# Design, Synthesis and Cytotoxic Evaluation Of Green Synthesized Metal Oxide Nanoparticles

Brita John C[1], Dr. P. Dhivya[2]
*Department of chemistry, Nirmala College for Women, Coimbatore*
[1]britajohn1301@gmail.com , [2]dhivsanto@gmail.com

***Abstract*** **- Nanoparticles synthesized from plants has a great impact on mankind in the field of medicine. Nanotechnology correlates nanoscience and its development in other fields. The combination of phytochemistry and nanomaterials has a low-cost and cost-effective approach than the traditional synthetic methodology. Zinc oxide, due to physical and chemical properties, is considered a proficient drug in medicinal field. An innovative approach of biosynthesized zinc oxide nanoparticles produced from natural source, such as plant extracts to reduce metal ions, which are readily scalable and non-toxic compared with physical and chemical methods is on trend. This work discusses about the zinc oxide nanoparticles which are synthesized organically from the *Adhatoda vasica* chloroform plant extract. The zinc nanoparticles are characterized by Scanning Electron Microscope (SEM), UV analysis, XRD analysis and their anti-oxidant activity were evaluated using DPPH assay. Thus the competence of the zinc oxide nanoparticles against oxidative problems is plausibly studied. The zinc oxide nanoparticles with varying concentrations of chloroform plant extract was analysed for its cytotoxic activity against MCF-7 and A549 cell lines.**

***Keywords*****: *Adhatoda vasica, Zinc oxide, nanoparticles, SEM, UV, XRD, cytotoxic activity,***

## I. INTRODUCTION

Nature is the greatest chemist and many attributes that remain undiscovered in plants are beyond the imagination of our best scientist.[1] As the world's population increases, the rate and variety of diseases are also increasing. From the ancient period, human beings are trying to control the growth of disease by new medicines and methods. But three fourth of the population cannot afford the recent advancement in the medical field. Therefore they have to rely upon the traditional medicines, which are derived from plants.

### A. *Adhatoda Vasica*

*Adhatoda vasica* is a medicinal plant which has been used in Ayurveda for the treatment of various ailments of respiratory tracts in both children and adults.[2] All the parts of the plant have been used for the therapeutic beneficiary effects from ancient times. It is used for the treatment of many diseases such as asthma, cold, cough, tuberculosis, bleeding piles, chronic bronchitis, rheumatism, malarial fever, gastro-intestinal disorders, hemorrhage, skin Diseases[3] etc. It acts as antispasmodic, expectorant, antithelmintic, anti-oxidant etc. *Adhatoda vasica* is an evergreen, gregarious, perennial shrub of nearly 1-6 m height with opposite ascending branches. The leaves are symbol, opposite, 7-19 cm long and 4-7 cm wide. Stem with yellowish bark. The plant grows throughout India and also found in Myanmar, Srilanka, Malaysia etc.[4]

### B. *Zinc Oxide Nanoparticles*

Zinc nanoparticles are more efficient than Zinc macro particles because, as the surface area increases the efficiency of the zinc particles also increases. As the size of the zinc particles decreases the surface are of the zinc particles get increased. And also less amount of zinc particles are required for the treatment. Globally, bacterial infections are recognized as serious health issue. New bacterial mutation, antibiotic resistance, outbreaks of pathogenic strains, etc. are increasing, and thus, development of more efficient antibacterial agents is demand of the time. Zinc oxide is known for its antibacterial properties from the time immemorial. It had been in use during the regime of Pharaohs, and historical records show that zinc oxide was used in many ointments for the treatment of injuries and boils even in 2000 BC. It is still used in sun screen lotion, as a supplement, photoconductive material, LED, transparent transistors, solar cells, memory devices, cosmetics, and catalysis. However, zinc powder inhaled or ingested may produce a condition called zinc fever, which is followed by chill, fever, cough, etc. Ready to eat food is more prone to infection by *Salmonella*, *Staphylococcus aureus* and *E. coli* which pose a great challenge to food safety and quality. The antimicrobial compounds are incorporated in the packed food to prevent them from damage. Antimicrobial packaging contains a nontoxic material which inhibits or slows down the growth of microbes present in food or packaging material.[5]

