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RESEARCH ARTICLE

Green synthesis Zinc Oxide nanoparticle using Allamanda cathartica leaf extract and their cytotoxic and antibacterial activity

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ABSTRACT

The objective of this study is to investigate the in vitro antibacterial and cytotoxicity activities of green synthesized zinc oxide (ZnO) nanoparticles using aqueous leaf extract of *Allamanda cathartica* (L). Zinc nitrate serves as a precursor while the aqueous leaf extract as chemical reducing agent. Green synthesized ZnO nanoparticles are confirmed by an analysis of the powder X-ray diffraction. The FTIR analysis indicates the existence of various functional groups in both the leaf extract and the Zno nanoparticles. The wavelength of the UV absorption was measured to a maximum of 360 nm. ZnO nanoparticles are measured for its crystalline form, shape and surface morphology using Field Emission Scanning Electron Microscopy (FE-SEM). The EDAX spectrum confirms that the ZnO nanoparticles contains zinc and oxygen. Green synthesized ZnO nanoparticles demonstrated significant antibacterial activity against clinical bacillus sp. The anticancer acvitiy of ZnO nanoparticles were studied against human breast cancer MCF7 cell and the proliferation of MCF7 cell was substantially reduced when compared with the control cell viability.

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INTRODUCTION

Antimicrobial resistance (AMR) challenges the successful prevention and treatment of an growing array of infections caused by bacteria, parasites, viruses and fungi. Antimicrobial resistance (AMR) develops when antimicrobial drugs such as antibiotics, antifungals, antivirals, antimalarials, and anthelmintics are exposed to microorganisms such as bacteria, fungi, viruses, and parasites. Microorganisms which develop antimicrobial resistance are sometimes called as "superbugs" [1]. Health expenses in USA have increased annually to the tune of 1.2 trillion USD anticipating an increase of antimicrobial resistance by 2050 [2]. Several people believe that AMR is primarily due to overconsumption of antibiotics but in reality, environmental conditions may cause AMR to spread more than antimicrobial usage. This happens, particularly in developing countries with limited access to clean water, unsanitary conditions and rudimentary waste management. These environmental conditions expose people to repeated and preventable contact with fecal matter, which can host millions of resistant genes and bacteria, including potentially incurable superbugs. Water bodies contaminated with resistant genes, bacteria and antibacterial agents from animals and

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human feces and antimicrobials are also the ideal cause for the emergence and spread of superbugs [3]. In recent decades metal oxide nanoparticles have evolved with a wide variety of biomedical applications [4,5]. Recent developments in green synthesis and bio-manufacturing of metal oxide nanoparticles have stimulated intensive work in the detection of newer applications of zinc oxide nanoparticles in the nanomedicine field. Nanoparticles of metal oxide such as zinc oxide and titanium oxide are now being used in many dermatological skin care products for sun screening effects [6].

The ZnO nanoparticles have recently been well researched and used as a possible antimicrobial concept. In recent years, oxide nanoparticles such as ZnO have gained a lot of attention because of its stability under different environmental conditions [7]. ZnO nanoparticles demonstrated antibacterial activity against Gram-positive, Gram-negative bacteria and even antibacterial activity against spores [8, 9]. ZnO nanoparticles are thought to be merely toxic, bio-safe, and biocompatible. The mechanisms of antibacterial activity of ZnO particles are not well known although some claims have been suggested that the production of hydrogen peroxide may be the key factor of antibacterial activity or the binding of ZnO nanoparticles to the bacterial cell surface due to electrostatic forces. The current work is an attempt to synthesize and validate its antimicrobial and cytotoxic activity with ZnO nanoparticles.

MATERIAL AND METHODS

Materials

All high purity chemical substances were obtained from Merck (Mumbai). The chemical mainly used in this study are Dulbecco's Modified Eagle's Medium (DMEM), PBS (Phosphate Buffered Saline), Penicillin-G, Streptomycin, L-glutamine, Ethidium bromide, Acridine orange and DMSO (Dimethyl sulfoxide). All these solutions used in the study were prepared under aseptic conditions, using double distilled water. Zn nano particles were elucidated by UV-Visible spectroscopy (Biospec-nano-230 V); Perkin Elmer (FTIR-00585); FE-SEM (TESCAN MIRA3 LMH Schottky FE-SEM (Japan); XRD (XPERT-PRO).

