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Biosynthesis of silver nanoparticles from skin of Hazara toad (*Bufo melanostictus*) and assessment of antibacterial activity

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SUMMARY

Current developments in nano-science and nanobiotechnology completely changed the method of analyze, treat and prevent different ailments in all features of life. Silver nanoparticles are one of the most vital and fascinating nonmaterial among several metallic nanoparticles that are involved in biomedical applications. The importance of bactericidal nonmaterial study is because of the increase in new resistant strains of bacteria against most potent antibiotics. The aim of current study was to synthesize silver nanoparticles from *Bufo melanostictus*, characterize and to evaluate the antibacterial and antibiofilm activities. The preliminary synthesis was noticed as color changed from light brown to dark brown which indicated the formation of silver nanoparticles. Characterization was carried out by UV-Visible spectroscopy; obtained range of absorbance was noted at 450 nm. The highest significant effective antibacterial was shown by *B. melanostictus* silver nanoparticles against *Proteus mirabilis* as 8.333 ±0.577 and *Klebsiella pneumonia* showed moderate inhibition and was recorded as 7.666 ± 0.667, *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed lowest zone of inhibition (5.234± 0.698 and 5.333 ± 0 .577 respectively). BMAqu showed no activity against these pathogens. It was concluded that *B. melanostictus* silver nanoparticles seem to be antibacterial agents.

Keywords: *Bufo melanostictus*, Nanobiotechnology, Spectroscopy, Bacteria

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INTRODUCTION

Nanotechnology has range 1 to 100 nm and is essential field of research that explains structure, design and synthesis of particles. Nanoparticles used in many fields such as health, makeup, foodstuff, cleanliness, biomedical sciences, industries, sight, electronics, drug-gene delivery (Colvin *et al*., 1994). Nanobiotechnology is a field of science that is fast developed, generating, modeling devices and production of chemical compositions, morphologies, restricted differences. Nanobiotechnology jointed new era and attain worldwide consideration because of large uses. It is used in a range of fields like medication, engineering, environmental sciences (Hoffman *et al*., 1992; Colvin *et al*., 1994).

Silver nanoparticles consist of huge amount of silver oxide. Universally used these particles are convex, slim sheets and geometric are also

common (Graf *et al*., 2003). Silver nanoparticles are essential particles that are related with biomedical applications. Silver nanoparticles have a great place in nanoscience, nanotechnology and nanomedicine. Following studies shows the peptides that kill the bacteria and fungi present in frog skin. Some major antibiotics are also discovered from frog skin for example, genus Rana (Channing, 2006). These particles due to its distinctive property present in makeup products, electronic components and biosensor materials. Some specific procedures are used for producing and maintaining the silver nanoparticles (Klaus-Joerger *et al*., 2001; Senapati, 2005).

Antibacterial effect of silver (Ag) has been most effective and enormously used in medicine (Brennan *et al.*, 2015). Silver synthesized through nanotechnology in to the silver nanoparticles (AgNPs) and has following properties, like physical chemical and biological. Currently, the uses of these particles on physically unfitness improvement and avoid infection effects that has more concentration (Xiang *et al.,* 2017). These particles have the great importance in the field of orthopedic improvement and the main role in infection control (Hardes, 2017). Since silver nanoparticles (AgNP) covered orthopedic implants performa hopeful approach to avoid these problems (Wang *et al.,* 2016).

Moreover, the antibacterial process of these particles have capability to generate reactive oxygen and free radical species as hydroxyl radical, hypochlorous acid, hydrogen peroxide, superoxide anion, and oxygen (Zhao *et al.,* 2017).Under normal situation, ROS is produced in cells and can be removed by antioxidant systems. During the inactivation of respiratory chain dehydrogenates and large ROS production, then AgNPs exert an antibacterial effect which constrained cell growth (Yuan *et al.,* 2017). Higher ROS lead to a cell death like response, DNA damage, lipid per oxidation and depletion of GSH (Hwang, 2012).

