



Enhanced anticancer potential of *Punica granatum* fruit extract in combination with Pheophorbide-a mediated photodynamic therapy on MCF-7 breast cancer cells

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Abstract

Combination therapy refers to the simultaneous administration of several therapeutic medications and treatment procedures with diverse biochemical and molecular targets and mechanisms of action. This is now used to treat a range of cancers. Furthermore, the search for less toxic natural alternatives has gained importance, which has led researchers' interest in phytotherapy-based combination therapies. *Punica granatum* leaf, fruit, seed, and peel extracts, like many other medicinal plants, contains a high concentration of phytochemicals like tannins, anthocyanidins, phenolics, and have shown to be effective in cancer treatment. In this study, the anticancer potentials of *P. granatum* extract were evaluated in combination with a chlorophyll derivative photosensitizer, pheophorbide-a mediated photodynamic therapy (PDT) against MCF-7, a human breast cancer cell line. The effects of single and combination therapy on the viability and morphology, were examined using, adenosine triphosphate (ATP) assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, and bright field and LIVE/DEAD assay respectively. The apoptotic cell death were determined by FITC-Annexin V/ Propidium Iodide assay, caspase, cytochrome-c, and Bax/Bcl-2 activity. This study demonstrated that combination treatment significantly inhibited the MCF-7 cell growth with an IC₅₀ value of 129 µg/mL compared to extract treatment alone at ~289 µg/mL at 24 h. Initiation of apoptosis indicated that it was mediated by the extrinsic and intrinsic pathways according to the enhanced levels of both caspase-8 and caspase-9 enzymes. Our findings suggest that a combination of phytocompound-based therapeutic modalities with different mechanisms of action has the potential to significantly improve cancer treatment efficacy when compared to single-agent therapy.

Keywords Anticancer · Breast cancer · Pheophorbide-a · Photodynamic therapy · Fruit extract · *Punica granatum*

Introduction

Cancer is considered one of the most serious diseases globally and new and advanced treatment modalities are continuously being investigated. According to the reports (global cancer statistics, GLOBOCAN), the sharp rise in cancer cases globally, putting an unprecedented level of emotional, physical, and financial pressure on patients, their families, healthcare systems, and communities (Soerjomataram and Bray 2021, pp. 2020–2070; Sung et al. 2021). In middle- and low-income countries, many cancer patients are not receiving reliable treatment because of the unpreparedness of healthcare systems to handle the daily increase in cancer incidence (Soerjomataram and Bray 2021, pp. 2020–2070; Sung et al. 2021). Current issues associated with conventional treatments such as surgery, chemotherapy, and

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radiotherapy include pulmonary toxicity, cancer-related fatigue, peripheral neuropathy, cardiotoxicity, cognitive, gastrointestinal, and musculoskeletal impairments, etc. (Nekhlyudov et al. 2022). As a result, a relatively safer and cost-effective treatment strategy is critical to address the alarming global rise in cancer cases as well as the major limitations of current treatments options. A wide range of medicinal plants are being studied for their phytotherapeutic properties due to their low-induced side effects and inherent sustainability. Natural products have recently gained the interest of researchers due to their multitargeted mechanisms of action, lower systemic toxicity, and potential to overcome drug resistance, thus can lessen side effects and improve the efficacy of cancer treatments. Over 65% of small-molecule anticancer drugs approved between 1981 and 2019 are derived from or inspired by natural sources, including widely used agents like paclitaxel, vinblastine, and camptothecin derivatives. Several other phytochemicals such as curcumin, resveratrol, betulinic acid, genistein, quercetin, and Epigallocatechin gallate are currently in Phase 2 or 3 clinical trials for various cancers (Gupta et al. 2012; Cragg and Pezzuto 2016; Choudhari et al. 2019; Newman and Cragg 2020; Feyisa et al. 2024; Ahmad et al. 2025).

Herbal extracts and medicinal plants were used to treat several different human ailments for many years, including digestive disorders, respiratory conditions, skin problems, infectious diseases, inflammation, cardiovascular conditions, neurological conditions, metabolic disorders, immune boosters and reproductive health (Awoke et al. 2024; Cock et al. 2024; Feyisa et al. 2024). Natural compounds have been given a lot of attention as cancer-preventive and therapeutic agents since several bioactive phytochemicals have been found to have anticancer properties and many of them have even received clinical approval (Thomford et al. 2018; Choudhari et al. 2019; Egbuna et al. 2019). Furthermore, natural product botanicals (0.4%), natural product derivatives (29.7%), and unaltered natural products (18.9%) account for the 247 antitumor drugs approved up to 2019; this demonstrates the significant probability for anticancer phytochemicals for future phytotherapies for cancer (Newman and Cragg 2020). Many patients employ natural or plant-based remedies in conjunction to traditional anticancer therapies to decrease the chemotherapy side effects, increase immune system function, manage illness symptoms, and promote overall health (Pezzani et al. 2019; Ma et al. 2021; Okem et al. 2023).

Due to the heterogeneous nature of cancer and the involvement of multiple targets and cancer-causing pathways, monotherapy has limited effectiveness in the treatment and prevention of cancer (Lin et al. 2020). Certain solid cancer types like Triple-Negative Breast Cancer,

Pancreatic Ductal Adenocarcinoma, Glioblastoma Multiforme, Non-Small Cell Lung Cancer and Ovarian Cancer are especially difficult to treat effectively with monotherapy due to their complexity, heterogeneity, aggressive behaviour, and high likelihood of developing resistance (Bhatia, Bhumika and Das 2020). Therefore, combination therapy is now frequently used to treat various types of cancer. Using multiple drugs or therapies has the significant advantage of lowering the drug doses, which can result in lesser resistance as well as retention of the same and/or higher therapeutic effectiveness (Lin et al. 2020). Certain plant products or extracts combined with chemotherapy have been shown the potential to increase susceptibility standard cytotoxic treatment, increase the combined impact of the two given treatments, overcome medical resistance, and strengthen the drug's efficiency in concentration and specifically target tumor cells with cytotoxic effects (Talib et al. 2022; Wang et al. 2023). Moreover, the combination of one or more natural compounds for cancer prevention and treatment is of great interest because this treatment regimen overcomes the use of chemical drugs.

A combination of drugs that target different disease pathways and/or mechanisms should be chosen in such a way that combination therapy does not impose any overlapping side effects or cross-resistance (Sauter 2020). Thus, combining plant products with cytotoxic and phototoxic efficacy can form a promising approach of combined phyto-phototherapy to combat cancer. Photodynamic Therapy (PDT) is a potentially effective clinically approved therapeutic alternative for the treatment of cancer. PDT causes cellular damage, induced by reactive oxygen species (ROS) which are produced within the cells and extracellular environment by the combination of photosensitizer (PS), light, and surrounding molecular oxygen (Correia et al. 2021). PDT offers several advantages over other conventional treatment modalities, including minimal systemic toxicity, the ability to target and eradicate specific tumors, and the ability to be used either in isolation or addition to alternative therapeutic approaches. Temoporfin-mediated PDT showed significantly improved local control and survival for patients with advanced head and neck squamous cell carcinoma with lesions ≤ 10 mm in depth who have tried other therapy modalities (Tan et al. 2010). A case study demonstrated that Hematoporphyrin- PDT not only alleviated obstructive symptoms and suppressed tumor growth in Colorectal cancer patients with reasonable safety but also enhanced systemic and local anti-tumor immunity by converting “cold” tumors into “hot” ones through immune cell recruitment and activation (Gu et al. 2022). This has been demonstrated to increase a patient's quality of life while increasing their average survival rate with minimal normal tissue toxicity

and invasion (Santos et al., 2019; Correia et al. 2021; Grin et al. 2022).

In our previous study, South African *Punica granatum* wonderful cultivar showed significant cytotoxic effects against breast cancer cells by inducing apoptosis (Fakudze et al. 2024). Based on that, we aimed to evaluate the efficacy of the combination of phytotherapy and PDT, to assess its impact on in vitro breast cancer cell line MCF-7. Pheophorbide-a is a natural chlorophyll-based tetrapyrrolic PS that exhibits strong absorption in the tissue-penetrating wavelength range of 650–700 nm for effective PDT (Xodo et al. 2012; Saide et al. 2020). The 650–700 nm range is considered a therapeutic window because it allows optimal tissue penetration with minimal absorption by endogenous chromophores and the photons of this energy can effectively photoactivate PSs for ROS generation (Plaetzer et al. 2009). This study evaluates the anticancer effects of methanolic extract of *P. granatum* arils in combination with pheophorbide-a mediated PDT on MCF-7 cell viability and morphology. Studies on intracellular localization of pheophorbide-a, and the underlying mechanism of cytotoxic action have been investigated by studying the expression of pro-apoptotic markers.

Materials and methods

General

Analytical-grade chemicals and solvents were utilized without any additional purification. Human Type Culture Collection's MCF7 (HTB-22) breast cancer cells acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA) were used in this study. High glucose Dulbecco's modified Eagle's medium (DMEM, D5796, SIGMA® Life sciences), Fetal bovine serum (FBS, F0679, SIGMA® Life sciences), Hanks' Balanced Salt solution (HBSS, 55037C, SIGMA® Life sciences) and 0.25% TrypLE solution (Gibco™, 12,563–029, ThermoFisher Scientific) were used. High purity grade Dimethyl sulfoxide (DMSO, D8418), Penicillin–streptomycin (P4333), Amphotericin B (A2942), and Methanol (179,337) were procured from Sigma-Aldrich. The stock solution of *P. granatum* methanolic extract and pheophorbide-a (16,072, Biocom Africa) was prepared in DMSO with an amount of 50 mg/mL and 1 mM respectively and were stored between 2–8 °C for further usage. *P. granatum* fruit was obtained from De Glebe Piketberg Farm, Western Cape, South Africa (32°54'21.3"S 18°45'21.7"E). Dr. Ashton Welcome at the University of Johannesburg herbarium stored and verified the authenticity of this fruit.

UV–Vis absorbance spectra

A spectrophotometer (Jenway 7315, Lasec, South Africa) was used to measure the UV/Vis absorbance spectra for methanolic extract, and PS was recorded from 200–800 nm with a 1 nm bandpass, with an initial blank reading with DMSO.

