Influence of Fruit Maturation on Phenolic Composition and Antibacterial Efficacy of Citrus Sinensis Peels

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The peel of Citrus sinensis was investigated as a potential source for the development of new antibacterial agents, addressing the growing global health concern posed by bacterial infections—a leading cause of mortality worldwide. This study aimed to evaluate the impact of fruit maturation on the total phenolic content and antibacterial potential of C. sinensis peel. Phenolic compounds, known for their antimicrobial properties, were the focus of this investigation. The peels of the selected plant were shade-dried for one week, ground into a fine powder, and subjected to both methanolic and aqueous extraction. Standard protocols were followed to carry out qualitative and quantitative analyses of phenolic content. The antibacterial activity of the extracts against Escherichia coli was evaluated using the agar well diffusion method and minimum inhibitory concentration (MIC) analysis. Among all samples, the third-week matured peels yielded the highest phenolic content (0.093 ± 0.08), while the first-week samples recorded the lowest (0.044 ± 0.02). The methanolic extract at a concentration of 1.5 mg/mL showed the largest zone of inhibition, whereas the aqueous extract at 1.0 mg/mL showed the smallest. Notably, the methanolic extract from the third-week peel exhibited the highest MIC values, achieving 80–100% inhibition of E. coli and significantly outperforming its aqueous counterpart. These results underscore the strong antibacterial potential of phenolic compounds in Citrus sinensis peel, particularly at the third week of maturation. The results support the use of plant-derived phenolics in the formulation of safe, effective, and natural antibacterial agents, potentially offering a valuable alternative to synthetic drugs with fewer adverse effects on human health.

Keywords: Phenolic Content, Maturation Effect, Peels, Antibacterial Activity

Introduction:

Citrus fruits, belonging to the Rutaceae family, are among the most widely cultivated fruits globally. Their peels are commonly repurposed for producing a range of non-food products, including bio-adsorbents, biofuels, biofertilizers, packaging materials, and activated carbon [1]. Citrus sinensis is the plant source of this beneficial essential oil. Known for its pleasant aroma as well as its strong antioxidant and antibacterial properties, it is widely used as an ingredient in various skin care products [2]. Bacterial infections are a leading cause of death and a significant contributor to global health challenges. Many phenolic compounds like polyphenols, gallic acid, and pyrogallol have antibacterial properties that are effective against bacteria and viruses [3]. These compounds work by inhibiting bacterial enzymes and toxins as well as disrupting the integrity of the bacterial membrane.

Citrus sinensis peels have higher concentrations of phenolic components, particularly phenolic acids and flavonoids, than in the peels of other Citrus fruit. Citrus peel has long been used in traditional medicine to treat a range of conditions, including eye infection, peptic ulcers,

respiratory issues, scurvy, and to aid in weight loss [4]. The antibacterial activity of secondary metabolites such as phenolic and flavonoid is primarily attributed to the presence of an aromatic ring with at least one hydroxyl group, which plays a key role in their mechanism of action. A primary mode of action is their capacity to form hydrogen bonds with proteins and enzymes in the bacterial cell wall and membrane, which can cause protein denaturation and compromise membrane structure [5]. This disruption increases membrane permeability, triggers leakage of intracellular components, and eventually leads to cell lysis. These compounds play a vital role in plant growth, reproduction, and defense against pathogens and herbivores. Citrus fruits, especially Citrus sinensis, have attracted growing interest for their high phenolic content and broad-spectrum bioactive properties, which in some cases can match or even exceed those of well-known medicinal plants such as Azadirachta indica and Ocimum sanctum. While these traditional herbs are renowned for their antimicrobial potential, citrus peels present a more accessible and environmentally friendly alternative, enriched with abundant flavonoids and phenolic acids [6]. Unlike many medicinal plants that maintain relatively stable phytochemical profiles, the phenolic composition of citrus peels undergoes significant changes during maturation, directly influencing their biological activity [7]. This maturation-linked variability makes citrus peels a versatile source of natural antimicrobials, with strong potential for use in food preservation and therapeutic products. Investigating these developmental shifts can guide the identification of the optimal harvest stage for maximum bioactivity.

