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Original Article

METABOLITE PROFILING, ANTIOXIDANT, AND IN VITRO WOUND HEALING ACTIVITIES OF *Citrus medica* L. AND *Citrus x microcarpa* Bunge PEELS AND LEAVES ESSENTIAL OILS

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ABSTRACT

Wound care is still dominated by the use of synthetic chemicals such as anti-inflammatory drugs, corticosteroids and antibiotics which have many shortcomings. One potential alternative to be developed is essential oils. Citrus are members of the Rutaceae family which contain essential oils. *Citrus medica* L. and *Citrus x microcarpa* Bunge are distributed in West Sumatra and research on wound healing activity is still lacking. Therefore, this study aims to identify the metabolite profiles of essential oils of two species of citrus using FTIR spectroscopy and GC-MS as well as evaluate their antioxidant activity and determine their wound healing activity. Essential oils were obtained by the hydrodistillation method. Metabolite fingerprinting and profiling were carried out by using FTIR and GC-MS methods. Antioxidant activity was determined using the ABTS and FRAP methods while wound healing used the MTT Assay and Scratch Assay. The result showed the secondary metabolites of *Citrus medica* peels (LPEO) and *Citrus medica* leaves (LLEO) essential oil contained D-limonene and citral. Meanwhile *Citrus x microcarpa* leaves (MLEO) contain ß-pinene, germacrene D, and elemol and *Citrus x microcarpa* peels essential oil (MPEO) consists of D-limonene, ß-pinene, and germacrene D. MLEO has stronger antioxidant activity than MPEO, LPEO and LPEO with ABTS and FRAP method with IC_{50} 197.051 µg/mL and 8.04 Fe (II)/mg sample and is followed by LLEO. Testing of wound healing activity using MTT assay showed that MPEO significantly increased cell proliferation. Meanwhile, the highest wound closure was found at a concentration of 0.1 µg/mL for MLEO followed by LPEO at 77.54% and 73.71% using scratch assay method. It can be concluded that essential oils of *Citrus medica* and *Citrus x microcarpa* can increase cell proliferation and migration so that they have potential to be developed as an alternative in wound healing.

KEYWORDS: citrus, essential oil, FTIR, GC-MS, wound healing, antioxidant

1. Introduction

A wound is a loss of epithelial integrity of the skin. When an injury occurs to skin tissue, healing, and cell regeneration occur automatically as a physiological response of the body [1]. The wound healing process is divided into three overlapping phases: inflammation, proliferation, and tissue remodeling. During the wound-healing process, there are interactions between various cytokine mediator cells and the extracellular matrix [2].

The current treatment methods for wounds include dressing, negative pressure therapy, surgery, hyperbaric oxygen therapy, and hydrogel. However, this approach has some limitations, such as not promoting wound healing, worsening pain by sticking to the wound, etc [3]. Synthetic chemicals such as anti-inflammatories, corticosteroids, and antibiotics are commonly used. Synthetic medicines are considered to have many disadvantages, such as high prices, side effects, and relatively lesser effectiveness for treating chronic wounds. In addition, they have not considerable wound healing properties and only act as antiseptics or induce angiogenesis [4]. Therefore, traditional medicine, such as essential oils, can be used as a safer and more economical alternative for treating wounds. Based on the literature, essential oils are reported to have biological activities, such as antioxidative and anti-inflammatory, which have been shown pre-clinically to heal wounds [5].

Antioxidant properties can increase wound healing activity by keeping ROS levels within the normal range. The wound healing process often involves radicals such as Reactive Oxygen Species (ROS). If ROS are produced in the right amount, they will function well in the wound-healing stage, but if they are made in excess, oxidative stress can occur, which can cause interference with the wound-healing process [6].

Citrus is a member of the Rutaceae family that contains essential oils. The Rutaceae family comprises 150,000 species from 150 genera in tropical and subtropical regions [7,8]. Many citrus species are well known and used as flavor enhancers in food, medicine, and personal care products. *Citrus* essential oils contain several chemical components, such as hydrocarbon group compounds, oxides, lactones, esters, alcohols, phenols, ketones, and aldehydes [9]. *Citrus* essential oils are reported to have various biological activities such as antimicrobial, antioxidant, anti-carcinogenic, anti-inflammatory. Previously, there were some citrus species reported to have wound-healing properties, including *Citrus x aurantiifolia* [10], *Citrus natsudaidai Hayata*, *Citrus obovoidea* Hort. ex Takahash and *Citrus reticulata* [10,11].

