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RESEARCH ARTICLE

Dietary supplementation of pomegranate extract alters the *Caspase-3* and *VEGF* promoter epigenetic methylation status and protein levels in breast cancer patients undergoing chemotherapy

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ABSTRACT

Background: Epigenetic modifications like promoter DNA methylation modulate the activity of cancer-associated genes. Pomegranate fruit extract (PE) possesses immense potential in cancer treatment. To the best of our knowledge, the effect of PE on DNA methylation status has not yet been investigated or reported before. **Aim**: to investigate the potential that PE exerts its anti-cancer effects by altering the expression of proapoptotic *Caspase-3* and proangiogenic *VEGF* genes via modulating their promoter methylation status in breast cancer (BC) patients receiving chemotherapy. **Material and Methods**: Bioinformatics analysis followed by Methylation-specific PCR were used to assess the methylation of *Caspase-3* and *VEGF* genes in 25 healthy females receiving 500 mg PE daily, 25 BC patients receiving a chemotherapy regimen (AC 60/600), and 31 BC patients receiving a combination of (AC 60/600) + 500 mg of PE. To investigate if methylation was the major effector on gene expression, protein levels for cleaved Caspase-3 and VEGF were measured by ELISA. Finally, since dietary antioxidants alter the pattern of DNA methylation, serum antioxidant power was measured using the FRAP assay. **Results**: PE dietary supplementation reduced the methylation of *Caspase-3* in patients receiving Chemotherapy + PE (12% to 3%), and increased the caspase-3 protein level by 74.2%. On the other hand, combination treatment slightly enhanced the methylation of the *VEGF* promoter and reduced its protein level by 36.99%. **Conclusion:** This study is the first that reports the modulation of DNA methylation status as a novel anti-cancer epigenetic mechanism of action for PE by pro-apoptotic and anti-angiogenic effects.

Keywords: Breast cancer; Caspase-3; Epigenetics; Pomegranate; VEGF

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INTRODUCTION

Globally, breast cancer (BC) is the most common malignancy in women and is responsible for the majority of cancer-related mortalities. (Siegel et al., 2012; Ferlay J, 2020). BC is the single most prevalent form of malignancy among women in Egypt. It is projected that there will be about 46,000 BC cases by 2050. If considering the mortality/incidence ratios for BC with developed countries, Egypt had nearly doubled the ratio (41% vs. 23%). Furthermore, with 11% death rate in 2020, the disease is already ranked as the 2nd leading cause of cancer-related death in Egypt, following liver cancer (cancer, 2020; Sung et al., 2021; Azim et al., 2023).

Chemotherapy is frequently advised to be used in combination with other therapies against breast cancer to overcome their side effects, these effects can range from transient and moderate to severe and long-lasting (Prieto-Callejero et al., 2020). Resistance to chemotherapy is not only common but also expected after treatment for long periods (Kannampuzha and Gopalakrishnan, 2023).

When apoptosis is disturbed, cancer cells can survive longer, therefore, accumulating further mutations that could increase invasiveness as a tumor grows. Caspase-3 is a cysteine–aspartic acid protease that, in response to specific extrinsic or intrinsic signals cleaves a wide variety of downstream substrates and induces different modes of cell death including apoptosis (Shoshan-Barmatz et al., 2023) pyroptosis, and necrosis (Wang et al., 2017). Several studies reported promising outcomes in the treatment of different malignancies using drugs inducing apoptosis and pyroptosis via induction of Caspase-3 (Suzuki et al., 2015; Boice and Bouchier-Hayes, 2020; Yan et al., 2021).

Another vital hallmark of cancer cells that could be targeted during cancer progression is sustained angiogenesis, which is considered the most crucial in the development and sustenance of metastasis (Lugano et al., 2020). More than a dozen distinct pro-angiogenic proteins have been identified including, the Vascular Endothelial Growth Factor (VEGF) which is considered the most crucial in the regulation of the angiogenesis process (Ghalehbandi et al., 2023). Because they prevent the development of blood vessels that promote tumor growth rather than the growth of tumor cells themselves, anti-VEGF drugs are among the most commonly used anticancer medicines in the clinic today (Albiges et al., 2023).

