Original Article

In-vitro Evaluation of Antifungal Activity of *Ganoderma lucidum* Against the Biofilm Producing *Candida* species

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ABSTRACT

Background: Bioproducts of mushrooms *Ganoderma lucidum* have multi beneficial effects for human welfare. **Objectives:** The aim of the present study was to evaluate antifungal activity of *Ganoderma lucidum* against the biofilm producing fungi. In this current study it carries 100 *Candida* species which forms biofilms were collected from the Immunocompromised and Immuno Suppressive patients. **Methods:** The fruit bodies of *Ganoderma lucidum* were collected and done the extraction at various concentrations with the help of Dimethyl sulfoxide (DMSO) and Methanol. The various concentrations of the extracts were applied by Proteinase and phospholipase enzyme secretion assay, Minimum Inhibitory Concentration (MIC) - XTT Assay and Minimal Fungicidal Concentration (MFC). **Results:** Our results showed the MIC and MFC of DMSO extract were shows highest significant value ($P<0.05$) against the antifungal activity than the methanolic extract of the *Ganoderma lucidum* among the various biofilm producing candida species. **Conclusion:** So we can be conclude that DMSO extract of the *Ganoderma lucidum* shows high significant factor against the various disease causing Candida species and its biofilm.

Key words: *Ganoderma lucidum*, Biofilms, Candida species, Proteinase, Phospholipase, DMSO and Methanol extracts.

INTRODUCTION

Edible Medicinal mushrooms have an established history of use in traditional oriental therapies and new antimicrobial drugs.[1,2,3] Modern clinical practice in Japan, China, Korea, and other Asian countries continues to relay on medicinal mushroom *Ganoderma lucidum* derived preparations.[4,5] One interesting aspect of its performance is antimicrobial effect due to the extracts derived from this mushroom which contain bacteriolytic enzyme, lysozyme and acid protease.[6] There are available in literature some studies reporting antimicrobial activity of different extracts of *Ganoderma lucidum*.[7,8,9] Immunocompromised and Immunosuppressive patients are prone to get secondary infections in that the main part belongs to the fungal infections. Fungi most commonly associated with such disease episodes are in the genus Candida, most notably *Candida albicans*, which causes both superficial and systemic disease. Non albican candida (NAC) like *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida kefyr*, etc are also causes various infections like Urinary tract infections, Vaginitis, Oropharyngeal infections, Onychomycosis, Candidemia, and other device related infections.[10,11,12] Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40%.[13] Nutrients, quorum-sens-

Submission Date: 13-04-2017;
Revision Date: 01-06-2017;
Accepted Date: 22-07-2017

DOI: 10.5530/ijper.51.4s.91

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ing molecules and surface contact are contributory factors of *C. albicans* (SC5314) are comprised primarily of yeast-form and hyphal cells, both of which are required for biofilm formation.[14] Biofilms are studied in a wide range of scientific disciplines including biomedicine.[15,16] Biofilm formation is also critical in the development of denture stomatitis, a superficial form of candidiasis that affects 65% of edentulous individuals.[17,18] These clinical observations emphasize the importance of biofilm formation to both superficial and systemic candidiasis and the inability of current antifungal therapy to cure such diseases. Estimates suggest that up to 80% of all microorganisms in the environment exist in biofilm communities.[19] Current treatments for *Candida species* infection consist of topical and systemic pharmaceutical antifungal agents, with triazoles being the first line of defense are effective.[20] The Proteinase and phospholipase enzyme are the virulence factors causes the cell damage of the host.[21] The present *in vitro* study screens that the modifications among those enzymes which causes by the antifungal activity of the Ganoderma extracts. Furthermore the MIC and MFC has been performed to screen the level of the growth inhibition of biofilm candida species. Therefore, this study aims to primarily evaluate the *in vitro* action of the antifungal activity of DMSO and Methanol extracts of the *Ganoderma lucidum* against *Candida species* which can produce the biofilms.[22]

MATERIALS AND METHODS

Sample Collection

About 162 samples were collected and screened for the candida infections have been illustrated in Table 1. Among 162 samples 100 isolates were screened and identified various Candida species in this present study. All the samples were collected from the both the immunocompromised and immunosuppressed patients who were admitted in the Sri Lakshmi Narayana Institute of Medical Science, Pondicherry. The isolates sample distributions were explained in the Table 2. All the isolates were grown in various media like Sabouraud dextrose agar, Chrom agar, Potato dextrose agar to get pure culture of the various candida species. All the isolates were speciated by screening of various tests like germ tube, carbohydrate assimilation test, Chlamydospore formation, Sugar fermentation, etc. About 09 candida species (*Candida albicans, Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida kefyr*, *Candida guilliermondi*, *Candida lusitaniae* and *Candida dubliniensis*) were isolated, speciated and treated against the crude extract of *Ganoderma lucidum* to screen antifungal activity.

