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Fabrication of nano-silver particles using *Cymodocea serrulata* and its cytotoxicity effect against human lung cancer A549 cells line

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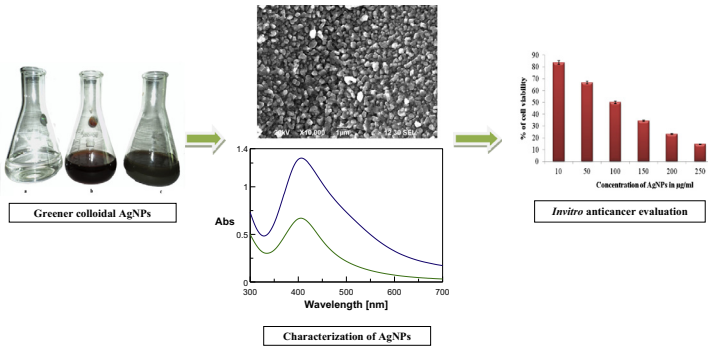
HIGHLIGHTS

- Greener colloidal silver nanoparticles (AgNPs) synthesis using *Cymodocea serrulata* as a potential marine bioresource.
- Physio-chemical characterization using UV–Visible spectrometer, SEM, TEM, XRD, DLS and FTIR.
- *In vitro* anticancer evaluation of AgNPs against human lung cancer A549 cells lines.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study reports, green synthesis of bioactive silver nanoparticles (AgNPs) under different temperature (60 °C, room temperature and 4° refrigerator) using the aqueous extract of sea grass *Cymodocea serrulata* as a potential bioreductant. Increased temperature fabricates more AgNPs compare to room temperature and refrigerator condition. At first the reduction of Ag^+ ions were confirmed through color change which produces an absorbance spectra at 420 nm in UV–Visible spectrophotometer. Additionally various exclusive instrumentations such as X-ray diffraction (XRD), Dynamic light scattering (DLS), scanning electron microscope (SEM) analysis and Transmission electron microscope (TEM) were authorizes the biosynthesis and physio-chemical characterization of AgNPs. From Fourier transform infrared spectroscopy (FTIR) analysis, it was identified that the water soluble fractions of the sea grass mainly responsible for reduction of ionic silver (Ag^+) into (Ag^0) nano-ranged particles and also they act as stabilizing agent to sustain the durability of NPs for long period of time. Further, synthesized AgNPs shows potential cytotoxicity against human lung cancer A549 cells (LD50–100 µg/ml). The overall results suggest that *C. serrulata* is a valuable bioresource to generate rapid and eco-friendly bioactive AgNPs towards cancer therapy.

Introduction

Nanobiotechnology, an emerging trend in material sciences which provides new improved nanoscale structures with different physio-chemical properties for various therapeutic applications

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[1]. Chemical and physical means of nanoparticle synthesis were commonly followed by researchers worldwide. However, physical methods are highly expensive and chemical methods involve in the use hazardous chemicals which cause harmful effects to the environment and its associated life forms [2,3]. Therefore, it is essential to develop reproducible environmentally benign green chemistry route to synthesis metal nanoparticle for their potential applications in nanomedicine [4]. Diverse biological entities such as plant extracts, algae, diatoms, heterotrophic human cell lines, biocompatible agents and microbial fractions have been exploited in the facile, rapid and cost effective metal nanoparticle synthesis [5]. Compared with all other natural products plant system is gaining much more interest because it does not require any elaborate process like multiple purification, maintenance of cell cultures and most importantly it is easy to scaled up for large scale synthesis and commercialization [6,7].

To synthesis of metal nanoparticles like silver, gold, copper, platinum, palladium, zinc and iron biogenic route will be preferred than chemical and physical methods due to their increased compatibility with maximum yield in shorter period of time [8]. Among different metals, silver nanoparticles (AgNPs) with a size range of <100 nm known as promising nanoproduct in many applications from cosmetics to therapeutics [9]. AgNPs are reported to possess anti-fungal, anti-inflammatory, anti-viral, anti-angiogenesis, anti-platelet activity and an effective antimicrobial agent against various pathogenic microorganisms [10]. Apart from its antimicrobial property, now a day's biogenic AgNPs have been explored in the field of nano-oncology to recognize its capability for the expansion of cancer treatment [11].

