Article

Exploring the Anti-Cancer Properties of Pomegranate Peel Aqueous Extract

Carla Luís [1,2,3,4], André P. Sousa [1,2,3,4], Raquel Costa [2], Ana T. Maduro [1,2], Patrick J. Pais [3,4,5], Sara Sá [1,2,3,4], Álvaro Gestoso [3,4], Flávia Fernandes [6], Eliana Jerónimo [6,7], Raquel Soares [1,2], Ruben Fernandes [2,3,4,8], Pilar Baylina [2,3,4,9] and Maria F. Duarte [6,7,*]

1 Biochemistry Unit, Biomedicine Department, FMUP—Faculty of Medicine of the University of Porto, 4200-450 Porto, Portugal; carlaluis@med.up.pt (C.L.); andre.mp.sousa2@hotmail.com (A.P.S.); teresamaduro98@gmail.com (A.T.M.); saravsa@ufp.edu.pt (S.S.); raqsoa@med.up.pt (R.S.)
2 I3S—Instituto de Investigação e Inovação em Saúde, 4200-135 Porto, Portugal; raquel.costa22@gmail.com (R.C.); ruben.fernandes@ufp.edu.pt (R.F.); pilarbaylina@ess.ipp.pt (P.B.)
3 FP-I3ID—Instituto de Investigação, Inovação e Desenvolvimento, FP-BHS—Biomedical and Health Sciences Research Unit, FFP—Fundação Fernando Pessoa, 4249-004 Porto, Portugal; patrickjspais@hotmail.com (P.J.P.)
4 HE-FP—Hospital Fernando Pessoa, CECLIN—Center of Clinical Studies, 4420-096 Gondomar, Portugal
5 ECVA—School of Life and Environmental Sciences, UTAD—University of Trás-os-Montes and Alto Douro, 5000-801 Vila Real, Portugal
6 Alentejo Biotechnology Center for Agriculture and Agro-Food (CEBAL), Instituto Politécnico de Beja (IPBeja), 7801-908 Beja, Portugal; flavia.fernandes01@gmail.com (F.F.); eliana.jeronimo@cebal.pt (E.J.)
7 MED—Mediterranean Institute for Agriculture, Environment and Development & CHANGE—Global Change and Sustainability Institute, CEBAL, 7800-309 Beja, Portugal
8 FCS—Faculty of Health Sciences, Fernando Pessoa University, 4200-150 Porto, Portugal
9 ESS—School of Health, IPP—Polytechnic Institute of Porto, 4200-072, Porto, Portugal
* Correspondence: fatima.duarte@cebal.pt

Abstract: The objective of this work is to evaluate the influence of pomegranate peel extract (PPE) in the behavior of breast cell lines (epithelial and tumor type) and related oxidative metabolism. Fruit-based functional foods have been the target of increasing scientific research for their physiological and pathophysiological properties. Pomegranate (*Punica granatum*) is a suitable example with both prophylactic and medicinal effects. MCF-7 cell line from tumor breast carcinoma, and MCF-10A cell line from normal epithelial mammary gland were used and subjected to different concentrations of PPE, ranging from 1 to 5 mM of gallic acid equivalents (GAE). Viability, proliferation, mobility, and cytotoxicity assays were performed along with the quantification of antioxidant enzymes, namely, catalase, superoxide dismutase (SOD) and reduced (GSH) and oxidized (GSSG) glutathione. We observed a decrease in viability and proliferation of MCF-7 cells, at higher concentrations of PPE, with no influence in epithelial cells. Interestingly, in a concentration-dependent manner, PPE triggered a significant decrease in migration on both cell lines, with a more pronounced effect in breast cancer cell line. Regarding antioxidant enzyme activity, on tumor cells higher concentrations of PPE decreased catalase activity and significantly increased SOD activity. Regarding GSH and GSSG, we observed different expression levels between MCF-7 and MCF-10A, with MCF-7 presenting lower levels compared to MCF-10A. GSH/GSSG ratio was notably higher in MCF-7 at 5 mM GAE. PPE exhibits anti-tumor effects without significantly affecting normal epithelial cells. Our work strengthens the potential antitumoral effect of PPE by reducing MCF-7 cell viability and proliferation through the imbalance of antioxidant enzymes.

Keywords: breast cancer; pomegranate peel extract; polyphenols; oxidative metabolism; oxidative stress; antioxidant activity

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1. Introduction

In the last two decades, nutrition as a science has progressed in an exponential way. Food is no longer the provider of nutrients, the energy workers of life sustaining functions of body growth and homeostasis, but rather one fundamental cornerstone in the complex network mechanism of the human body, both in health and disease [1]. Nutritional science uncovered innumerous research fields with extraordinary impact in human life and disease, like microbiome, food nanotechnology, public health (like preventive intervention against several chronic diseases), and specific nutrition niches like the ones used in sports and in clinical environment. One emerging field of nutrition is associated with functional foods: foods that promote a beneficial effect in the human metabolic functions [2].

