The Pharma Innovation Journal 2021; 10(12): 1421-1427

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(12): 1421-1427

www.thepharmajournal.com
Received: 01-09-2021
Accepted: 07-10-2021

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In vitro antifungal prospects of some Macrobasdiomycetes against *Fusarium oxysporum* infecting Cucumber

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Abstract
Cucumber (*Cucumis sativus* L.) family Cucurbitaceae is one of the vital economical crops of Asia and Africa. Cucumber plants are affected by various diseases; among this wilt incited by *Fusarium oxysporum* was found to be more predominant. The wilt-infected plant initially exhibited yellowing of leaves, wilting of plants, and vascular discoloration of the stem. The present study is framed to make use of the antifungal potential of *Ganoderma lucidum*, *Auricularia polytricha*, *Lentinus edodes*, *Schizophyllum commune*, *Trametes versicolor*, and *Auricularia auricula* against *Fusarium oxysporum* causing wilt disease of cucumber. Results of the dual plate technique revealed that the *Lentinus edodes*, *Schizophyllum commune*, and *Ganoderma lucidum* showed inhibition of radial mycelial growth of *F. oxysporum* with (55.00%, 50.33%, and 47.78% respectively). Although the crude filtrates of mushrooms were screened at different day intervals *viz.*, 10, 15, 20, 25, and 30 d. Among these, the crude extract at 20th day exhibited maximum inhibition of 54.07%, 52.96%, and 47.03% of mycelial growth of *F. oxysporum* by *L. edodes*, G. *lucidum*, and *S. commune*. Hence, further study towards the identification of diverse antifungal compounds will pave for the development of bio fungicide against plant diseases.

Keywords: Crude filtrate, dual plate, percent inhibition, and antifungal compounds

Introduction
Cucumber (*Cucumis sativus* L.) family Cucurbitaceae is one of the vital economical crops of Asia and Africa (Berke, 2002) [11]. Cucumber is native to tropical regions of Southern Asia, having been cultivated in India for over 3,000 years. India is one of the traditional producers of cucumber, the area under cucumber and gherkins cultivation was reported to be Cucumber is grown either in an open field or under-protected cultivation to safeguard from pests, diseases, and adverse growth conditions (Hanam *et al*., 1978) [19]. Cucumber plants are affected by several fungal diseases *viz*., downy mildew, powdery mildew, gray mold, root rot, white mold, Fusarium wilt, gummy stem blight, anthracnose, and black rot. Among these, soil-borne pathogen Fusarium is a major constraint that infects the plant at different growth stages and causes severe yield loss (Alymanesh *et al*., 2009 and Chehri *et al*., 2011) [3, 15]. Typical symptoms of wilt infection were observed in older plants and caused yellowing, stunting, and wilting. Once wilting occurred in the plant's death could result in 3 to 5 days. Affected plants might develop lesions on the lower stem and the fungus affecting the vascular system which resulted in browning. (Boughalleb *et al*., 2005) [12]. Continuous application of chemical pesticides and fungicides against pathogen cause environmental pollution, undesirable side effects on non-target organisms, residues in soil, and contamination of the water and food chains. Alternatively, several research scientists look forward through biologically active compounds produced by macro basidiomycetous to overcome the current situation. On this basis, mushrooms are known to produce various potent biologically active compounds like chitin, chitosan, terpenoids, glycoprotein, and other secondary metabolites (Arnone *et al*., 1997) [4]. These compounds are known to exhibit antifungal, antimicrobial, antibacterial, antiviral, antitumor, anti-nemic, anti-inflammatory, anti-allergic, anti-atherogenic, anti-diabetic properties (Cao *et al*., 2003; Imitaj and Lee 2007; Han *et al*., 2008; Aida *et al*., 2009; Akyuz *et al*., 2010 and Balakumar *et al*., 2011) [13, 21, 18, 1, 2, 9]. Owing to the current emphasis on the eco-friendly approaches for plant disease management, mushroom fungi can serve as a promising source of antimicrobials against plant pathogens as evidenced by the antimicrobial compound strobilurins and oudemansins from mushroom fungus were endorsed in wood-inhabiting fungi,

Strobilurus tenacellus and *Oudemansiella mucida* which is effective against powdery mildew and downy mildew of grapes (Bartett *et al.,* 2001) [10]. In the same way, Jeeva and Krishnamoorthy (2018) [22] reported that the antimicrobial activity of the culture filtrates of *Coprinus comatus* against *F. oxysporum* f. sp. *brachygibbosum*, *F. oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *cubense*.

