

ANTIDERMATOPHYTIC ACTIVITY OF GREEN SYNTHESISED ZINC OXIDE NANOPARTICLES USING *Cassia alata* LEAVES

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ABSTRACT

The green techniques are plying a major role in the synthesis of nanoparticles and in this present study we used the herbal plant *Cassia alata* for the green synthesis of zinc oxide nanoparticles. This reducing agent generates zinc oxide nanoparticles (ZnO NPs) from zinc acetate solution within 15 minutes. The synthesized ZnO NPs was characterized by UV-vis spectrophotometer shows surface Plasmon resonance band at 335 nm. Scanning electron microscope (SEM) image showed the size of the crystalline nanoparticles ranging from 30- 50 nm. Energy dispersive X-ray analysis (EDAX) confirms the presence of elemental Zn and O in the synthesized nanoparticles. FTIR revealed that presence of functional molecules like carboxylic acids, amine and nitro groups associated with synthesized oxide nanoparticles. Atomic force microscope (AFM) shows spherical shape of nanoparticles as well as aggregation also observed. Thus green synthesized nanoparticles were tested for their antidermatophytic potential against *Trichophyton mentagrophyte*, *Trichophyton rubrum*, *Epidermophyton floccosum*, *Microsporium audouinii*, and *Microsporium canis* and recorded percentage of inhibition, MIC and MFC values.

Keywords: Zinc oxide nanoparticles, green synthesis, *Cassia alata*, Antidermatophytic activity

INTRODUCTION

Dermatophytosis is common skin disease and also known as ringworm caused by fungal species mainly keratin digesting fungi. The mode of transmission of ringworm disease is skin to skin contact. The genera of dermatophytes are *Microsporum*, *Trichophyton* and *Epidermophyton* (White et al., 2008; Peres et al., 2010). Dermatophytes are evolved from soil residing keratinophilic organisms (Chuang et al., 2007). Dermatophytes cause infection mainly in hair, nails and skin and this disease can be mild or severe based on immune response of host cells (Abdel-Rahman, 2001; Macedo et al., 2005). Dermatophytes have the ability to produce proteolytic enzymes which are involved in hydrolyze of keratin (Akçaglar et al., 2011). Antifungal agents were used to heal the skin infection. After the skin healed, the treatment for ringworm is continued for 1-2 weeks. Common antifungals include Clotrimazole, Econazole, Ketoconazole, Miconazole, and Terbinafine. Terbinafine is a cream which applied in daily and it found to be an alternative. Usually it requires maximum one week to cure the disease compared to other antifungal agent but this remedy is expensive (Soares et al 2013).

Nanomaterials are the nanometre sized falls with ranges from 1-100 nm and these materials showed enhanced unique properties (Charinpanitkul et al 2008). Various types of nanoparticles are manipulated by physical, chemical and bio method. The nanoparticles are highly used as antimicrobial agents like antibacterial and antifungal activities against different types of disease causing pathogens (Soumya et al 2017, Venkat Kumar and Rajeshkumar 2017, Sujatha et al 2017, Rajeshkumar and Bharath 2017, Santhoshkumar et al 2017, Rajeshkumar 2016). Among other types of nanoparticles, metal oxide nanoparticles were having high antimicrobial activity due their increased surface area to volume ratio (Das Purkayastha and Manhar 2016). Zinc oxide nanoparticles are classified under metal oxide nanoparticles which is an n-type semiconducting metal oxide. ZnO NPs are much interested in research due to its wide range of applications in various fields of system and also they are inexpensive to fabricate, safe, simple and eco-friendly (Abdul et al 2014; Agarwal et al 2017). Due to the presence large band gap, ZnO nanoparticles exhibit catalytic activity, optic, antiinflammatory, and wound healing activity

(Mirzaei and Darrroudi 2017; Stan et al 2015). Moreover, ZnO NPs was mostly used in sunscreen lotions due to its UV filtering properties.

