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Synthesis, Characterization and Antimicrobial Activity of Zinc Oxide Nanoparticles Synthesized From *Calotropis procera*

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ABSTRACT

The aim of the study was to compare the yield, nature and antimicrobial activity of nanoparticles synthesized using *Calotropis procera* leaf extract. ZnO NPs synthesized were characterized by FTIR and SEM. It was evident from SEM images that the size of the particles obtained by biological method is ranging from 100-200 nm. Antibacterial study was carried out on human bacterial and plant bacterial pathogens and their MIC values were determined. The antibacterial activity towards human bacterial and plant pathogen showed good sensitivity towards the green synthesized ZnO NP's at all concentrations and maximum zone of inhibition occurred at the concentration of 30µg/mL. Minimum Inhibitory concentrations of NPs against human pathogenic bacteria and plant bacterial pathogens, shows that all tested microorganisms were completely inhibited at the concentration of 50 to 12.5µg/ml of nano-ZnO. The antifungal activity of ZnO NPs against fungi shows that different concentration of ZnO nanoparticles caused significant inhibition in the spore germination.

Keywords: *Calotropis procera,* Zinc Oxide nanoparticles, biological method, FTIR, SEM, antibacterial study, antifungal activity.

INTRODUCTION

Recent advance in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticles of any size and shape, have led to the development of new biocidal agents. Nano-materials are called "a wonder of modern medicine". [1] Nanoparticles interact with biological materials and established a series of nanoparticle / biological interfaces that depend on colloidal forces as well as dynamic bio physicochemical interactions. These interactions lead to the formation of new nanomaterial with control size shape, surface chemistry, roughness and surface coatings.

Zinc oxide (ZnO) is considered to be a technologically prodigious material having a wide spectrum of applications such as that of a semiconductor (Eg = 3.37 eV), magnetic material, electroluminescent material, piezoelectric sensor and actuator, gas sensor, constituent of cosmetics etc. Due to noble properties such as high refractive index, high thermal conductivity, binding energy, antibacterial and UV-protection of ZnO it could be used in various materials and products, including medicine, cosmetics, varistors, solar cells, rubber and concrete, foods. [2]

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piezoelectric sensor and actuator, gas sensor, constituent of cosmetics etc. Due to noble properties such as high refractive index, high thermal conductivity, binding energy, antibacterial and UV-protection of ZnO it could be used in various materials and products, including medicine, cosmetics, varistors, solar cells, rubber and concrete, foods. [2]

Use of biological organisms such as micro organisms, plant extract or plant biomass could be an alternative to chemical and physical methods for the production an eco-friendly manner on of nanoparticles. [3] *Calotropis procera* is a desert plant known as Madar in Greeco-Arab medicine. This plant is widely distributed in tropical and subtropical Africa and Asia. *C. procera* is a plant with good enough quantities of latex i.e. milky liquid. When any mechanical damages, their tissues are broken and secrete the milky latex, consisting of several biologically active compounds, including proteins, amino acids, carbohydrates, lipids, vitamins, alkaloids, resins, and tannins. Predominantly, milky latex contains several alkaloids of interest such as calotropin, catotoxin, calcilin, gigatin etc. [4] The aim of this study was to synthesize ZnO NP's from *C. procera* and study its antimicrobial activity against human, plant pathogens and common fungi.

Fig. 1: ***Calotropis procera***

Fig. 2: ZnO nanoparticles synthesized from ***Calotropis procera***

MATERIALS AND METHODS

Synthesis of ZnO NP's from plant

Materials and plant samples

Zinc nitrate [$Zn(NO_3)_2$] and glassware was purchased from Merck Chemical Reagent Co. Ltd. India. All glassware was washed with sterile distilled water and dried in and hot air oven before use. The plant material was collected from our college campus and was identified as *Calotropis procera (*family: *Asclepiadaceae).* The procedure for the synthesis of nanoparticles was referenced from literature. [5-7]

