Dicoma anomala enhances the zinc phthalocyanine tetrasulphonic acid ($ZnPcS_4$) mediated photodynamic therapy in breast cancer cells

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Abstract. Globally, cancer has been identified as one of the leading causes of death in both men and women. Breast cancer is the common type of cancer that affects women, and it is the second leading cause of cancer-related death. To completely eradicate cancer, multiple therapeutic options that target distinct disease processes must be applied. For many years, combination treatment has remained a therapeutic option for resistant cancers. In this research, we investigated the enhanced effect of zinc phthalocyanine tetrasulphonic acid ($ZnPcS_4$) mediated photodynamic therapy by using *Dicoma anomala* methanol root extract in breast cancer cells. *D. anomala* root methanol extract and $ZnPcS_4$ Photosensitizer (PS) was used to treat MCF-7 breast cancer cells at different concentrations (25, 50, and 100 µg/mL of *D. anomala* and 5, 10, 20, 40, and 60 µM of $ZnPcS_4$) in photodynamic therapy (PDT) using a diode laser of 680 nm at 10 J/cm^2 fluency. After 24 h of treatment, MCF-7 cells were analyzed for possible morphological changes, adenosine triphosphate (ATP) proliferation, and lactate dehydrogenase (LDH) cellular cytotoxicity. ATP proliferation rates and LDH cytotoxicity were analyzed to determine the anticancer effects of *D. anomala* and $ZnPcS_4$ mediated photodynamic therapy in monotherapy as well as in combination therapy. All experiments were performed 4 times (n=4) and results obtained were analyzed using SPSS statistical software version 27 at a 0.95 confidence interval. The outcomes from this study show that *D. anomala* significantly enhances the cytotoxic effects of $ZnPcS_4$ mediated PDT in breast cancer cells. In monotherapy, *D. anomala* root extract induced MCF-7 cell death, while in combination with $ZnPcS_4$, the plant extract significantly enhanced the PDT efficacy. Furthermore, the outcome from this research suggests the use of *D. anomala* root extract as a natural anticancer agent for the treatment of breast cancer.

1. Introduction

Cancer is a condition characterized by the unregulated proliferation of tumor cells. Despite the invention of new diagnostic, and treatment modalities, cancer has continued to be the leading cause of morbidity and mortality in both males and females [1]. Breast cancer is the most common and frequently diagnosed cancer in women. According to the 2020 GLOBOCAN report, the incidence rate of breast cancer is expected to increase from 2.63 million cases in 2020 to 3.19 million new cases by 2040 [2]. However, an increase in the incidence rate is attributed to many risk factors which may be intrinsic or extrinsic. Examples of intrinsic factors include mutations and random error during deoxyribonucleic acid (DNA) replication while extrinsic risk factors include radiation, chemicals, and smoking. There are various treatment modalities employed in the treatment of breast cancer. These include chemotherapy, immunotherapy, surgery, and radiotherapy. Although these therapies have been used in the treatment of various cancers, they are also known to elicit different side effects [3]. The increased side effects have led to the development of novel combination treatments [4].

Photodynamic therapy (PDT) is one of the emerging modalities that has shown potential in the treatment of various cancers such as breast cancer. This form of cancer therapy uses chemical compounds known as photosensitizers to initiate the generation of reactive oxygen species (ROS). When generated within tumor cells, ROS targets and induce oxidative stress of different organelles of the cells such as the nucleus, and the mitochondria [5]. In addition, many researchers are exploring the use of plant-derived anticancer compounds as a source of bioactive compounds as well as photosensitizers to

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be used in the treatment of different forms of cancer. *D. anomala* is an African medicinal plant that has been used in the treatment of various ailments [4]. Photodynamic therapy and plant-derived bioactive compounds may induce tumor cell death through various mechanisms which might be autophagy, apoptotic, necrotic, and cell cycle checkpoint arrest [6]. Most cancer studies are now concentrating on combining one or two therapeutic modalities to maximize efficacy with less side effects [7]. This study is aimed at evaluating the enhanced anticancer effects of zinc phthalocyanine tetrasulphonic acid ($ZnPcS_4$) mediated PDT with *D. anomala* on MCF-7 cancer cell line.

