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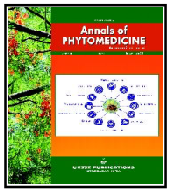

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Characterization of antimicrobial metabolites from medicinal mushrooms against Mango anthracnose pathogen *Colletotrichum gloeosporioides* (Penz.) Sacc.

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Abstract

Mango is prone to attack by many diseases and one among them is the anthracnose disease, caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. which cause both pre and post-harvest yield losses even up to 100 per cent based on prevailing wet humid weather condition. The present investigation has been focused on exploring the possibilities of identifying bioactive compounds from macro basidiomycetes against *C. gloeosporioides*. In the present study, different solvents such as ethyl acetate, diethyl ether, hexane, and chloroform at different concentrations (0.1 to 1 per cent) were used to extract secondary metabolites from culture filtrates of medicinal mushrooms, *Ganoderma lucidum* and *Auricularia. polytricha*. However, the ethyl acetate extracts of *G. lucidum* and diethyl ether fraction of *A. polytricha* at 1 per cent concentration showed maximum mycelial growth inhibition of 68.55 per cent and 58.55 per cent, respectively. Characterization of antimicrobial compounds from ethyl acetate extracted culture filtrates of *G. lucidum* through gas chromatography-mass spectrometry (GC-MS) indicated the presence of novel compound papaverine which recorded the highest peak area, 2.13 per cent with a probability of 90.74 per cent at RT 26.68. Further, the functional groups identified through fourier transform infra-red spectrophotometer (FT-IR) analysis revealed the nature of compounds belonging to aliphatic primary amine, amine salt, thiol, amide, conjugated ketone, alkane, and alcohol. The thin layer chromatography (TLC) studies performed with the ethyl acetate extracted culture filtrate of *G. lucidum* indicated the appearance of two bands at the R*f* value of 0.45 and 0.29. Further, testing of eluted bands through agar well diffusion assay showed 36.66 and 40.50 per cent inhibition of mycelial growth of *C. gloeosporioides*, respectively. The standard samples of papaverine (Source: Sigma Aldrich and Compound code: P3510-5g) tested against mycelial growth and conidial germination of *C. gloeosporioides* exhibited 100 per cent inhibition of mycelial growth of *C. gloeosporioides* at 1500 ppm and 3000 ppm in agar well diffusion assay and paper disc assay which confirmed the presence of antimicrobial metabolites from culture filtrates of *G. lucidum*.

1. Introduction

Mango is susceptible to various diseases, insect pests, and physiological disorders (Sayiprathap *et al.*,2018). The most destructive diseases are caused by fungi, bacteria, and phytoplasma which results in loss of a quantity of produce (Jha *et al.*,2010). Among that mango, anthracnose is one of the major devastating calamity due to its pre-harvest as well post-harvest infection and also widely distributed in all mango growing regions of the world. The disease causes a yield loss of 60 per cent, sometimes it extend up to 100 per cent under prevailing wet humid condition (Sharma *et al.*,2018). Under the current scenario, continuous and judicious application of fungicide against the pathogen has resulted in creating environmental pollution and health problem apart from that, it also leads to the loss of effectiveness of fungicide due to the build-up of fungicide resistance in the pathogen. As an alternative, scientists have started searching for antimicrobial bioactive compounds from various natural sources to overcome the current situation. In that context, mushrooms are in the limelight and evoked interest globally for their bioactive compounds that find their pharmaceutical and therapeutical values as well as their nutritive value (Poucheret *et al.*, 2006). Mushrooms are the natural blessing in the universe and they possess various natural compounds with bioactive potentials such as antifungal, antibacterial, antiviral, antitumor, antinemic, anti-inflammatory, antiallergic, antiantherogenic, antidiabetic properties (Lindequiste *et al.*,2005). In this background, the present study was framed with an aim to screen the antimicrobial activity of medicinal mushroom fungi against *C. gloeosporioides* and also involve the characterization of the antimicrobial compounds and identification of their functional groups which help us to develop fungicide formulation.

2. Materials and Methods

2.1 Collection of fungal cultures

Pure cultures of mango anthracnose pathogen, *Collectotrichum gloeosporioides*, and medicinal mushroom fungal cultures, *Ganoderma lucidum*, and *Auricularia polytricha* were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore.

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2.2 Extraction and testing of crude culture filtrates from mycelium of *Ganoderma lucidum* and *Auricularia polytricha*

The mycelial disc (9 mm) from 10 days old culture of *A. polytricha* and *G. lucidum* were placed into a 250 ml conical flask containing sterilized potato dextrose (PD) broth. The flasks were kept in an incubator cum shaker at 25°C with agitation at 150 rpm. After incubation, the mycelial mat was separated from the broth using Whatman filter paper No 1. Then, the culture filtrate was centrifuged at 10,000 rpm for 10 min. To avoid the bacterial contamination, the supernatant was then filtered through the 0.2 µm membrane filter. This extract was used as cell-free crude culture filtrates. The crude culture filtrates were collected from *A. polytricha* and *G. lucidum* at various periodical intervals, *viz.*, 10th, 15th, 20th, and 25th days of inoculation and tested for mycelial inhibition studies.

The extracted crude culture filtrate of *G. lucidum* and *A. polytricha* from various periodical intervals (10th, 15th, 20th and 25th days) were tested against the pathogen by agar well diffusion technique as described by (Stokes and Ridgway, 1980). The PDA medium was poured into the sterilized plates and allowed to solidify. Later, four wells were made at equal distance leaving 1cm space from the edge of the plates containing solidified medium using a sterilized cork borer. A 100 µl of crude culture filtrate was pipetted and poured into the wells. Using sterilized cork borer, the 9 mm mycelial disc of *C. gloeosporioides* from 10 days old culture was placed at the center of the plate and incubated at room temperature (28 ± 2°C). Sterile water was served as control instead of using culture filtrate.The results obtained from the mycelial inhibition test from culture filtrates of *G. lucidum* and *A. polytricha* at various periodical intervals against the test pathogen were taken for identification of the right stage for a collection of secondary metabolites (Jeeva and Krishnamoorthy, 2018). The percentage inhibition was calculated by using the formula of Vincent, (1947) as explained earlier.

