Biological Fabrication and Characterization of Zinc Oxide Nanoparticles Using *Gymnema Sylvestre* Potentially Produces Toxicity in Breast Cancer Cells -MCF-7

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Abstract

To study the biological fabrication and characterization of zinc oxide nanoparticles (ZnO NPs) using *Gymnema sylvestre* and their toxicity to breast cancer cells MCF-7. In the existing work, ZnO NPs were synthesized using leaf extract of the Indian medicinal plant *Gymnema sylvestre* and it was characterized by Particle size, Zeta potential, FT-IR, XRD and SEM analyses. This ZnO NPs to have potentially validated anticancer role in breast cancer cells MCF-7 *in vitro* approach. The plant-based synthesized ZnO NPs were evaluated against the inhibitory role on breast cancer cell lines. We significantly observed that ZnO-NPs induce efficient toxicity of MCF-7 cells by increasing ROS, mitochondrion membrane damage and apoptotic morphological alterations. These results stated that ZnO-NP induces Bax and Caspases and down-regulates Bcl-2 proteins in MCF-7 cells. Thus, the biologically synthesized ZnO NPs were identified as good performance to inhibit breast cancer cell growth even at low concentrations.

1. Introduction

Breast cancers have concern as furthestmost predominant cancer and also more aggressive type of cancer in the women worldwide [1]. Moreover, breast cancer has shown in global epidemic due to the highest metastatic condition. Closely, there are 250 000 breast cancer patients are diagnosed with metastatic condition in the United Nation, and 40 000 more patients were died for this disorder. [2]. In the developing countries especially in India, second most common cancer after cervix cancer [3]. Breast cancer could be referring to a malignant arrangement of tumour that has been settled from cells are highly proliferated in the breast tissue [4]. This kind of cancer being treated with several chemotherapy and radiation therapy that could be ends up in several side effects due to the nonexistence target specificity. This toxicity not only affects tumour tissues, but also disturbs healthy and normal cells [5]. These methods are reducing the risk of cancer but not ensure the complete eradication of the cancer cells [6]. Furthermore, reoccurrence of the disease has been pointed that use of chemotherapy can be failure or cancer cells become resistance against chemotherapy [7]. Chemotherapy based potential toxicity on the cancer cells are undergone increased side effects which are mainly associated with insufficient specificity for cancer cells that signifies a major restriction in this type of therapy. Therefore, it is very useful to find out a safe and effective approach to combat the life-threatening disease especially for treating breast cancer [8].

Research related to nanotechnology depended cancer therapy covers yonder drug delivery systems in the construction of new therapeutical potential pharmacological approaches. Advances in nanotechnology put forward the use of medicinal plants for the synthesis of various nanoparticles. It shall be used for treating various diseases owing to its low toxicity and increased target specificity. The nanoparticles fabricated with plant extracts were proven to demonstrate various biological activities [9]. Zinc oxide nanoparticle (ZnO NPs) is a key ingredient of several enzymes, ointments and sun screens that aids in treatment of pain and inflammation [10]. Synthesis of ZnO NPs belongs to metal nanomaterials and its exhibits promising pharmacological activities such as anti-bacterial, antitumoral, anti-viral due to its morphology, exposure time, particle size, concentration, and biocompatibility [11]. ZnO NPs, with their

exclusive properties such as biocompatibility with selective target specificity, enhanced cytotoxicity on the cancer cells and easy way to biosynthesis and auspicious anticancer agent [12].

Since ancient times, plants have been used in the treatment of human disease. In Ayurvedic system of medicine, several thousands of plants are being used for curing various diseases [13]. *Gymnema sylvestre* is one among such plants belongs to the family Asclepiadaceae and known as “Madhunashini” (sweet destructor) in Sanskrit language due to its anti-diabetic property. It has been used in the treatment of diabetes, obesity and snake bites. All these properties can be attributed to the bioactive phytochemicals present in this plant [14, 15]. With this perspective, the present work was carried out to synthesize ZnO NPs using the leaf extract of *G. sylvestre* and potentially validate anticancer role in breast cancer cells MCF-7 *in vitro* approach.

