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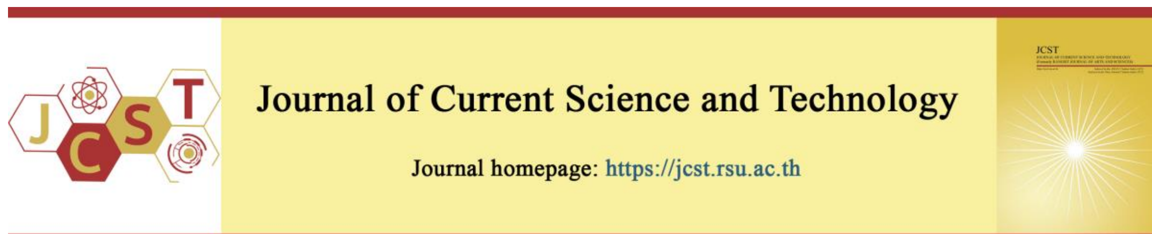

GC-MS Analysis, Antioxidant Activity and Antimicrobial Activity of Kaffir Lime (*Citrus hystrix* DC.) and Key Lime (*Citrus aurantifolia* (Christm.) Swingle.) Peel Essential Oils

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Abstract

This research studied the chemical compositions, antioxidant activities, and antimicrobial activities of kaffir lime (*Citrus hystrix* DC.) and key lime (*Citrus aurantifolia* (Christm.) Swingle.) peel essential oils extracted by hydrodistillation. GC-MS analysis of kaffir lime essential oil showed four major components as β-pinene (27.37%), β-phellandrene (22.69%), D-limonene (16.21%), and citronellal (12.43%), while in key lime essential oil the predominant component was D-limonene (45.91%), with two minor components as β-pinene (20.27%) and γ-terpinene (8.33%). Both essential oils scavenged free radicals, with amounts of sample required to decrease DPPH concentration by 50% (IC50) 93.20 and 57.90 mg/ml for kaffir lime and key lime essential oils, respectively. Both essential oils showed antibacterial activity against *Staphylococcus aureus* in the agar diffusion technique.

Keywords: *Citrus; Hydrodistillation; Essential oil; Antioxidant activity; Antibacterial*

1. Introduction

Essential oils are characteristic aroma products obtained from various plants which originate in leaves, buds, fruits, flowers, herbs, twigs, barks, woods, roots, and seeds. These oils have complex mixture compositions that may include volatile terpene compounds such as monoterpenes, sesquiterpenes and diterpenes (Edogbanya et al., 2019). Aromatic plants are used in the pharmaceutical, food, and fragrance industries. Methods of extracting essential oils (EOs) from various plants include cold expression, solvent extraction, "Enfleurage", hydrodistillation, steam distillation, supercritical fluid extraction, microwave-assisted extraction, controlled pressure drop process, and ultrasound-assisted extraction. However, the constituents of extracted essential oils may vary depending on the extraction method used (Stratakos & Koidis, 2016).

Essential oil extraction is widely performed in the citrus genus, which contains commercial plants belonging to the *Rutaceae* family that grow on all the continents but especially in tropical and subtropical regions and areas with the Mediterranean climate (Turan & Mammadov, 2021). Extracting essential oils from the citrus genus is well-documented. Frassinetti et al. (2011) studied the antibacterial and antioxidant activities of four essential citrus oils including bitter orange (*Citrus aurantium* L.), sweet orange (*Citrus sinensis* (L.) Osbeck), lemon (*Citrus limon* (L.) N.L. Burm.), and mandarin (*Citrus reticulata*

