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Green Synthesis and Evaluation of Pharmacological activities of Silver Nano particles Synthesised from Aqueous Leaf Extract of *Diospyroschloroxylon* (Roxb.)

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Abstract:

In recent years, biological precursor-based synthesis, particularly plant-based green synthesis, has drawn prime attention in the area of nanotechnology. The goal of this work is to look into the antibacterial, antioxidant, anti-diabetic and anti-inflammatory activity of silver nanoparticles (AgNPs) made from aqueous leaf extract of *Diospyroschloroxylon* (Roxb).This study can be broadly separated into three parts: the first extraction from *D chloroxylon*leaves, second characterization of AgNPsand third analysis of AgNPspharmacological properties. UV–Vis absorption indicated the synthesis of AgNPs, and the spectrum was seen at 411 nm. The existence of functional groups in the produced AgNPs was verified by FT-IR spectral peaks that conformvarious bio-active functional groups such as alcohol, carboxylic acids, aldehyde and secondary amines including aromatic skeleton. The NPs were characterized to be in rod shape with an average particle size of 26 nm and face-centred-cubic crystal having 75.81 % metallic silver. The results of pharmacological activities of synthesized AgNPs with *D chloroxylon*leaf extract have significant antimicrobial, antioxidant (IC50 of74.95µg/mL), anti-inflammatory(94.71µg/mL) and anti-diabetic (124.47µg/mL) activities. As a consequence, biosynthesized *Diospyroschloroxylon* (Roxb.) aqueous leaf extract mediated

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AgNPs shown outstanding pharmacological properties and have potential application in the fields of medical and pharmaceuticals for formulation of new drugs.

Key words:*Diospyroschloroxylon* (Roxb.), Green synthesis, Silver nanoparticles, pharmacological activities

Introduction:

The field of material sciences encourages obtaining materials of various types of nanoscale shapes and architectures.NPs with a size range of 1–100 nm, and different shapes provide unique chemical, physicaland optical properties. NPs can be synthesized with physical, chemical and biological methods [1]. These methods might have unique advantages and disadvantages depending on the end application. For example, physical methods might have some disadvantages when applied in microbiology. The methods can be time-consuming and constrain to specific requirements like high temperature or pressure, which might result unattractive owing to equipment and associated cost [2,3].A key advantage of chemical methods is the accessibility to get the NPs in suspension. After synthesis and purification, the NPs can immediately be accessible for functionality testing. However, in some cases, the synthesis procedure might result expensive owing to the material type used. For chemical synthesis methods applied in microbiology, the most critical point is the toxic effect of the NPs or by-products generated, especially when released to the environment [4]. On the contrary, biological methods (e.g., plant extract) utilize fewer toxic reactants and additives. The reaction can occur at room temperature without harsh or stringent reaction constraints. Plant extract can then provide low or no cytotoxicity when combined with NPs. Therefore, biological methods using plant extracts could be catalogued safe, eco-friendly and low-cost, representing a viable alternative for the production NPs [5,6].

Among various biosynthesized metal nanoparticles, silver nanoparticles (AgNPs) have emerged as the champion in the last two decades due to their unique biological, chemical, and physical properties [7] Although silver is toxic at higher concentrations, many studies have established that a lower concentration of $AgNO_3$ has higher chemical stability, catalytic activity, biocompatibility, and intrinsic therapeutic potential [8]. Silver nanoparticles are reported to have potential anticancer and antimicrobial activity. In fact, the slow and regulated release of silver from silver nanoparticles is one of the most striking advantages of

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these nanoparticles when compared with bulk metals and their salts. A combination of nanotechnology and traditional medicine is the mantra of the new-age bio-nanoformulations [9].

Diospyroschloroxylon (Green ebony persimmon) belongs to the family Ebenaceae and is a wild fruit-bearing plant indigenous to the Indian subcontinent. The unripe and ripe fruits have been eaten by tribal people. *Diospyroschloroxylon* is a small tree, with bark dark brown; the wood is yellowish-grey which is used in the preparation of agricultural implements and musical instruments. The leaves of this plant are used as fodder. The unripe and ripe fruits are globose, the size of a cherry, purplish when ripe, are eaten, and are very palatable. In India, *Diospyroschloroxylon* is naturally distributed in Maharashtra, Andhra Pradesh, Karnataka, and Tamil Nadu states.

