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Green synthesis of *Brassica campestris* mediated silver nanoparticles, their antibacterial and antioxidant activities

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Abstract
Here in the present work silver nanoparticles (AgNPs) were prepared via green method using *Brassica campestris* plant extract as a reducing as well as stabilizing agents. Synthesis of AgNPs via plant extract is an environmental friendly, simple and single step approach. Nanoparticles formation occurs at room temperature after the addition of plant extract to silver nitrate solution. The obtained AgNPs product was examined by using UV-visible spectroscopy, fourier-transform infrared spectroscopy (FT-IR), powder X-ray powder diffraction (PXRD), thermal gravimetric analysis (TGA), energy dispersive x-ray spectroscopy (EDX) and scanning electron microscope (SEM). SEM images of AgNPs showed that prepared nanoparticles spherical predominantly. EDX showed the elemental nature of AgNPs. XRD confirm the cubic structure of AgNPs, while the FT-IR analysis showed that presence of biomolecules on the nanoparticles surface.
Prepared AgNPs showed potent antibacterial activity against water borne pathogens *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) at a minimum inhibitory concentration (MIC) 50 µg/mL. The synthesized AgNPs demonstrate outstanding 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical activity.

Keywords: Silver nanoparticles, gemifloxacin, *E. coli*, *S. aureus*, antioxidant activity

1. Introduction
Green chemistry is the use of chemical techniques, routes and methodologies that decrease or eradicate the use or production of feedstock's, products, byproducts, reagents and solvents, etc. that are detrimental to human health or to their environment. By reducing the chemical hazards, then the risks by using or being exposed to those chemical will also be reduced. Chemical hazards cause acute or chronic toxicity including mutagenicity, flammability, carcinogenicity, corrosivity and explosivity. It is true that the role of chemistry is crucial in a fact that our next generation of materials, chemicals, and energy is more susceptible than the present generation. Worldwide demand for development of ecofriendly, cost-effective and novel approaches of chemical processes are required for pollution control [1, 2].
Nanoscience is the branch of science dedicated to the utilization and improvement of materials and devices ranging their size in nanometer scale. Nanoscience is one of the remarkable fields of science and technology that now a day being touted due to their potential improvement in human life. Research on nanotechnology is growing rapidly gradually, thus cover and making their impact in all fields of life [3, 4]. The optical and electronic properties of metal nanoparticles can be adjusted by controlling their shape, size, and their environment, providing a starting point for promising research in the fields of surface plasmon-based plasmonic or photonics [5, 6]. Due to unique chemical stability, high electrical conductivity, catalytic, antioxidant and antimicrobial activity the demand for AgNPs in various fields has increasing day by day [7]. Antimicrobial property of metal nanoparticles has been applied extensively to biomedical devices, clothing, water purification, cosmetics and other several household products. Possibly AgNPs may be attached on the cell membrane surface and by disturbing their respiratory functions and permeability of the cell membrane.

Silver collides not only interact with the surface of microbial cell membranes, but also can be penetrated inside bacterium cell and cause further cell damage. Silver ions can release by AgNPs, which bind with the biological macromolecules of the cell, such as proteins and enzymes [8-10]. Currently, AgNPs antimicrobial mechanism is not fully understood. Several studies are carried out which propose that metal nanoparticles attach on the surface of microbial cell by disturbing their membrane permeability and cell respiration function. It has been reported in the literature that biologically synthesized AgNPs possess elevated bactericidal, fungicidal and catalytic degradation activities than chemically synthesized AgNPs [8, 11]. Here in the current study the synthesized AgNPs nanomaterials were used to investigate the anti-bacterial activity against pathogenic bacteria *S. aureus* and *E. coli* and. Besides DPPH free radical antioxidant scavenging activity was also evaluated.

2. Experimental

2.1 Materials

Silver nitrate was purchased from Johnson Matthey Plc (England), nutrient agar used was purchased from Oxoid Ltd (England). Ascorbic acid used was from Merck and DPPH used was purchased from Sigma-Aldrich.

