Chemical Constituents and Toxicity Screening of Three Aromatic Plant Species from Peninsular Malaysia

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Medicinal and aromatic plants (MAPs) are widely valued for their aromas, tastes, and treatments for various human illnesses. The chemical constituents and toxicity content of three aromatic plant species, *Syzygium polyanthum* Wight (Walp.), *Monocarpia marginalis* (Scheff.) J. Sinclair, and *Chromolaena odorata* (L.) R.M. King & H. Rob, were determined, *via* gas chromatography (GC) with mass spectrometry (MS), and flame ionization detector (FID). Altogether, 116 compounds were identified in the essential oils and hexane and methanol extracts. Toxicity evaluations were carried out on human peripheral blood mononuclear cells (PBMCs). Three plant samples were found toxic to human PBMCs. The essential oils of *M. marginalis* and *C. odorata,* and the hexane extract of *C. odorata* yielded IC_{50} and LD_{50} values of 76 mg/mL and 6,913 mg/kg, 14 mg/mL and 3,684 mg/kg, and 2.45 mg/mL and 1,927 mg/kg, respectively. Based on the LD_{50} values, *M. marginalis* and *C. odorata* can be classified as slightly and moderately hazardous, respectively. A detailed toxicity evaluation *via* comet assay showed that *M. marginalis* and *C. odorata* induced significant DNA damage ($p < 0.05$). As for *S. polyanthum*, the species did not give any cytotoxic or genotoxic evidences.

Keywords: Syzygium polyanthum; Monocarpia marginalis; Chromolaena odorata; Cytotoxicity; Genotoxicity

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INTRODUCTION

As many as 20,000 species of medicinal and aromatic plants (MAPs) have been utilized for their medicinal and aromatic characteristics (World Health Organization 2002). At present, approximately 3,000 essential oils have been discovered, of which 300 are applied in various industries including pharmaceutical (Teixeira *et al.* 2013). The use of products derived from aromatic plants has become a trend among consumers (Raut and Karuppayil 2014) and is preferred in cosmetics, perfumes, confectionery food (Bialecki and Smadja 2014), and medicines (Dutra *et al.* 2016). Medicinal plants are significant sources of specific constituents that can be used as treatment for several illnesses. Certain compounds acquired from natural sources can be utilized as prototypes for the synthesis of pharmaceuticals with slight modifications to make them less or non-toxic (Sponchiado *et al.* 2016). Different parts of medicinal plants, such as the leaf and stem, contain different

active constituents. The amount and composition of essential oils and aroma-active compounds are influenced by their method of extraction and solvent used (Cowan 1999). In the industrial use of plant extracts for aromatic and medicinal product development, toxicity screening of the extracts is essential (Bakkali *et al*. 2008). Toxicity studies are conducted to differentiate the toxicity level of each composition of the plant species (Vigan 2010). The tests for toxicity at the cellular and genomic levels are crucial to address human safety when consuming and applying oils and extracts of plants (Sirikhansaeng *et al.* 2017).

Malaysia is endowed with bounties of plants that have aromatic and medicinal properties. *Syzygium polyanthum* Wight (Walp.), commonly known as bay leaf, *serai kayu* or *salam*, belongs to the botanical family Myrtaceae (Hamad *et al.* 2016). This species is a tree that can grow up to 30 m in height and 60 cm in diameter at breast height (dbh). It is widely distributed throughout Southeast Asia, including in the countries Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand, and Vietnam (Widyawati *et al*. 2015a). Local people acknowledge its medicinal values in fighting several illnesses, such as gastritis, hypercholesterolemia, skin diseases, and diabetes mellitus (Ismail *et al.* 2013; Widyawati *et al.* 2015b). The leaves are consumed by Malaysian natives in spices and local cuisines. Several bioactivities have been reported like antidiarrheal, antihypertensive, antimicrobial (Hidayati *et al*. 2017), and antioxidant (LeeWei and Ismail 2012). The leaf extracts mostly contain sesquiterpene compounds of eugenol- and methyl chavicol-types (Dalimartha 2000; Lumowa and Nova 2015). No cytotoxic activity has been reported towards 9KB (nasopharynx cancer), P388 (murine leukemia), and other cancer cell lines (Kusuma *et al.* 2011).

Monocarpia marginalis (Scheff.) J. Sinclair is a huge tree that reaches 30 m in height (Annonaceae). It is commonly known by natives as *mempisang* and can be found in lowland forests (Faridah-Hanum *et al.* 2001). This species is native to Malaysia, and has been extensively distributed throughout Borneo, Sumatera, and Thailand (Turner 2012). The ethnomedicinal properties of this species have been rarely studied. Two compounds that were previously isolated from *M. marginalis* essential oil are monocarpin and monomargine (Mahmood *et al*. 1993). Cytotoxic effect of the essential oil has been reported on P388 leukemia and KB human epidermal carcinoma cells (Taha *et al.* 2013).

Chromolaena odorata (L.) R.M. King & H. Rob is a perennial shrub (Asteraceae) (Suksamrarn *et al.* 2004) that grows extensively until 3 m to 7 m in height (Joshi 2013). It is frequently identified as 'Siam' weed, and is locally known as *kapal terbang*. This species has been introduced throughout Southeast Asia from its native Central and North American origins (Kouamé *et al.* 2013). This plant is traditionally utilized for skin diseases and wound healing treatments (Joshi 2013) because of the abundance of essential oil in the leaves and stem (Moni and Subramoniam 1960). It has a wide range of bioactivities such as analgesic, antihelmintic, antiinflammatory, antimalarial, antimicrobial, and antioxidant (Omokhua *et al.* 2016). The chemical constituents of the essential oil include pregeijerene, germacrene D, α-pinene, β-caryophyllene, vestitenone, β-pinene, geijerene, bulnesol, and trans-ocimene (Pisutthanan *et al.* 2006). Dichloromethane extracts of this plant had significant cytotoxic effect against Vero monkey kidney cells (Omokhua *et al.* 2017).

