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Synthesis and Characterization of Silver Nano Particles from Aqueous Leaf Extract of *Diospyrosebenum* (J.Koeniig) along with the evaluation of its Biopotency

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Abstract:

In the present study, the silver nanoparticles (AgNPs) were synthesized from aqueous leaf extract of *Diospyrosebenum* and characterized by UV–visible spectroscopy, FT-IR, SEM, TEM, EDX analysis. The results proved that the biosynthesized nanoparticles were uniformly dispersed, spherical shaped with an average particle size of 34 nm and with an atomic silver percentage of 79.13. In three planes, XRD was calculated to investigate the crystallinity and consistent spherical size (peaks at 2θ values of at 39.3^0, 44.5^0, 63.7^0 and 77.9^0) of the particles.The phytochemical and FT-IR analysis showed the presence of phenols, terpenoids and alkaloids in the synthesized AgNPs. The synthesized AgNPs exhibited significant free radical scavenging activity.Antibacterial properties of AgNPs were evaluated against clinically significant multidrug-resistant strains of two gram positive bacteria (*Bacillus subtilis*, *Bacillus cereus*), two-gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). AgNPs indicated significant antibacterial activity against pathogens tested.Significant antioxidant activity with an IC50 value of 72.66 µg/mL which was very close to standard (IC50 is 62.49 µg/mL). The synthesised AgNPs show remarkable anti-inflammatory and anti-diabetic activity. As a consequence, biosynthesized

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*Diospyrosebenum*extract mediated AgNPs shown outstanding effects and have the potential to be used as antibacterial, antioxidant, anti-inflammatory and anti-bacterial agents.

Key words:*Diospyrosebenum*, Green synthesis, Silver nanoparticles, antimicrobial activity, antioxidant activity, anti-inflammatory activity

Introduction:

Nanotechnology is the synthesis of particles with at least one dimension in 1-100 nm size range, resulting in high surface to volume ratios. As the particle size decreases, not only does the ratio of surface area to volume increase but also the physical, chemical and biological properties of the particles differ compared to their bulk counterparts [1]. Noble-metal nanoparticles exhibit incredible physicochemical, optoelectronic and biochemical characteristics. They are being used for various purposes in industrial and pharmaceutical applications [2]. Despite the existence of numerous metals in nature, only a few of them such as gold, silver, palladium and platinum are synthesized extensively in nanostructured form [3]. Among the above-mentioned metals, silver nanoparticles have attracted much attention due to their unique characteristics for utilizing in various applications including pharmaceutics, agriculture, water detoxification, air filtration, textile industries and as a catalyst in oxidization reactions [4]. Furthermore, their predominant property is their high antibacterial activity against a broad range of bacteria without any toxicity to animal cells [5].

Nanoparticles are traditionally synthesized broadly by physical and chemical procedures. Chemically prepared nanoparticles are not appropriate for medical usages due to hazardous chemicals binding on their surface. Furthermore, by-products produced in chemical routes are toxic for the environment. Physical routes for synthesis of NPs have some drawbacks, too. These methods require high energy and space, and are expensive [6]. Using biological systems such as microorganisms, plants, viruses and animal cell cultures is an alternative procedure for preparation of NPs [7].

Biosynthesis of NPs is eco-friendly, time affordable, cost effective. More importantly, the biosynthesized NPs are free of hazardous material on their surface. Also, they may be coated with bioorganic compounds that make them proper for medical applications. These give biosynthesis of NPs distinct advantages over conventional methods [8]. For example, NPs are used as a new tool for targeting in cancer therapy and the most important difficulty is toxicity

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of NPs synthesized by previous methods. Scientists overcome this issue using biologically synthesized NPs coated with biomolecules that are more biocompatible [9].

Although the exact mechanism of NPs biosynthesis by various plant extracts is ambiguous, it has been revealed that the biomolecules in plant extract such as protein, phenol and flavonoids play a significant role in the reduction of metals ions and capping the biosynthesized nanoparticles [10]. Culturing plants in controlled environmental systems presents an approach for the production of specific bioactive molecules in plants. Since these plants are not exposed to environmental turbulence, the metabolic production of these plants is not dependent on environmental changes [11].

