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Biocompatibility and antibacterial activity of the *Adathoda vasica* Linn extract mediated silver nanoparticles

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ABSTRACT

The aim of this study is to investigate the biocompatibility and anti-*Vibrio* efficacy of green synthesized silver nanoparticles (AgNPs) using an aqueous leaf extract of *Adathoda vasica* (*A. vasica*). The green synthesized silver nanoparticles were characterized by UV–vis, Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX). *A. vasica* AgNPs showed significant antibacterial activity against *Vibrio parahaemolyticus* in agar bioassay and well diffusion method. Further, nanoparticles interactions with bacteria and its antibacterial activity were confirmed by CLSM analysis. *In vivo* evaluation results confirmed that synthesized *A. vasica* AgNPs had good antibacterial efficacy and also nontoxic to the *Artemia* nauplii.

1. Introduction

The shrimp aquaculture industry is rapidly expanding and accounts for 15% of the internationally traded seafood products (FAO, 2014). Vibriosis is the presumptive cause of strong economic losses in the shrimp industry. Recently, the identification of certain strains such as *Vibrio parahaemolyticus*, a causative agent of early mortality syndrome (EMS) caused large scale losses in shrimp production in China, Vietnam, Thailand and Malaysia FAO, 2013 [1].

V. parahaemolyticus is a prevalent seafood-borne entero pathogen with the appearance of pandemic O3:K6 strains in 1996. It's also associated with mortalities in Siberian tooth carps, milk fish [2], abalone [3] and shrimp [4]. Fish result in by this classical *Vibrio* shows typical signs of a generalized septicemia with hemorrhage at the base of fins are frequently exophthalmia and corneal opacity in finfish [5]. Resistance to antimicrobial agents by *Vibrios* has emerged in recent years and is a major challenge for shrimp production [6]. *Vibrio* infection in the larval stages of cultured fish cause high mortality rate, sometimes it leads to the death of the entire population. Usage of vaccines, antibiotics, and chemotherapeutic for disease control resulted in bacterial resistance and cause potential hazards to the environment [7,8]. Therefore, aquaculture requires supports to overcome this bacterial disease by continuous research with scientific and technical innovation.

To keep sustainable production, health management approaches must go beyond antibiotics and chemotherapeutic and there has been urging for researchers to find a new drug against the pathogenic bacteria [9–11]. Recently, much attention has been paid towards the use of nanoparticles as another element set of antibiotics attributable to their distinct advantages over conventional antimicrobial agents [12]. A discipline of nanotoxicology should make an important contribution to the development of a sustainable and safe nanotechnology. Silver nanoparticles have gained much popularity because of their antimicrobial properties [13,14]. In recent years, synthesis of noble metal nanoparticles using various species of plants has arisen as a new trend [15]. Plant based nanoparticles are also reported to have antibacterial effect [16]. Biological route synthesis of nanoparticles has much attention focused by researchers because they are eco-friendly, economical and cost effective. Green synthesis of silver nanoparticles offers a simple and eco-friendly approach to form stable colloids of nontoxic AgNPs along with antibacterial activity.

In traditional medicine, different parts of *Adathoda vasica* Linn (known as vasaka), a shrub widespread in India, has been used for treating many human diseases. Vasaka is a bitter quinazoline alkaloid [17,18]. The leaves contain several alkaloids namely vasicinone, vasicinol, adhatodine, adhatonine, adhavasinone, anisotine

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and peganine. *A. vasica* leaf extract have been used for the anti-allergic, anti-asthmatic, anti-inflammatory, anti-microbial effect [19] and also used to control tuberculosis [20]. In this study, we synthesized biogenic-silver nanoparticles using A. *vasica* and evaluated for its antibacterial activities against *V. parahaemolyticus*. Furthermore, biocompatibility of the synthesized *A. vasica* AgNPs was confirmed using Artemia *napulii* as a model organism.

2. Materials and methods

2.1. Chemicals, medium and bacteria

The silver nitrate ($AgNO_3$), bacterial media and other components used in this study were purchased from Hi Media (Mumbai, India). *V. parahaemolyticus* (ATCC -17802) was obtained from the Department of Biotechnology, Alagappa University, Karaikudi.

2.2. Preparation of A. vasica leaf extract

The plant (*A. vasica*) was collected in and around Karaikudi, Tamil Nadu India and identified and confirmed with the help of field botanist. The leaves were washed thoroughly in distilled water to remove impurities and cut into small pieces. For extraction, the leaf materials (10 g) were weighed and mixed with 500 ml of deionized water and boiled at 60 °C for 10 min in a water bath. The prepared aqueous plant extract was allowed to cool and filtered with Whatman No. 1 filter paper and used for the further experiment.

2.3. Synthesis of silver nanoparticles

After standardization, 15 ml of clear leaf broth was added into the aqueous solution of 0.75 mM of $AgNO_3$ and the mixture was incubated in dark at room temperature. The change of colour solution indicates the formation of silver nanoparticles.