Materials and Methods

Collection, Isolation, and characterization of the pathogen

Collection and Isolation

Fusarium infected cucumber plants were collected from kariyamangalam, bandaravalli, adilam, irumathur areas of Dharmapuri district. For tissue isolation, the infected root portion along with healthy roots was cut into small pieces and surface sterilized with 1% sodium hypochlorite for 1 min and subsequently, three washings were given with sterile distilled water and air-dried. Then, they were placed in sterilized Petri dishes containing potato dextrose agar (PDA) medium by half-plate technique and incubated at the laboratory conditions at 25 ± 2ºC. The hyphal tips of fungi growing from these pieces were transferred aseptically to PDA slants and stored in refrigerated conditions for further studies. (Rangaswamy, 1972)

Morphological Characterization of Pathogen

Pure culture maintained on PDA medium was used for studying colony characters, color, growth rate, shape, and size of macroconidia, microconidia, chlamydospores, and mycelial characters of the pathogen were also observed based on Hussain *et al*., (2012) [20].

Pathogenicity test

The fungus isolated was multiplied in sand maize medium. Sand and ground maize seeds were mixed at the ratio of 19:1 respectively. The mixture was moisture with water and sterilized at 121 ºC at 15 psi for 2 h. The fungus was inoculated into sand maize medium and incubated for 15 days at room temperature (28º±2 ºC) for multiplication. Potting soil (Red soil: sand: cow dung manure @ 1:1:1 w/w/w) was sterilized in an autoclave at 121ºC at 15 psi for 2 h for consecutive days. The different fungi multiplied on sand maize medium were incorporated separately into the sterilized soil at the rate of 5% (w/w). Cucumber seeds were sown at the rate of 10 seeds/pot and maintained under glasshouse conditions. Three replications were maintained for each isolate and monitored regularly. Symptoms developed were observed 30 days after sowing for wilt. From the infected plants, the pathogen was reisolated and Koch's postulates were proved. Highly virulent isolates of pathogen obtained from infected seedlings were used for further studies

Molecular characterization of the pathogen

Genomic DNA was extracted from the suspension culture of *Fusarium* spp by the CTAB method (Chehri *et al.,* 2011 and Li *et al*., 2012) [15, 23]. The ITS (Internal transcribed spacer) regions of *Fusarium* spp were amplified with oligonucleotide primers ITS 1 - 5'- TCCGTAGGTGAACCTGCGC 3'and ITS 4 - 5' - TCCTCCGCTTATTGATATGC 3' (White *et al*., 1990). Amplification was performed in 20µl of reaction mixture containing 10 µl PCR master mix, 2 µl of forward primer and 2 µl reverse primer, DNA 4 µl and 2 µl of sterile water. The mixture was subjected to PCR in a Thermo cycler (BIORAD). The PCR cycle was performed with the initial denaturation step at 94º C for 5 min, 35 cycles of amplification denaturation 94ºC for 1 min, annealing ration step at 55ºC for 30 s annealing, and 72ºC for 30 s extension, a final extension was carried out at 72ºC for 10 min. The amplified PCR products were separated by electrophoresis (Genei Maxi Sub System 03-04, Genei, Bangalore, India) on 1.5 percent agarose gel using TAE buffer at 80 V constant current for 1h. The gel was stained with ethidium bromide, visualized on a UV-trans illuminator, and photographed in the gel documentation unit (Alpha Infotech Corp, USA). The sizes of the PCR products were determined in comparison with standard 100 bp molecular marker (Genei Pvt. Ltd., Bangalore, India).