The determination of aroma-active plants composition is important because each compound carries significant properties that can be manipulated for fragrance and pharmaceutical purposes. Many plant properties are still not examined for their potential of being poisonous and hazardous to humans; therefore, it is crucial to study the chemical constituents and toxicity properties of plant extracts. This study is the first report on the

cytotoxicity and genotoxicity of three selected plant species, *S. polyanthum*, *M. marginalis*, and *C. odorata*, on human peripheral blood mononuclear cells (PBMCs).

EXPERIMENTAL

Materials

Three aromatic plant species, *S. polyanthum*, *M. marginalis*, and *C. odorata*, were selected in this study. They were collected from the Ayer Hitam Forest Reserve (AHFR), Selangor, Malaysia. The AHFR is located within the Sultan Idris Shah Forestry Education Centre (SISFEC). The SISFEC is a field center at the Universiti Putra Malaysia (UPM) designed for teaching and learning. The AHFR is rich in aromatic and medicinal plants. These pleasant-smelling plants have served the local aborigine community in treating some illnesses. The leaf and stem parts were utilized for essential oil and crude extractions. Voucher specimens of these species were prepared and deposited in the Herbarium of Faculty of Forestry, UPM. The leaves and stems were air-dried for 7 d before being pulverized into powder and used in the extraction steps.

Extraction process- Hydrodistillation

Hydrodistillation was performed using a Clevenger-type apparatus. Fifty g of powdered material were immersed in 650 mL of deionized water in a distillation flask for 10 min, which was subsequently heated at 100 °C for 3 h. The volatile compounds were dissolved in 10 mL of hexane (J.T. Baker, Center Valley, PA, USA). The essential oils were collected, dried over anhydrous sodium sulphate to remove excess water, and stored in vials that were refrigerated prior to chemical analysis. The percentage yield of the essential oil was calculated according to Eq. 1 (Kasim *et al.* 2014).

$$Percentage\ of\ essential\ oil\ (\%) = \frac{Weight\ of\ oil\ (\mathrm{g})}{Weight\ of\ sample\ (\mathrm{g})}\ x\ 100 \quad (1)$$

Solvent extraction

Twenty g of powdered material were soaked separately in 200 mL of hexane or methanol (Merck, Darmstadt, Germany) for 3 d at room temperature under dark conditions. Then, the samples were filtered rapidly using Whatman No. 1 filter paper. The plant extracts were concentrated using a rotary evaporator (Rotavapor R-210, Buchi, Flawil, Switzerland) at 60 °C and 100 rpm to obtain the crude extracts, which were stored at -20 °C for further analysis. Similarly, the yield percentage of the extract was calculated on a dry weight basis, as shown in Eq. 1.

Methods

Gas chromatography (GC) analysis

Gas chromatography 7890A (GC) analyses were performed according to standard protocols (Agilent Technologies, Boeblingen, Germany) with mass spectrometry 5975C (MS) and flame ionization detector (FID). The compounds were separated on a 30 m × 0.25 mm × 0.25 µm fused silica capillary column bonded with 100% dimethylpolysiloxane (DB-1 ms, Agilent Technologies, Boeblingen, Germany). The helium gas acted as the carrier gas at a flow rate of 1.3 mL/min, and the injector temperature was set at 230 °C. The heating program increased the oven temperature to 60 °C, the temperature was held there for 3 min,

and it was then increased to 240 °C at 3 °C/min with a second hold for 5 min. An adequate amount of extract (1 µL) was injected with a splitless ratio. The detector temperature was held at 250 °C. An electron ionization system with an ionization energy of 70 eV was applied for MS, and compounds identification were done *via* the comparison with integrated National Institute of Standards and Technology (NIST) library. Meanwhile, compounds identification for GC-FID were done *via* comparison of Kovats retention indices. Quantification of the extracts for GC-MS was computed as the percentage of the peak area from the integration data.

Isolation of human peripheral blood mononuclear cells (PBMCs)

The PBMCs from a healthy donor were separated using Ficoll-Paque Plus (GE Healthcare, Bangkok, Thailand) as recommended by the manufacturer. The blood was centrifuged at 2,200 rpm for 40 min at room temperature. The PBMC plasma layers were discarded using sterile phosphate buffered saline (PBS) to remove the remaining erythrocytes. Fresh PBMCs with densities of at least 4×10^5 cells/mL were diluted with RPMI 1640 medium that contained 2.05 mM L-glutamine and 25 mM HEPES, supplemented with 10% fetal bovine serum (FBS, GE Healthcare, Bangkok, Thailand), 5 µg/mL phytohemagglutinin (PHA), 100 µg/mL streptomycin, and 100 U/mL penicillin. The RPMI medium and L-glutamine were from Gibco (Scoresby, Australia) and PHA, streptomycin, and penicillin were from Applichem (Chesire, UK).

Cytotoxicity assay

The cytotoxicity test on the PBMCs was conducted through the 3-(4,5-dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). In each well of a 96-well flat microtiter plate, 125 µL of PBMCs were seeded at a density of 1×10^6 cells/mL. From five working concentrations, 12.5 µL of the samples were added to their respective wells in triplicates and subsequently incubated in a humidified incubator (ThermoFisher, Paisley, UK) at 37 °C and 5% CO_2 for 4 h. Untreated cells were included as negative control. After incubation, the microtiter plates were centrifuged at 1,500 rpm in a laboratory centrifuge (LMC-3000, Biosan, Cardiff, UK) for 10 min, and the supernatants were discarded. Upon removal, 10 µL of MTT solution (Sigma, St. Louis, MO, USA) were added to each well. The plate was incubated for 4 h at 37 °C with 5% CO_2 in a humidified incubator. The formazan crystals that formed were solubilized in 100 µL of dimethyl sulfoxide (DMSO, Fisher Scientific, Loughborough, UK). The absorbance was recorded at 570 nm with a fluorescence microplate reader (SpectraMax M5 series, Molecular Devices, Sunnyvale, CA, USA). The cytotoxicity of the plant extracts was measured by the cellular reduction of the violet crystal formazan through the mitochondrial succinate dehydrogenase activity of the viable cells (Freshney 2010). The percentages of viable cells were calculated, and presented as cell viability (%) ± standard deviation (SD). The dose inducing 50% of cell viability (IC_{50}) was ascertained from the graph plotted from the sample concentration against the percentage of cell viability. The lethal dose required to kill 50% of a sample population (LD_{50}) was calculated from the IC_{50} values to determine the hazardous level of the plant extracts set by the World Health Organization (2004).

