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Phytochemical-assisted biosynthesis of silver nanoparticles from *Ajuga bracteosa* for biomedical applications

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Abstract

Silver nanoparticles (AgNPs) synthesized from plant extracts are widely used for the cure of many diseases from fever to cancers. Keeping in view the medicinal value of AgNPs, here we report a cost-effective phytochemical method for the biosynthesis of silver nanoparticles from *Ajuga bracteosa*. *A. bracteosa* is an important medicinal plant used to cure fever, appetite-loss, and cancer. Silver-nanoparticles were prepared from the aqueous extract of the plant. The methanolic extract of *A. bracteosa* (ABMF) was separated and n-hexane (ABHF) and chloroform (ABCF) fractions were obtained from the methanolic crude extract. The AgNPs were characterized by UV-Visible spectrophotometer, FTIR, XRD, and TEM. The total phenolic contents (TPC) and total flavonoid contents (TFC) in different fractions were determined and compared with AgNPs. The medicinal value of ABMF, ABHF, ABCF, and AgNPs was evaluated by antibacterial, antioxidant, anti-inflammatory, and cytotoxicity bioassays. The UV-visible spectrum showed a peak at 484 nm while FTIR results suggested strong capping of phytochemicals on AgNPs which was confirmed by a high amount of TPC and TFC. XRD analysis depicted a high degree of crystallinity and smaller size of AgNPs. TEM results showed spherical shaped AgNPs of size range 50 ± 12 nm. The biosynthesized AgNPs showed better antibacterial activity than plant extract fractions. Similarly, AgNPs have shown better antioxidant, cytotoxicity against cancer cell lines in-vitro, and anti-inflammatory activity in-vivo than a plant extract. The great medicinal value of *A. bracteosa* might be due to the presence of pharmacologically active phytochemicals such as diterpenoids, neo-clerodane flavonol glycosides, ergosterol, iridoid glycosides, phytocdysones, and other polyphenols. These phytochemicals surround the silver nanoparticles during green synthesis and therefore, this capping of phytochemicals over silver nanoparticles results in enhanced biomedical applications of plant extracts.

1. Introduction

Metallic nanoparticles (MNP)s have emerged as potential biomedical and engineering tools for various applications including imaging [1], catalysis [2], bio-sensing [3], and drug delivery [4] due to their peculiar physicochemical and optoelectronic features. Silver nanoparticles, the most utilized MNPs, are commonly employed in the pharmaceutical industry for the manufacture of creams and ointments to prevent wounds and burns related infections [5]. Silver nanoparticles (AgNPs) are also used in diagnostics, orthopedics, drug delivery and treatment of infections, wounds, and cancer [6, 7]. AgNPs are used in the development of new broad-spectrum antimicrobials against a variety of pathogenic microorganisms [8]. Moreover, AgNPs have shown...
antifungal, antioxidant, anti-inflammatory, antiviral, anti-angiogenesis, and antiplatelet activities previously [5, 9].

Currently, the MNPs are synthesized by methods that use toxic chemicals, costly apparatus, and hazardous conditions that restrict their large scale applicability in different biomedical areas. Therefore, cost-effective and eco-friendly techniques are urgently needed that could synthesize biocompatible, non-immunogenic, and biodegradable NPs for their therapeutic application to diverse diseases with more biocompatible, environment-friendly, and cost-effective methods. Phytochemicals-assisted green synthesis of NPs is a widely used approach for the fabrication of MNPs as it uses biocompatible ingredients such as plants and is carried out in bodily conditions. Furthermore, the biomolecules involved in the biosynthesis of nanoparticles act as functionalizing-ligands and adhere to nanoparticles making them more suitable for biological applications [10, 11]. Consequently, developing such procedures for the synthesis of nontoxic MNPs is a dire need of the hour.

