



Antioxidant and Antimicrobial Activity of Citrus Limetta

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Abstract: One of the most common fruit crops grown in tropical and subtropical areas is *Citrus limetta*. Upon processing, about 40-47% percent of the fruit's non-edible mass is discarded as waste that impacts the environment. The aromatic compounds called essential oils (EOs) are concentrated in oil glands found in leaves, flowers, and fruit peels. The study's primary objective was to evaluate the biological activity of fresh and heated peels extracted essential oil from *Citrus limetta* (CLEO). The study emphasized the determination of bioactive compounds from the CLEO antioxidant and antimicrobial activity assessment. CLEO's main volatile component was D-Limonene in both oils, with RA (%) values of 44.14 and 77.50 in fresh and heated peel oil, respectively. The antioxidant activity was comparable to the standard used for ascorbic. The activity (%) reached its highest ($83 \pm 1.53\%$) at the concentration of $100 \mu\text{L/mL}$ for fresh peel oil, which is higher than the heated peel oil. Comparative antimicrobial activity was determined to assess the effects of CLEO against ten bacterial pathogens, which implies that seven pathogens were significantly sensitive against heated oil. The current findings suggest that CLEO could be an impressive natural source with significant potential to show good biological activity in both fresh and treated forms. A complex composition with approximately 20 components in *Citrus limetta* essential oil has been scientifically proven beneficial in aromatherapy, medications, beverages, food color enhancers, and aromatic-nature infusions for personal health care.

Keywords: Antioxidant, Antimicrobial, Bioactive compound, Citrus, Essential oil, Heat treatment.

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I. INTRODUCTION

Citrus is one of the most widely grown fruit families globally. Citrus fruits have a significant antioxidant capacity and a protective effect on human health because they include an excellent store of nutrients, phytochemicals, and bioactive polyphenols, according to ¹. It is a great source of energy and vitamin C, and it may be eaten raw or pressed to make juice. Citrus juices are the most popular and widely consumed fruit juices globally ². Citrus fruit peel, which accounts for 50–55 percent of the fruit's weight, is thought to have some practical uses. It is a valuable source of biologically active substances like phenols, flavonoids, essential oils, carotenoids, organic acids, ascorbic acid, and vitamins ^{3,4}. The primary sources of antimicrobials that shield cells from numerous disease-causing pathogens are the phytoconstituents found in citrus peel essential oils. Microorganisms are the main source of disease and sickness in humans ⁵. One of the easiest-to-manage sources of such compounds is medical plants and their derivatives, such as essential oils (EO), which include a variety of phytochemicals with antibacterial action ⁶. The antibacterial activity of the EOs is affected by peeling before oil extraction since the activity depends on the types of chemical residues in the oil. Commercially, citrus peels are dried using a variety of drying techniques ⁷. Traditional solar drying is a useful technique for drying crops using solar energy ⁸. Another common technique is hot air drying, or "tray drying," which uses a continuous stream of hot air to dry food. It is frequently employed in the food industry. Still, it has several drawbacks, including a prolonged drying period, a slower drying rate, poorer thermal efficiency, loss of heat-sensitive compounds, and lower product quality ⁹. Foodborne illnesses caused by pathogenic microorganisms pose a significant threat to public health, often resulting in severe gastrointestinal infections and, in some cases, even fatalities ¹⁰. Conventional methods of controlling these pathogens, such as heat treatment and chemical preservatives, have limitations, including potential sensory alterations in food and the emergence of resistant strains. Therefore, there is a growing interest in exploring natural antimicrobial agents as alternative approaches to combat foodborne pathogens ¹¹. *Citrus limetta* essential oil (CLEO), derived from the peel of *Citrus limetta*, has garnered attention due to its potential antimicrobial properties. Previous research has demonstrated its effectiveness against various foodborne pathogens, including *Escherichia coli*, *Salmonella spp.*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Mycobacterium smegmatis* ¹². The antimicrobial activity of CLEO has been attributed to its ability to disrupt microbial cell membranes, interfere with essential metabolic processes, and inhibit microbial growth and

proliferation ¹³. In addition to its antimicrobial properties, using essential oils derived from plants like *Citrus limetta* offers potential advantages over synthetic antimicrobial agents. These essential oils are generally considered safe for consumption, environmentally friendly, and may provide additional health benefits due to their phytochemical composition ¹⁴. This study compares the volatile components, antioxidant activity, and antibacterial properties of essential oils derived from fresh and heat-treated *Citrus limetta* peels.