2.3 Testing the solvent extracted secondary metabolites from *G. lucidum* and *A. polytricha* against *C. gloeosporioides*

The efficacy of different solvents used for extraction of secondary metabolites from mushrooms possessing antimicrobial activity varied (Nithya *et al.*, 2013). For this purpose, the 20 days old culture filtrate of *G. lucidum* was extracted with various solvents (ethyl acetate, diethyl ether, chloroform, hexane) and made up to various concentrations of 0.1, 0.15, 0.2 per cent to test the desired concentration that could inhibit the maximum mycelial growth of *C. gloeosporioides*.

2.3.1 Extraction of secondary metabolites from *G. lucidum* and *A. polytricha* using solvents

Mycelial disc (9 mm) of *G. lucidum* and *A. polytricha* from 10 days old culture plate were taken separately and inoculated into the sterilized PD broth in a 250 ml conical flask. The flasks were kept incubated at 25°C with continuous agitation at 120 rpm for 20 days. Based on the previous experiments, the maximum antimicrobial activity was found to be reported at 20 days after inoculation. The extraction of metabolites through solvents was fixed at the 20th-day old culture filtrate. After incubation, the mycelial grown culture was separated using Whatman filter paper No 1. The mycelial mat was removed and the aqueous filtrate was collected in a separate conical flask, which is dissolved with an equal volume of solvent and further, the flasks were kept overnight at 150 rpm in a shaker. The layer of solvent and solution were separated using a separating funnel. Further, the solvent fraction was evaporated using a vacuum flask evaporator. The residue obtained during evaporation was dried and suspended in HPLC grade methanol. The final product of the solution was served as stock to analyze its antimicrobial activity against the pathogen.

2.4 *In vitro* antimicrobial activity of different solvent extracted antimicrobial metabolites from crude culture filtrates of *G. lucidum* against the growth of *C. gloeosporioides*

The different solvents (ethyl acetate, diethyl ether, hexane, and chloroform) extracts from crude cell-free mycelial filtrates of *G. lucidum* were tested separately against mycelial inhibition of *C. gloeosporioides* by using the agar well diffusion technique (Stokes and Rigdway, 1980). The extracts from various solvents (ethyl acetate, diethyl ether, chloroform, hexane) of *G. lucidum* was made into various concentration (0.1%, 0.15%, and 0.2%). The PDA medium was poured into the sterilized plates and allows the plates to solidify. After solidification, four well were made at equal distance leaving 1cm space from the periphery of the plate using a sterilized cork borer. 100 µl of solvent extracted culture filtrate was pipetted and poured into the well. Using sterilized cork borer, mycelial disc (9 mm) of *C. gloeosporioides* from 10 days old culture plate was placed at the center of the plate. Sterile water was served as control instead of using solvent extracted culture filtrate. The percentage inhibition was calculated by using the formula (Vincent, 1947).

The particular solvent extracts of crude mycelial extracts of *G. lucidum* possessing maximum antimicrobial activity against *C. gloeosporioides* were carried for further studies.

2.4.1 Testing different concentrations of ethyl acetate solvent extracts of *G. lucidum* mycelial filtrates on growth of *C. gloeosporioides*

Based on the previous experiment, ethyl acetate fraction of secondary metabolite showing the highest mycelial inhibition percentage against the pathogen was selected. To narrow down the effectiveness of ethyl acetate fraction, the concentration, was further increased up to 1 percentage to fix the maximum inhibitory concentration. The antimicrobial activity of different concentrations *viz.*, 0.05 %, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, and 1 % of ethyl acetate extracts of *G. lucidum* were tested against mycelial growth of *C. gloeosporioides* by agar well diffusion technique with three replications.

2.5 *In vitro* antimicrobial activity of different solvent extracted antimicrobial metabolites from crude culture filtrates of *A. polytricha* against *C. gloeosporioides*

The different solvents (ethyl acetate, diethyl ether, hexane, and chloroform) extracts from crude cell-free mycelial filtrates of *A. polytricha* were tested separately against mycelial inhibition of *C. gloeosporioides*. The extracts from various solvents (ethyl acetate, diethyl ether, chloroform, hexane) of *A. polytricha* was made into various concentration (0.1 per cent, 0.15 per cent and 0.2 per cent). Further, the different concentrations were tested separately against the mycelial growth of *C. gloeosporioides* by agar well diffusion. The particular solvent extracts of crude mycelial extracts of *A. polytricha* possessing maximum antimicrobial activity against *C. gloeosporioides* were carried for further studies.

2.5.1 Testing different concentrations of diethyl ether solvent extracts of *A. polytricha* mycelial filtrates on the growth of *C. gloeosporioides*

Based on a previous experiment diethyl ether fraction of secondary metabolite of *A. polytricha* showed the highest mycelial inhibition of *C. gloeosporioides*. To narrow down the effectiveness of the diethyl ether fraction, the concentration was further increased up to 1 percentage to fix the maximum inhibitory concentration.The antagonistic effect of different concentrations of diethyl ether solvent extracts from *A. polytricha*, *viz.*, 0.05%, 0.1%, 0.2 %, 0.4 %, 0.6 %, 0.8 % and 1 % were tested against mycelial growth of *C. gloeosporioides* by agar well diffusion method.

2.6 Identification of antimicrobial compound from mycelial extracts of *G. lucidum* through GC-MS, FT-IR, and TLC.

From the previous experiments, based on the maximum antifungal activity of ethyl acetate extracts of *G. lucidum* observed when compared to *A. polytricha*, an experiment was intended to identify the presence of bioactive molecules from solvent extracted mycelial culture filtrates of *G. lucidum* against *C. gloeosporioides*. For this purpose, the solvent extracts from mycelial culture filtrates of *G. lucidum* were subjected to gas chromatography-mass spectrometry (GC-MS) for identification of bioactive compounds. Furthermore, fourier transform - infra-red spectrophotometer (FT-IR) and thin layer chromatography (TLC) analysis were carried out to identify the functional group of such bioactive compounds.