Collection of *Ganoderma lucidum*

Ganoderma lucidum mushroom were collected from the field near Aurovile, MKV Organics, Puducherry. Taxonomy identification by their physical characteristics of the mushroom was done based on the Sporocarps and Basidiocarps.[23,24,25] Specimens collected from above mentioned place were brought to laboratory and rinsed with distilled water. Furthermore, dried under heater fan at 40°C for 2 hours and then preserved in freezer at -200°C to use for further processing. Ganoderma species was identified using keys provided.[26]

Powderization

The basidiocarps were cut in to small pieces, dried at 40°C for 48 hours and powdered. In each step, the fine product was dried to remove moisture and overcome the fungal contamination. The air-dried powder was stored in an air tight container for further use.[27]

Extract Preparation

The powder of dried *Ganoderma lucidum* was used for the preparation of mushroom extract. For the dimethyl sulfoxide (DMSO) extraction 50 g of mushroom powder were mixed with 100 ml of DMSO and placed on a shaker for 24 h at room temperature. The solution was filtered with 3M™ 740 Cartridge and then placed on the rotary evaporator vacuum, for 15 min at 37°C. Then the residue was stored at 4°C for further analysis. The methanolic extract was performed through Soxhlet apparatus of the experimental samples were prepared by adding 50 g of mushroom powder was subjected for extraction using of 100 ml of Methanol at room temperature for 24 hrs and filtered through Whatman No. 4 filter paper. The extracts were recovered by filtration and kept at 40°C in a rotary vacuum evaporator.[28] the residue was collected and store at 4°C for further use. The crude extracts were dissolved in both DMSO and Methanol to get various concentrations (150 μg/ml, 200 μg/ml, 250 μg/ml, 300 μg/ml and 350 μg/ml).

Screening of antifungal activity of mushroom extract

The antimicrobial activities of the extracts were determined by the Kirby-Bauer broth dilution method according to NCCLS standards.[29,30] The fungal cultures were incubated at room temperature for 48 hours in CHROM agar for the presumptive identification of several *Candida* species by using color reactions. For control *Candida albicans* ATCC SC5314 bioflim producer was used for antifungal activity.

Screening Proteinase and phospholipase enzyme secretion assay

Biofilms of Candida species were grown for 24 hrs in Yeast Nitrogen Base Medium (YNB) with 50 mM of glucose at 37°C in 5% CO_2 and treated with Ganoderma crude extract concentrations of 150 μg/ml - 350 μg/ml. Trypsin (for proteinase assay) and Phospholipase A2 (for phospholipase assay) were used to perform the assay. The control used in this assay was 1% ethanol. After 72 hrs of biofilm maturation, the enzyme secretion assays were performed on biofilms suspended in Phosphate Buffer Saline (PBS). The proteinase enzyme activity was determined by mixing the supernatant of the biofilm solution with 1% azocasein at 1 h at 37°C in 5% CO_2. Then, 500 ml of 10% trichloroacetic acid was added to stop the reaction. The solution was centrifuged for 5 min at 10,000 rpm and 500 ml of the supernatant was combined with 500 ml of NaOH, which was incubated for 15 min at 37°C in 5% CO_2. Absorbance was read in a spectrophotometer at 440 nm.[31,32,33] The phospholipase enzyme activity was determined by mixing the supernatant of the biofilm solution with phosphatidylcholine substrate for 1 hr at 37°C in 5% CO_2 and reading the absorbance in a spectrophotometer at 630 nm.[34]