Our work focused on pomegranate (*Punica granatum*), a widely consumed fruit with extraordinary health benefits and used since ancient civilizations [3]. Pomegranate plant is composed of different constituents (like the fruit, steams, leaves, flowers, or roots) which exert diverse pharmacologic activities and contain different polyphenol profile [3]. Within the present study, our focus was the external peel, since it represents about 50% of the fruit weight and is usually rejected to waste, regardless of its high levels of polyphenols and strong antioxidant capacity [4]. The family of phenolic compounds is a heterogeneous group of natural antioxidants and chemopreventive agents found in the human diet [5]. The pomegranate peel is mainly composed of gallic acid, epigallocatechin gallate, quercetin, rutin, anthocyanidins, and other flavanols, flavones, and flavanones [3]. Furthermore, the synergetic action of the global components seems to outperform the performance of the individual components when taken separately [6]. Polyphenols from pomegranate peel are involved in many wide-ranging molecular pathways due to their antioxidant, anti-inflammatory, antimicrobial, anti-obesogenic, and anticarcinogenic properties and they interfere in pathologies like diabetes and metabolic syndrome [7], cardiovascular diseases [8], male infertility [9], Alzheimer's [10], and some types of cancer [11]. All these pathologies share a common hallmark in which the polyphenols play an important role: oxidative stress. Oxidative stress and its associated pathways are responsible for the balance of reactive oxygen species (ROS). Excessive accumulation of ROS can lead to DNA damage, amino acid oxidation, protein damage, and lipid peroxidation, which subsequently cause cytotoxicity/dysmetabolism and subsequently cancer [12].

Breast cancer (BC) is frequently found as a model in the literature to investigate the potential protective effect of fruit extracts. Polyphenols have a significant role to play, not only because of the aforementioned mechanisms, which are applicable to all types of cancers, but they may also exert additional hormonal dependent functions like interfering with aromatase enzyme by disrupting estrogen synthesis [13]. Without overlooking that BC is still one of the most common cancers and the most frequent among women, it is a complex, heterogeneous pathology [14] and has been a recurrent research model in how oxidative stress can modulate cancer etiology.

Polyphenol-rich fruits have been frequently used in research as therapeutical agents in breast cancer with positive results [15]. Several studies have already demonstrated a correlation between pomegranate and antitumoral activity [16]. Results from in vitro studies conducted on several cell lines and using different pomegranate constituents have demonstrated its potential to decrease cell growth, proliferation, and modulate the inflammatory response. This suggests that pomegranate may exert anti-proliferative, anti-metastatic, anti-estrogenic, and pro-apoptotic effects [16].

While numerous studies have examined the outcomes of pomegranate extract, only a limited number have assessed the intracellular cascade linked to antioxidant enzymes. Therefore, our objective was to investigate the impact of pomegranate peel extract on both a tumor cell line and an epithelial breast cell line, as well as to examine the expression of key antioxidant enzymes.

2. Materials and Methods

2.1. Pomegranate Peel Extract (PPE)

Fruits were disinfected for 5 min in a sodium hypochlorite solution (200 mg/L), washed with tap water, dried at room temperature, and peeled manually. Pomegranate byproducts (peel and carpella membranes) were then cryopreserved in liquid nitrogen, and stored at −80 °C.

Afterwards, pomegranate byproducts were grounded in an electric domestic chopper (A320R1, Moulinex, Groupe SEB, Mayenne, France), mixed with distilled water in proportion of 1:1 and placed in ultrasound (Bransonic Ultrasonic 3510E-DTH, Danbury, CT, USA) during 20 min at 40 °C. The mixture was filtrated through four layers of gauze and the liquid phase collected and lyophilized.

2.2. Determination of Gallic Acid Equivalents

The Gallic Acid Equivalents was determined by using Folin–Ciocalteu. Briefly, 200 µL of Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to 100 µL diluted stock Pomegranate Peel Extract (PPE) solution. After vortex, 800 µL of 7.5% sodium carbonate (Sigma-Aldrich, St. Louis, MO, USA) was included in the mixture and stand for 2 h at room temperature in the dark. The absorbance was measured by a spectrophotometer (Multiskan Ascent, Thermo Fisher, Waltham, MA, USA) at 765 nm according to the method used by Ainsworth and Gillespie [17]. Total phenolic content was calculated by interpolation using a calibration curve of gallic acid (Merck, Darmstadt, Germany) dilutions (1 to 2 mM; $y = 0.8286x$; $R^2 = 0.9918$).

2.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Assay

For the free radical scavenging assessment of PPE was used the method based in Hatano and colleagues [18], named DPPH, which undergoes a hydrogen atom transfer reaction mechanism. Briefly, 270 µL of DPPH (Merck, Darmstadt, Germany) solution was added to 30 µL of the standards, extract, and blanks and allowed to react for 30 min at room temperature in the dark with agitation. Ascorbic acid (Merck, Darmstadt, Germany), within the range of 5–1500 µg/mL was used as standard.

For DPPH quantification of the stock solution we used the methodology based on the 96-well plate assay described by Horszwald and Andlauer [19]. In preparation for analysis, 10 mg of DPPH was dissolved in 250 mL of 80% methanol (Merck, Darmstadt, Germany) to create a DPPH solution. This solution was freshly prepared before the analysis. Afterwards, 200 µL of DPPH solution was added to 20 µL of the diluted samples, standard and blanks and allowed to react for 40 min at room temperature in the dark. Trolox (Acros organics, Thermo Fisher, Waltham, MA, USA) within the range from 0.05 to 2 mM was used as standards. The absorbance was measured by a spectrophotometer (Multiskan Ascent, Thermo Fisher, Waltham, MA, USA) at 515 nm. The results were expressed in mM Trolox Equivalents (TE).

2.4. Cell Cultures and In Vitro Experimental Design

Human breast carcinoma cells MCF-7 (ATCC HTB22, American Tissue and Cell Culture, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA), complemented with 10% Fetal Bovine Serum (Invitrogen Life Technologies, Carlsbad, CA, USA), 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA, EUA) and sodium bicarbonate at a concentration of 3.7 g/L (Merck, Darmstadt, Germany).

The non-tumorigenic epithelial MCF-10A cell line (ATCC CRL-10317, American Tissue and Cell Culture, USA) was cultured in DMEM/F12 medium (Gibco Life technologies, Thermo Fisher, Waltham, MA, USA) containing 10% horse serum (Invitrogen Life technologies, Carlsbad, CA, USA), 100 ng/mL cholera toxin (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 10 µg/mL insulin (Sigma-Aldrich, St.

