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Characterization ad evaluation of antibacterial potential of ZnO nanoparticle synthesized by *Vigna Mungo* and *Rhizobacteria*

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Abstract
In recent years, ZnO nanoparticles gained tremendous attention attributed to their unique properties. ZnO NPs exhibits antimicrobial properties. However, the properties of nanoparticles are depended upon their size and shape, which make them specific for various applications. The present study deals with the synthesis, characterization and evaluation of antibacterial potential of ZnO NPs synthesized by Vigna mungo and Rhizobacteria. The rhizobacteria have been isolated from the root nodule of V. mungo and has been morphologically, biochemically and molecularly characterized and identified to be Rhizobium sp. strain P4 and Bacillus flexus strain IFO15715. The GC-MS analysis of methanol leaf extract of V. mungo carried out for the detection and identification of bioactive compounds and this revealed phytol as the antibacterial compound while Squalene and Alpha – tocopherol have antioxidant and anti-tumor property. The antibacterial potential of ZnO nanoparticles and leaf extract of Vigna mungo were expressed by agar well diffusion assay. This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values. The results showed both methanol extract and zinc oxide nanoparticles harbor significant antimicrobial activity on most of the tested organisms. The synthesized nanoparticles from Rhizobium sp. were characterized by analytical techniques like SEM, XRD, FTIR, and UV-Vis.

Keywords: Vigna mungo, Zinc oxide nanoparticles, Nanotechnology, Antibacterial

Introduction
The field of nanotechnology is one of the most active research areas in modern materials science. Nanoparticles exhibit new or improved properties based on specific characteristics such as size, distribution and morphology. There have been impressive developments in the field of nanotechnology in the recent past years, with numerous methodologies developed to synthesize nanoparticles of particular shape and size depending on specific requirements. New applications of nanoparticles and nanomaterials are increasing rapidly [1].

There are various types of metal oxide including Titanium dioxide (TiO_2), Indium (III) Oxide (In_2O_3), Zinc oxide (ZnO), Tin (IV) and Silicon dioxide (SiO_2), where ZnO is one of the abundantly produced metal oxides after SiO_2 and TiO_2. ZnO is an inorganic material that exhibits unique properties including semiconductor, a wide range of radiation absorption, piezoelectric, pyroelectric and possesses high catalytic activity. In addition, ZnO has been listed as "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration (FDA 21CFR182.8991) due to its non-toxic properties. Consequently, this has extended the wide application of ZnO NPs in electronics, optics, biomedicine and agriculture. Nature has devised various processes for the synthesis of nano and micro-length scaled inorganic materials which have contributed to the development of relatively new and largely unexplored area of research based on the biosynthesis of nanomaterials. Synthesis using bio organisms is congruent with the green chemistry principles. Pulse crops occupy a high place in farming system because of their low water requirement and ability to withstand environmental stress. Pulses have the ability to fix atmospheric nitrogen through symbiosis with *Rhizobium*. Pulses have unique ability of deep root system mobilization of insoluble soil nutrients and bringing qualitative change in soil physical property, which make them known as soil fertility restorers [2, 3].

Plant roots release a wide variety of materials to their surrounding soil, including various alcohols, ethylene, sugars, amino acids, organic acids, vitamins, nucleotides, polysaccharides and enzymes. These materials create unique environments for the soil microorganisms. In 1904 the German agronomist and plant physiologist Lorenz Hiltner first coined the term "rhizosphere" to describe the volume of the soil present around the root [4].

The flavonoid inducers produced by the plant play a major role in the process by stimulating the *Rhizobium* to synthesize specific Nod factors that activate the host symbiotic processes necessary for root hair infection and nodule development.

The antibacterial activity of ZnO nanoparticles depends on the surface area and concentration, while the crystalline structure and particle shape have little effect. The smaller size of ZnO particles better is its antibacterial activity. Thus higher the concentration and larger the surface area of the nanoparticles, the better is its antibacterial activity. The mechanism of the antibacterial activity of ZnO particles is still not well understood. Some researchers have proposed in their study that the generation of hydrogen peroxide is the main factor of the antibacterial activity, while it also indicated that the binding of the particles on the bacteria surface due to the electrostatic forces could be another factor [5, 6].

Materials and methods

Preparation of plant extract

Fresh leaves of *Vigna mungo* were collected from the local regions of Thiruvananthapuram district, Kerala and washed several times with sterile distilled water and then dried using a hot air oven for 2-3 days and grinded to form powder. The powdered plant sample was sequentially extracted using three solvents namely hexane, butanol and methanol. The extracts were dried and the solvent-free extracts were stored in an air tight container for further study.

