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Effect of Protocatechuic Acid on Tamoxifen Efficacy and Oxidative Stress in Breast Cancer Cells: Implications for Combination Therapy

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Abstract

Breast cancer is a major global health concern, and resistance to chemotherapy poses a significant challenge. Traditional treatments like tamoxifen (TAM) induce oxidative stress, contributing to chemo-resistance. To enhance treatment effectiveness, exploring complementary options has become a target. Protocatechuic acid (PCA), an antioxidant compound with antitumor properties, has gained attention in this context.

This study evaluated the effect of PCA on MCF7 cell line and its impact on TAM efficacy. It assessed cytotoxicity, cell growth distribution, cell death indicators, reduced glutathione (GSH) levels, its related enzymes, and lactate dehydrogenase (LDH) activities. The combination of PCA and TAM demonstrated antagonistic cytotoxicity equal to 35.8%. Combining PCA with TAM protected against DNA damage in MCF7 cells. It altered cell cycle distribution, increasing cells in the S-phase and decreasing cells in the G2/M phase. The combination treatment also increased early apoptotic cells and reduced viable cells compared to TAM alone. Furthermore, the combination treatment upregulated antioxidant enzymes, including glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), and catalase (CAT), indicate enhanced cellular defense against oxidative damage. Additionally, the combination treatment increased LDH activity.

In conclusion, PCA effectively reduces oxidative stress in MCF7 cells, and combining PCA with TAM shows potential for enhancing treatment outcomes.

Keywords: Breast cancer; Protocatechuic acid; Tamoxifen; Glutathione; Oxidative stress

1. Introduction

Breast cancer, the most common cancer among women worldwide, ranks as the second most prevalent cancer overall. Despite advancements in treatment, resistance to chemotherapy remains a significant challenge, resulting in poor overall survival rates. As a result, researchers have been exploring complementary therapeutic options to enhance treatment effectiveness. Phenolic compounds, known for their potential anticancer properties, are found in various fruits, vegetables, and herbs and have gained attention in this field [1, 2].

Protocatechuic acid (PCA), a type of phenolic acid commonly found in nature, possesses antioxidant, pro-oxidant, and radical scavenging properties. It has been linked to several beneficial effects on human health, including anti-inflammatory, anti-bacterial, anti-tumor, and anti-apoptotic effects [1, 3]. PCA has shown promising therapeutic potential with low toxicity and minimal side effects [3, 4]. It has been demonstrated to induce apoptosis and enhance cytotoxicity in various cancer cell lines. These anti-tumor activities of PCA are associated with its antioxidant properties [5].

Cancer cells exhibit increased metabolic rates and produce high levels of reactive oxygen species (ROS) [1]. Traditional anti-cancer drug strategies aim to induce oxidative stress through pro-oxidant chemotherapy drugs or radiation. However, these approaches often lead to the development of chemo-resistance due to increased antioxidant defense

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mechanisms within cancer cells. Glutathione (GSH) and other antioxidants help maintenance of ROS at physiological levels, promoting cell survival and proliferation by activating redox signaling pathways and suppressing cell death caused by excessive ROS levels [6].

Reduced GSH, present mainly in the cytosol, plays a crucial role in maintaining intracellular redox balance as an antioxidant. It neutralizes free radicals and ROS and is essential for the activity of various antioxidant enzymes. GSH can be converted to its oxidized form, GSSG, which can be cytotoxic. The balance between GSH and GSSG within cells is a critical factor in regulating cellular functions. GSH also participates in detoxification processes by conjugating with xenobiotics through glutathione transferase (GST) enzymes [7] Additionally, GSH is involved in iron and sulfur metabolism and is essential for the activity of enzymes involved in DNA synthesis [6, 8].

Given its essential role in necroptosis, autophagy, apoptosis, and modulating GSH levels can have significant therapeutic effects. Decreased GSH/GSSG ratio and increased ROS generation have been associated with apoptosis and mitochondrial damage. Cancer cells employ various mechanisms to evade apoptosis and survive, including increased GSH production. Maintaining redox equilibrium is crucial for cancer cell survival, growth, and adaptation to treatment-induced stress. Overcoming the adaptive tolerance threshold of cancer cells can be a valuable strategy to combat chemotherapy-resistant cancer cells [6, 9].

Tamoxifen (TAM) is the gold standard drug for treating breast cancer. It induces oxidative stress, leading to cellular damage. Resistance to TAM can occur through mechanisms such as increased expression of proteins that activate genes associated with antioxidant defense, and elevated levels of ROS-protecting enzymes [10]. However, combining TAM with antioxidant therapy has shown promise in enhancing its effectiveness. For example, in a rat model of breast carcinogenesis, the administration of TAM along with riboflavin or niacin (Vitamin B), restored antioxidant activity and lipid peroxide levels, resulting in improved anticancer effects [10].

The objective of this research is to evaluate the effect of PCA on cancer cell oxidative stress conditions and its impact on the efficacy of TAM treatment. The study aims to assess different variables, including cytotoxicity, cell growth stages, cell death indicators, and their correlation with GSH levels and associated enzymes. The hypothesis is that PCA, as an antioxidant compound, can enhance the efficacy of TAM. The study also investigates the potential complementary effect of combining PCA and TAM with the addition of GSH as a potential adjacent therapy. Assuming that, GSH would enhance the efficiency of toxin control system.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Reduced glutathione≥*95 %* (GSH), oxidized glutathione≥*98%* (GSSG), and 1-chloro-2, 4-dinitrobenzene (CDNB) were purchased from Merck Company (Germany). Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), protocatechuic acid (3, 4-dihydroxy benzoic acid; PCA) and tamoxifen were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of the highest purity commercially available.

2.1.2. Cancer cell lines

The human breast cancer cell line (human Caucasian breast adenocarcinoma, MCF-7) was identified and performed by the Drug Bioassay-Cell Culture Laboratory, National Research Centre, Dokki, Cairo 12622, Egypt. All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, and Sanford, ME, USA). Cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) HCT116, 1% antibiotic-antimycotic mixture (10,000 U/mL potassium penicillin, 10,000µg/mL streptomycin sulphate and 25µg/mL amphotericin B) and 1% L-glutamine at 37ºC at 5% CO_2incubator. Cells were batch cultured for 10 days, then seeded at a concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37ºC for 24 h at 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated for 48 h either alone (negative control) or with the following treatments: 1-) different concentrations of PCA and TAM to achieve final concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL; 2-) IC_{25}concentration of PCA (96.8 µg/mL); 3-) TAM (0.8 µg/mL); and 4-) their co-treatments in absence and presence of GSH (100 µM).

2.2. Methods

2.2.1. Cell viability assay

Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan [11]. After 48 h of incubation, the medium was aspirated, 40uL MTT salt (2.5µg/mL) was added to each well and

incubated for a further 4 h at 37ºC under 5% CO_2. To stop the reaction and dissolve the formed crystals, 200µL of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37ºC [12]. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wavelength of 620nm. The percentage of change in viability was relative to the reading of negative control. The IC_{25} for each treatment was calculated. Accordingly, 96.8µg/mL and 0.8µg/mL for PCA and TAM, respectively, were used for subsequent studies.

2.2.2. Apoptosis/Necrosis analysis

Apoptosis and necrosis cell populations are determined using Annexin V-5- fluorescein isothiocyanate (Annexin V-FITC) apoptosis detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK) coupled with 2 fluorescent channels flow-cytometry. After treatment with test compounds for 48 h [TAM (0.8µg/mL) and the combination of TAM (0.8µg/mL) + PCA (96.8µg/mL)]; cells (10^5cells) were collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Then, cells were incubated in the dark with 0.5 mL of Annexin V-FITC/propidium iodide (PI) solution for 30 min in the dark at room temperature according to manufacturer protocol. After staining, cells were injected via ACEA Novocyte™ flow-cytometer (ACEA Biosciences Inc., San Diego, CA, USA) and analyzed for FITC and PI fluorescent signals using FL1 and FL2 (fluorescence channels) signal detector, respectively (λex/em488/530 nm for FITC and λex/em535/617 nm for PI). For each sample, 12,000 events are acquired and positive FITC and/or PI cells are quantified by quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA) [13-15].

