Green synthesis of AgNPs, characterization as an effective wound healing agent in the wound care after anorectal surgery

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Abstract

Thuja occidentalis is well-known for its ability to treat skin disorders. Although the plant extract of T. occidentalis has been shown to possess promising activity on skin infection, there is no report on T. occidentalis-based AgNPs for wound-healing therapy. Therefore, the present study was conducted to synthesize nanoparticles from T. occidentalis in a simple, facile and green method and to assess the ability of formulated nanoparticles in wound care management. The characterization of fabricated AgNPs revealed the particle size range of 40–80 nm, The UV-vis absorption peak at 443 nm and a zeta potential value of −24 mV, confirmed the development of stabilized AgNPs. In vitro cytotoxicity analysis carried out on MCF-7 and Vero cells through MTT assay revealed the cytotoxic effect in cancer cells. LD50 greater than 3000 mg kg\(^{-1}\) unveiled the less-toxic nature of fabricated nanoparticles. Furthermore, the phytosynthesized AgNPs were assessed for the wound healing property based on the size of lesions calculated from patients endured surgery (those who surpassed anorectal surgery). The injury site was found to be completely healed on 17th day on the AgNPs treated cases. The findings suggest that T. occidentalis-based AgNPs could be a better wound dressing material for chronic wounds. On the other hand, the obtained nanoparticles were found to be safe, efficient, non-toxic and eco-friendly. However, this study is limited to the molecular mechanisms.

1. Introduction

Skin is the initial line of protection to defend us from exterior toxic factors and it is the major organ in the human body [1, 2]. The barrier functioning of the skin is lost when the skin is burnt/damaged in any accidental situations [3]. Therefore, the skin, which is injured cannot defend the biological tasks of the interior organs and can be vulnerable towards bacterial infections [3–5]. Dressing the wound may fasten the biological rebuilding of skin as well as it can help in preventing dehydration and infections at the surface of the wound [6]. In addition, a perfect dressing of wound can also be responsible for appropriate biocompatibility [7]. Therefore, different dressing types of injuries in various kinds of forms were developed in recent times, which were primarily comprised of organic macromolecules, for example, cellulose, chitosan, collagen, alginate [2, 3, 8, 9].

Silver, both as nanoparticles (NPs) (AgNPs) or as other structures, exhibits exceptional anti-bacterial function in contrast to a wide range of microbes, for example, viruses, bacteria and fungus [10–13]. A significant advantage of silver when related with other antimicrobial agents is that it comprises less toxicity to mammalian cells whereas higher toxicity to microorganisms [14]. Recently, AgNPs owing to its strong antimicrobial functionality have attained great attention, and were extensively utilized in various medical instruments [15].

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AgNPs can influence the role of protein on the microbial cell membrane and interrupt microbial DNA in inhibiting the cell proliferation, therefore inhibiting microbial redevelopment [16]. In recent times, incorporating AgNPs into different dressings in order to advance wound healing is gaining an increasing interest. One of the previous analysis demonstrated the composite preparation of alginate–AgNPs for dressing the injury [17], which displayed decent anti-bacterial movement. Another report explained that AgNPs-loaded chitosan/collagen scaffolds were noticed to be bactericidal and stimulated wound healing through macrophage activation as well as by controlling the migration of fibroblasts [2]. In spite of the fact that efforts were being put for the developing this field, wound dressing comprised of alginate and collagen along with AgNPs has not yet been described.

Some studies have indicated the optimal conditions for nanoparticle production. Experimental variables such as temperature, pH, reaction time, plant extract volume and reagent concentration have been optimized through resonance surface methodology (RSM). This method provides fast and reliable optimization conditions in the preparation of nanoparticles as well as reduces the trial-and-error runs. The comprehensive investigation of Plantago major extract for AgNPs production has been described by Nikaeen et al. [18] using central composite design. In which they described the optimal conditions at 55 °C temperature, 9.9 pH, 1.5 ml volume of plant extract, 30 ml reagent concentration, and 60 min time for maximal synthesis. However, the effect of various experimental parameters such as pH, temperature, reaction time and reagent concentrations on synthesis of AgNPs were already explained in literature [18, 19]. It is reported that higher pH or plant extract concentration or reaction time results in formation of smaller and uniform NPs. On the other hand, higher temperature and lower reducing agents results in anisotropic or bigger sized NPs [18, 19].

