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Biogenic synthesis of Adhatoda vasica L. Nees mediated silver nanoparticles and their antibacterial, anticancer activity on Hep-G2 cell lines

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ABSTRACT

The present investigation has been studied with the green synthesis of silver nanoparticles (AgNPs) using medicinally valued Adhatoda vasica -Nees and to evaluate the antibacterial and anticancer activity against HEP-G2 (Human epithelium cells of liver cancer) cell lines. The UV-Vis spectroscopy results show a strong resonance centered on the surface of silver nanoparticles at 420 nm. Fourier transform infrared (FT-IR) spectroscopy study demonstrates A. vasica aqueous extract acted as the reducing and stabilizing agent during the synthesis. The X-ray diffraction (XRD) analysis confirmed that the synthesized AgNPs are single crystalline face-centered cubic in structure, average crystal size 21 nm. Scanning electron microscope–energy dispersive spectroscopy (SEM-EDS) image confirmed synthesis of relatively uniform nanoparticles. The EDS analysis of the nanoparticles dispersion, using a range of 2-4 keV, confirmed the presence of elemental silver, without any contamination. The antibacterial activities were carried out against pathogenic bacteria. The maximum zone of inhibition was observed in the synthesized AgNPs (10µg/mL) against *Staphylococcus sp.* (16mm), *Klebsiella sp.* (14.5mm). The cytotoxicity activity as evidence by MTT assay with HEP-G2 cell lines. The synthesized AgNPs are ready for the application in the field of nanomedicine against pathogenic bacteria and very good anticancer drug.

Keywords: Adhatoda vasica, silver nanoparticles, antibacterial, cytotoxicity, HEP-G2 cell lines.

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1. Introduction

Leaves of the *Adhatoda vasica*-Nees possess expectorant, bronchodilator, respiratory stimulant, antispasmodic, hypotensive, cardiac depressant, uterotonic, antimicrobial and hypoglycemic properties; roots and barks are expectorant, antispasmodic and antiseptic [1]. In recent years, nanotechnology has immense potential to revolutionize in the biomedical research by designing new and improved products for clinical diagnosis and therapy. Several noble metal nanoparticles such as silver, gold, copper and platinum were widely synthesized by employing various procedures including physical, chemical and biological methods. The physical and chemical routes of nanoparticles preparation have many disadvantages and are not eco friendly. Hence, researchers across the globe have searched for new and environmentally benign methods for the synthesis of biocompatible nanoparticles [2]. Recently, 'green synthesis' of nanoparticles using extracts of plants and microbes has gained importance because it could solve the problem of toxicity imposed by chemical methods. The silver nanoparticles (AgNPs) have been widely utilized in biology and medicine due to its attractive physiochemical properties [3]. AgNPs have been reported to exhibit number of pharmacological activities, including antibacterial activity [4,5] antifungal activity [6], anticancer activity [7], antiviral activity [8] antiplasmodium activity and mosquito larvicide activity [9], etc. nanoparticles delivery system has been proposed as colloidal drug carriers. The key advantages of nanoparticles are improved bioavailability by enhancing aqueous solubility, increasing resistance time in the body (increasing half life for clearance/increasing specificity for its cognate receptors and targeting drug to a specific location in the body (its site of action). AgNPs possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes [10]. It also has been used for antimicrobial activity and may locally destroy pathogenic organisms, without being toxic to the surrounding tissue. In medicines, Ag and AgNPs have a wide application including skin ointments and creams containing silver to prevent infection of burns and open wounds [11].

In anti cancer therapy, AgNP-induced reduce cell viability in various cell lines by causing apoptosis through the mitochondrial pathway [12]. The Ag-NPs accumulation in the liver could induce cytotoxicity via oxidative cell damage [12,13,14] and AgNPs may induce cytotoxicity in phagocytosing cells and monocytes [15-18].

The present study utilizes *A. vasica* a nontoxic bioresource for synthesizing AgNPs rapidly in a controlled manner and attempted to find the antibacterial, anticancer activity on HEP-G2 cell lines.

