*Int.J.Curr.Microbiol.App.Sci* (2022) *11*(09): 62-73

*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706* Volume 11 Number 09 (2022)**
Journal homepage: http://www.ijcmas.com

**Original Research Article** https://doi.org/10.20546/ijcmas.2022.1109.007

# Bio-synthesis of Zinc Oxide Nanoparticles; Characterization and Evaluating their Biofertilizer, Antimicrobial and Antioxidant Efficacy

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**Keywords**

Vitex negundo, HR-TEM, Vn-ZnO NPs, Antibacterial activity, DPPH activity

**Article Info**

***Received:***
08 August 2022
***Accepted:***
30 August 2022
***Available Online:***
10 September 2022

**A B S T R A C T**

The present work was carried out to the characterization and the evaluation of antimicrobial and anti-oxidant activity of Vn-ZnO NPs. The present research work focussed on the biosynthesized ZnO NPs by sodium hydroxide, zinc acetate and bio components of leaves of *Vitex negundo* in the reaction mixture was monitored in the precipitate from, which proved the formation of ZnO NPs. The biosynthesized nanoparticles were characterized by advanced tool viz. UV-Vis spectrophtometer, FT-IR, Particle size and zeta potential, X-ray diffraction and TEM along with EDAX. The UV-Vis peak was aquiered at 375nm. The zeta average 28 nm and the zeta potential value was -16.3mV.The average size of the nanoparticles was 34.46 nm and the minimum sized NPs read 16.2 nm by HR-TEM. Antibacterial activity of Vn-ZnO NPs was evaluated by examine against selected two gram negative (*Escherichia coli*, *Salmonella typhi*) and gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*). Antimicrobial activity of Vn-ZnO NPs was found to be more suceptable to the against gram negative bacteria. Strong DPPH activity was found by the Vn-ZnO NPs.

## Introduction

Production of nanoparticles by greener way using with plant materials, bacteria, fungi and algae allows for the huge-scale production of metal oxide nanoparticle with free of impurities (Yuvakkumar *et al.*, 2014). There has been tremendous enhancement in nanotechnology in the past decade because of its application in medicine, chemistry, biotechnology, food industry, textiles industry and agriculture (Singh *et al.*, 2008; Pelaz *et al.*, 2012). Growth in this area has opened new horizons in nanoscience, especially in drug delivery, gene delivery and bio-sensing etc. (Zhang and Guo, 2009). One of the unique properties that make nano-sized particles so interesting is their high surface- to- volume ratio (Yao *et al.*, 2002). Such kind of feature enables them to be more reactive than the bulk material as atoms on the surface tend to be more active than those at the center (Yin *et al.*, 2004). The

nanoparticles are synthesized by physical, chemical and biological methods. Different physical and chemical methods like hydrothermal, sol-gel synthesis, laser oblation, lithography etc. are requiring skilled labor and expensive equipment. Moreover, they have toxic effects that are hazardous to the health. The nanoparticles got via greener way are non-toxic, cost-effective, easy, eco-friendly and bio-degradable in nature as well as the method is safe and reduces the utilization of hazardous substances (Virkutyte and Varma, 2011; Iravani, 2011). Green synthesis nanoparticles comprises great properties, these are synthesized through every part of the plant such as root, leaf, stem-bark, twig and flower etc. Plant extracts have diverse bioactive molecules that help in the reduction and stability of the nanoparticles (Nilavukkarasi *et al.,* 2020). Current studies exhibit the significance of biosynthesis of metal oxide nanoparticles where oxides of metals like silver, copper, palladium, gold, nickel and zinc etc., are gaining importance (Moodley *et al.,* 2018; Chung *et al.,* 2017). Previous reports revealed on ZnO nanoparticles have been synthesized successfully from various plant extracts like *Aloe vera* (Chaudhary *et al.,* 2019), *Artocarpus gomezianus* (Suresh *et al.,* 2015), *Azadirachta indica* (Handago *et al.,* 2019), *Bauhinia tomentosa* (Sharmila *et al.,* 2018), *Camellia sinensis* (Shagufta *et al.,* 2018), *Cinnamomum verum* (Mohammad *et al.,* 2020), *Durantaerecta* (Shekhawat *et al.,* 2016), *Moringa oleifera* (Elumalai *et al.,* 2015), *Matricaria chamomilla*, *Olea europaea* and *Lycopersicon esculentum* (Solabomi Olaitan Ogunyemi, *et al.,*2019).

