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Evaluation of Antimicrobial efficacy of Silver Nanoparticles synthesized using Leaf Extract of *Citrullus Colocynthis*

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Abstract

This study focused on the investigation of antimicrobial effectiveness of green-synthesized silver nanoparticles (AgNPs), created using an extract from *Citrullus colocynthis* (Bitter Apple) leaves. The prepared AgNPs were tested for their antibacterial properties against the pathogens namely against gram positive- *Bacillus cereus* and gram negative *Pseudomonas aeruginosa* pathogenic bacteria.

Driven by their ready availability and the avoidance of toxic chemicals, the demand for green synthesis of metallic nanoparticles has grown in various sectors, including electronics, water purification, catalysis, chemistry, and pharmaceuticals.

The synthesized silver nanoparticles were characterized using UV spectroscopy, FT-IR, Zeta potential, and TEM. A color change from clear to colored upon mixing the plant leaf extract with an aqueous silver nitrate solution indicated the extract's ability to act as a reducing, capping, and stabilizing agent. Transmission Electron Microscopy (TEM) confirmed the nanoparticles' spherical shape, with a size range of 10–35 nm, and revealed their primary morphology and size distribution. FT-IR spectroscopy results, observed between 1000 and 4000 cm^{-1}, suggested the surface of the AgNPs was functionalized with free and bound amide groups, as well as polyphenols featuring an aromatic ring

Keywords: Citrullus colocynthis, Antimicroial property, Silver Nanoparticles,

1. INTRODUCTION

Nanoparticles (NPs) are generally defined as particles ranging in size from 1 to 100 nm. Historically, Bhasmas, an ancient Ayurvedic medicine, represent the earliest known use of nanoparticles, composed of herbomineral metallic compounds typically sized between 5 nm and 100 nm. For instance, gold nanoparticles of 56nm have been identified in Swarna Bhasma (Sarkar et al. 2010).

Modern nanoparticle synthesis employs chemical, physical, and biological (green) approaches, each with unique advantages and disadvantages. Chemical methods are widely used and include techniques like chemical precipitation, sol-gel processes, hydrothermal methods, and reverse micelle systems.The green/biological method is an increasingly important and attractive platform because it is cost-effective, uses non-hazardous chemicals at ambient temperatures and neutral pH, requires low maintenance, and relies on the biodegradable nature of metabolites. This method uses extracts from plants (leaves, fruits, herbs), fungi, or algae, as well as proteins, to serve as stabilizing and reducing agents (Awwad et al. 2012, Kumar et al. 2013). Various primary and secondary metabolites, such as proteins, vitamins, terpenoids, polyphenols, and eugenol, act as reducing, capping, and stabilizing agents in this process (Shankar et al. 2008).

Nanoparticles, including metallic (silver, gold, platinum, palladium), ceramic, magnetic, polymeric, and lipid-based types (Hussain et al. 2018), are used extensively in modern medicine for treatment, diagnosis, and prevention of ailments (Zahin et al. 2020, Khan et al. 2019). Their broad utility stems from unique properties such as: Small size and large surface-to-volume ratio,

ability to encapsulate drugs better penetration through cell membranes, target specificity and high tunable properties for ligand binding and increased circulatory longevity.

Silver nanoparticles (AgNPs), in particular, are widely applied across fields including medicine, water treatment, catalysis, textile engineering, and electronics due to their unique fungicidal and bacterial properties (Satyavani et al. 2011, Ahamed et al. 2010). They are common in consumer products like textiles, soaps, food, and paints.

The plant C. colocynthis has been studied for its antimicrobial, antilipidemic, antioxidant and anticancer properties (Gupta et al. 2018), and is rich in biomolecules like flavonoids, polyphenols, and alkaloids (Abu et al. 2018). In the green synthesis of AgNPs, these phytochemicals function as reducing, capping, and stabilizing agents, converting silver ions (Ag^+) into free silver. Surface functionalization of metallic nanoparticles by these phytochemicals can further enhance their efficacy as biomedical agents due to synergistic effects (Ahmad et al. 2019).Keeping in mind the different findings reported in literature

Silver nanoparticles (AgNPs) are well-established for their antimicrobial and antiseptic properties against various bacterial strains (Lopez et.al. 2003, Bhanvase et.al. 2015). More recently, AgNPs synthesized via green techniques have demonstrated potential antitumor and hepatocellular carcinoma activity against multiple cancer cell lines, including A375 (skin melanoma), HeLa (cervical), and Hep-2 cells (Shukla et.al. 2010, Mishra et.al.

