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Achieving a Sustainable Bio-Economy: Exploring the Antibacterial Efficacy and Phytochemical Properties of Two Indigenous Plants in Sri Lanka, *Citrus crenatifolia and Citrus reticulata*

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Abstract

Bio-economy offers a beneficial solution for sustainable development by rationalising the use of financial and human resources to improve productivity and environmental stewardship. The citrus family encompasses various plant species known for their antimicrobial properties, and different parts of these plants have been used in traditional Sri Lankan medicine for an extended period. However, information on the antibacterial efficacy of two indigenous citrus plants in Sri Lanka, *Citrus crenatifolia* and *Citrus reticulata*, is limited in the literature. Additionally, antibiotic resistance in bacterial pathogens is a global challenge that poses a significant threat to

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public health, highlighting the need for effective alternative strategies. The present study was conducted to investigate the antimicrobial properties and phytochemical composition of *C. crenatifolia* and *C. reticulata*. Active phytochemicals present in the fruit peels were extracted using an aqueous reflux method. The antimicrobial properties of the peel extracts were assessed using the agar well diffusion method against six common pathogens: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterococcus faecalis*. A 0.5 McFarland standard was used to standardise the bacterial inoculum, and double-distilled water served as the negative control. Diameters of inhibition zones were measured after incubation, and statistical analysis was performed using SPSS version 25. The independent sample t-test revealed a significant difference between the mean values of the control and both peel sample extractions against the selected pathogens ($p < 0.05$). However, no statistically significant difference in antimicrobial activity among the six microbes was observed for *C. crenatifolia* peel extract according to the one-way ANOVA test ($p > 0.05$), whereas a statistically significant difference was found for *C. reticulata* peel extract ($p < 0.05$). Tukey's post hoc analysis for *C. reticulata* indicated that the mean diameters of inhibition zones for *Staphylococcus aureus* differed significantly from those of *Pseudomonas aeruginosa* ($p = 0.044$) and *Klebsiella pneumoniae* ($p = 0.049$). The antibacterial activity of both *C. crenatifolia* and *C. reticulata* is supported by the presence of phytochemicals such as alkaloids, steroids, flavonoids, phenols, and tannins, which may act synergistically. These findings highlight the potential of these citrus peel extracts as valuable sources of antimicrobial agents, warranting further investigation to elucidate their specific mechanisms of action and potential applications in microbial control.

Keywords: *antibacterial efficacy, disk diffusion, inhibition zones, phytochemicals, pathogens, Citrus crenatifolia, Citrus reticulata*

Introduction

Antibiotics have undeniably revolutionized medicine, playing a pivotal role in saving countless lives by effectively combating infectious diseases. However, the emergence of antibiotic resistance presents a

formidable challenge to global public health in the modern era. The shortage of effective antibiotics against multidrug-resistant pathogens in routine clinical settings and the decline in the development of novel antimicrobial agents increase the urgency of this issue. Consequently, scientists are seeking novel compounds with potential antimicrobial properties to counteract antibiotic resistance. Although antibiotics are essential in treating various illnesses, their misuse or non-compliance may lead to potentially fatal side effects. Hence, implementing restrictions on antibiotic use is imperative to maintain human health over an extended period (Hasan et al., 2021).

Since 2000, the European Medicines Agency (EMA) and the American Food and Drug Administration (FDA) have been responsible for approving new antibiotics. However, due to the high costs involved in developing new drugs, estimated at approximately one billion US dollars, the pharmaceutical industry has significantly reduced its antibiotic research efforts. Consequently, antibiotic prescription rates and sales are lower compared to other drug categories, such as cardiovascular and anti-diabetic medications. Furthermore, the FDA now requires new antibiotics to demonstrate superiority over existing treatments (Marra, 2011).

