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Comparative Antifungal Mechanisms of Alcoholic and Nano-Ganoderma lucidum Extracts Against Aflatoxigenic Aspergillus Flavus

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ABSTRACT

The present study investigated the antifungal mechanisms of alcoholic and nano-formulated extracts of Ganoderma lucidum against aflatoxigenic Aspergillus flavus. The effects of both extracts on intracellular reactive oxygen species (ROS) production, cell membrane permeability, extracellular enzyme activities, and fungal ultrastructure were evaluated. ROS analysis revealed a marked increase in oxidative stress in treated fungal cells, with the nano-formulated extract producing the highest ROS accumulation compared with the alcoholic extract and untreated control. Increased membrane permeability was also observed, indicating severe disruption of cellular integrity. Scanning electron microscopy (SEM) demonstrated pronounced morphological alterations, including hyphal deformation, shrinkage, and surface damage, while transmission electron microscopy (TEM) revealed extensive ultrastructural changes such as membrane disruption, cytoplasmic disorganization, and degradation of intracellular organelles. In addition, both extracts reduced the activities of extracellular enzymes, including protease, lipase, and amylase, with the nano-formulated extract exhibiting greater inhibitory effects. The findings suggest that the antifungal activity of G. lucidum extracts is mediated through multiple mechanisms involving oxidative stress induction, membrane damage, enzymatic inhibition, and structural deterioration of fungal cells. The nano-formulated extract showed superior antifungal efficacy compared with the alcoholic extract, highlighting its potential as an environmentally friendly alternative for controlling aflatoxigenic fungi and reducing mycotoxin contamination in food systems.

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INTRODUCTION

Mycotoxigenic fungi constitute a major threat to food safety and public health because of their ability to contaminate agricultural commodities and produce toxic secondary metabolites known as mycotoxins. Among these fungi, *Aspergillus flavus* is considered one of the most important species due to its capacity to synthesize aflatoxins, particularly aflatoxin B1 (AFB1), which is recognized as one of the most potent naturally occurring carcinogens. Aflatoxin contamination of food and feed causes significant economic losses worldwide and poses serious health hazards to humans and animals, including hepatotoxicity, immunosuppression,

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mutagenicity, and carcinogenicity [1].

Conventional approaches for controlling mycotoxigenic fungi rely predominantly on synthetic fungicides. However, the extensive and prolonged use of these chemicals has raised concerns regarding environmental contamination, the emergence of resistant fungal strains, accumulation of chemical residues in food products, and potential adverse effects on human health. Consequently, increasing attention has been directed toward the development of environmentally friendly and sustainable alternatives derived from natural sources. Among these alternatives, medicinal mushrooms have attracted considerable interest because of their rich content of biologically active compounds with antimicrobial, antioxidant, and therapeutic properties [2].

Ganoderma lucidum (Reishi mushroom) is one of the most extensively investigated medicinal fungi and has been widely used in traditional medicine for centuries. Its biological activities are attributed to a diverse array of bioactive constituents, including triterpenoids, polysaccharides, phenolic compounds, flavonoids, sterols, and peptides. These compounds have demonstrated broad-spectrum biological activities, including antioxidant, antimicrobial, anti-inflammatory, and anticancer effects [3; 4].

Previous studies have also reported significant antifungal activity of *G. lucidum* extracts against several pathogenic and mycotoxigenic fungi, including Aspergillus flavus. The antifungal action of these extracts has been associated with disruption of cell membrane integrity, induction of oxidative stress, and interference with essential metabolic processes required for fungal growth and toxin production [5].

Recent advances in nanotechnology have opened new avenues for improving the efficacy of natural bioactive compounds. Nano-formulation of plant and fungal extracts can enhance their stability, solubility, bioavailability, and cellular uptake, thereby increasing their interaction with microbial targets. Owing to their small particle size and high surface-area-to-volume ratio, nano-formulated products frequently exhibit greater antimicrobial activity than their conventional forms. Despite the growing number of studies highlighting the potential of nano-formulated natural extracts as antifungal agents, the precise cellular and ultrastructural mechanisms underlying their inhibitory effects against mycotoxigenic fungi remain inadequately understood [6].

Therefore, the present study was designed to investigate and compare the antifungal mechanisms of alcoholic and nano-formulated extracts of Ganoderma lucidum against aflatoxigenic *Aspergillus flavus*. Particular emphasis was placed on evaluating oxidative stress generation through reactive oxygen species (ROS) production, alterations in cell membrane permeability, inhibition of extracellular enzymatic activities, and ultrastructural changes using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Elucidating these mechanisms may contribute to the development of effective, eco-friendly, and sustainable strategies for controlling mycotoxigenic fungi and minimizing aflatoxin contamination in food and feed systems.

