

Original Research Article

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Conquer Fluoroquinolone Multi-drug Resistant *Salmonella enterica*: Based on Biological Synthesis of Silver Nanoparticles using *Citrus sinensis* Peel Extract as an Alternative Therapeutic Pathway

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ABSTRACT

Antibiotic resistance is a worldwide health problem increasing with developing resistant strains. Therefore, there is a growing need to develop a simple, economic, commercially viable as well as the eco-friendly clean route for the synthesis of nanoparticles that can be used as nanodrug against multidrug resistance pathogens. To achieve this goal, peel extract of *Citrus sinensis* was implemented for nanoparticles synthesis in this study. The successful formation of silver nanoparticles has been monitored by ultraviolet–visible spectroscopy. Biosynthesized nanoparticles were characterized using transmission electron microscopy and X-ray diffraction spectra respectively. Seventy-two clinical *Salmonella* sp. isolates were isolated and fluoroquinolone resistance mechanisms of five isolates were studied. Cell wall modification, over expression of efflux pump and gene mutation in *gyrA* and/or *parC* genes were revealed. *In vitro* bio Ag-NPs showed effective bactericidal activity against forty-five *Salmonella* sp. with 40 µg/ml as minimum inhibitory concentration. Growth kinetics of *Salmonella typhimurium* showed a reduction in the number of viable cells after 24 h. According to transmission electron microscope, cell wall was damaged by pit formation. A membrane with such morphology exhibited a significant ($p < 0.05$) increase in permeability with increasing leakage of cytoplasm constitutes as proteins and nucleic acid.

Keywords

Salmonella enteric,
Antibiotic resistance,
orange peels extract,
Bio-AgNPs,
Inhibition
mechanism.

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Introduction

The emerging problem of antibiotic resistance, especially among Gram-negative bacteria, has become a serious threat to global public health (Kollef *et al.*, 2011). Various antibacterial classes used against antibiotic resistant bacteria have been losing their effectiveness and there has been a

successful development of new antibiotic agents targeting this class of pathogens (Talbot *et al.*, 2006).

Multi-drug resistant (MDR) strains as *Salmonella enterica* sp. were considered as major human and animal pathogen,

Salmonella sp. is the causative agent in the incidence of salmonellosis and typhoid fever. Several reports stated that food of animal origin was the main source of *Salmonella enterica* sp. Infection (Swartz, 2002).

Antimicrobial resistance, especially resistance to fluoroquinolones, has been evolved as a global health problem (Stoycheva and Murdjeva, 2006). Fluoroquinolone antibiotics represented the last line of defense against *Salmonella* infection in Egypt. *Salmonella enterica* sp. as gram-negative bacteria have been developed in different resistance mechanisms as mutations in the quinolone resistance-determining regions (QRDRs) of the target genes *gyrA*, *gyrB*, and *parC*, *parE*, encode both DNA-gyrase and topoisomerase IV, respectively, or/and low accumulation of the antimicrobial within the cell, due to over-expression of the AcrABTolC efflux pump (Fàbrega *et al.*, 2009) or/ and alteration in cell wall permeability, due to the loss one or more of outer membrane proteins. Therefore, there is much interest in finding ways to formulate new types of safe and cost-effective strategy to control such infection (Sondi and Salopek-Sondi, 2004).

Nanotechnology is an emerging rapidly growing technology with its application in life science, especially in biomedical device and biotechnology (Prabhu *et al.*, 2010). The synthesis of silver nanomaterials/nanoparticles extensively studied by using chemical, physical and biological method. The biological synthesis process offers a wide range of benefits for pharmaceutical and other biomedical applications.

Biological methods provide a non-toxic route with low-cost production and minimum time required (Kaviya *et al.*, 2011; Prathna *et al.*, 2011). The biological

synthesizing method comprises several biological systems as a plant extract, bacteria, fungi and enzymes (Savithamma *et al.*, 2011). Medicinal plants as biological route have been used for biological synthesis of silver nanoparticles, that contain capping and reducing agent involved in nanoparticles production as *Citrus limon* (lemon) (Prathna *et al.*, 2011); *Citrus sinensis* (Kaviya *et al.*, 2011).

Recently, there have been reports that silver nanoparticles have bactericidal activity against multidrug-resistant (MDR) strains of pathogenic bacteria that emerge in hospitals and the general community (Amirulhusni *et al.*, 2012; Rai *et al.*, 2012). Therefore, the principal objective of this work was to evaluate the influence of biological approaches for silver nanoparticles (AgNPS) synthesis as antimicrobial agents using peels extract of orange (*Citrus sinensis*) against MDR *Salmonella enterica* isolates in order to have a more profound understanding of the antibacterial mechanism of bio-Ag-NPs.

Materials and Methods

Bacterial strains

Pure cultures of seventy-two multidrug resistant *Salmonella enterica* isolates were obtained from different hospitals in Egypt and their identification has been done in Microbiology Laboratory using selective culture media, biochemical reaction, and serological test.

Antimicrobial susceptibility testing was done using a wide range of antibiotics (Sigma Aldrich, USA) according to Clinical and Laboratory Standards Institute (CLSI, 2015). MDR five *Salmonella enterica* isolates were chosen to study the mechanism of fluoroquinolone resistance.

Plant material and preparation of orange peel (*Citrus sinensis*) extract

The *Citrus sinensis* were collected from the local market at Giza, Egypt. All the experiments were done in triplicates. Double distilled water was used for the experiments. Fresh peels of *Citrus sinensis* were collected, washed thoroughly with double distilled water, and incised into small pieces. Preparation of orange peels extract was done as described previously (Kaviya *et al.*, 2011).

