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Tamanna Malik
Department of Chemistry, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India

VK Madan
MAP Section, Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India

Corresponding Author:
Tamanna Malik
Department of Chemistry, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India

Rapid and green synthesis of metallic/bimetallic nanoparticles using aqueous plant extract of Triphala with their excellent bio-efficacies

Tamanna Malik and VK Madan

Abstract
The synthesis of nanoparticles of variable sizes, shapes, chemical compositions and controlled dispersity and their potential use for human benefits are principally concerned with Nanotechnology. Although pure, well defined nanoparticles may be synthesized from the conventional chemical and physical methods, these methods are pretty expensive and critically dangerous to the environment. A wide range of microorganisms, plant extract or plant biomass can possibly be used as an alternative to chemical and physical methods for the fabrication of nanoparticles in an eco-friendly manner. Engaging plants in the synthesis of nanoparticles are evolving as beneficial in comparison to the microbes due to the presence of broad variability of bio-molecules in plants which may act as capping and reducing agents and thus increases the speed of reduction and stabilization of nanoparticles. Secondary plant metabolites (proteins, fatty acids, sugars, enzymes, phenolic, etc.) in the sources are intensely involved in both bio reduction of metallic ions to NPs and their stabilization. The biological methods opted for the synthesis of nanoparticles is safe, dynamic and energy efficient. NPs synthesized via biological methods are found to be more stable than others methods. Furthermore, functional groups i.e. polyols and carboxylic acid have also been alleged to be liable for the synthesis of NPs. Here we have reconnoitred an ingenious contribution for synthesis of silver, gold and bimetallic nanoparticles using fruit extract of the chosen medicinal plants. The procedure for the fabrication of stable AgNPs, AuNPs and Ag@AuNPs is rapid, simple and viable. Synthesized nanoparticles were characterized by various methods, viz. FESEM, TEM, XRD, EDX, DLS, UV–Vis and FTIR. Among various metal nanoparticles, AgNPs and AuNPs have several effective applications as antimicrobial, sensors and detectors besides their biomedical applications.

Keywords: Nanoparticles, extracts, metabolites, characterization, antimicrobial

Introduction
Nanotechnology has emerged as a promising multidisciplinary field with several applications which includes diagnostics, imaging and structural design. Various chemical and physical approaches are used in the synthesis of nanoparticles, which brings many threats to the ecosystem. To beat these threats, sustainable routes for the synthesis of nanoparticles were implemented. To opt for plants and plant metabolites out of other biological methods for synthesizing nanoparticles is the reason for which researchers are trying to explore mechanisms of bioreduction and metal ions uptake by plants. Noble metallic NPs, like Ag, Au, Pd, have attracted remarkable interest with in the scientific community [1, 2]. Here, green chemistry was employed for the synthesis of metallic/bimetallic nanoparticles (AgNPs, AuNPs/AgAuNPs) using fruit extracts of medicinal plants. Green synthesis is the most captivating and smart substitute to chemical synthesis as it offers more advantages. The secondary metabolites present in the plants are used as reducing and capping agents, which are non-toxic and eco-friendly. The process is moderately simple and cost-effective. A gold or silver salt is basically reduced by bio-molecules (phenols, alkaloids, proteins, etc.) present in the extracts of the medicinal plants.

The plant resources of our country have made a virtuous contribution to the progress of ancient Indian Material Medica. All the medicinal plants in one or the other way are utilized in the treatment of human diseases in traditional, folklore, Unani or Ayurveda system. Nanomaterials are seen as solution to numerous technological and environmental challenges within the field of drugs [3]. Within the contexts of worldwide efforts to the increasing demand of NPs must be amid green synthesis. It has been stated that NPs synthesized via greener route are non-toxic to humans and most effective against pathogens and other eukaryotic microorganisms at very low concentrations and that too with minimal side effects.

Metabolites such as proteins, fatty acids, sugars, enzymes, phenolics, tannins and flavonoids in these sources are responsible for both bio reduction of metal ions and their stabilization. Furthermore, functional groups i.e. polyols and carboxylic acid have also been alleged to be liable for the synthesis of NPs. The synthesis of nanoparticles via biological methods is safe, dynamic and energy efficient. NPs synthesized via biological methods are more stable than others methods. In ancient days also, particles were synthesized in eco-friendly method. Amongst these examples, gold as gold ash was utilized in Indian Ayurvedic/native medicine, and it had been called as globular gold particles whose size were upto 56-57 nm [4]. The development of green processes for the synthesis of NPs has been evolving into an essential branch of nanotechnology as green nanotechnology deals with the safe and eco-friendly methods of nanomaterials fabrication which might be considered as an alternate for the traditional physical and chemical methods.

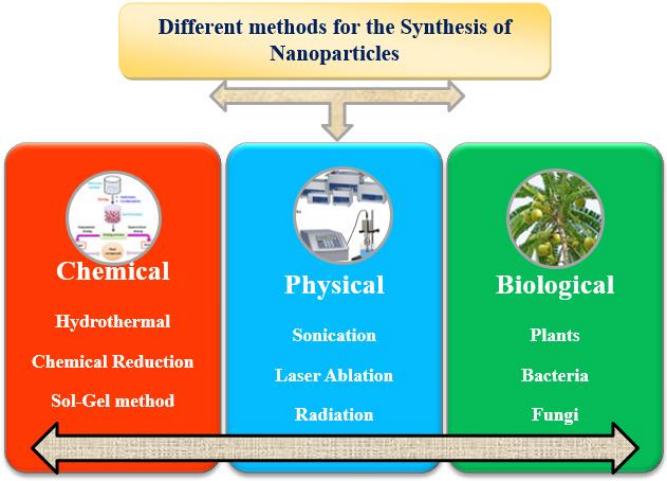

Fig 1: Different mode of synthesis of nanoparticles

The search of benign methods of synthesizing nanoparticles and antibacterial, antioxidant, and antitumor activity of natural products have been initiated recently under the studies of Green Chemistry. Biological methods for the synthesis are the viable alternative for the development of metallic nanoparticles where plant extract can be used for the synthesis of nanoparticles in the absence of any chemical ingredients. Amongst various noble metal nanoparticles, AgNPs and AuNPs have numerous uses such as antimicrobial agents, sensors and detectors besides their biomedical uses.

Over the past decade, herbal medicine has become a subject of world-wide importance, creating an impression on both world health and international trade. Medicinal plants lead a dynamic role in the healthcare system of large sizes of the world's population [5]. Different parts of a plant such as roots, leaves, barks, seeds or flowers of a plant can be used as different herbs [6].

Fig 2: Plant and Triphala powder whose extract was used as a reducing agent in synthesis

Triphala, a well-known Ayurvedic formulation, is used against a number of ailments since ancient times. It is an equi-proportional mixture of fruits of three medicinal herbs, Amla (*Emblica officinalis*), Bahera (*Terminalia belerica*) and Harad (*Terminalia chebula*). Triphala along-with its three fruit constituents show significant antimicrobial activity against several pathogens. Some of the chief potential uses of Triphala are free radical scavenging, antioxidant, anti-inflammatory and anti-carcinogenic.

The major constituents of *P. Emblica* are found to be tannins, flavonoids, and other phenolic compounds. Various ailments, such as anaemia, liver disease, dyspepsia, haemorrhage, jaundice and diarrhoea are cured by the consumption of the fruit of this plant [7]. The extracts of *P. Emblica* extract owns several biological activities, e.g. antimicrobial, anti-inflammatory and analgesic, antipyretic [8, 9].

