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Tribulus terrestris fruit's potential for synthesizing gold nanoparticles with remarkable biological prospects

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Abstract

Tribulus terrestris is a member of the family Zygophyllaceae commonly known as "puncture vine". The plant has been used traditionally as an analgesic and to relieve rheumatic pain, eye problems, sexual dysfunction and edema. The aim of this work was to test the use of *T. terrestris* fruit extract as a reducing agent in synthesizing gold nanoparticles (AuNPs), test their biological activities, and assess their suitability as a therapeutic agent by testing them for potential adverse effects on human cells. Indeed, we have performed the most comprehensive biological testing of AuNPs produced using *T. terrestris* extracts to date. The aqueous extract of dried powdered *T. terrestris* fruits was used for the reduction of hydrogen tetrachloroaurate (III) trihydrate ($AuCl_4 \cdot 3H_2O$). The fruit extract's phytochemical components effectively served as reducing, capping and stabilizing agents, resulting in the production of consistent and round-shaped AuNPs with a size range of less than 100 nm. The synthesized AuNPs were subjected to various physicochemical analyses, then evaluated for antibacterial, antifungal and antileishmanial activity, and subjected to hemagglutination, cytotoxicity and antioxidant bioassays. The AuNPs showed inhibition zones against several bacterial and fungal strains, and exhibited antileishmanial activity at high doses. The AuNPs demonstrated positive hemagglutination activity against human Red Blood Cells (RBCs) of blood groups A and B at 10 and 20 µg/mL, but no hemagglutination activity against groups AB and O at up to 40 µg/mL. The AuNPs showed no cytotoxicity against human RBCs at up to 40 µg/mL, suggesting that they may be suitable for use in a clinical setting. The antioxidant activity of the AuNPs was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the results indicated a high antioxidant potential.

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Introduction

Nanotechnology is a rapidly developing field that focuses on the synthesis and characterization of materials at the nanoscale, typically ranging from 1 to 100 nm. Because of their diminutive size and substantial surface area, nanoparticles possess a broad spectrum of uses in both physical and biological fields, including medicine, drug delivery, diagnosis, electronics, and communication.[1] Green synthesis methods, such as biological, polysaccharide, and irradiation methods, offer several advantages over conventional methods for producing nanoparticles. An increased interest in gold nanoparticles (AuNPs) has materialized because they have various potential roles in cancer therapy,[2,3] as carriers in the thermotherapy of biological targets,[4] as neuroprotectants,[5] in the enhanced delivery of antibiotics,[6] in the diagnosis of the SARS-CoV-2 virus and its proteins,[7] and in the detection and treatment of tuberculosis.[8]

Nanoparticles are synthesized using two main approaches, namely top-down and bottom-up (Figure 1).[9] Among them, the synthesis of AuNPs is a complicated and time-consuming process.[10,11] Top-down approaches involve chemical processes that pose a direct threat to the environment and produce toxic byproducts. On the

other hand, bottom-up approaches are more environmentally friendly and utilize biological methods for the fabrication of nanomaterials.[12] The bottom-up approach results in nanoparticles with a higher degree of homogeneity in terms of their chemical composition, compared to those synthesized using catalytic processes. However, the catalytic processes used for nanoparticle synthesis are affected by factors such as catalytic reaction, temperature, pressure, stabilizer, catalyst nature (enzymatic or reducing agent), and solvent type, which influence the properties of the resulting nanoparticles.[13] These properties include surface attributes, shape, and size, which in turn affect the behavior, toxicity, and transport in drug delivery systems and other applications.[14,15]

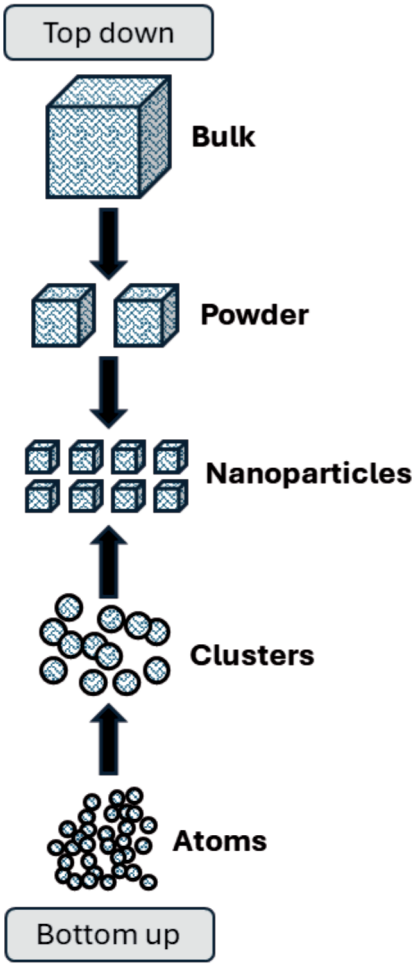

Figure 1. Nanoparticle synthesis using top-down and bottom-up approaches.

