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Original article

Biogenic synthesis of silver nanoparticles: Antibacterial and cytotoxic potential

Asma S. Algebaly [a], Afrah E. Mohammed [a,*], Nael Abutaha [b], Mudawi M. Elobeid [c]

[a] Department of Biology, Faculty of Sciences, Princess Nourah Bint Abdulrahman University, 84428 Riyadh, Saudi Arabia
[b] Bioproducts Research Chair, Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia
[c] Department of Silviculture, Faculty of Forestry, University of Khartoum, Khartoum North, 13314 Shambat, Sudan

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ABSTRACT

In green chemistry, the application of a biogenic material as a mediator in nanoparticles formation is an innovative nanotechnology. Our current investigation aimed at testing the cytotoxic potential and antimicrobial ability of silver nanoparticles (AgNPs) that were prepared using *Calligonum comosum* roots and *Azadirachta indica* leaf extracts as stabilizing and reducing agents. An agar well diffusion technique was employed to detect synthesized AgNPs antibacterial ability on *Pseudomonas aeruginosa, Escherichia coli*, and *Staphylococcus aureus* bacterial strains. Furthermore, their cytotoxic capability against LoVo, MDA-MB231 and HepG2 ca cells was investigated. For phyto-chemical detection in the biogenic AgNPs the Fourier-transform infrared spectroscopy (FT-IR) was considered. Zeta sizer, TEM (Transmission Electron Microscope) and FE-SEM (Field Emission Scanning Electron Microscope) were used to detect biogenic AgNPs' size and morphology. The current results showed the capability of tested plant extract for conversion of Ag ions to AgNPs with a mean size ranging between 90.8 ± 0.8 and 183.2 ± 0.7 nm in diameter. Furthermore, prepared AgNPs exhibited apoptotic potential against HepG2, LoVo, and MDA-MB 231-cell with IC_{50} ranging between 10.9 and 21.4 µg/ml and antibacterial ability in the range of 16.0 ± 0.1 to 22.0 ± 1.8 mm diameter. Activation of caspases in AgNPs treated cells could be the main indicator for their positive effect causing apoptosis. The current investigation suggested that the green production of AgNPs could be a suitable substitute to large-scale production of AgNPs, since stable and active nanoparticles could be obtained.

1. Introduction

Nowadays, a fast-growing field in science and nanotechnology is the formation of remarkable materials known as nanoparticles. Such materials have a vital role in different human activities due to their unique size properties that are more influential than Ag ions as proved in different investigations. Nanoparticles can be produced by several chemical and physical approaches, which have shown high efficiency in the production of nanoparticles. However, such techniques have disadvantages, such as the high cost of large-scale production and expected environmental pollution. Recently, the production of nanoparticles using biogenic materials such as plants, microbes and natural biomolecules, which is known as green chemistry, could be considered as an alternative technique to other methods due to their eco-friendly nature (Sastry et al., 2004; Mohanpuria et al., 2008; Gurunathan and Raman, 2013). Different literature has extensively described the ability of plant extracts and microbes for the positive production of AgNPs. Some studies used *Phoenix dactylifera, Ferula asafetida,* and *Acacia nilotica* (Mohammed et al., 2018); *Euphorbia milii* (de Matos et al., 2011); *Fusarium sp.* (Mohammed et al., 2018); *Bacillus licheniformis* (Kalimuthu et al., 2008); *Brevibacillus borstelensis* (Kumar et al., 2016) for safe AgNPs' production. Furthermore, the efficiency of AgNPs that are prepared using plant extracts has been noted, such as the cytotoxicity on breast cancer (Kathiravan et al., 2014; Lokina et al., 2014; Nagajyothi et al., 2014; Rathi Sre et al., 2015), as well as human lung cancer (Rathi Sre et al., 2015; Nagajyothi et al., 2014). HeLa cell lines' total death was also documented by Suman et al. (2013) when he studied the biogenic preparation of

* Corresponding author.
E-mail address: AFAMohammed@pnu.edu.sa (A.E. Mohammed).
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AgNPs using *Morinda citrifolia* root extract. 50% viability loss of MCF-7 was also noticed by Sathishkumar et al. (2014) and Gajendran et al. (2014) when they applied AgNPs prepared by *Dendrophthoe falcate* and *Datura inoxia;* respectively; discontinuing growth of the cell and decreasing DNA formation leading to apoptosis have been documented. The inhibitory impact on human breast cancer (MCF-7) cells migration has been well stated by Sathishkumar et al. (2016) for AgNPs that were prepared using *Alternanthera tenella* leaf extract. A recent study of biogenic AgNPs against LoVo cell lines confirmed the formation of apoptosis-like symptoms (Mohammed et al., 2018). Previous investigations presented AgNPs as good cytotoxic agents since they are connected with the cell reactive oxygen species' production and disturbance of mitochondrial membrane (AshaRani et al., 2009; Sanpui et al., 2011).

Moreover, antibacterial capability of AgNPs was reported against some clinical bacterial strains (Mohammed et al., 2018; Alqahtani et al., 2017; Guzman et al., 2012; Dubas et al., 2011). One of the proposed mechanisms of AgNPs on bacteria is their effect on cell morphology due to the membrane damage that may affect the permeability and transportation of cell-needed materials (Das et al., 2012).

In this study, a fast method to synthesize AgNPs using aqueous and ethanolic extracts of *C. comosum* roots and *A. indica* leaves as reducing and capping agents have been evaluated. *A. indica* (Neem) is abundant in India. It belongs to *Meliaceae* family and is famous for its medicinal uses (Subapriya and Nagini, 2005). *A. indica* has been previously studied for the formation of AgNPs (Verma and Mehata, 2016; Asimuddin et al., 2018). Since the phytochemicals in plant always act as stabilizing and reducing agents, such compounds may affect the biogenic AgNPs' morphology and size (Tripathy et al., 2010). Nevertheless, various factors such as genetic background may exhibit phytochemical variations (Srivastava et al., 2005; Vokou et al., 1993). Consequently, a comparative study for the same plants of different origins and their ability for AgNPs' formation is highly required. *A. indica* in the current study was obtained from Sudan. Arta, *C. comosum* L'Her as sub species of *Calligonum polygonoides* is related to the *Polygonaceae* family. It is a desert shrub with slow leaf development (Ahmed et al., 2016) and it is widely spread in the Kingdom of Saudi Arabia. A recent study (Mohammed, 2016) focused on *C. comosum* green leaves as a biomediator in the formation of AgNPs. Since different plant parts may contain different concentrations of phytochemicals, in the current study our focus was on plant roots as a bio-mediator in AgNPs' formation, in which a lack of information is observed. Generally, plants are regarded as a good source of active ingredients that reduce and cap biogenically synthesized AgNPs' such as phenols, enzymes and DNA (Jha et al., 2009). Ahmed et al. (2016) isolated a new flavonoid and kaempferol glycoside from Arta and tested their ability as cytotoxic agents against breast and liver cancer cells. Toxicity of AgNPs prepared from Arta using different extraction methods, might be supported by phytochemicals and it is likely associated to their size, shape as well as charge leading the particles to affect a particular site (Thorley and Tetley, 2013). Due to the significance of AgNPs in the treatment of cancer as well as the urgent need for cancer medications, the current work focuses on the cytotoxic efficiency of the biogenic AgNPs. Biogenic synthesis could better be used in medicine due to the natural sources of capping and reducing agents used. As a result, in this study *A. indica* and *C. comosum* were used for biogenic synthesis of AgNPs that were detected by TEM, SEM and zeta potential. Additionally, regarding the anticancer effect of AgNPs prepared by *A. indica* and *C. comosum* on LoVo, MDA-MB231 and HepG2 cancer cell lines, biogenic AgNPs triggered: i) Cell viability decline in a dose-dependent manner, ii) apoptotic cell death. Such findings were verified by cell morphology, staining by acridine orange/ethidium bromide, and activities of Caspase 3 and 7. The antibacterial ability of AgNPs was noted against different microbes. As far as we know, this investigation is considered to be the first source of information regarding *C. comosum* root extract as mediator in the biogenic AgNPs preparation.

