Daphne mucronata-mediated phytosynthesis of silver nanoparticles and their novel biological applications, compatibility and toxicity studies

Asma Shah, Ghosia Lutfulla, Kafeel Ahmad, Ali Talha Khalil, and Malik Maaz

ABSTRACT

This contribution reports the biosynthesis of silver nanoparticles (AgNPs) using aqueous leaf extracts of D. mucronata and their diverse applications. Synthesized AgNPs were characterized using diverse techniques, i.e. UV, XRD, EDS, SEM, TEM, FTIR, and TGA/DTA. These techniques confirmed the authenticity of the synthesized nanoparticles. The bimodulated AgNPs revealed the highest radical scavenging potential, i.e. 86.4% relative to plant extract at 600 μg/ml. Escherichia coli was found to be the most susceptible strain to AgNPs. Growth of vancomycin-resistant Staphylococcus aureus was also inhibited. Hemolytic activity revealed negligible hemolysis, indicating the biocompatible nature of biomodulated AgNPs. Furthermore, no mutagenic properties were shown by the biogenic AgNPs. Synthesized nanoparticles possessed promising insecticidal potential and had no phytotoxic activity. No haemagglutination was observed for biogenic AgNPs.

CONTACT

Kafeel Ahmad kafeelpbg@gmail.com

ARTICLE HISTORY
Received 18 January 2018
Accepted 16 July 2018

KEYWORDS
Daphne mucronata; silver nanoparticles; antimicrobial; phytotoxic; antioxidant

GREEN CHEMISTRY LETTERS AND REVIEWS
2018, VOL. 11, NO. 3, 318–333
https://doi.org/10.1080/17518253.2018.1502365

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

For many years, natural sources or components derived from these sources have been used as traditional remedies for the treatment of diseases and various ailments. More than 200,000 natural products have been documented in living organisms (1). Among them, the plant-derived chemical entities have been mostly studied (2). The interface of Nanoscience and medicinal plants has been recently explored for fabricating Nanoscale materials (3-6). Medicinal plants have rich phytochemistry, providing an exciting opportunity to fabricate nanomaterial of interest with novel shapes and properties. Plant extracts are valuable for the biosynthesis of metal and metal oxide nanoparticles (7,8). The potential application of AgNPs involves nanomedicine, drug delivery, cosmetics, and electronic and environmental protection applications (9,10). Silver nanoparticles are also famous for their antimicrobial activity and are commonly utilized in treatments of bacterial, viral and fungal infections throughout the world (11). The physical and chemical means of nanoparticles synthesis suffer from certain disadvantages (12,13). Chemical means involve the use of toxic chemicals, while physical means have energy requirements (14). In contrast, plant-based methods are cost-effective, rapid and benign.

*Daphne mucronata* is a wild hedge plant of family thymelaeaceae and its therapeutic properties are well established (15). Coumarins and diterpenes have been reported as main constituents of the plant. (16). In traditional Chinese medicine, the leaves and roots of *D. mucronata* are administered as purgative, abortifacient and anti-inflammatory drugs, and as a treatment for toothache and ulcers. The plant woody parts are used for the treatment of bone diseases (17). The plant has been used for the treatment of cancer and skin diseases (18). *D. mucronata* is also used for the treatment of skeletal-muscular problems and rheumatism (19,20). Limen of the plant is used for treating wound infections and smoke of the plant branches is used for muscle fatigue. Cooked leaves and decoction of the plant parts are used for curing menopause disorders, infertility, constipation and gynaecological problems (21). The woody part of the plant is finely ground to make a powder that is used to relieve eye pain (22).

Many plants (e.g. *Jatropha curcas*, *Acalypha indica*, *Emblica officinalis*, *Parthenium hysterophorus*, *Citrus limon* and *Morinda citrifolia*) have been explored for the phytofabrication of AgNPs (23-27). The fungus *Trichoderma viride*-mediated AgNPs were active against Gram-negative and Gram-positive bacteria when used with antibiotics in combination (28). In the current study, a complete green approach was adopted for the phytofabrication of AgNPs using the aqueous leaf extracts of *Daphne mucronata*. Complementing to the limited literature on the pharmacognostic potential of *Daphne mucronata*, this study was designed to investigate the pharmacological potential of the *Daphne mucronata* aqueous extract and derived silver nanoparticles.

Methodology

Synthesis of silver nanoparticles

Plant material was collected from Marghuzar, District Swat, Khyber Pakhtunkhwa, Pakistan and identified at Department of Botany, University of Peshawar, Pakistan. Aqueous extract was obtained by boiling 100 g of powdered *D. mucronata* leaves in 2000 ml of distilled water for 30 min. The filtrate was centrifuged two times at 10,000 rpm for 20 min at 4°C to eliminate cell debris. The supernatant was filtered using 0.2 μm filter. This filtrate was used for the biosynthesis of AgNPs (29). A complete green process was used to biosynthesize AgNPs using aqueous leaf extract and using silver nitrate (AgNO₃) as a precursor.

Reaction: For 1 mM AgNO₃ solution, 17 mg AgNO₃ was dissolved in 100 ml of distilled water. This AgNO₃ solution was mixed with the plant extract in 1:5 ratio. The appearance of brown color showed the reduction of Ag ions to AgNPs by flavonoids- and triterpenoids-rich leaf extract. The unbound phytochemicals were removed by centrifuging the mixtures at 4°C for 30 min at 10,000 rpm. The pellet was dissolved in distilled water. The reaction mixture was subjected to sonication for 2–3 min and then dried using a lyophilizer.

Characterization of silver nanoparticles

UV-Vis spectroscopy

A dual-beam spectrophotometer (UV-1100 Shimadzu) was used for the analysis of colloidal Ag solution to confirm the presence of AgNPs. The aqueous plant extract of *D. mucronata* leaves and derived AgNPs solution were scanned by diluting the reaction mixture 10 times with distilled water. The wavelength range used for scanning aqueous extracts of plant and AgNPs was 200–600 nm. The points of maximum absorbance regions show characteristic peaks that give information on charge transfer, purity of sample and analyte (30).

Fourier transform infra-red spectroscopy

The Fourier transform infra-red spectroscopy (FTIR) spectrophotometer equipment IRPrestige-21 (Shimadzu, Japan) was used to analyze aqueous extract and
derived AgNPs of plant material to study functional groups taking part in NP synthesis. The spectral range used for scanning samples was 4000 to 400 cm⁻¹.

**X-ray diffraction**

The crystalline natures of *D. mucronata* leaves derived AgNPs were determined by X-ray diffraction using X-ray diffractometer (JDX 3532, JEOL, Japan). The diffraction pattern was recorded by placing the sample in the sample holder. The instrument was operated at 20 to 40 kV at a wavelength of 1.5418 Å in θ to 2θ configurations, which gave the diffracted intensities in 2θ angle range of 10° to 90°. Crystal size of NPs was measured by using Debye Scherer equation as follows:

\[ D = \frac{0.94 \lambda}{\beta \cos \theta}, \]

where \( \beta \) is the full width at half maximum (FWHM), \( \lambda \) is the wavelength of X-ray used, \( D \) is the measure of the size of crystal that is perpendicular to the reflection planes and \( \theta \) is the angle of diffraction.