Blanco) from Perugia, Italy, extracted by the cold pressing method. All the essential oils had limonene as the main constituent and showed good antibacterial activity against both Gram-negative and Gram-positive bacteria. Antioxidant properties of all the essential oils were tested by the DPPH assay and showed good antioxidant activity depending on the concentration, while lemon oil gave the best antioxidant capacity. Aripin et al. (2015) analyzed the chemical composition and oral antimicrobial effect of five essential oils of orange peel including lime (*C. aurantifolia*), tangerine (*C. nobilis*), sweet orange (*C. sinensis*), lemon (*C. limon*), and kaffir lime (*C. hystrix*) collected from Indonesia. All essential oils were extracted by hydrodistillation. The major component of all the essential oils was D-limonene and they all showed antimicrobial activity against *Streptococcus mutans* (bacteria in plaque attached to the tooth surface), with the highest antimicrobial activity found in the essential oil of lime peel. Djenane (2015) reported on the extraction of three essential oils from citrus peel as fresh orange (*Citrus sinensis* L.), lemon (*Citrus limonum* L.), and bergamot (*Citrus aurantium* L.) from Algeria using the hydrodistillation method. Limonene was the main component of orange and lemon peel essential oils, while bergamot peel essential oil was dominated by two significant compounds, linanyle acetate (37.30%) and β-linalool (23.37%). The in vitro antimicrobial activities of the three essential oils were evaluated against *Staphylococcus aureus* (*S. aureus*), a bacterium that can produce toxins that often cause food poisoning. Results revealed that all the essential oils showed antimicrobial activity, with the lemon essential oil showing the highest antibacterial effects. Azman et al. (2019) investigated the antioxidant properties of three essential oils from citrus peel, selected from fresh and frozen peels of three citrus species including lemon (*Citrus limon*), key lime (*C. aurantifolia*), and musk lime (*C. microcarpa*) from Malaysia. Antioxidant properties were indicated by total phenolic content and total flavonoid content. All essential oils had antioxidant properties but frozen peel oils had higher antioxidant content than fresh peel oils. The musk lime peel essential oil had the highest total phenolic content and total flavonoid content.

Kaffir lime (*Citrus hystrix* DC.) and key lime (*Citrus aurantifolia* (Christm.) Swingle.) are widely used as functional foods and drinks in Thailand and also used in the flavor and cosmetic industries. Kaffir lime and key lime peels are treated as primary lime fruit by-products and discarded as waste into the environment. The previous research revealed that citrus peel essential oils exhibited good antimicrobial activities and antioxidant properties. Therefore, this research studied the chemical compositions and antioxidant and antimicrobial activities of the essential oils of kaffir lime and key lime peel against *S. aureus*. All essential oils were extracted by the hydrodistillation method. This method effectively separates high boiling point organic compounds of essential oils from plant materials under 100°C (Dangkulwanich & Charaslertrangsi, 2020).

2. Objectives

The objectives of this research were to study the chemical compositions, antioxidant activity, and antimicrobial activity of kaffir lime (*Citrus hystrix* DC.) and key lime (*Citrus aurantifolia* (Christm.) Swingle.) peel essential oils.

3. Materials and methods

3.1 Extraction process and density analysis

Samples of kaffir lime and key lime fruits were collected from a local orchard in Lopburi Province, Thailand. The fruits were washed under water, dried in the shade at room temperature, and the peel was cut into pieces. Essential oils of kaffir lime and key lime were extracted using a hydrodistillation apparatus with a modified Clevenger extension. The fresh peels were placed in a flask containing water and the unit was carried to boiling. The peel-water ratio was 1:3 (g:mL) and distillations were continued for 150 min. The vapor produced in the flask was passed through a condenser and condensed to essential oil and hydrosol. The essential oils were separated from hydrosol by decantation and dehydrated with sodium sulfate anhydrous (Na_2SO_4, 99%, AR, QRëC, New Zealand). The samples were kept in an amber glass vial, and stored at 4°C. The yield of the essential oils obtained (%) was determined using the following equation (1).

$$\text{Yield of essential oil (\%)} = \frac{\text{Weight of essential oil recovered}}{\text{Weight of citrus lime peels}} \times 100\% \quad (1)$$

The density (ρ) of the essential oils was determined by the pycnometer test using ASTM D1429-13 standard test methods. The pycnometer, also called a specific gravity bottle, is a bottle with a given volume measured accurately. Density was

calculated using equation (2) where m_a and m_b are the weight of a full bottle of essential oil and water (substance selected as a standard), and ρ_o is the density of the water at 28°C during the study. The specific gravity (S) is the ratio of the weight of the essential oil to the weight of an equal volume of water.