Green synthesis of MNPs by the use of different bio-reductants including microorganisms, plant extracts, marine organisms, and micro-fluids has been previously reported by different researchers [12, 13]. Plant materials are the most important bio-reductants and remained the frequent choice of many research groups due to easy handling and availability and are already been well-explored for the biosynthesis of different types of MNPs [12]. Moreover, plants are considered as a repertoire of several important phytochemicals such as phenolic and flavonoids compounds which may impart different biological activities to these nanoparticles. Many researchers have reported the green synthesis of AgNPs from plant extracts such as Erythrina suberosa [8], Azadirachta indica, Calendula officinalis, Lansium domesticum, Ziziphus jujube [12], Limonia acidissima [14] and Quercus semecarpifolia [15]. Green synthesized silver nanoparticles were found to be nontoxic and eco-friendly [16].

Ajuga bracteosa Wall. ex Benth. is a perennial medicinal herb of family Lamiaceae and is distributed from Azad Kashmir to Nepal at an elevation of 2000 m and sub-Himalayan tract [17]. This herb is well known for treating gout, palsy, rheumatism, and amenorrhea [17]. Its leaves are used to treat headaches, measles, pimples, stomach acidity, boils, burns, hypertension, jaundice, and sore throat [18] and to purify the blood. It has also shown anti-inflammatory, anticancer, and expectorant potential [19] and antimalarial activity [20].

Keeping in view the significance of phytochemical assisted green synthesis technique and medicinal value of A. bracteosa, here we report the biosynthesis of AgNPs from aqueous extract of A. bracteosa. AgNPs were characterized by UV–vis spectroscopy, FTIR, XRD, and TEM. The phytochemicals of plant extract were assessed and compared with AgNPs. We also report the therapeutic potential of AgNPs and plant extract by assessing antibacterial, anti-oxidant, and anti-inflammatory activities. Furthermore, the anticancer potential of AgNPs and extract was determined by the MTT assay. The results exhibited improved bio-activities of AgNPs might be due to capping of phytochemical around NPs surface but further studies are needed to investigate the chemistry of NPs.

2. Materials and methods

2.1. Preparation of extract

A. bracteosa Wall. ex Benth. was collected from district Kotli, Azad Kashmir, Pakistan. The plant sample was identified by a taxonomist in the Department of Botany, Mirpur University of Science and Technology, Mirpur, Azad Kashmir, Pakistan. Fresh plant parts were washed with water followed by drying under shade. For methanolic plant extract, the plant powder was soaked in methanol for 48 h. The sample mixture was shaken 5 to 6 times/day for proper mixing of samples and removal of undesired gases. After 2 days, the dissolved methanolic crude extract was separated through a muslin cloth and the remaining residue was extracted with fresh methanol twice in a similar way. The mixture was filtered and concentrated in vacuum by using a rotary evaporator at 40 °C. The methanolic extract of A. bracteosa given code ABMF. Then n-hexane (ABHF) and Chloroform (ABCF) fractions were separated from crude methanolic extract using separating funnel. Semiliquid fractions were dried and stored in the refrigerator for future use.

2.2. Green synthesis of AgNPs

The 20 g of sterilized fine powder was poured in 200 ml of d.H2O and heated at 60 °C–70 °C for 15 min. The extract was filtered and its 100 ml was mixed with pre-warmed 400 ml of 1 mM solution of AgNO3 with continuous stirring. The change in the color of the reaction mixture was recorded with a digital camera and quantitatively with UV–visible spectrophotometer. The reaction mixture was allowed to settle in the dark for 24 h and then centrifuged at 13 000 rpm for 20 min followed by washing once with d. H2O and thrice with ethanol followed by decanting supernatant and re-dispersing pellet. The washed pellet of AgNPs was dried in a desiccator with silica gel as a desiccant for more than 24 h and stored for further use.
2.3. Characterization of AgNPs

2.3.1. UV-Vis Spectroscopy

The optical properties and bio-reduction of Ag\(^+\) ions into AgNPs in the reaction mixture were determined with Optizen 3220 UV double beam UV–vis spectrophotometer. For this purpose, the samples of the reaction mixture were taken at 1, 5, 10, 15, 30 and 60 min after mixing of a plant extract with 1 mM AgNO\(_3\) solution and absorbance was recorded in the wavelength range 300–700 nm.

