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Synthesis, characterization, antimalarial, antitrypanocidal and antimicrobial properties of gold nanoparticle

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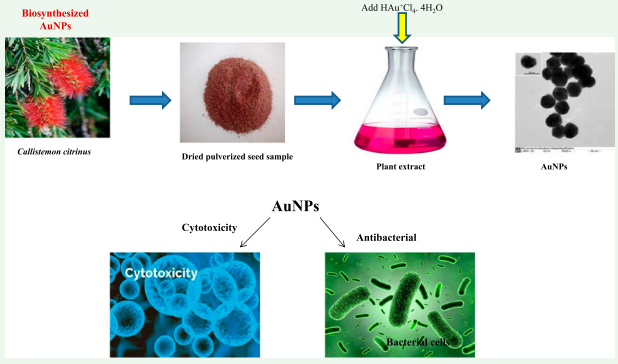
ABSTRACT

In this study, we report on the biosynthesis of gold nanoparticles (AuNPs) by using *Callistemon citrinus* (*C. citrinus*) seed extract as both reducing and capping agent as well as its characterization. Likewise, the *in vitro* antiparasitic activities of both biosynthesized gold nanoparticles and crude seed extract of the plant were evaluated. The formation of biosynthesized AuNPs was confirmed by a color change immediately when the seed extract was added to the gold chloride (III) solution. Characterization of the AuNPs was done using analytical tools like ultraviolet–visible spectroscopy, X–ray diffraction, scanning electron microscopy (SEM), energy dispersive X–ray (EDX), transmission electron microscopy (TEM) and Fourier transformed infra–red (FTIR). FTIR showed an absorption peak at 230 nm consistent with the absorption band for gold nanoparticles, the morphology and composition of AuNPs was ascertained by SEM and EDX micrographs; uneven spherical-shaped nanoparticles was established by SEM analysis, and an average particle size of about 37 nm was confirmed by the TEM analysis. The crude seed extracts exhibited antitrypanosoma activities with an IC50 of 11.06 µg/mL. Both the crude seed extract and AuNPs were inactive against plasmodial parasite, while the antibacterial assay showed that AuNPs is potent against gram positive and gram negative bacterial strains.



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AuNPs; TEM; SEM; FTIR; antiplasmodial; antitrypanosoma; cytotoxicity; *Callistemon citrinus*

1. Introduction

In the last ten years or more, metal nanoparticles have triggered serious interests among researchers as a result of their well-defined physical, chemical and biological properties. Natural products like biodegradable polymers (chitosan), bacteria, fungi and extracts of different plant genus are now adopted as stabilizing and reducing agents to serve as an alternative to the inorganic synthesis of nanoparticles (*1*–*3*). The reason for this is that green routes of nanoparticles synthesis are eco-friendly, simple, economical and comparatively reproducible (*4*, *5*).

Gold nanoparticles biogenesis via plant extracts is becoming more accepted due to their compelling antibacterial activity, low-risk for clinical research and the ease of gold salt reduction, the technique is straightforward, one step approach and it is profitable for large scale production.

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Gold nanoparticles possess electrical and harmonious optical characteristic, in addition to this, they display Surface Plasmon Resonance (SPR) which is employed in drug conveyance, trace detector and diagnostic probes. Gold nanoparticles through biogenic route possess superb biocompatibility and non-toxicity. Shapes of gold nanoparticles such as nanoprisms, nanotriangles, nanoplates, nanowires, nanocages, nanospheres, nanostars, nanobelts etc. have been reported, their shape and size have a great impact on their properties particularly optical properties (*6, 7*). More attractive optical characteristic is exhibited by triangular shaped nanoparticles than the spherical shaped (*8*).

Gold nanoparticles have transformed the field of medicine owing to its extensive application in direct drug delivery, imaging, analysis and curative purposes which is brought about by the enormously small size, solidity, tunable optical, non-cytotoxic, appreciative uppermost layer, physical and chemical properties of the gold nanoparticles (*7, 9*).

