Antioxidant, cytotoxic and antibacterial potential of biosynthesized nanoparticles using bee honey from two different floral sources in Saudi Arabia

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Nowadays, the innovative study of silver nanoparticles (AgNPs) is excessive since they have incredible biomedical applications. The current study aimed to find out the potential of honey from two different floral sources (Ziziphus spina-christi and Acacia gerrardii) as biogenic mediators to synthesize AgNPs and to evaluate their antioxidant, cytotoxic and antimicrobial abilities. Biogenic AgNPs were studied for particle characterizations and the expected biomolecules helped in the reduction process of silver (Ag) ions to AgNPs. Results demonstrated different size (50–98 nm) and potential /C0 42 and /C0 30 for AgNPs prepared using different biological materials, therefore different 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging free radicals were observed. Cytotoxic effect in a dose-dependent manner was detected against LoVo and HepG2 ca cells for biogenic AgNPs resulted from cell apoptosis that detected by caspase 3/7 activation in the treated cells compared to their corresponding controls. Furthermore, biogenic AgNPs suppressed the growth of Methicillin-resistant bacteria Staphylococcus aureus (Gram-positive) besides Escherichia coli and Pseudomonas aeruginosa (Gram-negative). The LC50 of AgNPs was between 12.8 and 19 µg/mL and the antibacterial capability was between 20.8 ± 1.2 and 15.6 ± 0.8 mm. Bacterial membrane disturbance was evident in the current study when treated bacteria were studied by field emission scanning electron microscopy (FE-SEM) in relation to untreated controls. Overall, the present findings indicated the possibility of simple green synthesis of AgNPs using honeybee, which are effective agents in some biomedical applications. Detailed future work is needed to further validate the results.