Detection of Fluoroquinolones resistance mechanisms of five MDR *Salmonella enterica* sp.

Efflux pump overexpression detection for five MDR *Salmonella enterica* was carried out using Cartwheel method as described previously (Martins *et al.*, 2010) with using ethidium bromide stock solution 50 ml/l (Sigma Aldrich, USA).

Outer membrane permeability was detected for five multidrug resistant *Salmonella enterica* isolates by detection of the outer membrane protein expression (omp-D, omp-F, and Opm-C) using electrophoretic mobility by 1.5% SDS-PAGE (Miró *et al.*, 2004).

Gene mutation: PCR amplification of *gyrA* and *parC* gene for five *Salmonella enterica* sp. was carried out using primers of *gyrA* and *parC* as described previously (Eaves *et al.*, 2004, 2002). Sequencing of PCR products was used to detect the mutations in QRDR of two *Salmonella enterica* sp. genes using Jena Gen GmbH Biotechnologie-Gentechnik-Diagnostik (Jena, Germany) using the BigDye™ Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) on a 3130 sequencer (Applied Biosystems) and the results were

analyzed using Bioedit software v.7 and by direct comparison with the gene sequences for the appropriate serotype using BLAST (PubMed) have reference strain with accession number (X78977) for *gyrA* and (AE008878) for *parC* genes.

Synthesis and characterization of silver nanoparticles using orange peels extract

For the synthesis of silver nanoparticles were prepared as described previously (Kaviya *et al.*, 2011). The bioreduction of Ag⁺ ion in solution was monitored using UV-visible spectrometer (Shimadzu, UK). Further characterization was done using X-Ray diffraction, which performed to determine the crystalline degree and the phases of the formed particles by DX-1000 X-ray powder diffractometer (Panalytical, Holand). The XRD pattern was recorded in a wide range of Bragg angles 2θ at a scanning rate of 2°min^{-1} , at a voltage of 40 kV and a current of 30 mA with Cu K α radiation (1.5405 Å). The size of the nanoparticles was calculated through the Debye-Scherrer's equation [1] (Borchert *et al.*, 2005).

$$D = k\lambda/\beta\cos\theta$$

D is the average crystal size, k is the Scherrer coefficient (0.89), λ is the x-ray wavelength ($\lambda = 1.5406 \text{ \AA}$), θ is the Bragg's angle (2θ), β is the full width at half maximum (FWHM) in radians. Transmission electron microscopy TEM (JEOL, SM2100) was used to determine the size and shape of synthesized silver nanoparticles. The zeta potential and size of particles were carried out using a Zetasizer ZS (Malvern, England).

Orange peels extract compound identification

The orange peel extract was analyzed using GC/MS analysis (Alignet, USA) and

identification of constituents was based on comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature (Adam, 2001).

Antimicrobial assay of bio-AgNPs against MDR *Salmonella enterica*

The silver nanoparticles synthesized using *Citrus sinensis* peel extract was tested for antimicrobial activity by agar well diffusion method against seventy-two clinical pathogenic multidrug resistant *Salmonella enterica* species isolated from different sources. Using a micropipette, 50 µl of nanoparticles solution was poured into each well on all plates. After incubation at 37 ±1°C for 24 h, the different levels of inhibition zone of bacteria were measured. Then one isolate, *Salmonella enterica* serovar Typhimurium (S.E.T. 17) was used as a template for further experiments.

Mechanism of inhibition

Growth kinetics

Growth pattern of *S. typhimurium* was studied. For organism preparation, *S. typhimurium* was grown overnight in Luria Bertina (LB) broth and at 37°C. Washed cells were resuspended in Luria Bertina (LB), and the optical density was adjusted to 0.1, corresponding to 10⁷ CFU/ml at 600 nm. *S. typhimurium* cells were treated with varying concentrations (10, 20, 30, 40 and 50 mg/l) of homogenized Ag-NPs then inoculated on a rotary shaker (180 rpm) at 37°C. Untreated culture flask was used as a control. Optical density was measured after every hour (up to 11 h) using UV- Visible

spectrophotometer at 600 nm (Sondi and Salopek-Sondi, 2004).

Time-dose dependent antibacterial activity

The bio silver nanoparticles were suspended in milliQ water to conduct the time and dose-dependent antimicrobial study as described previously (Tiwari *et al.*, 2008). Bio-AgNPs suspension was homogenized using ultrasonicator. *S. typhimurium* cells were treated with 2.0 ml of each concentration (10, 20, 30, 40 and 50 mg/L) of bio-AgNPs as well as with varying time intervals for each concentration (30 min, 2, 6, 12 and 24 h). Bacterial culture was serially diluted till 10⁶ dilution factors, then spread 100 µl culture homogeneously in Luria Bertina (LB) agar plates. All plates were incubated at 37 ±1°C for 24 h and the number of colonies grown on an agar plate was counted. Silver-free plates incubated under the same conditions were used as controls.

Studying of bio Ag-nanoparticles and *Salmonella enterica* Typhimurium (S.E.T 17) interaction using transmission electron microscope (TEM)

The interaction of bio-AgNPs with *S. typhimurium* (S.E.T 17) strain was studied using TEM, cells were grown in liquid LB medium to log phase at an optical density at 595 nm of approximately 0.5 at 37°C then cells were centrifuged at 1000 *xg* for 5 min at 4°C and mixed in phosphate buffer saline (PBS). Mixed 1.0 ml of bacterial solution with 1.0 ml of bio-AgNPs solution (40 mg/L) and incubated for 30 min. The effect of bio-AgNPs on the bacterial cells was monitored using High-Resolution Transmission Electron Microscope (HRTEM) operating at an accelerating voltage of 120 kV (Morones *et al.*, 2005).