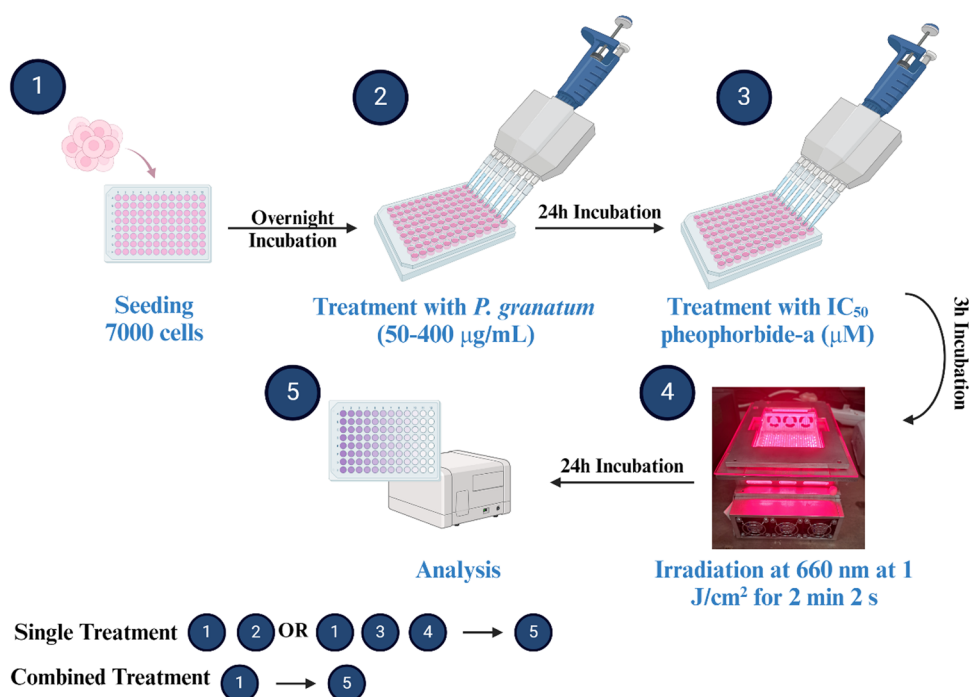
Cell culture

The monolayer of breast cancer cell line MCF-7 H1299 (ATTC® HTB-22) was grown and maintained in T75 tissue culture flask in DMEM growth medium supplemented with 10% FBS, Penicillin–Streptomycin at 1% and Amphotericin B at 1%. The cells were incubated in a humidified incubator (95%) at 37 °C with carbon dioxide at 5%. Experiments on cell culture were conducted using passages between 20–40 at 80% confluency. MCF-7 cells were seeded at 7×10^3 cells per well in 96-well plates and 2.5×10^5 cells per small culture plate, grown overnight as per the cell culture conditions mentioned.

Localization of pheophorbide-a

The colocalization of pheophorbide-a in different intracellular cell organelles were examined by fluorescence imaging using a fluorescent microscope (Carl Zeiss Axio Z1, Göttingen, Germany) equipped with ZIESS ZEN blue version 3.7.4 software. The nucleus, mitochondria, lysosomes, and endoplasmic reticulum were visualized using specific fluorescent organelle tracker dyes: Hoechst 33,342 (Invitrogen™, H1399, Cheshire, UK), carbocyanine-based MitoTracker® green FM probes (M7514), LysoTracker® green DND-26 acidotropic probes (L7526), and ER-Tracker™ Blue-White DPX dye (E12353). MCF-7 cells at seeding density of 1.5×10^3 were cultured on glass-bottom chamber slides (177,399, Nunc™ Lab-Tek™) overnight and were subsequently incubated with 2 µM pheophorbide-a in growth medium, for 3 h at 37 °C in the dark. The cells were washed two times with phenol red free 1X HBSS and stained with 100 nM MitoTracker, 5 µg/mL Hoechst 33,342, 500 nM ER-Tracker and 65 nM LysoTracker. After 30 min of incubation with Mito, Lyso, and ER tracking probes and 10 min with Hoechst 33,342, the cells were imaged. The following excitation and emission specifications were used to image the cells: Hoechst 33,342 ex/em 350 nm/461 nm (channel used: Hoechst 33,342, 455 nm), ER tracker at ex/em 430 nm/640 nm (channel used: ER tracker Blue-White DPX, 557 nm), Lysotracker at ex/em 504 nm/511 nm (channel used: Lysotracker Green DND-26, 509 nm), Mitotracker at ex/em 490/516 (channel used: Mitotracker Green FM,

Fig. 1 Single and combined treatment protocol with *P. granatum* fruit methanol extract and pheophorbide-a mediated PDT. The schematic representation is created with BioRender.com



512 nm), and pheophorbide-a at ex/em 668/698 (Alexa Fluor 660 channel, 691 nm) at a magnification of 200X.

Single and combined treatment

Based on our previous report, 24 h treatment of MCF-7 cells with methanolic extract of *P. granatum* arils is selected for single and combined treatment regimes, as this extract showed the potent cytotoxic activity amongst all the tested solvent extracts. The phototoxic efficacy of pheophorbide-a mediated PDT was determined by treating MCF-7 cells at varying concentrations of 0.1, 0.25, 0.4, 0.5, 0.6, 0.8, 1, 1.2, and 1.6 µM. Followed by 3 h of incubation period in dark, subsequent irradiation was done under the conditions as mentioned in next section. Cells were further incubated for 24 h. For combination treatment, cells were initially treated with methanol extract at 50–400 µg/mL for 24 h and followed by treatment with IC₅₀ dose of pheophorbide-a PDT with further incubation for 24 h. Following the proper incubation time, different experiments were carried out. The treatment protocol is represented in Fig. 1. For all experiments, untreated cells were used as the negative control to establish a reference to evaluate treatment effects. Additionally, PDT experiments included dark toxicity controls (cells treated with the pheophorbide-a but not exposed to light) and light toxicity controls (cells exposed to light without the pheophorbide-a treatment). Further solvent control (cells treated with the same volume of solvent used to dissolve the extract and pheophorbide-a) was included to rule out any solvent-induced cytotoxic effects.

Table 1 Irradiation parameter

Photosensitizer	<i>Pheophorbide-a</i>
Light source type	LED
Wavelength (nm)	660
Wave Emission	Continuous
Spectrum	Red (visible light)
Power (mW/cm ²)	7.08
Fluency (J/cm ²)	1
Irradiation duration	2 min 20 s

PDT parameters

After treatment of MCF-7 cells with pheophorbide-a for 3 h incubation, plates were placed in BioTHOR 660 Plate Illuminator (LED) and irradiated in the dark for 2 min 20 s. Irradiation parameters used are mentioned in Table 1.

MTT assay

Roche cell proliferation kit I (11,465,007,001, Germany) a colorimetric assay was utilized for the assessment of cell viability. The assay is based on the conversion of yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) to purple formazan crystals by living cells (Fakudze et al. 2024). After each treatment protocol, 100 µL of reagent was added in each well containing 1:9 ratio of MTT labelling agent and FBS free DMEM. The cells were incubated with the MTT (10 µL) reagent mix for 3 h followed by the subsequent addition of 100 µL of solubilization buffer. Plates were then incubated overnight in a humidified incubator. The sample absorbance was measured

at 570 nm with a standard wavelength of 650 nm using a microplate reader PerkinElmer, VICTOR Nivo™ (Separation Scientific, Johannesburg, South Africa). The percentage of cell viability was calculated with respect to the untreated control group.

ATP assay

Adenosine triphosphate (ATP) is present in live cells and can be utilized as an indicator of viable cells (Fakudze et al. 2024). Cells were treated according to the above-mentioned protocols and an ATP assay was carried out thereafter. Following the treatments, cells were washed with 1X HBSS twice and CellTiter Glo® reagent (Promega, G9682) at 50 µL and 1X HBSS at 50 µL was added to each well. Plates were incubated in the dark on a shaker for 25 min at room temperature. Luminescence reading was recorded in a microplate reader PerkinElmer, VICTOR Nivo™ (Separation Scientific, Johannesburg, South Africa). The percentage of cell viability was calculated with respect to the untreated control group.

Morphology: bright field and LIVE/DEAD

After treatment, cells were viewed under an inverted light microscope (Wirsam Olympus, CKX 41, Johannesburg, South Africa) with an attached digital camera (Olympus, C5060-ADUS, Johannesburg, South Africa) and CellSens 2.3 version imaging software for the assessment of cellular morphological alterations using brightfield setting. In the second set of experiments, the untreated/control cells, and cells undergone single and combined treatment were stained using the LIVE/DEAD® cytotoxicity kit (Invitrogen, L3224) for a qualitative cell viability analysis. A green, fluorescent dye calcein AM and a DNA-binding red fluorescent dye ethidium homodimer-1 are provided in the kit (LIVE/DEAD® cytotoxicity, L3224). Ethidium homodimer-1 dye only enters cells with damaged cell membranes and binds to nucleic acids while calcein AM dye is converted by intracellular esterase into fluorescent calcein in living cells, thus representing dead and live cells respectively. Briefly, the cell staining solution that included 1 µL of 1X HBSS containing 2 mL ethidium homodimer 1, and 1 µL calcein AM was prepared and then added to cells. After 10 min incubation, images were taken under a fluorescent microscope (Carl Zeiss Axio Z1, Göttingen, Germany) at ex/em 570 nm/602 nm (ethidium homodimer 1) and ex/em 488 nm/515 nm (calcein).

FITC annexin V/PI assay

The identification and quantification of the percentage of live, early and late apoptotic and necrotic cells was carried out by using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit II, 556,570. FITC-conjugated Annexin V protein complex binds to phosphatidylserine exposed on the outside of the plasma membrane of cells undergoing apoptotic cell death. Nucleic acid staining vital dye, propidium iodide can only pass through the membranes of dead and damaged cells, but cannot pass through the membranes of healthy, functioning cells. Following the treatment, cells were washed twice with 1 mL of 1X phosphate-buffered saline. Adherent cells were dissociated and resuspended in 1X binding buffer at a cell concentration of 1×10^6 . In each flow cytometry tube, 100 µL of cell suspension of different experimental groups was incubated with 5 µL Annexin V for 15 min at room temperature in the dark followed by the addition of 5 µL propidium iodide. This was followed by addition of 400 µL 1X binding buffer and samples were then analyzed using a flow cytometer (Becton Dickinson (BD) Accuri™ C6) with BD CSampler analysis software.

