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Efficient green synthesis of silver nanoparticles from *Caesalpinia bonducella* seeds and its antibacterial and cytotoxic effects: An *in vitro* study

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Abstract
The present study reports the successful synthesis of silver nanoparticles (Ag NPs) by plant-mediated green route and antimicrobial and anticancer activity investigated. The seed extract of locally sourced *Caesalpinia bonducella* acted as the reducing agents/capping agents. The CB seed AgNPs characteristics elucidated under UV–visible spectroscopy, Fourier transform infrared spectroscopy and transmission electron microscopy. The antimicrobial activity of the synthesized nanoparticles was tested against various pathogenic bacteria. Anticancer effect in Human breast cancer cell line MCF-7 was investigated. The synthesized AgNPs exhibited a maximum absorption at 455 nm and the Fourier transform infrared spectroscopy analysis confirmed the conversion of Ag+ ions to AgNPs due to the reduction by capping materials such as flavonoids and alkaloids of seed extract. The HR-TEM analysis revealed that they are spherical ranging 40nm. Significant growth inhibitions were found using analysis of variance (ANOVA), SPSS statistical tool at $P<0.05$. The highest activity of *CB seed* Ag NPs was against *P. aeruginosa*. Cytotoxicity of biosynthesized AgNPs against *in vitro* Human breast cancer cell line MCF-7 showed a dose-response activity with an IC50 value of 75µg/ml. Hence, the findings of this research suggest potential applications of the bionanoparticles as a candidate for therapeutic drugs because of their biogenic nature.

Keywords: *Caesalpinia bonducella* seed, CB seed AgNPs, FTIR, TEM, Anti microbial activity, anti cancer activity

Introduction
The emerging field of nanobiotechnology is envisioned to be one of the most fruitful areas of research in current science. The construction and characterization of noble metal nanoparticles such as gold silver and platinum is an upcoming field of research due to their vital applications in the fields of biotechnology, bioengineering, textile engineering, water treatment, metal-based consumer products and other areas [37]. Due to their utilization in various sectors, biomolecules found in the plant extracts can be employed to reduce metal ions to synthesize nanoparticles (NP) in green synthesis process [33]. Among them one of the most studied nanoparticles is silver nanoparticles, and it has greatly shown anti-neoplastic, anti-inflammatory and anti-bacterial effects in previous studies [15, 16]. Production of silver NPs using different medicinal plants for pharmaceutical and biological applications have been reported [26, 29].

Nowadays treating bacterial infection is risingly complicated because of the ability of the pathogens to develop resistance to available antimicrobial agents and existing antibiotics. Resistant pathogens tend to become broader infection control problems within hospitals and communities as well [42]. Resistant bacteria like *Staphylococci*, *Enterococci*, *Klebsiella Pneumonia* and *Pseudomonas* sp. are becoming more and more common [51]. To overcome this, novel strategies are required. The favorable approach was the use of natural antimicrobials individually, combination or synergistically and this set forth the way for the use of metal nanoparticles recently. Many studies evidenced that the cytotoxicity of synthesized Ag NPs is allied with their ability to produce reactive oxygen species (ROS) and initiating mitochondrial membrane disruption [3]. This cytotoxic property therefore manifest antitumor effects against lung cancer H1299 cells, breast cancer MCF-7 cells and glioblastoma U-87 cells [11, 10, 30]. AgNPs could be internalized by the cells through Trojan effect and inhibit the RNA polymerase activity and the gene transcription via a direct

reciprocal interaction [52]. Normal cells are less sensitive to AgNPs than the tumor cells [50]. The particle size and surface features of AgNPs are of very important biomedical considerations for AgNPs with smaller particle size seemed to have stronger penetration ability and greater toxicity for cancer cells [28]. Extracts of plants, deployed in green synthesis include active molecules acting as reducing and capping agents such as flavonoids, tannins, amines, aldehyde/ketone groups and polyols and proteins for Ag [26]. Formation of silver nanoparticles using black pepper leaf extract, olive leaf, cinnamon barks, grape seed extract, or papaya fruit extract have been reported [5, 39, 43, 14, 17].

