Asia-Pacific Journal of Science and Technology :Volume :31 Issue :01 Article ID :APST-31-01-03 Research Article

APST

Asia-Pacific Journal of Science and Technology

https//:www.tci-thaijo.org/index.php/APST/index

Published by Research and Innovation Department,
Khon Kaen University, Thailand

Antibacterial activity of calamansi, *Citrofortunella macrocarpa,* fruit waste extract against aquaculture pathogens

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Received 17 April 2024
Revised 26 August 2025
Accepted 30 September 2025

Abstract

Infectious diseases and multidrug-resistant microorganisms in global aquaculture have become major challenges in the industry that necessitate innovative solutions. This study investigated the antibacterial potential of Calamansi *Citrofortunella macrocarpa* fruit processing waste. The extracts were evaluated for their efficacy against known aquaculture pathogens, including *Streptococcus agalactiae*, *Aeromonas veronii*, *Edwardsiella tarda*, *Vibrio harveyi*, and *Vibrio parahaemolyticus*. Different drying methods (sun drying, dehydrator drying, oven drying) were used to obtain dried calamansi powder. The semi-solid extract was prepared via maceration with 95% ethanol. Extraction yields varied across drying techniques, with sun-dried samples yielding the highest (7%), oven-dried extracts at 6%, and dehydrator-dried samples the lowest (3%). Antibacterial activity was assessed via agar well diffusion, and potency was further confirmed by the minimum inhibitory concentration (MIC) method. Inhibition zones across pathogens ranged from 5.7 to 9.1 mm, compared to oxytetracycline as the positive control (14.6 mm). MIC assays revealed stronger antibacterial effects in sun-dried and dehydrator-derived extracts (256–512 mg/mL), while oven-dried extracts exhibited weaker activity (1024 mg/mL). Calamansi fruit waste emerges as a promising alternative for combating aquaculture pathogens, contributing to waste management and sustainable solutions. However, we show here that antimicrobial potential is significantly influenced by drying methods. Further research is crucial for identifying specific antibacterial compounds, optimizing application methods, and ensuring safety in aquaculture practices. This investigation contributes valuable knowledge for harnessing the antibacterial properties of calamansi waste in aquaculture, addressing the pressing concerns of infectious diseases and antibiotic resistance.

Keywords: Minimum inhibitory concentration, drying methods, *Streptococcus agalactiae*, *Aeromonas veronii, Edwardsiella tarda, Vibrio harveyi,* and *Vibrio parahaemolyticus*

1. Introduction

Challenges in treating infectious diseases and the emergence of multidrug-resistant microorganisms have led to an alarming increase in global aquaculture losses [1,2]. Consequently, there is a growing interest in exploring the potential of naturally active compounds, particularly antimicrobial agents, to combat these issues [3]. These natural compounds, such as phytocompounds, have become a focal point of research as potential alternatives to antibiotics in the fight against antimicrobial resistance. Bioactive agents, found in various plant species, offer

promising therapeutic potential [4]. Among the active compounds are alkaloids, terpenoids, pigments, polyphenols, quinones, lectins, tannins, essential oils, and polypeptides, which have demonstrated a range of beneficial effects, including antimicrobial properties, growth promotion, immune system enhancement, appetite stimulation, and stress reduction in fish [5,6,7].