2. MATERIALS AND METHODS

2.1 Materials

The *Citrus limetta* was obtained from local farmers at a local market in Kamrup District, Assam, India, and a botanist from the USTM Botany Department authenticated and verified them. Materials for chemical analysis (analytical grade) were purchased from Himedia A-516, Swastik Disha Business Park, via Vadhani Industrial Estate, L.B.S. Marg, Mumbai, 400 086, India., These include distilled water-methanol, DPPH, ascorbic acid, Mueller Hinton agar, Luria Bertani media, 1N NaOH, and 1N HCL. Bacterial pathogens, i.e., *Salmonella spp.*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Yersinia pestis*, *Yersinia enterocolitidis*, and *Listeria monocytogenes*, were obtained from the Microbiology section of the Department of Applied Biology, USTM, 793101.

2.2 Sample preparation

Fruit peels were peeled, cleaned in tap and distilled water to remove impurities, then chopped into small pieces. Essential oil (EO) extraction occurred under two sets of experimental circumstances.

2.3 Heat Treatment

The peels of *Citrus limetta* were directly cut and dried in an automatic tray dryer at 45°C for four hours. After heating, *Citrus limetta* peels were allowed to cool to ambient temperature before extracting essential oil.

2.4 Oil extraction

Essential oil was extracted using a Clevenger device following method 15; both sets were independently extracted after being roughly pulverized. Figure 1 describes the oil extraction process.¹⁵

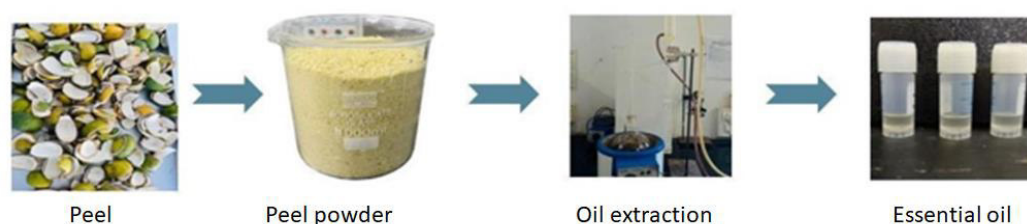


Fig 1: Schematic diagram of the extraction process of essential oil

2.5 Determination of percentage yield

The volume of the oil was recorded and expressed as oil content (%) as follows¹⁶.

$$\text{Oil content (\%)} = (\text{Volume of the oil} / \text{Weight of sample}) \times 100\%$$

2.6 GC/MS analysis of the oil

A Thermo Fisher Scientific model GC with a split/spitless injection port and a split ratio of 1/100, coupled with an ISQ7000 mass spectrometer system (electron impact mode), was also used with a 30 m × 0.25 mm, i.e., 0.25 μm film thickness, TG-5MS fused silica capillary column. The temperature program was isothermal for 3 min at 60 °C and then raised to 230 °C at 5 °C/min. The injector temperature was 290 °C, and the transfer line temperature was 230 °C. The column outlet was inserted directly into the ion source block from the GC outlet. Ultrapure helium was used as a carrier gas with a 1 mL/min flow rate. The injection volume was 0.2 μL. Peak area percentages were calculated automatically using Chromeleon™ Software provided along with instrument setup. Chromatographic analysis was performed in dilute

solutions of certain approximate strengths, qualitatively as per the method of Hou et al., 2019. Identification of components was confirmed by comparison of the experimental retention index and mass spectrum with those of authentic reference standards provided in the NIST Library as a component of Chromeleon™ Software.

2.7 Antioxidant Assay

2.7.1 DPPH scavenging activity

EOs of varying concentrations of 25, 50, 75, 100, 125, and 150 μL were made in methanol, followed by the addition of 2 mL of DPPH. An optical density measurement was made at 517 nm following a 30-minute incubation period at room temperature¹⁷. Utilizing the following formula, the scavenging activity was calculated:

$$\text{Scavenging activity (\%)} = (A-B)/A \times 100$$

A denotes DPPH absorbance, and B denotes the combined absorbance of fruit juice and DPPH.

2.8 Antimicrobial screening

Ten pathogenic bacterial strains, namely *Salmonella spp.*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Yersinia pestis*, *Yersinia enterocolitidis*, and *Listeria monocytogenes* were employed in this study. The test cultures were procured, inoculated in Luria-Bertani broth, and incubated at 37°C for 24 hours. The antimicrobial properties of all the isolates were determined by the Kirby Bauer disc diffusion method with little modification¹⁸ to confirm antimicrobial activity.

