IJPSR (2022), Volume 13, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 12 April 2021; received in revised form, 10 June 2021; accepted, 09 June 2021; published 01 February 2022

ANTI-BACTERIAL EFFICACY OF PHYTO-SYNTHESIZED ZINC OXIDE NANOPARTICLES USING *MURRAYA PANICULATA* L. LEAF EXTRACT

Ajay Kumar Tiwari [1], Saket Jha [* 2], Rahul Agrawal [3], Sheo Kumar Mishra [4], Ashok Kumar Pathak [5], Abhimanyu Kumar Singh [6], Anupam Dikshit [2] and Ram Rashile Awasthi [7]

Department of Physics [1], Nehru Gram Bharati (Deemed to be) University, Prayagraj - 221505, Uttar Pradesh, India.
Department of Botany [2], Centre of Food Technology [3], Institute of Professional Studies, University of Allahabad Prayagraj - 211002, Uttar Pradesh, India.
Department of Physics [4], Indira Gandhi National Tribal University Amarkantak - 484887, Madhya Pradesh, India.
Department of Physics [5], Ewing Christian College, University of Allahabad, Prayagraj - 211003, Uttar Pradesh, India.
Department of Physics [6], Shyama Prasad Mukherjee Government Degree College, University of Allahabad, Prayagraj - 211019, Uttar Pradesh, India.
Department of Physics [7], University of Lucknow, Lucknow - 226007, Uttar Pradesh, India.

Keywords:

Phyto-synthesis, ZnO NPs, Human pathogens, Anti-bacterial assay, Well diffusion methods, Tooth filling agent, etc.

Correspondence to Author:
Dr. Saket Jha

Department of Botany,
University of Allahabad Prayagraj - 211002, Uttar Pradesh, India.

E-mail: jhasaket90@gmail.com

ABSTRACT: Human beings always tried to protect their body from pathogenic microbes, whereas these pathogens always mimic and mutate themselves to infect humans. Nanotechnology is new and upgrading itself as well as enhancing its applicability in pharmaceuticals. Due to their nano-size structure, property drastically changes from their parent elements. Nanoparticles are used widely in biomedical sciences. Focusing on these perspectives, the present study investigates the impact of phyto-synthesized Zinc Oxide Nanoparticles (ZnO NPs) using *Murraya paniculata* leaf extracts. The characterization of synthesized ZnO NPs was 32 nm with a more optical stable showing peak value at 355 nm (UV-vis). Further, toxicological efficacy of ZnO NPs was checked against human pathogens *viz*., *Salmonella typhimurium* (causing typhoid), *Staphylococcus aureus* (causing tooth decay and rheumatic fever) and *Escherichia coli* (causing tooth decay in infants and dysentery). The anti-bacterial bioassay was done using a well diffusion method and compared with the standard drug streptomycin. The test pathogens were found more sensitive towards phyto- synthesized ZnO NPs with a Zone of Inhibition Diameter (ZID) value almost more than 22 mm for all three test pathogens as well as compared with earlier reports and found a potential herbal anti-bacterial agent. From the present investigation, the phyto-synthesized ZnO NPs showed promising results and may be used in dentistry as tooth filling agents and as nano-drugs in pharmaceuticals.

INTRODUCTION: The current global pandemic situation demands a focus on human health.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.13(2).864-71

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(2).864-71

As pathogenic microbes and human have been mostly antagonistic with each other and thus require a search of new possibilities to make pathogen resistant environment in the human body. More than 700 pathogens were found to invade our body *via* mouth [1]. The detection of these pathogens is time taking because of their symptomatic behavior and causes lethal many times, especially in children. Typhoid is first in this list and is a major concern to developing countries like India,

where people use drinking water from wells, rivers, ponds and stored tanks. Tooth decaying and dysentery are other diseases most common in children. The researchers are still searching for a noble and effective tool that kills these pathogens at very early stage [1].