2.6.1 Characterization of antimicrobial compound from ethyl acetate extracted mycelial filtrates of *G. lucidum* through GC-MS analysis

Characterization of biomolecules of cell-free culture filtrate (CFC) condensate and mycelial mat extract of *G. lucidum*. (Ethyl acetate and methanolic fractions) were done by GC-MS analysis. In this study, the trace GC Ultra and DSQII model MS from Thermo Fisher Scientific Limited were engaged for analysis. The instrument was set as follows: Injector port temperature set to 250°C, Interface temperature set as 250°C, source kept at 200°C. The oven temperature was programmed as available, 70°C for 2 min, 150°C @ 8°C /min. up to 260°C @ 10°C /min. The split ratio was set as 1:50 and the injector used was in splitless mode. The DB-35 MS non-polar column was used, whose dimensions were 0.29 mm OD x 0.25 µm ID x 30 meters length procured from Agilent Co., USA. Helium was used as the carrier gas at one Ml /min. The mass spectrum (MS) scan from 50 to 650 Da. The source was maintained at 200°C and < 40 motor vacuum pressure. The ionization energy was -70eV. The MS was also having an inbuilt pre-filter, which reduced the neutral particles. NIST4 and WILEY9 are the two inbuilt libraries was found in the data system for searching and matching the spectrum. Those compounds with spectral fit values equal to or greater than 700 were considered identification. Based on the MS data library and comparing the spectrum obtained through.

2.6.2 Identification of nature of antimicrobial compound from ethyl acetate extracted culture filtrates of *G. lucidum* through FT-IR analysis

The chemical nature of the antimicrobial compounds from ethyl acetate extracted mycelial culture filtrate fraction of *G. lucidum* was identified through FT-IR studies. The ethyl acetate solvent fraction of *G. lucidum* was prepared as same as mentioned (Jassco FT-IR 6800) and analyses were carried at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

2.6.3 Identification of antimicrobial from ethyl acetate solvent acetate extracted culture filtrates of *G. lucidum* through TLC studies

The ethyl acetate extracted solvent fractions of mycelial extracts of *G. lucidum* were spotted on the silica gel-coated TLC plates at the rate of 20 µl/spot.The spots were allowed to dry and the chromatograph was developed using a mobile solvent system, *viz* Chloroform: Methanol: Distilled water (30:4:1) as described by (Aryantha *et al.*, 2001). The plates were allowed to run until it reaches ¾th position of the plate, then the plates were observed under UV transilluminator at 254 nm. Bands observed in the UV lamp were marked and the R*f* value was calculated and recorded.

$$R_f \text{ value} = \frac{\text{Distance traveled by solute}}{\text{Distance travelled by solvent}}$$

2.6.4 Testing the antifungal activity of TLC eluted compound from ethyl acetate extracted mycelial filtrates of *G. lucidum* against *C. gloeosporioides*

The specific bands obtained through TLC were scrapped along with silica coating and suspended in 1 ml HPLC grade methanol. The solution was vortexed for 10-15 min and then further centrifuged at 10,000 rpm for 10 min. The compound associated with methanol were separated and filter through a membrane filter (0.2 µm), stored at 4°C used for further studies. The antimicrobial activity of the compound was tested against *C. gloeosporioides* by agar well diffusion technique.

2.7 Testing and confirming the antifungal activity of a standard sample of papaverine against *C. gloeosporioides*

From the GC-MS analysis, the compound papaverine from mycelium extracts with a high peak area percentage was identified as an antimicrobial compound. Hence, the standard sample papaverine was purchased from Sigma Aldrich chemicals. The antifungal activity of a standard sample of papaverine was tested against mycelial inhibition and spore germination activity of *C. gloeosporioides* agar well diffusion technique and spore germination assay.

2.7.1 Confirming the antifungal activity of papaverine against mycelial growth of *C. gloeosporioides* by agar well diffusion technique

The standard sample of papaverine was tested against the mycelial growth of *C. gloeosporioides* by agar well diffusion technique. Initially, the papaverine was prepared into different concentrations, *viz.*, 500, 1000, 1500, 2000, and 2500 ppm. About 15 ml of PDA medium was poured into a sterilized Petri plate and allowed to solidify. Wells were made at an equal distance leaving 1 cm from the corner of the Petri plate. A 100 µl of different concentrations of papaverine was added @ 100 µl per well. Sterile water served as a control. Incubate the plates at room temperature (28 ± 2°C) and observations were taken daily and the inhibition percentage of mycelial growth of pathogen was calculated by using the formula derived by Vincent (1947).

2.7.2 Testing the effect of papaverine on conidial germination of *C. gloeosporioides*

The antifungal activity of papaverine was tested against spore germination of *C. gloeosporioides* by using the cavity slide method. Conidia were collected from 10 days grown-up culture of *C. gloeosporioides*. The conidial suspension was prepared at a concentration of 1×10^6 conidia/ml. Based on the preliminary screening results obtained in the previous study, the best concentration of papaverine (1500 ppm) was taken for testing the conidial germination of C. *gloeosporioides*. A drop of conidia suspension of *C. gloeosporioides* and a drop of a standard sample of papaverine at the desired concentrations (1500 ppm) were added separately into the cavity slide and mixed well. The slides were incubated in the Petri plate containing moist cotton to maintain the humidity for germination of spores.The experiment was replicated four times. The conidial suspension suspended in sterile water served as a control. The conidia were incubated for 6, 12, 18, and 24 h at room temperature (28 ± 2ºC). Germination of the conidia was observed under a phase-contrast microscope. The per cent inhibition of conidial germination was calculated using the formula (Akhter *et al*., 2006).

Inhibition of conidial germination (%)

$$= \frac{\text{Total no. of conidia} - \text{No. of conidia germinated}}{\text{Total number of conidia}} \times 100$$

2.8 Statistical Analysis

All the experiments were performed in triplicate and the treatment mean differences were evaluated with the statistical analyses followed as suggested by (Gomez and Gomez, 1984). Statistical software SPSS was used for the analyses of the data. In the case of zero values, the data were arcsine transformed (1/4n) before statistical analysis.

