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Chemical Composition and Biological Activities of Essential Oil from *Citrus aurantiifolia* Peel Waste for Use in Aromatherapy Products

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Abstract

The processing of lime (*Citrus aurantiifolia* (Christm.) Swingle) results in considerable waste, including peels, seeds, and pomace. D-limonene belongs to the category of monoterpenoids, and it is one of the chemicals composed within lime oil. This study aims to explore the antioxidant and anti-inflammatory properties of essential oil extracted from lime peel waste to enhance its market value, with potential uses in natural fragrances for skin cosmetics and SPA products. Essential oils were extracted from lime peels using hydro distillation, and their properties were evaluated. Chemical profiling by gas chromatography-mass spectrometry (GC-MS) revealed D-limonene (51.45%) as the dominant compound among eleven identified constituents. The lime peel oil was found to be non-cytotoxic to normal human dermal fibroblast (NHDF) cells. Additionally, it demonstrated significant in vitro antioxidant activity, as shown by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and the reduction power measured through free radical scavenging activity (FRAP) tests. The essential oil also exhibited inhibitory effects against several skin pathogens, including *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), and *Candida albicans* (ATCC 10231). Significantly, the lime peel oil exhibited a concentration-dependent inhibition of nitric oxide production, with the 25 µg/mL concentration showing a greater reduction in nitric oxide levels compared to the other concentrations when assessed against the lipopolysaccharide-treated group. A massage oil formulation containing lime peel oil was created, demonstrating favorable physicochemical properties and stability at room temperature for 30 d. Overall, these results indicate that lime peel oil, sourced from waste, holds promising potential as a natural fragrance in skin cosmetic and SPA products, in addition to possessing significant antioxidant properties.

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Introduction

The *Citrus* genus includes a range of important fruits, such as oranges, mandarins, limes, lemons, and grapefruits. This genus, scientifically designated as *Citrus* L., is part of the Rutaceae family. *Citrus* fruits are among the most popular fruits consumed worldwide and possess significant economic importance (Lv et al., 2015). Lime (*Citrus aurantiifolia* (Christm.) Swingle) is an essential component of Thai cuisine, prominently featured in dishes such as Tom Yum soup, in various desserts, and in drinks. According to the Food and Agriculture Organization (FAO), Thailand produces approximately 130,000 tons of lemons and limes each year (Food and Agriculture Organization of the United Nations, 2015). After juice extraction, it is common for 50-55% of the peel, albedo, and seeds to be discarded as waste (Suri et al., 2022). Traditional waste management methods, such as landfilling, composting, and providing as animal feed, may not adequately address the disposal of citrus peel waste (Negro et al., 2017; Patsalou et al., 2019). Importantly, lime fruit waste is abundant in high-value compounds, offering numerous potential applications in both technological and health sectors. Lime peel is rich in various biologically active substances, including polyphenols, carotenoids, and essential oils (EOs) (Dosoky & Setzer, 2018). The incorporation of processed lime waste into cosmetics and SPA products could promote the sustainable recovery of functional ingredients while ensuring non-cytotoxic effects.

Lime peel is a significant source of high-value essential oils. The primary active compounds found in these oils include D-Limonene, γ-terpinene, β-pinene, α-pinene, and sabinene, with D-Limonene being the predominant component (Lin et al., 2019). Essential oils derived from lime peel are recognized for their antioxidant properties, as demonstrated by DPPH and ABTS assays (Al-Breiki et al., 2018), as well as their antimicrobial effects (Chanthaphon et al., 2008) and anti-inflammatory benefits observed in animal models (Amorim et al., 2016). Various extraction techniques are employed to obtain essential oils from lime peel, including hydro-distillation, ultrasonic-assisted extraction, and microwave-assisted extraction (Atti-Santos et al., 2005; Lin et al., 2019; Arafat et al., 2020). Notably, over 90% of lime peel essential oil is extracted commercially using traditional methods such as hydro-distillation and steam distillation. Furthermore, essential oils extracted from lime peel through distillation have not been reported to exhibit phototoxicity, indicating the absence of furocoumarins (National Toxicology Program, 2000).

The essential oil extracted from lime peels has a wide range of uses in the food, cosmetics, flavoring, and fragrance sectors. According to the U.S. Food and Drug Administration (FDA), they are recognized as safe flavoring agents. Additionally, lime, lemon, grapefruit, and orange oils are approved for use as flavoring agents in the United States Pharmacopeia (USP) Food Chemicals Codex (Burnett et al., 2019). According to the International Cosmetic Ingredient Dictionary and Handbook, citrus oils function only as a fragrance ingredient (Nikirakis & Kowcz 2023). A report by Opdyke (1974) has shown that the recommended concentration of distilled lime oil in products such as creams and lotions should not exceed 0.1% w/w, while in perfumes, it should be kept below 1.5% w/w. It is crucial to recognize that essential oils are insoluble in water; however, they can be mixed with alcohols, emollients, fats, and/or surfactants such as Tween 20 and PEG-40 hydrogenated castor oil before being added to formulations. Furthermore, essential oil (EO) derived from citrus lemon demonstrate positive effects on pain and anxiety, as examined in both male and female rats (Ceccarelli et al., 2004). Research conducted by Harahap et al. (2023) indicates that the application of lime essential oil as a topical treatment for athletes resulted in a significant reduction in lactic acid levels and pain intensity. Some studies have shown that essential oils have been used for muscle pain and inflammation such as eucalyptus, rosemary, geranium, patchouli, clary sage, lavender, marjoram, camphor, tea tree, black cumin, peppermint, ginger, and citrus oils (Hudson, 1999; Koonlaboot & Hongratanaworakit 2015; Harahap et al., 2023; Hongratanaworakit et al., 2018; Kanjanasilanont et al., 2022).

The essential oils extracted from these citrus cultivars have been reported to have anti-inflammatory effects. In previous studies, *C. obovoides* and *C. natsudaidai* reduced *P. acnes*-induced secretion of IL-8 and TNF-α in THP-1 cells, indicating anti-inflammatory effects (Kim et al., 2008). In another study, the anti-inflammatory effect of essential oil from *C. unshiu* was described (Kim et al., 2014). The essential oil was shown to suppress the production of inflammatory cytokines, including IL-1β, TNF-α, and IL-6, in LPS-activated RAW 264.7 macrophages.

