Biosynthesis of Silver Nanoparticles by *Punica Granatum* Peel Extract and their Biological Activity on different Pathogenic Bacteria

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Abstract

The present study was included the assessment of the antimicrobial activity of AgNPs synthesized by *Punica granatum* peel extract against pathogenic bacteria by testing warm aqueous *P. granatum* peel extract and silver nanoparticles. *Punica granatum* indicated potency for AgNP extracellular nanobiosynthesis after addition of silver nitrate (AgNO3) 4mM to the extract supernatant, in both concentrations (100mg and 50mg). The biogenic AgNPs showed potency to inhibit both gram-negative and gram-positive bacterial growth. Zons of inhibition in (mm) was lesser in gram-positive than gram-negative bacteria. The resulted phytogenic AgNPs gave higher biological activity than warm aqueous Punica granatum peel extract. The inhibition zone of the phytogenic AgNPs on E. coli reached 17.53, 22.35, and 26.06 mm at (0.1, 0.5, and 1) mg/ml respectively. While inhibition zones of Punica warm aqueous extract reached 5.33, 10.63, and 16.08 mm at the same concentrations. phytogenic AgNPs gave smaller inhibition zones in gram-positive than gram- negative. Cytotoxic activity of the phytogenic AgNPs was assayed in vitro agaist human blood erythrocytes (RBCs), spectroscopic results showed absorbance at 540 nm hemolysis was observed. In general, AgNPs showed least RBCs hemolysis percentage, at 1 mg/ml concentration, hemolysis percentage was (4.50%). This study, concluded that the *Punica granatum* peel extract has the power of synthses of AgNPs characterized by broad spectrum antimicrobial activity with cyto-toxicity proportional to AgNPs concentration.

Key Words: AgNP, Warm Aqueous Extract, Antimicrobial, Cytotoxicity, Characterization.

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Introduction

Plant catalysed nano-biosynthesis was proposed more advantageous than other bioprocesses as it is trouble-free maintaining and preserving of the extract synthesis medium. Plant extract catalysed nanobiosynthesis is a trouble-free, one-step method. Preparation of plant extract synthesis medium can be applied for larg-scale nanobio synthesis. (Veerasamy *et al*., 2011).

Nano-biosynthesis mediated by microorganisms is often more expensive than synthesis by plant extracts, phytocompounds-catalysed (bio-reductants) nanobiosynthesis using as bio-reductants is attaining a superior impetus, a diversity of plant parts, such as root, leaf, bark, fruit, fruit, callus, and peels extracts can be used (Rai and Yadav, 2013). Plan extracts include terpenoid, alkaloids, flavonoids, and phenolic compounds, these compounds play a crucial rule in bioreduction and stabilization of the synthesized nanoparticles (Dubey *et al*., 2009).

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Silver nanoparticles fabricated by microwave-assisted rapid green synthesis using fresh leaves of *Cymbopogan citratus*, founed to appear within 8-10 minutes (Zuas *et al*., 2014).

Among the various precious metals, silver is a good choice in biological systems, living organisms, and in medicine. Silver has been proven as an effective preventing and hindering tool for bacterial infections, in addition; silver exhibits wound healing, antibacterial and catalytic activity, chemical stability, and Good conductivity (Sharma et al., 2009). Investigations on AgNPs get the attention of the research community due to their application in optoelectronics, antimicrobial activity, and silver-embedded fabrics which applied in the sporting supliments. However, Economical, commercially viable and environmentally friendly synthetic methods for AgNPs synthesis are still needed (Aldujaili, *et al*., 2015).

