

Comparative Physicochemical and Biological Evaluation of Green-Synthesized Silver Nanoparticles from Four Traditionally Used Antidiabetic Medicinal Plants with in vitro Antidiabetic Assessment of their Combined Formulation

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ABSTRACT

Background: Diabetes mellitus is one of the important metabolic diseases linked to chronic hyperglycaemia and its long-term complications. These medicinal plants used traditionally for diabetes provide various phytochemicals which exhibit antioxidant and enzyme inhibitory properties. Medicinal plant extracts are used as eco-friendly supporting materials for green synthesis of silver nanoparticles (AgNPs) which may improve the physicochemical and biological properties of the AgNPs.

Aim: The present study was aimed to comparatively evaluate the physicochemical and biological characteristics of green-synthesized AgNPs prepared from *Eugenia jambolana*, “ *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea* and to assess the in-vitro antidiabetic potential of their combined polyherbal AgNP formulations.

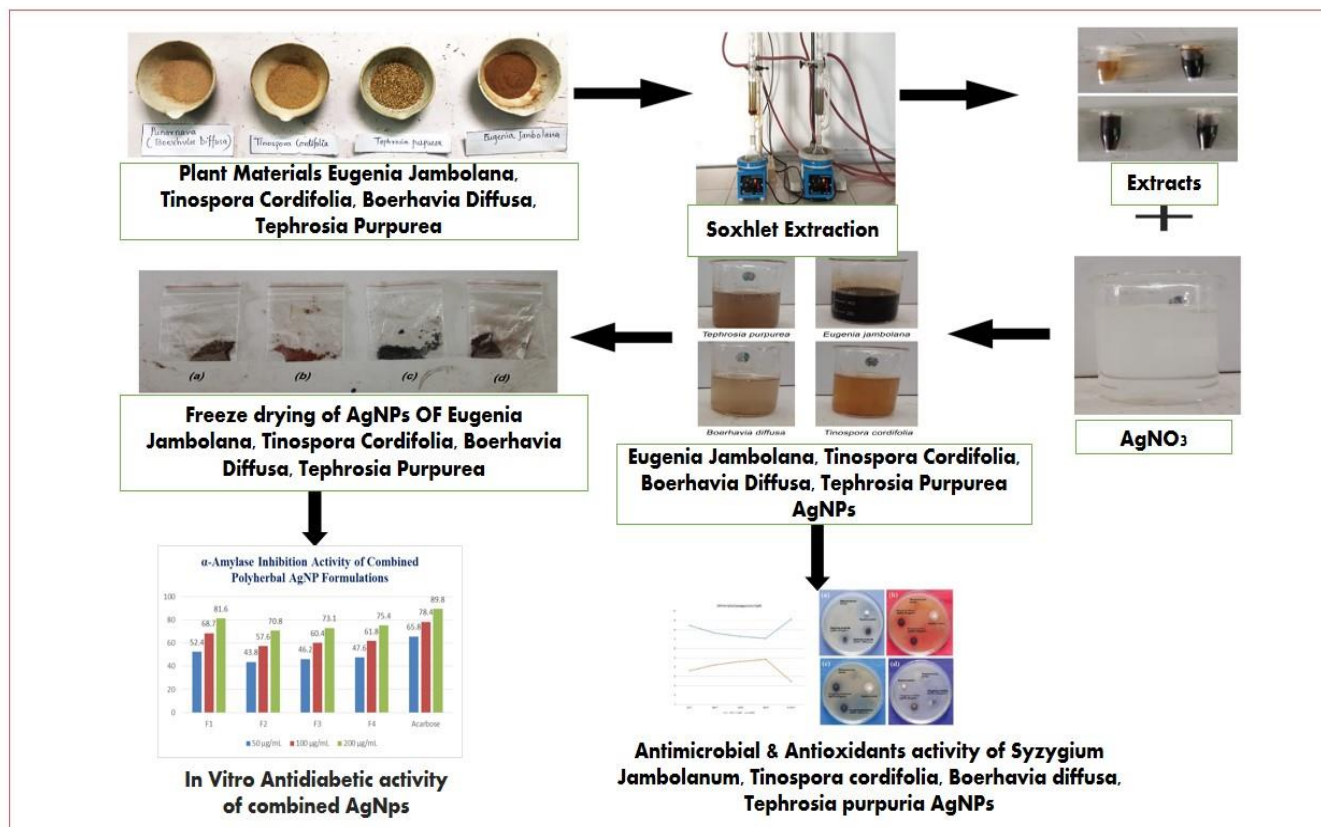
Methods: The selected medicinal plants were extracted using Soxhlet extractor in ethanol, the extracts were further used to synthesize AgNPs by green synthesis method using 1 mM silver nitrate. The synthesized nanoparticles were characterized by UV– visible spectroscopy, FTIR, particle-size and PDI analysis, SEM, zeta-potential analysis, DSC and XRD. Antibacterial activity was observed against the *E. coli* and *S. aureus* bacteria and antioxidant activity was investigated by the DPPH assay. The four combined formulations (F1-F4) were tested for their α -amylase and α -glucosidase inhibitory effects.

Results and Discussion: The synthesized AgNPs exhibited characteristic nanoscale properties, negative surface charge and differences in morphology and particle-size distribution depending on the plant. The DPPH inhibition was significantly high for *E. jambolana* AgNPs with $84.6 \pm 2.3\%$ and IC_{50} value of $36.2 \mu\text{g/mL}$. Formulation F1 exhibited the maximum inhibitory activities of α -amylase ($81.6 \pm 1.9\%$) and α -glucosidase ($85.4 \pm 1.9\%$) at a concentration of $200 \mu\text{g/mL}$.

Conclusion: It is concluded that plant mediated AgNPs seem to possess potential antioxidant, antimicrobial and in-vitro antidiabetic activity. F1 exhibited the highest enzyme-inhibitory activity and could be regarded a promising formulation for future mechanistic, toxicity and in-vivo studies.

KEYWORDS: Silver nanoparticles (AgNPs); *Eugenia jambolana*; *Tinospora cordifolia*; *Boerhavia diffusa*; *Tephrosia purpurea*. Antidiabetic activity.

Graphical abstract of proposed scheme



INTRODUCTION

Diabetes mellitus is one of the most important metabolic diseases of the modern times, the main feature of which is the maintenance of an abnormally high level of blood glucose caused by a lack of insulin secretion, an impairment of insulin activity or both. Chronic hyperglycaemia gradually impacts carbohydrate, lipid and protein metabolism and can result in vascular, neurological, renal and visual dysfunction. The growing prevalence of diabetes has spurred the efforts to identify new complementary therapeutic approaches that can act on several pathological mechanisms and have minimal side effects. In this context, the use of natural products and nanotechnology are two areas of present day biomedical research that are emerging as possibilities.[1][2]

Plants have been a significant source of pharmacologically active molecules for a long time. Therapeutic properties are mainly linked to their chemical composition, which includes chemically diverse compounds such as phenolic acids, flavonoids, tannins, alkaloids, terpenoids, glycosides, saponins and others specialized metabolites. Some of these compounds act as free-radical-scavengers, antimicrobial agents, anti-inflammatory, enzyme-modulators and glucose-regulators. The use of “*Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea* in conditions of disturbed glucose metabolism has been given special attention in the traditional knowledge. While these species are of great ethnomedicinal value, there is a need to systematically compare them on the bases of their chemical constituent and pharmacological activity.[3][4][5]

Eugenia jambolana traditionally known for use of its seeds and fruits in metabolic disorders, possesses a variety of polyphenolic and other secondary metabolites. The compound *Tinospora cordifolia*, traditionally used in formulations for metabolic and inflammatory disorders is known to have alkaloids, diterpenoids, glycosides and polysaccharide related compounds. *Boerhavia diffusa* is found to have various bioactive compounds such as alkaloids, flavonoids and phenolic compounds and *Tephrosia purpurea*, its phytochemical contains flavonoids and several biologically active compounds. Presence of various components of the species with different chemical composition gives a suitable background to compare their properties mediated by

nanoparticles. [6] [7]

Nanoscience is an opportunity to alter the functional behaviour of naturally derived compounds by altering their behaviour at the nanoscale. The silver nanoparticles have been studied in great detail due to their high surface to volume ratio, unique optical properties, surface reactivity and wide range of biological activities. More significantly, the properties of the nanoparticles may be manipulated by the type of material that is utilized during the production process. In the case of botanical extracts, the naturally occurring molecules might play the role of electron donors and also as surface associated stabilizing molecules in the process of nanoparticle formation. Thus, the generated particles can pick up a phytochemical corona which can affect dispersion, surface charge, morphology, and interaction with biological systems.[8][9]

The use of plant extracts for the synthesis of nanoparticles is also an alternative to the conventional chemical synthesis methods which may involve harsh reagents, high temperatures, organic solvents and other environmentally harmful factors. Biological synthesis can be done under relatively mild conditions and may minimize the need for synthetic reducing/stabilizing agents. But the characteristics of plant derived nanoparticles are not universal. The composition of the metabolites, efficiency of extraction, reaction parameters and capping interactions can result in significant variation in particle diameter, morphology, crystallinity, surface charge and aggregation behaviour. Therefore, comparative investigations are necessary and should not be assumed that the nanoparticles from both the botanical sources will have the same performance. [10]

These differences are set in the framework of comprehensive analytical characterization. The formation of nanoparticles can be detected by UV–Visible spectrophotometry by the effect of surface plasmon resonance and information about the chemical groups of the biomolecules attached to the surface can be obtained by FTIR spectroscopy. Dynamic light scattering allows evaluation of hydrodynamic diameter and distribution characteristics and polydispersity values indicate uniformity of the population. SEM allows to study the morphology and aggregation of particles. Zeta-potential measurements give information about the charge at interfaces and dispersion behaviour, while XRD gives information on crystalline phase and structural organization. In addition, DSC provides data on the thermal transitions and the stability of the phytochemical constituents associated with it. These nanostructures can be further analysed by complementary biological assays to reveal their functional significance. Antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* allows for comparison of activity towards representative Gram-negative and Gram-positive organisms. Evaluation of radical-scavenging capacity, using the DPPH assay, gives another perspective to biological evaluation, which is to determine the capacity of the preparations to neutralize a stable free radical. The IC_{50} value is a quantitative parameter that can be used to compare the relative antioxidant potency of four nanoparticle preparations. [11] [12] [13]

Carbohydrate-hydrolysing enzymes, especially α -amylase and α -glucosidase are targeted in the antidiabetic study. These enzymes are involved in the process of breaking down complex carbohydrates into absorbable sugars in a serial fashion. Their inhibition may help to slow down the digestion of carbohydrate and thus decrease the quick release of glucose after carbohydrate consumption. Thus, enzyme-inhibition models are valuable preliminary methods for screening compounds that have potential glucose-regulatory activity. Evaluation at various concentrations, along with IC_{50} calculation, allows the evaluation of inhibitory potency, and not only at one concentration. One significant point of the current study is the formulation of nanoparticles from all the four botanicals (combined formulations). The proportions of *E. jambolana*, *T. cordifolia*, *B. diffusa* and *T. purpurea* AgNPs were varied in the composition of F1, F2, F3 and F4. This kind of compositional variation allows to assess whether a specific combination has better enzyme-inhibitory properties. It is especially relevant, since the botanical material used may be chemically heterogeneous and thus provide different functional properties to a composite preparation. However, higher activities must be viewed as a formulation effect, until formal synergistic interaction studies are performed. [14] [15][16]

In this present work, a comparative study including botanical extraction, nano-fabrication, structural and surface characterization, antimicrobial screening, antioxidant assessment and in vitro anti-diabetic evaluation is established. Special attention is given to the correlation of the relationships between the characteristics of nanoparticles and biological responses and the relative performance of the four combined preparations. The results would be used to scientifically choose a promising polyherbal AgNP formulation for further mechanistic, toxicological, pharmacokinetic and in vivo study.[17]

MATERIALS AND METHODS

2.1. Chemicals, Reagents and Instruments

Silver nitrate (AgNO_3), Ethanol, were procured from merck (Germany). Reference standard acarbose, Ampicillin, DPPH, were obtained from Sigma-Aldrich (USA). All chemicals and reagents used analytical grade. Nanoparticle characterization and analytical evaluations were performed using a UV-Visible spectrophotometer (Shimadzu UV-1800), Fourier Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (DLS) analyzer, Scanning Electron Microscope (SEM), and X-ray Diffraction (XRD) system.

