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The Effect of Biosynthesized Zinc Oxide Nanoparticles Capped by *Scenedesmus Obliquus* on Apoptosis and Gene Expression of p53 and VEGF in Breast Cancer Cell Line

Running Title: Exploring the anti-cancer properties of biosynthesized zinc oxide nanoparticles from *Scenedesmus obliquus*

Zohreh Shirkhani* **and Simin Tajik Esmaeili**

Department of Plant Sciences, Faculty of Biological Sciences, Kharazmi University, Postal code 15719-14911, Tehran, Iran

*Corresponding author: Email: z.shirkhani@khu.ac.ir, 0000-0001-8058-7814, +982188329220

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ABSTRACT

Breast cancer is the most prevalent form of cancer in women. This condition poses a significant public health concern that requires further research to understand its prognosis better and proper treatment options. *Scenedesmus obiquus* algae has unique biological compounds that can be effective in the synthesis of nanoparticles. The objective of this study is to examine how the impact of ZnO NPs, which are produced by the *S. obliquus* algae, affects cell death and gene expression in a breast cancer cell line. MDA-MB-231 cells are a subtype of breast cancer cells known for their high aggressiveness and connection to the triple-negative breast cancer subtype. The impact of biosynthesized ZnO NPs (0, 800, 1600, and 2400 µg/ml) on apoptosis of the MDA-MB-231 cell line and VEGF and p53 gene expression in breast cancer cells was assessed. The absorption band at 362 nm exhibited an absorption maximum that confirmed the synthesis of ZnO NPs. Zinc oxide nanoparticles exhibited significant toxicity towards MDA-MB-231 cell lines, with an IC50 value of 1050 µg/ml. The findings indicated that 1600 µg/ml of ZnO NPs had the most potent inhibitory effect on the growth (71.1%) of MDA-MB-231cultures. The ZnO NPs increased the expression of p53 and reduced the expression of VEGF. This study demonstrates that nanoparticles created using *S. obliquus* algae, which possess anti-cancer properties, have potential effectiveness in treating cancer. However, it is essential to conduct appropriate *in vivo* studies to investigate its impact on additional biochemical indicators.

Keywords: Cell death, Green synthesis, MDA-MB-231 cell line, IC50

INTRODUCTION

Cancer has emerged as a critical global public health concern in contemporary times. Cancer development stems from disruptions in the normal cellular proliferation process, frequently arising due to genetic mutations or dysregulation of genes involved in controlling cell division. Breast cancer in developed countries accounts for about 10% of all cancers and 30% of women's cancers. Approximately 1 out of every 8 women are diagnosed with it. It is worth noting that more than 40% of breast cancer cases occur in women over the age of 50. Additionally, within this age group, around 65% of breast cancer-related deaths occur [1, 2]. The rise in the global incidence of breast cancer has emerged as a significant and concerning health issue. One of the challenges in effectively managing this disease is the development of drug resistance, whereby patients exhibit reduced responsiveness to various drugs with distinct mechanisms of action. Consequently, there is a pressing need to explore novel approaches for controlling cancer progression and identifying targeted therapies to address this issue [3, 4].

In recent years, there has been a growing interest in developing specialized nanoparticles to inhibit cancer tumors, making it a compelling area of research and a potential solution for cancer treatment [3, 4]. Nanoparticles exhibit distinct characteristics and behaviors compared to larger particles, which has led to their expanding application in various fields. Notably, nanoparticles with small particle sizes and high surface areas relative to their volume have demonstrated promising efficacy in cancer treatment. Zinc oxide nanoparticles possess distinct characteristics that set them apart from other types of nanoparticles, rendering them particularly suitable for

biomedical applications. These properties encompass optical, catalytic, semiconductor, piezoelectric, and magnetic attributes. Scientific investigations have demonstrated that the toxicity of zinc oxide nanoparticles induces cell death specifically in cancer cells. Furthermore, the release of Zn^{2+} ions from zinc oxide nanoparticles exhibits cytotoxic properties and triggers apoptotic activity in cells [5-8].