The synthesis of gold, nickel oxide and mercuric oxide nanoparticles using *Callistemon viminalis* have been reported (*10, 11*), in addition to this, the use of silver oxide and silver nitrate to prepare nanoparticles mediated by *C. citrinus* have also been documented (*12–14*).

The secondary metabolites embedded in plant such as terpenoids, tannins, flavonoids, proteins, amino acids, enzymes may be responsible for the bio-reduction of Au^{3+} ions into metallic Au^{1} nanoparticles. $HAuCl_4$. $4H_2O$ + extracts from plant Au NPs + by-products (*15*).

Callistemon citrinus (Curtis) Skeels (Myrtaceae), a commonly available plant in Eastern Cape of South Africa has a lot of therapeutic importance. It is known to be a rich source of compounds with bioactive components since it produces different secondary metabolites with important biological activities (*16*). Formation of strong antioxidant agents and free radicals are major expressions observed from the growth of this plant in different environment, this is made possible as a result of harsh growth conditions and combination of high oxygen concentration and light (*17*).

In previously published works, the antinociceptive, anti-inflammatory (*18, 19*), antimicrobial (*16, 20*), antioxidant (*16, 21*), antifungal (*22, 23*), cytotoxicity (*22*) and antihyperglycemic properties (*10*) of this plant were reported. Compounds with immense diversity like steroidal glycoside, triterpenoids (*24*), flavonoids (*25*), terpenoids (*16*), saponins and steroids (*26*) were isolated from this plant, in addition to this, the plant has been found to be very rich in phenolic content (*16*). Nanoparticles have been synthesized from *Callistemon* genus like *C. viminalis* (*10, 27, 28*).

This study reports on the synthesis and characterization of gold nanoparticles by the reduction of aqueous $HAuCl_4$ using the seed part of *C. citrinus* extract. This biosynthesized AuNPs was studied for its antimalarial activity against chloroquine sensitive (3D7) strain and their antitrypanocidal potential against *trypanosoma brucei brucei* (*T.b brucei*). The cytotoxicity of the AuNPs was also examined against HeLa (human cervical carcinoma cells) cell line.

Interestingly, no study to the best of our knowledge has reported the bio-synthesis of gold nanoparticles using *C. citrinus* seed extract with their antiplasmodial, antitrypanocidal and antimicrobial properties.

2. Materials and methods

2.1. Materials

Gold (III) chloride ($HAuCl_4$) solution was purchased from Merck, South Africa. All other reagents used in this study were of analytical grade.

2.2. Characterization

To determine the potential of the bio-synthesized nanomaterials towards plasmodial, trypanocidal and microbial strains, we carried out the characterization of the materials with different equipments. For instance, X-ray diffraction was obtained with Bruker D8 advanced x-ray diffractometer (XRD) in other to determine the crystallinity and size of the materials. The vibration of the materials was observed using Perkin-Elmer Universal ATR 100 Fourier Transformed Infra-Red spectrophotometer (FT-IR). Scanning electron microscope (SEM) and Electron diffraction spectrophotometer (EDS) images were obtained using JOEL JSM-6390 LVSEM. These provide information on the morphology and composition of the materials. The shape and particle size of the samples were confirmed by means of Transmission Electron Microscope (TEM) with model: JOEL 1210 transmission electron microscope at 100 kV accelerating voltage. Perkin-Elmer Universal absorption spectrophotometer was used to collect the absorption spectra of the materials.

2.3. Preparation of the plant extracts

The fresh seed of *C. citrinus* was air dried for about 21 days at room temperature, the dried seed was further pulverized using a mechanical blender (Polymix, PX-MFC 90D), 30 g of the grinded sample was added to 250 mL of distilled water, the mixture was shaken on an orbital shaker at 200 rpm for 24 h, it was filtered via

Whatman no 1 filter paper, the filtrate obtained was lyophilized into dry powder, kept in a centrifuge tube with a cover and preserved in the refrigerator at 4°C pending the time it was needed for the nanoparticles synthesis.

