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Standardisation, synthesis and characterisation of nanodrug from *Tabebuia rosea* leaves for breast cancer

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Abstract : In this paper silver nanoparticles (AgNP) were developed using *Tabebuia rosea* (TR) leaves as reducing agent and its anticancer potential against cancer cells was studied. The standardisation for synthesis of nanoparticle formation was carried out at different pH, temperature and reaction time. The AgNPs was studied using UV-Vis spectrum showed the high absorption band at 420 nm, size range of 49 to 98 nm, with an average size of 70 nm as determined by SEM, AFM. The energy dispersive x-ray spectroscopy (EDX) profile for AgNPs showed typical optical absorption peak approximately at 3 keV and it was found that O-H stretching of phenolic compounds and O-H, C=O stretchings in carboxylic acids are involved in formation of AgNPs studied by FTIR. NP showed specific toxicity on breast carcinoma T47D cells at 20 µg/mL concentration, showing almost 95 % cancer cell growth inhibition at 48hrs. Thus silver nanoparticles using *Tabebuia rosea* (TR) leaves have future application as nanodrug.
Keywords: Cytotoxicity, *Tabebuia rosea*, green synthesis, silver nanoparticles, Nanodrug.

Introduction

There is an increasing interest among researchers to explore the secondary metabolites of medicinal plants for use in medicine to treat diseases. But due to the advances in nanotechnology, the long process of isolating the drug compound and studying for its biological property is reduced. The green synthesis method of producing metal nanoparticles is said to be easy, eco friendly and cheap when compared to costly medicines. Thus the medicinal property of medicinal plants is explored for synthesis of silver nanoparticles. *Tabebuia* sp have a long history of use as traditional medicine for treating cancer due to its compound lapachol and its derivatives from bark of the tree[1] .

The essential oil of *Tabebuia rosea* leaf and bark is reported to be cytotoxic which may be due to the presence of o-xylene (2.13%), 2,4-dimethylhexane (1.03%), methyl cyclohexane (53.13%), methyl benzene (12.75%), 3-Pentene-2-one (0.11%)[2]. The earlier investigations on the phytochemical constituents of *Tabebuia rosea* leaves revealed the presence of saponins, tannins, phenolic acids, flavonoids and alkaloids[3]. The active compounds present in the *Tabebuia rosea* ethanolic leaf extract showed the presence of 2-furancarboxaldehyde, 5-hydroxy methyl (19.39%), 2-deoxy, D-erythropentose (11.01%), Santolina triene (8.28%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (6.07%), 7-Quinolinol (6.01%), phenol, 2-(2-methyl propyl) (5.41%) and Cinnamadehyde (2.42%)[4].The leaf extract has been reported for various activities such as antimicrobial[5], cytotoxicity on MOLT-4 cells[6], cytotoxicity on brine shrimps[7] and phytotoxicity on radish seeds[8]. Also the flowers of TR have been reported for antioxidant, cytotoxicity and phytotoxicity due to presence of volatile

constituents 4H- pyran-4–one, 2, 3- dihydro-3, 5- dihydroxy-6- methyl, 3, 4, 5-trimethyl phenol, thiocyanic acid, 2-propynyl ester and 2-propenoic acid, 3-(2-hydroxy phenol) compounds analysed by GC-MS[9].

Thus due to various medicinal reports on TR leaves have been explored for synthesis of silver nanoparticles. Silver have been used traditionally for curing various diseases due to its antibacterial properties. Recently silver is gaining a role in cancer therapy, biosensors, dressings, devices, formulations[10]. The objective was to characterize and study the anticancer drug synthesized from TR leaf extract.

Materials and methods

Plant material and preparation of the extract

Healthy leaves of TR were collected from Madurai Kamaraj University (MKU) campus, authenticated by Prof. K. Muthuchelian, Director, and Centre for Biodiversity and Forest Studies (CBFS), Madurai Kamaraj University and voucher specimens were deposited in the herbarium of CBFS of university (No.CBFS-01). The dry TR leaves was cut into small pieces and powdered finely. About 5g of the leaf powder was boiled for 10 min in 100 ml sterile double distilled water and further filtered through Whatman No. 1 filter paper and used for the present study.

Synthesis of silver nanoparticles

Silver nitrate ($AgNO_3$) was procured from Sigma-Aldrich (Bangalore, India) was used. For the synthesis of silver nanoparticles, $AgNO_3$ (2 mM) and aqueous plant extract (10 mg/ml) were mixed in different ratios. Briefly, 1 ml of plant extract was mixed with 9 ml of $AgNO_3$ (1:9 ratio). The subsequent mixtures were prepared by increasing plant extract and decreasing $AgNO_3$ volumes by 1 ml, until the final ratio of 9:1was attained. Furthermore, appropriate concentrations of $AgNO_3$ and plant extracts vice versa from 1 to 9 ratio mixed in a series of reactions for optimization of synthesis of silver nanoparticles, incubated overnight at room temperature in dark. The resultant yellowish brown solution indicated formation of silver nanoparticles. The silver nanoparticle synthesis was optimized at different temperature and pH. The reaction temperature was studied at 30 °C to 70 °C. The pH was studied in the range of 5, 6, 7, 8 and 9 by using 0.1 N HCl and 0.1 N NaOH respectively.