Plant collection

The *Allamanda cathartica* was collected from the environment at Vellapar, Palakkad, and Kerala, India, in April 2020 (10.69451447"N 76.58760309"E). Plant was authenticated by Prof. Dr. Jayaraman, Research Center for Plant Anatomy Director Institute of Herbal Botany, Chennai.

Preparation of plant extract

Plant leaves were washed with distilled water numerous times to remove any dust or particulate matter. The washed leaves were then dried at room temperature. Leaves were finely grounded into fine powder using the pulverizer [10, 11]. A cold maceration process was used to prepare the aqueous leaf extract. About 100 g of Allamanda cathartica Linn. leaf powder was soaked in one liter of distilled water and kept for 24 hours in a shaker at 30 ° C (100 rpm) under continuous stirring for thorough mixing. Then, the extract was purified and processed for further analysis at −4 ° C.

Phytochemical screening

Preliminary phytochemical screening of the leaf extract was performed to categorize active components present, using usual methods [12].

Green synthesis of zinc oxide nanoparticle

Green synthesis of zinc oxide nanoparticles was performed using the method described by Yasser A. Selim *et al*. About 50 ml of the plant extract was heated on a magnetic stirrer at 60-80 °C. When the extract temperature reaches 60 °C, 5 g hexahydrate of zinc nitrate (Zn (NO3)2.6H2O) was added and left for around 60 minutes until a white precipitate is developed. At a temperature of 60 °C, the mixture was left overnight in a hot air oven until a creamy paste was formed. This paste was further washed several times with a solution of distilled water: ethanol in the 3:1 ratio. Afterwards the paste was taken to a copper crucible cup and heated for 120 minutes in a furnace at 400 °C. The resulting white powder was then taken in a closed container for characterization.

Characterization of zinc oxide nanoparticle

Zinc oxide nano particles were examined by UV-Vis spectroscopic analysis in the range of 200-800 nm. FTIR was used to determine the structural features and particular phytochemical components involved in the reduction and stabilization of synthesized nano particles. Results in the range of 4000–400 cm−1 were estimated. In order to confirm the existence of ZnO and to analyze the crystalline structure and thickness, the sample in the form of powder was subjected to CuKα1-X Ray

diffractometer radiation at 40 kV and 30 mA with 2θ varying from 30 ° –140 °C. ZnO nanoparticles were suspended in the solution of ethanol and then coated on a gold grid which is then allowed to dry and subjected to analysis by the TESCAN MIRA3 LMH Schottky FE-SEM (Japan).

Antibacterial activity

Antibacterial behavior of ZnO nanoparticles has been checked with well plate and serial dilution methods against pathogenic bacteria [13, 14].

Bacterial strains (Clinical strains), Culture media

ZnO nanoparticles have been tested for antimicrobial properties against clinical *Bacillus* sp, *Staphylococcus aureus*, and *Enterococcus*. The microbial cultures were obtained from The Government Medical College, Tiruchirappalli, and Tamil Nadu. Muller-Hinton agar media was acquired from Himedia Pvt Bombay, India for microbial testing. The antibacterial activity was tested using a reader of the Himedia zone [15, 16].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) for zinc oxide nanoparticles

The micro-dilution processes were used to assess the antibacterial ability of the ZnO nanoparticle with the respective controls. A spectrophotometer (OD595=0.22) equal to 10^8 CFU / mL was used to align bacterial cultures to 0.22 optical density at 595 nm. Different concentrations of ZnO nanoparticles (200, 100, 50, 25, 12.5, 6.25 mg/ml) and standard drug (100, 50, 25, 12.5, 6.25, 3.125 mg/ml) were added in 2000 µL MIC tubes. 10^8 CFU/ml 100µL of the tested were added into each MIC test tube. MIC tubes were incubated overnight at 37 °C for 24hr.

Analysis of antimicrobial activity of ZnO nanoparticles using an electronic microscope

Bacterial culture of the MIC and MBC samples were centrifuged and collected in a test tube. Bacterial cells coated with non-reactive metal gold were observed under a scanning electron microscope (FESEM).