Screening of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was performed in the XTT reduction assay.[35,36] Biofilms were produced on commercially available presterilized, polystyrene, flat-bottom 96-well microtiter plates. 100 μL of C. *albicans* and other species of candida cell suspension was transferred into each well of a microtiter plate which contains Roswell Park Memorial Institute (RPMI) 1640 media, and the plate was incubated for 2 hours at 37°C in a shaker at 75 rpm to allow for adherence to the surface of the wells. Three wells of each microtiter plate were used as control in an identical fashion, except that no *Candida* suspensions were added. Following the adhesion phase, the cell suspensions were aspirated and each well was washed twice with 150 μL of Phosphate Buffer Saline (PBS) to remove loosely adherent cells. A total of 100 μL of RPMI 1640 was then transferred into each of the washed wells with a pipette, and the plates were incubated at 37°C in a shaker at 75 rpm. After the incubation media has been aspirated and both the extracts have been added separately of various concentrations 150 μg/ml – 350 μg/ml has mentioned earlier. The plate was incubated for 24 h at 37°C in 5% CO_2.[37] After the supernatant aspiration each well of 100 μl of XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) added and incubate in the dark area 2 – 3hrs at 37°C.[38,39] The visual inspection of the plates will typically demonstrate a gradient of orange color. Further read the plate in a microtiter plate reader at 490 nm. From the resulting colorimetric readings the fungal isolates and it OD Value has been calculated.

Minimal Fungicidal Concentration (MFC)

Minimum fungicidal concentration (MFC) was done through micro dilution method. The content of each well containing various concentrations of extracts corresponding to read after 72 h.[40] By sub culturing 20 μl of each well on Sabouraud Dextrose Agar and also in the Chrom agar after 24 h of incubation at 27°C, MFC concentration was determined as the lowest concentration of Ganoderma Extracts, showing no visible *C. albicans* growth on the agar plates.[41]

Data calculation

Treated (*T*) and control (*C*) Petri dishes were measured diametrically in three different directions till the fungal growth in the control dishes was nearly far-reaching. The percentage of growth inhibition (*I*) was calculated using the formula.[42]

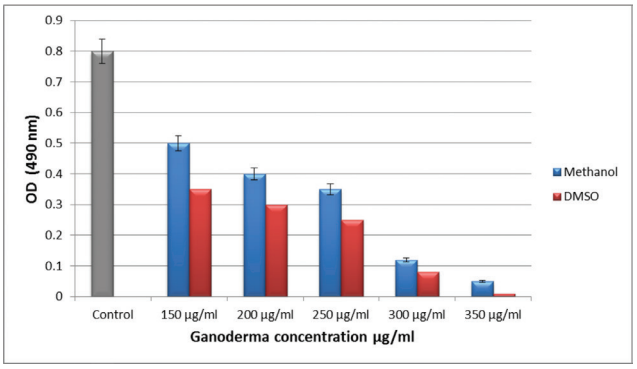
$$I\,(\%) = [(C - T)/C] \times 100$$

RESULT AND DISCUSSION

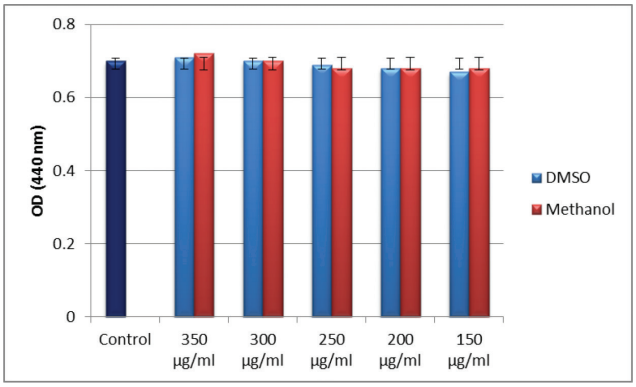
The present study tested the antifungal activity of crude extracts and their respective dilutions from medicinal mushroom belonging to *Ganoderma lucidum*. The medicinal mushroom was chosen based on either traditional usage, suggestive of antifungal activity. The incidence of *Candida albicans* and other species isolated from the immune compromised and immune suppressed patients has been reported to be 23% of patients with undergoing chemotherapy, 35% in diabetic patients and 42% of patients with HIV. Candida species plays a most common cause of nosocomial bloodstream infections. Patients who are critically ill and in medical and surgical ICUs have been the prime targets for opportunistic nosocomial fungal infections, primarily due to *Candida* species.

Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were established for DMSO and Methanolic extracts from each of the 09 species. From the total extracts evaluated by the growth inhibition percentage formula, 82 (82%) isolates were showed fungistatic activities and 78 (78%) isolates were showed fungicidal activity. The MIC values ranged from 150 to 250 μg/ml and MFCs values ranged from 150 to 200 μg/ml of the both the extracts. DMSO extracts of

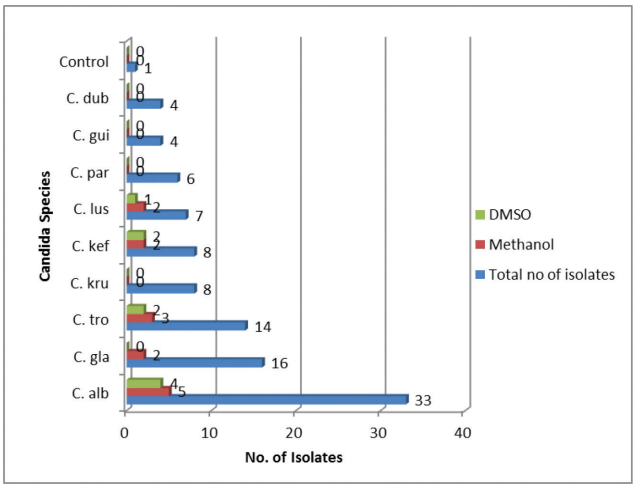
Table 1: Age and Sex Distribution of the Patients.				
S. No	Age Distribution of the Patients	Sex Distribution of the Patients		Total
		Male	Female	
1	≤ 10	17	14	31
2	11 - 20	8	5	13
3	21 - 30	9	12	21
4	31 - 40	8	15	23
5	41 - 50	10	7	17
6	51 - 60	9	8	17
7	≥ 60	22	18	40
Total		83	79	162



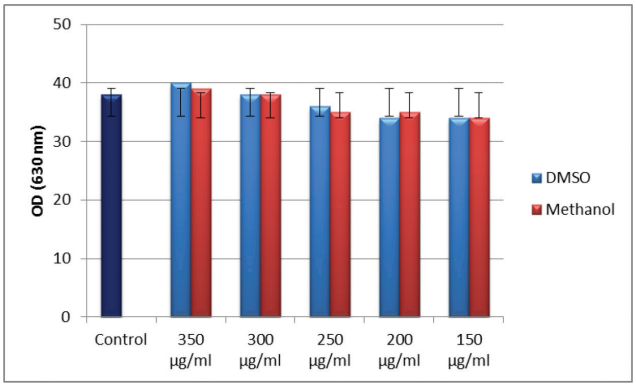
Bar Diagram: 1 – MIC (XTT Bioflim Reduction Assay).



Bar Diagram: 3 Proteinase enzyme secretion assay.



Bar Diagram: 2 MFC No. of Organisms grown.



Bar Diagram: 4 Phospholipase enzyme secretion assay.

Candida Species had the highest and significant value of MIC (150 µg/ml), while methanolic extracts of *Candida Species* had the highest MIC (250 µg/ml). The MIC of *C. tropicalis*, *C. kefyr*, *C. guilliermondii* and *C. dubliniensis* were 150, 200, 250 µg/ml, respectively, and these species whose two extracts showed fungistatic activity in all the concentrations, details are mentioned in the Table 3 and Bar diagram-1. The MFC of *C. krusei, C. parapsilosis, C. guilliermondii*, and *C. dubliniensis* and these species whose two extracts showed fungicidal activity in all the concentrations, datas are explained in the Table 4 and bar diagram-2. XTT assay for the bioflims production and to screen the antifungal activity of various crude extracts. The OD value of the MIC shows significant value in the lowest concentration of the various candida species. Both the extracts of the Ganoderma crude extract given various significant values. But the ratio of the both fungistatic and fungicidal