This research seeks to increase the utility of industrial waste produced during lime processing for

possible incorporation into cosmetic and SPA products. The essential oil extracted from lime peel waste was obtained via hydro-distillation, and its physicochemical properties were meticulously assessed. Following this, the chemical composition of the essential oil was examined using gas chromatography-mass spectrometry (GC-MS). To determine its appropriateness for cosmetic and SPA applications, various *in vitro* biological activities were evaluated, including antioxidant capacity, anti-inflammatory effects, antimicrobial properties, and cytotoxicity against normal human dermal fibroblast and RAW264.7 cells.

Materials and methods

1. Chemicals and reagents

DPPH (2,2-Diphenyl-1-picrylhtdrazyl), trolox and 2,4,6-tri (Z2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich Co., Ltd, USA. Ethanol (AR grade) was purchased from Merck Co., Ltd, Germany. Dimethyl sulfoxide (DMSO) was purchased from Fisher Co., Ltd, England. Hydrochloric acid, ferric chloride and sodium sulfate anhydrous were purchased from Qrec Co., Ltd, New Zealand. Ginger oil, plai oil, rosemary oil, peppermint oil, olive oil, mineral oil, jojoba oil and grapeseed oil were procured from Krungthep Chemi Co., Ltd, Thailand. Penicillin streptomycin, fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco Co., Ltd, USA. Normal human dermal fibroblast (NHDF) cells were purchased from ATCC Co., Ltd, USA. RAW 264.7 macrophage cell line was purchased from Elabscience Co., Ltd, USA. Nitric oxide assay kits were purchased from Thermo Fisher Co., Ltd, Austria. Sterile water was purchased from A.N.B. Laboratories Co., Ltd, Thailand. Griess reagent kits were purchased from Invitrogen Co., Ltd, Austria. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was procured from Amresco Co., Ltd, USA.

2. Preparation of lime peel and essential oil extraction

Lime peel waste (*Citrus aurantiifolia* (Christm.) Swingle) was sourced from food processing activities at Suan Dusit University, Suphanburi campus, Thailand, in November 2023. The peels were thoroughly washed with potable water and subsequently cut into smaller pieces. The extraction of essential oils from the lime peel was conducted following a modified version of the method described by Lin et al. (2019). Specifically, 600 g of lime peel were placed in a 2000 mL round-bottom flask, which was then filled with 1200 mL of distilled water and subjected to hydro-distillation for a duration of 3 to 4 h. Following this process, the upper organic layer containing the essential oil was collected, dried using anhydrous sodium sulfate, and filtered. The resulting essential oil was weighed, and the yield percentage was calculated. This assay was performed more than three repetitions in order to obtain a volume of 25 mL of lime peel oil. Finally, the essential oil was stored at -20°C for future applications.

3. Determination of physicochemical properties of essential oil from lime peel

The physicochemical properties of essential oil from lime peel were determined in terms of the physical appearance, color, odor, specific gravity at 20°C, refractive index at 20°C, and solubility in ethanol.

3.1 Physical appearance and color were assessed based on visual observation and odor was evaluated by the direct smelling of paper strips containing the oil compared with another paper strip dipped to the same depth in standard lime oil.

3.2 The specific gravity was determined using a pycnometer, which assesses the weight ratio of essential oil to water at an equivalent volume and temperature. A 10 mL pycnometer was utilized for this measurement. Initially, the empty pycnometer was weighed, after which it was filled with distilled water, ensuring that no air bubbles were present, and weighed again (M1). Subsequently, the pycnometer was cleaned with ethanol and dried. It was then filled with essential oil and weighed once more (M2). The specific gravity of the oil was calculated using the following formula [1]:

$$\text{Specific gravity} = M2/M1 \quad [1]$$

where M1 refers to the weight of water using 10 mL of pycnometer and M2 is the essential oil filled pycnometer weight.

3.3 The refractive index was determined by an oil testing refractometer (RND-025ATC, Jedto, Thailand).

3.4 Solubility of the essential oil was tested in 70%, 80%, and 90% ethanol by determining the minimum volume of ethanol required to completely dissolve one volume of oil without phase separation (v/v).

3.5 The L* a* b* color value was measured using a Chroma meter (CR-400, Konica Minolta, Thailand)

4. Compounds identification in essential oil from lime peel

The chemical composition of essential oil from lime peel was analyzed by gas chromatography-mass

spectrometry (GC-MS). The volatile compounds were identified using an Agilent GC equipped with a capillary column; DB-WAX 30m×0.25mm×0.25µm coupled to an Agilent MSD mass spectrometer (Agilent TechnologiesA, USA). The injector temperature was maintained at 210ºC with a split mode in the ratio of 50:1. The carrier gas flow rate was 1 mL helium/min. The electron energy was 70 eV at 250°C. The constituents were identified by matching their spectra with those recorded in an MS library (NIST MS Search 2.2 library). The relative content of each compound was calculated as a percent of the total chromatographic area.

5. Cytotoxicity to normal human dermal fibroblasts

A cytotoxicity assay was conducted to assess the viability of cultured fibroblast cells utilizing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, following the protocol established by Ala et al. (2018) with minor modifications. In this study, normal human dermal fibroblast (NHDF) cells served as the model for evaluating cytotoxicity. The normal human dermal fibroblast cells were trypsinized and plated at approximately $1x10^4$ cells/well in a 96-well plate, then cultured in complete growth media consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin. The cells were incubated at 37ºC with 5% CO_2 for 24 h. Following this incubation, the culture media was removed, and the cells were washed with sterile phosphate-buffered saline (PBS). The normal human dermal fibroblast cells were then treated with varying concentrations of lime oil (5, 10, 25, 50, 100, and 250 µg/mL) in comparison to a control group. Dimethyl sulfoxide (DMSO) was used as a solvent for lime peel oil in DMEM, and the final concentration of DMSO never exceeded 0.1%. After another 24-h period, the medium containing the treatments was carefully discarded, and the cells were washed again with sterile phosphate buffer saline (PBS) pH 7.4. Subsequently, the normal human dermal fibroblast cells were incubated with MTT solution at 37ºC in the dark for 2 h. After incubation, the medium was removed, and the formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO). The absorbance of each well was measured using a microplate reader (Synergy H1, BioTek, USA) at a wavelength of 570 nm. The percentage of cell viability was calculated using the following equation [2]:

$$\text{Cell viability (\%)} = (A_S/A_C) \times 100 \quad [2]$$

where A_S is the absorbance of the tested sample and A_C is the absorbance of the control (cell untreated with the tested sample)

6. Antioxidant capacity of essential oil from lime peel

6.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity of essential oil derived from lime peel was assessed following a previously established method (Yakaew et al., 2020) with minor modifications. In summary, 150 µL of a 0.2 mM DPPH ethanolic solution was combined with 75 µL of lime oil solution at a concentration of 1 mg/mL. The resulting mixture was incubated in the dark at room temperature for 30 min. Subsequently, the absorbance was recorded at 515 nm using a microplate reader (Biochrom, EZ Read 2000, England). The findings were reported as mg TE/g of lime oil, based on the calibration curve. This assay was conducted in triplicate.