In literature it was noticed that one work reported for the green synthesis of AgNPs using ripened fruit pulp of *Dchloroxylon*. No other work reported for green synthesis of nanoparticles using *Dchloroxylon* and hence in this work, we report different pharmacological activities of AgNPs synthesized by a green method using a *Diospyroschloroxylon*extract.Morphology, size, elemental analysis and electron diffraction pattern of nanoparticles were characterized.

Materials and Methods:

Materials and Chemicals:

The leaves of *Dchloroxylon* 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 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, diclofenac sodium and Gentamycin were procured from PiramalPharma Limited, Ennore, Chennai.

Preparation of aqueous leaf extractof *D chloroxylon*:

*Dchloroxylon*aqueous leaf extract was made using dried leaves. The leaves were powdered using a mortar and pestle to a uniform size and an accurately weighed 20 grams of leaves powder was boiled for 20 minutes in 250 mL of double distilled water at 60°C. Extracts were

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then filtered through nylon mesh and a hydrophilic 0.2 μmillipore filter to remove impurities. The *Dchloroxylon*aqueous leaf extract mediated AgNPs were synthesized using the extract produced [11].

Green synthesis of silver nanoparticles:

AgNPs were synthesised by reducing a silver nitrate solution at three concentrations of silver nitrate in the presence of the *Dchloroxylon*extract. A volume of 50 mL of $AgNO_3$ was added to 50 mL of *Dchloroxylon*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 [11].

Characterization of synthesised AgNPs:

The optical characterization of the synthesized AgNPs was done using a UV-visible spectrophotometer (JASCO, Japan). The NPs were dispersed in aqueous solution and the solution was scanned at a wavelength of 800-300 nm against distilled water as blank. The wavelength maximum of the UV- visible absorption spectra was evaluated and was compared with the existing literature. The functional groups of the plant biomolecules included in the biosynthesis of AgNPs were evaluated using an FT-IR spectrophotometer (Bruker, USA). The synthesized NPs pellet was prepared with KBr and the prepared sample was scanned using FT-IR in the scan range of 4000-500 cm^{-1}. The absorption bands observed in the FT-IR spectrum was compared with the standard FT-IR table and functional groups that involved in the formation of NPs was evaluated. A Field emission SEM (Nova Nanosem450, FEI, United States) was employed to assess the morphology as well as size of the formed AgNPs. An X-ray diffractometer (Rigaku Corporation) has been used to evaluate the crystalline nature and lattice composition of the synthesized AgNPs at a scanning rate of 2^{0}/min in the diffraction angles (2θ) from 20^{0}-80^{0}. Energy-dispersive X-ray spectroscopy (“RONTEC's EDX

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equipment, Model QuanTax 200, Germany") has been utilized to access the elemental structure of the synthesized AgNPs.

Evaluation of pharmacological activities of synthesised AgNPs

Antioxidant activity:

The antioxidant activity of AgNPs synthesised using aqueous leaf extract of *Dchloroxylon*was determined by DPPH free radical scavenging assay and assay was performed as per the procedure reported by Anand*et al*., 2020 [12]. In this, ascorbic acid was utilized as standard for evaluation of DPPH activity and the assay calculated using formula

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

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 activityof AgNPs synthesised using aqueous leaf extract of *Dchloroxylon* was determined by Inhibition of albumin denaturation assay and assay was performed as per the procedure reported by Govindappa*et al*., 2018 [13]. 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 *Dchloroxylon* was determined by α-Amylase inhibition assayand assay was performed as per the procedure reported by Govindappa*et al*., 2018 [13]. In this, Acarbose was utilized as standard and the assay calculated using formula I described above.