2. Materials and methods

2.1. Plants collection

Fresh *A. indica* leaves were collected from Khartoum, Sudan and the roots of *C. comosum* were obtained from the Riyadh, Saudi Arabia. At the Biology Department, Faculty of Sciences, Princess Nourah Bint Abdulrahman University, plant samples were identified, prepared, dried, milled to fine particles using a machine (IKA werke, GMBH and Co., Staufen im Breisgau, Germany) and deposited in sealed plastic bags for further study.

2.2. Silver nanoparticles (AgNPs) synthesis

About 10 g powder from each plant material was added to 100 ml solvent (water or ethanol, separately). For aqueous extract, the mixture was heated at 70 °C for 10 min for enzyme deactivation. Filter paper, Whatman at pore size 125 mm (No. 1, Maidstone, England,) was used for aqueous mixture filtration and for ethanolic extract after it was kept overnight. Heat treatment for ethanolic extract was performed for extract concentration and further use. Ninety ml of $AgNO_3$ (1 mM) was mixed with 10 ml from each plant extract and kept for 48 h at room temperature to allow reaction and development of AgNPs. Conversion process was observed for the mixture color change from yellow to dark. For repeatability, each sample was prepared thrice and stored for analysis at 4 °C.

2.3. Biogenic AgNPs characterization

Ultra-Violet Spectrophotometer, Zeta potential, FE-SEM and TEM (Peabody, MA, USA), were used to characterize the AgNPs biogenically synthesized as mentioned below:

2.3.1. UV spectrophotometer

At UV-2450 double-beam (200–800 nm), UV- spectrophotometer (Shimadzu, Tokyo, Japan) was used to detect the reduction process of Ag^+ ions.

2.3.2. Zeta potential

The biogenic AgNPs size was evaluated by a Zeta sizer device (Malvern, Worcestershire, UK).

2.3.3. Field Emission Scanning electron microscopy

External morphology of biogenic AgNPs was tested using FE-SEM technique (JEOL 7500FA JEOL, Peabody, MA, USA). About 8 μl from studied material was situated on 200 mesh grids including supporting film from a carbon (Agar Scientific, London, UK) and dehydrated. Additionally, EtOH was used to wash the sample, and then fixed on the SEM holder. 30 kV voltage was used for imaging.

2.3.4. Transmission electron microscopy

The shape and crystallinity of biogenic AgNPs were detected using TEM a JEOL JEM-1011 (JEOL, Peabody, MA, USA). A drop from the studied material (8 μl) was placed on a copper grid of 300 meshes coated with carbon. 200 kV voltage was used for imaging.

2.3.5. Fourier-Transform infrared spectroscopy

Possible biomolecules in the biogenic AgNPs were detected by FT-IR (Nicolet 6700 FT-IR Spectrometer, Waltham, MA, USA) at 500–4000 ^{cm} infrared absorption.

2.3.6. DPPH radical scavenging test

Antioxidant capability was determined as reported by Ghosh et al. (2013); in methanol 100 μM of DPPH (1, 1-Diphenyl-2-picrylhydrazyl) was prepared as a working solution. Twenty microliters of the extract and 180 μl DPPH were added to each well in a 96-well plate. Two hundred microliters of DPPH (no sample) were used as control. At 515 nm at 25 °C, the absorbance was measured after 30 min. The antioxidant ability of each fraction was assessed by the comparison between in its absorbance to that of the control. The scavenging activity was calculated as percentage applying the bellow equation:

$$\text{DPPH scavenging activity } (\%) = (A1 - A2)/A1 \times 100$$

where the control absorbance is A1 and the sample absorbance is A2.

Quercetin was used as positive control

2.4. Antibacterial activity of AgNPs evaluation

The well agar diffusion method was used to detect AgNPs' antibacterial action (Mohammed, 2015) against *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27584), and *E. coli* (ATCC 25922). On the Mueller-Hinton Agar, microorganisms were sub-cultured and 1.5×10^8 CFU/mL (0.2 ml) of bacteria were dispersed uniformly by sterilized swabs onto separate agar plates. Consequently, three wells (holes), using a sterile cork borer were made (4 mm diameter each) at bacterial inoculated agar surface. 0.2 ml of each extract (20 μg/ml) was placed in the prepared well under sterile conditions for extract diffusion into the agar; plates were stayed at room temperature for one hour. The negative control was sterile distilled water, thereafter; incubation process for 18–24 h was done at 37 °C. The clear areas around the holes were measured and considered as the inhibition zones.

Amoxicillin and Ciprofloxacin (30 μg/ml) were employed and placed into tested bacterial plates and their effect has been assessed by measuring the inhibition zone (mm) after incubation for 24 h of at 37 °C.

2.4.1. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Nutrient broth (NB) as a medium for micro dilution technique has been employed to determine the MIC and MBC values. About 10 μl of 5×10^8 CFU/mL bacterial strains was inserted separately to 10 ml of NB. Test tubes containing bacteria were incubated after being subjected to different concentrations of AgNPs for 24 h. MIC was obtained after the incubation by checking the turbidity of bacterial growth. The concentration inhibited 99% of bacterial growth is considered as the MIC and the lowest concentration that showed no bacterial growth is known as MBC (Das et al., 2012).

2.4.2. The tolerance level

AgNPs tolerance levels were calculated per bacterial strain with the formula below (May et al., 1998).

$$\text{Tolerance level} = \text{MBC}/\text{MIC}$$

Antibacterial ability of biogenic AgNPs was identified by indicating bactericidal and bacteriostatic factors for studied bacteria, which is known as Tolerance Level. Furthermore, to find out the mechanism of biogenic AgNPs on *S. aures* and *P. aeruginosa*, 10 ml NB having bacterial cell (5×10^8 CFU/ml) was exposed to AgNPs (MIC) for 2 h and then detected using FE-SEM h.

2.5. Cell culture

Human colon, human liver and human breast cancer cell lines (LoVo, HepG2, MDA-MB-231) were obtained from KSU, Riyadh, Saudi Arabia. Cell lines were maintained in high glucose Dulbecco's Modifed Eagle's medium (DMEM) 10% serum of fetal bovine (Gibco, Germany) and 1% penicillin-streptomycin solution (Thermo, Canada) at temperature of 37 °C and a CO_2 concentration of 5% in a humidified incubator.

