

In vitro Cytotoxicity Studies of Zn (Zinc) Nanoparticles Synthesized from *Abutilon indicum* L. against Human Cervical Cancer (HeLa) Cell Lines

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ABSTRACT

Background: The Zn nanoparticles synthesized from the plant sources are ecofriendly and are potent anticancer agents. **Objective:** The objective of the present work was to evaluate *In vitro* cytotoxic activity of Zn nanoparticles green synthesized from *Abutilon indicum* extract against HeLa cell lines (cervical cancer). **Methods:** The aqueous extract is prepared by cold extraction (maceration) using water as a solvent. Phytochemical analysis was done by using the standard procedures. Aqueous extract of *A. indicum* was used for synthesis of Zn nanoparticles. The nanoparticles were characterized by UV-Visible spectrometry and Scanning electron microscopy (SEM) techniques. *In vitro* cytotoxicity studies of Zn nanoparticles were done by MTT assay using HeLa cell lines. **Results:** The preliminary phytochemical results revealed that the aqueous extract of *A. indicum* contains broad spectrum of secondary metabolites like Tannins, Saponins, Glycosides, Flavonoids, Anthroquinones, Terpenoids and Steroids. The U.V spectrophotometric analysis of Zn nanoparticles displayed maximum absorption at 270 nm and scanning electron microscopic studies showed

that the nanoparticles size ranges from 50-500 nm. The MTT assay results revealed that the of Zn nanoparticles exhibits potent cytotoxicity against HeLa cell lines with IC₅₀ value of 45.82 µg/ml. **Conclusion:** Thus the present study concludes that Zn nanoparticles can be used as a potent drug in alternative therapy for treating the cervical cancer patients.

Key words: *Abutilon indicum*, Zn nanoparticles, MTT assay, Cervical cancer, Cytotoxicity

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INTRODUCTION

Nanoparticles are minute particles with dimension in 1-100 nm large, whose size is comparable to the biological molecules with its new and enhanced size-dependent properties compared to its bulk material, they penetrate more than larger substances and ideally interact with cellular structures.¹⁻³ These nanoparticles can be synthesized by chemical processes like pyrolysis, hydrothermal method, chemical precipitation etc. But these chemical processes cause pollution and are costly practices. Synthesis of metal oxide nanoparticles can be done using biological materials such as microbes and plants. Synthesis of nanoparticles using microorganism involves lengthy process of maintaining microbial cultures, intracellular synthesis and multiple purification steps. However using “green” methods in the synthesis of Zinc nanoparticles has increasingly become a topic of interest. Nanoparticles in recent days have revolutionized the modern medicinal practices because of their potential activities in treatment of various diseases.⁴ Plants have been used from centuries to treat various human diseases. Herbal drugs as compared to synthetic drugs have no or lesser side effects and are less expensive. Since plant mediated synthesis is easy and safe with one-step protocol and don't involve the use of harsh solvents or surfactant as the reducing agents, studies have suggested their use to be more ideal and compatible for their use in nanomedicine because of their stability in various biological media. The biomolecules present in plants mediates the synthesis of nanoparticles and also stabilize the nanoparticles formed with desired size and shape as well play a role in reducing the ions to the nanosize, and in the capping of nanoparticles.⁵ Numerous factors such as temperature, pH, concentration of extracts, concentration of raw material, etc. influence the reduction process of metal ions into the metal nanoparticles.⁶ Cancer is a class of disease where abnormal cells proliferate uncontrollably, producing

