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Antimicrobial Activity and Phytochemical Analysis of Solvent Extraction of *Citrus limon* Peels

Lingaraju Honnur Gurusiddappa[1]*, Christeena Varghese[1], Bharat Gowda[1], Shankramma Kalikeri[2]

[1]Department of Environmental Science, JSS Academy of Higher Education and Research, Mysuru, Karnataka – 570015, India.
[2]Division of Nanoscience and Technology, School of Life Sciences, JSS Academy of Higher Education and Research, Mysuru – 570 015, Karnataka, India.

ABSTRACT

Lemons make up the bulk of citrus fruits, and they're frequently employed in the juice business. These enterprises generate a lot of garbage because these peels contain a wide range of bioactive components, they may be used to create a variety of value-added products that help to reduce pollution. This study aims to determine the antibacterial activity and phytochemical analyses of Citrus limon peels extracted with a solvent. The antibacterial activity of C. limon peels was tested against Gram-positive bacteria like Staphylococcus aureus and Enterococcus faecalis, as well as Gram-negative like Salmonella typhi, utilizing different solvents including acetone, ethanol, ethyl acetate, and petroleum ether. To determine whether specific bio-reactive chemicals are present or absent in solvent extraction, a phytochemical analysis was performed. Antimicrobial tests such as agar well diffusion assay, the minimum inhibitory concentration (MIC), and the minimum bacterial concentration were performed, and the results revealed that the ethanol extract of C. limon peel was more effective than peel extracts prepared with other solvents. Essentially digesting chemical and organic waste, enzyme bio cleaner is a type of natural cleaning product that includes certain numbers and types of bacteria, as well as enzymes and microbial nutrients. The primary objective of the research is to create a natural cleaning agent from citrus fruit utilizing lemon.

Keywords: *Solvent extraction, Fermentation, Minimum bactericidal concentration (MBC), Minimum inhibitory concentration (MIC), Phytochemicals*

Corresponding author: Lingaraju Honnur Gurusiddappa
e-mail ✉ lingarajuhg@jssuni.edu.in
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INTRODUCTION

Lemons make up the bulk of citrus fruits, and they're frequently employed in the juice business. It is one of the most significant commercial fruits cultivated on all continents with a global output of 102.64 million tons (Patsalou *et al*., 2017). These enterprises generate a lot of garbage, which mostly consists of lemon peels. Because these peels contain a wide range of bioactive components, they may be used to create value-added products that help to reduce pollution. Peels contribute the most to this waste with 50-60% (Jimenez *et al*., 2020; Verma *et al.,* 2022).

Citrus peels are high in flavonoids, flavones glycosides, phenols, essential oils, and organic acids which are uncommon in other plants, according to studies (Kanedi *et al.,* 2021; Mitsagga *et al*., 2021). These chemicals are not only crucial for human health and the environment, but they also have a wide range of economic uses in the food and pharmaceutical sectors (Harfouch *et al*., 2019; Kryukova *et al*., 2021). Other active terpenes as well as aldehydes, esters, and alcohols contribute to the overall antimicrobial effects of the essential oils (Samarakoon *et al*., 2012). Citrus fruit has been used in traditional Asian medicine for thousands of years to treat gastrointestinal, bronchial, and asthmatic illnesses (Rafiq *et al*., 2018; Bottalico *et al*., 2020).

The most important fruits in the *Citrus* genus are lemon (*Citrus limoniuym*), lime (*Citrus aurantifulia*), grapefruit (*Citrus vitis*), tangerine or mandarin (*Citrus reticulata*), and sweet orange (*Citrus sinesis*) (Samarakoon *et al*., 2012). Flavedo or epicarp (colored peripheral surface) and albedo or mesocarp (white soft inner layer) are the two types of citrus peels (Muliarta & Darmawan, 2021). This research aimed to concentrate on minimizing the waste in the fruit juice manufacturing business. The combined efforts of waste reduction throughout the manufacturing process and product recovery significantly lower the waste and improve the environmental profile of the juice processing business. By analyzing the 3 chemical contents and calculating the yield percentage of crude phytochemicals, the study analyses the antimicrobial activity and fundamental scientific foundation for the usage of citrus peels (Henderson *et al*., 2018).