Phyto-fabrication practices are purely green synthetic hues and are considered a superior candidate among other biological derivartives as they are clean, eco-friendly, commercial, safe, conveniently applicable method for large-scale biosynthesis of metal NPs. Many plants are approved to mediate AgNPs biosynthesis and their underlying claims (Owaid et al., 2015). The accumulation rate of NPs vary with ion-reducing potential, and the plant extract reducing potential is proportional to the amount of various heterocyclic compounds and polyphenols such as Marigold (Calendula officinalis L.) belongs to the family of Asteraceae (Shamsi, 2015). Plan extracts include terpenoid, flavonoids, polysaccharides, and phenolic compounds, these, these compounds contribute to the reduction and stabilizing of AgNPs (Kumar and Yadav, 2009). Silver nanoparticles characterized by antimicrobial activity against multidrug-resistant (MDR) bacteria, and it is important to proposed as standby substitute for antibiotics in (MDR) infections (Franci et al., 2015). Nanobiotechnology is an emerging discipline of nanotechnology in which, physical and chemical procedures combined with biological practices to generate nano-scale particles with unique functions. Biological methods represents an economic substitute for chemical and physical methods for nanoparticles fabrication (Ahmad et al., 2016). Nanobiotechnology has been applied in many fields and succeeds in the treatment, diagnosis, monitoring, and control of diseases and cancer therapy (Drbohlavova et al., 2013).

Objectives

This research aims to:

- Synthesis of silver nanoparticles by aqueous Punica peel extract.
- Physical Characterization of nanosilver.
- Study evaluation of the biological activity of silver nanoparticles as antibacterial.

Methods

Selection of Pathogenic Bacteria

Standard clinical isolates (Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli) were isolated and identified according to Macfaddin protocols (Macfaddin, 2009) in the postgraduate laboratory for microbiology research in the Department of Biology, Faculty of Science, University of Kufa, and verified by Vitek-2 compact system GP and GN cards. The isolates were preserved at (−20°C) in brain heart infusion broth (BHI)-glycerol (20%), for subsequent investigation of antimicrobial activity of biogenic nanoparticles.

Preparation of Catalytic Plant Extract

Peels of pomegranate were set according to (Ghramh, et al., 2018). *Punica granatum* dried peels were powdered by electric grinder after ambient dehydration, the catalytic plant extract was prepared by warm water extraction of the powder. Twenty grams (20g) of peel powder was blended, with 100ml warm distilled water, the mixture was incubated at 24°C for 24 hrs with agitation. After 24 hrs incubation, the extract mix was clarified by Whatman No. 5 filter paper. The filterate was desiccated at 40 C for 2-3h using rotary evaporator. To prepare 1% stock solution, 1g of the dried extract was dissolved in 100ml of warm distilled water.

Biosynthesis of AgNPs

Synthesis mix was prepared by adding 1:1 AgNO3 4mM to the Punica granatum catalytic peel extract medium, as a substrate for AgNPs biosynthesis, the reaction mix was distributed in sterile screw-capped tubes and mixed well. AgNO3 was prepared in shade to avoid photo-oxidation. The resultant solutions were incubated for 24 hrs at 37°C, and 150 rpm in a shaking incubator.

After incubation, color change indicates nucleation and maturation of the AgNPs. Transcolored Punica peel extract was centrifuged at 10000 rpm for 10

minutes, the supernatant was poured and the residue resuspended in deionized distilled water and centrifuged, this washing process was repeated three times to get rid of supernatant residual, the deposited nanoparticle's pellet in the tube, was desiccated in an oven at 40 °C. The dessicated AgNPs was in vials for subsequent analysis (Aldujaili, et al., 2015).

Characterization of Silver Nanoparticles

Scanning Electron Microscopy (SEM)

Biogenic nanoparticles have been characterized distribution, morphology, and size by scanning electron microscopy (SEM) at an accelerated voltage 5-10 KV, low vacuum and various magnification powers, and, working distances 5-10 mm, and spot size 4, in the electron microscope unit/ Faculty of Science/ University of Kufa. (Caroling et al., 2013).

Energy Dispersive x-ray Spectroscopy (EDS)

Elemental analysis of the biogenic nanoparticles was analyzed using Braker SEM-linked EDS at accelerating voltage 10 KV, with spot size 5, and working distances 10mm (Sarvamangala et al., 2013) in the unit for electron microscopy/ Faculty of Science/ University of Kufa.