In vitro screening of antagonistic potential of some macro basidiomycetes against *Fusarium oxysporum*

Dual plate assay was conducted to evaluate *in vitro* antagonistic potential of macro basidiomycetes *viz*., *Ganoderma lucidum*, *Auricularia polytricha*, *Lentinus edodes*, *Schizophyllum commune*, *Trametes versicolor*, and *Auricularia auricula* were screened against *Fusarium oxysporum* (Dennis and Webster, 1971) [16]. The PDA medium was prepared and poured in a sterile Petri plate. A 9 mm mycelial disc of the antagonist was placed at the one edge of the Petri dish. On the opposite side, a 9 mm mycelial disc of the targeted pathogen was placed. The pathogen grown separately in monoculture served as control. The dual culture plates were incubated at 28 ± 2 °C for 7 d. Three replications were maintained for each treatment. Periodical observation of mycelial growth pattern towards the pathogen was taken. The percent inhibition was calculated by using the formula proposed by (Vincent, 1947) [26].

Percent inhibition of growth (PI) = C – T / C x 100 Where C is the growth of the pathogen in control (mm) and T is the growth of the pathogen in treatment (mm).

Table 1: Antagonistic ability of fungal organism was determined by the type of interaction

Type of interaction	Category	Antagonist index
Overgrowth of the pathogen over mushroom	A0	0.0
Mutual inhibition, in which neither of the organism was able to overgrow the other at mycelial contact	B	1.0
Color and boundary of fungal mycelium change at mycelium contact	B1	1.5
Presence of pseudosclerotial line at mycelial contact	B2	2.0
Mutual inhibition, in which neither of the organism was able to overgrow the other at a mycelial distance	C	2.5
Presence of pseudosclerotial line at a mycelial distance	C1	3.0
Overgrow of mushroom without initial deadlock	D	3.5
Partial replacement after initial deadlock with mycelial contact	E	4.0
Complete replacement after initial deadlock with mycelial contact	E1	4.5
Partial replacement after an initial deadlock at a distance	E2	5.0
Complete replacement after initial dead block at a distance	E3	5.5

Extraction and bioassay of antifungal molecules from *L. edodes* against *Fusarium oxysporum*

A 90mm mycelial disc of *L. edodes*, *G. lucidum*, and *S. commune* were inoculated in 250ml conical flask containing 100 ml sterilized Mushroom complete Broth (Glucose 20 g, MgSO4·7H2O 0.5 g, KH2PO4 0.46 g, K2HPO4 1.0 g, yeast extract 2 g, peptone 2 g, and water 1000 ml). The flasks were placed in an incubator cum shaker maintained at 25 °C and agitated at 120 rpm. The culture filtrates were collected from 10,15,20,25 days after incubation. The mycelial mat and crude filtrate were separated using Whatman no 1 filter paper. Consequently, the metabolites were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered through a membrane filter (0.2 µm) under the aseptic condition to eschew the bacterial contamination. Finally, the collected crude metabolites were tested against *F. oxysporum* for its antagonistic activity by poisoned food technique (Zentmeyer, 1955). A 100µl of crude metabolites were mixed with 100 ml of sterilized PDA medium was poured into a sterile Petri plate and allowed to solidify. At the center of the poisoned medium, a 9 mm mycelial disc of the target pathogen was placed and the Petri dish was incubated at 28 ± 2°C. A constant observation was taken and the percent inhibition of mycelial growth was calculated by using the formula as proposed by Vincent (1947) [26].