An infection is defined as, breaking the body tissues by infection-causing agent (Ghana, 2015). An infection is epidemic sickness, also famous as a contagious sickness. Following infectious agents are fungi, parasites, viroids and parasites etc. (Brown and Peter, 1987). Pathogens cause diseases when their existence was related with the common good physical host. Maximum human pathogens infect singly human host. Other pathogens create contagious infection in a host with low resistance. These infections maybe created by microorganisms when contact with host or when they unusually enter in to the body (Ryan and Ray, 2004).

In biology, a pathogen is the clear and largest sense that causes diseases. Pathogen is also called disease causing agent. This term explains briefly in 1880s (Casadevall and Pirofski, 2014). Small animals for example, worms and insect larvae, can also cause diseases. The lesson of pathogens disease is called pathology and the diseases created by these pathogens are called pathogenic diseases. Several diseases are not pathogenic like, Huntington's disease, this disease is inherited. So, pathogen is defined as an infectious germ (Alberts *et al.,* 2002). Algae are also nonpathogenic. Similarly, Protothecosis is found in animals because of a green alga. These are both non pathogenic (Satoh *et al*., 2010).

The range 0.15 and 700 μm length of bacterial mass are may or may not good to humans (Weiser and Jeffrey, 2013). Pathogenic bacteria create diseases through various methods. Firstly, these bacteria directly damage the host cell. Secondly create enough immune response then host cell also damaged. One bacterial disease is

tuberculosis; their bacterium is Mycobacterium tuberculosis which killed 1.5 million humans (Zumla *et al.,* 2015).

OBJECTIVES

- To biosynthesis of silver nanoparticles via skin of *Bufo melanostictus.*
- Silver nanoparticles of *B. melanostictus* are characterized by UV spectroscopy.
- To evaluate the antibiofilm assay.
- To evaluate antibacterial activity of silver nanoparticles of *B. melanostictus.*

MATERIALS AND METHODS

This examine has completed in “Department of Zoology, University of Women in Bagh, Azad Jammu and Kashmir, Pakistan”.

COLLECTION AND IDENTIFICATION OF HAZARA TOAD

Collection of Hazara Toad *(B. melanostictus)* was done from Poonch district, AJK, Pakistan. Identification of toad was done by the book named “Amphibians and reptiles of Pakistan” by Khan (2006).

EXTRACT PREPARATION

Extract were ready by means of the special method. The toad samples were strongly crushed by mortar and pestle. Distilled water was used in preparation of extract. The extract was made up to 100mL. Then extract was cleaned or filtered through “Whitman filter paper”. Through “Whitman filter paper” separates the cells and get a “pure extract”.

SYNTHESIS OF NANOPARTICLES

The Hazara toad (*B. melanostictus*) filtrates were used for synthesis of nanoparticles. Preparation of 1mM “silver nitrate” (0.0421 g) was included in “250ml” of water (distilled). Than stored in the “amber-colored” container to prevent the silver from “auto-oxidation”. For silver nanoparticles produce 90ml of 1mM silver nitrate solution was treated with 10 ml of aqueous extract and kept at room temperature. Brown color was appeared in the solution. This point showed that the formation of silver nanoparticles.

ANTIBACTERIAL ACTIVITY

In this activity, these particles that are synthesized, was performed against these microorganisms by agar well diffusion technique (Rios *et al.,* 1988). This action was performed adjacent to these pathogens. These strains of “bacteria” were gained from a Pathological lab, from hospital Islamabad, Pakistan. These “bacteria” were grown by means of an “inoculating loop” having bacteria in “nutrient broth” (25 ml) as well as keep warm in a shaker at “37ºC” for a full day. The “agar was mixed with all night culture at “45ºC” and after that put into the sterilized “Petri dishes”. These plates were set to the side at area in the “laminar flow” for solidify. The 3 wells having five millimeter diameter was arranged by means of uncontaminated “micropipette tip” (1.0 ml) in every one dishes, then uncontaminated “needles” were apply to eliminate the plug of agar. Approximately thirty µl of extract and these

particles (BMAqu and BMAgNps), solvent taken as control were put in each ready well and next located in an incubator for one to two days at “37ºC”. The bacterial growth was calculated later than 24 hours by calculating the diameter of the region of inhibition in millimeter (mm) (Seeley and Van Demark, 1962). Zones diameters were considered throughout scale if larger then 5mm. Results were showed as “zero” for no sensitivity, one to “five mm” for small sensitivity, “five to ten mm” for modest sensitivity, and “ten to twenty-five mm” for elevation of sensitivity.