Preparation of the leaf extract

Fresh leaves were collected from *Calotropis procera* plants in the college campus. The leaves were washed several times with water to remove the dust particles and then sun dried to remove the residual moisture. The extract used for the reduction of zinc ions (Zn^{2+}) to zinc nanoparticles (ZnO) was prepared by placing 50 g of washed fresh fine cut leaves in 250 mL glass beaker along with 100 mL of sterile distilled water. The mixture was then boiled for 60 minutes until the colour of the aqueous solution changes from watery to light yellow. The extract was cooled to room temperature and filtered using filter paper. The extract was stored in a refrigerator in order to be used for further experiments.

Preparation of Zinc Nanoparticles

For the synthesis of nanoparticle, 50ml of *Calotropis procera* leaves extract was taken and boiled to 60-80°C using a stirrer-heater. 5 grams of zinc nitrate was added to the solution as the temperatures reached 60°C. This mixture is then boiled until it reduced to a deep yellow colored paste. This paste was then collected in a ceramic crucible and heated in a hot air oven at 100°C for one day. A light yellow colored powder was obtained and this was carefully collected and packed for characterization purposes. The material was mashed in a mortar-pestle so as to get a finer nature for characterization.

Characterization of Zinc Oxide Nanoparticles by Fourier Transform Infrared Spectroscopy (FTIR)

The synthesized nanoparticles powder was subjected for FTIR analysis using KBr. FTIR analysis was done by using Shimadzu FTIR 8400s software KBR IR Solution. Fourier Transform Infrared Spectroscopy (FTIR) measurements are carried out to identify the possible biomolecules responsible for the reduction of the Zn^{+} ions and capping of the bio-reduced ZnO NPs synthesized by *Calotropis procera.*

Characterization of Zinc oxide Nanoparticles by Scanning Electron Microscope (SEM)

This study was undertaken to know the size and shape of Zinc oxide Nanoparticles biosynthesized using *Calotropis procera.* SEM analysis was done using SEI-11 30 machine. Thin films of the sample were prepared on a coated copper grid by just placing a very small amount of the sample on the grid. Then the film on the SEM grid was allowed to dry and the images of nanoparticles were taken.

Antimicrobial activity of synthesized ZnO nanoparticles

Synthesized metal oxide nanoparticle (ZnO) against human bacterial pathogens

Test organisms used for the analysis

Human pathogenic organisms *E. coli, P. aeruginosa, K. pneumoniae, S. aureus* were collected from Erode Diagnostic Laboratory, Erode. A loop of single colony of each test strain was inoculated in Nutrient agar (broth) and incubated in a temperature controlled shaker (120 rpm) at 30°C overnight.

Preparation of dilutions of synthesized compounds

10 mg of the each particle nanoparticles was weighed accurately and dissolved in 10 ml Saline solution (8.5 g of NaCl and 1000 ml of distilled water) giving a solution of 1 mg/ml concentration. 1 ml of the above solution was again diluted to 10 ml with Saline giving a solution of 100µg/ml concentration.

Synthesized metal oxide nanoparticle (ZnO) against plant pathogenic bacteria

Isolation of *Xanthomonas axonopodis pv. citri (Hasse) (Xac)* from citrus plant

A few typical lesions from the cankerous fruits were excised using a sterile razor in the laminar flow bench. The excised lesions are surfaced sterilized using 70% alcohol followed by serial washings with distilled water. Later the lesions were tweezed by using a sterile forceps or needle using aseptic conditions and left aside for 10 min, for release of the bacteria, loopful of tweezed bacteria was taken and streaked on the different selective media- *Xanthomonas* growth media (XGM) [Galactose-20 g, Yeast extract-10 g, Calcium carbonate-20 g, Agar agar-20 g, Distilled water-1000 mL]. The following culture media used for initial plating was Glucose Yeast extracts Peptone (GYP media) [Peptone-0.5 g, Yeast extract-0.5 g, Glucose-1 g, Agar agar-1.5 g, Distilled water-100 mL]. The bacteria were identified by the standard Gram's staining technique. The pathogen was identified biochemically by performing standard procedures explained in the laboratory manuals by Aneja (2003), Gunasekaran (2002). [8] IMVIC tests, fermentation of Carbohydrates, Catalase test, Hydrolysis of Gelatin, Casein Hydrolysis were used to identify the pathogen.

