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Research Article

Development of Nanodrug for Treatment of Breast Cancer Using *Mallotus Tetracoccus* Leaves- Standardisation, Synthesis and Characterisation

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Abstract

In this study, an ecofriendly, green method for synthesis of silver nanoparticles (AgNP) has been developed using *Mallotus tetracoccus* (MT) leaves as a reducing agent. The formation of AgNPs was standardised at pH 7 and 60 °C. UV-visible spectroscopy showed the high peak of absorption band at 420 nm. By atomic force microscopic (AFM) and scanning electron microscopic (SEM) observations, the size of the silver nanoparticles was found to be in the range of 46 to 100 nm, with an average size of 73 nm. The energy dispersive x-ray spectroscopic (EDX) profile of silver nanoparticles showed typical optical absorption peak approximately at 3 keV. FTIR spectroscopic study revealed that hydroxyl groups of phenols and carboxylic acids were involved in the formation of AgNPs. Through MTT assay, the cytotoxicity results showed that AgNPs were highly effective on human ductal breast carcinoma cell lines (T47D) (76.8 to 84.9%). Thus, the MT-synthesized NPs are said to possess significant anticancer activity on cancer cells and very less toxicity on normal cells, which suggests its further applications in medicine.

Keywords: Nanodrug; *Mallotus tetracoccus*; Green synthesis; Silver nanoparticles; Anticancer activity

Introduction

Mallotus tetracoccus (Roxb.) Kurz. of family Euphorbiaceae, found in Western Ghats of India. The common names include *Thavatta*, *Vatta*, *Vatta kumbil*, *Vetta kumbil* (malayalam), *Uppale mara* (kannada) and "vatta kanni" in Tamil. Several species of the genus *Mallotus* are a rich source of biologically active compounds such as phloroglucinols, tannins, terpenoids, coumarins, benzopyrans and chalcones [1, 2]. The reported bioactivities of the extracts or the individual chemical constituents isolated from this genus include antipyretic [3], anti-inflammatory, hepatoprotective [4], antioxidant and radical scavenging activities [5].

The GC-MS analysis of *Mallotus tetracoccus* ethanolic leaf extract have revealed the presence of Bis (2-ethyl hexyl) phthalate (46.78 %), 3-methyl-2-(2-

oxypropyl) furan (13.31%), E-8-methyl-9-tetradecen-1-ol acetate (6.63 %), Octadecanoic acid, 2-oxo (4.46 %) and Longiborneol (2.39%) [6]. The bark of *M. tetracoccus* have been reported for high antioxidant and antibacterial activity [7]. Also, the bark extract studied for GC-MS showed presences of thiocyanic acid, furfural and 4H- Pyran-4 –one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl compounds. The bark extract showed significant cytotoxicity and phytotoxicity on radish seeds [8].

Thus, the leaves of MT have been used for synthesis of silver nanoparticles. Since time immemorial, silve has been used for curing various diseases due to its antibacterial properties. Recently, silver is gaining a role in development of nanoparticles for cancer therapy, biosensors, dressings, devices, formulations etc. [9]. The objective of this study was to characterize and study the anticancer potential of silver nanoparticles synthesized from MT leaf extract.

Materials and Methods

Plant material and preparation of the extract

Fresh and intermediate leaves of *Mallotus tetracoccus* (Roxb.) Kurz was collected from Agasthiar Malai Biosphere Forest, Western Ghats, collected plant materials were identified and authenticated by the Director, Centre for Biodiversity and Forest Studies (CBFS), MKU, and voucher specimens were deposited in the herbarium of CBFS of university (No. AM-02). The dry leaves was cut into small pieces and powdered finely. About 5 g 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 ($AgNO_3$) 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 the formation of silver nanoparticles.

The above mentioned procedure was repeated for optimization of temperature and pH. 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. The reaction temperature was studied at 30 °C to 70 °C.

