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Tinospora Cordifolia plant leaf extract mediated Silver nanoparticles and their potential antimicrobial, antioxidant and *in vitro* anticancer activity on HepG2 Liver cancer cells.

Hindu C[a], Susmila Aparna Gaddam[b], Venkata Subbaiah Kotakadi[c]*, Varadarajulu Naidu Challagundla[d], and Josthna Penchalaneni[a]*.

[a]Department of Biotechnology, Sri Padmavathi Mahila University, Tirupati, AP. India
[b]Department of Virology, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, India
[c]DST-PURSE Centre, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, India.
[d]Department of Biotechnology, Dravidian University, Kuppam, AP. India

ABSTRACT

In the present study, we hereby report that the green synthesized *Tinospora Cordifolia* leaf extract mediated silver nanoparticles (TC-AgNPs) revealed versatile beneficial applications. The present work is an simple and fast method for green synthesis of silver nanoparticles (AgNPs) using 0.02 M silver nitrate and leaf extract of an significant medicinal plant *Tinospora Cordifolia* (TC). The synthesized TC-AgNPs were characterized by various spectroscopic methods, the results reveal that UV-visible absorption spectrum indicates peaks at 439 nm which confirms the green synthesis of TC-AgNPs. Fourier transform infrared spectroscopy (FT-IR) results specify the characteristic peaks plant bioactive molecules actively participated in reduction of their functional group**s** for capping and stabilization of silver nanoparticles. The particle size was between 5 nm to 35 ± 10 nm in dynamic light scattering analysis (DLS), with an average size of 14.9 nm with an Z-average 19.8 nm with an poly dispersed index (PI) of 0.187 indicating well dispersed in aqueous form, at the same time Transmission electron microscopy (TEM) analysis also reveal will similar type results, where the particles are spherical or roughly spherical the size range from 6 nm to 37 nm ± 5 nm. The TC-AgNPs were highly stable with negative zeta potential value −28.5 mV. The x-ray diffraction (XRD) data revealed that the particles are facets of face-centered cubic crystal structure of AgNPs. The TC-AgNPs have exhibit potential antioxidant activity and prosperous antibacterial activity against gram-positive and gram negative bacteria. The TC-AgNPs have also revealed potential anti-cancer activity *in vitro* on HepG-2 liver cancer cell lines. The anti- cancer activity of TC-AgNPs comparable to that of the standard drug was evidenced by various anticancer studies like MTT assay, ROS, Cell cycle, Apoptosis and Dual fluorescence studies. The anti-proliferative and cytotoxic studies on HepG2 Liver cancer cell line was carried by MTT assay, reveals that IC_{50} value was 56.32µg/ml and the other anticancer studies cell cycle, ROS studies and apoptotic studies revealed that the TC-AgNPs have excellent anticancer properties. The apoptotic cells were detected by Dual fluorescence assay and also by Flow Cytometry assay. From the above results it can conclude that the green synthesized TC-AgNPs can useful as future therapeutic agents to control antimicrobial resistance and different types cancers effectively.

Keywords: *Tinospora Cordifolia,* TC-AgNPs; Spectral analysis; Antibacterial activity; Antioxidant activity; Anticancer activity; HepG2 Liver cancer cell lines.

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Corresponding author

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INTRODUCTION

The 21st century is identified as the age of Nano-materials due their potential applications in various advanced areas of Medicine, Agriculture, and Industrial & Pharmaceutical technology. Since, few decades' scientists have been widely working in the area of Nanotechnology, using different noble metals for the synthesis of metal nanoparticles by diverse methods. These metal nanoparticles exhibit distinct biological, physical and chemical properties. These synthesized nanoparticles which are less than 100 nm in size are in front abundantly due to their novel applications in various areas like health care, food, medical and pharmaceutics. Apart from biomedical and therapeutic applications, they also play an important role in other applications like optical, electromagnetic, electrical, thermal, catalytic and biological properties [1-5].

Currently human population is facing major disease problem due to Cancer, Cancer is the main cause for high mortality rate worldwide. There are various types of cancer, generally they are classified by the tissue they originate in and they can be classified by the organ or body part they affect, such as breast, lung, colon or prostate etc., namely, Breast Cancer, Lung Cancer, Prostate Cancer, Colorectal Cancer, Melanoma, Bladder Cancer, Kidney Cancer, Uterine Cancer and Non-Hodgkin Lymphoma. Among the above cancers, Lung Cancer is the second most common cancer overall and a leading cause of cancer deaths in both men and women worldwide. The current epidemic statistics reveals that more than 75% of liver cancer incidents were reported from Asian countries, and highest mortality rate was observed in India and USA [6]. The important cancer of liver is Hepatocellular carcinoma (HCC) in most of the developing countries [7]. 'Hepatocarcinogenesis' occur due to the hepatocellular damage through reactive oxygen species (ROS), the ROS generate or lead to chronic inflammation in hepatocellular cells [8]. To combat with cancer the human body reacts by various methods to counteract against cancer, the most important such way is known as Apoptosis. During apoptosis, nuclear condensation and nuclear fragmentation are two main morphological changes that occur in the cells. These cells are phagocytized by macrophages. Therefore, induction of apoptosis in cancer can be targeted for the treatment of cancer [9-10]. From the ages, the traditional ayurvedic practitioners in India and other Asian countries medical practitioners have been using plant based formulations to treat cancer. These plants contains the major source of secondary metabolites including phenolic compounds such as flavonoids, quinones, coumarins, phenolic acids, lignans, stilbenes, tannins and also nitrogen-containing compounds like alkaloids, amines, betalains and they also consists of various number of vitamins and terpenoids which includes carotenoids and other metabolites [11-13]. Among these the flavonoid and phenolic acids are most important secondary metabolites capable of scavenging free superoxide radicals and reactive oxygen species (ROS), they also play an important role in promoting anti-aging, and also reducing the risk of cancer[14-16]. At the moment, different healing methods like chemotherapy, radiation therapy and surgery are now used for the treatment of liver cancer. Yet, a successful anti-liver cancer drug with no or minimum toxic effects are unavailable to date. India is having rich biodiversity of medicinal plants; these plants have not been investigated for both their chemical composition and bioactivities. Therefore, the current study was undertaken, to green synthesis silver nanoparticles by means of an important medicinal plant *Tinospora cordifolia* which is widely available an all parts of India. *Tinospora cordifolia* is an herbaceous climbing shrub native of India and South Asia, the extracts of leaves, roots and bark of which have widely been used extensively in traditional Indian Ayurveda medicine for a large number of diseases conditions including arthritis, diabetes, malaria, pain, liver diseases, urinary tract infection, and recently prevention or amelioration of COVID-19 infection. This plant sources can provide novel and effective anti-liver-cancer compounds as well as to characterize their anti-oxidant, antimicrobial and anti-cancer activities by the green synthesized silver nanoparticles by whole plant extract.

The silver nanoparticles have been widely green synthesized and studied, due to their size, shape, size distribution and surface area, which makes them has attractive and fulfilling nano-materials suitable for a range of potential therapeutic applications. Over the past three decades it is certainly evident that the Green synthesis metal nanoparticles and silver nanoparticles (AgNPs) have gained lot of interests due to their easy, bio-safe, low cost, and eco-friendly in nature [17-21]. The AgNPs were commonly synthesized using different parts of plant extracts by using plants leaves, flowers, fruits, seeds, stem, bark and roots etc. It is clearly known these plants parts are enriched with various types of bioactive phyto-constituents like polyphenols, flavinoids, tannins, alkaloids, saponins and glycosides etc., which are actively participated in bio-reduction and stabilization of silver nanoparticles. These AgNPs prove to exhibit thriving applications such as antimicrobial, antioxidant, anti-inflammatory & anticancer studies, anti-diabetic, wound healing and phyto-catalytic dye degradation [22-29].

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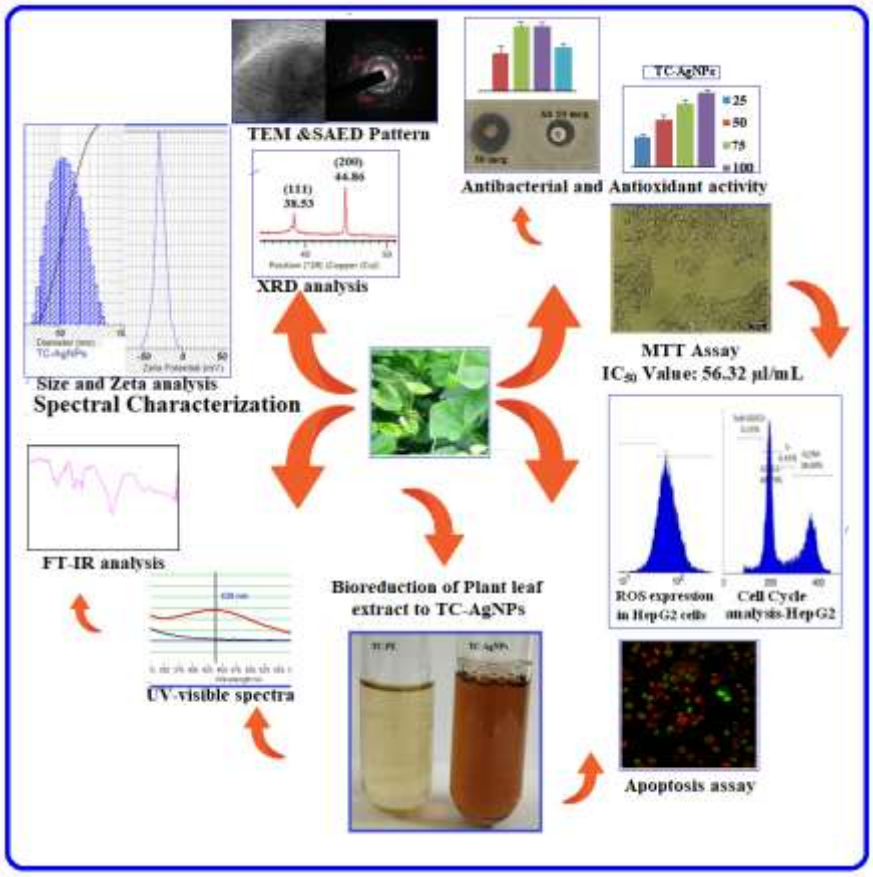
Keeping in view of above medical, industrial and pharmaceutical application of *Tinospora Cordifolia*, an significant attempt was made to green synthesis silver nanoparticles using plant aqueous extract. Currently quite a few researchers from all over the world have been extensively working on green synthesis of metal nanoparticles, for the development of biomedical applications to treat various illnesses in mankind and also for environmental protection and their impact on human health. So the current study was started to green synthesis of silver nanoparticles (AgNPs) by aqueous plant extract and these AgNPs were characterized by exclusively by various spectroscopic methods like UV-Visible spectroscopy, FTIR, Particle size & Zeta potential, XRD and TEM. To conclude, their importance in various bio-medicinal applications, studies like antibacterial activity, antioxidant activity and in-vitro cancer studies were carried out by green synthesized TC-AgNPs. The whole process of biosynthesis of *Tinospora Cordifolia* plant leaf extract mediated silver nanoparticles was shown in a schematic diagram as shown below



Schematic diagram of biosynthesis of *Tinospora Cordifolia* mediated silver nanoparticles and their spectral characterization and therapeutic applications

MATERIAL AND METHODS

Preparation of plant extract for Green Synthesis of silver nanoparticles

The whole plant of *Tinospora cordifolia* was collected from the herbs, shrubs and trees growing in and around of Thallakona forest region of Tirupati, Chittoor district of Andhra Pradesh State, India during the months of October and November. The whole plant along with leaves of *T. cordifolia* were collected and shade dried for a week at room temperature and then made into fine powder with the help of electric blender. The plant powder of *Tinospora cordifolia* extracts was prepared, by adding 10g of finely powder of whole plant along with leaves in a 500 ml Erlenmeyer flask with 250 ml of Milli-Q ultra pure distilled water and the mixture was heated at 70^0C for 45 min and left the sample at room temperature for overnight, next day morning the sample was then filtered through sterile muslin cloth followed by Whatman No.1 filter paper [30-31]. These filtrate solution was stored at 4^0C in refrigerator until further

use to other experimental studies. This filtrate solution was used as source of extract for preparation of silver nanoparticles nanoparticles and was utilized in subsequent procedures.

