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Research article

Antibacterial and Cytotoxicity of Silver Nanoparticles Synthesized in Green and Black Tea

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ABSTRACT

In this study, silver nanoparticles (AgNPs) have been successfully synthesized using green and black tea in a facile and inexpensive environmentally friendly process. The green and black tea extracts were verified to contain phytochemicals such as alkaloids, flavonoids, saponins, phenols and steroids. These phytochemicals in green and black tea served as reducing agent and as stabilizing and capping agents for the microwave-assisted synthesis of AgNPs from $AgNO_3$. The formation of the AgNPs were established by ultraviolet–visible spectroscopy (411 and 420 nm) and the physical were established with scanning electron microscopy (SEM) which indicated that the nanoparticles were spherical in shape with diameter between 30 nm and 50 nm. Different AgNPs concentrations of 680, 340, 170, 85 and 42.4 µg/disc were tested. AgNPs inhibited growth of Escherichia coli and Staphylococcus aureus. Half maximal inhibitory concentration (IC50) of AgNPs (with and without infusion of tea) by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay MTT (µM) that was required for 50% inhibition after 72 h was found at 400 to 454 µg /mL. AgNPs did not show any cytotoxicity against chicken embryo fibroblast cells. Tea extracts produced AgNPs showed promise as a non- toxic alternative for biological applications.

Keywords: Antibacterial activity, Cytotoxic, Green and black tea extracts, Green synthesis, Silver nanoparticles.

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INTRODUCTION

Worldwide interest in various aspects of nanotechnology research is on the rise as more and more diverse applications and developments of various types of nanoparticles are identified including therapeutics, diagnostics, sensing, drug/vaccine delivery, gene therapy, etc. [1]. One of the most important considerations before applying nanoparticles for biomedical applications is their toxicity [2]. Toxicity of some nanoparticles can be inferred from their physical characteristics such as their biocompatibility, chemical composition, size, shape solubility, surface chemistry, and concentration [3-5]. Among the nanoparticles that have received enormous attention are the silver nanoparticles (AgNPs) owing to their physicochemical attractive electronic and optical properties which enable a host of innovative applications [6]. The most harnessed attributes and properties of AgNPs are its strong disinfectant properties that had been used for antimicrobial disinfection, wound gauze, anti-odor sock and shirts, etc. [7].

Biological synthesis techniques using microorganisms and enzymes

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have been recommended as eco-friendly replacements for the chemical methods of synthesis [8]. The benefits of using plant or plant extract as reducing and coating agents during the synthesis of nanoparticles outweigh those of other biological methods, because it is a one-step synthesis process that takes a shorter process than that which involves bacterial cell culture, eliminates the use of bacterial cells that could leave toxic by-products, results in AgNPs that are potentially safe for human therapeutic use, and the process is amenable for large- scale manufacturing.

The process of producing silver nanoparticles involves reduction of silver nitrate in solution, microwave could be used as source of energy but other modes of heating are also used such as laser ablation, and there have been some environmentally benign synthesis methods [9,1]. The latter outweighs the rest because it is an eco-friendly rapid method for synthesizing nanoparticles. Plant or plant extracts had also been used for the synthesis of inorganic nanoparticles including silver nanoparticles [10,5].

In the current study, AgNPs were synthesized in aqueous leaf extract of green tea and black tea. These tea extracts served as the reducing agents in a microwave-assisted method. A microwave-assisted method had been described for the synthesis of AgNPs [11] which involved a dipolar mechanism and ionic transfer [12].

In comparison with conventional methods for synthesis of nanoparticles, microwave-assisted method provided small and uniform size monodispersed nanoparticles with uniform crystallinity [11,12]. Additionally, microwave-assisted synthesis of nanoparticles only requires low temperature compared with the conventional synthetic methods [11].

The toxicity of AgNPs had been reported in a wide-range of studies [13-16], however, there has been a scarcity of reliable and consistent data to categorically claim absolute AgNP toxicity [17]. Kim and Ryu [18] reported a high degree of apoptosis, oxidative stress, and genotoxicity to cell line due to exposure to AgNPs. A study carried out by Xu *et al* [13], indicated that AgNPs led to a high antimicrobial activity when administered at 5µg/mL. In addition, their study indicated that the production of reactive oxygen species (ROS) was the main reasonwhy AgNP antimicrobial activity was manifested.