2. Materials and methods

2.1. Collection of plant leaves

Adathoda vasica plant leaves were collected from Erode region, Tamil Nadu, India. The leaves were surface cleaned with tap water followed by distilled water. For removing the dust particles and allowed to air dry in room temperature.

2.2. Preparation of leaves extract

Twenty gram of sterilized leaf samples were taken and cut into small pieces. The finely cut leaves were placed in a 500 mL Erlenmeyer flask containing 100 mL of sterile double distilled water. Then, the mixture was boiled for 5 min (process of boiling leads to rupture of the cell walls in leaves and thus, release of inter cellular material into solution). After boiling, the mixture was cooled and filtered [19].

2.3. Synthesis of AgNPs

Bio-reduction of metal ions in solution was done through addition of leaf extract into the metal solution having known concentration. Silver nitrate ($AgNO_3$) was used as precursor for synthesizing the Green AgNPs. Five mL of leaf extract was added to 100 mL of 1 mM $AgNO_3$ aqueous solution in conical flask of 250 mL content at room temperature. The reaction was carried out for a period of 24 h. reduction was observed by the color change in the colloidal solution, which confirmed the formation of AgNPs [20,21].

2.4. Characterization of silver nanoparticles

2.4.1. UV-Vis spectra analysis

The formation and completion of AgNPs was characterized by UV-Visible spectroscopy by using Eleco UV-Visible spectrophotometer, Model

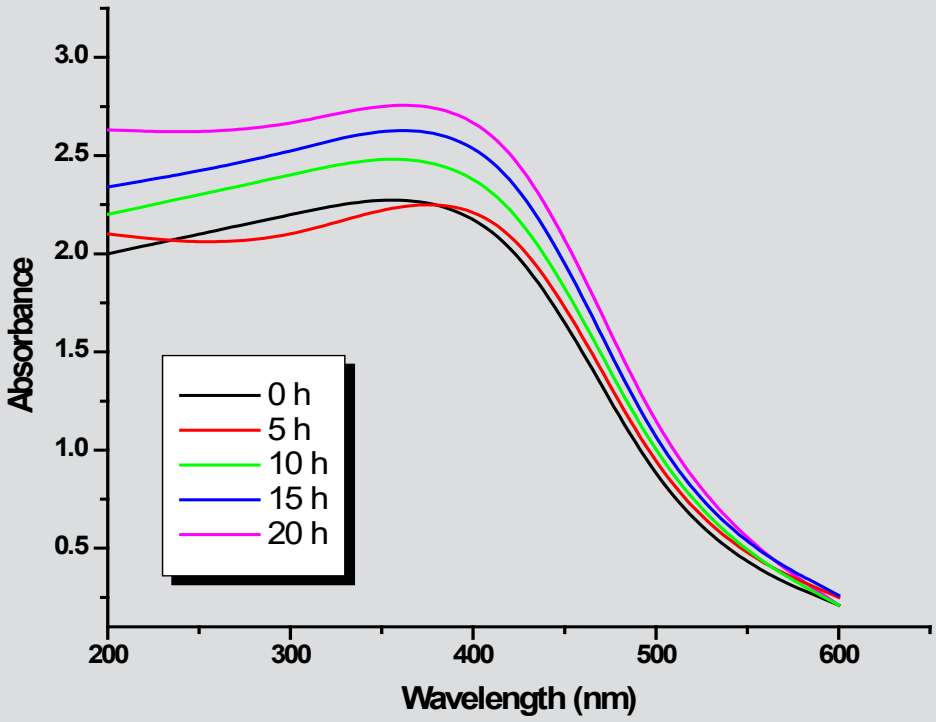

Fig.1. UV–Visible spectrum of biosynthesized AgNPs at different time interval, peaks shows at 420 nm.