6.2 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing capacity of essential oil derived from lime peel was assessed utilizing the ferric reducing antioxidant power (FRAP) assay, as described by Sulaiman et al. (2011), with minor modifications. In summary, the FRAP reagent was formulated by combining an acetate buffer (300 mM, pH 3.6), a 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ in a ratio of 10:1:1 (v/v/v). Subsequently, 190 µL of the FRAP reagent was combined with 10 µL of a lime oil solution (1 mg/mL). Following a 30-min incubation at 37°C, the absorbance was recorded at 593 nm using a microplate reader (Biochrom, EZ Read 2000, England). The results were quantified as mg TE/g of extract based on the calibration curve. This assay was performed in triplicate.

7. Evaluation of antimicrobial activity

7.1 Microorganisms studied

The bacterial and yeast/fungal organisms determined in this study contained 6 American Type Culture Collection (ATCC) bacterial strains. The ATCC bacterial and yeast/fungal strains were composed of *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (ATCC 10231). These microbials were obtained from the microbiology laboratory, Faculty of Science and Technology, Huachiew Chalermprakiet University.

7.2 Agar well diffusion assay

An agar well diffusion method was performed to

screen the in vitro antimicrobial activity according to the Clinical and Laboratory Standards Institute (CLSI) (Pate et al., 2015) with some modifications. Lime peel oil was dissolved using dimethyl sulfoxide (DMSO) and prepared at a concentration of 100 mg/mL (with 2% DMSO in the final concentration) (Permadi et al., 2024). Bacterial suspension was inoculated on a Mueller Hinton Agar (MHA) plate using a sterile cotton swab. Yeast/fungal suspension was inoculated on MHA supplemented with 2%w/v glucose and methylene blue dye (0.5 µg/mL). Wells were bored into the MHA plates using a sterile 8 mm cork-borer. The wells were filled up with 100 µL of lime peel oil. Levofloxacin (5 µg/disc) and fluconazole (25 µg/disc) were used as a positive control for bacteria and yeast/fungus, respectively while DMSO was used as a negative control. The bacterial plates were incubated in 35°C for 24 h and yeast/fungus was incubated for 48 h. The antimicrobial activity was evaluated by measuring inhibition zone diameters. This assay was done in triplicate.

7.3 Determination of minimal inhibitory concentration (MIC, mg/mL) and minimum bactericidal/fungicidal concentration (MBC/MFC, mg/mL)

The minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of lime peel oil were assessed using the broth microdilution technique, adhering to the guidelines set forth by the Clinical and Laboratory Standards Institute (Powell et al., 2016), with certain modifications. A bacterial suspension, approximately $1x10^5$ CFU/mL, and/or a yeast/fungal suspension, approximately $1x10^4$ CFU/mL, was combined with 1 mL of lime peel oil. The lime peel oil underwent two-fold serial dilution in Mueller Hinton broth, with concentrations ranging from 0.390625 to 200 mg/mL. Following incubation at 37°C for a duration of 24 to 48 h, the MIC was identified as the lowest concentration that entirely prevents visible bacterial growth. The MBC/MFC was established as the lowest concentration that completely inhibits bacterial growth on an agar plate. Each experiment was conducted in triplicate.

8. Anti-inflammatory activity

8.1 Cell culture

The murine macrophage cell line RAW 264.7 (EP-CL-0190, Elabscience, USA) was seeded in 96 well-plates at a density of $1x10^4$ cells/well in RPMI-1640 medium (GIBCO, Paisley, UK) supplemented with 10% v/v fetal bovine serum (GIBCO, Paisley, UK) and 1% v/v penicillin/streptomycin (GIBCO, Paisley, UK). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere for 24 h.

8.2 Cytotoxicity

A cytotoxicity assay was conducted to assess the viability of cultured RAW 264.7 macrophage cells utilizing the MTT assay, following a previously established protocol with some modifications (Lim et al., 2018). RAW 264.7 macrophages were seeded at a density of $1x10^4$ cells/well and incubated for 24 h. After this incubation period, lime oil was applied at various concentrations ranging from 0 to 250 µg/mL. Dimethyl sulfoxide (DMSO) served as the vehicle control, ensuring that the final concentration did not exceed 0.2% v/v. The treated cells were then incubated for an additional 24 h, after which the spent medium was discarded. Subsequently, 20 µL of MTT reagent (5.0 mg/mL) was introduced to each well and incubated at 37°C in a CO_2 incubator for 2 h. The absorbance of each well was measured at 570 nm using a microplate reader (Synergy H1, BioTek, USA).

8.3 Measurement of nitric oxide (NO) production

RAW264.7 macrophages were plated in a 96-well culture plate at a density of 5×10^4 cells/well (Kunnaja et al., 2019) and incubated for 24 h. Following this, the cells were pretreated with lime peel oil at varying concentrations (0-25 µg/mL) for 30 min, after which they were incubated with lipopolysaccharide (LPS) (1.0 µg/mL) at 37°C in a 5% CO_2 environment for an additional 24 h. The secretion of nitric oxide in the culture supernatant was assessed using the Griess reagent kit through the Griess assay (Hyun & Hyun, 2020), with an incubation period of 30 min at room temperature. The concentration of nitric oxide was quantified at 570 nm using a microplate reader (Synergy H1, BioTek, USA). Results were reported as nitrite levels (µM) relative to the nitrite levels measured in the DMSO control in LPS-treated cells. Indomethacin (50 µM) served as a positive control.

9. Formulation of aromatherapy massage oil

The massage oil formulation was created by combining essential oils such as lime peel oil, ginger oil, plai oil, rosemary oil, and peppermint oil with carrier oils, as detailed in Table 1. The carrier oils consisted of olive oil, mineral oil, jojoba oil, and grapeseed oil, mixed in a ratio of 3:4:1:2. The essential oils were blended with the carrier oils in a bottle that was light resistant. The bottle was then securely closed and shaken thoroughly until a uniform mixture was achieved.

