Anti -Inflammatory, Antioxidant and Antimicrobial Activity of Green Synthesized Silver Nanoparticles from Combined Leaf Extracts – An In-Vitro Approach

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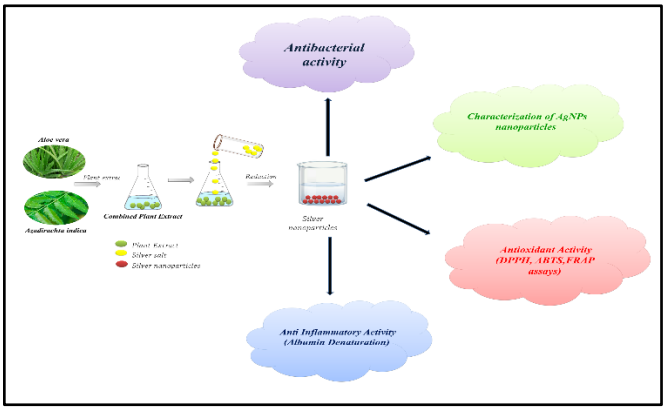

ABSTRACT

Green production of silver nanoparticles (AgNPs) utilizing plant extracts is now generally recognized due to its low cost, environmental friendliness, and low toxicity. In the present study, an attempt has been made to synthesize AgNPs by using combination of two different plant extracts namely *Azadirachta indica (Neem)* and *Aloe barbadensis (Aleo Vera)*. A simple green synthesis method was used for silver nanoparticles production using aqueous leaf extracts of *Azadirachta indica* and *Aloe barbadensis*in the combined form. Various characterization techniques were used to characterize the synthesized nanoparticles like UV-Visible spectrophotometer, FTIR, SEM and XRD. The *In-vitro* anti-inflammatory were done by Inhibition of albumin denaturation, *In-vitro* antioxidant activity was done by ABTS free radical scavenging assay , DPPH assay and Potassium Ferricyanide Reducing Power assay method then *In-vitro* antimicrobial studies against *Bacillus cereus*, *Actinomycetes Israeli, Proteus vulgaris, Streptococcus pyogenes, Corynebacterium diphtheria, Pseudomonas aeruginosa, Enterococcus faecalis and Staphylococcus aureus* were examinedat various concentrations (50,100,250,500µg). The results indicated that biosynthesized Silver nanoparticles (AgNPs) using aqueous leaf extracts of Azadirachta indica and Aloe barbadensis in combination work as stabilizing agents. Our research demonstrated the anti-inflammatory, antimicrobial and antioxidant efficacy of phytomediated silver ions in vitro.

Keywords: Combined leaf extract, Green synthesis, Aloe barbadensis, Azadirachta indica, Silver nanoparticles, anti-inflammatory, antioxidant activity and antimicrobial activity.

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INTRODUCTION

Nanotechnology is among the most important disciplines of scientific research in the twenty-first century. Noble nanoparticles are gaining popularity due to their attractive applications in multidisciplinary research[1]. There are different types of Nanoparticles available in which it is synthesized by various methods namely hybrid,

chemical, physical and biological method. While physical and chemical techniques of nanoparticle production are more common, the use of hazardous substances severely restricts their biological uses, especially in clinical domains [2]. Nanomaterials are used in tissue engineering to name a few[3,4]. Silver Nanoparticles are an important component of all metal Nanoparticles. Silver is one of the most often used nanomaterials[5,6]. Green synthesis has been considered as one of the most promising approaches for producing nanoparticles due to its biocompatibility, low toxicity, and environmental friendliness [7]. Inflammation is a complicated web of tissue disintegration, and repair that happens as a result of tissue damage[8]. Most anti-inflammatory medications on the market have a variety of issues, including effectiveness and unwanted side effects such as gastrointestinal tract diseases and other undesirable consequences, renal damage, and respiratory depression[9]. This circumstance emphasizes the need of developing safe, innovative, and effective analgesic and anti-inflammatory drugs[10,11]. Aloe gel has been shown to decrease agent-induced inflammation by increasing prostaglandin production and leucocyte infiltration, although it is less efficient against agent-induced inflammation[12,13]. Sliver Nano particles are well-known for having excellent antibacterial action against different pathogens such as bacteria, viruses, and fungus because of their size and large surface area [14]. The antioxidant activity of AgNps is well investigated by monitoring the scavenging of stable free radicals viz.2, 2'- diphenyl -1-pircyl hydrazyl (DPPH), and 2,2'-azino-bis-3-ethylbenzthiazoline -6-sulphonic acid (ABTS). AgNPs are recognized as antioxidant agents because silver favours two oxidation states (Ag^{+1} and Ag^{+2}) that depends on the reaction conditions, thus AgNPs may quench free radicals by accepting or donating electrons ,[15]. According to Bedlovikova et al the enhanced antioxidant potential of AgNPs than plant extract is due to the coating of phenolics, flavonoids, and terpenoids on surface which allow nanoparticles to act as singlet oxygen quenchers, hydrogen donors, and reducing agents [16,17].

