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Green synthesis of silver nanoparticles using Omani pomegranate peel extract and two polyphenolic natural products: characterization and comparison of their antioxidant, antibacterial, and cytotoxic activities

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Abstract

Background: Green synthesis of silver nanoparticles (AgNPs) has gained popularity due to the economical and eco-friendly approach associated with it. The aim of the study was to biosynthesize silver nanoparticles using pomegranate peel extract (PPE), quercetin (Q), and gallic acid (GA) and to evaluate their antioxidant, antimicrobial, and cytotoxic activities.

Results: A sharp color change from yellow to brown, appearance of a peak in a UV spectrum around 413–425 nm, and four intense peaks in XRD spectra matching with the reference silver crystal confirmed the formation of AgNPs. SEM and TEM analysis revealed particles to be mostly spherical with few aggregates. Average particle size distribution and zeta potential was found to be 43.6 nm and -18.01 mV, 31.6 nm and -21.5 mV, and 21.7 nm and -27.9 mV for Q-AgNPs, P-AgNPs, and GA-AgNPs, respectively. P-AgNPs showed excellent in vitro antioxidant activity (84.85–89.20%) and cytotoxic activity (100% mortality). P-AgNPs and GA-AgNPs exhibited good antimicrobial activity against the four bacterial strains comparable to the reference antibiotics but Q-AgNPs failed to inhibit the growth of *P. vulgaris*.

Conclusions: Omani pomegranate peel extract seems to be a potential and alternative source for the simple, one pot, eco-friendly green synthesis of extracellular stable AgNPs. The synthesized nanoparticles have the potential to be developed as possible antimicrobial agents and antioxidants.

Keywords: Green synthesis, Silver nanoparticles, Antioxidant, Cytotoxic, AgNO₃

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1 Background

Nanotechnology is an ever-growing discipline that has seen practical applications in the various fields including medical, pharmaceutical, food, and industrial sectors [1, 2]. It has been established that metal particles in the nanometer size range (1–100 nm) show discrete physico-chemical properties in comparison to their corresponding bigger size parent metals which is attributed in part to the greater ratio between their surface area and volume [3]. Amongst all the metal nanoparticles that have been synthesized, silver nanoparticles (AgNPs) remain to be the most widely studied and utilized nanoparticles in medical and pharmaceutical industries [4]. Multiple in vitro studies have demonstrated that AgNPs possess significant antimicrobial activity and some of these studies highlighted their potential in overcoming resistance to some bacterial strains [5, 6]. AgNPs have also displayed a role in anticancer therapy through both induction of apoptosis and targeted drug delivery of antineoplastic agents to the cancer cell site [7, 8]. While the mechanism of bactericidal action and cytotoxicity remains largely unclear, there's enough compelling evidence in the literature to suggest that method of synthesis and reducing agents used to synthesize them plays an important role in ascertaining the level of toxicity and antimicrobial efficacy of AgNPs [9, 10].

AgNPs can be synthesized through three different methods, viz., physical, chemical, and biological methods [11]. The former two processes have shown to be more cumbersome and toxic to the environment than the latter. Synthesis by chemical method employs chemicals usage which contributes significantly to the environmental pollution along with a higher energy demand to synthesize the AgNPs [12]. Physical method, in particular evaporation–condensation method, requires a large amount of energy to produce and maintain that increases the cost of synthesis. Henceforth, green synthesis

of AgNPs has gained more popularity in the past few years due to the economical and eco-friendly approach associated with it.

Various extracts of plant parts which are rich in secondary plant metabolites have been used for biosynthesis of AgNPs. These natural sources serve the purpose of both a reducing and capping agent, therefore eliminating the need to utilize two separate chemicals to reduce the metal ion and stabilize the nanoparticles, which is often the case in chemical synthesis of AgNPs [13]. Another advantage of biosynthesis of AgNPs using plant extracts or isolated plant constituents is the protective property it imparts to the AgNPs. Uncoated or chemically synthesized AgNPs are reported to induce oxidative stress in organisms, which in turn leads to cytotoxicity followed by cellular apoptosis [14]. Use of antioxidant plant extracts for the biosynthesis of AgNPs has greatly diminished their toxicity [15].

Pomegranate (*Punica granatum L.*) fruit is rich in polyphenolic phytochemicals which have been shown to exhibit excellent antioxidant properties [16, 17]. Pomegranates are among some of the major crops cultivated in the Sultanate of Oman. They are grown in the region of Jabal Al Akhdar where the temperature is cool and appropriate for optimum growth and development of the plant [18]. Pomegranate peel (PP) is an agro-waste constituting more than half of the weight of the fruit. PP are abundant sources of vitamins (A, B₆, B₉, and E), potassium, and oxalic acid. Pomegranate peels in Oman are traditionally used in the treatment of intestinal wounds and to aid weight loss. Phytochemical screening of Omani PP extract has revealed the presence of flavonoids and polyphenols, among other chemicals, as the major phytoconstituents [17]. Among the flavonoids and phenolics present in the pomegranate extracts, quercetin and gallic acid (Fig. 1) contribute significantly to their

