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Research Article

Bioactivity of Volatile Compounds Present in *Crinum zeylanicum* Leaf Extracts Identified by GC-MS for the Control of *Cercospora malayensis* Isolates of Okra (*Abelmoschus esculentus* L.) *In Vitro*

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ABSTRACT

Cercospora malayensis induced leaf spot is a dangerous okra disease that reduces crop yields. The aim of this study is to use leaf extract from *Crinum zeylanicum* to regulate the growth of *Cercospora malayensis in vitro*. The pathogen was isolated from okra leaves from the Meyomessala and Akonolinga localities that had typical disease symptoms. Phytochemical screening and GC-MS analysis of *C. zeylanicum* leaf extracts were carried out. The mycelial growth and germination of *C. malayensis* isolates 1 and 2 were evaluated with concentrations of 15, 30, 60, and 120 µL mL^{-1}, fungicide (3.33 g L^{-1}), and control. Minimum inhibition concentrations (MIC50 and MIC90) were evaluated. The results showed the presence of alkaloids, phenols, terpenoids and sterols in the leaf extracts. In acetone (AcE), methanol (ME) and aqueous (AqE) extracts, 32; 39 and 10 chemical compounds, respectively were found by GC-MS analysis. The most prevalent biochemically active compounds were n-Hexadecanoic acid (35.04 %), Cis-Vaccenic acid (31.76 %), Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester (26.63 %), 9,12-Octadecadienoic acid (Z,Z)-methyl ester (16.89 %) and 9,12-Octadecadienoic acid (Z,Z) (10.83 %). AqE, AcE and ME extracts at 120 µL mL^{-1} inhibited 100 % of mycelial growth and conidial germination of isolates 1 and 2 compared to the control. The lowest MICs (MIC50 and MIC90) were 6.79 and 10.98 µL mL^{-1} for isolate 1 and 7.48 and 11.22 µL mL^{-1} for isolate 2, respectively. *C. zeylanicum* it is possible to use extracts for their volatile biochemical substances in a *C. malayensis* control program.

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INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench.) is a plant species grown in tropical, subtropical and warm temperate regions (Saifullah & Rabbani 2009). It is a vegetable plant belonging to the Malvaceae family with controversial origins (West Africa, Ethiopia, South Asia) and cultivated for its succulent fruit as well as its rich nutrient content (Kahlon et al. 2007). Global okra production is projected to begin at around 9 million tons in 2021; Africa produces around 3.5 million tons (FAOSTAT 2024). Over the last ten years, Cameroon has recorded the highest okra fruit yield in 2016 with an estimated average of 78 763 tons, compared with 2022, with an estimated average of 77631.82 tons (FAOSTAT 2024). This decline in fruit yield production is due to non-compliance with good agricultural practices, the application of soil fertility, low-yielding varieties and biotic and abiotic stress (Dubey & Bhagat 1998; Ali & Hossain 2000).

Several diseases, such as fungi, hamper okra production (Raid & Palmateer 2006). Among fungal diseases, Cercospora leaf spot is one of the major diseases (Jukte et al. 2016). The genus *Cercospora* belongs to the Mycosphaerellaceae family, with two different *Cercospora* species including *C. malayensis* and *C. abelmoschi* that can cause the disease on economically important plants (Braun et al. 2014). Cercosporiosis with *C. malayensis* has been documented in tropical and subtropical Asian countries and also in West Africa where okra is grown, particularly during the rainy season (Beilharz et al. 2002; Braun & Freire 2004; Hernández-Gutiérrez & Dianese 2009). Disease symptoms are generally visible on the plant's leaves. All stages of plant development are affected by the disease. Infection begins when conidia reach the underside of okra leaves. The conidia germinate and emit mycelia that penetrate the leaf parenchyma through the stomata (Souza et al. 2015). The pathogen's mycelium spreads into the leaf parenchyma, damaging cells and causing their necrosis through the production of photosensitive toxins that degrade cell walls (Steinkamp et al. 1979; Daub 1982). This process resulted in the appearance of small, irregular, pale-brown spots on okra foliage. Leaf spots can enlarge and coalesce as the disease progresses on the plant, forming larger, irregular lesions leading to yellowing, wilting, defoliation and loss of vigour, resulting in yield losses ranging from 45 to 80 % (Chai et al. 2021).

Synthetic fungicides are abused by growers to control the disease in the field. In the long term, they give rise to resistance phenomena, cause environmental pollution and are toxic to humans (Watanabe-Akanuma et al. 2005; Perera et al. 2005). In the pursuit of better, non-polluting and health-promoting substitutes, biological control using natural substances of plant origin appears to be an alternative for protecting field crops (Ambang et al. 2011). The use of extracts from plants that are high in secondary metabolites (terpenoid compounds, phenolics, nitrogen compounds and saponins) as biopesticides has proved effective in managing crop diseases (Toka et al. 2023; Dida et al. 2024). Several studies have demonstrated the biopesticidal properties of certain plants (Mboussi et al. 2018; Atindo et al. 2020; Manga et al. 2021; Essomé et al. 2022). *Crinum zeylanicum* (Linn.) is a monocotyledonous perennial herb in the Amaryllidaceae family (Refaat et al. 2012). Like most plants, phytochemical studies of *Crinum* genus organs have identified biological activities (Tram et al. 2002). The purpose of this study is to use leaf extract from *Crinum zeylanicum* to regulate the growth of *Cercospora malayensis in vitro.*

MATERIALS AND METHODS

Isolation and identification of *Cercospora malayensis*

Pure isolates of *C. malayensis* were obtained on Potato Dextrose Agar (PDA) culture medium supplemented with Ampicillin 250 mg L^{-1} and chloramphenicol

col (200 mg L^{-1}) from leaves collected in the locality of Akonolinga (4°12 N, 11°24 E) and Meyomessala (2°43 N, 11°42 E) showing typical symptoms of the disease. Diseased leaves were washed several times with distilled water and disinfected with 90 % alcohol and sodium hypochlorite (10 %) for 2 min. Leaf fragments measuring approximately 2 mm^2 were placed in the middle of the Petri dish poured with PDA media and incubated in the dark for eight days 25 ± 2 °C (Hasanin & Hashem 2020). Emerging white mycelia of each fragments were observed under a light microscope and moved to new Petri dishes containing PDA culture medium for purification until pure cultures were obtained. Identification of the pathogen was based on macroscopic and microscopic observations of the morphological characteristics of the mycelium, conidia and an identification key (Hashem & Farrag 2005).

