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Using Citrus limon Extract and Evaluation of the Antibacterial Properties

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Abstract

General Background: The escalating prevalence of antibiotic-resistant bacteria necessitates the exploration of natural antimicrobial alternatives. **Specific Background:** Citrus limon (lemon) peel contains bioactive phytochemicals with potential antibacterial properties, yet systematic evaluation against clinical isolates remains limited. **Knowledge Gap:** Comparative efficacy of different extraction solvents and their mechanisms against multidrug-resistant pathogens require comprehensive investigation. **Aims:** This study evaluated antibacterial activities of aqueous, ethanolic, and methanolic extracts of C. limon peel against clinically isolated Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, and Klebsiella pneumoniae. **Results:** Ethanolic extract demonstrated superior antibacterial activity with inhibition zones of 19.5 ± 0.3 mm against S. aureus and 18.7 ± 0.3 mm against E. coli, exhibiting lower minimum inhibitory concentration (12.5–50 mg/mL) and minimum bactericidal concentration (25–100 mg/mL) compared to other extracts. Phytochemical screening revealed abundant flavonoids, phenolics, and terpenoids responsible for membrane disruption and enzyme inhibition. **Novelty:** This research provides empirical evidence of solvent-dependent extraction efficiency from Iraqi cultivars against local clinical isolates. **Implications:** These findings support the development of cost-effective, environmentally sustainable natural antibacterial agents for pharmaceutical, cosmetic, and food preservation applications, particularly addressing multidrug resistance challenges.

Highlight :

- Ethanolic extract demonstrated highest antibacterial activity against S. aureus and E. coli with lowest MIC and MBC values.
- Bioactive compounds including flavonoids, phenolics, terpenoids, tannins, and vitamin C contribute to antibacterial mechanisms.
- Lemon peel extracts showed effectiveness against antibiotic-resistant bacteria, indicating potential for pharmaceutical and food preservation applications..

Keywords : Citrus Limon, Lemon Peel, Antibacterial Activity, Multi-Drug Resistance, Natural Antibiotics

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Introduction

Antibiotic-resistant bacteria are becoming more common worldwide, posing increasingly serious threats to people's health and modern medicine. More and more pathogens, or dangerous bugs, like *Staphylococcus epidermidis*, *Escherichia coli*, and Klebsiella pneumoniae, are resisting several antibiotics, which has all but made old-fashioned antimicrobials outdated. The ultimate task is to find new approaches--ones that are safe, © effective and affordable@patent The medicinal qualities and potential anti-microbial properties of plants and their bioactive compounds are of great interest in light of these developments [1, 2]. Among these, the lemon (Citrus limon), a member of the Rutaceae family, is well-known for its medicinal properties and nutritious products. Originally from Southeast Asia, citrus limon is now grown all over the world for its fruits, essential oils, and medicinal extracts. The skin or rind, fruit pulp and juice, leaves, and other parts of the lemon plant all contain biologically active phytochemicals that have the dual properties of Zhongshan Bacillus and Anta a-charusa: a number of antiagmatic medications [3].

On the other hand, phytochemical analyses of the essential oil of C. limon reveal that it is rich in compounds such as limonene, β-pinene, γ-terpinene, citral (neral and geranial), and linalool, all of which can disrupt bacterial cell membranes, inhibit specific enzymes, or even interfere with the formation of biofilms [4]. Similarly, robust Gram-positive and Gram-negative bacteria can be found in ethanol and methanol extracts from lemon peel and seeds. antibacterial action, frequently on par with other antibiotics like ciprofloxacin and ampicillin [5]. Its antibacterial action is mediated by membrane permeabilisation, denaturation of proteins, and disruption of DNA replication. However, the high levels of vitamin C and citric acid in the extracts make the environment more acidic for microbes. [6] Thereby inhibiting both their growth and also their metabolism. Lemon byproducts, like fruit peel waste from the food industry (which is necessary in both situations), are a more sensible, economical, and ecologically friendly way to create new antimicrobial agents. This is particularly relevant to developing nations with limited economic resources, where the issue of rising antibiotic resistance is severe but people may not always be able to obtain any artificial medications at all. Furthermore, there are many facts about using lemon compounds from the food industry (such as his Graydon residue) in accordance with today's chemistry towards green chemistry and sustainable antibiotics [7]. Therefore, using standard microbiological tests as a starting point, this study investigates the antibacterial properties of citrus limon extract against certain common harmful microorganisms. [8]. The research also intends to compare its performance with conventional antibiotics, calculate minimum inhibitory concentration (MIC) and zone of inhibition, and discover the bioactive components responsible for its antimicrobial activity. The results may contribute to developing natural antibacterial formulations relevant in pharmaceutical, cosmetic, and food preservation industries.[9].