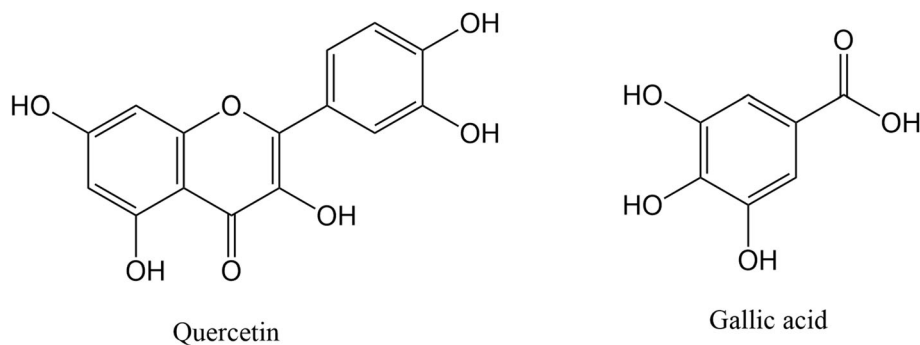


Fig. 1 Chemical structures of quercetin and gallic acid

content [19]. Quercetin is a flavonoid known to exhibit strong angioprotective, anti-inflammatory, and anticancer activity in humans [20]. Gallic acid is a polyphenolic acid having the potential clinical application as an anticancer and apoptosis-inducing agent. Additionally, its anti-inflammatory, antimicrobial, neuroprotective, nephroprotective, and hepatoprotective activities have been well established [21].

In green synthesis of AgNPs, the plant extract has a dual action of being both the reducing agent and the capping agent. Pomegranate peel extracts (PPE) elsewhere have been used for the synthesis of AgNPs and were shown to exhibit good antimicrobial activity [22–24]; however, literature review indicated that AgNPs prepared using Omani PPE have not yet been explored for their antioxidant and cytotoxicity activities.

The current study was designed to synthesize and characterize AgNPs using polyphenolic natural products such as pure quercetin and gallic acid and to compare their bioactivity and physical properties against AgNPs synthesized using PPE. We hypothesize that the AgNPs synthesized by using above three natural reducing agents would differ greatly in their physicochemical properties and thus would show differences in biological activities *viz.*, antimicrobial, cytotoxic, and antioxidant.

2 Methods

2.1 Chemicals, reagents, and microbiological supplies

Highly pure organic solvents, chemical substances of Sigma such as silver nitrate (AgNO_3), gallic acid (GA), quercetin (Q), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium hydroxide (NaOH), standard antibiotic discs (gentamicin 10 μg and cefuroxime 30 μg), and sterile Mueller-Hinton agar (MHA) media plates were procured through the local supplier. Brine shrimp eggs of San Francisco Bay Brand, USA, were used for cytotoxic activity. Bacterial cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Proteus vulgaris* were obtained from the Biology Department, College of Pharmacy, Sultanate of Oman.

2.2 Collection and preparation of pomegranate peel extract

Fresh pomegranate fruits were collected from a farm house in Jabal Al Akhdar (Green mountains) of Ad-dakhliya region in Oman during peak optimum growth season (August–September 2018). The fruits were identified by a faculty member and a voucher specimen was deposited in the Pharmacy lab for the future reference. The fruit was thoroughly washed using distilled water and the seeds were subsequently separated to obtain the peel. The isolated peels were dried under shade at the room temperature for 3 days. The dried peels were

ground into uniform coarse powder using a domestic blender.

Approximately 200 g of pomegranate peel powder upon extraction with ethanol (4–5 h, 90 °C) using a Soxhlet apparatus yielded a bright red color ethanolic pomegranate peel extract (PPE). The extract was concentrated under reduced pressure (45–50 °C) using a rotary evaporator to obtain the viscous mass (42 g; 21.0% w/w). The PPE was stored at 4 °C in the refrigerator until further use in the experiment.

2.3 Biosynthesis of AgNPs using PPE, quercetin, and gallic acid

In a 150-mL beaker containing 50 ml of 2.1 mM AgNO_3 , 2 mL of freshly prepared PPE (2.5% w/v in distilled water)/35 ml of 1mM quercetin (hydroalcoholic solution)/63 mL of 1 mM gallic acid was added with vigorous stirring at the room temperature. Sufficient quantity of 0.1 M NaOH solution was added to maintain the pH at 4–5. The change in the color of the solution in beaker from pale yellow to deep brown indicated the formation of the AgNPs by PPE/quercetin/gallic acid.

2.4 Characterization of biosynthesized AgNPs

The formation of AgNPs was confirmed by measuring the absorbance on a UV-Vis Spectrophotometer (UV Analyt-CT 8200) in the wavelength range of 200–600 nm and X-ray diffraction (XRD) spectrum measurement using a XRD powder method (MiniFlex 600, Rigaku) as per the standard experimental procedure. The zeta potential of the prepared AgNPs was measured using Zeta Analyzer (NICOMP™ 380 ZLS) and the average size distribution was measured using Malvern ZetaSizer. Surface morphology, shape of the nanoparticles, and elemental composition of AgNPs were analyzed through field emission scanning electron microscope (FE-SEM; JOEL JSM-7800F) operated at 15 kV with a working distance of 10 mm and by placing a single drop of aqueous AgNPs on a copper-coated grid for transmission electron microscope (TEM) imaging (JOEL JEM-1400).