The use of plants to treat various diseases dates back to ancient times and remains important in modern medicine. In addition, agricultural processes produce vast amounts of waste, yet plant-based by-products possess significant potential to combat diseases. Repurposing these residues as alternatives to fishmeal and soybean meal in aquafeed, fertilizers to improve live feed culture media, and sources of antimicrobial and immunostimulatory compounds provides a strategic approach to simultaneously address environmental waste management and health challenges [8]. Calamansi *Citrofortunella macrocarpa* is a member of the Rutaceae family and is known for its abundant essential oil content, making it a popular choice in beverages and condiments [9]. It is widely cultivated in tropical and subtropical regions, including Taiwan, China, Vietnam, Malaysia, and the Philippines, calamansi is considered an important fruit crop with its production continually increasing due to rising consumer demand [6,7]. As the citrus processing industry handles large quantities of calamansi fruit, substantial waste is generated annually, leading to significant disposal costs and potential environmental and human health issues if not managed appropriately [7,10]. However, studies have shown that this waste, particularly the pericarp, holds promise in terms of its potential for biotransformation and bioconversion of raw materials [11]. The abundance and cost-effectiveness of calamansi waste, along with the simplicity and affordability of its extraction, make it commercially viable [9]. Previous research has highlighted the antioxidant and antibacterial properties of *Citrus × macrocarpa* peel against a range of pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* [12]. Additionally, the peel demonstrated bacteriostatic activity against *Bacillus cereus*, indicating the presence of active compounds beyond flavonoids and sesquiterpenoids [13]. In their study, Lee & Najiah (2009) [14] identified a bioactive compound from *C. microcarpa*, which was revealed to be 2-hydroxypropane-1,2,3-tricarboxylic acid monohydrate and crude extract and evaluated the efficacy against 18 isolates including *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Streptococcus agalatiae*. The findings indicated that the isolated bioactive compound and crude extract exhibited promising antimicrobial activity suitable for aquaculture. Singh et al. (2021) identified the primary components of citrus peel essential oils as monoterpenes, sesquiterpenes, and their oxygenated derivatives. Specifically, limonene emerged as the major oil component found in the peel of various citrus species. Limonene is known for its wide array of beneficial properties, including antioxidant, anti-inflammatory, analgesic, antimicrobial, and anticancer effects, making it a valuable source of functional components with potential applications in various fields. Despite the investigations conducted by [14] on the antimicrobial activity of *C. microcarpa* extract against certain pathogenic agents in aquaculture, there is no available data regarding its effectiveness against *Vibrio harveyi* and *Vibrio parahaemolyticus*. Furthermore, Lee & Najiah (2009) [14] underscored the importance of additional *in vitro* and *in vivo* studies to further explore the antimicrobial properties of calamansi extract, particularly its potential efficacy against various fish pathogenic bacteria. Drying is one of the foremost and important steps during the processing of crops, medicinal plants, and herbs to preserve their properties [15]. It significantly influences the stability, concentration, and extractability of bioactive compounds. In citrus fruits, key phytochemicals such as flavonoids, essential oils, and organic acids are highly responsive to heat exposure, oxidative pressure, and water removal. The use of different drying methods could preserve or trigger the breakdown of such compounds. Existing studies have demonstrated that drying techniques significantly affect the antibacterial and antioxidant potentials of plant extracts through the alteration of bioactive component retention and availability [16, 17, 18]. Hence, more investigation is needed to explore better ways of drying plants and foods to retain more of their natural beneficial compounds. Doing so could significantly enhance the quality of the dried material, according to Belwal et al. (2022) [15]. Currently, there is no available data on the antibacterial activity of ethanol extracts derived from calamansi fruit waste subjected to different drying methods, which are likely to influence the potency of the extracts. Given the increasing utilization of industrial calamansi wastes, this study aims to investigate how various drying techniques affect the antibacterial activity of these extracts. Understanding these effects is essential to optimize processing methods and enhance the value of calamansi fruit waste. Therefore, this research aims to explore the antibacterial activity of calamansi fruit waste using various drying methods and their effectiveness against common pathogens in aquaculture. This study is expected to provide valuable insights into the potential applications of calamansi fruit waste in aquaculture, offering sustainable and innovative solutions to waste management challenges while maximizing the valorization of this abundant resource.

2. Materials and methods

2.1 Sample collection and preparation

The Calamansi Fruit Waste (CFW) was obtained from a local processing industry situated in Zamboanga Sibugay, Mindanao, which produces a large amount of calamansi waste daily and consequently faces waste

disposal problems due to the citrus byproduct. The samples were promptly transported to the laboratory and upon arrival, were subjected to a thorough washing process using running water to eliminate any dirt or dust. The samples were then stored at a temperature of 4º C until further processing. During the drying process, the CFW samples were thawed and subsequently subjected to three different drying methods sun drying, dehydrator, and oven at 40°C. The fresh-to-dry ratio was calculated to determine the total weight after drying, and the samples were placed in zip lock bags and stored in airtight containers. The plant extracts were prepared using 95% ethanol as the solvent, and a maceration process was conducted at room temperature for 6 days 1:10 dried calamansi to ethanol ratio. The resulting solvent filtrates will be gathered after each maceration cycle and concentrated at 40°C using a rotary evaporator to yield a dense, concentrated extract. At the final stage of pre-treatment, the percentage yield of extraction Y (%) was computed using the formula proposed by [19]:

$$Y(\%) = \frac{mass\ of\ dried\ sample\ after\ extraction\ proces}{dry\ mass\ of\ the\ fresh\ sample\ prior\ extraction} x\ 100 \quad (1)$$