Purification of Silver Nanoparticles

The broth containing nanoparticles was centrifuged, redispersed in sterile deionized water to get rid of any biological molecule. This process was repeated thrice to obtain better separation of entities from the metal nanoparticles. The purified pellet was then freeze dried using Lyophillizer (Micro Modulyo 230 freeze dryer, Thermo Electron Corporation, India).

UV–visible spectral analysis

The color change from green to brown was observed in the silver nitrate solution incubated with aqueous plant extract. The bioreduction of Ag nanoparticles was monitored by periodic sampling of aliquots (0.2 mL) of aqueous component and measuring the absorbance and spectrum of the solution in UV spectrophotometer (Shimadzu ,UV 2500,Japan), at a resolution of 1 nm between 300 and 600 nm.

Atomic Force Microscopy

A thin film of the sample was prepared on a cover slip by droping 0.1 ml of the sample on the slide, and allowed to dry for 30 minutes. The slide was then scanned with AFM (APE Research- model no: A100SGS). The AFM characterization was carried out in ambient temperature in non contact mode using silicon nitrate tips with varying resonance frequencies. These tips have spring constants of approximately 0.15 Nm^{-1} and are conical in shape with a cone angle of 20° and an effective radius of curvature at the tip of 10 nm.

Fourier transform infra-red (FTIR) spectroscopy

Silver NP dried powder (50mg) was analyzed using FTIR by drying and ground with KBr pellets and analyzed in a SHIZAMAZU model no 8400S spectrum instrument where spectrum was recorded in the region of 500 to 4500 cm^{-1}.

Scanning electron microscopy (SEM) & Energy dispersive X-ray analysis (EDX)

The lyophilized silver NPs were mounted on the copper stubs and the images were studied using scanning electron microscope (SEM). EDX analysis was done with (JEOL model-L6390) secondary electron detectors at an operating voltage of 30 kV.

Cytotoxic activity

Cell culture

Human ductal breast carcinoma cells (T47D) was obtained from the National Centre for Cell Science (NCCS), Pune, and maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS). For evaluation of the cytotoxicity, cell was seeded on a 96 well plate with a density of 1×10^4 cells/cm^2. Normal cells of L929 (human fibroblast cell line) was maintained in MEM (Minimum essential Medium) containing 10% fetal bovine serum (FBS) provided by Promo Cell Germany.

Assessment of cytotoxicity

Cytotoxicity of NTR was evaluated by using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium] assay. In MTT test viable cells reduce the tetrazolium component of MTT into purple colored formazan crystals. Stock solution of the samples were freshly prepared (1mg/1mL) and diluted with cell culture medium to the desired concentrations (20, 50 and 100 µg/mL). The compound with different concentration was added and incubated with phosphate buffer saline (PBS) resuspended cells, after attaining 90% confluency. Cells in media devoid of compound acted as the negative control and wells treated with Triton X-100 as the positive control for a period of 48 h. 5 mg of MTT (Sigma) was dissolved in 1 ml of PBS and filter sterilized. 10 µl of the MTT solution was further diluted to 100 µl with 90 µl of serum and phenol red free medium. 100 µl of the solubilisation solution (10% Triton X-100, 0.1 N HCl and isopropanol) was added to each well and incubated at room temperature for 1 h to dissolve the formazan crystals. The absorbance of the solution was measured at a wavelength = 570 nm using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were analyzed for each experiment.

Statistical analysis

The values are presented as mean ± SD (standard deviation) of triplicate measurements.

Results and discussion

Biosynthesis of silver nanoparticles

The silver nanoparticles are formed by reduction of silver nitrate to silver ions because of the reducing agents present in the plant extract, indicated by light yellowish color which turned to dark brown color after 24 hrs (Fig. 1). The size and shape of nanoparticles was examined by UV-Vis spectroscopy at range of 300–600 nm. Mixture of 6:4 (S6) of silver nitrate and plant extract gave optimized synthesis of silver nanoparticles of uniform size among the various combinations (Fig. 2A, 2B) studied at absorption spectrum of 420 nm characteristic for surface plasmon resonance for silver nanoparticles (Fig. 2C). The bioactive molecules present in the leaf extract of TR have played a role in the reduction of silver nitrate to silver, which can be reasoned due to the presence of saponins, tannins, phenolic acids, flavonoids and alkaloids[3], also because of the functional groups present in the tannins and phenolic compounds of the extract.

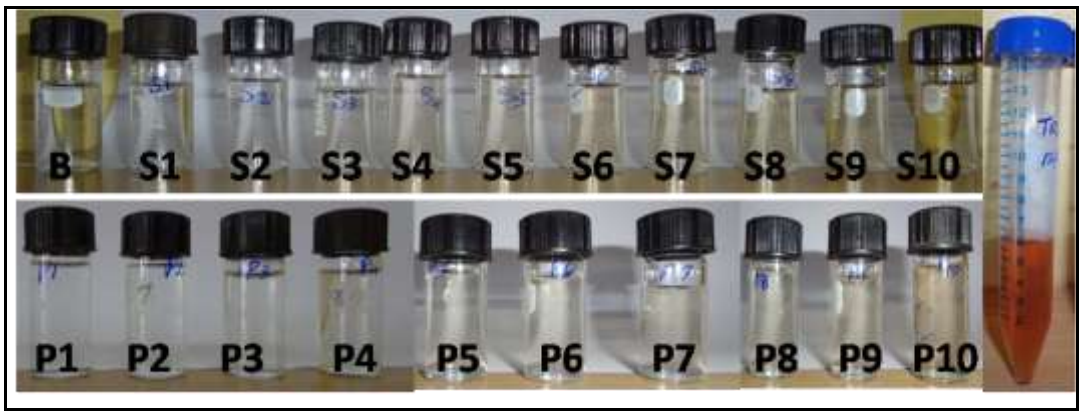


Figure1. The bottles containing silver nitrate solutions, TR plant extracts at different concentrations (A, B) denoted as S1-S10; P1-P10 respectively and bottle containing purified, concentrated silver nanoparticles (C).