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1. Introduction

One of the most critical problems in the field of medicine is the microbial resistance against a wide spectrum of diseases caused by various pathogenic agents. Progress and advances in science and research worldwide has given birth to nanotechnology, which has recently emerged as smart innovative alternative to help resolve such a problem of microbial resistance. Nanotechnology is currently assumed as a robust medical tool, which involves formation of different nanomaterials. For such formation, some metals have been employed such as copper, magnesium, gold and silver (Naveen et al., 2010). Currently, nanoparticles are successfully utilized in a variety of applications in medicine and pharmaceutical (Moghaddam et al., 2014; Makarov et al., 2014). Ag is the mostly used metal for the synthesis process of nanoparticles as evident in many previous studied (Sharma et al., 2012; Ashour et al., 2015; Devanesan et al., 2017; Mohammed et al., 2018). Formerly, the conventional methods adopted in the process of nanoparticles formation involve both chemical and physical approaches, electrochemical and photochemical reduction as well as heat evaporation method (Dubeya et al., 2010); however those techniques are associated with some drawbacks with regard to their harmfulness and the toxic hazards to human and other living organisms and the environment at large in addition to the high cost for the synthesis process (Moghaddam et al., 2014; Makarov et al., 2014). To address such constraints, science and research have been shifted towards seeking better alternative techniques for
nanoparticles formation. Plant extracts have been attempted as reducing and stabilizing agents coupled with specific metals and metal oxides for nanoparticles formations. Employment of plant extracts for this purpose has shown to have excellent merits over the former physico-chemical and other techniques as it is simple, cost-effective, safe, and eco-friendly (Zhang et al., 2008; Philip, 2010; Prasad, 2011; Kaviya et al., 2011; Kharisssova et al., 2013) and such a technique has been referred to as “green synthesis” or “green chemistry”. It has been suggested that AgNPs formation via this green technology involves three main phases: selection of solvent medium, selection of environmentally friendly reducing agent and selection of non-toxic substance for stability of AgNPs (Vaidyanathan et al., 2009). For formation of AgNPs by the green synthesis technique plant extracts as well as algae and fungi have been utilized as reported in numerous previous investigations (Vijayaraghavan and Nalini, 2010). AgNPs have been shown to be highly promising in various applications in different fields like drug and gene delivery, biological sensors, catalysis, electronics, energy storage, antimicrobial protection and biomedical treatments (Vidhu et al., 2011; Kannan and Subbalaxmi, 2011; Ghaseminezhada et al., 2012). Recently, the use of AgNPs as antimicrobial for a wide range of microorganisms has gained paramount concern as well as its elegant and successful employment as a cytotoxic agent against cancer cells (Mohammed et al., 2018). Recently, honey has been employed as a mediator in green synthesis of AgNPs for the treatment of different health disorders. Honey is generally regarded as a natural substance which possesses an active role in the inhibition process for many pathogenic organisms due to the fact that it is characterized with the presence of several active compounds, for instance flavonoids, glycosides, and phenolic acids (Chute, 2010). Moreover, some other investigations linked the inhibitory activity observed for several microorganisms to the low pH of honey (Bogdanov, 2008). To date, limited research work has been performed on its potential role in this process. In one study, Haiza and co-workers (Haiza et al., 2013) managed to produce AgNPs using local honey (Tualang) in Malaysia as a stabilizing agent. Honey has also been used by Ali and others (Ali et al., 2017) who evaluated the efficacy of honey in AgNPs synthesis. The authors have demonstrated that addition of AgNPs synthesized by honey to the stored maize grains caused significant reductions in the aflatoxin B1 toxin. Furthermore, antibacterial and cytotoxic effect against cancer cell were detected for the biogenic AgNPs (Mohammed et al., 2018; Alqahtani et al., 2017). Attraction between Ag ions positive charge and bacterial membrane negative charge may lead to AgNPs antibacterial activity via cell membrane disturbance and high affinity of Ag ions to react with vital enzymes and impair the replication of DNA (Shao et al., 2015). Furthermore, AgNPs may lead to the formation of reactive oxygen species (ROS) that is the main reason of bacterial cell membrane disturbance (Danilcau et al., 2006; Kim et al., 2007). On the other hand, the anticancer of AgNPs was early investigated which might be related to cell apoptotic induction via ROS that were considered to be cytotoxic agents (Sriram et al., 2010). As a novel line in nanotechnology, AgNPs (1–1000 nm) were useful in cancer diagnostic and treatment (Yezhelyev et al., 2006); therefore biogenic synthesis of AgNPs might be superior in medical applications since natural compounds are used with Ag ions as capping agents. In the present work, the principal goal was to evaluate the efficacy of honey as a mediator in the process of AgNPs formation. Honey was derived from two different floral sources naturally grown in Saudi Arabia, Z. spina-christi and A. gerrardii. To realize the goal in this study a set of parameters have been investigated using advanced techniques to characterize the prepared biogenic AgNPs besides studying their ability as anti-cancer and anti-microbial agents. To the best of our information, this comparative investigation is known to be a pioneer report regarding preparation of biogenic AgNPs using honey bee from different floral sources.