2.5.1. Culture conditions for cancer cell lines

An amount of 5×10^5 cells have been cultured overnight in plate containing 24 wells (NEST, China). Different concentrations of biogenic AgNPs were applied into the cell culture and the vehicle tested cells has been assigned as the negative control. After 48 h, cell incubated with 100 μl of (5 mg/ml) 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) for 2 h at 37 °C. Then, the medium has been aspirated ending with formazan crystals formation which have been dissolved in 0.1% HCL-methanol in a multi-well plate reader (Thermo Scientific™ Multiskan, China). Plates were measured at wavelength of 570 nm. The viability of the cells was assessed compared to control using the below equation:

$$\text{Viability}\% = \frac{\text{OD sample}}{\text{OD control}} \times 100$$

2.5.2. Acridine orange and ethidium bromide staining

Two fluorescent DNA dyes have been applied for staining (Acridine orange and ethidium bromide). Cells (5×10^5 cells/well) have been seeded in 24 well plates with an incubation period of a period of 24 h. Cells were then treated with extracts at IC_{50} value. Media were removed and phosphate buffer saline (PBS) has been used for cells washing. After all, cells have been treated by the staining media and incubated, then fluorescence microscopy (EVOS, USA) was used for assessment of the nuclear morphology. To differentiate among live, apoptotic and necrotic cells, double staining with these two dyes was carried out:.regular size green nucleus indicates live cells; condensed green, fragmented or shrunken nucleus indicate apoptotic cells; regular size red nucleus exhibit the necrotic cells.

2.5.3. Detection of caspase 3 and 7

Cells have been cultured and treated with the biogenic AgNPs at IC_{50} concentration in a 24 well plate and incubated for 24 h. After the incubation period, each cell was supplied with 2 μM Caspase-3/7 reagent (Invitrogen, USA), and thereafter kept in the dark for 30 min. at 37 °C. A fluorescent microscope (EVOS, USA) was used for plates observation.

3. Statistical analysis

Data was calculated as triplicate; Microsoft Excel 2013 was used to calculate means and standard deviations. The images of AgNPs detected using FE-SEM, TEM and Zeta size have been selected from one replicate. Graphs for cytotoxicity assay that show the IC_{50} were prepared for using origin 8 software (Northampton, MA).

4. Results and discussion

In the current study, AgNPs were formed when extracts from *C. comosum* roots and *A. indica* leaves were added to Ag ions separately. It has been well documented that extracts from plants containing phytochemicals, such as phenolic compounds, terpenoids,

flavonoids, may be involved in the conversion of Ag^{+} to AgNPs (Mohammed, 2016). Earlier investigations by Subapriya and Nagini (2005) identified phyto-molecules in the leaves of *A. indica*, such as polyphenols, flavonoids and amino acids which may help in such conversion. The main antioxidant composites isolated from *C. comosum* were flavonoids and anthraquinones (Ghazanfar, 1994; Kamil et al., 2000), which may affect such conversion. On the other hand, in this investigation, UV- spectra of the plant extract alone and AgNPs provided by the reduction of Ag ions identified absorption bands ranged from 430 to 470 nm. AgNPs synthesized using aqueous and ethanol from *C. comosum* showed 440 nm for aqueous extract and 470 nm for ethanolic extract. AgNPs synthesized using water and ethanolic extracts from *A. indica* showed 430 and 440 nm, respectively. The same trend from observations regarding *C. comosum* green shoot extract was also recorded (Mohammed, 2016). A range between 436 and 446 nm was recorded (Ahmed et al., 2016) for AgNPs prepared from *A. indica leaves*. On the other hand, zeta potential showed a size of 171.5 and 183.2 nm and mean zeta potential of −0.76 and 0.93 for AgNPs synthesized using water and ethanolic extract for the root of *C. comosum*, respectively (Table 1). In contrast, 105.2 nm and −6.62 mV were recorded for AgNPs prepared by *C. comosum* green shoot aqueous extract (Ahmed et al., 2016; Mohammed, 2016). Mean size of 90.8 and 136.2 and mean potential of −3.34 and 1.13 were recorded for AgNPs synthesized using water and ethanolic extract of *A. indica* leaves; respectively (Table 1). The size of 34 nm has been noticed for AgNPs synthesized using *A. indica* aqueous leaf extract (Ahmed et al., 2016). A negative zeta potential was observed only for AgNPs formed using aqueous extracts of *C. comosum* roots and *A. indica* leaves and was not observed for those prepared by ethanolic extract for both plant sources. Such information might specify higher stability for those AgNPs because the higher negative values exhibit the higher repulsion among particles. Consequently, stable AgNPs without gathering and accumulation might be achieved (Farhadi et al., 2017). Moreover, the extract phytochemicals might be adhered to Ag ions as a negative group providing them with the negative charge (Khatoon et al., 2017). A recent study by Mohammed et al. (2018) showed higher negative values for AgNPs prepared by ethanolic extracts than those prepared by aqueous extract from different plant types. Therefore, plant sources and extraction methods might be the main reasons for different sizes and potentials of biogenic AgNPs (Table 1). Further characterization of AgNPs was also recorded using TEM and SEM analysis (Fig. 1), TEM and their corresponding SEM images showed spherical shapes and some of them are in aggregate. The size of AgNPs obtained by TEM and SEM are in accordance with the size obtained by zeta sizer. Spherical shapes were also recorded for AgNPs prepared by *A. indica* leaves and *C. comosum* shoot (Ahmed et al., 2016; Mohammed, 2016). Regarding the FT-IR spectroscopy for AgNPs prepared by *C. comosum* and *A. indica,* they showed different functional groups since different peaks were observed indicating different biomolecules which might be responsible for Ag ions conversion to AgNPs. Furthermore, different employed extraction methods showed AgNPs with different FTIR absorption peaks. Peaks at 3267 and 3281 cm^{-1}, 1634 and 1641 cm^{-1} were recorded for AgNPs synthesized using water and ethanol for root of *C. comosum*, respectively and 3279 cm^{-1}, 3297 and 1635 were recorded for AgNPs prepared by both water and ethanol extracts of *A. indica* leaf. Peaks at 3550–3200 cm^{-1} might be corresponding to alcohol and phenol with hydrogen bond and 830–2695 may correspond to the aldehyde group. Peaks around 1635 cm^{-1} could be related to the amine NH band (Mohammed et al., 2018). 1634, 1641 and 1435 cm^{-1} peaks are the peaks recorded for the natural proteins, (Macdonald and Smith, 1996) suggesting the stability of the proteins after binding with Ag ions (Fayaz et al., 2010). Peaks at 1043 and 1044 cm^{-1} were only detected for ethanolic extracts of *C. comosum* and *A. indica,* respectively. Peaks at 1635.41 and 3249.83 cm^{-1} were recorded for AgNPs prepared by the green shoot of *C. comosum* (Mohammed, 2016). Peaks at 3389, 1635 and 1390 cm^{-1} were previously recorded (Verma and Mehata, 2016) for AgNPs prepared by *A. indica* leaf extract. Different peaks detected at the present work confirmed the existence of phenolic, aldehyde and proteins that may act as stabilizing and capping agents for AgNPs prepared from plant extracts. Terpenoids and flavanones are possible biomolecules may be present according to the peaks detected, which could react via their carbonyl groups with the Ag ions. Thirteen unknown flavonoids and kaempferol were isolated from Arta (Ahmed et al., 2016) which might support our findings according to the peaks detected. Furthermore, terpenoids may oxidize the aldehyde group to carboxylic acid in the biomolecules that may lead to a reduction process (Smitha et al., 2009).

