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Research Article

IN VITRO ANTICANCER POTENTIAL OF BIOSYNTHESIZED ZINC OXIDE NANOPARTICLES FROM THE SEAWEED *TURBINARIA CONOIDES*

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ABSTRACT

Objective: The objective of this study is to investigate the anticancer potential of zinc oxide nanoparticles (ZnO-NPs) and hydroethanolic extract of *Turbinaria conoides* (HETC) against Dalton's lymphoma ascites (DLA) cell line.

Methods: Nanoparticles were synthesized from the HETC. An ultraviolet-visible spectrophotometric analysis was performed to confirm the formation of ZnO-NPs. Size, morphology, and elemental composition of ZnO-NPs were also analyzed using scanning electron microscope-energy dispersive X-ray diffraction. The cytotoxic activity of ZnO-NPs and HETC was evaluated by 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue dye exclusion assays on DLA cells. The apoptosis inducing the effect was observed through acridine orange staining method (AO and EB) and DNA ladder assay.

Results: The results of *in vitro* cytotoxic studies by MTT and trypan blue dye exclusion assays on DLA cell line in the presence of ZnO-NPs showed an IC_{50} value of 23.13 and 25.81 µg/ml, respectively. The DNA ladder assay and AO/EB staining clearly demonstrated that the ZnO-NPs at 50 µg/ml concentration induced a maximum apoptosis in DLA cells when compared with HETC.

Conclusion: In the present study, the cytotoxic and apoptotic inducing effect of the synthesized ZnO-NPs and HETC were assessed, and it was found that ZnO-NPs possessed potent anticancer effect against DLA cells.

Keywords: Dalton's lymphoma ascites, *Turbinaria Conoides,* Apoptosis.

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INTRODUCTION

Nanobiotechnology, an emerging field of nanoscience, utilizes nano-based systems for various biomedical applications. This rapidly developing field of nanoscience has raised the possibility of using therapeutic nanoparticles in the diagnosis and treatment of human cancers. The nanoscale level of detection and treatment has advantages such as stability, extended shelf life, limited dose frequency, and high specificity [1]. Hence, nanocarriers have recently emerged as attractive candidates for drug delivery due to their efficacy and limited toxicity. Inorganic nanoparticles, notably, metal nanoparticles have raised much interest in research for its material properties, availability, capability, specific targeting, and sustained release [2]. They are most promising for their long-term use in medicine and have much attraction for potential applications in cancers [3].

Cancer is an abnormal type of tissue growth in which the cells exhibit an uncontrolled division, relatively in an autonomous fashion, leading to a progressive increase in the number of dividing cells [4]. It is a major public health problem worldwide and is the second leading cause of death in the world [5,6]. Cancer comprises more than 100 types of malignancy. Lymphoma is a disease of the lymphocytes (a type of white blood cell involved in immune responses) and the lymphatic system, which includes the spleen, thymus, and liver, as well as other lymphatic tissues. Dalton's lymphoma ascites (DLA) is a transplantable, poorly differentiated malignant tumor which appeared originally as lymphocytes in the mouse. It grows as both solid and ascitic forms [4].

Over the past few decades, there has been increased interest by pharmaceutical industries to discover the new drugs from the ethnobotanicals to provide new and alternative drugs to synthetic drugs for the treatment of dreadful diseases [7]. Marine algae are one of the natural resources in the marine ecosystem. They contain various biologically active substances which serve as a component for nutraceutical and pharmaceutical industry [8]. *Turbinaria conoides* belongs to the family of Sargassaceae. It produces a great variety of secondary metabolites having a broad spectrum of biological activities. Hence, the present study was aimed to evaluate the cytotoxic and apoptotic effect of the synthesized zinc oxide nanoparticles (ZnO-NPs) and hydroethanolic extract of *T. conoides* (HETC) against DLA cell line.

METHODS

Collection and preparation of seaweed

The brown seaweed, *T. conoides*, was collected from Mandapam coastal region, Gulf of Mannar, Southeast coast of India. The algal samples were washed thoroughly with running tap water followed by distilled water to remove adhering salts and associated biota. The washed samples were dried under shade at room temperature for a week. The dried materials were ground to fine powder using mixer grinder and stored in an airtight container for further analysis.

Preparation of algal extract

The crude algal extract (CAE) was prepared by adding 10 g of algal powder into 100 ml of 50% ethanol and kept in the rotatory shaker for 24 h, filtered, collected the solvent, and was used for further analysis.