Hoechst staining

Hoechst 33,342 is a cell permeable double-stranded DNA binding fluorescent dye. This nuclei stain is used to stain metabolically active or fixed cells and tissue and allows visualization of chromatin condensation and fragmentation of apoptotic cells. Following the treatment protocol, cells were washed twice with 1X PBS to remove unbound Pheophorbide-a and extract residues and cell debris. Cells were incubated in the dark with the staining solution containing 5 µL Hoechst 33,342 in 1 mL of 1X HBSS (phenol red free) for 10 min in a humidified incubator. Thereafter the staining solution was discarded, and the cells were washed three times with 1X PBS. The fluorescence of Hoechst 33,342 was visualized using Carl Zeiss Axio Z1 microscope using the Hoechst Channel (ex/em 350/461 nm) at a magnification of 200X.

Cell death analysis

Cell lysis

Following each treatment protocol, the cells were lysed using a lysis cocktail containing 100 µL of lysis buffer (Promega, G183A), 10 µL of protease (Thermo Scientific, 1,861,282), and 890 µL of 1X PBS. After dissociation, cells were washed twice with 1X PBS and incubated with 1 mL of lysis solution for 10 min in ice. The cell suspension was centrifugated at 1500 g for 10 min at 4 °C. The obtained

supernatant was used as cell lysate for ELISA assays. The untreated group was kept as a control in all assays.

Human Bcl-2 and Bax (ELISA) assay

Apoptotic protein concentration of Bax and Bcl-2 were evaluated utilizing an enzyme-linked immunosorbent assay with antibodies specific to Human B-cell leukemia (Bcl-2, Elabscience®, E-EL-H0114) and Human Bcl-2 Associated X Protein (Bax, Elabscience®, E-EL-H0562). Specific antibody-coated microplates included in the kit were used, whereby 100 µL of treated and untreated cell lysate was poured into each well and incubated for 90 min at room temperature. 100 µL of biotinylated detection antibody, specific to Human Bcl-2 and Bax proteins was added afterward and incubated for 60 min. Then 100 µL of Avidin-Horseradish Peroxidase was added and incubated for 30 min. Several wash cycles were performed after incubation periods using a wash buffer provided in the kit. Thereafter 90 µL of substrate solution was added followed by 50 µL of stop solution. The optical density (OD) of developed yellow coloured end product was determined at a wavelength of 450 nm using a multiplate reader PerkinElmer, VICTOR Nivo™ (Separation Scientific, Johannesburg, South Africa).

Cytochrome c

Enzyme-linked immunosorbent sandwich assay (Invitrogen™, 99-0040, Cheshire, UK) was used after cell treatment to evaluate the subsequent release of mitochondrial cytochrome-c in the cytoplasm. Cytochrome-c-specific antibody-coated micro-ELISA plates provided in the kit were utilized in this assay. 100 µL of treated and untreated cell lysate were added to wells followed by the addition of 50 µL of diluted biotin-conjugated antibody. The plates were incubated for 120 min at room temperature. 100 µL of Streptavidin-Horseradish Peroxidase was added in each well with an incubation time of 60 min. After each incubation period, cells were washed three times using the provided wash buffer, using 300 µL per well for each wash cycle. 100 µL of substrate solution was added in each well and allowed to incubate for 15 min. Stop solution of 100 µL was then added which was immediately followed by absorbance measurement of developed colour in each well at 450 nm wavelength using a microplate reader PerkinElmer, VICTOR Nivo™ (Separation Scientific, Johannesburg, South Africa).

Caspase-8 and caspase-9 activity analysis

Fluorometric caspase-3, caspase-8, and caspase-9 Multiplex Activity Assay Kit (Abcam, ab219915) was utilized to

determine the activity of caspase-8 and caspase-9 in treated and untreated cells. Since MCF-7 cells lack caspase-3 expression due to a CASP3 gene deletion, our study was focused on caspase-8 and caspase-9 as principal initiator caspases of the extrinsic and intrinsic apoptotic pathways, respectively. Assessment of these caspases enables determination of activation of the apoptotic pathway upstream of caspase-3 and provides insight into the cell death mechanisms induced by treatments. Therefore, only caspases 8 and 9 activity were assessed and quantified (Jänicke 2009). IETD-R110, and LEHD-AMC are green and blue fluorescence indicators provided in the kit for caspase 8 and caspase 9, respectively. As per the manufacturer's instructions 1×10^4 cells were plated in each of a black wall/clear bottom 96-well plate. Cells were either left untreated or treated accordingly, diluted 1X caspase assay loading solution at 100 µL was loaded into each well and incubated for 30 min at room temperature. The fluorescence intensity for caspase-8 and caspase-9 in each well was measured at ex/em of 490/525 nm and ex/em of 370/450 nm, respectively using PerkinElmer, VICTOR Nivo™ (Separation Scientific, Johannesburg, South Africa) microplate reader.

Statistical analysis

The data are represented as the mean and standard deviation of three or more independent experiments that were performed in triplicates. The student's t-test was used to compare data from various treatment groups with the untreated group. Data with p value of 0.05 or less was considered statistically significant. ($p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***)) indicates statistically significant differences between the mean values of the untreated and treated groups. Statistical analysis was carried out with IBM SPSS software version 29. The inhibitory concentration (IC) values are determined by fitting dose-response curves using nonlinear regression analysis with a sigmoidal (Dose-response curve) model using a semi-logarithmic graph. Confidence intervals of $\geq 95\%$ are used for the calculation of each IC_{50} and IC_{90} value.

Results

Spectroscopic characterization of methanolic extract and pheophorbide-a

Figure 2 shows the recorded absorption spectra of PS pheophorbide-a and *P. granatum* aril methanol extract in DMSO solvent. *P. granatum* aril methanol extract showed a strong absorption band at ~ 300 nm, which is outside the therapeutic window of PDT. The two distinct absorption

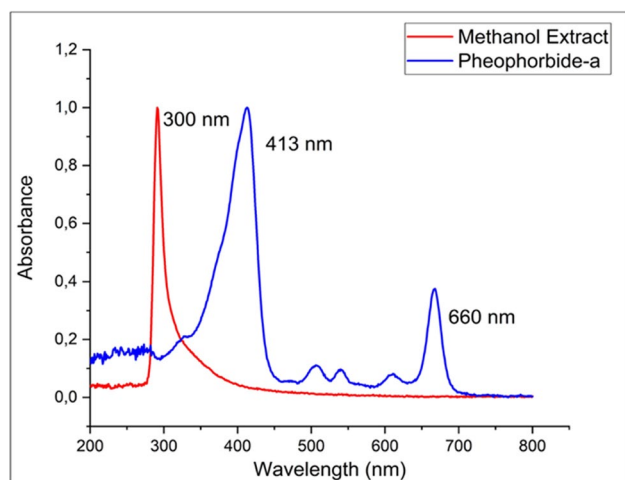


Fig. 2 Absorption spectra of *P. granatum* aril methanol extract and pheophorbide-a in DMSO

peaks observed with pheophorbide-a include a strong Soret band in the blue wavelength region with λ_{max} at ~413 nm and a Q band in the red wavelength region with λ_{max} at ~666 nm (Saide et al. 2020). The effective therapeutic window for PDT lies between 600 and 800 nm, and thus, irradiation at the ~666 nm Q band absorption peak is used for effective pheophorbide-a mediated PDT in this study.

Subcellular localization of pheophorbide-a

The microscopic images and fluorescence intensity profile of MCF-7 cells incubated with pheophorbide-a along with nuclear fluorescent dye Hoechst 33,342, and cell organelle probes Mito, ER, and Lyso tracker are presented in Fig. 3. The red fluorescence of pheophorbide-a was seen mostly in the cell cytoplasm, as represented by non-overlapping red fluorescence with blue fluorescence of Hoechst 33,342 both in the representative overlay images and fluorescence intensity profile [Fig. 3D, E]. As can be seen in Fig. 3h, m, the red fluorescent signals produced by pheophorbide-a in cells merged with those of ER-Tracker and Lysotracker with the highest and lesser extents respectively indicating their capacity to specifically target and localize to ER and a certain extent in lysosomes. While co-localization signals in mitochondria were not significant as can be seen in Fig. 3S & T.

Pheophorbide-a mediated phototoxicity

Figure 4 depicts the concentration-dependent pheophorbide-a-induced phototoxicity against MCF-7, as assessed by MTT assay. While no dark toxicity was observed in the used concentration range, treatment of cells with different concentrations of pheophorbide-a followed by light