2.2 Bacterial isolates

Five species of aquaculture pathogenic bacteria covering the Gram -positive Streptococcus agalactiae and Gram-negative *Aeromonas veronii, Edwardsiella tarda, Vibrio harveyi,* and *Vibrio parahaemolyticus* which were obtained National Fisheries Research and Development Institute-Fisheries Biotechnology Centre, Science City of Munoz, Philippines and *Vibrio parahaemolyticus* provided by the Aquaculture Department, Southeast Asian Fisheries Development Center in Tigbauan, Iloilo, Philippines. The bacteria were maintained on Tryptic Soy Agar (TSA) plates at 4 °C before the assay.

2.3 Preparation of microbial suspensions

The preparation of agar media was done according to the manufacturer's guidelines. The bacteria were subcultured on MHA and incubated at 37 °C for 24 h before inoculum preparation. A single microbial test loop was obtained from a pure culture and suspended in sterile physiological NaCl and homogenized using a vortex. The turbidity of the suspension was evaluated by comparing it to a 0.5% Mc-Farland standard against a black background and bright light. The test extract was dissolved in a dimethyl sulfoxide solution (DMSO) with a concentration of 1:1 mg/mL solvent and extract [12] with modification.

2.4 Antibacterial activity by agar well diffusion method

To evaluate the antibacterial effectiveness of the calamansi fruit waste, we conducted an assay using the agar well diffusion method, adapted from [20-22]. Mueller Hinton agar medium, heated to 40–45 °C, was poured into sterile petri plates and inoculated with various microbial cultures, allowing it to solidify for 15–20 minutes. Using a sterile borer, wells of five mm diameter were created in each plate. Then, 50 µL of CFW solvent stock solution extracts, with a concentration of 1000 mg/mL, were added to the wells, while 30 µL of broad-spectrum antibiotic Oxytetracycline containing 6.25 mg/mL served as the positive control. Dimethyl sulfoxide was used as the negative control solvent for each extract. The plates were then incubated at 37°C for 24 hours. Following incubation, the zone of inhibition was measured, excluding the wells (5 mm). Inhibition zones greater than 15 mm were classified as strong, 8 to 15 mm as moderate, and 1 to 8 mm as weak activities.

2.5 Determination of the minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) is a crucial measure of antimicrobial potency, representing the lowest concentration that can inhibit or kill specific microorganisms. In this study, twelve concentrations (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024) of CFW were prepared by dilution with sterile distilled water, including a drug-free control. MHA medium was prepared according to the manufacturer's instructions. Nineteen (22) mL volumes of molten agar were mixed thoroughly with 1 mL volume of the antimicrobial solution, making the total volume 20 mL, and poured into prelabeled sterile Petri dishes. Dilutions of the culture adjusted to a concentration of 10^6 –10^7 microorganisms per mL were prepared for use as inoculum in the MIC test. A 0.5 McFarland standard was utilized for visual comparison to adjust the suspension to a density of approximately 10^8 CFU/mL. Plates were inoculated within 30 minutes of standardizing the inoculum to avoid changes in density. A micropipette was used to inoculate plates with 0.5 µL spots, starting with the lowest concentration, and a control agar plate without any extract. The plates were allowed to dry at room temperature before inverting them for incubation at 35-37 ºC for 16 to 20 hours. Colony counting was performed the next day, adopting the method

outlined by Eucast (2000) [23], and the Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution [20] with modifications. Bacterial growth was monitored during this period, and when no growth was observed in the medium containing the lowest concentration of the test materials, the MIC was defined at this point of dilution [2].

