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Green tea leaves mediated ZnO nanoparticles and its antimicrobial activity

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Abstract

Plant mediated synthesis of ZnO Nanoparticles (NPs) have multiple advantages over conventional synthetic methods like easy, inexpensive, eco-friendly, nontoxic by-products and no critical conditions of temperature and pressure required. In present study, 9.1gm ZnO NPs were synthesized from 230 mL of 0.2 M Zinc acetate dihydrate and 100 mL of green tea leaves extract at room temperature (25^{o}C). The leaves extract was prepared by heating 10gm dried leaves in 100 mL of deionized distilled water at 80^{o}C for 2 hours. The synthesized ZnO NPs were dried at 40^{o}C for 24 hours and calcined at 100^{o}C for 1 hour. The agar well diffusion method was used to evaluate ZnO NPs for antimicrobial activity of selected pathogenic strains. A clear zone of inhibition was measured; 40.050mm ± 0.137 for *Staphylococcus aureus*, 36.150mm ± 0.304 for *Escherichia. coli* and 40.100mm ± 0.050 for *Aspergillus niger* that were comparably better results than standard antibiotic Gentamycin that showed 25mm and 26mm zone of inhibition for *Staphylococcus aureus* and *Escherichia coli* respectively. The minimum inhibitory concentration scored as 9.765µg ± 0.00, 9.531µg ± 0.00 and 5000µg ± 0.00 for *Staphylococcus aureus*, *Escherichia. Coli* and *Aspergillus niger* respectively, was documented low concentration than reported so far concentrations of green tea ZnO NPs.

Keywords: Green tea; Leaves; mediated; Nanoparticles, antimicrobial

Introduction

Nanotechnology is emerging as multidisciplinary field of science in which a wide range of metal nano nanoparticles (NPs) have been synthesized. The produced NPs have unique size with more surface area to volume ratio that promoted their reactivity with the surrounding molecules (Gunalana et al., 2012). Therefore conventionally both physical and chemical methods are used to synthesize them, however these methods have various demerits including expensive, toxic by-products, critical conditions of temperature and pressure and long-time of reactions etc (Geraldes et al., 2016). Whereas green synthesis of NPs; involve nontoxic, cheap and broadly available plant sources that are environment friendly (Salem et al., 2016). Those medicinal plants were preferably used in the synthesis of NPs that already documented for biomedical properties and having immense range of natural products (Agarwal et al., 2017). These bioactive phytochemicals are reacted to reduce metals into metal oxide and showing good stability in the formation of NPs (Mishra & Sharma, 2015). Noble metals like gold (Au) and silver (Ag) have been extensively used in biosynthesis NPs and medically evaluated. However very few reported work was available on inorganic metals such as Ti, Mg, Fe, Zn, S and Al. Among these metals; ZnO has got excellent position due its wide applications in various fields of science (Dhanemozhi et al., 2017).

ZnO NPs were reported to have better UV protection and enhanced opaqueness than TiO_2 NPs that was previously used for UV protections (Sundrarajan et al., 2015). These NPs showed elevated catalytic, photochemical and antimicrobial properties (Awwad et al., 2014). These NPs were documented to rupture the lipid bilayers of bacterium and fungal cell wall and revealed significant antibacterial and antifungal activity (Senthilkumar & Sivakumar, 2014). Beside this; ZnO NPs were also evaluated to have good antioxidant, anti- diabetic and anticancer property (Pattanayak et al., 2013). Thus these NPs depicted tremendous applications in biomedicines and microelectronics (Hasan et al., 2009). Production of ZnO

NPs is still infancy stage; therefore there is a gigantic scope of this work to synthesize and evaluate its antimicrobial activity.

In this present study, dried Green tea leaves were used to synthesize ZnO NPs. It is scientifically known as "*Camellia Sinensis*" famous for its Phenolic contents and high antioxidant activity (Saravanakkumar et al., 2016). This plant belongs to family Theaceae and is rich in bioactive phytochemicals that refers it as anti-septic, anticancer and antimicrobial agent for valuable medical drugs (Rani et al., 2014). Consequently, these properties are enhanced in the form of ZnO NPs; where these bioactive components are locked while reducing and capping of NPs (Malapermal et al., 2015). During the present work ZnO NPs were synthesized by using leave extract of *Camellia sinensis* (*C. sinensis*) and its antibacterial and antifungal activity was evaluated. Further produced ZnO NPs were also characterized by UV- visible, FTIR, XRD & SEM.