Many studies have described the beneficial properties of Caesalpinia *bonducella*, which is one of the oldest and the most popular plants used in medicine [23]. The therapeutic activity of *Caesalpinia bonducella seed* is due to different effective substances such as furanoditerpenes, phytosterinin, β-sitosterol, bonducellin, aspartic acid, arginine, citrulline, β-carotene, phenolics and flavonoids [53]. *Caesalpinia bonducella* is a thorny shrub, belonging to *Caesalpiniaceae family*, widely distributed in regions of Sri Lanka and Andaman and Nicobar Islands and in India they are found in tropical regions, near the sea-coasts, especially Bengal, Bihar, Mumbai, Rajasthan and whole of Southern India [23, 4]. The seeds are hard and dark brown and roots of this plant are also dark brown colour [13, 23]. It is a very valuable medicinal plant, because all its part contributes to the medicinal property which is utilized in traditional system of medicine to treat and cure various diseases of mankind [23]. The seeds of *C. bonducella* possess antistress hyperlipidaemic, anxiolytic, anti-inflammatory and immunostimulatory [22, 38, 46] activity. The therapeutic applications of ethanol and methanol extract of CB seeds have been reported [18, 47]. The importance of the present piece of work is viewed specially with respect to the novel aspect of surface capping of AgNP's with plant secondary metabolites of medicinal interest which may generate suggestive scope for research in the field of nanoparticles assisted metabolite pool. The aim of this study is to eliminate heavy chemicals in the design and manufacture of silver nanoparticles (AgNPs) from aqueous extracts of Caesalpinia bonducella seeds for antimicrobial applications. In addition, by synthesizing CB seed AgNPs it is expected to achieve cytotoxic effect. These green-synthesized silver nanoparticles of CB seed extract (CB AgNPs) were examined by Transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR) and UV–Visible spectrophotometer to determine, which compound of CB seed reduces Ag+ ions into Ag nanoparticles. The cytotoxic effect of CB AgNPs was analyzed by the zone of inhibition and the minimum inhibitory concentration on four different bacteria strains and the results were compared to standard drug. The present study also tried to validate the possible *in vitro* anti-proliferative effects of green synthesized CB seed Ag NPs against the MCF 7 breast cancer cell line.

Materials and Methods

Plant material and preparation of the extract

Caesalpinia bonducella seeds used as a reducing agent for synthesis of silver nanoparticle was purchased from the Madurai local market; Tamilnadu, India. Analytical grade silver nitrate ($AgNO_3$) was purchased from Sigma Aldrich, India, and used as received without further purification. Fresh seed powder 25gm was taken and dissolved in 100ml of deionized water and the solutions was stirred for 2hours at room temperature and subjected to centrifugation at 10,000 rpm for 10 min. After centrifugation, supernatant was collected and stored at 4 ^{0}C and used for silver nanoparticle synthesis.

Synthesis of silver nanoparticles

Silver nanoparticles were synthesized by the modified method of Prabhu *et al*. [37]. 4 ml of the seed extract was used as a reducing agent for silver nanoparticle synthesis. Add 20mL of aqueous solution of 0.1mM silver nitrate solution for reduction of silver ions for all the samples and incubated in the dark condition for 24 hrs. The reduction of silver ions was monitored by color change of the synthesized samples. AgNPs were collected by centrifugation. The collected CB seed AgNPs dried, powdered and stored for further analysis.

Characterization of synthesized Silver Nanoparticles

Synthesis of AgNPs was assured by measuring the UV–Vis spectrum of the reaction mixture. The absorption spectrum was recorded over the range of 200–800 nm using UV–Vis spectrophotometer. The measurements are recorded on Shimadzu Dual Beam Spectrometer (Model UV-2400PC). Morphology and size of the AgNPs were investigated by TEM images using S-3400 N model, Hitachi. A small amount of the sample was dropped on a carbon coated copper grid and drying under lamp to form a thin film of the sample. The interaction of the electrons transmitted through the specimen resulted in the formation of an image which is then magnified and focused onto an imaging device. FT-IR measurements were used to identify the possible biomolecules associated with AgNPs formation. The infrared spectrum of the sample was measured with KBr disk in the wavelength range of 4000–400 cm^{-1} using Shimadzu FTIR spectrophotometer.