The Bahera fruit contains condensed and hydrolysable tannins (such as gallic acid, ethyl gallate, and ellagic) as a chief component. *T. belerica* has been extensively used as a laxative and an astringent, and also as traditional medicine for several ailments such as fever, cough, diarrhoea, oral thrush, inflammation, dyspepsia, skin and liver diseases. Other biological activities of the fruit extract have been reported to possess antimutagenic effects, anti-HIV, antimalarial, antifungal and antimicrobial [10-12].

There are 14 hydrolysable tannins in the fruit of *T. chebula viz*. gallic acid, chebulic acid, punicalagin, asuarinin, chebulanin, corilagin, neochebulinic acid, terchebulin, ellagic acid, chebulagic acid, chebulinic acid, 1,6-di-O-galloyl-D-glucose, 3,4,6-tri-O-galloyl-D-glucose, and 1,2,3,4,6-penta-O-galloyl-D-glucose. *T. chebula* has been conventionally used as a laxative, diuretic, cardiotonic, digestive and antiseptic [13].

Here we have explored an inventive contribution for synthesis of silver, gold and bimetallic nanoparticles using fruit extract of Triphala powder: Amla, Bahera and Harad. A rapid, simple and viable method for the development of stable AgNPs, AuNPs and AgAuNPs is explored. In addition, the synthesis of nanoparticles using plant offers other advantages, such as

the utilization of safer and greener solvent (aqueous), decreased use of dangerous reagents (no reducing agent), milder response conditions (room temperature), feasibility, and their adaptability in use for medicinal, surgical, and pharmaceutical applications. Synthesized nanoparticles were characterized by various methods, such as FESEM, TEM, XRD, EDX, DLS, UV–Vis and FTIR.

Materials and Methods

Preparation of plant extract

Eight gram of powdered samples of Triphala powder and its fruits constituents were placed in a filter paper (Whatman No. 1) thimble in a classical soxhlet apparatus fitted with a 250 mL round bottom flask. The solvents (distilled water, methanol, ethanol and acetone) were added up to one and a half siphons i.e. approximately 150 ml. Extraction was performed at boiling temperature of respective solvent, solvent vapours move up to the column and after getting condensed in the condenser part, floods into the chamber housing thimble filled with plant samples. When this chamber was filled completely with solvent the siphon mechanism operates and the solvent containing some part of phytochemicals that got dissolved in solvent; empties this extract into round bottom flask containing solvent. Process was continued for 5h with completion of up to seven to eight cycles through siphon mechanism in case of ethanol as solvent. In case of water as solvent, time required for completion of one cycle was significantly more hence, with water as a solvent extraction was carried out for longer time with the completion of up to seven to eight cycles through siphon mechanism. After the completion of first extraction step, residue in thimble was again extracted twice (each extraction time 2h and 1h, respectively) with suitable amount of respective solvents. Filtrates of each solvent from three extraction steps were pooled and their volumes were noted. Aqueous extracts were used for the synthesis of AgNPs, AuNPs, AgAuNPs and estimation of total sugars, reducing sugars & non-reducing sugars whereas aqueous and other extracts were used for estimation of total phenolics, total flavonoids and for evaluation of DPPH free radical scavenging activity and total antioxidant capacity.

Synthesis of Nanoparticles using greener approach

The required chemicals, silver nitrate ($AgNO_3$) and chloroauric acid ($HAuCl_4.xH_2O$) were purchased from Sigma Aldrich, India. 1mM $AgNO_3$ and 1mM $HAuCl_4.xH_2O$ solution was prepared. In a typical synthesis for silver and gold nanoparticles using plant extract, the extract was taken in a burette and the precursor solutions were kept on a heating stirrer with 450 rpm at a varying temperature range of 60-80ºC in dark. While synthesizing AgAuNPs, equimolar solution of $AgNO_3$ and $HAuCl_4.xH_2O$ was used as the starting precursor for the synthesis of AgAu bimetallic nanoparticles. After the completion of synthesis of nanoparticles, the resulting solution is centrifuged at 8000 rpm for 25 minutes. The remaining solution (supernatant) was discarded with the help of pipette and the sediment is washed repeatedly with distilled water several times and finally with absolute ethanol. The sediment is dried in the oven at 60ºC for 4 hrs. The synthesized nanoparticles were then collected in the sample vial and correctly labeled. These were stored in freeze drying conditions till their characterization and further use/ testing their antimicrobial activity against the available pathogens.

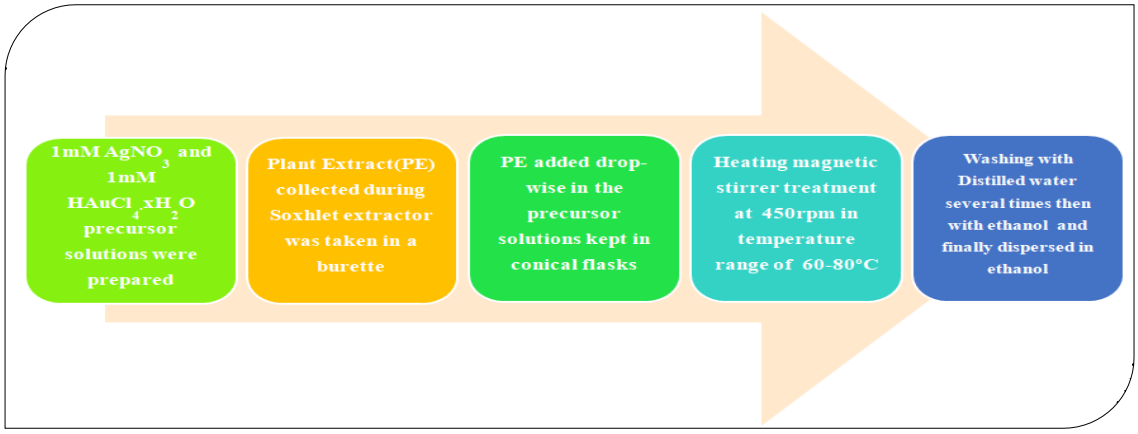

Fig 3: Experimental scheme for the bio-synthesis of nanoparticles using Greener approach

Characterization

X-ray Diffraction (XRD). The experimental samples were assessed in terms of the crystallinity, phase composition, and purity through X-ray diffraction (XRD) analysis on a Bruker D8 Advance diffractometer equipped with Ni-filtered Cu Kα radiation (λ = 1.5418 Å) in the 2θ range of 10−80° at a scanning rate of 0.02° per second. The search and match facility of JCPDS software was used in the determination of different phases present in the synthesized samples. The average particle size of the prepared samples was determined by using Scherrer's equation as follows; $D = 0.94 \lambda/\beta\cos\theta$, where D is the crystal size, λ is the wavelength of X-ray, θ is the Braggs angle in radians and β is the FWHM of the peak in radians.

Field-Emission Scanning Electron Microscopy (FESEM). The surface morphologies of all the synthesized samples were analyzed with the aid of field-emission scanning electron microscopy (FEI QUANTA 3D FEG) which operates at an accelerating voltage of 5 kV assembled with an energy dispersive X-ray spectroscopy (EDS) detector for elemental mapping. A small amount of experimental sample was put on carbon tape adhered to an aluminum stub and sputter-coated by an ultrathin layer of gold prior to inspection to prevent sample charging effects and observed at varied magnifications.

Transmission Electron Microscopy (TEM). TEM studies were employed to achieve detailed insight into the morphology of the as-synthesized nanoparticles. Specimens for TEM investigations were prepared by first dispersing the as-synthesized samples in ethanol using ultra-sonication and dropping one drop of the suspension onto a 400-mesh copper grid, coated with a holey carbon film. FEI Technai G2 20

operated at an accelerating voltage of 200 kV was used to record the images.

UV-Vis Spectroscopic measurement. The absorption spectra of aqueous precursors and synthesized AgNPs were recorded with UV−Vis spectrometer recorded on a Shimadzu UV-2450 spectrometer equipped with an integrating Assembly, over a wavelength range of 300−700 nm.