Genotoxicity assay

The genotoxic effects of the plants were tested on PBMCs through the comet assay (Singh *et al.* 1988). The comet assay was performed after obtaining the IC_{50} value. If the IC_{50} value was not available for a sample, the maximum concentration of the sample from

the cytotoxicity test was utilized. The PBMCs with the extracts were incubated and underwent lysis, phoresis, and washing steps. The negative control cells were incubated solely in the medium, and the positive control cells were treated under ultraviolet (UV) light for 30 min. Low melting agar (LMA, Sigma, St. Louis, MO, USA) and RPMI medium were immersed in boiling water until they melted. One hundred μL of LMA and 100 μL of the sample were inserted into a microcentrifuge tube and mixed well. One hundred μL of the mixture were placed onto a glass slide and covered with a sterile cover slip. The slide was kept at 4 °C for 10 min to prevent denaturation of the cell suspension. The slide was then placed in a jar containing lysis solution (8 M NaCl, 0.6 M Ethylenediaminetetraacetic acid (EDTA) at pH 8, 0.2 M tris, 0.1% triton X-100) and kept at 4 °C for 1 h. An electrophoresis buffer (6 mM EDTA at pH 10, 0.75 M NaOH) was added into the electrophoresis tank (Cleaver Scientific, Warwickshire, UK) without covering the slide surface. Electrophoresis was performed at 4 °C for 40 min. The power supply was turned on (26 V, 300 mA) for 25 min. Tris buffer (0.4 M at pH 7.5, Vivantis, Oceanside, CA, USA) was added at 4 °C for 5 min, which was repeated twice for slide washing purposes. Forty μL of the diluted 1 μg/mL ethidium bromide was added onto the slide and covered with a cover slip. The slide was left at 4 °C overnight. Comet images were captured using the LUCIA software (Version 5.8, Laboratory Imaging, Prague, Czech Republic) attached to the florescence microscope (Nikon, Tokyo, Japan) with a 200× magnification. Total images of 150 comets were acquired for each sample. The level of DNA damage was determined using the Comet Assay Software Project (CASP, Wrocław, Poland) to measure the Olive Tail Moment (OTM), which is the relative amount of DNA in the tail of the comet multiplied by the median migration distance. All of the values were expressed as the median, and the statistical analyses of the comet assay were measured using GraphPad Prism version 5.01 software (La Jolla, CA, USA) with the nonparametric Mann-Whitney test. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Yield Percentages of Essential Oils and Crude Extracts

In this study, the hydrodistillation and solvent extractions were performed to isolate the essential oils and crude extracts from the leaf and stem parts of three selected MAP species commonly found in Peninsular Malaysia (Table 1).

Table 1. Yield Percentages from the Leaf and Stem Extracts from Three Plants

Plant Species	Type of Extract	Yield Percentage (%)	
		Leaf	Stem
Syzygium polyanthum	EO	0.24	0.09
	HE	0.6	0.2
	ME	11.7	1.0
Monocarpia marginalis	EO	0.48	0.06
	HE	1.2	0.1
	ME	9.6	0.9
Chromolaena odorata	EO	0.8	0.18
	HE	1.5	0.4
	ME	12.9	2.1

EO: Essential oil; HE: Hexane; and ME: Methanol

Of the two plant parts, the leaf had the highest yield for all three species. *C. odorata* consistently produced the highest yield for the extraction methods used. The crude extraction using different solvents, *i.e.* hexane and methanol, had different yields. Methanol resulted in a higher yield percentage of crude extracts from the leaves and stems compared to hexane, at approximately 10% difference. This was due to hexane having a higher compound selectivity because it is a non-polar solvent (Aleksovski *et al.* 1998).

Identification of Chemical Constituents

The aromatic properties of the *S. polyanthum*, *M. marginalis*, and *C. odorata* leaves and stems, investigated by means of GC-MS and GC-FID, consisted of 116 compounds. Of these, 38 were found in the essential oils, and the rest in the solvent crude extracts. The qualitative and quantitative compositions of the essential oils with their percentage areas and retention indices (RI) on a DB-1 capillary column are shown in Table 2. The major constituent identified in the essential oils from the leaves of *S. polyanthum*, *M. marginalis*, and *C. odorata* was trans-β-nerolidol (30.87%), ledol (32.82%), and α-cadinol (4.82%), respectively. As for the stem, cubenol (14.15% in *S. polyanthum*) and n-hexadecanoic acid (50.73% in *M. marginalis* and 42.02% in *C. odorata*) were abundant in the three species.

In the solvent extracts, a total of 27 and 51 compounds were identified in the hexane (Table 3) and methanol (Table 4) extracts, respectively. The main constituents detected were trans-β-nerolidol in *S. polyanthum* (6.7%=leaf and 25.65%=stem), α-cedrene in *M. marginalis* (11.33%=leaf), n-hexadecanoic acid in *M. marginalis* (15.96%= stem) and *C. odorata* (9.32%=stem), and γ-muurolene in *C. odorata* (4.49%=leaf). Compound classes, such as fatty acid, monoterpenes, oxygenated sesquiterpenes, sesquiterpene hydrocarbons, and other derivatives, were found in the crude extracts. The sesquiterpenes were common in the essential oils and methanol extracts, but not in the hexane extracts except for *M. marginalis* stem.

