Sains Malaysiana 52(9)(2023): 2545-2557
http://doi.org/10.17576/jsm-2023-5209-06

In Vitro and *In Vivo* Assays of Selected Plant Extracts against Fruit Rot Fungi

(Asai *In Vitro* dan *In Vivo* Ekstrak Tumbuhan Terpilih terhadap Kulat Reput Buah)

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Received: 18 April 2023/Accepted: 5 September 2023

ABSTRACT

Post-harvest losses of crops in Malaysia is estimated at around 20%. Fungal infection is one of the primary causes of post-harvest loss in fruits. The common use of synthetic fungicide to combat fruit rot fungi poses negative impacts on human health and the environment. The objective of this study was to determine the efficacy of selected plant species as a safer alternative for post-harvest treatment. Ethanolic leaf extracts of *Piper sarmentosum, Psidium guajava,* and *Cymbopogon citratus* were tested against *Fusarium oxysporum* from tomato rot, *Fusarium proliferatum* from banana rot, and *Colletotrichum gloeosporioides* from mango rot. *In vitro* analysis was conducted using poisoned food bioassay and the percentage inhibitions of *Fusarium oxysporum* growth were 93.50% b*y Piper sarmentosum* extract, 46.68% by *Psidium guajava* extract*,* and 40.48% by *Cymbopogon citratus* extract. A test on a different fungus, *Fusarium proliferatum*, showed that *Piper sarmentosum, Psidium guajava*, and *Cymbopogon citratus* extracts displayed percentage inhibitions of 88.78%, 36.66%, and 22.44%, respectively. *In vitro* test on *Colletotrichum gloeosporioides* showed that *Piper sarmentosum* extract inhibited the fungal growth by 96.23%, while *Cymbopogon citratus* and *Psidium guajava* extract inhibited the fungal growth by 52.62% and 52.22%, respectively. The mechanism of action of these extracts appeared to be fungistatic. *Piper sarmentosum* was selected for further analysis by *in vivo* assay as it displayed the most significant anti-fungal action. The study was conducted using *Piper sarmentosum* leaf extract of various concentrations (25, 50, 50, and 100 mg/mL). The leaf extract displayed dose-dependent activity, with the highest reduction in disease severity observed at concentration of 100 mg/mL. The findings showed the potential use of *Piper sarmentosum, Psidium guajava*, and *Cymbopogon citratus* leaf extract as anti-fungal agent for post-harvest treatment to minimize fruit loss. *Piper sarmentosum* leaf extract showed comparable inhibitory action to commercial fungicide carbendazim.

Keywords: Anti-fungal*; Cymbopogon citratus*; *in vitro; in vivo; Piper sarmentosum; Psidium guajava*

ABSTRAK

Kehilangan hasil selepas tuaian di Malaysia dijangkakan sebanyak 20%. Jangkitan kulat merupakan antara punca utama kehilangan hasil buah selepas tuaian. Penggunaan meluas racun kulat sintetik untuk melawan kulat reput buah menyebabkan kesan negatif terhadap kesihatan manusia dan alam sekitar. Objektif kajian ini adalah untuk mengenal pasti keberkesanan ekstrak tumbuhan terpilih yang berpotensi untuk digunakan sebagai pengganti racun kulat untuk rawatan lepas tuai yang lebih selamat. Ekstrak etanol daripada daun *Piper sarmentosum, Psidium guajava* dan *Cymbopogon citratus* diuji ke atas *Fusarium oxysporum* daripada buah tomato yang reput, *Fusarium proliferatum* daripada buah pisang yang reput dan *Colletotrichum gloeosporioides* daripada buah mangga yang reput. Analisis *in vitro* dijalankan menggunakan kaedah bioasai makanan beracun dan peratus pengurangan pertumbuhan *Fusarium oxysporum* adalah sebanyak 93.50% oleh ekstrak *Piper sarmentosum*, 46.68% oleh ekstrak *Psidium guajava* dan 40.48% oleh ekstrak *Cymbopogon citratus*. Kajian ke atas kulat *Fusarium proliferatum* mendapati ekstrak *Piper sarmentosum, Psidium guajava* dan *Cymbopogon citratus*, masing-masing mengurangkan pertumbuhan kulat sebanyak 88.78%, 36.66% dan 22.44%. Ujian *in vitro* ke atas *Colletotrichum gloeosporioides* menunjukkan ekstrak *Piper sarmentosum* menghalang pertumbuhan kulat sebanyak 96.23%, manakala ekstrak *Cymbopogon citratus* dan *Psidium*

guajava masing-masing mengurangkan pertumbuhan kulat sebanyak 52.62% dan 52.22%. Mekanisme tindakan ekstrak tumbuhan ke atas kulat menunjukkan tindakan merencat pertumbuhan kulat. Ujian *in vivo* dijalankan menggunakan ekstrak *Piper sarmentosum* yang menunjukkan kesan anti-kulat paling ketara berbanding ekstrak lain. Uji kaji ini dilakukan menggunakan ekstrak dengan kepekatan berbeza (25, 50, 75 dan 100 mg/mL). Ekstrak *Piper sarmentosum* menggunakan etanol menunjukkan kesan bergantung kepada dos dan pengurangan tahap jangkitan penyakit tertinggi didapati pada kepekatan 100 mg/mL. Hasil kajian menunjukkan potensi penggunaan ekstrak *Piper sarmentosum, Psidium guajava* dan *Cymbopogon citratus* sebagai agen anti-kulat sebagai rawatan lepas tuai bagi mengurangkan kehilangan hasil buah. Ekstrak daun *Piper sarmentosum* terutamanya menunjukkan kesan perencatan kulat yang setanding dengan racun kulat komersial carbendazim.

Kata kunci: Anti-kulat; *Cymbopogon citratus*; *in vitro; in vivo; Piper sarmentosum; Psidium guajava*

Introduction

More than 600,000 metric tons of fruit is imported into Malaysia every year to meet the local demands (Department of Agriculture Malaysia 2021). The agrofood sector contributed approximately 3.5% to the national Gross Domestic Product (GDP) in 2019, with total land use of 5.63 million hectares. The National Agrofood Policy 2021-2030 (NAP 2.0) has been introduced to ensure the future of national food security. The policy aims to enhance the income of food producers, minimize food waste, improve the nutritional quality of food, and expand local participation in the agrofood sector (Ministry of Agriculture and Food Industries Malaysia 2021). Initiatives to increase agricultural output must be accompanied by measures to reduce food loss. Post-harvest loss of fruits and vegetables in Malaysia is estimated to be 20%. Produce loss is likely to be greater in less developed countries, and is estimated at around 42% (Mohamed 2017). Thus, post-harvest activities, from fruit-picking until consumption, play important roles in maintaining food quality and safety.

