Anti MRSA and Antiviral Evaluation of Nano Silver Against Avian Influenza virus

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Abstract:

Preparation of nano sized silver particles was performed by using flower extract of *Aerva javanica* (AJ). Conversion of Ag⁺ ions to nanoscaled Ag⁰ was carried out in 90 min reaction by green approach. Antiviral potential of nanosilver (NS) was evaluated against Avian Influenza Virus (AIV) strain H9N2, in 9-11 days old chicken embryonated eggs. Synthesized particles were also screened against four methicillin-resistant *Staphylococcus aureus* (MRSA) strains by well diffusion method. Formation of spherical particles was confirmed by SPR band at 428 nm. XRD analysis confirmed face centred cubic crystal structure of particles with average particle size of 15 nm as calculated by Debye–Scherrer’s formula. Remarkable anti AIV activity was observed from NS particles with IC₅₀ value 50 µg/ml and this value is 200 times greater control drug i.e., Amantadine. Synthesized particles were also screened against four methicillin-resistant *Staphylococcus aureus* (MRSA) strains by well diffusion method. Significantly high antiviral and antibacterial activities were observed from nanosilver particles against AIV H9N2 and all four MRSA strains.

Keywords: *Aerva javanica*, AIV H9N2, MRSA, Antimicrobial, Antiviral, flowers

I. Introduction

Avian Influenza (AI) belongs to highly contagious and extremely diverse group of viruses. Wild, aquatic birds are the natural hosts of these viruses. Domestic poultry and other birds including mammals can be infected by AI virus strains (Marchenko et al., 2012). Avian Influenza viruses (AIV) are classified into different strains depending on two surface proteins, known as hemagglutinin (HA) and neuraminidase (NA) (Nayak et al., 2010). These viruses are also classified on the basis of pathogenicity, some strains are considered as low pathogenic (LPAI) and some are considered as highly pathogenic avian influenza viruses (HPAI). The evolution of these strains is the result of two major processes, genetic drift and genetic shift. Many subtypes of AIV are known, but only few of them can cause infection in human. The AIV H9N2 strain is rarely involve in direct human infection but this subtype is considered as gene donor for other AIV strains that can cause human infection (Leong et al., 2008). Trachea is the favorite site for AIV replication and infected birds are more vulnerable to secondary infections. Severe respiratory tract infection cause poor ventilation, and result into death of birds. Some antiviral agents are found to be effective against AIV strains, which include Amantadine, Osteltamivir and Zanamivir. These drugs are potent anti AIV agents and reduce the seriousness and duration of the disease but they must be taken as soon as possible after the onset of symptoms (Boltz, Aldridge, Webster, & Govorkova, 2010). To the best of our knowledge, very few studies have been carried out to check antiviral potential of nano silver against AIV H9N2,

however these SNPs are already reported against feline calicivirus (FCV), murine norovirus (MNV) (Castro-Mayorga et al., 2017), human immunodeficiency virus type 1 (Elechiguerra et al., 2005) and hepatitis B virus (Lu et al., 2008). In addition to anti AIV studies, anti MRSA activity was also evaluated. SNPs are reported to show max bactericidal activity and biological compatibility (Knetsch & Koole, 2011) and it is assumed that mode of action of SNPs are based on Ag⁺ ions, which can inhibit bacterial growth through interference with DNA functions (Li, Leung, Yao, Song, & Newton, 2006). In humans, *S. aureus* can cause several types of infections including localized abscesses, superficial lesions, infections of central nervous system, urinary tract, blood, joints etc (Ansari, Khan, Khan, Sultan, & Azam, 2012). Among the available antimicrobial agents, investigated for bactericidal activity, nano sized silver particles augment as a promising antibacterial agent against drug resistant bacteria. They have wide applications as disinfectant, antiseptic and pharmaceutical applications due to their prominent antibacterial capability (Chaloupka, Malam, & Seifalian, 2010). In current study synthesized particles have been employed to evaluate their anti microbial potential against four resistant strains of *S. aureus* i.e., MRSA I, VI, VII and VIII with accession numbers KU662352, KR862285, KR862291 and KU662354 respectively.

As far as synthesis of silver nanoparticles is concerned, due to an ever-increasing demand to use safer methods in synthesis, medicinal plants are being widely used for the synthesis of nanoparticles. This plant mediated synthesis has got considerable attention for synthesis of size and shape controlled different nanoscale particles due to presence of natural compounds in plants that work both as reducing and capping agents required for this synthesis (Chung, Park, Seung-Hyun, Thiruvengadam, & Rajakumar, 2016). *Aerva javanica*, belonging to family Amaranthaceae, is known widely for its exceptional antioxidant, antibacterial and enzyme inhibitory uses. Flowers of *Aerva javanica* are reported to contain biochemical compounds such as sterols/terpenes, flavonoids, alkaloids, phenolics and sugars (Musaddiq et al., 2018) which make this source valuable for synthesis of nanoparticles.