C. *Cytotoxicity*

Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are an immune cell or some types of venom, e.g. from the puff adder (*Bitis arietans*) or brown recluse spider. Treating cells with the cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis).Cells undergoing necrosis typically exhibit rapid swelling, lose membrane integrity, shut down metabolism and release their contents into the environment. Cells that undergo rapid necrosis in vitro do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers. Apoptosis is characterized by well defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments. Cells in culture that are undergoing apoptosis eventually undergo secondary necrosis. They will shut down metabolism, lose membrane integrity and lyse. Normally plants show a great value in cytotoxic studies. Thus the present work deals with the analysis of the cytotoxic activity of the Zinc oxide nanoparticles which where biologically synthesized by the plant *Adhatoda Vasica.*[6]

## II. Materials and Methods

A. *Plant collection and identification*

The plant used in this study is *Adhatoda vasica. Adhatoda vasica* is a medicinal plant mainly used for the treatment of respiratory ailments.[7] Aerial parts of *Adhatoda vasica* were collected in the month of April 2019 from Peramangalam, Thrissur, Kerala. The collected plant material was authenticated by Botanical Survey of India located in Tamil Nadu Agricultural University, Coimbatore.

**Fig 1.** Image of *Adhatoda vasica* leaf

B. *Preparation of Crude Sample*

The plant was initially washed gently under the tap water to remove dust and soil, and then rinsed with distilled water. The leaves of *Adhatoda vasica* were separated, air dried for two weeks and coarsely powdered using mechanical blender and transferred into air tight container.[8]

C. *Preparation of Plant Extract*

Crude plant extract was prepared by Soxhlet extraction. About 50g of the powdered plant material was extracted with chloroform, which was run for 15 hours. The extract was concentrated in rotatory evaporator, dried in over and stored in air tight bottles.[9]

D. *Qualitative Phytochemical Analysis*

The extract was screened for the presence of bioactive compounds by standard methods as per the reported procedure.[10]The chloroform extract of *Adhatoda vasica* was tested for various bioactive phytoconstituents like proteins, flavanoids, anthraquinones, tannins, phenols, glycosides, saponins etc.

E. *Biosynthesis of Zinc oxide nanoparticles*

The aqueous leaf extract of *Adhatoda vasica* was added to 0.025 M aqueous zinc sulphate and the pH was adjusted to 12. The resulted solution was pale white in colour. After stirring, the precipitate was washed against distilled water followed by ethanol to get rid of the impurities. The solution was vaccum dried and used for characterization of ZnO-NPs.[11]

F. *Characterization of Zinc oxide nanoparticles*

The pure ZnO nanoparticles were characterized by UV – Vis spectroscopic analysis, Scanning Electron Microscope analysis and X ray Diffraction Analysis.

G. *Biological Activity*

*1) Anti-oxidant Activity*

1,1-diphenyl 2-picryl hydrazyl radical was used for the analysis of the anti-oxidant studies. This test was carried out to test the potential of the different concentration of the plant extract with the fixed concentration of the zinc oxide nanoparticles and thereby investigating its anti – oxidant activity. The test was conducted at Department of Zoology in Bharathiar University, Coimbatore.[12]

*2) Antimicrobial Activity*

Various concentrations of the extract were initially screened for their antimicrobial activity against the Gram-positive bacteria, methicillin resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria, *Pseudomonas aeruginosa* (MTCC 201) and fluconazole resistant *Candida albicans* (FRCA). Well diffusion assay was carried out to determine the antimicrobial activity[13]. The minimal inhibitory concentration (MIC) of the various concentration of the extract was examined. The test was conducted at Department of Zoology in Bharathiar University, Coimbatore.[14]