Fungal infection of fruit is a major cause of post-harvest loss that leads to significant food waste. For decades, synthetic fungicides have been used to inhibit fungal growth in crops. Benzimidazoles, sterol biosynthesis inhibitors, and aromatic hydrocarbons are examples of common fungicide groups, each with a unique mechanism of action (Cabral, Pinto & Patriarca 2013). However, there is growing concern over adverse impacts of synthetic fungicides on human health and the environment. Traces of fungicides are found in the soil, water, and plants, leading to toxic residues in food (Navale et al. 2021). Plant-based anti-fungal extracts can be used as safer and effective alternatives to synthetic fungicides as post-harvest treatment to protect fruits from decay by fungi and minimize food loss. The objectives of this study were to assess potential anti-fungal activity of *Piper sarmentosum, Psidium guajava,* and *Cymbopogon citratus* leaf extracts against fruit rot pathogens *in vitro*, and to evaluate the effectiveness of the selected plant extracts against fruit rot pathogens *in vivo*. The findings of this study may contribute towards target 12.3 of Sustainable Development Goals (SDGs), which is 'to halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses by 2030' (United Nations 2015).

Materials and Methods

Fungal cultures

Fusarium oxysporum (B713T), *Fusarium proliferatum* (B2433B), and *Colletotrichum gloeosporioides* (B3176M) previously isolated from tomato, banana, and mango rot, respectively, were used in the study. The fungi were obtained from the culture collection of Mycology Laboratory of Faculty of Science, Universiti Putra Malaysia. The fungal cultures were maintained on Potato Dextrose Agar (PDA) incubated at room temperature of around 28± 2 °C. Fungal colonies were sub-cultured after 7 days for anti-fungal assays.

Plant materials and extraction method

Fresh leaves of *Piper sarmentosum, Psidium guajava*, and *Cymbopogon citratus* were gathered locally or bought from the market. The leaves selected for use in the study were free of signs of physical damage and disease. The plant materials were thoroughly cleaned with running tap water to remove any dirt, followed by sterile distilled water for 15 minutes. The samples were dried in the oven at 50 ± 2 °C for 72 hours. Next, the dried plant materials were milled using a blender (Waring Commercial Products, USA). The fine powder obtained was stored in an air-tight container at 4 °C until further use.

To obtain plant extract, 30 g of powdered sample was soaked in 300 mL of 99% ethanol placed in plugged sterile conical flasks. The mixture was agitated at 150 rpm

and 28 ± 2 °C for 24 hours in an incubator shaker. The solution was next filtered using four layers of cheesecloth, followed by Whatman filter paper No. 1. The collected filtrate was evaporated to dry. The dried plant extracts were stored at 4 °C until further use.

In Vitro EVALUATION

In vitro assay was conducted using the poisoned food technique following the procedure described by Donlaporn and Suntornsuk (2010) and Gupta et al. (2007) with slight modification. Carbendazim and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). The commercial antifungal used in this study was dissolved in DMSO to the final concentration of (0.1%, v/v) to be used in positive control isolates (Zhou et al. 2015). The solution was sterilized using a syringe filter with a pore size of 0.22 µm. About 2 mL of the extract was mixed with 18 mL of cooled molten PDA per 90 mm sterile petri plate and left to solidify. A 5 mm diameter mycelial disc was obtained from the periphery of the 7-days old fungal colony using a sterile cork borer and inoculated at the center of the petri plate. All plates were incubated for 7 days at room temperature of around 28 ± 2 °C. Following incubation, the diameter of mycelial growth was measured. The experiment was conducted twice with five replicates for each plant extract tested in a completely randomized design. The percentage inhibition of fungal growth was calculated using the mean values according to the following formula:

$$\text{Percentage inhibition of fungal growth} = \frac{\text{Diameter of control (mm)} - \text{Diameter of treated sample (mm)}}{\text{Diameter of control (mm)}} \times 100$$

MODE OF ACTION OF ANTI-FUNGAL PLANT EXTRACTS

The fungicidal or fungistatic action of the plant extract was determined according to the method described by Chauhan et al. (2017) and Tabti et al. (2014). A 5 mm diameter mycelial disc from the treated sample was transferred to fresh PDA plate without any treatment. The cultures were incubated for another 7 days at room temperature of around 28 ± 2 °C. On day 7, fungal growth was observed. The plant extract was deemed to exhibit fungicidal activity if there was no growth, or fungistatic activity if there was fungal growth on the new plate.

In Vivo EVALUATION

In vivo assay was conducted using the standard wound inoculation method following the procedure described by Karim et al. (2015). Plant extract which displayed the highest inhibitory activity against each fruit rot pathogen in the *in vitro* assay was selected for the *in vivo* test. Plant extract of various concentrations (25, 50, 75, and 100 mg/mL) were prepared. The fruits purchased from the local fruit store were first cleaned under running tap water to remove dirt, and then soaked in 1% sodium hypochlorite for 2 minutes. The fruits were next rinsed twice with sterile distilled water. The washed fruits were dried using sterile filter paper. A wound of 2 mm depth and 3 mm length was made on the peel of each fruit at their equatorial region using a sterile cork borer. On the wounded area, 30 µL of plant extract was added and left for 2 hours. 1 mg/mL carbendazim was included as positive control and fruits with no treatment served as negative control following the procedure by Murad et al. (2022). Then, the wounded area was inoculated with a 3 mm mycelial disc. The fruit samples were stored in clear, covered plastic containers for 7 days at room temperature of around 28 ± 2 °C. On day 7, the lesion diameter on the fruits was measured as the following equation:

$$\text{Lesion diameter (mm)} = \frac{\text{Lesion length (mm)} + \text{Lesion width (mm)}}{2}$$

Using the mean lesion diameter obtained, the percentage of disease severity reduction was calculated as follows:

$$\text{Percentage of disease Severity reduction} = \frac{\text{Lesion diameter of control fruit (mm)} - \text{Lesion diameter of treated fruit (mm)}}{\text{Lesion diameter of control fruit (mm)}} \times 100$$

STATISTICAL ANALYSIS

Data from *in vitro* and *in vivo* analysis were statistically analyzed using Analysis of Variance (ANOVA) with IBM SPSS Statistics V28.0 for Windows Operating System. Tukey's Honest Significant Difference (HSD) test was performed to find the significant difference among means at the probability level of $p < 0.05$.

Results and Discussion

In Vitro EVALUATION OF PLANT EXTRACTS

Ethanolic leaf extracts of *Piper sarmentosum*, *Cymbopogon citratus*, and *Psidium guajava* displayed significant anti-fungal activities against pathogenic fungi *Fusarium oxysporum*, *Fusarium proliferatum*, and *Colletotrichum gloeosporioides* on treated media. However, the degree of inhibition against each fungus was different for each type of plant extract. *Piper sarmentosum* extract showed the greatest inhibitory action against all three fungi tested, compared to extracts of *Cymbopogon citratus* and *Psidium guajava* (Figure 1 & Table 1).