2.9 MIC determination

The Luria-Bertani broth was prepared for MIC determination. From this broth, 4.5 mL was added in each of the four test tubes labeled as A, B, C, and D, indicating the EO concentration used in each tube as 100%, 50%, 25%, and 12.5%, respectively. Sets of four test tubes containing 4.5 ml nutrient broth were prepared for all test microorganisms. Then, 0.5 ml of each sample concentration was added to the test tube. After this step, 0.05 ml test pathogen suspensions were inoculated in labeled test tubes. After inoculation, the test tubes were kept in a shaker incubator overnight at 37°C and observed for turbidity development, and O.D. values were recorded at 600 nm. MIC for a given sample is the concentration that showed the minimum absorbance¹⁹. It was determined by taking the absorbance and comparing the values at different concentrations.

3. STATISTICAL ANALYSIS

All experiments were performed in triplicate. The data represents the mean of triplicate values. The corresponding standard deviation was calculated. Origin pro8.5 was applied for the data analysis.

4. RESULTS AND DISCUSSION

4.1 Oil yield

Peels of *C. limetta* were used to extract the essential oil using

the cleverger apparatus. The current finding shows that the oil output (%) for fresh and heated peel-based oils was 0.331 and 0.461, respectively. The results are comparable to those of²⁰, who obtained a value of 0.313%, consistent with the general trend of²¹ findings. The oil quality would decline in yield, content, and antioxidant and antibacterial activity due to drying procedures that could harm heat-sensitive plant metabolites²². Therefore, fresh oil has a higher relative oil yield than oil that has been heat-treated.

4.2 Bioactive compounds

Figures 2a and 2b represent the chromatograms of heated peel and fresh peel essential oil, which constitute several peaks, where, as in both, the (CLEO) highest peak compound was found to be D-limonene. *Citrus limetta* is one of the key components of various citrus oils, such as D-limonene. D-Limonene compounds are highly volatile, easily oxidized, and have low solubility in water²³. D-limonene is a popular food component found in fruit juices and non-alcoholic beverages. It is generally considered safe (GRAS) in the Code of Federal Regulations. The chemo-preventive efficacy of D-limonene against multiple kinds of cancer is well-known²⁴. As shown in Table 1 and Table 2, D-Limonene was found at the higher level in fresh and treated peel oils, respectively. D-limonene comprises 77.5 (RA%) and 41.44 (RA%) for the heat-treated and fresh peel oil, making it an essential component of citrus peel oils. Cyclohexane, whose RA% was 10.07, was the second significant chemical identified. β-myrcene, β-pinene, Bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene, 3-Cyclohexene-1-methanol, a,a,4-trimethyl-, (R), (1S)-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene, 3-Furanacetic acid, dodecanal, cis-(-)-1,2-Epoxy-p-menth-8-ene, isodene, and p-Menth-8-en-1-ol were also found to be present. Fresh peels CLEO contain various chemical compounds, among which β-myrcene and β-pinene stand out as the second most significant compounds, constituting approximately 8.82% of the overall composition. The peels also contain Cyclohexane, Linalool, α-pinene, Decanal, 6-Octenal, 3,7-dimethyl-, Citronellol, Citral, Neral, and cis-Thujopsene. The components found were consistent

with the early reports in which limonene (RA%: 87.84) in moasmbi was identified as the main ingredient. However, terpinol, followed by other ingredients, was the second most important component. Other components were were

limonene, neral, geranial, pinene, caryophyllene, and neryl acetate. Limonene, linalyl acetate, terpinene, and linalool were the major components of citrus essential oil, according to the study^{25,26}.