Analyzing the phenolic content in Citrus sinensis peels can contribute to the development of effective, safe, and cost-efficient antibiotics [8]. Unlike many other plants, citrus peel extracts have demonstrated significant antibacterial activity against various bacterial isolates, including Staphylococcus aureus and Escherichia coli [9].

Objectives and Novelty Statement:

The present study uniquely examines the variations in phenolic composition throughout the ripening stages of C. sinensis peels and their influence on antibacterial activity, providing valuable insights for determining the optimal harvest time for functional and therapeutic uses. This study investigates how the different stages of fruit maturation influence the phenolic composition and antibacterial activity of Citrus sinensis peels, with an emphasis on identifying changes in bioactive compounds and their relationship to antibacterial potency.

Research Design and Methodology:

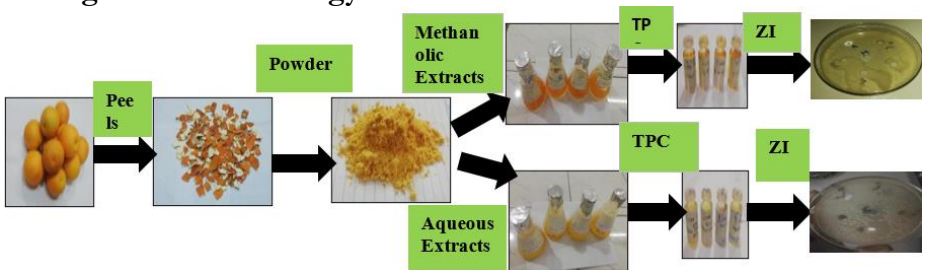

Figure 1. Flow sheet diagram of antibacterial activity of methanolic and aqueous extracts of total phenolic content of different weeks of peels of selected plant [TPC= Total Phenolic Content, ZI= Zone of Inhibition]

Sample Preparation:

The Experiment was conducted using a Completely Randomized Design (CRD). A total of 24 were collected from District Layyah. The oranges were washed with tap water, their peels were dried, and then ground into a fine powder.

Organoleptic Evaluation:

The plant part was subjected to organoleptic evaluation based on various sensory parameters. These included the color, general appearance, and external texture were assessed. External texture was evaluated by trained panelists based on parameters such as smoothness,

firmness, roughness, and uniformity.

Extraction Procedure:

Extraction was carried out using the maceration method. Fifteen grams of peel powder were extracted using 100 ml of a methanol and aqueous solvent in a 1:6 ratio at room temperature. The mixture was then filtered using Whatman filter paper No. 42, and the resulting filtrate and supernatant were collected and preserved at 4°C for further analysis.

Qualitative Test of Total Phenolic Substance:

Lead Acetate Test:

The 50 mg of plant peel powder was added to 5 ml of distilled water, followed by the addition of 3 ml of 10% lead acetate. The formation of a large white precipitate indicated the presence of phenolic content.

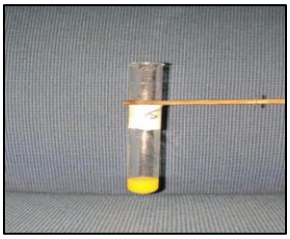

Figure 2. Lead acetate test for total phenolic content

Ferric Chloride Test:

Fifty milligrams of plant peel powder were dissolved in 10 milliliters of water. A few drops of 5% ferric chloride solution were then added to the diluted mixture. The appearance of a dark green color indicated the presence of phenolic compounds.

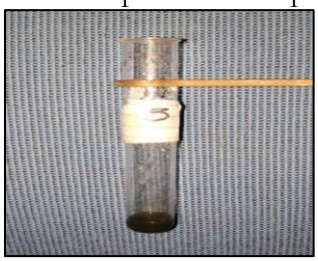

Figure 3. Ferric chloride test for total phenolic content

Measurement of Total Phenolic Content (TPC):

The total phenolic content was assessed using the method described in reference [10], with slight modifications to suit the experimental conditions. In brief, 400 µL of each extract was added with 4 mL of freshly diluted 9-fold Folin–Ciocalteu reagent. The mixture was then neutralized with 4 mL of 1 M sodium carbonate solution and allowed to proceed in the dark for 10 minutes, after which the absorbance was measured at 765 nm using a UV-Vis spectrophotometer. Methanol and Gallic acid were used as the blank and standard, respectively, and the results were recorded as mg GAE/mL extract.

