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Essential Oil from Lime Peel (*Citrus aurantifolia*) Grown in Long an Province, Vietnam: Chemical Composition and Biological Activities

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Abstract: The present study evaluated *Citrus aurantifolia* essential oil (CaEO) from lime peel grown in Long An province, Vietnam. Gas chromatography–mass spectrometry analysis identified six bioactive compounds, of which D-limonene (69.99%), β-Pinene (10.28%), and α-Pinene (13.08%) were the most abundant. Physicochemical properties, including relative density (0.9295), absolute density (0.9267 g/mL), acid value (0.5481 mg KOH/g), ester value (0.3123 mg KOH/g), and saponification value (0.8604 mg KOH/g), were determined. The antioxidant activity was evaluated using DPPH and ABTS assays, with IC_{50} values of 13.99 ± 1.84 and 1.52 ± 0.09 mg/mL, respectively, indicating significant free radical scavenging potential. The antibacterial effect against *Staphylococcus aureus* (S. aureus), *Bacillus subtilis* (B. subtilis), *Escherichia coli* (E. coli), and *Salmonella enteritidis* (S. enteritidis) was confirmed using the disk diffusion method. The inhibition zones were minimal (approximately 8 mm). In addition, CaEO exhibited a long-lasting aroma, making it suitable for industrial applications. These findings highlight the potential application of CaEO in food preservation, pharmaceuticals, and cosmetics, providing a sustainable approach to utilizing lime peel waste.

Keywords: Antioxidant activity, Antibacterial activity, *Citrus aurantifolia*, Essential oil.

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1. INTRODUCTION

Lime (*Citrus aurantifolia*) is one of the most widely grown citrus fruits in Vietnam, thriving in the tropical and subtropical climates of the region. The major lime-growing areas include Dong Thap, Tien Giang, and Ben Tre, which are known for their fertile soil and favorable climatic conditions (1).

Lime essential oil (CaEO), primarily extracted from the peel, is a valuable by-product rich in bioactive compounds such as D-limonene, β-pinene, and citral, which exhibit antibacterial, antioxidant, and antifungal properties (2). However, these properties depend on many factors, especially the extraction method. The extraction method significantly influences the yield and composition of essential oils. Jiang et al. (2011) reported that steam distillation, reflux extraction, and ultrasound-assisted extraction yielded at 0.16%, 2.18%, and 2.34%, respectively (3). Among these, D-limonene was the predominant component, accounting for 25.5% of the total composition, which plays a crucial role in the oil's bioactivity. Mohammed et al. (2014) also demonstrated that CaEO exhibits antibacterial activity against *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *E. coli* with inhibition zones ranging from 12 to 16 mm (4). Furthermore, CaEO exhibits strong antioxidant potential, with $IC_{50\text{-}DPPH}$=3.03 ± 0.019 mg/mL and $IC_{50\text{-}ABTS}$=4.27 ± 0.023 mg/mL, reinforcing its potential applications in various industries (5).

Due to these bioactive properties, CaEO is highly suitable for applications in food preservation, cosmetics, and pharmaceuticals (6). Despite being a popular refreshing beverage, particularly in Vietnam's hot climate, lime peels are frequently thrown away, raising environmental concerns. Utilizing these peels for essential oil extraction not only minimizes waste but also contributes to local development by creating value-added products from agricultural by-products (7).

Recent studies have further highlighted the antimicrobial and preservative properties of CaEO.

For example, Freche et al. (2022) demonstrated that CaEO effectively inhibits microbial growth, thereby extending the shelf life of food products (8). These findings underscore the need to characterize CaEO from different regions to optimize its applications. However, no studies have specifically investigated the physicochemical properties, chemical composition, and bioactive properties of CaEO derived from Long An. Given the unique soil and climate conditions of this region, an in-depth study on the biological properties of CaEO from Long An is essential for fully exploiting its potential in the food, pharmaceutical, and cosmetic industries.