2. Results and discussion

2.1. Characterization of AgNPs synthesis using honey

The outcomes from the present investigation showed that, addition of honey from the two different floral sources to the silver in the form of a nitrate (0.1 mM) provided brown color of the mixture that turned from yellow at the start point of addition, indicating the plasmon resonance excitation of AgNPs (Veerasamy et al., 2011). Mixture color changing in time-dependent manner was observed, after seven days keeping in dark condition. The dark brown color was clear and stable. UV–VIS Spectroscopy was used to observe Ag ions bio-reduction, peaks of 441 and 446 nm were detected for AgNPs prepared using honey from Z. spina-christi and A. gerrardii floral sources, respectively. Same trend of observations regarding color change and 425–450 nm absorption spectra were observed for AgNPs prepared from AgNO₃ (20–50 mM) using Egyptian honey (El-Desouky and Ammar, 2016). On the other hand, the biogenic AgNPs morphology, size and the surface shapes were investigated using SEM and TEM images. Fig. 1 shows SEM and TEM images, (a–c) shows AgNPs prepared by honey bee from Z. spina-christi, (b–d) shows AgNPs achieved by honey bee from A. gerrardii. Sphere-shaped with smooth edges and well-distributed AgNPs were obtained from honey bee from both floral sources when SEM and TEM were used. Hosny et al. (2017), El-Deeb et al. (2015) found same shape and characteristics for AgNPs prepared by honey. SEM-EDX was used for element analysis of the biogenic AgNPs prepared in the current study (Fig. 2) showing the oxygen and carbon as the main components. AgNPs nanostructure prepared from honey and contain carbon and oxygen besides the Ag was also stated by El-Deeb et al. (2015). Furthermore, biogenic AgNPs with size and zeta potential of 50.5 nm and −0.42 mV was noticed for those prepared from honey bee from Z. spina-christi and for those prepared from honey bee from A. gerrardii showed 78.2 nm and −0.41 mV. Low size (9–22 nm) were noted by El-Desouky and Ammar (2016) for AgNPs prepared using honey. Negative zeta potential of biogenic AgNPs was also recorded in different studies (Farhadi et al., 2017; Mohammed et al., 2018) which might be the main reason for AgNPs stability and distribution since it may exhibit the particles repulsion (Farhadi et al., 2017). Furthermore, negative ionizable groups in the biomolecules from honey attached to Ag ions may change them to negative ions (Khatoon et al., 2017). Honey from different floral sources showed AgNPs with no significant variations regarding zeta size and potential (Table 1).

2.2. Fourier transform infra-red spectroscopy

To identify the possible biomolecules in honey which contribute in the conversion of Ag ions to AgNPs, FT-IR was used. AgNPs prepared in this study by honey from Z. spina-christi showed a range of 1060–3266 cm⁻¹ and that from A. gerrardii had FT-IR in the range between 1636 and 3300 cm⁻¹ showing different biomolecules in each mixture. H-bonded OH groups stretching vibrations could be shown by peaks at 3268–3432 cm⁻¹ for phenol or glycosides (Prabu and Natarajan, 2012). The peaks 3266 and 3300 that have been observed for AgNPs prepared from honey bee from different floral sources (Farhadi et al., 2017; Mohammed et al., 2018) which might be the main reason for AgNPs stability and distribution since it may exhibit the particles repulsion (Farhadi et al., 2017). Furthermore, negative ionizable groups in the biomolecules from honey attached to Ag ions may change them to negative ions (Khatoon et al., 2017). Honey from different floral sources showed AgNPs with no significant variations regarding zeta size and potential (Table 1).
at both mixtures from different honey floral sources (Kong, 2007). Band near 1633 cm\(^{-1}\) were detected for natural protein showing that, binding with AgNPs didn’t change the honey protein nature (Macdonald and Smith, 1996). Bands detected in the current study suggested different components of honey that could be responsible for the conversion of AgNO\(_3\) into AgNPs. The study performed by Philip (Philip, 2010) suggested 181 different compounds from honey such as proteins, minerals and polyphenol that could be stabilized to reduce Ag ion to AgNPs. Regarding FT-IR results and results with the same line noted (El-Deeb et al., 2015; Murugaraj et al., 2013), conclusion may state that, the biomolecules from honey bee may attach to the AgNPs resulting in reducing, capping, stabilizing and improving the biological characteristics of prepared AgNPs. Commonly, the key factors in Ag ion reduction are the amino groups that easily attached to them (El-Deeb et al., 2015). Clear stability and low accumulation of the biogenic AgNPs are shown by TEM and SEM images could be related such bio-compounds from honey.