2. Methodology

2.1. Cell culture, and treatment

A breast cancer cell line (MCF-7) (ATCC® HTB 22) used in this research was obtained from American Type Culture Collection (ATTC). MCF-7 cells were cultured in a T175 culture flask containing Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% Amphotericin B, 1% Penicillin-streptomycin, and incubated at 37°C, 85% humidity, and 5% CO_2. Experimental cells were seeded at a seeding density of 5 x 10^5 in 3.4 cm^2 diameter culture plates. The IC_{50} for *D. anomala* plant extract was determined by treating cells with different concentrations of the extract (i.e., 25, 50, and 100 µg/mL), whereas IC_{50} concentration of $ZnPcS_4$ was calculated following 24 h treatment using 5, 10, 20, 40, and 60 µM concentrations of PS. Photodynamic therapy experiments were performed using the IC_{50} concentrations of PS with a 680 nm laser diode at the fluency 10 J/cm^2.

2.2. Cell morphology

Morphological changes of the cells were evaluated after 24 h of treatment with different concentrations of *D. anomala*, and $ZnPcS_4$ by using an inverted light microscope (Wirsan Olympus CKX 41).

2.3. Adenosine triphosphate (ATP) proliferation assay

The CellTiter-Glo® 3D luminescence reagent (Promega, G968A) was used to determine cellular proliferation and the amount of ATP in viable cells. To initiate cell lysis, 50 µL of reconstituted reagent was added to an equal volume of cell suspension and thoroughly mixed. The mixture was later incubated in the dark at room temperature for 10 min. After 10 min incubation, ATP luminescence was read by using PerkinElmer, VICTOR Nivo™.

2.4. Lactate dehydrogenase (LDH) cytotoxicity assay

Cyto Tox 96® Non-Radioactive Cytotoxicity assay (Promega, G179A) kit was used to measure the amount of cytosolic LDH released by cells with a damaged cell membrane. To estimate the levels of LDH, 50 µL of reconstituted reagent was added to an equivalent amount of cell suspension, mixed thoroughly, and incubated at room temperature in the dark for 30 min. The colorimetric mixture was read by using PerkinElmer, VICTOR Nivo™.

2.5. Statistical analysis

MCF-7 passage numbers between 16 and 21 were used to conduct all experiments. All sets of experiments were performed four times (n=4). Throughout the study, all the mean values of experimental groups were compared with the mean value of untreated MCF-7 cells. One-way ANOVA was performed with the aim of determining statistical significance between the control and experimental groups at 0.95 confidence interval. Statistical significances between the control and experimental groups in graphs are shown as p <0.05 (*), p <0.01 (**), and p <0.001 (***). All statistics were analysed by using SPSS statistical software version 27.

3. Results and discussion

3.1. Morphological changes

Morphological alterations in MCF-7 treated, and untreated cells are shown in Figure 1. Untreated MCF-7 cells (Fig. 1 A) displayed no aberrant changes in morphology. MCF-7 cells that only received laser (10 J/cm^2) treatment (Fig. 1 B) clearly suggests that laser alone does not have cytotoxic effects on MCF-

