https://doi.org/10.31925/farmacia.2019.4.16 **ORIGINAL ARTICLE**

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF BIOSYNTHESIZED SILVER NANOPARTICLES FROM *ULMUS WALLICHIANA* PLANCH. LEAF EXTRACT

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Abstract

Eco-friendly synthesis of metal nanoparticles using plant extract plays a vital role in nanotechnology. This study aimed to produce silver nanoparticles (SNPs) in an eco-friendly manner using leaf extract of *Ulmus wallichiana* Planch. When the leaf extract was treated with $AgNO_3$, the colour changed from yellowish to dark brown, indicated the formation of SNPs. The obtaining SNPs were pre-dominantly spherical, crystalline and a mean size of 54 nm. FTIR spectrum evidenced that plant's biomolecules acted as reducing agent. The antibacterial activity of SNPs was assessed against Gram positive (*Staphyllococcus aureus*) and Gram negative *(Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria and highest activity was found to be exerted at 1 mg/mL SNPs against *P. aeruginosa* (17.63 mm). They also showed profound antioxidant activity against DPPH. *Ulmus* mediated synthesis of SNPs is eco-friendly, non-toxic and cost effective with good antibacterial and antioxidant effect and can be used in biomedical applications.

Rezumat

Sinteza ecologică a nanoparticulelor metalice folosind extracte de plante joacă un rol vital în industria nanotehnologiilor. Acest studiu a urmărit biosinteza nanoparticulelor de argint (SNP) într-un mod ecologic, folosind extractul foliar de *Ulmus wallichiana* Planch. După tratarea extractului cu $AgNO_3$, culoarea s-a modificat de la galben la maro închis, indicând formarea nanoparticulelor. SNP-urile obținute au fost predominant sferice, cristaline și au prezentat dimensiunea medie de 54 nm. Spectrul FTIR a evidențiat faptul că biomoleculele din extract au acționat ca agent reducător. Activitatea antibacteriană a nanoparticulelor a fost evaluată asupra bacteriilor Gram pozitive (*Staphyllococcus aureus*) și Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa* și *Klebsiella pneumoniae*), iar cea mai mare activitate s-a înregistrat pentru doza de 1 mg/mL SNP asupra *P. aeruginosa* (17,63 mm). De asemenea, au prezentat o activitate antioxidantă pronunțată în testul DPPH. Sinteza mediată de *Ulmus* a nanoparticulelor de argint este ecologică, non-toxică și rentabilă, cu efect antibacterian și antioxidant, având potențiale aplicații biomedicale.

Keywords: collagen, minocycline, spongious delivery systems, freeze-drying

Introduction

Synthesis, characterization and application of nano-materials represent currently an essential area of research. Nanotechnology grips promising applications in many fields such as biomedical, bio-sensing, drug delivery, food packaging, cancer therapy, agriculture and cosmetics, and many more fields [1]. The materials having overall size of 100 nm are considered to be nanoparticles [2]. Nanoparticles exhibit larger surface area to volume ration, which is one of the most important and distinctive property [3].
Nanoparticles made from noble metals such as Au, Ag, Pt, and Pd are the most efficiently studied nano-particles nowadays. Among metals, silver nanoparticles have drawn much attention of the researchers due to their wide applications as antimicrobial [4]. They have been recognized to displaying solid antibacterial effect against both Gram negative and Gram positive bacteria [5, 6]. This antimicrobial proficiency of SNPs permits them to be appropriately engaged in several domestic stuffs such as home appliances, textiles, food packaging, dentistry and medical devices [7, 8].
Additionally, silver is a potent germicide agent that exhibits minor toxicity in human [9]. One of the utmost uses of SNPs is in topical liniments to avoid infection against open wounds and burn [10]. Development of eco-friendly, not toxic and low-cost methods for synthesis of SNPs of different morphologies is an essential feature of nanotechnology [11].
Although different type of physical and chemical methods, for the synthesis of SNPs, these methods have some drawbacks, like high cost, less efficient, slow synthesis and more importantly many toxic chemicals are involved. Therefore, there is a rising