THE ANTI-BIOFILM ASSAY

The “antibiofilm” action was calculated throughout a “crystal violet assay” with little modification (O'Toole, 2011). Bacterial growth was complete in “Boro-silicate tubes” (Minitek, USA) every tube consist of “30 mg/ml” of BMAqu and BMAgNPs and “2 ml of nutrient broth medium” next located in an incubator at “37°C” for the night. “Chloramphenicol” and “AgNPs” was in used as control. Meant for harmful control nutrient broth was in use. After that day “broth” was removed and discoloration of closed cells was complete by means of 0.1 percent “crystal violet” (125 µl). Tubes were next incubated for “10 to 15” minutes at heat after that rinsed with water thus color and additional single cells were detached. After that mixed these biofilm in 30 percent acetic acid and incubated for “10 to 15” minutes at heat. At “550 nm” mixed “crystal violet” was calculated by means of a “spectrophotometer”.

RESULTS AND DISCUSSIONS

When an aqueous extract of Hazara toad (*B. melanostictus)* BMaqu was mixed with the silver ions, $AgNO_3$ (1mM) solution changed from yellowish to brown, the final dark brown color appeared immediately. The common fact is, in aqueous solution silver nanoparticles have brown colour and shown excitation of surface plasmon vibrations (Nithya and Ragunathan, 2009). Deep brown color appeared while after boiling for minutes. This process showed that particles of silver were appeared, BMAgNPs using BMaqu extract. Yellowish-brown to dark brown color showed the biosynthesis of BMAgNPs.

CHARACTERIZATION TECHNIQUE

UV-Visible Spectrometry

In current study, UV-Visible spectrophotometer characterized the silver nanoparticles that were prepared biologically and chemically. The BMAgNPs were characterized in a “UV-Vis spectrophotometer (T80, PG instruments Ltd)” were used. Obtained range was present between “200-800” nm wavelengths. The range of absorbance was noted at 450 nm noted the BMAgNPs synthesis (Figure 2).

ANTIBECTERIAL ACTIVITIES

Silver nanoparticles that were synthesized whose antibacterial activity against clinical isolates was assayed by agar well diffusion method. From the current study, it was observed that BMAgNPs showed significant antibacterial activity. *Bufo melanostictus* showed high sensitivity and high antimicrobial activity against these four pathogens. In this present study, *Bufo melanostictus* extract showed no activity against four pathogenic bacteria. While *Bufo melanostictus* silver nanoparticles showed significant

activity against four pathogenic bacteria, *Proteus mirabilis*, *Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa.* Following studies shown that *Bufo melanostictus* silver nanoparticles were able to inhibit *Proteus mirabils* with maximum zone of inhibition, its value is 8.333± 0.577. Second, *Klebsiella pneumonia* showed moderate zone of inhibition and its value is 7.666 ± 0.667 next *Staphylococcus aureus have* lowest inhibition zone and its value is recorded that 5.234±0.698 and *Pseudomonas aeruginosa showed* also lowest zone of inhibition and its value is recorded that 5.333 ±0.577 respectively (Figure 1 and Table 1). Similar results were reported by (Savithramma *et al*., 2011).

Characterization of nanoparticles by broadly used technique, UV-visible spectrometry. This technique was used to determine the size and structure of nanoparticles. Absorption spectra of AgNPs has absorbance peak at 254 nm and 474 nm indicating the Ag nanoparticles present. Our results were constant with the report of Li *et al*., 2012, confirmed that powerful peak at 440nm. This technique was a very useful technique which characterized the synthesized silver nanoparticles. It was also used to maintenance of AgNPs (Sastry *et al*., 1998). Recently these particles have been accepted as antimicrobial means. These particles have the strong potential to kill the gram negative as compared to other bacteria (Shrivastava *et al.,* 2007).