II. Experimental

Collection of Plant Material and Preparation of Aqueous Extract; Fresh flowers of *A. javanica* were collected from Cholistan desert, Bahawalpur, Pakistan in September 2017 and voucher was deposited at Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur (AJ/CIDS-10-102) (Fig. 1a). Silver nitrate was purchased from the GERMISTON. All solvents used were of analytical grade and were purchased from Sigma Aldrich. The flowers were dried under shade at RT, ground to fine powder form and boiled in distilled water at 45°C for 60 min. The volume of the water was taken ten times more than weight of the plant material. After boiling, the solution was filtered and the extract was centrifuged. Finally, the extract was again filtered, and stored at 4°C for further use.

Synthesis of Silver Nanoparticles; To make silver nanoparticles of plant extracts, 90ml silver nitrate 1mm solution was mixed with 10 ml of plant extract and stirred magnetically for 120 min at 45°C. The gradual change in colour from yellowish to dark brown indicated that silver nanoparticles have been synthesized (Fig. 1b). The reaction vessel was kept at RT for whole night. The particles were settled via centrifugation at 3000 rpm for 30 minutes, separated and washed with distilled water. After washing the obtained particles were dried and stored for characterization and biological screening.

Figure 1; a) Plant of *Aerva javanica* b) Synthesized Silver NPs colloidal solution

For Characterization, Ultraviolet-visible spectral analysis was performed by Halo DB-20 UV-Vis Double Beam Spectrophotometer. XRD analysis was carried out on XPERT-PRO Diffractometer system.

Anti-Viral Assay of Nano Silver; Specific pathogen free 9-11 days old chick embryonated (CE) eggs were obtained from local hatchery located at Chak 13BC, Bahawalpur, Pakistan. High titer lyophilized AIV H9N2 was obtained from depository of department of Biochemistry & Biotechnology, The Islamia University of Bahawalpur. Eggs were inoculated through chorioallantoic route and incubated at 37 °C for 48 h (Sulaiman et al., 2011). After incubation, the allantoic fluids were collected and HA test was done (Wang et al., 2008). The whole procedure of inoculation and harvesting was done in Biosafety Cabinet type II.

To study antiviral activities of SNPs, equal volumes of SNPs and AIV H9N2 inoculum were used in inoculation studies. Normal saline was used as negative control, H9N2 without SNPs was used as virus control, DMSO was used as solvent control and Amantadine as used as positive control. Standard HA test was performed. High HA titer means more virus particles in solution and low HA titer means less/no virus particles in solution. The HA titers provide the basis to calculate efficacy of drugs in CE eggs. All steps of HA test were according to OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2012 (OIE, 2012). 50 µl PBS (pH 7.4) buffer was add in each well of round bottom titer tek plate. The IC₅₀ of NS was calculated by serial dilution method. The serially diluted SNPs were tested in CE eggs and dose dependant response was obtained.

MRSA Assay of Nano Silver; MRSA bacterial strains were refreshed from Glycerol stock on Nutrient broth (Sigma). 20 mL of sterilized nutrient broth was inoculated in 100ml conical flask was used to introduce 100 μ L of each bacterial strain. The cultures were grown in shaking incubator for 24 h. For antibacterial assay, bacterial strains were cultured on Muller Hinton (MH) agar medium (Sigma) (Abdallah, 2014).

All the Petri plates were autoclaved and 15mL MH agar medium was used in each plate and sterilized cotton swabs were used to inoculate cell suspension of each bacterial strain (108 cfu/ml) over agar medium surface. In each plate, wells of each size (8mm) were made by using sterilized cork borer and each test samples i.e., NS and AJ aq. extract were loaded in each well by sterile micropipette tips. Zones of inhibition were recorded in mm against each bacterial strain.

III. Result and Discussion

Characterization of Synthesized Material;

Plant mediated synthesis of nano silver is eco-friendly and cost effective method of rapid synthesis of NPs to be used in biological systems. Biological reducing agents occurring in plants i.e., reducing sugars, ascorbic acid, citric acid, flavonoids and other phenolics play an important role for the purpose. In current work aqueous extract of flowers of *A. javanica* was used for reduction of silver ions thus forming nano particles of silver which were characterized by UV-Vis spectroscopy and X-ray diffraction (XRD).

