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Morphological and molecular authentication, phytochemical composition and bioactivities of *Ganoderma multipileum* from Keralam, India

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Abstract
Ganoderma multipileum Ding Hou is a laccate polypore investigated here through an integrated taxonomic, phytochemical, antioxidant, nanoparticle-synthesis, and antibacterial study. Basidiomata were collected during a macrofungal survey at Government College for Women, Vazhuthacaud, Thiruvananthapuram, Keralamm, India, in June 2025. Identification was based on macroscopic and microscopic characters and nrITS sequence analysis. The sequence generated in the study was deposited in GenBank under accession PZ587000 and clustered with reference sequences of *G. multipileum* in a maximum-likelihood phylogeny, with 62% bootstrap support. Methanolic and aqueous extracts yielded 8.2% and 11.2%, respectively. Qualitative screening recorded several metabolite classes in the extracts. Total phenolic content was reported as 53 mg GAE/g extract for the methanolic extract and 51 mg GAE/g for the aqueous extract; total flavonoid content was 26 mg QE/g and 22 mg QE/g, respectively. Total terpenoid content was reported as 3.6% (w/w). The methanolic extract showed concentration-dependent activity in DPPH, ABTS, and FRAP assays. Green synthesis using the aqueous mushroom extract produced silver and copper nanoparticle preparations with UV–Visible absorbance maxima at 400–410 nm and approximately 560 nm, respectively. In agar disc diffusion tests against *Escherichia coli* and *Bacillus cereus*, the crude methanolic extract produced inhibition zones up to 15 mm; silver nanoparticle preparations produced zones up to 18 mm against *B. cereus*, while copper nanoparticle preparations produced a 20 mm zone against *E. coli*. The study documents the taxonomic identity and *in vitro* phytochemical, antioxidant, and antibacterial observations reported for *G. multipileum* collected in Keralamm.

Keywords: *Polyporales*, nrITS, phytochemical screening, antioxidant activity, silver nanoparticles, copper nanoparticles, antibacterial activity

Introduction
Ganoderma P. Karst. comprises wood-decaying polypores recognized for their ecological functions and longstanding medicinal and commercial interest. Species delimitation within the genus can be difficult because laccate basidiomata and microscopic characters overlap among taxa. Molecular approaches, particularly analysis of the nuclear ribosomal internal transcribed spacer (nrITS) region, have been used to distinguish taxa within the *G. lucidum* complex and to complement morphology (Wang *et al*., 2009; Cao *et al*., 2012; Zhou *et al*., 2015; Loyd *et al*., 2018) [6, 15, 20, 22].

Ganoderma multipileum Ding Hou is reported from tropical and subtropical Asia and has been documented from multiple woody hosts and geographical regions. Earlier studies describe its laccate, reddish-brown basidiocarps, trimitic hyphal system, and ornamented, double-walled basidiospores as useful characters for identification (Cao *et al*., 2012; Hapuarachchi *et al*., 2015, 2018; Umar *et al*., 2022; Nguyen *et al*., 2023) [6, 9, 10, 16, 19]. *Ganoderma* species are also known to contain diverse secondary metabolites, including phenolics, flavonoids, polysaccharides, and triterpenoids, and have been investigated for antioxidant and antimicrobial activities (Boh *et al*., 2007; Baby *et al*., 2015; Ferreira *et al*., 2009) [2, 4, 7].

Biological extracts have also been applied to the green synthesis of metallic nanoparticles. Fungal metabolites may participate in reduction and stabilization during synthesis, while silver and copper nanoparticle preparations have been examined for antimicrobial properties (Rai *et al*., 2009; Ahmed *et al*., 2016) [1, 17]. Against this background, the present study documented a *G. multipileum* collection from Government College for Women, Thiruvananthapuram, and investigated its morphology and nrITS-based phylogenetic placement, extractive yield, qualitative and quantitative phytochemistry, antioxidant activity using DPPH, ABTS and FRAP assays, green synthesis of silver and copper nanoparticles, and antibacterial activity against *E. coli* and *B. cereus*.

Materials and Methods
Collection and morphological identification
Basidiomata were collected during a macrofungal survey at Government College for Women, Vazhuthacaud, Thiruvananthapuram, Keralam, India, in June 2025. Field characters, including pileus, stipe, pore surface, and basidiocarp features, were recorded and specimens were photographed in their habitat. Macroscopic and microscopic characters were examined using standard taxonomic procedures. Thin hand-cut sections of dried basidiomata were stained with 1% Congo Red and mounted in 3% potassium hydroxide. Slides were examined with an Olympus CX43 compound microscope. Twenty mature basidiospores were measured for length and width, and the spore quotient was defined as length/width. Colour descriptions followed Kornerup and Wanscher (1978) [13].

Photomicrographs were captured using a Magcam DC10 digital camera attached to the microscope.

DNA extraction, nrITS amplification and phylogenetic analysis

Genomic DNA was isolated from fresh fungal material following Izumitsu *et al*. (2012) [12]. The nrITS region was amplified using primers ITS1 and ITS4 (White *et al*., 1990) [21], and PCR amplification and sequencing followed Kumar *et al*. (2018) [14]. The resulting sequence was deposited in GenBank under accession PZ587000. The sequence dataset included 26 taxa, with *Amauroderma calcitum* used as the outgroup. Multiple sequence alignment was performed using MAFFT and manually inspected and refined in BioEdit version 7.2.6.1 (Hall, 1999) [8]. Maximum-likelihood analysis was performed using the IQ-TREE web server with 1,000 ultrafast bootstrap replicates; K2P+G4 was selected as the best-fit substitution model according to the reported Bayesian Information Criterion. The tree was visualized using MEGA 11 (Tamura *et al*., 2021) [18].

Preparation of methanolic and aqueous extracts

For the methanolic extract, 10 g of dried mushroom powder was mixed with 100 mL methanol and shaken at room temperature for 24 h. The mixture was filtered through Whatman No. 1 filter paper, and the solvent was evaporated to obtain crude extract. The dried extract was weighed and stored in sterile glass bottles at 4°C. For the aqueous extract, 10 g of dried powder was mixed with 75 mL double-distilled water, heated at approximately 60°C for 6 h, and then shaken overnight. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was evaporated over a warm water bath. Extractive yield was calculated as (mass of dried extract / initial mass of powder) × 100.

