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To cite this article: Gunashekar Kalvakunta Subramanyam, Susmila Aparna Gaddam, Venkata Subbaiah Kotakadi, Hema Gunti, Sashikiran Palithya, Josthna Penchalaneni & Varadarajulu Naidu Challagundla (2023) Green Fabrication of silver nanoparticles by leaf extract of *Byttneria Herbacea Roxb* and their promising therapeutic applications and its interesting insightful observations in oral cancer, *Artificial Cells, Nanomedicine, and Biotechnology*, 51:1, 83-94, DOI: [10.1080/21691401.2023.2173218](https://doi.org/10.1080/21691401.2023.2173218)

To link to this article: <https://doi.org/10.1080/21691401.2023.2173218>



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Green Fabrication of silver nanoparticles by leaf extract of *Byttneria Herbacea Roxb* and their promising therapeutic applications and its interesting insightful observations in oral cancer

Gunashekar Kalvakunta Subramanyam^a, Susmila Aparna Gaddam^b , Venkata Subbaiah Kotakadi^c , Hema Gunti^d , Sashikiran Palithya^a , Josthna Penchalaneni^e  and Varadarajulu Naidu Challagundla^a 

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ABSTRACT

The present research was carried out to look into therapeutic insight of biosynthesized silver nanoparticles (AgNPs) by leaf extract of *Byttneria herbacea* Roxb (BH). The analysis of biosynthesized BH-AgNPs by UV-visible spectroscopy shows an intense surface plasmon resonance (SPR) peak at 422 nm initially and 437 nm after 30 min which certainly reveals the formation of BH-AgNPs. Fourier Infra-red Spectroscopy (FT-IR) reveals that BH-AgNPs are biosynthesized by using different bioactive compounds like O-H stretch of free hydroxyl alcohol and phenols, N-H bond of primary amines present in the leaf extract. Transmission Electron Microscope (TEM) analysis revealed that BH-AgNPs are almost spherical in nature with an average size range from of 2 nm to 12 nm. The particle size analysis by Dynamic Light Scattering (DLS) reveals that the BH-AgNPs are poly-dispersed in nature with an average size of 8 nm ± 2 nm, with a negative zeta potential value of -21 mV which reveals the biosynthesized BH-AgNPs are very stable. The BH-AgNPs (*Byttneria herbacea* -AgNPs) revealed excellent free radical scavenging activity and exceptional antimicrobial activity. The anti-proliferative and cytotoxic studies in KB oral cancer cells revealed biosynthesized BH-AgNPs can employ as future novel therapeutic agents in cancer treatment and other biomedical applications.

ARTICLE HISTORY

Received 4 October 2022
Revised 23 December 2022
Accepted 8 January 2023

KEYWORDS





Green AgNPs; spectral characterization; anticancer studies; MTT assay; Cell cycle; Apoptosis assay; Expression of Caspase 3; KB oral cancer cells

Introduction

The present era of research is mainly focussed on Nano-materials and Nanostructures derived from physical, chemical and biological sources that play an important role in nanotechnology for their promising industrial, pharmaceutical and biomedical applications. Over the past few decades, the metal nanoparticles fabricated from different noble metals exhibited distinct biological, physical and chemical properties. Among these metal nanoparticles silver nanoparticles (AgNPs) became the most commonly investigated, interesting and challenging nano-materials suitable for various potential therapeutic applications [1–2]. At present, the nano-sized particles less than 100 nm in size are gaining attention abundantly due to their new applications in various industries like medical, health care, food and pharmaceuticals. Presently, in the past two decades, it clearly revealed that silver nanoparticles attained great interest due to their shape, size and size distribution which plays an important role in optical, electromagnetic, electrical, thermal, catalytic and biological properties [3–6]. Currently, widespread research on silver

nanoparticles reveals excellent anticancer and antimicrobial properties [7–10].

Worldwide the cancer-related deaths are a major concern. Non-communicable diseases (NCD) like cancer is emerging as major public health problems in India. The most common cancers in men are lung cancer, mouth or oral cancer, stomach and oesophagus and among women wide spread cancers are breast cancer, cervix and uteri cancer. India is highly populated country with 1.417 million populations, according to a study in the year 2022 more than 1.39 million new cancer cases were registered and 7,70,230 deaths due to cancer in the year 2020. In India, the incidence of cancer cases is likely to increase from 1.46 million in 2022 to 1.57 million in 2025. Most prevalent cancer among the population is breast cancer, oral cancer, cervical cancer, lung cancer, stomach cancer and colorectal cancer. Oral cancer is the most common cancer among men and women in the world, Especially in India more than 16.1% of men and 10.4% of women are prone to oral cancer when compared with other cancers [11]. **Keeping in-view above oral cancer prevalence, we have initiated our present work.** The green synthesized metal

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nanoparticles exhibit numerous biomedical applications. Green synthesis of metallic nanoparticles has gained a lot of interest due to its bio-safe and eco-friendly with low-cost simple method [12–13]. This efficiently reduces the use of toxic chemicals to the maximum and protects the environment. In recent years, the green synthesis of silver nanoparticles was reported using different plant extracts which exhibit numerous functions such as antimicrobial, wound healing, anti-inflammatory and anticancer studies [14–19]. Recent studies revealed that silver nanoparticles (AgNPs) fabricated by aqueous leaf extracts of *Aristolochia bracteolata* revealed significant larvicidal and pupicidal efficiency on larvae and pupa of *Culex quinquefasciatus* mosquito vector. *Chloroxylon swietenia* leaf extract-mediated silver nanoparticles act as both food dye degrading and antibacterial agents against *Staphylococcus nepalensis* and *Pseudomonas stutzeri* food-borne pathogens. The leaf extract mediated AgNPs by *Ctenolepis garcini* L., showed good antibacterial activity against both gram-positive and gram-negative bacteria and also revealed excellent antioxidant activity followed by anticancer potential against HepG2 cell line. The silver nanoparticles with *Azadirachta indica* aqueous kernel extract have good anti-inflammatory activity as well as anti-diabetic activity. The biosynthesized of silver nanoparticles (AgNPs) from *Kigelia africana* leaves (Lam.) Benth. extract revealed good cytotoxicity against RINm5F insulinoma cell line and the expression of the Peroxisome proliferator-activated receptor gamma (PPAR γ) gene was upregulated in the RINm5F insulinoma cell line confirming the AgNPs have good anti-diabetic efficacy. [20–24]. The green synthesised silver nanoparticles are said have excellent anticancer properties which clearly disclose with the above studies in various cancers cell lines in both *in vitro* and *in vivo*. Though they are several studies on various cancers, very few studies are there in oral cancer anticancer studies. **So an important medicinal plant *Byttneria Herbacea*** leaf extract was selected for our study to green synthesis silver nanoparticles and to characterize them by different advanced spectroscopy methods and look into their biomedical applications like antibacterial activity, antioxidant activity and most importantly to explore the *in vitro* anticancer properties in KB oral cancer cell line.

