Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i1.2947

Research Article

Evaluation of *Morinda citrifolia* Leaf Extract Against *Phytophthora palmivora* in Controlling Stem Canker on Durian (*Durio zibethinus*)

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ABSTRACT

Durian is an economically important crop in Malaysia and has been identified as a new source of agricultural wealth valued at USD 1.58 billion. However, *Phytophthora palmivora* has been reported in all areas where durian has been planted and continues to pose a significant challenge for durian growers. The crop losses and control costs were estimated at 11.7% in 2020. Durian farmers have applied oomycete fungicides for more than 20 years to control stem canker, and extensive use of fungicides has resulted in the development of resistant *Phytophthora* isolates. This study aimed to evaluate four different leaf crude extracts of mengkudu (*Morinda citrifolia*) extracted using methanol, acetone, ethyl acetate, and hexane solvents against *P. palmivora*. GC-MS analysis of *M. citrifolia* leaves crude extract revealed the presence of squalene. In-vitro screening of the *M. citrifolia* plant extracts, the crude extracts of acetone and ethyl acetate revealed effective concentration 50% (EC_{50}) values of 36.115 and 38.095 mg/mL, respectively. PDA supplemented with acetone solvent at 60 mg/mL altered the morphology and inhibited the mycelial growth of *P. palmivora*. In an in-vivo bioassay screening level, treated plants of *M. citrifolia* extract of ethyl acetate 76 mg/mL had a lesion growth of 30.24 mm on the stem. This treatment was not significantly different from the control positive treatment applied with Ridomil fungicide. *Morinda citrifolia* leaf extracts using methanol, acetone, and ethyl acetate revealed it could be used as an alternative biocontrol agent for foliar spraying to reduce disease severity in durian seedlings against *P. palmivora*.

Key words: Antifungal activity, canker, durian, mengkudu, *Morinda citrifolia*, *Phytophthora palmivora*

INTRODUCTION

Durian (*Durio zibethinus*) is a sought-after fruit, especially in Asian countries. In Malaysia, durian has grown to be the most widely planted crop, occupying 41% of the country's total arable area, roughly 70,000 hectares. Durian, notably Musang King, is now regarded as a precious commodity and a new source of wealth for Malaysia. In Malaysia, this economically important crop, valued at USD 1.58 billion (Department of Agriculture, 2020), is the largest cultivated crop, making it a significant source of income for both small-scale and commercial farmers.

The stem canker disease caused by *Phytophthora palmivora* is notoriously challenging to control or eradicate. *P. palmivora* causes stem canker at nearly all phases of durian growth and is reported to have an estimated average loss of 20-30% in Malaysia (Misman *et al*., 2022). Many popular fungicide targets, like the process involved in ergosterol biosynthesis and cell walls made of chitin, are absent in the *Phytophthora* species (Kamoun *et al*., 2015). Several management measures, including phytosanitary, chemical control, cultural approaches, biological control, and genetic resistance, have been used to control the stem canker of durian. However, none have yielded satisfactory results or completely controlled the disease (Guest, 2007). Fungicide use necessitates precise control to avoid harm to human health and the environment and the emergence of fungicide resistance. Natural products, which are low toxicity and readily biodegradable into non-toxic products, offer a promising solution (Tripathi & Dubey, 2004). Plant extracts contain valuable secondary metabolites, which protect plants from oxidation and disease (Lima *et al*., 2019).

Morinda citrifolia is a medicinally valuable plant grown in the tropics for its fruits, leaves, and roots (Zin *et al*., 2002). The leaves are not only eaten as a vegetable (Zhang *et al*., 2016) but have also long been used to prevent various chronic diseases (Lim *et al*., 2016). Almost 200 phytochemicals have been identified and isolated from different parts of the *M. citrifolia* plant (Singh, 2012). *Morinda citrifolia* contains antifungal compounds such as anthraquinones (Takashima *et al*., 2007) and flavanols (Su *et al*., 2005). Both compounds inhibited *Phytophthora capsici* zoospores on cucurbits (Tala *et al*., 2018). This is in line with previous research showing that *M. citrifolia* can inhibit *Fusarium* sp. (Jayaraman *et al*., 2008), *Rhizoctonia solani* (Goun *et al*.,

Article History
Accepted: 18 October 2024
First version online: 27 March 2025
Cite This Article:
Nor Danial, N.D., Asib, N., Sadi, T. & Ismail. S.I. 2025. Evaluation of *Morinda citrifolia* leaf extract against *Phytophthora palmivora* in controlling stem canker on durian (*Durio zibethinus*). Malaysian Applied Biology, 54(1): 24-37. https://doi.org/10.55230/mabjournal.v54i1.2947

2003), and *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp (Mateus *et al*. 2017). Thus, the objectives of this study are to identify bioactive natural compounds in *M. citrifolia* leaf extracts extracted with methanol, acetone, ethyl acetate, and hexane and to evaluate in vitro and in vivo efficacy *of M. citrifolia* leaf crude extracts against mycelial growth of *P. palmivora* on durian seedlings, crude extracts against mycelial growth of *P. palmivora* on durian seedlings.

MATERIALS AND METHODS

Plant materials

Matured whole leaves of mengkudu (*Morinda citrifolia* L.) were collected from the landscaping area at Sultan Abdul Samad Library, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (3.002492, 101.706151). MFI 0199/21 is a voucher specimen deposited at the Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia. The preparation of plant materials followed the procedure of Zin *et al*. (2002) with minor modifications. The leaves were washed and cut into small pieces after being rinsed under running water. They were oven-dried for three days at 50°C before being ground into powder with a high-speed grinder machine. The powdered sample was kept at 28±1.5°C in an airtight container until further extraction processes.

Morinda citrifolia leaf crude extract preparation

Extraction procedures use selective solvents to separate active compounds from plant tissues. Solvents diffuse into the solid plant material during extractions and solubilize compounds with similar polarities. Analytical grade (99% minimum purity) organic solvents of methanol, acetone, ethyl acetate, and hexane were used in this study. The solvents were chosen based on their relative polarity: methanol > acetone > ethyl acetate > hexane. The polarity of the solvents can influence the quality and quantity of secondary metabolites in an extract and their composition. Minor modifications were made to the procedure described by Krishnaiah *et al*. (2012). Sample powder to organic solvents of methanol, acetone, ethyl acetate, and hexane (1.5 w/v) were used to extract the ground powder for 24 hr on an orbital shaker machine (Model 720, Protech) at 240 rpm. The resulting suspensions were filtered through Whatman no. 1 filter paper and evaporated to dryness in a rotary evaporator at 60 rpm and 50 degrees Celsius (°C). The crude extracts were kept at 4°C in an airtight bottle until further use. The extract yield (%) was calculated according to Truong *et al*. (2019).

$$\text{Extract yield (\%)} = \frac{\text{Weight of the extract after evaporating solvent}}{\text{Dry weight of sample}} \times 100$$

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The volatile chemical compounds in *M. citrifolia* crude leaf extract were investigated using gas chromatography-mass spectrometry (GC-MS). Natural Medicines and Products Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia conducted the analysis. The crude samples were diluted to 50,000 ppm with the same solvent used in extraction. Shimadzu Gas chromatography GC-2010 Plus (Kyoto, Japan) equipped with a Shimadzu QP-2010 Ultra mass selective detector was used for gas chromatography and mass spectrometry. The system includes a Rxi®-5ms (5% diphenyl + 95% dimethylpolysiloxane) electron capture detector column measuring 30 m (length) × 0.25 mm (internal diameter) × 0.25 m (thickness). At a constant flow rate of 1.0 mL/ min, ultra-high purity helium was used as the carrier gas. The oven temperature was gradually increased from 50°C (initial) to 300°C (final) at a rate of 3°C/min. With an interface temperature of 250°C and an ion source temperature of 200°C, the sample was injected at 250°C. The total running time was 90 min. The GC retention time was used to identify and characterize the chemical compounds in the crude extracts. MS spectra of separated components were identified using FFNSC1.3.lib, WILEY229.lib, and NIST11s.lib data libraries.