7 cells. When compared to untreated cells, various concentrations (25, 50, and 100 µg/mL) of *D. anomala* treated cells (Fig. 1 C-E) showed significant morphological changes. On the other hand, cells that were treated with different concentrations (10, 20, 40, and 60 µM) of $ZnPcS_4$ only (Fig. 1 F-J) displayed no morphological changes while cells that were treated with $ZnPcS_4$ + laser (Fig. 1 K-O) demonstrated significant morphological changes when compared to the control cells. Cells that were treated with $ZnPcS_4$ IC_{50} + laser (Fig. 1 P), *D. anomala* IC_{50} (Fig. 1 Q), *D. anomala* IC_{50} + laser (Fig. 1 R), and $ZnPcS_4$ IC_{50} + laser + *D. anomala* IC_{50} (Fig. 1 S) demonstrated changes in morphology when compared to untreated and laser only treated MCF-7 cells. Morphological changes observed 24 h post treatment include cell shrinkage, loss of membrane integrity, and cell detachment from culture plates. These morphological changes observed in the treatment groups of this study are suggestive of apoptotic activities.

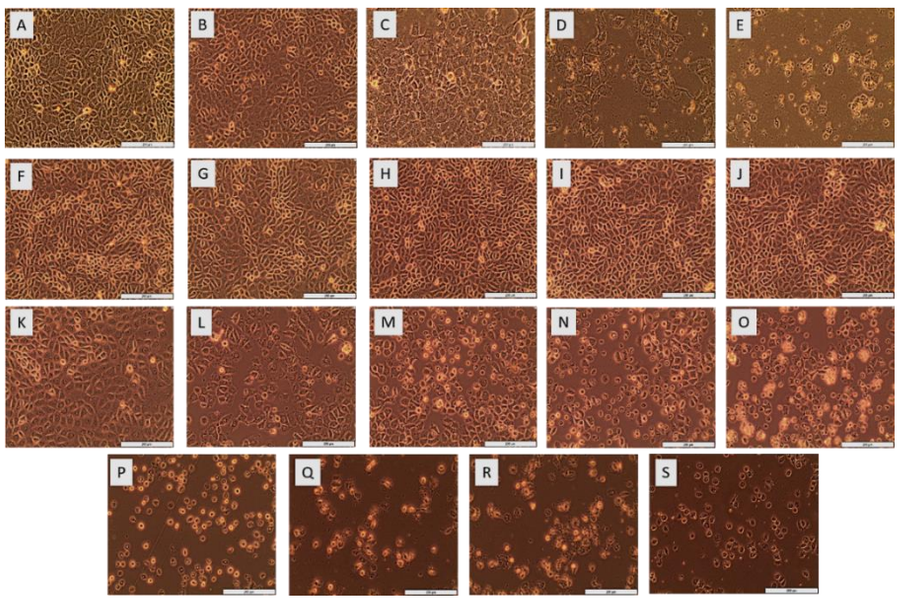

Figure 1. Morphological changes in MCF-7 cells after 24 h treatments. A) MCF-7 untreated cells; B) MCF-7 cells + laser; C) 25 µg/mL *D. anomala*; D) 50 µg/mL *D. anomala*; E) 100 µg/mL *D. anomala*; F) 5 µM of $ZnPcS_4$; G) 10 µM of $ZnPcS_4$; H) 20 µM of $ZnPcS_4$; I) 40 µM of $ZnPcS_4$; J) 60 µM of $ZnPcS_4$; K) 5 µM of $ZnPcS_4$ + laser; L) 10 µM of $ZnPcS_4$ + laser; M) 20 µM of $ZnPcS_4$ + laser; N) 40 µM of $ZnPcS_4$ + laser; O) 60 µM of $ZnPcS_4$ + laser; P) $ZnPcS_4$ IC_{50} + laser; Q) *D. anomala* IC_{50}; R) *D. nomala* IC_{50} + laser; S) $ZnPcS_4$ IC_{50} + laser + *D. anomala* IC_{50}.

