The Combined Anticancer Effect of Hypericin Mediated Photodynamic Effect and *Punica granatum* on MCF-7 Cells

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Abstract. As per cancer statistics, breast cancer is the most prevalent cancer in South African women, accounting for 22.6% of all cancers in women and 16% of cancer deaths in women. Phytochemicals/secondary metabolites are compounds found in plants that have antitumor, antiproliferative, and antioxidant effects used in traditional medicine. Current systematic studies on the use of phytochemicals for various diseases have proved to have significant success with reduced side effects. Photodynamic therapy (PDT) is a cancer therapeutic modality, which has been approved for treating various cancer types. Although being promising for cancer treatment, both phytomedicine and PDT suffers from their own limitations. Combination therapy is one of the avenues being explored to overcome those limitations with enhanced therapeutic outcome. This study aims to investigate the combined anticancer effect of IC_{50} dose of Hypericin mediated PDT with 1 J/cm^2 laser treatment at 594 nm to enhance the anticancer potential of *P. granatum* ethyl acetate extract against MCF-7 cells. Cytotoxicity in MCF-7 cells treated with extract, PDT alone and in combination was evaluated with 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Morphological changes were investigated using brightfield microscopy and cell death analysis using Annexin V/PI flow cytometry assay. The results showed enhancement in cytotoxicity (~90%) in combination treatment at a dose of 400 μg/mL of *P. granatum* aril ethyl acetate extract and 0.09 μM Hypericin-PDT in comparison to single treatment. In conclusion, *P. granatum* and Hypericin-mediated PDT in combination treatment for breast cancer is a viable treatment modality for further investigation.

1. Introduction

In the coming decades, regardless of the availability of resources, cancer, which is already the leading cause of death in many high-income countries, is expected to overtake other diseases as the leading cause of morbidity and mortality worldwide. The condition is worrisome particularly in low- and middle-income countries, which now bear 80% of the global burden of this illnesses [1]. Research and strategic frameworks have been developed to assist to mitigate and treat this disease. Breast cancer is one of the most common causes of death in women and has the leading incident rate [2]. Breast cancer mortality rates continue to increase every year despite the increased and advanced screening and treatment modalities [3]. Treatment modalities for breast cancer include surgery, hormone therapy, radiation, and chemotherapy [4]. These treatments have the benefit of cancer killing effects but also induces unwanted adverse effects. This has brought about the need for improved treatment remedies with little to no adverse effects.

Phytochemicals are natural products found in plants that in recent years gained interest in cancer treatment modalities due to their antioxidant, antitumor, and antiproliferative potentials with little to no toxic effects [5]. *P. granatum* is a medicinal plant with anticancer, antibacterial, antimicrobial, and antiproliferative properties and have been used in traditional medicine throughout the Asian region. In recent years extracts of this plant has been explored in cancer treatment in with positive treatment outcomes [6].

PDT is form a of minimally invasive cancer treatment which have gained interest as an alternative and palliative treatment modality recently [7]. The benefits of PDT include low invasiveness and low cost of treatment but has drawbacks such as low penetration and photosensitivity [8]. Hypericin is a naturally occurring compound isolated from *Hypericum perforatum* with phototoxic properties used in several PDT studies with significant cancer cell death induction [5]. Combination therapy is one of the avenues explored in cancer therapy to combat treatment limitations and results in a synergistic effect by inducing cancer cell apoptosis, inhibition of malignant growth, and cell cycle arrest [9]. This study aims to combine the therapeutic effects from Hypericin-PDT and *P. granatum* extract on breast cancer cells.