MATERIALS AND METHODS

Determination of Reactive Oxygen Species (ROS)

Intracellular reactive oxygen species (ROS) production in *Aspergillus flavus* cells treated with alcoholic and nano-formulated Ganoderma lucidum extracts was determined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Fungal cultures were grown under appropriate conditions and exposed to the tested extracts for the designated incubation period. Subsequently, fungal cells were harvested and washed three times with phosphate-buffered saline (PBS) to remove residual culture medium.

The washed cells were incubated with DCFH-DA solution for 30 min in the dark. Following cellular uptake, DCFH-DA was hydrolyzed to non-fluorescent DCFH, which was oxidized by intracellular ROS to form the fluorescent compound dichlorofluorescein (DCF). Fluorescence intensity was measured using a fluorescence spectrophotometer and expressed as an indicator of intracellular ROS accumulation. Increased fluorescence intensity was considered indicative of elevated ROS production [7].

Scanning Electron Microscopy (SEM)

Morphological alterations in fungal cells following treatment with alcoholic and nano-formulated G. lucidum extracts were examined using scanning electron microscopy (SEM). Treated fungal biomass was collected and washed repeatedly with PBS, followed by fixation in 2.5% glutaraldehyde. The samples were subsequently

rinsed with PBS and dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%).

The dehydrated samples were dried, mounted on aluminum stubs, and sputter-coated with a thin layer of gold to improve conductivity. The prepared specimens were then examined using a scanning electron microscope, and representative micrographs were recorded to assess treatment-induced surface morphological changes [8].

Transmission Electron Microscopy (TEM)

Ultrastructural changes in fungal cells were investigated using transmission electron microscopy (TEM). Following treatment, fungal samples were collected, washed with PBS, and fixed in 2.5% glutaraldehyde. Secondary fixation was performed using osmium tetroxide (OsO_4).

The samples were dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections were prepared using an ultramicrotome, mounted on copper grids, and stained with uranyl acetate and lead citrate. The sections were examined under a transmission electron microscope, and images were captured to evaluate intracellular ultrastructural alterations induced by the tested extracts [9].

Determination of Extracellular Enzyme Activities

The effects of alcoholic and nano-formulated G. lucidum extracts on extracellular enzyme production by A. flavus were evaluated by assessing protease, lipase, and amylase activities. Fungal cultures were inoculated onto specific media containing appropriate substrates for each enzyme. Casein-containing medium was used for protease activity, Tween-containing medium for lipase activity, and starch agar medium for amylase activity.

Following incubation, enzyme activity was assessed by measuring the diameter of the hydrolysis zone surrounding fungal colonies. For amylase determination, iodine solution was added to the starch medium after incubation, and the clear zone surrounding the colony was measured. Enzyme activity was expressed as the diameter of the substrate degradation zone and compared with the untreated control [10].

Cell Membrane Permeability Assay

The effect of alcoholic and nano-formulated G. lucidum extracts on fungal cell membrane integrity was assessed by measuring the leakage of intracellular components into the extracellular medium. Treated fungal mycelia were harvested, washed thoroughly with PBS, and resuspended in sterile buffer solution.

Following incubation, the absorbance of the extracellular medium was measured spectrophotometrically at 260 nm to quantify the release of intracellular materials. Increased absorbance values were considered indicative of enhanced membrane permeability and cellular leakage resulting from membrane damage. Untreated fungal cultures served as the control group [11].

RESULTS AND DISCUSSION

Reactive Oxygen Species (ROS) Production

The intracellular ROS levels in Aspergillus flavus increased significantly following treatment with

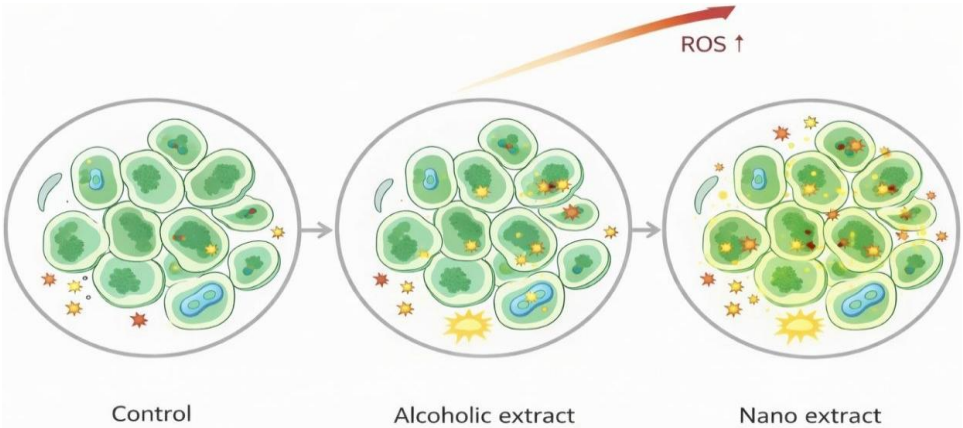

Fig. 1. Schematic illustration of increased reactive oxygen species (ROS) accumulation in Aspergillus flavus cells following treatment with alcoholic and nano-formulated Ganoderma lucidum extracts.

