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Research Article

Molecular Taxonomic Identification, Biosynthesis and *in vitro* Antibacterial Activity of ZNO Nanoparticles Using *Boerhavia diffusa* Against MRSA

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ABSTRACT
Plant mediated synthesis of nanoparticles is one of the cost effective and environmental friendly method for improving the antimicrobial activity of plant extracts. In the present study, biosynthesis of ZnO nanoparticles (ZnONps) has been demonstrated using an aqueous extract of *Boerhavia diffusa* leaves and the antimicrobial activity of synthesized nanoparticles was assessed in contrast to *Methicillin resistant Staphylococcus aureus (MRSA).* UV-visible spectroscopy and scanning electron microscopy (SEM) studies showed the formation of nanoparticles with an average size of 140 nm. The antibacterial and anti-biofilm properties were also evaluated with the green synthesized ZnONPs and found highly effective. The molecular identification by ribulose-bisphosphate carboxylase (rbcL) PCR authenticated the taxonomic status of *boerhavia diffusa*. This study proved that the ZnONPs could inhibit the activity of biofilms formed by MRSA strains and could be used as a potential antibacterial agent for cleaning and disinfection of MRSA in hospitals and other health care centers. In this regard, there is also an urgent need for further investigation of its use in cleaning and disinfection of equipments used in food industries and contagious materials.

Keywords: *Boerhavia diffusa*, *Methicillin resistant Staphylococcus aureus (MRSA),* ZnO nanoparticles (ZnONPs)

INTRODUCTION

Boerhavia diffusa is a tropical creeping root herb, with bioactive compounds in both the leaves and roots. The plant is named after of Hermann Boerhaave, a famous Dutch physician of the 18th century[1]. it is being used for its anti-diabetic, anti-viral, pain relief and diuretic properties. It sometimes suppresses the proliferation of immune cells, but is beneficial if the immune system is hyperactive. A potent antibacterial activity against gram positive and gram negative bacteria exposed by the leaves of *B. diffusa* might be because of the presence phytochemicals in the leaves. *Staphylococcus aureus* is a gram-positive coccal bacterium that is frequently found in the human respiratory tract and on the skin. Although *s. aureus* is not all the time, pathogenic, it is a common cause of skin infections (e.g. Boils), respiratory diseases (e.g. Sinusitis), and foodstuff poisoning. Disease-associated strains often promote infections by producing potent protein toxins, and show cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant forms of pathogenic *s. aureus* (e.g. MRSA) is a global problem in clinical medicine. *Methicillin resistant Staphylococcus aureus* (MRSA) is well-defined by the existence of a large mobile genetic element called staphylococcal cassette chromosome, mec (SCCmec). It has a mecA gene, which codes for an alternative penicillin binding protein, PBP2a, with low binding affinity to all β-lactams[6]. Today, nanotechnology is a common tool for the development of new cutting-edge applications in environmental protection, cosmetics, biology, and medicine. Nanoparticles like Zinc, Gold, and Silver belong to the class of metal oxides, which are characterized by photo catalytic and photo-oxidizing capacity against chemical and biological species[6]. Zinc oxide is known to be one of the multifunctional inorganic substances with effective antibacterial and antifungal activity[16,18,20]. Results of various study have proven that green synthesized ZnO nanoparticles showed more enhanced biocidal action counter to various pathogens when compared to chemical ZnO nanoparticles[19,21,23]. Antimicrobial actions of metal oxide (ZnO, MgO and CaO) powders against *staphylococcus aureus*, *escherichia coli*, and fungi were quantitatively evaluated in many studies[7,8]. Surface area to volume ratio of green ZnO nanoparticles are responsible for significantly greater antimicrobial activity, and therefore, it can be used effectively in agricultural and food safety applications and also they can

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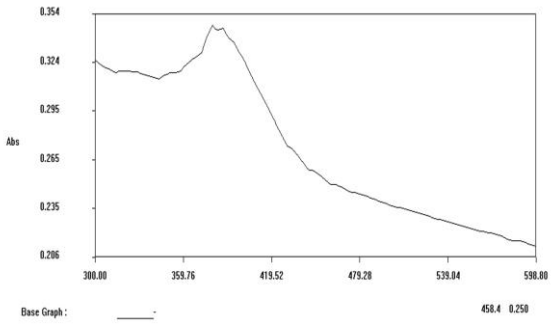