Preparation of *Crinum zeylanicum* leaf extracts

Crinum zeylanicum leaves collected in the locality of Akonolinga (4°12 N, 11° 24 E) were identified by an expert botanist from the National Herbarium. After three weeks of shade drying at room temperature, the leaves were processed into a powder with an electric grinder. The organic extracts were prepared by macerating of 500 g of powder in 5 L of solvent, represented here by Methanol and Acetone for 48 hours. The resulting mixes were filtered using filter paper and the filtrates concentrated in a rotary evaporator (Büchi R-200 Rotary Evaporator at 60 °C) until almost complete elimination of the extraction solvents. The extracts obtained were kept in a refrigerator at 4 °C until its needed (Vongsak et al. 2013). The aqueous extract of *C. zeylanicum* leaves was acquired by maceration of 50 g of powder in 100 mL of distilled water and filtered through a fine muslin cloth (Zibbu & Batra 2010).

Phytochemical screening of *Crinum zeylanicum* leaf extracts

The various extracts from *C. zeylanicum* leaves were subjected to phytochemical analysis to determine their chemical composition. Chemical compounds were identified by observing certain characteristics after testing each extract with specific dyes. The standard protocol described by Edeoga et al. (2005), Kumar et al. (2010), Tiwari et al. (2011) and Banu and Cathrine (2015) was used.

GC-MS analysis of *Crinum zeylanicum* leaf extracts

C. zeylanicum leaf extracts were analysed using a GC-MS apparatus (Agilent GC 7890A) coupled to a mass spectrometer (Agilent 5975C TAD VL MSD) equipped with an Elite-1 fused silica capillary column (30 m x 0.25 mm diameter, film thickness 0.25 µm) and helium as the carrier gas. The energy of the electronic ionisation system is 70 eV. After being kept at 150 °C for one minute at a rate of 20 °C min^{-1}, the oven reached a final temperature of 280 °C for 9 min. Total GC running time was 13 min. Separated constituents were identified by comparing their spectra with those of the National Institute Standards and Technology (NIST) database of over 62,000 models. The injected solution was prepared by mixing 1.5 g of anhydrous sodium acetate and 6 g of magnesium sulfate, autoclaved and cooled in a desiccator, with 10 mL of aqueous extract and 10 mL of acetonitrile. For the organic extract, 1 mL was mixed with 2 mL solvent (acetone or methanol) and 0.15 g magnesium sulfate. After that, the mixtures were vortexed and centrifuged at 4000 rpm (QuECHERS 2004). One microliter (1 µL) of each preparation was injected into the column.

In vitro test

From pure isolates 1 and 2 obtained from samples collected in the locality of Akonolinga and Meyomessala respectively, explants approximately 7 mm in

diameter were removed with a cork borer and placed in the middle of each Petri dish containing PDA medium poisoned with aqueous, methanol and acetone extracts at 15; 30; 60 and 120 µL mL^{-1} concentrations to the synthetic fungicide with active ingredient Metalaxyl 80 g kg^{-1} and Mancozèbe 640 g kg^{-1} at 3.33 g L^{-1} and to the control with no added extract or fungicide. From the stock solutions (500 µL mL^{-1}) of every extracted 50 mL of each extract and 100 mL of solvent, volumes of 1.8; 3.6; 7.2 and 14.4 mL were taken and added respectively to 28.2; 26.4; 22.8 and 15.6 mL of PDA for a final volume of 30 mL each, it was transferred at a rate of 10 mL per 90 mm Petri dish. For controls, a 10 mL solution of PDA medium was poured directly into each Petri dish. The medium enriched with the synthetic fungicide at the concentration of 3.33 g L^{-1} was prepared using the normal rate (50 g per 15 L of water). Tests were performed in triplicate for each pathogen. Incubation was carried out at 25 ± 2 °C, and mycelial growth of isolates was assessed from the second day after incubation (DAI) until the mycelia of each isolate completely filled the control Petri dishes, by taking measurements of the two perpendicular diameters traced on the Petri dishes back. The growth diameters were calculated and Inhibition Percentage (IP) was determined according to the formula used by Singh et al. (1993).

$$IP\ (\%) = (Mgc - Mgt/Mgc) \times 100$$

Where: IP = inhibition percentage; Mgc = mycelial growth in control; Mgt = mycelial growth in the treatment.

Evaluation of fungicidal or fungistatic activity

The fungicidal or fungistatic activity of *Crinum zeylanicum* leaf extracts was assessed by selecting Petri dishes in which the growth of pathogen isolates had been completely inhibited at the end of mycelial growth, and depositing them in a new culture medium containing no extract and/or fungicide. After 6 days, the Petri dishes were observed and if growth had resumed, the starting extract was qualified as fungistatic; if not, it was considered fungicidal (Toka et al. 2023).

Minimal inhibitory concentrations of the different extracts.

The Minimal Inhibitory Concentrations (MIC50 and MIC90) of the different extracts were ascertained by contrasting the Inhibitory Percentage values with the corresponding concentrations' (Ci) natural logarithm values (España et al. 2017).

$$IP = f\,(\ln Ci).$$

The inhibition percentage (IP) is determined for each treatment relative to the control after 8 days of mycelial growth catch, following the formula of Singh et al. (1993):

$$IP\ (\%) = (Dc\text{-}Dt)/Dc \times 100$$

Where: Dc= mean culture diameter measured in control; Dt= mean culture diameter measured in treatment.

The linear regression line Y = ax + b derived from the PI = f (ln Ci) function is used to determine MIC50 and MIC90, where Y = percentage inhibition, a = slope of line, MIC = ex and b = constant (Griffin et al. 2000).

Cercospora malayensis conidial germination test

The germination test was carried out with *C. malayensis* conidia obtained from a pure culture of isolates 1 and 2 aged 10 days contained in Petri dishes, by taking the mycelium of both isolates and mixing each in a quantity of sterile distilled water, then calibrated using the mallassez cell to obtain 2 x 10^4 conidia mL^{-1} (Fulano et al. 2016). The impact of extracts on conidial germination of *C. malayensis* isolates 1 and 2 compared with a synthetic fungicide was assessed by casting PDA culture medium supplemented with extracts and

fungicide onto slides. Conidial suspensions of 20 µL of *C. malayensis* were spread out on the slides using a micropipette in contrast to the control slides. Each preparation was repeated three times and kept in the dark at 25 °C for a minimum of 12 h. After 24 h the count of germinated conidia was made on a total of 100 conidia on three different zones of each slide using the photonic microscope (Widmer & Laurent 2006). Inhibition percentages (IP) of extracts against the two *C. malayensis* isolates were calculated applying the subsequent formula:

$$IP\ (\%) = (A-B)/A \times 100 \text{ (You et al. 2016)}$$

Where: A = Estimated number of conidial on control medium; B = Estimated number of conidial in the presence of the extract

Statistical analysis

Data collected for the parameters studied were subjected to a one-way ANOVA using R software version 4.0.1. Multiple comparison of means was determined by the Tukey HSD test when notable distinctions ($P < 0.05$) for one of the factors were found, and when normality of the data (Shapiro-Wilk test; $P > 0.05$) and homogeneity of variance (Bartlett test; $P > 0.05$) were verified.

