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Antimicrobial effect of silver nanoparticles synthesized from skin of *Laudakia agrorensis*

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SUMMARY

In this research developed commercial methods for the synthesis of silver nanoparticles utilizing specific concentrations, temperatures, and volumes. The antibacterial activity of 0.3mg nanoparticles against four human bacterial pathogens was tested. The results show that LAAgNPs (*Laudakia agrorensis* silver nanoparticles) are more effective against bacterial pathogens than LAAqu (aqueous extract of *Laudakia agrorensis*). The maximum zone of inhibition in LAAgNPs for antibacterial analysis is found in the bacterial pathogens Proteus mirabilis, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Some bacterial pathogens, such as *Klebsiella pneumoniae* and *Staphylococcus aureus*, represent a moderate zone of inhibition in LAAqu for antibacterial analysis, while *Proteus mirabilis* and *Pseudomonas aeruginosa* represent no outcome. The finding represents that the inhibitory impact was high against *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Antibiofilm observation also revealed that LAAgNPs performed LAAqu in terms of efficacy. LAAgNPs have the greatest potential to reduce biofilm formation for all four tested bacterial pathogens, whereas LAAqu have no such potential. As a result, LAAgNPs are more effective and can be used in future medical research.

Keywords: Nanoparticles, Nanotechnology, Agror agama, *Klebsiella pneumonia*

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INTRODUCTION

Nanotechnology is the utilization and the creation of devices, systems and materials, through 1 billionth of a meter in the length scale and when it apply in science of life it is known as nanobiotechnology (Jain, 2005). Nanotechnology is frequently recognized by two basically dissimilar methods “bottom-up and top-down”. In the ‘top-down’ structures of nanoscale formed by templating, lithographic techniques and machining, while ‘bottom-up’, used for formation of inorganic and organic substance into distinct structures, molecule by- molecule or “atom-by-atom” frequently by self organization or self-assembly. Researcher are involved in the formation of organic, hybrid and inorganic nanomaterials, for the purpose of utilization in nano-devices, nano-diagnostics techniques such as nanoparticle biolabels, nanoscale visualization, nanoparticle-based nucleic acid diagnostics, biochips/microarrays, Biobarcode assays,

nanoproteomic-based diagnostics, DNA nanomachines, nanoporetechnology, nanobiosensors, nanoparticle-based immunoassays, etc., are progressing admired in the ground of medical diagnostics. For mutual therapeutics and diagnosis purpose, nanodevice might be fixed as prophylactic and preventive measure. In the molecular diagnostics advantages of applied nanotechnology is due to its smaller size and faster reaction ability (Jain, 2007).

Nanotechnology is just like a rising ground of science which contain development and synthesis of a variety of nano-materials. Nanoparticles are those particles that have size 1- 100 nm and because of their smaller size these particles are entirely different from large materials. Diverse nanomaterials of metal are being formed by utilizing zinc, copper, magnesium, titanium, alginate, silver and gold. Nanoparticles are utilized for various purposes, using in various areas of industry formation like oxide and solar fuel battery for power storage, from medical treatments and in clothes or cosmetics (Dubchak *et al*., 2010).

Nanoparticles (NP) are incidental, natural and made from material like particles, that have size from 1–100 nm (Ema *et al*., 2017). Nanoparticles have two types (i) organic, and (ii) inorganic. Nanoparticles that are known as inorganic NPs include metallic nanoparticles (like Au, Cu, Al and Ag), magnetic nanoparticles (like Ni, Co and Fe) and semi-conductor nanoparticles (like ZnS CdS and ZnO) whereas organic nanoparticles include carbon nanoparticles (such as quantum dots, fullerenes, carbon-nano tubes) (Rafique *et al*., 2016). Engineered nanoparticles (ENPs) have high antimicrobial/fungicidal activity, high chemical reactivity, biocompatible surface properties and surface area to volume ratio (Gitipour *et al*., 2016).

Silver is utilized for dissimilar purposes in diverse forms. Form longer time silver has antibacterial properties that utilized for sterilize drinkable water by storage in silver bottol (Amato *et al.*, 2011; McGillicuddy *et al*., 2017). The Macedonians reported about the utilization of plates of silver to develop wound healing and Hippocrates also demonstrated that silver is used for curing of different kinds of ulcers. Silver utilization for antibacterial activity is not considered as a current progress (Nowack *et al*., 2011).

