

P-Coumaric Acid Induces Apoptosis in MCF7 Human Breast Cancer Cells and Suppresses Ras Gene Expression

Saremi¹ S, Kolahi M*, Tabandeh MR³ and Hashemitabar M⁴

¹Department of Cellular and molecular research center, faculty of medicine, ahvaz jundishapour university of medical sciences, ahvaz, iran

²Department of biology, faculty of science, shahid chamran university of ahvaz, ahvaz, iran

³Department of biochemistry and molecular biology, faculty of veterinary medicine, shahid chamran university of ahvaz, ahvaz, iran

⁴Department of Cellular and molecular research center, faculty of medicine, ahvaz jundishapour university of medical sciences, ahvaz, iran

***Corresponding author:** Maryam Kolahi, Department of biology, faculty of science, shahid chamran university of ahvaz, ahvaz, iran, Tel: +986133331045, +989166007160, E-mail: m.kolahi@scu.ac.ir

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1. Abstract

Breast cancer is the leading invasive cancer in women globally. This study was aimed at evaluating the anti-apoptosis of pca on the mcf-7 breast cancer cell line. Experiments were conducted in which the mcf-7 cell line was treated with pca which showed decreased cell viability, increase in ldh activity and caspase 3 activation. The results were evaluated with real time pcr and revealed that pca reduced the amount of h-ras and k-ras transcript in mcf-7 breast cancer cells. The amount of mrna gene bax, bearing caspase 3 and late apoptotic cells with annexin v+ and propidium iodide (pi+) characteristics increased significantly in the presence of pca and was dose dependent. It also retarded the relative expression of antiapoptotic gene; bcl2 in treated cells. The results suggest that pca exhibits anti-cancer properties against mcf-7 cells. Pca inhibited the growth of mcf7 cell. The optimum concentration of pca was 75-150 μ m. Pca can inhibit the growth of mcf-7 cells by reducing ras expression and inducing cell apoptosis. Our results suggest that pca could prove valuable in the search for possible inhibitors of ras oncogene functionality and gain further support for its potential utilization in the treatment of patients with breast cancer. Pca is safe and could complement current treatments employed for the disease.

2. Keywords: Apoptosis; Caspase; P-coumaric acid; N-ras oncogenes; H-ras oncogenes.

3. Introduction

Breast cancer is the leading invasive cancer in women globally. In

relation to its occurrence, it represents 16% of all female cancers and is also reflected in the 22.9% of invasive cancers in women. Another important point to note is that the disease does not only affect women but also men with a total of 18.2% cancer deaths being associated with breast cancer on a global scale. The percentage occurrence of breast cancer is interestingly much higher in developed countries in contrast to developing ones. This may be due to a number of factors with experts in the field postulating that differences in the lifestyles and consumption patterns of women from different demographic regions play a major role [1,2]. Different approaches are required to avoid and treat such a deadly disease. A number of treatment options are available for patients suffering from breast cancer. These may include the surgical removal of the infected breasts, application of various types of therapy inclusive of radiation, chemotherapy, hormone therapy as well as targeted therapy. In addition to the high cost of treatment, the majority of the treatment options may in some instances lead to side effects during treatment [3]. Apoptosis is the programmed death of cells in tissue. This procedure allows for various morphological modifications in the cells for example enhanced rates of cell budding and condensation, the production of apoptotic bodies that are membrane enclosed containing organelles that are well preserved. The most significant signs of cytotoxic antitumor agents is that of induction of apoptosis [4]. Phenylpropanoids such as plant secondary metabolites have been identified as powerful antioxidants being able to block cancer cells through various mechanisms including apop-

tosis [5]. *p*-coumaric acid (pca) a member of the large group of phenylpropanoid compounds is present in various fruits and vegetables that are consumed such as legumes (peanuts, navy beans), fruits (apples, tomatoes), vegetables (carrots), tea, garlic and is also found in vinegar and honey [6].

Current research focuses on the potential of new anticancer drugs that have the ability to target apoptotic defects [7].

Investigation of gene expression in breast cancer revealed that treatment with select phenylpropanoid compounds can lead to the death of cancer cells via apoptosis which was altered as a result of the dysregulation of the nuclear factor- κ B (NF- κ B) pathway, as revealed by a reduction in the expression of a number of κ B-regulated gene targets [8].

Ras proteins (H-, N-, and K-Ras) containing GTP-regulated molecular switches that are integrally involved in cell signaling pathways contributes to various aspects of cell response such as proliferation, differentiation, motility and death. In normal cells, Ras can be activated by extracellular stimuli through diverse cell surface receptors leading to the activation of the enzymes mitogen-activated protein kinase/Extracellular signal-Regulated Kinase (ERK), kinase (MEK)1 and MEK2. In many tumors, oncogenic mutations have reported excessive levels of Ras-GTP to mediate down regulation due to GAP and to stay constitutively GTP bound and active [9].

Previous research has shown the interference of Ras genes in several major pathways of apoptosis such as phosphatidylinositol 3-kinase (PI3-K) and NF- κ B, promotion via RASSF1/Nore1/Mst1 and the Raf/MEK/ERK pathways [10]. Research has revealed that Ras paradoxically mediates both pro and antiapoptotic signaling depending significantly on the type of cell and other contributing factors [11]. In normal cells, activated Ras leads to the protection of proapoptotic reactions allowing inactive oncogenes to respond to hyper proliferative signals, whereas in cancer cells, elevated levels of Ras has stimulated survival rather than death. The vital role of Ras proteins in cell proliferation has attracted much attention to their recruitment in Ras transformation [12]. Intensive efforts have been aimed at using anti-Ras strategies to inhibit oncogenic Ras in different cancers. Interestingly, screens with anti-Ras effect can be identified as Ras inhibitors with clinical function in cancer treatment in the future. Previous studies have provided significant support that Ras can induce growth and progression of breast cancer [13]. In the current study, the aim was to evaluate the cytotoxic and anti apoptotic effects of PCA and to determine their possible cell death properties on breast carcinoma cancer cells. Furthermore,

for the first time we investigated whether PCA can change the Ras and signaling pathways stimulated by Ras in human breast cancer cell lines via its effect on the rate of Ras gene expression.

