Antifungal efficacy of *Moringa oleifera* leaf and seed extracts against *Botrytis cinerea* causing gray mold disease of tomato (*Solanum lycopersicum* L.)

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Abstract

Drawbacks associated with the use of chemical fungicides to control plant pathogenic fungi such as *Botrytis cinerea* stimulate the need for alternatives. Therefore, the present study was carried out to determine the antifungal potentials of *Moringa oleifera* extracts against *B. cinerea*. Phytochemical analysis using qualitative chemical tests revealed the presence of huge amount of crucial phytochemicals compounds like phenolic compounds, alkaloids and saponins in the *M. oleifera* leaf extract. Antifungal bioassay of the crude extracts indicated better mycelial growth inhibition by methanol leaf extract (99%). The minimum inhibitory concentration (MIC) was 5 mg/ml with 100% spore germination inhibition and minimum fungicidal concentration (MFC) was 10 mg/ml with 98.10% mycelial growth inhibition using broth micro dilution and poisoned food techniques. Gas chromatography–mass spectrometry (GC-MS) analysis led to the identification of 67 volatile chemical compounds in the leaf extract with 6-decenoic acid (Z)- (19.87%) was the predominant compound. Further chemical elucidation of the crude extracts performed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) showed the presence of non-volatile chemical compounds, mostly flavones, flavonoids and phenolic acids (i.e. quercetin and kaempferol). Scanning electron microscopy and transmission electron microscopy analysis showed positive effect of *M. oleifera* leaf extract on the treated conidia and mycelium of *B. cinerea*. Findings revealed that irreversible surface and ultra-structural changes with severe detrimental effects on conidia and mycelium morphology compared to control treatment. Overall findings suggested that *M. oleifera* leaf extract is a promising candidate for biological control of fungal pathogens, thus limiting overdependence on chemical fungicides.

Keywords: antifungal activity, *Botrytis cinerea*, *Moringa oleifera*, phytochemicals.

Eficácia antifúngica de extratos de folha e de semente de *Moringa oleifera* contra *Botrytis cinerea* causador da doença do molde cinza de tomate (*Solanum lycopersicum* L.)

Resumo

As desvantagens associadas ao uso de fungicidas químicos para controlar fungos fitopatogênicos, como *Botrytis cinerea*, estimulam a necessidade de alternativas. Portanto, o presente estudo foi realizado para determinar o potencial antifúngico de extratos de *Moringa oleifera* contra *B. cinerea*. A análise fitoquímica usando testes químicos qualitativos revelou a presença de uma grande quantidade de compostos fitoquímicos cruciais, como compostos fenólicos, alcaloides e saponinas no extrato da folha de *M. oleifera*. O bioensaio antifúngico dos extratos brutos indicou melhor inibição do crescimento micelial pelo extrato de folhas de metanol (99%). A concentração inibitória mínima (MIC) foi de 5 mg/mL com 100% de inibição da germinação de esporos e a concentração fungicida mínima (MFC) foi de 10 mg/mL com 98,10% de inibição do crescimento micelial usando microdiluição em caldo e técnicas de alimentos envenenados. A análise por cromatografia gasosa-espectrometria de massa (GC-MS) levou à identificação de 67 compostos químicos voláteis no extrato da folha, sendo o ácido 6-decenoico (Z) (19,87%) o composto predominante. Elucidação química adicional dos extratos brutos realizada por cromatografia líquida com espectrometria de massa em tandem (LC-MS/MS) mostrou a presença de compostos químicos não voláteis, principalmente flavonas, flavonoides e ácidos fenólicos (ou seja, quercetina e kaempferol). As análises de microscopia eletrônica de varredura e microscopia eletrônica de transmissão mostraram efeito positivo do extrato de folhas de *M. oleifera* sobre os conídios e micélios tratados de *B. cinerea*. Os resultados revelaram a superfície irreversível e alterações ultraestruturais com graves efeitos prejudiciais sobre os conídios e a morfologia micelial, em comparação com o tratamento de controle. Os resultados gerais sugeriram que o extrato da folha de *M. oleifera* é um candidato promissor para o controle biológico de patógenos fúngicos, limitando assim a dependência excessiva de fungicidas químicos.

Palavras-chave: atividade antifúngica, *Botrytis cinerea*, *Moringa oleifera*, fitoquímicos.

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1. Introduction

Gray mold disease is caused by *Botrytis cinerea* Persoon: Fries [teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel] is a serious disease of tomato (*Solanum lycopersicon* L.) in Malaysia and in most areas of the world (Kim et al., 2016). The disease causes severe damage on fruits and vegetables at both pre- and post-harvest time. *Botrytis cinerea* is an ascomycetous fungus as well as a necrotrophic plant pathogen (Staats et al., 2005) that has been ranked second after *Magnaporthe oryzae* in the top 10 devastating fungal plant pathogens worldwide due to its broad host range (Shaw et al., 2016; Elad et al., 2016; Dean et al., 2012). The fungus is capable of infecting more than 200 plant species, including agriculturally important crop plants and harvested commodities, such as grapes, cucumbers, tomatoes, strawberries, cut flowers, bulb flowers, and ornamental plants (Parvu et al., 2010; Kars et al., 2005). The broad host range of *B. cinerea* leads to great economic losses, not only during crop growth but also during the storage and on transit of the harvested products (Elad, 2003). The fungus also kills it susceptible host cells by secreting lytic enzymes or toxic compounds and also produces an array of destructive enzymes and toxins that can conquer host defense mechanisms (Parvu et al., 2010; Makovitzki et al., 2007; Williamson et al., 2007). Gray mold occurs frequently in the tomato greenhouses in Cameron Highlands areas of Pahang in Malaysia resulting in reduced plant growth, premature leaves defoliation, fruit rot and consequently adverse effect on yields. Because of this, if the disease is not properly and effectively managed, it can be destructive enough to cause extensive yield losses leading to significant backwards in the tomato industries and/or tomato growers.

In a bid to control the disease, farmers depend on synthetic pesticides as their primary means for managing the disease, but the environment and human health have fared badly from the use of such synthetic chemicals. In addition, their use has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and possible side effects on human health through food chain (Hollomon, 2015; Ishii and Holloman, 2015; Enyiukwu et al., 2014; Aiying et al., 2011). In line with the public opinions and concerns related to the environmental and human safety, development of resistance by several strains of plant pathogens (i.e. *B. cinerea*) due to progressive use of such chemicals and increasing regulations on chemical pesticides use (Elliott et al., 2009), it is imperative to explore new alternatives that will reduce the overdependence on synthetic chemicals to manage the pathogen properly. Today, serious and much endeavours are put in place to give priority on pesticides of plant origin as there is a need to look for reliable and enduring solutions that care about the demands of man and environment (Taiga et al., 2008; Amadioha, 2002).