Materials and Methods

The plant material was procured from the local nursery near Gulberg and identified by Dr. Zaheer Uddin Khan, Distinguished Professor, Botany department, Govt. College University, Lahore, Pakistan. The fresh leaves were separated and dried under shade for 5 days. The dried leaves were grinded into power and stored in air tight jar for further work.

Synthesis of ZnO NPs

Leave extract was prepared by following Senthilkumar & Sivakumar, (2014) with some modifications as; 10 gm of dried leaves were heated in 100 mL deionized water at 80^{o}C with continuous stirring for 2 hours. It was cooled at room temperature (25^{o}C) and filtered by using whatman filter paper No. 40. Then clear extract was obtained by centrifugation at 4000 rpm for 10 minutes. Zinc acetate dihydrate solution (0.2 M) was freshly prepared and 230 mL was added to 100 mL of leave extract. With the instant formation of pale yellow ZnO NPs,

the reacted solution was dried at 40^{o}C for 24 hours and brown dried crystals were attained. These crystals were further calcined for 1 hour at 100^{o}C, cooled, weighed and stored in brown bottles for future investigations. Freshly prepared 100 mL leave extract was also dried at 40^{o}C for 24 hours for further analysis.

Antimicrobial studies

The synthesized ZnO NPs were evaluated for antimicrobial activity; well diffusion method (Hasan et al., 2009) was applied to screen famous pathogenic microbes. For antibacterial assay, gram positive bacteria *Staphylococcus aureus* (*S. aureus*) and gram negative bacteria *Escherichia. coli* (*E. coli*) were spread uniformly on 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 following well diffusion method in replicates (N= 3) for each microbial strain. This standard method for antimicrobial assay (Mishra et al., 2012) 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) and Mean value with standard deviation (SD) was calculated by descriptive analysis on SPSS statistics 17.0.

Characterization

The presence or absence of important bioactive molecules like alkaloids, flavonoids, phenols, carbohydrates, protein, terpenes and saponins were investigated by performing reported tests (Tiwari et al., 2011). The green tea mediated ZnO NPs were scanned for UV- visible spectroscopy between 200 – 500 nm to determine absorption maxima. The different functional groups in crude dried leave extract and ZnO nanoparticles were identified by FTIR (Agarwal et al., 2017) performed at Centre of Applied Chemistry, PCSIR Laboratories complex, Lahore, Pakistan. XRD and SEM analysis was achieved at Department of Chemistry, SBASSE Lahore University of Management Sciences, Lahore, Pakistan.

Results and Discussion

The green synthetic method of ZnO NPs 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 (Rani et al., 2014). Further such famous phytochemicals are involved in the inhibition mechanism of microbial pathogenic growth (Zhang et al., 2008). Therefore 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 area of research. In this present study, for the green synthesis of ZnO NPs; dried grinded leaves of *C. sinensis* were used to prepare extract and 230 mL of 0.2 M solution of Zinc acetate dihydrate was poured in 100 mL of fresh leave extract. Pale yellow 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 40^{o}C in oven for 24 hours and dried brown NPs were obtained by calcined at 100^{o}C for 1 hour. Thus green synthesized ZnO NPs were weighed as

9.1g/100 mL of leave extract. This is the first time reported concentration of green tea ZnO NPs with complete scheme of work.

Antimicrobial activity

The microbicidal activity of synthesized NPs (100mg/mL) was measured against pathogenic strains and found 40.050mm ± 0.137 zone of inhibition against *S. aureus*, 36.150mm ± 0.304 for *E. coli* and 40.100mm ± 0.050 for *A. niger* shown in fig. 2. 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 involve the disruption of cell membrane with the action of Zn^{+2} on its surface that ultimately cause the death of microbes (Gunalana et al., 2012). Further standard protocols were followed to measure MIC for the above mentioned strains and observed concentrations for *S. aureus* was 9.765µg ± 0.00, *E. coli* was 19.531µg ± 0.00 and *A. niger* was 5000µg ± 0.00. This minimum concentration of ZnO NPs required for antimicrobial activity as given in table 1 depicted the cost effectiveness of initially green synthesized ZnO NPs (91g/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, (2012) 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. 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.

