Research

IN VITRO ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF POLYSACCHARIDE EXTRACTED FROM MEDICINAL MUSHROOM *GANODERMA LUCIDUM*

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ABSTRACT

Ganoderma lucidum species is currently popular and used in the formulation of nutraceuticals and as functional foods. In this study, antioxidant activities of crude polysaccharides, polysaccharide fraction (GLP-F1-1 and GLP-F1-2) from *G.lucidum* were investigated. The antioxidant activity was measured by $DPPH^{\cdot}$ and Superoxide anions ($O^{\cdot}$) free radicals scavenging. The $DPPH^{\cdot}$ and $O^{\cdot}$ scavenging activity of crude polysaccharides, GLP-F1-1, GLP-F1-2 and ascorbic acid on the DPPH and $O^{\cdot}$ radical concentration- dependently increased and was 35.41%, 60.00%, 80.41% and 95.10%, 38.12%, 50.56%, 80.21% and 92.16% at the dose of 1mg/ml. The results indicated that the crude polysaccharide have a noticeable effect on scavenging free radicals, especially as high addition quantity. The antimicrobial activities of polysaccharides extract of fruit bodies from *G. lucidum* on selected five bacterial pathogens *E. coli, P. aeruginosa, S. aureus, K. Pneumoniae, S. mutants* and five pathogenic fungi *A. niger, C. albicans, M. mucedo, P. citrinum, and F. oxysporum* were investigated by the agar disk diffusion method. In *E.coli, P. aeruginosa* were more sensitive as compared with *S. aureus, K. Pneumoniae* and *S.mutants* and fungal activity *F. oxysporum, P.citrinum* shows good result as compared with *A. niger, C. albicans* and *M.mucedo*. Our findings suggested that *G.lucidum* polysaccharides could significantly enhance the antioxidant and antimicrobial activities.

KEYWORDS: *Ganoderma lucidum,* polysaccharide extract, Antioxidant activity and Antimicrobial activity.

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INTRODUCTION:

Antioxidants are considered important nutraceuticals on account of many health benefits[1-3]. Free radicals play an important positive physiological role and, at the same time, they may exert toxic effects[4]. Free radicals (Reactive oxygen species) are an entire class of highly reactive molecules derived from the metabolism of oxygen. Moreover, these radicals can cause extensive damage to cells and tissues, during infections and various degenerative disorders, such as cardiovascular disease, aging, and neurodegenerative diseases like Alzheimer's disease, mutations and cancer[5-7]. Free radicals participate in the regulation of signal transduction and gene expression, activation of receptors and as nuclear transcription factors [8-9]. They are playing a vital role in phagocytosis[4]. On the other hand, there is increasing evidence that free radicals may play a causative role in a variety of diseases including heart disease, cancer, aging, Parkinson's and Alzheimer's disease, impairment of immune function, cataracts and muscular degeneration in elderly people[10]. All organisms are protected against free radical damage by defence systems involving superoxide dismutase and catalase or ascorbic acid, tocopherol and glutathione[10 -11]. Improved antioxidant status may have an immunostimulatory effect[11].

Fungi are an important source of materials in traditional Chinese medicine. Higher basidiomycetes mushrooms have been used in folk medicine throughout the world since ancient times[12]. *Ganoderma lucidum*, a medicinal fungus belonging to the Polyporaceae family, is used extensively in traditional Chinese medicine. Modern studies have revealed that *Ganoderma lucidum* contain a variety of bioactive ingredients, including triterpenoids, polysaccharides, sterols, fatty acids, nucleosides and alkaloids[13], and possess multiple pharmacological activities, such as antitumor[14], Immunomodulation[15-16], anti-inflammatory[17], antiviral[18], antioxidant[19], anti-aging[20] and anti diabetic[21] effects. Due to its ability to cure many different diseases it received names like "Elixir of life", "Food of Gods", "Mushroom of Universe" [22-23]. Triterpenoids and polysaccharides are two main categories of the bioactive components from *G.lucidum* and it has been found previously that polysaccharides exert their effect mainly through an immunomodulatory mechanism[24-25].

Recent studies have indicated that components extracted from *G. lucidum* have a wide range of pharmacological actions including suppressing inflammation and scavenging free radicals[26]. Polysaccharides are potentially useful biologically active ingredients for pharmaceutical use, such as for immune regulation, for anti- radiation, anti blood coagulation, anti cancer, anti- HIV and hypoglycemic activities[27-29]. In addition its therapeutic effects, the methanolic extracts from *G.lucidum* and *G.tsugae* also possess antioxidant abilities[30]. Consumption of antioxidant-rich plants may help prevent cancer and other chronic diseases[31-32]. Antioxidants protect cellular components from oxidative damage, which is likely to decrease risk of mutations and carcinogenesis and also protect immune cells, allowing them to maintain immune surveillance and response. Various components of *G. lucidum*, in particular polysaccharides and triterpenoids, show high antioxidant activity in vitro[33 - 38]. Polysaccharides were also reported to protect the immune cells from oxidative damage[39]. Methanol extracts of *G. lucidum* were reported to prevent kidney damage (induced by the anticancer drug cisplatin) through restoration of the renal antioxidant defense system[40]. In vitro antioxidant activities of

G.lucidum polysaccharides was carried out by Jia and others using streptomycin–induced diabetic rats. The results indicated *G.lucidum* polysaccharides could significantly and dose-dependently increase nonenzymic/enzymic antioxidants and reduce lipid peroxidation[41].

