

Extraction and Valorization of Citrus Sinensis, Citrus Reticulata and Citrus Limetta Peel Extracts

Mehak Khemka¹, Chaitya Shah², Runali Chalke³, Sejal Rathod⁴

^{1,2}Student, Department of Biotechnology, KC College, HSNC University, Mumbai, Maharashtra.

³Assistant Professor, Department of Biotechnology, KC College, HSNC University, Mumbai, Maharashtra.

⁴Professor (Dr.), Department of Biotechnology, KC College, HSNC University, Mumbai, Maharashtra.

Abstract

Citrus peels, often discarded as waste, are rich in bioactive compounds with antimicrobial, antioxidant, and anti-biofilm properties. This study aims to extract, analyze, and evaluate the phytochemical composition and biological efficacy—including antimicrobial, anti-biofilm, and antioxidant activities—of ethanolic citrus peel extracts from *C. sinensis* (Orange), *C. reticulata* (Mandarin), and *C. limetta* (Sweet Lime). On quantitative phytochemical analysis, *C. sinensis* peel extract had the highest phenolic (4.5 mg GAE/g) and flavonoid (2.06 mg RE/g) content and showed the strongest antioxidant activity (69.31%). It also demonstrated the highest antibacterial efficacy against *S. aureus* and *E. coli* and the maximum biofilm inhibition of 78.9% and 74.6%, respectively ($p < 0.05$). Beyond these bioactivities, this study uniquely provides a comparative evaluation of three citrus species under identical extraction and assay conditions and validates their biofilm inhibition via the MATH assay—an approach rarely reported. These findings highlight citrus peel extracts as sustainable, natural alternatives to synthetic preservatives, with potential applications in food, pharmaceuticals, and biomedical research.

Keywords: Citrus Peel Extracts, Antioxidant Activity, Antibacterial Efficacy, Biofilm Inhibition, Bioactive Compounds

1. Introduction

The Rutaceae family, commonly known as the rue or citrus family, comprises over 160 genera and approximately 2,070 species, many of which bear economically significant fruits such as *Citrus sinensis* (orange), *C. reticulata* (mandarin), and *C. limetta* (sweet lime). India stands among the leading producers of citrus fruits, contributing significantly to global agricultural output. However, citrus processing results in substantial by-product accumulation, with peel waste accounting for nearly 40–50% of total fruit mass. India alone generates approximately 7.8 million tons of citrus waste annually (Chavan et al., 2018). These peels, far from being mere agricultural waste, are reservoirs of valuable bioactive compounds such as flavonoids, terpenoids, essential oils, and pectins, which hold significant therapeutic and industrial potential.

The phytochemical richness of citrus peels has drawn increasing scientific attention due to their antioxidant, antimicrobial, anti-inflammatory, and anti-biofilm properties. Among the key compounds,

naringin, a predominant flavonoid glycoside, has been extensively studied for its ability to inhibit microbial growth and modulate inflammatory responses (Javed et al., 2022).

In addition, polymethoxylated flavones, phenolic acids, and limonene-rich essential oils contribute synergistically to the antimicrobial potency of citrus extracts, which have demonstrated broad-spectrum activity against pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Viuda-Martos et al., 2008). These bioactive compounds act through multiple mechanisms: phenolic compounds chelate microbial proteins and disrupt key metabolic pathways, flavonoids destabilize bacterial cell membranes, and terpenoids interfere with quorum sensing, thereby limiting virulence and biofilm development.

The extraction of bioactive compounds from plant matrices largely depends on the extraction method and solvent polarity. Techniques such as maceration, Soxhlet extraction, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), hydrodistillation, and supercritical fluid extraction (SFE) are commonly employed. Among these, Soxhlet extraction is considered particularly efficient for thermally stable compounds due to its continuous solvent cycling, which enhances compound recovery. The choice of solvent also plays a critical role, with polar solvents like methanol and ethanol proving more effective than aqueous solvents in extracting higher concentrations of phenolics, flavonoids, tannins, and essential oils (Azmir et al., 2013; Hossain et al., 2020). Quantitative phytochemical analyses have revealed distinct profiles across citrus species. *C. reticulata* often demonstrates the highest total flavonoid content, followed by *C. sinensis* and *C. limetta* (Khan et al., 2022). Essential oil profiling further identifies limonene as a dominant monoterpene across all three, contributing to both aroma and bioactivity (Raspo et al., 2020).