Fruit solutions are an example of a natural cleaning product. Natural cleaning solutions are less expensive and damaging to the environment. Cleaning supplies are frequently utilized in daily life, whether it be for household chores, laundry, or other industrial uses. Since many of these products contain

substances made of petrochemicals, most conventional cleaning solutions employ chemicals that are relatively harmful to human health and the environment. Garbage enzymes are organic substances made by simply fermenting fresh vegetable and fruit wastes with water, brown sugar, and specialized yeast and bacteria (Satari & Karimi, 2018; Bahamid & Alhudaithi, 2022).

This study aims to determine the antibacterial activity and phytochemical analyses of *Citrus limon* peels extracted with a solvent. The antibacterial activity of *C. limon* peels was tested against Gram-positive bacteria like *Staphylococcus aureus* and *Enterococcus faecalis*, as well as Gram-negative like *Salmonella typhi*, utilizing different solvents including acetone, ethanol, ethyl acetate, and petroleum ether.

MATERIALS AND METHODS

Materials

Acetone, Ethanol, Ethyl acetate, Petroleum ether

Nutrient agar: Peptone - 5.00 g/L; Sodium chloride – 5.00 g/L; Meat extract – 1.50 g/L; Yeast extract – 1.50 g/L; Agar – 15.00 g/L; pH (at 25 °C) – 7.4 ± 0.2

Nutrient broth: Peptone - 5.00 g/L; Sodium chloride – 5.00 g/L; Meat extract – 1.50 g/L; Yeast extract – 1.50 g/L; pH (at 25 °C) – 7.4 ± 0.2

Microorganism selection

Extractions of *Staphylococcus aureus*; *Salmonella typhi*, and *Enterococcus faecalis* were used for antibacterial testing as Gram-positive and Gram-negative bacteria, respectively. The pure cultures of *Staphylococcus aureus*; *Salmonella typhi*, and *Enterococcus faecalis* are taken from the master plate and then subcultured into nutrient broth by inoculation technique. Overnight cultures of bacteria were prepared on nutrient broth.

Microbiological media and agar plate preparation

28 grams of nutrient agar was liquefied in one liter of distilled water. A small amount of agar-agar was added to it. The mixture was stirred to dissolve all the components. The mixture was autoclaved for 15 min. at 15 lbs pressure. The nutrient agar was cooled after autoclaving. Once the media is cooled, it is poured onto the Petri plates and allowed to solidify.

Collection of plant extracts

The peel of the plant used in the is *C. limon* (common name: Lemon). The peels were obtained from local juice shops. The peels were washed with distilled water to remove dust. They were then shade-dried at a temperature of 32-35 °C for 5 days. The dried peels were ground and made to powder. The powder was stored in a closed container.

Preparation of extracts

25 grams of dried and powdered orange peel was extracted using 100 ml of each solvent (acetone, ethanol, ethyl acetate, and petroleum ether). In a conical flask, the peel powder and solvents were placed and thoroughly mixed using a shaking machine for 48 hrs. at 150 rpm (except Petroleum ether- 24 hrs). The extracts were then filtered using the Whatman filter paper. The filtrate obtained was then dried using the open-dish evaporation method and the dried extract obtained was placed in small vials and stored in the refrigerator at 4 °C (El-Desoukey *et al*., 2018).

Estimation of percentage yield

The given formula was used to calculate the percentage yield of the extract that was obtained using different solvents (Giwa *et al*., 2018).

$$\text{Percentage yield (\%)} = \frac{\text{The weight of the extract recovered}}{\text{Weight of dry powder}} \times 100 \quad (1)$$

Antimicrobial activity/assay

Agar well diffusion assay

The extracts agar well diffusion assay was conducted to determine the antimicrobial assay. Two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and one Gram-negative bacterium (*Salmonella typhi*) were used to test for antimicrobial activity. Petri plates used for this assay were autoclaved and all the steps were carried inside the laminar airflow chamber. Nutrient agar was used as a nutrient medium for assay. Bacteria was poured and spread on the solidified nutrient agar and wells of 6 mm diameter were punched on the agar. 100 µL of 100 mg/ml of test samples were transferred to the labeled wells. The test samples were allowed to diffuse through the media and the plates were incubated in the upright position for 24 hrs at 37 °C. 1 mg/ml of ciprofloxacin was used as positive control and 25% Dimethyl sulfoxide (DMSO) was used as a negative control. The diameter of the zone of inhibition around the wells was measured to evaluate the antimicrobial assay.