**Energy-dispersive X-ray spectroscopy**

Elemental analysis of *D. mucronata* leaves derived AgNPs was performed by energy-dispersive X-ray Spectroscopy (EDS) using a scanning electron microscope (JSM-5910, JEOL) equipped with EDX Detector INCA Energy 200 (Oxford Instruments Analytical Ltd, UK).

**Scanning electron microscopy**

Analysis of shape, surface topology and morphology of AgNPs was performed through scanning electron microscopy (SEM) using a scanning electron microscope Zeiss (HR-SEM).

**Transmission electron microscopy**

Copper grid (carbon coated) was used for thin film preparation of the sample. The sample was kept for 5 min under a mercury lamp for drying. The Tecnai G2 (ThermoFisher Scientific) Transmission Electron Microscope was used for Transmission electron microscopy (TEM) analysis.

**Thermo gravimetric/differential thermal analysis**

Thermo gravimetric/Differential Thermal Analysis (TG-DTA) (Perkin Elmer Diamond Series, USA) was used to analyze *D. mucronata* leaves powder and derived AgNPs to study their thermal properties, stability, decomposition rates and physical processes like vaporization and sublimation.

**In vitro pharmacognostic properties**

The aqueous extracts of the *D. mucronata* leaves and derived AgNPs were investigated for pharmacognostic potential.

**Antioxidant activity**

The DPPH free radical scavenging potential was determined in the concentration range of 100, 200, 300, 400, 500 and 600 μg/ml. About 1 ml of test solution was added to 2 ml of reagent solution that was incubated in dark for 10 min. Absorbance was measured at 517 nm by UV–Vis spectrophotometer (Shimadzu UV-1601) (31). The curve of percent scavenging effect was plotted against sample concentrations that were used to determine 50% Effective Concentration (EC₅₀) (32). Ascorbic acid was used as positive control. For the determination of free radical scavenging potential, the following formula was used:

\[ \text{Percent inhibition of DPPH activity} = \frac{(A - B)}{A} \times 100. \]

In this formula, “B” represents test sample absorbance and “A” represents absorbance of the blank sample (31,33).

**Antibacterial activity**

**Determination of percent inhibition.** Agar well diffusion method was used for antibacterial activity (34,35). The strains used for antimicrobial studies included *Morganella morganii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, vancomycin-resistant *Staphylococcus aureus* (VRSA) and *Proteus vulgaris*. A 20 mg/ml stock solution of aqueous extracts and synthesized AgNPs was prepared in dimethylsulfoxide (DMSO). Turbidity of bacterial inoculum was adjusted according to 0.5% McFarland standard. Culture was inoculated on nutrient agar medium. Ciprofloxacin was used as a standard drug for *A. baumannii*, while Amoxicillin was used as reference drug for *E. coli*, *P. aeruginosa*, *S. aureus* and *P. vulgaris*. Ampicillin was used as a standard antibiotic for VRSA, Rifampicin was used as a standard for *M. morganii* and DMSO (1%) was used as a negative control. Wells were made into agar medium and 50 μl of the test sample was delivered into them. All plates were examined for any zones of growth inhibition after 24 h incubation at 37°C and zone diameters were measured in millimeters.

**Minimum inhibitory concentrations.** Minimum inhibitory concentrations (MIC) of the samples were determined against test bacteria according to the reported procedure with slight amendments (36). Stock solutions
Growth in sample (16 mg/ml) were prepared in DMSO. Different dilutions (0.04–14.0 mg/ml) of each extract were made in DMSO. Bacterial inoculum was added into each tube containing nutrient broth, which was then incubated for 24 h at 37° C. Results were estimated based on visible growth in test tubes in comparison to the negative control.

**Minimum bactericidal concentrations.** Minimum bactericidal concentrations (MBC) of each fraction and synthesized AgNPs were determined by a modified method of Spencer (37). In MIC assay, the plates with no visible growth were selected. Samples taken from them were sub-cultured on freshly prepared Sabouraud Dextrose Agar (SDA) plates and nutrient agar plates and incubated at 37°C for 48 h. The concentration of extract that did not show any growth was taken as MBC.

**Antifungal activity**

Antifungal activity of test samples was determined using linear mycelial growth inhibition (38) against *Candida albicans, Fusarium oxysporum, Aspergillus flavus, Aspergillus parasiticus, Penicillium digitatum,* and *Aspergillus niger.* SDA media was prepared and autoclaved. It was followed by incubation in sterile petri plates at 28°C for 24 h. A 67 µl of the test sample was added to SDA medium (heated, 50°C) to make slants. Fungal culture (5–7 days old) was incubated in test tubes for 7 days at 28 ± 1°C. Positive and negative controls were Miconazole and DMSO. Results were measured from linear growth on slanted test tubes (39).

\[
\text{Percent inhibition} = 100 \left(1 - \frac{\text{Growth in sample}}{\text{Growth in negative control}}\right) \times 100.
\]

**Haemagglutination activity**

Haemagglutination activity of test samples was performed at different dilutions (1:2, 1:4, 1:8 and 1:16), which were prepared in phosphate buffer (pH 7.4) from the stock solution (20 mg/ml of DMSO) against ABO blood groups of human erythrocytes. Stock solution was prepared by dissolving 20 mg of the test sample in 1 ml DMSO. Blood was taken from healthy volunteers and 2% RBC’s suspension was made in phosphate buffer through centrifugation. Sample (1 ml) from each dilution and RBC’s suspension were added and tubes were incubated at 37°C for 30 min followed by centrifugation. Positive and negative results were demonstrated by deposition of rough granules and smooth button formation, respectively (40).

**Cytotoxic activity**

Cytotoxic activity was tested as reported (41). *Artemia salina* was used to investigate the cytotoxic potential of plant extracts and derived silver nanoparticles. The eggs were developed in seawater using commercial salt mixture and double distilled water in a thin rectangular plastic bowl (22 × 32 cm). The plastic dish was asymmetrically partitioned by a perforated device. About 50 mg of eggs were spread in big partition. The small partition was kept open for light and incubated for 2 days at 37°C. After 2 days, nauplii were collected from the light exposed partition. The aqueous extract and derived NPs were tested against *A. salina.* For the preparation of stock solution, 5 mg test sample was dissolved in 5 ml of dimethylformamide. Different concentrations (10, 100 and 1000 µg/ml µl) of stock solution were transferred to vials. The solvent was evaporated from each vial and 38 g/l seawater solution was added. Then, 5 ml of seawater containing 10 shrimps was transferred to each vial. The vials were incubated at room temperature for 24 h. Number of surviving shrimps was calculated the next day. Dimethylformamide (2 ml) served as negative control and Etoposide served as positive control. Median lethal concentration (LD₅₀) of test samples was by probit analysis using Microsoft Excel. The test samples with LD₅₀ ≤ 1.0 mg/ml are known to exhibit toxic effects.