3.2. ATP proliferation assay

The level of metabolic activity of cells was determined by measuring cellular ATP concentration. The CellTiter-Glo® 3D luminous assay was used to assess the ability of cells to proliferate. All metabolically active cells contain ATP, which is a marker for cell viability and proliferation. Studies showed that, ATP can initiate cell differentiation and proliferation [8]. The levels of ATP in MCF-7 cells only remained high as shown in (Fig. 2 A-C). In comparison to control cells, cells treated with *D. anomala* methanol root extract and $ZnPcS_4$ mediated PDT demonstrated significant decrease in cellular proliferation. In *D. anomala* treatment model, all the three treatment groups demonstrated a significant decrease in ATP levels when compared to the untreated cells. The cells treated with increasing doses of *D. anomala* (25, 50, and 100 µg/mL) showed a dose-dependent significant decrease in ATP levels (Fig. 2 A). On the other hand, $ZnPcS_4$ alone treated cells showed no significant ATP levels. This suggests that $ZnPcS_4$ alone does not have cytotoxic effects when compared to $ZnPcS_4$ treated cells that received laser (680 nm) at 10 J/cm^2. MCF-7 cells treated with increasing doses of $ZnPcS_4$ (10, 20, 40, and 60 µM) showed a strong dose-dependent significance with p value < 0.001 when compared to untreated cells (Fig. 2 B). This suggests that the cytotoxicity effects of $ZnPcS_4$ is dependent on the exposure of MCF-7 cells treated

with $ZnPcS_4$ to a diode laser of 680 nm. In monotherapy, the two IC_{50} (*D. anomala* and $ZnPcS_4$ mediated PDT) concentrations demonstrated a strong significance with p value (<0.001) when compared to the untreated cells that showed higher ATP levels as shown in Fig. 2 C. The combination of the two IC_{50} (*D. anomala* and $ZnPcS_4$ mediated PDT) showed a significant decrease in ATP levels when compared to the control group (Fig. 2 C). Metabolically active cells undergoing proliferation produce high levels of ATP. The major source of cellular ATP occurs in the mitochondria via different metabolic processes such as beta-oxidation [9]. ATP levels drastically drop in cells which are in the late stages of apoptosis. In most cases, this is due to the loss of mitochondrial activities and increased activity of ATP dependent proteolytic enzymes [10]. This suggests that all cellular metabolic activities are dependent on the synthesis of ATP.