Louis, MO, USA), and 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA, EUA).

Cultured cell cells lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Treatments were performed using the appropriate corresponding medium, without supplementation and in a range from 1 to 5 mM of GAE concentrations from PPE. The assays were carried out in triplicate, each with three replicates.

2.5. Viability Assay (MTT)

MCF-7 and MCF-10A cell lines were seeded at a concentration of 1×10^5 cells/mL and 5×10^4 cells/mL, respectively. Treatment procedures were applied for 24 h. Viability protocol can be consulted in [20]. Assay was performed in triplicate, with 3 replicates. The mean of each trial was calculated and normalized to the control.

2.6. BrdU Proliferation Assay

MCF-7 and MCF-10A cell lines were seeded at a concentration of 1×10^5 cells/mL and 5×10^4 cells/mL, respectively, and cultured with standard treatments for 24 h. To quantify proliferation rate was used a colorimetric Cell Proliferation ELISA, BrdU (Abcam, Cambridge, UK), in accordance with the guidelines provided by the manufacturer. Assay was performed in triplicate, with 3 replicates. The mean of each trial was calculated and normalized to the control.

2.7. Injury Assay

Confluent monolayers of MCF-7 and MCF-10A cells in 24-well cell culture plates were subjected to a controlled wound using a 10 µL pipette tip. The resulting wound dimensions were captured using an inverted microscope (Nikon Instruments Inc., Melville, New York, NY, USA; 100× magnification) immediately after the injury and again 24 h following the treatment. Migrated distance was calculated by measuring the injury width with Image J software version 1.53k (U.S. National Institutes of Health, Bethesda, MD, USA). It was taken two measures from each well, performed in triplicate with 3 replicas. The migrated distance of the control was used as standard in the normalization.

2.8. Cytotoxicity Assay

MCF-7 and MCF-10A cell lines were seeded at a concentration of 1×10^5 cells/mL and 5×10^4 cells/mL, respectively, and cultured with standard treatments for 24 h. Cell secretome was used to quantify lactate dehydrogenase (LDH) with resource to Liquick Cor-LDH 30 kit (Cormay, Warsaw, Mazowieckie, Poland), as per the manufacturer's instructions. Spectrophotometric measurements were carried out employing Multiskan Ascent spectrophotometer (Thermo Fischer, Waltham, MA, USA). The assay was performed in triplicate with 3 replicates for each assay, results were presented by LDH activity (U/L).

2.9. Total Antioxidant Status

Total antioxidant status (TAS) was assessed using the secretome like previously described, using TAS Randox® assay (Randox laboratories, Crumlin, UK). Optical density was carried out on the Multiskan Ascent spectrophotometer (Thermo Fischer, Waltham, MA, USA). Results were presented using the concentration of Trolox, a vitamin E analog, in mM.

2.10. Protein Extraction

Total proteins were extracted using RIPA lysis buffer in triplicate, and each assay was performed with three replicates after a 24 h exposure to PPE treatments. Quantification of total protein was conducted using a BCA kit, specifically the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), following the manufacturer's instructions.

2.11. Catalase Activity Assay

Catalase activity was measured using the protein extraction in a colorimetric kit and the associated instructions (Abcam, Cambridge, UK). The results were obtained in mU/mL of catalase activity. Calculations were performed according to the manufacture instructions.

2.12. Superoxide Dismutase (SOD) Assay

SOD Activity was measured using the protein lysate with a SOD determination kit and the associated instructions (Sigma-Aldrich, St. Louis, MO, USA). The results were obtained according to the kit instructions, in percentage of inhibition of SOD activity.

2.13. Glutathione Assay

Reduced (GSH) and oxidized (GSSG) glutathione was calculated using a glutathione colorimetric assay kit by Biovision (Biovision, Waltham, MA, USA) according to the manufacturer's instructions. Concentration was calculated by interpolation using a calibration curve of GSH ($y = 0.0017x + 0.0113$; $R^2 = 0.9966$) and GSSG ($y = 0.1768x + 0.0095$; $R^2 = 0.9965$).

2.14. Statistical Analysis

The results are presented as the mean ± standard deviation (SD). Data analysis was performed using GraphPad Prism V8.1.2 (GraphPad Software Inc., La Jolla, CA, USA). The Kolmogorov–Smirnov test was conducted to confirm the normality of the data. Variations between samples were assessed using the Bonferroni test. For statistical analysis, an ordinary one-way ANOVA was applied to compare the control group with various concentrations of GAE within the same cell line. Significance was determined at a threshold of $p < 0.05$.

3. Results

3.1. Peel Pomegranate Extract Characterization

After extraction, total phenolic content and antioxidant activity of PPE was assessed. The phenolic content revealed a mean of 111.7 mg of gallic acid equivalents (GAE) per gram of extract. Regarding the antioxidant activity, DPPH method disclosed a mean of 251.3 mg of ascorbic acid equivalents (AAE) per gram of extract with an inhibitory percentage of 80.4 ± 0.90 (Table 1).

Table 1. Total phenolic content and antioxidant activity of PPE.

Parameters	Quantification
Total phenolic compounds (mg GAE/g)	111.7 ± 3.25
DPPH (mg AAE/g)	251.3 ± 7.97
DPPH (% inhibition)	80.4 ± 0.90

All values were presented in Mean ± Standard Deviation. GAE: Gallic Acid Equivalents; AAE: Ascorbic Acid Equivalents.