2003).. Furthermore, studies comparing synthesis methods, such as Nadagouda et al.'s work on iron nanoparticles using tea leaf-extracted polyphenols versus chemical methods, indicate that green-synthesized nanoparticles are often less toxic to human cell lines (e.g., keratinocytes)

Driven by these diverse and promising observations, the current study focuses on the green synthesis of silver nanoparticles using a leaf extract of Citrullus colocynthis (Bitter Apple). We proceed with characterizing these nanoparticles and assessing their antimicrobial activity against the pathogenic bacteria Gram-positive Bacillus cereus and Gram-negative Pseudomonas aeruginosa.

II. MATERIALS AND METHODS

2.1 Materials

Fresh Citrullus colocynthis leaves were sourced from the Regional Centre of Choudhary Charan Singh Haryana Agricultural University, Bawal, Rewari, India. For the experiments, analytical-grade silver nitrate ($AgNO_3$) was purchased from a commercial supplier, and distilled water was used exclusively. The study utilized the bacterial strains Bacillus cerus (MTCC- 1272) and Pseudomonas aeruginosa (PAO-1).

2.2 Preparation of Plant Extract

Citrullus colocynthis leaves were initially collected, thoroughly washed to remove surface contaminants, and then dried in an oven at 40^{o}C for three days to eliminate moisture. The dried leaves were subsequently pulverized into a fine powder using a grinder.

To prepare the plant extract, 20 g of the leaf powder was combined with 500 ml of distilled water in an Erlenmeyer flask and subjected to refluxing at 60 ^{o}C for 2 hours. Following the reflux, the solution was allowed to cool. Heavier biological materials were separated via centrifugation at 3000 rpm. The resulting supernatant was then filtered using Whatman No. 1 filter paper, and the obtained filtrate was refrigerated at 4^{o}C until its use in the silver nanoparticle synthesis.

2.3 Green Synthesis of Silver Nanoparticles AgNPs

Silver nanoparticles were synthesized by using 10 ml aliquots of the Citrullus colocynthis leaf extract, dropwise, into 100 ml of a (2 mM) $AgNO_3$ solution. This was performed with constant stirring at room temperature.

The successful formation of AgNPs was immediately evident by an instantaneous color change from white to brownish-red, a phenomenon characteristic of the Surface Plasmon Resonance (SPR) effect. The synthesized nanoparticles were then purified by ultra-centrifugation at 45000 rpm for 30 minutes using a REMI C-24 centrifuge (India). The collected pellets were washed three times with distilled water and subsequently redispersed in distilled water for use in further characterization analyses.

2.4 Characterization of Synthesized AgNPs

Ultraviolet-Visible Spectroscopy (UV-Vis)

Optical measurements were conducted using UV-Vis spectroscopy to track the bio-reduction of Ag^{+} ions. Aliquots of the reaction mixture were sampled periodically, and their spectra were recorded in the 300 - 600 nm range. The observed color change and the shift in the intensity of the UV-Vis absorbance peaks are attributed to the Surface Plasmon Resonance. Distilled water served as the reference blank for the spectral scanning.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed in the 500 – 4000 cm^{-1} range to identify the surface functionalization of the AgNPs by primary and secondary plant metabolites. AgNPs powder was placed on an ethanol-wiped surface, and infrared radiation was passed through the sample. The resulting absorption spectra were compared with values reported in the literature, which validated the surface functionalization of the silver nanoparticles achieved through the green synthesis method.

Transmission Electron Microscopy (TEM)

For morphology analysis, a drop of the washed, sonicated, and resuspended AgNPs sample was placed onto a copper grid. The solvent was allowed to evaporate under IR light for 5minutes. TEM images were captured by analyzing the interactions between the electron beam and the prepared sample.

III. Evaluation of Antimicrobial activity

3.1 Culture Media Preparation

The necessary quantities of nutrient agar and nutrient broth were prepared for microbial culturing and experimentation.