Various methods are currently used in ZnO nanoparticles like sol gel, laser ablation, solvothermal, thermolysis, and chemical vapour deposition techniques. The physical and chemical methods of production of nanoparticles are usually expensive and toxic to the environment and living systems. But the biological method offers simple and cost effective route of synthesis of nanoparticles. Moreover, green method of nanoparticles production offers, compatible, elimination of culture maintaining process, less time consuming and eco-friendliness. Currently, plant extract mediated synthesis method was achieved using different parts like bark, stem, root (Anand Raj and Jayalakshmy 2015), leaf (Sangeetha et al 2011; Elumalai and Velmurugan 2015), flower, fruit, peel (Mishra and Sharma 2015) and seed. The parts of plant has photochemical constituents like glucosides, apin, arthocyanins and quercetin which are act as reducing agents as well as involved in the stabilization of nanoparticles (Yedurkar et al 2016).

C. alata L is the member of Fabaceae family and can be grow n diverse habits. *C. alata* is an erect and herb which grows up to about 8 m tall with leathery compounded leaves. Traditionally, the species of *Cassia* exhibits antimicrobial property (Somchit et al., 2003) and it is used to treat ringworm and skin diseases in Thailand (Farnsworth and Bunyaprapatsara, 1992). The extract of leaves is considered as a good antioxidant agent, medicine for parasitic skin diseases, and is used in much eruptive and pustular skin (Phongpaichit et al 2004). In this study *C. alata* leaves extract was used to synthesis of ZnO nanoparticles and assessment of antidermatophytic activity against fungal species.

MATERIAL AND METHODS

Collection of plant

The plant *Cassia alata* was collected from Vandhavasi, Tamilnadu. Photo of this plant was taken from Vandhavasi

Preparation of plant leaf extract

About 20 g of *C. alata* leaves was finely chopped and washed with tap water followed by double distilled water. Leaves were boiled with 100 ml of distilled water in a beaker. After boiling extract was filtered using Whatman No 1 filter paper and collect the filtrate which is used to nanoparticles synthesis.

Green Synthesis of ZnO Nano`particles

In the typical synthesis of ZnO nanoparticles, 10 ml of plant extract was added into 90 ml of 1 mM Zinc acetate solution and kept it in stirring for constant mixing under room temperature. A color change of the solution was noted by visual examination confirming the synthesis of Zinc oxide nanoparticles.

Purification and Characterization of synthesized Zinc oxide nanoparticles

Colour change of bioreduction of Zinc oxide ions in aqueous solution was monitored by Double beam UV-vis spectrophotometer at different wavelength region from 320-700 nm. The bioreduced Zinc oxide ions were purified for further characterization studies by subjected to centrifugation at 10,000 rpm for 15 min. The purified Zinc oxide nanoparticle was morphologically characterized by using the Scanning Electron Microscope. Elemental analysis and crystalline structure of nanoparticles was examined by EDAX, respectively. Functional groups involved in the synthesis of zinc oxide nanoparticles were identified by FTIR spectrum. The study of morphological characters based on topography analysis was conducted by Atomic Force Microscope.

Anti-dermatophytic activity of ZnO nanoparticles synthesized by using *C. alata* leaves

Collection of dermatophytes

Dermatophytes like *Trichophyton mentagrophytes* (MTCC-7687), *Trichophyton rubrum* (MTCC-7859), *Epidermophyton floccosum* (MTCC-7880), *Microsporum audouinii* (MTCC- 8197), and *Microsporum canis* (MTCC- 3270) were purchased from MTCC, Chandigarh.

Antifungal activity by Agar well diffusion method

Antifungal activity of synthesized ZnO nanoparticles was assessed by the measuring of zone of inhibition of fungal growth around the well. Sterilized Potato dextrose medium was prepared and poured into petriplates. These sterile petriplates were allowed to solidify and swab the dermatophytes fungal spores using sterile cotton swab in each petriplates. In the culture inoculated plates, four wells were made with the diameter of 5 mm using gel puncture. Different concentrations of ZnO NPs (25, 50, and 75 μ l) were filled in each well and plates were incubated at 37°C for 24-48 h. After incubation, the different levels of zone of inhibition were measured around the well. The statistical analysis of standard error was calculated using triplicates of experiments (n=3). From this percentage of inhibition of nanoparticles was calculated by subtracting from control. Positive control is considered as 100% inhibition was compared with nanoparticles.