RESULTS

Identification of *Cercospora malayensis*

Two pure isolates of *C. malayensis* were obtained from fragments of okra leaves collected in the localities of Akonolinga and Meyomessala (Figure 1). The two isolates were identified morphologically by macroscopic and microscopic characters. The macroscopic characteristics of the two isolates observed in Petri dishes showed whitish mycelia on the upper and lower surfaces with a cottony appearance (Figure 1 A,C). Under the light microscope, conidia were continuous and elongated, subaigual to obtuse at the apex and truncate to obconical at the base with 3 to 8 septa and slightly curved (Figure 1 B,D). These morphological traits aligned with those of *C. malayensis* F. Stevens & Solheim, the causal agent of Cercosporiosis of okra.

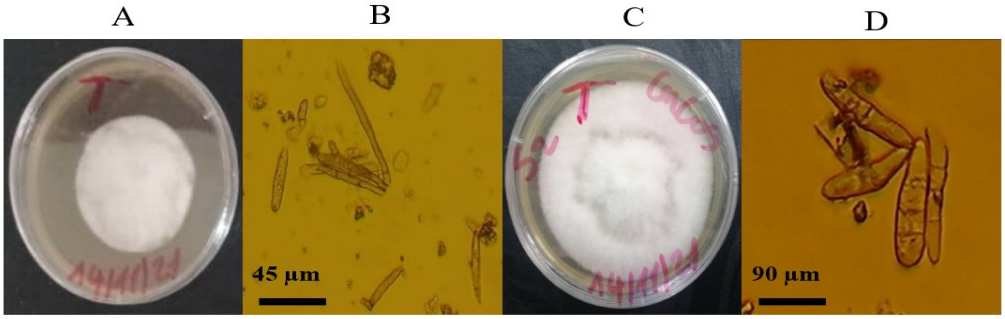

Figure 1. Pure isolates of *Cercospora malayensis*; A: Mycelium of *C. malayensis* isolate 1; B: Conidia of isolate 1 observed under a light microscope; C: Mycelium of *C. malayensis* isolate 2; D: Conidia of isolate 2 observed under a light microscope.

Phytochemical constituents of *Crinum zeylanicum* leaf extract

Phytochemical screening revealed the existence of of several families of compounds, including terpenes, sterols, phenols, saponins, flavonoids, oils, sugars, saponins and alkaloids. Alkaloids, flavonoids, sterols and terpenes are most abundant in the aqueous and methanol extracts. Sugars and oils are present in trace in the methanol extract. The methanol and aqueous extracts are the richest in compound families. The acetone extract is the poorest in chemical compound family, as only terpenes, sterols and oils are present. Terpenes, sterols and oils are present in all four extracts. Quinone and saponins are absent in all extracts (Table 1).

Table 1. Various natural products in the different *Crinum zeylanicum* extracts.

Products	Acetone extract	Aqueous extract	Methanol extract
Terpenes	++	+	+
Sterols	++	+	+
Phenols	-	+	+
Quinons	-	-	-
Saponins	-	-	-
Flavonoids	-	++	++
Alkaloids	-	+++	+++
Oils	+	+	T
Sugar	-	+	T

- Absent, + present, ++ moderate present, +++ highly present, T trace presence (Jafri et al. 2022).

GC-MS analysis of the various extracts

The chromatographic profile of the different *C. zeylanicum* leaf extracts showed variable results depending on the extraction solvents used. Peaks represent majority, minority and ultra-minority compounds (Figure 2). Majority chemical compounds constitute the highest peaks. Chemical compounds identified by mass spectrometry are grouped according to their bioactivity, molecular weight, molecular formula, and retention time (RT). In the acetone extract, 32 chemical compounds were detected, the most representative being: Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester (26.63 %), 9,12-Octadecadienoic acid (Z,Z) (10.83 %) and 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (10.37 %) (Table 2). In the methanol extract, 39 chemical compounds were detected (Table 3), the most abundant being: 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (16.89 %), Phytol (11.78 %), 3-Methyl-12-phenyl-8,9,10,12-tetrahydro-7H-benzo[b][4,7]phenanthrolin-11-one (8.91 %), 9-Octadecenoic acid, (E)- (8.28 %). Finally, in the aqueous extract, n-Hexadecanoic acid (35.04 %) and Cis-Vaccenic acid (31.76 %) were the most abundant, with 10 chemical compounds detected (Table 4).

In vitro effect of *Crinum zeylanicum* seed extracts against *Cercospora malayensis*

The suppression of mycelial development varied significantly of *C. malayensis* isolates 1 and 2 between different concentrations of *C. zeylanicum* leaf extracts, fungicide and control (Figure 3). For *C. malayensis* isolate 1, concentrations of 60 and 120 µL mL^{-1} inhibited mycelial growth by 100 % in both the methanol and acetone extracts. In contrast, the aqueous extract at 120 µL mL^{-1} showed little inhibition (53.04 %). The synthetic fungicide inhibited 100 % of the mycelial growth of *C. malayensis* isolate 1, compared with the control, where the inhibition percentage was null (Figure 3A). For *C. malayensis* isolate 2, 100 % inhibition was observed with methanol extract of *C. zeylanicum* leaves at concentrations of 60 and 120 µL mL^{-1}. With the aqueous and acetone extracts, inhibition of 94.52 % and 100 % respectively was recorded at a concentration of 120 µL mL^{-1} (Figure 3B). The synthetic fungicide inhibited 100 % of mycelial growth compared with the control, where growth was complete in Petri dishes after 8 days of incubation (Figure 4).

