Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

INVITRO TOXICITY OF SILVER NANOPARTICLES SYNTHESIZED BY USING CLOVES OF *SYZYGIUM AROMATICUM* AGAINST HEp-2 CELLS DERIVED FROM LARYNX CARCINOMA

VENUGOPAL KRISHNAN[*1] AND RAJAGOPAL KALYANARAMAN[2A]

[1,2a]Department of Biotechnology, Vels University, Pallavaram, Chennai-600117.

ABSTRACT

Present study reports sucessfull bio-synthesis of silver nanoparticles (AgNPs) using aqueous extract of cloves (*Syzygium aromaticum*) belonging to family Myrtaceae. The obtained nanoparticles were characterized by using UV–vis spectroscopy and transmission electron microscopy (TEM) and HR-TEM. The AgNPs were spherical, poly-dispersed and well scattered with a size ranging from 10 to 80 nm. The FTIR results confirmed the role of protiens liable for silver nitrate reduction into AgNPs. The bio-synthesized silver nanoparticles were checked for their cytotoxic property against HEp-2 cell line derived from larynx carcinoma. The results of MTT measures of AgNPs analyzed at different concentrations extending from 10 to 100µg/ml, demonstrated a considerable dose dependent toxicity of silver nanoparticles for HEp-2 cell line. The outstanding morphology appropriately relevant for anticancer property of silver nanoparticles against HEp-2 cells will have a beneficial impact of utilizing bio-synthesized nanoparticles for cancer therapy.

KEYWORDS: **Silver nanoparticles; biosynthesis; HEp-2 cell line; MTT assay; cytotoxicity**

VENUGOPAL KRISHNAN
Department of Biotechnology, Vels University, Pallavaram, Chennai-600117.

***Corresponding author**

INTRODUCTION

There exists a significant degree of difference in physico-chemical and biological properties of nanomaterials when compared to their counterpart bulk materials, although being similar in chemical compositions[1]. Silver nanoparticles (AgNPs) have been intensively studied among the various metal nanoparticles due to their well-known effectiveness in various fields[2]. The essential applications of silver nanoparticles in medical[3-6], food industries[7], agriculture[8], textile industries[9], water treatment[10], catalysis and surface-enhanced Raman scattering[11] etc. has demanded for creating an ecofriendly route for their mass production[12, 13]. Green synthesis of metals helps to develop the nano products and processes them to reduce or eliminate the use and generation of substances that are hazardous to human health and the environment. In this concern, the use of plants boasts of several advantages over commercial methodologies of nanoparticle synthesis. Moreover, plant extracts may act both as reducing agents and stabilizing agents in the synthesis of nanoparticles[14]. In the general way, a plant-extract-mediated bio-reduction for phytosynthesis of silver nanoparticles involves mixing the aqueous extract with an aqueous solution of the silver nitrate salt as described by various authors [14-19].The present study directs the advantageous of silver nanoparticles from silver nitrate through a simple green route utilizing the extract of Cloves (*Syzygium aromaticum*) as the reducing agent. Cloves are the aromatic flower buds of a plant in the family Myrtaceae and numerous restorative uses have been most broadly connected to toothache, and for mouth and throat aggravation. Cloves show antiseptic, antibacterial, antifungal and antiviral properties. Thus, the study proceeds with synthesis of silver nanoparticles utilizing *S. aromaticum* and their cytotoxicity of biosynthesized silver nanoparticles was studied against HEp-2 cells, human epithelial cells derived from a larynx carcinoma. However, the synthesis of silver nanoparticles utilizing cloves as biosource has not yet been studied.

MATERIALS AND METHODS

Materials

Silver nitrate ($AgNO_3$) (purchased from Hi Media Laboratories Pvt.Ltd. India), MTT (purchased from Hi Media Laboratories Pvt. Ltd. India) ,The HEP2 cancer cell line was collected from King Institute of Preventive Medicine and Research, Chennai, India,Dulbecco's modified Eagle's medium (DMEM: Hi media Laboratories, Mumbai, India) ,10% fetal bovine serum and 1%penicillin/streptomycin (Hi Media Laboratories Mumbai, India).

