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RESEARCH ARTICLE

ONE POT GREEN SYNTHESIS OF ZNO NANOPARTICLES USING AZOLLA EXTRACT AND ASSESSING ITS BIOLOGICAL ACTIVITIES

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ABSTRACT

Plant extract mediated bio reduction method can be used as an alternative to traditional chemical methods for the production of metal oxide nanomaterials. The present study, aimed to develop a rapid, eco-friendly method for the synthesis of ZnO NPs using Azolla leaf extract by microwave irradiation. The formation of nanocrystals was confirmed by employing standard characterisation studies such as UV-Vis spectroscopy, Scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction method (XRD). The antioxidant and antimicrobial activity of bio synthesized nanoparticles were evaluated & compared with chemically synthesised ZnO NPs for bio medical applications. Green synthesised ZnO NPs showed better antibacterial & antioxidant activity than chemically synthesised ZnO NPs.

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INTRODUCTION

Synthesis of nanoparticles using plant extracts is quite novel and leads to true green chemistry at a very affordable cost. It reduces the use of hazardous substances to human health and the environment. The low cost of cultivation, short production time & safety make plants an attractive platform for nanoparticle synthesis. Bio-molecules present in plant extracts can be used to reduce metals to nanoparticles (NPs) in a single-step green synthesis process. These bio-molecules not only acts as reducing agents for metal ion reduction but also as capping agents which helps to protect/ stabilize the NPs, thus improving their biological potential (Isaac, 2013; Yasin, 2013; Kumar *et al*., 2014; Yuvakkumar, 2014). Various plants differ in the concentration and composition of these biologically active components. The diversity in the morphology and size of nanoparticles synthesized from a variety of metal ions, using extracts of various plants make this bio route potentially attractive (Saxena, 2010; Khandelwal *et al*., 2010; Jain *et al*., 2009; Banerjee *et al*., 2014 and Veerasamy, 2010). Azolla Microphylla is a free floating aquatic fern found worldwide in wet lands traditionally used as a green manure. The phytochemical investigations show that Azolla is rich in phenols, tannins, sugars, flavanoids, steroids, proteins etc (Abraham and Vidhu Aeri, 2012; Mithraja, 2011). Phenolic compounds exhibit higher antioxidant potential and are good reducers of metal ions, thus favouring the green synthesis of nanoparticles. Further the higher content of proteins may help to stabilize the growth of nanoparticles . Flavanoids are of great importance for the bioactivities related to their antioxidant & anti inflammatory activities (Selvaraj, 2013). The potential use of transition metal oxide nanoparticles in catalytic, biomedical and waste water treatment applications is gaining interest in the research communities, largely due to their physical and chemical properties (Balamurughan *et al*., 2014and Jayalakshmi, 2014).

Among the transition metal oxide nano structures, ZnO nanoparticles have been of considerable interest because of the wide range of applications in various fields such as catalysts, antibacterial materials, luminescent materials, and photo catalyst (Divya, 2013; Shailaja, 2013; Sangeetha, 2011). One main shortcoming of conventional biological green methods is the longer reaction time. Microwave assisted synthesis is considered to be a valuable alternative in this regard. It is less time consuming, product obtained is of high quality, and there is no wastage of solvent because very less solvent are used for the synthesis (Barreto, 2013; Joseph *et al*., 2014 and Makhluf, 2005). In the present study, a low cost, single step microwave assisted green synthesis of ZnO nanoparticles using the natural

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plant Azolla was investigated. Synthesised nanoparticles were characterised by UV – vis spectroscopy, XRD, SEM & FTIR techniques. The biological activity of the green synthesised ZnO NPs were analysed & compared with that of conventional chemically synthesised ones.

MATERIALS AND METHODS

Methods of synthesis

Preparation of leaf extract

Fresh leaves of Azolla were collected from botanical garden and washed thoroughly with double distilled water and dried in shadow for 7 days and grind to fine powder. From this, 5g of leaf powder was taken & mixed with 50ml double distilled water and stirred well and reflux for 30 minutes in a 250ml RB flask. After refluxing the reaction mixture was cooled at room temperature and extract was filtered through Whatmann filter paper no.1 and kept in a refrigerator for synthesis.