The present selected plant species *Byttneria herbecea* Roxb (Figure 1(A)) is a procumbent aromatic plant that grows up to 215 cm with 4–10 cm long woody root-stocks which is

perennial in nature which has been collected from the Seshachal hills of Tirumala, Tirupati, Andhra Pradesh, India. It is also recognized as an endemic plant to the Indian Peninsular region by the Wildlife Institute of India, Dehradun s Envis Bulletin [25]. The identified plant was confirmed and authenticated [Herbarium No.NN2122/PURSESVU/2020] by Dr. N.Nagaraju, Associate Professor and Head Retd, Department of Botany, Sri Venkateswara Arts and Science College, Tirupati, Andhra Pradesh and a voucher specimen has been deposited in the department. The previous reports on the phytochemical analysis of different plant parts of *B. herbacea* revealed that the plant parts are rich in flavonoids, steroids, phenol, alkaloids, cardiac glycosides, tannins, fatty acid & lipid, volatile oil, carotenoids, saponin, gum & mucilage, anthraquinones, anthocynin & iridoids [26]. *Byttneria herbacea* Roxb., leaves, roots and stem are widely used as ethnic medicine to cure various disorders like dysentery and impaction by using leaves [27], treat leprosy[28], relief against asthma [29], retention of the placenta [30], treatment of fractures in limbs [31] for treatment of leucorrhoea and inflammation [32] wound healing, swellings and relief of body pains by root paste [33–35] and finally, they also possess good antioxidant potential [36]. National Medicinal Plant Board [37] enlisted *B. herbacea* as medicinal plant in India. Based upon the above biomedical properties the *Byttneria herbecea* Roxb is selected for further studies on green synthesis of silver nanoparticles.

2. Material and methods

2.1. Collection of plant material

Fresh leaves of *Byttneria herbacea* Roxb. were collected from Talakona forest region of Seshachala hills of Tirumala, Tirupati and they are washed thoroughly tap water quite a few times and finally followed by distilled water [9–10]. The washed leaves were air-dried on coarse filter paper for week-days, after that the dried leaves were macerated into a fine powder with the help of an electric blender.

2.2. Biosynthesis of BH-AgNPs

15 grams of fresh macerated leaf powder was added to 150 ml of Milli Q water and the sample was heated at 70° C

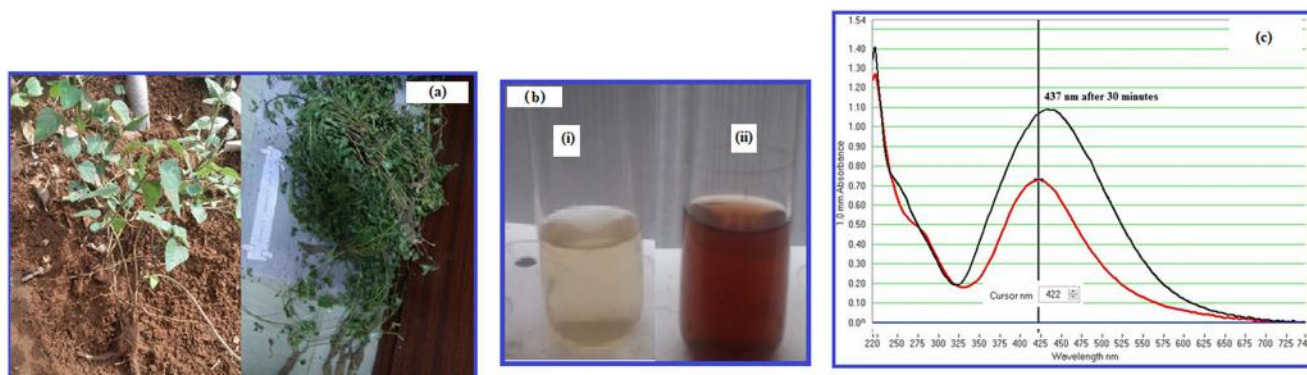


Figure 1. (a). *Byttneria herbacea* plant, (b). (i) Plant extract of *Byttneria herbacea* (ii). Green synthesized BH-AgNPs by leaf extract of *Byttneria herbacea* (c). UV-Visible absorption spectra of BH-AgNPs synthesized from *Byttneria herbacea* leaf extract with 0.02 M silver nitrate.

for one hour in a hot water bath and left overnight. Next day the aqueous leaf extract of *Byttneria herbacea* Roxb was the filter through muslin cloth followed by whatmann no 1 filter paper. This filtered extract was stored at 4 °C in the refrigerator for further studies.

The biosynthesis of silver nanoparticles (AgNPs) was carried out by the protocol [9–10]. The biosynthesized BH-AgNPs colloidal solution was centrifuged at 12000 rpm per 15 min, the obtained BH-AgNPs pellet was washed thrice with Milli Q water by repeating the centrifugation step to remove the unbound plant bio-molecules on the surface of the BH-AgNPs. The final pellet of biosynthesized BH-AgNPs was dissolved in sterile Milli Q water and used for further spectral characterization and biomedical applications.

2.3. Spectral Characterization of BH-AgNPs

The biosynthesized BH-AgNPs were further spectral characterized by various spectroscopic methods. The biosynthesized silver nanoparticles were analyzed in UV-visible Spectrometer, and recorded its surface Plasmon resonance (SPR) peak was by sampling 1 μ L – 3 μ L of the sample at regular time intervals at room temperature at the resolution of 1 nm between 220 nm –750 nm on the Nanodrop 8000 (Thermo Scientific, USA). Fourier transform infra-red spectroscopy (FTIR) analysis of *Byttneria herbacea* Roxb leaf extract and biosynthesized BH-AgNPs was carried out using Bruker Tensor 27 (Thermo Scientific, USA) to discover the bioactive compounds present in the leaf extract and which bioactive compounds are actively involved bio-reduction and biosynthesis of BH-AgNPs. Particle size and Zeta potential analysis were carried out by dynamic light scattering (DLS) method by using Nanopartica Horiba SZ-100, (Japan), to find out the size distribution and stability of biosynthesized BH-AgNPs in the purified aqueous sample. HR-TEM analysis was carried out to find out the shape and size of biosynthesized BH-AgNPs by coating a drop of the biosynthesized BH-AgNPs on carbon-coated copper grid, the sample was dried under a mercury lamp for 5 min prior to TEM analysis. Energy Dispersive X-ray (EDAX) analysis was carried-out to know the elemental percentage of silver ions and other elements present in biosynthesized BH-AgNPs. Both TEM and EDAX analysis was carried out using Transmission Electron Microscope (FEI-Tecnaï G2 20 Twin, Vellore Institute of Technology (VIT), Vellore.

2.4. Antimicrobial Analysis of BH-AgNPs

The antibacterial competence of bio-synthesized BH-AgNPs was determined against six bacterial strains of both gram-negative bacteria such as *Escherichia coli* (Donor) rifampin resistant, *Escherichia coli* (Mutant) streptomycin resistant, *Escherichia coli* (recipient) streptomycin resistant, (Medox, Biotech India Pvt. Ltd), *Escherichia coli* and gram-positive such as *Staphylococcus aureus* and *Bacillus megatorium*, by using Agar disc-diffusion method standardized by previous studies [38–39]

2.5. Antioxidant activity of BH-AgNPs by DPPH method

Free radical scavenging activity of the BH-AgNPs was determined by using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to the method described by Mittal et al. [40]. The DPPH stock solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol and stored at 20 °C. DPPH solution (2 ml) was added to 1 ml of a methanol solution containing test samples of leaf extract of *Byttneria herbacea* Roxb and biosynthesized BH-AgNPs at different concentrations of 25 μ g/mL, 50 μ g/mL, 75 μ g/mL, and 100 μ g/mL. The DPPH radical scavenging activity (RSA) was measured by determining the absorbance at 517 nm, using ascorbic acid as standard and the antioxidant activity.

$$\text{RSA (\%)} = \left[\frac{(\text{control absorbance} - \text{sample absorbance})}{(\text{control absorbance})} \right] \times 100$$