**Thrombolytic assay**

Reported procedure was followed for the determination of clot lysis (42). Apparently, healthy individuals having no previous history of anticoagulant therapy or oral contraceptive were selected for collecting whole blood (6 ml). For clot formation, 1 ml blood was dispensed into pre-weighed micro centrifuge tubes and the tubes incubated for 45 min at 37°C. After clot formation, serum was gently aspirated without unsettling the clot and the tubes were weighed again to calculate the weight of clot (Wtclot = W tf – W ti). The sample stock solutions of aqueous extract of leaves of *D. mucronata* and derived AgNPs were made as 1 mg/ml in water. From stock solutions, 20, 40, 60, 80 and 100 µl was added to each tube containing pre-weighed clot. Sterile distilled water (100 µl) was used as negative, and streptokinase (100 µl) was used as positive control. The tubes were incubated for 90 min at 37°C and clot lysis was observed. After clot disruption, the fluid obtained was removed carefully. Change in weight of tube was recorded by reweighing the tube. Percent clot lysis was calculated from the weight difference in the clot before and after lysis. Significance of test samples percent clot lysis in comparison to control was
determined by ANOVA following Tukey post-test. Data were expressed as mean ± standard deviation. The following formula was used to calculate percent clot lysis.

\[
\% \text{ Clot lysis} = \frac{\text{Weight of released clot}}{\text{Clot weight}} \times 100.
\]

**Hemolytic activity**

Spectrophotometric method reported for hemolytic activity was followed (43). Blood sample (5 ml) was taken from normal healthy individuals and centrifuged for 3 min at 1500 rpm. Blood pellet was washed in saline solution and sterile phosphate buffer (pH 7.2) three times. Normal saline solution (0.5%) was used for re-suspension of the pallet. 0.5 ml of aqueous extract and derived AgNPs at different concentrations (10, 50, 100, 200 and 250 µg/ml in saline) were added to blood cell suspension separately. The samples were incubated at 37°C for 30 min and then centrifuged for 10 min at 1500 rpm. At 540 nm, absorbance was measured to determine hemolytic activity. Triton X-100 was used as positive control and phosphate buffer (saline) as negative control. Percent hemolysis was calculated as

\[
\% \text{ Hemolysis} = \frac{\text{ABS} - \text{ABNC}}{\text{ABPC} - \text{ABNC}}.
\]

**Mutagenicity test**

Mutagenicity was determined by Muta-Chromplate kit (purchased from Environmental Biodetection Products Incorporation EBPI, Ontario, Canada). Fluctuation test was entirely performed in liquid culture and test kit was based on validated Ames bacterial reverse-mutation test. Chemicals used included 2 mg/ml bromocresol purple, Davis–Mingioli salt (concentrated 5.5 times), 0.1 mg/ml L-histidine, 40% D-glucose (w/v) and 0.1 mg/ml D-biotin. 2-nitrofluorine (30 µg/100 µl) and sodium azide (0.5 µg/100 µl) were used as sterile standard mutagens. These chemicals were purchased from EBPI.

Reagent mixture contained 4.75 ml D-glucose, 21.62 ml Davis–Mingioli salt, D-biotin (1.19 ml), bromocresol purple (2.38 ml), and L-histidine (0.06 ml) aseptically mixed in a sterile bottle. Distilled water, sample (plant extract and synthesized AgNPs), standard mutagen and reagent mixture were mixed in bottles by the quantity given in Table 1. *S. typhimurium* TA98 and *S. typhimurium* TA100 were the mutant strains provided by EBPI. Inoculation of bacteria in nutrient broth was followed by incubation for 18–24 h at 37°C. Culture broth (5 µl) was thoroughly mixed in bottles. The bottle contents were shifted to multichannel reagent boat and 200 µl aliquots of the mixture were poured into each well of the titration plate. Plate was prevented from evaporation by placing in an airtight polyethylene bag and incubated for 4 days at 37°C. First, the blank plate was observed, and the rest of the plates was read only when plates in blank were entirely purple in color. This indicated that the assay was not contaminated (44).

**Interpretation of results:** Test plates, standard and background were visually scored. Wells with yellow, partial yellow or turbid color were marked positive and wells with purple color were marked as negative. The number of positive wells was noted. Spontaneous mutation or background was shown by the background plate. The test sample was taken as toxic to test strain if all wells in plate showed purple color (Table 1). The test samples were considered mutagenic if a number of positive wells were more than twice the number of positive wells in background plate to account for the spontaneous mutation (45).

**Phytotoxic activity**

The phytotoxic bioassay of test samples was performed against *Lemna minor* as described previously (46). From stock solution (1 mg/ml) of aqueous plant extract and derived AgNPs, different amounts (10, 100 and 1000 µl) were separately transferred to sterilized Petri plates containing E-medium to give a final concentration of 10, 100 and 1000 µg/ml, respectively. Then, 10 *Lemna minor* plants were added to the plates and the plates incubated at 28°C in a growth chamber for 7 days. Results were taken on the seventh day by counting the number of damaged plants. Methanol was used as negative control and Paraquat [(C₆H₇N)₂]Cl₂ at 0.015 µg/ml was used as the standard herbicidal agent. The following equation was used to calculate percent regulation:

\[
\text{Percent regulation} = 100 - \left( \frac{\text{Fronds number in test sample}}{\text{Fronds number in negative control}} \times 100 \right).
\]

### Table 1. Set up of the fluctuation assay.

| Treatment                  | Mutagen standard | Sample | Reagent mixture | Deionized water | Salmonella test strain |
|----------------------------|------------------|--------|-----------------|----------------|-----------------------|
| Blank                      | –                | –      | 2.5             | 17.5           | –                     |
| Background                 | –                | –      | 2.5             | 17.5           | 0.005                 |
| Standard mutagen           | 0.1              | 0.005  | 2.5             | 17.4           | 0.005                 |
| Test sample                | –                | 0.005  | 2.5             | 17.5           | 0.005                 |
Criteria for phytotoxic activity were low inhibition (0–40%), moderate inhibition (40–60%), good inhibition (60–70%) and significant inhibition activity (above 70%)

**Insecticidal and anti-termite activity**
The insecticidal potential was determined against *Rhyzopertha dominica*, *Tribolium castaneum* and *Clinocottus analis* under proper aeration and feed (47-49). Anti-termite activity was performed against *Heterotermes indicola* as reported (50). A filter paper (Whatman No.1) was placed in petri plate according to the size of the petri plate. Then, 20 mg/ml of the test sample in methanol was poured into the petri plate. Methanol was allowed to evaporate. It was followed by spraying the plate with water to meet humidity requirements. Ten insects were transferred to separate plates for each sample using a clean brush. The rate of mortality in each plate was determined after 1, 2 and 3 days. For insecticidal activity, the positive control used was Permethrin (235.9 μg/cm²), while methanol was used as negative control. Termisolve B-PRO served as positive control in case of anti-termite activity. Abbott’s formula was used to calculate percent mortality.

\[
\text{% Mortality} = \frac{\text{Total number of dead insects/termites after treatment}}{\text{Total number of insects/termites before treatment}} \times 100.
\]