The green synthesis of nanoparticles utilizing biological materials as reducing agents encompasses a range of methods. These methods involve utilizing diverse substances such as microorganisms, marine organisms, micro-fluids, and plant extracts. These biological materials serve as effective reducing agents in the synthesis process, enabling the production of nanoparticles in an environmentally friendly manner. By harnessing the reducing capabilities of these natural sources, researchers have been able to develop sustainable approaches for nanoparticle synthesis, minimizing the use of hazardous chemicals and promoting eco-friendly practices [9-12].

Algae are a category of autotrophic organisms that hold significance in economic and ecological realms. They are utilized in nanoparticle synthesis due to their ability to efficiently accumulate metals, ease of cultivation and handling, adaptability to low temperatures, and minimal environmental toxicity. The widespread use of algae in this context is primarily attributed to their exceptional metal absorption capabilities, cost-effectiveness in production, and capacity to generate nanoparticles on a large scale [13].

Scenedesmus, a prevalent genus of freshwater algae, has gained prominence in microalgae biotechnology research due to its widespread occurrence and the convenience it offers in terms of cultivation, harvesting, and drying processes [14]. The extract derived from *Scenedesmus obliquus* algae has demonstrated potential anticancer properties against various cancer cell lines, including human breast MCF7, liver HePG2, colon HCT116, and human cervical adenocarcinoma HeLa [15-18]. Furthermore, this algae, known for its bioactive compounds, exhibits regenerative capabilities [19]. Based on the findings mentioned, the objective was to investigate the impact of ZnO nanoparticles (NPs) synthesized using *S. obliquus* algae on the expression of p53 and VEGF genes in breast cancer. The hypothesis posits that the algae extract may enhance the regeneration of nanoparticle synthesis and exert a positive influence on tumor genes, thereby impeding the proliferation of breast cancer cells.

MATERIAL AND METHODS

Reagents and Cell Lines

The necessary components for the polymerase chain reaction (PCR), including chemicals, restriction enzymes, and polymerases, were sourced from Fermentas, a division of Thermo Fisher Scientific Inc., located in Waltham, USA. The MDA-MB-231 triple-negative breast cancer cell line was procured from the National Cell Bank of Iran (NCBI), from the Pasture Institute of Iran in Tehran. The cells were cultured in Dulbecco's Modified Eagle medium, also obtained from Thermo Fisher Scientific Inc., supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cell cultures were maintained in a 5% CO2 environment at a temperature of 37 °C. Subcultures in the logarithmic growth phase were cultivated at 37 °C, washed with phosphate-buffered saline (PBS), and quantified using turbidimetric methods.

Algae Sampling and Preparation

Scenedesmus obliquus was procured from the Parsian Microalgae Company, Guilan, Iran. Within the laboratory setting, the morphological characteristics of *Scenedesmus obliquus* were confirmed, employing both visual examination and the assistance of the AlgaeBase valid identification key [20, 21]. To facilitate the extraction process and eliminate salts, the algae were immersed in water, with regular water replacement intervals. Following this, the samples were spread out and subjected to a drying period of ten days. For the extraction procedure, 100 g of dried algae powder was combined with 250 ml of deionized water and subjected to boiling for 60 min. Subsequently, the samples were allowed to rest for 48 h at the temperature of 35°C, after which they were filtered using filter paper [22].

Synthesis of Zinc Oxide Nanoparticles by *S. Obliquus*

In this experiment, a 10 ml solution of 2 mM zinc acetate dihydrate was combined with 5 ml of algae extract at room temperature. The resulting mixture was stirred continuously for one hour in a dark chamber at room temperature. The reduction of ions occurred rapidly, leading to a noticeable change in the solution's color from a

pale yellow hue to a dark brown shade, which remained stable. This color change indicated the successful formation of zinc oxide nanoparticles (ZnO NPs). To validate the synthesis of nanoparticles and the reduction process, spectra were obtained using a visible spectrophotometer (Perkin-Elmer Lambda 25, Boston, MA, USA) within the wavelength range of 300 to 750 nm. Additionally, the surface morphology of the synthesized ZnO NPs was examined using Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (FESEM-EDAX) via Quanta FEG 450.