4.1. Biogenic AgNPs antibacterial capability

A strong bactericidal effect of biogenic AgNPs against tested bacterial species was observed. The $AgNO_3$ solution showed lower antibacterial ability in relation to the aqueous and ethanolic plant extracts tested. Higher inhibition zones around the well in bacterial cultures were recorded for ethanolic extracts for both tested plants than for aqueous extracts. Since bacterial membrane permit only lipid soluble compounds to enter inside the cell, therefore the

Table 1
Biogenically synthesized AgNPs, size (nm), potential (mV) and the inhibition zone (mm) indicating antibacterial ability against tested strains.

Treatment	Average particle size (nm)*	Average Zeta potential (mV)*	Inhibition zone +gram (mm)	Inhibition zone − gram (mm)	
			S. aureus	*E. coli*	*P. aeruginosa*
Ag1	**171.5 ± 0.5**	**−0.76 ± 0.1**	**17.4 ± 0.6**	**18.8 ± 1.2**	**18.0 ± 0.9**
Ag2	**183.2 ± 0.7**	**0.93 ± 0.1**	**16.0 ± 0.1**	**16.5 ± 1.4**	**17.3 ± 0.2**
Ag3	**90.8 ± 0.8**	**−3.34 ± 0.3**	**21.1 ± 1.2**	**22.0 ± 1.8**	**19.9 ± 1.4**
Ag4	**136.2 ± 0.9**	**1.13 ± 0.2**	**18.3 ± 0.9**	**19.0 ± 1.3**	**16.6 ± 0.8**
C. comosum water extract	–	–	**11.1 ± 1.2**	**8.2 ± 1.3**	**7.0 ± 0.2**
C. comosum ethanol extract	–	–	**13.0 ± 0.7**	**10.2 ± 0.6**	**9.0 ± 0.1**
A. indica water extract	–	–	**9.0 ± 0.2**	**8.7 ± 1.0**	**8.0 ± 0.9**
A. indica ethanol extract	–	–	**10.8 ± 0.9**	**11.0 ± 0.3**	**10.0 ± 0.3**
Ag ions	–	–	**2**	**2.2**	**1.8**
Amoxicillin (AMO)	–	–	**22 ± 0.6**	**18 ± 1.4**	**0**
Ciprofloxacin (CIP)	–	–	**23 ± 1.7**	**34 ± 1.3**	**35 ± 1.8**

Presented values are means ± SD. The Ag1 indicates AgNPs synthesized using *C. comosum* root water extract, Ag2 = AgNPs prepared by *C. comosum* roots ethanolic extract, Ag3 = AgNPs prepared by *A. indica* water extract and Ag4 = AgNPs prepared by *A. indica* ethanolic extract.

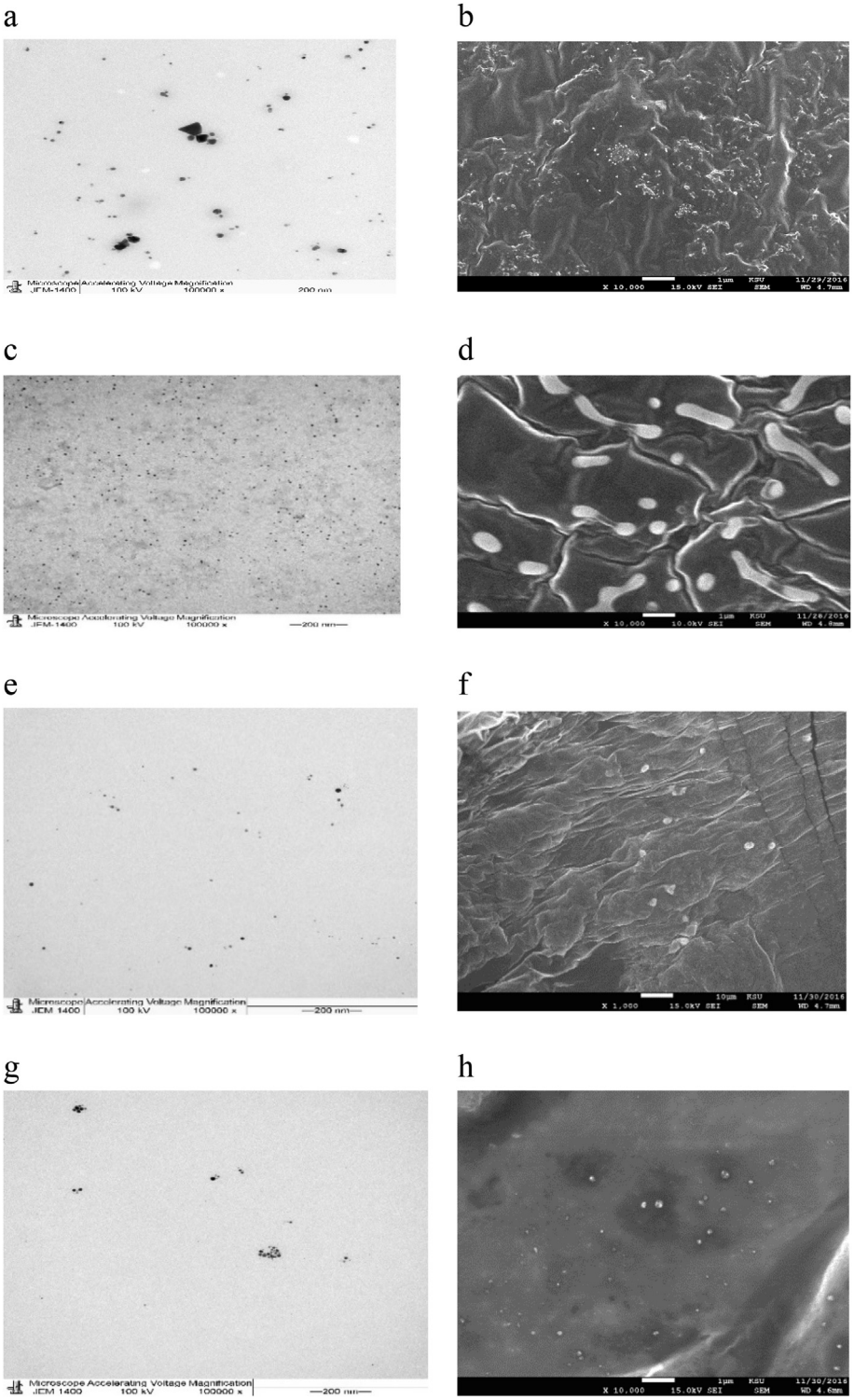

Fig. 1. TEM (a, c) and SEM (b, d) images for AgNPs covered with *C. comosum* phytochemicals from the aqueous and ethanolic extracts. For *A. indica* aqueous and ethanolic extracts, TEM and SEM images are indicated by (e, g) and (f, h), respectively. Magnification is 10,000× and the scale bar represents 1 μm for SEM images and 200 nm for TEM images.