Previous report [19] reported that immunological effect of silver nanoparticles *in vitro* and *in vivo* as an adjuvant. Their conclusion indicated that the AgNPs have significant adjuvant effect and the mechanism of this effect was mainly ascribed to the recruitment and activation of local leukocytes, especially lymphocytes, increased cytokines levels in mice, as well as increased concentration of IgG and phagocytes. Thus, in the present studies, biological methods were used to synthesize AgNPs using microwave-assisted method. The ultimate goal of this study was to evaluate the cytotoxicity of AgNPs that had been synthesized in tea leaf extracts on chicken embryo fibroblast (CEF) cell line as model cells. The antibacterial activities of the green synthesized AgNPs were tested on gram positive and gram negative bacteria.

MATERIALS and METHODS

Materials, chemicals and instruments

Green tea and black tea used in this study were purchased from the local market in Baghdad-Iraq**.** chloroform ,acetic anhydride, NaOH , potassium carbonate AgNO3, alcoholic ferric chloride, hydrochloric acid, H_2SO4, 3-5 dinitrobenzoic acid (Sigma-Aldrich-Germany). Chicken embryo fibroblast (CEF) cells were cultured in RPMI-1640 (Sigma-Aldrich-Germany) that was supplemented with 100 units/mL penicillin, 15% calf bovine serum, and 100 µg/mL streptomycin. Trypsin-EDTA (US biological, USA). fetal bovine serum (FBS), MTT (Sigma-Aldrich, USA), DMSO (Dimethyl Sulfoxide) (BDH, England). AgNPs electronic absorption spectra were established using spectrophotometer (PD-303, Apel, Japan). The silver nanoparticle size and shape analysis was performed using Scanning Electron Microscope (SEM) (Hitachi S-4500 SEM machine). The Zeta-potential was measured to establish the stability of the nanoparticles (Brookhaven NanoBrook ZetaPlus, USA). The signals were recorded using a microplate Reader (Organon Teknika Reader 230S, Austria).

Synthesis of AgNPs in black tea and green tea infusion

The extracts of green tea and black tea were prepared as follows. A 50 mg/L black tea extract was prepared by placing 1.5 g of black tea leaves in 250 mL of boiling distilled water for 20 min. From this volume, 60 mL of the extract was taken out with a pipette and diluted to 500 mL. The pH of the diluted extracts was adjusted to 10 by adding 500 mg of potassium carbonate to the reaction [20]. Green tea extract was prepared by adding 2.6 g of green tea in 200 mL DW water. The mixture was put in microwave for 60 sec before removing the leaves. For stock solution of black tea 1.3 g was used instead of 2.6 g. During the synthesis of the AgNPs in black tea, 67 mg of $AgNO_3$ were added directly into 200 mL of the black tea infusion, and for synthesis of the AgNPs in the green tea 54 mg of AgNO3 were added directly into 200 mL of green tea infusion extract with a pH adjusted to 10.

Phytochemical screening of the extracts

Around 2g of dried green and black tea leaves were weighed and added to distilled water. The extract-solutions were subjected to different phytochemical screening to distinguish the chemical components existent in the extracts using qualitative tests in comparison with standard chemical reagents. Standard techniques were used for testing tannins, saponins, flavonoids, alkaloids, steroids, terpenes, and glycosides.

Analysis of Phytochemical

Phytochemicals of the crude extract of green and black tea were qualitatively analyzed by standard methods. The methods described as follows.

Tannins

Solution of 10% alcoholic ferric chloride was mixed with 2 mL of extract. The development of green or blue color indicated the presence of tannins [21].

Alkaloids

Dragendorff's reagent and Mayer's reagent were used in crude extracts to test the presence of alkaloids. Briefly, 1 mL of extract was dissolved in 5 mL of hydrochloric acid (1%). Formation of white precipitation demonstrated the presence of alkaloids [22,23].