Usually a single plant extract is used to synthesize the Nanoparticles. Here in, we report the combination of plant extract to obtain the Nanoparticles, since it enriches the medicinal value of two different biological plants. In this work, silver nanoparticles were synthesized from aqueous leaf extracts of Azadirachta indica and Aloe barbadensis using a simple green synthesis process. Plant extracts work as a lowering agent as well as a capping agent. Furthermore, plant-mediated nanoparticle production is favored due to its low cost, one-step biosynthesis process, environmental friendliness, and safety for human medicinal application.

MATERIAL AND METHODS

Pre-treatment of Aloe barbadensis&Azadirachta indica

Azadirachta indica and Aloe barbadensis leaves were collected from the University campus and rinsed with running water to remove pollutants and other contaminated organic compounds from the leaves' surface before being double distilled and air dried at room temperature (Figure 1.).

(a) (b)

Figure 1. Photographs of the plants (a) Azadirachta indica (b) Aloe barbadensis

Preparation of Aloe barbadensis and Azadirachta indica leaf extract

About 20 gram of finely chopped A.indica and A. barbadensis leaves were ground separately and put together in a beaker holding 200 mL double distilled water and heated for 30 minutes. The extract was cooled and filtered with Whatman filter paper before being stored for future use.

Green synthesis of Silver Nanoparticles

In a 100ml volumetric flask, a 1 mM silver nitrate solution was produced. To decrease photo-activation of silver nitrate at room temperature, a known quantity of A. indica and A. barbadensis plant extract was added to a 1mM silver nitrate solution and stored in the dark. Then Ag^{+} was reduced to Ag^{0} which indicate Silver Nanoparticles formation by the solution colour change from colorless to brown.

Characterization of Silver Nanoparticles

The Nanoparticle solution obtained from the combined leaf extract of Azadirachta indica & Aloe barbadensis was refined by centrifugation repeatly at 20,000 rpm for 30 min monitored by re- dispersion of the pellet in distilled water. The colour shift visibly verified the production of silver nanoparticles. The Bio-Ag NP synthesized from A. indica and A.barbadensis leaf extract was characterized by UV, FTIR, SEM and XRD analysis. On a Perkin Elmer UV-Vis spectrophotometer were obtained as a function of reaction time. The chemical group changes were confirmed using the KBr pellet method and Fourier transform infrared spectroscopy (FT-IR, Jasco). The FTIR spectra were collected in transmission mode (4000 – 400 cm^{-1}) with a resolution of 4 cm^{-1}. XRD was used to identify the crystalline nature of silver nanoparticles. The X-ray patterns were acquired in two theta configurations with a range of 10° to 80°. These were carried out with the use of a PanalyticalX'pert Powder X' Celerator Diffractometer. SEM was used to determine the morphological feature.

Biological activities of Sliver Nanoparticles containing Azadirachta Indica and Aloe barbadensis

In vitro anti-inflammatory activity (Inhibition of albumin denaturation)

The anti-inflammatory efficacy of AgNPs biosynthesized from A. indica and A. barbadensis leaf extracts was investigated in vitro utilizing suppression of albumin denaturation,[18]. The reaction mixture contained test extracts and 1% aqueous solution of bovine albumin fraction, with the pH adjusted with a trace of 1N HCl. After incubating at 37°C for 20 minutes, the sample extracts were heated to 51°C for 20 minutes before being measured at 660nm. The experiment was repeated three times. The percentage inhibition of protein denaturation calculated was as follows:

$$Percentageinhibition = \frac{(Abscontrol - Abssample)}{Abscontrol} \times 100$$

In vitro anti-oxidant activity

DPPH Radical Scavenging Activity

The antioxidant activity of biosynthesized AgNPs derived from A. indica and A. barbadensis leaf extract was assessed by DPPH assay which is popular in natural product antioxidant studies. This assay is based on the theory that a hydrogen donor is an antioxidant which measures compounds that are radical scavengers. It shows the mechanism by which DPPH accepts hydrogen from an antioxidant [19].100µl of 0.1 mM of DPPH solution in methanol was added to 300 µl of sample solution at different concentration 25, 20, 15, 10 and 5 µg/mL. The mixtures was shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Ascorbic acid was used as the standard. The effect of radical scavenging activity calculated was as follow:

$$DPPHScavengingactivity = \frac{(Abscontrol - Abssample)}{Abscontrol} \times 100$$

ABTS free radical scavenging assay.