The present study focused on the producing of ZnO nanoparticles (ZnO NPs) by the eco-friendly approach, where using with the aqueous leaf extract of *Vitex negundo Linn* belonging to the family verbenaceae commonly known as Nirgundi. Nirgundi in Sanskrit means which protects the body from diseases (Raji, 2013). The plant is distributed allover India; altitude of 1500 m in the outer Himalayas and it is growing commercially as a crop in parts of Asia, Europe, North America and West Indies (Suganthi and Dubey, 2016). Root used in dyspepsia, colic, rheumatism, worms, boils and leprosy (Anonymous, 2003).The leaves of the Nirgundi are aromatic, tonic, vermifugediscutient and are useful in dispersing swelling of joints from acute rheumatism and of the tests from suppressed gonorrhoea. Leaves of the plant given with addition of long pepper in catarrhal fever with heaviness of head and dullness of hearing. The juice of the leaves is said to have the property of removing foetid discharges and worms from ulcers; fruit is nervine, cephalic and emmenagogue; dried fruits acts as a vermifuge (Kirtikar and Basu, 2008; Nadkarni, 2002).The flowers are useful in diarrhoea, cholera, fever, haemorrhages, hepatopathy and cardiac disorders and are seed of the plant considered useful in eye diseases in form of *anjan* (Sharma *et al.,* 2005).

Based on the above literature no work has been carried out. The present study, *Vitex negundo Linn* aqueous leaf extract was considered for the synthesis of nanoparticles. Therefore, this work is aimed to explore the application of *Vitex negundo Linn* leaf extract as a capping and reducing agent for the synthesis of Zinc Oxide nanoparticles (ZnO NPs) and evaluate the antibacterial activity of selected two gram negative and two gram positive along with anti-oxidant activity of the synthesized Zinc Oxide nanoparticles (ZnO NPs).

## Characterization of biologically synthesized ZnO NPs

Green synthesized zinc nanoparticles ZnO NPs was measured using with UV-Vis spectrophotometer nano drop range from ---. Frourier- Transform Infra-Red (FT-IR) spectra of synthesized ZnO NPs were analyzed with the compressed range from 4000 to 500 cm-1 with an ALPHA interferometer (ECO-ART), Bruker, Ettlingen, Karlsruhe, Germany, through KBr pellet method. Crystalline nature of metallic ZnO NPs was done by the X-ray Diffractometer (XRD), Shimadzu, XRD-6000 equipped with Cu Ka radiation source utilizing Ni filter at setting of 30 kV/30 mA. Synthesized ZnO NPs was analyzed by using Scanning Electron Microscopy (SEM) from FEI Quanta 200 FEG HR-

SEM. Morphology agglomeration pattern of ZnO NPs was analyzed with Transmission Electron Microscopy (TEM) equipped with EDAX (HF-3300 advanced 300 kV- Hitachi).

## Materials and Methods

### Collection of Plant Material

*Vitex nigundo* was grown in pots by using Mangampet byrites mine waste. After well enlarged the fresh leaves of the plant collected from glasshouse, department of Botany, Sri Venkateswara University, Tirupati. Leaves were washed with running tap water until the udmixture removed for thrice and followed by milli-Q water. Later all leaves were wiped through tisue paper, then cut small pieces and dry up to three weeks under shade dry environment to evaporate moisture content and finally ground fine powder using with electric blender until the further work.

### Preparation of plant extract

Leaf extract of the *Vitex nigundo* was prepared by mixing 20 g of fine leaf powder with 200 mL of Milli-Q water in 500 mL sterile Erlenmeyer conical flask and boiled for 30 min at $100^0$C. Later aqueous leaf extract was gathered in a separate sterile conical flask using with Whatman No.1 filter paper. The filtrate was used for characterization, antibacterial and anti-oxidant activities (Gibbs, 1974; Herborne, 1973).

### Chemicals

Zinc acetate dehydrate (99% purity) and sodium hydroxide pellet (99%) were purchased from Sigma-Aldrich chemicals (Banglore, India).

### Preparation of Zinc acetate solution

10 mM of 200 mL Zinc acetate solution was prepared by the Milli-Q water and it was taken into an sterile amber colored bottle, then it was kept $4^0$C until the synthesis.

### Green synthesis of ZnO NPs

Synthesis of ZnO NPs done by the aqueous leaf extract of *Vitex nigundo* was added to 10 mM Zinc acetate solution and maintained pH was 12. The resulted solution was changed from brown to pale white in color after heating for 30 min, later stirring the precipitation was washed by distilled water and followed by ethanol to get free of impurities. The solution was dried with the help of vacuum and this was utilized for the characterization, anti-bacterial and anti-oxidants activity of ZnO NPs.