Determination of minimum inhibitory concentration (MIC)

The final extraction concentration with no apparent microbial growth is known as minimum inhibitory concentration (MIC) (Guo *et al*., 2018). A sterile 96-well microtiter plate was labeled. To all the wells, a nutrient broth of 100 µL was added. Serial dilutions of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, and 50 mg/ml of test samples were prepared with a micropipette. 100 µL of the test sample was added to each well in ascending order and tips were discarded after each use. Finally, 25 µL of each bacterial suspension was added to every well. The plate was wrapped loosely with parafilm to avoid contamination and dehydration of bacteria. Positive control with vegetative broth and test organisms and negative control with nutrient broth were added. The plate was then kept in the incubator for 24 hrs at 37 °C. After 24 hrs the microtiter plate was read using a microtiter plate reader and the MIC of the extract was recorded.

Determination of minimum bactericidal concentration (MBC)

MBC was determined by taking a loopful of broth from the wells with no evident signs of bacterial growth. The broth was streaked onto the sterile nutrient agar plates by streaking. The plates were then incubated for 24 hrs at 37 °C. The concentration in which no visible growth of bacteria was seen was taken as the minimum bactericidal concentration.

Phytochemical analysis (Qualitative analysis)

- *Test for alkaloids*

2 ml of filtrate was mixed with 2 ml of distilled water and a continuous shaker. The presence of alkaloids was indicated by the formation of a 1 cm layer of foam.

- *Test for amino acids*

A few drops of Ninhydrin reagent were added to 2 ml of filtrate. The presence of amino acids was indicated by the appearance of a purple color.

- *Test for flavonoids*

2 ml of 2N sodium hydroxide was mixed with 2 ml of filtrate. The presence of flavonoids was indicated by the appearance of yellow color.

- *Test for steroids*

2 ml of filtrate was mixed with an equal volume of chloroform and a few drops of conc. Sulfuric acid. The presence of steroids is indicated by the formation of a brown ring.

- *Test for tannins*

2 ml of filtrate was mixed with a few drops of 0.1% ferric chloride. The presence of tannins was indicated by the formation of blue-black or brownish-green coloration.

RESULTS AND DISCUSSION

The solvent extract of the lemon peel using various solvents produced different outcomes in each of the tests conducted in this study. The percentage yield of the recovered extract was significantly varied when different solvents were used. The percentage yield of extracts obtained from various solvents is shown in **Figure 1**. The greatest percentage yield was ethanol (5.4%), followed by acetone extract (4.3%), ethyl acetate extract (2.3%), and petroleum ether (1.4%). The petroleum ether extract had the lowest percentage yield (1.4%). The variation in yield between various solvents explains the solubility of distinct plant components in various solvents. Studies conducted by Eldesukoy *et al*. (2018) showed that the percentage yield of ethanol extract from *C. limon* peels was greater than that of other citrus fruits such as *Citrus limetta*, *Citrus aurantifolia*, *Citrus reticulate*, and *Citrus maxima*.

