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Green Synthesis of Silver Nanoparticles Using *Piper nigrum* Concoction and its Anticancer Activity against MCF-7 and Hep-2 Cell Lines

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Abstract

In the present report, silver nanoparticles were synthesized using *Piper nigrum* extract for *in vitro* cytotoxicity efficacy against MCF-7 and Hep-2 cells. The silver nanoparticles (AgNPs) were formed within 20 minutes and preliminarily confirmed by UV-Visible spectroscopy. Further, it was characterized by FT-IR and HR-TEM. The UV-Visible analysis showed the strong broad peak located at 441 nm observed for Ag nanoparticles. TEM images of biosynthesized AgNPs pre-dominantly spherical shape with particle size in the range 20 nm. MTT assays were carried out using various concentrations of silver nanoparticles and *Piper nigrum* extract ranging from 10 to 100µg/ml. At various concentrations, biosynthesized silver nanoparticles showed a significant cytotoxic effect against both MCF-7 and Hep-2 cells compared to *Piper nigrum* extract. Therefore, the results reveal excellent applications of green synthesis of silver nanoparticles using *Piper nigrum*.

Keywords: *Piper nigrum*; Silver nanoparticles; UV-Visible spectroscopy; FTIR; HR-TEM; Anticancer activity

Introduction

Recently, metals nanoparticles have received considerable attention of researchers due to their wide unique properties as compared to bulk and possess immense applications in the fields of diagnostics, cell labelings, antimicrobial agents, drug delivery and cancer therapy [1]. Silver nanoparticles have established substantial consideration for different reasons such as valuable antimicrobial agent, reveals low toxicity etc. [2,3]. Plant mediated synthesis is gaining cost effective, economic, eco-friendly and aiding to scale up synthesis of nanoparticles. Biosynthetic processes are an effective way to prepare silver nanoparticles using plants or their extract in a restricted approach due to their dispersion, size and shape [4]. The plant based nanoparticles has enhanced the opportunity of using beneficial nanoparticles in the diagnosis and treatment of human cancers [5].

Piper nigrum (black pepper) is a spicy plant in which the whole plant is medicinally high in nutritional and therapeutic compounds. It possesses many medicinal properties such as antipyretic, anti-inflammatory, analgesic, and antimicrobial properties. Here, in the present study the concoction of *Piper nigrum* was employed to synthesize the silver nanoparticles and characterized by Fourier transform infrared spectroscopy (FTIR), UV-Visible spectroscopy, High resolution transmission electron microscopy (HR-TEM), further for the first time the anticancer activity against MCF-7 and Hep-2 cell lines were carried out.

Materials and Methods

Materials

Silver nitrate ($AgNO_3$) and MTT were purchased from Hi Media Laboratories Pvt. Ltd. India. The MCF-7 and Hep-2 cancer cell line was purchased from King Institute of Preventive Medicine and Research, Chennai, India.

Preparation of the *Piper nigrum* hot extract

Piper nigrum (fruit) were collected from the local market and authenticated. The *Piper nigrum* (fruit) was finely powdered using morter and pestle. The powder (20 g) was dissolved in 100 ml of millipore water and the mixture was boiled at 80°C for 10 min and then filtered through syringe filter (0.45 µ) [6,7].

Synthesis of silver nanoparticles

Piper nigrum concoction (10 ml) was added to 90 ml of 1 mM silver nitrate solution for the reduction of Ag^+ ions. Various temperatures such as RT, 40, 60 and 80°C were maintained using water bath to optimize the synthesis. The solution stirred at 1000 rpm for 10 minutes [8]. The color modulation was observed at various temperatures to ensure the formation of silver nanoparticles. The *Piper nigrum* seed concoction employed as a reducing and stabilizing agent for 1mM of silver nitrate [9,10].

Purification of biosynthesized silver nanoparticles

To remove the excess silver ions, the silver colloids were centrifuged at 10,000 rpm for 15 minutes and washed three times with millipore water. A dried powder of silver nanoparticles was obtained by freeze-drying in Alpha Christ 2.0 lyophiliser for further characterization.

