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Chemical composition and antibacterial activity of essential oil kaffir lime (*Citrus hystrix* DC) leaves from East Borneo

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Abstract

Kaffir lime (*Citrus hystrix* DC) leaves are a versatile plant with high commercialization potential. In the pharmaceutical industry, kaffir lime leaf essential oil is used to stimulate appetite, as a cosmetic ingredient, and as an antibacterial agent. Although most studies have concentrated on the essential oil of kaffir lime leaves, its components and biological activities need to be considered based on the growing location. The chemical components and biological activity of kaffir lime leaf essential oil are affected by differences in growth location. This research was conducted to determine the chemical composition and antibacterial activity of the essential oil of kaffir lime leaf essential oil from East Borneo. Fresh and dried kaffir lime leaves were extracted by steam distillation method and chemical compound analysis by Gas Chromatography-Mass Spectrometry (GC-MS) and the antibacterial activity of essential oils was analyzed by disc diffusion method. There are twenty-five compounds in the essential oil of fresh and dried kaffir lime leaves. The main components of fresh kaffir lime leave essential oil were citronellal (77.29%), linalool (4.88%), sabinene (4.83%), citronellol (3.41%), β-myrcene (1.23%), and citronellal (74.09%), sabinene (5.54%), linalool (3.59%), citronellol (3.56%), caryophyllene (2.26%) were the main components of the essential oil of dried kaffir lime leaves. The antibacterial activity of essential oils against *Staphylococcus aureus* ATCC 25925 and *Escherichia coli* ATCC 8733, with zones of inhibition of 21.75 and 19.17 mm, respectively. The minimum inhibitory concentration on all antibacterial activities was 6.25 µL/mL. The presence of strong antibacterial activity in dried and fresh kaffir lime leaf plant extracts was associated with the presence of citronellal compounds found in essential oil kaffir lime leaves

1. Introduction

Essential oils (EOs) produced from fruits, seeds, tubers, stems, and leaves have grown in importance over time to become one of the world's most important natural sources (Ramadan, 2019; da Silva *et al*., 2021). Monoterpenes, sesquiterpenes, and polyphenols are found in many plants that contain a volatile oil that can help to tackle the problem of resistant bacteria and medication residual dangers (Saxena and Kalra, 2011). Fungicidal, bactericidal, and virucidal properties are among the most well-known biological properties of EOs and are regarded as therapeutic, and their scent is used in the production of anti-inflammatory, analgesic, and antispasmodic medicines. These properties have remained intact to this day, and they are also helping scientists learn more about the molecular mechanisms that underpin the biological activities of essential oils, particularly their antibacterial (Hien *et al*., 2020). Steam/ hydro-distillation and simultaneous distillation/extraction are the most common methods for obtaining essential oils. The volatile chemicals are widely used and sought after in the food, fragrance, cosmetics, pharmaceutical, and winemaking industries (Magwa *et al.,* 2006). Furthermore, biological effects such as antibacterial, antioxidant, anticancer, antifungal, and insecticidal activity have been documented for volatile molecules

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(Derwich *et al.,* 2010). Furthermore, plant extracts were screened (for antibacterial activity) in the hopes of discovering novel sources as medications for the treatment of various ailments.

Combava (French), Kaffir Laim (Russian), Kahpiri Delhi (Sri Lanka), Naranja Puerco-Espín (Spanish), Bai Makrut (Thailand), Chanh Kaffir (Vietnam), Shauk Cho (Burmese), Ma Feng Gan (Chinese), Kobu Mikan (Japanese), Citrus hystrix (Danish), Kafir lime (Swedish), Kolumichai (India), Khi-Hout (Laos), Kabog (Philippines), Indonesische Citroenboom (Dutch), and Jeruk Purut (Indonesia) are all names for kaffir lime which is a medicinal plant endemic to Southeast Asia, especially Indonesia. Its aromatic leaves are used as a spice and for a variety of flavouring uses (Figure 1). Its leaves have been used to treat headaches, flu, fever, throats, poor breath, and indigestion for centuries. Previous research has found that kaffir lime leaves have antibacterial activities against the *Cutibacterium acnes* and antioxidant properties (Lertsatitthanakorn *et al.,* 2006), antiviral activity (Khan *et al.,* 2005) against the herpes virus, cytotoxicity against the cervix and neuroblastoma cancer cells, hepatoprotective activity against paracetamol-induced hepatotoxicity (Borusiewicz *et al.,* 2017), mosquito repellent properties, and anti-inflammatory activity against *C. acnes*, oedema-inducing compound on ICR mouse ears and shown to minimize acne scarring and acne blemishes (Dertyasasa and Tunjung, 2017). However, the biological activity and bioactive components of kaffir lime leaf as determined by place, climate, season, post-harvest, and processing have not been explored properly. The chemical composition and antibacterial activity of essential oils extracted from fresh and dry leaves of kaffir lime growing in East Borneo were investigated in this work.

