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Volatile Organic Compounds of Kaffir Lime (*Citrus hystrix* DC.) Leaves Fractions and their Potency as Traditional Medicine

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Previous studies have reported that a number of organic compounds are present in kaffir lime (*Citrus hystrix* DC.) leaf extracts. Further research is needed to purify these compounds and determine which are biologically active. The objective of this study is to identify the volatile organic compounds of kaffir lime leaf crude extracts and fractions and to study their bioactivity. Fractionation was performed by the double maceration method, using hexane as the second solvent. TLC was performed to analyze the qualitative separation, whereas the individual constituents were detected using GC-MS. Our results showed that chloroform and ethyl acetate crude extracts contained various volatile organic compounds such as fatty acids, fatty alcohols, prenol lipids, sterol lipids, terpenoids and long chain alkanes. Fractionation separated these compounds into non-hexane fractions, which contained less volatile compounds, and hexane fractions. The volatile compounds of non-hexane fractions were identified to be long chain alkanes, meanwhile the hexane fractions contained terpenoids, fatty acids, fatty alcohols, prenol lipids and sterol lipids. Palmitic acid and terpenoids, such as citronellyl propionate, nerolidol, citronella and caryophyllene oxide were found to be the most dominant bioactive compounds in chloroform and ethyl acetate crude extract and their hexane fractions, which were reported to possess cytotoxicity against cancer cells. Meanwhile in non-hexane fractions, long chain alkanes such as triacontane and hentriacontane were found to be the most dominant bioactive compound which also possessed cytotoxic effect. In conclusion, fractionation using the double maceration method yielded different volatile organic compounds composition with different biological activities. The crude extracts and fractions of kaffir lime leaves were potential to be developed as a traditional medicine for cancer treatment.

Keywords: Fractionation, Double maceration, Kaffir lime (*Citrus hystrix* DC.), Volatile organic compounds.

Traditional medicinal herbs have been widely used as a source of health treatments in many developed and developing countries[1]. Many modern medicines are produced indirectly from medicinal herbs[2]. Natural herbs provide easy availability, minimum cost, and minimum side effects[3], hence they are considered to be safer therapeutic agents. Kaffir lime (*Citrus hystrix* DC.) is a native medicinal herb in several Southeast Asia countries, including Indonesia. Its leaves have been traditionally used to treat headache, flu, fever, sore throats, bad breath and indigestion[4].

Many kinds of organic compounds have been detected in kaffir lime leaves. Flavonoids,

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tannin, saponin, glycoside, coumarine, bergamottin and pinene have been identified in kaffir lime leaf extracts[5]. In addition, phenolic acids, limonoids, glycerolipids and á-tocopherol were also reported as organic compounds from kaffir lime leaves[6]. Citronella was revealed to be the major volatile compounds of fresh kaffir lime extract, followed by linalool, caryophyllene, squalene, dihydrogeraniol and ²-citronellol[7]. However there is still no information about the volatile organic compounds of kaffir lime leaves originating in Indonesia.

Previous studies reported that kaffir lime leaves exhibited anti-bacterial[8] and antioxidant properties[7], antiviral action against the Herpes virus[9], cytotoxicity against cervix and neuroblastoma cancer cells[10], hepatoprotective action against paracetamol induced hepatotoxicity[6], usefulness as a mosquito repellent[11], and anti-inflammatory activities against *P. acne*[12] and edema inducing compound on ICR mouse ears[13]. In addition, kaffir lime leaf extracts are also reported to reduce acne scar formation and relieve acne blemishes[12].

The individual organic compounds from of kaffir lime leaves might each have different biological activities. Fractionation is needed to separate and purify those compounds, in order to determine each compound's biological activity and to determine which of these compounds appear to be more biologically active for specific health purposes. Many of the organic compounds of kaffir lime leaves detected from previous studies were non-polar. Hexane is used as the second solvent in double maceration method because of its very low polarity (0.009 out of 1.000)[14], hence it is expected to separate the non-polar compounds such as lipids from its more polar constituents. Therefore, the objective of this study is to separate and identify the volatile organic compounds of the chloroform and ethyl acetate extracts of kaffir lime leaves and their fractions. Furthermore this study will also determine each extract's potential as a natural medicine.

MATERIALS AND METHODS

Sample preparations and Extraction

Kaffir lime (*Citrus hystrix* DC.) leaves were collected from Candirejo Village, Borobudur, Magelang, Central Java, Indonesia. Only the spotless green to dark green leaves were harvested. The leaves were air dried at room temperature and ground to obtain simplicia powder. Extraction was done by maceration method. Leaf powder was soaked by 5 times volume of chloroform or ethyl acetate (Merck), as the first solvents, for 24 h with continuous shaking. The mixture was filtered and its residue was re-extracted three times using the same step. The total filtrate was evaporated to obtain crude extract paste.

Fractionation using double maceration

Double maceration was performed using hexane (e-Merck) as the second solvent. Hexane was added in 1:3 volume ratio compared to the accumulated filtrate volume from the first extraction. The crude paste and hexane mixture was homogenized for 30 min and then filtered using filter paper. The filtrate was evaporated to obtain hexane fraction, meanwhile the residue was collected as the non-hexane fraction. The crude extract paste and their fractions were gently blown under nitrogen gas for 5 minutes to remove any remaining solvent.