Today, a green anticancer approach based on chemotherapeutic agents discovered and extracted from medicinal herbs, plants, and fruits is receiving more attention than ever (Abu-Darwish and Efferth, 2018; Ijaz et al., 2018). The pomegranate (*Punica granatum* L.) fruit has been used for the prevention and treatment of a wide range of illnesses for ages in ancient societies (Sharma et al., 2017). Because it contains a significant number of hydrolyzable tannins and anthocyanins, pomegranate possesses potent antioxidant and anti-inflammatory properties (Singh et al., 2023). Several studies have demonstrated that the pomegranate extract (PE), seeds, and juice exerts anti-cancer potential by altering several key signaling pathways controlling cell division and proliferation (Malik et al., 2005; Adhami et al., 2009; Turrini et al., 2015; Amulya et al., 2021).

Deregulation of epigenetic homeostasis can result in altered gene expression and malignant cellular transformation. Global alteration in the epigenetic map is a distinguished feature of cancer (Maleknia et al., 2023). The administration of epi-drugs, either in standalone or in conjunction with immunotherapy or chemotherapy, has demonstrated strikingly favourable outcomes, such as the onset of anti-cancer implications, the reversal of drug resistance, and the improvement of the host's immune system (Lu et al., 2020; Mahmoud et al., 2021).

In general, the main objective of the current study is to enhance anti-cancer pro-apoptosis and anti-angiogenesis actions for adjuvant chemotherapeutic drugs by combination with PE as a dietary supplementation to BC patients during the course of their chemotherapy treatment.

Despite the numerous studies suggesting different anti-cancer mechanisms for pomegranate *in vitro* and *in vivo*, to the best of our knowledge, none has studied the potential effect of PE supplementation on the methylation status of cancer-associated genes. Therefore, this study aimed to investigate the effect of PE supplementation and chemotherapy on the *Caspase-3* and *VEGF* promoter methylation status and protein expression in BC patients receiving adjuvant chemotherapy.

SUBJECTS AND METHODS

Study design and patient recruitment

The current study is a prospective randomized controlled trial (RCT) (Zabor et al., 2020). involving 56 pathologically confirmed newly diagnosed female BC patients and 25 healthy females serving as control. The exclusion criteria included patients with metastatic or recurrent BC, pregnant or lactating women, and Patients with autoimmune diseases. Patients were recruited from those who were admitted to the Cancer Management & Research Department and the Experimental & Clinical Surgery Department, Medical Research Institute, Alexandria University, Egypt.

The study was approved by the local ethical committee of the Medical Research Institute (Code: IORG0008812). All participants signed an informed written consent for participation in the study. Demographic data and medical history were collected from all participants. All BC patients had complete surgical removal of the tumor and were randomly assigned to 2 groups:

The first included 25 patients who received a standard (AC 60/600) regimen (doxorubicin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2 every 21 days for six cycles) (Shulman et al., 2012; Anampa et al., 2015). The second included 31 patients who received a combination of standard (AC 60/600) plus 500 mg daily dose of pomegranate peel extract (Cat # 13567, Puritan's Pride, Inc. Holbrook, New York, U.S.A.) to the end of the 6 treatment cycles (Rastogi et al., 2008; Pantuck et al., 2015). Finally, 25 healthy subjects received the same 500 mg PE daily dose only, for the same treatment duration as in patient's groups, serving as control.

The Tumor-Node-Metastasis (TNM) BC classification system (Rosen and Sapra, 2023) was used for staging BC. This system was published by the American Joint Committee on Cancer (AJCC) (Giuliano et al., 2018) and is based on the size of the original tumor (T), degree of nodal engagement (N), and distant metastasis (M). Based on these parameters, the AJCC has established nine stages for BC (0, IA, IB, IIA, IIB, IIIA, IIIB, IIIC, and IV). The recruited BC patients in the current study were Stage IA (T1= tumor size ≤20 mm, N0 = no regional lymph node metastases, M0 = no evidence of distant metastases; IIB=T2 (>20 mm but ≤50 mm), N1 (metastases to moveable ipsilateral axillary lymph nodes), M0 or T3 (>50 mm), N0, M0; and IIIA with Larger size tumors with various combinations of lymph node involvement that are more extensive than stage II, but showing no distant metastases.

