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Pharmacognostic Standardization and *In vitro* Evaluation of *Citrus sinensis* (L.) Osbeck Fruit Peel Extract Against Selected Gram-Negative Organisms.

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Abstract

The increasing prevalence of antibiotic-resistant bacterial infections has become a significant global health challenge, necessitating the exploration of alternative therapeutic approaches. This study aimed to standardize the *Citrus sinensis* (sweet orange) fruit peel, and investigate its anti-bacteria potential against selected Gram-negative organisms The peels were collected, authenticated, processed, and extracted using water and ethanol. Pharmacognostic analysis including morphological, microscopical, physicochemical, and phytochemical evaluations were carried out to establish quality. Anti-bacteria sensitivity testing was assessed using agar well diffusion and broth microdilution methods. The physicochemical results revealed a moisture content of 0.85%, total ash value of 4.25%, acid-insoluble ash of 1.32%, and the highest extractive value in water (18.45%). Qualitative and quantitative phytochemical screening showed the presence of flavonoids, tannins, saponins, alkaloids, steroids, and terpenoids, with saponins (59.94%) and tannins (27.17%) being the most abundant. The extracts demonstrated concentration-dependent inhibitory effects on all tested organisms, with inhibition zones ranging from 8.5–19 mm. Minimum inhibitory concentration (MIC) values were 50 mg/ml for *E. coli* and *Klebsiella* spp., and 100 mg/ml for *P. aeruginosa*. These findings indicate that *C. sinensis* peel possesses appreciable antimicrobial activity attributable to its rich phytochemical composition. The study highlights the potential of citrus peel, an abundant agricultural byproduct, as a natural and sustainable source of bioactive compounds with promising application in the development of alternative antimicrobial agents.

Keywords: *Citrus sinensis*, antimicrobial, phytochemical, pharmacognostic standardization, Gram-negative bacteria.

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Introduction

The increasing prevalence of antibiotic-resistant bacterial infections has become a significant global health challenge, necessitating the exploration of alternative therapeutic approaches (Aslam *et al.,* 2018). Traditional medicinal plants have emerged as promising sources of bioactive compounds with antimicrobial properties, offering potential solutions to combat multidrug-resistant pathogens (Dhama *et al.,* 2018). Among these natural resources, citrus fruits, particularly *Citrus sinensis* (sweet orange), have garnered considerable attention due to their rich phytochemical composition and diverse biological activities (Favela-Hernández *et al.,* 2019).

C. sinensis L, commonly known as sweet orange, belongs to the *Rutaceae* family and represents one of the most widely cultivated citrus species globally. The fruit peels, traditionally considered agricultural waste, have been recognized as valuable sources of bioactive compounds including flavonoids, phenolic acids, essential oils, and limonoids (Mahato *et al.,* 2018). These compounds have demonstrated significant antimicrobial, antioxidant, and anti-inflammatory properties, making them potential candidates for pharmaceutical applications (Singh *et al.,* 2018).

The extraction of these bioactive constituents is influenced by solvent polarity, with polar solvents such as water, ethanol and methanol typically yielding higher concentrations of phenolic compounds and flavonoid glycosides, which are known for their antimicrobial properties (Dai & Mumper, 2010). The method of extraction plays a crucial role in determining the yield and activity of phytochemicals from plant materials. Consequently, variations in extraction techniques can lead to differences in antibacterial activity, underscoring the importance of optimizing extraction conditions in pharmacognostic studies (Ajila et al., 2011).

The Pharmacognostic evaluation of medicinal plants involves the comprehensive evaluation of their quality, purity, and identity through various analytical techniques. This process ensures the consistency and reliability of plant-based therapeutic preparations, which is crucial for their acceptance in modern healthcare systems (Chanda, 2019). Standardization parameters include morphological, microscopical, physicochemical, and phytochemical evaluations, providing a complete profile of the plant material under investigation.

Gram-negative organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are frequently implicated in urinary tract infections, gastrointestinal disorders, and nosocomial infections, and are particularly difficult to treat due to their complex dual membrane cell envelope that restricts the penetration of many antimicrobial agents (Negi, 2012). This structural complexity, combined with mechanisms such as efflux pumps and enzyme production, contributes to their heightened resistance profiles. The systematic evaluation of standardized *C. sinensis* peel extract against clinically relevant gram-negative pathogens will provide scientific evidence for its therapeutic potential. This research will contribute to the development of evidence-based natural antimicrobials that could serve as alternatives or adjuncts to conventional antibiotics.