Qualitative and quantitative phytochemical analysis

Qualitative screening followed standard procedures (Harborne, 1984) [11] and included tests for carbohydrates, proteins, alkaloids, flavonoids, steroids, terpenoids, saponins, phenols, coumarins, cardiac glycosides, and tannins. Total phenolic content was estimated using Folin–Ciocalteu reagent and a gallic acid standard; absorbance was measured at 760 nm after 30 min incubation at room temperature. Total flavonoid content was determined by the aluminium chloride colorimetric method using quercetin as the standard, with absorbance measured at 510 nm. Terpenoid content was estimated gravimetrically: 1 g of powder was soaked in 10 mL ethanol for 24 h, filtered and concentrated, and re-extracted with 10 mL petroleum ether. The recovered fraction was dried in a pre-weighed Petri plate and expressed as a percentage of the initial sample mass.

Antioxidant assays

The antioxidant potential of the methanolic extract of *G. multipileum* was evaluated using DPPH radical scavenging, ABTS radical cation scavenging, and ferric reducing antioxidant power (FRAP) assays. Ascorbic acid was used as the reference antioxidant in all three assays. The DPPH assay followed the modified method of Braca *et al*. (2001) [5]. A 0.1 mM DPPH solution was prepared by dissolving 3.94 mg of DPPH in 100 mL of methanol. The methanolic extract and ascorbic acid were prepared separately at 1 mg/mL, and aliquots of 50, 100, 150, 200 and 250 µL were made up to 1 mL with methanol. An equal volume (1 mL) of DPPH solution was added to each preparation, followed by incubation in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a UV–Visible spectrophotometer, with methanol and DPPH solution serving as the control mixture. Radical scavenging activity was calculated as a percentage, and the IC_{50} value was estimated from the relationship between inhibition and extract concentration.

For the ABTS assay, the stock solution was kept in the dark at room temperature for 16 h and diluted with methanol to prepare the working solution, whose absorbance was measured at 734 nm. The assay mixture consisted of 10 µL of extract at different concentrations and 200 µL of ABTS working solution. Following incubation at room temperature for 6 min, absorbance was recorded at 734 nm. Ascorbic acid was treated similarly to construct a reference standard curve. The assay was reported to have been performed in triplicate, and percentage radical scavenging activity and IC_{50} were determined from the absorbance values and concentration-response relationship, respectively, following Zhang *et al*. (2023) [23].

The FRAP assay was conducted according to Benzie and Strain (1996) [3]. Fresh FRAP reagent was prepared by mixing 10 mL of 300 mM sodium acetate buffer (pH 3.6), 1 mL of 10 mM TPTZ solution in 40 mM hydrochloric acid, and 1 mL of 20 mM ferric chloride solution. The reagent was maintained at 37°C before use. The reaction mixture contained 20 µL of methanolic extract and 150 µL of FRAP reagent and was incubated at room temperature. Absorbance was measured immediately at 593 nm. Ascorbic acid served as the positive reference, while the blank contained 20 µL of distilled water and 150 µL of FRAP reagent. The reducing response was calculated relative to the blank and reference absorbance.

Green synthesis and UV–Visible analysis of nanoparticles

An aqueous extract was prepared by heating 3 g of powdered mushroom material in 50 mL distilled water for 45 min with continuous stirring, cooling, and filtration through Whatman No. 1 filter paper. For silver nanoparticle synthesis, 10 mL extract was added to 90 mL of 1 mM silver nitrate solution and incubated at room temperature. For copper nanoparticle synthesis, 20 mL extract was mixed with 80 mL of 0.01 M copper sulfate pentahydrate solution and stirred at 80°C for 2 h. Colour changes were recorded. UV–Visible spectra were collected to assess absorbance peaks associated with nanoparticle formation.

Antibacterial activity

The antibacterial activity of the methanolic extract of *Ganoderma multipileum* and its biosynthesized silver and copper nanoparticles was evaluated against *Escherichia coli* (Gram-negative) and *Bacillus cereus* (Gram-positive) using the agar well diffusion method. The bacterial cultures were obtained from the laboratory culture collection and maintained on nutrient agar medium. Nutrient broth cultures were incubated at 37°C for 24 h before use. Mueller–Hinton agar medium was prepared by dissolving 3.8 g of the medium in 100 mL of distilled water, followed by sterilization at 121°C and 15 psi for 15 min. Approximately 20 mL of the prepared medium was dispensed into each sterile Petri plate. After solidification, the bacterial

inoculum was spread uniformly over the agar surface, and wells were made using a sterile cork borer. For the methanolic extract, the solvent was evaporated at room temperature, and 10 mg of the dried crude extract was dissolved in 2 mL of dimethyl sulfoxide (DMSO). The extract and biosynthesized nanoparticle preparations were tested at volumes of 25, 50, 75 and 100 µL. DMSO served as the negative control, while azithromycin (3 µL) was used as the positive control, as reported in the experimental results. The inoculated plates were incubated at 37°C for 24 h. Antibacterial activity was assessed by measuring the diameter of the clear zone of inhibition around each well using a scale, and the results were recorded in millimetres.

Results

Morphological identification and phylogenetic placement

Ganodema multipileum Ding Hou Fig. 1

The basidiocarps were annual, woody, laccate, and stipitate, occurring singly or in imbricate groups. The pileus was semicircular to fan-shaped, 15–20 cm broad and 1–3 cm thick, with a shiny, concentrically zoned reddish-brown to dark-brown surface and a whitish actively growing margin. The pore surface was white to cream when fresh and became brown after bruising or ageing; pores were circular to angular, 4–6 per mm. The context was corky to woody and brown to dark brown. The lateral to eccentric stipe reached 10–15 cm in length. The hymeniodermiform pileal crust consisted of thick-walled pigmented clavate to cylindrical terminal cells. Cuticular elements measured 46.26–74.52 × 6.04–7.83 µm. The hyphal system was trimitic, with skeletal hyphae measuring 64.68–150.20 × 3.28–7.95 µm and binding hyphae 87.93–156.28 × 3.12–6.41 µm. Basidiospores were ellipsoid to broadly ellipsoid, truncate, double-walled, brown and echinulate, measuring 8.95–11.41 × 5.69–7.63 µm. The specimen was reported from the base of a *Santalum album* stump, where it caused white rot.