4. Materials and Methods

4.1. Assay of Cell Viability Using the MTT Method

4.2. Cell Viability Assay Using MTT Method

The viability of the cells was assessed utilizing the mitochondria enzyme dependent reaction of 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In this assay, metabolic active cells deactivated the yellow tetrazolium salt MTT producing purple coloured formazan crystals. The resulting formazan was dissolved in DMSO and the absorbance determined spectroscopically at a wavelength of 570 nm. MTT was solubilized in phosphate buffered saline (PBS, pH 7.2) to a final concentration of 5 mg/mL. This solution (20 μ L) was added to each well and incubated for 4h. There after the supernatant from each well was cautiously removed, and DMSO (200 μ L) added to each well. The formazan produced was quantified spectroscopically by measuring the absorbance of the solution at a wavelength of 570 nm utilizing a micro titer plate reader (Bio-Tek SX2, Winooski, VT, USA). For the spectroscopic assay a test wavelength of 570 nm and a reference wavelength of 690 nm were utilized. Optical Density (OD) was determined by subtracting the absorbance reading at the reference wavelength from the absorbance reading at the test wavelength. The resulting data were expressed as a percentage of the control. Percentage growth inhibition and cell viability were calculated using Eqs. 1 and 2,

$$\text{Cell inhibition (\%)} = 100 - \{(A_t - A_b) / (A_c - A_b)\} \times 100 \quad (1)$$

$$\text{Cell viability (\%)} = \{(A_t - A_b) / (A_c - A_b)\} \times 100 \quad (2)$$

Where A_t is the absorbance of the test compound, A_b is the absorbance of the blank, and A_c is the absorbance of the control.

4.3. LDH Enzyme Assay

Lactate Dehydrogenase (LDH), a soluble cytosolic enzyme, is released upon loss of membrane integrity due to apoptosis. LDH activity can therefore be utilized to determine cell membrane integrity and serves as a means of assessing cell viability by measuring plasma membrane permeability (Haslam *et al.* 2000). The LDH activity in the cell supernatant was analyzed after centrifugation at 3000 g for a duration of 5 min and the LDH activity measured utilizing a LDH Assay Kit (Zist Chem, Tehran, Iran) based on the manufacturer's protocol. In the initial step of the reaction, LDH catalyzes the reduction of NAD^+ to NADH and H^+ through the oxidation of lactate to pyruvate. In the following step, diaphorase utilizes the newly formed

NADH and H^+ for the catalytic reduction of a tetrazolium salt (INT) to an intensely colored formazan, which absorbs strongly at 490nm to 520nm. The resulting data were expressed as a percentage of the control samples.

4.4. Caspase3 Enzyme Assay

The Caspase-3 assay was performed based on the double-antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA) to determine the level of cysteine 1 aspartate specific proteinases 3 (CASPASE-3) in treated MCF7 cells using human Cysteiny1 Aspartate Specific Proteinases 3(CASPASE-3) ELISA Kit (Hangzhou East-biopharm Co., LTD). Briefly, after treatment, cells were spun and cell pellets lysed with lysis buffer, followed by sample addition (40 μ l), and then both Caspase-3-antibody (10 μ l) and Streptavidin-HRP (50 μ l) were added. The plate was sealed with the provided membrane, with gentle agitation and incubation at 37 °C for 60 minutes after washing with 30x wash buffer. HRP substrate solution A (50 μ l) and HRP substrate solution B (50 μ L) were added to all wells. The reaction mixture was incubated for 10 min at 37 °C in the dark on a shaker. Stop solution (50 μ l) was added to all wells to terminate the reaction and the absorbance measured at 450nm using a plate reader (Bio-RAD Model 680). A standard calibration curve was prepared and the linear regression equation generated to calculate sample concentration.

4.5. RNA Preparation

Total RNA was extracted from MCF7 cells using Cinagen total RNA isolation kit based on the manufacturer's procedure (Cinagen, IRAN). Samples were suspended in dimethyl pyrocarbonate treated water and quantified by nanodrop spectrophotometry at a wavelength of 260 nm (Eppendorf, Hamburg, Germany). RNA with an optical density absorption ratio (OD260nm/OD280nm) of between 1.8 and 2.0 was used for Reverse Transcription (RT) reaction. Genomic DNA was eliminated by treatment of the isolated RNA (1 μ g) with 2 units of DNase I (Fermentas Inc, Vilnius, Lithuania).

4.6. Reverse Transcription – Polymerase Chain Reaction

Reverse transcription was performed in a final volume of 20 μ l by utilizing an AmpliSence cDNA synthesis kit (AmpliSens Enterovirus-Eph, Russia) based on the manufacturer's recommendations. PCR reactions were conducted in a 25 μ l reaction utilizing Taq DNA polymerase (Cinagen Co, Iran) and a thermal cycler (Eppendorf Mastercycler, Hamburg, Germany). The actual primers (Macrogen, Seoul, South Korea) utilized for gene amplification is shown in (Table 1). Thermal conditions for gene amplification were 35 cycles that consisted of denaturation at 95°C for 1 min, annealing at 58-60°C for

1 min, extension at 72°C for 1 min, with an initial denaturing step at 95°C for 10 min and final extension at 72°C for 10 min. Confirmation regarding the expression of the genes studied in MCF7 cells was obtained by visualizing the PCR product by agarose gelelectrophoresis (1%).