2.3.2. Fourier transmission infrared spectroscopy (FTIR)

To identify the biological agents responsible for the bio-reduction of silver ions and AgNPs stabilization, FTIR spectral analysis was determined with the Perkin-Elmer spectrum 65 FTIR spectrophotometer. The dried sample was minced with potassium bromide (KBr) pellets and analyzed with FTIR at a resolution of 4 cm\(^{-1}\) in the region of 4000 to 600 cm\(^{-1}\).

2.3.3. XRD spectroscopy

The crystalline size of prepared AgNPs was carried out using x-ray diffractometer (D8 Advanced BRUKER AXS GmbH, Germany) at 40 kV and 30 mA with CuK\(\alpha\)-radiation working between 10 and 80\(^\circ\) of 2\(\theta\) angles scanning at 2\(\degree\)/min.

2.3.4. Transmission electron microscopy (TEM)

TEM analysis was carried out for the determination of the morphology, shape, and size of AgNPs with HITACHI h-800, working at 200 kV. For the TEM-grid preparation, a drop of the reduced AgNPs solution was placed on a copper grid coated with carbon and drying under a lamp. The size of AgNPs was recorded by inbuilt software in the TEM.

2.3.5. Energy dispersive x-ray (EDX)

EDX is a technique used to assess the surface atomic distribution as well as the chemical elemental composition. Elemental analysis of AgNPs was assessed using the EDX detector attached to the SEM machine (Mira3-TESCAN).

2.4. Comparative studies of AgNPs and plant extracts

2.4.1. Phytochemical analysis

*A. bracteosa* contains a variety of phytochemicals including flavonol glycosides, neo-clerodane, diterpenoids, phytoecdysones, ergosterol, iridoid glycosides, and many other polyphenols [21]. These phytochemicals not only mediate the bioreduction of AgNPs but also adhere to these nanoparticles and improve the biomedical properties of nanoparticles. Here we quantified the polyphenols adhering to AgNPs and then compared them with polyphenolic contents of *A. bracteosa* extract.

2.4.1.1. Total phenolic content (TPC)

Folin–Ciocalteau reagent (FCR) process was used for the determination of TPC in plant fractions and AgNPs as reported by Ambreen *et al* 2019 [22]. The phenolic compounds present in samples were calculated from the calibration-curve of standard reference gallic acid. Methanolic solutions of different concentrations of 12.5, 25, 50, 100, 200, and 400 \(\mu\)gm\(\text{ml}^{-1}\) of gallic acid, ABMF, ABHF, ABCF, and AgNPs were prepared. Sample solutions (0.5 ml) were mixed with 2.5 ml of FC reagent (10%) and 2.5 ml of 7.5% sodium carbonate solution and incubated at RT for 30 min. The absorbance was taken at 765 nm with UV–visible spectrophotometer. Three readings were taken for each sample and the mean value was taken. TPC was recorded as mg of gallic acid equivalents/g of sample (mgGAE/g) with the formula:

\[
\text{TPC} = \frac{\text{Conc. of gallic acid} \times \text{Vol. of sample solution}}{\text{Weight of sample}}
\]

2.4.1.2. Total flavonoid content (TFC)

TFC in ABMF, ABHF, ABCF, and AgNPs was evaluated by following Ambreen *et al* 2019 [22]. Briefly, the sample solution (1 ml) was mixed with an equal volume of AlCl\(_3\) solution in ethanol and 5% sodium acetate solution (1.5 ml) and incubated at RT for 2.5 h. The absorbance of the reaction mixture was taken at 440 nm using a UV–vis spectrophotometer. Readings of samples were recorded in triplicates and an average of three analyses was taken. Different concentrations of 12.5, 25, 50, 100, 200, and 400 \(\mu\)g\(\text{ml}^{-1}\) of rutin as standard reference were prepared to obtain a calibration curve. Total flavonoid content was measured as mg/g of rutin equivalent (RE) by using a calibration curve.
2.5. Biological evaluation of AgNPs

2.5.1. In vitro studies

2.5.1.1. Antimicrobial activity
Antibacterial activity of AgNPs, ABMF, ABHF, and ABCF was tested using the disc diffusion method \(^{23}\) against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Prepared media was added in pre-sterilized petri dishes and kept at RT to solidify. Bacterial strains were swabbed evenly on separate plates with sterile cotton swabs and incubated at 37° overnight. The 10 mg ml\(^{-1}\) concentration of samples and control was used. The zone of inhibition was recorded in millimeters.