The nrITS sequence generated in the study (GenBank accession PZ587000) clustered within the *G. multipileum* clade with reference sequences JF915409, KJ143913 and KJ143914. The reported bootstrap support was 62% (Fig. 2). The clade was separated from other *Ganoderma* taxa included in the analysis,

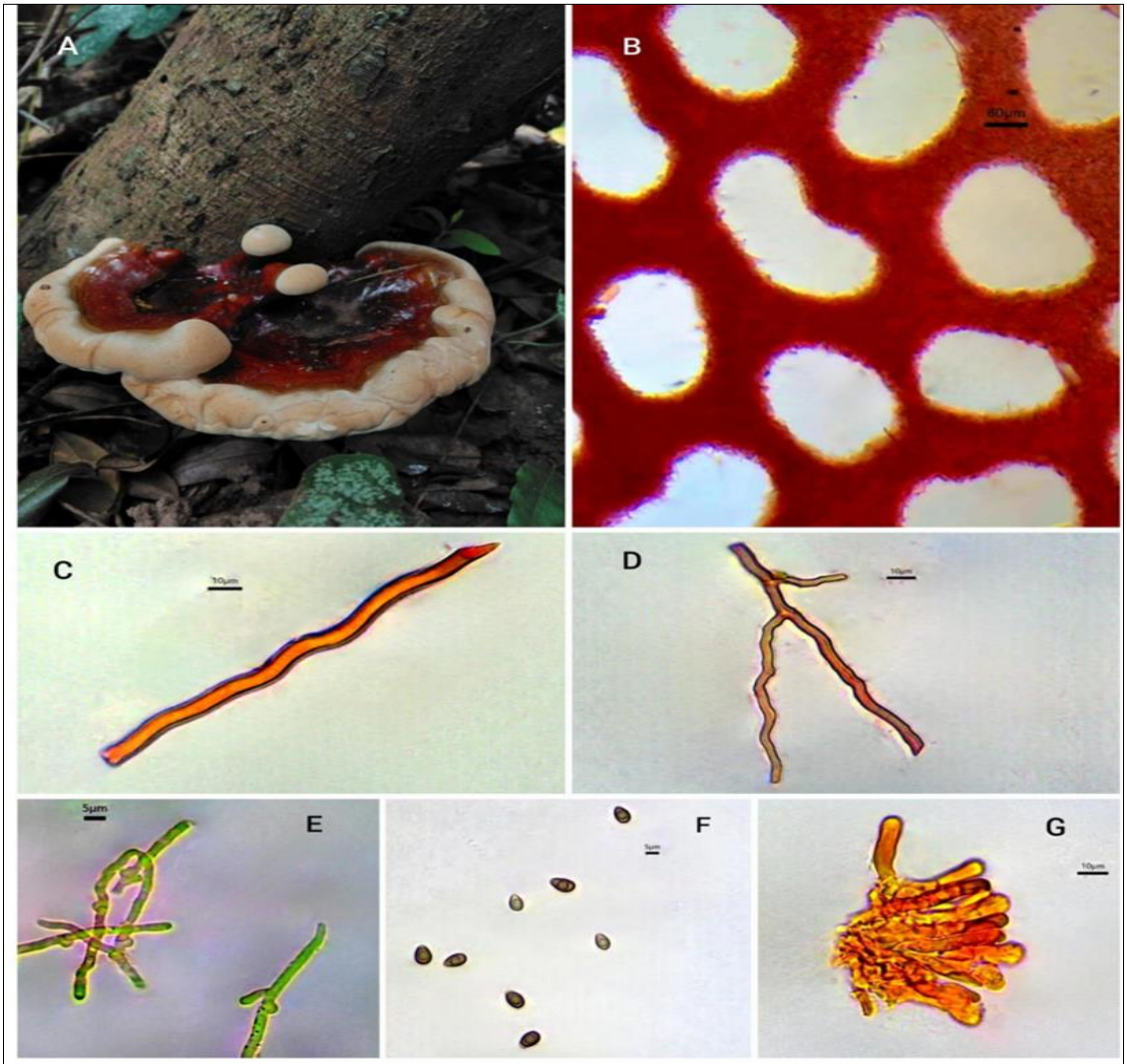


Figure 1: *Ganoderma multipileum* macro-micro morphology. A- habitat, B- hymenophore region, C- Skeletal hyphae, D- binding hyphae, E- Generative hyphae with clamp connections, F- Basidiospores, G- Modified hyphal elements in the pileal crust (pileipelis)