Cytoplasm leakage analysis

Cytoplasm leakage analysis for bio-AgNPs against treated *S. typhimurium* (S.E.T 17) cells was done. Cells were treated with bio-AgNPs followed by centrifugation at 12000 *xg* for 15 min and supernatant used to study protein and nucleic acid analysis (Tiwari *et al.*, 2008). The amount of nucleic acid released using a different concentration of bio-AgNPs (10, 20, 30, 40 and 50 mg/L) of treated cells was measured at 260 nm using a UV-Visible spectrophotometer. For preliminary identification of nucleic acid leakage, ninhydrin test showed a positive result. Protein leakage analysis was performed using Lowry assay (Lowry *et al.*, 1951). *S. typhimurium* (S.E.T 17) cells were treated with (10, 20, 30, 40 and 50 mg/L) of bio-AgNPs solution for 1, 2, 4 and 24 h. Leakage of protein was determined using Foline-Ciocateur's reagent at 650 nm after 20 min of incubation in the dark. BSA (Fluka, Biochemica) was used as a standard protein.

Statistical analysis

All samples analyzed using one-way analysis of variance (ANOVA) using IBM SPSS statistics 20. All analyses were repeated in triplicates. The generated data were subjected to analysis of variance (ANOVA) (Armitage and Berry, 1987). Categorical variables between mean values were established with a mean value comparison using LSD's test and were considered significant at $p < 0.05$.

Results and Discussion

Resistance profiles of *Salmonella enterica* isolates

The resistance profile of seventy-two *Salmonella enterica* sp. have been studied.

Out of seventy-two (72), forty-five *Salmonella enterica* isolates among five MDR isolates, especially resistant to quinolones and fluoroquinolones, have been chosen to study the resistance mechanism as shown in the online resources (ESM_1). The isolates were identified using the microbiological and serological method. The five isolates contributed three different resistant mechanisms as follow (Table 1): (1) altered membrane permeability as a result of Omp-F-deficient (40.0 KDa) and Omp-D (39.0 KDa) was lost in only four isolates. One isolate (*Salmonella enterica* serovar typhimurium) expressed Omp-C porin (35.6 KDa) with lower expression and missed in other isolates. As evidenced by cartwheel method, five *Salmonella enterica* isolates over expressed efflux pump of type Resistance-nodulation-cell division (RND).

Correspondingly, gene mutation of two *Salmonella enterica* isolates was studied. One *S. Enteritidis* (S.E.E 5) showed a double gene mutation in both genes *gyrA* and *parC* (Table 1). In *parC*, there was another mutation reported. On the other hands, another isolate *S. typhimurium* (S.E.T.17) showed a double *gyrA* gene mutation and *parC* has no mutation (Online resources ESM-2 and ESM_3).

Silver nanoparticles synthesis and characterization

The formation of silver nanoparticles was prepared by the addition of orange peels extract to silver nitrate solution, a color change from pale yellow to brown in about 20 min at 60°C was observed and UV-Visible spectrum of the silver nanoparticles showed a broad band at 432 nm (Fig. 1a). The zeta potential of the nanoparticles was -15 mV with negative charge formed.

The biosynthesized silver nanoparticles by employing orange peel extract were further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Fig. 1b). The broadening of Bragg's peaks indicates the formation of nanoparticles with a mean size of Ag-NPs were 20 ± 2 nm, according to equation [1]. Transmission electron microscope image of silver nanoparticles derived from orange peels extract was shown in Fig. 2. The morphology of the nanoparticles was spherical in nature (Fig. 2a) and under careful observation, it showed that the silver nanoparticles surrounded by a faint thin layer of other materials (Fig. 2b). The obtained nanoparticles in TEM have an average size of the particles synthesized was 20 nm with a size range from 20 to 30 nm of spherical shape and the few particles are agglomerated. However, some particles with diameters are longer than 60 nm may be formed.

Laser diffraction size analyzer provided Ag-NPs size production; the histogram of silver nanoparticles showed a variation in particle sizes and the average size estimated was 20 nm. It may be noted that the size of the synthesized silver nanoparticles obtained from TEM was in a good agreement with size obtained from XRD measurements.

Orange peels extract compound identification

Phytochemical analysis of orange peel extract represents a complex storehouse of the multitude of bimolecular like phenolics, ascorbic acid, flavonoids, sugars, carotenoids, essential oils, pectins and terpen especially limonene (online resources ESM_ 4) that have a vital role in biosynthesized of silver nanoparticles.

Biosynthesized Silver nanoparticles mechanism against *Salmonella enterica* sp

Biosynthesized Ag-NPs have an antimicrobial effect against thirty-six MDR *Salmonella enterica* isolates, among them four MDR *Salmonella enterica* sp. that contributed different mechanisms; while the other MDR *S. Enteritidis* (S.E.E 5) that contributed all porins deficient with overexpression of efflux pump and both *gyrA* and *parC* mutation, has not affected by bio-AgNPs.

Different parameters were studied against one selected isolate *S. typhimurium* (S.E.T.17) to reveal the bio- AgNPs effect. The growth curve of *S. typhimurium* (S.E.T.17) was exposed to different concentrations of bio-AgNPs (Fig. 3). In the presence of bio-AgNPs, the growth curve consisted of three phase lag, log, and stationary phase; however, decline phase could not be revealed, whereas in the absence of Ag-NPs, *S. typhimurium* (S.E.T.17) reached log phase rapidly, but with the addition of bio-AgNPs halted the growth and the partial complete inhibition of growth were detected at 50 mg/L concentration; indicating that MIC of bio-AgNPs towards *S. typhimurium* (S.E.T.17) was 40 mg/L.