### Characterization of ZnONPS

Biologically synthesized nanoparticles were analysed by using the recent techniques. ZnO NPs of *Vitex nigundo* was analysed by UV-Vis absorption spectra (UV-Vis spectro- photometer - Nano drop contain scan range 190 to 750 nm) to know which of the metallic nanoparticles were certainly involved in reduction of nanoparticles through Surface Plasmon Resonance (SPR)approach. To understand which phytoconstituents actually involved in the capping and stabilization of the nanoparticles was performed with the Fourier Transform Infra-Red (FT-IR, ECO ART), Bruker, Ettlingen, Karlsruhe, Germany by KBr pellet method. Dynamic light scattering (DLS) and zeta potential analysis was carried out by the Nanoparticle analyser (Horiba SZ 100, Japan).

Crystalline nature and calculate average size of the biogenic nanoparticles were observed by using an X-Ray Diffractometer (XRD, Shimadzu, XRD-6000)equipped with Cu kα radiation source using Ni as filter at a setting of 30kV/30mA. Size, shape, dispersed nature and agglomeration pattern of the synthesized nanoparticles analysed with HR-Transmission Electron Microscopy (TEM, HF-3300, 300 kV TEM/STEM, Hitachi).

### Antibacterial activity

The antibacterial study was carried out by using standard protocol followed by disc diffusion assay (Anonymous, 1996). The aqueous leaf extract of

synthesized VN- AgNPs was examined for antibacterial activity against selected two gram negative like *Escherichia coli*, *Salmonella typhi* and two gram positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*.

The bacterial stains were procured from Dept. of Microbiology, Sri Venkateswara University, Tirupati. For this 20µl of 50 µg/ml concentration of plant extract, 20 µl of 40 µg/ml of 10 mMZinc solution, 20µl of 50 µg/ml of 10 mM VN-ZnONPs and Amoxicillin standard drug were used.

All the solutions were applied on sterile separate filter paper discs (What manNo.1 filter paper disc with 7 mm diameter) and allowed to dry before positioned nutrient agar medium.

The entire activity was performed triplicates and incubated at $37^0$C for 24 hours. The formed zones diameter was measured in centimetres with the helping of scale and the results were tabulated.

## Anti-oxidant activity (DPPH free radical scavenging activity)

The in-vitro anti-oxidant activity of the VN-ZnONPs was calculated through 2,2'- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method as described previous (Mittal *et al.,* 2006). For this 4 g of DPPH was dissolved in 100 mL of methanol and stored at $20^0$C. By the stock solution, 2mL of solution was mixed with 1mL methanol solution containing test samples of*Vitex nigundo* leaf aqueous extract and VN-ZnO NPs at diverse concentrations like 25, 50, 75 and 100µg/ml.

DPPH free radical scavenging activity (RSA) was measured at 517 nm. Ascorbic acid was utilised as standard in the present work. The DPPH activity expressed $IC_{50}$ values. The percentage of free radical scavenging activity (RSA) was calculated with using following equation.

RSA (%) = ((control absorbance-sample absorbance) / control absorbance)X100.

## Results and Discussion

### Ultraviolet-visible spectroscopy (UV-Vis) analysis of Vn-ZnO NPs

The bioactive phyto –constituents from chief and rare medicinal plant species are extensively used directly or indirectly in find out new drug formulations. UV-Vis spectra analysis is a primary tool to detect of formation of Vn-ZnO NPs in the reaction mixture. Based on the previous reports, it was clearly revealed that the nanoparticles showed vary colors in aqueous solution because of its feature of surface Plasmon resonance (SPR) (Kreibig and Vollmer, 1995; Prathna *et al.,* 2011). The color of Vn-ZnO NPs colloidal solution changed in to ----- from brown color. Reduction of ZnO NPs ions were performed by the using UV-Vis spectroscopy with compressed scan range from 190 to 750 nm. The UV-Vis spectra absorption of biosynthesized nanoparticles peak was obtained at 375 nm. This is further confirmed that the synthesized solution contained ZnO NPs in the reaction mixture. Previous reports also similar to these results (Fig.1) (Jayachandran *et al.,* 2021). Thus this work, the formation and stability of the metallic nanoparticles were fully comprehensible through UV-Vis spectrum and SPR graph.