The saturated fatty acid, n-hexadecanoic acid is commonly found in the essential oils and crude extracts. It exerts anti-inflammatory function and is widely utilized in producing soaps, cosmetics, and for other pharmaceutical products (Aparna *et al.* 2012; Chaveerach *et al.* 2016); however, it is not aromatic. The aromatic properties in the three plant species are contributed mainly by the terpene group compounds, such as nerolidol, α-cadinol, α-cedrene, cubenol, ledol, and γ-muurolene, which were detected at high levels in both the essential oils and methanol extracts. Aromatic plants are known to contain mixtures of terpenoids, particularly monoterpenes (C10) and sesquiterpenes (C15), with a small fraction of diterpenes (C20) in some plants (Carson and Hammer 2011). Sesquiterpenes are classified as a major component in all plant species and are often utilized in making aromatherapy products such as perfumes, creams, soaps, fragrances, and flavors (Bakkali *et al*. 2008). Previous results demonstrate similar compounds in the essential oil of *C. odorata* leaf. This includes α-cadinol (2.19%), γ-cadinene (0.85%), δ-cadinene (3.5%), and germacrene D (2.1%) (Joshi 2013; Pitakpawasutthi *et al.* 2016).

Non-essential oils such as linoleic acid, methyl octadecenoate, and phytol were also isolated in the methanol extract because this method depends on the solubility of the solvent used (Richter and Schellenberg 2007). Phytol is known to have antiallergic, anti-inflammatory, and antioxidant effects (Sirikhansaeng *et al.* 2017).

Table 2. Chemical Constituents of the Essential Oils from the Leaves and Stems of Three Different Plant Species

Compounds	RI[a]	Relative Peak Area (%)					
		Syzygium polyanthum		*Monocarpia marginalis*		*Chromolaena odorata*	
		Leaf	Stem	Leaf	Stem	Leaf	Stem
Monoterpene							
p-Cymene	1013	-	-	-	2.00	-	-
Sesquiterpene							
Sesquiterpene hydrocarbon							
Copaene	1378	2.09	-	-	-	-	-
β-Cubebene	1390	-	-	-	-	0.67	-
Longifolene	1399	-	-	-	-	2.81	0.68
α-Guaiene	1437	0.47	-	-	-	-	-
allo-Aromadendrene	1445	-	-	-	-	1.11	-
β-Selinene	1466	0.46	0.20	1.15	-	0.33	-
γ-Gurjunene	1470	-	-	-	0.60	-	-
Germacrene D	1477	-	-	-	-	0.67	-
α-Selinene	1479	0.59	-	-	-	-	-
β-Guaiene	1483	-	0.19	-	-	0.76	-
α-Farnesene	1492	-	0.44	-	-	0.99	-
γ-Cadinene	1494	-	-	-	-	1.95	-
α-Elemene	1505	0.43	1.20	-	0.84	-	-
δ-Cadinene	1518	-	0.25	-	0.62	1.69	-
Oxygenated sesquiterpene							
Elemol	1520	-	0.53	1.80	-	-	-
Nerolidol	1543	0.54	2.30	0.90	0.73	0.96	-
trans-β-Nerolidol	1548	30.87	-	1.52	-	-	-
Ledol	1551	0.60	1.60	32.82	0.76	3.26	-
Globulol	1559	-	0.92	12.37	1.10	4.81	-
Spatulenol	1564	1.35	1.30	0.81	0.51	0.92	0.44
Viridiflorol	1568	2.26	1.14	-	0.63	1.28	-
β-Spathulenol	1577	0.51	0.36	2.49	5.77	0.20	0.40
Caryophyllene oxide	1578	1.42	2.65	1.32	1.76	1.60	1.27
Humulene oxide II	1599	2.27	1.65	1.94	3.48	1.30	-
Cubenol	1608	2.28	14.15	2.17	-	4.58	-
τ-Cadinol	1615	-	4.62	0.52	5.88	2.47	-
α-Cadinol	1622	5.58	6.92	4.81	1.19	4.82	0.59
β-Eudesmol	1623	-	-	0.85	3.73	3.78	-
τ-Muurolol	1631	-	0.81	5.71	1.28	1.94	-
Hinesol	1636	-	1.15	2.12	6.32	-	-
Cadalene	1653	-	2.07	0.67	-	0.68	1.49
cis-Farnesol	1661	-	-	0.95	-	-	-
Germacrone	1677	-	-	-	1.17	-	-
Farnesol	1707	6.23	0.63	1.11	0.71	0.70	0.54
Fatty acid							
Methyl tetradecanoate	1718	-	0.50	1.01	0.60	-	-
10-Methylnonadecane	1943	1.94	-	-	1.44	1.11	-
n-Hexadecanoic acid	1963	2.34	11.20	-	50.73	2.80	42.02

[a] retention indices using a DB-1 column; '-' indicates that the compound was not detected in the GC analysis

Table 3. Chemical Constituents of the Hexane Extracts from the Leaves and Stems of Three Different Plant Species

Compounds	RI[a]	Relative Peak Area (%)					
		Syzygium polyanthum		*Monocarpia marginalis*		*Chromolaena odorata*	
		Leaf	Stem	Leaf	Stem	Leaf	Stem
Monoterpene							
o-Cymene	1009	4.49	4.03	3.59	3.52	1.07	1.08
Sesquiterpene							
Sesquiterpene hydrocarbon							
Longifolene	1399	-	0.42	-	0.24	0.13	-
α-Cedrene	1404	-	-	0.28	-	-	-
allo-Aromadendrene	1445	-	-	0.15	-	-	-
α-Farnesene	1492	-	0.21	-	0.23	-	-
α-Cadinene	1522	-	-	0.14	0.12	-	-
Oxygenated sesquiterpene							
trans-β-Nerolidol	1548	-	0.95	0.75	-	-	-
Ledol	1551	-	-	-	2.18	-	-
Globulol	1559	-	-	-	0.52	-	-
Spatulenol	1564	-	-	0.20	-	-	-
β-Spathulenol	1577	-	-	0.45	-	-	-
Caryophyllene oxide	1578	-	-	-	0.51	-	-
Humulene oxide II	1599	-	0.22	-	1.11	-	-
Cubenol	1608	-	-	-	0.37	-	-
τ-Cadinol	1615	-	-	-	0.61	-	-
α-Cadinol	1622	-	-	-	0.32	-	-
β-Eudesmol	1623	-	0.29	-	0.31	-	-
Hinesol	1636	-	-	-	1.90	-	-
cis-Farnesol	1661	-	-	-	0.42	-	-
Farnesol	1707	0.36	0.70	0.57	0.66	0.17	0.14
Fatty acid							
Methyl tetradecanoate	1718	-	-	0.43	0.66	-	-
Tetradecanoic acid	1772	-	-	-	0.49	-	-
1-Octadecene	1793	-	-	0.58	0.38	-	-
Pentadecanoic acid	1869	-	-	0.19	0.32	-	-
10-Methylnonadecane	1943	0.70	-	1.57	-	-	-
n-Hexadecanoic acid	1963	0.40	1.09	0.32	0.91	0.74	0.35
Other							
Mesitylene	952	4.22	3.95	3.35	3.31	1.01	1.02