H. *Cytotoxic Activity*

To evaluate the cytotoxicity of different concentration of plant extract with fixed concentration of zinc oxide nanoparticles, the MTT experiments were carried out. Confluent monolayers of MCF-7 and A-549 cells were grown in 96-well tissue culture plates. Cells were incubated with samples1-8. The ability of the cells to cleave the tetrazolium salt MTT by the mitochondrial enzyme succinate dehydrogenase which develops a formazan crystal. Each concentration was replicated three times.

*1) Fluorescence microscopic analysis of apoptosis*

Approximately 1 μL of a dye mixture (100 mg/mL acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr) in distilled water) was mixed with 9 mL of cell suspension (1×105 cells/mL) on clean microscope coverslips. The cancer cells were collected, washed with phosphate buffered saline (PBS) (pH 7.2), and stained with 1 mL of AO/EtBr. After incubation for 2 min, the cells were washed twice with PBS (5 min each) and visualized under a fluorescence microscope. MCF-7 cells and A-549 cells were treated with the above methods for 48 h and then fixed with methanol/acetic acid (3:1, v/v) prior to washing with PBS. The washed cells were stained with 1 mg/mL DAPI for 20 min in the dark. Stained images were recorded with a fluorescent microscope with the appropriate excitation filter[15].

## III. Results and Discussions

### A. *Preliminary Phytochemical Analysis*

The results of the preliminary phytochemical analysis of the chloroform extract of *Adhatoda* vasica is tabulated in **Table 1**.

**Table 1** Phytochemical screening of leaf extract of *Adhatoda vasica*

| Bioactive Components | Characteristic Tests | Leaf Extract of *Adhatoda vasica* |
|---|---|---|
| Protein and Amino acids | Xanthoproteic Tests | - |
| Reducing sugar | Molisch's Test | - |
| Flavanoids | Lead Acetate Test | + |
| Anthraquinone | Borntragers Test | - |
| Tannins | Lead Acetate Test | + |
| Phenols | Ferric Chloride Test | + |
| Cardiac Glycosides | Keller – Kilani Test | - |
| Saponins | Foam Test | + |
| Steroids | Salkowski Test | + |
| Alkaloids | Mayer's Test | + |

### B. *Biosynthesis of zinc oxide nanoparticles*

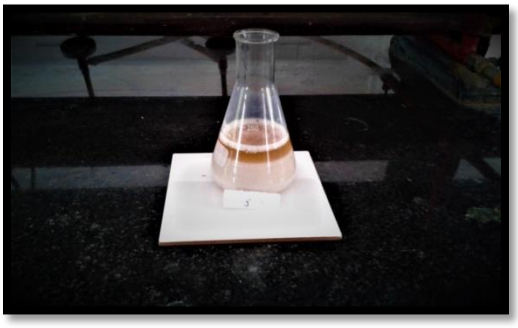


**Fig 2**. Image of zinc oxide nanoparticles

The *Adhatoda vasica* plant extract was used for the synthesis of zinc oxide nanoparticles as reducing agent. The resulted solution was chrome yellow in colour and the nanoparticle was pale white in colour.

### C. *Characterization of Zinc oxide nanoparticles*

#### *1) Evaluation of UV – Vis Spectroscopy Analysis*

The UV-Vis Spectrum of pure zinc oxide nanoparticles is shown **Fig 3.** The observation of the graph was in close agreement with the reported literature at 370nm.[16]