Plants are a valuable source of diverse biological molecules such as co-enzymes, vitamins, alcohols, flavonoids, and other intermediate organic biomolecules, which possess the ability to reduce metal ions and form metal nanoparticles.[16] The green synthesis method utilizing plant extracts for producing nanoparticles does not require strict control of temperature and pressure, making it a time-efficient process.[17] In these redox reactions occurring in plant parts, an excess of H^+ ions is also produced along with ATP due to the presence of secondary metabolites. Many coenzymes, including nicotinamide adenine dinucleotide (NAD), are present in living cells, which assist in redox reactions by transporting electrons. NAD occurs in two forms within cells: the oxidized form, NAD^+, accepts electrons from other species and becomes reduced, while the reduced form, NADH, acts as an electron pair donor as follows:

$NAD^+ + e^- \rightarrow NAD$
$NAD + H^+ \rightarrow NADH + e^-$
$2HAuCl_4 \rightarrow 2Au^+ + 4HCl$
$e^- + Au^+ \rightarrow Au^\circ$

The synthesis of AuNPs through a chemical process involves a series of three reduction steps of gold ions to gold atoms, followed by precipitation.[18] For example, algae contain polysaccharides rich in hydroxyl groups that reduce gold from Au(III) to Au(0) by oxidizing these hydroxyl groups to carbonyl groups.[19] The reduction reaction can be represented as follows:

$AuCl_4^- + 3R{-}OH \rightarrow Au^\circ + 3R = O + 3H^+ + 4Cl^-$

Tribulus terrestris is a widely distributed plant of the family Zygophyllaceae[20] that is commonly known as puncture vine due to the sharp horns that cover its fruit (Figure 2). It is an herbaceous, mat-forming plant that grows in tropical warm and dry climates all over the world. The sharp horns of *T. terrestris* can puncture air-

Figure 2. Fruit of *T. terrestris* (diameter 10-15 mm, length ~10 mm). A) Fresh fruit; B) Dried fruit.

filled objects such as vehicle tyres,[21] and they can injure the mouths of livestock that graze on the plants or any persons that accidentally step on them barefoot. However, in South Asia, the leaves and shoots of the plant are consumed as food materials and the plant has many potential nutritional and health benefits, including anti-diabetic, anti-cancer, anti-inflammatory, aphrodisiac, cardioprotective, and neuro-protective effects.[22,23] Different biochemical compounds have been extracted from *T. terrestris* fruit, including steroidal saponins, glycosides, flavonoids, tannins, amide derivatives, phytosterols, proteins, amino acids and terpenoids,[24,25] which have a wide range of biological properties.[26] For example, some of its active components, such as saponins and flavonoids, have been found to exhibit antioxidant, antiinflammatory, and antimicrobial properties.[27]

The first aim of this study was to demonstrate the synthesis of AuNPs using extract of *T. terrestris* fruits as a reducing, capping and stabilizing agent and to characterize the physical and chemical properties of the formed AuNPs using biophysical methods. Secondly, to test the biological activity of the synthesized AuNPs in terms of their potential use as an antimicrobial agent and whether or not they have adverse effects on human Red Blood Cells (RBCs). These tests would determine if the AuNPs have potential to be developed as therapeutic agents.

Materials and Methods

Ethical approval

This study was approved by the Ethical Board of Khyber Medical University (DIR/KMU-EB/000832/SC, 2022) and the Institutional Research and Ethical Review Board of Khyber Medical College (Peshawar) (298/DME/KMC, 2022). The study was performed in accordance with the National Bioethics Committee for Research (Pakistan) and with the Helsinki Declaration of 1975, as revised in 2013. All donors provided their informed written consent.

Sampling and handling of plant material

T. terrestris fruits were sampled from the Peshawar region of Khyber Pakhtunkhwa near to Sarasang and Chaghar Matti. The fruits were thoroughly washed using distilled water to remove any residual soil and sand. The fruits were air-dried under shade and then ground into a fine powder using an air-free circulation grinding mill with a mesh size of 60 (= 250 µm). The resulting powder was stored in air-tight jars for further analysis.

Plant extract preparation

The extraction of powdered *T. terrestris* fruits was done based on literature methods[28–30] with some necessary modifications, as follows. Measured amounts of the spiny fruits powder (10 or 20 g) were taken in conical flasks and mixed with distilled water in a 1:10 (w/w) ratio of dry mass to water. The flasks were tightly covered with aluminum foil and incubated at 45°C for 2 h in a shaking incubator. The mixture was then filtered twice using Whatman filter paper No. 1, resulting in a clear filtrate that was collected in conical flasks. The aqueous extract obtained from this process, which contains reducing compounds such as flavonoids and tannins,[29] was stored in small bottles and refrigerated at 4°C to preserve its integrity.

