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BIOSYNTHESIS OF ZnO NANOPARTICLES USING *OCIMUM BASILICUM* AND DETERMINATION OF ITS ANTIMICROBIAL ACTIVITY

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ABSTRACT

Plant mediated ZnO nanoparticles has now become prominent among other metal oxide nanoparticles as easy to synthesize, cheap and safe method with multiple applications. In present study 10% extract of *Ocimum basilicum* was used as reducing and capping agent for the synthesis of ZnO nanoparticles with 230 mL of 0.2 M Zinc Acetate dihydrate at room temperature (25°C). The synthesized ZnO nanoparticles were dried at 60°C, calcined at 100°C in oven and finally nanoparticles of weight 10.67g/100 mL of plant extract were weighed. Phytochemical analysis and FTIR characterization of plant extract and ZnO nanoparticles depicted strong presence of important bioactive components in plant extract while weak presence or absence was observed in ZnO nanoparticles supernatant layer. Nanoparticles IR-spectrum showed two prominent sharp peaks at 660.33 and 560.22 cm^{-1} of C- alkyl chloride and hexagonal ZnO that was totally absent in crude extract spectrum. Further XRD diffractogram illustrated highly crystalline face centered cubic ZnO nanoparticles structure having hexagonal wurtzite geometry with average size 30- 40 nm. Moreover, microbicidal activity of synthesized nanoparticles (100mg/mL) was measured against pathogenic strains and zones of inhibition against *Staphylococcus aureus*, *Escherichia coli* and *Aspergillus niger* were recorded as 1.05mm± 0.137, 36.15mm±0.304 and 24.10mm± 0.05, respectively. Minimum inhibitory concentration for *S. aureus* and *E. coli* was calculated same as 312.5µg±0.00 whereas for *Aspergillus niger* was 5000µg ±0.00. Thus a cheap and ecofriendly plant mediated complete method was developed that can be exploited on large scale synthesis to save the capita of Pakistan used to import antimicrobial drugs.

Key words: *Ocimum basilicum*; ZnO nanoparticles; antimicrobial activity; hexagonal wurtzite; zinc acetate dehydrate.

INTRODUCTION

Nanotechnology is emerging recently as new field of science that includes other basic disciplines such as biology, chemistry, physics and engineering work at nano scale level to create nano materials (Vaidyanathan *et al*. 2009). Whereas in nanobiotechnology, nano system is manufactured by utilizing biological materials and has enormous applications in life sciences. The nano system comprise of nano particles (NPs) with 1 to 100 nm size that increased its surface area for action and showed considerable benefits in agriculture, bio- engineering, electronics, automobiles pharmaceutics and cosmetics. These NPs showed exclusive optical, electrical, thermal, catalytic and antimicrobial properties (Parameswari, 2010). Various physical and chemical methods were used to manufacture metal nano particles. However, these methods have short comings; as they are more time consuming, use of toxic and non- biodegradable chemicals that are dangerous for biological systems. Therefore, plant mediated synthesis has got prominent position for NPs synthesis as it is inexpensive, nontoxic and eco- friendly alternative (Nadagouda and Varma, 2008).

Medicinal Plant extracts have bioactive constituents used in pharmaceutical formulations and are involved in reducing and capping of metal ions for the synthesis of NPs. Noble metals like Au and Ag NPs have been most extensively studied in past years with applications in biological system (Rajeshkumar *et al*. 2012; Sundrarajan *et al*. 2015). Little work is reported on the biosynthesis of some metals NPs like MgO, TiO_2, CuO, FeO_2, Al_2O_3 and ZnO (Moritz and Geszka-Moritz, 2013). From all these, ZnO NPs has got great attention in recent years with enormous applications as these are easy to synthesize, cheap and safe method (Pulit-prociak *et al*. 2016). Moreover, US FDA (United State Food and Drug Administration) recommended ZnO as (GRAS) which means generally recognized as safe (Sangani *et al*. 2015). It is widely used in biomedicine with enormous applications like drug delivery, anti- diabetic, anti- cancer and agricultural properties (Wodka *et al*. 2010). It has been widely used in cosmetic due to UV filtering

properties especially in Sunblock lotions (Malapermal *et al*. 2015).

