Green synthesized zinc oxide nanoparticles induce apoptosis by suppressing PI3K/Akt/mTOR signaling pathway in osteosarcoma MG63 cells

Satheeshkumar Subramaniyan1, Yoganathan Kamaraj1, Veenayohini Kumaresan1, Muthulakshmi Kannaiyan2, Ernest David3, Babujanarthanam Ranganathan4, Vijayanand Selvaraj3, and Agilan Balupillai3*

1Department of Microbiology, Faculty of Science, Annamalai University, Chidambaram, Tamil Nadu, India
2Department of Microbiology, Faculty of Science, Idhaya College for Women, Tiruvannamalai, Tamil Nadu, India
3Department of Biotechnology, Thiruvalluvar University, Serkkadu, Vellore, Tamil Nadu, India

Abstract

This study aimed to assess the apoptosis-inducing mechanism of zinc oxide nanoparticles (ZnO NPs) stabilized by Solanum xanthocarpum plant extract in human osteosarcoma MG63 cells. In the present study, we synthesized ZnO NPs from S. xanthocarpum extract and evaluated its anticancer mechanism on MG 63 cells. The synthesized ZnO NPs were characterized by ultraviolet spectroscopy, X-ray crystallography, transmission electron microscopy, energy dispersive X-ray, and Fourier-transform infrared spectroscopy analysis. The mean size of the synthesized ZnO NPs was 21.62 ± 7.45 nm and spherical in shape. The cytotoxicity of ZnO NPs on MG63 cells was determined by MTT assay. The Western blot analysis was carried out to examine the expression of apoptotic and autophagy-related proteins in MG63 cells. The findings of the study reveal that ZnO NPs treatment showed concentration-dependent cytotoxicity, increased lipid peroxidation, decreased antioxidant activity, increased reactive oxygen species generation, and increased DNA damage. In addition, ZnO NPs treatment increased the expression of apoptotic members such as p53, Bax, caspase-3, -8, and -9 while downregulating Bcl-2 expression in MG63 cells. Furthermore, ZnO NPs treatment suppressed the P13K/AKT/mTOR signaling pathway and increased the expression of LC3 and beclin-1 in MG63 cells. The present study demonstrated that ZnO NPs induced apoptosis and autophagy in MG63 cells through modifying apoptotic and autophagy-related proteins.

Keywords: Osteosarcoma; Solanum xanthocarpum; Zinc oxide nanoparticles; Apoptosis; Autophagy

1. Introduction

Osteosarcoma is a primary malignancy of bone tumor in humans, which has a high rate of metastasis[1]. Although a few detection and diagnostic methods have been developed, the severity and rapid metastasis of osteosarcoma probably contributes to the poor prognosis of people with the metastatic form of the malignancy[2]. Compared to single-
drug treatment like vincristine, nanoparticles can control cancer cell growth at various cell cycle stages, remove local or distant micro-metastases, and diminish the development of drug resistance. Among all malignancy cases, 40% have developed resistance to multidrug treatment after an early response to medication. In addition, 30% patients with multidrug resistance experience metastasis or recurrence within 5 years\(^9\). Disruption in transporter pumps, oncogenes, genes involved in tumor suppression, DNA repair mechanism, mitochondrial damage, autophagy, and epithelial-mesenchymal transition are some of the cellular events behind drug resistance\(^9\). Overall, the factors involved in drug resistance are complex and require further research. As a result, the underlying molecular processes and the involvement of biomarkers should be identified.

Nanotechnology has a significant impact on the development of anticancer and antibacterial drugs with higher efficacy. Nanoparticles with a size range of 1 – 100 nm offer unique properties due to their small size compared to their bulk counterpart, allowing them to be used in various industries, including electronics, energy, biomedicine, and healthcare. Physical, chemical, and biological techniques may be used to prepare nanoparticles, and they can be customized in size and form\(^6\). However, physical and chemical methods are rarely used for synthesis due to high cost and toxicity of the substances used. As a result, many researchers use biological approach to generate nanoparticles, including zinc oxide nanoparticles (ZnO NPs)\(^6\). The therapeutic abilities of ZnO NPs have caught the attention of scientists worldwide. A plethora of approaches for synthesizing ZnO NPs are available, allowing for easy control of ZnO NP size. Evidence showed that the level of cytotoxicity in cancer cells was related to the size of the ZnO NPs synthesized. Due to their semiconductor nature and distinct exterior characteristics, ZnO NPs cause cytotoxicity in malignant cells by generating reactive oxygen species (ROS), leading to oxidative stress and cell death\(^7\). Nonetheless, understanding the molecular mechanism involved in anticancer activity of ZnO NPs is deem important.