Antimicrobial Activity

Phytogenic AgNPs synthesized by (Punica) were examined for antimicrobial activity against standard gram-negative and gram-positive pathogenic bacteria (Table 1) using agar well diffusion technique (Bauer et al., 1966). Warm aqueous Punica granatum peel extract was used as a control. A suspension of each targeted bacteria (1.5x 10^8CFU/ml) has been prepared and standardized by McFarland standard (0.5N). Each bacterial isolate has been separately spread onto sterile Muller-Hinton Agar (MHA). With 0.8mm borer, four holes were made in every of the culture plates. One of the halls was loaded with 100 µl of AgNO3 4mM as positive control; 100µl of each concentration of the biogenic AgNPs [0.1, 0.5, 1] mg/ml were loaded to the remaining three holes. In a same way, same concentrations of the warm aqueous *Punica granatum* peel extract [0.1, 0.5, 1]mg/ml were loaded to another plate and incubated at 37°C for 24 h. Zones of inhibition was scaled in millimeters. This test was performed in triplicates.

Erythrocyte Toxicity

The hemolysis of blood was carried out as described by Bouma, (2002) as below: 30 µl of (50 – 60 µg/ml) silver nanoparticles solutions were mixed with 0.2 ml of healthy, non-smoker's blood. The mix were gently shaked for 5 seconds then 20 ml normal saline added (to avoid erythrocytolysis) and spinned at 3000 rpm for 10 minutes. 30 µl DMSO with normal saline and blood on the same ratio that used as positive control while the 100% hemolysis was validated by 100 folds dilutinion of the blood in distilled water. Spectroscopic absorbance has been read at 540 nm, Hemolysis percentage was evaluated by the equation:

Hemolysis%= (AT – AN) / (A 100% H –AN) x 100%

AT: Absorbance of test solution

AN: Absorbance of normal saline A100% H: Absorbance of 100 % hemolysis.

$$\%\ of\ Inhibition = \frac{(Abs\ of\ control - Abs\ of\ test)}{Abs\ of\ control} X\ 100$$

Results

Synthesis of AgNPs: This experience showed that the AgNPs cannot be synthesized by dark reaction in case of preparing in cold extract while boiled aqueous extract can be synthesized in gloom after 24hr. Aqueous Ethanol and methanol extract, prepared from the leaf of M. Spicata was used for rapid biosynthesis of silver nanoparticles under bright conditions with direct sunlight radiation. In addition to the chemical synthesis of nanoparticles induced by sunlight, light-induced biosynthesis of NPs has been investigated by many researchers (Dong et al., 2004). Among the variety of biosynthesis practices, light-induced phyto-nanosynthesis is supposed to be rapid. Zarchi et al. (2011) has reported the sunlight-induced biosynthesis of AgNPs by Andrachnea chordifolia extract, also reported the sunlight-induced AgNPs biosynthesis using the *Cassytha filiformis* aqueous extract. However, the mechanism of light-induced biosynthesis of nanoparticles is not clear (Jena et al., 2016). Silver nanoparticles were produced using solar-induced approach by aqueous garlic extract. Nanoparticles in this case were synthesized after 15 min when exposed directly to the sunlight (Rastogi and Arunachalam, 2011). The oxidation of phenols by pH change to phenolates was represented more easily oxidized than phenols in the extract of the plant (Zuman and Holthuis, 1988). The study differs from Kajani et al. (2014) who used the different

change of pH adjusted and temperature after adding silver nitrate to Taxus baccata extract, while Kahrilas et al. (2013) prepared silver nanoparticles by organ peel extract using microwave-assisted, by Fraxinus excelsior leaf extract (Parveen et al., 2016).