Isolation of rhizobacteria from *vigna mungo*

Healthy unbroken pink nodules were selected for the isolation. The Nodules were picked with sterile forceps. The collected nodules were kept in sterile polythene bags and transported to the laboratory for further investigation [7]. They were dipped in 0.1 % $HgCl_2$ or 3-5 % H_2O_2 for five minutes for surface sterilization of nodules.

The sterilized root nodules were crushed by adding 1 mL of sterile distilled water. This suspension was serially diluted up to 10^{-5}. The diluted suspensions 10^{-2}-10^{-4} were selected and 0.1 mL of suspension was inoculated in petri plates containing sterile Yeast Extract Mannitol Agar medium (YEMA) with congored. The inoculated plates were incubated at 30 ± 2°C for four days.

Morphological characterization of rhizobacteria

The bacterial isolates were grown on YEMA medium. After 24 to 72 hours, the colony morphology of isolates was studied with special consideration to the color, size of colonies, and their margins.

Biochemical characterization of rhizobacteria

The various biochemical characteristics viz., gram staining, motility test, indole production test, MR test, VP test, citrate utilization test, Oxidase test, GPA test, lactose assay, keto lactose test, starch hydrolysis test, gelatin hydrolysis test, flourescent assay, TSI test, Uresae test, Catalase test and nitrate reduction test were carried out [8-11].

Molecular characterization of rhizobacteria

The identification of virus requires metagenomic sequencing (the direct sequencing of the total DNA extracted from a microbial community) due to their lack of the phylogenetic marker gene 16S.

Synthesis of ZnO nanoparticles

Green synthesis of ZnO nanoparticles using *vigna mungo* leaf extract

2.4g of dried plant extracts of hexane, butanol and methanol were mixed with 12 mL of distilled water. 1 mL of leaf extract was added to the prepared solution of zinc acetate. 25 mL of 0.02 M NaOH was added drop wise to the solution to reach pH 12. The resultant white precipitate was filtered and washed repeatedly. Finally, a solid white powder was obtained after overnight drying of the purified precipitate at 60^0C in an oven [12].

Rhizobacteria mediated synthesis of ZnO nanoparticles

The bacterial isolates were inoculated on YEMA broth and incubated at 37^0C for 4 days. 1000 µL of cell extract was added drop by drop to 50 mL zinc acetate dehydrate ($Zn(CH_3COO).2H_2O$, 0.02 M). 0.1 M NaOH was added drop-wise to the constantly stirred solution until a faded white solution appeared. Then a pale white powder of ZnO nanoparticles was obtained after drying at 60°C in vacuum oven over night.

Chemical mediated synthesis of ZnO nanoparticles

Solution A contained 3.73 mmol of zinc acetate dihydrate dissolved in 40 ml of ethanol; solution B contained 7.22 mmol of NaOH dissolved in 320 µL of bi-distilled water and then in 25 mL of ethanol. Solution B was added drop wise to solution A under vigorous and constant stirring for 2.25 hour at 45, 50, 55, 60, and 65°C.

Antibacterial activity

The isolated three bacterial strains such as *Bacillus velezensis*, *Bacillus zanthoxyli* and *Klebsiella ozaenae* were used for determining the antibacterial activity of different plant extracts and nanoparticles.

Agar well diffusion assay

About 100 µL different plant extracts, green, chemical and rhizobacterial mediated synthesized nanoparticles were separately introduced into wells on each plates and allowed to diffuse at room temperature for 2 hours. 50 µL of DMSO was served as negative control and 25 µL of standard antibiotic solutions like Amoxicillin (AX) and streptomycin (SM) was used as positive control. The plates were then incubated at 37°C and zone of inhibition (mm) was observed after 24 hour of incubation.

Minimum inhibitory concentration (MIC)

100 µL of different concentrations (0.02µg/mL, 0.04µg/mL, 0.06µg/mL and 0.08µg/mL) of nanoparticles were added to the wells and allowed to diffuse at room temperature for 2 hours. The cultures were incubated at 37^0C for 24 hours [13].

Minimum bactericidal concentration (MBC)

The nanoparticles were diluted into various concentrations (0.06, 0.07, 0.08, 0.09, 0.1 and 0.5µg/mL) in sterile nutrient broth (5 mL) in test tubes. The 20 µL of *Bacillus velezensis* and *Klebsiella ozaenae* were added to all tubes and incubated at 37^0C for 24 hours. The lowest concentration in which has no single colony bacterial growth was taken as MBC [14].

GC-MS analysis

GC-MS analysis was carried out on a resolution GC-Agilent 7890 A and MS- Agilent 5975 C. The column used was DB5-

MS (30m x 25mm x 0.25µm).The temperature of the programme was 40^0C isothermal time; heating up to 250^0C with a heating rate of 40^0C/min. Helium was used as carrier gas with a flow rate of 1.0 ml /min. 1µl sample insertion volume was employed. The inlet temperature was controlled as 250˚C. The MS source temperature was 230^0C.