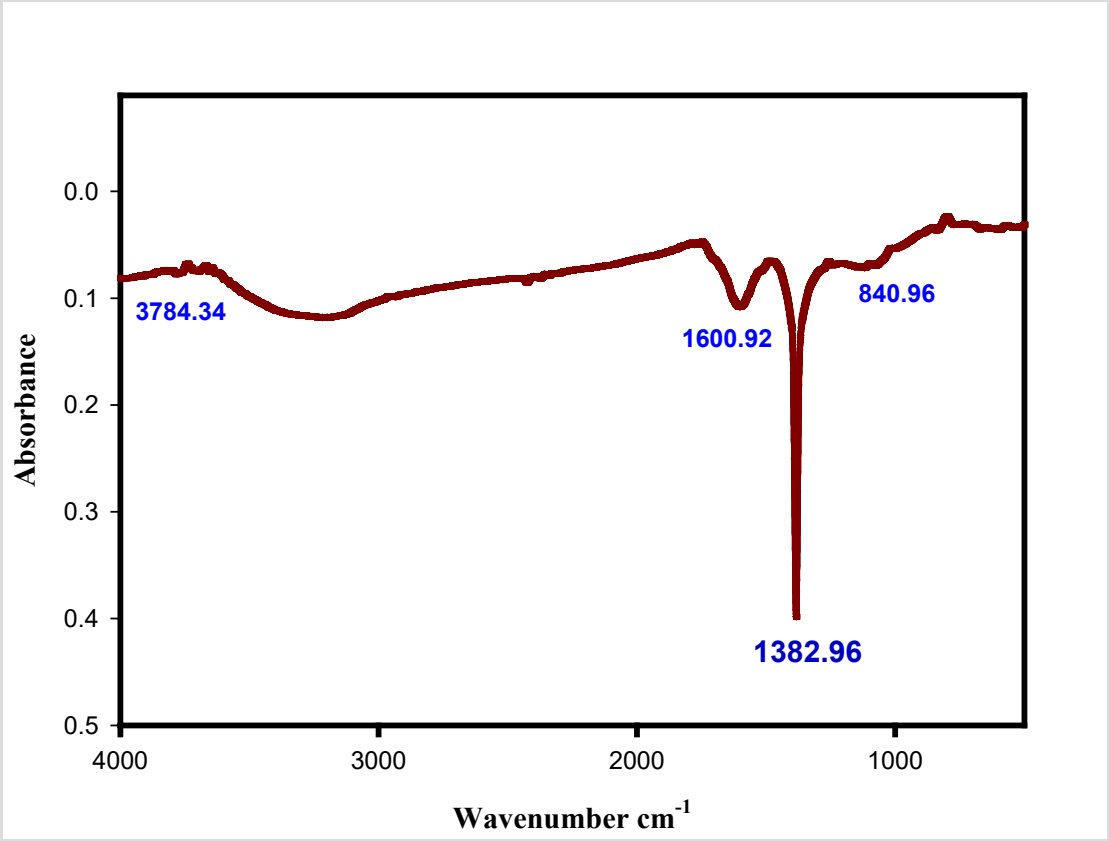

Fig.2. FT-IR spectra representing the functional groups associated with the reduction and stabilization of A. vasica mediated AgNPs.

164. The bio-reduction of the Ag+ ions in solution was monitored by periodical sampling of aliquots and the UV-Visible spectra of these aliquots were monitored as a function at time of reaction in 200-600 nm range operated at a resolution of 1nm. Distilled water was used as a blank.

2.4.2. Fourier transform infrared spectroscopy

FT-IR spectral analysis was carried out to identify the possible biomolecules responsible for the reduction of the Ag+ ions and the capping of the AgNPs synthesized by *A. vasica* extract. The dried AgNPs were analyzed by (Shimadzu) FT-IR Spectrometer. FT-IR spectrum was recorded over the range of (500-4000) cm^{-1} (with FTLA 2000 ABB).

2.4.3. X-ray diffraction

XRD was recorded in the 2 Θ range of 30–80 ° using XRD6000, (Shimadzu) of CuKa radiation, the energy of which was 8.04 keV and wavelength was 1.54 A°. The applied voltage was 40 kV and current was 25 mA. The crystallite size was estimated using the Scherer equation.

2.4.4. Scanning electron microscope and EDS measurements

SEM analysis was done using (Jeol JSM 6390 model) SEM machine. Thin film of the samples was prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid. Extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5mins.

In order to carry out EDS analysis, the bark extracts reduced AgNPs were dried and drop coated on to carbon film and performed on Jeol JSM 6390 model SEM instrument equipped with a Thermo EDS attachments.