Senthilkumar & Sivakumar, (2014) reported no zone of inhibition for *E. coli*, 5.300 ± 0.570 for *S. aureus* and 3 ± 1.00 for *A. niger* against C. *sinensis* ZnO NPs. Antimicrobial activity of black tea (*C. sinensis*) extract was performed by Vasudeo and Sonika, (2009) for different

pathogen bacterial strains and found zone of inhibition 14 ± 2 for both *E. coli* and *S. auerus*. The MIC calculated for chloroform tea extract was 25µg/mL. Boran et al. (2015) studied the antibacterial activity of Tea seeds against some famous pathogenic strains and reported the significant biocidal property of seeds. During this present study *C. sinensis* NPs depicted better antimicrobial activity than other researchers' findings and with low MIC. The high zone of inhibition with MIC was in agreement with documented literatures that ZnO NPs rupture lipid bilayer of bacterial and fungal cell wall with the ultimate death of microbes (Saravanakkumar et al., 2016).

Characterization

Some qualitative tests were performed to determine the presence or absence of important bioactive compounds in the crude leaves extract and in supernatant layer after ZnO NPs settlement; like alkaloids, flavonoids, carbohydrates, proteins, terpenes, phenols and saponins. 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 that bioactive constituents are involved in reduction and capping of metal oxides during NPs synthesis (Malapermal et al., 2015). Absence of protein might be related with its association with ZnO NPs synthesis and stabilization (Moghaddam et al., 2017).

UV- visible and FTIR analysis

The spectrum obtained by UV- visible spectroscopy as shown in fig. 3 represented characteristic peak of pure ZnO NPs with absorption maxima of 350 nm. The peak broadening was between 320- 380 nm which was in good agreement with the reported

literature (Awwad et al., 2014; Saravanakkumar et al., 2016; Datta et al., 2017). No other peak observed in spectrum indicating high purity and crystallinity of ZnO NPs (Santhoshkumar et al., 2017).

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. 4.

There is a broad stretch between 3000- 3600 cm^{-1} with absorption maxima at 3320 cm^{-1} that ascribed the stretching frequencies of amino and hydroxyl of alcohols and phenols. An absorption peak at 2910 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 might be associated that these functional groups are used to reduce Zn^{+2} into its oxide. Further when both spectrums compared it was revealed to have visible difference between absorption maxima and stretching frequencies. As ZnO NPs spectrum have two prominent sharp peaks at 610 and 520 cm^{-1} of C- alkyl chloride and hexagonal ZnO (Nalvolthula et l., 2014) that is totally absent in crude leaves extract spectrum. Moreover two weak peaks at 1650 cm^{-1} (carbonyl functional group in amide I and II) and 1430 cm^{-1} (C-N stretching frequencies of amide I and -CH_2- scissoring vibrations of proteins) appeared prominent and sharp in NPs spectrum. These results are in agreement with reported findings that proteins stabilize the NPs and also justified the absence of protein in supernatant layer of ZnO NPs during phytochemical studies. Two prominent peaks at 1017 cm^{-1} and 990 cm^{-1} corresponded to C-O vibrational stretching frequencies of alcohol and amino acids (Salem et al., 2016) and C-N stretch of amine respectively found in crude leave spectrum whereas two weak peaks appeared at 1050 cm^{-1} and 960 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 performing the job of capping, dispersing and stabilizing agents for NPs.