2.6 Statistical Analysis

One-way analysis of variance (ANOVA) and post hoc Duncan test were used to evaluate the differences of the inhibition zones (mean ± SD) among different extracts and pathogens. Prior to ANOVA, normality and homogeneity of variance tests were conducted to ensure data met the assumptions. All statistical analyses were performed using software R version 4.2.2. Statistical significance was designated as a p value < 0.05, and all experiments were performed in triplicate.

3. Results and discussion

During the preparation of the CFW samples in the drying process, it was observed that the sun drying method takes 2-3 days, depending on the weather and thermal conditions. For the dehydrator, the model allowed continued vertical heat and airflow for drying. This method was done only for 1 to 2 days at 45°C, and oven-dry at 45°C was dried for 4-5 days. Under different drying methods, the highest percentage of yield was achieved in CFW Sun drying 7% and Oven drying 6%, while Dehydrator drying 3% samples showed a smaller yield Table 1. According to [21], the overall yields of each sample from the extraction of *C. macrocarpa* waste indicate a significant mass loss during the extraction process. Possible reasons for the lower extraction yield may include loss of some sample recovered during the entire vacuum filtration and drying process. In this study, it was also observed that the yield of the extract was sticky in form. Similar results were reported earlier where the extraction using a rotary evaporator was stopped when a thick extract was obtained in the flask [12].

Table 1 Yield of calamansi fruit waste in different drying process.

Drying process	Fresh weight (g)	Dried weight (g)	Ethanol (mL)	Yield (%)
Sun Drying	10	0.7	100	7
Dehydrator	10	0.3	100	3
Oven Drying	10	0.6	100	6

Citrus is a widely cultivated fruit globally and has undergone extensive industrialization for various purposes, primarily valued for its nutritional advantages. However, the rapid pace of industrialization has presented challenges, leading to the disposal of parts such as calamansi fruit peel, pulp, and seeds into the environment as waste. This situation has created opportunities for exploring alternative methods to utilize this waste. Recent technological advancements have prompted researchers to seek alternatives for extracting potential therapeutic compounds from citrus fruit waste which directly addresses concerns related to waste management [21].

The utilization of calamansi fruit waste for antibacterial activity against aquaculture pathogens remains limited, and existing studies on its antibacterial activity have primarily focused on human pathogens, leaving room for further exploration. To address this research gap, the main objective of this study was to evaluate the ability of the ethanolic extract from calamansi fruit waste, utilizing different drying processes, to inhibit the growth of pathogenic bacteria in aquaculture. Also, it provides information on the potential of this undervalued waste to be used in the future as an alternative or supplement to common antibiotics in aquaculture.

The antimicrobial activity of the calamansi fruit waste methods was determined by agar well diffusion method. Results of the antimicrobial activity assessment of ethanolic extracts from CFW, subjected to different drying processes, showed good activity against the aquaculture pathogens (Figure 1 and 2). Statistical analysis result confirms that antibacterial activity of CFW against aquaculture bacterial pathogens differs among the drying processes (Figure 1). For *S. agalactiae*, both Sun drying (SD) and dehydrator drying (D) exhibited moderate activity with inhibition zones of 8.5±0.9 mm and 9.1±1.7 mm, respectively, while Oven drying (OD) displayed weaker activity with an inhibition zone of 5.7±1.7 mm. Oxytetracycline (OTC) demonstrated the highest activity with an inhibition zone of 14.6±1.0 mm while there were no inhibition zones in the negative controls DMSO. Statistical analysis confirmed significant differences between the means of SD, D, and OD, emphasizing the distinct antimicrobial effects of each drying process. Similar patterns were observed for *A. veronii*, *E. tarda*, *V. harveyi*, and *V. parahaemolyticus*, with OTC consistently exhibiting the highest activity and CFW extracts statistically differing from the drying processes.