Medicinal plants have served as rich sources of pharmacologically active substances. Herbs have been used in a diverse array of purposes, including medicine, nutrition, flavoring, dying, repellents, fragrances, cosmetic, charms, smoking and industrial uses. Today, herbs are still found in 40% of prescription drugs [12]. The genus *Diospyros* from Ebenaceaecontains 500 species of trees and shrubs and most of which are native to the tropics. Some members of the genus are valuable for their timber, particularly several species of ebony. Others are cultivated for their handsome foliage or edible fruit. *Diospyrosebenum* (J.Koenig) is commonly called as Ceylon ebony is a species of tree in the genus Diospyros and the family Ebenaceae. The tree produces valuable black wood.The edible fruits have medicinal properties as attenuant and lithontripic. In literature it was identified that that one work reported for the green synthesis of TiO_2 NPs using leaf extract of *Diospyrosebenum* and reported its antibacterial and photocatalytic degradation of crystal violet [13]. No other work reported for green synthesis of nanoparticles using *D ebenum* and hence in this work, we report different pharmacological activities of AgNPs synthesized by a green method using a *D ebenum* extract. Morphology, size, elemental analysis and electron diffraction pattern of nanoparticles were characterized.

Materials and Methods:

Reagents and Chemicals:

The LR grade chemicals like silver nitrate ($AgNO_3$), methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), dinitrosalicylic acid, α-amylase along with chemicals used for preparing microbial growth media were purchased from Sigma-Aldrich and were used without further purification. The standard compounds used in the study such as ascorbic acid, acarbose,

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diclofenac sodium and Gentamycin were procured from PiramalPharma Limited, Ennore, Chennai.

Collection, storage and extraction methods:

The leaves of *D ebenum*were collected in summer from Addateegala forest, East Godavari district, Andhra Pradesh. Collected leaves were dried up to constant weight in the dark at room temperature. The Leaves of *D ebenum*washed completely with distilled water and kept at ambient temperature for 2 days to dry up and then divided into small pieces and they powdered well with the grinder. To preparation the aqueous extract, 20 g powder of leaves in the form of separation with 250 mL distilled water of sterilizedplaced on the shaker at room temperature for 2 days. After this time, the extracts filtered with Whatman filter paper and in order to perform experiments were stored at 4C° [14].

Qualitative Phytochemical analysis:

The phytochemical evaluation of the aqueous extract prepared for the synthesis of AgNPs was carried as per the standard procedures described by Harborne 1973[15], Erum*et al*., 2015[16] and Rondon*et al*., 2018[17]. The colour change as per the procedure described in each studied test indicate the positive test which confirms the presence of the studied compound in the extract and no change in colour indicates negative test and confirms the absence of the studied compound in the extracts.

Green synthesis of silver nanoparticles:

The aqueous leaf extract of *D ebenum* mediated AgNPs were synthesised using silver nitrate as metal precursor and three concentrations of metal solution was utilized for synthesis. The volume of 50 mL of $AgNO_3$at selected concentration levels i.e 1 M, 0.1 M and 0.01 M was added separately to 50 mL of *D ebenum*aqueous extract (ratio 1:1) and incubated at ambient conditions for 2 min. Then the solutions were exposed under white light during 15, 30 and 60 min. The synthesis progress was monitored using UV–Visible spectrophotometer (JASCO, Japan) with a wavelength range from 800 to 300 with a resolution of 1 nm. The NPs formed in the reaction mixture was collected by centrifugation followed by heated at 80 °C for 8 h in a hot air oven. The resulting powder was rinsed multiple times with ethanol as well as distilled water. The obtained powder was dried and calcinated for 3 h at 350 ^{0}C in a muffle furnace. The resulting powder is a grounder and preserved as AgNPs and is used for characterization and activity studies [14].

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Characterization of synthesised AgNPs:

The expected crystal structure of the AgNPs was verified by performing XRD (X-ray diffractometer - Rigaku Corporation, Japan) and the analysis was conducted in the diffraction angle (2θ) scan range of 20 to 80° with a scan speed of 2°/min. The size, surface morphology, and elemental nanoparticle composition were assessed using the FESEM (FE-SEM - Nova, Nanosem-450, FEI, United States) and energy-dispersive X-ray spectroscopy (EDX - RONTEC's, QuanTax 200, Germany). By using FTIR (FT-IR - Bruker, USA) analysis, the functional groups that were derived from the plant extract and that are included in the nanoparticle formation were assessed. The FI-IR study was conducted between 4000 and 500cm^{-1} [18].

Pharmacological activities:

Antioxidant activity:

The DPPH free radical scavenging assay reported by Anand*et al*., 2020 [19] was adopted for evaluating the antioxidant activity of AgNPs synthesised using *D ebenum*aqueous leaf extract.In this, ascorbic acid was utilized as standard for evaluation of DPPH activity and the assay calculated using formula

$$\%\ \text{inhibition} = \frac{A1-A2}{A1} * 100 \qquad \text{- Formula I}$$

where A1 is the absorbance of solution without extract, A2 is the absorbance of solution with sample extract/standard.