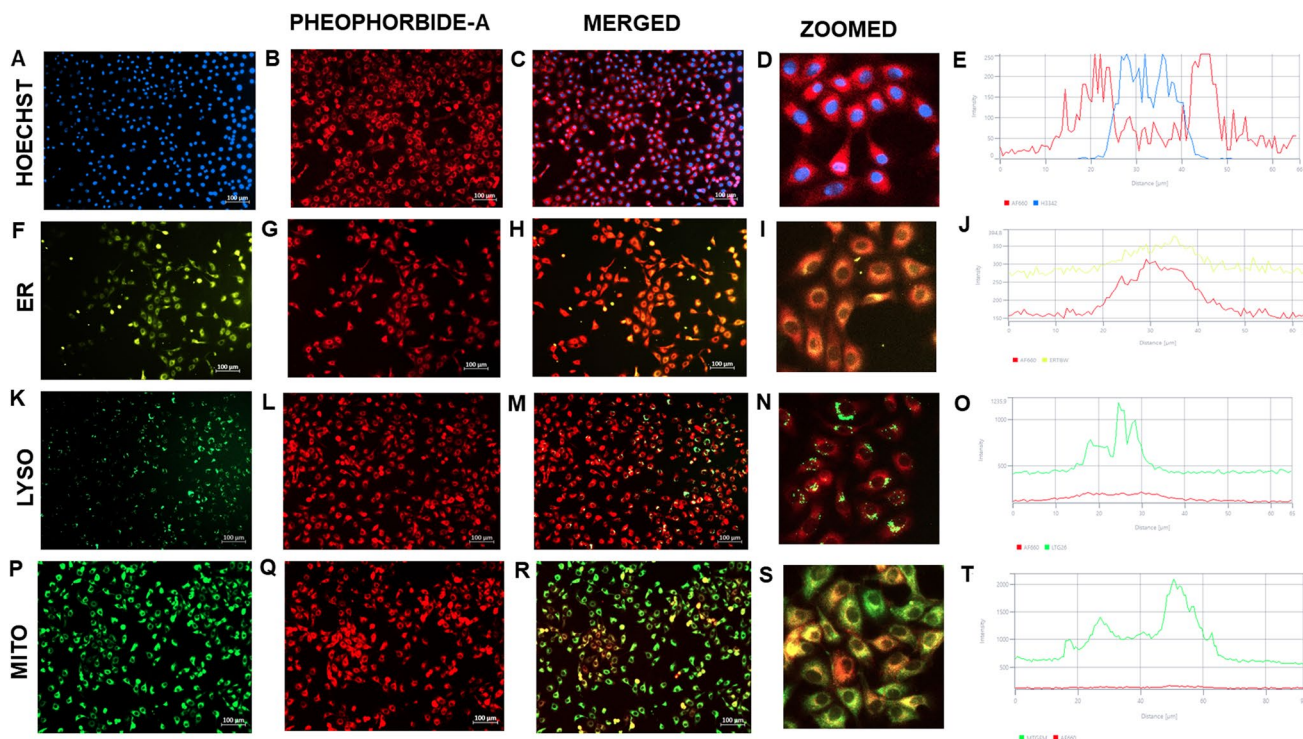


Fig. 3 A–T The subcellular localization of pheophorbide-a in MCF-7 cells. Representative fluorescence micrographs of MCF-7 treated with 2 μM pheophorbide-a co-stained with A Hoechst, F ER Tracker, K Lyso-tracker, and P Mito Tracker and their corresponding merged

images, the region of interest, and fluorescence intensity profiles (200X magnification, scale bar 100 μm). *Pheophorbide-a* red fluorescence present in the cell cytoplasm, ER, and lysosomes

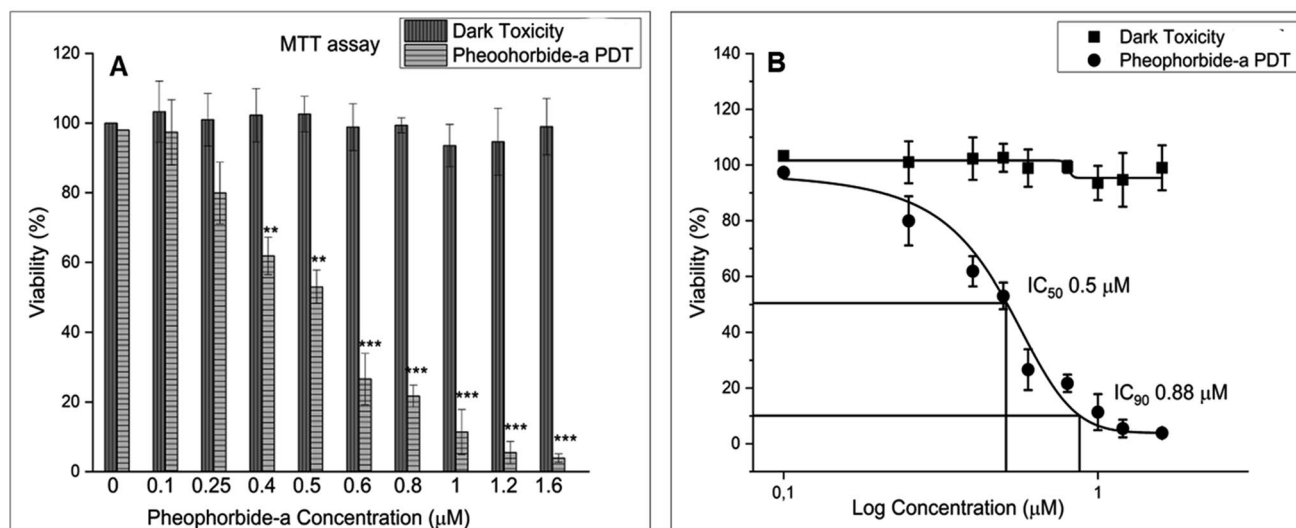


Fig. 4 Cell viability of MCF-7 cells following pheophorbide-a mediated dark and photo toxicity as determined by MTT assay. **A** phototoxicity induced by different concentrations of pheophorbide-a (0.1–1.6 μM) irradiated at 1 J/cm² and **B** dose–response curve of

pheophorbide-a induced phototoxicity. Data is represented as mean and standard deviation (n=3), performed in triplicates. (p<0.05 (*), p<0.01 (**), p<0.001 (***)) indicates statistically significant differences between the mean values of the untreated and treated groups

exposure at a fixed light dose of 1 J/cm² resulted in a concentration-dependent increase in phototoxicity (Fig. 4A). The light dose was optimized based on the gradient of cell phototoxicity observed over the used concentration range of pheophorbide-a. Concentration–response curve (Fig. 4B) shows that the IC₅₀ and IC₉₀ doses were found to be 0.5 μM and 0.88 μM, respectively. Based on the results, different concentrations of *P. granatum* methanol extract was combined with IC₅₀ dose of pheophorbide-a PDT. Additionally, as demonstrated in Fig. 6B, the ATP assay also demonstrated a concentration-dependent rise in phototoxicity with a roughly 90% reduction in cell viability at the highest pheophorbide-a (1.6 μM) concentration.

Combined treatment

The combined effect of anticancer effects of *P. granatum* methanol extract with pheophorbide-a mediated PDT was evaluated using MTT assay by treating the MCF-7 cells with various concentrations of methanolic extracts from 50 to 400 μg/mL for 24 h followed by 3 h of incubation with 0.5 μM of pheophorbide-a (IC₅₀) and irradiated at 1 J/cm². The results are shown in Fig. 5, whereby as compared to methanolic extract treatment alone (Fakudze et al. 2024), the loss in cell viability in the combinational group was higher in all used concentrations of methanolic extract. Further, the IC₅₀ and IC₉₀ dose for the combination treatment was calculated at the extract doses of 126 μg/mL and 400 μg/mL respectively (Fig. 5B). Similar combined treatment-induced cell viability was observed with ATP assay (Fig. 6B) with a significant reduction in cell viability at 200 μg/mL and

400 μg/mL as compared to single treatment with methanol extract. Based on the results, 400 μg/mL concentration of methanol extract treatment followed by 0.5 μM pheophorbide-a mediated PDT was selected as the optimum combinational concentration for further assays.

Treatment induced cellular morphological alteration

A bright field microscope was used to examine the morphological changes in both treated and untreated cells after single and combined treatments for indications of cellular damage and death, as shown in Fig. 7. The untreated group showed healthy cells with cell membranes left intact and of high yield (Fig. 7A). *P. granatum* extract (400 μg/mL) treated cells for 24 h displayed a decrease in cell numbers, while no drastic changes in cellular morphology were observed (Fig. 7B). While the cells undergone pheophorbide-a mediated PDT at IC₅₀ concentration (0.5 μM) showed significant number of cells with rounded up cellular morphology (Fig. 7C). Interestingly, in combination therapy group (Fig. 7D) a significant decrease in cell population was observed as compared to untreated and single treatment groups along with marked morphological alterations as represented by the rounded-up morphology, cell shrinkage, loss of cellular attachment and presence of cell debris.

LIVE/DEAD assay was performed to investigate the viability of MCF-7 cells subjected to single and combined treatment and representative fluorescence images are shown in Fig. 7. Strong green fluorescence cells showed healthy and live cells in the untreated group Fig. 7E. Single treatment

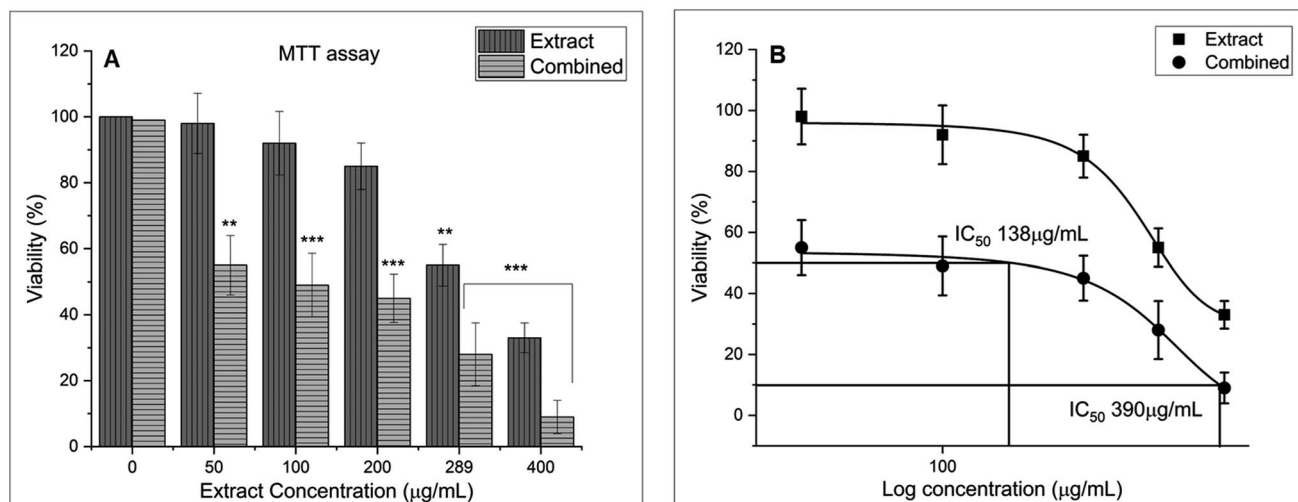


Fig. 5 Cell viability of MCF-7 cells following treatment, as assessed by MTT assay. **A** Single treatment with *P. granatum* aril methanol extract and combined treatment with IC₅₀ pheophorbide-a dose in the concentration range of 50–400 μg/mL and **B** dose–response curve of extract

alone and combined treatment groups. Data is represented as mean and standard deviation (n=3), performed in triplicates. (p<0.05 (*), p<0.01 (**), p<0.001 (***)) indicates statistically significant differences between the mean values of the untreated and treated groups