2.5 In vitro antioxidant assay

Antioxidant activity of the biosynthesized AgNPs was determined using DPPH free radical scavenging assay method. A 0.004% w/v solution of DPPH was prepared by dissolving 2 mg DPPH in 50 ml of methanol. Three different concentrations of the P-AgNPs, GA-AgNPs, and Q-AgNPs (5, 10, and 20 $\mu\text{g}/\text{mL}$) were tested for antioxidant activity. Quercetin (0.2, 0.5, and 1mM) was used as a positive control. In separate test tubes, 1 mL of each concentration of the AgNPs was mixed with 2 mL of freshly prepared 0.004% DPPH solution. Control sample contained methanol in place of AgNPs. The test tubes were

then left in the dark at room temperature for 1 h after which their absorbance was measured at 517 nm [25]. The percentage (%) inhibition of DPPH radical was calculated as per the following formula;

$$\% \text{inhibition of DPPH} = \left[\frac{(Ac - At)}{At} \right] \times 100$$

where Ac = absorbance of the control and At = absorbance of the test sample.

2.6 Antibacterial activity

Antimicrobial activity of the biosynthesized AgNPs was measured against two Gram-negative bacteria (*E. coli* and *P. vulgaris*) and two Gram-positive bacteria (*S. aureus* and *B. subtilis*) grown on MHA media. Well diffusion method was used to test the inhibitory effect of three different dilutions of AgNPs against the four bacterial strains. In sterile agar plates, wells were made using a 10-mm cork borer followed by streaking of the plates with bacterial broth. The wells were then filled with 10, 20, and 100 μg AgNPs solution. In the control agar plates, wells were loaded with AgNO_3 (1mM), quercetin (2mM), gallic Acid (2mM), and pomegranate extract for comparison purpose. Gentamicin 10 μg and cefuroxime 30 μg antibiotic discs were also added to each plate. The agar plates were incubated at 37°C for

24 h after which the zone of inhibition around each well was measured [26].

2.7 In vitro cytotoxicity assay

Brine shrimp lethality assay method was used to measure the cytotoxic effects of the biosynthesized AgNPs. Brine shrimp eggs were hatched in a glass tank using sea water and an aerator. The larvae were visibly alive and moving towards light source 72 h after placing in the water and were subsequently used. In three different test tubes, around 10 shrimp larvae were added along with 10-ml sea water. One milliliter of 0.1, 0.5, and 1 $\mu\text{g}/\text{mL}$ of all three samples of AgNPs was added to each test tube. A control test tube was prepared by omitting AgNPs. After 24 h, the numbers of dead and alive larvae were counted and percentage mortality was calculated [27].