### FT-IR analysis

Bio synthesized aqueous leaf Vn-ZnONPs analysis was done by the Fourier Transform Infra-Red (FT-IR) to know the feasible phyto-molecules responsible for the capping and stabilization of the nanoparticles (NPs). For this, the sample solution was analyzed in the scan range from 4000 to 500 cm-1 of spectra through FT-IR. The broad peaks were acquired at 1681.05cm-1 assigned to strong -C=O stretching conjugated ketone (belongs to the double bond region), 1446.19 and 865.24 these both are indicating to finger print region belongs to the mid-range IR spectrum. 1446.19 assigned to medium CH3 bend (Methylene) and the 865.24 assigned to the –C-H 1,3 di-substitution (meta-indicates benzene). This advises that the -C=O

ketone and 1446.19CH3 bend (Methylene) were participated to form of nanoparticles and these compounds act as capping and stabilization agents to prevent agglomeration in the reaction solution (Fig.3). Similar type of results was found in *Cayratia pedata* leaf aqueous ZnO NPs extract (Netala *et al.,* 2018).

## Dynamic light scattering (DLS) and zeta potential

DLS and zeta potential is an recent equipment to analyse the size and distribution of bio-synthesized nanoparticles. For this, the synthesized ZnO NPs were dispersed in 10 mL of distilled water. Owing to the Brownian motion of nanoparticles, light is dispersed at diverse intensities. Through this dispersed light intensities, DLS can be utilized to find the size of the nanoparticles. The zeta potential is used to estimate the stability, dissemination and aggregation levels of synthesized nanoparticles through repulsion effects induced by fluctuations in charge densities in the reaction mixture. The biosynthesized nanoparticles in the present work described 28.0 nm average size (Fig. 3 a) and -16.3 mV of zeta potential value (Fig. 3b). It assigned that the nanoparticles were well settled in the poly-dispersed juncture.

## X-Ray diffraction (XRD) analysis

XRD spectra clearly showed intensive peaks at $31.23^0$, $35.74^0$, $47.02^0$, $56.09^0$, $62.41^0$ and $67.52^0$ which all corresponded to (111), (200), (220), (222) and (311) respectively, which were significant agreement with the JCPDS file 89-1397. By this pattern confirmed that the nanoparticles were high purity and crystalline nature of the prepared Vn-ZnO NPs contains clear peaks.The XRD pattern results showed same accordance with previous study (Demissie *et al.,* 2020).

## TEM analysis

Biosynthesized Vn-ZnONPs were characterized by TEM, the tool provides micrographs and images at high magnifications (20nm). These images are helpful to find out the size, shape, surface morphology and distribution of the nanoparticles. Very small sized nanoparticles were recognized by the TEM. 20 nm scale bar studies of TEM micrographs of Vn-ZnONPs denotes that the biosynthesized nanoparticles are poly dispersed in nature, predominantly spherical in shape and no physical contact were seen between the particles i.e., no agglomeration was found. For theTEM analysis the ZnO NPs coated on copper grids and analysed by HR-Transmission Electron Microscopy (TEM, HF-3300 advanced with 300 kV TEM/STEM, Hitachi). The average size of the nanoparticles was 34.46 nm and the minimum sized NPs read 16.2 nm by HR-TEM(fig 5 and b). This type of results seen in ZnO NPs using leaf extract of *Lippiaadoensis* (Amrita Raj and Reena Lawerence, 2018).

## Antibacterial activity

The bio-fabricated Vn-ZnONPs efficacy were evaluated on two selected gram negative bacteria like *Escherichia coli*, *Salmonella typhi* and two gram positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. Amoxicillin standard drug used as a positive control, Zn acetate solution as a negative control and plant extract served as a control for compared. The results exhibited magnificent anti-microbial activity against gram negative bacteria; adequate activity was observed against gram positive bacteria. The zone of inhibition was shown in the table (table. 1, Fig.7--). Vn-ZnO NPs enter to the bacteria cell wall in to cytoplasm by diffusion and endocytosis when we added to the different bacteria. The Vn-ZnO NPs catalyse the cytotoxicity in the bacterial cell. In the order, the ROS (Reactive Oxygen Spaces) along with Vn-ZnO NPs acts on nucleus of the bacteria which stimulates the oxidation of nucleus and also catalyses chromosomal aberrations, finally causing the death of bacteria. The present study led to the set forward of new antibiotics against multidrug resistant bacteria by eco-friendly, cost-effective, easy green synthesis was carried out. These results were accordance with previous reports by various researchers (Bharathi *et al.,* 2018).

