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In-vitro Anticancer and Antioxidant Activity of Gold Nanoparticles Conjugate with *Tabernaemontana divaricata* flower SMs Against MCF -7 Breast Cancer Cells

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Abstract – Biologically stabilized gold nanoparticles were synthesized from the flower aqueous extract of *T. divaricata*. The synthesized nanoparticles were characterized by UV-Vis spectrophotometer, Zeta sizer, FTIR and TEM analysis. *T. divaricata* reduced gold nanoparticles having particle size and potential of 106.532 nm and -10.2 mV, respectively, with a characteristic peak of 550 nm in UV-visible spectrophotometer. FTIR graph after comparison between the crude flower extract and gold nanoparticles showed three major shifts in the functional groups. The morphology and size of the gold nanoparticles were examined by HRTEM analysis, which showed that most of the nanoparticles were nearly spherical with size of 100 nm. The gold nanoparticles synthesized demonstrated potent anticancer activity against MCF-7 cell line. The findings conclude that the antioxidant molecule present in *T. divaricata* may be responsible for both reduction and capping of gold nanoparticles which possess potential applications in medicine and pharmaceutical fields.

Key words: Anticancer, *T. divaricata,* Bioreduction, Breast cancer, Antioxidant, Gold nanoparticles

1. Introduction

Cancer is one of the most severe health problems worldwide [1]. Among the most common types of cancers, breast cancer is the leading cause of death in women [2]. Nanoparticles as drug carriers have new modalities for cancer therapy and diagnosis, because many anticancer drugs have been clinically applied to treat various cancers, but they cannot be used effectively due to poor cell penetration. Nanoparticles capped with hydrophobic therapeutic drugs enable to increase the efficiency of drug solubility [3]. Nanotechnology is a multidisciplinary field with medical science and biomedical engineering that offers a new avenue to medical diagnostics and drug delivery systems. The application of nanotechnology to medicine includes studying the interactions of nanomaterials with cells and tissues. Biodegradable polymers find a pivotal part in today's advancement of drug delivery as they can be degraded to non-toxic monomers inside the body [4]. Metals have been used for the biosynthesis of nanoparticles, including gold, silver, platinum, and zinc in order to minimize toxicity in drug delivery application [5]. A promising field has been opened wide by nanotechnology, called targeted drug therapy, which is hugely strengthened by gold nanoparticles as it is non-toxic to human cells. Biological methods of nanoparticle synthesis using microorganisms, enzymes and plants or plant extracts have been suggested as possible eco-friendly alternatives to chemical and physical methods [6]. Processes for making nanoparticles using plant extracts are readily scalable and are less expensive. Plant extracts may act both as reducing agents and stabilizing agents in the synthesis of nanoparticles [7].

Tabernaemontana divaricata (L.) R. Br. (Apocynaceae) is an evergreen glabrous tree found throughout the India, Australia and Polynesia. The tree species has special interest because of the presence of indole alkaloids, which are commonly used for anti-inflammatory, antifertility, antimicrobial and antioxidant activities [8]. In view of the vast application of this plant, the present study was investigated to determine the anticancer (against breast cancer MCF-7 cell lines) and antioxidant activity of *T. divaricata* reduced and capped gold nanoparticles as future therapeutic drug. The synthesized gold nanoparticles were characterized using various techniques.

2. Materials and Methods

2-1. Plant Materials

Flowers of *T. divaricata* were collected from Ramapuram, Tamil Nadu (India), and authenticated by Botanical Survey of Porur, Chennai, Tamil Nadu. Flowers were thoroughly washed with autoclaved distilled water, shade dried and powdered.

2-2. Synthesis of gold nanoparticle by *T.divaricata* conjugates

Glasswares were rinsed with deionized water before starting the synthesis. Tetrachloroauric acid acid ($HAuCl_4$ $3H_2O$) was obtained from Sigma-Aldrich Chemicals. One gram of flower powder was boiled in 10 ml of deionized water. It was filtered through Whatman No 1 filter paper. An aliquot of 500 µl was taken and added to 20 ml of 1 mM $HAuCl_4$. This mixture was kept for 24 hours at room temperature with stirring on a magnetic stirrer. The expected color change was observed in the colloidal solution and it was characterized further [9].