2.4. Synthesis of gold nanoparticles

The formation of gold nanoparticles was achieved by adding 12.5 mL of the plant extract to 90 mL of Gold (III) Chloride solution (0.001M), the mixture was incubated for 6 h with continuous stirring and the reaction was carried out in the dark to prevent unnecessary photochemical reactions. At the expiration of 6 h incubation time, the mixture was centrifuged at 15,000 rpm for 15 min at room temperature, the pellet obtained from the centrifuge process was washed twice with distilled water and air dried, and the obtained AuNPs was used for the different assays below.

2.5. Antimalarial assay

The *in vitro* antimalarial activity of the AuNPs was evaluated by measuring the malaria parasite viability (*Plasmodium falciparum* strain 3D7) using parasite lactate dehydrogenase (pLDH) method described by Makler et al. (*29*). Briefly, the AuNPs was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 20 mg/mL, 50 µg/mL from the stock solution earlier prepared was mixed with the parasite cultures in a 96-well plates, the resultant mixture above was incubated at 37°C in a CO_2 incubator for a period of 48 h. After the incubation period was completed, 20 µL of the culture was removed from all the wells and mixed with approximately 125 µL of a mixture of nitrotetrazolium blue chloride (NBT) /phenazineethosulphate (PES) and Malstat solutions in a new 96-well plate. A purple color will be generated if parasite lactate dehydrogenase (pLDH) is present.

The absorbance of the mixture in the new 96-well plates was recorded at 620 nm so as to estimate the amount of pLDH in each well plate. Finally, if the sample is able to bring down the parasite viability to less than 20% for the single concentration assay, the dose–response assay will be embarked on to ascertain the IC_{50} (50% inhibitory concentration). Chloroquine (20 µM) was employed as a positive control in this study.

2.6. Antitrypanocidal activity

The antitrypanosoma single concentration assay was also carried out in the same way antimalaria assay was done. In a nutshell a single concentration of 50 µg/mL of the sample acquired from the stock solution of 20 mg/mL was added to the *in vitro T.b brucei* culture in a 96-well plates, the mixture was subjected to 48 h incubation and the amount of parasite that can survive the drug contact were quantified by mixing resazurin-based reagents capable of being reduced to resorufin by living cells.

The resorufin can be quantified in a multi-well fluorescence plate reader because it is a fluorophore (Exc_{560}/Em_{590}). The result of the antitrypanosoma assay was checked in line with cell cytotoxicity. Pentamidine (a known drug for the management of trypanosomiasis) was used as a positive control drug standard.

2.7. Antibacterial activity

The zone of inhibition of the AuNPs was examined by agar well diffusion method as illustrated by Collin et al. (*30*); this method has been earlier described in our previous work Larayetan et al. (*16*). Briefly, the microbial cultures were inoculated in nutrient broth (Oxoid) and incubated for about 24 h at 37 ± 0.1°C. Adequate amount of Muller Hilton Agar (Oxoid) were dispensed into spotlessly clean petri dishes and allowed to congeal under aseptic situation by means of a sterilized cork borer, 6 mm diameter wells were uniformly distributed in the newly prepared and solidified Mueller Hilton agar (Oxoid) inside the petri dishes, thereafter, the bacterial culture was adjusted to 0.5 McFarland turbidity standard and the test microbes (0.1 mL) were inoculated using a sterilized swab on the outer surface of the solid medium in each of the petri dishes. Different concentrations of the AuNPs prepared from the stock (15.625–62.5 mg mL^{-1}) were introduced into each of the wells and labeled correctly. The inoculated petri dishes were incubated at 37°C for 24 h. The whole petri dishes were subsequently checked for zones of growth inhibition surrounding the individual wells and the average diameter of these zones was measured in millimeters.

2.8. Cytotoxicity assay

The cytotoxicity of the synthesized AuNPs was evaluated against HeLa (human cervix adenocarcinoma) cells by Keusch et al. method (*31*) with some modifications. In order to resolve maybe the samples were cytotoxic, they were incubated in duplicate along with 1×10^4 HeLa cells per well allotted in a 96-well plate for 48 h at 37°C in a 5% CO_2 incubator.