Phytosynthesis of Silver Nanoparticles by Plant Extract

Punica granatum extract found to be potent for extracellular biosynthesis of AgNPs by adding AgNO3 (4 mM) to each of the concentrations (100mg and 50mg) of hot water extracts. Transcoloration from yellow to reddish-brown in the reaction mixture after incubation for 24hrs at 37°C, and shking speed 150 rpm in a shaking incubator, indicates the presence of AgNPs in the *Punica granatum* peel extract reaction mix (figure 1). Change in the color and its intensity in the reaction mix, found to be increased with increasing the concentrations of (AgNO3) that were added to cell-free supernatant and when the pH of reaction mixt been elevated, the color was changed gradually by adding NaOH (1M).

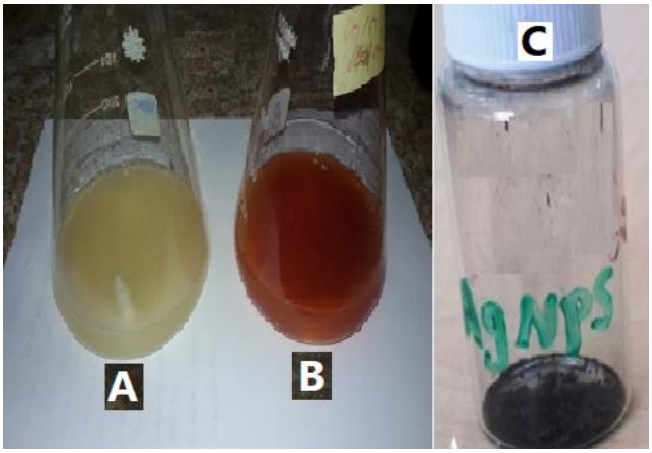

Figure 1. (A) Original colorof the extract (yellow) befor incubation and free of AgNO3. (B) Color change in to (reddish-brown) after incubation a shaking incubator (150 rpm). (C) dessicated AgNPs.

Characterization of Silver Nanoparticles

Scanning Electron Microscopy (SEM)

Scanning electron microscopy has been exploited to characterize shape, size, and distribution of the biogenic AgNPs. Well-dispersed, homogenous, almost spherical shaped nanoparticles with diameter ranging (30-100 nm), was observed (figure 2). According to SEM characterization results of AgNPs, Punica granatum peel extract showed potency for AgNPs biosynthesis distinct in their characteristics. It's found that the concentration of AgNO3 applied to the reaction mix play role in the shaping of the nanoparticles formed. The ideal AgNO3 concentration for AgNPs biosynthesis was 4mM among the applied concentrations.

EDS Analysis of the AgNPs

Formation of AgNPs were approaved by the presence of predominant elemental silver quantified by EDS analysis results. The optical absorbance peaks represent the selected elemental constituents. elemental silver (Ag^0) is the reduced form of silver nitrate ($AgNO_3$), so presence of Ag^0 in the extract provide an evidence for the reduction potential of *Punica granatum* peel extract supernatant. Strongest signal exhibited by Ag atoms, while medium signal was exhibited by oxygen and the delicate signals were from miscellaneous constituents. The weight percentage of elemental constituents of the AgNPs was (76.91%) silver and (23.09%) oxygen (figure 3). The optical absorption peak of AgNPs was detected at 3keV; the ideal absorbance of AgNPs.