lipid soluble compounds that dissolved in the ethanol might be the reason for such high activity for ethanolic plant extracts (Elemike et al., 2017). Currently, studied plant extracts have shown different abilities to convert Ag ions to AgNPs because of the different plant sources and different extraction methods used; therefore, particles differ in sizes and potential were detected which may lead to the stated different antibacterial abilities. AgNPs prepared by aqueous extracts from both plants showed higher antibacterial ability compared to those prepared by ethanolic plant extracts. Higher antibacterial ability was also correlated with the particle size;

the smaller the size, the higher the antibacterial action was observed although no significant variation was found in relation to higher particle size effect (Table 1). Moreover, AgNPs synthesized from *C. comosum* exhibited antibacterial ability against *S. aureus* more than 50% of amoxicillin and ciprofloxacin activity, but AgNPs synthesized from *A. indica* demonstrated more than 90% and 50% of amoxicillin and ciprofloxacin activities. Against *E. coli*, AgNPs prepared from aqueous extract of *C. comosum* roots showed the same activity of amoxicillin and more than 50% of ciprofloxacin activity. AgNPs prepared from *A. indica* leaves' water extract showed higher activity than the amoxicillin and more than 60% from ciprofloxacin activity. Interestingly, AgNPs in the current study showed high antibacterial activity against *P. aeruginosa* although no activity of amoxicillin and more than 50% of ciprofloxacin activity. Antimicrobial ability of AgNPs prepared by plant extract was highly investigated (Mohammed et al., 2018a, 2018b). Combined antibacterial ability of $AgNO_3$ and plant extract could be the main reason for higher antibacterial ability for their mixture in relation to each of them alone. The antibacterial effect might not be related to the bacterial structure of the cell since no clear observation was detected against different Gram-negative and Gram-positive bacteria. The mode of action of AgNPs on bacterial strains is not fully known. However, a cell membrane may undergo some changes leading to loss of permeability and ultimate cell death (Das et al., 2012). On the other hand, the noticed high antibacterial activity of AgNPs might be related to their ability to react with some significant enzymes and damaging DNA (Shao et al., 2015).

4.2. Determination of tolerance level

For MIC and MBC assessment, different concentrations from the biogenic AgNPs were prepared and applied to the tested bacterial strains. The MIC and MBC were 12.5 and 75 μg/mL, respectively. The bacteria susceptibility, tolerance or resistance to investigated compounds might be explained by MBC/MIC (Das et al., 2016). The biogenically synthesized AgNPs from the different plant types tested may act as a powerful bactericidal tool since the tolerance levels were less than 2 (Kamil et al., 2000).

4.3. Morphological study of bacteria using SEM images

In an attempt to find out the real mechanism of AgNPs against bacteria, two different strains that showed low tolerance to the investigated biogenic AgNPs, namely *S. aures* and *P. aeruginosa* were treated with biogenic AgNPs and subjected to SEM two hours after the treatment. Target microbes *S. aures* and *P. aeruginosa* were treated with AgNPs prepared by water extracts of *C. comosum,* (Fig. 2b and d). Control from both strains were examined (Fig. 2a and c) that showed smooth cells and stable morphology, but both treated bacteria showed morphology changes since the loss in membrane integrity was observed. The same trend in observations was also detected (Mohammed et al., 2018; Gopinath et al., 2015).

4.4. DPPH antioxidant activity test

To test the radical scavenging activity of biogenic AgNPs, scavenging of DPPH free radicals was detected at absorption peak of 520 nm. DPPH showed a positive correlation with the AgNPs concentration. The aqueously prepared AgNPs showed higher antioxidant activity in relation to those prepared by ethanolic extracts (Fig. 3), which might be related to their small size as well as negative charge. Thus, they showed greater antibacterial effect in relation to AgNPs prepared by ethanolic extracts for both plant types. A recent study by Azizi et al. (2017) indicated the correlation between negative charge and the greater affinity to reduce the DPPH when they studied the ability of *Citrullus colocynthis* for ZnO-NPs preparation. The same trend of observations was also observed by Mohammed et al. (2018) when they studied biogenic AgNPs. Small size AgNPs showed higher activity against tested microbes, which might be related to the larger surface area and higher antioxidant activity compared with bigger particle size.

4.5. In vitro cytotoxicity assay

Chemotherapy is among the most efficient therapeutic methods for the treatment of cancer. The side effects associated with the treatments are hard to tolerate for patients. Thus, there is a need for the development of effective and novel chemotherapeutic drugs, which are affordable, effective, and produce less toxicity. Medicinal plants contain an untold number of secondary metabolites with anticancer potentials that are cheap and less toxic. Recently, there is a growing concern for identifying anticancer drugs from medicinal plants (Greenwell and Rahman, 2015).

In this investigation, cytotoxic effect of the AgNPs synthesized using water and alcoholic extracts of *C. comosum* and *A. indica* was evaluated on HepG2, LoVo, and MDA-MB231 cells using the MTT assay. Fig. 4 shows the dose-response curve from which the half-maximal (IC_{50}) cytotoxic effects of AgNPs were estimated after 48 h of treatments. The results revealed that, all tested AgNPs showed cytotoxic effects in a dose-dependent manner indicating their potential as anticancer agents, but no cytotoxicity has been detected for AgNPs synthesized using ethanolic extract of *C. comosum*. IC_{50} of the AgNPs prepared by water extract of *A. indica* against HepG2, LoVo, and MDA-MB-231 were 21.2, 10.9 and10.9 μg/mL, respectively (Fig. 4). However, the IC_{50} values of the AgNPs prepared using aqueous extract of *C. comosum* against HepG2, LoVo, and MDA-MB231 were 21.4, 11.6 and 13.5 μg/mL respectively. The IC_{50}values of the AgNPs prepared using ethanolic extract of *A. indica* against HepG2, LoVo, and MDA-MB231 were

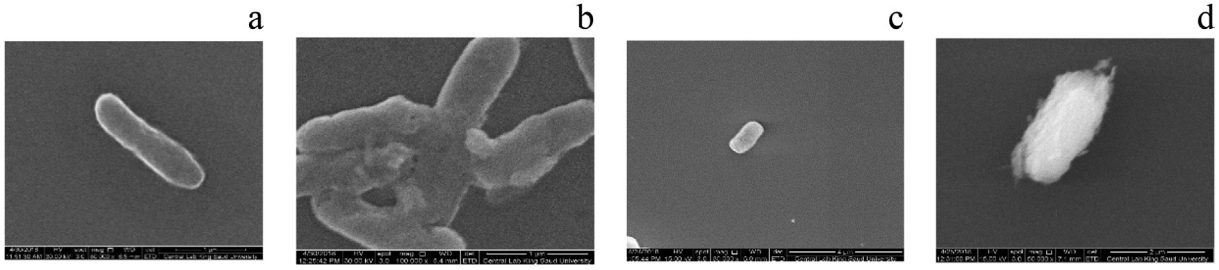

Fig. 2. Morphology of the AgNPs-tested bacteria using FE-SEM: morphological changes of *P. aeruginosa* (b) compared to untreated control (a) and *S. aureus* (d) compared to untreated control (c). treated cell (b and d) showed noticeable membrane damage.