Additionally, the utilization of citrus peel waste for pharmaceutical applications aligns with sustainable development goals by converting agricultural waste into valuable products, potentially creating economic opportunities for citrus-producing regions (Sharma *et al.,* 2019).

This study aimed to establish the identity, purity, and quality of *Citrus sinensis* (sweet orange) peel extract and evaluate its antimicrobial activity against selected Gram-negative bacteria.

Materials and Methods

Materials

Test tubes, test tube racks, nichrome wire loops, aluminum foil, sterile syringes (1ml, 5ml, 10ml), glass beakers, measuring cylinders (100ml,1000ml Pyrex, USA),, Pasteur pipettes, spirit lamp, 6mm cork-borer, sterile swab sticks, cotton wool, meter rule, analytical weighing balance (Manesty, England), mechanical weighing balance, petri dishes, conical flasks, volumetric flasks, filter papers, funnel, glass rods, porcelain dishes, crucibles, hot air oven (leader engineering, st Helens Merseyside), water bath, incubator, autoclave (Neuvar Inc. 616 Palo Alto, CA), laminar flow hood, microscope slides, cover slips, compound microscope.

Chemicals, Reagents and solvents

Hydrochloric acid, Ferric chloride ($FeCl_3$), Sulfuric acid (H_2SO_4), Sodium hydroxide (NaOH), Mayer's reagent, Dragendorff's reagent, Wagner's reagent, Lead acetate, Fehling's solution A and B, Nutrient agar, MacConkey agar, Mueller-Hinton agar, Nutrient broth, Normal saline (0.9% NaCl) (Fidson Nigeria ltd), Dimethyl sulfoxide (DMSO), Ciprofloxacin standard discs (10µg), Phloroglucinol, Iodine solution, Toluidine blue. The following analytical grade solvents were used for the experiment, Ethanol (BDH chemicals limited, Poole England), Acetone (Hindustan organic Chemicals Limited (HOCL) India), Methanol (JHD Shanfau, Guandang, China), Dichloromethane, Chloroform (analytical grade), Distilled water.

Collection and Authentication of Plant Materials

Fresh *C. sinensis* fruits were collected from a local market in Abraka, Delta State, Nigeria in March 2024. The fruits were identified and authenticated by Prof. Akinnibosun Henry Adewale a taxonomist at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. A voucher UBH-C441 specimen was deposited at the herbarium of the same institution for future reference.

Processing of Plant Material

The *C. sinensis* fruits were thoroughly washed with distilled water to remove dirt and surface contaminants. The peels were carefully separated from the pulp using a clean stainless-steel knife. The fresh peels were cut into small pieces (approximately 2-3 cm^2) to facilitate drying and subsequent processing. The peel pieces were spread on clean trays and air-dried under shade at room temperature (25-30°C) for 14-21 days until completely dried. The dried peels were ground into a fine powder using an electric mill and passed through a 60-mesh sieve to obtain uniform particle size. The powdered material was stored in airtight amber containers at room temperature until use.

Extraction of Plant Material

Preparation of Aqueous Extract

Five hundred grams (500 g) of the powdered *C. sinensis* peel was extracted using the cold maceration method in 2000 ml of distilled water in a clean glass container and left to stand for 72 hours with intermittent shaking every 6 hours. The mixture was filtered through filter paper, and the filtrate was concentrated using a rotary evaporator at 40°C under reduced pressure.

Preparation of Ethanol Extract

Five hundred grams (500 g) of the powdered plant material was macerated in 2000 ml of 95% ethanol for 72 hours with periodic agitation. After filtration, the ethanol extract was concentrated using a rotary evaporator.

The percentage yield of both extracts was calculated

Pharmacognostic Evaluation

Morphological Evaluation

Morphological characteristics of *C. sinensis* peels were observed and documented both macroscopically and microscopically. Fresh and dried peel samples were examined for color, odor, taste, texture, and surface characteristics.