Thin Layer Chromatography

Thin Layer Chromatography (TLC, e-Merck) was performed to observe the separation profile of kaffir lime leaves after fractionation. A solvent system of hexane: diethyl ether: acetic acid = 80 : 20 : 2 was used as the mobile phase. Silica Gel 60 F254 was used as the stationary phase. The TLC chamber was saturated by the solvent system for minimum 1 hour before running. Prior to running, each sample was prepared by diluting 10 µg of paste in 50 µL of solvent (hexane for the hexane fraction and chloroform/ethyl acetate for the non-hexane fraction and crude extract). After running, the plate was left dried and then observed under visible light and UV light at 254 nm and 366 nm wavelength.

Gas Chromatography-Mass Spectrometry

The GC-MS analysis was performed using GCMS-QP2010 SE (Shimadzu, Japan) instrument with AGILENT HP 1 MS column (30 m x 0.25 ID x 0.25 um film). Helium was used as the carrier gas at a constant flow of 3.0 mL/min. The oven temperature was initially set for 70 ^{0}C for 5 min and then increased gradually with a rate of 5 ^{0}C/min up to 310 ^{0}C as the injection temperature. Mass spectrometer was operated with electron ionization system with ionizing energy of 70 eV at

a 250 ^{0}C ion source temperature. Individual volatile compounds were identified by comparing their retention indices and mass spectra to the NIST 62 and WILEY 229 spectra libraries.

Table 1. Volatile compounds identified in chloroform crude extract and their biological activities

Peak Area (%)	Compounds Name	Groups	Biological Activity
17,24	9,12,15-octadecatrien-1-ol	Fatty alcohol	Antioxidant[15]
9,77	Citronellyl propionate	Terpenoids	Cytotoxicity and antioxidant[16]
8,32	Palmitic acid	Fatty acid	Cytotoxicity[17], anti-inflammatory [18], antibacterial and antifungal [19]
6,18	1,5,9-decatriene,2,3,5,8-tetramethyl	Alkene hydrocarbon	
4,76	Heneicosane	Long chain alkane	Cytotoxicity, antibacterial[20]
3,20	Neophytadiene	Alkene hydrocarbon	Antioxidant, analgesic, antipuretic, anti-inflammatory and antimicrobial[21]
2,75	14B-pregnane	Sterol lipid	
2,06	Squalene	Terpenoids	Antioxidant, antitumor against skin carcinogens, skin hydration[22], antibacterial[23]
2,02	Isophytol	Terpenoids	Antibacterial[24]
1,98	9-tricosene	Alkene hydrocarbon	
1,83	citronellyl acetate	Terpenoids	Cytotoxicity, antioxidant, antiproliferative[25]
1,64	tetradecanoic acid /myristic acid	Fatty acids	Anti-inflammatory[26], antioxidant, antibacterial[19]
1,48	Farnesol	Fatty alcohol	Cytotoxicity[27], antioxidant [27,28], antimicrobial [28]
1,25	9-octadecanoic acid/ Oleic acid	Fatty acid	Reduce Her-2/NEU overexpression in breast cancer[29], antibacterial[19]
2,18	(CAS) Phytol	Fatty alcohol	Cytotoxicity[30], anti-inflammatory[31], anti-neoceptive, antioxidant[32]
0,87	Cyclooctacosane	Alicyclic hydrocarbon	
0,81	3-eicosene	Alkene hydrocarbon	Cytotoxicity, antibacterial[20]
0,76	Citronella	Terpenoids	Cytotoxicity[33], antibacterial[34], Analgesic-like activity on mouse[35]
0,75	6-octen-1-ol	Alcohols	
0,74	Phenol,3,5-bis(1,1-dimethylethyl)-	Phenolic	
0,52	1-eicosanol	Fatty alcohols	
0,48	Benzene,1-methoxy-2-[(4-methoxyphenyl)methyl]	Aromatic hydrocarbon	
0,46	Spathulenol	Terpenoids	Cytotoxicity, anti-inflammatory, antioxidant[36]
0,38	trans-linalool oxide	Terpenoids	antioxidant and antibacterial[37] Anxiolytic-like effect[38]
0,37	hexanedioic acid/Adipic acid	Dicarboxylic acid	
0,36	Phytene	Diterpenoid alkene	
0,34	1,2-benzenedicarboxylic acid,bis (2-ethylhexyl)ester	Aromatic ester	Antioxidant[39]
0,24	Styrene	Aromatic hydrocarbon	
0,23	Eicosane	Long chain alkane	Cytotoxicity, antibacterial[24]

Table 2. Volatile compounds identified in chloroform non-hexane fraction and their biological activities

Peak Area (%)	Compounds Name	Groups	Biological Activity
30,02	Triacontane	Long chain alkane	Cytotoxic against melanoma B16F10-Nex2 cancer cell line [40]
29,85	Octacosane	Long chain alkane	Cytotoxic against melanoma B16F10-Nex2 cancer cell line [40]
10,17	Octadecyne	Alkyne hydrocarbon	
6,86	Nonacosane	Long chain alkane	Antibacterial[41], antioxidant[39]
6,27	1,7-nonadiene, 4,8-dimethyl	Alkene hydrocarbon	
3,58	1-octadecyne	Alkyne hydrocarbon	
1,73	Eicosyne	Alkyne hydrocarbon	