Table 2: *Candida* spp. Isolated from various clinical specimens.								
	Candida spp.	Urine	Oropharyngeal swab	Vaginal swab	Blood	CSF	Miscellaneous	Total and %
	C. albicans	12	04	05	04	02	06	33
	C. glabrata	05	03	02	03	02	01	16
	C. tropicalis	04	02	05	02	00	01	14
	C. krusei	03	03	00	00	00	02	08
	C. kefyr	02	02	04	00	00	00	08
	C. lusitaniae	01	02	00	02	00	02	07
	C. parapsilosis	02	03	00	00	00	01	06
	C. guilliermondii	01	01	02	00	00	00	04
	C. dubliniensis	00	02	02	00	00	00	04
Total		**30**	**22**	**20**	**11**	**04**	**13**	**100**

Table 3: Minimal inhibitory concentration (MIC) of crude extract from *Ganoderma lucidum* against *Candida sp.*		
Candida Species	**MIC no of isolates**	
	DMSO Extract in μg/ml 350 300 250 200 150	**Methanolic Extract in μg/ml 350 300 250 200 150**
C. albicans	+ + + + 02	+ + + 02 02
C. glabrata	+ + + + +	+ + + + 01
C. tropicalis	+ + + + +	+ + + + +
C. krusei	+ + + + 02	+ + 01 01 0
C. kefyr	+ + + + +	+ + + + +
C. lusitaniae	+ + + + 02	+ + + + 03
C. parapsilosis	+ + + + +	+ + + + 01
C. guilliermondii	+ + + + +	+ + + + +
C. dubliniensis	+ + + + +	+ + + + +

+; No OD Value

Table 4: Minimum fungicidal concentration (MFC) of crude extract from *Ganoderma lucidum* against *Candida sp.*		
Candida Species	**MFC no of isolates**	
	DMSO Extract in μg/ml 350 300 250 200 150	**Methanolic Extract in μg/ml 350 300 250 200 150**
C. albicans	Ng Ng Ng 01 03	Ng Ng Ng 02 03
C. glabrata	Ng Ng Ng Ng Ng	Ng Ng Ng Ng 02
C. tropicalis	Ng Ng Ng Ng 02	Ng Ng Ng 01 02
C. krusei	Ng Ng Ng Ng Ng	Ng Ng Ng Ng Ng
C. kefyr	Ng Ng Ng 01 01	Ng Ng Ng Ng 02
C. lusitaniae	Ng Ng Ng Ng 01	Ng Ng Ng Ng 02
C. parapsilosis	Ng Ng Ng Ng Ng	Ng Ng Ng Ng Ng
C. guilliermondii	Ng Ng Ng Ng Ng	Ng Ng Ng Ng Ng
C. dubliniensis	Ng Ng Ng Ng Ng	Ng Ng Ng Ng Ng

Ng; No Growth

activity of the DMSO crude extract shows high significant value ($P<0.05$) while compare to the methanolic extract of the *Ganoderma lucidum*. The MIC minimal value of *C albicans* show 02 isolates in the 150 μg/ml concentration at the DMSO extract but the 04 isolates were show in the methanol extract 02 isolate at 200 μg/ml and 02 at 150 μg/ml. The values were differed in the MFC 03 isolates in the DMSO extract 01 at 200 μg/ml and 02 at 150 μg/ml but the methanol extract show 05 isolates 02 at 200 μg/ml and 03 at 150 μg/ml. The

C. glabrata of the MIC and MFC are given significant value of the all the concentrations, the methanolic extract show 01 isolate in MIC and 02 isolate in the MFC at the 150 μg/ml concentration. The *C. tropicalis* and *C. kefyr* shows the significant value in the MIC at all the concentrations, while in methanolic extract of both the extracts of 200 – 150 μg/ml concentrations gives about 10 isolates. *C. krusei* and *C. parapsilosis* in MIC of both extracts show 06 isolates of the concentration between 150–250 μg/ml while in the MFC it shows significant value of the all the concentrations. *C. lusitaniae* in MIC shows 05 isolates, 02 isolates in the DMSO and 03 isolates at Methanol at 150 μg/ml while in MFC shows 03 isolates 01 isolate in the DMSO and 02 isolates at Methanol at 150 μg/ml. Isolates of *C. guilliermondii* and *C. dubliniensis* show significant ratio at fungistatic and fungicidal activity in both DMSO and methanolic extract of all the concentrations of both MIC and MFC assays. According to the results obtained, Proteinase and phospholipase enzyme assay has been performed to know the modifications among the enzymes produced by the biofilm Candida species. Supernatants of biofilms, which were treated with various concentrations of the DMSO and Methanolic Ganoderma extract, showed no significant difference in either the enzyme activity of proteinases and phospholipases, when compared to the Control group are described in the Bar diagram 3 and 4. Thus, there was no reduction in enzyme activities; such findings suggest no modulatory effects by the *Ganoderma lucidum* against the bioflim producers. Furthermore the extraction yields of DMSO and Methanol extracts are quite similar. However, greater efficiency in the extraction of solutes is not directly related to greater inhibition. In this regard, DMSO extracts proved to have more activity against Candida species than Methanolic extracts. Thus, it can be concluded that the DMSO extracts (200 μg/ml – 150 μg/ml) of *Ganoderma lucidum* in MIC and MFC showed higher significant value than those of the Methanol extract (250 μg/ml – 200 μg/ml). Also we conclude that the DMSO extract exhibit high significant of fungistatic and fungicidal properties that support their traditional use as antiseptics and treatment against the Bioflim producing candida species.