Various plant species have been reported to biosynthesize ZnO NPs which include *Garcinia mangostana*\(^8\), *Tamarindus indica*\(^9\), *Aeromonas hydrophila*\(^10\), and *Aloe barbadensis*\(^11\). *Solanum xanthocarpum* is a medicinal plant that belongs to the member of Solanaceae family. *S. xanthocarpum* has been used to treat various illnesses such as joint pain, inflammation, fever, and gastrointestinal diseases. Moreover, it has anti-tumorigenic, antioxidant, anti-inflammatory, diuretic, antipyretic, and antibacterial properties and can be used to treat sexually transmitted infections as well. The Solanaceae family plants contain a variety of compounds that are responsible for diverse bioactivity. The plant bioactive compounds encompassing glycoalkaloids, glycoproteins, polysaccharides, and polyphenolic substances that include gallic acid, catechin, protocatechuic acid, caffeic acid epicatechin, rutin, and naringenin were used for the preparation of ZnO NPs\(^12\). Several studies have been conducted to evaluate the anticancer activity of biologically prepared ZnO NPs; however, the formulation of ZnO NPs from *S. xanthocarpum* extract and its anticancer mechanism on osteosarcoma cells has not yet been studied. Hence, in this study, we synthesized ZnO NPs using *S. xanthocarpum* extract and investigate its molecular mechanism in inducing apoptosis in osteosarcoma MG63 cells.

## 2. Materials and methods

### 2.1. Chemicals

Zinc acetate, 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), non-fluorescent 2',7'-dichlorofluorescein diacetate (DCFH-DA), acridine orange (AO), ethidium bromide (EtBr), trypsin-ethylenediaminetetraacetic acid (EDTA), and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich, USA. Phosphate-buffered saline (PBS) solution (pH 7.2), Dulbecco’s modified eagle’s medium (DMEM), and all other chemicals were acquired from HiMedia Laboratories, India.

### 2.2. Preparation of plant extracts and synthesis of ZnO NPs

The disease-free fresh leaves of *S. xanthocarpum* were collected from the area around Jinan City, Shandong Province, China. The collected plant materials were splashed several times with pure water to remove dust particles. Further, the plant material was dried in shadow and minced into fine powder. 20 g of rinsed fine dried powder were combined with 100 mL of sterile water in a 250 mL flask to make the extract. After that, the mixture was boiled for 20 min, or until the aqueous solution color changed from watery to light yellow. Before being filtered using Whatman filter paper, the extract was left at room temperature to cool. The extract was then stored at room temperature for future analysis. For the amalgamation of ZnO NPs, 20 mL of the obtained extract was transferred into aqueous zinc acetate (3 mM, 80 mL). The reactant was incubated in the dark for 24 h using a mechanical stirrer. When zinc acetate was reduced entirely to ZnO NPs, the solution initially changed from colorless to dark brown. Afterward, the reaction solution was maintained at room temperature for another 24 h without shaking to precipitate the amalgamated nanoparticles thoroughly. Then, the settled particles were centrifuged for 10 min at 500 × g, and
the resulting residue was obtained and completely dried for further analysis.

2.3. Ultraviolet (UV)-vis spectra and size distribution analyses

After being harvested by centrifugation, the ZnO NPs were examined using UV-vis spectral analysis. The UV-vis spectrophotometer was used to record a spectral reading of amalgamated ZnO NPs at 300 – 700 nm wavelength (Hitachi, model U-2800). A light scattering crystallite size analyzer was used to explore the size and distribution of the prepared nanoparticles. After ultrasonication, ZnO NPs were placed into a sample stage, and then the size and its distribution were estimated using a computer-based dynamic light scattering analyzer.