2.2.3. Cell cycle distribution analysis

After treatment with test compounds for 48 h and paclitaxel (1 µM) for 24 h as positive control; cells (10^5 cells) were collected by trypsinization and washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4. Cells were re-suspended in 2mL of 60% ice-cold ethanol and incubated at 4ºC for 1 h for fixation. Fixed cells were washed twice again with PBS (pH 7.4) and re-suspended in 1 mL of PBS containing 50 µg/mL RNAase A and 10 µg/mL propidium iodide (PI). After 20 min of incubation in the dark at 37º C, cells are analyzed for DNA contents using flow-cytometry analysis using FL2 ($\lambda_{ex/em}$ 535/617 nm) signal detector (ACEA Novocyte™ flow-cytometer, ACEA Biosciences Inc., San Diego, CA, USA). For each sample, 12,000 events are acquired. Cell cycle distribution is calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA) [13-15].

2.2.4. DNA damage assay

Comet assay (Single-cell gel, SVG)

DNA fragmentation was measured by the alkaline comet assay, which is used to identify individual DNA migration patterns [16, 17]. The basic steps of comet assay are as follows: A layer of 1% ordinary agarose was first applied to conventional microscope slides. The cell suspension (lysate) was then combined with 70µL of 0.5% low melting point agarose and applied to the slides. All slides were immersed in chilled buffer solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, NaOH was added to pH 10; 1% Triton X-100, 10% DMSO were freshly added). Slides were kept in lysing solution for up to 24 h at 4 °C in the dark. Slides were then placed in an electrophoresis tank and placed in freshly prepared alkaline solution (300 mM NaOH and 1.0 mM EDTA, pH 13) for 20 min, then electrophoresis was performed for 30 min at 25 V (0.79 V). Slides were washed three times in neutralizing buffer for 5 min (0.4 M Tris, pH 7.5). Slides were fixed in cold 100% ethanol, dried in air, and stained with ethidium bromide. Slides were examined by fluorescent microscope (Carl Zeiss Axioplan with epifluorescence using filter 15 BP546/12, FT580, and LP590). The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 50 cells at a magnification of 400x using comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). Images of comets were taken using a closed-circuit digital camera (CCD). All samples were evaluated for the extent of DNA damage using the following parameters: tail length, tail DNA %, and tail moment.

2.2.5. Biochemical analysis

2.2.5.1. Preparation of cell lysate

After incubation, the medium was removed. Cells were scraped. The cells were washed twice with cold phosphate buffer saline. Cells were lysed in 0.1M potassium phosphate buffer, pH 8 containing 5mM EDTA and 5mM β-mercaptoethanol. The lysate was sonicated for 30 sec three times, centrifuged at 2000 rpm, and preserved at -20°C for further analyses.

2.2.5.2. Enzyme assays

Glutathione transferase activity

Glutathione transferase (GST; EC 2.5. 1.18) activity was determined according to the method

described by [18]. The reaction measured the increase in the concentration of the conjugation product of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm over 3 min at 27˚C.One unit of GST activity is equivalent to the amount of enzyme conjugating 1 µmole of CDNB in one minute. The extinction coefficient of the product was taken to be 9.6 $mM^{-1}cm^{-1}$.

Glutathione peroxidase activity

The activity of glutathione peroxidase (GPx; EC 1.11.1.9) was determined according to the method described by [19]. The assay reaction mixture contained in1mL volume, 50 mM potassium phosphate buffer, pH 7.0, 0.005 M EDTA, 0.075 mM H_2O_2, 5.0 mM GSH, 0.28 mM NADPH, 1 IU GR, and a suitable cell lysate volume. One unit is equivalent to the oxidation of 1 µmole of NADPH in 1 min, at 27°C. The extinction coefficient of NADPH was taken to be 6.22 $mM^{-1}cm^{-1}$.

Glutathione reductase activity

The activity of glutathione reductase (GR; EC 1.8.1.7) was determined spectrophotometrically at 27°C following the decrease in absorbance at 340 nm according to the method described by [20]. The assay reaction mixture volume of 1 mL contains 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and suitable cell lysate volume. One unit of GR activity is defined as the amount of enzyme that oxidizes 1 µmole of NADPH per minute.

Catalase activity

Catalase (CAT; EC 1.11.1.6) activity determination was carried out according to the method described by [21]. The method is based on monitoring the rate of decomposition of H_2O_2 at 27°C. For CAT activity determination, suitable cell lysate volume was added to 1 mL of substrate mixture, which consisted of 20 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0. The decomposition of H_2O_2 was followed by a decline in absorbance at 240 nm for 1 min. One unit of activity was defined as the calculated consumption of 1µmole of H_2O_2/min at 27 °C. The extinction coefficient of H_2O_2 was taken to be 43.6 M^{-1} cm^{-1}.

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH*;* 1.1.1.27) activity was measured spectrophotometrically at 340 of PCA and ascorbic acid with the anticancer drug TAM on the viability of MCF-7 cell lines after incubation for 48 h. IC_{25} concentrations of PCA and ascorbic acid were used in combination with TAM, nm by determining the rate of oxidation of NADH in the enzymatic conversion of pyruvate to lactate. The reaction was carried out in the potassium-pyruvate solution at 27°C as described by [22].

2.2.5.3. Total glutathione contents

The total glutathione content (GSH) was measured colorimetrically using the method of [23]**.** The cell homogenates were mixed with an equal volume of 13% Trichloroacetic acid (TCA). The precipitated proteins were removed by centrifugation at 2000 rpm for 10 min and the supernatant was used for the determination of total GSH level.

2.2.5.4. Total protein concentration

Protein concentration was determined by the method of [24] using bovine serum albumin as a standard.

2.2.5.5. Statistical analysis

All data obtained from each experiment were expressed as mean ± standard deviation (SD) or standard error (ES) from three independent experiments (n = 3 for each experiment). Statistical analysis was performed using the one-way analysis of variance (ANOVA) option in SAS 9.3. Significant differences among means were determined using Duncan's test. For the cell viability assay, statistical significance was assessed between samples and the negative control (vehicle cells) using the independent t-test in SPSS 11. Probit analysis was conducted using the SPSS 11 system to determine the IC_{25} and IC_{50} values. Two-variable data were analysed using the student's t-test for independent samples. The p-value ≤0.05 was considered statistically significant.

3. Result

3.1. The MCF-7 cytotoxicity (cell viability assay)

In this study, we evaluated the cytotoxicity of PCA (**Fig.1a**) and the antioxidant compound ascorbic acid on MCF-7 cells using a viability assay. The results, presented in **Fig. 1c**, demonstrated that the treatment with PCA at concentrations of 100 and 200 µM resulted in cytotoxic effects of 33% and 61.2%, respectively. Conversely, ascorbic acid showed a 100% cytotoxic effect on MCF-7 cell lines at the same concentrations. Importantly, neither PCA nor ascorbic acid showed any toxicity on normal skin fibroblast cell lines (BJI) (data not shown). Furthermore, we investigated the combined effect

and the results were compared to untreated cells. The co-treatment of PCA with TAM resulted in a cytotoxicity of 35.8%. Also, the co-treatment of ascorbic acid with TAM exhibited a cytotoxicity of

23.9%, indicating that the combination of ascorbic acid and TAM reduces the cytotoxic effects on MCF-7 cell survival compared to the individual treatments (**Fig. 1d**).