Preparation of *Phytophthora palmivora* isolates

Infected trees showing symptoms of water-soaking lesions were sampled. To obtain a pure culture of *P. palmivora* isolates, the outer layer of the bark surface was scrapped to obtain samples from the advancing lesion margin. Small pieces of bark containing the lesion margins were carefully removed using a machete. The small pieces (1 × 1 cm^2) were surface-sterilized in 0.5–1% sodium hypochlorite solution (NaOCl) for 30 s and washed three times using sterile distilled water. The tissue samples were blotted dry using sterile filter paper before being plated on P_{10}VP medium (Tsao and Ocana, 1969) containing 17 g/L corn meal agar (CMA), 0.4 mL/L pimaricin, 2 mL/L vancomycin and 100 mg powder pentachloronitrobenzene (PCNB). The plates were incubated for 2 days in the dark at 28±1.5°C. The growth of the hyphae was then cut and transferred onto V8 agar; 100 mL V8 vegetable juice; 1 g calcium carbonate; 16 g agar; and 900 mL distilled water to be incubated under the same conditions. Pure culture was viewed under a microscope for morphology characteristics. The morphology and molecular characteristics of *P. palmivora* were similar to the description of Latifah *et al*. (2018) and Bowman *et al*. (2007). For *in vitro* evaluation, the mycelium was used directly from the 7-day-old plate. As for the *in vivo* bioassay, the plates used were similar to the *in vitro* bioassay with the addition of zoospore preparation. This was done by pouring 5 mL of cold sterile distilled water onto the 14-day-old *P. palmivora* V8 plate. Zoospore was released with a sterile slide and counted using a hemocytometer to achieve 1 × 10^8.

In vitro screening of antifungal activities of *Morinda citrifolia*

Morinda citrifolia's antifungal activity was evaluated in vitro using the poison agar technique described by Pham *et al*. (2021). Treatments were prepared using crude extracts from solvent methanol, acetone, ethyl acetate, and hexane at 20, 40, 60, 80, and 100 mg/mL concentrations. The extracts were diluted using the same solvents in the extraction procedure to obtain the correct concentrations. In a conical flask, 15 mL of autoclaved V8 agar was cooled to 40°C and mixed with treatments for 30 min. The mixture was poured into a sterile Petri dish to solidify. Petri dishes containing V8 agar with no added treatments were the negative control, while Petri dishes containing the fungicide Ridomil at 439 mg/mL were the positive control. The concentration of

Ridomil fungicide used in this study referred to the fungicide label for controlling stem canker disease on durian. A 5-mm mycelial plug from the leading edge of a 7-day-old *P. palmivora* culture was cut using a sterile cork borer and placed on treated plates. Plates were incubated in the dark at 28±1.5°C for 72 hr. Colony diameters were measured, and percent inhibition of radial growth (PIRG) was calculated according to Živković *et al*. (2010). The inhibition of zoospores or zoosporangium at 50% was estimated by effective concentration (EC_{50}) value calculated using Probit analysis.

$$PIRG\ (\%) = \frac{R1 - R2}{R1} \times 100$$

Microscopic observation using a Scanning Electron Microscope (SEM)

SEM was used to compare the mechanism of inhibition in treated and negative control plates. The analysis was conducted at the Microscopic Unit (EM), Institute of Bioscience, Universiti Putra Malaysia, by Heckman *et al*. (2007). Specimens were prepared by cutting a 1 cm × 1 cm × 0.2 cm block from an agar plate. The mycelia were fixed with glutaraldehyde for 5 hr at 4°C (2.5%). The fixed specimens were washed three times for ten min each with sodium cacodylate (0.1M) Buffer. After being post-fixed in osmium tetroxide (1%) for 2 hr at 4°C, the specimens were rewashed with sodium cacodylate (0.1 M) buffer for three changes of 10 min each. The specimens were then dehydrated in serially diluted acetone (35%, 50%, 75% & 95%) for 10 min each and 100% acetone for three 15-min changes. For 1.5 hr, the specimens were dried in a critical point dryer. The dried specimens were taped to stubs and coated with gold in a high vacuum chamber using an ion sputterer (Baltic SCD005). Finally, the specimens were examined using a Scanning Electron Microscope, JSM-IT100 InTouchScopeTM (Jeol Ltd., Tokyo, Japan).

***In vivo* bioassay**

In vivo, bioassays were performed on acetone and ethyl acetate plates that demonstrated the highest in vitro antifungal activities. The extracts were treated according to their EC_{50} values. The treatment options were as follows: (i) the EC_{50} value, (ii) half the EC_{50} value, and (iii) double the EC_{50} value. Acetone and ethyl acetate extracts were tested at 18, 36, 72 mg/mL, and 19, 38, and 76 mg/mL, respectively. The negative control was sterile distilled water, while the positive control was Ridomil fungicide at 439 mg/mL, as specified for *P. palmivora*-caused durian disease. Each treatment had four durian seedlings replicated. The experiment was conducted at Universiti Putra Malaysia's Agriculture Faculty's Rain Shelter 2B. Thirty-two healthy 6-month-old Mousang King (D197) durian seedlings were purchased from BBshoppe in MARDI Serdang, Selangor, Malaysia. A handheld pressure sprayer was used to apply 50 mL of treatment per seedling for each experimental unit. Zoospores were collected by soaking 14-day-old *P. palmivora* in sterile water using a handheld pressure sprayer and increasing the concentration to 1×10^8. Then, 24 hr after treatment, 200 uL zoospore suspensions were dropped onto wounded intact and attached durian leaves. Each durian seedling receives three inoculated leaves. Every three days for up to 15 days, the lesion size (mm) was measured using a digital Electronic Digital Solar Calliper. A single-point inoculation on the main stem of the durian seedlings was performed using modified procedures described by Mohamed Azni *et al*. (2019). The stem was inoculated seven days after the treatment onto the same durian seedling mentioned earlier. The main stem of a durian seedling was wounded with a 4-mm cork borer and then inoculated with a 4-mm diameter *P. palmivora* mycelial plug. The inoculation site was covered with moist cotton and wrapped in parafilm to maintain the humidity. The progression of the infection on the leaves was recorded every three days. After ten days, the destructive sampling of the stem was performed.

Disease incidence (DI) was evaluated 15 days after inoculation based on the equation from Tabet Zatla *et al*. (2017). The disease severity index (DSI) was determined using the equation from Yusoff *et al*. (2020) 15 days after inoculation. Disease severity was evaluated based on a 1 to 4 scale as described by Latifah *et al*. (2018). The following is the scale: 1 = healthy leaf (no visible symptoms); 2 = initial infection with a leaf lesion less than 5 mm long; 3 = lesion dispersed around the leaf with a lesion length of 5-10 mm; and 4 = lesion progressed to the entire leaf with discoloration of a lesion length of more than 10 mm).

$$DI\ (\%) = \frac{\text{Total number of infected sample}}{\text{Total number of sample assessed}} \times 100$$

$$DSI\ (\%) = \frac{\sum(\text{Number of samples in the scale} \times \text{Severity scale})}{\text{Total number of samples assessed} \times \text{Highest scale}} \times 100$$

RESULTS

Yield of crude extract

Table 1 shows the percentage yield of crude extracts using methanol, acetone, ethyl acetate, and hexane. The extracts were dark green. Methanol crude extracts produced the highest percentage yield (11.07%), followed by ethyl acetate (3.43%), acetone (3.07%), and hexane (2.47%).