Plants commonly used in traditional medicine offer promising prospects for synthesizing new drug compounds capable of combating antibiotic-resistant bacteria. Their reliability, accessibility, and low toxicity make them a popular choice for novel medication development. Approximately 80% of the population in developing countries relies on plant-based herbal medicines. These medicinal plants possess abundant antimicrobial properties attributed to secondary metabolites or phytochemicals such as phenols, alkaloids, flavonoids, and essential oils (Hwang et al., 2001).

Among the various medicinal plants, citrus fruits and their derivatives have significant economic and medicinal value due to their versatile applications across industries, including food, cosmetics, and traditional medicine (Silalahi, 2002). Extracts derived from orange peels exhibit a wide range of therapeutic benefits, including the treatment of colic and digestive discomfort, potential anticancer properties, and immune system enhancement (Grosso et al., 2013). Additionally, orange peel extract is valued for its ability to address

vitamin C deficiencies, colds, scurvy, and viral and bacterial infections (Bernhoft, 2010).

Citrus fruit–derived compounds show promise against antibiotic-resistant bacteria, providing a possible remedy for the antimicrobial resistance health crisis. Significant fruits of the *Citrus* genus include *Citrus sinensis* (orange), *Citrus limon*(lemon), *Citrus reticulata* (tangerine), and *Citrus aurantifolia* (lime) (Singh et al., 1983; Anwar et al., 2008). Studies have demonstrated that orange peel extract exhibits potent antibacterial activity against intestinal pathogens (Mehmood et al., 2015) and effectively combats *Klebsiella pneumoniae* (Akdemir, 2015). Gram-positive bacteria, such as *Bacillus* species, are susceptible to the antibacterial properties of *Citrus macroptera*, while Gram-negative bacteria like *Escherichia coli* are also affected (Hasan et al., 2021).

Furthermore, research on traditional Sri Lankan plants, such as *Epaltes divaricata* and *Vetiveria zizanioides* revealed positive antibacterial activity against *Staphylococcus aureus* in their ethanol, hexane, and aqueous extracts (De Zoysa et al., 2019). Unlike conventional antibiotics, which can disrupt the body's natural flora and lead to unpleasant side effects (Zhang & Chen, 2019), components found in citrus fruits are safe for consumption even at high concentrations. Their affordability and widespread availability further enhance their potential, especially in developing nations with limited healthcare resources (Akanda et al., 2017).

Therefore, the main goal of the present study was to explore and analyse the antibacterial efficacy and phytochemical properties of two indigenous plants in Sri Lanka, *Citrus crenatifolia* and *Citrus reticulata.*

Methodology

Plant Collection and Authentication

C. crenatifolia and *C. reticulata* fruits were collected from Baddegama (6.1608892, 80.195025), in Sri Lanka (Figure 1). They were authenticated by the National Herbarium, Peradeniya, Sri Lanka. Upon thorough cleaning and drying, fruit skin was peeled, air-dried for 48 hours, and then was pulverised.

Figure 1: A*: Citrus crenatifolia*, B: *Citrus reticulata* trees

Aqueous Extraction

Sterile distilled water was added in a 1:1 ratio (g/mL) to 250 g of ground peel of both samples separately and was then refluxed for 30 minutes at 40°C. Filtered extractions were obtained into sterile labelled bottles using a Whatman No. 1 filter and stored at 4°C for further use of research.

Test Microorganisms for the Antimicrobial Assay

A total of six food-related bacteria were provided by the microbiology laboratory, CINEC Campus, Malabe, Sri Lanka. Stock cultures of microorganisms were prepared using culti loops from ATCC Culture collection; four Gram-negative bacteria i.e., *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Proteus mirabilis* (ATCC 35659) and *Pseudomonas aeruginosa* (ATCC 15442) and two Gram positive bacteria; *Staphylococcus aureus* (ATCC 23235) and *Enterococcus faecalis* (ATCC 29212). Subcultures of microbes were maintained from the stock cultures using four-way streaking.

