INVESTIGATING LOW DOSE OF WEED-MEDIATED SILVER NANOPARTICLES AGAINST PATHOGENIC BACTERIA

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ABSTRACT
Weeds are undesirable and unpleasant plants with robust growth. Around 18,000 weed species outcast native biodiversity and are responsible for tremendous economic losses. Plants were used to treat numerous ailments since ancient times. Antimicrobial resistance (AMR) is an emerging global health problem, where approximately 700,000 people lose their lives each year from AMR infections. The minimum inhibitory concentration of Biogenic silver nanoparticles against pathogenic bacterial strain has not been reported previously. This study aims to elucidate the lowest biogenic Alkanna tinctoria mediated silver nanoparticles dose to treat bacterial infections. Qualitative phytochemical analysis was performed using standard tests. The synthesized silver nanoparticles were characterized visually, UV–Vis spectroscopy and using X-ray diffraction. The minimum inhibitory concentration of biogenic silver nanoparticles was tested against gram-positive (Staphylococcus aureus) and gram-negative bacteria (Escherichia coli). Alkanna tinctoria leaf was positive for alkaloids, carbohydrates, saponins, amino acids, and fixed oil. The synthesized silver nanoparticles were in the range of 60 to 73 nm. Silver nanoparticles significantly affected E. coli as compared to S. aureus. The MIC for E. coli was recorded as 46.87 μg mL⁻¹ and 93.75 μg mL⁻¹ was effective against S. aureus. The search of naive plants as novel therapeutic agents along with nanotechnology can bring revolution in the field of drug development and medical sciences.

Keywords: Alkanna tinctoria, Antibacterial agent, Phytochemical analysis, Silver nanoparticles, Weed.


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INTRODUCTION

Weeds have undesirable characteristics, robust growth, and invasive nature (Hussain et al., 1989). They produce secondary metabolites to halt crop growth yet protect themselves against harsh environmental conditions (Rhodes et al., 1989). Commonly known metabolites like Saponins, fats, flavonoids, protein, and alkaloids act as reservoirs for the protection of plants and can be exploited in different therapeutics agents (Talib and Mahasneh, 2010). Certain plants have an antimicrobial activity such as the oil extract of black pepper, clove, oregano, thyme, and nutmeg against plants and animal pathogens (Anesini and Perez, 1993; Dorman and Deans, 2000). AMR is a major concern in today's era with the emerging resistance of microbes against diverse antibiotics termed multidrug resistance (MDR) which has exponentially increased at an alarming rate (Zaimuddin and Dale, 1990). This emerging resistance is due to overuse or misuse of prescribed drugs and the use of low-potent drugs by pharmaceutical companies. Medicinal plants are still considered the drug of choice to treat a wide range of infections among rural populations. Recently researchers are keen to identify effective plant metabolites to overcome MDR and AMR cases to combat bacterial resistance more effectively (D’Costa et al., 2011). According to World Health Organization (WHO) about 2000 plants were effective in treating AMR in 91 countries (Akinyemi et al., 2007). *Alkanna tinctoria* root is used as red dye agent. The alkanet belongs to the family of borage (Chew and Goh, 2011) and mainly grows in the Mediterranean region (Peiss, 2011). The red color is utilized in wines, alcoholic tinctures, vegetable oils and varnishes. Recently, the Alkannin compound isolated from this weed was used to treat abscesses and inflammations (Sengul et al., 2009).

