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Original Article

BIO-PROSPECTING THE *IN-VITRO* ANTIOXIDANT AND ANTI-CANCER ACTIVITIES OF SILVER NANOPARTICLES SYNTHESIZED FROM THE LEAVES OF *SYZYGIUM SAMARANGENSE*

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ABSTRACT

Objective: Green nanotechnology involves the tailoring of nanoparticles through a reliable and eco-friendly process making it suitable for a desired application. The current study is focussed on the biosynthesis of silver nanoparticles (AgNPs) using aqueous extract of *Syzygium samarangense* (Java Apple) leaves and to investigate their total antioxidant capacity (TAC), free radical scavenging activity and the anticancer activity.

Methods: The crude leaf extracts of *S. samarangense* was used to synthesize the AgNPs from 1 mM silver nitrate solution and the formation of AgNPs was confirmed by UV-Visible spectrophotometer, FT-IR and FESEM techniques. The TAC was determined by phosphomolybdenum method whereas the free radical scavenging activity was studied by H_2O_2 method. Cytotoxic activity was performed by MTT assay using the AgNPs against A549 cell lines.

Results: Biosynthesis of AgNPs was visually confirmed by observing the colour change from pale yellow to dark brown. UV-Visible spectral analysis showed silver Surface Plasmon Resonance band at 425 nm and the FT-IR peaks showed the presence of proteins and phenolic groups that are responsible for the stabilization of AgNPs. FESEM image showed the presence of AgNPs that were spherical shaped and poly dispersed. The efficiency of AgNPs as a source of good antioxidant and as a potential free radical scavenger was confirmed from the results of TAC and H_2O_2 assay. Further these nanoparticles showed reduced viability and increased cytotoxicity on A549 cell line in a dose dependent manner.

Conclusion: The present investigation suggests an impressive method for the biological reduction of silver to silver nanoparticles that can be fabricated into many valuable and replaceable therapeutic agents in the treatment of various lethal diseases.

Keywords: Silver nanoparticles, *Syzygium samarangense*, UV-Visible, FT-IR, FESEM, Antioxidant, Cytotoxic activity, MTT assay.

INTRODUCTION

Nanotechnology is one of the gloriously emanating, interdisciplinary areas of research with valuable commercial applications and would be a prevalent technology in the upcoming modern era. Nanotechnology focuses on the synthesis and development of nanometer scaled nanoparticles that are the building blocks of nanotechnology [1]. Until date, metallic nanoparticles are mostly prepared from noble metals such as Ag, Au, Pt and Pd [2].

Metal nanoparticles have been extensively studied because of their unique physical properties, chemical reactivity and potential applications in catalysis, biological labelling, bio-sensing, drug delivery, antibacterial activity, and antiviral activity, detection of genetic disorders, gene therapy and DNA sequencing [3]. Silver (Ag), among the noble metals is the metal of choice in the field of biological systems, living organisms and medicine [4].

The commonly used methods for the synthesis of nanoparticles are either chemical or physical but unfortunately many of these methods have several flaws such as consumption of high energy, expensive and also chemical method of synthesis comprises of carcinogenic chemicals that impart genotoxic effects in medical applications [5]. One of the key issues in nanoscience research is the integration of green chemistry principles to nanotechnology. The three main factors in nanoparticle preparation that should be considered for the development of green chemistry are: the use of an environmentally benign reducing agent, solvent choice and the use of a non-hazardous material for nanoparticle stabilisation [6].

This green chemistry approach towards silver nanoparticle (AgNPs) synthesis has many benefits such as cost effectiveness, ease of scale up, economic viability and eco-friendly nature [7]. In the last few decades, there has been a re awakening of interest in plants and plant derived products as a source of medicine. Certain reports featuring the role of plant constituents in the formation and stabilization of AgNPs have been published [8-10].

Free radicals or Reactive Oxygen Species (ROS) are highly reactive as they can interact with cellular molecules and metabolites leading to cellular damage [11]. Free radicals are recognized as the main products of lipid oxidation generating oxidative stress that plays a major role in the development of over 100 chronic diseases such as autoimmune disorders, aging, cardiovascular, neurodegenerative diseases and most importantly cancer [12, 13].

Cancer is a class of diseases in which a cell or a group of cells exhibit uncontrolled growth, invasion and metastasis [14]. Cancer is considered as one of the major death causes for humans. In 2008, particularly in the economically developing countries, cancer was accountable for 7.6 million deaths worldwide and its incidences are continuously increasing due to aging, growth of the world's population and cancer-causing behaviours [15]. There is an arising demand for anticancer therapies [16]. *In vitro* cytotoxicity testing procedures reduces the use of laboratory animals [17] and hence the use of cultured tissues and cells has increased [18].