Characterization of silver nanoparticles

The preliminary characterization of silver nanoparticles was carried out using UV-visible spectroscopy [11,12]. UV–Vis spectroscopy analysis was done using nanodrop 2000r in a scanning range of 200 nm to 800 nm. Millipore water was used as a blank. The interactions between protein-silver nanoparticles were analyzed by Fourier transform infrared spectroscopy (FTIR) in the range of 4000 to 400 cm-1 [11]. The TEM images of biosynthesized AgNPs were obtained for size and shape determination using libra 200 HR-TEM (m/s Carl Zeiss, Germany) operated at an accelerating voltage 120 kV and 200 kV. The AgNPs sonicated for 5 minutes and a drop of diluted sample placed onto the carbon-coated copper grid. The liquid fraction was allowed to evaporate at room temperature [13].

Cell lines and culture

Breast cancer cells (MCF-7), and Human Larynx Carcinoma cancer (Hep-2) cell lines were purchased from King Institute of Preventive Medicine and Research, ICMR, Chennai, India. It was cultured in Dulbecco's modified Eagle's medium (DMEM: Hi media Laboratories Mumbai, India), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hi Media Laboratories Mumbai, India). The cell lines were maintained at 5% CO_2 in CO_2 incubator [14]. Cultures were examined using an inverted microscope to evaluate the quality of confluency and confirming the absence of bacterial and fungal contaminants [15].

MTT assay

To determine the cytotoxic effect of silver nanoparticles and *Piper nigrum* extract, cell viability study was carried out with the MTT reduction assay. MCF-7 and Hep-2 cells were seeded in a 96-well plate at the density of 5×10^3 cells/well. The cells were allowed to attach and grown in 96-well plate for 24 h, in 200 µl of DMEM with 10% FBS [16]. After that the media were removed and replaced with the suspension of various concentrations of silver nanoparticles 10 to 100 mg/ml (minimum 4 wells were seeded with each concentration) and the cells were incubated for 48 h [17]. After the addition of MTT (10 ml, 5 mg/ml), the cells were incubated at 37°C for another 4 h. The medium was then removed, and 200 µl of DMSO added to each well. Optical density (OD) of the formazan product was read at 620 nm using multi well spectrophotometer [15,18]. The results were given as mean of four independent experiments. The OD value was subjected to sort-out percentage of viability by using the following formula,

Percentage of cell viability=

Statistical analysis

The grouped data were statistically evaluated using GRAPHPAD PRISM 6 software. Values are presented as the mean ± SD of the four replicates of each experiment.

Results and Discussion

Silver nitrate solution (Figure 1a) is colourless and after adding *Piper nigrum* plant extract to silver nitrate solution, the colourless silver nitrate solution became dark red in colour (Figure 1b). This confirms that the silver nitrate was reduced and transformed into silver nanoparticles.

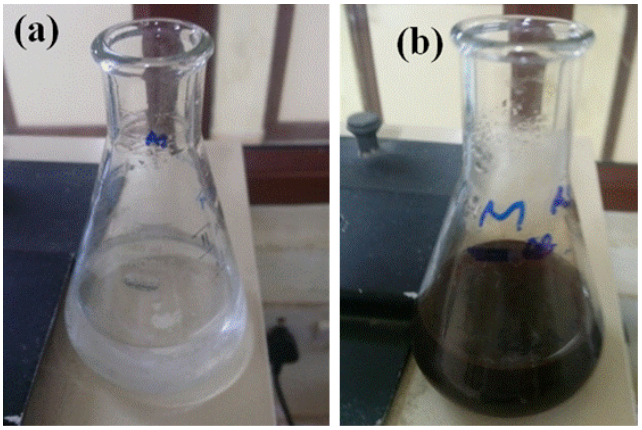

Figure 1: Visual observation of (a) silver nitrate solution (b) biosynthesized silver nanoparticles.

