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Green synthesis of gold nanoparticles using *Tribulus terrestris* extract and antibacterial activity against Gram-negative bacteria

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Aqueous extract of the plant *Tribulus terrestris* was used to reduce chloroauric acid for synthesizing gold nanoparticles (AuNPs). In this green synthesis, the reaction proceeded to give a red/purple color that was monitored by UV-vis spectrophotometry, where the formed AuNPs had an absorption band with λ_{\max} of 550 nm. In terms of the highest absorbance at 550 nm, reaction conditions were optimized at a temperature of 75 °C, at pH 7 and using a reaction time of 4 h. The integrity of the synthesized AuNPs was confirmed and their physical properties were characterized by Fourier-transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and scanning electron microscopy (SEM), which also showed evidence that plant metabolites contributed to capping or stabilization of the AuNPs. XRD spectra suggested a particle size of around 40 nm and SEM images revealed spherical and relatively uniform and disperse particles with a size of less than 0.1 μm . In a broth microdilution assay, the AuNPs showed inhibitory effects against Gram-negative *Escherichia coli*, but not against Gram-positive *Enterococcus faecalis*. The AuNPs showed no hemagglutination activity or cytotoxic activity against human blood cells, which is important for them to be explored as therapeutic antibacterial agents.



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Introduction

Gold nanoparticles (AuNPs) have various physical properties that make them suitable for a range of biomedical applications [1-3]. Highly stable AuNPs can be produced in different shapes and sizes, and they can be conjugated with different types of molecules, including drugs, proteins, nucleic acids, antibodies, enzymes and fluorescent dyes. Hence, AuNPs can be used for the targeted delivery of therapeutic agents such as drugs [4-9], proteins [10-12] and genes [13-16].

AuNPs possess unique electronic and optical properties, including the phenomenon of surface plasmon resonance (SPR) [17-22] which results from the interaction between an electromagnetic wave and conduction of electrons the metal. In the case of AuNPs, there is absorption of light in the blue-green region while red light is reflected to give a rich red colour. The properties of AuNPs allows them to be used for photothermal [23-25], photodynamic [26,27] and radiation [28-30] therapies in cancer treatment and as probes and contrast agents for the imaging modalities of X-ray, computed tomography (CT) and transmission electron microscopy (TEM) [31-36] AuNPs can also be used as biological and biomedical sensors [37-39] and in diagnostic tests including lateral flow bioassays [40-43] AuNPs can be used to detect cancers, heart disease, pregnancy, toxins and infectious agents, which recently includes the coronavirus COVID-19 [44-48].

AuNPs are traditionally synthesised in liquid medium by reducing chloroauric acid (HAuCl_4) using chemical methods. The dissolved acid is rapidly mixed with a reducing agent such as sodium borohydride or sodium citrate and the process results in reduction of gold ions (Au^{3+}) to neutral gold atoms (Au^0) [49-51]. As more gold atoms are produced, the solution becomes supersaturated and the gold precipitates as sub-nanometer particles. The size and shape of the resultant AuNPs can be controlled by using different reaction conditions of reducing agent, solvent, temperature, pH, reaction time and inclusion of additives, which also alters their electronic and optical properties. Whilst AuNPs themselves have low toxicity, the waste products from traditional chemical methods of synthesis can be highly toxic and produced in large quantities. Hence, there has been a shift towards using environmentally and biologically safe “green” and sustainable methods for synthesising AuNPs and other metal nanoparticles, especially by using microorganisms or plant extracts as the reducing agent [52-57]. Plants are particularly attractive for

synthesizing AuNPs because they are widely available in large quantities, inexpensive, non-toxic and rich in different types of organic compounds that can act as reducing and stabilizing/capping agents such as alkaloids, flavonoids, phenols, terpenoids, alcohols, sugars and proteins. The synthesis also does not require aseptic conditions. Whole plant extracts or extracts of leaves, fruits, rhizomes, peels, seeds, pollens, essential oils and gums from a range of different plants have been used for the green synthesis of AuNPs [58-60]. Furthermore, AuNPs synthesised using plant extracts have demonstrated antibacterial [61-63], antifungal [64-66] and antiviral [67-68] activities. The aim of this study, which was performed during the period month year to month year, was to synthesise AuNPs using extract of the plant *Tribulus terrestris*, characterise the synthesised AuNPs using biophysical techniques and test them for antibacterial activity.