The nanoparticles, due to their characteristic properties such as nano-crystal size and optical property, make them one of the best tools in nanotechnology [2, 3]. These nanoparticles having a maximum surface area to the volume and greater mechanical strength with optical stable make them more valuable [3]. These are widely used in almost all fields such as agriculture, pharmaceutical, biomedicine, robotics, *etc*. Due to this property, these bio-synthesized nanoparticles are used widely in drug delivery and noble formulation in medical sciences [4, 7].

Several metal oxide nanoparticles are introduced in which zinc oxide nanoparticles are considered suitable nanomaterial in dentistry and many bio medicine because of their potential possess antimicrobial, antioxidant, anti-cancerous, drug delivery, bio-sensing, *etc*. [7, 8]. Several methods are now introduced for making these nanoparticles in which phyto-synthesis and microbial synthesis are found eco-friendly and high toxic to pathogens [9, 19]. The Zinc oxide powders are used as tooth filling agents, and this powder can be replaced by ZnO NPs as having dual property (antimicrobial and smooth powder) along with its durability [9].

The phyto-synthesis is now used extensively due to non-toxic to human health and bio safe [17] but able to kills both (gram-positive and gram-negative) types of bacterial pathogens [9]. Phyto-synthesis of ZnO NPs provides a more suitable and efficient method. Nanoparticles interact with several biomolecules, and various microbes constitute the broad spectrum of research and unexplored till today [1]. Plant's secondary metabolite was used to reduce and stabilize the metal ions and leads to help in the reduction of their size during phyto-synthesis of metal oxide nanoparticles [20, 21]. Many plants were till today were used in the synthesis of ZnO NPs such as *Azadirachta indica* [22], *Aloe vera* [23], *Parthenium hysterophorus* [24], and *Pongamia pinnata* [25]. Phyto-synthesis provides wide dimensional and high potentially active nanocrystals [26] which show enhanced antimicrobial property [9, 27]. Due to their biocompatibility, these zinc oxide nanocrystals were widely used in various applications of medical sciences [28]. This manuscript emphasizes phyto-synthesis of ZnO nanoparticles using *Murraya paniculata* leaf extracts using hydro-thermal method [29]. The synthesized ZnO NPs were characterized, and anti-bacterial bioassay was performed against three human pathogens: *Staphylococcus aureus* (gram-positive), *Escherichia coli*, and *Salmonella typhimurium* (gram-negative) using agar well diffusion method [30, 31].

MATERIALS AND METHODS:

Phyto-synthesis of ZnO NPs: The preparation of ZnO NPs was accomplished following Bela *et al.*, [29] with some modifications. The 50 ml solution of Zinc acetate (0.45M) (was added dropwise in aqueous solution of 5 gm leaves of *Murraya paniculata* with continuous stirring (1200 rpm) at temperature of 60 °C for 2 h. The precipitate was centrifuged at 6000 rpm for 15 min and was thrice with ethanol and sterile water. The brown color precipitate was kept for 24 h in Hot Air Oven at 100°C [29]. The prepared nanoparticle was characterized with SEM for their morphological evaluation, FTIR for its stability, UV-vis for its optical properties, and XRD for their crystal size and grain structure.

Inoculum Preparation: The human pathogens *viz*., *Salmonella typhimurium* (MTCC-3219), *Staphylococcus aureus* (MTCC-7443), and *Escherichia coli* (MTCC-687) procured from MTCC, IMTECH, Chandigarh, Punjab, India. The inoculums were used from one-day-old cultures and the suspension was compared with 0.5 McFarland standard solution using spectrophotometer at Optical density 625 nm with value 0.08 - 0.13 prescribed by CLSI guidelines [32]. The inoculants were having a concentration of 5×10^5 CFU/mL used further for the well diffusion technique for their drug susceptibility test.