The ABTs free radical cation decolorization test was used to assess the in vitro antioxidant activity of biosynthesized AgNPs derived from A. indica and A. barbadensis leaf extract [20]. The reaction of 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1) produced a positive cation radical, which was stored in the dark for 12-16 hours at room temperature before use. The absorbance at 734 nm was measured to be 0.700 after diluting the ABTS•+ solution with methanol.

After 30 minutes of initial mixing, the absorbance was measured at 734 after the addition of 5l of Bio - Ag NP to 4 ml of diluted ABTS•+solution. Each experiment was preceded with a solvent blank. Every measurement was taken at least three times. The percent suppression of absorbance at 734 nm was calculated using the method below [21]

$$ABTS + scaveningeffects(\%) = \frac{(AB - AA)}{(AB)} \times 100$$

Where AB is ABTS radical + absorbance in methanol and AA is ABTS radical + absorbance in sample (extract/standard).

Potassium Ferricyanide Reducing Power Assay

The reducing capacity of extracts was investigated by using potassium ferricyanide-ferric chloride method [22]. 0.2 mL of each of the extracts at different concentrations, 2.5 mL of phosphate buffer (0.2 M, pH 6.6), and 2.5 mL of potassium ferricyanide $K_3Fe(CN)_6$ (1%) were mixed and incubated at 50°C for 20 min, to reduce ferricyanide into ferrocyanide. The reaction was stopped by adding 2.5 mL of 10% (w/v) trichloroacetic acid followed by centrifugation at 1000 rpm for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. The sample concentration providing 0.5 of absorbance (IC_{50}) was calculated by plotting absorbance against the corresponding sample concentration. Butylene Hydroxyl Toluene was used as a reference compound.

Antimicrobial Activity

The Biosynthesized Sliver nanoparticles was screened for antimicrobial activity by disc diffusion method, using nutrient agar media against gram-positive bacteria namely *Bacillus cereus*, *Actinomycetes Israeli, Staphylococcus aureus, Streptococcus pyogenes, Corynebacterium diphtheria and Enterococcus faecalis,* gram-negative bacteria namely *Proteus vulgaris and Pseudomonas aeruginosa.* Gentamicin was used as a reference standard. Petri plates containing 20 ml nutrient agar medium were seeded with 24 hr culture of bacterial strains and adjusted to 0.5 OD value according to McFarland standard. Well was cut and the different concentration (500, 250, 100 and 50 µg/ml) of the extract are added into the well. Then the plates were incubated at 37°C for 24 hrs till perfect growth was observed. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. The values were calculated using Graph Pad Prism 6.0 software (USA) [23].

RESULTS AND DISCUSSION

The AgNPs were created by combining the leaf extracts of two medicinal plants (A. indica and A. barbadensis) with a silver nitrate solution. The resultant nanoparticle solution was purified by centrifugation at 20,000 rpm for 30 minutes, followed by re-dispersion of the pellet in distilled water. Visual examination of the solution was used to assess the reduction of Ag ions (Ag+) to Ag NPs. Because of their small size, colloidal Ag NP solutions have a characteristic brown hue. Because Ag+ is a colorless solution, the production of Ag NPs has been seen by a change in colour (Figure2.).