Determination of minimum inhibitory concentration

The MIC of the synthesized zinc oxide nanoparticles was determined at different concentrations are 25-75 μ l/ml. In 250 ml conical flasks the media Saboroud dextrose broth was prepared and sterilized in autoclave. After sterilization different concentration of ZnO nanoparticles were added separately in each flask. To this, 0.1 ml of fungal inoculums spores (1×10^7 cfu/ml) was added in each flask and the flasks were incubated at 37°C for 24 to 48 hrs. The positive (antibiotic mixed) and negative (without ZnO nanoparticles) control was maintained. Minimum inhibition concentration was determined by observing turbidity. There is no turbidity or fungal growth was observed in broth is noted as minimum inhibitory concentration.

Determination of Minimum Fungicidal concentration

The in vitro minimum fungicidal activity (MFC) was determined described by Espinel-Ingroff et al. (2002). Different concentration of nanoparticles was mixed with fungal culture inoculated flasks were made subculture from each flasks and spread on PDA containing plates. Plates were incubated for 72 h at room temperature. After incubation, all the plates were observed that showed no visible growth is a 100% of inhibition. Lowest concentration that required inhibiting the growth of dermatophytes fungal species is considered as minimum fungicidal concentration (MFC).

RESULTS AND DISCUSSION

In the nanoparticles synthesis experiment, *C. alata* leaves extract was added into the 1 mM of Zinc acetate solution leads to the change in the color of the reaction solution. The leaf extracts of *C. alata* changed in to yellowish white precipitate

when challenged with 1mM Zinc acetate solution (Figure 1 (A) & (B)). *C. alata* leaf extract has been used for the reducing material as well as surface stabilizing agent for the synthesis of spherical shaped ZnO-NPs. Color change from white to pale yellow represents the synthesis of ZnO NPs.



Figure 1 Visual observation of ZnO NPs synthesis *C.alata* leaves extract (A) yellowish white colour at initial (B) final color change

UV-vis spectroscopy

The optical absorption spectra of green synthesized zinc oxide nanoparticles were recorded using UV – vis Spectrophotometer at different wavelength is in the range of 300 to 500 nm. Figure 2 shows the UV-vis absorption spectrum of zinc oxide nanoparticles. The spectrum showed the absorbance peaks at 335 nm which is corresponding to the characteristic band of zinc oxide nanoparticles (Imitan et al., 2009) synthesized by leaf extract of *C. alata* respectively.

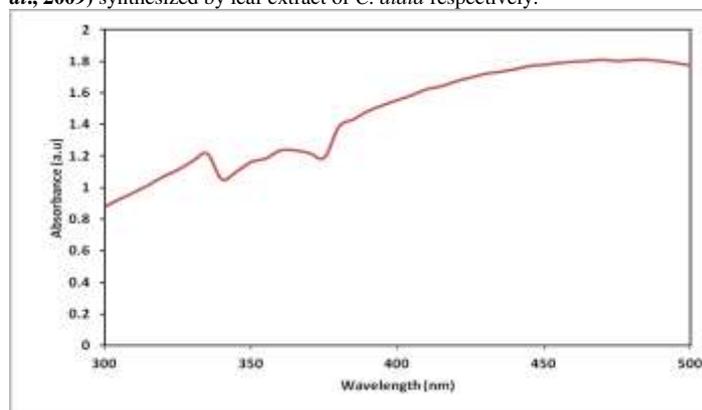


Figure 2 UV-vis spectrum of ZnO NPs synthesized by leaf extract of *C. alata*

Scanning Electron Microscope

The surface morphology and size of the zinc oxide nanoparticles was identified by Scanning Electron Microscope. SEM image had shown shape and size of the zinc oxide nanoparticles synthesized by using leaf extract of *C. alata* Figure 3(a) and (b) shows the surface morphology of the zinc oxide nanoparticles synthesized by using leaf extract of *C. alata* recorded under different magnifications. It shows mostly rod shaped ZnO nanoparticles as well as number of aggregates synthesized using leaf extract of *C. alata*. The SEM image showed that most of the nanoparticles are rod in shape formed within diameter range of 30 - 50 nm. Yedurkar et al (2016) synthesized ZnO nanoparticles are spherical in shape formed within diameter range of 80 - 130 nm using *Ixora coccinea* leaf extract.