Time-dose dependent method clarified the effect of bio-AgNPs on *S. enterica* Typhimurium (S.E.T.17) growth that significantly ($p < 0.05$) decreased CFU/ml with increasing concentration of bio-AgNPs (Fig. 4). After 2 h of incubation with bio-AgNPs, 60 % inhibition of viable cells were achieved at 20 mg/l and at 50 mg/l was sufficient to achieve 100 % inhibition after 24 h.

Indeed, TEM analysis (Fig. 5) confirmed the incorporation of bio-AgNPs into membrane structure. TEM showed the surface of native cells was smooth and intact, and some filaments around cells were obvious and clear that were peritrichous flagella. On the other hand, membrane of treated cells was damaged severely; many pits and gaps appeared in the micrograph (Fig. 5c), their membrane was disintegrated and cause damage at various sites that result in disturb its function, penetrate bacteria, and release of silver ions result in cell lysis.

To show the mechanism of bio-AgNPs on bacterial cells, leakage of nucleic acids and proteins (Figure 6) of *S. typhimurium* cells were studied. Bio-AgNPs penetrated cells and ninhydrin-positive materials, purine and pyrimidine, released in the suspension and their amount were significantly ($p < 0.05$) increased with increasing bio-AgNPs concentration and the maximum increasing were achieved at 4 h of incubation with bio-AgNPs (Fig. 6a).

Bio-AgNPs could enhance the leakage of protein (Fig. 6b) as a consequence of increasing membrane permeability. Initially, the amount of protein leakage in *S. typhimurium* (S.E.T 17) was as those in control and at 4 h of incubation, the protein leakage content was significantly increased ($p < 0.05$) with increasing bio-AgNPs concentration as compared with control.

In the present study, our goal was to confirm the effectiveness of biosynthesized AgNPs synthesized using *Citrus* peels extract as an antimicrobial agent and determine the mechanism of treatment action against MDR *Salmonella enterica* isolates from human that were represented as hospital and community threat.

Several studies mentioned that the outer membrane modification acts synergistically with enhanced active efflux systems to affect the level of the intrinsic and the acquired antibiotic resistance of gram-negative bacteria and/or genetic mutation in quinolone resistance-determining region of topoisomerase gene of *Salmonella enterica* isolates (Miró *et al.*, 2004; Rushdy *et al.*, 2013)

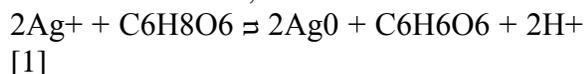
The present study highlights that at least two *Salmonella enterica* strains contributed three mechanisms of resistance with amino acid sequence conservation of the quinolone resistance-determining region of *gyrA* and *parC* at both codon S-83 or A-87 of *gyrA* (Kim *et al.*, 2011) and the mutations in *gyrA* are linked with a high resistance level of ciprofloxacin-resistant *Salmonella* isolates from humans and animals (Giraud *et al.*, 2006).

One *S. Enteritidis* strain (S.E.E 5) showed a mutation in both *gyrA* and *parC*; on the other hand, another *S. Typhimurium* strain (S.E.T 17) showed only three *gyrA* mutation. In gram-negative bacteria, gyrase was considered as a primary target of fluoroquinolones rather than topoisomerase IV (Piddock *et al.*, 1998), that is why mutations in *parC* are rarer in gram-negative bacteria and usually arise later than *gyrA* mutations.

The mutation of serine 83 (hydrophobic amino acid) resulted in a reduced level of fluoroquinolone binding gyrase-DNA complex; while substitution at codon 87 to glycine (small polar amino acid), led to the loss of negative charge, that is important in the quinolone gyrase interaction (Yoshida *et al.*, 1990).

The current discovery of the unique chemical power of non-toxic phytochemicals in the plant initiating nanoparticles formation is of paramount importance in the context of the production of silver nanoparticles for medical and technological applications. Several reports have been focused on *Citrus* peel extract that was treated as agro-industrial waste and have high economic and medicinal value such as in the food industry, cosmetics and folk medicine. *Citrus* peels were considered as a potential source of valuable secondary plant metabolite and essential oils which have natural antioxidant and antimicrobial properties (Saidani *et al.*, 2004).

The vast array of biomolecules (Guimarães *et al.*, 2010) in orange peel extracts like phenols and ascorbic acid have the advantages of nanoparticles Ag-NPs synthesis; hence, the reduction process of silver ion to produce Ag-NPs may be due to ascorbic acid that used as effective reduction as mentioned in the earlier reports (Konwarh *et al.*, 2011). Additionally, Konwarh *et al.*⁽³⁴⁾ illustrated the role of ascorbic acid as an effective reductant, as follow:



Additionally, another explanation was provided (Harborne, 1964) that phenolic compounds possess hydroxyl and carboxyl groups, which are able to bind to metals and that may be inactivated ions by chelating. According to Morgan *et al.* (Morgan *et al.*, 1997), this general chelating ability of phenolic compounds is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule.

Characterization of silver nanoparticles formed by orange peel extracts was identified as follows: the surface Plasmon

vibrations explain the brown color formation and it provides a convenient spectroscopic signature to indicate the formation of silver nanoparticles (Thirumurgan *et al.*, 2010); while broadening of peak in UV-V indicated that particles formed have small size and polydispersed (Kong and Jang, 2006).