3. Results

3.1 Testing the crude culture filtrate of mushroom fungi against mycelial growth of *C. gloeosporioides*

Based on the results, the crude culture filtrate *G. lucidum* and *A. polytricha* collected on the 10th day did not exhibit any antifungal activity against the pathogen tested. The crude culture filtrate collected from the 15th day onwards showed inhibition of mycelial growth of *C. gloeosporioides*. However, the culture filtrate collected on the 20th day showed maximum mycelial inhibition of 36.33 per cent and 47.11 per cent of *C. gloeosporioides* with respect to *G. lucidum* and *A. polytricha*, respectively (Table 1). From the result, it was concluded that the idiophase for the maximum production of antifungal metabolites from *G. lucidum* and *A. polytricha* could be the 20th day in the culture filtrate and it was found to be the crucial period for the extraction of metabolites under liquid state fermentation method. Hence, further studies were focused on the extraction of antimicrobial compounds from *A. polytricha* and *G. lucidum* on the 20th day of the extraction.

Table 1: Efficacy of crude culture filtrates of *G. lucidum* and *A. polytricha* against *C.gloeosporioides in vitro*

S. No.	Days interval	*G. lucidum*		*A. polytricha*	
		*Mean mycelial of the pathogen (mm)	Per cent inhibition over control	*Mean mycelial of the pathogen (mm)	Per cent inhibition over control
1	10th day	90.00 (71.61)	0.00	90.00 (71.61)	0.00
2	15th day	58.66 ab(49.96)	34.88	52.33 b(46.31)	41.88
3	20th day	57.33 a(49.19)	36.33	47.60 a(43.60)	47.11
4	25th day	59.33 b(50.35)	34.11	51.33 b(4.74)	43.00
5	Control	90.00 (71.61)	0.00	90.00 (71.61)	0.00
	SE(d)	1.265	-	1.235	-
	CD (*p*=0.05)	2.854	-	2.787	-

Values are the mean of four replications. Means followed by a common letter are not significantly different at 5% level by DMRT. Values in parenthesis are arcsine transformed values.

3.2 Testing the solvent extracted culture filtrate of *G. lucidum* and *A. polytricha* at different concentration against mycelial growth of *C. gloeosporioides*

In respect of cell-free culture filtrates of *G. lucidum*, all the concentrations of the solvents tested showed various degrees of mycelial inhibition percentage ranging from 31.11 to 55.55. Maximum inhibition of mycelial growth (41.55 to 55.55 per cent) was observed at 0.2 per cent concentration irrespective of the solvents chloroform, hexane, diethyl ether, and ethyl acetate. However, the ethyl acetate fraction of *G. lucidum* exhibited a mycelial inhibition percentage of 55.55 at 0.2 per cent concentration followed by diethyl ether, hexane, and chloroform fractions (47.77 per cent, 46.33 per cent, 41.55 per cent, respectively) (Figure 1). From the results, it was obvious that among the solvents used the ethyl acetate fraction of mycelial culture filtrate of *G. lucidum* showed maximum mycelial inhibition of *C. gloeosporioides*. To narrow down the effectiveness of the ethyl acetate fraction, the concentration was further increased up to 1 per cent to know the maximum inhibitory potential of the antimicrobial compound present in *G. lucidum* against the mycelium of *C. gloeosporioides*. The results revealed that the inhibition of mycelial growth of *C. gloeoporioides* was observed at all concentrations ranging from 40 per cent to 68.55 per cent inhibition. It is found that the inhibition

percentage of mycelial growth has been increased from 0.2 to 1 per cent concentration (55.55 to 68.55 per cent). However, the ethyl acetate extracts of *G. lucidum* at 1 per cent concentration showed maximum mycelial growth inhibition of *C. gloeosporioides* (68.55 per cent) when compared to control (Figure 2).

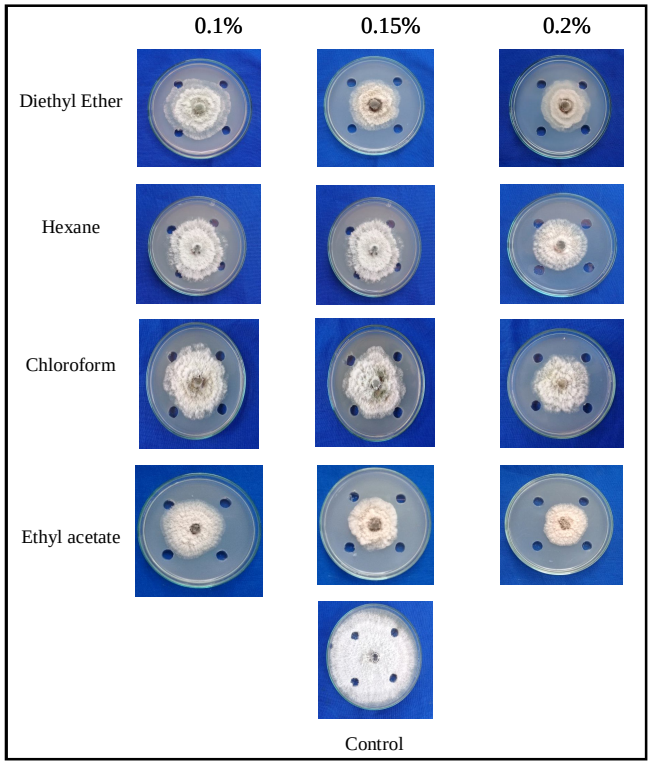

Figure 1: Antimicrobial effect of mycelial culture filtrate of *G. lucidum* using different solvents against mycelial growth of *C. gloeosporioides.*

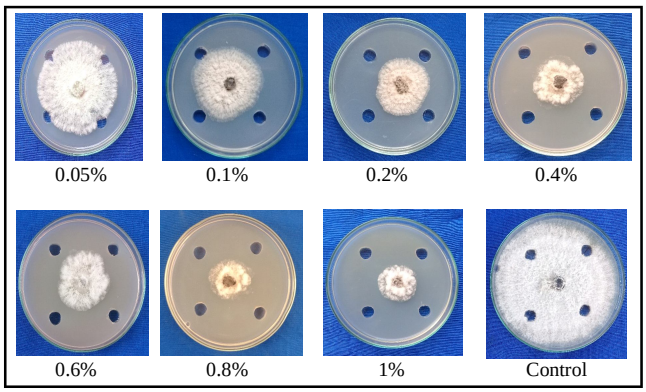

Figure 2: Testing the Ethyl acetate extracted crude mycelial extracts of *G. lucidum* against mycelial growth of *Colletotrichum gloeosporioides.*