Blood samples (3 mL) were collected from all individuals into EDTA-coated tubes (BD Biosciences), and serum used for clinical chemistry test parameters was isolated using standard procedures from all participants at the start and 48 hours after completion of the 6th AC cycle for patients and 105 days PE treatment for healthy subjects. Whole blood samples were also frozen at -20 °C for determination of caspase-3 and VEGF methylation status. No mortality was recorded among any of the participants during the whole course of the current study.

Measurement of serum biochemical parameters for assessment of liver and kidney function

The cobas® C501 automated clinical chemistry analyzer (Roche, Germany) was used to assess the liver and renal function by measurement of Alkaline Phosphatase (ALP), aspartate transaminase (AST), Alanine Transaminase (ALT), Urea and Creatinine.

Determination of antioxidant power

The ferric-reducing antioxidant power (FRAP) was measured in plasma to evaluate the effect of various treatments on oxidative stress status as previously described (Benzie and Strain, 1996). The FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} which interacts with 2,4,6-tri(2-pyridyl)- 1,3,5-triazine, resulting in a deep blue color that is read at 593 nm.

Analysis of Caspase-3 and VEGF promoter methylation status

The methylation status of the *Caspase-3* and *VEGF* promoter regions was assessed using the Methylation-specific PCR (MS-PCR). Initially, Genomic DNA extraction was performed from all the participant's whole blood samples using the GeneJET Genomic DNA purification kit (Cat # K0721, Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. The DNA concentration was measured by NanoDrop spectrophotometer and integrity by 0.8% agarose gel electrophoresis. Bisulfite conversion was performed using the EZ methylation-Gold™ kit (cat# D5005, Zymo Research), following the manufacturer's protocol. The MS-PCR reaction mixtures contained, 12.5 µL of (1×) Hot Start *Taq* 2X Master Mix (New England Biolabs), 1 µl (0.2 µM) of each primer, 100 ng of converted DNA and DNase-free water to a total volume of 25 µL. The PCR cycling conditions were: initial denaturation at 94 ℃ for 30 s, followed by 30

cycles at 95 ℃ denaturation for 30 s, 60 ℃ annealing for 60 s, 68 ℃ extensions for 1 min, and a final extension at 68 ℃ for 5 min. Finally, The MS-PCR products were separated on a 2% agarose gel yielding a 248 bp band for the methylated and unmethylated *Caspase-3*, and a 203 bp band for the methylated and unmethylated *VEGF* gene. The AccuBand 50 bp DNA ladder II (SMOBIO Technology, Inc) was used as a reference DNA size marker.

Bioinformatics tools and analysis

The Eukaryotic-Promoter-Database (https://epd.expasy.org/epd) was utilized for the determination of the promoter regions of the *Caspase-3* (GeneBank Accession: AY219866.1) and *VEGF* (GeneBank Accession: AH001553.2) genes. The Urogene MethPrimer software (Šestáková et al., 2019) (www.urogene.org/cgibin/methprimer/methprimer.cgi), was used in the identification of the promoter phosphate-linked cytosine-guanine pairs CpG-rich islands of *Caspase-3* (Figure 1A and VEGF Figure 1B). The MethMarker (Schüffler et al., 2009) was used in designing the methylated and unmethylated primers (Table 1). Most importantly, the 5' of each primer was designed specifically to terminate at a potential CpG that is located at a transcription factor binding site (TFBS) that was identified using the Genomatix MatInspector software tool (Genomatix, Ann Arbor, MI) (Cartharius et al., 2005). The location of the potential CpG sites, designed methylated and unmethylated primers, and the TFBS located in the promoter regions of the *Caspase-3* and *VEGF* genes are shown in Figures 2A and 2B, respectively.