Result and Discussion

Isolation and Identification of Pathogen

Fusarium wilt (*F. oxysporum*) was isolated from infected cucumber plant showing symptoms of yellowing of leaves, wilting of plants, and vascular discoloration of the stem (Plate 1a). The cultural character on PDA medium initially showed white mycelium submerged growth and yellowish color developed underneath of the plate and the color change of the medium was also observed from white to light yellow (Table 2 and Plate 1b). Microscopic structures like macroconidia, microconidia, and chlamydospores were observed from ten-day-old cultures. Macroconidia were hyaline, sickle-shaped with 3-4 septations and size of 9.08 to 11.56 µm (length) and 3.67 - 4.89 µm (width). Micro conidia were oval, hyaline, single, or bi-celled and their size ranged from 8.44 - 9.52 µm (length) and 3.89 - 4.50 µm (width). Chlamydospores were produced either on the terminal or intercalary (Plate 2a). Based on this morphological evidence, the pathogen was confirmed as *Fusarium* sp. (Fig 1). The number of microconidia was more as compared to macroconidia. Plentiful chlamydospores were observed terminally and intercalary. Morphological characteristics of *Fusarium* spp. play a crucial role in the early stage for differentiation among various *Fusarium* species. Owing to the current study the morphological studies related to suitable media for growth and sporulation of *Fusarium* spp concurrence with Asma *et al., (*2018). Based on the results *F. oxysporum* showed various cultural and morphological characteristics and the virulence isolate Foc 1 (sample 1) produced white colony and purple pigmentation. In general *F. oxysporum* produces dense aerial mycelium later purple color discoloration was noticed underneath of Petri dish and these findings agree with the work reported by Din *et al.,* (2020) [28].

On this basis, a total of 4 isolates has been taken from different areas of the Dharmapuri district were tested for pathogenicity to assess the virulent isolate. The result revealed that the sample taken from kariamangalam (sample 1) showed the highest disease incidence up to 85.01 percent Wilt infected plants showed the typical symptoms like yellowing, drying of leaves, and showed complete drying and wilting of plants on the 30th day. The virulence of the pathogens was calculated based on PDI (Table 2).

Molecular confirmation of *F. oxysporum* with ITS Primers

To identify the molecular systematics at the species level and even within species ITS region of fungal DNA was used. In this context, the ITS-rDNA region of the virulent fungal isolate was performed using universal primer ITS 1 and ITS 4. The *Fusarium* genus was amplified as a fragment of 550 bp corresponding to the region of the 18S-28S rRNA intervening sequence for *Fusarium* sp (Fig 1). Consequently, virulent isolates of *Fusarium* were sequenced and blasted in NCBI. It showed 98% homology with *Fusarium oxysporum* with the accession number of MZ268142. Hence, the pathogen was identified as *Fusarium*. Sequencing the ITS gene region is effective for identifying some species of *Fusarium* (AL-Taae and Al-Taae, 2019) [6].

In vitro* Antagonistic activity of macro basidiomycetes against *F. oxysporum

In our present study, among the six macro basidiomycetes tested, *Lentinus edodes* followed by *Ganoderma lucidum* and *Schizophyllum commune* showed reduced mycelial growth of *F. oxysporum* (40.50 mm, 44.70 mm, and 47.00 mm respectively) when compared to control (90 mm) with inhibition percent of 55.00, 50.33 and 47.78 respectively. Other mushroom fungi, *Trametes versicolor*, *Auricularia auricula,* and *Auricularia polytricha* also showed the least mycelial growth inhibition against *F. oxysporum* with 35.56 percent, 37.00 percent, and 28.11 percent respectively (Plate 3 and Table 3). Antagonistic interactions between different mushroom fungi and plant pathogenic fungi in dual cultures and various types of competitive interactions *viz.,* overgrowth of the pathogen, inhibition at mycelial contact, inhibition of mycelia at a distance, and partial or complete replacement of plant pathogenic fungi were reported (Badalyan *et al.,* 2002; Priya *et al*., 2019 and Gayathri *et al.,* 2020) [7, 24, 17]. In this context Bandalyan (2002) revealed that the 17 xylotrophic mushrooms were tested for their antagonistic activity against *Bipolaris sorokiniana*, *Fusarium culmorum*, *Gaeumannomyces graminis* var. *tritici*, and *Rhizoctonia cerealis*, results showed that *Pleurotus ostreatus*, *Hypholoma fasciculare*, *Ganoderma lucidum*, *Lentinus tigrinus*, and *Schizophyllum commune* was reported to be strong antagonistic activity against pathogen tested and inhibiting the activity of mushrooms and pathogenic fungi mainly comprised of interactions such as deadlock, consisting in mutual inhibition at a distance or mycelial contact, and replacement, consisting in initial deadlock followed by partial or complete overgrowth. Similarly, the interaction between 17 xylotrophic mushrooms and 4 mycoparasitic fungi was tested for antagonistic activity. Among all mushrooms, *Pleurotus ostreatus*, *Ganoderma* sp., *Flammulina velutipes*, and *Hypholoma fasciculare*, showed the highest competitive ability against mycoparasitic fungi Badalyan *et al.,* (2004) [8]. Gayathri *et al.,* (2020) [17] revealed that the mycelium of *Auricularia polytricha*, *Ganoderma lucidum*, *Pycnoporus sanguineous* and *Pleurotus florida* inhibited the radial mycelial growth and also have strong competitive ability against *Colletotrichum gloeosporioides*. In addition, interactions of mushroom fungi and pathogenic fungi in dual culture perhaps certain metabolite production that might be responsible for retardation of *F. oxysporum*. (Table 4).