Citrus has many species spread throughout the world. Two species, namely *Citrus medica* L., and *Citrus x microcarpa* Bunge can be found in West Sumatra. However, no reports were found regarding the wound-healing activity of the essential oils for both species. Therefore, this study aims to identify the metabolite profiles of peels and leaves of two species of citrus essential oils using FTIR spectroscopy and GC-MS, evaluate their antioxidant activity, and determine their wound healing activity.

2. Materials and Methods

2.1. Plant collection

The fruit peels and leaves of *Citrus medica* L and *Citrus x microcarpa* Bunge were collected in the Padang Pariaman District, West Sumatra, Indonesia. They were then identified at the Andalas Herbarium, which is part of the Department of Biology at Andalas University's Faculty of Mathematics and Natural Sciences in Padang. Subsequently, voucher numbers EH-001 and EH-002 were assigned to them.

2.2. Essential oils extraction

The *Citrus* fruits and leaves were freshly picked, washed, and sorted using tap water. They were then cut and placed into a distillation flask fitted with a Clevenger apparatus. The hydrodistillation process took approximately 5 hours. The essential oils were collected in a dark bottle, and Na_2SO_4 powder was added to remove residual water. Finally, the essential oils were stored at 4°C until they were ready to be used. The essential oil were characterized including yield, color, density and refractive index. The refractive index was measured using Abbe Refractometer®.

2.3. FTIR spectrum measurement

The FTIR spectra were obtained using a Thermo Scientific, Nicolet iS10 spectrometer. The sample was placed on the ATR surface and scanned at a wave number range of 4000 – 650 cm^{-1} at a temperature of 25°C. The scan was carried out with 32 scans and a resolution of 8 cm^{-1}. The resulting spectrum was adjusted to the previously measured background air. Each spectrum measurement was repeated three times. The collected IR spectrum was preprocessed using OMNIC software, including Atmospheric Correction and Smoothing. Finally, the functional groups were identified based on the literature.

2.4. Analysis of essential oils using gas chromatography-mass spectrometry (GC-MS)

The chemical components of the essential oils extracted from the peel and leaves of citrus fruit were identified using Gas Chromatography-Mass Spectrometry (GC-MS) with the conditions listed in (Table 1) [12]. The peaks obtained were identified using the "NIST library". The fragmentation patterns were compared with previously published articles and the NIST Chemistry Webbook to confirm the identification of the peaks.

Table 1. The GC-MS condition

Specification		Information
Instrument		GC Agilent® 7890A
Detector		MS Agilent® 5975C
Column		HP-5ms (Agilent®), diameter 0.25 mm, thick 0.25 µm, length 30 m.
Speed Genre gas		1 ml/minute
Gas Carrier		Helium
Temperature Detector	-	270°C, energy 1.25 kV
Temperature Column	-	50-300°C (temperature 50°C constant during 2 minutes, temperature raised until 80°C with increase 2°C/minute, then increased to 150°C in increments 5°C/minute, then increased to 200°C with an increase of 10°C/minute and then increased to 300°C with an increase of 20°C/minute, at a temperature of 300°C held constant for 5 minutes)
Temperature Injection	-	250°C (constant)
Pressure		70 kPa
Volume of injection		0.1 µL

2.5. Antioxidant activity

2.5.1. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) Method

Antioxidant activity using the ABTS method was carried out on 96-well plates. About 20 µL of essential oil with each series of concentrations (250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, and 15.625 µg/ml) were added to the wells. Then, 180 µL of ABTS solution was added to each well and absorbance was read using a Biochrom Assays UVM 340 Microplate Reader (Biochrom company) at a wavelength of 734 nm. Trolox was used as a positive control. All samples were performed in triplicate and the inhibition percentage and IC_{50} was calculated [13,14].