interest in developing eco-friendly methods for the synthesis of SNPs such as green chemistry procedures which eliminate the use of chemicals known as lethal to environment and also to the human health [12, 13]. Biological methods using bacteria, fungi and plants are beneficial in contrast to physical and chemicals approaches [14, 15]. In contrast to microorganism, plants are more advantageous as later reducing the cost and maintenance of culture media [16]. The phyto-chemicals exist in the plants extracts as a reducing agent and aid in the transformation of silver ions to silver nanoparticles [17, 18]. They do not embrace the use of costly and lethal chemicals and the raw material is inexpensive and effortlessly accessible [19, 20]. A survey of literature showed that SNPs fabricated from different plant extracts such as *Azedirachta indica*, *Ficus benghalensis* [21], *Amona reticulata* [22], *Iresine herbstii* [23], *Melia azedarach* [24] and *Berberis lcyium* [25] and many more have produced abundant attention in the nanomaterial formation.

Reactive oxygen species (ROS) and free radicals produced degenerative reactions that harm cells and macromolecules. Antioxidants are known to have protective effect against free radicals and ROS [26]. Many plant-based polyphenol substances such as alkaloid and flavonoid have already been reported as antioxidant [27, 28]. Recently it is studied that biosynthesized metal nanoparticles particularly SNPs also scavenge the oxygen based free radicals more effectively [29, 30].

Keeping in view the importance of SNPs, the present study aimed to synthesize SNPs from leaf extract of *U. walliachiana* as an eco-friendly approach and to examine their antibacterial efficiency against common pathogens both Gram positive (*S. aureus*) and Gram negative (*E. coli*, *P. aeruginosa* and *K. pneumoniae*) by disc diffusion method and the antioxidant activity by DPPH assay.

Materials and Methods

Preparation of plant extract

For the preparation of plant extract, fresh leaves of *Ulmus wallichiana* Planch. were collected from Rawalakot, Azad Kashmir, Pakistan. The plant was identified with the help of the Pakistan flora and the plant List. The voucher specimen was submitted to the Herbarium, University of the Poonch Rawalakot, Pakistan. The leaves were thoroughly rinsed with tape as well as distilled water to wash out any undesirable and dust particle from the surface of the leaves. The finely chopped leaves of 10 g were poured into a 250 mL Erlenmeyer flask with 100 mL distilled water and boiled for 10 min on hot plate for the extraction of biomolecules followed by cooling at room temperature. To remove the leaves debris, the extracted solution was filtered through Whatman filter paper no. 1 and the filtrate (leaf extract) was stored at 4ºC for further use for the synthesis of SNPs.

Synthesis of SNPs

For the phyto-fabrication of SNPs, 2 mL of plant leaf extract was reacted with 8 mL of 1 mM $AgNO_3$ solution in a test tube and incubated at room temperature for 24 hrs. Silver nitrate of Merck Company was purchased from Lahore. The development of SNPs in the mixture was monitored through colour change and UV-Vis spectroscopy. When Ag ions reduce to SNPs, the colour of the mixture changed to dark brown which is a clue of the SNPs synthesis. The colloidal solution was also scanned by UV-Vis spectroscopy in the wavelength range of 300 to 800 nm at a resolution of 1 nm after time intervals of 0, 5, 10 and 15 min and lastly after 24 h on a Perkin-Elmer Lambda 950, UK spectrophotometer. After the sign of the SNPs formation, the reaction solution was centrifuged for 10 min at 15,000 rpm in order to obtain them in a purified form. The purified SNPs were again re-dispersed in distilled water and centrifuged at the same condition. The process was repeated three times. The purified clean SNPs were characterized by scanning electron microscopy (SEM), X-ray diffraction analysis (XRD) and Fourier transform infrared spectroscopy (FTIR).