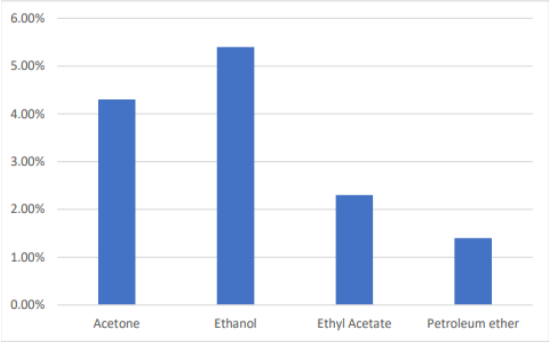

Figure 1. The percentage yield of *C. limon* peel extract

The color of the extract was brownish orange. Citrus peel extracts show considerable antibacterial activity against all of the species tested **(Table 1)**. Because of its high yield and antibacterial action, ethyl acetate is a good solvent for extracting antibacterial compounds. Because of the enhanced production, a higher antibacterial agent is linked to a higher concentration of certain phytochemicals. *C. limon* ethyl acetate extract had the maximum zone of inhibition (16 mm) against *S. aureus* and *S. typhi* (10 mm), followed by *S. aureus* (13 mm). Petroleum ether and ethanol both have antimicrobial properties. Petroleum ether has reduced antibacterial activity when compared to other solvents. This reveals that the solvents can extract antibiotic chemicals that are potentially dangerous to bacterial strains. As a result, different extracts may contain different antibacterial agents with different modes of action, or bacteria may develop a specialized metabolism to resist or adapt to their activity (Giwa *et al*., 2018). When compared to extracts from other solvents, ethanol extract contains more phytochemicals (alkaloids, tannins, flavonoids, and steroids), which may have boosted bacterial activity. The antibacterial effects seen in this investigation might be attributable to the phytochemical group's synergistic action (Guo *et al*., 2018).

Table 1. Zone of inhibition of *C. limon* peel extract for various bacterial strains (Well concentration = 100 mg/ml).

Bacteria	Zone of Inhibition			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
E. faecalis	-	14	13	13
S. aureus	13	15	16	12
S. typhi	9	9	10	-

The zone of inhibition obtained for ethanol, acetone, and ethyl acetate extract of *C. limon* peel against *S. aureus* bacteria is 9mm, 10mm, and 7mm respectively (Mubarak *et al*., 2020). In comparison to earlier research, the test organisms and solvents utilized in this investigation showed the following inhibitory zone: *E. faecalis* (acetone extract 0 mm, ethanol extract 14 mm, ethyl acetate extract 13 mm, petroleum ether extract 13 mm); *S. typhi* (acetone extract 9 mm, ethanol extract 9 mm, ethyl acetate extract 10 mm, petroleum ether extract 0 mm); *S. aureus* (ethanol 21 15 mm, acetone 13 mm, ethyl acetate, 16 mm, and petroleum ether against bacteria are 12 mm). Ciprofloxacin, the positive control had a larger inhibitory zone.

When compared to Gram-positive bacteria, Gram-negative bacteria were less susceptible to the solvent extraction of the peels. The insensitivity of diverse test organisms to the investigated solvent extracts was attributed to variations in the content and structure of Gram-positive and Gram-negative bacteria's cell walls (Shakya *et al*., 2019). Gram-negative bacteria are less susceptible to antibacterial agents because they have an outer membrane that inhibits hydrophobic chemicals from diffusing through the lipopolysaccharide coating (Kumar *et al*., 2011). The antibacterial activity of extracts obtained using several solvents indicated that not all phytochemicals with antimicrobial activity are miscible in one solvent (Oikeh *et al*., 2020).

The MIC and MBC of several solvent extracts of *C. limon* are shown in **(Tables 2 and 3)**. The extracts were shown to have significant antibacterial activity. The broth dilution technique was used to perform MIC. The broth approach, which is carried out in microtiter trays, has the advantage of requiring less effort for a larger number of duplicates and allowing for the use of very small amounts of test material and growth media. The MIC and MBC data also reveal that ethyl acetate extracts are more effective than extracts from other solvents. The following are the MIC values for different solvent extracts against various bacteria: *S. aureus* (acetone extract- 13 mg/ml, ethanol extract-