2.6. In vitro MTT assay for cytotoxic activity of BH-AgNPs

$$(\text{MTT} = "3 - (4, 5 - \text{Dimethylthiazol} - 2 - \text{yl}) - 2, 5 - \text{diphenyl tetrazolium bromide}")$$

Cell viability was quantified by performing MTT assay as described by well-known Scientists [9,10,41,42]. Seeded 200 μ L KB cancer cell suspension in a 96-well plate at the required cell density (20,000 cells per well) and allowed the cells to grow for about 24 h. After 24 h different concentrations of BH-AgNPs 25 μ L, 50 μ L, 100 μ L and 200 μ L along with standard drug Camptothecin 7.5 μ M concentration were added and incubated on the plate for 24 h at 37 °C in a 5% CO₂ atmosphere. After the incubation period, add MTT reagent to a final concentration of 0.5 mg/mL of total volume. The plate was wrapped with aluminium foil to avoid exposure to light, keep the plate in an incubator and incubate for 3 h and then 100 μ L of DMSO the solubilization solution was added. The plate was read in ELISA reader at 570 nm and 630 nm was used as a reference wavelength. **The IC₅₀ value** was determined by using a linear regression equation, i.e. Y = Mx + C. Here, Y = 50, M and C values were derived from the viability graph.

2.7. Analysis of cell cycle study by BH-AgNPs

KB oral cancer cells are cultured in a 6-well plate at a density of 2 x 10⁵ cells/2 ml and were added and incubated in a CO₂ incubator overnight at 37 °C for 24 h. Aspirate the spent medium and treat the cells with IC₅₀ value of BH-AgNPs at the concentration of 89.25 μ L/mL and along with standard drug Camptothecin 7.5 μ M concentration was added in 2 ml of culture medium, except in cell control and incubate the cells for 24 h. The cells were centrifuged and fixed with 1 ml of cold 70% ethanol. The cell pellet was washed twice with PBS to ensure that only DNA is stained with Propidium iodide (PI stains all nucleic acids), so the cell pellet is treated with 50 μ L of Ribonuclease A to get rid of RNA and finally add 400 μ L PI solution per million cells directly to cells and mix

well. Finally incubate the cells for 5 to 10 min at room temperature in dark and then analyze the samples by flow cytometry.

2.8. Dual fluorescence study of apoptosis by BH-AgNPs on KB oral cancer cell

The KB oral cancer cells are cultured in a 6-well plate explained as above. The stained cells were observed under a fluorescence microscope with a filter cube with Excitation 560/40 nm and Emission 645/75 nm for EtBr (Ethidium bromide) and Excitation 470/40 and Emission 525/50 for Acridine orange and Hoechst 33258 staining fluorescence microscopy Excitation 352/461 nm and Emission 510/40 nm. Images were overlaid using Image-J Software v1.48.

2.9. Apoptosis analysis of BH-AgNPs on KB cell lines

The KB oral cancer cells are cultured in a 6-well plate explained as above. At the end of the treatment, remove the medium from all the wells and give a PBS wash and add 5 μ L of FITC Annexin V and gently vortex the cells and incubate for 15 min at RT (25 °C) in the dark, after incubation finally add 5 μ L of PI and 400 μ L of 1X Binding Buffer to each tube and vortex gently and analyze the samples by flow cytometry immediately.

2.10. Caspase 3 expression study by BH-AgNPs on KB cell lines

The KB oral cancer cells are cultured in a 6-well plate explained as above. At the end of the treatment, remove the medium from all the wells and give a PBS wash. The cells were fixed in 1 ml of prechilled cold 70% ethanol. The cells are pelleted at a higher speed and then aspirate the supernatant careful not to lose the pellet, then wash the pellet twice with PBS and then finally add 5 μ L of FITC Caspase 3 antibody. Mix thoroughly and incubate for 30 min in the dark at Room Temperature (20° to 25 °C). The cells were washed with 1X PBS with 0.1% sodium azide once and then finally add 0.5 ml of PBS and mix thoroughly and analyze the samples by Flow Cytometry.

3. Results and discussion

3.1. Uv- Visible spectral analysis of BH-AgNPs

The UV visible data proved that the aqueous leaf extract of *Byttneria herbacea* has capability to change 0.02 M silver nitrate solution into the ionic form Ag^+ , the diluted colourless leaf extract reacts with 0.02 M silver nitrate solution and reduce to ionic form Ag^+ to Ag^0 which is confirmed by the colour change of the solution to dark brown colour (Figure 1B(a–b)). The surface plasmon resonance spectrum (SPR) peak of biosynthesized BH-AgNPs was obtained at 422 nm, the same sample was read after 30 min revealed the SPR at 437 nm (Figure 1(C)). The results were similar to the previous reports on the green synthesis of silver

nanoparticles from various plants extracts, the SPR peak was reported to increase its intensity with an increase in time intervals and concentration of AgNO_3 from 0.001 M to 0.005 M. Likewise, the biosynthesized AgNPs by different plant extract like leaf extract *Boerhavia erecta*, *Artemisia annua*, *Decaschistia crotonifolia*, and flower extracts of *Aerva lanata* and *Ferulago macrocarpa* [15, 43–46] revealed similar results.

3.2. Ftir analysis of biosynthesized BH-AgNPs

FTIR analysis of *Byttneria herbacea* leaf extract and biosynthesized BH-AgNPs were shown in (Figure 2(a,b)). The IR peaks for *Byttneria herbacea* leaf extract found at 3395.44, 1629.71, 510.17 cm^{-1} . The peak at 3395.44 corresponds to O–H stretch of free hydroxyl alcohol and phenols, the peak at 1629.71 cm^{-1} is due to N–H bond of primary amines. The biosynthesized BH-AgNPs showed some prominent peaks in FTIR analysis such as 3388.44, 1629.60 & 506.27 cm^{-1} . The peaks at 3388.44 cm^{-1} were due to O–H bonds of alcohol and phenols the intensity of the peak is reduced when compared with the extract, peak at 1629.60 cm^{-1} corresponds to the stretch of N–H bending of primary amines and 506.27 cm^{-1} due to aliphatic iodo compounds of C–I stretch. FTIR results revealed that the different bioactive molecules like flavonoids, terpenoids, quinuones, polyphenols and steroids of the aqueous leaf extract of *Byttneria herbacea* may perhaps involve in the reduction of silver nitrate to BH-AgNPs by capping and stabilization. The FTIR results were alike to previous reports on green synthesis of silver nanoparticles by various plant extracts of *Artemisia Annu*, *Decaschistia crotonifolia*, *Aerva lanta*, *Ferulago macrocarpa*, etc. [9–10, 38–39, 47–48].

3.3. Particle size and zeta potential analysis of biosynthesized BH-AgNPs

The particle size analysis of BH-AgNPs was carried out using Dynamic light scattering (DLS). The results were calculated by the scattered light intensity with time, the size of biosynthesized BH-AgNPs was in the range of 1 nm – 17 nm. 10% of particles were less than 1 nm, 50% of them were 2.2 nm and 90% of them were 15.7 nm in range with an average size found to be 8 nm \pm 2 nm (Figure 3(a)). The biosynthesized BH-AgNPs revealed poly disperse index (PDI) around 0.196, which concludes that the particles are polydispersed in nature. Zeta potential analysis is a physical property that gives the net surface charge of the nanoparticles. The net surface charge of biosynthesized BH-AgNPs was negatively charged which was detected to be around –21 mV shown in (Figure 3(b)). The results of the surface charge of BH-AgNPs revealed that they have a high zeta potential value of –21 mV which gives more stability to biosynthesized BH-AgNPs in a colloidal solution. It is concluded that BH-AgNPs are well dispersed without any agglomeration due to high zeta potential value [9–10, 17].