$$\rho = \frac{m_a}{m_b} \quad (2)$$

3.2 Chemical compositions

The chemical components of essential oil were identified by a gas chromatography-mass spectrometer (GC-MS). The GC-MS analysis was performed using a gas chromatography (Agilent Technologies, 7890B GC system) coupled to a triple quadrupole mass spectrometer (Agilent Technologies, 7000C GC/MS Triple Quad) equipped with a non-polar HP-5ms capillary column (30 m × 0.25 mm, 0.25 µm). For GC-MS detection, an electron ionization voltage of 70 eV was used in a scan range of 33 to 500 amu. The carrier gas was helium (He) with a flow rate of 1.0 mL/min. Injector and detector MS transfer line temperatures were set at 250°C and the ion source temperature was 230°C. The oven was set to 60°C for 10 min, and then the temperature was gradually increased to 220°C at a rate of 4°C/min.

Identification of essential oil components was performed by matching their spectral data with details in the NIST2011 libraries. Retention indices were identified by the Kovats index (Kováts, 1958), relative to the C8-C22 n-alkanes assayed using GC-MS under the same conditions as the oils. Composition percentages (%) of the essential oils were computed by the normalization method from the GC peak areas.

3.3 DPPH free radical scavenging activity

Antioxidant activities of the essential oils were assessed by the DPPH free radical scavenging assay. DPPH is a common abbreviation for the organic compound 2,2-diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$, AR, Sigma-Aldrich, USA), which is a stable free radical and pale violet in solution. DPPH free radicals can accept an electron or hydrogen radical to become a stable diamagnetic molecule, commonly used for the assessment of antioxidant potential (Singh et al., 2013). Essential oils were dissolved in methanol at concentrations ranging from 10 to 100 mg/mL. Tocopherol ($C_{29}H_{50}O_2$, ≥95.5%, Sigma-Aldrich, USA) was used as a positive control, and prepared by mixing with methanol at concentrations ranging from 0.002 to 0.020 mg/mL. Aliquots of 1 mL of these solutions were added to 3.0 mL of 0.1 mM DPPH solution in methanol and then incubated in darkness for 30 min. The absorbance of the samples was detected at 517 nm using a microplate reader (BioTek Instruments, Synergy™ HT). The DPPH scavenging activity (%) was calculated using equation (3) where A_0 is the absorbance of the control reaction (DPPH solution) and A_s is the absorbance of the samples. The IC50 values of the samples as the concentration required to scavenge 50% DPPH free radicals were calculated from the graph of concentration versus scavenging activity (Singh et al., 2013).

$$DPPH\ scavenging\ activity\ (\%) = \frac{(A_0 - A_s)}{A_0} \times 100\% \quad (3)$$

3.4 Antibacterial activity

The antimicrobial activity test was carried out using the agar diffusion method with CLSI M02-A11: Performance standards for antimicrobial disk susceptibility tests (clear zone test). The bacterial strain used was Gram-positive *Staphylococcus aureus* (ATCC 6538), provided by the American Type Culture Collection (ATCC).

Essential oils diluted with ethanol were prepared in six concentration variations: 0, 20, 40, 60, 80, and 100% (*v/v*). Paper discs 6 mm in diameter were impregnated with the essential oils and then placed on inoculated Mueller Hinton agar (MHA) media. The plates were incubated at 35 ± 2°C in an atmosphere of 5% CO_2 for 20 to 24 hours before measuring the zones of inhibition (clear zone). Antibacterial activity was determined by measuring the inhibition zone diameter around the dishes impregnated with essential oils. Each assay in this experiment was replicated three times, with the size of the inhibition zone calculated from three replicates. Negative controls (0% sample) were prepared using the same solvent (ethanol) used to dissolve the essential oils. The standard reference antibiotic, vancomycin (30 µg/disc) was used as a positive control to test the sensitivity of the microorganism tested.

4. Results and Discussion

4.1 Yield and density

Products from the kaffir lime peel and key lime peel extraction process formed two layers of essential oil on top of the container with hydrosol at the bottom. Hydrosol is a complex water-based

product mixture containing traces of essential oil and other water-soluble compounds (Shafie et al., 2022). Essential oils from fresh kaffir lime and key lime peels were obtained with yields (w/w) of 0.82% and 0.72%, respectively. Both essential oils were pale yellow and had a characteristic aroma, with densities of 0.88 g/cm^3 and 0.86 g/cm^3, respectively.