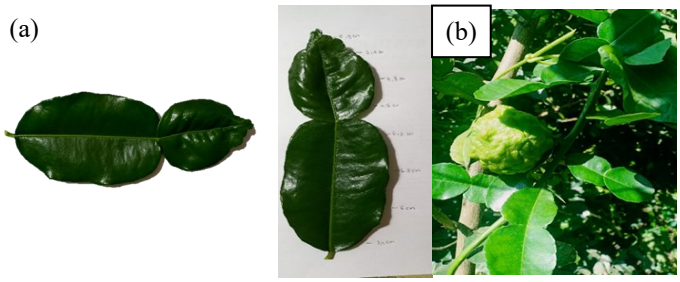

Figure 1. Kaffir lime leaves (a) and fruits (b) from East Borneo

2. Materials and methods

2.1 Sample preparations and extraction

Kaffir lime leaves were obtained in Indonesia's East Borneo village of Kembang Janggut (115º46'– 116º28' S and 0º02'–0º27' W). Only the leaves that were pristine green to dark green were plucked. To eliminate dust, the leaves were washed with water, and the contaminated leaves were separated. Before utilizing, the fresh leaves were cut, ground, and stored at room temperature in a vacuum-packed container. The leaves were air-dried at ambient temperature and crushed.

2.2 Isolation of essential oil

Hydrodistillation apparatus was used to extract the essential oil. After 5 hrs, the yield of the extraction was achieved. The distillate was then separated into two layers, one of which was oil and the other was water. A separating funnel was used to collect the distillate sample. The oil was separated from the lower layer, while the liquid or water from the upper layer was discarded. The essential oil was labelled and kept at 4^{o}C in a dark place (Kasuan *et al.,* 2013). Finally, antibacterial testing was performed on the extracted oil sample.

2.3 Physical properties analysis

The physical parameters of the plant extracts determined were extraction yield, refractive index, solubility in alcohol, and specific gravity. Hand-held Refractometer was used to assess the refractive index of plant extracts (Atago, Japan).

2.4 Gas Chromatography-Mass Spectrometry

The GC-MS analysis was carried out on a Shimadzu GCMS-QP2010 SE instrument with an AGILENT HP 1 MS column (Shimadzu, Japan) (30 m x 0.25 ID x 0.25 um film). At a steady flow rate of 3.0 mL/min, helium was used as the carrier gas. The oven temperature was adjusted to 60^{o}C for 5 mins, then steadily increased at a rate of 5^{o}C/min until the injection temperature reached 250^{o}C. At retention indices and mass spectra to the NIST 62 and WILEY 229 spectra libraries, the mass spectrometer was operated with an electron ionization system with ionizing energy of 70 eV.

2.5 Antibacterial activity

Disk diffusion was used for antibacterial activity testing. Muller Hinton agar (MHA) plates were swabbed with broth cultures of *Staphylococcus aureus* ATCC 25925 and *Escherichia coli* ATCC 8733. Inoculated plates were inoculated using sterile 6 mm filter paper (Whatman No.1) discs soaked with 100 µL/mL essential oil dissolved in sterile dimethylsulfoxide (DMSO). DMSO was used as a negative control. As a positive control, streptomycin (5 g/mL) was utilized. The plates were incubated overnight at 37±2^{o}C. The antibacterial activity was determined by measuring the inhibitory zones around the disc in diameter (mm). Three duplicates of the experiments were carried out (Naibaho *et al.,* 2012).