2.5. Antibacterial activity of AgNPs

The disc-diffusion assay Kirby–Bauer method [22] was used by many workers to determine the growth inhibition of bacteria by the AgNPs [20,23,24]. Antibacterial activity of the synthesized AgNPs was assessed against clinically isolated bacterial pathogens of *Staphylococcus sp.* MG87 (NCBI-Accession: KC688883.1), Uncultured *Klebsiella sp.* clone MASC-TSK (NCBI-Accession: KF649832.1) and *Bacillus sp.* BT MASC 1 (NCBI-Accession: KJ438150.1). All microorganisms were maintained at 4 ºC on nutrient agar [25]. The bacteria were cultured in Mueller-Hinton Broth (MHB) at 37 °C and prepared to the turbidity equivalent to 0.5 McFarland standards [26]. Then 100 µL of the bacterial suspension was spread on the Mueller-Hinton Agar (MHA). Sterile blank discs with 6 mm diameter (HiMedia, Mumbai) were impregnated with AgNPs synthesized at different silver nitrate concentrations so that to get a final nanoparticle concentration of 10 µg/disc. These were then placed on the surface of the test plate. A standard antibiotic disc was used as positive control (Ampicillin, 10µg/disc). The culture plates were incubated for overnight in an incubator at 37 °C. The diameters of the inhibition zones were measured in millimeters (mm). The experiment was repeated for three times to get an average value.

2.6. Invitro Cytotoxicity activity

2.6.1. Cell lines and culture condition

HEP-G2 liver cell lines were obtained from (NCCS, Pune University, India). These cells were maintained in DMEM (pH 7.4) with 10% FBS and streptomycin+ penicillin solution at 5% CO_2 at 37 ˚C.

2.6.2. Methylthiazolyl diphenyl-tetrazolium bromide (MTT) assay

To determine the cytotoxic effect of AgNPs, cell viability study was done with the conventional MTT-reduction assay with slight modifications [27]. Briefly, Hep-G2 cells 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 a 96-well plate for 24 h in 200 µL of DMEM with 10% FBS. After that the media was removed and replaced with suspension of various concentrations of AgNPs viz., 20-100µg/mL. Equal concentrations of *A. vasica* leaf extract were used as positive control and the cells were incubated for 48 h. After the addition of MTT-(3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) (10 µL, 5 mg/ml), the cells were incubated at 37 ˚C for another 4 h. Optical density of the formazan product was read at 495 nm using scanning multi well spectrophotometer. The results were given as mean of three independent experiments. Statistical analysis