Cells that remained alive after exposure to the drug were resolved by applying resazurin-based reagent to the mixture of extract and the Hela cells, the living cells reduced resazurin into resorufin and this was quantified in a fluorescence multi-well plate reader. The percentage

(%) cell viability was calculated from the resorufin fluorescence in compound treated wells relative to untreated controls (*32*).

2.9. Statistical analysis

Data analysis was carried out with the aid of origin software statistical computing system. This statistical package put into consideration the adjustment of the regression coefficient square, R^2 (*33*).

3. Result and discussion

3.1. Characterization

FTIR was employed for the determination of the functional groups on plant extract and AuNPs biosynthesized material (Figure 1).

The FTIR spectra of AuNPs showed vibration frequencies at about 1680 and 3400 cm^{-1} attributed to carbonyl stretch and N-H stretch vibrations respectively arising from amide bonds from protein. These stretching frequencies indicate that amino acid residues and protein peptides can effectively bind to the surface of metals thereby functioning as a coating agent on the surface of the nanoparticles (preventing agglomeration) as well as serving as reducing agent. The reducing ability ensured that a peak was observed at about 500 nm attributed to Au–O band. This Au-O band is however not observed in the spectra of the plant extract (Figure 1). Similar observation was reported by Abdel-Raouf et al. (*34*).

The successful synthesis of gold nanoparticles was confirmed by the change in color of the reacting solution brought about by the reduction of Au (III) to Au (I) in Gold (III) Chloride solution followed by scanning with UV–visible spectrophotometer. The UV–visible spectrophotometer measurement (Figure 2) showed an absorption band at 225 nm for gold nanoparticles formed by plant extract reduction. This band has been demonstrated to be the absorption band for gold nanoparticles with sizes ranging from 1 to 50 nm in previously reported study (*35*).

In order to determine the morphology and composition of the synthesized material, SEM and EDS micrographs of the bio-synthesized gold nanoparticles were recorded (Figure 3(A & B)). Irregular spherically shaped nanoparticles were seen to have been formed from the SEM micrograph (Figure 3(A)). Although few agglomerations of the particles were observed which could be as a result of the uneven distribution of the plant extract in solution, the particles formed were still well dispersed. EDS micrograph (Figure 3(B)) showed that pure AuNPs were obtained after the bio-reduction of gold chloride salt. This result conforms to the observations reported in the work of Thirumurugan et al. (*36*).

TEM image of AuNPs was observed to ascertain the morphology and size of the synthesized material (Figure 3(C)). It was revealed that a spherical shaped material was mainly formed. Other than these shapes, small amount of triangular and rectangular nano-shaped material was also formed. This result conforms to the result obtained when chemical method is

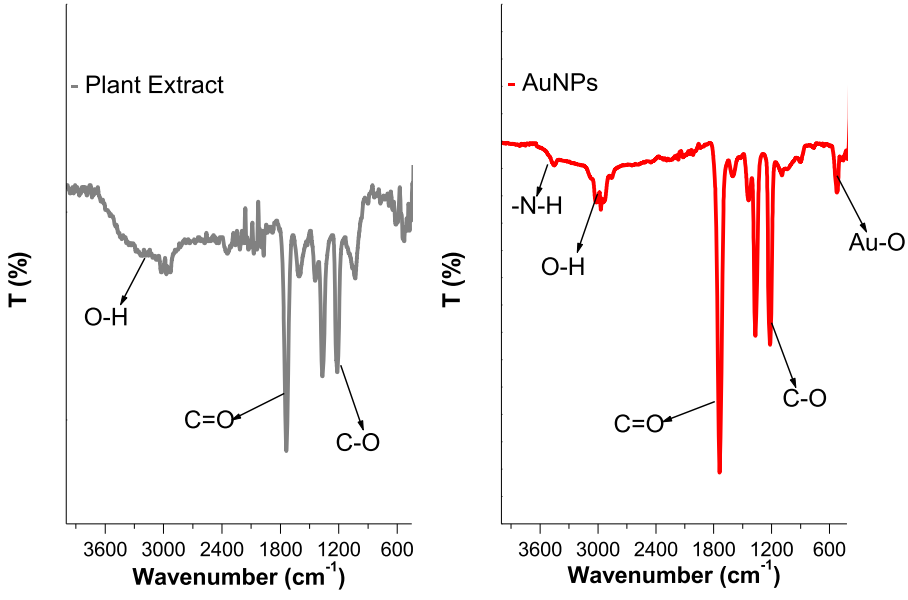

Figure 1. FTIR spectra of plant extract and gold nanoparticles.