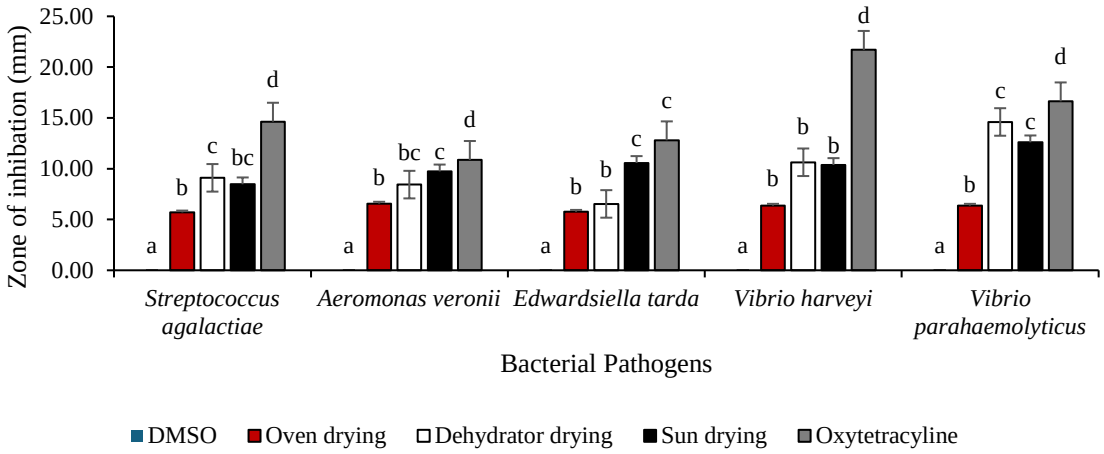

Figure 1 Graph of Antibacterial activity of calamansi fruit waste (CFW) in different drying processes against aquaculture pathogens. Values in the same row with different superscript letters (a, b, c, d) are significantly different according to Duncan's Multiple Range Test ($p < 0.05$).

These findings underscore the potential influence of drying methods on the antimicrobial potential of calamansi fruit waste extracts against aquaculture pathogens, providing valuable insights for further applications in the industry. The antibacterial activity of CPW extracts in all drying processes, however, showed statistically similar performance to OTC in inhibiting *A. veronii* and *E. tarda* for sundried and dehydrated *V. parahaemolyticus*. This suggests that CPW extracts can potentially be used as a substitute for OTC in eliminating these particular pathogenic bacteria. Roshni *et al.*, 2023 [24] suggest that the strong antibacterial activities of calamansi fruit waste ethanolic extract may be due to the presence of flavonoids. Flavonoids are among the major constituents of polyphenols found in different parts of Citrus fruits (skin, peels, seed, pulp membrane, and juice). Flavonoids have different biological properties antiviral, antifungal, and antibacterial activities [25]. While Zaim et al. (2023) [19] found that the alkaline reagent test conducted also indicated the presence of flavonoids in the peel samples of citrus fruits. In Husni et al. 2020 [12] study, hesperidin, a flavonoid from the flavanone subclass, which are low-molecular-weight polyphenolic substances found in citrus fruits, demonstrated the ability to inhibit the growth of *S. aureus*, *E. coli*, *E. faecalis*, and *P. aeruginosa*. The antibacterial properties of flavonoids are significantly influenced by their amphipathic characteristics [26]. The amphipathic nature of flavonoids allows them to interact with bacterial cell membranes, disrupting their structure and function. This interaction can lead to increased permeability and leakage of cellular contents, as well as interference with essential cellular processes, such as enzyme activity and DNA replication [27, 28]. These characteristics are crucial for their ability to fight bacterial infections.

