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14 **Synthesis and Antibacterial Characterization of Sustainable Nanosilver Using**
15 **Naturally-Derived Macromolecules**
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Abstract

Greener nanosynthesis utilizes fewer amounts of materials, water, and energy; while reducing or replacing the need for organic solvents. A novel approach is presented using naturally-derived flavonoids including Quercetin pentaphosphate (QPP), Quercetin sulfonic acid (QSA) and Apigenin Triphosphate (ATRP). These water soluble, phosphorylated flavonoids were utilized both as reducing agent and stabilizer. The synthesis was achieved at room temperature using water as a solvent and it requires no capping agents. The efficiency of the resulting silver nanoparticle synthesis was compared with naturally-occurring flavonoid such as Quercetin (QCR). Results show that QCR reduced Ag^+ faster followed by QPP, QSA and ATRP respectively. This is the first evidence of direct utilization of QCR for synthesis of silver nanoparticles (AgNPs) in water. The percentage conversion of Ag^+ to Ag^0 was determined to be 96% after 35 minutes. The synthesized nanoparticles were characterized using Transmission electron microscopy (TEM), Energy dispersive absorption spectroscopy (EDS), UV-vis spectroscopy, High resolution TEM (HR-TEM) with selected area electron diffraction (SAED). The particle sizes ranged from 2-80 nm with an average size of 22 nm and in the case of ATRP, the nanoparticle shapes varied from spherical to hexagonal with dispersed particle size ranging from 2-30 nm. Crystallinity was confirmed by XRD and the SAED of (111), (200), and the fringes observed in HRTEM images. Results were in agreement with the UV resonance peaks of 369-440 nm. The particles also exhibit excellent antibacterial activity against *S. epidermidis*, *E. coli* and *C. freundii* in water.

KEYWORDS: Flavonoids, green synthesis, nanoparticles, antibacterial, total environment

1. Introduction

The concept of sustainable nanotechnology involves the nanoscale control of synthesis and processing of matter without footprints that give rise to environmental degradation. Hence there is a search for synthetic methods that utilize fewer amounts of materials, water, and energy; while reducing or replacing the need for organic solvents. Notable developments include Safer-by-design concepts, biologically-inert SiO₂, microwave irradiation, and the use of biomass precursors [1, 2]. Safer by design concepts in Nano synthesis has been directed towards the use of plants that contain functional compounds that facilitate the reaction [3-6]. In this regard, a multitude of natural biomaterials have been employed in the synthesis of metal nanoparticles. Examples include blackberry, blueberry, pomegranate, and turmeric extracts, the basil plant, *Ocimum sanctum*, and the *Mangifera indica* leaf [4, 7, 8]. These studies have underscored the importance of environmentally benign method as a faster synthesis compared to the chemical reduction methods.

There is a significant interest in AgNPs due to their widespread applications in catalysis, biological tagging, optics, electronics and pharmaceuticals [3, 4, 7, 9, 10]. Silver is historically used as antibacterial with potential to address the problem of antibiotic resistance since it is highly toxic to micro-organisms [10]. Sondi and Salopek-Sondi, for instance, reported that silver nanoparticles (AgNPs) are potent microbial agents against *Escherichia coli* and of all antimicrobial agents, silver elements and nanoparticle compounds have been the most extensively tested [3,10].

A variety of physical, chemical, and biological techniques have been developed and used for the synthesis of AgNPs [8]. However, physical methods are costly and chemical synthesis involves