malignant tumors that invade surrounding healthy tissue. Cervical cancers are carcinomas of squamous cell, arising in the squamous (flattened) epithelial cells that line the cervix. Due to serious side effects of currently available cancer treatments-chemotherapy and radiation therapy, death rate is high. Studies have showed that plant derived nanoparticles can be used to treat cancer patients in the near future with minimum side effects. The Plant based Zn nanoparticles were used because of their inbuilt ability to penetrate tissue and cells and interact with the cancerous cells.^{7,8} This property of Zn-nanoparticles can be further engineered by functionalization with target proteins or chemical groups and rendering them benign to normal cells while retaining their cancer targeting and killing properties.⁹⁻¹¹ Studies demonstrate that cytotoxic property of Zn nanoparticles depends on their size i.e-smaller nanoparticles exhibit greater toxicity.^{12,13} The surface charge nature of Zinc nanoparticles is typically due to neutral hydroxyl groups attached to their surface.^{14,15} Also at lower pH Zn-nanoparticles gain positive charge from the environment, which then interact with negatively charged phospholipids on the membrane of cancer cells, thereby promoting cellular uptake, phagocytosis and cytotoxicity.¹⁶⁻¹⁸ Studies have showed that Zn nanoparticles exposure at particular concentration induces the production of various pro-inflammatory cytokines that elicitates Th1-mediated immune response which inturnenhances tumor cell killing through production of TNF-α (Tumor Necrosis Factor).¹⁹⁻²¹ *A. indicum* generally known as Country Mallow belongs to the family Malvaceae. It is spread throughout number of tropical and subtropical areas including India. It is a perennial shrub, softly tomentose and grows up to 3 m in height. It is found as a weed in sub-Himalayan tracts, hills up to 1200 m and in hotter parts of India. Almost all the parts of *A. indicum* are of medicinal importance

and used traditionally for the treatment of various ailments such as chest infection, urethritis, strangury, haematuria, leprosy, ulcer, toothache, inflammation of bladder, piles, laxative, in chronic cystitis, gleet and gonorrhoea, arthritis, seizures and in liver protection.²² There are limited numbers of reports available on the cytotoxic effect of Zn nanoparticles against cervical cancer, so the present study aims at profiling the same.

MATERIALS AND METHODS

Preparation of leaf extract

Fresh leaves were collected from *Abutilon indicum* plants in HSR Layout, Bangalore and it was identified and authenticated by taxonomist. A voucher specimen was deposited in The Oxford College of Pharmacy, Bangalore. The leaves were washed several times with water to remove the dust particles and then shade dried to remove the remaining moisture. The dried leaves were coarsely powdered and then placed in sterile distilled water in a conical flask so that all the crushed leaves were properly dipped in water. The conical flask was placed on a shaker for the cold extraction for 12-15 hrs. The extract was filtered using muslin cloth and the filtrate was poured into petriplate & dried using dessicator to form a thick jelly like extract.²³

Preliminary Phytochemical Analysis

Phytochemical analysis was performed using standard procedures by Harborne JB, 1998.²⁴

Synthesis of Zn Nanoparticles

Zinc acetate dihydrate and sodium hydroxide were used as the precursor material. The dried aqueous extract was dissolved in respective solvent, approximately 3 ml was prepared. Three sets of conical flasks with 50 ml distilled water each were prepared and labeled as 0.25 ml, 0.5 ml and 1 ml respectively. 0.02 M aqueous Zinc acetate dihydrate (0.219 gm) was added to each of the three flasks under vigorous stirring. After 10 min stirring aqueous leaf extract of *Abutilon indicum* were introduced into the above solution with the concentration of 0.25 ml, 0.5 ml, 1 ml to the three labeled flasks followed by the addition of aqueous 2.0M NaOH resulted in a white aqueous solution at pH 12. The pH meter was used for adjusting of pH. pH of the medium influence the size of Zn nanoparticles at great concern, which were then positioned in a magnetic stirrer for 2 hr. The precipitate were then taken out and washed repetitively with distilled water followed by ethanol to remove the impurities of the final product. Then a white powder of Zn nanoparticles was obtained after drying at 60°C in vacuum oven overnight.²⁵

Characterization of Zn Nanoparticles

The Zn nanoparticles were characterized by using a UV-Vis spectrometer, the nanoparticle suspension was prepared in deionized water; the sample was analyzed from 200 nm to 800 nm range in UV-Vis spectrophotometer. (UV-2450 Agilent)²⁶

SEM Observation of Zn Nanoparticles

The sample was prepared by placing a drop of Zn nanoparticles on gold coated copper grid for 5-6 min and subsequently drying in air, before transferring it to the microscope operated at an accelerated voltage of 10 KV (JOEL Model 5400).