2.2 Instrumentations

UV-Visible spectra were recorded in range of 300 to 800 nm. UV-visible spectrophotometer Shimadzu, UV-1800 (Japan) was also used for the synthesis of nanoparticles. FT-IR used was spectrum-100 FT-IR spectrophotometer Bruker (ALPHA, USA) using KBr pellets method in a fixed spectral range, i.e. 4000-400 cm^{-1}. TGA experiment was carried out on a SII Exstar 6000, TG/DTA6300 thermal analyzer instrument. FE-SEM JEOL (JSM-7600F, Japan) and EDX JEOL (JSM-7600F, Japan) systems were used for shape and confirmation of elemental analysis. Crystalline nature of the nanoparticles was investigated by JDX-3532 JEOL JAPAN X-ray Diffractometer.

2.3 Preparation of aqueous leaves extract

Fresh leaves of *Brassica campestris* were used for nanoparticles synthesis. 30g of *Brassica campestris* leaf were carefully washed with distilled water, and then grinded in 200 mL distilled water. Than filtered the juice of leaves through filter paper. The filtrate was stored in a refrigerator (-4 ºC).

2.4 Synthesis of silver nanoparticles

50 mL of 100 mM silver nitrate solution was mixed with 50 ml of aqueous extract of *Brassica campestris.* The reaction was placed at water bath at 50 °C temperature for the reduction of silver ions to nanoparticles. When the *Brassica campestris* extract come in contact with $AgNO_3$ solution, the change in color was observed after 30 minutes and the formation of nanoparticles was confirm from UV-visible spectroscopy. The change in color was observed from yellowish to dark red. The nanoparticles were centrifuge at 5000 rpm for 30 minutes and used for further experiment.

2.5 Antibacterial Assays

Antibacterial assays of AgNPs were conducted on gram-positive *Staphylococcus aureus* (*S. aureus*) and gram-negative *Escherichia coli* (*E.* coli) bacteria by standard disc diffusion method. Nutrient agar was used as cultivate medium for bacteria by dissolving 28 g of nutrient agar in 1000 mL of distilled water. The media was autoclaved and cooled. The media was poured in the petri plates and kept for 30 minutes for solidification. After 30 minutes the fresh cultures of inoculums (100 µl) of two different cultures were spread on to solidified nutrient agar plates. Four holes were made with the help of sterile cork borer in the solidified nutrient of each plate. Cultured agar plates were incubated at 37 ºC for 24 hours and after 24 hours of incubation the zones of inhibition were measured in mm. The assays were performed in triplicate.

2.6 Free radical quenching assay

The antioxidant property of AgNPs was carried out by monitoring the capability of quenching 1-1-diphenyl-2-picrylhydrazyl free radical into non-radical form. A reaction mixture containing 2 ml of 50 µM DPPH and 2 mL of the *Brassica campestris* extract synthesized AgNPs solution. Different concentrations of nanoparticles used were of 10, 50 and 100 µg/mL. The reaction was monitor and incubated at 37 ºC in dark for 40 min and the change in color was measured at 517 nm using UV-Visible spectrophotometer. Ascorbic acid of the same concentrations 10, 50 and 100 µg/ml was used as a positive control. Here we used 50 µM concentration of DPPH in consonance with the accuracy in measurements of spectrophotometer. Higher percentage of scavenging activity indicated lower absorbance of the reaction mixture. The antioxidant or free radical-scavenging activity (RSA) was calculated in percent inhibition by using the following equation [8].

$$\%RSA = \left[\frac{(A_{DPPH} - A_{Solution})}{A_{DPPH}}\right] \times 100 \quad (1)$$

3. Results and Discussion

3.1 UV-visible Studies of Silver Nanoparticles

In present work, the *Brassica campestris* leaf was used for stable AgNPs synthesis. Nanoparticles formation was confirmed using UV-visible absorption spectrophotometer. UV-visible absorption spectra were recorded after 30 minute after the reaction, which show maximum absorbance at 445 nm as shown in Fig. 1, which near to surface plasmon resonance of silver. The absorption at 445 nm shows the formation of AgNPs. It has been reported in the literature that silver show surface plasmon resonance from 440-450 depending upon their size [11]. The AgNPs surface plasmon band stability in the solution confirms that the particles are dispersed in aqueous phase with no sign of agglomeration. The stability of AgNPs could be due to a capping or stabilizing agent which probably may be proteins especially amino acids present in the plant extract into the reaction medium.

The hypothetical mechanism proposed for the reduction of silver ions by the hydroxyl groups of gallic acid and catech in of *Brassica campestris* leaves extract as shown in (Fig. 2) [45].