Commonly known as puncture vine, *T. terrestris* grows widely as an invasive weed in Pakistan, but it is also used as a medicinal herb [69]. This taprooted herbaceous plant grows as a summer annual and especially thrives on dry, loose, sandy soils, but also grows on other soils. Its yellow flowers develop into a fruit that bears hard burs with sharp spines (**Fig. 1**). Many of the chemical constituents identified in *T. terrestris* that provide its medicinal properties, such as steroidal saponins, flavonoids, alkaloids, glycosides, phytosterols, tannins, terpenoids, amide derivatives, amino acids, and proteins [70-72], all have the potential to serve as a reducing agent in the synthesis of AuNPs.



Fig. 1: *T. terrestris* plant with its yellow flowers (*top*) and its spined fruit (*bottom*)

Materials and Methods

Chemicals and reagents

All the chemicals and reagents used in the experimental work were analytical grade obtained from commercial suppliers and were further purified using standard protocols as necessary. Mueller-Hinton (Oxoid) was the nutrient broth used for the culturing of bacteria in liquid medium and on agar plates. Bacterial strains were obtained from Khyber Medical University, Peshawar.

Collection and processing of plant material

T. terrestris whole plants were collected from different locations of Peshawar, Khyber Pakhtunkhwa in April 2020. The plants were identified at the Department of Botany, Islamia College Peshawar. The collected plants were rinsed thoroughly with normal water and the soil and other residues were removed through rinsing with double distilled water. The plants were dried under shade conditions. The dried plant material was ground in a free air circulation grinding mill of mesh size 60 and the ground material was stored in air-tight jars.

Preparation of plant extract

A measured quantity (typically 10 or 20 g) of dried and powdered plant material was transferred to Erlenmeyer flasks and suspended in double distilled water with a dry mass: water ratio (w/w) of 1:10. The mouths of the flasks were closed with cotton wool and wrapped in aluminum foil, then the flasks were incubated at 45 °C in a shaking incubator for 2 h. The suspension was filtered twice through Whatman filter paper No.1 and the clear filtrate was collected in flasks. The extract was stored in amber bottles at 4 °C.

Synthesis of gold nanoparticles

The aqueous extract of *T. terrestris* was used for bioreduction of tetrachloroauric acid trihydrate (HAuCl₄.3H₂O) (Merck) to produce AuNPs. A 0.5% (w/v) stock solution of *T. terrestris* was prepared by dissolving powdered plant extract (5 mg) in double distilled water (100 ml), with heating for complete dissolution. For removal of bulk impurities, the plant extract solution was filtered and then centrifuged for 20 min at 4000 rpm and the clear supernatant was then used. A 1 mM solution of HAuCl₄.3H₂O was prepared using double distilled water. The aqueous solution of

HAuCl₄.3H₂O was reduced by mixing with the 0.5% plant extract in different ratios at room temperature (25 °C) with gentle stirring. The appearance of a red/purple color indicated the formation of AuNPs, which was monitored by UV-vis spectrophotometry at 550 nm. In order to obtain an effective synthesis and reproducibility of the AuNPs, the reaction parameters of temperature, pH and time were optimized.

Characterization of synthesized gold nanoparticles

The integrity of the synthesized AuNPs was confirmed and their physical properties were characterised using a range of spectroscopy and microscopy techniques. Ultraviolet-visible (UV-vis) spectra were recorded in the wavelength range of 250-800 nm using a double beam spectrophotometer (Lambda 25, Perkin Elmer). Surface plasmon resonance (SPR) spectra were recorded over the range 300-700 nm. Fourier-transform infrared (FTIR) spectra (Prestige-21 Shimadzu, Japan) were recorded over the wavenumber range 4000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹ over ten scans using IR Solution software. X-ray diffraction (XRD) was performed using a JDX-3532, JEOL (Japan) diffractometer. A layer of AuNPs was prepared by dipping a glass plate into a solution of the AuNPs. The instrument was operated at 20 to 40 kV and diffracted intensities were obtained in the Θ to 2Θ configurations. Data were collected in the 2Θ angles range of 10° to 80° with radiation of 1.5418 Å using a scan step of 0.02° and a scan speed of 0.5°/min. The XRD peak widths were used to find the crystalline domain. The AuNPs crystal size was measured using the Debye-Scherrer equation as follows: $D = 0.94\lambda / \beta \cos\theta$, where D is the crystal size perpendicular to the reflection planes, θ is the angle of diffraction, λ is the wavelength of X-ray used, β is the full width at half maximum (FWHM) height. For performing scanning electron microscopy (SEM), AuNPs were mixed with distilled water and a thin film was coated on to a copper grid using a sputter coater (SPI, USA) for 120 seconds, followed by drying with hot air at a temperature of 50 to 60 °C for 5 min. SEM images were taken using a JEOL-JSM 5910 instrument (Japan).