Preparation of Clove extract

The S. aromaticum (Cloves) were collected from the local market and authenticated. The S. aromaticum (Cloves) were finely powdered using mortar and pestle. The plant powder (20 g) was dissolved in 100 ml of millipore water and the mixture was bubbled at 80° C for 10 min followed by filtration through Whatman Grade No.1 filter Paper (11µm) and the broth was stored at low temperature till further use[20,21].

Bio-synthesis of silver nanoparticles

S. aromaticum concentrate (10 ml) was added to 90 ml of 1 mM silver nitrate solution in order to achieve reduction of Ag+ ions. Temperatures 60°C were maintained using water bath to optimize the synthesis. The solution stirred at 1000 rpm for 10 minutes [22]. The color change was observed at various temperatures to ensure the formation of silver nanoparticles. The *S. aromaticum* cloves extract was thus employed as a reducing and stabilizing agent for 1mM of silver nitrate [23,24].

Purification of silver nanoparticles

In order to remove the excessive silver ions and unwanted plant debris, 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 bio-synthesized silver nanoparticles

The preliminary characterization of silver nanoparticles was carried out using UV-visible spectroscopy [25, 26]. UV–Vis spectral investigation were carried out by using nanodrop 2000r working in scanning range of 200 nm to 800 nm. Millipore water was used as blank. The spectra recorded were then re-plotted using Origin 6.0 version. To verify the interactions between protein-silver nanoparticles, Fourier transform infrared spectroscopy (FTIR) in the range of 4000 to 400 cm^{-1} was used [25]. 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 [27].

HEp-2 Cell line culture

Human epithelial cells derived from a larynx carcinoma were collected from King Institute of Preventive Medicine and Research, 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). Further, the HEp-2 cell lines were maintained at 5% CO_2 in CO_2 incubator [28]. Cultures were routinely viewed under inverted microscope to evaluate the quantity of confluence and the absence of bacterial and fungal contaminants were confirmed [29].

MTT assay

To determine the cytotoxic effect of silver nanoparticles and *S. aromaticum* extract, cell viability study was done with the MTT reduction assay. HEp-2cells were seeded in a 96-well plate at the density of 5×10^3 cells / well. The cells were allowed to attach and were grown in 96-well plate for 24 h, in 200 µl of EMEM with 10% FBS [30]. After that the media was removed and replaced with suspension of various concentrations of silver

nanoparticles 10 to100 mg/ml (minimum 4 wells were seeded with each concentration) the cells were incubated for 48 h [32]. The addition of MTT (10 ml, 5 mg/ml) was followed incubation of cells at 37℃ for another 4 h. The medium was then removed, and 200 µl of DMSO was added to each well. The optical density of the formazan product was read at 620 nm using multi well spectrophotometer [29, 33]. The results were given as mean of four independent experiments. OD value was subjected to calculate the percentage of viability by using the following formula,

$$\text{Percentage of cell viability} = \frac{\text{OD value of treated sample (AgNPs)}}{\text{OD value of control sample}} \times 100$$

RESULTS

Formation of Silver Nanoparticles (AgNPs)