2.4. Transmission electron microscopy (TEM) and elemental analyses

The produced ZnO NPs were mixed in water, which was deionized previously, and one drop of the mixture was placed on the sample stage and dried under a vacuum before being coated with carbon. Tecnai G-10, an 80KV transmission electron microscope, was used to obtain micrographs of carbon-coated nanoparticles. The produced ZnO NPs were deposited on a section of micro-glass slip closed by a carbon layer for energy dispersive X-ray (EDX) studies, and then permitted to dehydrate at 37°C. The mimicked elements were studied using the spectrum of EDX (BrukerAXS Incorporation., USA).

2.5. X-ray crystallography (XRD) and Fourier-transform infrared spectroscopy (FTIR) examinations

The ZnO NPs were thoroughly dehydrated for XRD analysis before being bonded in an XRD condition by the prominent 30 kV at 20 mA between and radiation through an XRD analyzer (Philips Model PW1252/36). The scanning temperature range was set from 20°C to 80°C. FTIR measurements were used to investigate the surface functional group of chemicals coupled with phyto produced ZnO NPs (Shimadzu 8201PC, Japan). The sample was mixed with 10 mL of deionized water and examined in KBr pellets using FT-IR with a resolution of 4 cm⁻¹.

2.6. Cell culture

The osteosarcoma MG63 and normal Vero cell lines were obtained from ATCC. The cells were cultivated in DMEM 37°C in a 5% CO₂ and 95% air environment (humidified incubation). The S. xanthocarpum-stabilized ZnO NPs were dissolved in dimethyl sulfoxide (DMSO) before being treated with the cells.

2.7. Cytotoxicity of ZnO NPs on MG63 cells

The cytotoxic effect of produced ZnO NPs on MG63 and Vero cells was measured by MTT assay. The MG63 and Vero cells were inoculated (1 × 10⁴ cells/well) in a 96-well plate and cultivated for 24 h in a humidified environment. Except for control, the cells were introduced to increasing dosages of ZnO NPs for 24 h. Further, the cells were subsequently exposed to MTT (100 µL, 5.0 mg/mL in PBS) for 4 h in dark conditions to form formazan crystals. The resulting formazans in the wells were diluted by 100 µL of DMSO. The optical density value of the well was scrutinized by a microplate reader (Tecan Multimode Reader, Austria). Regression analysis was used to determine the ZnO NP concentrations.

2.8. Biochemical assays

The biochemical assays for lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were conducted to analyze the LPO and endogenous antioxidant activity in ZnO NPs-treated MG63 cells. The cell suspension was obtained by trypsinization after treatment and processed for biochemical studies. The LPO biomarker, thiobarbituric acid reactive substances (TBARS) activity was assessed by adapting the procedure suggested previously. The antioxidant activity in MG63 cells was determined by quantifying the antioxidant markers SOD, CAT, and GPx by adapting the aforementioned protocols. The cells that remained untreated were designated as control.

2.9. Determination of ROS

The ZnO NPs-induced ROS generation in MG63 cells was determined by staining the cells with DCFH-DA, which was then oxidized into fluorescent dichlorofluorescein (DCF). The cells were inoculated and grown (1 × 10⁴ cells) for 24 h in six-well plate. After treatment with 15, 30, and 45 µg/mL of ZnO NPs for 24 h, the cells were administered 100 µL of DCFH-DA for 10 min under dark conditions, then the cells were observed under a fluorescence microscope equipped with appropriate filters. The cells were trypsinized and the fluorescence intensity of the cell suspension was estimated using a spectrofluorometer (Shimadzu RF-5301 PC). The untreated wells served as a control group.

2.10. Examination of apoptotic morphological changes

The ZnO NPs-mediated apoptotic sign represents the hallmark of morphological alterations in MG63 cells and this sign was confirmed by staining with AO and EtBr. The cells were inoculated in six-well plate and incubated in humidified incubator, and treated with various doses (15, 30, 45 µg/mL) of ZnO NPs for 24 h under the same humidified...
ZnO NPs induce apoptosis in MG63 cells

2.11. DAPI staining

The ZnO NPs-induced nuclear condensation in MG63 cells were assessed by staining the cell with a DAPI stain. The cells were inoculated (1 × 10⁵ cells) and seeded in six-well plates for 24 h. Then, the cells were exposed to 15, 30, and 45 µg/mL of ZnO NPs for 24 h. The cells were gently washed and fixed with paraformaldehyde before being cleaned and fixed again with 70% ethanol. After that, the cells were exposed to DAPI (1 mg/mL) and kept for 20 min in the dark. The treated ZnO NPs and untreated control cells were observed under the fluorescence microscope for nuclear condensation.

