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Paradise Tree Extract (*Simarouba glauca*) Selectively Induces Cell Death, Enhances Efficacy of Common Chemotherapeutics and Reduces their Toxicity in *In-Vitro* and *In-Vivo* Models of Breast Cancer

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ABSTRACT

BACKGROUND: Although chemotherapeutics have proven to be effective in treating metastatic breast cancer, their limited target selectivity has resulted in adverse side effects, rendering them unsuitable for long-term usage. Alternatively, certain natural extracts provide a promising strategy to selectively target cancer while being safe to consume. Specifically, paradise tree (*Simarouba glauca*) has shown potential anti-tumour activity; however, its efficacy against cancer, mechanism of action, and interaction with standard chemotherapies have not been investigated.

METHOD: We have demonstrated the anti-tumour activity of ethanolic paradise tree extract (PTE) in triple-negative and ER-positive breast cancer cell lines and its interaction with chemotherapeutics when used in combination. The anti-tumour efficacy of PTE was evaluated through the expression of apoptotic biochemical markers as well as changes in cell morphology. For mechanistic studies, fluorogenic dyes were used to quantify reactive oxygen species production and mitochondrial membrane potential destabilization.

RESULTS: Our results have shown that PTE selectively triggers apoptosis in breast cancer cells while having limited effects on noncancerous cells. Importantly, we have found that PTE enhances the anti-tumour efficacy of chemotherapeutics, cisplatin and Taxol, when given in combination, while reducing their toxicity in noncancerous cells. Furthermore, PTE inhibits growth of human tumour xenografts in immunocompromised mice. Importantly, PTE in combination with Taxol and cisplatin had the best anti-tumour effect.

CONCLUSION: Our findings suggest that PTE could be a safe and effective treatment for breast cancer. Most importantly, as a supplement to chemotherapeutic regimens, it could enhance anti-tumour effects and reduce chemo-related toxicity.

KEYWORDS: Breast cancer; paradise tree; natural health products; apoptosis; oxidative stress; cisplatin; taxol; drug-drug interaction

Background

Among women, breast cancer remains the most prevalent form of cancer and accounts for 30% of all new cancer diagnoses [1]. Advanced screening methods and diagnostic tools have been developed for early detection and surgical procedures have been used for disease management [2]. However, complications can arise with surgical procedure as the tumour can disseminate, advancing the cancer to a late metastatic stage where surgical options are limited or futile [3]. Chemotherapeutic drugs are conventional treatments for advanced-stage breast cancer and are typically platinum-based, taxane-based or anthracyclines [4, 5]. However, they lack selectivity as they target features common to both cancerous and noncancerous cells, resulting in adverse side effects such as systemic toxicity [6]. Consequently, these treatments are limited to short-term usage only and do not lead to sustained remission [7]. Therefore, it is crucial to develop more selective, less toxic and well-tolerated treatment options that could be administered over long periods of time and lead to better quality of life.

Researchers have investigated the possibility of targeting cancerous cells through the exploitation of their specific cellular vulnerabilities. The rapid proliferation of cancerous cells requires a substantial amount of energy. Hyperactive energy production renders the cells vulnerable to oxidative stress and mitochondrial membrane destabilization [8]. Exploiting these vulnerabilities could result in selective apoptosis of cancer cells, a controlled physiological process that results in cell death [9]. Therapeutic agents capable of inducing apoptosis by acting on the vulnerabilities of cancer cells may serve as a better and more selective alternative for anti-cancer therapy. These agents would have limited or no effect on noncancerous cells, providing a long-term and sustainable health solution.

Natural health products (NHPs) are substances derived from various plant or food sources and have been traditionally known to possess medicinal properties and have widespread applications [10]. Although there is documented NHP usage for medicinal purposes dating back thousands of years, there is a lack of scientific validation and elucidation of potential mechanisms of action. Thus, these products are often overlooked as valid treatment options for several diseases. Despite various natural extracts having shown potent anti-tumour effects, such as *Taraxacum officinale* and *Cymbopogon citratus*, health care professionals are hesitant to suggest NHP usage to their patients to supplement their chemotherapeutic regimens due to the unknown interaction of NHPs with chemotherapy [11–14]. Therefore, it is necessary to evaluate and validate the medicinal properties of NHPs scientifically and investigate their interaction with chemotherapies for their advancement towards being considered as clinical treatment options. The utilization of natural extracts in conjunction with chemotherapeutic drugs could enhance the anti-cancer effect of the chemotherapy while also reducing its toxicity. This suggests a more sustainable, long-term approach to manage and control the progression of cancer [15].

Simarouba glauca, commonly known as Paradise Tree, has traditionally been utilized in herbal medicine for its antioxidant, hepatoprotective, and anti-cancer properties [16, 17]. Paradise tree extract (PTE) contains an abundance of bioactive compounds including quassinoids, alkaloids, flavonoids, cardinolides, glycosides, phenolic compounds, saponins, and fixed oils [18]. These compounds can display their activity independently or in combination with other compounds in the extract. Certain active components of PTE have shown potent anti-cancer activity. For example, glaucarubinone has shown anti-cancer activity via the downregulation of p21-activated kinases in pancreatic cancer [19]. As well, the alkaloid canthin-6-one and its derivatives were found to inhibit NF-κB, a key transcription factor that regulates immune response, inflammation, and cell growth pathways, making it a key protein in cancer development [20, 21]. Previous studies have indicated that PTE is active against human colon cancer, human oral epidermoid cancer, human hormone-dependent prostate cancer and human lung cancer cells [16]. However, its activity on breast cancer as well as its interaction with chemotherapy has not yet been investigated.

In this study, we investigated the potential anti-tumour properties of PTE against human estrogen-receptor positive (ER+) and triple-negative breast cancer cells. Triple-negative breast cancer is considered to be highly aggressive as it can rapidly metastasize and can be difficult to diagnose [22]. Our findings indicate that PTE induces apoptosis in breast cancer by increasing oxidative stress and by collapsing the mitochondrial membrane potential. PTE's efficacy against cancerous cells was both dose and time-dependent and its activity was selective to cancerous cells, without affecting non-cancerous cells. In addition, PTE demonstrated positive interactions with common chemotherapeutics, taxol, and cisplatin. PTE was able to enhance the anti-tumour effect of the chemotherapeutics while reducing their toxicity in normal healthy cells. Our results suggest that PTE can be used as a supplement to chemotherapeutic

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regimens in order to reduce the administered dose as well as the harmful side effects that come with chemo treatment. We further investigated the efficacy of PTE in triple-negative breast cancer xenograft models of immunocompromised mice. We observed reductions in tumour volume and maintenance of normal weight gain, indicating no obvious toxicity. Our findings show the potential of PTE as a potent and safe anti-cancer treatment which could supplement chemotherapeutic regimens in order to combat highly aggressive triple-negative breast cancer.

Method

Paradise Tree Extraction

Paradise tree (*Simarouba glauca*) was obtained from the University of Agricultural Sciences in Bangalore, India from Dr. Shyamsundar Joshi, professor of Botany in the form of dried leaves and twigs. The plant identification and certification is done by Dr. Joshi at the University of Agricultural Sciences [23]. The plant specimen from India has been certified in a previous publication by Jose et al. [24]. The total aerial part of dried paradise tree powder prepared from airdried leaves and twigs was extracted in 100% anhydrous ethanol (1 g leaf powder to 10 mL anhydrous ethanol) at room temperature overnight. Using gravity filtration (P8 coarse filter), followed by vacuum filtration (0.45 µm filter), the extract was filtered.