During this study medicinally famous herbal plant; *Ocimum basilicum* commonly known as Niazbo was used to prepare ZnO NPs. The plant bioactive phytochemicals have various health benefits and having natural antioxidant properties accomplished of neutralizing free radicals and reducing the brutality of diabetic, micro and macro vascular complications (Tejaswi *et al*. 2013). The extract of this plant was prepared to synthesize ZnO NPs that were characterized by FTIR, XRD and screened for antimicrobial activity.

MATERIALS AND METHODS

The plant material was collected from the garden of Govt. Post graduate College Gulberg, Lahore, Pakistan, identified and authenticated by Dr. Zaheer Uddin Khan; distinguish Prof. of Botany Govt. College University, Lahore. Voucher #GC. Herb. Bot. 3409. The fresh plant was washed with distilled water several times to remove dirt particles, dried under shade for 6 days and grinded sample was stored in air tight jars for further investigations.

Synthesis of ZnO NPs: Plant extract (shoot, leaves and flower) was prepared by following Ahmed *et al*. (2016) with some modifications as; 10 gm of dried plant material in 100 mL deionized water was heated for 2 hours at 80^{o}C with continuous stirring and cooled at room temperature (25^{o}C). A clear extract was obtained after centrifugation at 4000 rpm for 10 minutes. Further ZnO NPs were synthesized by following methodology of Senthilkumar and Sivakumar (2014) with some modification. Zinc acetate dihydrate (0.2 M) was freshly prepared in deionized distilled water and 230 mL was added to 100 mL of plant extract at room temperature (25^{o}C). Brown ZnO NPs were instantly appeared that thickened within minutes and the reacted solution was dried at 60^{o}C for 24 hours. The dried crystals were further calcined at 100^{o}C for 1 hour, cooled, weighed and stored in brown bottles for future investigations. Freshly prepared 100 mL leaf extract was also dried at 60^{o}C for 24 hours for phytochemical characterization.

Characterization: Bioactive phytochemicals like alkaloids, tannins, phenols, flavonoids, carbohydrates, proteins, terpenes and saponins were qualitatively tested in plant extract and ZnO NPs supernatant layer to observe their strong/ weak presence or absence by using important reported tests (Tiwari *et al*. 2011). Identification chemical bonds of functional groups in dried plant extract and ZnO NPs by FTIR was performed at Department of applied Chemistry, PCSIR, Lahore, Pakistan (Moghaddam *et al*. 2017). Further XRD studies were taken in the Department of Chemistry, SBASSE, Lahore University of Management Sciences, Lahore, Pakistan (Santhoshkumar *et al*. 2017).

Antimicrobial studies: The synthesized ZnO NPs were evaluated for antimicrobial activity; well diffusion method (Hasan *et al*. 2009) was applied. For antibacterial assay, the refresh day old culture of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were uniformly spread over nutrient agar plates having wells of 4 mm. ZnO NPs (100mg/mL) normal solution was introduced in the wells under sterilized conditions and incubated at 37^{o}C for 24 hours. The zone of inhibition (mm) around well was measured; same procedure was applied for pathogenic fungus: *Aspergillus niger* (*A. niger*) during antifungal assay. All the bacterial and fungal strains used in this study were obtained from the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. Gentamycin (100mg/mL) was used as standard antibiotic against bacterial strains and zone of inhibition (mm) was measured.

Minimum Inhibitory Concentration: Minimum Inhibitory Concentration (MIC) was measured by following well diffusion method in replicates (N= 3) for each microbial strain. This standard method for antimicrobial assay (Wiegand *et al*. 2008) was employed in tube serial dilutions of ZnO NPs (100mg/mL) in bacterial and fungal growth media. The pathogenic microbes were incubated at 37^{o}C for 24 hours and lowest inhibitory concentration was scored. All the experiments were applied in replicates (N= 3) to apply descriptive analysis on SPSS statistics 17.0 to calculate Mean value with standard deviation (SD).