Minimum inhibition concentrations of different extracts

The correlation test between the parameters studied demonstrated the existing relationships and enabled us to determine the Minimum Inhibitory Concentrations (MICs). Regression lines obtained with isolate 1 and aqueous extract (y = 12.076x - 2.2877), acetone extract (y = 33.371x - 49.508) and methanol extract (y = 25.95x - 14.907) all showed positive slopes. Similarly, positive regression lines were obtained with isolate 2 and the methanol (y = 32.016x - 42.135), acetone (y = 27.701x - 26.135) and aqueous (y = 24.605x -

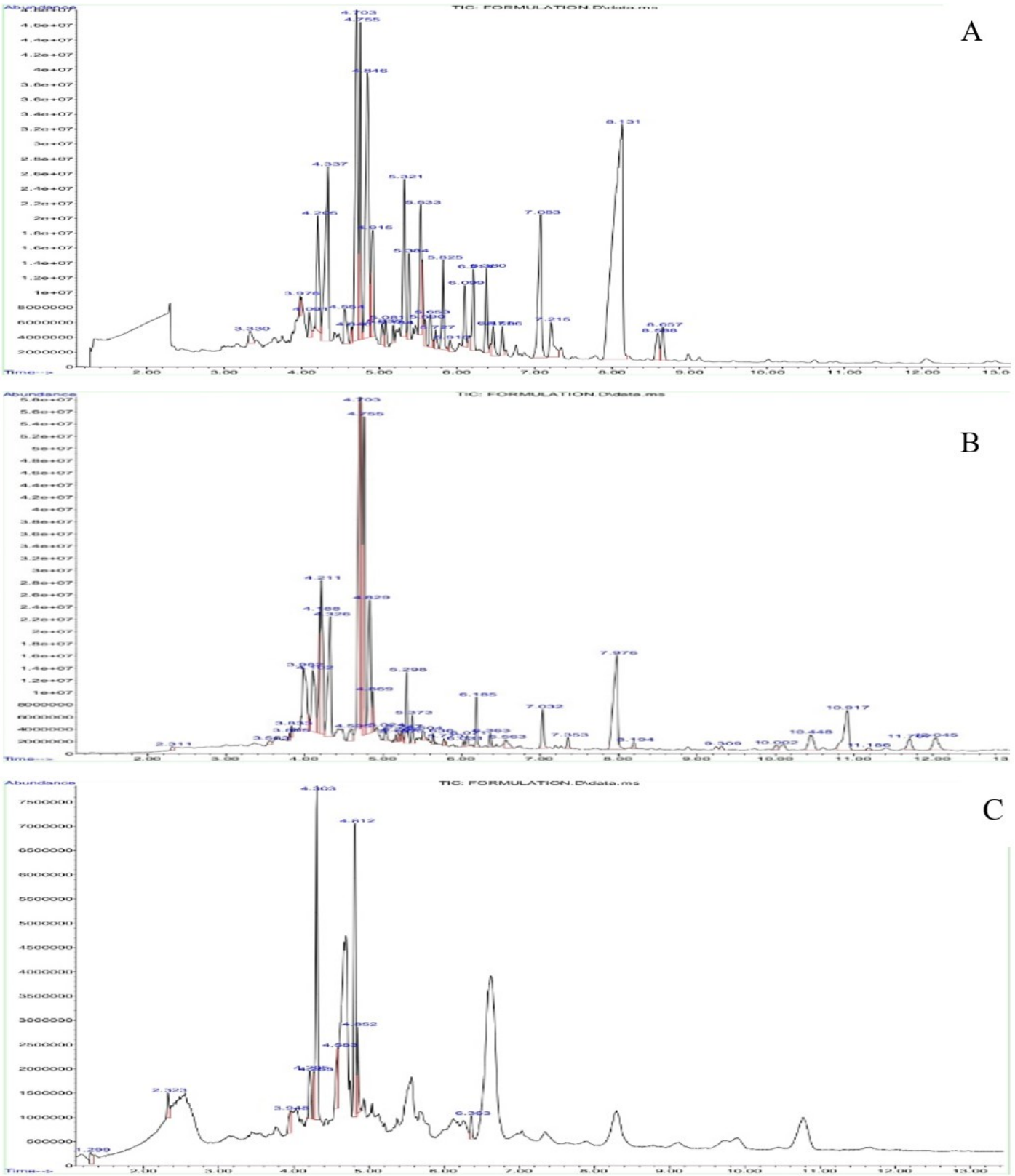

Figure 2. Chromatographic profile of *Crinum zeylanicum* leaf extract; A: Acetone extract, B: Methanol extract, C: Aqueous extract.

26.517) extracts of *C. zeylanicum* leaves. The lowest minimum inhibitory concentrations (MIC50) were acquired with the methanol extract (6.79 µL mL^{-1}) for *C. malayensis* isolate 1 and with the acetone extract (7.48 µL mL^{-1}) for isolate 2. For MIC90, low minimum inhibitory concentrations of 10.88 and 11.22 µL mL^{-1} were obtained for the methanol extract of *C. zeylanicum* leaves for *C. malayensis* isolate 1 and 2 respectively. The highest minimum inhibitory concentrations (MIC50 and MIC90) were recorded with the aqueous extract

Table 2. Chemical compounds present in the acetone extract of *Crinum zeylanicum* leaves identified by GC-MS.

Peak	RT (min)	Area (%)	Name of compound	MF	MW
1	3.33	0.55	2-Methyl-Z,Z-3,13-octadecadienol	$C_{19}H_{36}O$	280.00
2	3.98	0.36	(-)-trans-Pinane	$C_{10}H_{18}$	138.25
3	4.09	0.70	Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, [1S-(1.alpha., 2.beta. ,5.alpha.)]-	$C_{10}H_{18}$	138.25
4	4.21	3.66	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_3$	286.46
5	4.34	7.44	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42
6	4.55	1.19	1H-Phenalen-1-one	$C_{13}H_8O$	180.20
7	4.65	0.35	2-Methyl-4'-methoxy-benzophenone	$C_{15}H_{14}O_2$	226.27
8	4.70	10.37	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47
9	4.76	5.27	Phytol	$C_{20}H_{40}O$	296.00
10	4.85	10.83	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280.45
11	4.92	2.71	2,3,8-Trimethyl-1H,9H-pyrrolo[3,2-H]quinolin-6-one	$C_{14}H_{14}N_2O$	226.27
12	5.04	0.56	Acridin-9-amine, 1,2,3,4-tetrahydro-5,8-dimethyl-	$C_{15}H_{18}N_2$	226.32
13	5.08	0.58	1,1'-Biphenyl, 5-hydroxy-3,4'-dimethoxy-	$C_{14}H_{14}O_3$	230.26
14	5.18	0.29	2,4,6-Trimethyldecane-1,3,10-triol	$C_{13}H_{28}O_3$	232.36
15	5.32	3.74	Benzeneacetic acid, .alpha.-(acetyloxy)-2-methoxy-, methyl ester	$C_{12}H_{14}O_5$	238.24
16	5.38	1.70	Propanedioic acid, 3-methoxyphenylhydrazono-	$C_{10}H_{10}N_2O_5$	238.20
17	5.53	2.79	2-Propen-1-one, 1-(4-aminophenyl)-3-phenyl-	$C_{15}H_{13}NO$	223.27
18	5.59	0.46	4,5-Dihydro-N-(p-methoxyphenyl)-3-furamide	$C_{12}H_{13}NO_3$	219.24
19	5.65	0.87	4-Isopropenylcyclohexanone	$C_9H_{14}O$	138.21
20	5.73	0.41	Mesembrine	$C_{17}H_{23}NO_3$	289.40
21	5.83	2.01	Benzeneethanamine, N-(1-methylethylidene)-	$C_{11}H_{15}N$	161.24
22	5.91	0.33	2-[p-Cyanophenyl]-5-chlorobenzimidazole	$C_{14}H_8ClN_3$	253.68
23	6.09	1.48	4H-1-Benzopyran-4-one, 2,3-dihydro-7-hydroxy-2-(3-hydroxyphenyl)-	$C_{15}H_{12}O_4$	256.25
24	6.21	1.95	3-Hydroxy-4-nitrobenzaldehyde	$C_7H_5NO_4$	167.12
25	6.38	1.81	5-(p-Aminophenyl)-4-(p-tolyl)-2-thiazolamine	$C_{16}H_{15}N_3S$	281.40
26	6.47	0.82	Acetyl-caranine	$C_{18}H_{19}NO_4$	313.30
27	6.59	0.68	9,10-Anthracenedione, 2-hydroxy-	$C_{14}H_8O_3$	224.21
28	7.08	5.66	Daniquidone	$C_{15}H_{11}N_3O$	249.27
29	7.22	1.65	1H-Indene, 1-(1,5-dimethyl-2-hexenyl)octahydro-7a-methyl-, [1R-[1.alpha.(1R*,2Z),3a.beta.,7a.alpha.]]-	$C_{18}H_{32}$	248.25
30	8.13	26.63	Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester	$C_{17}H_8NO_2$	263.29
31	8.54	1.11	8-Hydroxy-6-methyl-8-phenyl-hexahydro-indolizin-5-one	$C_{11}H_{12}N_2S$	204.21
32	8.66	1.06	5-Ethoxy-6-methoxy-8-nitroquinoline	$C_{12}H_{12}N_2O_2$	248.23