both alcoholic and nano-formulated Ganoderma lucidum extracts compared with the untreated control. The absorbance value increased from 0.25 in the control group to 0.62 and 0.91 following treatment with the alcoholic and nano extracts, respectively (Table 1). The fluorescence images further confirmed these findings, showing a gradual increase in ROS accumulation from the control to the alcoholic extract treatment and reaching the highest intensity in fungal cells exposed to the nano-formulated extract (Fig. 1).

The elevated ROS production indicates that both extracts induced oxidative stress within fungal cells, leading to cellular damage through oxidation of lipids, proteins, and nucleic acids. The stronger ROS generation observed in the nano-extract treatment may be attributed to the enhanced surface activity and cellular interaction of nanoparticles, resulting in greater oxidative damage and antifungal efficacy. Similar findings have been reported for nanoparticle-mediated antifungal activity, where excessive ROS generation represents a primary mechanism of fungal inhibition [12].

Scanning Electron Microscopy (SEM)

SEM analysis revealed substantial morphological alterations in A. flavus following treatment with the tested extracts (Fig. 2). Untreated fungal cells exhibited normal morphology characterized by smooth and intact hyphae with regular surface architecture. In contrast, fungal cells treated with

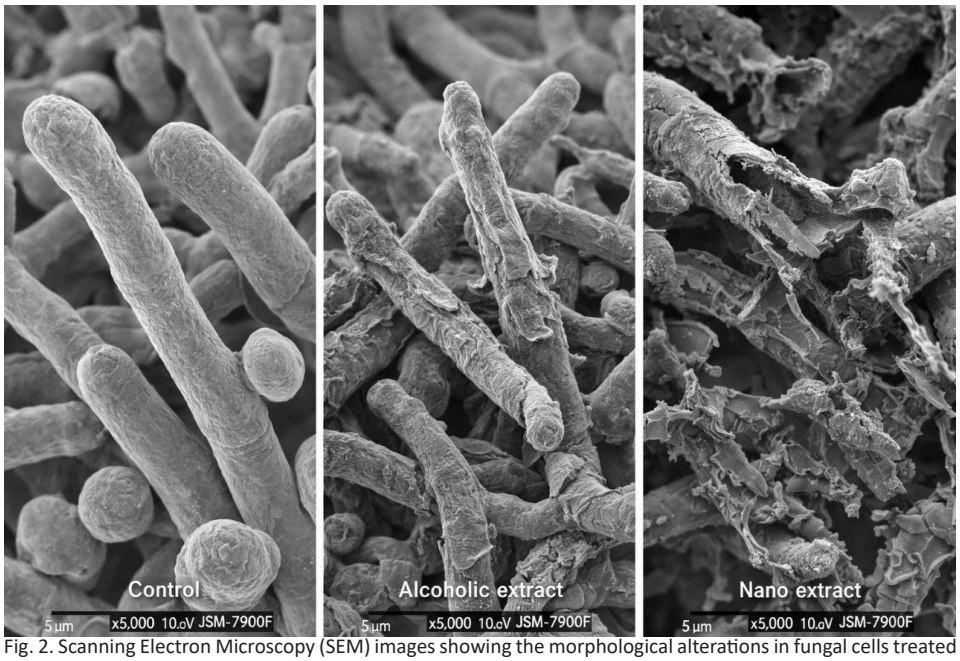

Fig. 2. Scanning Electron Microscopy (SEM) images showing the morphological alterations in fungal cells treated with alcoholic and nano-formulated extracts compared with the control.

Table 1. Reactive Oxygen Species (ROS) production in Aspergillus flavus following treatment with alcoholic and nano-formulated *Ganoderma lucidum* extracts.

No.	Treatment	ROS
1	Control	0.25
2	Alcoholic Extract	0.62
3	Nano Extract	0.91

the alcoholic extract showed noticeable surface deformation, shrinkage, and partial collapse of hyphal structures.

