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Research Article

Antifungal Activity of Roselle Decoction Residue Extract Against *Pestalotiopsis* sp.: Valorization of Agro-Industrial Waste

Nur Amirah Yusoff[1], Fauziah Tufail Ahmad[1,2], Aidilla Mubarak[1,2], Razifah Mohd Razali[3], Husni Hayati Mohd Rafdi[1,2]*

1. Faculty of Food Science and Agrotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
2. Research Program of Postharvest Research and Innovation, Food Security Research Cluster, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
3. Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

*Corresponding author: husni@umt.edu.my

ABSTRACT

This study investigates the antifungal activity of crude ethanolic extracts derived from roselle decoction residues, generated during calyx cordial and juice processing, against *Pestalotiopsis* sp., a postharvest fungal pathogen associated with fruit rot in wax apples. Valorizing this agro-industrial by-product presents an opportunity to develop sustainable antifungal agents. The experiment evaluated the efficacy of extracts prepared from both fresh and freeze-dried residues, namely roselle calyx (RC), decocted cordial residue (DCR), and decocted juice residue (DJR), at eight different concentrations (10 to 100,000 mg/L) using *in vitro* and *in vivo* assays. *In vitro* results revealed that freeze-dried samples exhibited significantly higher antifungal activity than fresh samples, with complete inhibition observed at lower concentrations (30,000 mg/L) for freeze-dried RC and DCR. DJR showed the lowest efficacy among all calyx sources. *In vivo* evaluation confirmed that coating wax apples with the most effective extracts reduced disease severity index (DSI) compared to both untreated and chitosan-treated controls. Notably, freeze-dried RC resulted in the lowest DSI, indicating strong protection against fungal infection. The antifungal effects were found to be dose-dependent and involved both fungistatic and fungicidal mechanisms. The superior efficacy of freeze-dried samples is attributed to the better retention of bioactive compounds such as anthocyanins and phenolics, which may disrupt fungal cell integrity and activate plant defense responses. This study supports the use of roselle decoction residues as a low-cost, eco-friendly alternative to synthetic fungicides, aligning with sustainable postharvest disease management strategies.

Key words: Antifungal activity, decoction residues, *Pestalotiopsis* sp., postharvest disease management, roselle, waste valorization

INTRODUCTION

The use of plant-derived crude extracts has emerged as a sustainable alternative to synthetic fungicides for managing postharvest diseases, which are often associated with environmental pollution, human health risks, and the development of fungicide-resistant strains (Neela *et al*., 2014; Meelah *et al*., 2017; Troncoso-Rojas *et al*., 2019; Ngibad *et al*., 2021). Various plant extracts rich in bioactive compounds have demonstrated broad-spectrum antifungal activity against phytopathogens such as *Aspergillus* spp., *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium* spp., *Penicillium* spp., and *Pestalotiopsis theae* (Pretorius *et al*., 2003; Saha *et al*., 2005; Neela *et al*., 2014; Bashar *et al*., 2015; Harikamal *et al*., 2015; Mahlo *et al*., 2016; Siti Fairuz, 2022; Salas-Gómez *et al*., 2023).

These pathogens are known to cause significant losses in fruit production through postharvest decay and spoilage (Agrios, 2005; Confortin *et al*., 2019; Oniha *et al*., 2021). *Pestalotiopsis* is classified as a genus of phytopathogenic fungi known to cause diseases in various plants, including fruit and ornamental crops. These fungi can lead to symptoms such as leaf spots, blights, and fruit rot, making them significant pathogens in agriculture (Maharachchikumbura *et al*., 2014). Managing phytopathogenic fungi is crucial for maintaining healthy crops and ensuring agricultural productivity. These plant fungal pathogens cause significant economic losses in the fruit industry and contribute to postharvest diseases (Mahlo *et al*., 2016).

Given its widespread occurrence and the difficulty of controlling *Pestalotiopsis* sp. with conventional fungicides, this pathogen poses a considerable threat to postharvest fruit quality. In wax apple (*Syzygium samarangense*), infections often manifest as fruit rot and necrotic lesions, leading to rapid spoilage during storage and transport. Its ability to survive in plant debris and infect through mechanical wounds increases its persistence and recurrence in orchard environments (Maharachchikumbura *et al*., 2014; Darapanit *et al*., 2021). The limited success of chemical treatments due to fungicide resistance and residue concerns has prompted interest in botanical alternatives with antifungal potential. However, little research has focused on the application of roselle decoction residues against *Pestalotiopsis* infections, particularly in tropical fruit systems.

Some of natural extract derived from plant based have demonstrated significant antifungal efficacy in various studies,

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including *Moringa oleifera* (Neela *et al*., 2014; Oniha *et al*., 2021), *Azadirachta indica* (Bashar *et al*., 2015; Ali *et al*., 2023), *Polyalthia longifolia* (Arora *et al*., 2015), *Aglaia odorata* L., *Chromolaena odorata* L., *Toona sureni* Merr (Ngibad *et al*., 2021), *Bucida buceras*, *Breonadia salicina* (Mahlo *et al*., 2016), and *Lupinus albescens* (Confortin *et al*., 2019). Most of these studies utilised fresh or air-dried plant materials that were cultivated or harvested specifically for experimental use.