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4 toxic materials and the generation of hazardous by-products. Conventional techniques use
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6 sodium borohydride (NaBH_4) and hydrazone as reducing agents. NaBH_4 is a potential source of
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8 caustic salt and flammable gases [4, 11, 12]. In order to alleviate such challenges, many
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10 researchers have turned to green chemistry to synthesize metal nanoparticles. One eco-friendly,
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12 biological, nanosynthetic method for AgNPs that is gaining significant attention from many
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14 researchers uses bacteria, fungi, yeast, and other natural sources. Unfortunately, a major
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16 disadvantage of using microbes is the challenge and difficulty of maintaining an aseptic
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18 environment throughout the synthetic process; it is also labor intensive and is not cost-effective
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20 [7-10].
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27 Another environmentally benign process for the synthesis of AgNPs uses plant and fruit extract
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29 in place of toxic chemicals and high maintenance microbes. Nadagouda and Varma conducted a
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31 study in which silver and palladium nanoparticles were synthesized using tea and coffee extracts
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33 and reported that the caffeine and polyphenols of coffee and tea form complexes with metal ions
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35 in solution while reducing them to the corresponding nanoparticles [3]. The synthesis of
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37 nanoparticles is a metal-ion reduction process. Many, if not all, of these plant extracts contain
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39 phenolic compounds such as curcuminoids and flavonoids [4, 9]. These phenols; also widely
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41 known for exhibiting antioxidant activity, are believed to be responsible for the reduction of Ag
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43 and Au cations during nanoparticle synthesis [7, 10].
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50 Ghosh et al. reported the synthesis of AgNPs via the reduction of silver cations using *Dioscorea*
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52 *bulbifera* tuber extract [10]. *D. bulbifera* is rich in water soluble polyphenolic compounds,
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54 especially flavonoids and catechin. The results of these studies all suggested that the active
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56 species involved in biochemical synthesis of nanoparticles is phenolics, or flavonoids, however,
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58 to date no structural information was given to justify the claim [8-10]. One study, reported the
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4 use of quercetin to synthesize gold nanoparticles but no silver nanoparticles could be generated
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6 in aqueous solution [9].
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10 It is worth noting that despite the broad literature demonstrating the preparation of AgNPs using
11 phenolics [3, 4, 8, 7-10]; no report has given any structural evidence or mechanistic elucidation
12 of the exact flavonoid responsible for the reduction of Ag^+ to Ag^0 . In this paper we report for the
13 first time the green synthesis of AgNPs using water soluble, phosphorylated and sulfonated
14 Quercetin, including QPP, QSA, and ATRP. Prior work suggests that QCR could not reduce
15 Ag^+ to Ag^0 [9]. This could be attributed to its lack of solubility in water. The application of noble
16 nanoparticles for the purification of water has received much attention and in particular the
17 antibacterial activities of nanoparticles have been studied extensively in the recent years [13-16].
18 This work also shows, for the first time that indeed the active flavonoids, (QCR, QPP, QSA and
19 ATRP) are the reducing agents leading to the formation of metal NPs which exhibit excellent
20 antibacterial activity and cytotoxic effect. **2. Experimental Section**
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38 **2.1. Chemicals**

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41 All chemicals were of analytical or reagent grade and were used as received from the vendors
42 without further purification. Methylene chloride, carbon tetrachloride (CCl_4), dibenzyl phosphite,
43 DMF, N, N-diisopropylethylamine (DIPEA), acetonitrile, silver nitrate 99% and methanol
44 solvents for reaction were bought as anhydrous grade in sure seal bottles from Sigma (St. Louis,
45 MO). Anhydrous Quercetin was purchased from MP Biomedicals, LLC, Solon, Ohio. Apigenin
46 was purchased from Indofine Chemicals Inc. (Hillsborough, NJ). 4-dimethyl aminopyridine
47 (DMAP), palladium 5% on activated carbon and silver nitrate were purchased from Sigma-
48 Aldrich, Milwaukee, WI. Dimethyl sulfoxide-D6 was from Cambridge Isotope Laboratories, Inc.
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4 MA. Methanol, hexane, ethyl acetate, sodium chloride (NaCl), anhydrous sodium sulfate
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6 (Na₂SO₄) and potassium dihydrogen phosphate (KH₂PO₄) were purchased from Fisher Scientific,
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MA. Methanol, hexane, ethyl acetate, sodium chloride (NaCl), anhydrous sodium sulfate (Na₂SO₄) and potassium dihydrogen phosphate (KH₂PO₄) were purchased from Fisher Scientific, Pittsburgh, PA. All reagents were prepared using Nanopure water with a specific resistivity of 18 MΩ cm. All reactions involving air or moisture sensitive reagents or intermediates were performed under Ar or N₂ atmosphere.

2.2. Instruments

Purification of products was carried out using Teledyne flash chromatography system. Analytical TLC was performed using 0.25 mm EM Silica Gel 60 F250 plates visualized by UV irradiation (254 nm). Melting point measured on Digi Melt Stanford Research Systems melting point apparatus MPA160 (USA). UV/Vis absorption spectra were obtained from an HP 8453 UV-visible diode array spectroscopy. ¹H, ¹³C, ³¹P NMR spectra were obtained using 600 MHz Bruker Avance. TEM measurements were carried out in a JEOL TEM 2100F. XRD was performed using Philips X'pert MPD.

