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Evaluation of Cytotoxic, Antimicrobial and Antioxidant Activities of Phyto-synthesized Silver Nanoparticles of *Tephrosia calophylla* Bedd.

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Abstract

The plant mediated synthesis of nanoparticles has significant application in biomedicine due to its novel properties and its eco-friendly nature. The present study deals with the biosynthesis of stable silver nanoparticles (SNPs) from aqueous rhizome extract of *Tephrosia calophylla* (TC). The synthesized nanoparticles are characterized by UV–Vis spectroscopy, Zeta potential, FTIR, XRD, TEM and EDAX methods. Colour change of the synthesized solution observed from Gray to brown indicates the formation of nanoparticles and UV–Vis surface plasmonresonance spectroscopy observed at 435 nm further confirmed the synthesis of nanoparticles as SNPs. FTIR spectroscopic studies confirm that phenols and proteins of rhizome extracts are mainly responsible for capping and stabilization of synthesized SNPs. The XRD data shows crystalline nature of nanoparticles and EDAX measurements reveals the presence of 69.56% Ag metal. Zeta potential at -19.9mV, negative value indicates the high stability of Nanoparticles. TEM microscopic analysis revealed that the size of synthesized SNPs ranges from 7 to 32.4 nm with spherical shape. Antimicrobial studies of AgNPs showed highest Zone of Inhibition against *Staphylococcus aureus* (32.75mm) among bacterial strains. Antioxidant activity of AgNPs shows 59.56%. Potential anticancer activity towards MDA MB 453 (human breast cancer cell line) cancer cell lines, shows 82.54% cell death with cell viability 17.46%. Overall, *T. Calophylla* is efficient in the synthesis of nanoparticles paves a way for better antimicrobial and anticancer therapeutic drug to be design by pharmaceuticals.

Keywords

Plasmon resonance, Ultraviolet-Visible (UV- Vis), Fourier Transform Infra-Red (FTIR), High Resolution Transmission Electron Microscopy (HR-TEM), X-ray Diffraction (XRD), Energy Dispersive X-ray (EDAX)

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INTRODUCTION

Fabaceae family members plays an important role in the field of herbal medicine. The genus *Tephrosia* is remarkably interesting having nearly 400 species [1]. In India about 37 species are distributed [2]. Only 13 species are reported from the state of Andhra Pradesh [3-5]. These *Tephrosia* species are most distributed in tropical and sub-tropical regions [6].

Phytochemical studies of *Tephrosia* species:

Phytoconstituents present in the *Tephrosia* genus manifested various biological activities such as antidiabetic, antiulcer, antidiarrheal, wound healing, anti-inflammatory, insecticidal, antiviral,

antiprotozoal, antifungal, antiplasmodial, and many other activities [7]. It was found that flavonoids were the most isolated and identified compounds in the genus, the other main classes of compounds include rotenoids, terpenoids, sterols, essential oils, and fixed oils. From *Tephrosia pumila* flavonoid as Praecansone, exists in two isomers [8]. Flavonoids in *T. Barbigera* with isopongaflavone and barbigerone [9]; *T.bracteolata* with isopongaflavone, Transtephrostachin, Transanhydrotephrostachin; *T.elongata* with elongatin [10]. *T.major* along with flavonoids sterol group β-sitosterol and stigmasterol, Triterpene lupeol [11]. *T.maxima* with flavonoid maxima flavanone A and maxima isoflavone A, B, C, D, E, F, G, H and J [12]. *T. procumbens* with rotenoid rotenone, sumatrol, praecansone A,B and obovatin [13]. *T.pumila* with flavonoid pumilanol, pumilaisoflavone A, B, C, D [8].*T.purpurea* with flavonoids Tephrosin, Pongaglabol, Purpureamethide, pongamol, Karanjin, Purpurenone, lanceolatin B, Quercitin etc. *T. Spinosa* flavonoid Spinochalcone A, B, C, fluvinervin A [14]. *T. tinctoria* with tephrowastin C , flemichapparin B [15-16]. *T. villosa* with Tephcalostan, villosin, villol, villinol, Tephrinone, villosone, Triterpenoid lupenone, Triterpene lupeol,sterol Stigmasterol [17]. Species of *Tephrosia* have been revealed the presence of number of phytochemicals which plays an important role in the control of vast range of diseases **Hepatoprotective:** *T. Purpura* stem methanol extracts [18] due to the presence of polyphenolic compounds and flavonoids [19]; **Antidiabetic:** *T. Calophylla* [20], *T. Purpurea* on diabetic induced cardiovascular complications and in the treatment of cataract due to the presence of flavonoid quercetin and rutin [21]. Antidiabetic activity of AgNPs of *T. Tinctoria* [22]. **Antiinflammatory:** ethonolic root extract of *T. Purpurea* [23]; Ethyl acetate extract of *T. sinapou* [24] pseudosemi glabrin isolated from *T. apollinea* possesses antiinflammatory activity [25]. *T. falciformis* root [26]; **Wound healing**: ethyl acetate extract of *T. purpurea* [27]**.**

Antioxidant: methonolic extract of *T. Calophylla* [20], *T. purpurea* supports effective cytotoxicity [28], *T. purpurea* [29,30], *T. vogelli* ethyl ether seed extracts [31]. Chlorooform leaf and aerial part extract of *T. villosa* [32] *T. tinctoria* leaf stem and root different extracts [33]. *T. apollinea* supported the oxidative stress and paraneoplastic symptoms caused by cancer [34, 35]. **Antiulcer:** *T. calophylla* [36] *T. purpurea* [37]. **Purgative:** activity by *T. vogalli* [38]. **Antihyperlipidemic:** *T. purpurea* [39]. **Anti-cancer:** *T. purpurea* [40]; *T. apollinea* isolated compounds prenylated flavones isoglabratephrin on three human cell lines and, effective on prostrate and pancreatic malignancies [41]. *T. tinctoria* callus extracts acts as antioxidant and anti proliferation apoptotic cell death on Hep G2 cells [42]. **Antifungal:** *T. purpurea* on 61 fungal isolates [43] *T. hildebradtii* against *Cladosporium cucumerinum* by root isolated component [44] *T. tinctoria* against *Aspergillus niger* and *Candida albicans* [15].

Antibactirial: *T. purpurea* ethanolic extract of roots on *S. aureus*, and on *P. aeruginosa, E. coli* [45]. *T. purpurea* methanolic extract on both gram positive and negative bacterial strains [46, 47] *T. vogelli* [48] *T. villosa* [49]. **Anthelmintic:** *T. calophylla* root extracts on *Pheretima posthuma* [50] *T. purpurea* [51]. **Larvicidal:** *T. egregia* [52] *T. purpurea* on larvae *Culex quinque fasiciatus* [53] *T. toxicaria* on larvae *Aedes aegypti* [54] *T. villosa* and *T. pumila* to control mosquitoes [55] *T. cinerea* oil against *A. aegypti* [56]. Flavonoids from seeds of *T. elata*, and *T. aequilata* antiplasmodial and larvicidal on *Marcua testularis*, *Spodopetra exemopta* and *Eldana sacchariana* [57, 58]. **Antifeedant:** *T. hidabrandtii* on the pest *Maruca testularis* [44] isolated compound benzofuran from *T.purpurea* towards H, Histamine [59]. Isolated prenylated flavonoid from *T. apollinea* against three major *Coleopteran* pests on grains [60] **Antiprotozol:** Isolated three flavonoids of *T. tinctoria* on parasitic *Trypanosome*, *Leishmania* and *Plasmodium* [61]

T. purpurea aqueous extract on diabetic abnormalities and cardiovascular complications and cataract [62] **Antiulergic:** *T. purpurea [*63] T. purpurea prevents cataract [64].