Gold nanoparticle synthesis

The general procedure for AuNP synthesis is outlined in Figure 3. The aqueous extract of the dried powdered *T. terrestris* fruit was used for reducing hydrogen tetrachloroaurate(III) trihydrate ($AuCl_4 \cdot 3H_2O$) (Merck; Darmstadt, Germany) to synthesize the AuNPs.[31] In a typical procedure, 5 mg of powdered fruit extract was mixed in 100 mL of double distilled water at a 0.5% (w/v) concentration and heated until complete dissolution was achieved. The solution was then filtered and subjected to centrifugation at 4000 rpm for 20 minutes to eliminate any contaminants. In addition, a 1 mM solution of the gold salt was prepared using double distilled water. The solution of $AuCl_4 \cdot 3H_2O$ was added to the 0.5% solution of plant extract in different ratios and shaken at 25°C to achieve thorough mixing. A red/purple colour appeared on formation of the AuNPs, and the colour change was monitored using UV-vis spectroscopy at a wavelength of 550 nm.[32]

Ultraviolet-visible (UV-vis) spectroscopy

UV-vis spectroscopy was performed on a double-beam spectrophotometer (Lambda 25, Perkin Elmer; Shelton, USA) in the wavelength range 250-800 nm. Surface plasmon resonance (SPR) spectra were obtained over the range of 300-700 nm.

Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on a Prestige-21 Shimadzu instrument (Japan) using a resolution of 4 cm^{-1} over ten scans in the wavenumber range 4000 to 400 cm^{-1}. FTIR data were analyzed by LabSolutions IR software.

X-ray diffraction (XRD)

XRD was performed on a JDX-3532 diffractometer (JEOL; Tokyo, Japan) operating at 20 to 40 kV, within the 2Θ angle range 10° to 80°, and using radiation 0.15418 nm, scan step 0.02°, and scan speed 0.5°/min. A thin layer of AuNPs was deposited on glass plates using the immersion technique and diffraction intensities were collected in Θ to 2Θ configurations. The crystalline domain of AuNPs was identified through peak widths and crystal size was estimated by the Debye-Scherrer equation: $D=0.94\lambda / \beta\cos\theta$, where D=crystal size perpendicular to the reflection planes, θ=angle of diffraction, λ=wavelength of X-ray, and β=full width at half maximum height.

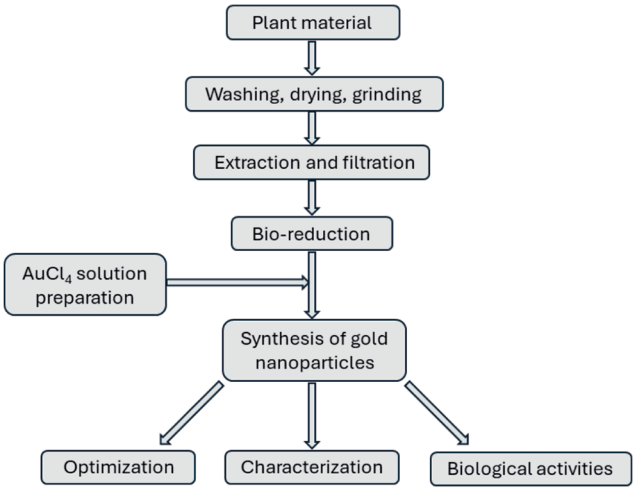

Figure 3. Gold nanoparticle (AuNP) synthesis.

Scanning electron microscopy (SEM)

SEM was performed on a JSM 5910 instrument (JEOL; Tokyo, Japan). To prepare the samples, a mixture of AuNPs and distilled water was used to coat a copper grid with a thin film, using a sputter coater (12150-AB, SPI Module™; West Chester, USA) for 120 seconds. After coating, the sample was air dried at a temperature of 60°C for 5 minutes.

Antibacterial activity

The antibacterial activity of the synthesized AuNPs was tested against the following pathogenic bacteria using an agar well diffusion assay: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Morganella morganii*, *Acinetobacter baumannii*, *Proteus vulgaris*, methicillin-resistant *Staphylococcus aureus* (MRSA). The test bacteria were already stocked in the laboratories at Khyber Medical University. These were cultured in nutrient broth (Difco™; Dilaco, Santiago, Chile) and refreshed on nutrient agar plates. Cultures were transferred again to nutrient broth and incubated at 37°C for 24 h. Test bacteria were then streaked on nutrient agar plates with a sterilized culture swab, and a sterile 6 mm borer was used to make the wells for the test samples. The test samples were prepared at a concentration of 3 mg/mL in dimethyl sulfoxide (DMSO) (<1%). Subsequently, 100 µL aliquots of test samples were introduced into different wells using a micropipette. The standard antibiotic Imipenem and DMSO were used as positive and negative controls, respectively. Plates were left undisturbed for 3-4 h for better diffusion and then incubated at 37°C for 24 h. The zone of inhibition around the wells was measured to the nearest mm and the percent inhibition by test samples was calculated using the formula: % inhibition = zone of inhibition of test sample/zone of inhibition of standard×100. The zone of inhibition for the negative control was zero mm.