The acid of citrus extracts in the preparation of the silver nanoparticles acting as a coating or shell for the silver nanoparticles that lead to a negative charge (Prathna *et al.*, 2011). Such behavior gave the bio silver nanoparticles, high stability which was confirmed by Zeta potential value that related to the stability of colloidal dispersions. XRD pattern was obtained is consistent with earlier reports (Kaviya *et al.*, 2011). Broadening of peaks in the XRD patterns of solids is attributed to the particle size effect (Jenkins and Snyder, 1996), that confirmed the smaller particle size formed and reflected the effects of the experimental conditions on the nucleation growth of the crystal nuclei (Becheri *et al.*, 2008).

The biosynthesized Ag-NPs have a spherical morphology observed under TEM was in accordance with previous reports (Kaviya *et al.*, 2011) and the faint layer formed was supposed as capping organic material from orange peel extract (Mallikarjuna *et al.*, 2011) and larger particles than 60 nm formed may be due to aggregation during preparation of the TEM holding grid (Guzmán *et al.*, 2008).

From the time-dose dependent result the minimum bactericidal concentration indicated that silver nanoparticles have a bactericidal rather than bacteriostatic effect (Amirulhusni *et al.*, 2012; Pal *et al.*, 2007) on the bacteria and as expected, the inhibition of bacterial growth depends on the number of initial cells applied in the test (10^7 CFU/ml).

French (French, 2006) postulated those effects as a bactericidal agent that preferred clinically. Hence, bacterial killing provided a radical solution to that problem, thereby improved clinical outcome and reduced the likelihood of the emergence of resistance as well as the spread of infection. If pathogens are killed rather than inhibited, the resistance mutations which appear as an antibiotic pressure consequence will be eliminated.

In addition, the size and shape of producing bio silver nanoparticles confirmed the bactericidal effect; in which the small size and high surface area to volume ratio allowed them to interact closely with microbial membranes. On the other hand, the bactericidal effect of silver nanoparticles decreases as the size increases (Pal *et al.*, 2007).

As a trail to understand the effect of biosynthesized Ag-NPs mechanism against MDR *S. typhimurium* (S.E.T 17), TEM showed that membrane integrity has been affected by bio-AgNPs as pits were formed in the membrane and recent studies proposed the same effect in of *E. coli*

membrane that related to metal depletion. Metal depletion causes the formation of irregular-shaped pits in the outer membrane and changed membrane permeability by the progressive release of LPS molecules and membrane proteins (Amro *et al.*, 2000; Sondi and Salopek-Sondi, 2004). Further, negatively charged Ag-NPs accumulated in the bacterial membrane increased the permeability of the membrane (Hwan *et al.*, 2011).

The bacterial membrane of MDR *S. typhimurium* (S.E.T 17) showed decreased permeability due to Omp-F and Omp-C porin-deficient and the bio-AgNPs could affect it and cause increased permeability and consequently cause cell death. On the other hand, the bacterial membrane of MDR *S. Enteritidis* (S.E.E 5) that showed Omp-D deficient, the bio-AgNPs had no effect recorded. Therefore, porins have a key role in allowing bio-AgNPs to exert their antibacterial effect. Our results were confirmed (Radzig *et al.*, 2013) that showed that porins Omp-F and Omp-C of *E. coli* mutant strains become 4-8 times more resistance to AgNPs.

Table.1 Contribution of fluoroquinolone resistance mechanism in five MDR *Salmonella enterica* isolates and correlated to its antimicrobial susceptibilities

<i>Salmonella enterica</i> sp.	MIC (µg/ml)	Resistance mechanism					
		Membrane permeability (protein deficiency)			Over expression efflux pump	Gene mutation	
		Omp-F	Omp-C	Omp-D		<i>gyrA</i>	<i>parC</i>
S. E.T 1	256	No expression	No expression	No expression	Over expressed	*ND	
S.E.E 5	>512	Expressed	Expressed (weak)	No expression	Over expressed	S83P A87S	S67C R76C C80R
S.E.T 17	512	No expression	No expression	Expressed	Over expressed	S83P A87G A119S	No mutation
S. E.T 19	128	No expression	No expression	No expression	Over expressed	*ND	
S. E.T 30	256	No expression	No expression	No expression	Over expressed	*ND	

*ND: Not determined

Fig.1 XRD pattern of silver nanoparticles. Silver nitrate (1mM) treated with orange peels extract.