Figure 1**:** UV Vis Spectrometry Analysis of ZnO Nanoparticles

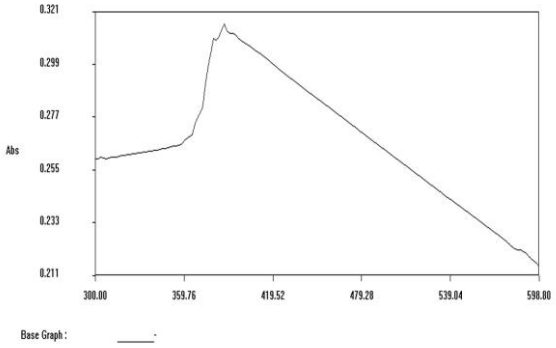

Figure 2**:** UV Vis Spectrometry Analysis of leaf extract and zinc oxide

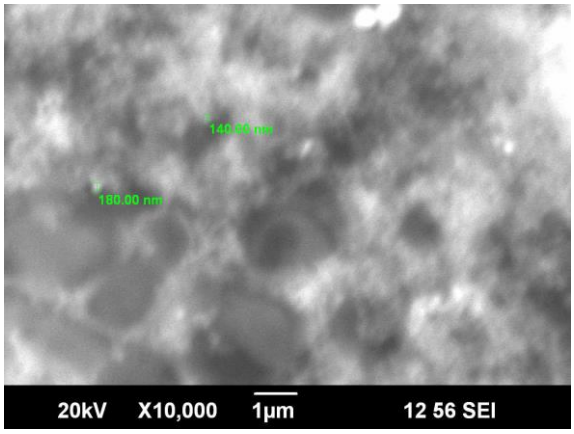

Figure 3: SEM analysis of nanoparticles of ZnO.

fulfill future medical concerns. rbcL gene, large subunit of ribulose 1.5 bisphosphate carboxylase/oxygenase was used for authentication of the plant species.

MATERIALS AND METHODS

Preparation of leaf extracts

Fresh leaves of *Boerrhavia diffusa* were collected from Cochin, Kerala, India. The leaves were washed several times with distilled water to remove dust particles. 50 g of the leaves were homogenized with mortar and pestle. The extract was transferred to a 250 ml glass beaker and boiled at 100°C for 45 minutes until the color changed to light yellow. The extract was cooled to room temperature and filtered using filter paper. The extract was stored in a refrigerator for further use.

Preparation of zinc nanoparticles

For the synthesis of nanoparticles, 4.5 g of ZnO was mixed with 1 ml of the leaf extract. This mixture was then boiled until it reduced to a deep yellow colored paste. This yellow paste was heated in a microwave oven for 30 seconds. A light yellow colored powder was formed and this was carefully collected and used for characterization.

Characterization of ZnONPs by UV- Vis spectroscopy and Scanning Electron Microscopy

The UV-Vis spectrum of the synthesized ZnO nanoparticles was carried out on a UV- Vis Spectrophotometer (Systronics, India). The absorption maximum of the Nanoparticles was scanned between 300 nm to 600 nm. The synthesized ZnO nanoparticles were structurally analyzed using a Scanning Electron Microscope (SEM) Carl Zeiss, Germany to confirm the size of the nanoparticles synthesized.

Antimicrobial and Antibiofilm studies

The antimicrobial and antibiofilm activity of the ZnONPs were studied against MRSA by Microtiter plate method. 40 µl of overnight culture of *Staphylococcus aureus* was inoculated in 96 well micro titer plates containing 150 µl mediums and 40 µl of distilled water and incubated for 24 hours at room temperature. After incubation, absorbance was measured to analyze the antimicrobial activity of the ZnONPs in comparison with the control wells, loaded with *Staphylococcus aureus* culture without ZnONPs. The inhibitory activity of the biofilm was checked by Crystal violet method. The biofilms formed were stained with 210 µl of 0.1% crystal violet solution (w/v) for 10 minutes, after which the dye was discarded, and the wells were rinsed twice with deionized water. The wells were allowed to air dry before solubilisation of the crystal violet with 210 µl of dimethyl sulphoxide. The antibiofilm activity was determined using a microplate reader (Thermoscientific, USA) at 595 nm.

