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Synthesis, Characterization and Evaluation of Antibacterial Efficacy, Antioxidant Potential of Silver nanoparticle using *Myrica nagi* leaf extract

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Abstract

In the present work, we report an environment friendly biosynthesis of silver nanoparticles using ethanolic extract of *Myrica nagi* leaves. This endangered tree with wide medicinal applications has rich amount of anti-oxidants along with other classes of chemicals. Various therapeutic compounds such as myricanol, myricanone, myricetrin, sitosterol, taraxerol are isolated from the various parts of the plant. In this process, reduction of Silver ions to silver nanoparticles was achieved by a bioactive compound from *Myrica Nagi* plant. The synthesized nanoparticles were characterized using UV-visible spectrophotometer, Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM) and FTIR. The formation, stability and particle size of Ag nanoparticles was characterized using UV-Vis spectrophotometer and Dynamic Light Scattering. Scanning Electron Microscopy (SEM) micrograph shows a uniform distribution of the particles with an average size of 50-60nm. FTIR analysis confirms the presence of hydroxyl, carboxyl and phenolic functional groups. Further, the antimicrobial activity of silver nanoparticles shows that these nanoparticles can be used as effective growth inhibitors against *E. coli*, *S.aureus and S. pyogenes* with zone of inhibition of 1.2, 1.3 and 0.8 cm respectively. The synthesized silver nanoparticle have a potential application in targeted drug delivery, wound healing and other medical applications. The antioxidant activity of AgNPs imparted by plant components was evaluated using DPPH assay and found to be comparable to standard ascorbic acid.

Keywords: Silver Nanoparticles; Myrica nagi; Scanning Electron Microscopy; Anti-Microbial

Introduction

Recent era has witnessed a revolution in the field of science due to intervention of Nanotechnology. It is considered as future science and is widely researched owing to its vast and diverse applications in biomedicines, catalysis, sensors, electronic and optical devices [1-3]. Nanoparticles in general exhibit very unique properties like optical, chemical, electronic, magnetic, catalytic and other physical and chemical properties compared to its bulk materials due to high surface to volume ratio [4-5]. Among all the nanoparticles, silver nanoparticles exhibit very high toxicity to microorganisms, owing to its biocidal effects on most of the species of bacteria including *E. coli*, *S. aureus* and *S. pyogenes*. Silver and its compounds show excellent antibacterial activity, high toxicity for microbes and less toxicity for mammalian cells compared to all other metals [6, 7]. The toxicity exhibited on the microbial cells is caused by the interaction of these metal ions with the enzymes of the microbial cells leading to metabolization of enzymes which in turn causes catabolism of the cells [8].

The properties of the silver nanoparticles (Ag Np) have been thoroughly investigated by taking into account different synthetic methods and all other properties. There have been numerous reports for chemical synthesis method for the preparation of silver nanoparticles with uniform sizes and shapes. This synthesis requires both strong and weak chemical reducing agents like sodium borohydride, sodium citrate and alcohols. These chemicals are mostly toxic, flammable and cannot be easily disposed off due to environmental issues [9]. Amongst all the synthesis methods, biological methods are considered as to be eco-friendly and non-toxic [10].

In recent times, the synthesis of silver nanoparticles has received a great interest due to its various application in different fields. Moreover, silver can be synthesized in several configurations like Nanoparticles (Zero-Dimensional), Nanorods (One-Dimensional) and Nanocubes (Three-Dimensional), which exhibit various potential applications in medicines, catalysis, water purification and biological labeling [11, 12]. Research is being done on the synthesis of silver nanoparticles using plant extracts, fungi and bacteria. Plant extracts are used more often for the synthesis due to their huge availability, medicinal properties and faster rate of synthesis. The plant leaf extracts of *Chenopodium album*, *Helianthus annus*, *Basella alba* [13], *Azardirachta indica* [14], *Medicago sativa* [15], *Aloe vera* [16], *Emblica officinalis* [17], *Acalypha indica* [18], *Garcinia mangostana* [19], *Capsicum annuum*, *Geraniumsp*, *Diopyros kaki*, *Magnolia kobus*, *Coriandrumsp, Carob leaf* [20], *Morinda tinctoria* [21] have been effectively used for silver nanoparticle synthesis and analyzed for its antimicrobial activity against various pathogenic organisms.

