Original Article

Unveiling of the Anti-Tumor Activity of Green Synthesized Zinc Nanoparticles against Ehrlich Solid Tumors in Mice

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ABSTRACT

Cancer, a disease threatening human life, is caused by the disturbance of the normal cell cycle, which results in the spontaneous growth of normal and malignant cells, the lack of differentiation between the two, and consequently malignant growths. Nowadays, various synthetic agents are applied for cancer therapy; nevertheless, reports have confirmed that these chemical agents are associated with various adverse complications. This experimental study was designed to assess the anti-tumor activities of zinc nanoparticles (ZnNPs) green synthesized by the *Astragalus maximus* (*A. maximus*) extract against Ehrlich solid tumors (EST) in mice. To induce the EST model, 0.2 ml of cell suspension was intramuscularly injected into the right thigh of the mice. Five days post-injection, the mice were assigned to five groups (eight mice each): EST mice treated with normal saline, EST mice orally treated with ZnNPs 10 mg/kg/day, EST mice orally treated with ZnNPs 25 mg/kg/day, and EST mice orally treated with ZnNPs 50 mg/kg/day for 14 days. Afterward, the volume of the tumor, tumor growth inhibition, body weight, tumor markers, oxidant/antioxidant markers, and tumor necrosis factor-alpha level were assessed in the tested mice. The results showed that after the treatment of EST mice with cyclophosphamide and ZnNPs at 10, 25, and 50 mg/kg, the volume of the tumor, and the serum amount of tumor markers (alpha-fetoprotein and carcinoembryonic antigen) were significantly reduced (P<0.001). It was found that ZnNPs at 10, 25, and 50 mg/kg markedly declined oxidative markers and increased the level of antioxidant enzymes (superoxide dismutase and glutathione peroxidase), compared to the control group, which received normal saline (P<0.001). To conclude, this study reported the unveiling of the anti-tumor activity of ZnNPs green synthesized by the *A. maximus* extract, mainly at a dose of 50 mg/kg against EST in mice. However, further supplementary studies are required to clarify all the anti-tumor aspects of these nanoparticles.

Keywords: Antioxidant, *in vivo*, Nanomedicine, Tumor, Zinc

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1. Introduction

Cancer, a disease threatening human life, is caused by the disturbance of the normal cell cycle, which results in the spontaneous growth of normal and malignant cells, the lack of differentiation between the two, and consequently malignant growths (1). Chemotherapy, as one of the main therapies, is applied alone or with other approaches, such as surgery, immunotherapy, and radiotherapy (2). Nowadays, various synthetic agents are applied for cancer therapy; nevertheless, reports have confirmed that these chemical agents are associated with various adverse complications, such as harming normal cells and organs, suppressing bone marrow function, as well as causing gastrointestinal disorders and alopecia (3). Therefore, the discovery of new anti-cancer agents with high efficiency and minimal toxicity has been particularly noticed by researchers in recent years. Nanoparticles (NPs) with a size of 1-100 nm and different morphological structures displayed various therapeutic applications for the treatment of various diseases (4). Current chemical and physical approaches to the synthesis of NPs have shown some limitations in that they are time-consuming, highly toxic, and environmentally damaging (5). Currently, herbal extracts called "green synthesis" are widely applied to the synthesis of NPs since they are highly reliable, tolerable, and environmentally friendly (6, 7). Zinc (Zn), as one of the main elements in human health, is meaningfully complicated in the function of several enzymes, the synthesis of protein, DNA, and RNA, as well as the growth and proliferation of cells (8). Zn nanoparticles (ZnNPs), due to their unique properties, are broadly used for the treatment and prevention of various diseases, mainly cancer (9, 10). In recent years, *in vitro* and *in vivo* anticancer effects of ZnNPs have been reported against a wide range of cancer cells in the liver, lung, breast, and skin; however, the results have been variable due to factors such as the synthesis method, the type of study, and the type of cancer cell (11, 12). The Ehrlich tumor is well-known as a mice mammary adenocarcinoma, which is characterized as a solid model of the tumor to study the efficacy of novel anti-tumor drugs (13, 14). This experimental study was designed to assess the anti-tumor activities of green synthesized ZnNPs against the Ehrlich solid tumor (EST) in mice.