4.7. Real Time PCR

To determine the levels of Ras and apoptotic gene expression in MCF7 cells treated with PCA, quantitative real-time PCR (qRT-PCR) was conducted using the ABI Step One plus real-time PCR detection system (ABI plus; Applied Biosystems, USA), and qPCR™ Green master kit for SYBR Green I® (Applied Biosystems, USA). The relative level of expression of gene transcripts was compared to the housekeeping gene, GAPDH. The actual primers (Macrogen, Seoul, South Korea) utilized for amplification of the genes were designed using Beacon Designer 7.1. Sequences of the primers utilized for gene amplification are listed in (Table 1). Real time PCR reactions were conducted utilizing the settings: Pre-incubation at 95°C for 5 min followed by 40 cycles at 95°C for 15 seconds and 45 seconds at 58-60°C in 45 cycles. Reactions were performed in triplicate. As negative control, a reaction without cDNA was conducted in parallel. Relative quantification was assessed according to the comparative $2^{-\Delta\Delta Ct}$. For the analysis of qRT-PCR results that were based on the $\Delta\Delta Ct$ method, StepOne™ software was utilized. The result of the gene expression was given by a unit less value based on the formula $2^{-\Delta\Delta Ct}$. Validation of the assay to check the primers of 6 genes showed similar amplification efficiencies as was previously described [14].

4.8. Apoptosis Study-Annexin V/Propidium Iodide

An annexin V apoptosis detection fluorescein isothiocyanate (FITC) kit (eBioscience, 88-8005-72) was utilized in the experiment. Briefly, after treatment, the cells were harvested and rinsed with 1 \times PBS, followed by digestion with Accutase (Gibco, A11105-01) at 37°C for 7 min. These were centrifuged at 300 \times g for 4 min and the resulting suspension discarded. Cells were washed once in 1 \times PBS, and once in binding buffer. Cells were resuspended in 1 \times binding buffer (100 μ l) at 5×10^6 /ml. FITC-conjugated Annexin V (5 μ l) and propidium

Table 1: Selected apoptotic related genes used in Real time PCR.

Gene	Accession number	Product size	Forward primer sequence	Reverse primer sequence
Bax	N M - 001291428	151	CCCGAGAGGCTCTTTTTC	GCCTTGAGCACCAGTTTG
BCL ₂	NM-000633	164	GCTCTAAATCCATCCAG	CCTCTCCATCATCAACTT
Caspase 3	N M - 004346.3	158	TGGTTCATCCAGTCGCTTTG	CATTCTGTTGCCACCTTTCG
KRas	N M - 004985.4	136	TAGGCAAGAGTGCCCTGACG	CCTCTTGACCTGCTGTGTCG
HRas	N M - 001130442.1	128	GAGGCTTCCTGTGTGTGT	GTCACACTGTTCCACCA
GAPDH	N M - 001256799.2	146	CCCATCACCATCTCCAGGAGC	CCAGTGAGCTTCCCGTTCAGC

Gene used for normalization.

iodide (5 μ l) were added to the cell suspension (100 μ l) and incubated for 15 min at RT. The reaction was terminated by centrifugation and the suspension removed. Cells were washed in 1 \times binding buffer, and the cells resuspended in 200 μ l of 1 \times binding buffer and analyzed by flow cytometry within 4h.

4.9. Statistical Analysis

Data analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). Two-way Analysis of Variance (ANOVA) and general linear model was fit to determine the effect of various concentrations of PCA and incubation times on each variable. One-way ANOVA was used to test differences between various means (post hoc analysis LSD test). All experimental data were presented as the mean \pm SD. The level of significance for all tests was set at $p < 0.05$.

5. Results

5.1. Cytotoxic Effects of PCA on MCF7 Cells

5.2. MTT and LDH Assay

The cytotoxic effects of PCA on the growth of MCF7 cells as determined by MTT assays are shown in (Figure 1). The treated cells with PCA when compared to the untreated control cells displayed a significant decrease in viability. Besides, treatment of MCF7 cells with PCA demonstrated cell growth inhibition in a dose dependent manner. At greater concentrations more cytotoxicity was detected. Evaluation of the cytotoxicity data revealed that the IC_{50} (dose required for 50% inhibition) of PCA on MCF7 cells was 40 mM for 24 hours ($p < 0.05$). The IC_{50} values powerfully supported the higher cytotoxic activity of PCA on MCF7 (Figure 1 (A-B)). The LDH enzyme leakage assay was also performed to test the given response after treatment with various concentrations of PCA. Similarly to the MTT assay, the LDH assay results displayed a dose dependent decrease in viability of MCF7 cells at 24 hours ($p < 0.05$) (Figure 2). The highest leakage of LDH was displayed in 37-70 mM PCA treatment.

Of note is that both bioactivity assays revealed PCA toxicity against the MCF7 cell line, regardless of their differences in mechanism. In the MTT assay, only the live cells decreased or reduced the MTT salt to purple formazan by the mitochondrial succinate dehydrogenase enzyme, while, in the LDH assay, the LDH enzyme results from distortion of cell membrane integrity in the culture medium.

5.3. Caspase-3 Assay

(Figure 3) shows the level of Caspase-3 protein in the MCF7 cell line treated with PCA based on ng per cell. Caspase-3 rates were

significantly increased in MCF7 cells treated with PCA at doses between 10-300 mM in comparison to untreated cells. Maximal Caspase-3 activity was detected in cells treated with 150 mM PCA (Figure 3) ($p < 0.05$).