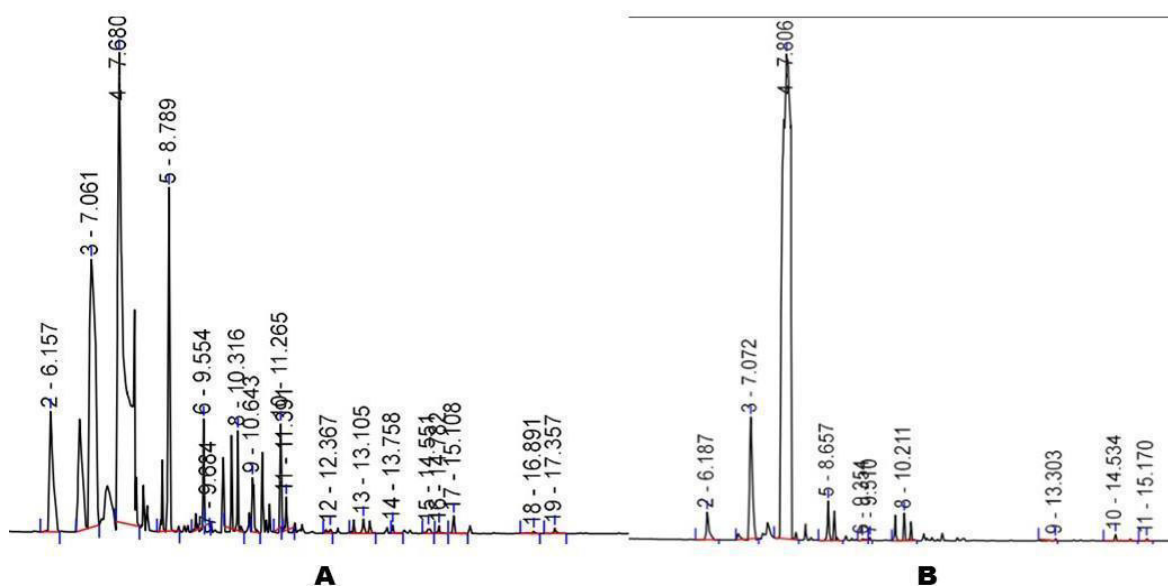
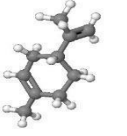
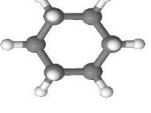
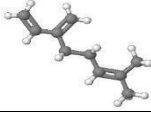
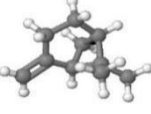

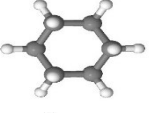



Fig 2: GC/MS chromatogram of *Citrus limetta*. (A: fresh peel's oil; B: heated peel's oil).

Except for the minor chemicals, such as linalool, terpinene-4-ol, and -terpineol, whose levels rose following steam distillation, the major components of the oils were comparable. Terpene alcohol concentrations were similarly greater in the whole fruit²⁷. Various treatment procedures have an impact on metabolites.²⁸ For example, at 85 and 95 °C, the level of terpineol is much higher than the sensory threshold level.

Table 1: Chemical composition of <i>Citrus limetta</i> oil from heated peels				
Sl. No.	RA%	RT	Compound	3D Structure
1	77.50	7.80	D-Limonene	
2	10.07	3.00	Cyclohexane	
3	6.75	7.07	β -Myrcene, β -Pinene, Bicyclo 3,1,1 heptane	
			β -Pinene	
4	1.85	8.657	Bicyclo 3,1,1 heptane	
			Cyclohexane	
5	1.70	10.211	3 Cyclohexane 1- methanol	

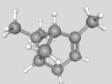
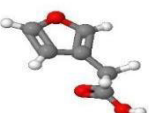

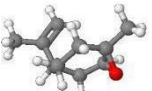
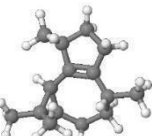

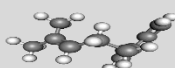
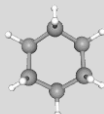
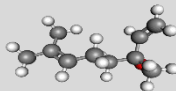
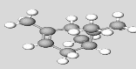
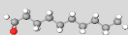
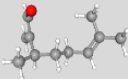
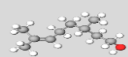
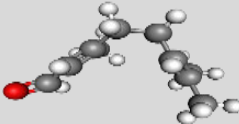

6	1.39	6.18	2,6,6- Trimethylbicyclohep-2-ene	
7.	0.30	14.53	3-furan acetic acid	
8.	0.19	13.30	Do-decanal	
9.	0.12	9.35	Cis-1,2 epoxy-menth-8-ene	
10.	0.08	15.17	Isoledene	
11.	0.06	9.51	p-menth-8-en-1-ol	

Table 2: Chemical composition of *Citrus limetta* oil from fresh peels

SL. No.	RA	RT	Compound Name	Compound 3D structure
1	41.44	7.680	D-Limonene	
2	27.20	7.061	β -Myrcene	
3	8.82	3.089	Cyclohexane	
4	8.13	8.789	Linalool	
5	5.87	6.157	α pinene	
6	2.13	10.316	Decanal	
7	1.43	9.554	6-Octenal, 3,7-dimethyl-, (R)-	
8	1.31	10.643	Citronellol	