Similarly, maximum inhibition of mycelial growth (35.22 to 46.33 per cent) was observed at 0.2 per cent concentration irrespective of the solvents diethyl ether, hexane, chloroform, and ethyl acetate. However, the diethyl ether extracts of *A. polytricha* showed maximum inhibition percentage (46.33 per cent) of mycelial growth of *C. gloeosporioides* followed by hexane (41.11 per cent), ethyl acetate fraction (38.88 per cent), and chloroform (35.22 per cent) at 0.2 per cent concentration (Table 2). Hence, to find out the effective concentration and maximum inhibitory potential, the concentration of diethyl ether extracts of *A. polytricha* was further increased up to 1 per cent. The result showed that the mycelial growth inhibition of *C. gloeoporioides* was observed at all concentrations ranging from 35.22 per cent to 58.55 per cent inhibition. However, the inhibition percentage was directly proportional to the higher concentration that exhibited maximum mycelial inhibition of *A. polytricha* to 58.55 percentage at 1 per cent concentration (Figure 3).

The result revealed that the ethyl acetate extracted secondary metabolites from mycelial extracts of *G. lucidum* showed maximum mycelial inhibitory potential than *A. polytricha* at 1 per cent concentration. Hence, *G. lucidum* was subjected to further studies.

Table 2: Testing crude mycelial culture filtrates of *A. polytricha* against mycelial growth of *C. gloeosporioides*

Solvents	Diethyl ether		Ethyl acetate		Chloroform		Hexane	
Concentration (%)	Mycelial growth (mm)	PI	Mycelial growth (mm)	PI	Mycelial growth (mm)	PI	Mycelial growth (mm)	PI
0.1	54.8 ^{bc} (47.73)	38.00	61.5 ^{b} (51.63)	31.66	68.5 ^{c} (55.86)	23.88	60.3 ^{c} (50.92)	33.00
0.15	53.3 ^{b} (46.87)	40.77	56.6 ^{a} (48.77)	37.77	61.1 ^{b} (51.41)	32.11	56.3 ^{b} (48.60)	37.44
0.2	48.3 ^{a} (44.00)	46.33	55.0 ^{a} (47.85)	38.88	58.3 ^{a} (59.48)	35.22	53.0 ^{a} (46.70)	41.11
Control	90.0 ^{d} (71.61)	0.00	90.0 ^{c} (71.61)	0.00	90.0 ^{d} (71.61)	0.00	90.0 ^{d} (71.61)	0.00
SE(d)	0.708	-	0.724	-	0.732	-	0.708	-
CD (p=0.05)	1.876	-	1.807	-	2.030	-	1.767	-

Values are the mean of four replications. Means followed by a common letter are not significantly different at 5% level by DMRT. Values in parenthesis are arcsine transformed values

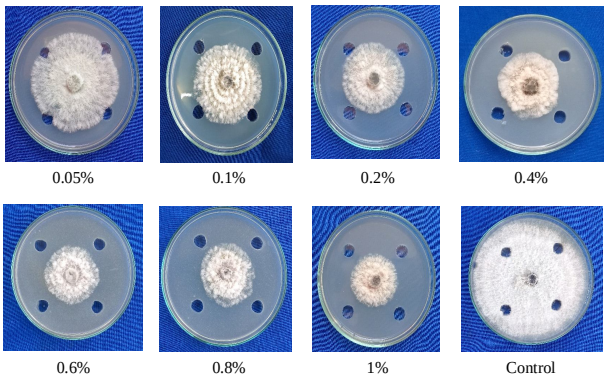

Figure 3: Testing diethyl ether extracted crude mycelial extract of *A. polytricha* against mycelial growth of *C. gloeosporioides.*

3.3 Identification and characterization of an antifungal compound from ethyl acetate extracts of mycelial filtrates of *G. lucidum* through GC-MS analysis

Characterization of biomolecules of cell-free culture filtrate condensate of *G .lucidum* (ethyl acetate fractions). GC-MS analysis revealed the presence of various compound such as Formic acid, 2-propenyl ester, 1, 4, 7, 10, 13, 16, 19 - Heptaoxa-2-cycloheneicosanone, Pentaethylene glycol,n-Hexadecanoic acid, Heptaethylene glycol, 9-Decanoic acid, 2,-dimethyl through GC-MS analysis. Among the compound, Papaverine is reported to have antiviral activity, n-Hexadecanoic acid with antibacterial and antifungal activity, Pentaethylene glycol, Heptaethylene glycol, and Formic acid, 2-propenyl ester, 1,4,7,10,13,16, 19- Heptaoxa-2-cycloheneicosanone with antifungal activity, and 9-Decenoic acid with antinemic property. Among several compounds characterized, papaverine has recorded the highest peak area of about 2.13 per cent with the probability of 90.74 per cent at RT 26.68 was used for further studies owing to its antimicrobial activity (Table 3).

3.4 Determination of functional groups of antimicrobial compounds of ethyl acetate fractions of *G. lucidum* mycelial culture filtrates through FT-IR analysis

FT-IR analysis of ethyl acetate solvent fraction of *G. lucidum* mycelial culture filtrates exhibited the distribution of functional group within the organic fractions. The spectrum indicated that the leading bands were observed in the regions between 632.537 and 3309.25 cm^{-1}. The peak in the spectrum reveals the presence of aliphatic primary amine, amine salt, thiol, amide, conjugated ketone, alkane, alcohol. The presence of different functional groups indicated the existence of a variety of potential biomolecules in mycelial culture filtrates of *G. lucidum* (Figure 4).

3.5 Identifying the bioactive molecules from ethyl acetate solvent extracts of mycelial culture filtrates of *G. lucidum* through TLC studies

The TLC analysis carried out for the ethyl acetate solvent extracts of *G. lucidum* mycelial culture filtrates using the mobile phase Chloroform: Methanol: Water at the proportion 30:4:1 indicated the appearance of two bright bands under a UV lamp at the R_f value of 0.45 and 0.39 (Figure 5a). Further, the bands (band1 and band 2) were eluted and their antimicrobial activity was tested against *C. gloeosporioides* using the agar well diffusion technique. The results indicated that bands 1 and 2 (R_f value-0.45 and 0.29 respectively) eluted from the TLC plate showed inhibition of mycelial growth of *C. gloeosporioides* in the range of 36.66 per cent and 40.50 per cent, respectively (Figure 5 b). Based on the result, it is obvious that band 2 possessed maximum antifungal activity against *C. gloeosporioides* when compared with band 1.