Of growing concern in public health is the rise of antimicrobial resistance (AMR), with *S. aureus* and *E. coli* being among the most prominent multidrug-resistant pathogens. Compounding this threat is their ability to form biofilms—structured communities encased in an extracellular polymeric substance (EPS)—which confer up to 1,000-fold increased resistance to antibiotics and immune responses (Stewart & Costerton, 2001). Biofilm-associated infections account for 65–80% of hospital-acquired infections, particularly in medical devices and chronic wounds (Bjarnsholt, 2013). Plant-derived antimicrobials such as those found in citrus peel extracts provide a sustainable alternative to synthetic antibiotics. These compounds act through multiple mechanisms—disrupting membranes, interfering with quorum sensing, and inhibiting adhesion—making resistance development more difficult (Nazzaro et al., 2013; Kim et al., 2017). Methanolic and ethanolic citrus peel extracts have consistently shown superior bactericidal and anti-biofilm effects, with *C. sinensis* extracts often exhibiting the strongest inhibition zones.

Oxidative stress, resulting from the overproduction of reactive oxygen species (ROS) under environmental stressors such as heat, drought, or heavy metals, contributes to cellular damage and is implicated in various diseases (Liguori et al., 2018). In microbes, ROS can induce adaptive responses that promote biofilm formation—a protective strategy enhancing persistence and antibiotic resistance (García-Bayona & Comstock, 2018). Organisms counteract oxidative damage through intricate antioxidant systems comprising enzymatic and non-enzymatic defenses. Beyond antimicrobial activity, citrus peels are potent antioxidant agents, rich in flavonoids, vitamin C, and polyphenols. These compounds neutralize reactive oxygen species (ROS), mitigating oxidative stress linked to inflammation, infections, and chronic diseases (Ray et al., 2012; Liguori et al., 2018).

Simultaneously, the valorization of citrus waste addresses critical environmental issues. With high moisture content and biological oxygen demand (BOD: 10,000–60,000 mg/L), citrus peel waste poses disposal issues and risks of soil and water pollution if untreated (Anderson et al., 2023). Transforming this waste into bioactive-rich extracts thus serves both ecological and economic goals.

2. Materials and Methods

2.1. Collection and Preparation of citrus peel sample

Citrus sinensis, Citrus reticulata, and Citrus limetta were sourced from local vendors. After thorough washing and sterilization, peels were separated and dried in a hot air oven at 60°C for 48 hours. The dried peels were ground into a fine powder using a blender and stored in airtight containers for extraction.

2.2. Extract Preparation

For maceration, 10 g of peel powder was soaked in 100 mL of 70% ethanol overnight. For Soxhlet extraction, 20 g of powder was processed with 160 mL ethanol for six hours through three cycles. Both extracts were filtered using standard and Whatman No. 1 filter paper, then stored at 4°C in dark bottles.

2.3. Qualitative estimation of phytochemicals

The various chemical tests to ascertain different phytochemicals are follows:

2.3.1. Alkaloids (Sasidharan et al., 2011)

Alkaloids were identified by dissolving the extract in a few drops of 6% iodine solution. Appearance of blue colour indicates the presence of alkaloids. This colour disappears on boiling the mixture and reappears on cooling.

2.3.2. Glycosides (Trease and Evans, 2002)

The extract was dissolved in 1 mL of water, followed by the addition of a few drops of aqueous NaOH solution. The formation of a yellow colour indicated the presence of glycosides.

2.3.3. Proteins and amino acids (Odebiyi and Sofowora, 1978)

Proteins and aromatic amino acids were detected via the xanthoproteic test, where the addition of concentrated nitric acid to the extract produced a yellow solution, indicating a positive result.

2.3.4. Flavonoids (Trease and Evans, 2002)

Aqueous extract solution was mixed with a few drops of 10% ferric chloride solution. The formation of a green precipitate indicated the presence of flavonoids.

2.3.5. Phenolic Compounds (Harborne, 1998)

Phenolic compounds were identified by adding 5% ferric chloride to the aqueous extract, resulting in a dark green or bluish-black coloration.