Cytotoxic activity

MCF7 Human breast cancer cells were obtained from the Cell Repository of the NCCS (National Center for Cell Sciences), Pune, and India. DMEM was used to preserve the cell line, which was complemented by 10% (FBS) Fetal Bovine Serum. Streptomycin (100 µg/ml) and Penicillin (100 U/ml) were applied to the medium to avoid bacterial contamination. The human cell medium was maintained in a humidified atmosphere of 5 percent CO2 at 37 ° C [17, 18].

Cell culture and MTT assay

The ZnO nanoparticles have been tested for cytotoxicity. The colorimetric assay for MTT (3-(4, 5- dimethylthiazol-2-yl – 2, 5-diphenyl tetrazolium bromide) was performed on a 96-well plate. Under sterile conditions, the whole procedure was carried out using a laminar air flow cabinet. MCF7 Human breast cancer cells were seeded in a 96-well at 5×10^4 cells / well and incubated overnight. After treatment with of ZnO nanoparticles for 48 hrs, the cells were removed, washed with cold PBS, and then treated at room temperature for 5 mins with a mixture of AO (100µg ml−1) and EB (100µg ml−1) in the ratio of 1:1. A 40x magnifications fluorescence microscope was used to observe the stained cells. The cells were extracted at the end of therapy and were washed with PBS three times. The cells were stained with acridine orange / ethidium bromide (AO / EB 1:1 ratio; 100 µg/ml) for 5 minutes separately and then the characters were studied at 40 x magnifications under a fluorescent microscope.

Statistical analysis

These values were expressed as mean ± SD. The results on cytotoxicity made statistical comparisons using a one-way variance analysis (ANOVA) followed by Duncan's Multiple Range Test (DMRT), using SPSS version 12.0 for windows (SPSS Inc. Chicago; http:/www.spss.com). If the p-value was less than 0.05 the values were deemed statistically important.

RESULTS

Phytochemical screening

All the results of the phytochemical investigations are shown in the Table 1. In the current investigation, the aqueous crude extract gave promising results for steroids, terpenoids which was established by Salkowski and Liebermann-Burchard's test. The presence of terpenoids, phenols and flavonoids were also confirmed in *Allamanda cathartica* aqueous extract.

Characterization of ZnO nanoparticle

During the synthesis, the change in solution color and the development of a yellowish-white precipitate was the indication of a reduction in zinc nitrate.

Table 1: Phytochemical constituents of *Allamanda cathartica* aqueous leaf extract.

S.No	Phyto-constituents	Chemical Test	Inference
1	Glycosides	Keller-killiani	+
2	Alkaloids	Dragendorff's reagent	+
3	Tannins	Ferric chloride	+
4	Saponins	Forthing	+
5	Flavonoids	Lead ethanoate	+
6	Steroids	Salkowski test	+
7	Terpenoids	Liebermann-Buchard's	+
8	Flavonols	Shinoda	+
9	Anthraquinones	Borntrager's	-
10	Carbohydrates	Molish's	+

(+) present, (-) absent

UV - Vis spectroscopic analysis

The development of ZnO nanoparticles within the 200–800 nm range was initially confirmed by UV spectroscopy. The absorption spectrum of green synthesized nanoparticles with zinc oxide shows a characteristic peak of 374 nm (Supplementary files)[19].

FTIR spectroscopic analysis

The FTIR analysis has been used as a confirmatory analysis for nano particles formation. This research provides an understanding of current molecules' vibrational and rotational modes, thus helping to classify the functional and potential phytochemical molecules involved in the reduction and stabilization of zinc oxide nanoparticles (Supplementary files).

XRD Analysis

XRD analysis of synthesized ZnO nanoparticles clearly indicates the crystalline structure of the synthesized nanoparticles (Supplementary files). Diffraction peaks have been observed at 2θ values 31.5, 34.5, 38.5, 42.5, 48.5 degrees.

FE-SEM analysis:

FE-SEM analysis was used to identify with the ZnO nanoparticles structure and size. The SEM analysis was carried out using TESCAN MIRA3 LMH Schottky FE-SEM (Japan) model. Microscopy of ZnO nanoparticles have shown that they have particle size of nano range (500nm), which is spherical and homogeneous in distribution. Zinc oxide nanoparticles looks like spherical and bullet shape under FE-SEM Microscope (Fig. 1).