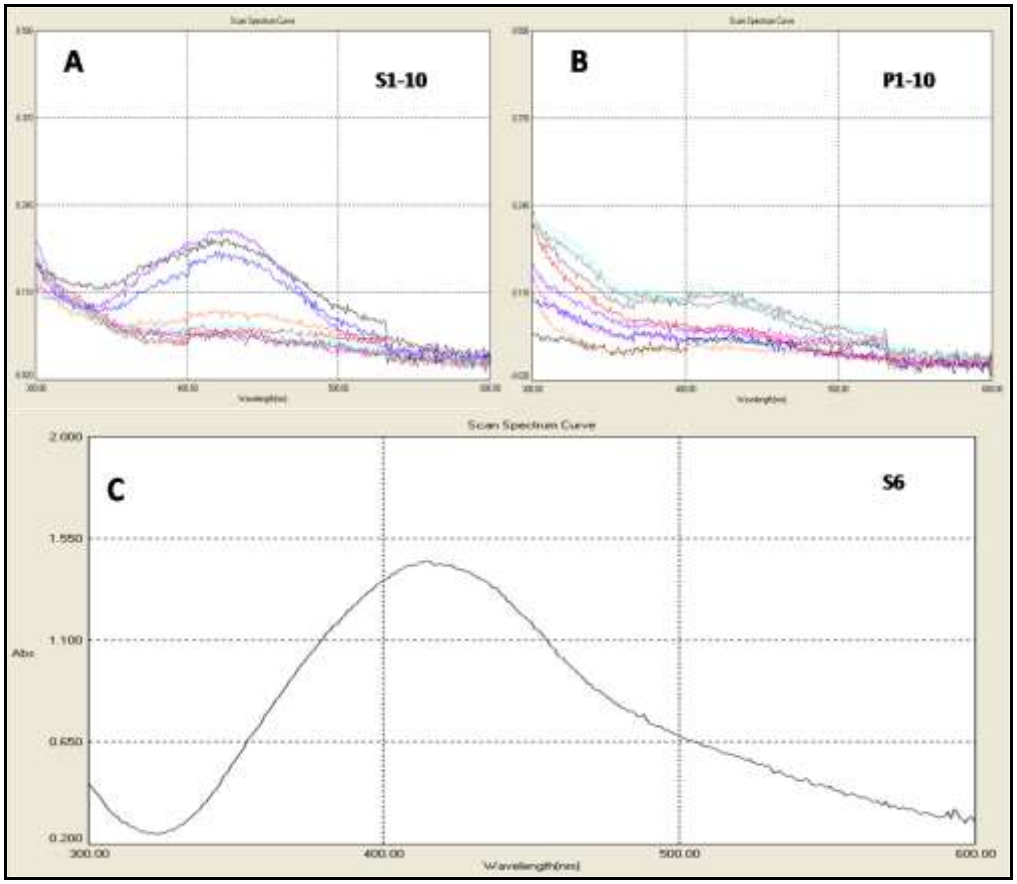


Figure2. UV-Visible spectrum of a mixture of solutions of silver nitrate S1-S10 (A), plant extracts P1-P10 (B) and standardised silver nanoparticles synthesized using *Tabebuia rosea* leaves.

Optimization of physicochemical parameters for synthesis of silver nanoparticles

The silver nanoparticles synthesis were standardized, optimized using parameters of pH and temperature, which plays very significant role in controlling shape and size of AgNPs. The pH 7 was found to synthesize non-agglomerated, monodispersed distribution of silver nanoparticles as indicated by their absorbance peaks at 420 nm confirmed by UV-Vis spectrum readings (Figure 3). For different temperature checked, optimal production was observed at 60°C, where high temperature favours the synthesis (Figure 3). Same temperature of 60°C has been reported during green synthesis of silver nanoparticles from *Tecomella undulata*[11].