Preparation of Pathogenic Test Strains Using 0.5 McFarland Standard

A solution of $BaCl_2 \cdot 2H_2O$ (1.175% w/v) was prepared by dissolving 1.175 g of anhydrous barium chloride in 100 mL of distilled water. A Sulfuric (1%) solution was prepared by mixing 1 mL of concentrated H_2SO_4 in 99 mL of distilled water to prepare a 0.5 McFarland solution. Then, barium chloride (0.5 mL) solution was added to a solution of sulfuric acid (99.5 mL) with constant stirring. After that, the

McFarland standard was thoroughly mixed using a magnetic stirrer to ensure homogenisation.

Screening for Antibacterial Activity

Antimicrobial activity was screened using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. Antimicrobial activity of *C. crenatifolia* and *C. reticulata* peels was assessed for *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*. The prepared pathogenic test strain was spread on Petri plates with Mueller-Hinton agar. A sterile glass spreader was used for this. Using a sterile cork borer (8 mm diameter), two wells were made in each Petri plate. This was done for the negative control and the extract conditions. Distilled water was loaded into the negative control well without overflowing, and the extract was loaded into the other well, again without overflowing. For each extract and each microbe, three replica plates were run. An Antibiotic Sensitivity Test (ABST) was performed previously with antibiotic disk panels to identify the sensitive antibiotics for each test microbe (Figure 2). Accordingly, the most suitable sensitive antibiotic for each test microbe was used. All plates were incubated for 24 hours at 37°C. After incubation, the diameter of the inhibition zones was carefully measured.

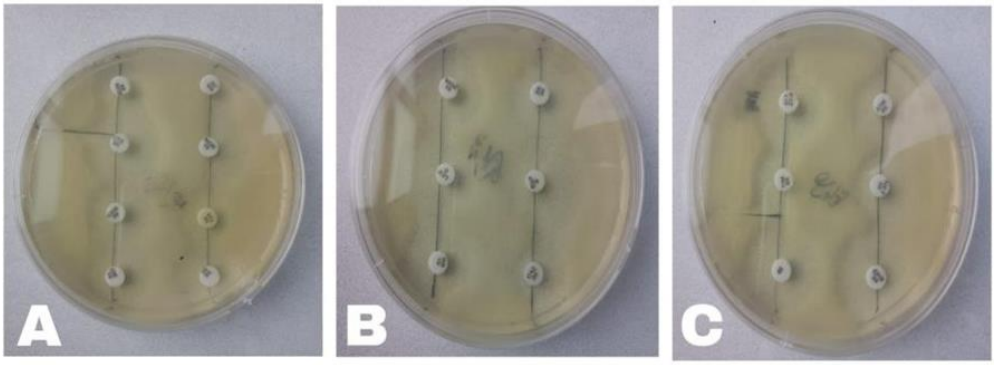

Figure 2: Antibiotic sensitivity Test (ABST), A: *Proteus mirabilis*, B: *Enterococcus faecalis*, C: *Staphylococcus aureus*

Phytochemical Assays

Determination of Flavonoids (Magnesium–HCl Reduction Test)

A few fragments of magnesium ribbon and 2 drops (0.1 mL) of concentrated HCl were added dropwise to each of the two sterile tubes

containing the plant peel extract (2 mL). A pink, red, or orange coloration indicates the presence of flavonoids (Aththanayaka et al., 2023).

Determination of Alkaloids (Mayer's Test)

Plant extract (1 mL) was added to two sterile tubes and dissolved in distilled water (1 mL). Two drops (0.1 mL) of Mayer's reagent were added to each tube. A cream or yellowish precipitate indicates the presence of alkaloids.