There are available in literature some studies reporting antimicrobial activity of different extracts of *Ganoderma lucidum* (Curtis) P. Karst from India[42-43] and China[44]. For evaluating the antibacterial effects of the mushroom, several in vitro and in vivo animal studies using *G. lucidum* were performed. Mice injected with *G. lucidum* extract (2 mg/mouse) 1 day prior to injection with *Escherichia coli* showed markedly improved survival rates (>80% compared to 33% in controls) [45]. In an in vitro study that used the disk assay[46], a chloroform extract of *G. lucidum* was investigated for its antibacterial effect on gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*) and gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*). Results showed that the extract had growth-inhibitory effects on two of the gram-positive bacteria with a minimal inhibitory concentration (MIC) of 8 mg/mL for *S. aureus* and *B. subtilis*. In another in vitro study, the direct antimicrobial effect of a *G. lucidum* water extract was examined against 15 species of bacteria alone and in combination with 4 kinds of antibiotics[47]. *G. lucidum* was found to be more effective than antibiotics against *E.coli, Micrococcus luteus*, *S. aureus*, *B. cereus*, *Proteus vulgaris*, and *Salmonella typhi*.

The objective of the present study was evaluating antioxidant and antimicrobial activities of methanol extract polysaccharides from the medicinal mushroom *G. lucidum*. The antioxidant activities were studied by the Superoxide anion radical-scavenging activity, free radical scavenging abilities of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) activity. Antimicrobial activities was tested in vitro, by the measuring the ager disk diffusion method.

MATERIALS AND METHODS

Chemicals

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from sigma- Aldrich (USA). Ascorbic acid and butylated hydeoxyanisole (BHA) were purchased from E.MERCK (India). Stock solutions of DPPH were prepared in methanol and methanol buffered with acetic acid buffer (0.1M, pH 5.5) respectively. Buffered methanol was prepared by mixing 40ml of 0.1M acetic acid buffer (pH 5.5) with 60ml methanol. The solvent and other chemicals were of analytical grade. The reaction tubes, in triplicate were wrapped in aluminum foil and kept at 30^{o}C for 30 min in dark.

Collection of *Ganoderma lucidum* fruit bodies

Matured *Ganoderma lucidum* fruiting bodies were collected from July 2013 to December 2013 on Maruthamalai hills region, Bharathiar University, Coimbatore, Tamil Nadu, India and authenticated by Botanical Survey of India, Coimbatore. They were identified with the aid of web based pictures and reference materials.

Isolation of *Ganoderma lucidum* polysaccharides (GLPs)

Sporocarps were cut into small pieces dried at 40-50^{0}C for 48h and powdered. Polysaccharides were isolated by the method of [48-50] with slight modification. The crushed powder (100g) was removed the impurities for 24h with 80% ethanol at room temperature. The extract was filtered and centrifuged at 7500rpm for 30min at room

temperature. The supernatant was concentrated in a rotary evaporator under reduced pressure at 50^0C and removed free protein layer by the use of method of sevage. At last the extract was subjected to the precipitation with fourfold volumes of ethanol. The crude polysaccharide was collected by centrifugation, washed with ethanol twice, and then freeze dried. The crude polysaccharides was dissolved in water and reprecipitated with equal volume of cetyl trimethal ammonium hydroxide and kept for overnight. The supernatant obtained was precipitated with chilled ethanol. After centrifugation the precipitate obtained was run thought DEAE cellulose column and eluted with deionized water. The precipitate thus obtained was lyophilized to get light brown polysaccharides (3.1g).

Purification and Fractionation of *G.lucidum* polysaccharides

The polysaccharide extract was firstly passed through a polyamide column (5.5cm×30 cm), and eluted with deionized water to adsorb proteins and pigments. The elution was collected, and then fractionated by anion-exchange chromatography on a column (2.6cm×37 cm) of DEAE Sepharose Fast Flow (Pharmacia). The column was firstly eluted with 20mM Tris–HCl (pH 8.0) at a flow rate of 1.25 mL/min. Then a stepwise elution from 0 to 1M NaCl in 20mM Tris–HCl (pH 8.0) was applied to the column. The seven collected fractions were denoted as F1–F7. These fractions were lyophilized respectively. The fraction F1 was dissolved in water and purified by size exclusion chromatography on a Sephacryl S-500HR (Pharmacia), eluted with deionized water and fractionated into two fractions (denoted as GLP-F1-1 to GLP-F1-2). These two fractions were collected and lyophilized respectively.

Antioxidant activity of Ganoderma lucidum polysaccharides

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging activity

The free radical scavenging activity of the purified polysaccharide fractions was measured by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) test according to the method of [51], with some modifications. 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical is the radical which has an unpaired valence electron at one atom of Nitrogen bridge [52]. Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay [53-55]. 100ml of a DPPH radical's solution in ethanol (60ml) was mixed with 100µL of sample (0, 0.25, 0.50, and 1.0) solution in ethanol (0.5-2µg/ml). A control, containing 100ml of DPPH solution and 60ml of ethanol was prepared. The mixture was incubated at room temperature for 30 min and then the absorbance was measured at 517nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. Ascorbic acid was used as positive controls. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following Eq. 1:

$$\text{Scavenging effect of DPPH (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the mixture containing extracts IC_{50} of reference antioxidant compounds. BHC and Ascorbic acid were used for comparison to IC_{50} of the polysaccharide extracts.

Superoxide anion radical-scavenging activity

Superoxide anion radical-scavenging activity was measured by a non-enzymatic method[56] modified slightly[57]. 0.025 ml of sample solutions with various concentrations (0, 0.25, 0.50, and 1.0) mg/mL) was treated with 0.1 ml of 25 mM phosphate buffer (pH 7.2), 2 mM NADH (0.025 ml) and 0.5 mM NBT (0.025 ml), and absorbance at 560 nm was measured as a blank value. After a 10 min incubation at ambient temperature with 0.025 ml of 0.03 mM PMS, the absorbance was again measured. Ascorbic acid was used as the positive control.

Antimicrobial activity of *G.lucidum* polysaccharide extract

Test microorganisms and growth media

Bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, staphylococcus aureus, klebsilla Pneumoniae, and staphylococcus mutants)* and fungal strains *(Aspergillus Niger, Candida albicans, Mucor mucedo, Penicillium citrinum, Fusarium oxysporum)* obtained from institute of Microbial Technology, Chandigarh. The bacterial and fungal stock cultures were inculcated for 24 hours at 37° C on nutrient ager and potato dextrose ager (PDA) medium respectively following refrigeration storage at 4° C. the bacterial strains were grown in Mueller-Hinton ager (MHA) plates at 37° C, whereas the yeast and moulds were grown in sabouraud dextrose ager and PDA media respectively. The stock culture was maintained at 4° C.

Determination of zone of inhibition method

In vitro antibacterial and antifungal activities were examined for methanol extract. Antibacterial and antifungal activities of Ganoderma lucidum polysaccharides extracts against five pathogenic bacteria and five pathogenic fungi were investigated by the ager disk diffusion method[58]. Antimicrobial activity testing was carried out using ager cub method. Each purified polysaccharide extract were dissolved in methanol, sterilized by filtration using sintered glass filter, and stored at 4° C. For the determination of zone of inhibition bacterial and fungal strains were taken as a standard antibiotic for comparison of the results. The polysaccharide extract were screened for their antibacterial and antifungal activities against the *Escherichia coli, Pseudomonas aeruginosa, staphylococcus aureus, klebsilla Pneumoniae, staphylococcus mutants* and the fungi *Aspergillus Niger, Candida albicans, Mucor mucedo, Penicillium citrinum, Fusarium oxysporum.* The set of five dilutions (10, 50, 100, 150, 200µg/ml) of *G.lucidum* polysaccharides extract and standard drugs were prepared in double distilled water using nutrient ager tubes. Mueller-Hinton sterile ager plates were seeded with indicator bacterial strains (10^8 cfu) and allowed to stay at 37° C for 3 hours. Control experiments were carried out under similar condition by using Gentamicin, Co- Trimoxazole and Roxithromycin for antibacterial activity and nystatin and griseofulvin for antifungal activity as standard drugs. The zones of inhibition around the disks were measured after 18 to 24 hours of incubation at 37° C for bacteria and 48 to 96 hours for fungi at 28° C. the sensitivities of the microorganisms species to the polysaccharides extract were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the ager surface around the disks, and values < 8mm were considered as not active against microorganisms.

RESULT

DPPH is a free radical compound that has been widely used to determine the free radical- scavenging ability of various samples[59]. The color of the DPPH radical solution becomes lighter and its absorbance goes down in the presence of an antioxidant compound[60]. The scavenging ability of crude polysaccharides, purified polysaccharides fractions is shown in Table 1 and compare with ascorbic acid as control standards. Figure 1 illustrates that the scavenging effect of crude polysaccharides, GLP-F1-1, GLP-F1-2 and ascorbic acid on the DPPH radical concentration- dependently increased and were 35.41%, 60.00%, 80.41% and 95.10% at the dose of 1mg/ml respectively. The results indicated that the crude polysaccharide have a noticeable effect on scavenging free radicals, especially as high addition quantity. The 50% inhibitory concentration [IC50] values for DPPH scavenging activities of the mushroom extracts, ascorbic acid compared and shown in Table 1, as calculated from the percentage inhibition versus log concentration of the extract curves.

The superoxide radical (O_2^-) is a highly toxic species could be generated by numerous biological and photochemical reactions. In addition directly attack important biological molecules, O_2^- may also decompose to form single oxygen and hydroxyl radicals, which may increase local oxidative stress and initiate cellular damage or lipid peroxidation and pathological incidents such as arthritis and Alzheimer's diseases[63]. In the present study, we investigated the scavenging capacity of *G.lucidum* polysaccharides against the superoxide anion free radicals. As illustrated in Fig 2, the superoxide anion radical scavenging effects of the crude polysaccharides, GLP-F1-1 and GLP-F2-1 increased with increasing concentrations. In particular, the crude polysaccharides extract had the highest scavenging activity, which was higher than that of L-Ascorbic acid.

Table 1: Scavenging effects of *Ganoderma lucidum* crude polysaccharides, GLP-F1-1 and GLP-F1-2 for DPPH and superoxide radicals.

Radical and extract Concentration mg/ml	GLCP	GLP-F1-1	GLP-F1-2	L-Ascorbic acid
DPPH				
0.25	25.40±1.1	53.60±1.7	70.12±1.6	75.37±1.0
0.50	29.64±1.2	62.13±1.3	80.34±0.9	87.11±0.3
1.00	36.88±1.9	63.12±1.2	90.17±0.8	92.81±0.2
O_2^-				
0.25	20.62±1.9	41.19±0.7	70.23±0.6	89.56±1.9
0.50	30.95±1.6	49.23±0.9	73.19±0.5	93.57±1.8
1.00	35.46±0.9	53.21±1.6	85.69±0.6	95.50±1.3

L= Ascorbic acid was used as a positive control. GLCP= Ganoderma lucidum crude polysaccharide; GLP-F1-1= Ganoderma lucidum polysaccharides fraction 1; GLP-F1-2= Ganoderma lucidum polysaccharides fraction 2.

Figure 1: DPPH radical scavenging activity of the crude polysaccharides, GLP-F1-1 and GLP-F1-2.

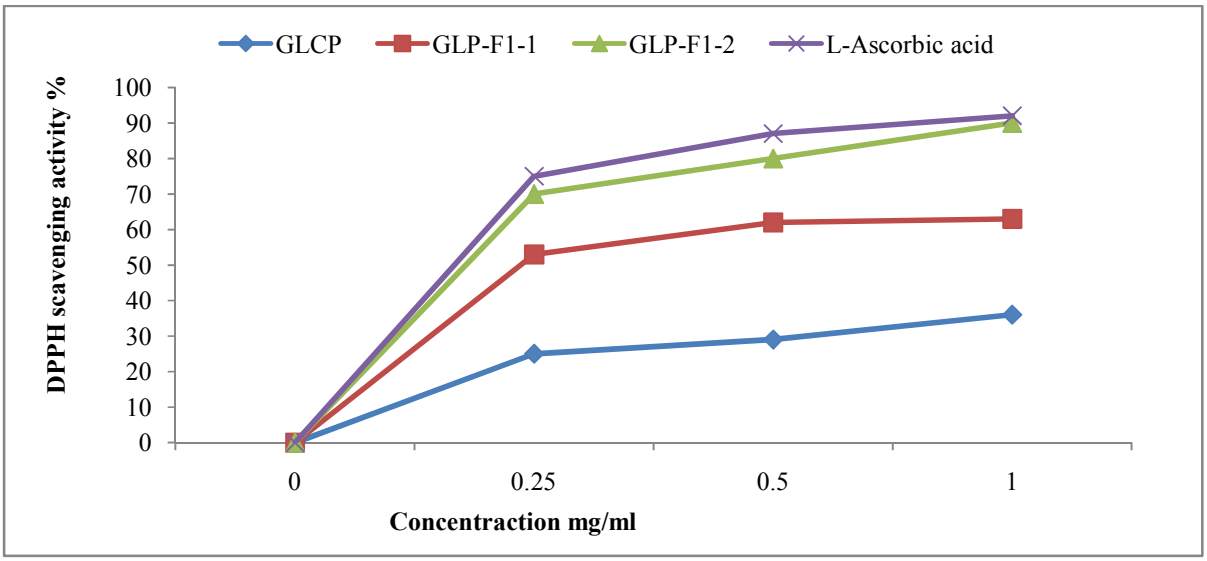
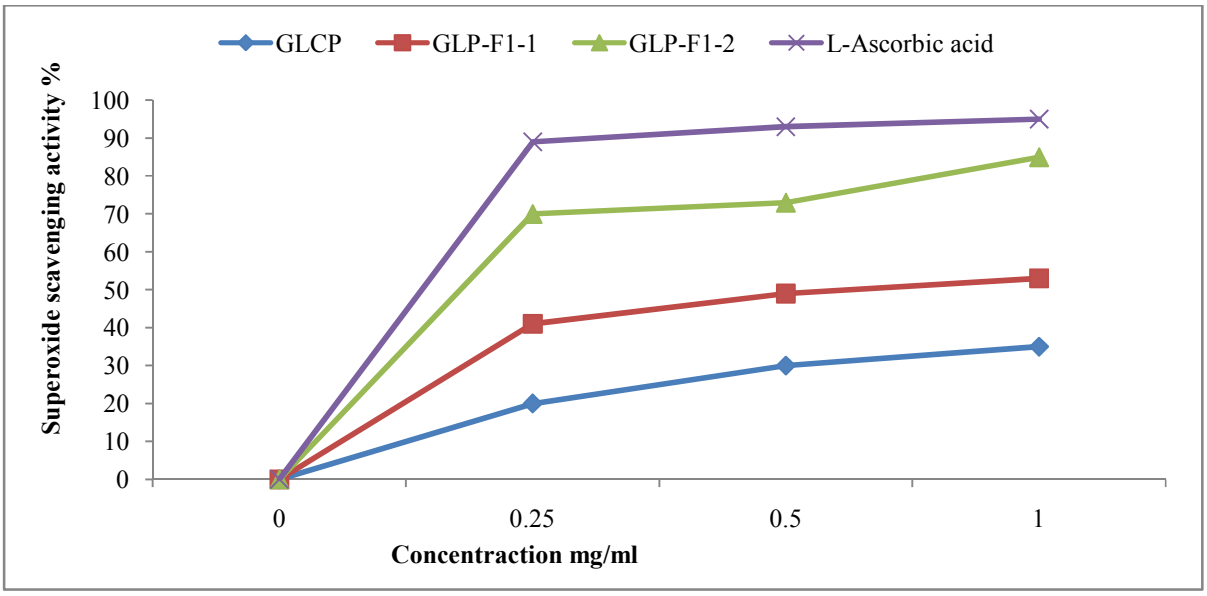


Figure 2: Superoxide anion radical scavenging activity of the crude polysaccharides, GLP-F1-1 and GLP-F1-2.



The antibacterial effect of the GLP from *G.lucidum* was tested against five species bacteria and five fungal species. Antibacterial and antifungal potential of GLP extract were assessed in terms of zone of inhibition of bacterial and fungal growth. The results of the antibacterial and antifungal activities are presented in Figure 3 and 4.

The antibacterial and antifungal activities of the GLP extract increased linearly with increase concentration of GLP extract (µg/ml). As compared with standard antibiotic drug (Gentamicin, Co- Trimoxazole and Roxithromycin, nystatin and griseofulvin), the results revealed that in the bacterial and fungal activity. E.coli, Pseudomonas aeruginosa were more sensitive as compared with Staphylococcus aureus, Klebsilla

Pneumoniae and Staphylococcus mutants and fungal activity Fusarium oxysporum, Penicillium citrinum shows good result as compare with Aspergillus niger, Candida albicans, and Mucor mucedo. The growth inhibition zone measured ranged from 9 to 21mm for all the sensitive bacteria, and ranged from 9 to 25mm for fungal strains (Figure 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12).

DISCUSSION

The IC50, meaning the concentration of antioxidant needed to decrease [by 50%] the initial substrate concentration, is a parameter widely used to measure the antiradical efficiency. The lower IC50 values show the higher antioxidant activity[61]. However the radical scavenging activity of the purified polysaccharide fractions was lower than that of ascorbic acid. Furthermore crude polysaccharide exhibited a relatively high level of radical scavenging activity. The crude polysaccharide purified further by the gel filtration column (GLP-F1-1 and GLP-F1-2) exhibited less antioxidant activity. The reason for this could be that the crude G.lucidum extracts were rich in antioxidant components, such as proteins, amino acids, peptides, phytosterols, ascorbic acid and microelements, which contributed to their antioxidant properties. Lin et al. found that methanolic extracts from other medicinal mushrooms were extremely effective in inhibiting the lipid peroxidation [6.41% for *Ganoderma lucidum*, 2.62% for *Ganoderma lucidum* and 2.30% for *Ganoderma tsugae* at 0.6 mg/mL] [62]. The result suggested that the *G. lucidum* polysaccharide is a good scavenger for DPPH radicals.

It has been suggested that the overall radical scavenging ability was related to the number of hydroxyl or amino groups in a polysaccharides molecule such as Chitosan[64]. It has been reported that the polysaccharide extracts of *G. lucidum* and *Grifola umbellata* possess superoxide radical scavenging activity[65].

Bacteria are the principle types of microorganisms that cause food- borne illness. Many natural compounds found in dietary plants, such as extracts of herbs and fruits, possess antimicrobial activities. The use of the natural extracts is a novel way to reduce the proliferation of microorganisms[66]. In the present study, we found that GLP from G.lucidum exhibited antimicrobial activity all of the tested bacteria and fungi. In *E.coli, Klebsilla Pneumoniae, Staphylococcus mutants*, the antibacterial activity of GLP showed more sensitive, compared with that in *Staphylococcus aureus* and *Pseudomonas aeruginosa* and fungal activity *Aspergillus niger* and *Fusarium oxysporum* were more sensitive, compare with that in *Candida albicans, Penicillium citrinum* and *Mucor mucedo*.

According to[67], *G.lucidum* and other *Ganoderma* species more often in combination with chemotherapeutic agents have been used to treat various bacterial diseases. Its polysaccharide components were found to be the bioactive principle which plays an important role in antibacterial activity. [68]observed maximum antibacterial activity of methyl australates, a derivative from *G. lucidum* against *E.coli* and *P. aeruginosa* followed by *S. aureus* while least zone of inhibition was recorded for *Bacillus* species. [69]have studied the influence of various extracts isolated from *G. lucidum* on *E. coli*, *Bacillus* species, *S. aureus* and *Salmonella* species. It is apparent from the present study that mushroom polysaccharides extracts from *G. lucidum* could be

employed to combat several diseases caused by pathogenic-microorganisms.