Preparation of ZnO nanoparticle

Chemical method

0.1M $Zn(NO_3)_2.6H_2O$ was prepared in 100ml standard flask. 50 ml of the above solution was taken in a beaker & placed in a magnetic stirrer-heater. 2M NaOH solution was added drop by drop under constant stirring with heating at room temperature till pH change to 12. It was magnetically stirred for 2 hours at room temperature. After the completion of reaction, the white precipitate formed was allowed to settle for overnight. The settled white precipitate was washed thoroughly with double distilled water and then centrifuged at 3000 rpm for 10 minutes. The obtained precipitate was dried in a hot air oven. During drying, complete conversion of $Zn(OH)_2$ into ZnO occurs. The above dried precursor was crushed in to powder. This powder was then calcinated in muffle furnace at 450^0C for 2 hrs. and stored in air tight container for the further analysis.

Microwave assisted green method

75 ml of 0.1M $Zn(NO_3)_2.6H_2O$ solution was taken in a 250ml beaker and added 20ml previously prepared Azolla leaf extract and stirred well. It was then placed in a domestic microwave oven operating at a power of 800W and frequency 2450 MHz. The solution was then subjected to microwave irradiation. The formation of ZnO nanoparticle was monitored by analysing the reaction mixture after 1, 2, 3, 4, 5, 6, 7minutes of microwave action. After the completion of reaction (8minutes), a white precipitate of ZnO NPs were obtained and was calcinated for 2hrs. at 450^0C. After calcination it was subjected to XRD measurements and SEM and FT-IR analysis.

Characterisation of Zinc oxide nanoparticles

UV-vis spectrum of nano zinc oxide particles

ZnO-NPs were re suspended in Millipore *TM* water and UV-Vis spectrum was recorded using LAMBDA 650-PERKIN ELMER spectrometer range from 200 nm to 800 nm.

X-RAY diffraction pattern of zinc oxide nanoparticle

The crystalline phase of the sample was identified at room temperature using RigakuMiniflex 600 X-ray diffractometer with CuK_α (λ = 1.5406 A^0) as the radiation source. The synthesised ZnO nanoparticle diameter was calculated using Debye-Scherrer formula $D= 0.8\ \lambda / \beta \cos\theta$ where λ is wave length of X-ray source (CuK_αline 1.541 A^0), β is the full with at half maximum (FWHM) in radians and θ is Bragg's diffraction angle.

SEM images of zinc oxide nanoparticle

The surface morphology of the prepared samples was evaluated by using JEOL JSM-6390 LV scanning electron microscope with a dynamic light scattering technique.

FT-IR spectroscopy of nano zinc oxide particles

The Fourier Transform Infrared spectral studies were carried out in KBr medium using Thermo Nicolet, Avatar 370 model FT-IR spectrometer in the range of 400-4000 cm^{-1} with resolution of $4cm^{-1}$.

Biological study of green synthesised zinc oxide nanoparticles

Antibacterial assay

The agar well diffusion method was used to screen the antibacterial activity of the green synthesised ZnONPs (Gunalan, 2012). The Gram-positive bacterial species *Staphylococcus aureus (S.aureus)* had been used in this study. 24hrs fresh culture was prepared and the standardized inoculum was made and used for the antibacterial assay. Approximately 20ml of molten and cooled media (Nutrient agar) was poured in sterilized petri dishes. The plates were left overnight at room temperature to check for the appearance of contamination. After inoculation and cultivation of target bacteria on top of nutrient agar, wells were placed in selected area on different plates. Agar wells of 5mm diameter were prepared with the help of a sterilized stainless steel cork borer. About 0.05 ml of various concentrations (1, 2, 3 mM) of two different ZnO nanoparticles were added in the wells. The plates containing the bacteria and zinc oxide nanoparticles were incubated at 37°C. The plates were examined for evidence of zones of inhibition which appear as a clear area around the wells. The diameter of such zones of inhibition was measured using a meter ruler and expressed in millimeter.

Antioxidant activity

The antioxidant activity of Zinc oxide nanoparticles were studied by the Ammonium Molybdate method (Syed Majid Bukhari *et al*., 2013).