Purification of silver nanoparticles

The broth containing nanoparticles was centrifuged at 15000 rpm for 15 min to obtain the pellet which was redispersed in sterile deionized water to get rid of any biological molecules. The process of centrifugation and redispersion in sterile deionized distilled water 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 colour change 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. The nanoparticle solution was diluted 20 times with deionized water to avoid errors due to high optical density of the solution.

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

For FTIR measurements, bioreduced silver NP dried powder was analyzed using FTIR. The samples were

dried, grounded with KBr pellets and analyzed in a SHIZAMAZU model no 8400S spectrum instrument. A disk of 50mg of KBr was prepared with a mixture of 2% finely dried sample, and was then examined under IR-spectrometer. Infrared spectrum was recorded in the region of 500 to 4500 cm^{-1}.

Scanning electron microscopy (SEM) and 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 1×104 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 the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium] assay. This colorimetric test is based on the selective ability of viable cells to reduce the tetrazolium component of MTT into purple coloured formazan crystals. Stock solution of the samples were freshly prepared (1 mg/1 mL) and diluted with cell culture medium to the desired concentrations (20, 50 and 100 mg). 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 hrs. 5 mg of MTT (Sigma) was dissolved in 1 ml of PBS and filter sterilized. 10 ml of MTT solution was further diluted to 100 ml with 90 ml of serum and phenol red free medium. 100 ml 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 hr to dissolve the formazan crystals. The absorbance of the solution was measured at a wavelength of 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

In this study, the AgNPs were rapidly formed soon after the addition of clear silver nitrate solution to the aqueous extract of MT leaves, which was indicated by the light yellowish color turning to the dark brown color after 24 hrs (Fig. 1). The UV-Vis spectroscopy examined the size and shape of nanoparticles at the range of 300–600 nm. Among the various combinations (Fig. 2(a), 2(b)) used for optimization of silver nanoparticle formation, the mixture of 6:4 (S6) of silver nitrate and plant extract gave the optimized absorption spectrum at 420 nm, which is

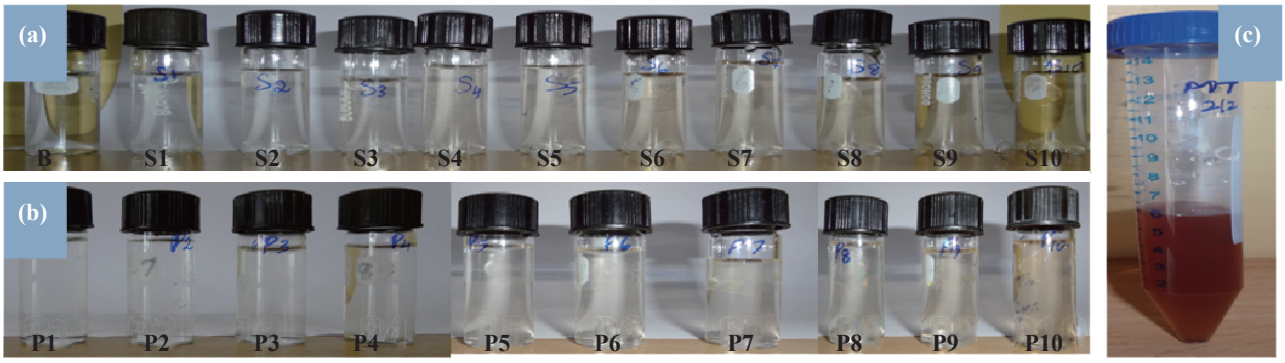


Fig. 1 The bottles containing silver nitrate solutions, plant extracts at different concentrations denoted as **(a)** S1-S10 and **(b)** P1-P10 respectively; **(c)** bottle containing purified, concentrated silver nanoparticles.