2.12. Determination of DNA damage (comet assay)

The gel electrophoresis method was used to determine the ZnO NPs-induced DNA damage in MG63 cells. The cells were obtained after administration with various doses (15, 30, and 45 µg/mL) of ZnO NPs. Then, the harvested cell suspension was transferred into a 1× PBS solution. After that, 10 mL of the suspensions were transferred to low melting 0.5 % agarose (60 mL), which was then laden into slides and allowed to solidify correctly. The slides were submerged in ice-cold lysis buffer for 1 h at 4°C after full solidification and then left for DNA to loosen up in electrophoresis solution for 30 min. The electrophoresis setup was operated at a continuous voltage of 22 V and 200 mA. Using 0.4 M Tris (pH 7.5), the slides were neutralized for 10 min, and then fixed using ethanol (70%). Further, slides were exposed to 0.5 mg/mL of ethidium bromide for 20 min in a dark room. The epifluorescence microscope captured the images using a 40× objective lens aided by a digital camera.

2.13. Determination of caspase 3, 8 and 9 activities

The ZnO NPs-induced modulations in caspase 3, 8, and 9 expressions in MG63 cells were assessed by adapting caspase assay as per manufacturer’s instructions. The MG63 cells were inoculated and grown in six-well plates for 24 h before being supplemented with different doses of ZnO NPs (15, 30, and 45 g/mL) for another 24 h. After the cells were collected, the cells were lysed by utilizing a lysis buffer containing 1 mM EDTA, 10 mM EGTA, 50 mM Tris-HCl, 10 mM digitonin, and 2 mM DTT. Further, the lysates were collected by centrifugation at 15,000 ×g for 1 h at 4°C, and then treated with caspase-3, 8, and 9 specific substrates in a 96-well plate with reaction buffer for 1 h at 37 °C. The caspase expressions were examined by detecting the optical density value at 405 nm wavelength using a spectrophotometric plate reader (BioRad, Tokyo, Japan), in adherence with steps described by Huang et al.[18] The assays were conducted in three independent experiments.

2.14. Western blot analysis

The cell suspension was obtained by trypsinization after a 24-h treatment with ZnO NPs. The collected suspension was processed for centrifugation and the pellet was collected. After rinsing with cold PBS, the pellet was lysed using RIPA lysis buffer (Pierce Biotechnology, IL, USA). The protein separation was achieved on a 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. After that, the membrane was blocked with 5% bovine serum albumin for 2 h at room temperature. Monoclonal primary antibodies (p53, Bcl-2, Bax, P13K, AKT, mTOR, LC3, beclin-1, P62, and β-actin 1:1000) were used to probe the membrane overnight at 4°C. The probed membrane was treated for 1 h with secondary antibodies that were horseradish peroxidase-conjugated. By following the manufacturer’s instructions, the protein bands were visualized using a chemiluminescence detection ECL kit (Amersham Biosciences, Buckinghamshire, UK).

2.15. Statistical analysis

The mean ± standard deviation of three replicates was used to display all results. One-way ANOVA analysis was used to analyze the significant differences between various groups using GraphPad Prism 5. P < 0.05, P < 0.01, and P < 0.001 were considered as statistically significance between different groups.

3. Results

3.1. UV-vis and particle size distribution analysis

The optical characteristics of metal oxide nanoparticles are highly dependent on the size, shape, and interaction of the constituents on the nanoparticles. After 48 h of incubation, the reaction solution changed from pale yellow to brown, indicating that ZnO NPs had been synthesized. The increased strong absorption arises at 380 nm in the UV-vis spectra readings affirms the amalgamation of ZnO NPs (Figure 1A). The formed ZnO NPs ranged from 5 to 60 nm and had a mean size of 21.62±7.45 nm in diameter (Figure 1B).