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2-3. UV-Visible spectroscopy studies

UV-Vis spectroscopy measurements of the tetrachloroauric acid solution and *T.divaricata* flowers extract reduced gold nanoparticles were carried out using UV-Vis spectrophotometer (UV-2450 Elico). Scanning was done from 200~800 nm.

2-4. Particle size analysis and zeta potential determination

The particle size ranges of the nanoparticles were determined using particle size analyzer (Malvern ZetasizerNano). Particle sizes were calculated based on measuring the time-dependent fluctuation of scattering of laser light by the nanoparticles.

2-5. Fourier Transform Infrared (FT-IR) Spectroscopy measurements

After complete reduction of $HAuCl_4$-ions by *T. divaricata,* the dispersion gold nanoparticles were centrifuged at 12000 rpm for 10 min at room temperature. The gold nanoparticle pellet obtained after centrifugation was resuspended in deionized water and centrifuged again to remove the traces of unbound flower extract present in the solution prior to FT-IR analysis. An FT-IR spectrum was taken to assess the involvement of possible capping by the plant extract. Measurements were carried out on a Perkin-Elmer FTIR Spectrum One spectrophotometer.

2-6. HR-TEM measurements

TEM samples of the gold nanoparticles were prepared by placing a drop over carbon-coated copper grids and allowing the solvent to evaporate. TEM measurements were performed on a Philips model 2400EX instrument operated at an accelerating voltage between 80–200 kV.

2-7. Anticancer activity

The MCF-7 breast cancer cell lines were obtained from the National Centre for Cell Sciences, Pune, India and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37 °C, 100% relative humidity, 5% CO_2 and 95% air. The culture medium was changed twice a week. The MTT assay [10] is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. Cells were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37 °C in humidified atmosphere with 5% CO_2.

The cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2×10^4 cells/well and allowed to attach overnight at 37 °C. The medium was then discarded and cells were incubated with different concentrations of the extract for 24 hours. After incubation, medium was discarded and 100 µl fresh medium was added with 10 µl of MTT (5 mg/ml). After 4 hours, the medium was discarded and 100 µl of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570 nm in a microtiter plate reader. The assay was performed for all ten fractions obtained from column chromatography.

Cell survival was calculated by the following formula:

Viability % = (Test OD/ Control OD) × 100

Cytotoxicity % = 100 - Viability%

2-7-1. Estimation of cell viability

Cell proliferation was measured using MTT assay. After 24 hours of treatment, 20 µl of MTT solution (5 mg MTT in 1 ml PBS) was added per well and incubated at 37 °C for 4 hours in 5% CO_2 atmosphere. Then, the medium was removed and washed with PBS; 200 µl of DMSO was added to each well. The intensity of the colored product was measured using an ELISA micro plate reader at 570/620 nm. The results were expressed as percentage of optical density of treated Vs control cells.

2.7-2. Estimation of catalase

Catalase activity was estimated by the method of Sinha [11]. To 0.1 ml of sample, 1 ml of phosphate buffer (0.01M, PH 7.0) and 1 ml of hydrogen peroxide were added and the timer was started. The reaction was arrested by the addition of 2 ml dichromate-acetic acid reagent (5% Potassium dichromate was prepared with dilute acetic acid (1:3 w/v in distilled water). Standard hydrogen peroxide (20 µM) was taken and treated similarly. The tubes were heated in the boiling water bath for 10 minutes. The green color developed was read at 570 nm using spectrophotometer.

