Current Trends in Biotechnology and Pharmacy
Vol. 14 (3) 289-301, July 2020, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2020.3.30

Green synthesis of silver nanoparticles from fractionated *Annona reticulata* leaf extract in different solvents and analysis of its antioxidant and antibacterial activity

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Abstract

Silver nanoparticles were synthesized from fractionated leaf extract in Hexane, Chloroform and Water. Synthesis of AgNPs was confirmed by change in color of leaf extract solution, followed by confirming of reduction of silver ions in the leaf extract by UV-Visible spectroscopy. The Surface Plasmon resonance (SPR) peak was observed from 400 to 450nm. The biosynthesized AgNPs were characterized by dynamic light scattering measurement (DLS), Zeta potential and Transmission electron microscopy (TEM). The XRD, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) analysis confirmed the crystalline nature of AgNPs and the presence of elemental silver. The size of the silver nano particles ranged from 10-50nm and were spherical in shape as found by DLS and TEM studies. The synthesized AgNPs showed higher antioxidant activity by DPPH assay as compared to the crude leaf extract. Antibacterial activity was higher in the synthesized AgNPs on observing the inhibition zone of Gram positive and Gram Negative bacteria.

Key words: AgNPs, Biosynthesis, Crystalline, Phytochemicals,Zeta potential.

Introduction

During the past decade production of Silver nanoparticles (AgNPs) has gained wide exposure due to its applications in different industries based on the unique physical, chemical, optical and biological properties of AgNPs. Biosynthesis of AgNPs can be carried out by Microorganisms and plant extracts or by physical and chemical methods. In case of Microorganisms, the synthesis is slow and time consuming, while in Physical method high energy radiation, microwave irradiation and inert gas condensation are used which is expensive . Whereas in chemical methods hazardous chemicals such as reductants, stabilizers and organic solvents, are used. The plant mediated biosynthesis of Silver Nanoparticles using plant extracts as a source of natural reductants has gained importance because of its higher stability, easier preparation procedure and no hazards to the environment as compared to chemical methods. The small size of AgNPs has paved the path to improve and gain more importance in pharmacological applications, electronics, optics, catalysis, food industry, agriculture, and textile industry and water treatment. Several studies have been carried out on synthesis of silver nanoparticles from plant extracts and it has been reported that the plant species is one main factor responsible for reduction of the silver ions. Apart from acting as reductants, Plant extracts also help in capping and stabilizing of nanoparticles (1).

Annona reticulata belongs to Annonaceae, a very large family of plants which comprise about 120 genera and more than 2000 species (2). *Annona reticulata*., is commonly known as

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Ramphal (Hindi), Ramasitapalam (Telugu), Ramachita (Tamil), Manilanilam (Malayalam), and Ramaphala (Kanada). It grows in tropical and subtropical region. It is a small tree with a height of seven to eight meters. Leaves of the plant are membranous, lanceolate, oblong lanceolate, acute, cuneate rounded at the base (3). It is a large evergreen shrub or small tree, grown commercially for its fruit.The leaves of *A. reticulata* have great medicinal properties due to the presence of phytochemicals like Annoreticuin, Solamin, Dopamine, Coclaurine, Acetogenin, Squamone (4). The leaves possess good antioxidant and antimicrobial activity. It is a medicinal plant used widely in ayurvedic system of medicine for the treatment of several diseases like dysentery, worm infections, hemorrhage, constipation, dysuria, and ulcer (5). *A. reticulata* possess various phytochemical constituents in Stem, Leaf and Root. The plant is reported to contain acetogenins which posses potential anticancer and anti-inflammatory property (6). Various plant parts like leaves, bark, seed and root are medicinally useful and they show many therapeutic activities as anticancer, CNS depressant, analgesic, antihyperglycemic, anti-inflammatory, antiproliferative, wound healing and antiulcer activity (7). The root contains aporphine alkaloids like liriodenine, norushinsunine, reticuline and one acetogenin neoannonin(8).

This is the first report of the biosynthesis of AgNPs using different leaf extract fractions in polar solvents like Hexane, Chloroform and Water. The Phytochemicals fractionated with different polar solvents of *A.reticulata* leaf were used to synthesize AgNPs. The synthesized AgNPs were characterized by UV-Vis spectroscopy, Dynamic Light Scattering (DLS), Zeta sensitizer, Fourier Transform Infrared spectroscopy (FTIR), X-ray diffraction (XRD) and Transmission Electron Microscopy (TEM). Various biological assays like Antioxidant and Antibacterial activities were carried out to evaluate their biomedical and pharmacological applications of *A.reticulata* leaf extract.

Materials and Methods

Silver nitrate was purchased from Avra synthesis Pvt.Ltd.,Nacharam, Hyderabad, Telangana State, India. Fresh leaves of *Annona reticulata* were collected from Mittapalem, Srinivasamangapuram village, Tirupathi region, Chittoor Disrict, Andhra Pradesh, India. The harvested leaf sample was authenticated by Dr. A. Mudhusudan Reddy, Assistant Professor, Department of Botany, Yogi Vemana University, Kadapa, Andhra Pradesh, India, and deposited in the herbarium with a voucher specimen number (AMR4855YVUH).