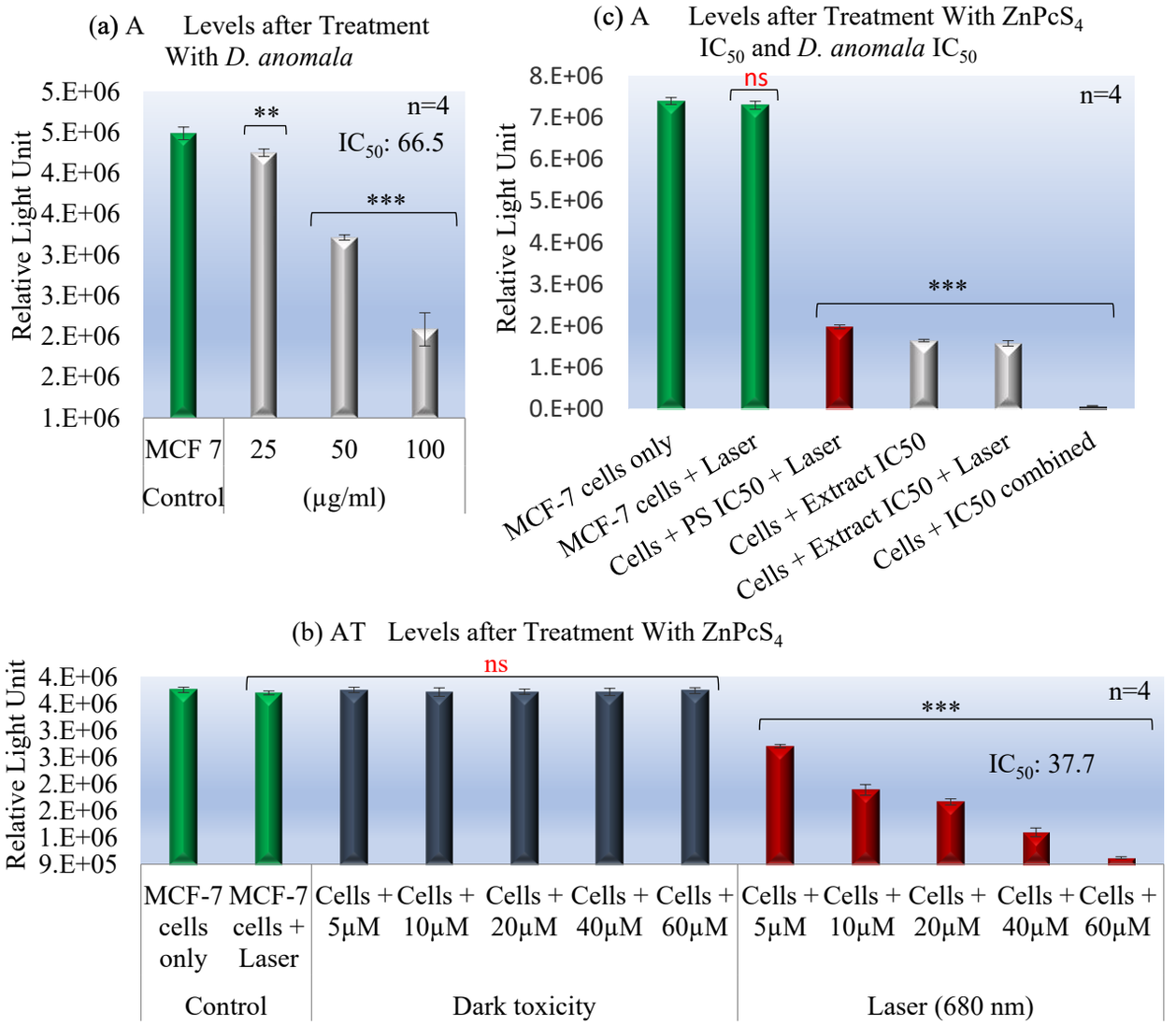

Figure 2. ATP proliferation effects of *Dicoma anomala* and $ZnPcS_4$ on MCF-7 cells. a) MCF-7 control cells showed a highest ATP levels while *D. anomala* root extract-treated cells showed a significant decrease in ATP levels and IC_{50} established was 66.5 µg/mL. b) MCF-7 control cell, laser only treated and $ZnPcS_4$ only treated cells showed a significant increase in ATP proliferation while $ZnPcS_4$ treated and laser irradiated cells showed a significant decrease in ATP proliferation and the IC_{50} established was 37.7 µM. c) A decrease in ATP proliferation was observed in IC_{50} (*D. anomala* and $ZnPcS_4$) treated MCF-7 cells as well as in combination of the two IC_{50} when compared to the control cells. The values depicted are mean plus or minus standard deviations (n=4). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences between the control and experimental groups.