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2.6 Minimum inhibition concentration value

MIC Kaffir lime leaf essential oil was tested by the disc diffusion method. Concentrations were made by diluting the sample with a concentration of 100 µL/mL with 1 mL of nutrient broth media where various concentrations were obtained, namely 50 µL/mL, 25 µL/mL, 12.5 µL/mL, and 6.25 µL/mL. After making the retailing series, aseptically inoculated at each concentration with 1.5 mL of bacterial suspension adjusted to standard turbidity of 0.5 McFarland or the number of colonies as much as 10^8 CFU/mL. Then the mixture of test media was vortexed until homogeneous and incubated with a shaker incubator at a speed of 120 rpm, room temperature and for 24 hrs. After incubation, the test medium was removed and 10 µL of the test medium was taken and planted in a nutrient agar medium, shaking the media to form a figure of eight so that the media solidified. After solidification, the media was incubated for 24 hrs to determine the number of bacteria in each concentration. The MIC is determined by the lowest concentration that inhibits bacterial growth.

3. Results and discussion

3.1 Physical properties of essential oils of kaffir lime leaves

The results of observations of yield value, refractive index, solubility in alcohol, and specific gravity of kaffir lime leaf extract were shown in Table 1. Hydrodistillation of the fresh and dry kaffir lime leaves yields 0.98 and 1.38% of essential oil, respectively. The EOs yield of dried kaffir lime leaves was 1.38% (w/w of dry weight) compared to fresh leaves of 0.98% (w/w of dry weight). The sample of kaffir lime leaves used in each method of the hydrodistillation process is 500 grams. The higher yield value of kaffir lime-dried leaves may be influenced by the less water content in the ingredients compared to the fresh leaf EOs. Pre-treatment such as drying at room temperature will evaporate the water contained in the kaffir lime leaves. Thus, the water contained in fresh leaves is more than in dried kaffir lime leaves. Therefore, with the same weight of the sample (500 g) the dry matter in the dried kaffir leaves is greater than in the fresh leaves, so the yield in the dried kaffir leaves is greater.

Table 1. Physical properties of EOs of kaffir lime leaves

Parameter	Yield % (w/w dry basis)	Refractive index	Specific gravity	Solubility in alcohol 95%
Dry leaves	1.38	1.419	0.98	1:03
Fresh leaves	0.98	1.486	0.89	1:04

The test of refractive index on EOs of kaffir lime leaves was intended for purity testing. The highest refractive index value was seen in fresh kaffir lime leaves with the same distillation methods. The refractive index values of the essential oil of fresh and dried kaffir lime leaves were 1.486 and 1.419, respectively. The viscosity and density of the essential oil determine the refractive index's high or low average value, therefore if the oil density is high, the refractive index will be high as well. Wijaya *et al.* (2000), reported that the essential oil produced from chopped kaffir lime leaves has a refractive index range of 1.4497-1.4506 while the essential oil produced from mashed kaffir lime leaves has a refractive index ranging from 1.4496-1.4529. The refractive index values in the analysis of EOs of kaffir lime leaves were found to be slightly similar to the refractive index of some herbal oils which were in the range of 1.472 and 1.462.

The alcohol solubility of kaffir lime leaf oil was carried out to determine the amount and concentration of alcohol needed to completely dissolve the essential oil. This is related to the quality of the essential oil.

The lowest alcohol solubility was found in dry leaf essential oil, which was in a ratio of 1:3 (1 mL EOs and 3 mL alcohol 95%), while the alcohol solubility of fresh kaffir lime essential oil with a ratio of 1:4 was slightly higher than that of dry leaf essential oil. This could be influenced by the chemical components found in kaffir lime leaf essential oil. Fresh kaffir lime leaf essential oil contains non-oxygenated terpenes, whereas dried kaffir lime leaf essential oil contains oxygenated terpenes. As a result, the lower the essential oil's solubility in alcohol, the higher the quality of the essential oil.