Anti-inflammatory activity:

The anti-inflammatory activity of AgNPs synthesised using aqueous leaf extract of *D ebenum* was determined by Inhibition of albumin denaturation assay and assay was performed as per the procedure reported by Govindappa*et al*., 2018 [20]. In this, diclofenac sodium was utilized as standard and the assay calculated using formulaI described above.

Anti-diabetic activity:

The anti-diabetic activity of AgNPs synthesised using aqueous leaf extract of *D ebenum* was determined by α-Amylase inhibition assay and assay was performed as per the procedure reported by Govindappa*et al*., 2018 [20]. In this, Acarbose was utilized as standard and the assay calculated using formula I described above.

Antimicrobial activity:

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The antibacterial potential of the synthesized AgNPs was evaluated using the agar well diffusion method reported by Pawar JS and Patil 2020 [21]. In this study, two gram positive bacteria namely *Bacillus subtilis* (MTCC – 1427) and *Bacillus cereus* (MTCC – 430), two-gram negative bacteria namely *Escherichia coli* (MTCC – 294) and *Pseudomonas aeruginosa* (MTCC – 1748) were selected. Gentamycin and distilled water were considered as positive and negative controls respectively and the results were expressed as millimetre (mm) of zone of growth inhibition observed for the studied concentration of the sample.

Results and Discussion:

The preliminary phytochemical analysis of the aqueous leaf extract of *D ebenum* shows positive test for major phytochemicals such as flavonoids, alkaloids, terpenoids and phenolic compounds. The leaf aqueous extract of *D ebenum* was used for the synthesis of AgNPs.The identification of colour change in the reaction mixture may be due to surface plasmon vibrations with the silver nanoparticles which confirm the bio-reduction of silver and the formation of AgNPs. The bio-reduction of silver and the formation of AgNPswas checked by using UV-visible spectrophotometer. The UV-visible absorbance spectra shows wavelength maxima cantered near 405 nm (Figure 1) indicating the reduction of silver nitarte to AgNPs. The similar type of UV absorption peak was reported in the literature and provides correlation with the present study [22,23].