3.3 LDH cytotoxicity assay

Lactate dehydrogenase (LDH) cytotoxicity assay is a colorimetric assay that determines cellular cytotoxicity by measuring the levels of LDH in a culture medium. LDH is an enzyme found in the cytoplasm of all living cells. It acts as a marker for cell membrane damage and is released into culture medium of cells undergoing apoptosis. In addition, the release of LDH into the culture media is directly proportional to the number of dead cells [6]. Cell membrane integrity of *D. anomala* and $ZnPcS_4$ treated MCF-7 cells was measured by using PerkinElmer, VICTOR Nivo™. The control cells and laser only ($10J/cm^2$) treated cells showed low levels of LDH when compared to cells that were treated with various concentrations of *D. anomala* and $ZnPcS_4$ mediated PDT. *D. anomala* treated MCF-7 cells induced the release of higher levels of LDH when compared to the control cells and laser ($10J/cm^2$) only treated cells. A dose-dependent significant increase in LDH levels was observed in *D. anomala* concentrations (25, 50, and 100 µg/mL) as shown in Table 1. In another experimental model, $ZnPcS_4$ treated cells that did not receive laser showed less LDH levels when compared to $ZnPcS_4$ treated cells that received laser (680 nm) at 10 J/cm^2. This suggests that the cytotoxic effects of $ZnPcS_4$ is dependent on concentration and exposure to laser light. MCF-7 cells that were treated with increasing doses of $ZnPcS_4$ (10, 20, 40, and 60 µM) showed high release in LDH levels when compared to the control cells as shown in Table 2. In monotherapy, the two IC_{50} (*D. anomala* and $ZnPcS_4$ mediated PDT) concentrations provoked a high release in LDH levels with a strong significance (p value <0.001) when compared to the untreated cells. The combination of two IC_{50} (*D. anomala* and $ZnPcS_4$ mediated PDT) showed a very strong significant increase in LDH levels when compared to the control group as shown in Table 3.

Table 1: LDH levels after treatment with *D. anomala.* Standard error ± of mean.

Groups	LDH levels
LDH positive control	1.004 ± 0.0220
MCF-7 control	0.54775 ± 0.0179
D. nomala (25 µg/mL)	0.6035 ± 0.0084 *
D. nomala (50 µg/mL)	0.75975 ± 0.0013 ***
D. nomala (100 µg/mL)	0.88275 ± 0.0194 ***

Table 2: LDH levels after treatment with $ZnPcS_4$. Standard error ± of mean.

Groups	LDH levels	
LDH positive control	1.1027 ± 0.023	
MCF-7 control	0.556 ± 0.020	
MCF-7 + Laser	0.5832 ± 0.014	
$ZnPcS_4$ concentrations	*Dark Toxicity*	*680 nm Laser ($10J/cm^2$)*
5 µM	0.5585 ± 0.016	0.669 ± 0.007 **
10 µM	0.5562 ± 0.020	0.7935 ± 0.024 ***
20 µM	0.5662 ± 0.0186	0.8487 ± 0.018 ***
40 µM	0.5585 ± 0.017	0.9745 ± 0.019 ***
60 µM	0.564 ± 0.019	1.0362 ± 0.042 ***

Table 3: LDH levels after treatment with $ZnPcS_4$ IC_{50} and *D. anomala* IC_{50}. Standard error ± of mean.

Groups	LDH levels
LDH positive control	1.9147 ± 0.0156
MCF-7 control	0.4625 ± 0.0177
MCF-7 + Laser	0.465 ± 0.0174
$ZnPcS_4$ + Laser	0.6167 ± 0.0086 ***
D. nomala IC_{50}	0.6287 ± 0.0166 ***
D. nomala IC_{50} + Laser	0.6325 ± 0.0109 ***
$ZnPcS_4$ IC_{50} + Laser + *D. anomala* IC_{50}	0.955 ± 0.0255 ***

4. Conclusion

Plants used in traditional medicine have been proven to have pharmacological properties and offer strong therapeutics, making complementary medicine essential to the medical sector. In conclusion, *Dicoma anomala* methanol root extract has demonstrated to have anticancer effects when administered in monotherapy as well as in combination with zinc phthalocyanine tetrasulfonate ($ZnPcS_4$) photosensitizer mediated photodynamic therapy in MCF-7 breast cancer cells. Furthermore, the cell death mechanism analysis will be warranted to explore the exact mechanisms of *D. anomala* in monotherapy as well as the combination therapy. This combination might potentially lower the effective therapeutic dosages of commercially prepared photosensitizers, lowering dose dependence in PDT. In drug development and cancer research, many natural chemicals serve as lead anticancer compounds. However, scientific validation is required for the discovery of anticancer properties in traditional medicinal plants and natural products.

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