Testing the effective crude culture filtrate of macro basidiomycetes against *F. oxysporum*

Based on the result obtained from the previous study *L. edodes*, *G. lucidum*, and *S. commune* were shown to be effective against *F. oxysporum*. On this consequence the crude filtrate of these macro basidiomycetes was collected at various periodical intervals 10th, 15th, 20th, and 25th days were tested to exploit the antimicrobial metabolites against *F. oxysporum*, Results revealed that the crude culture filtrate collected on the 20th day showed maximum mycelial inhibition of 42.21%, 40.11%, and 37.33% of *F. oxysporum* concerning *L. edodes*, *G. lucidum* and *S. commune* respectively (Table 5; Fig 2). The culture filtrate collected on the 10th day did not exhibit any antifungal effect against pathogen tested. However, the crude filtrate of *G. lucidum*, *A. polytricha*, and *L. edodes* collected on the 20th day exhibited maximum mycelial inhibition of *Colletotrichum capsici* (Priya *et al*., 2019) [24]. Our study concurrence with Gayathri *et al*., (2020) [17] reported that the culture filtrate collected from *G. lucidum* and *A. polytricha* on the 20th day exhibited the highest mycelial inhibition of 36.33% and 47.11% of *C. gloeosporioides*. From these findings, crude culture filtrate of macro basidiomycetes could secrete antifungal metabolites on the 20th day and inhibit the mycelial growth of *F. oxysporum.*

Table 2: Morphological and cultural characteristics of *Fusarium* spp.

S. No	Crop/ District	Place of Collection	Color of the culture in Petri dish	Macroconidia		Microconidia		Chlamydospores	Percent disease incidence
				Mean Length	Mean Width	Mean Length	Mean Width		
1	Cucumber Dharmapuri	Kariamangalam (Foc 1)	White mycelium with submerged growth and purple color developed at the back side of the Petri dish	18.65	4.72	13.22	4.31	Intercalary	85.01[a] (67.82)
2		Bandaravalli (Foc 2)	Fussy white mycelium submerged growth and yellowish color developed at the back side of the Petri dish	17.75	4.58	13.39	4.30	Intercalary	54.34[b] (47.49)
3		Adilam (Foc 3)	Submerged dull-white growth	18.52	4.56	13.71	4.29	Terminal and intercalary	48.90[c] (44.36)
4		Irumathur (Foc 4)	Creamy white to yellowish suppressed growth	18.84	5.86	13.03	3.91	Terminal	35.71[d] (36.68)

Table 3: Screening the antagonistic activity of macro basidiomycetes against *F. oxysporum* by dual culture assay

S. No	Treatment	Average radial mycelial growth of *F. oxysporum* (mm)	Percent Inhibition over control	Type of interaction	Antagonist index
1	*Schizophyllum commune*	47.00 (43.71)	47.78[c] (43.71)	B_1CE	11.5
2	*Ganoderma lucidum*	44.70 (42.70)	50.33[b] (45.18)	BC	10.0
3	*Lentinus edodes*	40.50 (39.88)	55.00[a] (47.87)	B_1BC	12.0
4	*Trametes versicolor*	58.00 (50.63)	35.56[e] (36.70)	C	7.5
5	*Auricularia polytricha*	56.70 (48.44)	37.00[d] (37.45)	BC	10.0
6	*Auricularia auricula*	64.70 (53.14)	28.11[f] (32.01)	A_0B	2.0
7	Control	90.00 (71.56)	0.0	-	-
SED		1.106	-	-	-
CD (p=0.05)		2.302	-	-	-
Total Antagonist index					56.0