More pronounced morphological damage was observed in cells treated with the nano-formulated extract, including severe distortion of hyphae, surface rupture, deep fissures, and complete loss of normal cellular morphology. These observations suggest that the nano-extract caused extensive damage to the fungal cell wall and membrane, thereby compromising cellular integrity. The enhanced activity of the nano-formulation may be attributed to its smaller particle size and increased surface area, which facilitate stronger interactions with fungal cell structures.

Transmission Electron Microscopy (TEM)

TEM examination demonstrated marked ultrastructural alterations in fungal cells following treatment with both extracts (Fig. 3). Control cells displayed intact cell walls, well-organized cytoplasm, and normal intracellular organelles. Treatment with the alcoholic extract resulted in partial cytoplasmic shrinkage, membrane irregularities, and moderate disruption of intracellular organization.

In contrast, fungal cells treated with the nano-formulated extract exhibited severe ultrastructural damage, including disruption of the cell wall and plasma membrane, cytoplasmic disintegration, extensive vacuolization, and degradation of intracellular organelles. These findings indicate that the nano-extract penetrated fungal cells and induced extensive intracellular damage, ultimately leading to cellular collapse. Similar ultrastructural alterations have been reported as indicators of

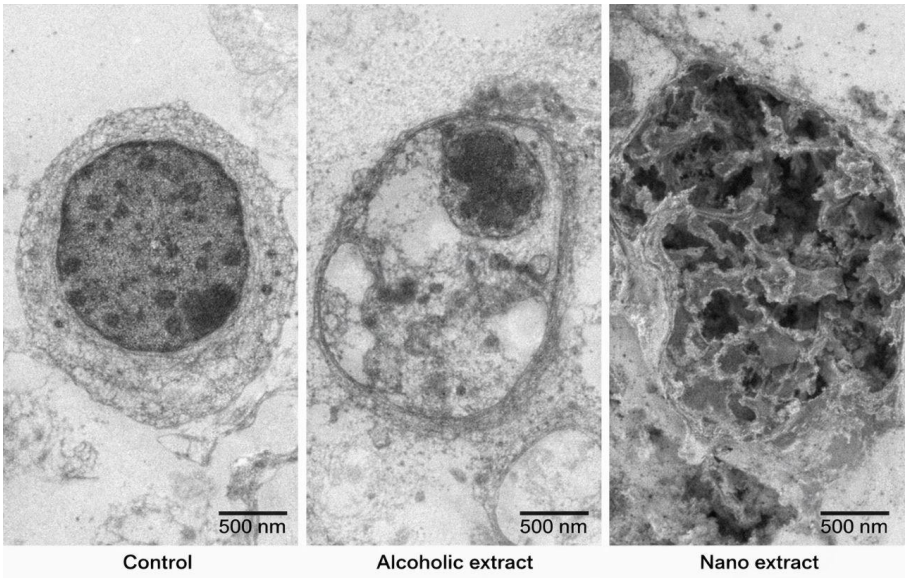

Fig. 3. Transmission Electron Microscopy (TEM) images showing the ultrastructural changes in fungal cells treated with alcoholic and nano-formulated extracts compared with the control.

Table 2. Effect of alcoholic extract and nano-formulated extract on the activity of extracellular enzymes in the fungus.

No.	Treatment	mm Protease	mm Lipase	Amylase mm
1	Control	22	20	24
2	Alcoholic Extract	14	13	16
3	Nano Extract	7	6	8

nanoparticle-induced fungal cell death [13].

Extracellular Enzyme Activities

The activities of protease, lipase, and amylase were markedly reduced following treatment with both alcoholic and nano-formulated extracts compared with the control (Table 2). The untreated fungal cultures exhibited the highest enzyme activities, as indicated by larger hydrolysis zones surrounding fungal colonies. Treatment with the alcoholic extract produced a moderate reduction in enzyme activity, whereas the nano-

Fig. 4. Effect of alcoholic and nano-formulated Ganoderma lucidum extracts on extracellular enzyme activities (protease, lipase, and amylase) in Aspergillus flavus compared with the control.

