Current Trends in Biotechnology and Pharmacy
Vol. 12 (3) 275-285 July 2018, ISSN 0973-8916 (Print), 2230-7303 (Online)

Characterization and Evaluation of Antibacterial, Antioxidant and Cytotoxicity of synthesised silver nanoparticles (AgNps) using chloroform crude callus extracts of *Wrightia tinctoria* (Roxb.)

Keerthi Priya Yadala and Asha S *
Department of Biotechnology, VFSTRU, Vadlamudi, Guntur Dist., A.P
*For correspondecne - sai848@gmail.com

ABSTRACT:

The present work established that the chloroform callus extract taken from the leaf midrib of *in vitro* grown seedling of *Wrightia tinctoria (Roxb.)* is very efficient for the synthesis of AgNPs. The produced silver nanoparticles were preliminarily conformed by UV Visible spectroscopy, SEM, TEM, FTIR spectroscopy and Energy Dispersion Spectrum. UV-Vis spectroscopy analysis revealed the peak at 425nm which corresponds to the surface plasmon resonance of AgNPs. The uniform spherical shape and size was detected by SEM and TEM. The size and stability of AgNps were detected using Dynamic light scattering (DLS), Zeta potential. The synthesised silver nano particles were evaluated for its antibacterial by agar well diffusion, antioxidant activities by DPPH assay and cytotoxicity on MCF-7 and HEK 29 were tested by MTT assay. The result indicated decrease in cell viability and cell growth inhibition. The present study highlighted the possibility of utilizing nanoparticles synthesised from *in vitro* derived callus of WTR for animal or human applications as it enhanced antibacterial, antioxidant activity and Cytotoxicity.

Key words: *WTR* leaf midrib callus, Silver nanoparticles, Bio-reductant, DPPH inhibition, Antibacterial activity, Antioxidant and Cytotoxicity.

Abbreviations :
AgNp - silver nanoparticles
AgNO3 - silver nitrate
UV-Vis - Ultraviolet Visible
FT-IR - Fourier transform infrared spectroscopy
SEM - Scanning electron microscopy
TEM - Transmission electron microscopy
EDS - Enzyme dispersion spectrum
DLS - Dynamic light scattering

INTRODUCTION

The synthesis of nanoparticles have achieved a significant recognition at present scenario because of their excellent applications in techno commercial products(1).Nanoparticles are smaller, ranges from 1 to 100nm which can exhibit high surface to volume ratio and differ greatly from those atoms and bulk materials (2).The nanoparticles has several advantages in numerous fields which includes health care, environmental health, food, cosmetics, biomedical science, energy science, chemical industries, electronics, nano drug and gene delivery (3,4,5); and are still charming the researchers to explore the nanoparticles in various new dimensions (6,7).The noble metal nanoparticles shows variety of applications in various fields. (8). Nanoparticles based treatment has been accepted clinically for diseases, vaccines and renal diseases (9).

Among various nanoparticles, silver nanoparticles have attracted significant consideration due to their effective antibacterial property which exhibits very less toxicity and in various *in vitro* and *in vivo* applications (10,11).

There are several conventional methods for the production of nanoparticles such as chemical, electrochemical (12) and photochemical (13). However, these methods are more expensive and also toxic which leads to biological risk. Bio-assisted production of silver nanoparticles by using plant crude extracts, enzyme and microorganisms pave the way to overcome such limitations. It is cost effective, economic, eco-friendly which is non toxic and free of chemicals (14) and have benefits that the plants are easily available everywhere. Moreover, the silver nanoparticles synthesized from plant extracts are rich in source of secondary metabolites (15) when compared to other methods. The plant extracts contains the organic compounds like carbohydrate, proteins, phenols, flavanoids, triterpenoids, alkaloids, tannins etc. These active ingredients are capable of donating electron and answerable for reduction of Ag to AgNps (16). In addition also aids for the superior antibacterial activity (18,19).