The therapy for cancer remains as a challenge regardless of the several modes of therapy such as chemotherapy, immunotherapy, and radiotherapy [19]. The delivery of chemotherapeutic drugs to cancer cells with minimal toxic effects on healthy tissues as well as the sustenance of their antitumor efficacy has been improved through numerous nano-technological approaches [20]. Many attempts have been made to use AgNPs as an anticancer agent and they have all turned up to be positive [21]. The size reduction of nanoparticles plays an important role in improving their bio-availability and compatibility for therapeutical applications in diseases like cancer [22]. Many plants derived products have been reported to show potent antitumor activity against several rodent and human cancer cell lines [23]. The global market for medical nanotechnology is expected to reach more than $3 billion within the next five years [24].

Keeping this in view, we have explored for the first time the *in vitro* free radical scavenging potential, total antioxidant capacity (TAC) and the anticancer activity of green synthesized silver nanoparticles (AgNPs) using aqueous leaves extract of *Syzygium samarangense* (Java Apple) that were characterised by UV-Visible spectrophotometer, FT-IR and FESEM techniques. The present

investigation can open a gateway for an exemplary method of AgNP synthesis from the leaves extract of *Syzygium samarangense* that can be modulated into a promising candidate as therapeutic drugs.

MATERIALS AND METHODS

Chemicals

DMEM medium and fetal bovine serum (FBS) was purchased from Sigma Chemical Co., USA. Trypsin, methyl thiazolyl diphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). Silver nitrate (purity>99.99%) as well as all other reagents and chemicals used in this study was of analytical grade and was obtained from Sigma Aldrich Mumbai.

Collection and maintenance of cell culture

A549 (Lung adeno carcinoma) cell line was obtained from Veterinary College, Vepery, Chennai. Cells were maintained in DMEM medium (Dulbecco's modifications of eugal's medium with L-glutamine & 4.5G/l glucose) supplemented with fetal bovine serum 100 units/ml of penicillin G and 0.1 mg/ml of streptomycin sulphate in a humidified atmosphere of a 5% CO_2 at 37 °C.

Procurement of plant materials

The fresh leaves of *Syzygium samarangense* (Java Apple) (fig. 1) were collected randomly from the areas of Western Ghats and were identified by a botanist from University of Calicut, Kerala, India.

Fig. 1: Photograph of *Syzygium samarangense* (Java Apple) fresh leaves

Preparation of plant extract

The collected leaves of *S. samarangense* were washed thoroughly 2-3 times in running tap water followed by sterile distilled water and were dried in hot air oven at 60 ºC overnight. The dried leaves were grounded well with the help of mortar and pestle and 5g of the powder was mixed with 100 ml of double distilled water (50 mg/ml). This solution was boiled in a water bath at 60 ºC to 80 ºC for one hour. The cooled solution was filtered through Whatmann filter paper no.1 and the filtrate was stored at 4 ºC.

Synthesis of herbal silver nanoparticles

Synthesis of silver nanoparticles was carried out in 250 ml Erlenmeyer flask containing 90 ml of 1 mM silver nitrate ($AgNO_3$) and 10 ml of leaf extract. The solution was kept at dark room at 37 ºC with continuous agitation at 100 rpm for 24-48 hrs for the reduction of Ag+ions. The colour change of the solution from pale yellow to dark brown indicates the synthesis of AgNPs.

Characterization of biosynthesized silver nanoparticles

The reduction of pure Ag+ions to Ag was monitored from the UV-Visible spectrum by taking small aliquots of AgNP solution diluted in distilled water and measuring the spectrum ranging from 200-700 nm using Shimadzu UV-1800 Spectrophotometer. Fourier-transform spectroscopy Perkin Elmer model was used for the analysis of the reduced silver. The spectrum was taken in mid-IR region of 400-4000 cm^{-1}. The sample was mixed with pure KBR crystals in the ratio of 1:100 and the pellet was fixed in the sample holder for the analysis. The surface structure and shape of the particles were analyzed by Field Emission scanning electron microscopic (FESEM) (JEOL JSM-6701F) analysis. The sample was prepared by placing a drop of AgNPs on gold coated copper grid and subsequently air dried, before transferring it to the microscope operated at 120 KV.