Flavonoids

A mixture of 1 mL of 5% of lead acetate and 1 ml of crude extract was prepared and kept for 2 min at room temperature. Formation of white precipitates indicated the presence of flavonoids [22, 23].

Phenols

Distilled water was used to prepare 5 mL of crude extract which was added to 3 mL of solution of lead acetate at a concentration of 10%. A dark green color indicated the presence of phenolic compounds [22,23].

Saponins

A 2 mL crude extract and 6 mL of DW were mixed in a test tube. The mixture was thoroughly mixed and the formation of foam indicated the presence of saponins [22, 23].

Steroids and Terpenoids

One milliliter of chloroform, few drops of concentrated H_2SO4, and 1ml acetic anhydride were added to 5 ml of the extract. The formation of red color indicated the presence of terpenoids and steroids [21].

Glycosides

A few drops of Keed reagent [3-5 dinitrobenzoic acid (0.5gm) dissolved in 25 mL of 95% methanol and a few drops of 1N NaOH solution was added to 5 ml of crude extract. A change in color of solution to blue indicated the presence of glycosides [24].

Characterization of the AgNPs

The UV–visible absorption of the resulting nanoparticles was scanned between 200–1200 nm to establish the maximum wavelength (λmax) of absorption. The λmax was used to estimate the diameter and the shape of the AgNPs. Scanning electron microscope (SEM) was used to establish the average size and the shape of the nanoparticles. The zeta potential was measured and was used to test the stability of the AgNPs.

Preparations of chicken embryo fibroblast (CEF) cells

Chicken embryo fibroblast (CEF) cells were cultured in RPMI-1640 that was supplemented with 100 units/mL penicillin, 15% calf bovine serum, and 100 µg/mL streptomycin. The cells were allowed to grow to about 85% confluence over 3 passages using Trypsin-EDTA pre-harvest detachment. The excess trypsin was neutralized with culture medium before using the cells. After harvest, the cell population was established using a hemacytometer after trypan blue staining. Fresh unstained cells were reseeded in 96- well plate that was incubated at 37°C before the MTT-cytotoxicity assay.

MTT-cytotoxicity assay

Freshly harvested cells were seeded at $1x10^4$ cells/well and incubated for 24 h at 37°C. When a confluent monolayer was achieved, the supernatant was aspirated from the well. Fresh aliquots of growth medium RPMI 1640 with 15% fetal bovine serum (FBS), antibiotic agent (penicillin, streptomycin), and AgNPs at various concentrations (150, 75, 37.5, 18.76, and 9.37µg/mL) were added respectively. Cell viability was measured after 72 h of exposure to the AgNPs by removing the medium, adding 28 µL of 2 mg/mL solution of MTT and incubating for 1.5 h at 37°C. After removing the MTT solution, the crystals remaining in the wells were solubilized by adding 130 µL of DMSO (Dimethyl Sulfoxide) followed by incubation at 37°C for 15 min with gentle shaking [25]. After checking that the crystals were completely dissolved, the absorbance was determined on a microplate reader at 450 nm (test wavelength). All assays were performed in triplicate. The LC50, which is the lowest concentration that kills 50% of cells was established [26].

Antimicrobial activity through Agar disks diffusion test

This method was performed in Luria-Bertani (LB) agar Petri dish. In brief, discs were saturated with different AgNPs concentrations of 680, 340, 170, 85 and 42.4 µg/disc. The discs were dried in air in sterile conditions. LB agar plates were swabbed with the microbe cultures at 1×10^6 cells. The plates were incubated for 24 h at 37ºC and the resulting inhibition zone was monitored [27].

RESULTS and DISCUSSION

Eliminating the unwanted toxicity and enhancing the antimicrobial activities of AgNPs is currently a preferred area of research because of its growing applications in various biomedical areas. Numerous environmentally friendly and biological methods of AgNPs synthesis have recently been reported that showed promise for future biomedical uses [13]. In this study, green and black tea extracts were used as substrate for the AgNP synthesis. The phytochemicals in green and black tea were screened by standard methods prior to use in AgNP synthesis. The results verified the qualitative presence of alkaloids, flavonoids, saponins, phenols and steroids whereas terpenoids and glycosides were absence in both extracts. These phytochemicals in green and black tea served as reducing agent and as stabilizing and capping agents for the microwave-assisted synthesis of AgNPs from $AgNO_3$.