4.2 Chemical composition

The GC-MS chromatograms of the essential oils of kaffir lime and key lime are shown in Figure 1. The x-axis represents the time required for the compound to reach the mass spectrometer detector, while the y-axis represents signal intensity. Identities of the components in both essential oils were assigned by matching their spectral data with details in the NIST2011 libraries. The retention index was identified by the Kovats index, as given in Table 1. Most of the identified substances in both essential oils were monoterpenes and sesquiterpenes.

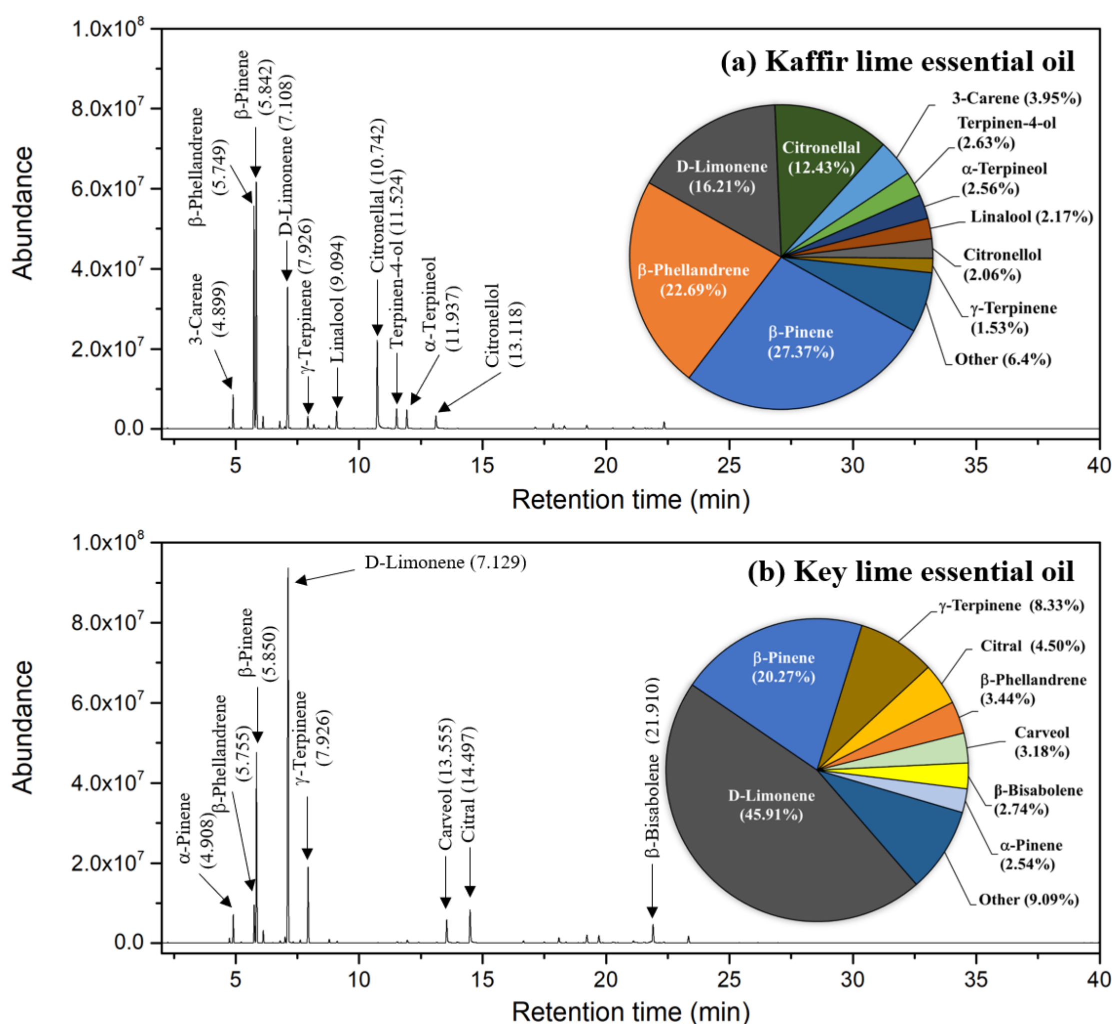

Figure 1 Chromatograms of (a) kaffir lime and (b) key lime essential oil by GC-MS analysis.