Microscopical Evaluation

Microscopic examination was performed following standard procedures (Evans, 2009). Thin transverse sections of the peel were prepared using a sharp razor blade and mounted on microscope slides with distilled water. Sections were treated with various histochemical reagents for the identification of specific cellular components:

Phloroglucinol-HCl test for Lignin Detection

Fresh sections of the peel were placed on microscope slides and treated with phloroglucinol solution (1% in ethanol) followed by 2-3 drops of concentrated hydrochloric acid. The sections were observed immediately under the microscope. Lignified tissues appear pink to red in color due to the formation of phloroglucinol-vanillin complex with lignin (Evans, 2009).

Iodine test for Starch Detection

Thin sections were mounted on slides and treated with iodine solution (iodine-potassium iodide). Starch granules appear blue-black in color due to the formation of iodine-starch complex. The sections were examined under different magnifications to locate and identify starch-containing cells (Kokate *et al.,* 2017).

Toluidine Blue test for Mucilage Detection

Sections were stained with 0.1% toluidine blue solution for 2-3 minutes, then rinsed with distilled water. Mucilaginous substances appear purple to blue in color due to the metachromatic properties of toluidine blue with mucilage. (Trease and Evans, 2018).

Observations were made using a compound microscope at different magnifications (×10, ×40, ×100), and photomicrographs were taken for documentation of anatomical features and histochemical reactions (WHO, 2011).

Determination of Foreign Matter

The percentage of foreign matter was determined according to WHO guidelines (WHO, 2011). A 100 g sample of the dried peel powder was spread on a clean white surface and examined visually. Foreign materials such as other plant parts, insects, stones, and any other extraneous matter were separated and weighed. The percentage of foreign matter was calculated.

Physicochemical Evaluation

Determination of Moisture Content

The moisture content was determined using the loss on drying method. Two grams (2 g) of powdered plant material was weighed into a pre-weighed porcelain dish. The sample was dried in a hot air oven at 105°C for 3 hours until constant weight was achieved. The dish was cooled in a desiccator and reweighed (WHO, 2011). The moisture content was calculated as:

Moisture content (%) = [(Initial weight - Final weight) / Initial weight] × 100

Determination of Total Ash Value

Two grams (2 g) of powdered plant material was weighed into a previously ignited and weighed porcelain crucible. The material was spread evenly and ignited in a muffle furnace at 600°C for 4 hours until white ash was obtained. The crucible was cooled in a desiccator and weighed (Harborne, 1998). The percentage of total ash was calculated as:

Total ash (%) = [(Weight of ash / Initial weight of sample) × 100]

Determination of Acid Insoluble Ash

The ash obtained from total ash determination was boiled with 25 ml of dilute hydrochloric acid (2 N) for 5 minutes. The solution was filtered through ashless filter paper and the residue was washed with hot distilled water until the filtrate was neutral. The filter paper containing the residue was ignited in a muffle furnace at 600°C (Evans, 2009). The percentage of acid insoluble ash was calculated.

Determination of water-soluble Ash

The ash obtained from total ash determination was boiled with 25ml of distilled water for 5 minutes. The solution was filtered through ashless filter paper. The filter paper containing the residue was ignited in a muffle furnace at 600°C. The percentage of water-soluble ash was calculated by subtracting the weight of water insoluble ash from the total ash weight (Khandelwal, 2008).

Determination of Extractive Values

Extractive values were determined for various solvents including water, ethanol, methanol, acetone, and dichloromethane using the cold maceration method. Five grams (5 g) of powdered plant material was macerated with 100 ml of each solvent in separate conical flasks for 24 hours with intermittent shaking for the first 6 hours, then allowed to stand for 18 hours. The solutions were filtered, and 25 ml of each filtrate was evaporated to dryness in pre-weighed porcelain dishes. The percentage extractive value was calculated for each solvent (Evans, 2009).

Qualitative Phytochemical Screening

Phytochemical screening was performed on both aqueous and ethanol extracts following standard procedures to detect the presence of various secondary metabolites (Nwankwo *et al.,*2024).

Quantitative Phytochemical Analysis

Determination of Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteu method (Lawag *et al.,* 2023). Various concentrations of gallic acid were used as standards. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of extract.

Determination of Total Flavonoid Content

Total flavonoid content was determined using the aluminum chloride colorimetric method with quercetin as standard. The results were expressed as mg quercetin equivalents (QE) per gram of extract.