Green Synthesis of silver nanoparticles

To the 5ml diluted filtrate, 10ml of 0.02 M silver nitrate ($AgNO_3$) was added and the sample was left at room temperature, until the colour of solution changed from pale light colour-less to light brown and subsequently dark brown. The solution containing TC-AgNPs was confirmed by the dark brown colour change. So, in the current study the green synthesis of silver nanoparticles (AgNPs) with *Tinospora cordifolia* (*T.cordifolia*) (TC-AgNPs) with whole plant extract was prepared without using any external toxic chemicals. Hence, this method is popularly known as 'Green synthesis' or 'Green Method' [30, 32]. The obtained AgNPs were used for further spectral characterization by UV-Vis, FTIR, Particle size analysis by DLS method and Zeta potential analysis, XRD and TEM and studied their biomedical applications like antibacterial, antioxidant, and anti-cancer studies. The biosynthesized and purified AgNPs were named as TC-AgNPs

Purification of TC-AgNPs

The green synthesized TC-AgNPs were purified by continues different centrifugal speeds as explained by Gaddam *et al* 2021 and Gunti H., *et al* 2023 [1, 25]. Finally the purified green TC-AgNPs pellets were dissolved in known volume of Milli-Q water and further used for spectral analysis like FTIR, Particle size analysis, Zeta potential analysis, XRD and Transmission electron microscopy (TEM). The remaining nanoparticles were used to study other biomedical and industrial applications.

Spectral Characterization of TC-AgNPs

The green synthesized TC-AgNPs along with **plant extract** were analyzed by Nanodrop-8000 UV visible spectrometry within the wavelength range of 220 nm -750 nm, (Thermo Scientifics, U.S.A. available at DST Purse Centre, Sri Venkateswara University, Tirupati). The TC-AgNPs were analyzed on Nanodrop-8000 spectrophotometer at a resolution of 1 nm to detect the surface Plasmon resonance (SPR) of green synthesized TC-AgNPs. Fourier transform infrared spectroscopy (FTIR) analysis of Corn silk extract and green synthesized TC-AgNPs were analyzed to detect which bioactive constituents of corn silk extract are actively involved bio-reduction and capping of green synthesized TC-AgNPs (Bruker Tensor 27, Thermo Scientifics, USA, available at Department of Physics, Sri Venkateswara University, Tirupati). The Particle size and Zeta potential analysis of green synthesized TC-AgNPs was carried out by dynamic light scattering (DLS) method by using Nanopartica Horiba SZ-100 (Japan), available at DST PURSE centre, Sri Venkateswara University, Tirupati. The size and surface charge or zeta potential of TC-AgNPs in the purified aqueous colloidal sample was analyzed to find out the size distribution of nanoparticles and the stability of green synthesized TC-AgNPs in Transmission Electron Microscopy (HR-EM) studies was performed by JEOL JEM Plus -2100 (SVU Tirupati). The images were taken by coating a drop of TC-AgNPs on a carbon coated copper grid.

Biomedical Applications of green synthesized silver nanoparticles TC-AgNPs

The green synthesized silver and zinc nanoparticles i.e. TC-AgNPs by whole plant extract of *Tinospora cordifolia* were spectral characterized by the above advanced techniques. Later on, they were tested for their various biomedical applications like the antimicrobial activity, antioxidant activity and the anticancer activity.

Antimicrobial activity assay by Disc diffusion method

The antimicrobial activity of green synthesized silver and zinc nanoparticles i.e. TC-AgNPs obtained from the whole plant extract of *Tinospora cordifolia* were tested by standard disc diffusion method standardized by Kotakadi., et al [31] [33]. The tested bacteria were maintained as the nutrient agar (Hi-media) slants. A loop full of culture from the slant was inoculated into the nutrient agar broth and incubated at 37^0 C for overnight. 0.1 ml of this culture was evenly spread on the sterile nutrient agar plate. Sterile disc of Whatman No.1 filter paper of about 5 mm diameter were prepared. The sterile disc were dipped in silver nanoparticles solution (10µl, 20µl and 30µl) and allowed to dry before being placed on the agar plate, along with the discs prepared from plant leaf extract and the standard disc of

Amoxyclav. The cultures were incubated at 37^0 C for 24 hours. The inoculated plates were incubated at 37^0 C for overnight, after incubation, the plates were observed for zones of inhibition surrounding the disc. Different levels of zones of inhibition were measured by scale using the antibiotic zone as standard scale and recorded. Zone of inhibition around the disc indicates that the compounds or synthesized nanoparticles which diffused into the agar from the disc inhibited the growth of the organism. The antimicrobial activity of silver nanoparticles was evaluated against Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* Gram-positive *Staphylococcus aureus, Bacillus megaterium* The bacterial strains of microorganisms used for the antibacterial activities were obtained from DST-PURSE Centre, S.V. University, Tirupati.

Antioxidant activity of the green synthesized TC-AgNPs

The antioxidant activity of the green synthesized TC-AgNPs obtained from the whole plant extracts of *T.cordifolia* were evaluated *in vitro* by three methods. The green synthesized AgNPs were used to access or evalutate the free radical scavenging activity against 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Nitric oxide (NO) and Hydrogen peroxide ($H_2O_{2)}$ radicals. Ascorbic acid is used as a standard control.

DPPH radical scavenging activity

The hydrogen atom or electron donation ability of the compounds was measured from the bleaching of the purple colored methanol solution of (DPPH) and used to detect radical scavenging assay. The method was followed by Mittal *et al* [34] and with slight modified procedure according to Gaddam *et al*.,[1]. The spectrophotometric assay uses the stable radical DPPH as a reagent. 1 mL of various concentrations of the test compounds i.e. the plant extracts and the green synthesized TC-AgNPs and TC-ZnONPs (25, 50, 75, and 100 µg/mL) in methanol were added to 4 mL of 0.004% (w/v) methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was measured against blank at 517 nm. The percent of inhibition (%) of free radical production from DPPH was calculated by the following equation. All the experiments were conducted and radical scavenging activity values were calculated from the absorbance values using the following formula.

$$\text{Radical Scavenging Activity } = \frac{(\text{Ac - As})}{(\text{Ac})} \times 100$$

Where 'A_C'stands for the absorbance of the control reaction (containing all reagents except the test compound i.e. the plant extracts and the TC-AgNPs and TC-ZnONPs) and 'A_S' stands for the absorbance of the test compound, the tests were carried in triplicate.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured by slightly modified methods by using a modified version of the method established by Mosmann [35]. Nitric oxide radicals (NO) were generated from sodium nitroprusside. 1 mL of sodium nitroprusside (10 mM) and 1.5 ml of phosphate buffer saline (0.2 M, pH 7.4) were added to different concentrations (25, 50, 75 and 100 µg/mL) of the test compounds (plant extracts and TC-AgNPs and TC-ZnONPs) and incubated for 150 min at 25° C and 1mL of the reaction mixture was treated with 1 mL of Griess reagent (1%) sulphanilamide, 2% H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride. The absorbance of the compounds was measured at 546 nm.

Hydrogen peroxide scavenging activity

H_2O_2 scavenging ability of the test compounds was determined according to the literature method Pick and Mizel [36] following the approach outlined by Sousa *et al* [37], Kotakadi *et al*., [38]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4) were added to different concentrations of (25, 50, 75 and 100 µg/mL) of the test compounds (i.e. whole plant leaf extract and TC-AgNPs 3.4 mL phosphate buffer were added to H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm.

Anticancer activity of the green synthesized TC-AgNPs

Materials and Reagents: Cell Lines

The HepG2 cell line is a human hepatoma cell line that is often used to study drug metabolism and hepatotoxicity. It is a pure cell line of human liver carcinoma that is used as a model for hepato cellular carcinoma (HCC) obtained from (NCCS, Pune, India).

Cell culture medium/Reagents

DMEM with high glucose medium - (Cat No:11965-084, Gibco, Invitrogen), Fetal Bovine Serum (#10270106, Gibco, Invitrogen), D-PBS (#D8537-500ml, Sigma Aldrich, St Louis, USA), Test compounds: TC-AgNPs and Doxorubicin (standard) (Cat No: D1515, Sigma Aldrich, St Louis, USA), DMSO (Cat No: 276855, Sigma), MTT (Cat No: M2128, Sigma Aldrich, St Louis, USA), Antibiotic-Antimycotic solution-100x (Cat No: 15240096, Gibco, Invitrogen), Trypsin EDTA (Cat No: T4174, Sigma), 96-well plate for culturing the cells (130188, BioLite, Thermo), 6well culture plate (130184, BioLite, Thermo), T25 flask (# 12556009, Biolite - Thermo), 50 ml centrifuge tubes (# 339652, Thermo Scientific), 1.5 ml centrifuge tubes (500010,TARSONS), 200 to 1000ul microtips (521020X, TARSONS), 2-200ul microtips (521010, TARSONS), 70% ethanol, BD Pharmingen Annexin V Apoptosis detection kit (Cat No: 556547, BD Biosciences, CA, USA), Falcon 5ml Round Bottom Polystyrene Test tube (Cat No: 352054, Corning), Propidium Iodide/RNase staining buffer (Cat No: 550825, BD Biosciences, CA, USA), Pre chilled Ethanol (China grade), Acridine orange hydrochloride (Cat No: 318337, Sigma aldrich, USA), Ethidium bromide (Cat No: E7637, Sigma Aldrich, USA) and 2,7-Dichlorofluorescein diaceate (H2DCFDA) Cat No: 20656, Cayman Chemicals, USA)

Equipment's

Analytical weighing balance (LCGC, RADWAG) Centrifuge (Remi: R-8C). Pipettes: 2-10µl, 10-100µl, and 100-1000µl. Inverted Biological Binocular Microscope (Cat No: CKX-41, Olympus), 37°C incubator with humidified atmosphere of 5% CO_2 (Healforce, China), 96 well microplate reader (ELX-800, BioTek, USA), Water bath (BioBee, India), Liquid Nitrogen Tank (Thermo Scientific), Hemocytometer (Rohem Instruments, India), Refrigerator (Samsung, India), 96well microplate absorbance reader (ELX-800, BioTek, USA), BD FACS Calibur (BD Biosciences, CA, USA), Ice bucket with cover. Generally, cells are more stable and tolerate insult better when they're cold. The cover keeps light out, which could bleach the fluorochromes and Fluorescence Microscope –LSM 880 live cell imaging confocal system, Carl Zeiss, Germany).

Software: GEN5, BioTeK (For Microplate Reader), BD Cell Quest Pro (ver.6.0), Image J (FIJI), ZEN BLUE Software (Carl Zeiss, Germany) and MS Excel sheet.

Maintenance of Cell line

The HepG2 cell line (Human liver carcinoma cell line) was purchased from NCCS, Pune, India. The cells were maintained in MEM with high glucose supplemented with 2 mM L-Glutamin, 1.5 g/L Sodium bicarbonate and 1 mM sodium pyruvate, 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-cultured for every 2 days. Passage numbers of HepG2 cells was 37 was used for the present study. After reaching 80% of density, cells were detached by using 0.025% trypsin and 0.01% EDTA (in D-PBS) solution. Cell viability and count were performed using hemocytometer. The appropriate density of cells was seeded in T25 flasks and cultured until further usage of cells to conduct cell-based assays.

Anticancer studies of TC-AgNPs

Anticancer studies was carried out on HepG2 liver cancer cells by TC-AgNPs by means of various methods like MTT assay, ROS assay, Cell cycle assay, Apoptosis by flow cytometry and Apoptosis by Dual fluorescence methods

MTT Assay of TC-AgNPs on HepG-2 Liver Cancer Cells (Cell viability and Cell proliferation studies by MTT assay)

The cytotoxicity effect of TC-AgNPs was analyzed on HepG2 liver cancer cells by performing the MTT assay [39]. MTT is a tetrazolium salt is converted into insoluble purple-colored formazan crystals by the action of lactate dehydrogenase enzyme released by mitochondria. Briefly, cells in 200µl of suitable media and at a density of 15,000 were plated in a 96-well plate and incubated at 37°C for overnight. After attachment of cells to the surface of cell culture plate, the spent medium was replaced with MEM having various working concentrations of Test compound (TC-AgNPs) (6.25, 12.5, 25, 50, 100µg/ml). After drug addition, cells were incubated for 24h at 37°C with 5% CO_2atmosphere. After incubation, cells were treated with 100µL of MTT (0.5 mg/mL) was added and incubated for 3hr at 37°C. DMSO (100µL) was used to dissolve the formazan crystals, and purple color was measured at 570 nm using microplate reader (ELX-800, BioTek, USA). The viability of cells treated with DMEM alone was considered as 100%.% cell viability is calculated using the below formula:

% cell viability=[OD of treated cells/OD of Untreated cells] * 100

The IC_{50} value was determined by using linear regression equation i.e. Y =Mx+C. Here, Y = 50, M and C values were derived from the viability graph.