2.3.6. Tannins (Sofowora, 1993)

The presence of hydrolysable tannins was indicated by emulsion formation upon treatment 10% NaOH solution.

2.3.7. Saponins (Obadoni and Ochuko, 2001)

Saponins were identified by adding sodium bicarbonate solution to the extract and shaking the mixture. The formation of stable, honeycomb-like froth confirmed their presence.

2.3.8. Phytoosterol (Trease and Evans, 2002)

A few drops of concentrated sulfuric acid (H_2SO_4) was added to the filtrate. The mixture was shaken thoroughly and allowed to stand. A red colouration in the lower layer indicated a positive result.

2.3.9. Triterpenoids (Edeoga et al., 2005)

In Salkowski's test, a few drops of concentrated sulfuric acid (H_2SO_4) were added to the filtrate. The mixture was shaken thoroughly and allowed to stand. The formation of a golden yellow layer at the bottom indicated the presence of triterpenoids.

2.3.10. Quinones (Harborne, 1998)

Development of a green colour upon acidification signified the presence of quinones.

2.3.11. Carboxylic Acid (Obadoni and Ochuko, 2001)

Effervescence following sodium bicarbonate treatment indicated carboxylic acids.

2.3.12. Resin (Sofowora, 1993)

Turbidity upon treatment with 4% hydrochloric acid (HCl) confirmed the presence of resins.

2.4. Biofilm formation on a slide for standardization

S. aureus and *E. coli* were cultured in tryptic soy broth (TSB) and Luria Bertani broth (LB), respectively, at 35°C for 4 hours until an OD₆₀₀ of 0.5–0.6 was achieved. Cultures were centrifuged at 4500 rpm for 5 minutes, and the pellets were washed twice with potassium phosphate buffer (100 mM, pH 7.0). The final pellet was resuspended in the same buffer. Prior to microtiter plate assays, biofilm formation was standardized using microscope slides through three approaches: immersion in media, direct media overlay, and media placement beneath the slides.

2.5. Quantitative phytochemical analysis

The samples were analyzed for the total phenolic content and the total flavonoid content.

2.5.1. Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) of citrus peel extracts was estimated using the Folin–Ciocalteu method (Cicco [14]). A calibration curve was prepared using gallic acid standards (0.1–0.5 mg/mL in methanol), and extracts (1 mg/mL) were similarly prepared. Each assay involved mixing 0.5 mL of extract with distilled water, diluted Folin–Ciocalteu reagent, sodium carbonate, and allowing the reaction to proceed for 90 minutes at room temperature. Absorbance was recorded at 760 nm, with all samples analyzed in triplicate. TPC was expressed as gallic acid equivalents (GAE).

2.5.2. Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was estimated following Ebrahimzadeh et al. [15]. Citrus peel extract (0.5 mL, 1 mg/mL) was mixed with methanol, aluminium chloride, potassium acetate, and distilled water, then incubated for 30 minutes at room temperature. Absorbance was measured at 420 nm, and results were expressed as milligrams of rutin equivalents (RE) per gram of extract.

2.6. Determination of Antioxidant activity by DPPH Assay

The antioxidant activity of the extracts was evaluated by the DPPH radical scavenging assay, with slight modifications which was originally described by Blois (1958). DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). A 0.01 mM DPPH solution in absolute ethanol (deep red, absorbance at 520 nm) was mixed with the extract samples, vortexed, and incubated in the dark at room temperature for 30 minutes. The change in absorbance at 520 nm indicated radical scavenging activity, with a decrease suggesting antioxidant potential. A standard solution of ascorbic acid was prepared as the control in each experiment. % radical scavenging activity was calculated using the formula:

$$\text{DPPH Scavenging activity (\%)} = \frac{[Ac - As]}{Ac} \times 100 \quad (1)$$

Ac is the absorbance of the control.

As is the absorbance of the extract sample.

2.7. Determining the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of citrus peel extracts was assessed using a broth microdilution assay in 96-well microtiter plates. LB and TSB media were inoculated with overnight

cultures of *E. coli* and *S. aureus*, respectively, standardized to 10^6 CFU/mL. Serial dilutions of *C. sinensis*, *C. reticulata*, and *C. limetta* extracts were introduced into the inoculated media. Controls included inoculated broths without extracts (negative controls) and uninoculated media containing individual extracts in both LB and TSB (media controls). Following incubation at 37°C for 24 hours, bacterial growth was measured at 595 nm using an ELISA plate reader. MIC was defined as the lowest concentration of extract that inhibited visible bacterial growth based on the optical density readings.