Anti-bacterial Bioassay: The anti-bacterial bioassay of biologically synthesized ZnO NPs was evaluated using the agar well diffusion method [31, 33, 34]. Using a sterile cotton swab, the prepared inoculum of test Pathogens *viz.*, *Salmonella typhimurium* (MTCC-3219), *Staphylococcus*

aureus (MTCC-7443), and *Escherichia coli* (MTCC-687) were swabbed on the surface of sterilized nutrient agar plates. Using a micropipette 100 µl of 1000 ppm concentration of biologically synthesized ZnO NPs was added to all four wells [24, 25]. Streptomycin was taken as a standard drug and for blank 100µl of sterile water was used. Quadruplets in each plate having a single dose of 1000 ppm and reading were compared with their standard, positive plates. The plates were incubated at 37ºC ± 2 ºC for 24 h placed in upright positions [31, 33, 35, 36]. The anti-bacterial activity was measured in the form of the zone of inhibition diameter (in mm). The recorded values were further statistically calculated by one-way ANOVA of the Duncan formula using SPSS 16.0. The significant value was $P > 0.05$.

RESULTS AND DISCUSSION:

Structural Analysis of ZnO NPs: The X-ray diffraction pattern of the phyto-synthesized ZnO NPs has been recorded by X-ray Diffractometer for 2Ɵ values ranging from 20º- 70º using CuKα radiation as shown in **Fig. 1**. The X-ray diffraction pattern of ZnO nanoparticles exhibits that ZnO nano-biomaterial has a spherical structure with agglomeration and having stable peaks [37]. These peaks have broadening, which implies that the ZnO nanoparticles have nanocrystalline nature. The calculated grain size of synthesized nanoparticles is found to be 25-35 nm range.

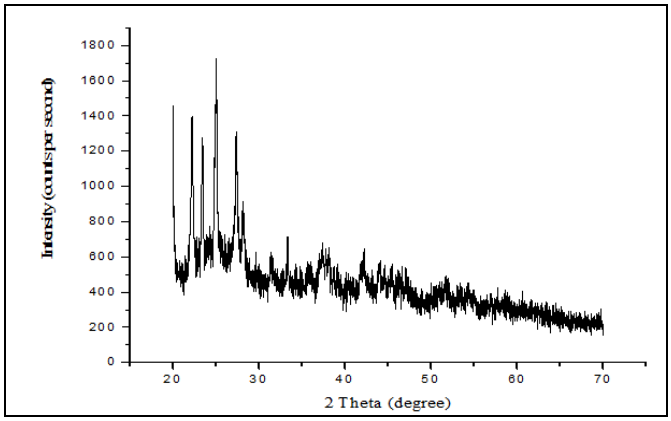


FIG. 1: X-RAY DIFFRACTION PATTERN OF PHYTO-SYNTHESIZED ZnO NANOPARTICLES

Scanning Electron Micrograph (SEM) Analysis: Scanning electron micrograph of ZnO nano-biomaterial showed spherical cluster shape. Particles are almost inconsistently distributed on the surface shown in **Fig. 2** A-D.

The increased magnification showed that cluster shape grains are clearly spherical clusters with granular shape background uniformly distributed throughout the surface.