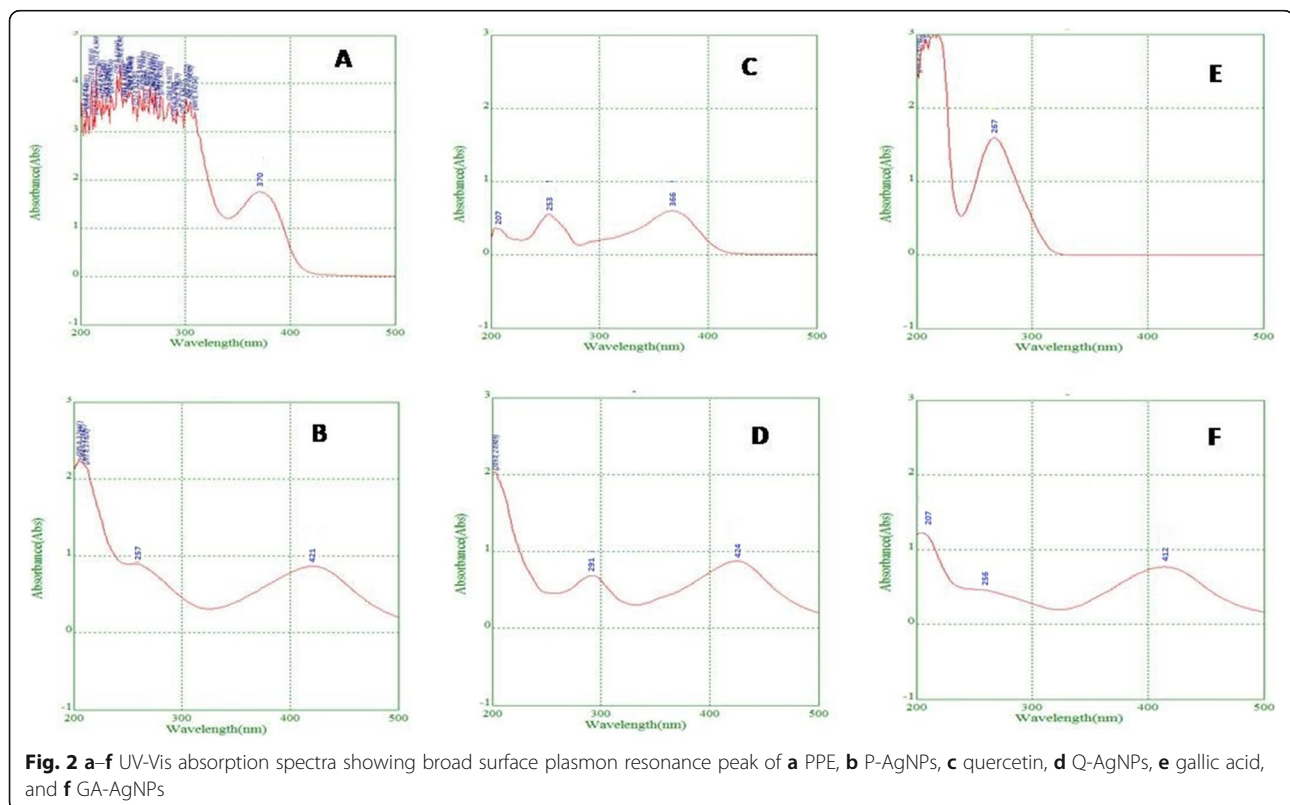
2.8 Statistical analysis

Each experiment was performed in triplicate and the results are expressed as mean \pm SD.

3 Results

3.1 Synthesis and characterization of AgNPs

The AgNPs were formed immediately upon addition of an alkali solution to the reaction mixture under vigorous stirring. A sharp color change from pale yellow to brown



in case of Q-AgNPs and P-AgNPs, and a change from colorless to brown in case of GA-AgNPs indicated the reduction of AgNO_3 to form nanoparticles [9]. The formation of eco-friendly AgNPs was confirmed by

recording their UV absorption spectrum in the 200–600 nm range and comparing them with the UV spectrum of PPE, quercetin, and gallic acid. Appearance of new broad surface plasmon resonance peaks at 425 nm, 421 nm,