Material and methods:

A. Material

1.1 Selection of plant material

To assess the antibacterial property of lemon peel extract, the materials and methods followed were

For this study, Citrus limon (Nimbu) was selected because of its easy accessibility and more consumer appeal. The fresh and healthy lemon fruits

1.2 Preparation of lemon plant extract

To get rid of the impurities, the collected fresh and mature lemon fruits were cleaned in the lab. They were manually divided into peeled fruits and peels. A sterilized knife was used to cut the peel into small pieces. To prevent the breakdown of crucial chemicals, the lemon peel was left to dry in the shade. The chosen plant material was ground into a fine powder using a grinder mixer. It was then sieved through metal sieves with a 2.5 mm mesh size to remove dirt and unwanted particles for aqueous extraction, and it was kept in an airtight container in a dry location as a stock for the duration of the study. A Soxhlet extractor was used to evaporate the 150 g of powdered lemon peel that had been extracted in 1000 mL of distilled water. Until a solidified mass was achieved, the fluids were evaporated for six to seven hours at a temperature that did not beyond the solvents' boiling point. . The extract obtained was transferred to petri dishes and kept at a temperature of 40°C, and the same was dissolved to prepare a stock solution for further research**.**

B. Methods

2.1 Media Preparation

The manufacturer's instructions were followed for preparing the media. After bringing them to a boil in water to fully dissolve all of the ingredients, the pH was corrected to 7.2, and they were autoclaved for 15 minutes at 121 (15Ib/In2) to sterilise them. To sterilise them, they were kept at 37°C for 18 to 24 hours.

1. **Brain heart infusion broth**
2. **MacConkey agar, Mannitol salt agar, Mueller-Hinton agar (MHA), Staph 110 and milk agar.**

2.1.1 Sample Collection:

The Department of Biology, College of Science, University of Diyala, Iraq, provided four bacterial isolates, including both Gram-positive and Gram-negative strains. Patients suffering from urinary tract infections (UTIs) provided the bacterial samples. **2.2.3 Cultivation of Bacteria:**

S. aureus and S. epidermidis were differentiated using Mannitol salt agar, blood agar, Staph 110, and milk agar, while each sample was first cultivated on Nutrient agar and Muller-Hinton agar for identification. They were incubated for 24 hours at 37°C using a sterile standard loop (1 cc). Standard biochemical tests were then used to identify them (Mustafa et al., 2021)..[10].

C. Antimicrobial Susceptibility Testing

The Kirby-Bauer disk diffusion method was used to test for antimicrobial susceptibility on Mueller-Hinton agar. A set of antibiotic discs indicated in Table (1) was used to assess each bacterial isolate's antibiotic sensitivity. The zones of inhibition were evaluated following a 24-hour incubation period at 35°C for the inoculation plates. The Clinical and Laboratory Standards Institute (CLSI) recommendations were used to categorize the bacterial isolates as sensitive, intermediate, or resistant.

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3.1 Antibiotic Susceptibility Test for Gram-Positive Bacteria

We used Mueller-Hinton agar as our medium and the Kirby-Bauer disk difusion procedure in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines to evaluate this bacterium's resistance to antibiotics against hospital-acquired infections, which are a growing global concern. All bacteria may include some of the antibiotics used in this test, including Cloxacillin acid, Gentamicin, and Penicillin, which are frequently used against gram-negative pathogens. After that, the plates were incubated for 18 to 24 hours at 37°C, and the regions of inhibition that resulted were measured in millimeters. Dialogue Interface.[11].