Table 3. Volatile compounds identified in chloroform hexane fraction and their biological activities

Peak Area (%)	Compounds Name	Groups	Biological Activity
22.7	Nerolidol	Terpenoids	Cytotoxicity, antibacterial[20]
13.42	Citronella	Terpenoids	Cytotoxicity[33], antibacterial[34], Analgesic-like activity on mouse[35]
9.78	Phytol	Fatty alcohol	Cytotoxicity[30], anti-inflammatory[31], anti-neoceptive, antioxidant[32]
8.43	Ergost-35-ene-3,5,6,12-tetrol	Sterol lipid	
6.43	Citronellol	Terpenoids	Cytotoxicity[42], antioxidant and antibacterial[37]
7.9	²-sitosterol	Sterol lipid	Cytotoxicity, antibacterial[24], anti-hypercholestrolemia[43]
5.43	Lupeol	Terpenoids	Cytotoxicity[44]
3.43	Hentriacontane	Long chain alkane	Cytotoxicity[45], anti-inflammatory[46]
2.41	Pentacosane	Long chain alkane	
1.82	Stigmasterol	Sterol lipid	Cytotoxicity and antioxidant[47]; antibacterial[48]
1.14	Spathulenol	Terpenoids	Cytotoxicity, anti-inflammatory, antioxidant[36]
1.13	Neophytadiene	Alkene hydrocarbon	Antioxidant, analgesic, antipuretic, anti-inflammatory and antimicrobial[21]
1.06	Caryophyllene oxide	Terpenoids	Cytotoxicity[49], analgesic-like activity on mouse[35], antimicrobial[50]
0.94	Squalene	Terpenoids	Antioxidant, antitumor against skin carcinogens, skin hydration[22], antibacterial[23]
0.9	9-Eicosyne	Alkyne hydrocarbon	
0.79	1,6-Octadiene, 2,5-dimethyl	Alkene hydrocarbon	
0.77	Myrcenol	Fatty alcohol	
0.73	Palmitaldehyde	Fatty aldehyde	
0.67	³-tocopherol	Prenol lipids	Cytotoxicity[51], antibacterial[52], antioxidant, neuroprotective effect[53]
0.67	Farnesol	Fatty alcohols	Cytotoxicity[27], antioxidant [27,28], antimicrobial[28]
0.51	Palmitoleic acid	Fatty acids	Lipid lowering activity, anti-inflammatory[54]
0.48	Terpineol	Terpenoids	Cytotoxicity, apoptosis inducing activity, inhibiting cell cycle at G1 phase[55]

Table 4. Volatile compounds identified in ethyl acetate crude extract and their biological activities

Peak Area (%)	Compounds Name	Groups	Biological Activity
10.13	Palmitic acid	Fatty acids	Cytotoxicity[17], anti-inflammatory[18], antibacterial and antifungal[19]
6.67	Caryophyllene oxide	Terpenoids	Cytotoxicity[49], analgesic-like activity on mouse[35], antimicrobial[50]
6.67	Citronella	Terpenoids	Cytotoxicity[33], antibacterial[34], Analgesic-like activity on mouse[35]
6.27	Lanost-7-en-3-one	Lipid sterol	
9.59	Citronellyl acetate	Terpenoids	Cytotoxicity, antioxidant, anti-proliferative[25]
3.96	Oleic acid	Fatty acids	Reduce Her-2/NEU overexpression in breast cancer[28], antibacterial[19]
3.85	Tetratetracontane	Long chain alkanes	
4.1	Phytol	Fatty alcohol	Cytotoxicity[30], anti-inflammatory[31], anti-neoceptive, antioxidant[32]
4.59	Citronellyl formate	Terpenoids	
3.3	Hexatriacontane	Long chain alkanes	
3.52	Farnesol	Fatty alcohol	Cytotoxicity[27], antioxidant[27,28],
3.58	1,7-Nonadiene, 4,8-dimethyl-	Alkene hydrocarbon	antimicrobial[28]
2.58	Palmitaldehyde	Fatty aldehyde	
2.23	Nerolidol	Terpenoids	Cytotoxicity, antibacterial[20]
2.4	Germacrene	Terpenoids	Cytotoxicity[36]
2.17	Stearyl aldehyde	Fatty aldehyde	
1.62	Stearyl vinyl ether		
1.33	Longipinenepoxide	Terpenoids	
1.31	1,4-Heptadiene, 3,3,6-trimethyl	Alkene hydrocarbon	
1.19	²-Sitosterol	Sterol lipid	Cytotoxicity, antibacterial[24], anti-hypercholestrolemia[43]
1.86	Citronellol	Terpenoids	Cytotoxicity[42], antioxidant and antibacterial[37]
1.07	1-Hexacosanal	Fatty aldehyde	
1.07	Globulol	Terpenoids	
1.02	a-Tocopherol	Lipid prenol	Cytotoxicity[51], antibacterial[52], antioxidant, neuroprotective effect[53]
1.54	Linalool-oxide	Terpenoids	
0.91	Limonene-oxide	Terpenoids	Analgesic-like activity on mouse[35]
0.91	Octacosane	Long chain alkanes	Cytotoxic against melanoma B16F10-Nex2 cancer cell line[40]
0.88	Patchulane	Hydrocarbon	
1.17	1,6-Octadiene, 2,5-dimethyl	Alkane hydrocarbon	
0.84	2-Dodecanol	Fatty alcohol	
0.79	Dichlorobenzene	Aromatic hydrocarbon	
0.71	Squalene	Terpenoids	Antioxidant, antitumor against skin carcinogens, skin hydration[22], antibacterial[23]
0.66	9-Eicosyne	Alkyne hydrocarbon	
0.65	Dihydromyrcenol	Fatty alcohol	Antibacterial, antifungal, cytotoxicity against colorectal, hepatilc and breast cancer cell[56]