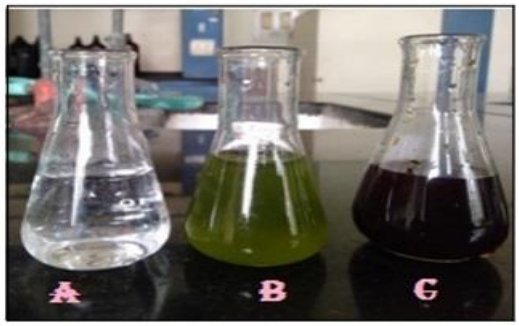


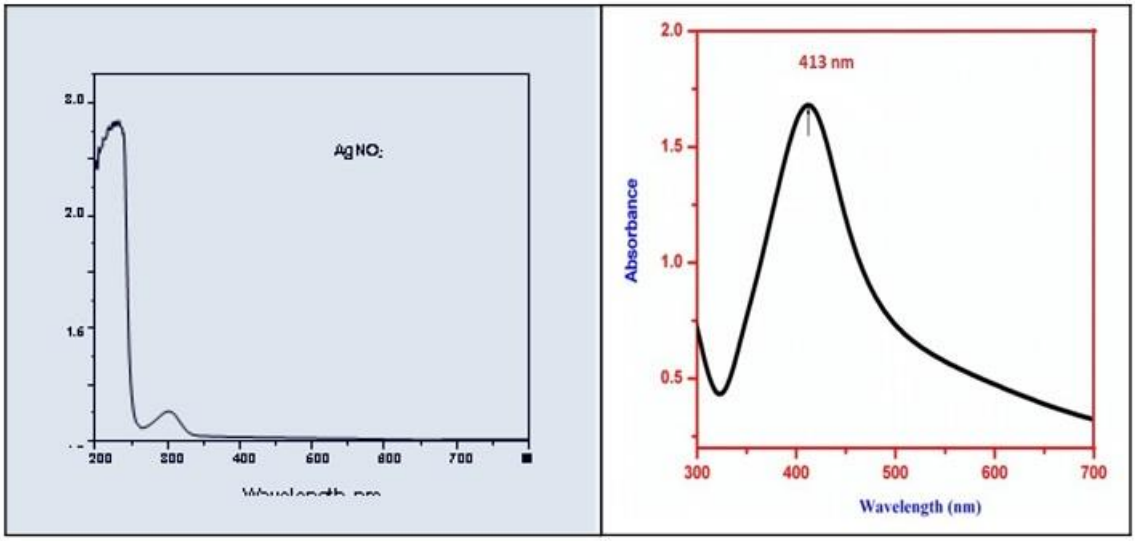
Figure 2. (A) Silver nitrate solution (B) Combined plant extract (C) Formation of Silver Nanoparticles of A. indica and A. barbadensis

Characterization

The silver nanoparticles were characterized using spectrum methods such as UV- Vis and FTIR, and their shape was examined using XRD and SEM.

UV Spectroscopy

The UV–Vis spectral analysis revealed a strong peak at 300 nm, which was linked with Ag+ ions in the solution (Figure 3a.). This is the first stage of the AgNO3 reduction process, in which Ag+ and NO3 - ions are separated. (Figure 3b.) is the absorption spectrum of Ag Nps and a peak is observed at 413 nm, assigned to SPR band of silver Nps.

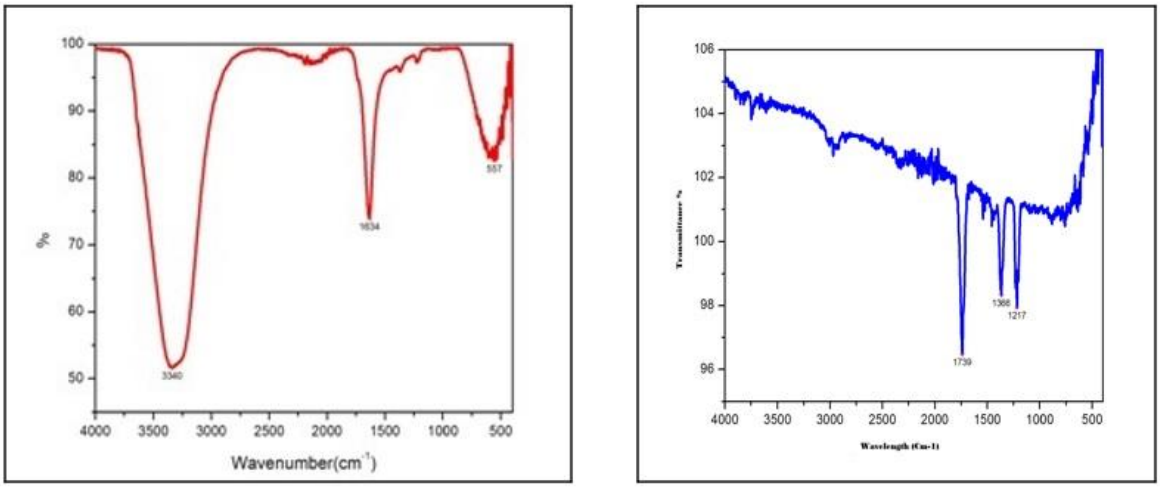


(a) **(b)**

Figure 3. Absorption spectrum of (a) silver nitrate solution and (b) silver nanoparticles from A. indica and A. barbadensis leaf extract

Fourier Transform Infrared spectral features

The use of Fourier Transform (FT) infrared spectrum measurement to detect the functional groups responsible for capping and reducing agent for the AgNPs generated by leaf extract has been approved. There were three separate bands visible (Figure 4.). The N—H stretching mode generated the intense broad band at 3271 cm-1, and the band corresponding to the O—H stretching mode implies protein connection. The CHO stretching mode in the amine group, which is commonly present in proteins, induces the strong band at 1637 cm-1, suggesting that proteins function as a capping agent for AgNPs, improving the stability of the Nanoparticles generated. A strong and broad peak at 386 cm-1, similar to Ag metal, was anticipated to appear. Figure 4.FT-IR spectra of A. indica and A. barbadensis biosynthesized silver nanoparticles.