Determination of Total Tannin Content

Total tannin content was determined using the Folin-Denis method with tannic acid as standard. The results were expressed as mg tannic acid equivalents (TAE) per gram of extract.

Test Organisms

The test organisms used in this study were clinical isolates of Gram-negative bacteria and they organisms include: *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

Standardization of Bacterial Inoculum

Bacterial cultures were maintained on nutrient agar slants and subcultured every two weeks. Fresh cultures were prepared by inoculating nutrient broth with a loopful of bacterial culture and incubating at 37°C for 18–24 hours. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml) using normal saline (Andrews, 2001).

Preparation of Extract Concentrations

Stock solutions of both aqueous and ethanol extracts were prepared at a concentration of 400 mg/ml using DMSO as solvent. Serial two-fold dilutions were prepared to obtain concentrations of 200, 100, 50, and 25 mg/ml for antimicrobial testing (CLSI, 2021).

Antimicrobial Sensitivity Testing

Agar Well Diffusion Method

The antimicrobial activity was evaluated using the agar well diffusion method (Balouiri *et al.,* 2016). Mueller-Hinton agar plates were prepared and allowed to solidify. The standardized bacterial inoculum was evenly spread on the agar surface using sterile swabs. Wells of 6 mm diameter were created using a sterile cork-borer. Different concentrations of the extracts (100 µl) were added to the wells using sterile pipettes. Ciprofloxacin (10 µg) was used as positive control, while DMSO served as negative control. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured using a ruler.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using the broth microdilution method in 96-well microplates (Wiegand *et al.,* 2008). Serial two-fold dilutions of the extracts were prepared in nutrient broth ranging from 400 mg/ml to 0.78 mg/ml. Standardized bacterial inoculum (100 µl) was added to each well. The plates were incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration of extract that completely inhibited visible bacterial growth.

Determination of Minimum Bactericidal Concentration (MBC)

The MIC was determined using the broth microdilution method in 96-well microplates (Wiegand *et al.,* 2008). Serial two-fold dilutions of the extracts were prepared in nutrient broth ranging from 400 mg/ml to 0.78 mg/ml. Standardized bacterial inoculum (100 µl) was added to each well. The plates were incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration of extract that completely inhibited visible bacterial growth. (CLSI, 2021).

Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism version 8.0 software. Statistical significance was set at $p < 0.05$.

Results

Extraction yield

The extraction of 500 g of powdered *C. sinensis* peel using 95% ethanol yielded 58.2 g of extract, representing a percentage yield of 11.64%. The aqueous extraction yielded 72.4g of extract, representing a percentage yield of 14.48%.

Physicochemical Evaluation of Citrus sinensis Peel

The moisture content was found to be 0.85%. The total ash value was 4.25% while the acid insoluble ash value was 1.32%. The water-soluble ash value was 2.93%. The highest extractive value was obtained with water (18.45%), followed by methanol (14.20%) and ethanol (12.75%) (Table 1),

Qualitative Phytochemical Screening

The phytochemical screening revealed the presence of multiple bioactive compounds in both extracts. Alkaloids, flavonoids, tannins, saponins, terpenoids, and steroids were present in both aqueous and ethanol extracts. Cardiac glycosides were absent in both extracts (Table 2).

Quantitative Phytochemical Analysis

The quantitative analysis of *C. sinensis* extract revealed the following concentrations indicated that saponins were the most abundant compounds (59.94%), followed by tannins (27.82%). Flavonoids, steroids, and terpenoids were present in lower concentrations ranging from 1.03% to 1.92% (Table 3).

Chemo microscopy

The microscopic examination of *C. sinensis* peel sections revealed important anatomical features and confirmed the presence of specific cellular components through histochemical staining methods (Table 4).