EDAX analysis:

The elemental analysis of the ZnO nanoparticle from the EDAX spectrum of the FE-SEM image was shown image supplementary files. The EDAX spectrum conform that zinc and oxygen were present in the nanoparticle. The percentage of molecular weight and atomic value of zinc and oxygen were observed to be 35.56, 34.77 and 1.4, 0.34 respectively.

Antibacterial activity

Study of antibacterial activity of ZnO nanoparticle by well diffusion (Kirby-Bauer) method

The antibacterial activity study of ZnO nanoparticles was quantitatively evaluated by the well-diffusion (Kirby-Bauer) method against the bacteria *Bacillus* sp, *Staphylococcus aureus,* and *Enterococcus*. Diameter of the zone inhibition was shown in Table 2.

Determination of MIC and MBC by serial dilution method.

The efficacy of the ZnO nanoparticles on the *bacillus* species were tested by calculating the MIC and MBC as shown in Table 2. MIC and MBC values were obtained from the ZnO nanoparticles of 1.250mg/mL, 0.625 mg/mL against *bacillus sp.* strain (Table 3).

Examination of antimicrobial activity of Zinc oxide nanoparticles *using Electron Microscope*

Antibacterial activity of ZnO nanoparticles were studied 1using serial dilution method against clinical *bacillus* sp. MIC and MBC bacterial samples were observed under the electronic microscope (Fig.2). Electron microscope scans showing

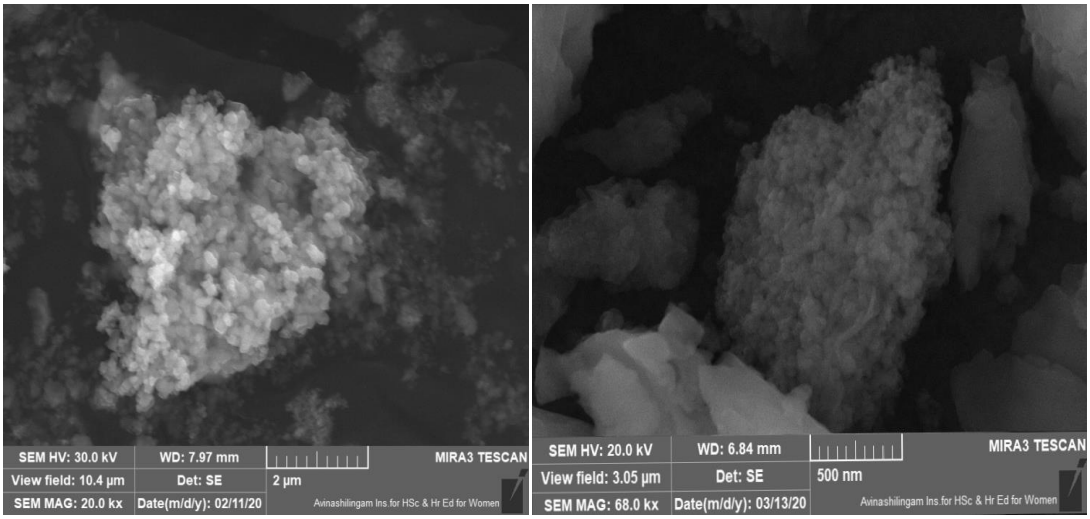


Fig. 1: FE-SEM images of Zinc Oxide nanoparticles

Table 2: Antibacterial activity of ZnO nanoparticles and standard drug against clinical *Bacillus sp*, *Staphylococcus aureus,* and *Enterococcus*

S.NO	Formulation/ Standard drug	Zone of Inhibition (mm)		
		Bacillus sp (n=2)	*Staphylococcus aureus* (n=2)	*Enterococcus* (n=2)
1	Amikacin 100µl (100µg)	24±5mm	14±5mm	16±5mm
2	zinc oxide nanoparticles 100µl (1000µg)	16±5mm	-	11±5mm

(-) Absent of Zone of Inhibition

Table 3**:** Target MICs µg/mL for pathogenic microorganism

S.NO	Formulation/ Standard drug	MIC (Minimum Inhibitory concentration)	MBC (Minimum bacterial concentration)
		(n=2)	
1	Amikacin (1000-3.90625)	31.25	15.625
2	zinc oxide nanoparticles (5000-78.125µg)	625	312.5

Note: ZnO nanoparticles showing significant activity against human pathogenic *bacillus* sp.