RESULTS AND DISCUSSION

UV-vis spectrum of nano zinc oxide particles

Confirmation of the green synthesised ZnO product in nanoscale was exhibited by the highly blue shifted absorption

maximum occurring around 325 nm (Fig.1). For bulk ZnO the absorption maximum usually around 385 nm approximately. nanoparticle diameter was calculated using Debye-Scherrer formula.

Table 1 . UV readings converted to mg vitamin C eq / gram weight

sample	Concentrations	Sl.No	mg vitamin C eq/g wt
		1	30
	0.001g/mL	2	36
		3	0
		1	16
Chemical method	0.002g/mL	2	10
		3	17
		1	7.333
	0.003g/mL	2	12.666
		3	12
	0.001g/mL	1	12
		2	36
		3	16
Green Method using Azolla		1	10
	0.002g/mL	2	9
		3	14
		1	10
	0.003g/mL	2	6.66
		3	12.66

Table 2. Statistical treatment for the antioxidant potential of ZnO NPs

Concentration	Sample	Mean	Std. Deviation	N
0.001g/mL	chemical	33.3333	3.05505	3
	Azolla	15.3333	3.05505	3
0.002g/mL	chemical	14.3333	3.78594	3
	Azolla	11.0000	2.64575	3
0.003g/mL	chemical	10.6663	2.90589	3
	Azolla	10.4400	2.03598	3

Table 3. Comparative study between conventional chemical and microwave assisted, green nano zinc oxide

	Time taken for synthesis	Particle size from XRD pattern (nm)	BIOLOGICAL STUDY	
			Antibacterial activity Vs*S.aureus*	Antioxidant activity
Chemical method	2 hours	12.49	Show less activity, ZOI max. 4mm for 1mM concentration	Show a better antioxidant activity at very low concentration & when concentration increases there is a considerable decrease in their antioxidant activity
Microwave method using Azolla leaf extract	8 minutes	19.41	Show better activity, ZOI max. 7mm for 3mM concentration	Show better antioxidant activity at lower concentration 0.001g/mL but there is no considerable change in antioxidant activity was noticed with increase of concentration.

X-RAY diffraction pattern of zinc oxide nanoparticle

XRD spectra of ZnO nanoparticles prepared by microwave method & by Chemical method were given in the following Figures (Fig. 2. a. & b). The obtained X-ray diffractogram was compared with standard data and were found matched with standard data (JCPDS data card no.75-0576 and 79-0207), which indicated the formation of ZnO nanoparticle. The peak intensity, position and width, full-width at half maximum (FWHM) data were obtained from the XRD pattern. The diffraction peaks located at 31.84^0, 34.52^0, 36.33^0, 47.65^0, 56.92^0, 63.06^0 and 68.17^0 has been cleanly indexed as hexagonal wurtzite structure. The synthesised ZnO The average particle size calculated have been found to be 19.41nm & 12.49 nm for the microwave method and chemical method respectively which was derived from the FWHM of more intense peak corresponding to (101) plane located at 36.19^0 using Scherrer's formula.

SEM images of zinc oxide nanoparticle

The SEM analysis of was used to determine the surface morphology and shape of Zinc oxide nanoparticles. In this study SEM images (Fig.3) has showed individual zinc oxide nanoparticles as well as a number of aggregates. The closer observations, magnification at X10,000 (Fig 3.c) revealed that

the surface of the product was smooth and shape of ZnO NPs was found approximately spherical.