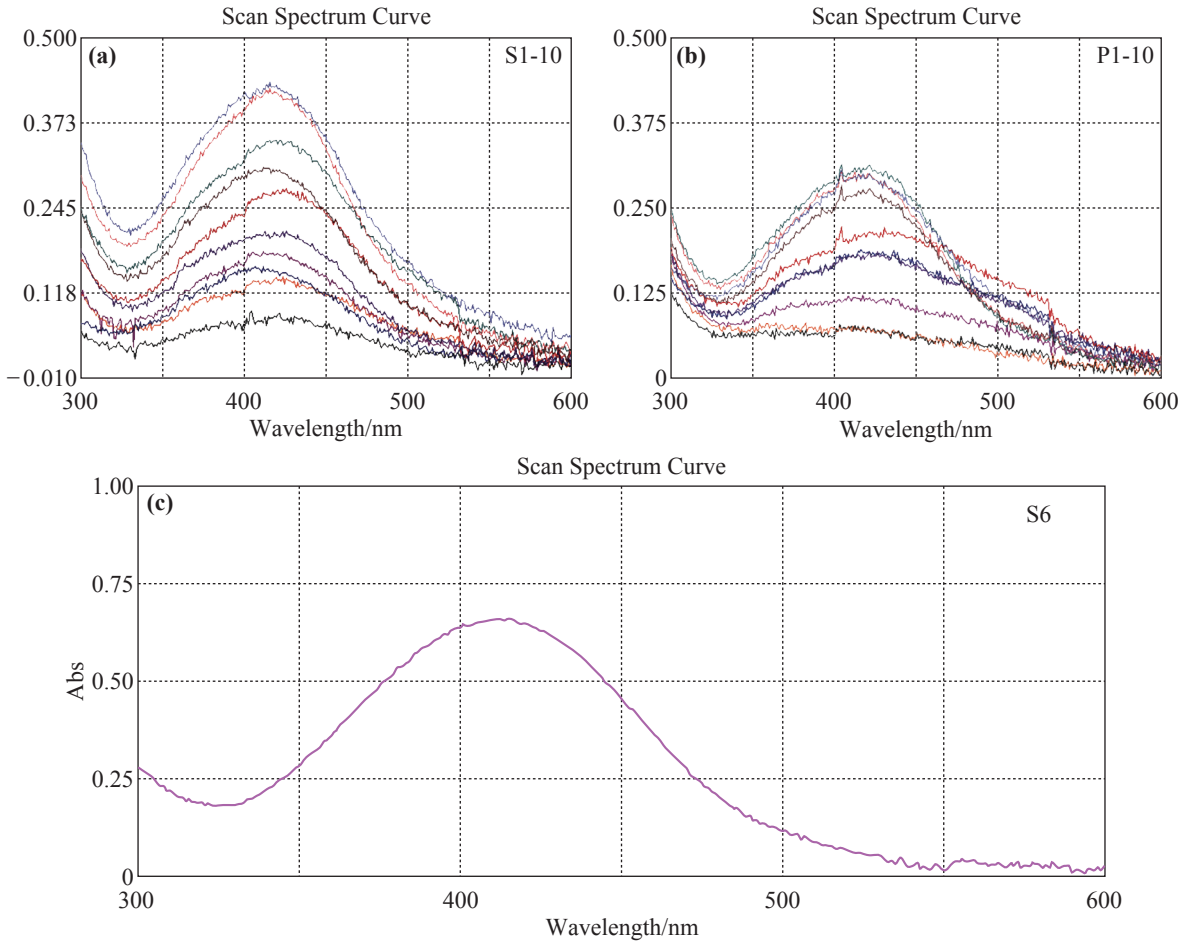


Fig. 2 (a) UV-visible spectrum of a mixture of solutions of silver nitrate S1-S10; **(b)** plant extracts P1-P10; **(c)** optimized silver nanoparticles synthesized using *Mallotus tetracoccus* leaves.

characteristic for surface plasmon resonance (SPR) of silver nanoparticles (Fig. 2(c)). Similar SPR of AgNPs synthesized by using seaweed *Padina tetrastromatica* showed an absorption peak at 424 nm (10). Here the biologically active molecules present in the leaf extract of MT have played a role in the reduction of silver nitrate to silver. The earlier investigations on the phytochemical constituents of *Mallotus tetracoccus* leaves revealed the presences of sugars, tannins, alkaloids, flavonoids, steroids, terpenoids and phenolic acids.