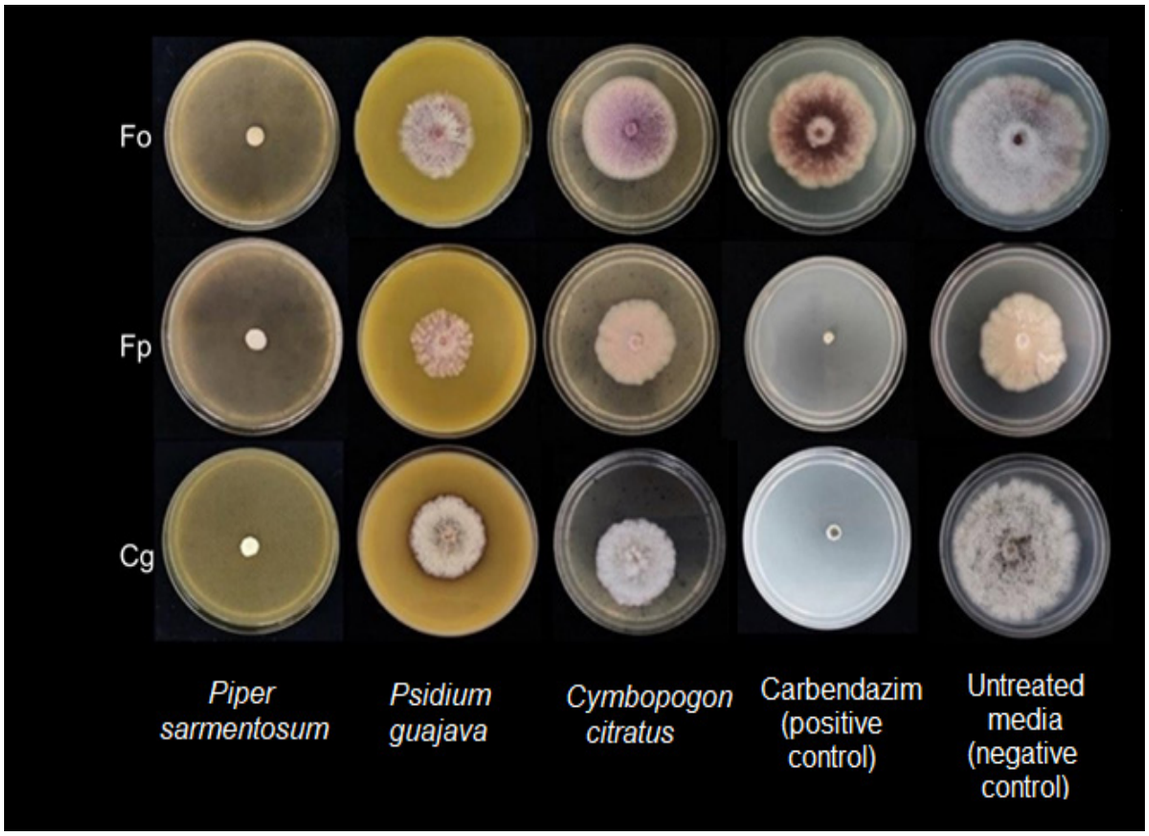


FIGURE 1. *In vitro* assay involving growth of *Fusarium oxysporum (Fo), Fusarium proliferatum* (Fp) and *Colletotrichum gloeosporioides* (Cg) on different media

TABLE 1. Mean diameter of fungal growth following 7 days incubation at room temperature of 28 ± 2 °C on various media in *in vitro* assay

Mean diameter of fungal growth (mm)			
Pathogen plant extract	*Fusarium oxysporum*	*Fusarium proliferatum*	*Colletotrichum gloeosporioides*
Piper sarmentosum	4.30 ± 0.30^{b}	4.50 ± 0.37^{b}	2.80 ± 0.20^{b}
Cymbopogon citratus	39.40 ± 2.01^{c}	31.10 ± 1.77^{c}	35.20 ± 1.69^{c}
Psidium guajava	35.30 ± 0.52^{c}	25.40 ± 0.16^{d}	35.50 ± 0.22^{c}
Carbendazim (Positive control)	52.20 ± 0.89^{d}	0 ± 0^{e}	2.40 ± 0.37^{b}
Untreated medium (Negative control)	66.20 ± 0.99^{a}	40.10 ± 0.43^{a}	74.30 ± 0.26^{a}

Each data value represents the mean of five replicates with two repetitions ± standard error. Mean values with different letters in each column for each fungus are significantly different at $p < 0.05$ according to Tukey's HSD test

The mean radial growth of pathogenic fungus *Fusarium oxysporum*, *Fusarium proliferatum,* and *Colletotrichum gloeosporioides* were observed to be the smallest on media supplied with *Piper sarmentosum*, followed by *Psidium guajava* and *Cymbopogon citratus* (Figure 2). The mean differences of inhibitory action of all three plant extracts and carbendazim against all tested pathogenic fungi were statistically significant ($p < 0.05$) compared to negative control (Table 1). The inhibitory action of *Piper sarmentosum* leaf extract was significantly different from *Psidium guajava* and *Cymbopogon citratus* leaf extracts (Table 1). The anti-fungal activity of *Psidium guajava* and *Cymbopogon citratus* leaf extracts were also significantly different compared to positive control, commercial fungicide carbendazim. Thus, the anti-fungal activity of all three ethanolic plant leaf extracts used in the current study were significantly different from carbendazim and showed superior ability to inhibit the growth of *Fusarium oxysporum*.

To determine the mode of action of the selected plant extracts, radial growth of fungi from media containing plant extracts were transferred to new plates containing media without any treatment. Since tested fungi resumed their growth on new untreated media, this indicates that ethanolic leaf extracts of *Piper sarmentosum, Psidium guajava*, and *Cymbopogon citratus* displayed fungistatic activity, similar to the mode of action of commercially used fungicide carbendazim. However, further analysis is required to determine the exact mode of action of the selected plant extracts. Based on the *in vitro* analysis, all three plant extracts tested displayed significant anti-fungal activity against *Fusarium oxysporum*, *Fusarium proliferatum,* and *Colletotrichum gloeosporioides* (Figure 3).