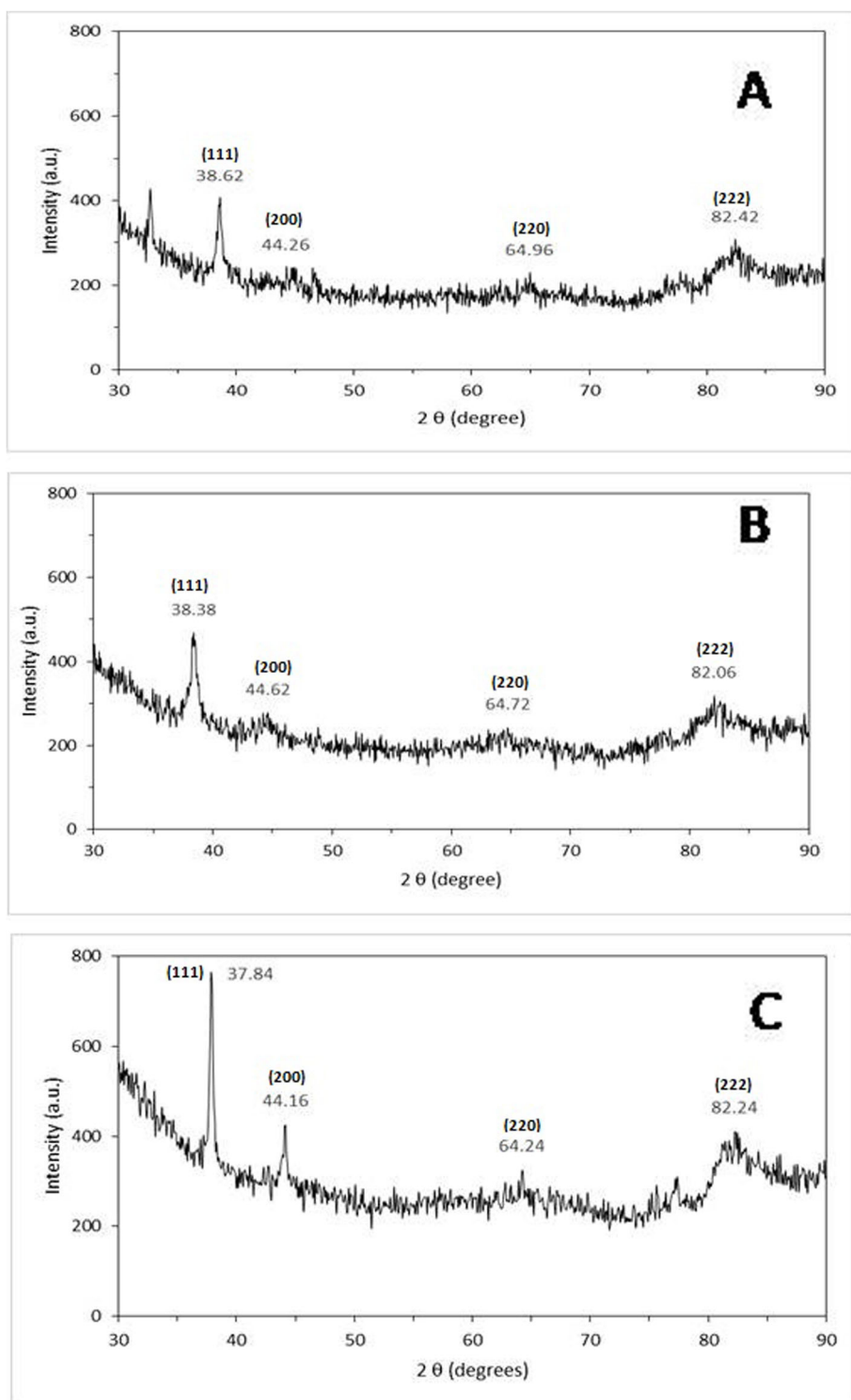


Fig. 3 a-c The XRD pattern of biosynthesized AgNPs. **a** P-AgNPs. **b** Q-AgNPs. **c** GA-AgNPs

and 413 nm in the UV-Vis absorption spectra (Fig. 2) for Q-AgNP, P-AgNP, and GA-AgNP, respectively are consistent with the literature data [11, 24].

Four intense peaks in the XRD spectra ($2\theta = 37.84\text{--}38.62^\circ$, $44.16\text{--}44.62^\circ$, $64.16\text{--}64.96^\circ$, and $82.06\text{--}82.42^\circ$) for all the three samples showed a pattern that matched with the reference cubic silver crystal further confirming formation of AgNPs (Fig. 3) [28]. Furthermore, the crystalline size of P-AgNPs, Q-AgNPs, and GA-AgNPs computed from XRD are noted to be 45.05 nm, 25.16 nm, and 32.15 nm, respectively. The values obtained in XRD were matched with JCPDS 4-0783 values for silver. The XRD spectrum for P-AgNPs showed an extra peak around 30 to 35° which is unaccounted for. This peak could possibly be due to a secondary phase. The high magnification imaging by SEM and TEM showed particles to be predominantly spherical with few aggregates (Figs. 4 and 5). The elemental EDX analysis illustrated high content, strong peaks for the silver. The minor peaks in the spectra may possibly be due to the biomolecules that could have bound to surface of AgNPs.

The average size distribution (Z-average diameter) of the particles was noted to be 43.6 nm for Q-AgNPs, 31.6 nm for P-AgNPs, and 21.7 nm for GA-AgNPs (Fig. 6) with a surface charge of -18.01 mV, -21.5 mV, and -27.9 mV respectively (Table 1).

3.2 Antimicrobial activity

P-AgNPs exhibited concentration-dependent good antimicrobial activity against the four bacterial strains. The activity was slightly better against Gram-positive bacteria, in particular *B. subtilis*, while *P. vulgaris* and *S. aureus* were noted to be resistant at lower concentrations of $10\ \mu\text{g}$. However, the antibacterial efficacy of P-AgNPs was found to be significantly lower than the PPE. GA-AgNPs showed bacterial growth inhibition comparable to that of P-AgNPs, but with a slightly more pronounced effect against *E. coli*. Q-AgNPs showed the antimicrobial activity only at $100\ \mu\text{g}$. *P. vulgaris* was

noted to be resistant to the AgNPs at all the three tested concentrations (Table 2).

3.3 In vitro antioxidant assay

The results of in vitro inhibition of DPPH free radicals by the biosynthesized AgNPs are presented in Table 3. P-AgNPs exhibited excellent antioxidant properties (84.85–89.20%) followed by Q-AgNPs (66.95–84.32%) and are comparable to positive control quercetin (92.74–95.32%). GA-AgNPs showed a moderate inhibition of DPPH radicals and thus lower antioxidant activity (52.06–63.28%). The DPPH radical scavenging activity of AgNPs at $20\ \mu\text{g}/\text{mL}$ was found in the following order: P-AgNPs>Q-AgNPs>GA-AgNPs.

3.4 In vitro cytotoxic activity

The results of Brine shrimp lethality assay of the AgNPs indicated that P-AgNPs caused 100% mortality of the larvae at $1\ \mu\text{g}/\text{mL}$ and are highly cytotoxic. The GA-AgNPs and Q-AgNPs showed 89.2 and 88.5% mortality respectively (Table 4).

4 Discussion

Formation of silver nanoparticles was confirmed through the observed color change and based on the shift in the λ_{max} of the peaks in the UV spectrum. Size and charge of the AgNPs varied depending on the reducing agent used. The size and charge of AgNPs synthesized using PPE falls somewhat in between the ones synthesized using quercetin and gallic acid. Contrary to our results, Joshi et al., in 2018, reported much lower zeta potential of -68.93 mV and a much larger average particle size distribution (57.7–142.4 nm) for the AgNPs synthesized using PPE [28]. However, the average particle size of 36 nm and zeta potential (-24.1 mV) of AgNPs biosynthesized by Jyoti et al., using aqueous leaves extract of *Urtica dioica* (Linn.), are consistent with the results obtained for P-AgNPs in our study [29]. The variation in the