0.54	Myrcenol	Fatty alcohol	
0.47	Dihydrobrassicasterol	Sterol lipid	
0.41	Melonal	Fatty aldehyde	
0.39	Eicosane	Long chain alkanes	Cytotoxicity, antibacterial[24]
0.37	Geraniol	Terpenoids	
0.32	1-octadecyne	Alkyne hydrocarbon	
0.28	a-Cubene	Terpenoids	
0.27	B-Pinene	Terpenoids	Cytotoxicity[57], analgesic-like activity on mouse and rat[35]
0.27	Geranyl Butyrate		
0.24	Calarene		
0.24	a-Cedrane	Terpenoids	
0.23	Cyclohexane	Alicyclic hydrocarbon	
0.22	a-Thujene	Terpenoids	
0.2	2-Dodecanal	Fatty aldehyde	
0.19	a-Calacorene		
0.18	1,5,9-DECATRIENE, 2,3,5,8-TETRAMETHYL		Alkane hydrocarbon

Table 5. Volatile compounds identified in ethyl acetate non-hexane fraction and their biological activities

Peak Area (%)	Compounds Name	Groups	Biological Activity
43.11	Hentriacontane	Long chain alkane	Cytotoxicity[45], anti-inflammatory[46]
35.37	Nonacosane	Long chain alkane	Antibacterial[41], antioxidant[39]
8.3	Octacosane	Long chain alkane	Cytotoxic against melanoma B16F10-Nex2 cancer cell line[40]
3.86	1,2,3-Propanetriol, monoacetate (Glycerol acetate)	Glycerolipid	
2.87	Tetradecanal (Myristaldehyde)	Fatty aldehydes	
2.72	Linalool-oxide	Terpenoids	

RESULTS AND DISCUSSION

Hexane in the TLC system solvent acted as the mobile phase which migrated the non-polar compounds further from the starting point. On the other hand, more polar compounds migrated slower due to their greater affinity towards the polar Silica Gel plate. The spot that appeared near the starting line indicated a polar compound. The polarity property decreased as the spot appeared further from the starting line. However, each spot detected in the chromatogram might not represent a single compound, but rather a group of compounds. The chromatogram was observed under UV light with wavelength of 254 nm and 366 nm because no clear spot was detected under the visible light (Fig. 1a-c and Fig. 2 a-c).

The chromatogram of chloroform crude extract, non-hexane and hexane fractions showed the appearance of 7, 5 and 12 spots respectively (Fig. 1d). Meanwhile in the ethyl acetate crude extract, its non-hexane and hexane fractions obtained 7, 4 and 13 spots respectively (Fig. 2d). The number of spots detected in the hexane fractions were higher compared to the chlorofomm and ethyl acetate crude extract, hence fractionation using hexane increased the number of organic compounds in the hexane fractions.

The non-hexane fractions of both extracts obtained fewer spots, indicating that the hexane fractions contained less numbers of organic compounds, compared to the hexane fractions. The spots in the non-hexane fractions were mostly detected near the starting line, suggesting that the non-hexane fractions contain more polar compounds. In addition, the hexane fractions (Fig.1b and Fig.2b) showed non-polar spots migrating further away from the starting line. The very non-polar spot of each hexane fraction (Fig.1b and 2b) also appeared to be thicker compared to

Table 6. Volatile compounds identified in ethyl acetate hexane fraction and their biological activities