The extract was evaporated using a RotoVap at 40°C and reconstituted in ethanol to obtain a final stock concentration of 200 mg/mL. The ethanolic extract was then passed through an Acrodisc 0.2 µm dimethyl sulfoxide (DMSO)-safe syringe filter in a biosafety cabinet. The preparation of the extract is similar to that of previously published work [25].

Cell Culture

The ER+ breast cancer cell line MCF-7 (ATCC® HTB-22™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30–2002™) supplemented with 10% (v/v) fetal bovine serum and 0.4% (v/v) gentamicin. The fetal bovine serum and the gentamicin were obtained from Thermo Scientific, Waltham, MA, USA, Cat. No. 12484-02, and Gibco BRL, VWR, Mississauga, ON, CA, Cat. No. 15710-064, respectively.

The triple-negative breast cancer cell line MDA-MB-231 (ATCC® HTB-26™) was cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30–2003™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.

NCM-460, the normal colon mucosa cell line (ATCC® CRL-1831™) was cultured in Medium M3 Base (Incell Corp. M300F-500) supplemented with 0.4% (v/v) gentamicin as well as 10% (v/v) FBS.

Assessment of Cellular Metabolic Activity and Viability: Annexin V and Propidium Iodide (PI) Binding Assay and WST-1 Assay

Annexin V and propidium iodide are dyes used as apoptotic markers. While Annexin V detects early apoptosis and cell permeabilization, while PI is used to monitor late cell death. Phosphate buffer saline (PBS) was used to wash the cells, which were then suspended in green fluorescent Annexin V AlexaFluor-488 (1:20) (Life Technologies Inc., Cat. No. A13201, Burlington, ON, Canada) with Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4), as well as red fluorescent propidium iodide (0.01 mg/mL, Life Technologies Inc., Cat. No. P3566, Burlington, ON, Canada). The samples were incubated in the absence of light at a temperature of 37°C. A Tali Image-Based Cytometer (Life Technologies Inc., Cat. No. T10796, Burlington, ON, Canada) was used to quantify a percentage of early apoptotic cells, late apoptotic cells, and necrotic cells (green, green and red, and red respectively). Atleast 13 random fields of the cell were analyzed using both the green and red channels (ex. 458 nm; em. 525/20 nm ex. 530 nm; em. 585 nm respectively).

Using LAS AF6000 software with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany) held at a magnification of 200×, a qualitative depiction of cell death was taken in the form of fluorescent micrographs. Monitored cells were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) to achieve a final concentration of 10 µM during the 15-minute incubation. The protocol followed is similar to that of previously published work [6].

In addition, a WST-1 assay for cell proliferation and viability was employed to assess toxicity of PTE on normal human fibroblast (NHF) cells. The cells were treated with a solvent control and increasing doses of PTE for 48 hours. Following which, the cells were incubated with WST-1 reagent (Catalog No. 05 015 944 001, Roche Diagnostics) for 4 hours at 37°C, according to the manufacturer's protocol. Absorbance readings of the formazan product were obtained 450 nm using a spectrofluorometer (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA).

Reactive Oxygen Species (ROS) Quantification

2',7'-dichlorodihydrofluorescein diacetate, or H_2DCDFA, was used to monitor the ROS generation in the whole cell. Once it enters the cell, reactive oxygen species oxidize H_2DCDFA and esterases deacetylate it, resulting in the fluorescent product 2',7'-dicholorofluorescein, or

DCF. This molecule exhibits excitation at 495 nm, and emission at 529 nm. The protocol used is similar to that described in previously published paper [6]. To begin, the cells were subject to a pretreatment of H_2DCDFA (20 µM, obtained from Sigma-Aldrich Canada, Cat. No. D6883, Mississauga, ON, Canada) over a period of 30 minutes at a temperature of 37°C, 5% CO_2, and in the absence of light. After being treated for the set durations, the cells were then collected and centrifuged for 5 minutes (3500×g). Finally, the cells were resuspended in Phosphate Buffer Saline. A Tali Image-Based Cytometer (Life Technologies Inc., Burlington, ON, CA, Cat. No. T10796) was used to quantify the percentage of cells that stained positive for DCF. Results are reflective of 13 random fields for each group displaying excitation at 458 nm and emission at 525/20 nm (the green channel).

Mitochondrial Potential Monitoring

In order to monitor the mitochondrial membrane potential (MMP), tetramethylrhodamine methyl ester, or TMRM (Gibco BRL; VWR; Cat. No. 89139-392), was administered at a concentration of 0.10 µM. The cells were initially incubated with TMRM. Next, the cells were collected, washed and resuspended using 1 × PBS. A Tali Image-Based Cytometer (Life Technologies Inc., Burlington, ON, CA, Cat. No. T10796) was used to analyze the results. Results are reflective of 13 random fields for each group displaying excitation at 530 nm and emission at 585 nm (the red channel). The protocol used is similar to that described in previously published [6].

Statistical Analysis

GraphPad 6.0 Prism software was used to conduct all statistical analyses in this experiment. Statistical significance was tested using a Two-Way Analysis of Variance (ANOVA). All experiments consisted of at least three independent trials.

Xenograft Models

10-week-old female immunocompromised Balb C Nu/Nu mice (Jackson Lab, USA) were used for the breast cancer study. The mice were kept in a pathogen-free rodent barrier facility held at a temperature of 22.7°C and 41.1% humidity. For this study, 3 million MDA-MB-231 cells were injected subcutaneously into the left flank of each mouse. A 200 µL solution containing equal parts of Corning Matrigel Basement Matrix (VWR Internation, Cat. No. 47743-715, Mississauga, ON, Canada) and cell solution was injected into each mouse. 23-gauge needles and 1 mL syringes were used for the injections. After allowing the tumours to establish, the 16 mice were randomly divided into 4 groups (4 cages): an untreated group (control), a PTE treatment group, a cisplatin treatment group, a Taxol cisplatin group and a PTE, Taxol-cisplatin combination group. Aqueous solutions of PTE (0.40 mg/mL) were placed into each cage for oral administration of the extract, given ad libitum in order to simulate human trial conditions. Intraperitoneal Taxol and cisplatin injections were conducted once per week on the mice from cages 3 and 4 for a total four injections. Volumes of 50 µL of 2 mM cisplatin and 20 µL of 2.5 mg/mL Taxol were administered. The weight of the mice was monitored two times per week for 4 weeks in order to obtain an indicator of the ability to tolerate the treatments. Tumour volumes were calculated on the final day of the experiment (after 4 weeks) using the modified ellipsoid formula: ½ (Length × Width^2). Mice were placed under anesthetic to minimize pain and were then sacrificed using a CO_2 chamber, followed by cervical dislocation, and tumour harvesting. All procedures were done in accordance with the animal protocols outlined in the University of Windsor research ethics board (AUPP # 17-15). Ethical approval was obtained by the Canadian Council on Animal Care (CCAC).

Evaluation of *In-Vivo* Apoptosis via TUNEL Assay

Following tumour harvesting, the control and PTE-treated tumours were formalin-fixed and subjected to sectioning. To detect the levels of apoptotic cell death caused by PTE *in-vivo*, the tumour tissues were analyzed using the *in situ* Click-iT™ Plus TUNEL Assay (ThermoFisher Scientific, Cat. No. C10617). The TUNEL assays were performed according to the manufacturer's protocol. In brief, paraffin embedded, formalin fixed sections of the breast tumour samples were deparaffinized in a series of xylenes and graded alcohols, were fixed in 4% paraformaldehyde for 15 minutes at 37°C and were permeabilized using Proteinase K. Then, the Terminal deoxynucleotidyl transferase (TdT) reaction mixture was incubated with the sample for 1 hour at 37°C as to allow for the TdT reaction to occur. The Click-iT™ Plus reaction was also performed which involved incubation with Click-iT™ Plus TUNEL reaction mixture for 30 minutes at 37°C. Stained sections were then observed under a Leica DMI6000 fluorescent microscope and photographed.