Preparation of Annona reticulata leaf powder: *A. reticulata* leaves were thoroughly washed with tap water, shade dried and then grounded into smooth powder and then transferred to air tight container and preserved at -20^{o}C in darkness till further use.

Extraction and fractionation of Annona reticulata leaf extract with hexane, chloroform and water *:* 500gm of *A.reticulata* leaf powder was soaked in 1000ml of 90% methanol for 48 hrs on rotary shaker for agitation at 35^{o}C. This leaf extract was later filtered and extracted by using Whatman filter paper No1. The filtrate was then concentrated on a rotary evaporator (Hidolph Germany make) at 35^{o}C temperature and under reduced pressure. This concentrated methanol extract of *A.reticulata* leaf was referred to as Crude (ArL Crude). The *A. reticulata* crude extract (ArL Crude) was dissolved in 500ml of distilled water and filtered to which 500ml of Hexane solvent was added this was followed by vigorous shaking. The layers were then allowed to separate by shaking for 6hr in a separating funnel (9). The separated hexane layer was collected and labelled as Fraction 1 *A. reticulata* Leaf Hexane Extract (F1-ArL HE). Similar procedure was followed and repeated with Chloroform solvent and this extract was labeled as Fraction 2 *A.reticulata* Leaf Chloroform Extract (F2-ArL CE), and the remaining aqueous extract was labeled as Fraction 3 *A.reticulata* Leaf Water Extract (F3- ArL WE). Each of the fractions obtained were dried and

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concentrated by using Rota vapor. The concentrated samples were collected into vials and stored at -20ÚC until further use.

Biosynthesis of silver nanoparticles (AgNPs) in fractionated A. reticulata leaf extracts : 100mg of all three fractions (hexane, chloroform and water) of leaf extracts were taken separately and added in individual flasks containing 100ml of 1mM $AgNO_3$ aqueous solution and then incubated in an oven at 80° C in darkness. This incubation leads to reduction of silver ions to AgNPs after 24hrs in darkness. The change in the color of the leaf extract fractions from green to dark brown in color indicated the formation of Silver Nano particles (10). The synthesized AgNPs in Hexane fraction of leaf extract was labelled as *A.reticulata* Leaf Hexane Extract AgNPs(ArL HE AgNPs), the synthesized AgNPs by chloroform extract was labelled as *A.reticulata* Leaf Chloroform Extract AgNPs(ArL CE AgNPs) and the synthesized AgNPs by water extract was labelled as *A.reticulata* Leaf Water Extract AgNPs (ArL WE AgNPs) respectively.

Characterization of synthesized AgNPs of A. reticulata leaf extract fractions : The reduction of silver ions in silver nitrate solution by *A. reticulata* leaf fractions was monitored by UV-visible spectroscopy (UV-1800 Shimadzu Co., Japan) ranging from 200 to 800nm at resolution of 1nm (11)

The particle size of the synthesized AgNPs were determined by Dynamic Light Scattering (DLS) technique and zeta potential was determined by using Zetasizer (HORIBA scientific sz-100) with a range of 0.1 to 10000nm at scattering of angle of 90° and 25°. For diameter measurement 1ml of sample was transferred to a plastic cuvette, and automatically equilibrated for 2min. For zeta potential analysis, 1ml of the sample was injected in to the zeta cell, and the measurement was repeated thrice.

The FTIR spectra analysis was carried out by JASCO FTIR-4700 with a range of 399.193$cm^{-1}$to 4000cm^{-1}.The synthesized AgNPs after the reaction were subjected to centrifugation at 10,000 rpm for 10min and then the precipitate was washed with deionized water to eliminate the uncoordinated biomolecules on the surface of AgNPs. The final product was collected and dried in a vacuum oven.

The X-ray diffraction (XRD) analysis of biosynthesized AgNPs was performed on the X-ray diffractometer (Brucker, D8 advance, Germany) operated at a voltage of 40kV and a current of 30mA at a wave length of 1.5406A, at scattering angle of 30°-80° range (12).

Transmission Electron Microscopy (TEM) studies was performed at 100kV, to determine the size and morphology of the biosynthesized AgNPs. Thus was done by taking one drop of the reaction mixture on silicon wafer and then the sample was evaporated and dried completely for further analysis.

Antioxidant assay (DPPH free radical scavenging activity) : 1, 1-Diphenyl 1-2-picrylhydrazyl (DPPH) free radical scavenging potential of phytochemical fractions and AgNPs of *A. reticulata* were evaluated by preparing 1mM of DPPH in Methanol solution (13). Phytochemicals of all fractions and AgNPs at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1mg) were added to 1ml of 1mM of DPPH solution. The reactions was carried out under dim light and incubated for 30min. The change in the color of the reaction mixture from purple to yellow was monitored and measured at 517nm. Ascorbic acid (Vitamin C) was used as reference and methanol was taken as blank.