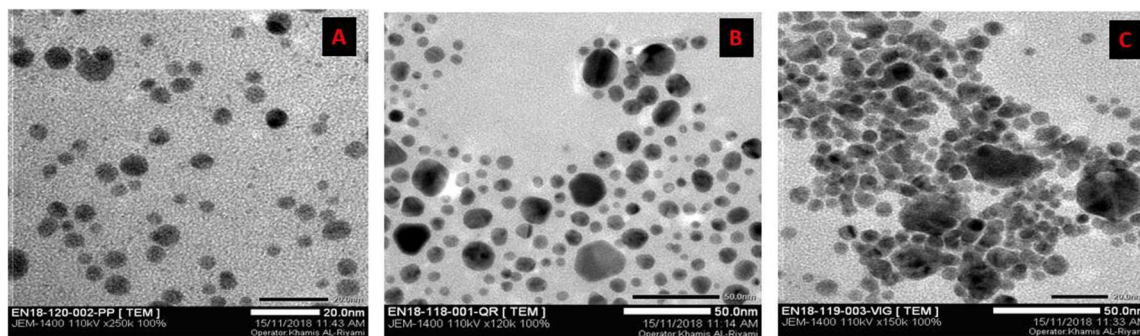
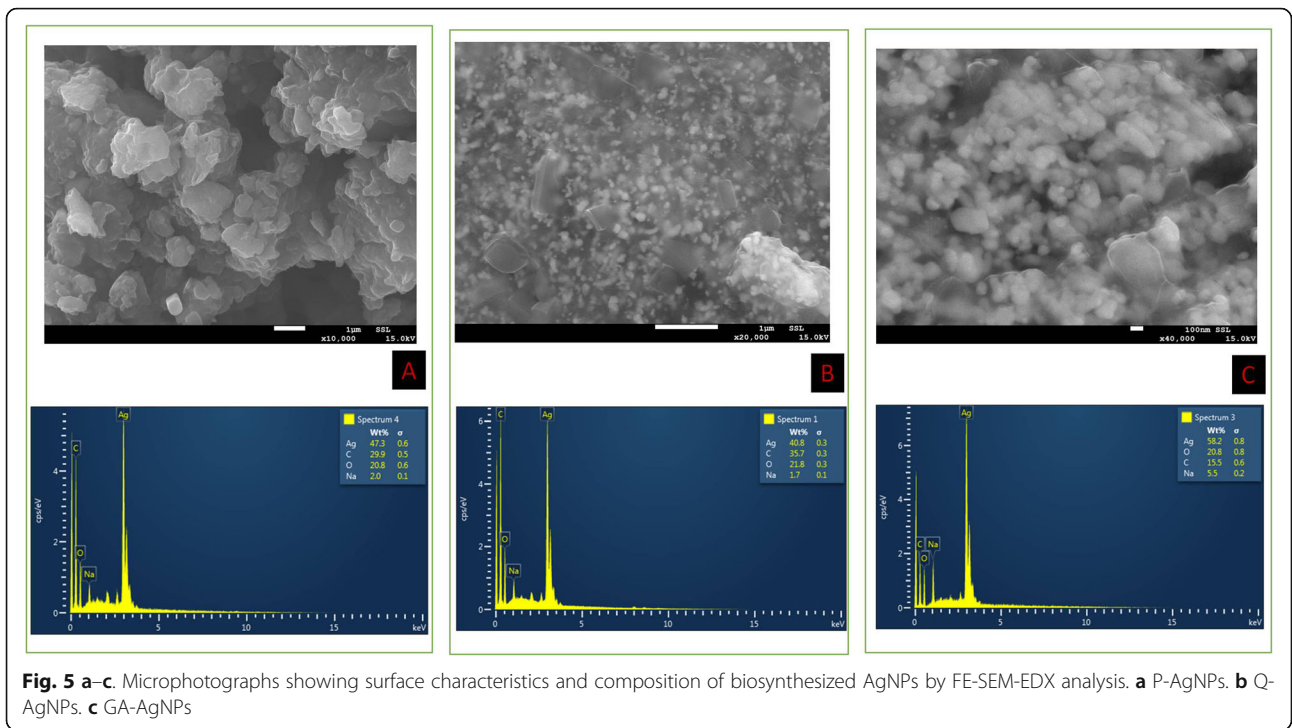


Fig. 4 a–c. TEM analysis of biosynthesized AgNPs. **a** P-AgNPs. **b** Q-AgNPs. **c** GA-AgNPs



size of three AgNPs could be due to the difference in the nature of reducing agents and chemical composition of PPE with respect to pure natural products, gallic acid and quercetin. The zeta potential values indicate good stability of AgNPs which could be

attributed to the natural bioactive compounds responsible for capping, reduction of silver ions, and stability of nanoparticles.