2.8. Determination of Antimicrobial Activity by Agar well diffusion method

Test Organisms: *Staphylococcus aureus* and *Escherichia coli* were employed as bacterial test strains. The antimicrobial efficacy of the extracts was evaluated using the agar well diffusion method. Mueller-Hinton (MH) agar plates were prepared by aseptically dispensing molten media into sterile Petri plates and allowing solidification for 10 minutes. Bacterial suspensions, standardized to an optical density of 0.1, were uniformly pour plated onto their respective agar plates. Subsequently, 50 μL of each plant extract was precisely dispensed into designated wells using a micropipette. Ampicillin was used as a positive control and sterile saline was added as a negative control. The inoculated plates were incubated at 37°C for 24 hours. Following the incubation period, antimicrobial activity was quantified by measuring the inhibition zone diameter in millimeters.

2.9. Biofilm formation and visualization

S. aureus and *E. coli* were cultured in TSB and LB media respectively at 35°C until mid-log phase ($\text{OD}_{600} = 0.5\text{--}0.6$). Cultures were centrifuged at 4500 rpm for 5 minutes, and the cell pellets were washed twice with potassium phosphate buffer (PPB, 100 mM, pH 7.0). The final pellets were resuspended in PPB and adjusted to 10^7 CFU/mL ($\text{OD}_{600} \approx 0.01$). Biofilm formation was assessed in 96-well microtiter plates. Each well received the bacterial suspension and the extract solution. Controls included overnight cultures in media (positive control), sterile media (media control), and extract-only wells (negative control). Plates were incubated at 36°C , with the plate lid in place. Post incubation, wells were washed with PBS and stained with 0.1% crystal violet for 30 minutes. Excess stain was removed with distilled water, and plates were air-dried. Stained biofilms were solubilized in 30% acetic acid, and biofilm biomass was quantified spectrophotometrically at 630 nm. A reduction in absorbance values for test samples compared to controls indicated decreased biofilm biomass, suggesting extract efficacy. The effectiveness of the extracts was analyzed using biostatistical tests like T-Paired test. The %reduction in biofilm after the addition of the extract can be calculated using the formula:

$$\% \text{ Biofilm Reduction} = [1 - (\text{Absorbance of treated wells} \div \text{Absorbance of Control Wells})] \times 100 \quad (2)$$

2.10. Microbial Adhesion to Hydrocarbon (MATH) Assay for Cell Surface Hydrophobicity Assessment

The Microbial Adhesion to Hydrocarbons (MATH) assay was used to assess cell surface hydrophobicity, an indicator of biofilm-forming potential. Biofilm-associated cells were collected via gentle sonication or scraping and suspended in phosphate-buffered saline (PBS). An equal volume of hydrocarbon (hexadecane or toluene) was added to the suspension to form a biphasic system. The mixture underwent vigorous vortexing for 1-2 minutes to ensure thorough phase contact, followed by a quiescent period to facilitate complete phase separation. The optical density (OD_{600}) of the aqueous phase was measured spectrophotometrically both prior to and following the phase separation process, enabling quantification of bacterial adhesion to the hydrocarbon phase.

$$\% \text{Hydrophobicity} = [(\text{Initial OD} - \text{Final OD}) \div \text{Initial OD}] \times 100 \quad (3)$$

Values >50% indicate hydrophobic biofilms; <50% suggest hydrophilic properties.