In contrast, increasing attention has recently been directed toward plant-derived wastes and by-products, such as peels, pomace, and decoction residues, as sustainable sources of bioactive antifungal compounds. These waste-derived materials retain substantial levels of phenolic compounds and other bioactive metabolites with antimicrobial potential, making them suitable for postharvest disease control applications (’Aqilah *et al*., 2023; Durmus, 2024). The extracts obtained from garlic peel and potato peel have also demonstrated notable antifungal efficacy against phytopathogens under both in vitro and in situ conditions, supporting their potential as eco-friendly alternatives to synthetic fungicides (Teixeira *et al*., 2023; Ikyenge *et al*., 2024).

The antifungal effectiveness of such plant-based extracts is strongly influenced by extraction method, solvent polarity, concentration, and target pathogen species (Jeff-Agboola & Onifade, 2016). Despite the variability in performance compared to synthetic fungicides, these natural formulations support sustainable agriculture by reducing chemical residues and ecological impact.

Roselle calyces are particularly rich in flavonoids such as anthocyanins, gossypeptin, hibiscritin, delphinidin 3-glucoside, and cyanidin 3-glucoside, which possess notable antifungal properties (Maryani & Kristiana, 2005; Sari *et al*., 2012). These secondary metabolites have shown inhibitory effects on fungal growth, including *Trichophyton rubrum*, by interfering with cell wall integrity and enzymatic pathways. Moreover, anthocyanins and phenolics enhance plant defense by stimulating antioxidant enzyme activity and reinforcing structural barriers. This could make roselle calyx extract a promising natural agent for controlling fungal diseases while promoting plant health. Thus, the objective of the present study was to evaluate *in vitro* and *in vivo* antifungal activities of roselle decoction residues, ethanolic crude extract of both sample states (fresh and freeze-dried) against *Pestalotiopsis* sp. in controlling fruit rot disease on wax apple fruits under laboratory conditions.

MATERIALS AND METHODS

Roselle decoction preparation

Roselle calyces of the Terengganu variety (UMKL-1) were obtained from Aslah Hibiscus, Aslah Supply and Services, Batu Pahat, Johor, Malaysia (1°56′01.1″N, 102°48′19.6″E). Freshly harvested calyces were transported on the same day to the Postharvest Laboratory, Faculty of Food Science and Agrotechnology, Universiti Malaysia Terengganu (5°24′35.1″N, 103°05′09.4″E). The calyces were selected based on uniformity in size, maturity, and absence of physical damage or defects, following standard postharvest quality criteria (Da-Costa-Rocha *et al*., 2014; AOAC, 2019).

Before processing, calyces were separated from the seed capsules and washed thoroughly with tap water to remove adhering impurities. Decoction was performed according to an industrial protocol provided by A.I. Agro Marketing Sdn. Bhd., Kuala Terengganu, Malaysia. Calyces were boiled in water at ratios of 1:1 (w/v) for cordial production and 1:2 (w/v) for juice preparation, and heated at approximately 100°C for 30 min. Following boiling, the liquid fractions were separated to obtain the beverage products, while the residual plant material was collected using a muslin cloth and gently pressed to remove excess liquid.

Two sample conditions were prepared: fresh and freeze-dried. Fresh residues were chopped into small pieces and used immediately to preserve their native moisture content and thermolabile constituents. For freeze-drying, the decocted residues were wrapped in aluminium foil, frozen at −80°C for 48 hr, and subsequently lyophilised using a freeze dryer (Labconco Corporation, USA) for 72 hr.

Untreated roselle calyces (RC) were included as controls and were analysed in both fresh and freeze-dried forms without undergoing decoction. All samples were stored at −30°C before analysis to minimise enzymatic activity, oxidative degradation, and microbial growth. The resulting decoction residues represent the principal by-products generated during industrial cordial and juice production and were therefore selected for evaluation as potential sources of natural antioxidants and antimicrobial agents for edible coating applications.

Crude extract preparation

The ethanolic crude extracts were prepared using the cold extraction maceration method. Fresh and freeze-dried samples, each weighing 200 g, were placed into a conical flask that was completely wrapped in aluminium foil to minimize light exposure. Pure ethanol (95%), pre-cooled at 5°C for 2 hr, was then added to the conical flask. The flasks containing the samples and ethanol were placed in an orbital shaker (Stuart SSL1, Cole-Parmer Ltd., United Kingdom) and shaken constantly at 150 rpm for 24 hr at room temperature. Then, the macerated ethanol solutions were filtered using filter paper (Whatman no. 2, UK), and all extracted samples were collected in a wrapped laboratory glass bottle and stored at −20°C. The remaining samples in the conical flask were extracted twice more, with all extracted samples collected in the same wrapped laboratory glass bottle. Subsequently, the extracted samples were concentrated using a rotary evaporator (Buchi R-300 EL, Büchi Labortechnik AG, Switzerland) to obtain crude ethanolic extracts. Finally, the crude ethanolic extracts were stored at −30°C before further analysis.

In vitro antifungal activity assay

The crude ethanolic extracts, namely, for both fresh and freeze-dried samples, are roselle calyx (RC), decocted cordial residue (DCR), and decocted juice residue (DJR). The crude extract was dissolved in 1 mL of sterile distilled water using a 0.4 µm syringe filter (Sartorius). Then, the extract solution was mixed with 15 mL of PDA in a sterile vial. The mixture was vortexed thoroughly and poured into a 90 mm petri dish. The agar medium was prepared individually for each crude extract at concentrations of 10, 100, 1000 (0.1%), 10,000 (1%), 30,000 (3%), 50,000 (5%), 70,000 (7%), and 100,000 mg/L (10%). The agar medium in petri dishes was allowed to solidify. These crude extracts were evaluated *in vitro* against the following poison food technique (Dhingra & Sincair, 1985).