Antifungal activity

The antifungal potential of the synthesized AuNPs was tested alongside the antifungal drug fluconazole against the fungal strains *Aspergillus niger*, *Candida albicans*, *Penicillium notatum* and *Aspergillus parasiticus* using an agar slant culture test. Suspensions of fungal isolates were freshly prepared by inoculating fresh stock from each isolate into separate Sabouraud's Dextrose Broth (SDB) (Sigma, Darmstadt, Germany). The inoculated tubes were incubated at 28°C for 24 h. Stock solutions of AuNPs and fluconazole were prepared at a concentration of 24 mg/mL in analytical grade DMSO. A 1 mL aliquot of the appropriate stock solution was added to 5 mL of Sabouraud Dextrose Agar (SDA) medium (Sigma, Darmstadt, Germany) in a test tube. The tubes were kept in a slanting position at room temperature overnight to allow solidification. On the following day the slants were streaked with the fresh fungal cultures. DMSO was used as the negative control. The tubes were incubated at 25°C for one week, then the zones of inhibition were measured to the nearest mm. The percent inhibition was measured by using the equation:

% inhibition = 100 - [(growth in test sample/growth in negative control) × 100].

Antileishmanial activity

The antileishmanial activity of the synthesized AuNPs was tested in an assay using established methods against the parasite *Leishmania tropica* .[33–35] The parasite was isolated from a patient at Khyber Medical College who had contracted cutaneous Leishmaniasis. The isolated parasite was grown in Roswell Park Memorial Institute (RPMI) medium (RPMI-1640 for cell culture; Sigma, Darmstadt, Germany) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco Premium FBS; Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 25°C. The number of parasites was adjusted to 1×10^5/mL by mixing media (4 mL) and parasite culture (1 mL), and cell counting was done using a hemocytometer under a high-resolution microscope (Leica DM500, Jena, Germany).[33,34] A 500 µg/mL stock solution of AuNPs was prepared in analytical grade DMSO. The activity was measured in 96-well plates where different concentrations of each stock solution (1.125 to 250 µg/mL) were taken in each well and mixed with 10% FBS. Into each well of the plate, parasite with 67.6×10^5 mL viable cell concentration was seeded. The plates were incubated at 25°C for 72 h, then living and dead cells were counted using a haemocytometer under a high-resolution microscope (Leica DM500, Jena, Germany). The experiments were done in triplicate and the results were taken as percent mortality (% inhibition). The drug amphotericin B was used as the positive control standard.[35]

Hemagglutination assay

The hemagglutination activity of the synthesized AuNPs was tested against different blood groups (A, B, AB and O). The whole blood of healthy subjects was taken through blood donations at Khyber Medical College. RBCs were isolated from the whole blood by centrifugation and then suspended to 2% in phosphate buffer (pH 7). A 1 mg/mL stock solution of AuNPs was prepared in analytical grade DMSO, which was further diluted to give working stock solutions. These were mixed in equal volumes with the RBC suspension (1 mL each) to give final AuNP concentrations of 5, 10, 20, 30 and 40 µg/mL. The mixture was incubated at room temperature for 30 minutes, and then examined for precipitate formation or granule deposition at the bottom of the tube.[36]

Cytotoxicity assay

The potential cytotoxic effects of the synthesized AuNPs were tested against RBCs based on an established method.[37] To prepare the test samples, a concentration of 500 µg/mL of AuNPs was dissolved in analytical grade DMSO using vortexing and sonication for 10 minutes to ensure homogeneity of the solution. Fresh blood from a healthy donor at Khyber Medical College was collected in an EDTA tube, then 200 µL were mixed with 9.8 mL saline. A 100 µL aliquot of this suspension was mixed with the same volume of the test sample in an Eppendorf tube (Lab Care, Karachi, Pakistan). The resulting mixture was incubated at 35°C for 1 h, allowing sufficient time for the AuNPs to interact with the RBCs. Unbound AuNPs were separated from RBCs by centrifugation (10,000 rpm, 10 minutes). The supernatant was isolated and the absorbance of the supernatant was measured at 405 nm using a UV-vis spectrophotometer (Lambda 25, Perkin Elmer; Shelton, USA). Isolation of the RBCs was achieved by centrifugation (14,000 rpm, 3 minutes), then the RBCs were washed three-times with saline. To ensure the reliability of the experiment, positive and negative controls were also prepared using Triton X-100 (1%) and saline (0.9%), respectively.