Table 1 The formula of aromatherapy massage oil

Components	Volume (mL)	Functions
Lime peel oil	0.50	Antioxidants, Anti-inflammatory, Antimicrobial, Aromatic compound
Ginger oil	1.25	Antioxidants, Aromatic compound
Plai oil	1.75	Cooling agent, Aromatic compound
Rosemary oil	0.50	Antioxidants, Aromatic compound
Peppermint oil	1.00	Cooling agent, Aromatic compound
Carrier oil	50.00	Emollient, Moisturizer

10. Characteristics test of the prepared aromatherapy massage oil

The assessment of physical characteristics includes the evaluation of organoleptic properties, pH, and viscosity of the formulated product. Organoleptic evaluation was performed by examining the color, appearance, visual clarity or turbidity and scent. The pH measurement was conducted with a pH meter (Starter B100, Ohaus Instruments, Shanghai, China), which was calibrated with a standard buffer solution. The L* a* b* values of the formulated massage oil were determined using a Chroma meter (CR-400, Centasia, Konica Minolta, Thailand). Viscosity was measured with a Brookfield viscometer (DV-I Prime, Brookfield LV, USA) at a speed of 30 rpm, utilizing spindle number 63.

11. Stability test of aromatherapy massage oil

The physical stability of the prepared massage oil was assessed based on its appearance, color, odor, pH, and viscosity. The formulation was stored in airtight containers and assessed under accelerated conditions, which included a heating-cooling cycle (45°C for 24 h followed by 4°C for 24 h, repeated for 6 cycles), storage at room temperature (30±5°C) for 30 d, and exposure to sunlight for 30 d. After this storage period, the formulas were taken out and allowed to return to room temperature before evaluating their physical characteristics in comparison to the initial state.

12. Statistical analysis

Data were expressed as mean values ± standard deviation (SD) using Microsoft Excel 2013. Statistical analysis of the data was analyzed by performing a one-way analysis of variance (ANOVA) and Duncan post-hoc tests using SPSS program (SPSS ver. 22.0 for Windows, SPSS Inc., Chicago, IL, USA) and the statistical significance found was $p<0.05$.

Results and discussion

1. Physicochemical properties of essential oil from lime peel waste

The process of extracting essential oil from lime peel waste through hydro-distillation is illustrated in Fig. 1. The yield of essential oil obtained from the lime peel was measured at 0.41±0.02% w/w. The findings were consistent with previous research carried out by Visakh et al. (2022) and Permadi et al. (2024). Visakh et al. (2022) reported a yield of lime peel oil from waste at 0.48±0.12%, whereas the study by Permadi et al. (2024) indicated that the yield of lime peel oil was 0.48±0.014%. Both studies employed hydro-distillation extraction techniques. The essential oil derived from lime peel appeared as a yellowish liquid, characterized by a distinct fresh citrus aroma (Fig. 1D).

Organoleptic analysis of physicochemical properties is an important method to assess the quality of the essential oil. The important physicochemical properties of essential oil include specific gravity, refractive index, and miscibility in ethanol. The result of the physicochemical analysis is presented in Table 2. The color value (L* a* b*) was similar under a visual investigation. The specific gravity of essential oils is generally less than 1, indicating that they are lighter than water and will float on its surface (Gamarra et al., 2006). Obtained result found that the specific gravity of essential oil from lime peel ranged from 0.8428 to 0.8628. The refractive index value of essential oil from lime peel ranged from 1.464 to 1.466. Essential oil is characterized by high refractive indices (the refractive index of pure water at 20°C is 1.333). The findings were consistent

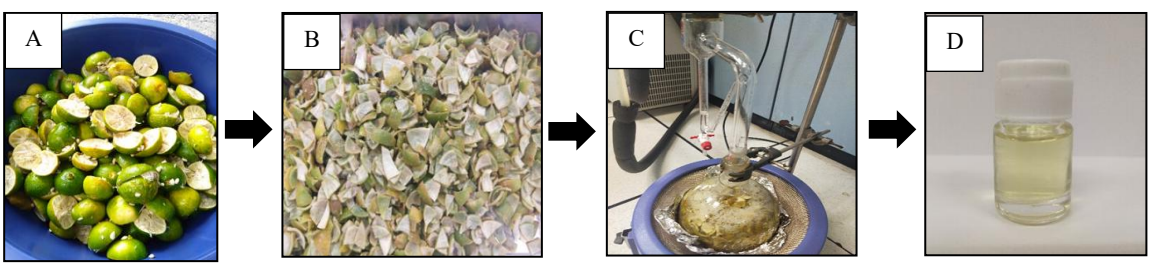

Fig. 1 The extraction process of essential oil from lime peel waste using hydro-distillation; A: lime peel waste, B: peels were washed and cut into small pieces, C: hydro-distilled for 3-4 h, and D: visual appearance of essential oil extracted from lime peel.

with those previously documented, which indicated that the specific gravity and refractive index values of distilled lime peel oil ranged from 0.855 to 0.863 and from 1.474 to 1.477, respectively (Burnett et al., 2019). Furthermore, this suggests that the presence of D-limonene and α-pinene, which exhibit specific gravity values between 0.837 and 0.847 and 0.858 and 0.861, respectively, as well as refractive index values ranging from 1.470 to 1.474 and from 1.464 to 1.468, respectively, may be contributing factors. Lime peel oil also showed a proficient solubility in ethanol at concentration of 90%, which was 1.0 mL of oil in 5.0 mL of 90% ethanol. It was based on visual transparency and lack of phase separation. This study may be caused by the higher content of monoterpene in the oil, which makes it possible for the oil to dissolve in ethanol. The results were mirrored previous research (Arafat et al., 2020; García-Fajardo et al., 2023).