2. Materials And Methods

2.1. Materials

Zinc nitrate (SD-Fine chemicals), MCF-7 cells, (National centre for cell sciences, Pune), DMEM, PBS, Trypsin (Hyclone), MTT, (Himedia laboratories); DPPH (Sigma-Aldrich Fine Chemicals).

2.2. Plant sample collection and preparation of ZnONPs

Leaves of *G. sylvestre* were visually identified from the Acharya N G Ranga Agricultural University, Tirupati, Andhra Pradesh, India. Fresh and sterile leaves weighing about 5 grams were cut into fine pieces and was rinsed with distilled H₂O and boiled with H₂O for 15 min at 70°C. Boiled mixture was filtered using Whatman No. 1 filter paper and the supernatant of leaf extract was collected and 90ml of 1M zinc nitrate solution was added. The reaction mixture was left for overnight. The colour change from colour less to pale yellow colour indicate the formation of ZnO NPs [16].

2.3. Characterization of nanoparticles

In this study, the colour changing ability reaction combination was well-thought-out as the pilot event to detect the nanoparticle synthesis. FT-IR spectrum analysis was used by FT-IR-ALPHA interferometer instrument (Bruker, Germany) and the scanning range between 500 to 4000 cm⁻¹. Horiba nanoparticle analyser was employed to regulate the exact average size and Zeta potential values of the synthesized nanoparticles. The average size of bio fabricated ZnONP-GS was determined by XRD-6000-Shimadzu Analytical, India, Scanning Electron Microscope (SEM- FEIQuanta, 200 FEGHRSEM equipped with EDAX).

2.4. Cell culture

MCF-7 human breast cancer cell lines were routinely maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 2mM/L glutamine, 10% foetal bovine serum (FBS) and 10 µg/ml of ciprofloxacin in a 5% CO₂ atmosphere at 37°C [17].

2.5. Cytotoxicity assay

The cytotoxic activity of the ZnO NPs were screened by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide) assay [18]. The MCF-7 cells (0.2×10^6 /well) were equally seeded in 96 well plates and kept 24 hours incubation at 37°C in 5% CO_2 . After grown the cells, it can be treated with different concentrations of the ZnO NPs viz. 1, 2, 3, 4, 5, 10, 20, 30 and 40 $\mu\text{g/ml}$ respectively. After 24 hours of incubation with the test compounds, 0.02 ml of MTT reagents were equally added to each well and incubation was continued for another 4 hours. Moreover, 0.02 ml of DMSO was equally given to all the wells to solubilise the formazan crystals and the colorimetric absorbance reading was detected by Micro plate reader at 570 nm [19].

2.6. DCFH-DA staining

Inner cellular ROS fabrication was considered by fluorescence dye, DCFH-DA, DCFH-DA penetrate cells passively and transformed to DCFH, which responses by reactive oxygen species for the formation of fluorescent DCF. MCF-7 cells stayed scattered on the top edge of cover slips in 24 well plates and supplemented with ZnO NPs for 60 mins. Then cells were nurtured with $25\mu\text{M}$ DCFH-DA for about half an hour. The fluorescence concentration was resolute at 490nm in excited level and 540nm emission level by means of fluorescence microplate reader (Sunnyvale, California, USA) similarly [20].

2.7. Acridine orange/ethidium bromide staining

To envisage the nuclear variations and characteristics of apoptosis, that is apoptotic body formation, dual staining was done by standard protocol [21]. ZnO NPs implored apoptotic variations in MCF-7 cells which were suspected by staining methods like acridine orange and ethidium bromide. Shortly, 1×10^6 cells were dispensed in 6-well plate then maintained MCF-7 cells were managed through ZnO NPs which is reserved for development or incubation period. Subsequent to development phase, the cells stood rinsed with ice cold phosphate buffered saline then discoloured with $10\mu\text{L}$ of acridine orange and $10\mu\text{L/mg}$ ethidium solution at room temperature and saved for gestation for about 45mins. The discoloured apoptotic and viable cells were scrutinized by glowing microscope [22].