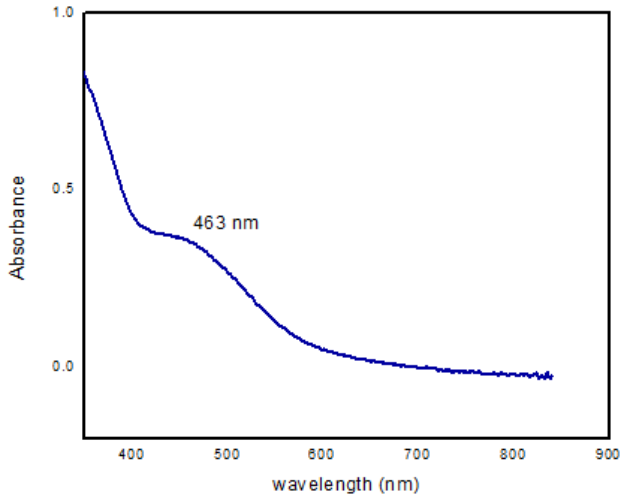
Silver nitrate solution is colorless and extract of Cloves is dark red in color. After adding *Syzygium aromaticum* extract to Silver nitrate solution, the solution became grayish red in color after incubation. The color change confirms the synthesis of silver nanoparticles in the solution.

UV–Vis spectra analysis

The color change demonstrating the presence of Ag nanoparticles was further characterized by UV-Vis spectrophotometer. The absorption peaks shows the UV-Vis spectra of silver nanoparticle formation at temperature using *Syzygium aromaticum* nanoparticle extract 60°C in aqueous medium. The intense SPR band observed at 463 nm (Fig.1) confirms the synthesis of silver nanoparticles. Our results confirm the observations of Shalini *et.al* [34].

Figure 1

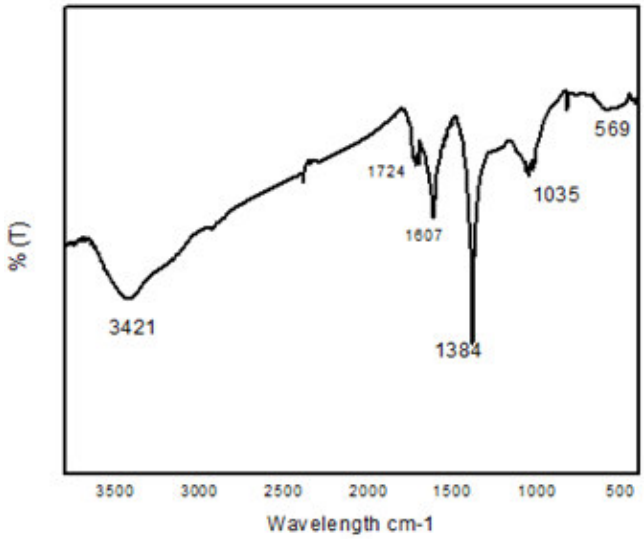
UV-Vis spectra analysis of biosynthesized AgNPs.



FTIR analysis

The FT-IR transmission spectra of silver nanoparticles from *S. aromaticum* are represented in Figure 3. FTIR spectra of AgNPs demonstrated the peaks around but not limited to 3421, 1724, 1607, 1384, 1035, and 569 cm^{-1} . The band at 3421 cm^{-1} corresponds to ‘‘polymeric’’ OH stretching mode. The 1724 cm^{-1} peak corresponds to normal aldehyde group. The absorption at 1607 cm^{-1} represents amide and Open chain imino (- C= N-). The 1384 cm^{-1} peaks relates to trimethyl or "tert-butyl" (multiplet). The 1035 cm^{-1} peak corresponds to the C-C stretch and the aliphatic fluoro compounds C- F stretch. The peaks at 569 cm^{-1} corresponds the aliphatic iodo compounds, C-I stretch, alcohol and OH out-of-plane bending. This confirmation proposes that the protein particles could perform the capacity of the arrangement and adjustment of AgNPs in the aqueous medium [21]. This also confirmed that the compounds did not disturb the synthesis of silver nanoparticles.

Figure 2
IR spectra of biosynthesized AgNPs using Syzygium aromaticum



Cytotoxicity analysis of HEp-2 cell line

The cyotoxicity of the silver nanoparticle and *Syzygium aromaticum* extract was studied against the HEp-2 cell line by MTT assay. Figure 3 shows the cytotoxicity of AgNPs and *Syzygium aromaticum* against Hep-2 cell lines. The cytotoxicity impact on growth cell was examined 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 (IC_{50}) estimation of the phytomediated AgNPs was recorded at 58µg/ml against HEp-2 cells as shown in figure 4. This study demonstrates that the dosage needed was less for the cancer cell line. In fact, silver nanoparticles may stimulate reactive oxygen species and effect in damage cellular components which lead to cell death.