3.1.1 Nutrient Agar Preparation

Nutrient agar medium, essential for solid-surface microbial growth, was prepared by dissolving 28g of the commercial powder in 1 liter of distilled water. The mixture was then dispensed into conical flasks, which were plugged with cotton wool. Sterilization of the medium was achieved by autoclaving at 121ºC for 15 minutes under 15 psi of pressure.

Following sterilization, the molten nutrient agar was cooled slightly and then aseptically poured into sterile plastic Petri plates within a sterile environment (e.g., a laminar flow hood) to prevent contamination. The poured plates were left undisturbed to solidify and were incubated at 37ºC for 24 hours. Plates showing no microbial growth (uncontaminated) were subsequently used for culturing.

3.1.2 Nutrient Broth Preparation

Nutrient broth was used for liquid suspension culturing, shaking incubation, and standardization of the microbial cultures. This medium was prepared by dissolving 13 g of the nutrient broth powder in 1 liter of distilled water. Portions of the resulting liquid were dispensed into both conical flasks and test tubes 15 ml in the latter.

Sterilization followed the same protocol as the agar. The sterile nutrient broth in test tubes was later utilized for the standardization of the microbial cultures.

3.2 Microorganism Handling and Culturing

The antimicrobial efficacy of the silver nanoparticles (AgNPs), synthesized using a green method, was tested against two bacterial strains: Bacillus cereus and Pseudomonas aeruginosa.

The microbial stock cultures were revived by streaking them onto fresh nutrient agar plates using a sterile inoculation loop under a laminar flow hood. These primary streaked cultures were incubated at 37^{o}C for 24 hours and were again sub-cultured and incubate for 24 hour incubation at 37^{o}C.

These second-generation streaked cultures were then used to inoculate nutrient broth in flasks. The broth cultures were incubated at 37^{o}C for 18 hours with continuous agitation 200 (rpm) on a shaking incubator to achieve a standardized, actively growing liquid culture.

3.3 Agar Well-Diffusion Method

The antimicrobial activity of the synthesized silver nanoparticles (AgNPs) was evaluated using the Agar Well-Diffusion Method. An 8 hour-old liquid culture of the respective test bacteria was used to uniformly swab the surface of a sterile nutrient agar plate using a sterile cotton swab, creating a bacterial lawn. Wells with a diameter of 7 mm were created in the inoculated agar using a sterile gel puncture tool. Three different concentrations (5 ug, 20 ug and 30 ug of green synthesized AgNPs were added to the wells. The plates were then left at room temperature for the nanoparticles to diffuse into the agar.

The plates were then incubated at 37^{o}C for 24 hours. Average activity index was calculated by measuring the zone of inhibition (mm) in different fixed directions by maintaining triplicates.

IV. RESULTS AND DISCUSSION

4.4 UV- Visible studies

To confirm the synthesis of AgNPs via the green method, UV-Vis spectroscopy was performed, and the resulting spectra are shown in Fig. 1. The successful formation of silver nanoparticles was visually apparent by an immediate color change of the solution from clear to colored following the addition of the plant leaf extract to the aqueous silver nitrate. An absorption peak centered at 450 nm was detected for the AgNPs, which were synthesized using 6 ml of *Citrullus colocynthis* leaf extract. The presence of this peak in the range 400-500 nm, corresponds to the longitudinal surface 796lasmon resonance (SPR) characteristic of silver nanoparticles, provides confirmation of their successful synthesis (Singh et.al.2014). The precise location of this 796lasmon absorption band, which typically appears between 380–500 nm, is influenced by the size and shape of the nanoparticles (Peng et.al.1995).