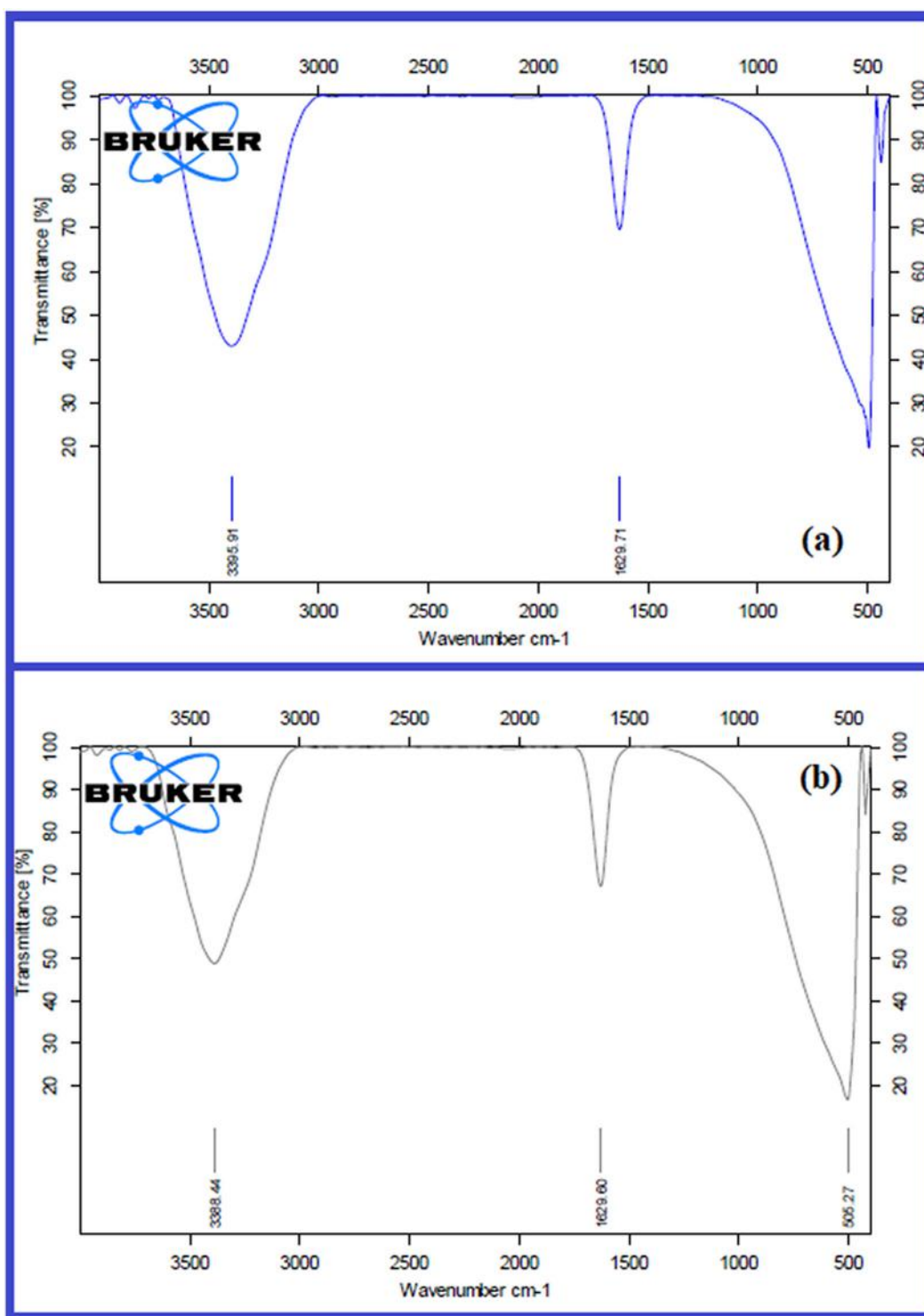


Figure 2. (a). FTIR absorption spectra of *Byttneria herbacea* leaf extract and Fig. 2. (b). BH-AgNPs synthesized from *Byttneria herbacea* leaf extract with 0.02 M silver nitrate.

3.4. Tem and EDAX analysis of biosynthesized BH-AgNPs

The size and morphology of biosynthesized BH-AgNPs were carried out by transmission electron microscopy, the results reveal the sizes of BH-AgNPs were range of 1 nm to 8 nm \pm 2 nm with spherical shape with an average size 8 \pm 2 nm, (Figure 4(A)) apparently the results were similar to DLS data. Similar type of result was observed from an earlier report [17]. The crystalline nature of biosynthesized BH-AgNPs was

further detected by selected area diffraction pattern (SAED) by TEM analysis showing well-defined sharp spots in a symmetric manner around the bright spots. The Energy dispersive X-ray analysis of BH-AgNPs reveals the elemental composition of the biosynthesized BH-AgNPs, it reveals the highest proportion peak of Ag obtained at 3.0 KeV, followed by some minor peak of C, O, Cu. EDAX data shows the weight percentage of elemental silver is 69.53%, Cu 13.25%,

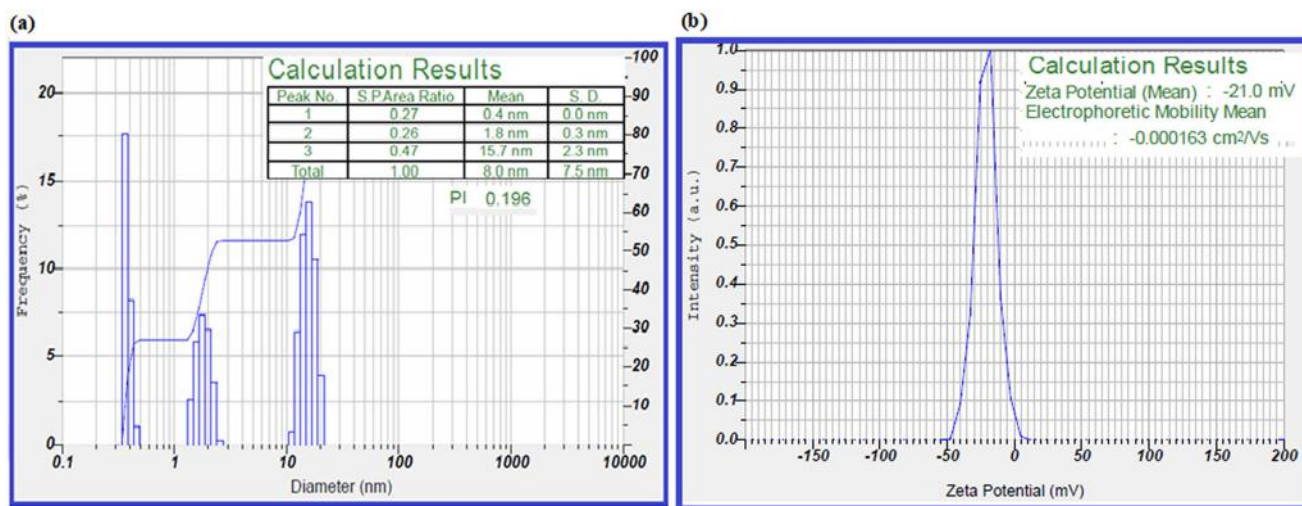


Figure 3. (a). Particle size analysis of BH-AgNPs biosynthesized with leaf extract of *Byttneria herbacea* Fig.3. (b). Zeta Potential analysis of BH-AgNPs biosynthesized with leaf extract of *Byttneria herbacea*.