About the Selected Medicinal Plant

The medicinal plant *Tephrosia Calophylla* (Fig 1) was selected for the synthesis and charecterization of Silver Nanoparticles and their bioactivity through antibacterial sensitivity, antioxidant radical scavenging and cytotoxicity studies on human breast cancer cell lines

T. calophylla is a perennial rhizomatous sub shrub, mainly available in the Talakona forest area under shade. Leaves simple, parallel venation, Petiole winged, flowers light pink, pods compressed. Locally known as Adavi Vempali / Kommu vempali [65]. *Tephrosia Calophylla* used traditionally in folk medicine. According to Ayurveda, the plant is useful as an anti-helminthic, anti-pyretic and as well as an alexiteric drug. It is also active against leprosy, ulcers, cures diseases of the liver, spleen, heart and blood. According to the Unani system of medicine, the root is diuretic, allays thirst, enriches blood, cures diarrhea, it is also useful in bronchitis, inflammations, boils and pimples. Leaves are tonic to

intestines, and a promising appetizer [66]. Hepatoprotective [67]: Antiplasmodial [68]: Anti cancer [69]:Anthelmintic activity [48,70]: Antiulcer activity [36]: Antimicrobial [47]:

T. calophylla with Coumestan like Tephcalostan and Tephcalostan B, C, D and flavoniods like 7-O-methylglabranin, Calophione A, Kaempferol glucopyranoside [61]. From *Tephrosia calophylla* 23 different compounds were isolated of which 18 known and 5 are new. Flavonoids like (2S) – 5 – hydroxyl -7, 4-di-O-(γ, γ-dimethylallyl 1- flavanone and 6-hydroxy-E-3-(2, 5-dimethoxybenzylidine) 2', 5'-dimethoxyflavonone [71]. Tephcalostan is a new coumestan devivative isolated from the whole plant extract of *T. calophylla* and two known flavonoids, 7 – O – methylglabranin and kaempferol 3 – O – β – D - glucopyranoside [72]. Calophione – A (abenzylderivative), 1 - (6'-Hydroxy-1', 3'– benzodioxol - 5'- y) - 2- (6-hydroxy – 2 – isopropenyl - 2, 3 – dihydro – benzofuran - 5- yl) – ethane -1, 2dione and Tephcalostan - B, C, and D are three coumestan derivatives which were isolated from the roots of *T. calophylla* [73].Betulinic acid was isolated from *T. calophylla* having anticancer and anti-HIV activity [74].

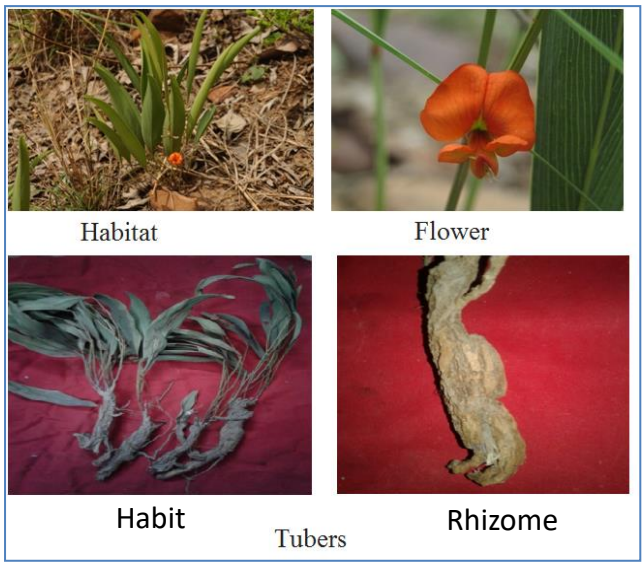


Fig 1: *Tephrosia calophylla*

MATERIALS AND METHODS

Plant collection, extract preparation and Synthesis of AgNPs

The ***Tephrosia calophylla*** plant materials was collected from Talakona area of Chittoor District of Andhra Pradesh, India and are identified with the help of Flora of the Presidency of Madras [75] and cross checked by herbarium (voucher no.409) deposited in Department of Botany, Sri Venkateswara University, Tirupati [76]. The rhizomes were washed several times with tap water to remove the dust particles and shade dried to evaporate the residual moisture. Then made into fine powder with the help of electric blender. The rhizome extract was prepared, by adding 5g of fine powder in a 500 ml Erlenmeyer flask with 100 ml of Milli Q ultra-pure distilled water and the mixture was heated at 70^0C for 30 min and then filtered through sterile muslin cloth followed by whatmann No.1 filter paper. This filtrate solution was used as source of extract for preparation of silver nanoparticles and was utilized in subsequent procedures. To the 5 ml aqueous extract, 50ml of 1 mM Ag $(NO_3)2$ was added and the sample was left at room temperature, until the color of solution changed from gray color to light brown.

Characterization of silver nanoparticles:

The bio-reduction of pure Ag+ ions done with the rhizome extract of ***T. Calophylla*** was monitored periodically by sampling of the 1µl and the optical absorbance of silver nanoparticles suspended in distilled water was recorded on UV–Vis Spectrophotometer (Nanodrop 8000 UV-Vis spectrometer) at 220–750nm wavelength range. The expiriments were carried out at room temperature on spectrophotometer at a resolution of 1 nm. Particle size and zeta potential measurement experiments were carried out by using a Nanopartical (Horiba Nanopartica SZ-100 instrument). Fourier-Transform Infra-Red (FT-IR) spectra of synthesised SNPs were analyzed in the

range of 4,000 to 500 cm-1 with an IRAFFINITY-1,IR by ATR method. Crystalline nature of metallic silver nanoparticles was examined using an X-ray diffractometer (XRD) from Bruker, D8 advance, Germany. XRD-6000 equipped with Cu, Ka radiation source using Ni as filter at a setting of 40 kV/30 mA. Transmission electron microscopy (TEM) technique was used to visualize the morphology of the AgNps. The 200 kV ultra-high-resolution transmission electron microscope (FEI-TECNAI G2 20 TWIN).TEM Grid was prepared by placing 5 µL AgNp Solution on Carbon- Coated Copper grids and drying under lamp [77-83]

Antioxidant Activity [DPPH]:

DPPH (2,2-diphenyl-1-picryl hydrazyl) free radical scavenging method involves the stock solution prepared by dissolving 4 mg of DPPH in 100 ml of methanol and stored at 20 °C. 2 ml of this solution was added to 1 ml of ***T.calophylla*** rhizome aqueous extract and *T. calophylla* AgNPs at different concentrations (25- 100µg/ml). Ascorbic acid was used as a standard. Where RSA is Radical scavenging activity, *Ac* is the absorbance of the control, and *as* is the absorbance of the sample or standard [84]

$$\text{Radical Scavenging Activity} = \frac{(Ac-As)}{(Ac)} X100 \rightarrow (1)$$

Antimicrobial studies of SNPs:

The antimicrobial activity of green synthesized silver nanoparticles from *T. calophylla* rhizome extract was analyzed against two Gram positive bacterial strains like *Bacillus subtilis*, *Staphylococcus aureus* and Two Gram negative bacterial strains like *Escherichia coli*, *Klebsiella pneumoniae*, as well as two fungal strains like *Aspergillus niger*, *Candida albicans* by Disc diffusion method [85]. The antimicrobial activity with green synthesized SNPs and comparative studies were made with plant rhizome extract as a positive control, 1mM $Ag(NO_3)2$ as negative control and Streptomycin/Flucanozole (10mg) as the standard. Sterile discs of 7 mm size were prepared from whatman No.1 filter paper and 20 µl of each extract was loaded on separate discs with the help of micro pipette and allowed to air dry for one hour in aseptic conditions. Freshly prepared nutrient agar media for bacterial culture substrate was poured into sterile petriplates and allowed 30 minutes for solidification. The plates were swabbed with microbial cultures and placed the previously prepared discs; the experiment was carried out in triplicates. The plates were incubated at 37 °C for 24 to 48 h then the zone of inhibition was measured

Anticancer activity:

AgNPs of *T.calophylla* was subjected to MTT 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colored water soluble tetrazolium dye (MTT) to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm [86, 87]. MDA MB 453 (human cancer cell line) cell line is procured from National Centre for Cell Sciences (NCCS), Pune, India. The Dulbecco's Modified Eagle's Medium with high glucose is used to growing up 2×10^4 cells per well in 96-well plates and incubated in 5% CO_2 atmosphere at 37^0C for 24 h supplemented with 2 mM/L glutamine, 10% Foetal Bovine Serum (FBS) with 10 μg/ml of *Ciprofloxacin* [88].