2.5.2. Ferric ion reducing antioxidant power (FRAP) method

Antioxidant activity using the FRAP method was carried out on 96-well plates. FRAP reagent was prepared by mixing 300 mM acetate buffer pH 3.6, TPTZ (tripyridyltriazine) (10 mM in 40 mM HCl), and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1. The reagent was pre-

warmed at 37°C for 30 minutes before adding to well. Essential oils were dissolved in DMSO to produce a concentration of 125 µg/ml, then 20 µL of diluted samples were put into the well and mixed with 180 µL of FRAP reagent and left for 5 minutes. Absorbance was measured using a Biochrom Assays UVM 340 Microplate Reader (at a wavelength of 595 nm). A standard $FeSO_4$ 300 µM – 18.75 µM standard curve was used to calculate the FRAP value. Ascorbic acid was used as a positive control. Triplicate measurements were performed [13,14].

2.6. In vitro wound healing activity

2.6.1. Fibroblast proliferation activity with MTT assay method

The proliferation of fibroblast cells was evaluated using 3-(4,5)-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. About 180 µl of fibroblast cell suspension (10^4 cells/well) was added to each well. The plate was kept in a CO_2 incubator at 37°C for twenty-four hours with 5% CO_2. Next, 20 µl of essential oil with concentrations of 100, 10, 1, and 0.1 mg/ml in DMSO was added to the well. Dimethyl sulfoxide (DMSO)-containing RPMI medium was added to the well as the negative control. After that, the 96-well plates were incubated for 24 to 48 hours at 37°C with 5% CO_2. Subsequently, each well received 100 µl of MTT (0.5 mg/ml) and was incubated for 4-6 hours at 37°C with 5% CO_2. Viable cells will respond and generate purple formazan. The formazan crystals that were generated were dissolved in 100 µl of DMSO. Then, the absorbances were measured for each well at the wavelength of 550 nm and the percentage of fibroblast cell proliferation was calculated. The assay was repeated for three times.

2.6.2. Fibroblast Cell Migration with Scratch Assay Method

Fibroblast cells were cultivated in 24-well plates with 10^5 cells per well until confluence was reached. A straight wound was then created with the yellow tip. Well was rinsed with 500 µl of PBS (Phosphate Buffer Saline) to remove cellular debris. Then, 1 ml of each test solution (0.1 µg/ml; 1 µg/ml; and 10 µg/ml in DMSO) was added to each well, except for the control, which received 1 ml of DMEM-containing solvent (DMSO). The plate was placed in an incubator and incubated at 37 °C with 5% CO_2. An inverted microscope was then used to observe cell migration at 0, 24, and 48 hours and the percentage of wound closure was calculated. The assay was repeated three times.

2.6.3 Data Analysis

The data were analyzed by Minitab version 21 using one-way ANOVA.

3. Results

3.1. Physical characteristics

The physical characteristics of peels and leaves of *C. medica* and *C. x microcarpa* essential oil included yield, color, refractive index, and density are listed in Table 2. The physical appearance of citrus oil is as seen in Fig 1.

(a) (b) (c) (d)

Fig 1. Essential oil of (a) *Citrus medica* L. peels (b) *Citrus medica* L. leaves (c) *Citrus x microcarpa* Bunge peels (d) *Citrus x microcarpa* Bunge leaves.

3.2. FTIR spectroscopy

FT-IR spectra were obtained at wave numbers of 4000-650 cm^{-1}(Fig. 2) The peak at 3386 cm^{-1} was caused by O-H vibrations. The spectra show prominent peaks at 2965-2855 cm^{-1}, assigned to symmetric and antisymmetric C-H stretching of alkane chain CH_2 and CH_3 groups in *Citrus* peels and leaves essential oils. Additionally, a peak at wave number 1678-1644 cm^{-1} indicated the presence of C=C alkenes in both essential oil samples. C-H bending can be observed at wave numbers 1436-1376 cm^{-1}. It was found that there was a vibration peak at wave number 914 cm^{-1} which indicated the presence of C=C bending in both samples. At wave number 885 cm^{-1} stretching of alkane chain CH_2 and CH_3 groups could be seen in *Citrus* peels and leaves essential oils. Additionally, a peak at wave number 1678-1644 cm^{-1} indicated the presence of C=C alkenes in both essential oil samples. Meanwhile, with the same functional group, there was a medium vibration peak at wave number 797 cm^{-1}, indicating the presence of C=C stretching, trisubstituted [15,16].