**Results**

**Biosynthesis and physical characterization**
Diverse techniques were used for monitoring the biosynthesis and characterization of synthesized nanoparticles. The progress of AgNPs formation was visualized by a color change after 30 min of heating at 40°C. Appearance of brown color indicated the synthesis of NPs. Surface Plasmon resonance at 425 nm and the color change were considered as the first indication of the successful biosynthesis of AgNPs. Figure 1(A) indicates the UV absorption peak of the plant extracts and silver nanoparticles, while their corresponding EDS spectra are indicated in Figure 1(B,C). Figure 1(C) indicates the EDS spectra of AgNPs, which clearly indicate the formation of AgNPs. The EDS spectra also indicate the presence of elements like Mg, Na, Al and Cl in the leaf extracts, while no such elements are indicated in the EDS spectra of AgNPs indicating the purity of the AgNPs. XRD spectra confirmed the formation of AgNPs (face-centered cubic) while its crystal size was determined by Debye–Scherer’s equation as shown in Table 2. Crystal size of *D. mucronata* leaves derived AgNPs was 12 nm. Diffraction peaks were observed at 38.1°, 44.25°, 64.45° and 77.4°; referring to 111, 200, 220 and 310 planes of fcc (face-centered cubic) Ag, respectively. Figure 2(B) indicates the FTIR spectra of plant extracts and derived AgNPs. Flavonoids- and triterpenoids-rich *D. mucronata* leaves aqueous extract FTIR spectra showed a wide
peak at 3334.92 cm\(^{-1}\), which confirmed the presence of OH group. Similarly, a sharp peak was observed at 2920.23 cm\(^{-1}\) that corresponds to C–H methylene asymmetric stretching. Another characteristic peak was observed at 2850.79 and 1614.42 cm\(^{-1}\) demonstrating C–H methylene symmetric and aromatic C–C groups. On the other hand, in case of AgNPs, the peak at 1614 cm\(^{-1}\) was dissolved, while the OH (hydroxyl group) peak at 3334.92 cm\(^{-1}\) and C–H peaks at 2920.23 and 2850.79 cm\(^{-1}\) were also dissolved. Hence, it is confirmed that reduction of AgNO\(_3\) was due to OH group in case of \(D.\) mucronata leaves derived AgNPs. The peaks at 1066.64 and 1041.56 cm\(^{-1}\) became wide, showing a wide peak at 1271.09 cm\(^{-1}\) that corresponds to –C–O groups of polyols like terpenoids, flavones and

Table 2. Size deduced from XRD spectrum.

| Hkl | Peak observed at 2θ | Size (nm) |
|-----|---------------------|----------|
| 111  | 38.09               | 13.5     |
| 200  | 44.24               | 11.2     |
| 220  | 64.30               | 15.0     |
| 311  | 77.3                | 11.2     |
| Average |                   | 12.7     |

Figure 2. (A) X-ray diffraction studies; (B) FTIR spectra of plant extracts and derived silver nano particles.
polysaccharides. It indicates that polyols were the capping material involved in the synthesis of AgNPs. The morphology of the AgNPs was studied using high resolution scanning electron microscopy. The morphology of the AgNPs appeared spherical. The HR-SEM images are indicated in Figure 3(A,B). The spherical morphology of the biogenic NPs was also confirmed by the TEM image, as indicated in Figure 4. The size of the nanoparticles was also determined using TEM by digitizing the image through image J software. The particles size (8–16 nm) appeared to be in the range as calculated using XRD and Scherer formula. The thermal properties of bio-inspired AgNPs were studied using TGA/DTA. The TGA spectra were recorded in the range of 0–1000°C as shown in Figure 5. The sample was heated in a ceramic container at the rate of 10°C/min. The dominant weight loss occurred in the range of 400–500°C, while the weight loss was negligible below 400°C and above 500°C. The DTA curve indicated an intense exothermic peak which relates to the crystallization of AgNPs between 400 and 500°C. The earlier minor weight loss events may be caused by the evaporation of the moisture or degradation of the aromatic or phenolic components. The TGA/DTA results of AgNPs are indicated in Figure 5(A,B) while the same experiment was repeated with the plant powder as indicated in Figure 5(C,D). The breakdown events ∼220°C and ∼340°C further support the concept of phytochemical stabilization of the NPs.

Figure 3. HR-SEM images of biosynthesized AgNPs. (A) HR-SEM image of Aqueous extracts of D. mucronata and (B) HR-SEM image of derived AgNPs.
In vitro pharmacognostic properties

Antioxidant activity
Figure 6 indicates free radical scavenging potential of plant extracts and its derived AgNPs. Highest percent antioxidant activity (91.99%) was shown by aqueous fraction followed by AgNPs (85.4%) at 600 µg/ml. The EC50 of leaves extracts and derived AgNPs are presented in Table 3. Lowest EC50 value (322.83 µg/ml) was observed for AgNPs and highest EC50 value (350.34 µg/ml) was observed for an aqueous fraction. The EC50 and percent antioxidant activity have an inverse relationship.

Antibacterial activity
Figure 7 indicates percent inhibition of bacterial strains by plant aqueous extracts and derived AgNPs. The MIC50 and MBC values for aqueous extracts and AgNPs are indicated in Table 4. Aqueous portion of D. mucronata leaves showed moderate antibacterial activity against A. baumannii (43.47%) and P. vulgaris (44%). Low antibacterial effect was observed against E. coli (33.33%), P. aeruginosa (38.89%), S. aureus (36.53%), and VRSA (32%) while no antibacterial activity was observed against M. morganii. The D. mucronata leaves derived AgNPs showed significant antibacterial activity against E. coli (81.48%). Good antibacterial activity was observed against S. aureus (76.92%), P. vulgaris (76%), P. aeruginosa (74.07%), A. baumannii (65.21%), M. morganii (65.21%) and VRSA (64%).

Antifungal activity
Results of the antifungal activity of leaves extracts of D. mucronata and synthesized NPs are shown in
Table 5. Results showed that *D. mucronata* leaves derived AgNPs exhibited antifungal activities only against *C. albicans* and *A. niger*. The *D. mucronata* leaves derived AgNPs showed highest antifungal activity (49.5% growth inhibition) against *C. albicans*. *D. mucronata* leaves derived AgNPs showed 20% growth inhibition of *A. niger*.

**Haemagglutination activity**

In the current study, the *D. mucronata* extracts and their derived AgNPs showed no haemagglutination activity.

**Table 3. EC₅₀ values for DPPH free radical scavenging**

|            | EC₅₀ value (µg/ml) |
|------------|-------------------|
| Aqueous    |                   |
| *D. mucronata* leaves derived AgNPs | 350.34             |
| Vitamin C (Standard) | 342.50             |

**Figure 5.** (A,B) TGA/DTA curves of AgNPs; (C,D) TGA/DTA curves of plant powder.

**Figure 6.** DPPH free radical scavenging potential.

**Figure 7.** Percent inhibition of the various bacterial strains by aqueous extracts and biogenic AgNPs.
Cytotoxic activity

Figure 8(A) represents the cytotoxic activity of *D. mucronata* leaves extract and derived AgNPs. Least percent mortality (73.33% with LD50 = 90.213 µg/ml) was observed for aqueous *D. mucronata* leaves extract. Cytotoxicity of AgNPs was recorded with higher activity (73.33% with LD50=5.074 µg/ml) against brine shrimp in comparison to aqueous extract at the concentration of 10, 100 and 1000 µg/ml.