2.2. Collection and Authentication of Plant Materials

Eugenia jambolana was collected in summer from the fruit ripe and seed mature stage from the forest areas of Western Maharashtra. *Tinospora cordifolia* was collected from the vines that climb up Neem tree in central India during early monsoon when, stem alkaloids are at their peak. *Boerhavia diffusa* roots and aerial parts were collected from cultivated fields in Maharashtra during post monsoon season as they possess highest concentration of alkaloids and flavonoids. *Tephrosia purpurea* was gathered at the early flowering stage from the profusely growing areas in the semi-arid Maharashtra. The collected specimens were identified by 'Government of India, Ministry of Environment Forests & Climate Change, Botanical Survey of India'.

2.3. Preparation and Preservation of Plant Samples

After collection, of all plant materials were washed with distilled water to remove adhering soil and other contaminants. The materials were shade dried to maintain the phytochemical integrity and then coarsely powdered mechanically with grinders. To preserve the quality of dried plant powder before extraction and analytical studies, containers storing the powder were protected from light and moisture.

2.4. Extraction Procedure

In the present study, the plant materials, *Eugenia jambolana*, *T. cordifolia* stems, *T. purpurea* whole plant and *B. diffusa* roots were extracted separately using ethanol as extraction solvent. Dried and coarsely powdered plant samples were exhaustively extracted using Soxhlet extraction technique. The extract obtained were filtered and then concentrated under reduced pressure in a rotary evaporator at a temperature of not more than. The concentrated extract was then dried to get the semisolid/dry extracts and kept in airtight containers in until further use.

2.5. Green Synthesis of Silver Nanoparticles (AgNPs)

The silver nanoparticles of all selected plant extracts were synthesized using a green synthesis approach with plant extracts as reducing and stabilizing agents. In brief 10 ml of plant extract was mixed with 90 ml of aqueous silver nitrate solution (1mM AgNO_3) while stirring continuously at room temperature. Silver nanoparticles were formed as indicated by visible colour change due to surface Plasmon resonance. The reaction mixture was left for 24 hours so that all the silver ions were reduced. The nanoparticles generated were separated out by centrifugation at 10000 rpm for 20 minutes followed by repeated washing with distilled water to remove any unreacted material. A cleaned-up nanoparticles was then dried and stored for further investigation and formulation.

2.6. Characterization of Synthesized AgNPs

The entire process was done to make sure that the synthesized silver nanoparticles were formed, morphologically, size distribution, crystalline structure and stable. UV-visible spectroscopic reports were used

to determine the synthesis of nanoparticles and revealed that a sharp peak of SPR is obtained at 420-450 nm which is a characteristic indication of AgNPs formation. FTIR spectroscopy was used to confirm the presence of functional groups (e.g. -OH, -C=O and -NH) and thus the use of compounds derived from plants to stabilize nanoparticles and modify their surfaces, while polydispersity index (PDI) was used to assure monodispersity and uniformity of the diameter of the nanoparticles. All the selected extract resulted in the formation of a large nanoscale population as shown in the particle size measurement of the synthesized AgNPs. The SEM images of the synthesized AgNPs showed that the surface of the nanoparticles was smooth and had a spherical shape, while the XRD pattern of AgNPs showed that they were crystalline and exhibited characteristic diffraction peaks corresponding to the face-centered cubic (fcc) structure of silver. The higher value of surface charge obtained from zeta potential analysis indicated good stability of the particles in the form of a colloid because of the electrostatic repulsive forces.

2.7.Evaluation of Antibacterial Assay

One of the standard methods used to determine the antibacterial activity of produced silver nanoparticles is the disc diffusion technique, this method alone gives both qualitative and quantitative information of the inhibitory property of silver nanoparticles against disease causing bacteria. Two common example organisms that often are used to make such assays are *Escherichia coli* (*E. coli*) which is a Gram-negative bacterium and *Staphylococcus aureus* (*S. aureus*) which is a Gram-positive bacterium. The bacteria are clinically and commonly relevant pathogens that cause many infections hence ideal candidates of antimicrobial screens. [18] The disc diffusion method involves uniformly inoculating plates with bacterial culture and placing sterile filter paper discs containing a known concentration of AgNPs on the plates. After incubation AgNPs penetrate into the agar and interact with the bacterial cells and inhibit the growth of the bacteria. The more the bacteria are inhibited, the larger the diameter of the clear zone around the disc, and thus the greater the extent of the antibacterial activity.

2.8.Antioxidant Activity

2.8.1. DPPH Antioxidant Assay

The free radical scavenging activity of antioxidants could be assessed by the widely used 2, 2 diphenyl 1 picrylhydrazyl (DPPH) assay. DPPH is a stable purple colored free radical with strong absorption at 517 nm. After adding an antioxidant, it donates its electron or hydrogen atom to DPPH, which reduces it to a yellow colored diphenyl picrylhydrazine, causing the absorbance to be reduced. The DPPH activity assay is performed using different concentrations of AgNPs and a fixed concentration of DPPH solution and incubation in the dark. The amount of the decrease in absorbance is measured spectrophotometrically. Percentage inhibition of DPPH radicals and half-maximal inhibitory concentration (IC₅₀) value, which is the concentration of AgNPs for scavenging 50% of DPPH radicals is calculated. [19][20]

2.9. In Vitro Anti-diabetic Activity of Combined Formulation.

α -Amylase and α -glucosidase inhibitory activities were assessed to determine the in vitro antidiabetic potential of the combined polyherbal formulations of silver nanoparticles (F1-F4). The percentage inhibition was determined for each formulation at concentrations of 50, 100 and 200 μ g/mL. IC₅₀ values were determined as a measure of inhibitory potencies. Concentration-dependent inhibition of both enzymes was observed for all formulations. Of the formulations tested, F1 showed the greatest inhibitory activity, suggesting that it has a relatively higher potential to inhibit the activity of carbohydrate-digesting enzymes. These results can be used to further explore combined formulation as a potential in vitro antidiabetic drug.

Ingredient	F1	F2	F3	F4
Mg				
<i>Eugenia jambolana</i> AgNPs	30	20	10	40
<i>Tinospora cordifolia</i> AgNPs	20	30	40	10

<i>Boerhavia diffusa</i> AgNPs	10	40	20	30
<i>Tephrosia purpurea</i> AgNPs	40	10	30	20
Total	100 mg	100 mg	100 mg	100

RESULTS

In the current study, the physicochemical properties and biological activities of the AgNPs synthesized using four traditionally used antidiabetic medicinal plants (AMPs) were assessed. Observations involve the nanoparticle formation, particle size, polydispersity, surface charge, morphology, thermal properties, crystallinity, antibacterial activity and antioxidant potential. In addition, antidiabetic activities of the combined polyherbal silver nanoparticle formulations were evaluated by α -amylase and α -glucosidase inhibition assays at various concentrations. The results of these investigations are summarized in the sections that follow.

3.1. Collection, Authentication, preparation of Plant Materials

The selected plants were thoroughly washed in distilled water to remove external medium (soil and microbes) from all the collected parts of the plants. Next, the plant materials were shade dried at room temperature to prevent heat-sensitive metabolites from being lost. The grinding was done using a mechanical grinder and the powdered materials were stored in hygienic containers, which were airtight and lightproof. The selected plants were authenticated by “Government of India, Ministry of Environment Forests & Climate Change, Botanical Survey of India” [21]

3.2 Extraction

The ethanolic extracts of all four plant materials, namely, *Syzygium jambolanum*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea* were prepared through Soxhlet extraction and were visually appreciated with different color and consistency having different phytoconstituents extracted. 95% ethanol was very effective in that the solvent colour deepened during extraction and the siphon tube was de-coloured, indicating that the extraction was complete. The semisolid residues derived from rotary evaporation showed good extractability of the polar and semi-polar metabolites such as phenolics, flavonoids, glycosides, tannins and alkaloids.

Table 1: Physical Characteristics of Plant Extracts Used for Green Synthesis of Silver Nanoparticles

Sr. No.	Plant Extract	Color	Appearance / Consistency
1	<i>Boerhavia diffusa</i>	Light brown	Clear, moderately viscous
2	<i>Syzygium jambolanum</i>	Blackish	Dense, highly pigmented
3	<i>Tinospora cordifolia</i>	Deep brown	Thick, uniform solution
4	<i>Tephrosia purpurea</i>	Dark brown	Opaque, concentrated extract

3.3 Green Synthesis of Silver Nanoparticles (AgNPs)

All four plant extracts exhibited colour change which is the characteristic feature of the formation of the silver nanoparticles (AgNPs). *Syzygium jambolanum* showed the darkest color indicating that there was a higher number of nanoparticles being formed or aggregated, with *Tephrosia purpurea* exhibiting moderate nanoparticle formation. The color of *Boerhavia diffusa* was pale, suggesting lower concentration of nanoparticles or well dispersed smaller nanoparticles, while the color of *Tinospora cordifolia* was yellow-orange-brown, which indicated the formation of stable nanoparticles. The differences could be attributed to the phytochemical composition, reducing capability and capping ability of the plant extracts.

3.4 Characterization of Synthesized AgNPs

3.4.1 UV-Visible Spectroscopy

The UV–Visible spectra of the synthesized silver nanoparticles using *Eugenia jambolana*, *Tinospora*

cordifolia, *Boerhavia diffusa* and *Tephrosia purpurea* are shown in Fig. 1. The spectra were obtained in the range of about 300-800nm. The four AgNP preparation had different absorption profiles, which can be attributed to differences in AgNP concentration, particle-size distribution, surface chemistry, and the type of phytochemicals bound to the surface of the AgNP.

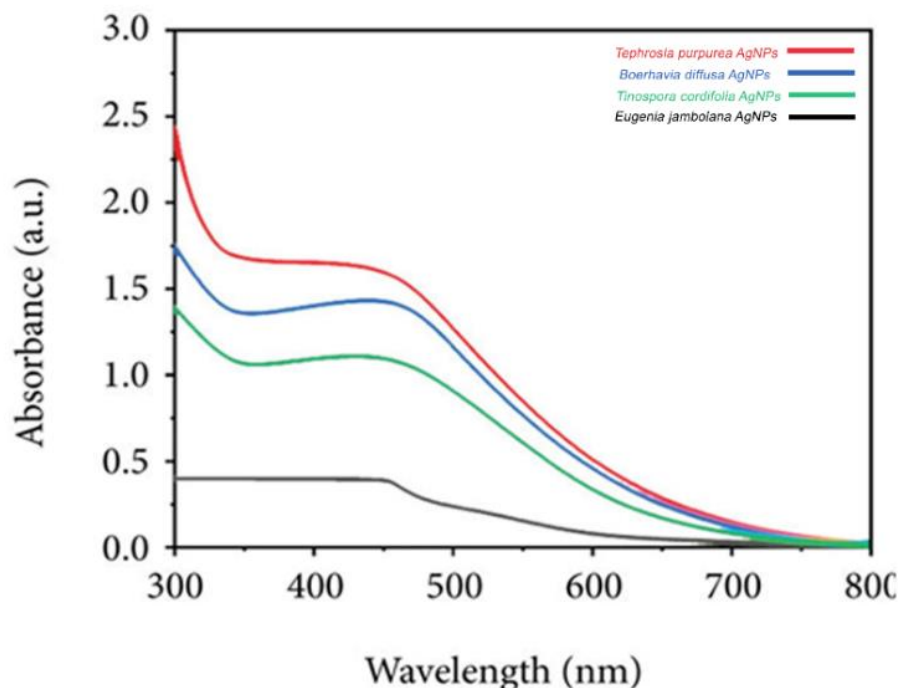


Fig 1: UV-Visible spectroscopy of AgNPs containing *Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea*

Eugenia jambolana, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea*. The absorption data of all four preparations showed the typical absorption of silver nanoparticles in the visible spectrum, especially in the region of 420– 460 nm, suggesting the formation of silver nanoparticles through surface plasmon resonance. The absorbance of *T. purpurea* AgNPs showed comparatively higher absorbance, followed by *B. diffusa* and *T. cordifolia* while *E. jambolana* showed comparatively lower absorbance. The wide spectral profiles may indicate variations in concentration, size range, aggregation of nanoparticles, and type and concentration of phytochemicals bound to the surface. The gradual decrease in absorbance towards 700– 800 nm suggests a decrease in light scattering at longer wavelengths. In summary, the observed UV– Visible profiles indicate effective plant mediated synthesis of AgNP and the variation of optical property of the synthesized nanoparticles with varying species.