2.3. Cytotoxic effect of the biogenic AgNPs

Combination of honorable metals and biomolecules may have an interest in some biomedical applications (Jeyaraj et al., 2013), therefore biogenic AgNPs could be applied in the treatment of cancer or bacterial infection as an alternative agent or complementary medicine. Using MTT test, biogenic AgNPs showed anti-proliferative ability against HepG2 cancer cells with LC\(_{50}\) of 15.8 and 14.1 µg/mL indicating potential anticancer agents for those prepared using honey from Z. spina-christi and A. gerrardii, respectively. Positive correlation between the high concentration of AgNPs and suppression level of cancer cell was also observed for both biogenic agents studied. Against colon cancer, El-Deeb et al. (2015) demonstrated anticancer ability of honey-prepared AgNPs with 58.6% suppression ability and a concentration of 50 µg/ml showed total MCF7 cell death (Jeyaraj et al., 2013). Different observation with regard to the AgNPs cytotoxicity level might be more related to their size, the type of tested cell and the biomolecules attached to them since secondary metabolites

Fig. 1. SEM images (a, b) the corresponding TEM images (c, d) of AgNPs capped with the biomolecules of honey bee from Z. spina-christi (a - c) and A. gerrardii (b - d). Magnification is 20,000 and scale bar represents 1 and 10 µm for SEM images and 200 nm for TEM images.
may have anticancer ability. The mechanism of AgNPs against cancer cells is not completely understood, but cell oxidative stress caused by generation of ROS might be related to uptake of AgNPs by the cell. Apoptosis is considered as one of the biological mechanisms that disturb the abnormal cell and a good indicator in cytotoxicity research (Aigner, 2002). Mostly, chromatin condensation is one of the apoptosis signs in cancer cells (Ciniglia et al., 2010).

### 2.4. Morphological evaluation by AO/EB double staining

In the current study, by using AO/EB double staining for HepG2 48 h after the application of biogenic AgNPs, nuclear morphology was checked under fluorescent microscopy. Results revealed that untreated cells were uniform and green and no apoptotic sign was detected (Fig. 5), but yellow green granular and unequally localized nuclei were clear in apoptotic cell in early stage (Fig. 6).

[Fig. 2. SEM-EDX images (a, b) and the corresponding element analysis images (c, d) of AgNPs obtained using honey bee from Z. spina-christi (a - c) and (b - d) for those obtained using honey bee from A. gerrardi.]
but cells that contain uneven orange nuclei considered as necrotic cells increased in volume (Fig. 6). Such information suggesting the cell death was appeared due to apoptosis. Same trend of observation was recorded for apoptosis detect in Osteosarcoma using Dual AO/EB Staining (Liu et al., 2015).

### 2.5. Activation of caspase-3/7 in HepG2 cell lines

Above prediction was additionally defined by Caspase-3/7 test. Proteolytic enzymes caspases are efficient in apoptosis (Mcllwain et al., 2015). Botho caspase3/7 take place during apoptosis making cleavage, however each of them direct role is not known (Slee et al., 1999). In the current study, CellEvent®Caspase-3/7 kit that once the fluorescently labeled DEVD peptide is disjointed releases fluorescence light was used to find out the apoptotic ability of AgNPs prepared by honey from different floral sources. Green bright fluorescence light was used to find out the apoptotic ability of AgNPs prepared using honey from both floral sources. Green bright fluorescence in the current study was clear for HepG2 cells treated with biogenic AgNPs (Fig. 3). Expected signaling of caspase by biogenic AgNPs might happen leading to cell death. Apoptosis induction is the main way for cell destruction by cancer drugs (Liu et al., 2015). Biogenic AgNPs tested here showed anti-proliferative effect against HepG2 cells that showed cellular apoptosis and cell reduction in a dose-dependent manner. High detected level of caspase 3 might be the reason for apoptosis and cell death as a result to biogenic AgNPs treatment.