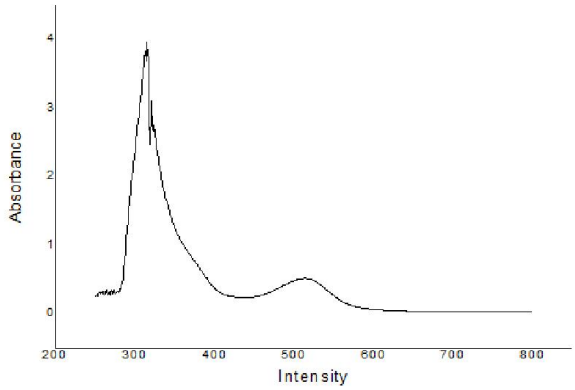
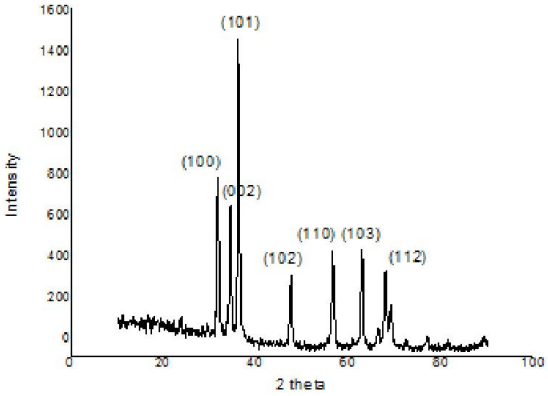
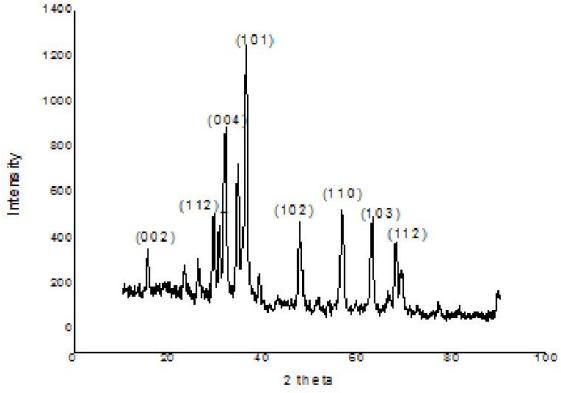


Fig. 1. UV- vis spectrum of ZnONPs prepared by microwave-method



(a)



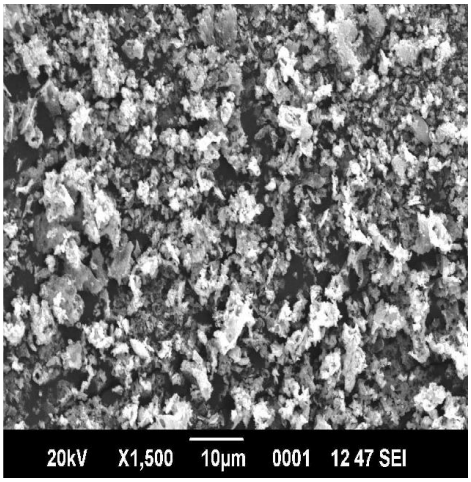
(b)

Fig. 2. XRD spectra of ZnO NP prepared by (a)microwave method (b) by Chemical method

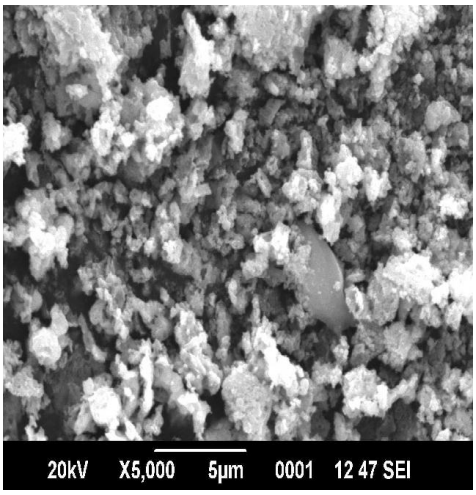
FT-IR spectroscopy of nano zinc oxide particles

The FT-IR spectrum revealed the involvement of biomolecules in the synthesis process which act as the reducing as well as stabilising agent of ZnO NPs**.** The peaks observed at 3427 cm^{-1}, and 3435.53 cm^{-1} (Fig.4 & 5 respectively) make 3427 cm-1 in the same line are may be due to O-H stretching vibrations assigned to phenols & the water adsorbed on the metal surface.

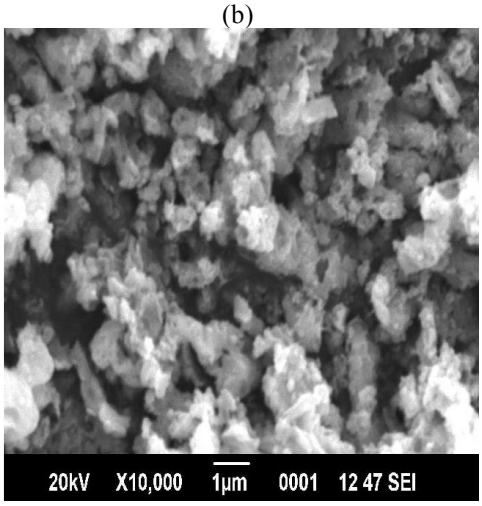
In case of FT-IR spectrum of microwave method (Fig.4) the additional band at 1627cm^{-1} is attributed to C=C stretch in aromatic ring and C=O stretch in polyphenols. In the IR spectrum the bands at the 3427 cm^{-1} is due to the stretching vibrations of O-H groups in water, alcohol and phenols and N-H stretching in amines.