3.4.2. FTIR spectroscopy

The Fourier Transform Infrared (FTIR) spectra of the four-plant mediated AgNP preparations are shown in Fig. 2. The functional groups present in the synthesized nanoparticles and the involvement of biomolecules present in plants for the reduction and stabilization of Ag nanoparticle were identified by FTIR analysis. These spectra exhibited a number of absorption bands spread over the region of about 4000-500 cm⁻¹. The variations in the intensity and peak position of the four samples suggest the differences in the phytochemical composition of the corresponding plant extracts and its interaction with the AgNP surface.

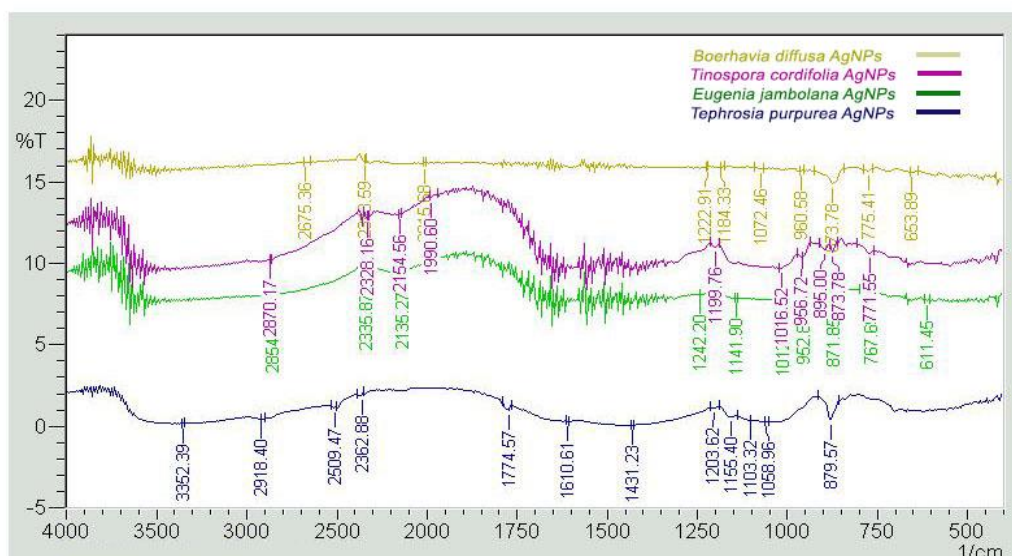


Fig 2: FTIR Spectra of AgNPs containing of *Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea*

The four synthesized AgNPs were investigated by FTIR, and the spectra are shown in Fig. 2. Characteristic functional group vibrations were observed in all the samples, indicating the interaction of plant derived biomolecules with the surface of the nanoparticles. The presence of hydroxyl containing phytoconstituents were indicated by broad bands at around 3360– 3410 cm^{-1} , which are attributed to the O– H stretching. Bands near $\sim 2900 \text{ cm}^{-1}$ are due to C–H stretching vibrations in aliphatics and bands near $\sim 1600\text{--}1650 \text{ cm}^{-1}$ may be due to C=O, C=C or amide. Other fingerprint region bands, especially at $1000\text{--}1200 \text{ cm}^{-1}$, suggest the presence of C– O containing groups. The differences in the intensities and position of the peaks for four AgNPs indicate different phytochemical composition. These functional groups could be involved in the reduction of silver ions and stabilization of the nanoparticles as well as capping.

Table 2: FTIR Peak Assignments of Synthesized AgNPs

Sample	Peak Position (cm^{-1})	Functional Group	Possible Compound Type
AgNPs <i>E. Jambolana</i>	3410	O–H stretching	Flavonoids, polyphenols
	1635	C=O stretching	Proteins, esters
AgNPs <i>T. Cardifolia</i>	3365	□OH stretching	Terpenoids
	1650	Amide I band	Protein capping
AgNPs <i>B. diffusa</i>	3402	O□H stretching	Saponins, alcohols
	1050	C□O stretching	Ether linkages
AgNPs <i>T. purpurea</i>	3360	O□H stretching	Phenols, flavonoids

The FTIR spectroscopy revealed the presence of characteristic functional groups of synthesized AgNPs, which indicated the participation of plant derived biomolecules to the process of AgNP formation and stabilization. The *E. jambolana* AgNPs exhibited stretching at 3410 cm^{-1} attributed to O– H and 1635 cm^{-1} attributed to C=O which indicated the presence of phenolics, flavonoids, proteins, and ester associated compounds. *T. cordifolia* had O– H bands at 3365 cm^{-1} , and amide-I bands at 1650 cm^{-1} , which are indicative of hydroxyl containing compounds and protein mediated capping, respectively. *B. diffusa* showed O–H and C–O bands at 3402 and 1050 cm^{-1} , respectively. In the case of *T. purpurea*, the O–H stretching was observed at 3360 cm^{-1} corresponding to the presence of phenolic and flavonoid components.

3.4.3. Particle Size Measurement

Dynamic light scattering (DLS) was used to determine the particle size of the synthesized silver nanoparticles (AgNPs). Nanoparticles showed nanoscale hydrodynamic diameters and there were differences between the four plants mediated preparations. The mean diameter of the *Tinospora cordifolia* AgNPs was found to be 67.9 nm, *Boerhavia diffusa* AgNPs was 84.8 nm, *Tephrosia purpurea* AgNPs was 59.1 nm and *Eugenia jambolana* AgNPs was 51.1 nm. The respective PDI confirmed the size distribution and dispersion which is necessary for formation of nanosized particles for further evaluation of their physicochemical and biological characteristics.

3.4.3.1. *Tinospora cordifolia* AgNPs

The *T. cordifolia*-mediated AgNPs were subjected to particle-size analysis that measured the hydrodynamic diameter, size distribution, polydispersity, and dispersion, assessing the uniformity and stability of nanoparticles.

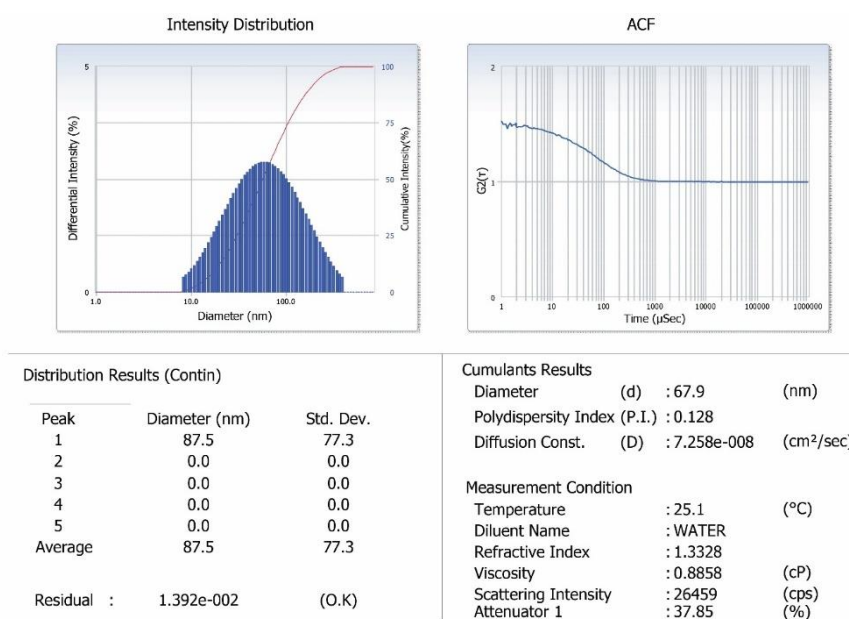


Fig. 3: Particle size of *Tinospora cordifolia* AgNPs

The cumulant analysis revealed a distinct nanoscale particle population with a mean hydrodynamic diameter of 67.9 nm of the AgNPs synthesized using the extract of *Tinospora cordifolia*. The intensity distribution curve revealed a single prominent peak at ~87.5 nm indicating the presence of a single, dominant and homogeneously distributed nanoparticle population. The polydispersity index (PDI = 0.128) is quite low, which means that the size distribution is limited. The result indicated that the phytoconstituents of *T. cordifolia* were successful in stabilizing the nanoparticles in the synthesis process. There is a broad standard deviation (77.3 nm) but the presence of a single main peak indicates that the system is predominantly monodisperse with a few larger aggregates, which are typical in nanoparticles biologically encapsulated. The autocorrelation function (ACF) showed a smooth exponential decrease, which further proved that the particles in suspension were stable and diffused evenly. The results indicate that *T. cordifolia* extract can be used as a good reducing and capping agent as it yields stable and well-dispersed AgNPs in a desired nanoscale range which can be employed in further physicochemical and biological studies.

3.4.3.2. *Boerhavia diffusa* AgNPs

For the particle-size characteristics, the average hydrodynamic diameter, distribution pattern, polydispersity and dispersion behaviour were determined, which indicates the uniformity of the nanoparticles of *B. diffusa* AgNPs.

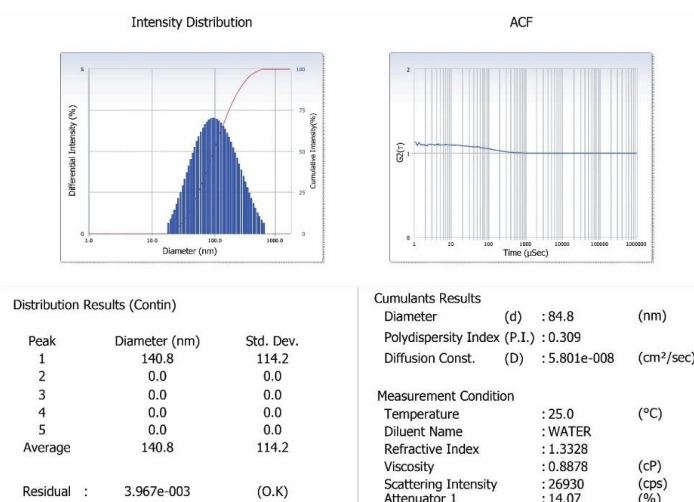


Fig. 4: Particle size of *Boerhavia diffusa* AgNPs

The particle size investigation of silver nanoparticles synthesized by *Boerhavia diffusa* extract showed the presence of a broad size range of particles, which were well defined in the nanoscale. The intensity distribution profile indicated one dominant peak of which the average size was 140.8 nm. This resulted in most of the nanoparticles being in the higher end of the nanoscale. The distribution curve of the particles is bell shaped showing consistent particle formation; however, a relatively high standard deviation of 114.2 nm confirmed the presence of particles of different size. This is a regular phenomenon during the synthesis of nanoparticles by plants due to the natural variation in the ability of phytochemicals to cap nanoparticles. The mean diameter obtained by using the cumulant method confirmed the presence of a large fraction of smaller nanoparticles throughout the solution. The results for a moderate polydispersity index (P.I. = 0.309) indicated that the sizes were more dispersed. This could be due to some particles becoming agglomerated or due to particle type generated during the synthesis. Throughout the decay pattern of the autocorrelation function (ACF), the nanoparticles remained well-dispersed and did not settle out too much. The particle size characteristics show that *Boerhavia diffusa* phytoconstituents may decrease and stabilize silver ions. This results in the generation of a mixed size population of nanoparticles which are stable for further physicochemical and biological testing.[22]

3.4.3.3. *Tephrosia purpurea* AgNPs

The hydrodynamic diameter, particle-size distribution, polydispersity index and dispersion characteristics of the *T. purpurea* AgNPs were measured by particle-size analysis, which provides an idea of the quality of nanoparticle synthesis.