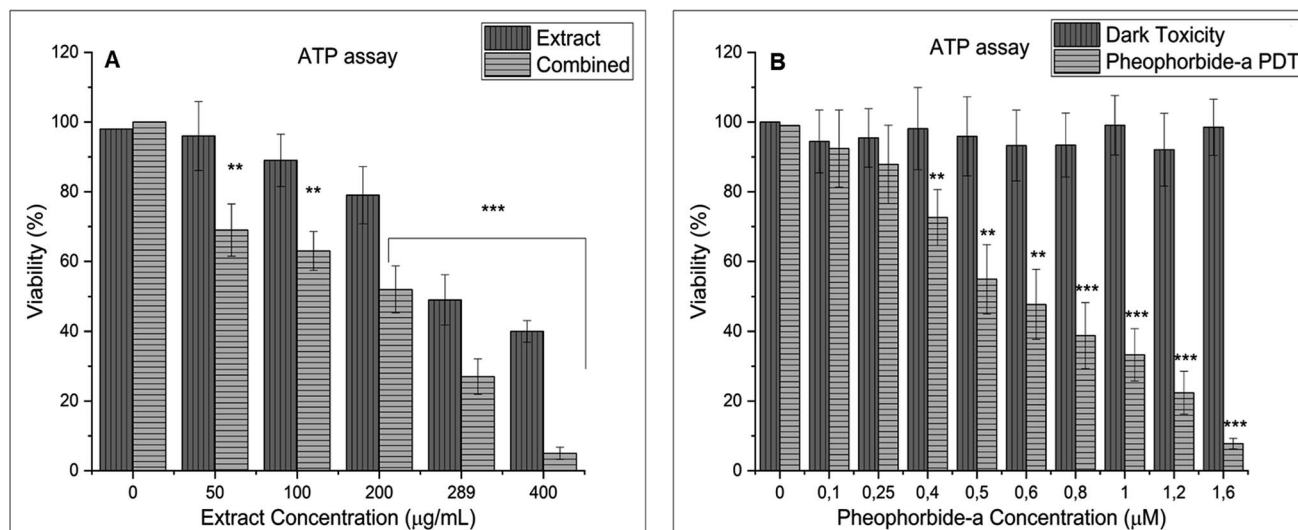


Fig. 6 Cell viability of MCF-7 cells following treatment, as assessed by ATP assay. **A** *P. granatum* aril methanol extract in single and combined treatment in the concentration range of 50–400 μg/mL, and **B** Pheophorbide-a mediated dark and photo-toxicity at concentrations

range of 0.1–1.6 μM. Data is represented as mean and standard deviation (n=3), performed in triplicates. (p<0.05 (*), p<0.01 (**), p<0.001 (***)) indicates statistically significant differences between the mean values of the untreated and treated groups

of *P. granatum* at 24 h (Fig. 7F) exhibited reduced number of cells while pheophorbide-a PDT (Fig. 7G) revealed morphologically altered green fluorescent cells and red fluorescent nuclei present, representative of dead cells. The combined treatment group (Fig. 7H) clearly showed a substantial decrease in cell viability as compared to the single treatment with almost insignificant green fluorescence along with significantly higher percentage of dead cells as represented by red fluorescent nuclei.

Treatment induced cell death

The double-staining technique using FITC Annexin V and PI was used to assess the possible induction of apoptosis and/or necrosis in response to single and combined treatments on MCF-7 cells. As shown in Fig. 8e, the percentages of the examined cells were split into four groups and displayed as a bar graph, live cells were represented by FITC Annexin V⁻/PI⁻; early and late apoptotic cells as FITC Annexin V⁺/PI⁻, and FITC Annexin V⁺/PI⁺, respectively and necrotic cells as FITC Annexin V⁻/PI⁺. Untreated control cells

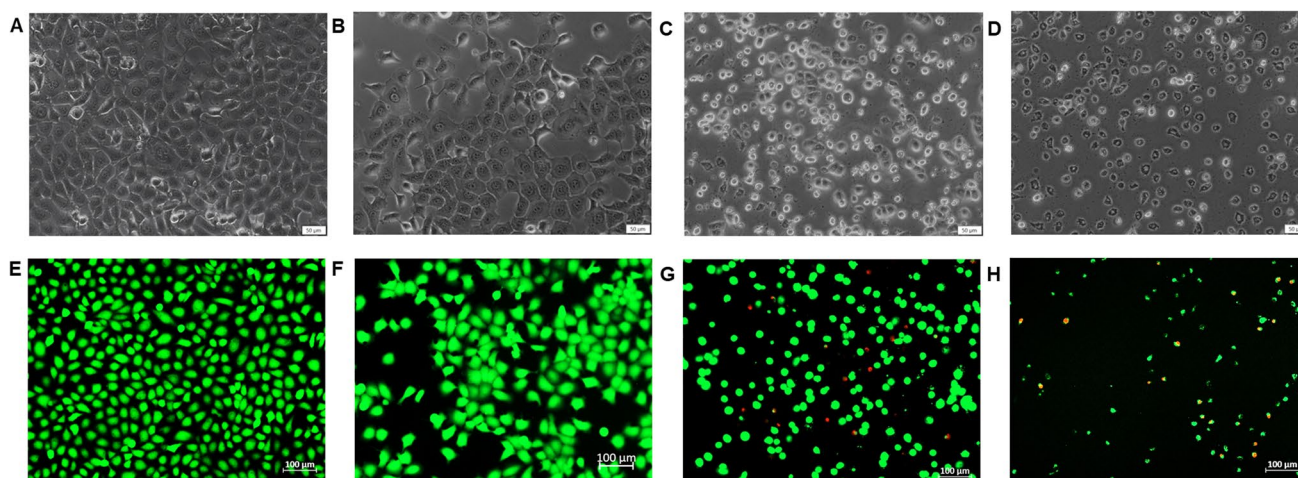


Fig. 7 Representative brightfield and fluorescent micrographs demonstrating changes in the morphology of MCF-7 cells following single and combined treatment. **A–E** Untreated cells, **B–F** *BP. granatum* aril methanol extract treatment at 400 $\mu\text{g/mL}$ for 24 h, **C & G** pheophor-

bide-a mediated PDT at IC_{50} , and **D–H** combination treatment with methanol extract (400 $\mu\text{g/mL}$, 24 h) followed by pheophorbide-a mediated PDT at IC_{50} (200X magnification, scale bar 50 μm and 100 μm respectively)

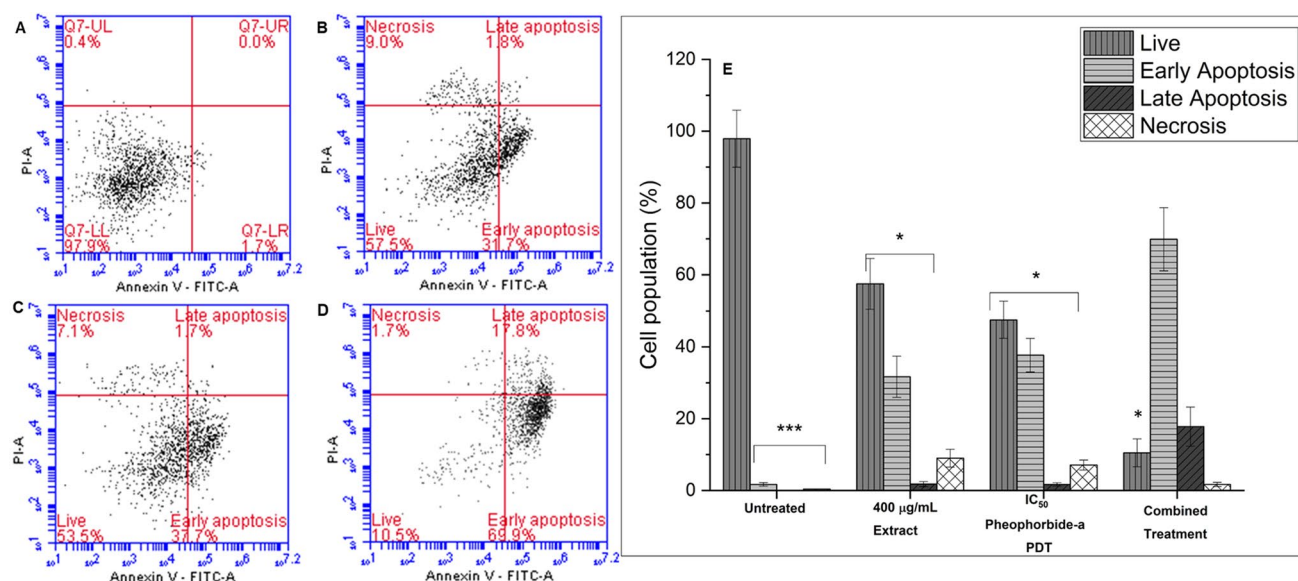


Fig. 8 Representative dot plot (**A–D**) and bar graph **E** of FITC Annexin V/PI-stained cells following single and combined treatment. **A** Untreated cells, **B** *BP. granatum* fruit methanol extract treatment at 400 $\mu\text{g/mL}$ for 24 h, **C** IC_{50} of pheophorbide-a PDT, **D** combina-

tion treatment with methanol extract (400 $\mu\text{g/mL}$, 24 h) followed by pheophorbide-a mediated PDT at IC_{50} and **E** percentage of live, apoptotic (early and late) and necrotic cell populations for all experimental groups

(Fig. 8a) showed ~98% of viable cells. Single treatment groups with cells treated with 400 $\mu\text{g/mL}$ methanol extract for 24 h and IC_{50} pheophorbide-a dose showed a decrease in viable cell population as represented by ~57% and ~47% respectively. Single treatment groups showed a significant increase in early apoptotic cell populations as compared to the untreated group. Combined treatment showed marked difference in viable cell population by ~90%, and early and late apoptotic cells at ~70% and ~18%, respectively. Further, a negligible % of necrotic cell population in all the treatment conditions, suggested that apoptosis is the main

mechanism of cell death induced by both single and combination treatment.