### 2.6. DPPH radical scavenging test

For biogenic AgNPs antioxidant action assessment, scavenging of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radicals were identified at absorption peak 520 nm. Different concentrations were used from biogenic AgNPs and the DPPH exhibited positive correlation with the AgNPs concentration. AgNPs synthesized using honey from both studied floral sources displayed high antioxidant activity (Fig. 4). However AgNPs prepared by honey from Z. spina-christi showed higher antioxidant ability than those prepared by honey from A. gerrardii, this could be linked to their smaller particle size and therefore higher cytotoxic effect was found. Same line of result was noted (Mohammed et al., 2018) when they investigated biogenic AgNPs.

### 2.7. Antibacterial ability test

A bactericidal action of AgNPs prepared by honey from different floral sources against Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli was found. Higher zones of inhibition in bacterial cultures around AgNPs wells were noted for those prepared from Z. spina-christi honey than those prepared from A. gerrardii honey (Table 1). Different detected AgNPs particle sizes could be the main reason for different antibacterial capabilities since negative correlation between particle size and antibacterial level was observed (Table 1). Additionally, AgNPs prepared using honey from Z. spina-christi revealed about 95% of amoxicillin and 79% of Cefuroxime and 86% of Ciprofloxacin abilities to suppress S. aureus. More than 100%, 88% and 64% of amoxicillin, cefuroxime and ciprofloxacin, respectively against E. coli were detected, regarding P. aeruginosa activity was clear for such AgNPs whenever, amoxicillin, Cefuroxime showed no activity and more than 50% of Ciprofloxacin activity was clear. 85%, 70% and 77% of amoxicillin, cefuroxime and ciprofloxacin activities, respectively were stated for AgNPs prepared using honey from A. gerrardii against S. aureus, respectively were stated for AgNPs prepared using honey from A. gerrardii against S. aureus were detected, more than 100%, 76% and 55% of amoxicillin, cefuroxime and ciprofloxacin, respectively were stated against AgNPs prepared using honey from A. gerrardii against S. aureus were detected, more than 100%, 76% and 55% of amoxicillin, cefuroxime and ciprofloxacin, respectively were stated against E. coli. Good activity was clear when compared with amoxicillin, cefuroxime activity against P. aeruginosa while more than 50% of ciprofloxacin activity was clear. High antibacterial activity observed for biogenic AgNPs, which might be related to the combination process of honey activity and Ag ions activity. Silver nitrate is an effective antimicrobial agent leading to cell death via blocking of different biological functions such as respiration, transport systems and DNA and RNA transcriptions (Dakal et al., 2016). Furthermore, interference of Ag ion and AgNPs with disulfide bonds in protein may change its 3D construction that affects the microbial function (Lok et al., 2006). Our current study is in the same line with other previous works that showed greater antibacterial activity of AgNPs in relation to Ag ions alone (Cavassin et al., 2015; Franci et al., 2015) (which is good since bacterial Ag resistance has been reported (Randall et al., 2012). Although the mode of action against microbes for Ag ions in both forms is expected to be the same, but high distribution surface area of AgNPs increase its ability against microbes. In the current study, although not significant, but AgNPs prepared by honey from both floral sources showed higher antimicrobial against Gram-negative bacteria compared to Gram-positive ones, which might suggest a relationship between cell wall structure and antimicrobial effect. Hard penetration of AgNPs in Gram-positive bacteria could be because of the dense peptidoglycan with negative charge (Dakal et al., 2016). Penetration of AgNPs inside bacterial cell may induce intracellular metabolites excretion that lead to abnormal cell structure (Gopinath et al., 2015).