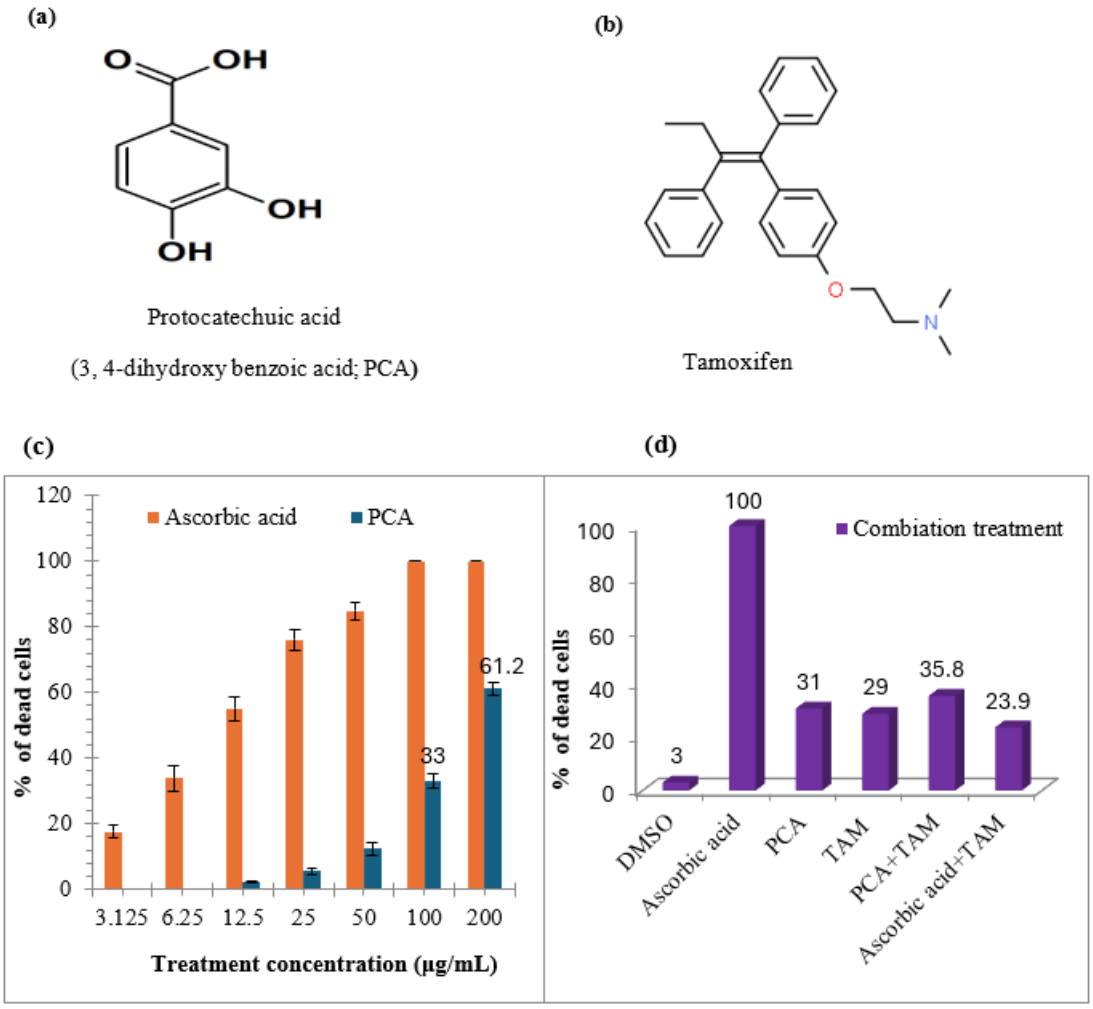

Figure 1: The chemical structure of a) PCA and b) tamoxifen was drawn using the ACD/ChemSketch 2018.2.1 program (ACD/Labs, Toronto, ON, Canada). c) The cytotoxic effect of PCA and ascorbic acid on MCF-7 cell lines at different concentrations (3.125-200 µg/mL). d) Cytotoxicity of TAM at IC_{25} value (0.8 µg/mL), combined with PCA at IC_{25} value (96.8 µg/mL) and ascorbic acid at IC_{25} value (3.8 µg/mL) in MCF-7 cells treated with DMSO as a negative control.

3.2. Evaluation of PCA and combination treatments on genotoxicity and DNA damage of MCF-7 cells

In our study, DNA damage parameters including tail length, proportion of DNA in the tail, and tail moment were evaluated in MCF-7 cells treated with PCA, TAM, and their combination, with or without the addition of GSH. The results noted that treatment with PCA alone had no significant effect on the three DNA damage parameters examined. However, when PCA was combined with TAM, a significant reduction in all three DNA damage parameters was observed compared to TAM treatment alone. This indicates that the combination of PCA and TAM has a synergistic effect in reducing DNA damage in MCF-7 cells. On the other hand, the addition of GSH to the combination treatment of PCA and TAM showed no significant effect on the DNA damage parameters examined **(Table1**).

Table 1: Evaluation of PCA and co-treatments of PCA+TAM on MCF7 genotoxicity and DNA damage parameters.

Treatment	Tail Length (px)	%DNA in Tail	Tail Moment
Untreated cells	0.82±0.199^{c}	0.99±0.48^{b}	0.01±0.004^{b}
Protochatuic acid (PCA)	1.00±0.19^{c}	0.62±0.33^{b}	0.02±0.014^{b}
Tamoxifen (TAM)	5.72±0.48^{a}	6.58±1.02^{a}	0.50±0.1^{a}
ssPCA + TAM	2.46±0.246^{b*} P= 0.0001	3.028±0.75^{b*} P= 0.0061	0.11±0.028^{b*} P= 0.0003
PCA + TAM + GSH	2.54±0.27^{b}	2.35±0.47^{b}	0.09±0.03^{b}

-Tail length is measured from the centre of the head to the end of the tail (µm); %DNA in Tail = 100* Tail DNA intensity/ cell DNA intensity; and Tail moment (µm) =tail length x % of DNA in the tail.
-The values represent the mean ± SE of 50 independent experiments.
-Values with different letters within the same column indicate significant differences at level P≤0.05.
-* P values of PCA+TAM co-treated cells showed statistically highly significant differences from the TAM-treated cells by Student's t-test.

3.3. Evaluation of PCA + TAM co-treatment on MCF7 cell cycle distribution phases

The results of PCA + TAM co-treatment on cell cycle distribution showed an increase in the S-phase (20.10±0.52) and a decrease in the number of cells in the G2/M phase (18.63±1.20) compared to the TAM treatment. There is no noticeable effect on G1 and sub-G1 populations (**Fig. 2& Table 2**).

Table 2: Effect of PCA + TAM co-treatment on MCF7 cell cycle distribution phases

Treatment	Cell cycle distribution (cell population %)			
	G1 (%)	S–phase (%)	G2/M (%)	G0/ G1 (%)
Untreated cells	47.65±1.66^{b}	25.63±1.21^{a}	21.36±0.25^{a}	1.54±0.23^{a}
TAM	54.27±1.10^{a}	17.75±0.31^{c}	22.31±1.12^{a}	1.06±0.12^{b}
PCA+TAM (IC_{25}+IC_{25})	57.17±1.55^{a}	20.10±0.52^{b*} P=0.0025	18.63±1.20^{b*} P=0.0178	1.19±0.07^{ab}

- The number of cells in the G0/G1, S, or G2/M phases was expressed as a percentage.
-Cell populations are presented for each phase of the cell cycle for negative control (untreated cell), positive control (TAM), and MCF-7 cells co-treated with PCA (96.8 µg/mL) +TAM (0.8 µg/mL) at the IC_{25} values for 48 h. Data represents the mean ± standard deviation (SD) (n = 3). TAM: tamoxifen and PCA: protochatuic acid.
- Values with different letters within the same column indicate significant differences at level P≤0.05.
-*P values of PCA+TAM co-treated cells showed statistically significant differences from the TAM treated cells by Student's t-test.
- Cell cycle phases:
G_0/G_1 phase representing non-proliferating cells.
S-phase indicates proliferating cells that are undergoing DNA synthesis
G_2/M-phase indicates the final phase of cell replication (mitosis).