3.3. Chemical composition of citrus essential oil

The terpenoids are major compounds often found in essential oil. Three essential oils (LPEO, LLEO, MPEO) contained D-limonene as major constituents, while in MLEO ß-pinene was found as the main constituent. The chemical profiling for four essential oils are as seen in Table 3.

There were differences in the composition of the essential oils for both *Citrus*. Terpenes can be divided into hydrocarbon terpenes and oxygenated terpenes. The classification of terpenes in the four essential oils can be seen in Table 4. Essential oil of leaves and peels of *C. medica* is dominated by oxygenated monoterpenes. Meanwhile, leaves of *C. x microcarpa* contained sesquiterpene hydrocarbons as the main class of terpene. In addition monoterpene hydrocarbons were found in high amount at *C. x microcarpa* peels.

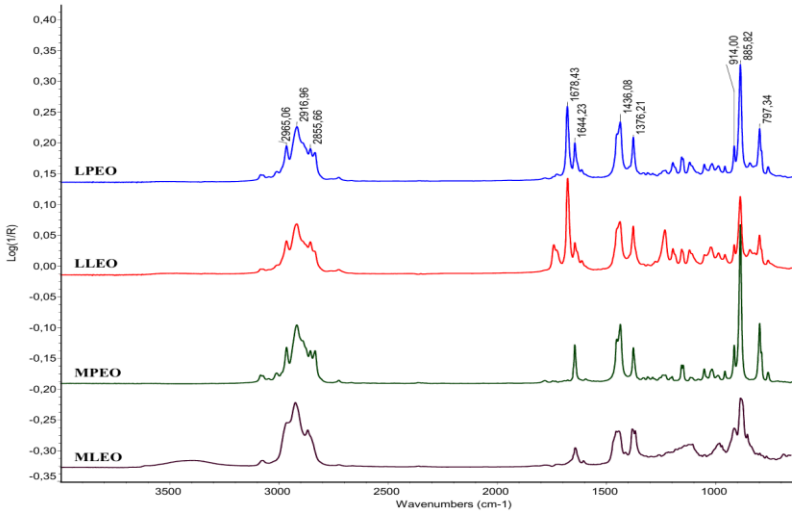


Fig 2. FTIR spectra of peels and leaves of C*itrus* species essential oils.

Table 2. Characteristic of peels and leaves essential oils

Physical characteristics	Peels		Leaves	
	Citrus medica L.(LPEO)	*Citrus x microcarpa* Bunge (MPEO)	*Citrus medica* L. (LLEO)	*Citrus x microcarpa* Bunge (MLEO)
Yield (% v/w)	0.17	0.37	0.49	0.51
Colour	Colorless	Colorless	Pale yellow	Colorless
Density(g/ml)	0.8202	0.8200	0.8492	0.8430
Refractive index	1.4751	1.4714	1.4717	1.4914

Table 3. Chemical profiling of two species citrus essential oils

Chemical Compounds	Relative Percentage (%)			
	Leaves		Peels	
	C. medica	*C. x microcarpa*	*C. medica*	*C. x microcarpa*
D-Limonene	**30.16**	2.52	**41.86**	**24.53**
Citral	**14.72**	0.00	**19.60**	0.41
ß-Pinene	0.00	**17.12**	0.00	**7.61**
Z-Neral	**13.29**	0.00	**15.16**	0.00
Germacrene D	0.39	**14.92**	0.45	**6.22**
Elemol	0.30	**9.11**	0.00	0.72
ß-Eudesmol	0.00	**8.57**	0.00	1.81
Geranyl acetate	**7.61**	0.00	0.79	0.00
Linalool	1.48	**6.93**	1.66	**8.70**
γ-Eudesmol	0.00	**5.80**	0.00	2.23
Nerol acetate	3.44	0.00	0.44	0.00
Caryophyllene	3.04	3.41	2.20	0.00
Methylheptenone	2.60	0.00	0.94	0.00
(R)-Citronellal	2.38	0.00	0.00	0.00
ß-Myrcene	2.19	0.33	2.99	0.00
(R)-α-Pinene	0.64	2.07	1.08	**5.10**
ß-Ocimene	1.86	0.00	1.05	0.66
Terpineol	0.31	0.41	1.26	**8.34**
α-Pinene	0.00	0.00	0.00	**7.63**
Geraniol	1.07	0.00	2.22	0.00
Isogeranial	1.76	0.00	1.82	0.00
Citronellol	0.27	0.11	0.00	0.34