### Table 1

| Treatment | Average particle size (nm)* | Average Zeta potential (mV)* | Inhibition zone + gram (mm) | Inhibition zone – gram (mm) |
|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Ag1       | 50.5 ± 0.7                  | -0.42 ± 0.1                 | 194 ± 6.6                   | 228 ± 1.2                   |
| Ag2       | 98.2 ± 0.9                  | -0.40 ± 0.3                 | 170 ± 0.1                   | 190 ± 1.3                   |
| Honey1    | –                           | –                           | 10.1 ± 1.2                  | 11.2 ± 1.1                  |
| Honey2    | –                           | –                           | 12.0 ± 0.7                  | 9.2 ± 0.6                   |
| Ag ions   | –                           | –                           | 2                           | 2.2                        |
| Amoxicillin (AMO) | –                  | –                           | 20 ± 0.6                    | 18 ± 1.4                    |
| Cefuroxime (CXM) | –              | –                           | 24 ± 0.9                    | 25 ± 1.9                    |
| Ciprofloxacin (CIP) | –            | –                           | 22 ± 1.7                    | 34 ± 1.3                    |

Data expressed as mean ± SD. Ag1 = AgNPs prepared by honey from Z. spina-christi, Ag2 = AgNPs prepared by honey from A. gerrardii, Honey 1 = honey from Z. spina-christi, honey 2 = honey from A. gerrardii.
2.8. Determination of tolerance level

Against bacterial strain tested, MIC and MBC level were determined for AgNPs using different concentrations. A range between 12.5 and 75 µg/mL were the MIC and MBC for studied AgNPs that reflect their bactericidal ability since the value of 2 or less were recorded for tolerance level (Woods and Washington, 1995; Mohammed et al., 2018) showed same trend of observations. Furthermore, bacterial strains S. aures, E. coli and P. aeruginosa were examined by FESEM (JEOL JSM-7001F, Germany) to check bacterial cells morphological modifications of before and after AgNPs treatment. MIC of biogenic AgNPs prepared using honey from Z. spina-christi was added to the tested bacteria and after 1 h test was done. Membrane compact loss lead to some morphological alterations in all treated bacterial strains was noticed in comparison to untreated ones that showed constant morphology and smooth cell wall, same line of results was observed (Mohammed et al., 2018; Gopinath et al., 2015).

3. Materials and methods

3.1. Honey samples and materials collection

The honey samples from Z. spina-christi and A. gerrardii were purchased from Riyadh region, Saudi Arabia. Sealed glass containers were used to store the samples until further uses at room temperature. AgNO₃ was obtained from Somatco Company, Riyadh, Saudi Arabia.

3.2. Honey bees for green synthesis of AgNPs

For biogenic synthesis of AgNPs by Ag ion reduction, one ml of honey was added to 5 ml AgNO₃ (1 mM). Combined substances were homogenized and incubated under dark conditions for 72 h at 30°C. Conversion of the mixture color to brown is considered to be as the first indicator for AgNPs formation. Prepared materials were stored at 4°C for further AgNPs characterization and applications.
3.3. AgNPs detection and characterization

The optical properties of the biogenic AgNPs were studied using UV-2450 double-beam (200–800 nm) UV-spectrophotometer (Shimadzu, Tokyo, Japan). FT-IR (Nicolet 6700 FT-IR Spectrometer, Waltham, MA, USA) was used to find out the expected biomolecules in biogenic AgNPs at 500–4000 cm\(^{-1}\) resolution. Biogenic AgNPs structure was observed and imaged using transmission electron microscope (TEM), a JEOL JEM-1011 (JEOL, Peabody, MA, USA) and field emission scanning electron microscopy (JEOL 7500FA JEOL, Peabody, MA, USA) for their size and shape determinations at 200 kV and 30 kV voltage, respectively. Mean size distribution and zeta potential were examined using a zeta sizer instrument (Malvern, Worcestershire, UK).

![Cell apoptosis images](image1.png)

**Fig. 4.** Cell apoptosis images captured using fluorescence microscope (200×). HepG2 cells were treated with IC\(_{50}\) for 24 h and stained with caspase3/7. (Control) untreated cells, (1 and 2) cells treated with AgNPs prepared using honeybee from Z. spina-christi, (first raw) and A. gerrardii, (second raw).