3.2. TEM and EDX analysis

The TEM observation shows that the ZnO NPs were spherical and cylindrical. The ZnO NPs size had ranged...
from 5 nm to 60 nm. This suggests that the particles were synthesized with near-size uniformity (Figure 2A). The elemental properties of produced ZnO NPs were assessed using the EDX patterns of ZnO NPs. The EDX profile of ZnO-NPs displays only zinc and oxygen atoms, showing that the ZnO-NPs synthesized was impurity-free. Since the identification lines for the principal emission energies for zinc and oxygen match the peaks in the spectrum, we consider that zinc has been accurately identified (Figure 2B).

3.3. XRD analysis

The XRD analysis reveals the nature of the produced ZnO NPs. The peaks raised in XRD at 31.77°, 34.44°, 36.26°, 47.52°, 56.58°, 62.85°, and 67.91° correspond to the lattice plane of (100), (002), (101), (102), (110), (112), and (201), respectively, suggesting the spherically shaped crystal structure of the nanoparticle (Figure 2C).

3.4. FTIR analysis

FTIR spectral examination was employed to determine the association of bioactive molecules with the zinc ions. The unique signals were acquired from the substance-distinct vibrations of the ZnO NPs-coupled biomolecules (Figure 3). The band at 3424-3452 cm⁻¹ assisted in determining the functional groups associated with nanoparticles. The strong, deep absorption peak at 3424 cm⁻¹ represents the stretching of alcohol groups O-H. The C=C stretching vibrations of primary amines were responsible for the absorption band at about 1632 cm⁻¹. The O-H vibrations of aromatic groups were responsible for the vibration bands observed at 1382 cm⁻¹. The 1110 cm⁻¹ band shows the existence of C-O stretching in alcohol, carboxylic acids, an ester, and other group compounds. The band verifies the stretching vibration of ZnO NPs at 452 cm⁻¹.

3.5. Cytotoxic effect of ZnO NPs on MG63 cells

The ZnO NPs-induced cytotoxic effect on MG63 and normal Vero cells were determined by MTT assay. The administration of ZnO NPs has attenuated the cell proliferation in MG63 cells in a dose-dependent manner. The concentration of ZnO NPs required for 50% inhibition of MG63 cells was recorded as 28.12 ± 0.42 µg/mL. As a result, we selected dosages of 15, 30, and 45 µg/mL for further investigations. ZnO NPs showed no significant cytotoxicity in normal Vero cells (Figure 4).

3.6. Effect of ZnO NPs on antioxidant enzymes and LPO

Apoptotic hallmarks in cancer cells are characterized by increased LPO activity and diminished antioxidant activity. Depending on the doses used, the TBARS activity has considerably enhanced in cells incubated with ZnO NPs compared to cells that remained untreated. The levels of SOD, CAT, and GPx in ZnO NPs-exposed MG63 cells were significantly attenuated depending on the quantities of ZnO NPs used compared to untreated cells (Figure 5A).

3.7. Effect of ZnO NPs on ROS activity

The augmentation of ROS production in cancer cells is a prominent hallmark of oxidative stress-induced apoptosis. The DCF fluorescence emission of control and ZnO NPs-administered cells was assessed, and the results are presented in Figure 5B. The augmented green fluorescence depth was observed in ZnO NPs-exposed MG63 cells according to the doses utilized, indicating ZnO NPs-induced ROS production. The DCF fluorescence depth in MG63 cells is depicted graphically (Figure 5C). These results suggest that the high fluorescence intensity is due to the augmented intracellular ROS activity in MG63 cells induced by ZnO NPs.
3.8. Effect of ZnO NPs on apoptotic morphological changes

Cell shrinkage and membrane blebbing are essential hallmarks of apoptosis in cancer cells. ZnO NPs induced apoptotic changes in MG63 cells morphology, which were evaluated using a dual staining method after 24 h of treatment. Compared to the control cells which displayed morphologically distinct healthy cells, the number of detached cells with shrunken shapes increased in ZnO NPs-treated cells. The orange-colored, round-shaped cells in the ZnO NPs-exposed group (45 µg) indicate the late apoptotic cells, evidenced by membrane damage and damaged nuclei in MG63 cells (Figure 6A).