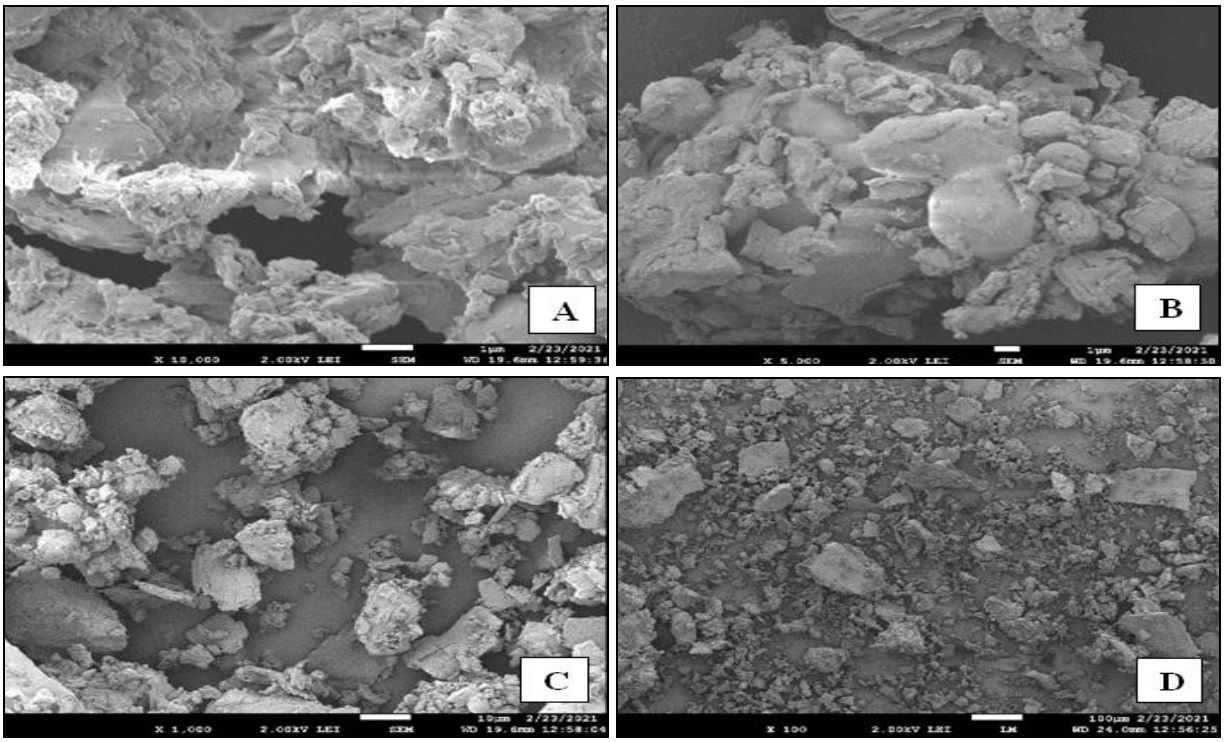


FIG. 2: SCANNING ELECTRON MICROGRAPH OF PHYTO-SYNTHESIZED (*M. PANICULATA* LEAVES) ZnO NANOPARTICLES. MAGNIFICATION AT X10K (A), X5K (B), X1K (C) AND X100 (D)

UV-Visible Absorption Analysis: The synthesized nanoparticles having absorption peak value at 355 nm in UV-vis spectra and is shown in **Fig. 3**.

The entire properties of nano-materials depend on its nano-size and crystal structure. Also optical property plays a vital role in stable nanoparticles and is done by UV-visible spectroscopy [38].

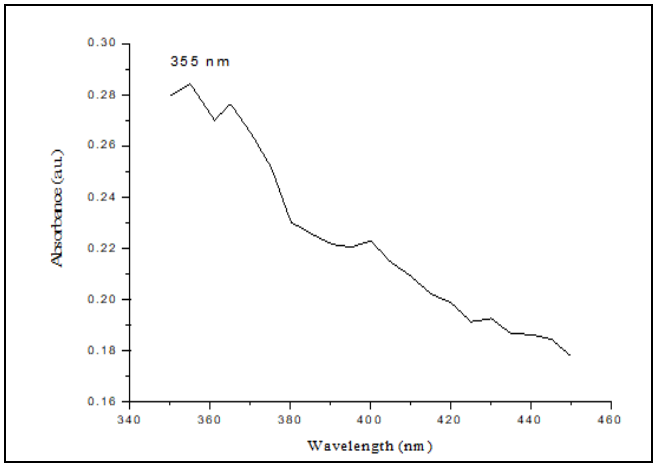


FIG. 3: UV-VISIBLE SPECTRA OF PHYTO-SYNTHESIZED (*M. PANICULATA* LEAVES) ZnO NANOPARTICLES

The significant sharp absorption ZnO NPs indicate the mono disappeared nature [39]. The nano-crystals distribution of synthesized nanoparticles with particle radius of 17 nm having bandgap enlargement [40].