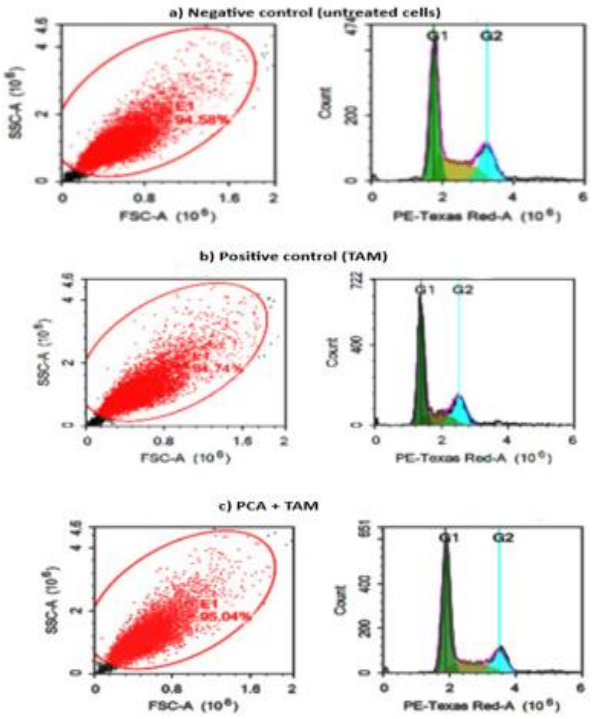

Figure 2: Cell cycle distribution analysis of the (**a**) untreated cells, (**b**) TAM treated cells; (**C**) PCA+TAM co-treated cells using the Annexin V-FITC flow-cytometer technique of three independent experiments which displayed similar results. Cell cycle phases:
G_0/G_1 phase representing non-proliferating cells.
S-phase indicates proliferating cells that are undergoing DNA synthesis
G_2/M-phase indicates the final phase of cell replication (mitosis).

3.4. Evaluation of PCA + TAM co-treatment on MCF7 cell apoptotic and necrotic parameters

The results of the combination of PCA + TAM treatments on the percentage of cells between late apoptotic and necrotic stages showed no significant effect. A significant decrease in the percentage of viable cells (96.32±0.21) and increase in early apoptotic cells was observed (0.56±0.1) compared to untreated cells (0.21±0.04) and single TAM-treated cells (0.36±0.03; P = 0.05) (**Fig. 3&Table 3**).

Table 3: Effect of PCA + TAM co-treatment on MCF7 cell apoptotic and necrotic parameters.

Treatment	Cell percentage (%)			
	Viable cell (Q3)	Early apoptosis (Q4)	Late apoptosis/ early necrosis (Q2)	Late necrosis (Q1)
Untreated cells	97.57±0.5^{a}	0.21±0.04^{b}	1.517±0.11^{d}	0.71±0.11^{c}
TAM	97.14±0.11^{a}	0.36±0.03^{b}	2.01±0.11^{cd}	0.49±0.07^{c}
PCA+TAM (IC_{25}+IC_{25})	96.32±0.21^{b} P= 0.0039	0.56±0.10^{a*} P=0.0049	2.54±0.21^{c}	0.59±0.06^{c}

-The percentage of viable (Q3), early apoptotic (Q4), late apoptotic/early necrotic (Q2), and late necrotic cells (Q1) concerning negative control (untreated), positive control (TAM), and MCF-7 cells co-treated with PCA (96.8 µg/mL) +TAM (0.8 µg/mL) at IC25 valuesfor 48 h.
-Data represents the mean ± standard deviation (SD) (n = 3).
-Values with different letters within the same column indicate significant differences at level P≤0.05.
- TAM: tamoxifen and PCA: protochatuic acid. Means with the same letter are not significantly different at p≤0.05.
-* P values of PCA+TAM co-treated cells showed statistically highly significant differences from the TAM treated cells by Student's t-test.

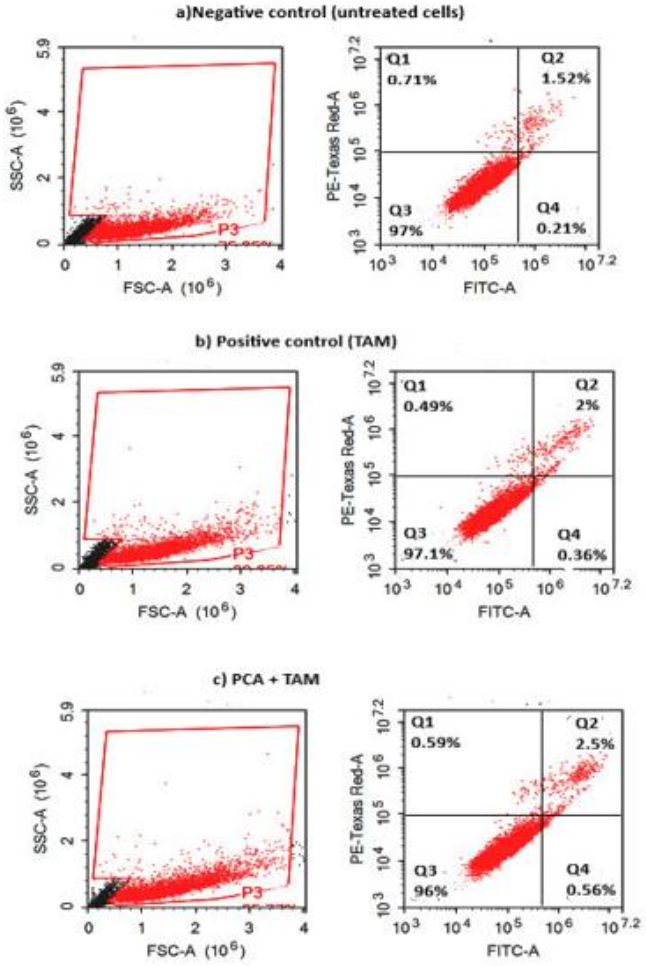

Figure 3: Dot plot of apoptotic and necrotic parameters using Annexin V-FITC flow-cytometer technique. Data represent three independent experiments that showed similar results. Quadrant image of the (**a**) untreated cell, (**b**) TAM treated cells; (**C**) PCA+TAM treated cells.
The lower left quadrant represents **intact viable cells (Q3)**. The lower right quadrant represents **early apoptosis phase (Q4):** This phase denotes early phase apoptotic cells; cells just initiated the apoptosis programmed cell death process with their cell membrane still intact. The upper right quadrant represents **late apoptosis phase (Q2):** This phase denotes late phase apoptotic cells; cells initiated the apoptosis programmed cell death process with their cell membrane perforated (damaged). The upper left quadrant represents **necrosis phase (Q1):** This phase denotes cells undergoing a non-programmed cell death process (necrosis) with their cell membrane perforated (damaged).

3.5. Evaluation of PCA and its combined treatment with TAM on MCF7cellular antioxidant defense system and enzymes involved in redox balance maintenance, detoxification, and oxidative stress response

Effect of PCA treatment on GSH levels, activities of GSH related enzymes, catalase (CAT) activity, and LDH activity; the cell viability indicator; was determined in MCF7 cell lines. Cells were also treated with IC_{25} TAM. A significant increase in GSH level (0.37 ± 0.013 µmol/mg protein, P = 0.0001) associated with a more than 3-fold decrease in GPx, GR and CAT activities compared to untreated cells was reported. A significant 10-fold reduction in LDH activity was observed compared to untreated MCF7 cells (**Fig.4**). While LDH activity significantly increased to 0.06±0.0053 µmol/min/mg protein (P=0.0002) compared to untreated cells as a result of TAM treatment. An increase in GSH level was also observed (0.67 ± 0.013 µmol/mg protein, P = 0.0001). Decreased activities of CAT, GPx, and GR have also been reported after TAM treatment. Treatment of cells with both PCA and TAM had almost no effect on GST activity, as indicated by insignificant p values (**Fig.4**).