Synthesis of AgNPs in black tea and green tea extracts

In this study, AgNPs were synthesized in the presence of black tea and green tea extracts. As illustrated in Table 1, the phytochemical screening of green and black tea extracts exhibited the presence a complex mixture of phytochemical components. The black tea extracts contained polyphenol and other substances like thearubigins, gallocatechin, epigallocatechin epicatechin, catechins, and flavanols [20] that served as reducing agents for $AgNO_3$. The green tea extract contained catechins and flavanols [20] which served as reducing agents for the $AgNO_3$. These bioorganic contents also served as capping agents during the nanoparticle synthesis. The mechanisms of action of the bioorganic molecules have been reported to provide antioxidant activity giving the ability to stimulate reduction of Ag^+ ions to Ag atoms in the formation of the AgNPs [15]. Both green tea and black tea have been used for the preparation of AgNPs as reducing agents [20].

Table 1. Phytochemical screening of leaf extracts of green and black tea

Phytochemical	Green and Black tea
Alkaloids	+
Flavonoids	
Saponins	
Phenols	
Steroids	
Terpenoids	-
Glycosides	

In the present study, the extracts from both dried black tea and green tea yielded a greenish solution during microwave-assisted synthesis of nanoparticles.

Characterization of the AgNPs

At end of the AgNPs synthesis, the presence of nanoparticles was detected through the UV-Vis spectra. The absorption peaks were recorded in the range of 411 nm to 420 nm for the black tea and green tea synthesized AgNPs, respectively (**Fig. 1**). The tea-leaves extract reduction of the silver ions into AgNPs was exhibited by the color change of the silver nitrate solution from colorless to brown within 60 seconds in the microwave. The control $AgNO_3$ solution (without the leaves extract) that was subjected to the same microwave conditions did not show any change in color. During synthesis, the addition of the tea extracts was accompanied by an immediate change in color which indicated the beginning of the reduction of the $AgNO_3$. The color of the reaction went from colorless to yellowish tinge which became darker over time. The color change indicated the formation of silver nanoparticles as confirmed by the UV-Vis spectra. In **fig 1**, the spectra showed a significant peak around 400 nm which had been previously reported for the synthesis of AgNPs [12]. **Fig. 2 a,b** showed that AgNPs were about 30 nm and 50 nm in size for the black tea and green tea synthesized AgNPs, respectively.

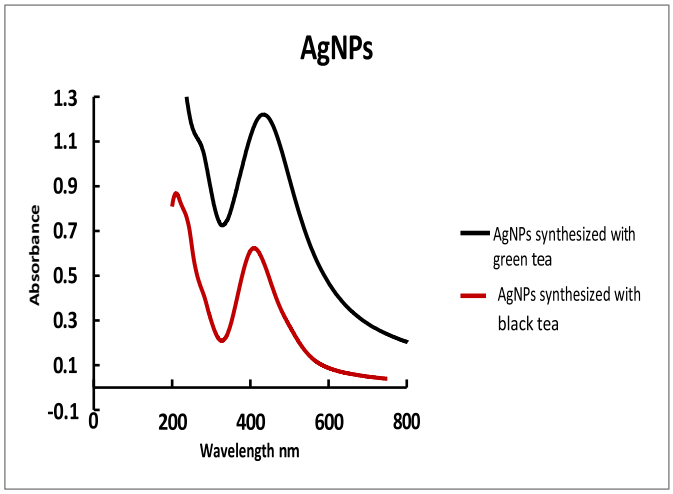

Fig 1. UV–vis absorbance for green tea-extract synthesized and black tea-extract synthesized AgNPs.