Phytotoxic activity

Variable phytotoxic activities were observed for *D. mucronata* leaves aqueous extract and its derived AgNPs. The leaves aqueous extract has 50% phytotoxic activity at 20 mg/ml while at lower concentrations its activity was 10%, 30% and 50% at 10, 100 and 1000 µg/ml. The derived AgNPs do not manifest any phytotoxicity. The results are indicated in Figure 8(B).

Thrombolytic activity

Results of thrombolytic activity of plant extracts and derived AgNPs are indicated in Figure 9(A). Positive control (streptokinase) showed 68.77% lysis activity and negative control (distilled water) showed negligible lysis activity (3.91%). Results showed that aqueous extract of leaves and derived AgNPs possess 15.9% and 25.8% thrombolytic activity respectively at 100 µg/ml. The derived AgNPs showed high thrombolytic activity in comparison to aqueous extracts, as shown in Figure 9(B).

Hemolytic activity

Figure 10 shows hemolytic activity results. No hemolysis was observed for the negative control (PBS). Complete lysis (100%) of human erythrocytes was observed for the positive control (Triton X-100). The aqueous extracts and derived nanoparticles showed no hemolytic activity; therefore, aqueous extract of *D. mucronata* leaves and synthesized AgNPs could be considered biocompatible at low concentrations.

### Table 4. Antibacterial Activity of *D. mucronata* leaves and derived AgNPs.

| Test organism | MIC50 and MBC (mg/ml) of *D. mucronata* | *D. mucronata* leaves aqueous extract | *D. mucronata* leaves derived AgNPs |
|---------------|-----------------------------------------|--------------------------------------|-------------------------------------|
| A. baumannii  | 5.74                                    | 2.72                                 | 3.8                                 |
| E. coli       | 6.18                                    | 1.96                                 | 2.76                                |
| P. aeruginosa | 6.42                                    | 2.52                                 | 3.8                                 |
| S. aureus     | 6.22                                    | 2.32                                 | 3.56                                |
| M. morganii   | –                                       | 2.76                                 | 3.92                                |
| Vrsa          | 6.34                                    | 2.8                                  | 4.2                                 |
| P. vulgaris   | 5.1                                     | 2.4                                  | 3.68                                |

### Table 5. Antifungal activity of *D. mucronata* leaves and derived AgNPs.

| Name of fungi | Negative control | Positive control | Percent linear growth inhibition |
|---------------|------------------|------------------|----------------------------------|
| C. albicans   | 0                | 100              | 0                                |
| F. oxysporum  | 0                | 100              | 0                                |
| A. flavus     | 0                | 100              | 0                                |
| A. parasiticus| 0                | 100              | 0                                |
| P. digitatum  | 0                | 100              | 0                                |
| A. niger      | 0                | 100              | 20                               |

Figure 8. (A) Cytotoxicity of plant extracts and their derived AgNPs; (B) phytotoxicity.
Mutagenicity test
Results of mutagenicity test are given in Table 6. For the TA-98 strain of S. typhimurium, the background was non-mutagenic and non-toxic with 5/96 positive wells and the standard was highly mutagenic with 85/96 positive wells. Aqueous extract was non-toxic and non-mutagenic (0/96 positive wells) to the bacterial strain. Similarly, the derived AgNPs were non-mutagenic and non-toxic (10/96 wells positive) to the bacterial strain. For the TA-100 strain of S. typhimurium, the background was non-mutagenic and non-toxic with 10/96 positive wells. The standard was highly mutagenic with 87/96 positive wells. Aqueous extract of leaves of D. mucronata and derived AgNPs were non-mutagenic and non-toxic to the bacterial strain with 5/96 and 6/96 positive wells. None of the samples exhibited mutagenic potential; therefore, they could be safe for use in drug development.

Insecticidal activity and anti-termite activity
Insecticidal and anti-termite potentials are highlighted in Figure 11. The D. mucronata leaves derived AgNPs showed 30%, 40% and 50% insecticidal activity against Tribolium castaneum, Rhizopertha dominica and Clinocotus analis respectively. While on day 3, 100% insecticidal activity was manifested by the biogenic silver nanoparticles. Insecticidal potential of biogenic AgNPs was higher when compared to D. mucronata leaves aqueous extract, as shown in Figure 11(A,B).

On the first day, D. mucronata leaves extracts indicated moderate anti-termite activity (50%) while on the second day, the extract indicated higher anti-termite activity (70%). On the third day, complete mortality was observed (100%). The derived AgNPs also indicated 100% termicidal potential at day 3. The biogenic silver nanoparticles appeared to be more effective when compared to the extracts. The results are indicated in Figure 11(C).

Discussion
Various physical and chemical methods can be used for synthesizing silver nanoparticles, but they are either time-consuming, costly or hazardous. Even in one of the reports, it was indicated that toxic chemicals used during

| Sample                              | TA98 No. of +ve wells/total number of wells | Result  | TA100 No. of +ve wells/total number of wells | Result  |
|-------------------------------------|------------------------------------------|---------|-------------------------------------------|---------|
| Background                          | 5/96                                     |         | 10/96                                     |         |
| 2-nitrofluorone                     | 85/96                                    | Mutagenic|                                          |         |
| NaN₃                                | –                                        | –       | 87/96                                     | Mutagenic|
| D. mucronata leaves aqueous extract | 0/96                                     | Non-mutagenic | 5/96 | Non-mutagenic|
| D. mucronata leaves derived AgNPs   | 10/96                                    | Non-mutagenic | 6/96 | Non-mutagenic|
synthesis may remain adsorbed to nanoparticles, which hinder widespread applications in therapeutics (51). Using a green method, AgNPs were biosynthesized for the first time using *D. mucronata* leaf aqueous extracts. The plant has well-established therapeutic uses in traditional medicine while preliminary research indicated the presence of valuable chemical entities such as phenols, coumarins and diterpenes. Such bioactive secondary metabolites are considered responsible for reduction and stabilization of nanoparticles.

In the present study, significant antioxidant activity was observed. Silver and gold nanoparticles served as strong nitric oxide, superoxide, hydroxyl and DPPH radical scavengers in comparison to their respective metal oxide (52). *Dalbergia spinosa*-mediated AgNPs showed much greater quenching abilities than that of control, highlighting the potential application of AgNPs in the field of pharmaceuticals (53).

