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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *PIPER NIGRAM* LEAF EXTRACTS AND ITS CYTOTOXIC ACTIVITY AGAINST HEP-2 CELL LINE

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ABSTRACT

Silver nanoparticle has attracted considerable interest due to their extensive applicability in various areas such as electronics, catalysis, chemistry, energy and medicine. Metallic nanoparticles are traditionally synthesized by wet chemical techniques, where the chemicals used are quite often toxic and flammable. In the present study, we describe a cost effective and eco-friendly technique for green synthesis of silver nanoparticles from 1mM $AgNO_3$ solution through the extract of *Piper nigram* leaf as reducing as well as capping agent. Nanoparticles were characterized using UV–vis absorption spectroscopy, FTIR, and SEM. SEM analysis showed the spherical nanoparticles with 19.7- 82 nm in size. Further these biologically synthesized nanoparticles were also exhibiting excellent cytotoxic effect on HEp-2 cell lines.

Keywords: *Piper nigram*, nanoparticle, cytotoxicity, HEp-2 cells.

INTRODUCTION

In recent times, nanomaterials have gained significant attention throughout the world today and there is an increasingly frenetic search for new nanomaterials and methods to make them. There have been impressive developments in the field of nanotechnology in the recent past, with numerous methodologies formulated to synthesize nanoparticles of particular shape and size depending on specific requirements. Metal nanoparticles have received considerable attention in recent years because of their unique properties and potential applications in catalysis, photonics, optoelectronics, biological tagging and pharmaceutical applications. Their performance depends critically on their size, shape and composition.

Silver Nanoparticles, as a significant member of the noble metal nanoparticles, are excellent substrates for Surface Enhanced Raman Scattering (SERS) [1] to probe single molecules, and are excellent as catalysts for accelerating some chemical reactions [2]. A number of approaches are available for the synthesis of silver NPs. For example, silver ions are reduced by chemical [3] electrochemical [4], radiation [5], photochemical methods [6], Langmuir–Blodgett [7,8] and biological techniques [9]. In the chemical synthesis methods the reactants and starting materials used in these reactions are toxic and potentially hazardous. In contrast, synthetic methods based on naturally occurring biomaterials provide an alternative, environmental-friendly means of obtaining these nanoparticles.

Biosynthesis of silver nanoparticles using microorganisms like bacteria [10,11], fungi [12-14] and yeast [15] are exploited for the synthesis of nanoparticles. However, exploration of the plant systems as the potential nanofactories, has heightened interest in the biological synthesis of nanoparticles. In the earlier work Justin *et al*, 2012 [16] reported the biosynthesis of nanoparticles using plant leaf extracts and their potential application. They studied bioreduction silver ions by extracts of Piper longum [16] and neem leaf [17]. Further, synthesis of silver nanoparticles using extracts of various plants like *Aloe vera* [18], *Cinnamon zeylanicum* [19], *Stevia rebaudiana* [20], Papaya [21] were reported. Most of the above research on the synthesis of silver or gold nanoparticles utilizing plant extracts employed broths resulting from boiling fresh plant leaves. Whereas, Huang et al. exploited the synthesis of silver and gold nanoparticles using the sundried Cinnamomum camphora leaf extract [22]. The synthesis and its application of nanorods using *Coscinium fenestratum* extract was also reported [23]. The rapidly developing field of nanotechnology will result in exposure of nanoparticles to humans via several routes (e.g., inhalation, ingestion, skin, etc.). Nanoparticles can translocate from the route of exposure to other vital organs and penetrate cells. Toxicity studies to determine the deleterious effects of nanoparticles on living cells are required. The present study was aimed at rapid synthesis of silver nanoparticles using aqueous leaf extract of *Piper nigram* and evaluation of their anticancerous activity using Hep-2 cell line.

MATERIAL AND METHOD

Plant material and preparation of the Extract

Freshly collected *P. nigram* leaves were shade dried and powdered. 5 g of the leaf powder was boiled for 10 min. in 100 ml sterile distilled water and filtered through Whatman No.1 filter paper (pore size 25 µm). The filtrate was further filtered through 0.6 µm sized filters, stored in refrigerated condition and used for the further study.

Synthesis of silver nanoparticles

1mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 10 ml of *P. nigram* extract was added into 90 ml of aqueous solution of 1 mM Silver nitrate for reduction into Ag^+ ions and incubated overnight at room temperature in dark.

UV-vis Spectra analysis

The reduction of pure Ag^+ ions was monitored by measuring the UV-vis spectrum of the reaction medium after overnight incubation, after diluting a small aliquot of the sample into distilled water. Silver nanoparticles (AgNPs) are soluble in distilled water and the colour changes were observed visually. A yellowish brown colouration was noticed at the synthesis phase. The concentration of AgNP produced was measured using a Systronics UV double-beam spectrophotometer (model 2201), at a resolution of 1 nm, between 200 and 600 nm using 10-mm-optical-path-length quartz cuvettes.

FTIR (Fourier-transform IR)

Studies on the samples were carried out using Nicolet Impact 400 FTIR spectroscopy to ensure the formation of silver nanoparticles.