Antimicrobial Activity

Nano-ZnO was tested *in vitro* for their antimicrobial activities against bacterial strains by the agar diffusion technique. About 20 ml of sterile Nutrient agar (HiMedia Laboratories Pvt. Limited, Mumbai, India) was poured into the sterile petriplates. Total plates were swabbed with the overnight culture (10^8 cells/ml) of human pathogenic bacteria *viz. E. coli, P. aeruginosa, K. pneumoniae, S. aureus* and plant pathogenic bacteria *Xanthomonas axonopodis pv. citri (Hasse) (Xac).* The solid medium was gently punctured with the help of cork-borer to make a well. Finally the nanoparticals sample with various concentrations (10µl, 20µl, 30µl) of ZnO NPs were added from the stock into each well and incubated for 24 h at 37 ± 2°C. After 24 h of incubation, the zone of inhibition was measured and expressed as millimeter in diameter.

Table 1: Zone of Inhibition of related ZnO NP's against Human Bacterial Pathogens

S. No	Test Organism	Zone of Inhibition (in mm)		
		10µl	20µl	30µl
1	*E. coli*	9	12	15
2	*P. aeruginosa*	10	10	14
3	*K. pneumoniae*	12	14	16
4	*S. aureus*	16	18	21

Minimum Inhibitory Concentration (MIC)

The antimicrobial activities of the samples were evaluated through the determination of the minimum inhibitory concentration (MIC) by the micro dilution method in culture broth. For both the antibacterial assays, the compounds were dissolved in saline (50 mg/ml). Further dilutions were prepared at the required quantities of 50, 25, 12.5, and 6.2µg/ml concentrations. The minimum inhibitory concentration (MIC) values were determined using the method of serial dilutions. The Nutrient Broth, which contained tested samples and controls, were inoculated with approximately 5×10^5 cfu/ml of actively dividing bacterial cells. The cultures were incubated for 24 h and 48 h at 30°C on a metabolic rotary shaker (220 rev/min), and the growth was monitored visually and spectrophotometerically (at 540 nm). In order to ensure that the solvent had no effect on bacterial growth, a control test was also performed containing inoculated broth supplemented with only saline at the same dilutions used in our experiments and found inactive in culture medium.

Synthesized metal oxide nanoparticle (ZnO) against fungi

Isolation and identification of fungal isolate

Fungal samples- *Aspergillus sps* and *Penicillium sps* were procured from Department of Microbiology, Maharaja Co-education Arts and Science College, Erode, Tamil Nadu. The fungal samples were grown in Sabouraud dextrose agar. The pure culture was maintained on PDA broth media (Hi media Laboratories Ltd. Bombay, India) at 25±1°C.

Antifungal activity

To determine the antifungal activity of ZnO nanoparticles against cultured food borne fungal samples. About 20 ml of sterile PDA media (HiMedia Laboratories Pvt. Limited, Mumbai, India) was poured into the sterile petriplates. From PDA broth the loop of culture was inoculated and then well was formed in the medium. The ZnO nanoparticles were taken at different concentrations like 100µl, 200µl, and 300µl was added into the well. Further, the plates were incubated at room temperature and after 48 hours of incubation the growth inhibition was examined by the formation of zone.

RESULTS AND DISCUSSION

Preparation of Plant extract and Synthesis of ZnO nanoparticles

Calotropis procera plant leaves were collected and the leaves were used for biosynthesis of ZnO NPs. During synthesis, leaf extract (50 ml) was added to zinc nitrate

(5 g) boiled and reduced to deep yellow colored paste. This paste was then collected in a ceramic crucible and heated in an air oven at 100°C for one day, the dried sample material was mashed in a mortar-pestle to form a light yellow colored powder was obtained.