[a] retention indices using a DB-1 column; '-' indicates that the compound was not detected in the GC analysis

The solvent extraction process depends on the solubility behavior of the extracted components according to the solvent polarity, while hydrodistillation is attributed to their steam volatility. Different polarities of solvents isolate different groups of compounds (Cowan 1999). Non-polar solvents extract alkaloids, terpenoids, fatty acids, and coumarins, semi-polar solvents extract flavanols, flavonoids, and terpenoids, while polar solvents isolate polyphenols, terpenoids, tannins, and saponins (Aqil *et al*. 2010). As a result, non-essential oils were also acquired during our extraction steps. Essential oils are volatile because they evaporate extensively through exposure to normal temperatures (Inoue and Craker 2014). Hence, low or non-volatile components cannot be classified into the essential oil group.

Table 4. Chemical Constituents of the Methanol Extracts from the Leaves and Stems of Three Different Plant Species

Compounds	RI[a]	Relative Peak Area (%)					
		Syzygium polyanthum		*Monocarpia marginalis*		*Chromolaena odorata*	
		Leaf	Stem	Leaf	Stem	Leaf	Stem
Monoterpene							
Nerol	1210	1.47	0.45	-	-	-	0.47
Sesquiterpene							
Sesquiterpene hydrocarbon							
α-Longipinene	1338	-	-	0.27	-	0.24	-
α-Cubebene	1346	0.21	1.39	1.54	0.62	0.22	0.52
α-Ylangene	1362	-	4.40	-	-	1.32	0.12
β-Maaliene	1375	4.93	-	-	-	-	-
Copaene	1378	0.35	-	1.42	-	0.49	-
β-Elemene	1388	-	-	-	1.21	0.27	0.73
β-Cubebene	1390	-	-	-	1.17	0.20	0.34
Longifolene	1399	0.27	-	-	1.94	1.20	0.32
α-Cedrene	1404	-	-	11.33	-	3.85	1.31
α-Gurjunene	1410	-	4.03	-	-	0.42	-
Isocaryophyllene	1419	-	1.19	0.20	-	0.32	-
α-Guaiene	1437	0.55	-	-	-	-	-
β-Gurjunene	1433	0.56	-	-	-	0.74	-
Aromandendrene	1439	4.96	-	1.38	0.60	1.17	-
allo-Aromadendrene	1445	-	0.75	-	-	0.10	0.25
α-Humulene	1447	1.12	-	-	-	-	-
γ-Muurolene	1462	-	-	-	-	4.49	0.79
β-Selinene	1466	3.82	-	-	-	0.13	-
α-Amorphene	1469	-	-	0.93	-	0.51	-
γ-Selinene	1472	0.57	-	-	-	-	-
Germacrene D	1477	3.86	0.72	1.39	0.43	1.11	0.19
α-Muurolene	1484	1.05	0.60	-	-	-	-
α-Farnesene	1492	0.53	-	-	1.31	0.37	-
Oxygenated sesquiterpene							
Elemol	1520	-	-	0.37	-	0.28	-
Nerolidol	1543	-	-	-	-	1.15	0.20
trans-β-Nerolidol	1548	6.70	25.65	6.30	0.56	2.07	0.17
Ledol	1551	-	0.89	-	-	1.67	-
Globulol	1559	-	1.23	-	0.56	2.88	-
Spatulenol	1564	0.42	1.40	8.16	0.64	0.53	0.45
Viridiflorol	1568	0.35	1.83	-	-	-	-
β-Spathulenol	1577	0.42	0.52	0.77	-	-	0.17
Caryophyllene oxide	1578	-	1.81	0.67	-	0.85	0.49
Humulene oxide II	1599	-	0.88	2.20	-	0.96	0.21
Cubenol	1608	0.43	1.86	-	-	-	-
τ-Cadinol	1615	0.82	1.49	0.78	0.75	0.36	0.13
α-Cadinol	1622	-	-	0.98	-	0.67	-
β-Eudesmol	1623	3.76	1.75	0.83	-	0.84	-
τ-Muurolol	1631	-	0.54	-	-	-	-
Hinesol	1636	-	4.28	-	-	1.05	-
Cadalene	1653	-	-	1.07	-	0.29	0.17
cis-Farnesol	1661	-	-	0.88	-	-	-
Germacrone	1677	0.46	-	0.54	-	-	0.40
Farnesol	1707	1.89	4.98	0.71	0.48	-	-

Fatty acid							
Methyl tetradecanoate	1718	1.36	-	1.01	-	0.39	-
Tetradecanoic acid	1772	0.46	-	-	0.51	0.19	-
Pentadecanoic acid	1869	0.45	-	-	-	1.29	0.60
n-Hexadecanoic acid	1963	2.90	2.80	2.80	15.96	4.32	9.32
Linoleic acid	2107	-	-	2.25	-	2.00	1.83
Methyl octadecanoate	2109	-	-	0.73	-	-	-
Other							
Phytol	2105	2.77	-	3.37	-	2.97	4.01

[a] retention indices using a DB-1 column; '-' indicates that the compound was not detected in the GC analysis

In this study, compounds that possessed RI values of 2,000 and above were discovered in the crude extracts, but not in the essential oils. This correlates to a previous study by de Castro *et al.* (1999), who reported that compounds with an RI > 2,000 are classified as non-essential oils.