Antimicrobial activity:

The antibacterial potential of the synthesized AgNPs was evaluated using the agar well diffusion method reported by PawarJS and Patil 2020 [14]. 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:

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The beneficial aspect of the nanotechnology field was shifted rapidly from laboratory to large scale industrial production and our proposed method provide a green synthetic approach for the synthesis of AgNPs using aqueous leaf extract of *D chloroxylon*as a biological reduction agent. In the process synthesis of AgNPs, the formation of NPs was initiated by the addition of aqueous plant extract and silver metal solution. The initiation of reaction was confirmed by observing the colour change from light yellow to dark brown in the reaction mixture. It is also observed that the intensity of the colour was increased by passing the incubation time confirms that the NPs formation was increased with increase in time. Further the functional group analysis, optical and structural characterization of synthesised NPs was evaluated.

Functional group analysis:

The synthesized AgNPS were analysedby using FT-IR spectroscopy for the evaluation of the possible functional groups of bioactive molecules from the aqueous leaf extract of the *D chloroxylon*that are actively involved in the formation of NPs. The FT-IR transmittance bands were recorded at 3595 cm^{-1} and O-H stretching in alcohol and intermolecular bonded alcohol respectively whereas band at 3249 cm^{-1} represents O-H stretching in carboxylic acids. The presence of aldehyde and secondary amine groups in the synthesised NPs was confirmed by noticing band at 2789 cm^{-1} and 3327 cm^{-1}which represents C-H stretching and N-H stretching in aldehyde and secondary amine respectively. The presence of aromatic skelition in the NPs was confiremed by observing bands at 1673 cm^{-1}, 1587 cm^{-1} and 1225 cm^{-1} which represents C-H bending, C=C stretching and C-N stretching in aromatic compounds. The FT-IR spectrum was presented in figure 1.

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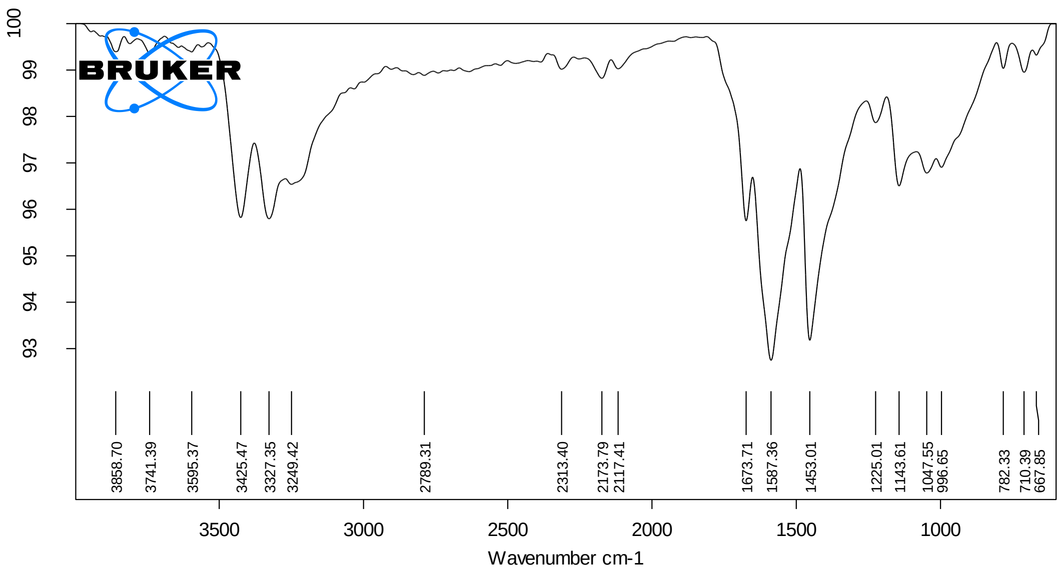

Figure 1: FT IR spectra of AgNPs

Optical absorption study:

The UV-Vis absorption spectroscopy is an important technique to monitor the formation and stability of metal NPs in aqueous solution. The absorption spectrum of metal NPs is sensitive to several factors, including particle size, shape, and particle–particle interaction (agglomeration) with the medium. The optical absorption ability of synthesized AgNPs was determined using a UV visible spectrophotometer and the scanning spectra were given in figure 2. The photocatalytic ability of the synthesized nanoparticles will depend on the light absorption nature of the nanoparticles. The AgNPs show high absorption in the visible region and synthesised AgNPs have a distinctive emission peak at 411 nm. This finding proved that the particles are the nano sized particles that shows the characteristic absorption maxima. A similar UV – visible absorption pattern was observed for the AgNPs reported in literature [15,16].