XRD studies

The spectrum of green synthesized ZnO NPs is given in fig. 5(b) and observed prominent peaks that were in fair agreement with reported literature of international Centre of Diffraction Data card (JCPDS- 36- 1451). Whereas diffractogram of green tea leaves extract (fig 5a) depicted no such peak pattern was found in ZnO NPs, moreover no characteristic peaks of ZnO was noticed. Both spectrums were quite different and revealed presence of ZnO NPs formation in fig. 5(b) after reacting extract with Zinc acetate. The peaks in ZnO NPs spectrum were appeared with 2θ ranging from 20- 70 with noticeable diffraction angles of 23.2°, 24.1°, 26.2°, 28.1°, 32.2°, 34.45°, 36.29°, 46.22°, 55.19° and 67.15° that indexed size of crystals with prominent indices plane of ZnO (hkl) as (100), (002), (101), (102), (110) and (112). Thus average size of NPs was ranged between 30– 40 nm, calculated by Debye–Scherrer equation (Saravanakkumar et al., 2016). These results of XRD were compatible with reported researchers' work that confirmed the prepared ZnO sample is highly crystalline, having hexagonal wurtzite crystalline structure calculated by Bragg Equation, $\lambda=2d\sin\theta$ (Agarwal et al., 2017). The typical XRD pattern revealed that the sample contains Zinc Oxide nanoparticles. XRD pattern of synthesized metal oxide nanoparticles showed a high crystallinity of sample level with diffraction angles which correspond to the characteristic face centred cubic. XRD patterns were analysed to determine peak intensity, position and width ((Moghaddam et al., 2017). Vennila et al. (2016) also reported similar results of XRD Diffractogram with diffractions angles at 23.4°, 27.9°, 35.3° and 44.3°; indexed (111), (220), (101) and (311) plane that corresponded to face centred cubic ZnO plane. Moreover many researchers found XRD diffractogram in good agreement with data cards JCPDS- 36- 1451 (Awwad et al., 2014; Saravanakkumar et al., 2016), JCPDS- 5- 0566 (Vidya et al., 2013),

JCPDS- 89-1397 (Joel & Badhusha, 2016) and JCPDS- 01- 079- 0207 ((Nalvolthula et al., 2014).

SEM analysis

The images gained by SEM analysis represented the morphologies of green tea mediated resultant ZnO nanosheets; as fig. 6 (a) showed the surface image of primary particles merged together to yield bigger sized secondary particles. Although larger quantities of phytochemicals in leaves extract synthesized nanosheets with quite surface; fig. 6 (b- d) demonstrated pretty dense morphology of nanoflowers randomly oriented overlapping. Several thinner sheets aggregated to form nanosheet networks, where individual sheets seem to have lateral dimension of less than 1μm. The present results of SEM are in good agreement with Awwad et al. (2014) documented green synthesized ZnO nanosheets and nanoflowers with average size 500 nm and average thickness of 8 nm. Whereas Saravanakkumar et al. (2016) found the images of nanostructures that are highly aggregated, irregular as well as uniform hexagonal plates. Datta et al. (2017) revealed biosynthesized NPs' SEM results as irregular structure of radical, cylindrical and spherical particles aggregated in small cluster.

Conclusion

Thus ZnO nano particles were synthesized by using green tea leaves extract that effectively inhibit microbial growth. Moreover during characterization UV visible spectral peak at 350 nm confirmed the purity of ZnO NPs and FTIR results documented clearly the capping, reducing and stabilizing phytochemicals found in green tea. XRD diffractogram revealed characteristic peak of ZnO NPs with size range 30- 40nm those coalesced to organize in nanosheets as depicted in SEM images.