Antimicrobial assay and MIC determination

Antibacterial activity of AgNPs was assayed against different pathogenic bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella dysenteriae, Neisseria gonorrhea, Vibrio cholera.* The Antimicrobial activity of silver nanoparticles synthesized using CB seed extract was determined on Muller & Hinton Agar (Hi-Media Pvt. Ltd. Mumbai) using Kirby-Bauer disk diffusion method [27].Test pathogens were spread on the test plates- Muller Hinton agar (MHA) for bacteria using sterile swabs. Sterile wells were made with the help of a sterile cork borer at aseptic conditions. Samples (100µg) were added to the wells at aseptic conditions. Stock solutions of the extracts were prepared using DMSO. The test plates were incubated and the zone of inhibition (in mm diameter) was read and taken as the activity of the extract against the organisms. Ampicillin was used as a standard antibacterial antibiotic. Each experiment was repeated three times and the mean diameters of the inhibition zones were recorded in millimeters.

A minimum inhibitory concentration (MIC) test was conducted with the synthesized AgNPs. Various concentrations of seed extract-synthesized AgNPs (0.5 - 10ul) from the stock of 100mg/ml were prepared with Dimethyl sulphoxide (DMSO) and subsequently mixed with 100µl of 24 h-old individual bacterial pathogens. Each mixture was incubated at 37ºC for 48 h, and the visible turbidity was observed in each concentration in order to calculate MIC [55].

Evaluation of cytotoxic activity on MCF-7

Human breast cancer cell line (MCF-7) was seeded in 96-well

tissue culture plate. After 24 h of cell attachment in plate, different concentrations of nanoparticle were added to culture medium and incubated for 24 h at 37 °C. Non-treated cells were used as control. Incubated cultured cells were then subjected to tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [1]. MTT which is the determinant of cell viability was added at a final concentration of 0.5 mg/ml and the cells were incubated at 37 °C for 3.5 h. Then formazon dissolved with 150 µl of DMSO in each well. The color changes were measured using a ELISA reader. The rate of survival was determined by using the following formulae: Cell viability (%) = (1-ODA1/OD Ao)/100, where Ao = Absorbency of control cells and A1 = Absorbency of treated cells [22].

Statistical analysis

All the experiments were carried out in triplicate and the results were expressed as the mean. The results were expressed as mean ±SD. Statistical significances of difference throughout this study were calculated by one-way variance analysis.

Results

Visual observation & UV- Visible spectroscopy

As the Aqueous seed extract was mixed with aqueous solution of 0.1 mM silver nitrate, it started to change colour from colourless to brown due to reduction of silver ions; which indicates the formation of silver nanoparticles (Fig.1A).Evaluating the synthesis of AgNP using Aqueous extract of CB seeds show prominent peak around ʎmax at 455nm at within 24 hours.(Fig.1B) Based on colour change and UV-Vis spectral analysis, aqueous extract-based synthesized C*aesalpinia bonducella seeds* AgNPs were taken for further analysis.

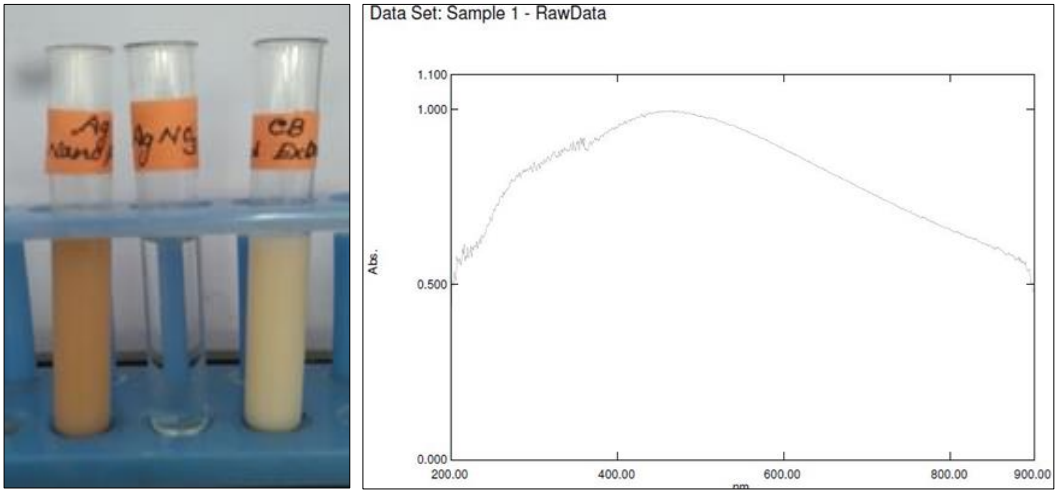

Fig 1: A) Visual Characterisation of CBAgNPs B) UV Visible absorption spectra of synthesized silver nanoparticles, showing the surface plasmon resonance peak of silver nanoparticles at 455 nm