3.9. Effect of ZnO NPs on chromatin condensation in MG63 cells

DAPI staining was used to decide whether ZnO NPs caused condensation in chromatin and nuclear fragmentation in cancer cells. These hallmarks in ZnO NPs-exposed cells were determined by DAPI staining, which indicates that the ZnO NPs caused nuclear damage in MG63 cells. However, the untreated cells remained with intact nuclei (Figure 6A).
ZnO NPs induce apoptosis in MG63 cells

**Figure 5.** (A) Effect of ZnO NPs on LPO marker (TBARS) and antioxidants SOD, CAT, and GPx in MG63 cells. (B) The effect of ZnO NPs on ROS activity in MG63 cells. The images show that the increased green fluorescence on ZnO NPs-treated cells indicates the increased ROS activity in MG63 cells. (C) The graphical presentation of intracellular ROS production was detected by spectrofluorometer. The bars represent the mean ± standard deviation of three experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control group. The scale bar is 50 µm.

**Figure 6.** (A) The effect of ZnO NPs on apoptotic morphological changes, nuclear fragmentation, and DNA damage in MG63 cells. Apoptotic morphological changes were determined by AO/EB staining, DAPI staining explores the nuclear condensation and fragmentation in MG63 cells. The comet assay shows the DNA damage in MG63 cells. (B) The effect of ZnO NPs on the activities of caspase-3, -8, and -9 in MG63 cells. The graphical illustration shows the percentage of caspase activity. The bars represent mean ± standard deviation of three experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control group. The scale bar is 50 µm.
3.10. Effect of ZnO NPs on DNA damage
The comet test demonstrated that the ZnO NPs caused DNA damage in MG63 cells. In untreated control cells, we observed normal, intact DNA in the electrophoresis plot. On the contrary, the damaged increased head DNA was detected in ZnO NPs-exposed cells. The increased DNA head DNA was directly associated with increasing concentrations of ZnO NPs (Figure 6A).

3.11. Effect of ZnO NPs on caspase-3, -8, and -9
The expression of caspase-3, -8, and -9 in MG63 cells was ascertained by standard protocols. When comparing ZnO NPs-administered MG63 cells to control cells, the levels of caspase-3, -8, and -9 were increased in a concentration-dependent manner. The graphical representation of caspase-3, -8, and -9 is depicted graphically (Figure 6B).

3.12. Effect of ZnO NPs on p53, Bcl-2, and Bax protein expression
The expression of apoptotic proteins induced by ZnO NPs in MG63 cells was determined using Western blots and the relevant antibodies. The treatment of produced ZnO NPs significantly increased the expression of p53 and Bax proteins but dramatically decreased the expression of Bcl-2 proteins, according to concentrations used (Figure 7A and B).

3.13. Effect of ZnO NPs on PI3K/Akt/mTOR and LC3 signaling pathway
Cell cycle, proliferation, apoptosis, and autophagy are all regulated by the PI3K/Akt/mTOR signaling cascade. The function of the PI3K/Akt/mTOR cascade in ZnO NPs-induced apoptosis in MG63 cells was investigated utilizing phosphorylated antibodies in a Western blot assay. Western blot analysis was used to quantify the expression of apoptosis-related proteins in ZnO NPs-treated MG63 cells. In comparison to untreated cells, the injection of ZnO NPs significantly reduced the expression levels of p-P13K, p-AKT, and p-mTOR, depending on the concentrations applied (Figure 8A and B). The activation of multiple conjugation processes mediate the lipidation of LC3 onto cell membranes, thereby transforming LC3-I to LC3-II. The LC3-positive puncta indicative of this lipided form is required for autophagosome formation. The treatment of ZnO NPs at the doses utilized in MG63 cells caused the conversion of LC3-1 to its lipitated LC3-2 form, as shown by Western blot results. Furthermore, ZnO NPs treatment considerably increased beclin1 expression. Nevertheless, another autophagy marker, P62, was dramatically decreased by MG63 treatment at doses determined (Figure 8C). The relative expression of LC3, beclin-1, and P62 versus β-actin is depicted in the graph (Figure 8D).