Table 1 The content of compounds (%) of kaffir lime and key lime essential oils identified by GC-MS analysis.

No.	Compound	Formula	Retention index (RI*)	Peak area (%)	
				Kaffir lime	Key lime
1	α-Phellandrene	$C_{10}H_{16}$	934	0.28	0.47
2	3-Carene	$C_{10}H_{16}$	941	3.95	0.5
3	α-Pinene	$C_{10}H_{16}$	942	-	2.54
4	Camphene	$C_{10}H_{16}$	957	0.18	0.11
5	β-Phellandrene	$C_{10}H_{16}$	979	22.69	3.44
6	β-Pinene	$C_{10}H_{16}$	983	27.37	20.27
7	α-Terpinene	$C_{10}H_{16}$	1021	1.22	0.24
8	m-Cymene	$C_{10}H_{14}$	1030	0.27	0.68
9	D-Limonene	$C_{10}H_{16}$	1034	16.21	45.91
10	Eucalyptol	$C_{10}H_{18}O$	1038	0.16	0.06
11	γ-Terpinene	$C_{10}H_{16}$	1064	1.53	8.33
12	1,2-Oxolinalool	$C_{10}H_{18}O_2$	1077	0.12	-
13	Terpinolene	$C_{10}H_{16}$	1091	-	0.41
14	Linalool	$C_{10}H_{18}O$	1100	2.17	0.23
15	Citronellal	$C_{10}H_{18}O$	1157	12.43	-
16	endo-Borneol	$C_{10}H_{18}O$	1170	0.12	0.03
17	Terpinen-4-ol	$C_{10}H_{18}O$	1181	2.63	0.18
18	α-Terpineol	$C_{10}H_{18}O$	1193	2.56	0.43
19	Citronellol	$C_{10}H_{20}O$	1231	2.06	-
20	cis-Geraniol	$C_{10}H_{18}O$	1232	-	0.29
21	Carveol	$C_{10}H_{16}O$	1245	-	3.18
22	Citral	$C_{10}H_{16}O$	1274	-	4.5
23	δ-Elemene	$C_{15}H_{24}$	1342	-	0.44
24	Citronellol acetate	$C_{12}H_{22}O_2$	1357	0.24	-
25	α-Copaene	$C_{15}H_{24}$	1379	0.70	-
26	β-Cubebene	$C_{15}H_{24}$	1392	0.44	-
27	β-Elemene	$C_{15}H_{24}$	1394	-	0.39
28	Caryophyllene	$C_{15}H_{24}$	1423	0.53	1.09
29	α-Bergamotene	$C_{15}H_{24}$	1439	-	1.05
30	Humulene	$C_{15}H_{24}$	1458	0.11	0.13
31	Bicyclo[4.4.0]dec-1-ene,2-isopropyl-5-methyl-9-methylene-	$C_{15}H_{24}$	1484	0.26	0.35
32	Patchoulene	$C_{15}H_{24}$	1487	0.03	0.12
33	Eudesma-4(14),7(11)-diene	$C_{15}H_{24}$	1497	-	0.11
34	Elixene	$C_{15}H_{24}$	1498	0.14	-
35	β-Chamigrene	$C_{15}H_{24}$	1508	0.1	-
36	β-Bisabolene	$C_{15}H_{24}$	1510	-	2.74
37	γ-Gurjunene	$C_{15}H_{24}$	1560	-	0.95
38	δ-Cadinene	$C_{15}H_{24}$	1526	1.04	-

RI*: Retention index relative to *n-alkanes* (C8-C22) in the nonpolar HP-5ms capillary column (identification by Kovats index)