To address this gap, the present study aims to analyze the chemical composition and biological properties of CaEO extracted from limes grown in Long An. This research not only provides new insights into the value of CaEO but also promotes the utilization of lime by-products, contributing to sustainable development in the citrus industry.

2. MATERIALS AND METHODS

2.1. Plant Extraction

CaEO was extracted from the peel of *Citrus aurantifolia*, a lime variety grown and harvested in Long An province, Vietnam (Coordinates: 10°25′21″N, 106°28′45″E). The essential oil extraction was according to the procedure described by Long et al. (2023) (9). After being processed into powder and juice, the peel essential oil (EO) was obtained by steam distillation at 100 °C for 3 h. The extraction efficiency of EO in this process was about 0.5% (w/w). The EO obtained after extraction was stored in dark glass bottles at room temperature to maintain its quality and long-term effectiveness.

2.2. Bacterials Strains

In this study, four bacterial strains were used: two Gram-positive bacteria, *S. aureus* (ATCC 33591) and *B. cereus* (ATCC 11778); and two Gram-negative bacteria, *E. coli* (ATCC 25922) and *S. enteritidis* (ATCC 13072). These bacterial strains were provided by the Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City.

2.3. Chemicals

The chemicals used in the study included 2,2-diphenyl-1-picrylhydrazyl (DPPH, ≥97%, Sigma, USA), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, ≥98%, Sigma, USA), and dimethyl sulfoxide (DMSO, ≥99.5%, China). Also, the media used for growing cultures and testing antibacterial properties, like Mueller–Hinton agar and nutrient broth from HiMedia in India, along with other chemicals, were of analytical grade.

2.4. Evaluation of The Physicochemical Properties of CaEO

According to ISO 279 (1998) (10), the relative density (RD) was determined by the proportion of the mass of a given volume of the EO to the mass of an equal volume of distilled water at 20 °C, while the absolute density (AD) was determined by the proportion of the mass of a given volume of the EO to the same volume. In addition, the freezing point (FP) was determined following ISO 1041 (1973) (11), 5 mL of the obtained CaEO was added to the test tube, which was then placed into a freezing container. The temperature of the freezing container was gradually lowered until the EO appeared to crystallize. The FP was recorded at that moment.

The acid value (AV) was determined according to the procedure of ISO 1242 (2023) (12). The obtained CaEO (1 g) was dissolved in 5 mL of 96% ethanol, and a few drops of 1% phenolphthalein were added to the mixture. The KOH solution (0.1 M) was used to titrate this mixture until it turned pink. The AV was calculated using the equation below:

$$\mathrm{AV} = \frac{\mathrm{V_{KOH}} \times 0.1 \times 56.1}{\text{Mass of essential oil}} \quad (12)$$

For the determination of the saponification value (SV), 2 g of EO and 25 mL of the ethanol solution of KOH (0.5 M) were mixed in a glass flask (250 mL). The mixture was then heated for 60 min in the condenser system. Subsequently, 25 mL of distilled water and a few drops of 1% phenolphthalein were added to the mixture. The HCl solution (0.5 M) was used to titrate this mixture until it turned colorless (13). The SV was calculated using the following formula:

$$\mathrm{SV} = \frac{(\mathrm{V_{Blank}} - \mathrm{V_{Sample)}} \times 0.5 \times 56.1}{\text{Mass of essential oil}} \quad (13)$$

The difference between SV and AV represents the ester value (EV):

$$\mathrm{EV} = \mathrm{SV} - \mathrm{AV} \quad (14)$$

2.5. Fragrance Retention (FR) of CaEO

The fragrance retention (FR) of the EO was determined based on the concentration and FR duration following the methods described by Mahajan (2022) with minor modifications (14). The EO was diluted in 96% ethanol to 20%, 40%, 60%, 80%, and 100% (v/v). The three drops of solution were applied to a scent test paper and left to distribute evenly. The time until the scent fully disappeared under normal conditions was recorded to evaluate fragrance retention.