![Antioxidant ability](image2.png)

**Fig. 5.** Antioxidant ability of AgNPs prepared using honey from Z. spina christi (Ag1) and that prepared from A. gerrardii (Ag2). Different concentrations were used.
3.4. DPPH radical scavenging assessment

The antioxidant activity was measured according to Ghosh et al. (2013). 100 μM of DPPH was prepared in methanol and 20 μL of biogenic AgNPs and 180 μL DPPH working solution were mixed together in a 96-well plate. 200 μL of DPPH alone was used as control. Absorption was taken after 30 min at 25 °C and 515 nm. Antioxidant ability of each mixture in the well was evaluated by comparing its absorbance to that of the control. Calculations were done as a percentage of scavenging activity using following formula:

\[
\text{DPPH scavenging activity(\%) = } \frac{[A1 - A2]}{A1} \times 100
\]

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

3.5. Cytotoxicity of biosynthesized AgNPs

Liver carcinoma (HepG2) cell lines for human were maintained from King Saud University, Riyadh, Saudi Arabia. Cells were obtained in high glucose Dulbecco's Modified Eagle's medium (DMEM), enhanced with fetal bovine serum 10% (Gibco, Germany) in a humidified incubator and 1% penicillin streptomycin (Thermo, Canada) at 37 °C and 5% CO₂. 5 x 10⁵ cells were seeded into 24-well plate (NEST, China) overnight. Cells were treated with different concentrations of biogenic AgNPs for 48 h and cells treated with vehicle only were considered as negative control. Then cells were incubated with 100 μL of 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) for 2 h at 37 °C after 48 h of treatment. Thereafter, the medium was aspirated and formazan crystals formed were dissolved in 0.1% HCL-methanol and plates were read at 570 nm in a multi-well plate reader (Thermo Scien-
tic™ Multiskan, China). The optical density (OD) obtained was used to find out the viability (%) compared to control using the following formula:

\[
\text{Viability} = \frac{\text{OD sample}}{\text{OD control}} \times 100
\]

3.6. Staining with acridine orange/ethidium bromide

Fluorescent DNA dyes, Acridine orange and ethidium bromide (Sigma) were used. Double staining with these dyes permits to differentiate among the different types of affected cells: live cells will be a green nucleus of stable size; apoptotic cells will be condensed in green, contracted or split nucleus; and necrotic cells will show a red nucleus of regular size or larger. Briefly, cells were grown in 24-well plates (5 × 10^5 cells/well) and incubated for 24 h. IC50 of biogenic AgNPs was used against the target cells. Media was washed and cells and phosphate buffer saline (PBS) was used for cleaning. Finally, AO/EB solution was added to the cell and incubated, then nuclear morphology was assessed by fluorescence microscopy (EVOS, USA).

3.7. Detection of caspase 3/7

Cell Event Caspase-3/7 Green Detection Reagent (Invitrogen, USA) was used to find out caspase 3/7. It is amino acid with four peptides, connected to a nucleic acid binding dye. After activation of caspase-3/7 in apoptotic cells, the DEVD peptide is cleaved enabling the dye to bind to DNA and produce a bright, fluorescence response. For this purpose, cells were cultured in a 24-well plate and treated with the biogenic AgNPs extracts at IC50 concentration for 24 h. The control was included where only the vehicle (methanol) was used. After 24 h, each well received Caspase-3/7 reagent (2 μM), then incubation at 37 °C for 30 min in a dark condition. Plates were then detected under fluorescent microscope (EVOS, USA).