5.4. Gene Expression Study

The degree of gene expression in MCF7 cells after they were treated with PCA was compared with the control samples (untreated MCF7 cells) and the results expressed as fold change. The fold changes of H-Ras in MCF7 cells treated with PCA at doses between 75-150 mM in relation to untreated cells decreased from 1.0032 to 0.268 and 0.460 at 24 hours, respectively ($p < 0.05$) (Figure 4A). The level of K-Ras expression was lesser in a dose-dependent manner in MCF7 cells, 24 hours after exposure to PCA. Minimum K-Ras expression was found in cells that were treated with 150 mM PCA. K-Ras expression showed significant difference at 300 mM PCA as compared to the control treatment (Figure 4B) ($p < 0.05$).

An increase in fold change of proapoptotic gene, Bax, from 1.015 at untreated cells to 2.367, 5.598, 8.565, 3.471 and 4.441 at 24 hours was observable in the treated MCF7 cells at doses of 10 to 300 mM respectively, with the greatest Bax expression being found in cells that were treated with 75 mM PCA (Figure 4C) ($p < 0.05$). The fold change of antiapoptotic gene, Bcl-2, was however significantly reduced from 1.006 for untreated cells to 0.746 and 0.515 at doses of 75 to 150 mM respectively. The most reduction of Bcl-2 expression was shown in cells treated with 150 mM PCA (Figure 4D) ($p < 0.05$). The Bax/Bcl-2 ratios of the mRNA levels are recorded in (Figure 4E). The ratios were increased towards the doses of 37 to 150 mM in comparison to untreated cells. As (Figure 4E) shows, the highest ratio was observed for 150 mM PCA (Figure 4E) ($p < 0.05$). The fold changes of Caspase 3 in treated MCF7 cells also increased significantly in doses of 75 to 300 mM. The most expression of Caspase 3 was detected in cells treated with 150 mM PCA (Figure 4F) ($p < 0.05$).

5.5. Annexin V/Propidium Iodide Study

A significant difference ($p < 0.05$) was observed in cell viability, early apoptosis, and late apoptosis of MCF7 cells after treating with PCA for 24 hours (Figure 5). The incubation of MCF7 with 10 mM PCA (24 hours) decreased cell viability to 60.45 \pm 1.31%, 30.92 \pm 1.8% and 10.98 \pm 2.05% for early apoptosis, late apoptosis and necrosis cells respectively. Increase in PCA concentration to 35 mM, resulted in a further decrease in cell viability to 60.19 \pm 0.13%, 25.85 \pm 1.51 and 15.13 \pm 0.21% of early apoptosis, late apoptosis and necrosis in MCF7 cells, respectively, was identified. A similar decrease in cell viability and increase in apoptotic cells was found in MCF7 cells

after treating with 35 mM PCA with an incubation period of 24 hours. Cells treated with 35 mM PCA showed $60.52 \pm 0.25\%$, $25.82 \pm 1.19\%$, and $15.58 \pm 0.52\%$ of early apoptosis, late apoptosis and necrosis, respectively. Incubation of MCF7 with PCA (75 mM) after 24 hours however resulted in decreased cell viability to $5.51 \pm 0.54\%$ with $85.55 \pm 1.17\%$ and $10.03 \pm 0.14\%$ of early apoptosis, late apoptosis and necrosis, respectively (Figure 5). Higher doses of PCA (150 mM) showed $25.15 \pm 0.12\%$, $65.85 \pm 0.85\%$, and $10.15 \pm 0.10\%$ of early apoptosis, late apoptosis and necrosis, respectively, in MCF7 cells after 24-hour incubation. At the highest dose (300 mM) $70 \pm 1.22\%$, $20 \pm 1.32\%$ and $10 \pm 1.15\%$ early apoptosis, late apoptosis and necrosis, respectively, in MCF7 cells was observed after 24-hour incubation.