ROS Assay of TC-AgNPs on HepG2 Liver Cancer Cells Using H2DCFDA Staining

HepG2 cells were seeded in a 6-well plate at a density of 0.5×10^6 cells per 2 mL of medium and incubated overnight at 37°C in a CO_2 incubator. After 24 hours, the used medium was removed, and the cells were rinsed with 1 mL of 1X PBS. The cells were treated with the test compound for 24 hours, with one well of cells serving as a negative control. After treatment, the medium was collected into 12 × 75 mm polystyrene tubes, and the cells were washed with 500 µL of PBS, which was retained. Following this, 250 µL of trypsin-EDTA solution was added, and the plates were incubated at 37°C for 3–4 minutes. The culture medium was then returned, and the cells were harvested into the tubes. The cells were centrifuged for 5 minutes at 300 × g at 25°C, and the supernatant was discarded. The cells were washed twice with PBS and resuspended in 10 µM H2DCFDA working solution at 1×10^6 cells/mL, incubating for 30 minutes at 37°C, protected from light. After centrifugation at 150 × g for 5 minutes, the supernatant was discarded, and the cells were gently resuspended in 400 µL of pre-warmed DPBS. Flow cytometry analysis was performed using a flow cytometer with a 488 nm laser for excitation and 535 nm detection (FL1) to measure ROS expression [40].

Cell cycle Assay of TC-AgNPs on HepG-2 Liver Cancer Cells

Culture cells in a 6-well plate at a density of 2×10^5 cells/2 ml were added and incubated in a CO_2 incubator overnight at 37°C for 24 hours. Aspirate the spent medium and treat the cells with IC_{50} value of TC-AgNPs at the concentration of 56.32 µl/mL and along with standard drug Doxorubicin 1µg/mL concentration were added in 2 ml of culture medium, except in cell control and incubate the cells for 24 hours. The cell pellet was washed twice with PBS to ensure that only DNA is stained with Propidium iodide (PI stains all nucleic acids), so the cell pellet is treated with 50µl of Ribonuclease A to get rid of RNA and finally add 400µl PI solution per million cells directly to cells and mix well. Finally incubate the cells for 5 to 10 minutes at room temperature in dark and then analyze the samples by flow cytometry [3, 18].

Apoptosis Assay of TC-AgNPs on HepG-2 cell lines by Flow Cytometry

The HepG2 liver cancer cells are used in this study. The cells are cultured in a 6-well plate at a density of 0.5×10^6 cells/2 ml and incubated in a CO_2 incubator overnight at 37°C for 24 hours. Aspirate the spent medium and treat the cells with IC_{50} value of TC-AgNPs at the concentration of 56.32 µl/mL and along with standard drug Doxorubicin 1µg/mL concentration, except in cell control in 2 ml of culture medium and incubate the cells for 24 hours. Add 5 µl of FITC Annexin V and gently vortex the cells and incubate for 15 min at RT (25°C) in the dark, after incubation finally add 5 µl of PI and 400 µl of 1X Binding Buffer to each tube and vortex gently and analyze the samples by flow cytometry immediately according to procedure of Subramanyam et al 2021&2023 [3, 18].

Dual fluorescence Apoptosis assay on HepG-2 Liver Cancer Cells by TC-AgNPs

The HepG2 liver cancer cells are cultured in a 6-well plate at a density of 2 x 10^5 cells/2 ml and incubate in a CO_2 incubator overnight at 37°C for 24 hours. Aspirate the spent medium and treat the cells with IC_{50} value of TC-AgNPs at the concentration of 56.32 µl/mL and along with standard drug Doxorubicin 1µg/mL concentration, except in cell control in 2 ml of culture medium and incubate the cells for 24 hours. The stained cells were observed under fluorescence microscope with filter cube with Excitation 560/40 nm and Emission 645/75 nm for EtBr (Ethidium bromide) and Excitation 470/40 and Emission 525/50 for Acridine orange and Hoechst 33258 staining fluorescence microscopy Excitation 352/461 nm and Emission 510/40 nm**.** The images were overlay using Image J Software v1.48 according to the protocol of Gaddam et al 2021& 2024 [1, 32].

RESULTS AND DISCUSSION

Preparation of leaf extract of *Tinospora cordifolia*

Ever since, from the decades a variety of plant materials are exploited for the green synthesis of metal nanoparticles is considered as a 'Green Technology or Green Method', offering abundant advantages in terms of environmental friendliness, cost-effectiveness, biocompatibility, controllable synthesis of nanoparticles and there is no use of any harmful chemicals. At the same 'green synthesis' emerged as a promising and sustainable alternative to traditional methods like chemical and physical methods [32, 21]. The green synthesis of silver nanoparticles using silver nitrate ($AgNO_3$) solution, by simple green method using the *Tinospora cordifolia* leaf extract which acts as a reducing and as a capping agent [21, 22, 30]. The leaves of *Tinospora cordifolia* along with herbaceous stem were collected from herb and shrubs growing in and around of forest region of Tirumala, Tirupati, Chittoor Dist., Andhra Pradesh state, India during the months of October and November, as shown in [Fig.1(a)]. The leaves along with herbaceous stem of *Tinospora cordifolia* were collected and shade dried for a week at room temperature and then the leaves were collected and made into fine powder with the help of electric blender. The plant leaf extracts of *Tinospora cordifolia* was prepared, by adding 5g of finely powdered leaves of *Tinospora cordifolia* in a 500 ml Erlenmeyer flask with 250 ml of Milli Q ultra-pure distilled water and the mixture was heated at 70^0C for 45 min and then filtered through sterile muslin cloth followed by Whatman No.1 filter paper. This filtrate solution was used as source of extract for preparation of silver nanoparticles and was utilized in subsequent procedures.

Figure 1(a): *Tinospora cordifolia* plant

Green synthesis of Silver nanoparticles by leaf extract of *Tinospora cordifolia*

To the 5ml diluted filtrate, 10ml of 0.2 M $AgNO_3$ solution was added and the sample was left at room temperature, until the color of solution changed from pale light color to light brown and subsequently dark brown. The solution containing silver nanoparticles of *Tinospora cordifolia* (TC-AgNPs) was confirmed by the dark brown color [Fig.1(b)]. So, in the current study the green synthesis of silver nanoparticles (TC-AgNPs) with *Tinospora cordifolia* (*T. cordifolia*) plant leaf extracts was prepared without any external toxic chemicals so this method is popularly known as 'Green synthesis or Green Method'.

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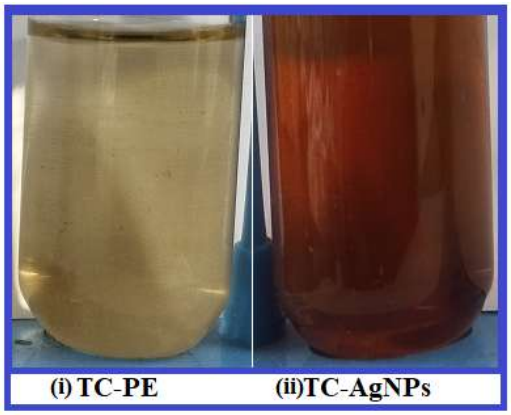

Figure 1(b): Green synthesis of TC-AgNPs (i) Aqueous plant leaf extract of *Tinospora cordifolia* (ii) Green synthesized TC-AgNPs

Similarly, the light brown colorless leaf extract of *Tinospora cordifolia* (*T.cordifolia*) was also converted into dark brown color after the addition of the 0.2 M $AgNO_3$ solution within few minutes. Therefore it is confirmed the reduction of $AgNO_3$ to silver nanoparticles (TC-AgNPs). The formation of TC-AgNPs using the plant leaf extract of *T.cordifolia* was shown in [Fig.1(b)] and the synthesis was carried out without using any other external toxic chemicals. Therefore, the color change indicates the formation of silver nanoparticles viz. TC-AgNPs by parts of plant sources. It is undoubtedly evident that the nanoparticles are reduced within 1 minute to 3 minutes of time. So, it is concluded that the method we followed to synthesis of nanoparticles was very simple and inexpensive and rapid without any external toxic chemicals. Hence it is concluded as eco-friendly, simple, safe and rapid method known as "Green Synthesis" and "Green Future" [31, 33, 41-43]. The green synthesized TC-AgNPs were used for further spectral characterization by various advanced spectroscopic methods and further used to study their biomedical applications like Antioxidant, Anti-bacterial and Anti-cancer studies on HepG2 liver cancer cell lines.

Spectral characterization of green synthesized TC-AgNPs

The spectral characterization of the green synthesized silver nanoparticles i.e TC-AgNPs obtained from the plant leaf extract of *T.cordifolia* comprised of the UV-Vis spectroscopy, FTIR analysis, DLS particle size analysis, Zeta potential analysis, XRD analysis and the TEM & SAED analysis.

Ultra violet–visible spectroscopy of the green synthesized TC-AgNPs

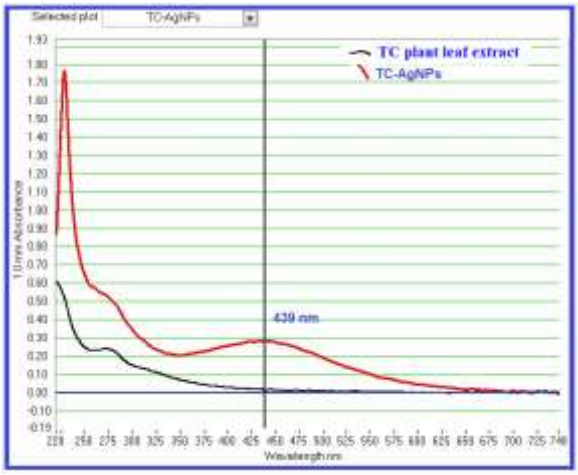

Figure.1.(c): UV-visible spectra of *T.cordifolia* leaf extract and Green synthesized TC-AgNPs.

UV-visible spectroscopy is a simple and straightforward method for the finding out and detection and confirming the formation of nanoparticles. The bio-reduction of Ag^+ ions by using the plant leaf

extracts of *T.cordifolia* were monitored from time to time by sampling of the 1 µl aliquots and the optical absorbance was recorded on the Nanodrop 8000 UV-vis spectrophotometer in 220 – 750 nm wavelength range. The UV-visible spectra of the bio reduced TC-AgNPs solutions showed an absorbance peaks at 439 nm [Fig.1(c)] which is characteristic surface plasmon resonance (SPR) peak of silver nanoparticles and therefore confirmed their green synthesis.

The size and shape of the TC-AgNPs, reflects on the absorbance peaks. It is well known the that the SPR range of silver nanoparticles is between 390nm to 470 nm, earlier reports on AgNPs also reveals the same results that the SPR of AgNPs between 400nm to 450 nm are small and spherical between the range of 20nm to 100nm [18, 23, 30-33, 44-45]. In the present study the SPR of the biosynthesized TC-AgNPs is 439 nm [Fig.1(c)], it indicates that the green synthesized silver nanoparticles (TC-AgNPs) are morphologically small and spherical in shape. These TC-AgNPs were further confirmed by TEM analysis and DLS particle size analysis for their morphological characters [23, 33], 46-48].