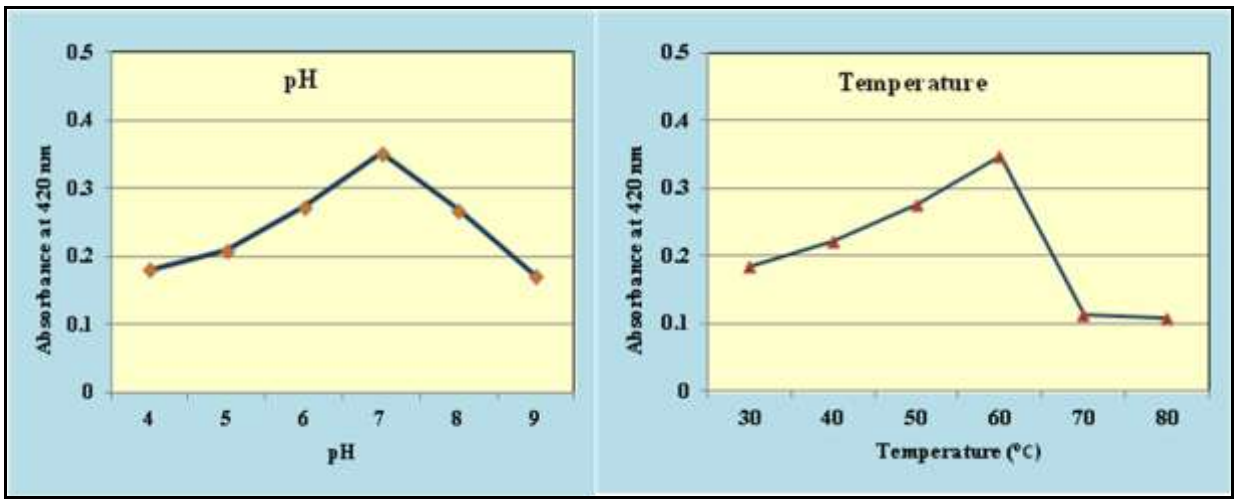


Figure3. Absorption spectrum for silver nanoparticles synthesized using *Tabebuia rosea* leaves at different pH and Temperature.

Analysis of Silver nanoparticle in AFM

The surface morphology of optimized Ag NPs was studied using atomic force spectroscopy, were almost spherical in shape confirmed by absorbance spectrum (Figure 4). The size of the silver nanoparticles studied from AFM showed size in the range of 49 to 98 nm, with an average size of 70 nm. The particles were found to be monodispersed and non-agglomerated due to the binding of some stabilising agents present in the TR extract. Figure 4 shows two and three dimensional view of sample surface over a 2 x 2 µm and at 5 µm scan that shows non-agglomerated, monodispersed biosynthesized silver nanoparticles.

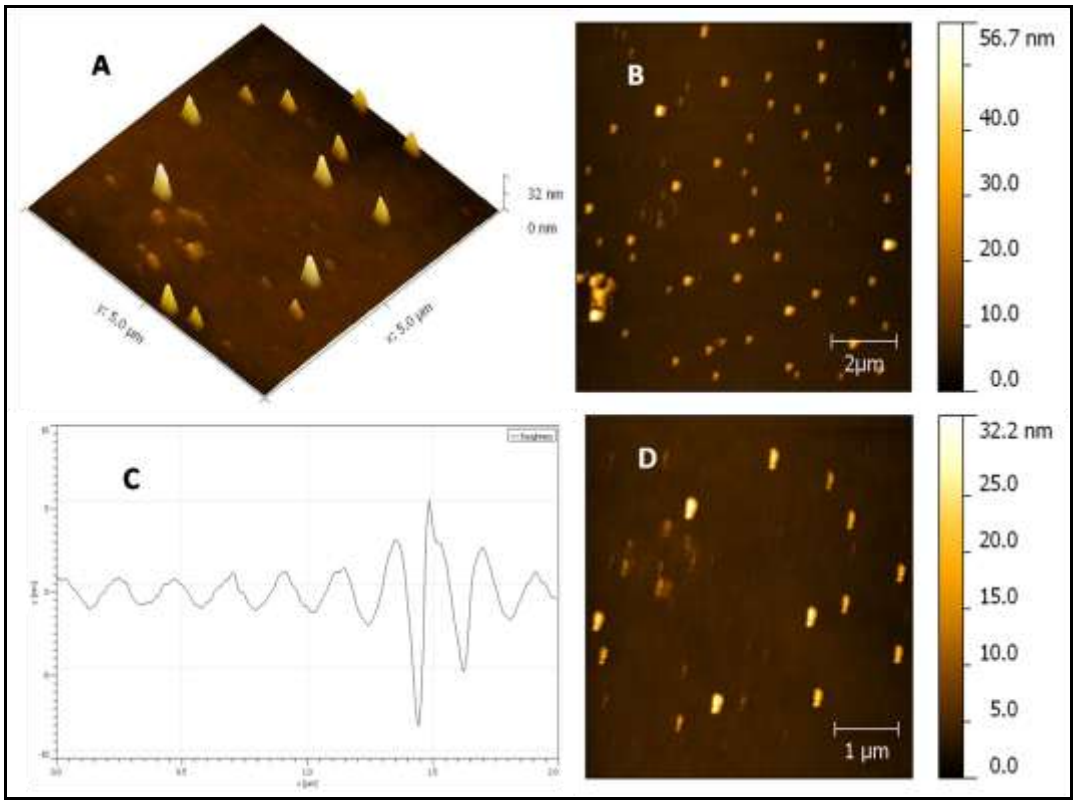


Figure 4. AFM images obtained from lyophilized sample of silver nanoparticles obtained from *Tabebuia rosea*, indicating the two dimensional images at magnification of 2µm (B), 1 µm (D), roughness data of nanoparticles at 2 µm (C) and three dimensional image of nanoparticles size in the range of 46 to 100 nm (A).