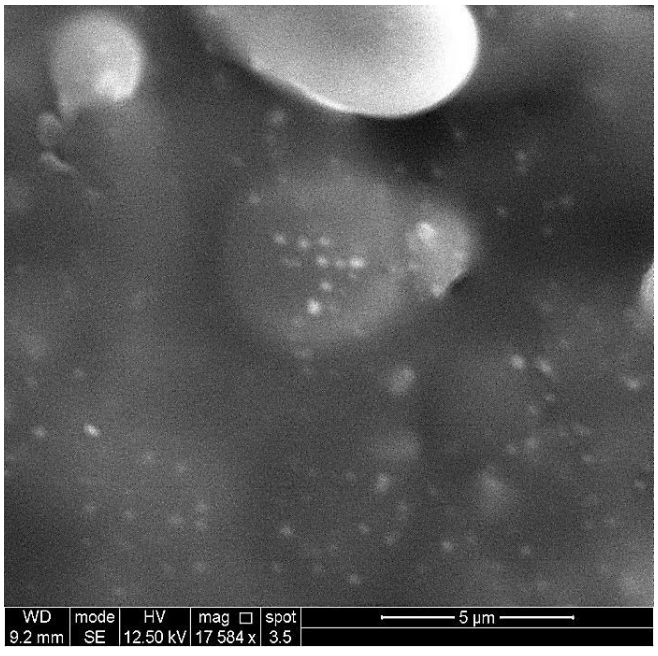

Figure 2. SEM electron micrograph of silver nanoparticles biosynthesized by Punica peel extract showed homogenous, spherical shaped AgNPs, with mean size between (30-100nm)

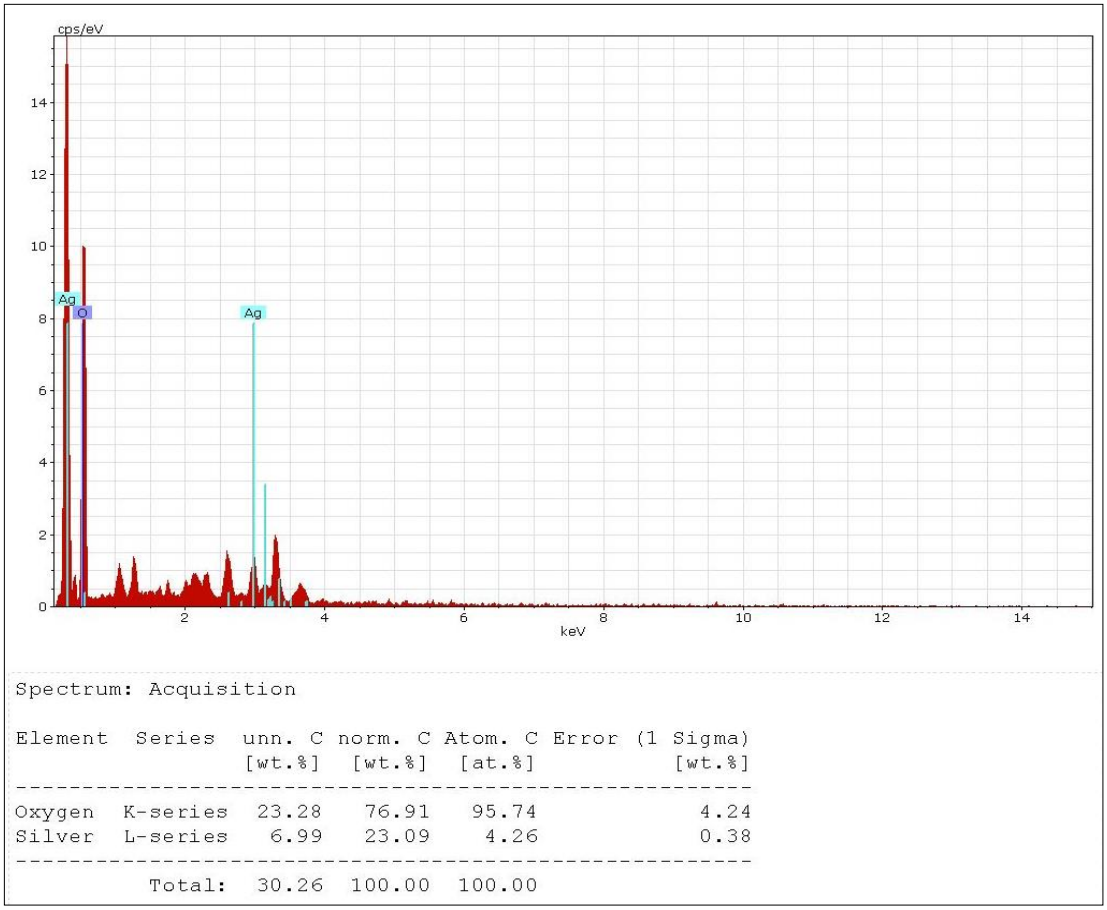

Figure 3. EDS analysis of AgNPs synthesized Illustrated strong signal from Ag, medium signal from O2 the optical absorbance peak of Ag was observed at 3keV