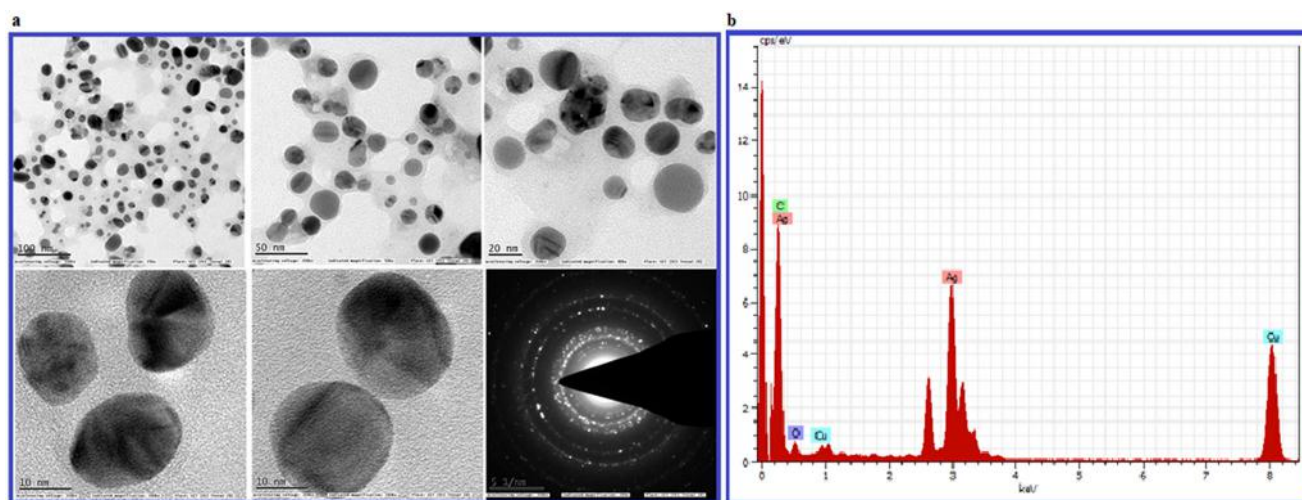


Figure 4. a. TEM images of biosynthesized BH-AgNPs at different magnification (a) 100 Kx (b) 50 Kx (c) 20Kx (d) 10Kx (e) 10 Kx and (f) SAED pattern showed four diffraction rings Fig. 4. b. EDX analysis of biosynthesized BH-AgNPs.

carbon 16.25% and oxygen 0.98% (Figure 4(b)). The results were similar to our previous reports [9–10].

3.5. Bactericidal activity of biosynthesized BH-AgNPs

Presently many researchers are working on antimicrobial activity using different metal nanoparticles because the bacterial strains are becoming very resistant to antibiotics. So it is important to find out an alternative source for the future against these bacterial strains which can overcome the present problem. So in the present study, an attempt has been made to study the antibacterial activity of biosynthesized BH-AgNPs. The results revealed that the biosynthesized BH-AgNPs showed excellent antibacterial activity against six bacterial species viz., Gram -ve antibiotic-resistant *Escherichia coli* (Donor strain) rifampin resistant, and *Escherichia coli* (Recipient strain) streptomycin-resistant and *Escherichia coli* (mutant strain) streptomycin resistant and *Escherichia coli* and Gram +ve *Staphylococcus aureus*, *Bacillus megatrium*. The zone of inhibition (ZOI) of BH-AgNPs at different

concentrations of 10 mcg, 20 mcg, 30 mcg and 40 mcg along with standard antibiotic Amoxyclav 30 mcg (Himedia-SD063) in different bacterial cultures was shown in Figure 5(a) and ZOI values in mm were measured and tabulated in Table 1 by comparing with standard antibiotic Amoxyclav 30 mcg. From the above results, it is concluded that the BH-AgNPs have an excellent and efficient antibacterial activity when compared with standard antibiotics shown in Figure 5(b). It also reveals that lower concentrations revealed an excellent zone of inhibition which equivalent to the concentration of standard antibiotic. The higher concentrations of BH-AgNPs were superlative than the standard antibiotic Amoxyclav 30 mcg. According to previous studies, it's well known that metal nanoparticles exhibit very good antibacterial activity, especially silver nanoparticles revealed superior antibacterial activity due to their physicochemical properties. The shape and size of silver nanoparticles play an important role in these processes, they cause lethal effect by three mechanisms, when they come in contact with the surface of bacteria, they attach to the cell surface and disturb the cell wall

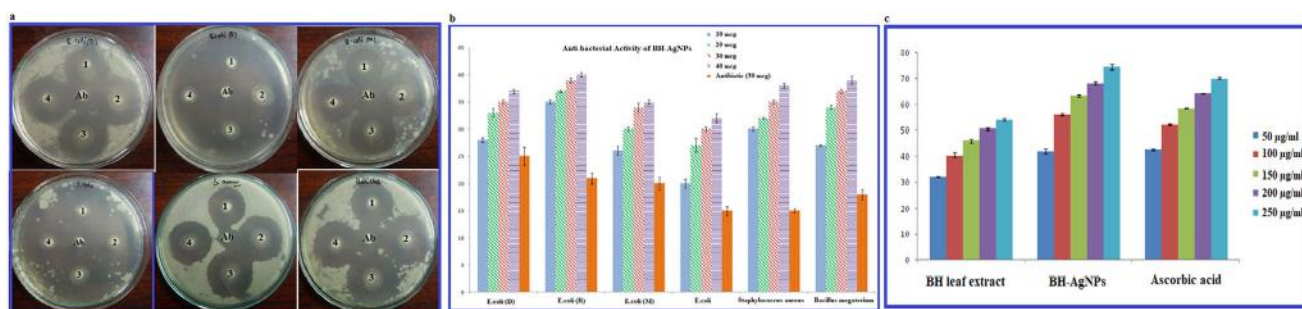


Figure 5. a. Antibacterial activity of biosynthesized BH- AgNPs on Gram negative antibiotic resistant *E.coli* strains *E.coli* Mutant strain, *E.coli* Donor strain, *E.coli* recipient strain, and gram positive *Staphylococcus aureus* and *Bacillus megaterium* at different concentrations of 10mcg, 20 mcg, 30mcg and 40mcg and centre Ab-Antibiotic (Amoxyclav 30mcg, Himedia-SD063) Fig 5.b. Graphical representation of Antibacterial activity of green synthesized BH- AgNPs on Gram negative antibiotic resistant *E.coli* strains *E.coli* Mutant strain, *E.coli* Donor strain, *E.coli* recipient strain, and gram positive *Staphylococcus aureus* and *Bacillus megaterium* and antibiotic Amoxycillin 30 mcg Himedia-SD063 Fig 5.c. Antioxidant activity of biosynthesized BH- AgNPs by DPPH method $\mu\text{g/mL}$.

Table 1. Antimicrobial Activity of BH-AgNPs.

S.No.	Bacterial Species	10 μl (10 mcg)	20 μl (20 mcg)	30 μl (30 mcg)	40 μl (40 mcg)	Antibiotic Amoxyclav (30 mcg)
1	<i>E.coli</i> (D) rifampin resistant	28	33	35	37	25
2	<i>E.coli</i> (R) streptomycin resistant	35	37	39	40	21
3	<i>E.coli</i> (M) streptomycin resistant	26	30	34	35	20
4	<i>E.coli</i>	20	27	30	32	15
5	<i>Staphylococcus aureus</i>	30	32	35	38	15
6	<i>Bacillus megatorium</i>	27	34	37	39	18

Table 2. DPPH antioxidant activity of biosynthesized BH-AgNPs.