3.8. Antibacterial action of AgNPs

Agar well diffusion method was performed to find out the antibacterial action of AgNPs (Mohammed, 2015) against S. aureus; P. aeruginosa and E. coli. Mueller-Hinton agar was used to microbial growth and into a single agar plate, 2.5 × 10^5 CFU/ml (0.2 ml) of each bacteria species was equally spread using sterile swabs. Thereafter, three wells (4 mm diameter each) were prepared at each agar surface using a sterile cork borer. 0.2 ml AgNPs was placed in the well under sterilized conditions, plates were kept for 1 h at room temperature for the diffusion of biogenic AgNPs into the agar. Distilled water was considered as negative control, thereafter; all plates were incubated for 18–24 h at 37 °C. Inhibition zones around the wells were measured. On the other hand, a micro dilution method in NB was used to find out the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). About 2.5 × 10^5 CFU/ml (10 μL) of bacterial strain was added separately to NB (10 ml) and strains was tested with different AgNPs concentrations and incubated for 24 h. MIC was determined by turbidity checking of bacterial growth after the incubation. The concentration that suppresses bacterial growth (99%) is known as the MIC and the concentration that totally killed bacteria growth is the MBC (Das et al., 2012). With the aid of the following formula, tolerance levels for the tested bacteria in relation to AgNPs were calculated (May et al., 1998) indicating their bactericidal ability.

\[
\text{Tolerance} = \frac{\text{MBC}}{\text{MIC}}
\]

3.9. AgNPs synergistic antibacterial potential

Using a disk diffusion method, Amoxicillin, Cefuroxime and Ciprofloxacin were used to investigate the synergistic antimicrobial effect of biogenic AgNPs against S. aureus, P. aeruginosa, and E. coli (Finegold and Baron, 1996). 1 ml of AgNPs was added to the disks of antibiotic then located inside the bacterial plates. Synergistic activity of biogenic AgNPs in combination with antibiotics was evaluated after incubation process for 24 h at 37 °C as an inhibition zone (mm).

3.10. Assessment of bacterial cell membrane damage

In a trial to find out the actual mode of action of AgNPs against studied microbes, the treated bacteria were checked using FE-SEM to detect any change in the bacterial structure. AgNPs from Z. spina-christi honey was added at MIC to the bacterial growth then cultures were incubated for 1 h at 37 °C. The treated cells were subjected to centrifugation for 10 min at 6000 r.p.m and 4 °C. PBS was used for pellet washing at pH 7, then fixed with glutaraldehyde 8% and Sorensen's phosphate buffer (SPB) for 1 h. Osmium tetroxide 4% (OsO4) and H2O mixed together (1:1) volume and used to fix the samples. Water and ethanol were used for washing and dehydrating the sample after 24 h, then assessment under FESEM (JEOL JSM-7001F, Germany) was done.

3.11. Statistical analysis

In the present study, evaluations were done in triplicates; expressed values were as mean ± SD. The statistical program SPSS version 11 was used to perform one-way ANOVA. Student's t-test at (P < 0.05). One sample image for biogenic AgNPs was chosen from TEM, SEM and zeta size images. Representative pictures are supplemented with scales.

4. Conclusion

Drug resistance concern related to cancer drug and antibiotics enhanced the scientific research to find out alternatives to such chemical compounds. Ag in different forms showed ability to suppress microbial and cell growth. Green synthesis of AgNPs using honey from two different floral sources is an efficient and cost-effective technique. Biogenic prepared AgNPs showed a cytotoxic effect against HepG2 cells and antibacterial against some Gram (−) and Gram (+) bacteria. Higher antioxidant ability of AgNPs enhanced their ability to suppress microbial and HepG2 cells growth. Exact mechanisms need to be further confirmed since hints in the current study are given such as, apoptosis in HepG2 cells since caspases increased in treated cells and degradation of microbial cell wall was clear when tested under scanning electron microscope. In the current study, positive correlations between particle size, DPPH antioxidant activity, cytotoxic effect and antimicrobial efficacy were stated.

Author contributions

J.S.A. and A.E.M planned the experiments, accomplished the experiment, and A.E.M. wrote the paper.

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Declaratıon of Competing Interest

The authors declare no conflict of interest.

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