Treatment induced DNA damage

Using a fluorescent DNA-binding dye, Hoechst 33,342, the dying cells' nuclear morphology was examined to determine the kind of cell death induced by single and combined treatment. This assay allows the detection of apoptosis-mediated nuclear morphological changes, such as chromatin condensation, nuclear fragmentation, and damage. As

shown in Fig. 9A the nuclei of untreated cells are round and homogeneously stained. The methanol extract treatment of MCF-7 for 24 h showed similar nuclear morphology as the untreated group with very few cell nuclei with condensed, fragmented and/or degraded nuclei (Fig. 9B). While higher cell population in pheophorbide-a PDT group showed the features with condensed and damaged nuclei (Fig. 9C). As can be seen in Fig. 9D, the morphological changes of cell nuclei were more obvious in the combined treatment group, indicating enhanced apoptotic cell death effects.

Treatment induced apoptotic protein expression

Following the single and combined treatment, the induced apoptotic cell death in MCF-7 cells was further confirmed by measuring the cytochrome c levels released, Bax/Bcl-2 ratio, and the activation of caspase-8 and caspase-9 enzymes as shown in Fig. 10. A fixed time point between 4–6 h post-irradiation was selected, based on literature evidence

indicating peak apoptotic activation within this time period (Granville et al. 1999). Compared to untreated groups, Bax protein showed a relative increase while Bcl-2 protein showed a decline in both single and combined treatment groups. Additionally, all treated groups showed a ratio of >1 for Bax/Bcl-2 relative expression (Fig. 10B). Remarkably, compared to other single treatments, the combined group exhibited the highest Bax/Bcl-2 ratio, indicating a strong activation of the apoptotic pathway. Figure 10C shows the relative concentration of cytochrome-c proteins in the treated groups relative to the untreated MCF-7 cells. It was observed that the cytosolic concentration of cytochrome-c increases significantly in both single and combined groups, whereby the combined group showed the largest relative increase, approximately three times higher. The relative activity levels of initiator caspases, caspase-8 and caspase-9, in the single and combined treatment groups were significantly higher than the untreated group. The combined group showed the most enhanced levels of caspase-8 and

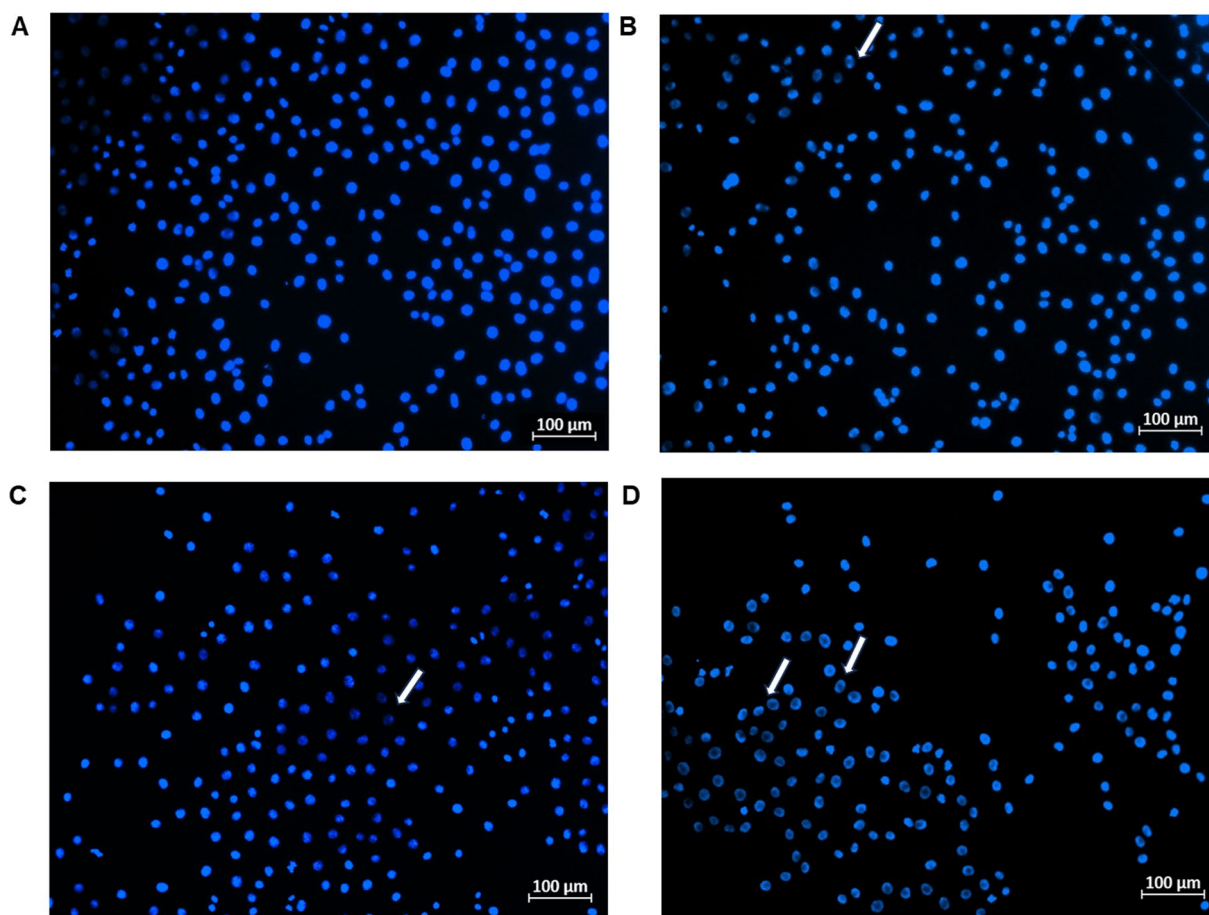


Fig. 9 Representative fluorescence images of nuclear morphology analysis by Hoechst staining following single and combined treatment **A** Untreated cells, **B** *P. granatum* fruit methanol extract treatment at 400 µg/mL for 24 h **C** pheophorbide-a mediated PDT at IC_{50} , and **D** combination treatment with methanol extract (400 µg/mL, 24 h) fol-

lowed by pheophorbide-a mediated PDT at IC_{50} . Chromatin condensation and nuclear fragmentation are indicated by white arrows. (200X magnification, scale bar 100 µm). The white arrow represents chromatin condensation, nuclear fragmentation, and damage

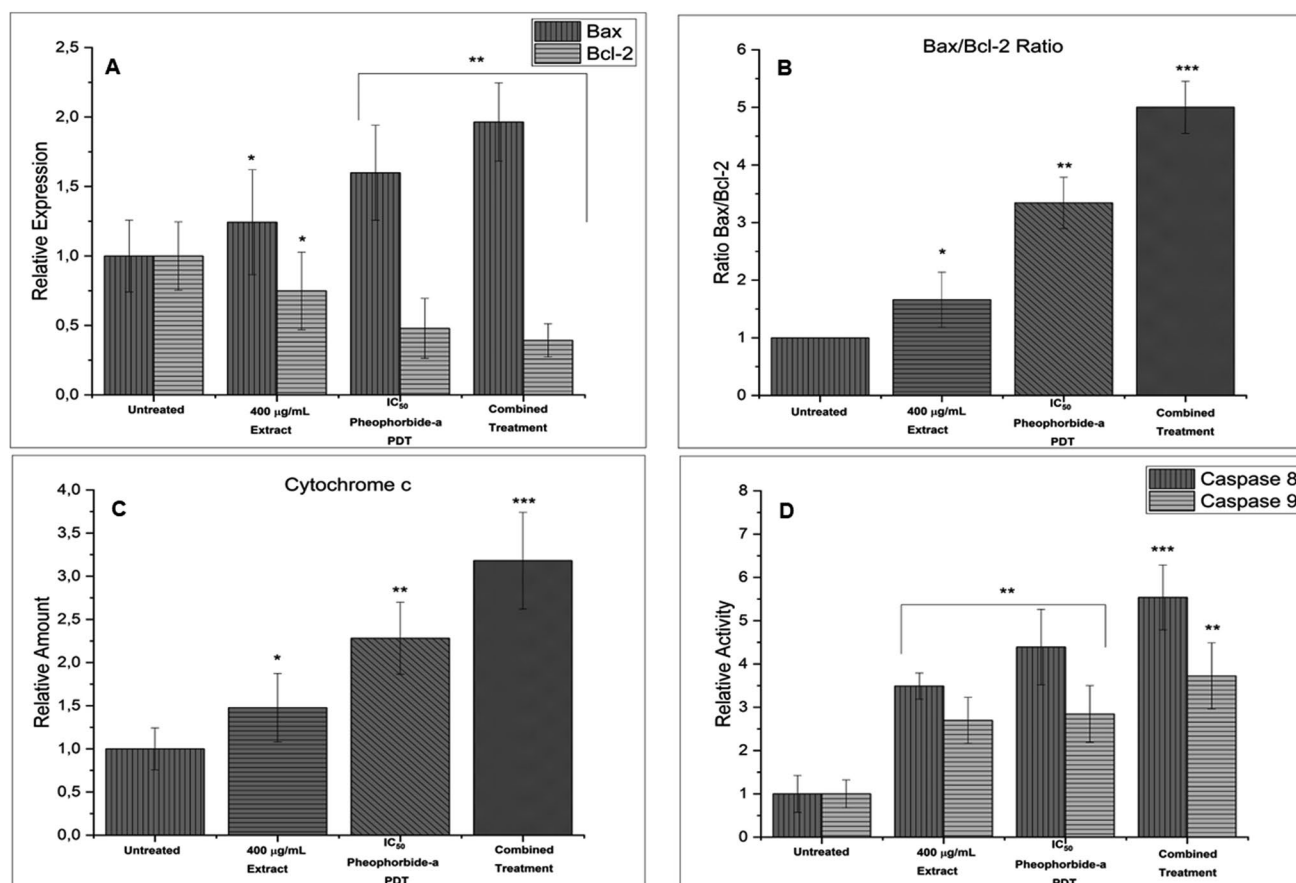


Fig. 10 Effect of single and combined treatment on Bax and Bcl-2 protein levels, release of cytochrome-c, and activity of caspase-8 and caspase-9 in each experimental group: (untreated cells, *P. granatum* fruit methanol extract treatment at 400 µg/mL for 24 h, IC₅₀ of pheophorbide-a PDT, and combination treatment with methanol extract (400 µg/mL, 24 h) followed by pheophorbide-a mediated PDT at IC₅₀. **A** Bax

and Bcl-2 relative protein expression, **B** Bax/Bcl-2 ratio, **C** cytoplasmic cytochrome-c relative amounts and **D** caspase 8 and 9 relative activity. Data is represented as mean and standard deviation (n=3), performed in triplicates. ($p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***)) indicates statistically significant differences between the mean values of the untreated and treated groups

caspase-9, by ~5.5 and ~3.7 fold, respectively with respect to untreated group.