Several reports have described the green synthesis of nanoparticles through biomolecule-[20–26] and plant extract-based approaches [26–31]. This green method of synthesis provides advantages over chemical methods. The technique is simple, cost effective and leads to rapid production of nanoparticles [32–37]. Also, this process is scalable from small-scale to large-scale production of nanoparticles. The nanoparticles synthesized through green method have been witnessed for antioxidant, antimicrobial, and anticancer activities [38–47].

In the current study, we investigated the T. occidentalis extract mediated synthesis of AgNPs. The prepared AgNPs were studied by using various spectroscopic and microscopic techniques. Furthermore, the wound healing studies of AgNPs exposed to surgery patients, those who surpassed anorectal surgery revealed the effective wound healing potential of AgNPs.

2. Materials and methods

2.1. Plant extract preparation
The leaves of T. occidentalis were obtained and thoroughly washed using deionized water for removing any impurities on the surface, dried and then crusher was utilized to coarsely powder the dried T. occidentalis leaves. Deionized water of 100 ml was utilized to boil 10 g of dried plant material and then Whatman filter paper was used to filter the extract in order to obtain the purified form of the mixture.

2.2. Synthesis of AgNPs
Optimized conditions were followed to achieve the fabrication of AgNPs, where aqueous plant extract volume of about 3 ml was added to 10 ml of aqueous silver nitrate (1 mM) and then stored at room temperature (i.e., 24 °C) for four hours for the formation of AgNPs [19, 48]. Initially the formation of AgNPs was known by the visual color change of the reaction mixture, which later was identified with the help of UV-vis spectroscopy.

2.3. Phytochemical analysis of T. occidentalis leaf extract
(a) Evaluation of Tannin: The method of Follins–Dennis spectrophotometer [49] was utilized for the evaluation of Tannin.
(b) Evaluation of Flavonoid: The method reported in an earlier study [50] was used for the evaluation of Flavonoid.
(c) Evaluation of Phenol: the Follins method, which was reported in an earlier study [49] was utilized to evaluate the content of Phenol.
(d) Evaluation of Phytate: the content of phytic acid was evaluated by using an earlier reported method [51] with slight modifications.
(e) Evaluation of Saponin: The method reported in an earlier study [52] was used for the evaluation of Saponin content.
(f) Evaluation of Hydrogen Cyanide (HCN): The method of modified alkaline picrate colorimeter was utilized to evaluate HCN [53].

(g) Evaluation of Vitamin A (Retinol): The official methods of the Association of Official Analytical Chemists [53] are utilized to evaluate the vitamins in the leafy vegetables.

2.4. Characterization of AgNPs
Hitachi H-7500, Japan was utilized to capture the TEM images at 1,50,000 × to 3,00,000 × of magnification range. A JEOL 6335F FE-SEM microscope that was equipped with a Thermo Noran energy dispersive spectroscopy (EDS) detector was utilized to obtain the FE-SEM micrographs. The same instrument was used for EDS elemental composition measurements. A Scintag 2000 PDS diffractometer with Cu KR radiation operated at 45 kV beam voltage, and 40 mA current was utilized to record the patterns of powder x-ray diffraction (XRD). Furthermore, a Jasco V530 UV-vis spectrophotometer was utilized to obtain the UV–vis absorption spectra. The electrophoretic mobility of particles (EMP) was used to measure zetapotential of the formulated nanoparticles (Zetasizer Nano, Malvern).

2.5. Stability of AgNPs
The stability of formulated AgNPs was investigated in citrate (1 mM) and KH₂PO₄ buffer (0.3 mM, pH 7.2). UV-visible absorption spectrum was also recorded during the experiments [54].

2.6. Cell culture
Breast cancer cell, MCF-7 and normal African green monkey kidney epithelial cells, Vero, were obtained from xxx and cultured in DMEM medium supplemented with glucose and pyruvate. Cells were incubated at 37 °C with 5%CO₂ for 24 h.