RT: retention time; MF: molecular formula; MW: molecular weight

(11.77 and 20.77 µL mL^{-1}) and (8.45 and 12.88 µL mL^{-1}) respectively, for isolate 1 and 2 of *C. malayensis* (Table 5).

Fungicidal and fungistatic activity of extracts

The fungicidal and fungistatic activity of *C. zeylanicum* leaf extracts was studied with Petri dishes at the highest concentration (C4 = 120 µL mL^{-1}), which showed total inhibition (Table 6). After 10 days of incubation, no renewed growth of *C. malayensis* isolates 1 and 2 was observed with explants taken from Petri dishes poisoned with methanol (MeE) and acetone (AcE) extracts of fungicidally active *C. zeylanicum* leaves. The aqueous extract (AqE) of *C. zeylanicum* leaves was fungistatic for *C. malayensis* isolates 1 and 2.

Effect of *Crinum zeylanicum* leaf extracts on germination of *Cercospora malayensis* conidia

The germination test for *C. malayensis* conidia demonstrates that there is a substantial difference ($P < 0.05$) between *C. zeylanicum* leaf extracts at different concentrations, the synthetic fungicide and the control (Figure 5). With *C. malayensis* isolate 1, low conidial germination of 46.56 %, 45.57 % and 42.22

Table 3. Chemical compounds present in the methanol extract of *Crinum zeylanicum* leaves identified by GC-MS.

Peak	RT (min)	Area (%)	Name of compound	MF	MW
1	2.31	0.23	Benzeneacetic acid, .alpha.-ethyl-, ethyl ester	$C_{12}H_{16}O_2$	192.25
2	3.55	0.31	1,19-Eicosadiene	$C_{20}H_{38}$	278.52
3	3.81	0.20	Methyl 13-methyltetradecanoate	$C_{16}H_{32}O_2$	256.42
4	3.83	0.32	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	256.42
5	3.98	5.55	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	$C_{10}H_{18}$	138.25
6	4.10	4.97	11-Hexadecen-1-ol, acetate, (Z)-	$C_{18}H_{34}O_2$	282.46
7	4.12	3.69	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270
8	4.21	7.71	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_3$	286.45
9	4.33	7.94	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42
10	4.59	1.00	2-Butyloxycarbonyloxy-1,1,10-trimethyl-6,9-epidioxydecalin	$C_{18}H_{30}O_5$	326.4
11	4.70	16.89	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47
12	4.76	11.78	Phytol	$C_{20}H_{40}O$	296
13	4.83	8.28	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	282.5
14	4.87	0.84	Octadecanoic acid	$C_{18}H_{36}O_2$	282.5
15	5.02	0.57	Acridin-9-amine, 1,2,3,4-tetrahydro-5,7-dimethyl-	$C_{15}H_{18}N_2$	226.32
16	5.17	0.18	3-Hydroxydecanoic acid	$C_{10}H_{20}O_3$	188.26
17	5.20	0.27	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292.00
18	5.25	0.30	cis-5,8,11-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	320.5
19	5.29	2.63	6-Amino-2,4-dimethylphenol	$C_8H_{11}NO$	137.18
20	5.37	0.98	Methyl 18-methylnonadecanoate	$C_{21}H_{42}O$	326.6
21	5.50	0.48	Tricyclo[3.3.3.0(1,5)]undec-6-ene-2,3,6-tricarbonitrile	$C_{14}H_{13}N$	223.27
23	5.77	0.25	1,19-Eicosadiene	$C_{20}H_{38}$	278.52
24	6.03	0.20	Triacontyl acetate	$C_{32}H_{64}O_2$	480.8
25	6.07	0.43	4H-1-Benzopyran-4-one,2,3-dihydro-7-hydroxy-2-(3-hydroxyphenyl)-	$C_{15}H_{12}O_4$	256.25
26	6.19	1.67	Methyl 20-methyl-heneicosanoate	$C_{23}H_{46}O_2$	354.6
27	6.36	0.51	Bis (2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6
28	6.56	0.71	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	$C_{30}H_{50}O$	426.7
29	7.03	1.89	3-Methyl-1-phenethyl-3,4-dihydro-isoquinoline	$C_{18}H_{19}N$	249.3
30	7.35	0.51	Tetracosanoic acid, methyl ester	$C_{20}H_{50}O_2$	382.7
31	7.98	8.91	3-Methyl-12-phenyl-8,9,10,12-tetrahydro-7H-benzo[b][4,7]phenanthrolin-11-one	$C_{23}H_{20}NO_2$	340.4
32	8.19	0.33	Squalene	$C_{30}H_{50}$	410.7
33	9.31	0.22	Stigmastan-6,22-dien, 3,5-dedihydro-	$C_{29}H_{46}$	394.6
34	10.02	0.36	Thiazole, 4-[1,1'-biphenyl]-4-yl-2-methyl-	$C_{16}H_{13}NS$	251.3
35	10.45	1.65	Stigmasteryl tosylate	$C_{36}H_{54}O_3S$	566.9
36	10.92	4.3	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-	$C_{31}H_{50}O_2$	454.7
37	11.19	0.18	Ergosta-4,6,22-trien-3.beta.-ol	$C_{28}H_{44}O$	396.6
38	11.71	0.98	Stigmastan-3,5-diene	$C_{29}H_{48}$	396.7
39	12.05	1.48	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol	$C_{27}H_{46}O$	386.7

RT: retention time; MF: molecular formula; MW: molecular weight.