Table 2 Physicochemical characteristics of essential oil extracted from lime peel waste

Physicochemical characteristics	Results
Visual color	Yellowish liquid
Odor	Fresh citrus, intense
Specific gravity at 20°C	0.8528±0.01
Refractive index at 20°C	1.465±0.001
L* a* b* color	L*=59.88±0.19, a*=-0.57±0.03, b*=-0.73±0.07
Solubility in ethanol	1 mL in 5 mL of 90% ethanol

2. Chemicals composition in essential oil from lime peel

Eleven compounds of essential oil were identified in lime peel waste, according to GC-MS analysis and are presented in Table while the gas chromatography–mass spectrometry (GC-MS) chromatogram is depicted in Fig. 2. Of those compounds, the content of D-limonene was the highest (51.45%), followed by those of α-Pinene (16.59%) and γ-Terpinene (8.44%), respectively. The results reflected previous research (Al-Breiki et al., 2018; Lin et al., 2019). According to Al-Breiki et al. (2018), the essential oil extracted from the fresh peels of *Citrus aurantifolia* in the North Al-Batinah region was found to comprise D-limonene (54.44%), E-citral (7.28%), Z-citral (5.81%), γ-terpinene (5.60%), and E, E-α-farnesene (5.27%). In contrast, Lin et al. (2019) reported that the essential oil obtained from fresh lime peels had limonene as its primary component (42.35%), followed by γ-terpinene (15.44%), β-pinene (12.57%), α-pinene (3.12%), neryl acetate (2.2%), and sabinene (2.12%). Meanwhile, Visakh et al. (2022) documented that the chemical composition of lime peel oil derived from waste included D-limonene (32.43%), followed by α-terpineol (14.75%) and α-pinene (11.50%), respectively. Monoterpene components of lime peel oil could be described as "top note" because they were sharp and could be perceived immediately upon application (Sadgrove & Jones 2015). Monoterpene synthases manage various volatile compounds from *Citrus* essential oils. As noted by Al-Breiki et al. (2018), the yield, chemical composition, and characteristics of the essential oils extracted from the peel of Omani lime (*Citrus aurantifolia* L.) cultivated in three distinct regions of Oman exhibited variations. These differences may be attributed to factors such as geographical distribution, environmental conditions, irrigation practices, and harvest timing, all of which can influence the medicinal properties of the oils.

Table 3 Chemical composition of lime oil from peel waste

No.	Retention (RT, min)	Compound name	% Composition
1	3.022	4-Carene	6.29
2	4.109	α-Pinene	16.59
3	5.078	β-Thujene	1.47
4	6.072	D-Limonene	51.45
5	6.301	β-Phellandrene	0.6
6	7.473	γ-Terpinene	8.44
7	27.565	Geranyl isobutyrate	0.68
8	31.295	Terpinen-4-ol	3.05
9	38.040	trans-2-Decalone	1.78
10	40.673	β-Santalol	6.78
11	43.069	Geraniol	2.91

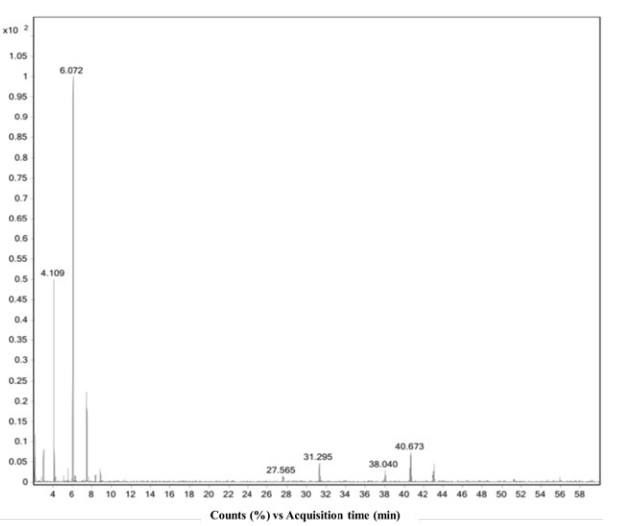

Fig. 2 Gas chromatography–mass spectrometry chromatogram of essential oil from lime peel waste

3. Cytotoxicity to normal human dermal fibroblasts

One of the key criteria for evaluating the cytotoxicity of lime peel oil is cell viability. The cytotoxic impact of lime peel oil on normal human dermal fibroblast (NHDF) cells was assessed using the MTT assay. The results are presented as a percentage of cell viability, with the concentrations of the extracts illustrated in Fig. 3. All concentrations tested (ranging from 5.0 to 250 µg/mL) did not compromise the viability of normal human skin fibroblasts after a 24-h incubation period, with cell viability remaining above 70%. This finding indicates that the lime peel oil, at the concentrations examined, is not toxic to normal human skin fibroblasts in vitro. These results serve as a foundational guideline for product formulation development, particularly in identifying the suitable concentration of the extract for application. According to the studies by Hattingh et al. (2023) and Lopez-Garcia et al. (2014), a cell survival rate exceeding 70% categorizes the tested substance as non-cytotoxic. In contrast, survival rates from 40% to 60% indicate moderate cytotoxicity. A survival rate below 40% signifies strong cytotoxicity.

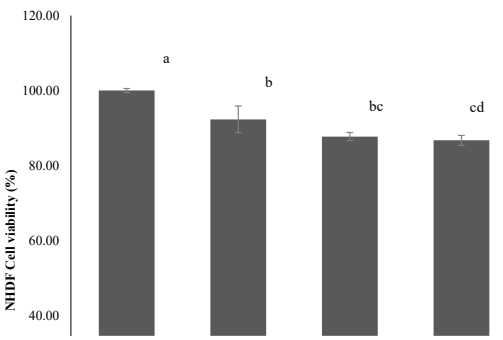

Fig. 3 The percentages of NHDF cell viability after exposure to lime peel oil for 24 h. Each bar represents mean ± standard deviation (SD) of triplicate (n=3). The different letters represent statistical significance ($p<0.05$).