This shown that, this bacterium was inhibited a great extent by silver nanoparticles than Gram positive bacteria. These particles were extremely poisonous in Gram-negative as contrast to other bacteria due to the cell wall difference. Due to this difference silver nanoparticles showed the significantly less effect on Gram-positive bacteria growth. These particles have higher surface area that provides different properties (Lok *et al*., 2007). The larger external region of these particles that come close to the microbial cells, increasing the penetration and attraction in the membrane of cell and consequently their antibacterial action (Choi and Hu, 2008). Furthermore the nanoparticles that are stabilized by the molecules may influence the action with polyvinyl alcohol (PVA). Silver nanoparticles that are stabilized showing elevated antimicrobial properties against *Pseudomonas aeruginosa* and *Staphylococcus aureus* than polyvinylpyrrolidone (PVP) that are stabilized (Pencheva, 2012**).** Method of bactericidal effect of these particles was not very famous. It was showed that after the treatment with these particles the proteins of cell become inactive. Silver has been very famous for antibacterial activities for the last ten years. Specially, the silver nanoparticles that inhibiting the enzymatic and respiratory activities of bacteria by apply its own effect. Similarly, AgNPs changed the DNA replication and S-H bonds of proteins (Guzman *et al.,* 2008). AgNPs are also action on reactive oxygen species development and stop the proteins and respiratory enzymes. The bactericidal efficiency of the synthesized AgNPs was studied against subculture strain of *A.veronii through* agar well diffusion method, the inhibition zone was observed. It is obvious from our results that if the microbes have been treated with Ag ions then microbes DNA stop its replication capability and their maintenance in medium as colloid and changed the phosphotyrosine profile of proteins and stop its development.

The bactericidal efficiency of the synthesized BMAgNPs was studied against human bacterial pathogen through agar well diffusion method, the inhibition zone was observed. It is obvious from our results that gram negative bacteria showed high

antimicrobial activity as compared to gram positive bacteria. Earlier work had been done by only the skin and mucous of *Bufo melanostictus*. So it is the first approach synthesizing the AgNPs in the skin of *Bufo melanostictus*.

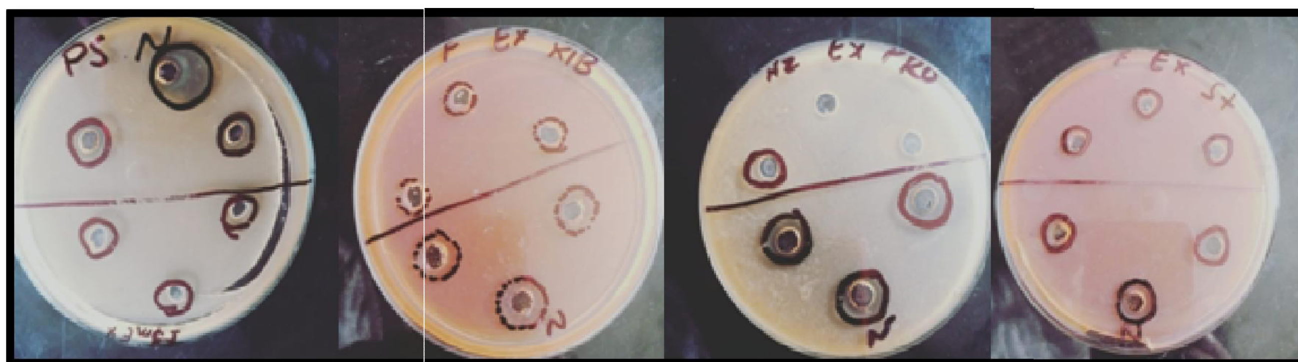

Figure 1: Zone of inhibition against pathogens.