Green synthesis of ZnO-NPs from CAE

20 ml of the CAE was heated at 50°C for 10 min, and 50 ml of 91 mM of zinc acetate solution (1 g of zinc acetate was dissolved in 50 ml of distilled water) was added dropwise. This was then placed on a magnetic stirrer for 2 h. Then, the precipitate was collected by centrifugation at 16,000 rpm for 10 min at 4°C. The pale white precipitate was then taken out and washed with distilled water followed by ethanol to get free of the impurities. The ZnO-NPs were obtained after drying at 60°C in an oven overnight, and the sample was stored for further studies.

Characterization of ZnO-NPs
The obtained ZnO-NPs were measured for its maximum absorbance using UV-visible spectrophotometry. The optical property of ZnO-NPs was determined through ultraviolet and visible absorption spectroscopy in the range of 280–420 nm. External morphology, i.e. the shape of the nanoparticles was characterized by scanning electron microscope (SEM). Elemental analysis was obtained from energy dispersive X-ray diffraction (EDX), which was attached with SEM.

Anticancer potential of the synthesized nanoparticles and the algal extract
The cytotoxic activity of ZnO-NPs and HETC was evaluated by 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue dye exclusion assays on DLA cells. The apoptosis inducing the effect was observed through acridine orange-ethidium bromide dual staining method (AO/EB) and DNA fragmentation assay.

Determination of cytotoxicity by MTT assay [9]
DLA cells aspirated from DLA bearing mice were adjusted to a concentration of 1×10^6 cells/ml, plated onto 96-well flat bottom culture plates, and treated with various concentrations of ZnO-NPs and HETC. Incubated for 24 h at 37°C in a 5% CO_2 incubator and added 10 ml of MTT to each well which was further incubated for 4 h at 37°C. The formazan was dissolved in 100 ml of dissolving buffer and the absorbance was read at 595 nm and the percentage cytotoxicity was calculated.

Determination of cytotoxicity by trypan blue dye exclusion technique [10]
For the assay, DLA cells aspirated from DLA-bearing mice were adjusted to a concentration of 1×10^6 cells in 0.1 ml phosphate-buffered saline (PBS) (pH 7.4). To about 0.8 ml of PBS, add 0.1 ml of cell suspension containing 1×10^6 cells and treated with different concentrations of ZnO-NPs and HETC. Incubated for 3 h at 37°C and added 0.1 ml trypan blue dye. Applied a drop of trypan blue-cell mixture to a hemocytometer, and the stained (non-viable) and unstained (viable) cells were counted separately under a microscopic field. The percentage cytotoxicity was calculated.

DNA ladder assay
The induction of apoptosis was studied by DNA ladder assay [11]. 1×10^6 DLA cells were lysed in 250 µl lysis buffer containing 50 mM Tris HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid, 0.1 M NaCl, and 0.5% sodium dodecyl sulfate. The lysate was incubated with 0.5 mg/ml RNase A at 37°C for 1 h, and then with 0.2 mg/ml proteinase K at 50°C overnight. Phenol extraction of this mixture was carried out, and DNA in the aqueous phase was precipitated by 25 µl (1/10 volume) of 7.5 M ammonium acetate and 250 µl (1/1 volume) isopropanol. DNA electrophoresis was performed on a 1% agarose gel containing 1 µg/ml ethidium bromide at 70 V, and the DNA fragments were visualized by exposing the gel to ultraviolet light, followed by photography.

Detection of apoptotic morphological changes by AO/EB dual staining method
To investigate the cytotoxicity induced by apoptosis, AO and EB dual staining method was performed [12]. DLA cells were isolated from DLA bearing mouse and washed with PBS. 1×10^6 cells/ml were treated with 50 µg/ml of ZnO-NPs and HETC for 24 h at 37°C, and 5% CO_2 level was maintained. After the incubation, cells were washed in PBS. Subsequently, the cells were stained with 20 l EB (100 g/ml) and 20 l AO (100 g/ml) in a ratio of 1:1. After washing, the cells were resuspended in PBS. The cells were then examined on a slide under a fluorescence microscope, and the images were captured.

RESULTS AND DISCUSSIONS

Biosynthesis of ZnO-NPs using Brown seaweed
ZnO-NPs were synthesized from the CAE of *T. conoides* by the green method, which is more reliable and less toxic when compared with chemical synthesis. The formation of pale-white color within 3 h of preparation indicated the synthesis of ZnO nanoparticles.