4. Antioxidant capacity of essential oil from lime peel

The antioxidant capacity of essential oil from lime peel was studied according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect, and the reducing power was determined through free radical scavenging activity (FRAP) assays. The researchers' results found the lime oil showed the antioxidant capacity which was the amount of DPPH scavenging and ferric reducing power expressed as trolox of 19.77±0.03 mgTE/g of lime oil and 193.81±41.47 mgTE/g of lime oil, respectively. The antioxidant activity of the tested lime peel oil might be related to the presence of monoterpenes, particularly D-limonene α-Pinene and γ-terpinene, which are the main compounds of lime peel oil and have been reported to have a good antioxidant activity (Conforti et al., 2007). D-limonene comprises two isoprene units and has two double bonds. Compounds made up of the isoprene structure possess antioxidant properties (Erasto & Viljoen, 2008). The results obtained were consistent with previous studies, notably those by Al-Breiki et al. (2018), which revealed that essential oils derived from the lime peel of the North Al-Batinah, Al-Sharqia, and Muscat regions displayed considerable antioxidant activity, with percentages ranging from 12.72-58.22%, 13.45-50.89%, and 12.14-59.87%, respectively. In contrast, ascorbic acid exhibited an antioxidant activity ranging from 31.22-96.12%, as assessed by the DPPH assay. Furthermore, additional studies have shown that the DPPH scavenging effect of lime peel oil fluctuated between 10.65% and 66.44% at concentrations of 0.08–3.46 mg/mL, with an IC_{50} value of 2.36 mg/mL (Lin et al., 2019). Research conducted by Yang & Park (2025) indicated that essential oils derived from the peels of *Citrus* cultivars through hydro-distillation exhibit reducing power activities, as assessed by the FRAP method. The findings presented by Visakh et al. (2022) indicated that essential oils derived from *Citrus* peel waste, such as *C. aurantifolia, C. limon, C. limetta,* and *C. reticulata*, can be regarded as a source of phytochemicals possessing insecticidal and antioxidant properties, as demonstrated by DPPH, ABTS, and FRAP assays.

5. Antimicrobial activity of lime peel oil

The antimicrobial activity of essential oils is partly due to their lipophilic character that leads to interaction of its constituents with the lipid layer, eventually disrupting the cell membrane integrity of microorganisms and causing death of microorganisms (Costa et al., 2010). In this study, antimicrobial activities of essential oil from lime peel against pathogenic; *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 10231) was evaluated using agar well diffusion assay and broth microdilution assay.

5.1 Agar well diffusion assay

As observed in Table 4, the inhibition zone diameter was observed for lime peel oil (100 mg/mL) and positive controls; levofloxacin (5 µg/disc) for bacteria and fluconazole (25 µg/disc) for yeast/fungus. Results showed that the highest inhibition zone diameter of lime peel oil determined against *C. albicans* was 22.6±1.2 mm, followed by against *S. aureus* was 21.4±1.3

mm and against *P. aeruginosa* which was 19.7±2.5 mm, respectively. The visible clear zone of lime peel oil, negative control (dimethyl sulfoxide, DMSO) and positive controls are as shown in Fig. 4.

Table 4 The inhibition zone diameter of lime peel oil and positive controls (levofloxacin and fluconazole)

Samples	Inhibition zone diameter (mm.±S.D)			
	E. coli	*P. aeruginosa*	*S. aureus*	*C. albicans*
Lime peel oil (100 mg/mL)	17.0 ± 3.2[b]	19.7 ± 2.5[b]	21.4 ± 1.3[b]	22.6 ± 1.2[b]
Levofloxacin (5 µg/disc)	30.3 ± 0.6[a]	27.2 ± 0.9[a]	33.5 ± 0.8[a]	-
Fluconazole (25 µg/disc)	-	-	-	31.1 ± 0.9[a]

Remark: Values are given as mean ± S.D of triplicate. The different superscript letters in the same column represent significant differences when compared with each positive control at p<0.05.

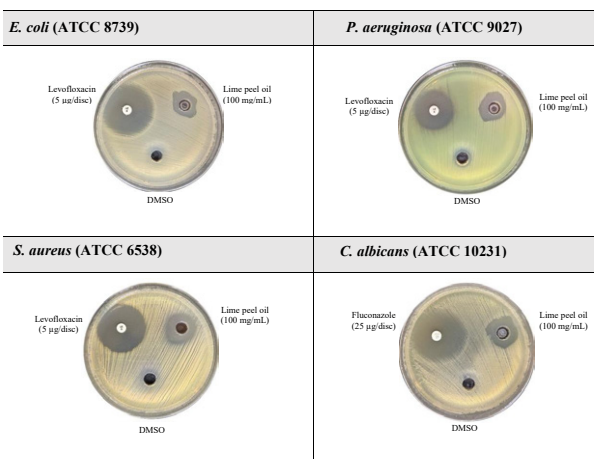

Fig. 4 Visible clear zone of lime peel oil, negative controls and positive controls

5.2 MIC and MBC/MFC

Table 5 indicates that lime peel oil exhibits minimum inhibitory concentrations (MICs) between 6.25 and 50.0 mg/mL, with minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) ranging from 12.5 to 50 mg/mL. Notably, lime peel oil had the strongest antifungal action on *C. albicans* yeast, with a MIC of 6.25 mg/mL and MBC/MFC of 12.5 mg/mL, as evidenced by the lowest recorded MIC and MBC/MFC values.

Table 5 Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of lime peel oil

Bacteria and yeast/fungal	MIC (mg/mL)	MBC/MFC (mg/mL)
E. coli (ATCC 8739)	12.5±0.00[b]	12.5±0.00[c]
P. aeruginosa (ATCC 9027)	50.0±0.00[a]	50.0±0.00[a]
S. aureus (ATCC 6538)	12.5±0.00[b]	25.0±0.00[b]
C. albicans (ATCC 10231)	6.25±0.00[c]	12.5±0.00[c]

Remark: Values are given as mean ± S.D of triplicate. The different superscript letters in the same column represent significant differences when compared with each microorganism at p<0.05.

The oil extracted from the peel of *C. aurantifolia* is extensively recognized for its bioactive properties, particularly its antimicrobial effects. The largest diameter of the inhibition zone for lime peel oil, as determined against *C. albicans*, aligns with the findings of Mohammed et al. (2024). Their investigation into the antimicrobial properties of *Citrus* essential oils (including grapefruit, lime, and orange) involved measuring the inhibition zones against specific tested organisms. They reported that lime peel oil exhibited the most significant inhibition zone against *P. aeruginosa, S. aureus, B. subtilis* and *C. albicans*, whereas orange essential oil demonstrated the largest inhibition zone against *E. coli*. Furthermore, Thonglem et al. (2023) indicated that lime essential oil had a pronounced effect on *S. aureus*, with an inhibition zone measuring 22.67 mm. This finding is consistent with the research conducted by Al-Breiki et al. (2018), which revealed that lime peel oil was more effective against *S. aureus* compared to *E. coli.*