Determination of Steroids (Liebermann–Burchard Test)

Each plant extract (2 mL) was added to two sterile test tubes. Chloroform (2 mL) and acetic anhydride (2 mL) were added to each tube and mixed well. Concentrated sulfuric acid (2 mL) was then added slowly down the side of the test tube to form a layer beneath the plant extract–acetic anhydride mixture. The interface between the two layers was observed for a colour change. The appearance of a green, blue, or violet coloration within 2 minutes indicates the presence of steroids in the plant extract (Aththanayaka et al., 2023).

Determination of Saponin (Froth Test/Foam Test)

For the foam test, 5 mL of plant extracts were added to two sterile tubes and vigorously shaken for 30 seconds. Formation of a layer of foam that persists for 30 minutes indicates the presence of saponins.

Determination of Phenol (Ferric Chloride Test)

Plant extracts (2 mL) were added to two sterile tubes. Three drops (0.15 mL) of neutral ferric chloride (5%) solution were added to each test tube and mixed gently by swirling. The formation of a green, blue, black, purple, or brown colouration indicates the presence of phenolic compounds (Aththanayaka et al., 2023).

Determination of Tannins (Ferric Chloride Test)

Plant extracts (1 mL) were added to two sterile test tubes, and 1 mL of ferric chloride (5%) was added to the same tubes. The presence of tannins was indicated by the formation of a greenish-black colour.

Data Analysis

Data was analysed using SPSS version 25. An independent t-test was performed to test whether there is a significant difference between the

antibacterial activity of *C. crenatifolia* peel and *C. reticulata* peel against the selected pathogenic bacteria; *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. One way ANOVA was carried out to test whether there is a significant difference in antimicrobial activity among the six microbes for the peel of *C. reticulata* extract. Two-way ANOVA was carried out to test the significant effects for both the independent variable, extract and bacteria as well as their interaction.

Results

Comparison of the Activity of *C. crenatifolia* and *C. reticulata* Peel Extract against Tested Pathogenic Bacteria

Upon incubation of the two extractions, peel extract of both *C. crenatifolia* and *C. reticulata* in Mueller Hinton Agar plates against selected pathogenic bacteria, inhibition zones were clearly visible around the wells (Figure 3 and Figure 4). This revealed their antibacterial activity against test pathogenic bacteria. Both extracts demonstrated enhanced antibacterial activity against the selected pathogenic bacteria *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Both extracts showed activity against all the tested pathogenic bacteria and ranged between 20.66 mm and 31.16 mm.

When considering *C. reticulata*, the zone of inhibition ranged between 20.66 mm and 24.66 mm. The highest inhibition zone (Mean = 24.66 mm) was observed for *Proteus mirabilis*, while the lowest inhibition zone (Mean = 20.66 mm) was observed for *Klebsiella pneumoniae*. *C.*

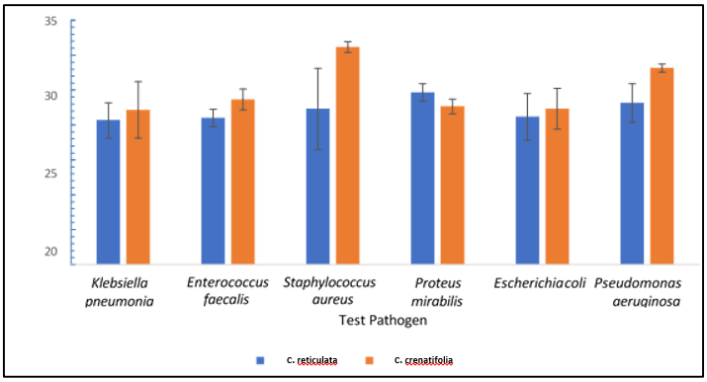

Figure 3: Mean inhibition zone diameters ± SD of *C. crenatifolia* and *C. reticulata* peel extracts against *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*

crenatifolia's zone of inhibition ranged between 22.16 mm and 31.16 mm. It exhibited the highest antibacterial activity against *Staphylococcus aureus* with a notable mean of 31.16 mm inhibition zone and showed its lowest efficacy against *Klebsiella pneumoniae* with a mean diameter of 22.16 mm.