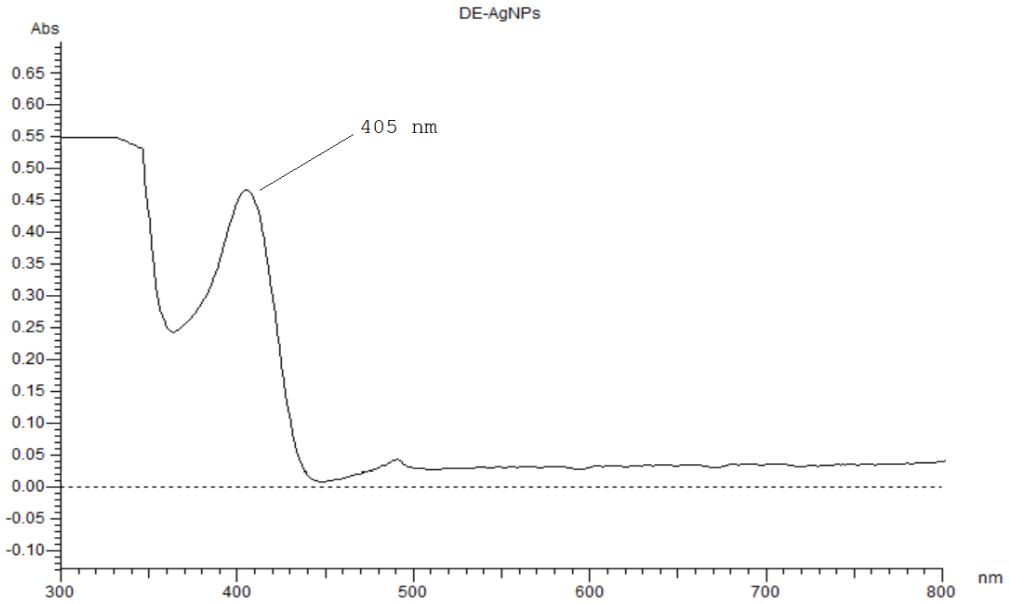

Figure 1: UV-visible absorption of spectra of AgNPs

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The surface analysis of AgNPs synthesized using *D ebenum*as bio-stabilizing agent gives an idea about the involvement of biomolecules that are responsible for the reduction and the capping of Nano composites. The FT-IR spectra of the AgNPs (Figure 2) shows absorption peaks due to bio molecules present in the plant extract and reflects the complex nature of the NPs. The FT-IR spectra shows peak at 3397 cm^{-1} and 2930 cm^{-1} corresponds toN-H stretching in aliphatic primary amine and amine salt respectively. Band at 2877 cm^{-1} and 2975 cm^{-1} corresponds toO-H stretching in carboxylic acid and alcohol respectively. Intense band at 1740 cm^{-1} represents C-H bending in aromatic compound and 1324 cm^{-1} represents C-N stretching in aromatic amine. Results of FT-IR analysis of AgNPs confirms that flavonoids, phenolic compounds, terpenoidsand alkaloids are the chemical constitutes shows positive in the preliminary screening of the aqueous extract of *D ebenum*may be acts as reducing, stabilizing and dispersing agent for AgNPs formation.

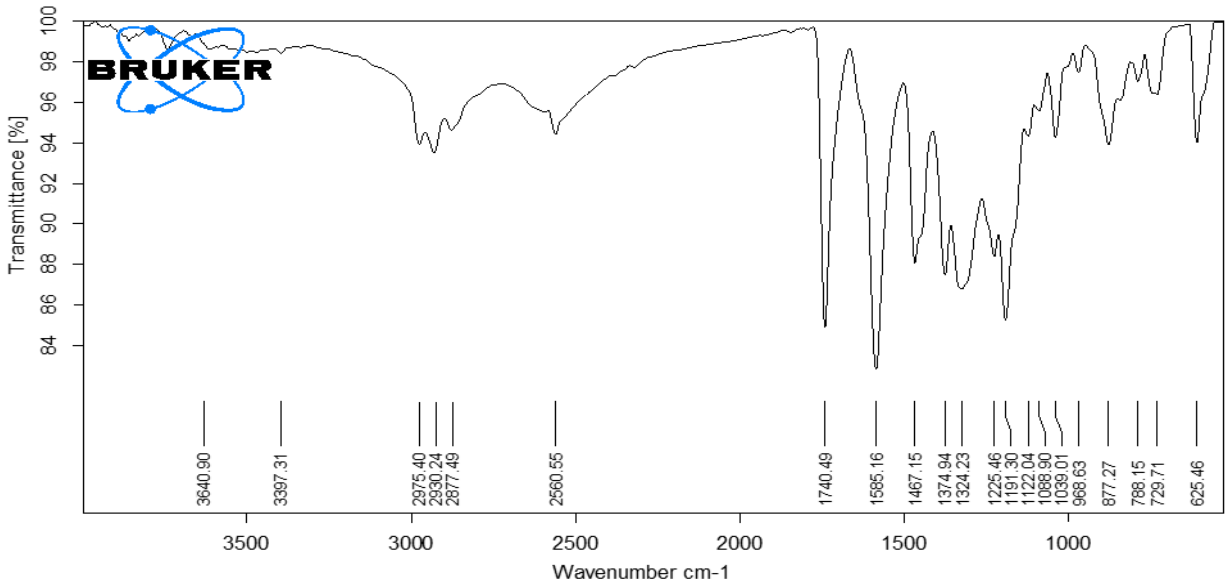

Figure 2: FT-IR analysis spectrum of AgNPs

The micrograph observed in SEM analysis of synthesized AgNPs is shown in figure 3. The SEM micrograph shows agglomerations of the NPs and the shape of the NPs was observed to be circular with rough surface morphology and dispersed as clusters. The size of the obtained AgNPs was in 15-50 nm size range.

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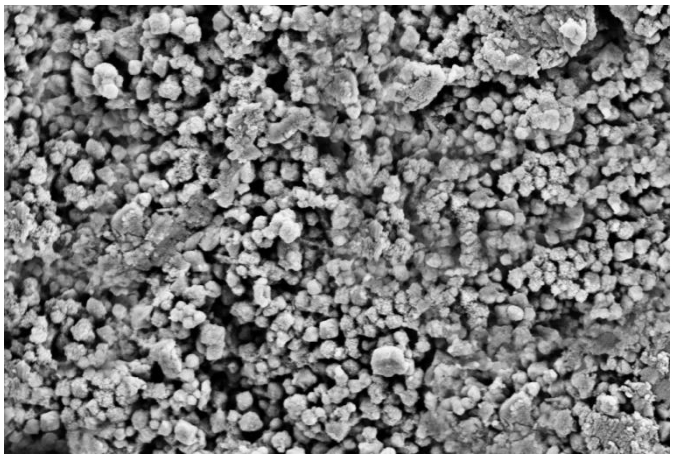

Figure 3: SEM photo of AgNPs.