% was recorded in the methanol extract at concentrations of 15, 30 and 60 µL mL^{-1} respectively. With the aqueous extract, conidial germination was observed at 15 µL mL^{-1} (88.88 %), 30 µL mL^{-1} (87 %) and C3 = 60 µL mL^{-1} (39.78 %), and with the acetone extract at 15 µL mL^{-1} (29 %) and 30 µL mL^{-1} (17.33 %). With *C. malayensis* isolate 2, germination rates of 37.11 %, 20.44 % and 16.88 % were recorded with the methanol extract at concentrations 15, 30 and 60 µL mL^{-1} respectively. The aqueous extract recorded a germination of 95.55 %, 76.67 %, 32.22 % and 13.33 % at concentrations 15, 30 and 60 µL mL^{-1} respectively. In general, the synthetic fungicide and 120 µL mL^{-1} recorded no conidial germination compared with the control, where germination was 100 %. Conidial germination in the control, methanol extract and aqueous extract at 120 µL mL^{-1} is shown in figure 6.

Table 4. Chemical compounds present in the aqueous extract of *Crinum zeylanicum* leaves identified by GC-MS.

Peak	RT (min)	Area (%)	Name of compound	MF	MW
1	1.299	1.91	Chlorfenapyr	$C_{15}H_{11}BrClF_3NO_2$	407.61
2	2.323	2.81	Pentanoic acid	$C_5H_{10}O_2$	102.13
3	3.948	2.88	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.40
4	4.205	5.37	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_3$	286.456
5	4.263	4.03	cis-9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254.41
6	4.303	35.04	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42
7	4.583	7.77	Gamma.- Sitosterol	$C_{29}H_{50}O$	414.7
8	4.812	31.76	Cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.5
9	4.852	6.31	Octadecanoic acid	$C_{18}H_{36}O_2$	282.5
10	6.363	2.11	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6

RT: retention time; MF: molecular formula; MW: molecular weight

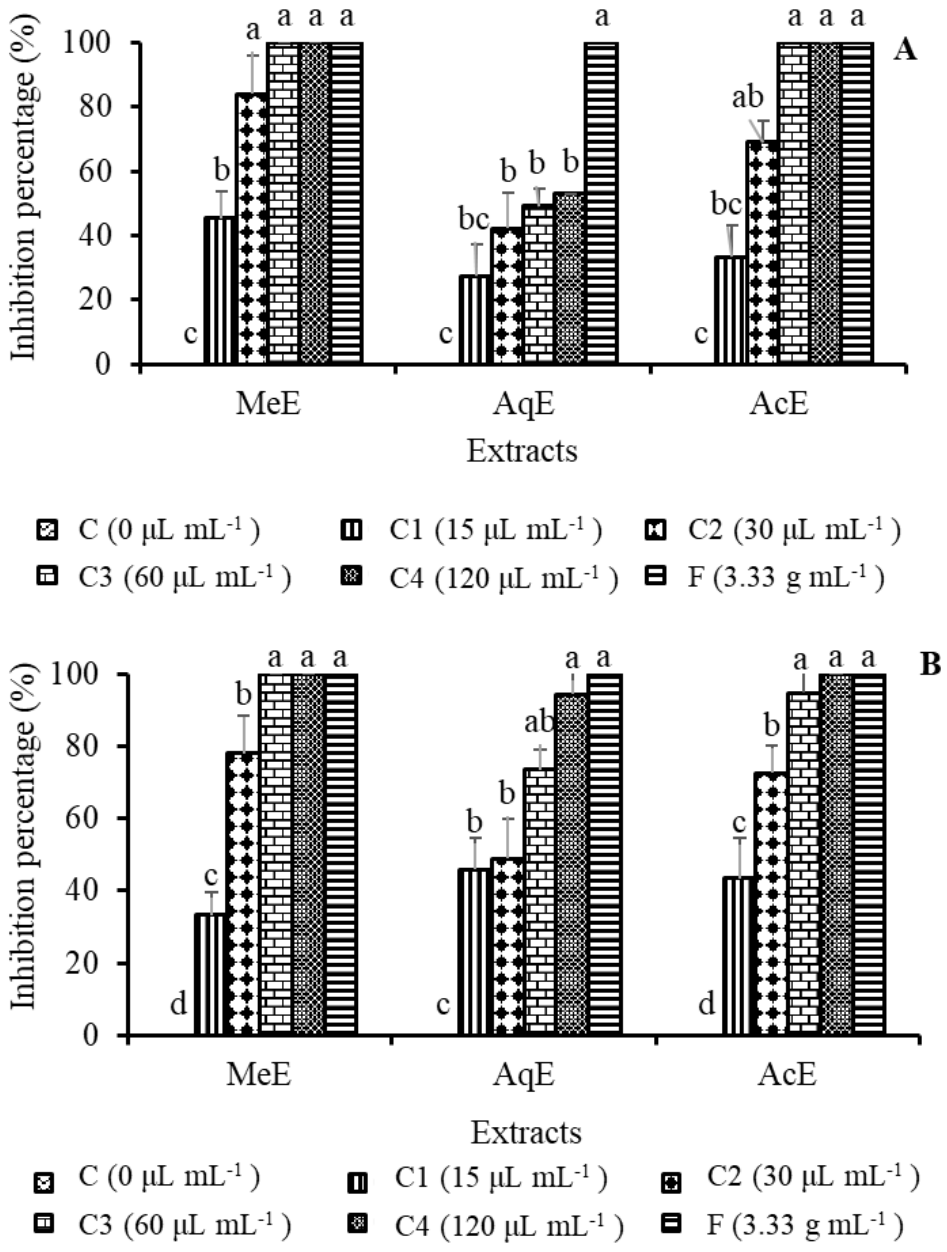

Figure 3. Inhibition of mycelial growth of *Crinum zeylanicum*; A: Isolate 1 obtained from leaves from Akonolinga; B: Isolate 2 obtained from leaves from Meyomessala; AqE: aqueous extract; AcE: acetone extract, MeE: methanol extract; F: fungicide; C: control.