(a)



(b)



(c)

Fig. 3. SEM images of nanoZnO prepared by microwave method at different magnifications

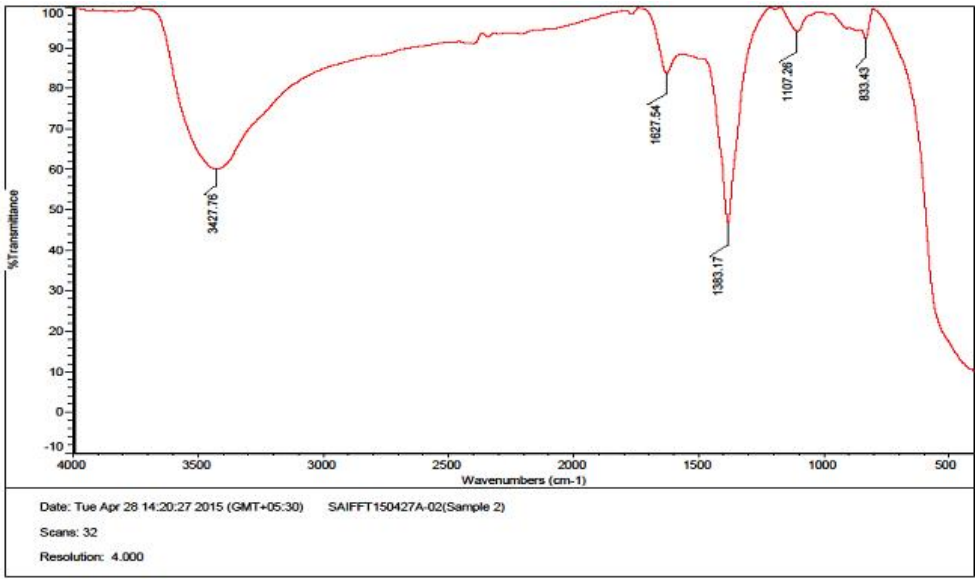


Fig.4. FT-IR spectra of ZnO NPs prepared by microwave green method

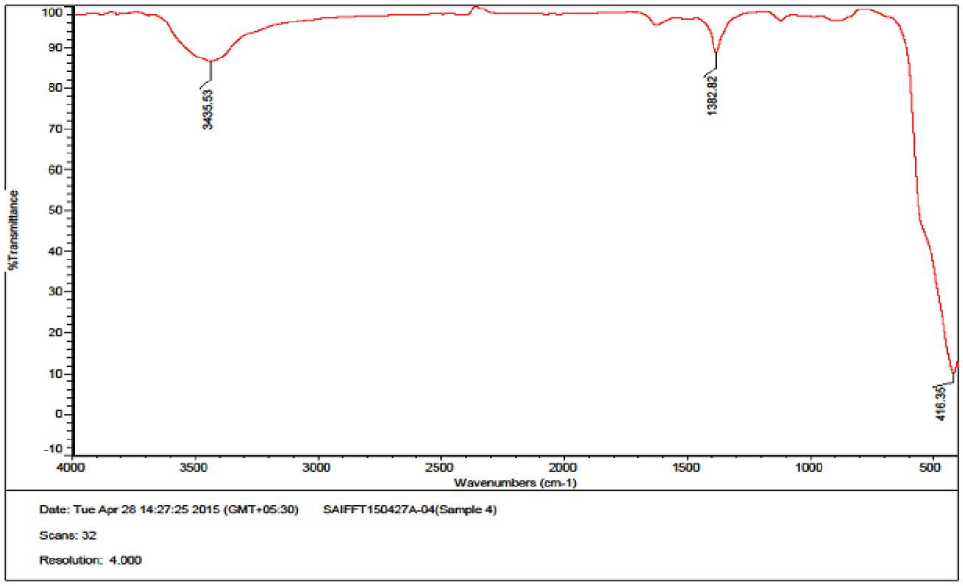
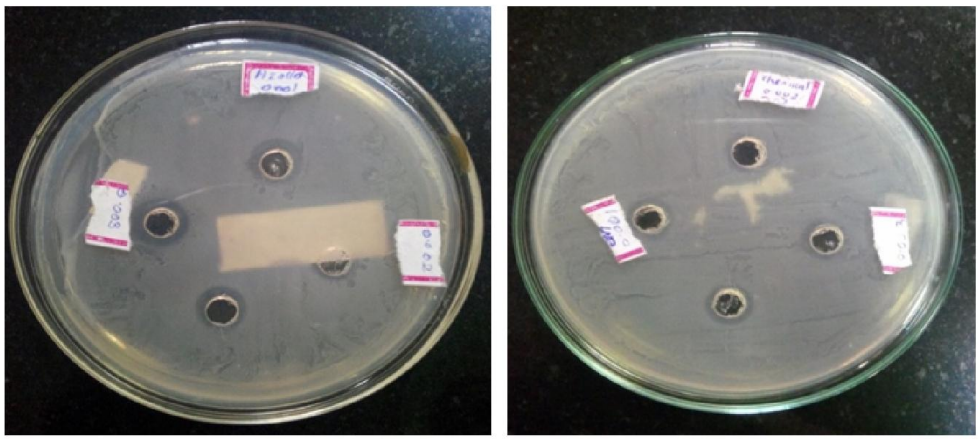


Fig. 5. FT-IR spectra of ZnO NPs prepared by chemical method



(a) **(b)**

Fig. 6. Antibacterial activity of ZnO NPs against *S.aureus* at different concentrations (a) ZnO NPs synthesised by microwave method and (b) by chemical method

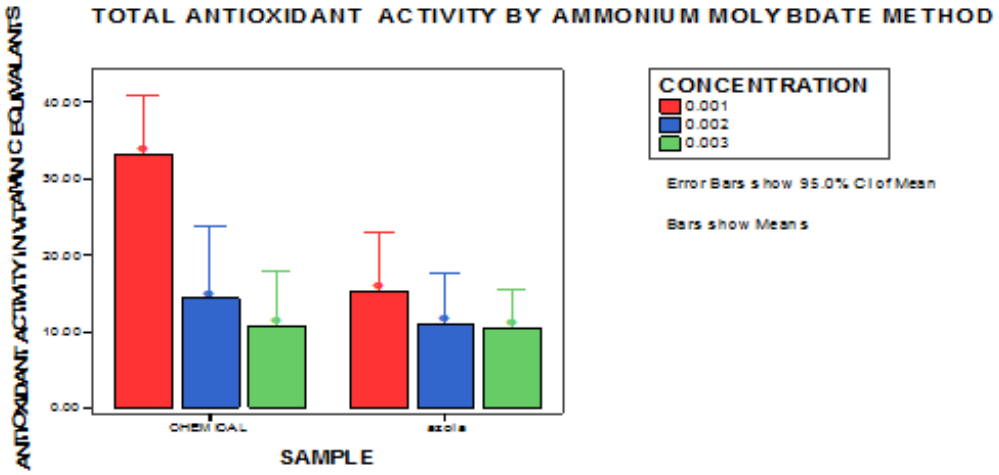


Fig.7. Total antioxidant activity by Ammonium Molybdate method

The C-N stretch of amide-I in protein gives the band at 1382 cm^{-1}. Comparison between spectra of chemically synthesised sample to the green synthesised samples of ZnONPs revealed that the presence of phenolic group of molecules are responsible for the reduction process and the polyphenols, amines and amide linkages in protein are responsible for the stabilisation of the ZnO NPs**.** Metal oxides generally give absorption bands in finger print region i.e below 1000 cm^{-1} arising from inter-atomic vibrations. Thus it can be concluded that the peak appearing at 416.35 cm^{-1} (range 400-500cm^{-1}) in the IR spectrum corresponds to the characteristic peak of ZnO molecule.