2.5.1.2. DPPH assay
The antioxidant activity of ABMF, ABHF, ABCF, and AgNPs was evaluated by DPPH scavenging activity following Hatano et al. \(^{24}\). Briefly, different concentrations of the samples (50–250 μg ml\(^{-1}\)) in methanol were prepared. The sample solution (1 ml) was added in 1 ml of DPPH (0.2 mM) solution in methanol. The resultant mixture was mixed robustly and placed in the dark for 30 min. The absorbance was taken at 517 nm against a blank. Then the scavenging activity was determined with the following equation:

\[
\text{% Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

Where \(A_{\text{control}}\) is the value of the control (having all other reagents excluding test sample) and \(A_{\text{sample}}\) is the value of the sample.

2.5.1.3. ABTS assay
Free-radical scavenging activities of ABMF, ABHF, ABCF, and AgNPs were also determined using ABTS radical-cation de-colorization assay \(^{25}\). Briefly, the ABTS solution was prepared by mixing 44 μl of potassium persulfate solution (3.75%) in d. H\(_2\)O with 9.7 mg of ABTS in 2.5 ml of d. H\(_2\)O and keeping in the dark at RT for about 15 h. The ABTS working solution was prepared by adding a stock ABTS solution (1 ml) with a 50% ethanol solution (88 ml) in water. Different concentrations of samples ABMF, ABHF, ABCF, and AgNPs were prepared and 25 μl of these sample solutions were added in the working solution of ABTS (250 μl) and kept for 4 min. Then, absorbance was taken at 734 nm with UV–visible spectrophotometer. The results were recorded as ascorbic acid equivalent used as a standard.

2.5.1.4. Cytotoxic activity
Human HCT-116 and HT-29 colon cancer cell lines were selected to assess the cytotoxic effects of AgNPs. HCT-116 and HT-29 cells were cultured and maintained in DMEM media and kept at 37 °C under 5% CO\(_2\) incubation throughout the experiment. MTT assay was carried out to assess the percentage growth inhibition of HCT-116 and HT-29 cells. Colon cancer cells seeded at a density of 5000 cells/well were treated with AgNPs and crude extract of *A. bracteosa* at different concentrations of 125, 100, 75, 50, 25 μg ml\(^{-1}\) for 72 h. MTT dye was added into respective wells of 96-well plate and incubated for 4 h. Then the media was discarded and formazan crystals were diluted by adding DMSO. Finally, absorbance was recorded by a spectrometer, and the percentage viability was determined.

2.5.2. In vivo studies

2.5.2.1. Anti-inflamatory activity
The anti-inflammatory activity of ABMF, ABHF, ABCF, and AgNPs was assessed using carrageenan-induced paw-edema assay according to a previously used protocol by Winter et al. 1962 \(^{26}\). Briefly, six groups (n = 6) of inbred rabbits of weight 500–1250 g were used in this experiment. Rabbits were fasted overnight prior to the test. The 1st, 2nd, 3rd, and 4th groups were given ABMF, ABHF, ABCF, and AgNPs respectively at a dose rate of 100 mg per kg of body weight dissolved in sodium CMC (0.1%). The animals of groups five and six were given reference drugs Indomethacin in 50 mg kg\(^{-1}\) and Ibuprofen at 100 mg kg\(^{-1}\) respectively. After 30 min of sample administration, 100 μl of 1% solution of carrageenan in PBS was administered into the sub-plantar region of the right hind paw of each test animal. The reduction in paw volume was taken through a plethysmometer at a 30 min interval for 3 h. The % protection was measured with the help of the following formula:

\[
\text{% Protection} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100
\]
2.6. Statistical analysis
The data was analyzed using Graphpad prism 7.0 and the graphs were plotted. The values were expressed as Mean and Standard Error of Mean (Mean ± SEM). P-value was calculated to measure the significance of the change in parameters observed for different groups.