In the present study, the percentage inhibition conferred by *Piper sarmentosum* leaf extract ranges between 88.78% against *Fusarium proliferatum,* 93.50% against *Fusarium oxysporum*, and 96.23% against *Colletotrichum gloeosporioides* (Figure 3). This

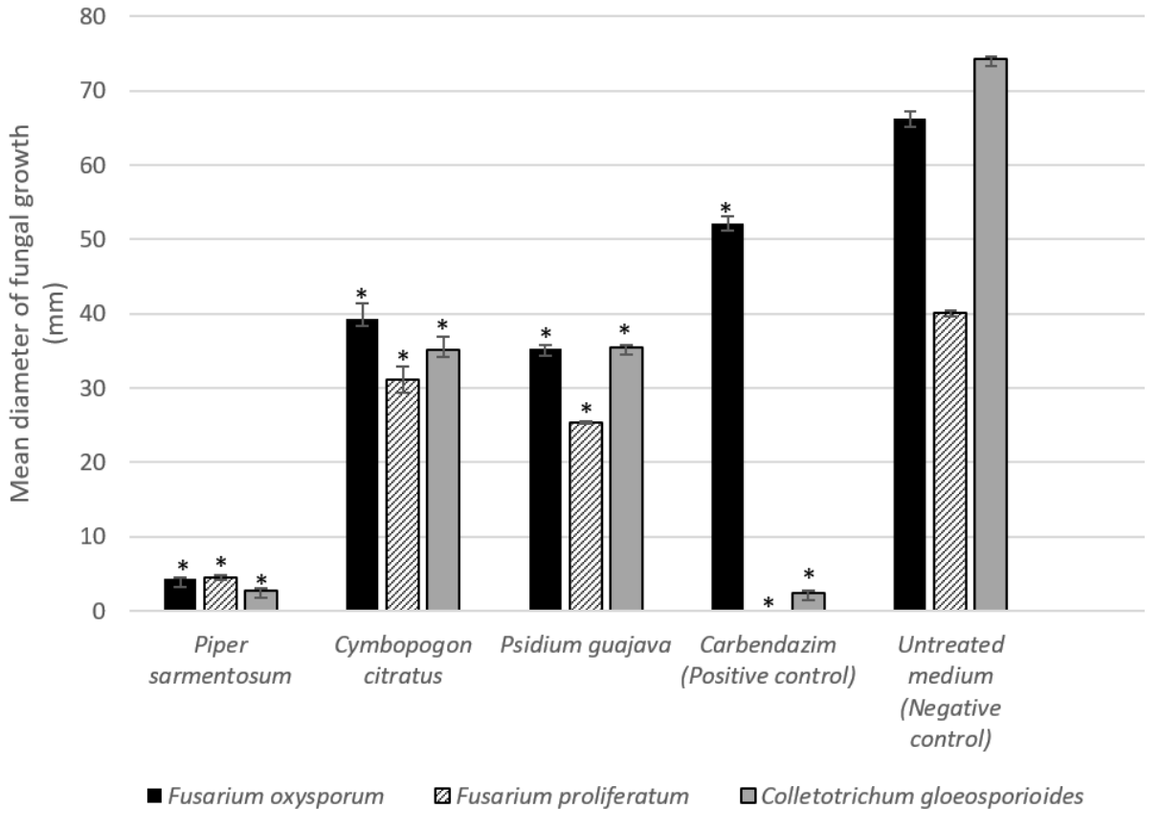


FIGURE 2. The mean diameter of fungal growth on different media in *in vitro* assay. Data are means of five replicates with two repetitions with error bars indicating standard error (SE). Asterisk (*) indicates the mean difference was statistically significant compared to untreated medium (negative control) at $p < 0.05$ according to Tukey's HSD test

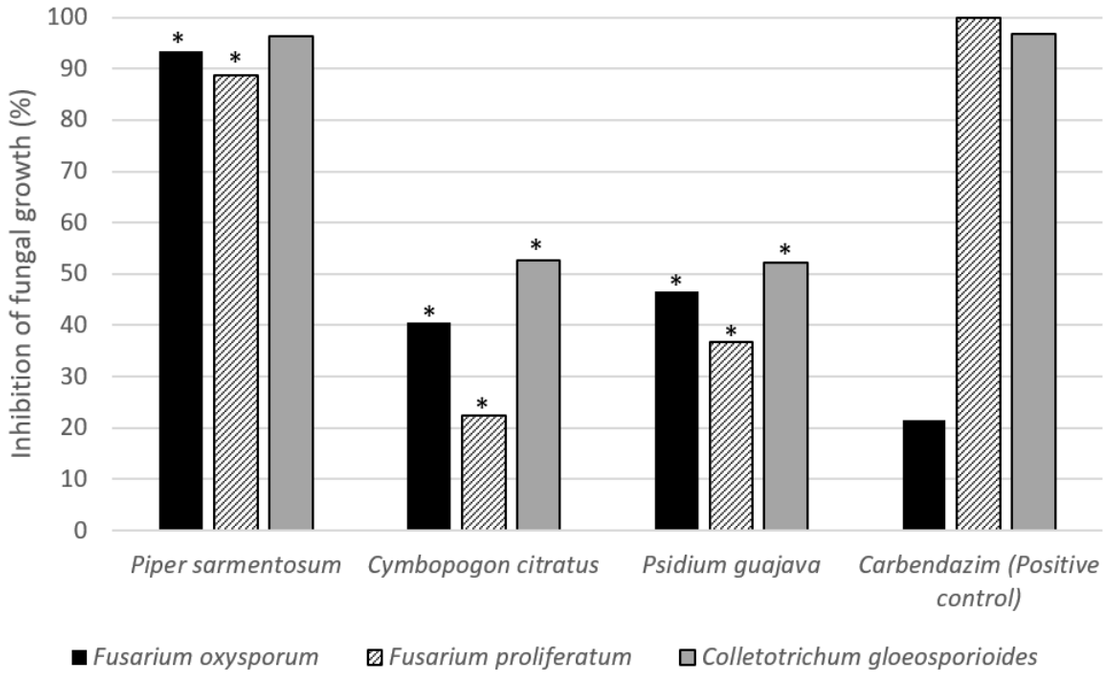


FIGURE 3. Percentage inhibition of plant extracts against pathogenic fungi compared to positive control carbendazim in *in vitro* assay. Asterisk (*) indicates the mean difference was statistically significant compared to positive control carbendazim at $p< 0.05$ according to Tukey's HSD test

showed that *Piper sarmentosum* extract is comparable to commercially used fungicide carbendazim, which served as positive control. As such, *Piper sarmentosum* leaf extract has the potential to be used as an alternative for post-harvest treatment of fruits to prevent decay caused by fungal infection. The findings from the present study were supported by an *in vitro* analysis performed by Sanit (2020), in which ethanolic extract of *Piper sarmentosum* displayed between 42% and 97% inhibition of *Colletotrichum gloeosporioides* mycelial growth at various concentrations.