Nanotechnology involves the use of metals in nanoparticles with sizes ranging from 10-100 nm. These nanoparticles are more active and robust than bulk material (Rolland et al., 2005), facilitating nanoparticles to efficiently trap and link drugs for targeted activity (Langer, 2000). Biologically mediated synthesis of nanoparticles is considered a safe, effective and eco-friendly method with robust effect (Ahmed et al., 2011; Pum and Sleytr, 1999). Polymeric nanoparticles coated with polyethylene glycol (as a widespread circulating substance) permit the drug to persist in the body for longer durations at the target site (Bhadra et al., 2002; Kommareddy et al., 2005). Thus, efficiently releasing the required dose of the drug to the targeted site to attain a desirable therapeutic effect (Vila et al., 2002). Previously, various nanoparticles of Gold, Carbon, Zinc, and Silver and their alloy have been synthesized using plant extracts (Chandran et al., 2006; Shanker et al., 2003). Plant extracts concentrated with antioxidants, reactive metabolites, enzymes (Wu et al., 2008) and polysaccharides (Collera-Zuniga et al., 2005) are utilized for nanoparticle synthesis (Ahmad et al., 2009). Silver (Ag) has been used for 2000 years due to its antimicrobial activity (Salwson et al., 1992). Ag nanoparticles ruptures the cell wall or halts its synthesis and disrupt cell transduction which leads to cell lysis (Prabhu et al., 2012).

Here, we aimed to evaluate the lowest dose of biogenic *Alkanna tinctoria*-mediated silver nanoparticles required to treat bacterial infections. We evaluated the qualitative phytochemical properties of *Alkanna tinctoria* leave extract. Silver nanoparticles were synthesized using leaves of *Alkanna tinctoria* and tested against gram-positive and gram-negative bacteria. Finally, the lowest dose of biogenic silver nanoparticles was identified to check the effective dose with inhibitory properties.

MATERIAL AND METHODS

Plant collection

The fresh plant of *Alkanna tinctoria* was collected from Balochistan Agriculture Research and Development Center Quetta (BARDC). The leaves were separated from the plant material and washed with tap water and distilled water to remove all dirt and debris (Fig. 1)
Qualitative Phytochemical Analysis

The secondary metabolites such as Alkaloids, proteins, carbohydrates, Saponins, fats and oil in *Alkanna tinctoria* was identified using the qualitative phytochemical test. Hager's Test was performing to detect the presence of Alkaloids. The leaves were mixed with 5 mL of HCl then the solution was filtered, after filtration hager's reagent (comprised of aqueous solution of saturated picric acid) about 2 mL was added (Yadav et al., 2011). The presence of Saponins in the leaf was identified using standard protocol. Leave extract was diluted with distilled water up to 20 mL to form the solution. The diluted solution was shaken for approximately 15 min (Yadav et al., 2011) and results were observed. Fehling's test was used to identify carbohydrate level in plant leaves. The leave extract was mixed with distilled water and solution was filtered. About 1 mL of Fehling solution A (34.66 g of CuSO₄·5H₂O distilled water) was added to 1ml of filtrate, followed by 1 mL of Fehling solution B (Potassium sodium tartrate of 173 g and NaOH of 50 g mixed in the distilled water). The final mix was kept in water bath until color changes (Yadav et al., 2011). To identify the presence of protein and amino acid, Ninhydrin test was performed. About 100 mg of leaves were mixed in 10 mL of distilled water and filtered. About 2 mL of filtrate solution was mixed with 2 drops of ninhydrin solution (10 mg of ninhydrin dissolved in the 200 mL of acetone). To identify fixed oil and gas, spot test was performed. Plant leaves extract was placed and pressed on plain white paper (Mir et al., 2013).

Synthesis of silver nanoparticles

Fresh leaves of plants were washed with distilled water to remove all the debris. The plant extract was prepared by adding plant leaves (2.5 g) and distilled water (50 mL) in water bath at 60 °C for 4 hours. The solution was further cooled down and purified through Whatman filter paper (Singh et al., 2008). About 1 mM silver nitrate (AgNO₃) solution with concentration 7.64 mg 45 mL⁻¹ of distilled water was prepared in Erlenmeyer flask and constantly stirred for 2 hours at room temperature. The flask was covered with aluminum foil to prevent photo degradation. About 5 mL of plant extract was added onto 45 mL of AgNO₃ drop wise until color changes (Singh et al., 2008).