Table 1. Percentage yield of different crude extracts of *Morinda citrifolia* leaf

Solvent extract	Weight of dried sample (g)	Weight of crude extract (g)	Yield (%)
Methanol	100	11.07	11.07
Acetone	100	3.07	3.07
Ethyl acetate	100	3.43	3.43
Hexane	100	2.47	2.47

Chemical constituent from *Morinda citrifolia* extracts by GC-MS analysis

The most polar methanol extract of *M. citrifolia* leaves identified six chemical compounds contributing to the six peaks (Figure 1a). Squalene (peak five at 76.96 min retention time) and Hexadecanoic acid (peak two at 52.85 min retention time) were identified as the main chemical constituents, with an area of 41.5% and 15.1%, respectively. The acetone extract gave 12 peaks in the chromatogram (Figure 1b). The extract contains a mixture consisting mainly of squalene. The top five primary chemical compounds were identified as Squalene (Peak 7 at 77.08 min retention time), Hexadecanoic acid (Peak 3 at 53.11 min retention time), Linolenate (Peak 6 at 58.86 min retention time), Diacetone alcohol (Peak 1 at 6.26 min retention time) and Phytol (Peak 5 at 57.69 min retention time) with areas of 36.12%, 14.77%, 14.24%, 12.62% and 7.62% respectively. GCMS analysis of the crude extract of ethyl acetate led to the identification of 9 compounds (Figure 2a). The main constituents were Squalene (Peak 4 at 77.08 min retention time), Phytol (Peak 2 at 57.72 min retention time), and Alpha-Tocopherol (Peak 6 at 84.33 min retention time) with an area of 51.94%, 18.34%, and 11.00% respectively. The nonpolar hexane crude extract showed 17 peaks in the GC-MS chromatogram (Figure 2b & Table 2), identified according to their retention time. The compounds found comprised mainly of squalene at peak 12 with a retention time of 77.06, identified as a significant chemical constituent (51.3%), followed by Phytol (15.1% at 57.70 min retention time), Alpha-Tocopherol (7.52% at 84.31 min retention time), and Triacontane (4.55% at 83.46 min retention time). This result aligns with findings by Lima *et al*. (2019), where *M. citrifolia* leaves extracted with hexane had obtained squalene as the most significant component.

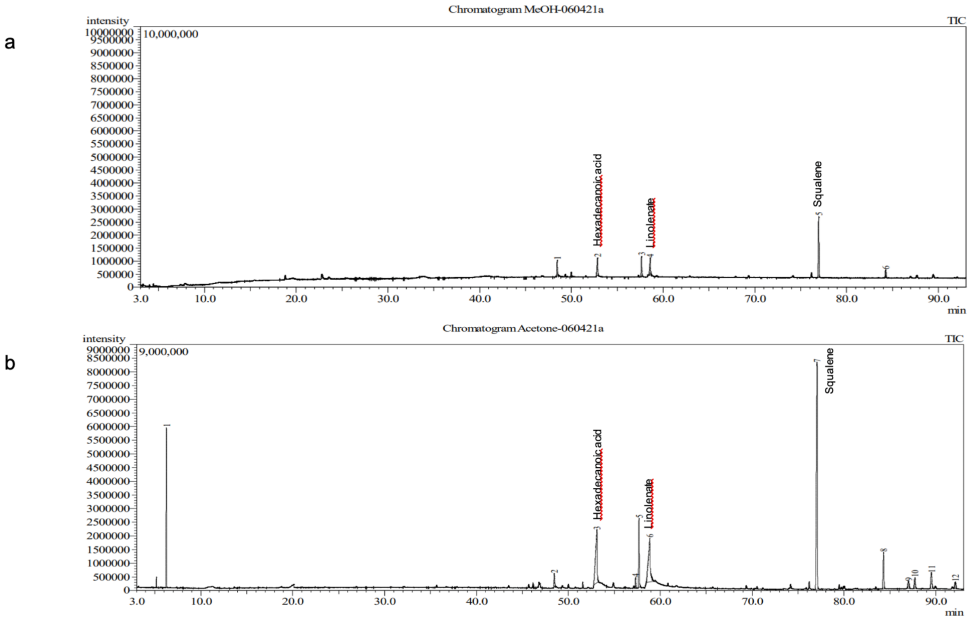

Fig.1. GC-MS chromatogram of *Morinda citrifolia* leaves extract using methanol (a) and acetone (b).

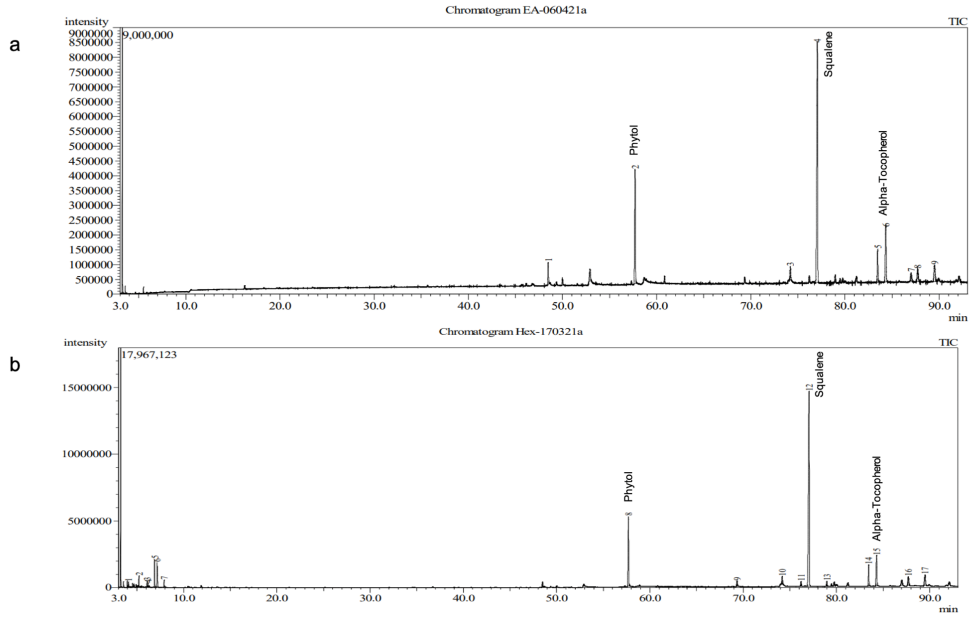

Fig. 2. GC-MS chromatogram of *Morinda citrifolia* leaves extract using ethyl acetate (a) and hexane (b).

Table 2. Chemical constituent of *Morinda citrifolia* leaf extracts

Solvent	Peak	Compound	Molecular formula	Retention time (min)	Retention index	Area (%)	Chemical group
Methanol	1	Neophytadiene	$C_{20}H_{38}$	48.45	1836	10.06	Diterpene
	2	Hexadecanoic acid	$C_{16}H_{32}O_2$	52.85	1963	15.05	Fatty acid
	3	Phytol	$C_{20}H_{40}O$	57.64	2109	13.06	Diterpene
	4	Linolenate	$C_{19}H_{32}O_2$	58.59	2139	14.38	Fatty acid
	5	Squalene	$C_{30}H_{50}$	76.96	2815	41.50	Triterpene
	6	Alpha-Tocopherol	$C_{29}H_{50}O_2$	84.25	3134	5.94	Vitamin E
Acetone	1	Diacetone alcohol	$C_6H_{12}O_2$	6.26	843	12.62	Ketones
	2	Neophytadiene	$C_{20}H_{38}$	48.47	1836	1.29	Diterpene
	3	Hexadecanoic acid	$C_{16}H_{32}O_2$	53.11	1970	14.77	Fatty acid
	4	9,12,15-Octadecatrienoic acid	$C_{19}H_{32}O_2$	57.31	2098	1.04	Fatty acid
	5	Phytol	$C_{20}H_{40}O$	57.69	2111	7.62	Diterpene
	6	Linolenate	$C_{19}H_{32}O_2$	58.86	2148	14.24	Fatty acid
	7	Squalene	$C_{30}H_{50}$	77.08	2820	36.12	Triterpene
	8	Alpha-Tocopherol	$C_{29}H_{50}O_2$	84.31	3137	4.91	Vitamin E
	9	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	87.02	3247	1.30	Phytosterol
	10	Stigmasterol	$C_{29}H_{48}O$	87.72	3273	1.82	Stigmastane
	11	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	89.51	3341	2.97	Phytosterol
	12	9,19-Cyclo-9.beta.-lanostane-3.beta.,25-diol	$C_{30}H_{52}O_2$	92.12	3440	1.31	Triterpene
Ethyl acetate	1	Neophytadiene	$C_{20}H_{38}$	48.48	1837	3.19	Diterpene
	2	Phytol	$C_{20}H_{40}O$	57.72	2111	18.34	Diterpene
	3	Ethyl Linoleolate	$C_{20}H_{36}O_2$	74.19	2701	2.25	Fatty acid
	4	Squalene	$C_{30}H_{50}$	77.08	2820	51.94	Triterpene
	5	Triacontane	$C_{30}H_{62}$	83.47	3101	4.94	Alkane
	6	Alpha-Tocopherol	$C_{29}H_{50}O_2$	84.33	3138	11.00	Vitamin E
	7	Cholest-5-en-3-ol	$C_{27}H_{46}O$	87.02	3247	1.62	Phytosterol
	8	Stigmasterol	$C_{29}H_{48}O$	87.73	3274	2.65	Phytosterol
	9	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	89.52	3342	4.07	Phytosterol
Hexane	1	Cyclopentane	C_7H_{14}	4.08	755	0.27	Alkane
	2	Octane	C_8H_{18}	5.21	810	1.00	Alkane
	3	Cyclopentane	C_8H_{16}	6.08	838	0.46	Alkane
	4	Cyclohexane	C_8H_{16}	6.18	841	0.38	Alkene
	5	Ethylbenzene	C_8H_{10}	6.89	863	3.25	Hydrocarbon
	6	Benzene	C_8H_{10}	7.16	872	4.00	Hydrocarbon
	7	Benzene	C_8H_{10}	7.90	895	0.90	Hydrocarbon
	8	Phytol	$C_{20}H_{40}O$	57.70	2111	15.10	Diterpene
	9	Hexadecanoic acid	$C_{19}H_{38}O_4$	69.33	2512	1.20	Fatty acid
	10	Methyl (Z)-5,11,14,17-eicosatetraenoate	$C_{21}H_{34}O_2$	74.18	2700	1.76	Fatty acid
	11	9-Octadecenamide	$C_{18}H_{35}NO$	76.19	2782	1.03	Fatty acid
	12	Squalene	$C_{30}H_{50}$	77.06	2819	51.33	Triterpene
	13	Nonacosane	$C_{29}H_{60}$	78.96	2899	0.89	Hydrocarbon
	14	Triacontane	$C_{30}H_{62}$	83.46	3100	4.55	Alkane
	15	Alpha-Tocopherol	$C_{29}H_{50}O_2$	84.30	3136	7.52	Vitamin E
	16	Stigmasterol	$C_{29}H_{48}O$	87.70	3273	2.74	Phytosterol
	17	gamma.-Sitosterol	$C_{29}H_{50}O$	89.49	3341	3.61	Phytosterol