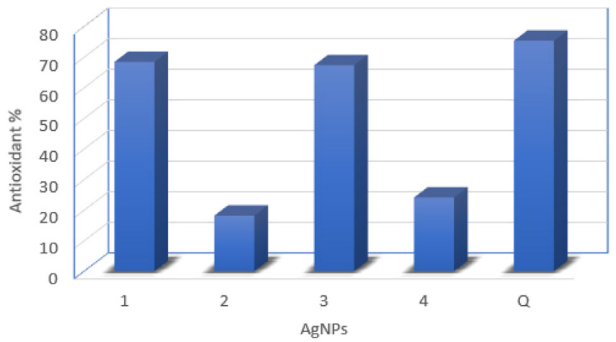

Fig. 3. Scavenging ability of different biogenically synthesized AgNPs on DPPH, 1 indicates scavenging ability of AgNPs synthesized using water extract of *C. comosum*, 2 for AgNPs synthesized using ethanol extract of *C. comosum*, 3 for AgNPs synthesized using water extract of *A. indica*, 4 for AgNPs synthesized using ethanol extract of *A. indica*. Q for the positive control Quercetin.

16.4, 11.9 and 11.3 μg/mL, respectively. The most promising activity against HepG2 cell line was in the AgNPs prepared using ethanolic extract of *A. indica* while the most promising activity against LoVo and MDA-MB231 was in the AgNPs prepared using aqueous extract of *A. indica*.

Our study showed higher cytotoxicity compared to Asimuddin et al. (2018) who revealed the biosynthesized AgNPs at the concentration of 250 μg/ml showed more than 60% of cell viability, however, it was similar to the cytotoxicity of other researchers who reported 50% cytotoxic effect on MCF-7 cell lines of 10 μg/ml AgNPs prepared by *A. indica* (Mittal et al., 2016). However, these differences in the cytotoxicity could be attributed to cell lines variations. Since cytotoxic activity of *A. indica* was more promising than *C. comosum*, further investigation will be carried out using only *A. indica* extracts.

4.6. Morphological evaluation by AO/EB double staining

Apoptosis is characterized by different morphological features including nuclear fragmentation, chromatin condensation, and membrane blebbing (Thompson, 1995). In the current study, Figs. 5, 6, 7 showed the nuclear fragmentation and chromatin condensation in LoVo, MDA-MB-231 and HepG2 cells, respectively by AO/EB double staining after 48 h of treatment with the aqueous and ethanolic extract of *A. indica*.

A comparison of nuclear morphology by dual staining (AO/EB) using fluorescent microscopy revealed that treated cells with AgNPs prepared using *A. indica* extracts- revealed nuclear morphological changes. No apoptosis was detected in the control group since uniform green cells were clear (Figs. 5–7A), while apoptotic cells showed granulated yellow-green, concentrated and unevenly located nuclei (Figs. 5–7C), and necrotic cells were marked with orange nuclei (Figs. 5–7C). This helped in predicting that apoptosis induced cell death rather than necrosis. The prediction was further confirmed by Caspase-3/7 assay.

4.7. Caspase-3/7 stimulation in cancer cell lines

The most effective significant effector compounds in apoptosis are proteolytic enzymes Caspases (McIlwain et al., 2015). To study the apoptotic potential of AgNPs prepared using *A. indica* aqueous and ethanolic extracts, caspase 3/7 stimulation was evaluated by the CellEvent™Caspase-3/7 kit that emits fluorescent light when the fluorescently labeled DEVD peptide is fragmented. Both the aqueous and the ethanolic extract showed bright green fluorescence for treated cells (LoVo, MDA-MB-231 and HepG2) compared to control cells (Figs. 5–7). Therefore, it can be inferred that death of the cells took place due to caspase signaling by the AgNPs. This confirms the results obtained with AO/EB staining and demonstrates that AgNPs prepared using *A. indica* aqueous and ethanolic

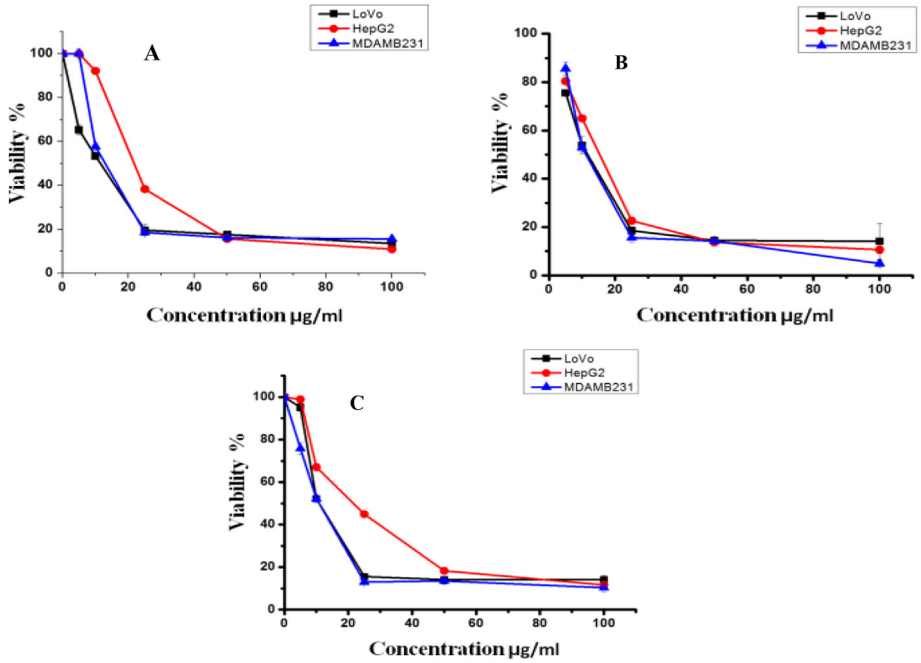

Fig. 4. Cytotoxic ability for the AgNPs prepared by using extracts of *C. comosum* and *A. indica* against HepG2, LoVo, and MDA-MB231 cell lines after 48 h of treatment. A: is the IC_{50} for AgNPs prepared by aqueous extract of *C. comosum*, B and C: for those prepared using ethanolic and aqueous extracts of *A. indica*, respectively.