2. Materials and Methods

2.1. Green Synthesis of ZnNPs

ZnNPs were green synthesized using the precipitation technique with the *Astragalus maximus aqueous* (*A. maximus*) extract, based on the method described in previous studies (13, 14).

2.2. Characterization of ZnNPs

The surface plasmon resonance of ZnNPs was measured using a UV-vis spectrophotometer (Shimadzu UV2550, Japan) to check the alteration of the Ag ions to ZnNPs. The synthesis of NPs by the *A. maximus* extract was also confirmed by an X-ray diffraction (XRD, 2000 APD, Italy) analysis. Some physical characterizations of ZnNPs, such as size and shape, were evaluated by a scanning electron microscope (SEM) (Mira3, Czech).

2.3. Cell Culture

The Ehrlich ascites tumor cell line was prepared from the American Type Tissue Culture Collection (Manassas, USA), and the cells were adjusted into 2.5×10^6 cells/ml in sterile saline solution using a Neubauer hemocytometer (15).

2.4. Animals and Induction of EST in Mice

In total, 48 Balb/C mice aged 40-50 weeks with a body weight of 25-30 g were selected to induce the EST model. They were handled in line with the recommendations of the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. To induce the EST model, 0.2 ml of cell suspension was intramuscularly injected into the right thigh of the mice (16).

2.5. Study Design

Five days post-injection (16), the mice were assigned to five groups (eight mice each), as follows (Suppl. 1): 1) EST mice treated with normal saline; 2) EST mice orally treated with ZnNPs 10 mg/kg/day for 14 days; 3) EST mice orally treated with ZnNPs 25 mg/kg/day for 14 days; 4) EST mice orally treated with ZnNPs 50 mg/kg/day (17) for 14 days; and 5) EST mice treated with cyclophosphamide (CYC) 50 mg/kg/day for three days (16).

2.5.1. Sample Collection

After treatment, all mice were euthanized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). After that, blood samples were collected from their hearts and then centrifuged at 5000 rpm for 15 min to collect the era. Tumors were aseptically collected and weighed, and their size was documented (16).

2.6. Tumor Growth Inhibition

On day seven of therapy, the volume of tumor (VT) and the rate of tumor growth inhibition (TGI) were estimated by the Vernier caliper, according to previous studies (18).

2.7. Evaluating Tumor Markers

In this survey, the serum levels of alpha-fetoprotein (AFP) and carcinoembryonic antigen tumor (CAE) were assessed using the Mouse alpha Fetoprotein ELISA Kit (ab210969, abcam, USA) and Mouse CEA ELISA Kit (Elabscience, USA), based on the manufacturer's protocols, respectively.

2.8. Evaluation of the Oxidative Stress Markers

Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric) (ab118970) and Nitric Oxide Assay Kit (ab272517) were used to assess the tissue level of malondialdehyde (MDA) and nitric oxide (NO) production as oxidative stress markers, based on the manufacturer's protocols, respectively.

2.9. Evaluation of Antioxidant Enzymes

The levels of glutathione peroxidase (GPx) and superoxide dismutase enzyme activity (SOD) were determined using the Glutathione Peroxidase Assay Kit (Colorimetric) (ab102530) and Superoxide Dismutase Activity Assay Kit (Colorimetric) (ab65354), based on the manufacturer's protocols, respectively.

2.10. Assessment of Anti-Inflammatory Cytokines

The level of tumor necrosis factor-alpha (TNF-α), as the main proinflammatory cytokine, was assessed by the Mouse TNF-α ELISA Kit (ab208348), based on the manufacturer's protocols.

2.11. Statistical Analysis

The obtained data were analyzed using one-way ANOVA and t-tests in SPSS software (version 26.0). $P<0.05$ was considered statistically significant.