Results

Initial Assessment of Paradise Tree Extract's Efficacy Against Breast Cancer Cell Lines

This study evaluated PTE's ability to induce apoptosis in MDA-MB-231 and MCF-7 breast cancer cells

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following 24-hour and 48-hour treatments through the analysis of fluorescent apoptotic markers. Both cell lines were fluorescently stained with Annexin V (AV) and Propidium Iodide (PI) apoptotic markers. AV detects cells undergoing the early stages of apoptosis, marked by the externalization of phosphatidylserine. PI detects cell death by intercalating with DNA, indicating cell permeabilization. Late apoptotic cells stained positive for both AV and PI, while those that stained negative for both were considered viable.

Following 24-hours and 48-hours, treatments of PTE were able to induce apoptosis in breast cancer. At the 24-hour time point, reduced cell viability in both cell lines was statistically significant at 0.20 mg/mL and 0.40 mg/mL of PTE (Fig. 1A, B). At a 48-hour treatment time point, PTE significantly reduced cell viability in triple-negative breast and ER+ cells, even at lower concentrations of the extract. The anti-tumour activity of PTE was dose-dependent at both time points; apoptosis increased as the treatment concentration was increased. Dose dependency was more apparent at a 48-hour time point.

Qualitative analysis of MDA-MB-231 and MCF-7 cells was conducted using morphological assays (Fig. 1C). Both cell lines were fluorescently stained with AV (green) and PI (red), as previously mentioned. Cells were additionally counterstained with Hoechst (blue), a fluorescent dye that stains the nuclei of live or dead cells. Micrographs were taken following 48-hour treatments of PTE and chemotherapeutics, either individually or in combination. Apoptotic markers visible by microscopy include fluorescent cell staining as well as morphological changes such as cell shrinkage, membrane blebbing, and nuclear condensation.

Interaction of Paradise Tree Extract with Conventional Chemotherapies, Taxol and Cisplatin, in Combination Treatments

MDA-MB-231 and MCF-7 cells were treated with increasing doses of common chemotherapeutics, taxol and cisplatin, to compare the level of cell death of common chemotherapeutics in the presence and absence of PTE. In both cell lines, both taxol and cisplatin induced significant levels of apoptosis (Fig. 2A, B). Cisplatin was able to induce cell death in a more dose-dependent manner compared to taxol.

To test the efficacy of PTE in conjunction with common chemotherapeutics, combination assays were conducted following 48-hour treatments. MDA-MB-231 and MCF-7 cells were treated with taxol and cisplatin in the presence or absence of 0.1 mg/mL PTE at a 48-hour time point (Fig. 2A, B). This concentration was selected because alone, it induced about 25% cell death which allowed for the quantification of potentiation of cell death in the presence of chemo in combination therapy. If a higher dose of extract was selected, there would be a saturation of cell death and it would be difficult to discern any enhancing effect. To analyze the induction of apoptosis, both image-based cytometry and fluorescence microscopy were utilized.

In the MDA-MB-231 triple-negative breast cancer cell line, the combination of 0.1 mg/mL PTE with varying levels of chemotherapy led to significant increases of apoptotic cell death as compared to chemotherapy on its own. It was observed that the second lowest combination dosage of cisplatin (1 μM with 0.1 mg/mL PTE) was able to induce a similar level of apoptosis as that caused by a higher dose of cisplatin alone (5 μM). This indicates that a similar level of apoptosis can be achieved with a 5-fold decrease in chemotherapeutic concentration. In addition, the lowest taxol combination treatment (0.01 μM with 0.1 mg/mL PTE) showed similar efficacy to the highest individual taxol treatment (0.5 μM). This indicates that similar results can be achieved by combining PTE with a 50-fold decrease in the dose of taxol. These results were further verified through qualitative analysis of apoptotic marker fluorescence and cell morphology. Apoptotic fluorescence and apoptotic cell morphology were found to increase in combination treatments as compared to individual chemotherapeutic treatments (Fig. 2C).

In the ER+ breast cancer cell line, MCF-7, similar results were observed as PTE significantly increased the anti-tumour efficacy of the chemotherapeutic when used in combination. With both taxol and cisplatin, a dose-dependent decrease in cell viability was observed with the combination treatments. For cisplatin, greatest enhancement was observed at the second highest combination treatment (5 μM + 0.15 mg/mL PTE) (Fig. 3A, B). These results were also confirmed through fluorescent microscopy (Fig. 3C).

Effects of Paradise Tree Extract on Normal Human Cells, Individually and in Combination Treatment with Chemotherapeutics

PTE's selectivity for cancerous cells was assessed after the treatment of Normal Colon Mucosal cells (NCM-460). Due to a lack of cholera toxin, we were unable to effectively grow a cell-culture of MCF-10, a normal breast tissue cell line. The cells did not grow at a sufficient rate for timely *in-vitro* experimentation. Therefore, we substituted MCF-10 with Normal Colon Mucosa (NCM) cells, another valid model that is representative of non-cancerous human cells. Noncancerous cells underwent individual and combination treatments of PTE and chemotherapeutics. Cells treated with