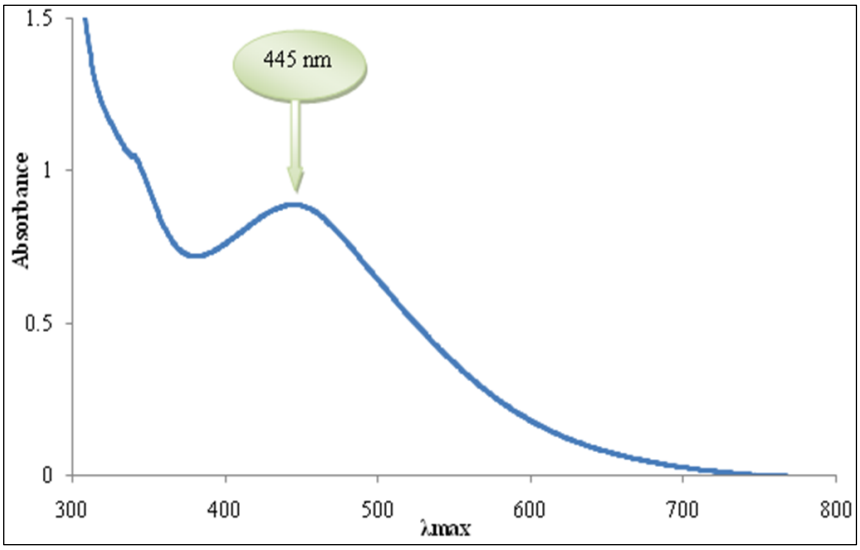


Fig 1: UV-visible spectrum of *Brassica campestris* extract mediated AgNPs showing intense strong absorbance peak at 445 nm.

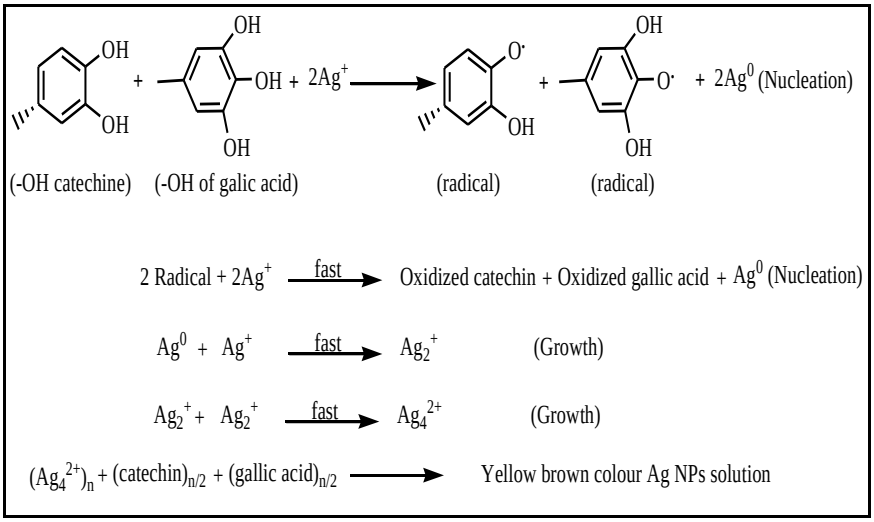


Fig 2: A proposed hypothetical mechanism for the reduction of Ag^+ ions by the hydroxyl groups of gallic acid and catechin of *Brassica campestris*.

