

Antifungal Activity of Green Synthesized Silver Nanoparticles with Natural Reducing Agents against *Aspergillus* and *Penicillium* Species

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Abstract

Green synthesis technology is a novel biological strategy for producing nanomaterials that are widely used in biomedical applications. This study investigated the antifungal potential of green-synthesised silver nanoparticles (AgNPs), using activated carbon with mucus from the garden snail *Cornu aspersum* (AgNPs-AC) or tea tree essential oil (AgNPs-TT) as reducing agents. The agar well diffusion method and resazurin-based micro-dilution viability assays were employed to determine the inhibitory effect on the growth of novel nanocomposites against amphotericin-B-resistant strains belonging to the *Aspergillus niger* and *Penicillium griseofulvum* species. Formulations containing tea tree oil (AgNPs-TT) were found to have a considerably higher antifungal effect against the test strains than those containing activated carbon (AgNPs-AC). Replacing activated charcoal with tea tree oil was shown to enhance the effectiveness of the nanoparticles and extend the duration of their action. The findings reveal a synergistic interaction between snail mucus and essential oil, as well as the critical role of a higher Ag⁺ concentration. The growth inhibition rate after treatment with these new nanoformulations is comparable to or exceeds that obtained with Nys. Consequently, AgNPs produced using mucus and plant essential oils are a promising source for novel therapeutic formulations.

Keywords: Ag-nanoparticles, green synthesis technology, *Cornu aspersum* mucus, tea tree essential oil, antifungal effect

Резюме

Технологията „зелен синтез“ е нова биологична стратегия за производство на наноматериали, които се използват широко в биомедицината. Целта настоящата работа е да проучи противогъбичния потенциал на сребърни наночастици (AgNPs), получени чрез „зелен синтез“, чрез използване на активен въглен и слуз от градинския охлюв *Cornu aspersum* (AgNPs-AC) или етерично масло от чаено дърво (AgNPs-TT) като редуциращи агенти. Чрез метода на дифузия в агарови ямки и микроразредящи тестове на базата на резазурин, е определен инхибиращият ефект на новите наноконпозити върху растежа на устойчиви на амфотерицин-В щамове, принадлежащи към видовете *Aspergillus niger* и *Penicillium griseofulvum*. Установено е, че формулировките, съдържащи масло от чаено дърво (AgNPs-TT) имат значително по-висок ефект срещу тестовите щамове, отколкото тези, съдържащи активен въглен (AgNPs-AC). Замяната на активния въглен с масло от чаено дърво повишава ефективността на наночастиците и удължава продължителността на тяхното действие. Доказано е синергичното взаимодействие между слузта от охлюв и етеричното масло, както и критичната роля на по-високата концентрация на Ag⁺. Скоростта на инхибиране на растежа след третиране с използваните нови наноформулировки е сравнима или надвишава тази, получена след третиране с нистатин. Резултатите демонстрират перспективността на сребърни наночастици, съдържащи слуз и растителни етерични масла като обещаващ източник за нови терапевтични формулировки.

Introduction

In recent decades, the medical and ecological importance of fungal diseases has attracted greater attention. Published data indicate that these diseases affect more than 1 billion people worldwide. Fungal infections account for around 6.5 million invasive cases and approximately 3.8 million fatalities globally each year (Chaudhary and Thakur, 2025). The reasons for this situation are very complex. However, the increasing resistance of fungal strains to existing antifungal drugs is one of the main reasons (Denning, 2022; Tan *et al.*, 2022; Gurajala and Challapilla, 2026).

Invasive mycoses are a serious problem due to resistance to antifungal treatments. Furthermore, the spread of pathogens through global trade, increased travel, and climate change has made fungal infections uncontrollable (Vitiello *et al.*, 2023). Critical fungal infections are predominantly found in immunocompromised individuals, including those infected with Human Immunodeficiency Virus (HIV) or suffering from Severe Combined Immunodeficiency (SCID). These problems also arise in patients with endocrine and metabolic disorders, as well as in those receiving antineoplastic chemotherapy or immunosuppressive treatment following organ transplants (Casadevall, 2018). Numerous fungal infections among COVID-19 patients have been documented globally (Chandley *et al.*, 2022).

Among the fungal pathogens most responsible for severe systemic mycoses are species of several genera, mainly *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Candida*, *Blastomyces*, *Sporothrix*, *Trichoderma*, *Penicillium*, *Trichophyton*, etc. (Denning, 2022; Vitiello *et al.*, 2023). Among them, *Aspergillus* spp. and *Penicillium* spp. have been recognized as the causative agents of serious mycological diseases.

Aspergillosis is among the most widespread invasive mold infections, demonstrating a range of clinical forms. *Aspergillus fumigatus* is responsible for most of these infections; however, there have also been cases of infections caused by non-*fumigatus* *Aspergillus* species (Tan *et al.*, 2022; Mianrood *et al.*, 2025). Additionally, *A. niger* is of particular significance in human pathogenesis, especially in relation to respiratory infections (Calvo *et al.*, 2026). Despite being less virulent overall, *A. niger* causes 5–22.3% of *Aspergillus* infections, with a greater incidence of non-invasive diseases including otomycosis and colonization in pre-existing cavities (Franconi *et al.*, 2024; Rafique *et al.*, 2026). It is frequently found in indoor air and envi-

ronmental sources, and its frequency in respiratory samples is rising, especially in people with immunocompromised conditions.

Penicillium is one of the largest genera within the fungal kingdom, encompassing several widely recognized species. This genus has been observed to inhabit a wide variety of substrates and habitats ([Guevara-Suarez *et al.*, 2016). Cases of *Penicillium* spp. infections have been identified in humans, mainly immunocompetent and immunosuppressed individuals (Geltner *et al.*, 2013; Sharma and Nonzom, 2021). The clinical presentation varies based on the source of the infection. Respiratory infections are common and may include pneumonitis, pneumonia, pulmonary fibrosis, localized fungal aggregates, and pulmonary abscesses. Reported cases of invasive infections include endocarditis, peritonitis associated with peritoneal dialysis, brain abscesses, endophthalmitis, and cutaneous infections (Orós *et al.*, 1996; Mehta *et al.*, 2021).

