancer cell lines HepG2, MCF7, and A549, which are important in providing a more scientific basis for the use of this plant in the healthcare industry. Future studies are also required to evaluate the side effects, safety, and efficacy of the essential oil from *Alphonsea* species to facilitate their clinical applications as modern medicines for human health.

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