Specific gravity values of essential oil from dried and fresh kaffir lime leaves were 0.91 and 0.89 g/mL, respectively. The lower density value of fresh kaffir leaves oil is suspected because it contains light fractions in the form of terpene compounds that have a small number of carbon atoms and a small number of double bonds. The specific gravity value produced is close to the standard specific gravity value of EOs leaf in general, which is 0.696-1.188. Specific gravity is one of the important criteria for the quality and purity of essential oils. The findings of this study's observations based on reference libraries revealed high-quality essential oils since they were within a range that was consistent with the literature. Wijaya *et al.* (2000) reported that the difference between increasing and decreasing size methods did not have a significant effect on the average value of kaffir lime leaves essential oil types. The specific gravity of the essential oil of chopped kaffir lime leaves was 0.8569-0.8605, not much different from the density of the oil with mashed kaffir lime leaves at

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0.8562-0.8641. This means that the range of specific gravity values does not go beyond the current norm.

3.2 Volatile compound essential oils of kaffir lime leaves

DE techniques were used to separate EOs from fresh and dried leaves of kaffir lime, which were slightly yellow and had a moderate woody odour. Table 2 lists the constituents of volatile chemicals recovered by DE techniques from kaffir lime leaves. There were 25 components identified that comprised 98.86%, and 99.82% of the total oils both from fresh and dried leaves, respectively. The most abundant components of essential oils from fresh kaffir lime leaves were citronellal (77.29%), β-linalool (4.88%), sabinene (4.83%), β-citronellol (3.41%), β-myrcene (1.23%), linalyl acetate (1.18%), and germacrene (1.10%). The most abundant components of EOs from dried kaffir lime leaves citronellal (74.09%), sabinene (5.54%), β-linalool (3.59%), citronellol (3.56%), β-caryophyllene (2.26%), β -citronellyl acetate (1.80%), β-myrcene (1.57%), and linalyl acetate (1.38%). The main component of citronellal is 80.83% (Simanjuntak *et al*., 2021), and 80.86% (Wulandari *et al*., 2017) which comes from the EOs of dried kaffir lime leaves, almost the same as the results of the research conducted. Citronellal was also found as the main component of dry leaf EOs through the distillation process, but the percentage of components was different (Lestari *et al*., 2015; Warsito *et al*., 2017). Furthermore, Hien *et al*. (2020) reported that the main component of fresh kaffir lime EOs was citronellal 85.436% of the time. The major compounds of EOs from fresh leaves were contrasted with dried leaves, which were citronellal and sabinene. The other compounds such as β-ocimene, limonene, α-pinene, β-pinene, geraniol, α-copaene, α-humulene, germacrene b, α-cadinene, nerolidol, and caryophyllene oxide were also found in trace amounts. However, the compounds of nerol were not found in the fresh leaves, and also isogeraniol in the dried leaves, which may be due to the differences in samples and pretreatment. Depending on the pretreatment, the spice's volatile profile can change. When measured by the amount of citronellal produced, the drying process had no discernible effect. This may be influenced by the loss of volatile substances during the drying process, resulting in a lower percentage. As a result, the percentage of volatile components produced will differ depending on the pretreatment method used. These modifications may have a major impact on the

Table 2. Volatile compounds identified in EOs of fresh and dry kaffir lime leaves.

Compounds	RI Value [a]	% relative peak area		Group	Odor Description**
		Fresh leaves	Dry leaves		
α-pinene	5.050	0.17	0.24	Terpenoids	pine, resin, turpentine
Sabinene	5.642	4.83	5.54	Monoterpenoids	pepper, turpentine, wood
β-pinene	5.725	0.29	0.32	Terpenoids	pine, resin, turpentine
myrcene	5.842	1.23	1.57	Monoterpenoids	Cannabis, lemongrass, and bay
Limonene	6.508	0.29	0.32	Terpenoids	lemon, orange
β-ocimene	6.742	1.01	1.26	Terpenoids	herb
β-linalool	7.575	4.88	3.59	Terpenoids	flower, lavender
Citronellal	8.442	77.29	74.09	oxygenated monoterpenes	fat
Citronellol	9.567	-	3.56	Terpenoids	rose
β-citronellol	9.892	3.41	0.14	Terpenoids	rose
Geraniol	9.983	0.51	0.38	Monoterpenoids	rose, geranium
β-citronellyl acetate	11.400	0.22	1.80	oxygenated hydrocarbon	rose, dust
Nerol	11.567	-	0.18	monoterpenoid	wood, flower, wax
Linalyl acetate	11.833	1.18	1.38	Monoterpenoids	rose, dust
α-copaene	11.950	0.21	0.20	monoterpen	wood, spice
Germacrene	12.133	1.10	0.24	Terpenoids	wood, spice
caryophyllene	12.625	0.16	2.26	oxygenated hydrocarbon	wood, spice
α-humulene	13.092	0.57	0.43	monoterpen	wood
Isogeraniol	13.075	0.48	-	monoterpenoid	rose
Isocaryophyllene	13.567	0.25	0.37	sesquiterpene	wood
Germacrene b	13.658	0.21	0.50	Monoterpenoids	wood, earth, spice
α-cadinene	13.933	0.17	0.37	Monoterpenoids	wood
Nerolidol	14.308	0.25	0.39	Sesquiterpenoid	wood, flower, wax
Carryophyllen Oxide	14.842	0.15	0.30	hidrokarbon seskuiterpen	herb, sweet, spice
Total		98.86	99.82		