2-8. Calculation

$$\text{Enzyme activity } (\mu \text{ moles of } H_2O_2 \text{ utilized/min/g protein}) = \frac{\text{A OD} \times \text{std conc. } (\mu \text{ mol})}{\text{Enzyme (ml)} \times \text{std OD} \times \text{Protein (mg/ml)}}$$

2-8-1. Assay for NO production

Nitric oxide radical inhibition was estimated using Griess Illosvoy reaction [12]. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Stock solution of leaf extracts and ascorbic acid (standard) was prepared at the concentration of 1 mg/ml. The reaction mixture contains 2 ml of sodium nitroprusside (10 mM), 0.1 ml of phosphate buffer saline and samples at different concentration (25, 50 and 75 µg). Standard solution obtained from stock solution was incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1ml of sulfanilamide (1%) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride (0.1% W/V) was added, mixed and allowed to stand for 30 min at 25 °C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Blank consisted of all the reagents, except for the extract or standard solution which was substituted with water. The annihilation activity of free radicals

was calculated in % inhibition according to the following relation:

$$\text{Inhibition \%} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2-8-2. Estimation of lipoperoxides

2-9. Preparation of cell lysate

Cells were harvested from culture flasks after the removal of medium which contained the floating dead cells, washed with PBS and centrifuged at 1500 × g for 10 min at 4 ºC. Cell pellets were resuspended in 0.5 ml of PBS and then sonicated on ice three times for 30 seconds each. The total extract was centrifuged at 3000 × g for 15 min at 4 ºC. Aliquots of the supernatant were used for enzyme assays.

2-10. Lipid peroxidation

MDA, an *in vitro* marker of lipid peroxidation, was assessed by using the method described by Del Rio [13]. To 200 µl of sample with a protein concentration of 2 mg/ml, 700 µl of 0.1 NHCl was added and the mixture was incubated for 20 min at room temperature. Then 900 µl of 0.025 M thiobarbituric acid (TBA) was added and the mixture was incubated for 65 min at 37 ºC. Finally, 400 µl of 10 mM PBS was added. The fluorescence of MDA was recorded using a 520/549 (excitation/emission) filter. A calibration curve with MDA in the range 0.05~5 µM was used to calculate the MDA concentration. The results were expressed as n moles of MDA/mg protein.

3. Results and Discussion

Nanomedicine is the latest advancement in which the world has seen nanoparticles being the prime part of therapeutic and diagnostic agents [14]. Nanoparticulate materials are made up of a wide variety of materials, and their small size, unique physicochemical properties and biological action thereby requires proper characterization through standard analytical techniques to encounter those challenges [15]. A thorough understanding of the nanomaterials through their physicochemical characterization, stability measurement, bio-distribution and toxicity assessment, both in-vitro and in-vivo studies is essential. A rational characterization approach prior to application is an integral part of clinical translation.

The synthesized *T. divaricata* reduced gold nanoparticles were initially confirmed by visual observation on the color change from pale yellow to purple pink due to reduction of gold ions during the reaction (Fig. 1a). The appearance of purple color is a clear indication of the formation of gold nanoparticles in the reaction mixture. It is well known that Au nano particles exhibit reddish brown color in aqueous solution due to excitation of surface plasmon vibrations. The color exhibited by metallic nanoparticles is due to the coherent excitation of all the "free" electrons within the conduction band, leading to an in-phase oscillation which is known as surface plasmon resonance-SPR [16].

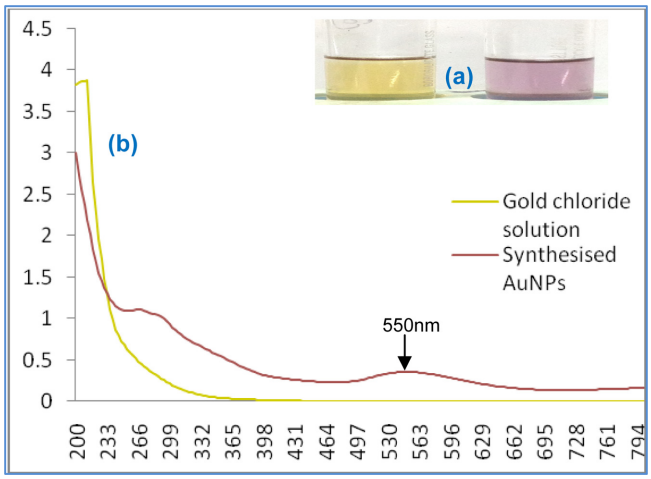

Fig. 1. (a) Optical photograph of gold chloride solution and gold nano particle colloidal solution after 24 hours of reduction (b) UV-Visible spectroscopy showing spectrum the of gold chloride solution at initial timeand colloidal solution of AuNPs biore-duction of by *T. divaricata* flower extract after 24 hours.