Organisms present in the earth producing number metabolites to protect and withstand themselves in different ecological conditions. These chemical constituents have enormous significance for the surviving humanity in various manners. Interestingly, marine organisms produce novel chemicals with unique structural and functional features to withstand extreme variations in pressure, salinity and temperature [12]. It is very obvious that most of the bioactive compounds isolated from marine flora shown to be a potential precursors in the development of innovative and improved drugs for wide range of biological activities. However, many of these compounds are considered to be stupendous anticancer drugs that commonly inhibit the progression of cancer cell proliferation via apoptosis and inhibition of cell cycle [13]. Generally, seagrasses are the marine flowering plants that widely distributed in the marine environment. A variety of bioactive principles have been identified and several drugs have been developed from seagrasses and its by-products. *Cymodocea serrulata* belongs to *Cymodoceaceae* family mostly used in folk medicine for malaria, cough and stomach troubles. Moreover it is a perennial seagrass with male and female plants which have commonly existed throughout the tropical Indo–West Pacific regions. It possesses stupendous biological activities like antimicrobial, antioxidant, cytotoxicity and antifouling activities [14–16]. In this article, we report the green synthesis of colloidal AgNPs using the aqueous leaf extract of *C. serrulata* as a reducing agent. Further the cytotoxicity effect of synthesized AgNPs was examined against the human lung cancer cell line towards the development of new nano-drug formulations for cancer treatment.

Experimental

Chemicals

Silver nitrate ($AgNO_3$), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Penicillin, Streptomycin, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Himedia (Mumbai, India).

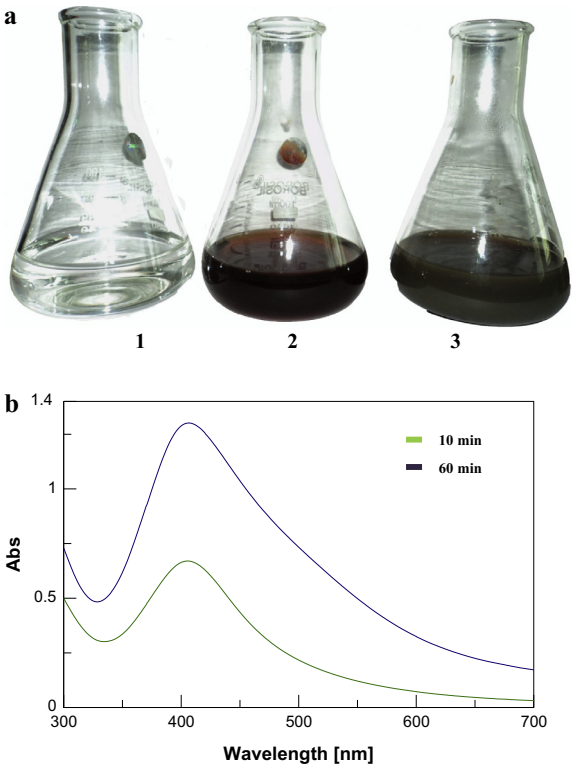

Fig. 1. (a) Formation of dark brown color indicates the generation of AgNPs (1) silver nitrate ($AgNO_3$), (2) leaf extract of *C. serrulata* and (3) synthesized AgNPs. (b) UV–Visible spectroscopy shows intense absorbance peak at 420 for synthesized AgNPs at 60 °C.