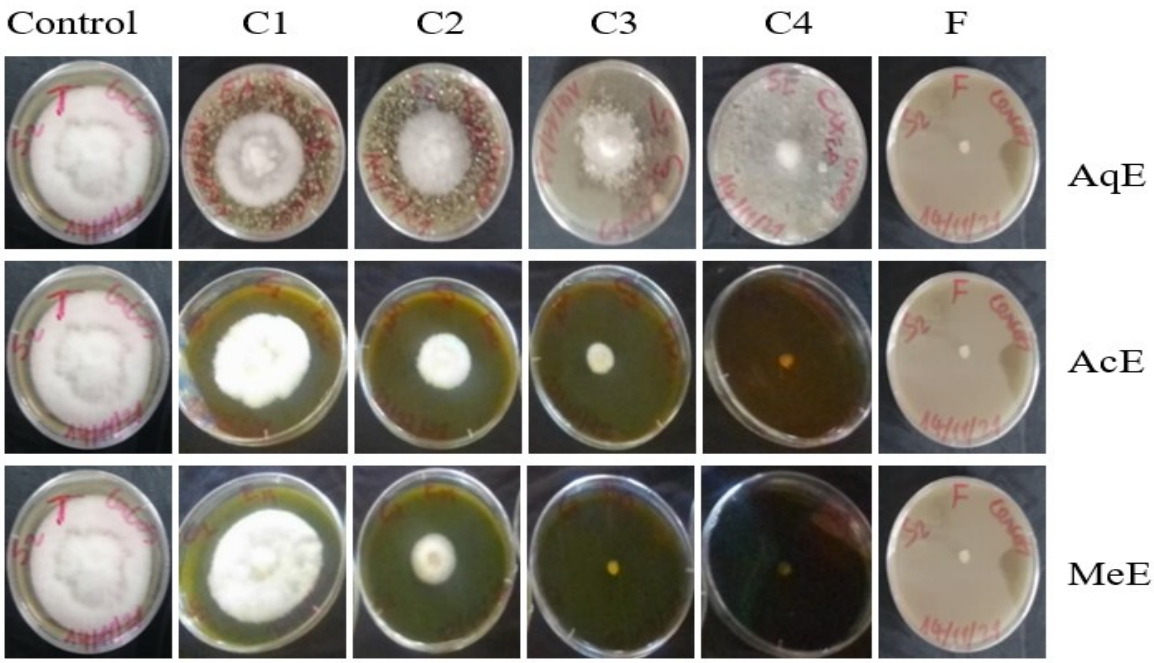

Figure 4. Inhibitory effect of organic and aqueous extracts of *Crinum zeylanicum* leaves on mycelial growth of *Cercospora malayensis* isolate 2 after 8 days incubation. C1: 15 µL mL^{-1}; C2: 30 µL mL^{-1}; C3: 60 µL mL^{-1}; C4: 120 µL mL^{-1}; F: 3.33 g L^{-1} fungicide C: Control; AqE: aqueous extract; AcE: acetone extract, MeE: methanol extract; F: fungicide; C: control.

Table 5. Minimum inhibition concentration MIC 50 and MIC 90.

Pathogen agent	Extracts	MIC 50	MIC 90
Cercospora malayensis (Isolate 1)	Methanol extract	6.79	10.98
	Aqueous extract	11.77	20.77
	Acetone extract	8.10	11.36
Cercospora malayensis (Isolate 2)	Methanol extract	7.83	11.22
	Aqueous extract	8.45	12.88
	Acetone extract	7.48	11.39

Table 6. Fungicidal and fungistatic effect of *Crinum zeylanicum* leaf extracts

Agent pathogen	Extracts (120 µL mL^{-1})	Activities
Cercospora malayensis (Isolate 1)	Methanol extract	Fungicide
	Aqueous extract	Fungistatic
	Acetone extract	Fungicide
Cercospora malayensis (Isolate 2)	Methanol extract	Fungicide
	Aqueous extract	Fungistatic
	Acetone extract	Fungicide

DISCUSSION

Pure isolates of *C. malayensis* obtained from samples collected in Akonolinga (Isolate 1) and Meyomessala (Isolate 2) showed morphological characteristics similar to those of *C. malayensis* F. Stevens & Solheim, the causal agent of Cercosporiosis of okra. In Petri dishes, the macroscopic characteristics of *C. malayensis* filaments show white, cottony mycelia on both the top and bottom surfaces. Indeed, Farrag (2011), Ju et al. (2020), Park et al. (2017), and Chai et al. (2021) describe *C. malayensis* mycelial filaments as off-white, flat and aerial colonies. Microscopic study shows continuous, elongated conidia, truncate to obconical at the base, with three to eight septa, and subacute to obtuse at the apex. Ju et al. (2020) working on the first report of *C. malayensis* causing leaf

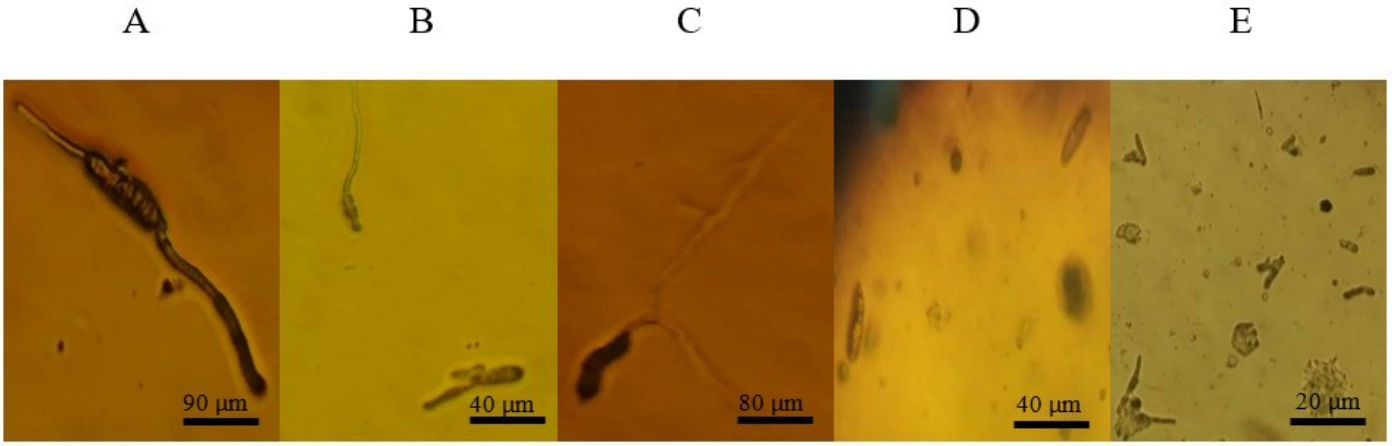

Figure 6. Effect of extracts on germination of *Cercospora malayensis* conidia; A, B and C: Germination of conidia in control; D: Conidia not germinated at concentration C4 = 120 µL mL^{-1} in methanol extract and E: in aqueous extract of *Crinum zeylanicum* leaves.

spots on okra in Korea described the conidia as acicular, hyaline, subacute to obtuse at the apex, truncate to obconic at the base, 2 to 20 septate, with a swollen hilum at the tip, and measuring 34 to 280 × 3.4 to 6.5 µm. Also, Crous and Braun (2003) and Świderska-Burek (2015) present conidia as singly or in acropetal chains, amero to scolecosporeous and pigmented or hyaline.