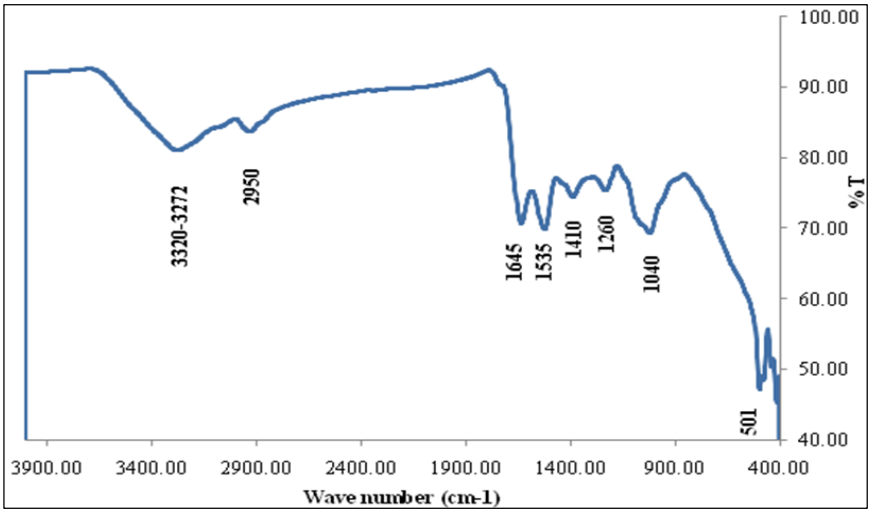
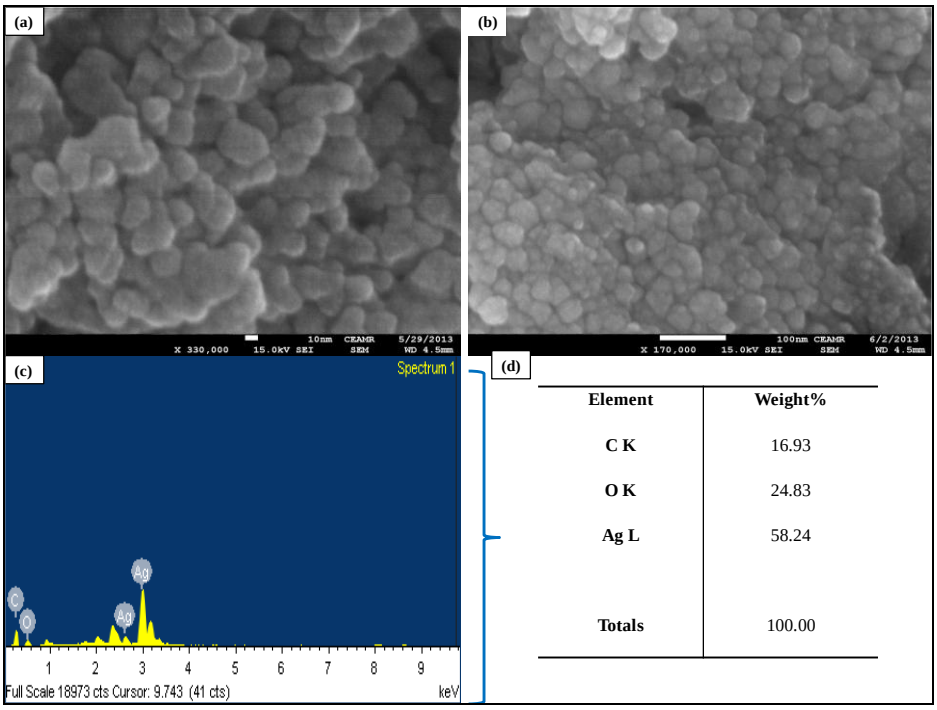


Fig 3: FT-IR spectrum of *Brassica campestris* synthesized Ag NPs



Element	Weight%
C K	16.93
O K	24.83
Ag L	58.24
Totals	100.00

Fig 4: FE-SEM of images of Ag NPs (a) high (b) low resolution images of *Brassica campestris* synthesized Ag NP (c) high (d) EDX spectrum of *Brassica campestris* synthesized Ag NPs