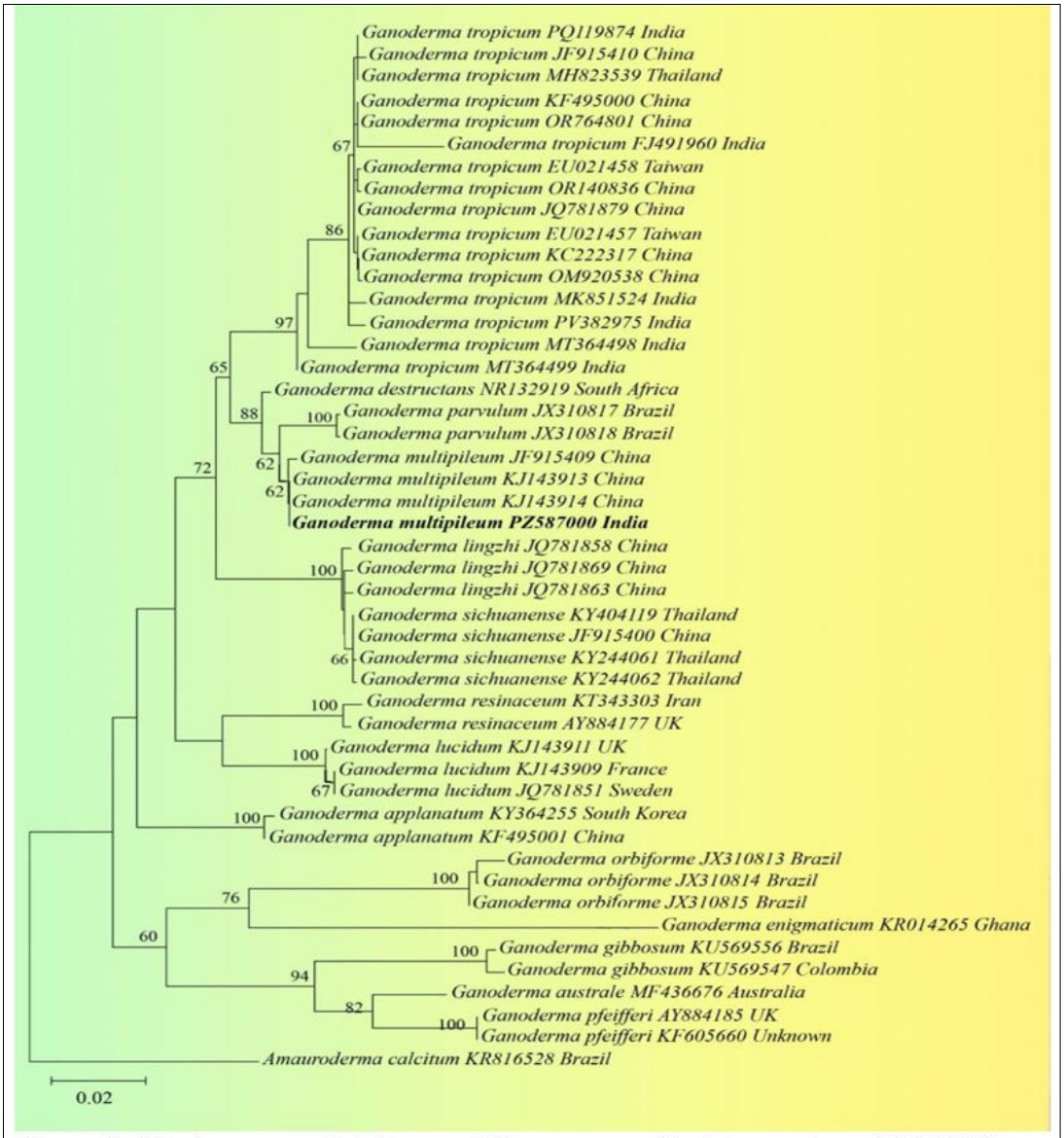


Figure 2: ML tree generated from nrITS sequences. Bootstrap values (ML>82%) are indicated above branches. *Ganoderma multipileum* from present study is indicated in bold. GenBank accession numbers and geographical locations are given after the species name.

Extractive yield and qualitative phytochemical screening

The aqueous extract had an extractive yield of 11.2%, compared with 8.2% for the methanolic extract.

Qualitative phytochemical screening results are summarized in Table 1 and shown in Fig. 3 and 4. The methanolic extract tested positive for proteins (HNO_3 test), flavonoids (H_2SO_4, lead acetate and NaOH tests), terpenoids (Salkowski test), saponins (froth test), phenols ($FeCl_3$ test) and cardiac glycosides (Keller–Kiliani test). The aqueous extract tested positive for carbohydrates (Molisch's test), proteins (HNO_3 test), alkaloids (Wagner's test), flavonoids (lead acetate and NaOH tests), saponins and cardiac glycosides. The remaining test–extract combinations were negative as recorded in the table.

Table 1: Qualitative phytochemical screening of aqueous and methanolic extracts.

Constituent	Test	Aqueous	Methanolic
Carbohydrates	Benedict's	−	−
Carbohydrates	Molisch's	+	−
Protein	Biuret	−	−
Protein	HNO_3	+	+
Alkaloids	Dragendorff's	−	−
Alkaloids	Wagner's	+	−
Flavonoids	H_2SO_4	−	+
Flavonoids	Lead acetate	+	+
Flavonoids	NaOH	+	+
Steroids	Liebermann–Burchard	−	−

Terpenoids	Salkowski	−	+
Saponins	Froth	+	+
Phenols	$FeCl_3$	−	+
Coumarins	$FeCl_3$	−	−
Cardiac glycosides	Keller–Kiliani	+	+
Tannins	$FeCl_3$	−	−

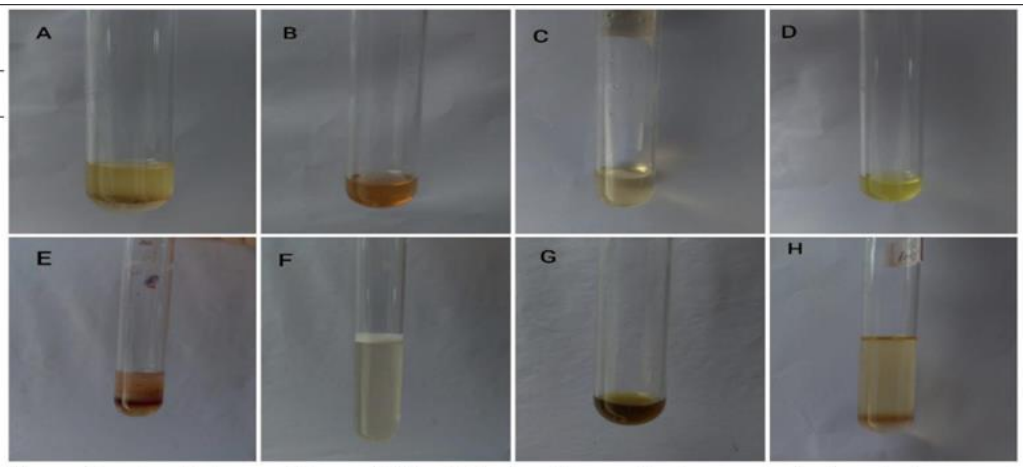


Figure 3: visual observations obtained during the preliminary phytochemical screening of the methanolic extract of *Ganoderma multipileum*, indicating the presence of various phytochemical constituents. A-protein (HNO_3 test), B-Flavanoid(H_2S0_4), C-Flavanoid (Lead acetate test), D-Flavanoid (NaOH test), E-Terpenoid (Salkowski test), F-Saponin (Froth test), G-Phenol ($Fecl_3$ test), H-Cardiac glycosides (killer-killani test)