Determination of total antioxidant capacity

The total antioxidant capacity (TAC) was evaluated by the phospho molybdenum method [25]. The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml of the AgNP solution at different concentrations (250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml) was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of control, 0.3 ml of water was used in place of AgNP solution. The tubes containing the reaction solution were capped and incubated in a boiling water bath at 95 °C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695 nm using a UV-Vis Spectrophotometer (Shimadzu UV-1800). *S. samarangense* leaves extract and Ascorbic Acid was used as the control. The percentage antioxidant capacity were calculated using the following formula:

% Antioxidant Capacity = 1-Abs (sample)/Abs (control) x100

Where, Abs (control) was the absorbance of the control and Abs (sample) was the absorbance of the solution containing AgNP solution. IC_{50} was calculated using regression analysis in MS excel by plotting the % antioxidant versus different concentrations. Each experiment was carried out in triplicates and the results were recorded as a mean % antioxidant activity±SD.

Determination of free radical scavenging activity by H_2O_2 Assay

The ability of the AgNP solution to scavenge hydrogen peroxide was determined by following the method of Ruch *et al.* [26]. A solution of hydrogen peroxide (H_2O_2) (40 mM) was prepared in phosphate buffer (pH 7.4). 4 ml of the AgNP solution at different concentrations (250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml) was added to 0.6 ml of previously prepared H_2O_2 solution. The absorbance of the solution was measured at 230 nm after 10 min against a blank containing phosphate buffer without H_2O_2 using UV-Vis Spectrophotometer (Shimadzu UV-1800). *S. samarangense* leaves extract and Ascorbic Acid was used as the control. The percentage of H_2O_2 scavenging by the extracts was calculated using the formula:

% scavenging [H_2O_2] = 1-Abs (sample)/Abs (control) x100

Where, Abs (control) was the absorbance of the control at 230 nm and Abs (sample) was the absorbance of the solution containing AgNP solution at 230 nm. IC_{50} was calculated using regression analysis in MS excel by plotting the **%** scavenging versus different concentrations. Each experiment was carried out in triplicates and the results were recorded as a mean % scavenging activity±SD.

Determination of *in vitro* cytotoxic activity

Cytotoxic activity of the biosynthesized silver nanoparticles against A549 cell lines was determined by MTT assay [27]. A549 cells (1×10^5/well) were plated in 24-well plates and incubated in 37 °C with 5% CO_2 condition. The medium was then discarded and cells were incubated with different concentrations of AgNP solution (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) for 24 hours. After the 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 microtitre plate reader. Cyclophosphamide was used as a standard. IC_{50} was calculated using regression analysis in MS excel. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells/A570 of control cells × 100

The % cytotoxicity was calculated using the following formula:

Cytotoxicity % = 100–Viability%

Statistical analysis

Values were expressed as mean±SD Statistical analysis was performed using the statistical software Graph Pad prism 5.0. Statistical difference in meaning was analysed using one way ANOVA. $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Synthesis of herbal silver nanoparticles

Initially, silver nitrate was colourless without the extract that was taken as the control. During the synthesis of silver nanoparticles (AgNPs), it was visually observed that after the addition of the leaf extract resulted in the colour change of the silver nitrate solution from pale yellow to dark brown indicating the synthesis of AgNPs (fig. 2). A report from [28] states that upon addition of silver ions into the filtered cell free filtrate of the sample, changes its colour from almost colourless to brown with intensity increasing during the period of incubation. It is well known that AgNPs produces a yellowish-brown colour in the solution due to excitation of Surface Plasmon Resonance (SPR) vibrations that in turn is due to the presence of free electrons [29].

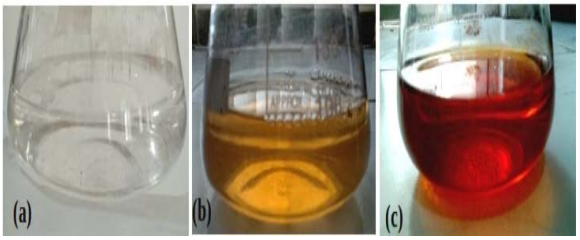

Fig. 2: (a) $AgNO_3$ solution (control) (b) *S. samarangense* (Java Apple) leaf extract (c) Synthesized AgNPs

Characterization of biosynthesized silver nanoparticles

In the UV-Visible spectroscopic analysis, it was observed that the maximum spectrum was obtained at 425 nm (fig. 3) due to the surface plasmon peak of green synthesized AgNPs from the leaves of *S. samarangense* extract. An UV-Visible spectrum is one of the important and easier techniques to verify the formation of metal nanoparticles provided the surface plasmon resonance exists for the metal [30]. It was studied that the frequency and width of surface plasmon absorption depend on the size and shape of nanocolloids in an aqueous suspension [31].