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectrum was used to identify the functional groups of the active components based on the peak value in the region of infrared radiation. The results of FT-IR peak values and functional groups are presented in Fig 2. The FT-IR spectra of CB AgNPs shows interaction of biomolecules having intensive peak at 496 to 3127 Cm-1 (Fig 2). The biomolecules such as alkenes, carboxylic acid, nitro groups, amines, ethers and alkyl halides are responsible for the formation of silver nanoparticles (Fig.2).The FTIR spectroscopy gives the large amount of information's about the molecular atmosphere of the carbon-based materials on surface of the nanoparticles. In this work FTIR establish the organic biomolecules of the seed extracted amino silver nanoparticles by simple chemical method. The broad band at 3294 cm-1 corresponds to the strong stretching vibrations of hydroxyl group (–OH) of phenolic compounds; The sharp two intense peaks 2924 $cm^{-1}$and 2854 cm^{-1} shows the O-H stretching bonds which is more number water molecules are presents, aggregation of silver ions with the aldehyde and C-H stretching bonds respectively. The asymmetric carbon-carbon (C-C) stretching mode was present around 1743 cm^{-1} and 1658 cm^{-1}shows discrete C-N; C-C stretching modes due to its presence of proteins. Peaks probably towards the FT-IR region is capable to be a stable molecular stretching due to its detached energy level. Peaks around 1064 cm^{-1} was experienced by C-N stretching vibrations and C=CH_2 out of bending vibrations around 686 cm^{-1}. Ag-N prospects around 500 cm^{-1} assigned as silver atoms augmentations with respect to the (Methoxide-compound) stretching vibrations.

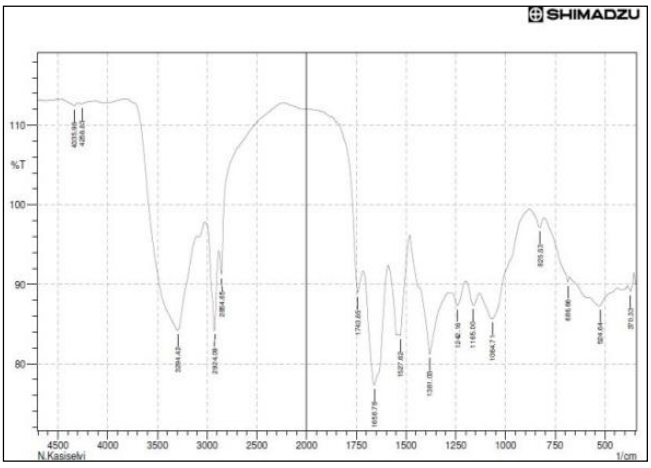

Fig 2: Fourier Transform Infrared Spectroscopy spectrum of CBAgNPs.

TEM

Figure 3 shows the TEM micrograph of the synthesized Ag nanoparticles. It is observed that most of the Ag nanoparticles were spherical in shape. A few agglomerated Ag nanoparticles were also observed in some places, thereby

indicating possible sedimentation at a later time. Fig. 3 shows the particle size frequency histogram taken from a large number of micrographs. It is evident that there is variation in particle sizes and the average size estimated was 40 nm.

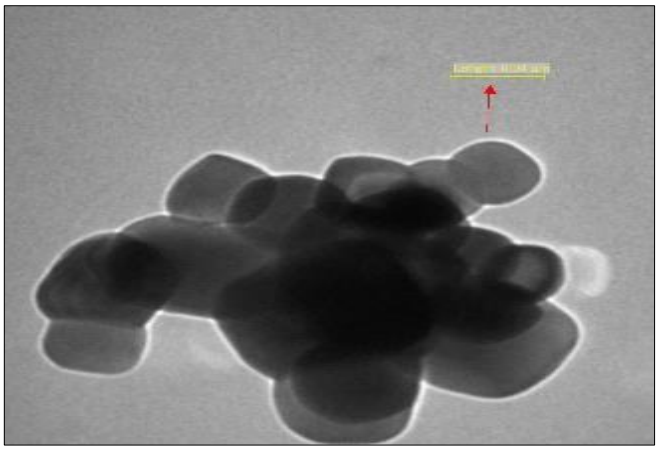

Fig 3: TEM images of Ag nanoparticles.