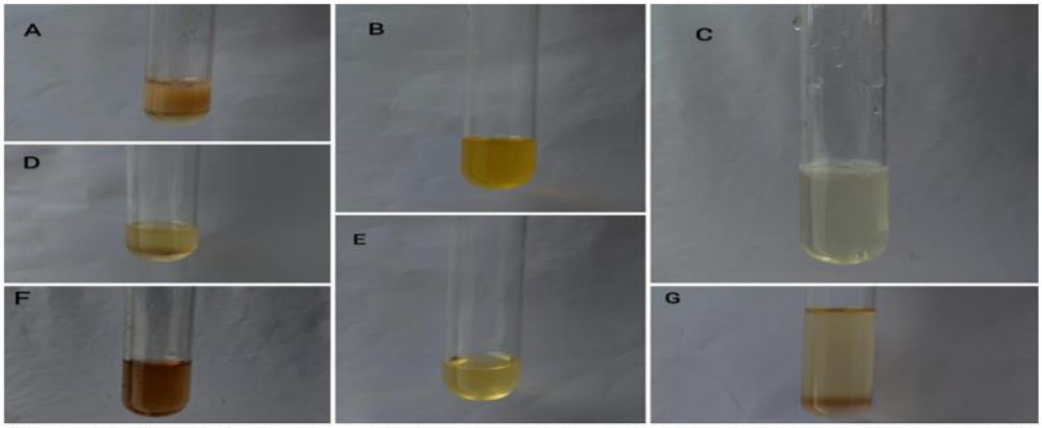


Figure 4: visual observations obtained during the preliminary phytochemical screening of the aqueous extract of *Ganoderma multipileum*, indicating the presence of various phytochemical constituents. A- Carbohydrates (Molisch test), B-Flavanoids (NaOH test), C-Saponin (Froth test), D-Protein (HNO_3 test), E- Flavanoids (lead acetate test), F- Alkaloids (Wagner's test), H-Cardiac glycosides (keller killani test)

Quantitative phytochemical estimation

Quantitative estimation gave total phenolic contents of 53 mg gallic acid equivalents (GAE)/g extract for the methanolic extract and 51 mg GAE/g for the aqueous extract. Total flavonoid contents were 26 mg quercetin equivalents (QE)/g extract and 22 mg QE/g extract, respectively.

The maximum absorbance at 510 nm was 0.30 for the methanolic extract and 0.219 for the aqueous extract. The reported terpenoid recovery was 0.036 g from 1 g of mushroom powder, corresponding to 3.6% (w/w). The gallic acid and quercetin calibration absorbances increased across the tested standard ranges.

Antioxidant activity

In the DPPH assay, extract inhibition increased from 13.33% at 50 µg/mL to 62.50% at 250 µg/mL, with an approximate reported IC_{50} of 200 µg/mL. In the ABTS assay, inhibition increased from 5.13% at 25 µg/mL to 66.67% at 150 µg/mL, with a reported IC_{50} of approximately 120–130 µg/mL. In the FRAP assay, extract absorbance increased from 0.215 at 25 µg/mL to 0.880 at 400 µg/mL. Relative activity values ranged from 73.12% to 78.57% across the tested concentrations. (Fig. 5 & 6)

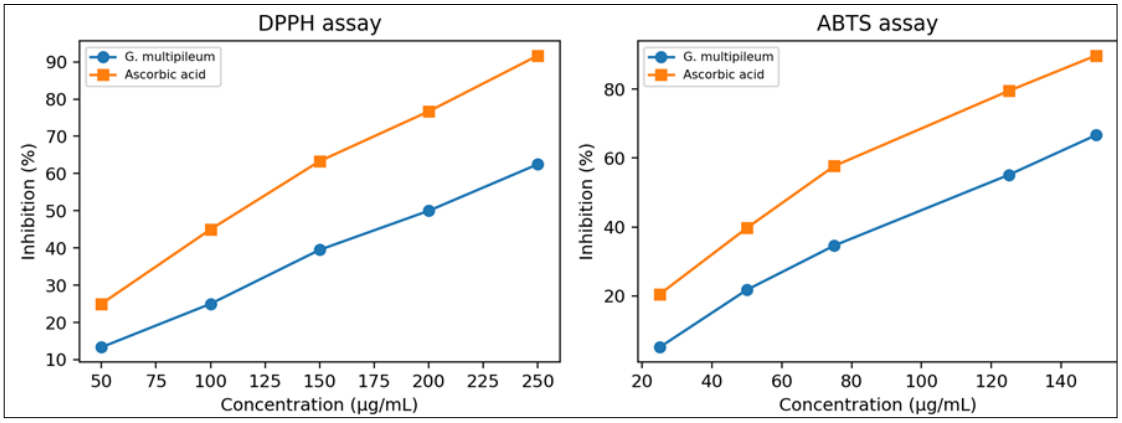


Fig 5: Concentration-response data for DPPH and ABTS radical scavenging.

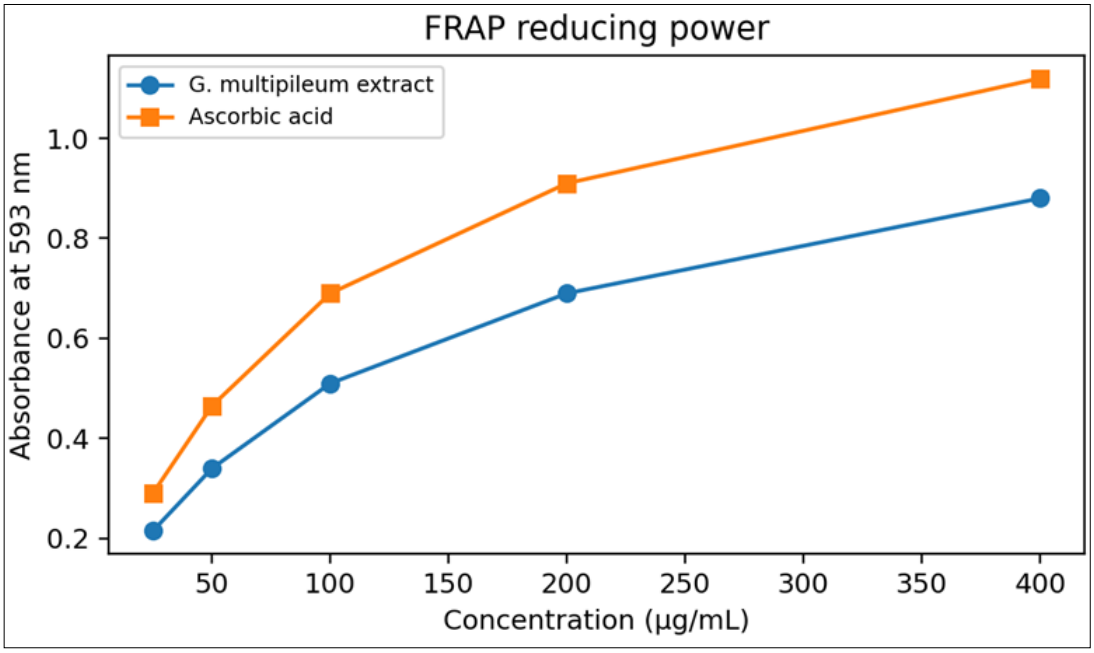


Fig 6: FRAP absorbance response of the methanolic extract and ascorbic-acid reference.