3. Results

3.1. Characterization of Green Synthesized ZnNPs

The synthesis of ZnNPs was confirmed through the UV-vis spectral examination, with the absorption peak being in the range of 296 nm (Figure 1A). Using the XRD analysis, the peaks of 101, 201, 203, 300, and 112 were displayed at 26.3°, 37.1°, 46.3°, 53.9°, and 62.4°, respectively (Figure 1B). The results of the SEM analysis showed that green synthesized ZnNPs displayed a round appearance with various sizes ranging from 25 to 90 nm; however, the majority of these particles were in the size range of 45 to 55 nm (Figure 1C).

3.2. Evaluation of Body Weight

Following the treatment of EST mice with CYC and ZnNPs at 10-50 mg/kg, the body weight of the tested mice was markedly reduced ($P<0.05$), compared to the control group, which received normal saline (Figure 2). The maximum reduction in body weight was observed ($P<0.05$) in the mice treated with ZnNPs at 50 mg/kg.

3.3. Tumor Growth Inhibition

As shown in figure 3, after the treatment of EST mice with CYC and ZnNPs at 10, 25, and 50 mg/kg, the VT was dose-dependently reduced ($P<0.05$). The TGI values for ZnNPs at 10, 25, and 50 mg/kg were 42.4%, 54.3%, and 72.3%, respectively.

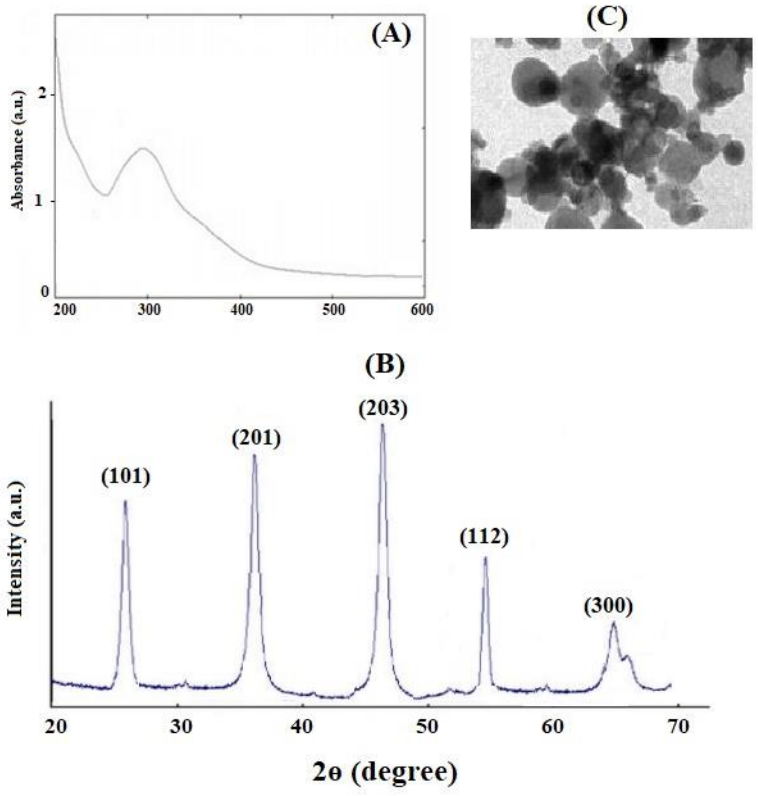

Figure 1. Characterization of green synthesized zinc nanoparticles by UV–vis spectral examination (A), X-ray diffraction (B), and scanning electron microscope analysis (C).