Normally, three different methods are used for the synthesis of nanoparticles, such as biological physical and chemical methods. By using a tube furnace at distinctive pressure, nanoparticles are synthesized through the process of evaporation-condensation in the physical method (Kruis *et al*., 2000). In the physical approach pyrolysis and spark discharging are utilized for the formation of silver NPs (Tien *et al*., 2008). Among physical methods the benefits are radiation used as reducing agents, absence of uniform distribution, no dangerous chemicals used, low yield are required and large amount of energy use, solvent contamination and are rapid (Shameli *et al*., 2010; Elsupikhe *et al*., 2015). Traditional study of herptiles is known as ethno-herpetology. Cross-cultural traditional studies are critical for understanding human populations and their utilization of biodiversity (Alves and Rosa, 2005; Altaf *et al.*, 2017; Altaf *et al.*, 2018; Altaf *et al.*, 2020). These animals are usually used to treat joint pain, backbone pain and impotency (Altaf *et al.*, 2018).

Chemical methods employ organic solvents or water to synthesize the Ag nanoparticles (Tao *et al*., 2006). This process occurs in three major steps of mechanism, like reducing agents, stabilizing/capping agents and metal precursors. On

the whole, the lessening of silver salts involve into two stages (1) subsequent growth and (2) nucleation. Commonly, Ag nanoparticle can be formed by two approach, classify as “bottom-up” and “top-down” (Deepak *et al*., 2011). The “top- down” approach involve in the synthesis of large metals with succeeding stabilization by means of defensive agents (Mallick *et al*., 2004). The “bottom-up” approach contains electrochemical methods, sono-decomposition and chemical reduction. The main benefit of chemical approach is large yield, as compared to physical approach that contain small yield. Chemical approach is hazardous and toxic (Mallick *et al*., 2004). The synthesis of silver NPs with unique sizes is very difficult process (Malik *et al*., 2002). The objectives of the research are to prepare the “AgNPs” utilizing skin extract of Agror agama, “characterize the AgNPs” by using “Ultra Violet-Visible Spectroscopy”, to know “antimicrobial activity” and “antibiofilm assay”.

MATERIAL AND METHOD

Data were compiled in “Department of Zoology, Women University of Bagh, Azad Jammu and Kashmir, Pakistan”.

COLLECTION SPECIMENS

The “*Laudakia agrorensis”* was taken from “Bagh, Azad Jammu and Kashmir, Pakistan”. The lizard was recognized using book "The Amphibians and Reptiles of Pakistan" by Khan (2006).

PREPARATION OF EXTRACT

Reena's modified technique was used to make the extract (2014). Using a mill and pestle, the lizard skin sample was coarsely mashed. Using double-distilled Milli-Q water, the extract was prepared up to 100mL. Following that, the extract was filtered using "Whatman N0. 1 filter paper" to remove the “tissue rubbles” and get a “pure extract”.

PREPARATION OF SILVER NANOPARTICLES

The filtrate of *Laudakia agrorensis* was employed in the manufacture of nanoparticles. To avoid silver auto-oxidation, “1 mM silver nitrate” was prepared in “250ml” of distilled water and kept in an amber coloured container. In a typical silver nanoparticle production, “10 ml” of liquid extract was used with “90 ml” of “1mM silver nitrate solution” and stored at “room temperature”. Following that, the creation of dark brown hue was used to identify the making of silver nanoparticles.

CHARACTERIZATION OF SILVER NANOPARTICLES

The first stage in characterizing biologically produced nanoparticles (LAAgNPs) was performed using a “UV–Visible spectrophotometer”. The spectra were noted from “200 to 800 nm”.

IN VITRO ANTIBACTERIAL ACTIVITY

The antibacterial efficacy produced silver nanoparticles to treat four harmful bacteria were tested using the agar well diffusion technique (Rios *et al.,* 1988). Staphylococcus aureus, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* (human

clinical bacteria), and Proteus Mirabilis were tested for antibacterial efficacy. These bacterial strains were collected at Al Khidmat Hospital Bagh's Pathalogical Laboratory in Azad Kashmir, Pakistan. To grow bacteria, "nutrient broth" medium and "nutrient agar" media were utilized. The bacteria were prepared by "inoculating a loop" with bacteria in "nutrient broth 25 ml" and incubating it whole night in a "rotary shaker" at "37^{o}C" for "24 hours".