UV-Vis spectral analysis

UV-Vis spectra of synthesized AgNPs from *Piper nigrum* extract at different temperature conditions were as shown in the Figure 2. The color change depicts the existence of the formation of silver nanoparticles in the *Piper nigrum* extract. It shows that the UV-Vis spectra of silver nanoparticle formation at different temperature using *Piper nigrum* nanoparticle extract (i) Room temperature (RT), (ii) 40°C, (iii) 60°C, (iv) 80°C in aqueous medium. The surface plasmon resonance bands of colloidal silver for different temperatures were observed in the range 420 to 446 nm. At higher temperature (80°C), intense SPR band observed compared other samples. The strong SPR broad peak observed at 441 nm which confirms the formation of silver nanoparticles.

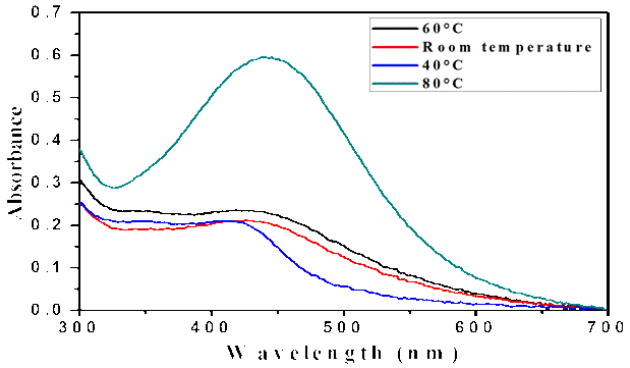

Figure 2: UV-Visible analysis of synthesized AgNPs from *Piper nigrum* extract at different temperature conditions.

FT-IR analysis

FTIR spectrum of *Piper nigrum* is depicted in Figure 3a. The assignments of *Piper nigrum* observed at 3421 cm^{-1}, 2933 cm^{-1}, 2388 cm^{-1}, 1632 cm^{-1}, 1388 cm^{-1}, 1221 cm^{-1}, 1021 cm^{-1}, 565 cm^{-1} and 521 cm^{-1}. The band at 3421 cm^{-1} which corresponds to normal "polymeric" OH stretching mode. The peak at 2933 cm-1 associated to the methylene C-H asymmetric and symmetric stretching mode. The peak at 2388 cm^{-1} indicates the symmetric stretching of alkanes and the peak at 1632 cm^{-1} attributed to the C=O stretching mode of ketones. The peak at 1388 cm^{-1} corresponds to the N=O stretching of nitro groups of leaf extract. The 1021 cm^{-1} peak corresponds to the C-C stretch and the aliphatic fluoro compounds C-F stretch. The peaks at 521 cm^{-1} and 565 cm^{-1} which are corresponds the aliphatic iodo compounds, C-I stretch, alcohol and OH out-of-plane bending.