Table 4. Classification of terpenes in *Citrus medica* and *Citrus x microcarpa* essential oils

Class of Compound	Relative Percentage (%)			
	Leaves		Peels	
	C. medica	*C. x microcarpa*	*C. medica*	*C. x microcarpa*
Monoterpene Hydrocarbon	34.14%	27.43%	46.98%	48.59%
Oxygenated Monoterpene	53.87%	8.49%	48.37%	26.36%
Sesquiterpene Hydrocarbon	6.47%	32.59%	3.42%	9.43%
Oxygenated Sesquiterpene	1.12%	28.84%	1.21%	6.11%
Others	0.31%	2.66%	0.00%	9.52%
Total	100%	100%	100%	100%

3.4. Antioxidant activity

Antioxidant activities of essential oil were assessed using ABTS and FRAP. Table 5 displays the antioxidant activity of four essential oils. Trolox was used as the positive control in ABTS method. The study revealed variations in the antioxidant activity between the essential oils derived from the peels and leaves of *Citrus medica* L. and *Citrus x microcarpa* Bunge. The essential oil of *Citrus x microcarpa* Bunge leaves (MLEO) exhibited the highest antioxidant activity. Conversely, MPEO, LPEO, and LLEO demonstrated very weak antioxidant activity. The FRAP value of MLEO was closer to the positive control (ascorbic acid). However, three other essential oils showed FRAP values significantly different from ascorbic acid. MLEO has the highest FRAP value of 8.04 and is followed by LLEO of 7.75 µmol Fe (II) /mg.

Table 5. Antioxidant activity of peels and leaves citrus essential oils

No	Sample		IC_{50} (ppm)	Ferrous Equivalent (µmol Fe (II) /mg)
1.	Peels	*Citrus medica* L.	1931.909	6.38
		Citrus x microcarpa Bunge	3011.624	4.57
2.	Leaves	*Citrus medica* L.	1132.663	7.75
		Citrus x microcarpa Bunge	197.051	8.04
3.	Positive Control	Ascorbic acid	-	10.02
		Trolox	125.036	-

3.5. In vitro wound healing activity

3.5.1. Fibroblast proliferation activity with MTT assay method

The fibroblast cell proliferation activity of *Citrus* essential oils and the positive control (curcumin) are shown in Fig. 3. The results of one-way ANOVA showed a p value <0.05, indicating that there was a significant difference between the test samples in the percentage of proliferation. MPEO showed the highest fibroblast cell proliferation with a 100 µg/ml concentration. However, LLEO and curcumin (CUR) at a concentration of 100 µg/ml showed toxic effects on fibroblast cells, where the percentage of proliferation was below 85%. Meanwhile, three other essential oils did not show a toxic effect on fibroblast cells.

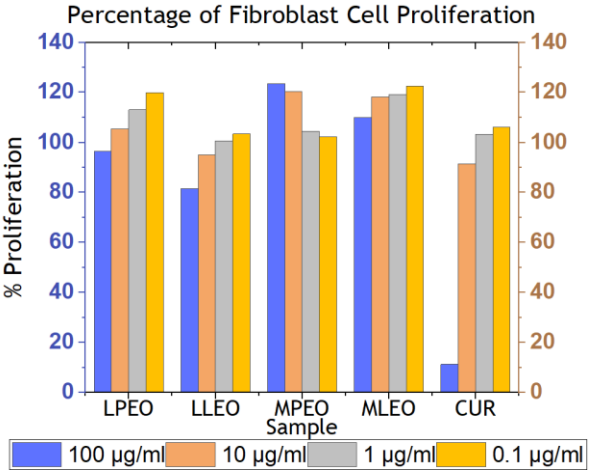


Fig 3. Percentage of fibroblast cell proliferation after treating with citrus essential oils and curcumin.