DLS measurement. The hydrodynamic size distributions and polydispersity index (PDI) of nanoparticles were analyzed by using dynamic light scattering (DLS) instrumentation (Malvern Zetasizer Nano ZS90)

FTIR analysis. The chemical compositions of plant extract and as synthesized nanoparticles (Ag, Au and AgAuNPs) were studied using FTIR spectrometer (Thermo Electron Scientific Nicolet 380). The solutions were characterized within the range 4000–500 cm^{-1} using KBr pellet. For removal of any free biomass residue or unbound extract on the surfaces of the NPs, the NPs were repeatedly washed with distilled water; subsequently the product was centrifuged at 9000 rpm for 30 min and dried. The purified NPs were mixed with KBr powder and pressed into a pellet for measurement. A reference blank KBr pellet was used for making background correction.

Results and Discussion

By suitably modulating the experimental parameters such as $AgNO_3$, $HAuCl_4.xH_2O$ precursor concentration as well as by varying the temperature, the formation of controlled sized silver, gold and bimetallic nanoparticles was achieved. The presently synthesized nanoparticles are thoroughly characterized by XRD, FESEM, TEM, EDS, DLS, UV-Vis and FT-IR studies and provide an ideal platform to study the antimicrobial activity against certain bacteria and fungi. These can be used as a suitable catalyst in various chemical reactions.

The activity results of AgNPs, AuNPs and AgAuNPs obtained with optimized conditions, accompanied by comparison with the individual plant extract which were used as a reducing agent and capping agent during the course of reaction (Triphala powder: Amla, Bahera and Harad) are discussed here.

XRD ANALYSIS

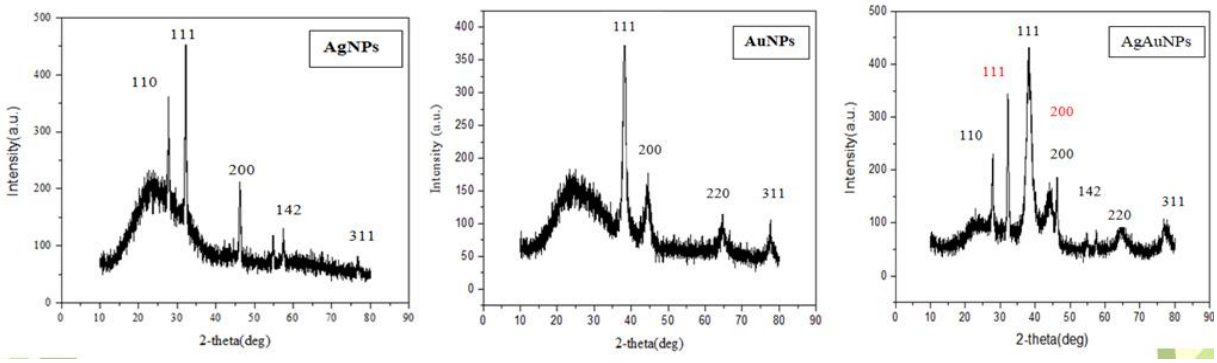

Fig 4: Experimental diffraction patterns of biosynthesized silver and gold nanoparticles samples AuNPs (a), and AgNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

In the XRD pattern of AgNPs, diffraction peaks at 29.16°, 38.13°, 44.21°, 64.47°, 77.37° can be assigned to face-centered cubic (FCC) metallic silver (identified by PDF-2 ref. 4-0783). The intense reflection at 111, in comparison to the other four, may indicate the growth direction of the nanocrystals. It is clear that the AuNPs formed were crystalline in nature. The XRD pattern showed four distinct peaks at 38.16, 44.33, 64.51 and 77.54 which could be indexed to (1 1 1), (2 0 0), (2 2 0) and (3 1 1) planes of cubic AuNPs, respectively, and were in conformity with the JCPDS database (JCPDS no. 00-004-0784). In case of AgAuNPs, the diffraction peaks of both Ag and Au are observed. The hump observed at 2-theta (22º) in all the three diffraction patterns is attributed to the plant extract/residue. No other additional reflections other than the Ag lattice reflections clearly indicates that the green synthesized Ag NP lattice was unaffected by other molecules in the plant extract. Elemental silver and gold peak found in the EDX study and elemental mapping which are in accordance with the XRD results. The average particle size of as-synthesized AgNPs obtained using the green approach varied between 40–70 nm as calculated by using Debye-Scherrer equation.

Fesem Analysis

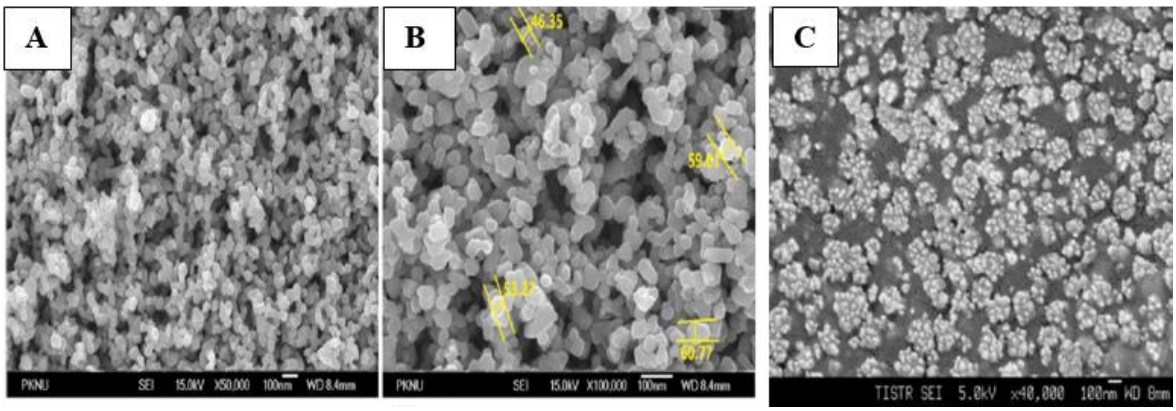

Fig 5: Experimental FESEM images of biosynthesized silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

Morphologies and chemical composition of prepared materials were analyzed by FESEM studies. The synthesized nanoparticles were of polygonal shape, mainly spherical. The morphological alignment of the silver nanoparticles is not changed after the accumulation of gold nanoparticles during the synthesis of bimetallic nanoparticles. It can be predicted that Ag@Au core-shell heterostructure is formed. Clear evidence of core−shell formation is indicated by the observed shift of the XRD diffraction peaks to higher angles in the core−shell structures which is due to lattice shrinkage of the Ag core with the growth of Au shell. The elements present in the nanoparticles were further confirmed using TEM-EDX and elemental mapping. The size of as synthesized nanoparticles was found to be in the range of 40 to 70 nm.