The elemental composition of *D ebenum*extract mediated AgNPs was confirmed by EDX analysis and the spectrum was given in figure 4. The EDX study of the chemical composition of AgNPs confirms that Ag was observed at an atomic percentage of 79.13 %, the other elements carbon and oxygen were detected in the EDX spectra with an atomic percentage of 16.85 and 4.02 % respectively suggest that the NPs are formed with Ag. The presence of carbon and oxygen in the NPs is originated from the bio-active chemical constituents present in the aqueous extract of *D ebenum*. The atomic % of silver was found to be high. Oxygen composition was low than the carbon. There is no detection of other elements in the EDX spectra suggest that no impurities were present in the formed NPs.

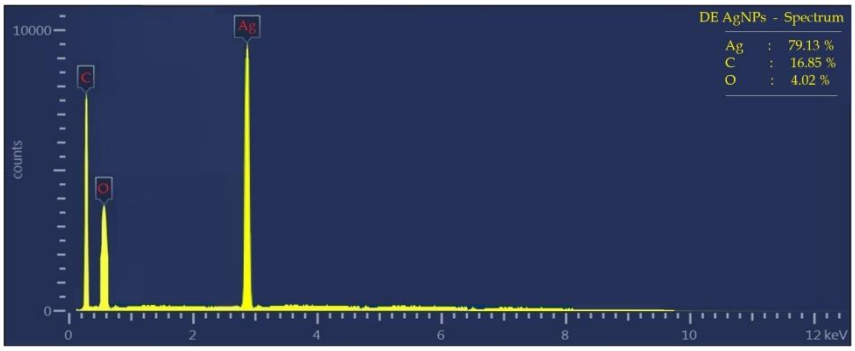

Figure 4: EDX profile of AgNPS

The crystalline nature and phase orientation of the synthesized AgNPs was determined using XRD studies. The XRD pattern shows the peaks position with 2θ values of 39.3^0, 44.5^0, 63.7^0 and 77.9^0(Figure 5) are indexed as (111), (200), (220) and (311) planes corresponds to (111), (200), (220) and (311) planes which confirm the formation of well face-cantered-cubiccrystalline silver and it is well evident with the “Joint Committee on Powder Diffraction Standards” (#21-1272). Scherrer's equation was used to compute the

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nanoparticles' size of the particles, and it was determined to be 26 nm. In the XRD spectra, no peaks corresponding to other phases was detected confirms that AgNPs were pure and other phases were involved in the structure.

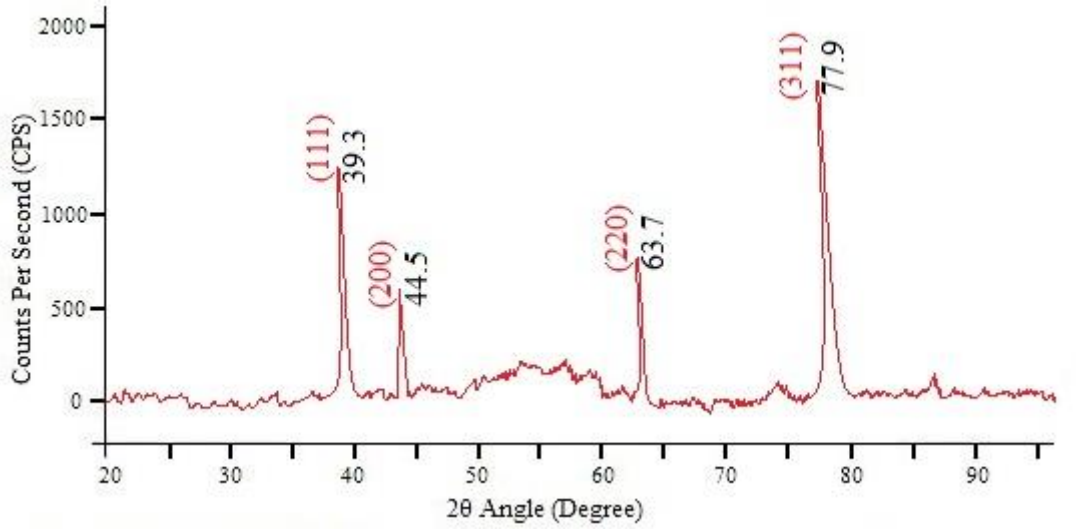

Figure 5: XRD spectra of CuO NPs

The TEM analysis was performed to evaluate the surface morphology of the produced AgNPs. The TEM analysis confirms that the particles were uniform in shape and shape of most of the particles was confirmed to be round. The clear observation of the surfaces of the particles confirms that the particles have smooth surfaces. The collected particles were determined to have an average size of 34 nm by calculation and confirmation. The TEM report was depicted in figure 6.