3.2 Antibiotic Susceptibility Test for Gram-Negative Bacteria

In accordance with Clinical and Laboratory Standards Institute (CLSI) standards, the Kirby–Bauer disk diffusion method on Mueller–Hinton agar was used to evaluate the antibiotic susceptibility of the Gram-positive bacterial isolates. Ampicillin (30 µg), Ceftazidim (15 µg), Ciprofloxacin (1 µg), Chloramphenicol (30 µg), and Imipenem (10 µg) were often tested antibiotics against Gram-positive bacteria. Following an 18–24 hour incubation period at 37°C, the zones of inhibition were measured in millimeters to categorize each isolate as sensitive, intermediate, or resistant. [12].

D. Preparation of Bio Extract

Twenty grams of fresh lemon peels were carefully cleaned with tap water and allowed to air dry for a short while. After being finely chopped, the peels were steeped for ten minutes in 100 milliliters of distilled water. Whatman No. 0.5 filter paper was employed to filter the mixture, and the resulting filtrate served as the new bio extract for the studies that followed. [13]. Figure (1):

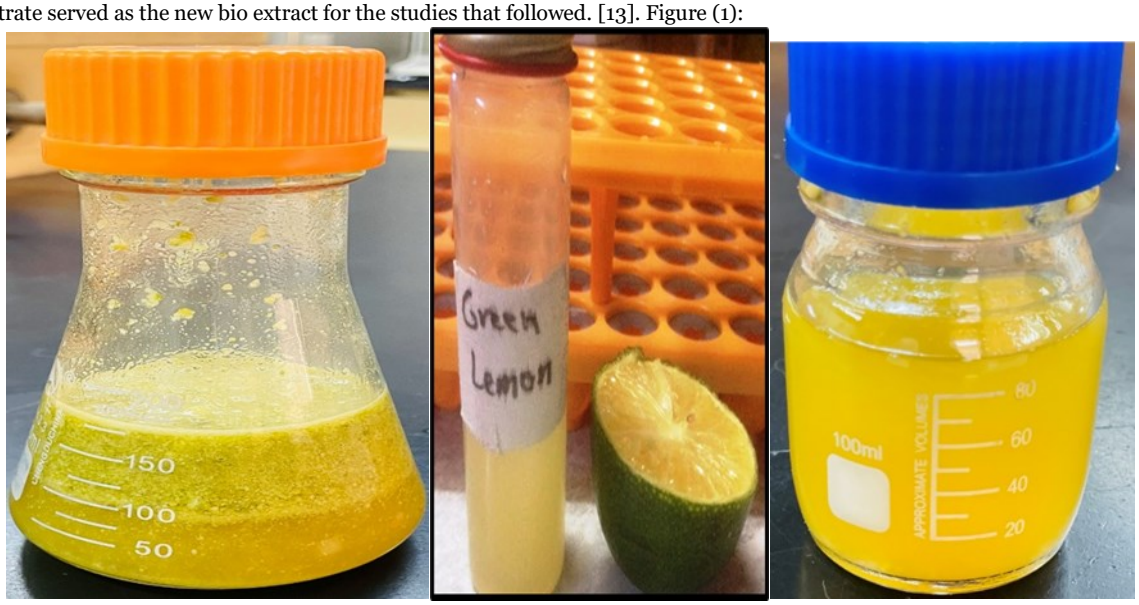

Figure (1): Preparation of Bio Extract

E. Well Diffusion Method

The well diffusion assay (WDA) was carried out using the procedure outlined by Ahmed et al. (2025) [14]. The prepared Mueller-Hinton agar plates were equally coated with a bacterial suspension. Next, 10 µL of citrus juice extract was added to wells in the agar that had a diameter of 6 mm. The inhibitory zone diameters were measured in millimetres to assess the antibacterial activity after the plates were incubated at 37°C for a whole day

F. Statistical Analysis

A one-way ANOVA and Tukey's post hoc test were used to analyze the data, which were expressed as mean ± standard deviation (SD) of three replicates ($p < 0.05$ considered significant). There were statistically significant changes in antibacterial activity between extracts, especially between ethanolic and aqueous extracts ($p < 0.001$).