A total of 44 compounds were identified from the *M. citrifolia* leaves extract obtained by the four solvents (Table 2). The identification of the compounds is arranged according to their elution order on silica capillary columns (Yusoff *et al*., 2020). For all solvents, the dominant component is squalene. The second primary compound for methanol and acetone extracts was Hexadecanoic acid, followed by Linolenate. Both ethyl acetate and hexane extracts had Phytol compound as the second principal component, followed by Alpha-Tocopherol.

In vitro screening of antifungal potential of *Morinda citrifolia*

Crude extractions of *M. citrifolia* using methanol, acetone, ethyl acetate, and hexane were tested against *P. palmivora* at seven different concentrations (Figure 3). As shown in Table 3, each type of crude extract had a different level of inhibition rate at different concentrations.

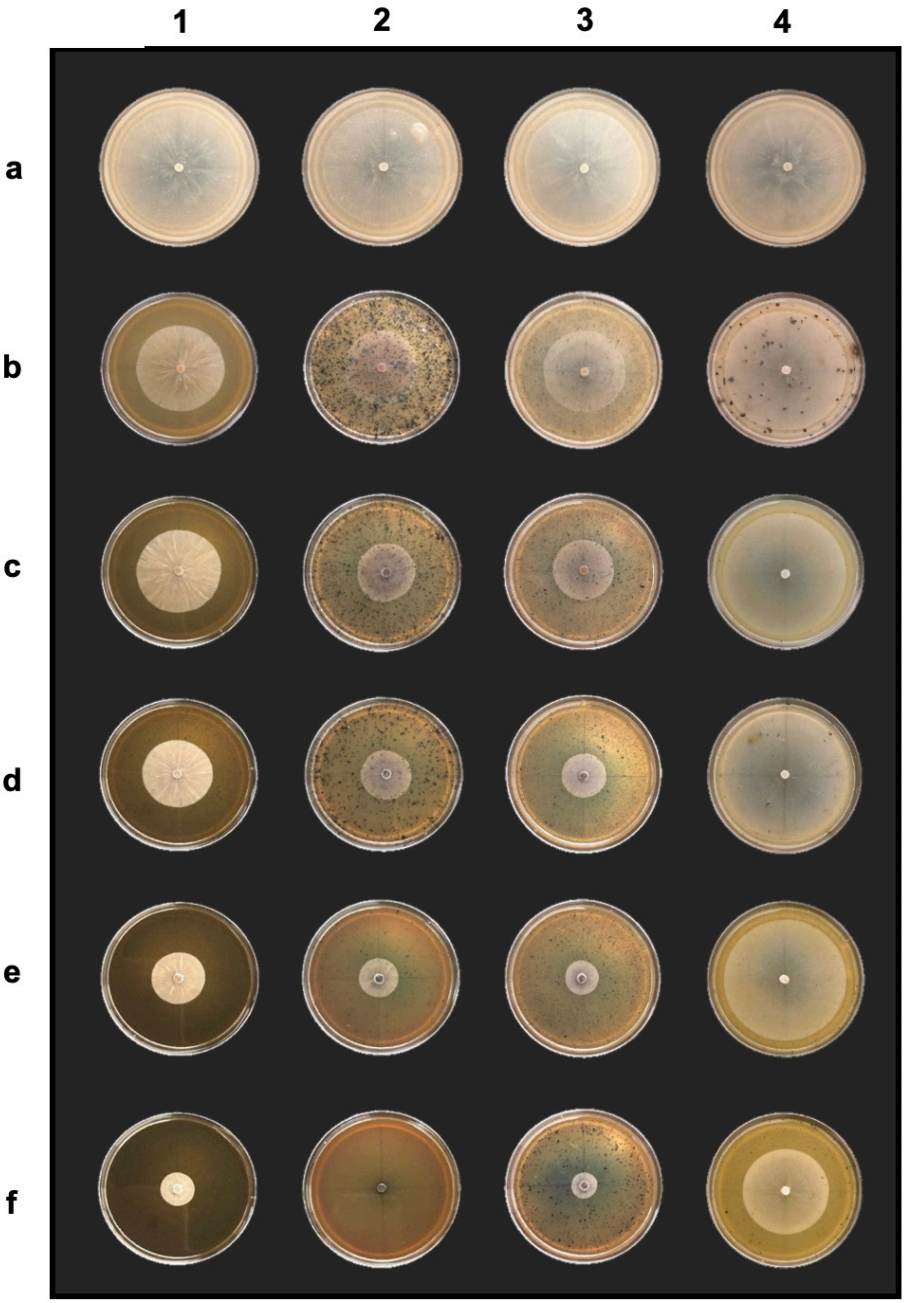

Fig. 3. *In vitro* antifungal activity of *Morinda citrifolia* leaf extracts against *Phytophthora palmivora* after three days of treatment. The numbers represent the solvents that were used. Methanol = 1, Acetone = 2, Ethyl acetate = 3 and Hexane = 4. The letters denote the concentration. a = 0 mg/mL; b = 20 mg/mL; c = 40 mg/mL; d = 60 mg/mL; e = 80 mg/mL; f = 100 mg/mL; f = 100 mg/mL.