The plant world is rich store house of natural chemicals that could be exploited for use as biopesticides (Li Destri Nicosia et al., 2016; Satish et al., 2007). *Moringa oleifera* is a medicinal plant that is now emerging as a safer and more compatible alternative to chemical fungicides. All parts of the plant including roots, flowers, bark, stem, leaves, seeds and essential oils possess antimicrobial properties and are therefore being used for medicinal and other purposes (Dwivedi and Enespa, 2012; Anwar et al., 2007). Chemical analysis of these morphological parts showed that they are containing a profile of important minerals, vitamins, protein and a large quantity of phytochemical compounds that have biological activity and can potentially be used to retard the effects of microorganisms (Arora and Onsare, 2014; Satish et al., 2013; Gurjar et al., 2012). Recent reports on phytochemical analysis of the *M. oleifera* leaf revealed the presence of various antioxidant compounds like ascorbic acid, fatty acids and phenolic acids (Vongsak et al., 2014; Alhakmani et al., 2013). Other compounds reported present in *M. oleifera* includes quercetin, zeatin, β-sitosterol, kaempferol, caffeoylquinic acid (Sharma and Paliwal, 2013) and myricetin, rutin, chlorogenic acid, glycosides niaziminin and niazin (Mbikay, 2012). These compounds are reported to possessed antimicrobial properties (Arora et al., 2014; Arora et al., 2013; Dwivedi and Enespa, 2012), antifungal (Batista et al., 2014; Kadhim and Al-Shammaa, 2014), antioxidant (Verma et al., 2009), antibacterial, antiulcer (Arora and Onsare, 2014; Belay and Sisay, 2014), anti-inflammatory, diuretic (Cáceres et al., 1992; Krishnamurthy et al., 2015) and anticancer (Pinto et al., 2015). Batista et al. (2014) and Gifoni et al. (2012) reported the antifungal inhibitory activity of *Moringa*-Chitin Binding Protein (*Mo*-CBP$_3$) isolated and purified from the seeds of *M. oleifera* against mycelial growth and spore germination of *Fusarium solani* at minimum inhibitory concentration (MIC) concentration (0.05 mgmL^{-1}) with irreversible ultra-structural changes.

Although many reports are available on the efficacy of *M. oleifera* extracts as bio-fungicide against plants pathogenic fungi, further investigation are required to understand the *M. oleifera* extracts effect on *B. cinerea*. Therefore, the objectives of the present study were to carry out phytochemical analysis of methanolic leaf extract of *M. oleifera*, evaluate its antifungal activity against *B. cinerea* and examine the ultrastructural changes in *B. cinerea* conidia and mycelia at MIC concentration of *M. oleifera* methanolic leaf extract.

2. Material and Methods

2.1. Plant materials

The plant material (*M. oleifera*) was obtained from Demonstration Farm 2 of the Universiti Putra Malaysia (UPM), Selangor, Malaysia. The plant was taxonomically identified and confirmed by Dr. Shamsul Khamis from the Institute of Bioscience (IBS) Universiti Putra Malaysia (UPM), Malaysia. Voucher specimen (SK 3029/16) was deposited in the Herbarium of Biodiversity, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM), Malaysia.

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2.2. Pure culture and spore preparation

Pure culture *B. cinerea* isolates of BCH02 and BCH07 were used in the present study selected as the most virulent strains from the previous study (Tijjani et al., 2016). These isolates were previously isolated from infected tomato fruits with typical symptoms of gray mold disease from a greenhouse in Cameron Highlands, Pahang, Malaysia. The identities of the isolates were confirmed based on morphology; gene sequence analysis of the internal transcribed spacer (ITS) region and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The gene sequences were deposited in the National Centre for Biotechnology Information (NCBI) database with the following accession number; KU992694 (BCH02) and KU992698 (BCH07) for ITS gene and KY201457 (BCH02) and KY201460 (BCH07) for G3PDH gene. The concentration of the conidial suspension used was adjusted to 10^6 conidia/ml using Haemocytometer (Abd-Alla and Haggag, 2010).

2.3. Preparation of plant extracts

2.3.1. Solvents extraction

Organic solvents with different polarity were used for small-scale extraction which includes methanol, ethanol (polar), ethyl acetate (semi-polar) and hexane (non-polar) to determine the best solvent for extraction. The extraction was done by dissolving

100 g of dry powdered leaf of *M. oleifera* into 500 ml of methanol and ethanol in 1000 ml conical flask each and then shaken with a mechanical shaker (Orbitron, infors HT Switzerland) for 72 hours to allow proper extraction. The macerates were filtered with Whatman's no. 1 filter paper (ALBERT^R) after 3 days of immersion. The solvents were evaporated from the crude extracts to reduce the solvent concentration with a rotary evaporator (HahnShin Scientific Co., Taiwan). The semi-liquid crude extracts from the rotary evaporator flask were filtered with a sterilized 0.45 syringe filter (Sartouris® Syringe filters) to remove unwanted materials. Same procedure was followed for the powdered *M. oleifera* seed.

2.3.2. Aqueous extraction

This extraction was performed with water as the solvent by dissolving 100 g of dried leaf of *M. oleifera* in 1 litre of sterilized distilled water in 2 litre conical flasks, vigorously shaken and left for 24 hours at 28±2°C. After 24 hours, the mixture was filtered with two layered muslin's cheese cloth. The obtained filtrate was then centrifuge at 6000 rpm for 15 minutes (Avanti J-26 XPI centrifuge, Beekman Coulter, USA). The supernatant collected was filtered again with a Whatman's no. 1 filter paper (ALBERT^R) and then followed by dry-freezing at 54°C in 010 vacuum mbar with a modulyod freeze drier (Valupump VLP). The crude extracts were filtered with a sterilized 0.45 syringe filter (Sartouris® Syringe filters) to remove unwanted materials. The final material was maintained in sterile airtight glass bottles and stored in a refrigerator at 4°C. Same procedure was followed for the powdered *M. oleifera* seed.

The percentage yield of the crude extracts was calculated following the method of Mushore and Matuvhunye (2013) (Formula 1) below:

$$\% \text{ of yield weight} = \frac{(W_2) - (W_1)}{(W_0)} \times 100 \qquad (1)$$

Where: W_0: weight of initial dried sample
W_1: weight of empty flask
W_2: weight of the extract and flask

2.4. Antifungal bioassay

Poisoned food technique was used to screen the best plant solvent extract. Ten different plant solvent fractions were screened viz: *Moringa* leaf methanol extract (MLM), *Moringa* leaf ethanol extract (MLE), *Moringa* leaf ethyl acetate extract (MLEA), *Moringa* leaf hexane extract (MLH), *Moringa* leaf distilled water extract (MLDW), *Moringa* seed methanol extract (MSM), *Moringa* seed ethanol extract (MSE), *Moringa* ethyl acetate extract (MSEA), *Moringa* seed hexane extract (MSH) and *Moringa* seed distilled water extract (MSDW). Potato dextrose agar (PDA) plates were amended, with 100 mg/ml of plant extracts from each solvent. The mixture was swirled to enhance efficient miscibility of the agar and the extracts. Five-day old fungal cultures of *B. cinerea* isolates (BCH02 and BCH07) isolates were aseptically punched with a sterile cork borer of 6 mm (0.6 cm) diameter and the fungal discs were placed at the centre of PDA gelled plates. Perpendicular lines were drawn at the bottom of each plate and the point of intersection was taken as the centre of the plate to ease precise measurement. The plates were incubated at 28°C for 5-7 days until the control plates were filled. The diameter of the colony was recorded on daily basis by measuring the two paradoxical circumference of the colony growth along the lines drawn at the bottom of each plate. Percentage inhibition of radial mycelial growth (PIGR) was evaluated by measuring the colony diameter of plates with plant extract (poisoned plates) and plate with solvent (non-poisoned plates) and calculated using Formula 2 given below:

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100 \qquad (2)$$

Where: R_1: average measurement in the control
R_2: average measurement in the treatment