2.7. MTT assay
To determine the effect of formulated AgNPs on cell proliferation, MCF-7 and Vero cell lines were assessed for cytotoxicity through MTT assay. In brief, about 1 × 10⁴ cells per well were cultured in 96-well plate to form a monolayer. After 24 h incubation, the medium was replaced with fresh medium containing different concentrations of AgNPs (1 to 1000 μg ml⁻¹). The cells were further incubated for 24 h and the medium from the wells were emptied after post-incubation. The cells in the wells were mixed with 200 μl of MTT reagent and incubated for 4 h. Metabolically viable cells reduces the yellow tetrazolium dye to purple crystals. This insoluble formazan crystals were collected and suspended in DMSO (100 μl). Absorbance was quantified at 540 nm [55].

3. Wound healing activity
We examined the injured part of the surgery patients with 64 kg of weight. Patients were classified into control and experimental groups. Each group contains 10 patients and all the experimental data was represented with ± standard deviation. Washed muslin cloth was used as a property to heal the injury. In order to remove the bacterial contamination, muslin cloth of about half a meter was taken and we autoclaved it. Approximate quantity of AgNPs were coated evenly in the muslin cloth. Later, the formed and known AgNPs were taken and coated in particular region of the muslin cloth. Furthermore, this cloth was dressed in the injured part of the surgery patient. The dressing material of the patient was changed every day. We calculated the fraction of injury closure daily. The wound was retracted on a millimeter scale in order to measure the injured portion [56]. The percentage of injury cured was measured by the proportion of wound closure from original wound area with the help of the below formulae.

\[ \text{Percentage closure} = \left(1 - \frac{(A - D)}{(A - O)} \right) \times 100 \]

Where \((A - D)\) = wound portion on equivalent days and \((A - O)\) = wound portion on the zeroth day.

We calculated the average and SD values.

3.1. Statistical analysis
The Student’s t test was utilized to perform the Statistical analysis. Data was represented as mean ± standard deviation.
4. Result and discussion

4.1. Phytochemical analysis of T. occidentalis leaf extract

Table 1 shows the results of the antioxidant study of T. occidentalis leaves and displays the mean percentage values of the antioxidants present in the leaf extract. The studied T. occidentalis leaves have riboflavin, vitamin C, vitamin A, Hydrogen cyanide, phenol, phytate, saponins, tannin, with the following percentage mean values of 12.40 mg/100 g, 64.11 mg/100 g, 16.58 mg/100 g, 0.0132%, 12.24%, 0.096%, 3.61%, 0.435%, 1.51%, and 1.34%. These results are noticed to be in agreement with an earlier study in which the leaf of Telfaria occidentalis was used to study the antioxidant activity.

The metabolites such as flavonoids, phenols, alkaloids, tannins, and saponins are considered to be the major phytocomponents that are involved in the capping and reduction process during the fabrication of nanoparticles. These metabolites contain functional groups such as hydroxyl, carbonyl, and amine which is considered to react with the metal ion such as silver to reduce from Ag⁺ to Ag⁰. Several plant-based AgNP fabrications have reported that these molecules play a significant role in capping and reduction process.

When the plant extract is gradually mixed with the silver nitrate solution, the functional groups such as hydroxyl, carbonyl, and amine from the plant metabolites (flavonoids, phenols, alkaloids, tannins, and saponins) react with the silver ions of silver nitrate solution and thereby it initiates the reduction process (in which the silver ion is reduced from Ag⁺ to Ag⁰ and promotes nucleation. This event will be perceived by the gradual color change of the solution mixture. The resulting small molecule then progressively develops into thermodynamically stable AgNPs through a process referred as growth of AgNPs. At this stage, the biomolecules from plant extract coats over the surface of the nanoparticles, acting as a capping and stabilizing agent.

This event precisely regulates the growth, agglomeration, and physicochemical parameters of the fabricated nanoparticles. Amphiphilic surfactants such as saponins and tannins act as a capping agent. It contains polar head section which interacts with the nanometals and the non-polar hydrocarbon tail section that interacts with the surrounding medium.