In addition, the strains belonging to the genera *Aspergillus* and *Penicillium* also manifested a high tolerance to the existing antifungal agents (Guevara-Suarez *et al.*, 2016; Bottery *et al.*, 2024; Mianrood *et al.*, 2025). Recently, reports have indicated the emergence of drug-resistant species of *Aspergillus* and *Penicillium* due to extended exposure to antifungal agents (Hashem *et al.*, 2022; Sharma *et al.*, 2024; Oso *et al.*, 2025). In response to the growing importance of this problem, nanotechnology has emerged as a promising alternative (Yassin *et al.*, 2023; Singh *et al.*, 2024). Metal oxide nanoparticles are attracting increasing interest due to their energy efficiency and cost-effectiveness. They offer solutions to a variety of challenges in sectors such as healthcare, agriculture, cosmetics, food production, and optics. Silver nanoparticles (AgNPs) have been recognised as effective antifungal agents (Hashem *et al.*, 2022; Matras *et al.*, 2022). They can inhibit fungal cell development using a wide range of mechanisms. These include damaging phosphate groups in DNA, disrupting plasma membrane permeability, interacting with sulfhydryl groups in proteins and enzymes, and producing reactive oxygen species (ROS) (Li *et al.*, 2022; Liu *et al.*, 2024).

Green synthesis technology is a new biological approach to creating nanomaterials. The producing NPs have been found to be more effective, environmentally friendly, cost-effective, and non-hazardous (Pourakbar *et al.*, 2026; Singh *et al.*, 2026). This technology involves the use of biomolecules from various organisms such as bacte-

ria, yeast, mold, microalgae, and plants (Ali *et al.*, 2020; Guo *et al.*, 2022; Anjum *et al.*, 2026; Raj *et al.*, 2026). Plant extracts are the most commonly used in green synthesis. However, using bodily secretions from living organisms is far more beneficial, as the organisms are not destroyed and can be safely returned to their natural environment, helping to preserve biodiversity (Mane *et al.*, 2021).

In this context, mollusc body fluids are highly suitable for incorporation into nanoparticles. Data on the effective use of garden snail mucus-based nanoparticles have been published (Mane *et al.*, 2021; El-Zawawy and Mona, 2021; Consoli *et al.*, 2024). Snail mucus contains proteins, peptides, amino acids, hyaluronic acid, glycolic acid, vitamins, and antioxidants. It also contains metal ions (e.g. Cu, Zn, and Fe), as well as lactic acid, metalloproteinases, and their inhibitors (Aouji *et al.*, 2023).

Our previous publications demonstrate the significant antibacterial and antifungal activity of mucus from the garden snail *Cornu aspersum*, distributed in Bulgaria (Velkova *et al.*, 2024). Furthermore, incorporating *C. aspersum* mucus into the composition of nanoparticles as a reducing and stabilising agent increases the degree of inhibition of the growth of pathogenic Gram-positive and Gram-negative bacteria (Dolashka *et al.*, 2024; Todorova *et al.*, 2025).

The present study was designed to compare the antifungal activity of two types green synthesized silver nanoparticles (AgNPs) using as reducing agents: 1) activated carbon with mucus of the garden snail *Cornu aspersum*; 2) mucus from *C. aspersum* and essential oil from tea tree (*Melaleuca alternifolia*) against amphotericin B (AmB)-resistant strains belonging to the species *A. niger* and *P. griseofulvum*.

Materials and Methods

Sample preparation and characterization

The carbon adsorbent (AC) was produced via the pyrolysis of peach pit shells, following the methodology described by Dolashka *et al.* (2024). The mucus was collected using a patented technology from garden snails *C. aspersum*, grown in Bulgarian organic farms (Dolashka and Atanasov, 2015). The purified mucus, as described in Dolashki *et al.* (2020) and Velkova *et al.* (2024), was separated by ultrafiltration through membrane filters with a pore size of 20 kDa (Dolashki *et al.*, 2020; Velkova *et al.*, 2024). The obtained fraction with MW >20 kDa was used for the green synthesis of MeNPs in the presence of activated carbon from peach shells or in a mixture with tea tree *M. alternifolia* oil. The synthesized nanoparticles and adsorbents used in this study are presented in Table 1.

Synthesis of silver nanoparticles on activated carbon (AgNPs-AC)

Silver nanoparticles (AgNPs) were synthesized using a “green” biological route. A mixture of 45 mL pure snail mucus and 10 mL of 50 mM AgNO₃ was stirred mechanically at room temperature. The formation of AgNPs was visually confirmed by a distinct color change in the solution. Two variations of silver-carbon composites were obtained. AgNPs-AC1A: 25 mL of the synthesized AgNPs was centrifuged at 6 000 rpm for 15 min at 4°C. The resulting precipitate was washed three times and subsequently mixed with 0.1 g of AC in 10 mL of distilled water (dH₂O). AgNPs-AC2: The sample was prepared by directly mixing 25 mL of the crude AgNPs solution with 0.1 g of AC without prior centrifugation or washing.

