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 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 activity 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.
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
dифрактометром с излучением 40 кВ и 30 мА с углами 30 ° – 140 °. Наночастицы ZnO были поднесущими в раствор этилового спирта и затем нанесены на золотую подложку, которая затем высушена и подвергнута анализу с помощью сканирующего электронного микроскопа TESCAN MIRA3 LMH Schottky FE-SEM (Япония).

**Antibacterial activity**

Антибактериальное поведение наночастиц ZnO было проверено с помощью теста на культуральной пластине и последовательного разведения против патогенных бактерий [13, 14].

**Bacterial strains (Clinical strains), Culture media**

Наночастицы ZnO были протестированы на антибактериальные свойства против клинических бактерий Bacillus sp, Staphylococcus aureus, и Enterococcus. Микробные культуры были получены из Госпиталя медицинского колледжа, Тиручираппули, и Тамил Наду. Муллер-Ниптон агаровая среда была приобретена у Himedia Pvt Bombay, Индия для микробного тестирования. Антибактериальная активность была исследована с помощью читателя Himedia зона [15, 16].

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

Микропроба была использована для оценки антибактериальной активности наночастиц ZnO с контролем. Адаптиционный спектрофотометр (OD595=0.22) равен 10⁶ CFU / mL был использован для альпинизации бактериальных культур к 0,22 оптической плотности при 595 нм. Разные концентрации ZnO наночастиц (200, 100, 50, 25, 12,5, 6,25 и 3,125 мг/мл) и стандартный антибактериальный препарат (100, 50, 25, 12,5, 6,25, 3,125 мг/мл) были добавлены в микробные тюбик. 10⁶ CFU/ml 100µL из теста были добавлены в микробный тюбик. MIC тюбик был инкубирован на 37 °C на 24 ч.

**Analysis of antimicrobial activity of ZnO nanoparticles using an electronic microscope**

Микробные культуры микробного и MBC образца были центрифугированы и собраны в пробирку. Бактериальные клетки, покрытые нереактивным металлом, были исследованы под сканирующим электронным микроскопом (FESEM).

**Cytotoxic activity**

MCF7 человеческие раковые клетки были получены из библиотеки NCFS (National Center for Cell Sciences), Пуне, Индия. DMEM была использована для сохранения клеточного полевого, которое было сбалансировано на 10% (FBS) Fetal Bovine Serum. Streptomycin (100 µg/ml) и Penicillin (100 U/ml) были добавлены к среде для предотвращения бактериальной контаминации. Человеческая клеточная культура была поддерживалась в влажной атмосфере с 5% CO2 при температуре 37 °C [17, 18].

**Cell culture and MTT assay**

Значения были выражены как среднее ± SD. Результаты тестирования цитотоксичности были сопоставлены с помощью одноочного анализа (ANOVA) [19, 20] методом Duncan’s Multiple Range Test (DMRT), используя версию 12.0 SPSS для Windows (SPSS Inc. Chicago; http://www.spss.com). Если p-значение было меньше 0.05, эти значения были признаны статистически значимыми.

**RESULTS**

**Phytochemical screening**

Все результаты фитохимических исследований представлены в Таблице 1. В настоящее время, исследование, водах дикой экстракт давал обещающие результаты для стероидов, терпеноидов, которые были установлены с помощью Salkowski и Liebermann-Burchard’s тест. Присутствие терпеноидов, фенолов и флавоноидов также было подтверждено в Allamanda cathartica водном экстракте.

**Characterization of ZnO nanoparticle**

В процессе синтеза, изменение цвета раствора и развитие желтого осадка наночастиц было признаком снижения зинк оксида.
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 confirm 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

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

<table>
<thead>
<tr>
<th>S.No</th>
<th>Phyto-constituents</th>
<th>Keller Killiani</th>
<th>Forthing</th>
<th>Lieberman-Burchard’s</th>
<th>Shinoda</th>
<th>Borntrager's</th>
<th>Molish's</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycosides</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Alkaloids</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tannins</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Saponins</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Flavonoids</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Steroids</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Terpenoids</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Flavonols</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Anthraquinones</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Carbohydrates</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(+) present, (-) absent
Table 2: Antibacterial activity of ZnO nanoparticles and standard drug against clinical Bacillus sp, Staphylococcus aureus, and Enterococcus

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Formulation/ Standard drug</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacillus (n=2)</td>
</tr>
<tr>
<td>1</td>
<td>Amikacin 100µl (100µg)</td>
<td>24±5mm</td>
</tr>
<tr>
<td>2</td>
<td>Zinc oxide nanoparticles 100µl (1000µg)</td>
<td>16±5mm</td>
</tr>
</tbody>
</table>

(*) Absent of Zone of Inhibition

Table 3: Target MICs µg/mL for pathogenic microorganism

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Formulation/ Standard drug</th>
<th>MIC (Minimum Inhibitory concentration) (n=2)</th>
<th>MBC (Minimum bacterial concentration) (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amikacin (1000-3,906/25)</td>
<td>31.25</td>
<td>15.625</td>
</tr>
<tr>
<td>2</td>
<td>Zinc oxide nanoparticles (5000-78,125µg)</td>
<td>625</td>
<td>312.5</td>
</tr>
</tbody>
</table>

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

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

ACKNOWLEDGEMENT

The authors would like to thank Anna University, BIT Campus, Tiruchirappalli, Center for Biotechnology and Phyto Pharmacognosy Research, Coimbatore and Sanjo College of Pharmaceutical Studies for providing the requisite facilities.

CONFICT OF INTEREST

Authors declare no conflict of interest.

FUNDING SUPPORT

Nil

ABBREVIATIONS

DMEM: Dulbecco's Modified Eagle's Medium
PBS: Phosphate Buffered Saline
DMSO: Dimethyl Sulfoxide
MIC: Minimum Inhibitory Concentration
MBC: Minimum Bacterial Concentration
NCSS: 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

REFERENCES