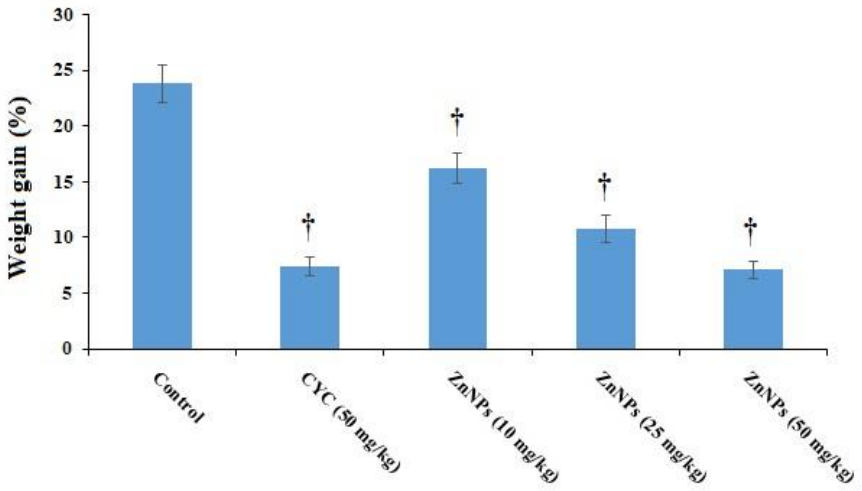

Figure 2. Evaluating of body weight after treatment of EST mice with cyclophosphamide (CYC) and green synthesized zinc nanoparticles (ZnNPs) at 10-50 mg/kg. †: P<0.001, significant difference in comparison with control group received normal saline.

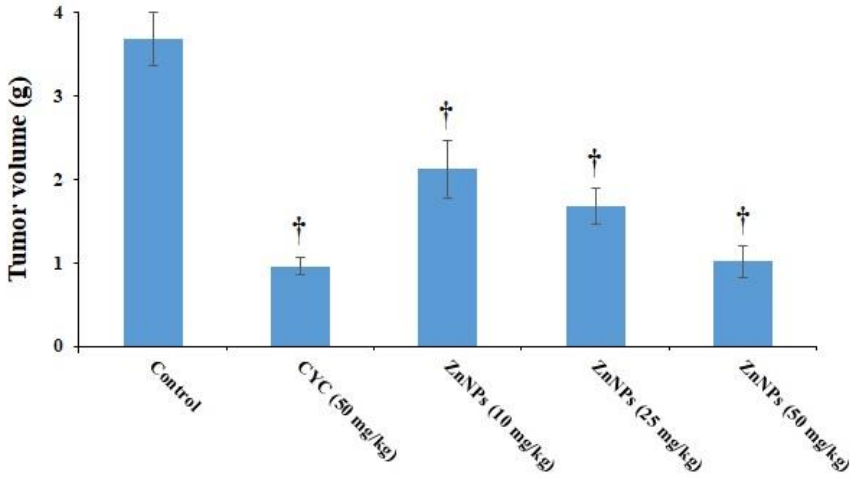

Figure 3. Evaluating of tumor weight after treatment of EST mice with cyclophosphamide (CYC) and green synthesized zinc nanoparticles (ZnNPs) at 10-50 mg/kg. †: P<0.001, significant difference in comparison with control group received normal saline.

3.4. Level of Tumor Markers

The EST results showed a considerable increase in the serum amount of AFP and CEA, while after the treatment of EST mice with CYC and ZnNPs at 10, 25, and 50 mg/kg, the serum amount of AFP and CEA was dose-dependently decreased (P<0.05), compared to the control group, which received normal saline (Figure 4).

3.5. Evaluation of Oxidative Markers

The EST caused a significant increase in the MDA and NO, while after the treatment of EST mice with CYC and ZnNPs at 10, 25, and 50 mg/kg, MDA and NO values were markedly declined, compared to the control group, which received normal saline (Figure 5).

3.6. Evaluation of Antioxidant Markers

The EST caused a significant increase in GPx and SOD, whereas after the treatment of EST mice with CYC and ZnNPs at 10, 25, and 50 mg/kg, the levels of GPx and SOD were markedly increased (P<0.05) (Figure 6).

3.7. Evaluation of TNF-α level

As shown in figure 7, the inducement of EST caused a considerable rise in the TNF-α level; however, after the treatment of EST mice with CYC and ZnNPs at 10, 25, and 50 mg/kg, the TNF-α level was obviously reduced (P<0.05).