Quantitative detection of Caspase-3 and VEGF protein levels

Serum cleaved Caspase-3 and VEGF protein levels were measured using a human caspase-3 instant ELISA kit from (eBioscience, USA), and Boster's human VEGF ELISA kit (BOSTER, Wuhan, China), respectively, following the manufacturer's instructions.

Sample size calculation

We conducted a power analysis to determine whether the differences between the groups were clinically significant (Moerbeek and Teerenstra, 2016; Thotambailu et al., 2019). A sample size of 25 participants was required for each group to achieve 90% power and a 95% confidence interval. The power analysis determined the minimum sample size needed to detect a difference between the groups, considering the test's power and the confidence interval. With 25 participants in each group, there is a 90% chance of detecting a statistically significant difference, assuming the confidence interval is 95% (Pourhoseingholi et al., 2013).

Statistical analysis

Statistical analysis was carried out using the SPSS software, version 20 (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as mean ± standard deviation. Comparison between the three studied groups regarding continuous variables was done using a one-way analysis of variance (ANOVA) and the paired t-test was used for comparing the data between two groups (before and after treatment) for normally distributed data. Whereas, qualitative variables were compared using the Chi-square test. All statistical tests were two-sided, and differences between groups were considered statistically significant when $P \leq 0.05$.

RESULTS

Demographic and Clinical characteristics of study subjects

The current study included a total of 81 participants. The first group included a total of 25 healthy control females who received PE supplement. The second group comprised 56 BC patients who were randomly divided into two sub-groups: 25 patients who received chemotherapy alone and 31 patients, who received a combination of chemotherapy + PE. Considered a major risk factor for BC, the selected control subjects were of a matched age compared to the BC patients included in the current study, this was confirmed by the data showing no significant difference between the mean age of the control group and the BC patients' groups ($p = 0.272$), (Table 2). Similar to age in being a risk factor for BC development, healthy control subjects enrolled in this study had approximate body mass index (BMI) values compared to BC patients as indicated by the statistically non-significant difference in the mean BMI values for the studied groups ($p=0.531$).

The difference between the control group and the patients' groups regarding the family history of BC was statistically significant (p = 0.001). Data showed that there was no significant difference (p≥0.5) between the two patients groups concerning all clinical characteristics such as histological type, grade, pathological stage, tumor size, vascular invasion, and ER, PR, and HER-2 receptor status, which was an essential finding that confirmed the randomization of individuals divided between the two patients' groups. Overall, data confirm that the tested groups had no significant difference concerning the demographic and clinical status before treatment.

Effect of Treatment on Hematological, Liver, and Kidney Function Parameters

The effect of chemotherapy and PE supplementation on hematological parameters is shown in Table 3. No significant difference was detected in hemoglobin levels between the control + PE or the Chemotherapy + PE subjects before and after administration of PE. However, hemoglobin levels were significantly (≤ 0.001) reduced after treatment with chemotherapy. On the other hand, hemoglobin levels improved as a result of adding PE to the standard chemotherapy treatment, and the difference between hemoglobin levels before and after treatment was not significant. RBCs count significantly increased in the control group after pomegranate administration (p ≤ 0.001). Whereas, the number of RBCs decreased significantly after receiving the standard chemotherapy alone or chemotherapy +PE treatment (p=0.02 and 0.009, respectively).

On the other hand, data summarized in Table 4 indicated that there was no significant change in the levels of AST and ALT before and after treatment protocols in all groups. However, the overall comparison between groups indicated a significant difference between groups regarding ALT values (p=0.02). Serum alkaline phosphatase levels were significantly reduced in the control group after administration of PE (p=0.006). With respect to renal function tests, blood urea significantly increased in the patients' group after receiving the standard chemotherapy treatment (p = 0.04), whilst, there was no significant difference in the other groups where PE was administered before and after treatment. Except for the control group which showed a significant (p=0.007) increase after PE treatment, there was no meaningful change in creatinine levels before and after treatment in other groups.