Treatment of Cells with Synthesized Nanoparticles

Four concentrations (0, 800, 1600, and 2400 µg/ml) were prepared using nanoparticles in RPMI 1640 culture medium. To ensure uniformity and proper mixing of the extract with the medium, 12 drops of DMSO (dimethyl sulfoxide) were added. MDA-MB-231 cells were then cultured in a 96-well plate containing the culture medium mixed with the algae extract. The plate was subsequently incubated at a temperature of 47 °C.

Examination of Cell Viability

The cell line was subjected to a 48-hour incubation period with the algal extracts at various concentrations (0, 800, 1600, and 2400 µg/ml). To assess cell viability, the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was employed [23].

Assessment of Cell Death

The ability of synthesized nanoparticles to cause cell death was assessed in a laboratory setting. After a 48-hour exposure to the nanoparticles, the cells were collected and analyzed using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit from Bioligand, San Diego, following the manufacturer's instructions. The cells, treated with ZnO NPs at a density of 1 x 106 cells/ml, were washed with phosphate-buffered saline (PBS) and then recovered through centrifugation (2000 rpm, 6 min). Next, they were mixed in 100 µl of phosphate buffer and incubated with Annexin V-FITC and PI for 15 minutes in the dark at room temperature. The cells were ultimately collected and analyzed using an FC-500 flow cytometer from Beckman Coulter in less than 1 hour, with analysis performed using Beckman CXP software.

Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR)

The control and treated MDA-MB-231 cells (1×10^5) were trypsinized, centrifuged at 4400 rpm, and then rinsed with 1x PBS. The collected cells were subsequently subjected to RNA extraction, followed by conversion to cDNA. To determine the expression levels of VEGF and p53 mRNA in the treated and control cells, Applied qPCR Biosystems (Foster City, CA, USA) was utilized, following the methodology outlined by Livak & Schmittgen (2001). The housekeeping gene, GAPDH, was employed as a control gene. Primer sequences were designed using Primer 3 plus, as detailed in Table 1.

Table 1 The primer specifications used in this study

Gene	Forward Primer (5´ to 3´)	Reverse Primer (3´ to 5´)
VEGF	CACCATCGACAGAACAGTCC	GAATCCAATTCCAAGAGGGA
p53	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
GAPDH	TGTGTCCGTCGTGGGGCTGA	CCTGCTTCACCACCTCGCTGA

Statistical Analysis

Statistical analyses were conducted using SPSS 25 software. A one-way ANOVA analysis was employed to assess significant differences, with a p-value < 0.05 considered statistically significant. Each treatment was replicated three times in all assays.

RESULTS

UV–Visible Spectroscopic/Fe-SEM Analysis of ZnO NPs

In this study, it was observed the reduction of zinc oxide ions through visual characteristics and visible-ultraviolet spectrum. The ultraviolet-visible spectrophotometry data obtained at 1 nm wavelength, as shown in Fig. 1a,

revealed that the absorption band at 362 nm exhibited an absorption maximum of 0.68 A. This finding confirms the presence of a plasmo-genic resonance absorption band of ZnO NPs (Fig. 1a). The morphology of the nanoparticles was analyzed using scanning electron micros b , which showed that the ZnO NPs had a hexagonal shape with a size of less than 60 nm (Fig. 1B).