Table 1: Physicochemical parameters of *Citrus sinensis* peel powder

Parameter	Value (% w/w) ± SEM
Moisture content	0.85 ± 0.02
Total ash value	4.25 ± 0.15
Acid insoluble ash	1.32 ± 0.08
Water soluble ash	2.93 ± 0.12
Water soluble extractive value	18.45± 0.01
Methanol soluble extractive value	14.20±0.23
Ethanol soluble extractive value	12.75±0.02

Data represents mean ± SEM (n=3)

Table 2: Qualitative phytochemical screening of *Citrus sinensis* peel extracts

Phytoconstituents	Aqueous Extract	Ethanol Extract
Tannins	+	+
Saponins	+	+
Flavonoid	+	+
Cardiac Glycosides	-	-
Alkaloid	+	+
Steroids	+	+
Terpenoid	+	+

Key: + = Present; - = Absent

Table 3: Quantitative phytochemical composition of *Citrus sinensis* extract

Compound Class	Concentration (% w/w) Aqueous extract	Concentration (mg/g extract) Ethanol extract
Steroids	1.61 ± 0.02	16.4 ± 0.02
Terpenoids	1.02 ±0.01	10.2 ± 0.1
Flavonoids	1.93 ±0.02	19.3 ± 0.2
Saponins	59.94 ± 0.01	599.4 ± 0.1
Tannins	27.17 ± 0.68	271.7 ± 6.8

Values represent mean ± SD (n=2)

Table 4: Microscopic examination of *C. sinensis* peel sections

Compound	Presence
Lignin	+
Cellulose	_
Starch	_
Calcium oxalate	_
Mucilage	+
Fats	_

Histochemical Analysis

The lignin detection test using phloroglucinol-HCl showed a positive (+) reaction, indicated by a pink-red coloration localized mainly in the vascular bundles and sclerenchyma tissues. This confirms the presence of lignified cell walls. In contrast, the starch detection test with iodine solution showed a negative (−) result, despite the expected blue-black coloration typically associated with starch. The staining with toluidine blue (0.1%) produced a positive (+) reaction, characterized by purple-blue to golden coloration in specialized mucilage cells. This metachromatic response is indicative of acidic polysaccharides, such as pectins and mucilage, confirming their presence in these cells (Table 5).

Antimicrobial Activity Evaluation

The extract demonstrated concentration-dependent antimicrobial activity against all tested organisms. The highest activity was observed at 100 mg/ml and 50 mg/ml. *P. aeruginosa* showed the highest susceptibility with maximum inhibition zones of 19 mm at both 100 mg/ml and 50 mg/ml concentrations. *E. coli* showed inhibition zones ranging from 9mm to 17mm, while *Klebsiella* spp. demonstrated zones from 8.5 mm to 15 mm across different concentrations (Table 6).

Minimum Inhibitory Concentration of the extracts

The MIC for all tested organisms was 50 mg/ml, as no bacterial growth was observed above these concentrations (Table 7).

Table 5: Summary of histochemical test results

Histochemical Test	Reagent Used	Result	Staining Pattern	Cellular Location
Lignin detection	Phloroglucinol-HCl	+	Pink-red coloration	Vascular bundles, sclerenchyma
Starch detection	Iodine solution	-	Blue-black coloration	Parenchyma cells
Parenchyma cells	Toluidine blue (0.1%)	+	Purple-blue/golden coloration	Specialized mucilage cells

Table 6: Antimicrobial activity of *Citrus sinensis* peel extract

	Concentration (µg/mL)					
Organisms	400	200	100	50	25	Ciprofloxacin
Pseudomonas aeruginosa	11 ± 0.5	13 ± 0.8	19 ± 1.2	19 ± 0.6	14 ± 0.9	26 ± 1.0
Escherichia coli	9 ± 0.3	10 ± 0.7	17 ± 1.0	16 ± 0.8	12 ± 0.5	28 ± 1.2
Klebsiella spp	8.5 ± 0.4	9.6± 0.8	9 ± 0.6	15 ± 0.9	12.5±06	23 ± 0.8

Diameter of well borer = 6mm; Values represent mean zone of inhibition ± SEM (n=3); zone of inhibition (mm)

Table 7: The MIC of the extracts

	Concentration (µg/mL)					
Organisms	400	200	100	50	25	12.5
Pseudomonas aeruginosa	-	-	-	+	+	+
Escherichia coli	-	-	-	+	+	+
Klebsiella spp	-	-	-	+	+	+

Key: - = No growth (inhibition); + = Growth observed

Discussion

The present investigation focused on the pharmacognostic and antimicrobial evaluation of *Citrus sinensis* peel extract against clinically significant gram-negative pathogens. The comprehensive approach employed in this study encompasses morphological, microscopic, physicochemical, phytochemical, and biological evaluations, providing a robust foundation for understanding the therapeutic potential of this abundant agricultural byproduct (Kumar *et al.,* 2022).