Molecular identification of Boerhavia diffusa by rbcL-PCR

The rbcL primer set consisted of forward primer 5'-ATGTCACCACAAACAGACTAAAGC-3', and the reverse primer 5'-GTAAAATCAAGTCCACCRCG-3'. The PCR master mix (25µL) was prepared containing 1X assay buffer with 1.5 mM $MgCl_2$, 5p moles of each primer, 200mM dNTPs, 1.5 U Taq DNA polymerase and 50 ng of template DNA. To check DNA contamination, a negative control was set up excluding templates DNA from the reaction mixture. PCR was carried out on a Surecycler (Agilent Technologies, India) and each cycle consist of initial denaturation at 94°C for 3 min, then 30 cycles of DNA denaturation at 94°C for 30 s, annealed at 65°C for 30 s and extension at 72° C for 1 min followed by final extension step at 72°C for 2 min. The PCR products were run on ethidium bromide stained 1% agarose gel with 100 bp DNA ladder to visually as the marker.

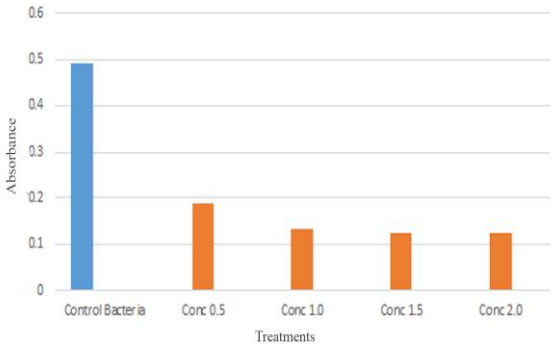

Figure 4: Effectiveness of antimicrobial activity

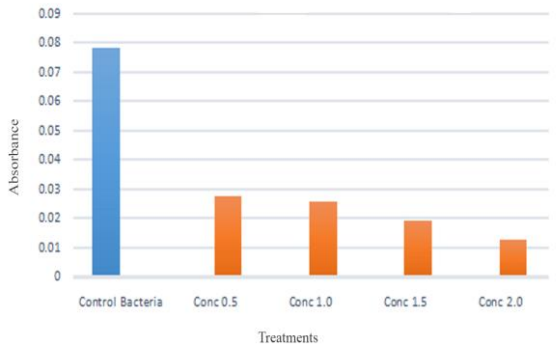

Figure 5: Effectiveness of antibiofilm activity

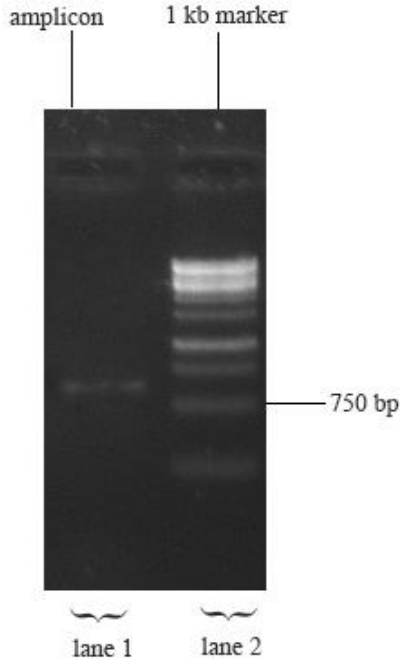

Figure 6: Agarose Gel Electrophoresis of amplified PCR products (lane 1 – amplicon, lane 2 -1kb marker)

RESULTS AND DISCUSSION

UV-Vis spectrum analysis

The UV-Vis spectrum of ZnO Nanoparticles is shown in Figure (1) and Figure (2). Confirmation of the synthesized ZnO nanoparticles was exhibited by the blue shifted absorption maximum at 360nm.

SEM Analysis

The Scanning Electron Microscopic (SEM) images confirm the size and shape of the synthesized ZnO nanoparticles. Figure 3 represents the SEM images of ZnO nanoparticles. The obtained powder of ZnO nanoparticles were spherical and well agglomerated with a particle size range of 140 nm whereas a size range less than 100 nm was found in the current study on the effect of commercially obtained ZnONPs in *staphylococcus aureus*[25].