Peak Area (%)	Compounds Name	Groups	Biological Activity
14.23	Palmitic acid	Fatty acids	Cytotoxicity[17], anti-inflammatory[18], antibacterial and antifungal[19]
7.08	Lanost-7-en-3-one	Sterol lipids	
6.24	Phytol	Fatty alcohols	Cytotoxicity[30], anti-inflammatory[31], anti-neoceptive, antioxidant[32]
4.65	Citronella	Terpenoids	Cytotoxicity[33], antibacterial[34], Analgesic-like activity on mouse[35]
4.47	Tetratetracontane	Long chain alkanes	
4.36	1,7-Nonadiene, 4,8-dimethyl-	Alkene hydrocarbon	
3.2	Hexatriacontane	Long chain alkanes	
3.18	Oleic acid	Fatty acids	Reduce Her-2/NEU overexpression in breast cancer[28], antibacterial[19]
3.07	Caryophyllene oxide	Terpenoids	Cytotoxicity[49], analgesic-like activity on mouse[35], antimicrobial[50]
2.9	Linoleidic acid methyl ester	Fatty acids Ester	
2.55	Thunbergol	Terpenoids	
2.02	Undecylenic aldehyde	Fatty aldehyde	
1.74	2-Decyn-1-ol	Alcohol	
1.56	Globulol	Terpenoids	
1.54	3-Buten-2-ol, 2,3-dimethyl-	Branched alcohol	
1.5	Palmitaldehyde	Fatty aldehyde	
1.64	Myrcenol	Fatty alcohols	
4.02	Citronellol	Terpenoids	Cytotoxicity[42], antioxidant and antibacterial[37]
1.13	Farnesol	Fatty alcohols	Cytotoxicity[27], antioxidant[27,28], antimicrobial[28]
1.13	Widdrol	Aromatic hydrocarbon	
1.50	Neophytadiene	Alkene hydrocarbon	Antioxidant, analgesic, antipuretic, anti-inflammatory and antimicrobial[21]
1.06	Stearic acid	Fatty acids	Antibacterial[19]
0.97	²-sitosterol	Sterol lipids	Cytotoxicity, antibacterial[24], anti-hypercholestrolemia[43]
0.94	Citronellyl propionate	Terpenoids	Cytotoxicity and antioxidant[16]
0.91	2-Hexyl-1-decanol	Alcohols	
0.79	Squalene	Terpenoids	Antioxidant, antitumor against skin carcinogens, skin hydration[22], antibacterial[23]
0.74	8-Hexadecanol	Fatty alcohol	
0.67	Eicosane	Long chain alkanes	Cytotoxicity, antibacterial[24]
0.61	Geranyl linalool	Terpenoids	
0.61	n-hentriacontanol	Fatty alcohol	
0.47	Heptacosane	Long chain alkanes	
0.43	Caryophyllene	Terpenoids	Antimicrobial and antioxidant[58]
0.4	Dihydromyrcenol	Fatty alcohols	Antibacterial, antifungal, cytotoxicity against colorectal, hepatilc and breast cancer cell[56]
0.36	Methyl Palmitate	Fatty acids Ester	
0.34	Dihidrobrassicosterol	Sterol lipids	
0.32	3-Hexen-1-ol	Fatty alcohols	
0.29	Citronellyl acetate	Terpenoids	Cytotoxicity, antioxidant, antiproliferative[25]

0.26	±-tocopherol	Lipid Prenol	Cytotoxicity[51], antibacterial[52], antioxidant, neuroprotective effect[53]
0.24	±-copaene	Terpenoids	
0.23	4,5-dimethyl-3-heptanol	Branched alcohol	
0.23	Germacrene	Terpenoids	Cytotoxicity[36]
0.19	Trans-dodec-5-enal	Fatty aldehyde	
0.16	Linalool	Terpenoids	Analgesic-like activity on mouse[35]
0.25	Dihydrocarveol	Terpenoids	
0.15	Methyl Citronellate	Terpenoids	
0.15	n-octyl phthalate	Dicarboxylic ester	
0.11	±-Muurolene	Terpenoids	
0.1	ä-Cadinene	Terpenoids	

the crude extract; this may indicate an increasing content of non-polar compounds in the hexane fraction compare to crude extract.

The GC-MS chromatogram of kaffir lime leaf chloroform extract exhibited the presence of 45 peaks in crude extract (Fig.3), 9 peaks in non-hexane fraction (Fig.4) and 35 peaks in hexane fraction (Fig.5). Meanwhile the ethyl acetate crude extract, its non-hexane and its hexane fraction showed a total of 65 peaks (Fig.6), 7 peaks (Fig.7) and 70 peaks (Fig.8) respectively. The number of peaks differ in each extract because of the different polarity of each solvent. Ethyl acetate, which was more polar than chloroform, dissolved more organic compounds. Each peak detected in the GC-MS analysis represented an individual compound however, different peaks Table 4.
might result in similar compounds.

The five dominant volatile compounds of chloroform crude extract were 9,12,15-octadecatrien-1-ol, citronellyl propionate, palmitic acid, 1,5,9-decatriene-2,3,5,8-tetramethyl and heneicosane (Table 1). These compounds belong to fatty alcohols, terpenoids, fatty acids and long chain alkane classes of compounds. However none of these compounds were detected in non-hexane or hexane fraction. The chloroform non-hexane fraction was dominated by long chain alkane compounds, which were of triacontane, octacosane and nonacosane (Table 2). Meanwhile the chloroform hexane fraction was dominated by terpenoids, phytosterol and fatty alcohol compounds which were nerolidol, citronella, phytol, ergost-35-ene-3,5,6,12-tetrol, citronellol and β-sitosterol (Table 3).