Fourier transform infra-red (FTIR) spectral analysis of TC-AgNPs

Consequent to the UV-Visible spectral analysis, FTIR analysis was approved to find out which bioactive phyto-constituents have participated in the reduction of AgNO3 to Ag^+ ions i.e. (Ag^0) AgNPs. The FTIR analysis was performed by using Brucker Tensor 27 Thermo Scientifics, USA available at department of Physics, Sri Venkateswara University, Tirupati. The *T. cordifolia* plant extract exhibit clear bands at wave numbers 3905.22, 3856.77, 3841.10, 3823.45, 3805.26, 3754.71, 3692.67, 3676.88, 3653.16, 3468.72, 2921.41, 2851.42, 1721.83, 1657.42, 1622.69, 1461.78, 1384.75, 1316.19, 1105.99, 799.47, 516.04, 492.38, 458.33, 447.83, 429.79 and 412.32 cm^{-1} respectively **Fig.2(a).**

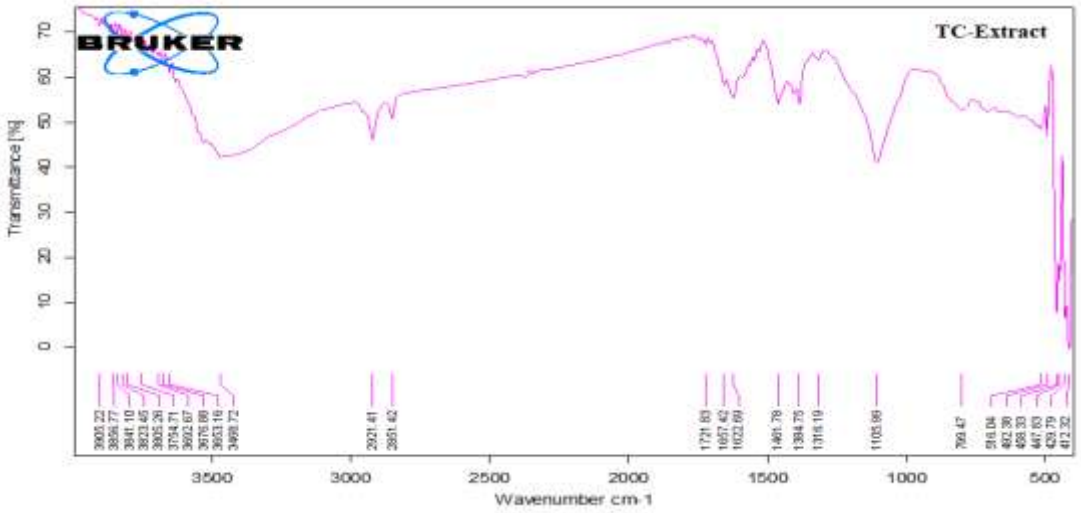

Figure 3(a): FTIR spectrum of the aqueous plant extract of *T. Coridifolia*

T.cordifolia is an important medicinal plant, the herbaceous stem along with leaves consists of major bioactive compounds such as flavonoids, alkaloids, saponins, resins, etc. So, in the present study reveals that the carboxylic acids, hydroxy, ether, alcohol, etc. are responsible for the reduction of the Ag^+ ions to the Ag^0 ions and also act as the capping and the stabilizing agents of TC-AgNPs. The FTIR spectral analysis of TC-AgNPs [Fig.2(b] showed intensive peaks at 3840.02, 3465.42, 2920.45 cm^{-1}, 1623.56, 1384.15, 1106.03, 498.79, 462.95, 430.89 and 415.23 cm^{-1} respectively. The sharp peaks at, 3840.02 cm^{-1}, 3465.42 cm^{-1}, 2920.45 cm^{-1} can be assigned to hydroxyl group of alkanes such as O-H and C-H stretching of the –alkenes group, 1623.56 cm^{-1} can be assigned to C=C stretching of the alkenes group, 1384.15 cm^{-1} can be assigned to Amide II stretching, 1106.03 cm^{-1} can be assigned to amide III stretching can be assigned to –C–O–C–stretching and –C=C– of the amide group [49-50]. Therefore, the FTIR analysis clearly revealed that phytochemicals like amides, carboxylic acids, ether groups, hydroxyl groups and alcohol groups present in the plant leaf extract were mainly responsible for the reduction of silver ions (Ag^+) into nanoscale silver nanoparticles (Ag^o), from the above results it may be concluded that Alkaloids, tannins, cardiac glycosides, flavoinoids, saponins, steroid and glycosides. Especially alkaloids of *Tinospora cordifolia* such as choline, tionosporin, isocolumbin palmatine, tetrahydropalmatine and magnoflorine. The major bioactive compounds in leaves and stem reveals compounds like 11-hydromustakone, N-methyl-2- pyrrolidine, N- formylannonain, cordifoliaside, A magnoflorine, and tinocordiside are

responsible major bioactive compounds responsible for capping and efficient stabilization TC-AgNPs by acting as capping agents [22]. Therefore, the FTIR analysis reveals that phytochemicals such as Alkaloids, tannins, cardiac glycosides, flavoinoids, saponins, steroid and glycosides. etc. and water-soluble carbohydrates may be the major bioactive compounds that were responsible for the reduction of Ag^{+} ions to Ag^{0} ions, and also for the capping and efficient stabilization of the biosynthesized TC-AgNPs. Similar types of reports were reported by Kotakadi *et al.*[31] and others [51-53].

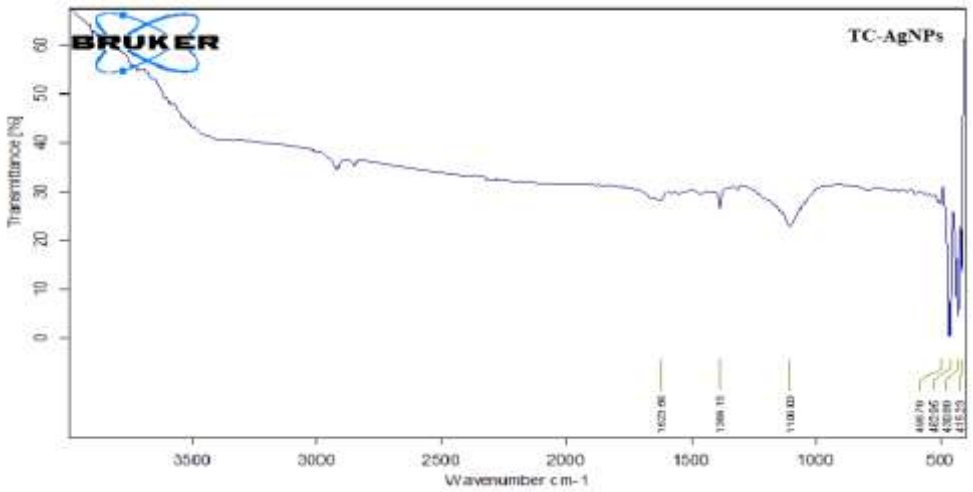

Figure 2(b): FTIR spectrum of the green synthesized TC-AgNPs

Dynamic Light Scattering (DLS) particle size analysis of TC-AgNPs

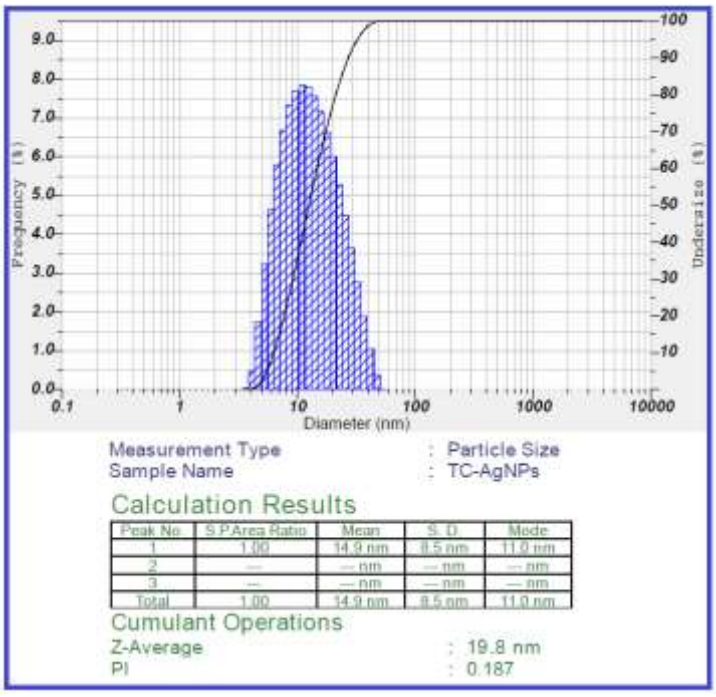

Figure 3(a): Particle size distribution of TC-AgNPs

The DLS particle size of the green synthesized TC-AgNPs was detected by the intensity and laser diffraction method using the colloidal solutions of TC-AgNPs, which is poly-dispersed in nature. The poly-disperse index (PI) value in the range of 0.1 to 0.4 then they are said to be well dispersed and moderate in nature and if the PI are more than 0.4 then the nanoparticles are broad in size and shape. In the present study the particle distribution of synthesized TC-AgNPs were in the range 5 nm to 35 ± 10 nm in dynamic light scattering analysis (DLS), with an average size of 14.9 nm with an Z-average 19.8 nm with an poly dispersed index (PI) of 0.187 indicating well dispersed in aqueous form [Fig.3(a)]. The Z average of the

nanoparticles was around 19.8 nm. 10 % of the particles are in the range of 3 nm to 15 nm, 50% are in the range of 15 to 35 nm and 90% are in the range of 15.4nm to 35.6 nm. Similar results were observed in TEM. The DLS analysis results reveal that results are in correlation with UV-Vis analysis of TC-AgNPs that is if the SPR region of AgNPs is in between 400 to 470 nm, the nanoparticles are said to be small and spherical in size [33,41,54].

Zeta potential analysis of the green synthesized TC-AgNPs

Zeta potential is the measurement of electrostatic charge present on the surface of synthesized nanoparticles and the potential difference between the nanoparticles and the dispersed solution. It's also known has the potential difference between the solutions in which the nanoparticles are dissolved containing oppositely charged ions around the nanoparticles. The zeta potential is the most important parameter for detecting the stability of nanoparticles. Higher the zeta potential indicates greater electrostatic repulsion and increased stability. The zeta potential greater than +30 mV and -30 mV are very stable. The values also represents whether the nanoparticles are cationic or anionic in nature, which is an important property, how the nanoparticles interact with cellular membranes and other body fluids like mucus. The most important application is used to evaluate the stability of nano-dispersions, determine surface coatings and also to detect the nanoparticle-cell interactions.

In the current study, the Zeta potential analysis of the green synthesized TC-AgNPs was also carried out, the result revealed that the TC-AgNPs have high negative zeta potential which is around -28.5 mV [Fig.3(b)], because of its high negative potential value the silver nanoparticles were highly stable without any agglomeration in the colloidal form.

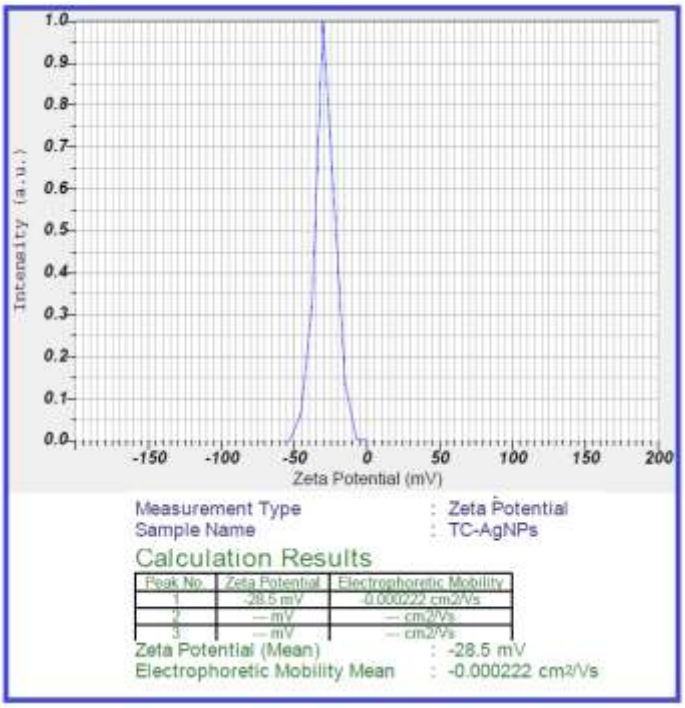

Figure 3(b): Zeta potential of green synthesized TC-AgNPs

The earlier reports on green synthesized nanoparticles having zeta potential values in the range of ±25 mV to ±30 mV are highly stable, whereas the nanoparticles within the range of ±15 mV to ±25 mV are moderately stable and ±10 mV to ≤ ±10 mV are not stable after longer time. So it is concluded that the synthesized TC-AgNPS are highly stable with -28.5 mV of zeta potential value.

X-ray diffraction analysis (XRD) of the green synthesized TC-AgNPs

XRD is a significant spectroscopic technique used to evaluate the crystalline nature of green synthesized TC-AgNPs was obtained by the XRD analysis. The XRD pattern showed that TC-AgNPs are

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face centered cubic structure in nature [Fig.4(a)] and that three characteristic diffraction peaks indexed to 38.53 (111), 44.86 (200) and 65.22 (220) and 78.33 (311) planes of face centered cubic crystal lattice of Silver (JCPDS card No. 89-3722) the results were according to researcher on silver nanoparticles [23, 55].