2.6. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

The chemical composition of CaEO was analyzed using gas chromatography–mass spectrometry (GC-MS). An aliquot of 1 µL CaEO was injected into the instrument (Agilent Technology 5977E MSD) using an autosampler and an Agilent 7890A GC system. Analysis was performed using a Carbowax 20MTM column (30 m × 0.25 mm × 0.25 µm) and helium carrier gas at a constant flow rate of 10 mL/min, with a split ratio of 10:1. The injection rate was set to 250°C. The individual heating program was as follows: hold at 50 °C for 2 min, increase to 250 °C at a rate of 10 °C/min, hold at this temperature for

5 min, increase to 280 °C, and hold for 3 min. Mass spectra were recorded in electron ionization (EI) mode at an energy of 70 eV.

2.7. Determination of The 2,2-diphenyl-1-picrylhydrazyl (DPPH) Antioxidant Activity of CaEO

The antioxidant capacity of CaEO was evaluated by the free radical scavenging activity (RSA) using the DPPH method, following the procedure described by Quyen and Quoc (2024), with minor modifications (15). The EO was dissolved in ethanol (96%) to create different concentrations. Then, 0.3 mL of EO solution was mixed with 2.7 mL of 0.1 mM DPPH solution and left to rest at room temperature in the dark for 30 min. The color loss of DPPH was measured using a Thermo Scientific™ Genesys™ 20 Visible Spectrophotometer (USA) at 517 nm. Vitamin C was used as a control. The percentage of inhibition was calculated based on the CaEO concentration to estimate the 50% inhibitory concentration (IC_{50}). The antioxidant capacity (AC) was calculated using the following formula:

$$\%\mathrm{DPPH}_{\mathrm{RSC}} = \frac{\mathrm{A}_{\mathrm{control}} - \mathrm{A}_{\mathrm{sample}}}{\mathrm{A}_{\mathrm{control}}} \times 100 \quad (15)$$

where $\mathrm{A}_{\mathrm{control}}$ is the absorbance of the DPPH solution; and $\mathrm{A}_{\mathrm{sample}}$ is the absorbance of CaEO solution in the presence of DPPH solution.

2.8. Determination of Antioxidant Activity in CaEO Using ABTS

The experiments were performed based on the method described by Biskup et al. (2013) with minor modifications (16). The ABTS solution was standardized by dissolving 7 mM ABTS and 2.45 mM potassium persulphate in stored water. This service was mixed in a 1:1 ratio and reacted in the dark at room temperature for 16 h to form ABTS radicals (ABTS radical cation). After 16 h, the ABTS solution was diluted with stored water until an absorbance of 0.70 ± 0.02 was obtained at 734 nm. Then, 0.1 mL of EO solution was prepared at various concentrations and mixed with 3 mL of ABTS solution. The solution was adjusted to a final volume of 5 mL using ethanol and then kept in the dark at room temperature for 6 min. After this period, the absorbance was measured at 734 nm. The percentage of inhibition was determined based on the CaEO concentration, and the IC_{50} value (the concentration required to achieve 50% inhibition) was subsequently calculated. The antioxidant capacity (AC) was calculated using the following formula:

$$\%\mathrm{ABTS}_{\mathrm{RSC}} = \frac{\mathrm{A}_{\mathrm{control}} - \mathrm{A}_{\mathrm{sample}}}{\mathrm{A}_{\mathrm{control}}} \times 100 \quad (16)$$

where $\mathrm{A}_{\mathrm{control}}$ is the absorbance of the ABTS solution; and $\mathrm{A}_{\mathrm{sample}}$ is the absorbance of CaEO solution in the presence of ABTS solution.