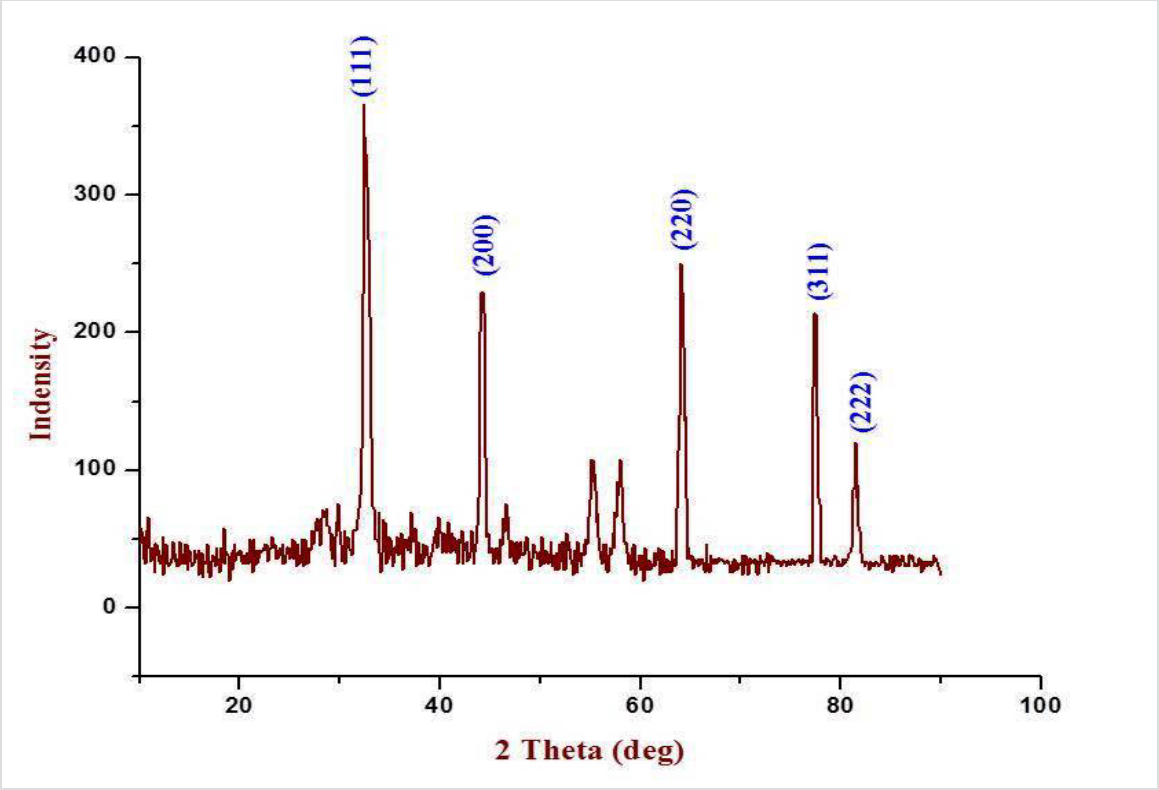

Fig.3. XRD pattern of AgNPs synthesized using A. vasica extract.

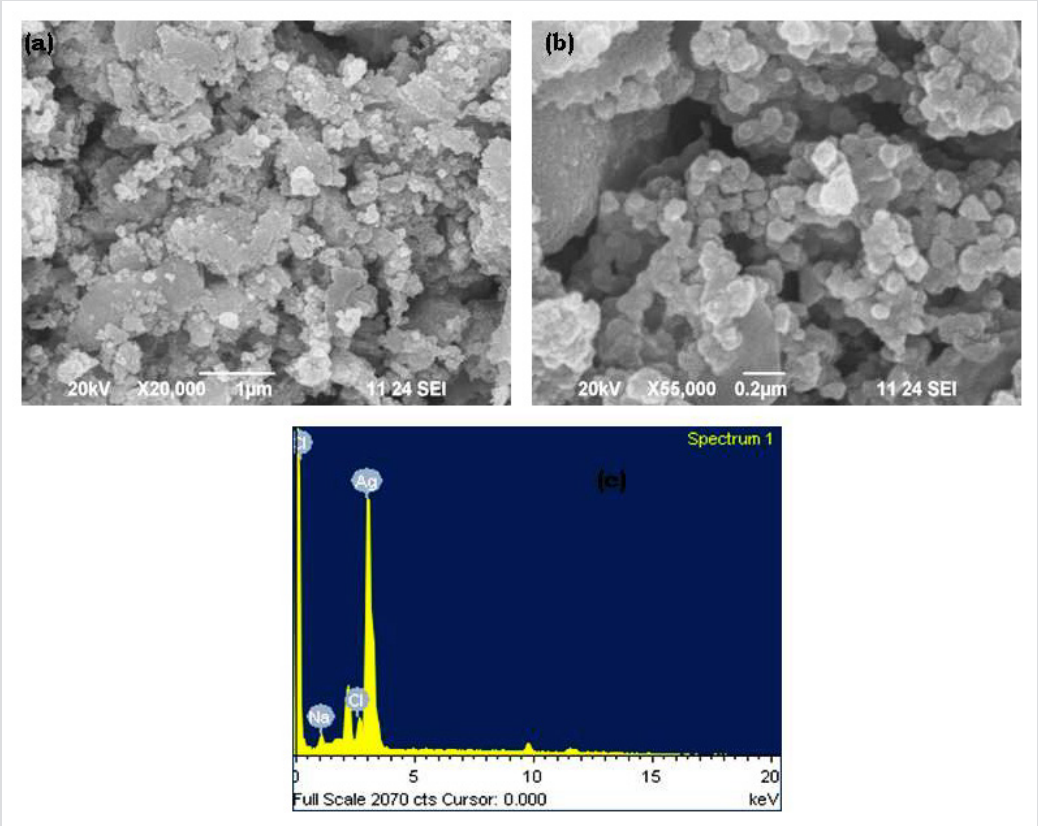

Fig.4. (a,b) SEM images of AgNPs show Spherical in shape. (c) SEM-EDS spectrum of AgNPs. A strong pesk at 3keV confirms the presence of Ag