Antioxidant activity

The antioxidant activity of the synthesized AuNPs was evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. A stock solution of AuNPs was prepared by dissolving 10 mg in 1 mL of

methanol. Further dilutions were made to obtain concentrations of 100, 200 and 300 µg/mL. Next, 1 mL of each concentration was mixed with 2 mL of freshly prepared DPPH solution, and the mixture was incubated in a dark room for 10 minutes. The absorbance of each sample was measured at 517 nm using a UV-visible spectrophotometer (Lambda 25, Perkin Elmer; Shelton, USA) to determine the free radical scavenging effect and antioxidant activity of the samples.[38] The percent inhibition of the DPPH activity was calculated using the following equation: % inhibition = {(A0 – A1) / A0)}×100%, where A0 is the absorbance of the control sample, and A1 is the absorbance of the test sample. A curve of percent inhibition was plotted, and the EC_{50} value was determined as the concentration where the scavenging reached 50%. A negative control sample was prepared using 1 mL of methanol and 2 mL of DPPH solution and vitamin C was used as the positive control standard.[39]

Statistical analysis

Where appropriate, measurements were performed in triplicate and data were represented as the mean±standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) using GraphPad Prism software. P-values of ≤0.05 were considered statistically significant.

Results

Synthesis and characterization of gold nanoparticles

In this study, AuNPs were produced by utilizing aqueous extracts of *T. terrestris* fruit, used as a reducing agent. Formation of AuNPs was evident by observing a change in colour of the aqueous reaction mixture from yellow to red/purple. The AuNPs had a UV-vis absorption spectrum with a maximum at a wavelength of ~530 nm (Figure 4A). When the SPR of colloidal AuNPs was recorded over time (Figure 4B), the optimum conditions for their formation were a temperature of 25°C, pH of 7 and reaction time of 4 h. FTIR analysis identified the functional groups involved in bonding, stabilizing and capping the AuNPs with some characteristic peaks (Figure 5A). These included a broad peak with a minimum of

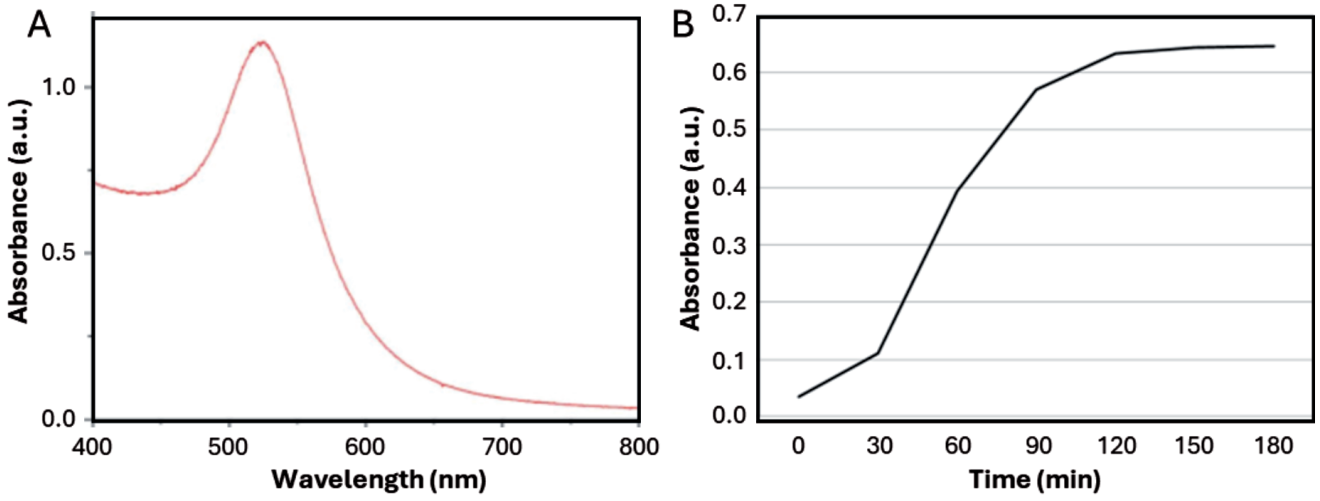

Figure 4. UV-vis absorption analysis of AuNPs synthesized using *T. terrestris* fruit powder extract. A) UV-vis absorption spectrum of AuNPs; B) Example SPR of colloidal AuNPs monitored over time at 530 nm. Where a.u.=absorbance units.