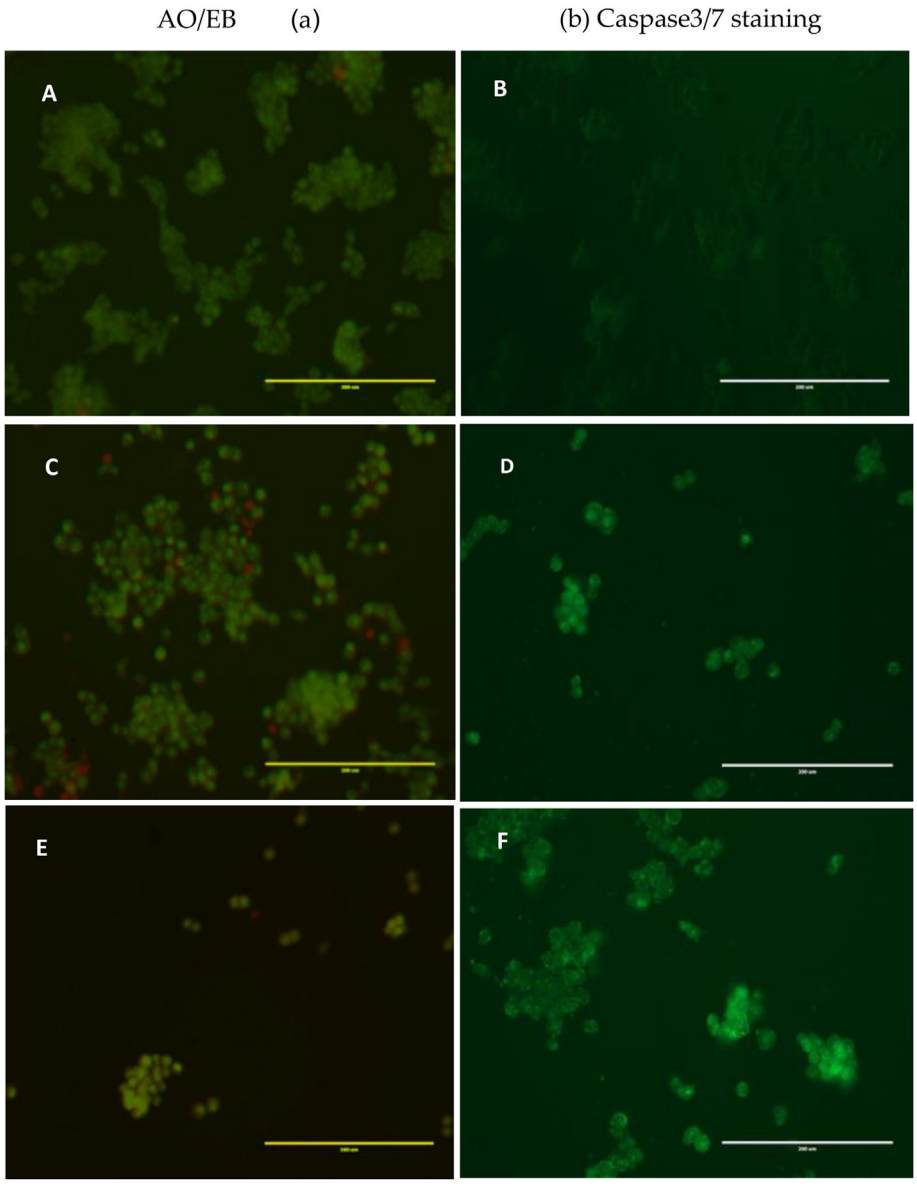

Fig. 5. Fluorescence microscope (200×) was used for cell apoptosis imaging. IC_{50} was applied on LoVo cells for 24 h then acridine orange-ethidium bromide used for staining (column (a) and caspase 3/7 (column (b). (A and B) untreated control, (C and D) are the treated cells with AgNPs prepared using *A. indica* ethanolic extract, and (E and F) are treated cells with AgNPs prepared using *A. indica* aqueous extract.

extract and *C. comosum* aqueous extract induced apoptosis. Substances that induce apoptosis are perfect candidates for cancer treatment. The main mechanism by which anticancer drugs destroy cells is induction of apoptosis (Ji Yubin et al., 2017). Natural materials were described to enhance apoptosis. The cytotoxic potential of AgNPs prepared using *A. indica* aqueous and ethanolic extracts and *C. comosum* aqueous extract were investigated and the cell viability and morphology of treated LoVo, MDA-MB-231 and HepG2 cells were assessed. Our results revealed that AgNPs prepared using *A. indica* aqueous and ethanolic extracts as well as *C. comosum* aqueous extract exerted promising cytotoxic effects against all cell lines tested by causing noticeable changes in morphology associated with a reduction in the cells number in a dose-wise and apoptosis. Regarding the morphological signs of observed apoptosis in response to AgNPs prepared in the current study, the apoptotic effects of AgNPs in all cell line cells were further clarified by investigating AO/EB staining and caspase-3/7 activation. Caspases refers to the step wise stimulation of a cascade of proteases that lead to apoptosis. Initiators and effectors are the two classes of caspase including caspase-3 and -7 as the effector caspases (Pirnia et al., 2002). Cells apoptotic pathway may be affected by caspase-3; therefore, high caspase 3 level in the treated cell by AgNPs may help in cell death during apoptosis. Such information proposed that active AgNPs against different cell lines that were investigated may enhance apoptosis in caspase dependent style (Sriram et al., 2010). Cell death due to apoptotic action caused by AgNPs against Jurkat cells was presented by Gurunathan and Raman (2013) and Mollick et al. (2014). Other researchers have