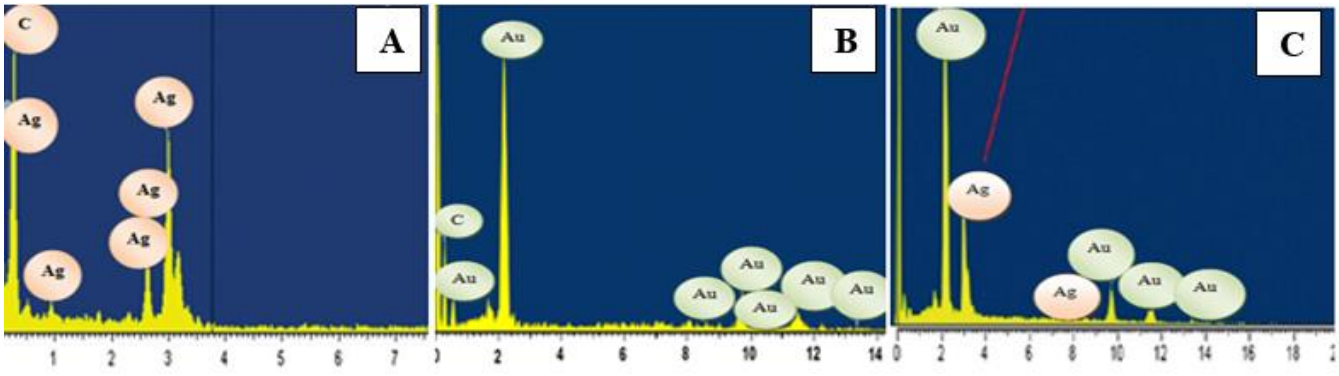

Fig 6: EDX Spectra of biosynthesized silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

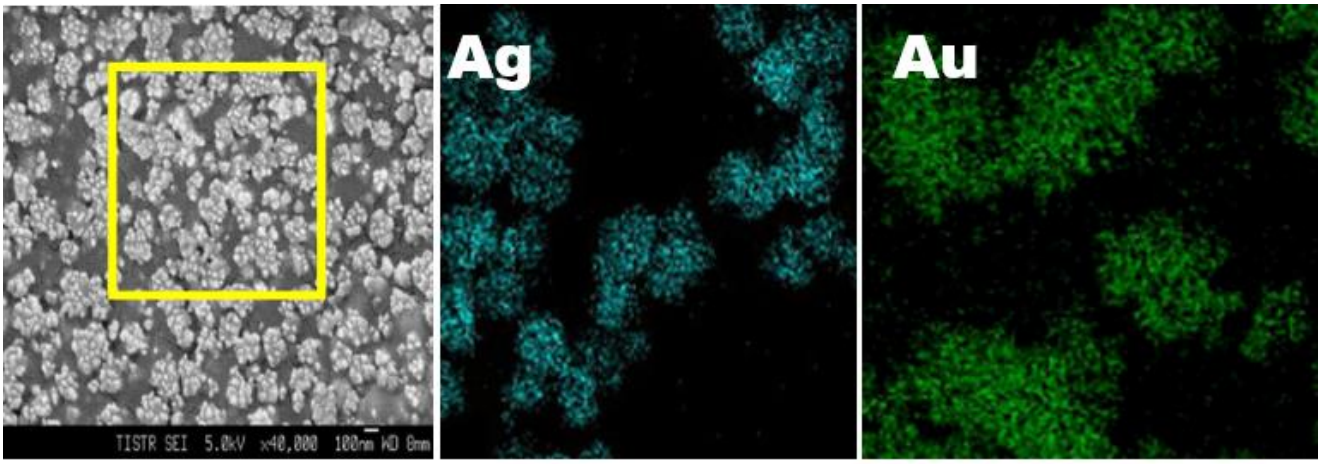

Fig 7: Display of the Ag@Au core-shell nanomaterials with elemental mapping results biosynthesized silver and gold nanoparticles in AgAuNPs using aqueous extract of Triphala

X-ray EDS was conducted during HRTEM analysis for elemental analysis. The result represents the presence of Au and Ag in the samples of as synthesized nanoparticles.

TEM (Transmission Electron Microscopy) Studies

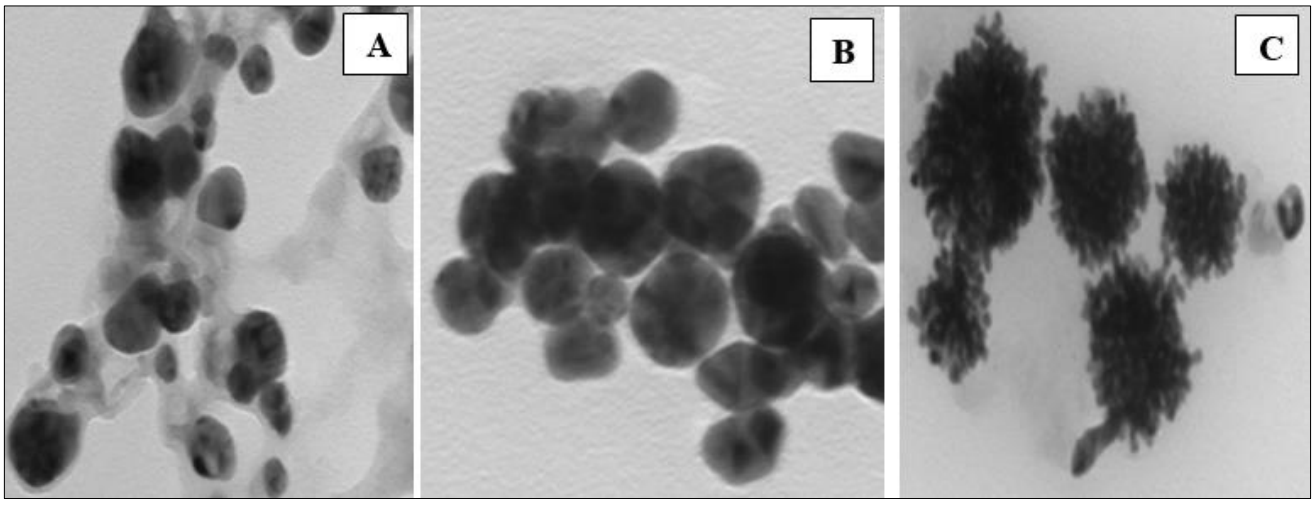

Fig 8: Experimental HRTEM images of silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

The morphology and particle size of the green synthesized NPs were studied by TEM. Figure 8 shows the as-synthesized NPs. Figure 8A and B clearly show the spherical morphology of the well dispersed NPs, with a size range of 40–60 nm. The lateral anisotropic modifications (irregularities in the shapes) in the nanostructures were formed due to the lack of protective biomolecules in the plant extract used. Some irregularities in the morphologies of the synthesized NPs indicates that the plant were effectively involved in the synthesis as well as controlled their formation.

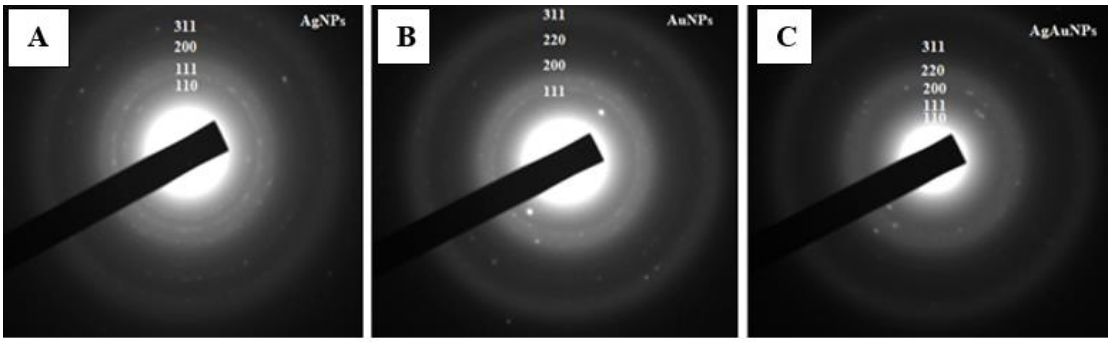

Fig 9: Experimental SAED pattern of silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

SAED (Selected area electron diffraction) analysis of the biosynthesized nanoparticles showed concentric diffraction rings. The SAED data indicates that the green synthesized nanoparticles are of polycrystalline nature. The darker and lighter region seen in the HRTEM shows the different planes and symmetry of the nanoparticles. The SAED pattern shows that the formed NPs were polycrystalline in nature and the patterns were assigned as (1 1 1), (2 0 0), (2 2 0), (3 1 1) and (2 2 2) Bragg's reflections which are in strong agreement with the XRD pattern obtained as earlier. The biosynthesized nanoparticles were of polyhedral morphology.