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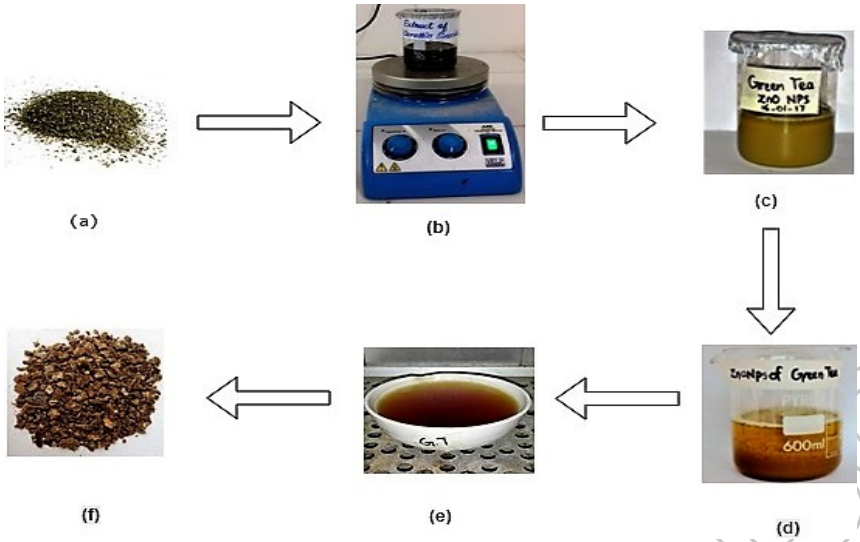
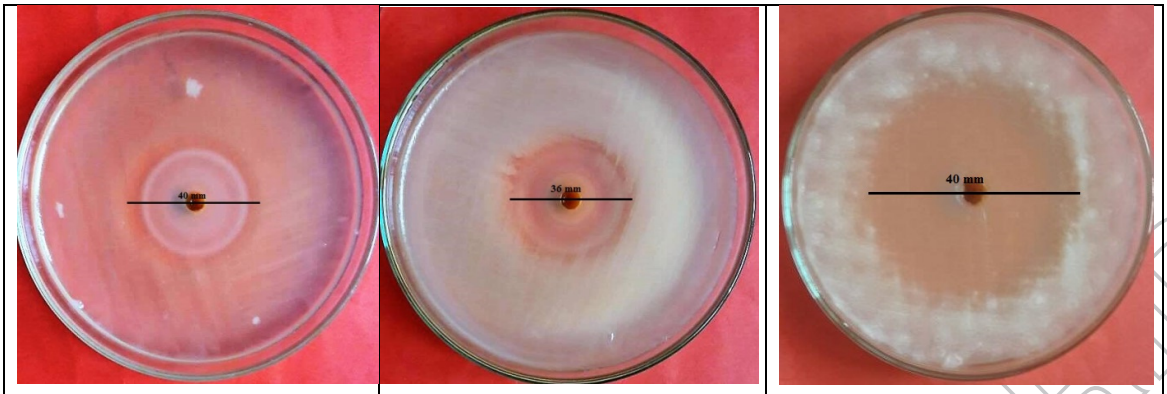


Figure 1: Flow sheet diagram representing green synthesis of ZnO NPs (a) dried grind leave of *C. sinensis* (b) Leave extract (c) synthesis of ZnO NPs (d) settled ZnO NPs having supernatant layer (e) drying of ZnO NPs at 40^{o}C (f) dried and calcined at 100^{o}C ZnO NPs



(a) (b) (c)

Figure 2: Zone of inhibition (mm) measured for antimicrobial activity of Green tea ZnO NPs evaluated on (a) *Staphylococcus aureus* (b) *Escherichia. coli* (c) *Aspergillus niger*

Table 1: Zone of inhibition measured by Well Diffusion Method and MIC for Antimicrobial Activity of green synthesized ZnO Nps against pathogenic microbial strains of clinical sources.

Microbial strain	**Aq. Extract of *Camellia sinensis* leaves (100 mg/mL)**	**ZnO NPs of *Camellia sinensis* leave (100 mg/mL)** ± SD	**Gentamycin (Std. Antibiotic) (100 mg/mL)**	**MIC** ± SD
Staphylococcus aureus	No zone	40.05mm ± 0.137	25 mm	9.765µg ± 0.00
Escherichia coli	No zone	36.15mm ± 0.304	26 mm	19.531µg ± 0.00
Aspergillus niger	No zone	40.10mm ± 0.05	-------	5000µg ± 0.00