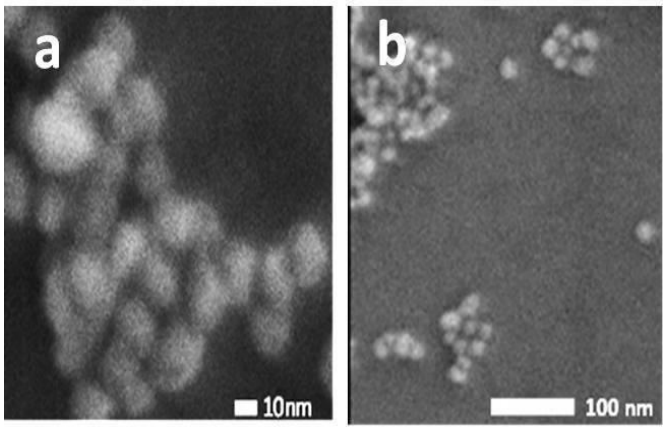

Fig 2. SEM image of green tea-extract synthesized AgNPs (a) and black tea-extract synthesized AgNPs (b).

The long-term stability of the tea-extract synthesized colloidal AgNPs was monitored through the zeta potential which was used to establish the properties of the surface of the AgNPs. The predominance of either positive or negative surface charges in nanoparticles has been generally claimed as neces- sary for the stability of colloidal inorganic nanoparticles [28]. The inorganic nanoparticles with a large positive or negative zeta potential had been shown to repel each other, thereby, preventing aggregation. It has been reported that low absolute zeta potential values lead to aggregation and flocculation due to the absence of repulsive forces [29]. The Zeta potential of the tea-extract synthesized colloidal AgNPs were −30 mV and -19mV, respectively. **Fig. 3** shows that AgNPs were stable as exhibited by a zeta potential that did not change significantly over a period of 30 days. This stability may be attributed to the complex composition of the tea extracts that may have acted as coating agents for the AgNPs. In comparison with other published processes for the synthesis of AgNPs, the current preparations did not require additional steps for coating to make the AgNPs stable and to prevent aggregations [30]. The tea extracts synthesized AgNPs stayed in colloidal dispersion without additional treatment after synthesis. However, in order to be able to store these AgNPs for extended periods of time, further shelf-life studies must be carried out over periods 6, 12, 18, and maybe as much as 24 months.

MTT-cytotoxicity assay

Most commercially available AgNPs were toxic to both human cells and bacterial cells at sufficiently high concentrations [1,31]. In order for AgNPs to be more valuable for biomedical applications, they need to be less or non-toxic to human cells. This study addressed of the toxicity of AgNPs in order to expand its antimicrobial applications to biomedical applications as well. Having prepared the AgNPs in human friendly tea extracts, it was expected to get non-toxic nanoparticle preparations. Thus, the cytotoxicity of the tea extracts synthesi- zed AgNPs , at various concentrations at 9.37,18.75,37.5,75 and 150 µg/mL, respectively, were tested on Chicken embryo fibroblast CEF and the toxicity was evaluated by MTT assay. The results shown in **fig 4** indicated that the viability of the CEF cells were not significantly affected by the presence of the various concentrations of the AgNPs in comparison with the control which were not exposed to the nanoparticles. Even at the highest concentration tested which was 150 µg/mL of AgNPs, the change in response compared with the untreated cells was very insignificant. A previous study on the use of AgNPs for wound healing and tissue renewal showed that the viability was significantly reduced after a 1 h exposure to 10 µg/mL of 50 nm commercial AgNPs as reported by Hackenb- erg *et al* [32]. A separate study using100 µg/mL of AgNPs at 10 and 20 nm diameter that were synthesized by growing Ag onto 5 nm gold-seed particles and stabilized by citrate buffer did not show toxicity for progenitor human adipose-derived stem cells after incubation for 24 h [32]. These differences may be attributed to different synthesis methods, different capping and reducing agents, degree of purification, different sizes, disseminations and different shells. The variations in cell lines may have contributed to the variations in results as well. Furthermore; the absence of full characterization of the AgNPs after addition into the cell medium was common to all of the reported studies. Thus far, there has been no consensus on the *in vitro* toxicity of silver nanoparticles (AgNPs). However, contrary to the report of Hackenberg *et al*. [32], tea extract synthesized AgNPs reported here showed no toxicity to the