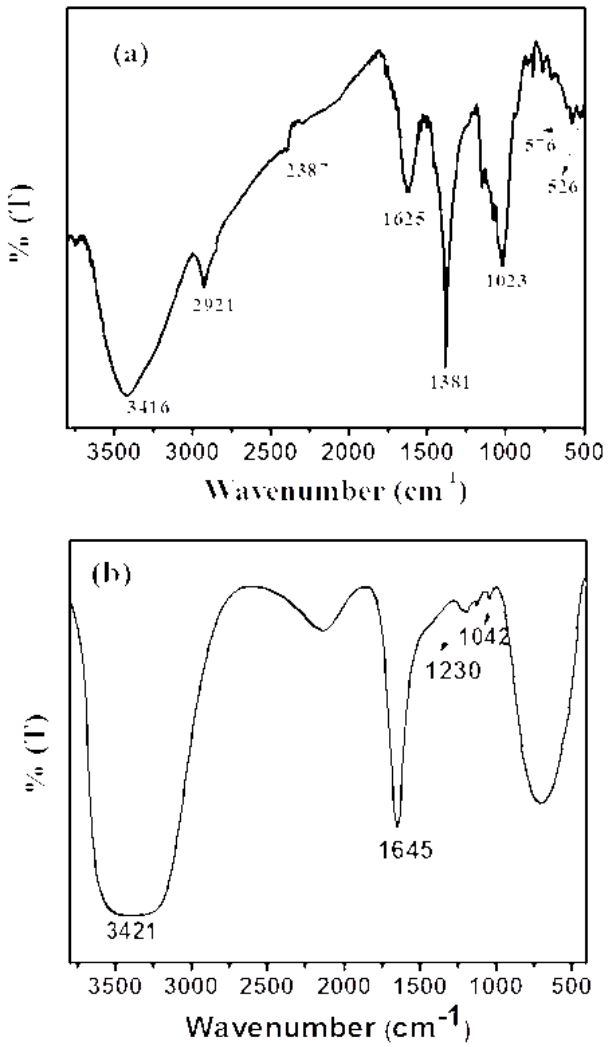

Figure 3: (a) FTIR spectrum of *Piper nigrum* and (b) FTIR spectrum of biosynthesized silver nanoparticles.

FTIR spectrum of AgNPs *Piper nigrum* is as shown in the Figure 3b. Addition of silver nitrate, still, we have the band at 3421 cm^{-1} which corresponds to "polymeric" OH stretching mode. An intense peak observed at 1645 cm^{-1} attributed to the C=O stretching mode of ketone (Figure 3b). The peaks at 1230 cm^{-1} and 1026 cm^{-1} which are corresponding to the amine C–N stretching, and the C-C stretching and the aliphatic fluoro compounds C-F stretching respectively present in the AgNPs and the peaks suppressed in comparison with *Piper nigrum.* The stretching vibration of nitro group of leaf (1388 cm^{-1}), iodo compounds OH group out of plane bending (521 cm^{-1}, 565 cm^{-1}) are completely suppressed due to the addition of silver nitrate, which means the complete reduction and stabilization of the silver nanoparticles. Here, we confirm the modulated transmittance percentage of ketone, fluoro compounds and amine groups play a vital role for the bioreduction of silver nitrate to silver nanoparticles [6,10].

HRTEM analysis

HRTEM is a powerful tools to analysis the properties of the materials at the atomic level. HRTEM image of AgNPs is shown in the Figure 4. The particle size of AgNPs is approximately in the range of 20 nm to 40 nm. The nanoparticles are embedded in a dense matrix which may be the organic stabilizing components of *Piper nigrum* extract. The shape of silver nanoparticles is spherical.

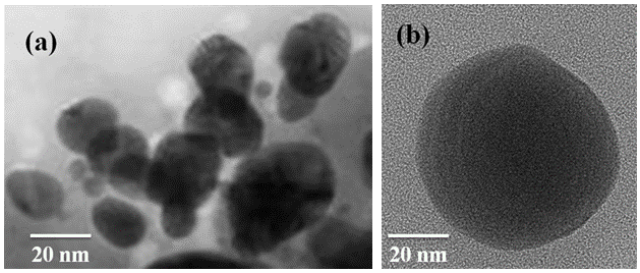

Figure 4: (a) HRTEM image of AgNPs and (b) Magnified portion of AgNps.

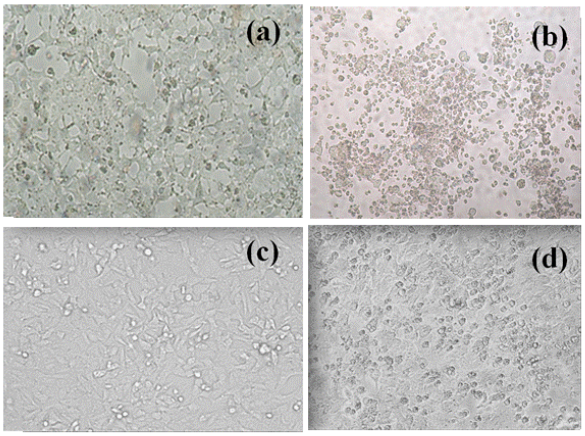

Figure 5: Cytotoxicity of the green synthesized silver nanoparticles against the MCF7. (a) control, (b) cytotoxic and the Hep2 cell line (c) control, (d) cytotoxic.