Table 3. The effect of crude *Morinda citrifolia* extraction using methanol, acetone, ethyl acetate, and hexane at different concentrations on the PIRG of *Phytophthora palmivora* mycelial growth 3 days after incubation

Concentration (mg/mL)	PIRG			
	Methanol	Acetone	Ethyl acetate	Hexane
0	0.00 ± 0.00 ^{g}	0.00 ± 0.00 ^{f}	0.00 ± 0.00 ^{g}	0.00 ± 0.00 ^{c}
20	24.82 ± 0.61 ^{f}	36.38 ± 0.40 ^{e}	30.90 ± 0.97 ^{f}	0.00 ± 0.00 ^{c}
40	27.88 ± 0.39 ^{e}	49.50 ± 0.52 ^{d}	51.36 ± 0.98 ^{e}	0.00 ± 0.00 ^{c}
60	40.26 ± 0.52 ^{d}	58.52 ± 1.85 ^{c}	64.12 ± 0.81 ^{d}	0.00 ± 0.00 ^{c}
80	55.92 ± 1.12 ^{c}	68.80 ± 1.78 ^{b}	69.24 ± 0.90 ^{c}	0.00 ± 0.00 ^{c}
100	69.08 ± 0.78 ^{b}	92.80 ± 0.00 ^{a}	80.50 ± 1.52 ^{b}	22.53 ± 4.87 ^{b}
Ridomil at 439 mg/mL	92.80 ± 0.00 ^{a}	92.80 ± 0.00 ^{a}	92.80 ± 0.00 ^{a}	92.80 ± 0.00 ^{a}

PIRG is expressed as mean ± SE (standard error). Means with different letters within the same column are significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) Test.

Mycelial growth on V8 agar plates treated with Ridomil fungicide (positive control) had the highest PIRG of 92.8% for the entire crude extract and exhibited 100% inhibition in mycelium growth. The antifungal activity of crude extract acetone at 100 mg/mL was not significantly different from Ridomil, indicating that crude extract treatment had the most potent antifungal activity. Inhibition of *P. palmivora* mycelia growth was also observed in a concentration-dependent manner for crude extracts of methanol, acetone, and ethyl acetate (Figure 4).

The EC_{50} for treatments using crude methanol, acetone, and ethyl acetate extracts is 66.52, 36.12, and 38.10 mg/mL, as shown in Table 4. However, the Probit analysis application could not compute the EC_{50} for crude hexane extracts due to insufficient PIRG data. Crude extracts of acetone and ethyl acetate had the lowest EC_{50} values. In a study by Choi (2017), crude extracts of *Dipsacur asper* extracted using acetone and ethyl acetate had the highest inhibition, suppressing the development of *Phytophthora infestans*.

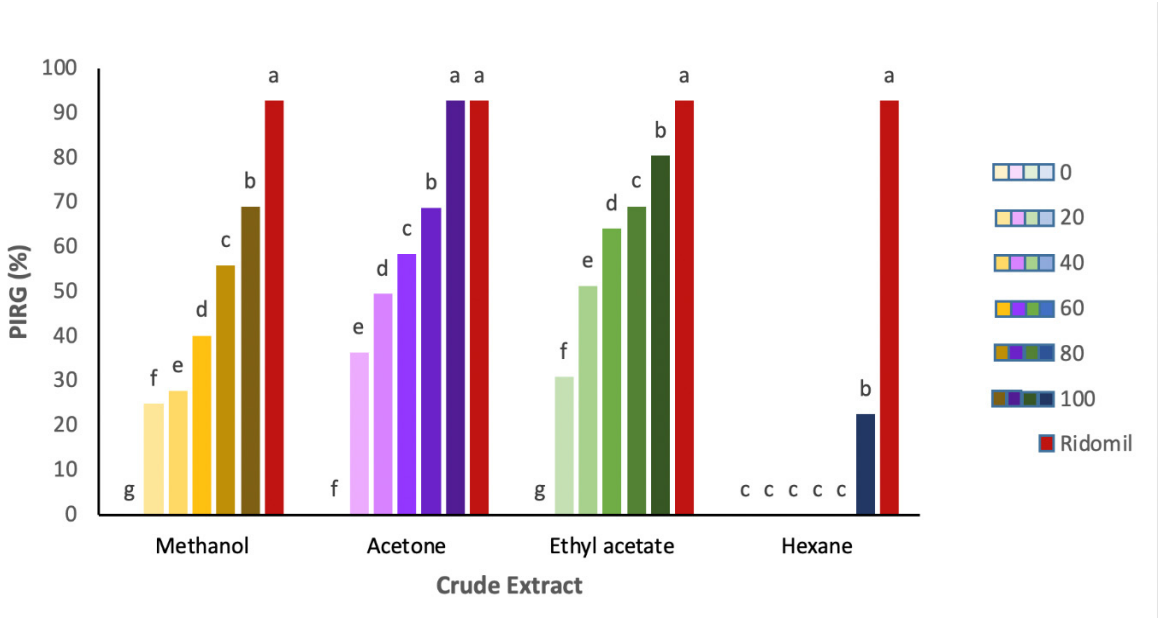

Fig. 4. Effect of crude extraction of Morinda citrifolia at different concentrations on the PIRG of *Phytophthora palmivora* after 3 days incubation. Means with the same letter within each crude extraction are not significantly different at p≤ 0.05 using Tukey's Studentized Range (HSD) Test.

Table 4. The mean effective concentration (EC_{50}) that inhibited mycelial growth by 50% for *Phytophthora palmivora* determined using Probit analysis

Crude extract	EC_{50} (mg/mL)	95% Confidence Limits	
		Lower	Upper
Methanol	66.52	40.41	225.16
Acetone	36.12	0.25	63.60
Ethyl acetate	38.10	31.47	44.23

Effect of *Morinda citrifolia* leaf crude extract on the mycelial morphology of *Phytophthora palmivora*

Microscopic observation on experimental plates using a scanning electron microscope helps obtain detailed information about mycelium morphology and interactions. Based on the results, crude extracted using acetone at 60mg/mL altered the morphology of *P. palmivora*. Figures 5c and d show SEM images of this fungus's mycelia, which were deformed, clumped, and had excessive branching. Furthermore, the mycelia surface had numerous hyphal tops, whereas the untreated plates in Figures 5a and 5b had a smooth shape and were evenly distributed.

In vivo antifungal activities of *Morinda citrifolia* crude extract against *Phytophthora palmivora*

Acetone and ethyl acetate extracts were tested in vivo for antifungal activity against *P. palmivora* on wounded intact and attached durian leaves because they had the lowest EC_{50} values for in vitro bioassay. The in vivo bioassay revealed that 100% of the leaves inoculated with *P. palmivora* developed symptoms (negative control treatment) (Table 5). Ethyl acetate treatment at 19 mg/mL reduced disease severity to 52.08%. Meanwhile, disease incidence ranged from 0 to 41.67% for the other treatments.

Table 5 shows leaf samples in the negative control treatment had a disease severity index of 56.25% on a scale of 2 to 3. As shown in Figures 6a, 6f, 6g, and 6h, the leaf samples exhibited symptoms of water-soaking lesions with a yellow halo discoloration around the lesion. *Morinda citrifolia* leaves treated with acetone (18, 36 & 72 mg/mL) and ethyl acetate (38 & 76 mg/mL) showed a significantly lower percentage of disease severity index when compared to the negative control. These treatments showed similar disease reduction using the commercial fungicide Ridomil. Thus, *M. citrifolia* leaf extract treatments could be an alternative fungicide, acting as a natural fungicide. Inoculation of *P. palmivora* onto the leaves of healthy durian seedlings resulted in water-soaking lesions three days later, as shown in Table 6. The developed lesions were limited to the inoculation site. *Phytophthora palmivora* water-soaking lesions were mainly small (0.19 - 4.03 mm) on day 15, as shown in Figure 6. The severity

of the infection is affected by the rate at which the inoculum spreads (Drenth & Guest, 2004). After 15 days of inoculation, the lesion size of the control negative treatments measured 4.03 mm. This result was comparable to ethyl acetate treatment at 19 mg/mL with a lesion growth of 3.08 mm and no antifungal properties. At day 15, there was no disease development in the control-positive treatment, which used a commercial fungicide (Ridomil). Acetone treatment at 36 and 72 mg/mL effectively controlled *P. palmivora*, with lesion development of only 0.19 mm and 0.00 mm, respectively.