			Citral	
9	1.18	11.265		
			Neral	
10	0.16	14.782	cis-Thujopsene	

4.3 Antioxidant activity

Plants that can scavenge free radicals and act as antioxidants can be used medicinally and as food additives. The current study uses the DPPH radical scavenging method to assess the antioxidant potential of oil from fresh and heated peels. The DPPH test determines how well an essential oil can neutralize the stable free radical DPPH by giving up an electron or a hydrogen atom²⁹. Ascorbic acid was utilized at the concentration of 50µg/ml as a positive control, which showed an antioxidant capacity of 97.85±0.97%, higher than the antioxidant properties of both types of oils. As depicted in Table 3, fresh CLEO demonstrated greater antioxidant activity, with a percentage of activity of 83±1.53% at a concentration of 100µL/mL. In contrast, heat-treated CLEO exhibited comparatively lower antioxidant activity, 70.6±0.67%, at a 150 µL/mL concentration. The antioxidative activity seen could be attributed to monoterpane

hydrocarbons, particularly terpinolene - and -terpinene, but undoubtedly, none have a stronger antioxidative effect than oxygenated monoterpenes. This behavior is likely caused by significantly activated methylene groups in these molecules³⁰. The results of the current study have demonstrated that citrus oil has natural antioxidant properties that are comparable to those of ascorbic acid. This very effective antioxidant agent is frequently employed in food technology. Research into naturally occurring antioxidants for use in foods or medications has seen a noticeable increase in attention as a replacement for synthetic antioxidants, whose usage is restricted due to their potential toxicity³¹. The stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was subjected to the scavenger effect at 25, 50, 75, 100, 125, and 150 µL/mL concentrations. Results are given as a percentage decrease in absorbance at 517 nm compared to the control. Positive controls included ascorbic acid. Each value corresponds to the mean and standard error of three experiments.

Table 3: Table represents the antioxidant activity (%) of both heats treated and fresh CLEOs in different concentration

Concentration (µL/mL)	Fresh CLEOs	Heated CLEOs
25	30.2±1.33	22.3±1.43
50	42.7±0.97	26.2±0.57
75	55.8±1.56	35.7±0.98
100	83.5±1.53	60.7±1.13
125	79.3±1.89	60.5±0.98
150	78.2±2.33	70.6±0.67
175	78.0±1.56	69.8±2.98
200	72.9±1.32	69.1±2.35

4.4 Antimicrobial assay

Salmonella spp, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Yersinia pestis*, *Yersinia enterocolitis*, and *Listeria monocytogenes* were the ten bacteria strains that were tested for EOs antibacterial activity. These bacterial strains used for testing are pathogens commonly associated with human foodborne illnesses³²⁻³⁴. The antibacterial ability of several citrus essential oils has been shown in earlier studies which reported that CEO is effective against *Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus*, and other bacteria. The presence of bioactive compounds supports the antimicrobial activity. Citrus peel oils' antibacterial capabilities is due to monoterpene components, of the oils' active ingredients³⁵. This study extracted ten bacterial pathogens and their response to CLEO from heat-treated and fresh peels. The findings revealed that seven pathogens showed significantly higher sensitivity to CLEO from heat-treated

peels, while three pathogens were more sensitive to CLEO from fresh peels. This difference could be attributed to the higher percentage of terpenoids (84.44%) in the CLEO extracted from heated peels, compared to the lower percentage (79.42%) in CLEO from fresh peels. Consequently, these results strongly suggest that the antibacterial activity of citrus is primarily influenced by the bioactive compounds present, especially at higher concentrations in heat-treated peels. Table 3 presents the Minimum Inhibitory Concentration (MIC) values of heat-treated and fresh EOs against ten pathogenic microbes. For *S. aureus*, *Salmonella*, and *B. subtilis*, the MIC value was the lowest at 0.025 (µL/mL) for both heat-treated and fresh EO. As for *E. coli*, *S. enterica*, *B. cereus*, *Y. pestis*, *Y. enterocolitis*, *L. monocytogenes*, and *K. pneumoniae*, the MIC value was determined to be 0.05 (µL/mL) for both types of EO. It indicates the fresh EO's optical density (O). This is higher than heat-treated EO. This observation suggests that heat-treated EO exhibits a higher efficacy in inhibiting the growth of these microbes.