3.5.2. Fibroblast cell migration with scratch assay method

Citrus essential oil was further evaluated for fibroblast cell migration using the scratch assay. Scratch on fibroblast cell layer represented a wound model. The percentage of wound closure was calculated at incubation times of 24 and 48 hours. The results of the percentage of wound closure are illustrated in Fig 4. The results of the study showed an increase in wound closure at 24 hours and 48 hours. The results revealed a significant effect ($p < 0.05$) of the concentration variations of each test sample on the percentage of wound closure. MLEO demonstrated fastest wound closure at a concentration of 0.1 µg/ml, achieving a wound closure percentage of 77.54%. As the concentration of *Citrus medica* L. increased, there was a decrease in wound closure, with LPEO exhibiting better activity than LLEO. Figure 5 illustrates wound closure after treatment with citrus essential oils (a) and in the control group (b).

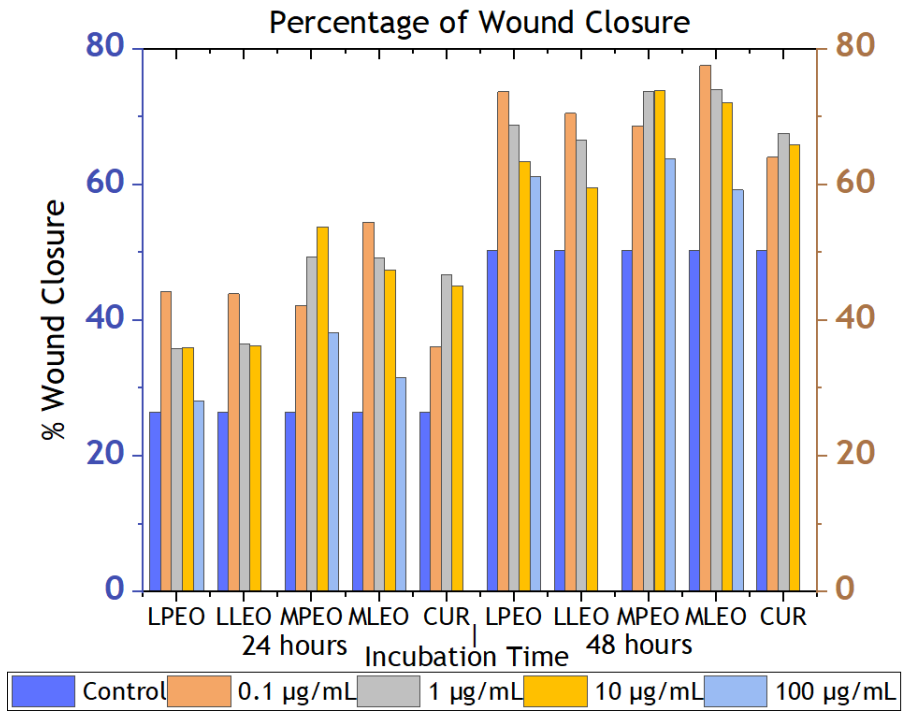
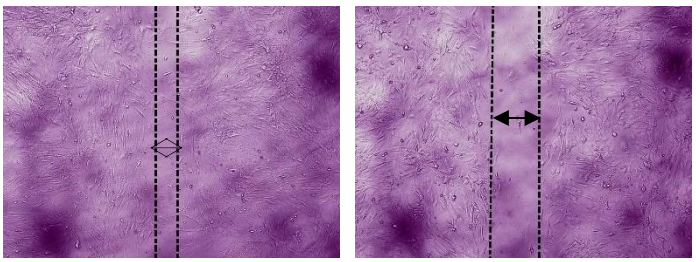


Fig 4. Percentage of wound closure after treating with citrus essential oils and curcumin



(a) (b)

Fig 5. Wound closure after (a) being given *Citrus x microcarpa* Bunge leaves essential oil was 77.54% better than (b) control with wound closure of 50% after incubation for 48 hours.