Antimicrobial activity of P-AgNPs compared to PPE was lower than expected. This could be due to the

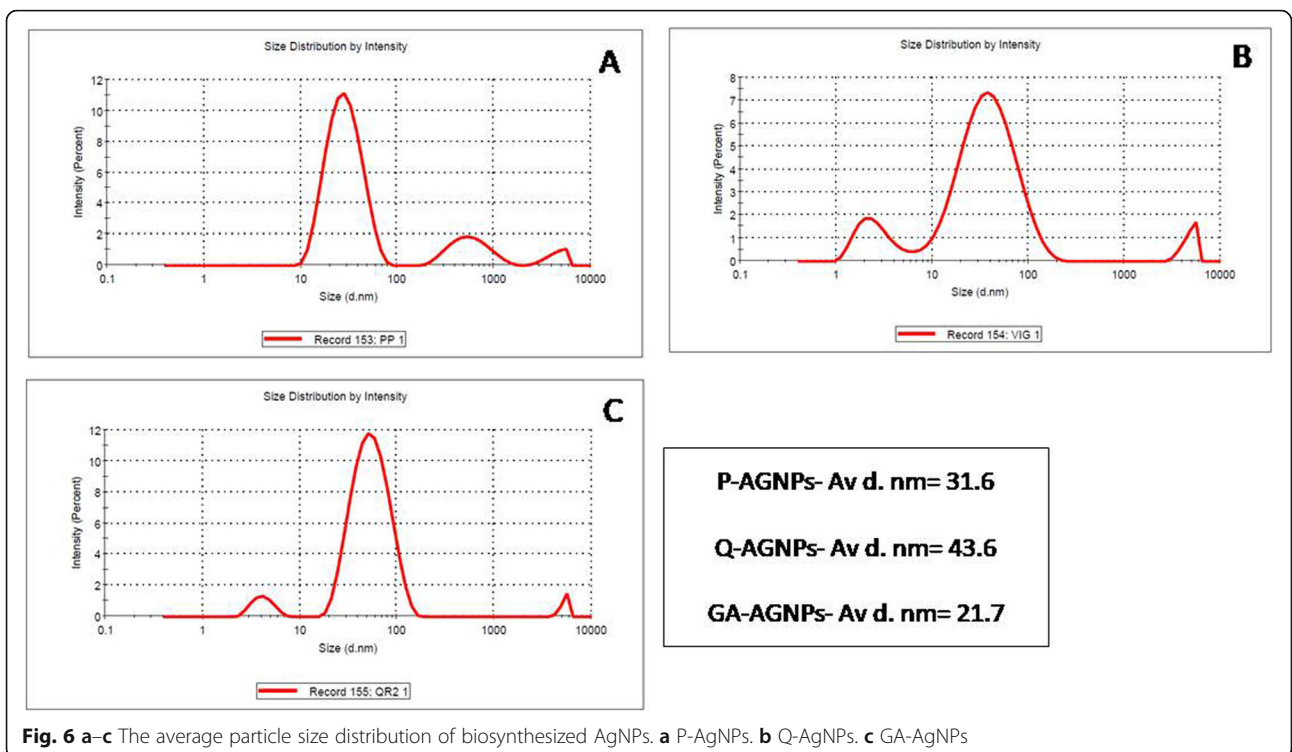


Table 1 Zeta potential and average size distribution of eco-friendly biosynthesized AgNPs

| | Average sizedistribution (nm) | Zeta potential (mV) |
|----------|-------------------------------|---------------------|
| Q-AgNPs | 43.6 | - 18.01 |
| P-AgNPs | 31.6 | - 21.5 |
| GA-AgNPs | 21.7 | - 27.9 |

presence of other bioactive phytoconstituents in the PPE which would have exerted an additive effect against microbial growth. The presence of a negative surface charge on the nanoparticles which correspond closely to the charge of the bacterial cell wall could have also been a driving factor in decreased efficacy through repulsion and subsequent decreased uptake by the bacteria [30]. The slightly more pronounced inhibitory effect of GA-AgNPs against *E. coli* could be attributed to their relatively thin cell walls that might have facilitated the entry of the GA-AgNPs [31]. This result is of quite significance as GA solution did not inhibit bacterial growth. Q-AgNPs antimicrobial activity at higher concentration can be attributed to their large particle size which prevents their penetration of the bacterial cell wall.

The antibacterial results of our study are in agreement with the result obtained in a previous study [32] (Table 2). The AgNPs have been reported to exert bactericidal activity by multiple mechanisms of action such as by attacking the respiratory chain and cell division leading to cell death, alteration of cell membrane permeability, release of polysaccharides, and membrane potential [33]. Various studies have shown that a negative charge imparted on

Table 2 Antibacterial activity of biosynthesized AgNPs

| Sample | Concentration | Zone of inhibition (mm) | | | |
|-------------------|---------------|-------------------------|--------------------|----------------|--------------------|
| | | <i>S. aureus</i> | <i>B. subtilis</i> | <i>E. coli</i> | <i>P. vulgaris</i> |
| Q-AgNPs | 100 µg | 11.7 | 14 | 12.4 | - |
| | 20µg | - | - | - | - |
| | 10µg | - | - | - | - |
| P-AgNPs | 100 µg | 15 | 15.2 | 15.2 | 14.7 |
| | 20µg | 14.2 | 14 | 13.2 | - |
| | 10µg | - | 13.2 | 12.6 | - |
| GA-AgNPs | 100 µg | 15.2 | 14.7 | 17.7 | 14.7 |
| | 20µg | - | 13.9 | - | - |
| | 10µg | - | - | - | - |
| AgNO ₃ | 2mM | 20 | 20 | 19 | 13.2 |
| Quercetin | 1mM | 12.2 | 15 | - | - |
| Gallic Acid | 1mM | - | - | - | - |
| PPE | 2.1% w/v | 23 | 22 | 21.8 | 17.8 |
| Gentamicin | 10 µg | 24.4 | 35 | 24.9 | 24.3 |
| Cefuroxime | 30 µg | 19.3 | - | 12.2 | 19.3 |