Anti-bacterial Activity:

To evaluate antibacterial activity following groups were established

Negative Control: Methanol or distilled water solvent

Positive Control: A standard antibiotic

Treatment Groups: Different concentrations of C. sinensis peel extract were tested to assess dose-dependent effects:

T1: 0.5 mg/mL of plant extract

T2: 1.0 mg/ mL of plant extract

T3: 1.5 mg/mL of plant extract

Preparation of Culture Medium:

Agar Well Diffusion Method:

The antibacterial activity of Citrus sinensis peel was evaluated. Wells were punched into the agar plates using a No. 4 cork borer, each with a diameter of 8 mm. Peel extracts at concentrations of 0.5 mg/ml, 1.0 mg/ml, and 1.5 mg/ml were then carefully poured into the respective wells. Amoxicillin was used as a positive control, and solvents were used as a negative control on the same plate. After incubation at 37°C for 24 hr, the subsequent clear zones surrounding the well were measured. The presence of clear zones of growth inhibition around the wells indicated that Citrus sinensis peel extracts possess antibacterial activity.

Minimum Inhibitory Concentration:

In the MIC assay, E. coli was cultured on solid growth medium plates containing six wells. The first well contained an antibiotic, the second held methanol, the third served as an empty control, and the remaining three wells were filled with plant extract at concentrations of 0.5, 1, and 2 mL, respectively. After a 24-hour incubation period, the MIC was assessed by measuring the clear zones of inhibition using a ruler or scale. An inverse relationship was observed between the MIC values and the diameter of the inhibition zones—the greater the susceptibility of E. coli to the antimicrobial agent, the lower the MIC and the larger the zone of inhibition.

$$MIC = control - treatment/control$$

$$MIC = T1 - T2 / T1$$

T1, used as the control, did not show any inhibition compared to the treatments, such as plant extracts and antibiotics, which exhibited noticeable antibacterial activity.

Statistical analysis:

The data was analyzed statistically for ANOVA using a Completely Randomized Design (CRD) and was expressed as mean± standard errors.

Results:

Table 1. Traditional uses of the peel of Citrus sinensis (sweet Oranges)

Use Category	Traditional / Ethnobotanical Application	Region / Culture	Preparation / Notes
Digestive Aid	Used to relieve indigestion, bloating, and loss of appetite	Traditional Chinese Medicine (TCM), Ayurveda	Dried peel used in teas or decoctions [11].
Aromatherapy	Used to reduce anxiety, stress, and uplift mood	Europe, Middle East	Peel oil is used in essential oils or potpourri [12].
Cold & Cough Remedy	Remedy for colds, cough, and respiratory issues	India, Southeast Asia	Peel boiled in water or used in herbal mixtures [13].
Skin Care	Used for treating acne, brightening skin, and removing blemishes	India, Africa	Peel powder is applied as face masks or scrubs [13].
Insect Repellent	Used to deter insects	Various traditional rural communities	Peel burned or oil extracted for topical use [14].
Anti-inflammatory	Used to reduce inflammation and treat minor wounds	Mediterranean, South America	Poultices made from fresh or dried peel [11].
Flavoring Agent	Used in traditional sweets and beverages	Worldwide	Candied or dried peel is used in

			desserts and teas [12].
Mouth Freshener	Used to freshen breath and improve oral hygiene	India, Middle East	Chewed raw peel or made into powder for mouth rinse [12].
Traditional Medicine	Used for treating heartburn and nausea	Middle Eastern folk medicine	Dried peel brewed as an herbal infusion [12].
Spiritual Use	Used in rituals and for warding off negative energy	Caribbean, African diaspora religions	Peel included in spiritual baths or burned as incense [12].