Cytotoxic Effects of the Plant Extracts

A toxicological assessment is essential to clarify the possible risks associated with human usage of plants. Toxicity testing is a required step before a specific plant material can be considered safe for human consumption and other applications (Mellado-García *et al.* 2017). The cytotoxicity and genotoxicity tests were designed to detect the extent of damage caused by the constituents in a plant on human cells at the cellular and genomic levels, respectively. The materials used in a toxicity assessment are crucial, particularly the procedures used and type of cells tested (Nabeshi *et al.* 2011). The PBMCs consisting of lymphocytes are commonly chosen for conducting cytotoxicity and genotoxicity testing of plant extracts because they are vulnerable to free radicals, which results in damaging effects (Heaton *et al.* 2002). Furthermore, the cells are considered a suitable indicator of the actual state of the body (Kassie *et al.* 2000).

In this study, the *in vitro* cytotoxicity of *S. polyanthum, M. marginalis*, and *C. odorata* leaf extracts were evaluated using human PBMCs. The leaf extracts were utilized because they are rich in active compounds. The data obtained from this assay revealed the dose response relationship with regards to the cytotoxic properties of the plant species. There was a gradual decrease in the cell viability of the PBMCs with increased concentration of the plant extract (Fig. 1). Only the essential oils of *M. marginalis* and *C. odorata*, and the hexane extract of *C. odorata* had cytotoxicity effect to PBMCs (cell viability < 50%) (Table 5). Their IC_{50} and LD_{50} values were calculated and indicated in the respective figures (Fig. 1). The essential oils and extracts from *S. polyanthum* did not cause any significant cell injury (Figs. 1a, 1b, and 1c), while it was observed in the essential oil of *M. marginalis* (Fig. 1d). The essential oil and hexane extract of *C. odorata* were considered toxic with a gradual decrease in cell proliferation (Figs. 1g and 1h), while methanol extract was non-toxic, at least at the cellular level (Fig. 1i). From the LD_{50} values, it can be concluded that the essential oil of *M. marginalis* had a Class III toxicity level (slightly hazardous), while the essential oil and hexane extract of *C. odorata* were both Class II (moderately hazardous).

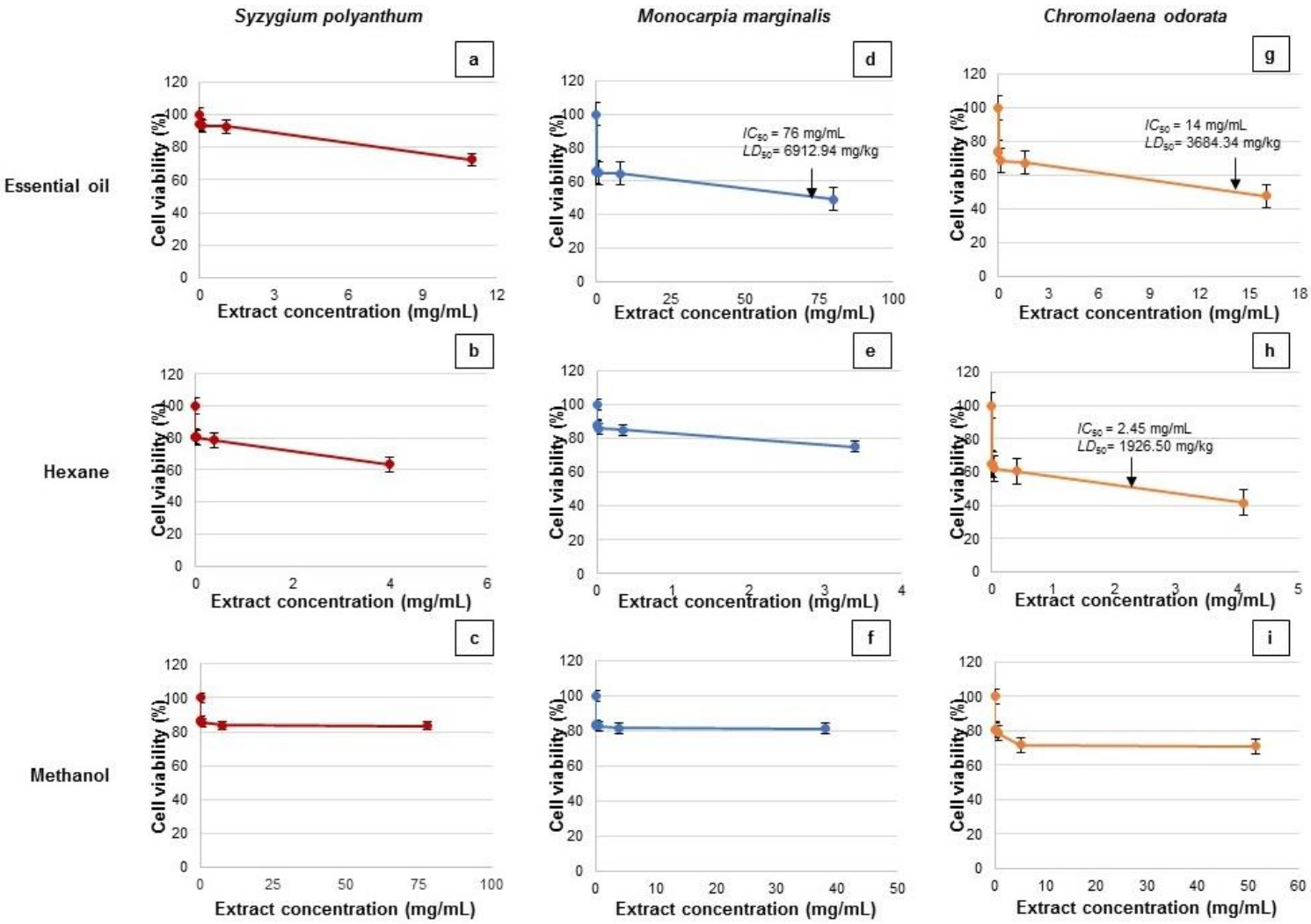

Fig. 1. The cytotoxicity effects of the three aromatic plant species from Malaysia. The left column is *Syzygium polyanthum*, the middle column is *Monocarpia marginalis*, and the right column is *Chromolaena odorata.* The top row is the essential oil, the middle row is the hexane extract, and the bottom row is the methanol extract.