Afterwards medium was expelled and treated with different concentrations (12.5, 25, 50, 100 and 200 µl/ml) AgNPs of *T.calophylla* incubated for 24 hrs. Further, remove the spent media and add 100 µl of MTT reagent with the 0.5 mg/ml concentration and incubate the plate for 2.5 hrs for the reaction. Later, remove MTT reagent completely and add 100 µl of 100% Dimethyl sulfoxide (DMSO) to solubilize the formazone crystals completely and measure the absorbance at 570 nm using 96 well Plate reader. The 0.1% of DMSO used to dissolve the nanoparticles and set as negative control and 15 µM *Camptothecin* treated cell lines were set as positive control. The initial experiment was maintained for 0 to 24 hrs of timeline period with 12 hrs of time gap period to check probability of cell toxicity. It provides specific time course period to allow functional cell mortality to understand the experiment in a flexible and adaptable way. According to the results, significant cytotoxicity was observed at 24 hrs at 37^0 C incubation period. The percentage of cell viability was calculated by the following formula [89]

$$\text{Percentage of Cell viability} = \frac{\text{OD value of treated cell lines}}{\text{OD value of control}} X\ 100 \rightarrow (2)$$

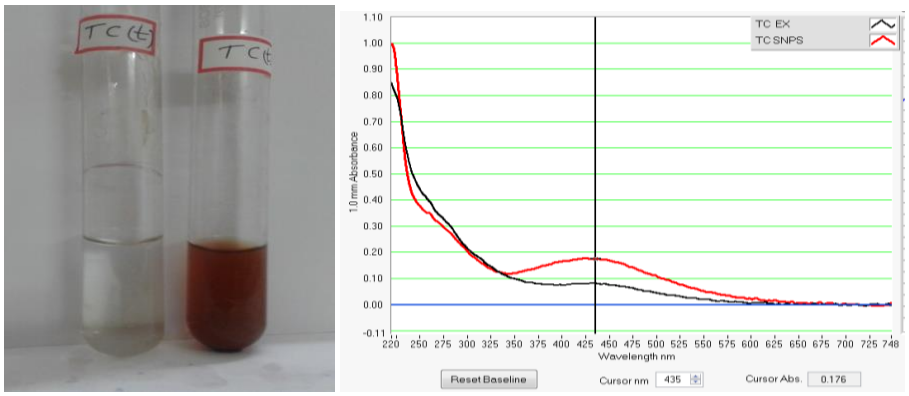
RESULTS:

Characterization of AgNPs of *T. calophylla*

UV–visible spectral analysis:

The gray colored fresh aqueous rhizome extract of *T. Calophylla* has an ability to convert silver nitrate solution into the ionic form. In this reaction, the rhizome extract reacts with 0.001M silver nitrate solution and changes to light brown color. The SPR spectrum peak of biosynthesized *Tephrosia calophylla* (TC)-AgNPs was obtained at 435 nm (Figure 2 (a–b)). Previous studies reported that lower concentration (1 ml) of plant extract results quasi-

spherical nanoparticles and higher concentrations of plant extract results in symmetrical nanoparticles [90].



(a) **(b)**

Fig 2: (a) Colour change gray to brown. (b) UV-VIS analysis of synthesized SNPs shows peak at 435nm.

Fourier Transform infra-Red (FTIR) analysis of the biosynthesized TC-AgNPs:

FTIR analysis of biosynthesized TC-AgNPs **(Fig3).** the peaks observed at **3275.13 cm^{-1}** for – C = C – H (terminal) bond of alkynes, **2981.80 cm^{-1}** for O – H (Stretch) bond of Carboxylic acids, **1629.85 cm^{-1}** for N – H (Bend) bond of amines, **1517.98 cm^{-1}** for N – O (asymmetric stretch) bond of Nitro compounds, **1330.88 cm^{-1}** for C – N (Stretch) bond of aromatic amines, **1197.79 cm^{-1}** for C – O (Stretch) bond of alcohol, **1072.42 cm^{-1}** for C – N (Stretch) bond of aliphatic amines, **1020.34 cm^{-1}** for C – O (Stretch) bond of Carboxylic acid, **825.53 cm^{-1}** for C – H (bend) bond of alkynes, **744.52 cm^{-1}** for C – H (oop) bond of aromatic, **594.08 cm^{-1}** for C – Br (Stretch) bond of alkyl halides. The results reveal that different phyto-constituents like carbohydrates, starch, tannins, flavonoids, and polyphenols of the rhizome extract have been actively participated in reduction of silver nitrate to TC-AgNPs, in capping and in stabilization of the nanoparticles. Bioactive compounds consist different functional compounds which are reduced in the process of biosynthesis of TC-AgNPs [91].

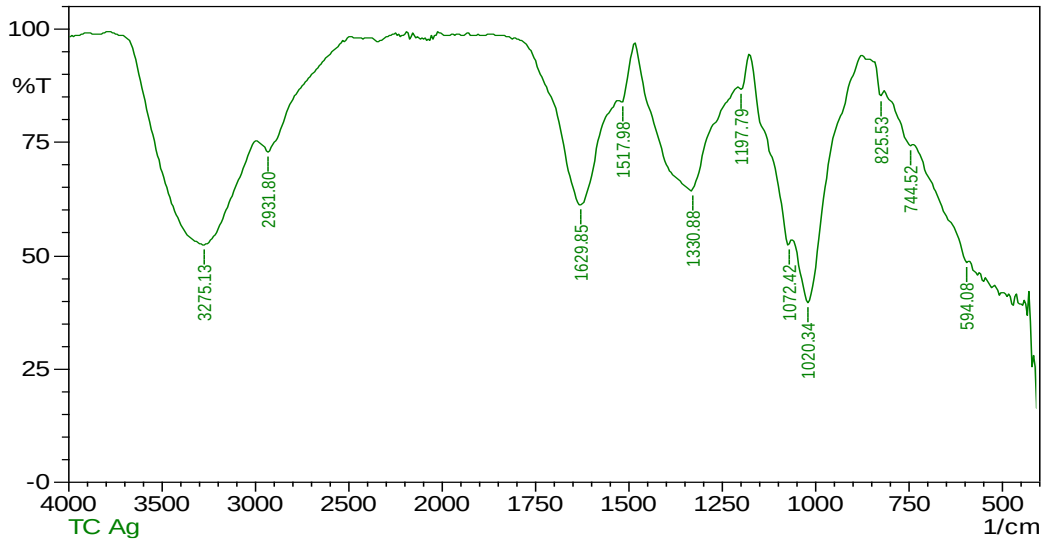


Fig 3: FTIR spectra of green synthesized **AgNPs** from aqueous rhizome extract of ***T. calophylla***

Zeta potential analysis of the biosynthesized *T. calophylla*-AgNPs:

Zeta potential is an essential parameter for the characterization of stability in aqueous nanosuspensions minimum of ± 30 mV Zeta potential values are required for indication of stable nanosuspension [92]. Zeta potential at-19.9 mV, of *T. Calophylla* AgNPs **(Fig 4)** negative value indicates the high stability of Nanoparticles. So, this result clearly indicated that the particles are fairly stable due to the electrostatic repulsion. FTIR spectrum of synthesized SNPs was carried out to know the

possible biomolecules responsible for capping and stabilization of nanoparticles. It is well known that the zeta potential value with greater than 20 mV and less than –20 mV has more electrostatic repulsion to remain stable in solution. The stability of any biosynthesized AgNPs also depends upon their pH value, with an increase in pH, the value of zeta potential of AgNPs increases [93]. The stability of nanoparticles also depends on the shape. Spherical shaped AgNPs were more stable compared with hexagonal AgNPs [94].