Organoleptic Evaluation:

The 4th week extract of C. sinensis peels showed the highest yield (71.60 %), and the 3rd week C. sinensis peel extract exhibited the lowest yield (68.45 %) (Table 2).

Table 2. Percentage yield of aqueous extraction of Citrus sinensis peel extract

Plant material (C. sinensis Peel)	Powder (g)	Crude extract (g)	Yield (%)	Texture	Colour
1st week orange peel	168	119	70.8 %	Pasty	Light Yellow
2nd week orange peel	170	118	69.41 %	pasty	Light Yellow
3rd week orange peel	168	115	68.45 %	Pasty	Light Yellow
4th week orange peel	162	116	71.60 %	pasty	Light Yellow

The 1st (44.04 %) and 3rd weeks (41.66 %) extracts of C. sinensis peels exhibited the highest and lowest yield, respectively (Table 3).

Table 3. Percentage yield of methanolic extraction of Citrus sinensis peel extract

Plant material (C. sinensis Peel)	Powder (g)	Crude extract (g)	Yield (%)	Texture	Colour
1st week orange peel	168	74	44.04 %	Pasty	Dark Orange
2nd week orange peel	170	73	42.94 %	pasty	Dark Orange
3rd week orange peel	168	70	41.66 %	Pasty	Dark Orange
4th week orange peel	162	71	43.82 %	pasty	Dark Orange

Estimation of Total Phenolic Content:

Table 4 illustrates the total phenolic content of Citrus sinensis peel extracts—both methanolic and aqueous—measured weekly over a four-week maturation period. The phenolic concentration varied with the fruit's maturity stage. The highest levels were observed during the third week, with methanolic extracts containing 0.093 ± 0.08 and aqueous extracts 0.071 ± 0.05, indicating a peak in phenolic accumulation at this stage. This increase is likely due to heightened biosynthesis of phenolic compounds during active fruit development, serving as a defense mechanism against oxidative stress and microbial attack. In comparison, lower phenolic contents were recorded during the early (1st and 2nd week) and late (4th week) stages, suggesting that both immaturity and over-ripening may hinder phenolic synthesis or accelerate degradation. These findings highlight the critical role of optimal harvest timing in maximizing phenolic yield for therapeutic and nutraceutical applications.

Table 4. Total phenolic content of methanolic and aqueous extracts of C. sinensis peel

Peel of C.sinensis according to weeks	Total Phenolic Content (Methanolic extract) mg/ml	Total Phenolic Content (Aqueous extract) mg/ml
1st week C. sinensis Peel	0.047±0.03	0.044±0.02
2nd week C. sinensis Peel	0.044±0.01	0.055±0.04
3rd week C. sinensis Peel	0.093±0.08	0.071±0.05
4th week C.sinensis Peel	0.049±0.03	0.066±0.07

The zone of inhibition was measured with the rule, and the data given in the heat map is in mm. This clustered heat map (Figure 4) illustrated the antimicrobial efficacy of methanolic extract volumes (0.5, 1.0, 1.5 mL), along with positive/negative and solvent controls, over four weeks. Each row represents a specific treatment, each column corresponds to a week, and the color intensity indicates the zone of inhibition (with warm reds signifying strong inhibition and cool blues indicating weak or no inhibition). The clustering revealed that certain extract volumes and time points grouped due to similar inhibitory patterns. Notably, the 1.5 mL extracts and control treatments formed distinct branches, separating them from the lower-volume treatments—highlighting consistent differences in antimicrobial activity. This visual organization facilitated the quick identification of treatments with similar time-dependent efficacy trends and clearly showed how extract performance evolved week by week. (Figure 4).