2.8. Western blot analysis

Protein expression studies were done by western blotting technique in accordance with standard protocol. Crucial apoptotic intracellular signalling markers such as, Bax, caspase-3, 9 and Bcl-2 were examined by western blotting. Presently, 1×10^6 cells were treated with ZnO NPs and the proteins were extracted from cells by RIPA buffer that comprises cocktail protease enzyme. The concentration of protein was restrained by Nanodrop products, Thermo Fischer scientific, United States of America. Later the proteins were located to SDS-PAGE (10%) and the gel was reallocated to membrane (PVDF). Then the membrane was jammed with bovine serum albumin (5%) for more than 2hrs. Again, membranes were pampered with a suitable primary antibody and it kept for 24hrs incubation at 4°C . Moreover, it was further nurtured through apt conjugated secondary horseradish peroxidase antibodies for 1hr. Resultant bands were recognized by using improved chemiluminescence underlying substance and band illumination were scrutinized by software image J [23].

2.9. Statistical Examination

All experimentations stayed approved out in tierce self-destructive trials and the outcomes were expressed as the mean $\pm$ standard deviation (Mean $\pm$ SD) by means of one-way analysis of variance (ANOVA). Standards of $P < 0.05$ were symptomatic of substantial alterations and modifications.

3. Results And Discussion

3.1. Synthesis of ZnO Nanoparticles from *Gymnema sylvestre* (ZnONP-GS) and its characterization studies

Plants are instrumental in treating human diseases in almost every system of medicine worldwide [24]. Recent advances in nano biomedicine emphasize on the importance of green synthesis of metal nanoparticles using medicinal plants. This approach is advantageous over the physical and chemical methods due to safety, cost effectiveness and absence of toxic residues [25]. The synthesis of metal nanoparticles such as Ag, Au, Zn etc., have potential biological application especially it may inhibit cancer cell growth [26]. In this present work, we elaborately demonstrate the biologically synthesis and characterization of ZnO NPs from the medicinal plant *Gymnema sylvestre* and it was evaluated in anticancer potential in MCF-7 breast cancer cells [27].

The biological synthesis of ZnO NPs from *Gymnema sylvestre* was initially conformed by the colour change of the reaction mixture from colourless to brown colour indicated the synthesis of ZnO NPs preliminarily. Then, the synthesized nanoparticles were exhibits as strongest UV absorbance peak at 300 nm (Fig. 1). Moreover, the particle size of the synthesized nanoparticles was analysed by Zeta potential and particle size analysis. The particle size and Zeta potential analysis revealed that 81.1nm average size (Fig. 2). and -25.1 mv zetapotential value (Fig. 3) of the synthesized nanoparticles. reported the synthesis of Mg^{2+} doped ZnO NPs using the leaf extract of *Gsylvestre*. From the results it can be concluded that the synthesized nanoparticles are stable [28].

Moreover, active and functional biomolecules are present in the ZnO NPs synthesized from *G.sylvestre* which are identified and analysed from FTIR spectrum and it is shown (Fig. 4).The FTIR spectrum exhibits the peaks at 3415cm^{-1} were allotted to the extending vibrations of hydroxy groups; primary and secondary amines groups were presented in the synthesized nanoparticles respectively. The presented peaks were directly equivalent to protein and enzymes molecules or polysaccharides are found in the cell biomass. The peak at $2926, 2854, 2358$ and 2330cm^{-1} were owed to symmetric and asymmetric stretching shaking of sp^3 hybridized. The peak at 1612 and 1313cm^{-1} were allocated to $\text{C}=\text{O}$ extending vibrations of the carbonyl group in ketones, aldehydes and functional carboxylic acids. Moreover, the peak at 1163 and 1055cm^{-1} were allocated to vibration of $-\text{C}=\text{C}-$ aromatic ring stretching. In addition to this band at 995cm^{-1} resembles to metal binding interact with carboxylic ($\text{M}\leftrightarrow\text{C}\equiv\text{O}$) groups, this functional group might be acts template, reducing agent and capping of nanocrystals [29].