Characterization of ZnO nanoparticles

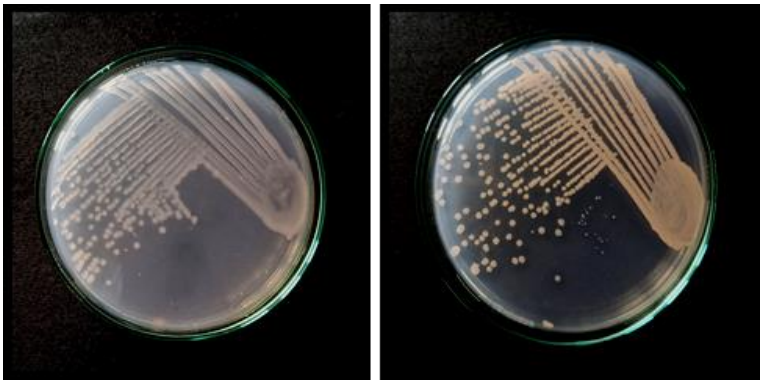
The synthesized nanoparticles were characterized by various techniques. They are SEM, XRD analysis, FTIR spectroscopy and UV–Vis spectroscopy.

Result and discussion

The results of morphological characteristics of rhizobacterial isolates were summarized in Table 1 and shown in fig 1. The rhizobacterial isolates were appear dominant growth on YEMA medium and two isolates were designated as RN001 and RN002. The isolates were slow growers and growth obtained after 2-3 days. The RN001 failed to absorb Congo red in the medium.

Table 1: Morphological characterization of Rhizobacteria

Isolate name	Colony characteristics on YEMA						
	Size	**Shape**	**Texture**	**Opacity**	**Pigmentation**	**Margin**	**Elevation**
RN001	Medium	Circular	Mucoid	Translucent	White	Entire	Raised
RN002	Small	Circular	Smooth	Opaque	Pink	Undulate	Flat



(a) RN001 (b) RN002

Fig 1: Isolation of rhizobacteria on YEMA medium

The RN001 colony morphology on YEMA medium was mostly circular, mucoid, white and translucent. They showed highly mucoid on after 3 days of incubation at 28°C. The RN002 colony was circular, smooth, pink and opaque.

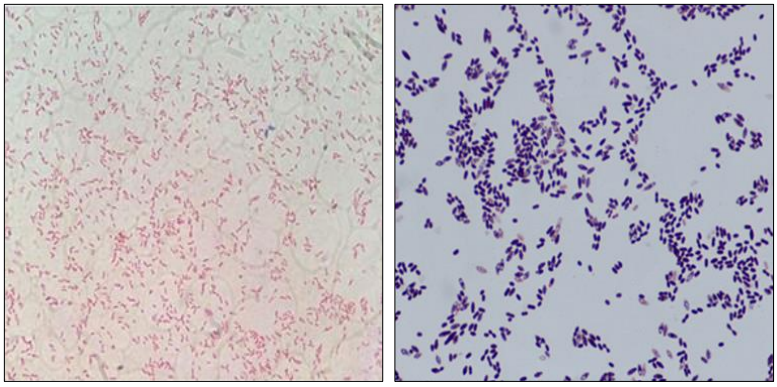
Biochemical characterization of Rhizobacteria

The biochemical characteristics of the isolated organisms are summarized in the Table 2. The bacterial isolate RN001 was aerobic, Gram-negative, rod-shaped and motile (fig 2). The isolate RN001 showed positive reactions in Methyl Red (MR), Starch hydrolysis, Triple Sugar Iron (TSI) agar, Nitrate reduction, Glucose Peptone Agar (GPA), Catalase, Oxidase, Urease test and Lactose assay. They were negative to Indole production, Voges-Proskauer (VP), Citrate utilization, Gelatin hydrolysis, Keto - lactose test, and Flourescent assay. The bacterial isolate RN002 was aerobic, Gram-positive, rod-shaped and motile. The cells contained endospores. The isolate RN002 showed positive reactions in Indole production, Citrate utilization, Starch hydrolysis, Triple Sugar Iron (TSI) agar, Gelatin hydrolysis, Glucose Peptone Agar (GPA), Oxidase, Catalase test and Lactose assay. They are negative to Methyl Red (MR), Voges - Proskauer (VP), Urease, Nitrate reduction, Keto - lactose test, and Flourescent assay.