3.2. Total Phenolic Content and Antioxidant Activity of Stock Solution

To perform the cell-based assays, a soluble stock solution (200 mM) was prepared, and the total phenolic content and the antioxidant activity was once again quantified. Total phenolic content concentration mean was 155.81 mM of GAE, and the antioxidant activity mean was 308 mM of Trolox equivalents (TE) (Table 2).

Table 2. Total phenolic content and antioxidant activity of stock solution of PPE.

Parameters	Quantification
Total phenolic compounds (mM GAE)	1237.9 ± 73.82
DPPH (mM TE)	307.9 ± 3.56

All values were presented in Mean ± Standard Deviation. GAE: Gallic Acid Equivalents; TE: Trolox Equivalents.

3.3. Cell-Based Assays

Although viability and proliferation are two distinct cell characteristics, in our study they both reveal a similar pattern. PPE significantly inhibits both viability and proliferation in a dose dependent manner in tumor cell line MCF-7 with no significant alteration in non-tumor cell line MCF-10A (Figure 1a,b). Additionally, PPE revealed no cytotoxicity, with no release of LDH in cell medium after membrane disruption (Figure 1c).

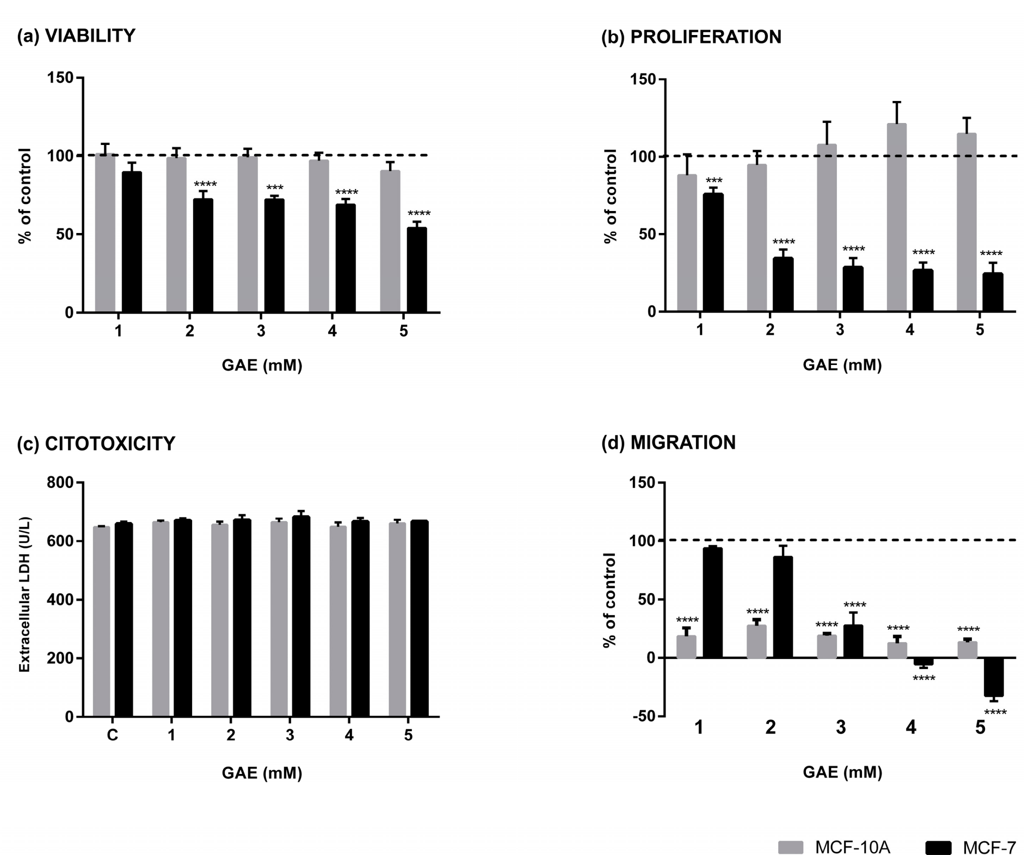

Figure 1. Effect of Pomegranate Peel Extract in cell cultures. (**a**) Viability performed by MTT; (**b**) Proliferation using BrdU assay; (**c**) Cytotoxicity through LDH assay and (**d**) Migration according to the results of the injury assay. Statistical analysis was conducted through ordinary one-way ANOVA, comparing each condition to the control. Significance was set at $p \leq 0.05$. (*** $p \leq 0.001$; **** $p \leq 0.0001$). Legend: GAE—Gallic Acid Equivalents.

In the injury assay we observed a significant decrease in the migration in both MCF-10A and MCF-7 in a dose dependent manner. At concentrations of 4 and 5 mM of GAE, MCF-7 cells exhibited a reversal of the initial damage, indicating a possible anti-metastatic effect. (Figure 1d).

3.4. Oxidative Metabolism

Results from the total antioxidant status assay show that TE were congruently higher in MCF-7 when compared with MCF-10A (Figure 2a). These results suggest that the two cell lines present different oxidative metabolisms.