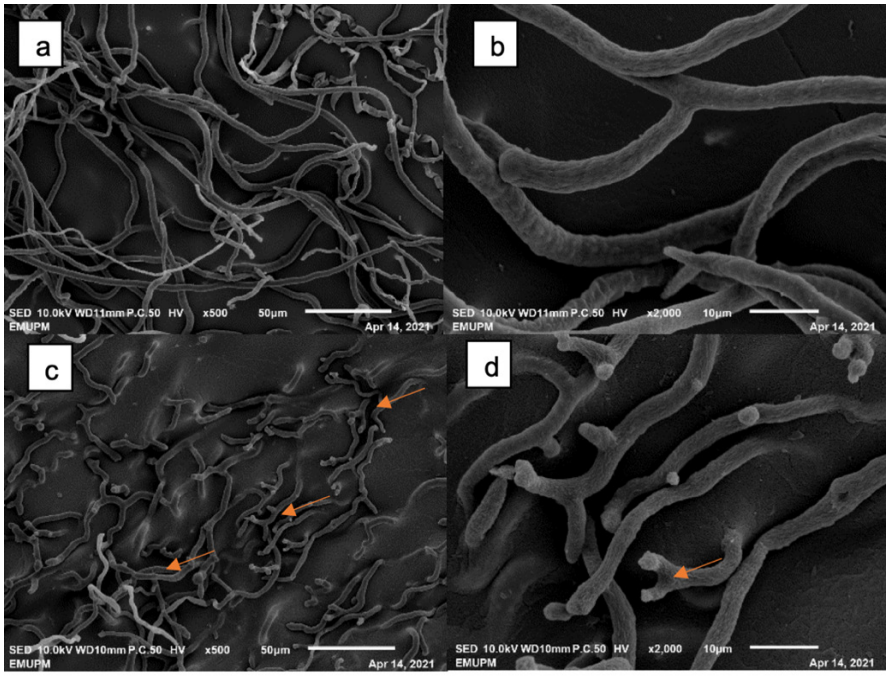

Fig. 5. SEM analysis of the effects of acetone crude extract on *Phytophthora palmivora* mycelial morphology at 60 mg/mL. Smooth surface, uniform and intact structure for the control negative plate at x500 (a) and x2000 magnification (b); Mycelial ruptured and deformed at x500 (c) and x2000 magnification (d).

Table 5. The incidence and severity of *Phytophthora palmivora* (mm) disease on treated durian seedlings 15 days after inoculation in a glasshouse

Treatment		Disease incidence (%)	Disease severity index (%)
T1	Negative control (SDW)	100.00 ± 0.00 ^{a}	56.25 ± 2.08 ^{a}
T2	Positive control (Ridomil)	0.00 ± 0.00 ^{b}	25.00 ± 0.00 ^{b}
T3	Acetone 18 mg/mL	25.00 ± 15.96 ^{b}	31.25 ± 3.99 ^{b}
T4	Acetone 36 mg/mL	8.33 ± 8.33 ^{b}	27.09 ± 2.09 ^{b}
T5	Acetone 72 mg/mL	0.00 ± 0.00 ^{b}	25.00 ± 0.00 ^{b}
T6	Ethyl acetate 19 mg/mL	83.33 ± 9.62 ^{a}	52.08 ± 6.25 ^{a}
T7	Ethyl acetate 38 mg/mL	41.67 ± 21.52 ^{b}	37.50 ± 7.22 ^{b}
T8	Ethyl acetate 76 mg/mL	25.00 ± 13.61 ^{b}	31.25 ± 3.99 ^{b}

Disease incidence and disease severity index are expressed as mean ± SE (standard error). Means with different letters within the same column are significantly different at $p< 0.05$ using Tukey's Studentized Range (HSD) Test. SDW = sterile distilled water

Table 6. Size of the lesion caused by *Phytophthora palmivora* on durian leaves

Treatment		Lesion size (mm)					
		Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
T1	Control negative (SDW)	0.00 ± 0 ^{a}	2.36 ± 0.31 ^{a}	3.42 ± 0.44 ^{a}	3.60 ± 0.45 ^{a}	3.78 ± 0.46 ^{a}	4.03 ± 0.44 ^{a}
T2	Control positive (Ridomil 439 mg/mL)	0.00 ± 0 ^{a}	0.00 ± 0 ^{d}	0.00 ± 0 ^{c}	0.00 ± 0 ^{d}	0.00 ± 0 ^{d}	0.00 ± 0 ^{c}
T3	Acetone 18 mg/mL	0.00 ± 0 ^{a}	0.31 ± 0.17 ^{cd}	0.40 ± 0.21 ^{c}	0.49 ± 0.26 ^{cd}	0.50 ± 0.27 ^{cd}	0.51 ± 0.27 ^{bc}
T4	Acetone 36 mg/mL	0.00 ± 0 ^{a}	0.12 ± 0.17 ^{d}	0.18 ± 0.18 ^{c}	0.19 ± 0.19 ^{d}	0.19 ± 0.19 ^{d}	0.19 ± 0.19 ^{c}
T5	Acetone 72 mg/mL	0.00 ± 0 ^{a}	0.00 ± 0 ^{d}	0.00 ± 0 c	0.00 ± 0 ^{d}	0.00 ± 0 ^{d}	0.00 ± 0 ^{c}
T6	Ethyl acetate 19 mg/mL	0.00 ± 0 ^{a}	1.45 ± 0.47 ^{b}	2.21 ± 0.57 ^{b}	2.46 ± 0.64 ^{b}	2.61 ± 0.67 ^{b}	3.08 ± 0.63 ^{a}
T7	Ethyl acetate 38 mg/mL	0.00 ± 0 ^{a}	0.99 ± 0.32 ^{bc}	1.52 ± 0.48 ^{b}	1.53 ± 0.60 ^{bc}	1.58 ± 0.62 ^{bc}	1.64 ± 0.63 ^{b}
T8	Ethyl acetate 76 mg/mL	0.00 ± 0 ^{a}	0.31 ± 0.26 ^{cd}	0.44 ± 0.30 ^{c}	0.50 ± 0.34 ^{cd}	0.54 ± 0.37 ^{cd}	0.70 ± 0.39 ^{bc}
		-	**	**	**	**	**

Lesion size (mm) on durian leaves is expressed as mean ± SE (standard error). Means with different letters within the same column are significantly different at $p< 0.05$ using Tukey's Studentized Range (HSD) Test. SDW = sterile distilled water