Calotropis procera has been used for the first time as a reducing material as well as surface stabilizing agent for the synthesis of ZnO nanoparticles. The photographs of the *Calotropis procera* plant and the synthesized ZnO NPs powder are as shown in Fig. 1, 2**.** The light yellow coloured ZnO NPs arise due to capping action of biomolecules of green leaf extract on the surface of the nanoparticles.

FT-IR analysis

FT-IR spectroscopy is the measurement of absorption of IR radiations by a sample plotted against the wavelength. The interpretation of the IR spectrum involves the correlation of the absorption bands (vibrational bands) with the chemical compounds in the sample. The FTIR spectrum *Calotropis procera* - ZnO NPs are shown in Fig. 3. The band located near 621.08 to 692.44 cm^{-1} can be attributed to the Zn-O stretching mode. The absorption peak 952 cm^{-1} illustrates the saturated primary alcohol, chemical bonding, crystal structure and relative intensities of the IR bands of the carbonate group and band located near 1022.27 cm^{-1} can be attributed to presence of C-N amines, Medium absorption in the region 1581^{-1}415 cm^{-1} implies the presence of aromatic ring. 3116 cm^{-1} represents the presence of hydrogen-bonded O-H stretch. Broad IR bands at 692.44 cm^{-1}, 952.84cm^{-1}, 1022.27cm^{-1}, 1411.89 cm^{-1}, 1442.75cm^{-1}, 1562.34cm^{-1}, 3116.97cm^{-1} indicating presence of hydroxyl group, aromatic group, amine group, saturated primary alcohol, and carbonate group.

SEM analysis

SEM analysis was done using Hitachi SEI1130 SEM machine. Thin film of sample were prepared on a carbon coated copper grid by just dropping a very small amount of sample on the grid , extra solution was removed using a blotting paper and then the film on the SEM grid allowed to dry by putting it under a mercury lamp for 5 minutes.

The SEM image showed relatively granular, spongy shape nanoparticle. The low magnified observation Fig. 4(a), 4(b) shows that the morphology is nano structured ranging from 0.2-2µm in diameter. Fig. 4(c), 4(d) represents that the obtained products are composed of near granular, spongy shape morphology with the average size in the range from 1µm- 0.5µm. Closer observation of 4(c), 4(d) **s**hows that the nanoparticles have granular, spongy morphology ranging particle size from 100-200 nm.

Antibacterial activity

Synthesized metal oxide (ZnO) nanoparticles against pathogenic strains from human

Antibacterial study was carried out on four clinically isolated strains from Erode Diagnostic Laboratory (Erode) namely, *E. coli, P. aeruginosa*, *K. pneumoniae, S. aureus* had been used in the assay. Green synthesis of ZnO NP's samples from *Calotropis procera* was tested against four human bacterial samples collected from the laboratory by the agar diffusion technique. The antibacterial activities of ZnO NPs *E. hirta* against the pathogenic strains are shown in Fig. 5. The zone of inhibition of related NPs at various concentrations (10µl, 20µl, and 30µl) against human bacterial pathogens were represented in Table 1.

All Gram-negative and gram positive bacteria had shown good sensitivity towards the green synthesized ZnO NPs at all concentrations and maximum zone of inhibition occurred at the concentration of 30µl**.** The zone of inhibition for pathogenic strain was decreased at the increased concentration of ZnO nanoparticles and the maximum inhibition of growth was obtained at 30µl.

