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Determination of flavonoid content and antibacterial activity of crude ethanolic extract from calamansi (*Citrus macrocarpa*) peels against Staphylococcus aureus

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Abstract

The rising problem of antimicrobial resistance (AMR) has substantially decreased the effectiveness of conventional antibiotics, highlighting the urgent necessity to identify new antibacterial agents from natural sources. Staphylococcus aureus, including methicillin-resistant strains, is still a prominent cause of community-acquired and nosocomial infections, particularly in developing countries such as the Philippines. Calamansi (Citrus microcarpa), a fruit traditionally used in traditional medicine, contains many bioactive comopunds, including flavonoids and terpenoids, which have been proven to have antibacterial properties. This study seeks to test the antibacterial potential of a crude ethanolic extract produced from calamansi peels against S. aureus, and to investigate the impact of extraction process on antibacterial property. Fresh calamansi peels were collected, oven-dried, ground, and macerated with 80% ethanol prior to Soxhlet extraction. Phytochemical testing confirmed the presence of flavonoids. Disk diffusion assay was performed to assess its antibacterial activity at 20% w/v concentration. 1% DMSO was utilized as the negative control, and doxycycline (30 mcg/disc) as the positive control. The positive control significantly inhibited S. aureus (32.00 ± 5.80 mm), but the crude ethanolic extract did not exhibit any measurable inhibition. These data emphasize the need of improving extraction procedures to increase the stability and recovery of bioactive ingredients such as limonene. The study highlights the scientific importance of negative findings and lays the groundwork for future research using different solvents or essential oil-based extraction methods to better assess the antibacterial properties of calamansi.

Keywords: Antimicrobial Resistance; Natural Products; Phytochemicals; Complex Mixtures; Calamansi

1. Introduction

The emergence of antimicrobial resistance (AMR) has resulted in a progressive decrease in antibiotic efficacy, complicating the treatment of infections and underscoring the urgent need for novel therapeutic agents. Staphylococcus aureus remains a major human pathogen, responsible for a variety of community-acquired infections, such as pneumonia, septic shock, and endocarditis [1]. Drug-resistant strains, including methicillin-resistant (MRSA) and vancomycin-resistant S. aureus (VRSA), are significant contributors to nosocomial infections. A prospective study conducted in 2010 at a provincial tertiary hospital in the Philippines reported over 90 cases of S. aureus infections, of

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which more than 40% were identified as MRSA by 2012 [2]. More recently, a 2024 study found MRSA carriage in approximately 43% of otorhinolaryngology physicians at a tertiary hospital in Manila [3]. Collectively, these data underscore the alarming prevalence of S. aureus infections and their potential impact on the healthcare system.

Plant-based compounds constitute a potential source for antimicrobial discovery due to their structural diversity, which allows for the development of novel drugs, as well as their capacity for molecular promiscuity—the ability to interact with multiple targets—which may confer additive or even synergistic antibacterial effects [4]. Calamansi (Citrus microcarpa) is a rich source of ascorbic acid and essential oils, particularly limonene and pinene, which exhibit various biological activities against oxidation, cancer, inflammation, and microbial infections. Consequently, calamansi has long been used in traditional medicine, especially for managing pulmonary conditions such as coughs and colds [5]. D-limonene, the primary bioactive compound in calamansi, exhibits antibacterial properties through multiple mechanisms, including cell membrane disruption, inhibition of cellular ATP synthesis, and interference with gene expression, such as the dpeE1 gene involved in cell wall synthesis. Additionally, D-limonene inhibits quorum sensing, thereby impairing biofilm formation. Such properties suggest that calamansi could serve as a locally available and sustainable supplement to standard antibiotic therapies.

However, findings on the antibacterial properties of calamansi are focused on essential oils as the key compounds are more efficiently obtained through this form due to their volatile nature [5]. As a result, the United Nations (UN) Sustainable Development Goals and World Health Organization (WHO) highlight the need to explore natural antimicrobial agents as a sustainable strategy to combat AMR [6].

This study seeks to test the antibacterial potential of crude ethanolic extract from calamansi peels against S. aureus to assess its potential for further research aimed at developing clinically translatable strategies to combat AMR.

2. Materials and Methods

2.1. Ethical Considerations

This study was performed solely in vitro using bacterial strains, and thus did not require ethical clearance. The entire experimental process adhered to standard and internationally accepted laboratory protocols for in vitro studies.