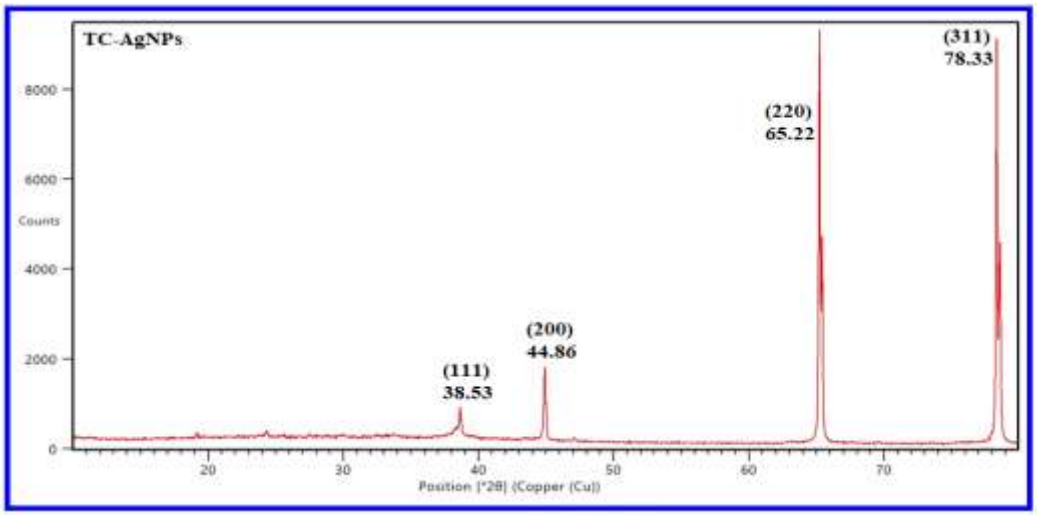

Figure 4(a): XRD spectral data of green synthesized TC-AgNPs

The XRD analysis of all the three green synthesized silver nanoparticles TC-AgNPs reveals that they are crystalline in nature with face centred cubic structure [55-57].

Transmission electron microscopy (TEM) analysis of green synthesized TC-AgNPs

TEM analysis was performed to determine the shape and size of the green synthesized TC-AgNPs TEM analysis was carried out using HR-TEM (200 kV) JOEL- JEM 2100 Plus - High resolution Transmission Electron Microscope (DST PURSE Centre, SVU, Tirupati). The HR- TEM image analysis showed that the green synthesized TC-AgNPs [Fig.4(b)], were round in shape the TC-AgNPs size was in-between 5 nm to 50 nm with an average size of 13.9 nm ± 5 nm of the results were similar to DLS particle size analysis. The SAED pattern analysis also revealed that the green synthesized TC-AgNPs had different planes of cubic crystal lattice which were already confirmed by XRD analysis.

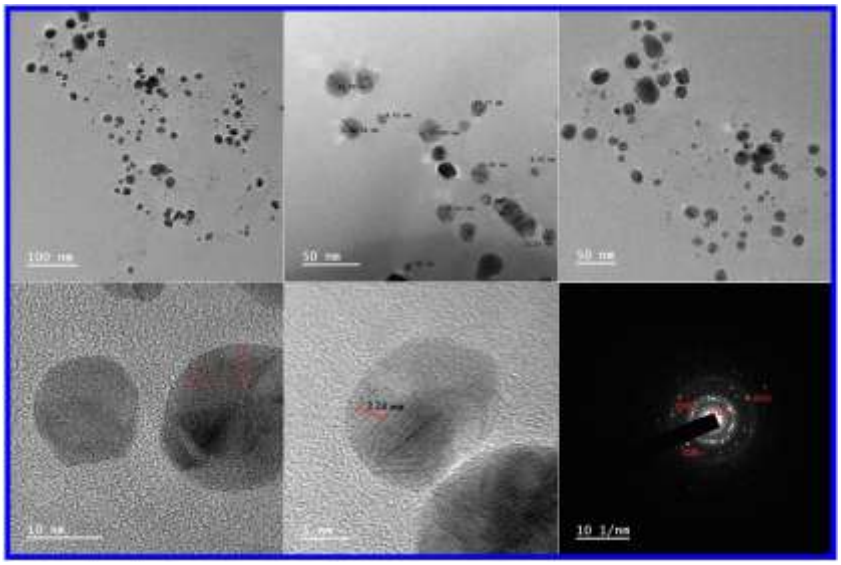

Figure 4(b): HR TEM analysis of green synthesized TC-AgNPs at magnification of 100 nm , 50 nm, 10 nm and 5 nm scale bar and SAED pattern showed four diffraction rings

The SAED pattern analysis also revealed that the green synthesized TC-AgNPs had different planes of cubic crystal lattice which were already confirmed by XRD analysis were similar to earlier studies [1,5, 18,22-23, 32].

Therapeutic Biomedical Applications of TC-AgNPs

The therapeutic biomedical applications of the green synthesized silver nanoparticles i.e TC-AgNPs obtained from the plant leaf extract of *Tinospora cordifolia* was carried out. This comprised of the Antimicrobial activity, Antioxidant activity and the Cancer activity followed according to other researchers [1, 5, 18, 32, 38,55].

Antimicrobial activity of the green synthesized TC-AgNPs

In nature, it is well known that the antimicrobial activities of medicinal plants are associated with the differences in their contents of bioactive compounds present in them [58-60, 32]. The previous reports revealed that alkaloids and flavonoids were the major responsible compounds for the antimicrobial activities in higher plants. It is also known that secondary metabolites such as tannins and other phenolic compounds were also classified as active antimicrobial agents. At the same time, it is reported that the tannins play a major role in preventing the development of microorganisms by the precipitation of the microbial protein, thereby inhibiting the growth of many fungi, yeasts, bacteria and viruses. The current study was carried out to detect the therapeutic biomedical importance of the green synthesized TC-AgNPs from leaf extract of *Tinospora cordifolia*. The antimicrobial activity was performed against four bacterial species *viz.* Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* Gram-positive *Staphylococcus aureus, Bacillus megaterium* by the Kirby – Bauer disc diffusion method [1, 32-33, 54].

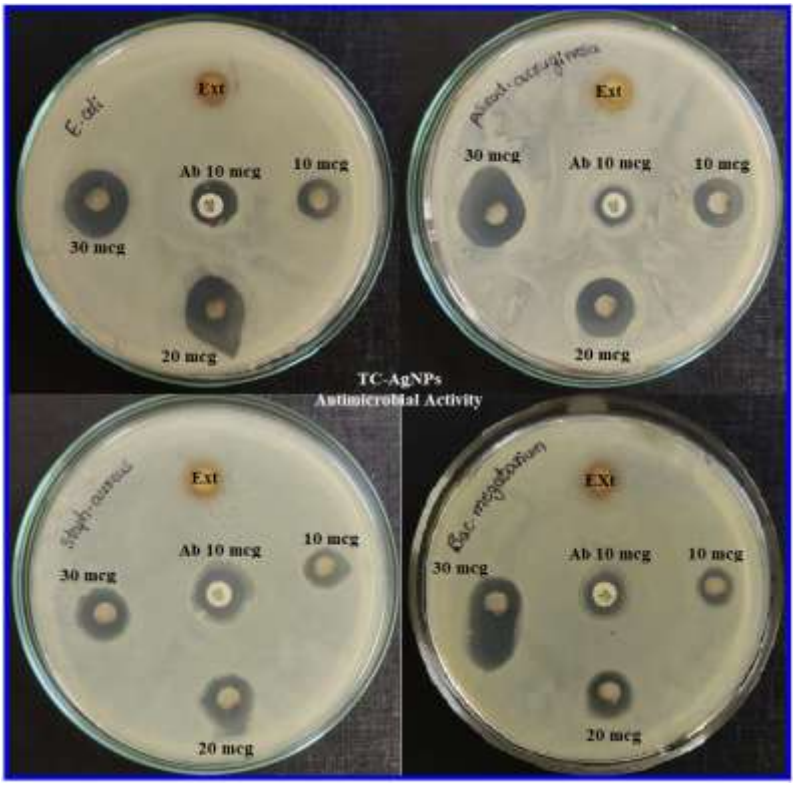

Figure 5(a): Antimicrobial activity of green synthesized TC-AgNPs against

Gram –ve bacteria: *Escherichia coli, Pseudomonas aeruginosa* and
Gram +ve bacteria: *Staphylococcus aureus, Bacillus megaterium*

The green synthesized TC-AgNPs from leaf extract of *Tinospora cordifolia* were used to study the antibacterial activity against four bacterial species *viz.* Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* Gram-positive *Staphylococcus aureus, Bacillus megaterium* by the Kirby – Bauer disc diffusion

method. The green synthesized TC-AgNPs exhibited very good antibacterial activity against both gram negative and gram positive bacteria. The results revealed the *E. coli*, *P. aeruginosa* showed zone of inhibition (ZOI) of diameters 11 mm (10 mcg), 15 mm (20 mcg),16 mm (30 mcg) and 13 mm(10 mcg), 15 mm (20 mcg),, 19 mm (30 mcg) respectively and Gram-positive *Staphylococcus aureus, Bacillus megaterium* with the zone of inhibition (ZOI) of diameters 11 mm (10 mcg), 12 mm (20 mcg), 13 mm (30 mcg) and 10 mm (10 mcg), 12 mm (20 mcg),, 23 mm (30 mcg), respectively when compared to the standard drug Amoxicillin (10mcg) revealed ZOI as follows 14 mm against *E. coli*, 12 mm for against *P. aeruginosa* and 13 mm against *Staphylococcus aureus* and 11 mm against *Bacillus megaterium* respectively. From the above results the green synthesized TC-AgNPs showed very good antibacterial activity against Gram +ve and Gram –ve bacteria [Fig.5 (a)]. The TC-AgNPs exhibited almost equivalent activity with that of Amoxycillin at the concentration 10mcg [Table-1]. The results revealed that the antibacterial activity is in dose dependent manner.

Table 2: Anti bacterial Activity of TC-AgNPs

SNo	Bacteria	*Tinospora cordifolia* shoot extract	TC-AgNPs (10 mcg)	TC-AgNPs (20 mcg)	TC-AgNPs (30 mcg)	Antibiotic (Amx 10 mcg)
			Zone of Inhibition in mm (ZOI)			
1	*E.coli*	Nil	11 mm	15 mm	16 mm	14 mm
2	*Pseud. aeruginosa*	Nil	14 mm	15 mm	19 mm	13 mm
3	*Staph. aureus*	Nil	11 mm	12 mm	13 mm	13 mm
4	*Bac. Megatarium*	Nil	10 mm	12 mm	23 mm	15 mm

*The table indicates the Mean values of the triplicates with standard deviation.

The results were similar to the earlier reports [1, 5, 18-19, 28, 30-32, 45]. The results are also represented in graphical form which were shown in Fig.5. (b).

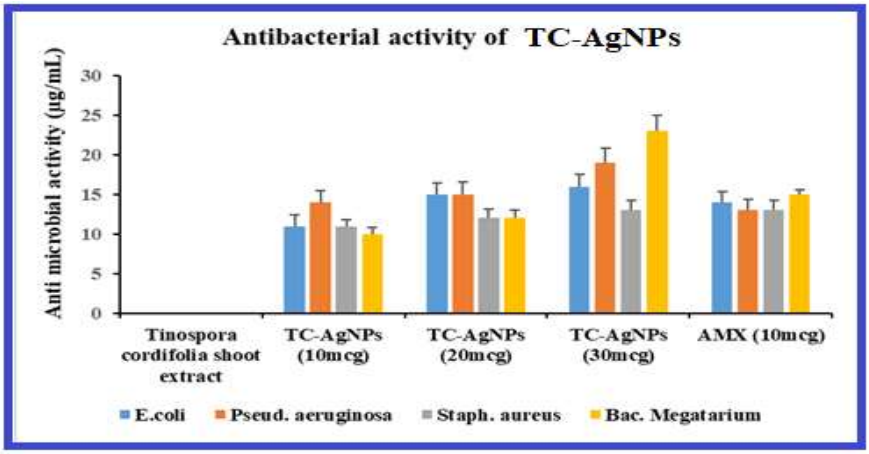

Figure 5(b): Antimicrobial activity of green synthesized TC-AgNPs
*The table indicates the Mean values of the triplicates with standard deviation

Antioxidant activity of the Green synthesized TC-AgNPs

The antioxidant activity of both TC-ZnONPs and TC-AgNPs was carried out by using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to the method described by Gaddam et al. and Kotakadi et al. with slight modifications. The Nitric oxide scavenging activity was carried out by a tailored method of Ferreira et al and H_2O_2 scavenging ability of the *Tinospora cordifolia* plant extract and green synthesized TC-AgNPs was examined according to the modified method of Pick and Mizel. The antioxidant activity of the green synthesized Zinc oxide nanoparticles and silver nanoparticles i.e. TC-AgNPs obtained from the plant extract of *Tinospora cordifolia*. Three different methods were used to detect the antioxidant activity as follows DPPH antioxidant activity, Hydrogen peroxide antioxidant activity and the Nitric oxide antioxidant activity [1, 21, 32, 61-62].