The sample antibacterial activity is considered significant when MIC values are <2 mg/ml, good when 2 ≤ MIC ≤ 3 mg/ml, moderate when 3 ≤ MIC ≤ 6 mg/ml and weak when MIC > 6 mg/ml. Consequently, the activity (MIC of 1.96 mg/ml) observed for AgNPs could be considered significant against *E. coli*. Moderate or weak antibacterial activities (2 ≤ MIC ≤ 7 mg/ml) were observed for the test samples against *P. aeruginosa*, *A. baumannii*, *E. coli*, *M. morganii*, *S. aureus*, *P. vulgaris* and VRSA strains. The present study provides data on the ability of AgNPs to fight against pathogenic bacteria. These AgNPs could be further exploited for use in antimicrobial drug development as a starting material. Large surface area to volume ratio of nanoparticles makes them highly reactive due to which they play an important role in bacterial growth inhibition. Cell penetration may cause damage by interaction with sulfur and phosphorous-containing compounds like protein and DNA (71). Cell wall of microbes was found to be adversely affected by “pits” formation upon interaction with AgNPs (54). In the current studies, good antibacterial activity was observed for AgNPs. Antifungal effects of AgNPs and their hemolytic effects on erythrocytes were studied. It was concluded that due to membrane disruption by AgNPs, fungal cells were destructed, and low hemolytic potential was observed as previously reported (55). Liu et al. revealed that novel nano-composite synthesized in N, N-dimethylformamide had biocidal activity against *Bacillus subtilis*, *Escherichia coli* and *Candida albicans* (56). The biocompatibility of these nanoparticles in these cells was determined by the hemolysis test (56). These studies are in line with present research as moderate antifungal and no hemolytic activity was observed. Atherothrombotic diseases (myocardial or cerebral infarction) are caused by a thrombus in blood vessels. Thrombolytic agents are used to dissolve the clots formed already in blood vessels; however, some serious and fatal consequences could occur. Since ancient times, herbal preparations have been exploited for the treatment of several diseases, although in some cases, toxicity has been observed. In a study, organic extracts were investigated for thrombolytic properties, which possessed minimal or no toxicity (57).

**Conclusion**

In the present study, *D. mucronata* aqueous leaf extract was successfully used as chelating as well as stabilizing agents for the biosynthesis of spherical shaped silver nanoparticles of size 12 nm. The plant extract and synthesized AgNPs could be exploited in therapeutic and diagnostic applications. Further research on scaling up and optimizing the synthesis of nanoparticles from lab scale to pilot scale will be fruitful in determining the industrial as well as the commercial potential of these nanoparticles. Moreover, biogenically synthesized silver-based nanocomposites can be helpful in further diversifying the applications of biogenic silver nanoparticles.

**Acknowledgments**

AS, ATK, GL and KA performed and designed the experimental part. KA, AS and ATK drafted the manuscript. GL, KA and MM reviewed and significantly improved the manuscript. AS, GL conceived the idea.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

The authors are indebted to the valuable support of Higher Education Commission of Pakistan, Pakistan Academy of Sciences, National Research Foundation, South Africa, iThemba Labs.
Notes on contributors

Asma Shah, is PhD scholar at the Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan. Her research interests include nanobiotechnology and exploitation of endogenous plants for production of nanoparticles that could be exploited in various applications for medical and diagnostic purposes.

Professor Ghosia Lutfullah, is professor of Biochemistry and has diverse research interests in the field of Biotechnology, medicinal plants and, Microbiology Biochemistry. She has been awarded with several awards in recognition of her contribution to the field of Biotechnology and microbiology.

Dr. Kafeel Ahmad, is assistant Professor at the Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan. He has obtained his PhD degree from the University of Leicester, UK in the field of Biotechnology and Molecular Biology. His research interests include Molecular Biology, Biotechnology, Metabolic Pathway Engineering, Molecular Farming and Microbiology.

Ali Talha Khalil, is assistant professor of Biotechnology, has interests in the use of nanoscience for various biomedical and energy storage applications. He is an active academician and a researcher. He is also keen to work on the need of ethics, safety and security of cutting edge science.

Malik Maaza, is professor in UNISA, also holds the UNESCO UNISA Chair in Nanoscience and Nanotechnology. He has significant contribution to material sciences and optics. He is involved with various international academies and associations in supporting nanotechnology-based research work. His work is cited frequently all over the world. Due his outstanding and supportive role in the promotion of science in the developing countries he has been recently awarded ‘World award of Education.

References

[1] Füllbeck, M.; Michalsky, E.; Dunkel, M.; Preissner, R. Natural Products: Sources and Databases. Nat. Prod. Rep. 2006, 23 (3), 347–356.
[2] Cragg, G.M.; Newman, D.J.; Snader, K.M. Natural Products in Drug Discovery and Development. J. Nat. Prod. 1997, 60 (1), 52–60.
[3] Ovais, M.; Khalil, A.T.; Raza, A.; Khan, M.A.; Ahmad, I.; Islam, N.U.; Saravanan, M.; Ubaid, M.F.; Ali, M.; Shinwari, Z.K. Green Synthesis of Silver Nanoparticles via Plant Extracts: Beginning a New Era in Cancer Theranostics. Nanomedicine 2016, 11 (23), 3157–3177.
[4] Barabadi, H.; Ovais, M.; Shinwari, Z.K.; Saravanan, M. Anticancer Green Bionanomaterials: Present Status and Future Prospects. Green Chem. Lett. Rev. 2017, 10 (4), 285–314.
[5] Ovais, M.; Nadhman, A.; Khalil, A.T.; Raza, A.; Khuda, F.; Sohail, M.F.; Islam, N.U.; Sarwar, H.S.; Shahnaz, G.; Ahmad, I. Biosynthesized Colloidal Silver and Gold Nanoparticles as Emerging Leishmanicidal Agents: An Insight. Nanomedicine 2017, 12 (24), 2807–2819.
[6] Khalil, A.T.; Ovais, M.; Ullah, I.; Ali, M.; Shinwari, Z.K.; Maaza, M. Biosynthesis of Iron Oxide (Fe3O4) Nanoparticles via Aqueous Extracts of Sageretia thea (Osbeck) and Their Pharmacognostic Properties. Green Chem. Lett. Rev. 2017, 10 (4), 186–201.
[7] Li, S.; Shen, Y.; Xie, A.; Yu, X.; Qiu, L.; Zhang, L.; Zhang, Q. Green Synthesis of Silver Nanoparticles Using Capsicum annuum L. Extract. Green Chem. 2007, 9 (8), 852–858.
[8] Khalil, A.T.; Ovais, M.; Ullah, I.; Ali, M.; Jan, S.A.; Shinwari, Z.K.; Maaza, M. Bioinspired Synthesis of Pure Massicot Phase Lead Oxide Nanoparticles and Assessment of Their Biocompatibility, Cytotoxicity and In-vitro Biological Properties. Arab. J. Chem., in press.
[9] Tolaymat, T.M.; El Badawy, A.M.; Genaidy, A.; Scheckel, K.G.; Luxton, T.P.; Suidan, M. An Evidence-based Environmental Perspective of Manufactured Silver Nanoparticle in Syntheses and Applications: A Systematic Review and Critical Appraisal of Peer-reviewed Scientific Papers. Sci. Total Environ. 2010, 408 (5), 999–1006.
[10] Ahamed, M.; AlSalhi, M.S.; Siddiqui, M. Silver Nanoparticle Applications and Human Health. Clin. Chim. Acta 2010, 411 (23), 1841–1848.
[11] Sambhy, V.; MacBride, M.M.; Peterson, B.R.; Sen, A. Silver Bromide Nanoparticle/Polymer Composites: Dual Action Tunable Antimicrobial Materials. J. Am. Chem. Soc. 2006, 128 (30), 9798–9808.
[12] Khalil, A.T.; Ovais, M.; Ullah, I.; Ali, M.; Shinwari, Z.K.; Khamlich, S.; Maaza, M. Sageretia thea (Osbeck) Mediated Synthesis of Zinc Oxide Nanoparticles and its Biological Applications. Nanomedicine 2017, 12 (15), 1767–1789.
[13] Khalil, A.T.; Ovais, M.; Ullah, I.; Ali, M.; Shinwari, Z.K.; Maaza, M. Physical Properties, Biological Applications and Biocompatibility Studies on Biosynthesized Single Phase Cobalt Oxide (Co3O4) Nanoparticles via Sageretia thea (Osbeck). Arab. J. Chem., in press.
[14] Matinise, N.; Fuku, X.; Kaviyarasu, K.; Mayedwa, N.; Maaza, M. ZnO Nanoparticles via Moringa oleifera Green Synthesis: Physical Properties & Mechanism of Formation. Appl. Surf. Sci. 2017, 406, 339–347.
[15] Zaidi, A.; Bukhari, S.M.; Khan, F.A.; Noor, T.; Iqbal, N. Ethnobotanical, Phytochemical and Pharmacological Aspects of Daphne mucronata (Thymeleaceae). Trop. J. Pharm. Res. 2015, 14 (8), 1517–1523.
[16] Khodadadian, Z.; Hassanpour-Ezatti, M.; Mousavi, S.Z.; Asgarpanah, J. Analgesic and Anti-inflammatory Potential of Aerial Parts of the Daphne mucronata Royle Extract in Mice: Opioid-independent Action. Asian Pac. J. Trop. Biomed. 2016, 6 (3), 198–201.
[17] Rasool, M.A.; Khan, R.; Malik, A.; Bibi, N.; Kazmi, S.U. Structural Determination of Daphne, A New Coumarinolignan from Daphne Mucronata: Note. J. Asian Nat. Prod. Res. 2010, 12 (4), 324–327.
[18] Lavidia, K.; Miri, R.; Jahromi, R.B.N.K. A Preliminary Study on the Biological Activity of Daphne mucronata Royle. DARU J. Pharm. Sci. 2003, 11 (1), 28–21.
[19] Murad, W.; Ahmad, A.; Gilani, S.A.; Khan, M.A. Indigenous Knowledge and Folk use of Medicinal Plants by the Tribal Communities of Hazar Nao Forest, Malakand District, North Pakistan. J. Med. Plants Res. 2011, 5 (7), 1072–1086.
[20] Hamayun, M. Traditional Uses of Some Medicinal Plants of Swat Valley Pakistan. Indian J. Tradit. Know. 2007, 6, 636–641.
[21] Mosaddegh, M.; Naghibi, F.; Moazzenni, H.; Pirani, A.; Esmaeili, S. Ethnobotanical Survey of Herbal Remedies
Traditionally Used in Kohgiluyeh va Boyer Ahmad Province of Iran. J. Ethnopharmacol. 2012, 141 (1), 80–95.