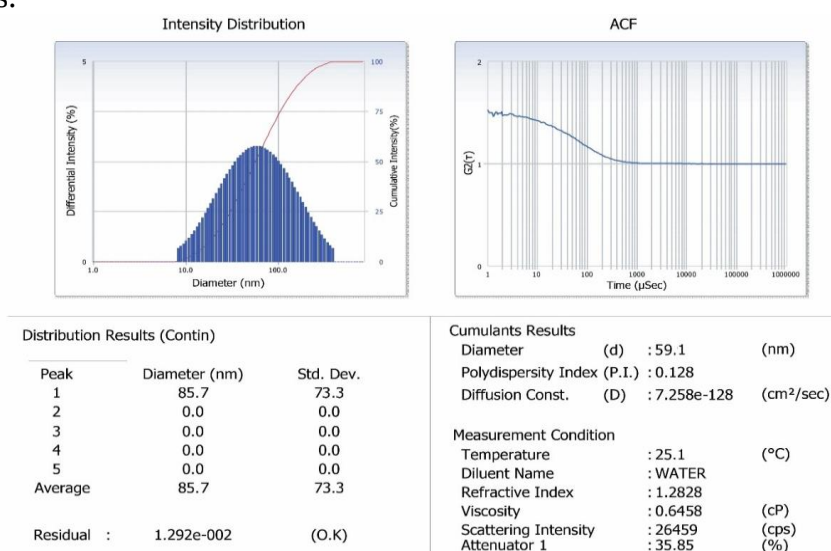


Fig. 5: Particle size of *Tephrosia purpurea* AgNPs

The particle size analysis of silver nanoparticles prepared using *Tephrosia purpurea* extract demonstrated that the majority of the particles were nanoscale and only one peak was observed which corresponds to 85.7 nm suggesting the particles are of the same size. The intensity distribution curve was smooth and bell-shaped, indicating that the nucleation and particle growth were stable during the synthesis process. The average particle size of 85.7 nm, with a standard deviation of 73.3 nm, shows that there are a lot of nanoparticles and a few larger particles. This is a typical problem with plant-based fabrication as biomolecular capping and biomolecular stabilization are not always consistent. The evidence that there are well-dispersed particles in the lower nanoscale zone is further supported by the cumulant derived mean diameter of 59.1 nm. A low polydispersity value (P.I. = 0.108) means that the nanoparticles that were made were mostly the same size and didn't clump together too much. The autocorrelation function (ACF) also showed a smooth decay pattern indicating that the particles were stable and the scattering behavior was constant. The particle size profile revealed that the phytochemicals from *Tephrosia purpurea* are effective in reducing and stabilizing the nanoparticles to a controllable size which can be utilized for further studies and biological application.

3.4.3. 4. *Eugenia jambolana* AgNPs

The particle-size profile of *E. jambolana* AgNPs was evaluated for its hydrodynamic diameter, size distribution, polydispersity, and colloidal dispersion characteristics, which have contributed to the evaluation of nanoparticles' stability.

The particle size measurement of silver nanoparticles produced using *Eugenia jambolana* extract showed the presence of a large population of nanoparticles, in the nanoscale range, with a predominant peak at 107.0 nm corresponding to medium sized nanoparticles. This intensity distribution curve was well-defined in the shape of a bell, indicating that the nucleation process occurred regularly and that the natural variation as the particles grew followed. The high standard deviation (105.5 nm) shows that the sample had a wide range of sizes. This indicates that, apart from the main population of nanoparticles, there were some larger particles or clusters. This is common occurrence in phytochemical mediated synthesis owing to the complex mixtures of capping and stabilizing biomolecules. The mean particle diameter obtained from the

cumulant is 51.1 nm which shows that a considerable portion of the particle is in the lower nanoscale region. The polydispersity index (0.312) showed that the system was moderately polydisperse, which backed up the mixed size distribution shown in the intensity plot.

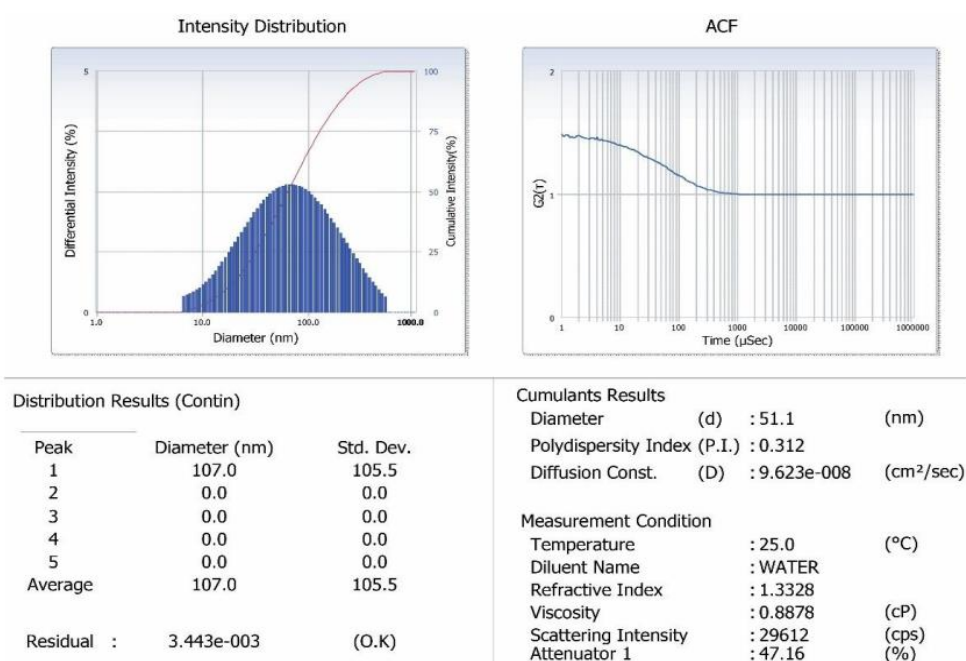


Fig. 6: Particle size of *Eugenia jambolana* AgNPs

This ACF curve showed a monotonically decreasing trend in the particles moving and scattering behaviour was stable. No sudden changes were found which would indicate serious aggregation. In general, our results show that *Eugenia jambolana* extract is good at lowering and stabilizing silver ions, making nanoparticles with a wide yet stable size range that may be used for further research and practical purposes.

3.4.4. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was carried out to assess the surface morphology, shape and aggregation pattern of green-synthesized silver nanoparticles (AgNPs). The SEM images showed that there were some morphological differences between the four plant-mediated AgNPs. The *Eugenia jambolana* AgNPs were observed to have irregularly shaped particles with moderate surface clustering. AgNPs were mostly spherical and polyhedral in shape with relatively uniform distribution and limited aggregation for *Tinospora cordifolia*. The AgNPs synthesized using *Boerhavia diffusa* showed a dense granular structure with aggregation. On the other hand, *Tephrosia purpurea* AgNPs revealed less aggregation, well dispersed and relatively uniform nanoparticles. SEM revealed that nanoparticles are successfully formed with varying their morphology and aggregation based on the plant. [23]

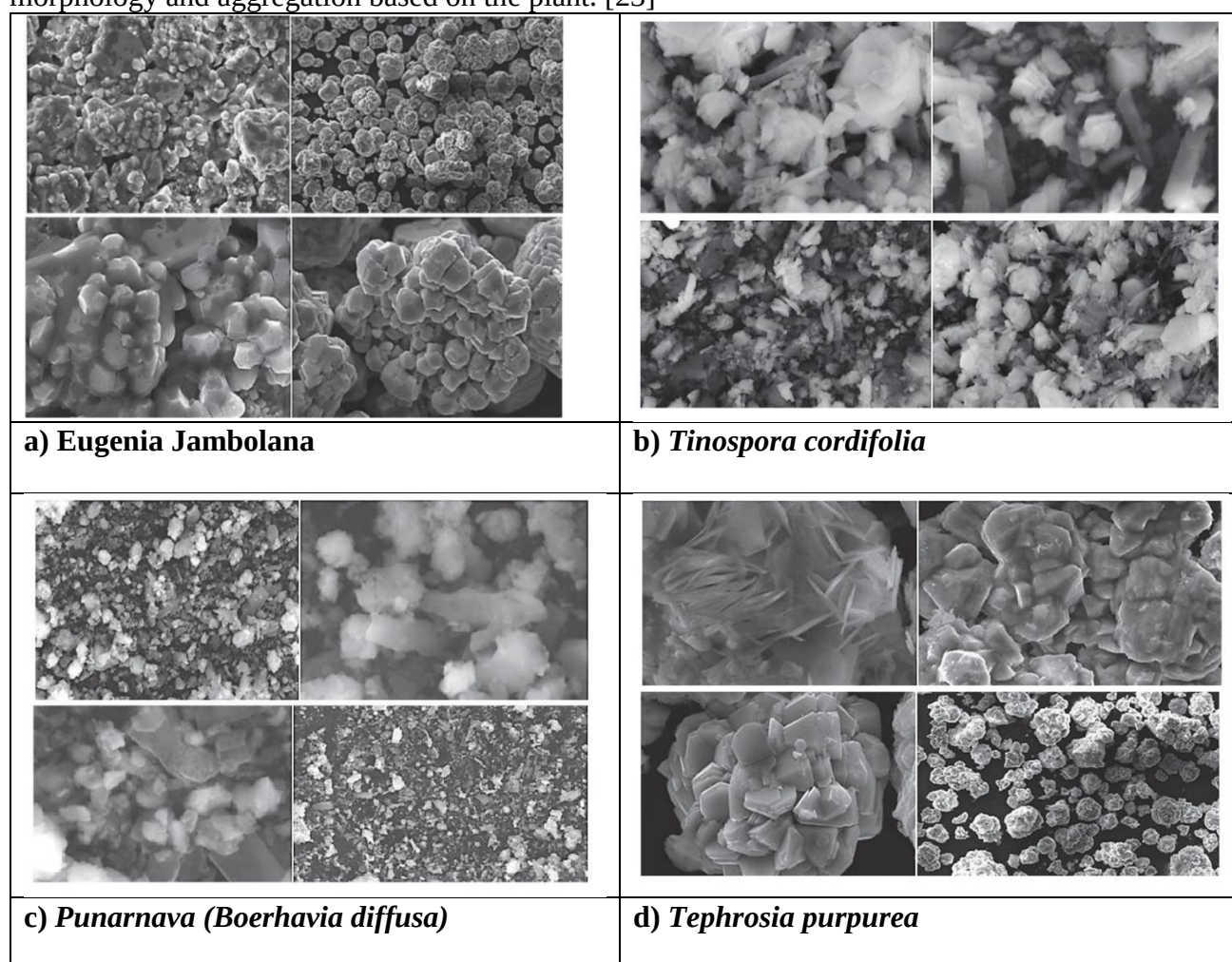


Fig 7: Scanning Electron Microscopy (SEM) analysis of AgNPs synthesized of a). *Eugenia jambolana*, b). *Tinospora cordifolia*, c). *Punarnava (Boerhavia diffusa)* and d). *Tephrosia purpurea*

The SEM micrographs in Fig. 7 clearly show that the AgNPs obtained from different medicinal plants have different morphologies. The *Eugenia jambolana* AgNPs (a) showed a high number of nanoparticles that are mostly irregular to near spherical shapes with significant aggregation and clustering. The *Tinospora cordifolia* AgNPs (b) exhibited relatively irregular and non-uniform particle sizes and shapes, suggesting a certain degree of heterogeneity in the nanoparticles. AgNPs from *Boerhavia diffusa* (c) exhibited dense granular distribution, Acta Sci., 27(3), 2026

with high degree of aggregation and slightly rough surface. *Tephrosia purpurea* AgNPs (d) exhibited well-defined clusters with rounded-to-irregular-shaped particles and comparatively organized distribution. The observed morphological difference could be attributed to the differences in plant derived reducing and capping phytochemicals used for the synthesis of the nanoparticles.