4. Discussion
Most currently available anticancer medications fail to reach their intended target and are harmful to human health. Therefore, it is necessary to identify effective and safer anticancer medicines or formulations[18]. The ZnO NPs are a popular metal-based nano-formulation that demonstrates a significant cytotoxic effect on many cancers, including cervical, breast, lymphomas, leukemia, bone, brain, and colon cancers[19]. The selective cytotoxicity of ZnO NPs, which inhibits the proliferation of cancer cells while boosting the growth of non-dividing healthy cells, indicates that increasing sensitivity to ZnO NPs can cause cell death. The ZnO NPs have been widely investigated for anticancer treatments against quickly dividing malignant cells due to their intrinsic selective toxicity. The stimulation of extreme ROS generation serves as the major factor in triggering apoptosis[20].

In this study, ZnO nanoparticles using *S. xanthocarpum* extract were produced. The ZnO NPs were first observed by measuring UV absorbance, which revealed an enhanced peak at 380 nm, consistent with a study by Santhoshkumar et al.[21]. The existence of various saponin, alcohol, phenol, and amine groups of chemicals responsible for stabilizing ZnO NPs were observed by the FTIR investigations of the produced ZnO NPs. According to the researchers, the nanoparticles are guarded by the phytochemicals found in *S. xanthocarpum* leaf extract. Saponins, alkaloids, glycosides, phenols, and flavonoids were commonly found in *S. xanthocarpum*. When zinc acetate is used as a precursor, the ZnO NPs form tiny sphere-shaped particles that gather like bullets over time. We employed zinc acetate as a precursor, which was reduced by *S. xanthocarpum* extract and resulted in nano-sized spherical nanoparticles. In theory, zinc and oxygen have a stoichiometric mass of 80.3% and 19.7%, respectively[22].
The activation of excessive free radicals generation, which acts as the key mechanism to trigger apoptosis in tumor cells, could advance the proposed mechanism of nanoparticles-induced cytotoxicity in cancerous cells\cite{21}. In the current research, the treatment of ZnO NPs induced augmented ROS production in MG63 cells according to the concentrations administered. The increased activity of LPO is a significant example of oxidative stress activated by the extreme generation of endogenous oxidative free radicals in cancer cells. The induced apoptotic features in cancer cells include the augmented LPO with diminished endogenous antioxidant activity\cite{25}. SOD is a crucial antioxidant in cellular metabolism that protects against indigenous free radicals. In cancer and asthmatic cases, the SOD activity was reduced compared to healthy adults\cite{26}. In the current investigation, we noticed the diminished antioxidant activities and increased ROS activities in ZnO NPs-administered MG63 cells. It may be due to an overabundance of magnesium ions released into the cancer cell microenvironment. In malignant cells, the release of magnesium ions leads to an increased intracellular ROS activity, which activates apoptosis through the apoptotic signaling pathway. Oxidative stress generated by ROS can result in the expression of apoptotic protein p53, inducing DNA, protein, and lipid damage in cancer cells. ROS-mediated cell regulation will be regulated by oxidative modifications of oxidative transcriptional regulators and intermediary signaling molecules\cite{27}.

Numerous studies proposed that the ZnO NPs exert significant cytotoxic activity on various human cancer cell lines by inducing increased ROS activity while diminishing the activities of intracellular antioxidants. The loss of mitochondrial membrane integrity resulted in the opening of outer membrane pores, release of cytochrome-c and activation of caspase proteins. It is well established that MMP-related cell death was triggered by an imbalance in the Bcl-2/Bax ratios and the stimulation of caspase-9\cite{28}. Most anticancer drugs characteristically destroy DNA and form DNA breaks to induce apoptosis\cite{29}. As a result, the generation of ROS in nanoparticle-exposed cells was
ZnO NPs induce apoptosis in MG63 cells

In cancer cells, autophagy is considered an alternative therapeutic target[37]. Autophagy entails the production of autophagosomes, which encircle and encapsulate injured organelles or cellular detritus before fusing with lysosomes to destroy their contents[38]. Autophagy is defined by the involvement of LC3 in autophagosomes and the alteration of LC3-I to LC3-II[39]. According to Western blot results, administration of ZnO NPs in MG63 cells promoted the conversion of LC3-I to LC3-II in a dose-dependent manner. LC3-II binds to P62, a protein involved in protein trafficking to the proteasome and autophagic breakdown of ubiquitinated protein aggregates. When autophagy is defective, p62 accumulates and is normally destroyed by autophagosomes[40]. ZnO NPs treatment also reduced the expression of p62 levels in MG63 cells in this investigation. The effect of ZnO NPs on apoptosis induction in MG63 could be utilized for conceiving an alternative chemotherapeutic formulation containing the currently available anti-cancer drugs to treat osteosarcoma. However, proper in vivo experiments should be carried out to explore its effect on other biochemical markers.