The smaller size of NPs facilitates easy entry into the microbial cell membrane and enables inhibition mechanisms to occur inside the cell. ZnO NPs generate hydrogen peroxides which chemically interact with membrane proteins and lipid bilayers. [9] The antimicrobial activity of these NPs may involve both the production of reactive oxygen species (ROS) and the accumulation of NPs in the cytoplasm on the outer membranes. ROS causes membrane dysfunction [10] and cell death by oxidizing the membrane lipids. [11]

Minimum Inhibitory Concentration (MIC)

The MIC of the agent is the concentration at which the solution becomes turbid. Here, ZnO NP's suspensions with different concentrations were tested, in the range of 50, 25, 12.5, and 6.2µg/ml concentrations and the results are given in Table 2. The data shows that all tested microorganisms were completely inhibited at the concentration of 50 to 12.5µg/ml of nano-ZnO. In 6.2µg/ml concentrations microbial reductions are not occurred in the case of 10^{-4} serial dilution because lower concentration of ZnO NPs does not reduce the microbial number (Fig. 6).

The percentage microbial reduction with the ZnO from *Calotropis procera* against the four species of bacteria is shown in Table 2**.** The MIC of nano-ZnO against *S. aureus* and *K. pneumonia,* were the best affect at the both strains (50µg/ml), followed by *Pseudomonas aeruginosa* (gram negative), *E. coli* (gram negative), (50µg/ml). The moderated effect had been showed by *S. aureus, K. pneumoniae* and *P. aeruginosa, E. coli* 25, 12.5µg/ml concentrations of ZnO NPs from *Calotropis procera.*

ZnO NP's continue to release peroxides into the medium even after the surface of the dead bacteria are completely covered by ZnO NP's, so it showing high bactericidal efficacy The MIC was defined as the lowest concentration required arresting the growth of the bacteria at the end of 24 h of incubation. [12] Absorbance measurements are not as accurate as plate counts for the determination of viable bacteria but they can give a rapid estimate of cell numbers. Absorbance measurements (turbidity) are commonly used for minimum inhibitory concentration (MIC). [13]

Table 2: MIC of ZnO NP's against Human Bacterial Pathogens

Test Organism	Serial dilution	MIC for *C. procera*					
		O.D. after 3 hours (540 nm)		Reduction %	O.D. after 24 hours (540 nm)		Reduction %
		Control	ZnO		Control	ZnO	
E. coli	10^{-1}	1.394	0.763	0.631	1.88	0.689	1.191
	10^{-2}	1.392	0.768	0.624	1.936	0.658	1.278
	10^{-3}	1.298	0.801	0.497	1.901	0.8	1.101
	10^{-4}	1.229	1.005	0.224	2.012	0.985	1.027
P. aeruginosa	10^{-1}	1.712	0.758	0.954	2.363	0.555	1.808
	10^{-2}	1.333	0.948	0.385	2.369	0.985	1.384
	10^{-3}	1.716	1.482	0.234	2.001	0.855	1.146
	10^{-4}	1.455	1.623	-0.168	2.222	0.901	1.321
K. pneumoniae	10^{-1}	1.333	0.632	0.701	1.88	0.458	1.422
	10^{-2}	1.455	0.942	0.513	1.945	0.458	1.487
	10^{-3}	1.385	1.021	0.364	1.885	0.552	1.333
	10^{-4}	1.33	1.712	-0.382	1.753	0.789	0.964
S. aureus	10^{-1}	1.685	1.217	0.468	2.459	0.422	2.037
	10^{-2}	1.398	0.942	0.456	2.156	0.568	1.588
	10^{-3}	1.321	0.622	0.699	2.741	0.496	2.245
	10^{-4}	1.369	0.551	0.818	2.234	0.321	1.913