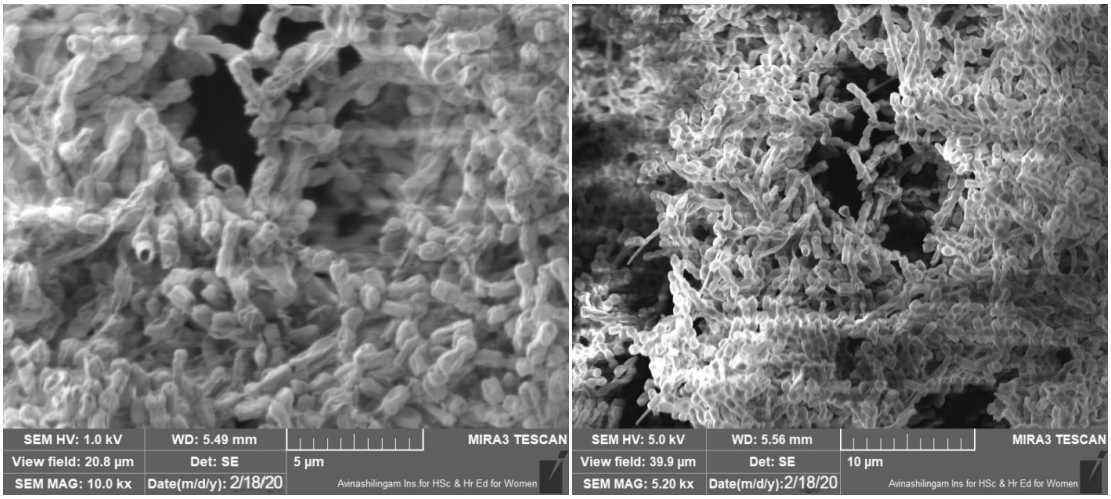


Fig. 2: (a) Electron microscope scans showing morphological changes at MIC concentration bacterial cells. (b) Healthy cells present at MBC concentration bacterial cells.

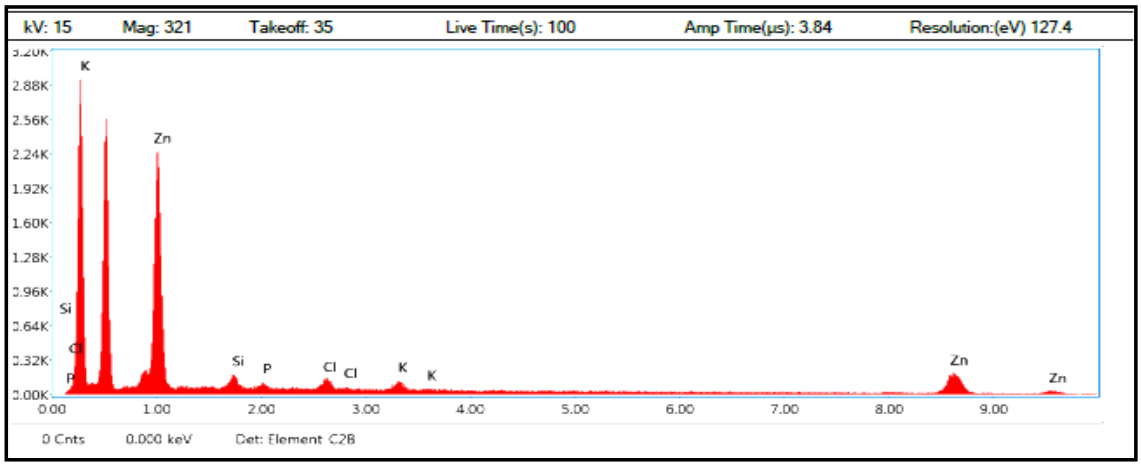


Fig. 3: EDAX analysis of Zinc Oxide nanoparticles treated bacterial cells.