2.4. Characterization of silver nanoparticles

The reduction of pure Ag^+ ions was monitored by measuring the UV–vis spectrum of the reaction medium at different time intervals (5 min to 3 h) in Shimadzu UV-1800, measured between the wavelengths of 200–700 nm. In order to identify the AgNPs associated biomolecules, Fourier Transform Infrared (FTIR) spectral analysis was performed to the washed and purified AgNPs powder on the thermo scientific Nicolet 380 FTIR spectroscopy. Two milligrams of the sample were mixed with 200 mg KBr (FTIR grade) and pressed into a pellet and placed into the sample holder of FTIR spectra. To obtain good signal to noise ratio, 256 scans of AgNPs were taken in the range of 400–4000 cm^{-1} and the resolution was kept at 4 cm^{-1}. To determine the nature of AgNPs, the air dried powder was analyzed in XRD (X'PERT- PRO.PAN analytical Netherland) operating in transmission mode at 40 kV and 30 mA with Cu K radiation. Further to evaluate the size and shape of the synthesized nanoparticles TEM was performed on Technite 10 Philips instrument on carbon coated copper grids with an accelerating voltage of 80 Kv. SEM analysis was measured using Hitachi-S-3000H and characterization of intermediate compounds which formed during the silver ions reduction with the plant extract was carried out by EDX(Hitachi S3000H).

2.5. Antibacterial activity

The pathogenic bacterium was pre-grown in nutrient broth at 37 °C for 24 h. Sterilized Muller Hinton Agar (MHA) medium with different concentration of AgNPs (10, 20, 30, 40 and 50 μg/ml) was poured into petri dishes. In the agar bioassay method, 100 μl of *V. parahaemolyticus* at the density of 10^5 CFU/ml was spread on the lawn of MHA medium and incubated at 37 °C for 24 h and observed for the antibacterial effect. Positive and negative control plates were also maintained for comparison. In agar well diffusion assay, 100 μl nutrient broth culture of targeted bacterium (10^5 CFU/ml) was used to prepare bacterial lawns. Agar wells of 6 mm diameter were prepared with the help of a sterilized stainless steel cork borer. Different concentrations of AgNPs (10, 20, 30, 40 and 50 μg/ml) were inoculated into the wells made on MHA. The plates were allowed for incubation at 37 °C for 24 h.

2.6. Confocal laser scanning microscopic studies

For CLSM analysis, different concentrations of AgNPs were added into bacterial suspension of 10^5 CFU/ml. After 24 h incubation, the bacterial cell suspensions were centrifuged at 5000 rpm for 5 min. The supernatant of these samples were discarded and the remaining bacterial pellets were suspended in 0.5 ml prepared fluorescent marker solution (Acridine orange 1 μl and 1 ml of PBS). The remaining pellet was suspended into 200 μl PBS and incubated for 15 min. Then the incubated bacterial suspension was centrifuged at 5000 g for 5 min to remove the excess dyes. The antibacterial activity was observed under CLSM (Model: LSM710) (Carl Zeiss Jena, Germany). The 488-nm Ar laser and a 500–640 nm band pass emission filters are used to excite and detect the stained cells. Control and treated bacterial cell images were recorded and processed using Zen 2009 image.

2.7. In vivo analysis

In vivo experiments were executed with freshly hatched *Artemia* nauplii (San Francisco Bay Brand, San Francisco, CA, USA). To hatch out the *Artemia* nauplii, one hundred milligrams of decapsulated cysts were kept in 100 ml of sterile seawater with the salinity of 30 ppt and were well aerated for 24 h. The freshly hatched healthy *Artemia* nauplii were collected and used for challenging test by standard method [21,22]. Briefly, healthy *Artemia* nauplii were divided into 7 groups in triplicate and each group contains 30 numbers of nauplii. The experiment was performed with 24 well plates in 5 ml of 30 ppt sea water for 48 h to assess the antibacterial efficacy. *V. parahaemolyticus* 10^5 CFU/ml were used in this experiment with the concentration of 50 μg/ml AgNPs based on the minimum bactericidal concentration determined. The nauplii survival rate was observed at 6 h intervals over the period of 48 h and percentage was calculated by following this formula: Survival rate (%) = [number of live nauplii at the 6 h intervals/number of nauplii at the time of inoculation] X100.

3. Results

3.1. Synthesis of plant based AgNPs

A. vasica was used for the reduction of $AgNO_3$ into nanoscale level. The synthesis of AgNPs was confirmed based on the color

Experimental setup

Group I	Control (without any application)
Group II	AgNPs (50 μg/ml) alone.
Group III	Plant extract (50 μg/ml) alone.
Group IV	*V. parahaemolyticus* alone (50 μg/ml).
Group V	*V. parahaemolyticus* + AgNPs (50 μg/ml)
Group VI	*V. parahaemolyticus* + plant extract (50 μg/ml).
Group VII	$AgNO_3$ alone (50 μg/ml).