CONCLUSION

The results obtained from this work showed that extracts of *Ganoderma lucidum* medicinal mushroom screened exhibit antifungal effects against biofilm producing *Candida* sp. In particular, while comparing to the Methanolic extract and DMSO extract, DMSO offer effective bioactive compounds for growth inhibition of the Candida and its Biofilm. Even at low concentrations, these species showed antifungal activity neither than the commercial fungicide according to the previous studies. So we can be conclude that DMSO extract of the *Ganoderma lucidum* shows high significant factor against the various disease causing Candida species and its Biofilm.

ACKNOWLEDGEMENT

I thank Dr. R. Chidambaram, who aided to screen the *Ganoderma lucidum* and I also thank Dr. R. Srikumar for reviewing the manuscript for the publication.

CONFLICT OF INTEREST

None of the authors has a conflict of interest to disclose.

ABBREVIATIONS USED

NAC: Non albican candida, **MIC:** Minimum Inhibitory Concentration, **MFC:** Minimum Fungicidal Concentration, **DMSO:** Dimethyl Sulfoxide, **YNB:** Yeast Nitrogen Base Medium, **PBS:** Phosphate Buffer Saline, **XTT:** (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, **RPMI:** Roswell Park Memorial Institute Medium.

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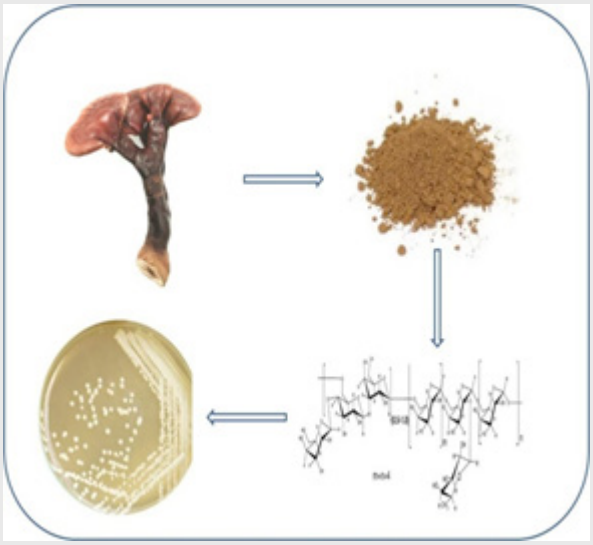
SUMMARY

- *Ganderma lucidum* is an edible mushroom has more Medicinal values.
- Dimethyl sulfoxide (DMSO) and Methanol Extracts of *Ganderma lucidum* were treated against the Candida albicans and Non albicans Organisms which causes both superficial and systemic infections.
- Screened for Proteinase and phospholipase enzyme secretion assay, Minimum Inhibitory Concentration (MIC) - XTT Assay and Minimal Fungicidal Concentration (MFC)
- DMSO extract of the *Ganoderma lucidum* shows high inhibition factor against the various disease causing Candida species and its biofilm.

PICTORIAL ABSTRACT



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Cite this article: kumar NC, Jayalakshmi G, Chidambaram, Srikumar R. *In-vitro* Evaluation of Antifungal Activity of *Ganoderma lucidum* Against the Biofilm Producing *Candida* species. Indian J of Pharmaceutical Education and Research. 2017;51(4S):S623-S30.