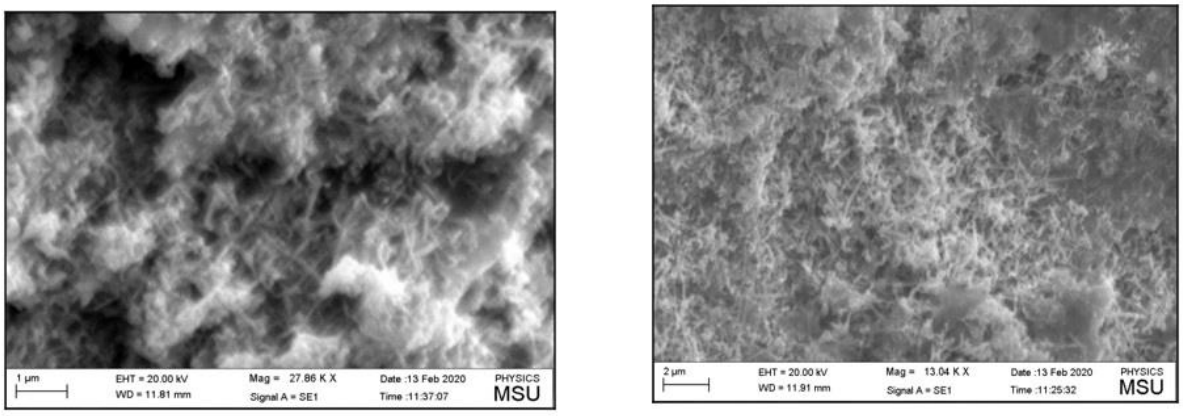


(a) **(b)**

Figure 4. FT-IR spectra of (a) Combined plant extract (b)biosynthesized Ag NPsof A. indica and A. barbadensis.

Scanning Electron Microscopy analysis

The morphology of the synthesized Nps are determined using SEM analysis. (Figure 5.) shows the SEM images of synthesized Ag Nps of nano wires with an average length of ¬ 15nm.



(a) **(b)**

Figure 5. SEM Images of biosynthesized Ag NPs with different magnifications (a) 1µm (b) 2 µm

X-ray diffraction (XRD pattern) Analysis

XRD analysis was used to determine the crystalline structure of AgNPs extracted from the leaves of A. indica and A. barbadensis. The X-ray diffraction pattern (Figure 6.) displays four primary distinctive diffraction peaks, suggesting face-centered cubic Ag crystals (blue lines). 38.12°, 44.35°, 64.56°, and 77.48° are the diffraction peak values at 2. Peaks from other phases were also found, indicating that the nanoparticles are single phase Ag with cubic structure. The average crystallite size computed using Scherrer's equation for the width of the (111) peak is 15- 19 nm, which agrees with the particle size measured by SEM images. The unassigned peaks are caused by the crystallisation of bioorganic species found in plant extract.

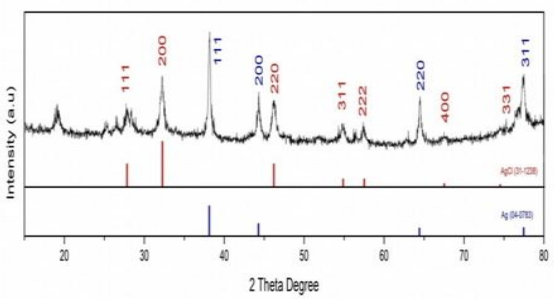


Figure 5. X-ray diffraction

Biological activities of Sliver Nanoparticles containing Azadirachta Indica and Aloe barbadensis

The synthesized silver NPs are characterized and it is very essential to study the biological activity of these particles to exploit commercially for pharmaceutical applications. In the present work, anti-inflammatory and anti-oxidant activities of the Bio-synthesized Ag NP of combined extract are evaluated.

In vitro anti-inflammatory activity (Inhibition of albumin denaturation)

Inflammation is a key event in many infections and is the first line of defence. However, the inflammation if chronic may lead to disorders and pose a major health issue. Currently, the drugs in use for inflammation are palliative in nature but mostly exhibit side effects. Nanoparticles may aid in decreasing toxicity and adverse effects by reducing dose and size. As a result, we examined the anti-inflammatory efficacy of bio-AgNPs produced from A. indica and A. barbadensis. Protein denaturation is a condition in which the natural structure of a protein is destroyed owing to chemical, stress, or heat action. Inflammation has been linked to protein denaturation and the loss of biological activities. A. indica and A. barbadensis bio-AgNPs inhibited albumin denaturation in a dose-

dependent manner, with 66 and 68 percent inhibition, respectively. (Figure 7.) for20 and 25 µg/ml. The inhibition was on par with the standard drug aspirin (68%) at 100 µg/ml. Figure 7 .Effect of biosynthesized Ag NP of A. indica and A. barbadensis on albumin denaturation. Values represent mean ± SD of triplicates.