The ethyl acetate crude extract was dominated by terpenoids and fatty alcohol compounds which were palmitic acid, citronellyl acetate, caryophyllene oxide, citronella and Lanost-7-en-3-one (Table 4). Similar compounds were

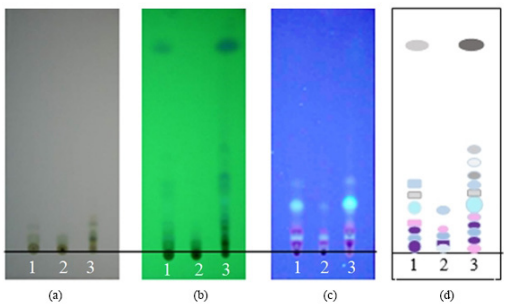

Fig. 1. TLC profile of kaffir lime leaves chloroform extract and fractions. (a) visible light, (b) UV light at 254 nm, (c) UV light at 366 nm and (d) representative diagram. Note: 1: chloroform crude extract, 2: chloroform non-hexane fraction, 3: chloroform hexane fraction

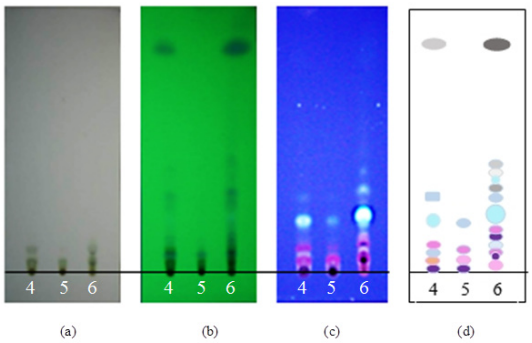

Fig. 2. TLC profile of kaffir lime leaves ethyl acetate extract and fraction. (a) visible light, (b) UV light at 254 nm, (c) UV light at 366 nm and (d) representative diagram. Note : 4: ethyl acetate crude extract, 5: ethyl acetate non-hexane fraction, 6: ethyl acetate hexane fraction

detected in the hexane fraction however, with different peak areas. The ethyl acetate hexane fraction was dominated by palmitic acid, Lanost-7-en-3-one, phytol, citronella, caryophyllene oxide and citronellyl acetate (Table 6). Meanwhile the non-hexane fraction was dominated by long chain alkane hydrocarbons, which were octacosane, hentriacontane and nonacosane (Table 5). Hentriacontane and nonacosane, on the other hand, were not detected in the ethyl acetate crude extract.

Fractionation by the double maceration method separated the organic compounds of kaffir lime leavf extracts based on like-dissolve-like principle. The non-polar constituents were more soluble in the hexane fraction. The more polar constituents, on the other hand, were left insoluble as the non-hexane fraction.

The GC-MS analysis in this study was performed to detect volatile organic compounds of kaffir lime leaf extracts. Each compound has a different boiling point and polarity, resulting in different retention times and mass spectra which are then used to identify a single compound. The number of peaks detected in ethyl acetate crude extract was higher compared to chloroform crude extract. Ethyl acetate has a polarity index of 4.4 whereas chloroform's polarity index is 4.1[59]. Both solvents are considered as moderately polar[60]. Ethyl acetate dissolved more organic compounds