Assay for antibacterial activity

Antibacterial activity was tested by a broth microdilution assay performed in 24-well plates. Mueller-Hinton nutrient broth (600 μ l) was added to each well in the plate along with a fresh overnight culture of the test bacteria *E. coli* or *E. faecalis* (300

µl). Synthesised AuNPs at different concentrations or an antibiotic (trimethoprim sulphamethoxazole or ciprofloxacin) as positive control were added to the wells as appropriate with gentle mixing, then the plate was incubated at 37 °C for 18-24 h. The plates were monitored for bacterial cell growth.

Hemagglutination assay

Haemagglutination activity of the synthesised AuNPs was tested against human red blood cells (RBCs) of all blood groups. A stock solution of AuNPs was prepared at 1 mg/ml in DMSO. Different concentrations in different ratios (1:2, 1:4, 1:8, 1:16) were then prepared from the stock solution. RBCs from whole blood of a healthy person were separated by centrifugation, then a 2% suspension was prepared in phosphate buffer (pH 7). In a test tube 1 ml of test sample and 1 ml of RBC suspension were mixed and incubated for 30 min at room temperature, and then centrifuged. The bottom of the tube was examined for granule deposition.

Cytotoxicity assay

A 500 mg sample of AuNPs was dissolved in DMSO with vortexing to mix followed by sonication for 10 min. Fresh blood taken from a healthy person was transferred to an EDTA tube and mixed by inverting. RBCs were obtained by centrifugation at 14,000 rpm for 3 min and these were washed three-times with saline until the supernatant was clear. A 200 µl aliquot of RBC suspension was added to 9.8 ml of saline, then a 100 µl aliquot of this suspension was mixed with a 100 µl aliquot of test samples in an Eppendorf tube. Triton X-100 (1%) and saline (0.9%) were used as positive and negative controls, respectively. The mixtures were incubated at 35 °C for 1 h and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected, and its absorbance was measured at 405 nm.

Results and Discussion

The principal objectives of this study were to prepare AuNPs using a green synthesis approach by reduction with extracts of the plant *T. terrestris*, to characterize the synthesised AuNPs using biophysical methods, and to test their potential as antibacterial agents.

Synthesis of gold nanoparticles

T. terrestris whole plants were collected from different locations of Khyber Pakhtunkhwa. The plants were rinsed with water, dried under shade conditions and then ground to a powder form. Aqueous extracts of the plant material were used for bioreduction of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ to form AuNPs. During the bioreduction reaction, in which ionic gold (Au^{3+}) is reduced to metallic gold (Au^0), a red/purple colour appeared on formation of the AuNPs. A control reaction was also performed without addition of plant extract and in this case, there was no observable colour change withstanding over 24 h. Phytochemical components of the plant extracts are responsible for reduction of the metal ions in forming the AuNPs and also for stabilising them. For example, the polyphenol groups of flavonoids, tannin, proteins and polysaccharides provide electrons for reducing the metal ions. The phenolic groups display keto-enol tautomerism and it is through de-bonding of the hydroxyl bond of the enol form that electrons or reactive hydrogen are released to reduce the metal ions. The reactions are initially very fast and then become slow because of the capping action of the reducing proteins and polysaccharides in the plant extracts.

UV-vis spectrophotometry was used to monitor the bioreduction reaction and to confirm successful synthesis of the nanoparticles. The developed UV-vis absorption spectrum of the synthesised AuNPs had a maximum absorbance of around 550 nm (**Fig. 2**). This was compared with the plant extract alone, which had a yellow/straw colour and a maximum absorbance at less than 300 nm. In terms of highest absorbance, the optimum temperature for synthesising the AuNPs was 75 °C, the optimum pH was 7 and the optimum reaction time was 4 h (

Fig. 3).

Biophysical characterization of gold nanoparticles

The successful synthesis of AuNPs derived through bioreduction with *T. terrestris* plant extract was confirmed by using further biophysical techniques.