The prepared ZnO nanoparticles exhibit an absorbance peak at 355 nm, which corresponds to the particle size of 33.82 nm. Earlier researchers were found the size up to 62-94 nm (*Catharanthus roseus* leaf extract) [41]; 15-20 nm (*Bambusa Vulgaris* leaf extract) [42]; 25-30 nm (*Sambucus ebulus* leaf extract) [43] and 12-46 nm (*Hibiscus subdariffa* leaf extract [29].

These results have been confirmed by the SEM and XRD values. The nanoparticles were agglomeration (clusters) of spherical and crystalline structure [44, 45].

FTIR Analysis: FTIR analysis was carried out for the functional groups present in the synthesized ZnO NPs, shown in **Fig. 4**.

The phyto-synthesized sample of ZnO NPs exhibits several absorption bands such as 1559, 1447, 1343, 1023, 954, 695, 617, 518 cm^{-1}. The broad absorption band peak at 1015 cm^{-1} is ascribed to the broadening pulsation of C-N bond. The absorption band at 1559 cm^{-1} is ascribed to 1º, 2º alcohol in-plane curve or pulsation and corresponding to the aromatic nitro compounds vibration modes.

The absorption peaks located at 1559, 1447, 1343, 1023, 954, 695, 617, 518 cm^{-1} are due to stretching vibrations as reported earlier [46].

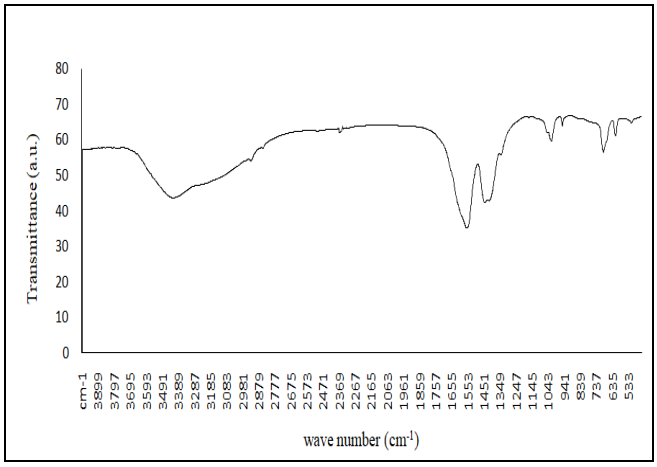


FIG. 4: FTIR VALUE OF PHYTO-SYNTHESIZED (*M. PANICULATA* LEAVES) ZnO NANOPARTICLES

The scanning electron micrograph (SEM) and XRD value calculated from Debye-Scherer formula ($D= 0.9\lambda/ \beta \cos\Theta$) found with particle sizes 33.82 nm to be good nano-biomaterial **Fig. 1** and **2** with their agglomeration of spherical crystal shapes **Fig. 2**.

The phyto-synthesized sample of ZnO NPs exhibits several absorption bands such as 1559, 1447, 1343, 1023, 954, 695, 617, 518 cm^{-1}. The synthesized nanoparticles having an absorbance peak value at 355 nm **Fig. 3**.

Anti-bacterial Bioassay of Phyto-synthesized ZnO NPs: The phyto-synthesized ZnO NPs were further tested against human pathogens with the concentration of 1000 ppm by using the agar well diffusion method. The degree of susceptibility was measured as given by Tomova *et al.* [47, 48].

The present result observed that *S. typhimurium* found more sensitive against phyto-synthesized ZnO NPs as compared to remaining test pathogens.

A similar result was earlier stated by Levin-Reisman *et al.* [49] The significance level was 95% ($p < 0.05$).