Antibacterial activity

CBAgNPs showed maximum antibacterial activity against *Pseudomonas aeruginosa* (120mm). Moderate activity was shown against *Salmonella typhi* (60mm), *Neisseria gonorrhea* (60mm), *Shigella dysenteriae* (50mm).The least activity was shown against *Vibrio cholerae* (35mm) and Escherichia Coli (40mm).The MIC of CBAgNPs against all the microorganism tested was 100mg/ml for P*seudomonas aeruginosa, Salmonella typhi, Shigella dysenteriae, Neisseria gonorrhoeae, Vibrio cholerae, Escherichia Coli*, respectively in Table 1. MIC observation against *Pseudomonas aeruginosa, Shigella dysenteriae, Salmonella typhi, Neisseria gonorrhoeae, Escherichia Coli, Vibrio cholerae* for CBAgNPs at various concentrations of 0.5 - 10 µl were performed in 18 hours duration. The least concentration of 10µl was found to be effective. Turbidity was noticed and observed after 24 hours in the tubes containing the different concentrations. An absence of organism was confirmed by streaking the contents of the tube. The MIC of 9.5 mg/ml against *P. aeruginosa and V. cholerae* followed by 9.3 and 9.2 mg/ml against *Neisseria gonorrhoeae and Shigella dysenteriae*. However, the MIC towards *Salmonella typhi* and *E. coli* was 8.7mg/ml.

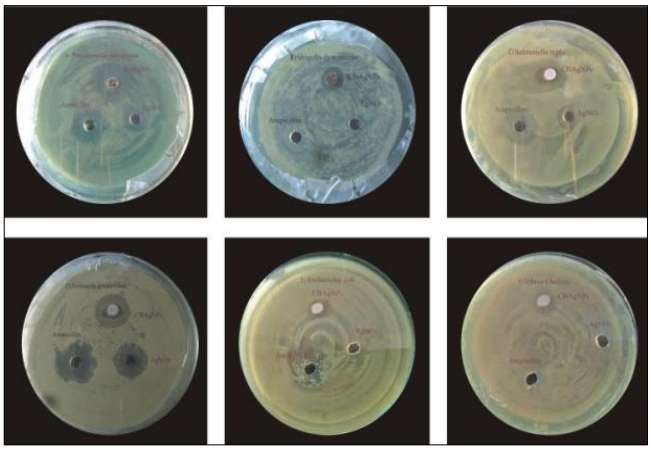

Fig 4: Antimicrobial activity of silver nanoparticles against Gram positive and Gram negative bacteria. a) *Pseudomonas aeruginosa*, b) *Shigella dysenteriae* c*) Salmonella typhi*, d) *Neisseria gonorrhoeae* e) *Escherichia coli* f) *Vibrio cholera*

Table 1: MIC of CB seed AgNPs against some pathogenic organisms. *All values represented in the table are average of results of three separately conducted experiments.

Species	CB AgNPs(mg/ml)
Pseudomonas aeruginosa	9.5
Shigella dysenteriae	9.2
Salmonella typhi	8.7
Neisseria gonorrhoeae	9.3
Escherichia Coli	8.7
Vibrio cholera	9.3

Cytotoxicity of CB seed AgNPs

The *in vitro* cytotoxicity of the AgNPs was evaluated MCF7 cell lines at different concentrations. Our cytotoxicity analysis of the sample shows a direct dose-response relationship; cytotoxicity increased at higher concentrations. The IC_{50} value was plotted by taking the concentration of AgNPs on X axis versus percentage of cell viability on Y axis (Fig.5). The samples demonstrated a considerable cytotoxicity against the MCF7.MCF-7 cells proliferation was significantly inhibited by AgNPs with an IC_{50} value of 78µg/ml.