Myrica nagi synomous *Myrica esculenta* Buch. Ham. of Myricaceae family is commonly known as Kaiphal is one among the ecological and economically important species of Himalayan region. It is one of the top exported medicinal plant of India [22]. The bark is rich in tannin, saccharine matter and salts. The species is known for its triterpenoids and tannins in treating cardiovascular diseases. The triterpenoids from the leaves are known to inhibit the Angiotensin I-converting enzyme. In literature survey, the HPTLC chromatogram of the ethanolic extract of leaves possess number of compounds which are yet to be investigated. Hence, preparation of silver nanoparticles from the leaf extracts would be a boon to the medical field. This study will provide a new horizon to this unexplored species which is highly neglected. The ethanolic extract of *Myrica nagi* aerial parts has shown antiallergic and anti-inflammatory potential in mice with the dose of 75 and 150 mg/kg. [23]. Species in this genus have a symbiotic relationship with certain soil micro-organisms, by forming nodules on the roots of the plants and by fixing atmospheric nitrogen. Some of this nitrogen is utilized by the growing plant but some can also be used by other plants growing nearby. Hence, in addition to its medicinal value, the tree is useful in stabilizing nitrogen-depleted soils. [24].

In the present work, we report a simple and effective environmental friendly method for the synthesis of highly stable Ag nanoparticles, where in ethanolic leaf extract of *Myrica nagi* is used as a reducing and capping agent, which in turn exhibits less toxicity for mammalian cells compared to wet chemical synthesis.

Material and methods

Chemicals

Silver nitrate was purchased from Sigma Aldrich, Delhi, India. The bacteriological media were purchased from Chemical and Scientific instruments, Delhi.

Microorganisms and Media

The strains of *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus pyrogens* were used. Nutrient agar was used for growth and maintenance of bacterial strains. Nutrient broth was used for preparation of suspension cultures. Mueller Hinton Agar (MHA) was used to assess antibacterial activities.

Collection of Leaves

The leaves of *Myrica nagi* were collected from Thalkedar Forest, Pithoragarh, Uttarakhand, India from the altitude of 2005 m, N: 29°31'53.6" and E: 80°12'14.1".

Extraction Procedure

Cold extraction of leaves was carried out using ethanol. 100 g of powdered leaves were macerated in 300 mL ethanol with intermittent shaking for three days. It was filtered with muslin cloth and then through Whattman no.1 filters paper, residue was further extracted two times by using the same fresh solvent and finally all the filtrates were pooled together [25]. Solvent from the filtrate was removed using rotary evaporator under reduced pressure and low temperature. The yield of each extract was weighed and stored at 4 C until used. The yield percentage was 7.21.

Biosynthesis of Silver nanoparticles

1mM Silver Nitrate was prepared in 50 ml of distilled water and it was subjected to constant stirring for 10 minutes. 5 ml of the prepared ethanolic extract of *Myrica nagi* was added drop-wise over a period of 5 minutes and left to stir for another 10 minutes. The final reaction mixture was centrifuged at an rpm of 4500 and the supernatant was used for further analysis.

Characterization of Ag nanoparticles

Figure 1 shows the formation and stability of the synthesized sample which was observed using a UV-Vis spectrophotometer (UV-VIS, VSI-501 spectrophotometer) after a fixed interval of time.Fig.1 shows the UV-visible spectra of synthesized Ag nanoparticles. Sample displays a peak around 430nm, which corresponds to the characteristic peak of Ag nanoparticles due to surface plasma resonance. The absorption spectrum clearly indicates the particles shows stability even after 12 hrs.

SEM analysis of Silver nanoparticles

The surface morphology and size of the as synthesized Ag nanoparticles were evaluated using Scanning Electronic Microscopy (SEM). The surface energy and surface area are the effective parameters for antibacterial activities. The agglomeration reduces the antibacterial activities due to the less surface area. Therefore, the investigation of surface area, morphology and disparity of nanoparticles is vital. The morphology of the material was investigated using scanning electron microscope (Zeiss EVO 18) at room temperature. Figure 2 shows the Scanning Electron Microscopy (SEM) image of the synthesized nanoparticles. The image indicates that the particles were spherical in nature and uniform in size. The average radiuses of the particles are of 50- 60 nm. The particles are also mono-dispersed in nature.