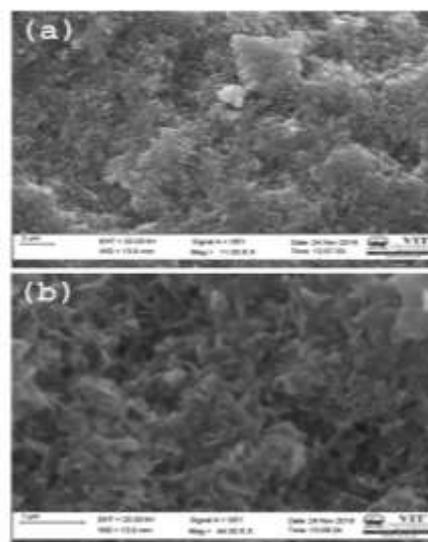


Figure 3 SEM image of ZnO NPs synthesized from *C. alata* leaf extract at different scale bar (a) 2 µm (b) 1 µm
EDX

EDAX spectrum (Figure 4) revealed that the other elements along with Zinc are identified in the synthesized ZnO nanoparticles. Figure shows the EDAX analysis, confirmed the presence of metallic zinc oxide in biosynthesized ZnO NPs. The composition obtained from EDAX analysis was Zinc and Oxygen at the Plasmon resonance peak of 8.5eV and 1eV, respectively. Additionally, Calcium and carbon was observed in *C. alata* leaf extract mediated synthesized ZnO nanoparticles. The presence of carbon and calcium in trace amount indicates the involvement of plant phytochemical groups in reduction and capping of the synthesized ZnO nanoparticles (Raut et al 2013).

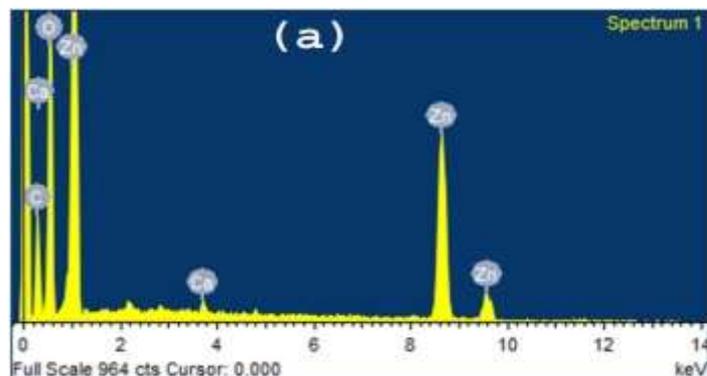


Figure 4 EDX Spectrum of ZnO nanoparticles synthesized from *C. alata*

FT-IR analysis

FT-IR spectra used to identify the functional group of *C.alata* leaf extract involved in ZnO NP synthesis illustrated peak in the range of 450–4000 cm⁻¹ (Figure 5). Broad peak obtained at 3282.31 corresponded to OH stretching vibrations of carboxylic acids, peak in the range of 1409.74 cm⁻¹ corresponded to CC stretch in aromatic ring. Weak peaks obtained at and 1017.85 cm⁻¹ and 643.62 cm⁻¹ demonstrated the presence of and C-O stretch in alcohols and C-Br stretch alkyl Halide (Table 1). Similarly, some researchers resulted that alcohol, aliphatic amine, phenols and carboxylic acids are the functional groups associated with nanoparticles (Rastogi and Arunachalam, 2011; Das et al., 2011; Awwad et al., 2013; Jamdagni et al 2016).

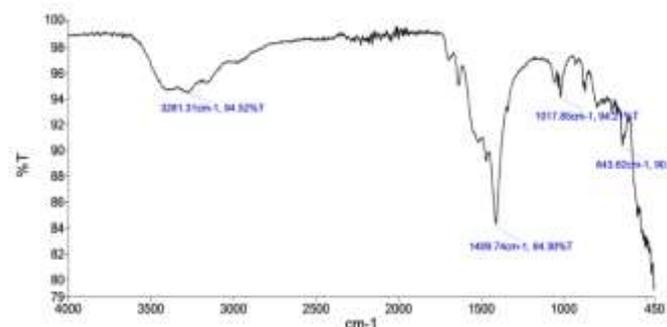


Figure 5 FT-IR Spectrum of ZnO NPs with plant leaf extract of *C. alata*

AFM analysis

AFM analysis is gives us insight about the topography, roughness of nanoparticles. AFM imaging was conducted in different magnification ranges of 5 and 25 µm. AFM (Figure 6(a) and (b)) image clearly demonstrate smooth nanoparticle with capping of phytochemicals over the surface of nanoparticles which are synthesized by leaf extract of *C. alata* (Femi et al 2011).