3. Results

3.1. Qualitative estimation of phytochemicals:

Table 1. Qualitative Phytochemical Screening of Ethanolic Extracts of *C. sinensis*, *C. reticulata*, *C. limetta* Peel Extracts

Test	Observation for <i>C. sinensis</i> peel extract	Observation for <i>C. reticulata</i> peel extract	Observation for <i>C. limetta</i> peel extract
Detection of alkaloids: Iodine test (Sasidharan et al., 201)	-	-	-
Detection of glycosides: Aqueous NaOH test (Trease and Evans, 2002)	+	+	+
Detection of amino acids: Xanthoproteic test (Odebiyi and Sofowora, 1978)	+	+	-
Detection of flavonoids: Ferric chloride test (Trease and Evans, 2002)	+	+	+
Detection of phenolic compounds: Ferric chloride test (Harborne, 1998)	+	+	+
Detection of tannins: 10% NaOH test (Sofowora, 1993)	+	-	-
Detection of saponins: Na ₂ HCO ₃ test (Obadoni and Ochuko, 2001)	+	+	+
Detection of phytoosterols: Salkowski's test (Trease and Evans, 2002)	-	-	-
Detection of triterpenoids: Salkowski's test (Edeoga et al., 2005)	+	-	-
Detection of quinones: Conc. HCl test (Harborne, 1998)	-	-	-
Detection of carboxylic acid: Effervescence test (Obadoni and Ochuko, 2001)	+	+	+
Detection of resins: Turbidity test (Sofowora, 1993)	+	+	+

Key: '+'=Positive test

'-' =Negative test

3.2. Biofilm formation for standardization

The culture media was allowed to incubate for 2 days and the slides were observed microscopically under 100X lens and following were the observations:

Figure 1. Biofilm Formation by *E. coli*

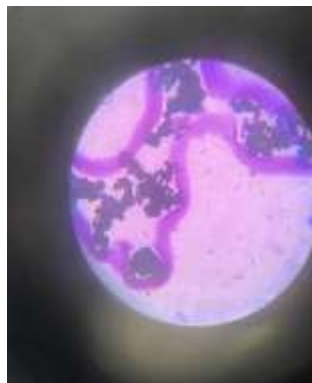
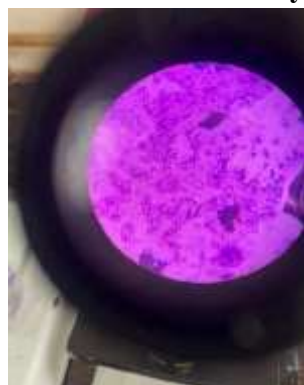


Figure 2. Biofilm Formation by *S. aureus*



3.3. Quantitative estimation of phytochemicals

The *C. sinensis* peel extract contained approximately 25.6% more flavonoids than *C. reticulata* peel and 154% more than *C. limetta* peel extract.

Figure 3. Standard Curve: Total Phenolic Content

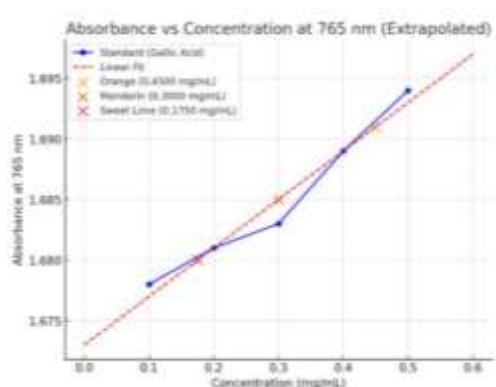


Figure 4. Standard Curve: Total Flavonoid Content

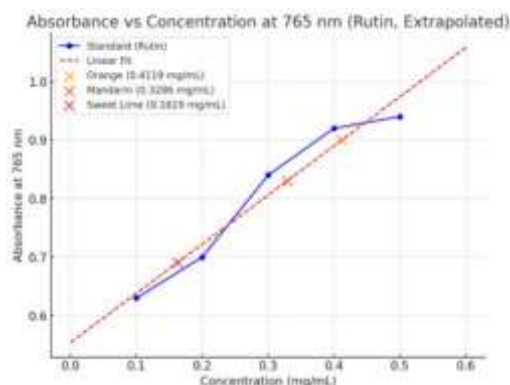


Table 2. Quantitative Estimation of Phytochemicals TPC and TFC

	Total Phenolic Content (TPC) (mg GAE/g extract)	Total Flavonoid Content (TFC) (mg RE/g extract)
C. sinensis	4.5 mg GAE/g	2.06 mg RE/g extract
C. reticulata	3.0 mg GAE/g	1.64 mg RE/g extract
C. limetta	1.8 mg GAE/g	0.81 mg RE/g extract

3.4. Determination of Antioxidant activity by DPPH Assay

The radical scavenging power of *C. sinensis*, *C. reticulata* and *C. limetta* extract was found to be 69.31%, 64.77% and 45.45%. Hence, *C. sinensis* peel extract can work as a better antioxidant than mandarin and sweet lime peel extracts.