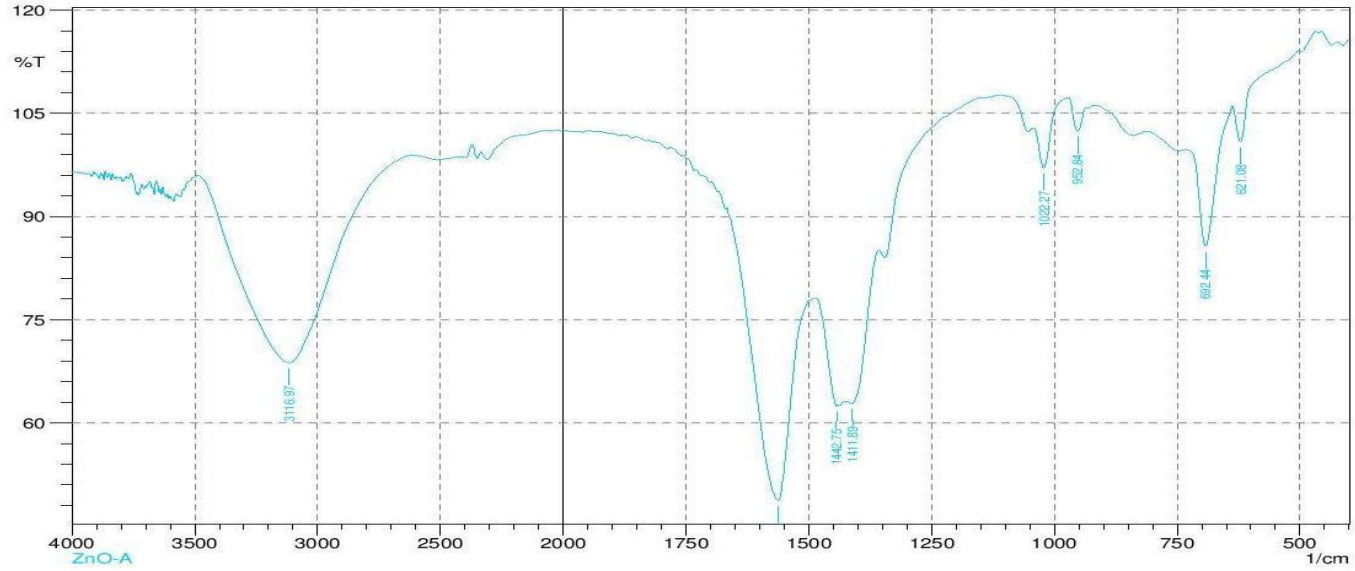

Fig. 3: FTIR Spectrum of ZnO NP's synthesized from *Calotropis procera*

Synthesized metal oxide (ZnO) nanoparticles against plant bacterial pathogens isolated from citrus canker disease affected plant.

The following are the results indicating the morphological (Table 3) and biochemical characterization (Table 4) of *Xanthomonas axonopodis*. On Nutrient agar media, after 48 h of incubation small, round mucous yellow to orange colonies were observed and biochemical characterization photos are shown in (Fig. 7). On *Xanthomonas* selective media and Glucose yeast extract peptone media, yellow colored appear against white background of the media. Bacterial cells retained pink color after staining, so it was identified as gram negative bacteria.

Table 3: Morphological identification

Test	Organism
Configuration	Small and round
Margins	Smooth
Surface	Mucous
Pigmentation	Yellow
Opacity	Opaque
Gram's reaction	-ve rods with slight bulged ends

Table 4: Biochemical characterization

Indole test	-ve
Methyl Red Test	+ve
Voges-Proskauer test	+ve
Citrate utilization test	+ve
Gelatin utilization test	+ve
Catalase test	+ve
Glucose	+ve for acid and –ve for gas production

Table 5: Zone of Inhibition of related ZnO NP's against Plant Bacterial Pathogens

S. No	Test Organism	Zone of Inhibition (in mm)		
		10µl	20µl	30µl
1	*Xanthomonas axonopodis*	6	8	16

Table 6: Antifungal activities of ZnO NPs from *C.procera* against fungal sps

S. No.	Test Organisms	Zone of Inhibition (mm) of ZnO NP's from *C. procera*		
		100µl	200µl	300µl
1	*Pencillium sps*	-	15	17
2	*Aspergillus sps*	18	25	28

Antibacterial activity of ZnO NPs against *Xanthomonas axonopodis*

The zone of inhibitions of ZnO NPs against *Xanthomonas axonopodis* is shown in the (Table 6).