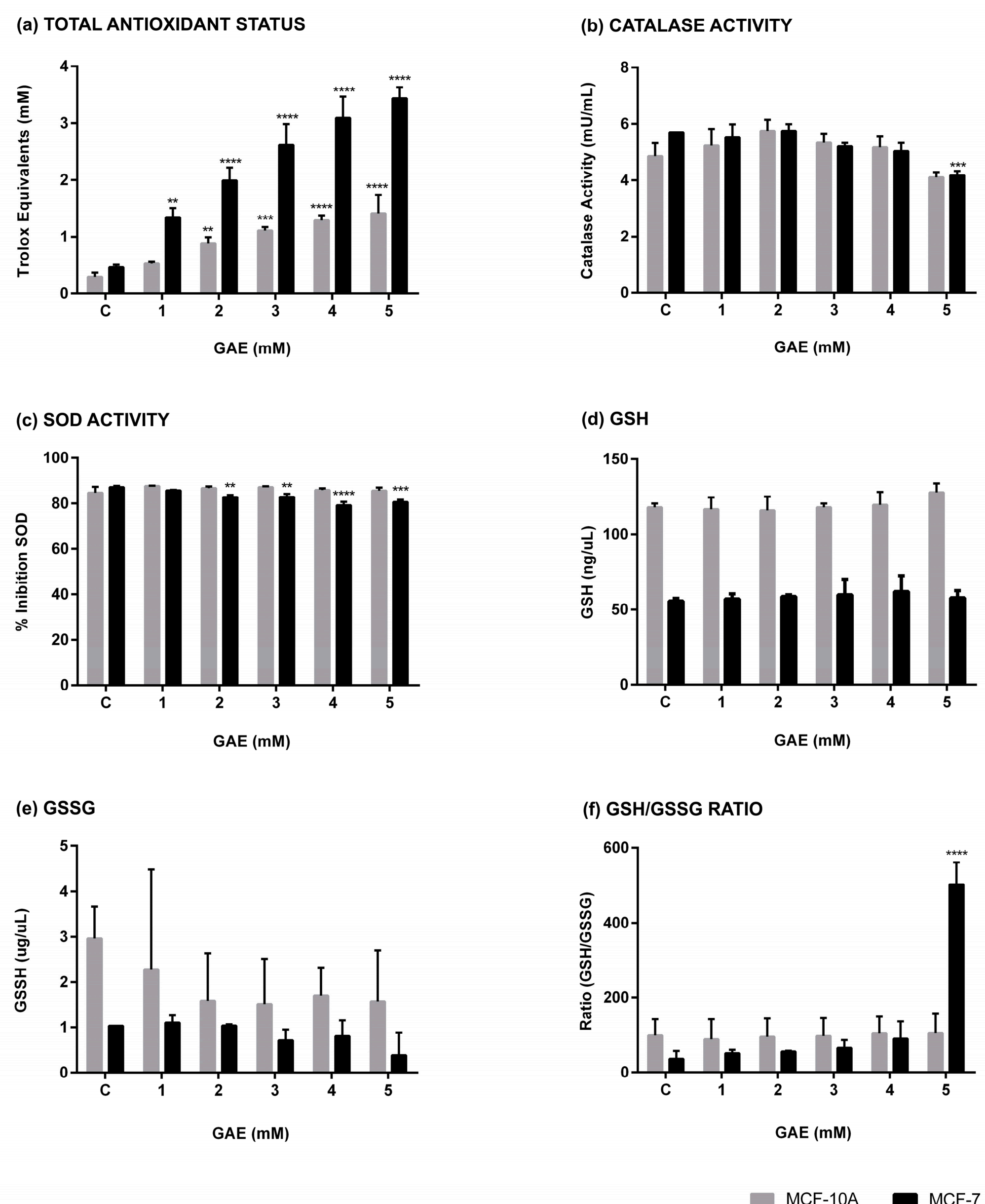

Figure 2. Effect of pomegranate peel extract on oxidative metabolism. (**a**) Total antioxidant status; (**b**) catalase activity; (**c**) SOD activity inhibition; (**d**) reduced glutathione (GSH) quantification; (**e**) oxidized glutathione (GSSG) quantification; and (**f**) GSH/GSSG ratio. Statistical analysis was conducted through ordinary one-way ANOVA, comparing each condition to the control. Significance was set at $p \leq 0.05$. (** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Legend: GAE—Gallic Acid Equivalents.

Catalase was significantly decreased in MCF-7 cell lines at a concentration of 5 GAE (Figure 2b). A decrease in SOD inhibition levels was observed in MCF-7 cells at all concentrations, except in 1 GAE (Figure 2c), meaning that SOD activity is increasing in a dose dependent manner.

Glutathione levels were unbalanced when comparing MCF-7 with MCF-10A even in the control, since both GSH and GSSG levels were much lower in MCF-7 than in MCF-10A, we assumed this was due to the intrinsic tumor metabolism. Although with no significant differences for GSH and GSSG, when we performed the ratio GSH/GSSG, 5 GAE revealed a significant and exponential increase at 5 GAE in tumor cells.

4. Discussion

Some authors preserve the idea that functional foods should be classified only by their preventive character instead of its medicinal potential [21,22]. Pomegranate, considered a functional fruit for its antioxidant capacity, also demonstrates a medicinal role against many non-communicable diseases like diabetes [23] and cancer [24]. The assessment of PPE antioxidant activity assayed by DPPH assay, where the DPPH odd electron of nitrogen atom is reduced by receiving a hydrogen atom from the tested antioxidants, demonstrated that PPE has an effective antioxidant activity as reflected by the inhibition of the free radical DPPH (80.4 ± 0.90).

Pomegranate is a complex fruit, in which each component presents a different nutritional composition, all of them with promising therapeutic results in different types of cancer. Seidi and colleagues evaluated the impact of pomegranate seed extract in different tumor cell lines: MCF-7 (breast carcinoma), PC3 (prostate adenocarcinoma); SKOV3 (ovarian carcinoma) and A549 (lung carcinoma). The results reveal that low concentration of seed extract (starting at 5 μg/mL) induce strong anti-proliferative effects in all cancer cell lines, with a growth inhibition between 78.46% (for A549 cell line) and 85.2% (for SKOV3 cell line) [25]. There have been other studies conducted using pomegranate juice with HT-29 cells (colon cancer cell line) which resulted in a phosphorylation decrease in the NF-kB/p65 subunit and in a 79% suppression of TNFα-induced (COX)-2 protein expressions [26].