Fourier transforms infrared spectroscopy (FTIR)

FTIR analysis reveals the functional groups involved in reduction of silver nitrate to nanosilver for nanoparticle formation. The FTIR spectrum of the silver nanoparticle showed bands at 3423, 2918, 2848, 2368, 1593, 1375, 1020, 678 cm^{-1} but highly intense broad absorbance peak was observed at 3423 cm^{-1} characteristic of the O-H stretching of phenolic compounds (Figure 5). Also at 1020 and 2918 cm^{-1} corresponds to the stretch vibration of amine and C-H stretch (alkane H). Thus the silver nanoparticles are formed by interacting with the functional groups of secondary metabolites of *TR* extract such as alkaloids, alkanes, amines, alcohols, phenols and carboxylic acids.

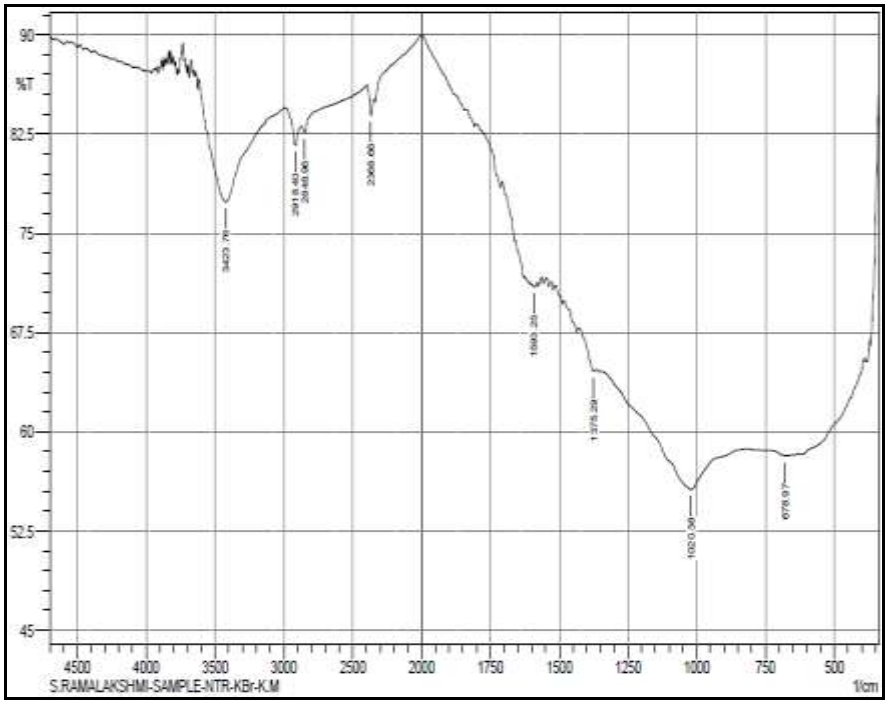
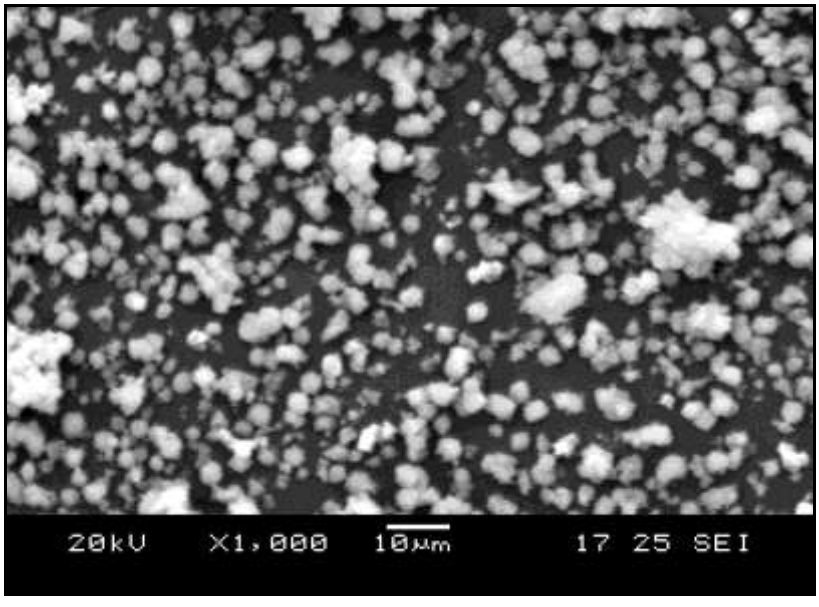


Figure 5. FTIR spectrum for silver nanoparticles synthesized using *Tabebuia rosea* leaf extract.

SEM and EDX profile

Scanning electron microscope images of the silver nano powder showed spherical morphology without agglomeration (Figure 6), with size distribution of 70 nm similar to that found in AFM.