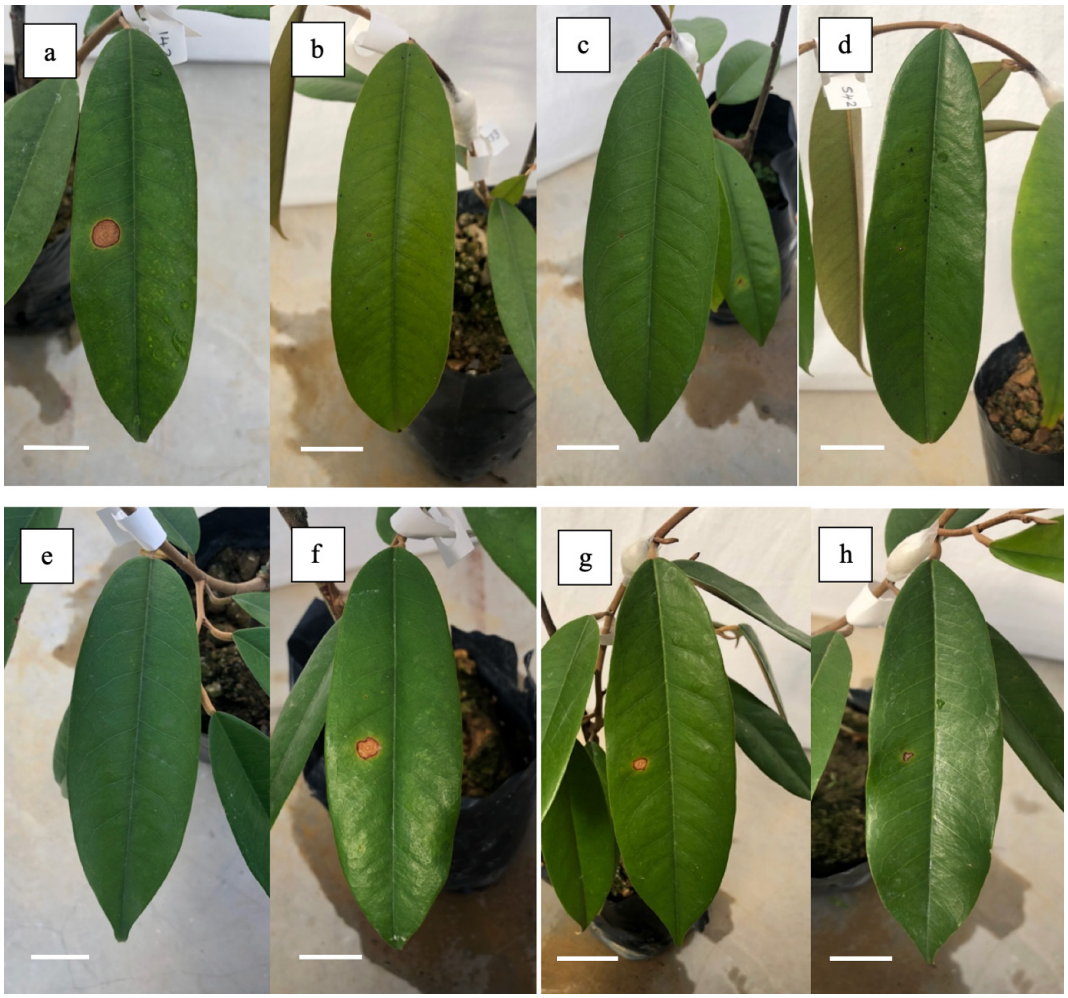

Fig. 6. *In vivo* test via artificial inoculation on durian leaves 15 days after inoculation. Inoculated leaves showed dark to light brown water-soaking lesions around the inoculation site. Control negative (a), Acetone 18 mg/mL (b), Acetone 36 mg/mL (c), Acetone 72 mg/mL (d), Control positive (Ridomil fungicide) (e), Ethyl acetate 19 mg/mL (f), Ethyl acetate 38 mg/mL (g) and Ethyl acetate 76 mg/mL (h). Scale bar 2 cm.

Inoculation on the main stem resulted in *P. palmivora* infection, with brown water-soaking discoloration at areas of mycelium insertion. Symptoms appeared seven days after inoculation. On day 10, the seedlings's outer bark layer was removed during destruction sampling to reveal lesion progression, as shown in Figure 7. According to Table 7, the control negative treatment on durian seedlings demonstrated significantly similar lesion progression to acetone treatment at 18 mg/mL. Both treatments increased pathogen colonization into epidermal tissues compared to *M. citrifolia* extracts of acetone 36 and 72 mg/mL and ethyl acetate 38 and 76 mg/mL. At the same time, Ridomil fungicide was used for positive control treatments.

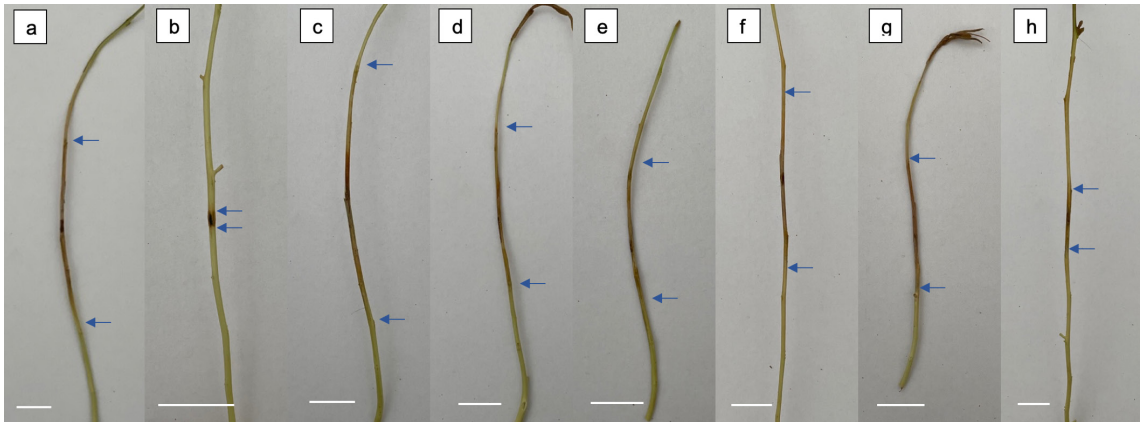

Fig.7. In vivo test via artificial inoculation on durian stem using destructive sampling. Symptoms developed on wounded stems caused by Phytophthora palmivora are indicated by dark brown necrotic lesions after 10 days of inoculation. Control negative (a), Control positive (Ridomil) (b), Acetone 18 mg/mL (c), Acetone 36 mg/mL (d), Acetone 72 mg/mL (e), Ethyl acetate 19 mg/mL (f), Ethyl acetate 38 mg/mL (g) and Ethyl acetate 76 mg/mL (h). Scale bar = 2 cm

Table 7. Lesion growth of *Phytophthora palmivora* (mm) on durian stem after 7 days of inoculation at glasshouse

	Treatment	Lesion diameter (mm) developed on the stem
T1	Control negative (SDW)	104.81 ± 23.96 [a]
T2	Control positive (Ridomil)	9.49 ± 4.67 [d]
T3	Acetone 18 mg/mL	107.89 ± 9.60 [a]
T4	Acetone 36 mg/mL	58.62 ± 8.02 [bc]
T5	Acetone 72 mg/mL	52.08 ± 8.08 [bc]
T6	Ethyl acetate 19 mg/mL	85.43 ± 4.94 [ab]
T7	Ethyl acetate 38 mg/mL	56.21 ± 11.43 [bc]
T8	Ethyl acetate 76 mg/mL	30.24 ± 3.32 [cd]

Lesion diameter (mm) developed on the stem is expressed as mean ± SE (standard error). Means with different letters are significantly different at $p< 0.05$ using Tukey's Studentized Range (HSD) Test. SDW = sterile distilled water

DISCUSSION

The differences in yield percentage between the four crude extracts are due to the different solvents used for extraction, which may affect the solubility of extractable components due to the plant's varied chemical composition (Hsu *et al*., 2006). According to Turkmen *et al*. (2006) and McDonald *et al*. (2001), solvent is one of the most important parameters influencing yield percentage. This result is because differences in the polarity of the extraction solvents can result in a wide range of bioactive compound levels in the extract (Truong *et al*., 2019). Methanolic extract had a higher extraction yield than acetone, ethyl acetate, and hexane extracts, indicating that highly polar solvents have a higher extraction efficiency. In this study, the plant material *M. citrifolia* contains a higher yield percentage of polar compounds soluble in highly polar solvents such as methanol. This result is consistent with the findings of Jayaraman *et al*. (2008), who reported that the extraction yields obtained from *M. citrifolia* extracts were obtained in the following order: methanol > ethyl acetate > hexane. As a result, methanol with a relatively strong polarity has the best extraction efficiency (Zhu *et al*., 2020). Out of the four solvents used, extracts using hexane, a non-polar solvent, had the highest yield of 17 total compounds. This result might be because the compound extraction yield was affected not only by the solvent types but also by the chemical and physical structure of the plant samples (Zhu *et al*., 2020). The visible difference between the four solvents is that the presence of hydrocarbons is higher in hexane extract. The non-polarity of the solvent justifies this. According to Suchinina *et al*. (2011), the polarity and viscosity of the extraction solvents affect the composition and concentrations of the specific compounds in the plant material used. Thus, each solvent could extract different compounds, leading to different products and indicating different applications for the extracts (Lima *et al*., 2019).

A total of 44 compounds were found in the GC-MS analysis of *M. citrifolia* leaves crude extract using methanol, acetone, ethyl acetate, and hexane solvents. Squalene is reported to be the most abundant constituent of each crude extract. Similar findings by Lima *et al*. (2019) on leaves and fruit pulp of *M. citrifolia* also found squalene to be the most prominent compound in both plant samples. Squalene belongs to the chemical class triterpenes, which is a hyponym of terpene and plays a crucial role as an antioxidant (Micera *et al*., 2020), anticancer, antibacterial, and antifungal (Reddy & Couvreur, 2009). Squalene at a high concentration can disturb the structure of cellular membranes and thereby interfere with essential membrane functions (Gnamusch *et al*., 1992). Terpene compounds associated with antifungal activity against *Phytophthora* spp. In a study done by Camele *et al*. (2012), three Mediterranean aromatic plants (*Verbena officinalis, Thymus vulgaris, and Origanum vulgare*) consisting of mainly terpenes had a 100% inhibitory effect on *Phytophthora citrophthora*.