SEM analysis of silver nanoparticles

A scanning electron microscope (JEOL 6380A; Tokyo, Japan) was used to record the micrograph images of synthesized AgNPs.

Cytotoxicity assay

The cytotoxicity assay of the prepared silver nanoparticle was measured using HEp-2 cell line by MTT test [24]. The cells were seeded in 24 well tissue culture plates at a density of 1 x 10^6, allowed to attach for 24 h and treated with different concentration (1.95-500 μl/ml) of AgNPs. After the AgNP treatment the medium was changed and the cells were washed twice with MEM without FCS to remove the dead cells, the cells were incubated with 200 μl (5mg/ml) of MTT for 6-7 h in 5% CO2 incubator for cytotoxicity. Cell viability was marked by the conversion of the tetrazolium salt MTT to a coloured formazan by the mitochondrial dehydrogenases. Colour development was measured photometrically using a spectrophotometer at 595nm after cell lyses in DMSO. The untreated cells absorbance was used as a control reference. The viability was calculated using the following formula.

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \times 100$$

RESULT AND DISCUSSION

The chemical reduction of aqueous solution of silver nitrate is one of the most widely used methods for the synthesis of silver colloids. In the present study, the formation of silver nanoparticles by *P.nigram* extract was investigated. The appearance of a yellowish brown colour in the reaction vessels suggested the formation of silver nanoparticles [25]. The plant extract after addition of aqueous 1 mM silver nitrate was subjected to optical measurements by UV-vis spectrophotometer. This analysis showed an absorbance peak at 420 nm (Fig.1), which was specific for the Ag nanoparticles.

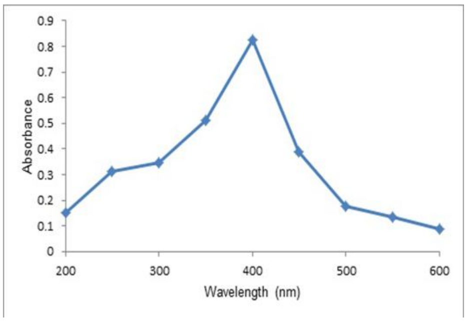

Figure 1. UV-visible spectra of *Piper nigram* extract containing silver nanoparticles (AgNPs).

The sample was stored in a refrigerator and used for further characterization studies. The FTIR spectrum (Fig.2) of Ag nanoparticles showed distinct peaks 1660 cm^{-1}, which represent the involvement of C=N in plane vibrations of aminoacides, 1032 – 1225 cm^{-1} represent the involvement of C-N in plane vibrations of aliphatic amines. The above bonds commonly occur in proteins indicating the presence of proteins as ligands for silver nanoparticles, which increases the stability of nanoparticles synthesized.

A peak at 1225 cm^{-1}, most propably from the C-O group of polyols, indicating that polyols are playing major role in the reduction of silver nanoparticles. Lim et al. [26] identified the alkaloids like pelliterine, piperidine, piperine and pellitorine in *P.nigram* and *Piper betle*. The above

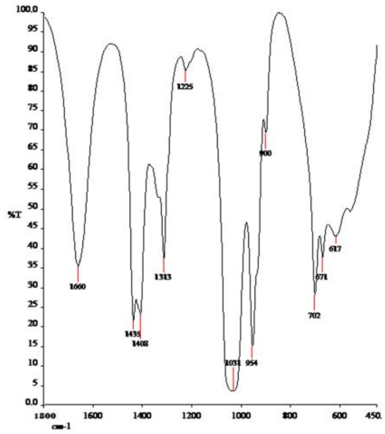

Figure 2. FTIR spectra of silver nanoparticles (AgNPs).

alkaloids may be responsible for the reduction of $AgNO_3$ into AgNPs. Rest of the bands showed resemblance to alkenes (617 -702 cm^{-1}) and aromatic (900 cm^{-1}) groups, which is present in the plant extract may have an effect in the synthesis of nanoparticles.

The possible mechanism of biosynthesis of nanoparticles by biological system was reductases and any other equivalent reductants as reported earlier [27]. The nitrate reductase from Fusarium oxysporum has been documented to catalyze the reduction of $AgNO_3$ to silver nanoparticles utilizing NADPH as reducing agent [28]. Several naphthoquinones and anthraquinones having very high redox potentials have been reported from F. oxysporum that could act as an electron shuttle in metal reduction [29]. Although such systems were not repeated in plant mediated synthesis nanoparticles, the phytochemical constituents are attributed to the formation of nanoparticles. Caffeine and theophylline present in tea extracts were also reported to catalyze the synthesis of nanoparticles [30]. Phyllanthin from Phyllanthus amarus was also reported as the capping ligands in the synthesis of silver nanoparticles [31]. Quercetin and polysaccharides have been used for silver nanoparticle synthesis [32]. Quercetin belongs to a group of plant pigments called flavonoids, and the other active constituent of the phytochemicals in the *P.nigram*, may be responsible for the nanoparticles synthesis.