Discussion

Numerous studies have demonstrated that phytochemicals exert chemopreventive or chemosuppressive effects by regulating molecular pathways involved in cancer growth and progression through diverse mechanisms, including enhancing antioxidant defenses, inactivating carcinogens, inhibiting epithelial-to-mesenchymal transition, suppressing cell proliferation, triggering apoptosis and cell cycle arrest, downregulation of anti-apoptotic proteins, prevention of epigenetic alterations, and reduction of oxidative stress-induced DNA damage as well as promoting immune regulation (Choudhari et al. 2019; Ahmad et al. 2025).

In our previous study, we investigated the phytochemical content and antitumor potential of South African Wonderful

cultivar fruit (*Punica granatum* L.). The methanolic extract of arils with the highest phenolics and flavonoid content showed the most significant cytotoxic potential against MCF-7 induced by apoptotic cell death (Fakudze et al. 2024). Phytochemicals have demonstrated an ability to improve anticancer activity (Kim et al. 2014; Chen et al. 2018; Dou et al. 2018) and overcome drug resistance in pre-clinical studies when combined with other therapies like chemotherapy. Other than the advantage of overcoming the induced negative effects, combination therapy with plant products enhances the therapeutic effect in a synergistic or additive way along with the reduction in delivery doses, drug dose toxicity, and lessening drug resistance (Aziz et al. 2023; *Combination Anticancer Therapies Using Selected Phytochemicals—PubMed*, <https://pubmed.ncbi.nlm.nih.gov/36080219/>). In this regard, the anticipated cross-resistance and overlapping negative effects of these compounds should be considered when creating a combination experimental model (Nikanjam et al. 2017). Interestingly, as per

our knowledge this is the first study of its own for combination of *P. granatum* fruit extract with PDT.

Although *in vitro* studies cannot confirm *in vivo* biological activity, they do provide a rationale for using plant extracts for anticancer efficacy. However, caution must be taken when interpreting these findings, as several bioactive metabolites, which are frequently produced by intestinal or hepatic metabolism, may differ from those first found in the extract, and many chemicals utilized *in vitro* may not be accessible *in vivo*. Ellagic acid (EA), one of the major ingredients of pomegranate fruit extract, reached a C_{max} of 31.9 ng/ml in human plasma 1 h after ingestion but was rapidly cleared from the bloodstream within 4 h, when administered with 180 ml of pomegranate juice containing 25 mg EA and 318 mg ellagitannins (ETs). This is due to EA's poor water solubility and tendency to accumulate in intestinal epithelial cells (Seeram et al. 2004). The same group in a different pharmacokinetics clinical trial reported a EA C_{max} of 18.64 ng/mL, T_{max} of 0.98 h, AUC of 50.07 ng/h/mL, and a half-life (t_{1/2}) of 0.75 h in human subjects who consumed 180 mL of pomegranate juice containing 318 mg of ETs (Seeram et al. 2006). Another study reported the results following consumption of pomegranate extract by healthy human volunteers in the form of capsules containing 800 mg of extract per dose, providing 330.4 mg of punicalagins and 21.6 mg of EA. EA and its microbial metabolites urolithins A and B, their glucuronides, and dimethyl ellagic acid-glucuronide were detected in plasma, though punicalagins were not. These metabolites, likely formed in the colon by gut microbiota, appeared in plasma between 2 to 24 h post-ingestion, with inter-individual variability indicating variation in metabolism or prior dietary intake. Pharmacokinetics studies showed mean C_{max} for EA in plasma was 33.8 ± 12.7 ng/mL, with a T_{max} of 1 h post-ingestion (Mertens-Talcott et al. 2006). While the study conducted to study *in vivo* distribution of the pheophorbide A in tumor bearing rats, indicated its primary accumulation in the reticuloendothelial system, followed by the gut, lungs, and pancreas, with minimal presence in the skin. Notably, it showed prolonged tumor retention with a high tumor-to-pancreas ratio at 24 h, suggesting a favorable absorption spectrum and lower skin toxicity needed for PDT (Evrard et al. 1994). While *in silico* study with SwissADME predicted limited gastrointestinal absorption for the pheophorbide-a, likely due to its high molecular weight and substrate for P-glycoprotein, and demonstrated bioavailability of 56%, indicating that a substantial amount of the administered dose enters systemic circulation (Paul et al. 2024).

Since polyphenolic phytochemicals are known to absorb substantially in the 250–350 nm range, they are principally responsible for this UV-region absorption of the methanolic extract of the fruit (Anouar et al. 2012). The presence of this

peak does not directly contribute to conventional PDT light activation. The extract's role in our combination treatment study was to work in concert with the PDT-activated agent, as a chemotoxic agent with a different mode of action than PDT to achieve a synergistic treatment outcome. Thus, the relevance of use of a potent PS like pheophorbide-a is due to its strong Soret band about ~410 nm and distinctive Q bands between 500 and 700 nm, with most notable absorption peak at 665–670 nm. This Q band is ideal for efficient ROS production and light penetration into biological tissues since it lies well within the therapeutic window (600–800 nm), which can improve the efficacy of treatment (Ethirajan et al. 2010).

Colocalization study results showed that pheophorbide-a, a well-known hydrophobic PS, accumulated in lysosomes and the ER but not in the mitochondria (Fig. 3) (Röder et al. 2000). It is commonly known that receptor-mediated endocytosis causes more hydrophobic PS to bind with low-density lipoproteins and accumulate in endosomes and lysosomes (Rosenkranz et al. 2000; Benov 2014; Van Straten et al. 2017). Our results are consistent with studies conducted by Moret et al. whereby they showed that the pheophorbide-a was primarily located in the ER in MDA-MB-231 cells and induced apoptotic cell death following light irradiation (Moret et al. 2021). Notably, in contrast to other reports in the literature, we were unable to detect mitochondrial localization (Tang and Liu 2009a; Choi et al. 2014; Yoon et al. 2014). Treatment results are significantly impacted by the subcellular localization of PS. Upon PDT-induced oxidative stress, lysosomal membrane permeabilization results in release of cathepsins and other hydrolytic enzymes, and ER stress, all of which can cumulatively trigger apoptotic cell death. Studies have shown that, depending on the subcellular localization of the PS, different pathways are activated. For instance, lysosomal accumulation triggers p38 MAPK and mitochondrial apoptosis, while ER-localization disrupts ER homeostasis and activates Ca²⁺ signaling and the unfolded protein response via PERK. These primary signals can be amplified through caspase-dependent and independent pathways, with later-stage events potentially involving secondary pathways leading to mitochondrial cell death (Mosserova and Kralova 2012). Delivery of therapeutic compounds to precisely the subcellular site of action leads to increased efficacy and reduced toxicity. Localization in the mitochondria and ER, which are crucial functional organelles in cells, can cause synergistic effects to cancer treatment through maximizing therapeutic indices, lowering the overall drug dose, and these multiple techniques alter the drug's initial mode of action, which then may lead to lowered chances of multidrug resistance.

Pheophorbide-a mediated PDT induced strong phototoxicity against MCF-7 cells at a light dose of 1 J/cm²,

with IC_{50} and IC_{90} concentrations of 0.5 μ M and 0.88 μ M, respectively (Fig. 4). Further, the cell viability was assessed for combination therapy, whereby different methanol extract concentrations (0–400 μ g/ml) were combined with pheophorbide-a PDT at IC_{50} dose (0.5 μ M, 1 J/cm²). As represented in Figs. 5 and 6, the effectiveness of pheophorbide-a mediated PDT showed a dose-dependent increase in combined treatment as compared to extract alone treatment. In the combination treatment, the IC_{50} dose was reduced to 129 μ g/mL compared to single extract treatment of ~289 μ g/mL and ~229 μ g/mL at 24 and 48 h respectively (Fakudze et al. 2024). Interestingly an IC_{90} dose of 400 μ g/mL was achieved with the combination dose, whereby 90% cell death was not observed with the methanol extract alone even at the highest concentration of 400 μ g/mL within 48 h of the treatment period (Fakudze et al. 2024). A probable underlying mechanism for enhanced cytotoxicity in combination treatment can be explained as the synergistic interactions between the phytochemicals in the extract and the pheophorbide-a induced PDT. The phytochemicals present in the extract may sensitize cancer cells by modulating signaling pathways, whereby ROS generated during PDT directly harm cellular components, increasing cell death. When combined, these complementary processes have a stronger cytotoxic effect than when used as a single treatment. These results highlight the therapeutic potential of combination methods to improve efficacy at therapeutically feasible doses by overcoming the drawbacks of monotherapies, such as resistance development and partial cell death.

ROS generation is the central mechanism by which PDT exerts cytotoxic effects, leading to cancer cell death. Pheophorbide-a is a potent generator of singlet oxygen, making it highly effective as a PS in PDT (Wang et al. 2014). Thus, the photoactivation of pheophorbide-a leads to an increase in ROS, resulting in loss of cell viability. On the other hand, studies have shown that certain dietary phytochemicals demonstrate dual functionality, behaving as antioxidants or pro-oxidants depending on their concentrations and the oxidative state of the cellular environment (Fernando et al. 2019). Thus, based upon their activity, they can significantly influence the effectiveness of ROS-mediated cancer treatment at the molecular level. Thus, polyphenols, flavonoids, and anthocyanins present in *Punica granatum* aril extract could be acting as prooxidants under the concentrations used in this study (Fakudze et al. 2024). This redox modulation can amplify oxidative stress in cancer cells, thereby enhancing PDT efficacy.

The morphological analyses of MCF-7 cells using bright field microscopy provided additional evidence for the cytotoxic effect of single and combined treatments (Fig. 7 A-D). Both the single treatment and the combination treatment resulted in notable morphological alterations in MCF-7

cells relative to untreated control cells. The combination treatment group showed the highest cell toxicity as indicated by the majority of the free-floating cells, detached and rounded cells revealing the primary apoptotic features. The results were further confirmed with LIVE/DEAD fluorescent assay; compared to the single treatment, the combined treatment group (Fig. 7H) demonstrated a markedly higher percentage of dead cells, as indicated by fluorescent red nuclei, and nearly negligible green fluorescence, indicating a substantial decrease in cell viability.