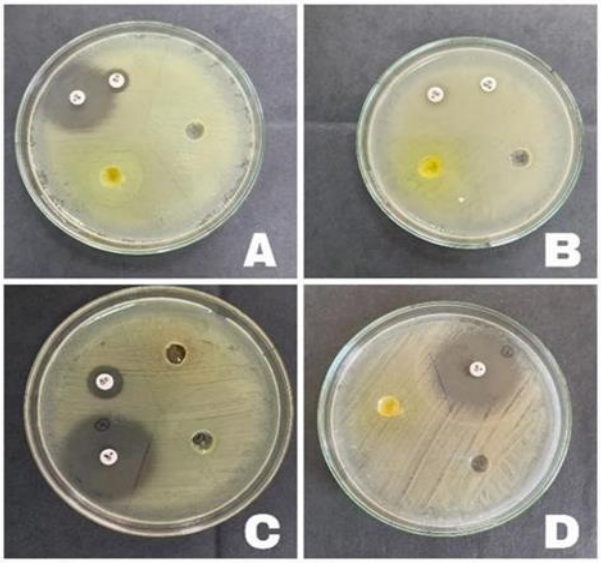

Figure 4: Inhibition zones observed in Mueller-Hinton agar plates for microbes (A: *Pseudomonas aeruginosa* against *C. crenatifolia*, B: *Proteus mirabilis* against *C. crenatifolia*, C: *Pseudomonas aeruginosa* against *C. reticulata*, D: *E. coli* against *C. reticulata*).

All tests were conducted in three replicates, and the values were reported as the mean. According to the statistical analysis of data obtained during the independent sample t-test, it was observed that there was no significant difference between the antibacterial activity of *C. crenatifolia* peel and *C. reticulata* peel against the selected pathogenic bacteria (*Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*) ($p = 0.115 > 0.05$).

Additionally, there was no statistically significant difference in antimicrobial activity among the six microbes according to the one-way ANOVA test for the peel (p = 0.271) of *C. reticulata* extract. However, there was a statistically significant difference in the antimicrobial activity of *C. crenatifolia* peel extract (One-Way ANOVA: p = 0.021). This suggests that different tested microorganisms responded differently to the *C. crenatifolia* peel extract's ability to limit microbial development.

Tukey's Post-Hoc analysis (Table 1) conducted for *C. crenatifolia* extract revealed that the mean diameter of inhibition zones of *Staphylococcus aureus* with *E. coli* (p = 0.049) was significantly different. These results offer insightful information about the possible differences in the effects of *Citrus crenatifolia* extract on different strains of bacteria, information that may help with the targeted usage of the extract in particular antimicrobial formulations or applications.

Table 1: *Tukey's Post Hoc Analysis*

Test Microbes	P Value from Post-Hoc Analysis
S. aureus & P. aeruginosa	0.853
S. aureus & P. mirabilis	0.060
S. aureus & E. faecalis	0.113
S. aureus & E. coli	0.049
S. aureus & K. pneumoniae	0.853
P. aeruginosa & P. mirabilis	0.348
P. aeruginosa & E. faecalis	0.546
P. aeruginosa & E. coli	0.294
P. aeruginosa & K. pneumoniae	0.269
P. mirabilis & E. faecalis	0.999
P. mirabilis & E. coli	1.000
P. mirabilis & K. pneumoniae	1.000
E. faecalis & E. coli	0.995
E. faecalis & K. pneumoniae	0.991
E. coli & K. pneumoniae	1.000

The results obtained through a two-way ANOVA (Table 2) revealed significant effects for both the independent variable, extract and bacteria, as well as their interaction. Individual effects of extract (p =

0.005) and bacteria (p = 0.011) were observed to be significant. This suggests that there are statistically significant differences in the dependent variable among different extracts and bacterial types that were being analyzed. Further, interaction between extract and bacteria (p = 0.044) was found to be significant as well. This denotes the varying nature of the relationship between the extract and the dependent variables, depending on the specific bacteria being tested.