Combination treatment of PCA+ TAM significantly increased the level of GSH (0.71± 0.008 µmol/mg protein, P=0.0001), GPx (0.34 ±

0.03 µmol/mg protein, P=0.009), GR (0.12 ± 0.007µmol/mg protein, P=0.008), GST (0.19 ± 0.04 µmol/mg protein, P=0.028) CAT (0.027±0.006 mmol/mg protein, P=0.0064) and LDH activity (0.08±0.0048 µmol/mg protein, P=0.0001) compared to TAM treatment alone. Addition of 100 µM GSH to this co-treatment resulted in decreased GSH level and GPx, CAT and LDH activities with GR and GST activities unchanged (**Fig.4**).

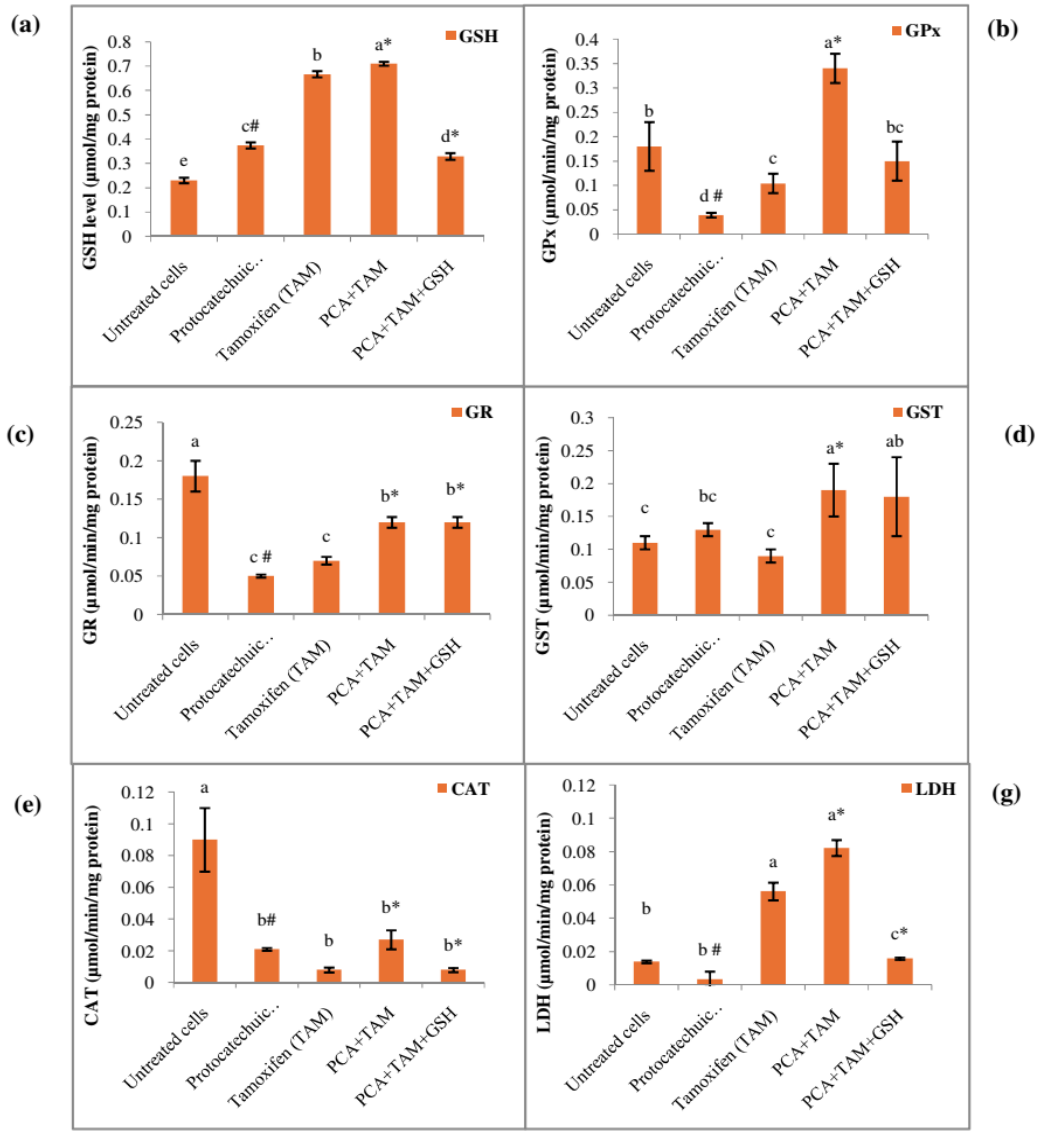

Figure 4: Effect of PCA on the levels of glutathione (GSH), antioxidant enzyme activities and viability of MCF-7 cells. The values represent the mean ± SD of three independent experiments. Values with different superscript letters within the same column indicate significant differences at level P≤0.05. * P values of PCA+TAM co-treated cells showed statistically significant differences from the TAM treated cells by Student's t-test. # P values of PCA treated cells showed statistically significant differences from the untreated cells by Student's t-test. Error bars represent standard deviation or standard error. GSH level was expressed as µmol/mg protein and enzyme activities of GST, GPx, GR, and LDH were expressed as µmole /min/ mg protein and CAT activity was as mmole /min/ mg protein.GSH: reduced glutathione; GST: glutathione transferase; GPx: glutathione peroxidase: GR: glutathione reductase; CAT: catalase and LDH: lactate dehydrogenase.

4. Discussion

PCA is a naturally occurring compound possesses antioxidant and anti-inflammatory properties and has been studied for its potential anticancer effects [1]. In the present results, PCA alone exhibits a cytotoxicity of approximately 33% and 61.2% at concentrations of 100 and 200 µM, respectively. The cytotoxicity of PCA suggests that it can induce cell death in the tested MCF7 through mechanisms such as direct cytotoxic effects, modulation of cellular signalling pathways, or interference with cellular metabolism.

It was demonstrated that PCA exhibits antitumor activity by increasing apoptosis and inhibiting invasion and metastasis in various cancer cell lines, including MCF-7 human breast cancer cells, A549 lung cancer cells, HepG2 liver cancer cells, cervical cancer cells, and LNCaP prostate cancer cells [25]. According to the findings of [26], PCA extracted from Hibiscus plants induced apoptosis in human leukemia cells by reducing the phosphorylation of retinoblastoma (RB), a tumor suppressor protein involved in essential cellular processes such as proliferation, differentiation, senescence, and apoptosis. Additionally, PCA decreased the expression of Bcl-2 (B-cell lymphoma 2); a regulatory protein involved in cell death, in human leukemia cells. The rate of cell death depends on the concentration and duration of PCA treatment, reaching 70% cell death at 2 mM after 48 h [27].

Concerning the combination treatments, our results highlight the effects of combining PCA or ascorbic acid with TAM on MCF-7 cell proliferation (**Fig. 1c**). The combination of PCA (35.8% toxicity) and ascorbic acid (23.9% of toxicity) with TAM appears to reduce cytotoxicity compared to single treatments. The goal of combining ascorbic acid with TAM is to increase cytotoxicity and prevent cancer cell proliferation. The reduced cytotoxicity observed with such combinations is therefore considered a negative finding. Combination therapy may have an antagonistic effect, reducing the inhibitory effect on cancer growth compared to single TAM treatment. Also, vitamin C with TAM has a greater antagonistic effect on TAM efficacy than PCA.