The freshly made "nutrient agar" was combined with the "overnight culture" at "45^{o}C" before being placed into the "Petri plates". These were placed in the laminar flow at room temperature to solidify. Three 5 mm diameter wells were formed in each plate using a sterile micropipette tip (1ml), and the agar plug was removed using sterilised needles. A control solvent, were put into each prepared and after that placed in an incubator at "37^{o}C for "24-48 hours". Bacterial growth was determined after "24 hours" by estimating the width of the zone of inhibition in millimetres (mm) (Seeley and VanDemark, 1962). If the zone diameter was higher than 5mm, it was determined using a scale (Hammer, 1999). Outputs were showed as "zero" for "no sensitivity", "*1-5 mm" for "low sensitivity", "**>5- 10 mm" for "moderate sensitivity" and "***>10- 25 mm" for "high sensitivity".

ANTI-BIOFILM ASSAY

The "antibiofilm activity" was determined using a "crystal violet test" with minor changes (O'Toole, 2011). Bacterial growth was carried out in "Boro-silicate tubes", with lal tube containing "30 mg/ml" of "LAAqu" and "LAAgNPs" and "2 ml" of "nutritional broth medium". The tubes were then put in "incubator" at "37°C" in whole night. As a control, "chloramphenicol" and "silver nitrate" were used. Only nutritional broth was used as a negative control. The broth medium was removed the next day, and adherent cells were stained with "0.1 percent" crystal violet. These were after that incubated for "10 to 15 minutes" at "room temperature" before being washed with water to remove the "dye" and any additional adhered cells. The biofilms were then mixed in "30% acetic acid" after that "incubated" at "room temperature" for "10 to 15 minutes". A spectrophotometer was used to quantify the concentration of dissolved crystal violet at "550 nm".

RESULTS AND DISCUSSION

SYNTHESIS OF AgNPs OF *LAUDAKIA AGRORENSIS*

When silver ion start to mix with aqueous extract of *Laudakia agrorensis* (LAAqu), the reduction process take place. LAaqu extract had yellowish color before the addition of $AgNO_3$. After adding $AgNO_3$ in the LAaqu extract and then boiling it for 15- 20 min the color of LAAqu changed into dark brown that indicate the formation of silver nanoparticles of *Laudakia agrorensis* (LAAgNPs) (Figure 4.1).

Surface plasmon resonance (SPR) is liable for brown color solution. Due to excitation of surface plasmon sensations in AgNPs, it starts to show a yellowish color in igneous solution (Jae and Beom, 2009). The process of Ag ions decline because of color change that is helpful for nanoparticles examination (Sastry *et al*., 1997; Sastry

et al., 1998). As a result, the changes from Ag ions to nanoparticles of silver were observed through spectroscopic techniques and change in color.

CHARACTERIZATION OF *LAUDAKIA AGRORENSIS*

UV- Visible Spectrometry

In the current study UV-Visible spectrophotometer was used to characterize the LAAgNPs that were synthesized biologically. The range of absorbance was noted that confirmed the synthesis of LAAgNPs (Figure 1).

UV-visible spectroscopy is utilized for observing the consistency and formation of AgNPs (Sastry *et al*., 1998). UV technique is simple, rapid and takes little time for measuring the SPR of diverse kind of nanoparticles (Alhede *et al*., 2011). Free electron is moving in the valence band and the conduction band of AgNPs that are very close to one another. "Surface Plasmon Resonance" is formed due to these free moving electrons (Das *et al*., 2009). The inclusion of "AgNPs" based on the "chemical environment", "dielectric medium", and "size of particle" that all are very important in "AgNPs" absorption (Link and Sayed *et al*., 2003). The SPR of metallic nanoparticles of different sizes are less than 99 nm. The constancy of AgNPs that are prepared from biogenic method remains same more than one year (Sastry *et al*., 1997).