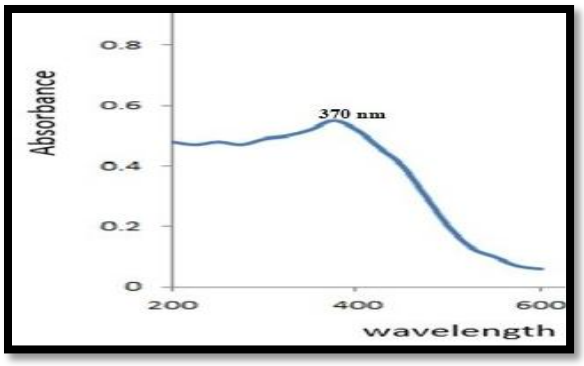
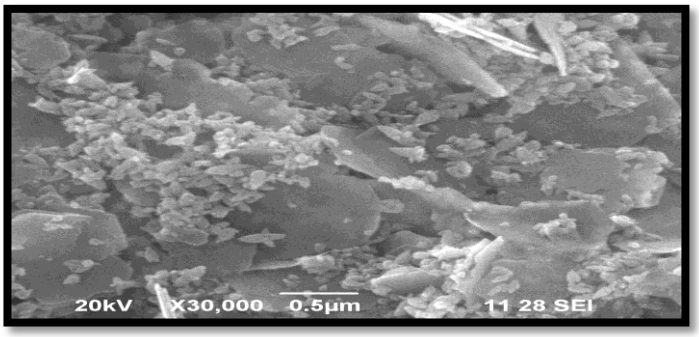



**Fig 3.** UV-Vis graph of zinc oxide nanoparticle

*2) Evaluation of Scanning Electron Microscope Analysis*

The surface analysis revealed the shape of the biosynthesized nanoparticles to be flower bundle shape as shown in **Fig**.4 & 5.



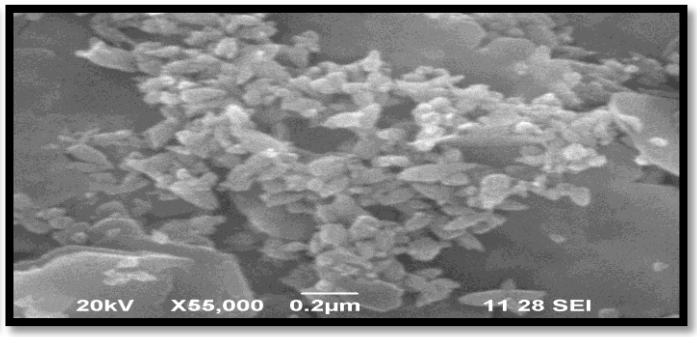




**Fig 4** & **Fig 5** SEM image of zinc oxide nanoparticle.

*3) Evaluation of XRD Analysis*

**Table 2** Crystallinity of the zinc oxide nanoparticles

| NANO MATERIAL | Total intensity of stronger peak (counts) ($I_c$) | Total intensity of Broader peak (counts)($I_a$) | % Crystallinity, $\%\chi=(I_c/(I_c+I_a)) \times 100$ |
|---|---|---|---|
| Zinc oxide nanoparticles | 4851.05 | 2489.02 | 66.08% |

From the analysis, the percentage of crystallinity of zinc oxide nanoparticles is 66%.

Particle size calculation by using Debye Scherrer's Equation

$$D = \frac{K\lambda}{\beta \cos\theta}$$

D = Size of the particle
K= Debye Scherrer's Constant = 0.94
$\lambda$ = Wavelength value = 0.15406

$\beta$ = Full Width Half Maximum for the diffracted peak = $5.253x10^{-3}$

$\theta$ = Bragg's angle for the peak = $1.82010^{-1}$

$$D = \frac{(0.94)(0.01547)}{(5.25310-3)\cos(18.20)} = 27.90nm$$

Therefore, the size of the particle of 27.90nm which confirms that the synthesized particle is nano.