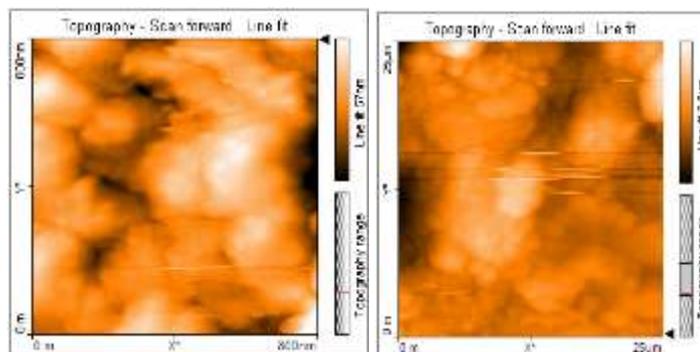


Figure 6 AFM images of *C. alata* mediated synthesis of ZnO nanoparticles
Antidermatophytic activity of ZnO NPs synthesized by using *C. alata* leaf extract

The results of antifungal sensitivity of ZnO nanoparticles synthesized by using extract of *C. alata* leaves against the five different dermatophytic fungal strains are interpreted in table 1-3 and Figure 7. Results clearly demonstrate that the nanoparticles showed anti- bacterial effect in a dose-dependent manner (Figure 7 and Table 1). Maximum zone of inhibition was observed against the dermatophytes *T. rubrum*. Minimum zone of inhibition was observed against *M. canis*. Zone of inhibition obtained using nanoparticles was much lower than the standard disc used which depicts the need of further engineering of nanoparticles to obtain desirable effects. The entire tests were done in triplicate. The zinc oxide nanoparticles are inhibits the microbial growth in in-vitro antimicrobial activities.

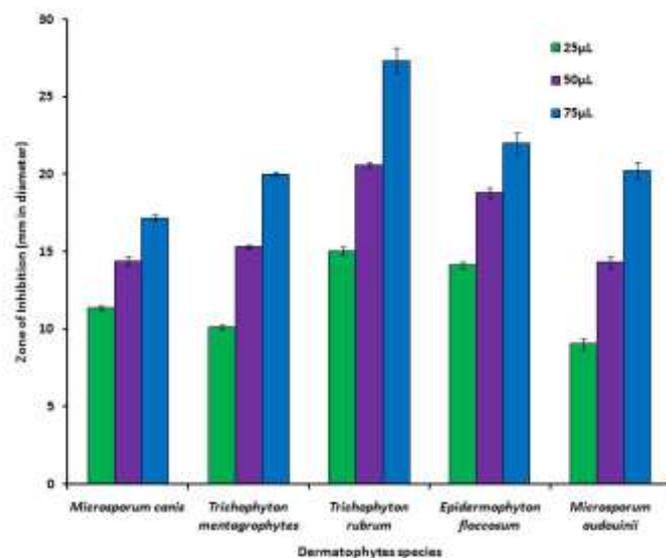


Figure 7 Anti-dermatophytic activity of ZnO NPs synthesized using the extract of *C. alata* leaves against dermatophytes at different concentrations

Table 1 Zone of inhibition of ZnO NPs synthesized using the extract of *C. alata* leaves against dermatophytes

Dermatophytes used	Zone of Inhibition (mm in diameter)		
	25µL	50µL	75µL
<i>M. canis</i>	11.37±0.15	14.43±0.29	17.20±0.19
<i>T. mentagrophytes</i>	10.17±0.15	15.33±0.13	20.03±0.13
<i>T. rubrum</i>	15.03±0.27	20.63±0.17	27.37±0.83
<i>E. floccosum</i>	14.17±0.23	18.83±0.33	22.03±0.67
<i>M. audouinii</i>	09.07±0.35	14.33±0.37	20.25±0.54

In this study, zone of inhibition was increased as increasing the volume of the dosage of ZnO nanoparticles. However, antifungal activity is directly proportional to dosage of ZnO nanoparticles i.e. antifungal activity is increased as a dose dependent manner. Among the two different plants mediated synthesized ZnO nanoparticles, *C. alata* leaves mediated synthesized nanoparticles shown significant activity against all the fungal strains.