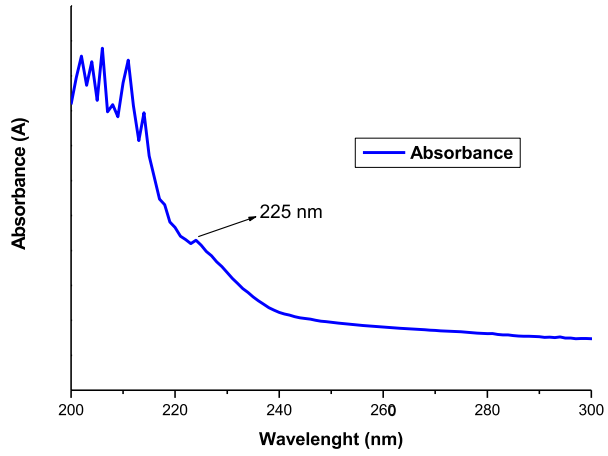

Figure 2. Absorption spectra of gold nanoparticles.

used to synthesize AuNPs. For instance, AuNPs obtained by the reduction of $HAuCl_4$ with cetyl trimethyl ammonium bromide (CTAB) shows that upon examination of the material under TEM, different shapes of the synthesized material were obtained (*37*). Size determination obtained from the average of the measurement of the diameters of the individual particles by the instrument indicates that an average particle size of around 37 nm was obtained for this bio-synthesized material.

XRD analysis of the gold nanoparticles observed from 2θ values between 20° to 100° (Figure 4) indicates that AuNPs was synthesized. Strong diffraction peaks at 2θ values of 38.5° characteristics of AuNPs was observed

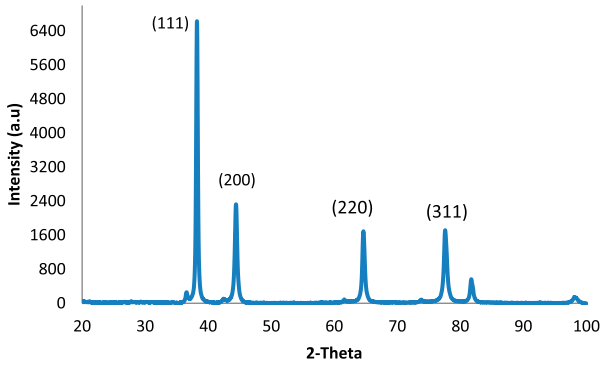

Figure 4. XRD spectra of AuNPs mediated by *C. citrinus*.

in the diffractogram. Also, diffraction peaks at 2θ values of 44.4°, 64.5° and 77.5° confirms the successful synthesis and the crystalline nature of the material. Furthermore, employing the Scherrer formular (*38*), the size of 35.5 nm was obtained for the synthesized nanoparticles; this result complements the data obtained for the size of this material using the transmission electron microscope. Similar result was reported in previously published paper (*39*).

3.2. Antiplasmodial properties

The antiplasmodial activity of the gold seed nanoparticle was also examined, the % viability at (91.58 ± 8.04%) were discovered to be inactive as they were not able to bring reduction of pLDH to less than 20%.