UV-visible spectral analysis
UV-visible spectral analysis recorded the optical absorption spectra of the samples when the size of the particle is reduced to the nanoscale. Fig. 1 shows the UV-visible absorption spectrum of ZnO-NPs. The absorption spectrum was recorded for the sample in the range of 280–420 nm. The spectrum showed the absorbance peak at 370 nm corresponding to the characteristic band of ZnO-NPs. The UV-visible spectrum showed the absorbance peak at 340 nm corresponding to the characteristic band of ZnO-NPs [13].

SEM analysis
The morphology of the synthesized nanoparticles was examined using SEM. Fig. 2a and b reveals the surface morphology of the synthesized ZnO-NPs under different magnifications. The SEM image showed that most of the nanoparticles are spherical with a diameter ranging from 70 to 120 nm.

EDX analysis
The EDX study was carried out for the synthesized ZnO-NPs to elucidate the elemental composition. EDX confirms the presence of zinc and oxygen signals of ZnO-NPs as depicted in Fig. 3.

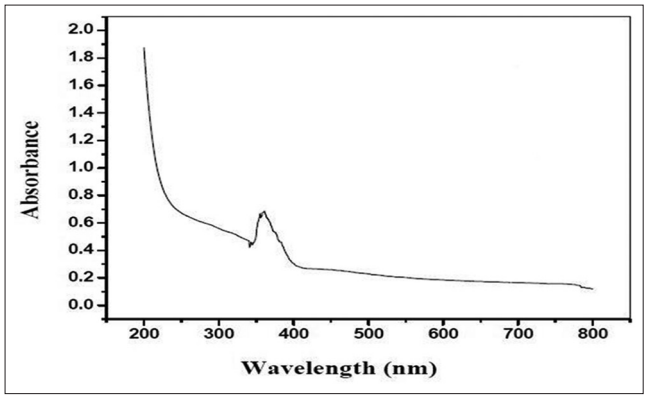

Fig. 1: UV-visible spectrum of synthesized zinc oxide nanoparticles

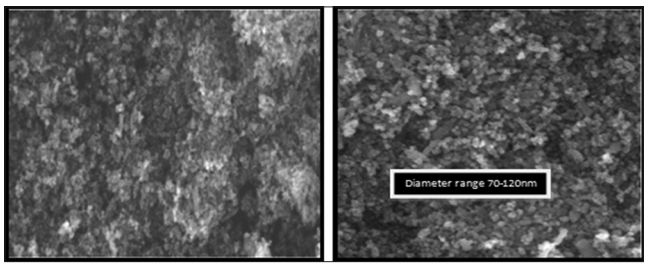

Fig. 2: Scanning electron microscope image of the synthesized zinc oxide nanoparticles

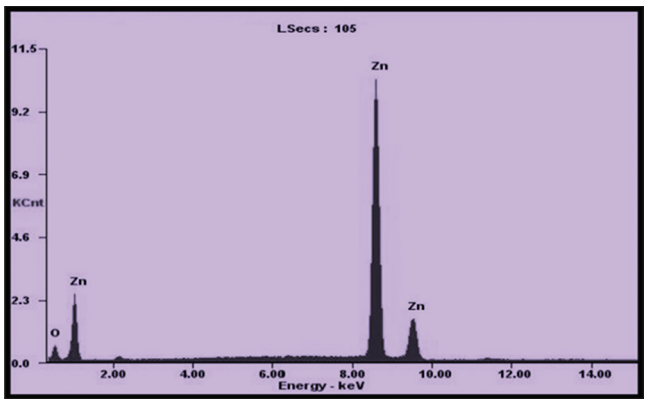

Fig. 3: Energy dispersive X-ray diffraction spectrum of synthesized zinc oxide nanoparticles

The results revealed that the peaks correspond to the optical absorption of the produced nanoparticle. The elemental analysis of the nanoparticle yielded 77.32% zinc and 22.68% oxygen which proved that the produced nanoparticle is in its highest purified form.

The observed results are in good rapport with the SEM-EDX analysis of ZnO nanoparticles synthesized using *Spathodea campanulata* [14].

Anticancer potential of the synthesized nanoparticles and the hydroethanolic extract

Determination of cytotoxicity by MTT assay

Cytotoxic effect on DLA cell line was investigated by MTT assay, and the results were shown in Fig. 4. Cells were treated with ZnO-NPs and HETC at different concentrations ranging from 10 to 50 µg/ml, and then, the percentage of cytotoxicity was analyzed. The IC_{50} value was found to be 23.13 µg/ml for ZnO-NPs and 37.13 µg/ml for HETC. The result emphasized that ZnO-NPs showed maximum concentration-dependent cytotoxicity to DLA cells when compared with the hydroethanolic extract. Our results are in good accordance with Sriram *et al.* [1] who reported that the AgNPs also showed dose-dependent cytotoxicity against DLA cells.