2. Methodology

2.1. Cell culture, experimental groups and treatment

The MCF-7 breast cancer cell line (ATTC® HTB-22) was used in this study. It was grown in a monolayer in a T75 tissue culture flask in Dulbecco's Modified Eagles's Medium (DMEM) and supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% amphotericin B. Cells were incubated under the conditions of 95% humidified incubator operating at 37 °C and 5% carbon dioxide (CO_2). Seeding density of control and experimental groups was 7000 cells per well in 96 well plates. After 24 h of incubation, 24 h and 48 h treatment of cells with various concentrations (50, 100, 200, and 400 µg/mL) of *P. granatum* alone and PDT with 0.0125, 0.025, 0.1 and 0.2 µM concentrations of hypericin treated for 3 h was carried out. The highest concentration of *P. granatum* (400 µg/mL) and IC_{50} dose (0.09 µM) of Hypericin groups were used for combined treatment. Laser treatment was carried out using semiconductor diode laser at 594 nm wavelength, 1 J/cm^2 fluency. Cytotoxic studies were done after completion of all treatments.

2.2. Morphology

The cellular morphological alterations were evaluated for each group using Olympus CKX 41 inverted microscope following the treatment.

2.3. Cell viability

Roche cell proliferation kit (11465007001) was used to determine cell viability after treatment. MTT reagent (10 µL) was added to each well in 90 µL of culture media containing cell and incubated for 3 h. Thereafter 100 µL of solubilization buffer was added and plate was incubated overnight. Absorbance was measured using multiplate reader at 570 nm.

2.4. Apoptosis assay

FITC Annexin V/PI (Becton Dickinson, 556570 FITC Annexin V Apoptosis Detection Kit II) assay was used to evaluate and quantify percentage of apoptotic and necrotic cells. After treatment, cells were prepared as per manufactures instructions and Annexin V protein complex was added, followed by addition of propidium iodide (PI) as vital dye. Measurement was carried out using flow cytometer (BD Accuri™ C6) BD CSampler analysis software.

2.5. Statistical analysis

In this study each experiment was performed in triplicate and repeated thrice independently. The experimental groups were compared to MCF-7 cells control and data expressed as mean and standard error. The student's *t*-test was used as an analysis tool for determining the difference in control and experimental groups with 95% confidence interval. Data was investigated using Sigma Plot v.14.0 software. Statistically significant values were expressed as $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

3. Results

3.1. Absorption spectra

Absorption spectra of *P. granatum* aril ethyl acetate extract and Hypericin were recorded in DMSO. Results showed that the extract doesn't represent any absorption peak in the visible region between 400-800 nm but at 300 nm as represented in Figure 1 (a) thus, cannot be useful in PDT. Therefore, the cytotoxicity effects of the extract were combined with the phototoxic effect of Hypericin which has a strong absorption peak at 600 nm as shown in Figure 1 (b).

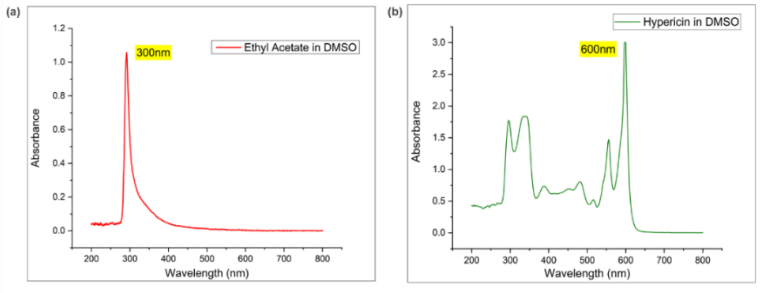

Figure 1. Absorption spectra of (a). P. granatum aril ethyl acetate extract and (b) hypericin recorded in DMSO as solvent.

3.2. Cell viability assay

3.2.1. *Single treatment*

Cell viability assay showed cytotoxicity at 400 µg/ml concentration of *P. granatum* extract at 24 and 48 h represented in Figure 2 (a). However, as shown in Figure 2 (b), the induced cytotoxicity by extract was less than 50%, thus IC_{50} dose cannot be calculated.