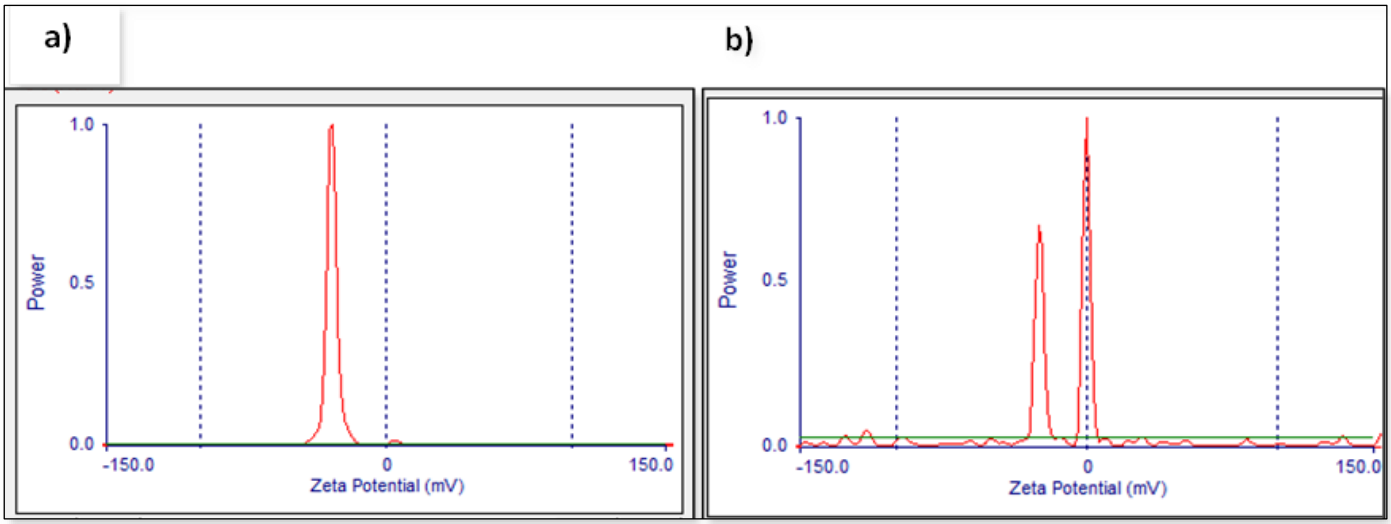

Fig 3. Zeta potential of freshly prepared green tea-extract synthesized AgNPs (a) and black tea-extract synthesized AgNPs (b) with ζ values −30 mV and -19mV, respectively.

model cells which was based on the report by Yang *et al.* [35] which indicated that polyphenol found in tea infusion could inhibit tumor cell. According to Yang *et al*., the green method of synthesis of AgNPs diminished the concerns regarding the danger of extreme exposure to treatment by reducing their cytotoxicity making them as a prospective material for food packaging, wound dressing applications, and for inhibiting cancer cells. The half maximal inhibitory of AgNPs concentration (IC50) by MTT (μM) that was required for 50% inhibition was found between 400 and 454 μg /mL after 72 h. According to IC 50 values the silver nanoparticles synthesized in this study can be classified as non-cytotoxic material.