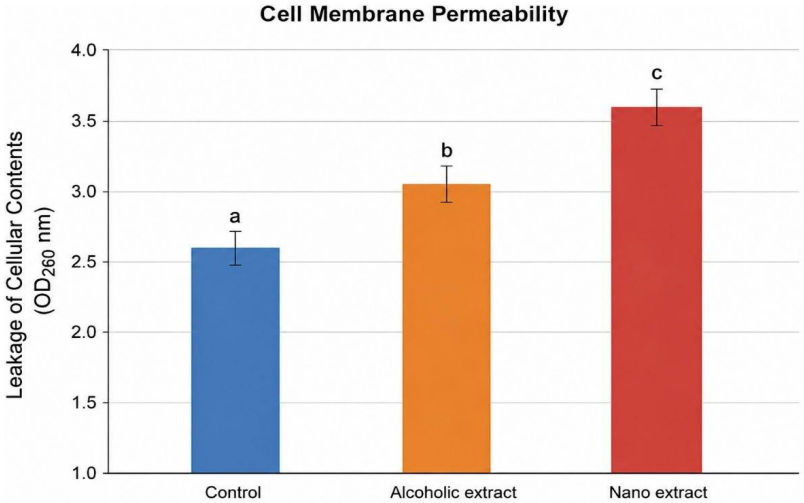

Fig. 5. Effect of alcoholic and nano-formulated Ganoderma lucidum extracts on cell membrane permeability of Aspergillus flavus measured as leakage of cellular contents at 260 nm.

Table 3. Effect of alcoholic and nano-formulated Ganoderma lucidum extracts on cell membrane permeability of Aspergillus flavus measured at 260 nm.

No.	Treatment	Cell Membrane Permeability (Absorbance at 260 nm)
1	Control	0.18
2	Alcoholic Extract	0.47
3	Nano Extract	0.83

extract caused a substantial inhibition, resulting in minimal hydrolysis zones.

The suppression of extracellular enzyme production may significantly impair fungal growth and pathogenicity by limiting nutrient acquisition from the surrounding environment. Furthermore, oxidative stress induced by the extracts may contribute to enzyme inactivation and disruption of protein biosynthesis, thereby reducing the secretion of hydrolytic enzymes required for fungal metabolism [14].

Fig. 4 illustrates the reduction in extracellular enzyme activities (protease, lipase, and amylase) following treatment with alcoholic and nano-formulated extracts compared with the control.

Cell Membrane Permeability

A significant increase in cell membrane permeability was observed in fungal cells treated with both extracts compared with the untreated control (Table 3). The nano-formulated extract produced the highest membrane permeability, indicating severe membrane damage and leakage of intracellular constituents into the extracellular medium.

The increased membrane permeability can be attributed to ROS-mediated lipid peroxidation and direct interactions between nanoparticles and membrane components. Damage to membrane integrity disrupts cellular homeostasis and promotes leakage of essential intracellular molecules, ultimately leading to growth inhibition and cell death. These findings are consistent with the SEM and TEM observations, which demonstrated extensive structural damage in treated fungal cells. (Fig. 5) illustrates the significant increase in cell membrane permeability in Aspergillus flavus following treatment with alcoholic and nano-formulated Ganoderma lucidum extracts, with the nano-formulated extract exhibiting the highest permeability.

Proposed Antifungal Mechanism

Collectively, the findings suggest that the antifungal activity of alcoholic and nano-formulated Ganoderma lucidum extracts is mediated through a multi-target mechanism involving ROS overproduction, oxidative stress induction, membrane disruption, increased cellular leakage, inhibition of extracellular enzymes, and severe morphological and ultrastructural damage. The nano-formulated extract exhibited superior antifungal efficacy compared with the alcoholic extract, highlighting the potential of nanoformulation as an effective strategy for enhancing the antifungal properties of G. lucidum against aflatoxigenic fungi.

CONCLUSION

The present study demonstrated that both alcoholic and nano-formulated Ganoderma lucidum extracts exhibited effective antifungal activity against aflatoxigenic Aspergillus flavus, with the nano-formulated extract showing superior efficacy. The antifungal mechanism was associated with the induction of intracellular reactive oxygen species (ROS), resulting in oxidative stress and subsequent cellular damage. Increased ROS accumulation was accompanied by enhanced cell membrane permeability, indicating disruption of membrane integrity and leakage of intracellular components. Furthermore, both extracts significantly affected fungal morphology and ultrastructure, as confirmed by SEM and TEM analyses, which revealed deformation of hyphae, disruption of cell walls and membranes, cytoplasmic disorganization, and extensive intracellular damage. The treatments also reduced the activities of extracellular enzymes, including protease, lipase, and amylase, thereby impairing fungal metabolic functions and nutrient acquisition. Collectively, these findings suggest that the antifungal activity of G. lucidum extracts involves multiple interconnected mechanisms, including oxidative stress induction, membrane disruption, enzymatic inhibition, and structural deterioration of fungal cells. The enhanced effectiveness of the nano-formulated extract highlights the potential of nanotechnology to improve the antifungal properties of natural bioactive compounds. Therefore, nano-G. lucidum extract may represent a promising eco-friendly alternative for the control of aflatoxigenic fungi and the reduction of mycotoxin-related contamination in food and agricultural products.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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