Scanning Electron Microscopy

The surface morphology of the synthesized SNPs was observed in Tescan Mira 3X field emission scanning electron microscope (FESEM). Previously purified SNPs were sonicated in Milli Q water until a dilute suspension of SNPs is formed. A small drop of suspension was placed on carbon tape fastened on the sample (aluminium) tubs and permitted to fully dry below a mercury lamp. These tubs were then examined in FESEM at an accelerating voltage of 20 kV.

X-ray diffraction analysis

The crystallinity and mean crystalline domain size were determined through XRD. The purified SNPs were freeze dried and powder was subjected to XRD analysis on Bruker D8 Diffractometer with Cu K_α X-ray source of 1.54 Å wavelength. The diffraction pattern was obtained at 2θ value between the ranges of 10 to 80 with 2º Å *per* min scan rate.

Fourier transform infra-red spectroscopy

FTIR was used to identify the functional groups of compounds acted as reducing and stabilized agent in the synthesis process of SNPs. The freeze-dried powder of SNPs was analysed in FTIR (Perkin-Elmer Spectrum 100, USA) and the spectrum was recorded between the ranges of 4000 to 400 cm^{-1} at a resolution of 1 cm^{-1}.

Antibacterial activity

The bactericidal capability of SNPs was assessed against common drug resistant human bacterial pathogens including Gram positive (*Staphyllococcus aureus*) and Gram negative (*Escherichia. coli*, *P. aeruginosa* and *K. pneumoniae*) bacteria by disc diffusion method

[31]. The bacterial pathogens were obtained from Combined Military Hospital (CMH), Muzaffarabad, Pakistan. The experiment was performed in completely randomized design (CRD) with three replicates. Nutrient agar medium was prepared by adding 28 g of nutrient agar in 1000 mL distilled water for the cultivation of bacteria. The culture medium and all other requirements were sterilized in an autoclave at 121ºC for 15 min. A loop of each bacterium from overnight fresh culture was dipped in 10 mL distilled water in separate test tubes to make bacterial inoculum. A 1 mL of each bacterial inoculum was taken in corresponding sterile Petri plates. Sterile nutrient agar medium was poured in each Petri plate, mixed well by gently shaking and kept at room temperature for solidification. The filter paper discs of 6 mm were prepared, sterilized and supplemented with SNPs (1 mg/mL), aqueous leaf extract (1 mg/mL), ampicillin (100 µg/mL) as positive control and distilled water as negative control, and placed on solid agar medium at their labelled positions. For the growth of the bacteria, the Petri plates were incubated at 37ºC for 24 h and then the zone of inhibition was measured in millimetre around each disc using the meter scale.

Minimum inhibitory concentration (MIC) of SNPs

The lowest amount of the antimicrobial agent that inhibits the growth of bacteria after 24 h is known as MIC. A serial dilution of SNPs such 1, 0.5, 0.25 and 0.125 mg/mL was made and tested by disc diffusion method. The experiment was performed same as in antimicrobial activity. After 24 h of incubation, zones of inhibition were measured around the discs.

Antioxidant Activity

The antioxidant activity of biosynthesized SNPs and plant extract was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [32]. A stock solution of DPPH was prepared by adding 7 mg DPPH in 100 mL 95% methanol. The leaf extract and SNPs were mixed in methanol to make their stock solution (0.1 mg/mL). From this stock solution, 2, 4 and 6 mL of each leaf extract and SNPs were taken in 3 separate test tubes and, by serial dilution, the final volume of each test tube was raised to 10 mL with same solvent whose concentration was then 20 µg/mL, 40 µg/mL and 60 µg/mL respectively. Freshly prepared DPPH solution was added in each of the test tube containing leaf extract and SNPs, vortexed vigorously and incubated for 30 min in dark at room temperature. The optical density of each of the sample was recorded at 517 nm using UV-Vis spectrophotometer (Perkin-Elmer Lambda 950, UK). The antioxidant activity was assessed by calculating the % inhibition using the following formula:

$$\%\ \text{scavenging activity} = \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100,$$

where, OD control is the optical density of DPPH + methanol and OD sample is the optical density of DPPH + sample (leaf extract and SNPs). The IC_{50} was calculated by using the regression line equation against different concentrations of tested samples.