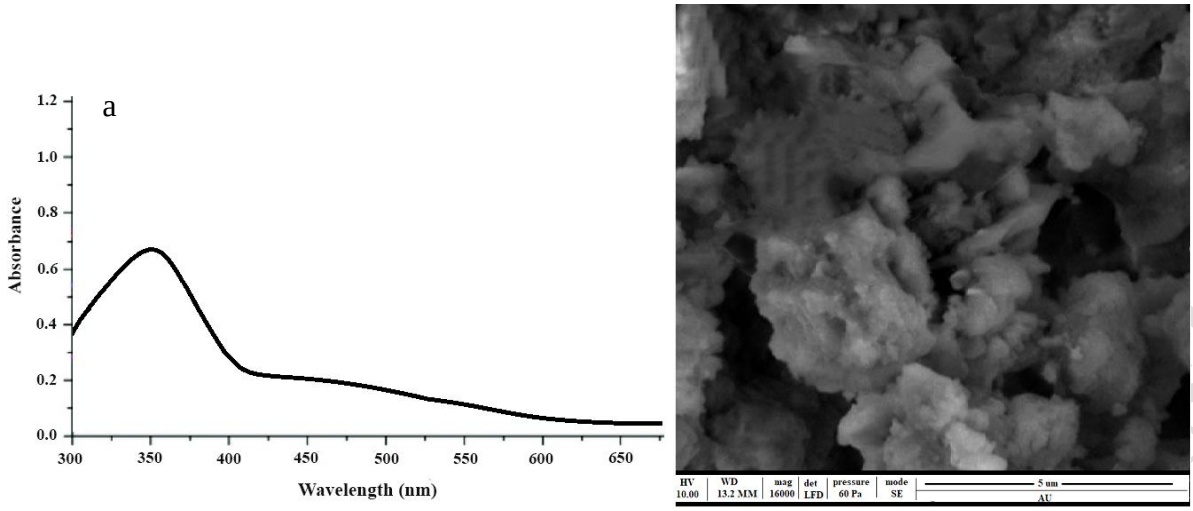

Fig. 1 a.UV–vis spectra of the biosynthesized ZnO NPs capped by S. obliquus extracts; b. FE-SEM image

Toxicity Results of ZnO NPs by MTT Assay Method

The findings illustrated in Fig. 2 demonstrate that cell proliferation is suppressed considerably in a concentration-dependent manner following 48 hours of exposure to nanoparticles. The calculation for the 50% inhibitory concentration (IC 50) yielded a value of 1050 µg/ml.

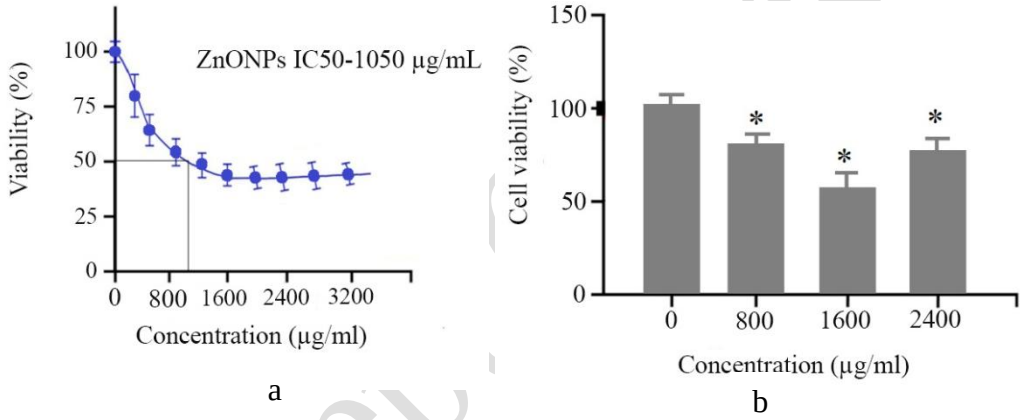

Fig. 2 The effect of algae extracts on cell viability. a. The effect of concentrations on the induction of cell death (IC50); b. MDA-MB cells were treated with ZnO NPs (0, 800, 1600, and 2400 µg/ml) for 48 h and assessed by MTT Assay

Apoptotic Cell Death Assay

Cells were cultured with or without nanoparticles at various concentrations for 48 hours and then treated with Annexin V/PI staining. The results demonstrated that the ZnO NPs could induce cell death at different levels depending on the concentration. The results showed that in the group treated with 0, 800, 1600, and 2400 µg/ml of ZnO NPs, apoptosis was 1.44%, 9.23%, 71.1%, and 50.23%, respectively. Overall, the concentration of 1600 exhibited the greatest increase in apoptosis (Fig. 3).

Real-Time Quantitative Reverse Transcription PCR Analysis

The levels of VEGF and p53 mRNA in MDA-MB-231 cancer cells were determined through the application of Real-Time qRT-PCR. The results showed a significant downregulation ($p < 0.05$) of VEGF gene expression in the treated cells compared to the untreated cells (Fig. 4a). Conversely, the expression of the tumor suppressor gene p53 was significantly increased ($p < 0.05$) in cells that were treated with ZnO NPs (Fig. 4b).