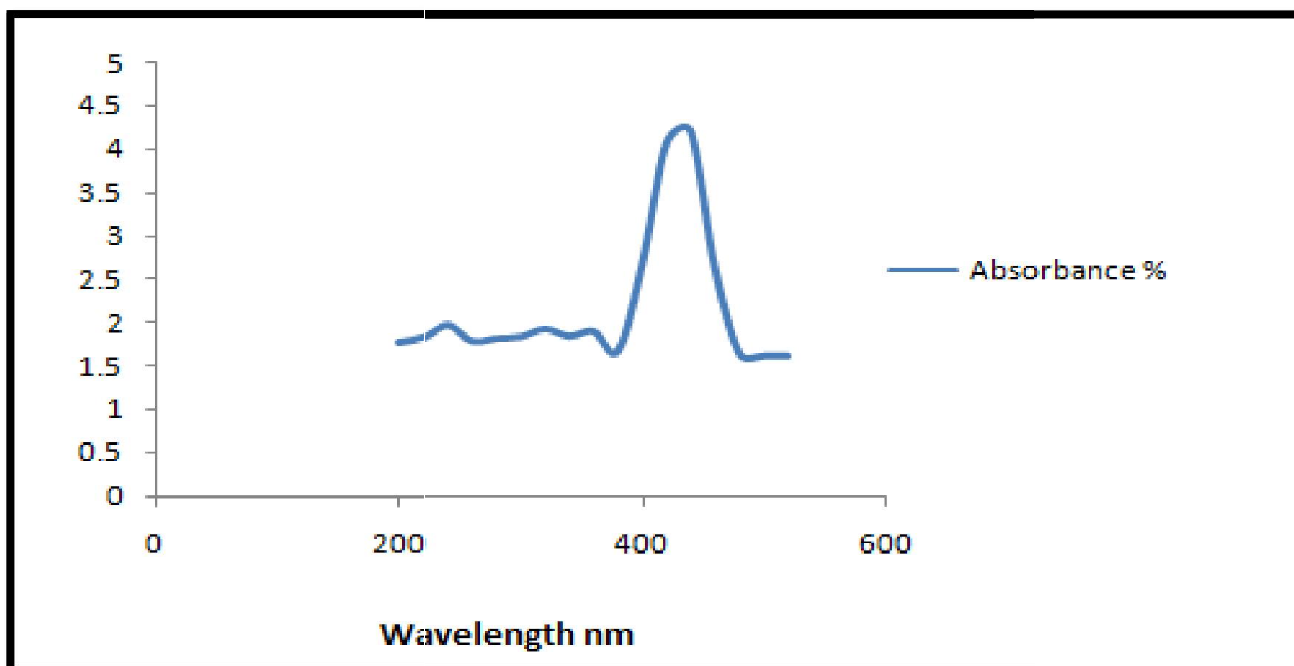

Figure 2: Obtained peak of wave length.

ANTIBIOFILM ACTIVITY OF *B. MELANOSTICTUS*

This activity was performed to find out the capability of *B. melanostictus* and its synthesized silver nanoparticles to controlled biofilm activity of bacteria *P. aeruginosa*, *S. aureus*, *K. pneumonia* and *P. mirabilis*. In present study, the effect of BMAqu and BMAgNPs on biofilm formation was practiced via crystal violet assay. BMAgNPs and BMAqu that were synthesized was tested the inhibitory activities by means of four microbial isolates *S. aureus, P. mirabilis, K. pneumonia and P. aeruginosa.* Following the bacterial pathogens are general pathogens entered the formation of biofilm. BMAgNPs keep the ability to change biofilm of a number of

pathogenic bacteria. Our result showed that experienced bacteria were not capable to show biofilm in the occurrence of BMAgNPs. It was concluded, BMAgNPs showed huge strength to decrease biofilm formation at a significant level (Table 2).

Table 1: Antibacterial activity from toad nanoparticles.

Test sample Pathogen	**Antibacterial of toad nanoparticles**
Pseudomonas aeruginosa	5.333 ±0.577
Klebsiella pneumonia	7.666 ±0.667
Proteus mirabilis	8.333±0.577
Staphylococcus aureus	5.234±0.698

Table 2: Antibiofilm activity of BM skin extract and BM AgNPs.

Tested organism	BMAgNPs	BMAqu
Staph	0.101667±0.01756	1.006±0.314
Pseudo	0.017333±0.00058	1.044±0.253
Proteus	0.015667±0.00058	1.063±0.173
Kleb	0.013333±0.00058	0.855±0.124

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