4. Discussion

Each essential oil has different physical characteristics. LPEO, MLEO, MPEO have a distinctive odor and are colorless, while LLEO has a slightly pale yellow color of the peels and leaves. The yield was varied from 0.17% w/v to 0.51% w/v. The density and refractive index of essential oils obtained can be seen in Table 2. The density of the essential oil obtained is in accordance with the density characteristics of essential oils in the range of 0.8-1.17 g/mL. The refractive index value obtained also meets the refractive index characteristics of essential oils with a range of 1.42-1.61 [17].

The results showed that there were differences in components between *Citrus medica* L. and *Citrus x microcarpa* Bunge. In *Citrus medica* L, the main chemical components of the essential oil of peels and leaves are D-limonene, citral and neral. Meanwhile, the chemical components contained in both *Citrus x microcarpa* Bunge essential oils in the fruit skin and leaves are B-pinene and germacrene D. The previous study was also found D-limonene in *Citrus* essential oil, but the percentage was different. Previous study showed that fruit peel essential oil of *Citrus medica* L originated from Sylhet, Bangladesh contains components such as isolimonene (39.78%), limonene (21.78%), and citral (23.12%), neryl acetate (2.51%), 4-terpineol (0.80%), and 1,3,6-octatriene,3,7-dimethyl-,(Z)- (0.43%). Meanwhile, the essential oil of the leaves contains erucylamyde (28.43%), limonene (18.36%), citral (12.95%), Mehp (8.96%), neryl acetate (5.23%), citronellal (4, 39%), geranyl methyl ether (1.42%), and 2,6-octadien-1-ol,3,7-dimethyl-, acetate (Z) (5.23%) [18]. Another study in Malaysia revealed that the essential oil of *Citrus x microcarpa* contained limonene (94%), ß-myrcene (1.8%), linalool (0.4%), and terpineol (0.3%). Meanwhile, the leaves contain hedycaryol (19.0%), α-sesquiphellandrene (18.3%), α-eudesmol (14.4%), ß-pinene (13.4%), ß-eudesmol (8.6%), and linalool (6.1%) [19].

Citrus species possess diverse and uneven levels of metabolites. Variations in the chemical composition of essential oils can occur across many aromatic plant species. Variations in the chemical composition of the citrus oil could be caused by internal factors, such as genetics. Differences in genetic makeup in different species cause different metabolite profiles. In addition, the quantity of chemical content can be influenced by external factors such as pH, light, temperature, soil nutrient content and plant parts [20].

Antioxidant activity was categorized based on the IC_{50} value. If the IC_{50} value was less than 50 µg/mL, the antioxidant activity was considered very strong. If the $IC5_0$ value was between 50-100 µg/mL, it was classified as strong antioxidant activity. A sample was said to have

moderate antioxidant activity with an IC_{50} value of 100-150 µg/mL, and an IC_{50} of 150-200 µg/mL was categorized as weak. If the IC_{50} value exceeded 200 µg/mL, it was classified as very weak antioxidant activity [21]. The study showed that three citrus oils (MPEO, LLEO, LPEO) can be categorized as very weak, except for MLEO which has weak antioxidant activity. The positive control, Trolox, is considered to have moderate antioxidant activity.

In previous study it was found that essential oil extracted from the peel of *Citrus medica* L. var Acidica was able to effectively inhibit free radicals by 59.64% using the DPPH method [22]. Another study reported that essential oil extracted from the same citrus species (*Citrus medica* L. var Sarcodylactyls) had the ability to scavenge free radicals by 54.1% using the ABTS method and 26.4% using the DPPH method. However, the antioxidant activity of essential oils extracted from *Citrus medica* L. leaves has not been extensively studied [23]. The essential oil of *C. microcarpa* peel was not active as an antioxidant with an IC_{50} value of 3247 µg/mL while *C. microcarpa* leaves have very strong antioxidant activity with an IC_{50} value of 25.3 µg/mL [24,25]. The result obtained was in accordance with previous research, the antioxidant activity of *C. microcarpa* leaves essential oil was stronger than *C. microcarpa* peel.