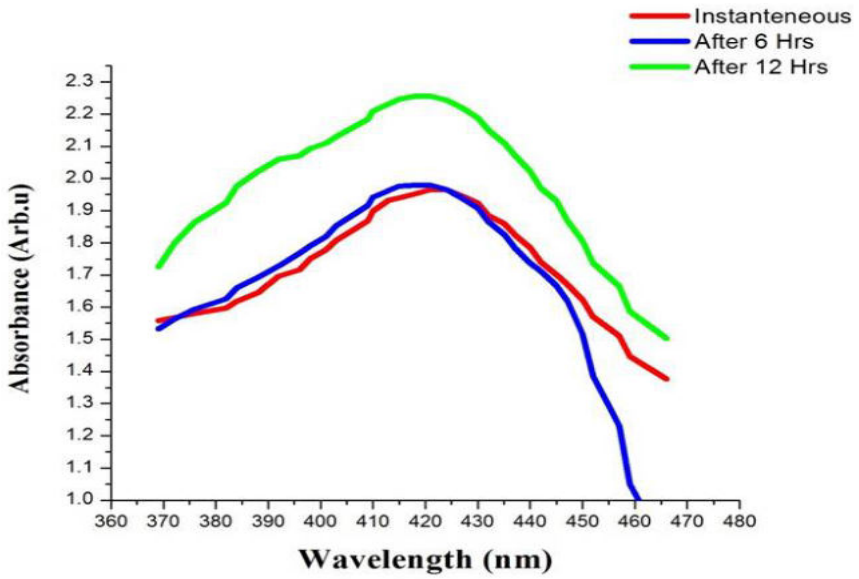

Figure 1: UV Visible Spectrum of Ag Nanoparticles synthesized.

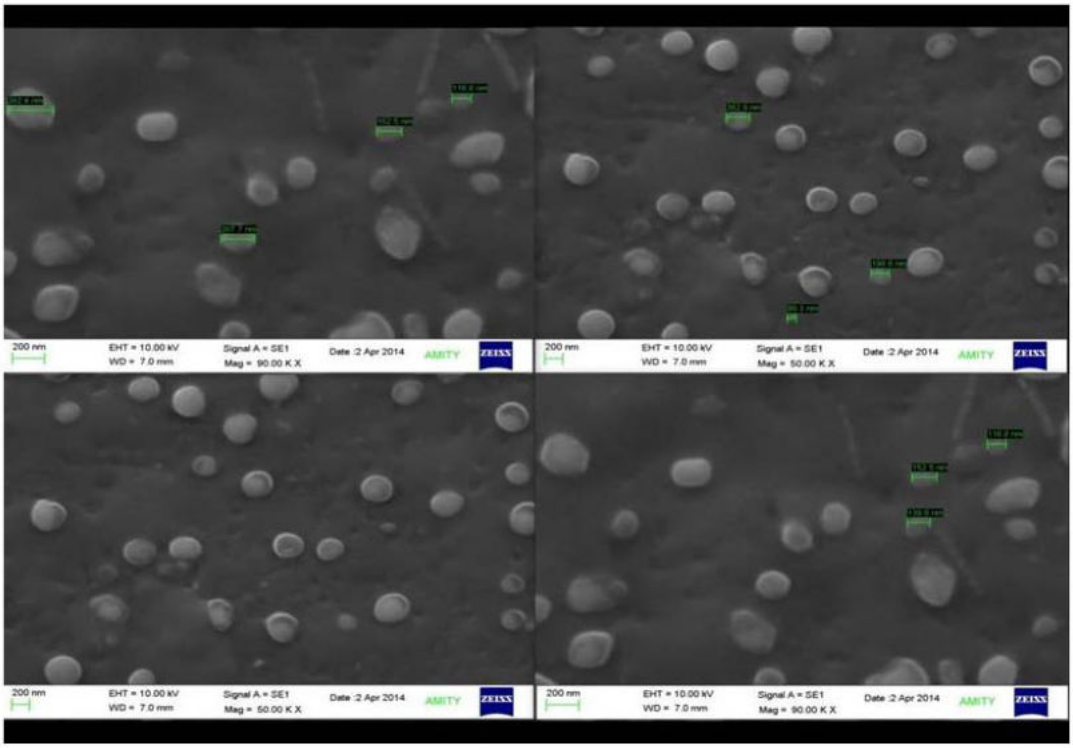

Figure 2: SEM Micrograph showing the uniformly distributed spherical nanoparticles of size ranging between 50- 60nm.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The binding properties of silver nanoparticles perepared by *Myrica nagi* leaves extract were investigated by FTIR analysis. Infrared spectra were recorded in KBr pellets in the region 4000 – 400 cm^{-1} on a Shimadzu FTIR-8300 spectrometer at Delhi University, Delhi.