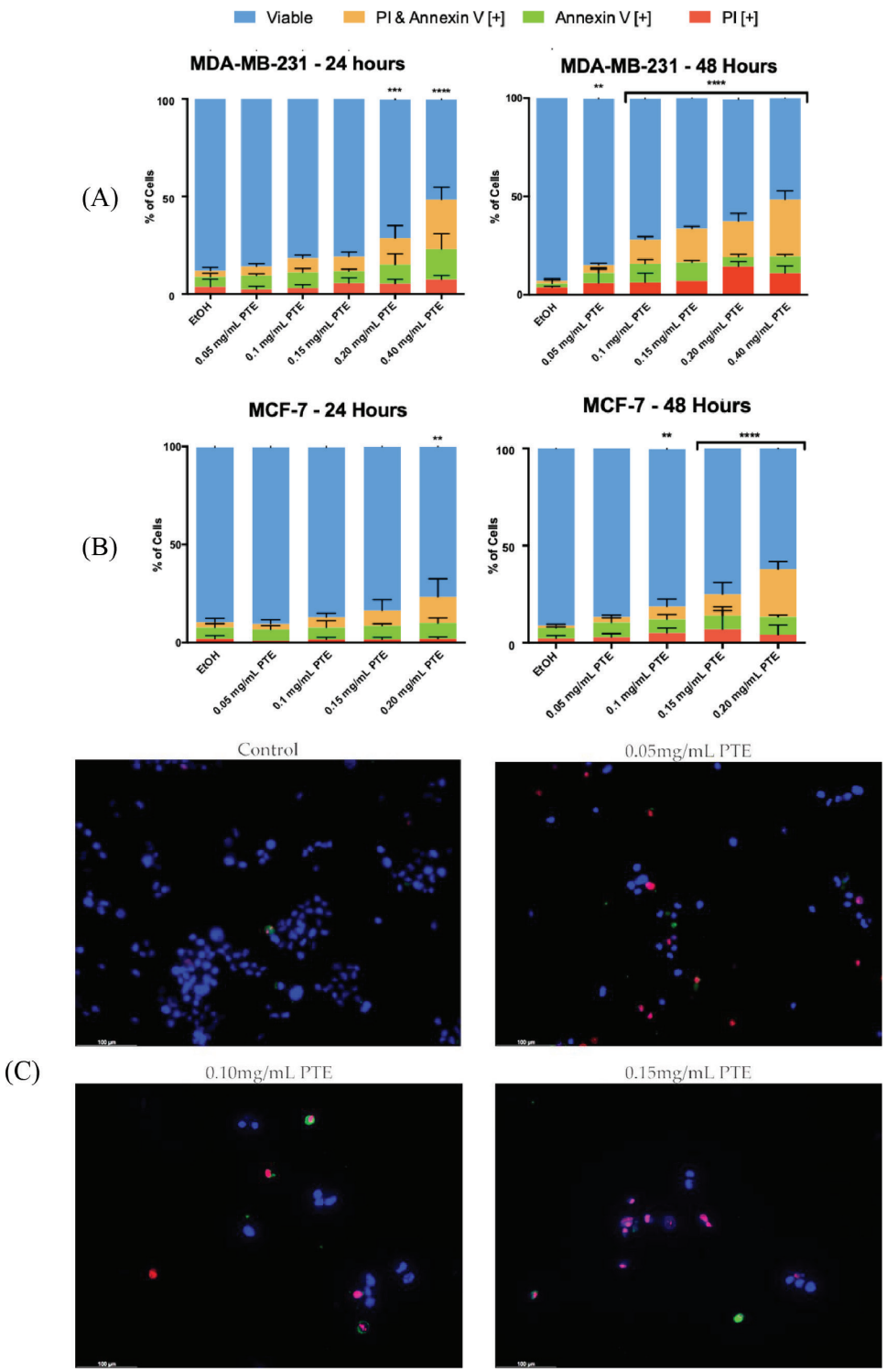

Figure 1. PTE induces apoptosis in *in-vivo* breast cancer models. Breast cancer cell lines (A) MDA-MB-231 and (B) MCF-7 were treated with various concentrations of PTE and assessed at 24-hour and 48-hour time points. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. PTE treatment groups are compared with a negative control of 100% anhydrous ethanol. (C) MDA-MB-231 micrographs of various PTE concentrations at 48-hours. Fluorescent images stained with Annexin V (green), PI (red), and Hoechst (blue) at 200× magnification. Scale bar is 100 microns. Images are representative of three independent experiments. Statistical calculations were performed using Two-Way ANOVA multiple comparison. *$p < 0.05$ vs Control; **$p < 0.01$ vs Control, ****$p < 0.0001$ vs Control.

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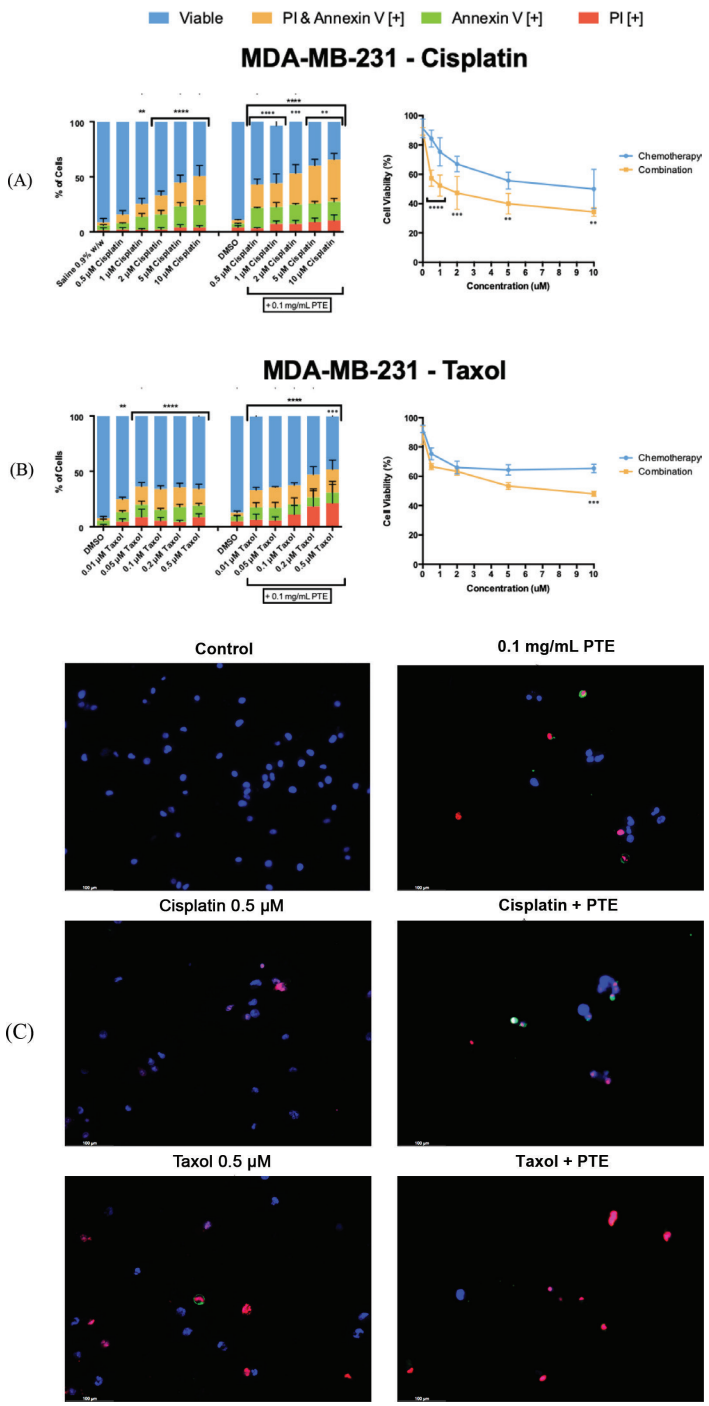

Figure 2. Supplementation of PTE extracts to chemotherapeutics significantly reduced cell viability in triple-negative breast cancer. MDA-MB-231 cells were treated with varying concentrations of (A) cisplatin and (B) taxol on their own and in combination with 0.10 mg/mL of PTE (left). The percentage of viable cells was graphed for both individual chemotherapeutic treatments and PT combination treatments (right). Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. PTE treatment groups are compared with a negative control of DMSO. (C) MDA-MB-231 micrographs of individual and PT combination chemotherapeutic treatments at 48-hours. Fluorescent images stained with Annexin V (green), PI (red), and Hoechst (blue) at 200× magnification. Scale bar is 100 microns. Images are representative of three independent experiments. Statistical calculations were performed using Two-Way ANOVA multiple comparison. *$p < 0.05$ vs Control; **$p < 0.01$ vs Control, ****$p < 0.0001$ vs Control. •$p < 0.05$ vs Individual Chemotherapy Treatment, ••$p < 0.01$ vs Individual Chemotherapy Treatment, ••••$p < 0.0001$ vs Individual Chemotherapy Treatment.