Table 1. NPs used in experiments

AgNPs -AC	AgNPs-muc (<i>C. aspersum</i> mucus and AgNO ₃)
AC	Activated carbon
AgNPs-AC1	AgNPs-muc centrifuged + AC
AgNPs-AC2	AgNPs-muc + AC
AgNPs-TT	Mixture (<i>C. aspersum</i> mucus + essential oil from <i>M. alternifolia</i>)
AgNPs-TT1A	AgNPs (45 ml mixture + 5 ml 50 mM AgNO ₃) + TT
AgNPs-TT1B	AgNPs (45 ml mixture + 5 ml 50 mM AgNO ₃) + TT
AgNPs-TT2A	AgNPs (40 ml mixture + 10 ml 50 mM AgNO ₃) + TT
AgNPs-TT2B	AgNPs (40 ml mixture + 10 ml 50 mM AgNO ₃) + TT
AgNPs-TT3B	AgNPs (30 ml mixture + 20 ml 50 mM AgNO ₃) + TT

Synthesis of modified silver nanoparticles from tea tree (AgNPs-TT)

To investigate the influence of essential oils, a biomatrix consisting of 100 mL of *C. aspersum* mucus and 20 mL of *M. alternifolia* (tea tree) oil was prepared. Different volumes of 50 mM AgNO₃ were added to this biomatrix under continuous stirring at room temperature. The resulting products were separated into supernatant (A) and pellet (B) fractions by centrifugation. AgNPs-TT1 sample was synthesized with 45 mL of biomatrix and 5 mL of 50 mM AgNO₃, yielding two fractions: AgNPs-TT1A (supernatant) and AgNPs-TT1B (pellet). A mixture of 40 mL biomatrix and 10 mL 50 mM AgNO₃ was used to synthesize the AgNPs-TT2 sample, separated into AgNPs-TT2A (supernatant) and AgNPs-TT2B (precipitate). The main fraction collected after mixing 30 mL biomatrix and 20 mL 50 mM AgNO₃ was AgNPs-TT3B (precipitate). After separation, all samples were dried before further experimental use.

Strains and growth conditions

The fungal strains *A. niger* and *P. griseofulvum*, belonging to the Mycological collection of the Stephan Angeloff Institute of Microbiology, Sofia, were used in the experiments. They were selected as AmB-resistant strains in our previous study (unpublished data). Long-term preservation of these fungi was carried out in the Microbank system (Prolab Diagnostics, Richmond Hill, Canada), consisting of sterile vials that contain 25 porous, colored beads and a cryopreservative fluid at -80°C. Before use, the conidiospores were grown on potato-dextrose agar (PDA) medium at 28°C for 7 days. Preparation of standardized spore suspension

Inoculum suspensions were prepared from fresh, mature (5- to 7-day-old) cultures grown on PDA. The resulting spores were added to a sterile solution of Triton X-100 and adjusted to a concentration of 2-6x10⁴ spores/mL. Ten mL of this suspension was incubated with or without AgNP in a shaking thermomixer (Eppendorf Thermomixer comfort) at 28°C for 24 h (Yang et al., 2016).

Antifungal activity assessment

Agar diffusion assay (ADM)

Antifungal activity was assessed using a diffusion technique on a potato-dextrose agar (PDA) growth medium. Treated spore suspensions (50 µL) of each fungal strain were inoculated in the centre of a PDA plate in a 90 mm Petri dish and cultivated at 28°C for 144 hours. Fungal growth was assessed by measuring the colony diameter every 24 hours

and taking photographs of each Petri dish for image analysis. The percentage of mycelial growth inhibition (MGI) was calculated according to the following formula:

$$\text{MGI}(\%) = \frac{C-T}{C} \times 100$$

where MGI (%) is the percentage of mycelial growth inhibition by the tested compounds, C is the diameter of fungal growth on control, and T is the diameter of fungal growth on treated PDA.

Broth microdilution method (BDM)

In general, 96-well plates were prepared by adding 50 µL of potato dextrose broth (PDB) supplemented with 50 µL of the treated spore suspension and 30 µL of 0.02% resazurin. Then plates were incubated for 72 hours at 28°C. The inhibitory effect was determined by direct observation of growth and change of the dye color from blue to pink to purple. As control samples for each variants were used as follows: 1) medium + untreated spore suspension; 2) medium + untreated spore suspension + NYS.

Statistical analysis

The results obtained in this investigation were evaluated from at least three repeated experiments to represent the mean. The error bars indicate the standard deviation (SD) of the mean of triplicate experiments. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test. For the statistical processing of the data, the version of the ANOVA software built into the Origin program (OriginPro 2019b, 64-bit) was used.

Results

To study the inhibitory effect against the fungal strains *A. niger* and *P. griseofulvum*, activated carbon and two types of AgNPs synthesized by "green synthesis" of a mixture of mucus from the garden snail *C. aspersum* with MT>30 kDa and 50 mM AgNO₃, to which a carbon adsorbent (AgNPs-AC) or essential oil from tea tree *M. alternifolia* (AgNPs-TT) was added, were used. Two types of carbon composites were obtained after mixing AB from peach shells (Dolashka et al., 2024) with AgNPs (a mixture of mucus from the snail *C. aspersum* and 50 mM AgNO₃), formed after centrifugation (AgNPs-AC1) and without centrifugation (AgNPs-AC2). The second type of AgNPs, tested as growth inhibitors of fungal strains *A. niger* and *P. gryseofulvum*, were obtained after synthesis of nanoparticles from snail mucus and es-

sential oil with different concentrations of 50 mM AgNO₃: 5 ml (AgNPs-TT1A; AgNPs-TT1B); 10 ml (AgNPs-TT2A; AgNPs-TT2B) and 20 ml (AgNPs-TT3B), respectively, the supernatant and precipitate.