Dynamic light scattering/ Particle size analyzer

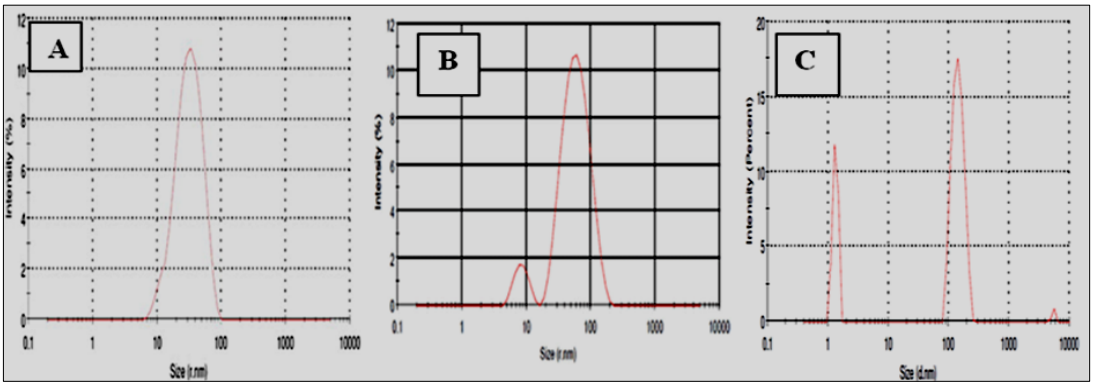

Fig 10: Particle size distribution by intensity of silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

The average particle size distributions of the bio synthesized NPs are in the range of 40-60 nm, which is in good correlation with Electron micrographs and XRD spectra. The histogram of particle size distribution of the biosynthesized NPs obtained after the reaction time at pH 7.0. The particle size range was determined to be 45–70 nm by representative FESEM micrographs Stability is another important parameter ensuring the area of application in which nanoparticles can be incorporated. The stability of silver nanoparticles varies as per the synthesis technique, storage conditions and also with stabilizing and capping agent used in the synthesis process. The negative zeta-potential along with the particle size distribution confirms for the stability, well dispersion of as synthesized nanoparticles due to the presence of secondary metabolites which act as the reducing and capping agent. There may be some difference in the size of these synthesized nanoparticles from electron micrographs to DLS due to particles agglomeration.

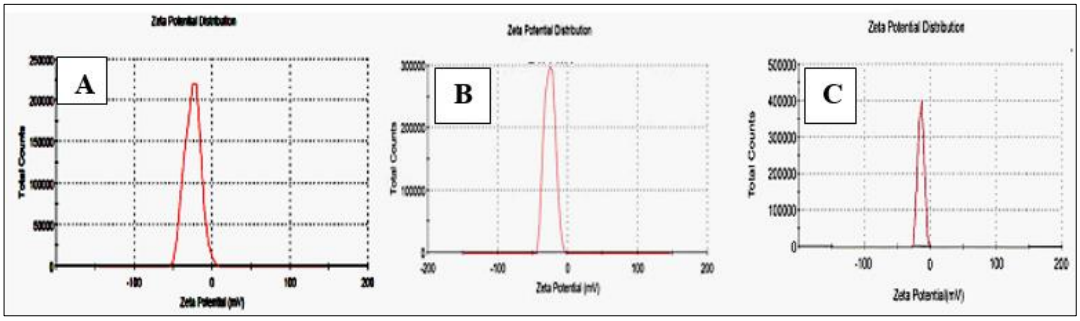

Fig 11: Zeta potential statistics of silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using Triphala aqueous extracts using aqueous extract of Triphala

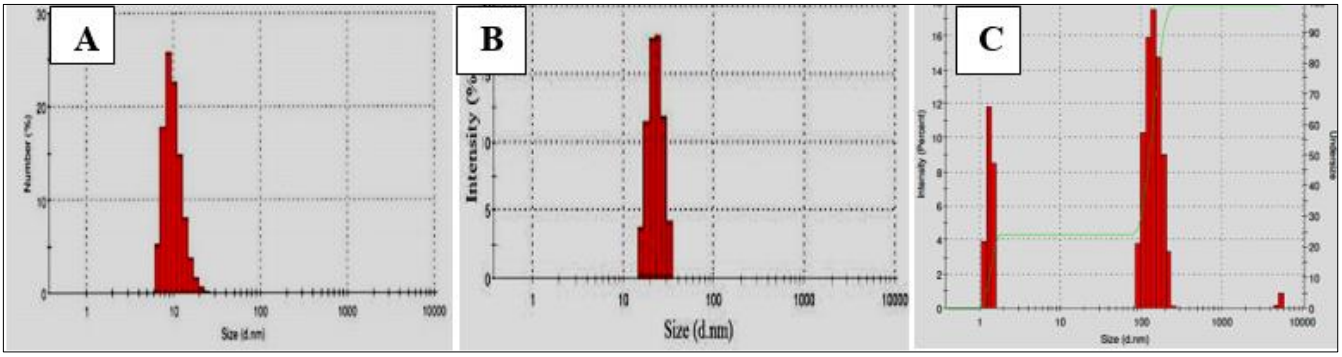

Fig 12: Intensity peak statistics of silver and gold nanoparticles samples AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala UV-VIS Absorbance spectrum:

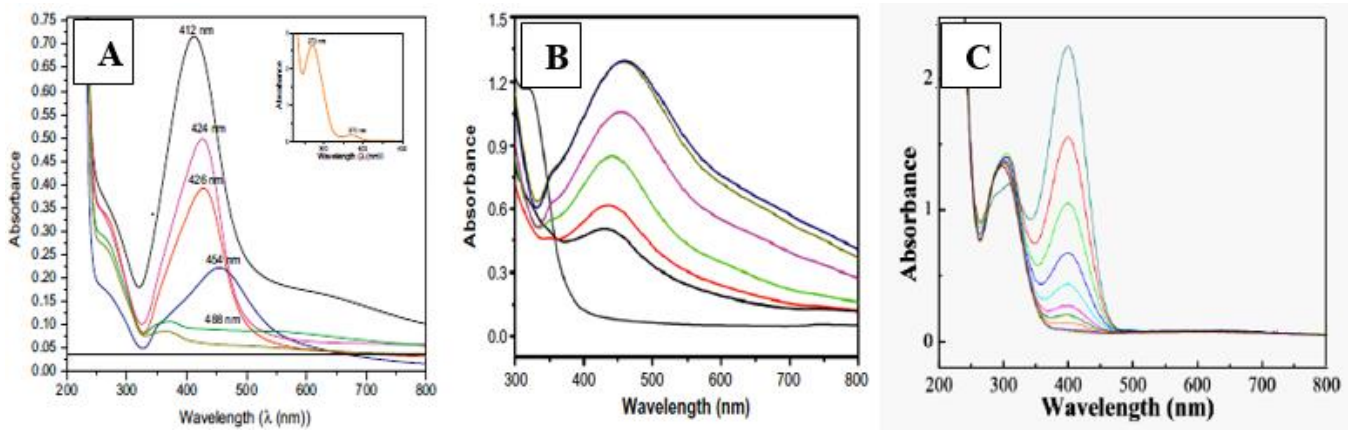

Fig 13: UV-Vis spectra of silver and gold nanoparticles samples at different time intervals AgNPs (a), and AuNPs (b) and AgAuNPs(c) using aqueous extract of Triphala

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. Therefore, the aqueous bio-reduction of Ag^{+} and Au^{3+} ions can be effectively monitored by a UV-Vis spectrophotometer at different time intervals with the spectra of plant extract (inset A). It was observed that a broad Surface Plasmon Resonance appeared at λ_{max} 468 nm for AgNPs, 430 nm for AuNPs and 416 nm for AgAuNPs. The peaks get shifted to lower wavelength, giving rise to a blue shift of the SPR band (toward a shorter wavelength) with time. The sharpness of the absorption band with increasing time of the reaction was due to the better stabilization of the NPs' surfaces as the plant extracts also act as surface functionalizing ligands, which was added drop-wise. This is also reflected by the color change of the diluted pure AgNPs solution from light to dark brown, pure AuNPs solution from yellow to pink and then brownish-pink as the plant extract concentration increased overtime.