Moreover, antioxidant activity was evaluated using FRAP method. This method involved the reduction of Fe(III) to Fe(II) by antioxidant compounds in the presence of the tripyridyltriazine (TPTZ) ligand, which results in the formation of a colored Fe(II) complex. TPTZ is a ligand with a maximum absorption at a wavelength of 593 nm [16,26]. This test was conducted in an acidic conditions because it reduces the ionization potential, which encourages electron transfer, increasing the redox potential and shifting the reaction mechanism [27]. The Fe(III) complex produced with TPTZ is usually yellow, but when Fe(III) was reduced to Fe(II) by antioxidants, the complex changes color to blue [28].

In this study, there were differences in antioxidant activity between the essential oils of the peels and leaves of *Citrus medica* L and *Citrus x microcarpa* Bunge. The higher FRAP value indicated higher antioxidant activity. Ascorbic acid as a positive control had the highest FRAP value compared to citrus essential oils. MLEO has the highest FRAP value of 8.04 and is followed by LLEO of 7.75 µmol Fe(II) /mg. The antioxidant activity of essential oil extracted from the leaves was better than peels. The antioxidant activity of essential oils is mainly due to the content of their main compounds. However, small percentages of compounds in the mixture must be taken into consideration due to interactions between compounds which can also influence biological activity [29].

The MPEO increased fibroblast cell proliferation at higher concentrations, while MLEO, LPEO, and LLEO increased fibroblast cell proliferation at lower concentrations. Each essential oil showed wound healing activity, resulting in a significant difference in the percentage of wound closure compared to the control after 24 and 48 hours of incubation. The width of the wound gap decreases as cell migration increases over time. The highest wound closure was found in MLEO at a concentration of 0.1 µg/mL followed by LPEO at 77.54% and 73.71%.

D-limonene was found as major compound in LLEO. D-limonene acted as an anti-inflammatory agent which can accelerate the proliferation phase. Apart from that, limonene also supported fibroblast proliferation and collagen formation which is a part of wound healing process. Besides, limonene can modulate the activity of antioxidant enzymes and protect cells affected by oxidative stress. However, high levels of D-limonene can also cause decreased proliferation. Therefore, as the concentration increases, it can lead to a decrease in fibroblast cell proliferation [30].

The essential oil of *Citrus x microcarpa* leaves showed to have the highest antioxidant activity. Antioxidant activity has been preclinically proven to be able to heal wounds [5]. Antioxidants can keep ROS levels within the normal range. The wound healing process produces ROS as a by-product. Low amounts of ROS have a good impact on the wound healing process, but excessive production can cause oxidative stress that inhibits wound healing [6].

Essential oils can be toxic at high concentrations, possibly due to the toxic nature of the constituent. Citral was found in citrus essential oil and it was known to cause toxicity at high concentrations (>30 µg/kg/day). *C. medica* contained high percentage of citral which may contributed to toxicity effect to fibroblast cell at higher concentration. Whereas, the essential oil of peels and leaves of *Citrus x microcarpa* did not contain citral, so the fibroblast cells were still viable at the oil concentration of 100 µg/ml [30].

Essential oils trigged cell migration by increasing cell adhesion [5]. Several studies have reported that essential oils such as lavender essential oil can increase EGF as a signaling molecule responsible for the rate of contraction and epithelialization of wounds through stimulating the migration of fibroblast and epithelial cells. Apart from that, rosemary and eucalyptus essential oils also have the potential to increase the rate of cell migration. Certain terpene was reported to promote cell migration. Conversely, a number of studies discovered that the dose of terpene molecule or essential oil (from different species) inhibits cell migration in a dependent manner. Thus, the essential oils or terpene compound either stimulate or inhibit cell migration in a dose-dependent manner. This suggests that a higher concentration will likely have a less pronounced influence on migration [5].

5. Conclusions

Citrus x microcarpa Bunge has a major component that is different from *Citrus medica* L. Essential oils from *Citrus x microcarpa* Bunge leaves have the strongest antioxidant activity among four citrus essential oils with ABTS and FRAP methods. Testing of wound healing activity using MTT assay and scratch assay methods showed that essential oils of *Citrus medica* L. and *Citrus x microcarpa* Bunge increased the proliferation and migration of fibroblast cells significantly ($p<0.05$) so that they had potential to be developed as an alternative for wound healing.

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