Minimum inhibitory concentration (MIC) is the lowest concentration of medicament in which bacterial growth cannot be visibly or spectrophotometrically detected in liquid media. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) are the lowest dilution of medicament that kills at least 99.9% of the initial inoculum of bacteria on solid media. In addition, the ratio of minimum bactericidal concentration (MBC) to minimum fungicidal concentration (MFC) is used to determine the killing ability of an antimicrobial compound in relation to its inhibitory capacity. A ratio of MBC/MFC less than or equal to 4 suggests a bactericidal or fungicidal effect, meaning the agent destroys the microorganisms. A ratio greater than 4 suggests a bacteriostatic or fungistatic effect, meaning the agent inhibits the growth of the microorganisms but does not necessarily destroy them. Obtained results for the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) indicated that lime peel oil exhibited a greater inhibitory effect on the fungi/yeast *C. albicans* compared to other bacteria, with a MIC of 6.25 mg/mL and MBC/MFC of 12.5 mg/mL, respectively. It means that lime peel oil can inhibit *C. albicans* and the ratio of MBC/MFC provided greater than 4 was considered to have a bacteriostatic or fungistatic effect. According to the study conducted by Permadi et al. (2024), it was reported that

lime peel oil exhibited MICs and MBC/MFCs ranging from 0.20 to 6.25 mg/mL and 0.39 to 12.5 mg/mL, respectively. They confirmed that lime peel oil displays enhanced fungicidal activity against *T. viride*, with the lowest recorded MIC (0.2 mg/mL) and MBC (0.39 mg/mL) values. This increased efficacy is linked to the variety of active chemical constituents found in lime peel oil, with D-limonene being a key component. D-limonene has been shown to effectively combat several foodborne bacterial and fungal pathogens, such as *A. niger, C. falcatum, S. aureus,* and *L. monocytogenes*. According to Han et al. (2020), D-limonene can induce membrane damage, increase membrane permeability, and result in the leakage of cellular contents in *L. monocytogenes*.

6. Anti-inflammatory activities

6.1 Measurement of cell viability

Cytotoxicity was assessed in RAW264.7 cells treated with lime peel oil using the MTT assay, with various concentrations ranging from 5.0 to 250 µg/mL, as illustrated in Fig. 5. The viability of the cells treated with lime oil was compared to the control group, which exhibited 100% viability (untreated cells). The results indicated that lime peel oil decreased the viability of RAW264.7 cells in a concentration-dependent manner. At all tested concentrations (5.0-250 µg/mL), a significant reduction in cell viability was observed ($p<0.05$) compared to the control group, which showed cell viabilities of 85.99±2.03%, 83.96±1.50%, 76.55±1.68%, 66.50±2.29%, 55.43±4.79%, and 49.64±1.33%, respectively. Notably, lime peel oil at concentrations of 5-25 µg/mL maintain cell viability above 70%, indicating a lack of toxicity towards RAW264.7 cells. Consequently, lower concentrations (5, 10, and 25 µg/mL) were chosen for subsequent investigations into the reduction of nitric oxide (NO).

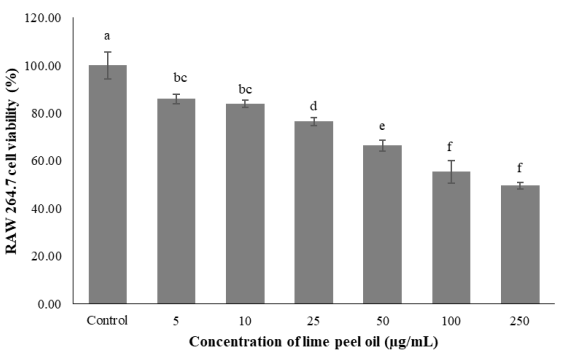

Fig. 5 The percentages of RAW 264.7 cell viability after exposure to lime peel oil for 24 h. Each bar represents mean ± standard deviation (SD) of triplicate (n = 3). Different letters represent statistical significance ($p<0.05$).

6.2 Measurement of nitric oxide (NO) production

Macrophages play a crucial role in modulating immune responses and inflammatory processes. An overproduction of nitric oxide is linked to inflammatory diseases and can cause oxidative damage to cells and tissues, resulting in genetic mutations and nerve injury (Sharma et al., 2007). Therefore, reducing nitric oxide synthesis may help prevent the development of inflammation-related disorders. In this investigation, nitric oxide was generated by stimulating the RAW264.7 mouse macrophage cell line with lipopolysaccharide (LPS), and its levels were quantified using the Griess reagent, which detects nitrite ions (NO^{2-}) in the culture medium, as illustrated in Fig. 6. The study examined the inhibitory effects of essential oil derived from lime peel waste on the production of pro-inflammatory mediators by measuring nitric oxide levels in lipopolysaccharide-stimulated RAW 264.7 cells. The nitric oxide concentration in the group treated with lipopolysaccharide was found to be 16.10±0.86 µM. In contrast, indomethacin, serving as a positive control, significantly ($p<0.05$) reduced nitric oxide production compared to the lipopolysaccharide-treated group (1.57±0.30 µM) and in relation to each concentration of lime peel oil tested. The lime peel oil demonstrated a concentration-dependent suppression of nitric oxide production, with the 25 µg/mL concentration being able to reduce the nitric oxide production higher than the other concentrations when compared to the lipopolysaccharide-treated group, as there was no statistically significant difference ($p>0.05$) among the three concentrations, although it was less effective than the indomethacin (the positive control). By observing changes in inflammatory markers after LPS exposure in cells pretreated with the oil, the results obtained from the research's experimental design indicated that the study focused on the protective effect of lime peel oil, rather than its anti-inflammatory properties in relation to inflammation.

Currently, there is no documented evidence regarding the impact of lime (*C. aurantifolia (Christ.)* Swingle) peel on nitric oxide production in RAW264.7 cells. According to the findings reported by Moraes et al. (2024), the commercial essential oil derived from the steam distillation of cold-pressed oil from *C. aurantifolia (Christ.)* Swingle fruits demonstrated anti-inflammatory properties, specifically a reduction in TNF-α and NF-κB levels in dystrophic muscle cells by 87% and 36%, respectively. This research's findings indicate that the essential oil derived from the peel of *C. aurantifolia*

exhibits anti-inflammatory effects as a protective or mitigating effect on inflammation, attributed to the presence of geranial compounds, D-limonene, and α-terpinene. This aligns with previous studies demonstrating that lime peel oil significantly decreases the levels of protein extravasation and nitric oxide (NO) production at a dosage of 100 mg/kg. The anti-inflammatory mechanism of this essential oil involves the reduction of cell migration, cytokine production, and protein extravasation induced by carrageenan (Amorim et al., 2016).