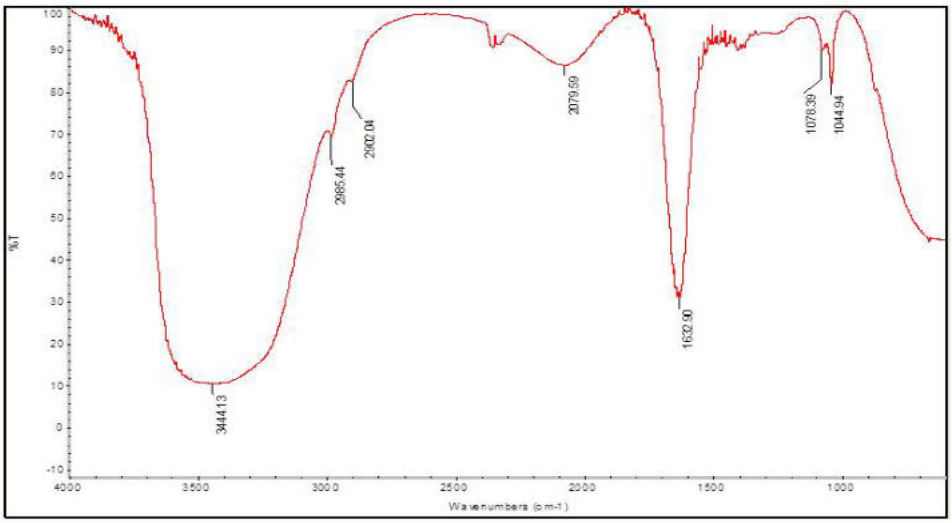

Figure 3: FTIR Spectrum of Ag Nanoparticles.

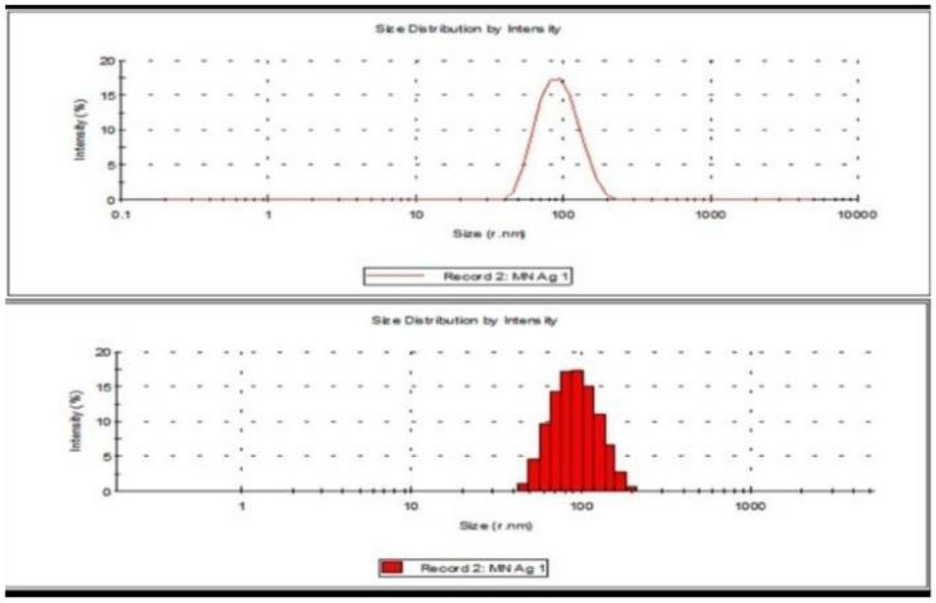

Figure 4: DLS Spectra of Ag Nanoparticles.

DLC Analysis

The average particle size was analyzed by Dynamic light scattering system (DLS) (Malvern).Figure4 shows the average particle size of the synthesized Ag nanoparticles. The measurement reveals the average particle size was 100nm and the particles were mono-disperse in nature.