S.No.	Concentration	<i>Byttneria Herbacea</i> Leaf Extract	BH-AgNPs	Ascorbic acid
1	50 $\mu\text{l/mL}$	32.16	42.01	42.67
2	100 $\mu\text{l/mL}$	40.44	56.12	52.23
3	150 $\mu\text{l/mL}$	46.12	63.48	58.64
4	200 $\mu\text{l/mL}$	50.64	68.26	64.34
5	250 $\mu\text{l/mL}$	54.12	74.78	70.26

and enter the bacterial cell and cause disturbance in permeability and respiration. Thus induce DNA damage and ROS stress and also stops the production of ATP, which finally lead to uncontrollable ion transport through the cell membrane and finally leads to bacterial cell death. So it is concluded that BH-AgNPs exhibited exceptional antibacterial activity due to their spherical shape and very minute size of less than 10 nm. So BH-AgNPs can be useful as excellent antibacterial agents in the future [9–10, 13–14, 17, 46–50]. As per recent studies, Chloroxylon *swietenia* leaf extract-mediated silver nanoparticles act as very good antibacterial agents against *Staphylococcus nepalensis* and *Pseudomonas stutzeri* food-borne pathogens in which the nanoparticles are varied shape and size. The AgNPs biosynthesized by *Ctenolepis garcini* L. also showed good antibacterial activity against both gram-positive and gram-negative bacteria with different sizes and shapes [21–22].

3.6. Dpph antioxidant activity of biosynthesized BH-AgNPs

It is well known that certain types of unstable molecules consist oxygen, which readily reacts with other molecules in a cell, these unstable molecules are known as reactive oxygen

species (ROS) which readily and easily reacts with DNA, RNA, proteins and lipids of the cell and cause cell death. These different reactive oxygen species cause oxidative stress in various human cells and cause disorders like inflammation, cancer and ageing and other neurodegenerative disorders. So it is very important and useful to find out suitable antioxidants which can reduce ROS. In the present study, *in vitro* antioxidant activity of the BH-AgNPs was determined by DPPH free radical scavenging assay. The results revealed that BH-AgNPs exhibited concentration-dependent scavenging activity against both DPPH [Table 2 & Figure 5c]. *Byttneria herbacea* leaf extract (BH-LE) and BH-AgNPs showed the maximum inhibition of 54.12% and 74.78%, respectively against DPPH radicals at the highest concentration of 250 $\mu\text{g/mL}$ used in this assay. The DPPH activity of BH-AgNPs is greater than the standard ascorbic acid 70.26% at the highest concentration. From this assay, it can be concluded that the BH-AgNPs have efficient scavenging activity. There are several other studies reported previously, where the silver nanoparticles exhibited excellent antioxidant activity. [9–10, 14–15, 48, 51]. So, it reveals that the biosynthesized BH-AgNPs can be useful as future antioxidant agents.

3.7. In vitro MTT assay for cytotoxic activity of BH-AgNPs

The results of MTT assay revealed the cytotoxic effect and cell viability of KB oral cancer cell lines treated with BH-AgNPs and Camptothecin (standard drug). The cells were treated with BH-AgNPs for 24 h with a range of concentrations (Figure 6(A)). The IC_{50} value was calculated after 24 h. The IC_{50} value was calculated by using a linear regression equation, i.e. $Y = Mx + C$. Here, $Y = 50$, M and C values were

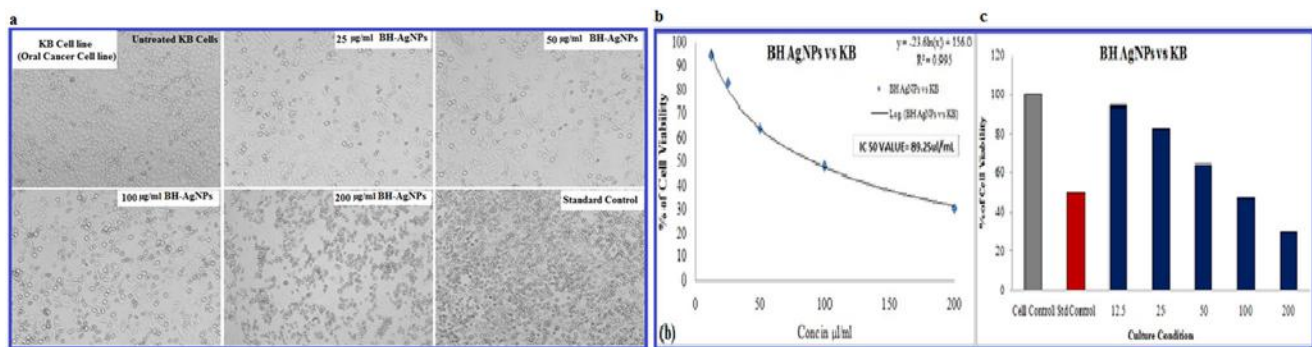


Figure 6. a. MTT assay of cytotoxic studies of green synthesized BH-AgNPs on KB –Human oral carcinoma cell line and its Morphological changes of KB cells observed under an inverted light microscope. a. Untreated KB cell line b. KB cell line cells treated with 25 µg of BH-AgNPs c. KB- cell line treated with 50 µg of BH-AgNPs d. KB cell lines treated with 100 µg of BH-AgNPs e. KB cell lines treated with 200µg of BH-AgNPs and f. Standard control (Camptothecin 7.5 µM). Fig.6.b and c. Comparative percentage of Cell viability studies of green synthesized BH-AgNPs on KB –Human oral carcinoma cell line by MTT assay and the IC₅₀ VALUE is calculated to be = 89.25µL/mL.