Statistical analysis

The experiments were performed in completely randomized design (CRD) with three replicates. The data was subjected to analysis of variance (ANOVA) using MSTAT C software and means were compared by Duncan's multiple range test (DMRT).

Results and Discussion

Synthesis and characterization of SNPs

SNPs were synthesized from the aqueous leaf extract of *U. wallichiana*. When the aqueous leaf extract was added in $AgNO_3$ solution, the colour of the reaction was started to change from yellowish to reddish brown, indicating the SNPs synthesis (Figure 1).

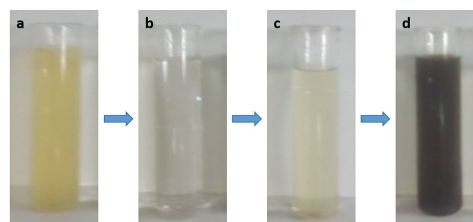

Figure 1.

Visual observation of formation of SNPs from the leaf extract of *U. wallichiana*: (a) leaf extract; (b) $AgNO_3$ solution; (c) leaf extract + $AgNO_3$ at zero time and (d) leaf extract + $AgNO_3$ after 24 h

The change of the colour in the reaction mixture is the first visual evidence of the SNPs formation [30]. A reddish brown colour is a characteristic feature of the SNPs in the solution [33]. A rapid change in colour (just within 5 min) was observed which indicates the increased rate of reaction. This change within the optical properties of the colloidal solution is also linked with the change in Surface Plasmon Resonance (SPR) excited when Ag^+ changes to Ag^0 [34]. This formation of SNPs as indicated by the colour change was further supported by taking UV-Vis spectra of the colloidal solution as a time function (Figure 2). The spectra were recorded after 0, 5, 10 and 15 min and finally after 24 h. There was no absorption band at zero time but when reaction time proceeded to 5 min, a characteristic SPR band was centred at 439 nm. After 10 and 15 min, the SPR bands were developed at 446 and 450 nm respectively. For observing the stability of SNPs, the reaction was left for 24 h and the absorption spectrum was recorded showing an SPR band at 456 nm. The intensity of absorption was increased with the passage of time, indicating the increased concentration of SNPs in the solution. It is a fact well established that, in the aqueous solution, SNPs exhibit absorption spectrum in the wavelength range of 400 to 500 nm [35]. It was

also proposed that number and width of absorption bands are associated with the shape and the size distribution of the synthesized SNPs in the solution [36]. A single SPR band in the wavelength range of 300 to 700 nm corresponds to pre-dominantly spherical particles [37]. It was also observed in our study that SPR band shifted from 439 to 456 nm (red shift) with the reaction time. This red shift suggested an increase in the mean size of SNPs [38].

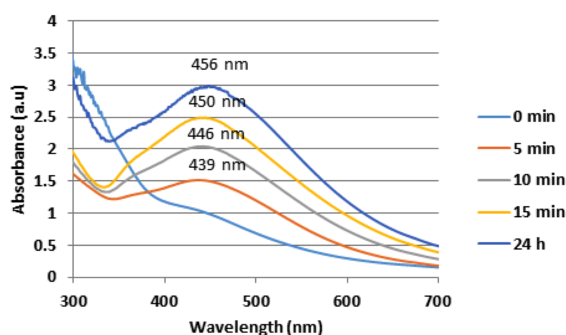

Figure 2.
UV-Vis spectra of colloidal solution of leaf extract of *U. wallichiana* and $AgNO_3$ at different time of intervals such as 0, 5, 10 and 15 min and finally after 24 h

After the evidence of formation of SNPs by colour change and UV-Vis spectroscopy, they were further confirmed by analysing their surface morphology in FESEM. Figure 3 shows the SEM micrograph of SNPs which manifests that most of the particles are predominantly spherical with mean size of 54 nm. Due to small size, the individual particles were aggregated to clusters [39]. Earlier studies also support the result of SEM [40, 41]. The biosynthesized SNPs were further characterized by XRD which gives information about the crystalline nature and the phase of nanoparticles.