Rapid formation of NPs was due to higher availability of polyphenols and flavonoids. Moreover, plant extracts not only reduced the M^{n+} to their respective metallic and bimetallic nanoparticles, but acted as capping/stabilizing agent. Due to excitation of surface plasmon vibrations in the synthesized nanoparticles, their aqueous suspension appeared reddish brown, brownish pink. The reduction of M^{n+} ions to MNPs and bimetallic nanoparticles were monitored using UV-Vis spectroscopy. Figure 13 shows the UV-Vis spectrum which confirms the formation of metallic and bimetallic nanoparticles. The absorption band in the range of 410-470 nm was indicative of anisotropic nanoparticles formation.

Fourier-Transform Infrared Spectrum

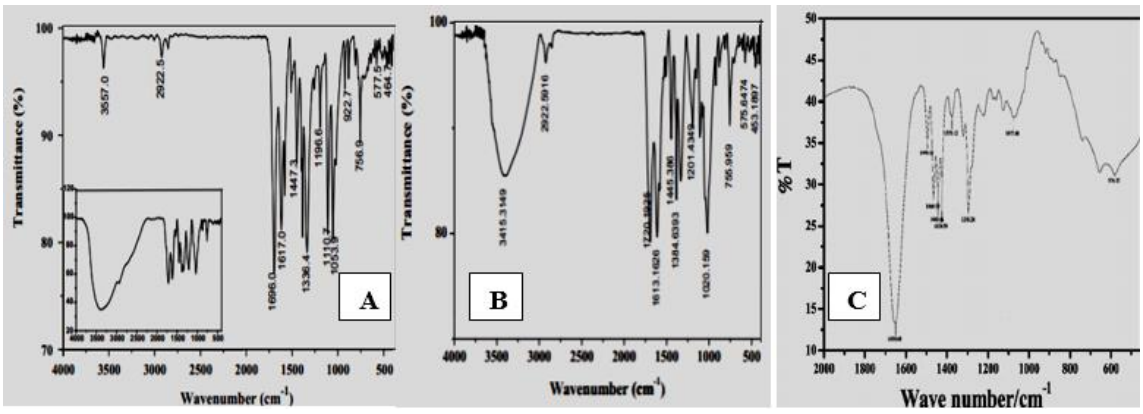

Fig 14: FTIR spectra of silver and gold nanoparticles samples AgNPs (A), and AuNPs (B) and AgAuNPs (C) along with Plant extract in inset of A using aqueous extract of Triphala

Fourier-transform infrared spectroscopic analysis confirmed that the plant extracts not only acted as a bio-reductant but also functionalized the NPs' surfaces to act as a capping ligand to stabilize them in the solvent. The dual role of the plant extract as a bio-reductant and capping agent was confirmed by FT-IR analysis of the as-prepared NPs. The sample for the infrared analysis was carefully prepared to exclude any possibility of the presence of any unbound plant extract residue. For this purpose, the as-prepared NPs after final workup were dispersed again in distilled water via 30 minutes of sonication, and subsequently centrifuged at a speed of 9000 rpm for 30 minutes, and this process was repeated twice to isolate the pure NPs and exclude the presence any unbound ligand. The similarities between the spectra of plant extract and as-synthesized nanoparticles in Figure 14, with some marginal shifts in peak position, clearly indicate the presence of residual plant extract in the sample as a capping agent to the AgNPs in 13A. Detailed analysis of the plant extract spectra strongly suggested the presence of flavonoids and polyphenols, apart from other phytochemicals, which were mainly responsible for the formation of the AgNPs and AuNPs by reducing the $AgNO_3$ and hydrated $HAuCl_4$. FT-IR analysis shows IR bands at 3557, 2922, 1696, 1617, 1447, 2336, 1196 and 1053 cm^{-1}. The band at 3557 cm^{-1} corresponds to –C=O overtone, the peak at 2922 corresponds to aliphatic –C–H stretching. The bands appeared at 1696 and 1617 cm^{-1} are due to –C=O and–C=C stretching, respectively. The bands appeared at 1447, 1336 and 1053 cm^{-1} can be assigned to –C–N, –C–O and –C–O–C stretching, respectively. The peak at 1196 cm^{-1} is due to –C–O–H bending. The absence of –OH stretching and appearance of –C=O overtone in the FT-IR spectrum of purified AgNPs indicate that the polyphenols present in the aqueous extract were responsible for reduction of Ag^{+} to Ag and the oxidised form of polyphenols binds the Ag nanoparticles via –C=O coordination. Although glucose contains carbonyl group, it may not take part in stabilisation process of AgNPs, because FT-IR spectrum of purified AgNPs powder did not show any –OH stretching except –C=O overtone at 3557 cm^{-1}. The spectrum of the plant extract shows the absorption peaks at 3746 and 3410 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (-OH) and the peak at 2943 cm^{-1} indicates the presence of C−H. The absorption peaks situated around 1753, 1622, and 1407 cm^{-1} are the characteristic peaks for the C−H, C−C, and C−O stretching, respectively, of the aromatics. The bands at 1264 and 1077 cm^{-1} indicate the presence of C−O stretching of alcohols, carboxylic acids, and ester and ether groups. Notably, the absorption band at 1974 and 512 cm^{-1} in the metallic NPs spectrum, in addition to peaks present in the plant extract spectrum, represents the MNPs' banding with oxygen from the hydroxyl groups in *P. Emblica*, *T. chebula*, *T. belerica* and Triphala powder compounds. Further, the absorption peak centered at 1729 cm^{-1} points toward the formation of a new C=O group that is either an aldehyde, ketone, or COOH. This strongly suggests that the reduction of Ag and Au was carried out by some hydroxyl groups that get oxidized at the expense of Ag and Au because Ag and Au are reduced.

Antimicrobial activity

The antimicrobial activity was tested against the human pathogens (bacteria/fungi) using aqueous extract of *E. officinalis*, *T. belerica*, *T. chebula* and Triphala powder and as synthesized nanoparticles by standard protocol.

Preparation of test solutions for antimicrobial activity

The minimum amount of Dimethyl Sulfoxide (DMSO) was used in preparing a stock solution of 100mg/ml of each extract and the volume is made up with distilled water. Further dilutions of 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml were made up from the stock solution for determining their antifungal and antibacterial activity.

Antifungal activity

Test Organisms

Human pathogenic fungi; *Candida Albicans*, *Aspergillus flavus* and *Aspergillus niger* were used for evaluating antifungal activity of the extracts of *E. officinalis*, *T. belerica*, *T. chebula* and Triphala powder as well as the synthesized nanoparticles (Ag, Au and Ag@Au) using these crude extracts. Test organisms were procured from the Department of Biochemistry, IIT Delhi.