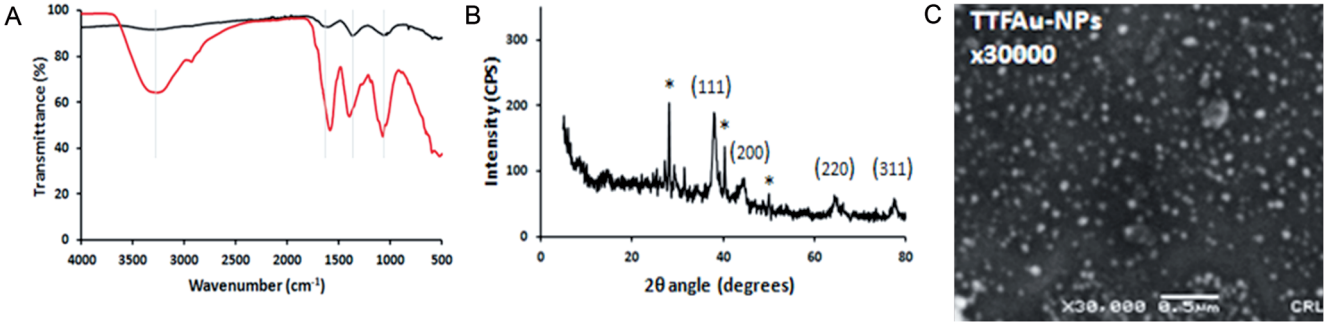

Figure 5. Analysis of AuNPs synthesized using *T. terrestris* fruit powder extract. A) FTIR spectra of AuNPs (red) and *T. terrestris* fruit extract (black). The vertical grey lines illustrate the alignment of peaks; B) XRD spectrum of AuNPs. (111), (200), (220) and (311)=standard Bragg reflections. Where CPS=counts per second, asterisks=peaks likely due to bioorganic compounds on the surface of the AuNPs. C. SEM image of AuNPs at 30,000× magnification.

~3,300 cm^{-1} from OH groups, sharp peaks at 2,925 and 2,855 cm^{-1} from the C-H bond of alkanes, and peaks at 1,650 and 1,360 cm^{-1} from the $–NH_2$ groups of amino acids and the –C=O groups of flavonoids and tannins. When comparing the spectrum of the plant extract with that of the AuNPs, a shift in positions of the –C=O and $–NH_2$ peaks indicated successful formation of AuNPs. XRD analysis confirmed the crystalline structure of the synthesized AuNPs (Figure 5B), where reflection peaks of 38.1°, 44.3°, 64.4° and 77.5° at 2θ angles corresponded to standard Bragg reflections of (111), (200), (220), and (311), respectively, and suggested a Face-Centered Cubic (FCC) structure. When the size of the AuNPs was estimated from the peak Full Width at Half Maximum (FWHM), the average size was around 40 nm. SEM analysis under 30,000× magnification suggested that the AuNPs were roughly spherical and relatively uniform with a diameter of <100 nm (Figure 5C).

Biological activities of synthesized gold nanoparticles

The synthesized AuNPs showed antibacterial activity against different pathogenic bacteria with inhibition ranging from 44.4% to 89.0% (Table 1). The highest inhibition was against MRSA (89.0%) and *S. aureus* (73.0%).

When the antifungal activity of the synthesized AuNPs was tested against different pathogenic fungi using fluconazole as a comparative standard, there was 50% inhibition against *Aspergillus niger*, *Candida albicans* and *Aspergillus parasiticus*, and 40% inhibition against *Penicillium notatum* (Table 2; SD are not available because measurements were performed in duplicate).

The antileishmanial activity of the synthesized AuNPs was tested *in vitro* against the isolated parasite *L. tropica*. In this assay, the drug amphotericin B was used as standard, which produced over 100% inhibition at a concentration of 250 µg/mL. The AuNPs produced antileishmanial activity at all tested concentrations in comparison to the standard. The data fitted best to a two-site binding model with K_d values for high affinity and low affinity sites of 1.45±0.47 and 104.70±45.43 µg/mL for amphotericin B, and 2.60±1.17 and 289.60±172.40 µg/mL for the AuNPs, respectively (Figure 6). Under microscopic examination the effect of the AuNPs was leishmanicidal rather than leishmanistatic, as the parasites were found to be dead.

The AuNPs exhibited positive hemagglutination activity against RBCs of blood groups A and B at 10 and 20 µg/mL, but no hemagglutination activity was observed against blood groups AB

Table 1. Antibacterial activity of synthesized AuNPs from *T. terrestris* fruit. Standard, Imipenem; MRSA, methicillin-resistant *Staphylococcus aureus*. Measurements were made to the nearest mm.