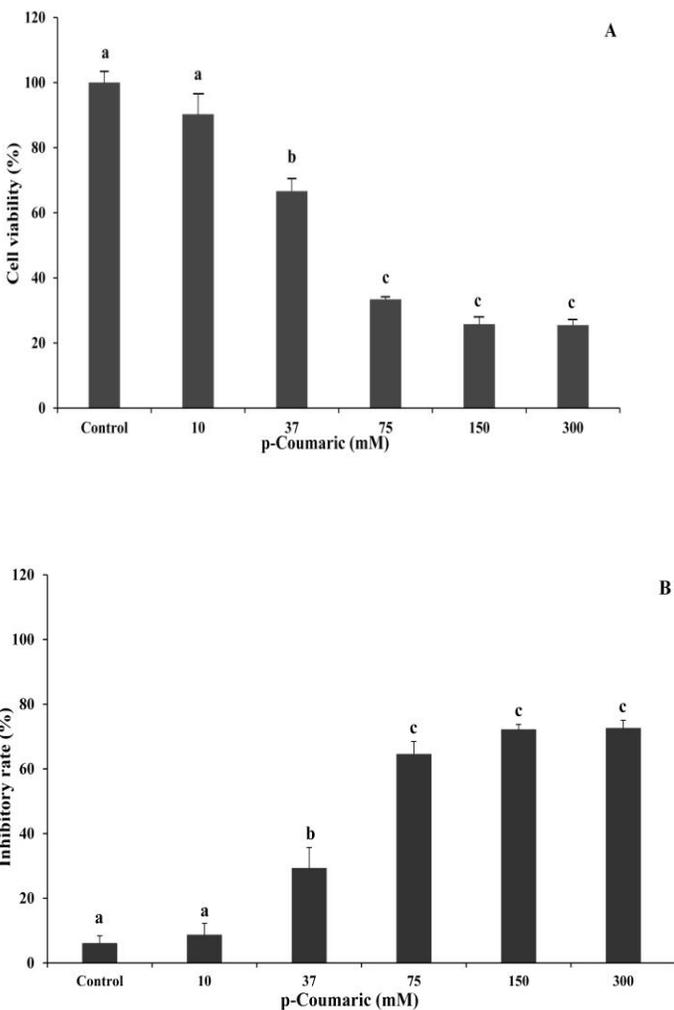


Figure 1: The percentage of growth inhibition and viability of MCF7 cells treated with PCA using the MTT assay. A: The mean percentage of cell viability and B: Inhibitory rate of PCA at various concentrations compared with control group after 24 h treatment. Letters denote significant differences among values at $p < 0.05$.

Only the percentages of early apoptosis, late apoptosis and necrosis in MCF7 cells between the two different concentrations (35-70 mM) were significantly different, indicating that by increasing PCA concentration, viable cells were moderated, and an increased number of cells experienced late apoptosis at 24 hours of incubation. As shown in Fig 6, MCF-7 cells were exposed to various concentrations of PCA (10-300 mM) for 24 hours after preparation with the annexin kit and staining with PI with Annexin V being studied utilizing a florescent microscope. With the increase in the concentration of PCA in a dose-dependent manner, the viability of cells can be reduced. The greatest impact of PCA was observed at concentrations of 150-300 mM that results in significant induced indications of apoptosis. Propidium iodide staining showed increased accumulation of the cells in the last stages of apoptosis or Annexin V+ and Propidium Iodide (PI+) (Figure 6).

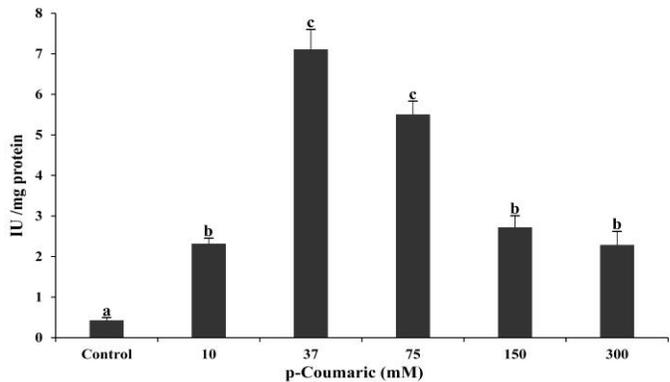


Figure 2: The enzyme activity of LDH in MCF7 cells exposed to PCA. Cells were exposed to 10 and 300 mM PCA for 24 h at 37°C. Results are represented as mean \pm SD for three independent experiments, each with a minimum of three cultures. Letters denote significant differences among values at $p < 0.05$.

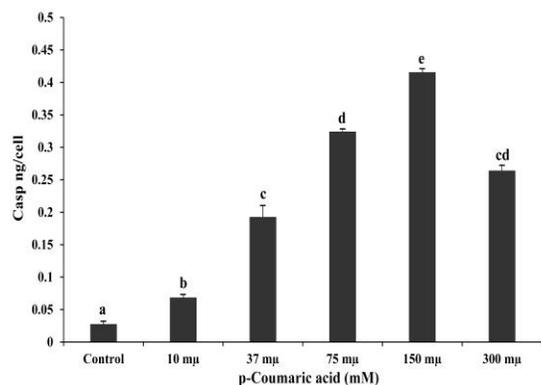


Figure 3: Inhibitory effect of PCA induced caspase-3 in MCF7 cells were treated with 10 and 300 mM PCA for 24 h. Results are represented as mean \pm SD for three independent experiments, each with a minimum of three cultures. Letters denote significant differences among values at $p < 0.05$.

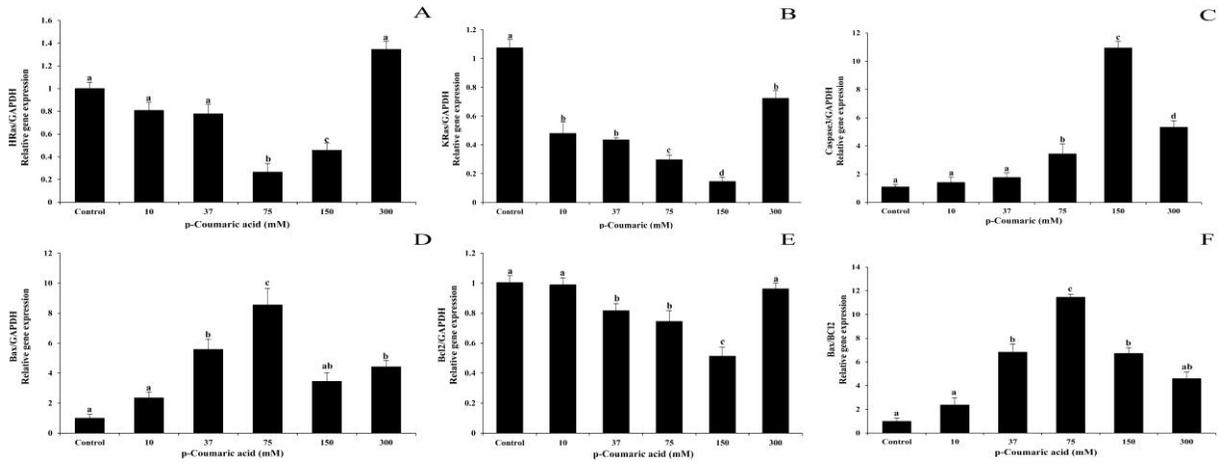


Figure 4: Relative expression of H-RAS, K-RAS, Bax and Bcl2 genes in MCF-7 cells treated with 10 and 300 mM *p*-coumaric acid for 24 h. Results are represented as mean ± SD for three independent experiments, each with a minimum of three cultures. Expression values were normalized to those of GAPDH. Data were expressed as the mean fold difference (mean ± SD). Letters denote significant differences among values at *p* < 0.05.