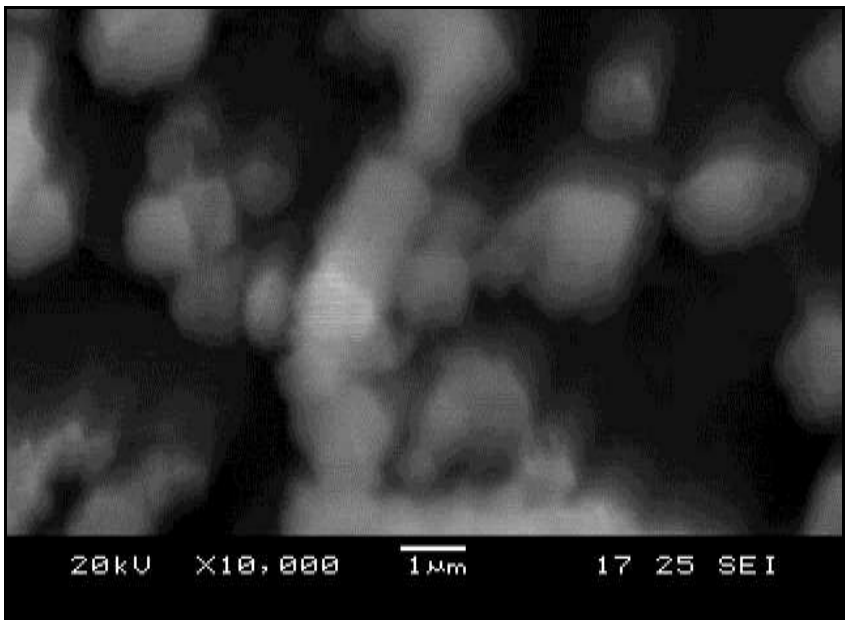
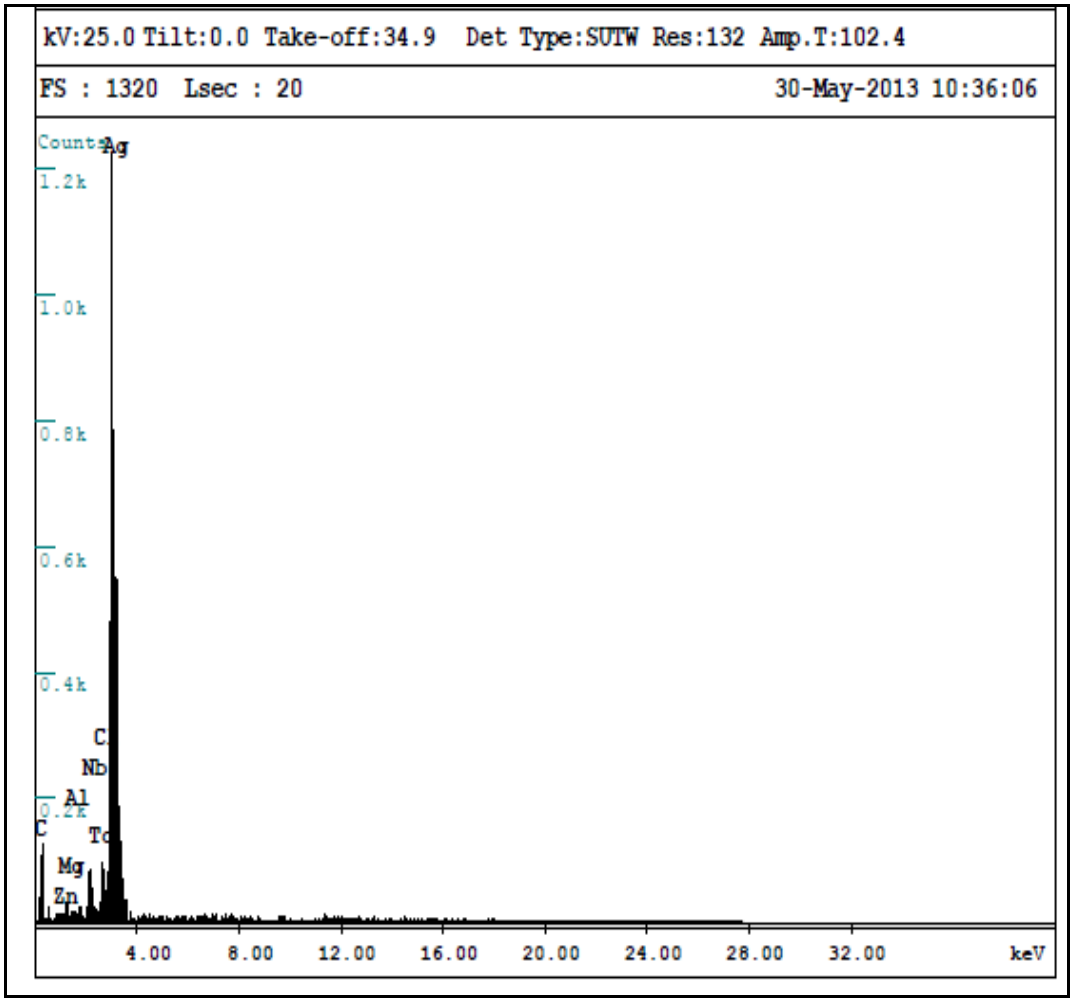


Figure 6. SEM images of silver nanoparticles synthesized using *Tabebuia rosea* leaf extract at 10 µm and 1 µm magnification.



EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	24.32	72.24	0.1053	1.1726	0.3692	1.0000
ZnL	1.22	0.67	0.0038	0.9793	0.3178	1.0016
MgK	1.12	1.64	0.0035	1.1090	0.2796	1.0035
AlK	0.31	0.41	0.0013	1.0767	0.3769	1.0067
NbL	3.99	1.53	0.0338	0.9346	0.8843	1.0263
TcL	0.81	0.29	0.0072	0.9293	0.9273	1.0337
ClK	0.96	0.97	0.0083	1.0747	0.7635	1.0532
AgL	67.27	22.25	0.6147	0.9059	1.0087	1.0000
Total	100.00	100.00				

Figure 7. EDX images of silver nanoparticles synthesized using *Tabebuia rosea* leaf extract.