Green synthesis of silver and copper nanoparticles

Addition of the aqueous mushroom extract to silver nitrate changed the colour from pale yellow to reddish-brown. UV–Visible analysis showed a maximum absorbance at 400–410 nm (0.510), with absorbance decreasing at longer wavelengths and reaching zero at 700–800 nm. During copper nanoparticle preparation, the solution changed from light blue to dark greenish-brown. UV–Visible analysis showed a maximum absorbance of 0.287 at approximately 560 nm, followed by lower absorbance at longer wavelengths. (Fig. 7 to 10)

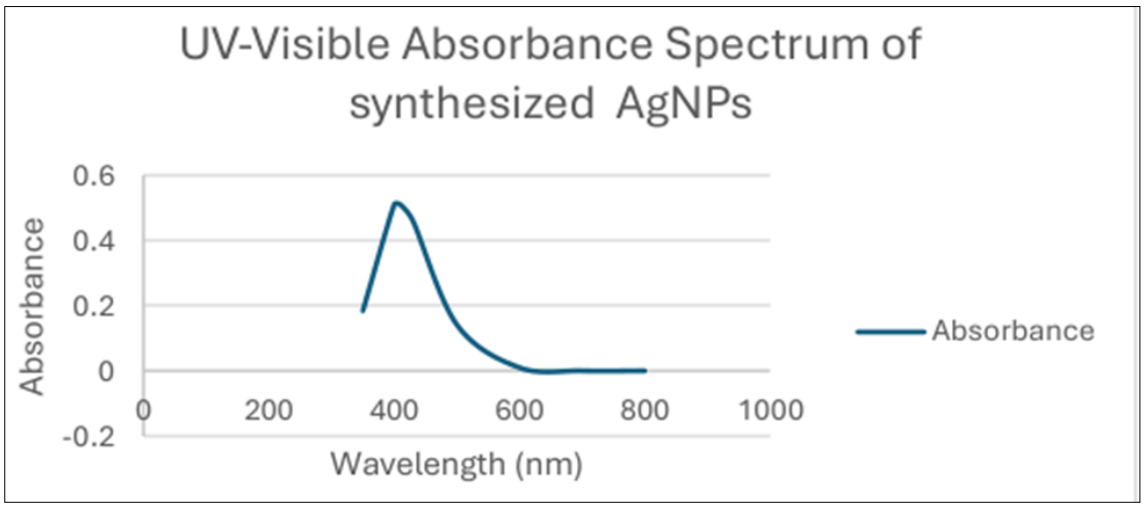


Fig 7: visible absorbance spectrum of synthesized AgNPs

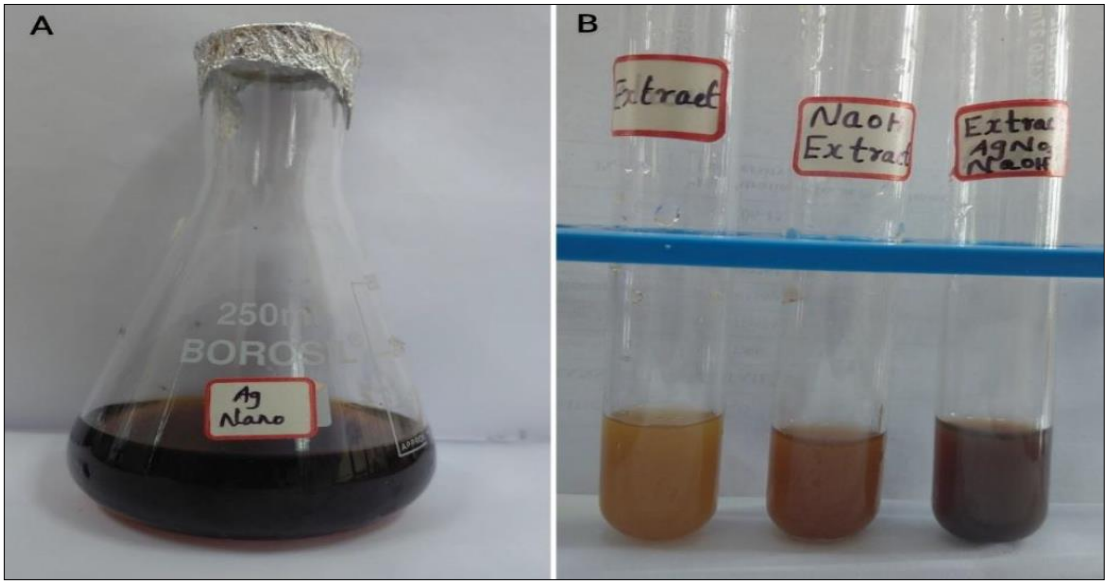


Figure 8: colour change obtained on green synthesis of AgNPs. A-Synthesized silver nanoparticles (AgNPs) solution. B-Visual comparison of the plant extract, NaOH-treated extract, and the final synthesized AgNPs reaction mixture.