2.5. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

2.5.1. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the crude extract was determined following the method of Broekaert et al., (1990) using broth micro-dilution technique or microtitre plate (96 wells). Potato dextrose broth (PDB), (Difco™, Becton, USA) was prepared 24 g/l, autoclaved at 121°C for 15 min and used for the determination of the MIC. Ten different concentrations ranging from 50 mg/ml – 5 mg/ml were screened along with benomyl (0.2 mg/ml) as a standard

control practice (SCP). In each well, 100 µl of PDB, 50 µl of plant extract and 40 µl of fungal spore suspension (1×10^6 spore concentration ML^1) were loaded. For SCP 50 µl of benomyl was loaded and 50 µl of methanol for the negative control instead of plant extract. Treatments were replicated three times and arranged in completely randomize design. The plates were incubated for 24 hours at 37°C (Lourens et al., 2004). Measurements were taken at 620 nm using spectrophotometric microtitre plate reader (MULTI SKAN GO). Growth inhibition (PGI) was determined based on the Formula 3 below:

$$PGI = \frac{\Delta C - \Delta T}{\Delta C} \times 100 \qquad (3)$$

Where: ΔC: corrected absorbance of the control micro culture at 620nm
ΔT: corrected absorbance of the treatment micro culture at 620nm

2.5.2. Minimum fungicidal concentration (MFC)

Poisoned food technique was used to determine minimum fungicidal concentration (MFC). Potato dextrose agar (Difco™, Becton, USA) was prepared 39 g/l, autoclaved at 121°C for 15 min and used for the determination of the MFC. Ten different concentrations ranging from 100 mg/ml – 10 mg/ml diluted from the stock crude extract were tested along with benomyl (0.2 mg/ml) as a SCP and distilled water as a negative control. About 15 ml of the PDA was poured into each petri plate and prior to solidification each concentration of the extract was incorporated into each plate, gently swirled to obtain uniform miscibility of agar and the extract. Then 5-days-old fungal cultures of *B. cinerea* isolates (BCH02 and BCH07) isolates were aseptically punched with a sterile cork borer of 6 mm diameter and the fungal discs were placed at the centre of PDA gelled plates. Perpendicular lines were drawn at the bottom of each plate and the point of intersection was taken as the centre of the plate to ease precise measurement. The plates were incubated at 28°C for 5-7 days until the control plates were filled. The diameter of the colony was recorded on daily basis by measuring the two paradoxical circumferences of the colony growth along the lines drawn at the bottom of each plate. Percentage inhibition of radial mycelial growth was evaluated by measuring the colony diameter of plates with plant extract (poisoned plates) and plate with solvent (non-poisoned plates) and calculated using the Formula 4 given below:

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100 \qquad (4)$$

Where: R_1: average measurement in the control
R_2: average measurement in the treatment

2.6. Phytochemical analysis of M. oleifera methanol leaf extracts

2.6.1. Preliminary qualitative tests

Crude methanolic leaf extract of *M. oleifera* was subjected to preliminary phytochemical analysis using chemical tests for qualitative detection of the presence of phytochemical compounds including alkaloids, flavonoids, tannin, saponin, phenolic compounds, terpenoid, triterpenoids, steroids, and anthraquinone. The summary of the procedures for the chemical tests was shown in Table 1.

2.6.2. Gas chromatography–mass spectrometry (GC-MS) analysis for volatile compounds

The method of Gao et al. (2014) was followed to perform the GC-MS analysis for volatile compounds present in the methanolic leaf extracts of *M. oleifera.* Briefly, Shimadu QP-2010 GC-MS system that consist of gas chromatograph interfaced with mass spectrometer and equipped with a Zebron ZB5-MS capillary fused silica column (30 m × 0.25 mm I.D. × 0.25 m film thickness) was used for the analysis. The oven temperature was started from 70°C (isothermal for 3 min), with an adjustment of 10°C/min, to 240°C, ending with 10 min isothermal at 300°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 440 Da. Total GC running time is 40 min. Other running conditions used were as follows: carrier gas, helium (99.999%), with a flow rate of 1 ml/min; injection volume of 1 µl was used with split ratio of 10:1, injector temperature, 250°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 440 Da. Total GC running time is 40 min. The mass spectrum of the obscured constituents was compared with those stored in the National Institute of Standards and Technology (NIST) Mass Spectral Library built up by using pure substances and the mass spectra from the published literature. The compound's name, percentage, retention time, molecular formula, molecular weight and chemical structure of the materials were established. GC-17A version 3 programming software was utilized to handle the mass spectra and chromatogram.

2.6.3. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis for non-volatile compounds

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis was performed using an AB Sciex 5500Q Trap (Linear Quadrupole Hybrid Ion Trap Mass Spectrometer, AB Sciex, Toronto, Canada) mass spectrometer operating in electrospray ionization (ESI) negative mode and hyphenated with an Agilent 1290 ultra-high liquid chromatography system. The high purity gas for the mass spectrometer was set at 40 psi gas source, 40 psi for the heating gas and for collision gas with a source temperature of 500°C. The setting for electrospray ionization voltage was set to 4500 kV. The collision energy to attain fragmentation was set at 35 eV with a spread of +/- 15 eV. Mass range for MS/MS scan was set from 50-1000 m/z while mass range for full scan was set from 100-1000 m/z while scan speed was set at 1000 m/z per second. A Phenomenex Synergy Fusion RP (100 mm × 2.1 mm i.d., 3 µm particle size, Phenomenex, CA, USA) was used to obtain separation. The mobile phase was made up of aqueous ammonium formate (5 mmol/L) with 0.1%

Table 1. Chemical tests for some phytochemical compounds from methanolic leaves extracts of *M. oleifera*.

Phytochemical compound	Test type	Procedure	Reference
Tannins	Ferric chloride test	-About 0.5 g of methanolic extract was dissolved into 10ml of distilled water and filtered. - 4-6 drops of 10% ferric chloride was then added into the filtrate.	(Harbone, 2001)
Alkaloids	Dragendorff's test	- Methanolic extract (0.2 g) was put into a conical flask, followed by the addition of 20 ml of sulphuric acid diluted in methanol. - The mixture was then filtered and treated with 2 drops of Dragendorff's reagents (Potassium bismuth iodide solution).	(Ghani, 1998; Harbone, 2001).
Flavonoids	Ammonium test	-About 10 ml of ethyl acetate was added to 0.2 g of the extract and heated in water bath for 4 min. -The mixture was allowed to be cooled and then filtered. - 1 ml of diluted ammonia was shaken with 4 ml of the filtrate and the layers were allowed to separate.	(Harbone, 2001; Sofowora, 2005)'
Saponnins	Froth test	-About 20 ml of water was added to 0.25 g of the extract in 100 ml beaker, boiled and then filtered. -The filtrate (5 ml) was diluted with 20ml of distilled water and shaken vigorously. -This was the allowed to stand.	(Harbone, 2001; Khalil et al., 2013; Sofowora, 2005).
Steroids/ Triterpenoids	Liebermann-Burchardt test.	-Methanolic extract (1 ml) was boiled with 2 ml of chloroform and allowed to cool. -Then 1-3 drops of concentrated sulphuric acid were added, shaken and allowed to stand.	(Khalil et al., 2013).
Phenolic compounds	Ferric chloride test	-3 drops of 1% ferric chloride was added to 3ml portion of methanolic extract.	(Harbone, 2001: Khalil et al., 2013).
Anthraquinone	Borntrager's test	-10ml of benzene was added to 4 g of the methanol extract, shaken and filtered. -Then 5 ml of 10% ammonia solution was added to the filtrate, shaken and allowed to stand.	(Kadhim and Al-Shammaa, 2014).
Terpenoids	Salkowski test	-The extract (2ml) was dissolved in chloroform (2ml) and then evaporated to dryness. -Sulphuric acid (2ml) was added and boiled for 2 minutes.	(Kadhim and Al-Shammaa, 2014; Khalil et al., 2013).

formic acid (solvent A) and acetonitrile with ammonium formate (5 mmol/L) with 0.1% formic acid (solvent B). The compounds were separated with the following linear-programmed solvent gradient: 0 minute (10%), 10 minutes (95% B), 2 minutes (95% B) then equilibrating to 10% B for 3 minutes. The flow rate for the column was set at 0.25 mL/min while the column temperature was set at 40°C and injection volume at 10 µl.