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

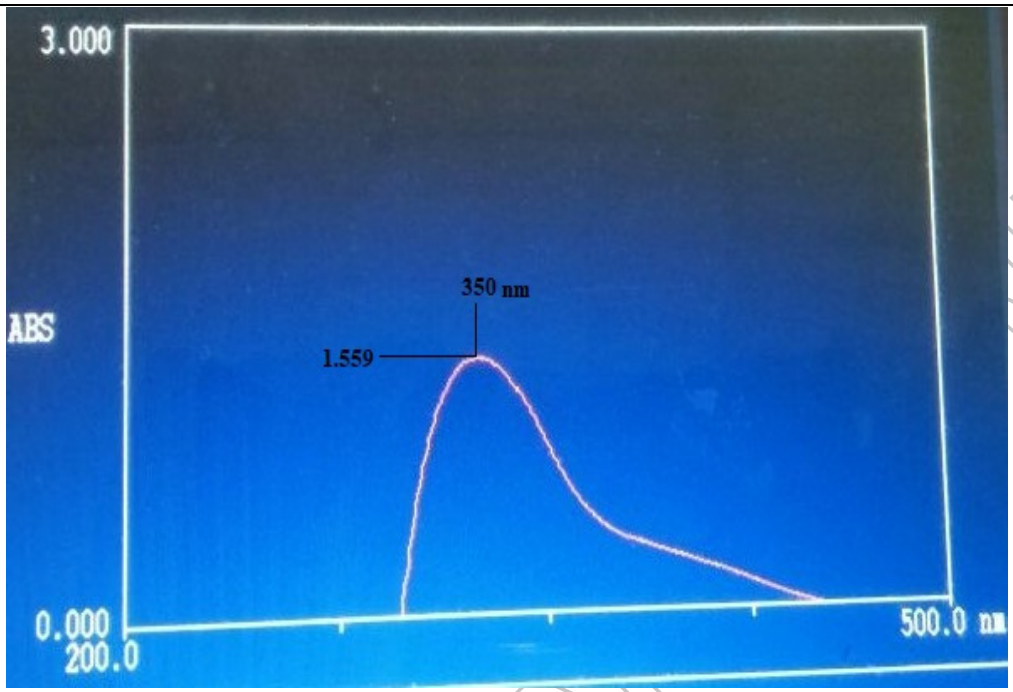


Figure 2: UV visible spectrum of green tea ZnO NPs showing characteristic absorption between 320- 380 nm and λmax at 350nm.