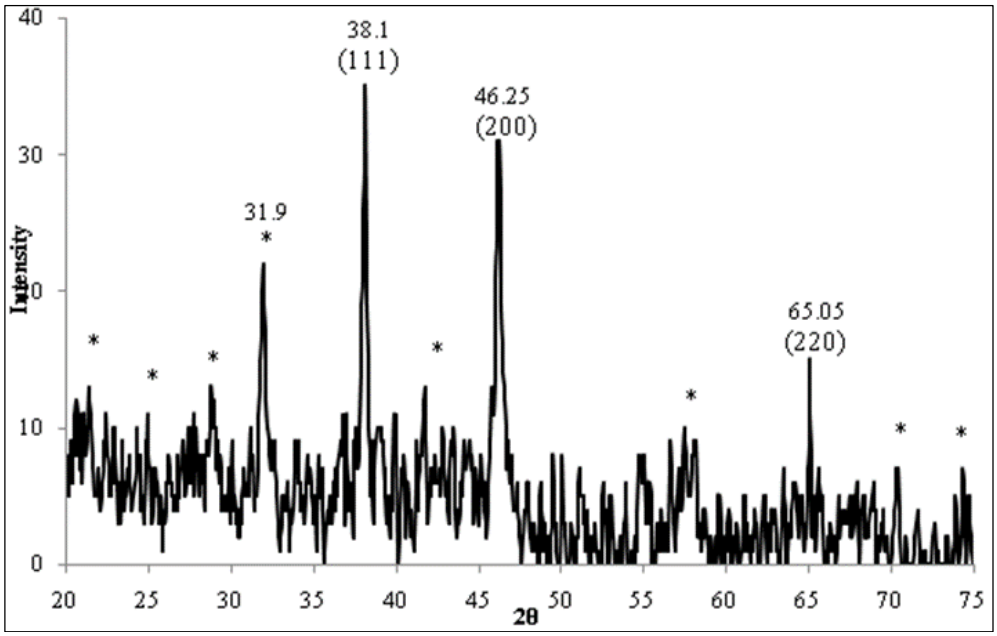


Fig 5: XRD pattern of *Brassica campestris* synthesized Ag NPs

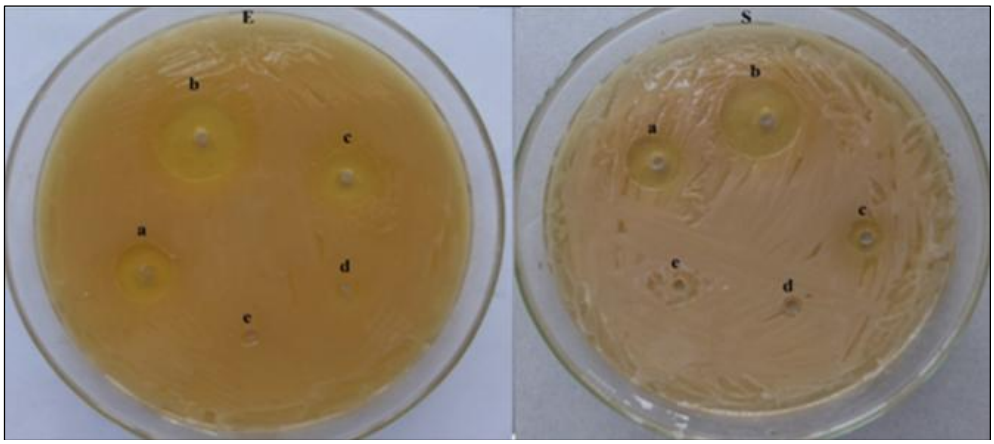


Fig 6: Antibacterial activities of discs 25 µl/hole AgNPs and gemifloxacin solution on (E) *E. coli* and (S) *S. aureus*, a = gemifloxacin, b = gemifloxacin + AgNPs, c = AgNPs, d = silver nitrate, and e = plant extract

3.2 FT-IR spectroscopic Studies of Silver Nanoparticles

FT-IR spectroscopy has becoming an important tool for identification of the functional groups of biomolecules responsible for stabilization and reduction of nanoparticles. The major absorbance bands present in the spectrum of Brassica campestris prepared AgNPs were at 3320-3275, 2950, 1645, 1535, 1410, 1260, 1040 and 501 cm^{-1}, respectively as shown in (Fig. 3). The broad band's observed from 3320-3275 cm^{-1} is the characteristic of alcohols and phenolic compounds hydroxyl functional group and various carbonyl groups of the extract. The bands at 2950 cm^{-1} could be assigned to stretching vibrations of methylene groups. The bands at 1645 and 1535 cm^{-1} is the characteristic bending vibrations mood of the amide I and II linkages of the proteins, respectively. The observed bands at 1410 cm^{-1} can be assigned to the C-N stretching vibrations mood of aromatic amines. The sharp peak at 1260 cm^{-1} corresponds to C-O stretching vibration of carboxylic acids. The peak at 1040 cm^{-1} corresponds to the C-N aliphatic amines stretching vibrations [12-15]. A strong peak observed at 501 cm^{-1} may be corresponds to the ligands-metal stretching frequency that are arise due to the capping of biomolecules on the nanoparticles surfaces [8]. Thus, one can conclude that the biological molecules (protein, alcohols and phenolic compounds) could probably perform the function of both reductions as well as stabilization agents of the AgNPs in aqueous medium.