Table 3 In vitro antioxidant activity of biosynthesized AgNPs against DPPH free radicals

| | % inhibition of DPPH (mean±SD) | | |
|-----------|--------------------------------|--------------------------|--------------------------|
| | 20 µg/mL | 10 µg/mL | 5 µg/mL |
| Q-AgNPs | 84.32 ± 1.19 | 83.93 ± 1.95 | 66.95 ± 1.61 |
| P-AgNPs | 89.20 ± 0.31 | 85.90 ± 0.88 | 84.85 ± 0.88 |
| GA-AgNPs | 63.28 ± 2.67 | 61.07 ± 1.83 | 52.06 ± 0.61 |
| Quercetin | 95.32 ± 1.3 (1 mM) | 93.43 ± 0.76 (0.5 mM) | 92.74 ± 0.94 (0.2 mM) |

AgNPs can significantly exhibit cytotoxicity [30, 34]. Size-dependent toxicity has been shown to be more prominent in particles less than 50 nm. Smaller particles can penetrate through the cell membrane at a much larger extent owing to the greater surface area, leading to more interaction with the membrane.

Antioxidant activity results of the present study are in agreement with previously published reports demonstrating beneficial effects of AgNPs in combating oxidative stress [35]. The higher activity demonstrated by P-AgNPs could again be due to the presence of unreacted bioactive phytoconstituents present in PPE.

5 Conclusion

The present study demonstrates a simple, one pot, eco-friendly biosynthesis of AgNPs using Omani pomegranate peel extract and two of its polyphenolic constituents, namely quercetin and gallic acid as reducing agents. The phytochemicals (flavonoids, alkaloids, glycosides, terpenoids) present in the pomegranate peel extract acted as better reducing and capping agents in comparison to the pure quercetin and gallic acid for the formation of stable AgNPs. A peak in the UV spectrum at 421 nm and in the XRD at $2\theta = 38.62, 44.26, 64.96, \text{ and } 82.42$ confirmed the formation of P-AgNPs. Further characterization by SEM and TEM analysis showed particles to be predominantly spherical with few aggregates, an average size distribution of 31.6 nm and a surface charge of -21.5 mV. Beside physicochemical advantages of P-AgNPs over Q-AgNPs and GA-AgNPs, these have shown improved antibacterial, antioxidant (84.85–89.20% inhibition of DPPH over 5 to 20 µg/mL) and promising cytotoxic properties (100% mortality) in

Table 4 In vitro cytotoxic activity of biosynthesized AgNPs in Brine shrimp lethality test

| | % Mortality | | |
|----------|-------------|------------|------------|
| | 1 µg/mL | 0.5µg/mL | 0.1µg/mL |
| Q-AgNPs | 88.5 ± 2.7 | 78.2 ± 3.9 | 56.7 ± 2.4 |
| P-AgNPs | 100 | 93.6 ± 4.5 | 77.3 ± 5.2 |
| GA-AgNPs | 89.2 ± 3.4 | 83.6 ± 3.1 | 67.6 ± 2.8 |

Brine shrimp lethality assay. The other advantages of using peel extract for the biosynthesis include cost effectiveness as pomegranate peels are agro-waste, energy efficient, and their ability to protect the environment leading to lesser waste and safer products. The biosynthesized AgNPs using pomegranate peel therefore seems to offer viable alternative to chemical synthesis. However, further studies are required to ascertain the benefits of P-AgNPs over traditional AgNPs.

Abbreviations

AgNPs: Silver nanoparticles; AgNO₃: Silver nitrate; GA: Gallic acid; Q: Quercetin; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; NaOH: Sodium hydroxide; MHA: Mueller-hinton agar; PPE: Pomegranate peel extract; XRD: X-ray diffraction; FE-SEM: Field emission scanning electron microscope; TEM: Transmission electron microscope; GA: Gallic acid; P-AgNPs: Pomegranate-coated silver nanoparticles; GA-AgNPs: Gallic acid-coated silver nanoparticles; Q-AgNPs: Quercetin-coated silver nanoparticles; EDX: Energy dispersive X-ray analysis

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Authors' contributions

PG: Planning, experiment performance, writing the manuscript. RC: Planning, experiment performance, writing the manuscript. VR: Planning, experiment performance, writing the manuscript. IA: Analysis of data, reviewing, and editing of MS. SK: Supervised the experiment, performed major corrections in the experiment, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

Authors declare no conflict of interest.

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