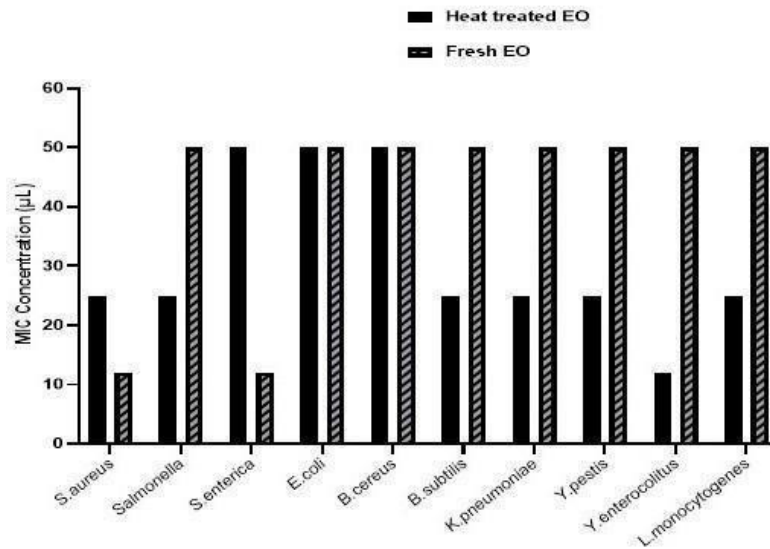


Fig 3: MIC values for heat and fresh CLEOs against bacterial pathogens.

Table 3. MIC values with their respective OD for Heat and Fresh CLEOs against bacterial pathogens.

Test Organism	MIC (% v/v) (µL/mL)		Absorbance at 600 nm	
	Heat treated EO	Fresh EO	Heat treated EO	Fresh EO
<i>E. coli</i>	0.05	0.05	0.796±0.003	1.374±0.006
<i>S.aureus</i>	0.025	0.025	0.296±0.001	1.942±0.004
<i>Salmonella</i>	0.025	0.025	0.609±0.004	1.984±0.002
<i>S.enterica</i>	0.05	0.05	0.433±0.002	1.500±0.004
<i>B.cereus</i>	0.05	0.05	0.700±0.003	2.061±0.003
<i>B.subtilis</i>	0.025	0.025	0.769±0.002	1.250±0.070
<i>Y.pestis</i>	0.05	0.05	0.678±0.003	1.438±0.009
<i>Y.enterocolitus</i>	0.05	0.05	0.444±0.022	1.773±0.004
<i>L.monocytogenes</i>	0.05	0.05	0.517±0.002	1.612±0.005
<i>K.pneumoniae</i>	0.05	0.05	0.182±0.001	0.917±0.003

Prior studies have provided evidence of the antimicrobial properties exhibited by numerous citrus essential oils^{33,36}. In another study, the MIC value varied from 0.039 to 2.5 mg/mL³⁰. The antimicrobial effects of EOs are attributed to the bioactive compound D-Limonene, which contributes to its bactericidal properties³⁷.

5. CONCLUSION

In conclusion, the current study focused on extracting essential oil from *Citrus limetta* peels using the clevenger apparatus. The oil yields were found to be higher in fresh peel-based oil compared to heat-treated peel-based oil. The major component identified in both types of oil was D-limonene, followed by other compounds such as cyclohexane, β-myrcene, and β-pinene. It also explored the antioxidant potential of the oils using the DPPH radical scavenging method. Fresh CLEO demonstrated greater antioxidant activity than heat-treated CLEO, indicating the presence of effective natural antioxidants in the oil, particularly monoterpenes. Furthermore, the antibacterial activity of the oils was evaluated against ten bacterial strains commonly associated with foodborne illnesses. It was found that the antibacterial efficacy varied between the fresh and heat-treated oils, with

some pathogens showing higher sensitivity to one type of oil over the other. This difference in antibacterial activity was attributed to the varying concentrations of bioactive compounds, particularly terpenoids, in the oils.

6. AUTHORS CONTRIBUTION STATEMENT

Dr. Sony Kumari and Mr. Pranab Jyoti Koch conceptualized and designed the study, curated the data, and prepared the original draft. Mr. Muzaharul Islam and Anisur Rahman performed the experimentation. Mr. Rahel Debbarma, Nahid Nasrin, and Mamoni Sikdar provided valuable inputs toward the design of the manuscript. All the authors read and approved the final version of the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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