3. Results and discussion

3.1. Characterization of silver nanoparticles
In the current study, AgNPs were successfully biosynthesized using plant extract of *A. bracteosa*. The appearance of brownish color as shown in figure 1(a) indicated the formation of AgNPs which achieve final and mature form. The color change was due to surface plasmon resonance (SPR) and bioreduction of Ag\(^{+}\) ions by phytochemicals \[27\]. The characterization of biosynthesized AgNPs was done with UV–vis spectroscopy, FTIR, XRD, EDX and TEM which confirmed spherical nanoparticles with size in the nanoscale range.

3.1.1. UV–vis analysis of AgNPs
The biosynthesis of AgNPs from *A. bracteosa* extract was observed and established with UV–visible spectroscopy. The gradual increase in the intensity of absorption peaks was observed with the passage of time. The characteristic color change was due to the excitation of the SPR in the AgNPs. In figure 1(b), the broader absorption peak at 5 min indicates that the initiation of AgNPs synthesis while a characteristic broad peak of AgNPs at 417 nm observed after 30 min indicates the maturation of AgNPs. Many previous reports observed maximum absorbance at 452, 452, 420, 427, and 475 nm for AgNPs prepared from plants *Ocimum sanctum*, *Terminalia chebula*, *Piper nigrum*, *Syzygium cumini*, and *Foeniculum vulgare* respectively \[28\]. At 60 min interval, a well-defined absorption peak appeared at 484 nm. The shifting of the SPR peak of silver nanoparticles from 417 nm to 484 nm is also known as redshift which indicates complete reduction and that the size of prepared nanoparticles increases with more reaction time. Moreover, a broad UV spectrum ranging from 350 nm to 490 nm is an indication of the poly-dispersed nature of biosynthesized AgNPs \[27\].

3.1.2. FTIR analysis of AgNPs
FTIR was carried out to determine the functional groups of biomolecules linked to the synthesized AgNPs. Figure 2(a) shows a strong band at 3247.4 cm\(^{-1}\) corresponding to –N–H or O–H stretching of carboxylic acids while bands at 2853 and 2921.2 cm\(^{-1}\) correspond to the sp\(^{3}\)-hybridized C–H stretching of alkane and aldehyde groups \[29\]. A peak at 1702.0 cm\(^{-1}\) represents the C=O stretching of ester or saturated aldehyde while a week peak at 1533 cm\(^{-1}\) and a strong peak of 1616.5 cm\(^{-1}\) corresponds to C=C stretching of alkene and aromatic ring. Peaks at 1228.5, 1350.1, and 1439.3 cm\(^{-1}\) represent the C–N and C–C stretching of alkanes while strong
bands at 1017 cm\(^{-1}\) and 1092.4 cm\(^{-1}\) correspond to ether linkages suggesting the presence of flavanones adsorbed on the surface of the MNPs [30]. A weak peak at 1146.8 cm\(^{-1}\) corresponds to O–prim–C stretching [31, 32]. Thus, FTIR analyses suggest that the functional groups including phenol/alcohol, aromatic amine, and carbonyl groups from \textit{A. bracteosa} mediated the bio-reduction and capping followed by stabilization of biosynthesized AgNPs. \textit{A. bracteosa} has been reported to contain various bioactive compounds such as flavanol glycosides, neo-clerodane, diterpenoids, phytoecdysones, ergosterol, iridoid glycosides [21] which supports our FTIR analysis. This also suggests a strong capping of biomolecules on the synthesized AgNPs. Many researchers reported the presence of bioactive phytochemicals in AgNPs and their cappings [10, 11, 27].