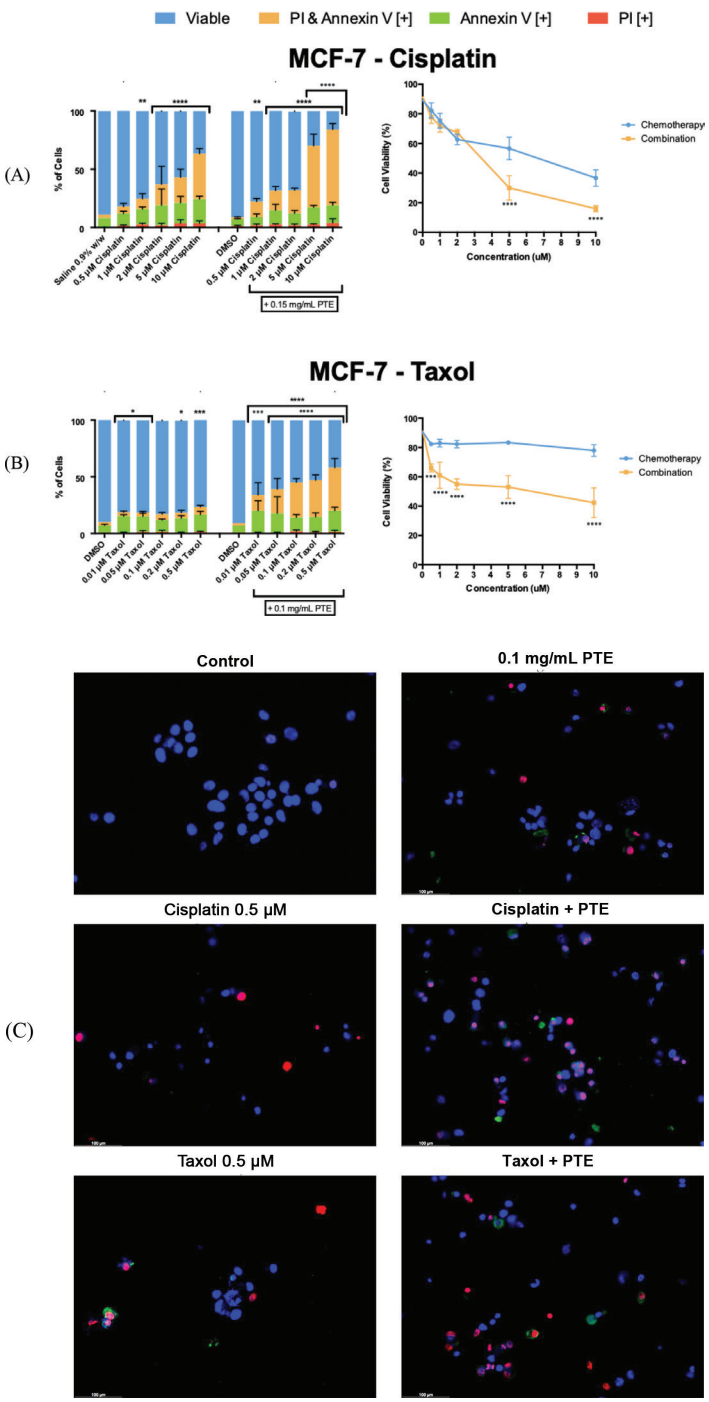

Figure 3. Supplementation of PTE to chemotherapeutics significantly reduced cell viability in ER-positive cells. MCF-7 cells were treated with varying concentrations of (A) cisplatin and (B) taxol on their own and in combination with 0.15 mg/mL and 0.10 mg/mL of PT respectively (left). The percentage of viable cells was graphed for both individual chemotherapeutic treatments and PT combination treatments (right). Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. PT treatment groups are compared with a negative control of DMSO. (C) MCF-7 micrographs of individual and PT combination chemotherapeutic treatments at 48-hours. Fluorescent images stained with Annexin V (green), PI (red), and Hoechst (blue) at 200× magnification. Scale bar is 100 microns. Images are representative of three independent experiments. Statistical calculations were performed using Two-Way ANOVA multiple comparison. *$p < 0.05$ vs Control; **$p < 0.01$ vs Control, ****$p < 0.0001$ vs Control. •$p < 0.05$ vs Individual Chemotherapy Treatment, ••$p < 0.01$ vs Individual Chemotherapy Treatment, ••••$p < 0.0001$ vs Individual Chemotherapy Treatment.

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0.10 mg/mL of PTE did not undergo significant apoptosis when compared to the control groups (Fig. 4). Chemotherapeutics, taxol and cisplatin, induced significant cell death in noncancerous cells. NCM-460 cells treated with a combination of PTE and chemotherapeutics were not significantly different than control groups. Further, the combination treatment groups induced apoptosis in normal cells at a significantly lower rate than individual chemotherapeutic treatment groups.

Apoptotic Mechanism Analysis of Paradise Tree Extract Through Assays Analyzing Reactive Oxygen Species (ROS) Production and Mitochondrial Membrane Potential Destabilization

PTE has many active compounds which may interact on multiple pathways, either individually or in combination. An ROS quantification assay was used in order to investigate PTE's ability to induce apoptosis by the induction of oxidative stress (Fig. 5). Breast cancer cells were pre-treated with PTE and chemotherapeutics either together or on their own. Three hours after treatment, endogenous ROS levels were quantified using fluorescent reagent H_2DCDFA, which fluoresces green upon oxidation. In addition, both cell lines were treated with or without N-Acetyl Cysteine (NAC), a known inhibitor of ROS. Cells treated with 0.10 mg/mL of PTE showed a significant increase in ROS production. When administered alone, taxol and cisplatin were not able to induce statistically significant ROS production compared to the control. However, chemotherapeutics administered in combination with PTE significantly increased ROS levels. Further, ROS production shown in the combination groups was statistically higher when compared to their respective individual chemotherapy treatment. These results were consistent in both cell lines (Fig. 5A, B).

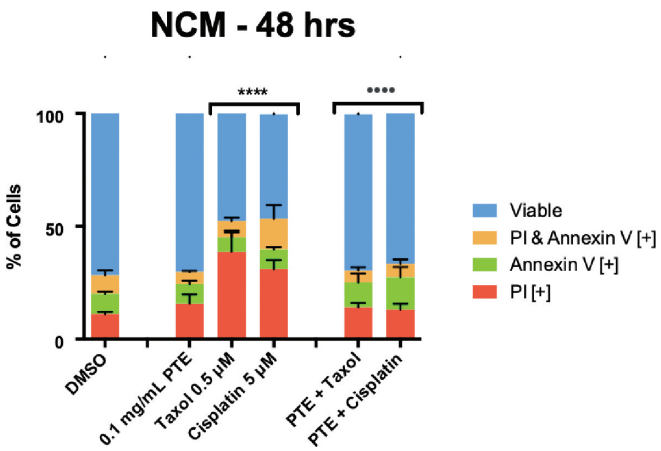

Figure 4. PTE is selective for breast cancer cells and reduces the toxicity associated with chemotherapeutics. NCM-460 cells were treated with 0.10 mg/mL of PTE, 5 µM of cisplatin, and 0.50 µM of taxol and assessed at 48-hours. PTE combination chemotherapeutic treatment groups were not statistically different compared to the DMSO negative control. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and Pi (blue). Values are expressed as a mean ± SD from three independent experiments. *p < 0.05 vs Control; **p < 0.01 vs Control, ****p < 0.0001 vs Control. •p < 0.05 vs Individual Chemotherapy Treatment, ••p < 0.01 vs Individual Chemotherapy Treatment, ••••p < 0.0001 vs Individual Chemotherapy Treatment.

Mitochondrial dysfunction leads to the permeabilization of the mitochondrial membrane, followed by the induction of apoptosis. Once again, cells were treated with PTE and chemotherapeutics either together or on their own. Tetramethylrhodamine methyl ester (TMRM) dye was used to quantify mitochondrial membrane potential (MMP) (Fig. 6). Cells treated with PTE and chemotherapeutics, either individually or alone, experienced significant mitochondrial depolarization at a 48-hour time point. Chemotherapeutics administered individually did not significantly affect MMP. However, both individually and in combination with chemotherapies, PTE was able to significantly destabilize the mitochondria in both MDA-MB-231 and MCF-7 cell lines (Fig 6A, B).