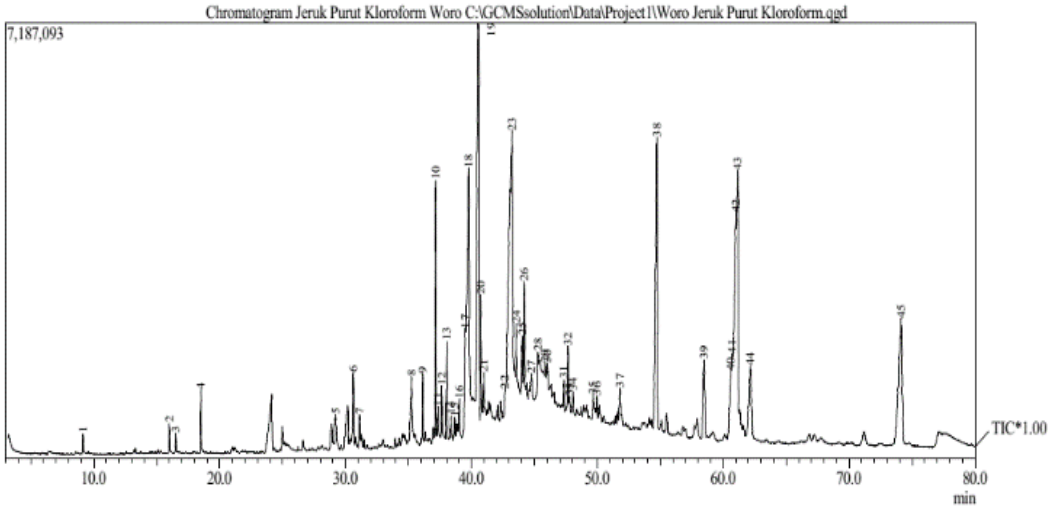

Fig. 3. GC-MS chromatogram of chloroform crude extract

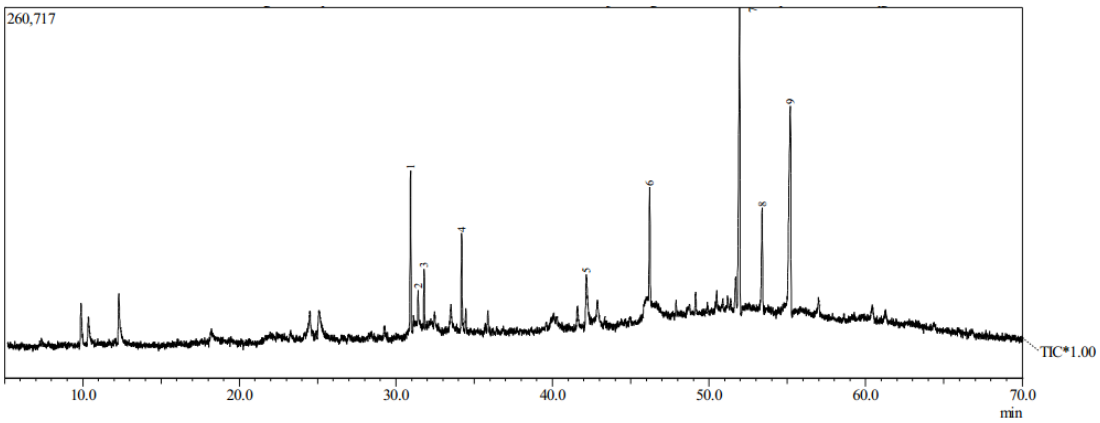

Fig. 4. GC-MS chromatogram of chloroform non-hexane fraction

due to its hydrogen bonding acceptor and high dipolarity-polarizability properties,making it a stronger solvent[59].

TLC and GCMS results showed similar phenomena. TLC chromatograms suggested that the non-hexane fraction contained more polar compounds, while the hexane fraction contained more non-polar compounds. Kaffir lime leaves have been reported to contain glycosides, flavonoids, saponin and tannin[5] which are considered more polar than terpenoids. In addition, other polar compounds such as flavonone glycosides, namely hesperidine and neohesperidine[61] , as well as some phenolic acids such as vanillic acid, coumaric acid and benzoic acid[62] were also detected in kaffir lime leaves. However, none of these polar compounds were detected in this study. This may be because polar compounds tend to be less volatile due to their high boiling point. Other detection methods, such as LC-MS might be needed for further analysis in order to determine the non-volatile compounds of kaffir lime fractions.

In this study, the diversity of volatile compounds detected in the non-hexane fractions of

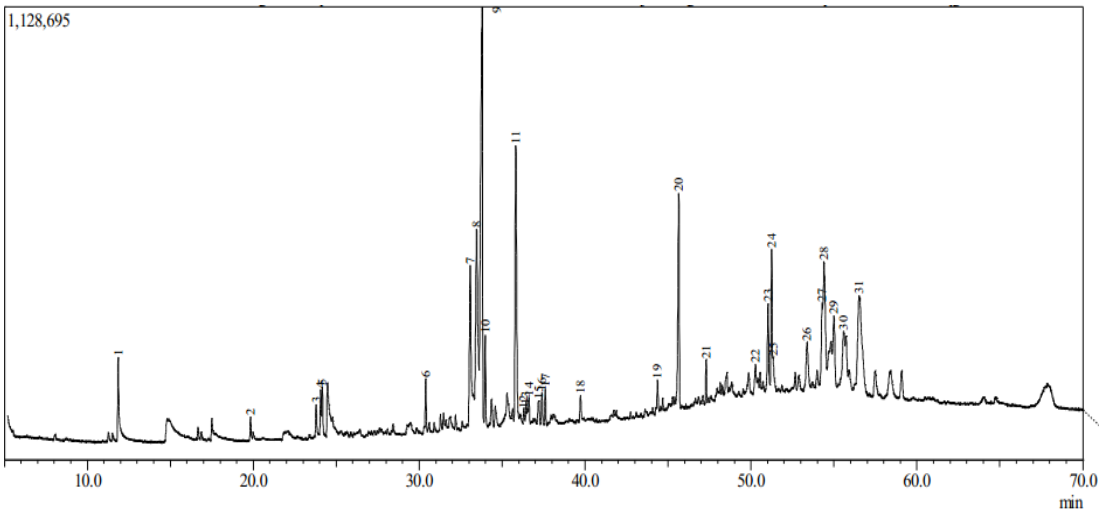

Fig. 5. GC-MS chromatogram of chloroform hexane fraction

Fig. 6. GC-MS chromatogram of ethyl acetate crude extract

both extracts were lower compared to the hexane fractions. This may suggest that the majority of organic compounds contained in the non-hexane fractions were polar and non-volatile compounds, hence they were not detected using GC-MS.

Hexane fractions of both chloroform and ethyl acetate extract were revealed to contain more varieties of non-polar volatile compounds compared to the non-hexane fractions. Fatty acids, terpenoids, fatty alcohols and sterol lipids composed the majority of volatile organic compounds detected. These compounds possess various biological activities. Palmitic acid, the most dominant saturated fatty acid detected in this study, is reported to induce apoptosis in human leukemic cell lines[17]. Saturated fatty acids in general were also reported to possess antibacterial activity against methicillin-resistant *Staphylococcus aureus*[63]. Phytol, as the major fatty alcohol detected, is reported to have various medicinal bioactive properties, including anti-allergy, anti-inflammatory[64], anti-schistosomiasis[65], antineoceptive, anti-oxidant activities and anticancer activities[32]

Terpenoids were detected as the major constituents of volatile secondary metabolites in kaffir lime leaves. Terpenoids have been reported to exhibit anticancer activity in cervical cancer cells[66], oral squamosa carcinoma[67] and induce cell death in breast and prostate cancer[68]. In addition, terpenoids, as the major components of essential oils in plant, were reported to have anti-oxidant,