3.1.3. X-ray diffraction (XRD) analysis
XRD analysis of the synthesized AgNPs from \textit{Ajuga bracteosa} extract revealed eight peaks at 27.59°, 31.88°, 46.05°, 54.55°, 57.13°, 67.24°, 74.13° and 76.47° that showed variation in the peak area and length as shown in figure 2(b). Unpredicted crystalline structures were also observed at 31.88° and 46.05° because of the presence of organic biomolecules in the \textit{A. bracteosa} extract. Similarly, Kumar and Yadav (2009) [33] and Jeeva et al 2014 [34] prepared nanoparticles from plant extracts and observed crystalline peaks at 32.28°, 46.28°, 54.83°, 67.47°, and 76.69° which in turn supports our findings. Moreover, we observed a face-centered cubic structure of AgNPs when compared with an online database (JCPDS: 00-041-0788). The highly intense peaks revealed a high degree of crystallinity of AgNPs while broadness of diffraction peaks can be linked to an extremely small size of nanoparticles [35].

3.1.4. Transmission electron microscopy
TEM was used to determine the size, shape, and morphology of prepared AgNPs, and results are shown in figure 3. It revealed that the majority of the biosynthesized AgNPs were spherical while some nanoparticles were found to be rough in shape and the size of AgNPs was 50 ± 12 nm as shown in figure 3(a) and (b). Slight agglomeration was also observed. The spherical shape can be correlated with the single SPR spectrum observed in the UV–vis spectrophotometry. The biosynthesis of spherical-shaped AgNPs from the leaf-extracts of \textit{Euphorbia hirta} [36] and \textit{Ceratonia siliqua} [37] determined by SEM analysis was observed. Many previous studies observed the size of AgNPs prepared from plant extracts of \textit{Citrus limon} [38], \textit{Carica papaya} [32], \textit{Nelumbo nucifera} [39] and \textit{Eucalyptus hybrida} [40] were 50, 60, 80 and 90 nm respectively.
3.1.5. EDX analysis

The EDX spectrum of the biosynthesized AgNPs is shown in figure 3(c) which depicts the presence of silver as the major element. AgNPs showed a typically strong peak at 3 keV which is the characteristic of metallic silver due to surface plasmon resonance [41]. Figure 3(c) shows the quantitative information of prepared AgNPs. Moreover, figure 3(c) also shows the occurrence of other elements like C, Fe, Mg, Ca, Cl, and Na which was also observed by other researchers [42].

3.2. Comparative studies of AgNPs and plant extract

3.2.1. Phytochemicals analysis

Phenolic phytochemicals are very important secondary metabolites of plants which are involved in providing health benefits to both plants and animals. More than 8000 phenolic compounds have been discovered so far and about 4000 of these phenolic compounds are flavonoids [43, 44]. Polyphenols are commonly used in health, pharmaceutical, cosmetic, and food industries as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-allergic, and anti-cancer agents [45, 46]. The key biomolecules found in plants are polyphenols, flavones, sugars, terpenoids, aldehydes, ketones, carboxylic acids, and amides which are responsible for the bio-reduction of metal NPs [47].

3.2.1.1. Total phenolic contents

In the current study, polyphenols present in different fractions of plant extract of A. bracteosa and those which oxidized during nanoparticle synthesis were quantified using FC reagent, and results are expressed as gallic acid equivalents per gram of extract are shown in figure 4(a). The TPC of the various fractions of Ajuga bracteosa prepared in methanol (ABMF), n-hexane (ABHF), chloroform (ABCF) and silver nanoparticles (AgNPs) were 12.5 ± 0.12, 15.3 ± 0.15, 13.6 ± 0.1 and 17.8 ± 0.2 mgGAE/g respectively. The amount of TPC quantified from AgNPs was comparatively higher as compared to the TPC of ABMF, ABHF, and ABCF. Similar results have also been reported about the presence of a higher amount of TPC in AgNPs of crude extracts of various plants as compared to their methanolic, hexane, and chloroform fractions. For instance, Zahra et al [48] observed a higher amount of TPC content in AgNPs as compared to methanolic, hexane, and chloroform fractions of the plant used to synthesize AgNPs. According to some researchers, it is the é donating potential of polyphenols that facilitate the formation of AgNPs by bioreduction of Ag⁺ to Ag⁰ and further stabilize AgNPs suspension by

Figure 3. The scanning electron micrographs of: (a) biosynthesized AgNPs at low resolution to focus spherical shape (b) AgNPs at high resolution indicating the size of AgNPs in the range of 50 ± 12 nm. (c) The EDX spectrum of the biosynthesized AgNPs depicting the presence of silver as the major element with a strong peak at 3 keV which is the characteristic of metallic silver due to surface plasmon resonance. The EDX spectrum also shows the occurrence of other elements like C, Fe, Mg, Ca, Cl, and Na.
adsorbing quinoids produced during the reaction by oxidation of phenolic groups on the surface of nanoparticles [4, 49, 50].