GC-MS analysis identified 27 components in the kaffir lime essential oils. The four major components were β-pinene (27.37%), β-phellandrene (22.69%), D-limonene (16.21%), and citronellal (12.43%), eluted at retention times of 5.842, 5.749, 7.108, and 10.742 min, respectively. Other constituents had significant levels such as 3-carene (3.95%), terpinen-4-ol (2.63%), α-terpineol (2.56%), linalool (2.17%), citronellol (2.06%), and γ-terpinene (1.53%). Seventeen compounds were found at trace levels with peak areas below 1.5%. Aripin et al. (2015) investigated kaffir lime essential oil collected from Indonesia and extracted by hydrodistillation. They detected limonene (31.24%), β-pinene (13.81%), and citronellal (13.41%) as the main components, while β-phellandrene was found at trace levels of 1.43%. Kasuan et al. (2013) investigated the composition of kaffir lime essential oil collected from Malaysia and extracted by steam distillation at uncontrolled temperature. Their results showed that the essential oil of kaffir lime was mainly composed of β-pinene (32.97%), sabinene (31.22%) and limonene (20.69%), while citronellal (7.8%) was a minor component. An et al. (2021) analyzed the chemical composition of kaffir lime essential oil collected from Vietnam. They found that kaffir lime essential oil was mainly composed of β-pinene (33.939%), sabinene (22.875%), D-limonene (15.847%) and citronellal (14.791%). Kaffir lime essential oils from many countries have β-pinene and D-limonene as the main components. Sabinene was the main component of kaffir lime essential oil extracted by steam distillation but was not found in kaffir lime essential oil extracted by hydrodistillation, while β-phellandrene was found only in kaffir lime essential oil extracted by hydrodistillation.

The GC-MS analysis of key lime essential oils identified 29 components. The predominant component was D-limonene (45.91%) at a retention time of 7.129 min, with two minor components as β-pinene (20.27%) and γ-terpinene (8.33%) at retention times of 5.850 and 7.926 min, respectively. Other components as citral (4.5%), β-phellandrene (3.44%), carveol (3.18%), β-bisabolene (2.74%), and α-pinene (2.54%) were found in significant levels, with another 21 components at trace levels (< 1.5%). In other research, limonene was often detected as the predominant component in key lime essential oils. For example, Chisholm et al. (2003) extracted essential oils from key lime in Southern Florida by solvent extraction for 1 hour. They determined that the major component was limonene (32.6%) and minor components were α-terpineol (12.5%) and β-pinene (6.3%), while Gamarra et al. (2006) found that limonene was the major component (50%) of key lime essential oil from Northern Peru extracted by hydrodistillation. Spadaro et al. (2012) analyzed the chemical composition of key lime essential oil from Italy extracted by hydrodistillation. Their results showed that this essential oil was composed mainly of D-limonene (53.8%), with minor components as γ-terpinene (16.5%) and β-pinene (12.6%).

4.3 DPPH free radical scavenging activity

After adding tocopherol (positive control) and both essential oils to the DPPH solution, the samples were incubated in the darkness for 30 minutes. The color of the DPPH solution changed from light violet to light yellow. These changing occurred because the antioxidant substance reduced DPPH free radicals to diphenyl picryl hydrazine (Molyneux, 2004). The DPPH scavenging activities of tocopherol and both essential oils are shown in Figure 2. Data revealed that these essential oils acted as scavenging free radicals. The DPPH scavenging activities of kaffir lime and key lime essential oils were 1.83-75.33% in 10-100 mg/mL and 15.99-76.74% in 10-100 mg/mL, respectively. Scavenging activity increased at higher essential oil concentrations. Key lime essential oils showed significantly ($p < 0.05$) higher activity than kaffir lime essential oils, with amounts of sample required to decrease DPPH concentration at 50% (IC50) 93.20 and 57.90 mg/mL for kaffir lime and key lime essential oils, respectively. It can be seen that both essential oils showed less antioxidant activity when compared to tocopherol as the positive control (IC50 value of 14.96 μg/mL).