**Table.1** Antimicrobial activity of *Vitex nigundo* aqueous leaf extract biologically synthesized Vn-Zno NPs, Zn acetate solution and antibiotic Amoxicillin.

| Bacteria | Extract | ZnO (40 µg/ml) | ZnO NPs (50 µg/ml) | Amoxicillin |
|---|---|---|---|---|
| ***Escherichia coli*** | 0 | 16.25±1.11 | 19.25±0.63 | 25±0.71 |
| ***Salmonella typhi*** | 0 | 16±1.08 | 17.5±0.96 | 24.5±0.29 |
| ***Bacillus subtilis*** | 8.25±0.48 | 13.5±0.65 | 15.5±0.65 | 24.25±0.48 |
| ***Staphylococcus aureus*** | 0 | 12.5±0.87 | 14±0.41 | 22.5±0.50 |

**Fig.1** Uv-Vis spectra analysis spectrum of aqueous leaf Vn- ZnO NPs peak

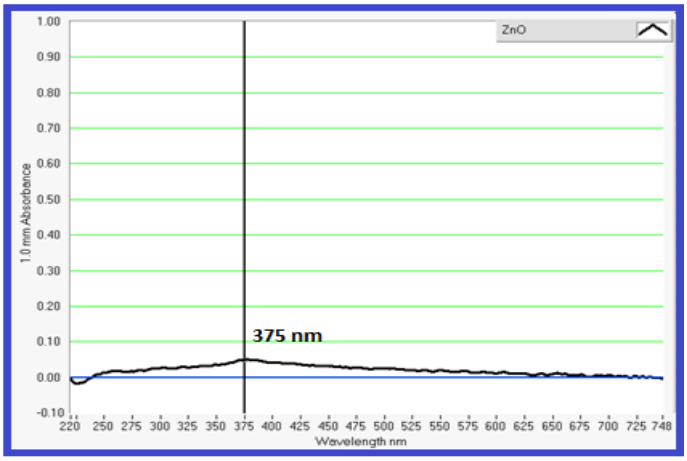



**Fig.2** a). Particle size Vn- ZnO NPs
b). Zeta potential of Vn- ZnO NPs
indicates the formation of biogenic ZnO NPs from the leaf extract

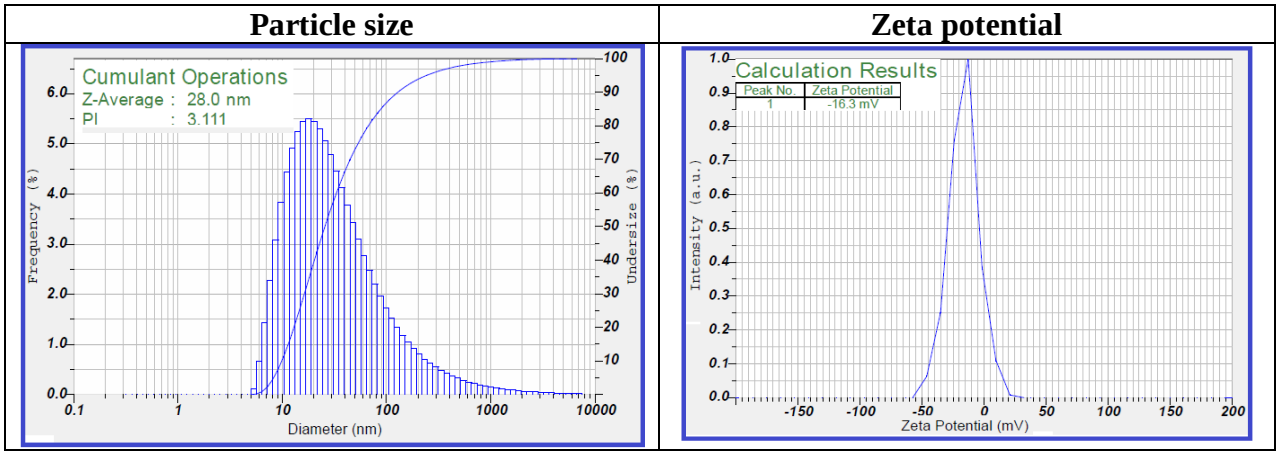


**Table.2** Anti-oxidant activity of *Vitex nigundo* leaf extract and biologically synthesized Vn-ZnO NPs

| Constituents | 25 µg/ml | 50 µg/ml | 75 µg/ml | 100 µg/ml | IC50 |
|---|---|---|---|---|---|
| **ZnONPs** | 35.5±0.25 | 51.16±0.8 | 65.24±0.66 | 72.4±0,74 | 48.86 |
| **Plant sample** | 25.35±0.60 | 30.28±0.56 | 40.64±0.78 | 53.40±0.48 | 93.63 |
| **Ascorbic Acid** | 44.44±1.40 | 57.64±0.47 | 65.65±1.50 | 77.54±0.60 | 28.12 |