The XRD graph of the zinc oxide nanoparticle is shown below in the **Fig 6.**

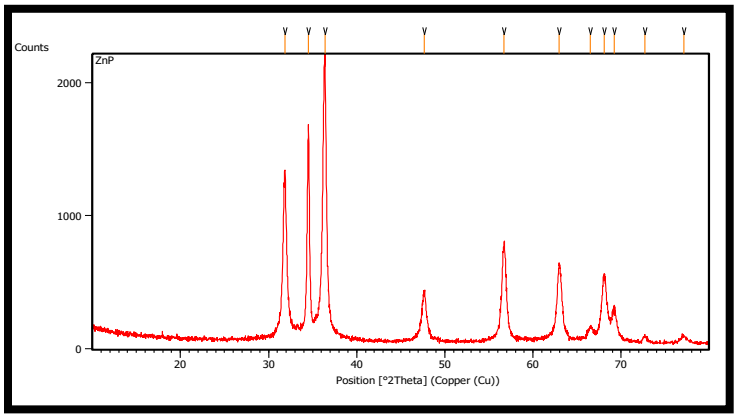



**Fig 6.** The XRD graph of Zinc oxide nanoparticle

D. *Biological activity*

*1) Evaluation of anti-oxidant activity*

The result of anti-oxidant activity is tabulated in the **Table 3.**

**Table 3** Antioxidant activity of *Adhatoda vasica* with DPPH assay.

| SAMPLE | % of inhibition DPPH radical |
|---|---|
| 0.1% of plant extract + zinc nanoparticles | $39.21 \pm 2.82$ |
| 0.25% of plant extract + zinc nanoparticles | $47.86 \pm 2.56$ |
| 0.5% of plant extract + zinc nanoparticles | $53.62 \pm 2.31$ |
| 0.75% of plant extract + zinc nanoparticles | $59.32 \pm 2.64$ |
| 1.0% of plant extract + zinc nanoparticles | $66.52 \pm 2.98$ |

*2) Evaluation of Antimicrobial Activity*

The leaf extract of *Adhatoda vasica* was tested with two human bacterial pathogens, the Gram-positive Methicillin resistant *Staphylococcus aureus* (MRSA) and Gram- negative *Pseudomonas aureus* (MTCC 201) and one human yeast pathogen, Fluconazole Resistant *Candida Albicans* (FRCA) by well diffusion assay. The analysis of anti-oxidant activity is represented in **Table 4**.

**Table 4** Antimicrobial Activity of the leaf extract of *Adhatoda vasica* in different strains.

| NAME OF THE ORGANISMS | Zone of Inhibition | Sample concentration (mg/ml) | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 30 | 40 |
| *Staphylococcus aureus* | 19 | 7 | 9 | 12 | 17 |
| *Pseudomonas aeruginosa* | 21 | 7 | 10 | 12 | 16 |
| *Candida Albicans* | 19 | 6 | 9 | 11 | 15 |

The anti-microbial analysis showed a moderate activity against the pathogens. The MIC results of various concentrations of the extract revealed a greater inhibition against MRSA and MTCC 201 and FRCA. The MIC values of the extract are represented in the **Table 5.**

**Table 5** Minimum Inhibitory Concentration of the synthesized compounds against MRSA, MTCC 201, FRCA

| Entry | Minimum Inhibitory Concentration (MIC) | | |
|---|---|---|---|
| | $G^{+ve}$ strain | $G^{-ve}$ strain | Fungal Strain |
| 10mg/ml | 17.3 | 16.2 | 15.4 |
| 20mg/ml | 12.3 | 14.6 | 7.8 |
| 30mg/ml | 8.4 | 6.8 | 7.2 |
| 40mg/ml | 4.5 | 5.4 | 6.9 |

E. *Cytotoxic assay*

The samples **1-8** was evaluated for its anti-proliferative potential using MTT assay for 24 h and 48 h treatment. The compound inhibited the proliferation of all the cell lines in a dose dependent and time dependent manner. $IC_{50}$ value was calculated from the growth curve as 20µg/mL concentration as inhibitory concentration ($IC_{50}$) for MCF-7 and A-549 cells. The sample 4 (60% of plant extract and zinc oxide nanoparticles) was most effective against A-549 cells. The sample 1 (100% of plant extract and zinc oxide nanoparticles) was most effective against MCF-7. The phase contrast micrographs supported the MTT results as there was decrease in cell density when treated with different doses of the compound. Significant numbers of cells were found to be formless, detached and floating at higher concentrations of the compound in the treated cells.