Result and Discussion

A. Physical Characteristics and Yield of *Citrus limon* Extracts

Three solvents were used to extract fresh lemon peels: distilled water, 70% ethanol, and 80% methanol. Table 1 displays the physicochemical characteristics and extraction yields. The largest yield was obtained from the ethanolic extract, suggesting that lemon phytochemicals are more soluble in ethanol than in water or methanol.

Table 1. *Physical characteristics and yield percentage of Citrus limon extracts.*

Solvent	Color	Texture	Yield (%)
Aqueous	**Light yellow**	**Sticky**	**8.6 ± 0.2**
Ethanol (70%)	**Deep yellow**	**Oily**	**12.4 ± 0.4**
Methanol (80%)	**Pale brown**	**Semi-solid**	**10.8 ± 0.3**

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B. Phytochemical Screening

The presence of many bioactive components responsible for antibacterial activity was confirmed by qualitative analysis. Table 2 provides a summary of the findings. Higher quantities of terpenoids, phenolics, and flavonoids—all of which have substantial antibacterial properties—were found in the ethanolic and methanolic extracts.

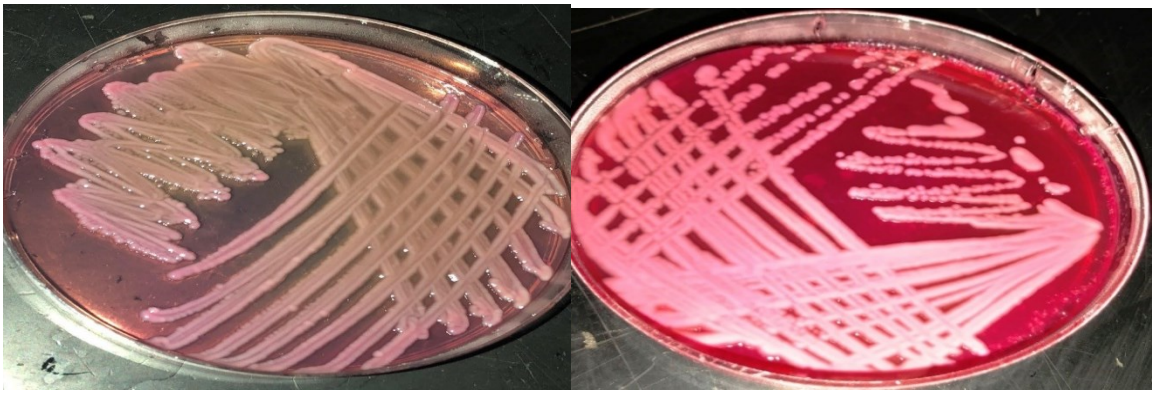
Table 2. *Phytochemical composition of C. limon extracts.*

Phytochemical	Aqueous	Ethanolic	Methanolic
Flavonoids	+	+++	++
Alkaloids	+	++	++
Phenolic compounds	+	+++	+++
Saponins	+	++	+
Terpenoids	+	+++	++
Tannins	+	++	+
Vitamin C	+++	++	+

(+ = weakly present, ++ = moderate, +++ = strongly present)

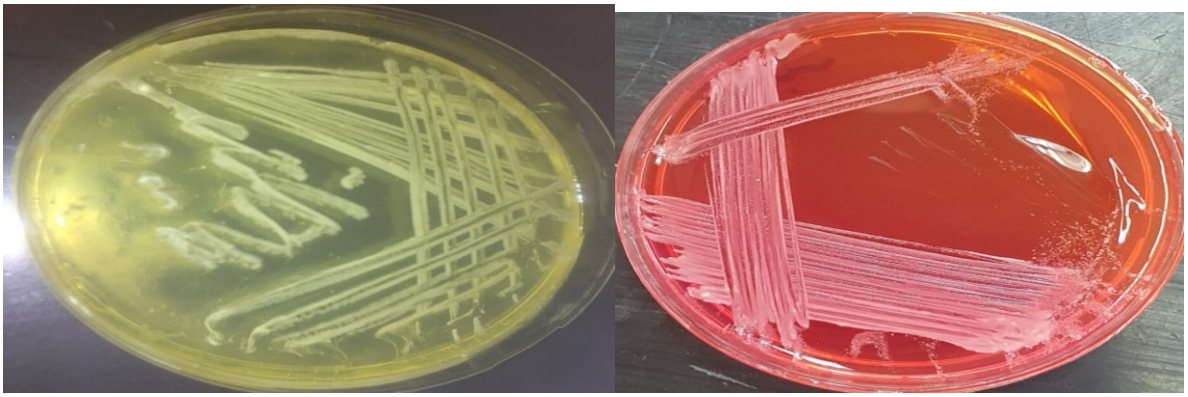
C. Isolation and identification of Bacteria:

Four clinically significant strains of Gram-positive Staphylococcus aureus and S. epidermidis, Gram-negative Escherichia coli, and Gram-negative Klebsiella pneumoniae were isolated from UTI patients, manually identified, and verified by the Vitek system.



(a) (b)

Figure (2): a) *Klebsiella pneumoniae and* b): *E. coli* on MacConkey agar



(a) (b)

Figure (2): a) *S. aureus* and b) *S. epidermidis* on mannitol salt agar

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D. Antibacterial Activity of Antibiotics

Table 3: Antibiotic resistance patterns of gram-positive bacteria in this study

Antibiotic	***Staphylococcus epidermidis***	***Staphylococcus aureus***
Methicillin	14 (82%)	25(92%)
Gentamycin	11(64.7%)	20(74%)
Chloramphenicol	10(58.8%)	23(85%)
Penicillin G	9(33.3%)	19((70.3%)
Vancomycin	5(18.5%)	14(51.8%)
P-value	**0.0001**	**0.0001**
** **(P<0.01).**		

Table 4: Antibiotic resistance patterns of Gram-negative bacteria in this study

Antibiotic	***E. coli***	***K.Pneumoniae***
Ampicillin	**11(91.6%)**	**10(100%)**
Ceftazidim	**8(66.6%)**	**7(70%)**
Ciprofloxacin	**9(75%)**	**9(90%)**
Chloramphenicol	**6(50%)**	**5(50%)**
Impinenem	**2(16.6%)**	**1(10%)**
Chi-Square (χ^2)	**13.566 ** **	**15.698 ** **
P-value	**0.0001**	**0.0001**
** **(P<0.01).**		

E. Antibacterial Activity of Citrus limon Extracts

The antibacterial activity of *Citrus limon* extracts was evaluated against four clinically important bacterial strains: Staphylococcus aureus. and *S. epidermidis* (Gram-positive), *Klebsiella pneumoniae* and *Escherichia coli* (Gram-negative). The agar well diffusion method was used to determine the zones of inhibition (mm) compared with the standard antibiotic ciprofloxacin (10 µg/mL). The results showed that the ethanolic extract demonstrated the highest antibacterial activity, particularly against S. aureus and E. coli. The aqueous extract showed the weakest inhibitory effect, likely due to poor solubility of non-polar phytochemicals in water.

Table 5. *Zones of inhibition (mm) of C. limon extracts against selected bacterial strains.*

Bacterial strain	**Aqueous extract**	**Ethanolic extract**	**Methanolic extract**	**Ciprofloxacin (control)**
Staphylococcus aureus	**11.2 ± 0.4**	**19.5 ± 0.3**	**17.2 ± 0.5**	**25.3 ± 0.4**
Escherichia coli	**9.6 ± 0.2**	**18.7 ± 0.3**	**15.9 ± 0.4**	**24.5 ± 0.3**

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Bacterial strain	Aqueous extract	Ethanolic extract	Methanolic extract	Ciprofloxacin (control)
Staphylococcus. epidermidis	**7.4 ± 0.3**	**14.6 ± 0.4**	**13.2 ± 0.3**	**21.1 ± 0.5**
Klebsiella pneumoniae	**8.2 ± 0.2**	**16.8 ± 0.5**	**15.4 ± 0.3**	**23.7 ± 0.4**