% of DPPH scavenged = (A ctrl – A test) / A ctrl x 100

A ctrl = Absorbance of the control reaction

A test = Absorbance of the test sample with DPPH

Antibacterial activity : The bacterial strains were obtained from Institute of microbial Technology (IMTEC), Chandigarh, India. The antibacterial

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assay was performed by disc diffusion method (14) against pathogenic bacteria like *Staphylococcus aureus* (MTCC-87)and *Bacillus subtilis* (MTCC 10619) which are (Gram-positive) and *Salmonella ebony* (MTCC 3384) and *Salmonella enteric* (MTCC 3858) which are Gram-negative bacteria. The bacteria were cultured in nutrient broth and kept for incubation at 35°C in an rotary shaker for 24hr. After incubation, 100µl of bacterial culture was uniformly spread on the surface of the freshly prepared nutrient agar petridishes with the help of sterile 'L' shaped glass rod. Simultaneously presterilized circular filter paper discs with a diameter of 25mm are dipped in 10µl of each sample leaf extracts with a concentration of 1mg and were placed on the surface of the bacterial plated petridish. Standard antibiotic Ampicillin (1mg/ml) was used as a positive control. Eventually the zone of inhibition was measured after incubation time of 24hr at 37°C.

Results and Discussion

UV-Visible spectroscopy analysis: The initial formation of AgNPs was monitored by change in color of all leaf extracts samples in all three solvents. The color was initially green and later turned to dark brown (Fig.1).

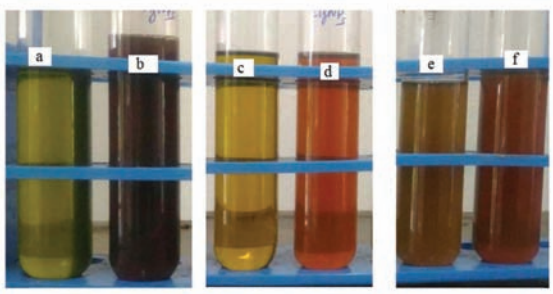

Fig. 1: Synthesis of AgNPs in *A.reticulata* leaf extracts in different solvents.

a. Leaf extract in Hexane
b. Synthesis of AgNPs in Hexane extract
c. Leaf extract in Chloroform
d. Synthesis of AgNPs in Chloroform extract
e. Leaf extract in Water
f. Synthesis of AgNPs in Water extract

The biosynthesized AgNPs exhibited a strong absorption Surface Plasmon resonance (SPR) band by UV visible spectral analysis as shown in (Fig.2). The well-defined SPR absorption at 446 nm, confirmed the synthesis of ArLHEAgNPs (Fig 2.a). SPR absorption peak at 439 nm, confirmed the synthesis of ArLCEAgNPs (Fig2.b) and SPR absorption peak at 441 nm, confirmed the synthesis of ArLWEAgNPs (Fig2 c).

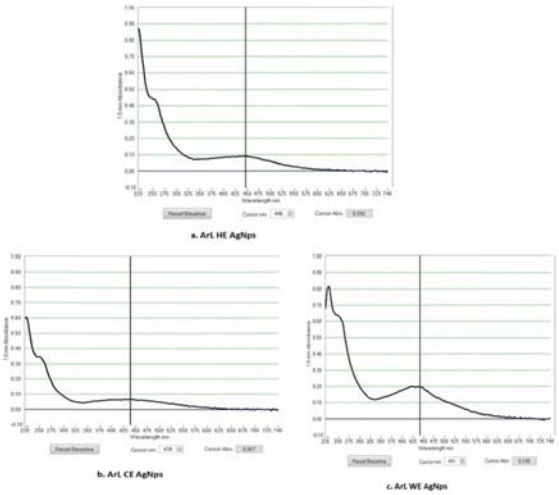

Fig. 2: UV–Vis absorption spectra of the synthesized AgNPs by *A.reticulata* leaf extract

(2.a) *A.reticulata* Hexane Extract AgNPs Shows the SPR absorption peak at 446 nm.
(2.b) *A.reticulata* Chloroform Extract AgNPs Shows the SPR absorption peak at 439 nm.
(2.c) *A.reticulata* Water Extract AgNPs Shows the SPR absorption peak at 441nm.