The energy dispersive x-ray spectroscopy profile for silver nanoparticles showed strong silver signal (67.27 %) at 3 keV due to surface plasmon resonance[12] along with signals for C, Al, Zn, Mg, Al and Cl (Figure 7) confirming the formation of silver nanoparticles. In this study the AgNP synthesized from plant extracts are stabilized because of the active phytochemicals which act as stabilizing and capping agents which make the particles stable for long time proved by other researchers also[13, 14].

In vitro assessment of biosynthesized AgNPs cytotoxicity

Thus the cytotoxicity study results on normal human fibroblast cells (L929) and human ductal breast carcinoma cells (T47D) are given as percentage of cytotoxicity (Figure 8). The anticancer activity of crude ethanolic extract on normal and cancer studies was compared with nanoparticles prepared from plants. The results proved that when compared to NTR, TR leaf extract displayed cytotoxicity activities lesser. The cytotoxicity values of TR on normal cells were found to be from 12.67 to 30.69 % respectively. Also the cytotoxicity on tumour cells were found to be from 20.98 to 58.34 % respectively (Figure 8). NTR exhibited the highest activity on cancer cells (88.32 to 96.57 %) but it also displayed high toxicity on normal cells (20.43 to 50.68 %) at concentrations of 20 to 100 µg/ml (Figure 8).

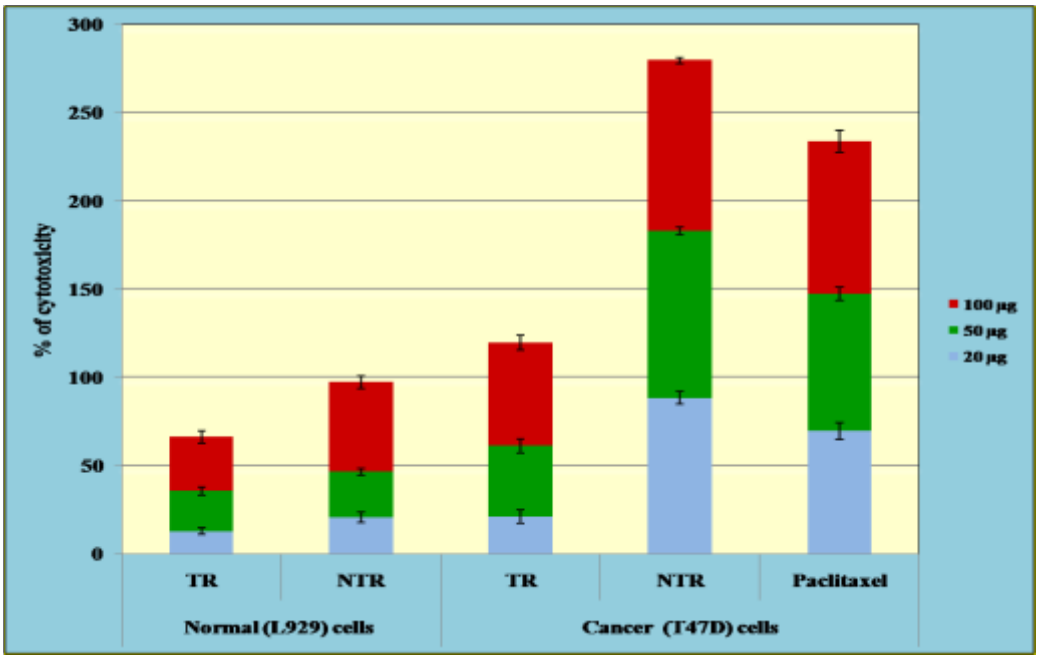


Figure 8. MTT assay results confirming the *in vitro* cytotoxicity effects of TR leaf extracts and AgNPs from TR against the normal L929 cells and cancer T47D cells at 48 h.

Studies on nanodrug from *Mallotus tetracoccus* showed anticancer activity on breast cancer cells, and silver nanoparticles were agglomerated with size of 73 nm[15]. Similar study reports show that these AgNPs are effective at lower concentrations[16]. The reported anti cancer properties of AgNPs whose possible mode of activity has been reported due to oxidative stress, lipid peroxidation, generation of free radicals[17, 18], interrupting ATP synthesis, mitochondrial damage resulting in genotoxicity[19]. The AgNP with DNA finally leads to cell cycle arrest in the G2/M phase[20] also it involves mitochondria-dependent jun-N terminal kinase (JNK) pathway involved in AgNP toxicity leading to cell death[21].

Conclusion

The green synthesis of silver nanoparticles using *Tabebuia rosea* leaf extract was found to be very effective against breast cancer. The optimized synthesis of silver nanoparticles were standardised at pH 7 and temperature 60^{o}C. FTIR analysis of nanoparticle sample indicates the involvement of hydroxyl and carboxyl functional groups of phenols taking part in reduction of silver nitrate to silver nanoparticles. The nanoparticles showed spherical morphology with size range of 49 to 98 nm, also giving strong silver signal for EDX analysis. The AgNPs synthesized from TR leaf showed upto 90 % cytotoxicity at concentrations of less than 20 µg/mL. Thus further investigations (*in vivo* studies) are needed for proving this nanodrug efficiency for applying in cancer therapy.