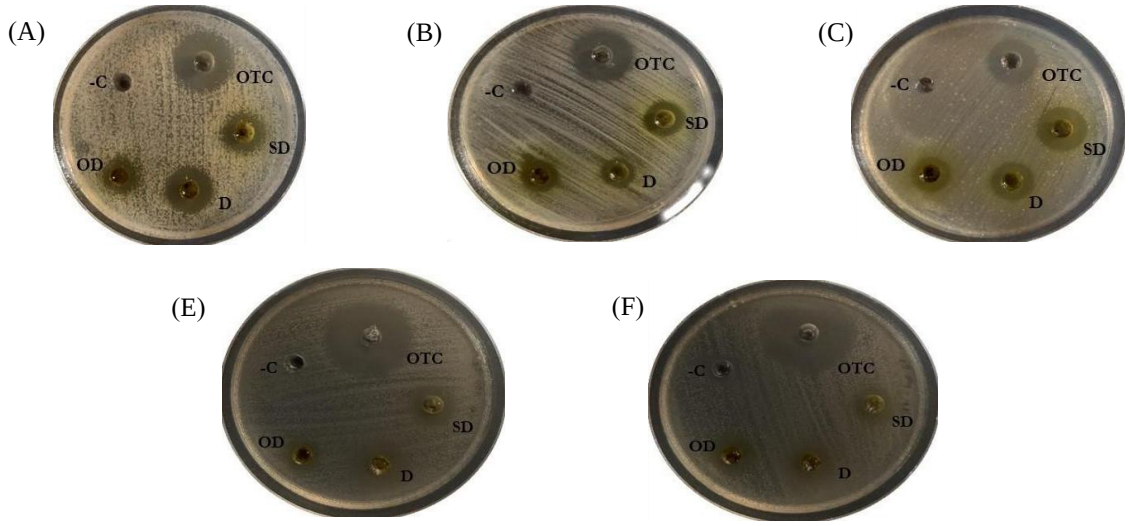

Figure 2 Inhibition zones of calamansi fruit waste extracts from different drying methods against aquaculture pathogens by agar well diffusion method (A) *S. agalactiae*, (B) *A. veronii*, (C) *E. tarda*, (D) *V. harveyi*, and (E) *V. parahaemolyticus*. Treatments C (DMSO), OTC (Oxytetracycline), SD (Sun-dried), D (Dehydrated), OD (Oven-dried).

The agar dilution method was employed for determining MIC. As stated by [24], the minimum inhibitory concentration refers to the smallest concentration of an antimicrobial substance, such as plant extracts or agents with bactericidal, bacteriostatic, or antifungal properties, which can hinder the growth of microbial pathogens following an overnight incubation period. A notable advantage of the agar dilution method lies in its ability to simultaneously test numerous bacterial isolates under uniform conditions, as highlighted by Wiegand et al. (2008) [22]. In the study by [14], MIC for *A. hydrophila*, *E. tarda*, and *S. agalatiae* were reported as 31.6 mg/mL, 15.6 to 62.5 mg/mL, and 31.6 mg/mL, respectively, for 2-Hydroxypropane and 1,2,3-tricarboxylic acid. The MIC value for the crude extract against these bacteria ranged from 7.8 to 31.3 mg/mL. The compounds 2-hydroxypropane-1,2,3-tricarboxylic acid monohydrate was identified as responsible for the antimicrobial activity of the aqueous extract of *C. microcarpa*. The MIC value of the *C. microcarpa* crude extract was lower than that of both isolated and synthetic citric acid against the bacterial isolates, indicating its efficiency. In the current study, the MIC values for calamansi fruit waste extracts against *S. agalactiae*, *A. veronii*, *E. tarda*, *V. harveyi*, and *V. parahaemolyticus* were consistently 512 for Sun Dried and Dehydrated extracts, with a lower MIC (256) for Dehydrated extract against *V. harveyi* and *V. parahaemolyticus* (Table 2). Conversely, the Oven Dried extract showed higher MIC values (1024), indicating potentially reduced antimicrobial effects. Therefore, based on the result antimicrobial agent extracted from calamansi especially waste required a high concentration dosage to exhibit antimicrobial activity. Additionally, the prolonged drying of OD samples can be attributed to the loss of some nutrients and compounds, potentially affecting the antibacterial potential of the extract. It was stated by [29] that extended drying periods can also lead to higher losses of unblanched tissues due to enzymatic browning. In this study, the browning of CFW was observed in oven-dried samples. Additionally, [30] investigated the effects of drying processes on total phenolics, antioxidant activity, and flavonoid contents of common Mediterranean herbs. Their findings suggest that oven drying has significantly reduced herbs' total phenolics, antioxidant activity, and flavonoid content compared to other drying methods. Considering these factors and the results of the antibacterial experiment, sun-dried and dehydrator drying can be the best alternative for retaining biochemical compounds responsible for its antibacterial effect due to faster drying compared to other drying methods. However, considering that the choice of drying method depends on various factors such as the type of product, availability of dryer, energy consumption, cost of dehydration, and quality of dehydrated product, research on this area can potentially optimize drying technology to reduce postharvest losses [29]

Table 2 Minimum inhibitory concentration (MIC) (mg/mL) values of ethanolic extracts from calamansi fruit waste in different drying processes against aquaculture pathogens.