15 mg/ml, ethyl acetate extract- 16 mg/ml, petroleum ether extract- 12 mg/ml); *S. typhi* (acetone extract- 9 mg/ml, ethanol extract- 9 mg/ml, ethyl acetate extract- 10 mg/ml, petroleum ether extract- 0mg/ml); *E. faecalis* (acetone extract- 0 mg/ml, ethanol extract- 14 mg/ml, ethyl acetate extract- 13 mg/ml, petroleum ether extract- 13 mg/ml). MBC values if various solvent extracts against different bacteria are as follows: *E. faecalis* (acetone extract- 50 mg/ml, ethanol extract- 50 mg/ml, ethyl acetate extract 100 mg/ml, petroleum ether extract- 0 mg/ml); *S. aureus* (acetone extract- 50 mg/ml, ethanol extract- 75 mg/ml, ethyl acetate- 1 mg/ml, petroleum ether extract- 0 mg/ml); *S. typhi* (acetone extract- 1 mg/ml, ethanol extract- 75 mg/ml, ethyl acetate extract- 50 mg/ml, petroleum ether extract- 0 mg/ml). Less activity at low extract concentrations might imply that the active ingredient concentrations in the extracts are insufficient to have any antibacterial action (Abdella *et al*., 2019). To battle infections caused *by E. faecalis, S. typhi*, and *S. aureus*, ethyl acetate extract of the peel may be chosen to over-extract from other solvents (Teneva *et al*., 2019).

Table 2. MIC value of *C. limon* peel extract for various bacterial strains

Bacteria	Extract of Citrus limon peel (mg/ml)			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
E. faecalis	-	14	13	13
S. aureus	13	15	16	12
S. typhi	9	9	10	-

*Well concentration at 100 mg/ml

Table 3. MBC values of *C. limon* peel extract for various bacterial strains

Bacteria	Extract of *C. limon* peel (mg/ml)			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
E. faecalis	50	50	100	-
S. aureus	50	75	1	-
S. typhi	1	75	50	-

A phytochemical study revealed the presence of numerous components in lemon peels. The presence of different phytochemicals such as tannins, saponins, essential oils, flavonoids, and 22 phenolic compounds in lemon peel is connected to its antibacterial effect (Hsouna *et al*., 2017; Gorski *et al*., 2022). The phytochemical analysis findings are shown in **(Table 4)**. Extracts from various solvents included phytochemicals of diverse types. All extracts included significant amounts of alkaloids, steroids, flavonoids, amino acids, and tannins (Musa & Hafiz, 2019). All solvents were able to dissolve alkaloids, steroids, flavonoids, amino acids, and tannins in this study. In the extracts of several solvents, amino acids were absent (Egbuonu & Osuji, 2016).

Table 4. Phytochemical analysis (qualitative analysis) of *C. limon* peel extracts

Phytochemicals	Solvents			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
Alkaloids	+	+	+	+
Amino acids	-	-	-	-
Flavonoids	+	+	+	-
Steroids	+	-	-	+
Tannins	+	+	+	+

- Indicates the absence of phytochemical; + indicates the presence of phytochemical

The polarity of the chemical and the solvent might explain the presence and absence of distinct components in the extracts. Tannins, steroids, flavonoids, and alkaloids are secondary active metabolites found in plants that contribute to lemon peel's antibacterial activity (Rehab *et al*., 2018; Nawaz *et al*., 2020). *C. limon* peel contains flavonoids and tannins, indicating that the peel has antioxidant effects. Tannins are also said to have antibacterial properties (Mostafa & Essaway, 2021).

CONCLUSION

Fruit waste recycling is one of the most important strategies for repurposing it in several innovative ways, resulting in new products and meeting crucial demands in human, animal, and plant nutrition, as well as the pharmaceutical industry. *C. limon* peel extracts made with various solvents were tested for antibacterial activity and phytochemical content against test organisms. Although the kind and number of active components in each extract are not immediately apparent, they are encouraging; nonetheless, further research with pure compounds is required before a firm judgment can be made about the bioactive molecules that contribute to antimicrobial activity. However, further research is needed to understand how the bioactive component confers antimicrobial properties to the orange peel extract and how these extracts might be used to treat pathogenic bacteria like *S. typhi*. Four different fermentation samples namely T1, T2, T3, and T4 were prepared. Results obtained from PH and sugar analysis showed that T2 and T4 were more effective bio cleaners.

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