Preparation of medium

The most extensively used fungal medium, Potato Dextrose Agar (PDA) consists of the following:

- Water 1000ml
- Potatoes 250g
- Dextrose 20g
- Agar powder 20g

250g of washed and chopped potatoes in approx. 1 litre distilled water were boiled for 30 minutes in order to prepare Potato infusion and then strain the broth through muslin cloth. The total volume of the suspension was made 1 litre by Distilled Water. After the addition of 20g dextrose and 20g agar powder, the sterilization of the medium is done by autoclaving at 15 lbs for 20 minutes. In order to prevent the growth of unwanted bacteria, Gentamycin (50mg/L) was also added.

Test of Antifungal Activity

Out of all the available methods, the most common poisoned food technique by Grooves and Moore 1962, Tuit, 1969 was used for the assessment of antifungal activity. The test fungi were grown on PDA medium. The desired concentration/amount of extracts in 1 ml of DMSO was subsumed aseptically into 99 ml aliquots of sterilized PDA medium which was cooled at 45ºC. After pouring each lot of medium in Petri dishes, these were allowed to solidify. DMSO (~1ml) in the medium was taken as a control.

A 5mm mycelial disc cut from the periphery of 2-3 days old fungal colonies was used for the inoculation of each dish at the centre. These inoculated Petri dishes were incubated in the dark at 25±2ºC for 48-72 hrs and colony diameters were measured periodically till the control dishes were nearly completely covered with fungus growth.

The whole test is performed in triplicates with three dishes containing only the solvent and no toxicant.

Antibacterial activity

Test Organisms

The bacterial cultures of two gram negative and two gram positive namely *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 6633) were used for evaluating the antibacterial activity of the extracts of *E. officinalis*, *T. belerica*, *T. chebula* and Triphala powder as well as the synthesized nanoparticles (Ag, Au and Ag@Au) using these crude extracts. Test organisms were obtained from the Department of Biochemistry, IIT Delhi.

Preparation of medium

For the growth of bacteria, NA (Nutrient agar) medium is used which consists of the following:

- Peptone 5g
- Beef extract 3g
- NaCl 5g
- Agar-Agar 20g
- Distilled water 1 litre

All the constituents were dissolved in distilled water and the final volume was made to 1 litre.

Test of Antibacterial activity

The Thornberry's method of zone inhibition was adopted for testing the extracts for their bactericidal activity. The bacterial suspensions were prepared using 48h old culture. The bacterial growths from five slots were taken and mixed in 100ml sterilized distilled water aseptically.

The molten medium was cooled to 45ºC, and poured aseptically in sterilized Petri plates. For levelled distribution of the medium it was rotated gently and allowed to get solidified. Different concentrations of the test extracts (10, 20, 30, 40 and 100 µg/ml) were prepared from the stock solution by taking appropriate amount and the dilution is done using DMSO.

10 mm diameter circular paper discs were prepared from Whatman's filter paper No. 1. The petri plates containing these discs were autoclaved at 15 lbs pressure for 20 minutes. One set for the control and two paper discs for each concentration of extract were used. These discs were soaked in different concentration of the test compounds. The excess solution absorbed by the paper discs was removed by holding them vertically by sterile forceps. Such dipped discs were transferred aseptically to Petri plates containing bacterial suspension spread over the surface and media.

Such Petri plates were kept at 5 ºC inversely for two hours for better diffusion of the chemicals in agar medium. Later on, the incubation of Petri plates was done at 25±2 ºC for 48 h. The zone of inhibition (in mm) for each concentration of the samples was recorded after 48 h of incubation.

Antimicrobial Activity

Promoting a disease free; hale and hearty life, the Mother Nature has gifted mankind a boon in the form of medicinal plants. Numerous medicinal plants are present in a collection of herbal preparations of the Indian traditional health care system (Ayurveda) named Rasayana, recommended for their interesting antioxidant and antimicrobial activities. Triphala is a Thai traditional herbal formulation composed of *Emblica officinalis*, *Terminalia belerica* and *Terminalia chebula*.

Amongst these natural therapeutic agents, Amla showed the highest antibacterial activity followed by Triphala powder, Bahera and Harad. In case of Antifungal activity, it was Bahera which showed the highest activity against the available test organisms followed by Amla, Harad and Triphala powder. Phyto-synthesized metallic nanoparticles have many applications such as antimicrobial, biomedical, agriculture, bio-insecticides, catalyst, biosensor, etc. The antibacterial activities were found to be inversely related to the average sizes of synthesized nanoparticles. Bimetallic nanoparticles possessed better antimicrobial activity as compared to monometallic nanoparticles. Gram negative (*E. coli and P. aeruginosa*) bacteria were more susceptible to gram positive (*S. aureus and B. subtilis*) bacteria. The order for antibacterial efficacies is as follows: Ag@AuNPs> AgNPs>AuNPs. In case of antifungal activities, AuNPs were better than AgNPs i.e. the order is Ag@AuNPs> AuNPs>AgNPs. *A. niger* was most susceptible fungal organism as compared to *A. flavus* and *C. albicans* to the action of these plant derived therapeutic agents. Green synthesis of AgNPs, AuNPs and bimetallic nanoparticles by using plant extracts gained significant interest over the years due to the remarkable antibacterial and anticancer properties of these nanoparticles. The excellent antibacterial activity of silver and gold nanoparticles corresponds to their large surface area-to-volume ratio which enables larger presence of atoms on the surface and thus, in turn, larger contact with the environment. In addition to this, the Nano sizes of these particles make penetration through cell membrane easier, interacting with intracellular materials and finally resulting in cell destruction in the process of multiplication.

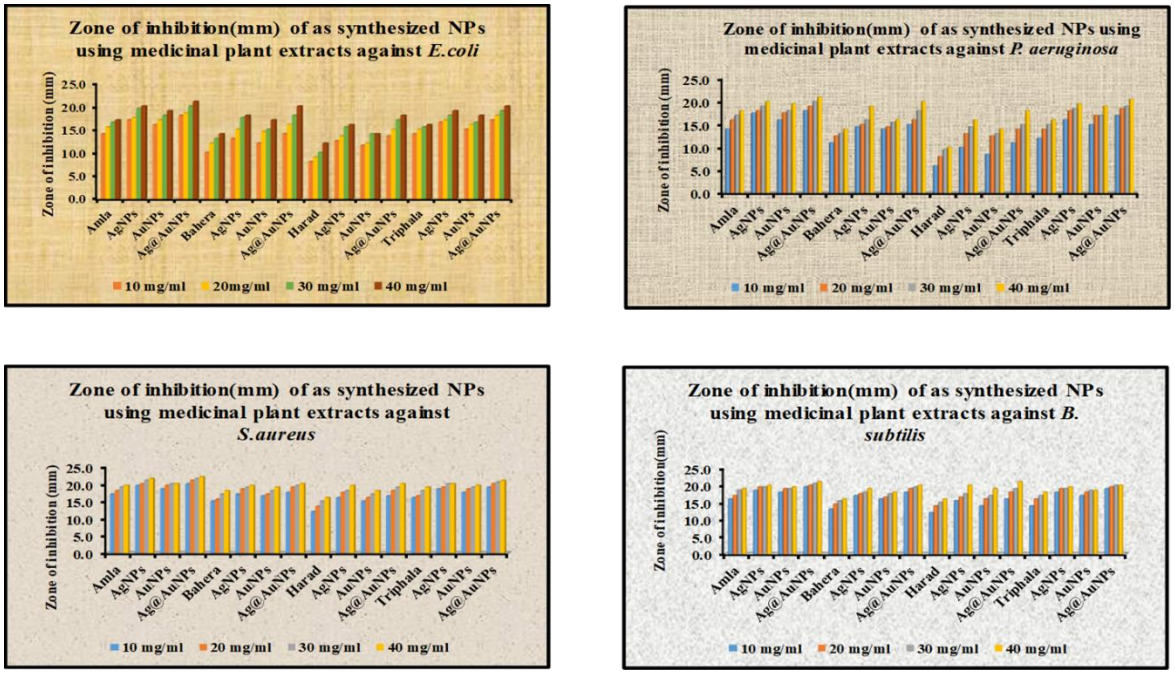

Fig 15: Zone of inhibition (mm) of as synthesized NPs using medicinal plant extracts against the test organisms (*E. coli*, *P. aeruginosa*, *S aureus* and *B. subtilis*).