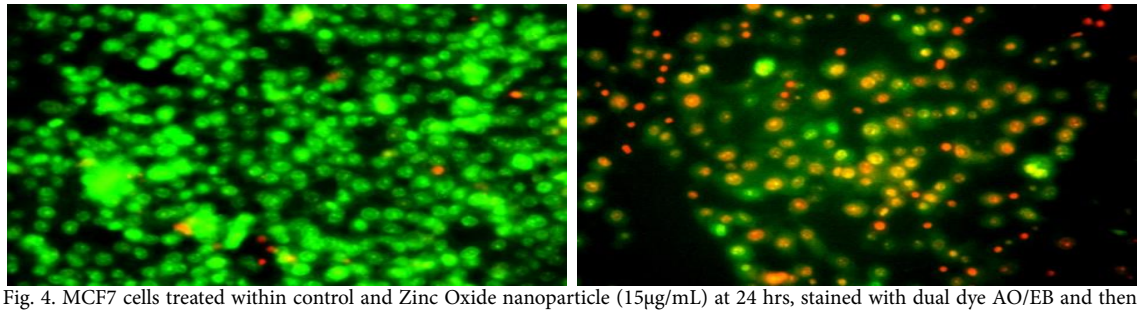


Fig. 4. MCF7 cells treated within control and Zinc Oxide nanoparticle (15µg/mL) at 24 hrs, stained with dual dye AO/EB and then analyzed by fluorescence microscopy. Living cells normal green nucleus appeared; early apoptotic condensed or fragmented form of yellow colour nucleus with chromatin, late apoptotic chromatin condensation or fragmentation orange-stained nuclei and necrotic cells (uniformly red-stained cell nuclei)

morphological changes at MIC concentration and Healthy cells present at MBC concentration.

EDAX analysis

ZnO nanoparticles treated with microbial culture were subjected to elemental analysis by using EDAX of the FE-SEM. The image was shown in Fig. 8. EDAX spectrum confirms that the bacterial culture was having zinc element (Fig. 3).

Cytotoxic activity (MTT assay)

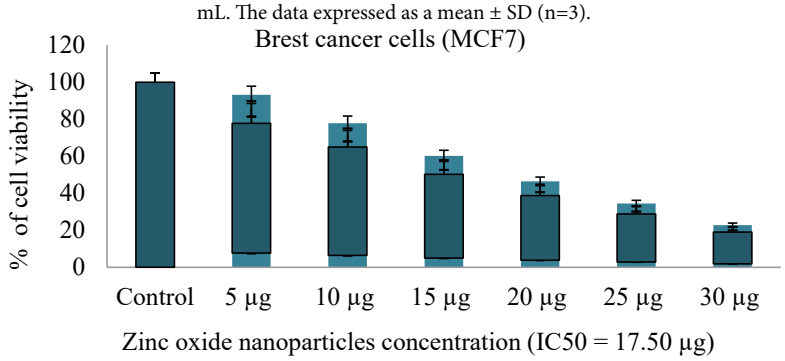
Cytotoxic activity was evaluated for *Allamanda cathartica* mediated Zinc oxide nanoparticle against MCF7 Human breast cancer cells with the different concentration range from 5µg/mL, 10µg/mL, 15µg/ml, 20µg/mL, 25µg/ml and 30µg/ml. After 48hrs the cell viability analysis was determined. Fig. 9 shows the altered morphology of MCF7 cells after dose dependent treatment with ZnO nanoparticle. ZnO nanoparticles (17.50µg) were significantly reduced the proliferation of MCF7 cell comparison with the control cell viability (Fig. 4 and 5).

DISCUSSION

In the present analysis, ZnO nanoparticles were bio-synthesized from *Allamanda cathartica* leaves. Microscopical characterizations of ZnO nanoparticles have shown that they have particle size in the nano range; they are spherical and homogeneous in distribution. The size, shape and arrangement of the ZnO nanoparticles were observed under FE-SEM. ZnO particles grow slowly, form small spherical structures and accumulate like bullets. This agglomeration is due to the polarity and electrostatic attraction of nanoparticle zinc oxide.

The ZnO nanoparticles showed antibacterial activity against clinical pathogenic bacillus sp. The values of MIC and MBC obtained from nanoparticles of ZnO were 1.250 mg / mL and 0.625

Fig. 5. Percentage of MCF cell viability of control and Zinc Oxide nanoparticles by MTT assay at different concentrations from 5-30 µg/mL. The data expressed as a mean ± SD (n=3).