Our results are consistent with the findings of [10] study that vitamin C supplements have detrimental effects on MCF-7 cells during treatment. What we found in our results contradicts the theory of synergistic activity of antioxidants, which can be used in different fields. For example, in the pharmaceutical industry; the use of a mixture of antioxidants with some drugs increases therapeutic effectiveness and reduces cell damage [8].

In our study, the results of DNA damage parameters indicated that PCA alone did not have a significant effect on DNA damage in MCF-7 cells. However, when combined with TAM, PCA showed a significant protective effect against DNA damage in these cells. Interestingly, the addition of GSH did not appear to enhance this protective effect.

The study conducted by [29] also revealed that PCA exhibits an anti-genotoxic effect by reducing the cytogenetic potential of carbon tetrachloride (CCl_4) in rats. Exposure to CCl_4 resulted in a significant increase in micronuclei occurrence in the bone marrow of male mice, which may be attributed to DNA breaks or deficiencies in DNA double-strand repair mechanisms, potentially leading to deletions. The anti-genotoxic effect of PCA represented in increased DNA repair mechanisms has also been demonstrated in other studies conducted by [28-29]. It is believed that the antioxidant properties of PCA and its ability, as a polyphenolic compound, to scavenge reactive oxygen species (ROS) contribute to its anti-genotoxic effect.

Commonly used DNA damage parameters, such as tail moment, % of DNA in the tail, and tail length, are indicative of DNA damage and the efficacy of anticancer therapies. However, the results of our study indicated suboptimal efficacy of combination therapy in inducing DNA damage and killing cancer cells. In our study, the combination of PCA and TAM exhibited lower values in these parameters compared to TAM alone, implying a reduction in DNA damage induction and cancer cell killing. This reduction suggests an antagonistic effect between PCA and TAM, which is undesirable when the objective is to effectively eliminate cancer cells. Given the weak effect of PCA on DNA damage, the effect of the PCA+TAM co-treatment on cell cycle distribution, apoptotic and necrotic stages was tested.

The cell cycle is a tightly regulated process that includes different stages controlling cell proliferation and DNA replication, ensuring the proper reproduction of eukaryotic cells. To maintain homeostasis, cell growth and cell death must be carefully regulated and balanced. Changes in cell distribution among these stages can provide valuable insights into the effects of treatments on cell cycle progression and cell proliferation [30].

In the present study, the cytological results obtained in the treated MCF7 cells, as relatively high concentrations (approximately 200 µM) were required to produce an inhibition effect on cell growth. Thus, it can be concluded that PCA alone does not have a significant impact on these cellular processes.

However, when PCA was combined with TAM, we observed alterations in the cell cycle distribution. Specifically, the co-treatment of PCA and TAM led to an increase in the percentage of cells

in the S-phase (DNA synthesis phase) and a decrease in the G2/M (preparation for cell division) phase compared to TAM treatment alone (**Table 2**). These findings suggest that the combination of PCA and TAM affects the progression of the cell cycle in MCF7 cells.

The observed increase in the S-phase cell percentage indicates that more cells in the co-treated group were undergoing DNA synthesis. This increase may suggest heightened replication activity or DNA damage repair in response to the combined treatment. TAM is known to induce DNA damage in cancer cells, and the activation of DNA repair pathways could potentially explain the observed increase in the S-phase population. On the other hand, the decrease in the G2/M cell percentage suggests a disruption in the progression of cells from the G2 phase to the mitosis (M) phase. This change in the phases of cell cycle distribution may affect cancer cell division processes. The cell cycle distribution results were confirmed through the DNA damage assay conducted in this study (**Table 1**).

The results of our study showed that the combined treatment of PCA and TAM did not have a significant effect on late apoptotic and necrotic parameters. However, we observed a notable decrease in the percentage of viable cells and increase in early apoptotic cells compared to TAM-treated cells (**Table 3**). This finding suggests that the combined treatment may have a protective effect against necrosis, leading to decreased percentage of viable cells and increase in early apoptotic cell populations. These results are not in line with the findings of [4]. In their study, quercetin increased cell viability, improved cell number and proliferation rate, and reduced LDH levels in cells exposed to hydrogen peroxide and an Alzheimer's disease cell model.

Enhanced LDH enzymatic activity is associated with the development of various forms of cancer, including breast cancer. Therefore, low levels of lactate in the extracellular environment can cause inhibition of cell motility. Quercetin has been reported to inhibit LDH activity resulting in a significant reduction in extracellular lactate level, cell proliferation, motility and invasion [31].

The protective action of PCA and quercetin against apoptosis may be attributed to their ability to counteract oxidative stress and modulate intracellular signaling pathways responsible for caspase activation. Modulating apoptosis is a key mechanism by which polyphenols exert their protective effects against degenerative diseases [33]. Furthermore, the beneficial role of PCA and quercetin in tissue damage and inflammation has been associated with the suppression of necroptosis signaling pathways, highlighting the potential of plant polyphenols to provide protection [4].

The combination therapy of TAM with antioxidants, such as riboflavin and niacin, has been shown to enhance the anticancer efficacy of TAM in a model of DMBA-induced breast cancer in Sprague-Dawley rats [10]. Co-administration of TAM with these antioxidants restored lipid peroxidation levels and antioxidant activity, resulting in enhanced antitumor activity. Thus, PCA+TAM combination in the present study appears to modulate apoptotic pathways toward the programmed cell apoptosis and affect cellular viability.

In the context of cancer, tumors consist of heterogeneous cells that adapt to their microenvironment through metabolic changes and reprogramming. Cancer cells often exhibit higher levels of oxidative stress, primarily due to the increased production of reactive oxygen species (ROS) [1, 32]. The excessive production of ROS can lead to the oxidation of GSH, resulting in an imbalance in the GSH/GSSG ratio. The GSH/GSSG redox system is critical for maintaining proper cellular redox status and is involved in cell proliferation, differentiation, signal transduction, and apoptosis [34]. Phenolic compounds like PCA have been shown to scavenge ROS and induce the expression of cellular defense antioxidant and detoxification enzymes, including superoxide dismutase, GPx, GR, and GST [1, 32].

In the present study, single treatment with PCA or TAM resulted in increased GSH levels while reducing the activities of GPx, GR, and CAT in MCF7 cell lines. Additionally, PCA treatment significantly decreased LDH activity, while TAM treatment increased LDH activity. However, GST activity was not significantly affected by either treatment.

These findings suggest that single PCA or TAM treatments can influence the redox status of MCF7 cells and modulate the activities of antioxidant enzymes. The decrease in antioxidant enzyme activities, along with the decrease in LDH activity following PCA treatment, provides evidence for the antioxidant effect of PCA in reducing ROS production and glycolysis, which are typically associated with cancer cell growth. These changes may reflect alterations in cellular metabolism and energy production.

Several studies have reported similar results. For instance, the study by [4] that demonstrated the pre-treatment with PCA reduced LDH and malondialdehyde (MDA) levels in rat hepatocytes exposed to tert-butyl hydroperoxide, highlighting the antioxidant properties of PCA. Another study by [33] showed the increase GSH levels in response to PCA treatment, indicating its ability to enhance

endogenous antioxidant defences. Moreover, the study by [34] found that PCA exhibited growth-inhibitory effects on ovarian cancer cells by increasing intracellular GSH levels, reducing ROS levels, decreasing cell viability, and arresting cells in the G2/M phase. Another study by [35] demonstrated that PCA acts as a potential dual-acting agent by inhibiting phase I enzymes (e.g., cytochrome P450) and activating phase II enzymes (e.g., GST).