UV-Visible spectroscopy is one of the most widely used techniques for structural characterization of silver and gold nanoparticles [17]. The bio-reduction of nanoparticles was monitored periodically by UV-Vis spectrum at regular different time intervals [18]. A UV-Vis spectrograph of the colloidal solution of gold nanoparticles was recorded as a function of time. The UV-Vis spectrometric readings were recorded at a scanning speed of 200 to 800 with the absorbance maximum peak at 551 nm for *T. divaricata* reduced gold nano particles (Fig. 1b).

For zeta sizer measurement the Malvern instrument is used to measure three characteristics of particles or molecules in a liquid medium, such as measurement of the size, zeta potential of nanoparticles and electrophoretic mobility of proteins. *T. divaricata* reduced gold nanoparticles have particle size and potential of 106.532 nm and –10.2 mV, respectively (Fig. 2a, 2b). The negative zeta potential value of –10.2 mV for gold nanoparticles provides the necessary repulsive forces for the particles to remain stable in solution. Thus the *T. divaricata* reduced gold nanoparticles were stable for more than three months when stored in a refrigerator.

FTIR measurements were carried out to identify the possible biomolecules in the *T. divaricata* flower extract responsible for the reduction of $AuCl_4$- ions and also the capping agents responsible for the stability of the biogenic nanoparticle solution. Fig. 3a and 3b represent the FTIR spectrum of *T. divaricata* flower extract and *T. divaricata* flower extract reduced gold nanoparticles with the absorption bands at 2881 cm^{-1}, 2746 cm^{-1}, 2391 cm^{-1}, 2239 cm^{-1} and 1637 cm^{-1}. The shift in the C=C stretching and C-C stretch deformation frequency (2239 cm^{-1}) to lower wave numbers (1637 cm^{-1}) followed by the disappearance of the 1637 cm^{-1} resonance indicate the facilitation of the binding of C-C group of alkynes with the gold nanoparticle surface. In addition to the above supportive evidence, the 2881 cm^{-1}

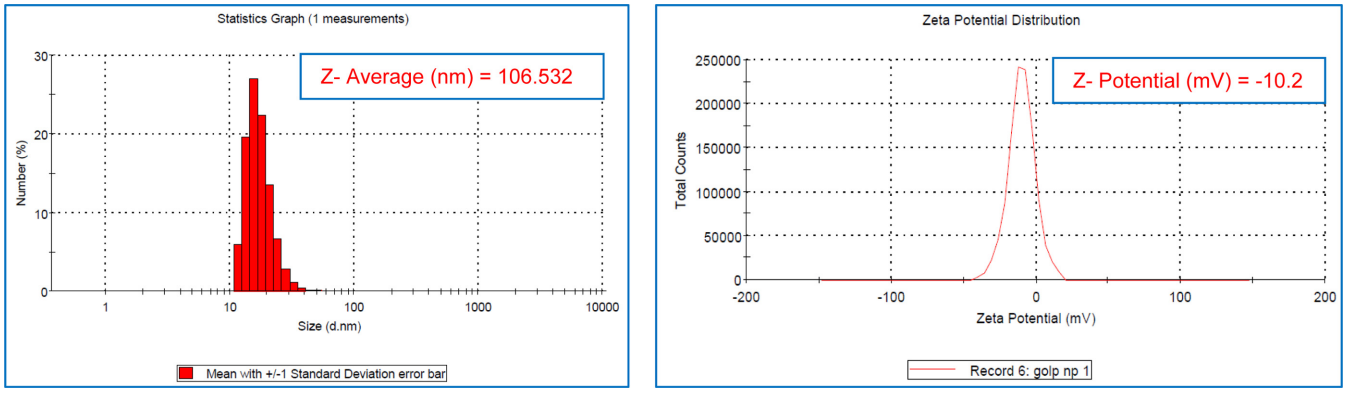

Fig. 2.(a) Zeta Particle size measurement (b) zeta potential measurement of *T. divaricata* leaf extract reduced gold nanoparticles.