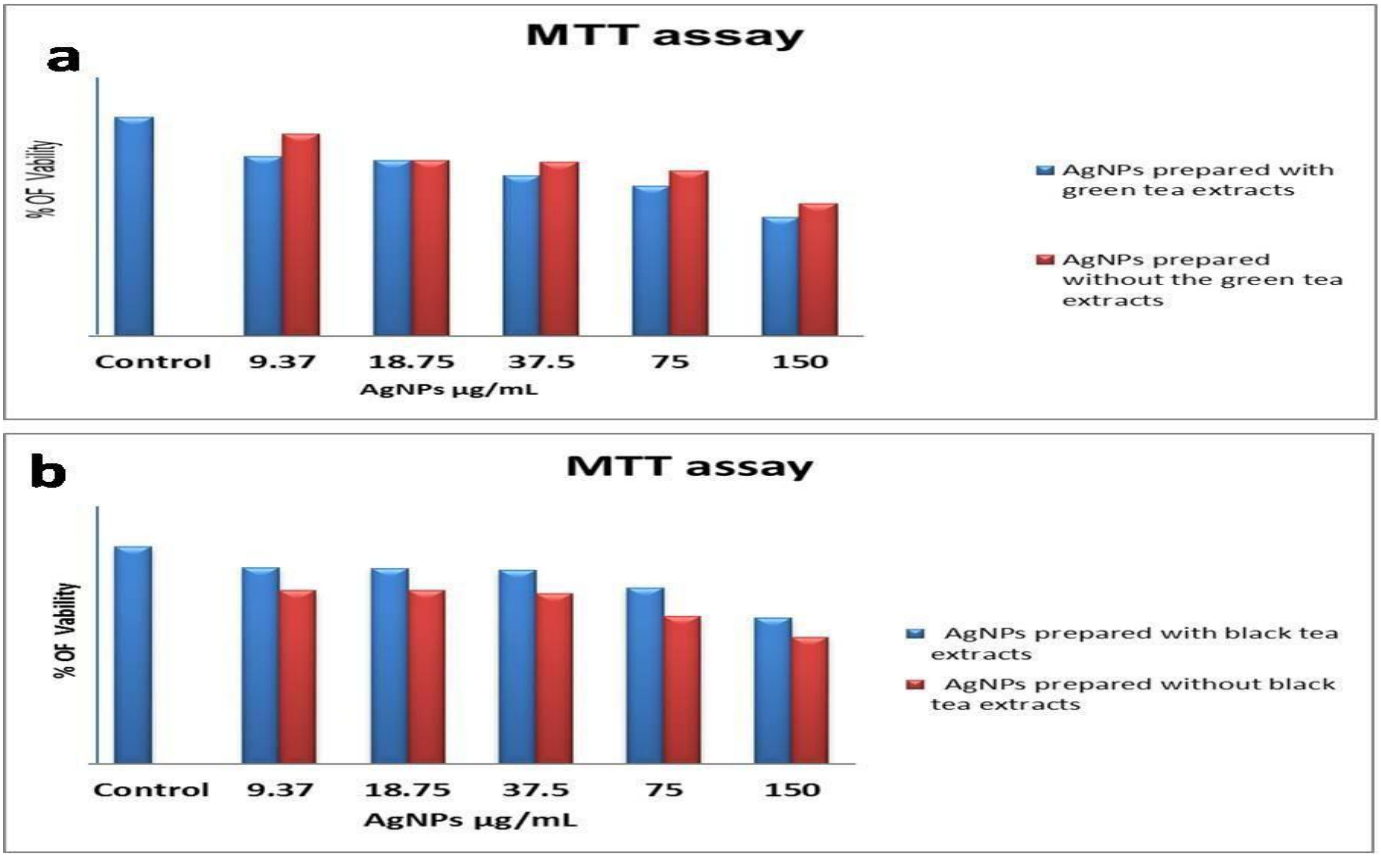

Fig 4. MTT assay to evaluate the effects of AgNPs prepared with and without green tea extracts on the viability of CEF cells after 72 hr of incubation (a); AgNPs prepared with and without black tea extracts on the viability of CEF cells after 72 hr of incubation (b).

Agar Disk Diffusion tests

The antimicrobial activity of the tea extract synthesized AgNPs was tested against two known human pathogens which were *E. coli* and *S. aureus*. AgNPs were used at concentrations of 680, 340, 170, 85 and 42.4 µg/mL for *E. coli*, and at 680, 340, 170, 85, 42.4 µg/mL for *S. aureus*. AgNPs showed significant inhibition zones as shown in **table 2** with the biggest shown at 680 µg/mL for *E. coli*. Most commonly reported mechanism of action of AgNPs against bacteria was through attachment of the AgNPs to the cell membrane which disturbs its function causing the nanoparticles to penetrate the outer membrane through the releas of Ag+ ions [30,34] (**Fig 5**).