Antibacterial Activity

Antibacterial activity of AgNPs biosynthesized by Punica granatum peel extract supernatant were examined for their antibacterial activity following Agar well diffusion method. Different concentrations of AgNPs were examined. The results showed antimicrobial activity against both gram-negative and gram-positive bacterial growth. Inhibition zones founed to be smaller in gram-positive than gram-negative bacteria (table 1). AgNPs produced by Punica peel extract expressed higher antibacterial activity than warm aqueous Punica granatum peel extract. The inhibition zone of the biogenic AgNPs on E. coli measured 17.53, 22.35, and 26.06 at the concentrations 0.1, 0.5, and 1 mg/m respectively. While the inhibition zone of warm aqueous Punica granatum peel extract measured 5.33, 10.63, and 16.08 at the same concentrations. Biogenic AgNPs revealed greater inhibition zones in gram-negative than in gram-positive bacteria.

Table 1. Inhibition Zone of clinical bacteria in (mm) of different concentrations of warm aqueous Punica peels extraction and AgNPs synthesize by Punica peels extract

Type of extract	Conce. mg/m Punica	Inhibition zone rate (mm)		
		E.coli	P. aeruginosa	S.aureus
Punica peels extraction	0.1	5.33	1.00	3.14
	0.5	10.63	11.0	13.23
	1	16.08	20.10	17.50
AgNPs synthesize by Punica peels extract	0.1	17.53	15.05	10.14
	0.5	22.35	22.5	13.23
	1	26.06	24.09	17.50

Cytotoxic Activity of Silver Nanoparticles

Cytotoxicity of AgNPs was assayed in vitro using human blood erythrocytes (RBCs). Table (2) showed the value of absorbance at 540 nm and hemolysis present by silver nanoparticles, in general, silver nanoparticles show low RBCs hemolysis percent (4.50%) at concentration 1 mg/ml aqueous Punica. This study, concluded an increase in AgNP-induced hemolysis (erythrocyt destruction) proportional to

the increase in AgNPs concentrations after direct contact with human RBCs, possibly caused by membrane destruction. Investigation of erythrocyte cytotoxicity is recommended together with antibacterial activity (Golubeva et al., 2010).

Table 2. Hemolytic activity of silver nanoparticles

	Concentration	Absorbance at 540	Hemolysis (%)
AgNPs (Punica)	1 Mg/ml	0.066	4.50
	0.5 mg/ml	0.062	4.84
	0.1 Mg/ml	0.059	4.24

Discussion

Biosynthesis of Silver Nanoparticles

Biosynthesis of nanoparticles by plant extract supernatant. The use of biological entities for nano-fabrication is an proliferate research area get scientist's attention, proposing plant extracts as probable sustainable nano-factories (Iravani, 2011). When plant extracts exposed to metal ions, they develop a remarkable ability to fight against metal stress as biotechnological applications. Recently, it has been agreed that phyto-extracts have been concidered as possible bio-industries for synthesis of metallic nanoparticles. (Mukherjee et al., 2002). Results of this research endeavor to utilize the potency of Punica hot water extract for synthesis of AgNPs. Upon adding AgNO3 under dark conditions to the Punica hot water extract, the medium color was changed from off white to brown, indicating the presence of AgNPs in the reaction mix as a result of Ag+ reduction to Ag^0 (metal Ag) by the reductants (secondary metabolites) in the extract reaction mix. The resulting color reflected by AgNPs is resulted by excitation of the entire coherent free electrons in the interface within the conduction band, resulting in SPR (surface plasmon resonance) (Sreedevi et al., 2015).