Table 2: Biochemical characterization of Rhizobacteria

Size	**Shape**	**Texture**	**Opacity**	**Pigmentation**	**Margin**	**Elevation**

Sr. No.	**Biochemical tests**	**Isolate name**	
		RN001	**RN002**
1	Gram staining	Gram negative	Gram positive
2	Motility test	Motile	Motile
3	Indole production test	Negative	Positive
4	Methyl Red (MR) test	Positive	Negative
5	Voges – Proskauer (VP) test	Negative	Negative
6	Citrate utilization test	Negative	Positive
7	Nitrate reduction test	Positive	Negative
8	Urease test	Positive	Negative
9	Triple Sugar Iron (TSI) agar test	Positive; K/A	Positive; K/A
10	Glucose Peptone Agar (GPA) test	Positive	Positive
11	Lactose assay	Positive	Positive
12	Keto lactose test	Negative	Negative
13	Starch hydrolysis test	Positive	Positive
14	Gelatin hydrolysis test	Negative	Positive
15	Flourescent assay	Negative	Negative
16	Catalase test	Positive	Positive
17	Oxidase test	Positive	Positive



(a) RN001 (b) RN002

Fig 2: Gram staining results of rhizobacteria

Molecular characterization of Rhizobacteria

A single band of high-molecular weight DNA has been observed. 16S rRNA gene was amplified by 16S rRNA F and 16S rRNA R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDTv3.1Cycle sequencing kit on ABI3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence (fig 3).

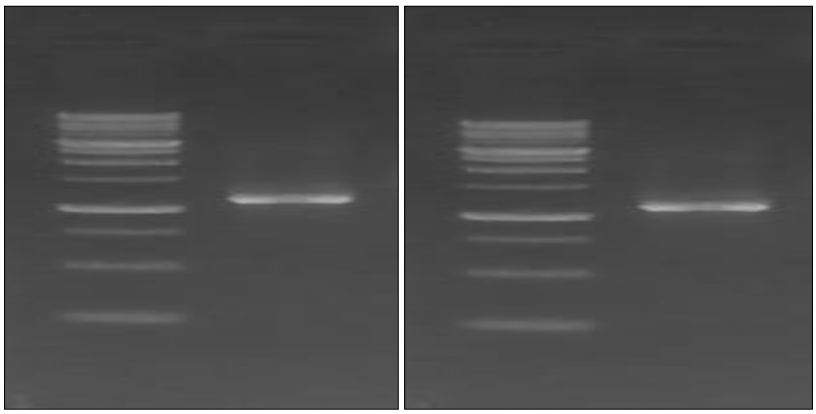


Fig 3: Agarose gel showing the band of isolate RN001 and RN002

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.38899409 for RN001 and the optimal tree with the sum of branch length = 0.23306337 is shown for RN002. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1017 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Based on sequence homology and phylogenetic analysis, RN001 was identified to be *Rhizobium* sp. strain P4 and the RN002 was identified to be *Bacillus flexus* strain IFO15715 (fig 4 & 5).