A number of studies have also shown the effectiveness of *Piper sarmentosum* extract against other types of fungi. Ibrahim and Emlee (2020) reported that aqueous extract of *Piper sarmentosum* with concentration of 100 mg/mL displayed inhibitory action against *Fusarium verticillioides*, a primary cause of fungal infection in maize. 100% ethanol extract of *Piper sarmentosum* has also been shown to display very strong anti-fungal action against the growth of *Microsporum gypseum* and strong anti-fungal action against *Candida albicans* (Khusnul et al. 2019). Chanprapai and Chavasiri (2017) found that the dichloromethane and methanol extracts of *Piper sarmentosum* leaf exhibited anti-fungal activity against rice pathogenic fungi, *Rhizoctonia solani* and *Bipolaris oryzae*, but the extract of *Piper sarmentosum* fruit showed an even higher inhibitory action.

Phytochemical analysis of *Piper sarmentosum* leaf ethanolic extract showed the presence of alkaloids, steroids, tannins, and flavonoids (Sakilan, Demayo & Opanasopit 2019). Gas chromatography-mass spectrometry analysis showed presence of compounds such as β-asarone (37.37%), α-Asarone (30.39%), and myristicin (24.26%) as the primary constituent (Ibrahim & Emlee 2020). Rajput and Karuppayil (2013) suggested that the mechanism of action of asarone is by reducing ergosterol content in the fungal plasma membrane. The exact anti-fungal mode of action of myristicin is still unknown. However, Das et al. (2020) reported reduction in ergosterol content from the plasma membrane of *Aspergillus flavus* that leads to cellular ion leakage following treatment with *Myristica fragrans* essential oil, which contains myristicin as one of the principal components.

In the present study, the percentage inhibition of fungal growth displayed by *Psidium guajava* extract ranges between 36.66% against *Fusarium proliferatum*, 46.68% against *Fusarium oxysporum*, and 52.22% against *Colletotrichum gloeosporioides* (Figure 3). Previous study by Senbua and Wichitwechkarn (2019) also showed similar findings, whereby 10,000 ppm ethanolic *Psidium guajava* extract displayed around 35% inhibition against *Fusarium proliferatum*. Silva, Yerena and Necha (2021) reported that ethanolic extract of *Psidium guajava* leaf displayed percentage inhibition between 40.5 and 59.0% against mycelial growth of *Colletotrichum* spp.

Ethanolic extract of *Psidium guajava* has also been shown to inhibit the growth of various other fungi. Senbua and Wichitwechkarn (2019) reported that the plant extract displayed higher inhibitory action against *Curvularia geniculata, Curvularia lunata, Curvularia verruculosa, Fusarium solani, Aspergillus sclerotiorum*, and *Aspergillus fumigatus*, ranging between 40.0% and 64.4% inhibition. The percentage inhibition of mycelial growth was observed to be lower, between 12% and 14%, against *Penicillium citrinum* and *Aspergillus unguis.*

Phytochemical screening of the ethanolic extract of *Psidium guajava* leaf showed presence of numerous compounds such as alkaloids, flavonoids, phenols, quinones, saponin glycosides, steroidal glycosides, tannins, and terpenoids (Manikandan et al. 2016). Gas chromatography – mass spectrometry analysis showed that the most abundant compounds in the extract were trans-nerolidol (20.97%), caryophyllene (18.76%), and beta-caryophyllene oxide (9.55%) (El-Sesy & Mahran 2020). de Oliveira et al. (2020) suggested inhibitory activity of nerolidol against *Trichophyton rubrum* by disruption of the cell membrane. Yang et al. (2021) suggested that the mechanism of action of caryophyllene was by buildup of reactive oxygen species (ROS) in the fungal hyphae which harms the cells. Caryophyllene oxide, another primary constituent of *Psidium guajava* leaf extract, was suggested to inhibit mitochondrial electron transport chain, whereby the primary target of the compound is complex I in mitochondria (Magharri et al. 2015).

Similar to other tested plant extracts, ethanolic extract of *Cymbopogon citratus* leaf also displayed significant inhibitory action against fungal growth based on the findings in the current study. The percentage inhibition of mycelial growth ranges from 22.44% against *Fusarium proliferatum*, 40.48% against *Fusarium oxysporum*, and 52.62% against *Colletotrichum gloeosporioides* in *in vitro* analysis (Figure 3). Previous study by Dakole et al. (2016) also showed anti-fungal activity of the *Cymbopogon citratus* ethanolic extract. The inhibitory action of the plant extract ranges from less than 10% at 195.313 µg/mL to complete inhibition at 12,500 µg/mL. The mechanism of action of the plant extract was also reported to be fungistatic, similar to the observation in the current study. In a study by Santos et al. (2019), the ethanolic extract of *Cymbopogon citratus* leaf was able to completely inhibit the mycelial growth of *Colletotrichum gloeosporioides* at concentration of 8%. Murad et al. (2021) reported that aqueous extract of *Cymbopogon citratus* leaf displayed inhibitory action against mycelial growth of *Fusarium oxysporum* at 17.18% and *Fusarium proliferatum* at 32.21%.

Various studies have also shown the anti-fungal activity of *Cymbopogon citratus* extract against other fungi. Dakole et al. (2016) reported the inhibitory action of essential oil, ethanolic, and cold water extracts of *Cymbopogon citratus* against *Phytophthora infestans.* In another study by Nyamath and Karthikeyan (2018), the methanol, ethanol, cold water, and hot water extracts of *Cymbopogon citratus* showed inhibitory action against *Aspergillus niger* and *Colletotrichum musae* at various strengths.

Phytochemical screening of ethanolic extract of *Cymbopogon citratus* leaf by Falah, Ayunda and Faridah (2015) showed presence of alkaloids, flavonoids, phenols, saponins, steroids, and tannins. Gas chromatography – mass spectrometry analysis showed the most abundant compound in the ethanol extract was butylated hydroxytoluene at 29.24% (Gazwi 2019). The anti-microbial activity of anti-oxidants such as butylated hydroxytoluene is still largely unknown, but they have been associated with disruption of membrane permeability, leakage of cytoplasmic content, and inhibition of formation of nucleic acid (Naqvi et al. 2019).