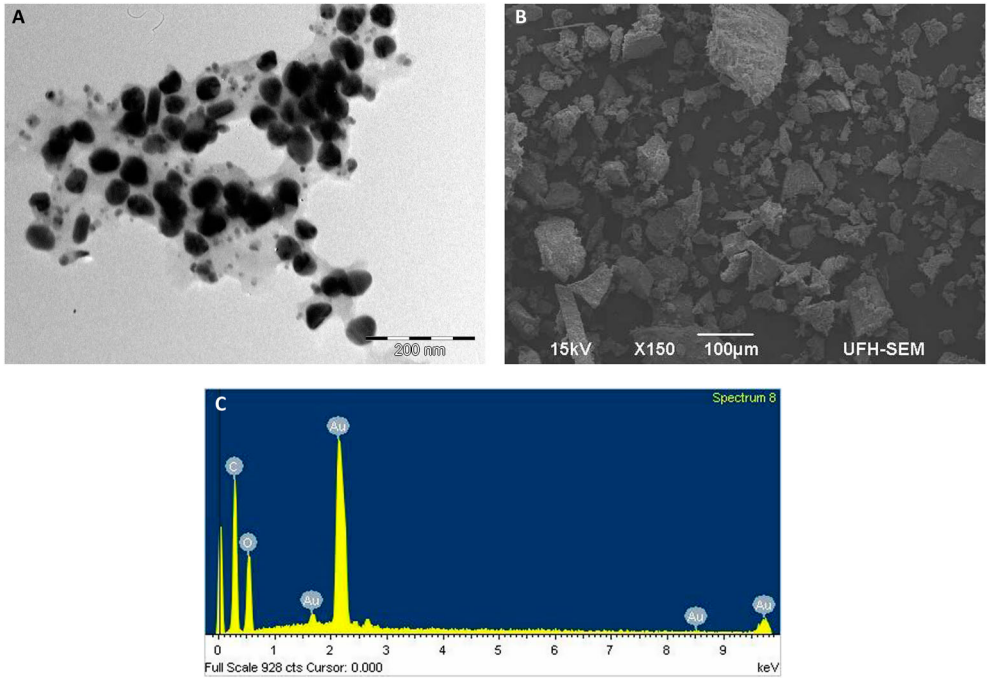

Figure 3. Images of AuNPs under (A) SEM (B) EDS and (C) TEM.

3.3. Antitrypanosomal and cytotoxicity activities

The gold synthesized nanoparticles from the seed extract of *C. citrinus* was investigated with *T.b brucei* assay, it was revealed that the percentage (%) viability at 50 µg/mL was (103.19 ± 0.56%) and this value was not able to bring a considerable reduction to about 20% of the trypanosome parasites and are considered inactive, but the crude seed from which it was synthesized was able to reduce trypanosome parasites to (0.54 ± 0.01%) which was considered very active (*14*). The crude seed was further subjected to dose–response test to ascertain the concentration of the compound needed to kill 50% of the parasites in the culture. It was discovered from the dose–response curve that the IC_{50} of the crude seed was 11.06 µg/mL (Figure 5). An existing literature document by Bero et al. (*40*) stated that IC_{50} value of ≤ 20 µg/mL are considered as good or very potent, while IC_{50} of between 20–60 µg/mL are considered as fair and the $IC_{50} > 100$ µg/mL is termed not active. Based on this observation the crude seed showed very good activity against trypanosome parasites.

The AuNPs (seed) was tested against HeLa (human cervix adenocarcinoma) cells at an exact concentration of 50 µg/mL. It was found that the synthesized gold (seed) nanoparticle was not cytotoxic since it was not able to reduce the viability of HeLa cells to below 50% (89.66 ± 1.55%), one can reliably establish from the above result that the killing of the parasite cultures was not as a result of general cytotoxicity of the seed AuNPs.

3.4. In vitro *antibacterial activity*

The aqueous extract of the seed of *C. citrinus* plant brought about the reduction of gold ions; this may be due to the presence of high amount of eucalyptol with α-terpineol and terpinen-4-ol present in the seed as reported by Larayetan et al. (*16*). The *in-vitro* antibacterial potency of the synthesized gold seed nanoparticles were evaluated against three gram negative and four-gram positive bacterial strains (*Escherichia coli 0157:H7:ATCC 35150, Vibro alginolyticus DSM 2171, Salmonella typhi ACC, Staphylococcal enteritis ACC, Staphylococcus aureus ACC, Listeria Ivanovii ATCC 19119* and *Mycobacterium smegmatis ATCC 19420*). It was discovered that the AuNPs has a broad spectrum of activity against the bacteria strains.