Wrightia tinctoria (Roxb.) is an deciduous tree which belongs to the family Apocynaceae (native to India and Burma). (17) having medicinal importance in curing human ailments like tuberculosis, psoriasis etc.,

This is the first report on synthesis of silver nanoparticles using callus extract of *Wrightia tinctoria* (Roxb.). Since we used plant based biological materials (callus of *Wrightia*) for the synthesis of nano particles they could be safer for human or animal applications.

MATERIALS AND METHODS:

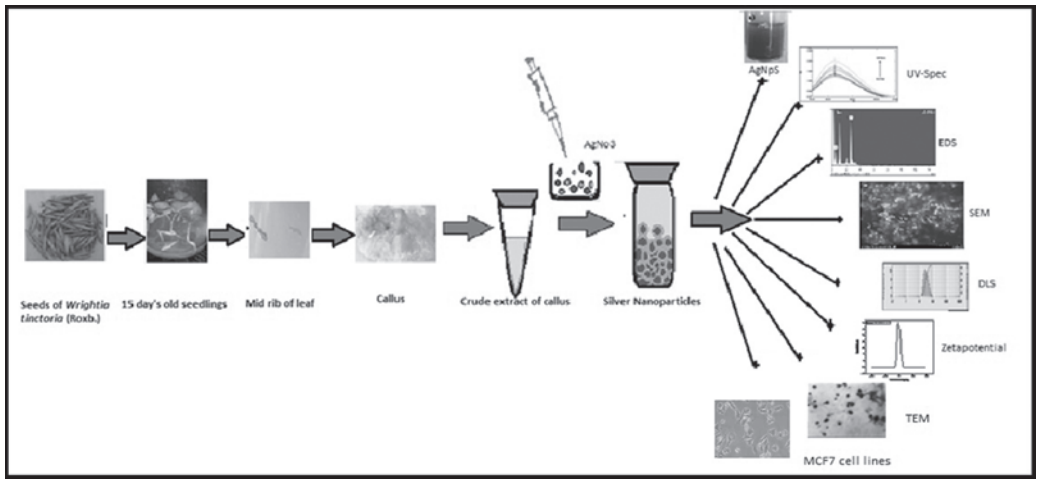
Chemicals and Micro Organisms : The Chemicals required for this study i.e., MS medium, hormones, nutrient medium and DPPH were purchased from Hi-media (Mumbai, India). The reagents were prepared according to standard protocols. pH of the MS media were maintained to 5.8 and 7.4. The strains of bacteria were obtained from the microbial type culture collection (MTCC, Chandigarh, India). The codes of the bacterial culture are as follows: *Pseudomonas aeruginosa* (10636), *Staphylococcus aureus* (6908), *Bacillus subtilis* (1305), *Pseudomonas putida* (1194), *Proteus vulgaris* (744), *Escherichia coli* (9537), *Klebsiella pneumonia* (10309), *Salmonella paratyphi* (ATCC 9150), *Shigilla boydii* (ATCC 9207) and *Enterococcus feacalis* (MTCC 459).

Collection of Plant Materials : The fruits of *WTR* were collected from the Botanical garden of Yogi Vemana University, Kadapa, Andhra Pradesh, India. The gathered fruits were dried at room temperature for 20 days. The seeds were separated from the dried seed pods and stored at room temperature for further use.

Raising of Aseptic Seedlings and Callus Initiation : The dried seeds of *WTR* were first washed gently with tap water for 10 minutes, followed by Tween-20 in distilled water for several times. The seeds were then treated with 1% sodium hypochlorite for 2 min subsequently washed with 70% of alcohol for 60 Sec and rinsed well in distilled water for three times. The sterilized seeds were then dried on a sterile Whatman filter paper and were inoculated on to the MS basal medium pH of 5.8 (20).The leaf midrib of *in-vitro* grown seedlings were excised under aseptic conditions and inoculated on MS medium amended with growth regulators such as KIN, NAA, 2,4-D and BAP in different concentrations. The cultures were incubated at 25^{o}C in dark condition for initial period of one week followed by exposing to photoperiod of 16 h light followed by 8 h dark which resulted in the initiation of callus.