2.3. Synthesis of QPP, QSA and ATRP

The synthesis of QPP, ATRP followed the procedure described in literature [17] with slight modification in use of equivalents in chemicals for phosphorylation. However in the case of QPP and ATRP catalytic hydrogenation at ambient temperature under pressure of hydrogen was used for debenzylolation purposes [18]. The synthesis of QSA followed procedure in literature [19].

2.4. NMR – Characterization

All H¹ –NMR and C-¹³ NMR spectra were measured on a Bruker 600 MHz spectrometer while P-NMR spectra were recorded on AC300 at 121.5 MHz using DMSO-d₆ or CDCl₃ as solvent

with 85% HPO_3 as the external reference[18]. Five aromatic protons were identified to occur between $\delta \sim 6.19$ ppm – $\delta \sim 7.68$ ppm. 15 chemical shifts were identified on the C^{13} -NMR spectrum with the carbonyl-C being assigned the chemical shift of $\delta \sim 175.85$ ppm. Both ^1H and C^{13} -NMR data were consistent with the calculated theoretical chemical shifts values using ChemDraw. Table S1 is a summary of the major peaks from QCR, QPP and QSA for proton, carbon and phosphorus NMR. From these results, QPP and QSA have at least 84000 and 2700 fold enhancement in solubility over the unmodified QCR respectively.

2.5. Synthesis of Silver Nanoparticles

Four oven dried vial was cooled down and then purged with nitrogen. Four vials were each charged with 5 ml of Dimethyl Formamide (DMF), 5 mg AgNO_3 and then 2 mg; 3 mg, 4 mg and 5 mg QCR respectively were added and then capped immediately. To optimize the conditions the experiment was set up at room temperature as follows;

1. 5 mg AgNO_3 + 5 mL DMF + 2 mg QCR
2. 5 mg AgNO_3 + 5 mL DMF + 3 mg QCR
3. 5 mg AgNO_3 + 5 mL DMF + 4 mg QCR
4. 5 mg AgNO_3 + 5 mL DMF + 5 mg QCR

It was observed that the color change (reddish brown color) occurred immediately for QCR confirming that silver nanoparticles were formed. Quercetin is largely soluble in organic solvents and hence the use of DMF for comparison with water soluble derivatives. Quercetin is very soluble in DMF but poorly soluble in water. The Quercetin derivatives QPP, QSA and apigenin

derivative ATRP are soluble in water and hence 5.89×10^{-4} M silver nitrate solution was prepared using water as a solvent. Four vials were each charged with 5 ml of 5.89×10^{-4} M of AgNO_3 solution then 2 mg, 3 mg, 3 mg, 4 mg of QPP, QCR, QSA and ATRP respectively were added separately to each vial and then capped immediately the solution was left at room temperature. The color change occurred after 8.5 hours, 1, 2 and 5 days for QCR, QPP, QSA and ATRP respectively (Figure S1). The samples for TEM were prepared by adding drops of silver nanoparticle solutions onto carbon-coated copper grids to form film layer coat on the grid and then allowed to dry before carrying out measurements on a JEOL TEM 2100F instrument.

2.6 Antibacterial Studies

Antibacterial activity of AgNP was tested for gram positive and gram negative bacteria. Utilizing Mueller Hinton Agar 2, different concentrations of silver nanoparticles (20 $\mu\text{g/mL}$ to 0.2 $\mu\text{g/mL}$) were applied to *Staphylococcus epidermidis* ATCC® 12228™, *Escherichia coli* ATCC® 25922™ and *Citrobacter freundii* ATCC® 8090 as model microorganisms. The goal of using different concentrations of bacteria were to (i) evaluate whether the observed antimicrobial property of AgNPs depends on the concentration of bacteria inoculated onto the agar, and (ii) to check if the AgNPs show bacteriostatic and bactericidal effects depending on the nature of the AgNP and its microbial concentration.