These findings imply that the consumption of pomegranate juice, which leads to a decrease in inflammatory markers, may have a significant impact on colon cancer. Pomegranate fruit extract was also evaluated in skin cancer progression. SKU-1064 (skin fibroblasts) cell line was exposed to ultraviolet induced damage when treated with pomegranate extract it was observed that at high concentrations ranging from 500 to 10,000 mg/L, pomegranate fruit exhibited protective effects by reducing NF-κB expression, decreasing caspase-3, and increasing DNA repair in G0/G1 [27]. Focusing on peel extracts, Negi and colleagues demonstrated that peel extract have both antioxidant and antimutagenic effects [28]. Another study by Sadik and Asker corroborated the antitumor effect of peel extract in Ehrlich ascites carcinoma cells (EACC) [29].

A recent academic work assembled information from studies performed in breast cancer regarding extracts of the different pomegranate components [16]. In cell-based studies, results were concomitant and revealed that pomegranate acts as: anti-proliferative, pro-apoptotic, anti-metastatic and antioxidant—all that corroborated by our present findings [16]. Moreover, pomegranate also reveals metabolic functions like anti-estrogenic activity, anti-aromatase, anti-angiogenic, and enhancement of tamoxifen effect [16]. Our cell-based results were concordant with previous studies since a selective decrease was observed in cell viability, proliferation, and migration of tumor cells at higher concentrations of PPE [30–32], with no cytotoxicity for epithelial cells.

To better understand the mechanism behind the anti-tumoral capacity of PPE, we further analyzed the antioxidant enzymatic activity. It is well established that ROS (including singlet oxygen radical, superoxide radical, hydrogen peroxide and hydroxyl radical), not only play an important role in carcinogenesis due to DNA damages but are also considered mediators of central cellular mechanisms like proliferation and apoptosis [33]. Recent studies support that the redox state in tumor cells is so distinctive that modulation of ROS should be considered in the therapeutical strategy [34].

To maintain redox homeostasis, healthy cells take advantage of a robust antioxidant defense system from which we focused in the enzymatic system which includes the catalase, SOD, and glutathione complex system to detox ROS into harmless compounds like water or molecular oxygen. Catalase is responsible for the decomposition of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). SOD catalyzes the dismutation of the superoxide anion (O_2.) into hydrogen peroxide (H_2O_2). Finally, glutathione peroxidase (GPx), glutathione reductase (GR) system is a hydrogen peroxide scavenger and cooperates to detoxify H_2O_2 into H_2O.

Pomegranate appears to be potent antioxidant, since it affects the redox homeostasis, especially in tumor cells. In epithelial cells no significant alterations were observed on antioxidant enzymes, although we observed that the total oxidant status was constantly increasing (with an increase concentration of Trolox Equivalents) revealing the antioxidant capacity (reducing ability) of pomegranate. In tumor cells, homeostatic reducing capacity was also increased but in a more pronounced manner. It is well established that oxidative stress is a cancer hallmark [35]. In breast cancer, ROS are increased, and these byproducts mediate several mechanisms in tumor progression, drug resistance, metastization, and epigenetic and genetic alterations [36]. Our data demonstrated that pomegranate had a greater impact on tumor cells perhaps due to its intrinsic oxidative state. Moreover, the fact that the PPE effects are much more pronounced in breast cancer cells renders them potential agents to target cancer, with no significant (toxic) roles in host cells.

We were not able to clarify whether pomegranate acts upstream to the redox internal system or interacts directly in the enzyme system. It should be expected that with the increasing of a stressor mechanism both SOD, CAT, and GPx would also increase. With GPx increase, GSH would decrease and inversely GSSG should increase. Since it was observed a decrease in CAT and an increase in GSH/GSSG ratio, the cell enzyme system did not seem to be recruited. Since antioxidant capacity was increased one should ask where antioxidant ability came from. Thus, phenolic compounds from pomegranate may have a role in reducing homeostasis. Our observations sustain that the balance of the tumor redox homeostasis promotes tumor regression.

Despite the promising results that pomegranate has been demonstrating, the variables that interfere with the component composition of the fruit must be considered. Pomegranate composition can be modulated not only by external factors like soil, climate, and harvest season [37] but also by individual characteristics such as the microbiome, which can interfere on the pomegranate phytochemical absorption rates as well as in the source of metabolites [38]. Additionally, polyphenol uptake is known to interfere in gut microbiome and vice versa, and the outcomes are not fully understood.

5. Conclusions

Pomegranate is a complex fruit with various components, each having distinct nutritional compositions, all showing promise in treating different types of cancer. This study found that non-toxic levels of PPE significantly reduced viability, proliferation, and migration in MCF-7 breast cancer cells while influencing oxidative status differently in epithelial non-tumoral and cancer cell lines.

To understand the anti-tumoral capacity of PPE, this study also analyzed antioxidant enzymatic activity. ROS play a role in carcinogenesis, and pomegranate appears to modulate redox homeostasis, particularly in tumor cells. In breast cancer, where ROS levels are elevated, pomegranate had a more pronounced impact, possibly due to its intrinsic oxidative state. PPE seems to promote tumor regression by influencing the tumor's redox balance.

Nevertheless, due to the challenges posed by uncontrolled variables, there is still much work to be performed to establish PPE as a viable therapeutic agent. Further research is necessary, alongside with extraction methods standardization to advance our understanding of its potential benefits.