2.9. Determination of The Antibacterial Activity (AA) of CaEO

The antibacterial activity (AA) was determined using the paper disk diffusion method based on the method described by Carović-Stanko et al. (2010) with some modifications (17). First, 100 µL of bacterial suspension (0.5 McFarland standard concentration, equivalent to approximately 1.5×10^8 CFU/mL) was evenly spread onto Mueller–Hinton agar (MHA) medium using an inoculating loop. Sterile paper disks (6 mm in diameter) were inoculated with 5 µL of the EO and then they were placed on MHA medium surface, while gentamicin (10 µg/disc) and 5% (v/v) dimethyl sulfoxide (DMSO) solution were used as positive and negative controls, respectively. The plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the paper disk.

2.10. Data Analysis

Analysis of variance (ANOVA) and comparison of means were performed using Statgraphics Centurion 20 (StatPoint Technologies, Inc.) software, with a 95% confidence level ($p \leq 0.05$) determined using the least significant difference (HSD) method. The results are shown as the mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Determination of Physicochemical Properties of CaEO

The physicochemical properties of CaEO are summarized in Table 1. The CaEO was a pale yellow, transparent liquid with a characteristic aroma and slight flame. The physicochemical parameters of CaEO were measured as follows: pH value, 3.47; RD, 0.9295 ± 0.0021; AD, 0.9267 ± 0.0021 g/mL; AV, 0.5481 ± 0.0211 mg KOH/g; SV, 0.8604 ± 0.0095 mg KOH/g; and EV, 0.3123 ± 0.0084 mg KOH/g. These characteristics indicate that CaEO belongs to the group of light EOs, is rich in terpenes, and has a very low free acid content. Ester compounds also account for a negligible proportion (18).

Compared to the Algerian CaEO, the pH value was 6.0, which was much higher than the pH of 3.47 for CaEO in this study. In terms of RD, the Algerian EO had a mass of only 0.894 g/mL, which was lower than the 0.9267 g/mL observed for CaEO. The AV value of the Algerian EO reached 2.10 mg KOH/g, which was higher than the 0.5481 mg KOH/g observed for CaEO (19). Also, a different study on CaEO (*Citrus aurantifolia*) from Nigeria found an SV value of 130.37 mg KOH/g, which is much higher than the 0.8604 mg KOH/g found for CaEO in this study. These differences may be caused by differences in chemical composition, plant origin, and environmental conditions.

CaEO stands out for its outstanding ability to retain fragrance. The FR duration ranged from 5.75 (with 20% EO) to 57.21 hours (pure EO). This characteristic makes CaEO a priority choice in the fragrance and cosmetic industries, where the ability to retain a long-lasting scent plays an important role.

Moreover, compared to other EOs in the same group, CaEO has shown advantages in terms of stability, pleasant scent, and versatility, making it suitable for many applications.

Table 1: Physicochemical properties of *Citrus aurantifolia*.

No.	Physicochemical properties	Value
1	pH	3.47 ± 0.12
2	Freezing point (FP, °C)	< −18°C
3	Relative density (RD)	0.9295 ± 0.0021
4	Absolute density (AD, g/mL)	0.9267 ± 0.0021
5	Acid value (AV, mg KOH/g EO)	0.5481 ± 0.0211
6	Saponification value (SV, mg KOH/g EO)	0.8604 ± 0.0095
7	Ester value (EV, mg KOH/g EO)	0.3123 ± 0.0084
8	Fragrance retention (FR, h):	
	20% EO	5.75 ± 0.47
	40% EO	18.66 ± 0.98
	60% EO	29.76 ± 1.45
	80% EO	41.12 ± 2.61
	100% EO	57.21 ± 3.27