Table 2: *Two-way ANOVA*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	320.576[a]	11	29.143	3.847	.003
Intercept	20045.840	1	20045.840	20045.840	.000
Sample	73.674	1	73.674	9.724	.005
Bacteria	143.951	5	28.790	3.800	.011
Sample * Bacteria	102.951	5	20.590	2.718	.044
Error	181.833	24	7.576		

Phytochemical Analysis of Peel Extracts

Flavonoids, alkaloids, steroids, phenols, and tannins were present in both peel extracts of the plants. However, saponin was lacking in both peel extracts (Table 3 and Figure 5).

Table 3: *Phytochemical Screening Characteristics*

Chemical Constituent	*C.crenatifolia* Peel Extract	*C. reticulata* Peel Extract
Flavonoids	+	+
Alkaloids	+	+
Steroids	+	+
Saponin	-	-
Phenol	+	+
Tannis	+	+

+ = presence of target analyte - = no detectable analyte

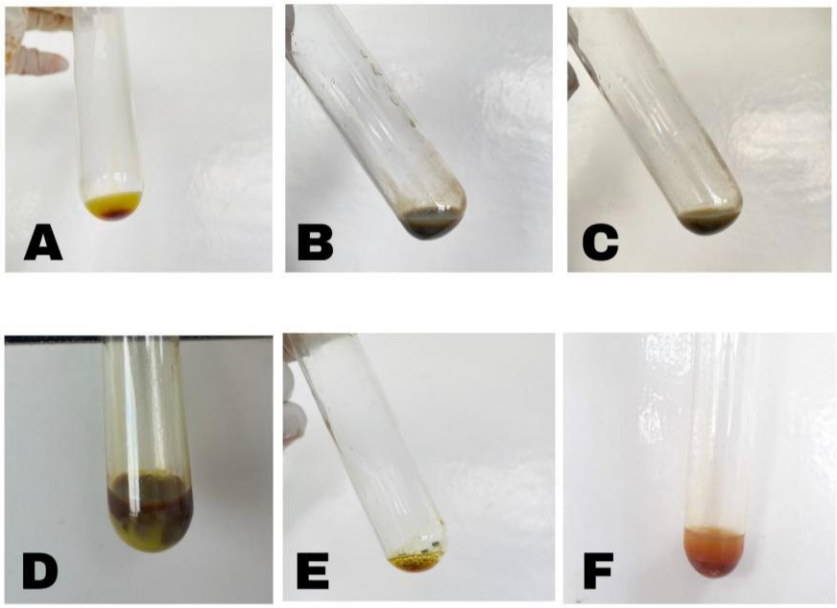

Figure 5: A: Steroid (*C. crenatifolia*), B: Tannins (*C. reticulata*), C: Phenol (*C. reticulata*), D: Alkaloid (*C. crenatifolia*), F: Flavanoids (*C. crenatifolia*), F: Alkaloid (*C. reticulata*)

Discussion

The emergence of multidrug-resistant bacteria has prompted scientists to research novel compounds with potential antibacterial properties. According to various studies conducted in several

countries, it has been confirmed that extractions prepared from various parts of medicinal plants may be beneficial in the production of antibiotics (Abouzeed et al., 2013). There is limited data published on the antibacterial properties of peels of *Citrus* spp. Further, no data have been published regarding antibacterial properties of peels of *Citrus crenatifolia* and *Citrus reticulata*.