The sizes of the spherical Ag nanoparticles were found to be in the range of 21.4 to 82.0 nm (Fig.3a,b).

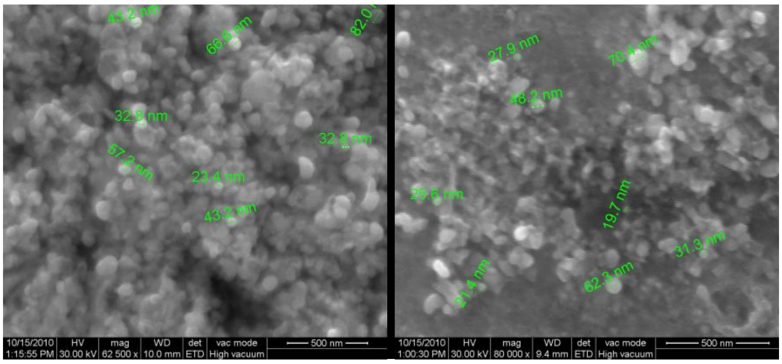

Figure 3a,b SEM images of silver nanoparticles.

The cytotoxic effect of Ag-nanoparticle was determined using Hep-2 cell lines by MTT-assay (Fig.4). Significant cytotoxic effect (90.02%) was observed at 500 µg/ml concentration of AgNP, whereas, at 31.25 µg/ml 47.06 % death (52.94% viability) was observed (Fig.5). At the same time $AgNO_3$ showed a maximum of 64.8% death at 500 µg/ml concentrations and the LD 50 of 50.8% death at 250 µg/ml concentrations was also observed. This killing effect was

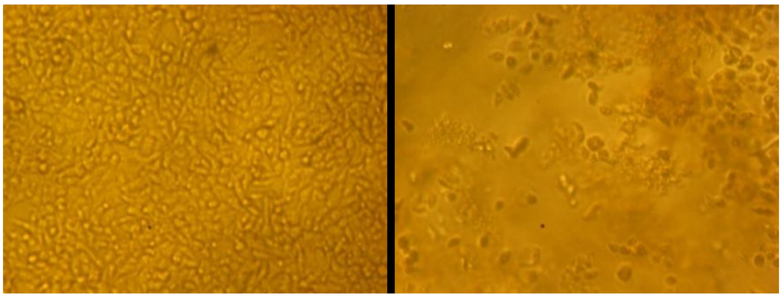

Figure 4. Image of Hep-2 cell line a). Control b). Treated with silver nanoparticles.

identified due to the formation of ROS. ROS typically include the superoxide radical, hydrogen peroxide and the hydroxyl radical, which cause damage to cellular components such as lipids, DNA and proteins and eventually lead to death. Earlier studies conformed that the *in vitro*

antiproliferative property of piperidine from P.nigram against HEp2 cancer cell line [33]. Lim et al. [26] reported that the extract of *P. nigrum* and *P. betle* have cytotoxicity activity against HL60 and HeLa cell line. Since lower doses of AgNPs reduced by plant extracts are efficient in exerting cytotoxic effect on HEp-2 cell line, the present investigation suggests that AgNPs may potentially prove to be useful as nanomedicine for anti cancerous drug preparations.

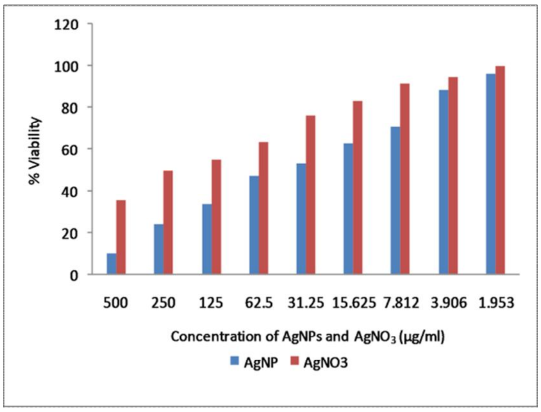

Figure 5. Cytotoxicity studies of silver nanoparticles and silver nitrate on Hep-2 cell line.

The enhanced cytotoxic effect may be due to the presence of piperidine as a capping agent in AgNPs. Since leaves of P.nigram are used in this study, it is a cost effective and ecofriendly technology for the disposal of waste biomass into wealth. Further studies are required inorder to analyse the mechanism behind the effects observed and the effects of AgNPs on mammalian immune system.

CONCLUSION

The present study demonstrated the green synthesis of silver nanoparticles using the extract of *Piper nigram* in room temperature. The piperidine and other bioactive components present in *P.nigram* may be responsible for the synthesis of silver nanoparticles. In the present study silver nano particles have exhibited a significant cytotoxic effect on HEp-2 cell lines. It may be due to the presence of piperidine in the nanoparticle as a reducing agent. Nanomaterial usage will continue to increase rapidly and widely in areas such as cosmetics, pharmaceuticals and other industrial applications. Accurately assessing the toxicity and safety of these nanomaterials to human health is of upmost importance. Therefore, further studies are needed in order to analyze the mechanism behind the effects observed and the effects of AgNPs on mammalian immune system.

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