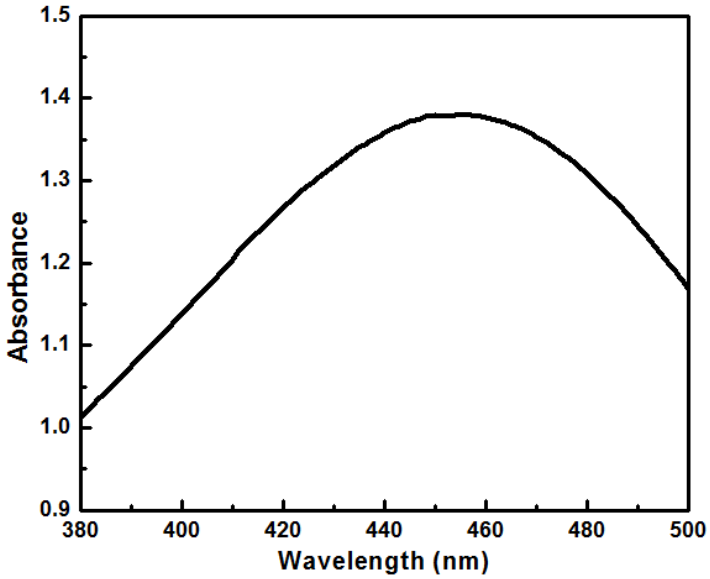

Fig. 1: UV-Vis spectra of *Citrullus colocynthis.* AgNps

4.5 FTIR Studies

Deepak et. al. studied the FTIR absorption spectra of AgNPs synthesized using leaves extracts of *Citrullus colocynthis* in their previous studies which is shown as Fig.2.

The absorption peak observed at 1023 cm-1 likely corresponds to the C-N stretching vibrations found in aliphatic amines, alcohols, or phenols, suggesting the presence of polyphenols (Wahl et al. 1995). At 1231 cm-1, the absorbance peak may indicate the presence of the amide III group, while the peak at 1515 cm-1 represents the symmetric stretching vibrations of carboxylate ion groups (–COO-) from amino acid residues featuring free carboxylate groups in proteins (Songa et al. 2009). Additionally, the peak at 1610 cm-1 can be attributed to carbonyl groups (C=O) from polyphenols such as catechin, epicatechin, theaflavin, and gallate (Huang et al. 2007). The absorption bands at 2920 cm-1 most likely correspond to C-H stretching vibrations. The peak at 3255 cm-1 indicates the presence of polyphenolic hydroxyl groups (-OH) linked to alcohols, geraniols, or carbohydrates, while the peak at 866 cm-1 may represent C-H vibrations from aromatic rings, highlighting the involvement of free catechin (Elemike et al. 2017). The results suggest that the molecules bound to the silver nanoparticles may contain free or bound amide groups, which are likely situated within the aromatic rings. This leads to the conclusion that the compounds associated with silver nanoparticles could be polyphenols featuring aromatic rings and bound amide regions. Overall, the FTIR bands indicate the presence of metabolites such as tannins, flavonoids, catechins, lignans, sterols, and other polyphenols in the leaf extracts, which not only play a role in the synthesis of silver nanoparticles but also serve as stabilizing agents for these nanoparticles (Singh et al. 2009).