MTT ASSAY

Chemicals

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's

Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

HeLa (Cervical carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For Cytotoxicity studies, each weighed test drugs were separately dissolved in DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 × 10⁵ cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitreplate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) were added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100$$

RESULTS

The preliminary phytochemical analysis inferred the presence of different classes of phytoconstituents which are depicted in Table 1.

Characterization of Zn Nanoparticles

The Zn nanoparticles synthesized were observed by change of colour to white precipitate, the precipitate was dried and used for UV spectrophotometer analysis Graph 1 shows the U.V spectrophotometer result with maximum absorption 270 nm.

SEM Analysis

The Scanning Electron Microscope (SEM) analysis was used to determine the structure of the reaction products that were formed. SEM

Table 1: Preliminary phytochemical analysis of aqueous extract of *A. indicum*

| SL NO. | TEST | INFERENCE |
|--------|----------------|-----------|
| 1 | Reducing Sugar | + |
| 2 | Tannin | + |
| 3 | Alkaloid | - |
| 4 | Saponin | + |
| 5 | Glycosides | + |
| 6 | Steroid | + |
| 7 | Flavanoids | + |
| 8 | Anthroquinones | + |
| 9 | Terpenoides | + |

Table 2: MTT assay results showing cell viability (%) based on concentration

| SL.No. | Concentration in (µg/ml) | Cell Viability (%) | |
|---------|--------------------------|--------------------|----------------|
| | | Sample | Standard(5-FU) |
| 1 | 5 | 89.540 | 70.126 |
| 2 | 10 | 79.714 | 42.364 |
| 3 | 20 | 69.889 | 37.562 |
| 4 | 30 | 58.795 | 33.891 |
| 5 | 40 | 54.358 | 30.456 |
| 6 | 50 | 50.396 | 25.755 |
| Control | - | 100 | - |

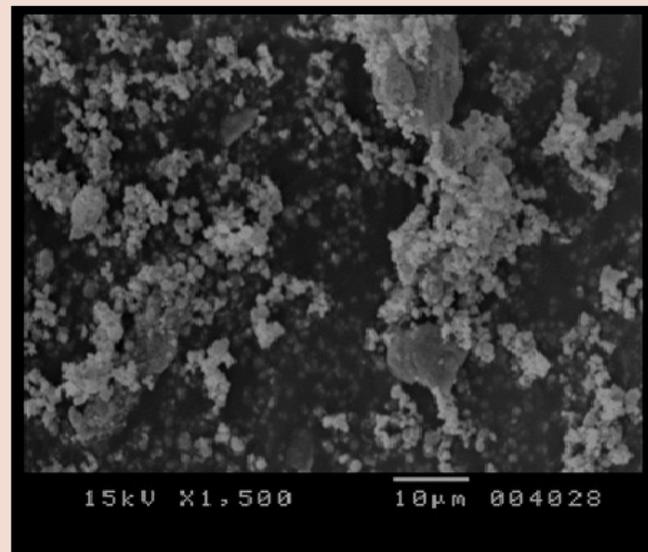
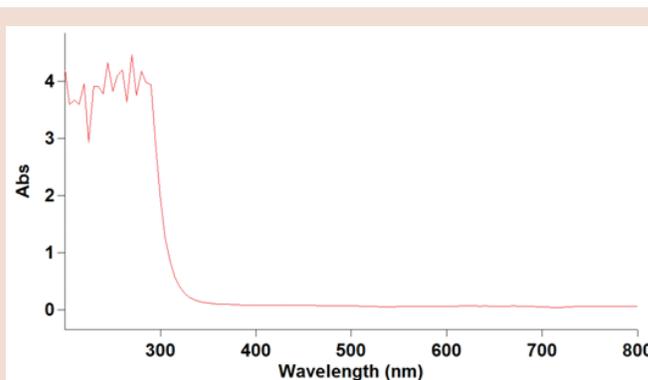