Effect of PE supplementation on methylation status of Caspase-3 and VEGF promoter

Bioinformatics software tools were used for the identification of the potential CpG sequences within the Caspase-3 and VEGF promoter sites as well as the identification of the TFBS and design of the MS-PCR primers that were used for the investigation of their promoter methylation status before and after PE supplementation to control subjects as well as BC patients receiving chemotherapy + PE, or chemotherapy alone regimen.

Interestingly, within the same individual, PE treatment has caused a remarkable reduction in the intensity of the methylated Caspase-3 bands in both control + PE and Chemotherapy + PE groups, an effect that was oppositely observed in chemotherapy only which caused an increase in methylated amplicon in the same patient (Figure 3A). The effect of treatment on the intensity of the MS-PCR amplified bands from the VEGF promoter was less apparent compared to the Caspase-3 gene (Figure 3B).

The results shown in Figure 4 summarize the methylation status data as a number of patients with methylated or unmethylated promoter regions for both the *Caspase-3* and *VEGF* genes before and after the different treatments. The data indicates a trend to decrease in the number of individuals with methylated *Caspase-3* promoter in both the control subjects from 7 participants representing (28%) before treatment to 5 participants representing (20%) after treatment, and in patients receiving Chemotherapy +PE from 4 patients representing 12% to single patient representing 3% after the combination treatment. Interestingly, the number of patients receiving the chemotherapy regimen alone showed an increase in the methylation status of the *Caspase-3* gene from 2-7 patients representing a patient percentage increase from 8% to 28% after single chemotherapy treatment (Figure 4A).

Table 1. Primers used for methylation-specific polymerase chain reaction showing M (methylated) and U (Unmethylated) primer sequence.

Gene	Primer	Primer Sequence 5'- 3'	Product size (bp)
***Caspase-3* (M)**	F R	GGGGAGTCGGGTCGAGTTTTAGGGC AATATATTCCAACGCCAACCCCGCCTCCCG	248
***Caspase-3* (U)**	F R	GGGGAGTTGGGTTGAGTTTTAGGGT AATATATTCCAACACCAACCCCACCTCCCA	248
***VEGF* (M)**	F R	GGCGCGTGTTTTTGGATAGAGTTTTCGGGGGC AACCCCGCCCCCGACCCGCCCCGAAAAACG	203
***VEGF* (U)**	F R	GGTGTGTGTTTTTGGATAGAGTTTTTGGGGGT AACCCCACCCCCAACCCACCCCAAAAAACA	203

Table 2. Demographic and clinical characteristics of the studied control and patient groups

Variable		Control + PE (n=25)	Chemotherapy (n = 25)	Chemotherapy + PE (n = 31)	Total (n = 56)	p
Histological type	IDC ILC IMC	NA	12 (48%) 5 (20%) 8 (32%)	18 (58%) 5 (16.2%) 8 (25.8)	30 (53.6%) 10 (17.9%) 16 (28.5%)	0.754
Histological Grade	GII GIII	NA	20 (80%) 5 (20%)	21 (67.7%) 10 (32.3%)	41 (73.2%) 15 (26.8%)	0.372
Pathologic Stage	IA IIB IIIA	NA	1 (4.0%) 6 (24.0%) 18 (72.0%)	1 (3.2%) 9 (29.0%) 21 (67.8%)	2 (3.6%) 15 (26.8%) 39 (69.6%)	0.909
ALN status	Negative Positive	NA	6 (24.0%) 19 (76.0%)	8 (25.8%) 23 (74.2%)	14 (25.0%) 42 (75.0%)	1.00
Vascular invasion	Negative Positive	NA	7 (28.0%) 18 (72.0%)	9 (29%) 22 (71%)	16 (28.5%) 40 (71.5%)	1.00
Tumor size (cm)	< 2 > 2	NA	5 (20%) 20 (80%)	5 (16.2%) 26 (83.8%)	10 (17.8%) 46 (82.2%)	0.738
Tumor location	Right Left	NA	10 (40%) 15 (60%)	12 (38.8%) 19 (61.2%)	22 (39.3%) 34 (60.7%)	1.00
ER status	Negative Positive	NA	4 (16%) 21 (84%)	6 (19.3%) 25 (80.7%)	10 (17.8%) 46 (82.2%)	1.00
PR status	Negative Positive	NA	4 (16%) 21 (84%)	10 (32.3%) 21 (67.7%)	14 (25.0%) 42 (75.0%)	0.219
HER-2 status	Negative Positive	NA NA	15 (60.0%) 10 (40.0%)	18 (58.0%) 13 (42.0%)	33 (58.9%) 23 (41.1%)	1.00
Age (years)		47.04 ± 7.6	48.92 ± 7.5	50.5 ± 8.4	NA	0.272
Positive BC Family history		1 (4.0%)	10 (40%)	15 (48%)	NA	≤ 0.001***
BMI (kg/m²)		30.01 ± 4.67	31.63 ± 5.10	31.13 ± 5.73	NA	0.531