3.2. Chemical Composition of CaEO

GC-MS detected 6 main components in CaEO (Table 2). The analysis indicated that CaEO was mainly comprised of monoterpene hydrocarbons. The main component was D-Limonene (69.99%), a monoterpene ring with a characteristic citrus scent. Similar to previous studies, such as Miller et al. (2011), this ratio ranged between 60 and 75% (21). They play an important role in antibacterial, anti-inflammatory, and antioxidant properties and are widely used in the food and pharmaceutical industries. The second most abundant component was α-Pinene (13.08%), followed by β-Pinene (10.28%), which are monoterpenes with high antibacterial and anti-inflammatory properties commonly found in EOs derived from the Rutaceae family (22). The α-Pinene and β-Pinene content in this lime peel EO sample was superior to that of some CaEOs from the Mediterranean region, which typically range from 5 to 10% (23). The Sabinene (3.63%) and α-Myrcene (2.00%) contents in the sample were also consistent with other studies, contributing to the complex aroma and anti-relaxant properties (24). Although present in low proportions, γ-Terpinene (1.02%) plays a significant role in the antioxidant activity of EO (25).

Table 2: Chemical composition of *Citrus aurantifolia* essential oil.

No.	Compounds	Molecular formula	RT (min)	Content (%)
1	α-Pinene	$C_{10}H_{16}$	3.27	13.08
2	β-Pinene	$C_{10}H_{16}$	3.92	10.28
3	Sabinene	$C_{10}H_{16}$	4.00	3.63
4	α-Myrcene	$C_{10}H_{16}$	4.25	2.00
5	D-Limonene	$C_{10}H_{16}$	4.60	69.99
6	γ-Terpinene	$C_{10}H_{16}$	4.94	1.02

3.3. Determination of The Antioxidant Activity

Table 3 shows that the antioxidant activity of CaEO was significantly lower than that of vitamin C when determined using both the DPPH and ABTS methods. Specifically, with the DPPH method, the IC_{50} of CaEO was 13.99 mg/mL, while that of vitamin C was only 6.04 µg/mL, indicating a significant difference. Similarly, using the ABTS method, the IC_{50} of CaEO was 1.52 mg/mL, whereas vitamin C exhibited a much lower value of 3.12 µg/mL, confirming its superior antioxidant activity compared to CaEO.

When compared to other studies, the $IC_{50\text{-DPPH}}$ value of CaEO in this study (13.99 mg/mL) was higher than that of Algerian CaEO (7.41 mg/mL) (26), indicating lower antioxidant activity. However, the $IC_{50\text{-ABTS}}$ of our CaEO (1.52 mg/mL) was significantly lower than that of Algerian CaEO (53.6 mg/mL) (26), suggesting a higher ABTS inhibitory ability. Additionally, CaEO from Tunisia also exhibited a lower $IC_{50\text{-DPPH}}$ value of 4.1 mg/mL (27), suggesting it had a stronger DPPH scavenging ability than that of CaEO in this study. Differences in antioxidant activity could be attributed to variations in chemical composition influenced by geographic location, climate, and extraction methods (28). Differences in the content of bioactive compounds, particularly D-limonene, γ-terpinene, α-pinene, etc., strongly affect antioxidant activity. The higher IC_{50} value in our study suggests that the CaEO from Long An (Vietnam) may have a different chemical composition compared to those from Tunisia and Algeria.

Overall, although CaEO exhibits weaker antioxidant activity than vitamin C, it still holds potential as a natural antioxidant source. Further studies should investigate the impact of extraction methods and environmental factors on its bioactive composition to optimize its application in the food and pharmaceutical industry.

Table 3: Antioxidant activity using DPPH and ABTS methods.

Test sample	$IC_{50\text{-DPPH}}$	$IC_{50\text{-ABTS}}$
Vitamin C (µg/mL)	$6.04^{a} \pm 0.35$	$3.12^{b} \pm 0.26$
CaEO (mg/mL)	$13.99^{b} \pm 1.84$	$1.52^{a} \pm 0.09$

Different letters (a, b) in the same column indicate significant differences ($p \leq 0.05$) between samples.