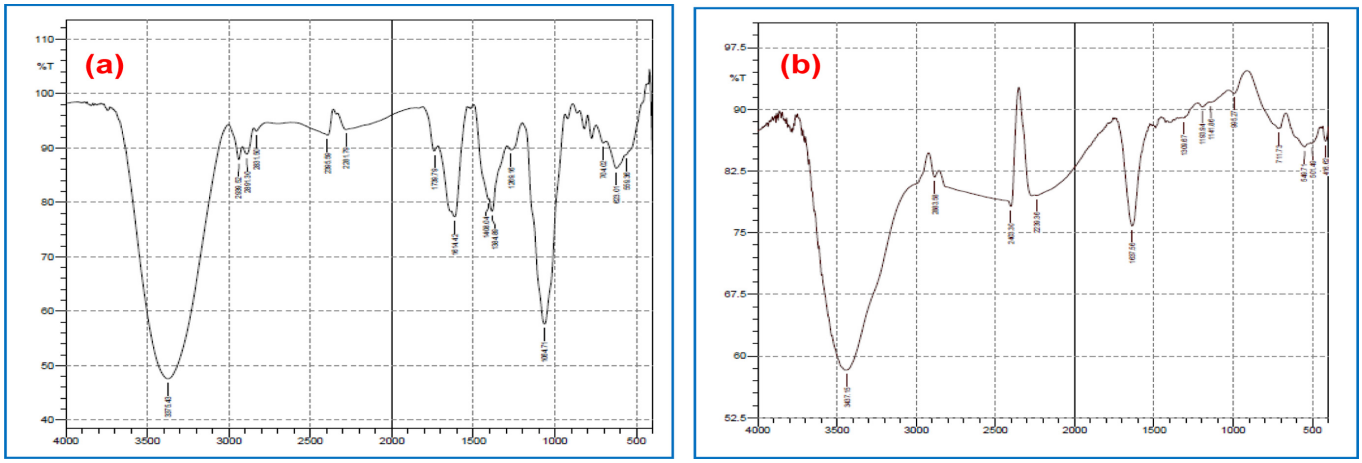

Fig. 3. Fourier transform infrared (FT-IR) absorption spectra (a) *T. divaricata* flower extract spectra, (b) *T. divaricata* flower extract reduced gold nanoparticles spectra.

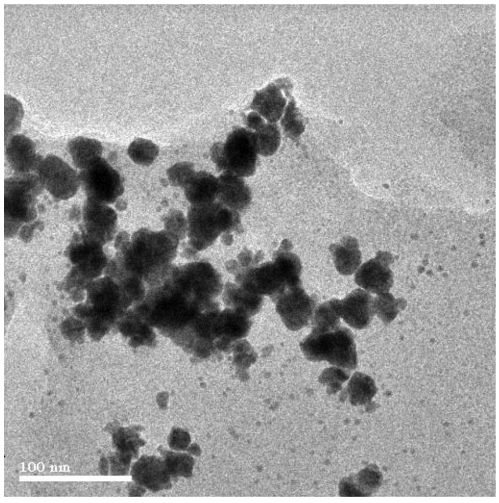

Fig. 4. HR TEM image of *T. divaricata* flower extract reduced gold nanoparticles.

feature shifts to 2746 cm^{-1} due to the binding of the carbonyl group with gold nanoparticle surface. The morphology and size of the gold nanoparticles were examined by HR-TEM analysis. It shows that most of the nanoparticles were nearly spherical with size of 100 nm (Fig. 4).

According the previous finding, tannins present in the *Terminalia catappa* leaf, and flavonoids, carbohydrates and glycosides molecules present in fruit extract of *Lantanacamara* were responsible for the reduction of the metal ions and the stabilization of nanoparticles[19, 20]. Current researches for cancer treatment are not fully effective. In view of this, there is interest in the discovery of novel anticancer drug from plant sources. MCF-7 cell lines retained various properties of differentiated mammary epithelium. The results for cell growth inhibition of the extract at various concentrations against breast cancer MCF-7 cell lines was determined (Fig. 5). As the concentration increases, there is a decrease in the cell growth inhibition, but it was found to be much less with only 88.85% growth inhibition at 75 µg. The IC_{50} value was more than 100 µg/ml and the regression value was difficult to analyze. The results obtained showed that synthesized gold nanoparticles by flower aqueous extract of *T. divaricata* have anticancer activity. Synthesized gold nanoparticles of *T. divaricata* were tested for cytotoxic activity on MCF-7 cell lines using MTT assay where 75 µg of sample was not sufficient for showing activity against cancer cell line. If we provide 100 µg of gold nanoparticles, it shows good result when compared with 75 µg of sample. Synthesized gold nanoparticles of *T. divaricata* tested for cytotoxicity on MCF-7 cell lines showed moderate anticancer activity (Fig. 6a, 6b). Whereas the antioxidant