5. Conclusion

We synthesized ZnO NPs from S. xanthocarpum leaves extract and investigated their anticancer activities on human osteosarcoma MG63 cells. Based on our findings, ZnO NPs induced ROS-mediated apoptosis and DNA damage in MG63 cells. Furthermore, ZnO NPs influenced the expression of apoptotic proteins, such as p53, Bax, Caspase-3, Caspase-8, and Caspase-9. Moreover, ZnO NPs induced apoptosis and autophagy in MG63 cells by inhibiting the P13K/AKT/mTOR signaling pathway and increasing the expression of pro-apoptotic proteins, such as p53, Bax, Caspase-3, Caspase-8, and Caspase-9. As per a previous report, when HepG2 cells were treated with ZnO NPs, the expression of p53 and Bax was notably high, while anti-apoptotic Bcl-2 members were inactivated[31].

The PI3K/Akt/mTOR signaling cascade is the most commonly impaired in cancer cells[36]. Most studies conducted on cancer cells found an increase in Akt protein expression from 50% to 70% due to PI3K/AKT/mTOR signaling[35]. It is shown that a drug that suppresses the overexpression of PI3K/AKT/mTOR signaling molecules may be a potent drug for the treatment of lung cancer. Therefore, we evaluated the effect of ZnO NPs on the inhibition of PI3K/AKT/mTOR signaling in the MG63 cells. The PI3Ks consist of three types of lipid kinases, with class IA PI3Ks being the most frequently altered in cancer cases. Receptor tyrosine kinases activate PI3K, and active PI3K activates AKT further. AKT activation then phosphorylates downstream PDK1 and mTOR molecules, and subsequently activating transcription factors involved in cell survival, growth, and proliferation[30]. In the present study, ZnO NPs effectively induced apoptosis and autophagy by inhibiting PI3K/Akt/mTOR signaling pathway. Hence, ZnO NPs are considered a potent anticancer candidate to treat osteosarcoma.

Acknowledgments

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Agilan Balupillai  
Data curation: Muthulakshmi Kannaiyan  
Formal analysis: Ernest David, Babujanarthanam Ranganathan, Vijayanand Selvaraj
Investigation: Satheeshkumar Subramaniyan, Yoganathan Kamaraj, Veenayohini Kumaresan

Resource: Agilan Balupillai

Writing – original draft: Satheeshkumar Subramaniyan, Yoganathan Kamaraj, Veenayohini Kumaresan, Agilan Balupillai

Writing – review and editing: Yoganathan Kamaraj

References


ZnO NPs induce apoptosis in MG63 cells


https://doi.org/10.1016/j.reffit.2017.05.001


https://doi.org/10.1080/17518253.2018.1547925


https://doi.org/10.1016/j.reffit.2017.05.001


https://doi.org/10.7314/apjcp.2012.13.6.2753


https://doi.org/10.1093/jpp/rgab015


https://doi.org/10.1016/j.nantod.2010.02.001


https://doi.org/10.1007/s10495-012-0705-6


https://doi.org/10.1038/sj.onc.1208332


https://doi.org/10.1038/nrm2308


https://doi.org/10.1038/bjc.1998.257


https://doi.org/10.1016/j.ijbiomac.2020.02.007


https://doi.org/10.1200/jco.2011.29.15_suppl.3035


https://doi.org/10.1016/j.lcr.2014.06.006


https://doi.org/10.3892/ol.2017.6953


https://doi.org/10.1016/j.bjcb.2010.07.003


https://doi.org/10.1016/j.biochi.2007.08.014


https://doi.org/10.1093/emboj/19.21.5720


https://doi.org/10.1016/j.cell.2009.03.048