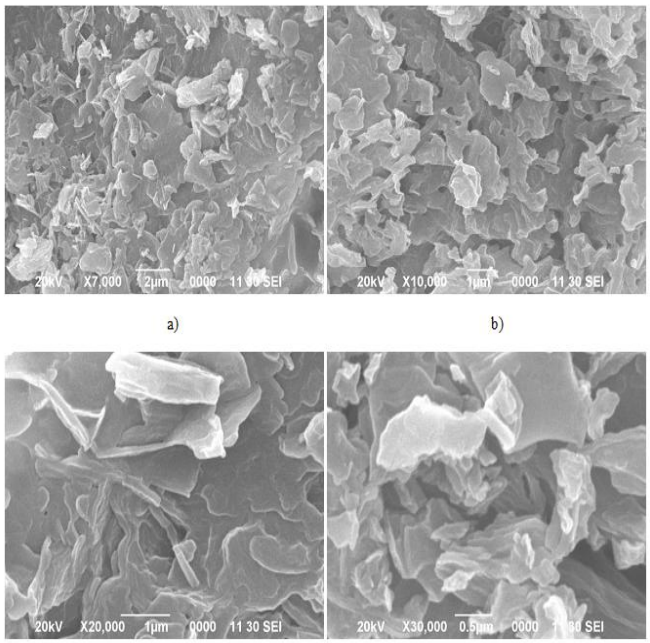

Fig. 4: SEM Images of ZnO NP's synthesized from *Calotropis procera*

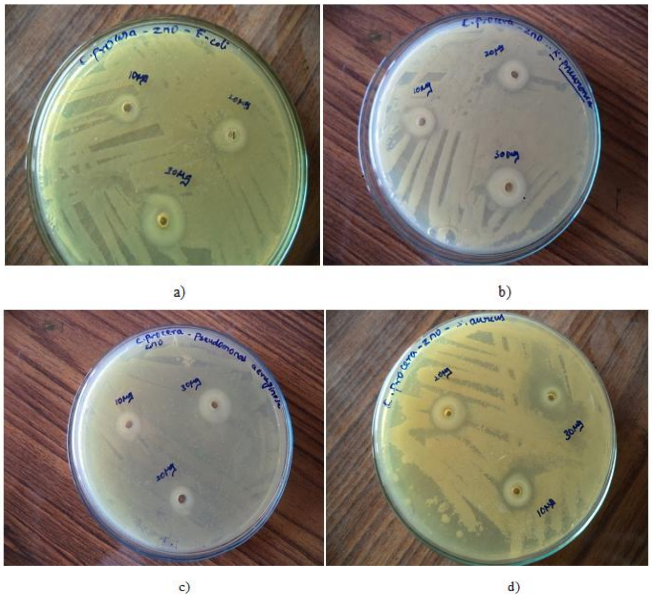

Fig. 5: Antibacterial activity of ZnO NP's against a) *E. coli* ,b) *K. pneumoniea*, c) *P. aeruginosa*, d) *S. aureus*