FTIR spectroscopy reports on different chemical bonding groups, so it can be used to demonstrate successful synthesis of the AuNPs. FTIR also reveals possible functional groups and biomolecules that are responsible for capping or stabilising the AuNPs. An FTIR spectrum of the synthesised AuNPs was compared with that of the plant extract before the reduction reaction (

Fig. 4). In the spectrum of the AuNPs the broad peak with a minimum at around 3300 cm^{-1} is due to the O-H stretching vibration of phenolic or alcoholic compounds. The sharp peaks at 2925 and 2855 cm^{-1} are likely to be due to asymmetric stretching vibrations of the C-H bond of alkanes. The peaks at around 1650 and 1360 cm^{-1} are due to $-\text{NH}_2$ groups of amino acids and $-\text{C}=\text{O}$ groups of flavanoids and tannins. Indeed, there was a shift in the $-\text{C}=\text{O}$ peak when comparing the plant extract with the synthesised AuNPs. There was also a shift in the $-\text{NH}_2$ peak when comparing the plant extract with the AuNPs. These observed shifts indicate successful synthesis of the AuNPs and suggest that flavonoids, amino acids, proteins and sugars contribute to bioreduction of the gold ions and capping or stabilisation of the synthesised AuNPs. The significant peak at around 1070 cm^{-1} is due to C-O and C-O-C groups of alcohols, ethers, carboxylic acids and esters, which did not shift on formation of the AuNPs.

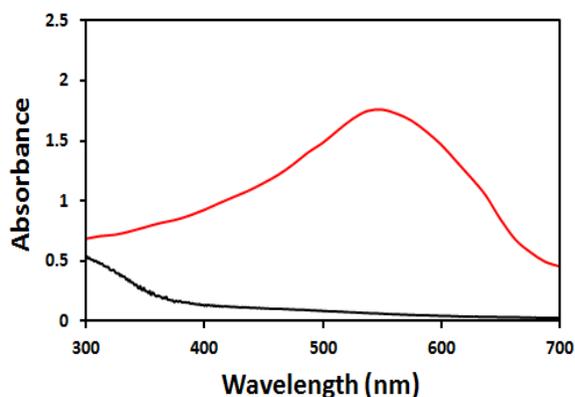


Fig. 2: UV-vis absorption analysis of synthesised gold nanoparticles. UV-vis absorption spectra of *T. terrestris* whole plant extract (black) and synthesised AuNPs (red).

The crystallinity of the AuNPs was confirmed by XRD, where the spectrum showed reflection peaks of 38.1° , 44.3° , 64.4° and 77.5° at 2θ angles, corresponding to standard Bragg reflections of (111), (200), (220), and (311), respectively (**Fig. 5**). The standard 'd-values' of these samples correlate well with the observed 'd-values' from the JCPDS data card 04-0784 for gold and confirm the formation of AuNPs. Considering the Bragg peaks of the angular positions, a face-centered cubic (FCC) structure can be assigned to the AuNPs. The peak at 38.1° was most intense, suggesting preferential crystal growth in the (111) direction. The prominent peaks at 28.4° and 40.6° may originate from bioorganic compounds occurring on the surface of the AuNPs, possibly from

the capping reaction. The size of the nanoparticles was estimated from the peak full width at half maximum (FWHM) and calculated using Scherrer's equation: $D = K\lambda/(\beta\cos\theta)$. Where K is the Scherrer's constant value of 0.94 selected due to the cubic and crystalline form of the nanoparticles, D is the mean size of the ordered crystalline domains, β is the full width at half maximum intensity of the peak, θ is the Bragg angle and λ is the wavelength of the X-ray. The average size of the AuNPs was around 40 nm.