The crystal nature-based structure is often crucial conformation of ZnO nanomaterials which are determined by X-Ray diffraction (Fig. 5). In this study, obtains several crystal-based aspects of ZnO nanofabricated material. The X-Ray diffraction design of ZnO nanofabricated material are wurtzite hexagonal phase which designates the well indexed XRD peaks that has corresponding to the plane's values such as (34699), (30566), (44739), (64831) (77739) and (81869). This present result has been implying that the products comprised of pure phases. Moreover, the effective diffraction peaks were found more rigorous and narrower that implying a respectable crystalline structure of Zn nanofabricated products. The respectable of size range of ZnO nanofabricated material was from 20 nm to 100nm [30].

As shown in the (Fig. 6) demonstrates the surface and shape with size morphology of ZnO were characterized from the microscopical studies of SEM. This study evident that ZnO NPs were spherical and irregular in shape and were poly-dispersed. The measured average size was 50µm, Occasional agglomeration of the ZnO NPs has been observed. These all the characterization studies are scientifically evident that present nanoparticles are ZnO [31].

*3.2. Cytotoxic effect and intracellular ROS generation in ZnO nanoparticles synthesized from *Gymnema sylvestre* against breast cancer MCF-7 cells*

The ZnO NPs can be easily biodegraded or shall take part in nutritional cycle of the body [32]. These nanoparticles exhibit discriminating cytotoxicity over cancer cells [33]. ZnO NPs have ability to stimulate oxidative stress in cancer cells, which has been found to be one of the prime mechanisms of cytotoxicity. This property may be attributed to the semiconductor nature of ZnO NPs [34]. The cytotoxic event of ZnO NPs synthesized from *Gymnema sylvestre* in the breast cancer MCF-7 cells were evaluated by studies of MTT assay. Here we found that increasing concentration of ZnO NPs at the ranges from 10–100 µg for 24 hrs incubation notably reduces the cell viability in MCF-7 cells. The IC₅₀ value of the tested ZnONP-GS exhibits 36 µg/ml. In the current work, we have chosen IC₅₀ value of 36 µg/ml and IC₂₅ value of 50 µg/ml of ZnONP-GS for further molecular studies [35]. (Fig. 7).

It also induces ROS generation and leads to oxidative stress, eventually ends up in cell death when the anti-oxidative capacity of the cell is exceeded [36]. DCFH-DA staining are highly used for detection of ZnO NP-GS treated MCF-7 cells. Untreated MCF-7 cells showed there is no significant production ROS staining. ZnO NP-GS treated MCF-7 cells showing magnificent production of ROS for 24 hours incubation by dose depended manner. This result indicated that synthesized ZnONP-GS induces oxidative stress in MCF-7 cancer cells. Our results also conform that ZnO NP produces cytotoxicity and high over production of ROS in MCF-7 cells that can be led to oxidative stress. Several in vitro studies witnessed the ZnO NPs shows selective cytotoxic activity against the cancer cells [37]. Moreover, reported the anticancer activity of silver nanoparticles bio functionalized using aqueous extract of *G.sylvestre* against HT29 human colon adenoma cancer cells. Hanley also stated that ZnO NPs exhibited 28–35 times selective toxicity over cancerous cells when compared with normal cells [38]. ZnO NPs selectively kill cancer cells by inferring selective localization and selective cytotoxicity towards them [39]. (Fig. 8).

3.3. ZnONP-GS induces the production of ROS and apoptosis in MCF-7 cells

High over production of ROS arbitrated oxidative stress leads to the programmed cell death [40]. Apoptosis is a crucial programmed cell death which has actively eliminated the cancer cells through whether intrinsic or extrinsic apoptotic signalling pathway [41]. In this present work, ZnONP-GS treatment associated morphological changes of apoptotic MCF-7 cells were test by double staining of acridine orange and ethidium bromide staining. Here, we noticed ZnONP-GS treated cells showed a greater number of apoptotic cells significantly in dose depended manner. Conversely, untreated MCF-7 cells showing there is no significant apoptotic cells conformed by green fluorescence staining [42]. (Fig. 9).