The mycelial agar plugs from the pure culture with 6 mm diameters were aseptically placed at the center of the petri dish containing different concentrations of crude extracts. Then, the plates were put into an incubator (Model LM-575RD, Yihder Technology Co., Ltd., Taipei, Taiwan) and incubated at 25 ± 2°C for five days to produce full-plate growth of mycelium with conidia. Six replicates were used for each treatment. The radial growth of the pathogenic fungus was recorded after five days of incubation when the fungus in the control was covered on the whole agar plate. The percentage inhibition of radial growth (PIRG) was calculated by using the formula of Yusoff *et al*. (2020) as Equation 1. Where x = average of mycelial in control plates; y = average growth in extracted treated plate.

$$\mathrm{PIRG} = \frac{\mathrm{x} - \mathrm{y}}{\mathrm{x}} \times 100 \qquad \text{- Equation 1}$$

***In vivo* antifungal activity assay**

The preparation method for fruits and fungal plugs used in the *in vivo* bioassay was similar to that of the *in vitro* bioassay. The fresh and premium quality of wax apples was washed under running tap water and air-dried for 2 hr. Then, the wax apples were surface sterilized using 70% (v/v) ethanol for 1 min, 1% sodium hypochlorite (NaOCl) for 1 min, and then rinsed with sterile distilled water for 2 min. Then, they were air-dried in a laminar flow at ambient temperature for 30 min. After that, the wax apples were dipped into the most effective extract solutions that from the most effective extract solutions from highest antifungal activity from *in vitro* bioassay for 30 sec. For the negative control, the wax apples were dipped in sterile distilled water. Meanwhile, for the positive control, the wax apples were dipped in chitosan 1%. All treated fruits were wounded with a sterile needle at the equatorial side. The mycelial agar plugs from 7 days culture was place on the fruits. Each of the treated wax apples was placed on separated plastic box containing water to keep the moisture and incubated at 25 ± 2°C. All inoculated fruits were visually assessed daily, and the symptoms were recorded. Each treatment was replicated six times, and each replication consisted of 18 fruits. At the end of storage duration, the disease severity index (DSI) was determined and calculated as Equation 2. Where, a = disease scale; n = number of fruits in a specific scale; A = highest disease scale; B = total number of fruits.

$$\mathrm{DSI} = \sum \frac{\mathrm{a} \times \mathrm{n}}{\mathrm{AB}} \times 100 \qquad \text{- Equation 2}$$

The DSI was calculated using the Townsend-Heuberger equation (Townsend & Heuberger, 1943; Chiang *et al*., 2017). The fungal growth symptoms were observed visually using the following scales, as shown in Table 1.

Table 1. Disease severity scale of disease assessment for wax apple

Scale	Rating	Description
0	No infection	No visible symptoms on the fruit
1	Mild infection	1 – 25% of the area covered by slight necrotic and fungal mycelia
2	Moderate infection	26 – 50% of the fruit area is covered by necrotic and fungal mycelia
3	Severe infection	51 – 75% of the fruit is necrotic with the presence of a spore mass
4	Very severe infection	> 76% necrotic tissue with fungal mass, and the fruit appears soft and decayed

Experimental design and statistical analysis

For the *in vitro* bioassay, the study was conducted in a completely randomized design (CRD) with six replications. The test was arranged in factorial analyses consisting of sample state (fresh, and freeze-dried), calyx source (RC, DCR & DJR) with eight concentration levels (10, 100, 1000 (0.1%), 10,000 (1%), 30,000 (3%), 50,000 (5%), 70,000 (7%), and 100,000 mg/L (10%)). The experimental design and treatment combinations are summarized in Table 2.

Table 2. Experimental design of the antifungal assay

Factor	Level	Description
Experimental design	–	Completely randomized design (CRD)
Replications	6	Six replicates per treatment
Sample state (S)	2 levels	Fresh, Freeze-dried
Calyx source (CS)	3 levels	RC (roselle calyx), DCR (decoction residue), DJR (juice residue)
Extract concentration (C)	8 levels	10, 100, 1,000, 10,000, 30,000, 50,000, 70,000, 100,000 mg/L
Experimental layout	Factorial	S × CS × C

For the *in vivo* bioassay, the study was conducted in the CRD with six replications. The treatment involved including sterile distilled water (negative control), chitosan (positive control), fresh RC, fresh DCR, freeze-dried RC, and freeze-dried DCR. The data were analyzed using ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post hoc test with a significance level of $p \leq 0.05$. All data analysis was carried out in SAS Studio 3.81 (SAS Institute Inc., Cary, NC, USA., 2024).

RESULTS AND DISCUSSION

***In vitro* antifungal activity assay**

Table 3 revealed significant interaction effects among sample state (S), calyx source (CS), and concentration (C) on PIRG (%) values. These interactions indicate that the influence of one factor on PIRG depends on the levels of the other factors. The

interaction between sample state and calyx source (S × CS) was significant, suggesting that the effect of sample state on PIRG depended on the specific calyx source. Freeze-dried samples exhibited higher PIRG values than fresh samples when RC or DCR was used. However, for DJR, this trend was either less pronounced or reversed, with both sample states (fresh and freeze-dried) showing lower PIRG values. This emphasizes the significance of considering both sample states and calyx source when interpreting PIRG values.