4.2. Synthesis and characterization of AgNPs

Depending on its strong anti-oxidant content, T. occidentalis extract was efficiently utilized as a green resource of reducing agent in the current study of synthesizing AgNPs. Initially, the development of AgNPs was identified depending on the visual color change from colorless to brown color in the first 4 h. Later, the fabrication of well stabilized AgNPs was confirmed by the UV-visible (UV-vis) peak (AgNP characteristic band) identified at 443 nm (as shown in figure 1). Some of the factors such as the temperature, the shape of the particle and the dielectric constant of the medium majorly affects the peak positions of UV-Vis spectrum. This recommends that the morphology of AgNPs is determined by the poly-dispersibility of the fabricated AgNPs i.e., the shape of the UV-vis peak. The broad asymmetrical Surface Plasmonic Resonance (SPR) band suggests that the fabricated AgNPs were anisotropic.

Figure 2 represents the XRD patterns of the formed AgNPs. The AgNPs were exceptionally crystalline and its diffraction peaks were equivalent to the Face Centered Cubic (FCC) phase of metallic Ag. The reported value of the lattice constant (a = 4.0862 Å) and the calculated value (a = 4.0602 Å) are in accordance with each other. Except for the broad peak value at about 2θ = 25° possibly from natural moieties existing in the extracts, these patterns depict diffraction peaks equivalent to oxides or impurities.

The FESEM images of the formed AgNPs fabricated using T. occidentalis extracts were represented in figure 3. The fabricated AgNPs formed nanoclusters within the extract matrix and these formed nanoclusters are very much isolated from one another. The formed AgNPs were mostly uniform along with narrow size.
distribution. The micrographs of High-Resolution Transmission Electron Microscope (HR-TEM) revealed that the synthesized AgNPs are spherical in shape along with size existed in the range from 40–80 nm.

The FESEM equipped with the detector of EDS was used to determine the elemental composition of the crushed mixture. The EDS spectrum of the fabricated AgNPs was represented in figure 4(A). The spectrum confirms that the fabricated NPs were predominantly comprised of Ag. On the other hand, figure 4(B) exhibited the Zeta potential data for prepared AgNPs which showed the negative surface charge of AgNPs found to be
−24 mV and this negative surface charge may be because of the surface adsorbed phytochemicals of plant extract that are capped onto the surface of formed AgNPs.

The magnitude of zetapotential divulges the stability of nanoparticles. Indeed, zetapotential in the range of +25 mV to −25 mV are reported to have high degrees of stability. Lower zetapotential will lead to agglomeration due to Van der Waals attraction between particles [67]. The higher magnitude of zetapotential accounts for the high stability of the formulated AgNPs. The electrostatic interaction between the nanoparticles and capping agents determines the surface charge of the formulated nanoparticles. Here, the zetapotential measured through electrophoretic mobility of particle (EMP) using Zetasizer Nano revealed the magnitude of −24 mV.

4.3. Stability of AgNPs

The long-term stability of formulated AgNPs in citrate and KH₂PO₄ buffer was investigated (figure 5). As evident from figure 5 there is no agglomeration or degradation in the prepared AgNPs. The nanoparticles were found to be stable for 6 months. In addition, the UV−visible absorption spectrum substantiated the results through plasmon band position. This finding suggests the prodigious stability of synthesized AgNPs.

4.4. Cell viability assay

The effect of fabricated AgNPs on cell proliferations were investigated using MTT reagent. The cytotoxicity profile after 24 h exposure of MCF-7 and Vero cells to different concentrations of formulated AgNPs were presented in figure 6. The AgNPs showed dose-dependent cytotoxicity on MCF-7 cells. However, Vero cells did not exhibit significant cytotoxicity even up to 1000 μg ml⁻¹ concentration of AgNPs, compared to MCF-7 cells. Many studies have indicated the differences in uptake of nanoparticles due to the variations (such as size, shape, charge and surface area) emanating from the methods of nanoparticle synthesis. Apart, the cell types also play a critical role in uptake of nanoparticles. Particularly, the cancer cell types with different growth characteristics permits diffusion of nanoparticles into cells than normal cell types [19, 68, 69].
4.5. Size of the lesions