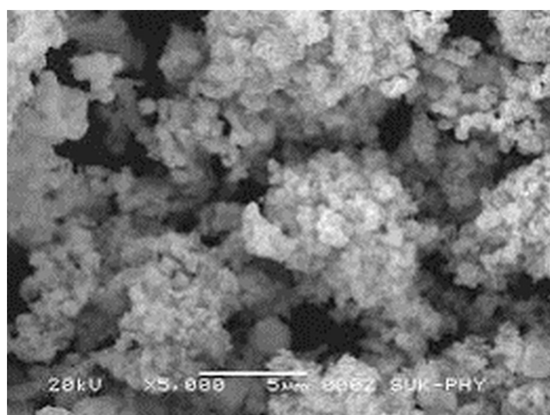

Figure 3.
The morphology and size of the purified SNPs showed by the SEM micrograph

The diffraction pattern is shown in Figure 4, it manifests diffraction peaks at 2 values of 37.95°, 44.05°, 64.30° and 78.45° which corresponds to (111) (200), (220) and (311) crystalline planes of face centred cubic silver compared with the standard powder diffraction card, silver file (JCPDS No. 04-0783). Our results are also supported by previous studies as well [30, 42]. The functional groups of compounds, present in the plant extract, involved in the chemical transformation of Ag ions to SNPs were identified with the help of FTIR spectrum of SNPs as shown in Figure 5. The transmission bands were centred at 1010, 1100, 1430, 1550, 1650, 2025, 2987 and 3406 which are associated with –C–O– phenolic groups, C=C aromatic amides, N-H bending vibrations of primary amines and O-H stretching vibrations of phenolic compounds. The presence of functional groups indicates that SNPs are capped by the biomolecules of plant extract. These biomolecules served as reducing agent and reduced Ag ions to SNPs. It is a well fact established that biomolecules such as phenol, alkaloid, flavonoid and protein present in plant extract synthesized SNPs [41, 43].

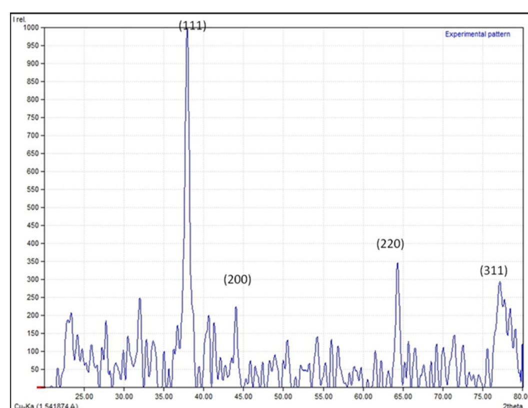

Figure 4.
The crystalline nature of SNPs demonstrated by the XRD pattern

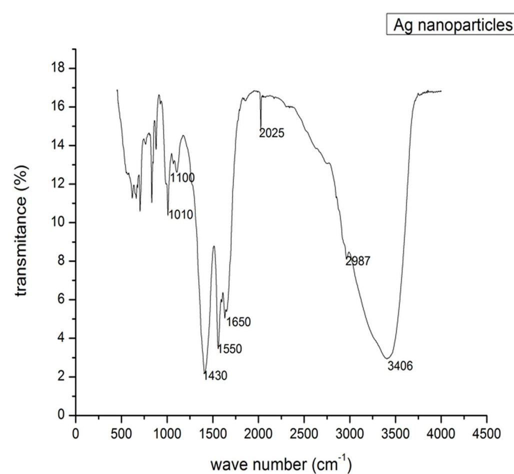

Figure 5.
FTIR spectrum of SNPs

Antibacterial activity of SNPs
Many antibiotics and other antibacterial agents have been used to inhibit the growth of common human pathogens in various industries. But these antibacterial

sources are now facing a problem of multidrug resistant power in pathogens. Therefore, there is a need to focus on alternative ways to overcome the microbial drug resistance. In this research, we scrutinized the antibacterial power of the biosynthesized SNPs against common human pathogens both Gram positive (*S. aureus*) and Gram negative (*E. coli*, *P. aeruginosa* and *K. pneumoniae*) bacteria.