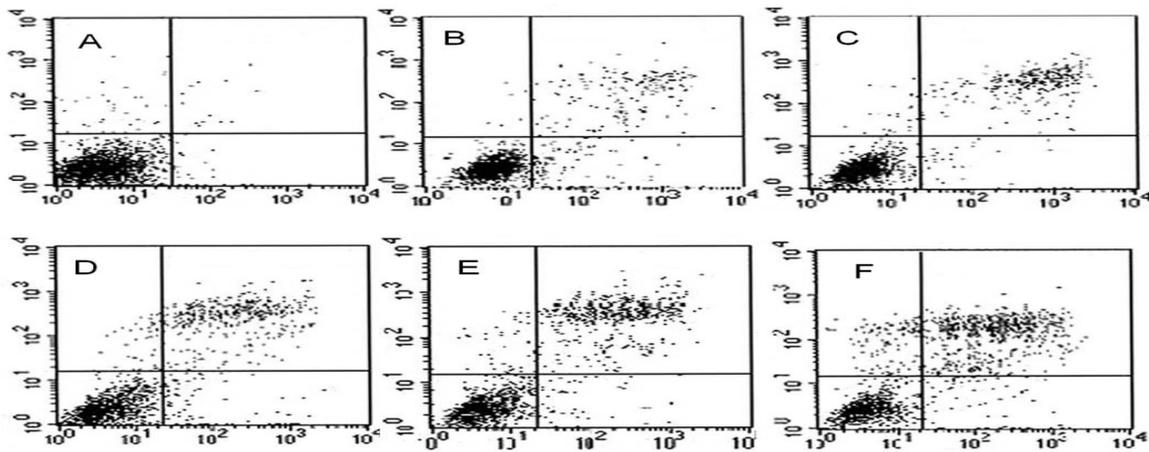


Figure 5: Annexin V FITC/PI staining used to assess the mode of cell death. PCA treated MCF7 cells (a) Control (b) 10 mM, (c) 37 mM, (d) 150 mM, (e) 150 mM and (f) 300 mM for 24 hours showed an increased percentage of apoptotic population after 24 h incubation. The population of early, late apoptotic and necrosis cells in the control group was found to be lower compared with experimental groups. Living cells or Annexin- V/FLUOS (-)/PI (-) [LL] are seen in the lower left quadrant. Cells that are Annexin V/FLUOS (+)/PI (-) [LR] are apoptotic (lower right). The cell population with Annexin V/FLUOS (+)/PI (+) [UR] has been described as necrotic or advanced apoptotic (upper right) and Annexin V/FLUOS (-)/PI (+) [UL] may be bare nuclei cells in late necrosis, or cellular debris (upper left).

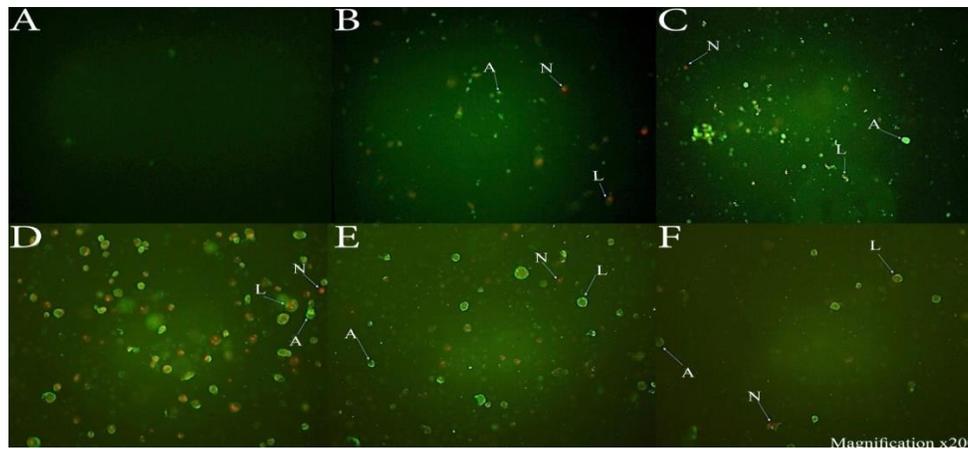


Figure 6: The results of fluorescent microscopic study MCF7 cells treated with (a) Control (b) 10 mM, (c) 37 mM, (d) 150 mM, (e) 150 mM and (f) 300 mM for 24 hours after staining with PI and Annexin V. Living cells or Annexin- V/FLUOS (-)/PI (-) [LL] are without color. Cells that are necrosis are red, Annexin V/FLUOS (-)/PI (+) [LR]. Early apoptosis cells are green, Annexin V/FLUOS (+)/PI (-) [UR] and late apoptotic cell are green and red Annexin V/FLUOS (-)/PI (-) [UL].