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References

1. Shahidi, F. Nutraceuticals, Functional Foods and Dietary Supplements in Health and Disease. *J. Food Drug Anal.* **2012**, *20*, 78. [CrossRef]
2. Temple, N.J.; Wilson, T.; Jacobs, D.R., Jr.; Bray, G.A. *Nutritional Health: Strategies for Disease Prevention*; Springer: Berlin/Heidelberg, Germany, 2023; ISBN 3031246632.
3. Jurenka, J. Therapeutic Applications of Pomegranate (*Punica granatum* L.): A Review. *Altern. Med. Rev.* **2008**, *13*, 128–144. [PubMed]
4. Gözlekçi, Ş.; Saraçoğlu, O.; Onursal, E.; Özgen, M. Total Phenolic Distribution of Juice, Peel, and Seed Extracts of Four Pomegranate Cultivars. *Pharmacogn. Mag.* **2011**, *7*, 161–164. [CrossRef] [PubMed]
5. Zhang, H.; Tsao, R. Dietary Polyphenols, Oxidative Stress and Antioxidant and Anti-Inflammatory Effects. *Curr. Opin. Food. Sci.* **2016**, *8*, 33–42. [CrossRef]
6. Lansky, E.P. Beware of Pomegranates Bearing 40% Ellagic Acid. *J. Med. Food.* **2006**, *9*, 119–122. [CrossRef]
7. Habtemariam, S. *Medicinal Foods as Potential Therapies for Type-2 Diabetes and Associated Diseases: The Chemical and Pharmaco-Logical Basis of Their Action*; Academic Press: Cambridge, MA, USA, 2019.
8. Haghighian, M.K.; Rafraf, M.; Moghaddam, A.; Hemmati, S.; Jafarabadi, M.A.; Gargari, B.P. Pomegranate (*Punica granatum* L.) Peel Hydro Alcoholic Extract Ameliorates Cardiovascular Risk Factors in Obese Women with Dyslipidemia: A Double Blind, Randomized, Placebo Controlled Pilot Study. *Eur. J. Integr. Med.* **2016**, *8*, 676–682. [CrossRef]
9. Boroujeni, M.B.; Shahrokhi, S.S.; Birjandi, M.; Abbaszadeh, A.; Beyranvand, F.; Hamoleh, S.; Zandbaf, Z.; Gholami, M. Effects of Pomegranate Peel Extract on Histopathology, Testosterone Levels and Sperm of Testicular Torsion—Detorsion Induced in Adult Wistar Rats. *J. Complement. Integr. Med.* **2017**, *14*, 20170009. [CrossRef]
10. Morzelle, M.C.; Salgado, J.M.; Massarioli, A.P.; Bachiega, P.; de Oliveira Rios, A.; Alencar, S.M.; Schwember, A.R.; de Camargo, A.C. Potential Benefits of Phenolics from Pomegranate Pulp and Peel in Alzheimer's Disease: Antioxidant Activity and Inhibition of Acetylcholinesterase. *J. Food. Bioact.* **2019**, *5*, 136–141. [CrossRef]
11. Moghazy, A.M.; Khader, M.S.; Saleh, N. Antitumor and Antimicrobial Activities of Pomegranate (*Punica granatum*) Dried Powder Peel in Vitro. *Curr. Sci. Int.* **2019**, *2019*, 764–775.
12. Visconti, R.; Grieco, D. New Insights on Oxidative Stress in Cancer. *Curr. Opin. Drug Discov. Dev.* **2009**, *12*, 240–245.
13. Ain, H.B.U.; Tufail, T.; Bashir, S.; Ijaz, N.; Hussain, M.; Ikram, A.; Farooq, M.A.; Saewan, S.A. Nutritional Importance and Industrial Uses of Pomegranate Peel: A Critical Review. *Food. Sci. Nutr.* **2023**, *11*, 2589–2598. [CrossRef] [PubMed]
14. Harbeck, N.; Penault-Llorca, F.; Cortes, J.; Gnant, M.; Houssami, N.; Poortmans, P.; Ruddy, K.; Tsang, J.; Cardoso, F. Breast Cancer. *Nat. Rev. Dis. Primers* **2019**, *5*, 66. [CrossRef] [PubMed]
15. Ramos, S. Cancer Chemoprevention and Chemotherapy: Dietary Polyphenols and Signalling Pathways. *Mol. Nutr. Food. Res.* **2008**, *52*, 507–526. [CrossRef] [PubMed]
16. Moga, M.A.; Dimienescu, O.G.; Bălan, A.; Dima, L.; Toma, S.I.; Bîgiu, N.F.; Blidaru, A. Pharmacological and Therapeutic Properties of *Punica granatum* Phytochemicals: Possible Roles in Breast Cancer. *Molecules* **2021**, *26*, 1054. [CrossRef] [PubMed]
17. Ainsworth, E.A.; Gillespie, K.M. Estimation of Total Phenolic Content and Other Oxidation Substrates in Plant Tissues Using Folin—Ciocalteu Reagent. *Nat. Protoc.* **2007**, *2*, 875. [CrossRef] [PubMed]
18. Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two New Flavonoids and Other Constituents in Licorice Root Their Relative Astringency and Radical Scavenging Effects. *Chem. Pharm. Bull.* **1988**, *36*, 2090–2097. [CrossRef]
19. Horszwald, A.; Andlauer, W. Characterisation of Bioactive Compounds in Berry Juices by Traditional Photometric and Modern Microplate Methods. *J. Berry Res.* **2011**, *1*, 189–199. [CrossRef]
20. Luis, C.; Duarte, F.; Faria, I.; Jarak, I.; Oliveira, P.F.; Alves, M.G.; Soares, R.; Fernandes, R. Warburg Effect Inversion: Adiposity Shifts Central Primary Metabolism in MCF-7 Breast Cancer Cells. *Life Sci.* **2019**, *223*, 38–46. [CrossRef]