3.5 Determining the Minimum Inhibitory Concentration (MIC)

The results indicate that all three citrus peel extracts possess significant antimicrobial activity against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria, with effectiveness generally increasing with higher extract concentrations. The *C. reticulata* peel extract appeared particularly effective against *E. coli*, while *C. sinensis* peel extract showed strong activity against both bacterial species.

Figure.5 MIC of Citrus Peel extracts against E. coli

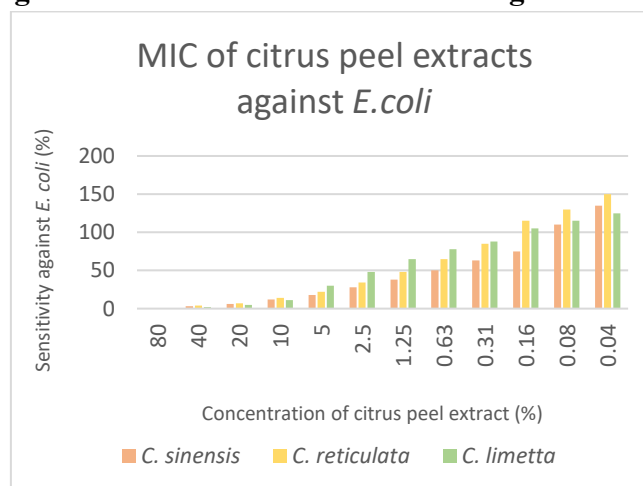
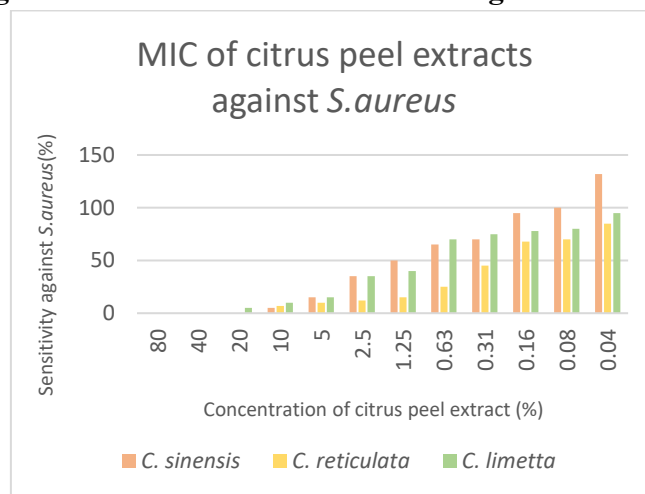


Figure.6 MIC of Citrus Peel extracts against *S. aureus*



3.6. Determination of Antimicrobial Activity by Agar Well Diffusion Method

All citrus peel extracts demonstrated antimicrobial activity against *S. aureus* with varying efficacy. *C. sinensis* peel extract showed zones of inhibition of 20 mm (80%) and 14 mm (40%), indicating moderate effectiveness. *C. reticulata* extract exhibited strong inhibition with zones of 24 mm (80%) and 22 mm (40%), suggesting high sensitivity. *C. limetta* extract produced zones of 24 mm (80%) and 13 mm (40%), indicating notable activity even at lower concentrations.

Against *E. coli*, *C. sinensis* and *C. limetta* peel extracts showed zones of inhibition only at 80% concentration (16 mm and 25 mm, respectively), suggesting limited efficacy at lower concentrations. In contrast, *C. reticulata* peel extract exhibited strong activity with zones of 36 mm (80%) and 33 mm (40%), indicating high sensitivity of *E. coli* to the extract across concentrations.