6. Discussion

The current study evaluated the effectiveness of PCA on the apoptosis process in MCF7 cells and changes in gene expression involved in apoptosis. The effects of antioxidants from herbal phenylpropanoids on cancer cell are of significant interest to researchers. The role of these compounds in apoptosis and their impact on oncogenes was evaluated in the present study. It is not yet known whether phenolic compounds of herbal origin can have an effect on oncogenes as Ras genes. To the best of our knowledge, this is the first report on the ability of PCA to inhibit the gene expression of Ras genes. This research has revealed that phenolic compounds in conjugation with their role as antioxidants in cancer cells could also change the expression of genes that are an integral part of the molecular pathway of cancerous cells. Studying the expression of Bax and Bcl2 genes as the index genes in cancer cells and assessment of Caspase 3 activity as a biochemical indicator of apoptosis were deemed necessary to determine the mode of action and mechanism of PCA in our study. In addition, by studying Caspase activity, one can assess the apoptosis path (internal and external). The viability assay revealed that the IC_{50} of PCA in MCF-7 cells was approximately 40mM. Real time PCR analysis showed that PCA decreased the levels of H-Ras and K-RAS transcript in MCF-7 breast cancer cells. Maximal inhibitory effect of PCA was observed on K-Ras expression at a concentration of 150mM. PCA at a dose of 75-150mM significantly induced apoptosis after 24h treatment, resulting in increased Bax and Caspase 3mRNA levels and late apoptotic cells with Annexin V+ and Propidium Iodide (PI+) characteristic features based on the incidence of protein bands associated with Annexin. It also inhibited the relative expression of antiapoptotic gene; Bcl2 in treated cells. An evaluation of the ratio of gene expression of Bax/bcl2 as an indicator of the apoptosis status revealed that PCA at a dose of 37-150 mM leads to an increase in this ratio that represents the progress of apoptosis in treated cancer cells. Overall, a survey of the data showed that PCA is one of the most abundant herbal ingredients used in human nutrition. PCA reduced gene expression of H-Ras and K-Ras which is one of the most critical growth factors in cancer cells and mutations in the occurrence of many tumors. Our findings are similar to Jagathan et al. who investigated the influence of PCA on human colorectal carcinoma cells. HCT 15 cells treated with PCA gave rise to an accumulation at the G1 phase of the cell cycle. In this study it was observed that there was growth inhibition by PCA on treated HCT 15 cells which is due to ROS generation and a plunge in mitochondrial membrane potential. These data were supported by Propidium iodide and YO-PRO-1 staining and photomicrograph

and scanning electron microscope observations [15]. PCA also has an antiproliferative effect on Caco-2 and MCF7 cells [16, 17]. Mitochondrial malfunction is one of the earlier events of apoptosis and is induced in PCA treated cancer cells mediated by ROS generation [18]. Some other phenolic phytochemicals such as EGCG and Resveratrol could improve p53 activation by the Ras/MAPK kinase/MAPK pathway. Data from this research shows that PCA resulted in the up-regulation of Bax and down-regulation of Bcl2 in treated cells and that both of them are affected by p53 activation in the apoptosis process [19,20]. Nakayama et al. showed that phenolic components such as cinnamic acid, have inhibitory effects on DNA fragmentation initiated by hydrogen peroxide stress in V79 cells [21]. Enhanced expression of Bax, Caspase-8, Caspase-9 and Fas genes and reduction in Bcl-2 and Bcl-xL gene expression in myocardial cells treated with PCA has been reported [22]. Caspase 9 is an initiator Caspase that induces the stimulation of effector Caspases, which includes Caspase 3 and Caspase 7 [23, 24]. The activation of Caspase 9 supports the data that was obtained from HT-144 cells that were treated with cinnamic acid. Micronuclei resulted from chromosomal breakage and/or whole chromosomal loss in NGM and HT-144 cells treated with phenolic components [22]. Researchers following the discovery of anticancer drugs postulate that inhibition of Ras proteins may be due to compounds having the ability to remove K-Ras from the plasma membrane as Ras proteins are thought to be fixed to the plasma membrane [25].

Use of herbal phenolic compounds on cancer cells showed that some of them including quercetin, can induce specific functions of K-Ras-p53 such as suppressing K-Ras-induced cancer [26]. Huang in 2013 proved that quercetin prevents mTOR signaling by stopping PI3K and Ras activity, activating AMPK, and up regulating TSC1 [27]. Currently scientists have studied some molecular pathways, as well as the PI3K/AKT/mTOR pathway for the treatment of cancer and anti-cancer drug design. The PI3K/AKT/mTOR pathways play a critical role in the malignancy of human tumors and their resulting growth, proliferation, and metastasis [28]. Bharate et al in 2012, reviewed 157 natural products in order to study their capability to regulate K-Ras post-translational targets. They showed that the polycarboxylic class of natural products has more potential as anticancer agents through change of K-Ras signaling [29]. Researchers recommend that compounds possessing polyphenol hydroxyl, a new source in the Mevalonate pathway, can inhibit the function of Ras proteins in Human Solid Tumor Cell Lines as a novel source of FPTase inhibitors. Chen et al proposed that the anticancer effect of the phenolic compound, Salvia miltiorrhiza derivative, may be connected to prevention of P21 Ras membrane association and augmentation of gap junction intercellular

communication [30].

7. Conclusion

Our results suggest that although PCA may be applicable for *in vitro* toxicity, research in human breast cancer MCF-7 cells through engaging in the apoptosis pathway could also change the outcome of gene and protein expression. PCA inhibited the growth of MCF7 cells, with the optimum concentration of PCA being 75-150mM. This finding suggests that PCA can affect the activity of some critical enzymes utilized in cancer therapy. Our results propose that PCA may be a new source which may prove valuable in the search for potential inhibitors of Ras oncogene function as anti-Ras drugs. However, due to limited bioavailability and pharmacokinetics, innovative formulations or chemical alterations of PCA are required for clinical cancer treatment.