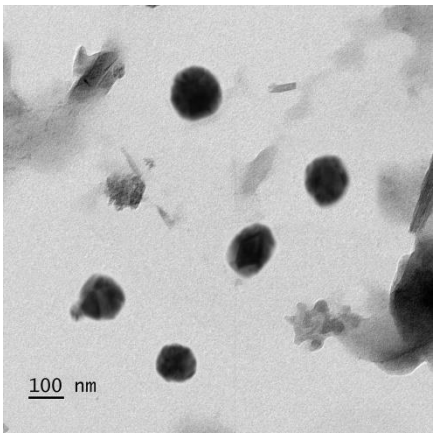

Figure 6: TEM analysis image of AgNPs

Pharmacological activities of synthesised AgNPs

Antioxidant activity:

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The antioxidant activity of the green synthesized AgNPs was evaluated using DPPH radical scavenging assay. The DPPH scavenging activity of AgNPs using ascorbic acid as standard antioxidant is shown in Figure 7. As reflected from the figure, the antioxidant properties of the samples increased with increasing concentrations of AgNPs in the range 10–100 µg/mL resulting in increase in percentage DPPH radical scavenging abilities. A dose dependent pattern was observed in the scavenging activity, which increased with increase in dose of the AgNPs. Nevertheless, the scavenging potential of the AgNPs was comparatively lower than that of the standard but significantly higher than aqueous leaf extract. The IC50 was noticed to be 62.49µg/mL, 72.66µg/mL and 142.92µg/mL respectively for standard, AgNPs and aqueous leaf extract.

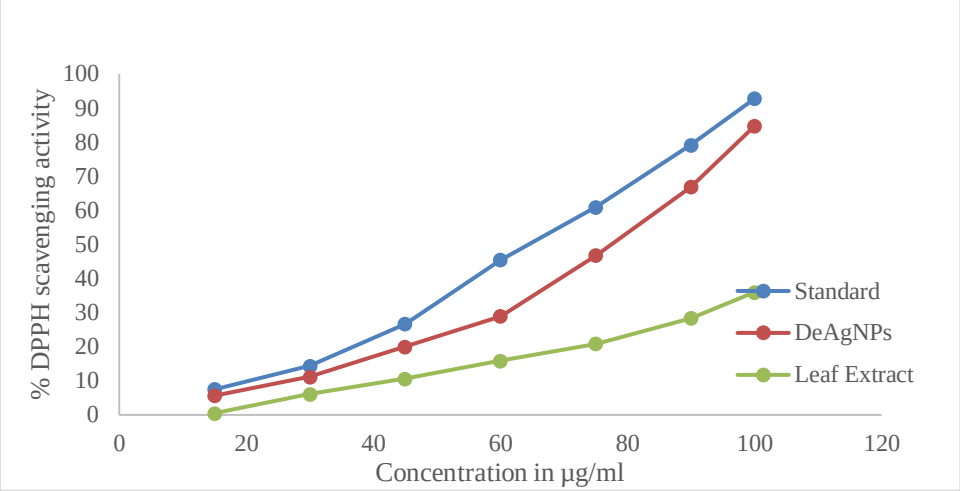

Figure 7: Antioxidant activity results

Anti-inflammatory activity:

This study finding exhibited a concentration dependent inhibition of protein (albumin) denaturation by the *D ebenum*leaves extract and AgNPs. The lowest activity of *D ebenum*leaves extract, AgNPs and Diclofenac sodium were 24.15 %, 16.02 % and 0.38 % in theconcentration of 20µg/mL respectively, while the highest activity of *D ebenum*leaves extract, AgNPs and Diclofenac sodium were 89.05 %, 64.45 % and 25.44 % inthe concentration of 120µg/mL respectively. The half inhibition concentration (IC50) of *D ebenum*leaves extract, AgNPs and diclofenacsodium were 52.46µg/mL, 87.21µg/mL and 222.18µg/mL respectively. From the present study it can be confirmed that AgNPs showed marked in vitro anti-inflammatory effect against the denaturation of protein (Figure 8)