The results of antibacterial activity of SNPs, plant extract, positive control (ampicillin) and negative control (distilled water) are presented in Table I. Statistically a significant difference was found among the tested bacteria as well as among the treatments. The highest inhibitory activity was shown by SNPs (1 mg/mL) against *P. aeruginosa* (17.63 mm) followed by *E. coli* (17.60 mm), *S. aureus* (17.00 mm) and *K. pneumoniae* (14.67 mm). The antibacterial activity of ampicillin was 15.67, 16.00, 16.37 and 6.80 mm against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae* respectively. The plant extract and distilled water displayed no antibacterial activity. Among the bacteria, *P. aeruginosa* was more sensitive towards SNPs followed by *E. coli*, *S. aureus* and *K. pneumoniae*. From our results it is clearly observed that biosynthesized SNPs from *U. wallichiana* leaf extract have higher antibacterial potential when compared to ampicillin.

Table I

Antibacterial activity of SNPs, plant extract, positive control and negative control against four common human pathogens

Names	**Zone of Inhibition in millimetres (mm)**				
	Ampicillin	**AgNPs (1 mg/mL)**	**Leaf aqueous extract**	**Distilled water**	**Means**
S. aureus	16	17.13	0	0	8.28 ^{A}
E. coli	15.67	17.60	0	0	8.32 ^{A}
P. aeruginosa	16.37	17.63	0	0	8.50 ^{A}
K. pneumoniae	6.80	14.67	0	0	5.37 ^{B}
Means	13.70 ^{B}	17.18 ^{A}	0 ^{C}	0	

The same letter (s) in a row or column represents non-significant results at p = 0.05 by DMRT

Table II

MIC of SNPs against four common human pathogens

Name	**Zone of Inhibition in millimetres (mm)**				
	AgNPs (1 mg/mL)	**AgNPs (0.5 mg/mL)**	**AgNPs (0.25 mg/mL)**	**AgNPs (0.125 mg/mL)**	**Means**
S. aureus	17.13	13.80	10	0	10.23 ^{B}
E. coli	17.60	14.70	7.40	0	9.93 ^{BC}
P. aeruginosa	17.63	14.40	11.20	7.40	12.66 ^{A}
K. pneumoniae	14.67	11.80	6.06	0	8.13 ^{C}
Means	13.70 ^{A}	16.75 ^{B}	8.70 ^{C}	**0**	

The same letter (s) in a row or column represents non-significant results at p = 0.05 by DMRT

SNPs were further tested for their MIC value and results are shown in Table II. Previously tested 1 mg/mL SNPs were further diluted in distilled water in serial dilution of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL in four separate test tubes. The MIC of SNPs was 0.125 mg/mL against *P. aeruginosa* and 0.25 mg/mL against all other tested bacteria.

The biosynthesized SNPs have shown very profound antibacterial activity against both Gram positive and Gram negative bacteria even at very low concentration [44]. Lesser concentration of SNPs is considered benign for the human consumption but lethal against the microorganisms [14]. In our study, Gram negative bacteria were found more susceptible to SNPs. Previously SNPs have also inhibited the growth of negative bacteria more intensely than Gram positive bacteria because Ag^{+} possesses positive charge which interact with negatively charged cell membrane of Gram negative bacteria with better affinity, creating holes in the membrane, thus restricting their function [45, 46].