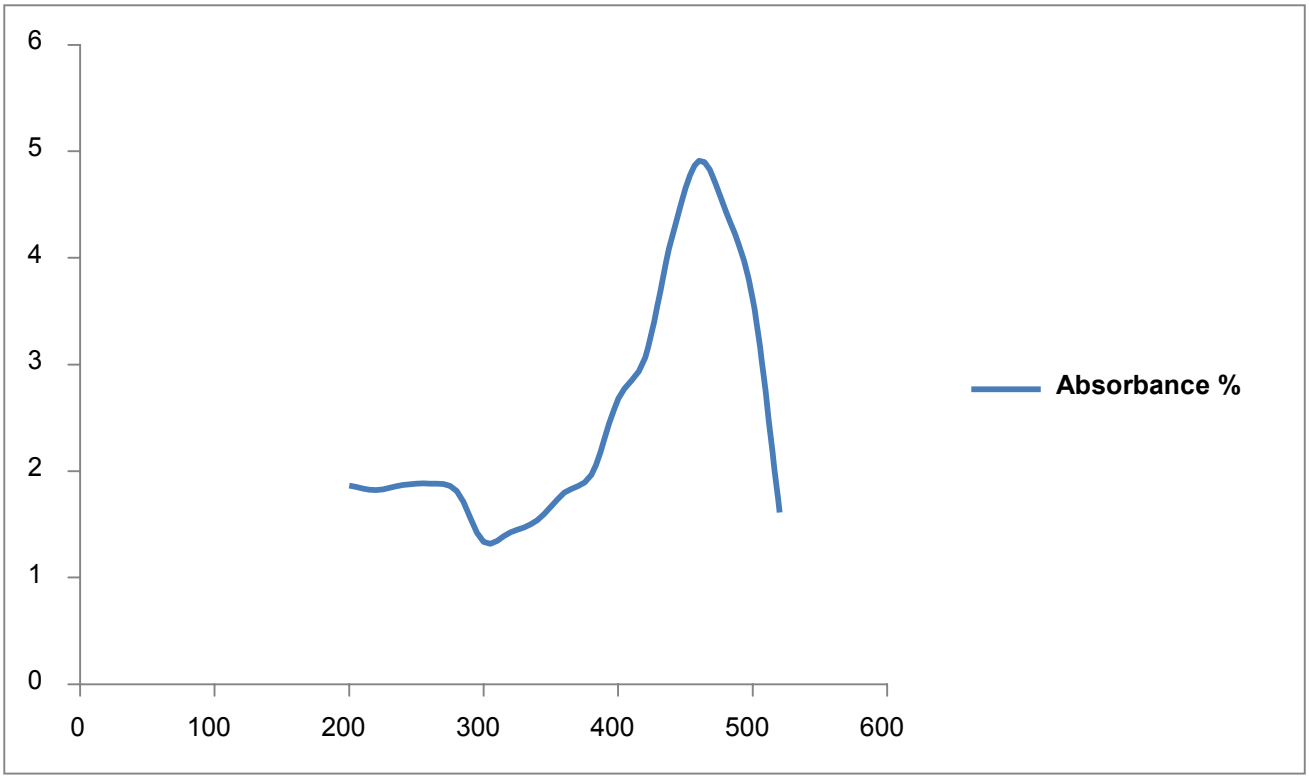

Figure 1: Characterization of silver nanoparticles synthesized from *Laudakia agrorensis* via UV-vis spectrophotometer.

Antibacterial Effect

The current study began with an agar well diffusion experiment to investigate the antibacterial activity of biologically produced "silver nanoparticles of *Laudakia*

agrorensis" (LAAgNPs) and "its aqueous extract" (LAAqu) against four disease-causing bacteria. LAAgNPs were shown to have a strong bactericidal impact against all tested bacterial pathogens in the current investigation. LAAgNPs had considerable antibacterial activity at "0.3 mg/ml" concentration (Table 1).

Inhibitory Effect of LAAqu and LAAgNPs

When compared to LAAqu, LAAgNPs demonstrate better sensitivity against all four pathogenic bacteria in the current study. All four pathogenic gramme negative, "*K. pneumoniae, P. aeruginosa*, *P. mirabilis*" and gramme positive, "*S. aureus*" bacteria examined had a zone of inhibition. Because "LAAqu" showed "no results" against two gramme negative bacteria, "*P. aeruginosa* and *P. mirabilis*", "LAAgNPs" showed the "highest zone of inhibition" against all four tested "pathogenic bacteria", ranging from 91.732mm to 145.527mm, as compared to LAAqu, which showed a zone of inhibition between 95.196mm and 102.544mm.LAAgNPs showed maximum zone of inhibition against pathogenic bacteria *P. mirabilis* had a zone of inhibition of 145.527mm, *P. aeruginosa* had a zone of inhibition of 124.577mm, and *K. pneumoniae* had a zone of inhibition of 112.309mm, but LAAgNPs had a moderate zone of inhibition of 91.732mm against S. aureus. LAAqu had a moderate zone of inhibition against *K. pneumoniae* (102.544mm) and *S. aureus* (95.196mm), but no findings against *P. aeruginosa* or *P. mirabilis* bacteria (Table 1).