RESULTS AND DISCUSSION

The plant mediated method of ZnO NPs synthesis is a recent approach that is the elucidation of a cheap, eco-friendly and scale up synthetic method. Medically important plants have such phytochemicals which act to stabilize and reduce metal oxides for the synthesis of NPs with controlled shape and size (Agarwal *et al*. 2017). Further such famous phytochemicals are reported to involve inhibitory growth mechanism in pathogenic microbial strains (Zhang *et al*. 2008). Hence, such plant mediated NPs free from toxins can have vast scope in the field of biomedical science, food and cosmetics industries, consequently this study now become a foremost research area for researchers. Keeping this in mind a medically renowned plant *Ocimum basilicum* commonly known as “Niazbo” was selected and identified. Further 230 mL of 0.2 M solution of Zinc acetate dihydrate was poured in 100 mL of fresh plant extract at room temperature (25^{o}C) and brown ZnO nanoparticles were instantly appeared that grew larger within seconds and finally settled down leaving supernatant layer which was also taken for phytochemical

investigation. The visual diagram of all the procedure in the form of flow sheet was presented in fig. 1. Further the produced NPs were dried at 60°C in oven for 24 hours and dried brown ZnO NPs were obtained. Moreover, NPs were calcined at 100°C for 1 hour. Thus green synthesized ZnO NPs were weighed as 10.67g/100 mL of plant extract. This is the first-time reported concentration of *Ocimum basilicum* ZnO NPs with complete scheme of work.

Characterization: Well-known qualitative tests were performed to determine the presence or absence of important bioactive compounds like alkaloids, flavonoids, carbohydrates, proteins, terpenes, phenols and saponins; in the crude leaves extract and in supernatant layer after ZnO NPs settlement. There was strong presence of all above bioactive phytochemicals in leave extract whereas weak presence of most phytochemicals was noticed in supernatant layer. During screening proteins and phenols were absent in supernatant layer, although saponins were strongly present in both extracts. The weak presence or absence of these natural products was in agreement with reported literature of Malapermal *et al*. (2015) that bioactive constituents are involved in reduction and capping of metal oxides during NPs synthesis. Tejaswi *et al*. (2013) documented triterpenes, eugenol and flavonoids as important ingredients in plant extracts responsible for the formation of silver NPs. Absence of protein might be related with its association with ZnO NPs synthesis and stabilization (Nalvolthula *et al*. 2014).

FTIR analysis: Further FTIR of crude leaves extract and ZnO NPs also depicted the compatible results with other researchers' findings and different pattern of peaks were observed in both dried moieties as shown in fig. 2.

There is a broad stretch between 3000- 3500 cm^{-1} with absorption maxima at 3337.88 cm^{-1} that ascribed the stretching frequencies of amino and hydroxyl of amine, alcohols and phenols. A weak absorption peak at 2926.32 cm^{-1} represented the symmetric and asymmetric stretching of aliphatic functional group (CH_3 and CH_2). When these two peaks are compared with the IR-spectrum of ZnO NPs, these stretching became narrow with decrease of peak broadening in NPs spectrum and absorption maxima is shifted to 3160.45 cm^{-1}, might be associated that these functional groups are used to reduce Zn^{+2}. Further by comparing both spectrums, visible difference between absorption maxima and stretching frequencies were found. As ZnO NPs spectrum has two prominent sharp peaks at 660.33 and 560.22 cm^{-1} of C-alkyl chloride and hexagonal ZnO (Salem *et al*. 2016) that is totally absent in crude plant extract spectrum. Moreover, peak at 1584.32 cm^{-1} (carbonyl functional group in amide I and II) and two peaks at 1393.76 cm^{-1}; 1264.2 cm^{-1} (C-N stretching frequencies of amide I and -CH_2- scissoring vibrations of proteins) reducibly appeared with absorption maxima at 1570.25 and 1412.34 cm^{-1} in NPs spectrum. These results are also in agreement with reported findings that proteins stabilize the NPs and also a justification of protein absence during the phytochemical analysis in supernatant layer of ZnO NPs. A prominent peak at 1051.29 cm^{-1} and a weak peak at 865 cm^{-1} corresponded to C-O vibrational stretching frequencies of alcohol and amino acids (Moghaddam *et al*. 2017) and C-N stretch of amine respectively found in crude leave spectrum whereas two reduced weak peaks appeared at 1020.65 cm^{-1} and 958.43 cm^{-1} in ZnO NPs spectrum. The presence of some sharp and prominent peaks in crude extract spectrum and absence or weak presence in ZnO NPs spectrum suggested that those functional groups of active ingredients were performing the job of capping, dispersing and stabilizing agents for NPs. Wonsawat, 2014 found during research study that Basil leaves extract contained active reducing agents those reduce metal ions into metal NPs.