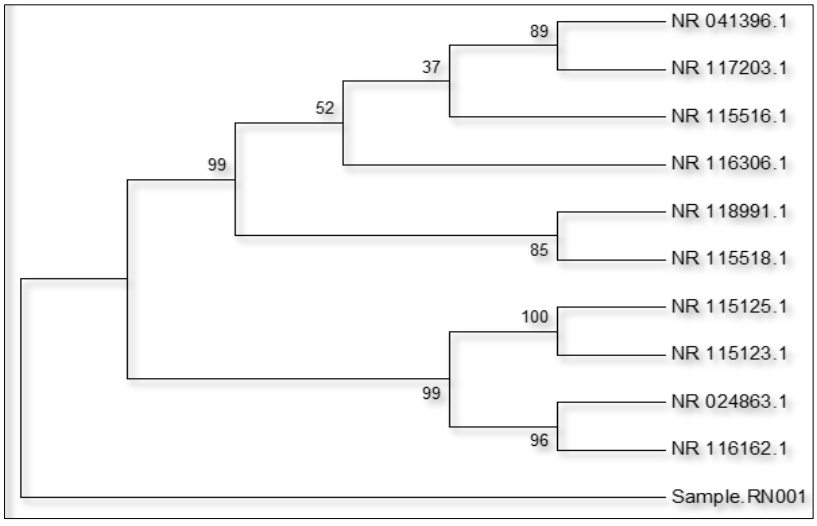


Fig 4: Taxonomic tree of isolate RN001

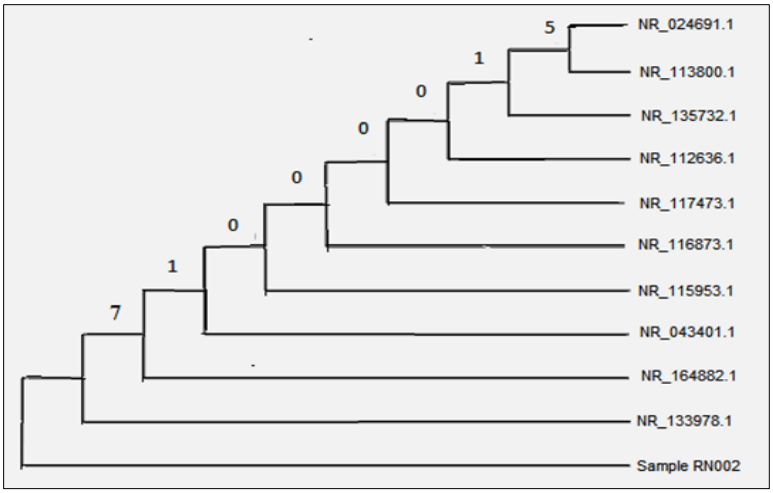


Fig 5: Taxonomic tree of isolate RN002

Antibacterial activit

DMSO was served as negative control and standard antibiotic solutions like Amoxicillin (AX) and streptomycin (SM) was used as positive control. The antibacterial activity of different plant extracts of *Vigna mungo* in different test organisms showed antibacterial activity in the range between 14 to 24mm (fig 6). The methanol extract (VMNP) showed highest zone of growth inhibition against *B. zanthoxyli* (24 mm) followed by *B. velezensis* (14 mm). The antibacterial activity of green synthesized zinc oxide nanoparticles in different test organisms showed broad spectrum antibacterial activity in the range between 13 to 25 mm (fig 7). Among them methanol mediated synthesized zinc oxide nanoparticles (VMNP) showed highest zone of growth inhibition against *K. ozaenae* (25 mm), *B. zanthoxyli* (21 mm) and *B. velezensis* (15 mm).The antibacterial activity of rhizobacterial synthesized NPs showed antibacterial activity in the range between 11 to 22 mm against test organisms (fig 8). Among them the pellet of both *Rhizobium* sp. (NWP) and *Bacillus flexus* (NPP) showed broad spectrum antibacterial activity in the range between 15 to 22 mm. Microbial synthesized zinc oxide nanoparticles showed greater antimicrobial potential when compared with chemically synthesized zinc oxide nanoparticles.

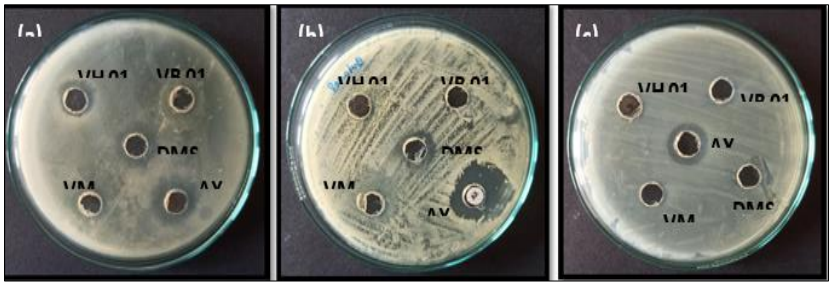


Fig 6: Antibacterial activity of plant extracts against (a) *B. velezensis* (b) *B. zanthoxyli* (c) *K. ozaenae*

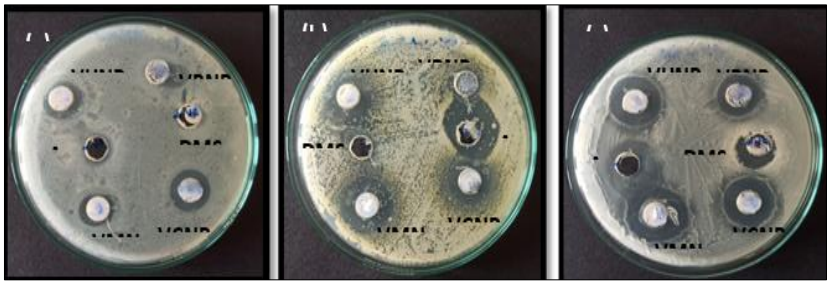


Fig 7: Antibacterial activity of green synthesized NPs against (a) *B. velezensis* (b) *B. zanthoxyli* (c) *K. ozaenae*