3.3 FE-SEM studies of silver nanoparticles

In order to known about the surface morphology size and shape of the prepared AgNPs, FE-SEM was used. FE-SEM images of *Brassica campestris* synthesized AgNPs were found to be spherical in shape having no aggregation as shown in (Fig. 4). It has been confirmed from the image also that the synthesized nanoparticles are capped by biomolecules as the edges of the nanoparticles are looking coarse. The FE-SEM images of the nanoparticles show that the shape of AgNPs are spherical and oval and are monodispersed. The size of the *Brassica campestris* extract synthesized AgNPs determined from FE-SEM images was found within the range of 10-25 nm (mean size of about ~19 ± 6 nm) and are monodispersed lacking aggregation. The formation of nanoclusturs may be cause due the presence of elevated concentration of biologically active molecules in the colloidal solution. It has been reported that the optical and electronic properties of metal nanoparticles has considerably changed by changing the shape of the nanoparticles.

3.4 EDX Investigation of silver nanoparticles

For elemental analysis confirmation the prepared nanoparticles were subjected for EDX analysis, giving an additional confirmation for the reduction of silver ions to elemental silver collides. EDX spectrum of the synthesized AgNPs is shown in the (Fig. 4d). The crystalline natures of AgNPs are clearly shown from the EDX pattern, which is reduced by *Brassica campestris* extract from silver ions. A strong signal peak for silver was observed at 3 keV, which confirm the formation of AgNPs. Due to surface plasmon resonance metallic AgNPs usually show absorption peak approximately at 3 keV [16]. The spectrum also shows weak signal peak for carbon and oxygen, which may be originating from the biomolecules that are capping the surface of AgNPs. The EDX analysis obtained from the *Brassica campestris* synthesized AgNPs confirmed the presence of AgNPs and showed strong signal energy peaks for silver atoms in the range 2.6-3 keV.

3.5 XRD Studies of synthesized silver nanoparticles

The XRD patterns of biosynthesized AgNPs (Fig. 5) showed four intense peaks in the whole spectrum of 2θ value ranging from 20 to 75. The XRD patterns showed three distinct diffraction peaks at 2θ position of 38.1, 46.25 and 65.05 corresponding to characteristic Bragg peaks of (111), (200) and (220) which correspond to the face centered cubic of AgNPs [17, 18]. The low intensity and broadness of the peaks reveals small crystallite size of the AgNPs. From the XRD data the average crystallite size of the nanoparticles was calculated using the Scherrer equation, where the average particle size is calculated by the FWHM of the most intense peak. The average crystallite size of nanoparticles was thus found about ~21 ± 6 nm, ably supported by FE-SEM analysis too. These results indicate that AgNPs synthesized using *Brassica campestris* leaf produced crystallite in nanosize. Some unidentified crystalline peaks (21.35º, 28.75º, 31.9º, 41.7º, 57.45º, 70.45º) are also apparent in literature in which the XRD pattern includes the relevant 2θ range. These unassigned peaks (*) are due to the crystallization of bio-organic molecules that act as capping agents which are present in the extract.

3.6 The antibacterial activity of silver nanoparticles

Use of chemical antifoulants to control biofilms is easy and frequently used. However, resistances are shown by large number of microorganisms in biofilms to many antimicrobial materials that are active against planktonic cells. The toxic and environmental risk of contaminated antimicrobial agents must be noted. In biofilms silver can be used for the control of microorganisms. However, the major drawback in the use of ionic silver is that it is inactivated very easily by precipitation and complexation. So alternately zero-valent AgNPs can be used as a valuable source instead of ionic silver. AgNPs have high germicidal effects and are most effective against lower organisms with no harm to higher animals. In addition, AgNPs also possess active anti-fungal activity, anti-viral activity, anti-inflammatory activity and anti angiogenic activity. AgNPs and their ions by generation of free radicals effect on bacterial cell resulting in initiation of oxidative stress by producing reactive oxygen species (ROS). The ROS irreversibly harm bacteria (e.g. by damaging their cell membrane, mitochondria, and DNA) resulting in death of bacterial cell [19]. Only few mechanisms exist about antibacterial activity of metals nanoparticles. The antibacterial action of metals nanoparticles may result in damaging of bacterial membrane or cell wall or may be responsible for changes in their cellular organelles. Though, it should also be noted that most of proposed mechanisms are hypothetical which need further research and investigation [20].