Optimization of physicochemical parameters for synthesis of silver nanoparticles

The formation of silver nanoparticles was optimized using different parameters: pH and temperature. PH and temperature play a very significant role in the synthesis of silver nanoparticles by controlling their shapes and sizes. The reaction medium was checked at different pH values for the production of optimized silver nanoparticles. PH 7 was said to give small, highly dispersed silver nanoparticles as indicated by their absorbance peaks at the nanometer scale, which was confirmed by the UV-Vis spectrum readings (Fig. 3). Among the different temperatures checked, the optimal production was observed at 60 °C, where high temperature favoured the synthesis (Fig. 3). Similar green synthesis of AgNPs was reported at the temperature of 60 °C using leaf extracts of *Tecomella undulata* (11).

Analysis of silver nanoparticles in AFM

The surface morphology of AgNPs synthesized using leaf extract of MT was studied by AFM. The obtained morphology revealed the fact that the synthesized silver nanoparticles were almost spherical in shape, which was confirmed by the absorbance spectrum (Fig. 4). The sizes of the silver nanoparticles as observed by AFM were found to be in the range of 46 to 100 nm, with an average size of 73 nm. The particles were polydispersed and agglomerated due to the binding of some stabilising and capping agents

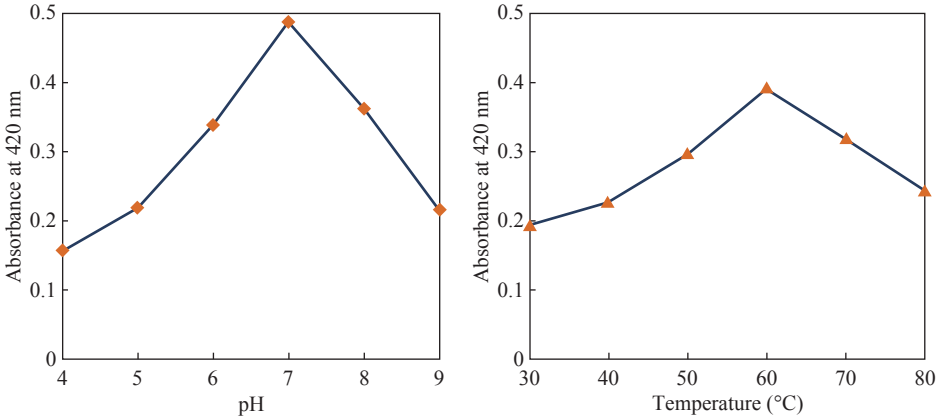


Fig. 3 Absorption spectrum for silver nanoparticles synthesized using *Mallotus tetracoccus* leaves at different pH values and temperatures.