While the current findings contradict the findings of [36], that found that PCA exerted anti-cancer effects via inducing oxidative stress and apoptosis. In this study, 100 µM PCA inhibited colon cancer cell (CaCo-2) proliferation and produced apoptotic and/or necrotic cell death. Lower concentrations of PCA (1, 25 and 50 M) enhanced the percentage of apoptotic cells without altering cell viability. As a result, PCA exerts its action via oxidative/antioxidant imbalance in a dose-dependent way. In contrast to that, PCA (50 and 100 mg/kg) can significantly relieve ROS production and lipid peroxidation in myocardial tissue in type 2 diabetes (T2D) [37]. Such findings may be explained by the dual antioxidant and pro-oxidant activity of several phenolic compounds, including PCA, investigated in [38]. The pro-oxidant activity, which is measured by the amount of H_2O_2 production, was found to be high at 52.99±3.30 µM. Such study highlighting the pro-oxidant properties of PCA which is attributed to the number and configuration of hydroxyl groups present in the compounds. This difference in results between studies raises the question: Does this compound act as an antioxidant or as a cause of oxidation i.e. pro-oxidant?

In the context of specific cancer types, PCA has been shown to contribute to the apoptotic and autophagy cell death of active myeloid leukaemia cells by stimulating ROS generation [1]. Additionally, PCA has been found to prevent apoptotic induction and inhibit the growth of pancreatic tumour cells through ROS-dependent down regulation in human osteosarcoma cell lines. The study by [39] reported that PCA-mediated osteosarcoma cellular apoptosis was linked to ROS production. While PCA has demonstrated anti-proliferative activity by triggering ROS production, it is important to note that its anti-tumour effects may vary across different cell types. Further research is needed to fully understand the consistency and effectiveness of PCA as an anti-tumour agent in various cellular contexts.

In the present study, the combination treatment of PCA and TAM demonstrated significant increases in the levels of GSH, GPx, GR, GST, CAT, and LDH activity compared to TAM treatment alone. However, the addition of 100 µM GSH to this co-treatment resulted in decreased GSH levels, GPx, CAT, and LDH activities, while GR and GST activities remained unchanged.

These results indicate that combined treatment of PCA and TAM enhances the adaptive response of cancer cells to cope with the harmful oxidative conditions induced by TAM treatment through increased antioxidant capacity. While this combined treatment maintains the effect of TAM on MCF7 cells. This adaptation response is demonstrated by the levels of LDH activity compared to TAM treatment alone and untreated cells. In addition, this was confirmed by the cytotoxic effect previously observed in our results for PCA + TAM (35.8% of dead cells) compared to the reduction of single TAM treatment (29% of dead cells) (**Fig. 1 c**). These indicate protection from TAM-induced oxidative damage with the up-regulation of antioxidant molecules and the antagonistic effect.

Tumors consist of heterogeneous cells that can dynamically adapt their microenvironment through genetic/epigenetic changes and metabolic reprogramming. The uncontrolled proliferation and resistance to apoptosis are hallmarks of tumor cells. Additionally, increased oxidative stress caused by ROS is a prominent feature of cancer cells [32].

Resistance to therapy is a common occurrence in cancer cells, with increased antioxidant capacity playing a crucial role. Chemo-resistance, a complex phenomenon, contributes to a poor prognosis for cancer patients. Various mechanisms contribute to cancer cell resistance to chemotherapeutic drugs, including drug inactivation, induction of efflux transporters, inhibition of apoptosis, cell cycle deregulation, enhanced DNA repair, and genetic and epigenetic alterations in cellular oxidative metabolism. Notably, increased GSH levels and activation of GSH-related enzymes have been reported to support tumor growth and counteract the efficacy of therapy, contributing to the development of chemo-resistance [6]. Targeting the metabolic and antioxidant pathways that contribute to redox balance in cancer cells shows promise as an anticancer therapy, particularly considering that conventional therapies rely on the accumulation of ROS to exert cytotoxic effects [32].

The effects of oxidative stress on the efficacy or resistance to TAM are multifaceted and influenced by various factors. Tumor cells can develop resistance to TAM by enhancing protection against ROS through increased antioxidant enzyme activity. However, combining TAM with antioxidants has been shown to enhance its anticancer activity. Conflicting results have been observed in different breast cancer models. For example, the accumulation of vitamin C by tumor

cells has been shown to protect against the effects of TAM. Therefore, the association of oxidative stress and antioxidants with the efficacy of TAM is not consistently linked to resistance or efficacy, highlighting the need for further investigation [10].

5. Conclusion

Our study revealed that antioxidant PCA treatment alone may protect cancer cells and interfere with TAM mechanisms by reducing oxidative stress. Importantly, this treatment did not impact DNA integrity or induce cell death through apoptosis or necrosis. In contrast, co-treatment of TAM with PCA increased oxidative stress levels, stimulated antioxidant enzymes, and enhanced apoptosis. The combination treatment may exhibit complex interactions within the cellular environment, interfere with the metabolism of TAM, leading to unexpected outcomes. However, the interpretation of these results requires further investigation by using higher concentrations of TAM and PCA to fully elucidate the effects observed in the present study.

6. Competing interests

The authors declare that they have no competing interests.

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8. References

1. Cadena-Iñiguez J, Santiago-Osorio E, Sánchez-Flores N, Salazar-Aguilar S, Soto-Hernández RM, Riviello-Flores ML, Macías-Zaragoza VM, Aguiñiga-Sánchez I. The Cancer-Protective Potential of Protocatechuic Acid: A Narrative Review. Molecules. Mar 23;29 (7):1439 (2024). doi: 10.3390/molecules29071439. PMID: 38611719; PMCID: PMC11012759.
2. Rabelo ACS, Guerreiro CdA, Shinzato VI, Ong TP, Noratto G. Anthocyanins reduce cell invasion and migration through Akt/mTOR down regulation and apoptosis activation in triple-negative breast cancer cells: A systematic review and meta-analysis. Cancers 15: 2300 (2023). https://doi.org/10.3390/cancers15082300.
3. Yüksel M, Yıldar M, Başbuğ M, Çavdar F, Çıkman Ö, Akşit H, Aslan F, Akşit D. Does protocatechuic acid, a natural antioxidant, reduce renal ischemia reperfusion injury in rats? Ulus Travma Acil CerrahiDerg 23:1-6 (2017). http://doi: 10.5505/tjtes.2016.20165.
4. Xiao K, Zhou M, Lv Q, He P, Qin X, Wang D, Zhao J, Liu Y. Protocatechuic acid and quercetin attenuate ETEC-caused IPEC-1 cell inflammation and injuryassociated with inhibition of necroptosis and pyroptosis signaling pathways. J Anim Sci Biotechn 14:5 (2023). https://doi.org/10.1186/s40104-022-00816-x.
5. Buskaran K, Bullo S, Hussein MZ, Masarudin MJ, MohdMoklas MA, Fakurazi S. Anticancer molecular mechanism of protocatechuic acid loaded on folate coated functionalized graphene nanocomposite delivery system in human hepatocellular carcinoma. Materials 14:8-17 (2021). https://doi.org/10.3390/ma14040817.
6. Valenti GE, Tasso B, Traverso N, Domenicotti C, Marengo B.Glutathione in cancer progression and chemoresistance: an update. Redox Exp Med 2023: e220023 **(2023)**. https://doi.org/10.1530/REM-22-0023.
7. Singh RR, Reindl KM. Glutathione S-transferases in cancer. Antioxidants 10: 701 (2021). https://doi.org/10.3390/antiox10050701.
8. Carreon-Gonzalez M, Alvarez-Idaboy JR. The Synergy between glutathione and phenols—phenolic antioxidants repair glutathione: closing the virtuous circle-A theoretical insight. Antioxidants 12: 1125 (2023). https://doi:10.3390/antiox12051125.
9. Buskaran K, Bullo S, Hussein MZ, Masarudin MJ, MohdMoklas MA, Fakurazi S. Anticancer molecular mechanism of protocatechuic acid loaded on folate coated functionalized graphene nanocomposite delivery system in human hepatocellular carcinoma. Materials 14:8-17 (2021). https://doi.org/10.3390/ma14040817.
10. Ahmed NS, Samec M, Liskova A, Kubatka P, Saso L. Tamoxifen and oxidative stress: an overlooked connection. Discov Oncol 12:17 (2021). https://doi: 10.1007/s12672-021-00411-y.
11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63 (1983). https://doi: 10.1016/0022-1759(83)90303-4.
12. Thabrew MI, Hughes RD, McFarlane IG. Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J Pharm Pharmacol 49: 1132–1135 (1997). https://doi: 10.1111/j.2042-7158.1997.tb06055.x.PMID: 9401951.
13. Alaufi OM, Noorwali A, Zahran F, Al-Abd AM, Al-Attas S. Cytotoxicity of thymoquinone alone or in combination with cisplatin (CDDP) against oral squamous cell carcinoma in vitro. Scientific Report; 7:13131 (2017). https://doi:10.1038\s41598-017-13357-5.
14. Bashmail HA, Alamoudi AA, Noorwali A, Hegazy GA, AJabnoor G, Choudhry H, Al-Abd AM. Thymoquinone synergizes gemcitabine anti-breast cancer activity via modulating its apoptotic and autophagic activities. Sci Rep 8: 11674 (2018). https://doi: 10.1038/s41598-018-30046-z.
15. Fekry MI, Ezzat SM, Salama MM, Alshehri OY, Al-Abd AM. Bioactive glycoalkaloides isolated from Solanum melongena fruit peels with potential anticancer properties against