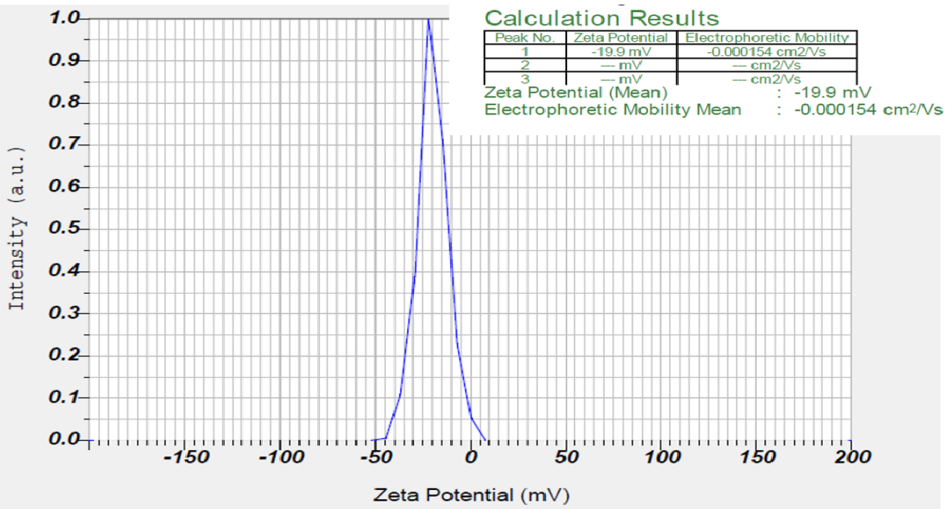


Fig 4: Zeta potential of *T.calpohylla*

XRD Analysis:

The XRD results of the synthesized silver nanoparticles of *T. Calophylla* spectrum shows distinct diffraction peaks at 2θ = 38.20°, 44.40°, 64.60°, 77.60° and 81.76 in the experimental diffractogram have been identified due to silver metal and corresponding to *hkl* values (111), (002), (022), (113) and (222) respectively (**Fig.5)**. Thus, confirmed that the resultant particles in the prepared sample are silver nanoparticles having a face-centered cubic (fcc) crystal structure. The diffractograms have been compared with the standard powder diffractogram card of JCPDS silver file no. 89-3722.

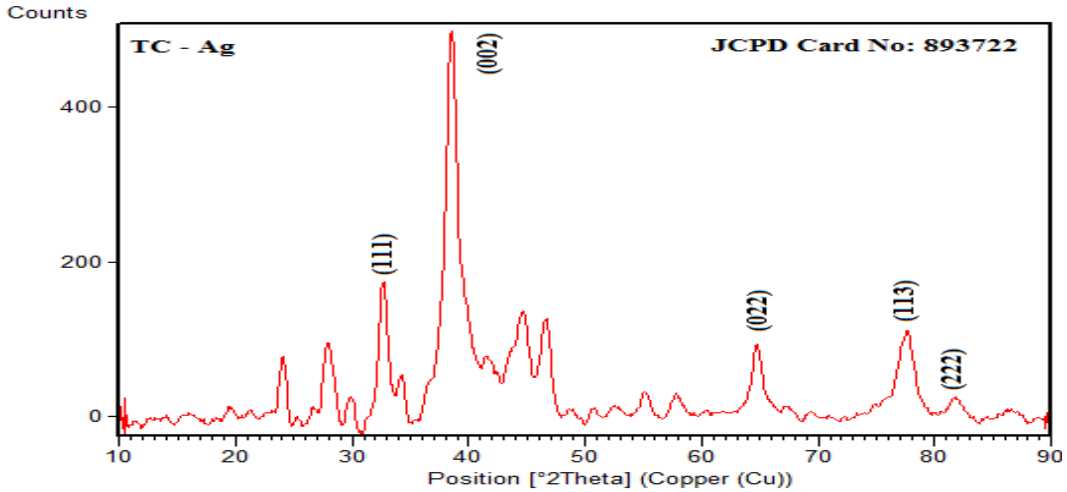


Fig 5: XRD pattern of green synthesized SNPs from rhizome extract of ***T. calophylla***.

High-Resolution Transmission Electron Microscopy (HR-TEM) Analysis:

HR-TEM was used to analyze the morphology and size of the silver nanoparticles. The selected area of electron diffraction (SAED) of *T. Calophylla* AgNPs shows clear diffused concentric rings **(Fig.6)**, which are due to crystalline and polycrystalline spots. These results indicate that the synthesized nanoparticles were crystalline in nature. The HR-TEM image of biosynthesized silver nanoparticles showing the lattice fringes quite clearly like projections of tunnels. 100-nm scale bar studies of TEM

micrographs of SNPs signify that the synthesized nanoparticles are polydispersed, predominantly spherical in shape, owing 6.7–32.4 nm size and are not in physical contact with each other i.e., no agglomeration of nanoparticles were seen.

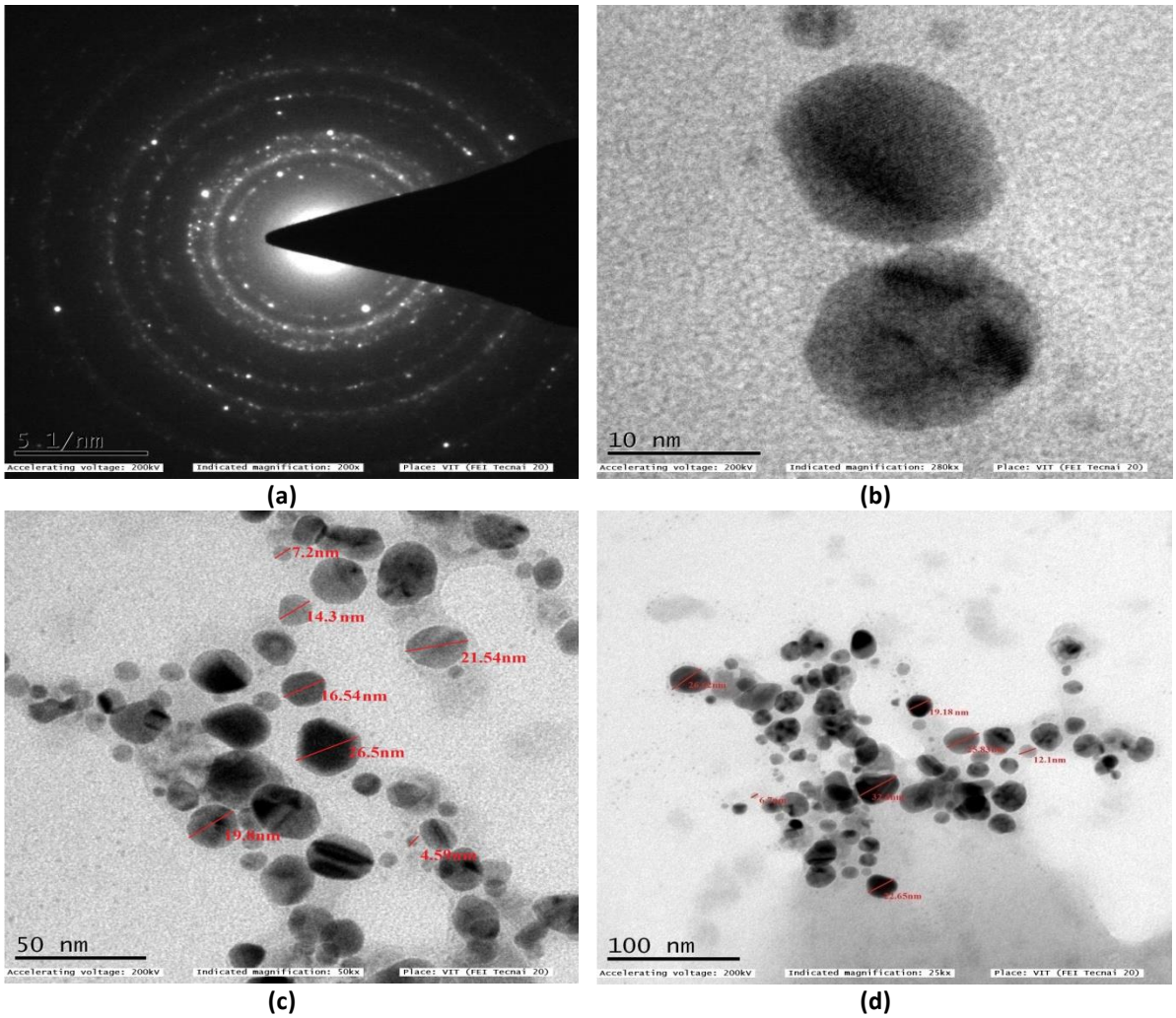


Fig 6: (a) Selected area electron diffraction (SAED) of green synthesized SNPs, (b) 10 nm resolution SNPs. shows mostly spherical shaped (c) 50 nm resolution nanoparticles with 4.59-26.5nm size. (d) 100 nm resolution with 6.7-32.4nm shows mostly spherical shaped nanoparticles.

Energy Dispersive X-ray (EDAX) Analysis:

The energy-dispersive spectrum of the synthesized AgNPs, which illustrates the presence of Ag as the ingredient element. Generally, metallic AgNPs show a strong signal peak at 3keV, due to the surface plasmon resonance [95]. The quantitative information of biosynthesized AgNPs of *T. Calophylla* EDAX analysis demonstrated the presence of 69.56% of strong silver, which shows an absorption peak at 3 keV **(Fig.7 &Table1)** along with different elements and their weight percentages like copper 30.44% without any contaminants. All the peaks of the silver are observed and assigned. Also, one strong signal peaks for Cu (Copper) appeared in EDAX data, which are due to the carbon coated copper grid.

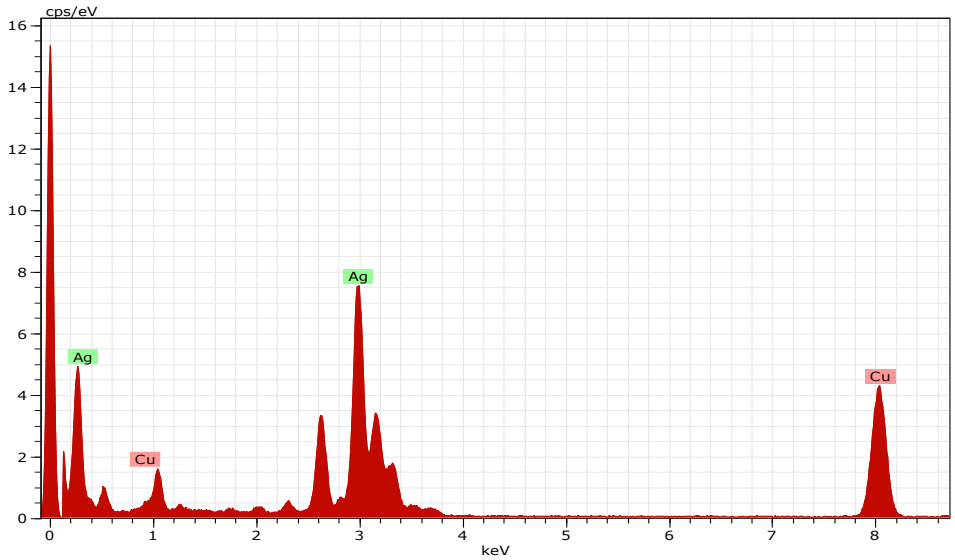


Fig 7: EDX analysis of green Synthesized **AgNPs** of ***T. calophylla***

Table 1: EDAX Spectrum: Spectrum 446-TC - **AgNPs**

Elements	Series	Net	unn. C [wt. %]	corm. C [wt. %]	Atom. C [wt. %]	Error (3 sigma) [wt. %]
Copper	K – series	41444	30.44	30.44	42.62	2.86
Silver	K - series	79811	69.56	69.56	57.38	20.96
		Total	100.00	100.00	100.00	

The EDAX analysis of synthesized sample shows **69.56%** of silver metal along with **30.44%** of copper. **69.56%** of silver indicates the sample having high purity of silver nanoparticles

Antioxidant Analysis (DPPH): AgNPs *T. calophylla*

The aqueous rhizome extract and synthesized AgNPs of *T. calophylla* showed better antioxidant potential when compare to standard ascorbic acid by DPPH scavenging assay method. Different concentrations ranging from 25-100 µg/ml of *T. calophylla* rhizome aqueous extract and *T. calophylla* AgNPs; Ascorbic acid was taken as a positive control to compare the percentage activity of the aqueous rhizome extract and silver nanoparticles **(Fig. 8.)** The antioxidant activity was increased in dose-dependent manner. The highest percentage activity was exhibited at 100 µg/ml *T. calophylla* rhizome extract (33.4) < *T. calophylla* AgNPs (59.56) < Ascorbic acid (76.6) **(Table 2)**. It is concluded that silver nanoparticles of *T. calophylla* rhizome extract possesses good DPPH activity when compared to that of rhizome extract alone. The antioxidant activity of AgNPs by the DPPH method shows a strong absorption band at 517 nm.

Table 2: Antioxidant activity of ***T. calophylla*** **- AgNPs**

Concentration	Extracts (%)	AgNPs (%)	Ascorbic acid (%)
25 µg/ml	12.02 ± 0.16	13.64 ± 0.28	46.36 ± 0.08
50 µg/ml	17.14 ± 0.15	18.56 ± 0.26	58.42 ± 0.06
75 µg/ml	25.02 ± 0.1	35.42 ± 0.42	68.16 ± 0.28
100 µg/ml	33.4 ± 0.16	59.56 ± 0.39	76.6 ± 0.62

Values of average of triplicates ± S.E

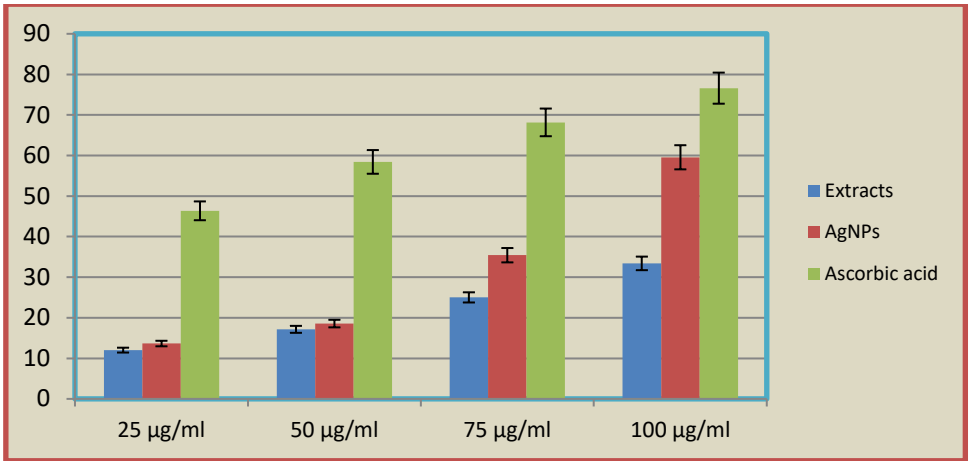


Fig 8: Antioxidant activity of Phytosynthesized **AgNPs** of ***T. calophylla***

Antimicrobial Activity: AgNPs *T. calophylla*

The green synthesized silver nanoparticles of *T. Calophylla* were assessed for antimicrobial activities against two gram positive and two-gram negative bacterial strains as well as two fungal strains. Among the bacteria the highest Inhibition zones were observed on *Staphylococcus aureus* (32.25 mm) followed by *Klebsiella pneumoniae* (30.50 mm), *Escherichia coli* (28.28) and *Bacillus subtilis* (28mm) Whereas in the case of fungi, the highest zone of inhibition were observed against *C.albicans* (15.75mm) The aqueous extract of *T. calophylla* and Ag $(NO_3)2$ showed a limited zone of inhibition compared to TC-AgNPs **(Figs. 9 (a & b), 10(a & b), &Table 3&4)**. The above result clearly showed that the synthesized AgNPs have potent antibacterial activity against microbial pathogens. In this study, the zone of inhibition was less against fungal strains when compared with bacterial strains. Among the bacteria, a zone of inhibition was lesser in gram negative bacteria when compared with gram positive bacterial strains.

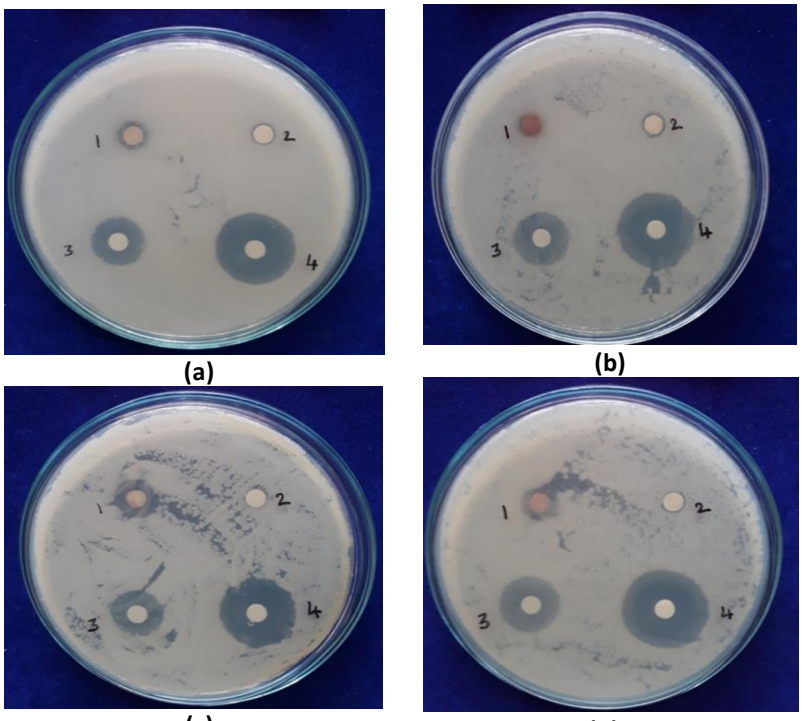


Fig 9a : (a) *Escherichia coli,* **(b)** *Klebsiella pneumonia,* **(c)** *Bacillus subtilis* and **(d)** *Staphylococcus aureus* **Note: 1)** Aqueous Extract **2)** Ag$(NO_3)2$ **3)** AgNPs **4)** Streptomycin

Table 3: Effect of different extracts and green synthesized copper oxide nanoparticles of ***T. calophylla*** on bacterial Strains

Name of Organism	Aqueous Extract	$Ag(NO_3)2$	AgNPs	Streptomycin
Escherichia coli	13.75 ± 0.25 ***	11.75 ± 0.48 ***	28.25 ± 0.48 ***	37.00 ± 0.58
Klebsiella pneumoniae	13.00 ± 0.41 ***	11.50 ± 0.29 ***	30.50 ± 0.29 ***	40.00 ± 0.41
Bacillus subtilis	18.75 ± 0.48 ***	13.25 ± 0.25 ***	28.00 ± 0.41 ***	39.75 ± 0.48
Staphylococcus aureus	18.50 ± 0.29 ***	12.00 ± 0.41 ***	32.75 ± 0.75 ***	42.25 ± 0.63

All the data are expressed as **mean ± SEM:** ***p<0.01, **p<0.02, *p<0.03 as compared to Control group, n=4; (One – Way ANOVA followed by Dunnett's test).

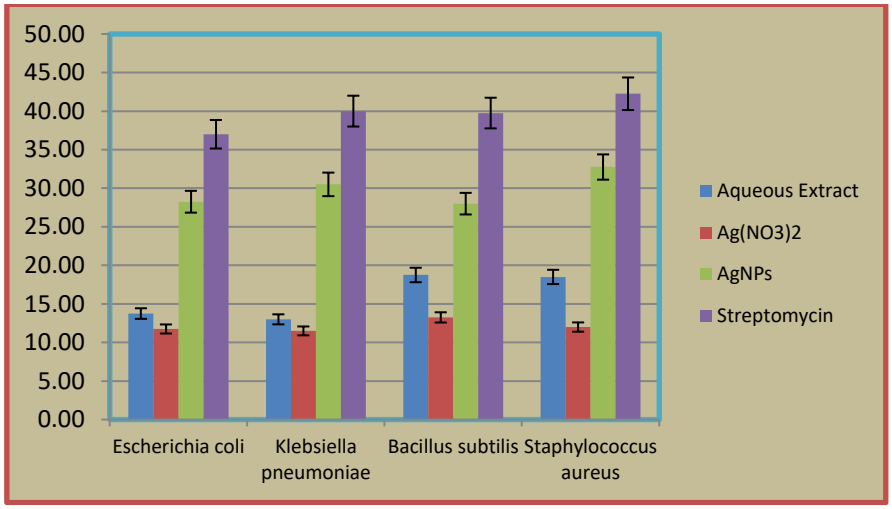


Fig 9b: Zone of inhibition of different extracts and AgNPs of ***T. calophylla*** on bacterial strains.

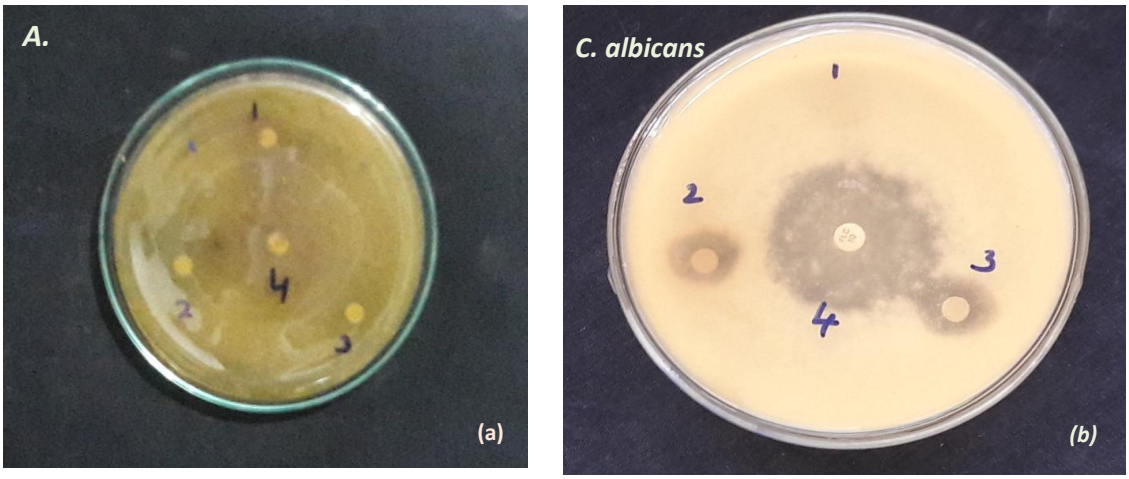


Fig 10a: Antifungal activity of aqueous extracts and **AgNPs** of ***T. calophylla*** 1) Aqueous Extract 2) $Ag(NO_3)2$ 3) AgNPs 4) Fluconazole

Table 4: Effect of green synthesized **AgNPs** of ***T. calophylla*** on fungal strains.

Name of Organism	Aqueous Extract	$Ag(NO_3)2$	AgNPs	Fluconazole
Aspergillus niger	6.28 ± 0.02 ***	7.4 ± 0.07 ***	9.60 ± 0.08 ***	14.25± 0.63
Candida albicans	6.83± 0.03 ***	8.35± 0.09 ***	15.75 ± 0.63 ***	26.00± 0.71

All the data are expressed as **mean ± SEM:** ***p<0.01, **p<0.02, *p<0.03 as compared to Control group, n=4; (One – Way ANOVA followed by Dunnett's test).

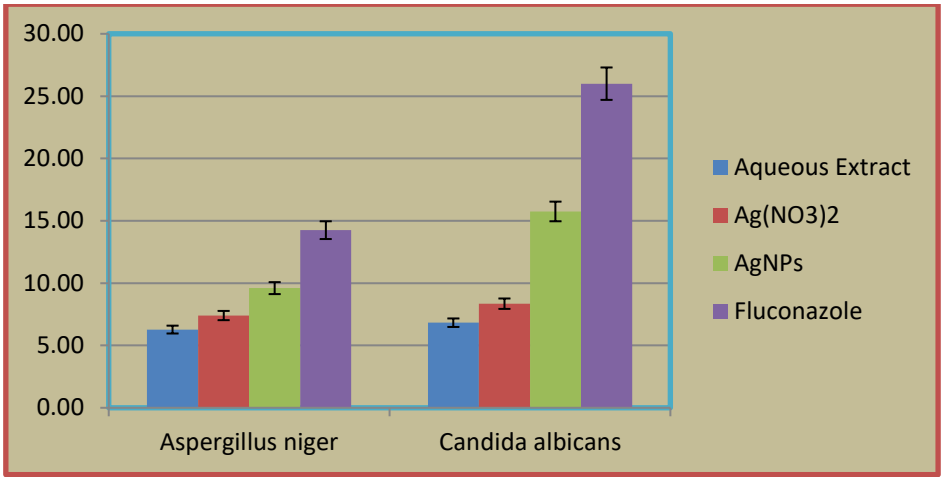
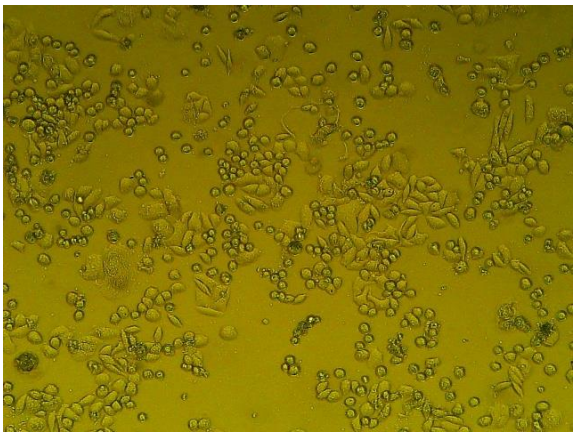


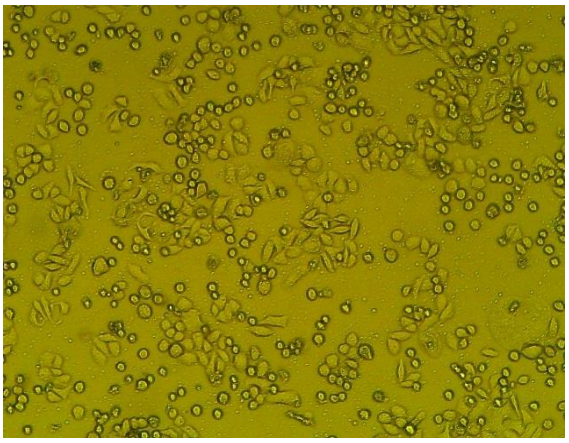
Fig10b: Zone of inhibition of AgNPs of ***T. calophylla*** on fungal strains.

Anticancer activity *T. calophylla* –AgNPs:

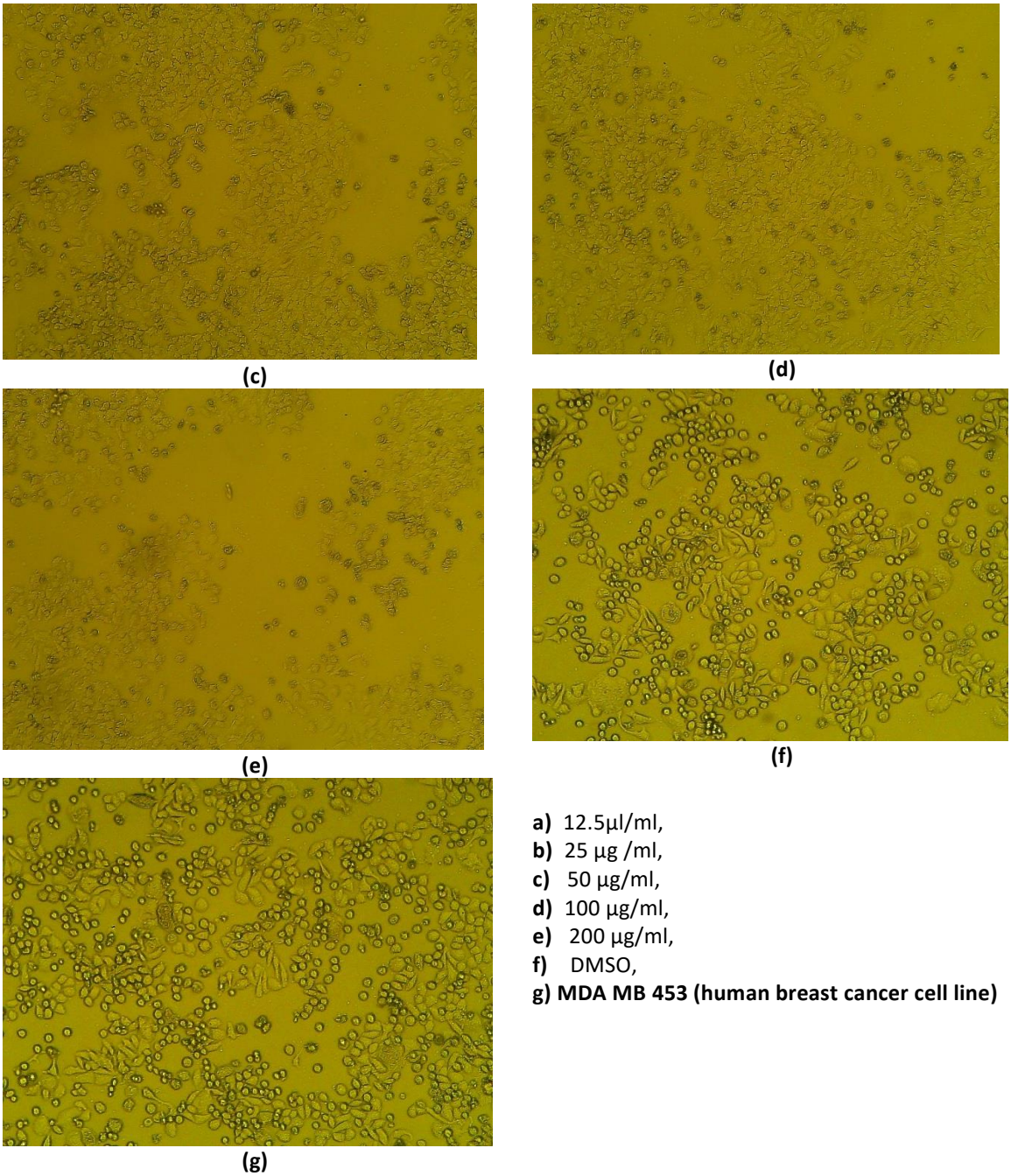
The MDA MB 453 (human cancer cell line) cell line was used for cytotoxicity analysis by reading Formazan crystals formed by the reaction of mitochondrial dehydrogenase by MTT assay. At 48 hrs of incubation period, a significant abatement in cell viability was observed against the treated cell lines, when the concentration of AgNPs was increased from 12.5, 25, 50, 100 and 200 µg/ml. DMSO was used as a positive control to exhibit 100% of healthy proliferated cells **(Figs. 11a&11b, & Table 5).** The 50 µg/ml concentration (IC_{50}) of AgNPs may have the capability to reduce 50% of treated cell lines when compared with negative control. The cytotoxicity of nanoparticles may depend on the small size and spherical shape of the particles [69]. The Observations in Statistical data of Cytotoxicity study by ELISA Reader suggesting us that against MDA MB 453 (human cancer cell line) cells, TC-AgNPs showing good cytotoxic potential properties with the IC IC_{50} Concentrations 69.11µg/ml. But, there is no report on AgNPs synthesized from *T. Calophylla* to attribute anticancer activity against MDA MB 453 (human breast cancer cell line) cell lines. From this study, the green synthesized AgNPs from rhizome extract showed small size spherical shaped particles, which exhibit strong cytotoxic activity against MDA MB 453.



(a)



(b)



(c) **(d)** **(e)** **(f)** **(g)**

a) 12.5µl/ml,
b) 25 µg /ml,
c) 50 µg/ml,
d) 100 µg/ml,
e) 200 µg/ml,
f) DMSO,
g) MDA MB 453 (human breast cancer cell line)

Fig 11 a: Anticancer activity of synthesized AgNPs.

Table 5: Anticancer effect of ***T. calophylla*** **AgNPs** on the MDA MB453 cell lines

Concentration (µg/ml)	Absorbance (O.D)	Cell viability (%)	Cell Death (%)
DMSO	0.833	100	0
12.5	0.662	79.47	20.53
25	0.546	65.54	34.46
50	0.37	44.41	55.59
100	0.2585	31.03	68.97
200	0.0955	17.46	82.54
Camptothecin	0.41	14.21	85.79

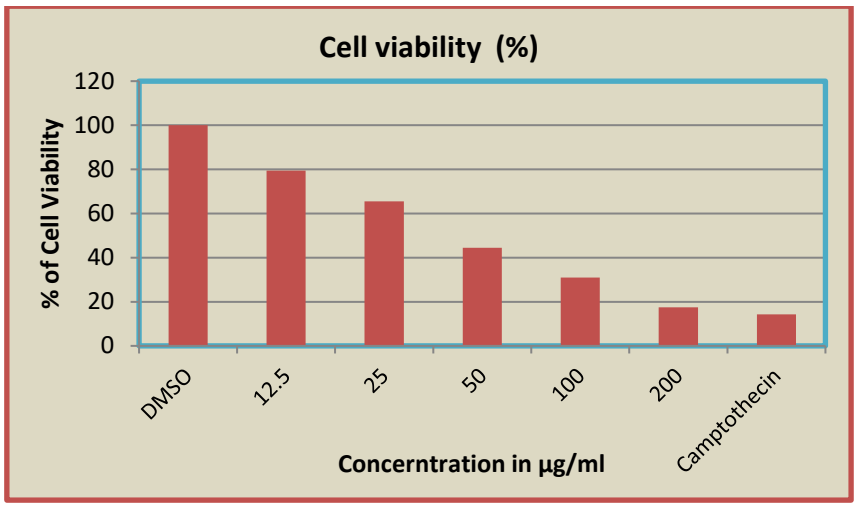


Fig 11b: Anticancer activity of AgNPs from rhizome extract of ***T. calophylla***

DISCUSSION

T. Calophylla is having hepatoprotective activity [67]. *T. calophylla* roots chloroform extracts showed antibacterial and antifungal activity, at 200 mg/ml may be due to the presence of isoflavones like caloisoflavones [47]. The cytotoxicity on RAW & HT-29 cell lines by *T. calophylla*, showed significant activity [20]. Leaf extract of *T. purpurea* synthesized AgNPs (silver nanoparticles) showed effective antimicrobial activity against *Pseudomonas spp.* and Penicillium *spp.* [52]. Different flavonoids isolated from *T. calophylla* are responsible for antiprotozoal activity on *Trypanosoma*, *Leishmania* and *Plasmodium* parasites [68]. *T. calophylla* contains a wide variety of flavonoids and isoflavonoids. According to Ayurveda, this plant exhibits several medicinal properties such as antihelmintic, antipyretic, antiulcer, antimicrobial, anticancer and hepatoprotective activity. It is also active against leprosy, ulcers, and used as alternative to cure the diseases of the liver, spleen, heart and blood. The roots having diuretic, enriches blood, cures diarrhea and is useful in bronchitis, inflammations, antidiabetic, boils and pimples. Leaves are tonic to intestines and a promising appetizer [66].

CONCLUSION AND RECOMMENDATIONS

In this investigation we report a green chemistry approach for the synthesis of Ag NPs using *T. calophylla* rhizome extract. This is simple, reliable, clean method which promotes green industrial level product of Ag NPs and environmentally friendly route for synthesis of benign nanoparticles. Peaks in XRD profile and bright spot array in the SAED pattern evidenced the crystalline nature of the Ag NPs. The presence of flavonoids and proteins are possibly the key factors for the formation of Ag NPs. FTIR spectrum confirms the biomolecules are responsible for reducing and capping of Ag NPs. From TEM measurements, the size of Ag NPs was found to be 7-32 nm is in good agreement with XRD results. Moreover, synthesized Ag NPs were pure and showed good antimicrobial and anticancer activity leads to go high potential uses in biological applications.

T. calophylla having a vast amount of secondary metabolites such as Coumestan flavonoid, Quercetin and Rutin shows significant activities such as Hepato protective, antimicrobial, anti protozoal, anti cancer and anti HIV, anthelmintic, and anti ulcer activities. The synthesized nanoparticles of *T. calophylla* may be tested for the therapeutic applications of various biological activities for the better treatment with biosynthesized components.

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