Characterization of AgNPs

Formation of biogenic nanoparticles predicted by trans-coloration from off-white into brown, the characteristic for AgNPs as a result of SPR. SEM used to characterize the size the physical properties of the yield nanoparticles (distribution, size, and shape), experimental results exhibited well-dispersed, almost spherical nanoparticles with mean diameter (30-100 nm) (Figure 2) these results in agreement with the results of (Aldujaili et al., 2015). Results of EDS revealed abundance of Ag^0, suggesting the reduction of Ag+ into Ag^0 in the synthesis medium. Silver weight percentage was 76.91%. Optical absorbance peak was detected at 3keV, the ideal absorbance for AgNPs.

Antimicrobial Activity of AgNPs

Antibacterial activity assay of the biogenic AgNPs expresses greater activity against G-ve compared with G+ve bacteria. Highest inhibition zone among examined G-ve bacteria was (26 mm) for E. coli, while among examined G+ve bacteria, the maximum inhibition zone was (13mm) for S. aureus at the same concentration. Difference, possibly reflects the variability in cell wall architecture among G-ve and G+ve bacteria. Cell wall of Gram-ve comprises of an outer membrane, thin peptidoglycan layer, and. In comparison; Gram+ve cell wall comprises of lipoteichoic acid including a layer of thick peptidoglycan, in addition to the cell membrane (Taglietti et al., 2012). Differences in the sensitivity of examined bacteria upon exposed to AgNPs as in E. coli, and *P. aerogenosa*, the inhibition zone of these bacteria was (26, 17.5) and (21, 10), mm at highest and lowest concentrations respectively, this might reverted to the differences in species-linked susceptibility mediated by the synergistic activity of several intrinsic factors, termed (intrinsic resistome) (Blake and Neill, 2013). The positive charge on the silver ion is the reason for antimicrobial activity because it can attract negative charge of plant extract via electrostatic interactions. Perhaps these attractions outweighs other factors, like nanoparticle shape and size which can affect the death of bacterial cells (Abbaszadegan et al., 2015). Silver ions liberated from AgNPs is the key mechanism of AgNPs (Aldujaili, et al., 2015). Silver ions Ag^+ attach to proteins and nucleic acids negative charge triggering structural changes and cell wall, membrane, and nucleic acids deformation in the bacterial cells. Ionic silver Ag+ interacts with functional groups in some electron donors such as indoles, thiols, imidazoles, phosphates, and hydroxyls. Silver nanoparticles damage membranes causing release of oxidative stress factors like (ROS), thus powerful bactericidal free radicals formed (Wu et al., 2014). Silver ions or small AgNPs may cause ribosomal denaturation leading to suppression of protein synthesis in addition to blockage of gene expression and replication when AgNPs interacted with the bacterial genetic materials, (Jung et al., 2008). Furthermore; nanoparticles has been observed to modulate signal transduction in

bacterial cell as a result of dephosphorylation of tyrosine residues in the peptide substrates, which results in blocking of signal transduction leading to slowdown or stop bacterial growth (Shrivastava et al., 2007). When silver ions interacted with sulfhydryl groups (–SH) in the nucleoprotein causing DNA unwinding. Silver nanoparticles interfere with hydrogen bonding leading to inhibition of cell division (Davod et al., 2011). Some enzymes are inhibited by silver ions particularly type II NADH dehydrogenase in the respiratory chain, which is implied as a candidate production site for reactive oxygen species (Matsumura et al., 2003).

Conclusions

Peel extract of *Punica granatum* contains reducing and stabilizing compounds, crucial for the synthesis of silver nanoparticles. Hot water can be serve as cheap, and abundant solvent for extraction and AgNPs biosynthesis in plant samples. In addition; an increase in the biological activity of the biogenic nanoparticle against pathogenic bacteria compared to the traditional extract of Punica.

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