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

Table 4: Testing the efficacy of crude culture filtrates of *G. lucidum*, *S. commune*, and *L. edodes*

S. No	Day's interval	*G. lucidum*		*S. commune*		*L. edodes*	
		Mean mycelial of the pathogen (mm)	Per cent inhibition over control	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	63.67[d] (52.93)	29.25	68.00[d] (55.58)	24.44	68.00[d] (55.57)	24.44
2	15th day	55.67[c] (48.26)	38.14	59.00[c] (50.18)	34.44	56.00[c] (48.44)	37.77
3	20th day	42.33[a] (40.58)	52.96	47.67[b] (43.66)	47.03	41.33[a] (39.99)	54.07
4	25th day	53.67[bc] (47.10)	40.36	59.33[c] (50.38)	34.07	58.00[c] (49.60)	35.55
5	30th day	70.67[de] (57.41)	21.47	78.33[e] (62.33)	12.96	66.00[d] (54.39)	26.00

6	Control	90.00[f] (71.56)	0.0	90.00 (71.56)	0.0	90.00[f] (71.56)	0.0
7	SE(d)	2.785	-	2.361	-	2.066	-
8	CD (p=0.05)	6.205	-	5.260	-	4.191	-

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.

Table 5: Interaction of macro basidiomycetes and *F. oxysporum*

Macro basidiomycetes with *F. oxysporum*	Nature of Interaction
Auricularia auriculata	There is no inhibition zone was observed at the zonal point and both mushroom fungi and pathogen were mutually inhibited
Lentinus edodes	An inhibition zone was formed and the; both mushroom and pathogen did not grow each other
Trametes vesicolor	A prominent Inhibition zone was observed.
Schizophyllum commune	A thick mat of hyphae was formed at the zonal point and later pathogen gets hyper parasitized by the mushroom fungi
Auricularia polytricha	A slight inhibition zone was observed at the contact point
Ganoderma lucidum	A clear inhibition zone was observed at the contact point; both mushroom fungi and pathogen did not grow each other