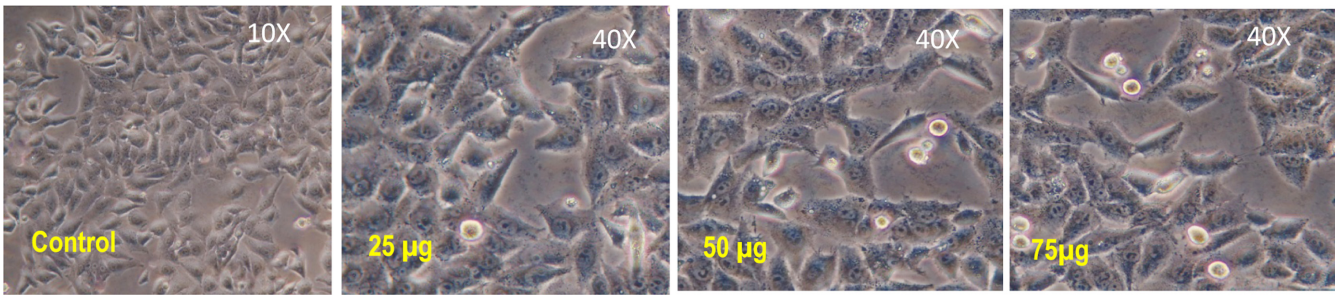

Fig. 5. Microscopic observation of MCF-7 cell line treated with different concentrations of *T. divaricata* flower extract reduced gold nanoparticles and control cells without treatment.

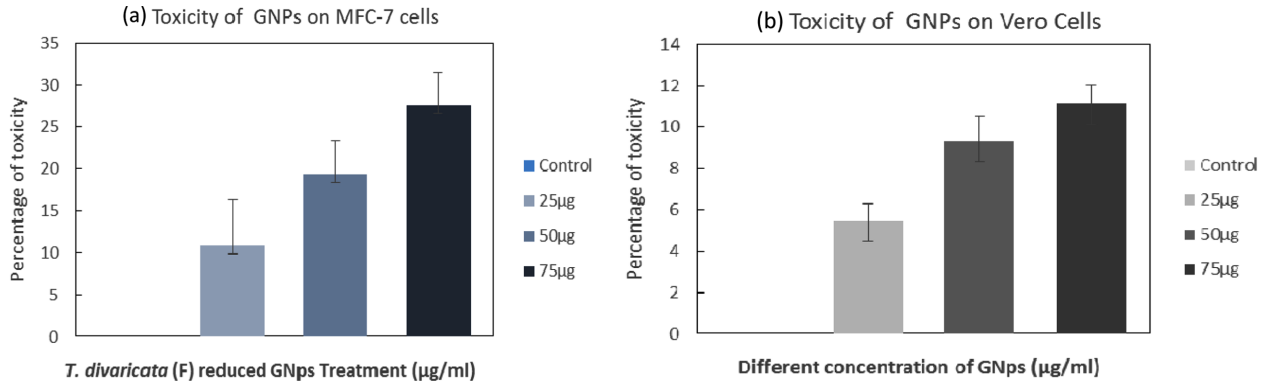

Fig. 6. Graph showing cytotoxicity of *T. divaricata* flower extract reduced gold nanoparticles on (a) MCF-7 cell lines (b) Vero cell lines.