Characterization of silver nanoparticles

Silver nanoparticles were characterized visually as the color changes from colorless to dark brown in color. This change in color confirms reduction of Ag⁺ ions to Ag particles (Prasad et al., 2011). The colloidal solution was tested between 300nm to 700 nm using UV-vis spectrophotometer (Jenway, model, 6305) to confirm synthesis of biogenic Ag nanoparticles. The colloidal silver nanoparticles solution was centrifuged for 20 min at 13,000 rpm to obtain pure particles. The supernatant was discarded and pellet containing Ag nanoparticles
were washed with distilled water and centrifuged. This process was repeated thrice to obtain purified Ag particles. The purified Ag nanoparticles were dried in hot air oven at 80 °C for 3 days. About 1gm of dried nanoparticles was given to Department of Petroleum and Gas Engineering to confirm Ag nanoparticles using X-ray diffraction technique (XRD). The results were matched with standard silver nanoparticles (JCPDS file No. 04-0783).

**Antibacterial activity of silver nanoparticle**

The antibacterial activity of biogenic Ag nanoparticles was identified against *Staphylococcus aureus* (Gram positive) and *Escherichia coli* (gram negative). The isolates were grown on a nutrient broth and incubated for 18 hours at 37 °C. The bacterial growth on broth was matched to 0.5 McFarland solution. About 100 µL of respective strain containing 1.5 x 10^8 bacteria was spread onto the agar plates using spreader, and 4 wells were made using cork-borer using aseptic technique. About 100 µL of each solution [plant extract (2.5 g50 mL^-1), distilled water, AgNO_3 (7.65 mg 45 mL^-1), and Ag nanoparticles (108 µg mL^-1)] was added onto respective wells (Table 1). The plates were incubated for 18 hours at 37 °C. Each experiment was repeated thrice with three replicates (Pal et al., 2007). The zone of inhibition was recorded using measuring scale in mm and analyzed using Prism software.

<table>
<thead>
<tr>
<th>Wells</th>
<th>Solution</th>
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<tbody>
<tr>
<td>A.</td>
<td>Distilled water (Negative control)</td>
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<tr>
<td>B.</td>
<td>AgNO_3 solution (Positive control)</td>
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<tr>
<td>C.</td>
<td>Extract of <em>Alkanna</em> (Positive control)</td>
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<tr>
<td>D.</td>
<td>Silver (Ag) Nanoparticles</td>
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**Minimum inhibitory concentration (MIC)**

Minimum inhibitory concentration is the least amount of antimicrobial agent required to halt the growth of microorganisms. To determine MIC for silver nanoparticles against gram-positive (*S. aureus*) and gram-negative (*E. coli*) broth serial dilution method was used. Positive control comprised broth media and respective bacterial cultures to ensure the ability of bacteria to grow in a medium. While negative control comprised only broth media to confirm the sterility of media and equipment. To determine MIC in *S. aureus* and *E. coli* 6 test tubes were taken. About 200 µL of respective strain was added in all test tubes containing 2ml of broth except for negative control. About 2mL of Ag nanoparticles was added onto the first tube, and further serially diluted into each tube. The test tubes along with their respective replicates were incubated at 37 °C for 18 hours. After 18 hours the test tubes were poured onto nutrient agar for *S. aureus* and EM agar for *E. coli* to determine its growth. The Petri plate without bacterial growth is the minimum amount of Ag nanoparticles required to halt bacterial (Wiegand et al., 2008).

**RESULT AND DISCUSSION**

**Phytochemical analysis**

The phytochemical compounds like alkaloids, carbohydrates, proteins, Saponins fats and oil in *Alkanna tinctoria* were measured using a qualitative method (Bruneton, 2007). Alkaloids are bioactive compounds in plants, usually a product of metabolism. It acts as a nitrogen reservoir and protects the plants from predator attacks. Hager’s test confirmed the presence of Alkaloids as the leave extract changed to yellow (Fig. 2c). Previous research was conducted on *Terminalia bellerica* (Leaves), *Tinospora cordifolia* (Leaves), *Tinospora cordifolia* (Stem) and *Xanthium strumarium* (Leaves) confirmed the presence of flavonoids, proteins,
Carbohydrates and Saponins. Whereas, *Terminalia bellerica* was positive for alkaloids and terpenoids (Yadav et al., 2011).