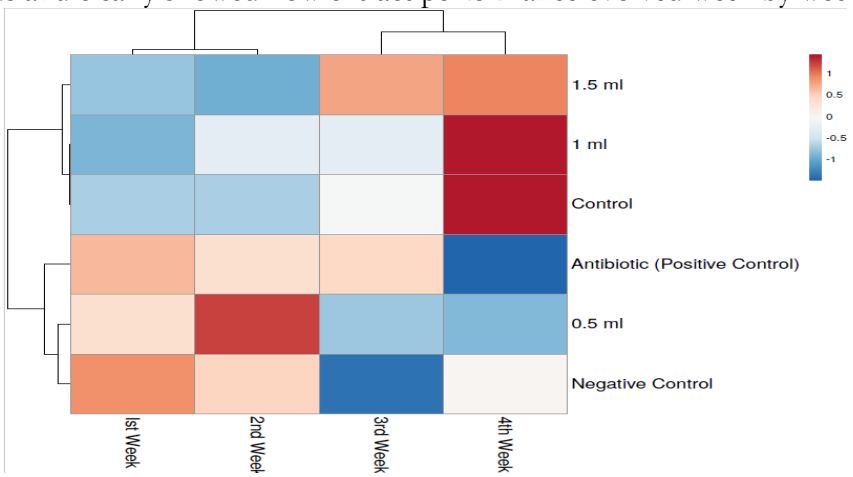

Figure 4. Comparative analysis of the heat map plot of the antibacterial potential of methanolic extracts of the total phenolic content of different weeks of selected plants against E coli

The heat map (Figure 5) exhibited a comparative overview of the antibacterial activity of aqueous extracts at different concentrations (1 mL and 1.5 mL) tested against various bacterial strains, along with appropriate controls. Color intensity within each cell represented the level of antibacterial effect, with deeper red shades indicating strong inhibition and blue shades reflecting low or no activity. The 1.5 mL concentration exhibited notably stronger antibacterial effects, nearly matching the efficacy of the antibiotic positive control, as shown by the prominent red coloration. In contrast, the 1 mL concentration and the negative control generally showed weaker or negligible activity, indicated by blue or lighter-colored cells. Hierarchical clustering (dendrograms) grouped bacterial strains and treatments based on similar response patterns, highlighting the influence of both extract concentration and bacterial susceptibility. Overall, the heat map clearly demonstrated a dose-dependent antibacterial effect of the aqueous extract (Figure 5).

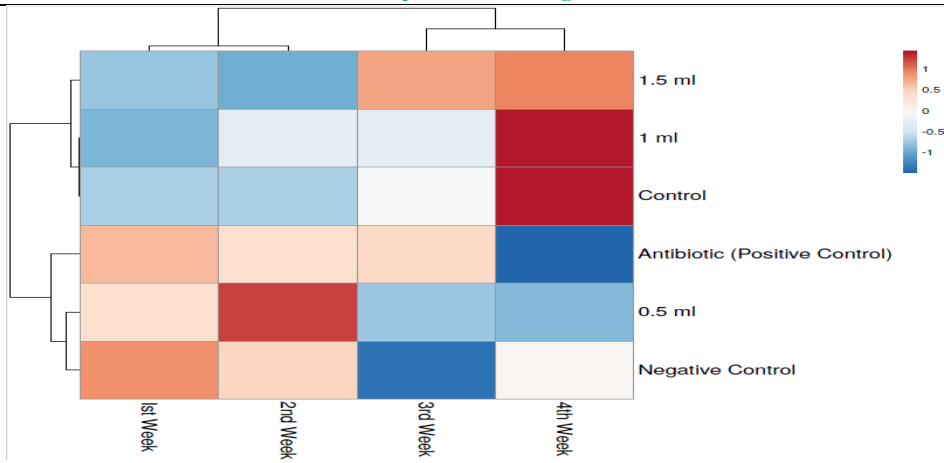

Figure 5. Comparative analysis of the heat map plot of the antibacterial potential of aqueous extracts of the total phenolic content of different weeks of selected plants against E coli

Minimum Inhibitory Concentration:

Table 5 presented the percentage-based MIC (minimum inhibitory concentration) values for methanolic and aqueous plant extracts tested over four consecutive weeks, highlighting the effectiveness of each extract volume (0.5 mL, 1.0 mL, 1.5 mL) in inhibiting microbial growth relative to solvent-only controls (set at 100%) and antibiotic positive controls (approximately 31%). Overall, methanolic extracts demonstrated stronger and more consistent antimicrobial activity compared to aqueous extracts. For instance, the 0.5 mL methanolic extract initially achieved 89–90% inhibition but declined significantly by week 4, whereas the 1.0 mL methanol extract peaked sharply at 87% in week 4—possibly indicating time-dependent stabilization or delayed release of active compounds. In contrast, aqueous extracts showed more variable results; the 0.5 mL aqueous extract, for example, increased from moderate inhibition (56%) to a strong effect (91%) by week 4. These week-to-week variations suggest dynamic shifts in extract potency influenced by factors such as compound degradation, solvent-specific solubility, or microbial resistance. The consistent behavior of the control groups supports the reliability of the assay and underscores the critical impact of solvent type, extract concentration, and storage duration on antimicrobial efficacy.

Table 5. Minimum inhibitory concentration of methanolic and aqueous extracts of the total phenolic content of different weeks

Sr. No.	Concentrations	Methanolic Extract (MIC) (%)				Aqueous Extract (MIC) (%)			
		Ist Week	2nd Week	3rd Week	4th Week	Ist Week	2nd Week	3rd Week	4th Week
1	0.5ml	89	90	85	31	56	38	85	91
2	1.0 ml	31.25	37.25	37.25	87.00	68.75	56.25	56.25	31.25
3	1.5 ml	25.50	25.30	25.50	25.50	56.25	56.25	31.25	30.00
4	Control	100	100	100	100	100	100	100	100
5	Negative Control	98	96	96	89	50	56	80	65
6	Antibiotic (Positive Control)	31	31	31	13	25	31	31	31

Discussion:

Medicinal plants have a number of therapeutic properties, including anticancer, antibacterial, anti-inflammatory, antimalarial, antiviral, and analgesic properties. According to [15], secondary metabolites like phenolic and flavonoid from Citrus sinensis are considered significant parts of the pharmacological effects of this plant.

Phenolic compounds are widely used in various areas of research on plants due to their significant biological roles. In humans, these compounds play a vital role in defense mechanisms, offering anti-inflammatory and antioxidant properties. Several factors influence the extraction of phenolic compounds from plant materials, including solvent polarity, the age of the material, extraction technique, particle size, and environmental conditions, in addition to the pretreatment process. The Folin-Ciocalteu method, widely employed for phenolic quantification, is based on the formation of a blue-colored complex in an alkaline medium. This complex can be measured spectrophotometrically at a wavelength of 765 nm. In plants, an increase in phenolic phytoalexins enhances resistance against infections [16]. Various studies have reported that Citrus sinensis peel possesses notable antibacterial properties capable of targeting multiple harmful pathogens. The present study also confirmed the strong antibacterial and antifungal potential of Citrus sinensis, suggesting its promising application in the pharmaceutical industry.

The methanolic extract of peels of the mentioned plant from the 4th week exhibited the maximum yield (71.60%), followed closely by the 1st week methanolic extract (70.80%). In contrast, the lowest yield was recorded in the 3rd week aqueous extract (41.66%). The higher yields observed in methanolic extracts, particularly from the 1st and 4th weeks, can be attributed to methanol's superior ability to dissolve phenolic compounds, flavonoids, and essential oils. As a polar organic solvent, methanol penetrates plant tissues more effectively than water, enhancing the extraction of bioactive compounds. On the other hand, the lower yields in aqueous extracts are likely due to the limited solubility of certain metabolites in water and compositional changes in more mature peel samples. These solvent-dependent variations in extraction efficiency align with findings from previous studies on citrus peel extractions.,[16]; [17].

The third-week peel exhibited the highest phenolic content in both methanolic (0.093 ± 0.08) and aqueous (0.071 ± 0.05) extracts, indicating a peak in phenolic accumulation at this stage of fruit maturation. This increase is likely attributed to enhanced phenolic biosynthesis during active developmental phases, functioning as a defense mechanism against oxidative stress and microbial invasion. In contrast, the 1st, 2nd, and 4th weeks showed lower phenolic levels, possibly due to limited synthesis in immature peels or degradation of phenolic compounds in overripe tissues. [17].