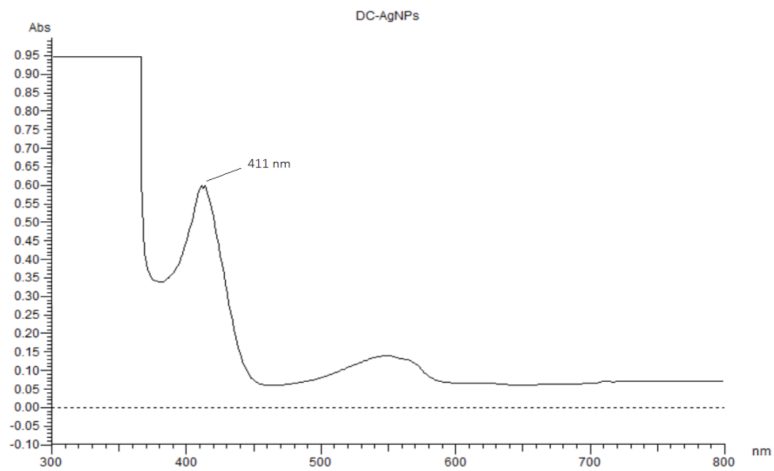

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Figure 2: UV absorption spectra of AgNPs

Structural characterization:

The use of SEM made it possible to provide information on the size and shape of the AgNPs and hence HR-SEM analysis of the synthesized AgNPs was performed to evaluate the size and shape of the NPs. The SEM data demonstrate that the nanoparticles were equally spread on the surface and that aggregate nanoparticles formed. Nanoparticlessize range between 28-46 nm has been seen in the micrograph, which shows the synthesis of AgNPs aggregates. The SEM micrograph of the synthesized AgNPs was given in figure 3

Figure 3: SEM micrograph of AgNPs

The homogeneity of the synthesized AgNPs and its elemental distribution of elements were investigated using EDX spectroscopy and figure 4 shows the EDX spectrum of the AgNPs synthesized using *D chloroxylon*aqueous leaf extract. In the EDX analysis, it generates the X-Ray Spectrum in the scan range and an X-Ray is generated by the element corresponding to its energy level. The EDX spectrum of AgNPs shows high intense peak at Kα of 2.94keV which was attributed to metallic silver confirms the existence of silver in the green synthesized nanoparticles. The peaks corresponding to oxygen (O-Kα) and carbon (C-Kα) were detected in the EDX spectra which correspond to the plant biomolecules which are included in the green synthesis mechanism. The % composition of silver in NPS was found to be 75.81 %. Thus, it can be confirmed that the synthesized NPs having high percentage of elemental silver along with oxygen and carbon.

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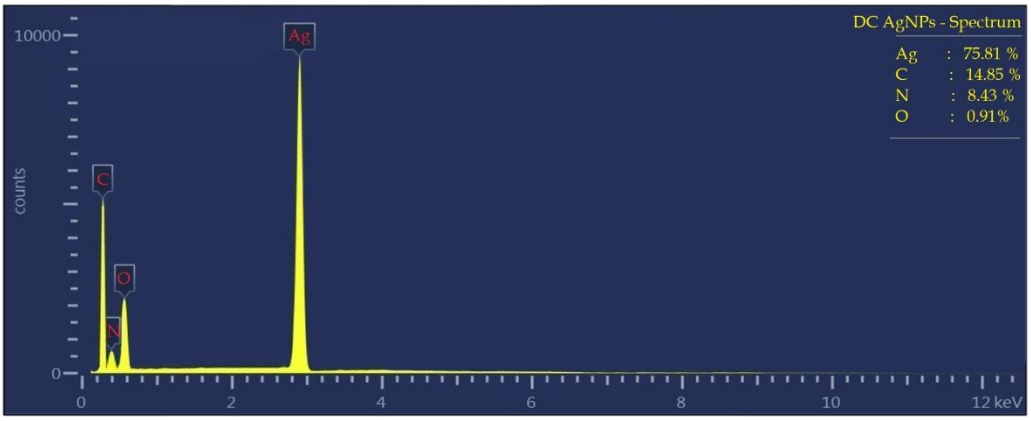