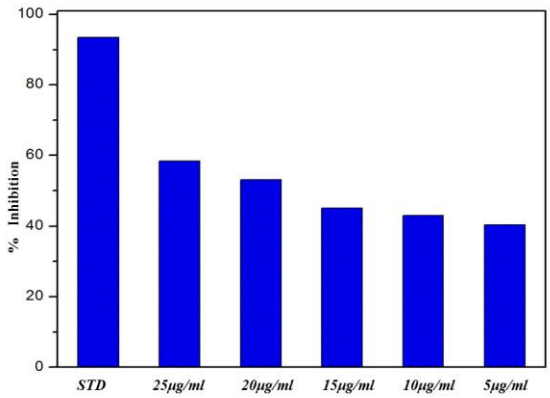


Figure 7. Inhibition of Albumin Denaturation Assay of biosynthesized Ag NPs of A. indica and A. barbadensis

In vitro anti-oxidant activity

Antioxidant activities might be related by the presence of flavonoids, alkaloids in the extract of plants used for the study. This means a reduction in antioxidant activity may result in a reduction in the metabolite concentration of plants during Nanoparticles formation. The surface area of silver is large, which means more plant chemical substances are added to the active surface. As a result, the shell response phenomenon in the NP is elevated by bio AgNPs (due to an adsorbed antioxidant moiety on the surface).

DPPH Radical Scavenging Activity

DPPH is a persistent free radical with a distinctive absorbance of 517 nm that lowers dramatically when exposed to radical scavengers, resulting in a stable diamagnetic molecule. Biosynthesized Ag NP of A. indica and A. barbadensis show the IC50 value of 132 which indicates the potent DPPH radical scavenging potential of the extract. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. (Figure 8.) shows the DPPH radical scavenging activity of biosynthesized Ag NP by A. indica and A. barbadensis.

ABTS radical scavenging assay

. The bio AgNP of A. indica and A. barbadensis quenched the free radicals induced by ABTS. As the concentration increases, the ABTS free radical scavenging activity also increases (Figure 8.). Maximum inhibition of 19.65% was observed for the maximum concentration tested. With transition metal ions, ABTS + forms complexes. The intensity of the ABTS + green hue in the presence of metal ions reflects this.

When ABTS is combined with potassium persulfate, the ABTS+ chromophore is formed, which transforms ABTS to its radical cation. This radical cation has a blue colour and absorbs light with a wavelength of 734 nanometers. ABTS+ reacts with the majority of antioxidants, including phenols, thiols, and vitamin C. [24]. Bio-AgNP derived from A. A. indica and Barbadensis inhibited the formation of ABTS radicals in an intensity manner.

Several assays used to measure total antioxidant capacity of antioxidants in food components or biological samples are also based on the electron transfer process. The following reaction serves as the foundation for this assay:

$$Probe + Electronfromantioxidant \rightarrow Reducedprobe + oxizisedantioxidant$$

The dark blue 2,2-azino –bis (3-ethylbenzothiazoline-6-sulfonate)radical cation (ABTS+) is converted by an antioxidant into colourless ABTS, which may be evaluated spectrophotometrically in the ABTS + radical scavenging test (an electron transfer –based assay). Metmyoglobin was coupled with hydrogen peroxide to produce ferrylmyoglobin, which then interacted with ABTS to form the ABTS+ radical cation in the new test. Other antioxidants that can be used to oxidize ABTS include manganese dioxide, potassium persulfate, and horseradish peroxides. Because antioxidants can convert this radical cation into ABTS, in the presence of antioxidants in the extract, the ABTS+ radical cation's blue green colour changed to colourless (lower absorbance) Figure 8. ABTS free radical scavenging activity of Ag NP biosynthesized by A. indica and A. barbadensis.

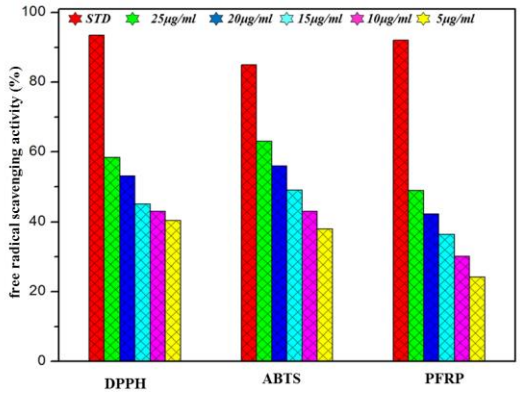


Figure 8. Different methods of Antioxidant Activity of biosynthesized Ag NPs of A. indica and A. barbadensis

Potassium Ferricyanide Reducing Power Assay

The antioxidant activity is complex mechanism and cannot be evaluated by single method. It is essential to carry out more than one method to assess the anti-oxidant potential. The biosynthesized AgNP of A. indica and A. barbadensis showed reducing power activity however lesser than the standard Butyl Hydroxy Toluene (BHT).The reducing power of the sliver nanoparticles indicates its potential antioxidant activity by reduce ferric salt (Fe^{3+}) to ferrous salt (Fe^{2+}) by electron transfer reaction [25]. Figure 8. Reducing power activity of biosynthesized AgNP of A. indica and A. barbadensis. Values represented as mean ± SD of triplicates.