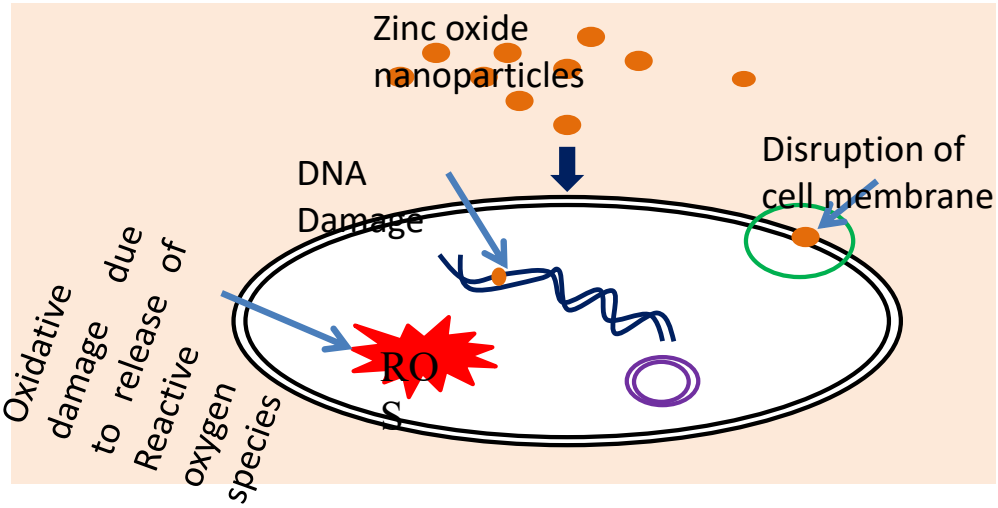


Fig. 6. Various mechanism actions of Zinc Oxide nanoparticles against bacteria

mg / mL for bacillus species strain. The increased antibacterial activity was attributed to a high surface to volume ratio [20, 21]. A MIC and MBC concentration bacterial cell was observed under the electronic microscope (FE-SEM). Bacterial cell morphological changes were observed in MIC concentration (Fig.2a) and healthy bacterial cells were observed at MBC concentration (Fig. 2b). EDAX analysis conformed that Zinc was present in the bacterial cells. ZnO nanoparticles effectively inhibit the growth of clinical *bacillus* species which may be due to cell wall damage, oxidative damage (release of reactive oxygen species) and DNA cleavage (Fig.6). The cytotoxic activity conducted against human breast cancer MCF7 cells showed significant cytotoxic effect. Additional investigations were required to confirm these *in vitro* assays and to characterize the molecular mechanism causing biological activity. Our present findings showed a significant *in vitro* antibacterial and anticancer activity against clinical pathogenic bacillus species and MCF7 breast cancer cells.

CONCLUSION

In this study, ZnO nanoparticles were prepared by *Allamanda cathartica* leaves aqueous extract using Zinc nitrate. Various methods have been used for identification of ZnO nanoparticles using UV-Visible spectroscopy (Biospec-nano-230 V); Perkin Elmer (FTIR-00585); FE-SEM (TESCAN MIRA3 LMH Schottky FE-SEM (Japan); XRD (XPERT-PRO). FESEM confirmed the presence of ZnO nanoparticles. ZnO nanoparticles showed attractive antibacterial activity and cytotoxic activity against clinical bacillus sp and human breast cancer cells MCF7. Further, the findings of the present study

need to be substantiated by testing these products for *in vivo* analysis.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Nil

ABBREVIATIONS

DMEM: Dulbecco's Modified Eagle's Medium
PBS: Phosphate Buffered Saline
DMSO: Dimethyl Sulfoxide
MIC: Minimum Inhibitory Concentration
MBC: Minimum Bacterial Concentration
NCCS: National Center for Cell Sciences
MTT: 3-(4, 5- dimethylthiazol-2-yl– 2, 5-diphenyl tetrazolium bromide
AO: Acridine Orange
XRD: Fourier-transform Infrared Spectroscopy
FE-SEM: Field Emission Scanning Electron Microscopy
EDAX**:** Energy Dispersive X-Ray Analysis

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