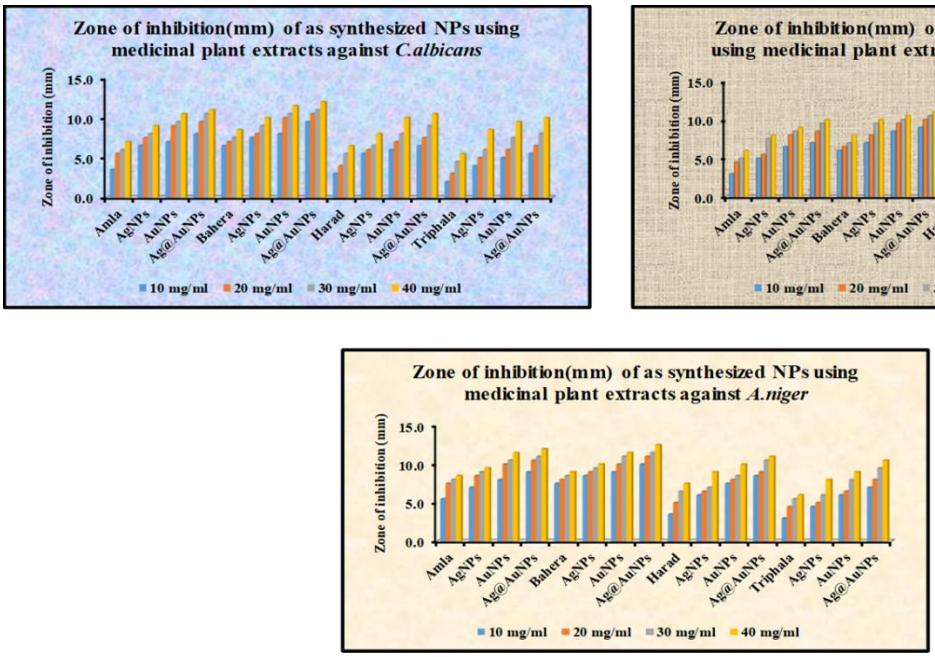

Fig 16: Zone of inhibition (mm) of as synthesized NPs using medicinal plant extracts against the available pathogens (*C. albicans*, *A. flavus*, *A. niger*

The smaller nanoparticles have more antibacterial activity due to them providing more surface exposure to the bacterial membrane [15, 20]. The positive charge of Ag^{+} interacts with the negative charge on the cell wall of bacteria which leads to changes in cell wall morphology and increase in the cell permeability or leakage of the cell which consequently results in cell death [16, 19].

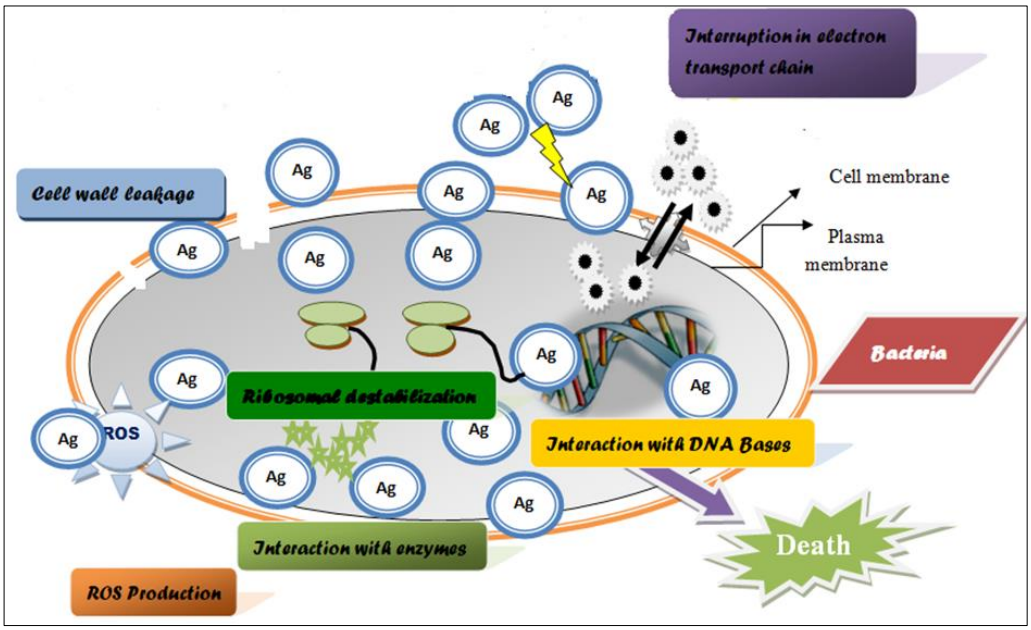

Fig 17: Diagrammatic representation of mode of action of AgNPs for antibacterial activity

AgNPs have more affinity to interact with phosphorous and, sulfur-containing biomolecules present in extracellular (membrane protein), and intracellular components (DNA bases, protein); these biomolecules affect cell division, respiration, and ultimately, the survival of the cell. Other investigations have reported that the Ag^{+}, which has an affinity for nitrogen and sulfur, can inhibit and disrupt protein structures by binding to thiol and amino groups [17]. The interaction of nanoparticles with thiol group may be responsible for the induction of reactive oxygen species (ROS), which leads to the inhibition of respiratory enzymes and, consequently, death [14, 21]. Silver ions act as an antibacterial by interacting with the peptidoglycan cell wall and plasma membrane [18] and also prevent bacterial DNA replication by interfering with sulfhydryl groups in protein. The use of these compounds as therapeutic agents will open a chapter of cheaper and safe herbal substitute of antibiotics and may prove as a tool to check the problem of increasing antimicrobial resistance in pathogens.

Conclusion

We have reported the synthesis of silver nanoparticles by Triphala powder: *P. Emblica*, *T. Belerica* and *T. Chebula* extracts, which provide simple and efficient ways for the synthesis of nanomaterials. Silver and gold nanoparticles prepared in this process are quite fast and of low cost. The

biosynthesized nanoparticles in an economical, efficient and eco-friendly process were characterized by UV-Vis, FTIR, FESEM, TEM-EDX, Elemental mapping, Particle size analyzer (DLS) and XRD techniques. The FESEM and TEM image suggests that the particles are of mainly spherical shaped of size 40-70 nm. The presence of Ag^{+} in silver nanoparticles, Au^{3+} in gold nanoparticles and Ag^{+}-Au^{3+} in bimetallic AgAu nanoparticles was confirmed using the XRD pattern, EDS Studies, Elemental mapping techniques. The FTIR study suggests that the Phyto-constituents play an important role in the stabilization of silver nanoparticles, which was further confirmed by negative zeta potential in DLS studies. The amount of plant material was found to play a critical role in the size dispersity of NPs. It is found that lower amounts of plant material were sufficient to bring about reduction. This eco-friendly method could be a competitive alternative to the conventional physical/chemical methods used for synthesis of silver, gold & bimetallic nanoparticles and thus has a potential to use in biomedical applications and will play an important role in opto-electronics, medical devices, and antimicrobial agents in near future.

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Conflict of interest
The author declares that there is no conflict of interest, financial or otherwise regarding the publication of this paper.

Abbreviations: NPs: Nanoparticles

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