PE Pomegranate extract, IDC Invasive ductal carcinoma, ILC Invasive lobular carcinoma, IMC Invasive mammary carcinoma, ALN Axillary lymph node, ER Estrogen receptor, PR progesterone receptor, HER-2 human epidermal growth factor receptor 2, BC breast cancer BMI: Body mass index

Table 3. Hemoglobin level and RBCs count before and after treatment.

	Variable	Control + PE (n=25)	Chemotherapy (n = 25)	Chemotherapy + PE (n = 31)	P1
Hemoglobin (gm/dL)	**Before**				0.43
	Mean ± SD.	11.69 ± 1.22	11.92 ± 0.94	11.69 ± 1.54	
	After				≤ 0.001
	Mean ± SD.	11.96 ± 1.18	10.85 ± 1.16	11.06 ± 1.23	
	P2	0.4780	≤ 0.001	0.05664	
RBCs ($\times 10^6/\mu L$)	**Before**				≤ 0.001
	Mean ± SD.	4.06 ± 0.222	4.67 ± 0.45	4.51 ± 0.41	
	After				0.023
	Mean ± SD.	4.55 ± 0.23	4.36 ± 0.45	4.25 ± 0.45	
	P2	≤ 0.001	0.02605	0.009	

P1: p-value for comparing the studied groups by one-way ANOVA test.
P2: p-value for comparing between before and after treatment in the same group by paired t-test.

Table 4. Liver and kidney function parameters before and after treatment.

Variable		Control + PE (n=25)	Chemotherapy (n = 25)	Chemotherapy + PE (n = 31)	P1
Serum AST (U/L)	**Before**				
	Mean ± S.D.	25.08 ± 1.9	25.68± 6.06	25.5 ± 4.6	0.88
	After				
	Mean ± S.D.	25.26 ± 2.7	28.16 ± 4.47	26.7 ± 6.5	0.12
	P2	0.7552	0.1343	0.3834	
Serum ALT (U/L)	**Before**				
	Mean ± S.D.	27.64 ± 5.54	31.28 ± 9.41	28.4 ± 9.8	0.288
	After				
	Mean ± S.D.	26.92 ± 2.97	32.96 ± 10.7	29.4 ± 7.6	0.02
	P2	0.5075	0.52	0.71	
Serum Alkaline phosphatase (U/L)	**Before**				
	Mean ± S.D.	75.72 ± 8.26	90.68 ± 21.4	80.54 ± 20.58	0.01
	After				
	Mean ± S.D.	66.0 ± 15.4	92.7± 17.3	98.8 ± 16.45	≤ 0.001
	P2	0.006	0.63	≤ 0.001	
Blood urea (mg/dl)	**Before**				
	Mean ± S.D.	19.76 ± 3.04	20.76 ± 4.2	25.1 ± 6.1	≤ 0.001
	After				
	Mean ± S.D.	20.92 ± 5.04	22.8 ± 2.7	25.0 ± 4.6	0.002766
	P2	0.3641	0.04352	0.8657	
Serum creatinine (mg/dl)	**Before**				
	Mean ± S.D.	0.81 ± 0.10	0.826 ± 0.08	0.85 ± 0.20	0.62
	After				
	Mean ± S.D.	0.88 ± 0.07	0.88 ± 0.11	0.88 ± 0.12	0.98
	P2	0.007346	0.05195	0.42	

P1: p-value for comparing between the three studied groups (one-way ANOVA)
P2: p-value for comparison between before and after treatment for the same group. (Paired t-test).