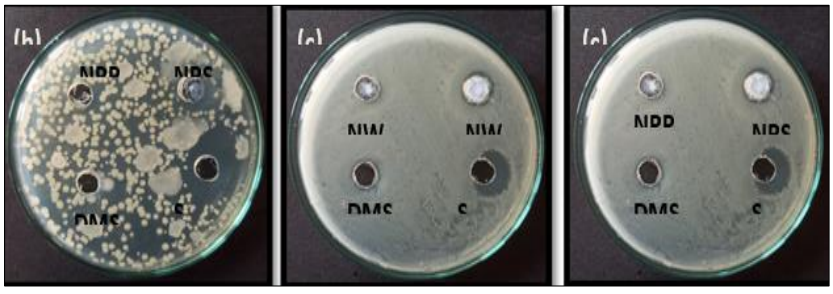


Fig 8: Antibacterial activity of rhizobacterial synthesized NPs against (a) *B. velezensis* (b) *B. zanthoxyli* (c) *K. ozaenae*

Minimum inhibitory concentration (MIC)

The MIC was determined against two bacterial strains such as *B. velezensis* and *K. ozaenae*. The NPs synthesized from the pellet of *Rhizobium* sp. expressed minimal MIC against *B. velezensis* (0.04 µg/mL) followed by *K. ozaenae* were showed in table 3.

Table 3: MIC results

	Diameter of zone of inhibition (in mm)	
Concentration (µg/mL)	**B. velezensis**	**K. ozaenae**
0.02	nil	nil
0.04	12	15
0.06	19	20
0.08	23	22

Minimum bactericidal concentration (MBC)

The MBC results revealed that zinc oxide nanoparticles of *Rhizobium* sp. significantly inhibit the growth of selected test organisms (table 4). The zinc oxide nanoparticles expressed minimal MBC value against *Bacillus velezensis* (0.5 µg/mL) followed by *Klebsiella ozaenae.*

Table 4: MBC results

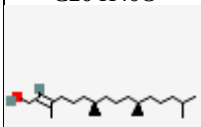
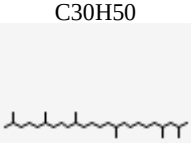
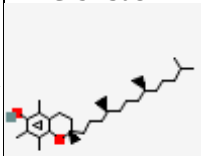
Concentration (µg/mL)	**Number of colonies**	
	Bacillus velezensis	**Klebsiella ozaenae**
Control	TNTC	TNTC
0.06	326	TNTC
0.07	262	298
0.08	125	280
0.09	63	119
0.1	29	60
0.5	nil	nil

GC-MS Analysis

The spectrum of the compounds was correlated with the database of spectrum of identified compounds were stored in the GC-MS library. The summary of analysis is given in Table 5. The GC-MS study of leaf extract of *Vigna mungo* plant have shown many phytochemicals. The major components present in the methanol extract of *V. mungo* along with the molecular formula, molecular weight, and retention time are presented in Table 5. The methanol extract of *Vigna mungo* leaves showed the content of Phytol, 2,6,10,15,19,23 –hexamethyltetracosane and Alpha-tocopherol. These compounds have been frequently attributed to their antioxidant, antitumor and antimicrobial activity [7].

Vigna have antioxidant properties and are able to manage and cure different diseases linked with free radical generation [9].

Table 5: GC-MS Analysis of *Vigna mungo* leaf extract

Sl. No:	**Retention Time (RT)**	**Area percentage (%)**	**Compound**	**Molecular Formula And structure**	**Molecular weight (g/mol)**	**Bioactivity of compound**
1	56.770	16.88	Phytol	C20 H40O 	128.1705	Antioxidant and Antimicrobial activity
2	68.560	55.56	2,6,10,15,19,23 - hexamethyl - tetracosane (Squalene)	C30H50 	410.73	Antioxidant and Antitumor activity
3	73.292	27.56	Alpha- tocopherol (Vitamin E)	C29H50O2 	430.71	Antioxidant activity

Characterization of ZnO nanoparticles

SEM analysis

These images (Fig. 9) demonstrated that zinc oxide nanoparticles are spherical in shape and each particle was aggregation of many smaller particles [3]. Claimed that the morphology of ZnO nano-powder using zinc acetate is smoother than in zinc nitrate. Moreover, precursor concentration plays a great role on morphological features of nanoparticles. According to Pourrahimi *et al*. (2014), ZnO can be form in different structures due to the type of precursors that has been used [11].