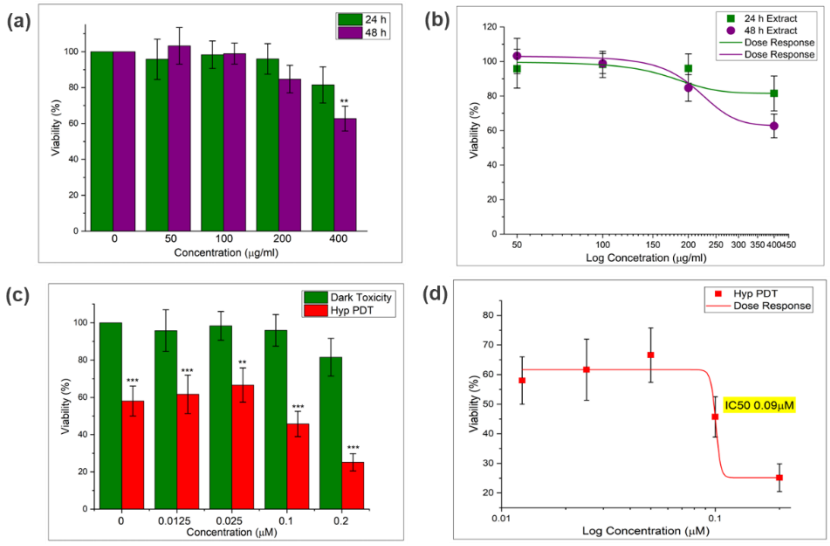

Figure 2. Effect of single treatment on cell viability of MCF-7 cells. (a) P. granatum aril ethyl acetate extract treatment in concentration range of 50-400 µg/ml, (b) dose response curve of P. granatum aril ethyl acetate extract induced cytotoxicity, (c) phototoxicity induced by different concentration of hypericin (0.0125- 0.2 µM) irradiated at 1 J/cm^2 and (d) dose response curve of hypericin induced phytotoxicity. (p <0.05 (*), p < 0.01 (**) or p < 0.001 (***)).

Cells treated with Hypericin followed by irradiation after 3 h, showed a significant decrease in cell viability with all concentrations (0.001-0.2 µM) as shown in Figure 2 (c). The dark toxicity group included Hypericin treatment of cells for 3 h without any light irradiation, showing no significant toxicity in cells. The IC_{50} of Hypericin was determined to be at 0.09 µM concentration (Figure 2 (d)). The effect of DMSO as solvent on cells was eliminated through vehicle control which showed no toxicity in cells.

3.2.2. Combined treatment

Combined treatment showed a significant decrease in cell viability as represented in Figure 3 (a). MCF-7 cells were treated with ethyl acetate extract (400 µg/mL) for 24 h followed by Hypericin-PDT at 0.001-0.2 µM concentrations. An increase in the concentration of Hypericin caused a significant decrease in cell viability. As shown in Figure 3 (b) the calculated IC_{50} dose of 0.06 µM for combination treatment is lower than effective concentration of Hypericin PDT (IC_{50} ~ 0.09 µM) and extract treatment alone.