Determination of particle size by Dynamic Light Scattering (DLS) analysis: The synthesized AgNPs of *A. reticulata* Leaf extract was analyzed by DLS to determine the partical size and diameter.The average particle size is as shown in Table.1 and Fig .3. The results have shown that the average particle size of ArL HE AgNPs is

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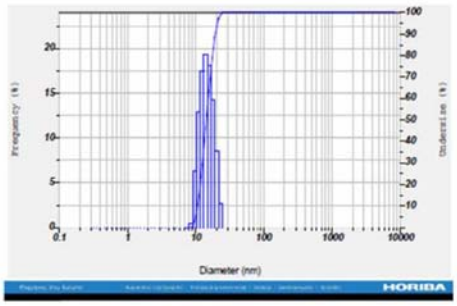
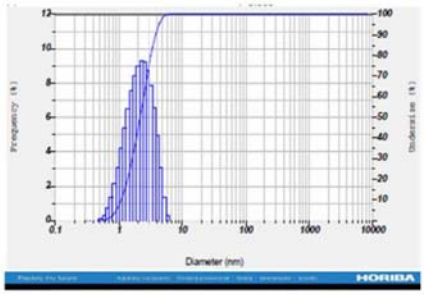
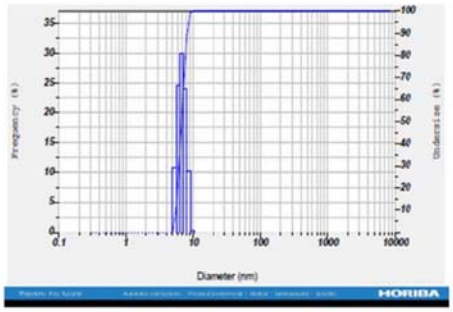


a. ArL HE AgNps



b. ArL CE AgNps



c. ArL WE AgNps

Fig. 3. Particle size of AgNPs synthesized by fractionated *A. reticulata* leaf extract. (a) Hexane Extract AgNPs (b) Chloroform Extract AgNPs (c) Water Extract AgNPs.

Table. 1. The average particle size of biosynthesized AgNPs from *A.reticulata* leaf extract

S.No	AgNPs of different solvent extracts	Particle size
1.	Hexane Extract AgNPs (ArL HE AgNPs)	14.1nm
2.	Chloroform Extract AgNPs (ArL CE AgNPs)	2.3 nm
3.	Water Extract AgNPs (ArL WE AgNPs)	6.8 nm

Table.2. The Zeta potential of AgNPs synthesized from fractionated *A. reticulata* leaf extract in different solvents.

S.No	AgNPs of different solvent extracts	Zeta potentialin mv
1.	Hexane Extract AgNPs (ArL HE AgNPs)	-28.0 mv
2.	Chloroform Extract AgNPs (ArL CE AgNPs)	-31.3 mv
3.	Water Extract AgNPs (ArL WE AgNPs)	-30 mv

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14.1nm, ArL CE AgNPs is 2.3nm and particle size of ArL WE is 6.8nm.

Zeta potential : The Zeta potential of the AgNPs of *A. reticulata* leaf extract fractions in Hexane, Chloroform and water was analyzed and is as shown in Table.2 and Fig.4.(a,b,c) shows the graphical representation of the Zeta potential. The negatively charged Nanoparticles prevented the agglomeration formation and lead to long term stability of the nanoparticles. In this study the synthesized AgNPs showed very high value of zeta potential and confirms the existence of repulsive force among the particles thus increasing the long term stability of the AgNPs.

FT-IR analysis : FT-IR analysis with synthesized AgNPs in different solvents to investigate the surface chemistry composition of AgNPs capped by the biomolecules in *Annona reticulata* leaf extract showed different peaks which is because of different functional groups involved in the synthesis and stabilization of AgNPs (Fig.5.a,b,c) various functional groups involved in the formation of AgNPs leaf extract in different solvents.

Table. 3. The various functional groups involved in the formation of AgNPs in different solvents (a) Hexane extract (b) Chloroform Extract and (c) Water Extract.

AgNps Sample Different solvents	FTIR peak (cm^{-1})	Functional group	Reference no
a. ArL HE AgNPs	3272.92	N–H stretching of amide II	15
(Hexane)	2941.98	–C–H stretching of –CH2 of Protein	16
	2128.37	CC or CN triple bond	17
	1618.19	C–C stretching	18
	1328.06	C–O stretching/ O–H	19
	1080.52	C–OH of the phenols	20
b. ArL CE AgNPs (chloroform)	3334.34	N–H stretching of the secondary amide of the protein	21
	2320.60	N–H/C–O stretching	22
	2093.32	CC or CN triple bond	23
	1608.74	C–O/aromaticC–C stretching	24
	1337.04	C–O stretching/ O–H	25
	1064.38	C–OH of the phenols	26
c. ArLWEAgNps(Water)	3300.43	N–H stretching of the secondary amide ofthe protein	27
	2914.47	–C–H stretching of –CH2 ofProtein	28
	2320.90	N–H/C–O stretching	29
	2128.37	CC or CN triple bond	30
	1618.19	C–O/aromaticC–C stretching	31
	1314.15	C–O stretching/ O–H	32