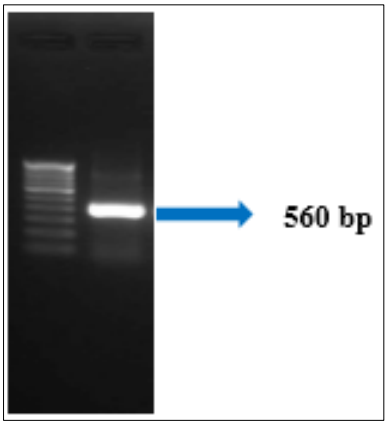

Fig 1: Molecular Characterization of *Fusarium oxysporum*

Plate 1a: Symptoms of Fusarial wilt

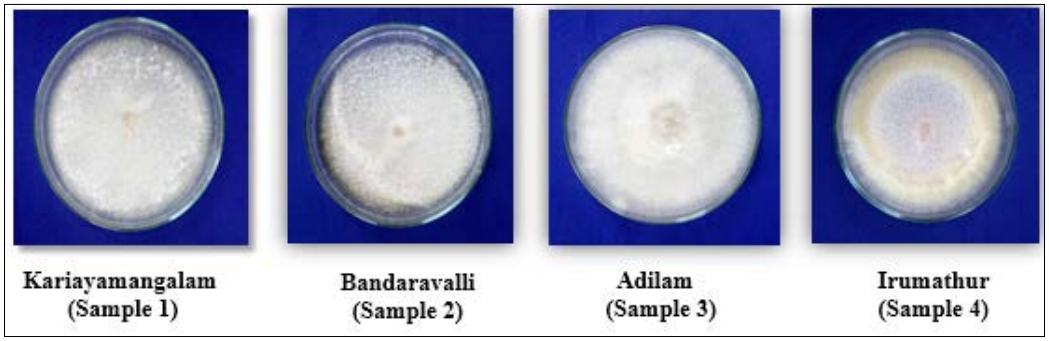

Plate 1b: Collection of isolates

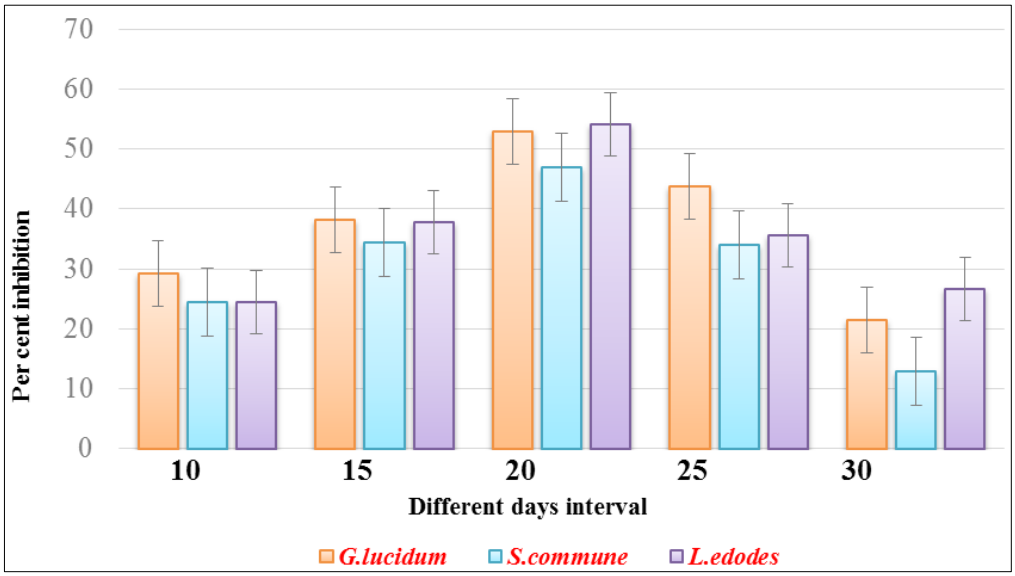

Fig 2: Testing the efficacy of crude culture filtrates of *G. lucidum*, *S. commune*, and *L. edodes*

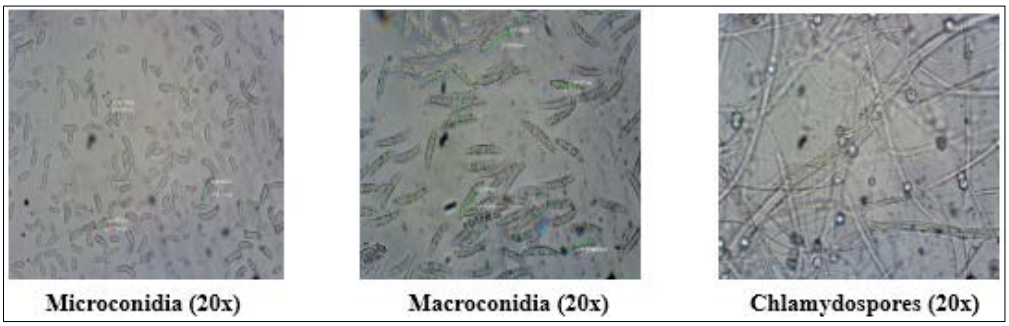

Plate 2a: Morphological character *of Fusarium oxysporum*

Plate 3: *In vitro* efficacy of macro basidiomycetes against *F. oxysporum* by dual plate technique

Conclusion

Finally, it is concluded that the antagonistic activity of macro basidiomycetes by dual culture assay and bioassay of crude metabolites indicated the presence of antimicrobial compounds. Such crude metabolites were subjected to GCMS for identification of the compounds.

Acknowledgment

The author thanks the University for providing facilities through the scheme UGC-SAP DRS1 on Mushroom biotechnology to carry out the research work.

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