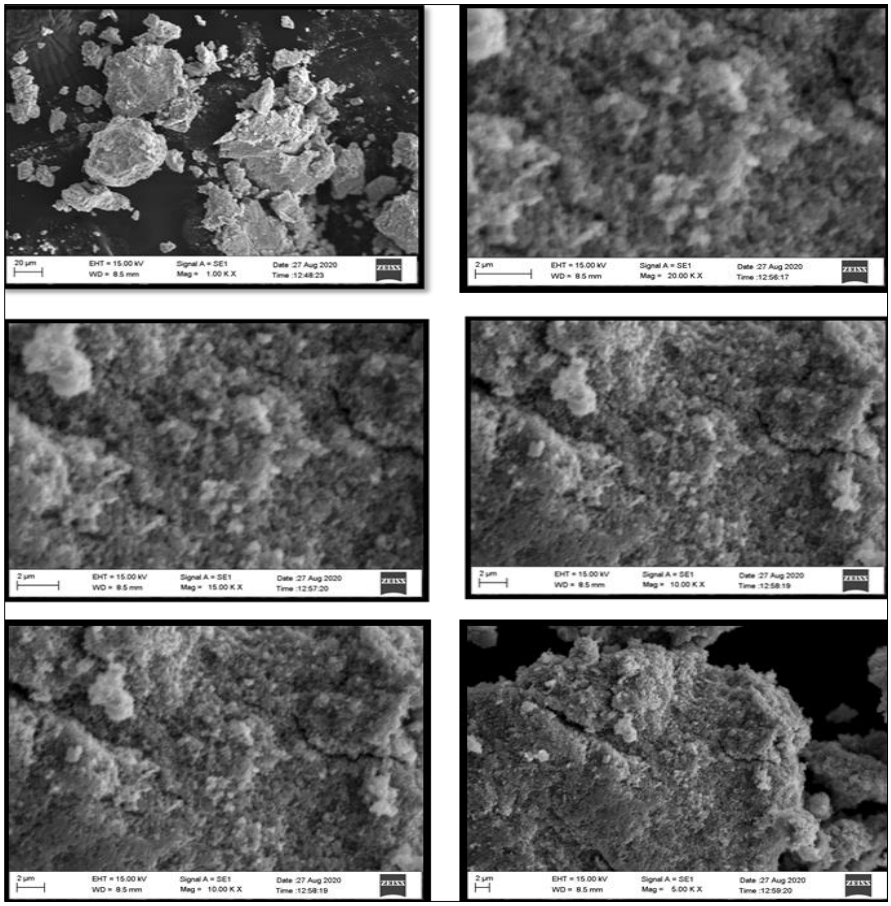
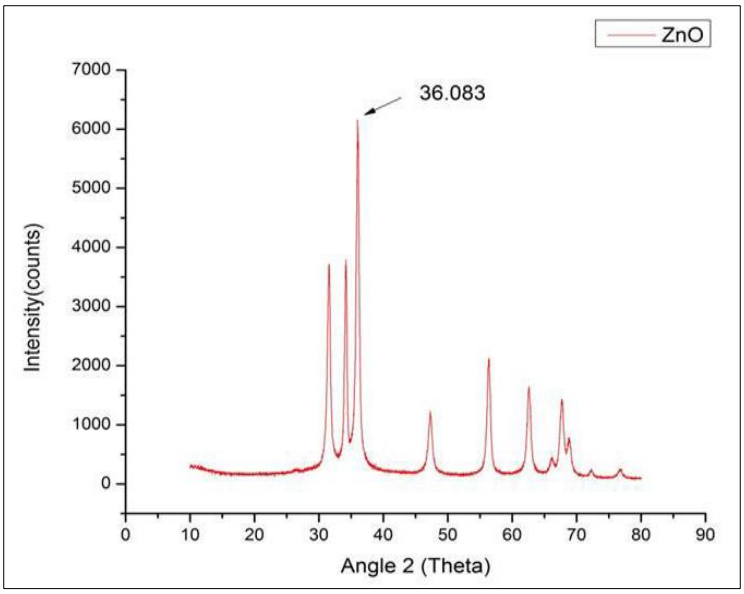


Fig 9: SEM analysis of ZnO NPs synthesized from the pellet of *Rhizobium* sp.

XRD analysis

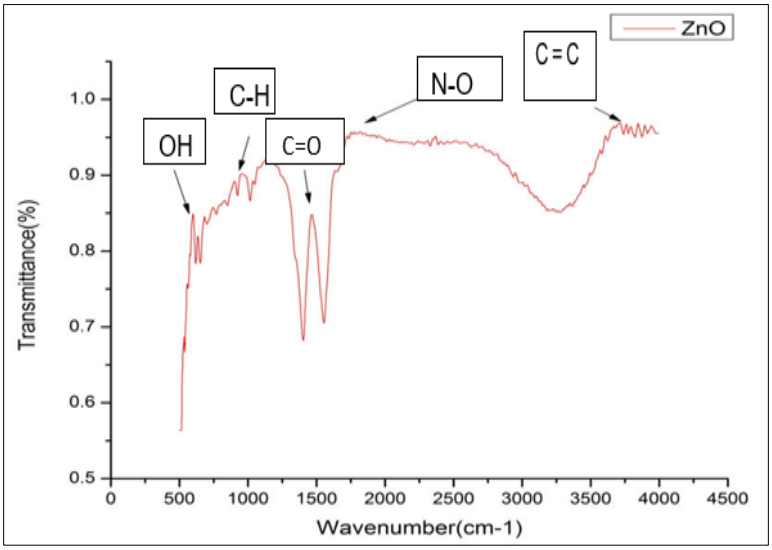
Graph 1 showed the XRD pattern of ZnO NPs synthesized by the *Rhizobium* sp. XRD shows 2θ values at 31.548^0, 34.197^0, 36.017^0, 47.292^0, 56.360^0, 62.622^0, 66.15^0, 67.729^0, 68.820^0, 72.309^0 and 76.739^0. It also confirms the synthesized nano powder was free of impurities as it does not contain any characteristics XRD peaks other than zinc oxide peaks.