Fig. 2. The phytochemical analysis of (a) *Alkanna tinctoria* leave extract was positive for (b) carbohydrate (c) Alkaloid (d) Saponins (e) proteins and amino acids (f) fat and oil.

Carbohydrates are the product of photosynthesis and are utilized to obtain energy. It is also vital in the formation of cellulose (McPherson and Williams, 1998). The Fehling’s test was performed to qualitatively determine the presence of carbohydrates, as indicated by the violet ring on the top extract (Fig. 2b). Saponins are a nonstructural assorted type of compound present in different types of plants. It protects the plant from fungi and other microbes by acting as antifeedants (Sexena et al., 2013). Foam-like layer conformed presence of saponins (Fig. 2d). Another study further confirmed phytochemical compounds in Ward Taifi (Taif rose) such as flavonoids, terpenoids, glycosides and Saponins (Abdul Hameed et al., 2013). These phytochemical compounds act as an antioxidant, anti-inflammatory, antimicrobial drugs and anti-hepatotoxic compounds. WHO previously recommended that medicinal plants are the best therapeutic drugs. Traditional herbal medicines are still preferred by 80% of the population in developing countries, however, necessary steps are required to explore the efficiency, properties and its physiological activity on an individual (Baser, 1992). Proteins and amino acids are vital for several functions such as: the biosynthesis of hormones, enzymes, proteins pumps and channels (Garcia-Olmedo et al., 1995). Ninhydrin test confirmed the presence of amino acids and proteins as the colour of the extract changed from pale yellow colour to purple (Fig. 2e). Plants reserve its energy in the form of fats and oil. So, the unusable energy is converted into fats and oil (Coulston, 1999). Spot test confirmed the presence of fats in the leaves as an oil stain was on the paper (Fig. 2f).
Characterization of silver nanoparticles

The visual characterization is the first step to confirming the synthesis of Ag nanoparticles. The pale-yellow colloidal solution turned into dark brown which indicated the end of the reaction. This dark solution confirmed the reduction of Ag+ ions to Ag nanoparticles (Fig. 3). Another study confirmed that reduction can be caused by compounds like caffeine and theophylline in Acalypha indica (Krishnraj et al., 2010). Similarly, Ag nanoparticles are formed due to compounds such as flavanones and terpenoids derived from neem leaves extract. The stimulation of surface plasmon vibration showed yellowish darker fluid of Ag nanoparticles (Shankar et al., 2004). The diversity of antioxidant metabolites in plants vigorously hunts free radicals (responsible for damage to the structure of cells) and neutralizes them. Phenol has oxidation-reduction properties by absorbing and removing a free radical through its antioxidative action (Saeed et al., 2012). The Alkanna tinctoria root extract has phenol that has cinnamic acid and caffeic acid in a hydro form that acts as a reducing agent of AgNO3 to form Ag nanoparticles (Abdel-Latif et al., 2013).

Fig. 3. Synthesis of Ag nanoparticles. The color of the colloidal Ag nanoparticles changes from pale yellow (a) to ultimately dark brown (b) confirms the synthesis of Silver nanoparticles.