Phytochemical screening of *C. zeylanicum* leaf extracts revealed the existence of multiple chemical families, including: terpenes, sterols, phenols, flavonoids, oils, sugars, saponins, alkaloids. These families of secondary compounds have been listed in numerous works as possessing antifungal activity (Rosen & Stein-Gold 2016; Ramírez-Gómez et al. 2019; Yusoff et al. 2020; Zulbayu et al. 2021; Sukdee 2023). Phenols, terpenes and flavonoids disrupt pathogen membranes, inactivate enzymes, bind to adhesins, and create complexes with the cell wall. Tannins bind to proteins, inhibit enzymes and alkaloids embed themselves in the cell wall (Gurjar et al. 2012). GC-MS results of *C. zeylanicum* leaf extracts show the presence of numerous chemical compounds that exhibit biocidal activity. The difference in the detection of chemical compounds in *C. zeylanicum* leaf extracts would stem from the polarity of the solvents used. Ngo et al. (2017) show that differences in solvent polarity can help explain differences in the solubility of plant active ingredients. The most dominant compounds being Quinoline-7-carboxylic acid, 2-phenyl-, methyl ester (26.63 %), 9,12-Octadecadienoic acid (Z,Z) (10.83 %) and 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (10.37 %) in acetone extract, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (16.89 %), Phytol (11.78 %) in methanol extract and n-Hexadecanoic acid (35.04 %), Cis-Vaccenic acid (31.76 %) in the aqueous extract. Studies have shown that the compounds present in *C. zeylanicum* leaf extracts have antifungal, insecticidal and antibacterial activity (VasudhaUdupa et al. 2021; Toka et al. 2023; Dida et al. 2024).

Crinum zeylanicum leaf extracts tested at different concentrations against *C. malayensis* significantly inhibited its mycelial growth. Concentrations C3=60 µL mL^{-1} and C4=120 µL mL^{-1} of the methanol and acetone extract and C4=120 µL mL^{-1} of the aqueous extract inhibited 100 % of the mycelial growth of isolates 1 and 2, as did the synthetic fungicide Metalaxyl 80 g kg^{-1} and Mancozeb 640 g kg^{-1}. Tram et al. (2002) reported on the biological activities of bulbs, leaves, roots and flowers of the *Crinum* genus. The authors showed that *C. zeylanicum* bulbs are rich in alkaloids, which regulate plant growth and are active against phanerogamic parasites. Also, the antifungal properties of leaf extracts from the leaves of this plant could be due to chemical compounds possessing biological properties (Berkov et al. 2011). Studies have shown that certain chemical compounds (n-Hexadecanoic acid, Octade-

canoic acid, Hexadecanoic acid, methyl ester, Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, 9,12-Octadecadienoic acid (Z,Z)- etc.) possess antifungal properties (Alawode et al. 2021; Vuerich et al. 2023). The precise way that chemical substance work present in plant extracts is unclear, but it is likely that these chemical compounds form complexes with polysaccharides and proteins associated with an outer layer of fungal cells, which can lead to destabilization of cell membrane function, resulting in pathogen death (Rongai et al. 2017). In contrast, Yoshimi et al. (2022) and Da et al. (2019) demonstrated that plant extracts rich in chemical compounds disrupt the synthesis of β-1,3-glucan, an integral part of many fungi's cell walls of many fungi.

The low minimal inhibitory concentrations (MIC50 and MIC90) confirmed the efficacy of *C. zeylanicum* leaf extracts. Jasso de Rodríguez et al. (2006) pointed out that the inhibitory capacity of plant extracts results from differences in the composition and concentration of bioactive compounds in the plant. The fungicidal and fungistatic activity of extracts at a concentration of 120 µL mL^{-1} showed that only the aqueous extract was fungistatic, while the methanol and acetone extracts were fungicidal. Indeed, Boli et al. (2021), Toka et al. (2023), and Dida et al. (2024) report fungicidal activity with organic extracts of *Anona muricata*, *Azadirachta indica*, *Balanites aegyptiaca*, *Thevetia peruviana* against *Cercospora malayensis*, *Fusarium oxysporum* and *Phytophthora infestans*. *C. zeylanicum* leaf extracts inhibited conidial germination of *C. malayensis* isolates 1 and 2 with no germination at the concentration of 120 µL mL^{-1} of organic, aqueous and synthetic fungicide extracts. Reports by Abayhne and Chauhan (2016) showed that the methanol extract and ethanol extract of *Cymbopogon citratus* and *Datura stramonium* directly inhibit germination of *P. infestans* zoospores. Rashid et al. (2004) also showed that aqueous extracts of *Azadirachta indica* inhibited germination of *Phytophthora infestans* sporangia. According to Baltussen et al. (2020), conidial germination is a fundamental stage in fungal development. Due to their high content of secondary compounds, the extracts render conidial germination conditions unfavorable, keeping them dormant.

CONCLUSION

The goal of this study is to use leaf extract from *Crinum zeylanicum* to regulate the growth of *Cercospora malayensis in vitro*. Extracts of *C. zeylanicum* leaves revealed the presence of terpenes, sterols, phenols, saponins, flavonoids, oils, sugars, and alkaloids. GC-MS analysis of the extracts unveiled a wealth of chemical compounds with biocidal activity. The extracts inhibited mycelial growth and conidial germination of *C. malayensis* similar to the synthetic fungicide at a concentration of 120 µL mL^{-1}. The lowest MICs were recorded with the methanol and acetone extracts. *C. zeylanicum* leaf extracts can be used for integrated crop disease control strategy.

AUTHOR CONTRIBUTION

Ndongo B., Conceptualisation; Akong M.E. Original draft preparation; Ngatsi P.Z., Formal Analysis data, Writing –Review- Editing; Kuate W.N.T., Methodology.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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