The heat map analysis of both methanolic and aqueous Citrus sinensis peel extracts revealed a clear dose-dependent antibacterial effect, strongly influenced by extract concentration and duration of exposure. Higher extract volumes, especially at 1.5 mL, consistently produced larger zones of inhibition—represented by deeper red shades—comparable to those of the antibiotic positive control. In contrast, lower volumes (such as 1 mL) and negative controls formed separate clusters, displaying significantly weaker or minimal antibacterial activity (blue shades). This underscores the critical role of extract concentration in microbial inhibition. Hierarchical clustering further reinforced these findings by grouping treatments and bacterial strains with similar inhibition patterns over time, suggesting that both bacterial susceptibility and the concentration of bioactive compounds contribute to the observed effects. Notably, methanolic extracts generally exhibited more consistent and broader antibacterial activity than aqueous extracts, likely due to methanol's superior ability to solubilize phenolic and flavonoid compounds.

The Citrus sinensis fruit peel methanolic extract demonstrated inhibitory effects against Escherichia coli. [18]. Methanol extract produced significantly larger inhibition zones than aqueous extract—e.g., E. coli: methanol 17 mm vs aqueous −7 mm; S. aureus: methanol 19 mm vs aqueous 10 mm [19]. The methanolic extracts are more potent and showed stronger dose-dependent effects than aqueous extracts. These compounds are known to disrupt microbial membranes, inhibit enzymatic activity, and chelate essential metal ions, contributing to their antimicrobial efficacy [20];[21], thereby enhancing the observed antibacterial effectiveness.

Additionally, the week-to-week variations in activity indicate that phenolic content in Citrus sinensis peels fluctuates with the fruit's maturation stage, potentially affecting the efficacy of the extracts over time. The result of the selected plant is similar to previous knowledge that highlighted the highest antibacterial properties enriched with phenolic compounds [22]; [23]. Overall, the integration of heat map visualization and hierarchical clustering offers a powerful tool for quickly identifying the most effective treatment groups and understanding the time-dependent behavior of natural extract potency.

The MIC data collected over four weeks demonstrated that methanolic extracts consistently exhibited greater antimicrobial efficacy than aqueous extracts, supporting previous findings that methanol is highly effective in extracting phenolic and flavonoid compounds responsible for microbial inhibition [24]. Notably, the 0.5 mL methanolic extract showed strong initial inhibition (89–90%) but declined by week 4, whereas the 1.0 mL methanol extract peaked at 87% in the final week—suggesting a delayed release or stabilization of bioactive constituents. In comparison, aqueous extracts displayed more variable activity, with some instances of increased inhibition over time—for example, the 0.5 mL sample rose from 56% to 91%—potentially due to gradual solubilization of hydrophilic compounds or microbial adaptation to stress. These time-dependent variations underscore the influence of solvent type, bioactive compound stability, and extract concentration on antimicrobial performance. These findings are consistent with the trends observed in the heat map and hierarchical clustering analyses, which revealed distinct dose- and time-dependent response patterns. The use of reliable controls further reinforces the conclusion that the antimicrobial efficacy of Citrus sinensis peel extracts is closely linked to their phytochemical composition and the methods used for extraction and handling—highlighting the crucial role of phenolic content in plant-derived antimicrobial agents. [25];[26].

Conclusion:

The peel of Citrus sinensis was rich in phenolic compounds known for their effective antibacterial properties, particularly against Escherichia coli. In the current study, the methanolic extract of the peel of the selected plant from the third week of maturation demonstrated the strongest antibacterial activity against Escherichia coli, outperforming the corresponding aqueous extract. With bacterial infections continuing to pose significant global health challenges and contribute to high mortality rates, there is an urgent need for alternative therapeutic options. Due to its potent antibacterial effects and natural origin, C. sinensis peel shows great potential for the development of novel antimicrobial agents. Increasingly, research is focusing on plant-derived compounds like phenolics, which exhibit strong antimicrobial properties with minimal or no adverse effects on human health. The result of a recent study indicates that C. sinensis peel could be a promising source for the development of safe and effective next-generation antibacterial treatments.

Competing Interest: The data included in this article do not involve any competing interests.

Author Contributions: MA designed and supervised all the research experiments, while NM conducted the experimental work.

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