XRD studies: The diffractogram of plant mediate ZnO NPs given in fig. 3 had noticeable prominent peaks appeared between 2θ (degree) against intensity. The prominent indices plane of ZnO (hkl) calculated as (100), (002), (101), (102), 110), (103) and (112) corresponding to diffraction angles of 32.2°, 34.21°, 36.31°, 45.87°, 52.10°, 60.52° and 66.98° indexed the size of crystals. The average size of crystals was between 30- 40 nm estimated by Debye–Scherrer equation (Santhoshkumar *et al*. 2017). Moreover, the spectrum was in agreement with documented diffraction data card (JCPDS-36-1451) and also fairly comparable with reported literature of other researchers (Irshad *et* al. 2018). Further Bragg Equation (Senthilkumar and Sivakumar, 2014) was used to calculate crystal structure that was found highly crystalline face centered cubic having hexagonal wurtzite geometry. The similar results were also reported by Vennila *et al*. (2016).

Antimicrobial activity: The microbicidal activity of synthesized NPs (100mg/mL) was measured against pathogenic strains as shown in fig. 4 and found 31.05mm± 0.137 zone of inhibition against *S. aureus*, 36.15mm±0.304 for *E. coli* and 24.10mm± 0.05 for *A. niger* given in the table. These results documented better antibacterial activity of produced ZnO NPs than the standard antibiotic; Gentamycin (100mg/mL) that showed zone of inhibition 25mm against *S. aureus* and 26mm for *E. coli*. The biocidal action of ZnO NPs revealed their mechanism that involved the disruption of cell membrane with the action of Zn^{+2} on its surface that ultimately cause the death of microbes (Gunalan *et al*. 2012).

Further standard protocols were followed to measure MIC for the above mentioned strains and observed concentrations are given in the table for *S. aureus* and *E. coli* were same of 312.5µg±0.00 and *A.*

niger was 5000µg ±0.00. This minimum concentration of ZnO NPs required for antimicrobial activity as given in the table depicted the cost effectiveness of initially green synthesized ZnO NPs (10.67g/100 mL) and its application in antimicrobial activity. Some researchers also studied mode of inhibitory action of ZnO NPs for microbial growth, as Mishra and Sharma (2015) documented cell damage caused by these NPs with the presence of protein and nucleic acid of nutrient agar, Femi *et al*. (2011) demonstrated the surface binding of NPs with thiol group of glycoproteins one the cell wall of microbes and decreases the permeability with subsequently lyses of cell to inhibit cell growth (Hasan *et al*. 2009). Gunalan *et al*. (2012) also explained the damage of cell membrane with leakage of protein, minerals and genetic material by the interaction of ZnO NPs with microbial strains.

Rout *et al*. (2012) measured zone of inhibition (mm) of *Ocimum sanctum* silver NPs against *E. coli*, *S. aureus* and *A. niger* as; 14±0.33, 19±1.30 and 12.24±0.03 respectively. Sivaranjani and Meenakshisundaram, (2013) synthesized silver NPs using *Ocimum basilicum* leaves extract and estimated 11 mm zone of inhibition against *Pseudomonas aereginose* however no zone was observed for *E. coli* and *Bacillus subtilis*. Singhal *et al*. (2011) calculated MIC of silver NPs 0.314 and 1.25 mg/mL for *E. coli* and *S. aureus* respectively. During this present study *Ocimum basilicum* ZnO NPs depicted better antimicrobial activity than other researchers' findings and with low MIC.