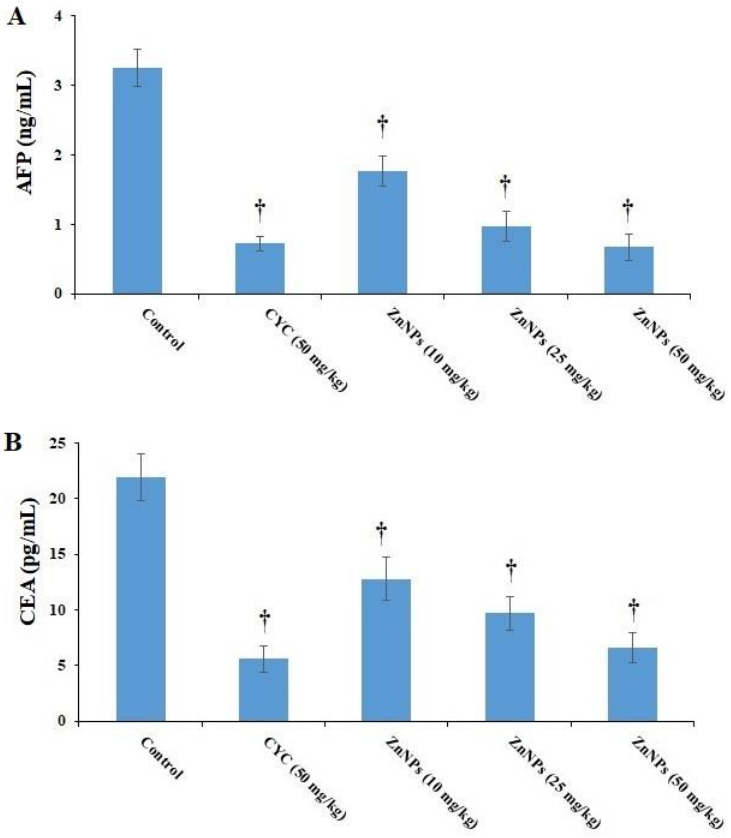

Figure 4. Evaluating of (A) alpha-fetoprotein (AFP) and (B) carcinoembryonic antigen tumor (CAE) after treatment of EST mice with cyclophosphamide (CYC) and green synthesized zinc nanoparticles (ZnNPs) at 10-50 mg/kg. †: P<0.001, significant difference in comparison with control group received normal saline.

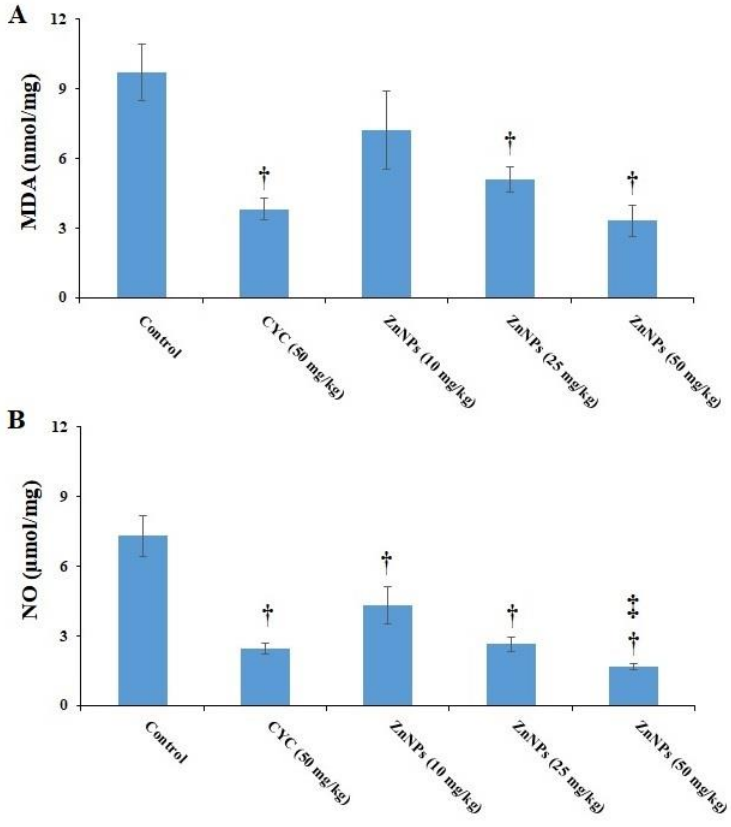

Figure 5. Evaluating of (A) lipid peroxidation (MDA) and (B) nitric oxide (NO) after treatment of EST mice with cyclophosphamide (CYC) and green synthesized zinc nanoparticles (ZnNPs) at 10-50 mg/kg. †: P<0.001, significant difference in comparison with control group received normal saline. ‡ significant difference in comparison with CYC.