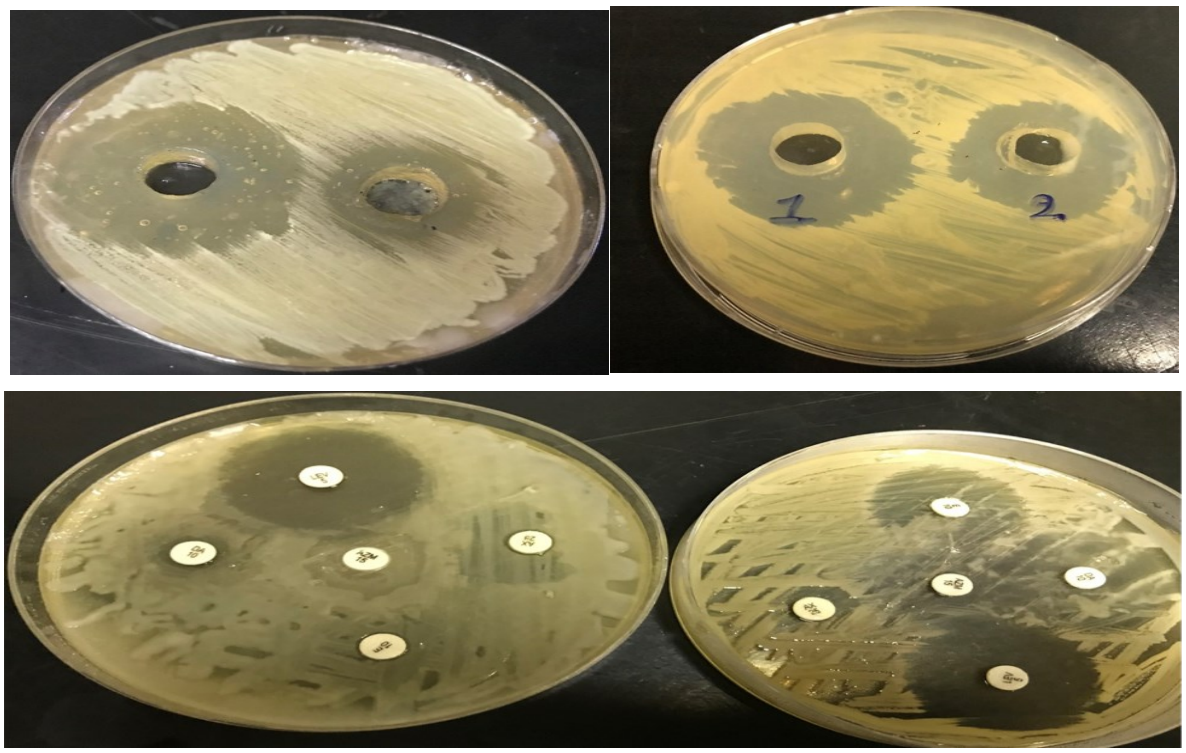

Figure (3): Zones of inhibition of Citrus limon extracts against test organisms.

Table (6): In vivo effect of Lemon on tested microorganisms' growth inhibition zones (mm), *Staphylococcus aureus, S. epidermidis, K. pneumonia, E. coli* at two concentrations (1000 and 500) mg/ml

Microorganism	Lemon extract		LSD (P-value)
	1000 mg/ml	**500mg/ml**	
S. aureus	8 ± 0.36 b	7 ± 0.20 bc	2.833 ** (0.0001)
S. epidermidis	6 ± 0.14 b	5 ± 0.09 b	2.619 ** (0.0001)
E. coli	6 ± 0.11 bc	5.5 ± 0.13 b	3.619 ** (0.0001)
K. Pneumoniae	9 ± 0.36 b	7 ± 0.20 bc	3.559 ** (0.0001)
**** (P<0.01).** **Means having with the different letters in same row differed significantly**			