2.7 Cell Cytotoxicity Studies

Cytotoxicity of AgNPs was tested at concentrations between 5-20 $\mu\text{g/mL}$ on IEC-6 cells in DMEM medium with 0.1 $\mu\text{g/mL}$ Bovine insulin, 10% FBS and 1% penicillin & streptomycin mixture. Cytotoxicity of Nanoparticles was tested via non-cancerous immortalized rabbit

intestinal cell line, IEC6 cells. Different concentrations of AgNPs were tested for 10^4 cells/mL for 24 h incubation at 37 °C under 5 % CO₂ concentration.

3. RESULTS AND DISCUSSION

3.1. UV- Vis characterization

Uv/Vis Spectroscopy plays an important role in confirmation of formation of nanoparticles in aqueous medium. The formation of AgNPs was depicted by formation of reddish brown color as shown in Figure 1a and Figure S1. The formation of reddish brown color is a spectroscopic characteristic sign that AgNPs have been formed. It has been established that the color of AgNPs is due to surface plasmon resonance caused by collective oscillation of free conduction electrons majorly induced by an interacting electromagnetic field [20]. The surface plasmon of AgNPs occurred at 520 nm for QCR reacted with AgNO₃ in DMF (Figure1a), 434 nm for QCR reacted with AgNO₃ in deionized nanopure water (Figure 1b) and 430 nm, 404 nm and 399 nm for QPP, QSA and ATRP reacted with AgNO₃ in water (Figure S2). It is worth noting the reduction of Ag⁺ by QCR was instant in DMF while it took 8.5 hours for the Ag⁺ to be reduced by QCR in water. The high solubility of Quercetin in DMF facilitated the faster formation of AgNPs.

Figure 1a. UV-Vis spectra of AgNPs derived from QCR. Inset is the characteristic reddish brown color depicting formation of AgNPs.

Figure 1b Uv –vis spectra of formation of AgNPs from reaction of 3 mg of QCR (9.926×10^{-6} M QCR) with 5.89×10^{-4} M AgNO_3 solution in deionized nanopure water from time $t= 0$ min to $t= 29$ hours.

Studies have illustrated that the experimentally measured surface plasmon absorption bands of silver solutions in water vary greatly in position ranging from 375 to 405 nm with the true position of silver in water being about 382 nm [20-23].

Figure 1c. UV-Vis spectra of temperature dependence on formation of AgNPs derived from QSA.

Figure 1c clearly depicts that silver nanoparticles were formed at room temperature with the highest absorbance at 80°C. Increase in temperature led to increase in absorbance of AgNPs (Figure 1c). This clearly demonstrates that although the formation of AgNPs from these flavonoid derivatives is favored by increase in temperature, AgNPs could also be formed at room temperature (25°C) and hence the synthesis of the nanoparticles could be carried out at room temperature thereby reducing the cost of production. Flavonoids are not only responsible for the reduction of Ag^+ to Ag^0 but they also enhance efficient stabilization of nanoparticles [24]. QCR reduced Ag^+ instantly forming AgNPs and the characteristic brown color of AgNPs is observed. This is the first evidence of direct use of QCR for Ag nanoparticles formation from AgNO_3 solution in water. Earlier reports show that AgNPs cannot be formed from aqueous solution [9].

After about 7 days, black precipitation occurred on the glass walls of the vial forming a lustrous silver coating (Adsorption of metallic silver on the walls).

Figure 1d. Uv-vis absorption spectra of AgNPs after reaction at room temperature (50°C) of 5.89×10^{-4} M AgNO_3 solution with 4.5×10^{-3} M QPP recorded at different times.

Figure 1e. Reaction progress depicting percentage conversion of AgNPs with time.

Figure 1d illustrates the formation of AgNPs with time as the reaction of QPP with AgNO_3 progressed at 50°C. After 5 minutes the silver peak occurred at 450 nm and the reaction reached completion after 40 minutes. The percentage conversion of Ag^+ to Ag^0 (Figure 1e) was

calculated based on results from Figure 1d. It is worth noting that QPP reduces Ag^+ to Ag^0 and 90% efficiency conversion was realized after 30 minutes while 96% conversion was realized after 35 minutes.

3.2. TEM Characterization

The TEM images of AgNPs obtained from ATRP, QCR, QSA and QPP are shown in Figure 2 (a) to (d) respectively.