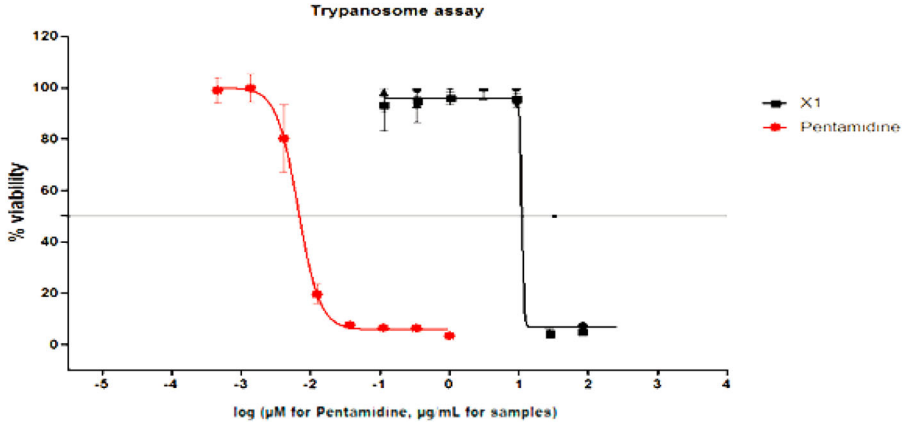

Figure 5. Dose–response curve for trypanosome assay X1: (Crude seed).

Table 1. Zone of inhibition of the synthesized AuNPs from *C. citrinus* and the standard drug.

Microorganism concentration	Positive control Ciprofloxacin (mg mL^{-1})			Au Seed nano (mg mL^{-1})			Dimethyl Sulfoxide (DMSO) (Negative Control)
	62.5	31.25	15.625	62.5	31.25	15.625	
Gram negative bacteria strains							
E. coli 0157:H7:ATCC 35150	35.0 ± 4.0	30.0 ± 4.0	18.0 ± 0.9	15.0 ± 3.0	12.0 ± 3.0	7.0 ± 3.0	0.4 mL VG
Vibroalginolyticus DSM 2171	33.0 ± 2.0	25.0 ± 2.0	13.0 ± 0.4	13.0 ± 2.0	12.0 ± 5.0	9.0 ± 4.0	0.4 mL VG
Salmonella typhi ACC	35.0 ± 1.0	32.0 ± 0.6	18.0 ± 3.0	15.0 ± 0.5	9.0 ± l.0	7.0 ± 2.0	0.4 mL VG
Gram positive bacteria strains							
Staphylococcal enteritis ACC	40.0 ± 5.0	32.0 ± 1.0	17.0 ± 0.2	20.0 ± 0.5	13.0 ± 0.1	9.0 ± 0.4	0.4 mL VG
S. aureus ACC	20.0 ± 2.0	15.0 ± 0.0	11.0 ± 2.0	20.0 ± 4.0	10.0 ± 1.0	8.0 ± 2.0	0.4 mL VG
Listeria Ivanovii ATCC 19119	35.0 ± 6.0	30.0 ± 0.2	22.0 ± 1.0	15.0 ± 2.0	12.0 ± 2.0	10.0 ± 1.0	0.4 mL VG
M. smegmatis ATCC 19420	35.0 ± 6.0	32.0 ± 0.0	24.0 ± 1.0	13.0 ± 0.0	11.0 ± 1.0	9.0 ± 0.3	0.4 mL VG

Zone of inhibition (millimeter), ACC (AEMREG CULTURE COLLECTION), ATCC (AMERICAN TYPE COLLECTION CENTER), values are mean ± SD, n = 3, VG (Visible growth).