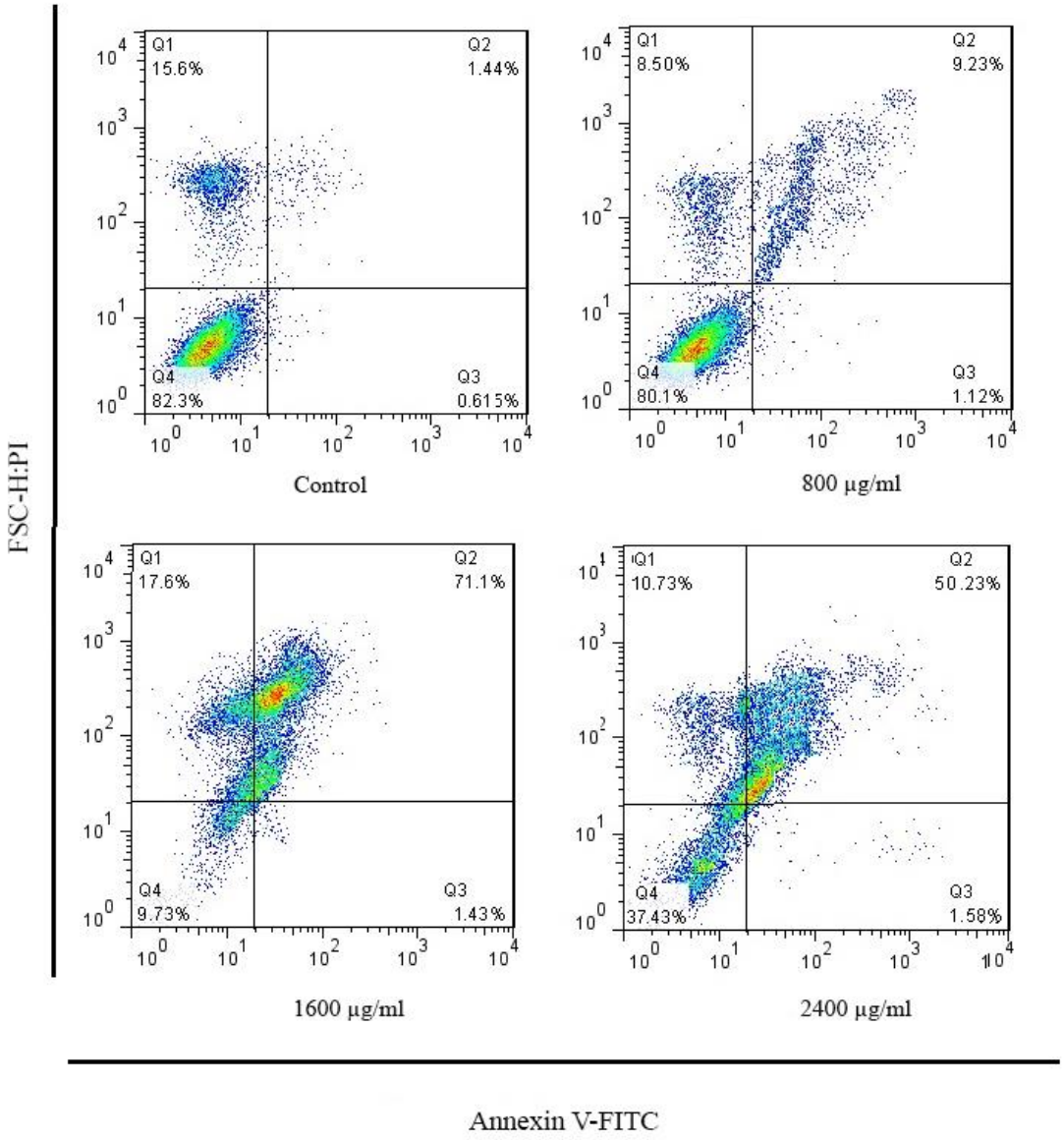

Fig. 3 The effect of different concentrations of ZnO NPs on cell apoptosis in laboratory conditions. Flow cytometry dot plots show the percentage of apoptotic cells (Annexin V+/PI+) after 48 hours of treatment