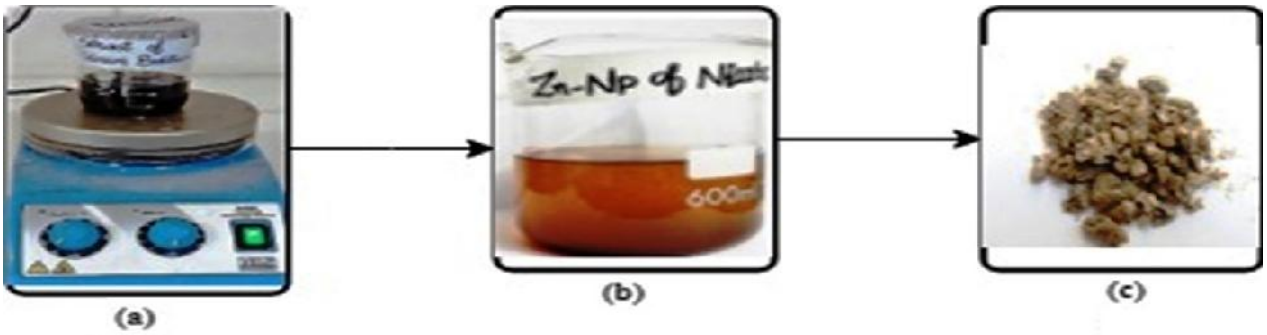


Figure 1. Visual diagram of Ocimum basilicum ZnO NPs biosynthesis; (a) plant extract preparation (b) synthesis of ZnO NPs (c) dried and calcined ZnO NPs.

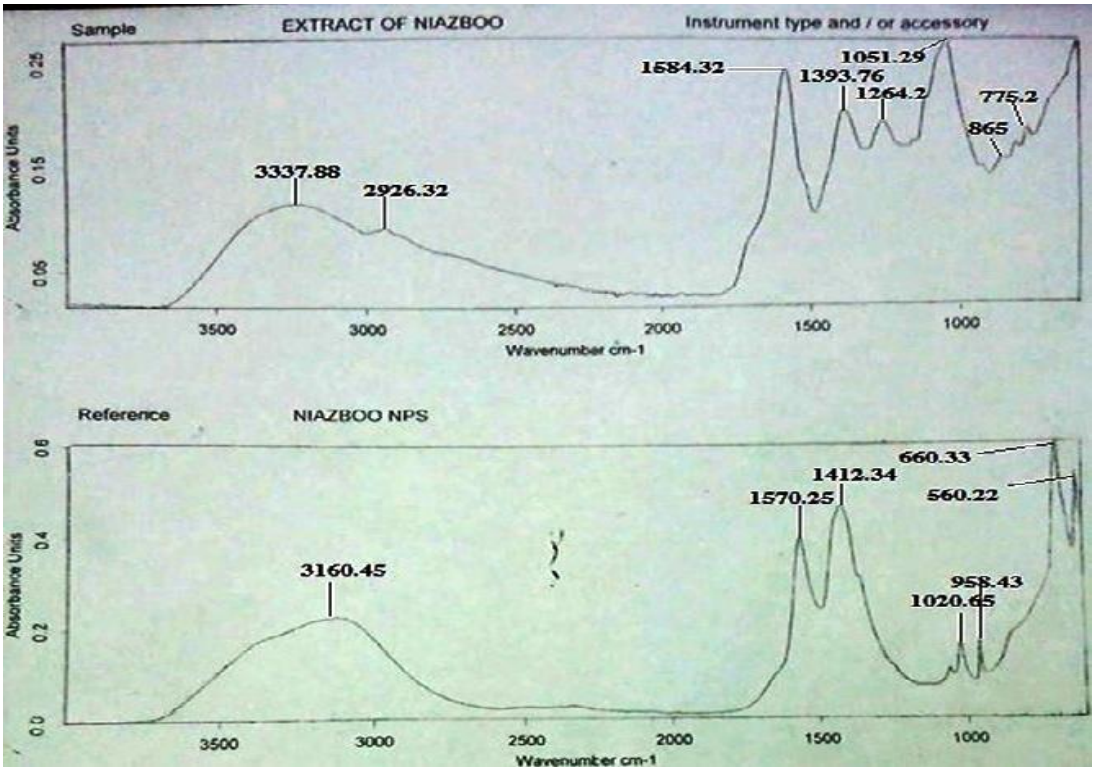


Figure 2. FTIR of crude dried leaves extract and its ZnO NPs representing characteristic functional groups peaks.