Experimentation

Antimicrobial activity

Preparation of Inoculum: The gram positive (*Staphylococcus aureus*, Streptococcus pyogenes) and gram negative bacteria (*Escherichia coli*) were pre-cultured in nutrient broth overnight in a shaker incubator at 37 C.

Anti-bacterial Activity: The ethanolic plant extract and silver nanoparticle prepared from the ethanolic plant extract were tested by the disc diffusion method. The test microorganisms were seeded into respective medium by spread plate method 200 µl with the 24hrs cultures of bacteria growth in nutrient broth. After

solidification, the filter paper discs impregnated with the extracts were placed on test organism-seeded plates. *E.coli, S.aureus and S. pyogenes* were used for antibacterial test. The antibacterial assay plates were incubated at 37 C for 24h. The diameters of the inhibition zones were measured.

Figure 5: (a) *Zone of Inhibition against E.coli.* (b) *Zone of Inhibition against Streptococcus pyogenes.* (C) *Zone of Inhibition against Staphylococcus aureus.*

Figure 6: Colour change in reaction mixture (Silver nitrate + *Myrica nagi* leaves extract) (a) at 0 hours; (b) after 2hours

Antioxidant Activity Assay

DPPH (2, 2-diphenyl-l-picryl hydrazyl) radical scavenging assay

The free radical scavenging capacity of the plant extracts was determined using DPPH. 3ml DPPH solution absorbance was taken immediately at 517 nm for control reading and methanol as a blank. To 0.1 ml of different volume level of test samples 3.9 ml of DPPH was added in each test sample. Absorbance was taken at 517 nm in UV-visible spectrophotometer using methanol as a blank. The absorbance was measured at different time intervals at 517 nm for two hours or until the absorbance become steady. The free radical scavenging was calculated using the following equation:

Free radical scavenging activity (%) = $Ab_{(B)}$-$Ab_{(S)}$/ $Ab_{(B)}$ 100

Preparation of DPPH solution

6 10^{-5} mol/L DPPH in methanol. It was protected from light by covering the beaker with aluminium foil.

Preparation of test samples

Required quantity of test samples was dissolved in methanol to give the concentration of 25, 40, 55, 70, 85 and 100µg/ml.

Preparation of standard stock solution

Ascorbic acid was used as standard for the study and its stock solution was prepared in the concentration of 1000 µg/ml in methanol. It was prepared freshly and used immediately for the study. From the stock solution, different concentration viz. 25, 40, 55, 70, 85, 100 µg/ ml were prepared in methanol and used for antioxidant studies.

Table 1: Antioxidant assay

		% Inhibition		
Concentration	Ascorbic acid	Ethanolic extract	Silver nano particle	
25	32.46	30.646	40.97	
40	49.23	41.068	45.91	
55	56.16	45.8509	52.84	
70	67.14	59.912	61.69	
85	87.14	72.225	77.62	
100	94.76	85.334	90.69	

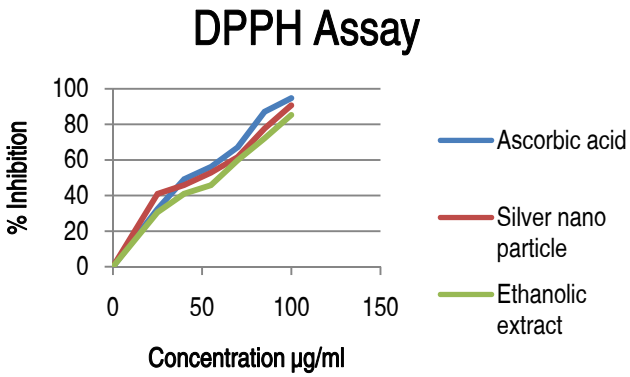

Figure 7: Antioxidant assay of ethanolic extract and silver nanoparticles

Result and Discussion

The present paper demonstrated the synthesis of Ag nanoparticles by simple and effective biological methods. The ethanoic extract of Myrica nagi is used as a reducing and stabilizing agent. Formation of AgNPs by reduction of silver nitrate during exposure to *Myrica nagi* leaves extract can be easily monitored from the change in colour of the reaction mixture. Silver nanoparticles bear a characteristic yellow brown colour due to the excitation of surface Plasmon vibrations. The change in reaction mixture after 2 hours is presented in Figure 6 which indicates the formation of AgNPs. This formation indicates that silver ions in reaction medium have been converted to elemental silver having the size of nanometric range.