was calculated using SigmaPlot 10.0 (SYSTAT Software, Inc., Chicago, IL, USA). Data were analyzed with one-way analysis of variance (ANOVA). The statistical significance of the experiments were considered *p<0.05; **p<0.01.

3. Results and discussion

3.1. Green Synthesis of AgNPs

The first indication of nanoparticles formation is colour change. A clear yellowish brown colour was formed due to the reduction of silver ion within 30 min when 1mM $AgNO_3$ was added into the leaf extract of *A. vasica*, which indicates the synthesis of AgNPs. The intensity of yellowish brown colour was increased with the incubation period and it was due to the excitation of surface plasmon vibrations [21].

3.2. Characterization of AgNPs

3.2.1. UV-Visible spectra analysis

The colour of synthesised AgNPs clearly changes to reddish brown within 24 h of incubation at room temperature and corresponding UV–Visible absorption spectrum of AgNPs was recorded in Fig.1. The spectra of AgNPs showed maximum absorption at 420 nm ranging from 0 min to 20 h to the surface plasmon resonance of the formed AgNPs. Similarly, Sudhakar et al., [20] have recently reported that *Acromus calamus* rhizome extract effectively synthesized AgNPs at 420 nm.

3.2.2. Fourier transform infrared spectroscopy

FTIR measurement was carried out to identify the possible biomolecules in *A. vasica*, leaf extract responsible for capping leading to efficient stabilization of the AgNPs (Fig.2). The IR spectrum of AgNPs manifests prominent absorption bands located at 3784.34, 1600.92, 1382.96, and 840.96 cm^{-1}. The results of the FTIR studies further confirm that the compounds present in the *A. vasica* leaf extract reduced $AgNO_3$ into AgNPs. This result showed similarity with those reported by Sivakumar et al. [28].

3.2.3. X-ray diffraction

XRD was employed to confirm the crystalline nature of the particles, and the XRD displayed numbers of Braggs reflections that may be indexed on the basis of the face centered cubic structure of silver [29,30]. The sample of AgNPs could be also characterized by X-ray diffraction analysis of dry powders. The diffracted intensities were recorded from 10˚ to 90˚ at 2 theta angles (Fig. 3). Four different and important characteristic peaks were observed at the 2 h of 38.1˚, 44.3˚, 64.5˚, and 77.4˚ that correspond to (111), (200), (220), and (311) planes, respectively. All the peaks in XRD pattern can be readily indexed to a face centered cubic structure of silver as per available literature (JCPDS, File No. 89-3722). The average crystal size of the silver crystallites was calculated from the FWHMs of the diffraction peaks, using the Scherrer equation. The size of the crystallite in different planes of silver was determined as 21 nm. Similar results were reported by Sivakumar et al. [28].

3.2.2. Scanning electron microscope and EDS

The Fig.4 represents the SEM image recording from drop coated films of the AgNPs synthesized with *A. vasica* -Nees leaf extract. The SEM image showed spherical and relatively uniform shape of nanoparticles formation. Analysis through Energy dispersive X-ray (EDS) spectrometers confirmed the presence of elemental silver signal of AgNPs.

3.2.3. Antimicrobial Activity of AgNPs

The antibacterial activity assay of *A. vasica* leaf extract synthesized AgNPs showed effective inhibitory action against pathogens such as *Staphylococcus* sp. MG87, *Klebsiella* sp. clone MASC-TSK, and *Bacillus* sp. BT MASC 1 (Fig. 5). Greater antimicrobial activity was observed against the *Staphylococcus* sp. (16mm), *Klebsiella* sp. (14.5mm). The Bacillus sp. was found to be less susceptible to AgNPs than the other bacteria. The antimicrobial activity of A. vasica extract based synthesized AgNPs is statistically significant. However, A. vasica extract green synthesized AgNPs showed the greatest antimicrobial activity against the tested microorganism. The antibacterial activity of the silver nanoparticles may be centered on permeability of bacterial cells due to cell wall layers or its charges [31-33].