Bacterium	Standard	AuNPs	
	Inhibition zone (mm)	Inhibition zone (mm)	% Inhibition
Escherichia coli	27±0	15±0	55.5
Pseudomonas aeruginosa	27±0	12±0	44.4
Staphylococcus aureus	26±0	19±0	73.0
Morganella morganii	22±0	12±0	54.5
Acinetobacter baumannii	23±0	13±0	56.5
Proteus vulgaris	25±0	11±0	44.0
MRSA	19±0	17±0	89.0

Table 2. Antifungal activity of synthesized AuNPs from *T. terrestris* fruit. Negative control, DMSO; positive control, fluconazole. SD are not available because measurements were performed in duplicate.

Fungal strain	Growth inhibition (%)		
	Negative control	Positive control	AuNPs
Aspergillus niger	0.0	100.0	50.0
Candida albicans	0.0	100.0	50.0
Penicillium notatum	0.0	100.0	40.0
Aspergillus parasiticus	0.0	100.0	50.0

Table 3. Hemagglutination activity of synthesized AuNPs against different blood groups.

Blood group	Concentration of AuNPs (µg/mL)	Hemagglutination activity
A	10	Positive
A	5	Negative
B	20	Positive
B	10	Positive
AB	30	Negative
O	40	Negative
O	20	Negative

and O at up to 40 µg/mL (Table 3). The synthesized AuNPs showed no cytotoxicity when tested against human RBCs at concentrations up to 40 µg/mL.

When the antioxidant potential of the crude *T. terrestris* fruit extract and the synthesized AuNPs were tested using the stable free radical DPPH, both showed antioxidant activity compared with the standard vitamin C. The fruit extract, AuNPs and vitamin C produced EC_{50} values of 235.98±2.23, 319.07±2.98 and 174.45±1.67 µg/mL, respectively.

Discussion

AuNPs have garnered significant attention due to their unique properties and potential applications in various fields.[2–8] In this study we successfully produced AuNPs utilizing *T. terrestris* fruit extract as a reducing agent. In the UV-vis spectrocopy analysis of the synthesized AuNPs, there was an absorption peak maximum at approximately 530 nm (Figure 4A), which is a characteristic resonance resulting from metabolites-to-metal charge transfer. The maximum resonance observed corresponds to the surface plasmon vibrations in the synthesized AuNPs. When the SPR of colloidal AuNPs was monitored over time using UV-vis spectroscopy (Figure 4B), the maximum SPR absorption was observed at 530 nm after 150 minutes of reaction time. A slight increase in particle size was observed with prolonged reaction time, which could be attributed to the nucleation of initially formed particles and further deposition of AuNPs onto their surfaces. Furthermore, a slow deposition process was observed due to chemical reduction. These findings were consistent with those reported in the seed-mediated growth of gold nanostructures.[40] FTIR analysis of the AuNPs confirmed the involvement of specific functional groups in their synthesis and stabilization. XRD analysis confirmed the crystalline nature of the synthesized AuNPs and was consistent with a FCC crystal structure. The XRD data also estimated the average size of the AuNPs to be around 40 nm. Consistent with this, SEM images of the synthesized AuNPs suggested that they had a particle size of less than 100 nm.

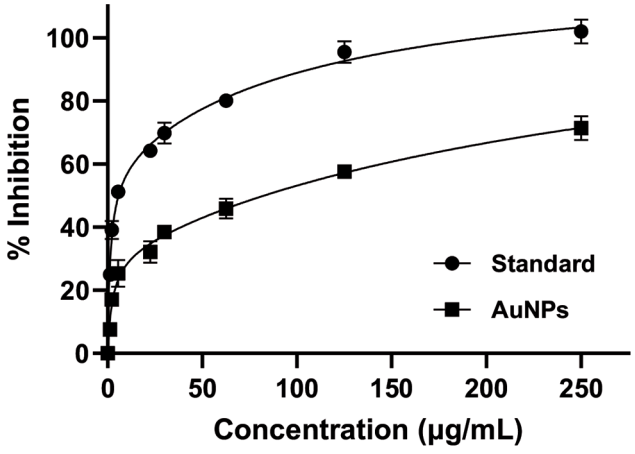

Figure 6. Antileishmanial activity of synthesized AuNPs from *T. terrestris* fruit. Fitting of data to a two-site binding model, where the standard was amphotericin B. The K_d values for high affinity and low affinity sites were 1.45±0.47 and 104.70±45.43 µg/mL for amphotericin B, and 2.60±1.17 and 289.60±172.40 µg/mL for the AuNPs, respectively.

Our observations indicated that the synthesized AuNPs were stable and possessed unique properties potentially suitable for diverse applications in the fields of nanotechnology and biomedicine.