**Fig.3** Frourier- Transform Infra-Red (FT-IR) spectra of bio-synthesized Vn- ZnO NPs

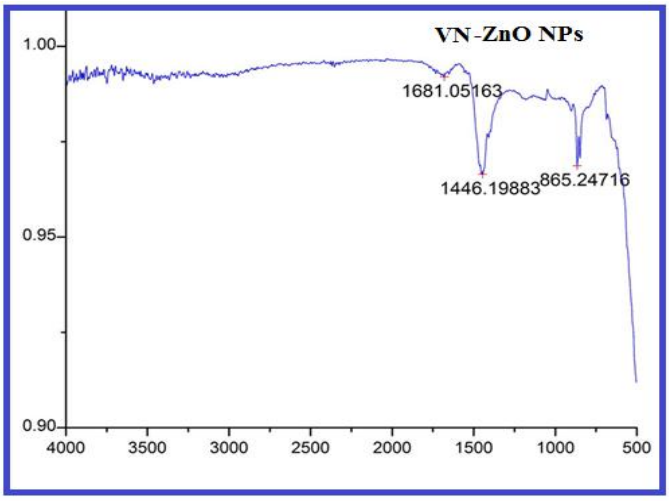



**Fig.4** XRD pattern analysis of bio-synthesized aqueous leaf source of VN-ZnO NPs

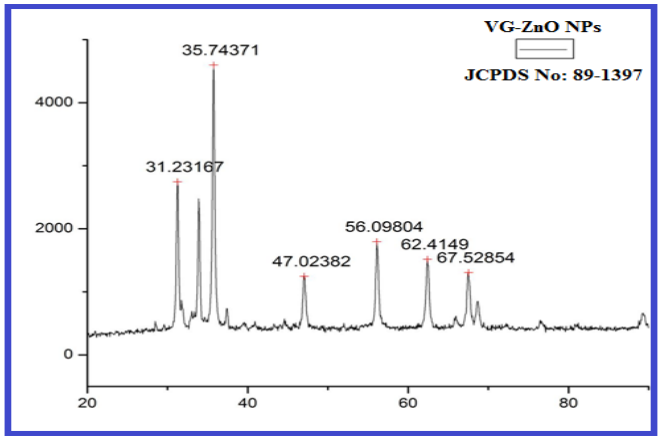


**Fig.5** a). TEM images at different magnifications b).EDAX

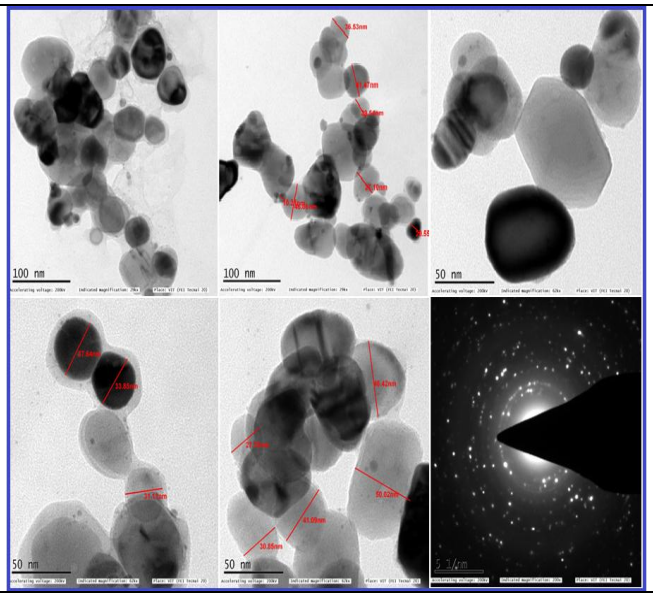
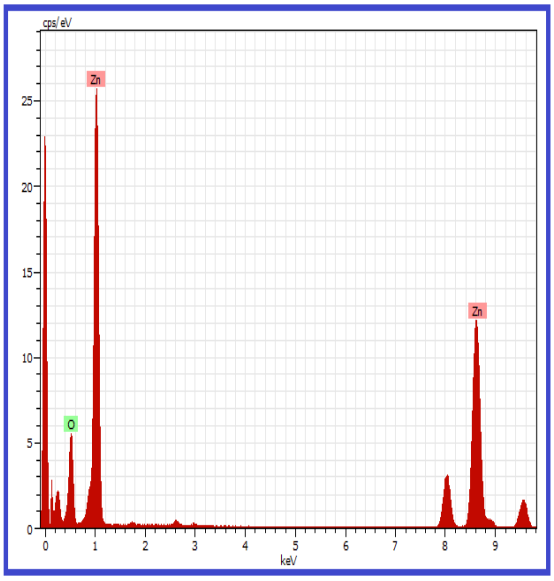
| a). TEM Images | b). EDAX |
|---|---|
|  |  |
| The average size of the nanoparticles was 34.46 nm | |