Antimicrobial activity

The biosynthesized AgNP of A. indica and A. barbadensis extracts exhibited marked anti-microbial activity against all the microorganisms, Among the gram positive bacteria tested in the present experiment *Bacillus cereus*, *Actinomycetes Israeli, and Enterococcus faecalis* was more susceptibility to the extract, where *Staphylococcus aureus, Streptococcus pyogenes, Corynebacterium diphtheria* was moderately susceptibility to the extract. In the gram negative bacteria *Proteus vulgaris* was more susceptibility to the extract, where *Pseudomonas aeruginosa* was moderately susceptibility to the extract. Figure 9a shows antimicrobial activity for Gram positive bacteria of biosynthesized Ag NPs of A. indica and A. barbadensis and Figure 9b shows antimicrobial activity for Gram negative bacteria of biosynthesized Ag NPs of A. indica and A. barbadensis, The zones of inhibition produced by AgNPs against the different eight species are given in the Figure 10

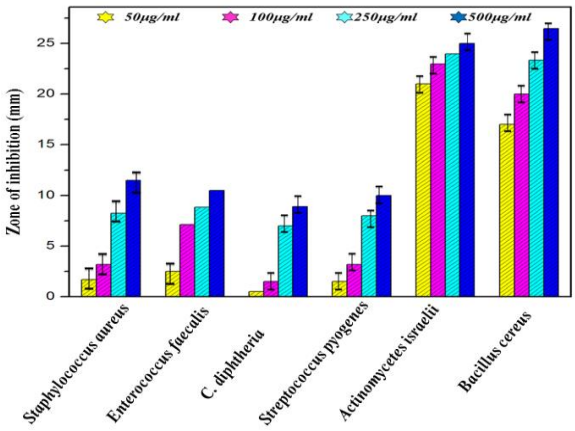


Fig.9a: Antimicrobial activity for Gram positive bacteria of biosynthesized Ag NPs of A. indica and A. barbadensis

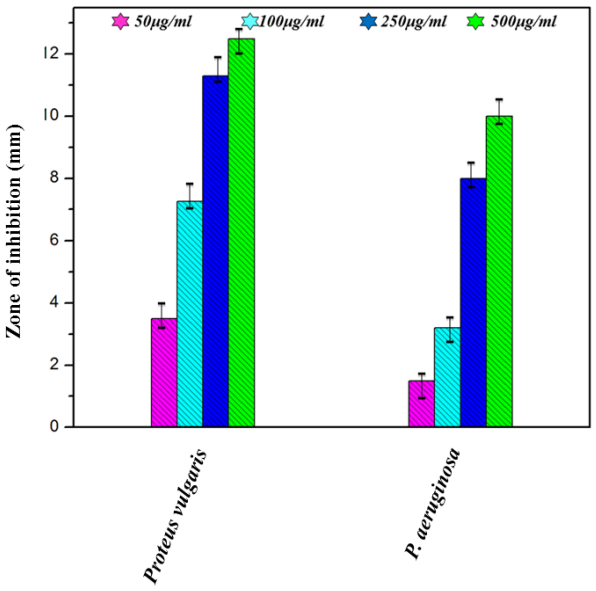


Fig .9b: Antimicrobial activity for Gram negative bacteria of biosynthesized Ag NPs of A. indica and A. barbadensis