**Table 7** Cytotoxic activity of samples at various concentrations (µg/ml)

| COMPOUND NAME | Concentration | MCF-7 | A-549 |
|---|---|---|---|
| Sample 1 | 100% of plant extract and zinc oxide nanoparticles. | 13± 1.0 | 12.5± 1.0 |
| Sample 2 | 90% of plant extract and zinc oxide nanoparticles. | 18± 1.0 | 16± 1.0 |
| Sample 3 | 75% of plant extract and zinc oxide nanoparticles. | 17± 1.0 | 12± 1.0 |
| Sample 4 | 60% of plant extract and zinc oxide nanoparticles. | 19± 1.0 | 11.5± 1.0 |
| Sample 5 | 50% of plant extract and zinc oxide nanoparticles. | 21± 1.0 | 17.5± 1.0 |
| Sample 6 | 25% of plant extract and zinc oxide nanoparticles. | 19± 1.0 | 22± 1.0 |
| Sample 7 | 15% of plant extract and zinc oxide nanoparticles. | 15± 1.0 | 18± 1.0 |
| Sample8 | 0% of plant extract and zinc oxide nanoparticles. | 19± 1.0 | 21± 1.0 |
| Doxorubicin | Standard Medicine | 12.5± 1.5 | 14± 1.5 |

$IC_{50}$ – Values of respective Compounds (at 24 hrs)

*1) Morphological analysis*

Selected breast cancer cells (MCF-7) and lung cancer cells (A-549) were incubated with the compound. The membrane blebbing and alterations induced by the compound were observed at the $IC_{50}$ concentration after 24h and 48h time exposure.

Similarly, prolonged exposure to the compound at the same concentration resulted in shrinkage and blebbing of the cell membrane. Whereas the untreated control cells were not shown any adverse effect.

*2) AO/EtBr and DAPI staining*

No aggregation of the sample 4 (60% of plant extract and zinc oxide nanoparticles) was observed during the assay because most of the sample 4 (60% of plant extract and zinc oxide nanoparticles) was dissolved at these low concentrations in A-549 cell lines. Whereas no aggregation of the sample 1 (100% of plant extract and zinc oxide nanoparticles) was observed during the assay because most of the sample 1 (100% of plant extract and zinc oxide nanoparticles) was dissolved at these low concentrations in MCF-7 cell lines. After the treatment with $IC_{50}$ concentration of compound, the induction of apoptosis was assessed for MCF-7 and A-549 cells by fluorescence microscopy stained with Acridine Orange/Ethidium Bromide (AO/EtBr). The Acridine Orange penetrated the normal cell membrane and the cells were observed as green fluorescence; whereas in apoptotic cells and apoptotic bodies observed a nuclear shrinkage, nuclear damage and blebbing as orange colored bodies. Necrotic cells were observed as red color fluorescence due to their loss of membrane integrity when viewed under a fluorescence microscope. Similarly nuclear dye DAPI also exhibits nuclear fragmentation in treated cells. The loss of cell adherence property and severe membrane disintegration were found in MCF-7 and A-549 cells treated with the compound. This further confirms that the compound induced detachment of cell through activating membrane precipitating proteins.