Nanoparticles contain optical characteristics which are considered sensitive for the size, and refractive index that’s why UV-visible is considered as an important tool to confirm nanoparticles (Gao et al., 2007). The UV-vis spectrophotometers confirmed Ag nanoparticle formation as displayed by the sharp peak at a range of 400 nm to 450 nm (Fig. 4) (Gao et al., 2007). X-ray diffraction (XRD) is a key tool by which the probing structure of nanomaterials was determined (Westesen et al., 1993). XRD was used for the further characterization of bio-synthetic Ag nanoparticles. XRD provides data about the crystalline structure, nature of state and size of the crystal (Westesen et al., 1993). The sharp peak at 380, 64.670 and 77.120 corresponds to peak positions at 111, 220, and 311 according to Bragg reflections. The average size for silver nanoparticles was obtained using Full-Width Half-Maximum (FWHM) using Debye-Scherer’s formula for synthesized Ag nanoparticles ranging between 60-73 nm (Fig. 5). Previously, researchers have synthesized Ag nanoparticles from lavender, alkanet and artichoke and have also confirmed the synthesis of silver nanoparticles using XRD with peaks at 38°, 44° and 77° respectively (Mehboob et al., 2021).
Figure 4. UV-Visible Spectrophotometer. The silver nanoparticles at the initial stage (a) of synthesis while the final reaction (b) shows peaks at 450nm confirms the presence of Ag nanoparticles.

Antibacterial activity

*E. coli* strains are considered microbiota of the Gastrointestinal (GIT) tract in human beings, however in some cases, they cause ailments like diarrheal infection, abdomen discomfort, temperature, and sometimes nausea. The ranges of infections are colon infection, urinary tract infection (UTI), tourist diarrhea, and cholecystitis, contagions clinical infections like pneumonia and meningitis (Griffin and Tauxe, 1991). Our results showed that Ag nanoparticles were effective in inhibiting the growth of *E. coli* as compared to positive and negative controls. *E. coli* is normal flora yet pathogenic and causes infections like pneumonia and UTI. The Ag nanoparticles showed significant (P≤0.05) effect on *E. coli* (2.10±0.20) as compared to plant leaf extracts (0.80±0.0), control group (0.0±0.0), and AgNO₃ solution (0.45± 0.35) (Fig. 6).

The antibacterial activity of Ag nanoparticles inhibited the growth of *E. coli* and *S. aureus* rather than AgNO₃ or plant extract. However, the antibacterial activity of Ag nanoparticles was more
effective against gram-negative bacteria due to structural differences in cell walls between the two bacteria. Secondly, charge on gram negative’s cell wall (such as *E. coli*) is negative due to carboxyl, amino group and phosphate group and Ag nanoparticles have positive charge, therefore this attraction helps in attachment of Ag nanoparticles to cell membrane—allowing its penetration into the cells (Yun'an *et al.*, 2018). As a result, reactive oxygen species (ROS) are produced, disrupting signalling pathways. Meanwhile, exposure of silver nanoparticle is not safe for human cells because of its induction of genotoxicity, cytotoxicity, and inflammation of cells (Varier *et al.*, 2019).

**Fig. 6.** Antibacterial activity of biosynthetic Silver nanoparticles on *E. coli*. (a) illustration of the zone of inhibition in *E. coli* (b) Where silver nanoparticles showed highest zone of inhibition against Positive and negative controls.

*Staphylococcus aureus* causes infections like impetigo, boils, and other life-threatening diseases such as: toxic shock syndrome, osteomyelitis, and endocarditis. Ag nanoparticles (1.75±0.55) were significantly (*P*≤0.05) effective in inhibiting *S. aureus* as compared to plant extract (1.80±0.50), control and AgNO₃ solution (0.0±0.0) (Fig. 7). *S. aureus* is responsible for Hospital and community-acquired infections such as endocarditis, urinary tract infection, and pneumonia of cystic. The virulence factors of *S. aureus* such as Factor A, secreted protein B and C, toxin I initiates infection and are known to cause toxic shock syndrome). The surface of cell membrane helps in its attachment to host cell and produces a slime layer upon attachment, also using antiphagocytic microcapsule to protect itself from drugs. However, amid infection particular enzymes such as protein breaking enzymes, lipid breaking enzymes and elastases help bacteria invade the host and other sites (Gordon and Lowy, 2008). The resistivity of *S. aureus* is due to the mecA gene that encodes for a protein that binds with penicillin which inhibits the activity of beta-lactam antibiotics. Whereas nosocomial ailments are the major cause of death ratio caused due to MRSA as it is more virulent than *S. aureus* (Shurland *et al.*, 2007).
Antibacterial activity of biosynthetic Silver nanoparticles on *S. aureus*. (a) Illustration of the zone of inhibition in *E. coli* (b) Where silver nanoparticles showed highest zone of inhibition against Positive and negative controls.