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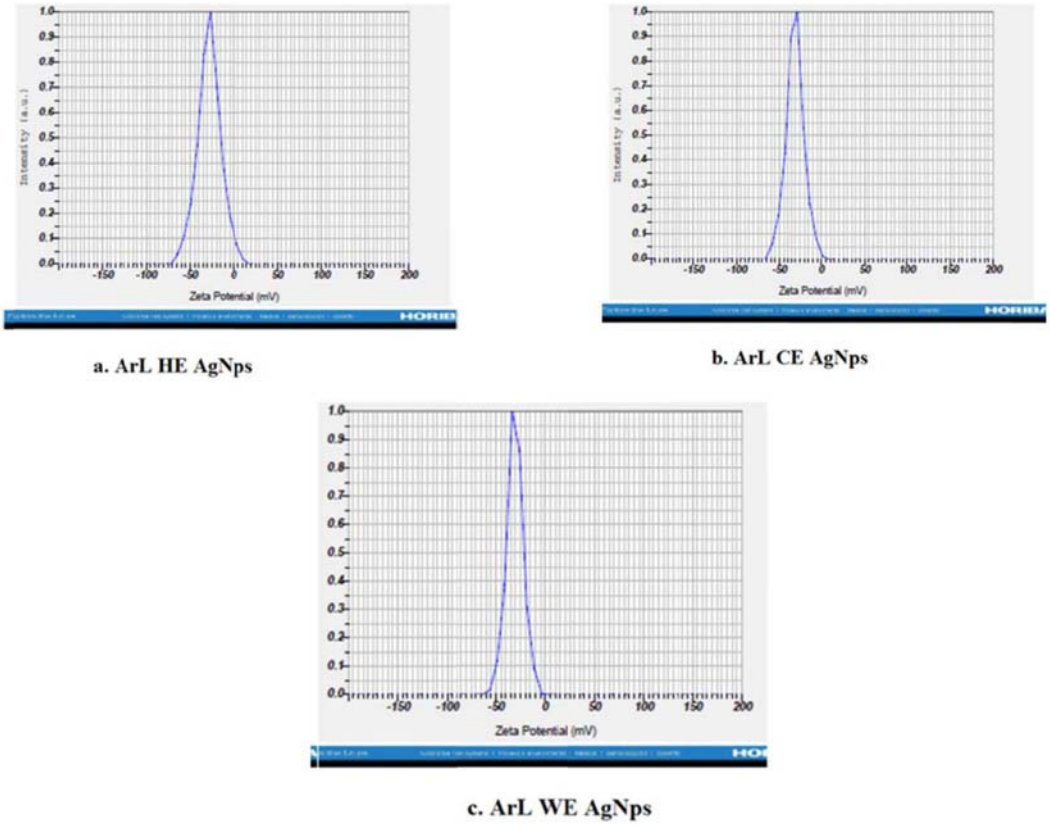

Fig. 4. Zeta potential of AgNPs synthesized by fractionated *A.reticulata* leaf extract in different solvent. (a) Hexane extract AgNPs (b) Chloroform Extract AgNPs (c) Water extract AgNPs.

(a) A.reticulata Hexane Extract AgNPs

(b) *A.reticulata* Chloroform Extract AgNPs
(c) *A.reticulata* Water Extract AgNPs.

XRD Studies : The crystalline nature of the biosynthesized AgNPs was confirmed by XRD pattern as displayed in Fig. 6. These patterns revealed four diffraction peaks at the 2è values of the three samples at 38.31$^{æ\%}$, 44.58$^{æ\%,}$ 64.71$^{æ\%}$, 77.71$^{æ\%}$, and 81.92$^{æ\%}$ .This could be indexed to (111), (200), (220), (311), and (222) crystallographic planes respectively. This suggested that all the peaks of the prepared AgNPs corresponded to face-centered cubic (FCC) lattice phase of silver and showed consistency with the standard JCPDS (File No 04-0783) data.

TEM Analysis : The TEM Micrographs revealed the spherical morphology of all the AgNPs. The AgNPs segregated based on their diameters which ranged diameters ranging from 10nm-100nm as seen in the (Fig.7).

Antioxidant activity (1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity) : Anti oxidant activity if different solvent fractions (F1 ArL HE, F2 ArL CE and F3 ArL WE), of *A.reticulata* leaf extract and its synthesized AgNPs varied as in Table.4 and Fig.8. The best antioxidant activity was observed at 1.0 mg conc., (97.84%) in AgNPs synthesized in water extract and crude leaf water extract sample (97.516%). Among all the synthesized AgNPs in different solvents, the lowest antioxidant activity was