To further, analyze the cell death mechanism in both single and combination treatment, several biochemical and molecular marker-based assays were carried out. FITC Annexin V/PI assay (Fig. 8) indicated induction of significant apoptotic cell death in all treatment groups. However, the combined treatment resulted in a significant increase in early and late apoptotic cell populations, of ~70% and ~18% respectively in comparison to all single treatment groups. These results are consistent with DNA damage detection assay with Hoechst 33,342 fluorescent staining as shown in Fig. 9. Whereby, as compared to untreated and single treatment groups, a greater number of cells in the combined treatment group displayed nuclear morphological characteristics such as DNA condensation and damage in dying cells, indicating apoptotic mode of cell death.

Several studies have suggested that different intracellular cell death pathways can be triggered in a cell type-specific manner by pheophorbide-a PDT. For example, pheophorbide-a mediated PDT-induced generation of ROS has been shown to induce apoptosis through the mitochondrial-mediated pathway in several human cancer cells such as uterine carcinosarcoma, pigmented melanoma, colon cancer, pancreatic cancer, hepatocellular, head and neck cancer cells and Jurkat leukemia (Hajri et al. 1999; Jin et al. 2000; Lee et al. 2004; Tang et al. 2006, 2009a, b; Chung et al. 2009; Tang and Zhang 2009b). While Yoon et al. showed that pheophorbide-a PDT leads to the activation of both autophagy and apoptotic pathways in the human breast, oral squamous carcinoma, non-melanoma, and melanoma skin cancer cell lines (Bui-Xuan et al. 2010; Ahn et al. 2013; Yoon et al. 2014). While in another study pheophorbide-a PDT was shown to cause G0/G1 cell cycle arrest in androgen-sensitive human prostate adenocarcinoma cell line (Gheewala et al. 2018). These results indicate that pheophorbide-a PDT may trigger different or overlapping pathways of cell death, depending on the cellular environment, its subcellular distribution, delivered PS concentration, and light dose as well as intracellular uptake limits.

Following the initiation of apoptosis by the different treatments in MCF-7 cells, alterations in the expression of the cytochrome-c, Bax (pro-apoptotic), Bcl-2 (anti-apoptotic), and caspase 8 and 9 proteins (Fig. 10) were found in order to

understand probable molecular pathways that caused cells to undergo apoptosis. There are two main ways in which apoptosis is initiated: intrinsically through the mitochondria or extrinsically via death receptors, or DR. In contrast to the extrinsic pathway, which is triggered by death ligand binding to its appropriate receptor and subsequent caspase-8 activation, the intrinsic pathway is activated by cytochrome-c release from the mitochondria following inner mitochondrial membrane disruption and subsequent caspase-9 activation. Caspases-3 and -7, which are the ultimate agents of apoptotic cell death, are triggered by a sequence of signaling cascades that are initiated in these two pathways (Elmore 2007). It is commonly known that proteins belonging to the Bcl-2 family are essential for either initiating or blocking intrinsic apoptotic pathways (Qian et al. 2022). Combining treatments in this study resulted in enhanced levels of the proapoptotic protein Bax and a decline in the levels of the antiapoptotic Bcl-2 protein, which raised the ratio of Bax/Bcl-2. The increase in the Bax to Bcl-2 ratio causes the mitochondrial membrane to become porous, which allows cytochrome c release from the mitochondria and to enter the cytoplasm, where it triggers a series of caspase activation events that induce apoptosis (De et al. 2023). Notably, the increased Bax to Bcl-2 ratio and cytoplasmic cytochrome-c in the combined treatment was the highest with respect to single treatment groups. Further, the combined treatment additionally displayed a comparable chain of events denoted by increased cytochrome-c in the cytoplasm levels and increased caspase 8 and 9 activities.

The caspases, which are classified as either initiator or executioner caspases, include caspases -8 and -9 (initiators) and caspases-3 or -7 (executioners). During the leaking of mitochondrial cytochrome c into the cytoplasm, caspase-9 is triggered, while caspase-8 initiator triggers the extrinsic pathway (Elmore 2007). In our previous report, cells treated with methanol extract showed enhancement of caspase 9 levels, suggesting that the mitochondria-induced intrinsic pathway was involved in facilitating apoptosis. Furthermore, the amount of caspase 8 was also noticeably greater in treated cells than in untreated cells, indicating that the extrinsic apoptotic pathway was likely also involved (Fakudze et al. 2024). Interestingly, other than the heightened activity of caspase 9, pheophorbide-a mediated PDT at IC₅₀ dose also showed a relative incline in caspase-8 activity (Fig. 9). This result is quite interesting compared to common characteristic of PDT-induced apoptosis which generally results in the quick release of cytochrome c from the mitochondria into the cytosol and the activation of caspase-9 pathway (Robertson et al. 2009). Pyropheophorbide-a methyl ester was reported to mediate PDT-induced apoptosis primarily via the mitochondrial caspase-9/-3 pathway intrinsic pathway (Matroule et al. 2001; Tian et al. 2006). While in another

study, a PSMA inhibitor-conjugate of pyropheophorbide-a was shown to trigger apoptosis specifically through the caspase-8/-3 pathway (Liu et al. 2010).

The difference in the subcellular localization and PDT-generated ROS can be partially attributed to the difference in the activation of distinct apoptotic pathways (Liu et al. 2010). As pheophorbide-a was observed to localize partially in the lysosomes, the PDT-mediated disruption of lysosome and eventual release of cathepsins B and D, activation of proapoptotic protein Bid can be speculated to be involved in the activation of the intrinsic apoptotic pathway (Maharjan and Bhattarai 2022a). Furthermore, localization of pheophorbide-a in ER can also be responsible for PDT-induced ER stress to induce cell death, as also reported in other studies (Bui-Xuan et al. 2010; Gheewala et al. 2018).

Interestingly, the extent and type of ROS produced also determines the type of apoptotic caspase pathway involved. Certain studies have demonstrated that singlet oxygen mediated selective activation of p38 and caspase 8. This in turn signals the cleavage of Bid, which is further able to link to extrinsic and intrinsic apoptotic pathways. The active Bid protein causes depolarization of the mitochondrial transmembrane potential and releases cytochrome c, mediating the activation of downstream caspases (Zhuang et al. 2000; Salmerón et al. 2018). Furthermore, direct activation of the extrinsic apoptotic pathway can also be mediated by photo-generation of singlet oxygen which promotes the Fas receptor's ligand-independent oligomerization, which binds to the adapter protein FADD and causes the activation of caspase 8 (Morita et al. 1997; Zhuang et al. 2000; Maharjan and Bhattarai 2022b). Thus, pheophorbide-a with a high singlet oxygen quantum yield of ~0.69, is strongly speculated to be involved in activation of extrinsic apoptotic pathway along with the intrinsic pathway (Fernandez et al. 1997). Apoptosis in MCF-7 cells is mediated by caspases-9/8, 7, and 6 due to the lack of caspase-3 expression (Liang et al. 2001).

When compared to single treatment groups, the combined treatment group showed the highest activity of both caspase 8 and caspase 9 representing the involvement of intrinsic and extrinsic apoptosis pathways in MCF-7 cell death by this treatment (Fig. 10D). Our study showed strong synergistic/additive effects of using *P. granatum* extracts in combination with pheophorbide-a mediated PDT. There are very few reports on the combination of plant extracts or phytochemicals with PDT. One study demonstrated the lung cancer (A549) cell line's cytotoxicity, DNA damage, and apoptosis were all markedly and dose-dependently enhanced by the catechin combined with zinc phthalocyanine (ZnPcS4)-mediated PDT (Senapathy et al. 2020). Another study conducted with *Dicoma anomala* root extracts combined with ZnPcS4-mediated PDT to produce cytotoxic effects on lung (A549) and breast (MCF-7) cancer cells, which led to the

upregulation of apoptotic proteins (p38, p53, Bax, caspase 3, caspase 8, and caspase 9) (Chota et al. 2022a; b). Aziz et al., also reported the synergetic effect of the combination of *Ficus Carica* and *Ficus racemose* extract with photosense-mediated PDT resulting in a pronounced cytotoxic effect, suppressed cell migration and cell apoptosis in rhabdomyosarcoma cells (Aziz et al. 2021; Aziz et al. 2023).

Although a direct comparison with conventional chemotherapeutic treatments such as doxorubicin or paclitaxel was not included in our current investigation, the literature indicates that these chemotherapeutic drugs, used as the first line of treatment for breast cancer, despite their high effectiveness, are frequently linked to dose-limiting toxicities and multidrug resistance (Crown et al. 2004; Okem et al. 2023; Kaveh Zenjanab et al. 2024). On the other hand, a combination of phytochemical-based treatment as used in this study provides a more effective treatment strategy that may result in selective cytotoxicity and less induced toxicities, at comparatively lower doses. Even though this is a preliminary study, the results demonstrate the therapeutic value of combining plant-based bioactive compounds with PDT as a complementary or alternative strategy, particularly when traditional treatments show limited responses or have serious adverse effects. The relative effectiveness, safety, and clinical transferability of this strategy will need to be confirmed by additional preclinical in vivo studies. Future studies are essential to confirm the therapeutic efficacy, pharmacokinetics, bioavailability, and safety profile of the combination therapy.

Conclusion

In summary, the study showed that compared to single treatments, pheophorbide-a mediated PDT showed enhanced cancer cell death by apoptosis in combination with methanolic extract of arils of *P. granatum* fruit. This combination approach showed the potential to lower the dosage of both extract and PS and thus have the potential to improve overall therapeutic outcomes. Further investigation on the mechanism and the targeted pathways is warranted to thoroughly understand the chemopreventive, chemotoxic, phototoxic, and enhanced effects to guide the potential adjunctive therapeutic applications of a combination of different plant-based phytochemicals.

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Data availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval University of Johannesburg Health Sciences Research Ethics Committee approved the study. The approval document is REC-1401-2022.

Consent for publication Not applicable.

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