3.2.1.2. Total flavonoid contents
The total flavonoid content (TFC) was assessed by a standard rutin calibration curve. Total flavonoids in ABMF, ABHF, ABCF, and AgNPs were 7.0 ± 0.3, 8.3 ± 0.1, 8.9 ± 0.3, and 12.7 ± 0.4 mgRE/g respectively (figure 4(b)). It is clearly evident in figure 4(b) that AgNPs had a significantly higher amount of flavonoids as compared to plant extracts. Many other studies also reported that water-soluble flavonoids may involve in the reduction of silver ions indicating phytochemicals as good bio-reducers [49, 51].

3.2.2. In vitro studies
Different parameters of biological activities like antibacterial, anticoagulant, antioxidant, anti-inflammatory, and anti-cancer of different fractions and AgNPs of Ajuga bracteosa were applied that proved AgNPs have shown the best results in comparison with frictions of the plant.

3.2.2.1. Antibacterial activity
The antibacterial activity of the ABMF, ABHF, ABCF, and AgNPs was tested for both Gram-positive (S. aureus and B. subtilis) and Gram-negative (E. coli and P. aeruginosa) bacteria. Based on the zone of inhibition produced, the AgNPs prepared from A. bracteosa extract showed good antibacterial activity against both Gram-positive and Gram-negative bacterial strains as compared to other fractions of A. bracteosa as shown in figure 5. As reported earlier, various extracts of A. bracteosa leaves, roots, and bark have shown activity against many bacterial strains. In many previous reports, a similar pattern of antibacterial activity was observed by AgNPs biosynthesized from Vitex negundo extract and Acorous calamus rhizome extract against gram-negative and gram-positive bacterial strains [27, 52]. Silver nanoparticles were known as powerful antibacterial agents [53]. The AgNPs show antibacterial property by attaching to the bacterial cell membrane [54] because it is the site of the respiratory...
chain, energy-transducing systems, and transport of molecules and therefore, any change in the structure of the membrane would eventually result in inhibition of bacterial growth. The AgNPs showed better antimicrobial activity because of their very large surface-area giving more room to contact the cell wall of microorganisms [55].

3.2.2.2. Antioxidant activity

The antioxidant activity of biosynthesized AgNPs was compared to plant extract preparations determined by DPPH and ABTS in vitro assays. AgNPs may possess antioxidant potential due to the existence of silver in two oxidation states $\text{Ag}^{+}$ and $\text{Ag}^{2+}$ or phytochemicals capped on silver nanoparticles. All fractions of A. bracteosa showed pronounced antioxidant activity which might be related to the presence of abundant phytoecdysteroids reported by Kayani et al 2016 [56].

3.2.2.3. DPPH assay

The methanolic, n-hexane, chloroform extracts of A. bracteosa and silver nanoparticles showed IC-50 values of 35.2, 23.3, 31, and 21 $\mu$g ml$^{-1}$ respectively. AgNPs of the plant showed the best DPPH radical scavenging activity $(\text{IC}_{50} = 21.6 \ \mu\text{g ml}^{-1})$ in comparison with other solvent fraction of plant as shown in figure 6(a). This might be due to the enhanced activity of phenols after the formation of AgNPs. Phenolic biomolecules present in plant extracts are responsible for their high antioxidant activity and act as reducing agents which in turn play a central role in the biosynthesis of silver nanoparticles [57].