Figure-1 (b): SEM image of Zn Nanoparticles 1500X



Graph 1: Absorption maximum of Zn nanoparticles

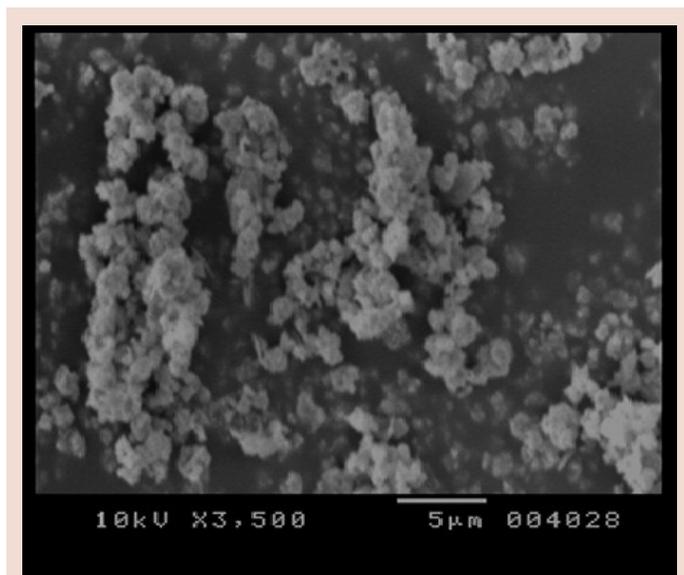
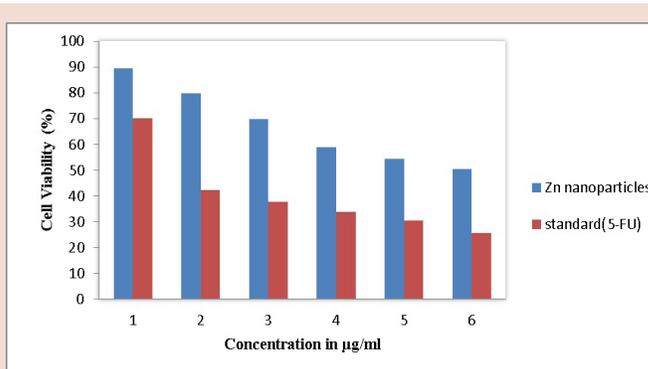


Figure-1 (a): SEM Image of Zn nanoparticles 3500X

image has showed scattered zinc particles as well as a number of aggregates. The SEM image showed broad range of relatively spherical shaped nanoparticles in the range of 50-500 nm in size (Figure 1a).

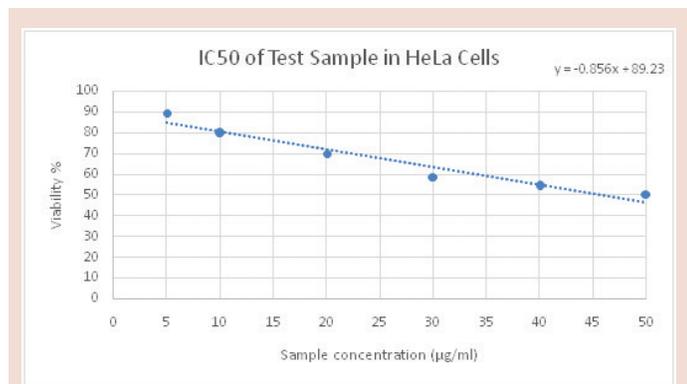
The nanoparticles with different concentration (5-50 µg/ml) were checked for their cytotoxicity against HeLa cell lines for cervical cancer using MTT assay (Table 2). The IC₅₀ value was then calculated from the graph with % Viability vs Drug Concentration which was found to be 45.82 µg/ml. The effect was compared with control and standard (5-Fluorouracil). Results show dose dependent cytotoxicity at higher concentrations there is significant cell mortality (Graph 2).