21. Huggett, A.C.; Schilter, B. Research Needs for Establishing the Safety of Functional Foods. *Nutr. Rev.* **1996**, *54*, S143. [CrossRef]
22. Charalampopoulos, D.; Wang, R.; Pandiella, S.S.; Webb, C. Application of Cereals and Cereal Components in Functional Foods: A Review. *Int. J. Food. Microbiol.* **2002**, *79*, 131–141. [CrossRef]
23. Sohrab, G.; Ebrahimof, S.; Sotoudeh, G.; Neyestani, T.R.; Angoorani, P.; Hedayati, M.; Siasi, F. Effects of Pomegranate Juice Consumption on Oxidative Stress in Patients with Type 2 Diabetes: A Single-Blind, Randomized Clinical Trial. *Int. J. Food. Sci. Nutr.* **2017**, *68*, 249–255. [CrossRef] [PubMed]
24. Zare, M.; Shaverdi, H.; Ebrahimi Vosta Kalaee, S. Anti-Cancer Effects of Pomegranate Seed Oil on Esophageal Cancer Cell Line (KYSE-30). *Gene. Cell. Tissue.* **2021**, *8*, e108995. [CrossRef]
25. Seidi, K.; Jahanban-Esfahlan, R.; Abasi, M.; Abbasi, M.M. Anti Tumoral Properties of *Punica granatum* (Pomegranate) Seed Extract in Different Human Cancer Cells. *Asian. Pac. J. Cancer Prev.* **2016**, *17*, 1119–1122. [CrossRef]
26. Adams, L.S.; Seeram, N.P.; Aggarwal, B.B.; Takada, Y.; Sand, D.; Heber, D. Pomegranate Juice, Total Pomegranate Ellagitannins, and Punicalagin Suppress Inflammatory Cell Signaling in Colon Cancer Cells. *J. Agric. Food. Chem.* **2006**, *54*, 980–985. [CrossRef] [PubMed]
27. Lisbeth, A.; Noratto, G.; Hingorani, L.; Talcott, S.T.; Mertens-Talcott, S.U. Protective Effects of Standardized Pomegranate (*Punica granatum* L.) Polyphenolic Extract in Ultraviolet-Irradiated Human Skin Fibroblasts. *J. Agric. Food. Chem.* **2008**, *56*, 8434–8441. [CrossRef]
28. Negi, P.S.; Jayaprakasha, G.K.; Jena, B.S. Antioxidant and Antimutagenic Activities of Pomegranate Peel Extracts. *Food. Chem.* **2003**, *80*, 393–397. [CrossRef]
29. Sadik, M.S.; Asker, M.M.S. Antioxidant and Antitumor Activities of Pomegranate (*Punica granatum*) Peel Extracts. *World. J. Pharm. Sci.* **2014**, *2*, 1441–1445.
30. Kim, N.D.; Mehta, R.; Yu, W.; Neeman, I.; Livney, T.; Amichay, A.; Poirier, D.; Nicholls, P.; Kirby, A.; Jiang, W.; et al. Chemopreventive and Adjuvant Therapeutic Potential of Pomegranate (*Punica granatum*) for Human Breast Cancer. *Breast. Cancer. Res. Treat.* **2002**, *71*, 203–217. [CrossRef]
31. Dikmen, M.; Ozturk, N.; Ozturk, Y. The Antioxidant Potency of *Punica granatum* L. Fruit Peel Reduces Cell Proliferation and Induces Apoptosis on Breast Cancer. *J. Med. Food.* **2011**, *14*, 1638–1646. [CrossRef]
32. Shirode, A.B.; Kovvuru, P.; Chittur, S.V.; Henning, S.M.; Heber, D.; Reliene, R. Antiproliferative Effects of Pomegranate Extract in MCF-7 Breast Cancer Cells Are Associated with Reduced DNA Repair Gene Expression and Induction of Double Strand Breaks. *Mol. Carcinog.* **2014**, *53*, 458–470. [CrossRef]
33. Waris, G.; Ahsan, H. Reactive Oxygen Species: Role in the Development of Cancer and Various Chronic Conditions. *J. Carcinog.* **2006**, *5*, 14. [CrossRef]
34. Kong, H.; Chandel, N.S. Regulation of Redox Balance in Cancer and T Cells. *J. Biol. Chem.* **2018**, *293*, 7499–7507. [CrossRef] [PubMed]
35. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer. Discov.* **2022**, *12*, 31–46. [CrossRef] [PubMed]
36. Malla, R.; Surepalli, N.; Farran, B.; Malhotra, S.V.; Nagaraju, G.P. Reactive Oxygen Species (ROS): Critical Roles in Breast Tumor Microenvironment. *Crit. Rev. Oncol. Hematol.* **2021**, *160*, 103285. [CrossRef] [PubMed]
37. Melgarejo-Sánchez, P.; Núñez-Gómez, D.; Martínez-Nicolás, J.J.; Hernández, F.; Legua, P.; Melgarejo, P. Pomegranate Variety and Pomegranate Plant Part, Relevance from Bioactive Point of View: A Review. *Bioresour. Bioprocess.* **2021**, *8*, 2. [CrossRef]
38. George, N.S.; Cheung, L.; Luthria, D.L.; Santin, M.; Dawson, H.D.; Bhagwat, A.A.; Smith, A.D. Pomegranate Peel Extract Alters the Microbiome in Mice and Dysbiosis Caused by Citrobacter Rodentium Infection. *Food. Sci. Nutr.* **2019**, *7*, 2565–2576. [CrossRef] [PubMed]

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