Figure 4: EDX spectra of AgNPs

The surface morphology of the produced AgNPs was evaluated using TEM examination. The TEM analysis confirms that the particles were uniform in shape with fewer agglomerates identified. The shape of most of the particles was confirmed to be rod like shape. 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 26 nm by calculation and confirmation. The TEM report was depicted in figure 5.

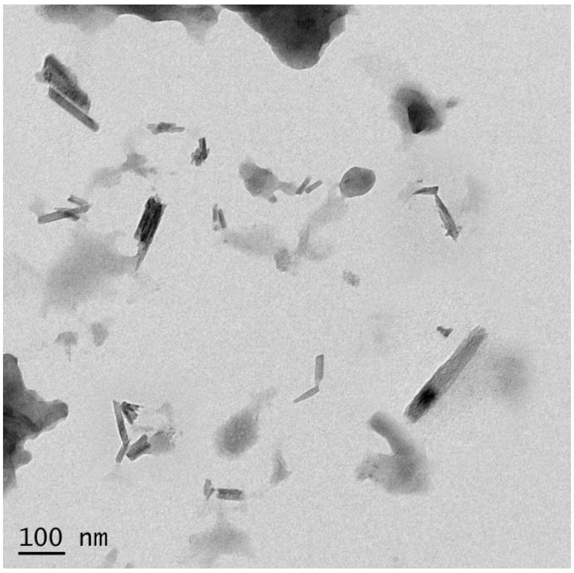

Figure 5: TEM analysis image of AgNPs

Thecrystallinity and phase purity of the synthesized AgNPs was confirmed by XRD studies and the XRD spectrograph observed in the study was given in Figure 6. The XRD patterns of AgNPs shows diffraction peaks at 38.7, 43.9, 64.1 and 77.8 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

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Standards" (# 21-1272). Scherrer's equation was used to compute the nanoparticles' size of the particles, and it was determined to be 26 nm.

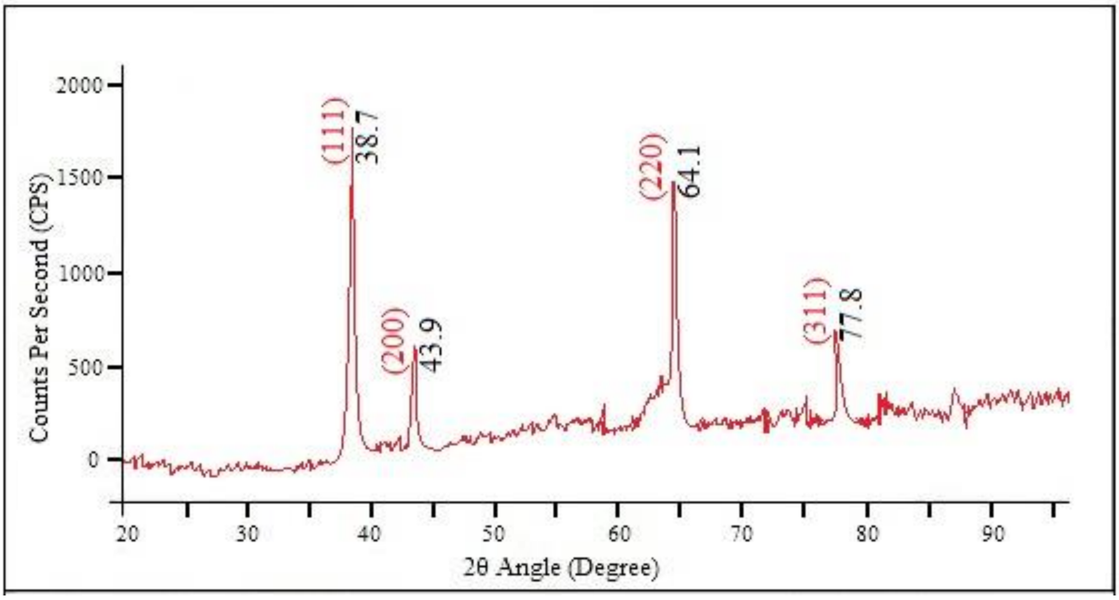

Figure 6: XRD spectra of AgNPs

Pharmacological activities of synthesised AgNPs

Antioxidant activity:

Oxidative stress is playing a major role in the onset of diabetes. Hyperglycemia also stimulates the formation of free radicals which damages the endogenous antioxidant defence system in diabetic patients. So the particles with high antioxidant activity will be a better source for the treatment of diabetes. There areevidences with experimental, clinical, and epidemiological studieswhich showed that antioxidants were useful in the treatment of diabetes and its complications. DPPH assay is one of themost important methods for checking the antioxidant activity of the molecules. The synthesized silver nanoparticles in this study showed a better DPPH scavenging activity and it was dose dependent (Figure 7). The IC50 concentration was noticed to be 62.49µg/mL, 74.95µg/mL and 156.61µg/mL respectively for standard ascorbic acid, AgNPs synthesised and aqueous leaf extract respectively. It showed that the synthesized silvernanoparticles have good antioxidant activity which may be dueto the presence of functional groups from aqueous leaf extract of *D chloroxylon*in the synthesized silver nanoparticles.

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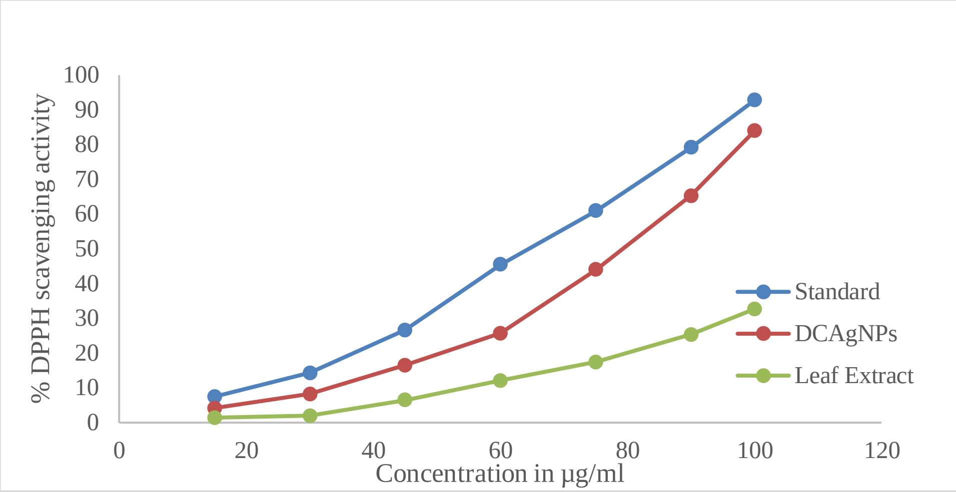

Figure 7: Antioxidant activity results

Anti-inflammatory activity:

Protein denaturation is the well-known reason for inflammatory responses. Denaturation of proteins results in the production of autoantigens which cause serious inflammation in rheumatoid diseases. In the present study,inhibitory effect of biosynthesized AgNP against protein/albumin denaturation was evaluated. These AgNP exhibited significant inhibition of albumin denaturation even at low concentrations as compared to the standard drug, Diclofenac sodium salt (Figure 8). The IC50 values for aqueous leaf extract, AgNP and Diclofenac sodium salt were 160.70µg/mL, 94.71µg/mL and 52.46µg/mL respectively. From the above results, it can be stated that synthesized AgNP are capable of controlling the