Figure 2. TEM images of AgNPs obtained using (a) QSA (b) QCR (c) ATRP and (d) QPP

From the results, it is clearly depicted that the AgNPs obtained from QCR, QPP, QSA and ATRP were spherical in shape and the particles sizes range from 2-80 nm and 5-80 nm for QCR and QSA respectively. The nanoparticle shapes varied from spherical to hexagonal for the case of with dispersed particle size ranging from 2-30 nm. The average size of AgNPs derived from ATRP was determined to be 10.25 nm (Figure S3). For QPP agglomerated particles of size range 5-50 nm of spherical shape with an average size of 23 nm (Figure S4) were formed. The high resolution TEM images (Figure-3 (a)-(d)) showed good crystalline structure of the nanoparticles. Particles were internally twinned as shown in the HRTEM images. Single crystalline particles were formed oriented in same way. Crystallinity was confirmed by the SAED spectra and the fringes observed in HRTEM images depicting that Quercetin was a good reducing agent for Ag^+ in water.

Figure 3. High resolution TEM images of AgNPs obtained using (a) QSA (b) QCR (c) ATRP and (d) QPP.

Twinned nanoparticles with 5 fold symmetry can be seen from HRTEM (Figure-3). HRTEM images show that the nanoparticles were largely spherical in shape and also reveal internally twinned particles. The presence of well-defined lattice fringes in HRTEM demonstrates the crystalline nature of the AgNPs as depicted in Figure 3.

EDS spectra were employed to confirm formation of AgNPs as depicted in Figure 4. The EDS spectra clearly indicate the formation of Ag and this is demonstrated by the Ag peaks observed in the spectra. However, since AgNPs were drop coated on carbon coated copper grid, the peaks for Cu and C are basically from the grid used.

Figure 4. Energy dispersive absorption spectroscopy of AgNPs obtained from (a) QPP (b) QCR (c) ATRP.

3.3. X-ray Diffraction Characterization

XRD studies normally reveal the crystalline nature of sample by yielding a pattern showing numbers of Bragg's reflections which are usually indexed based on face centered cubic structure of the particle of interest. Figure 5 shows the XRD pattern of Ag nanoparticles derived from QPP. In this pattern 2 distinguished peaks were observed at $2\theta = 38.28^\circ$, 44.43° which can be indexed to correspond to (111) and (200) Braggs reflections of face centered cubic Ag nanoparticles. The diffraction peaks are in agreement with the standard value as obtained from Joint Committee on Powder Diffraction Standards (JCPDS) card No. 04-0783 [8, 25,26].

Figure 5 (a) XRD pattern of AgNPs derived from QPP

From the XRD results it is clearly evidenced that the silver nanoparticles derived from naturally derived flavonoids are crystalline in nature. Although XRD data did indicate the presence of crystal structure for some of the AgNPs, the presence of amorphous particle may still be

possible. The flavonoid derivatives are able to reduce and stabilize the AgNPs formed. The peaks at $2\theta = 38.28^\circ, 44.43^\circ$ generally correspond to silver peaks.

The size of the Ag nano particles can be estimated by using Debye-Scherrer formula [4, 7, 10, 8, 11, 12]; $d = K\lambda/\beta \cos\theta$, Where; d = the crystalline particle size of silver nanoparticles; K = the Scherrer constant (values always range from 0.9-1 usually taken for a cubic system); λ = the wavelength of X-ray radiation ($1.5406\text{\AA}$) which is used in XRD; β = is the full width at half the maximum intensity of the diffraction peak of (111) Bragg reflection generally in radians and θ = the Bragg angle.

The formula was used to calculate the average particle size which was determined to be about 22 nm which is in agreement to AgNPs synthesized from past green synthesis studies from plants [5, 6]. It is worth noting that the XRD patterns and the crystalline nanoparticle size of about 22 nm confirmed that our nanoparticles synthesized from naturally derived flavonoids were crystalline in nature. The results are consistent with the sizes of AgNPs obtained from TEM results (Figure S4).

Figure 5b. Selected Area Electron Diffraction (SAED) crystallinity of AgNPs derived from QPP

The SAED pattern confirmed the crystalline nature of the nanoparticles. This is clearly evidenced by the bright circular spots (Figure 5b) which are assigned to correspond to (111) and (200) Bragg's reflection planes. Studies based on SAED pattern revealed similar trends [8] of the pattern and hence we were able to synthesize the Ag nanocrystals obtained from naturally-derived flavonoids.