In Vivo EVALUATION OF PLANT EXTRACTS AGAINST FRUIT ROT PATHOGENIC FUNGI

Based on *in vitro* findings, *Piper sarmentosum* extract displayed superior inhibitory action against tested fungi compared to *Cymbopogon citratus* and *Psidium guajava* extracts and was selected for *in vivo* analysis. The assay was conducted to determine the efficacy of the anti-fungal plant extract on living fruit samples based on synergistic interaction with whole, complex cells. Findings in the current study showed that treatment of *Piper sarmentosum* extract on fruit wounds were able

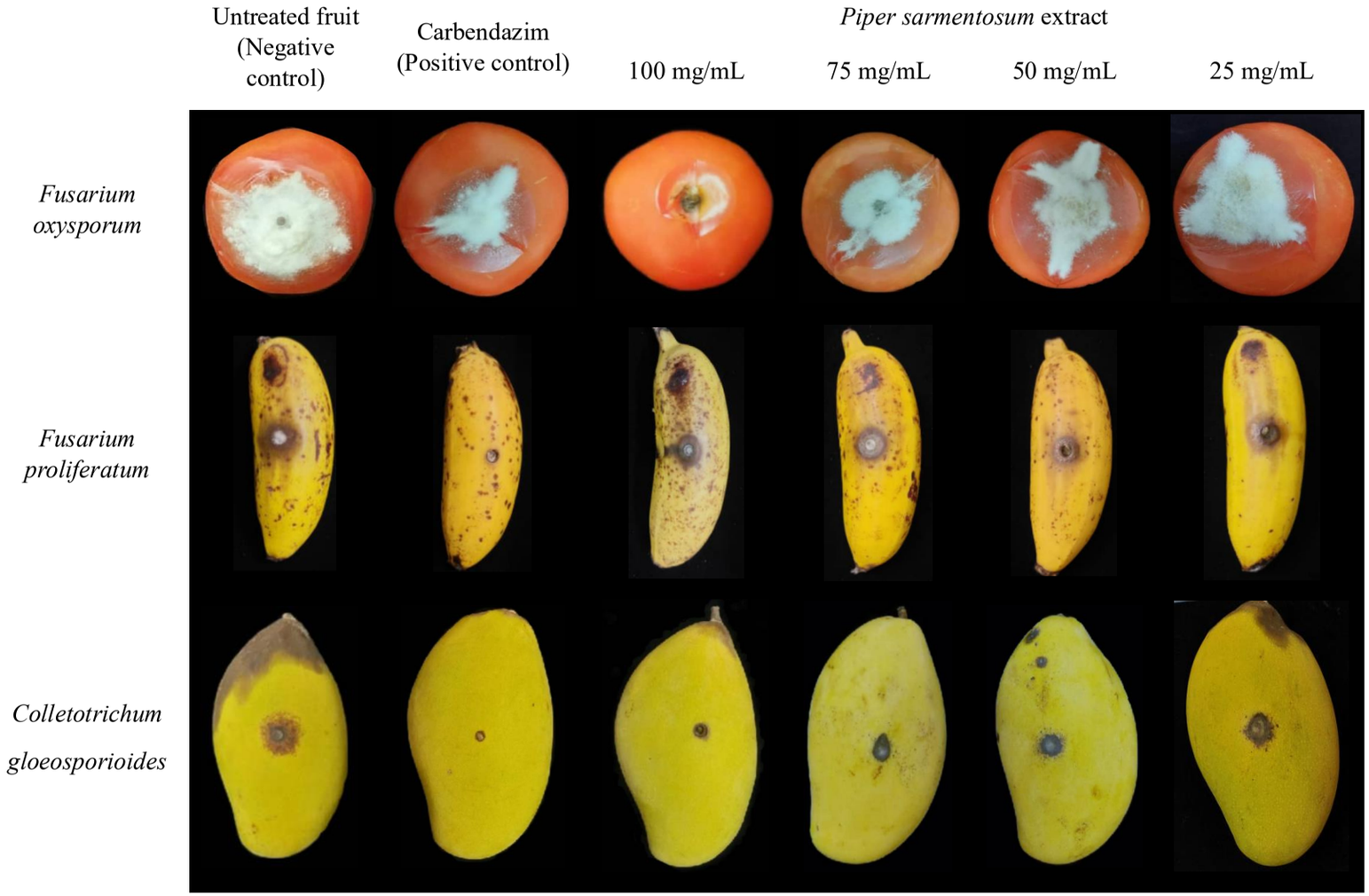
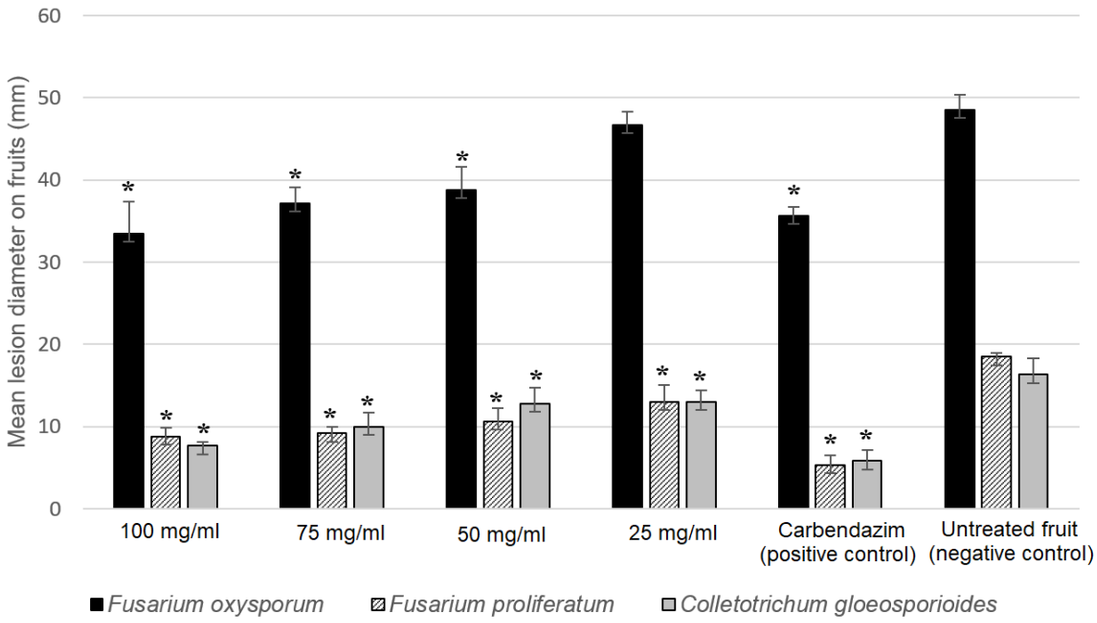


FIGURE 4. *In vivo* analysis of different concentrations of *Piper sarmentosum* leaf ethanolic extract against pathogenic fungi *Fusarium oxysporum, Fusarium proliferatum*, and *Colletotrichum gloeosporioides*

to inhibit mycelial growth and reduced the severity of disease caused by fungal infection (Figures 4 & 5).