Preparation of extract

Fresh samples of *C. serrulata* were collected from Mimisal, Pudukkottai district southeast coast of Tamilnadu, India. Collected sea grass were surface sterilized thoroughly with distilled water to remove the contaminant and shade dried for 10 days, after that the dried leaves were grounded into fine powder using kitchen blender. For synthesis of silver nanoparticles, 10 g leaf powder was taken and mixed with 100 ml of distilled water and kept in boiling water bath at 60 °C for 20 min. After cooling, the extracts were filtered with Whatman filter paper No. 1 and stored in refrigerator at 4 °C for further studies [17].

Synthesis of silver nanoparticles

The aqueous solution of 1 mM silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. To attain efficient synthesis 5 ml of *C. serrulata* extract was added into 95 ml of aqueous solution of 1 mM silver nitrate and kept in different temperatures such room temperature, 60 °C and refrigerator at 4 °C. The reaction mixtures were monitored spectrophotometrically at every 20 min interval for 0–60 min. Generation of AgNPs was observed by change in the color of reaction mixture at different conditions.

Physio-chemical characterization of AgNPs

The reduction of pure Ag^+ ions were measured spectrophotometrically, for that small aliquots of the samples was diluted with distilled water and absorption maxima was scanned using JASCO V-650 UV–Visible spectrophotometer at the wavelength of 300–700 nm. Further, synthesized AgNPs were purified by

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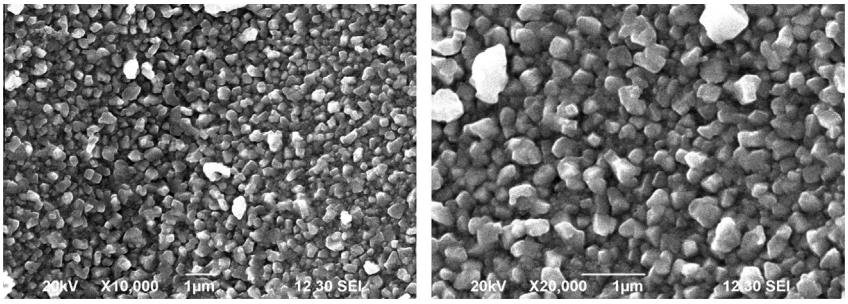

Fig. 2. Scanning electron microscopic image shows the polydispersed AgNPs with the mean size of below 100 nm.

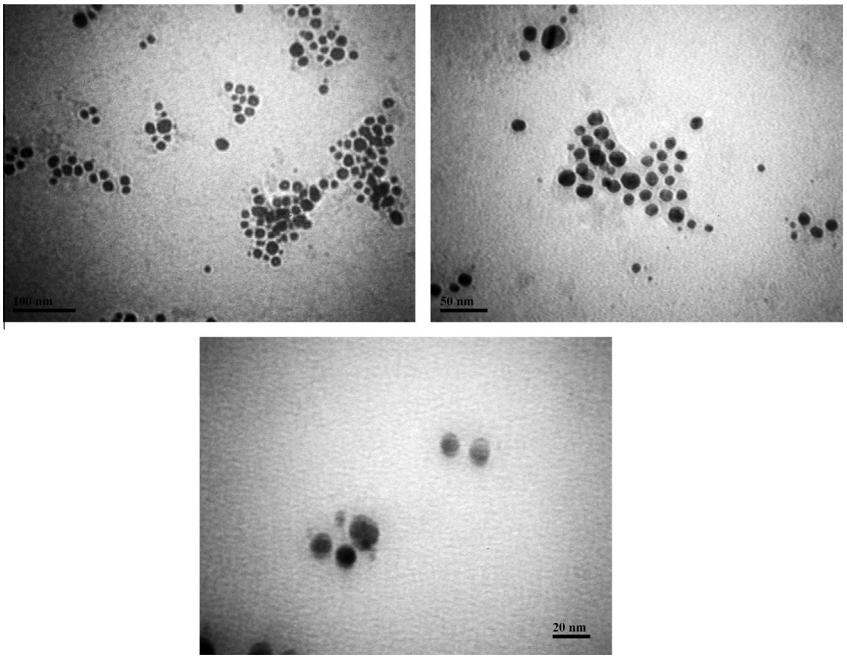

Fig. 3. TEM micrograph showing the morphometric features of synthesized AgNPs. The size range of the particles was found to be 5–25 nm.

repeated centrifugation to remove silver ions and other unbound debris present in the extract, after separation the pellet was mixed distilled water and freeze dried and used for further analysis. Mean particle size and morphology of synthesized AgNPs was measured by the Jeol JSM-6480 LV SEM machine. For TEM analysis, briefly the colloidal AgNPs were separated and allowed for sonication, a drop of this solution was used to make a thin layer on the copper coated grid and dried. The morphometric features of the AgNPs were measured at different magnification using PHILIPSTECNAI10 transmission electron microscope. To carry out XRD the sample was drop-coated onto an aluminum plate by just dropping a small amount of sample on the plate frequently, allowed to dry and finally a thick coat of sample was prepared. The XRD measurement was performed on a Shimazdu, model LabX-XRD-6000 instrument operated at a voltage of 20–30 keV and a current of 30 mA with Cu Kα radiation with a wavelength of 1.5418 Å. From DLS (Zetasizer 6.20, Malvern) it is possible to determine the size distribution and stability of colloidal AgNPs moving in the reaction medium. FTIR analysis was carried to study the surface chemistry of AgNPs and to know the responsible plant biocompound for stabilization process. To perform FTIR analysis sample was mixed with KBr powder and pelletized after drying the spectra were recorded using Perkin Elmer make model spectrum RX1 (wavelength range between 4000 cm^{-1} and 400 cm^{-1}). Concentration of synthesized AgNPs was measured with inductively coupled plasma atomic emission spectroscopy (ICP–OES Perkin Elmer Optima 5300 DV model).

In vitro anticancer studies of synthesized AgNPs

Cell culture

Human lung cancer cell line (A549) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin and incubated at 37 °C in 5% CO_2 atmosphere.

MTT assay

In MTT assay the A549 cells were exposed (1×10^4) in 96-well plate and incubated for 24 h at 37 °C a humidified atmosphere of 95% air and 5% CO_2 [18]. Subsequently, the green synthesized AgNPs were treated with various concentrations (10–250 μg/ml) against A549 lung cancer cells, control cells were treated with DMSO. After 36 h incubation, 20 μl of MTT (dimethyl thiazolytetrazolium bromide) solution were added to each well and incubated for 4 h and then 200 μl of DMSO were added. The absorbance was measured at 570 nm using Elisa reader.

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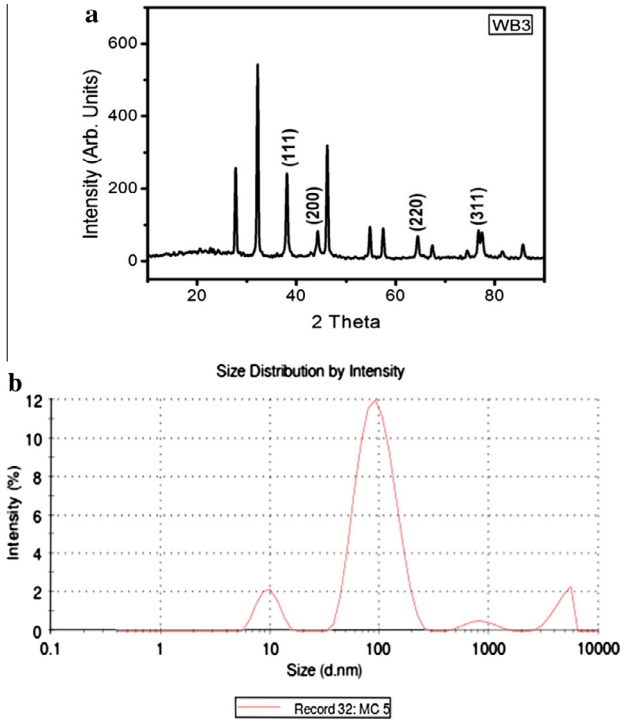

Fig. 4. (a) XRD pattern of the AgNPs produce planes at (111) and (200) corresponds to face centered cubic crystalline AgNPs. (b) DLS showing the size distribution intensity of synthesized AgNPs.

Statistical analysis

The data were subjected to one-way Analysis of Variance (ANOVA) was done for the expressing significance of the present study. Statistical significance was accepted at a level of $p < 0.05$. The statistical analysis was done using SPSS software Version 16 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Several approaches have been employed to obtain a better synthesis of AgNPs such as chemical and biological methods. Recent reports on biological synthesis of AgNPs revealed nonobiotechnological potential of several pharmacologically important plant materials that have been successfully explored for metal nanoparticle synthesis. Several products from the biological origins were useful surviving humanity and its associated life in various aspects like drug development and nutritional adequate. Particularly plant is the warehouse several bioactive compounds, the interaction between plant biochemical and inorganic nanoparticles tend to be hopeful area in nanoscience and technology [19].

Synthesis of silver nanoparticles

In this present study, the biochemical principles of seagrass *C. serrulata* such as protein, tannin, sterol, flavonoid and alkaloids have been screened for its potential to synthesis of bioactive AgNPs in short reaction time. Various bio active metabolites present in the leaf extract reduces ionic silver (Ag^+) into nanoscale, it was confirmed with the formation of yellowish brown[1] color (Fig. 1a) mainly due to the excitation of Surface plasmanoic vibrations [20]. No color change was observed 1 mM $AgNO_3$ solution (control) without seagrass extract. Our data highly correlate with the result of [21] where synthesized AgNPs using leaf and fruit extracts of *C. guianensis* Aubl. produces yellowish brown color at different reaction temperature. The UV–Vis spectra of AgNPs synthesized by *C. serrulata* are shown in (Fig. 1b) as the incubation time get increased the synthesis rate of AgNPs is also get increased. Temperature plays crucial role in the synthesis process, synthesized AgNPs at water bath temperature (60 °C) produces intense SPR spectra at 420 nm whereas a broad spectrum was noticed for AgNPs synthesized in room temperature (28 ± 2) and refrigerator (4 °C) conditions. In an earlier study [17] it was proved that increasing the temperature release more silver ions that results high yield of AgNPs, but the temperature does not have any effect on the plant biocompound present in surface of generated AgNPs.

Physio-chemical characterization of silver nanoparticles

Aggregated spherical AgNPs with the size of below 100 nm were noticed in scanning electron microscope (Fig. 2). Similar phenomenon has been reported by [22] where the synthesized AgNPs using the leaf extract of *Crataegus douglasii* fruit shown spherical particles with 29.28 nm size. TEM micrographs at different magnification shows the spherical AgNPs with a thin layer of biocompound coating on the surface, size range between 5 and 25 nm (Fig. 3). In TEM micrographs synthesized AgNPs were found polydispersed in nature, the presence of stabilizing agent prevents the aggregation [23]. The XRD peaks at 38.04°, 44.19°, 64.1° and 77.09° correspond to the (111), (200), (220) and (311) planes are observed which may be indexed as the band for face centered cubic (fcc) structure of silver (Fig. 4a). The XRD pattern thus clearly illustrates that the AgNPs synthesized by the present green method are crystalline in nature [24] with the average size of 15 nm. From DLS the average mean size of AgNPs predicted to be 73.24 nm (Fig. 4b) denotes the stability of AgNPs [25]. Involvement of stable bio compound in relation to nanoparticles synthesis has been identified with the transmittance vibrations of FTIR. Dried *C. serrulata* showed the transmittance at different wavenumbers such as 3423 cm^{-1}, 2076 cm^{-1}, 1637 cm^{-1} and 697 cm^{-1}, which corresponds to the N—H stretching vibration, SH structure (organo silicon compounds), C═C structure (Alkanes) and C═S (stretching vibration sulfides) respectively (Fig. 5a). Synthesized AgNPs at 60 °C also produces the transmittance at 3428 cm^{-1}, 2077 cm^{-1}, 1637 cm^{-1}, 1371 cm^{-1}, 1231 cm^{-1}, 1108 cm^{-1}, 684 cm^{-1} (Fig. 5b). FTIR results of both aqueous extract and synthesized AgNPs displays variation in the transmittance due to the interaction of biomolecules with AgNPs, the same result shows the presence of phenolic compounds such as flavonoids, tri terphenoids present in the present in the leaf extract which may possibly influence the reduction and stabilization of silver nanoscale particles [26]. Based on the ICP–OES results the concentration of AgNPs was quantified to be 51 mg/L.

Cytotoxicity studies

The synthesized AgNPs demonstrated a considerable cytotoxicity against human lung cancer A549 cells lines at different concentration (50–250 μg/ml). In this report, there was a direct dose–response relationship with tested cells at higher concentrations. More than 80% of cell death was observed in 250 μg/ml concentrations of AgNPs. Hence, the presence of 100 μg/ml of colloids AgNPs is sufficient to inhibit the 50% of A549 lung cancer cells (Fig. 6). Recently cytotoxicity effect of biologically synthesized AgNPs has been reported against different cancer cell lines such as human cervical carcinoma (HeLa) [27], human breast cancer (MCF-7) [28], human lung cancer (A549) [29], human Epithelium cells of liver cancer (HEP G2) [30] and human acute promyelocytic leukemia (HL-60) cell lines [31]. In this report, we found that

[1] For interpretation of color in 'Fig. 1', the reader is referred to the web version of this article.

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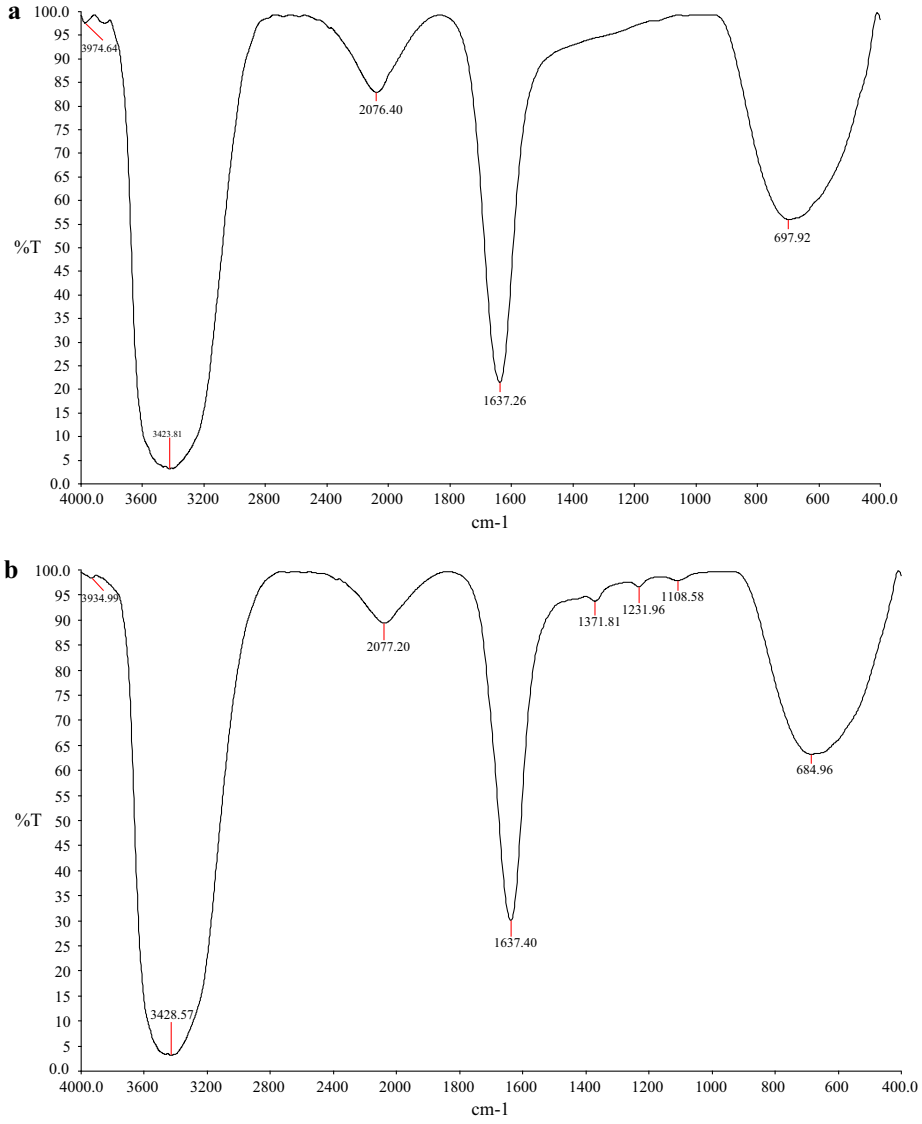