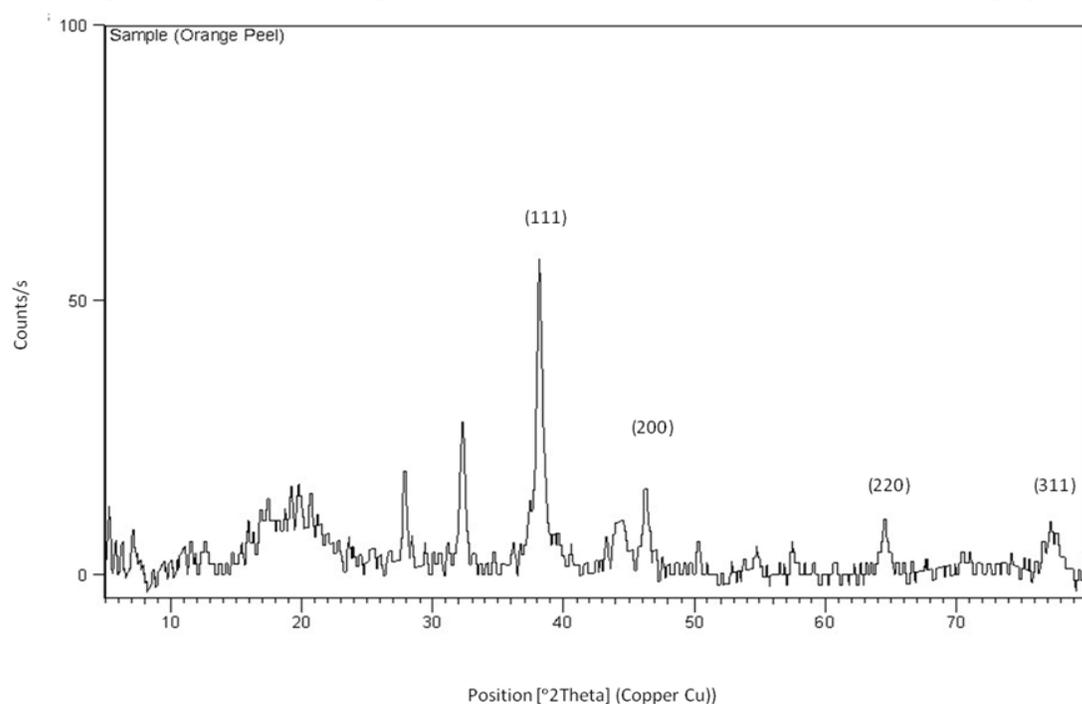


Fig.2 HRTEM of spherical bio silver nanoparticles. Bio-AgNPs derived from orange (*Citrus sinensis*) peels extract. (a) spherical nanoparticles (b) silver nanoparticles surrounded by a faint thin layer of other materials.

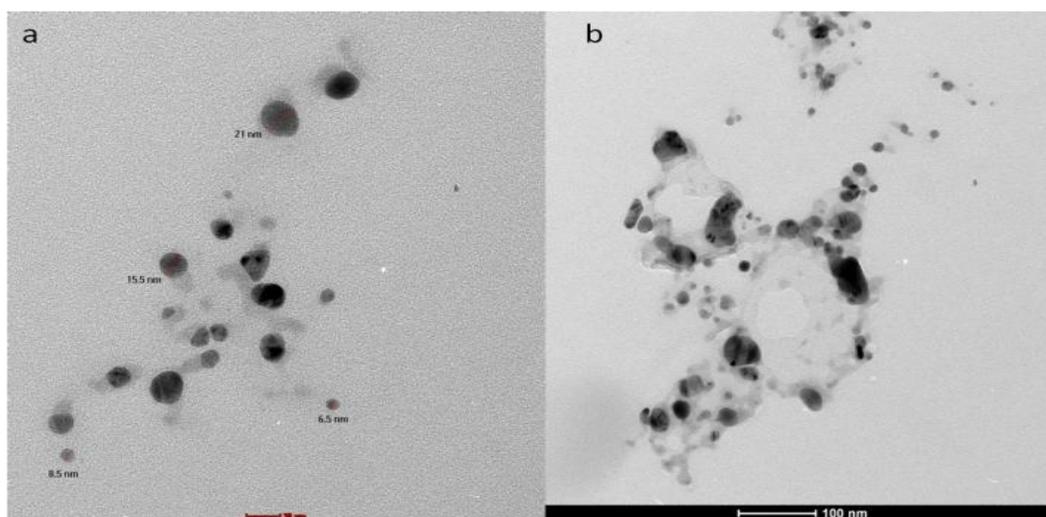


Fig.3 Growth curve of *Salmonella enterica typhimurium* (S.E.T. 17) treated with different concentration (10, 20, 30, 40 and 50 µg/l-1) of bio Ag-NPs of orange (*Citrus sinensis*) peels extract.