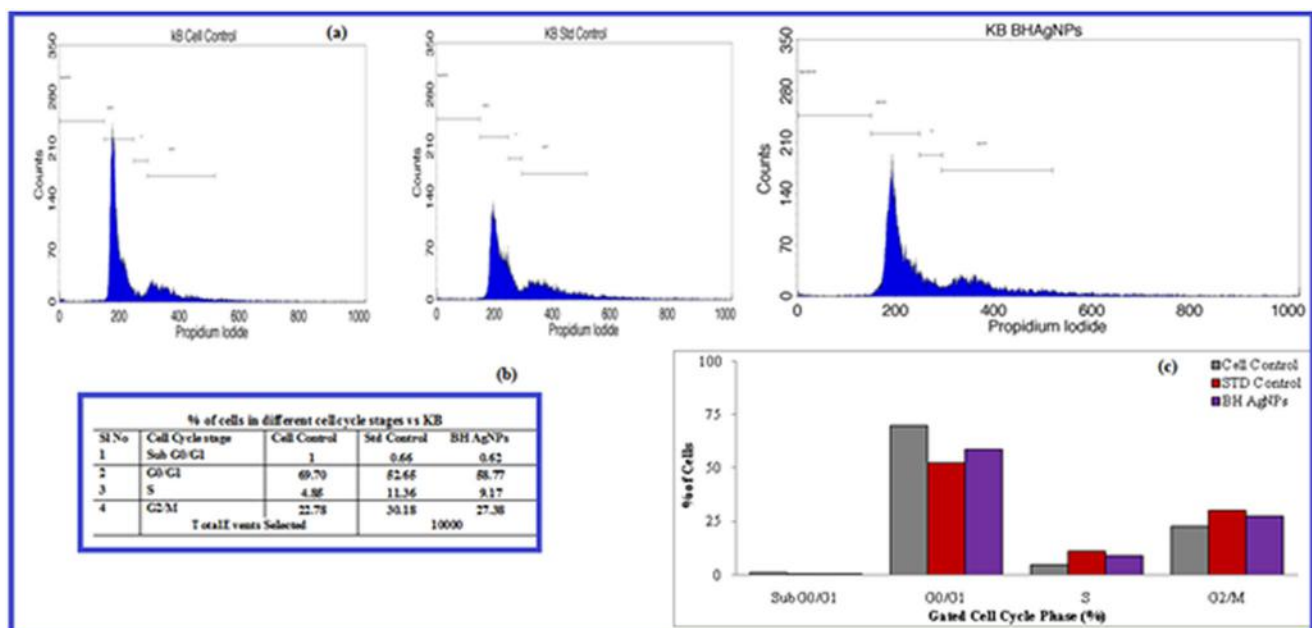


Figure 7. a.b.c. Cycle cell assay of green synthesized BH-AgNPs on KB –Human oral carcinoma cell line (a): Flow cytometric histograms showing the phases of cell cycle distribution in the KB cell line treated with BH AgNPs with IC₅₀ values and standard drug camptothecin at IC₅₀ value compared to the control. (b) Table showing the % of Cells gets arrested in the different stages of their life cycle. (c) Overlay showing the % of cells gets arrested in the different stages of their life cycle.

derived from the viability graph (Figure 6(b,c)). The IC₅₀ value of BH-AgNPs was calculated by statistical data of cell cytotoxicity studies reveal that IC₅₀ value of 89.25 µL/mL. The cytotoxicity of biosynthesized BH-AgNPs is dosage-dependent manner, as the concentration increase the cell viability decreases shown in Figure 6(b,c). The results showed that BH-AgNPs have anticancer nature and revealed good cytotoxic potency against KB human Oral cancer cells. Similarly, *Nepeta deflersiana*-mediated silver nanoparticles show toxicity against Human Cervical Cancer Cells (HeLa), *Seripheidium quettense*-mediated green synthesis of biogenic silver nanoparticles also showed very good theranostic applications against cancer [52–53]. The AgNPs by leaf extract of *Ctenolepis garcini L* revealed excellent anticancer potential against HepG2 cell line and the biosynthesized silver nanoparticles (AgNPs) from *Kigelia africana* leaves (Lam.) Benth. extract revealed good cytotoxicity against RINm5F insulinoma cell line. [22, 24]. So in keeping view of the above studies, it's

very essential to determine the molecular mechanism behind anticancer properties of the BH-AgNPs and conducted further studies.

3.8. Cell Cycle assay by BH-AgNPs on oral cancer cell lines

The BH-AgNPs detected have substantial cell inhibition, the study was conducted by selecting the IC₅₀ concentrations of 89.25 µL/mL, against the KB cell lines and evaluated Cell Cycle study results by Flow Cytometry. The cells treated with Std Control and BH-AgNPs with a concentration of 89.25 µL/mL. The results revealed follows: the % of Cells get arrested in the different stages of their life cycle. In Sub G0/G1 phase (Apoptotic phase), **1%**, **0.66%** and **0.62%** of cells get arrested in Untreated, Standard and BH AgNPs respectively. In G0/G1 phase (Growth Phase), **69.70%**,

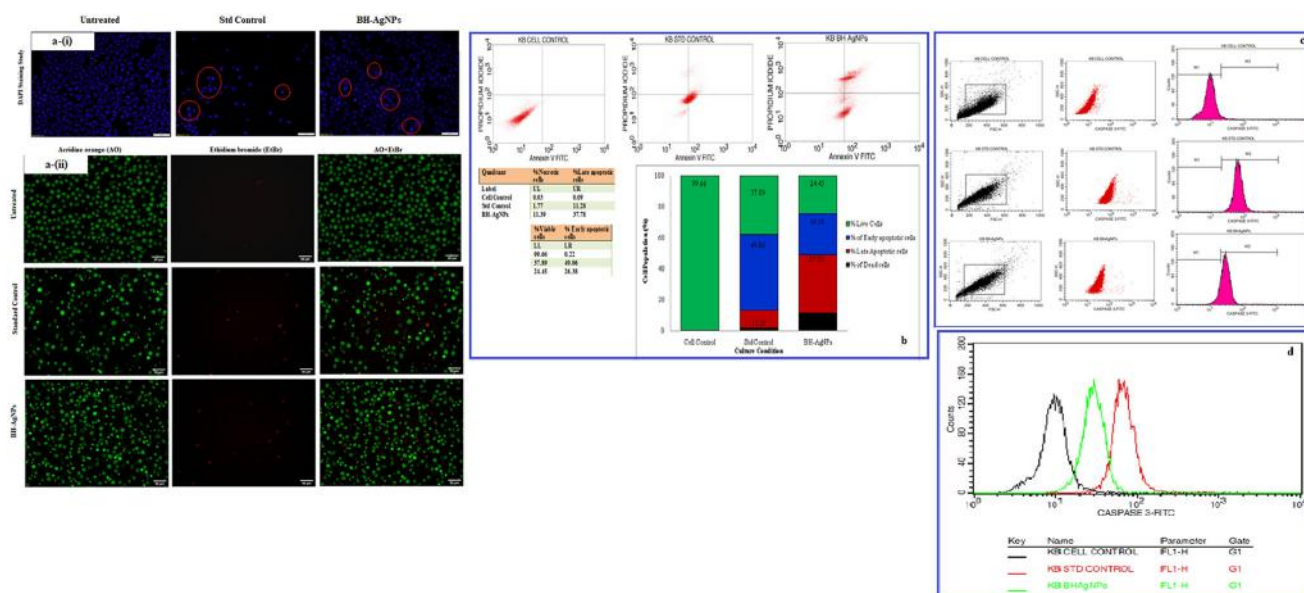


Figure 8. a(i). Effect of green synthesized BH-AgNPs on nuclear damages in KB oral cancer cells were observed with Hoechst 33258 staining under fluorescence microscopy. (A) Control (untreated), (B) Standard Control (7.5 $\mu\text{g/mL}$) and (C) BH-AgNPs (89.25 $\mu\text{g/mL}$) for 24 h. Fig.8. a(ii). Analysis of Dual Fluorescent staining of Acridine orange and Ethidium bromide on KB oral cancer cells of (A) Untreated, (B) Standard Control and (C) BH-AgNPs used to identify apoptosis-associated changes of cell membranes during the process of apoptosis and also to accurately distinguish cells in different stages of apoptosis Fig. 8.b. Apoptotic assay of green synthesized BH-AgNPs on KB –Human oral carcinoma cell line by Flow cytometry assay Figure. 8.c. d. Expression of Apoptotic protein Caspase 3 analysis on KB –Human oral carcinoma cell line treated with green synthesized BH-AgNPs.