Fourier transform infrared (FTIR) spectroscopy is a chemical analytical technique, which measures infrared intensity versus wavelength (wave number) of light. It is used to determine the nature of associated molecules of plant extracts with nanoparticles. The FT-IR spectrum of the bio reduced AgNPs synthesized from the leaves of *S. samarangense* extracts (fig. 4) shows the peak values at 3411 cm^{-1} is assigned for the O-H stretching of H-bonded alcohols and phenols. The peak value at 2134 cm^{-1} corresponds to carboxylic acids and their derivatives (C=O). The peak value 1641 cm^{-1} corresponds to the N-H bending of primary amines. As plant molecules adsorbed on the surface of the AgNPs, the amide groups intend to form stronger bonds with Ag atoms, which will break most of the H-bonds between the N–H groups and lead to the narrowing and blue-shifts of the amide bond. A literature reports that proteins can bind to AgNPs through free amine groups in the proteins [32]. This result suggests that the biological molecules could possibly be involved in a function for the formation and stabilization of AgNPs in an aqueous solution.

FESEM analysis was used to confirm the size, shape and morphology of the synthesized AgNPs. FESEM provided further insight into the structural and size details of the AgNPs. The FESEM image showed the presence of silver nanoparticles that are predominantly spherical shaped and poly dispersed (fig. 5). Formation of clusters is due to the presence of elevated concentration of bioactive compounds in the colloids, a similar phenomenon has been reported [33].

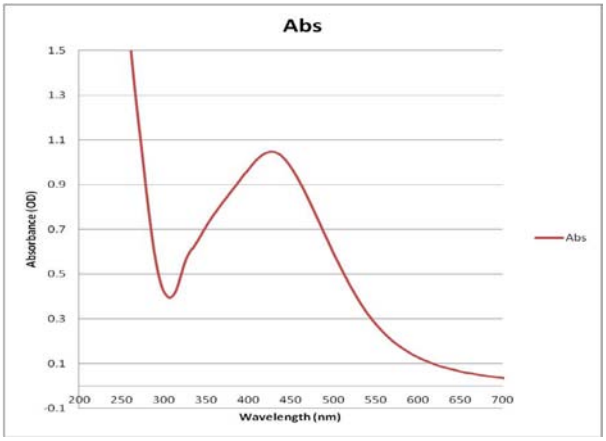

Fig. 3: UV-Visible absorption spectrum of AgNPs synthesized from *S. samarangense* leaf extract

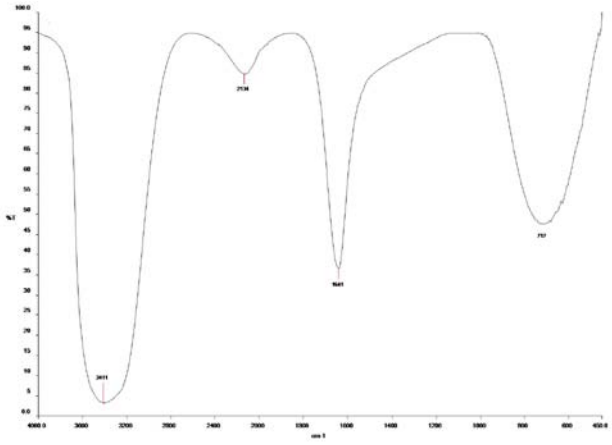

Fig. 4: FT-IR spectrum of biosynthesized AgNPs from *S. samarangense* leaf extract

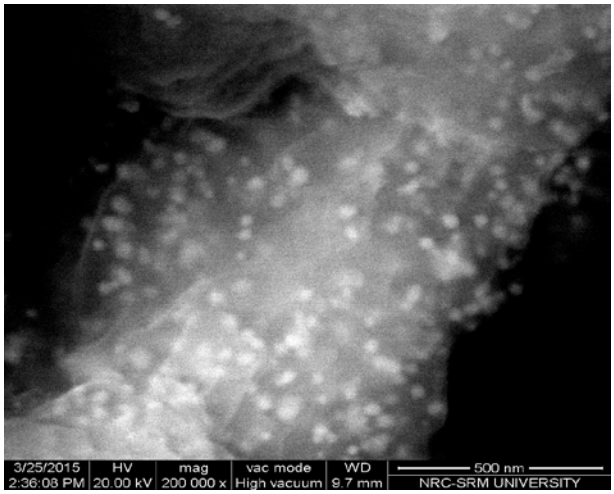

Fig. 5: FESEM showing spherical AgNPs synthesized from *S. samarangense* aqueous leaf extract

Determination of total antioxidant capacity

The total antioxidant capacity (TAC) was based on the reduction of Mo (VI) to Mo (V) by the different extracts and subsequent formation of green phosphate/Mo(V) complex at acid pH. It

evaluates both water-soluble and fat-soluble antioxidants (total antioxidant capacity). It was revealed that the antioxidant capacity of the AgNPs, plant extract and the ascorbic acid standard showed a concentration depended effect. The observed % antioxidant effect of AgNPs and the standards on the total antioxidant capacity decreases in the following order: AgNPs>Ascorbic acid>Plant extracts. The IC_{50} value was calculated for all the three extracts and was found to be 83.26±0.72µg/ml for AgNPs that was lesser than ascorbic acid (table 1) indicating that AgNPs are good antioxidant source. Antioxidant capacity of ascorbic acid has been used as a reference standard from which plant extracts with potential antioxidant activity are compared [34].

Table 1: Total antioxidant capacity of AgNPs showing % Antioxidant capacity at various concentrations

Concentration (µg/ml)	% Antioxidant activity		
	Synthesized AgNPs	*S. samarangense* Leaves extract	Ascorbic Acid
250	67.38±1.05	63.55±0.94	65.72±0.43
500	73.46±0.87	67.92±0.53	73.98±1.40
750	80.44±1.07	74.62±0.77	77.30±1.11
1000	88.10±0.85	83.19±0.98	85.25±1.07
IC_{50} (µg/ml)	83.26±0.72	102.46±0.73	88.29±0.71

Values are in mean±SD of three replicates (n=3)

Determination of free radical Scavenging Activity by H_2O_2 assay

In hydrogen peroxide free radical scavenging assay, it was revealed that free radical scavenging activity of the extract is in the increasing trend with the increasing concentration of the plant extract, ascorbic acid and AgNPs. The observed % scavenging activity of the three extracts was found to be decreasing in the following order: Plant extract>AgNPs>Ascorbic acid. The IC_{50} value for the green synthesized AgNPs was 12.58±0.93µg/ml (table 2). Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+}ions to form hydroxyl radical and this may be the origin of many of its toxic effects [35]. It can also be observed that the % scavenging activity of *S. samarangense* (Myrtaceae family) leaves extract showed the highest activity that could be due to the presence of various quercetin glycosides.

Table 2: H_2O_2 free radical scavenging activity of AgNPs showing % scavenging at various concentrations

Concentration (µg/ml)	% Free radical scavenging activity		
	Synthesized AgNPs	*S. samarangense* leaves extract	Ascorbic acid
250	78.13±0.49	80.88±1.05	70.99±0.89
500	81.67±0.88	86.65±0.82	72.84±0.87
750	87.11±0.36	89.09±0.99	79.08±0.96
1000	92.13±0.86	93.34±0.80	81.89±0.98
IC_{50} (µg/ml)	12.58±0.93	11.85±1.04	27.88±0.30

Values are in mean±SD of three replicates (n=3)

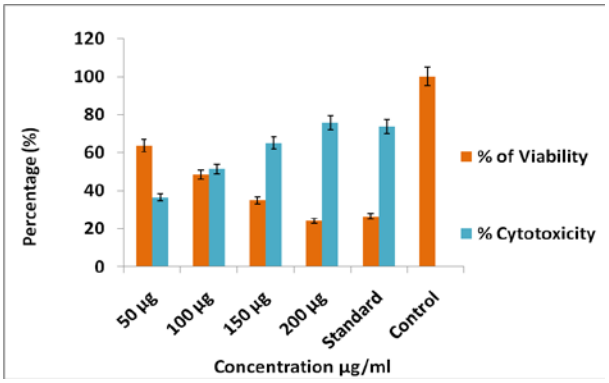

Fig. 6: Cell viability and cytotoxicity in A549 cells induced by exposure to different concentration of AgNPs determined by MTT assay