2.7. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis

2.7.1. Scanning electron microscopy (SEM) analysis

Scanning electron microscopy (SEM) was performed to determine the antifungal efficacy of the plant extracts (crude methanolic extract of *M. oleifera*) on the structural integrity of *B. cinerea* cells at the surface level. The samples of the test fungus were prepared for SEM examination following the method described by Hasan et al. (2014), starting from primary fixation to coating and finally viewing. First, the samples were cut (1 cm³) from the margin of actively growing PDA fungal culture treated with plant extract using a sterilized razor blade. The samples were then fixed with modified Karnovsky's fixative (Karnovsky, 1965) containing 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer solution (pH 7.2) and left at 4°C overnight. The samples were washed with 0.1 M sodium cacodylate buffer in 3 changes after (30 mins.) each, followed by post-fixation in 1% osmium tetroxide in 0.2 M PBS for 2 hours, dehydrated through series of graded acetone (35, 50, 75, 95%) for 10 minutes each and 100% for 15 minutes. After

this, the specimens were stick on to stub with double tape, critical point dried in CO_2, gold-coated using sputter ion coater (5-10 nm) and observed with a scanning electron microscope (SEM: JSM−5610LV, JOEL, Japan) at the IBS, University Putra Malaysia.

2.7.2. Transmission electron microscopy (TEM) Analysis

Transmission electron microscopy (TEM) was performed to confirm the SEM finding and also to determine the ultrastructural changes on the *B. cinerea* cells as a result of exposure to crude methanolic extract of *M. oleifera*. The samples of the test fungus were prepared for TEM examination following the method described by Sasidharan et al. (2012), starting from primary fixation to staining and finally viewing. The specimens were cut (1 mm^3) from the margin of actively growing PDA fungal culture treated with plant extract using a sterilized razor blade. The samples were then fixed with modified Karnovsky's fixative (Karnovsky, 1965) containing 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer solution (pH 7.2) and left at 4°C overnight. The samples were washed with 0.1 M sodium cacodylate buffer in 3 changes after (30 mins.) each, followed by post-fixation in 1% osmium tetroxide in 0.2 M PBS for 2 hours, dehydrated through series of graded acetone (35, 50, 75, 95%) for 10 minutes each and 100% for 15 minutes. The specimens were infiltrated with acetone and resin mixture at the ratio of 1:1 for 4 hours, 1:3 overnight and 100% resin overnight again. After infiltration, the specimens were embedded in beam capsules filled with Spurr's resin. Ultra-thin sections (approximately 1 µm in thickness) of the specimens were cut with a glass/ diamond knife using an ultra-microtome. The sections were mounted on copper grids and were stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) for 10 min each. Finally, the sections were observed or viewed with a transmission electron microscope (TEM MODEL JEM 2100 field emission electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) in IBS, University Putra Malaysia.

3. Results

3.1. Effect of solvents on the percentage extraction yield

From our results it is evident that the analysis of the percentage yield of the crude extracts obtained from *M. oleifera* leaf and seeds with different solvents showed that methanol gave the highest yield (14.16%) from leaf (Table 2), followed by ethanol (10.23%) and the lowest yield was observed in distilled water (1.71%). Similarly, in the seeds, methanol gave the highest yield (11.54%), followed by ethanol (6.41%) and the lowest yield was observed in hexane (1.27%). Methanol solvent gave the highest yield compared to other solvents.

3.2. Antifungal bioassay and determination of MIC and MFC of the plant extracts

Figure 1 presented the results of antifungal bioassay of different solvent crude extracts from both *M. oleifera* leaf and seeds. The results indicated that, methanol crude extracts from both *M. oleifera* leaf and seeds gave better growth inhibition than other solvents. The highest growth inhibition was observed under both isolate BCH02 (>99.00%) and BCH07 (>99.00%) by methanol leaf extract, 99.00% in isolates BCH07 by methanol seed extracts, followed by 98.00% growth inhibition by ethanol leave and seed extracts as observed in both isolates. However, the lowest growth inhibition was observed in MSDW (28.00%) under isolate BCH07. All extracts irrespective of plant part (leaves or seeds) inhibit fungal mycelial growth compared to the control.

MIC and MBC of *M. oleifera* were determined against BCH02 and BCH07 isolates of *B. cinerea* (Table 3). Methanol leaf extracts of *M. oleifera* showed the best performance in terms of MIC and MFC against the isolates. Both isolates (BCH02 and BCH07) had their most sensitivity to methanol leaf extracts of *M. oleifera*, which showed the MIC to be 5 mgml⁻¹ and MFC 10 mgml⁻¹. These results suggested that the antifungal compounds activity of *M. oleifera* is mainly concentrated in the leaf.

3.3. Phytochemical profiling

3.3.1. Preliminary phytochemical analysis

The preliminary phytochemical analysis of the methanolic leaves extract of *M. oleifera* carried out with various

Table 2. Extraction yield percentage of *M. oleifera* crude extracts from different solvents.

Part of plant	Solvents	Percentage yield (%).
Leaf	Methanol	14.16
	Ethanol	10.23
	Ethyl acetate	5.24
	Hexane	2.3
	Distilled water	1.71
Seed	Methanol	11.54
	Ethanol	6.41
	Ethyl acetate	3.43
	Hexane	1.27
	Distilled water	2.83

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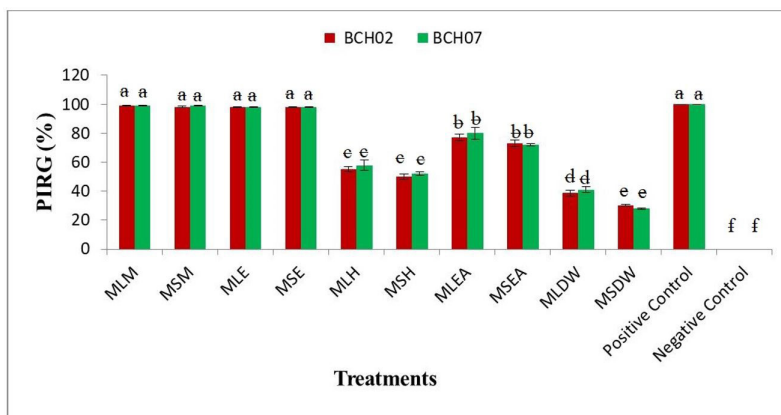*Braz. J. Biol., 2021, vol. 81, no. 4 pp.1007-1022*

Figure 1. The percentage inhibition of radial mycelial growth (PIRG) of antifungal bioassay activities of different extracts of *M. oleifera* on two *B. cinerea* isolates. Means followed by the same letters within each chart's column are not significantly different at P≤0.05 according to Turkey Studentized Range (HSD) Test.

Table 3. Minimum inhibitory concentration and minimum fungicidal concentrations of *M. oleifera* leaf extracts against *B. cinerea* isolate (mgml^{-1})

Part of plant	Solvent	BCH02		BCH07	
		MIC	MFC	MIC	MFC
Leaf	Methanol	5.00	10.00	5.00	10.00
	Ethanol	10.00	20.00	15.00	30.00
Seed	Methanol	10.00	10.00	10.00	10.00
	Ethanol	15.00	20.00	15.00	30.00

Table 4. Chemical tests for phytochemical compounds of methanol leaf extracts of *M. oleifera*.

Phytochemical compound	Chemical/colour tests	Results/observations	Inferences
Tannins	Ferric chloride test	-Greenish black colour	+
Alkaloids	Dragendorff's test	-Development of an orange colour	+
Flavonoids	Ammonium test	-Yellow colour in bottom layer	+
Saponnins	Froth test	-Stable froth on standing	+
Steroids/ Triterpenoids	Liebermann-Burchardt test.	-Red colour top and yellow colour on bottom	+
Phenolic compounds	Ferric chloride test	- Development of violet or dark blue colour	+
Anthraquinone	Borntrager's test	- Development of red colour	+
Terpenoids	Salkowski test	-Formation of red colour at the lower layer indicates the presence of Steroids	+

+ = indicates the presence of phytochemical compounds.

chemical or colour tests for qualitative detection of the presence of phytochemical compounds were presented in (Table 4). Our analysis revealed the presence of alkaloids, flavonoids, tannin, saponin, phenolic compounds, terpenoid, triterpenoids, steroids, and anthraquinone in *M. oleifera*. The presence of the phytochemical compounds indicated the antifungal potentials of *M. oleifera*.

3.3.2. Active compounds profiling using GC-MS

The chemical compounds present in the crude methanol leaves extract of *M. oleifera* were identified by GC-MS analysis. A total of 67 different components were identified and the chromatogram of the components with their peaks was shown in Figure 2. The active principal components with higher peak percentages were 6-decenoic acid (Z)- (19.87%), 2-dimethyl (trimethylsilylmethyl) silyloxymethyltetrahydrofuran (11.90%), beta.-l-rhamnofuranoside (11.07%), malonic acid (6.22%), n-hexadecanoic acid (5.87%), 1,3-propanediol (3.77%), benzeneacetonitrile (3.12%), octadecanoic acid/ Stearic acid (2.95%), 1,2,3,5-cyclohexanetetrol (2.38%), docosanoic acid (2.24%), cyclohexanecarboxylic (2.20%), 2-furancarboxaldehyde (2.14%) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (2.11%). Their retention time (RT), molecular weight (MW), molecular formula, and chemical structure were presented in Table 5. The volatile chemical components, their chemical groups and biological activity were also presented in Table 6.

Active compounds profiling using LC-MS/MS

In an analysis conducted with LC-MS/MS to determine the non-volatile components available in the methanol leaf extract of *M. oleifera,* it is evident that methanol crude leaf
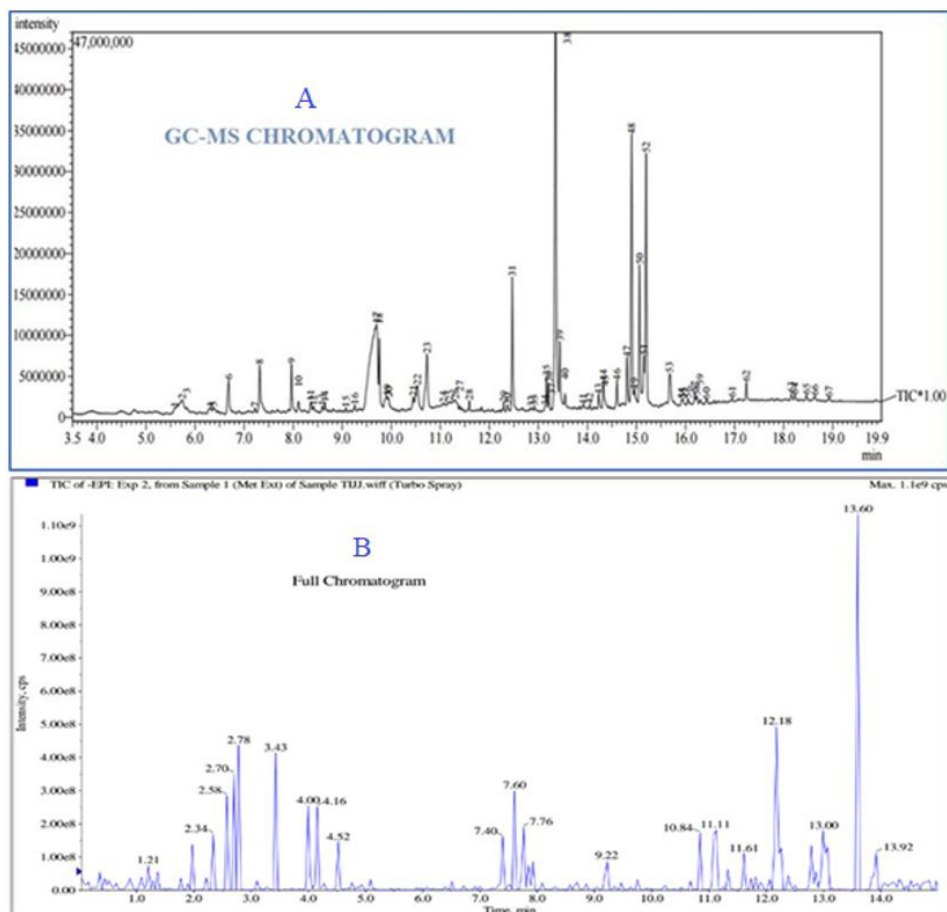
Figure 2. Chromatogram of methanol leaf extracts of *M. oleifera*. (**A**) GC-MS chromatogram of volatile active compounds. (**B**) LC-MS/MS chromatogram of non-volatile active compounds.

extract of *M. oleifera* was containing some non-volatile chemical compounds as presented in Table 7. The results of the analysis revealed the presence of phenylvaleric acid, caffeic acid, quinic acid, apigenin-6,8-di-C-β-D-glucopyranoside, apigenin 6 C glucoside, quercetin-3-O-glucoside dimer and kaempferol 3-o glucoside dimer as the predominant compounds and their retention time, molecular formula and molecular weight were given also in Table 7. The peaks in the LC-MS/MS chromatogram (Figure 2) confirmed the presence of compounds due to the large amounts of peaks in the chromatography.

Effect of methanol leaf extract of M. oleifera on the surface and ultrastructure of B. cinerea examined with SEM and TEM

3.4 Observation with SEM

Examination of the changes on the mycelium and conidia of *B. cinerea* treated with *M. oleifera* methanol leaf extract at MIC concentration through SEM micrographs revealed that *M. oleifera* have caused severe detrimental effects on the morphology of both mycelium (Figure 3b, c and d) and the conidia (Figure 4b and c) compared to the controls (Figure 3a and 4a). The effects observed include abnormal

growth, lysis, shrinkage, disruption, aggregation, reduced hyphal length and diameters and pore formation on both mycelium (Figure 3b, c and d) and spores (Figure 4b and c) of the samples treated with crude methanol leaves extracts. The control treatments micrograph for mycelium (Figure 3a) and spore (Figure 4a) were observed with normal growth and smooth surfaces.

3.5 TEM observation

Figures 5a-d presented the results of TEM on the effect of *M. oleifera* methanol leaf extract at MIC concentration. The results showed that the untreated conidium of *B. cinerea* (control) grew normally with the ultra-structural components (Figure 5a). The TEM micrographs for the control conidium appeared with a regular cell wall of about 300 to 400 nm (thickness) having a two-layer structure, plasmalemma, cytosol (cytoplasm matrix) with nucleus and nucleolus, lipids; mitochondria and vacuole. The external layer of the cell wall was thin and electrodense; thick inner wall, less and uniform electrodense. The plasmalemma tightly adhered to the cell wall. The cytosol was distributed uniformly, and the nucleus was spherical. The mitochondria were usually ovoid, numerous and had an average electron density. Lipid and glycogen

Table 5. Various volatile compounds identified in methanol leaf extracts of *M. oleifera* by GC-MS

Peak No.	RT (min)	Height (%)	Chemical compound	Molecular weight (g/mol)	Molecular formula	Chemical structure
8	7.317	2.11	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	144.12	$C_6H_8O_4$	
9	7.967	2.14	2-Furancarboxaldehyde	188.18	$C_{10}H_8N_2O_3$	
17	9.700	3.77	1,3-Propanediol	76.09	$C_3H_8O_2$	
18	9.758	3.13	Benzeneacetonitrile	117.15	C_8H_7N	
23	10.725	2.38	1,2,3,5-Cyclohexanetetrol	148.16	$C_6H_{12}O_4$	
31	12.467	5.87	n-Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	
38	13.333	19.87	6-decenoic acid (Z)-	170.25	$C_{10}H_{18}O_2$	
39	13.433	2.95	Octadecanoic acid/Stearic acid	284.5	$C_{18}H_{36}O_2$	
47	14.808	2.20	Cyclohexanecarboxylic acid	128.17	$C_7H_{12}O_2$	
48	14.900	11.90	2-Dimethyl(trimethylsilylmethyl)silyloxymethyltetrahydrofuran	246.49	$C_{11}H_{26}O_2$	-
50	15.058	6.22	Malonic acid	104.06	$C_3H_4O_4$	
51	15.150	2.24	Docosanoic acid	340.6	$C_{22}H_{44}O_2$	
52	15.192	11.07	beta.-l-Rhamnofuranoside, 5-O-acetyl-thio-octyl-	334.5	$C_{16}H_{30}O_5$	

RT=retention time.

were the main intracellular nutrient reserves. In contrast to the controls, the micrographs of the treated *B. cinerea* conidia revealed that *M. oleifera* methanol leaf extract at MIC, acts by causing irreversible ultra-structural changes to the conidia (Figures 5b-d). It was observed that the TEM micrographs clearly showed that there were destruction of the organelles and precipitation of the entire cytoplasmic (cytosol) content (Figures 5b-d). More so, the cell wall was observed to have a slightly irregular shape, shrinked and highly permeable (Figures 5b and d). The external layer of the cell wall was more electrons dense; the plasmalemma was mostly destroyed or did not adhere to the cell wall (Figures 5c-d). It was also observed that there was a reduced periplasmic space between the cell wall and the altered cytoplasm.

Table 6. Biological activity of different compounds identified in leaf of *M. oleifera* by GC-MS analysis.

| Peak No	Chemical compound	Chemical group	Biological activity
8	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	New compound	No activity reported
9	2-Furancarboxaldehyde		Antimicrobial, Antifungal, Antioxidant
17	1,3-Propanediol	-	Antimicrobial
18	Benzeneacetonitrile		Cytotoxicity.
23	1,2,3,5-Cyclohexanetetrol	-	Cytotoxicity, Antineuropathatic
31	n-Hexadecanoic acid	Palmitic acid	Antimicrobial, Antibacterial Antifungal. Antioxidant, Nematicide, Antiandrogenic
38	6-decanoic acid (Z)-	Stearic acid	Antioxidant, insectifuge, Cancer preventive.
39	Octadecanoic acid/Stearic acid	Stearic acid	Antimicrobial, Antifungal.
47	Cyclohexanecarboxylic acid	Aromatic compound	Antimicrobial
48	2-Dimethyl(trimethylsilylmethyl)silyloxymethyltetrahydrofuran	New compound	No activity reported
50	Malonic acid	Dicarboxylic acid	Antimicrobial Anticancer
51	Docosanoic acid	Saturated fatty acid	Antioxidant, Antimicrobial
52	beta.-l-Rhamnofuranoside, 5-O-acetyl-thio-octyl-	Glycoside	Antimicrobial
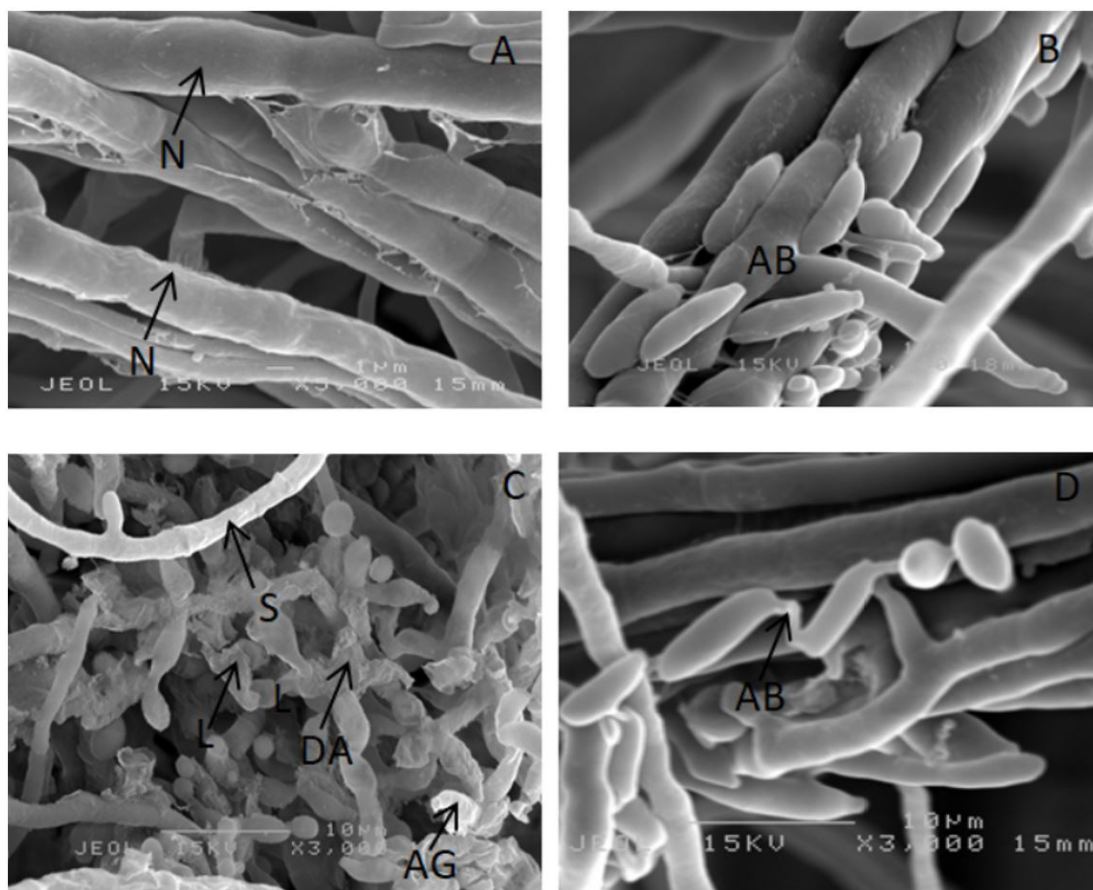
Figure 3. Effect of *M. oleifera* methanol leaf extract on mycelium of *B. cinerea*. (**A**) Scanning electron micrographs of control treatment and (**B-D**) Scanning electron micrographs of treated samples. N= normal growth and smooth surface; S=shrinkage; L=lysis; AB=abnormal growth; DA=disruption and aggregation; AG=aggregation.

Table 7. Non-volatile active compounds identified in *M. oleifera* methanol leaf crude extract by LC-MS/MS analysis.

Peak No.	tR/min	[M-H] (Error (ppm))	Compound name	Molecular formula	Molecular weight (g/mol)
1	0.927	174.93	Phenylvaleric acid	$C_{11}H_{14}O_2$	178.23
2	1.210	377.25	Caffeic acid	$C_9H_8O_4$	180.16
3	1.290	191.55	Quinic acid	$C_7H_{12}O_6$	192.17
10	3.433	592.61	Apigenin-6,8-di-C-β-D-glucopyranoside (Vicenin II)	$C_{27}H_{30}O_{15}$	594.52
11	3.998	431.60	Apigenin 6 C glucoside	$C_{27}H_{30}O_{15}$	594.52
12	4.159	928.25	Quercetin-3-O-glucoside dimer	$C_{21}H_{20}O_{12}$	464.38
14	4.524	896.31	Kaempferol 3-o glucoside dimer	$C_{21}H_{20}O_{11}$	286.24
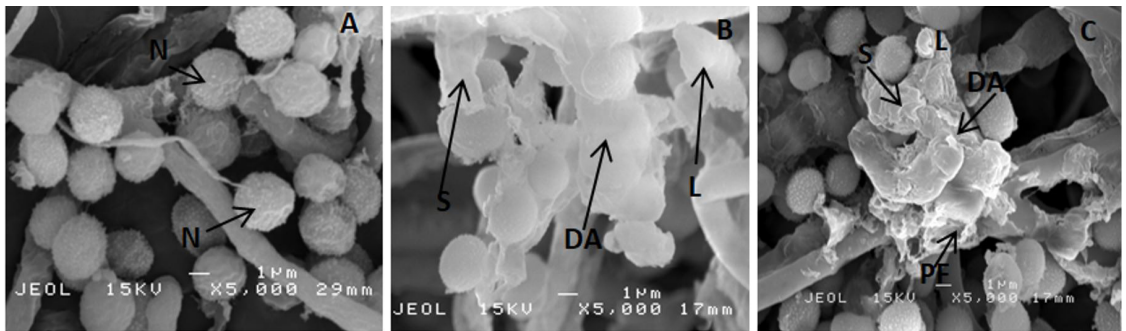
Figure 4. Effect of *M. oleifera* methanol leaf extract on conidia of *B. cinerea*. (**A**) Scanning electron micrographs of control treatment and (**B** and **C**) Scanning electron micrographs of treated samples. N=normal growth and smooth surface; S=shrinkage; L=lysis; DA= disruption and aggregation; PF=pore formation.
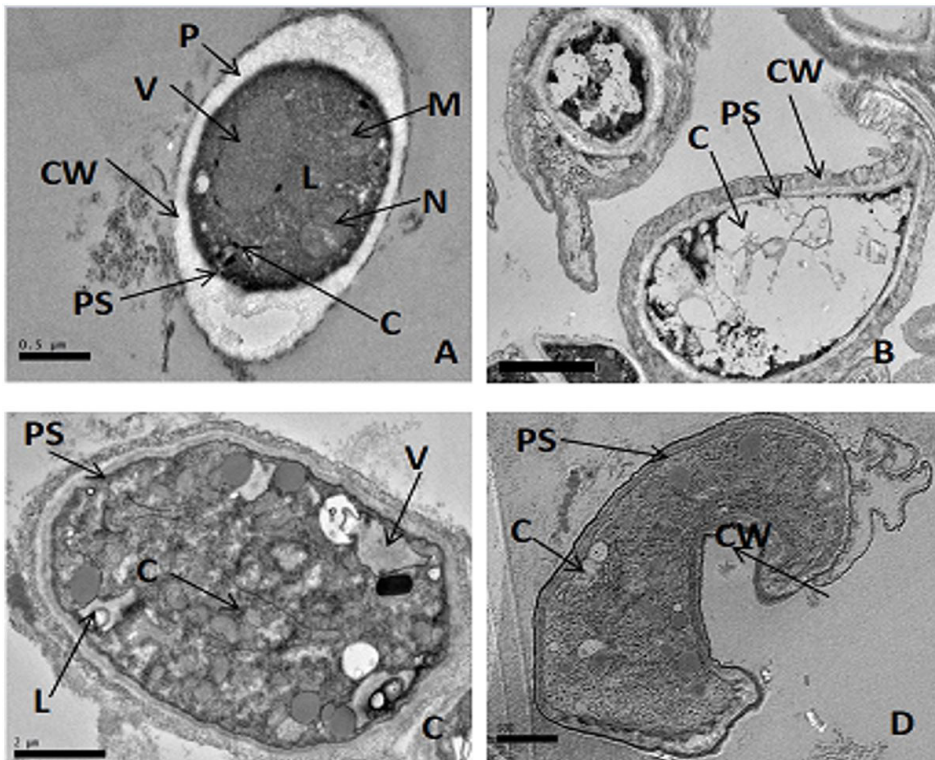
Figure 5. Transmission electron micrograph of treated *B. cinerea*: (**A**) Cross section of untreated *B. cinerea* conidium with the normal ultra-structural components; (**B-D**) Cross section of *B. cinerea* conidium treated with *M. oleifera* methanol leaf extract at the minimum inhibitory concentration (MIC) with irreversible ultra-structural changes. CW = cell wall; PS = periplasmic space; P = plasma lemma; C = cytoplasm; M = mitochondrion; V = vacuole; N = nucleus; L = lipids.

4. Discussion

Moringa oleifera was chosen for this study based on the previous reports that indicated its antifungal and antimicrobial properties effective against various phytopathogens (Emad El Din et al., 2016; Batista et al., 2014; Belay and Sisay, 2014; Díaz Dellavalle et al., 2011). Considering the efficacy of the solvent extracts and the morphological part of the plant used (seeds or leaf), the results on the bioassay showed that organic solvent extracts proved to have high antifungal activity against *B. cinerea* isolates than the aqueous extract irrespective of the morphological part of the plant used. Although, it was noticed that methanol leaf extracts have more antifungal efficacy, in general, organic solvents showed high efficiency in the extraction of water-soluble biomolecules with antifungal activity. However, this could be due the fact that when plant materials were dissolved in the distilled water, some hydrolases and phenolases were released and could have modulating effects on the activity of the active principles in the extracts. Our findings were in accordance with Emad El Din et al. (2016), Gurjar et al. (2012) and Das et al. (2010), who reported that the chemical properties of the solvent, the extraction method used and the various structural and compositional manners of the natural plant products led to extract solvent system with specific differences. Variations in polarity among solvents have been reported to elucidate the differences in solubility of active plant properties, hence differences in the degree of activity (Díaz Dellavalle et al., 2011).

Crude plant extracts are generally a combination of active and non-active compounds. In the present study MIC was obtained at 5 mg/ml with growth inhibition of up to 100% comparable to synthetic fungicide benomyl at 0.2 mg/ml concentration, while the MFC was 10 mg/ml with 98.10% growth inhibition. Our results indicated that the MFC of the crude extracts assessed were obtained at higher concentration (10 mg/ml) than in the MIC assays (5 mg/ml), but not at lower concentration. This finding agrees with the earlier report of Banso et al. (1999) that indicated that antifungal compounds found in the plant extracts were fungistatic at lower concentrations, while becoming fungicidal at higher concentrations of the extracts. In addition, growth inhibition activity of the crude plant extracts might be due to the synergistic effect of various active compounds that are in higher or small proportion in the crude extract but not due to the action of a single active compound (Rashid et al., 2016; Pinto et al., 2015; Díaz Dellavalle et al., 2011; Banso et al. 1999). *In-vitro* study on the diametric growth inhibition of the *B. cinerea* isolates by methanol crude extracts of *M. oleifera* has confirmed the antifungal efficacy of the plant. The antifungal activity of the morphological parts of *M. oleifera* found in the reported findings could be as a result of the presence of phenolic compounds, tannins, alkaloids, flavonoids, terpenoids, triterpenoids, saponins (Elad et al., 2016; Pinto et al., 2015; El-Mohamedy and Abdalla, 2014; Tijjani et al., 2014). These phenolic constituents caused the denaturing of enzymes that could

impede the amino acids involved in the germination of the spore (Cushnie and Lamb, 2005).

GC-MS analysis of the crude methanol leaf extract was performed and a total of 67 chemical compounds were identified. In comparison with the findings reported previously, most of the compounds identified from this study have also been reported somewhere in other species. For instance, 6-decenoic acid (Z)- was identified as the major compound (19.87%) of methanol extract of *M. oleifera* leaf and this compound was also reported as a major compound in the methanolic leaves extracts of *Clerodendrum viscosum*, *Indigofera suffruticosa* and *Trichilia connaroides* (Ghosh et al., 2015; Elezabeth and Arumugam 2014), n-hexadecanoic acid in the methanolic leaves extracts of *Justicia adhatoda* and *Lantana camara* (Jayapriya and Shoba 2015; Swamy et al., 2015), 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one in methanolic extracts of agarwood leaves (Khalil et al., 2013). The identified phytocompounds obtained many biological properties. For example, n-hexadecanoic acid - palmitic acid (R/T 12.467) have biological properties such as an antiandrogenic, antioxidant, antibacterial, hypocholesterolemic, antifungal, nematicide, pesticide and lubricant activities (Mirali et al., 2016; Sermakkani and Thangapandian, 2012). 6-decenoic acid (Z)-acid- Stearic acid (R/T 13.333) was reported to have properties that includes antioxidant, insectifuge and cancer preventive (Elezabeth and Arumugam, 2014).

The analysis of the compounds conducted with LC-MS on methanolic leaf extract showed that the major non-volatile constituents of the identified chemical compounds were phenylvaleric acid, caffeic acid, quinic acid, apigenin-6,8-di-C-β-D-glucopyranoside (Vicenin II), apigenin 6 C glucoside, quercetin-3-O-glucoside dimer and kaempferol 3-o glucoside dimer which are mostly flavones, flavonois and phenolic acids. Previous studies have indicated the protective effects of flavonoids against many infectious and degenerative diseases such as cancers, cardiovascular diseases and other age-related diseases (Khaleel et al., 2016; Kumar and Pandey 2013; Agati et al., 2012). In accordance with previous reports quinic acid was the major chemical constituents in *Mussaenda frondosa* Linn. with antioxidant and antimicrobial properties (Vadivel and Gopalakrishnan, 2011). Parvu and Parvu (2011) reported the antifungal activity of quercetin-3-O-glucoside and kaempferol 3-o glucoside isolated from *Allium fistulosum* and *A. schoenoprasum* subsp. *schoenoprasum* leaves and flowers against *B. cinerea*, *B. paeoniae*, *A. niger*, *F. oxysporum* f.sp. *tulipae*, *F. oxysporum* f. sp. *gladioli, Penicillium expansum*, *P. gladioli*, *Heterosporium pruneti*, and *Sclerotinia sclerotiorum*.

Botrytis cinerea usually survive in such structures as mycelia and/or conidia and sclerotia in the host plants or their debris. For this reason, we studied the changes that occurred at ultrastructural level in the conidia of *B. cinerea* produced as a result of their exposure at MIC concentration of methanol leaf extract of *M. oleifera*. Under normal circumstances, the cell wall of *B. cinerea* conidia have

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two layers and dark appearance because of the melanin pigment that protects the conidia from ultraviolet light and probably enzymatic action. Furthermore, the *B. cinerea* conidia contain 3 to 18 nuclei (Pacirc et al., 2013; Epton and Richmond, 1980). The surface of *B. cinerea* conidia and other *Botrytis* spp. have many short protuberances (200 to 250 nm) which are visible under SEM and TEM. When being treated at MIC concentration of methanol leaf extract of *M. oleifera*, the shapes and sizes of the mycelia and conidia changed with severe detrimental effects on their morphology which include abnormal growth, lysis, shrinkage, disruption, aggregation, reduced hyphal length and diameters, hypertrophy and pore formation. Similarly, irreversible ultra-structural changes occurred on the conidia that include the destruction of organelles and precipitation of the entire cytoplasmic (cytosol) content, irregular cell wall shape, destruction plasmalemma and a reduced periplasmic space between the cell wall and the altered cytoplasm. Previous studies have indicated that the functional integrity of fungal cell components is needed to keep up its viability and germination quality (Pacirc et al., 2013). However, the precipitation of the cytoplasm and the destruction of the cytoplasmic organelles and nucleus led to the loss of viability and germination quality of *B. cinerea* conidia treated with methanol leaves extract of *M. oleifera* at the MIC concentration. Additionally, the methanol leaf extract caused irreversible changes that abrogate the cell wall's barrier capacity and the possibility of activating enzymes bound to the cell wall (Pacirc et al., 2013; Parvu et al., 2008; Isaac, 1992).

In conclusion, our results showed that methanol leaf extract of *M. oleifera* have strong antifungal efficacy on mycelial growth and spore germination of *B. cinerea* due to severe ultrastructural changes at MIC concentration. Several volatile and non-volatile chemical compounds were identified by GC-MS and LC-MS/MS analysis. Availability of these compounds depends on the organic solvent and the extraction method used. These compounds have been theorised to possess among others, cytotoxicity, antioxidant and antimicrobial properties against *B. cinerea* as confirmed by the results of the present study. The antifungal effect of the studied plant extract recommends it as a promising candidate for biological control of fungal pathogens, thus limiting the overdependence on chemical fungicides.

Acknowledgements

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