Similarly, the interaction between sample state and concentration (S × C) revealed that the effect of sample state on PIRG varies across different concentration levels. At higher concentrations (10,000 to 100,000 mg/L), freeze-dried samples consistently exhibited higher PIRG values than fresh samples. However, at lower concentrations (10 to 1,000 mg/L), this difference was minimal, with both sample states exhibiting similarly low PIRG values. This suggests that the influence of sample state on PIRG is more significant at higher concentration levels.

The interaction between calyx source and concentrations (CS × C) further emphasizes that the effect of concentration on PIRG depends on the calyx source. Across all concentration levels, RC consistently exhibited higher PIRG values than DJR. However, the difference between RC and DCR was more significant at higher concentrations, whereas at lower concentrations, the variation was not significant. This suggests that the impact of calyx source on PIRG is more evident at higher concentrations.

The most complex interaction observed was among sample, sample state, calyx source, and concentration (S × CS × C). The significance of this interaction implies that the combined effects of these factors on PIRG cannot be predicted solely from their individual effects. The extent of PIRG variation across sample state and calyx source was dependent on concentration. For instance, the difference in PIRG between fresh and freeze-dried samples was more pronounced for RC at high concentrations than for DCR at low concentrations. This emphasizes the need to consider all three factors together when analyzing PIRG responses.

Table 3. Two-way analysis of variance (ANOVA) for response variables

Source of variation	Sum of square	Mean square	F value	Pr > F
S	8805.9721	8805.9721	3385.04	<.0001
CS	47524.6530	23762.3265	9134.30	<.0001
C	392717.3632	56102.4805	21565.9	<.0001
S × CS	2763.8756	1381.9378	531.22	<.0001
S × C	5178.9866	739.8552	284.40	<.0001
CS × C	21431.6998	1530.8357	588.46	<.0001
S × CS × C	11103.4322	793.1023	304.87	<.0001

Significant ($p \leq 0.05$), very significant ($p \leq 0.01$), highly significant ($p \leq 0.001$), S: sample state, CS: calyx source, C: concentration, PIRG: percentage inhibition of radial growth. (n=18).

Figure 1 and Table 4 showed that the antifungal activity of roselle decoction residues was assessed across various concentrations ranging from 10 mg/L to 100,000 mg/L for both fresh and freeze-dried samples against the isolated fungal pathogen (HSD = 3.3898, p<0.0001). At lower concentrations (10 mg/L to 1000 mg/L), the antifungal activity varied considerably. The ethanolic crude extracts, freeze-dried RC, DCR, and DJR also exhibited low antifungal activity at low concentrations (10 mg/L to 1000 mg/L), with 1.38 ± 0.94%, 0.87 ± 0.92%, and 1.43 ± 1.63%, respectively. As increasing the concentration increased to 1000 mg/L, the inhibition for freeze-dried RC also increased to 11.15 ± 2.06%, freeze-dried DCR to 10.48 ± 4.35%, and freeze-dried DJR to 3.79 ± 0.62%. Meanwhile, at 10 mg/L, fresh RC and DCR showed significantly lower inhibition percentages of 4.18 ± 1.52% and 1.85 ± 0.59%, respectively, while fresh DJR exhibited inhibition at 0.99 ± 0.21%. As the concentration increased to 100 mg/L, fresh RC inhibition rose to 8.09 ± 1.35%, whereas fresh DJR inhibition slightly increased to 1.30 ± 0.62%.

At medium concentrations (10,000 mg/L & 30,000 mg/L), both freeze-dried RC and DCR showed a substantial inhibition of 79.23 ± 0.79% and 72.29 ± 1.29% at a concentration of 10,000 mg/L, respectively, while freeze-dried DJR showed 6.96 ± 1.54% inhibition. At 30,000 mg/L, both freeze-dried RC and DCR achieved complete inhibition (100 ± 0.00%), similar to fresh samples at 70,000 mg/L. At 10,000 mg/L, fresh RC and DCR had inhibition percentages of 48.65 ± 0.75% and 43.03 ± 3.91%, respectively, while fresh DJR remained minimal at 15.54 ± 0.74%. This trend continued at 30,000 mg/L, where fresh RC and DCR showed high antifungal activity with inhibition percentages of 89.10 ± 0.87% and 84.24 ± 0.83%, respectively. At a concentration of 70,000 mg/L, both fresh RC and DCR achieved complete inhibition (100 ± 0.00%), indicating maximum antifungal activity, whereas fresh DJR increased significantly to 40.26 ± 3.14%. At the highest concentration of 100,000 mg/L, both freeze-dried RC and DCR maintained complete inhibition. Freeze-dried DJR continued to exhibit antifungal activity, with inhibition increasing to 87.83 ± 0.53%.

In this present study, data analysis revealed that the freeze-dried samples exhibited a greater level of antifungal activity. Freeze-dried samples achieved complete inhibition at 30,000 mg/L, whereas fresh samples required 70,000 mg/L to reach similar efficacy. This suggests that the freeze-drying process preserves the integrity and potency of bioactive compounds more effectively and better than other drying methods, as evidenced by various studies before (Nguyen *et al*., 2022a; Nguyen *et al*., 2022b; Cid-Ortega & Guerrero-Beltrán, 2022), resulting in improved antifungal properties. Thus, these findings may emphasize the potential of roselle decoction residues as effective natural antifungal agents against *Pestalotiopsis* sp., with freeze-drying identified as a superior edible coating formulation for enhancing antifungal efficacy.

The obtained results also revealed that both fresh RC and DCR exhibited a dose-dependent antifungal activity, with maximum efficacy observed at higher concentrations. This observation aligned with prior research indicating the potential of extracts as natural antifungal agents when used at appropriate concentrations (Papoutsis *et al*., 2018; Boukaew *et al*, 2022; Boukaew *et al*, 2023a; Boukaew *et al*, 2023b). It was worth noting that the antifungal activity of fresh samples was notably lower than that of freeze-dried samples at the same concentrations. This difference highlights the influence of the drying method on the effectiveness of bioactive compounds.

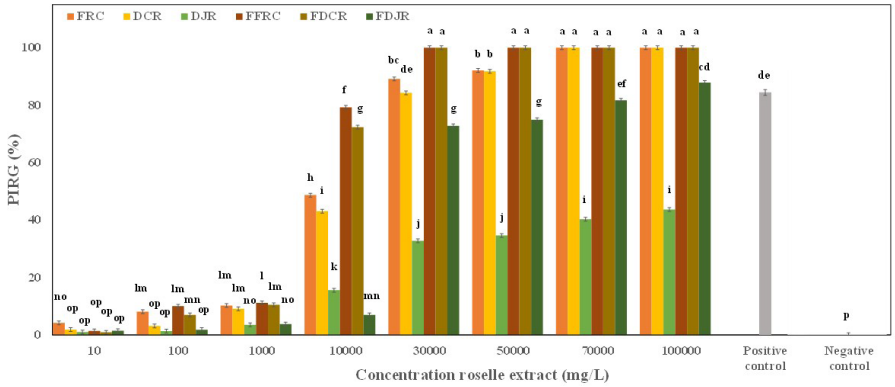


Fig. 1. Effect of crude ethanolic extracts of roselle decoction residues (FRC, DCR, DJR, FFRC, FDCR, and FDJR) at concentrations of 10, 100, 1,000, 10 000, 30 000, 50 000, 70 000, and 100 000 mg/L on PIRG of the isolated fungal pathogen compared to inhibition of positive (chitosan 1%) and negative controls. Error bars represent the standard error of the mean (SEM). Means with different letters are significantly different at the 5% level according to the HSD test. FRC: fresh roselle calyx, DCR: fresh decocted cordial residue, DJR: fresh decocted juice residue, FFRC: freeze-dried roselle calyx, FDCR: freeze-dried decocted cordial residue, FDJR: freeze-dried decocted juice residue. (*n*=18).

Table 4. Antifungal activity of crude ethanolic extracts at different concentrations against the isolated fungal pathogen

Calyx source	Concentrations (mg/L)							
	10	100	1000	10, 000	30, 000	50, 000	70, 000	100, 000
Fresh RC								
Fresh DCR								
Fresh DJR								
Freeze-dried RC								
Freeze-dried DCR								
Freeze-dried DJR								

RC: roselle calyx, DCR: decocted cordial residue, DJR: decocted juice residue.

Nguyen *et al*. (2022) also supported that freeze-drying can preserve bioactive compounds in the samples, which may enhance their effectiveness. Consequently, lower concentrations in freeze-dried samples can result in greater antifungal activity. In addition, Boukaew *et al*. (2023b) also found that freeze-dried culture filtrates of *Streptomyces philanthi* exhibited significant antifungal activity, which was attributed to the retention of active compounds during the freeze-drying process. This supported the current study's observation that freeze-drying enhanced the bioactive profile of roselle decoction residues.

The superior antifungal activity of freeze-dried roselle samples may be attributed to several mechanisms. Freeze-drying effectively preserved the cell structure and concentration of bioactive compounds, which may enhance their interaction with fungal cells. This hypothesis was supported by Urban *et al*. (2023), who observed that freeze-drying improved the antifungal activity of nystatin by enhancing its complexation with BB-cyclodextrin, thereby increasing its efficacy against *Candida* spp. Similarly, Juhari *et al*. (2021) also found that freeze-drying better maintained the cell structure of roselle calyx compared to other drying methods, making it a suitable choice for preserving sample integrity.

In addition, Lema *et al*. (2022b) also reported that freeze-drying of roselle calyx resulted in significantly higher retention of antioxidant activity and anthocyanin content compared to sun and oven drying methods. This finding aligned with our results, suggesting that the freeze-drying process preserved the antifungal compounds within the roselle decoction residues, thus enhancing their activity against *Pestalotiopsis* sp. This was also supported by Santangelo *et al*. (2024), who claimed that freeze-drying preserved the antifungal activity of bioactive peptides derived from bread waste, highlighting the general effectiveness of

this method in retaining bioactive properties.

The antifungal activity observed in freeze-dried roselle samples was also consistent with the findings of studies on other plants, such as *Codium bursa* (Jerković *et al*., 2019), where freeze-drying was found to enhance antifungal effects against *Fusarium* spp. In summary, the present study highlights the effectiveness of freeze-drying in preserving and enhancing the antifungal activity of roselle decoction residues. These findings contribute to the growing body of evidence supporting the utilization of freeze-drying as a superior method for processing botanical extracts for antifungal applications. Future research should focus on identifying the specific bioactive compounds that are responsible for the observed activity and optimizing the extraction and drying processes to maximize their effectiveness.

In addition, the crude extract from the present study was known for its richness in secondary metabolites, such as flavonoids, which exhibit antifungal properties by inhibiting the growth of fungal pathogens through fungistatic effects observed *in vitro*. The present study demonstrated that the roselle decoction residues extracted from both fresh and freeze-dried samples exhibit both fungicidal and fungistatic properties. At lower concentrations, the extract exhibited fungistatic activity, whereas at higher concentrations, it became fungicidal. Similar observations had been reported by de Billerbeck *et al*. (2001), Sari *et al*. (2012), and Jeff-Agboola & Onifade (2016).

Crude plant extracts have been recognized as potential antifungal agents against *Neopestalotiopsis* and *Pseudopestalotiopsis*, offering an alternative to synthetic fungicides. In a previous study, plant materials were washed, air-dried at ambient temperature, ground, and extracted using ethanol prior to antifungal evaluation. Among the six tested plant extracts, clove and turmeric exhibited the highest antifungal efficacy, achieving complete mycelial inhibition at a concentration of 10,000 mg/L. Roselle extract displayed moderate inhibitory effects, while mangosteen was the least effective (Darapanit *et al*., 2021).

In contrast, the current study employedfreeze-dried roselle extract, which better preserves thermolabile and bioactive compounds. Under these conditions, complete inhibition was achieved at 30,000 mg/L, whereas fresh extracts required a higher concentration, 70,000 mg/L, for similar efficacy. The enhanced antifungal activity observed in the freeze-dried samples may be attributed to the concentration of bioactive compounds during the drying process, as previously suggested for other plant extracts with antimicrobial properties (Agarwal *et al*., 2001; Duduk *et al*., 2015).

The comparative efficacy of crude plant extracts and chemical fungicides further reinforces the potential of natural alternatives for fungal management. Azoxystrobin + tebucolnazole and prochloraz exhibited 100% inhibition of *Neopestalotiopsis* and *Pseudopestalotiopsis*, while captan showed lower effectiveness (Darapanit *et al*., 2021). These findings suggest that while roselle extract possesses antifungal activity, its higher required concentration compared to clove and turmeric indicates a relatively weaker potency. However, given the growing concerns over fungicide resistance and environmental impact, plant-derived antifungal agents remain promising candidates for sustainable disease management strategies in agricultural systems (Saha *et al*., 2005; Barman *et al*., 2015).

In vivo antifungal activity assay

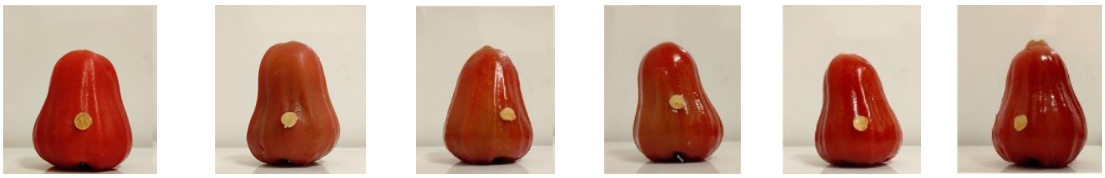
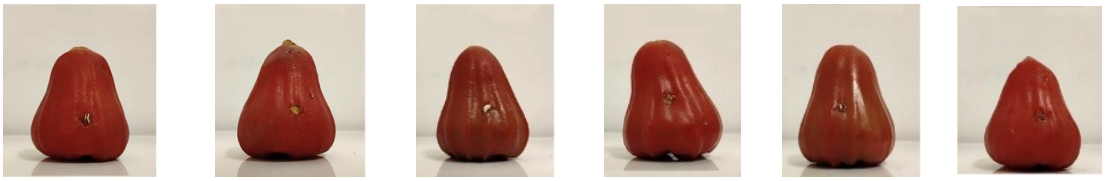
The disease severity varied depending on the treatment applied to the infected fruits. The DJR was not included in the *in vivo* trial, as it showed the lowest antifungal activity across all tested concentrations in vitro. Therefore, only the most effective extract formulations, RC and DCR, in both fresh and freeze-dried forms, were selected for in vivo evaluation at a concentration of 30,000 mg/L (3%), based on the minimum effective dose determined *in vitro*. This indicates that different treatments had different levels of effectiveness in controlling the disease. Table 5 shows the percentage of DSI on wax apple fruits treated with different treatments. In general, all treatments resulted in a severity scale of 1, indicating a mild infection category (Table 6) (HSD = 1.5329, $p<0.0001$).

Table 5. Percentage of disease severity index on wax apple-treated fruits

Coating formulation	DSI (%)
Negative control (F1)	6.33 ± 1.36 [a]
Positive control (F2)	2.78 ± 0.83 [b]
Fresh RC (F3)	1.08 ± 0.70 [c]
Fresh DCR (F4)	2.93 ± 0.70 [b]
Freeze-dried RC (F5)	0.31 ± 0.76 [c]
Freeze-dried DCR (F6)	0.77 ± 0.70 [c]

Values are expressed as mean ± standard deviation (SD). Means with different letters are significantly different at the 5% level according to the HSD test. (n=108).

Table 6. Effect of edible coating formulations on fruit rot of wax apples

Storage duration	Coating formulations					
	F1	F2	F3	F4	F5	F6
Day 0						
Day 7						

F1: negative control, F2: positive control, F3: fresh RC, F4: fresh DCR, F5: freeze-dried RC, F6: freeze-dried DCR, RC: roselle calyx, DCR: decocted cordial residue (*n*=108).

The negative control exhibited the highest DSI (6.33 ± 1.36%), highlighting significant disease severity. At day 7, the negative control exhibited early disease symptoms primarily at the apical region of the wax apple, characterized by colour fading, surface softening, and the onset of tissue maceration. These symptoms are indicative of initial fungal colonisation and tissue degradation, which contributed to the higher DSI recorded for this treatment.

In contrast, both freeze-dried RC and DCR treatments exhibited significantly lower DSI compared to the negative and positive controls, with values ranging from 0.31 ± 0.76% to 0.77 ± 0.70%. Similarly, fresh RC and DCR treatments were effective in reducing DSI with values ranging from 1.08 ± 0.70% to 2.93 ± 0.70%. Notably, freeze-dried RC treatment showed the most significant reduction in DSI (0.31 ± 0.76%), indicating its potential as a highly effective method for controlling disease in wax apple fruits. In addition to this, freeze-dried RC, DCR, and fresh RC showed comparable efficacy, resulting in an effect equivalent to a reduction in disease severity.

While fresh DCR also effectively reduced disease severity, it exhibited a slightly higher DSI, signifying it might be less effective. Meanwhile, the positive control (chitosan) demonstrated a significantly lower DSI (2.78 ± 0.83%), comparable to the fresh DCR but lower than the negative control. This suggests that chitosan is also an effective treatment for reducing disease severity in wax apple fruits. These findings also suggest that both fresh and freeze-dried treatments are effective in reducing disease severity in wax apple fruits, with freeze-dried RC appearing to be the most effective, followed by freeze-dried DCR and fresh RC. However, the disease severity of all treated fruits constituted a mild infection. Further research is needed to confirm these findings and explore the long-term effects of these treatments on wax apple fruit quality and yield.

According to Graybill *et al*. (1997), fungistatic activity inhibited the growth and reproduction of fungi without killing them by interfering with their metabolic processes, specifically disrupting protein synthesis and energy production. This inhibition slowed down fungal spread but did not cause immediate cell death, allowing for the possibility that fungi may resume growth once the influence of the extract decreases. While fungicidal activity not only inhibited fungal growth but also killed fungal cells. Elevated levels of active compounds lead to severe damage to fungal cell membranes and disrupted essential cellular processes, resulting in irreversible cell death.

As the concentration of 30, 000 mg/L (3%) *in vitro* effectively inhibited fungal growth in freeze-dried samples, making it the best treatment option. Throughout the observations in the present study, the probability that roselle decoction residue extracts exhibited both fungicidal and fungistatic properties was higher since freeze-dried samples achieved complete inhibition at lower concentrations, whereas fresh samples required higher concentrations for comparable efficacy. Therefore, this concentration was used as a benchmark for assessing efficacy *in vivo*.

The results indicated that, even after 7 days of inoculation, this concentration inhibited fungal growth on the wax apples, with no visible growth or only minimal amounts detected, resulting in a DSI with a severity scale of 1, indicating a mild infection category (Table 6). This dual behaviour enhances the efficacy of the extracts in disease management, providing fungistatic effects for prolonged suppression and shifting to fungicidal effects for complete eradication. This adaptability makes roselle decoction residue extracts promising for natural antifungal treatments in agriculture, supporting a sustainable approach to effectively managing plant diseases, particularly *Pestalotiopsis* sp.

Although studies investigating the *in vivo* antifungal efficacy of roselle calyx extracts or their residue-derived extracts were limited, research on other fruit waste extracts showed potential as natural antifungal agents. For instance, the antifungal activity of *Prosopis glandulosa* (mesquite) extracts has been evaluated against phytopathogenic fungi, demonstrating significant growth inhibition in both *in vitro* and *in vivo* assays. The mesquite extracts effectively suppressed fungal infections on treated plants, highlighting its potential as a biofungicide (López-Anchondo *et al*., 2021).

Similarly, research on citrus fruit waste has revealed promising antifungal properties. Extracts derived from dropped *Citrus reticulata* fruits exhibited strong antifungal activity against phytopathogenic fungi responsible for rice diseases, suggesting their applicability as biofungicides in agricultural disease management (Heena *et al*., 2025). Furthermore, plant waste extracts, including garlic peel extract, have shown significant efficacy in reducing lesion sizes caused by *Colletotrichum acutatum* in apples during ex situ assays, further supporting their potential as sustainable alternatives to synthetic fungicides (Teixeira *et al*., 2023). These findings collectively emphasized the need for further exploration of fruit waste-derived biofungicides, including roselle calyx and their residue-derived extracts, to develop environmentally friendly solutions for crop protection.

In the present study, the presence of secondary metabolites, such as anthocyanins and phenolic compounds, in extracts

from roselle decoction residues applied to inoculated wax apples may contribute not only to direct antifungal action but also to the induction of plant defense responses, enhancing the plant's resistance to pathogens and resulting in the highest reduction in disease incidence of fruit rot. The possible reason for these results was that the concentration level of antifungal compounds from roselle decoction residue extracts was sufficient to control the inoculum of *Pestalotiopsis* sp. on wax apples.