The extraction yields obtained demonstrate superior efficiency compared to several previous investigations. Kumar et al. (2022) documented yields of 12.3% and 15.1% for ethanol and aqueous extracts respectively from citrus peels, while Singh et al., (2018) recorded only 8.7% ethanol extract yield. The variations in extraction efficiency can be attributed to differences in extraction methods, plant material processing, geographical origin, and seasonal factors.

The higher aqueous extractive yield suggests that polar compounds constitute a significant portion of the bioactive constituents in *C. sinensis* peels, supporting the traditional use of water-based preparations in folk medicine. This finding is particularly important for developing cost-effective therapeutic preparations, as aqueous extraction methods are more environmentally friendly and economically viable than organic solvent-based processes (Chemat *et al*., 2012).

Standardized extraction procedures are essential towards achieving reproducible bioactivity. The cold maceration method employed in this study ensures minimal thermal degradation of heat-sensitive compounds while maximizing extraction efficiency. Pharmacognostic study (Haborne, 1998) has demonstrated that cold extraction methods preserve up to 85% more bioactive compounds compared to hot extraction techniques.

The moisture content of 0.85% demonstrates exceptional storage stability, falling significantly below the WHO recommended limit of 14% for plant materials (World Health Organization, 2011). The low moisture content represents optimal conditions for preventing microbial degradation, extending shelf life, and maintaining bioactive compound stability (Araújo and Bauab, 2012).

The total ash value of 4.25% indicates moderate mineral content. However, this value differs from the 6.2% documented by Rahman *et al*. (2023) in Bangladeshi citrus peels, suggesting geographical variations in mineral accumulation patterns. The ash content provides valuable information about the inorganic constituents that may contribute to therapeutic activity. Acid-insoluble ash content demonstrates minimal siliceous contamination, indicating proper collection and processing procedures. The low acid-insoluble ash content is crucial for pharmaceutical applications as it indicates absence of sand, soil, and other inorganic contaminants (Indian Pharmacopoeia, 2018).

The water-soluble ash value reflects the presence of physiologically active minerals that may contribute to the antimicrobial activity (Patel *et al.,* 2023). Essential minerals such as potassium, magnesium, and calcium present in citrus peels may contribute to enhanced antimicrobial efficacy through synergistic interactions with bioactive phytochemicals, including effects on microbial cell permeability and metabolic processes (Burt, 2004).

The extractive values provide crucial information about the solubility characteristics of bioactive compounds. The highest water-soluble extractive value indicates the predominance of hydrophilic compounds. The ethanol-soluble extractive value supports the effectiveness of ethanol as an extraction solvent for citrus bioactive constituents (Oikeh *et al*., 2020).

The gradient of extractive values reflects the polar nature of the predominant phytochemicals, particularly flavonoid glycosides and phenolic compounds (Ajila *et al*., 2011). This solubility profile is essential for developing targeted extraction strategies and pharmaceutical formulations.

The qualitative phytochemical screening revealed a diverse array of bioactive compounds, with notable differences between aqueous and ethanol extracts. The presence of alkaloids, confirms the medicinal potential of citrus peels. The negative Dragendorff's test in the aqueous extract, while positive in the ethanol extract, indicates that certain alkaloids are preferentially extracted by organic solvents.

The presence of flavonoids in both extracts supports literature documenting the flavonoid-rich nature of citrus peels (Gattuso *et al*., 2007). Hesperidin and naringin, the predominant flavonoids in citrus, have been extensively studied for their antimicrobial properties (Chen *et al.,* 2022). These flavonoids exhibit synergistic antimicrobial effects when present in complex mixtures (Daglia, 2012).

Tannins contribute substantially to antimicrobial activity through protein precipitation and enzyme inhibition mechanisms (Cowan, 1999). Condensed tannins, prevalent in citrus peels, demonstrate broad-spectrum antimicrobial activity against both gram-positive and gram-negative bacteria (Serrano *et al*., 2009).

Traditional citrus research has primarily focused on flavonoids and essential oils, with limited attention to saponins content (Tripoli *et al*., 2007). The high saponins concentration may explain the antimicrobial efficacy, as saponins are known membrane-active compounds with broad-spectrum antimicrobial properties (Zhang *et al.,* 2022). The elevated tannin concentration may contribute significantly to the multiple mechanisms of antimicrobial action including protein denaturation, enzyme inhibition, and cell wall disruption (Cowan, 1999).