Table 3: SEM Morphology analysis of Plant-Mediated AgNPs

Plant Extract	SEM Morphology	Aggregation Pattern	Phytochemical Influence
<i>Eugenia jambolana</i>	Irregular nanoparticles, variable shapes; clustered deposits	Moderate aggregation; surface clustering	High polyphenolic & tannin content causing fast nucleation and partial aggregation
<i>Tinospora cordifolia</i>	Spherical to polyhedral nanoparticles; uniform coating	Lower aggregation; more uniform spread	Presence of mucilage, glycosides strong capping & steric stabilization
<i>Boerhavia diffusa</i>	Dense granular morphology; highly compact structures	High aggregation; packed nanoclusters	Saponins & flavonoids reduce quickly dense agglomerates
<i>Tephrosia purpurea</i>	Fine, porous, evenly dispersed nanoparticles	Minimal aggregation; porous distribution	Controlled reduction by flavonoids stable, smaller nanoparticles

The SEM analysis showed that the morphology of the AgNPs prepared with the four medicinal plant extracts was distinguishable. The AgNPs of *Eugenia jambolana* presented irregular-shaped nanoparticles and moderate surface clustering which is probably related to the high polyphenolic and tannin content of the extract. The AgNPs of *Tinospora cordifolia* exhibited mostly spherical to polyhedral shapes with relatively less aggregation, indicating the good stabilization capacity of mucilage and glycosidic components. AgNPs of *Boerhavia diffusa* showed dense aggregation and granular structures, which might be attributed to the faster reduction and capping of the AgNPs by the saponins and flavonoids. *Tephrosia purpurea* AgNPs, on the other hand, showed fine, porous and evenly distributed nanoparticles with minimum particle aggregation, suggesting the controlled formation of nanoparticles and their stabilization by the flavonoid-rich phytoconstituents. In all, the SEM results suggest that the phytochemical composition of plants had a significant effect on the morphology and aggregation behavior of nanoparticles.

3.4.5. Zeta Potential Measurement

The surface charge and colloidal stability of the synthesized silver nanoparticles (AgNPs) were determined by zeta potential measurement. It is a good indicator of the electrostatic repulsion between nanoparticles and a prediction of the nanoparticles' tendency to aggregate during storage or application. The zeta-potential of AgNP suspension was measured under controlled measurement conditions using a zeta -potential analyzer. The obtained values were used to compare the stability and surface properties of AgNPs obtained by the different medicinal plant extracts. [25]

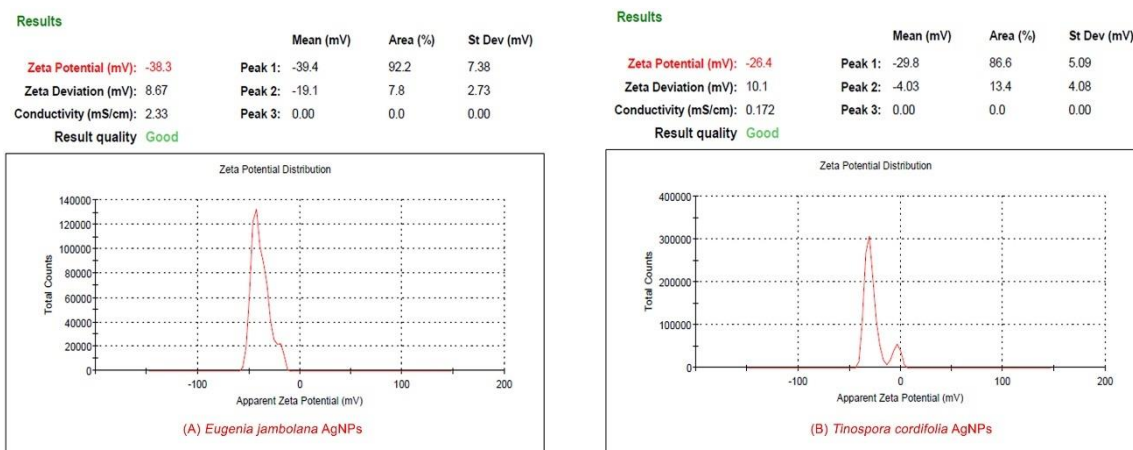


Fig 8: Zeta Potential of AgNPs synthesized of a) *Eugenia jambolana*, b) *Tinospora cordifolia*.

The zeta potential graphs of the AgNPs obtained from *Eugenia jambolana* and *Tinospora cordifolia* show that the surfaces of the nanoparticles are negatively charged. The negative potential of *E. jambolana* was more negative -38.3mV, which indicated that the colloidal stability was excellent, and the negative charge of *T. cordifolia* was comparatively less negative, -26.4mV, but still good stability because of the combination of electrostatic and steric stabilization.

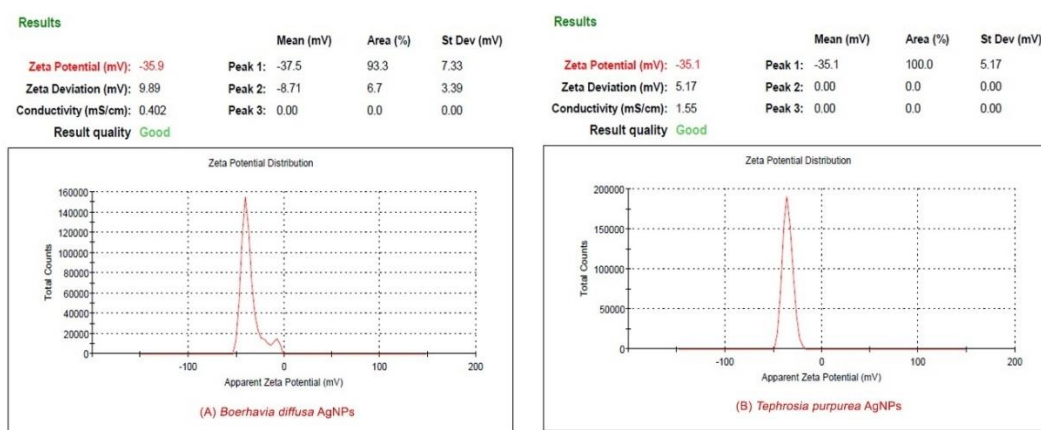


Fig 9: Zeta Potential of AgNPs synthesized of a) *Boerhavia diffusa* b) *Tephrosia purpurea*.

The zeta potential profiles of *Boerhavia diffusa* and *Tephrosia purpurea* AgNPs revealed the highly negative surface charge, suggesting the high colloidal stability of the synthesized AgNPs. The zeta potential of *B. diffusa* was -35.9 mV, and *T. purpurea* had a -35.1 mV with 100% peak area indicating uniform surface capping and stable dispersion of the nanoparticles.

Table 4: Zeta Potential Comparison of Plant-Mediated Silver Nanoparticles

Plant Extract	Zeta Potential (mV)	Main Peak (mV)	Peak Area (%)	Zeta Deviation (mV)	Stability Interpretation
<i>Eugenia jambolana</i>	-38.3	-39.4	92.2%	8.67	Excellent stability due to strong negative charge from polyphenols
<i>Tinospora cordifolia</i>	-26.4	-29.8	86.6%	10.1	Good stability , supported by electrostatic + steric stabilization (mucilage)
<i>Boerhavia diffusa</i>	-35.9	-37.5	93.3%	9.89	Highly stable , strong capping by saponins and flavonoids

<i>Tephrosia purpurea</i>	-35.1	-35.1	100%	5.17	Excellent stability , monodisperse distribution indicating uniform capping
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The zeta potential data showed that the plant mediated AgNPs had different surface charges and colloidal stability. The zeta potential of *Eugenia jambolana* was the most negative (−38.3 mV) and it was characterized with excellent electrostatic stability. Also, *Boerhavia diffusa* (−35.9 mV) and *Tephrosia purpurea* (−35.1 mV) were found to have highly stable nanoparticles with effective surface capping. The value of *Tinospora cordifolia* (−26.4 mV) indicated good but comparatively weaker electrostatic stabilization. The high peak areas also showed uniform particle populations and the low deviation for *T. purpurea* showed its surface characteristics and dispersion stability.

3.4.6 Differential Scanning Calorimetry (DSC)

The DSC thermograms of AgNPs of ethanolic extracts of *S. jambolanum*, *B. diffusa*, *T. cordifolia* and *T. purpurea* showed distinct endothermic transitions, which indicated the thermal behaviour and stability of the phytoconstituents.

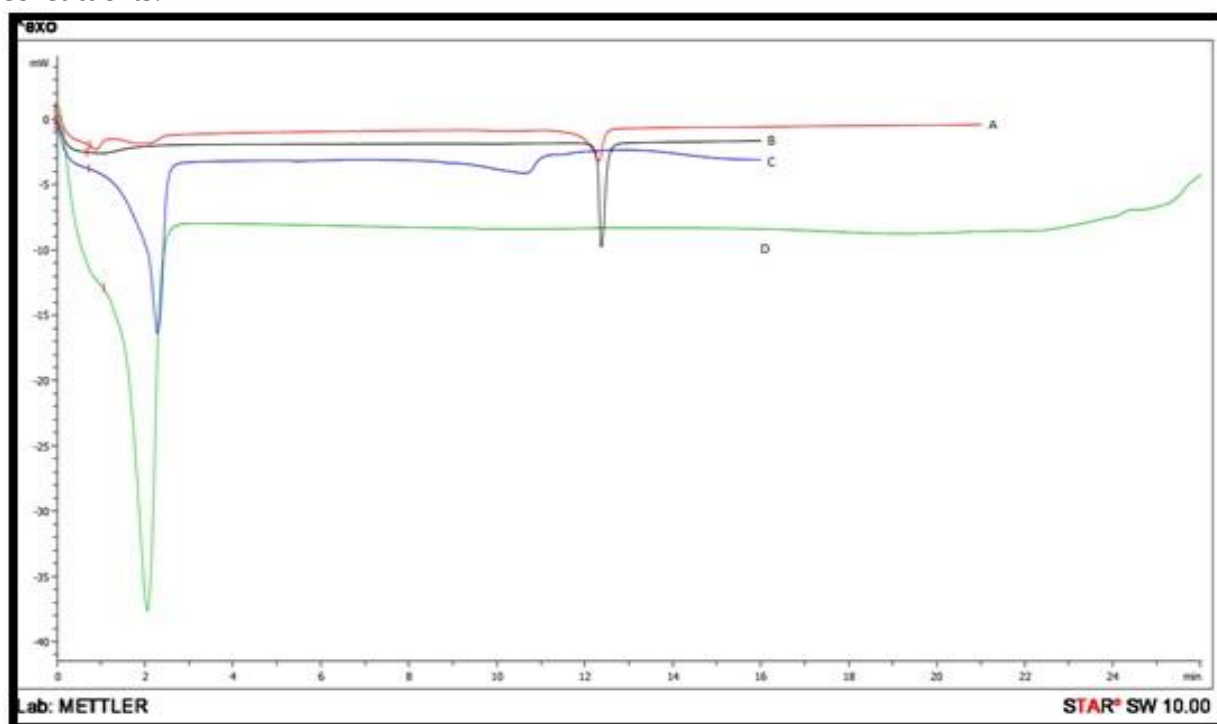


Fig 9: DSC thermograph of Ethanolic extract of a) *Syzygium jambolanum* b) *Boerhavia diffusa* c) *Tinospora cordifolia* d) *Tephrosia purpurea*