When tested in various assays, the synthesized AuNPs showed some potentially very useful biological activities. The AuNPs showed inhibitory activity (44.0% to 89.0%) against several strains of both Gram-negative and Gram-positive pathogenic bacteria, with the highest activity against the potentially lethal hospital-aquired MRSA. The synthesized AuNPs also showed 40 or 50% inhibitory activity against different pathogenic fungi. Furthermore, the synthesized AuNPs demonstrated antileishmanial activity against the isolated parasite *L. tropica*.

For the AuNPs to be potentially used as therapeutic agents in a clinical setting, it would be important that they did not have adverse effects on human cells. When the hemagglutination activity of the AuNPs was evaluated against different blood groups (A, B, AB, and O), there was positive activity against blood groups A and B at at 10 and 20 µg/mL, but no activity against blood groups AB and O. When the cytotoxic activity of the AuNPs was evaluated against RBCs at different concentrations, no activity was observed even at the highest concentration tested (40 µg/mL), with cell viability remaining above 80%. This suggests that the synthesized AuNPs may have potential use as a therapeutic agent in the fields where AuNPs have already shown applications without causing harm to human cells (e.g. cancer therapy,[2,3] thermotherapy of biological targets,[4] neuroprotection,[5] enhanced antibiotic delivery,[6] detection and treatment of viral and bacterial infections[7,8]).

The only other study that used *T. terrestris* fruits for producing AuNPs was reported by Gopinath *et al.*[41] They used the aqueous extract of powdered *T. terrestris* fruit materials, purchased from a local market in Chennai (India), to give AuNPs with average sizes of 7 nm (GNP7) and 55 nm (GNP55), from reactions using 1 mM and 2 mM $HAuCl_4$, respectively. The synthesized AuNPs demonstrated antibacterial activity against several multidrug resistant *Helicobacter pylori* strains (UM37, UM38, UM67, UM77, UM119 and UM158) with GNP55 having greater effects. The minimum inhibitory concentrations (MICs) of GNP7 and GNP55 ranged from 16.00 to 21.50 µg/mL and their minimum bactericidal concentrations (MBCs) ranged from 18.75 to 24.50 µg/mL. Both GNP7 and GNP55 showed no toxicity against human adenocarcinoma cell lines at the MIC of *H. pylori*.[41]

Molani *et al.* used aqueous extract of whole *T. terrestris* plant powder to reduce $HAuCl_4$ in the production of AuNPs.[42] Their optimised reaction conditions employed a pH of 3 and a temperature of 80°C to give AuNPs with an estimated particle size of 6 to 25 nm. They observed that a larger number of AuNPs with a smaller diameter are formed at lower pH and higher temperature, which is consistent with the larger size of AuNPs estimated from our study (~40 nm and <100 nm) using reaction conditions of pH 7 and 25°C. The synthesized AuNPs from Molani *et al.* were subjected to only one biological assay. The AuNPs showed antibacterial activity against both Gram-positive *S. aureus* and Gram-negative *E. coli*.[42]

Zhao *et al*. synthesized AuNPs using *T. terrestris* flower extract. In addition to subjecting the AuNPs to a DPPH anti-oxidant assay, they were tested in a cytotoxicity assay against an acute leukemia cell line (THP-1), showing good effects.[43] Al-Taee *et al.* synthesized AuNPs (diameter 17-25 nm) from chloroauric acid, using trisodium citrate as the reducing agent, that showed strong antibacterial activity against clinical MRSA isolates. These AuNPs were not used in any other biological assays, including testing for toxicity against human cells.[44]

In our study we have reported the most comprehensive biological testing of AuNPs produced using *T. terrestris* extracts to date.

Our testing results and those of others have demonstrated that such AuNPs have potential use as antibacterial, antifungal and antiparasitic agents with no adverse effects on different blood groups of human RBCs, depending on the concentration used. The AuNPs could also be tested in other fields such as drug delivery,[45,46] medical implants[47,48] and medical imaging.[49,50] Further research is needed to explore these possibilities and to test the safety of the AuNPs *in vivo*.

Conclusions

This study demonstrates that *T. terrestris* fruit powder aqueous extract can serve as an efficient and eco-friendly reducing, capping, and stabilizing agent for the synthesis of AuNPs. The results contribute to the knowledge for the development of green nanotechnology by providing an alternative to traditional chemical methods for synthesizing AuNPs. Because the synthesized AuNPs demonstrated broad antimicrobial activity against pathogenic strains of bacteria, fungi and parasites, but not adverse effects against human RBCs up to certain concentrations, they have potential therapeutic applications. They could also be explored as potentially useful agents in drug delivery, medical implants and medical imaging. In addition to further testing of the therapeutic potential of the AuNPs, and their safety *in vivo*, further research could focus on harnessing the AuNPs for specific purposes or optimizing the synthesis process for desired nanoparticle properties.

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