The dress material on the wound of the patient was changed every day in order to prevent the bacterial growth around the wound. The dress material on the wound was checked for any contagions after removing it off. Furthermore, the injury was washed, cleaned followed by measuring and recording the width and length of the wound. This method was followed every day in order to confirm the healing of the injury. We have recorded the width and length of the wound as 5.75 and 11.29 mm, respectively on the first day. On performing this treatment every day, we have noticed the contraction of the wound with weak response of inflammatory. Outmost change was noticed in the wound on its 10th day, the width and length of the wound contraction was noticed to be 2.50 and 4.89 mm, respectively. While in the group of control, we have noticed the width and length of the wound contraction as 3.61 and 8.59 mm, respectively. The injury was observed to be closed completely on its 17th day with width and length as 0 and 0.03 mm on the AgNPs coated muslin cloth, while 2.19 mm width and 6.65 mm length in the control group. Through these responses, it is significant that when compared with the control, the AgNPs treated injury displayed a speedy epithelialization. This is because of the fact that the AgNPs are potential enough to change the cytokine cascade, which can enhance the appearance of the wound by immunomodulation.

On the 17th day, when related with the control, the wound treated with AgNPs displayed significant decrease in the area of scratch with speedy upsurge in the area of cell-covered (as shown in table 2). Hence, the combination of AgNPs prepared with the aqueous extract of Delonix elata could be the better injury healer with less inflammation and less formation of scar through the cytokine modulation [70].

In fact, metals such as silver has excellent bactericidal and bacteriostatic activities. When the fabricated AgNPs combine with the collagen it enhances the antibacterial activity as well as demolish the quorum-sensing-mediated biofilm architecture at the site of injury. It has been reported that AgNPs get oxidized into silver ions under in vivo condition. The oxidized silver ion exhibits antibacterial activity by integrating and penetrating the bacterial cell membrane. Many studies have indicated the generation of free radicals by AgNPs in bacterial cells and its effective killing mechanism in both Gram-positive and Gram-negative bacteria. Thus, it plays a significant role in microbial clearance at the site of injury, which otherwise might interrupt with the normal healing process [71–73]. The study using dermal cells has unveiled the role of AgNPs in suppression of inflammatory cytokines and oxidative stress [74]. Thus, it has been envisaged that the biocompatible and green-synthesized AgNPs produced from T. occidentalis exerts a significant bactericidal activity against microbes. However, the comprehensive molecular mechanism of action involved in AgNP synthesized from T. occidentalis needs to be elucidated at future research.

5. Conclusions

In the current study, we have investigated the T. occidentalis extract mediated AgNPs and their antibacterial activity. The prepared AgNPs were found to exhibited a particle size in the range of 40–80 nm, UV-Vis peak of 443 nm and a zeta potential value of −24 mV, confirming the formation of stabilized AgNPs. Furthermore, the wound healing studies of AgNPs exposed to surgery patients, those who surpassed anorectal surgery revealed the
effective wound healing potential of AgNPs. All these results suggested that the prepared AgNPs can act as a better wound dressing material for curing wounds after anorectal surgery.

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Data availability statement

No new data were created or analysed in this study.

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| Time (number of days) | Control Width | Control Length | Experiment Width | Experiment Length |
|----------------------|---------------|----------------|------------------|-------------------|
| 1                    | 5.38          | 10.58          | 5.75             | 11.29             |
| 2                    | 5.29          | 10.49          | 5.22             | 10.89             |
| 3                    | 5.22          | 10.39          | 5.10             | 8.51              |
| 4                    | 4.89          | 10.29          | 4.85             | 8.39              |
| 5                    | 4.49          | 9.99           | 4.02             | 7.62              |
| 8                    | 4.18          | 9.19           | 3.32             | 6.69              |
| 9                    | 4.05          | 9.09           | 2.92             | 5.18              |
| 10                   | 3.61          | 8.59           | 2.50             | 4.89              |
| 11                   | 3.19          | 8.49           | 1.52             | 4.72              |
| 12                   | 2.98          | 7.59           | 1.08             | 4.21              |
| 15                   | 2.69          | 7.13           | 0.10             | 3.23              |
| 16                   | 2.36          | 6.73           | 0                | 0.75              |
| 17                   | 2.19          | 6.65           | 0                | 0.03              |
| 18                   | 1.98          | 6.39           |                 |                   |
| 19                   | 1.59          | 6.03           |                 |                   |
| 22                   | 1.18          | 5.39           |                 |                   |
| 23                   | 0.59          | 5.19           |                 |                   |
| 24                   | 0.49          | 4.49           |                 |                   |
| 25                   | 0.29          | 4.22           |                 |                   |
| 26                   | 0.03          | 3.79           |                 |                   |
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