Figure 3
Cytotoxicity of the cells

A control cells **B cells showing cytotoxicity**

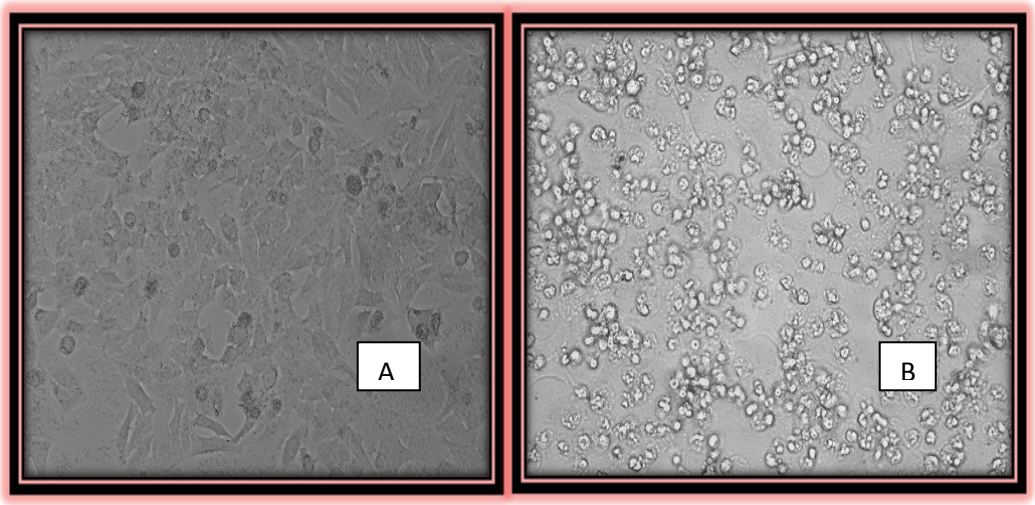
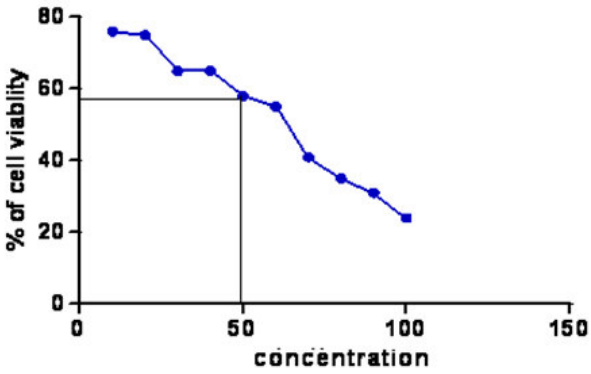


Figure 4

Cytotoxicity evaluation of bio-synthesized AgNPs at various concentrations against cancer (HEP2) cell line. The figure demonstrates the Inhibitory Concentration (IC_{50}) value of the phytomediated AgNPs recorded a 58µg/ml against HEP2 cells.



DISSCUSSION

Present study demonstrates bio-synthesis of silver nanoparticle by using *S. aromaticum* clove extract and their cytotoxicity against HEp-2 cells, human epithelial cells derived from a larynx carcinoma. UV–Vis spectroscopy confirmed the preliminary formation of silver nanoparticles at 60°C which is inagreement with previous studies[34]. Our FTIR observations demonstrated that the protein particles of *S. aromaticum* extract induce reduction of silver nitrate solution to form AgNPs in the aqueous medium sucessfully. The size of AgNPs was confirmed by TEM analysis which illustrates spherical shape of AgNPs with size ranging from 10 to 80 nm Synthesized AgNPs appeared polydispersed and well scattered. The synthesized silver nanoparticles and *S. aromaticum* extract compared and showed promising anticancer activity against HEp-2 cells, human epithelial cells derived from a larynx carcinoma.