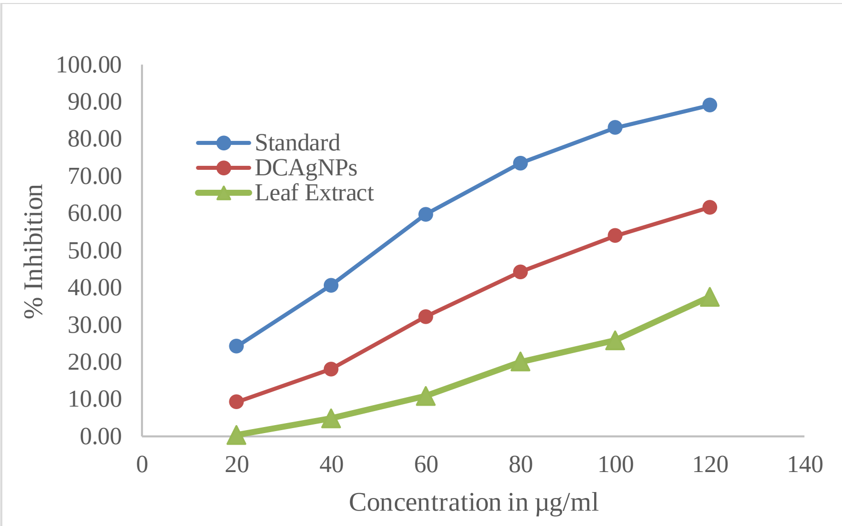

Figure 8:Anti-inflammatory activity results

Anti-diabetic activity:

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Theα-amylase is an important enzyme in carbohydrate metabolism. Inhibition of α-amylase is one of the best approaches toreduce the blood sugar level. Amylase inhibitors or starch blockerswill prevent the absorption of dietary starches by the body. So, itwill reduce the usual rise in blood sugar levels by the consumption of carbohydrates. It was reported that the green synthesizedsilver nanoparticles will be acted as amylase inhibitors for the reduction of blood sugar level. The silver nanoparticles synthesised in this study showed significantly higherlevel of a-amylase inhibition activity than the plant extract in all the tested concentration. The IC50 values for aqueous leaf extract, AgNP and standard were 231.03µg/mL, 124.47µg/mLand 88.31 µg/mL respectively.As the concentration of silvernanoparticles increased, the percentage of inhibition was also increased significantly in a dose dependent manner (Figure 9).

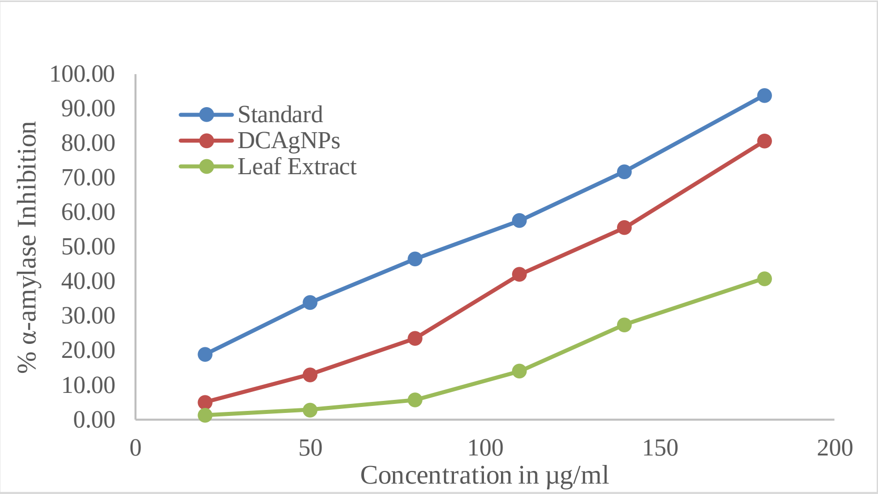

Figure 9:Anti-diabetic activity results

Anti-bacterial activity study:

The anti-bacterial activity of the green synthesized AgNPs was evaluated using agar plate well diffusion method and results were compared with standard gentamycin. The results confirm that the AgNPs having significant activity on the growth inhibition of bacteria in the study. The high inhibition activity at a low dose was observed for *Bacillus subtilis* that shows zone of inhibition of 5.4 mm at a very low dose of 10 µg/mL concentration. At a very high dose of 100 µg/mL, the inhibition zone was found to be very elevated for all the bacterial strains in the study. The antibacterial activity of AgNPs confirms that they are particularly efficient towards gram-positive bacteria whereas less potent towards gram-negative bacteria. This confirms that the green synthesized AgNPS was identified to be having potential anti-microbial activities. It has been confirmed that the NPs have the capacity to hold fast to the

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bacterial cell membrane and penetrate the cytoplasm. The results of the zone inhibition study of AgNPs were presented in figure 10 and and agar plates show the zone of inhibition were given in Figure 11.