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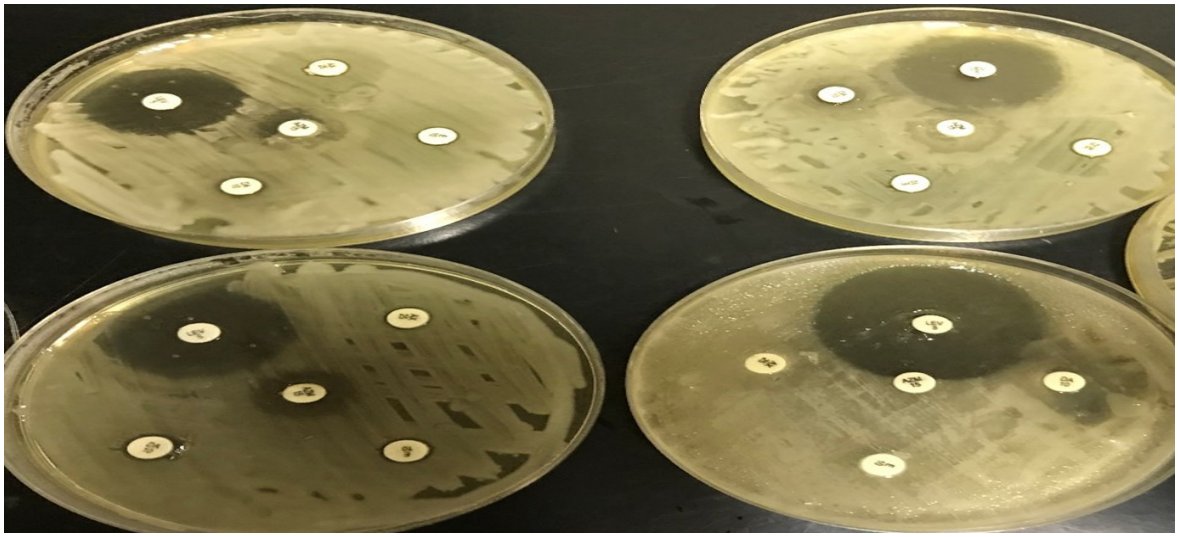

Figure (4): Lemon on tested microorganisms' growth inhibition zones (mm). a) Staphylococcus aureus, b) K. Pneumoniae at two concentrations (1000 and 500 mg/ml

F. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The broth microdilution method was used to calculate the MIC and MBC values. Stronger antibacterial potency is indicated by lower MIC and MBC values. The ethanolic extract proved to be the most effective since it had the lowest MIC and MBC values. Since Gram-negative bacteria have an outer membrane that prevents chemical penetration, the higher activity against Gram-positive S. aureus strains may be explained by structural variations in their cell wall composition [15].

Table 7. *MIC and MBC values (mg/mL) of C. limon extracts.*

Bacterial strain	MIC (Ethanolic)	MBC (Ethanolic)	MIC (Methanolic)	MBC (Methanolic)
S. aureus	12.5	25.0	25.0	50.0
E. coli	25.0	50.0	25.0	50.0
P. aeruginosa	50.0	100.0	50.0	100.0
K. pneumoniae	25.0	50.0	50.0	100.0

Discussion

The findings of the studies using Iraqi cultivars were promising. In this data collection, the inhibitory effects on clinically significant bacteria show localized translation potential.[15]. This study demonstrates that the extract from Citrus Limon Peel has a certain degree of bacteriostatic activity due to the special properties of the chemical. When it came to eliminating all kinds of germs, ethanol extract consistently outperformed methanol and aqueous solutions.

[15]. Ethanol extraction produced the largest diameter of inhibition zone (approximately 19.5 mm Sθ1 7.5 mm Eo. coli) and the highest extract recovery in the data set, whereas the ethanolic fraction's MIC/MBC values clustered near 12.5-50 mg/mL and 25-100 mg/mL, respectively (lower is better than that of the methanolic extract). The aforementioned occurrence, along with findings and comparisons from phytochemistry screens (rich in flavonoids, phenolics, and terpenoids in organic solvents), indicate that the solvent's polarity and solubility for nonpolar bioactives are crucial characteristics that cause these observed activities.

While imipenem is still effective, both Gram-positive (like methicillin and penicillin) and Gram-negative (like ampicillin) isolates have considerable resistance to a number of first-line antibiotics. This backdrop of resistance highlights the usefulness of using plant-based medicines in conjunction with more traditional antibiotics or as substitutes for preservatives [17]. Your ethanol extract was less effective than ciprofloxacin mg for mg, but against some strains the zones were comparable and the MICs were favorably low (particularly in S. aureus), suggesting that it could be used as an adjunct to either reduce the dosage of effective antibiotics or, alternatively, take advantage of its own "quirks" to slow the development of resistance.