2.2. Reagents

Ethanol (molecular weight: 46.07 g/mol; GPR grade) was procured from Belman Laboratories (Quezon City, National Capital Region, Philippines). Dimethyl sulfoxide (molecular weight: 78.13 g/mol; ≥99.5% purity) was sourced from Sigma-Aldrich (St. Louis, Missouri, USA). Reagents for phytochemical screening were provided by the laboratory of Lourdes College, Inc. (Cagayan de Oro City, Misamis Oriental, Philippines). Unless otherwise specified, all reagents and materials used in the study were of analytical grade.

2.3. Instruments

The peels were dried in a hot air oven (PSO-451, Presto Stanvest Pvt. Ltd., Haryana, India), then processed into a fine powder using a cutting mill (Wiley™ Model 4, Thomas Scientific, New Jersey, USA). The powdered peels were extracted using a Soxhlet apparatus (LSOA-A11, Labtron Equipment Ltd., Surrey, United Kingdom), and the resulting extract was concentrated with a rotary evaporator (RV 10™, IKA®, Selangor, Malaysia). For the antibacterial assay, test microorganisms were placed in an electro-thermostatic incubator (DRP-9082, Senxin®, Shanghai, China).

2.4. Preparation of Plant Material

Fresh calamansi fruits were procured in February 2025 from Cogon Public Market, Cagayan de Oro City, Misamis Oriental. A voucher specimen was submitted to Far Eastern University's herbarium for taxonomic validation. The fruits were washed with distilled water, cut into halves, and the juice was extracted. The peels were then oven-dried at 60 °C for 24 hours following the method of Sun et al. [7], which ensures effective drying while preserving flavonoid content. The dried peels were subsequently ground into a fine powder using a cutting mill.

2.5. Preparation of Crude Ethanolic Extract (CEE)

145.45 g of dried, ground peels were subjected to Soxhlet extraction for 7 hours using approximately 3 L of 80% ethanol. The extracts were then concentrated at 60 °C for 5 hours with a rotary evaporator, resulting in a dark green, viscous residue. The crude ethanolic extract was refrigerated in an amber, airtight container until ready to use.

2.6. Phytochemical Screening for Flavonoids

To test for flavonoids, 1 mL of CEE was transferred into labeled test tubes and subjected to standard qualitative tests, as described below.

2.6.1. Alkaline Reagent Test

Five drops of 10% sodium hydroxide solution were added to the extract, resulting in a bright yellow color. Following the addition of dilute hydrochloric acid, the color faded, showing the presence of flavonoids.

2.6.2. Ammonia Test

5 mL of dilute ammonia solution and 10 drops of strong hydrochloric acid were added to the extraction. The appearance of a yellow color suggested the presence of flavonoids.

2.6.3. Shinoda Test

10 drops of strong hydrochloric acid and a few magnesium turnings were added to the extract. Flavonoids were detected when a red or orange color developed.

2.6.4. Zinc Test

10 drops of strong hydrochloric acid and zinc dust were mixed into the extract. The appearance of a red color suggested the presence of flavonoids.

2.7. Determination of Antibacterial Activity

The disk diffusion method was performed to test the CEE's antibacterial property against Staphylococcus aureus (BIOTECH 1582).

2.7.1. Preparation of Inoculum

The test strain was subculture on freshly prepared Mueller–Hinton agar (MHA) plates and incubated at 37 °C for 24 hours. To adjust turbidity to a 0.5 McFarland standard (about 1.5×10^8 CFU/mL), well-isolated colonies were suspended in nutrient broth and visually or spectrophotometrically measured. The suspension was thoroughly mixed using a vortex mixer, then utilized within 1 hour of preparation.

2.7.2. Disk Diffusion Assay

Freshly prepared MHA (20 mL per plate) was added to sterilized Petri dishes and allowed to harden. A sterile cotton swab was used to distribute a standardized bacterial inoculum equally across the agar surface. Sterile paper discs (6 mm in diameter) were aseptically placed on the inoculated agar and impregnated with 20 µL of the CEE at a concentration of 20% w/v. The plates were incubated at 37 °C for 24 hours. Zones of inhibition (ZOI) were measured in millimeters with a digital Vernier caliper, following Clinical and Laboratory Standards Institute (CLSI) criteria. Doxycycline (30 mcg/disc) was employed as the positive control, and 1% dimethyl sulfoxide (DMSO) as the negative control.

2.8. Statistical Analysis

The antimicrobial assay was performed in triplicate, with data provided as mean ± SD. The statistical analyses were performed using SPSS software (version 20; IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to compare antibacterial activity between treatments, with $p < 0.05$ indicating statistical significance.