Antimicrobial activity of the ZnO Nanoparticles

The antimicrobial activity of ZnO-NPs was determined by using Microtiter plate method and the absorbance values read using an ELISA reader. The graphical representation of the effectiveness of antimicrobial activity of ZnO-NPs against MRSA is shown Figure (4). The Figure (4) shows that conc 1.0 µg/mL is enough to reduce the effectiveness of the antimicrobial activity as compared to the control the activity is reduced more than half. At this concentration the absorbance is shown to be 0.13. Vani.c *et al* reported that, when the concentration of nanoparticles increases, the zone of inhibition also increases[25]. This may be due to the destructive effect of ZnO nanoparticles with the cells and increased production of active oxygen, such as H_2O_2, lead to cell death.

Antibiofilm activity of the ZnO Nanoparticles

The Antibiofilm activity of ZnO-NPs was determined by using Microtiter plate method. The effectiveness of antibiofilm activity of ZnO-NPs against MRSA is shown in Figure (5). In addition to antimicrobial activity, the antibiofilm activity of ZnONPs nanoparticles against MRSA isolate was studied (Figure: 5). *Methicillin resistant Staphylococcus aureus* (MRSA) Infection is caused by a strain of *Staphylococcus aureus* bacteria that's become resistant to the antibiotics commonly used to treat ordinary staph infections. MRSA infection may result in a number of clinical manifestations, including bacteraemia, endocarditis, sepsis, and death. Given its resistance to therapy with multiple antibiotics, MRSA infection is often difficult to treat. A higher rate of biofilm formation is directly linked with the drug resistance pattern of MRSA[26]. The ZnO nanoparticles coated surfaces could inhibit bacterial biofilm formation, thereby increasing the antibiotic exposure[24]. This sensitive activity of the ZnONPs showed their potential in controlling biofilm formation even at 0.5mg/ml concentration. The antibioflim activity has been reduced to the maximum at a concentration of 2.0. At this concentration, the absorbance is shown to be 0.014. Thus, it has been identified that the best concentration to reduce the effectiveness of antibioflim activity was 2.0.

Isolation of Plant DNA

To confirm the plant by molecular taxonomy, the plant DNA was isolated and agarose gel electrophoresis was performed. The amplification of the isolated DNA had been done using rbcL PCR. The result of the rbcL PCR is shown in the Figure (6).

After PCR amplification, sequence similarity searches were done by BLAST analysis to identify the plant in the NCBI website. The NCBI- BLAST result confirmed that

the plant is *Boerrhaavia diffusa*. The sequences of the rbcL gene were deposited in GenBank nucleotide and the accession number is KT966747. Further, in the present study, ZnONPs against the MRSA isolate from the medicinal species *Boerhavia diffusa* were prepared. MRSA is caused by a strain of *S.aureus* bacteria that has become resistant to antibiotics commonly used to treat ordinary *staphylococcus* infections. Mostly, these occur in people who have been in hospitals or other health care settings (Nosocomial infections). Therefore, this study would certainly pave the way for future development in pharmaceutical industries, healthcare, etc.

CONCLUSION

The present study demonstrated the green synthesis and the potential of ZnONPs from *Boerhavia diffusa*, as a plant drug against pathogenic MRSA isolates. The use of plant extracts avoids the usage of harmful and toxic reducing and stabilizing agents. The ZnO nanoparticles were synthesized by microwave mediated heating of leaf extracts and ZnO. In the present study, the complex activity of the ZnONPs counter to biofilm formation and the microbial activity of *Staphylococcus aureus* was found in the Microtiter well even at smaller concentrations. The current study concludes that *Boerhavia diffusa* mediated ZnONPs could inhibit the pathogenic activities of Multi-Drug Resistance (MDR) *Staphylococcus aureus* and therefore, the use of plant extracts for the production of ZnONPs seems to be a good alternative to conventional sanitizing agents in hospitals and clinical labs. The plant material responsible for the reduction and stabilization of nanoparticles may be further studied and standardization with many other plant extracts may reveal the wide spectrum of antibiotic property of traditional indigenous plants with wide applications.

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