52.65% and 58.77% of cells get arrested in Untreated, Standard and BH AgNPs respectively. In S phase (synthetic phase), **4.85%, 11.36% and 9.17%** of cells get arrested in Untreated, Standard and BH-AgNPs respectively. On the other hand, in G2/M phase, **22.78%, 30.18% and 27.38%** of cells get arrested in Untreated, Standard and BH-AgNPs respectively. BH-AgNPs exhibit prominent Cell Cycle phase arrest similar to the std Control, Camptothecin on KB cells. Figure 7(a,b,c). In the present study also the BH-AgNPs showed cell cycle arrest at G2/M stage which leads to induced cell death, thus proves to BH-AgNPs as effective anticancer agents against oral cancer cells. A report on *Nepeta deflersiana* extract-mediated AgNPs also showed that the cell cycle and apoptosis were induced by SubG1 arrest and lead to apoptotic/necrotic cell death in HeLA cells [52]. Similar type of results were observed by AgNPs biosynthesized by *Hydrastis canadensis* and *Thuja occidentalis* induce differential cytotoxicity through G2/M arrest in A375 cells [54]. Our earlier leaf extract of *Argyria nervosa* also showed cell cycle arrest at the G2/M stage, further leads to induced cell death [10]. The present study concludes that BH-AgNPs was confirmed to be a competent anticancer agent against KB oral cancer cells.

3.9. Dual fluorescence assay by BH-AgNPs

The results revealed the apoptotic nature of BH-AgNPs by fluorescent staining when compared with treated KB cells to untreated cells, they show more number of early apoptotic cells and late apoptotic cells and necrotic cells with chromatin condensation and blebbing of the cell membrane in the human oral carcinoma cells was very clearly observed (Figure

8(a). The final results were similar to that of standard drug Camptothecin treated cells indicating that BH-AgNPs have very good anticancer activity. Similarly, the biosynthesized AgNPs using leaf extract of *Flemingia wightiana*, *Saccharina japonica* and Asian Spider Flower showed apoptotic cells and late apoptotic cells with blebbing of the cell membrane, chromatin condensation, followed by necrotic cells [55–57].

3.10. Apoptotic assay by annexin V-FITC using BH-AgNPs

The apoptosis assay of BH-AgNPs by flow cytometry was shown in Figure 8(b). The results revealed that KB cancer cell lines treated with BH-AgNPs at conc. 89.25 $\mu\text{g/mL}$ and Camptothecin (7.5 μM) along with controls reveal that early apoptosis is 26.38% for BH-AgNPs and 49.06% for Camptothecin is considerably lower. But the rate of late apoptosis induced by both BH-AgNPs and Standard Camptothecin was 37.78% and 11.28% which was appreciably increased, but the percentage of necrotic cells was also appreciably increased as follows 11.39% for BH-AgNPs and 1.77% for Standard control. It is well known that KB oral cancer cells which show early apoptosis are FITC Annexin V positive and PI negative; but in case of late apoptosis both FITC Annexin V and PI positive. We hereby concluded that the KB cells treated with IC_{50} concentration of BH-AgNPs and Camptothecin (7.5 μM) reveals that the total apoptosis cells in Q1(75.55% of necrotic cells or dead cells) Q2 (62.11% of late apoptosis cells) and Q4(% of early apoptosis) and Q3 (% of live cells) 24.45% and 37.89% indicating that the BH-AgNPs can be an outstanding anticancer agent against KB oral cancer cells. The biosynthesized silver nanoparticles (BH-AgNPs) showed significant apoptotic nature then the

standard drug shown in (Figure 8 (b)). The final observations and results recommended and concluded that the test compound, BH-AgNPs induces significant necrosis and in human oral cancer cells. The previous reports on *in vitro* studies in MCF 7 cancer cells and other cancer cell lines by silver nanoparticles also revealed similar results [58–59]. So, we recommend that the BH-AgNPs may have good therapeutic potential against human oral cancer; further preclinical studies have to be done to confirm the mechanism of action on KB oral cancer cells.

3.11. Expression of caspase 3 in KB oral cancer cell line

Finally, an important experiment was performed to find out the expression of apoptosis protein caspase 3. The caspase 3 expression was evaluated with the dose of IC₅₀ BH-AgNPs at conc. 89.25 µg/mL and standard drug (Camptothecin) at the concentration of 7.5 µM. The results after 24 h were represented in Figure 8(c, d). From the histogram of the results it's clear that the cells are gated in M1 and M2 phase, of which M1 refers to negative expression and M2 phase refers to positive expression of Caspase 3. So in the current study, both the treated and untreated cells were gated in both phases and analyzed using Cell Quest Software, Version 6.0. The analysis of the study revealed that Caspase 3 expression is very low in untreated cells, i.e. 9.90 Mean Fluorescence Intensity (MFI), but its very high in the case standard drug, i.e. 71.16 MFI, whereas the cells treated with biosynthesized BH-AgNPs revealed 50.04 MFI of Caspase 3 expression after 24 h. From the results, BH-AgNPs treated cells showed content and significant apoptotic potential with high expression of Caspase 3. The expression of apoptotic protein caspase 3 was high with the standard drug Camptothecin when compared with BH-AgNPs. Similar type of results was observed by earlier researchers who worked on the expression of Caspase 3 by silver nanoparticles synthesized by *Argyrea nervosa* leaf extract [10]. An another important study on Caspases expression was done by using silver nanoparticles green synthesized by *Mentha arvensis* on different cancer cells like MCF-7 and Hep 2 cells also revealed the induction of apoptosis by caspase-9, which confirms the activation of caspase 3 and induce oxidative stress in cancer cells and cause cell death due apoptosis [59]. So, in the present study, we can conclude that the biosynthesized BH-AgNPs comprise promising therapeutic prospects against human oral cancer-derived diseases. Even though there is a range of studies on *in vitro* anti-cancer effect of AgNPs on diverse cancers, other than very few studies were done on KB oral cancer cell lines.

4. Summary and conclusions

In the current study, it is concluded that biosynthesized AgNPs by *Byttneria herbacea* have revealed SPR band initially at 422 nm and 437 nm after 30 min, which confirms the green synthesis of BH-AgNPs. The FT-IR analysis confirmed that various bioactive compounds of leaf extract functional groups have actively participated in the reduction of BH-AgNPs. TEM data revealed the particles are in size range from 1 nm ± 2 nm

to 10 nm ± 2 nm, and the results were similar to that of Dynamic Light Scattering results. The BH-AgNPs was stable with a negative zeta potential value of –21 mV. The biosynthesized BH-AgNPs also revealed significant free radical scavenging activity and outstanding antimicrobial activity against gram +ve and gram –ve bacteria. The cytotoxic and anti-proliferative studies against KB oral cancer cells reveal an astounding cytotoxic effect with an IC₅₀ value 89.25 µg/ml. Additional studies were carried out on KB oral cancer cells by BH-AgNPs to look for insights into its anticancer effects by means of Cell cycle assay, Apoptosis assay and Caspase activity by flow cytometry. The final results revealed that the biosynthesized BH-AgNPs showed magnificent beneficial applications by arresting the cell cycle and inducing apoptosis in KB oral cancer cell lines. So we conclude that the BH-AgNPs have possible therapeutic potential against human oral cancer-derived diseases and can employ as novel anticancer therapeutic agents. Finally, we report that there are very few studies are noticed KB oral cancer cell lines using AgNPs.

Acknowledgements

The authors GKS and VSK are grateful to DST PURSE centre, Sri Venkateswara University, Tirupati, for providing the facility to carry out a part of the research.

Authors Contributions

The authors GKS, VSK and VNC work planning and design; Conceptualization VSK and VNC; Execution- GKS VSK, SAG and GH; Data analysis VSK, VNC, JP, SAG; Draft Preparation- GKS VSK, SAG and VNC; editing and revision- VSK, VNC, JP

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the present work featured in this article

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Data availability statement

All the data related to our research article is present in the manuscript submitted

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