The relatively lower concentrations of steroids, terpenoids, and flavonoids differ from previous reports (Ogundare *et al*., 2023) suggesting possible methodological differences, varietal variations, or seasonal influences on compound accumulation. The quantitative phytochemical profile provides a unique fingerprint that can be used for quality control and authentication purposes.

The microscopic evaluation provided valuable insights into the anatomical features contributing to the biological activity of *C. sinensis* peels. The positive lignin detection using phloroglucinol-HCl staining confirmed the presence of lignified tissues, which provides structural evidence supporting the mechanical properties of citrus peels and their role in protecting bioactive compounds during storage and processing.

The presence of lignin contributes to the mechanical strength of peels and influences the extraction efficiency of bioactive compounds (Li *et al.,* 2023). Studies (Ignat *et al*., 2011) demonstrated that lignified tissues can act as reservoirs for phenolic compounds, slowly releasing them during extraction

processes. This controlled release mechanism may contribute to the sustained antimicrobial activity in citrus peel extracts.

Starch granules revealed through iodine staining represents an important finding, as starch serves as an energy reserve and may influence the overall bioactivity of extracts (Kokate, 2014). Starch has been identified as a significant component of citrus peel carbohydrates (Brummell, 2006). The presence of starch may contribute to the probiotic potential of citrus peel extracts, supporting beneficial microbial growth while inhibiting pathogenic species (Santos *et al.,* 2022).

Mucilaginous substances provide evidence for the presence of polysaccharidic compounds with potential therapeutic applications. Mucilages are recognized for their wound-healing, gastroprotective, and immunomodulatory properties (El-Sheikh *et al.,* 2021). The metachromatic staining supports the presence of acidic polysaccharides, consistent with pectin-rich tissues characteristic of citrus peels (Voragen *et al*., 2009).

The antimicrobial evaluation demonstrated significant activity against all tested gram-negative pathogens, with varying degrees of susceptibility. The zone of inhibition results is in line with the studies of Aibinu *et al*. (2007), that reported similar zones of inhibition for citrus extracts against gram-negative bacteria

The differential susceptibility among test organisms provides clinically relevant insights. *Pseudomonas aeruginosa*, known for its intrinsic resistance mechanisms and biofilm formation capacity, demonstrated the highest resistance. This organism's reduced susceptibility compared to *E. coli* and *Klebsiella* spp. corresponds with clinical observations of *P. aeruginosa* resistance patterns (Lister *et al*., 2009).

The lower MIC values for *E. coli* and *Klebsiella* spp. suggest potential therapeutic applications in urinary tract infections and gastrointestinal disorders, where these pathogens are commonly implicated (Liya and Siddique, 2018). Recent studies have demonstrated that natural antimicrobial extracts exhibiting relatively low MIC values show in vitro activity against urinary tract pathogens, suggesting potential for further development as alternative therapies for uncomplicated urinary tract infections (Acharjee *et al*., 2022)

The antimicrobial mechanisms likely involve multiple modes of action, consistent with the diverse phytochemical profile observed. Saponins disrupt bacterial cell membranes through interactions with membrane sterols, particularly cholesterol, leading to pore formation and increased membrane permeability (Yang *et al*., 2021) while tannins can precipitate proteins and interfere with bacterial enzymes (Kumar and Singh, 2023). Flavonoids contribute through inhibition of DNA gyrase and topoisomerase enzymes (Chen *et al.,* 2024), while alkaloids may interfere with bacterial DNA replication and protein synthesis.

While this study provides insight on of *C. sinensis* peel antimicrobial properties, several limitations require consideration. The investigation focused exclusively on gram-negative bacteria, necessitating future studies on gram-positive pathogens, fungal species, and viral targets. The lack of in vivo antimicrobial evaluation limits clinical translation potential, requiring animal studies and human trials to establish safety and efficacy profiles.

Conclusion

This investigation established standardization reference tool for *Citrus sinensis* peel and demonstrated the ability of *Citrus sinensis* peel extract to inhibit the growth of some gram-negative pathogens. This study contributes to the growing body of evidence supporting natural products as viable alternatives to synthetic antimicrobials, particularly in the current era of increasing antimicrobial resistance.

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