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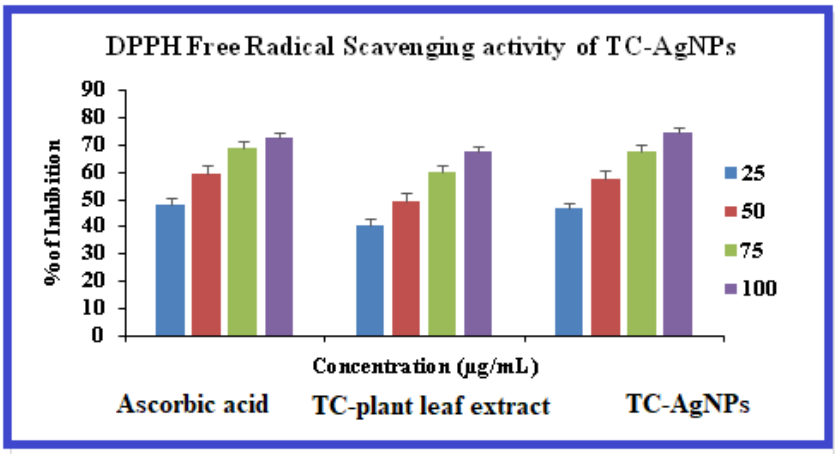
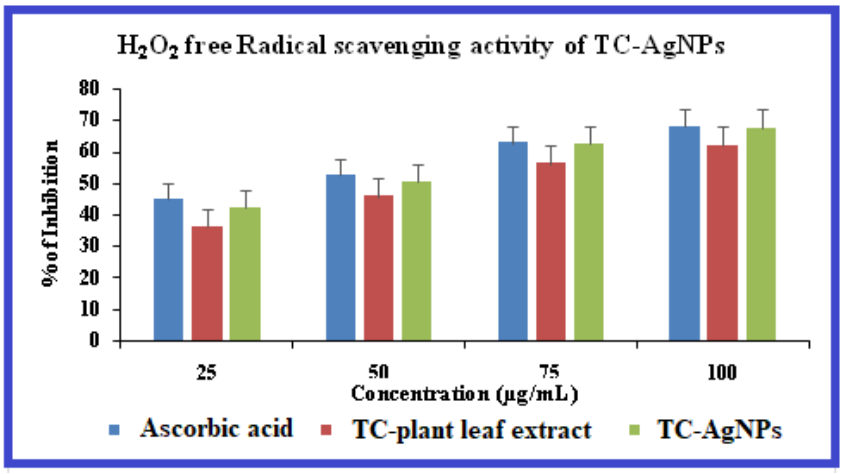
Several scientists have been constantly working for the past few decades to find out suitable antioxidants by using various plant sources and their bioactive compounds as natural antioxidants to protect the humans from quite a lot of human disorders which are caused mainly by oxidative stress. The cells that consist of unstable molecules such as oxygen, these oxygen molecules voluntarily react with other molecules in the cellular system and form reactive oxygen species (ROS), which is dangerous sign for the living system. These ROS molecules without difficulty react with DNA, RNA, proteins and lipids of the cell and cause cell death. Thus continuous reaction further cause oxidative stress in different human cells and cause various disorders like inflammation, cancer and ageing and other neurodegenerative disorders etc. So it is very essential to find out sensible and suitable antioxidants which can diminish ROS. So, depending on the necessity and significance of antioxidants several researchers have been working on plant bioactive components, and they also started working on metal nanoparticles which are synthesized by plant sources exhibit superior antioxidant activity as an alternative sources of antioxidants. In the present study, in vitro antioxidant activity of the TC-AgNPs was determined by different free radical scavenging assays.

In vitro antioxidant activity of TC-AgNPs by DPPH, H_2O_2 and NO methods

In the present study antioxidant activity of *Tinospora cordifolia* plant extract TC-PE and TC-AgNPs was determined by three important assays namely DPPH assay, H_2O_2 assay and NO scavenging assays. The results were shown in (Table.2 (i) and Fig.4.c) DPPH assay (Table.2(ii) and Fig.4.c) H_2O_2 free radicals assay and Table.2(iii) and Fig.4.c) Nitric oxide assay for both the *Tinospora cordifolia* plant extract TC-PE and green synthesized TC-AgNPs. The results revealed that DPPH assay is the best antioxidant assay with highest inhibition of free radicals at the percentage of 67% and 74% for both (TC-PE) and TC-AgNPs. Similarly, the H_2O_2 antioxidant assay reveals that both the TC-PE and TC-AgNPs show good scavenging activity of 62.14% and 67.62% against H_2O_2 radicals respectively for TC-PE and TC-AgNPs. Lastly in NO free radical assay, TC-PE and TC-AgNPs exhibited very good scavenging activity of 60.18% and 65.64% against NO free radicals, the results were tabulated in Table.2 and also the results are represented in graphical diagram as follows Fig.4.(c)





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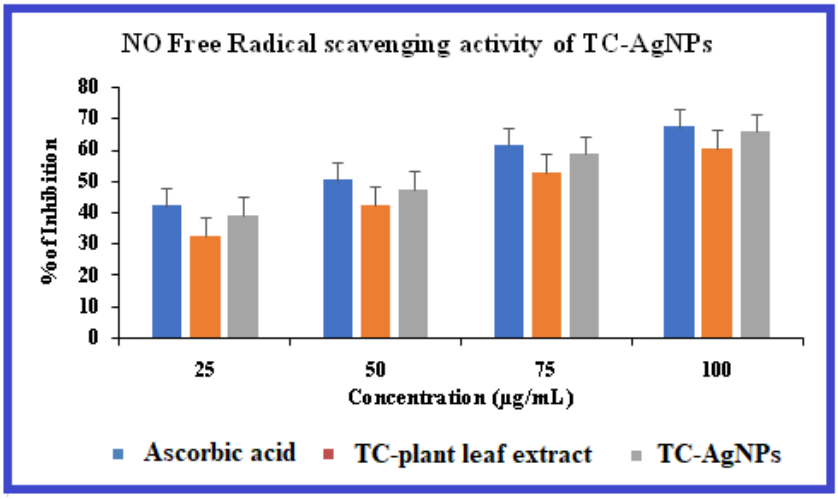


Figure 5(c): Antioxidant activity of green synthesized TC-AgNPs by DPPH, H_2O_2 and NO methods
(*The graph indicates the Mean values of the triplicates with standard deviation)

Table 2: Antioxidant activity of TC-AgNPS

(i): DPPH Free Radical Scavenging activity of TC-AgNPs, (ii) H_2O_2 i*n-vitro* antioxidant activity of TC-AgNPs and (iii) Nitric Oxide *in-vitro* antioxidant activity of TC-AgNPs

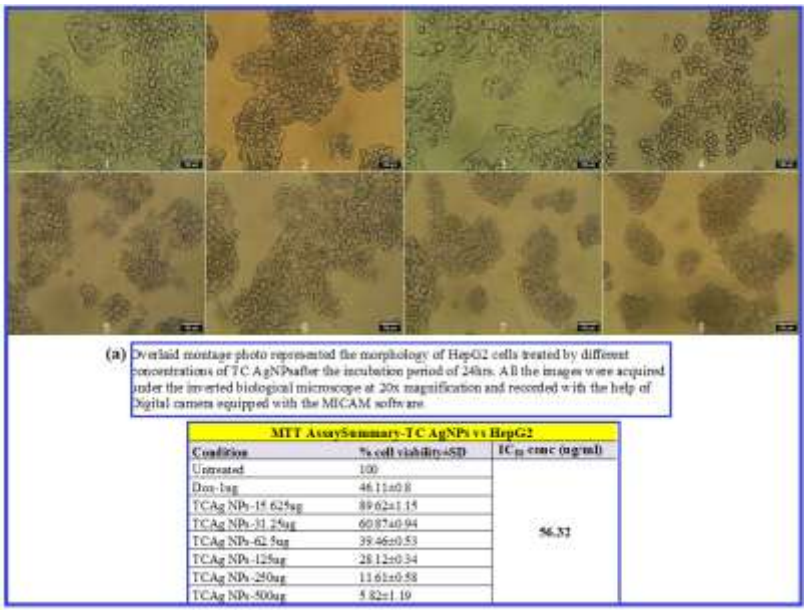
(i)DPPH method	Free Radical scavenging activity ± SD (%)			
Sample name	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Ascorbic acid	48.02±0.06	59.42±0.17	68.42±0.16	72.12±0.12
T. cordifolia leaf extract	40.16±0.02	49.04±0.14	60.02±0.18	67.12±0.44
TC-AgNPs	46.36±0.22	57.12±0.64	67.16±0.02	74.18±0.36
(ii)H_2O_2 method	Free Radical scavenging activity ± SD (%)			
Sample name	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Ascorbic acid	45.02±0.26	52.46±0.34	62.76±0.24	68.18±0.12
T. cordifolia leaf extract	36.18±0.56	45.82±0.48	56.18±0.02	62.14±0.26
TC-AgNPs	42.18±0.22	50.47±0.30	62.42±0.18	67.62±0.18
(iii)NO method	Free Radical scavenging activity ± SD (%)			
Sample name	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Ascorbic acid	42.02±0.18	50.24±0.16	61.36±0.14	67.18±0.12
T. cordifolia leaf extract	32.46±0.14	42.34±0.16	52.68±0.92	60.18±0.18
TC-AgNPs	38.96±0.12	47.34±0.16	58.48±0.12	65.64±0.18

In view of the above results it is clearly understood that the antioxidant activity was dose dependent manner in all the three methods, and the data revealed that the DPPH showed superior antioxidant activity when compared with other two methods, followed by H_2O_2 free radical assay and NO antioxidant activity. Finally, it is concluded that efficient DPPH scavenging activity of TC-AgNPs could be due to the bioactive compounds like Alkaloids, tannins, cardiac glycosides, flavoinoids, saponins, steroid, glycosides, water-soluble carbohydrates, polyphenols and proteins of TC-PE which are present plant extract. The Flavoinoids, polyphenols, saponins, glycosides, water-soluble carbohydrates and alcohols might have actively participated in the green synthesis of TC-AgNPs. The above bioactive components might be involved in the coating and capping of the TC-AgNPs. Similar type of results were also determined the free radical scavenging activity of plant aqueous extracts and green synthesized AgNPs,

by different method like DPPH [63], H_2O_2 and NO antioxidant activity. Plants are rich in antioxidant compounds like the presence of flavonoid, total phenols, tannin, carotenoids, lycopene [64], carbohydrates, triterpenoids, glycosides, phenolic compounds steroids, canthin-6-one and β-caroline alkaloids and other classes of phyto constituents like tannins, flavonoids and phenolic compounds. These important plant compounds give up hydrogen atoms from their hydroxyl groups to free radicals and form stable phenoxy radicals [1, 5, 18, 21, 32].