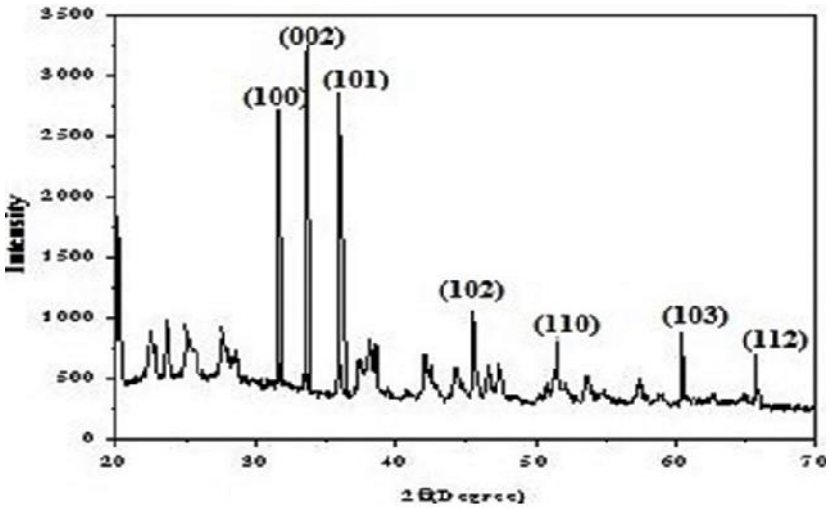
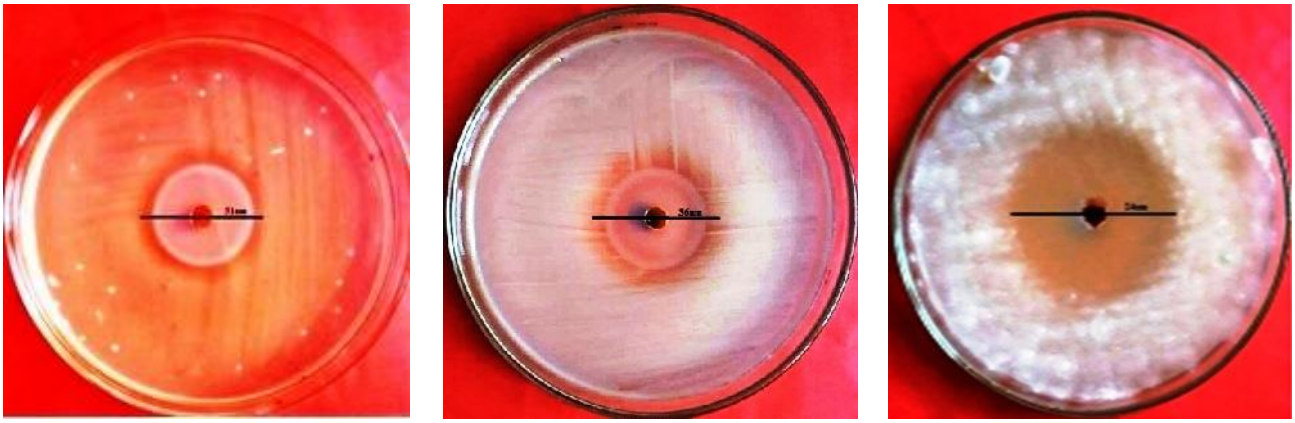


Figure 3. XRD diffractogram of Ocimum basilicum ZnO NPs.



A **B** **C**

Figure 4. Zone of inhibition measured for antimicrobial activity of *Ocimum basilicum* ZnO NPs evaluated on (a) *S. aureus* (b) *E. coli* (c) *A. niger*

Table. Determination of zone of inhibition measured by Well Diffusion Method and MIC of *Ocimum basilicum* ZnO NPs against pathogenic microbial strains

Microbial strain	Aq. Extract of *Ocimum basilicum* leaves (100 mg/mL)	ZnO NPs of *Ocimum basilicum* leave (100 mg/mL) ±SD	Gentamycin (Std. Antibiotic) (100 mg/mL)	MIC± SD
S. aureus	No zone	31.05mm± 0.137	25 mm	312.5µg±0.00
E. coli	No zone	36.15mm± 0.304	26 mm	312.5µg±0.00
A. niger	No zone	24.10mm± 0.05	-------	5000µg ±0.00

Where no. of treatments (N) =3, Standard deviation= SD

Conclusion: ZnO nano particles were synthesized by using *Ocimum basilicum* plant extract that effectively inhibit pathogenic microbial strains of clinical source. Moreover, during characterization, FTIR of ZnO NPs documented clearly the capping, reducing and stabilizing phytochemicals found in crude extract of plant. XRD diffractogram revealed characteristic peak of ZnO NPs with size range 30- 40nm.

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