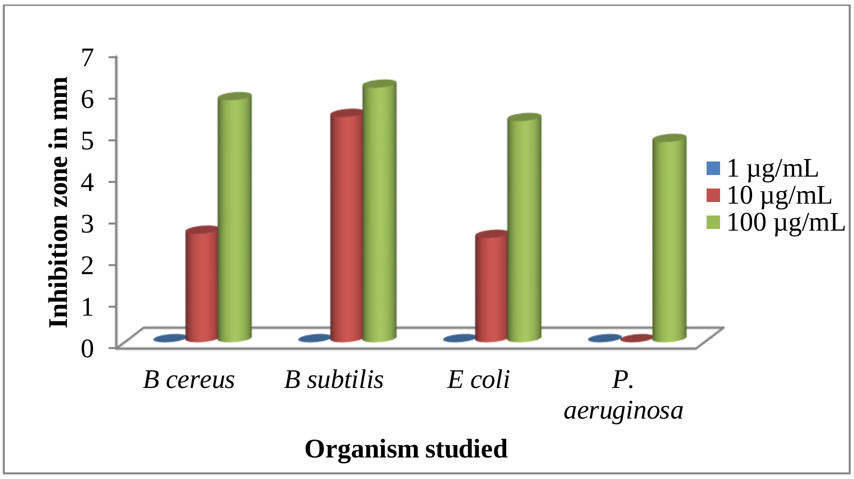

Figure 10: Anti-bacterial growth inhibition study comparison results

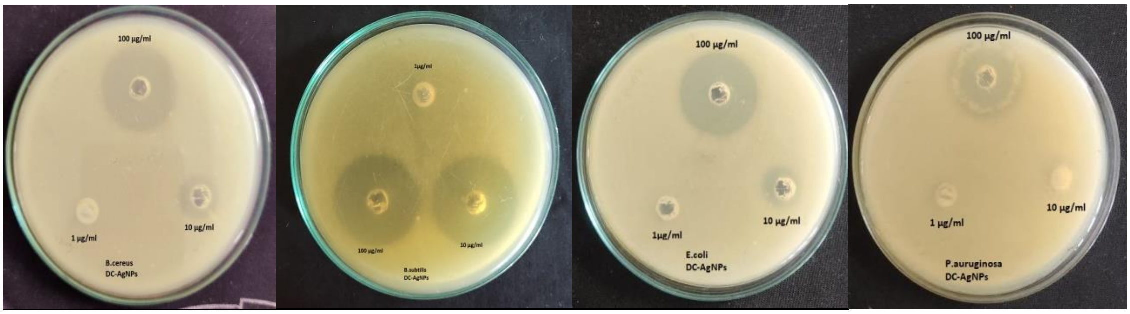

Figure 11: Agar plate well diffusion study results

Conclusion:

This analysis leads to some useful conclusions, most important of which, the potent therapeutic phytochemicals concerned in plant-based nanoparticle fabrications are biocompatible for various biological purposes. Green synthesis of AgNPsutilizing *D chloroxylon* aqueous leaf extract as reducing and capping agent can able to reduce silver ions into AgNPs without the use of harsh conditions. In a dose-dependent manner, the produced silver nanoparticles were examined for antioxidant, anti-diabetic, anti-inflammatory and antibacterial activity against pathogenic bacteria. In an overall conclusion, this study offers another dimension to the medicinal hub plant *D chloroxylon*. Its capacity to successfully synthesize AgNPs may improve antioxidant defense mechanisms and could also be utilized in the pharmaceutical industry.

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