**Fig.6** Graphical representation of DPPH Anti-oxidant activity by biologically synthesized VN-ZnO NPs

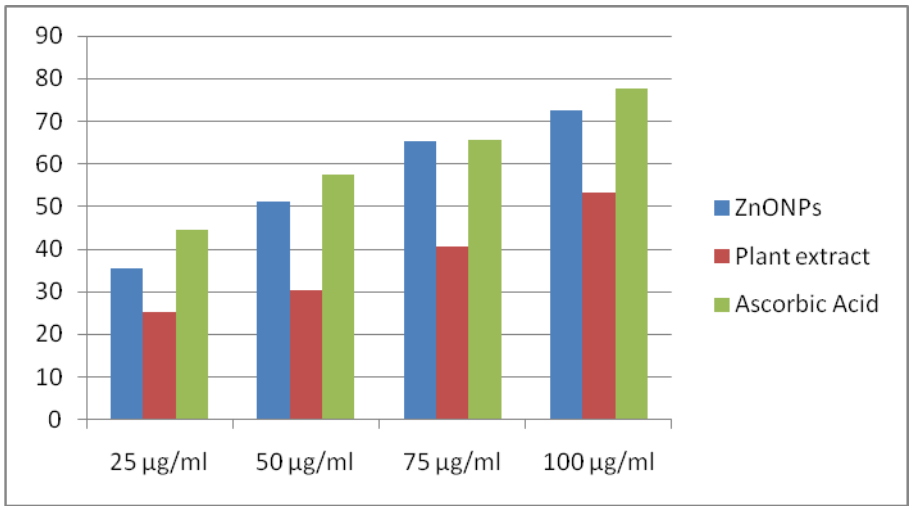


**Fig.7** Antimicrobial activity of biologically synthesized Vn-ZnO NPs against Two gram negative bacteria and two gram positive bacteria. 1. Plant extract 2. Zn acetate solution 3.ZnO NPs 4.Antibiotic Amoxicillin.

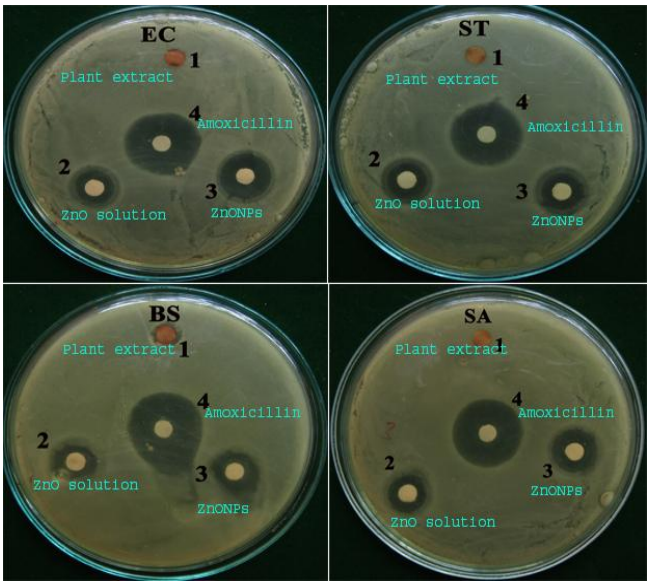
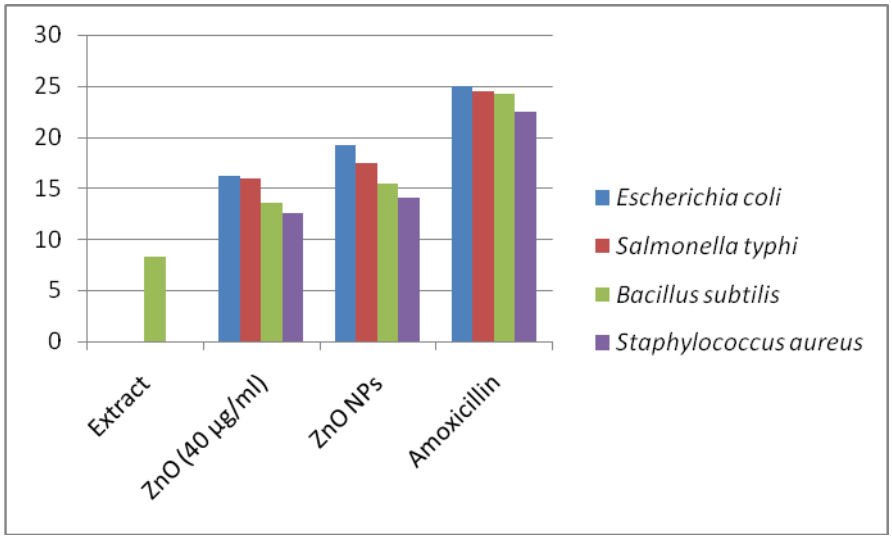