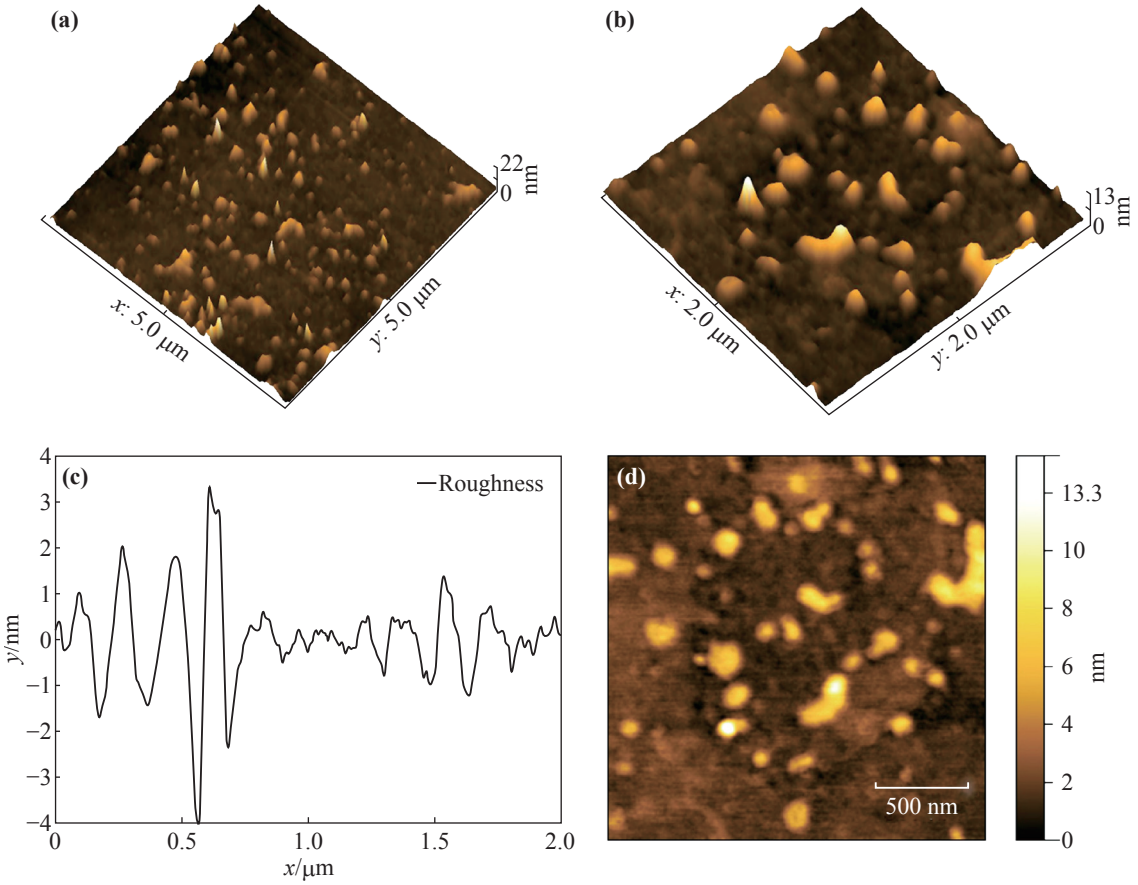


Fig. 4 AFM images obtained from the lyophilized sample of silver nanoparticles obtained from *Mallotus tetracoccus*, indicating the three dimensional images at the magnification of **(a)** 5 µm and **(b)** 2 µm; **(c)** roughness data of nanoparticles at 2 µm; **(d)** two dimensional image of nanoparticles with sizes in the range of 46 to 100 nm.

present in the MT extract. Figure 4 shows two and three dimensional views of the sample surface at the scan sizes of 2 µm×2 µm and 5 µm×5 µm, depicting the agglomerated, polydispersed distribution of silver nanoparticles.

Fourier transforms infrared spectroscopy (FTIR)

FTIR analysis gives an idea of the functional groups involved in reduction of silver nitrate to nanosilver. The FTIR spectrum of the silver nanoparticle showed bands at 3425, 2922, 2854, 2368, 1593, 1018, 667 and 609 cm^{-1}. Highly intense broad absorbance peak was observed at 3425 cm^{-1} characteristic of the O-H stretching of phenolic compounds (Fig. 5). The bands at 2922, 2854 represents the C-H stretch (alkane H),