TABLE 1: ANTI-BACTERIAL BIOASSAY AND DEGREE OF SUSCEPTIBILITY TEST OF PHYTO-SYNTHESIZED ZnO NPs AGAINST TEST PATHOGENS

Pathogens	ZnO NPs Mean ± Error	Streptomycin Mean ± Error	Degree of susceptibility
E. coli	36.775 mm ± 1.27 mm	25.5 mm ± 0.57 mm	Sensitive
S. typhimurium	32.375 mm ± 1.96 mm	28.2 mm ± 0.63 mm	Sensitive
S. aureus	24.35 mm ± 0.43 mm	26.6 mm ± 0.42 mm	Sensitive

Note: Sensitive = ≥ 21 mm; Intermediate = 16-20 mm; Resistant = ≤ 15 mm [49].

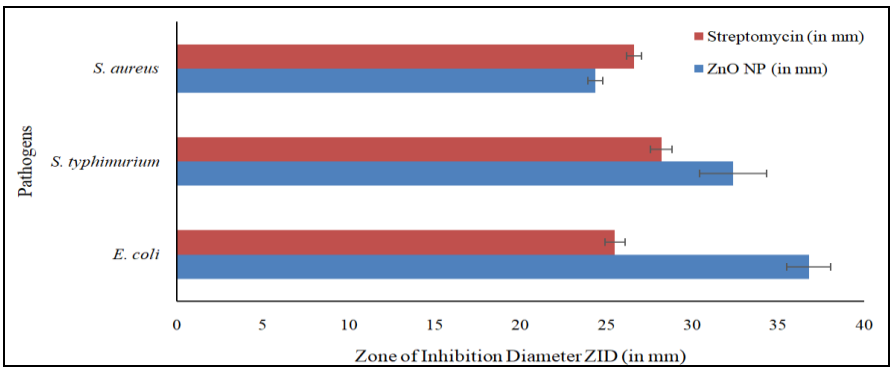
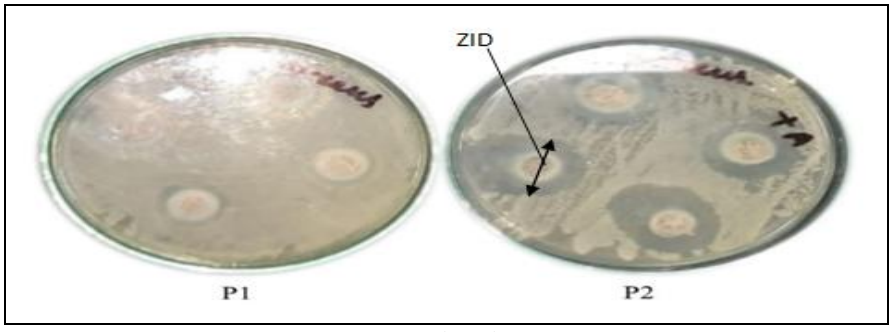


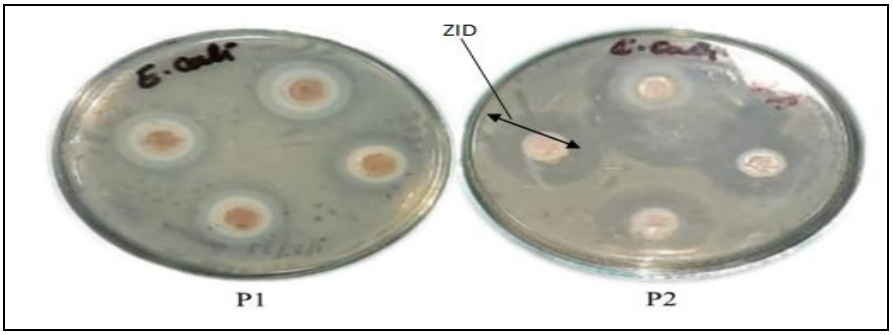
FIG. 5: ANTI-BACTERIAL BIOASSAY OF ZnO NPs AGAINST TEST PATHOGENS (VALUES IN MM)