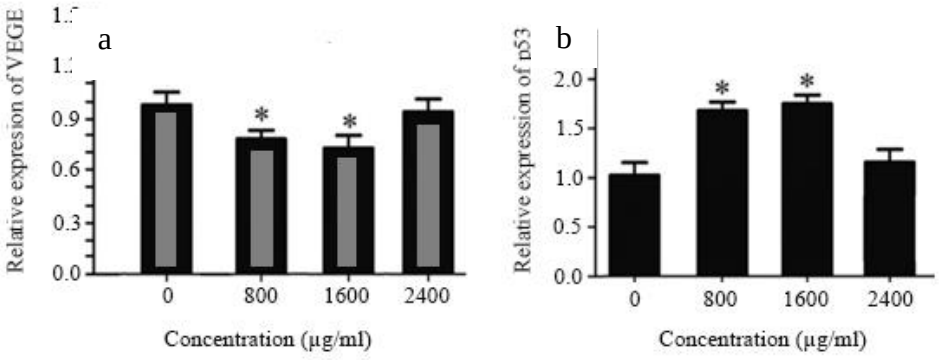

Fig. 4 The relative expression level of (a) VEGF and (b) p53 in MDA-MB-231 cancer cells treated with different concentrations of ZnO NPs. * indicate a significant difference between the experimental groups and control ($P \leq 0.05$). (means ± SD, n = 3)

DISCUSSION

Breast cancer ranks among the top three most prevalent cancers globally. Detecting breast cancer in its early stages offers a chance for a potential cure. Treatment options have advanced significantly in recent years, leading to a decrease in the intensity of therapy for both local and systemic treatment. There is now a growing emphasis

on exploring alternative therapies in the field of breast cancer treatment [25, 26]. The average diameter of human cells is around 10 μm. However, the components within cells are significantly smaller, falling within the sub-micron size range. Proteins, which are even smaller, typically measure just 5 nm in size, similar to the smallest manmade nanoparticles. This comparison highlights the potential of nanoparticles as tiny probes that could enable the observation of cellular processes with minimal disruption [27, 28]. While nanoparticles can be created from a variety of materials, it is crucial to take into account compatibility and toxicity when designing nanomaterials for use in biomedical applications [29].

The significance of nanoparticles has garnered researchers' interest in devising optimal approaches for synthesizing nanoparticles across different scientific domains [23]. In recent years, the utilization of biological methods in fabricating metal nanoparticles has gained considerable attention owing to its advantages over conventional chemical methods [30]. Metal-based nanoparticles are particularly notable among the different types of nanoparticles for their outstanding performance and convenience in imaging techniques [31].

Zinc oxide (ZnO) is classified as a 'GRAS' (generally recognized as safe) substance by the Food and Drug Administration (FDA). This difference has made ZnO a popular and favored material in nanomedicine [29]. Many studies have shown the anticancer properties of ZnO NPs, but their therapeutic efficiency has been different in different studies [32-35]. The selective cytotoxicity of ZnO NPs, which hinders the growth of cancer cells while promoting the development of non-dividing normal cells, suggests that enhanced susceptibility to ZnO NPs can lead to cellular demise. ZnO NPs have been extensively studied for their potential in combating rapidly dividing cancerous cells because of their inherent selective toxicity. The induction of high levels of reactive oxygen species (ROS) plays a crucial role in initiating apoptosis [36]. Considering that in the past, due to its pigments and biological compounds, the regenerative properties of *S. obliquus* have been proven [19], in this research, the aqueous extract of this algae was used. With the growth of various cancer types and the initiation of their successful treatment profile, recent study has been conducted to measure the anticancer performance of ZnO NPs synthesized with *S. obliquus* algae.