Determination of cytotoxicity by trypan blue dye exclusion assay

Cytotoxic activity of ZnO-NPs and HETC on DLA cells was assessed by Trypan blue dye exclusion method. Fig. 5 represents the percentage cytotoxicity of the DLA cells at different concentrations of ZnO-NPs and HETC ranging from 10 to 50 µg/ml. The sample showed the viable cells which remained unstained by trypan blue and were counted with the use of a hemocytometer. The IC_{50} of the ZnO-NPs and HETC was found to be 25.81 µg/ml and 38.78 µg/ml on DLA cell lines. When compared with the hydroethanolic extract, ZnO-NPs exhibited maximum cytotoxicity in a dose-dependent manner. Khairunnisa and Karthik [15] also reported that *Hymenodictyon excelsum* bark extract was found to have cytotoxic effects against DLA cells which further supported our study.

DNA ladder assay

The antitumor activity was further confirmed by DNA ladder assay which is an indicator of late apoptosis [2]. Fig. 6 revealed the electrophoretic pattern of the DNA fragmentation assay. In DLA control group (Lane 2), DNA fragmentation ladders were not observed while minimum fragmentation was noticed in HETC-treated group (Lane 3 at 50 µg/ml concentration). The group treated with standard drug and ZnO-NPs (50 µg/ml) exhibited extensive double-strand breaks, thereby yielding a ladder appearance which confirmed apoptosis. The 100 bp ladder (Lane 1) served as a marker. This implied that ZnO-NPs possess maximum apoptotic inducing effect by causing DNA damage to DLA cells when compared with hydroethanolic extract. Our results are in good rapport with the work done by Sriram *et al.* [1] who reported that AgNPs induced a apoptosis by cellular DNA fragmentation.

Detection of apoptotic morphological changes by AO/EB dual staining method

To confirm whether the cytotoxic effect is induced by ZnO-NPs and HETC involve apoptosis, morphological changes in the nanoparticle-treated and untreated cells were observed through AO/EB dual staining method (Fig. 7). AO is a cationic dye that enters only live cells and stain DNA, and hence, the live cells were observed as green under blue emission. On the contrary, ethidium bromide stains DNA in the cells undergoing apoptosis, and hence, apoptotic cells appeared orange in color [16]. Untreated (DLA control) cells emitted green fluorescence indicating normal nucleus with intact cell membrane. The HETC-treated cells emitted green fluorescence which might be due to the

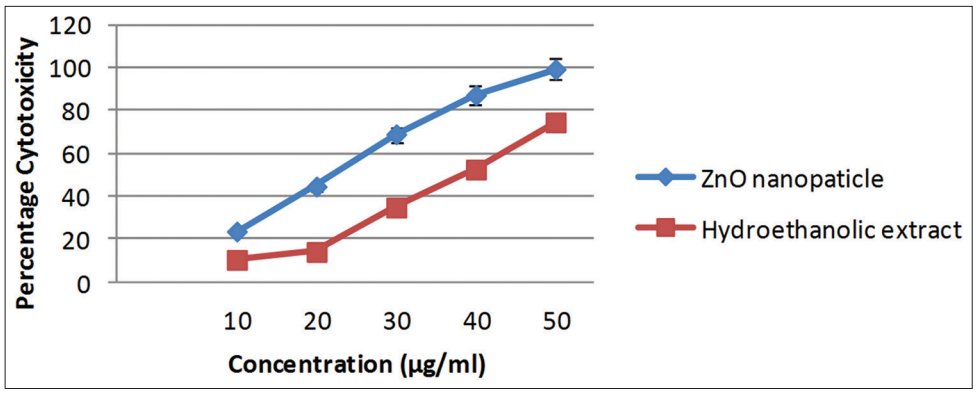

Fig. 4: Determination of percentage cytotoxicity using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values were expressed as mean±standard deviation for triplicates