8. Conflict of Interest

The authors report no declarations of interest.

9. Acknowledgements

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References

- Kazemi A, Ramezanzadeh F, Nasr-Esfahani M, Saboor-Yaraghi A, Nejat S, Rahimiforoshani A. Impact of environmental tobacco smoke exposure in women on oxidative stress in the natural follicle and assisted reproduction outcomes. *J Res Med Sci.* 2013; 18: 688-94.
- Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C et al. GLOBOCAN 2012 v1.1, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2014.
- Scharl A, Kuhn T, Papatthemelis T, Salterberg A. The right treatment for the right patient—personalised treatment of breast cancer. *Geburtshilfe Frauenheilkd.* 2015; 75: 683-91.
- Safarzadeh E, Shotorbani SS, Baradaran B. Herbal medicine as inducers of apoptosis in cancer treatment. *Adv Pharm Bull.* 2014; 4: 421-7.
- Jafarian-Dehkordi A, A--Emami S, Saeidi M, Sadeghi H. Cytotoxicologic Studies of the Extracts of Iranian *Juniperus sabina* and *Platycladus orientalis* on Cancer Cells. *J Res Med Sci* 2004; 9: 205-9.
- Pei K, Ou J, Huang J, Ou S. p-Coumaric acid and its conjugates: dietary sources, pharmacokinetic properties and biological activities. *J Sci Food Agr.* 2016; 96:2952-62.
- Wong RS. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res.* 2011; 30: 87.
- In LL, Azmi MN, Ibrahim H, Awang K, Nagoor NH. 1' S-1'-acetoxyeugenol acetate: a novel phenylpropanoid from *Alpinia conchigera* enhances the apoptotic effects of paclitaxel in MCF-7 cells through NF- κ B inactivation. *Anticancer Drugs.* 2011; 22: 424-34.
- McCormick F. K-Ras protein as a drug target. *J Mol Med.* 2016; 94: 253-8.
- McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Montalto M, Cervello M et al. Mutations and Deregulation of Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR Cascades Which Alter Therapy Response. *Oncotarget.* 2012; 3: 954-87.
- Hueber AO, Evan GI. Trap to catch unwary oncogenes. *Trends Genet.* 1998; 14: 364-7.
- Eckert LB, Repasky GA, Ulku AS, McFall A, Zhou H, Sartor CI et al., Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res.* 2004; 64: 4585-92.
- Gysin S, Salt M, Young A, McCormick F. Therapeutic Strategies for Targeting Ras Proteins. Santos E, ed. *Genes Cancer.* 2011; 2: 359-72.
- Livak KJ. ABI Prism 7700 sequence detection system. Foster City, CA: User bulletin 2. PE. Applied Biosystems. 1997; 2: 1-36.
- Jaganathan SK, Supriyanto E, Mandal M. Events associated with apoptotic effect of p-coumaric acid in HCT-15 colon cancer cells. *World. J. Gastroenterol.* 2013; 19: 7726-34.
- Janicke B, Onning G, Oredsson SM. Differential effects of ferulic acid and p-coumaric acid on S phase distribution and length of S phase in the human colonic cell line Caco-2. *J. Agric. Food Chem.* 2005; 53: 6658-65.
- Kolahi M, Tabandeh M, Saremy S, Hosseini S, Hashemitabar M. The study of apoptotic effect of p-coumaric acid on breast cancer cells MCF-7. *JSSU.* 2016; 24: 211-21.
- Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H.

- et al., Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell*. 2006; 10:241-52.
19. Chen C, Yu R, Owuor ED, Kong AN. Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch Pharm Res*. 2000; 23: 605-12.
 20. Shih A, Davis FB, Lin HY, Davis PJ. Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *J Clin Endocrinol Metab*. 2002; 87: 1223-32.
 21. Nakayama T, Yamada M, Osawa T, Kawakishi S. Inhibitory effects of caffeic acid ethyl ester on H₂O₂-induced cytotoxicity and DNA single-strand breaks in Chinese hamster V79 cells. *Biosci Biotechnol Biochem*. 1996; 60:316-8.
 22. Prince PS, Roy AJ. p-Coumaric acid attenuates apoptosis in isoproterenol-induced myocardial infarcted rats by inhibiting oxidative stress. *Int J Cardiol*. 2013; 168: 3259-66.
 23. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Ann Rev Cell Dev Biol*. 1999; 15: 269-90.
 24. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 1997; 91: 479-89.
 25. Cho KJ, van der Hoeven D, Hancock JF. Inhibitors of K-Ras plasma membrane localization. *Enzymes*. 2013; 33 Pt A: 249-65.
 26. Lee SJ, Jung YS, Lee SH, Chung HY, Park BJ. Isolation of a chemical inhibitor against K-Ras-induced p53 suppression through natural compound screening. *Int J Oncol*. 2009; 34: 1637-43.
 27. Huang S. Inhibition of PI3K/Akt/mTOR signaling by Natural Products. *Anticancer Agents Med Chem*. 2013; 13: 967-70.
 28. Mabuchi S, Kuroda H, Takahashi R, Sasano T. The PI3K/AKT/mTOR pathway as a therapeutic target in ovarian cancer. *Gynecol Oncol*. 2015; 137: 173-9.
 29. Bharate SB, Singh B, Vishwakarma RA. Modulation of k-Ras signaling by natural products. *Curr Med Chem*. 2012; 19: 2273-91.
 30. Chen XG, Shuzo O, Li Y, Han R. Inhibition of farnesyl protein transferase, H-ras oncogene expression and P21ras membrane association by natural products in human solid tumor cell lines. *J Asian Nat Prod Res*. 1998; 1: 29-51.