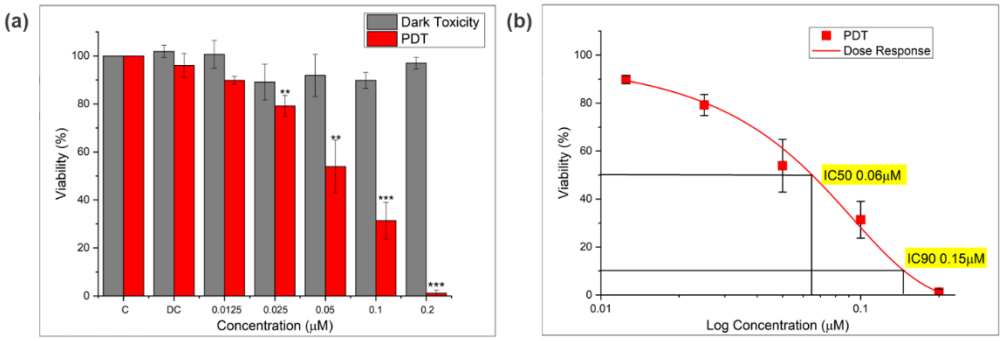

Figure 3. Effect of combination treatment. (a) Cell viability percent of MCF-7 cells treated with 400 µg/ml P. granatum aril ethyl acetate extract and different hypericin concentrations (0.001-0.2 µM) irradiated at 1 J/cm^2. (b) Dose response curve of induced cytotoxicity by combination treatment. (p <0.05 (*), p < 0.01 (**) or p < 0.001 (***)).

3.3. Morphological alterations

Morphological changes of untreated and treated cells were observed for signs of cell death and representative images for each group is shown in Figure 4.

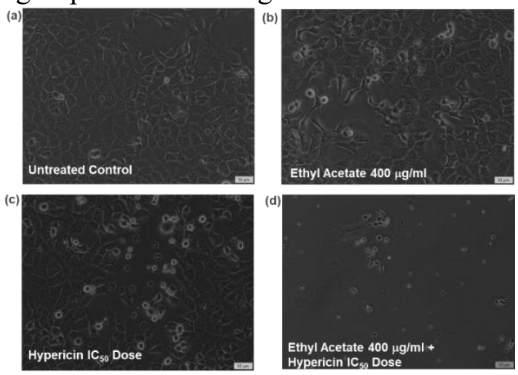

Figure 4. Morphological alterations of MCF-7 cells after treatment. Representative images of (a) Untreated cells, (b) P. granatum aril ethyl acetate extract treatment at 400 µg/ml for 48 h, (c) hypericin-PDT IC_{50} at 0.09 µM and (d) Combination treatment with P. granatum aril ethyl acetate extract at 400 µg with hypericin PDT IC_{50}. (200X Magnification).

Alone treatment with *P. granatum* aril ethyl acetate extract at 400 µg/mL (figure 4 (b)) on MCF-7 cells showed modest induction in cell damage while Hypericin-mediated PDT at IC_{50} of 0.09 µM (figure 4 (c)) showed relatively more cells with damaged and rounded up morphology when compared to the untreated cells (Figure 4 (a)). Combination treatment showed significant number of cells with altered and damaged morphology along with a significant decrease in cells' population (Figure 4 (d)).

3.4. FITC Annexin V/PI assay

FITC Annexin V/PI assay was carried out to show the percentage of live, early apoptotic, late apoptotic, and necrotic cells following the different treatment. A representation of cell death for each treatment group is shown in Figure 5. As shown in Figure 5 (b), untreated MCF-7 cells showed ~ 98% of live cells while single treatment with *P. granatum* aril ethyl acetate extract at 400 µg/mL for 24 h showed a significant decrease in live cells (~22%) with a marked increase in cells in early apoptosis (~20%).

Combination treatment showed only ~56% of live cells with increase in percentage of late apoptotic (~18%) and necrotic (~22%) cells with ~ 4% of early apoptosis cells.