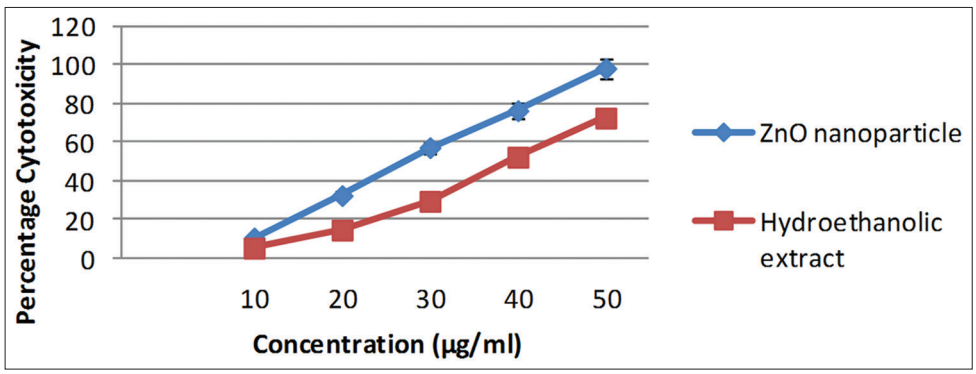

Fig. 5: Determination of percentage cytotoxicity using trypan blue dye exclusion assay. Values were expressed as mean±standard deviation for triplicates

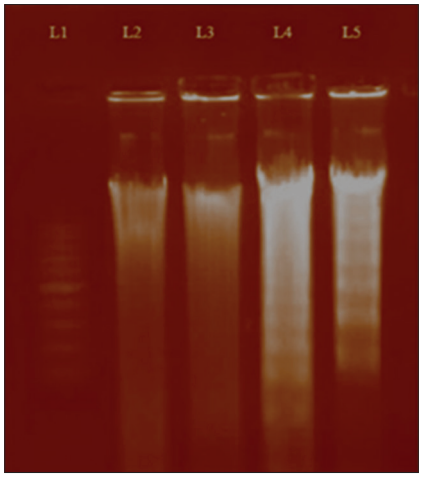

Fig. 6: Determination of antitumor activity by DNA ladder assay. Lane 1: 100 bp DNA marker; Lane 2: Dalton's lymphoma ascites (DLA) cell line (untreated); Lane 3: DLA cell line treated with hydroethanolic extract of *Turbinaria conoides* (50 µg/ml); Lane 4: DLA cell line treated with zinc oxide nanoparticles (50 µg/ml); Lane 5: DLA cell line treated with doxorubicin (10 µg/ml)

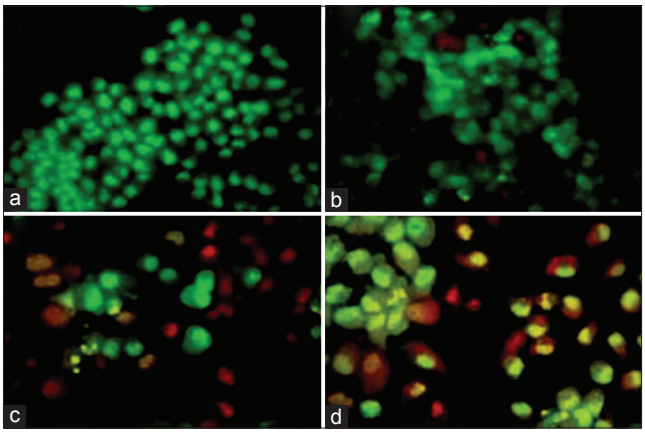

Fig. 7: Effect of zinc oxide nanoparticles (ZnO-NPs) on apoptotic morphological changes in Dalton's lymphoma ascites (DLA) cells. (a) DLA cell line (control); (b) DLA cell line treated with HETC (50 µg/ml); (c) DLA cell line treated with ZnO-NPs (50 µg/ml); (d) DLA cell line treated with 5-fluorouracil (10 µg/ml)

permeabilization of a cytoplasmic stain that stained the viable cells. The ZnO-NPs-treated cells exhibited reddish-orange fluorescence indicating the loss of membrane integrity, cellular shrinkage, membrane blebbing, and typical nuclear fragmentation which might be due to apoptosis, and the results were also compared with to that of the standard. The findings revealed that the HETC exhibited minimum apoptotic inducing effect when compared to ZnO-NPs. Our study was in accordance with the work done by Antony *et al.* [2] who also reported that the AgNPs derived from *Ficus religiosa* induced apoptosis and morphological changes in DLA cells.

CONCLUSION

Our findings clearly demonstrated that exposure of Dalton's lymphoma cells to the biosynthesized ZnO-NPs exhibited the maximum cytotoxic and apoptotic inducing effects when compared with the hydroethanolic extract of *T. conoides.* The results are found to be promising and are further required to elucidate the detailed molecular mechanism regulated by the ZnO-NPs to combat cancer.

CONFLICTS OF INTERESTS

Declared none.

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