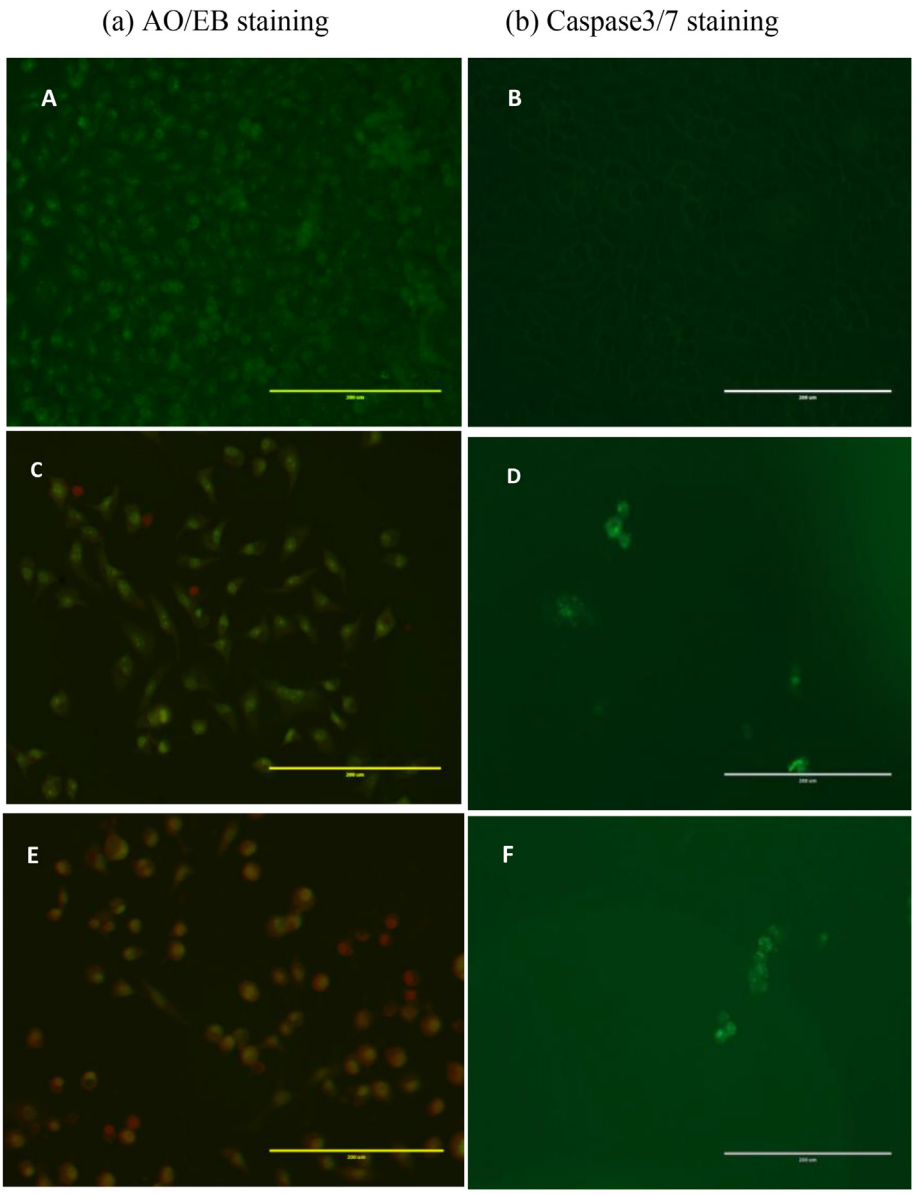

Fig. 6. Fluorescence microscope (200×) was used for cell apoptosis imaging. IC_{50} was applied on MDA-MB231 cells for 24 h and stained with acridine orange-ethidium bromide (column (a)) and caspase 3/7 (column (b)). (A and B) untreated cells, (C and D) cells treated with AgNPs prepared using *A. indica* ethanolic extract, and (E and F) cells treated with those prepared using *A. indica* aqueous extract.

also reported similar findings that *A. indica* encourages apoptosis in various cancer cell through both extrinsic and intrinsic pathways (Sharma et al., 2017; Priyadarsini et al., 2010; Hao et al., 2014). For instance, nimbolide, limonoids, azadirachtin induced apoptosis in different cancer cell lines such as HeLa, MCF-7 and MDA-MB-231 cells (Priyadarsini et al., 2010; Elumalai et al., 2012). It was also reported that the antioxidants' activity of azadiracht in and nimbolide can simultaneously target many molecules involved in apoptosis that offer a huge potential as anticancer therapeutic agents (Priyadarsini et al., 2010). Taken together, our result warrants further investigation on *A. indica* and *C. comosum* as potential chemotherapeutic agents including the assessment of their activity using animal models.

5. Conclusion

Medication resistance concerns linked to cancer drugs and antibiotics motivated researcher to discover substitutes to chemical therapeutic agents. Silver ion in various structures showed the power to suppress the growth of microbes and cancer cell lines. Green production of AgNPs utilizing plant extract is an economical and cost-efficient method. Biogenic prepared AgNPs showed a cytotoxic effect against LoVo, MDA-MB231 and HepG2 ca cells and antibacterial against some bacterial strains. Increased caspase in tested cell lines and the bacterial cell wall decomposition could be indicators for AgNPs mechanism that should be further investigated.

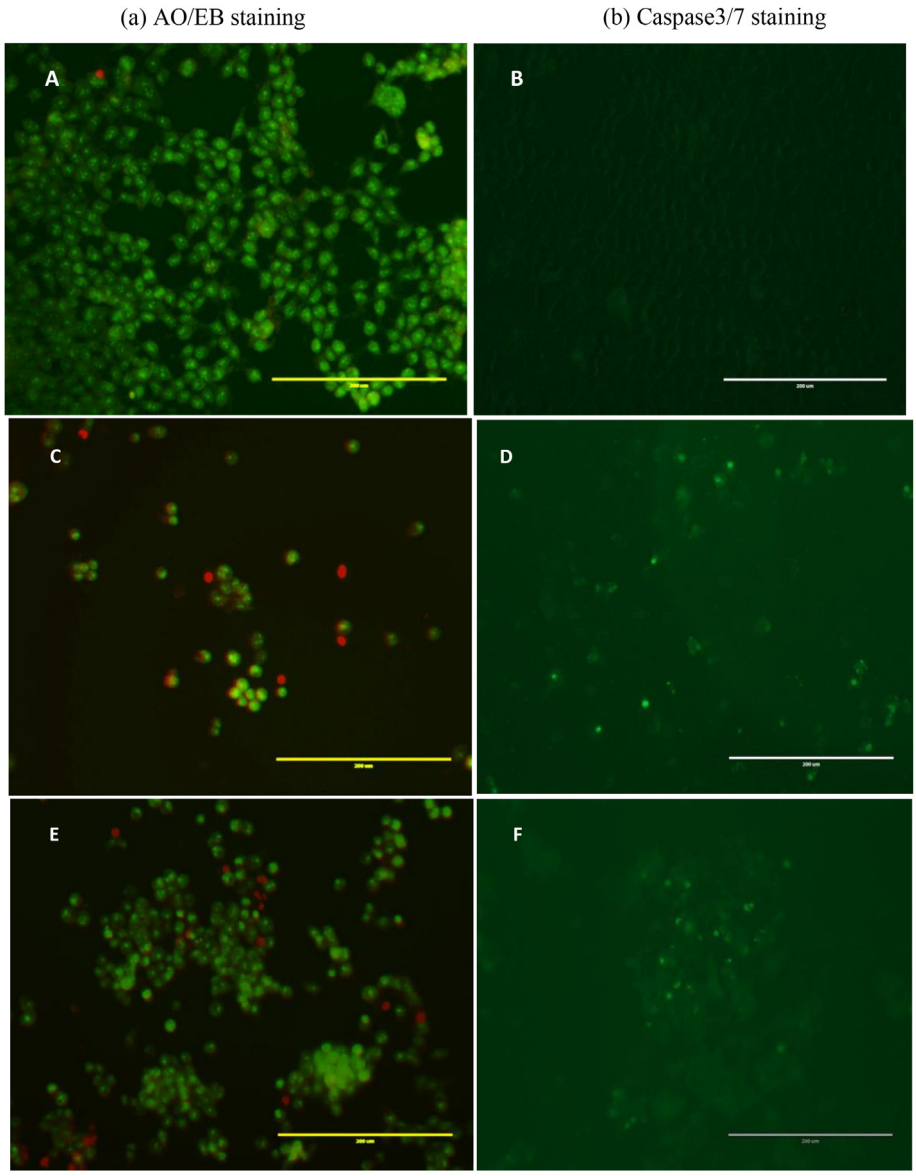

Fig. 7. Fluorescence microscope (200×) was used for cell apoptosis imaging. IC_{50} was applied on HepG2 cells 24 h and stained with acridine orange-ethidium bromide (column (a)) and caspase 3/7 (column (b)). (A and B) untreated cells, (C and D) cells treated with AgNPs prepared using *A. indica* ethanolic extract, and (E and F) cells treated with those prepared using *A. indica* aqueous extract.

Author contributions

A.E.M. and A.S.A planned the experiments, A.E.M, A.S.A., and N. A. accomplished the experiment, A.E.M, M.M.E and N.A. prepared the first draft, A.E.M and M.M.E wrote the final version.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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