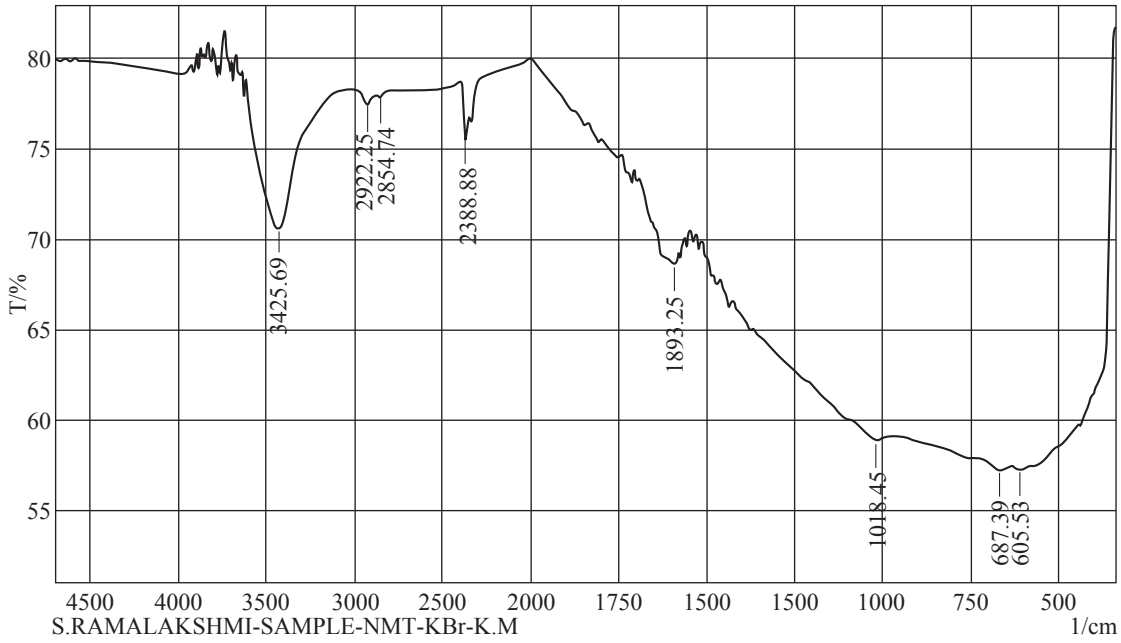
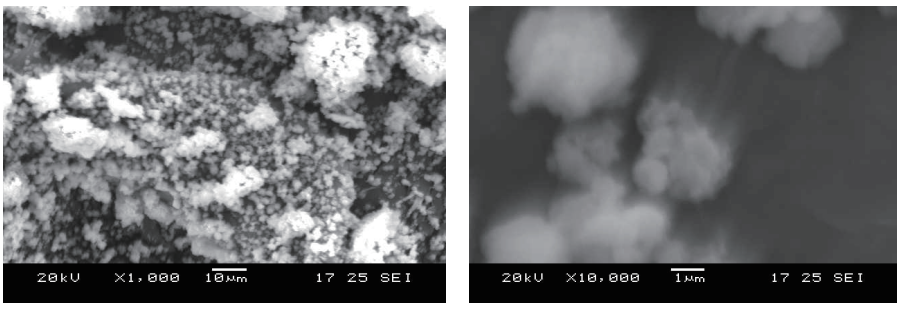


Fig. 5 FTIR spectrum for silver nanoparticles synthesized using *Mallotus tetracoccus* leaf extract.

O-H stretching in carboxylic acids and bands at 2368 and 1593 corresponds to C=O stretch in carboxylic acid respectively. Also the absorbance peak at 1018 cm^{-1} corresponds to the stretch vibration of C=C and C-N stretching vibrations of amine. Thus the silver nanoparticles are formed due to the interactions with the active compounds (secondary metabolites) of MT extract such as sugars, tannins, alkaloids, flavonoids, steroids, terpenoids and phenolic acids with silver nitrate to form nanoparticles. Thus the hydroxyl groups of phenols and carboxylic acids are involved in formation of AgNPs.

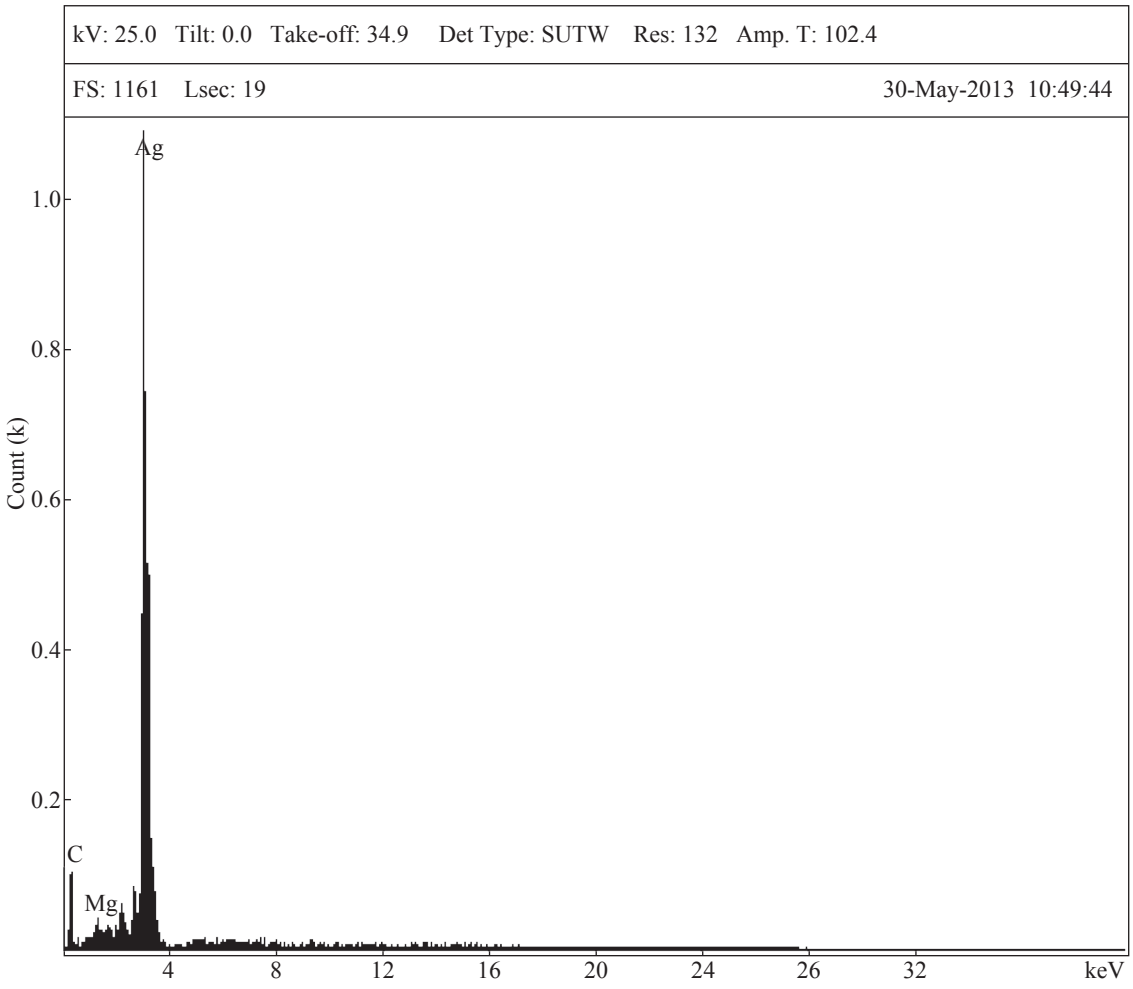
SEM and EDX profiles

SEM images were obtained from the lyophilised sample of silver nanoparticle synthesized from the leaf extract of MT (Fig. 6). Scanning electron microscope images of the silver nano powder showed spherical morphology with agglomeration. The obtained size distribution was similar to that found in AFM. The energy dispersive X-ray spectroscopy profile for silver nanoparticles showed strong silver signal along with signals for C and Mg, which may be due to the X-ray emission of the attached active molecules present on the surface of the silver nanoparticles (Fig. 7). Thus, the EDX reveals strong signal (78.38%) in the silver region, confirming the formation of silver nanoparticles. Metallic silver nanocrystals generally show typical optical absorption peak approximately at 3 keV due to surface plasmon resonance [12]. Various study reports on AgNp synthesized from plant extracts showed that several stabilizing agents act as capping agents and make the particles stable for a long time [13, 14].



(a) **(b)**

Fig. 6 SEM images of silver nanoparticles synthesized using *Mallotus tetracoccus* leaf extract at **(a)** 10 µm and **(b)** 1 µm magnifications.



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

Element	wt%	at%	K-Ratio	Z	A	F
C K	20.04	67.83	0.1163	1.1897	0.4876	1.0000
MgK	1.58	2.64	0.0048	1.1250	0.2674	1.0036
AgL	78.38	29.54	0.7623	0.9220	1.0549	1.0000
Total	100.00	100.00				

Element	Net Inte.	Bkgd Inte.	Inte. Error	P/B
C K	27.58	0.61	4.39	45.25
MgK	10.51	2.13	8.24	4.93
AgL	510.01	2.34	1.00	218.28

Fig. 7 EDX images of silver nanoparticles synthesized using *Mallotus tetracoccus* leaf extract.

In vitro assessment of biosynthesized AgNPs cytotoxicity

Today, assays based on cell culture play an important role in the screening of compounds for various anticancer studies. Thus, the results of cytotoxicity study on normal human fibroblast cells (L929) and human ductal breast carcinoma cells (T47D) are given as cytotoxicity percentage. NMT showed very less toxicity on normal cells (4.4 to 10.6 %) at concentrations of 20 to 100 µg, whereas it exhibited highest cytotoxicity (76.8 to 84.9 %) on tumour cells (Fig. 8). Thus, the fact that AgNPs show higher cytotoxicity of 80-95 % at concentrations of 20 to 100 µg represents its potential to effectively kill cancer cells.

Similar study results from earlier researchers also show that silver nanoparticles synthesized from plants can effectively kill cancer cells at lower concentrations

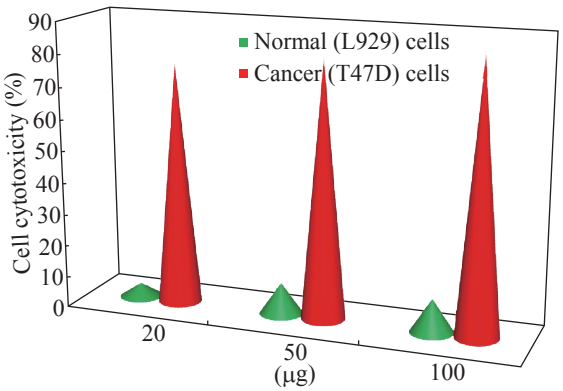


Fig. 8 MTT assay results confirm the *in vitro* cytotoxicity effect of AgNPs against the normal L929 cells and cancer T47D cells at 48 hrs.

[15]-[18]. The possible mode of the activity of silver nanoparticles on cancer cells has been reported by various researchers, that is, the created oxidative stress leads to depletion of GSH and induction of ROS, LPO, SOD and catalase in a dose dependent manner [16]-[18]. This stress causes interruption of ATP synthesis and mitochondrial damage, resulting in genotoxicity [22]. There is an interaction of AgNP with DNA, leading to cell cycle arrest in the G2/M phase [23]. There is also an increase in the activities of caspases, level of pro-inflammatory cytokines (interleukin-1b (IL-1b) and interleukin-6 (IL-6)) in the treated cancer cells [21]. Reports also show that there is the involvement of mitochondria-dependent jun-N terminal kinase (JNK) pathway in AgNP toxicity [24]. Finally the cancer cells undergo apoptosis.

Conclusions

This study reports the green synthesis of silver nanoparticles using *Mallotus tetracoccus* leaf extract. The synthesis of silver nanoparticles was standardised at pH 7 and the temperature of 60 °C. The silver nanoparticles were characterized using AFM, FTIR and SEM with EDX. FTIR analysis of the nanoparticle sample indicates the involvement of hydroxyl and carboxyl functional groups of phenols in the reduction of silver nitrate to silver nanoparticles. The nanoparticles showed a spherical morphology with the size range of 49 to 98 nm, and gave a strong silver signal in the EDX analysis. The AgNPs synthesized from MT leaf showed up to 85% cytotoxicity on cancer cells and very less toxicity on normal cells at the concentrations studied. Thus, further investigations are needed to prove its potential in *in vivo* studies for its application in cancer therapy.

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