3. Results and Discussion

3.1. Qualitative Phytochemical Screening

Table 1 presents the results of phytochemical tests conducted to identify the presence of flavonoids, confirming their presence in the CEE of calamansi peels. Members of the Rutaceae family, including calamansi, are well-established to contain polyphenolic compounds such as flavonoids, which contribute to their antioxidant, anticancer, and chemotherapeutic properties. Palma et al. [8] characterized the essential oil of calamansi using gas chromatography–mass spectrometry (GC–MS) and identified D-limonene as the predominant constituent, accounting for approximately 93% of the oil, along with limonene oxide and other terpenes and aldehydes. The essential oil showed strong

antineoplastic activity against the MCF-7 cell line (IC50 = 7.98 ± 1.77 µg/mL). Goh et al. [9] reported limonene contents exceeding 90% in calamansi peel oil samples from both Indonesia and the Philippines, along with additional bioactive compounds such as myrcene and β-pinene. Collectively, the presence of these compounds emphasizes calamansi's therapeutic potential and lends support to its historic ethnomedical use. Furthermore, these findings serve as a platform for future research into the clinical application of calamansi extracts' biological activity.

Table 1 Qualitative detection of flavonoids in the crude ethanolic extract of calamansi peels

Test	Result
Alkaline Reagent	+
Ammonia	+
Shinoda	+
Zinc	+

(+) present; (-) absent

3.2. Results of Antibacterial Assay

The results of the antibacterial assay in Table 2 show that the CEE of calamansi peels did not produce a detectable ZOI against S. aureus at a concentration of 20% (w/v). In contrast, the positive control, doxycycline, showed significant ZOIs across all trials, confirming the susceptibility of S. aureus and the reliability of the test protocol. Although calamansi has been widely reported to exhibit antimicrobial activity, the CEE did not demonstrate measurable antibacterial effects against S. aureus under the conditions employed in this study. This discrepancy may be attributed to solvent-dependent differences in the extraction of bioactive compounds. In a comparative analysis of calamansi peel extracts made with solvents of different polarity (methanol, ethanol, acetone, hexane, and water) against Gram-positive (Bacillus subtilis and Streptococcus spp.) and Gram-negative (Escherichia coli and Salmonella spp.) bacteria, Rubato et al. [10] found that methanolic extracts had the strongest antibacterial activity, with zones of inhibition up to 26 mm. The increased polarity of methanol has been shown to improve the extraction of polar compounds such as flavonoids, which are well known for their antibacterial activities. Another potential reason for the observed discrepancy is the loss or degradation of bioactive constituents during the extraction process. Key antibacterial compounds, such as limonene and pinene, are highly volatile and may be partially lost during drying, extraction, or solvent evaporation. Previous research has shown that steam distillation is the most reliable method for obtaining essential oils from calamansi [8, 11, 12, 13]. Consequently, the differences in antibacterial activity observed may largely reflect the impact of the extraction method on the stability and retention of these volatile compounds, underscoring the importance of selecting an appropriate extraction technique to preserve their bioactivity. Overall, these findings highlight the complexity of antibacterial efficacy in calamansi extracts, implying that solvent selection and extraction procedure are crucial in influencing outcomes. Importantly, the current result is consistent with previous observations that crude ethanolic extracts may lack enough antibacterial efficacy unless different solvents or extraction procedures are used.

Table 2 Antibacterial activity of crude ethanolic calamansi peel extract against S. aureus using the disk diffusion method

Sample	Concentration	ZOI (mm)
CEE	20% w/v	-
Doxycycline	30 mcg	32.00 ± 5.80 mm
DMSO	1%	-

4. Conclusion

The study found that, while calamansi contains bioactive phytochemicals, their antibacterial property is heavily reliant on the extraction method used. The lack of observable antibacterial activity in the crude ethanolic extract indicates that ethanol-based extraction combined with prolonged heat exposure may be insufficient for preserving or concentrating the specific constituents responsible for antibacterial effects, particularly volatile terpenoids. These data demonstrate that the mere existence of phytochemicals does not always translate into biological efficacy. These findings underscore the importance of carefully aligning target bioactivity and extraction process when investigating plant-based treatments against AMR. The work contributes to the growing realization that negative or null antibacterial findings are

scientifically significant because they assist refine experimental methodologies and prevent overgeneralization of antimicrobial claims based only on traditional use or phytochemical profiles. To appropriately assess antibacterial potential, future studies should prioritize extraction methodologies that maximize active ingredient stability and bioavailability, as well as use more specific fractionation strategies.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper's content.

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