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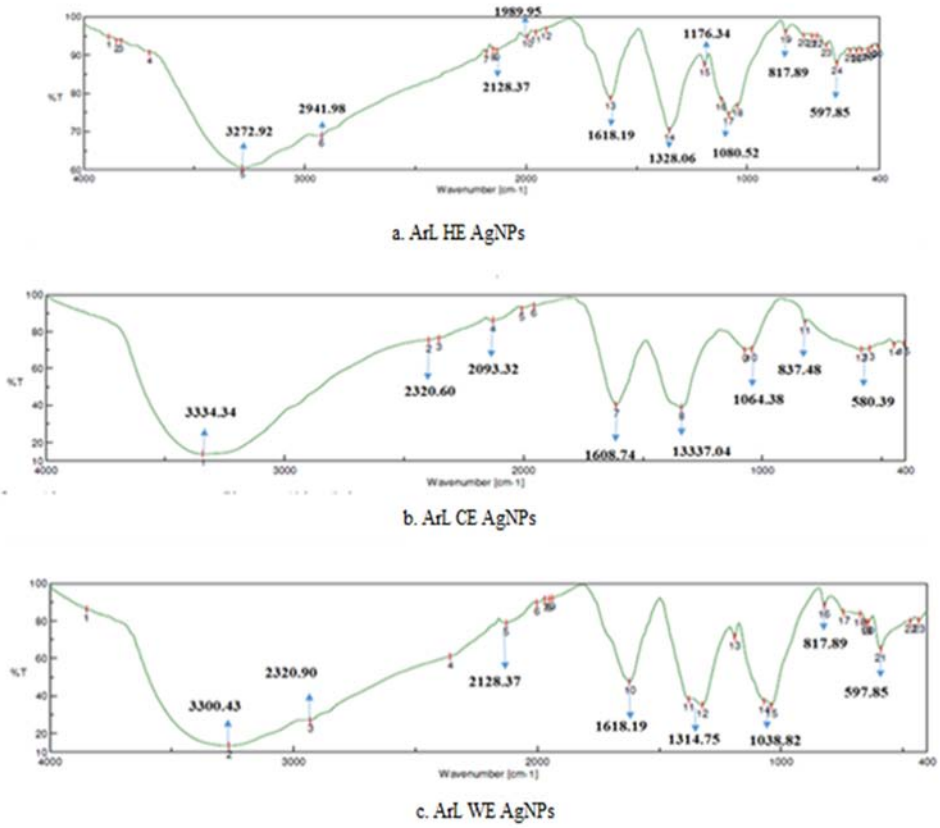

Fig. 5. Shows the various functional groups observed by the different peaks of AgNps Synthesized by *A. reticulata* leaf extracts in different solvents.

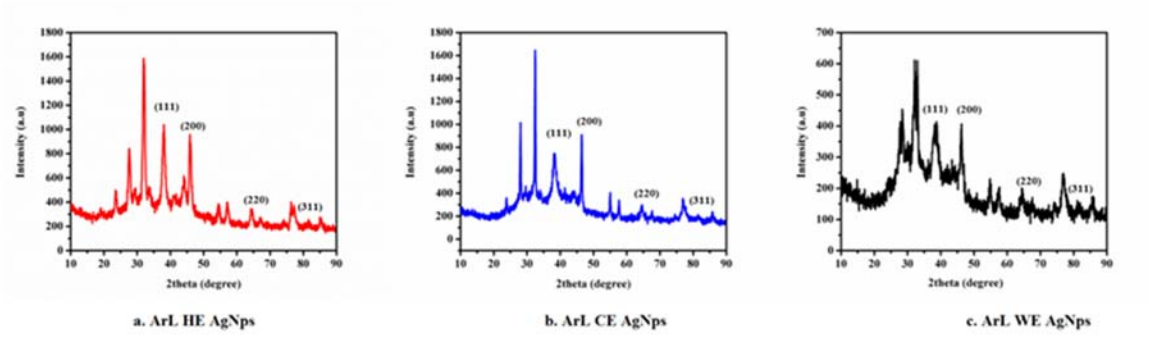

Fig. 6. XRD pattern studies confirming the crystal nature of the AgNPs by showing the Braggs peaks corresponding to(111), (200), (220) and (311) planes of (a) *A.reticulata* Hexane Extract AgNPs (b) *A.reticulata* Chloroform Extract AgNPs (c) *A.reticulata* Water Extract AgNPs.

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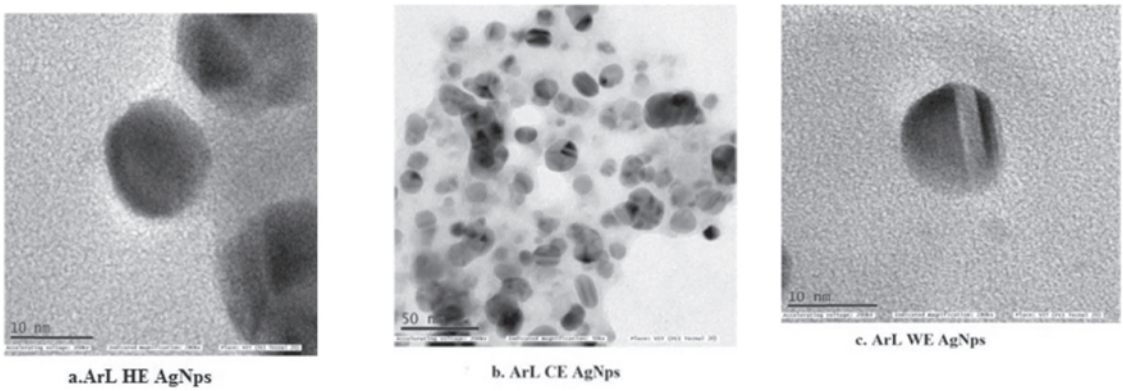

Fig. 7. Shows TEM micrograph of synthesized AgNPs in different solvent fractionated *A.reticulata* Leaf extracts.(a) *A.reticulata* Hexane Extract AgNPs (b) *A.reticulata* Chloroform Extract AgNPs (c) *A.reticulata* Water Extract AgNPs.