Initially indication for SNPs formation was obtained by UV/VIS absorption spectrum, proved to be sensitive to formation of SNPs, as these particles show pronounced SPR band around 400 nm due to collective excitation of conduction electrons in a metal. The electronic transitions due to Ag^+ ion appear in the range of 200 and 230 nm. Color of metal particles is believed to be due to scattering and absorption effects of visible light. Mie's theory explains that nano crystals with small spherical shapes, show a single Plasmon's band, but anisotropic crystals may exhibit more than one bands depending on their shapes. Generously proportioned metal colloidal dispersions, usually exhibit broad or additional bands in absorption spectra due to multiple Plasmon's excitation.

The UV/VIS spectrum of prepared nanoparticles in our study displayed only one good symmetric absorption peak at 422 nm indicating complete reduction of Ag^+ to Ag^0 confirming uniform particle size (Fig 2a).

Crystal structure of particles was evaluated by their XRD pattern that exhibited the main peaks at 2θ 38.2°, 44.3°, 64.6° and 77.5° degree corresponding to the (111), (200), (220) and (311) planes respectively, a typical pattern of face centred cubic structure of AgNPs. Presence of such unidentified crystal structures have previously been reported in green synthesis of nano particles. The average crystalline size of the particles was estimated to be 15 nm using Debye–Scherrer's equation i.e.,

$$d = \frac{K\lambda}{\beta \cos \theta}$$

Fig.2 a) UV/VIS spectrum of SNPs b) XRD pattern of SNPs prepared from Aqueous Extract of *A. javanica* flowers

Antiviral Evaluation of Silver Nanoparticles; Threat causing viral infections in living organisms are controlled by several preventive measures including development of vaccine, synthesis of new potent antiviral drugs and using plant extracts as antiviral drugs. Ability of viruses to mutate and develop resistance against available drugs create an urgent need to develop new and more potent antiviral agents. In continuous search of such agents nanosized silver particles were screened to check their antiviral potential against AIV H9N2. Remarkable anti AIV activity was observed from NS particles with IC_{50} value 50 $\mu\text{g/ml}$ and this value is 200 times greater control drug i.e., Amantadine. This enhanced effect can be attributed to the nanosize diameter of silver i.e., $\sim 15 \pm 1$ nm and the enhanced surface reactivity, making them more effective. Also in situ green synthesis of nanosilver and their stabilization by natural stabilizers existing in plant material could also enhanced their anti viral potential preventing the aggregation of nanosized silver particles. All the values are recorded in Table 1.

Table 1: Antiviral activity of AgNPs against AIV H9N2

Tested Sample		*HA Titer	IC_{50}
AgNP	-----	0	50 $\mu\text{g/ml}$
-ve control	Normal Saline	1024	--
Solvent control	DMSO	1024	--
+ve control	Amantadine**	0	10 (mg/ml)

*0-8 strongly effective drug (no growth or very limited growth of virus), 16-32 effective drug (limited growth of virus, drug has controlled viral growth effectively), 64-128 moderately effective drug (drug is not able to control growth of virus very efficiently but still able to control growth to some extent), 256-2048 drug ineffective(no control of growth of virus),** Standard Anti-AIV drug

Antimicrobial Evaluation of Silver Nanoparticles; Silver nanoparticles (AgNP) have attracted attention in recent years due to their broad antibacterial spectrum and long-term antibacterial activity (Nanda & Saravanan, 2009; Parashar, Parashar, Sharma, & Pandey, 2009). Although the antibacterial mechanisms of AgNP are still controversial, the view point that the antibacterial effect of silver ions released from AgNP is greater than that of AgNP has been gradually accepted. It has been demonstrated that silver ions can increase membrane permeability and produce reactive oxygen species (ROS) to damage cell walls, subsequently causing bacterial death (Kim et al., 2007). Anti microbial evaluation against four resistant strains of *S. aureus* i.e., MRSA I, VI, VII and VIII with accession numbers KU662352, KR862285, KR862291 and KU662354 respectively revealed with zones of inhibition 16.8, 19, 17 and 18 mm respectively comparable to that of standard drug. Results are summed up in Table 2.

Table 2: Antimicrobial Activity of AgNPs against MRSA strains (Zone of inhibition in mm)

Sr. No.	Tested sample		KU662352	KR862285	KR862291	KU662354
01	Flower Extract	Aq.	12.7±0.15	11±0.9	10±0.5	11±0.2
02	Silver Nanoparticles		16.8±0.20	19±0.16	17±0.8	18±0.9

V. Conclusions

The Study concludes that nanosilver is effective antiviral as well as anti MRSA candidate and their potential can be related to the nanosize diameter of silver. In situ green synthesis of nanosilver and their stabilization by natural stabilizers existing in plant material are also contributing factors for their activity by preventing the aggregation of nanosized silver particles. Antimicrobial potential can also be attributed to the fact that silver ions can increase membrane permeability and produce reactive oxygen species (ROS) to damage cell walls, subsequently causing bacterial death.

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Conflict of interest

The authors declare no conflict of interest.

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