Hexadecanoic acid found in *M. citrifolia* leaves had a high area percentage from solvents of methanol and acetone. Hexadecanoic acid is a fatty acid with vital biological roles, such as being involved in β-oxidation for energy, cell membrane composition, and stress tolerance (Pang *et al*., 2015). As a result, genes involved in fatty acid metabolism may have essential biological functions such as regulating the growth and pathogenicity of the *Phytophthora* pathogen. A high level of hexadecanoic acid contributes to a decreased membrane fluidity and permeabilization. Thus, disrupting the normal function of the cell wall of *Phytophthora* (Liu *et al*., 2020)

Linolenate is a form of triglyceride ester of linolenic acid, a naturally occurring fatty acid. These fatty acids have excellent antioxidant and radical scavenging activity (Erasto *et al*., 2007). Linoleic acid is a substrate for the production of oxylipins thought to be involved in regulating fungal development (Herman, 1998). In 2004, Walters *et al*. tested linolenic acid against plant pathogenic fungi, *Rhizoctonia solani*, *Pythium ultimum*, *Pyrenophora avenae*, and *Crinipellis perniciosa,* and it showed that the biomass production of all tested pathogens reduced significantly. This finding is consistent with the findings by Kumar *et al*. (2013), *Ceriops decandra* leaves extracts had 8.62% linolenic and showed antifungal solid activity against *Pythium aphanidermatum*, *Rhizoctonia solani*, *Pyricularia oryzae*, *Curvularia oryzae*, and *Fusarium oxysporum*. Both studies used *Pythium* spp., an oomycete like *Phytophthora* spp. as test subjects in the mentioned screening. Hence, extracts containing this compound have the potential antifungal activity against *P. palmivora*.

Phytol belongs to the diterpene family and is a component that is relevant to the crude extract of *M. citrifolia*. Plant extract-derived diterpenoids reduce the number of fungal mitochondria, which could impact reactive oxygen species and ATP production (Haque *et al*., 2016). In a study on combating *Ganoderma boninense*, a disease in oil palm, a phytol component extracted from seaweed was highlighted to have remarkable potential as an antifungal property (Aziz *et al*., 2019). Phytol acts as an antimicrobial by leaking K+ ions from cells, directly disrupting the membrane tissue (Inoue *et al*., 2005). As a result, the fungus's mitochondria become dysfunctional. Masi and Evidente (2021) reported that a diterpene compound isolated from *Diplodia cupressi* completely inhibited the mycelial growth of *Phytophthora cambivora*.

Alpha-tocopherol is a naturally sourced vitamin E that is a free radical scavenger, preventing lipid peroxidation in biological systems (Rani *et al*., 2007). Due to this role, it can cause cell membrane perturbations, resulting in damage to essential components for the reliability of the membrane, thereby allowing an increase in permeability (Andrade *et al*., 2014). According to reports, tocopherols are critical in protecting against pathogens (Ghimire *et al*., 2017). The use of isomers of vitamin E was

reported to distort the functions of bacteria *Staphylococcus aureus, Pseudomonas eaeruginosa*, and *Escherichia coli* (Andrade *et al*., 2014). In a study by Ghimire *et al*. (2017), the transgenic leaf extract of *Codonopsis lanceolate* had increased antimicrobial activity with the increase of Alpha-tocopherol content. For oomycetes specifically, reports of *Dittrichia viscosa* (L.) having high content of tocopherols (Rhimi *et al*., 2018) were able to control foliar disease caused by *Phytophthora infestans* (Wang *et al*., 2004).

Different solvents will extract a variety of compounds with varying polarities. Methanol solvent was used to extract the chemical groups of flavonoids, polyphenols, and glycosides. (Rauha *et al*., 2000). Acetone is an excellent solvent for extracting phenolics, flavonoids, alkaloids, and terpenoids (Truong *et al*., 2019). Hexane extracts semi-polar and nonpolar compounds like fatty acids, terpenoids, and fats (Tiwari *et al*., 2011). Both polar and nonpolar plant extract components contribute to antifungal activity against *P. palmivora*. Jayaraman *et al*. (2008) found that the plant's chemical makeup may account for the varying antifungal effectiveness of *M. citrifolia* leaf extract when employing different solvents.

Mycelial growth of *Phytophthora palmivora* was reduced significantly by crude extracts of methanol, acetone, and ethyl acetate in a concentration-dependent manner (Figure 4). This result is consistent with the findings of John *et al*. (2016) and Krzyko-upicka *et al*. (2019), who discovered that increasing the crude extract concentration increases the level of antifungal compounds. Such reduction in mycelia growth may be related to the enzymatic activity of the extract, which disrupts cell wall structure and blocks membrane synthesis (Li *et al*., 2014). The differences in antifungal activity of *M. citrifolia* leaf extracts extracted with different solvents could be attributed to differences in chemical composition (Ma *et al*., 2019). Studies have shown that extracts' secondary metabolites (such as phenolics, flavonoids, and terpenoids) play critical roles in antimicrobial action against various pathogenic microorganisms (de Araujo Gomes *et al*., 2020). The crude hexane extracts were ineffective in controlling *P. palmivora*, possibly due to the crude's lack of antifungal compounds. This result is consistent with an antifungal assay in which crude extracts of *M. citrifolia* fruit against nine pathogenic fungal strains in hexane solvent had the lowest percentage of inhibition (Jayaraman *et al*. 2008).

For *in vitro* bioassay evaluation, *M. citrifolia* leaves crude extracted with acetone and ethyl acetate had the best EC_{50} values of 36.12 and 38.10 mg/mL, respectively, where the treatments have altered the morphology and restricted the growth of *P. palmivora* through microscopic observation using a scanning electron microscope, the fungus' morphological change is directly related to the secondary metabolites produced by the plant extract, which served as an antifungal agent to prevent fungal growth. There is a direct correlation between the morphological alteration of the fungus and the secondary metabolites generated by the plant extract, which functioned as an antifungal agent to restrict fungal growth (Pusztahelyi *et al*., 2015).

According to the *in vivo* bioassay, the negative control treatment developed 100% canker symptoms, while the other treatments had a disease incidence ranging from 0 to 41.67%. This result shows that the specific intracellular receptors are essential for the expression of the antifungal activity of the crude extract. These chemical constituents can detect sites of action in the pathogen. They would, therefore, bind on their receptors, thus inducing responses of fungicidal effects such as suppression of general metabolism or alteration of the fungus membrane and inhibition of respiration (Essomé *et al*., 2022). In this study, positive control using Ridomil commercial fungicide had 0% disease incidence, as did Acetone treatment at 72 mg/mL. This finding also demonstrates that the tested crude extract treatments have a bright future in developing a biological control product.

A study by Freeman and Beattie (2008) discovered that no infection was obtained at the inoculation site without wounding because the plant's outer barriers were still intact; hence, wounding will expedite disease infection. Unlike true fungi, oomycetes lack the melanin and septin necessary for producing appressoria. Instead, *P. palmivora* infiltrates its hosts by hyphal slicing through the host surface at an oblique angle (Bronkhorst *et al*., 2021). Therefore, in durian fields, wounds are thought to play a role in the natural infection of *P. palmivora* (Mohamed Azni *et al*., 2019).

CONCLUSION

The two highest acetone and ethyl acetate concentrations resulted in significantly lower lesion development on the durian stem. The concentration of antifungal components in the plant extract was adequate for controlling the *P. palmivora* pathogen. Nevertheless, it is essential to note that the bioactivity of plant extracts can be attributed to the main constituents alone or in combination.

ACKNOWLEDGMENTS

The Malaysian Ministry of Higher Education funded this study under the Fundamental Research Grant Scheme (FRGS), grant project code FRGS/1/2019/WAB01/UPM/02/20. The authors would also like to thank Universiti Putra Malaysia and the Malaysian Agricultural Research and Development Institute (MARDI) for providing research facilities for this study.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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