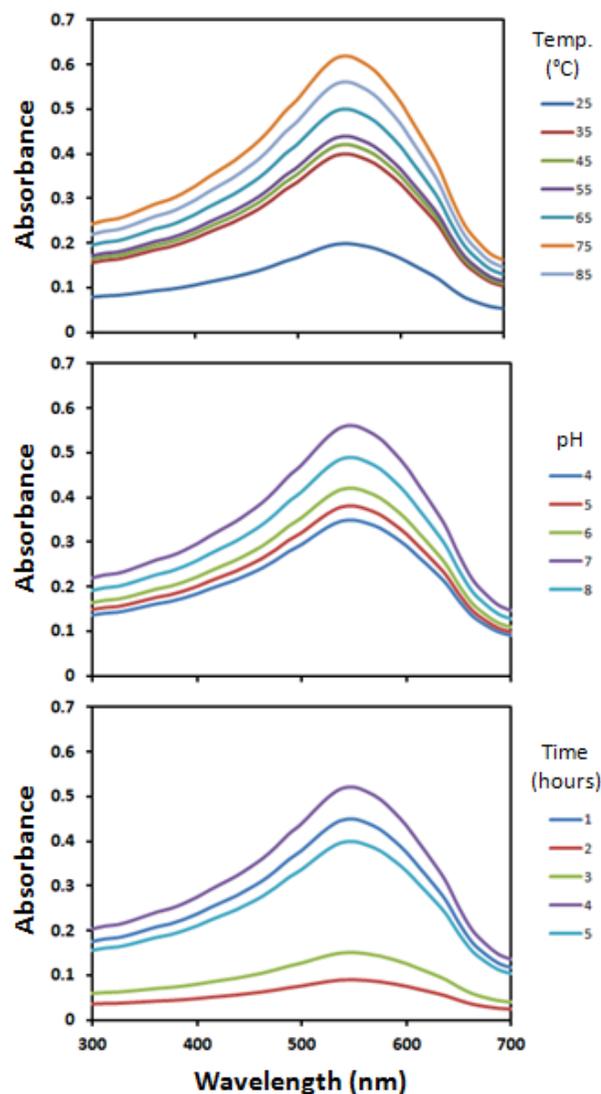


Fig. 3: Optimisation of conditions for synthesis of gold nanoparticles. Absorbance measurements of reaction mixture at 550 nm using different temperatures (pH 7, 4 h) (top), pH values (25 °C, 4 h) (middle) and reaction times (25 °C, pH 7) (bottom).

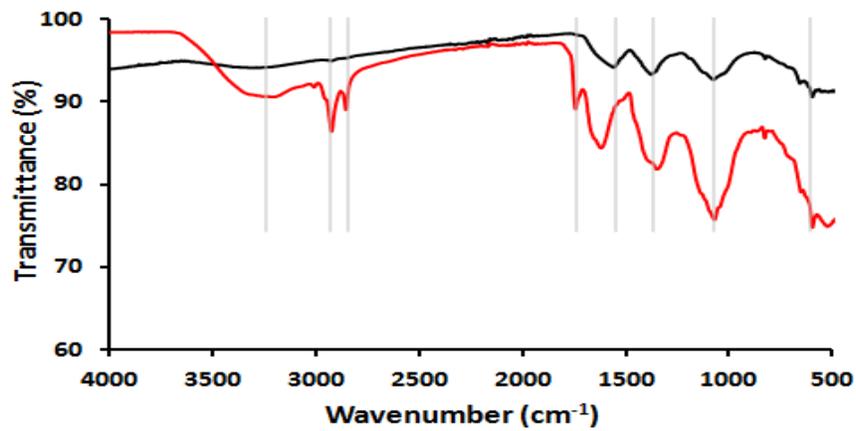


Fig. 4: FTIR analysis of synthesized gold nanoparticles. FTIR spectra of *T. terrestris* whole plant extract (black) and synthesized AuNPs (red).

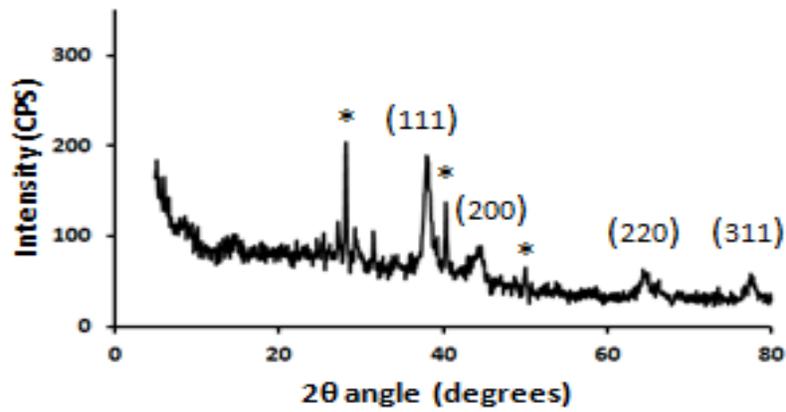


Fig. 5: XRD spectrum of synthesised gold nanoparticles. (111), (200), (220) and (311) = standard Bragg reflections. Asterisks = peaks likely due to bioorganic compounds on the surface of the AuNPs.