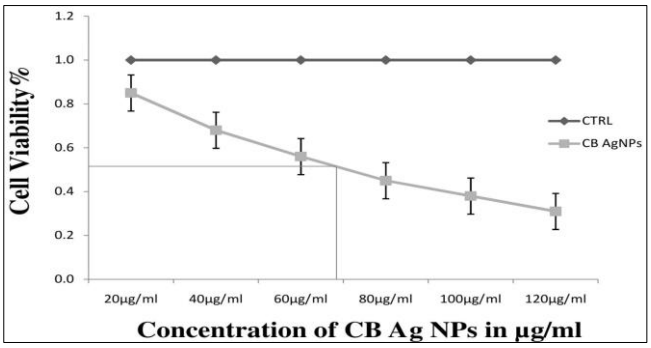

Fig 5: Cytotoxicity developed by CB seed AgNPs on MCF7 cells

Discussion

UV-Visible Spectroscopy

The UV/Visible spectrum for prepared CB seed AgNPs is shown in the Fig.1B. The corresponding curve of the absorption peaks are slow scanned between 200 to 800 nm with 1nm interval time and profound visible region was focused. Due to the surface plasma resonance the broad absorption peak is detected with two major peak points at 290 &455 nm respectively. Meanwhile as the prepared silver nanoparticles have wide band gap and the maximum absorption reached at 455 nm as aforementioned. After the maximum intensity of the broad peak, because of its continuous energy loss of discrete silver atoms have been perceived up to 800nm. The maximum light absorption from the spectra visuals the observed silver nanoparticles have been agglomerated and dispute the average particles size around 200-400 nm. With this broadened silver nanoparticle spectrum is highly light dependent which is experienced from the confined LASER source amplitude [36]. A slight deformation in the continuous energy defeat from 890 nm due to its accepting band gap energy experienced by the overcoming of energy loss. [45].

Fourier Transform Infrared Spectroscopy (FT-IR): The Fourier Transform Infra-Red spectroscopy reveals the chemical composition of the samples such as biological samples, organic chemicals, semiconducting materials, polymers, gases and lubricants etc., FTIR spectra of CB AgNPs suggested the presence of different compounds.

Variety of phytoconstituents such as steroids, phenolic, flavonoids, saponins, terpenoids, flavonoid, tannins and saponins have been reported in methanolic extract of C. bonducella [18]. Shankar *et al*. [44] have reported the reducing potential of terpenoids for metal ions to form complexes through the oxidation of aldehyde groups to carboxylic acids. This is probably due to the involvement of aromatic compounds such as phenol compounds in the CB extract in capping and stabilizing the CBAgNPs. Plant derived polyphenolic compounds such as tannic acid is reported to have reducing potential towards silver metal for synthesis of nanoparticles [48]. It is also evident from the above results that compounds other than phenolics and flavonoids are also involved in nanoparticles formation.

TEM

Electron microscopy analysis of the CB seed AgNPs showed that the Ag nanoparticles have size of about 40 nm which is in good agreement with the shape of the SPR band in the UV-Visible spectrum and are well dispersed on the globular CB seed support, prohibiting their agglomeration. This dispersion combined with the small size allows these Ag particles to exploit their entire active surface—the smaller the particle the higher its activity [34]. Many other authors have illustrated the descriptive images of the silver nanoparticles with their size and structural details using TEM analysis [40].

Antibacterial activity

The antibacterial activities of CB seed AgNPs showed that the nanoparticles had varying degree of antibacterial activity towards the test organism. This hypothesis is confirmed also by the MIC test as well as zone of inhibition Fig.4 and 6. In both cases, CB seed AgNPs suspension clearly affected the bacterial growth. It was also found that rise in the concentration of the whole nanoparticle yielding their respective increase in the zones of inhibition. This linear relationship between the concentrations of nanoparticle and zones of inhibition could be that the nanoparticles were able to diffuse into the inoculated nutrient agar. Bankar *et al*. [6] reported the antibacterial activity of AgNPs using *E. coli*, *Shigella* sp. Peptidoglycans composed of a thick layer of bacterial cell wall, consisting of linear polysaccharide chains cross-linked by short peptides thus forming more rigid structure leading to difficult penetration of the AgNPs [8]. Indeed, Gogoi and co-authors [12] obtained similar bactericidal results using green synthesis of silver nanoparticles based on alcoholic flower extract of *Nyctanthes arbortristis*. This high bactericidal activity is certainly due to the silver cations released from AgNPs that act as reservoirs for the Ag bactericidal agent [35]. Therefore, Eby *et al*. [9] and Krishnaraj *et al*. [24] observed that AgNPs were widely used in antibacterial coatings in processing of medical instruments and food industries for packaging. The biologically synthesized AgNPs using different plant extracts also showed a similar potent bactericidal activity [34]. Arunachalam *et al*. [2] evinced that AgNPs are extensively used in the pharmaceutical and medical industries as they have shown inhibitory activities against various microorganisms. According to the reports of Kasi *et al* [21] they have also been used in balms, topical ointments to avert infections following burn wounds which are in agreement with our results. AgNPs can easily reach the nuclear content of bacteria, and as they are present in the greatest surface area, therefore, the contact with bacteria is the greatest [27].