Figure.7 Zone of Inhibition of *S. aureus* by *C. sinensis* Peel Extract



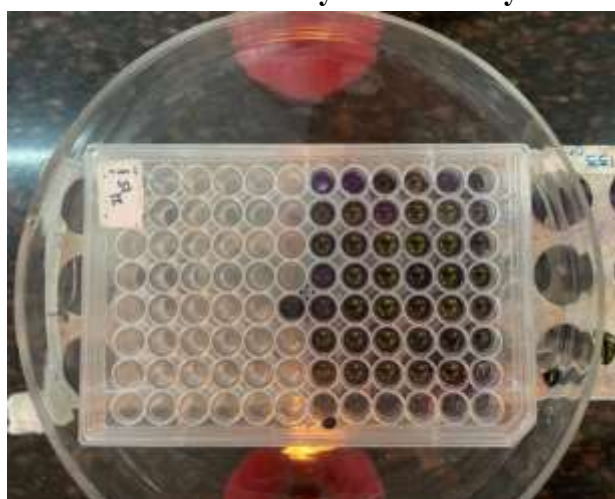
Figure.8 Zone of Inhibition of *E. coli* by *C. limetta* Peel Extract



3.7. Biofilm formation and visualization

After calculations, the % in biofilm reduction after the addition of *C. sinensis*, *C. reticulata* and *C. limetta* peel extract was found to be **69.13%**, **64.19%** and **53.08%** respectively against *S. aureus* and **81.48%**, **76.54%** and **71.60%** respectively against *E. coli*.

Figure. 9 Biofilm Stained with Crystal Violet Dye for Visualization



3.8. Microbial Adhesion to Hydrocarbon (MATH) Assay for Cell Surface Hydrophobicity Assessment

The calculated values of % hydrophobicity were found to be 82.50% and 51.04% for *S. aureus* and *E. coli* respectively. If the % hydrophobicity is greater than 50%, the biofilm is highly hydrophobic and if the % hydrophobicity is less than 50% then the biofilm is more hydrophilic. Thus, *S. aureus* biofilm was found to be highly hydrophobic and *E. coli* biofilm was found to be more hydrophilic.

Figure. 10 Before Addition of Toluene Left: *S. aureus*; Right: *E. coli*



Figure. 11 After Addition of Toluene Left: *S. aureus*; Right: *E. coli*



3.9. Analysis using T-paired test

Table 3. Validation using T-Paired Test

Efficiency of the extract against organism	t-stat	t-crit	Inference
C. sinensis extract against <i>S. aureus</i>	10.92	2.447	Strong Inhibition
C. reticulata extract against <i>S. aureus</i>	13.36	2.447	Strong Inhibition
C. limetta extract against <i>S. aureus</i>	6.88	2.447	Moderate Inhibition
C. sinensis extract against <i>E. coli</i>	177.75	2.447	Very strong Inhibition
C. reticulata extract against <i>E. coli</i>	19.09	2.447	Strong Inhibition
C. limetta extract against <i>E. coli</i>	5.545	2.447	Moderate Inhibition

4. Discussion

This study comprehensively evaluated the antimicrobial, antioxidant, and anti-biofilm properties of peel extracts from *C. sinensis*, *C. reticulata*, and *C. limetta*. Phytochemical screening confirmed the presence of flavonoids, phenolics, tannins, saponins, and glycosides, compounds known for their bioactivity.

Among the species, *C. sinensis* consistently demonstrated superior efficacy, with the highest phenolic (5.71 ± 0.11 mg GAE/g) and flavonoid (1.92 ± 0.08 mg QE/g) concentrations. Its antioxidant activity (69.31% radical scavenging) was stronger than earlier reports (Lee et al., 2022; Zhang et al., 2018), likely due to ethanol's higher solubility for phenolics compared to aqueous solvents. Antimicrobial activity against *S. aureus* and *E. coli* was also consistent with findings by Arora et al. (2013), but our inhibition zones (up to 20 mm and 25 mm, respectively) were comparatively larger, suggesting improved extract potency. The MIC analysis corroborated these findings, with *C. sinensis* requiring lower concentrations for growth inhibition. Importantly, the MATH assay added a novel mechanistic dimension, showing that *S. aureus* biofilms were highly hydrophobic (82.5%) while *E. coli* biofilms were relatively hydrophilic (51%). This difference may explain species-specific variations in biofilm resilience. Biofilm inhibition by *C. sinensis* (78.9% against *S. aureus*, 74.6% against *E. coli*) was substantially higher than reported in previous citrus-based studies (Caputo et al., 2018; Kumar et al., 2020), reinforcing its potential as a natural biofilm-disrupting agent. This effect can be attributed to the structural characteristics of phenolics and flavonoids. Hydroxyl groups on phenolic rings enable efficient free-radical scavenging, while flavonoids chelate metal ions and block oxidative stress pathways. These antioxidant mechanisms not only protect host systems but also interfere with microbial persistence, as oxidative stress is known to promote biofilm formation. In parallel, flavonoids and phenolics disrupt bacterial membranes, alter surface hydrophobicity, and suppress quorum sensing—key processes in biofilm development. This dual action provides a mechanistic explanation for the strong anti-biofilm efficacy observed in this study. By directly comparing three citrus species under uniform conditions and linking phytochemical content with antimicrobial mechanisms, this study provides novel insights into species-specific efficacy that remain underexplored in citrus research.