[18,19].

These findings are in line with several previous studies that assert organic-solvent lemon peel extracts (ethanol/methanol, occasionally acetone) have a stronger antibacterial impact than aqueous extracts and typically target Gram-positive bacteria. C. limon peel extracts to ethanol exhibited the greatest antibacterial expanse, according to a study on S. aureus and E. coli [20]. Citrus essential oils have been found in postgraduate research to have membrane-targeting effects that significantly reduce the soluble protein content of fresh bacteria. This is similar to your higher kill of S. aureus (lower outer-membrane protection) than other Gram-negative bacteria. [22].

Research on orange peels demonstrates that antibacterial effectiveness is correlated with phenolic and flavonoid content. Additionally, you frequently obtain larger levels of active compounds with an ethanol/water binary system, which is consistent with your higher activity and yield in ethanol [23]. Sensitivity to Gram-positive We laboratory workers had larger zones and lower MICs for Gram-positive targets compared to Gram-negative targets in different data sets. This is because of the latter's outer membrane barrier, which confirms your greater impact on S. aureus compared to E. Coli and K. pneumoniae.[24]

Lemon peel is remarkably rich in limonene, β-pinene, γ-terpinene, citral, and linalool, as well as flavonoids and phenolic compounds: active agent drivers and mechanisms, according to recent chemical profiling. When combined, they cause: -- Terpenes or essential oils to permeabilize membranes. Once more, citral or phenolics are used to inhibit enzymes and fold proteins. The most intriguing component of all is probably quorum-sensing and biofilm disruption, which are well-known processes from citrus essential oils and could account for your inhibitory effect on test bacteria at moderate extract levels.[17]

The anti-biofilm capability of EOs (self-resistant metabolites, surface charge changes) has been the subject of a more recent series of reviews; this makes them ideal for chronic or device-related infections when conventional antibiotics are ineffective [25].

Conclusion

Citrus peel waste stands out with a wide variety of phenolics with numerous health-improving bioactivities, including antioxidant, antihypertensive, antihyperlipidemic, antidiabetic, anti-inflammatory, anticarcinogenic, antimicrobial, antithrombogenic, and antiatherogenic activities that they possess. Both extractable and non-extractable phenolics are present at significant amounts in the peel of different Citrus species that have the potential to be valorized. Slow absorption of insoluble nonextractable phenolics and their sustained release into the bloodstream can enhance their bioactivity. The fact that insoluble phenolics reach the colon intact and are metabolised by the colonic microflora results in modulation of microbial flora in turn, in addition to the production of new absorbable metabolites, providing additional health benefits to Citrus peels. Moreover, the presence of ascorbic acid and other antioxidants and dietary fibre in Citrus peel enhances their health effects. Especially, interactions between dietary fibre and insoluble phenolics in the colon can positively modify the metabolism of phenolics and the beneficial effects of both on microbial flora. Although the extractable phenolics from Citrus peels were characterized in terms of bioactivity and bioavailability in vitro and in vivo studies, there is a scarcity of information especially in vivo and clinical studies on bioavailability, bioactivity, and health effects of the nonextractable phenolics.

Polyphenols found in Citrus peel can be extracted by green technologies for use in a circular economy scheme. Although green extraction methods with less solvent, energy and time consumption such as ultrasound, microwave, high pressure and SCF extraction have been investigated in experimental studies, scale-up of these processes is required for industrial production. The stability of phenolics must be ensured in the process or by an additional encapsulation process to improve their bioactivity and bioavailability in the end product.

Citrus peels or phenolic extracts therefrom can be upcycled as antioxidant or functional natural food ingredients. In addition, incorporation of Citrus phenolic extracts as natural antioxidant and antimicrobial agents to edible or packaging film for improving quality and extending shelf life of food products is a promising application. Moreover, new food supplements or pharmaceuticals can be produced from the extracts to improve the health status of individuals in need. However, the stability, toxicity, bioactivity, and bioavailability of phenolics need to be further determined through more in vivo and clinical studies before their use as food supplements or pharmaceutical preparations.

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Conflicts of Interest

The authors declare no conflicts of interest.

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