Syzygium jambolanum (curve A) had a shallow endothermic peak, suggesting the presence of moderately stable phenolic and flavonoid components with relatively lower moisture loss. *Boerhavia diffusa* (curve B) exhibited more distinct endothermic phenomenon, indicating the melting or degradation of compounds like punarnavine, boeravinones and allied bioactive molecules. *Tinospora cordifolia* (curve C) had a more pronounced endothermic transition, indicating the decomposition of thermolabile components (alkaloids, glycosides and diterpenoids). The endothermic peak for the decomposed proteins, flavonoids and other heat-labile phytochemicals present in the extract was the most intense peak for the sample of *Tephrosia purpurea* (curve D). Results of thermal transitions varied between four extracts, representing differences in compositional complexity, purity and inherent thermal stability of the extracts. In general, the DSC profiles give useful information about the physicochemical properties of each extract, for the formulation that will follow, in particular for heating, drying and lyophilization.

3.5 Antimicrobial and Antioxidant Activity of AgNPs

The antimicrobial and antioxidant activities of the biosynthesized silver nanoparticles (AgNPs) of *Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea* were investigated using standard in vitro assays. The antimicrobial activity was evaluated by disc diffusion method against *Escherichia coli* and *Staphylococcus aureus*, zone of inhibition was measured at 50 and 100 µg/mL..

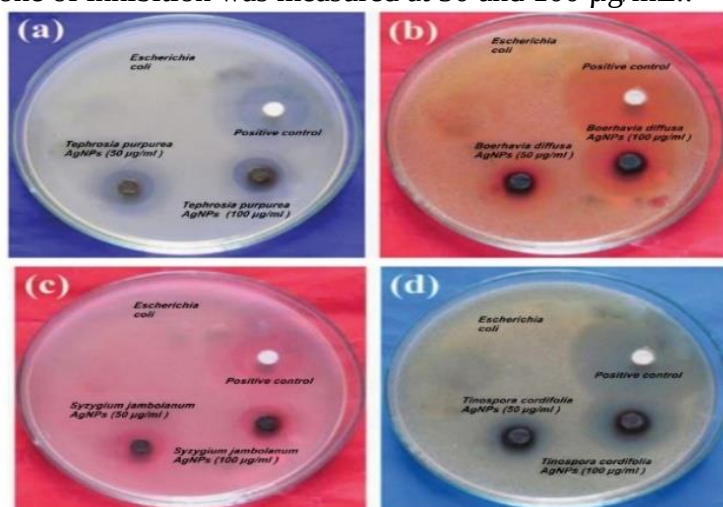


Fig 10: Antimicrobial activities of AgNPs utilizing disc diffusion technique (zone inhibition assay) of *Escherichia coli* against 50 µg/ml and 100 µg/ml.

AgNPs of all the concentrations exhibited concentration-dependent antibacterial activity, and *T. cordifolia* AgNPs exhibited strong activity against *E. coli* and *S. aureus*. The DPPH radical-scavenging assay was used to determine antioxidant potential. *E. jambolana* AgNPs had the lowest IC₅₀ and highest antioxidant activity among the tested nanoparticles, thus suggesting stronger free-radical-scavenging potential.

Table 5: Zone of Inhibition of AgNPs against *E. coli*

Sr. No.	Plant Extract AgNPs	Zone of Inhibition (mm) at 50 µg/mL	Zone of Inhibition (mm) at 100 µg/mL	Observation
1	Ampicillin	23 mm		Reference standard
2	<i>Tephrosia purpurea</i>	13±1 mm	19±1 mm	Good
3	<i>Boerhavia diffusa</i>	11±1 mm	21±1 mm	Strong
4	<i>Syzygium jambolanum</i>	12±1 mm	18±1 mm	Moderate Good
5	<i>Tinospora cordifolia</i>	14±1 mm	22±1 mm	Very Strong

The results showed that zone of inhibition was found to be concentration dependent for all the AgNPs in which the zone of inhibition was found to increase at 100 µg/mL than at 50 µg/mL for all the AgNPs. The AgNPs obtained from *Tinospora cordifolia* exhibited the highest activity with inhibition zone of 14 ± 1 mm and 22 ± 1 mm, respectively, which was very close to the inhibition zone of the reference drug, ampicillin (23 mm). *Boerhavia diffusa* also showed strong activity at 100 µg/mL (21 ± 1 mm). Comparatively moderate antibacterial effects were observed on *Tephrosia purpurea* and *Syzygium jambolanum*. Generally, the results suggest that the efficacy is higher with the increasing concentration of AgNP.

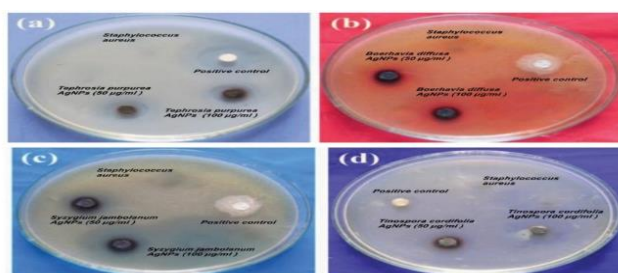


Fig 11: Antimicrobial activities of AgNPs utilizing disc diffusion technique (zone inhibition assay) of *Staphylococcus aureus* against 50 µg/ml and 100 µg/ml.

Table 6: Zone of Inhibition of AgNPs against *S. aureus*

Sr. No.	Plant Extract AgNPs	ZOI (mm) at 50 µg/mL	ZOI (mm) at 100 µg/mL	Observation
1	Ampicillin	18 mm		Positive Control
2	<i>Tephrosia purpurea</i>	16±1 mm	18±1 mm	Good
3	<i>Boerhavia diffusa</i>	11±1 mm	15±1 mm	Moderate Good
4	<i>Syzygium jambolanum</i>	15±1 mm	22±1 mm	Very Strong
5	<i>Tinospora cordifolia</i>	13±1 mm	20±1 mm	Strong

The results showed that all the synthesized AgNPs showed antibacterial activity with the inhibition activity increased at 100 µg/mL as compared to 50 µg/mL concentration. The results showed that *Syzygium jambolanum* AgNPs exhibited the maximum activity against *S. aureus* (15 ± 1 to 22 ± 1 mm), which is greater than the activity of ampicillin (18 mm), *Tinospora cordifolia* (13 ± 1 and 20 ± 1 mm) and *Tephrosia purpurea* (15 ± 1 and 18 ± 1 mm), while *Boerhavia diffusa* showed comparatively moderate activity.

3.6 DPPH Antioxidant Activity

Table 7: Antioxidant Activity of Synthesized AgNPs (DPPH Assay)

AgNPs	% Inhibition @100 µg/ml	IC ₅₀ (µg/mL)
AgNP-EJ	84.6 ± 2.3	36.2
AgNP-TC	76.4 ± 2.5	42.5
AgNP-BD	72.9 ± 2.9	46.1
AgNP-TP	70.8 ± 3.2	48.3
Ascorbic Acid	91.4 ± 2.0	24.6

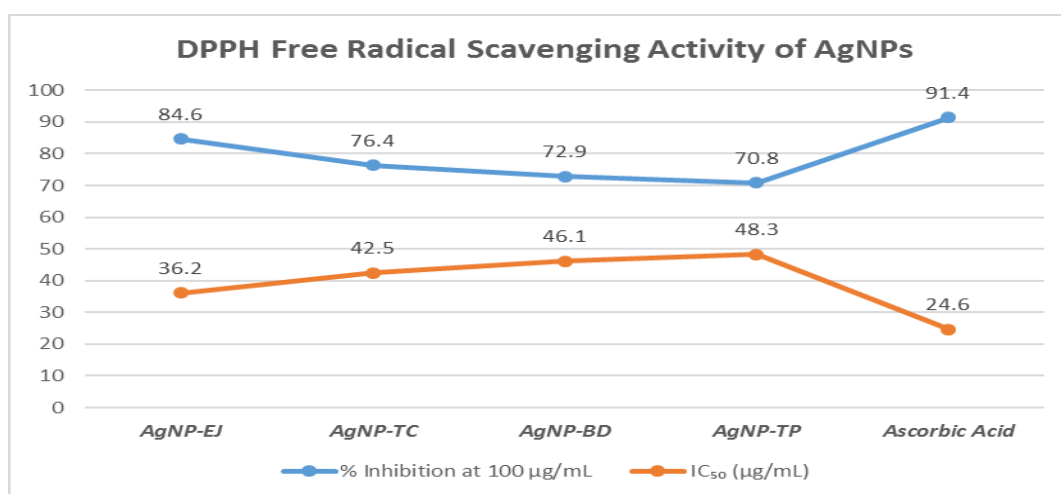


Fig 12: Graphical Presentation of antioxidant activity

The free-radical scavenging antioxidant ability of the green-synthesized silver nanoparticles (AgNPs) was assessed by DPPH free-radical scavenging assay. AgNPs showed a significant antioxidant activity in all four preparations, which increased with the concentration of nanoparticles. The AgNPs obtained from *Eugenia jambolana* displayed the highest DPPH radical-scavenging activity ($84.6 \pm 2.3\%$) followed by AgNPs from *Tinospora cordifolia* ($76.4 \pm 2.5\%$), *Boerhavia diffusa* ($72.9 \pm 2.9\%$) and *Tephrosia purpurea* ($70.8 \pm 3.2\%$) at concentration of 100 $\mu\text{g/mL}$. The corresponding IC_{50} values were 36.2, 42.5, 46.1, and 48.3 $\mu\text{g/mL}$, respectively. Ascorbic acid showed $91.4 \pm 2.0\%$ inhibition with an IC_{50} of 24.6 $\mu\text{g/mL}$. So, the *E. jambolana* AgNPs exhibit best antioxidant activity of the synthesized nanoparticles.

3.7. Lyophilization of AgNPs

Clear differences in the color and texture of the freeze-dried silver nanoparticles were observed for the four plant extract used, which suggests the differences of phytochemical content and surface chemistry of nanoparticles. The *Boerhavia diffusa* AgNPs appeared as brownish-grey powder in which the particles were evenly distributed. This indicates that reduction and stabilization of the particles by the extract, rich in flavonoids and alkaloids, was very good. The *Tinospora cordifolia* AgNPs had a reddish-brown hue that was typical of diterpenoids and glycosides, which probably helped with strong capping and stability. However, the powder of *Syzygium jambolanum* AgNPs was dark, close to black in colour. This is because they are rich in polyphenols which produce very well reduced and colourful nanoparticles. A brown to dark-brown colour for the *Tephrosia purpurea* AgNPs was observed, and the reason for this was that they were rich in flavonoids and phenolics. The capability of the lyophilisation method to obtain dry, free-flowing powders without any apparent aggregation indicates that the process is successful and that the colloidal AgNPs can be stored for a longer period with the possibility to be incorporated into the polyherbal Polyherbal AgNPs-tablet formulation.