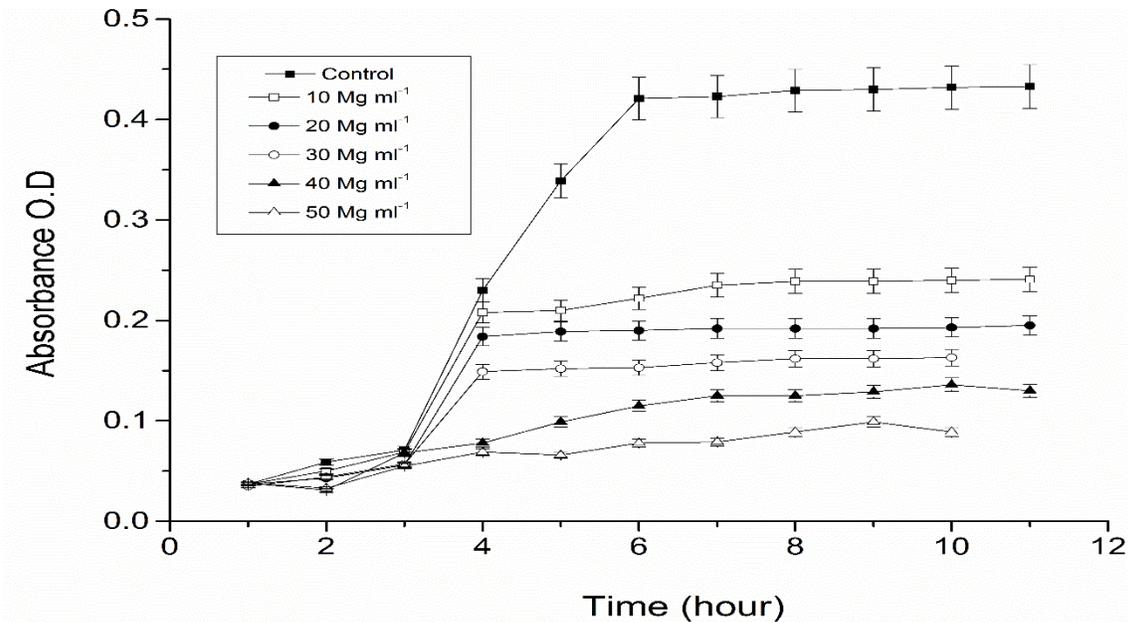
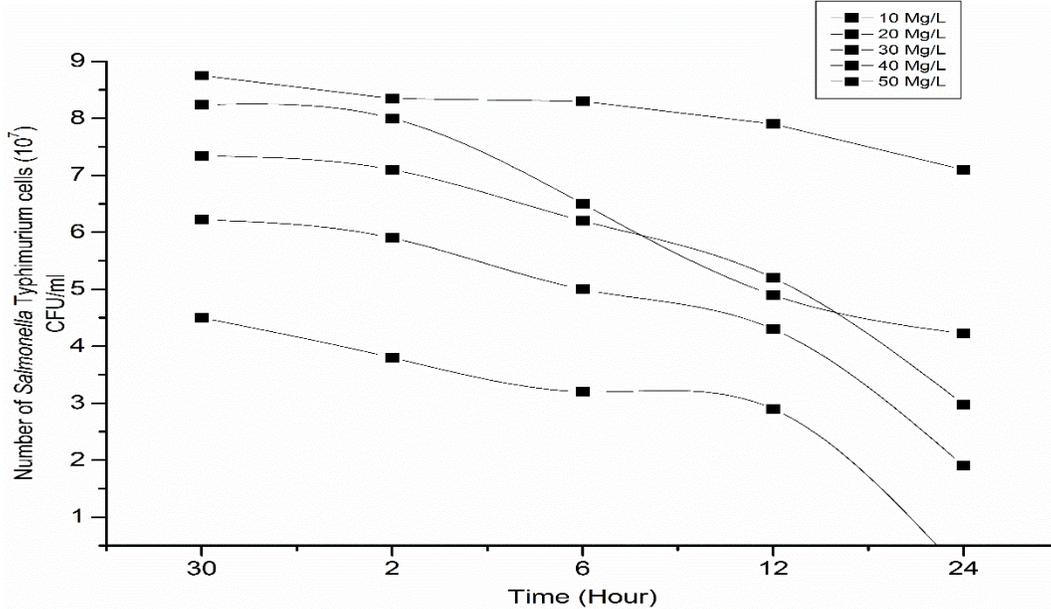


Fig.4 Number of viable cell counts of *Salmonella enterica typhimurium* (S.E.T. 17) treated with different concentration (10, 20, 30, 40 and 50 $\mu\text{g l}^{-1}$) of bio Ag-NPs of orange (*Citrus sinensis*) peels

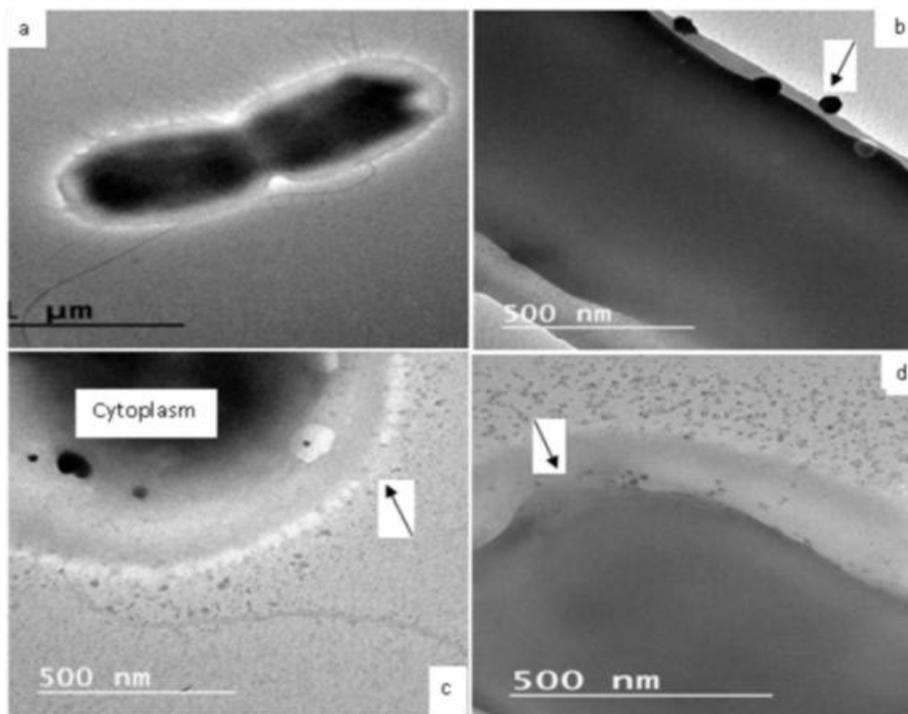


Fig.5 HRTEM images of *Salmonella enterica typhimurium* (S.E.T. 17) cell wall. (a) Untreated *Salmonella enterica Typhimurium* flagella can be seen. (b) spherical bio AgNPs appear as dark irregular pits on the cell surface part of the bacteria cell membrane. (c) The cell membrane is damaged in multiple location. Arrows indicated partially damaged membranes. (d) Incorporation of bio AgNPs into cell membrane and enlarged parts of cell membrane indicated damage with bio AgNPs surrounded cells.