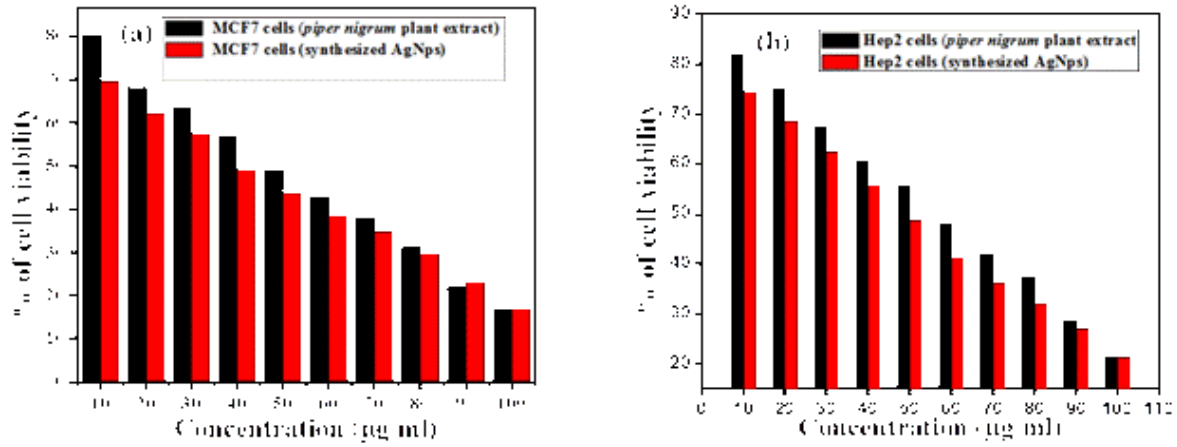

Figure 6: (a) Efficacy of *Piper nigrum* and biosynthesized AgNps against MCF7 cells at different concentration and (b) Efficacy of *Piper nigrum* and biosynthesized AgNps against Hep2 cells at different concentration.

Cytotoxicity analysis

The cytotoxicity of the silver nanoparticle and *Piper nigrum* extract was studied against the MCF7 (Figures 5a and 5b) and Hep2 cell line by MTT assay (Figures 5c and 5d). The cytotoxicity effect of cancer cell was studied at different concentration (10 µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 90 µg, 100 µg). The inhibitory concentration (IC50) value of the phytomediated AgNPs observed at the concentration of 52 µg/mL against MCF7 cells and *Piper nigrum* plant extract were observed as 54 µg/ml. This study shows that the minimum dose showed good anticancer activity. The Inhibitory Concentration (IC50) value of the phytomediated AgNPs was recorded at 43 µg/ml against Hep-2 cells [19,20]. The bar diagram of efficacy of *Piper nigrum* and biosynthesized AgNps against MCF7 cells at different concentration (Figure 6a). The bar diagram of efficacy of *Piper nigrum* and biosynthesized AgNps against Hep2 cells at different concentration as shown in the Figure 6b. In fact, silver nanoparticles may stimulate reactive oxygen species and effect in damage cellular components which lead to cell death [21].

Conclusion

We report a simple, facile, inexpensive, eco-friendly and green synthesis of silver nanoparticles from the *Piper nigrum* in aqueous medium without employing manmade chemicals. The UV–Vis spectroscopy and FT-IR analysis is confirmed the preliminary confirmation of the formation of silver nanoparticles. TEM image showed spherical shape with an average particle size of 20-40 nm. The biosynthesized silver nanoparticles and *Piper nigrum* extract showed promising anticancer activity against breast cancer cells (MCF-7) and human pharynx cancer cell line (Hep-2). From the study, it can be concluded that the silver nanoparticles synthesized using plant possess high anticancer activity against cell lines which further suggested the potential therapeutic use of these nanoparticles.

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