In-Vivo Analysis of Paradise Tree Extract's Efficacy and Tolerability in Triple-Negative Breast Cancer Xenograft Tumour Models and Determination of Tumour Growth Inhibition Mechanism

Extensive *in-vitro* work on PTE's effect against breast cancer cell lines warranted further investigation on the extract's anti-tumour capability. Balb C Nu/Nu immunocompromised mice were subcutaneously injected with MDA-MB-231 cells on both flanks. After allowing the tumours to establish, the 16 mice were randomly divided into 4 groups: an untreated group (control), a PTE treatment group, a cisplatin and Taxol treated group, and a PTE, cisplatin, and Taxol combination group. The extract was orally administered to the mice via a bottled solution and they were allowed to drink ad libitum. Cisplatin and Taxol were injected intraperitoneally every week, giving a total of 4 injections.

Results shown in Figure 7B show that animals fed with PTE had reduced tumour volume and weight compared to the untreated control. There was a reduction in

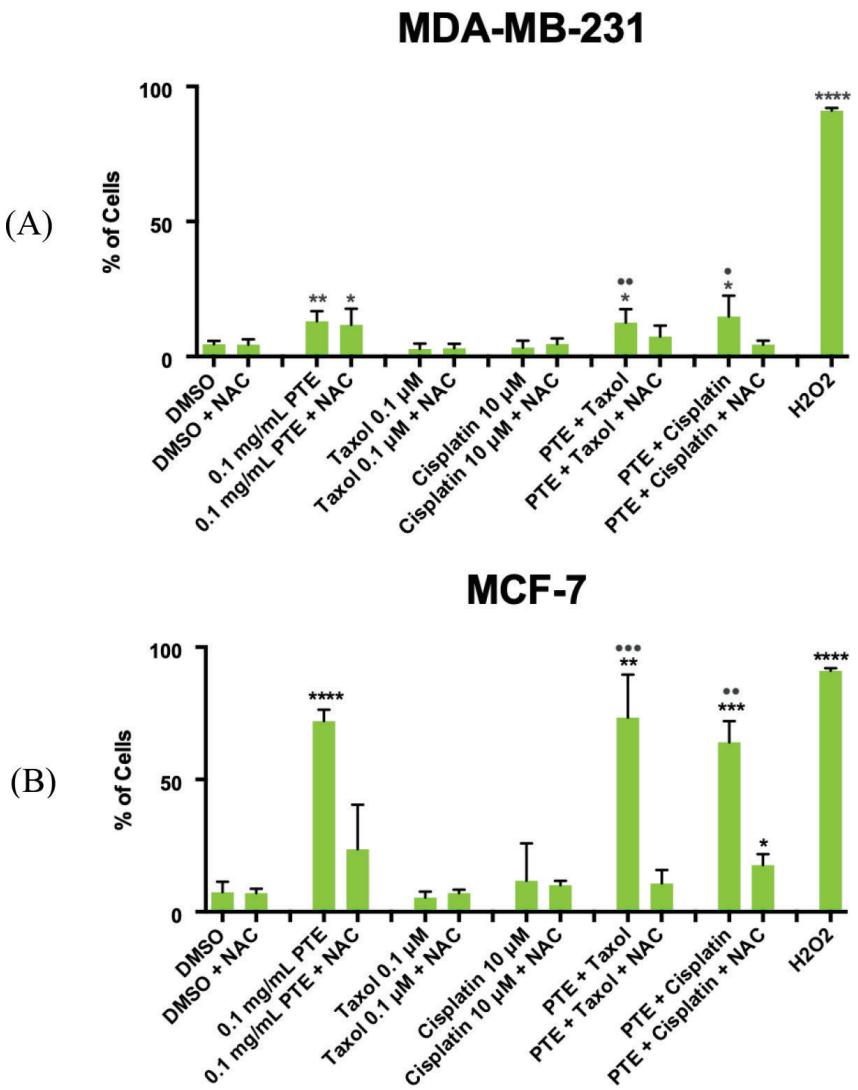

Figure 5. PTE is causes on the production of oxidative stress to induce apoptosis. (A) MDA-MB-231 and (B) MCF-7 breast cancer cells were treated with H_2DCDFA following treatments with chemotherapeutics taxol and cisplatin individually and in combination with 0.10 mg/mL PTE, with or without the antioxidant NAC for 3 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with DCF. Treatment groups were compared with the negative control DMSO. Values are expressed as a mean ± SD from three independent experiments. *p < 0.05 vs Control; **p < 0.01 vs Control, ****p < 0.0001 vs Control. •p < 0.05 vs corresponding treatment without antioxidant NAC, ••p < 0.01 vs corresponding treatment without antioxidant NAC, ••••p < 0.0001 vs corresponding treatment without antioxidant NAC.

the tumour volume and weight in the animals treated with cisplatin and Taxol. Importantly, the combination of PTE along with cisplatin and Taxol led to significant reductions in the tumour weight (Fig. 7A and 7B). During the 4 week period, there was no apparent change in the mice body weight in any of the 3 treatment groups compared to the control indicating that all treatments were well tolerated. Any weight gain or loss was statistically insignificant (Fig. 7C).

To investigate if the inhibition of tumour growth in PTE-treated animals was due to induction of apoptosis of xenografted tumour cells, both control and PTE-treated tumour samples were sectioned and subjected to TUNEL assay. This assay analyzes DNA fragmentation as an apoptotic marker by estimating and staining 3' hydroxyl group DNA breaks for visualization. As indicated in Figure 7, the number of TUNEL-positive stained apoptotic cells was significantly higher in PTE-treated samples indicating significant DNA fragmentation and apoptosis as compared to control samples. These results suggest that xenografted tumour cells in PTE-treated animals were undergoing marked apoptosis and that

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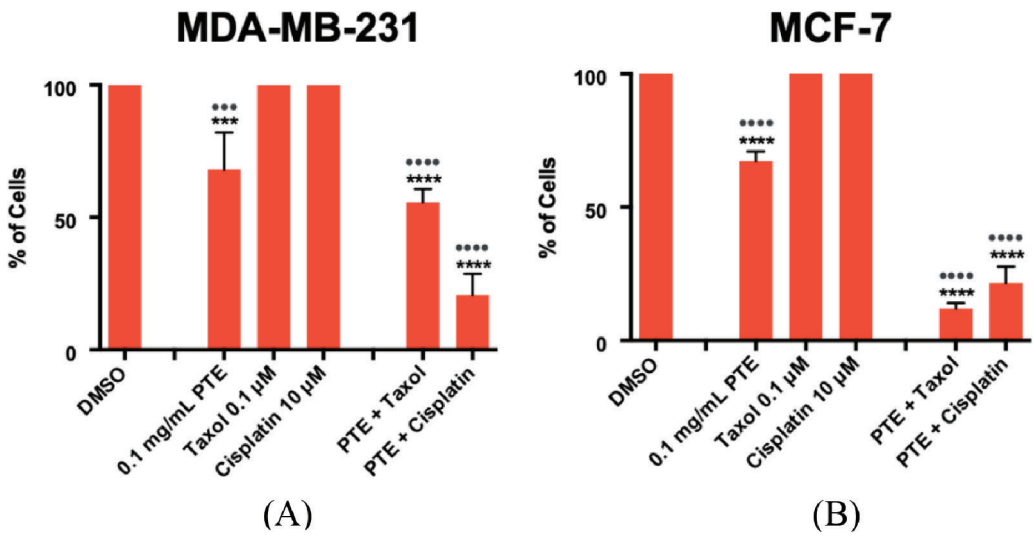

Figure 6. PTE is causes depolarization of mitochondrial membrane potential to induce apoptosis. (A) MDA-MB-231 cells and (B) MCF-7 cells were treated with chemotherapeutics taxol and cisplatin individually and in combination with 0.10 mg/mL PTE and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with mitochondrial membrane potential (TMRM, fluoresces red). Treatment groups were compared with the negative control DMSO. Values are expressed as a mean ± SD from three independent experiments. *p < 0.05 vs Control; **p < 0.01 vs Control, ****p < 0.0001 vs Control. ·p < 0.05 vs Individual Chemotherapy Treatment, ··p < 0.01 vs Individual Chemotherapy Treatment, ····p < 0.0001 vs Individual Chemotherapy Treatment.

reduction in tumour growth was in fact due to apoptotic death induced by PTE.