Ghasemi, P.A.; Momeni, M.; Bahmani, M. Ethnobotanical Study of Medicinal Plants Used by Kurd Tribe in Dehloran and Abdanan Districts, Ilam Province, Iran. Afr. J. Tradit. Complement Altern. Med. 2013, 10 (2), 368–385.

Ankamwar, B.; Damle, C.; Ahmad, A.; Sastry, M. Biosynthesis of Gold and Silver Nanoparticles Using Emblica Officinalis Fruit Extract, Their Phase Transfer and Transmetallation in an Organic Solution. J. Nanosci. Nanotechnol. 2005, 5 (10), 1665–1671.

Bar, H.; Bhui, D.K.; Sahoo, G.P.; Sarkar, P.; De, S.P.; Misra, A. Green Synthesis of Silver Nanoparticles Using Latex of Jatropha curcas. Colloids Surf. A. 2009, 339 (1), 134–139.

Parashar, V.; Parashar, R.; Sharma, B.; Pandey, A.C. Parthenium Leaf Extract Mediated Synthesis of Silver Nanoparticles: A Novel Approach Towards Weed Utilization. Dig. J. Nanomater. Biostuct. 2009, 4 (1), 45–50.

Krishnaraj, C.; Jagan, E.; Rajasekar, S.; Selvakumar, P.; Kalaichelvan, P.; Mohan, N. Synthesis of Silver Nanoparticles Using Acalypha indica Leaf Extracts and its Antibacterial Activity Against Water Borne Pathogens. Colloids Surf. B. 2010, 76 (1), 50–56.

Prathna, T.; Chandrasekaran, N.; Rai, D.; Mukherjee, A. Biomimetic Synthesis of Silver Nanoparticles by Citrus Limon (Lemon) Aqueous Extract and Theoretical Prediction of Particle Size. Colloids Surf. B. 2011, 82 (1), 152–159.

Fayaz, A.M.; Balaji, K.; Girilal, M.; Yadav, R.; Kalaichelvan, P.T.; Venkatesan, R. Biogenic Synthesis of Silver Nanoparticles and Their Synergistic Effect with Antibiotics: A Study Against Gram-positive and Gram-negative Bacteria. Nanomed. Nanotechnol. 2010, 6 (1), 103–109.

Basavegowda, N.; Sobczak-Kupiec, A.; Malina, D.; Yathirajan, H.; Keerthi, V.; Chandrashekar, N.; Dinkar, S.; Liny, P. Plant Mediated Synthesis of Gold Nanoparticles Using Fruit Extracts of Ananas comosus (L) (Pineapple) and Evaluation of Biological Activities. Adv. Mater. Lett. 2013, 4 (5), 332–337.

Mitra, B.; Vishnudas, D.; Sant, S.B.; Annamalai, A. Green-synthesis and Characterization of Silver Nanoparticles by Aqueous Leaf Extracts of Cardiospermum helicacabum Leaves. Drug Invent. Today. 2012, 4 (2), 340–344.

Khalil, A.; Dababneh, B.F.; Al-Gabbiesh, A.H. Antimicrobial Activity Against Pathogenic Microorganisms by Extracts From Herbal Jordanian Plants. J. Food Agric. Env. 2009, 7 103–106.

Kanatt, S.R.; Chander, R.; Sharma, A. Antioxidant Potential of Mint (Mentha Spicata L.) in Radiation-processed Lamb Meat. Food Chem. 2007, 100 (2), 451–458.

Kurca, A.; Arslan, E. Antioxidant Capacity and Total Phenolic Content of Selected Plants from Turkey. Int. J. Food Sci. Tech. 2008, 43 (11), 2038–2046.

Tepe, B.; Daferera, D.; Sokmen, A.; Sokmen, M.; Polissiou, M. Antimicrobial and Antioxidant Activities of the Essential oil and Various Extracts of Salvia Tomentosa Miller (Lamiaceae). Food Chem. 2005, 90 (3), 333–340.