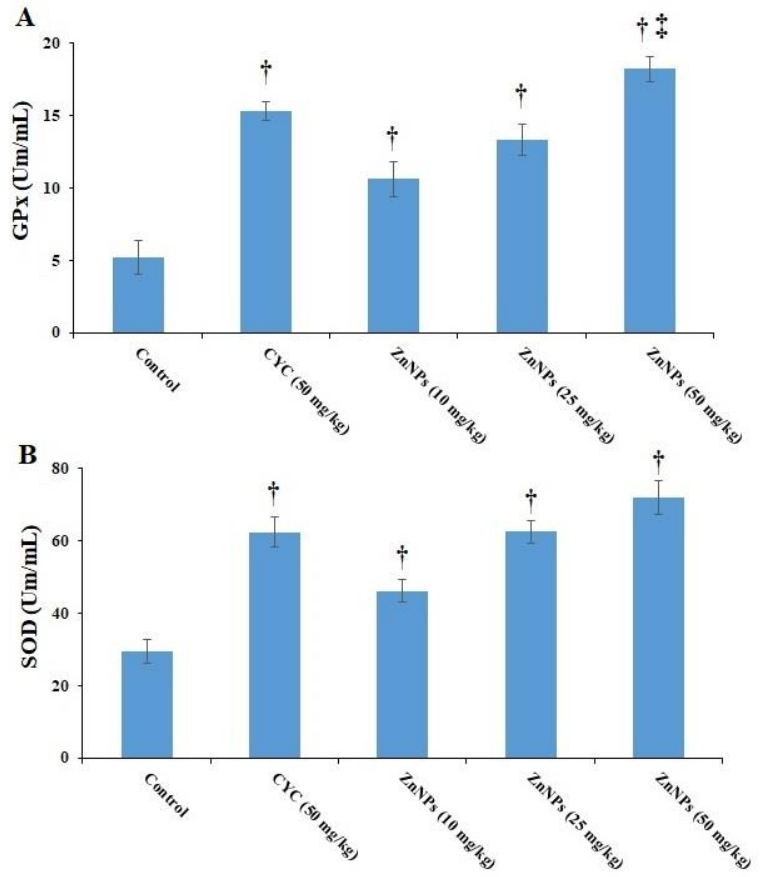

Figure 6. Evaluating of (A) glutathione peroxidase (GPx) and (B) superoxide dismutase enzyme activity (SOD) after treatment of EST mice with cyclophosphamide (CYC) and green synthesized zinc nanoparticles (ZnNPs) at 10-50 mg/kg. †: P<0.001, significant difference in comparison with control group received normal saline. ‡ significant difference in comparison with CYC.

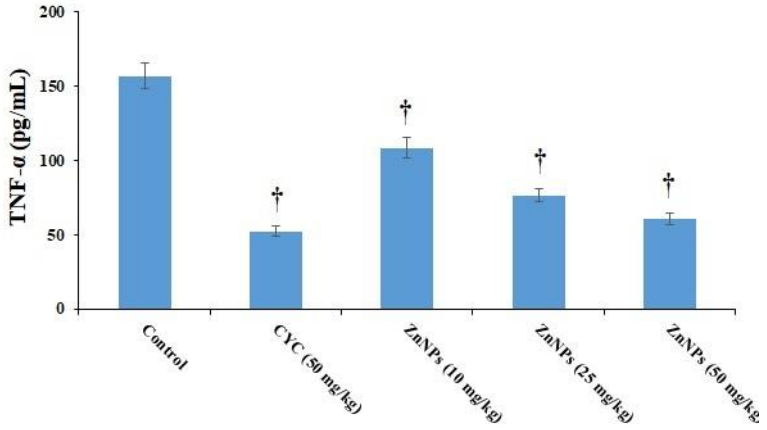

Figure 7. Evaluating of TNF-α level after treatment of EST mice with cyclophosphamide (CYC) and green synthesized zinc nanoparticles (ZnNPs) at 10-50 mg/kg. †: P<0.001, significant difference in comparison with control group received normal saline.