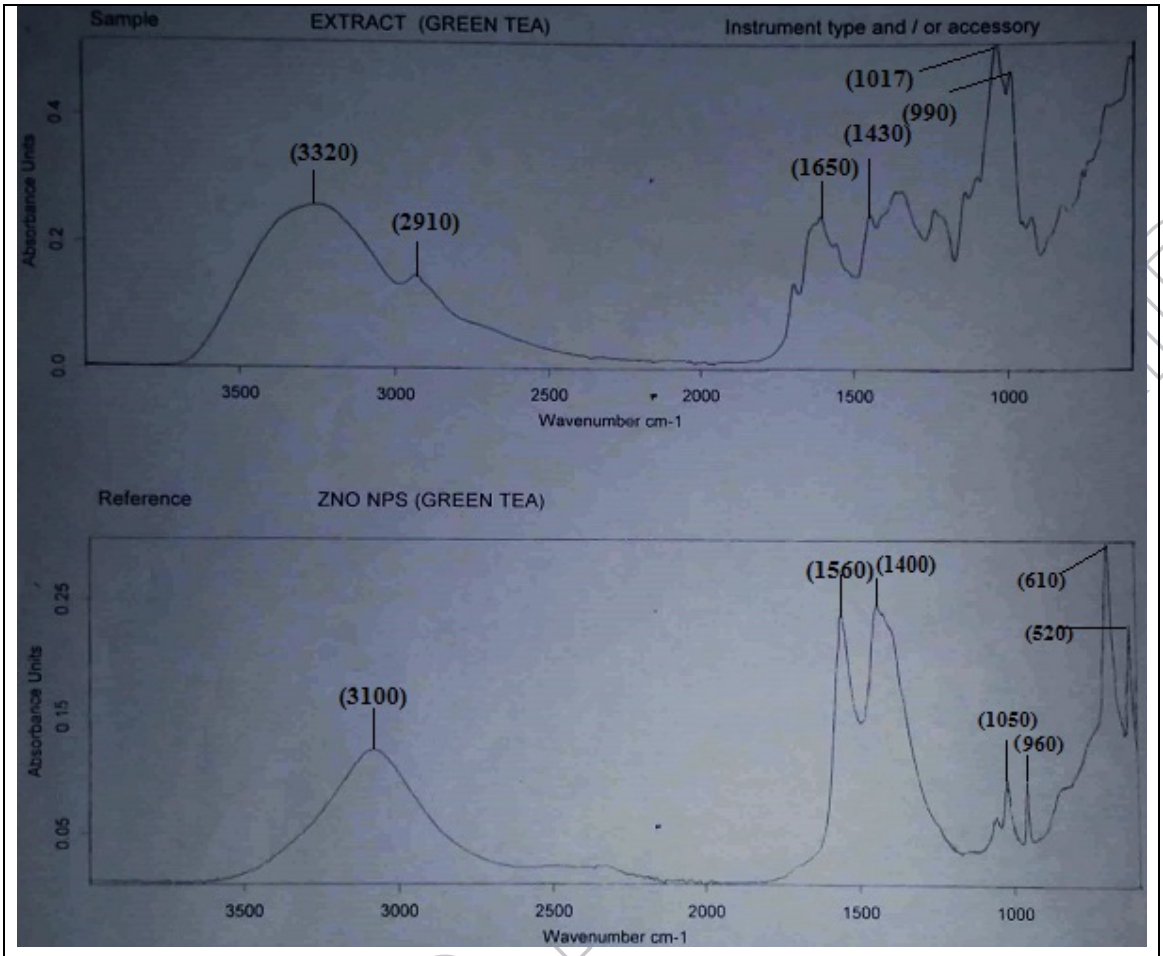


Figure 4: FTIR of crude dried extract of *Camellia sinensis* and its ZnO NPs representing characteristic functional groups peaks