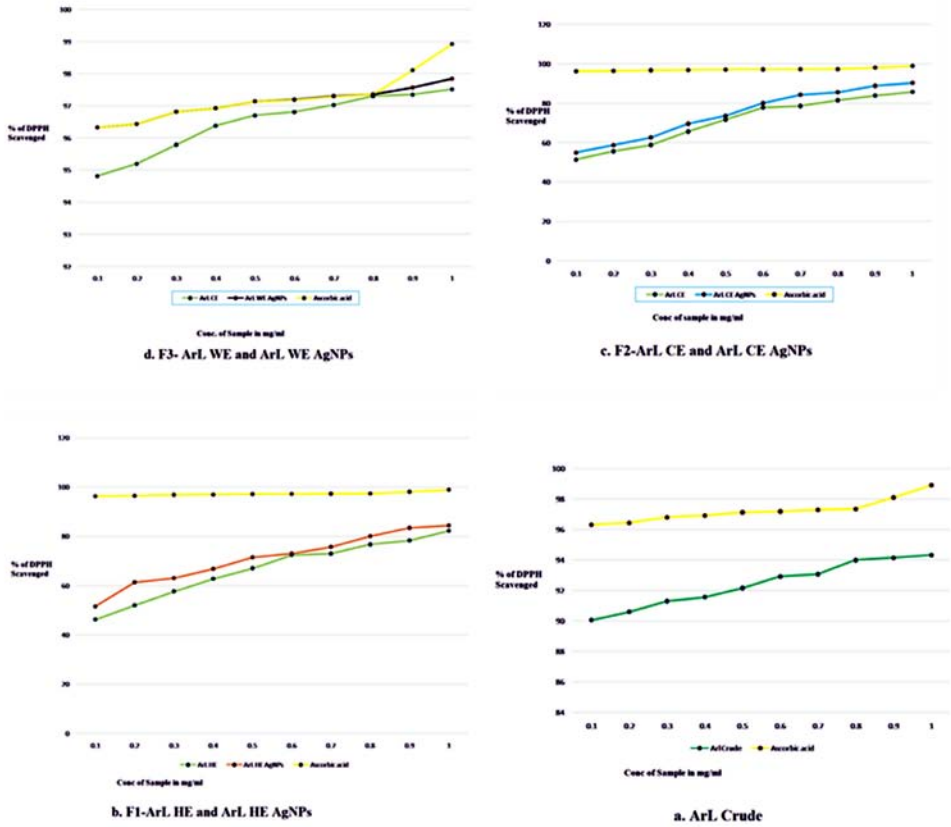

Fig. 8. shows the graphical representation of antioxidant activity of fractionated *A.reticulata* leaf extracts and its synthesized AgNPs.

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Table.4: Showing the percentage DPPH radical scavenging activity of A. reticulatafractionated leaf extracts and its synthesized AgNPs.

Conc.,of Samplein mg/ml	%of DPPH Scavenged byArLCrude	%of DPPH Scavenged by F1ArLHE	%of DPPH Scavenged by ArL HE AgNps	%of DPPH Scavenged by F2ArLCE AgNPS	%of DPPH Scavenged by ArLCE WE	%of DPPH Scavenged by F3ArL	%of DPPH by ArLWE AgNps	%of DPPH Scavenged by Ascorbic Acid
0.1	90.064	46.220	51.619	51.349	55.021	94.816	96.328	96.328
0.2	90.604	51.943	61.339	55.561	58.747	95.194	96.436	96.436
0.3	91.306	57.667	63.067	58.747	62.635	95.788	96.814	96.814
0.4	91.576	62.742	66.792	65.604	69.600	96.382	96.922	96.922
0.5	92.170	67.008	71.436	71.652	73.596	96.706	97.138	97.138
0.6	92.926	72.408	73.002	77.807	80.075	96.814	97.192	97.192
0.7	93.088	73.002	75.647	78.617	84.287	97.030	97.300	97.300
0.8	94.006	76.727	80.021	81.479	85.529	97.300	97.354	97.354
0.9	94.168	78.347	83.369	83.909	88.768	97.354	97.570	98.110
1	94.330	82.235	84.341	85.745	90.280	97.516	97.840	98.920

ArL = Annonareticulata leaf, HE = Hexane extract, CE = Chloroform, WE = Water extract