**Minimum Inhibitory Concentration (MIC)**

The minimum amount of solution to halt the growth of microorganisms was also evaluated using the MIC of Ag nanoparticles. Our results showed that Ag nanoparticles at 93.75 μg mL⁻¹ was effective against *S. aureus*, and 46.87 μg mL⁻¹ of Ag nanoparticles were effective against *E. coli* (Fig. 8 and 9). Ag particles also adhere to ribosomes and harm protein synthesis, thereby inhibiting protein synthesis (Xiu et al., 2012). Free radical by Ag nanoparticles makes it an effective biocide for resistant strains. About 70% of infections are caused by *S. aureus* whereas 18% are community-based and 59.8% are hospital infections (Raho and Abouni, 2015).

**Fig. 8.** Minimum Inhibitory Concentration (MIC) of 93.75 μg mL⁻¹ Ag nanoparticles was effective against *S. aureus*.
Resistance to existing antimicrobial agents especially trivial antibiotics is currently hitting the world, to combat its efforts cost-effective, nearly nontoxic products are constantly produced by pharmaceutical companies. Nanoparticles are thought to be an emerging effective strategy against existing resistance. Ag nanoparticles can fight up to 650 types of diseases. The general mechanism of action is attachment to the cell membrane, to the protein component of the membrane and DNA structure, resulting in function alteration impacting the membrane, nucleic acids (DNA), and protein content of the cell and oxidative stress mounting as a result of reactive oxygen species release with effects on the cell membrane, nucleic acids (DNA), and protein content of the membrane. Minimal inhibitory concentration is the lowest concentration of a chemical that prevents the growth of bacteria. Minimal bactericidal concentration (MBC) is the lowest concentration of a chemical that results in bacteria death. Previous research supported the use of MIC and MBC of Ag nanoparticles. Bacteriostatic activity of biogenic Ag nanoparticles toward B. subtilis, K. pneumoniae, E. coli, S. aureus, and S. infantis was recorded at low concentration (6.25 μg mL⁻¹); however, for P. mirabilis and P. aeruginosa, this value reached 50 μg mL⁻¹ and 25 μg mL⁻¹, respectively. For E. coli and P. aeruginosa, the Ag nanoparticles exhibited the lowest MBC at 50 μg mL⁻¹. Bactericidal activity of biogenic Ag nanoparticle for K. pneumoniae, P. mirabilis, B. subtilis and S. aureus was reported around 100 μg mL⁻¹ (Buszewski et al., 2018; Crisan et al., 2021).

CONCLUSIONS

Alkanna tinctoria is positive for carbohydrates, amino acids, proteins, saponins, alkaloids, fats and oil. The characterization was done by UV-vis and XRD which confirmed the synthesis of silver nanoparticles with a size range of 63-70nm. The synthesized Ag nanoparticles were tested to check antimicrobial properties that showed a significant effect on E. coli and S. aureus. The minimum dose of Ag nanoparticles to inhibit the growth was more effective on gram-negative as compared to gram-positive due to a thick peptidoglycan layer or some intrinsic factors. The biogenic synthesis of Ag nanoparticles is eco-friendly, economical, and effective in inhibiting bacterial growth at a lower dose. Therefore, further studies are required to check the effectiveness of these nanoparticles as nontoxic drugs for future use. The robustness and non-toxic nature of Ag nanoparticles in human tissues can be further investigated by comparing its efficacy with some other drugs.
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REFERENCES CITED


Bruneton, J. Pharmacognosy, phytochemistry, medicinal plants. 1995, Lavoisier publishing


Raho, G., &Abouni, B. (2015). Escherichia coli and Staphylococcus aureus most common source of infection. The Battle against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs; Méndez-Vilas, A., Ed.