3.2.4. Determination of cell viability by MTT assay

The AgNPs synthesized by using *A. vasica* against induced cell damage in HEP G2 cell line

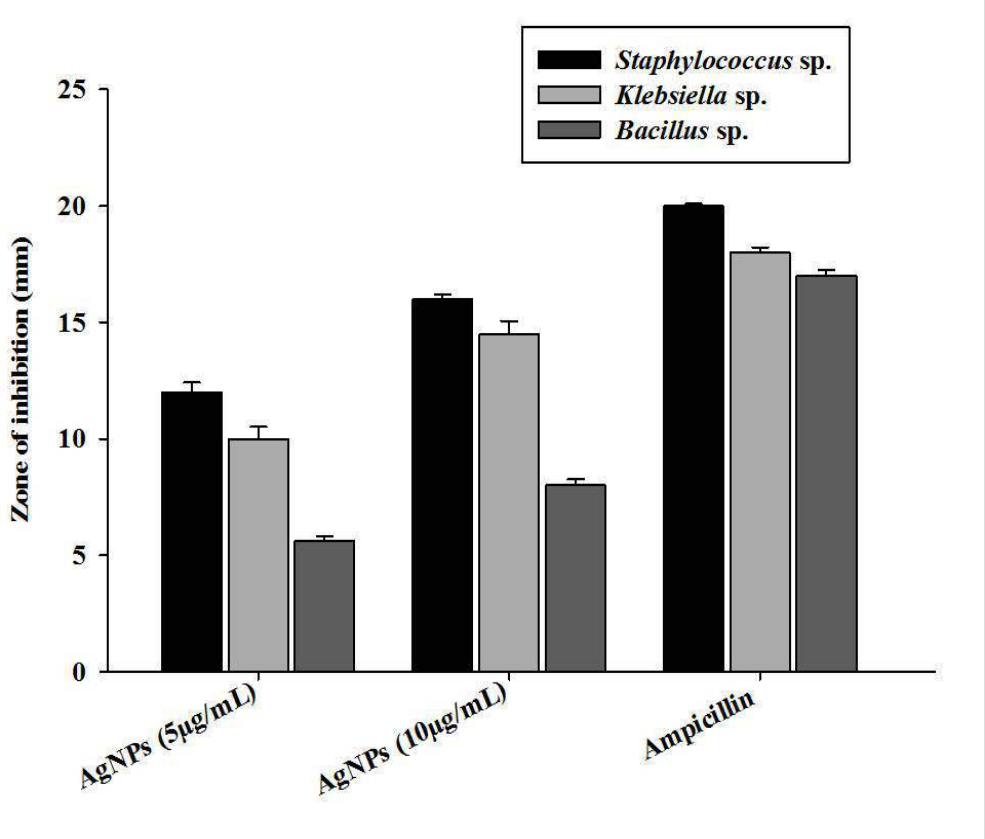

Fig.5. Antimicrobial activity of A. vasica mediated AgNPs. Error bars indicate standard deviation of means, where absent, bars fall within symbols