Table 8: Characteristics of Freeze-Dried AgNPs

Sr. No.	Plant Extract AgNPs	Color After Lyophilization	Texture / Appearance
1	<i>Boerhavia diffusa</i>	Brownish grey	Fine, smooth powder
2	<i>Tinospora cordifolia</i>	Reddish-brown	Soft, uniform powder
3	<i>Syzygium jambolanum</i>	Black	Thick, dense powder
4	<i>Tephrosia purpurea</i>	Brown to deep brown	Grainy but free flowing

The lyophilized AgNPs prepared from all four plant extracts exhibited significant differences in colour and texture depending on the phytochemical makeup and surface chemistry of the nanoparticles. The AgNPs synthesized by *Boerhavia diffusa* were brownish-grey in color and were fine powdered which showed uniform stabilization of the particle by its flavonoids and alkaloids. The powder of *Tinospora cordifolia* was found to be reddish-brown, soft and uniform, which could be attributed to glycosides and diterpenoids, which are essential for efficient capping of nanoparticles. *Syzygium jambolanum* produced a good dark brown powder, which has a large number of tannins and anthocyanins, and thus good reducing power. In contrast, *Tephrosia purpurea* produced a brown to deep brown, coarse and loose powder, suggesting a stable formation of nanoparticles in moderate polyphenolic conditions. More importantly, the lyophilisation of these AgNPs not only helped in improving their stability and shelf life but also enabled the incorporation of these AgNPs into polyherbal formulations for antidiabetic potential as each of these plants individually has been traditionally recognised for their glucose lowering potential.

3.8 Characterization of Lyophilization of AgNPs

The synthesized AgNP dispersions were then lyophilized to make stable dry AgNP preparations for storage and formulation. The lyophilized AgNPs were characterized to evaluate the crystalline structure and structural integrity of the AgNPs following lyophilization. The observation of the crystalline nature of the nanoparticles was confirmed by X-ray diffraction (XRD).

3.8.1. X-Ray Diffraction (XRD)

The lyophilized AgNPs were analyzed to determine the crystalline structure and the phase characteristics using X-ray diffraction analysis. The diffraction pattern was analysed for reflections of face centered metallic silver. The peaks observed gave insight into the crystallinity, the orientation of the crystal and the preservation of the structure of the nanoparticles after lyophilization. [25]

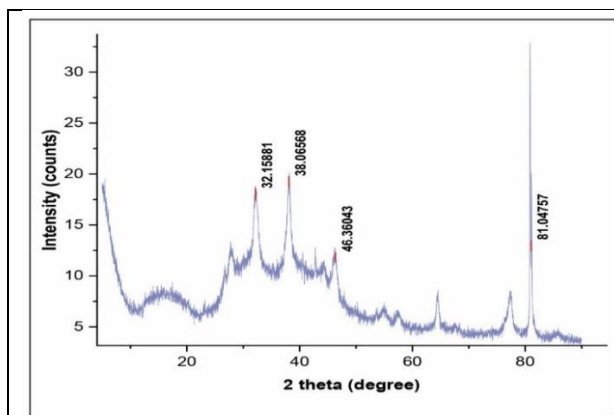


Fig 13: XRD of *Boerhavia diffusa* Lyophilized AgNPs

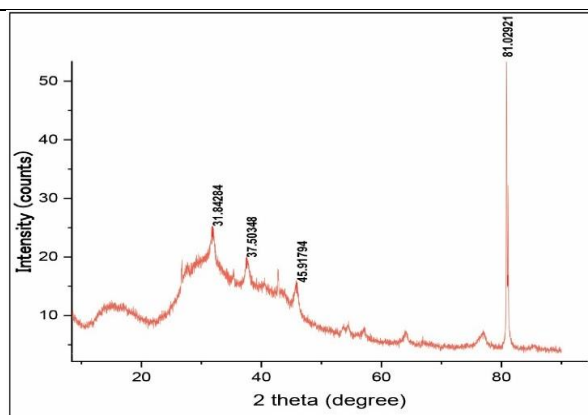


Fig 14: XRD of *Tinospora cordifolia* Lyophilized AgNPs

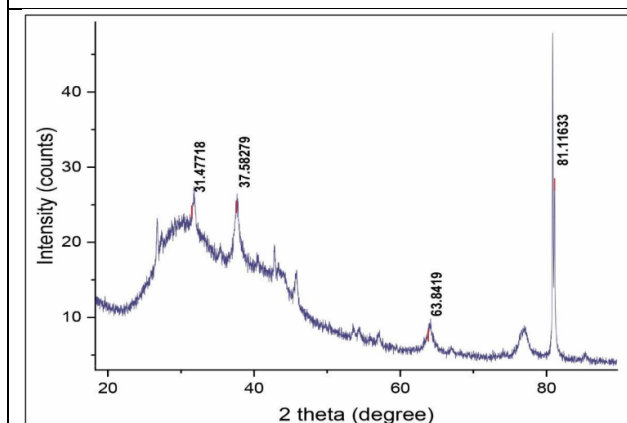


Fig 15: XRD of *Syzygium jambolanum* Lyophilized AgNPs

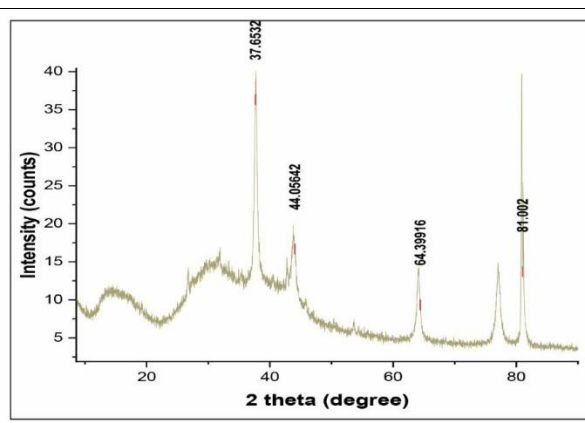


Fig 16: XRD of *Tephrosia purpurea* Lyophilized AgNPs

This is a case of XRD reflections coming from crystalline silver nanoparticles after lyophilization. The strong peak at ~37-38° with a corresponding plane of (111) corresponds to the characteristic structure of fcc metallic Ag⁰. The reflections at additional positions at 44-45°, at 63-65° and at 80-82° are the (200), (220) and (311) planes, respectively, which further confirms the crystalline nature of the synthesized nanoparticles. There is a minor peak at 31–33° which might be due to the presence of phytochemical residues or capping related components.

Table 9: XRD Peak Interpretation for Lyophilized AgNPs

2θ (Approx.)	Crystal Plane (hkl)	Phase / Structure	Significance
31–33°	(110) / secondary planes	Possible metabolite-associated peak	Often linked to organic residues or mixed phytochemical interaction during capping.
37–38°	(111)	fcc silver (Ag ⁰)	Most intense peak; indicates dominant crystal orientation and successful biosynthesis.

44–45°	(200)	fcc silver (Ag ⁰)	Confirms crystal growth and stability of metallic silver.
63–65°	(220)	fcc silver (Ag ⁰)	Supports multi-plane crystallinity, showing well-developed nanoparticles.
80–82°	(311)	fcc silver (Ag ⁰)	Higher-order reflection confirming high crystallinity and purity.

Table 9 shows the typical XRD peaks of the lyophilized crystalline silver nanoparticles. The peak at around 37–38° corresponding to the (111) plane is prominent and it is the characteristic fcc structure of the metallic Ag⁰. There are other reflections at 44–45°, 63–65° and 80–82° which match the (200), (220) and (311) planes, respectively, thereby exhibiting the crystallinity of the synthesized nanoparticles. A minor peak is observed around 31–33° which could correspond to some phytochemical residues or the components related to capping. In general the results show that the crystallinity is preserved after lyophilization.

Table 10: Combined Formulation of Polyherbal AgNPs.

Ingredient	F1	F2	F3	F4
Mg				
<i>Eugenia jambolana</i> AgNPs	30	20	10	40
<i>Tinospora cordifolia</i> AgNPs	20	30	40	10
<i>Boerhavia diffusa</i> AgNPs	10	40	20	30
<i>Tephrosia purpurea</i> AgNPs	40	10	30	20
Total	100 mg	100 mg	100 mg	100 mg

Four polyherbal AgNP formulations were prepared by the use of *Eugenia jambolana* AgNP, *Tinospora cordifolia* AgNP, *Boerhavia diffusa* AgNP and *Tephrosia purpurea* AgNPs respectively and the composition of all the formulations was presented in Table 10. The total amount of AgNPs in each formulation was 100 mg; however, the percentage of each individual AgNP varied. F1 had the highest percentage of *T. purpurea* AgNPs, F2 had the highest percentage of *B. diffusa*, F3 had the highest percentage of *T. cordifolia* and F4 had the highest percentage of *E. jambolana*. Using these formulations, the antidiabetic potential of these formulations could be compared.

3.9. In Vitro Antidiabetic Activity

The four combined polyherbal AgNP formulations (F1– F4) were tested for in-vitro antidiabetic activity through the study of α -amylase and α -glucosidase inhibition. The formulations were comprised of varying amounts of AgNPs synthesized using *Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea*. The individual AgNPs had already exhibited significant biological activity, especially AgNP-EJ that exhibited the highest DPPH radical-scavenging activity ($84.6 \pm 2.3\%$) and lowest IC₅₀ value (36.2 μ g/mL) and AgNP-TC exhibited strong activity against *E. coli* and *S. aureus*. Hence, a combined formulation was tested to see if any of the various nanoparticle preparations would help to increase the inhibition of carbohydrate-digestive enzymes.

3.9.1. α -Amylase Inhibition Activity

The four formulations showed concentration dependent α -amylase inhibitory effect. The inhibitory activity was dose responsive as it increased with concentration. F4 had high inhibitory activity while F1 had the highest α -amylase inhibition among the formulations. F1 exhibited the highest inhibition followed by F4, F3 and F2 at a concentration of 200 μ g/mL. The higher activity of F1 might be correlated with its composition of the four plant AgNPs particularly the high ratio of *Eugenia jambolana* AgNPs (30 mg) and *Tinospora cordifolia* AgNPs (20 mg) which had proved comparatively higher antioxidant activity in individual nanoparticle

evaluation.