Table 3: Characterization of antimicrobial compounds from ethyl acetate solvent extracts from the mycelial culture filtrate of *G. lucidum*

S.No.	RT	Compound weight (g/ml)	Molecular formula	Molecular	Biological activity
1.	4.07	Formic acid,2-propenyl ester	46.025	CH_2O_2	Antifungal activity against *Candida albicans and C. guillermondii*
2.	15.61	1,4,7,10,13,16,19 Heptaoxa-2-cycloheneicosanone	322.35	$C_{14}H_{26}O_8$	-
3.	16.86	Pentaethylene glycol	238.28	$C_{10}H_{22}O_6$	Antifungal activity against *C. graminicola* and *Fusarium* spp
4.	26.68	Papaverine	375.80	$C_{20}H_{21}NO_4$	Antifungal activity and antimicrobial activity
5.	20.68	n-Hexadecanoic acid	256.43	$C_{16}H_{32}O_2$	Antifungal, antibacterial
6.	25.47	Heptaethylene glycol	326.38	$C_{14}H_{30}O_8$	Antifungal activity against *C. graminicola* and *Fusarium* spp.

3.6 Confirming the antifungal activity of papaverine (Standard sample) against mycelial growth of *C. gloeosporioides* by agar well diffusion technique

From the GC-MS analysis, the bioactive compound papaverine from mycelial extracts of *G. lucidum* was identified with a high peak area percentage. Hence, the standard sample of papaverine was purchased from Sigma Aldrich chemicals (Compound code: P3510-5 g). tested against mycelial inhibition and spore germination activity of *C. gloeosporioides* indicated that all the concentrations of papaverine showed inhibition of mycelial growth of *C. gloeosporioides.* Interestingly, the compound exhibits a higher degree of inhibition even at a lower concentration. However, 1500 ppm

was the minimum concentration that was sufficient enough to completely inhibit the mycelial growth of *C. gloeosporioides*. Moreover, the mycelial pattern of the pathogen in treatment was observed. The morphological changes of cultural characters were observed as breakage of the mycelial disc, formation of thin mycelial pattern with inhibition zone, complete growth arrestment whereas control showed normal growth of mycelium in concentric patterns (Figure 6).

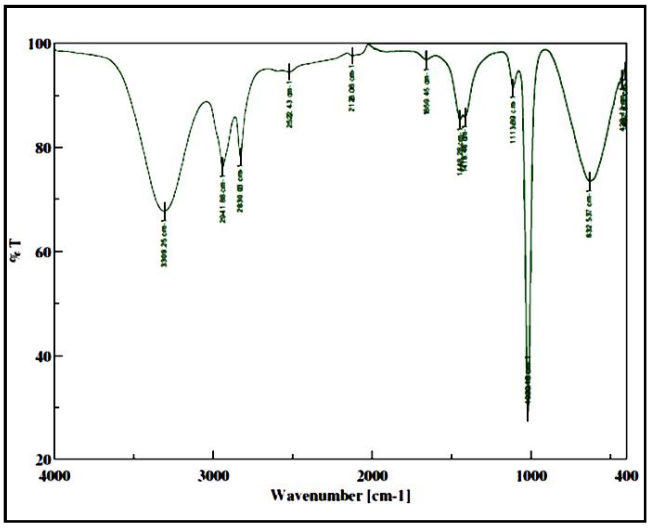

Figure 4: Identification of functional groups of compounds from Ethyl acetate solvent fractions of *G. lucidum* mycelial culture filtrates.

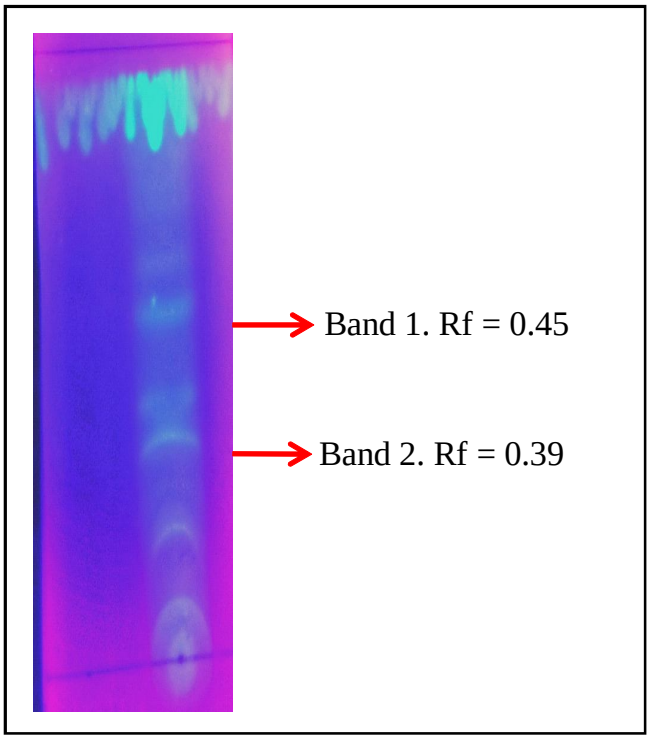

Plate 5a: Detection of antimicrobial compounds from ethyl acetate extracts of *G. lucidum* culture filtrates through TLC.