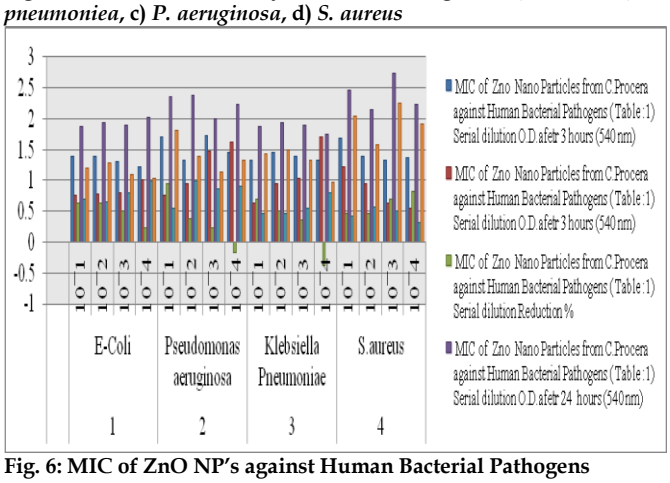

Fig. 6: MIC of ZnO NP's against Human Bacterial Pathogens

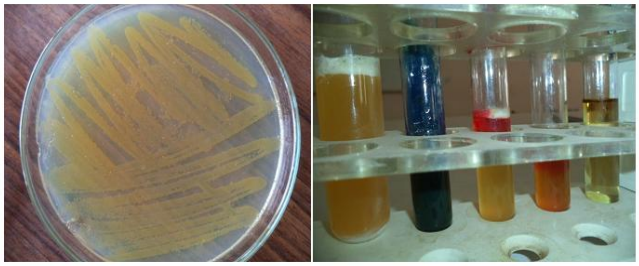

Fig. 7: *Xanthomonas axonopodis* and Biochemical tests

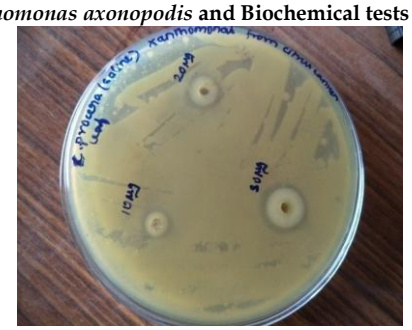

Fig. 8: Antibacterial activity of ZnO NP's against *Xanthomonas axonopodis*

Fig. 9: Antifungal activities of ZnO NP's against a) *Aspergillus* sps b) *Penicillium* sps

ZnO NPs have showed highest zone of inhibitions (16nm) and (18nm) respectively at 30µg/ml against *Xanthomonas axonopodis* (Fig. 8). The zone of inhibition for plant pathogenic strains was decreased at the increased concentration of ZnO nanoparticles and the maximum inhibition of growth was obtained at 30µg. However, studies related with the metal oxide nanoparticles against *Xanthomonas axonopodis* pathogens are too limited**.** Hence, the present study has been made an attempt to find out the novel antibacterial agents from metal oxide nanoparticles for the disease free agricultural management systems.

Regarding to the impact of nano-materials as plant pathogenic antibacterial agent, recent research has been studied nanosilver utilization of bio-control of plant pathogenic bacteria. They investigated the effects of three different kinds of liquid nano-silver against different plant pathogenic bacteria (*Clavibacter, Erwinia, Pseudomonas, Ralstonia, and Xanthomonas* genera) and they revealed that nano-silver could be used to control the plant pathogenic bacteria.

Antifungal assay

The fungal samples were grown in Sabouraud dextrose agar. The pure culture was maintained on PDA broth media at 25±1°C. Identification of the fungal isolates was carried out by morphological and microscopic examination the fungal samples are identified as

Aspergillus sps. and *Pencillium sps.* The zone of inhibition of related ZnO NP's at various concentrations (100µl, 200µl, and 300µl) against fungi were represented in Table. 6**.** It was clear from the results, that different concentration of ZnO nanoparticles caused significant inhibition in the spore germination. The highest inhibition was observed at highest concentration of nanoparticles followed by lower concentration of nanoparticles repetitively (Fig. 9).

Chitra *et al*., 2013 reported effect of ZnO nanoparticles against the fungus *Aspergillus niger.* Herein, the maximum inhibition of fungal growth was achieved at 400µl exhibits the increased concentration of ZnO nanoparticles resulting in the decreased growth rate of *Aspergillus niger*, whereas in *Penicillium expansum* (ZnO nanoparticles treated cells) the release of protein, carbohydrates and lipids through the damaged cell membrane results in the decreased amount of proteins, carbohydrates and lipids in fungal cells leads to death of the cells. [14-16]

The present study indicates that the *C. procera* ZnO nanoparticles had strong antimicrobial activity against the tested human and plant bacterial pathogens along with the fungal pathogens.

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