

Original Research Article

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Antifungal Activity of Chinese Caterpillar Fungus (*Ophiocordyceps sinensis* Berk.) against Anthracnose Disease on Banana

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ABSTRACT

Tamilnadu is one of the leading banana grower in the country. Eighty-two per cent of fruit production in India is shared by the banana. Banana fruits after harvest are infected by a series of post-harvest pathogens. Among them, anthracnose disease caused by the pathogen *Colletotrichum musae* is the very dexterous disease. It may cause fruit loss up to 80 per cent. Many fungicides are used to control this disease. The method of applications like dipping, spraying and top dressing is commonly practised. An alternate approach of the application of biological control agents to manage the pathogens is now come into the light. This present study was done to put forth the effect of antifungal activity of a potential biocontrol agent *Ophiocordyceps sinensis* and its mycomolecules against the post-harvest pathogen *C. musae*.

Keywords

C. musae, Post-harvest disease, *O. sinensis* and Mycomolecules

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Introduction

In India, the southernmost state, Tamil Nadu account the 10 per cent of fruit production of India. The major fruit crops in Tamil Nadu is banana (*Musa* spp. Family: Musaceae) and mango (*Mangifera indica* L. Family:

Anacardiaceae). In Tamil Nadu, banana occupies 82 thousand hectares with the production of 32.05 lakh metric tonnes. The districts like Erode, Thoothukudi, Dindigul, Coimbatore and Kanyakumari are leading the banana cultivation and production (Department of Horticulture and Plantation

Crops, 2019). Post-harvest losses in India is secularly about 20-30 per cent of its annual production (Ahmed and Palta, 2016; Chen *et al.*, 2017). Post-harvest losses due to the invasion of fungal pathogens are leads to the major economic loss and makes the fruit unavailable for consuming. In banana, the anthracnose disease causing pathogen is quiescent in nature and causes latent infection during immature fruits and produces black to brown spots with orange colour acervuli on the central portion of the spot (Krauss *et al.*, 1998; Cordeiro *et al.*, 2005; Sivakumar and Bautista-Baños, 2014; Zhimo *et al.*, 2016, Caroline Lopes Damasceno *et al.*, 2019). Post-harvest diseases can be controlled by use of fungicides as sprays or dips, incorporated in wax or impregnated in packaging materials. Alternative methods to reduce the usage of chemical fungicides like heat treatment (Lurie, 1998), ozone treatment (Kim *et al.*, 1999) and biocontrol agents (Wisniewski and Wilson 1992). The interest of usage of natural volatiles produced from the plants, botanicals and mycomolecules from fungi has also increases markedly (El-Ramady *et al.*, 2015; Mahajan *et al.*, 2014; Song *et al.*, 2007). *Ophiocordyceps sinensis* (syn: *Cordyceps sinensis* (Berk.) Sacc. Family: *Ophiocordycepsitaceae*, Phylum: Ascomycota), commonly called as “Chinese caterpillar fungus” (Sung *et al.*, 2007), is one of the most valued mushroom fungus in pharmaceutical industry for its antioxidant and anti-inflammatory properties. This fungus can know to parasitize the larvae of the ghost moth (Pegler *et al.*, 1994; Wang, 1995 and Yao, 2004).

The report says that the perfect stage of this mushroom is *Beauveria*, *Metarhizium* and *Paecilomyces*. Exploration of the mycomolecules which are collectively produced by this fungus can able to control the soil borne pathogens, nematodes as well as the harmful insect (Yue *et al.*, 2013).

Materials and Methods

Collection and maintenance of fungal cultures

Virulent isolates of banana post-harvest disease anthracnose caused by *C. musae* MT071509 was collected from the stock culture maintained at Department of Plant pathology, TNAU. Slant culture of *O. sinensis* was collected from Mushroom Research and Training Centre, Department of Plant Pathology, TNAU, Coimbatore.

All the collected isolates were subculture in potato dextrose agar medium (PDA) by following the standard procedure. Pure culture of each microbial isolates was maintained under the refrigerated condition at Department of Nano Science and Technology.

Pathogenicity

Spore suspension of *C. musae* was prepared by using the well sporulation 15 days old culture plate. The culture plate was flushed with sterile distilled water and scraped by the sterile brush.

The spore collected was examined under the microscope to standardise the spore population as 10^4 conidia per ml of water. Then the conidial suspension is added with 0.05 per cent Tween 20.

The spore suspension of *C. musae* was inoculated to the mid-portion of the banana fingers harvested at 80 per cent maturity from the orchard, TNAU. The spore suspension was inoculated with minor pinprick with sterile needle and inoculated without pinprick. Uninoculated fruits were kept as control. Three replications were maintained and the pathogens were re-isolated from the disease symptom expressing on fruits to confirm Koch's postulates.

Antifungal activity of *O. sinensis* against *C. musae*

Antifungal activity of *O. sinensis* was tested against the post-harvest pathogens *Colletotrichum musae* through dual plate technique. A mycelial disc of pathogen was placed on the side of Petri plate, one cm away from the margin. On the opposite side a mycelial disc of *O. sinensis* was also placed one cm away from the margin of the Petri plate. The experiment was replicated thrice and the plates were kept at incubation under room temperature ($28 \pm 2^{\circ}\text{C}$). Ten days after incubation, inhibition zone and per cent inhibition (PI) of mycelial growth of pathogens were recorded using the formula

$$\text{PI} = \frac{\text{C} - \text{T}}{\text{C}} \times 100$$

Whereas,

C = mycelial growth of pathogen in control plate (without *O. sinensis*)

T = mycelial growth of pathogen in treated plate (with *O. sinensis*)

Extraction of mycomolecules from *O. sinensis*

Actively grown mycelium of was inoculated in MC broth (pH adjusted to 5.5) and incubated in orbital shaker (Obitek, India) at 25°C for 20 days at continuous shaking at 150 rpm (Akshaya, 2016). After 20 days of incubation, the culture was homogenised and centrifuge at 10,000 rpm for 10 minutes. The supernatant was collected to the fresh conical flask and the pellet containing cellular debris and mycelium was discarded. Equal volume of ethyl acetate was added to the supernatant and incubates overnight at orbital shaker at 25°C with 150 rpm. The fractionate solution was separated using the separating funnel and the upper phase was collected. The collected

fraction was subjected for condensation in vacuum flash evaporator at 50°C boiling temperature; 150 rpm; 400 psi vacuum pressure. The condensate was collected in the Petri plate and dried using desiccator. The dried condensate was scrapped using HPLC grade methanol for further analysis.

Antifungal activity of mycomolecules *O. sinensis* against *C. musae*

Antifungal activity of mycomolecules extracted from *O. sinensis* was tested against the post-harvest pathogens *C. musae* through agar well diffusion assay. A mycelial disc of pathogen was inoculated on the centre of the Petri plate. 20 μL of Mycomolecules of *O. sinensis* at different concentration 1000, 2000, 3000 and 4000 ppm were inoculated in the three well and water as control. The experiment was replicated thrice and the plates were kept at incubation under room temperature ($28 \pm 2^{\circ}\text{C}$). Ten days after incubation, inhibition zone and per cent inhibition (PI) of mycelial growth of pathogens were recorded using the formula

$$\text{PI} = \frac{\text{C} - \text{T}}{\text{C}} \times 100$$

Whereas,

C = mycelial growth of pathogen in control plate (without *O. sinensis*)

T = mycelial growth of pathogen in treated plate (with *O. sinensis*)

GC-MS analysis of mycomolecules of *O. sinensis*

Characterization of biomolecules of *O. sinensis* was done by GC – MS analysis using Thermo Scientific Trace GC Ultra chromatograph system (Thermo Fischer Scientific, Austria) coupled to Thermo Scientific DSQ II quadruple mass

spectrometer. Mycomolecules of *O. sinensis* was separated using a TG-SQC capillary column (15 m in length, 0.25mm I.D. and 0.25 µm film thicknesses). Helium gas was used as a carrier gas with a flow rate of 1.0 mL/min and split mode was used with the split flow of 10 mL. The injector temperature was set at 207°C. The column temperature programs consisted of the following: initial temperature of 50°C (held 1 min), increased to 150°C at a rate of 25°C/min. After each injection, the column temperature was increased to 250°C and then held for 7.0 min to remove the residues that were potentially retained in the column. The transfer line temperature and MS source temperature were 265 and 200°C, respectively. The sample extraction and introduction were fully automated using a Triplus RSH Head Space Autosampler. The volume of syringe used was 2.5mL and needle length was 65mm. The 20mL headspace vials were incubated for 1 min in the agitator with temperature 300°C. Filling and injection speed was maintained at 20mL/min. Pre-injection and post-injection flush were given using nitrogen gas to avoid contamination. The time for pre-injection and post-injection flushing was 5s and 30s respectively.

Results and Discussion

Morphological characterization of *O. sinensis* and *C. musae*

The fungal isolates collected are sub cultured in PDA medium. The cultured organisms show the typical morphological characters for its identifications. The sub-cultured isolate of *C. musae* MT071509 shows the grey whitish mycelium with slight orange colour which denotes the induction of the production of acervuli (Plate 1a). pure culture of *O. sinensis* shows that the pure milky white mycelium with orange yellow headed raised basidiocarp. This shows that the available culture is

typically *O. sinensis* (Plate 1b). When compared the results with the findings of Petre *et al.*, (2009), there is a slight modification in our isolates that yellowish colour of the colony. Cunningham *et al.*, 1950 reveals that the *O.sinensis* is closely related to that of *C. militaris*.

Pathogenesis of *C. musae*

Anthraxnose is the post harvest disease on banana caused by *C. musae* (Chakravarty 1957; Meredith 1960; Jeger *et al.*, 1995; Jones and Slabaugh, 1998). The spore suspension of *C. musae* (10^4 conidia / mL) was inoculated in the mid portion of the bananavar Grand Naine fruit. The inoculated fruits exhibit the symptoms at 10 days after inoculation. The typical symptoms exhibit the brown to black colour sunken lesion with orange colour acervuli at the centre of the lesion (Plate 2a and 2b). The spore suspension inoculated fruit with pinprick exhibits the symptoms but the inoculated fruit without pinprick and control fruits doesn't shows any symptoms. The pathogenesis of *C. musae* is also visualised under Scanning electron microscope. It clearly shows that the pathogen can colonise the surface of the banana fruit peel and the inoculated spore becomes viable and germinate in the surface of the fruit (Plate 2C). Jones and Slabaugh (1998) stated that, it doesn't cause infection only on fruits, it also causes infection on bracts, flowers, petioles and leaves of banana plants. Sutton and Waterson (1970), reported that the *C. musae* can also cause the infection in apple, mango, avocado and guava.

Antifungal activity of *O. sinensis*

Antifungal activity of *O. sinensis* against *C. musae* was tested by dual plate method. The result shows that, the *O. sinensis* 10 days after inoculation can inhibit the mycelial growth of the pathogen *C. musae*.

Table.1 List of Compounds Identified in GC-MS Analysis of mycomolecules extracted from *O. sinensis*

S. No	Compound name	Retention time	Functions
1	Ethane, 1-chloro-2-nitro-	3.62	Anticancer Activity
2	Silane, triethyl(2-phenylethoxy)-	1.29	Hydrosilylation
3	1-Monolinoleoylglycerol trimethylsilyl ether	0.12	Antimicrobial Antioxidant Antiinflammatory Antiarthritic Antiasthma, Diuretic
4	9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	0.01	Anti-Inflammatory, Hypocholesterolemic Cancer Preventive, Hepatoprotective, Nematicide Insectifuge, Antihistaminic Antieczemic, Antiacne, 5-Alpha Reductase Inhibitor Antiandrogenic, Antiarthritic, Anticoronary, Insectifuge
5	1,8-Dioxa-5-thiaoctane, 8-(9-borabicyclo[3.3.1]non-9-yl)-3-(9-borabicyclo[3.3.1]non-9-yloxy)-1-phenyl	0.02	Reducing Agent
6	Quinoline, 1,2-dihydro-2,2,4-trimethyl-	0.06	Antioxidant
7	Phenol, 2,4-bis(1,1-dimethylethyl)	0.08	Antifungal Activity
8	Cucurbitacin B, dihydro-	0.02	Anticancer Activity
9	2,5,5,8a-Tetramethyl-4-methylene-6,7,8,8a-tetrahydro-4H,5H -chromen-4a-yl hydroperoxide	0.13	
10	Isocalamendiol	0.18	Anticancer Activity
11	Widdrolhydroxyether	3.18	Antimicrobial Activity
12	2-Cyclohexene-1-carboxylic acid, 2-(7-hydroxy-3-methyl-1,3-octadienyl)-1,3-dimethyl-4-oxo-, methyl ester, [R-[R*,S*-(E,E)]]-	0.02	
13	Corymbolone	0.13	Antiplasmodial Activity
14	Hexadecanoic acid, ethyl ester	0.16	Antioxidant Hypocholesterolemic Nematicide Pesticide Lubricant Antiandrogenic
15	1,9-Dioxacyclohexadeca-4,13-diene-2-10-dione, 7,8,15,16-tetramethyl	0.62	Antibacterial
16	1b,4a-Epoxy-2H-cyclopenta[3,4]cyclopropa[8,9]cycloundec[1,2-b]oxiren-5(6H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-3,6,8,8,10a-penta methyl	0.13	
17	Phthalic acid, butyl tetradecyl ester	0.41	Anhydration Activity

18	Dasycarpidan-1-methanol, acetate (ester)	0.26	Antifungal, Anticancer, Antiinflammatory
19	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	0.48	
20	Palmitoleic acid	1.31	Ant Antitumor, Anti-Inflammatory
21	n-Hexadecanoic acid	12.05	Antioxidant Hypocholesterolemic Nematicide Pesticide Lubricant AntiandrogenicFlavorHemolytic5-Alpha Reductase Inhibitor
22	Oleic Acid	0.16	Ant Antitumor, Anti-Inflammatory
23	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, trans-	0.02	
24	Dasycarpidan-1-methanol, acetate (ester)	0.03	
25	Oxiraneoctanoic acid, 3-octyl-, cis-	0.02	Antiinflammatory
26	10-Acetoxy-2-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydro-2 H-picene-4a-carboxylic acid, methyl ester	0.05	
27	Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5á)-	0.18	Antifungal, Anticancer, Antiinflammatory
28	3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tet rone	0.55	Antiinflammatory
29	9(11)-Dehydroergosteroltosylate	2.53	Fluorescent Property

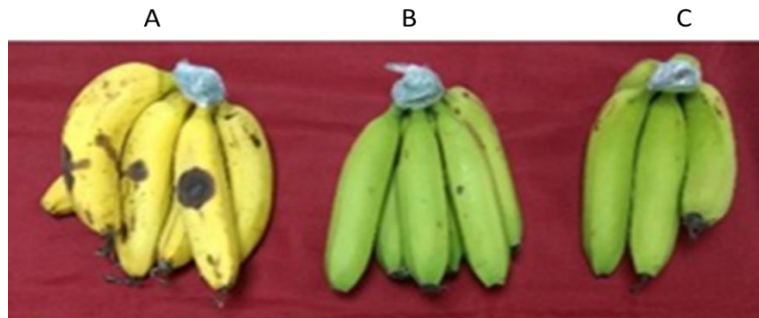
Plate.1a Pure culture of *Colletotrichum musae*



Plate.1b Pure culture of *Ophiocordyceps sinensis*



Plate.2a Pathogenesis of *C. musae*



A – Fruits inoculated with *C. musae* with pinprick
B - Fruits inoculated with *C. musae* without pinprick
C - Control

Plate.2b SEM image of Pathogenesis of *C. musae* on the epidermis of Banana fruit var. Grand Naine

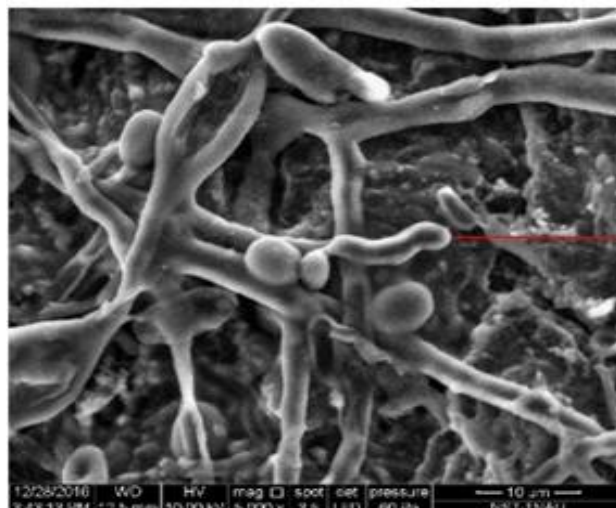
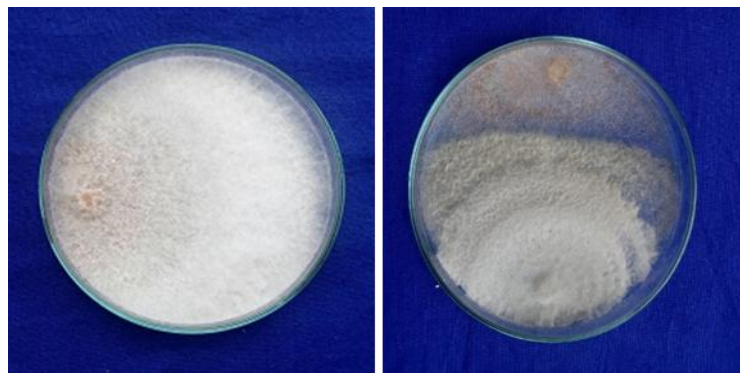


Plate.2c Characteristics symptom of anthracnose produced in Banana var Grand Naine



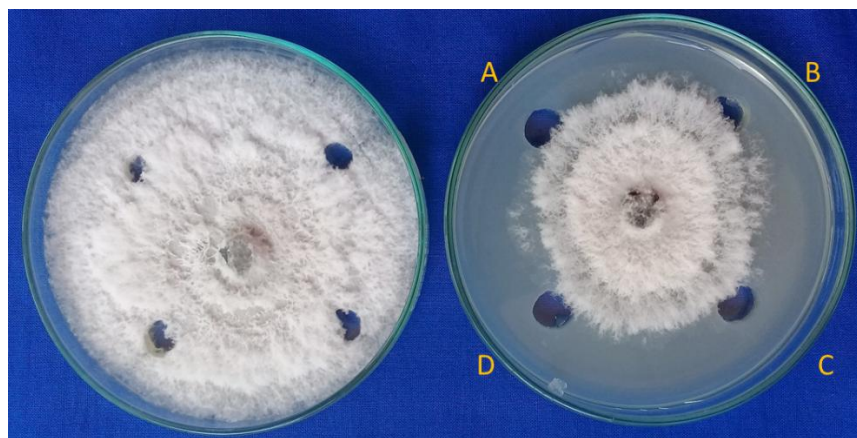
Plate.3 Dual culture technique with *O. sinensis* against *C. musae*



Control

Dual culture with *O. sinensis*

Plate.4 Agar well diffusion test with mycomolecules of *O. sinensis*



Control

Agar well diffusion with Mycomolecules

- A- 1000 ppm of crude mycomolecule
- B- 2000 ppm of crude mycomolecule
- C- 3000 ppm of crude mycomolecule
- D- 4000 ppm of crude mycomolecule

Figure.1 Antifungal activity against mycelial growth of *C. musae*

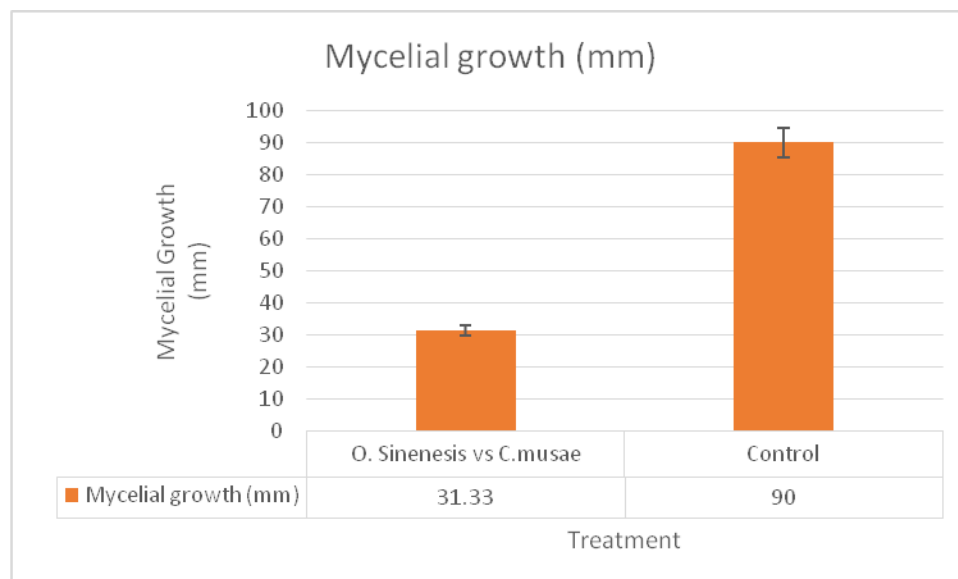
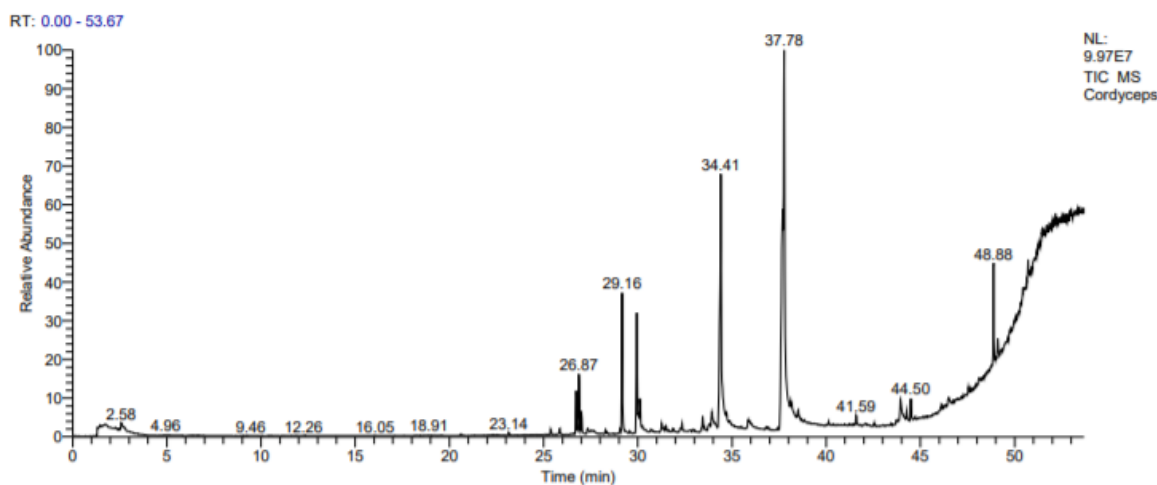


Figure.2 Chromatogram of compounds present in mycomolecules of *O. sinensis*



The growth of the biocontrol fungus *O. sinensis* is 56 mm and the mycelial growth of the pathogen *C. musae* is only 31.33 cm. The inhibition zone recorded is 2 mm. The percent inhibition of the mycelial growth is 65.19 per cent (Figure 1 and Plate 3). Pandey (2010) had observed very strong competitive interaction and inhibition at mycelial contact (37.5 per cent) followed by inhibition and replacement (30.00 per cent) and inhibition at a distance (5 per cent), when *F. oxysporum*

and *F. solani* were challenge inoculated with macrofungi like *Coprinus* sp., *Pycnoporus* sp., *Cordyceps* sp. and *Polyporus* sp.

Antifungal activity of mycomolecules against *C. musae*

The mycomolecules extracted from the broth culture of *O. sinensis* was tested against *C. musae* by agar well diffusion method. The results declared that, the overall mycelial

growth was inhibited when compare to control but there is no clear zone of inhibition formed. The mycelial growth of the pathogen is uniform in all concentrations. At once, the control reaches 90 mm of mycelial growth, the treated reaches only 7 cm of growth (Plate 4).

Antifungal compounds present in crude extract of mycomolecules from *O. sinensis*

The ethyl acetate fraction of mycomolecules from *O. sinensis* is subjected to GC-MS analysis. It is found that the total of 29 compounds is present. Among the 29 compounds, 1-Monolinoleoylglycerol trimethylsilyl ether (0.12 %), 9,12,15-Octadecatrienoic acid, 2,3-bis [(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)- (0.01 %), Phenol, 2,4-bis(1,1-dimethylethyl) (0.08 %), Widdrol hydroxyether (3.18 %), Dasycarpidan-1-methanol, acetate (ester) (0.26 %), n-Hexadecanoic acid (0.16 %), 1,9-Dioxacyclohexadeca-4,13-diene-2-10-dione, 7,8,15,16-tetramethyl (0.62 %) and Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5 α)- (0.18%) are responsible for the antifungal nature of the mycomolecules (Table 2, Figure 1). A number of bioactive compounds obtained from *Cordyceps* spp have also been reported to possess multiple pharmacological properties including anti-fungal, anti-microbial, anti-tumor, anti-inflammatory and immunomodulatory activities (Jiang *et al.*, 2002; Schuffler and Anke, 2009; Colombo and Ammirati, 2011; Qian *et al.*, 2012).

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