Fig. 5. FTIR transmittance of (a) leaf extract of *C. serrulata* and (b) synthesized AgNPs.

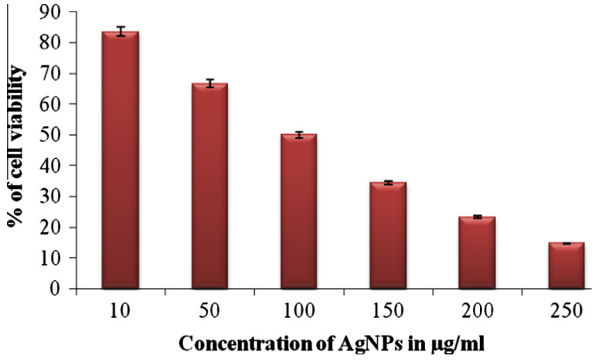

Fig. 6. Cytotoxicity effect of synthesized AgNPs shows 100% mortality at 250 µg/ml with the IC-50 value of 100 µg/ml.

100 µg/ml of green synthesized colloidal silver nanoparticles inhibits more than 50% of lung cancer cells. The improved cytotoxicity is mainly due to its easy permeability to the cellular barriers and its strong affinity towards biological macromolecules, in addition it release reactive oxygen species that cause damages to cellular components via intracellular oxidative stress [32]. Cell viability assay clearly explains the cellular response to a toxicant, it clearly implicates that nanoparticles induced cytotoxicity was absolutely based on their size, shape and surface chemistry [33]. NPs induced cellular toxicity was begin with the cellular uptake process through clathrin-dependent endocytosis and macropinocytosis [34]. Recently, it was reported [27] that synthesized AgNPs using *Podophyllum hexandrum* leaf extract triggers cellular toxicity in treated HeLa cells. Interestingly, they have noticed that synthesized AgNPs alters the expression of various apoptotic regulators which results in mitochondrial dysfunction and cell death. The overall results suggested that engineered colloidal AgNPs using *C. serrulata* leaf extract as a nanofactory system shown immense anticancer property to human lung cancer (A549) and can display potential application in cancer therapy. Further studies are needed to reveal the exact molecular mechanism involved in the cytotoxicity effect of biogenic nanoparticles.

Conclusion

To sum up, in the present investigation we successfully developed a greener route to synthesis colloid AgNPs through exploiting

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medicinally important sea grass *C. serrulata* as a potential renewable bioresource. Synthesized nanomaterials have shown improved physio-chemical properties like size, shape and surface chemistry. Phenolic bioactive principles of *C. serrulata* actively involves in the reduction and stabilization of colloidal AgNPs. Moreover, the colloidal AgNPs have showed promising *in vitro* cytotoxicity potential against human lung cancer A549 cell lines, this will be helpful to find an alternative avenue for human cancer therapy.

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