Antifungal activity measurement by ADM

Antifungal activity of AgNPs-AC

The *in vitro* antifungal activity of AgNP using *C. aspersum* mucus (mol mass >30 kDa) and activated carbon (AgNP-AC) towards *A. niger* was evaluated using ADM methods. A time-dependent reduction of *A. niger* colony size and percentage of inhibition rate after 144 hours of treatment is noticeable in Fig. 1. All treatments illustrated different degrees of inhibition of mycelial growth. Macrophotographs of the plates showed that the negative control variant exhibited the typical characteristics of *A. niger* development, including thick, fluffy mycelial growth and sporulation (Fig. 1A). In the case of treatment with formulation AC alone,

negligible differences in growth reduction were observed compared to the control group. The greatest limitation of fungal colony growth was observed after the spores were treated with formulation AgNPs-AC2 after 24 hours of cultivation. The growth inhibitory tendency was confirmed by the calculated percentage of inhibition rate (Fig. 1B).

Figure 2A shows the growth changes of *P. griseofulvum* when exposed to various formulas containing active carbon, compared to the control group. Growth inhibition of 100% was recorded after 24 hours for treatments with formulations AgNPs-AC1 and AgNPs-AC2.

However, the antifungal effect decreased substantially during the subsequent hour of cultivation. While the AgNPs-AC1 treatment achieved an inhibition rate of approximately 35%, the AgNPs-AC2 treatment attained an inhibition rate of approximately 55% (Fig. 2B). Meanwhile, the AC variant exhibited a rapid decrease in inhibition rate,

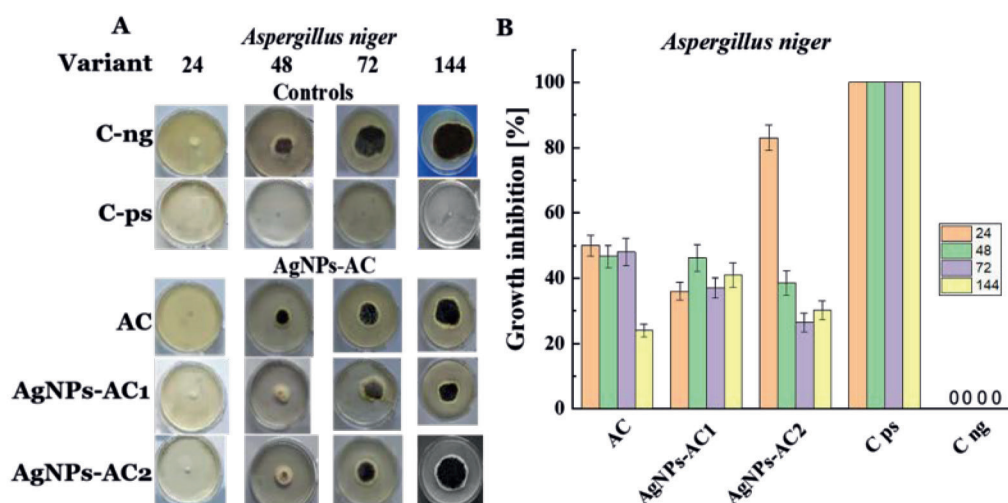


Fig. 1. Time-dependent inhibitory effect of AgNPs-AC (AgNPs-AC1 and AgNPs-AC2) against *A. niger*. A – colony growth reduction; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C ps - positive control (PDA + AgNP); AC – activated carbon.

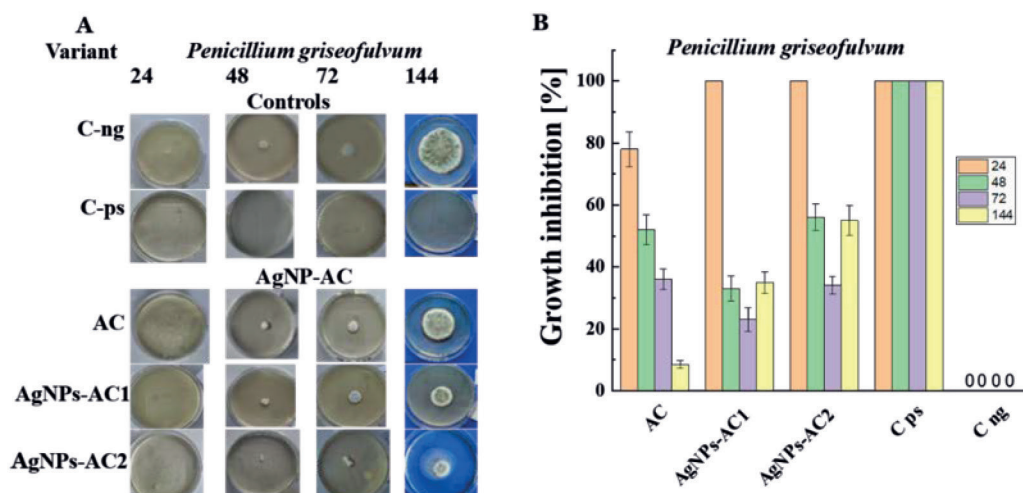


Fig. 2. Time-dependent inhibitory effect of AgNPs-AC (AgNPs-AC1 and AgNPs-AC2) against *P. griseofulvum*. A – colony growth reduction; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C ps - positive control (PDA + AgNP); AC – active carbon.

dropping from 70% to 8.5% during the treatment period.

Antifungal activity of AgNPs-TT

The combination of AgNPs and tea tree extract demonstrated an enhanced antifungal effect against *A. niger* in comparison to variants incorporating organic carbon-based nanoparticles (Fig. 3).

The measurements of the mycelial growth zone of the fungus in Petri plates (Fig. 3A) were used to obtain the readings illustrated in Fig. 3B. In the initial 24-hour period, variants AgNPs-TT1B and AgNPs-TT2B exhibited the most pronounced antifungal activity, achieving 100% growth inhibition. These were followed by variants Ag-

NPs-TT1A, AgNPs-TT2A, and AgNPs-TT3B, which demonstrated 70-80% inhibition rates during this time (Fig. 3B). After 48 hours of cultivation, the efficacy of the treatment was found to be reduced by approximately 40–50%. An exception to this pattern is variant AgNPs-TT3B, which exhibits a higher level of inhibitory activity (approximately 60%). Furthermore, AgNPs-TT1B, AgNPs-TT2B, and AgNPs-TT3B exhibited a delay in sporulation. An extended cultivation period of 144 hours led to a diminished effect on growth and spore formation.

In vitro antifungal activity was assessed by measuring the zone of inhibition and calculating the percentage inhibition over a period of several

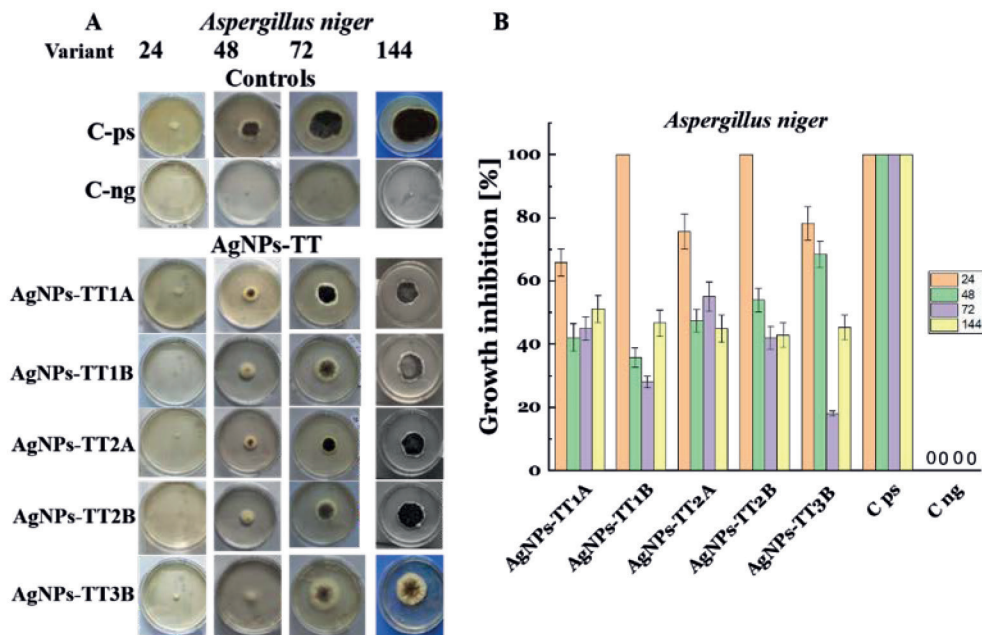


Fig. 3. Antifungal activity of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B, and AgNPs-TT3B - AgNPs-TT) against *A. niger* dependent on the NPs content and duration of cultivation. A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + AgNP).

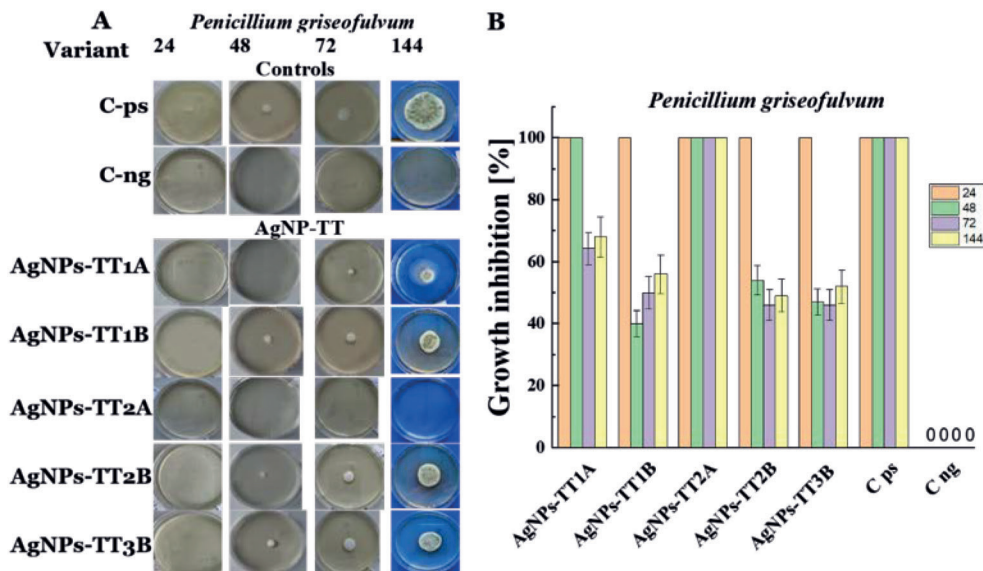


Fig. 4. Growth inhibition of *P. griseofulvum* by treatment with different variants of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B, and AgNPs-TT3B). A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + AgNP);

days (Fig. 4). The data show that the *P. griseofulvum* test strain was more sensitive to AgNPs-TT than the *A. niger* strain. Variant AgNPs-TT2A exhibited complete growth inhibition over the entire 144-hour period. The same 100% inhibition rate was observed for variant AgNPs-TT1A after 24 and 48 hours of cultivation, and for variants AgNPs-TT1B, AgNPs-TT2B, and AgNPs-TT3B after 24 hours. Moreover, a notable delay in the sporulation process was observed for a duration of 48 hours in all the variants used.

Antifungal activity measurement by BDM

In subsequent experiments, AgNPs-AC and AgNPs-TT were evaluated as antifungal agents using a resazurin-mediated microtiter plate assay towards *A. niger* and *P. griseofulvum*. The color shift of the resazurin indicator, as measured by the ELIZA reader, was utilized for the purpose of evaluating the fungal growth after treatment with the corresponding NPs. Nystatin was used as a positive control.

Antifungal activity of AgNPs-AC

The potential antifungal activity of synthesized AgNPs-AC was illustrated in Fig. 5. Formulations AgNPs-AC1 and AgNPs-AC2 demonstrated the capacity to impede the proliferation of *A. niger* only for a duration of 24 hours, with inhibition rates of 37.69 and 34.44%, respectively. In contrast, the Nys variant demonstrated a more pronounced antifungal effect.

The same formulations, AgNPs-AC1 and AgNPs-AC2, exhibited a more pronounced inhibitory effect on *P. griseofulvum* in comparison to the *A.*

niger strain (Fig. 6). The data obtained following a 72-hour cultivation period were found to be comparable to those exhibited by the Nys treatment. The highest antifungal effect was achieved at 24 hours, at which point the suppression of strain development reached 72%, 81%, and 82% for AgNPs-AC1, AgNPs-AC2, and Nys, respectively.

Antifungal activity of AgNPs-TT

The ability of AgNPs-TT to inhibit the growth of the *A. niger* strain is demonstrated in Fig. 7. It revealed the various sensitivity of the fungal strain to the tested nanoparticles, as well as the effectiveness of different formulations according to their content.

The formulations AgNPs-TT2A and AgNPs-TT2B exhibited an inhibitory effect (approximately 70%) that was comparable to that of the control agent Nys over a period of 48 hours. Subsequent to these results, AgNPs-TT1A, AgNPs-TT1B, and AgNPs-TT3B were observed to cause an inhibition rate of between 33 and 36% over the initial 24 hours of cultivation.

Green-synthesised AgNPs-TT exhibited better antifungal activity against *P. griseofulvum* than *A. niger* (see Fig. 8). All formulations resulted in a high degree of inhibition rate over the entire cultivation period (144 h). The data confirmed that the effect was comparable to that of the control agent Nys. In addition, formulations AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, and AgNPs-TT2B exhibited higher growth inhibition in comparison to the Nys for the initial 24 hours. It is also noteworthy that AgNPs-TT2B demonstrated more pronounced antifungal potential than that evaluated for the Nys.

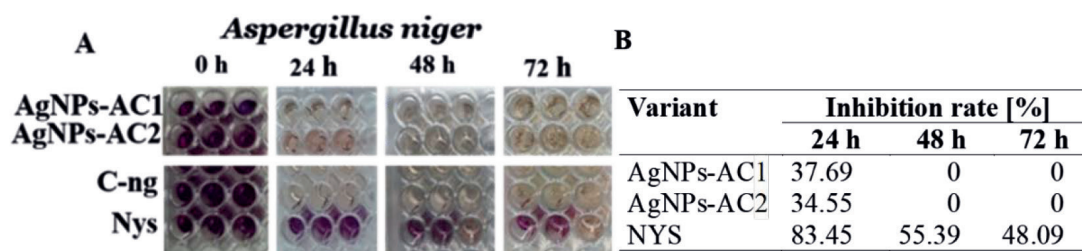


Fig. 5. Resazurin microdilution assay for the antifungal activity of AgNPs-AC (AgNPs-AC1 и AgNPs-AC2) towards *A. niger*. A – resazurin plates; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys).

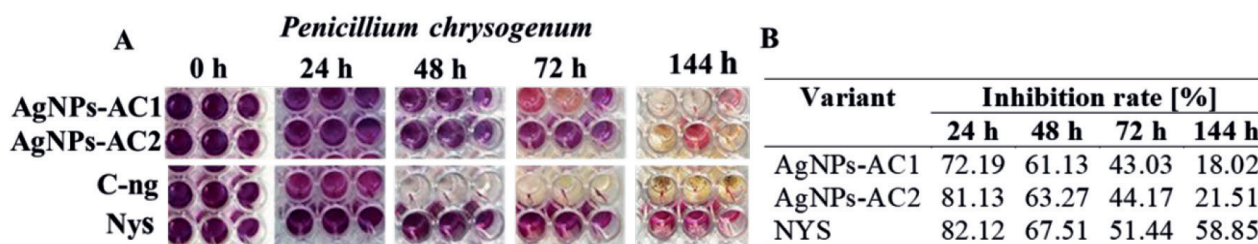


Fig. 6. Antifungal effect of AgNPs-AC (AgNPs-AC1 and AgNPs-AC2) in terms of color intensity of resazurin towards *P. griseofulvum*. A – resazurin plates; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys).

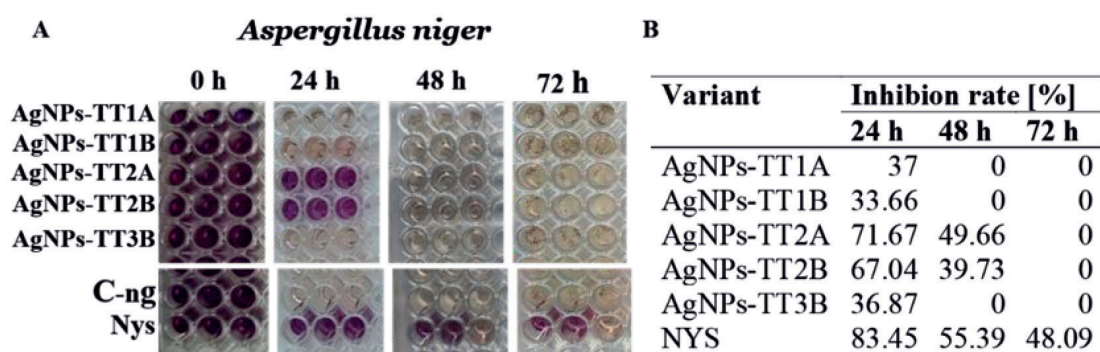


Fig. 7. Resazurin assay of the antifungal activity of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B и AgNPs-TT3B) towards *A. niger*. A – resazurin plates; B - inhibitory rate (%); A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys).

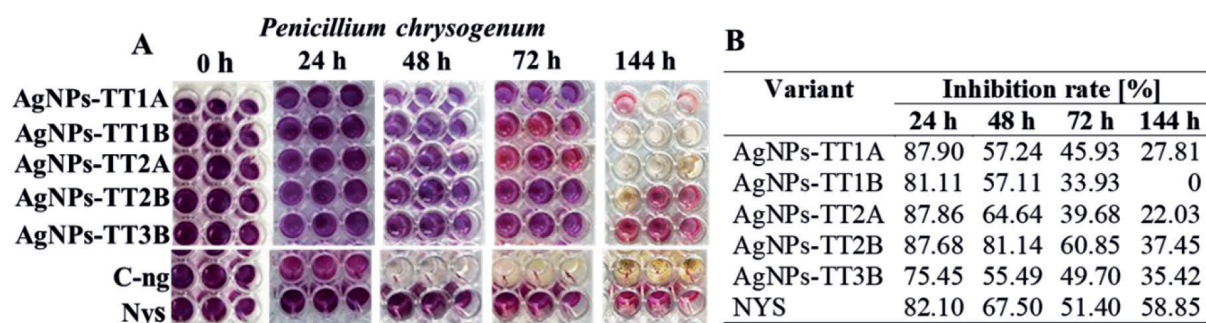


Fig. 8. Antifungal activity of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B, and AgNPs-TT3B) assessed based on the microdilution method against *P. griseofulvum*. A – resazurin plates; B - inhibitory rate (%); A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys)

Discussion

A considerable number of potentially pathogenic fungal strains are regarded as hazardous due to their resistance to antifungal agents that are frequently utilized for the treatment of such infections. In recent years, this novel health problem has attracted increased attention, coinciding with the emergence of drug resistance in bacterial and viral pathogens. Factors contributing to the development of antifungal resistance may include intricate interactions among environmental conditions, virulence factors, and gene expression alterations (Vitiello *et al.*, 2023). The widespread use of fungicides has also been cited as a significant cause of resistance. Consequently, there exists an immediate necessity for the development of new and effective drugs to address resistant fungal strains (De Francesco, 2023; Gurajala and Challapilla, 2026; Singh *et al.*, 2026). Nanomaterials offer a promising alternative to conventional antifungal drugs [Hashem *et al.*, 2022; Bottery *et al.*, 2024; Anjum *et al.*, 2026].

The present study provides new information on the effects of two distinct types of AgNPs, synthesized *via* a green methodology, on AMB-resistant strains belonging to the species *A. niger* and *P. griseofulvum*. As potential inhibitors of the growth

of fungal strains *A. niger* and *P. griseofulvum*, AgNPs synthesized by “green synthesis” with active components, a mixture of mucus from garden snail *C. aspersum* with MT>30 kDa and activated carbon (AgNPs-AC) or tea tree *M. alternifolia* (AgNPs-TT) were tested. The conducted analyses proved a weak inhibitory effect of two types of carbon composites obtained after mixing AC from peach shells (Dolashka *et al.*, 2024) with AgNPs (a mixture of mucus from snail *C. aspersum* and 50 mM AgNO₃) after centrifugation (AgNPs-AC1) and without centrifugation (AgNPs-AC2).

The influence of Ag-ion concentration on the growth inhibition of fungal strains was established by testing the second type of AgNPs, as inhibitors of the growth of fungal strains *A. niger* and *P. griseofulvum*. The results of the tests synthesized AgNPs-TT (supernatant and precipitate) with different concentrations of Ag⁺: 5 mM (AgNPs-TT1A; AgNPs-TT1B); 10 mM (AgNPs-TT2A; AgNPs-TT2B), and 20 mM (AgNPs-TT3B) AgNO₃ show the synergistic effect of snail mucus and essential oil, as well as the importance of higher concentrations of Ag⁺.

A comparison of two types of Ag-nanoparticles was conducted. Formulations containing mu-

cus from *C. aspersum* and tea tree oil (AgNPs-TT) were found to have a substantially higher antifungal effect against the test strains than those comprising activated carbon (AgNPs-AC). The replacement of activated charcoal with tea tree oil has been demonstrated to enhance the efficacy of the nanoparticles, as well as prolong the duration of their action. Furthermore, *P. griseofulvum* demonstrated greater sensitivity to both NPs than *A. niger*. Based on the results obtained by ADM, it can be concluded that the order of efficacy for *A. niger* was as follows: AgNPs-TT2B, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT1A, and AgNPs-TT3B for AgNPs-TT and AgNPs-AC2, AgNPs-AC1 for AgNPs-AC. Concurrently, the efficacy ranking for *P. griseofulvum* was AgNPs-TT2A, AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2B, AgNPs-TT3B, and AgNPs-AC2, AgNPs-AC1 for AgNPs-TT and AgNPs-AC, respectively. It is important to note the extended period of 100% growth inhibition, which was observed at 144 and 48 hours after the treatment of *P. griseofulvum* with AgNPs-TT2A and AgNPs-TT1A, respectively.

A comparison of the aforementioned data (ADM) with the results obtained by the BMD method revealed that both methods correctly evaluated the antifungal properties of the AgNPs used. The higher sensitivity of the *P. griseofulvum* strain to the nanoparticles used compared to the *A. niger* strain was confirmed.

The main finding of this study is that the evaluated antifungal activity is comparable to, or even higher than, that of Nys. Because of its toxicity, the application of Nys is limited, highlighting the necessity for alternative antifungal medications. Thus, synthesized AgNPs containing mucus and plant essential oil could be estimated as a potential source of novel therapeutic formulations.

The effect of silver ions was examined in combination with the potential of *C. aspersum* mucus and activated carbon (AgNPs-AC). As can be seen in Figures 1 and 2, the silver nanoparticles synthesised in the mucus matrix demonstrated higher antifungal activity towards both test strains compared to the control particles (AC). The green synthesis of metal nanoparticles using mollusc-secreted mucus has been the subject of a limited number of studies (Di Filippo *et al.*, 2022; Ganesan *et al.*, 2024). Our previous studies have revealed the potent antibacterial properties of nanoparticles using a reducing agent from *C. aspersum* mucus (Daskalova *et al.*, 2023; Dolashka *et al.*, 2024; Todorova *et al.*, 2025a,b). Mane *et al.* (2021) reported the synthesis

of silver nanoparticles from the mucus of the territorial snail *Achatina fulica*, which exhibited antifungal activity against *A. fumigatus*. Green-synthesised AgNPs from fish mucus (*Cyprinus carpio*) significantly inhibited the mycelial growth of *A. niger* and *S. cerevisiae* (Tasleem *et al.*, 2024). Similar results were demonstrated by Fe₂O₃ NPs using *Lissachatina fulica* snail mucus as an antimicrobial agent (Ganesan *et al.*, 2024). The authors noted the bio-reduction and bio-stabilization effects of the mucus during the synthesis of the NPs.

In recent years, there has been growing interest in using plant extracts to reduce silver ions during green nanoparticle synthesis (Munir *et al.*, 2016; Huo *et al.*, 2019; Fatimah *et al.*, 2022; Eker *et al.*, 2025). Essential oils, which are secondary plant metabolites, contribute to enhanced pharmacological activities of nano-formulations. Our results confirmed this suggestion. Using a combination of *C. aspersum* mucus and *M. alternifolia* oil in the green synthesis of AgNPs enhanced the antifungal activity against *A. niger* and *P. griseofulvum* and had an extended inhibitory effect. Moosavy *et al.* (2023) reported that green-synthesized gold and silver nanoparticles using *Mentha spicata* essential oil demonstrated higher antimicrobial activity in comparison with chemically synthesized NPs. Lavender oil significantly reinforces the antifungal activity of AgNPs, displaying remarkable broad-spectrum efficacy against the agricultural pathogens *Colletotrichum musae* and *Alternaria alternata* (Abri *et al.*, 2026). Green synthesis of AgNPs from *Origanum vulgare* essential oil demonstrated enhanced antifungal activity compared to sole oil application, improving its stability, water solubility, and dispersion (Pourakbar *et al.*, 2026). The utilization of citron juice (*Citrus medica*) for the production of copper nanoparticles also demonstrated significant inhibitory effects against the pathogens *F. graminearum*, *F. culmorum*, and *F. oxysporum* (Shende *et al.*, 2021).

While the exact molecular antifungal mechanisms of nanoparticles remain unclear, the antifungal potential of AgNPs can be attributed to their capacity to bind to hyphae, thereby inflicting damage to the fungal cells (Hashem *et al.*, 2022). It is probable that Ag⁺ primarily impacts the functionality of membrane-associated enzymes, particularly those found within the respiratory chain (Singh *et al.*, 2026). Furthermore, AgNPs have been shown to inactivate intracellular enzymes *via* the mechanism of competitive inhibition (Hashem *et al.*, 2022). It has also been documented that there is an effect on the

expression of genes responsible for many proteins, as well as the disruption of DNA replication. In addition, NPs have the capability to generate reactive oxygen species (ROS), consequently causing DNA damage, lipid peroxidation, and protein denaturation (Mansoor *et al.*, 2021; Wang *et al.*, 2022; Li *et al.*, 2024). This action, which involves various factors, is further strengthened by phytochemical capping agents, particularly phenolic compounds and tannins discovered in plant extracts that synergistically affect fungal growth and metabolism (Moretti *et al.*, 1998). According to Irshad *et al.* (2024), green-synthesized NPs can generate ROS, causing oxidative stress damage, including disruption to cellular integrity and osmotic balance, and a decrease in pathogenicity. In addition, NPs can induce cell death *via* a caspase-dependent route, implying their potential to trigger apoptosis. The application of mucus as a reducing agent in green synthesis of metal-containing NPs provides additional antimicrobial and anti-inflammatory properties (Mane *et al.*, 2021). The accelerated leakage through fungal membranes of proteins, reducing sugars, DNA, and RNA has been evaluated after treatment.

The antifungal activity of AgNPs-TT is likely enhanced by the complex mixture of compounds in tea tree essential oil, such as primarily terpene hydrocarbons, including monoterpenes and sesquiterpenes, with key components like terpinen-4-ol, gamma-terpinene, and alpha-terpinene (Carson *et al.*, 2006). These hydrophobic substances have the potential to compromise the integrity and permeability of fungal plasma membranes, resulting in the leakage of essential cellular components and ultimately leading to cell death (Zhang *et al.*, 2015).

In this context, variations in efficacy against *A. niger* and *P. griseofulvum* may be species-specific. Similar differences in the antifungal effect of AgNPs with *Lavandula angustifolia* essential oil on *Botrytis cinerea* compared to *Fusarium proliferatum* have been reported by Abri *et al.* (2026). The authors proposed that this variance may result from intrinsic differences in fungal membrane composition, the existence of efflux pumps, or variations in cell wall structure that influence the penetration of the oil's bioactive components.

Conclusion

The increasing prevalence of drug-resistant fungal infections makes the development of novel therapeutic strategies crucially necessary. This study demonstrates a rapid, cost-effective approach to synthesising AgNPs using *C. aspersum* mucus and *M. alternifolia* oil as efficient reducing agents.

Investigations show the significant potential of green-synthesised AgNPs-AC and AgNPs-TT as antifungal agents against AmB-resistant *A. niger* and *P. griseofulvum* strains. The growth inhibition rate following treatment with these novel nano-formulations is equal to or higher than that achieved with Nys. Consequently, AgNPs synthesised using mucus and plant's essential oils could be considered a promising source for innovative therapeutic formulations.

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