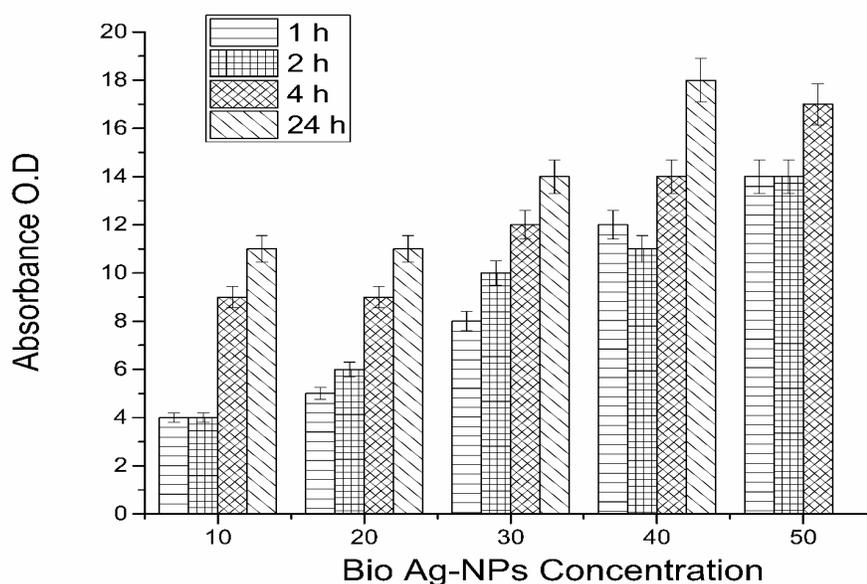
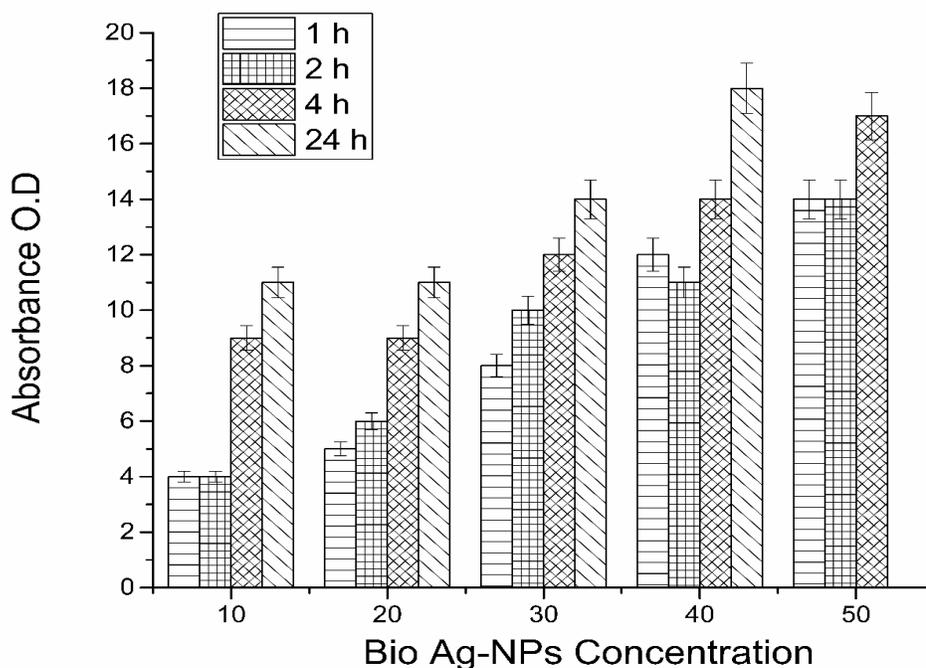


Fig.6 leakage of cytoplasmic nucleic acid of *Salmonella enterica typhimurium* (S.E.T. 17) with different concentration (10, 20, 30, 40 and 50 $\mu\text{g-1ml}$) of bio Ag-NPs of orange peels extract after different interaction time 1 h, 2 h, 4 h and 24 h.



The increasing leakage of nucleic acid and protein indicated that increasing membrane permeability. The injury in the cell membrane, causing the release of nucleic acid material and proteins and these results were in agreement with that stated in different studies (Tiwari *et al.*, 2008). However, the mode of action of silver nanoparticles is similar to that of silver ions, which complex with electron donor groups containing sulfur, oxygen or nitrogen atoms that are normally present as thiols or phosphates on amino acids and nucleic acids (McDonnell, 2007).

Moreover, several studies revealed the role of silver ions in bacteria cells as denaturing the 30s ribosome subunit, suppressing the expression of enzymes and proteins essential to ATP production (Matsumura *et al.*, 2003),

inhibiting respiratory enzymes, thereby inducing the production of reactive oxygen species (Matsumura *et al.*, 2003) that cause bacteria death. Therefore, the present study proved that bio-AgNPs produced by orange peel extract have an effective way to control certain pathogenic bacteria involved in infectious disease (Rai *et al.*, 2012; Tiwari *et al.*, 2008).

In conclusion, the present work demonstrated a simple, eco-friendly, economic green route as an alternative way of chemical synthesis for the AgNPs synthesis. We have shown the importance of silver nanoparticles in the nosocomial and community environment as bactericidal by rupturing the membrane of bacterial cells at low concentration and bind to intracellular material and causes cell death. Therefore,

biological silver nanoparticles can be recommended as an effective broad-spectrum bactericidal agent.

Abbreviation

S.E.T.	<i>Salmonella enterica</i> serovar Typhimurium
S.E.E.	<i>Salmonella enterica</i> serovar Enteritidis
MDR	Multi-drug resistance
Bio-AgNPs	Biosynthesized silver nanoparticles
OMP	Outer membrane protein

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