3.4. Proposed reduction mechanism

Quercetin is a naturally occurring flavonoid with strong antioxidant properties and is able to chelate with metal ions. Studies have revealed that naturally derived flavonoids are able to reduce chromium (VI) to chromium (III) [18, 27] and are able to form complexes with metals such as copper [28]. Flavonoids can also directly scavenge molecular species of active oxygen. The antioxidant action of flavonoids resides mainly in their ability to donate electrons or hydrogen atoms.

Scheme 1. Proposed mechanism of reduction of Ag^+ to Ag^0 using quercetin

The formation of enol form of the quercetin freely liberates reactive hydrogen which is responsible for the conversion of Ag^+ to Ag^0 (Scheme 1). Recently a study proposed that Luteolin, a flavonoid reduces silver ions via same mechanism [7].

3.5 Antibacterial Activity

The antibacterial activity of AgNPs synthesized from naturally-derived macromolecules was conducted on *Escherichia coli*, *Staphylococcus epidermidis* and *Citrobacter freundii*. Although silver metal is a well-known antimicrobial agent, the mechanism of its bactericidal effect has been a subject of study. Some literature sources suggests that the antibacterial activity may be probably due to electrostatic attraction between negatively charged cell membrane of microorganism and positively charged nanoparticles.^{29,30} Silver nanoparticles possess a good chemical stability, increased surface area and small size which enhances faster interaction with microorganisms.^{31,32} Furthermore, it has been reported that the particles may interact with the outer membrane and may cause degradation, structural changes leading to death³³. However it is now clear that the toxicity of AgNPs may be explained via three possible mechanisms. The first mechanism may involve the release of silver ions which can interact with enzymes via thiol groups. This mechanism was established as the major pathway relevant in antibacterial activity involving Ag^+ ions.^{34,35} In this case, the major role of electrostatic attraction is critical. The second mechanism may involve the generation of reactive oxygen species which is fundamental in determining nanosilver toxicity.^{36,37} The third and final mechanism could be related to the penetration of nanoparticle into cell membrane leading to degradation and structural change.^{34,37}

The study had two controls labelled (a) and (d). In this case a and d had different bacterial cultures. Control (a) was inoculated with one drop of 10^2 cfu *E. coli* in 20 μL while control (d)

was inoculated with two drops of 10^4 cfu *E. coli* in 20 μ L. When the control (d) was compared with (e) and (h), it can be observed that 200 ng/mL AgNPs possessed minor inhibitory effect while 800 ng/mL (h) and 2 μ g/mL AgNPs made the colonies discernible and provided strong inhibitory effect (Figure 6a).

Figure 6a. Silver toxicity on *E.coli* ATCC® 25922™, (a) Control, (b) 200 ng/mL, (c) 800 ng/mL, (d) Control (e) 200 g/mL, and (f and h) 2 μ g/mL, (g and i) 3 μ g/mL. The control plate (a) and AgNPs treated plates (b) and (c) were incubated with one drop of 10^2 and 10^3 cfu *E.coli* in 20 μ L. The control (d) and the AgNPs treated plates (e), (f), (g), (h) and (i) were inoculated with two drops of 10^4 and 10^5 cfu *E.coli* in 20 μ L.

In the case of 24 h incubation, *E.coli* colonies were not visible in plates. The plates (j) and (k) were treated with 8 and 20 μ g/mL AgNPs and inoculated with four drops of 10^7 cfu *E.coli* in 20 μ L. Despite these concentrations, no visible colony formation was seen at 72 h incubation.

Figure 6b. AgNPs toxicity on *C. freundii*. (a) Control, (b) 200 ng/mL, (c) 2 µg/mL, (d) 3 µg/mL, (e) 5 µg/mL, and (f) 8 µg/mL AgNPs treated plates.

In all cases, *C. freundii* was introduced into the plates as two drops of 10^4 and 10^5 cfu in 40 µL carriers. As shown in Figure 6b, the 200 ng/mL AgNPs treatment did not provide any significant toxicity on *C. freundii* growth while AgNPs started showing toxicity at 2 µg/mL concentration. However, 2 and 3 µg/mL AgNPs treatments did not inhibit the growth of *C. freundii* completely. Rather they inhibited the growth at over 99% in the case of 10^4 cfu inoculations while the inhibitory effect was less than 50 % in the case of 10^5 cfu inoculations (Figure 6b). In the case of 5 µg/mL treatment (e), any colony formation was not seen at 72 h incubation. With 8 µg/mL AgNPs treatment (f) did not allow any bacterial colony formation at 72 h incubation in the case of 10^6 cfu inoculation in 100 µL.