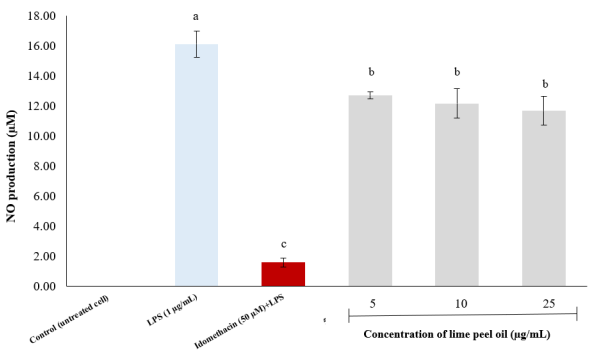

Fig. 6 Nitric oxide production in RAW264.7 macrophage cells after treated with lipopolysaccharide (LPS), indomethacin, and lime peel oil at concentration levels of 5.0 µg/mL, 10 µg/mL and 25 µg/mL for 24 h. Each bar represents mean ± standard deviation (SD) of triplicate (n = 3). The different subscript letters on the chart in each sample represent significant differences at $p<0.05$.

7. Stability of aromatherapy massage oil

Aromatherapy massage oil, formulated by blending essential oils with carrier oils, exhibited distinct physical properties. The initial formulation displayed a yellow hue, a characteristic aroma, visual clarity is achieved, and turbidity is absent, with homogeneous consistency attributed to the effective solubility of both the essential and carrier oils. This solubility is a result of the oils sharing similar polarities (Ma'arif et al., 2023). The total volume of the formulation is 55 mL including 0.5 mL of lime peel oil (0.9% in 100 mL), which has been dissolved in the carrier oil. For massage oils, it is advised to use a maximum concentration of 2% lime oil, which is obtained through hydro-distillation. In contrast, Persian cold-pressed lime essential oil is known to be phototoxic. For leave-on topical formulations, the International Fragrance Association (IFRA) and the Essential Oil Safety Edition 2nd by Robert Tisserand and Rodney Young suggest a dermal maximum concentration of 0.7% for Persian cold-pressed lime essential oil. If this guideline is exceeded in topical applications, it is advisable to avoid sun or UV exposure for a minimum of 12 h. (Tisserand & Young, 2013). Furthermore, the report by Opdyke (1974) suggested that the concentration of distilled lime oil in perfume products should be kept under 1.5% w/w, while in creams or lotions, it should not exceed 0.1% w/w. The stability of the massage oil was assessed under accelerated conditions and results indicated that the color remained unchanged across all tested conditions, while the aroma remained stable at room temperature, although slight alterations were noted after exposure to heating-cooling and sunlight (as detailed in Table 6). No phase separation was observed. The findings are illustrated in Fig. 7.

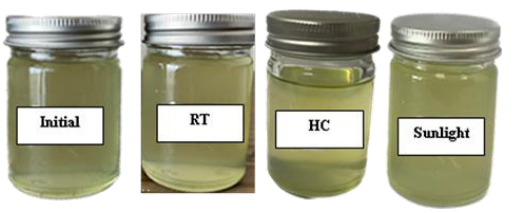

Fig. 7 The physical appearance of the aromatherapy massage oil after storage at room temperature (RT), heating-cooling cycle (HC) and sunlight compared with the initial condition.

Table 6 presents the findings regarding the physicochemical stability of prepared massage oil after being subjected to room temperature storage, heating-cooling cycles, and exposure to sunlight. The analysis focused on pH, viscosity, and L*, a*, b* values in comparison to the initial state. The initial pH of the massage oil was recorded at 5.13±0.07, suggesting that the product is suitable for application on skin. This finding aligns with previous studies suggesting that topical products should maintain a pH that is neither excessively acidic, which may irritate the skin, nor overly alkaline, which can lead to dryness and scaling (Ma'arif et al., 2023). The stability assessment revealed that the pH of the massage oil under the 6 heating-cooling cycles did not exhibit significant changes ($p>0.05$) from the initial measurement. Additionally, the viscosity and L* and a* values remained stable ($p>0.05$) compared to the original formulation throughout the 30 d of storage at room temperature. However, these parameters showed slight variations during the six heating-cooling cycles and the 30 d of sunlight exposure, with significant differences observed ($p<0.05$).

Table 6 Physicochemical stability of the aromatherapy massage oil

Conditions	Odor	Viscosity (cP)	pH	Color		
				L*	a*	b*
Initial	Specific aroma	22.30±0.17^{b}	5.13±0.07^{ab}	23.17±0.10^{a}	0.82±0.01^{a}	6.24±0.03^{b}
RT	Specific aroma	22.19±0.02^{b}	5.17±0.03^{a}	22.73±0.57^{a}	0.78±0.03^{a}	6.12±0.10^{c}
HC	Slight changed	23.20±0.30^{a}	5.11±0.01^{ab}	21.39±0.03^{b}	-1.31±0.04^{c}	7.33±0.03^{a}
Sunlight	Slight changed	19.93±0.21^{c}	5.08±0.02^{b}	23.21±0.02^{a}	-1.03±0.05^{b}	4.60±0.02^{d}

Remark: Values are given as mean ± S.D of triplicate. Different letters represent statistical significance ($p<0.05$). RT: room temperature; HC: heating-cooling cycles

Conclusion

In the current investigation, essential oil derived from lime peel waste through hydro distillation was found to predominantly contain D-Limonene, with α-pinene as the second most prevalent component, as determined by GC-MS analysis. The lime peel oil demonstrated no cytotoxic effects on normal human dermal fibroblasts (NHDF) (ranging from 5.0 to 250 µg/mL). Furthermore, the oil exhibited significant *in vitro* antioxidant activity, evidenced by both the DPPH scavenging ability and power reduction. The lime peel oil also displayed notable antimicrobial properties against *Candida albicans*. Additionally, its anti-inflammatory effects were highlighted by the inhibition of nitric oxide (NO) production, an inflammatory mediator, which is advantageous for cosmetic applications in skincare. An additional study is scheduled to investigate the biological activities (antioxidant, antimicrobial, and anti-inflammatory) of the developed massage oil. Additionally, the bioactivity of the product will be assessed following a storage duration of 30 d. Collectively, these findings suggest that lime peel oil, sourced from waste, has potential as a natural active agent in skin care and SPA products. Moreover, future studies will focus on examining skin irritation associated with SPA products, which will encompass a satisfaction test involving lime peel essential oil, in addition to conducting clinical trials on skincare items.

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