Preparation of Chloroform Callus Extracts : The *in vitro* grown friable calluses were dried at 40^{o}C and then grinded in a blender. The fine powdered callus was stored in a glass container for further use. About 10 g of the powdered crude callus was used for extraction with chloroform in a soxhlet apparatus for 2 days and followed by rotary evaporator to get fine powder extract and named it as callus chloroform extract.

Synthesis of AgNps : In a clean sterile 250 ml Erlenmeyer flask, 10 ml of crude callus extract was assorted with 90 ml of 1mM $AgNO_3$under dark conditions. The mixture was then incubated at 37^{o}C on shaker incubator under dark conditions until the colour reform from light yellow to brown.



The bio synthesized nanoparticles were then complying by UV Visible spectrophotometer.

Characterization : The biological reduction of $AgNo_3$ to AgNps was observed periodically using UV-Vis spec (UV-1800 240V Shimadzu, made in Japan) and the absorbance values was noted at a range of 200-700nm. AgNps suspension was dried and made into powder. 1mg fine powder was used for the characterization of SEM and EDS using the instruments JEOLJSM 6360A (SEM), and JEOLJSM 1600(EDS). A fine coater for uniform coating of Platinum on the sample. Analysis was carried out on JEOLJED-2300 Analysis Station. The structure of nano size particles was observed by TEM Hitachi h-7500. The nano particles were coated on to the carbon coated copper grid and observed under various magnifications using transmission electron microscope. The size distribution were identified by using Dynamic light scattering, Zeta potential, EDS and FTIR,

Antibacterial Assay : Pathogenic strains from MTTC were used in this assay. Bactericidal activity was done by using Agar well diffusion method. Bacterial cultures were cultured in nutrient broth for 24h at 37°C. These bacterial strains were spread on nutrient agar medium by L-shaped rod and wells were made using cork borer. The samples viz, chloroform callus extract, silver nanoparticles, silver nitrate, +ve control as ampicilin and -ve control as DMSO were loaded to the wells carefully and was incubated for 24h at 37°C. The inhibition zone diameter was measured in centimeter. The experiment was repeated for three times and the graphs was plotted using graphpad prism 5.

Antioxidant Activity : The free redical activity was performed by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method. The various concentrations of synthesized silver nanoparticles from callus extract are dissolved with 3 ml of the DPPH and ethanol. The combination was vigorously assorted and kept in dark condition for 30mins. The absorbance of optical density was taken at 517 nm by using UV-Vis spectrophotometer against standard ascorbic acid as +ve control and blank DPPH as -ve control. The free radical scavenger activity was calibrated by a formula given below (21).

$$\text{Inhibition } \% = I_0\text{-}I/I_0 \times 100$$

Where I_0 was the absorbance of control/or blank and I was the absorbance of sample.

Cytotoxicity: Viability of MCF-7 and HEK293 cells was assessed by the MTT Assay with six different concentrations of extracts in triplicates. Cells were trypsinized. The trypan blue assay was performed to know the viable cells in cell suspension. Cells were counted using hemocytometer and seeded

at density of 5.0 X 10 ^{3} cells / well. 100µl media was poured into 96 well plate and incubated at 37^{0}C for overnight. After incubation, the old media was replaced with 100µl of fresh media along with different concentrations of test compound in representative wells in 96 plates. After 48 hrs., the drug solution was discarded and the fresh media was added with solution of MTT (0.5 mg / mL^{-1}) to each well and incubated for 3 hrs at 37^{0}C. Precipitates were formed at the end of incubation time. The reduction of MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria was observed. Compute the solubilised crystals at 570 nm using micro plate reader. The percentage inhibition of growth was quantified using the formula. The concentration of test drug needed to inhibit cell growth by 50% values is produced from the dose-response curves for each cell line using origin software (22).

$$\% \text{ Inhibition} = \frac{100 \text{ (Control - Treatment)}}{\text{Control}}$$

RESULTS and DISCUSSION

Callus Initiation : The tender leaf midrib explants from the *in vitro* grown seedlings were used for the initiation of callus. The callus was grown in almost all the combinations and concentrations, tested. Among different concentrations and combinations of hormones tested for callus induction, the friable callus from MS medium supplemented with NAA (2.5mg/l) + (KIN 1mg/l) (Fig-1) has chosen for the study.

The friable light greenish callus obtained from the 4th week of tissue culture were used for the photochemical analysis. The chloroform extract of *in vitro* grown callus showed the presence of carbohydrates, phenols, phytosterols, saponins, alkaloids, xantho proteins, quinine, tannins and coumarin. There is a rich amount of phytochemicals because of the actively dividing cells present in callus. Various biomolecules present in the callus acts as catalyst by helping in bio reduction and stabilization of synthesized nanoparticles (23).

The friable callus from MS medium supplemented with NAA 2.5mg/l+ KIN 1mg/l was chosen for the synthesis of AgNps.

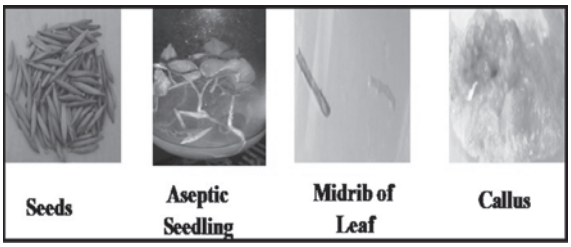


Fig. 1: Different Stages of Callus Initiation

Synthesis of AgNps : The visual observation of colour modification was noted after the treatment with callus extract of *WTR* to $AgNO_3$ solution with continuous shaking at room temperature (Fig. 3). The colour changed from pale green to brown colour. The callus extracts did not show any change in colour in the absence of $AgNO_3$ (Fig.4). The change of colour to light brown is mainly due to diminution of $AgNo_3$ to AgNps and the excitation of plasmon resonance of the AgNps (24). It may also due to the bioactive compounds present in the sample (25).

UV Spectrophotometer : Due to the size of nanoparticles, UV-Vis spectrum revealed an absorption maxima at 425nm. The absorbance (max) also showed blue tendency (distribution of size>35nm) with a peak at 425nm (distribution of

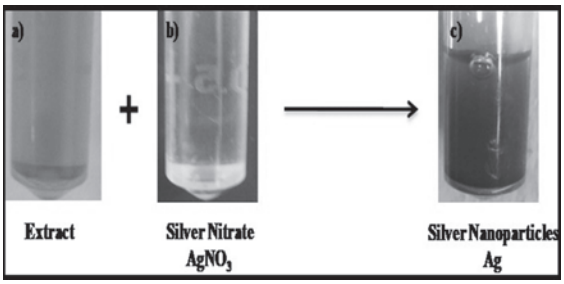


Fig. 3. Synthesis ofSilver Nanoparticles (a) *Wrightia tinctoria* (Roxb.) *Callus Extract* (Yellow colour) *(b)* $AgNO_3$ Solution and (c) Synthesized Silver Nanoparticles (Brown colour).

size =/<35nm) because of the various sized nanoparticles which confirms the formation of bio-reduced silver nanoparticles (26). SNps peaks at 420-480 (27) and 440-460 (28) UV were observed. These noble metal nanoparticles exhibited a strong absorption peak at 425nm in visible range which is named as surface plasmon resonance peak. The bioactive compounds in callus extract aids in the stabilization of synthesized silver nanoparticles. The absorption peaks at 425 nm gets sharper and colour intensity increased with incubation time which results in the formation of larger amount of nanoparticles (29).

SEM and EDS

The SEM analysis showed the formation of the properly distributed (or) dispersed and morphologically stable AgNps obtained from the callus. As shown in Fig. 5, synthesized Ag nanoparticles seemed to be roughly spherical ranging in the size from 35nm to 89nm.The results suggested that, the callus extract of *WTR* act as good bio-reductant for synthesis of AgNps. The surface plasmon resonance (max) observed in the UV-Vis spectroscopy because of the size which resemblance with the SEM. Further, the EDS gives both qualitative and quantitative information about the presence of elemental silver metal (37 %).

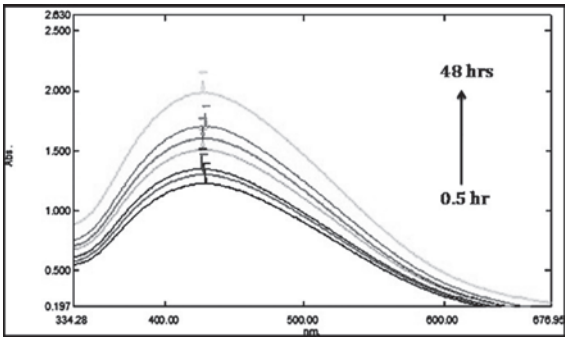


Fig. 4. UV Spectral Analysis of Silver Nanoparticles Synthesised from *In Vitro* Derived Callus of *Wrightia tinctoria* (Roxb.) Mid Leaf Vs Incubation time (0.5 h to 48 hrs).

TEM analysis : The transmission electron microscopy reveals the presence of well dispersed nano particles obtained from chloroform callus extracts, acts as capping agents and the formed nanoparticles. Most of the particles that are synthesised are in nano range which are round in shape and the diameter size ranges from 25 to 80nm (Fig.6). By increasing the concentration of callus extract, the size of the particle is decreased.(30)

DLS and Zeta potential : The size of the monodispersed silver nano particles is around 25 to 100nm in diameter which was measured by DLS. The histogram bars indicate the percentage of the volume for the AgNps.The wide range distribution of AgNps was quantified from 20-95nm. The calculated mean average particle size distribution of AgNps is 59.9nm (Fig.7) (31,32). The Zeta potential of the synthesised silver nanoparticles was found as a sharp peak at -14.2mV. It is recommended that the medium

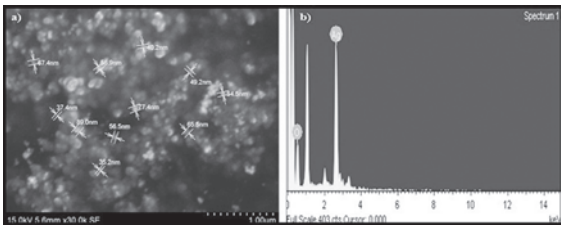


Fig. 5. (a) Scanning Electron Microscopic image (b) Energy Dispersive Spectrum of Ag nanoparticles Synthesized Using Callus Extract of *Wrightia tinctoria* (Roxb.)

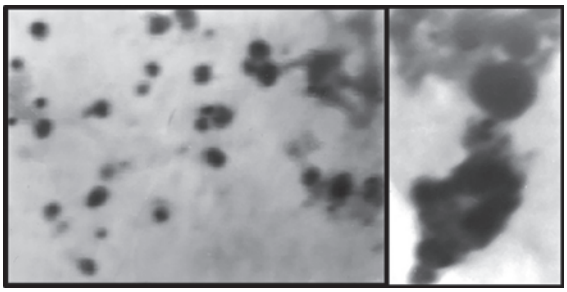


Fig. 6. (a) Transmission Electron Microscopic image of Ag nanoparticles Synthesized Using Callus Extract of *Wrightia tinctoria* (Roxb.)

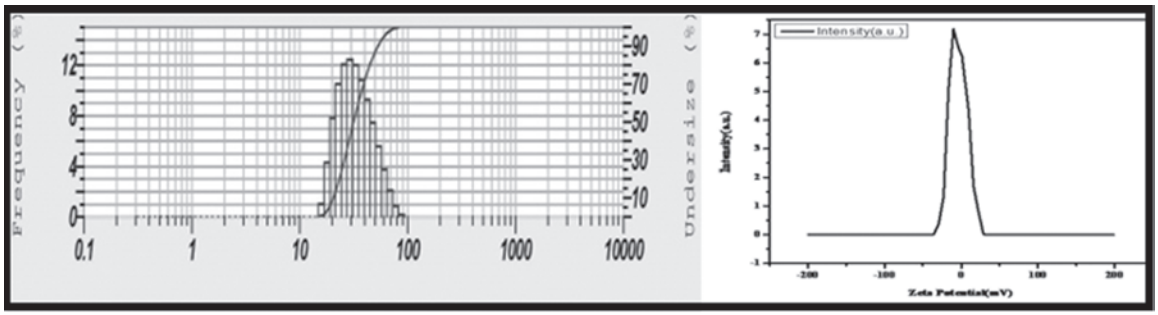


Fig. 7. Dynamic light scattering and Zeta potential of Ag nanoparticles Synthesized Using Callus Extract of *Wrightia tinctoria* (Roxb.)

dispersed silver nanoparticles with negative charge confirms the repulsion among the particles which proves that the AgNps are stable.

Fourier Transform Infrared (FTIR) Spectroscopy : The bioactive compounds act as an bio-reductant and capping agent for the creation of AgNps. The chemical interaction between silver and biological compounds present in the callus extract was investigated by the FTIR spectra. The FTIR spectra of callus extract of *WTR* was presented in Fig 8. Biological compounds present in the callus extract were also investigated by the FTIR spectra. The appearance of peaks at 2850 cm^{-1} and 2341 cm^{-1} was ascertained to aliphatic group and –C-H stretching of alkanes respectively. Similarly, the bands at 1384 cm^{-1} corresponds to nitro N-O bending respectively. Further, the peak at 578 cm-1 correspond to the alkylhalides (C-Cl) and the characteristic OH stretching vibrations of phenol/carboxylic group present in extract was observed at 3471 cm-1. Moreover, the appearance of bands at 1631 cm-1 is assigned to stretching mode of C=O stretching or amide bending. The peak at 1114 cm^{-1}corrosponds to C-O-C stretching of aromatic ring. With this study, it may be inferred that the presence of all these bioactive compounds of *WTR* callus extracts acts as a capping agent and aids in the bio-reduction /stabilization of silver nanoparticles (33,34).

Antibacterial Activity: The synthesized AgNps were tested against ten pathogenic bacteria viz., *Pseudomonas aeruginosa, Staphylococcus aureus, Pseudomonas putida, Bacillus subtilis,*

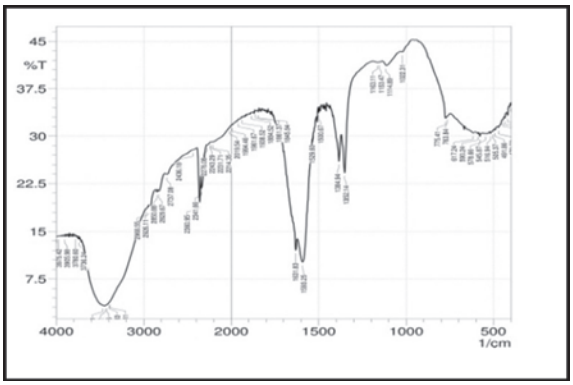


Fig. 8. FTIR Spectra of *Wrightia tinctoria* (Roxb.) Callus Extracts and Synthesized Silver Nanoparticles.

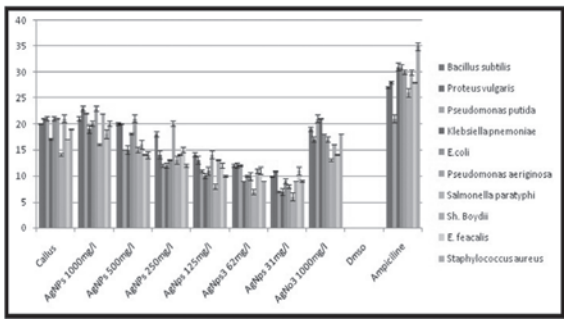


Fig. 9. Zone of inhibition (mm) of Callus, Silver Nanoparticles, Silver, Antibiotic, DMSO *against* ten Different Human Pathogens.

Escherichia coli, Salmonella paratyphi, Klebsiella pneumoniae, Proteus vulgaris, Shigilla boydii and Enterococcus feacalis. Due to the large surface area of silver nanoparticles it is showing efficient antibacterial activity. The synthesized nanoparticles demonstrate a superior antibacterial activity against 10 pathogenic bacteria. The antibacterial activity of AgNps against bacteria is as follows as *Sh. boydii > Proteus vulgaris> Pseudomonas aeruginosa> Pseudomonas putida> Bacillus subtilis > E-coli> Klebsiella pneumonia> Staphylococcus aureus > Enterococcus feacalis >Salmonella typhi*. Moreover, the inhibition zone of synthesized silver nanoparticles, callus, silver and antibiotics was compared against all the 10 bacteria and the values are tabulated in Fig.9. It was found that zone of inhibition of synthesized silver nanoparticles is more than that of callus and silver.The leaf derived silver nanoparticles of *Wrightia tinctoria* (Roxb.) showed effect on *Staphylococcus aureus*, *Klebsiella pneuminiae* (35).

Antioxidant Activity : The antioxidant activity of *WTR* leaf callus assisted silver nanoparticles was estimated by using DPPH assay, The percentage of inhibition was calculated based on the absorbance values taken from UV-Visible spectrophotometer at 517 nm. The synthesized silver nanoparticles showed very good percentage of free radical scavenging activity than that of *in vitro* derived callus extract. The values are tabulated in Fig.10 and Table 1. The valuable secondary metabolites present in the plant extracts showed antioxidant activity (36). These secondary metabolites may involve in the inhibition of the oxidative chain reaction as a result of oxidation of the molecules. Due to redox property of phenols, it may be showing antioxidant activity and in turn plays a role in neutralizing oxygen free radicals, quenching of singlet and triplet oxygen. The anti oxidant activity has been attributed by the capacity of the phyto compounds in donating H- ions (37).

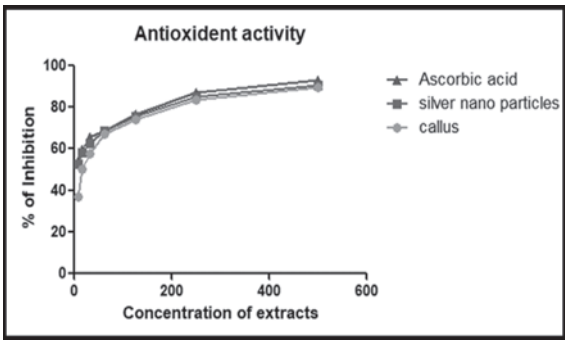


Fig. 10. Percentage of Inhibition of Free Radical Scavenging Activity of *Wrightia tinctoria* (Roxb.) Callus, Silver Nanoparticals and with the Standard.

Table. 1. Percentage of Inhibition of Free Radical Scavenging Activity of *Wrightia tinctoria* (Roxb.) Callus and Silver Nanoparticals Compared with the Standard.

Name of the Extract/ Standard	**Antioxidant activity IC50 (μg/ml)**
Chloroform Extract	17.04±0.5
Silver nanoparticles	13.70 ±0.8
Ascorbic acid	6.499±0.87

Cytotoxicity: The cytotoxicity of the crude and silver nanoparticles of callus extracts of *WTR* on MCF-7 cells from human breast cancer and normal cells HEK 293 cells was investigated by MTT assay. Based on the dose of extract the cell growth as well as the viability of cells were decreased. The IC_{50} values of MCF 7 for crude chloroform extract, silver nanoparticles, commercial nanoparticles and standard cisplatin were 62.21, 104.96, 46.192 and 3.609 where as for HEK 293 no activity on cells was detected. When compared to the crude extract, silver nanoparticles demonstrated more anti oxident, and antiproliferative activity(Fig.11). The bark extract of *WTR* cytotoxic activity on MCF-7 cell lines and HL-60 cell line (38); leaf methanolic extract of *WTR* showed 50% effect on Huh5.2 cell line (39). Petroleum ether and ethyl acetate fractions of *WTR* are more potent on MCF-7 cell lines (40).

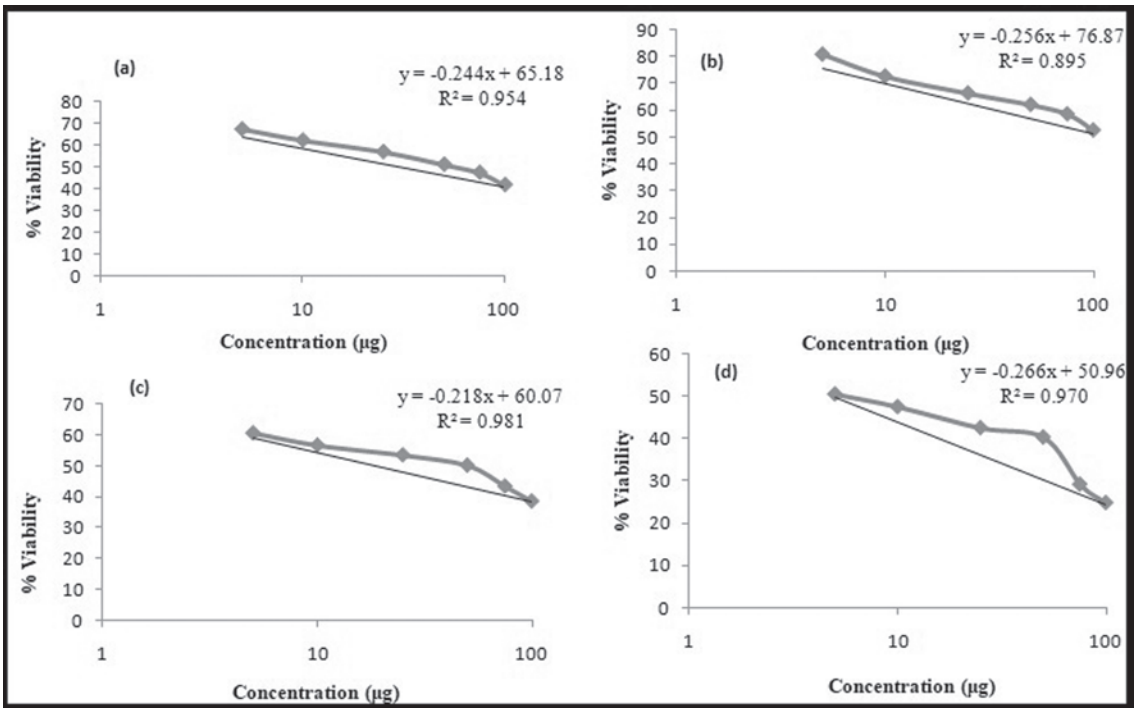


Fig. 11: Cytotoxic Effect of the (a) crude exract, (b) Synthesised Silver nanoparticles (c) Nanoparticles (d) cisplatin on MCF 7 Cell Line

Table. 2. Cytotoxic effect of callus crude extracts of Wrightia tinctoria (Roxb.) and silver nanoparticals compared with the nanoparticles and Cisplatin on MCF 7 and HEK293 Cell Lines

S. No.	Sample Description	IC_{50}(µg) MCF 7	HEK293
1	Crude extract	62.21	ND
2	Nanoparticles	104.96	ND
3	Silver nanoparticles of callus	46.192	ND
4	Cisplatin	3.609	-

CONCLUSION :

The bioreduction of Ag^+ ions by the *in vitro* derived callus of *WTR* leaf midrib leaf has been demonstrated. It is believed that *in vitro* derived callus extract are rich in phyto-chemicals which can act as a bioreducing agents for the production of AgNps with a size ranging from 10-100nm. The resulted AgNps were preliminarily categorized by UV–Vis spectroscopy, FTIR and SEM equipped with EDS. The silver nano particles synthesized from callus extract are eco friendly and efficient. It showed enhanced antibacterial activity against ten bacteria and also efficient antioxidant activity. Antibacterial studies of synthesized plant mediated-silver nanoparticles on human pathogens pave a way to develop nano-medicine against various human and veterinary pathogens.

Acknowledgements

We thank Dr. T. Chandra Sekhar, Dept. of Biotechnology, Yogi Vemana University for helping in collection of Wrightia tinctoria (Roxb.) R.Br. fruits. We are grateful to the Management,VFSTR (Deemed to be University) for sanctioning “Seed

Grant" for carrying out the work and the encouragement.

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