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References

1. Gentry AH. A Synopsis of Bignoniaceae Ethnobotany and Economic Botany, Annuals of Missouri Botanical Garden, 1992, 79: 53-64.
2. Oloyede GK, Oladosu IA, Shodia AF, Oloyade OO. Cytotoxic Effects of *Tabebuia Rosea* Oils (Leaf and Stem Bark). *Archives. App. Sci. Res*., 2010, 2: 127-130.
3. Sathiya M, Muthuchelian K. Studies on Phytochemical Profile and Antibacterial Activity of Ethanolic Leaf Extract of *Tabebuia rosea* (Bertol.) DC. Ethnobotanical Leaflets., 2008,12 :1153-57.
4. Ramalakshmi S, Muthuchelian K. Analysis of bio-active constituents of the ethanolic leaf extract of *Tabebuia rosea* (Bertol.) DC by Gas Chromatography-Mass Spectrometry. *I. J. ChemTech. Res*., 2011, 3: 1054-59.
5. Binutu OA, Lajubutu BA. Antimicrobial potentials of some plant species of the Bignoniaceae family. *Afr. J. Med. Sci.*, 1994, 23: 269-73.
6. Sathiya M, Muthuchelian K. Antitumor Potential of Total Alkaloid Extract from *Tabebuia rosea* (Bertol.) DC. Leaves on MOLT-4 Cells, *In Vitro. Nature and Science.*, 2010, 8: 77-85.
7. Ramalakshmi S, Saravanan S and Muthuchelian K. Radical scavenging activities and cytotoxic studies on different extracts of Tabebuia rosea leaves. A Treatise on Medicinal Plants and Herbal Products published by Virudhunagar Hindu Nadar's Senthilkumara Nadar College. Pg No.143-150, 2013 (ISBN NO. 978-93-81723-18-0).
8. Ramalakshmi S, Muthuchelian K. Phytotoxicity studies on leaf fractions of leaf fractions of *Tabebuia rosea* (Bertol.) DC. Indian journal of science., 2015, 15(48): 189-201.
9. Ramalakshmi S, Muthuchelian K. Studies on cytotoxicity, phytotoxicity and volatile profile of flower extract of *Tabebuia rosea* (Bertol.) DC. *Medicinal plants-I. J. Phytomed. Related Ind.*, 2012,4: 154-161.
10. Prabhu S, Poulose EK. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects, I. Nano. Letters., 2012, 2:125-132.
11. Chaudhuri SK, Chandela S, Malodia L. Plant Mediated Green Synthesis of Silver Nanoparticles Using *Tecomella undulata* Leaf Extract and Their Characterization. Nano Biomed. Eng., 2016, 8(1): 1-8.

12. Magudapathi P, Gangopadhyay P, Panigrahi EK, Nair KGM, Dhara S. Electrical transport studies of silver nanoparticles embedded in glass matrix, Physica B., 2001, 299: 142–146.
13. Song JY, Kim BS. Rapid biological synthesis of silver nanoparticles using plant leaf extracts, Bioprocess. Biosyst. Eng., 2009, 32: 79–84.
14. Singh M, Kalaivani RC, Anand M, Ramakritinan CM, Kumaraguru AK. Rapid Biomimetic Synthesis Of Silver Nanoparticles From Asiatic Mangrove (*Rhizopora Mucronata*) And Its Antimicrobial Activity Against Clinically Isolated Pathogens. Proceedings of the "International Conference on Nanoscience, Engineering and Technology" (ICONSET 2011), Kalpakkam, 28-30 November, 2011.
15. Ramalakshmi S, Muthuchelian K. Development of nanodrug for treatment of breast cancer using *Mallotus tetracoccus* leaves - standardisation, synthesis and characterization. *Nano Biomedicine engineering.*, 2016, 8(2): 82-90.
16. Das S, Das J, Samadder A, Bhattacharyya SS, Das D, Khuda-Bukhsh AR. Biosynthesized silver nanoparticles by ethanolic extracts of *Phytolacca decandra*, *Gelsemium sempervirens*, *Hydrastis canadensis* and *Thuja occidentalis* induce differential cytotoxicity through G2/M arrest in A375 cells. Colloids and Surfaces B: Biointerfaces., 2013, 101: 325– 336.
17. Jeyaraj M, Rajesh M, Arun R, MubarakAli D, Sathishkumar G, Sivanandhan G, Kapil Dev G, Manickavasagam M, Premkumar K, Thajuddin N, Ganapathi A. An investigation on the cytotoxicity and caspase-mediated apoptotic effect of biologically synthesized silver nanoparticles using *Podophyllum hexandrum* on human cervical carcinoma cells. Colloids Surf. B., 2013,102: 708–717.
18. Suliman AO, Ali D, Alarifi S, Harrath AH, Mansour L, Alwasel SH. Evaluation of cytotoxic, oxidative stress, proinflammatory and genotoxic effect of silver nanoparticles in human lung epithelial cells. Environ. Toxicol., 2013, 21:315-335.
19. Foldbjerg R, Dang DA, Autrup H. Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. Arch. Toxicol.,2011, 85: 743–750
20. Asharani PV, Mun GLK, Hande MP, Valiyaveettil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS nano., 2009, 3: 279–290.
21. Hsin YH, Chen CF, Huang S, Shih TS, Lai PS, Chueh PJ. The apoptotic effect of nanosilver is mediated by a ROS- and JNK- dependent mechanism involving the mitochondrial pathway in nih3t3 cells. Toxicol. Lett., 2008, 179: 130–139.

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