Table 2. Inhibition zones of different concentrations of tea-extract synthesized AgNPs.

Bacterial types	AgNPs prepared with black tea extracts		AgNPs prepared with green tea extracts	
	Concentration µg/mL	Inhibition zone in mm	Concentration µg/mL	Inhibition zone in mm
E.coli	680	18mm	680	16mm
	340	18mm	340	14mm
	170	15mm	170	12mm
	85	13mm	85	9 mm
	42.4	11mm	42 .5	9 mm
	21.25	9mm	21.25	No effect
	85	6mm	85	No effect
S.aureus	680	14 mm	680	13 mm
	340	12 mm	340	10 mm
	170	9 mm	170	8 mm
	85	6mm	85	No effect

This result was also found by Ranjitham *et al*. [35] using biosynthesized silver nanoparticles prepared with aqueous extract of Cauliflower florets which exhibited antimicrobial activity against four human pathogens which are *Klebsiella*

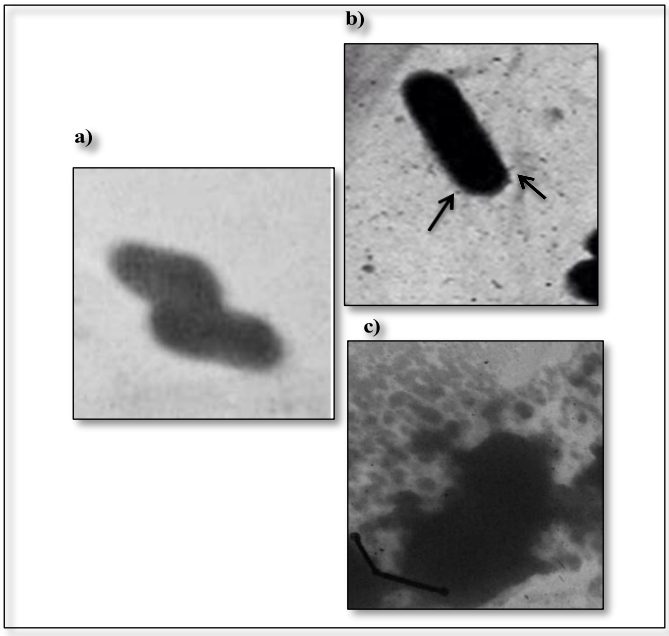

Fig 5. TEM image of E. coli (a); E. coli mixed with tea-extract synthesized AgNPs (Bacteria − AgNP) after 6 h (b); E. coli mixed with tea-extract synthesized AgNPs (Bacteria − AgNP) after 24h (c).

Pneumonia, E. coli, S. Saprophyticus, and *Bacillus cereus*. Meanwhile, Shameli *et al*. [36] investigated the action of AgNPs synthesized by green method by using polyethylene glycol (PEG) under moderate temperature as antibacterial agents. They found that AgNPs had good activity against gram negative and positive bacteria.

The current study described the synthesis of AgNPs using black and green tea extracts to eliminate the use of potentially toxic reducing or coating agents. Tea extract synthesized AgNPs were characterized by UV–Vis spectrophotometer, SEM, and Zeta potential techniques. The characterization tea- extract synthesized AgNPs studies showed spherical shaped AgNPs with diameters between 30-50 nm. These particles showed antibacterial activities against *S. aureus* and *E. coli.* The results of the cytotoxicity studies indicated that the viability of the CEF cells were not significantly affected by the presence of the various concentrations of the AgNPs in comparison with the control which were not exposed to the nanoparticles. Even at the highest concentration tested which was 400 µg/mL of AgNPs, the change in response compared with the untreated cells was very insignificant. Eliminating the unwanted toxicity and enhancing the antimicrobial activities of AgNPs was achieved by preparing the AgNPs in tea extracts. The green method of synthesis of AgNPs showed no toxicity to the human model cell line, thereby, diminishing concerns regarding the cytotoxicity. Thus, the tea-extract synthesized AgNPs hold promise as possible material for food packaging, wound dressing applications, and for other biomedical applications.

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Conflict of interest

The authors declare that they have no conflict of interests.

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