hepatocellular carcinoma cells. Sci Rep 9:1746 (2019). https://doi: 10.1038/s41598-018-36089-6.
16. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu J-C, Sasaki YF. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35: 206-221 (2000). https://doi: 10.1002/(sici)1098-2280(2000)35:3<206: aid-em8>3.0.co;2-j.
17. Nandhakumar S, Parasuraman S, Shanmugam MM, Ramachandra Rao KR, Chand P, Bhat BV. Evaluation of DNA damage using singlecell gel electrophoresis (Comet Assay). J PharmacolPharmacother 2: 107–111 (2011). https://doi: 10.4103/0976-500X.81903.
18. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130-7139 (1974).
19. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Lab Clin Med 70:158–169 (1967).
20. Zanetti G. Rabbit liver glutathione reductase: purification and properties. Arch BiochemBiophys 198: 241-246 (1979).
21. Aebi H. Catalase in vitro, Methods Enzymol 105: 121-126 (1984).
22. Fountain JA, Parks ME, Dickey A, McKee RW. Lactate dehydrogenase isoenzymes in tissues of normal and Ehrlich-Lettré ascites tumor-bearing swiss mice. Cancer Res 30: 998-1002 (1970).
23. Saville B. Colorimetric method for total thiol determination. Analyst 83: 670-672 (1958).
24. Bradford MM. A rapid and sensitive method for quantization of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72: 248-254 (1976).
25. Yin MC, Lin CC, Wu HC, Tsao SM, Hsu CK. Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: potential mechanisms of action. J Agric Food Chem 57:6468-73 (2009). http://doi: 10.1021/jf9004466.
26. Tseng TH, Kao TW, Chu CY, Chou FP, Lin WL, Wang CJ. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. BiochemPharmacol 60:307-315 (2000). http://doi: 10.1016/s0006-2952(00)00322-1.
27. Dung NC, Ngoc HT, Ninh PTM, Phu DH, Hiep DM, Suong NK. Studying on cytotoxic activity of ethyl acetate extracts and isolated substances from cultured Isaria cicadae F0004 in Vietnam against the MCF-7 cell lines and Jurkat cell lines. IOP Conf. Series: Earth Environ Sci 947: 012037 (2021). http://doi:10.1088/1755-1315/947/1/012037.
28. Anter J, Romero-Jiménez M, Fernández-Bedmar Z, Villatoro-Pulido M, Analla M, et al. Antigenotoxicity, cytotoxicity, and apoptosis induction by apigenin, bisabolol, and protocatechuic acid. J Med Food 14: 276-283 (2011). http://doi:10.1089/jmf.2010.0139.
29. Adeyanju AA, Silas F, Chibuisi AG, Babatunde OA. Protective effect of protocatechuic acid in genotoxicity-induced by carbon tetrachloride: A preliminary study. Adv Clin Toxicol 8:000273(2023). http://doi:10.23880/act-16000273
30. Buskaran K, Bullo S, Hussein MZ, Masarudin MJ, MohdMoklas MA, Fakurazi S. Anticancer molecular mechanism of protocatechuic acid loaded on folate coated functionalized graphene nanocomposite delivery system in human hepatocellular carcinoma. Materials 14:8-17 (2021). https://doi.org/10.3390/ma14040817
31. Khajah MA, Khushaish S, Luqmani YA. The effect of lactate dehydrogenase inhibitors on proliferation, mobility and invasion of breast cancer cells *in vitro* highlights a new role for lactate. Mol Med Rep 29:12 (2024). http://doi: 10.3892/mmr.2023.13135.
32. Varì R, Scazzocchio B, Santangelo C, Filesi C, Galvano F, D'Archivio M, Masella R, Giovannini C. Protocatechuic Acid Prevents oxLDL-Induced Apoptosis by Activating JNK/Nrf2 Survival Signals in Macrophages". Oxid Med Cell longev 2015:1-11 (2015). https://doi.org/10.1155/2015/351827.
33. Sharifi-Rad J, Seidel V, Izabela M, Monserrat-Mequida M, Sureda A, Ormazabal V, Zuniga FA , Mangalpady SS, Pezzani R, Ydyrys A, Tussupbekova G, Martorell M, Calina D, Cho WC. Phenolic compounds as Nrf2 inhibitors: potential applications in cancer therapy. Cell Commun Signal 21:89 (2023). https://doi.org/10.1186/s12964-023-01109-0 .
34. Xie Z, Guo Z, Wang Y, Lei J, Yu J. Protocatechuic acid inhibits the growth of ovarian cancer cells by inducing apoptosis and autophagy. Phytother Res 32:2256-2263 (2018). http://doi: 10.1002/ptr.6163.
35. Punvittayagul C, Luangsuphabool T, Wongpoomchai R. Protocatechuic acid as a potent anticarcinogenic compound in purple rice bran against diethylnitrosamine-initiated rat hepatocarcinogenesis. Sci Rep 12:10548 (2022). http://doi: 10.1038/s41598-022-14888.
36. Acquaviva R, Tomasello B, Di Giacomo C, Santangelo R, La Mantia A, Naletova I, Sarpietro MG, Castelli F, Malfa GA. Protocatechuic Acid, a Simple Plant Secondary Metabolite, Induced Apoptosis by Promoting Oxidative Stress through HO-1 Down regulation and p21 Up regulation in Colon Cancer Cells. Biomolecules 11:1485 (2021). http://doi: 10.3390/biom11101485.
37. Bhattacharjee N, Dua TK, Khanra R, Joardar S, Nandy A, Saha A, De Feo V, Dewanjee S. Protocatechuic Acid, a Phenolic from *Sansevieria roxburghiana* Leaves, Suppresses Diabetic Cardiomyopathy via Stimulating Glucose Metabolism, Ameliorating Oxidative Stress, and Inhibiting Inflammation. Front Pharmacol. 8:251 (2017). doi: 10.3389/fphar.2017.00251.
38. Guneidy RA, Zaki ER, Gad AAM, Saleh NSE, Shokeer A. Evaluation of phenolic content diversity along with antioxidant/pro-oxidant,

glutathione transferase inhibition, and cytotoxic potential of selected commonly used plants. Prev Nutr Food Sci 27:282-298 (2022). http://doi: 10.3746/pnf.2022.27.3.282.
39. Mei Q, Chen X, Liu W. Protocatechuic acid induces apoptosis in human osteosarcoma cells by regulating P13K/AKT/ROS pathway. Sains Malays 51:1167-1179 (2022). http://doi.org/10.17576/jsm-2022-5104-18.