In the present study, the extraction method employed to extract active compounds was the reflux method in an aqueous medium. When determining the efficacy of the active compounds in *C. crenatifolia* and *C. reticulata* peels, the extraction method plays a crucial role. Water is a polar solvent, and it is effective in extracting phytochemicals from plants such as flavonoids, phenolic compounds, and organic acids (Adeeyo et al., 2023). The solubility of different chemicals in the plant matrix is affected by the solvent's polarity, which makes it easier for those compounds to be released into the extraction medium. As a result, a wide range of bioactive chemicals may be extracted from peels of *C. crenatifolia* and *C. reticulata* using water as the solvent in our extraction procedure.

Further, the profile and amount of extracted chemicals can be greatly impacted by solvent selection. Hexane and ethyl acetate are examples of non-polar solvents that can be used to extract lipophilic molecules only, leaving behind polar components (Rout et al., 2026). On the other hand, polar and non-polar components can be extracted from a wider variety of substances using highly polar solvents such as methanol or ethanol (Gomez-Garcia et al., 2017). Using water as the extraction solvent in this study has allowed the extraction of a wide range of active ingredients. By keeping a steady temperature and enabling the continuous extraction of chemicals from the plant material, the reflux approach significantly improves extraction efficiency. The extended heating under reflux conditions guarantees complete extraction of heat-stable chemicals (Rout et al., 2026).

The findings of this study demonstrated that aqueous extractions from peels of *C. crenatifolia* and *C. reticulata* showed positive antibacterial properties against *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* when assayed in Mueller-Hinton Agar. Diameters of zones of inhibition ranged between 20.66 mm and 31.16 mm. According to the criteria adopted by the Medicinal and Aromatic

Plant Research Institute, National Centre for Research, Khartoum, Sudan, a sample is considered inactive when the inhibition zone is < 9 mm, partially active at 9–12 mm, active at 13–18 mm, and very active at > 18 mm. Both *C. crenatifolia* and *C. reticulata* peel extracts demonstrated a very active state against all the tested bacteria.

The preliminary phytochemical investigation revealed the presence of flavonoids, alkaloids, steroids, phenols, and tannins in both peel extracts, while saponins were absent. These bioactive compounds are known to exert antimicrobial effects through various mechanisms. Flavonoids disrupt microbial cell membranes, inhibit enzymes crucial for microbial growth, and impede microbial DNA replication. Alkaloids exhibit antimicrobial activity by disrupting cellular processes in bacteria (Cowan, 1999). Steroidal compounds can disrupt microbial cell membranes (Rahman, 2000). Phenolic compounds precipitate microbial proteins and interfere with microbial metabolism (Nazzaro et al., 2013). Tannins inhibit microbial enzymes and disrupt cell membranes (Cushnie & Lamb, 2011). These findings confirm that *C. crenatifolia* and *C. reticulata* are rich sources of phytochemicals with significant antibacterial potential.

Being economically friendly, it becomes more practical and safer for the environment to use such botanical compounds against microbes. This approach would be a beneficial solution through the rational use of finances and human resources to achieve better productivity and fruitfulness. Hence, this warrants microbial control entities to rethink “biological wealth for economic prosperity” with environmentally friendly control approaches for medically important microbes.

Conclusions and Recommendations

The study examined the antibacterial properties of aqueous extracts from the peels of *C. crenatifolia* and *C. reticulata* against various pathogenic bacteria, showing strong efficacy against both Gram-positive and Gram-negative organisms. The presence of phytochemicals such as flavonoids, alkaloids, steroids, phenols, and tannins in the extracts highlights their medicinal value. The statistical analyses support the significant antibacterial activity, particularly of *C. crenatifolia* against certain bacterial species.

The study underscores the potential of *C. crenatifolia* and *C. reticulata* peel extracts as natural sources for developing new antibacterial agents, which are crucial in combating antibiotic-resistant bacteria. Future research should focus on isolating and characterising the active compounds responsible for antibacterial effects and exploring their potential applications in food preservation, safety, and agriculture.

Data Availability

Data sharing is NA. The datasets supporting the conclusions of this article are included in the article.

Conflicts of Interest

There are no conflicts of interest.

Acknowledgments

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