In the present study, the combine effect (synergistic effect) of *Brassica campestris* mediated AgNPs was studied to commercially available antibiotic gemifloxacin using standards disc diffusion method against two human pathogen the gram positive *S. aureus* and gram negative *E. coli*. Synergistic effect is refers to increased in an intensity due to the combination of two substances than the sum of the individual effects on an organism. (Fig. 6) shows the antibacterial activities of the *Brassica campestris* synthesized AgNPs, antibiotic gemifloxacin, *Brassica campestris* leaf extract and synergistic effect of AgNPs with antibiotic gemifloxacin. The zones of inhibition diameter (mm) against

test strains of antibiotic with and without AgNPs are shown in (Table 1). The increase in fold area was calculated by the equation $(B^2-A^2)/A^2$ where 'A' and 'B' were zones of inhibition for antibiotic and antibiotic + AgNPs respectively [21]. As shown from (Fig. **7**) the antibacterial activity of antibiotic gemifloxacin is increased in combination with AgNPs against gram negative *E. coli* as compared to gram positive *S. aureus*. Thus, the synergistic activity observed was better in gram negative *E. coli* as compare to gram positive *S. aureus*. The high activity of AgNPs against gram-negative bacteria then gram-positive was attributed to their perspective cell walls structure of microorganisms. The cell walls of gram negative bacteria in comparison to gram positive a thin lipopolysaccharide and a peptidoglycan layer. The lipopolysaccharides are made up of lipids covalently linked with negative charge polysaccharides, but permeability barrier to a positive charge AgNPs is very weak. As a compare to gram negative the cell wall of gram positive bacteria is composed of polysaccharide linear chains which are cross-linked by short peptides and a thick peptidoglycans layer to form rigid three dimensional structures. Gram positive are embedded in double membrane. This rigidness of gram positive membrane makes it hard for nanoparticles on penetrating or attach to the cell wall [22]. It has been reported in the literature that silver has greater affinity to react with sulfur present in cell membrane or phosphorus containing biomolecules (phospholipids and DNA). Thus sulfur present in the amino acids of membrane protein and phosphorus present inside the nucleus (DNA) are liable to be the privileged place for the binding of AgNPs. From the literature it has been shown that AgNPs creating breakages in the double stranded DNA molecules of bacteria [22]. It has been also reported in the literature that irregular ahaped pits are caused by metal depletion in the outer membrane by metals nanoparticles and in this way membrane permeability are changed, which cause successive release membrane proteins and lipopolysaccharide molecules [23].

3.7 Antioxidant Activity of Silver and Copper Nanoparticles

In order to elevate the antioxidant activity of the *Brassica campestris* synthesized AgNPs, free radical scavenging activity of aqueous leaf extract of *Brassica campestris* and AgNPs was evaluate by DPPH assay. As shown in (Fig. 8) shows that aqueous extract of *Brassica campestris* and AgNPs demonstrate excellent DPPH radical inhibitory activity, indicating that a source for antioxidants. For free radical scavenging activity different concentrations (10, 50, 100 µg/ml) of aqueous leaf extract of *Brassica campestris*, AgNPs and positive control ascorbic acid were used to react with equimolar concentration of 50µM DPPH solution. Here we used 50µM concentration of DPPH in consonance with the accuracy in measurements of spectrophotometer. Antioxidant activity of *Brassica campestris* leaf extract, AgNPs and ascorbic acid was found to increase in a dose-dependent manner. As a compare to positive control ascorbic acid, AgNPs exhibit stronger free radical scavenging activity. Most importantly, *Brassica campestris* mediated AgNPs exhibit excellent antioxidant activity with 65% DPPH scavenging activity. *Brassica campestris* mediated AgNPs show higher DPPH scavenging activity. However, the aqueous leaf extract exhibit lower antioxidant activity then previously reported in the literature [24]. Silver colloids exhibit higher antioxidant activity than *Brassica campestris* leaf extract at all the tested concentrations. It is evident that the resulting excellent antioxidant activity of the Ag and Cu NPs is not only due to the stabilizing agents (biomolecules present in the *Brassica campestris* extract), but it is also due to the elemental copper and silver.

4. Conclusions

In the present study it has been demonstrated that *Brassica campestris* aqueous leaf extract act as a natural, low-cost, fast, and ecofriendly biological reducing agent for Ag NPs synthesis at room temperature. FTIR spectrum shows that leaf extract act as a stabilizer agent for the green synthesized AgNPs It was concluded from the XRD data that the *Brassica campestris* aqueous leaf extract AgNPs were crystalline in nature. As evidenced by antibacterial and free radical quenching activities, silver collides are more active than their synthesized plant extract and corresponding salts. This is the first report revealing that *Brassica campestris* extract can reduce the metal ions into their corresponding metal nanoparticles.

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