3.2.2.4. ABTS assay

In the present work, ABMF, ABHF, ABCF, and AgNPs were evaluated for their ABTS radical cation scavenging activities. IC-50 values of samples range from 31 to 47 $\mu$g ml$^{-1}$. Among all extracts, the lowest IC-50 value was observed for AgNPs of A. bracteosa as shown in figure 6(b) which indicates the higher antioxidant potential of synthesized AgNPs. Many researchers have shown similar high antioxidant activities of AgNPs prepared from plant extracts with ABTS [57, 58]. This is might be due to cappings of bioactive compounds such as polyphenols over AgNPs which enhanced the antioxidant of AgNPs many folds.

3.2.2.5. Cytotoxicity activity

The therapeutic potential of AgNPs for treating cancer is well known as they penetrate immediately in cancer cells causing cell lysis. Silver nanoparticles are found to be very effective cancer cell growth-inhibiting agents in many types of cancers especially lung, breast, glioblastoma, hepatic, and ovarian [59]. Here in this study, the anticancer potential of silver nanoparticles biosynthesized from A. bracteosa was tested on two colon cancer cell lines (HT-29 and HCT-116) at various dose concentrations of 25 to 125 $\mu$g ml$^{-1}$. The increase in dose concentration of silver nanoparticles and PE continuously decreased the cell viability of both kinds of cancer cells as shown in figure 7(a). However, the inhibitory effect of AgNPs and PE was more pronounced on HCT-116 as compared to HT-29. IC-50 values obtained for AgNPs and PE were (114.8 ± 7.7 versus 196 ± 13.9 $\mu$g ml$^{-1}$) for HT-29 and (69.1 ± 11.3 versus 103.6 ± 14.5 $\mu$g ml$^{-1}$) for HCT-116 respectively as shown in figure 7(b). These values showed that the anticancer potential of AgNPs against HCT-116 was within the clinically acceptable concentration of 100 mg l$^{-1}$ [27]. Many previous studies reported the cytotoxic effect of biosynthesized AgNPs from the extract of J. dolomiaea against HeLa and MCF-7 cancer cell lines [58], from Artemisia turcomanica leaf extract against gastric cancer cell line (AGS) in a dose- and time-dependent manner [60] and from Morinda citrifolia against HeLa cell lines [61] in vitro.
3.2.3. In vivo study

3.2.3.1. Anti-inflammatory assay

Carrageenan induced Paw edema is a non-specific inflammation which is the result of various mediators [62] and a decrease in paw volume is an indication of anti-inflammatory effects. Carrageenan paw edema is highly sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and thus frequently used for assessing new anti-inflammatory agents [26]. The reduction in inflammation by test samples ABMF, ABHF, ABCF, and AgNPs were measured in % and compared with reference drugs Indomethacin (50 mg kg$^{-1}$) and Ibuprofen (100 mg kg$^{-1}$) as shown in figure 8. The results showed the higher anti-inflammatory potential of AgNPs (89.1 ± 2.6%) at 100 mg kg$^{-1}$ as compared to standard COX-1 inhibitor Ibuprofen 69.0 ± 3.6% and selective COX-1 and COX-2 inhibitor Indomethacin 86.7 ± 2.4% after three hours of carrageenan induction. Moreover, all the fraction of A. bracteosa relatively showed better edema protection activity with ABMF (82.5 ± 2.9) being the most potent followed by ABCF (71.6 ± 3.8) and ABHF (65.7 ± 4.0). The high anti-inflammatory activity of plant extract fractions and AgNPs might be linked with phytoecdysteroids present in A. bracteosa [56].

4. Conclusions

Herbal medicine’s importance is well recognized in the medical field today due to multiple beneficial and limited side effects on human health. Modern and advanced medication leads are required for the cure of diseases like cancer and chronic wounds. Herbal extracts and metallic nanoparticles prepared Ajuga bracteosa could be one of the therapeutic agents for the delayed wound healing complications. Here, we evaluated the healing influence of at present well-acknowledged herbal plant A. bracteosa leaf extracts and AgNPs prepared from their leaf on different bacterial strains as well as the influence of these formulations on wounds induced artificially in the skin.
of rabbits. A marked degree of bactericidal effects was displayed by leaf extracts and AgNPs but AgNPs proved more lethal on both gram-positive and negative bacterial strains.

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Disclosure

All authors confirm that no conflicts of interest exist.

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