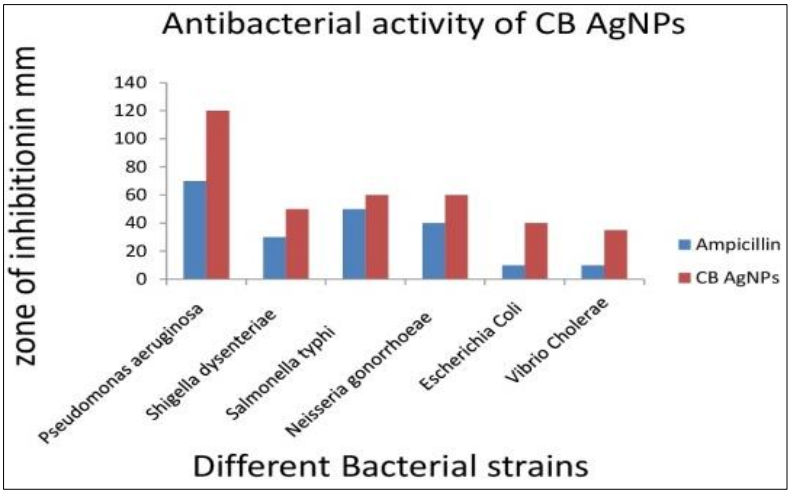

Fig 6: Each value was mean of inhibitory zones of CB seed Ag NPs on five bacteria. Values with different superscripts in each concentration are significantly different (p<0.05) by two-factor.

Anticancer Activity

The cytotoxic effect of AgNPs on cell viability has a major role in anticancer activity thereby reducing the disease progression. As shown in Fig. 5, the synthesized AgNPs of size 40 nm had dose-dependent cytotoxic effects on MCF7 cells *in vitro*. About 50% of MCF 7 cells died when treated with AgNPs at the concentration of 75 µg/ml. The enhanced cytotoxicity of CBseed AgNPs may be due to their size which facilitates their subsequent penetration in tumor cells. Supporting these studies Sriram *et al*. [50] reported that cytotoxic effect of AgNPs on cell viability has a major role in antitumor activity, thereby reducing disease progression. Cellular internalization of silver might provide the basis for the cytotoxicity of AgNPs [7, 49]. Our results also coincides with the findings of Mercy ranjitham *et al*. [31] who have reported that increased cytotoxicity of silver nanoparticles synthesized from aqueous extract of fresh Cauliflower floret on MCF-7 breast cancer cell line in a dose dependent manner. The cytotoxic effects of silver were the results of active physiochemical interaction of silver atoms with the functional group of intra cellular proteins as well as with the nitrogen bases and phosphate groups in DNA. Further research efforts are deserved to elucidate the latent mechanism and comprehensive study of molecular mechanism and *in vivo*

effects of AgNPs on breast cancer. Active anticancer components mostly the flavonoids in CBAgNPs known to exhibit a consistent antitumor effect by blocking abnormal expression of multiple apoptotic genes such as Bax and Bcl.

Conclusion

Developing biocompatible molecule as an antimicrobial and anticancer agent is one of the novel approaches in the field of medicine using nanotechnology. In the present work, we have proposed for the first time a simple, high efficiency, ecofriendly green synthesis method for AgNPs using *Caesalpinia bonducella* seed extract as reducing and stabilizing agent. The biosynthesized nanoparticles have been characterized by TEM, FT-IR, and UV-VIS spectroscopy and appears to be spherical, single crystalline with a narrow particle size range of 40 nm. The synthesized AgNPs revealed good bactericidal activities against gram-positive and gram-negative bacteria. AgNPs exhibited a strong inhibitory effect on the breast cancer MCF 7 cells. CB seed AgNPs might be a potential active candidate for breast cancer treatments. Further research should be focused on the comprehensive study of molecular mechanism and *in vivo* effects of CB seed AgNPs on breast cancer.

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