There are several proposed mechanisms regarding the action of SNPs to inhibit to growth of bacteria. It is found that size and shape of nanoparticles determine the antibacterial activity as spherical shaped and smaller sized nanoparticles have been studied to show good antibacterial activity [47, 48]. The smaller is the size of SNPs, the larger is the surface area, resulting a high antibacterial activity [49]. It is believed that SNPs release Ag in the ionic form on the surface of bacteria which create inhibitory effect on their growth [50]. SNPs interact with cell membrane and disturb the cell membrane permeability and metabolic pathway. They penetrate through cell membrane, interact with chromosome, bind to DNA and block DNA replication [14]. In *E. coli* it is reported that SNPs decrease enzymatic activity by damaging cell membrane and cause death of *E. coli*. Biosynthesized SNPs are new alternatives to antibiotics and other antibacterial agents, because former has less probability of evolving resistance [51].

Antioxidant Activity

The antioxidant potential of biosynthesized SNPs, plant extract and standard (ascorbic acid) was determined by DPPH radical scavenging assay. The biosynthesized SNPs efficiently scavenged free radicals of DPPH as

compared to plant extract and ascorbic acid (Figure 6). The antioxidant activity of SNPs was 36.44, 53.76 and 81.24 % at 20, 40 and 60 µg/mL concentration respectively.

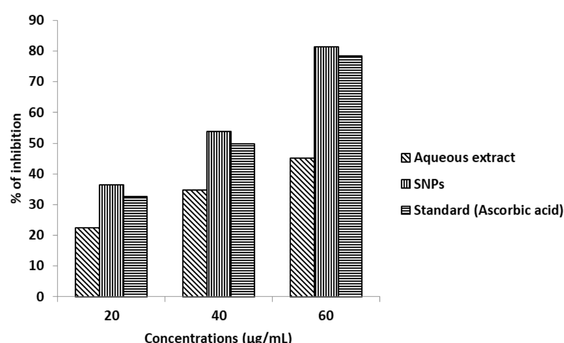

Figure 6.
Free radical scavenging activity of SNPs, plant extract and ascorbic acid against DPPH radicals

The DPPH results were further interpreted by the calculation of IC_{50} (the concentration required to inhibit 50% of the DPPH) value of SNPs and plant extract (Figure 7). SNPs exhibited IC_{50} value (33.62 µg/mL) better than ascorbic acid (36.91 µg/mL). The IC_{50} value of plant extract was 68.19 µg/mL. The lowest IC_{50} value of SNPs indicates their higher antioxidant activity. A variety of bioactive compounds such as polyphenols, alkaloids, proteins etc. those are present in the *U. wallichiana* [52] leaves act as hydrogen donor to free radical and thus break the chain reaction of free radical [53]. The enhanced antioxidant activity of biosynthesized SNPs is due to the capping of these biomolecules.

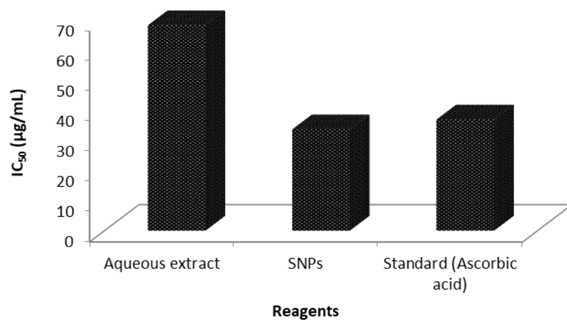

Figure 7.
IC_{50} value of SNPs, plant extract and standard control (ascorbic acid)

Conclusions

In this research, we developed an environmentally sustainable process for the synthesis of SNPs from leaf extract of *Ulmus wallichiana* for the first time and it synthesized SNPs within five minutes, a very fast synthesis. The synthesized SNPs were in predominantly spheroid shape with mean size of 54 nm. The biomolecules present in plant extract served as reducing, capping and stabilizing agent of SNPs. When these synthesized SNPs were applied to find their antibacterial and antioxidant activity, they effectively inhibited the growth of common human pathogens and also potentially scavenged DPPH radicals. In conclusion, this synthesis process of SNPs is rapid, non-toxic, low cost and sustainable with environment. Due to effective antibacterial and antioxidant activities, the synthesized SNPs can be used in biomedical applications.

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