Determination of *in vitro* cytotoxic activity

The cytotoxicity of the biosynthesized AgNPs was studied against the A549 cell lines by MTT assay. The cytotoxicity effect of synthesized silver nanoparticle was evaluated at different concentration (50µg, 100µg, 150µg and 200µg/ml). In this analysis, the % viability as well as the % cytotoxicity of AgNPs showed a direct dose-response relationship; cytotoxicity increased at higher concentrations (fig. 6). The IC_{50} value calculated for the green synthesized AgNPs is 87.37µg/ml. The cytopathic effect of AgNPs on A549 cells was analyzed using an optical microscope (fig. 7). Untreated cells appeared elongated, attached smoothly on the culture surface. Some cells were grouped together to form colonies. Following treatments with AgNPs for 24 hrs, the cells became rounded and lost cell contacts. It was found that the % viability of AgNPs at 200µg was 24.22% and for cyclophosphamide (standard) was 26.48%, suggesting that increasing concentration of the synthesized AgNPs>200µg could be effective than the standard drug.

The cytotoxic effect of silver can be explained as a result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins as well as with the nitrogen bases and phosphate groups in DNA [36]. Another report demonstrated that AgNPs serve as antitumor agents by decreasing progressive development of tumour cells. They suggested that AgNPs can induce cytotoxic effects on DLA cells, inhibiting tumour progression and thereby effectively controlling disease progression without toxicity to normal cells [37]. Zolghadri and co-workers demonstrated that SNPs provide a relatively high hydrophobicity inside bovine haemoglobin which causes a transition from alpha helixes to beta sheets and leads to partial unfolding and aggregation of the protein [38].

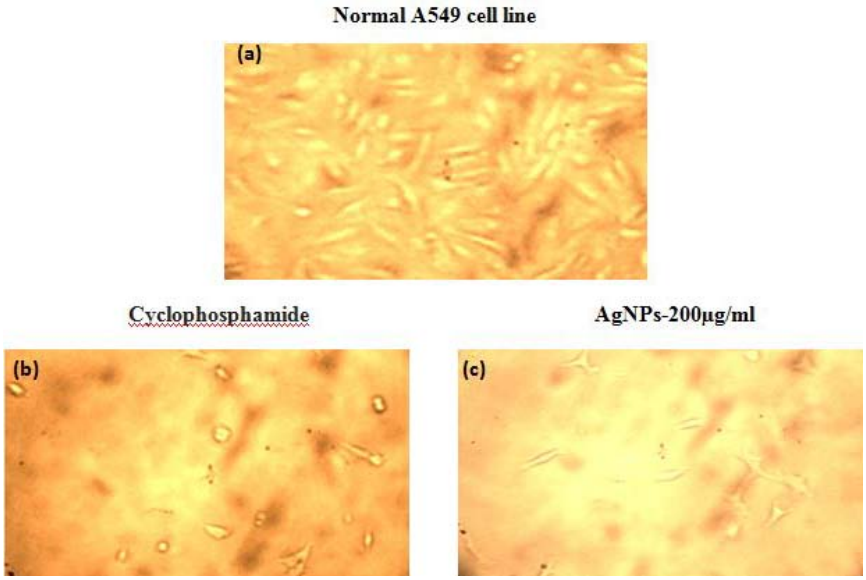

Fig. 7: Microscopic examination of morphological changes in A549 cells (a) Control (b) treated with Cyclophosphamide (c) treated with biosynthesised AgNPs

CONCLUSION

To windup, the present study reports a simple, cost effective and efficient synthesis of silver nanoparticles from the leaves of *S. samarangense* (Java Apple) through the integration of green chemistry. Confirmation from the visual property as well as from the characterization with UV–Visible spectrophotometer, Fourier transmission infrared (FT-IR) and Field Emission scanning electron microscopic (FESEM) provides a primary evidence for the formation of AgNPs. Results from the *in vitro* Total Antioxidant Capacity and Free Radical Scavenging by H_2O_2 method revealed the efficiency of AgNPs as a source of good antioxidant and to combat the free radicals that are the potent cause of oxidative stress. This investigation also explored the potential anticancer activity of green synthesized AgNPs against A549 tumour cell lines using MTT assay. Our results suggest that with the aid of metal based nanoparticles and through the better understanding of certain molecular mechanisms, there leads to a expansive range of applications in future such as targeted drug delivery to the tumour cells. Thus, an *in vivo* analysis of the exact mechanism through which AgNPs inhibit signalling cascades responsible for the development and progression of the disease would be an astounding progress in the field of nanomedicine and fabricate these agents as an effective alternative to anti-cancer drugs.

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CONFLICT OF INTERESTS

Declared None

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