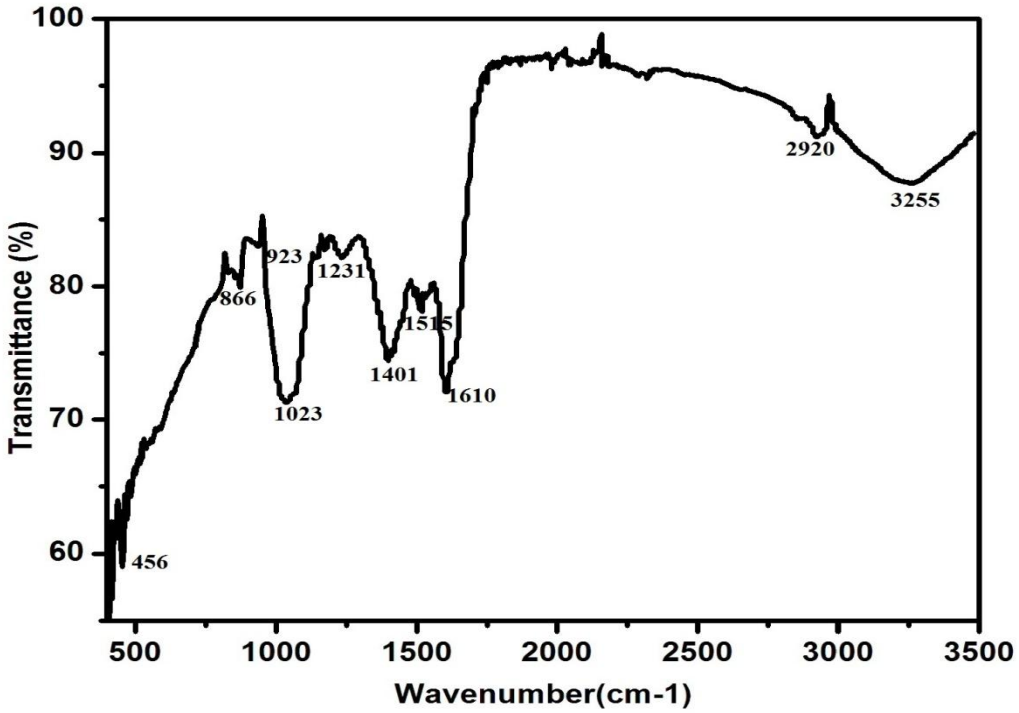

Fig. 2: FTIR spectra of *Citrullus colocynthis* AgNps (Deepak et.al.)

4.6 TEM Studies

Fig. 3 shows the representative TEM images of silver nanoparticles synthesized using *Citrullus colocynthis* leaves extract. TEM images reveal the primary morphology, shape and size distribution of synthesized AgNPs. TEM images reveal that synthesized AgNPs were spherical in size having size in the range of 10 – 35 nm.

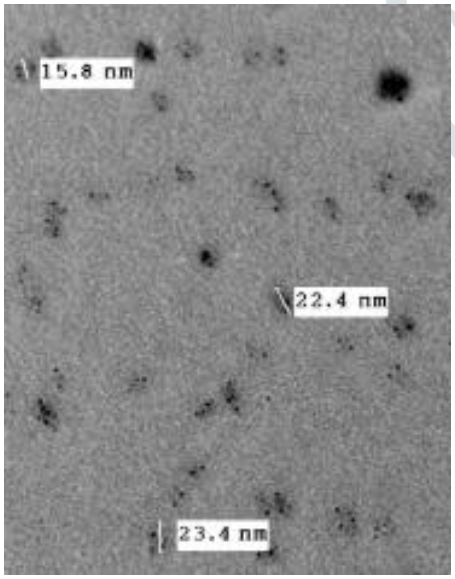

Fig. 3: Representative TEM images of AgNps synthesized using 6ml of leaf extract.

4.7 Zeta Potential Studies

The stability of nanoparticles in an aqueous suspension is directly related to their zeta potential. As an indicator of surface charge, a higher zeta potential whether positive or negative creates greater electrostatic repulsion between particles, which prevents agglomeration (Haier et.al. 2014, Chaudhuri et.al. 2016). The measured zeta

potential of -42.32 mV for the synthesized silver nanoparticles (AgNPs) suggests high stability, demonstrating that negatively charged metabolites have successfully functionalized their surface

V. ANTI MICROBIAL ACTIVITY

Fig. 4: Represent the zone of inhibitions against (A) *Bacillus cereus* and (B) *Pseudomonas aeruginosa.* Whereas (1) represent pure plant extract, while (2, 3, 4) represent 5ug, 20ug and 30ug concentration of AgNPs respectively.

Fig.4 shows the antimicrobial activity of AgNPs synthesized using leaf extract of *Citrullus colocynthis* against gram-postive as well as gram-negative bacteria strains. The zone of inhibition increases with increase in concentration of AgNPs. The AgNPs shows the higher antimicrobial efficacy at all concentration against gram negative bacteria (*Pseudomonas aeruginosa)* compared to gram positive bacteria (*Bacillus cereus)*. The smaller zones of inhibition observed against Gram positive bacteria (Bacillus Cerus) may be due to reduced liability and diffusibility of AgNPs owing to electrostatic repulsions between Peptidoglycan molecules of Gram positive bacteria and negatively charged AgNPs (Loza et.al. 2014). It is pertinent to point out here that the AgNPs synthesized in the present work using leaf extract of *Citrullus colocynthis* showed significant antimicrobial activity against the bacterial strains namely *Bacillus cerus,* and *Pseudomonas aeruginosa*

VI. CONCLUSION

Silver nanoparticles synthesized with a green approach are environmentally friendly and can replace toxic and costly physical and chemical methods. Silver nanoparticles synthesized with plant extracts are therefore better agents for the mitigation of microbial toxicants and this current study concludes that AgNPs synthesized using *Citrullus colocynthis* can be used for mitigation of microbial toxicants

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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