[a] RI calculated based on n-alkane standard (C8 - C20), * Kovat index, **odor description from flavornet

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essential oil's fragrance qualities (Chatterjee *et al.,* 2015). Differences in environmental factors, weather, and genetic characteristics could explain the existence of several volatile compounds at low levels (Taktak *et al.*, 2021). In the study, there are differences in the composition and percentage of compounds produced. This reinforces the theory that differences in the growth environment have an impact on the quality of essential oils produced (Nur *et al.,* 2019).

3.3 Antibacterial activity of essential oils of kaffir lime leaves

The results showed the antibacterial activity of kaffir lime leaves EOs is shown in Table 3. Several treatments were used in this investigation, including negative control (DMSO), a positive control (Streptomycin), and a concentration of kaffir lime leaves EOs. The inhibitory power of the clear zone that was generated was measured and studied. The negative control (DMSO was used as a solvent to make the test concentration) did not produce a clear zone on either type of test bacteria. Because there was no inhibition in the negative control test, this solvent had no inhibitory effect on bacterial growth. Streptomycin, the positive control, demonstrated a clear zone in *S. aureus* ATCC 25925 (23.21 mm) and *E. coli* ATCC 8733 (17.12 mm) bacteria in this research. Streptomycin resistance levels are thought to be effective at inhibiting the growth of *S. aureus* ATCC 25925 and *E. coli* ATCC 8733 bacteria. The antibacterial activity of the EOs was evaluated against gram-positive bacteria like *S. aureus* ATCC 25925 as well as gram-negative bacteria such as *E. coli* ATCC 8733. Antibacterial activity tests of fresh and dried EOs of kaffir lime leaf showed strong antibacterial activity in the minimum inhibitory concentration (MIC). The essential oil of dried and fresh kaffir lime leaves had the highest inhibitory zones on *S. aureus* ATCC 25925 and *E. coli* ATCC 8733 bacteria, measuring 21.75 mm, 20.89 mm, 20.17 mm, and 16.18 mm, respectively. Essential oil of dried kaffir lime leaves inhibits *S. aureus* ATCC 25925 and *E. coli* ATCC 8733 bacteria more than fresh leaf essential oil. This indicated that *S. aureus* ATCC 25925 was a more sensitive microorganism than *E. coli* ATCC 8733. When an essential oil is used with antibiotic treatment, it has a synergistic impact against multidrug-resistant *S. aureus* ATCC 25925, and in many situations, the MIC is reduced significantly (Chouhan *et al.,* 2017). An extract of 20% concentration of kaffir lime leaves dried in an oven at 45^{o}C demonstrated antibacterial activity with an inhibition zone of 8.3 mm against *S. aureus*, according to Maimunah *et al.,* (2020). Alcohol extract and kaffir lime leaf water inhibited *Aspergillus niger* (14 mm) and *Candida albicans* (27.20 mm), with MIC values of 10.23 g/mL for *C. albicans* and 47.86 g/mL for *A. niger*. The chemical composition of EOs in the aqueous extract and ethanol extract of kaffir lime leaves, where limonene is the main compound (34.32%) and has a major influence on the inhibition of the two fungi, may be affecting the high antifungal inhibition (Soffian *et al*., 2017). The EOs in this investigation had an oxygenated monoterpene composition, with citronellal accounting for 77.29% (wet leaves) and 74.09% (dry leaves). Citronellal is the main compound (80.04%) produced from kaffir lime plants, according to Srisukh *et al.* (2012), and it inhibits *Streptococci* bacteria, *Streptococcus pneumonia*, *Haemophilus influenza*, *S. aureus* (methicillin-resistant and sensitive), and *Acinetobacter baumannii* at MIC ranges of 0.06–68 mg/mL (kaffir lime leaves) and 0.03–17.40 mg/mL (kaffir lime fruit). The rise of drug-resistant microbes is a rising public health concern around the world, and researchers should continue to look for new antimicrobial chemicals. Antimicrobial agents derived from natural sources could be one of the answers.