CONCLUSION

From the present study, it can be concluded that the silver nanoparticles synthesized using plant possess significant anticancer activity which suggests their potential therapeutic application. Further studies are needed to demonstrate the mechanism of action of AgNPs in normal and cancer cells *in vivo* for their application in cancer therapy.

REFERENCES

1. Chaloupka K, Malam Y, Seifalian AM. Nanosilver as a new generation of nanoproduct in biomedical applications. Trends Biotechnol. 2010; 28: 580–88.
2. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv. 2009; 27:76–83.
3. Krutyakov YA, Kudrinskiy AA, Olenin AY, Lisichkin GV. Synthesis and properties of silver nanoparticles: advances and prospects. Russ Chem Rev. 2008; 77:233.
4. Prow TW, Grice JE, Lin LL, Faye R, Butler M, Becker W, Wurm EMT, Yoong C, Robertson TA, Soyer HP, Roberts MS. Nanoparticles and microparticles for skin drug delivery. Adv. Drug Deliv. Rev. 2011; 63: 470–491.
5. Chaudhry Q, Castle L. Food applications of nanotechnologies: an overview of opportunities and challenges for developing countries. Trends Food Sci. Tech. 2011; 22: 595.
6. Nair R, Varghese SH, Nair BG, Maekawa T, Yoshida Y, Sakthi Kumar D. Nanoparticulate material delivery to plants. Plant Sci. 2010; 179: 154–163.
7. Kelly FM, Johnston JH. Colored and functional silver nanoparticle wool fiber composites. ACS Appl. Mater. Interfaces 2011; 3: 1083–1092.
8. Dankovich TA, Gray DG. Bactericidal paper impregnated with silver nanoparticles for point-of-use water treatment. Environ. Sci. Technol. 2011; 45: 1992–1998.
9. Sharma VK, Yngard RA, Lin Y. Silver nanoparticles: Green synthesis and their microbial activities. Adv in Coll and Interf Sci. 2009; 145: 83–96.
10. Rivas L, Sanchez-cartos S, Garcia-Ramos JV, Marcillo G. Growth of silver colloidal particles obtained by citrate reduction to increase the Raman enhancement factor. Langmuir. 2001; 17: 574–577.
11. Maity D, Bain MK, Bhowmick B, Sarkar J, Saha S, Acharya K. In situ synthesis, characterization, and antimicrobial activity of silver nanoparticles using water soluble polymer. J of Appli Poly Sci. 2011; 122 (4): 2189-2196
12. Ohno K, Koh K, Tsujii Y, Fukada T. Fabrication of ordered arrays of gold nanoparticles coated with high-density polymer brushes. Angewandte Chemie International Edition. 2003; 42: 2751–2754.
13. Zhang Z, Patel RC, Kothari R, Johnson CP, Friberg SE, Aikens PA. Stable silver clusters and nanoparticles prepared in polyacrylate and inverse micellar Solutions. J of Physl Chem. B. 2000; 104: 1176–1182.