Discussion

In this paper, we have shown that PTE selectively induces apoptosis in both, ER-positive MCF-7 and the more aggressive triple-negative MDA-MB-231 breast cancer cell lines. PTE was able to significantly reduce the cell viability of cancer cells *in-vitro* in a dose and time-dependent manner (with IC-50 of approximately 0.4 mg/mL PTE), without exhibiting toxic effects on normal human cells (no toxicity observed even at highest dose of PTE as per results from WST-1 cell viability assay – data not shown). These results suggest a very high selectivity for PTE towards inflicting cell death against breast cancer cells. Additionally, when administered in combination, PTE was able to enhance the anti-tumour effects of chemotherapeutics, taxol and cisplatin, while reducing chemotherapy-related toxicity in normal cells. This positive interaction with chemotherapeutics suggests PTE's potential to serve as a safe and effective supplemental therapy to conventional treatments. PTE induced apoptosis by increasing ROS production and by disrupting MMP in breast cancer cells, and these effects were enhanced in the combination treatment with chemotherapeutics. Furthermore, PTE was well-tolerated in mice over long periods of time as there was no change in normal weight gain in PTE-treated animals. In human tumour xenograft mouse models, PTE was able to reduce tumour weight on its own and significantly when combined with low dose chemotherapy treatment. These findings are especially significant for patients who present with triple-negative breast cancer which is often more aggressive as these cells are harder to sensitize to treatment. PTE was able to sensitize these aggressive cancer cells in *in-vitro* models in addition to decreasing overall cellular toxicity in non-cancerous cells. Thus, PTE could have potential to be developed as an adjuvant to chemotherapy treatments, providing a more sustainable and long-term treatment plan for breast cancer patients.

Natural extracts possess multiple phytochemicals, providing extract-based therapeutic methods with some key advantages over isolated pure compounds. The numerous bioactive compounds present in an extract can target many different pathways within the cell at once and may demonstrate interactions that cause potentiation or inhibition of particular cell pathways, which could explain the extract's selective anti-tumour activity as well as its protective activity towards normal cells [26]. Previous work has shown that purified phytochemicals from dandelion root extract were effective against cancerous cells only at very high

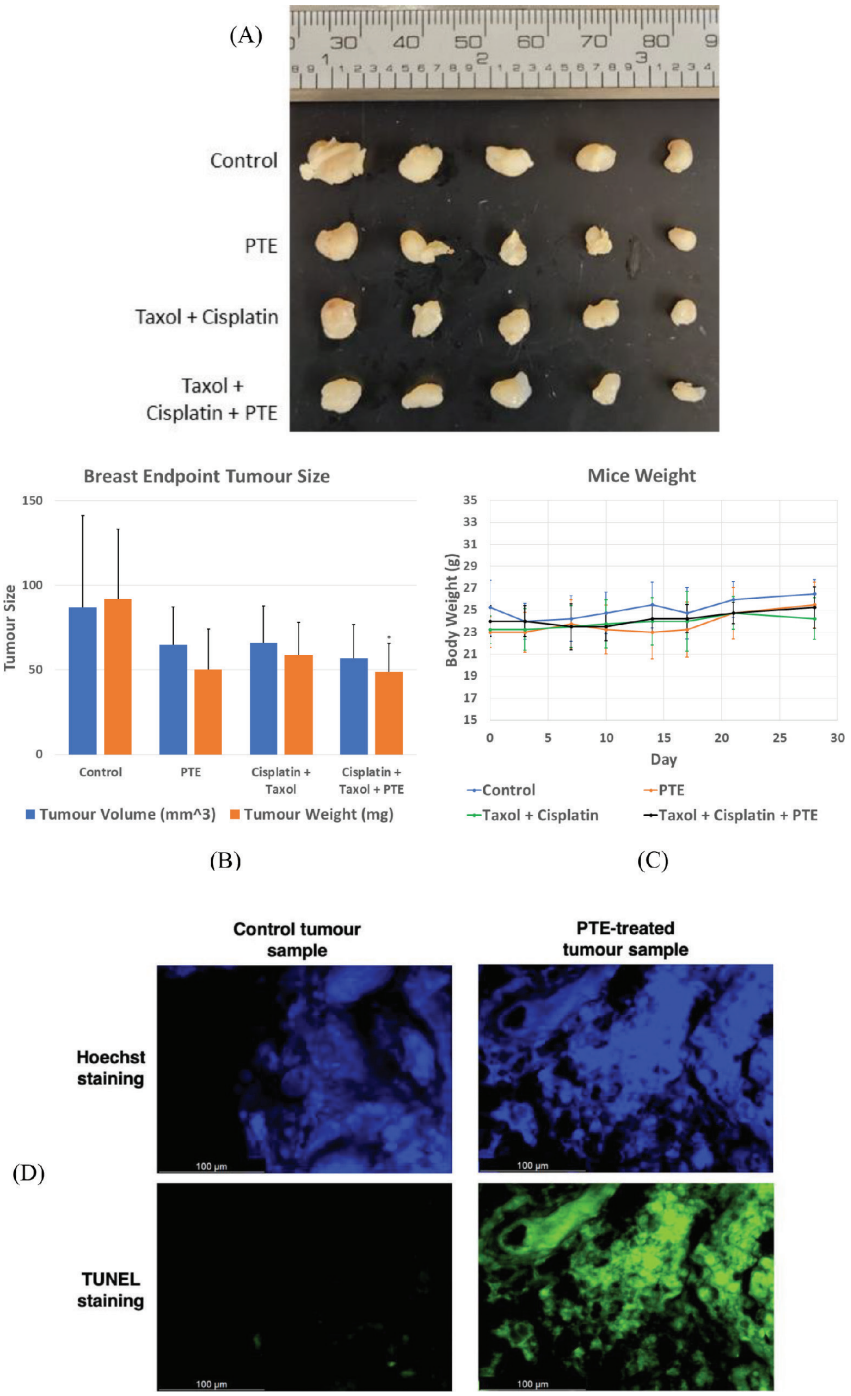

Figure 7. PTE is able to reduce tumour weight and remain well-tolerated in immunocompromised mice. The anti-tumour efficacy of orally administered PTE with or without intraperitoneally administered cisplatin and Taxol is assessed on MDA-MB-231 xenograft tumour growth. Immunocompromised mice were subcutaneously injected with the triple-negative breast cancer on their flanks, and tumours were allowed to establish. An aqueous solution of PTE was orally administered to the mice ad libitum. The chemotherapeutic treatment was administered via weekly intraperitoneal injections. Tumour volume and mouse mass were measured two times per week. (A) Photographs of triple-negative tumours were taken after extraction from the animals. (B) The tumour volume and mass of MDA-MB-231 was measured at the endpoint of the experiment. (C) The weight profile of the mice is shown over a 4-week period. Results are representative for the Control (n = 4), the orally administered PTE group (n = 4), the Taxol + cisplatin group (n = 4), and the extract and chemotherapy combination group (n = 4). Statistical analysis was done using One-Way ANOVA. *$p < 0.05$ vs tumour volume of the control. (D) To determine the mechanism of tumour growth inhibition, TUNEL assay was used to qualitatively depict apoptotic levels of control versus PTE-treated samples.