***i)* AO/EtBr staining of A-549**

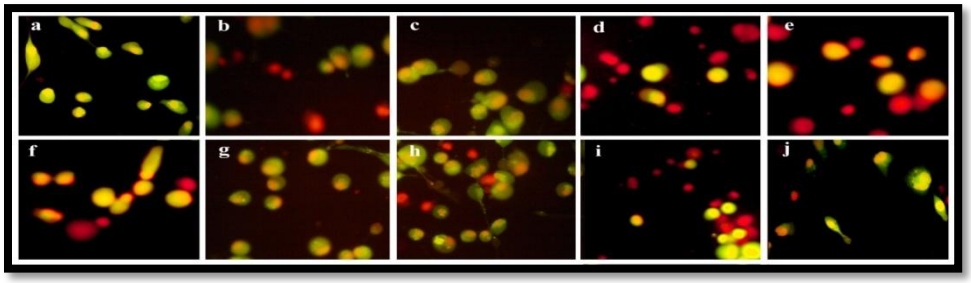



**Fig 7**. Fluorescence microscope image of A549 cells stained with AO/EtBr. Live cells appeared green in colour and Dead cells appeared red in colour. Cells were cultured with **a** (Live Cells), **b**(100% of plant extract and zinc oxide nanoparticles), **c**(90% of plant extract and zinc oxide nanoparticles), **d** (75% of plant extract and zinc oxide nanoparticles), **e** (60% of plant extract and zinc oxide nanoparticles), **g** (50% of plant extract and zinc oxide nanoparticles), **h** (25% of plant extract and zinc oxide nanoparticles), **i** (15% of plant extract and zinc oxide nanoparticles), **j** (0% of plant extract and zinc oxide nanoparticles), **f** (Doxorubicin, Standard Medicine).

***ii)* AO/EtBr staining of MCF-7**

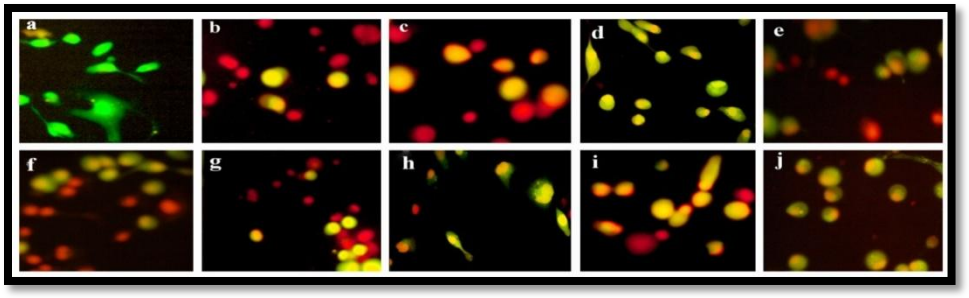



**Fig 8**. Fluorescence microscope image of MCF-7 cells stained with AO/EtBr. Live cells appeared green in colour and Dead cells appeared red in colour. Cells were cultured with **a** (Live Cells), **b** (100% of plant extract and zinc oxide nanoparticles), **c** (90% of plant extract and zinc oxide nanoparticles), **d** (75% of plant extract and zinc oxide nanoparticles), **e** (60% of plant extract and zinc oxide nanoparticles), **g** (50% of plant extract and zinc oxide nanoparticles), **h** (25% of plant extract and zinc oxide nanoparticles), **i** (15% of plant extract and zinc oxide nanoparticles), **j** (0% of plant extract and zinc oxide nanoparticles), **f**(Doxorubicin, Standard Medicine).