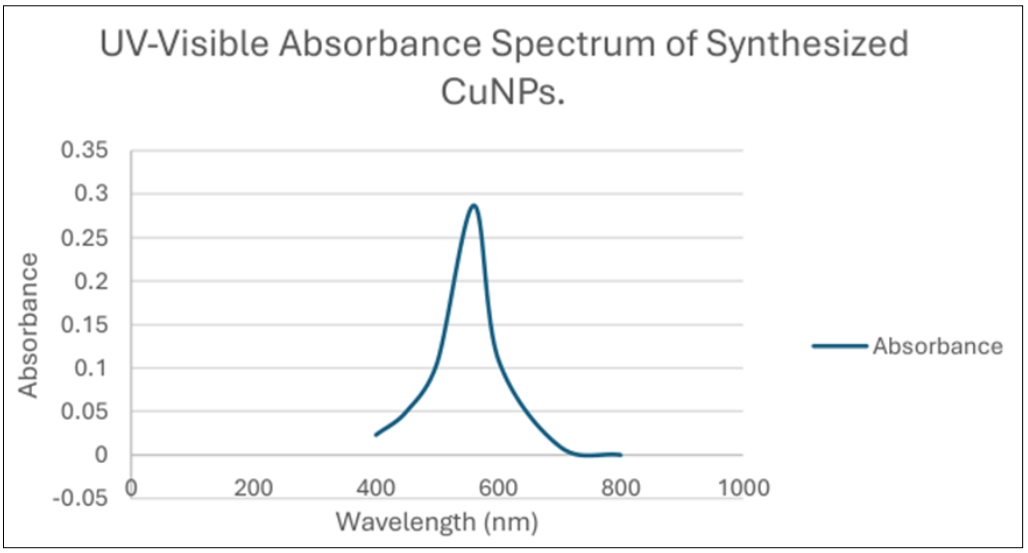


Fig 9: UV – visible Absorbance spectrum of synthesized CuNPs

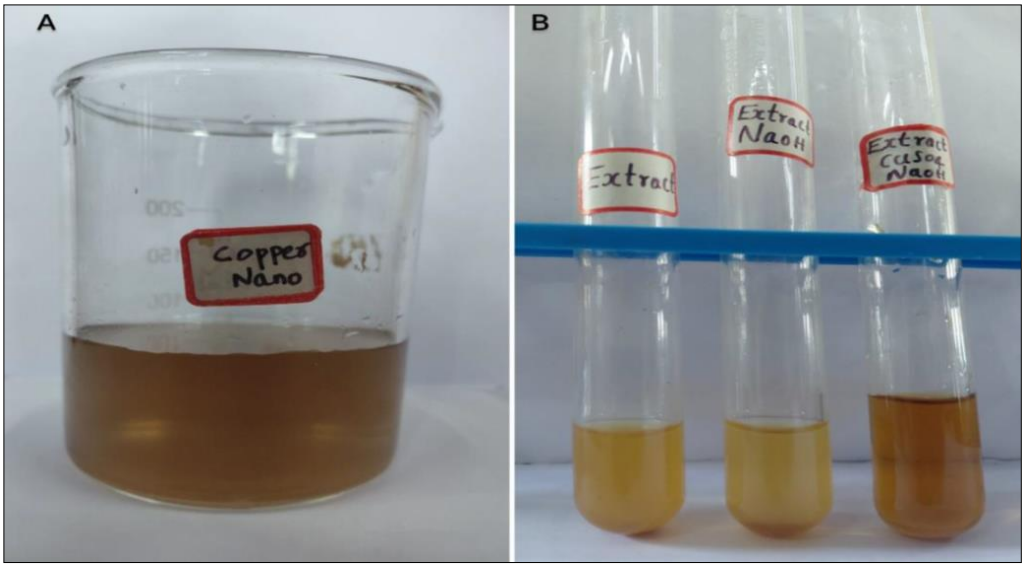


Fig 10: Colour change obtained on green synthesis of CuNPs. A- Visual comparison of the plant extract, NaOH-treated extract, and the final synthesized AgNPs reaction mixture. B- Visual comparison of the plant extract, NaOH-treated extract, and the final synthesized CuNPs reaction mixture

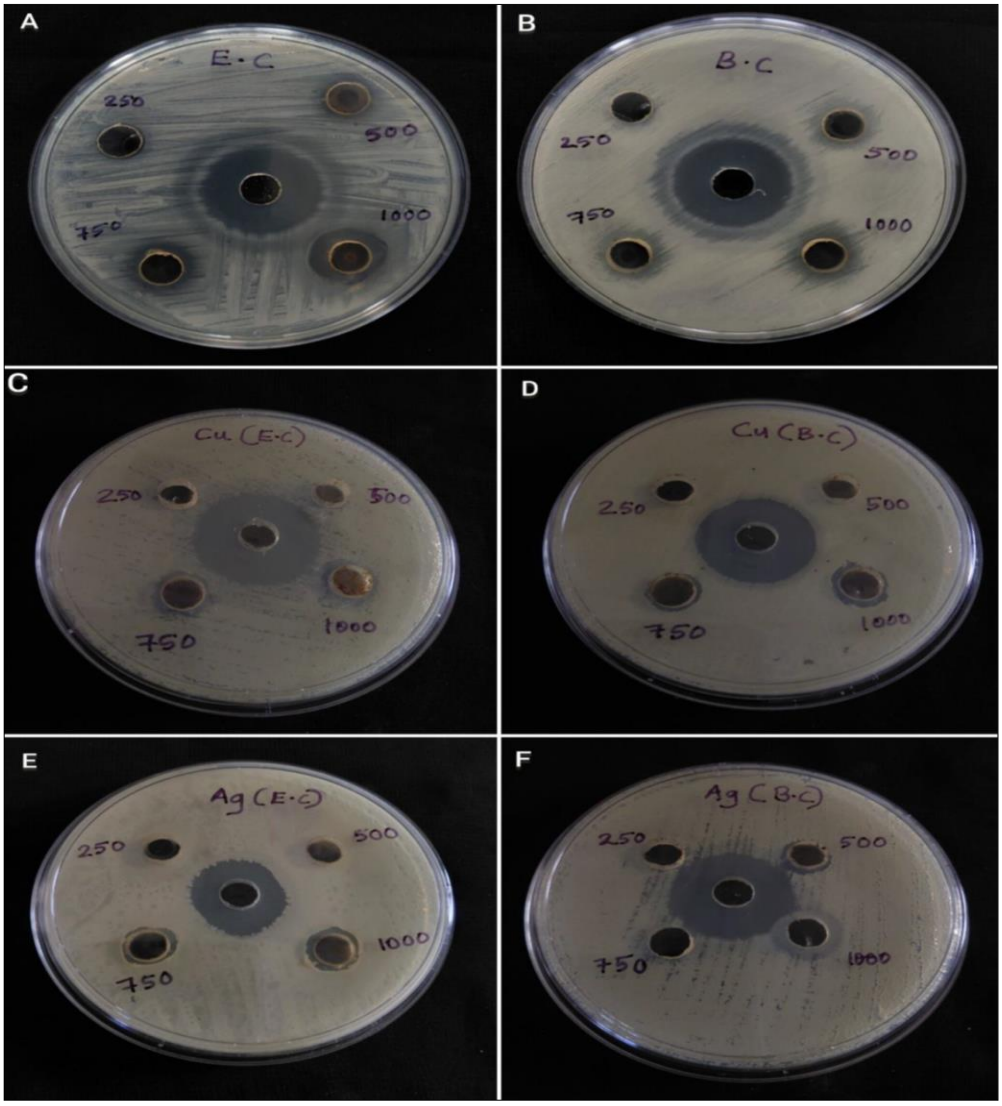


Fig 11: zone obtained on antibacterial activity. A and B: Antibacterial activity of methanolic extract of *Ganoderma multipileum* against *Escherichia coli* and *Bacillus cereus.* C & D-Antibacterial activity of green-synthesized copper nanoparticles (CuNPs) against *Escherichia coli* and *Bacillus cereus*. E & F Antibacterial activity of green-synthesized silver nanoparticles (AgNPs) against *Escherichia coli* and *Bacillus cereus.*