Various nanoparticles have been produced using *S. obliquus*, and the results demonstrated that this algae can enhance the effectiveness of treatment and yield favorable outcomes. Sharif *et al.* (2023) discovered that nanocomposites synthesized with *S. obliquus* induced apoptosis in 87.11% of treated cells. Additionally, cell cycle arrest was observed in 94.77% of cells [37]. Numerous research studies have illustrated that enhancing the qualities of ZnO NPs through the integration of substances and metabolites present in plants and algae can augment the effectiveness of its anticancer characteristics [38, 39]. The present study has substantiated the ability of ZnO NPs to impede the proliferation of breast cancer cells. The nanoparticles were successful in suppressing 71.3 cells in the G2 phase. Subramaniyan *et al.* (2022) showed the *Solanum xanthocarpum*-stabilized ZnO NPs induced cytotoxic effects on MG63 cells. Histopathological findings indicated that the use of ZnO NPs can hinder tumor growth and extend the lifespan of affected animals. Furthermore, ZnO NPs demonstrated a significant increase in the expression of miR-34a and miR-125b and played a crucial role in the PI3K/AKT/mTOR pathway [39]. Cellular processes like changes in apoptosis, cell growth, and how cells react to cancer therapies are significantly impacted by pro-apoptotic and anti-apoptotic proteins. The majority of the ways in which anti-cancer medications trigger cell death in cancerous cells involve increasing the expression of pro-apoptotic proteins while reducing the expression of anti-apoptotic proteins [40].

In this research, the significance of p53 as a crucial gene in controlling cell cycle and growth was examined. Molecular evidence has convincingly demonstrated that p53 effectively regulates cellular responses to various forms of stress, such as DNA damage, shortage of nucleotides, oxidative stress, activation of oncogenes, and lack of oxygen [41, 42]. The levels of apoptotic proteins triggered by ZnO NPs in MG63 cells were assessed through Western blot analysis with specific antibodies. Findings indicated that the administration of ZnO NPs notably elevated the levels of p53 and Bax proteins while substantially reducing the levels of Bcl-2 proteins [39].

Findings showed that treatment with ZnO NPs increased Bax, the tumor suppressor protein (p53), the suppression of tissue lipid peroxidation marker (MDA) levels, and neoangiogenesis marker (VEGF) [32, 33, 43]. Nanocomposite fabricated by *S. obliquus* extract induced a 2.65-fold increase in the expression of the BAX gene, a 1.25-fold increase in the Bcl-2 gene, and a 1.95-fold increase in the CASP8 gene compared to the control [37].

Other studies demonstrated that nanoparticles produced with *S. obliquus* exhibited high efficacy by inhibiting CAD, CASP8, and p53 in cancer cells [44].

It also demonstrated that ZnO NPs effectively decreased the expression of VEGF. This discovery suggests that the extracts of *S. obliquus* contain suitable compounds for environmentally friendly synthesis of ZnO NPs, which could be further explored in the future. It is worth mentioning that previous studies have reported the synthesis of nanoparticles using other types of algae, so identifying the optimal composition is crucial [45, 46]. Advances in modern immunotherapy have allowed for the use of bioinformatics techniques to identify messenger molecules, with a focus on directly inhibiting signaling pathways in cancer [47]. The interconnection of cellular pathways may explain the reduction in angiogenesis and VEGF expression in cancer cells. Additionally, the increased expression of p53, an anti-cancer protein, highlights the role of *Scenedesmus* aqueous extract in suppressing cancer cell proliferation. Microalgae are promising candidates for nanoparticle synthesis due to their abundant secondary metabolites that act as capping and reducing agents. The successful application of microalgae-synthesized nanoparticles in the biomedical field demonstrates the positive impact of these metabolites on the physicochemical properties of nanoparticles. However, further research is needed to fully understand the exact role of these nanoparticles in the human body and to progress from laboratory-based studies to clinical trials.

CONCLUSION

Synthesized ZnO NPs had a reducing effect on VEGF in breast cancer cells and considerably boosted p53 expression. The toxic behavior of ZnO NPs on MDA-MB-231 was linked to a reduction in cancer cell proliferation and survival. The results suggest that ZnO NPs capped by *S. obliquus* extracts have the potential to be utilized as an anti-breast cancer medication in conjunction with other cancer drugs. The examination and evaluation of this matter in clinical studies can be crucial in validating this concern.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interests.

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