14. Gauri Y, Thieuleux C, Mehadi A, Reye C, Corriu RJP, Gomez-Gallardo S. In situ formation of gold nanoparticles within thiol functionalized HMS-C16 and SBA-15 type materials via an organometallic two-step approach. Chem of Mat. 2003; 15:2017–2024.
15. Moura MRD, Mattoso LHC, Zucolotto V. Development of cellulose based bactericidal nanocomposites containing silver nanoparticles and their use as active food packaging. J Food Engg. 2012; 109: 520–524.
16. Hebeish A, Hashem M, Abd El-Hady MM, Sharaf S. Development of CMC hydrogels loaded with silver nano-particles for medical applications. Carbohy Poly. 2013; 92: 407–413.
17. Bankar A, Joshi B, Kumar AR, Zinjarde S. Banana peel extract mediated novel route for the synthesis of silver nanoparticles. Collo. Surf A 2010; 368:58–63.
18. Stiger RM, Gorer S, Craft B, Penner PM. Investigations of electrochemical silver nanocrystal growth on hydrogen-terminated silicon (1 0 0). Langmuir. 1999; 3:790–798.
19. Kora AJ, Arunachalam J. Assessment of antibacterial activity of silver nanoparticles on *Pseudomonas aeruginosa* and its mechanism of action. Wor J of Microb and Biotech. 2011; 27: 1209–1216.
20. Mayya KS, Schoeler B, Caruso F. Preparation and organization of nanoscale polyelectrolyte-coated gold nanoparticles. Advan Funct Mat. 2003; 13: 183–188.
21. Tan Y, Jiang L, Li Y, Zhu D. One dimensional aggregates of silver nanoparticles induced by the stabilizer 2-Mercaptobenzimidazole. J of Phy Chem B. 2002; 106: 3131–3138.
22. Tanori J, Pileni MP. Control of the shape of copper metallic particles by Smetana, using a colloidal system as template. Langmuir 1997; 13: 639–646.
23. Wang H, Qiao XJ, Chen S, Ding S. Preparation of silver nanoparticles by chemical reduction method Colloids Surf. A: Physicochem. Eng. Aspects 2005; 256: 111.
24. Abid JP, Wark AW, Brevetm PF, Girault HH. Preparation of silver nanoparticles in solution from a silver salt by laser irradiation. Chem Commun. 2002; 7: 792–793.
25. Pol VG, Srivastava DN, Palchik V, Slifkin MA, Weiss AM, Gedanken A. Sonochemical deposition of silver nanoparticles on silica spheres. Langmuir 2002; 18: 3352–3357.
26. Esau SR, Roberto SB, Ocotlan-Flores J, Saniger JM. Synthesis of AgNPs by sonochemical induced reduction application in SERS. J Nanopart Res. 2010; 9: 77.
27. Hunng HH, Ni XP, Loy GL, Chew CH, Tan KL, Loh HC. Photochemical formation of silver nanoparticles in poly(N-vinylpyrrolidone). Langmuir 1996; 12(4): 909–912.
28. Malune A, Kohon JY, Takeda Y, Kondow T. Structure and stability of silver nanoparticles in aqueous solution produced by laser ablation. J of Phy Chem. B 2000; 104(35): 8333–8337.
29. Wei X, Luo M, Li W, Yang L, Liang X, Xu L *et al*. Synthesis of silver nanoparticles by solar irradiation of cell-free *Bacillus amyloliquefaciens* extracts and $AgNO_3$. Biores Tech. 2012; 103: 273–278.
30. Peng H, Yang A, Xiong J. Green, microwave-assisted synthesis of silver nanoparticles using bamboo hemicelluloses and glucose in an aqueous medium. Carboh Polym 2013; 91: 348–355.
31. Yu YY, Chang SS, Lee CL, Wang CRC. Gold nanorods: Electrochemical synthesis and optical properties. J Physi Chem. B 1997; 101(34): 6661–6664.
32. Zhu JJ, Liu SW, Palchik O, Kottypin Y, Gedanken A. Shape- controlled synthesis of silver nanoparticles by pulse sonoelectrochemical methods. Langmuir 2000; 16: 6396–6399.
33. Starowicz M, Stypuła B, Banaś J. Electrochemical synthesis of silver nanoparticles. Electrochem Commun 2006; 8(2): 227-230.
34. Shalini Chauhan and Mukesh Kumar Upadhyay. Fruit based synthesis of silver nanoparticles-an effect of temperature on the size of particles, Rec Res in Sci and Tech. 2012; 4(5):41-44.