Graph 1: XRD analysis of ZnO nanoparticles

FTIR spectroscopy

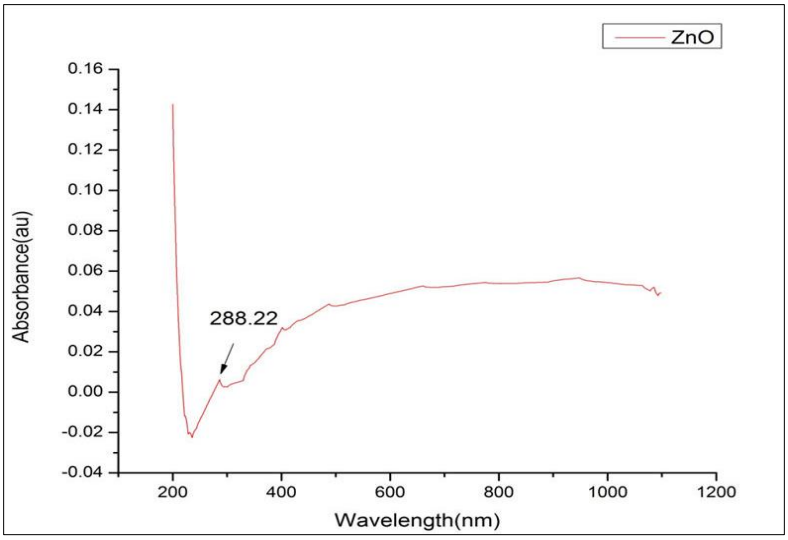
FTIR spectra (Graph 2) of the synthesized nanoparticles were recorded in the 500–4500 cm^{-1} range. The absorption at 1227 cm^{-1} indicates the presence of hydroxyl group. The absorption at 1687 indicates the presence of alkane groups. The absorption at 1593 indicates the presence of carbonyl groups. The weak absorption at 2015 is due to C=C stretching vibration. The absorption peaks at 2686 and 3120 indicates the presence of amide and aromatic alcohol. They could possibly enhance the stabilization of ZnO nanoparticles in the aqueous medium.



Graph 2: FTIR spectrum of ZnO nanoparticles

UV-VIS spectroscopy

Graph 3 shows the UV-Vis absorption spectrum of zinc oxide nanoparticles. The absorption spectrum was recorded for the sample in the range of 280 - 420 nm. The spectrum showed the absorbance peak at 288 nm corresponding to the characteristic band of zinc oxide nanoparticles. On the surface of nanoparticles, the electron clouds are present which are able to oscillate and absorbs the electromagnetic radiation at a particular energy, energy corresponding to the photons of 288 nm. This resonance is known as surface plasmon resonance (SPR).



Graph 3: UV-Vis spectrum of ZnO nanoparticles

Conclusion

The present study shows that Zinc oxide NPs were successfully synthesized by the biological method. ZnO NPs can easily be synthesized using rhizobacteria as the biological system. Among them Rhizobium sp. can be manipulated under controlled conditions and has great potential for extracellular and intracellular synthesis of metallic nanoparticles. This method is simple, cheap and without danger of any contaminants and pollution and produced large amount of stable nanoparticles. From the above study, the antibacterial potential of zinc oxide nanoparticles as well as leaf extract of V. mungo are also verified. The antibacterial activity was investigated against three test microorganisms such as Bacillus velezensis, Bacillus zanthoxyli and Klebsiella ozaenae. The GC-MS analysis of methanol leaf extract of V. mungo reveals the presence of Phytol, Squalene and Alpha - tocopherol. These studies demonstrate that ZnO nanoparticles and leaf extract of V. mungo have a wide range of antibacterial activities toward various potential pathogenic bacteria which could be applied in nanodrug formulations. On the basis of overall results achieved during this study, we can conclude that biosynthesis of ZnO NPs through V.

mungo and rhizobacteria is much safer and ecofriendly than the physical and chemical methods.

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