Khan, I.; Ahmad, K.; Khalil, A.T.; Khan, J.; Khan, Y.A.; Saqib, M.S.; Umar, M.N.; Ahmad, H. Evaluation of Antileishmanial, Antibacterial and Brine Shrimp Cytotoxic Potential of Crude Methanolic Extract of Herb Ocimum basilicum (Lamiaceae). J Tradit Chin Med 2015, 35 (3), 316–322.

Banso, A. Phytochemical and Antibacterial Investigation of Bark Extracts of Acacia nilotica. J. Med. Plants Res. 2009, 3 (2), 082–085.

Spencer, J.F.; de Spencer, A.L.R. Public Health Microbiology: Methods and Protocols, Vol. 268. Humana Press Inc.: Totowa, NJ, 2004.

Ahmad, K.; Khalil, A.T.; Khan, Y.A.; Somayya, R. Antifungal Phytotoxic and Hemagglutination Activity of Methanolic Extracts of Ocimum basilicum. J. Tradit. Chin. Med. 2016, 36 (6), 794–798.

Ahmad, B.; Bashir, S.; Azam, S.; Ali, N. Screening of Acacia Modesta for Antifungal, Anti-termite, Nitric Oxide Free Radical Scavenging Assay and Brine Shrimp Cytotoxic Activities. J. Med. Plants Res. 2011, 5 (15), 3380–3386.

Ahmad, B.; Azam, S.; Bashir, S.; Ahmad, J.; Hussain, F. Screening of Acacia modesta for Haemagglutination, Antibacterial, Phytotoxic and Insecticidal Activities. J. Med. Plants Res. 2011, 5 (14), 3090–3096.

Ahmed, Y.; Sohrab, M.H.; Al-Reza, S.; Tareq, F.S.; Hasan, C.M.; Sattar, M. Antimicrobial and Cytotoxic Constituents From Leaves of Sapium baccatum. Food Chem. Toxicol. 2010, 48 (2), 549–552.

Lakshmi, K.S.; Sangeetha, D.; Sivamani, S.; Tamarlarasan, M.; Rajesh, T.; Anandraj, B. In Vitro Antibacterial, Antioxidant, Haemolytic, Thrombolytic Activities and Phytochemical Analysis of Simarouba glauca Leaves Extracts. Int. J. Pharm. Sci. Res. 2014, 5 (2), 432.

Shabbir, M.; Khan, M.R.; Saeed, N. Assessment of Phytochemicals, Antioxidant, Anti-lipid Peroxidation and Anti-hemolytic Activity of Extract and Various Fractions of Maytenus royleanus Leaves. BMC Complement Altern. Med. 2013, 13 (1), 143.

Razak, M.F.A.; Aidoo, K.E. Toxicity Studies of Eurycoma longifolia (Jack)-based Remedial Products. Asian J. Pharm. Clin. Res. 2011, 4 (3), 1256–1267.

Gilbert, R. The Analysis of Fluctuation Tests. Mutat. Res. Environ. Mutagen. Rel. Subj. 1980, 74 (4), 283–289.

Hussain, F.; Hameed, I.; Dastagir, G.; Khan, I.; Ahmad, B. Cytoxicity and Phytotoxicity of Some Selected Medicinal Plants of the Family Polygonaceae. Afr. J. Biotechnol. 2010, 9 (5), 770–774.

Khaliquzzaman, M.; Khatun, M.; Talukdar, D. Growth of Tribolium confusum Duv. on Wheat Flour with Various Yeast Levels. Int. Pest Control. 1994, 36 (5), 128–130.

Park, T.; Frank, M.B. The Fecundity and Development of the Flour Beetles, Tribolium confusum and Tribolium castaneum, at Three Constant Temperatures. Ecology 1948, 29 (3), 368–374.

Dastagir, G.; Hussain, F. Phytotoxic and Insecticidal Activity of Plants of Family Zygophyllaceae and Euphorbiaceae. Sarhad J. Agric. 2013, 29 (1), 83–91.

Salihah, Z.; Khatoon, R.; Khan, A.; Sattar, A. In A Termite Trap, NIWA-TERMAP, for Capturing Large Population of Field Population of Heterotermes indicola, pp. 395–400, Proceedings: 13th Pakistan Congress of Zoology, Islamabad, March 31 –April 1, 1993; 1993.

Prabhu, S.; Poulose, E.K. Silver Nanoparticles: Mechanism of Antimicrobial Action, Synthesis, Medical Applications, and Toxicity Effects. Int. Nano Lett. 2012, 2 (1), 32.

Ramamurthy, C.; Padma, M.; Mareeswaran, R.; Suyavaran, A.; Kumar, M.S.; Premkumar, K.; Thirunavukkarasu, C. The Extra Cellular Synthesis of
Gold and Silver Nanoparticles and Their Free Radical Scavenging and Antibacterial Properties. *Colloids Surf. B.* 2013, 102, 808–815.

[53] Muniyappan, N.; Nagarajan, N. Green Synthesis of Silver Nanoparticles with *Dalbergia spinosa* Leaves and Their Applications in Biological and Catalytic Activities. *Process Biochem.* 2014, 49 (6), 1054–1061.

[54] Sondi, I.; Salopek-Sondi, B. Silver Nanoparticles as Antimicrobial Agent: A Case Study on *E. Coli* as a Model for Gram-negative Bacteria. *J. Colloid Interface Sci.* 2004, 275 (1), 177–182.

[55] Kim, K.-J.; Sung, W.S.; Suh, B.K.; Moon, S.-K.; Choi, J.-S.; Kim, J.G.; Lee, D.G. Antifungal Activity and Mode of Action of Silver Nano-particles on *Candida albicans*. *Biometals* 2009, 22 (2), 235–242.

[56] Liu, L.; Yang, J.; Xie, J.; Luo, Z.; Jiang, J.; Yang, Y.Y.; Liu, S. The Potent Antimicrobial Properties of Cell Penetrating Peptide-conjugated Silver Nanoparticles with Excellent Selectivity for Gram-positive Bacteria Over Erythrocytes. *Nanoscale* 2013, 5 (9), 3834–3840.

[57] Rahman, M.A.; Sultana, R.; Emran, T.B.; Islam, M.S.; Rahman, M.A.; Chakma, J.S.; Rashid, H.-u.; Hasan, C.M.M. Effects of Organic Extracts of six Bangladeshi Plants on In Vitro Thrombolysis and Cytotoxicity. *BMC Complement Altern. Med.* 2013, 13 (1), 25.

[58] Li, Y.; Chen, D.H.; Yan, J.; Chen, Y.; Mittelstaedt, R.A.; Zhang, Y.; Biris, A.S.; Heflich, R.H.; Chen, T. Genotoxicity of Silver Nanoparticles Evaluated Using the Ames Test and in Vitro Micronucleus Assay. *Mutat. Res. Genet. Toxicol. Environ. Mutagen* 2012, 745 (1), 4–10.

[59] Kim, J.S.; Sung, J.H.; Ji, J.H.; Song, K.S.; Lee, J.H.; Kang, C.S.; Yu, I.J. In Vivo Genotoxicity of Silver Nanoparticles After 90-day Silver Nanoparticle Inhalation Exposure. *Saf. Health Work.* 2011, 2 (1), 34–38.