***iii)* DAPI staining of A-549**

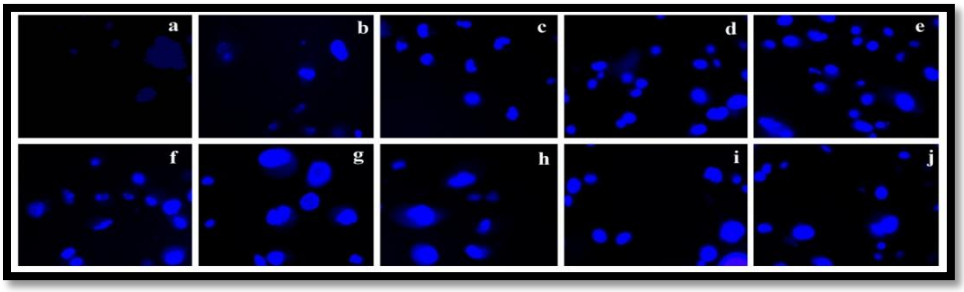



Fig 9**.** Fluorescence microscope image of A549 cells stained with DAPI. Live cells appeared dark blue in colour and Dead cells appeared blue in colour. Cells were cultured with **a** (Live Cells), **b** (100% of plant extract and zinc oxide nanoparticles), **c** (90% of plant extract and zinc oxide nanoparticles), **d** (75% of plant extract and zinc oxide nanoparticles), **e** (60% of plant extract and zinc oxide nanoparticles), **g** (50% of plant extract and zinc oxide nanoparticles), **h** (25% of plant extract and zinc oxide nanoparticles), **i** (15% of plant extract and zinc oxide nanoparticles), **j** (0% of plant extract and zinc oxide nanoparticles), **f** (Doxorubicin, Standard Medicine).

***iv)* DAPI staining of MCF-7**

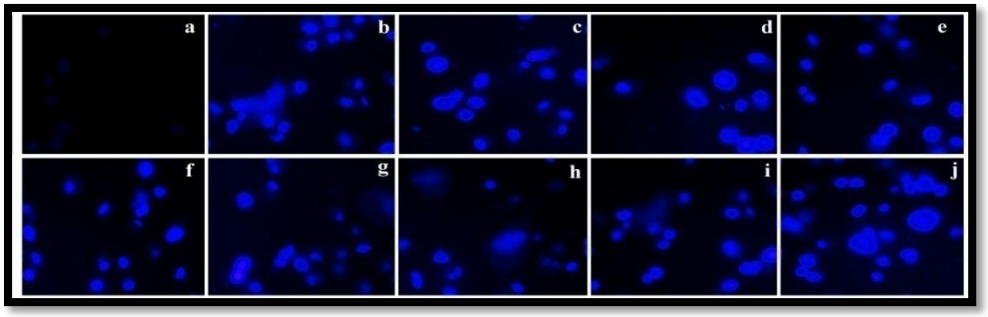



**Fig 10.** Fluorescence microscope image of MCF-7 cells stained with DAPI. Live cells appeared dark blue in colour and Dead cells appeared blue in colour. Cells were cultured with **a** (Live Cells), **b** (100% of plant extract and zinc oxide nanoparticles), **c** (90% of plant extract and zinc oxide nanoparticles), **d** (75% of plant extract and zinc oxide nanoparticles), **e** (60% of plant extract and zinc oxide nanoparticles), **g** (50% of plant extract and zinc oxide nanoparticles), **h** (25% of plant extract and zinc oxide nanoparticles), **i** (15% of plant extract and zinc oxide nanoparticles), **j** (0% of plant extract and zinc oxide nanoparticles), **f** (Doxorubicin, Standard Medicine).

## IV. CONCLUSION

The current study reports an efficient synthesis of Zinc oxide nanoparticles from *Adhatoda vasica*. The pilot study involves the screening of *Adhatoda vasica* for the phytoconstituents present. The characterization of the zinc oxide nanoparticles were carried out by Scanning Electron Microscope (SEM), UV analysis and XRD analysis. The particles synthesized formed are in the nano size and crystalline in nature. The particles prepared were examined for antimicrobial and anti-oxidant analysis. The synthesized zinc oxide nanoparticles exhibited proficient activity. From the cytotoxic analysis, the combination of the selected plant extract and bio-synthesized zinc oxide nanoparticles showed a very good efficiency against breast cancer cells and lung cancer cells. From the above study, the plant *Adhatoda vasica* was found to have a number of biological activities and therefore it is an eminent plant which is the need of the hour.

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