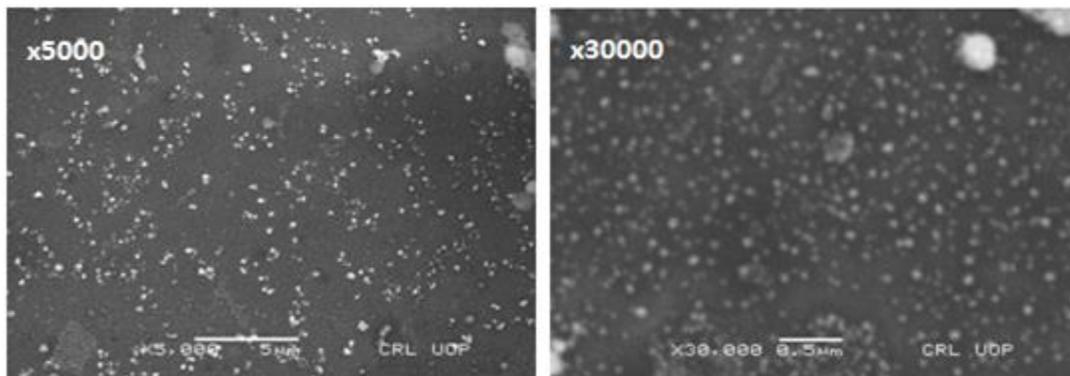


Fig. 6: SEM images of synthesized gold nanoparticles. At x5000 and x30000 magnification.

SEM images of the synthesised AuNPs revealed spherical and relatively uniform and disperse particles with a size of less than 100 nm, as seen under x30,000 magnification (Fig. 6). The SEM images were further evidence for successful synthesis of the AuNPs.

Antibacterial activity of gold nanoparticles

The antibacterial activity of the synthesised AuNPs was tested by broth microdilution assay using *E. coli* (Gram-negative) and *E. faecalis* (Gram-positive) as test organisms (

Table 1). The AuNPs caused inhibition of growth of *E. coli* and therefore antibacterial effects on this organism. This was compared with visible growth in the negative control sample and inhibited growth in the antibiotic positive control sample. The same inhibitory effects of the AuNPs were not observed with *E. faecalis*, which may be due to differences in cell wall structure in the organisms, with the Gram-negative one being permeated or broken down more easily. For example, the cell walls of Gram-positive bacteria contain teichoic acid whilst the outer membranes of Gram-negative bacteria contain lipopolysaccharides and phospholipids, giving them a different charge. The Gram-positive cell wall is also formed by a thicker and more rigid peptidoglycan layer than that of Gram-negative bacteria. Furthermore, the AuNPs showed no haemagglutination activity against all human blood groups and no cytotoxic activity against human blood cells (not shown). These observations are important for the AuNPs to be explored as therapeutic antibacterial agents.

Table 1: Summary of antibacterial effects of synthesized gold nanoparticles by broth microdilution assay.

Test sample	<i>E. coli</i>	<i>E. faecalis</i>
TTW AuNPs	Inhibited	Growth occurred
TTF AuNPs	Inhibited	Growth occurred
Positive control	No growth	Less growth
Negative control	Visible growth	Visible growth

Note: TTW = *T. terrestris* whole plant, TTF = *T. terrestris* fruit, Positive control = antibiotic added, Negative control = no AuNPs or antibiotic added.

Conclusions

We have demonstrated the successful green synthesis of AuNPs using *T. terrestris* extract as the reducing agent. Formation of the AuNPs was evidenced by a colour change during the synthesis reaction and by analysis of the product using biophysical methods. The *T. terrestris*-derived AuNPs showed antibacterial

effects against Gram-negative *E. coli*, but no effect against Gram-positive *E. faecalis*. Importantly, the AuNPs showed no haemagglutination activity or cytotoxic effects against human red blood cells, so they have potential for use as a therapeutic antibacterial agent. Future work should optimise a rigorous procedure for preparing the *T. terrestris*-derived AuNPs that showed antibacterial effects and they should be tested on a wider range of Gram-negative and Gram-positive bacteria. The antibacterial AuNPs may have applications in medicine, medical implants, dentistry, cosmetics, biosensors and agriculture. The potential antifungal, antioxidant, antiviral, anti-inflammatory, anticancer and antidiabetic properties of the optimised AuNPs could also be investigated.

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Conflict of interest

The authors declare no conflict of interest.

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