The anti-bacterial bioassay revealed that the phyto-synthesized zinc oxide nanoparticles were showing potential anti-bacterial activity against test pathogens with their Zone of Inhibition Diameter values are 36.775 mm ± 1.27 mm for *E. coli*, 32.375 mm ± 1.96 mm for *S. typhimurium* and 24.35 mm ± 0.43 mm for *S. aureus* as compared to standard drug streptomycin (500 ppm) showed 25.5 mm ± 0.57 mm, 28.2 mm ± 0.63 mm and 26.6 mm ± 0.42 mm respectively. The anti-bacterial activity of phyto-synthesized ZnO NPs at concentration 1000 ppm was analyzed on the basis of Zone of Inhibition diameter **Fig. 5**, **6,** and **Table 1**. The present data of *E. coli* with a value of 36.775 mm and *S. aureus* with a value of 24.35 mm was found more significant as compared to an earlier study performed by Narayan *et al.* [50] with chemically synthesized ZnO NPs with value for *E. coli* 17 mm and *S. aureus* 21 mm respectively [50]. From the present investigation, the used concentration of ZnO NPs (1000 ppm) was found very low from the experiment performed by Nazoori and Kariminik [51] used the concentration of 2500 ppm for the chemically synthesized ZnO NPs.

A. ZONE OF INHIBITION DIAMETER (ZID) OF ZnO NPs AGAINST *S. TYPHIMURIUM*



B. ZONE OF INHIBITION DIAMETER (ZID) OF ZnO NPs AGAINST *S. AUREUS*



C. ZONE OF INHIBITION DIAMETER (ZID) OF ZnO NPs AGAINST *E. COLI*

FIG. 6 A, B AND C: ANTI-BACTERIAL BIOASSAY OF THE PHYTO-SYNTHESIZED ZnO NANOPARTICLE WITH CONCENTRATION OF 1000 PPM TESTED AGAINST ALL THE THREE TEST PATHOGENS

Farzana *et al.*, [52] performed their experiment using purchased biologically synthesized ZnO NPs and reported 13.2 mm inhibition diameter against *E. coli* at 1000 ppm. The present study found more potential from the study of Farzana *et al.* [52] at the same concentration (1000 ppm) with ZID value 36.775 mm against *E. coli* [52]. Thus, the present study found more and relevant from earlier studies. Many researchers reported biologically synthesized ZnO NPs are more effective as compared to chemically synthesized as well as these are bio-safe and non-hazardous [52]. The present study demonstrated that all three pathogens are highly sensitive to ZnO NPs as accordance to Reddy *et al.* [53] and Narayana *et al.* [50] ZnO NPs easily penetrate the cell wall of both types of tested bacteria (Gram-positive and gram-negative) and able to damage the cellular metabolism of tested pathogenic bacteria. This mechanism was confirmed experimentally by Padmavathy and Vijayaraghvan [54], Feng *et al.* [55] and Furno *et al.* [56].

CONCLUSION: The present study found eco-friendly and highly potential against tested pathogens by showing excellent bacteriostatic activity. Phyto-synthesized ZnO NPs may serve as safe nano-drug which may be used as therapeutic agents due to their extensive pharmaceutical properties. ZnO NPs may also be used as tooth filling agents in dentistry.

ACKNOWLEDGEMENT: The authors are thankful to Head, Department of Botany for providing lab facilities and the director of Nehru Gram Bharati deemed to be university Prayagraj; for providing assistance and support.

CONFLICTS OF INTEREST: The authors are having no conflicts of interest.

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How to cite this article:
Tiwari AK, Jha S, Agrawal R, Mishra SK, Pathak AK, Singh AK, Dikshit A and Awasthi RR: Antibacterial efficacy of phyto-synthesized zinc oxide nanoparticles using *Murraya paniculata* L. leaf extract. Int J Pharm Sci & Res 2021; 13(2): 864-71. doi: 10.13040/IJPSR.0975-8232.13(2).864-71.