Graph 2: Graph showing the concentration of sample against the cell Viability

DISCUSSION

A. indicum is an important medicinal plant used traditionally to treat many human ailments. In the present study the preliminary phytochemical analysis of *A. indicum* revealed the presence of broad range



Graph 3: Graph showing IC₅₀ Value of Zn nanoparticles against HeLa cell lines

of phytoconstituents like flavonoids, tannins, saponins, anthroquinones, terpenoides, glycosides and steroids (Table 1). These phytoconstituents are used as potent drugs in treating diabetes, cancer, inflammation and as potential antioxidants.²² The plant phytochemicals with anti-oxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles. The Zn nanoparticles were synthesized by using zinc acetate dihydrate as the precursor material with the plant extracts. These Zn nanoparticles when subjected to UV-Vis characterization showed the maximum absorption at 270 nm (Graph 1). SEM analysis revealed that the nanoparticles were spherical in shape and found as aggregates in the range of 50-500 nm (Figure 1). When these Zn nanoparticles subjected to cytotoxicity assay exhibited potential cytotoxicity against HeLa cell lines with IC₅₀ value of 45.82 µg/ml (Graph 3). The cytotoxicity was found to be in a dose dependent manner (Graph 2). These Zn nanoparticles may selectively kill the cancer cells by apoptosis induced by the production of reactive oxygen species (ROS) via p53 pathway.²⁷ This ability of the Zn nanoparticles to inhibit the growth of cancer cell can be used in the treatment of cervical cancer.

CONCLUSION

The Zn nanoparticles synthesized using *A. indicum*, showed positive results for cytotoxic activity with an IC₅₀ value of 45.82 µg/ml. These positive results confirmed the cytotoxic potential of the Zn nanoparticles against cervical cancer. Thus Zn nanoparticles can be used as a potent drug in alternative therapy for treating the cervical cancer patients in the near future. Further potential of cytotoxicity against cancer can be enhanced by *In vivo* studies in animal models and human volunteers.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The author declare no conflict of interest.

ABBREVIATION USED

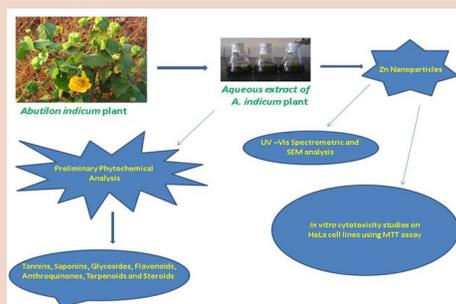
Zn: Zinc; **HeLa:** Henrietta Lacks; **SEM:** Scanning Electron Microscope; **UV-Vis:** Ultraviolet-Visible; **MTT:** 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; **nm:** Nanometer; **Th1:** TNF-α: Tumor Necrosis Factor; **hrs:** Hours; **ml:** Milliliter; **M:** Molar; **gm:** Gram; **min:** Minutes; **KV:** Kilovolts; **DMEM:** Dulbecco's Modified Eagle's Medium; **EDTA:** Ethylene Diamine Tetra Acetic acid; **DMSO:** Dimethyl Sulfoxide;

FBS: Fetal Bovine Serum; **IU/ml:** International Units/milliliter; **µg:** Microgram; **TPVG:** Trypsin Phosphate Versene Glucose; **cm:** centimeter; **mg:** milligram; **µl:** microliter; **OD:** optical density; **5-FU:** 5-Fluoro Uracil; **IC:** Inhibitory Concentration.

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PICTORIAL ABSTRACT



SUMMARY

- *Abutilon indicum* is a potent medicinal plant used in traditional medicine.
- The preliminary phytochemical analysis revealed the presence of broad spectrum of secondary metabolites.
- Zn nanoparticles synthesized from the aqueous extract of *A. indicum* showed strong cytotoxic activity against HeLa cell lines.

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