The smallest lesion on tomato fruits caused by the pathogenic fungus *Fusarium oxysporum* was observed on samples treated with 100 mg/mL *Piper sarmentosum* extract (Figure 5). This was followed by treatments with carbendazim, 75, 50, and 25 mg/mL extracts. The largest diameter of lesion was observed on untreated tomato fruits which served as negative control. Statistical analysis with ANOVA showed that *Piper sarmentosum* extract with concentrations of 50, 75, and 100 mg/mL were able to significantly reduce disease severity in tomato fruit infected with *Fusarium oxysporum* (Figure 5). The mean difference between carbendazim and *Piper sarmentosum* extract with concentration of 50, 75 and 100 mg/mL were not significantly different, thus comparable to one another (Table 2).



Data are means of three replicates with two repetitions. Error bars indicate the standard error (SE). Asterisk (*) indicates the mean difference was statistically significant compared to negative control (untreated fruits) at $p < 0.05$ according to Tukey's HSD test

FIGURE 5. Mean lesion diameter of infected fruits following treatment with various concentrations of ethanolic *Piper sarmentosum* leaf extract compared to carbendazim (positive control) and untreated fruits (negative control) in *in vivo* assay

TABLE 2. Percentage reduction of disease severity in infected fruits following treatment with various concentrations of ethanolic *Piper sarmentosum* leaf extract compared to positive control carbendazim

	Percentage reduction of disease severity (%)			
	Concentration	*Fusarium oxysporum* (Host: Tomato)	*Fusarium proliferatum* (Host: Banana)	*Colletotrichum gloeosporioides* (Host: Mango)
Piper sarmentosum	100 mg/mL	30.93^{c}	52.27^{c}	53.03^{a,c}
	75 mg/mL	23.36^{b,c}	50.43^{c}	38.76^{c}
	50 mg/mL	19.94^{b}	42.32^{c}	21.43^{b}
	25 mg/mL	3.77^{a}	29.73^{b}	20.39^{b}
Carbendazim (Positive control)	1 mg/mL	26.45^{b,c}	71.19^{a}	64.30^{a}

Each data value represents the mean of three replicates with two repetitions. Values with different letters in each column for each fungus are significantly different at $p < 0.05$ according to Tukey's HSD test

In vivo assay on banana samples infected with pathogenic fungus *Fusarium proliferatum* showed treatments with various concentrations of *Piper sarmentosum* extract and carbendazim resulted in significantly different mean lesion diameter compared to the negative control. This indicated that *Piper sarmentosum* extracts with concentration of 25, 50, 75, and 100 mg/mL were able to significantly reduce lesion diameter in infected banana. The smallest mean lesion diameter was observed on mangos treated with carbendazim as positive control (Figure 5). Statistical analysis with ANOVA showed that the mean lesion diameters of all treated mangos were significantly different compared to untreated control. Thus, *Piper sarmentosum* with concentration of 25, 50, 75, and 100 mg/mL were able to significantly reduce lesion diameter in mango samples. Analysis with Tukey HSD indicated that there was no significant difference between treatment with carbendazim and 100 mg/mL *Piper sarmentosum* extract (Table 2). Hence, this showed that they are comparable to one another and 100 mg/mL *Piper sarmentosum* extract showed superior anti-fungal activity, similar to commercially used fungicide carbendazim.

Ethanolic extracts of *Piper sarmentosum* leaf with concentrations of 50, 75, and 100 mg/mL were able to significantly reduce disease severity in tomato samples infected with pathogenic fungus *Fusarium oxysporum* at 19.94%, 23.36%, and 30.93%, respectively (Figure 6). Meanwhile, the 25 mg/mL plant extract only displayed 3.77% disease severity reduction. Infected tomatoes treated with commercial fungicide carbendazim which served as positive control displayed 26.45% reduction in disease severity. Statistical analysis with Tukey HSD showed that the mean difference between 50, 75, and 100 mg/mL *Piper sarmentosum* leaf extract and carbendazim were not significantly different (Table 2). Thus, this indicated that the anti-fungal action of 50 mg/mL *Piper sarmentosum* extract against *Fusarium oxysporum* was similar to commercial fungicide carbendazim, showing the potential of this plant extract to be used as an effective post-harvest treatment to prevent tomato rot. By using lower concentration of plant extract for post-harvest treatment, less plant materials are required to obtain the extract which helps reduce resources and cost.

In vivo analysis of *Piper sarmentosum* leaf ethanolic extract on another pathogenic fungus, *Fusarium proliferatum* showed significant reduction in disease severity for all concentrations tested. The percentage reduction in disease severity caused by the plant extract with concentration of 100 mg/mL was at 52.27%, 75 mg/mL at 50.43%, 50 mg/mL at 42.32%, and 25 mg/mL at 29.73% (Figure 6). The mean difference between

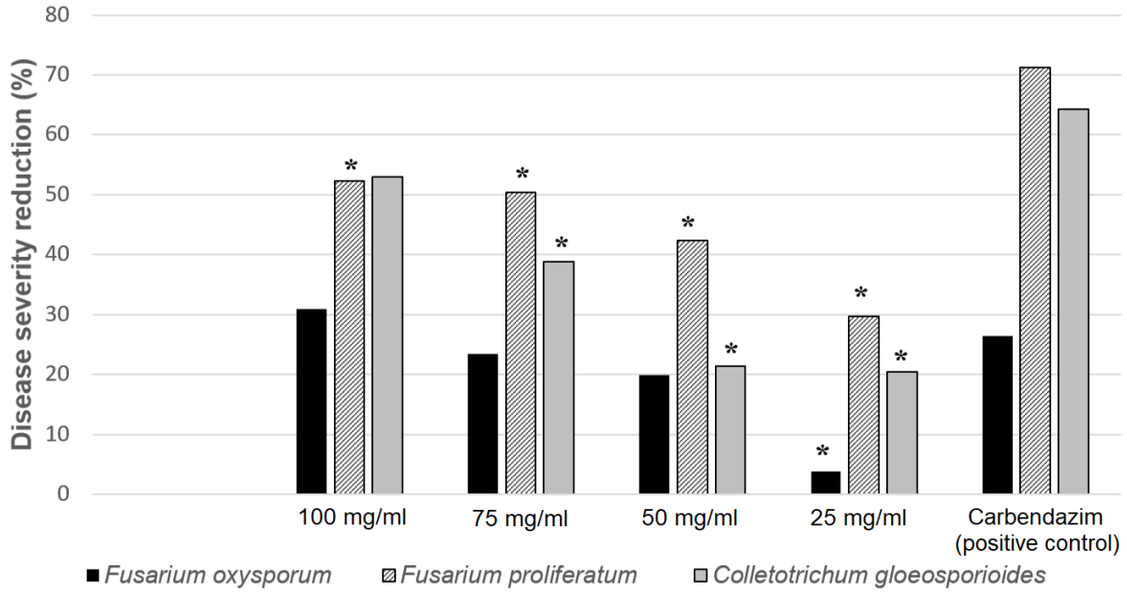


FIGURE 6. Percentage reduction of disease severity on infected fruits following treatment with various concentrations of ethanolic *Piper sarmentosum* leaf extract compared to positive control carbendazim in *in vivo* assay. Asterisk (*) indicates the mean difference was statistically significant compared to positive control carbendazim at $p < 0.05$ according to Tukey's HSD test