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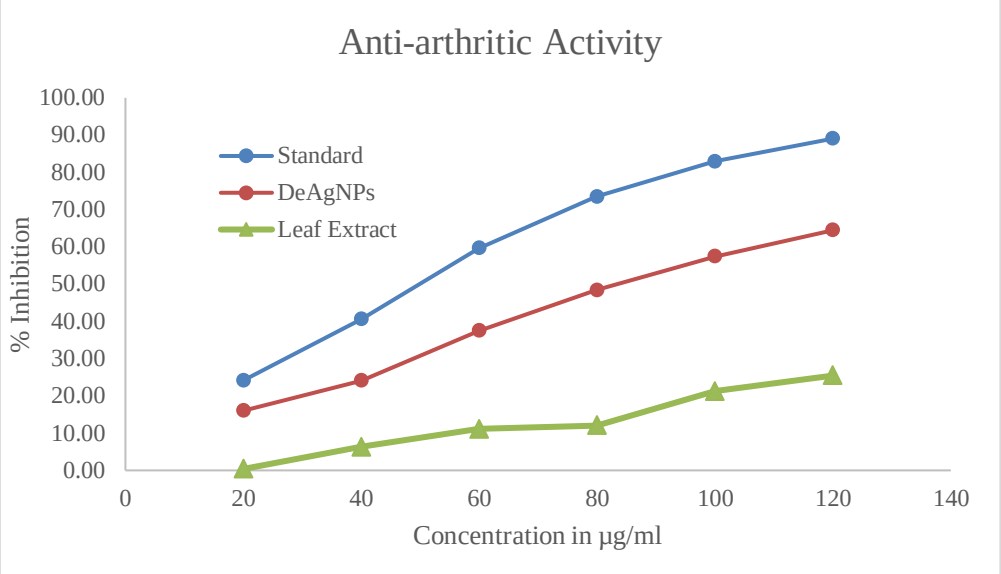

Figure 8:Anti-inflammatory activity results

Anti-diabetic activity:

The DPPH radical scavenging activity of AgNPs was displayed in Figure 9. The results confirmed that the synthesized AgNPs are free radical scavengers. As the concentration increased, the antioxidant activity increased in a dose-dependent manner. The AgNPs at concentrations ranging from 20 to 180 µg/mL exhibited the antioxidant activity of 10.08 % to 74.50 % with an average IC50 value of 124.45 µg/mL. However, the AgNPs exhibited lower scavenging activity of DPPH than the standard ascorbic acid (IC50 value 88.31 µg/mL) whereas significant high activity than aqueous leaf extract of *D ebenum*(IC50 value 250.25µg/mL)

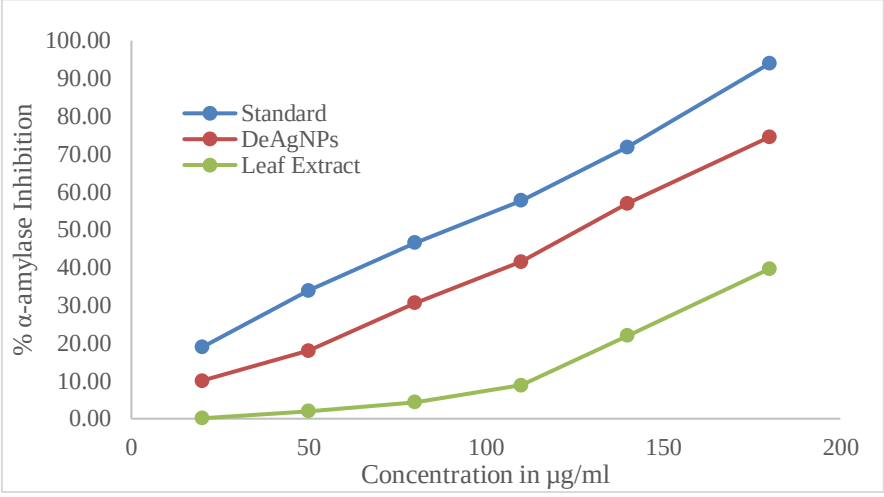

Figure 9:Anti-diabetic activity results

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Anti-bacterial activity study:

The antibacterial activities of the biofabricatedAgNPs by *D ebenum*leaf against two gram positive bacteria namely *Bacillus subtilis* and *Bacillus cereus*, two-gram negative bacteria namely *Escherichia coli* and *Pseudomonas aeruginosa*. Figures 10 present the comparative bacterial activity results whereas figure 11 show the inhibition zone of the biosynthesised AgNPs against the bacterial strains studied. It is clear from the obtained results that as the concentration of the biosynthesised AgNPs increased, the zone of inhibition increased gradually and besides, the biosynthesised AgNPs exhibited more excellent antibacterial property against two-gram negative bacteria. The potent antibacterial properties of AgNPs may be attributed to the released silver ions, which could have an interaction with microorganisms by means of their attaching to the surface of the cell membranes of bacteria, penetrating into the bacterial cells, and affecting the membrane permeability and respiration. In the bacterial cells, AgNPs could even interact with sulfur- and phosphorus-containing compounds like DNA to give rise to the deadly impairment of microorganisms

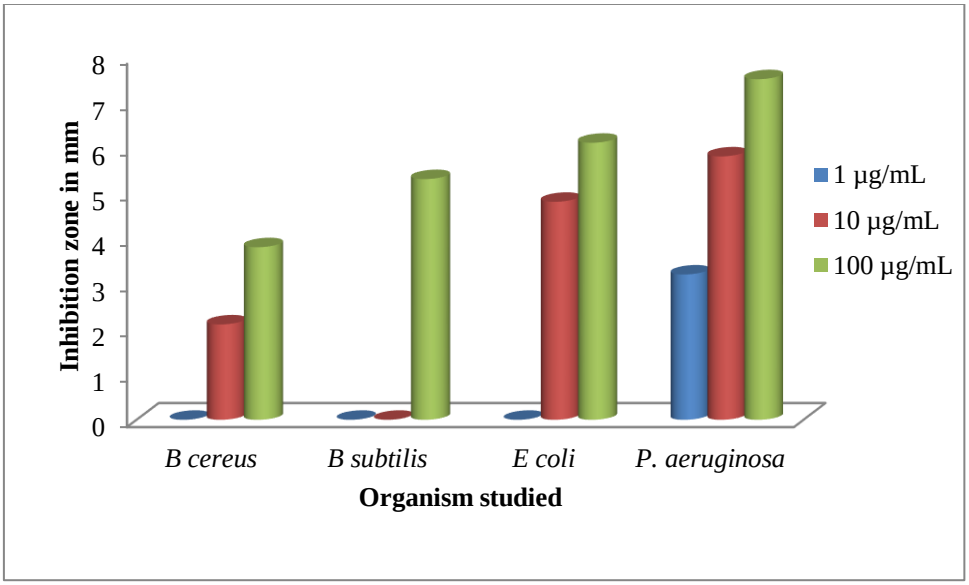

Figure 10: Anti-bacterial growth inhibition study comparison results

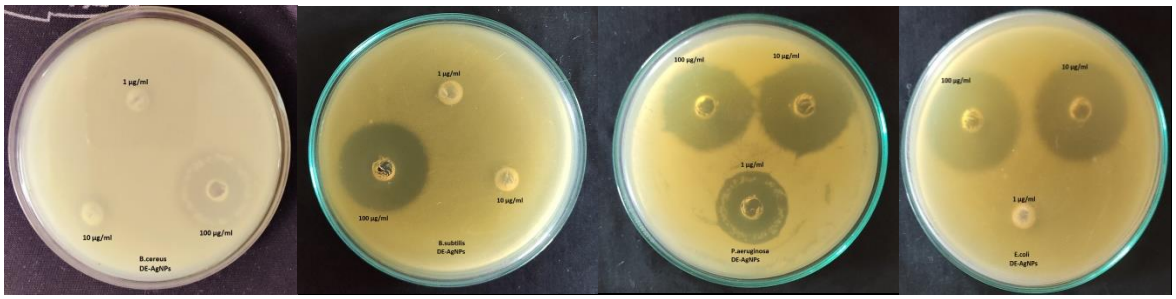

Figure 11: Agar plate well diffusion study results

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Conclusion:

The silver nanoparticleshave been successfully synthesized from the aqueous extract of *D ebenum* and were characterized using standard analytical procedures. The synthesized AgNPs were uniformly dispersed, spherical in shape, and crystalline in nature with the nanometer size range. The presence of functional groups corresponds to phenols, terpenoids and alkaloids were detected in the synthesized AgNPs. The biosynthesized AgNPs are the best free radical scavenger and showed higher levels of antioxidants activity. Moreover, the synthesized AgNPsshow enhanced the anti-inflammatory and anti-bacterial activity. Hence, it can be concluded that NPs synthesised in this study have the potential to be developed as promising pharmacological agent for antibacterial, anti-inflammatory, anti-diabetic and antioxidant agents. However, further in vivo studies were required to confirm its applications as safe therapeutic agent in the current era of developing safe medicine.

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