Bacterial Pathogen	Sun Dried (mg/mL)	Dehydrated (mg/mL)	Oven Dried (mg/mL)
S. agalactiae	512	512	1024
A. veronii	512	512	1024
E. tarda	512	512	1024
V. harveyi	512	256	512
V. parahaemolyticus	512	256	512

Furthermore, variations in MIC values between studies could be attributed to factors such as extraction methods, sample quality, geographic origins, and transportation conditions. As per Lee & Najiah (2009) [14], the antimicrobial capability of *C. microcarpa* extract may exhibit tolerance to a broad range of temperatures. Interestingly, this study revealed that the drying process plays a significant role, holding substantial potential to impact the observed differences in antibacterial properties. Therefore, it is essential to take the drying process into account when targeting specific antibacterial compounds.

4. Conclusions

In conclusion, the extraction process of calamansi fruit waste, employing various drying methods, demonstrated varying yields, with Sun drying and Oven drying yielding the highest percentages. However, a higher yield does not necessarily translate directly to increased antibacterial potency of the extract. This phenomenon was observed in dehydrated samples, which, despite having a lower extraction yield, demonstrated stronger antibacterial activity compared to oven-dried samples. The reduced efficacy in oven drying may be attributed to prolonged drying time and oxidation occurring at 40 °C. Additionally, it is recommended to measure the moisture content of the dried powder, as this parameter may influence the extraction yield and bioactivity of the resulting extract. The ethanolic extract from CFW exhibited significant antibacterial activity against aquaculture pathogens, with distinct effects observed among different drying processes. Sun drying and dehydrator drying showed moderate activity against *S. agalactiae*, *A. veronii*, *E. tarda V. harveyi, and V. parahaemolyticus*, while Oven drying displayed weaker activity. The choice of drying method significantly influenced the antimicrobial potential, emphasizing the need for careful consideration in processing. The MIC of the extracts were consistent for Sun Dried and Dehydrated extracts but higher for Oven Dried, indicating potentially reduced antimicrobial effects. The study highlights the potential of calamansi fruit waste as an

alternative source of antibacterial agent for aquaculture pathogen, but further research is essential to identify specific antibacterial compounds, optimize utilization, and ensure safety in aquaculture practices. Additionally, the influence of drying methods on antimicrobial properties can be an effective approach in maximizing the efficacy of calamansi fruit waste extracts while taking into account its economic viability.

5. Acknowledgements

This research was funded by the Department of Science and Technology - Science Education Institute Science and Technology Regional Alliance of Universities for National Development (STRAND), and the Department of Science and Technology, Philippine Council for Agriculture and Aquatic and Natural Resources Division (DOST PCAARD – IARRD) Fruit Waste Project "Utilization of Fruit Processing Waste as a source of Prebiotic and Immunostimulants for the Development of Healthy and Improved Aquaculture Feeds" of Mindanao State University at Naawan, Naawan, Misamis Oriental. We would also like to express our gratitude to the National Fisheries Research and Development Institute-Fisheries Biotechnology Centre, Science City of Munoz, Philippines for allowing us to use the laboratory facilities for this study.

6. Conflicts of interest

The authors have declared that no conflict of interest exists.

7. Author contributions

All authors contributed significantly to the completion of this study. FFFagutao led the conceptualization, design, funding acquisition, experiment execution, data collection, analysis, drafting, editing, and project administration. FLPedroso contributed to the study design, funding acquisition, experimental work, data analysis, manuscript drafting, editing, and project coordination. CHChoresca, Jr. and CMACaipang were involved in conceptualization, data interpretation, manuscript writing, editing, and project oversight. MJSalutan participated in all aspects of the research, including design, funding, experimentation, data handling, analysis, writing, and administration. HTaka contributed to the study design, data analysis, manuscript drafting, an editing. All authors reviewed and approved the final version of the manuscript.

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