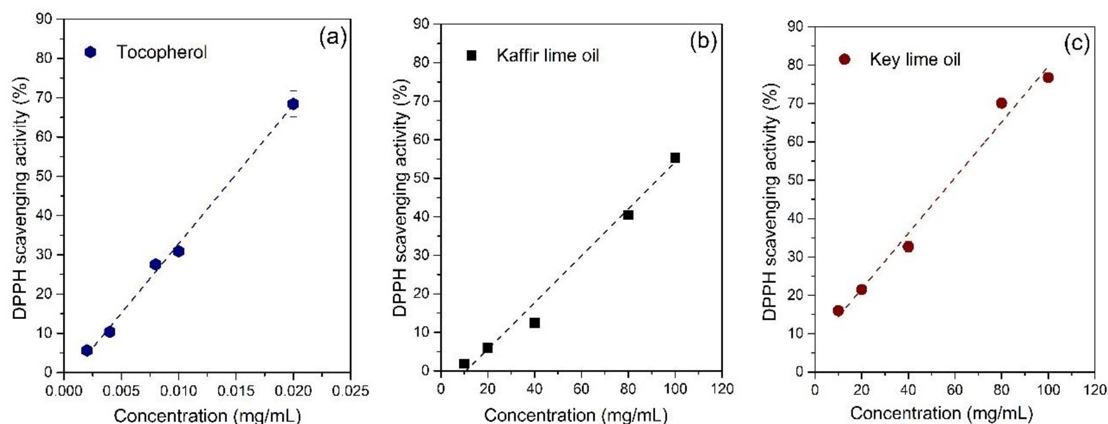

Figure 2 DPPH scavenging activity (%) versus concentration (mg/mL) of (a) tocopherol (positive control), (b) kaffir lime and (c) key lime essential oils.

Previous studies recorded lower IC50 values for kaffir lime and key lime essential oils. For example, Lin et al. (2019) reported on key lime essential oil extracted by steam distillation and measured according to the DPPH assay with IC50 value of 2.36 mg/ml, while Chit-aree et al. (2021) extracted essential oils from unripened and ripened kaffir lime peels from Chanthaburi Province, Thailand by hydrodistillation. Their results gave antioxidant activity by DPPH assay with IC50 values of 36.14 and 31.62 mg/ml, respectively. Klangpetch et al. (2016) reported very high antioxidant activity of kaffir lime essential oils extracted by soaking in ethanol and ethyl acetate, with IC50 values of 1.5 and 0.5 mg/mL, respectively while IC50 values of key lime essential oils were 4.0 and 3.2 mg/mL after soaking with ethanol and ethyl acetate, respectively.

4.4 Antibacterial activity

The antibacterial effects of kaffir lime and key lime essential oils were tested against *S. aureus* ATTC 6538. Both essential oils diluted with ethanol were prepared at six concentrations: 0, 20, 40, 60, 80, and 100% (*v/v*). These conditions were then denoted as ET00 (negative control), KA20, KA40, KA60, KA80, and KA100, respectively for kaffir lime essential oils and KE20, KE40, KE60, KE80, and KE100, respectively for key lime essential oils. The antibacterial activities (*S. aureus*) of both essential oils as 0 (negative control), 20, and 100% samples and vancomycin (positive control) using the disc diffusion method are shown in Figure 3. The ET00 sample did not present an inhibitory effect against any of the test microorganisms in the control treatment. The clear zone of the experiment indicates antibacterial effects against *S. aureus*. There was the clear zone in the positive control (30 mg vancomycin) treatment because of its antibiotic properties. It can be noted that both essential oils had antibacterial effects against *S. aureus*. These essential oils showed antibacterial activity depending on the concentrations of D-limonene, β-pinene, and other components. The antimicrobial activities of the essential oils may result from the cumulative effects of D-limonene, β-pinene and some other components or flavonoids and phenolic compounds present (Edogbanya et al., 2019; Aripin et al., 2015). The inhibition zone results of both essential oils at various concentrations (0, 20, 40, 60, 80, and 100%) were tested for antimicrobial activity against *S. aureus*, with results shown in Table 2. At 100% the kaffir lime essential oil had the highest effect on *S. aureus* with an inhibition zone of 28.00 mm. At 60% key lime essential oil had the highest effect on *S. aureus* with an inhibition zone of 22.67 mm. Inhibition zones of kaffir lime and key lime essential oils at 60% had similar values, while 60% of the lime essential oils extracted by the hydrodistillation technique showed higher antibacterial effect against *S. aureus* than oil extracted by cold maceration (7.50 mm) (Edogbanya et al., 2019).