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doses [27]. However, dandelion root extract as a whole has been shown to have significant activity against multiple cancer cell lines [12, 28]. Although the overall treatment concentrations of extract therapeutics may appear high, a significant portion of the extract is composed of materials abundant in plants such as sugars and salts [8]. Therefore, bioactive compounds, although present in lower concentrations in whole extracts, display significantly higher anti-cancer activity due to positive drug-drug interactions between compounds. Additionally, the interaction alone between bioactive compounds in a whole extract has shown reduced overall toxicity as it has been found that some compounds may inhibit the toxicity of others when administered together [29]. Since these extracts comprised of several bioactive compounds are highly efficacious against cancer and are relatively non-toxic, they could be suitable for long-term use during cancer treatment.

PTE selectively reduced breast cancer cell viability by inducing apoptosis through exploiting metabolic vulnerabilities of cancer cells. Cancer cells generally display increased intracellular levels of ROS [30, 31]. Some therapeutic agents increase ROS levels beyond the limit that can be resolved by cellular protection mechanisms, thus inducing apoptotic cell death [32, 33]. It was found that PTE treatment led to an increased production of ROS in cancer cells, thus triggering apoptosis in both MDA-MB-231 and MCF-7 breast cancer cell lines (Fig. 5). This effect was further enhanced when used in combination with conventional chemotherapeutics, indicating that increased oxidative stress could be one of the key mechanisms of PTE's anti-tumour activity. PTE was also shown to exploit the differences between cancerous and non-cancerous mitochondrial activity. The Warburg effect explains that cancerous cells prefer metabolism via glycolysis which leads to a buildup of lactic acid within the cytosol [34, 35]. An acidic cytosol is one of the multiple factors leading to the hyperpolarization of cancerous mitochondria [36]. Therapeutics that target this mitochondrial membrane potential (MMP) do so by initiating an internal stress stimulus which causes pro-apoptotic proteins to translocate to and permeabilize the outer mitochondrial membrane. Once permeabilized, the mitochondria become depolarized/destabilized and apoptogenic factors from the intermembrane space are released into the cytosol of the cell, leading to apoptotic induction [37]. For both breast cancer cells, PTE was able to permeabilize and destabilize the mitochondria causing the induction of apoptosis (Fig. 6). In addition, when PTE was used in combination with chemotherapeutics, an increased depolarization was observed. Ability of PTE to selectively induce apoptosis by targeting these specific metabolic/mitochondrial vulnerabilities of cancer cells suggests that PTE has potential of serving as a selective and efficacious anti-cancer treatment.

We have shown for the first time through a proof-of-concept study that oral administration of PTE reduces growth of triple-negative breast cancer xenograft *in-vivo*. Our experiments *in-vivo* confirm the conclusions gathered from our *in-vitro* work that PTE treatment displays significant anti-tumour activity against MDA-MB-231 cells. Taxol and cisplatin had minor effects in reducing tumour volume which was enhanced when combined with PTE. To determine whether PTE-treated tumour cells were undergoing apoptotic or necrotic cell death, a TUNEL assay was conducted to visualize late-stage apoptosis by DNA fragmentation. Indeed, the TUNEL assay proved that the inhibition of xenografted tumour growth was due to apoptotic tumour cell death induced by PTE and that the degree of apoptosis in PTE-treated samples was significantly increased as compared to the control sample (Fig. 7). In an earlier experiment, higher doses of chemotherapy resulted in severe toxicity leading to all animals being euthanized before completion. As a result, we used lower doses of chemotherapy (50 µL of 2 mM cisplatin and 20 µL of 2.5 mg/mL Taxol) in the subsequent experiment. However, the reduction in tumour weight in the xenograft model indicated that PTE treatment could be effective against triple-negative breast tumours both alone and in combination with standard chemotherapy. As seen in previous work, we have used weight loss as an indicator of the toxicity associated with chemotherapeutics [25]. Our findings show that both the PTE and combination treatment groups displayed similar weight profiles to control mice, indicating that the treatment was generally well-tolerated (Fig. 7C).

Paradise tree extract, a natural health product, has the potential to serve as a complementary anti-cancer therapy as it is highly selective and efficacious. PTE targets specific vulnerabilities of cancer cells including mitochondrial dysfunction and oxidative stress. With the combination of PTE and chemotherapeutics, we observed increased mitochondrial dysfunction and reactive oxygen species in cancer cells as well as decreased toxicity to normal healthy cells. Thus, PTE exerts a cytotoxic anti-cancer effect while remaining protective to noncancerous cells. This enhanced and targeted anti-tumour response suggests PTE as a promising supplemental treatment in eradicating cancer cells. The anti-tumour activity displayed by PTE individually and in combination with chemotherapy allows

for the administration of significantly lower doses of cytotoxic chemotherapeutics, while achieving comparable if not enhanced cell death in addition to decreased adverse effects. Furthermore, combination approaches target multiple cancer cell vulnerabilities, which may be able to decrease the probability of the cancer to acquire chemoresistance. Given the selective anti-tumour activity on multiple pathways, the enhancement of chemotherapeutic efficacy, and the minimization of adverse effects, PTE presents a promising adjuvant therapeutic strategy against breast cancer.

Conclusions

In this study, we have demonstrated that paradise tree extract effectively and selectively targets breast cancer. In cellular breast cancer models, PTE targets vulnerabilities exclusive to cancer cells. PTE induces apoptosis by increasing oxidative stress and by dissipating mitochondrial membrane potential. When utilized in combination with chemotherapeutics, PTE was able to enhance anti-tumour efficacy while reducing toxic effects on noncancerous cells. The addition of PTE to chemotherapeutic regimens minimized the chemotherapeutic dosage while still achieving comparable, if not enhanced cell death. Thus, PTE has the potential to reduce the drug-related toxicity associated with chemotherapeutics.

In mice xenograft models, we have shown that PTE suppresses tumour growth and is well-tolerated. Our results indicate positive interactions of PTE with Taxol and cisplatin resulting in significantly reduced tumour weight. Overall, these results *in-vivo* and *in-vitro* suggest that a PTE-based therapeutic regimen may improve the quality of life and even extend the prognosis of cancer patients. Our findings show that it is crucial to further investigate the specific cell proteins and pathways targeted by PTE in order to provide the scaffold for potential novel therapeutics.

List of Abbreviations

ABC: ATP-Binding Cassette
AV: Annexin V
DCF: 2′,7′-Dicholorofluorescein
DMSO: Dimethylsulfoxide
ER+: Estrogen-Receptor Positive
H_2DCDFA: 2′,7′-Dicholorofluorescein Diacetate
MMP: Mitochondrial Membrane Potential
NAC: N-Acetyl Cysteine
NCM: Normal Colon Mucosal
NHF: Normal Human Fibroblasts
NHP: Natural Health Product
PI: Propidium Iodide
PTE: Paradise Tree Extract
ROS: Reactive Oxygen Species
TdT: Terminal Deoxynucleotidyl Transferase
TMRM: Tetramethylrhodamine Methyl ester

Declarations

Ethics Approval and Consent to Participate

All procedures were done in accordance with the animal protocols outlined in the University of Windsor research ethics board (AUPP # 17-15). Ethical approval was obtained by the Canadian Council on Animal Care (CCAC).

Consent for Publication

Not applicable

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Author Contributions

Conceptualization, S.P.; methodology, S.P.; software, A.P., C.R.; validation, A.P., C.R.; formal analysis, A.P., C.R.; investigation, A.P., C.R., D.W., A.R., L.P., C.V.; resources, S.P.; data curation, A.P., C.R.; writing—original draft preparation, A.P., C.R.; writing—review and editing, S.P.; visualization, A.P., C.R.; supervision, S.P.; project administration, A.P., C.R.; funding acquisition, S.P. All authors have read and agreed to the published version of the manuscript.

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