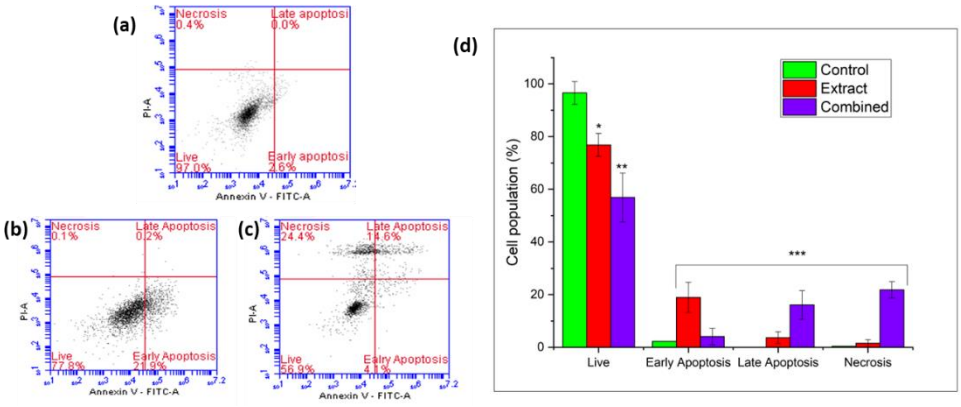

Figure 5. FITC Annexin V/PI assay. Representative histogram of (a) untreated MCF-7 cells (b) P. granatum aril ethyl acetate extract (400 µg/ml) treatment for 48 h (c) combination treatment (P. granatum aril ethyl acetate extract at 400 µg/ml + hypericin-PDT at IC_{50}) and (d) percentage of live, early apoptotic, late apoptotic and necrotic cells populations for each experimental group. ($p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***)).

4. Discussion

P. granatum extracts have shown the therapeutic effects against different types of cancer, including breast cancer [10]. In this study the ethyl acetate extract was found to be moderately cytotoxic at a concentration of 400 µg/ml at 48 h treatment (Figure 2(a)). Our study is the first report on evaluation of cytotoxic potential of South African cultivar of *P. granatum*. Cytotoxicity induced by extracted fraction from *P. granatum* arils can be explained due the presence of bioactive phytochemicals in the fruit.Others studies with *P. granatum* extracts on MCF-7 cells have reported, 320 µg/ml of methanol extract of peel treated for 48 h induced ~65% loss of cell viability [11], while IC_{50} dose of 377.88 µg/ml of ethanol extract of peel and 49.08 µg/ml of arils juice extract from P7 -Izmir 1513 Turkish cultivar was determined at 24 h [13]. However, the results on investigation on combination treatment utilizing extracted *P. granatum* arils phytochemicals with ethyl acetate fraction and Hypericin-mediated PDT against breast cancer cells showed strong cytotoxic effects, compared to single treatment with extract and Hypericin-PDT (Figure 3(a)). Cell viability and morphological results (Figure 4) clearly showed Hypericin-PDT significantly enhanced the cytotoxicity induced by extract alone, almost reaching a cell death of ~90% with 0.15 µM of Hypericin-PDT in combination with 400 µg/mL extract treatment. Further, the FITC Annexin V/PI assay also showed increase in cell death percentage with almost ~40% of cells undergoing either apoptosis or necrosis in combined treatment group. This showed the combination therapy can induce both apoptosis and necrosis in MCF-7 cells, which can be due to additive effect of both the treatment. Although no study has been reported for combination of *P. granatum* extract with PDT, while one study have reported the combination effect of pomegranate extracts (40 µg/mL) and genistein (~20 µg/mL) on MCF-7 cells which induced apoptotic cell death in ~50% cell population, which was more effective than single treatments [14]. Although no direct comparison can be made from this study, but it can be theorised that effect of Hypericin-PDT causes extreme cell damage which promotes the cells to undergo necrosis as compare to programmed apoptotic pathway induced by extract alone.

5. Conclusion

In conclusion, combination treatment with plant based non-phototoxic and phototoxic phytochemicals can be potential strategy for treatment of cancer with the advantages of less drug delivery doses, better therapeutic outcome and minimally induced unwanted toxicity.

Acknowledgements
The authors sincerely thank the South African Research Chairs initiative of the Department of Science and Technology and the National Research Foundation (NRF) of South Africa (Grant No 98337), South African Medical Research Council (Grant No. SAMRC EIP007/2021), South African Development Community (SADC), as well as grants received from the NRF Research Development Grants for Y-Rated Researchers (Grant No. 137788), Council for Scientific Industrial Research (CSIR)-National Laser Centre (NLC), African Laser Centre (ALC), and University Research Committee (URC).

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