Table 5. Cytotoxicity Test of Different Plant Extracts Against Human PBMCs

Plant Species	Type of Extract	Maximum Extract Conc. (mg/mL)	IC_{50} Value (mg/mL)	Percentage of Cell Viability (%)
Syzygium polyanthum	EO	11	ND	72.6 ± 0.5 - 94.0 ± 0.6
	HE	4	ND	59.8 ± 0.1 - 85.6 ± 0.2
	ME	78	ND	83.5 ± 0.2 - 86.9 ± 0.2
Monocarpia marginalis	EO	80	76	49.4 ± 0.2 - 65.5 ± 0.1
	HE	3	ND	75.1 ± 0.2 - 87.6 ± 0.2
	ME	38	ND	81.2 ± 0.2 - 83.3 ± 0.2
Chromolaena odorata	EO	16	14	47.5 ± 0.1 - 73.9 ± 0.1
	HE	4	2.45	41.7 ± 0.1 - 65.2 ± 0.1
	ME	52	ND	70.9 ± 0.3 - 80.9 ± 0.2

The values are represented as the percentage of cell viability ± SD of three replicates; EO: Essential oil; HE: Hexane; ME: Methanol; and ND: Not determined

To investigate the cytotoxicity effects of the plant extracts, the PBMCs were treated with various extract concentrations, and the cell viability was measured through a MTT assay. The MTT substances react with the enzyme succinate dehydrogenase in living mitochondria cells, which produces purple formazan crystals. The amount of formazan produced is directly proportional to the number of viable cells (Mosmann 1983). In the present investigation, the essential oils of *M. marginalis* and *C. odorata*, and hexane extract of *C. odorata* exhibited *in vitro* cytotoxicity with IC_{50} values that ranged from 2.45 mg/mL to 76 mg/mL. This correlates with a previous cytotoxic study of the essential oil from *M. marginalis* against KB human epidermal carcinoma cells and P388 leukemia cells with an IC_{50} value of 0.7 mg/mL (Taha *et al.* 2013). However, the other six leaf extracts were relatively safe as the cytotoxicity testing using human lymphocytes indicates no major toxicity at the cellular level.

The LD_{50} toxicity classification is based on the oral and dermal hazardous levels set forth by the World Health Organization (2004). The LD_{50} value is defined as a statistical estimation of the number of toxicant (mg) per bodyweight (kg) required to induce the death of 50% of a large population of test animals. This assay is useful and convenient in revealing the cytotoxicity of plant materials. It is suitable to perform the cytotoxicity test within 24 h to 72 h, and it is capable of affecting the cell metabolism and function without killing the cells rapidly (Ciapetti *et al.* 1993). In addition, the reproducibility and versatility of the MTT assay has advantages for toxicity testing and cell culture applications. It evaluates the survival and proliferation based on the functional state of the cell mitochondria. This versatility has been displayed in the reduction of cell viability and cytotoxicity quantitation (Edmondson *et al.* 1988).

Genotoxic Effects of the Plant Extracts

The comet assay is a sensitive biological assay employed to measure DNA damage in PBMCs in human. DNA damage is indicated by the breaking of the DNA strands and is represented by the median of the olive tail moment (OTM) in the comet tail in relative to the total amount of DNA. The PBMCs treated with *S. polyanthum* extracts had no detachable or comet tail (Fig. 2a), while extracts from the other two plant species caused significant DNA damage to the PBMCs ($p < 0.05$), as shown from the increased amount of damaged DNA tails (Table 6).

Table 6. Level of DNA Damage Expressed as Olive Tail Moment (OTM) in PBMCs After Treatment with Different Plant Extracts

Plant Species	Type of Extract	Parameter	
		OTM	*p*-value*
Syzygium polyanthum	EO	0.16	0.3938
	HE	0.12	0.1940
	ME	0.12	0.0921
Monocarpia marginalis	EO	3.31	< 0.0001
	HE	3.22	< 0.0001
	ME	2.88	< 0.0001
Chromolaena odorata	EO	2.58	< 0.0001
	HE	1.27	< 0.0001
	ME	5.50	< 0.0001
Positive control		5.73	< 0.0001
Negative control		0.20	-

*$p < 0.05$ indicates significant difference in comparison with the negative control; EO: Essential oil; HE: Hexane; and ME: Methanol