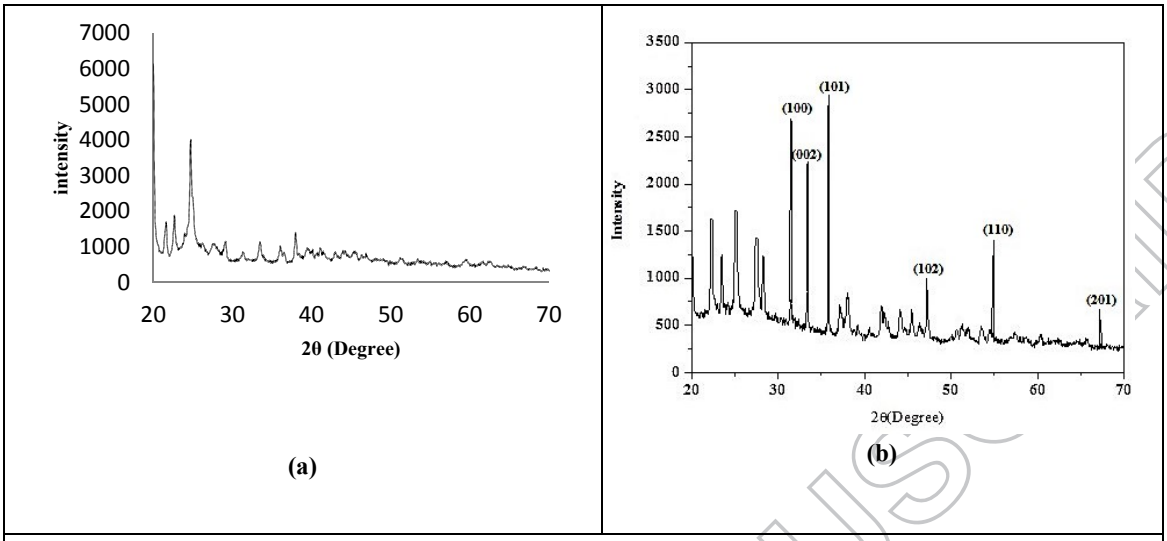


Figure 5: XRD diffractograms; (a) green tea leaves extract (b) green tea ZnO NPs

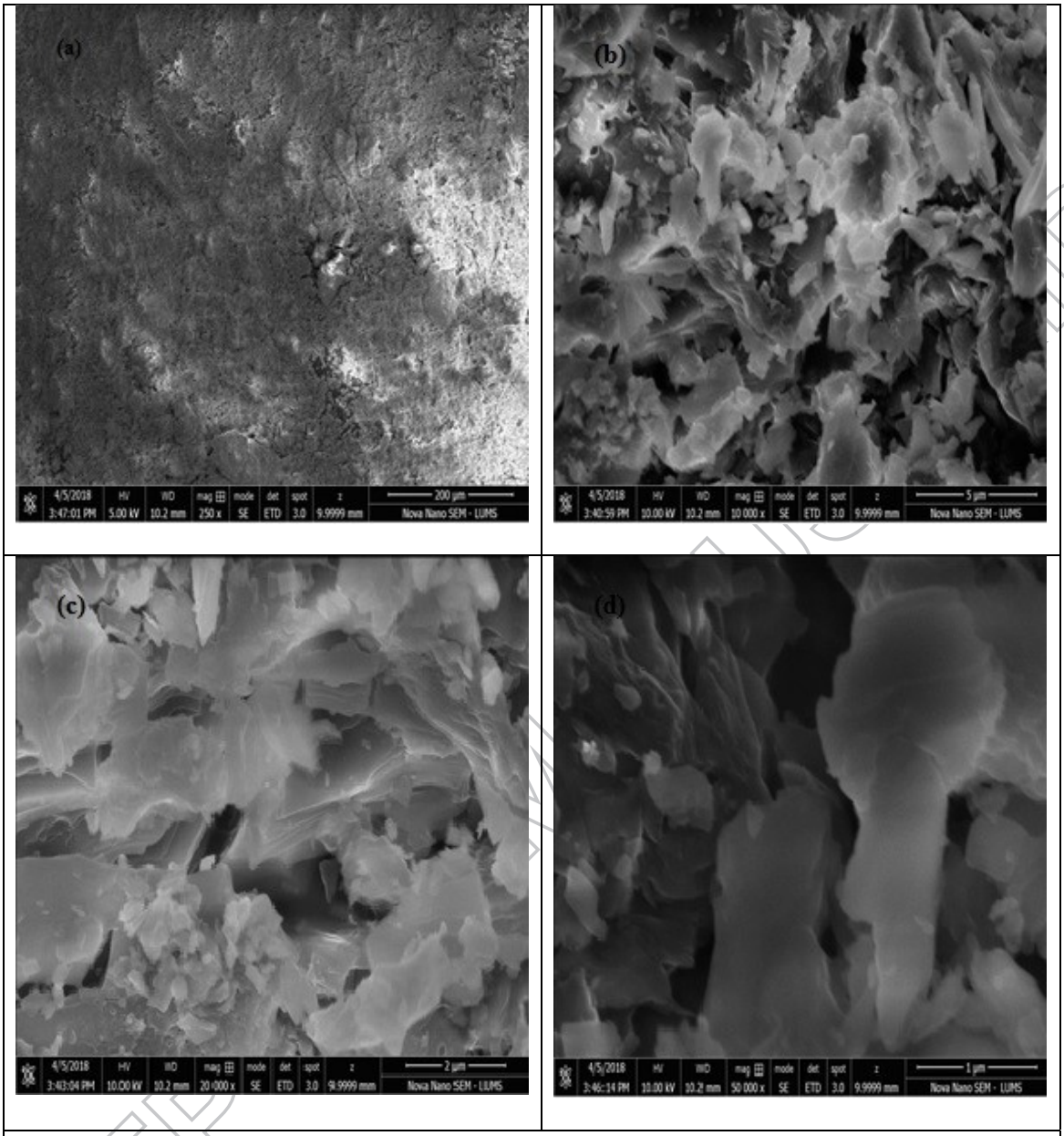


Figure 6: SEM images of Green Tea mediated ZnO nanosheets; (a) surface image of nanosheets (b- d) numerous thinner sheets accumulated to form a nanosheet networks.

Public Interest Statement

Since plant extracts having bioactive constituents are commonly used in pharmaceutical formulations due to their medicinal applications. These bioactive constituents can be involved in reducing and capping of metal ions for the synthesis of nanoparticles (NPs). A few reported works is available on the biosynthesis of some metals NPs like MgO, TiO_2, CuO, FeO_2, Al_2O_3 and ZnO. 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. Plant mediated NPs free from toxins can have vast scope in the field of biomedical science, food and cosmetics industries. These NPs are most promising as they show good antibacterial and antifungal properties due to their large surface area to volume ratio, became a current interest in the researches due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains.

Biography

Shagufta Irshad is associate professor at Department of chemistry, Govt. Postgraduate College for Women, Gulberg Lahore, Pakistan. She is PhD in Biochemistry from Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan. She received her MSc and MPhil from Department of Chemistry, Govt. College University, Lahore, Pakistan. Her research interests are: essential amino acids production by Fermentation, phytochemical studies, green synthesis of nanoparticles, characterization, antioxidant, antimicrobial and toxicity evaluation.

Cover page image: