Antifungal and Demelanizing Activity of *Ganoderma lucidum* on Some Pathogenic Fungi, and Electron Microscopic Study of Fungi Damages

Sayeh Jamadi Khiyabani[1], Pegah Shakib[2], Mahboobeh Madani[1*], Seyed Masoud Hashemi Karouei[3]

1. Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran.
2. Department of Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.
3. Department of Microbiology, Babol Branch, Islamic Azad University, Babol, Iran.

***Corresponding author:** Mahboobeh Madani. Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran.

Email: mmadani66@gmail.com.

Abstract

In the last two decades, fungal diseases have increased for various reasons, including weakened immune systems, overuse of antibiotics, antifungals, and corticosteroids, as well as increased longevity. *Ganoderma lucidum* has a variety of biologically active compounds with medicinal functions. The present study aimed to investigate the antimicrobial and demelanizing activity of *Ganoderma lucidum* on somepathogenic fungi and to assess the damage by scanning electron microscopy(SEM). In this study,After preparing methanolic and chloroform extracts of *Ganoderma lucidum* ,antifungal activities of methanolic and chloroform extracts were separately evaluated on *Sporothrix schneckei, Alternaria alternata, Mucor, Penicillium, and Trichophyton violaceum* based on growth inhibition percentage. Also, inhibition of melanin production of *Ganoderma lucidum* was investigated on these fungi. Furthermore, the damage caused by the extracts on these fungi was studied by scanning electron microscopy(SEM). According to the results, the mean percentage of growth inhibition of methanolic extract were 67.25±1.02, 100.00±.00, 27.92±3.00, 100.00±.00, and 100.00±.00 for *Sporothrix schneckei*, *Alternaria alternata*, *Mucor*, *Penicillium*, and *Trichophyton violaceum*, respectively, and 83.82±14.16, 100.00±.00, 47.92±3.61, 100.00±.00, and zero for the chloroform extract. *Ganoderma lucidum* inhibited the production of melanin with *Penicillium*. Based on scanning electron microscope(SEM) observations, changes in morphology, production of pores shrinkage at the surface of the mycelium and conidia, and a decrease in the number of conidia were observed. According to the results, *Ganoderma lucidum* is a valuable mushroom with an antifungal effect against fungal pathogens and might be used to treat these diseases. However, more research is needed to investigate the possible side effects of consuming it.

Keywords: *Ganoderma lucidum*, Antifungal effect, Demelanizing, Scanning electron microscope(SEM).

1. Introduction

Fungal pathogens are very challenging because they are eukaryotes and have evolutionary relationships with their human hosts (1). The emergence of drug resistance is an evolutionary issue. A ubiquitous process based on natural selection for organisms that have an improved ability to survive and reproduce in the presence of drugs. This is not only a great threat to human health but also has significant economic consequences. In this regard, fungi are of interest for their medicinal value as well as their infectivity. Many medicinal mushrooms have a long history in traditional medicine, contributing to a healthy diet due to the presence of rich sources of vitamins, minerals, and proteins. More than 100 medicinal functions are produced by fungi. Among these, we can mention antioxidant, anti-cancer, anti-diabetic, anti-allergic, anti-cholesterol, antimicrobial, and detoxification activities, strengthening the immune system, and protecting the liver, heart, and blood vessels (2). Currently, almost 270 species of mushrooms with different therapeutic properties are used in the traditional medicine of many countries, such as Japan, China, Korea, and Russia. Among these, we can refer to *Ganoderma lucidum*, *Lentinola edudos*, and *Inunotus oblicuso*as some examples (3,4). *Ganoderma lucidum* belongs to *Basidiomycete* branch, *Aphyllophorales* order, *Ganodermataceae* family, and *Ganoderma* genus. This fungus is found as a saprophyte or an optional parasite of plants and can be found in nature mainly on live and dead wood of leafy species growing under high humidity and invisible lighting conditions (5,6). *Ganoderma lucidum* has bioactive compounds including polysaccharides, triterpenoids, proteins, and sterols with therapeutic properties, such as anti-cancer, anti-microbial, anti-viral, antioxidant,and anti-diabetic activities. These active ingredients are responsible for protecting the cardiovascular system, whitening the skin, and strengthening effective immune cells, among others (3-5).

In 2020, Rostami Nejad et al., by investigating the antimicrobial effect of *Ganoderma lucidum* mushroom extract on *Candida albicans* isolated from lung samples using two methods: disk diffusion and microdilution, showed that *Ganoderma lucidum* extract showed antifungal effects against *Candida albicans* and could be used as an alternative to antibiotics in the treatment of this type of infection(7). In 2023, Mousavi et al. reported that *Ganoderma lucidum* methanolic extract (GLME) has attracted much attention due to its exceptional antimicrobial and anticancer properties, which can be tuned by carefully controlling the content and concentration of the initial extraction. In this study, they described in detail its antimicrobial performance against Gram-positive and Gram-negative bacterial strains *(Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa*). The results showed that the antibacterial activity of this product against *E. coli* was higher than that of streptomycin, with the diameter of the zone of inhibition being 44±0.09 mm and 30±0.11 mm, respectively(8).
The present study aimed to investigate the antimicrobial and demelanizing activity of *Ganoderma lucidum* on somepathogenic fungi and to assess the damage by scanning electron microscopy(SEM).

2. Materials and methods

Ganoderma lucidum was purchased from Islamic Azad University, Najafabad Branch. After sterilization with 70% ethanol and sterile water, the desired mushroom was dried, powdered, and prepared for extraction (9).

2.1. Extraction

The amount of 10 grams of *Ganoderma lucidum* mushroom powder was poured separately in 50 ml of methanol and chloroform (99% Merck) and kept at -20º C for seven days. Then, 150 ml of methanol and chloroform were added to the solution. The resulting solution was placed in an ultrasonic bain marie at a temperature of 50º C with a

wavelength of 59 kHz for 20 minutes. Next, the Arlene-containing mushroom solution, methanol, and chloroform were placed on a shaker at 130 rpm for 48 hours. Finally, the desired extracts were filtered by filter paper No. 4 and placed in an oven to dry for 24 hours at a temperature of 45ºC (10).

2.2. Preparation of extract solution

8 mg of the methanol extract was mixed with 12 ml of sterile water and 6 mg of chloroform extract was mixed with 12 ml of water, to prepare the methanol and chloroform extract solutions, respectively. 5% dimethyl-sulfoxide and Tween 80 (20 microliters) were used to dissolve the extract. The concentrations prepared for each of the extracts were considered to be 66.66 mg/ml and 50 mg/ml, respectively (10).

2.3. Tests to determine the sensitivity of fungus to extracts

For determining the growth inhibition percentage (GIP) of moldy fungi, first, 2 ml of each of the methanol and chloroform extracts were specifically mixed with 13 ml of Sabouraud Dextrose Agar (SDA) culture medium in a plate. After the mediums were transformed and sealed cold, *Sporothrix schneckei*, *Alternaria alternata*, *Mucor*, *Penicillium*, and *Trichophyton violaceum* were directly inoculated in the center of each plate. As a control, inoculation was done in the SDA medium without the desired extracts for each of the fungi. The control and treatment plates were placed in the incubator at 25º C. Then, the diameter of control and treatment colonies was measured once every 24 hours for 5 days. All test steps were repeated 3 times for each of the extracts (11).

2.4. Preparation of samples of moldy fungi to examine the damage caused to the fungus by SEM

In the first step, the mold was cut from the site using a sterile scalpel and placed in 2.5% glutaraldehyde at 4°C for 1 hour for initial fixation. In the second step, it was washed with 0.1 M sodium cacodylate buffer, and for secondary fixation, it was placed in 1% tetroxydaosmium solution at room temperature for 1 hour and washed again with 0.1 M sodium cacodylate buffer.

Finally, the last step was performed by passing the sample through 25, 50, 70, 90, and 100 % of ethanol each for 10 minutes as a dehydration agent and then drying the samples at ambient temperature.

After preparation, the sample was attached to a sample holder using double-sided carbon conductive glue and then covered with gold coating by the sputtering method, and imaging was done using a Scanning Electron Microscope(SEM) (12). Damage investigation was accomplished by SEM for each of *Sporothrix schneckei*, *Alternaria alternata*, *Mucor*, *Penicillium*, and *Trichophyton violaceum* fungi.

2.5. Investigating the activity of *Ganoderma lucidum* fungus on the inhibition of melanin production by mold fungi

To determine the depigmentation of mold fungi by methanolic and chloroform extracts of *Ganoderma lucidum*mushroom, 2 ml of the extracts separately were mixed with 13 ml of SDA medium in a plate. After cooling the medium, *Sporothrix schneckei*, *Alternaria alternata*, *Mucor*, *Penicillium*, and *Trichophyton violaceum* were planted separately in the center of each plate. As a control, each fungus was cultured separately in the SDA without the *Ganoderma lucidum* extracts. The control and treatment plates were placed at room temperature. Then, the fungi were compared with the control in terms of pigment production, and depigmentation was observed by a light microscope (10,13).

2.6. Data analysis

The mean and standard deviation values were determined based on the results of Man-Whitney and Friedman test comparing the percentage of growth inhibition between two methanolic and chloroform extracts.

3. RESULTS

3.1. Investigating the antimicrobial effect of *Ganoderma lucidum* mushroom on the pathogenic fungi

- Inhibition of *Mucor*

According to the Mann-Whitney test, comparing the GIP between two methanolic and chloroform extracts, it was found that on all the first (p=0.046), second (p=0.050), third (p=0.046), fourth (p=0.050), and fifth (p=0.046) days, the GIP of the chloroform extract was significantly higher than the methanolic extract (Fig. 1).

- Inhibition of *Penicillium*

Based on the Mann-Whitney test, it was discovered that in comparison with the methanolic extract, the GIP of the chloroform extract was significantly lower on the second (p=0.043), third (p=0.046), fourth (p=0.043), and the fifth day (p=0.050). On the first day, there was no significant difference between the growth percentage of the two extracts (p=1.00) (Fig. 1).

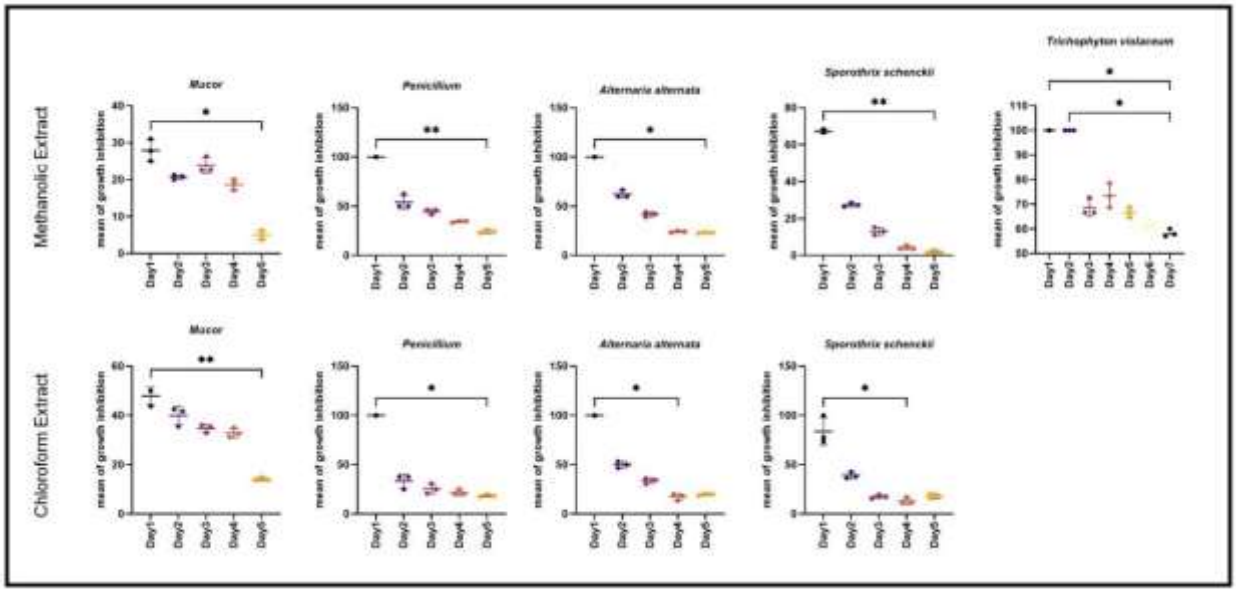

Fig.1 Mean (± standard deviation) growth inhibition percentage of *Mucor*, *Penicillium*, *Alternaria alternata*, *Sporothrix schenckii*, and Trichophyton *Violaceum* by the methanolic and chloroform extracts of Ganoderma *lucidum* from the first to the fifth day Significant differences are marked with the sign *

- Inhibition of *Alternaria alternata*

According to the Mann-Whitney test, comparing the percentage of growth inhibition between the intended extracts, it was found that on the second (p=0.046), third (p=0.050), fourth (p=0.050), and the fifth day(p=0.050) the GIP of the chloroform extract was significantly lower than methanolic extract. And on the first day, no significant difference was observed between the GIP of the two extracts (p=1.00) (Fig. 1).

- Inhibition of *Sporothrix schenckii*

According to the Mann-Whitney test, comparing the percentage of growth inhibition between ~~two~~ the methanolic and chloroform extracts, we found that in the first (p=0.046), second (p=0.050), third (p=0.050) p=0), fourth

(p=0.046), and the fifth day(p=0.050) the GIP of the chloroform extract was significantly more than the methanolic extract (Figure 1).

- Inhibition of *Trichophyton Violaceum*

According to the analysis of results by Friedman's test, there was a significant difference in the GIP of the methanolic extract between the first and seventh days. (p=0.007) Wilkason's post hoc test was performed with Bonferroni adjustment for two-by-two comparisons between seven days of measurement. The results are demonstrated in Figure 1. A noticeable inhibition effect was found on this fungus by the chloroform extract **(**Figure 1).

3.2. Electron microscope images of fungi treated with methanolic and chloroform extracts of *Ganoderma lucidum*

When fungi are exposed to *Ganoderma lucidum* extracts, the mycelium is wrinkled or crushed. The hyphae are collapsed with holes created in the surface. This indicates the destruction of the cell membrane and the emptying of the cell cytoplasm as a result. The SEM images could clearly illustrate the mentioned damages. These are shown in Figures 2-3 for the *Mucor, Penicillium, Alternaria alternat, Sporothrix schenckii, and Trichophyton violaceum.*

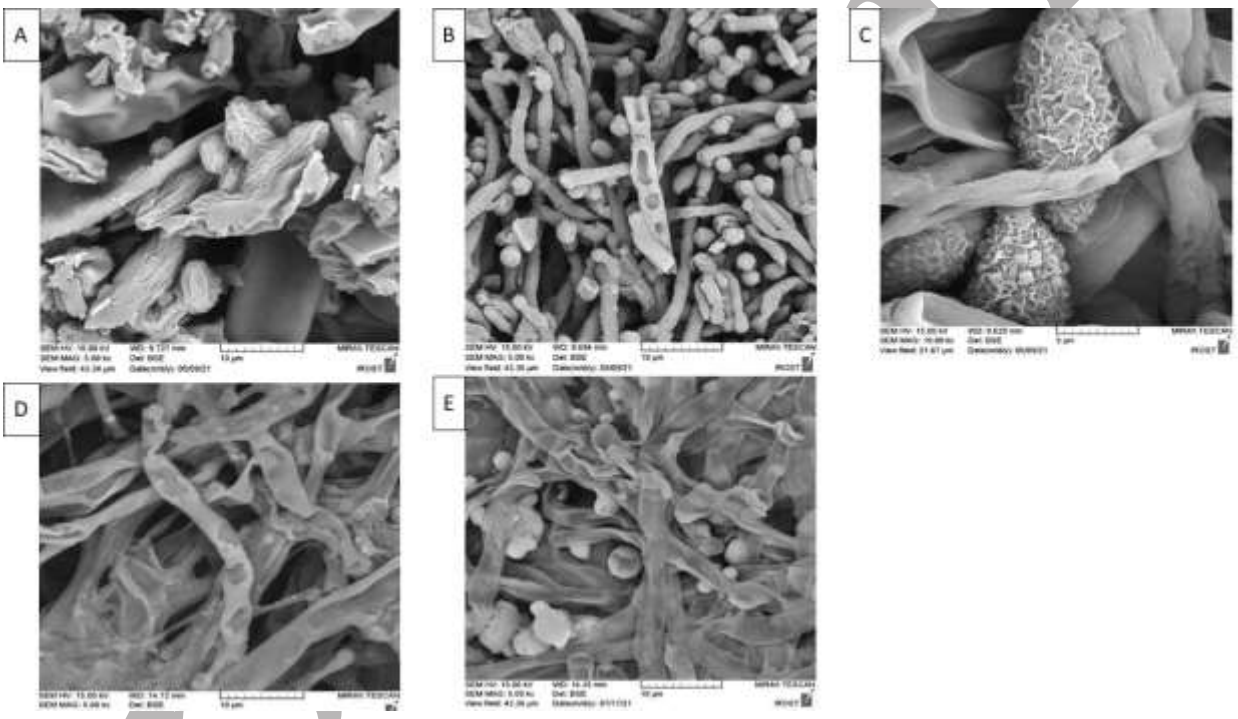

Figure 2 .SEM images of *Mucor* (a), *Penicillium* (b), *Alternaria alternat* (c), *Sporothrix schenckii* (d) *and Trichophyton violaceum* (e) structural damages caused by the methanolic extracts of *Ganoderma lucidum*

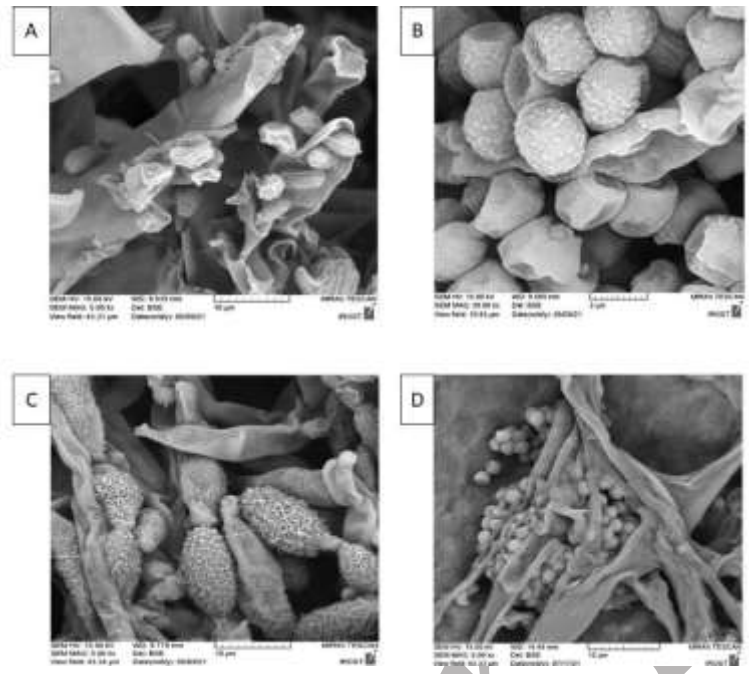

Figure 3 .SEM images of *Mucor* (a), *Penicillium* (b), *Alternaria alternat* (c), *and Sporothrix schenckii* (d) structural damages caused by the chloroform extracts of *Ganoderma lucidum*

3.3. Investigating the ability of *Ganoderma lucidum* to inhibit the melanin production of mold fungi

Morphological changes in the melanization of *Penicillium* fungus and depigmentation by methanolic extract are evident in Figure 4. Morphological changes of conidiophores revealed that the tested fungus is melanized. To better detect the depigmentation activity, microscopic slides were taken from the control and the methanol extract. Also, *Sporothrix*, *Alternaria*, *Mucor*, and *Trichophyton violaceum* fungi were tested, with no results of depigmentation observed (Figure 4).

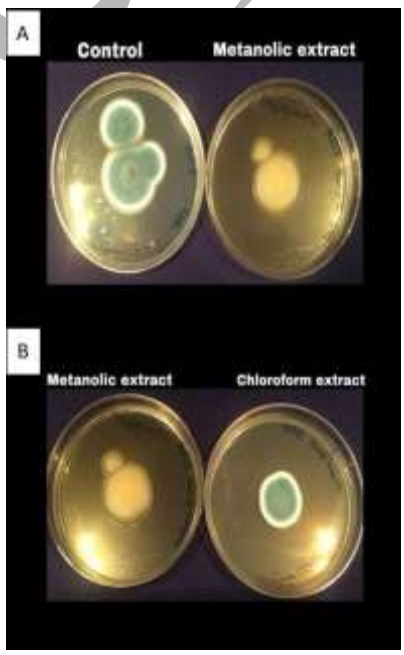

Figure 4. Morphological changes and melanization in *Penicillium* culture treated with methanol extract compared with *Penicillium* culture without extract (control) and chloroform extract

4. DISCUSSION

Today, many antifungal drugs are used to treat pathogenic fungal infections which have side effects. *Ganoderma lucidum* is used as an effective medicine to treat various diseases and infections in China and other Asian countries. Studies have shown that this mushroom contains various chemical compounds including polysaccharides, triterpenoids, alkaloids, and ergosterol (14,15). The results of this research revealed that the methanolic and chloroform extracts of *Ganoderma lucidum* benefited from an inhibitory effect on diverse variants of mold fungi.

In this study, a comparison was made between methanolic and chloroform extracts on five consecutive days at 25ºC, and the results showed that the maximum inhibitory effect for *Mucor and Sporothrix* was for the chloroform extract at different times. In cases of *Penicillium*, *Alternaria*, and *Trichophyton violaceum*, most of the inhibitory effect pertained to the methanolic extract.

Specifically, the maximum growth inhibition percentage of *Ganoderma lucidum* mushroom extracts on Mucor on the first five days was 27.92±3 and the minimum growth inhibition percentage on the fifth day was 1.25±5 for the methanolic extract. For the chloroform extract, the maximum GIP on the first day was 47.92±3.61 and the minimum inhibition percentage on the fifth day was 14.17±0.72. The comparison of the GIPs for *Penicillium* during the same period revealed that the highest inhibitory effect for both extracts was on the first day with 100% inhibition, and the lowest effect on the fifth day was 27.27±1.70 for the methanolic extract and 27.27±1.70 for the chloroform extract.

Comparing different days for both extracts, the results showed that the methanolic extract had a higher percentage of inhibition than the chloroform extract. The results of the GIP for *Alternaria alternata a* were reported as 100% inhibition for the first day in both extracts, while on the second day, the maximum inhibition was 62.22±3.85 for the methanolic extract. The results showed that the methanolic extract had the greatest inhibitory effect on this fungus.

The investigation carried out on *Sporothrix schenckii* revealed that the best inhibitory effect was by the chloroform extract with a GIP of 82.82±14.16 on the first day and 17.8±2.43 on the fifth day, while for the methanolic extract, these values were 67.25±1.02 on the first day and 20.3±0.79 on the fifth day. Also, while no growth percentage was observed for *Trichophyton violaceum*in the chloroform extract, the methanolic extract showed highly effective onthis fungus. Based on the results obtained in this research, it is probable that some morphological changes are made in the cell wall of the fungi by the *Ganoderma lucidum* metabolites thatfinally prevent their growth.

Helano et al. 2013 (6) showed that the antimicrobial activity of medicinal mushrooms can be due to compounds with high and low molecular weights. They also believe that the grapholine terpene compound have the highest antifungal activity. Also, phenolic acid and its derivatives such as hydroxybenzoic acid have been identified in *Ganoderma lucidum*, which could have antifungal activity on fungi such as *Aspergillus fumigatus, Aspergillus niger*, etc. These compounds can affect the membrane of fungal cells and prevent the growth of fungi. During the research of Azadbakht et al. 2015(16) methanolic solvent extracts compounds such as anthocyanins, saponins, tannins, flavons, phenols, polyphenols, lactones, triterpenoids, glycosides, and resins. Chloroform solvents can also extract effective compounds such as terpenoids, flavonoids, anthraquinones, glucosinolates, cannabinoids, steroids, and alkaloids from the plant. The extraction of such compounds with a special molecular structure that can penetrate the fungal cell can have high antifungal activity against pathogenic fungi. It is possible that methanol and chloroform solvents could efficiently extract the antimicrobial compounds of *Ganoderma lucidum*.

The results of the present study showed that the chloroform extract of *Ganoderma lucidum* had a greater inhibitory effect on *Mucor, Sporothrix schenckii and Trichophyton violaceum* than the methanol extract, while a lesser effect was observed on *Penicillium* and *Alternaria alternata*. These findings are consistent with the results of Rostaminejad et al. (2020), who found that *Ganoderma lucidum* extract showed significant anti-*Candida albicans* effect and had a low MIC(7). Also, the study of Mousavi et al. (2023) showed that the methanol extract of *Ganoderma lucidum* inhibits a wide range of bacteria and fungi, and the effect of organic extracts is stronger than that of aqueous extracts(8). In a review study titled Ganoderma lucidum: A look at antimicrobial and antioxidant properties with secondary metabolite development have also stated that organic extracts and ganodermin protein are able to inhibit the growth of fungal spp such as *Fusarium oxysporum, Trichoderma viride* and *Alternaria alternata*(17). SEM images in the present study showed that the extracts caused hyphal disintegration and cell membrane perforation, which is consistent with the antifungal mechanisms reported in these references. Our findings on the effect of methanol on reducing melanin production in *Penicillium* are also noteworthy and are in line with previous reports on the demelanizing activity of *Ganoderma lucidum* glucan sulfate (18). Also, the observation of significant daily differences in the growth inhibition rate indicates the time and concentration dependence of the effect of the extracts, which is consistent with the MIC results and the effect of boiled extracts in the studies of Idu et al. (19). Therefore, the results of this study not only confirm the broad antifungal effect of organic extracts of G. lucidum, but also provide preliminary evidence of the antimelanin activity of these extracts and highlight the importance of using natural compounds with a broad spectrum of activity in the treatment of fungal infections.

Therefore, it can be stated that the mold GIP results of methanolic and chloroform extracts compared to other extracts in our research are consistent with most of the research done by others, presenting more reasons for the higher efficacy of Ganoderma mushroom extracts on fungi growth inhibition.

The antioxidant, antibacterial, and DNA protection activity of the protein extracted from *Ganoderma lucidum* shows that the protein extracts of *Ganoderma lucidum* mycelium and fruit body have antibacterial activity. In the amount of tested protein, 25 micrograms of fruit body extract protein and 115 micrograms of mycelium extract protein were obtained. It was found that *Ganoderma lucidum* fruit body protein extract can inhibit all tested bacteria except *Staphylococcus aureus*, while the extract *Ganoderma lucidum* mycelium protein can inhibit all tested bacteria except *Pseudomonas aeruginosa* (20). According to the studies conducted by researchers, *Ganoderma lucidum* G2 mushroom has proteins with antimicrobial properties on a wide range of Gram-positive and Gram-negative bacteria, especially on *Staphylococcus epidermidis* (21).

Studies have shown that the pigment of mushrooms plays an important role in protecting mushrooms against immune cells. By producing pigments, fungi inhibit the toxic oxygen metabolites produced by the host's macrophages and neutrophils, thus avoiding the host's immune system. The production of pigment by the fungus contributes to pathogenicity inside the host cell (6). Polysaccharides or sulfated glucans are usually found in marine organisms, but recently they have also been reported in the structure of fungi. Glucan sulfate with the structure of beta-1,3-D-glucan extracted from *Ganoderma lucidum* mycelium shows antimicrobial activity against pathogenic bacteria and fungi. This structure was also non-cytotoxic for human cells. Glucan sulfate in *Ganoderma* is soluble and has significant therapeutic activity compared to non-sulfated *Ganoderma* Glucan. As a result, *Ganoderma lucidum* having such compounds has different biological activities, including antifungal effect and depigmentation activity (13). Genes responsible for melanin production in fungi including alb1, ketide synthetase PKS, arp1, citalone reductase, arp2, hydroxynaphthalene reductase, abr1, abr2, laccase, and ayg1 are of unknown function. As a result, compounds such as sulfated glucans likely inhibit and prevent the production of melanin production genes in mushrooms (10). Helano et al. 2013 (6)and Wan-Mohtar et al. 2017 (13) investigated the potential of *Ganoderma lucidum* methanol extract on demelanization of *Aspergillus niger* for 72 hours. The colour of *Aspergillus niger* changed from black to white. In the present research, morphological changes during the demelanization process showed that the number of *Penicillium* conidia under the treatment with the methanol extract of *Ganoderma lucidum* decreased compared to the control. Also, the colony morphology changed from mold to mucoid and the colour of the colony from blue-green to

colorless. Therefore, it is possible that the methanol extract of *Ganoderma lucidum*, having compounds such as sulfated glucans, is directly involved in inhibiting or modifying polyketide synthetase type 1. However, more studies should be done to investigate the mechanism of demelanization. Also, the depigmentation ability of other moldy fungi such as *Sporothrix schneckei*, *Alternaria alternata*, *Mucor*, and *Trichophyton violaceum* by *Ganoderma Lucidum* were investigated here, but no results were observed.

The main function of the cell wall is to provide the shape and integrity of the cell, acting as an osmotic barrier. Hence, observation of changes in the cell structure of bacteria and fungi by SEM can help to clarify the mode of action of antimicrobial agents, including mushroom extracts. In addition, much attention has been paid to the use of natural antimicrobial compounds derived from natural sources such as plants, animals, bacteria, algae, and fungi to reduce the harm of microorganisms, and improve the quality and shelf life of food (22). According to the research by Turecka et al. (2018) (23), antifungal substances prevent the formation of an essential lipid in the cell membrane of the fungus called ergosterol and avoid the formation of beta-glucan or chitin in the mushroom wall. They cause abnormality, cell accumulation, malformation, inhibition of growth, and ultimately death of the fungus.

Some phenolic compounds, such as tannins, have a toxic effect on mushrooms when combined with mushroom proteins. Also, among other phenolic compounds such as phenolic acid, flavonoid, xanthone, anthocyanin, and anthocyanins, it was lipophilic and despite this characteristic, it affects the membrane compounds and prevents the exchange of substances (24). Also, the observations of scanning electron microscope in the research of Muniyappan show that the extract of *Ganoderma lucidum* can stop the germination of *Colletotrichum gloeosporioides* and cause contraction, contraction of mycelium and abnormality in the spores of this fungus (25). In this research, the effect of methanolic and chloroform extracts of *Ganoderma lucidum* on *Sporothrix schenckii, Alternaria alternata, Mucor, Penicillium, and Trichophyton violaceum*molds was investigated by electron microscope. Abnormal unevenness on the surface of conidia and mycelium is visible in the photos taken. The results show that the myceliums of the five fungi in question are shriveled, fragmented, and narrowed, which indicates membrane damage and plasmolysis of cell contents. Also, the conidia of mold fungi treated with two mushroom extracts are depressed and the perforation of the conidia is visible.Regarding the morphological changes shown by the electron microscope and also investigating the inhibitory effect of methanolic and chloroform extracts of *Ganoderma lucidum* on the destruction of the spore cell wall and hyphae of *Sporothrix schenckii, Alternaria alternata, Mucor, Penicillium, and Trichophyton violaceum* fungi in the present study, we found that the extracted compounds cause morphological changes in the fungi structure and significantly inhibit their growth. *Ganoderma lucidum* methanolic extract changed the *penicillium* colony morphology from mold to mucoid and the colour of the colony from blue-green to colorless.

DECLARATIONS

Ethics Approval

There were no human subjects or animal experiments in our study.

Competing Interests

The authors have declared that no competing interests exist.

Authors' Contributions

Study concept and design:M.M

2- Acquisition of data:S.J.K

3- Analysis and interpretation of data:S.J.K., S.M.H.K

4- Drafting of the manuscript:M.M.,S.J.K

5- Critical revision of the manuscript for important intellectual content: P.SH

6- Statistical analysis: S.M.H.K

8- Study supervision:M.M

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Availability Of Data And Materials

All relevant data are within the paper and its supporting information files.

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