Table 1: Antibacterial activity of LAAqu and LAAgNPS against bacterial pathogens.

Test sample pathogens	**LAAqu**	**LAAgNPs**	**Choramphenicol (10ug)**
Staphylococcus aureus	9±5.196	9±1.732	15±0
Klebsiella pneumonia	10±2.544	11±2.309	21.3±3.21
Pseudomonas aeruginosa	0	12±4.577	25±5
Proteus mirabilis	0	14±5.527	17.3±0.57

Kuppulakshmi *et al*. (2008) noted the results in the previous study of antibacterial activity of *Channa punctata* and *Cirrhinus mrigala* are showed in the maximum inhibition zone was noted against "*Vibrio cholera* and *Staphylococcus aureus"* while least inhibition was noted against "*Salmonella paratyphi"*. On the other hand bacteria show a significant inhibition in their growth in the order of *Salmonella typhi*, *Lactobacillus vulgaris*, *Pseudomonas aeruginosa, Proteus mirabilis*, *Klebsiella oxytoca, Escherichia coli and Klebsiella pneumoniae* respectively.

Why Gram Negative Bacteria are More Resistant to Antibiotics

Outer cover consists of three layers in the Gram-negative bacteria. The "first layer" is the external membrane, a unique feature and a protective that distinguish "Gram-negative bacteria" from "Gram-positive bacteria". Outer membrane contains phospholipid and proteins that known as outer membrane proteins (OMPs) and others that allowed the passage of tiny molecules such as ike small saccharides and amino acids. The 2nd layer is the v cell wall that made up of peptidoglycan which is a stiff exoskeleton (Silhavy *et al*., 2010). The 3rd layer is the "inner membrane" that is a

phospholipidbilayer that is dependable for multi-functional process such as biosynthetic, structure and transport functions (Murray *et al*., 2005).

The external wall of "gram-negative bacteria" is the chief motive for struggle to a broad variety of antibiotics such as quinilons, colistins, β-lactams. Some antibiotics able to pass the external membrane to entrée their targets, such as hydrophobic drugs able to cross through by a diffusion way, while water loving antibiotics such as β-lactams cross through vancomycin and porins, can't cross the outer membrane because of its structure that delay it from use any of these passages. Gram-positive bacteria don't have this significant layer, that make in the Gram-negative bacteria extra resistant to antibiotics as compared to Gram-positive ones (Datta and Gupta, 2019).

Anti-Biofilm Assay

In this study the affect of "LAAqu and LAAgNPs" on "biofilm formation" were noted via "crystal violet assay". These results showed that all the observed "4 bacteria" were not capable to prepare biofilm in the occurrence of "LAAgNPs" and "chloramphenicol" while "LAAqu" "4 tested bacteria" were capable to form "biofilm". Outputs showed that "LAAgNPs" had high potential to decrease biofilm as judge against to "LAAqu" but all the tested groups represent high potential to decrease "biofilm formation" at a stage of significant (Table 2).

Table 2: Mean and STD of anti-biofilm activity of LAAqu and LAAgNPS against bacterial pathogens.

Test organism	**Mean and STD of LAAgNPs**	**Mean and STD of LAAqu**	**Pathogen**	**Chloramphenicol**
S. aureus	0.448±0.0133	0.256±0.0321	1.45	0.12
P. aeruginosa	0.442±0.0233	0.256±0.0422	1.45	0.023
P. mirabilis	0.452±0.0252	0.269±0.0433	1.05	1.430
K. pneumoniae	0.184±0.0288	0.229±0.0334	1.542	0.027

Biofilms are bacterial complex netwok (Johnson and Reddish, 2007). This soft layer provide friendly climate for microorganisms development (Rao *et al*., 2005). Inner "biofilm" microscopic organisms turn into competent and sweeping to line with one another through intracellular connection as consequence indicate the variable environment (Clutterbuck *et al*., 2007). Inside these changes character of surface and foundation of microbial cells are normally important and different microbial diseases are caused the effect on biofilm arrangement that showed defense from diverse antitoxins (Shi and Zhu, 2009). Biofilm is useful for all microbs just like (1) separation towards addition rich zone (colonization), (2) defensive against negative circumstances in have guard, (3) biofilms frequently expand as "biofilms" and "planktonic" society are an in vitro abscond, (4) employment of a variety of pleasant advantages (network) (Jefferson, 2004).

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