The recorded inhibitory effect was highest for *Staphylococcal enteritis ACC* and *S. aureus ACC* (20.0 ± 0.5 and 20.0 ± 0.4 mm) at a concentration of 62.5 mg/mL and was found to compete favorably well with the standard drug used as positive control while the lowest inhibitory action of (13.0 ± 2.0 & 13.0 ± 0.0 mm) was documented for *Vibro alginolyticus DSM 2171* and *M. smegmatis ATCC 19420*. The highest inhibitory effect on gold seed nanoparticle AuNPs was contrary to our previous work on silver seed nanoparticles(AgNPs) were it was documented that the AgNPs from the seed had one of the least inhibitory activity of 15.0 ± 4.0 on *S. aureus ACC* (*14*). The smaller size of the AuNPs coupled with the large surface area could be responsible for the enhanced membrane permeability and subsequent cell damage (*41*) (Table 1).

4. Conclusion

AuNPs was successfully synthesized from the seed extract of *C. citrinus*. It was tested against *plasmodial*, *trypanosoma* parasites as well as some bacterial strains. The size determination via TEM analysis indicate an average size of about 37 nm and SEM analysis showed that most of the nanoparticles synthesized were irregular spherical shaped. Interestingly, the synthesized AuNPs was also found not to be cytotoxic to HeLa cells, although it was not active against *plasmodial* and *trypanosomal* parasites, but it was able to inhibit all the bacterial strains used confirming the usage of the seed plant as an excellent source for naturally occurring cytotoxic and antimicrobial drugs.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Notes on contributors

Larayetan Rotimi received his Ph.D. in Chemistry at the University of Fort Hare, South Africa. Dr Larayetan has co-authored more than 13 publications in reputable and scientific journals. He is a member of South African Chemical Society and Chemical Society of Nigeria. He has presented several papers at national and international conferences. He received the best oral presentation (2017) at the 3rd African International Biotechnology and Biomedical conference held in Kenya. His research interests are on natural product, biogenic synthesis of nanoparticles and organic synthesis.

Mike O. Ojemaye holds a Ph.D. degree in Chemistry. He is presently a Post–Doctoral Research Fellow at the South Africa Medical Research Council; Microbial Water Quality Monitoring Centre at the University of Fort Hare, South Africa. His research interests are in the area of Bio–Inorganic, Material and Environmental Chemistry. He has eight published articles and has presented several papers at national and international conferences and seminars. He is a recipient of the South Africa Medical Research Council study support (2015–2017). He also served as Quality Assurance Executive for five years. He is a member of the South African Chemical Institute, South African Catalysis Society and Associate member of the Royal Society of Chemistry, UK.

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Alexander Sadimenko is a Professor of Chemistry. He has taught and conducted research in different universities including Rostov State University, Addis Ababa University, National University of Lesotho and University of Fort Hare. His area of specialization is in Physical Chemistry and has published over 200 research articles and several papers presented at national and international conferences and seminars. He has also supervised and graduated several doctoral and master students. Prof Sadimenko was responsible for the designing of a new curriculum on Pure and Applied Chemistry at the University of Fort Hare in 2003. He serves as a referee for Elsevier and Kluwer journals in Chemistry and Applied Chemistry. He is a member of the Electrochemical Society of South Africa as well as the Catalysis Society of South Africa.

Prof Anthony I. Okoh holds a Ph.D. degree in Microbiology. He served as Head of the Department of Biochemistry and Microbiology at the University of Fort Hare (UFH) from 2009 to 2015. He is currently the Leader of the Water Research Niche Area of the university; Director, SAMRC Microbial Water Quality Monitoring Centre; and the Deputy Dean of the Faculty of Science and Agriculture in the same University. His research interests fall within the aegis of Applied and Environmental Microbiology including water/wastewater quality, emerging pollutants, reservoirs of antibiotic resistance and bioactive compounds of health and biotechnological importance. He has published over 330 journal articles and graduated 41 Ph.D. and 57 Master's degree students. He is a recipient of the UFH Vice-Chancellor Emerging Researcher Award; the Vice-Chancellor Senior Researcher Medal; and Achievers Award of the Biotechnology Society of Nigeria. Prof Okoh also served as President of the prestigious South Africa Society for Microbiology (2011–2013). He is a member of the Academy of Science of South Africa (ASSAF); Fellow of the Water Institute of Southern Africa; and Fellow of the prestigious African Academy of Sciences (AAS); and currently has an h-index of 41 and i-10 index of 145.

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