Table 11. α - Amylase Inhibition Activity of Combined Polyherbal AgNP Formulations

Formulation	50 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$	200 $\mu\text{g/mL}$
F1	52.4 $\pm$ 1.5	68.7 $\pm$ 1.7	81.6 $\pm$ 1.9
F2	43.8 $\pm$ 1.3	57.6 $\pm$ 1.6	70.8 $\pm$ 1.7
F3	46.2 $\pm$ 1.4	60.4 $\pm$ 1.6	73.1 $\pm$ 1.8
F4	47.6 $\pm$ 1.4	61.8 $\pm$ 1.7	75.4 $\pm$ 1.8
Acarbose	65.8 $\pm$ 1.2	78.4 $\pm$ 1.4	89.8 $\pm$ 1.5

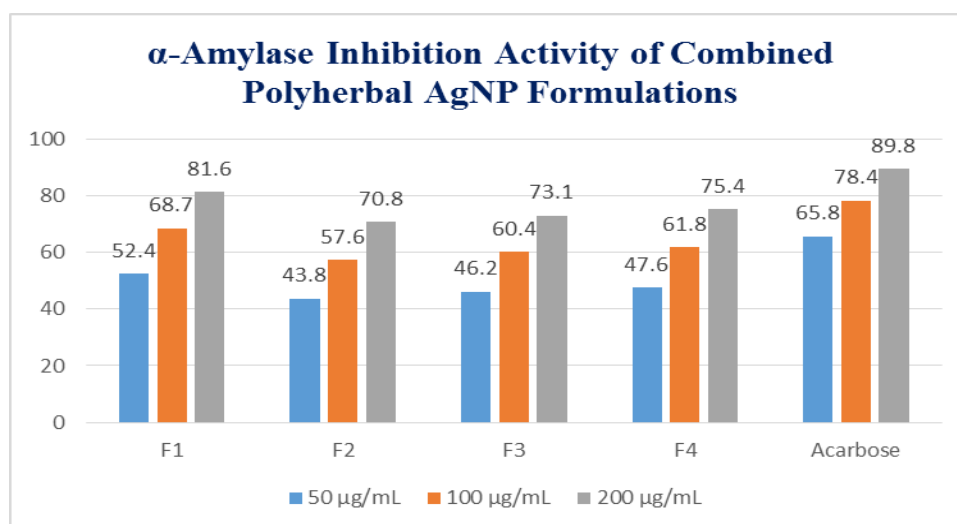


Figure: 17: α -Amylase Inhibition Activity of Combined Polyherbal AgNP Formulations

All four combined polyherbal AgNP formulations were found to inhibit α -amylase in a concentration dependent manner as shown in Table 11 and Figure 17. F1 showed the highest inhibitory activity with 52.4% at 50 $\mu\text{g/mL}$ to 81.6% at 200 $\mu\text{g/mL}$, followed by F4, F3 and F2. Acarbose exhibited a comparatively higher inhibitory activity of 65.8%, 78.4% and 89.8% respectively. These results suggest that rise in the formulation concentration led to higher enzyme inhibition where the F1 formulation exhibited the superior inhibitory potential against α -amylase among the formulations. The concentration-dependent inhibition is in line with known in vitro α -amylase screening methods.

3.9.2. α -Glucosidase Inhibition Activity

The same trend of concentration dependent inhibition was seen in the inhibition of α -glucosidase. The formulations were combined and showed a concentration dependent inhibitory activity. F1 was the most active formulation of the four and F4 had high α -glucosidase inhibitory activity. At 200 $\mu\text{g/mL}$, F1 produced 85.4 $\pm$ 1.9% inhibition, followed by F4 (79.8 $\pm$ 1.8%), F3 (77.2 $\pm$ 1.8%) and F2 (74.6 $\pm$ 1.7%).

Table 12. α -Glucosidase Inhibition Activity of Combined Polyherbal AgNP Formulations

Formulation	50 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$	200 $\mu\text{g/mL}$
F1	57.8 $\pm$ 1.5	72.6 $\pm$ 1.7	85.4 $\pm$ 1.9
F2	48.9 $\pm$ 1.3	62.8 $\pm$ 1.5	74.6 $\pm$ 1.7
F3	51.0 $\pm$ 1.4	65.4 $\pm$ 1.6	77.2 $\pm$ 1.8
F4	52.7 $\pm$ 1.4	67.9 $\pm$ 1.6	79.8 $\pm$ 1.8
Acarbose	74.5 $\pm$ 1.3	85.7 $\pm$ 1.4	93.6 $\pm$ 1.4

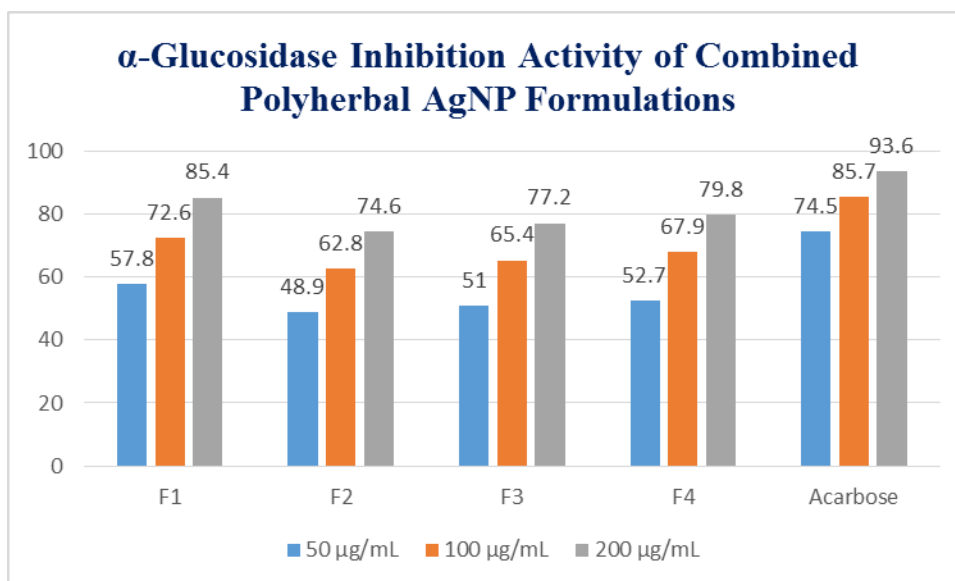


Figure 18: α-Glucosidase Inhibition Activity of Combined Polyherbal AgNP Formulations

Table 12 and Figure 18 show that the α-glucosidase inhibitory activity of all the combined polyherbal AgNP formulations increased with increasing concentration. F1 showed maximum activity with 57.8% at 50 µg/mL and 85.4% at 200 µg/mL followed by F4, F3 and F2. Acarbose was more inhibitory activity than the formulations with 74.5% and 93.6% at 50 and 200 µg/mL respectively. Overall, F1 exhibited the strongest α-glucosidase inhibitory activity, suggesting higher inhibitory activity on carbohydrate hydrolysis and subsequent glucose release.

Table 13. Comparative IC₅₀ Values of Combined Polyherbal AgNP Formulations

Formulation	α-Amylase IC ₅₀ (µg/mL)	α-Glucosidase IC ₅₀ (µg/mL)
F1	105.7 ± 3.1	91.4 ± 2.5
F2	146.8 ± 4.1	129.5 ± 3.8
F3	134.6 ± 3.8	119.3 ± 3.5
F4	128.4 ± 3.6	112.7 ± 3.2
Acarbose	54.6 ± 1.9	38.7 ± 1.5

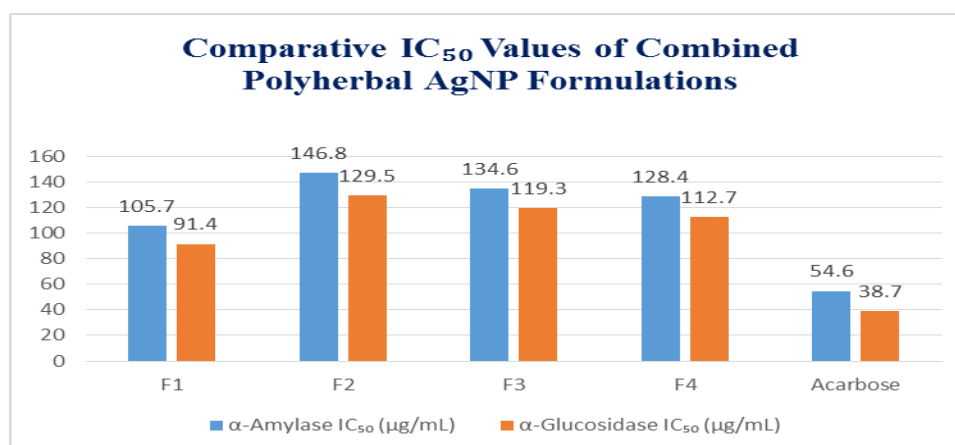


Figure 19: Comparative IC₅₀ Values of Combined Polyherbal AgNP Formulations

Table 13 and Figure 19 show the comparative IC₅₀ values of combined polyherbal AgNP formulations for α -amylase and α -glucosidase. As observed, F1 showed the lowest IC₅₀ values (105.7 μ g/mL for α -amylase and 91.4 μ g/mL for α -glucosidase) among the experimental formulations, demonstrating the most potent inhibitory activity. The IC₅₀ value of F4, F3 and F2 were in ascending order. Acarbose was the most potent with an IC₅₀ value of 54.6 μ g/mL compared to 38.7 μ g/mL. The smaller the IC₅₀ value, the greater the enzyme inhibition.

DISCUSSION

The present study revealed that, the green-synthesized silver nanoparticles (AgNPs) synthesized using *Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa*, and *Tephrosia purpurea* exhibited unique biological and physicochemical properties. The variation among the nanoparticles could be attributed to the phytochemical constituents of the corresponding plant extracts that may affect the reduction, capping, surface properties, morphology and biological activity of the nanoparticles.

The antioxidant results were quite diverse for the different AgNPs produced. At 100 μ g/mL, *E. jambolana* AgNPs exhibited the highest DPPH radical-scavenging activity (84.6 $\pm$ 2.3%), followed by *T. cordifolia* (76.4 $\pm$ 2.5%), *B. diffusa* (72.9 $\pm$ 2.9%), and *T. purpurea* (70.8 $\pm$ 3.2%). The corresponding IC₅₀ values were 36.2, 42.5, 46.1, and 48.3 μ g/mL, respectively. While ascorbic acid exhibited higher activity (91.4 $\pm$ 2.0%; IC₅₀ 24.6 μ g/mL), the high activity of *E. jambolana* AgNPs suggests that phytochemicals derived from plants make a significant contribution to antioxidant activity.

The formulations also showed good enzyme inhibitory activity when combined. At 200 μ g/mL, F1 produced the highest α -amylase inhibition (81.6 $\pm$ 1.9%), followed by F4 (75.4 $\pm$ 1.8%), while F1 also showed the highest α -glucosidase inhibition (85.4 $\pm$ 1.9%), followed by F4 (79.8 $\pm$ 1.8%). F1 exhibited 30 mg of *E. jambolana* AgNPs and 20 mg *Tinospora cordifolia* which might suggest that the presence of this comparatively strong antioxidant activity played a role in the overall performance of the combination.

The results indicate that mixture of AgNPs from various medicinal plants might exhibit synergistic biological activity. The results of F1 were superior to that of F4, F3, F2 revealing that formulation composition can significantly affect the antidiabetic enzyme inhibition. But more mechanistic and dose-response studies would be necessary to determine the contribution of individual phytochemicals and to verify the therapeutic relevance of these findings.

CONCLUSION

In the current investigation, the researchers find that green synthesis of silver nanoparticles (AgNPs) using traditionally used antidiabetic medicinal plants such as *Eugenia jambolana*, *Tinospora cordifolia*, *Boerhavia diffusa* and *Tephrosia purpurea* is a promising approach. The synthesized nanoparticles exhibited unique physicochemical properties, which were influenced by the plant derived phytochemicals for the formation, stabilization, morphology and surface properties of nanoparticles. The highest antioxidant activity among the individual AgNPs was observed for *E. jambolana* which produced DPPH radical-scavenging activity of 84.6 $\pm$ 2.3% at 100 μ g/mL, followed by *T. cordifolia* (76.4 $\pm$ 2.5%), *B. diffusa* (72.9 $\pm$ 2.9%) and *T. purpurea* (70.8 $\pm$ 3.2%). There was also antimicrobial activity against *E. coli* and *S. aureus*, but this varied depending on the plant origin and concentration. Besides, the blended polyherbal AgNP formulations showed good α -amylase and α -glucosidase inhibitory properties. These results suggest that plant mediated AgNPs could be a potential platform for further evaluation of the polyherbal nanotechnology approach for antidiabetic activity especially F1 combination. Further in-vivo, in-mechanistic, toxicity and formulation-stability studies, however, are required prior to any consideration of a therapeutic use.

Conflict of interest

The authors have no conflict of interest to declare.

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Authors Contribution

Asif Husain Ansari: Conceptualization, writing original draft; methodology, data curation, formal analysis, investigation, writing review and editing.

Sufiyan Ahmad: Conceptualization, writing original draft; Funding acquisition, methodology, data curation, formal analysis, investigation, project administration, supervision, writing review and editing.

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