Biological study of zinc oxide nanoparticles

Antibacterial assay

The antibacterial activities of ZnO NPs were investigated against Gram-positive bacterial strain using agar-well diffusion technique & were shown in Fig.6 The particular concentrations of ZnONPs which show the inhibition towards Gram-positive bacteria *S.aureus* were found (3mM & 1mM) respectively for microwave method and chemical method. The maximum Zone of Inhibition (ZOI) was 7mm for 3mM in the case of azolla method Fig.6(a) and 4mm for 1mM in the case of chemical method Fig.6 (b). From the comparison of results it was evident that green synthesised ZnO NPs exhibit higher antibacterial activity against pathogenic strain than chemically synthesised one. The small size and greater surface area of contact with the microbial pathogens provide enhanced antibacterial activity to ZnO nanoparticles

Antioxidant activity

The total antioxidant capacity of the ZnO NPs was determined by Ammonium Molybdate method using vitamin c as standard. An aliquot of 0.2 mL sample ZnO NPs with different concentrations (0.001 g/mL, 0.002 g/mL & 0.003 g/mL)was combined with 2.0 mL of the reagent (0.6 M sulfuric acid, 28.0 mM sodium phosphate and 4.0 mM ammonium molybdate). Experiments were conducted in triplicate for each sample. The blank solution was made mixing 2.0 mL of the reagent solution with appropriate volume of the same solvent used to dissolve the sample. The tubes were capped and incubated at 95 °C for a period of 90 minutes. The sample and blank were left on the shelf for half an hour to cool down to room temperature . The absorbance sample was measured against blank solution at 695 nm. This UV readings were compared with standard readings and determined the corresponding milligram vitamin C equivalent per gram weight (mg vitamin C eq/gwt) .The total antioxidant activity of the samples were expressed as mg vitamin C eq/gwt. shown in the Table 1.

Statistical Study

The above results were subjected to statistical treatment as shown in the Table 2. An error bar diagram was drawn by using these results (Fig.7). The diagram clearly indicated the antioxidant potential of Zinc oxide NPs

Error Bar Diagram

Error bar diagram indicates that chemically synthesised ZnO nanoparticles show better antioxidant activity at very low concentrations & when concentration increases there is a considerable decrease in their antioxidant activity to low value. In the case of green synthesised ZnO NPs, they also show better antioxidant activity at lower concentration of 0.001g/mL but no significant change in antioxidant activity was noticed with increase of concentration. This may be due to the phytochemicals bound to the ZnO-NPs, acting as capping agents to prevent agglomeration and provide stability to the medium and there by maintaining their bioactivity.

An 'equivalence' study between conventional and microwave assisted, green nano zinc oxide

Comparative study on the time requirement and application potential of microwave assisted bio synthesised nano zinc oxide particles with conventionally synthesised nanoparticles were given in the following Table 3. The formation of stable nanoparticles occur rapidly by microwave method, making it one of the fastest bio-reducion methods. On comparing the

results, the nanoparticle size (D) was found below 20 nm in both the methods . Antibacterial study revealed that green synthesised ZnO NPs show better antibacterial inhibition towards Gram-positive bacteria S.aureus than chemically synthesised ZnO NPs. The chemically synthesised ZnO NP showed better antioxidant activity at very low concentrations & when concentration increases a considerable decrease in their antioxidant activity was noticed. In the case of green synthesised ZnO NPs, they showed antioxidant activity at lower concentration 0.001g/mL but there is no considerable change in antioxidant activity when concentration increases.

Conclusions

The present work demonstrated a low cost, rapid, green synthetic approach for the preparation of ZnO nano particles. ZnO nano particles were synthesised by conventional chemical method & by microwave assisted green route using azola plant extract as a reducing agent. The microwave assisted bioreduction was completed within 8 minutes making it one of the fastest biosynthesis route reported so far. The characteristics of the obtained ZnO nanoparticles were studied using UV – Vis spectroscopy, Fourier transform infrared spectroscopy, Scanning electron microscopy & XRD. The biological application of such synthesised ZnO nanoparticles were analysed by antibacterial & antioxidant study. Green synthesized ZnO nanoparticles are found to have superior antibacterial & antioxidant activity than chemically synthesised ZnO nanoparticles.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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