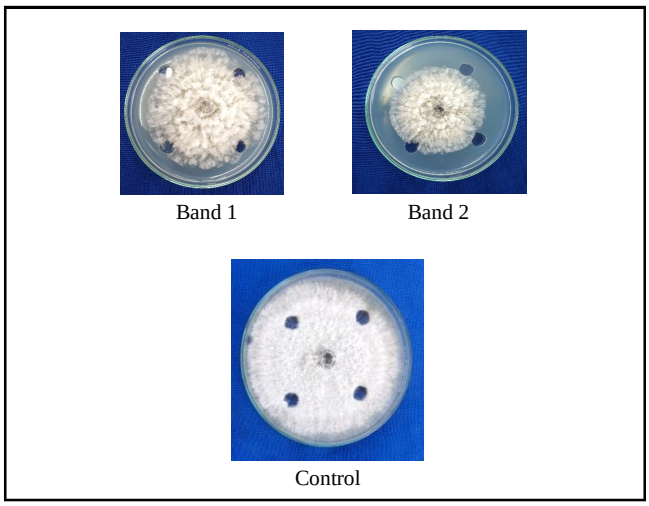

Plate 5b: Antimicrobial activity of compound eluted from TLC

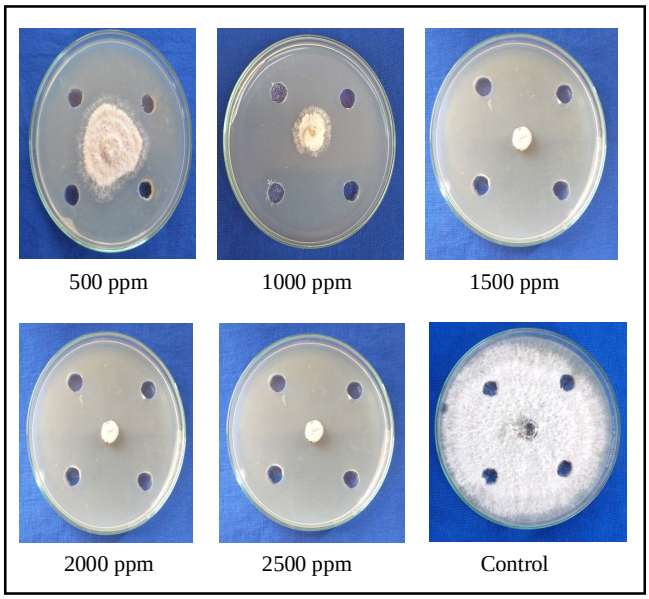

Figure 6: Testing the antifungal efficacy of papaverine at different concentrations against mycelial growth of *C. gloeosporioides* by agar well diffusion assay.

3.6.1 Testing the effect of papaverine (Standard sample) at an optimum concentration (1500 ppm) on conidial germination of *C. gloeosporioides*

The antifungal activity of papaverine tested against *C. gloeosporioides* by cavity slide method revealed that the papaverine at 1500 ppm effectively reduced the conidial germination of *C. gloeosporioides* after 6 h of incubation, while the untreated conidia showed reasonable germination at this time. After 12 h of incubation in papaverine, the germination of conidia was only 13.77 numbers with a 51.34 per cent reduction in conidial germination per cent whereas that of control conidia was 26.82 in number. After 24 h, the germination of spores incubated in papaverine was only 26.74 with 53.11 germination per cent while germination of the untreated conidia of *C. gloeosporioides* was higher in number (50.34). Therefore, it was concluded that the standard sample of papaverine possesses both antifungal as well as inhibition of spore germination (Table 4).

Table 4: Testing the effect of papaverine (standard) on germination of spores of *C. gloeosporioides*

Time interval	Conidial germination of *C. gloeosporioides* at 1500 ppm		Reduction in germination over Control (%)
	Control (No.)	Treatment (No.)	
6 h	0.00(0.57)	0.00[c](0.57)	0.00
12 h	26.82(31.19)	13.77[b](21.77)	51.34
24 h	50.34(45.19)	26.74[a] (31.12)	53.11
SE(d)	-	0.317	-
CD (*p*=0.05)	–	0.903	-

Values are the mean of four replications.
Means followed by a common letter are not significantly different at 5% level by DMRT.
Values in parenthesis are arcsine transformed values.

4. Discussion

Fungal secondary metabolites are playing major role in inhibiting growth of food borne and clinical pathogens. An understanding on the antifungal metabolites of macrobasiomycetes against plant pathogens is the need of current situation owing to the harmful effects of fungicides. In the present investigation, testing of crude culture filtrates from *G. lucidum* and *A. polytricha* at various periodical intervals 10th, 15th, 20^{th,} and 25thdays against *C. gloeosporioides* indicated that the crude culture filtrate collected on 10thday did not exhibit any antifungal effect against the target pathogen. The culture filtrate extracted from the 20th day showed maximum mycelial inhibition of 36.33 % and 47.11% of *C. gloeosporioides* with respect to *G. lucidum* and *A. polytricha,* respectively which show that maximal secretion of antifungal metabolites takes place on the 20th day. In a similar study, Imtiaj and Lee (2007) analyzed the cell-free culture filtrates of *Cordyceps sobolifera, Pycnoporus cinnabarinus, P. coccineus, Oudemansiella mucida,* and *Sterium ostrea* collected on the 20th day against *Botrytis cinerea, C. gloeosporioides,* and *C. miyabeanus*. The result depicted that the culture filtrate of *O.mucida* showed higher mycelial inhibition of 63.01 per cent in *C. gloeosporioides* while in *B. cinerea*, the culture filtrate of *C. sobolifera* had exhibited a higher mycelial inhibition percentage of 43.48 and in the case of *C. miyabeanus*, the culture filtrate of *O. mucida* showed higher inhibition percentage of 63.01%, followed by *P. coccineus* (59.90 %). Priya *et al.* (2019) reported the antimicrobial activity of crude culture filtrate collected on the 20th day from *L. edodes, G. lucidum, C. sinensis,* and *A. polytricha* against *C. capsici* when tested by agar well diffusion assay. Jeeva and Krishnamoorthy (2018) reported that the cell-free culture filtrate (CFC) of *Coprinopsis sinensis* collected on the 20th day after inoculation showed maximum mycelial inhibition of *Fusarium brachygibbosum* (28.11 per cent), *F. o.* f. sp. *cubense* (39.33), and *F. o.* f. sp. *lycopersici* (31.00 per cent) as compared to other days. Similarly, the cell-free culture filtrates of *C. comatus, L. edodes, Tremella aurantialba,* and *Clitocybe* spp. collected on the 20th day had exhibited good antifungal effects against *Phytophthora capsici* (Chen and Huang, 2010). It is obvious to note the result which indicate that the mushroom fungi *G. lucidum* and *A. polytricha* secreted minimum quantities of bioactive compound in the culture filtrate since the inhibition percentage was only up to 47.11%. On the other hand, this led us to take up a study on the extraction of bioactive antifungal metabolites from the culture filtrate by using various polar and non-polar solvents. Nithya *et al.* (2013) mentioned that the efficacy of antimicrobial activity of secondary metabolites was varied based on the polarity of the solvent. Moreover, the selection of solvent is a crucial factor for the extraction of metabolite as it is based on the chemical nature and dissolving capacity of a specific compound. Based on the polarity, different solvents such as ethyl acetate, diethyl ether, hexane, and chloroform were selected and the effectiveness against the target pathogen was observed by the mycelial inhibition test.The antimicrobial screening of mycelial extracts of *G. lucidum* and *A. polytricha* showed that the inhibition percentage was directly proportional to the higher concentration. However, the ethyl acetate and diethyl ether fraction of G. *lucidum* and *A. polytricha* exhibited maximum inhibition of 68.55 per cent and 58.55 per cent at an increased concentration of 1 per cent which would have extracted more of phenols, terpenoids, enzymes, and proteins. Ethyl acetate performs well in the secondary metabolite extraction because it contains two important chemical and biological characteristics together with medium polarity and minimum toxicity which can help to extract many biologically active compounds (polar and non-polar) as reported by Kumar *et al.* (2013). Moreover, *Ganoderma* spp. found to perform differently based on the solvent used for secondary metabolite extraction. This is evidenced by the findings of (Jonathan and Fasidi, 2003) where they reported that the methanol and ethanol extraction of metabolite performed well against the target pathogen than the water extract. On that basis Fujita *et al.*(2005) reported that the ethanol-based solvent performed better in extracting bioactive compounds and also (Cowan, 1999) stated that more active compounds not soluble in water but highly soluble in low polarity compound which yields effective extraction of bioactive compound. Shahid *et al.* (2016) found that the acetone extract fraction of *G. lucidum* exhibited maximum antifungal activity than a methanolic fraction as evidenced by inhibition against *Fusarium oxysporum* (64%) and 57% inhibition against *Alternaria alternata* at 20% concentration. Stojkoviæ *et al.* (2013) indicated that the ethanolic extract of *C. comatus* showed antifungal activity against *Aspergillus fumigatus, A.ochraceus, A.versicolor Trichoderma viride, Penicillium funiculosum, P. ochrochloron,* and *P. verrucosum* var. *cyclopium.* Similar to our results, Radhajeyalakshmi *et al,*(2010) extracted of antimicrobial compound from *A. polytricha, L. edodes* and *V. volvacea* using chloroform, ethyl acetate, ether and methanol against plant pathogenic fungus such as *Alterneria solani, Rhizoctonia solani, C. capsici* and *Pythium aphanidermatum* and the reported that ethyl acetate was the best solvent with a maximum inhibition of 45 mm against the targeted pathogen.

In this study, the ethyl acetate solvent extracts from mycelial culture filtrates of *G. lucidum* subjected to GC-MS for identification of bioactive compounds revealed that the presence of various compounds, and among them papaverine recorded the highest peak area of about 2.13% with a probability of 90.74 per cent at RT 26.68. Furthermore, papaverine is known to possess antiviral activity and is used in human medicine (www.ncbi.com). The peak in the spectrum of the FT-IR analysis revealed the presence of different functional groups, *viz.*, aliphatic primary amine, amine salt, thiol, amide, conjugated ketone, alkane, alcohol which is an indication for the existence of a variety of potential biomolecules in mycelial culture filtrates of *G. lucidum*. Similar to this study, Elumba *et al*. (2013) carried out the FT-IR analysis of the solvent fraction of *G. lucidum* revealed the presence of various functional groups include primary amine, amine salt, thiol, ketone, alkane, and alcoholic group. Further, in our study, the TLC analysis for the ethyl acetate solvent extracts of mycelium of *G. lucidum* indicated the appearance of two bright bands with R_f value 0.45 and 0.29 and both the band 1 and 2 eluted from the TLC plate showed the inhibition of mycelial growth of *C. gloeosporioides* in the range of 36.66 per cent and 40.50 per cent, respectively. Similarly, Priya (2019) carried the TLC analysis for the chloroform fraction of *G. lucidum* revealed the presence of a band with R*f* value 0.27 that had antifungal activity against *C.capsici*, fruit rot pathogen of chilli.

The compound papaverine is a benzylisoquinoline alkaloid that is reported to have strong antimicrobial activity and also act against viral pathogens in humans (Aiyegoro and Okoh, 2009), but it is so far not exploited against fungal pathogens. The standard sample of papaverine tested against *C. gloeosporioides* by agar well diffusion technique at different concentrations (500 to 3500 ppm) indicated that the 1500 ppm concentration was the minimum concentration required for the complete inhibition of mycelial growth of *C. gloeosporioides*. Papaverine induced different monocultural changes including breakage of the mycelial disc, dense mycelial growth at the center of the plate, mycelial malformation, formation of the thin mycelial pattern, distortion, and restricted growth of mycelium and. also, the conidial germination inhibition was tested at different hours intervals (from 6 h to 24 h interval) by cavity slide method and the result showed that although the conidia germinated at 24 h of incubation the germination percentage was significantly reduced to 53.11 per cent when compared to control. Probably, this is the first report of papaverine exhibiting antifungal activity against *C. gloesporioides*.

5. Conclusion

Macrobasidiomycetes contain ample bioactive compounds which needs to be properly tapped and identified for the management of plant diseases. The export of mangoes are greatly affected due to anthracnose disease that deteriorates the fruit qulaity. Also, fungicide treated mangoes are not preferred in the international market so an alternate ecofriendly strategy identification of green chemicals will be the only solution in future. In this context, from the present study, it is well proven that for *G. lucidum* secretes secondary metabo-lites that possess antifungal compound papaverine that could be used further to develop fungicidal formulations for the management of *C. gloeosporioides* of Mango.

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Conflict of interest

The authors declare that there is no conflicts of interest relevant to this article.

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