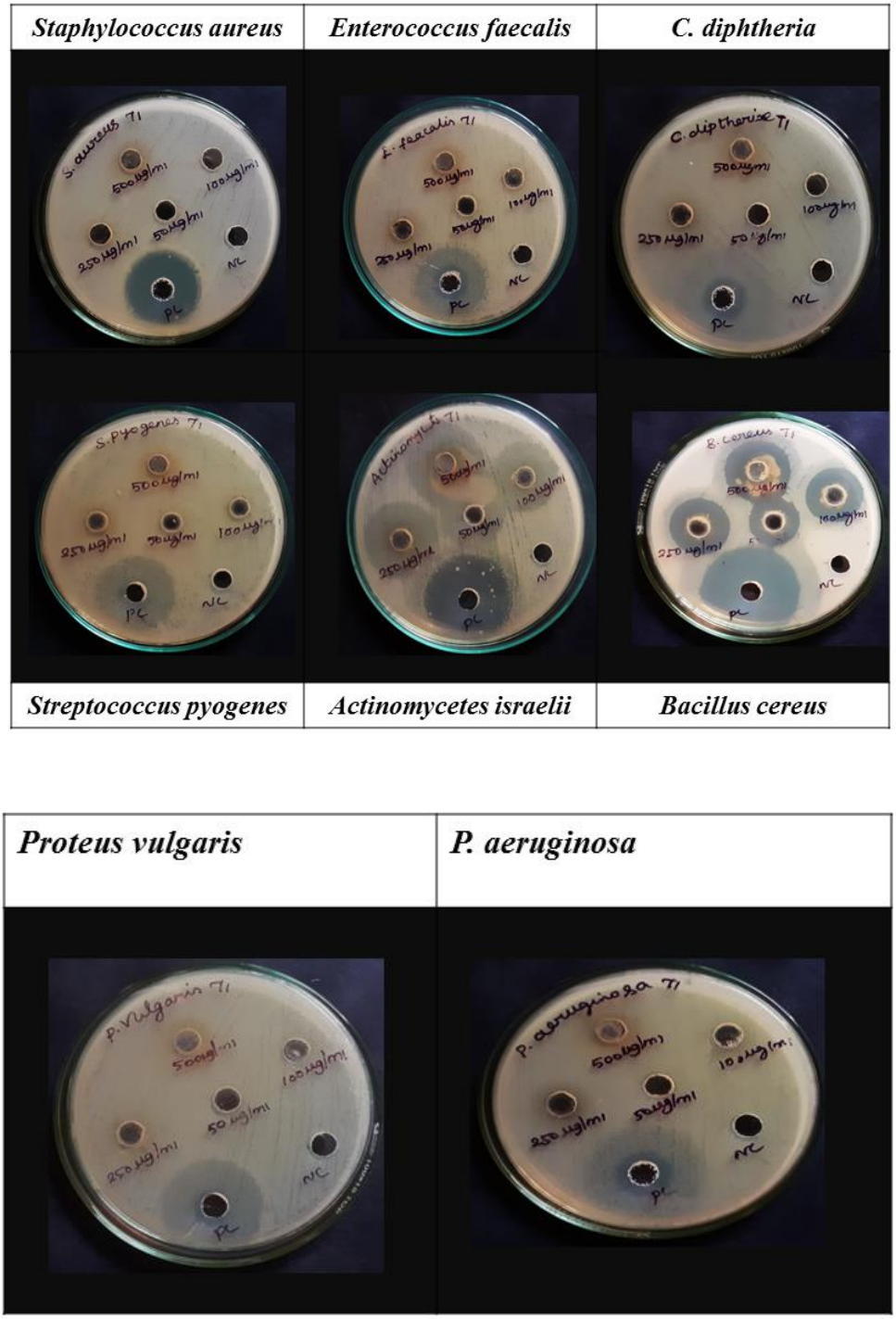


Fig.10: Zone of Inhibition of biosynthesized Ag NPs of A. indica and A. barbadensis

CONCULSION

Biosynthesized AgNP from A. indica and A. Barbadensis were evaluated for in vitro anti-inflammatory and antioxidant and antimicrobial properties. Suppression of albumin denaturation, reduction of free radical in DPPH assay, ABTS radical scavenging test, and Potassium Ferricyanide reducing power assay in vitro indicated that the bio generated AgNP of A. indica and A. Barbadensis possesses anti-inflammatory and antioxidant properties. The antibacterial activity showed efficient inhibition on both gram-negative and gram-positive bacteria. Based on these results, we can conclude

that silver nanoparticles synthesized from A. indica and A. Barbadensis have a faster rate of synthesis, higher yield with a smaller size, high antibacterial activity against both gram-negative and gram-positive bacteria, efficient antioxidant and anti inflammatory activity which indicates the bio synthesized AgNP have potent biological activity. In present study we found that combined leaf extract of Azadirachta Indica & Aloe Barbadensis act as a best source for the formation of Silver Nanoparticles which show the dose dependent activity. The green synthesized combined leaf extract of Azadirachta Indica & Aloe BarbadensisAgNP shows excellent anti- inflammatory, antioxidant and antimicrobial activity when compared with individuals. It confirmed that this synthesis method was simple, fast, eco-friendly, and nontoxic methods. Our findings suggest that biosynthesized silver nanoparticles derived from plants will provide unique prospects for the advancement of Nano medicine, and our bio AgNP have the potential to evolve into pharmaceutical goods for biomedical purposes.

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