Figure 1 shows UV-vis absorption spectrum of silver nanoparticles. There are a number of secondary metabolites which are responsible for the broadening of the Plasmon bond in *Myrica nagi* extract which are also shown in the spectrophotometric range. At 430 nm silver surface Plasmon resonance peak is shown which is steadily increasing in intensity as a function reaction time. The absorption spectrum clearly indicates the particles shows stability even after 12 hrs. No change in the peak clearly suggest the stability of the silver nanoparticles being formed. The spherical structure of silver nanoparticles is further confirmed by SEM Figure 2.

The stability was also tested along with the efficacy. The solution after storing for two months, were studied and it was found that no precipitation has taken place. Observations were taken regularly in UV-Vis absorption properties of the solution. There was no shift in the absorbance intensity as well as absorption maxima indicating the particle size to be same as recorded earlier.

Figure 3 shows the FTIR analysis results of the AgNPs. Functional group analysis of the synthesized nanoparticles was carried out with the help of FTIR. A broad intermolecular hydrogen bonded O-H stretch was seen at 3444 cm^{-1}. Peak at 2985.44 cm^{-1} and 2902.04 cm^{-1} denotes –C-H stretch (alkane H). A broad peak at 2079 cm^{-1} depicts the presence of =C-H group. A peak at 1632.90 cm^{-1} confirms C-C stretch seen in aromatic rings for the synthesized Ag nanoparticles. Depending on the FTIR peak range obtained it can be concluded that the stabilization is attained due to presence of phenolic and aromatic compounds present in the extract.

Figure 4 shows the DLS of Ag nanoparticles. The image reveals that the particles are monodisperse in nature with an average particle size. The antimicrobial nature of the bark [26] and fruits [27] are well known. This is the first time that antimicrobial assay of leaves extract is done. Silver nanoparticle of *Myrica nagi* ethanolic extract posseses potential antibacterial activity against *E.coli, S. pyogenes* and *S.aureus*. When tested by the disc diffusion method, it had shown significant activity against *E.coli* (Figure 5a), *S. pyogenes* (Figure 5b) and *S.aureus* (Figure 5c) with zone of inhibition of 1.2, 1.3, 0.8 cm respectively. This study shows that the silver nanoparticles have antibacterial activity against E.coli, Streptococcus pyogenes, Staphylococcus aureus. This study in future can be utilized for testing on other future gram positive and gram negative bacterias for finding out the efficacy and better utilization of the synthesized nanoparticles. It can be also researched for targeted drug delivery.

The radical-scavenging activity of the ethanol extracts of *Myrica nagi* was estimated by comparing the percentage inhibition of formation of DPPH radicals with that of ascorbic acid. The

ethanolic extracts showed moderate antioxidant activity when compared with ascorbic acid (Table 1). The DPPH radical scavenging activity of ethanolic extract increased with increasing the concentration (Figure7). [28]

Natural antioxidants those are present in medicinal plants which are responsible for inhibiting the harmful consequences of oxidative stress [29]. Many plants extract exhibit efficient antioxidant properties due to their phytoconstituents, including phenolics. This method has been extensively used for screening antioxidants, such as polyphenols. The antioxidant effectiveness in natural sources has been reported to be mostly due to phenolic compounds. Phenolic compounds may contribute directly to antioxidative effect of the extracts. The free radical scavenging activity of acetone and methanolic extracts were confirmed in the present investigation.

The control (standard ascorbic acid) and the plant extracts showed their maximum activity at: control (94.76%), ethanol (85.33%) and silver nanoparticle (90.96%). DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals. The results revealed that the silver nanoparticle of ethanolic extract of *Myrica nagi* exhibited the highest radical scavenging activity with 90.69 % (Table 1). The secondary metabolites in the plant are responsible for several pharmacological activities [30-34]. In the present paper, we report for the first time antioxidant activity of silver nanoparticles capped with plant extracts possessing free radical scavenging activity.

Conclusions

The present study provides strong evidence for environment friendly synthesis of silver nanoparticles using ethanolic extract of *Myrica nagi* leaves. The investigation confirms the high antioxidant potential of ethanolic extract *Myrica nagi* leaves and its silver nanoparticles.

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