3.4. Determination of The Antibacterial Activity (AA) of CaEO

Table 4 shows that CaEO exhibited significantly lower antibacterial efficacy than gentamicin against both Gram-negative and Gram-positive bacteria. For Gram-negative bacteria, the inhibition zone diameters of CaEO against *E. coli* (8.52 ± 0.62 mm) and *S. enteritidis* (8.45 ± 0.29 mm) were much smaller than those of gentamicin (16.11 ± 0.56 mm and 10.67 ± 0.27 mm, respectively). Similarly, for Gram-positive bacteria, CaEO demonstrated weaker antibacterial activity, with inhibition zones of 8.11 ± 0.73 mm for *S. aureus* (compared to 14.48 ± 0.83 mm for gentamicin) and 7.99 ± 0.55 mm for *B. cereus* (compared to 20.04 ± 0.98 mm for gentamicin). Overall, the antibacterial sensitivity of CaEO in this study was classified as "not sensitive" due to its inhibition zones being approximately 8 mm (29).

Compared to some raw materials from other regions, there are notable differences in antibacterial properties, with Egyptian CaEO demonstrating significantly higher antibacterial activity, showing inhibition zone diameters of 32 mm for *S. aureus* and 49 mm for *P. aeruginosa* (30). Similarly, Ben Hsouna et al. (2017) reported higher inhibition zones for *S. enteritidis* (18 mm), *B. cereus* (24 mm), *E. coli* (15 mm), and *S. aureus* (22 mm) using essential oils from Tunisia, suggesting variations in antibacterial efficacy based on plant origin, extraction method, and chemical composition (27). The lower antibacterial activity of CaEO in this study may be attributed to its chemical composition. Limonene, the dominant compound in CaEO, has been reported to exhibit antibacterial properties, but its efficacy depends on concentration and interactions with other bioactive compounds (31).

The antibacterial activity of EOs is primarily linked to their hydrophobicity, allowing them to penetrate bacterial cell membranes, disrupt lipid bilayers, and increase membrane permeability. This process leads to ion leakage, loss of intracellular components, and ultimately, bacterial cell death (32). These mechanisms explain why EOs are widely applied in food preservation despite variations in their antibacterial potency.

Table 4: Antibacterial zones of CaEO.

Test strains	Diameter of the inhibitory zones of gentamicin (mm)	Diameter of the inhibitory zones of CaEO (mm)
E. coli	$16.11^{Ca} \pm 0.56$	$8.52^{Db} \pm 0.62$
S. enteritidis	$10.67^{Ab} \pm 0.27$	$8.45^{Ca} \pm 0.29$
S. aureus	$14.48^{Bb} \pm 0.83$	$8.11^{Ba} \pm 0.73$
B. cereus	$20.04^{Db} \pm 0.98$	$7.99^{Aa} \pm 0.55$

Within a row (a–b) or a column (A–D), different letters denote significant differences ($p < 0.05$) between samples or microorganisms, respectively.

4. CONCLUSION

This study extracted and characterized *Citrus aurantifolia* essential oil (CaEO) from lime peels in Long An province, Vietnam, highlighting its chemical composition and bioactive potential. GC-MS analysis identified six key compounds, with D-limonene (69.99%), β-Pinene (10.28%), and α-Pinene (13.08%) as the predominant constituents. The physicochemical properties of CaEO, including relative and absolute density, acid value, ester value, and saponification value, were determined to assess its stability and quality. Antioxidant activity was confirmed through DPPH and ABTS assays, with IC_{50} values of 13.99 ± 1.84 and 1.52 ± 0.09 mg/mL, respectively, indicating strong free radical scavenging capacity. However, the antibacterial activities of CaEO were relatively low, with inhibition zones ranging from 7.99 to 8.52 mm against *S. aureus*, *B. subtilis*, *E. coli*, and *S. enteritidis*. Additionally, the long-lasting aroma of CaEO enhances its applicability in various industries. These findings suggest that CaEO could be a valuable natural additive in food preservation, pharmaceuticals, and cosmetics, offering an eco-friendly approach to utilizing lime peel waste while increasing the economic value of local lime products.

5. CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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