This statement was supported by the relatively low disease severity recorded in the present study, where only mild symptoms of fruit rot were observed. The anthocyanin and total phenolic contents of the roselle decoction residues have been previously reported (Yusoff *et al*., 2024), while changes in these compounds in response to fungal inoculation are currently under review in a separate study. Therefore, these data are not reproduced here to avoid duplication, but they provide supporting evidence for the antifungal efficacy observed in this study.

Furthermore, Jeff-Agboola & Onifade (2016) also found that the antifungal activity was attributed to the bioactive compounds present in the plant, such as polyphenols, flavonoids, and organic acids, which can interfere with fungal cell membranes and metabolic pathways. Their findings suggest that the antifungal action of plants may involve attacking the cell wall and retracting the cytoplasm in the hyphae, ultimately leading to the death of the mycelium and inhibiting spore germination.

According to Mandal (2010), phenolic accumulation and lignin deposition have been observed in eggplant roots as part of their defensive response to pathogens, demonstrating the protective role of these secondary metabolites in reinforcing plant cell walls. The anthocyanins and phenolics activate signalling pathways crucial for plant defense. They acted as signalling molecules in the phenylpropanoid pathway, which generates a range of defense compounds, including antimicrobial substances, in response to pathogen attacks (Yadav *et al*., 2020). This activation allowed for a more robust defense mechanism, enhancing the plant's ability to manage oxidative stress and other environmental pressures (Cimino, 2016; Yadav *et al*., 2020).

Furthermore, the presence of anthocyanins and phenolics was also linked to the upregulation of defense-related enzymes such as peroxidases and phenylalanine ammonia-lyase (PAL), which are essential for synthesizing additional protective compounds. These enzymes play a central role in plant responses to stress and pathogen attacks, contributing to a more resilient defense system (Yadav *et al*., 2020; Zhao *et al*., 2021). The increased activity of PAL and peroxidases has been observed in strawberry fruit development, where anthocyanins and phenolics help regulate ripening and pathogen resistance (Cheng & Breen, 1991).

Treutter (2005) and Albert *et al*. (2023) previously reported that flavonoids derived from the phenylpropanoid pathway were essential compounds in plant defense with a significant role in protecting plants from microbial pathogens. These defense-related flavonoids were classified into preformed and induced groups based on their synthesis timing and mode of action. Preformed flavonoids were synthesized during the normal growth and development of the plant, forming a consistent protective layer against microbial invasions (acting as the primary defense). However, induced flavonoids were synthesized in response to environmental stressors, including microbial attacks, making them an adaptive component of the plant's defense system.

To the best of my knowledge, no research work has been reported on specifically detailing PAL activity in roselle calyx and its residues. However, related studies on roselle highlight its rich phenolic content and bioactive compounds such as flavonoids and anthocyanins, which are typically associated with upregulated PAL activity in many plants. PAL is commonly activated in the presence of phenolic compounds and contributes to their synthesis; a pathway often associated with plant defenses and responses to stress. Studies in other plants suggest that increased phenolic and flavonoid content is linked with enhanced PAL activity, particularly in plants rich in antioxidants (Mandal, 2010; Yadav *et al*., 2020), like roselle calyx and its residues.

To date, research on PAL has primarily focused on postharvest degradation, with no specific role in pathogen-related responses. This indicates that anthocyanins are involved in activating antioxidant enzymes in response to stress and biochemical changes occurring during the deterioration of roselle calyx (Lema *et al*., 2022a). Overall, the flavonoids and other phenolic compounds play significant roles in strengthening cell walls and activating signalling pathways that trigger defense-related enzymes in plants. These compounds contributed to various physiological processes, including defense mechanisms against both biotic and abiotic stresses. Based on these observations, the bioactive compounds present in the roselle decoction residue extracts may enhance the structural integrity of plant tissues by promoting lignin formation, thus improving mechanical strength and resilience against pathogens.

CONCLUSION

In conclusion, this study demonstrates that ethanolic crude extracts of roselle decoction residues can inhibit the growth of *Pestalotiopsis* sp., indicating their potential for controlling fruit rot disease in wax apples. Among the calyx sources in *in vitro* analysis, all treatments exhibited the most effective results against *Pestalotiopsis* sp., except for DJR in both sample states (fresh and freeze-dried). DJR exhibited lower antifungal activity against the fungus isolated from wax apples, necessitating higher concentrations to achieve the same effectiveness as the other treatments. However, among fresh and freeze-dried samples, the freeze-dried samples of roselle decoction residues demonstrated superior antifungal activity, achieving complete inhibition at a lower concentration compared to the fresh samples, which required a higher concentration for similar efficacy. Based on the most favourable results obtained from *in vitro* studies, *in vivo* analysis was subsequently conducted to determine the efficacy of the formulation in treating fruit rot disease in wax apples. The treatments involved modifying the fungal morphology and inhibiting its growth. The chemical constituents in these crude extracts may serve as a natural antifungal agent. Therefore, these findings proved that roselle decoction residues provide effective protection for wax apples and may help to overcome the problem of fruit rot disease. However, there is a notable lack of studies comparing the *in vivo* antifungal efficacy of fresh and freeze-dried roselle calyx extracts or their residue-derived extracts. Similarly, while certain fruit waste extracts have demonstrated promising antifungal properties, direct evaluations of their efficacy in fresh versus processed forms remain scarce. Further investigation into the *in vivo* antifungal potential of roselle calyx extracts could provide valuable insights for developing natural, sustainable alternatives to synthetic fungicides in agricultural disease management.

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ETHICAL STATEMENT
Not applicable

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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