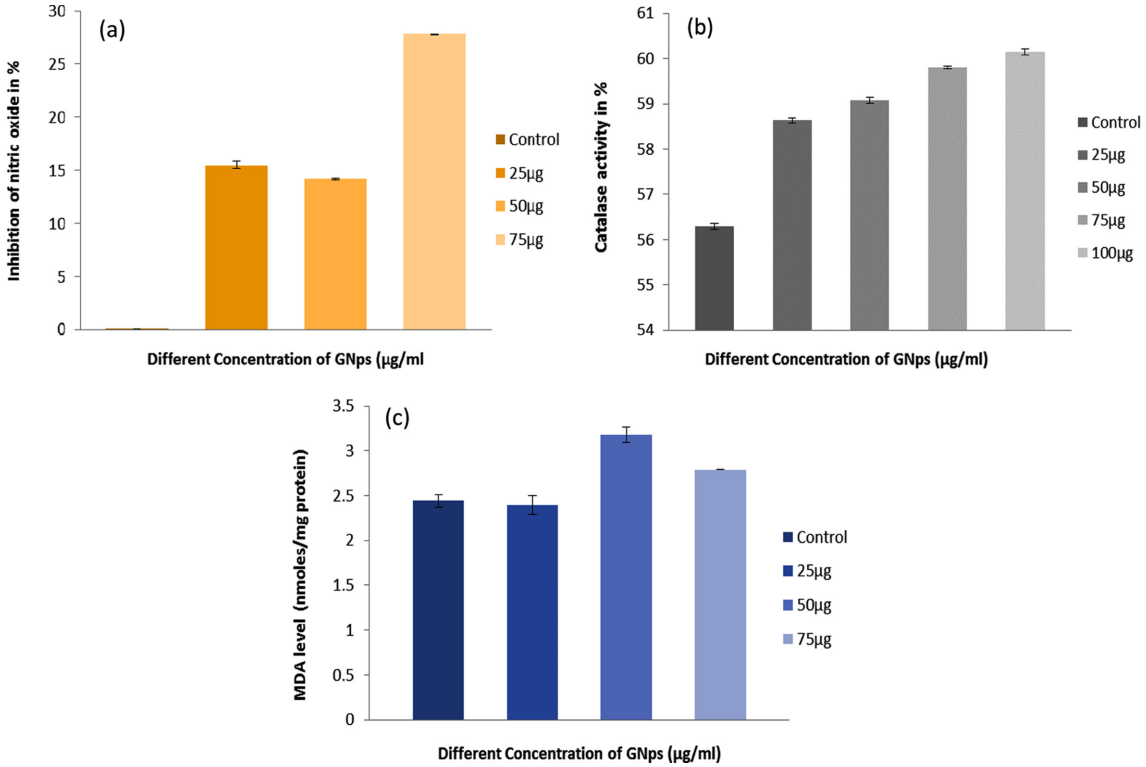

Fig. 7. Effect of *T. divaricata* flower extract reduced gold nanoparticles: (a) nitric oxide inhibition, (b) Catalase activity and (c) lipid peroxidase activity.

activity of the *T. divaricata* reduced gold nanoparticles showed better results with dose dependent manner (Fig. 7a-7c). Poornima and Gopalakrishnan [21] reported that *T. divaricata* has a very good antioxidant and anticancer effect against clear cell renal cell carcinoma induced with DEN and Fe-NTA in male Wistar albino rats, and it can be used for the purpose of anticancer therapy. Hence the results con-

firm that the antioxidant molecule is responsible for both reduction and capping of gold nanoparticles.

4. Conclusion

The fresh flower extract of *T. divaricate* reduced the gold ions and synthesized the nanoparticles. The green synthesized nanoparticles were analyzed by UV-Spectrophotometer at 551 nm. Spherical gold nanoparticles of 100 nm were observed after TEM analysis. Further FT-IR analysis of extract revealed the presence of various biomolecules, which provides stability to gold nanoparticles. The results obtained showed that synthesized gold nanoparticles by flower aqueous extract of *T.divaricata* have anticancer activity on breast cancer MCF-7 cell lines with IC_{50} value of more than 100 μg/ml. The present investigation reveals that the gold nanoparticles synthesized from flower aqueous extract of T. *divaricata* have medicinal applications, especially for anticancer profiling.

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