Anticancer studies of the TC-AgNPs on HepG2 Liver cancer cell lines

***In vitro* MTT Assay to determine Cytotoxic Activity of TC-AgNPs on HepG2 Liver cancer cell line**



(a) Overlaid montage photo represented the morphology of HepG2 cells treated by different concentrations of TC AgNPsafter the incubation period of 24hrs. All the images were acquired under the inverted biological microscope at 20x magnification and recorded with the help of Digital camera equipped with the MICAM software

MTT AssaySummary-TC AgNPs vs HepG2

Condition	% cell viability±SD	IC_{50} conc (ng/ml)
Untreated	100	
Dox-1ug	46.11±0.8	
TCAg NPs-15.625ug	89.62±1.15	
TCAg NPs-31.25ug	60.87±0.94	56.32
TCAg NPs-62.5ug	39.46±0.53	
TCAg NPs-125ug	28.12±0.34	
TCAg NPs-250ug	11.61±0.58	
TCAg NPs-500ug	5.82±1.19	

Figure 6 (a): MTT assay of TC-AgNPs against HepG2 Liver cancer cells

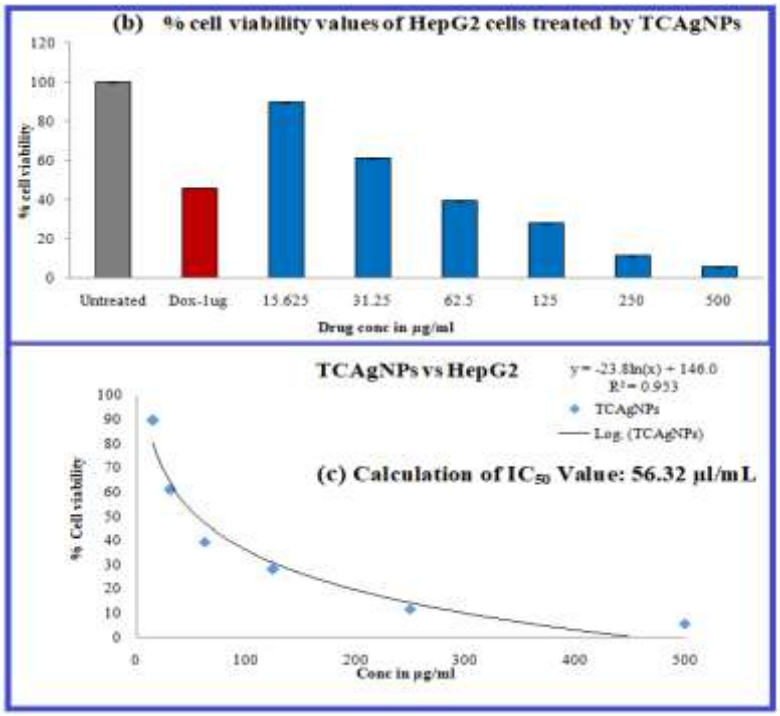


Figure 6 (b): % of Cell viability of HepG2 Liver cancer cells against TC-AgNPs at different concentrations (c) Calculation of IC_{50} Value: 56.32 µl/mL

The results of MTT assay revealed the cytotoxic effect and cell viability of HepG2 Liver cancer cell lines treated with TC-AgNPs and Doxorubicin 1 µg/mL (standard drug). The cells were treated with TC-AgNPs for 24 hours with a range of concentrations (Fig.6.a.). The IC_{50} value was calculated after 24 hours. The IC_{50} value was calculated by using linear regression equation i.e. Y =Mx+C. Here, Y = 50, M and C values were derived from the viability graph (Fig.6.b&c). The IC_{50} value of TC-AgNPs was calculated by statistical data of cell cytotoxicity studies reveals that IC_{50} value of 56.32 µL/mL. The cytotoxicity of green synthesized TC-AgNPs is dosage dependent manner, as the concentration increase the cell viability decreases shown in Fig.6.b&c. The results showed that TC-AgNPs have anticancer nature and revealed good cytotoxic potency against HepG2 human liver cancer cells. Likewise, the silver nanoparticles synthesized by leaf extract of *Ctenolepis garcini L* revealed excellent anticancer potential against HepG2 cell line with an IC_{50} of 33.78 µg mL^{-1} [65-66]. Similarly silver nanoparticles (AgNPs) from *Kigelia africana* leave (Lam.) Benth. extract revealed good cytotoxicity against RINm5F insulinoma cell line. Other plant like *Nepeta deflersiana* mediated silver nanoparticles show toxicity against Human Cervical Cancer Cells (HeLA) and *Seripheidium quettense* mediated green synthesis of biogenic silver nanoparticles also showed very good theranostic applications against cancer [67-68].

ROS Assay of TC-AgNPs on HepG2 Liver Cancer Cells Using H2DCFDA Staining

Cell-permeant 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) is a widely used ROS indicator. The reduced non-fluorescent fluorescein H2DCFDA can be oxidized and converted into fluorescent 2', 7'- dichlorofluorescein (DCF) by intracellular ROS. In the present study, TC AgNPs treated HepG2 cells significantly increased the ROS expression similar to the reference std drug, Doxorubicin (for HepG2 cells) and confirmed that TC AgNPs enhance the apoptosis mediated through cellular oxidative stress induced apoptosis in human liver cancer cells.

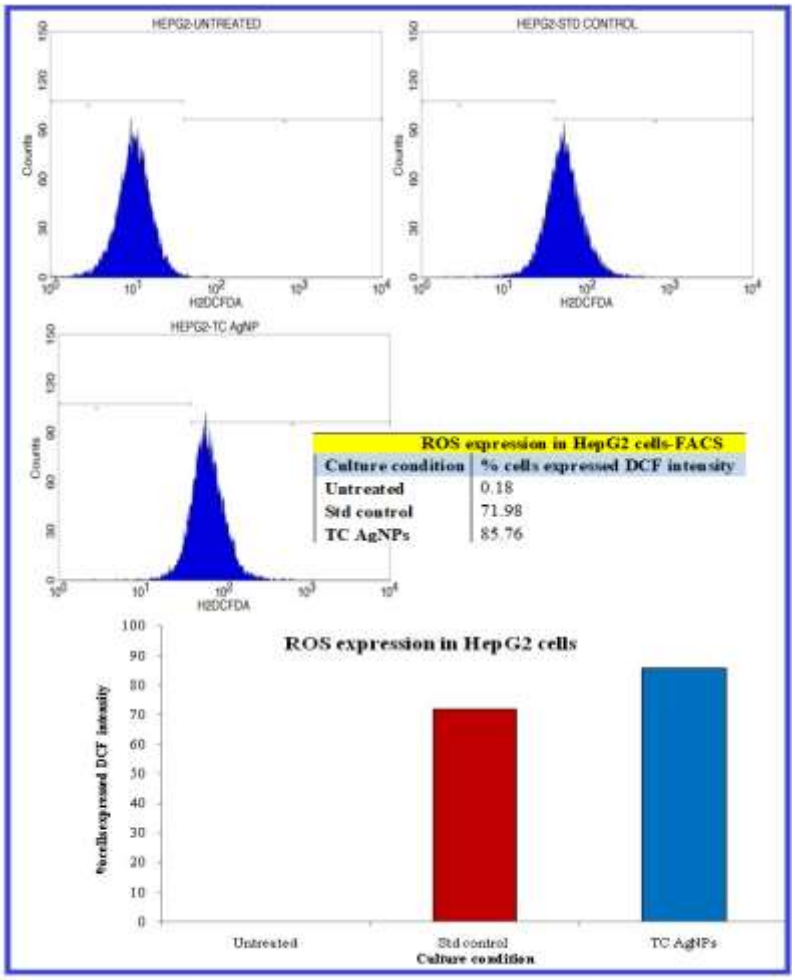


Figure 7: FACS-ROS expression study: H2DCFDA staining

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ROS analysis: % cells expressed ROS expression in the Untreated and treated with Std control and TC AgNPs groups in HepG2 cells by flow cytometry measured in DCF units

The results of ROS expression reveals that TC-AgNPs have more expression then the standard drug was observed in HepG2 liver cancer cell lines. Similar types of results were observed by silver nanoparticles biosynthesized by various parts plants extracts.

Cell cycle Assay of TC-AgNPs on HepG2 Liver Cancer Cells

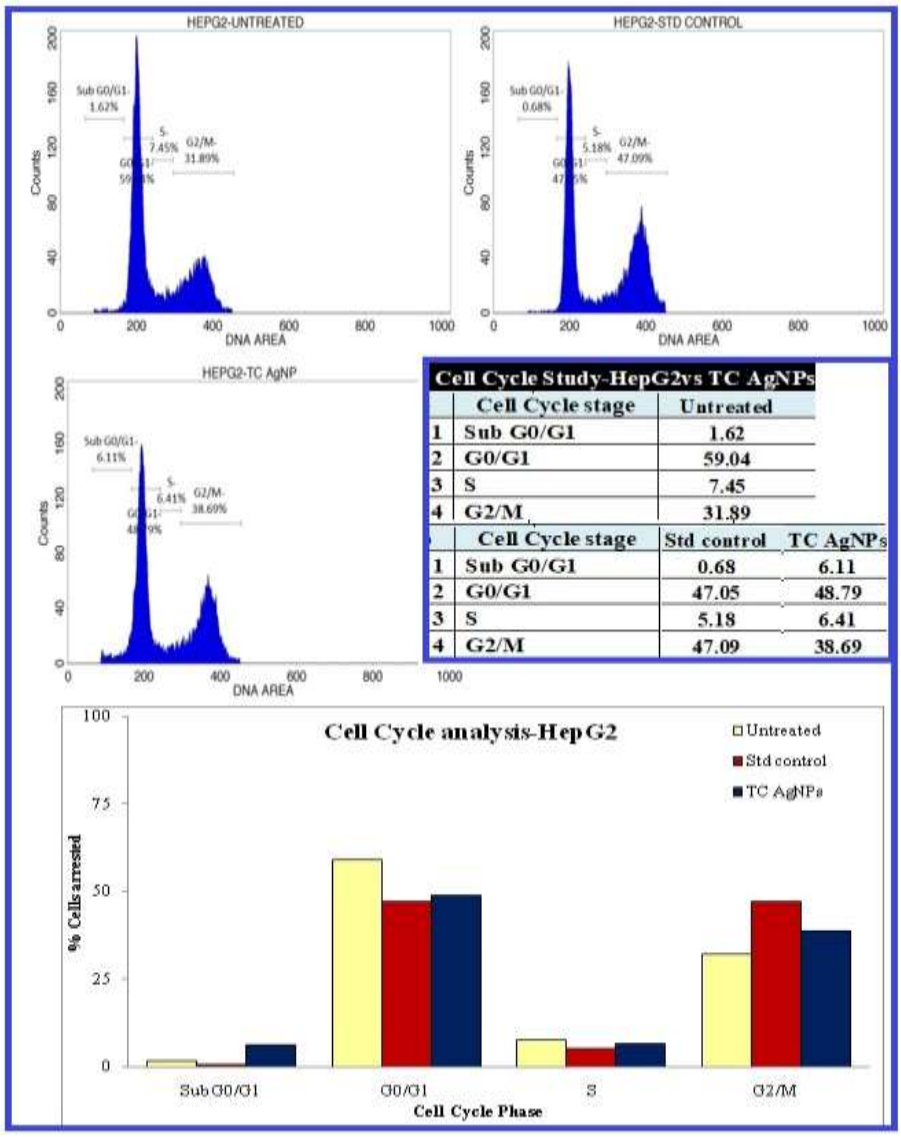

Figure 8: Cell cycle analysis of HepG2 liver cancer cells treated with TC-AgNPs along with Std. Control with Flow cytometry

Cell cycle analysis using propidium iodide (PI), that is using the fluorescent nucleic acid dye to identify the proportion of cells that are in one of the three interphase stages of the cell cycle. It is used as stain in flow cytometry to evaluate cell viability or DNA content in cell cycle analysis. PI has two disadvantages; it stains all double stranded nucleic acids so the cells have to be incubated with RNase to remove any double stranded RNA and the dye is excluded by the plasma membrane so that the cells have to be fixed or permeabilised before adding the dye. On the ploidy of HepG2 cells, in Sub G0/G1 phase (Apoptotic phase), 1.62%, 0.68% and 6.11% cells get arrested in Untreated, Std control and TC AgNPs with IC_{50} concentration respectively. In G0/G1 phase (Growth Phase), 59.04%, 47.05% and 48.79% cells get arrested in Untreated, Std control and TC AgNPs with IC_{50}concentration respectively. In S phase (synthetic phase), 7.45%, 5.18% and 6.41% cells get arrested in Untreated, Std control and TC AgNPs

with IC_{50}concentration respectively shown in Fig.8. On the other hand, in G2/M phase, 31.89%, 47.09% and 38.69% cells get arrested in Untreated, Std control and TC AgNPs with IC_{50} concentration respectively. The HepG2 cells treated with TC AgNPs with IC_{50} concentration showed the increased % cells at Sub G0/G1 and G2/M phases by inhibiting the % cells at G0/G1 phase as compared to control group. Hence the cell cycle got arrested at Sub G0/G1 and G2/M phases in treated group of HepG2cells. It's evident that the compound, TC AgNPs won't allow the cells to undergo cell division or proliferate by G2/M phase arrest and may be considered as a potent anti-liver cancer drug. Doxorubicin with 1ug/ml was used as a Std control against the HepG2cells which also arrested the cells at G2/M phases respectively. Similar type of results were observed by silver and nanoparticles synthesized by *Argyeria nervosa* (An) leaf extract, the An-AgNPs with a concentration of 58.64 mg/ml respectively are showing a high % of cells at S and G2/M stages when compared to untreated cells [5]. The AgNPs green synthesized by *Hydrastis canadensis* and *Thuja occidentalis* also induce differential cytotoxicity through G2/M arrest in A375 cells which are similar to our results [69], AgNPs green synthesized by *Nepeta deflersiana*, also revealed that the cells in SubG1 arrest and lead to apoptotic/necrotic cell death in cervical cancer cell lines [70] Silver–palm pollen nanocomposite also showed the morphological changes associated with cell death and accumulation of dead cells in sub-G1 phase [71].