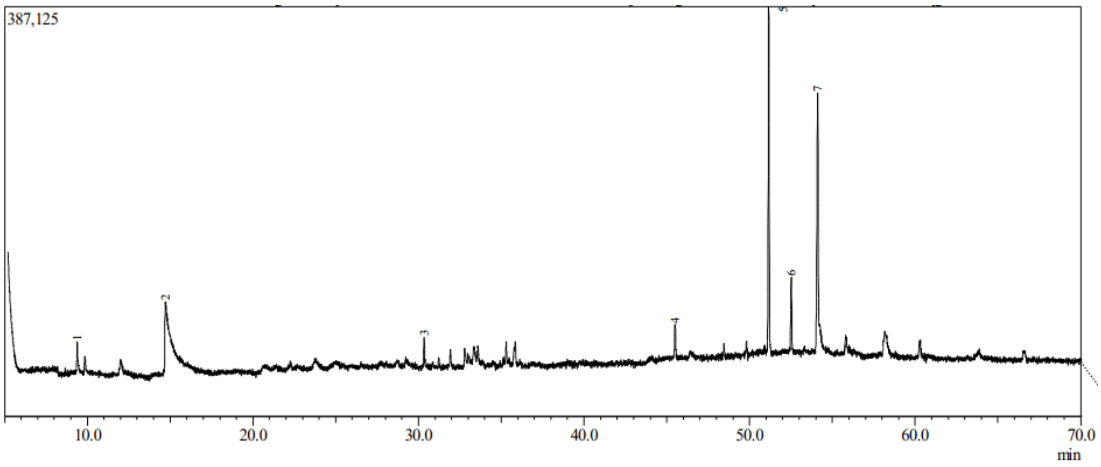

Fig. 7. GC-MS chromatogram of ethyl acetate non-hexane fractionl

Fig. 8. GC-MS chromatogram of ethyl acetate hexane fraction

antimicrobial[69] repellent, insecticidal[70] antifungal[71] antihelminthic against *Haemonchus contortus*[72] antiviral against HPV (Herpes simplex virus)[73] and antibacterial activity against respiratory tract pathogens[74].

Sterol lipids, or phytosterols specifically, also made up a large component of kaffir lime leaf extract fractions. Phytosterols are reported to have lipid lowering activities and thus reduce the risk of coronary and cardiovascular diseases[75]. Phytosterols reduce the total cholesterol and low density lipoprotein (LDL) levels in blood by inhibiting cholesterol absorption in intestinum[75]. In addition, phytosterols also inhibited cancer cell growth and induced apoptosis in cancer cell[76], thus making its potential as anticancer agent.

Some minor compounds were also detected in the hexane fractions which were fatty aldehydes, prenol lipids and aromatic hydrocarbons. Prenol lipids such as tocopherol is widely reported to possess cytotoxicity[51], antioxidant and anti-bacterial activity[52] and also exhibit a neuroprotective effect against H_2O_2 and BSO oxidative stress[53]. However, no biological activity information of the fatty aldehydes and aromatic hydrocarbon compounds have been reported.

The non-hexane fractions of both extracts revealed the presence of aliphatic hydrocarbon such as long chain alkanes, which have also been reported to possess several biological activities. Triacontane, octacosane and hentriacontane have potential as anticancer agents. Triacontane and octacosane were reported to be cytotoxic against melanoma B16F10-Nex2 cancer cell line[40], meanwhile hentriacontane induced cell death in lymphoma cancer cell line[45] as well as possessing anti-inflammatory activity[46] by inhibiting the activation of caspase-1 pro-inflammatory agents[45]. In addition, another long chain alkane detected in both non-hexane fractions, nonacosane, was also reported to have anti-bacterial and antioxidant properties[39,41].

Groups of fatty acids, fatty alcohols, prenol lipids, terpenoids, phytosterols and long chain alkenes were also detected in the crude extracts. However they appear in different amounts and composition compared to their fractions. Several compounds were only detected in their fractions and were missing in the crude extracts. It has been suggested that fractionation using the double maceration method yields different volatile organic compounds, possibly because different solubility in hexane separates the larger and complex molecules of crude extracts into their simpler constituents. This may be followed by further fractionation to purify a single compound. In addition, double maceration also affected the composition of kaffir lime volatile compounds through the increased or decreased abundance (peak area) of some individual compounds.

Generally, the volatile organic compounds of kaffir lime leaf crude extracts and fractions possess various biological activities, including anticancer (cytotoxicity, apoptosis inducing activity, anti-proliferative), antimicrobial (antibacterial, antifungal, antiviral), antioxidant, anti-inflammatory, lipid lowering effect, anxiolytic-like effect, anti-neoceptive and analgesic-like effect which make each of them potential to be developed as a protective or therapeutic agents for various health care applications. However further research still needs to be done in order to examine their biological activities through both *in-vitro* and *in-vivo* studies.

In conclusion, the double maceration method separated the volatile organic compounds of kaffir lime crude extracts into non-hexane and hexane fractions. Non-hexane fractions contained more long chain alkanes. Meanwhile the hexane fractions contained more secondary metabolite terpenoids and lipids (fatty acids, fatty alcohol, fatty aldehydes, prenol lipids and sterol lipids). Fractionation using the double maceration method yielded different volatile organic compounds with different biological activities, and it will be useful to further investigate these for their potential use as medicinal therapeutic agents.

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