**Fig.8** Graphical representation of antibacterial activity through bio-synthesized Vn- ZnONPs




## ZnO Nanoparticles from Leafs Extracts of *Rosa indica*

### 2, 2- diphenyl-1- picrylhydrazyl (DPPH) anti-oxidant activity

Bio-synthesized Vn-ZnO NPs was ascertained by DPPH approach. The activity depends on the reduction of DPPH radical from DPPH to DPPH-H, a hydrogen donating anti-oxidant. The in-vitro anti-oxidants activity and half maximal inhibitory concentrations ($IC_{50}$) values of the *Vitex nigundo* leaf extract biologically synthesized Vn-ZnO NPs were described in the table--. The results revealed that the DPPH anti-oxidants activity increased by the concentration of the test solutions. Plants contain rich bio-active compounds like flavonoids and tannins belongs to the phenolic compounds along

with other polyphenols, which are a crucial group of phyto constituents that acts as primary anti-oxidants of free radical scavengers (Khan and Sultana, 2006). The maximum free radical scavenging activity was observed in the Vn-ZnO NPs with 77.54% at 100 µg/mL concentrations and lowest activity was seen 35.5% at 25µg/mL respectively (Table 2 and Fig. 8).

The present study on green synthesis of ZnO NPs through leaves of *Vitex nigundo*. In this reaction leaves aqueous extract was utilised as a capping agent for the stabilization of the biological synthesized nanoparticles. For the preparation of the ZnO NPs we followed simple, cost-effective, eco-friendly approach, less chemicals and were used and biologically safe method. This is a highly effective way of synthesis which involves non-toxic and very conventional method which leads to digging out for further routes of environmentally friendly nanoparticles. UV-Vis (scan range from 190 to 750 nm.) spectra analysis is a primary tool to detect of formation of Vn-ZnO NPs in the reaction mixture, by this tool at 375 nm we acquired peak. FT-IR results revealed about conjugated ketone, 1,3 di-substitution(meta- indicates benzene) and Methylene were participated to form of nanoparticles and these compounds act as capping and stabilization agents to prevent agglomeration in the reaction solution.The crystallinity of the synthesized nanoparticles was proven from XRD analysis. The morphology, shape, size and agglomeration pattern of the ZnO NPs was analysed by HR-TEM. The average size of the synthesized NPs was 34.46 nm, lowest size of the Vn-ZnO NPs was 16.2 nm and the highest size was 50 nm, sherical shaped, without any agglometation was found and EDAX was revealed about the Zn percentage (26%) and Oxide percentage (6%) in the reaction mixture. The biologically synthesized Vn-ZnO NPs was exhibited significant antimicrobial activity against both gram negative (*Escherichia coli*, *Salmonella typhi*) and gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*). Hence the higher bacterial growth zone of inhibition was recorded towards gram negative bacteria when compare the gram positive bacteria by the small sized phtyo-synthesized nanoparticles. Diverse concentrations of aqueous leaf sourced Vn-ZnO NPs showed strong anti-oxidant activity.

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**How to cite this article:**

Ganta Nalini and Tartte Vijaya. 2022. Bio-synthesis of Zinc Oxide Nanoparticles; Characterization and Evaluating their Biofertilizer, Antimicrobial and Antioxidant Efficacy. *Int.J.Curr.Microbiol.App.Sci.* 11(09): 62-73. doi: https://doi.org/10.20546/ijcmas.2022.1109.007