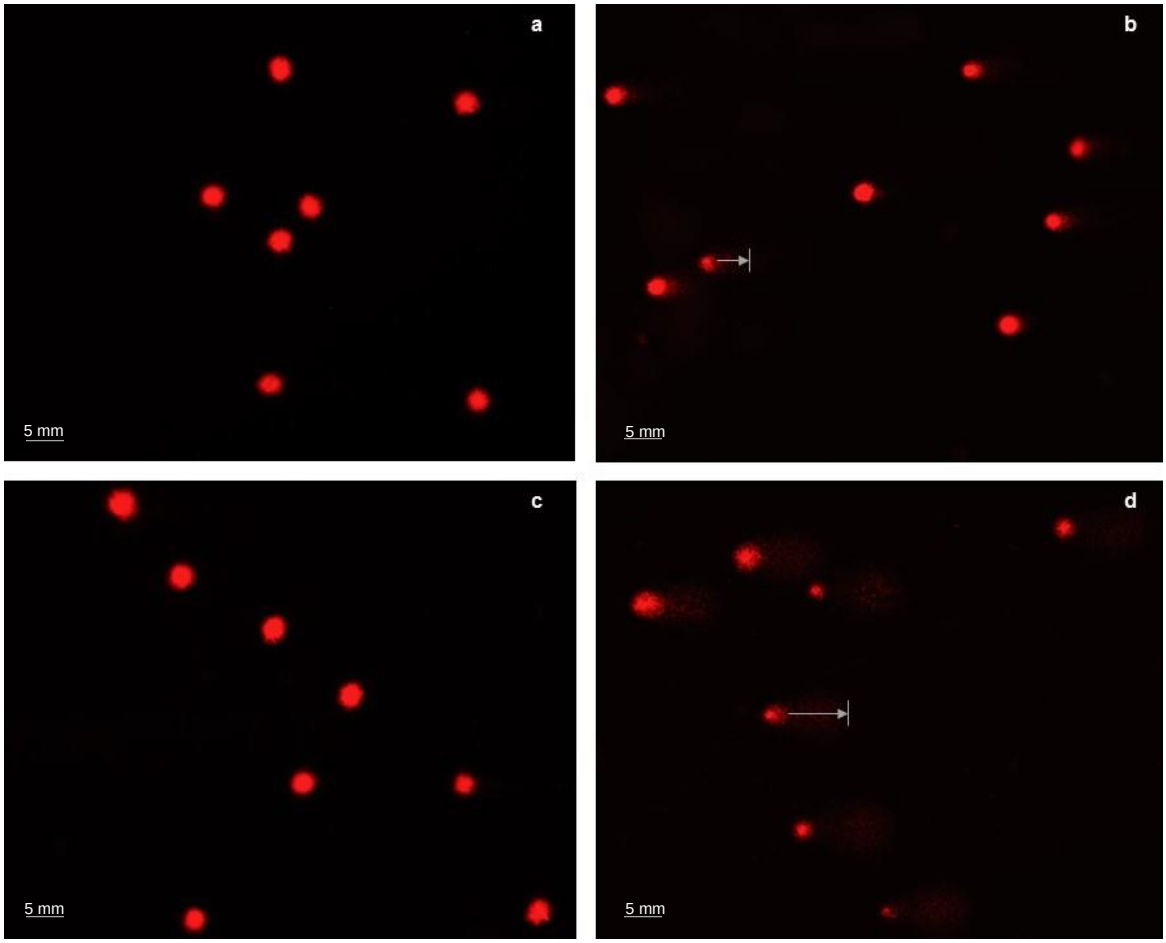

Fig. 2. Comet assay images of PBMCs; human PBMCs were treated with *Syzygium polyanthum* hexane extract (a) and the essential oil of *Monocarpia marginalis* (b). Healthy untreated cells appeared as round whole cells (c), while UV-damaged cells appeared as comet-like shapes (d). The images were captured using the image analysis system attached to a flourescence microscope at 200× magnification equipped with a 560 nm excitation filter, 590 nm barrier filter, and a CCD video camera PCO. The arrows indicate the range of DNA fragmentation resulting from cell damage.

When the cells treated with *M. marginalis* and *C. odorata* extracts were observed under a microscope, DNA fragmentation with comet-like appearances were detected, as shown in Fig. 2b. In this assay, the untreated cells acted as a negative control (Fig. 2c), while the cells exposed to UV light served as a positive control (Fig. 2d).

The genotoxicity test is performed to evaluate DNA damage of the PBMCs from exogenous application of a specific plant extract through the comet assay, which is also known as single cell gel electrophoresis (Singh *et al.* 1988). The DNA migration from the head region into the tail region indicates the number of DNA strand breaks (Yedjou and Tchounwou 2007). The concentrations used in the comet assay were selected based on the cytotoxicity test. If the extracts did not exhibit cytotoxicity, the maximum concentration was employed instead (Chaveerach *et al.* 2016). In this study, six out of the nine tested samples exhibited genotoxicity. All of the *M. marginalis* and *C. odorata* essential oils and extracts had induced DNA damage ($p < 0.05$). The migrating DNA, if damaged, is observed as a comet-like structure with a tail forming at one end (Fig. 2d). This is known as OTM, which can then be calculated after comparing the images with negative and positive controls. The negative control is represented by untreated cell characterized by the absence of a massive DNA breakage, concluding with no DNA migration phenomenon; hence, the nucleoids are spherical. The positive control is represented by UV-treated cell typified by the progression in DNA strand breakage and thus a DNA tail can be seen expanding out from the nucleoid, resulting in a comet-like structure (Tice *et al.* 2000; Musa *et al*. 2012).

The comet assay has several advantages as it is rapid, simple, sensitive, and produces quantitative results in investigating DNA damage (Lin *et al.* 2014). Moreover, only a small number of cells are needed per sample to detect the DNA damage levels (Rojas *et al.* 1999; Speit and Hartmann 1999). Even though the assay requires a few days and comet image analysis could be biased, still it is regarded a remarkably useful tool for measuring DNA damage and repair in genetic toxicology (Wood *et al.* 2010). Hexane and methanol extracts from *M. marginalis* and methanol extract from *C. odorata* had no cytotoxic effects to PBMCs, but instead had genotoxic effects. This is because cell viability at the genomic level is more sensitive than at the cellular level (Tice *et al.* 2000). In contrast, none of the *S. polyanthum* extracts had shown cytotoxic or genotoxic effects; therefore, this species is safe for human consumption and applications.

CONCLUSIONS

1. The essential oils and crude extracts of the three plant species are rich in aroma active compounds from the terpene group such as α-cadinol, α-cedrene, cubenol, farnesol, ledol, nerolidol, muurolene, and others.
2. The essential oils of *M. marginalis* and *C. odorata*, and the hexane extract of *C. odorata* were toxic to human's PBMCs by inhibiting cell survival and proliferation.
3. From the LD_{50} values, only the *M. marginalis* essential oil belongs to Class III (slightly hazardous), while the *C. odorata* extracts belong to Class II (moderately hazardous). All other extracts were relatively safe without major toxicity at the cellular level.
4. The comet assay indicates *M. marginalis* and *C. odorata* of having high cytotoxic and genotoxic potentials. These two species induced a substantial amount of DNA damage in the PBMCs.

5. Of the three plant species, only *S. polyanthum* had no cytotoxic and genotoxic effects on the PBMCs.
6. These findings identified the chemical constituents and potential toxicity effects of the three plant species to human, and may serve as a benchmark for their application in the fragrance, food, and pharmaceutical industries.

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