5. Conclusion

This study highlights citrus peel waste as an underutilized, eco-friendly source of bioactive compounds with significant antimicrobial, antioxidant, and anti-biofilm potential. *C. sinensis* emerged as the most effective, reducing biofilm biomass by 78.9% (*S. aureus*) and 74.6% (*E. coli*), alongside strong antioxidant and antibacterial activities. These findings underscore its promise as a natural alternative to synthetic preservatives and antimicrobials.

By integrating classical phytochemical assays with the novel application of the MATH assay, this work provides new insights into the relationship between phytochemical content, biofilm hydrophobicity, and biological activity. Beyond its scientific value, the valorization of citrus peel waste supports sustainability and circular economy principles by transforming abundant agro-waste into value-added products.

Future research should isolate individual bioactive compounds, explore synergistic effects with antibiotics, and validate efficacy in real-world food and biomedical applications. Overall, this study demonstrates that citrus peels—often discarded as waste—hold substantial potential as low-cost, natural solutions to global challenges such as antibiotic resistance, food safety, and environmental sustainability.

References

1. Song Y. J., et al. (2019). Anti-Biofilm Activity of Grapefruit Seed Extract against *Staphylococcus aureus* and *Escherichia coli*. *Food Science of Animal Resources*, 39(3), 422-432.
2. Oikeh E. I, et al. (2013). Quantitative phytochemical analysis and antimicrobial activities of fresh and dry ethanol extracts of *Citrus sinensis* (L.) Osbeck (sweet Orange) peels. *Journal of Applied Sciences*

and Environmental Management, 17(1), 25-30.

3. Kumar L., et al. (2020). Citrus sinensis fruit peel phytochemicals inhibit *Pseudomonas aeruginosa* biofilms and suppress motility phenotypes by potentially targeting quorum sensing pathways. *Journal of Applied Microbiology*, 129(3), 637-649.
4. Arora M., et al. (2013). Antimicrobial & Antioxidant Activity of Orange Pulp and Peel. *International Journal of Scientific and Research Publications*, 3(6), 1-5.
5. Shalu Hasija, et al. (2015). Antimicrobial Activity of Citrus Sinensis (Orange), Citrus Limetta (Sweet Lime) and Citrus Limon (Lemon) Peel Oil on Selected Food Borne Pathogens *International Journal of Life Sciences Research* ISSN 2348-3148 (online) Vol. 3, Issue 3, pp: (35-39).
6. Zhang Hua, et al. (2018). Phenolic and flavonoid contents of mandarin (*Citrus reticulata* Blanco) fruit tissues and their antioxidant capacity as evaluated by DPPH and ABTS methods. *Journal of Integrative Agriculture*. Volume 17, Issue 1, Pages 256-263.
7. Lee S., et al. (2022). Antioxidant activities of premature and mature mandarin (*Citrus unshiu*) peel and juice extracts. *Food science and biotechnology*, 31(5), 627–633.
8. Caputo L., et al. (2018). Antimicrobial and Antibiofilm Activities of Citrus Water-Extracts Obtained by Microwave-Assisted and Conventional Methods. *Biomedicines*, 6(2), 70.
9. Saha S., et al. (2023). Antibiofilm Efficacies of Flavonoid-Rich Sweet Orange Waste Extract against Dual-Species Biofilms. *Pathogens (Basel, Switzerland)*, 12(5), 657.
10. Ashraf H., et al. (2024). Phytochemical and antioxidant profile of citrus peel extracts in relation to different extraction parameters. *International Journal of Food Properties*, 27(1), 286–299.