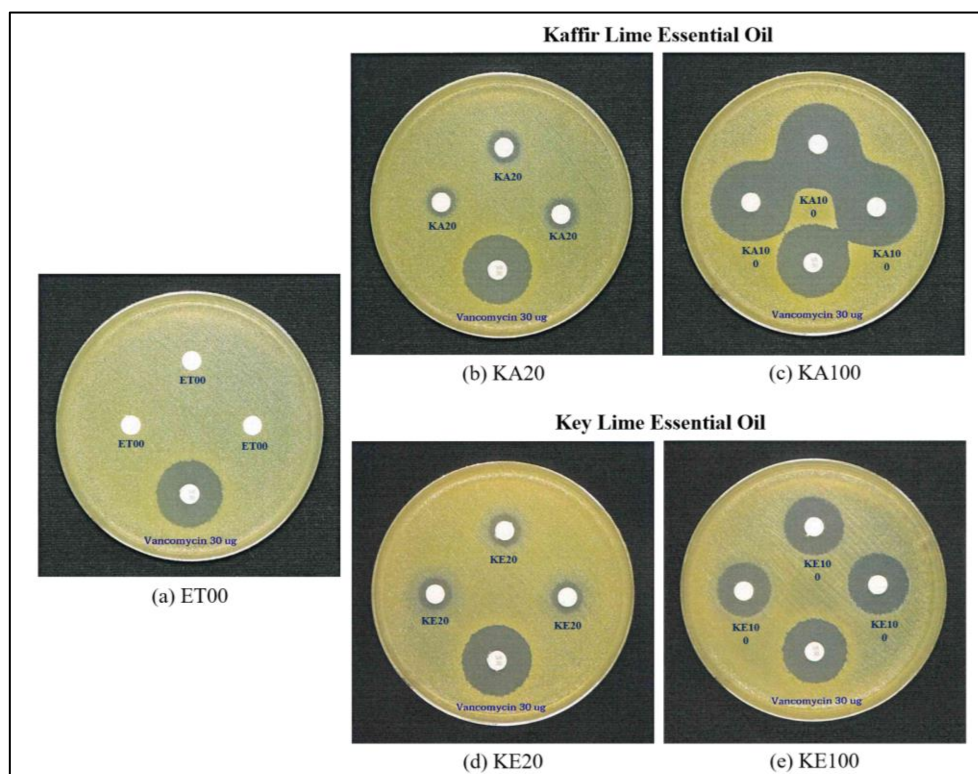

Figure 3 Antibacterial activity (*S. aureus*) of (a) Ethanol (ET00 sample, negative control), kaffir lime essential oil with (b) KA20 and (c) KA100 samples, and key lime essential oil with (d) KE20 and (e) KE100 samples using the disc diffusion method.

Table 2 Diameter of inhibition zone against *S. aureus* of essential oil extracts of kaffir lime and key lime at different concentrations using the paper disc diffusion method.

Concentration (%)	**Zone of inhibition (mm)**	
	kaffir lime oil	**key lime oil**
0	0.00	0.00
20	10.00	11.00
40	16.00	15.67
60	23.00	22.67
80	23.00	19.33
100	28.00	19.33

5. Conclusions

In Thailand, kaffir lime and key lime fruits are widely used as functional foods and drinks and also in the flavor and cosmetic industries. The peels of these limes are discarded as waste in the environment. This study extracted kaffir lime and key lime peel waste by hydrodistillation. Kaffir lime essential oil contained four major components as β-pinene (27.37%), β-phellandrene (22.69%), D-limonene (16.21%), and citronellal (12.43%), while essential oil of key lime had the predominant component as D-limonene (45.91%) with two minor components as β-pinene (20.27%) and γ-terpinene (8.33%). The kaffir lime and key lime peel essential oils scavenged free radicals with an inhibitory effect on bacterial strains against *S. aureus*. Furthermore, 60% key lime essential oil extracted by the hydrodistillation method had greater antibacterial activity than oil extracted by the maceration method at the same concentration, while the hydrodistillation technique did not have solvent (n-hexane) residue compared to the maceration technique. Results suggested hydro-distillation as a safer and healthier technique than maceration for essential oil production and application as cosmetics and pharmaceuticals.

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