50, 75, and 100 mg/mL *Piper sarmentosum* extract was not significant (Table 2). This indicated that lower concentration *Piper sarmentosum* leaf extract, at 50 mg/mL, can be used as anti-fungal treatment on banana samples which is still able to produce significant anti-fungal effect. However, none of the concentrations tested were able to produce greater reduction in disease severity compared to the positive control carbendazim at 71.19%. Though the anti-fungal activity of *Piper sarmentosum* leaf ethanolic extract was lower than the commercial fungicide against *Fusarium proliferatum*, further research can be conducted to evaluate possible combinations of the extract with different bioactive compounds to produce superior plant-based fungicide. Previous study conducted by Seepe et al. (2020a) showed that combination of extracts obtained from different plants increased the anti-fungal activity by up to 80 times compared to the use of individual plant extract. This indicated possible synergistic or additive interactions between the bioactive compounds present in the combined extracts.

In vivo assay was also conducted using another pathogenic fungus, *Colletotrichum gloeosporioides*, one of the primary causes of anthracnose in mango. The findings from current study showed that all concentrations tested were able to significantly reduce disease severity in infected mangoes (Table 2). The ethanolic extract of *Piper sarmentosum* leaf with concentrations of 25, 50, 75, and 100 mg/mL resulted in 20.39%, 21.43%, 38.76%, and 53.03% reduction in disease severity respectively (Figure 6). Though positive control carbendazim produced 64.30% disease severity reduction, the mean difference between 100 mg/mL leaf extract and carbendazim was not significantly different (Table 2). This indicated the effectiveness of 100 mg/mL *Piper sarmentosum* leaf ethanolic extract as natural-based anti-fungal agent which can be used as a safer alternative to commercial fungicide for treatment of mangoes against infection by *Colletotrichum gloeosporioides*.

The findings in the current study showed that *Piper sarmentosum* leaf ethanolic extract exhibited dose-dependent activity against the tested pathogenic fungi. The higher the concentration of the leaf extract, the smaller the mean lesion on the wounded fruit samples. This is because extract with higher concentration contained a higher amount of bioactive compounds which were able to inhibit the growth of pathogenic fungi (Park et al. 2009).

In vivo analysis showed lower inhibitory action against pathogenic fungi compared to *in vitro* analysis. The finding was similar to the previous study by Angraeni et al. (2019). They reported higher inhibition of *Colletotrichum gloeosporioides* in *in vitro* analysis using *Pseudocydonia sinensis* seed extract. The percentage fungal inhibition ranges between 77.69% and 81.40% at concentrations of 0.5% to 1.5% extract. However, *in vivo* analysis produced reduction of disease severity on infected mango samples by just 21.88 - 32.63% for the different concentrations of extract tested. Seepe et al. (2020b) also reported similar observation, in which the minimum inhibitory concentration (MIC) of plant extracts tested with concentrations of less than 0.1 mg/mL in *in vitro* assay were not even effective at higher concentration of 2.5 mg/mL in *in vivo* analysis. According to Alzamora, Tapia and Malo (2000), this discrepancy may be caused by several factors. One of them is that components of the fruit may interfere with anti-fungal activity of the extract used as treatment. Another reason is the pH of the fruit may also affect the activity of the bioactive compounds. Apart from that, the extract may not be equally distributed in the fruit wound during treatment process. Another possible reason could be due to low solubility of the extract, leading to reduced anti-fungal activity observed *in vivo* compared to *in vitro* assays.

So far, there is currently no reports available in previous literature about evaluation of *Piper sarmentosum* leaf extract against *Fusarium oxysporum*, *Fusarium proliferatum,* and *Colletotrichum gloeosporioides* on fruit samples in *in vivo* assay. Though numerous studies evaluating anti-fungal activity of plant extracts have been conducted previously, most of them were limited to only *in vitro* analysis. Further investigation in *in vivo* analysis was not always conducted to support and validate the findings observed from *in vitro* assay. Therefore, more research should be conducted for highly potent anti-fungal plant materials in *in vivo* analysis.

Conclusion

The findings from *in vitro* analysis showed that ethanolic extracts obtained from *Piper sarmentosum, Psidium guajava,* and *Cymbopogon citratus* leaf showed significant anti-fungal activity against the tested pathogenic fungi. However, the degree of inhibition was observed to be different for each type of plant extract. *Piper sarmentosum* leaf ethanolic extract has been shown to produce the greatest inhibition of mycelial growth against all tested fungi. The anti-fungal activity of *Piper sarmentosum* extract was significantly higher than commercial fungicide carbendazim against *Fusarium*

oxysporum, slightly lower than carbendazim against *Fusarium proliferatum*, and produced similar level of inhibition as carbendazim against *Colletotrichum gloeosporioides*. As such, *Piper sarmentosum* leaf ethanolic extract was selected for further analysis *in vivo*. *In vivo* evaluation showed that *Piper sarmentosum* leaf extract was able to significantly inhibit fungal growth on fruit samples. *Piper sarmentosum* extract with concentrations of 50, 75, and 100 mg/mL produced a similar level of anti-fungal activity as carbendazim against *Fusarium oxysporum* on tomato fruits. Similar level of inhibitory action as carbendazim was also observed with 100 mg/mL *Piper sarmentosum* extract against *Colletotrichum gloeosporioides* on mango fruits. However, the inhibitory activity of *Piper sarmentosum* extract was lower compared to carbendazim against *Fusarium proliferatum* on banana fruit samples. These findings indicated the potential use of *Piper sarmentosum* leaf ethanolic extract as a safer, plant-based anti-fungal agent for use in agriculture as post-harvest treatment against fruit-rot by fungi. In turn, prevention of fruit disease caused by fungal infection will help extend shelf life of fruits and reduce food loss, thus enhancing food security.

ACKNOWLEDGEMENTS

The present study was supported by the Ministry of Higher Education (MOHE), Malaysia under a Fundamental Research Grant Scheme (FRGS/1/2018/STG03/UPM/02/12) awarded to Nur Ain Izzati M.Z. The authors wish to thank the Department of Biology, Faculty of Science, UPM for providing the technical supports and laboratory to make this study possible.

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