4. Discussion

Every year, 2.3 million new cases of breast cancer are reported in the world among adult women. Breast cancer is the first or second leading cause of female cancer deaths (19). Chemotherapy, as one of the main strategies for controlling cancer, is associated with various adverse complications, such as harming normal cells and organs, suppressing bone marrow function, as well as causing gastrointestinal disorders and alopecia (2, 3). This study aimed to assess the anti-tumor activities of green synthesized ZnNPs against the EST, a mice mammary adenocarcinoma, which is characterized as a solid model of the tumor to study the efficacy of novel anti-tumor drugs. The findings showed that ZnNPs had an absorption peak in the range of 296 nm. However, they displayed a round appearance with various sizes ranging from 25 to 90 nm, and most of these particles were in the size range of 45-55 nm. The size and morphology are the main factors affecting the stability and biological properties of synthesized NPs (6). The findings revealed that after the treatment of EST mice with CYC and ZnNPs at 10, 25, and 50 mg/kg, the VT and serum amounts of tumor markers (AFP and CEA) were dose-dependently reduced. It has been proven that NPs can be used in cancer therapy to cover active pharmaceutical agents and drugs and deliver them to the tumor location more efficiently (20). In recent years, several studies have been conducted on the anti-tumor effects of some organic and non-organic NPs, such as silver NPs, gold NPs, and polymeric NPs. However, their results have been different and sometimes contrasting due to factors including the synthesis approach, the way of use, and the type of NPs, which can affect their efficiency (21-23). Seyed et al. reported that zinc oxide-caffeic acid nanoparticles (ZnO-CA NPs) had potent *in vitro* anticancer effects on the human breast (MCF-7). The *in vivo* anticancer effects of ZnO-CA NPs against EST-bearing mice decreased vascular cell adhesion molecule 1 level by reducing the tumor size and the down-regulation of B-cell lymphoma 2 and nuclear factor kappa B gene expressions, leading to the downregulation of phosphorylated-extracellular-regulated kinase 1 and 2 protein expression, DNA fragmentation, and a recognizable peak at sub-G0/G1, indicating dead cells' population in cancer tissues (24). Another study conducted by Ghanem showed that zinc oxide nanoparticles (ZnO NPs) and thymoquinone, alone or combined, displayed potent anticancer activity in EST by reducing tumor cell viability and ascites fluid volume, as well as the downregulation of Bcl2 protein expression, increasing Beclin 1 and INFγ, and decreasing IL-13 (25). These findings suggested that ZnNPs can control EST in mice, probably by triggering apoptosis and improving the immune system. Based on the results, ZnNPs at 10, 25, and 50 mg/kg markedly declined oxidative markers and increased the level of antioxidant enzymes (SOD and GPx), compared to the control group, which received normal saline. Considering the activity of ZnNPs on oxidant/antioxidant markers, Saddick et al. (2017) showed that ZnNPs significantly increased the mRNA expression of GST, GR, GPx, CAT, and SOD genes, yet markedly declined the mRNA expression of MDA in the brains of *Oreochromis niloticus* and *Tilapia zillii* (25). To conclude, this study reported the unveiling of the anti-tumor activity of ZnNPs green synthesized by the *A. maximus* extract, mainly at the dose of 50 mg/kg against EST in mice. However, further supplementary studies are required to clarify all the anti-tumor aspects of these NPs.

Acknowledgment

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Authors' Contribution

FI performed the tests and collected the data. SR supervised and managed the work. FI drafted the manuscript. All authors approved the final version to be published.

Ethics

The Ethical Committee of the Visveswarapura Institute of Health Sciences, Karanataka, Rajiv Gandhi University of Health Science, Bengaluru, India, approved the protocol of this study with No. 381610205.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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