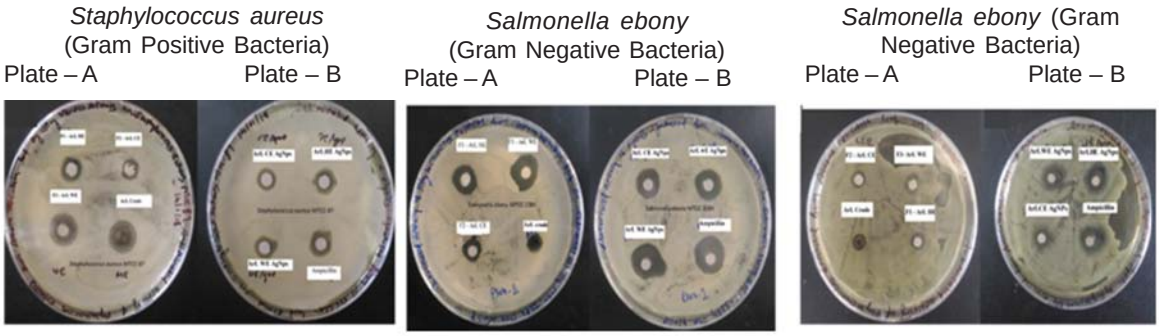

Fig. 9. Showing the Zone of inhibition of *A. reticulata* fractionated leaf extracts and its synthesized AgNPs towards the Gram positive and Gram negative bacteria

observed in AgNPs synthesized in Hexane leaf extract(84.34%). The antioxidant activity increased with an increase in concentration of leafextract . The lowest concentration used of leaf extract in different solvents is (0.1mg). The hexane extract (F1 fraction) showed the least Antioxidant activity (46.220%) and the highest antioxidant activity in concentration (0.1mg) of water extract. Synthesized AgNPs all the synthesized AgNPs showed a better response (84.341, 90.280 & 97.840) as compared to the crude leaf extract showed a steady increasing antioxidant activity with increase in leaf sample concentration(0.1 to 1.0mg conc.,). The F3 fraction of *A.reticulata* leaf extract in water and its synthesized AgNPs antioxidant activity (97.516% and 97.840%) was very near to the Ascorbic acid(98.920%).

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Anti-bacterial activity : After incubation period for 24hr, growth (ZOI) was observed around discs impregnated with AgNPs and leaf fractions. Fig.9 shows the plating of bacteria with the test samples. Plate a. with normal leaf fractions Whereas plate b. with synthesized AgNPs and Ampicillin against Gram positive and Gram-negative bacteria. ArL WE AgNPs showed Maximum ZOI against *Stephylococcus aureus* (5.43±0.08) , *Salmonella abony*(6.26±0.08), *Salmonella enteric* (6.3±0.05). The results of Antibacterial activity has shown that ArL WE AgNPs showed the highest activity against the bacteria . he Zone of inhibition of samples were measured and is shown in Table.5. It is clear from the obtained results that the AgNPs showed a higher antibacterial activity compared to the normal plant extract samples.

Plate. a: *A. reticulata* leaf extract fractions,

Plate.b: Synthesized AgNPs by *A. reticulata* leaf extract fractions

Conclusion:

Plant mediated biosynthesis of silver Nanoparticles was performed by using various fractions of *Annona reticulata.*L leaf extract fractionated by Hexane, Chloroform, and Water solvents . It was found that various phytochemical constituents of leaf extract were responsible for the formation of AgNPs. The size, morphology, crystalline nature and stability of AgNPs were characterized by using advanced techiniques like UV Visible spectroscopy, Dynamic light scattering, zeta potential, FTIR, X-Ray diffraction and Transmission electron microscope. The synthesized AgNPs were spherical in shape with size ranging 10 to 100nm, as observed in TEM and XRD. Among three different fractions, aqueous leaf extract fraction of *A. reticulata* has shown good antioxidant and antibacterial activity. The synthesized AgNPs have also shown enhanced activity of phytoconstituents compared to the normal leaf extract fractions due to the decrease in the size of the particle and increase in the

Table .5. Antibacterial activity of different extracts and their synthesized AgNPs against gram positive and gram negative bacteria.

ZOI = Zone of Inhibition

Samples	***Stephylococcusaureus*** (MTCC-87) **ZOI in (mm)**	***Salmonella abony*** (MTCC 3384) **ZOI in (mm)**	***Salmonella enteric*** (MTCC 3858) **ZOI in (mm)**
ArL Crude	2.4±0.15	2.43±0.08	2.23±0.08
F1-ArL HE	3.3±0.05	4.2±0.05	3.3±0.05
F2-ArL CE	2.56± 0.14	3.26±0.03	4.3±0.05
F3-ArL WE	3.73±0.08	5.13±0.16	4.36±0.03
ArL HE AgNps	5.33±0.12	5.5±0.05	6.26±0.08
ArLCEAgNps	3.33±0.08	5.2±0.05	5.46±0.06
ArL WE AgNps	5.43±0.08	6.26±0.08	6.3±0.05
Ampicillin	6.73±0.12	8.16±0.08	7.63±0.08

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DOI: 10.5530/ctbp.2020.3.30

surface volume ratio. Thus, the study concluded that plant mediated biosynthesis of AgNPs is a cost effective and ecofriendly which would establish its importance in biomedicine.

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