Percentage of zone of inhibition

Terbinafine is used as positive control and its complete antifungal activity against pathogens is considered as 100% of inhibition. It was compared with Zinc oxide nanoparticles synthesized by using *C. alata* leaves. *C. alata* synthesized

nanoparticles shows 100 % of activity against *T. mentagrophytes* and *T. rubrum* and minimum percentage of activity (64%) was noted against *E. floccosum*. *C. alata* leaf extract mediated synthesized ZnO NPs have the ability to complete control against all the pathogens above 60% of inhibition.

Minimum Inhibitory Concentration and MFC determination

The results of antifungal activity of ZnO nanoparticles synthesized using *C. alata* leaves showed good activity on all the strains of *M. canis*, *T. mentagrophytes*, *T. rubrum*, *E. floccosum* and *M. audouinii* tested at different concentrations. The *C. alata* ZnO NPs exerting more higher activity as revealed by mean diameter of zone of inhibitions, minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) (Tables 2). However, MIC and MFC values of 25.0 and 25.0 µl/ml were recorded for *C. alata* ZnO NPs respectively against *T. rubrum* strain. From this results *C. alata* ZnO NPs shows more fungicidal activity at minimum concentration.

Table 2 Percentage of zone of inhibition by *C. alata* leaves mediated synthesized ZnO nanoparticles against dermatophytes

Dermatophytic fungi	% Zone of Inhibition	
	Control	<i>C. alata</i> ZnO NPs
<i>M. canis</i>	20	14 (72%)
<i>T. mentagrophytes</i>	18	18 (100%)
<i>T. rubrum</i>	22	22 (100%)
<i>E. floccosum</i>	19	15 (64%)
<i>M. audouinii</i>	21	19 (92.2%)

*Control is considered as 100% inhibition. Percentage of inhibition was calculated by determining differences between control and ZnO NPs.

Minimum Inhibitory Concentration and MFC determination

The results of antifungal activity of ZnO nanoparticles synthesized using *C. alata* leaves showed good activity on all the strains of *M. canis*, *T. mentagrophytes*, *T. rubrum*, *E. floccosum* and *M. audouinii* tested at different concentrations. The *C. alata* ZnO NPs exerting more higher activity as revealed by mean diameter of zone of inhibitions, minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) (Tables 3). However, MIC and MFC values of 25.0 and 25.0 µl/ml were recorded for *C. alata* ZnO NPs respectively against *T. rubrum* strain.

Table 3 Minimum inhibitory and fungicidal concentrations of the ZnO nanoparticles

Microorganism	MIC/MFC (µl/mL)			
	Control		<i>C. alata</i> ZnO NPs	
	MFC	MIC	MFC	MIC
<i>M. canis</i>	50	53	25	25
<i>T. mentagrophytes</i>	50	59	50	55
<i>T. rubrum</i>	25	33	25	30
<i>E. floccosum</i>	75	78	100	100
<i>M. audouinii</i>	25	27	25	35

Nf<100 – No fungicidal activity below 100 µl/ml

CONCLUSION

Green method of production of oxide nanoparticles is a great attention in the research field. By using of leaf extract in nanoparticles synthesis is a simple, eco-friendly, and also the extract act as reducing agent as well as stabilizing agent. In this study, ZnO nanoparticle was prepared by using aqueous extract of *C. alata* leaves. UV vis spectrum shows the absorption peak at 335 nm and the SEM image shows the morphological characters of synthesized nanoparticles. While examining the effect of zinc oxide nanoparticles for their antifungal potential against dermatophytic species, these showed good activity against tested fungal human pathogens. Moreover there is a need to develop the formulation regarding fungicidal activity will be achieved through further research.

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