Apoptosis Assay of TC-AgNPs on HepG2 Liver Cancer Cells (CLSM-Live/Dead staining study: AO/EtBr staining)

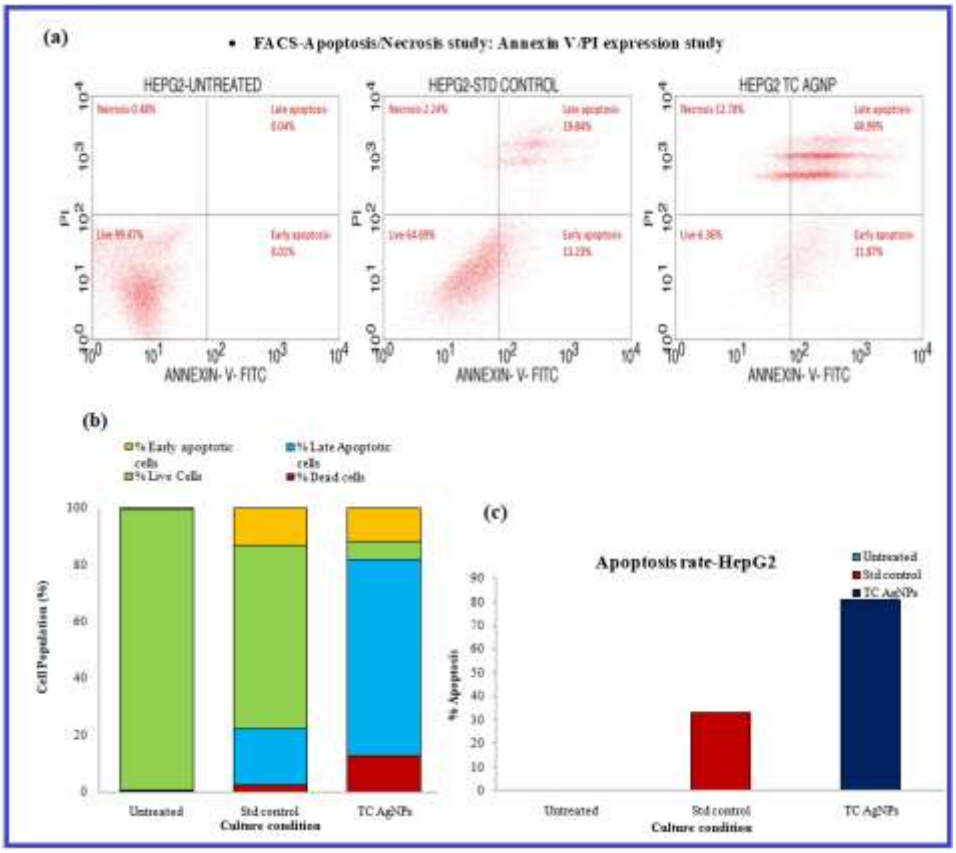

Figure 9: (a) Quadrangular plots represented the Annexin V/PI expression in HepG2cells in the presence and absence of TC AgNPs and Std control by Flow Cytometry. Acquisition was done by using BD FACS calibur and data analyzed by BD Cell Quest Pro Software (Version: 6.0). Here, Annexin V- FITC - Primary Marker, PI- Propidium Iodide (Secondary fluorescence Marker). (b)Side scatter graph represented the distribution of %apoptotic cells, % Necrotic cells and % live cells in treated and non-treated conditions of HepG2 cells using Annexin V/PI staining by Flow cytometry method. (c)Bar graph represented the %apoptotic cells in treated and untreated conditions of HepG2cells by Flow cytometry

Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenance of tissue homeostasis. The apoptotic program is characterized by certain morphologic

features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and inters nucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. In the current study, the observations of apoptosis study suggested us that the TC AgNPs significantly enhanced the 80.86% of apoptosis and 12.78% of necrosis in HepG2 cells after the 24hours of exposure respectively. TC AgNPs showed the similar kind apoptotic potential against Human liver cancer cells with high % apoptosis and moderate rate of necrosis similar to the Doxorubicin after the 24 hours of treatment. Doxorubicin caused the 33.07% Apoptosis and 2.24% Necrosis in HepG2 cells after the 24hours of treatment shown in Figure 9.

Apoptosis Assay of TC-AgNPs on HepG2 Liver Cancer Cells by CLSM-Live/Dead staining study: AO/EtBr staining)

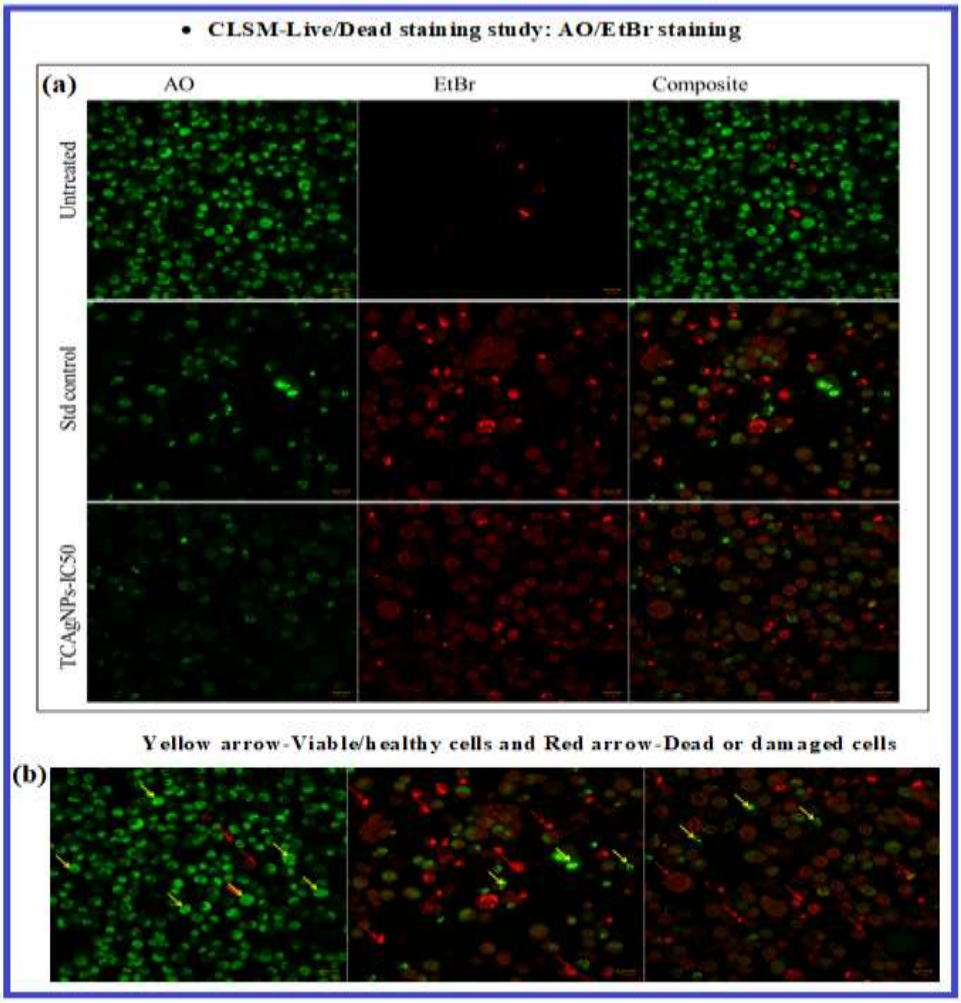

Figure 10: (a)Composite overlaid image represented the Acridine orange (AO) and Ethidium bromide (EB) dual staining study of HepG2 cells in Untreated, Std control and TC AgNPs treated with IC50 concentration represented the changes in nuclear morphology of cells. AO represents viable cells (green) and EtBr represents dead cells (red color). All the images were captured at 25x magnification. (b) Acridine orange (AO) and Ethidium bromide (EB) dual staining study of HepG2 cells in Untreated, Std control and TC AgNPs treated with IC_{50} concentration represented the changes in nuclear morphology of cells. AO represents viable cells (green) and EtBr represents dead cells (red color). All the images were captured at 25x magnification.

The additional Apoptosis studies by TC-AgNPs was further confirmed the mechanism of cell death using the treatments at their IC_{50} concentrations. The dual acridine orange / ethidium bromide (AO/EB) assay allows for the sensitive fluorescent detection of live, early and late apoptotic cells and necrotic cells. The AO stains live cells by intercalating with the cellular DNA to emit a green fluorescence, whereas EB stains dead cells resulting in the emission of a yellow-to-red fluorescence by depending on the stage of apoptosis [72-75]. The results revealed the apoptotic nature of TC-AgNPs by fluorescent staining when compared with treated HepG2 liver cancer cells to untreated cells, they show more number of early apoptotic cells and late apoptotic cells and necrotic cells with chromatin condensation and blebbing of cell membrane in the human liver cancer cells was very clearly observed [Fig.10.a-b]. The final results were similar to that of standard drug Doxorubicin treated cells indicating that TC-AgNPs have very good anticancer activity. Likewise, the other nanoparticles like gold nanoparticles also reveals cytotoxicity in the human cancer cell lines like HeLa and MCF-7, and the non-cancer HEK293 highlight the potential target by dual-drug AuNP nanocomplexes in providing safe and efficient delivery of anticancer drugs with reduced side effects *in vitro* [72]. In conclusion to the study, the TC-AgNPs might have induced apoptosis by oxidative stress-mediated pathway?, Further studies are needed to be carried out to expose the exact mechanism of action.

SUMMARY AND CONCLUSIONS

In the current study, the green synthesized TC-AgNPs revealed versatile beneficial applications. The synthesized TC-AgNPs were characterized by a variety of spectroscopic methods, the results reveal that UV-visible absorption spectrum indicates SPR (Surface Plasmon resonance) peaks at 439 nm which confirms synthesis of silver nanoparticles i.e. TC-AgNPs. FT-IR analysis results identify that several characteristic peaks plant bioactive molecules are actively participated in reduction of their functional group**s** for capping and stabilization of TC-AgNPs. The particle size was between 5 nm to 35 ± 10 nm in dynamic light scattering analysis (DLS), with an average size of 14.9 nm with an Z-average 19.8 nm with an poly dispersed index (PI) of 0.187 indicating well dispersed in aqueous form, at the same time HR-TEM analysis also reveal will similar type results, where the particles are spherical or roughly spherical the size range from 6 nm to 37 nm ± 5 nm. The TC-AgNPs were highly stable with negative zeta potential value −28.5 mV. The x-ray diffraction (XRD) data revealed that the particles are facets of face-centered cubic crystal structure of AgNPs, which also further confirmed by HR-TEM SAED pattern with diffraction fringes which are confined silver nanoparticles. The TC-AgNPs have exhibit potential antioxidant activity and thriving antibacterial activity against gram-positive and gram negative bacteria. The TC-AgNPs have also revealed potential anti-cancer activity *in vitro* on HepG-2 liver cancer cell lines. The anti- cancer activity of TC-AgNPs comparable to that of the standard drug was evidenced by various anticancer studies like MTT assay, ROS, Cell cycle, Apoptosis and Dual fluorescence studies. The anti-proliferative and cytotoxic studies on HepG-2 Liver cancer cell line was carried by MTT assay, reveals that IC_{50} value was 56.32µg/mL. Overall in summary, all the observed results are satisfactory and double confirmed the anti-liver cancer effect of TC AgNPs molecule by causing the apoptosis, increasing the rate of ROS and arresting the Sub G0/G1, G2/M phase arrest (key phase of anti-cancer drug properties) and increasing the rate of Apoptotic bodies respectively. From the above results it can concluded that the green synthesized TC-AgNPs can useful as future therapeutic agents to control antimicrobial resistance and different types cancers effectively. At the same TC-AgNPs have possible therapeutic potential against human liver cancer derived diseases and can employ as novel anticancer therapeutic agents. We also report that there are very few reports on HepG2 liver cancer cell lines using silver nanoparticles.

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Authors Contribution

The authors confirm contribution to the paper as follows: Study conception and Design: HC, JP and VSK Data collection: HC and SAG, Experimental: HC, SAG &VSK & JP: Green synthesis and Spectral Characterization; HC & SAG (Antioxidant studies) and (Antimicrobial studies) and (Anticancer studies) HC, SAG & VSK: MTT assay, ROS assay and Cell cycle and Cytotoxic studies and Dual Fluorescence Analysis and Interpretation of Results: JP, VNC and VSK, Draft Manuscript preparation: HC, JP & VSK; Final Manuscript editing: VSK and VNC

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