Figure 3: Antibacterial activity against *Escherichia coli*

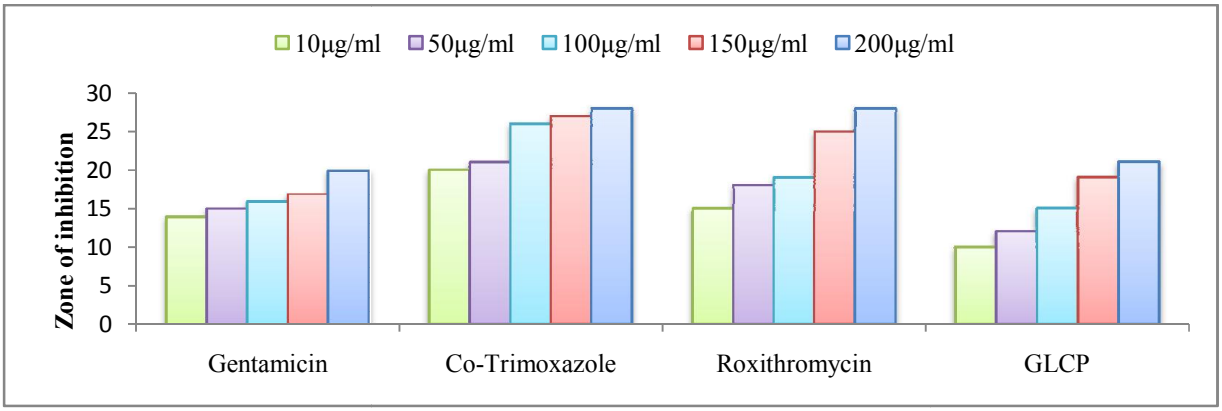


Figure 4: Antibacterial activity against *Pseudomonas aeruginosa*

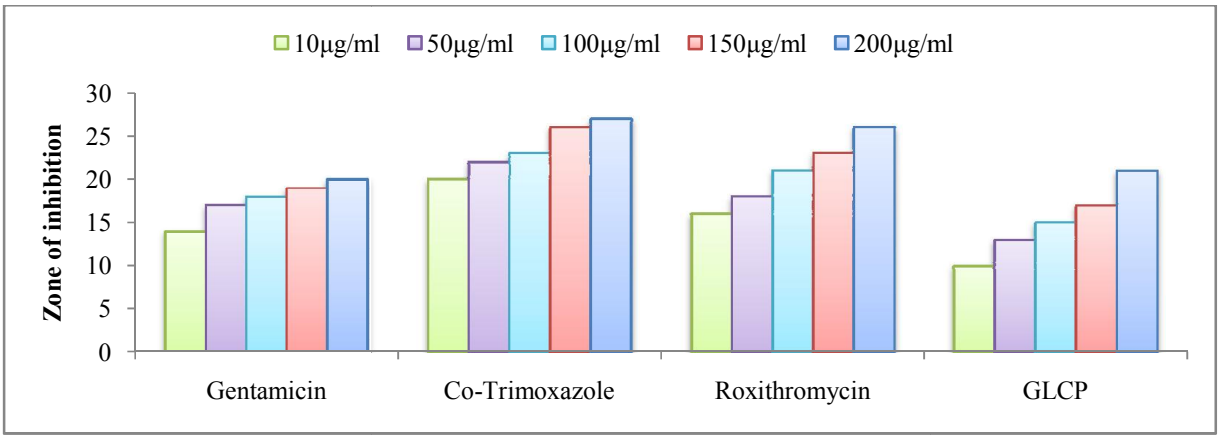


Figure 5: Antibacterial activity against *Staphylococcus aureus*

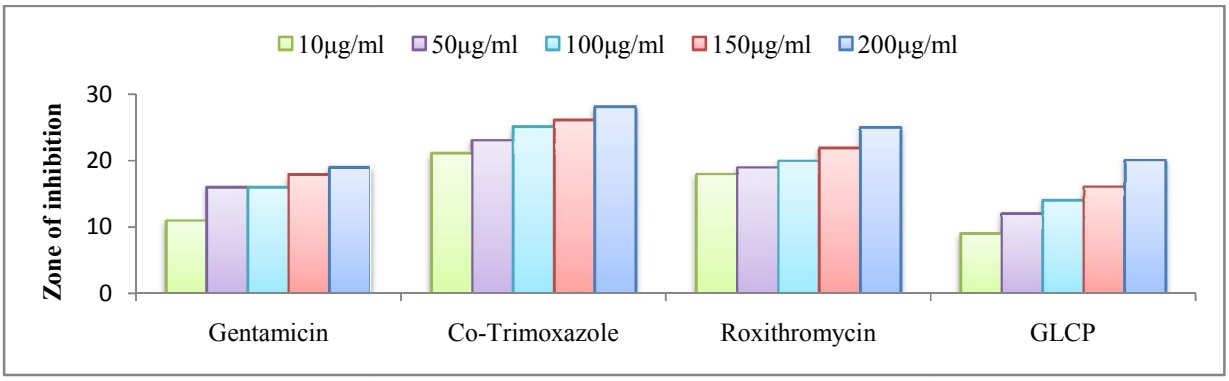


Figure 6: Antibacterial activity against *Klebsilla Pneumoniae*

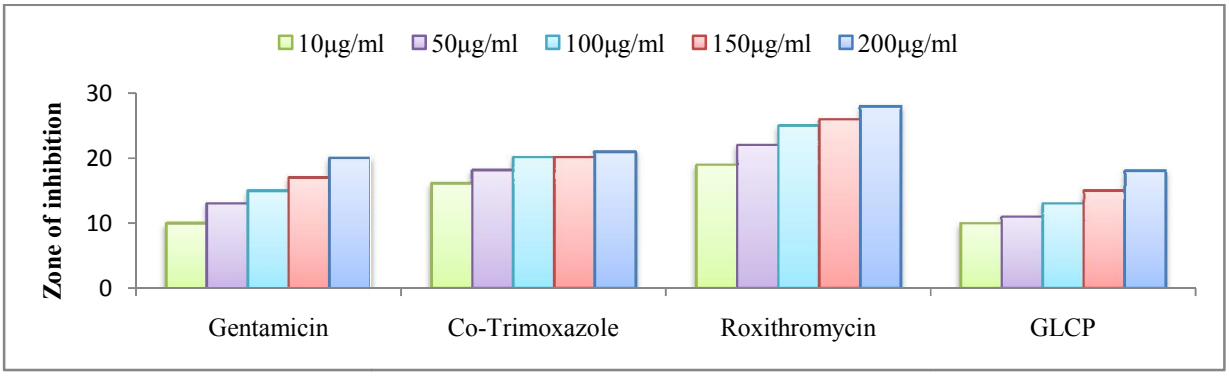


Figure 7: Antibacterial activity against *Staphylococcus mutants*

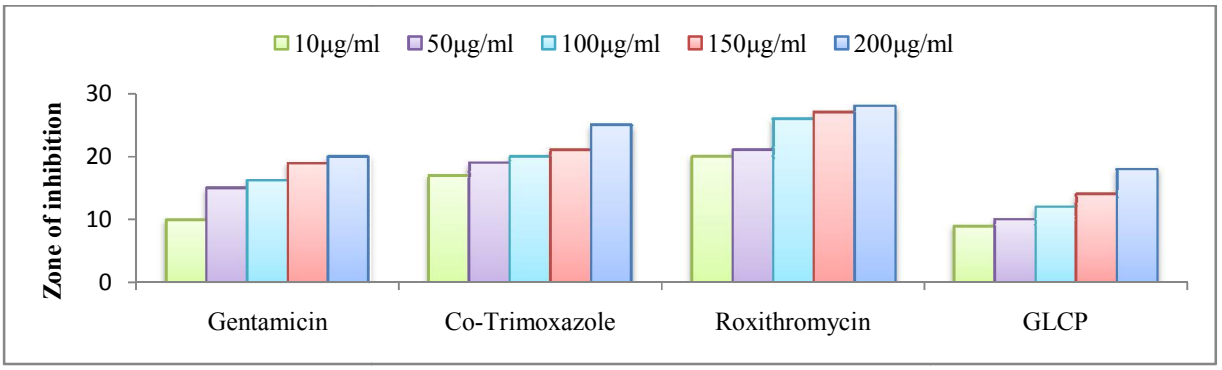


Figure 8: Antifungal activity against *Aspergillus niger*

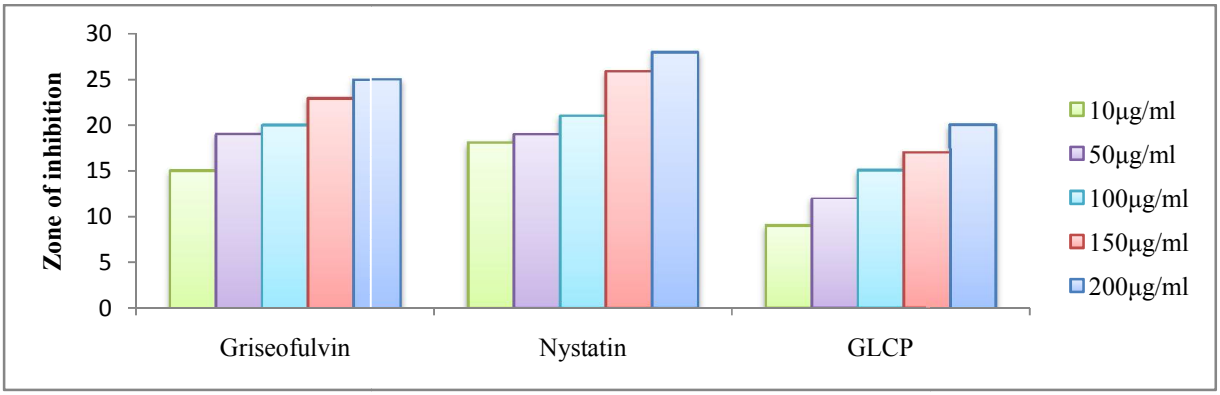


Figure 9: Antifungal activity against *Candida albicans*

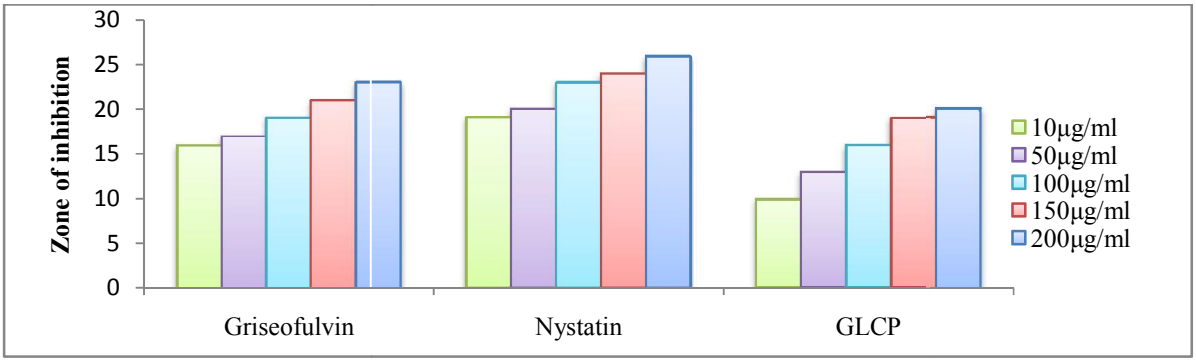


Figure 10: Antifungal activity against *Mucor mucedo*

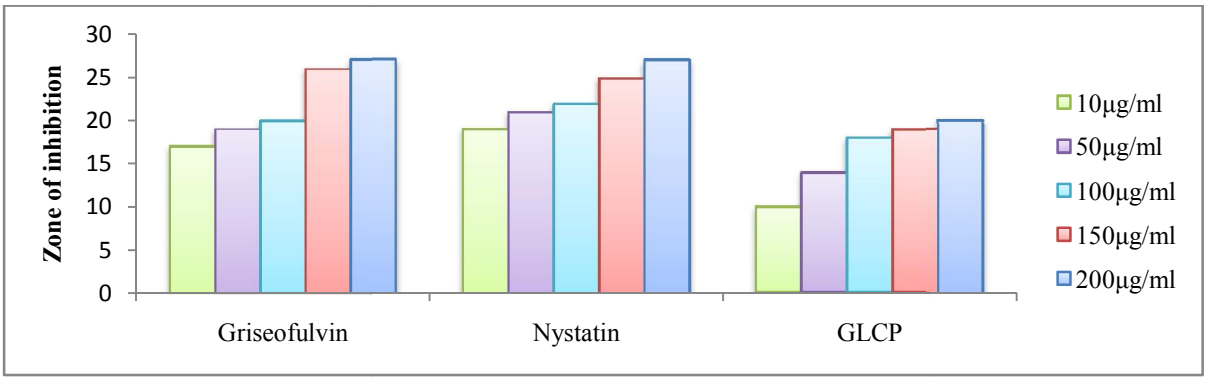


Figure 11: Antifungal activity against *Penicillium citrinum*

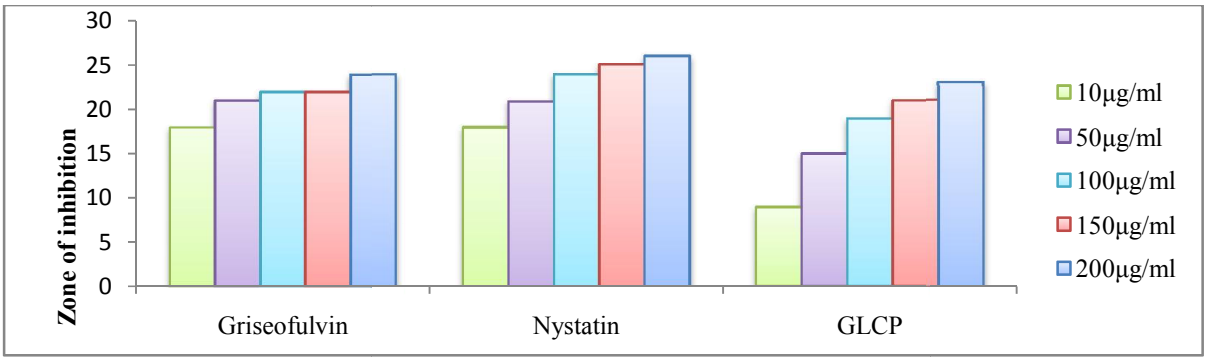
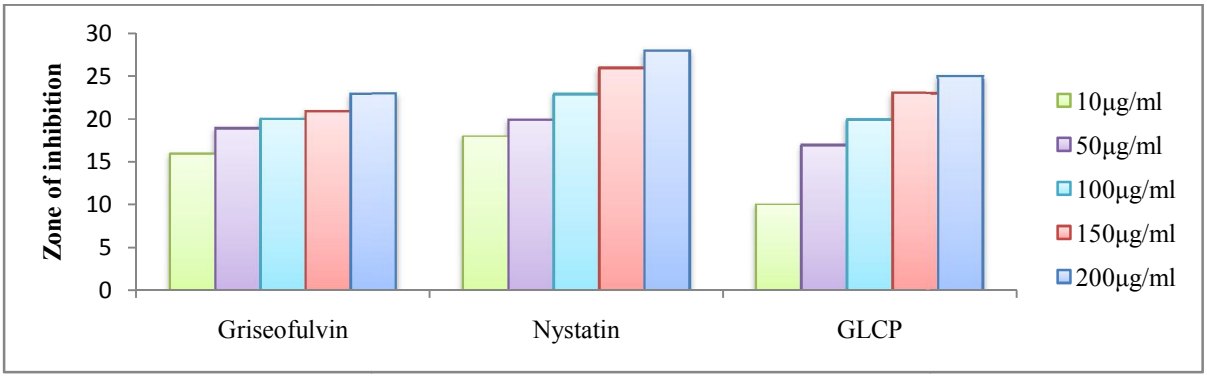


Figure 12: Antifungal activity against *Fusarium oxysporum*



CONCLUSION

Measurement of antioxidant properties of methanol crude polysaccharide extracts, polysaccharides fractions (GLP-F1-1 and GLP-F1-2) of the fruiting bodies of *G.lucidum* showed relatively crude polysaccharide extracts high antioxidant activities. The activity was in descending order: G.lucidum > GLP-F1-1 > GLP-F1-2).The results of the present study suggest that polysaccharides extracts of medicinal mushrooms *G.lucidum* act as natural antioxidant and antimicrobial properties. Polysaccharides extract may be good sources for the development of antioxidant food additives.

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