Antibacterial activity

The crude methanolic extract and the silver and copper nanoparticle preparations were tested against *E. coli* and *B. cereus.* The methanolic extract produced no inhibition zone at 25 µL, with zones of 12, 14 and 15 mm against *E. coli* at 50, 75 and 100 µL, respectively. Against *B. cereus*, the corresponding zones were absent at 25 µL and measured 11, 14 and 15 mm at 50, 75 and 100 µL. Silver nanoparticle preparation produced no zone against *E. coli* at 25 and 50 µL, followed by 15 and 17 mm at 75 and 100 µL. Against *B. cereus*, it produced no zone at 25 µL and zones of 13, 14 and 18 mm at 50, 75 and 100 µL. Copper nanoparticle preparation produced no zone against *E. coli* at 25 and 50 µL, followed by 15 and 20 mm at 75 and 100 µL; against *B. cereus*, zones were absent at 25 and 50 µL and measured 14 and 17 mm at 75 and 100 µL. Results are provided in Table 2.

Table 2: Inhibition zones (mm) reported for methanolic extract, silver nanoparticles and copper nanoparticles.

Preparation	Organism	25 µL	50 µL	75 µL	100 µL
Methanolic extract	*E. coli*	No zone	12	14	15
Methanolic extract	*B. cereus*	No zone	11	14	15
Silver nanoparticles	*E. coli*	No zone	No zone	15	17
Silver nanoparticles	*B. cereus*	No zone	13	14	18
Copper nanoparticles	*E. coli*	No zone	No zone	15	20
Copper nanoparticles	*B. cereus*	No zone	No zone	14	17

Discussion

The specimen was characterized using both morphology and nrITS sequence analysis. The reported laccate pileus, trimitic hyphal system, and double-walled echinulate basidiospores align with the diagnostic character combinations discussed for *G. multipileum* and related

laccate *Ganoderma* species (Cao *et al*., 2012; Hapuarachchi *et al*., 2015, 2018; Richter *et al*., 2015; Loyd *et al*., 2018) [6, 9, 10, 15]. In the molecular analysis, the study sequence grouped with *G. multipileum* reference sequences with 62% bootstrap support. This combined morphological and molecular account follows the general approach used in previous taxonomic studies of *Ganoderma* (Wang *et al*., 2009; Zhou *et al*., 2015; Nguyen *et al*., 2023) [16, 20, 22].

The aqueous extraction yield (11.2%) exceeded the methanolic yield (8.2%), while the methanolic extract had the higher reported total phenolic and flavonoid contents. The qualitative screening recorded different patterns of positive reactions between the two solvents. Solvent-dependent recovery of extractable constituents is a recurring consideration in phytochemical investigations, and the recorded values provide a comparative account of these two preparations. The terpenoid fraction was reported as 3.6% (w/w). Previous work on *Ganoderma* has described diverse triterpenoids and other metabolites, including lanostane-type compounds, in species of the genus (Boh *et al*., 2007; Baby *et al*., 2015 [2, 4]; Binh *et al*., 2018; Welti *et al*., 2015) [2].

The extract showed increasing DPPH and ABTS inhibition with increasing concentration, while FRAP absorbance also increased across the tested range. The reported antioxidant assays provide three complementary *in vitro* readouts: radical scavenging with DPPH and ABTS and reducing capacity with FRAP. Such assays have been applied in studies of wild mushrooms and *Ganoderma*-derived extracts (Braca *et al*., 2001; Benzie and Strain, 1996 [3, 5]; Re *et al*., 1999; Ferreira *et al*., 2009 [7]; Campi *et al*., 2021). The observed activity can be attributed to phenolic and flavonoid constituents.

Colour changes and UV–Visible spectra were reported for the silver and copper nanoparticle preparations. The silver preparation showed an absorption maximum at 400–410 nm and the copper preparation at approximately 560 nm. Fungal and other biological extracts have been investigated as reducing and stabilizing media for nanoparticle synthesis, including silver nanoparticles (Rai *et al*., 2009; Ahmed *et al*., 2016) [1, 17].

In the antibacterial assay, inhibition zones increased with the tested volume for all three preparations. The largest recorded zone was 20 mm for the copper nanoparticle preparation against *E. coli*, while the largest recorded zone for the silver nanoparticle preparation was 18 mm against *B. cereus*.

Conclusion

The study documented a *Ganoderma multipileum* collection from Government College for Women, Thiruvananthapuram, using morphological characters and nrITS sequence analysis. The aqueous and methanolic extracts yielded 11.2% and 8.2%, respectively. The methanolic extract had reported total phenolic and flavonoid contents of 53 mg GAE/g and 26 mg QE/g, compared with 51 mg GAE/g and 22 mg QE/g for the aqueous extract; total terpenoid content was reported as 3.6% (w/w). The methanolic extract showed concentration-dependent responses in DPPH, ABTS and FRAP assays. Aqueous mushroom extract was used to prepare silver and copper nanoparticle preparations, with reported UV–Visible absorbance maxima at 400–410 nm and around 560 nm, respectively. In agar disc diffusion assays, the nanoparticle preparations produced inhibition zones against *E. coli* and *B. cereus*, with maximum reported zones of 20 mm and 18 mm, respectively.

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Declarations

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Conflicts of interest: The authors declare that they have no conflict of interest.

Author contributions: All authors contributed equally to the conceptualization, methodology, investigation, data analysis, interpretation of results, and preparation of the manuscript. All authors reviewed and approved the final version of the manuscript.

Data availability: The nrITS sequence is reported as deposited in GenBank under accession PZ587000. Other data are presented in the article and its figures/tables.

Ethics approval: Ethical approval was not required for this study, as it did not involve human participants or experimental procedures involving animals.

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