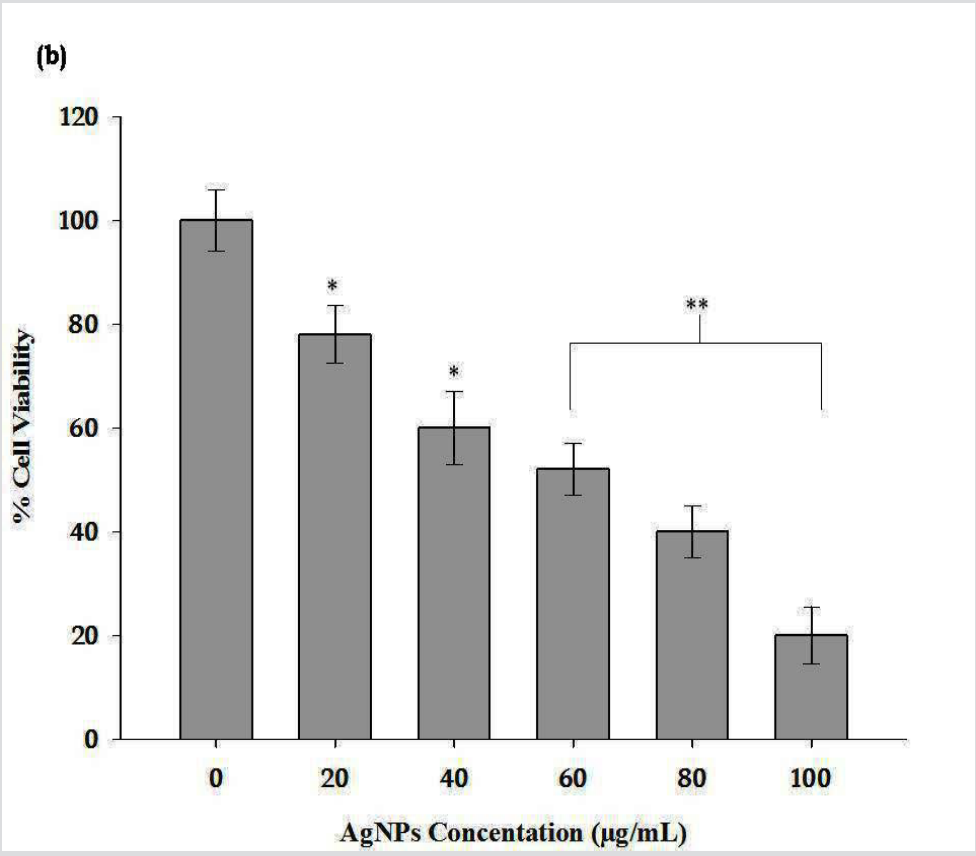

Fig.6. Cytotoxic effect of AgNPs on growth of HEP-G2 cell lines
(a) a 96-well plate image of MTT assay, the cytotoxicity is indicated by color reduction; (b) dose dependent reduction of cell viability at 595nm (*p<0.05; **p<0.01)

was assessed by estimating the percentage cell viability Tryphan blue dye uptake and measuring viable cells in the medium Fig. 6 (a). The average percentage cell growth inhibition in HEP G2 cell was found to be dose dependent in nature. The cytotoxic nature of *A. vasica* synthesized AgNPs was assed using MTT assy, and Fig 6 (b) shows the the percentage of live cells after various concentration (20-100 µL) of AgNPs treatment. Increasing of AgNPs increased the percentage of cell growth inhibition. The cell death (18%) was reported at 20 µg/mL and gradually increased according to the concentration and reached the maximum (78%) at 100 µg/mL. Inbathamizh et al. [34] showed the viability of HEP G2 cells was found to be 16.39% at 1 mg/mL concentration of AgNPs. Aravinthan et al. [21] recently reported the toxicity of phytosynthesized AgNPs in splenocytes, and found that the cytotoxicity is directly proportional to an increasing concentration of nanoparticles.

Conclusions

In conclusion, we developed a simple, green, and efficient route to synthesize AgNPs by treating silver ions with *A. vasica* extract at room temperature without using any harmful reducing, capping or dispersing agents. *A. vasica* plays an important role as the biofriendly reducing and stabilizing agent reduces the cost of production, and the environmental impact. The synthesized AgNPs showed more antibacterial activity and cytotoxicity activity as evidence by MTT assay with HEP-G2 cell lines. The present research work showed that the synthesized AgNPs using such *A. vasica* extract are ready for the application in the field of nanomedicine against multidrug resistant pathogenic bacteria.

Author Contributions

Arumugam Sengottaiyan conceived the research idea; Thangaswamy Selvankumar, Balakrishnan Senthikumar and Koildhasan Manoharan designed the experiments, analyzed the data and wrote the manuscript; Chinnappan Sudhakar, Muthusamy Govarthanan and Kandasamy Selvam performed the experiments; Thangaswamy Selvankumar joined the final discussions; Arumugam Sengottaiyan had primary responsibility for final content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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