The minimum inhibitory concentration (MIC) values of EOs of kaffir lime leaves are shown in Table 3. The observed MIC values indicated that EOs fresh leaf had higher efficiency than EOs dried orange leaf. The EOs had a low MIC value against gram-positive and gram-negative bacteria at 6.25 µL/mL. If each extraction showed a different MIC, it maybe because the difference in the extraction procedures yields different chemical compounds, and in the end, may contribute to differences in antibacterial activity. The changes in the antibacterial activity of the investigated EOs of kaffir lime leaves might be attributed to the different chemical compositions of the oils. In addition, the basis of varying

Table 3. Antibacterial activity and MIC of kaffir lime leaves at 100 ppm.

Method of extraction	Inhibition zones (mm)[1]		MIC value (µL/mL)	
	Staphylococcus aureus ATCC 25925	*Escherichia coli* ATCC 8733	*Staphylococcus aureus*	*Escherichia coli*
DE-fresh leaves	21.75	20.17	6.25	6.25
DE-dry leaves	20.89	16.18	6.25	6.25
Streptomycin (5 mg/mL)	23.21	17.12	6.25	6.25
DMSO	Negative	Negative	Negative	Negative

DE: distillation/extraction

[1]Inhibition zone including the diameter of the paper disc (6 mm)

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degrees of sensitivity of test organisms of bacteria may be due to the intrinsic tolerance of microorganisms and the nature and combinations of phytocompounds present in the EOs (Naibaho *et al.,* 2012). Overall, the essential oil of fresh and dried kaffir lime leaves tested showed high antibacterial activity against gram-positive bacteria. *E. coli* ATCC 8733 bacteria showed the strongest bacteria while *S. aureus* ATCC 25925 was the more sensitive bacteria to the test. The composition of essential oils, the functional groups present inactive components, and their synergistic interactions are all factors that influence their activity. The antibacterial mechanism of action differs depending on the kind of EOs or the microorganism strain utilized. Gram-positive bacteria are more vulnerable to EOs than gram-negative bacteria, as is widely known. This is because gram-negative bacteria have a rigid outer membrane that is rich in lipopolysaccharide (LPS) and more complex, limiting the diffusion of hydrophobic compounds through it, whereas gram-positive bacteria are surrounded by a thick peptidoglycan wall that is not dense enough to resist small antimicrobial molecules, allowing easier access to the cell membrane. Furthermore, because of the lipophilic ends of lipoteichoic acid present in the cell membrane, Gram-positive bacteria may facilitate the penetration of hydrophobic EO compounds (Chouhan *et al.,* 2017). Thus, this study showed that gram-positive bacteria were more sensitive than gram-negative to the essential oil of dried and fresh kaffir lime leaves.

4. Conclusion

Different condition extraction showed different chemical and microbiological profiles of kaffir lime leaf oil. The potential antibacterial activity of fresh and dried EOs of kaffir lime leaves from East Borneo from all samples was evaluated using two separate methods, the analysis of the zone of inhibition and MIC. The results showed that dried kaffir lime leaf EOs was more effective in reducing bacterial growth than fresh EOs of kaffir lime leaf. The presence of strong antibacterial activity of dried and fresh kaffir lime leaf plant extracts was associated with the presence of citronellal compounds found in EOs of kaffir lime leaves. To develop EOs as alternative antibacterial medicines, more research is needed on their method of action, synergistic effects with essential oils from other herbal plants, and in vivo adverse effects.

Conflict of interest

The author declared no conflict of interest.

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