In addition, proapoptotic biomarkers such as Bax, caspases and antiapoptotic protein Bcl-2 are highly regulating apoptosis [43]. Apoptosis has been documented as dispensation over several machineries, including amendment of the intracellular mitochondrial depended pathway to stimulate the caspase cascade activation [44]. When the over production of ROS sprightly induces proapptotic mediators leads to apoptosis [45]. ZnO NPs treatment arbitrated apoptotic protein appearance was studied using western blotting analysis. As (Fig. 10) confirms an extensively increased protein expression of Bcl-2 and broadly decreased expression of Bax, caspase-9 and 3 protein in untreated breast cancer cells. Suggestively, the expression of anti-apoptosis marker Bcl-2 was experientially to be less whereas the expression of proapoptotic mediators were relatively quite higher expression in ZnONPs-GS treatment in the MCF-7 cells. There are numerous documentation has been reported strongly that ZnONPs from several plant extracts induces proapoptotic markers in several cancer cell lines [46]. This result indicates that ZnONPs-GS induces proapoptotic mediators leads to cell death [47].

Conclusion

Based on the results of this work, we concluded that the biologically synthesized ZnO nanoparticles from leaf extract of the medicinal plant *G.sylvestre* have been characterized. The studies from UV Spectra, Zeta potential, FTIR, SEM concluded that conforms the synthesis of ZnO NPs produced from *G.sylvestre*. Then, the synthesized ZnONPs-GS have distinct role in the breast cancer MCF-7 cells by the producing toxicity, ROS and apoptosis. Moreover, our molecular experiments suggested that ZnO NPs -GS induces proapoptotic markers in MCF-7 cells.

Declarations

Conflicts of interest

None

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Figures

Figure 1

UV–Vis spectra of synthesized ZnONPs from *Gymnemasyvestre* .The strong absorption spectrum of ZnONPs exhibited at 300 nm.

Figure 2

Particle size analysis of ZnONPs synthesized using the leaf extract of *Gymnemasyvestre*

Figure 3

Zeta potential analysis of ZnONPs synthesized using the leaf extract of *Gymnemasyvestre*

Figure 4

FTIR analysis of ZnONPs synthesized using the leaf extract of *Gymnemasylvestre*

Figure 5

Figure 6

SEM analysis of ZnONPs synthesized using the leaf extract of *Gymnemasylyvestre*

Figure 7

The cytotoxic effect of ZnONPs-GS on MCF-7 cells was determined by MTT assay.

A) Fluorescence microscopic image for ZnONPs-GS on intracellular ROS generation. Percentage of ROS generation was detected by spectrofluorometer. B) The data represent mean $\pm$ SD of triplicate, * P <0.05 as compared with the control group.

Figure 8

The effect of ZnONPs-GS on intracellular ROS generation was analyzed in MCF-7 cells by using DCFH-DA staining.

A) Fluorescence microscopic image for ZnONPs-GS on apoptotic morphological status by acridine orange and ethidium bromide staining. B) Bar diagram represents percentage of apoptosis. The data represent mean $\pm$ SD of triplicate, * $P < 0.05$ as compared with the control group.

Figure 9

ZnONPs-GS induces apoptotic morphological changes in MCF-7 cells.

Western blots images for Bax, Bcl-2 caspase-9 and caspase-3 in MCF-7 cell lines. Lane 1: Control; Lane 2: ZnONPs-GS (36 $\mu\text{g/mL}$); Lane 3: ZnONPs-GS (50 $\mu\text{g/mL}$). The interests of proteins were normalized to respective β -actin loading control. The data represent mean $\pm$ SD of triplicate, * $P < 0.05$ as compared with the control group.

Figure 10

ZnONPs-GS induces apoptotic marker expression in MCF-7 cells