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Evaluation of Antimicrobial and Proliferation of Fibroblast Cells Activities of Citrus Essential Oils

Suryati Syafri, Elidahanum Husni, Nurul Wafiqah, Fitrah Ramadhan, Sovia Ramadani, Dachriyanus Hamidi*

Department of Biology Pharmacy, Faculty of Pharmacy, Universitas Andalas, Padang, West Sumatera, Indonesia

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***Correspondence:** Dachriyanus Hamidi, Faculty of Pharmacy, Universitas Andalas, Kampus Limau Manis, Padang, West Sumatra, 25163 Indonesia. E-mail: dachriyanus@phar.unand.ac.id
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Abstract

BACKGROUND: Citrus species produce essential oils (EOs) containing various chemical components that show many pharmacological activities.

AIM: The purpose of this study is to evaluate the chemical content and antimicrobial activity of EO extracted from fruit peels and leaves of *Citrus x aurantifolia* ("Asam sundai") and *Citrus aurantifolia* (lime EOs extracted from fruit peels and leaves).

METHODS: The EO was extracted by the hydrodistillation method. The chemical content was determined using gas chromatography in tandem with mass spectroscopy (GC-MS). Antibacterial activity was performed using broth microdilution method, while proliferation of fibroblast cell was carried out using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay.

RESULTS: The main constituent of the EO of "asam sundai" peel (MAKS) and lime peel (MAKN) was l-limonene, while the EO of "asam sundai" leaves (MADS) was γ-terpinene. MAKN EOs showed stronger antibacterial activity than MAKS and MADS with minimum inhibitory concentration values of 3.12 mg/ml against *S. aureus*, MRSA, and *Pseudomonas aeruginosa* and 6.25 mg/ml for *Streptococcus mutans* and *Escherichia coli*. Meanwhile, the highest fibroblast cell proliferation activity showed by MAKS EO at concentrations of 10, 1, and 0.1 g/ml with a percentage of the proliferation of more than 100%.

CONCLUSION: It can be concluded that the different species of citrus have different chemical compositions and different biological activities.

Introduction

Citrus is a member of the family Rutaceae and grows almost all over the world. The Rutaceae family contains approximately 150,000 species from 150 genera found in tropical and subtropical areas [1], [2]. According to FAO, citrus is one of the among the most valuable plants grown worldwide. In 2019, it was reported that the total worldwide production of citrus was 143,755.6 million metric tons [3].

Many citrus species are well known and used as flavor enhancers in foods, medicine, and personal care products. The most popular types of citrus are Sweet oranges (*Citrus sinensis* Osbeck), Mandarins (*Citrus reticulata* Blanco), Grapefruits (*Citrus paradisi* Macfadyen), Lemons (*Citrus limon* Burmann), and Limes (*Citrus aurantifolia* Swingle). Flavonoids, limonoids, coumarins and furanocoumarins, sterols, essential oils (EO), organic acids, and alkaloids are among the many biologically active secondary metabolites found in members of this genus [4].

Recently, a lot of attention has focused on exploring plant extracts' antimicrobial activities, particularly EOs. The citrus EOs consist of 85%–99% volatile substances and 1%–15% non-volatile substances [5]. It contains approximately 400 different types of compounds, which vary according to (a) species, varieties, and cultivars, (b) cultivation, (c) extraction method, and (d) separation technique [6], [7].

Citrus EOs have long been used in traditional medicine. It has a diverse variety of chemical constituents such as hydrocarbon compounds, oxides, lactones, esters, alcohols, phenols, ketones, and aldehydes [8]. Citrus EOs have been shown to have a variety of biological properties and food preservative [9]. Furthermore, EOs are considered safe for human consumption generally recognized as safe [10].

Two types of citrus are grown and used by the people of West Sumatra, namely *Citrus aurantiifolia* and *Citrus x aurantiifolia*. *Citrus x aurantiifolia* is a hybrid cross between lime (*Citrus aurantiifolia*) and *Citrus hystrix* and is known as "asam sundai." Conventionally, the people of West Sumatra mix the sundai fruit juice, lime juice, and coconut oil to treat coughs. Besides that, both are also used as cooking spices. However, the utilization of the leaves and peels is still lacking. The peels that are frequently discarded

contain potential chemical constituents such as EOs [11], [12], [13].

Many articles report the chemical content and antibacterial properties of lime oil (Citrus aurantiifolia), but the activity on fibroblast cell proliferation has not been reported (Jain *et al.*, 2020). Likewise, with Citrus x aurantiifolia ("Sundai Acid"), there is still little research on the EO of this plant. Therefore, this study investigates the chemical content of EO of the peels and leaf EOs of lime and "asam sundai," which were grown at West Sumatera, and evaluates the antibacterial activity and fibroblast cells proliferation activity.

Materials and Methods

Sample collection

Samples of "asam sundai" peels and leaves (*Citrus x aurantiifolia*) were collected from farmers' gardens in the Kamang area, Ampek Angkek, Agam regency, West Sumatra, Indonesia. While the peels of lime (*Citrus aurantiifolia*) were obtained from a farmer's garden in Padang, West Sumatra Indonesia. The ripe fruits and green leaves were only used in this study. The samples were identified in the ANDA Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang.

Test microorganisms

The test microorganisms used were *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* FNCC 9027, *Escherichia coli* ATCC 8739, *Streptococcus mutans* ATCC 25175, and *Methicillin-Resistant Staphylococcus aureus* (MRSA) ATCC 43300 and were obtained from Research Laboratory, Faculty of Pharmacy, Andalas University.

Essential oil extraction

The EOs of peels and leaves were extracted using the hydrodistillation method. Fresh fruit and leaves were washed with tap water to remove dirt. The fruits were carefully peeled then the skin and leaves were chopped and transferred into a distillation flask. The distillation process took 4 h to complete. The EOs were collected and preserved in dark bottles. Then, sodium sulfate (Na_2SO_4) powder was added to EOs to remove the remaining water. The EOs were stored in a refrigerator at 4°C for further use.

Analysis of essential oils using gas chromatography-mass spectrometry

Chemical components of EOs were determined using gas chromatography-mass spectrometry (Shimadzu GCMS-QP 2010 SE) and an RTX1 column. Helium was used as the carrier gas, with a flow rate of 1mL/min. The temperature ranged from 50°C to 300°C (the temperature was constant at 50°C for 2 min then increased to 80°C at a rate of 2°C/min, then to 150°C at a rate of 5°C/min, then to 200°C at a rate of 10°C/min and then to 300°C at a rate of 20°C/min, at a temperature of 300°C was held constant for 5 min). The injector and detector temperatures were 250°C and 270°C, respectively, and the detector energy was 1.25 kV. The pressure was 70 kPa. 1 μL of samples was injected. The compound was identified using the "WILEY library" available in the Gas Chromatography-Mass Spectrometry (GC-MS) software.

Antibacterial activity of EOs

Antibacterial activity was assessed using the broth microdilution technique. The bacterial subculture was suspended in sterile 0.9% NaCl solution, and then the turbidity was adjusted according to the McFarland standard of 0.5. The bacterial suspension was diluted in Mueller–Hinton Broth (MHB) media with a ratio of 1:150 to produce a bacterial concentration of 1 × 106 cfu/ml. A total of 50 μl of MHB medium were transferred into all wells of the 96-well plate. After that, 50 μl (50 mg/ml) of the test solution was added to the first row and diluted to give 25, 12.5, 6.25, 3.1251, 1.262, 0.781 mg/ml concentrations. Ciprofloxacin as positive control was transferred to the well, and then 50 μl of bacterial suspension was added except for wells for sterility control and bacterial growth. The 96-well plate was then incubated for 18–24 h at 37°C. Then, 40 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well at a final concentration of 0.5 mg/ml, and the plate was incubated at 37°C for 30 min. After 30 min, a color change was observed (colorless to purple). The minimum inhibitory concentration (MIC) was determined, which showed no color change. The test was repeated three times [14].

Fibroblast culture and isolation

Fibroblast cells were isolated from the fetal muscle of mice aged 10–14 days. The fetal body was finely chopped using a sterile knife, then put into the Falcon tube, which already contained 5 ml of phosphate-buffered saline (PBS), then centrifuged at a speed of 200 G for 5 min. The supernatant was discarded and added 5 ml of PBS, then centrifuged three times. After that, 0.25% trypsin EDTA was added, warmed as much as 2 ml, then vortexed for a while. Then, the falcon tube containing cells and PBS was put into a Water Bath at 37°C for 5 min. Then, 10 ml of

complete RPMI medium was added (10% Fetal Bovine Serum + 1% Penicillin-Streptomycin + 1% Amphotericin B/Fungizone antibiotics) centrifuged at 200 G for 5 min. The supernatant was discarded, and 12 ml of complete RPMI medium was added. Cell suspensions were grown into 6-well plates and incubated at 37°C with 5% CO_2 for two days [15].

Fibroblast proliferation activity

The activity of citrus EOs on proliferation of fibroblast cell was carried out using the MTT assay method on a 96-well plate. A total of 180 μl of fibroblast cell suspension were included in each well (density of 10,000 cells/well). The plate was incubated for 24 h in a CO_2 incubator at 37°C with 5% CO_2. Then, 20 μl was added to the well with concentrations of 100 μg/mL, 10 μg/mL, 1 μg/mL, and 0.1 μg/mL. RPMI media containing dimethyl sulfoxide (DMSO) was added to the well for negative control. After that, the 96-well plates were incubated for 24–48 h in a CO_2 incubator at 37°C with 5% CO_2. Next, 100 μl MTT (0.5 mg/mL) was added to each well and incubated for 4–6 h in an incubator at 37°C with 5% CO_2. Viable cells will react to form purple formazan. The formed formazan crystals were dissolved in 100 l of DMSO. The absorption of each well was then measured with a microplate reader at 550 nm. The test was carried out three times [16].

Data analysis

Statistical analysis was performed using Minitab version 19 software for one-way and two-way ANOVA followed by Tukey analysis. Before data analysis, the data were evaluated to determine that the data were normally distributed. The significant level of the p value was $p < 0.05$

Results

Extraction of citrus EOs by hydrodistillation produced a pale yellow EO with a yield of 1.1% for fruit peel and 0.6% for “asam sundai” leaves. In contrast, the peels of lime produce a result of 0.24%. Analysis of the chemical profile of citrus EOs by GC-MS showed that the components in the fruit peel and leaves of “asam sundai” and lime peel differed in terms of the number of compounds and the percentage of compounds in each EO.

The total ion chromatograms of the three EOs are shown in Figure 1a-c. Comparison of the chromatograms of the three EOs showed that there were more peaks that appeared in the MAKN and MADS chromatograms compared to the MAKS EOs. This means that these two oils contain more chemical components than MAKS.

Table 1 shows the chemical profile of the EO of fruit peel (MAKS) and “asam sundai” leaves (MADS), and lime peel (MAKN). All three EOs (MAKS, MAKN, and MADS) contain l-limonene as the main component, where the l-limonene content of MAKS is greater (53.71%) than MAKN (36.68%). In addition, MAKN does not contain γ-terpinene compounds. Meanwhile, both MAKS and MADS EOs contain γ-terpinene, the main compound for MADS (37.08%).

Table 1: Chemical profiling of citrus essential oils

Compound	Percentage of relative area (%)		
	MAKS	MAKN	MADS
Limonene	53.71	36.68	7.20
γ-Terpinene	16.47	0.00	36.41
2-β-Pinene	10.95	16.23	5.01
Cymol	5.04	2.38	21.03
α-Pinene	2.14	1.87	2.76
β-Phellandrene	1.55	0.35	0.70
β-Myrcene	1.19	0.63	0.88
2,2-Dimethoxypropane	0.86	1.25	0.00
β-Fenchyl alcohol	0.84	0.00	0.33
α-Terpinolene	0.82	0.00	2.00
Diacetone alcohol	0.78	1.11	0.00
Terpinen-4-ol	0.67	4.25	0.42
β-Bisabolene	0.20	1.83	2.18
Linalool	0.14	1.37	2.30
β-Ocimene	0.11	0.00	2.20
α-Terpineol	0.00	12.37	0.00
E-Citral	0.00	1.36	0.00
α-Bergamotene	0.00	1.33	0.42
Z-Citral	0.00	1.15	0.00
(-)-Caryophyllene oxide	0.00	1.01	0.00
1-Isoprenyl methyl benzene	0.00	0.00	1.23
Trans-β-Caryophyllene	0.00	0.00	0.88

* MAKS: EOs of “asam sundai” peels, MAKN: EOs of lime peels, MADS: EOs of “asam sundai” leaves, Eos: Essential oils.

The antibacterial activity of citrus EOs is illustrated in Figure 2. The results showed a significant inhibition difference among three citrus oil toward test bacteria. The three citrus oils inhibited the growth of Gram-negative and Gram-positive bacteria. The lowest MIC value means more active EOs. MAKN EO showed stronger antibacterial activity than MAKS and MADS with MIC values of 3.12 mg/ml toward *S. aureus*, MRSA, and *P. aeruginosa* and 6.25 mg/ml for *S. mutans* and *E. coli*. However, MAKS showed the lowest inhibition toward test bacteria, whereby the MIC value was 125 mg/ml for all test bacteria except for the MIC value of *S.aureus* was 62.5 mg/ml.

The fibroblast cells proliferation of citrus EOs is shown in Figure 3. All three citrus oils, MAKS, MAKN, and MADS at a concentration of 100 μg/ml, showed toxicity to fibroblast cells where the percentage of cell proliferation was between 55% and 59%. Meanwhile, at lower concentrations of 10, 1, and 0.1 g/ml, MAKS oil showed that fibroblast cell proliferation was more than 100%, while MAKN and MADS at 10 and 1 g/ml proliferative activity were still below 100%. Statistical analysis showed that MAKS oil significantly enhanced fibroblast proliferation ($p < 0.05$) compared to MAKN and MADS with more than 100% at the concentration of 10 and 1 μg/ml. Furthermore, there were no significant differences in fibroblast cell proliferation between concentration levels of 0.1 and 1 g/ml.

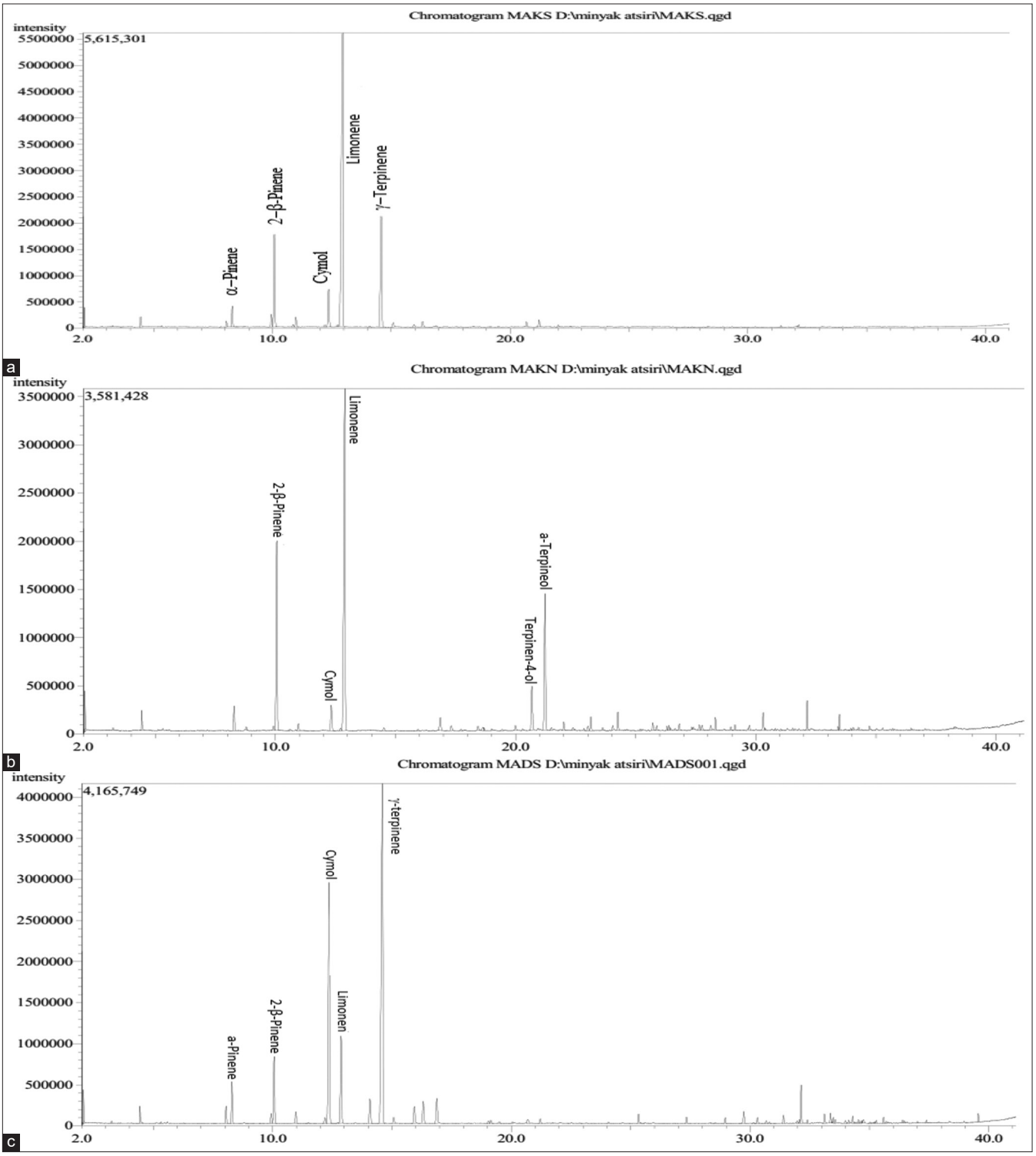

Figure 1: Total ion chromatogram of citrus essential oils. Total ion chromatogram of citrus essential oils A= EOs of "asam sundai" peel (MAKS), B= EOs of lime peel MAKN, C= EOs of "asam sundai" leaves MADS

Discussion

Several factors influenced the yield of EOs, including extraction method, duration time of distillation, and sample preparation [17]. The yield of lime oil (MAKN) obtained in this study (0.24%) differed from other studies, which were 0.83%. This study showed that three citrus oils have the same major constituents, while minor components were different for each. It was supported by a study on EOs of the peel containing D-limonene (38.94%) and β-pinene (26.66%) [17]. Another study of peels and leaves of lime collected in Brazil found limonene (77.5%) as main constituents followed by linalool (20.1%), citronellal (14.5%), and citronellol (14.2%) [18]. The chemical components contained in EOs were influenced by various factors such as geographical conditions where the plant grows, fruit maturity, harvest

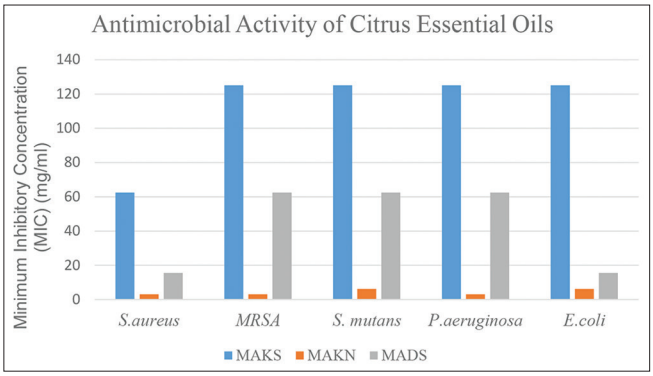

Figure 2: Minimum inhibition concentration of citrus essential oils. The asterisks (*) showed significant differences ($p < 0.05$)

time, the weather during growth and harvest time, plant genetics, method, and duration of EO extraction [8]. As a result, EOs derived from the same plant may differ in chemical composition and bioactivity.

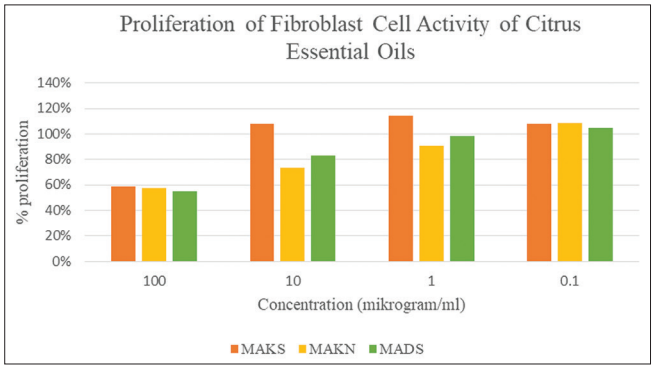

Figure 3: Percentage of proliferation of fibroblast cells of citrus essential oils. The asterisks (*) showed significant differences ($p < 0.05$)

The citrus EOs contain more than 90% volatile compounds, consisting of monoterpenes and sesquiterpenes [12]. The EOs of MAKS and MADS were dominated by monoterpene compounds, which were about 92% and 79%, while MAKN contains only about 59% monoterpenes. Besides that, they also have oxygenated monoterpenes about 21%, and the rest are sesquiterpene compounds. This finding was supported by other studies which found differences in chemical profiles in the peel, leaves, and flowers of citrus species [4], [18].

The variation in chemical constituents and concentration of each component led to different biological activities. The antibacterial activity of some citrus oils has been proven and used in food, cosmetics, and medicine [19]. The MAKN oil was reported to have the highest activity toward test bacteria. It was similar as reported by a study on five citrus species, namely Lime (*Citrus aurantifolia*), Tangerine (*Citrus nobilis*), Sweet Orange (*Citrus sinensis*), Lemon (*Citrus limon*), and Kaffir Lime (*Citrus hystrix*) revealed that lime oil had the highest activity against S.mutans [17]. Citrus EO inhibited *S. aureus* growth more effectively than *E. coli*. The penetration of EOs into the cell wall *S. aureus* easier than into *E. coli* due to the simpler composition of their cell wall. The cell walls of Gram-positive bacteria are composed of peptidoglycan and teichoic acid. Gram-negative bacteria's cell wall on the other hand is made up of peptidoglycan, lipoprotein, an outer membrane, and lipopolysaccharide. Gram-negative bacteria are protected from penetrating polar compounds by the presence of a lipopolysaccharide layer. Meanwhile, leaves EOs MADS had the same sensitivity to *S. aureus* and *E. coli* and weaker to *S. mutan*, *P. aeruginosa*, and MRSA. *S. mutan* and *P. aeruginosa* bacteria have stronger resistance to physical and chemical environments than other bacteria [20].

Limonene is found as the major constituent in some Citrus species, inhibiting bacterial growth [12]. It is said to have a broad spectrum of action, specifically inhibiting the growth of Gram-positive and Gram-negative bacteria. Although limonene is abundant, it is not solely responsible for Citrus oils' antibacterial activity. The presence of other minor components can increase the antibacterial activity of EOs, providing a synergistic relationship between minor and major components at concentrations that lead to the effectiveness of antibacterial activity [21], [22].

Furthermore, other components g-terpinene and -pinene, monoterpene compounds, were shown to have broad-spectrum antibacterial effect against Gram-positive and Gram-negative bacteria as well as to inhibit the growth of growth. TB bacteria *(Mycobacterium tuberculosis)* [23], [24]. This β-pinene had the bactericidal effect on methicillin-resistant *Staphylococcus aureus* (MRSA) within 6 h after exposure to this compound [24]. The presence of oxygenated terpene group compounds such as -terpineol and citral in MAKN oil may contribute to the highest inhibition of these oils compared to others [25]. Mechanism of action of citral could be through inducing changes in ATP concentration and membrane hyperpolarization, causing the difference in the action potential and reducing the pH of the cell resulting in cell disruption [25], [26], [27].

These three EOs showed enhancing proliferation of fibroblast cells. It was supported by the study in South Korea on two Citrus species, namely Citrus obovoidea Hort. ex Takahash and Citrus natsudaidai Hayata. They found that the EOs of these two citruses at a concentration of 0.1 μl/ml had a percentage of proliferation or viability against human fibroblast cell lines above 85% [28]. Monoterpene compounds present in EOs, such as -terpineol, borneol, thymol, genipin, and aucibin, have been shown to have wound healing activity [29].

Conclusion

The chemical profiles of the three citrus EOs differ in terms of constituent type and percentage. l-limonene was the primary constituent of the EOs of "asam sundai" peel (MAKS) and lime peel (MAKN),

while the essential oil of “asam sundai” leaves (MADS) was γ-terpinene. The antibacterial activity of lime peel EO (MAKN) was stronger than other EOs. The proliferation of fibroblast cell activity showed that MAKS EO had a proliferation percentage of more than 100% at 0.1, 1, and 10% concentrations.

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