Figure 6c. Toxicity of AgNPs on *S. epidermidis*. (a) Control, (b) 800 ng/mL (c and e), 2 µg/mL (d and f), 3 µg/mL and (g) 5 µg/mL AgNPs treated plates.

In all cases, *S. epidermidis* was introduced into the plates as two drops of 10^4 and 10^5 cfu in 20 µL carrier. 800 ng/mL AgNPs treatment did not possess strong significant toxicity on *S. epidermidis* growth at 24 h incubation. In the case of 2 (c) and 3 (d) µg/mL AgNPs treatment, no colony formation was observed at 24 h while 72 h incubation showed abundant colony formation especially for 10^5 cfu inoculation (Figure 6c). However, 3 µg/mL AgNPs (f) treatment inhibited *S. epidermidis* growth at over 99.9% whereas at 72 h incubation with 5 µg/mL AuNPs treatment did not inhibit *S. epidermidis* growth at all.

AgNPs were tested for their potential bacteriostatic and bactericidal effects. This was the main reason of using different inoculum concentrations since selecting only one bacteria concentration did not give any clear idea about whether the AgNPs possess either bactericidal or bacteriostatic properties or both. The AgNPs was found to possess bacteriostatic and bactericidal effects. However, these effects were directly dependent on both NPs concentration and inoculum concentration. The age of the bacteria used in the toxicity study was kept in the log phase.

According to the results shown in Figures 6b and 6c, 5 $\mu\text{g/mL}$ AgNPs exhibited significant growth inhibition at 10^5 cfu inoculum. Even though the concentrations below 5 $\mu\text{g/mL}$ AgNPs showed some bactericidal effects, their effect on the bacteria were more bacteriostatic in nature. Based on these results, 5 $\mu\text{g/mL}$ AgNPs can be accepted as a strong bactericidal agent to eliminate the tested bacteria at up to 10^5 cfu inoculum. In the case of 8 $\mu\text{g/mL}$, the concentration can be increased to 10^6 and 10^7 cfu inoculum. Altering the addition of AgNPs to the agar did not provide any significant difference.

The antibacterial activity of the synthesized AgNPs was compared with AgNO_3 salt by dissolving the AgNO_3 solution in Mueller Hinton Agar at different concentrations. Results showed that AgNO_3 salt solution at 1 $\mu\text{g/mL}$ concentration was enough to wipe-out each of 100 μL of 10^7 cfu/mL *E.coli*, *C. freundii* and *S. epidermidis* microbial inoculums [Figure S5]. This is expected because silver ions have better natural interaction with microbes. As discussed in the Supplementary Section, adding AgNPs to the agar before autoclaving slightly increased the antibacterial activity of the nanoparticles. The particles were also tested for cytotoxic activities as depicted in the supplementary section (Figure S6 and S7).

4.0 Conclusion

We have successfully synthesized greener AgNPs for the first time using flavonoid derivatives QPP, QSA and ATRP using water as the solvent and without using any capping agents. UV-vis spectrometry was used to confirm the synthesis of AgNPs based on the presence of plasma resonance peak while TEM and HRTEM were employed to determine the shape and size of silver nanoparticles. The particle size ranged from 2- 80 nm depending on the flavonoid used. XRD results confirmed that the Ag nanoparticles obtained from naturally- derived flavonoids

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4 were crystalline in nature with average nanoparticle size of 22 nm. The greener synthetic
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6 approach is also an indication that it could be used at industrial scale as a synthetic protocol. The
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8 synthesized AgNPs exhibited excellent antibacterial activity against *E.coli*, *S. epidermidis* and *C.*
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26 27 **Appendix A: Supporting data.**

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30 Additional information showing major NMR chemical shifts and solubility of QCR, QSA and
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32 QPP derivatives; color changes depicting the formation of silver nanoparticles; UV/Vis spectra
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34 of silver nanoparticles peaks derived from QPP, QSA and ATRP and histogram showing the
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36 average particle size. The cytotoxicity studies results are also available. These materials are
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38 available free of charge via Internet at <http://dx.doi.org/.....>
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