Table 5. Caspase-3 and VEGF protein levels before and after treatment.

	Protein level (pg/mL)	Control + PE (n=25)	Chemotherapy (n = 25)	Chemotherapy + PE (n = 31)	P_1
Caspase-3	**Before**				
	Mean ± SD	640.24 ± 584.0	1249.9 ± 611.1	780.9 ± 580.0	0.001***
	After				
	Mean ± SD	719.0 ± 523.4	1020.84 ± 691.55	1360.74 ± 745.37	0.002**
	P_2	0.59	0.163	0.002**	
	Change (%)	+12.3%	-18.3%	+74.2%	
VEGF	**Before**				
	Mean ± SD.	190.46 ± 93.74	335.86 ± 92.51	281.93 ± 92.17	0.001***
	After				
	Mean ± SD.	176.15 ± 66.23	269.76 ± 82.49	177.63 ± 66.94	0.001***
	P_2	0.285	0.009***	≤0.001***	
	Change (%)	- 7.6 %	- 19.6 %	- 36.99 %	

P_1: p-value for comparing between the studied groups (One-way ANOVA)
P_2: p-value for comparing between before and after (Paired t-test)
*: Statistically significant at p ≤ 0.05, **: Statistically significant at p ≤ 0.01
***Statistically significant at p ≤ 0.001

Epigenetically, gene methylation is considered as a major gene-silencing action, therefore, the decrease in the intensity of the *Caspase-3* amplicons as well as the number of patients with methylated *Caspase-3* after treatment with PE reflects a hypomethylation of the promoter region which would consequently result in an upregulation in caspase-3 expression and therefore explains the pro-apoptotic action for PE.

On the other hand, the effect of treatment on the methylation status of the *VEGF* promoter region was less evident when compared to the effect described for the *Caspase-3*. The number of individuals with methylated *VEGF* promoter remained unchanged (2 subjects) within the control group after treatment with PE. Chemotherapy single treatment increased the number of patients with methylated *VEGF* promoter from a single patient representing 4% to 2 patients representing 8% within the treatment group. Finally, the combined chemotherapy + PE treatment caused an increase in the number of patients with methylated *VEGF* promoter from 3 to 4 patients which represents a 10% to 13% increase within the group (Figure 4B).

Collectively, the promoter methylation data displayed in Figures 3 and 4 demonstrate a significant alteration in the methylation pattern of the Caspase-3 promoter and a less apparent change in the VEGF promoter after supplementation of PE to both control and BC patients.

Effect of treatment on the Caspase-3 and VEGF protein levels

To confirm the MS-PCR analysis methylation data, and further investigate if the modulation of the methylation for the *Caspase-3* and *VEGF* genes was a major mode of action for the regulation of transcription of these two genes, the effect of treatment on the Caspase-3 and VEGF protein levels was also examined by ELISA assay, presented in Table 5. With respect to caspase-3, only combination treatment of PE with chemotherapy resulted in a statistically significant increase in the level of caspase-3 ($p = 0.002$) and with a potent mean protein increment of 74.2%, while the difference in caspase-3 protein levels was not statistically significant before and after treatment in the control +PE and the chemotherapy alone groups ($p=0.59$ and 0.163, respectively), and a protein level change of +12.3% and -18.3%, respectively.

On the other hand, concerning VEGF protein levels, the administration of PE to the control subjects resulted in a minor (-7.6%) non-significant ($p=0.285$) decrease and a highly significant ($p\leq0.001$) potent reduction(-36.99%) after combination treatment with chemotherapy compared to the sole treatment with chemotherapy (-19.6% reduction, $p=0.009$). Notably, the ELISA results supported our theory that PE treatment modulates the promoter methylation state of the Caspase-3 and VEGF genes, hence modulating their expression.

The effect of PE treatment on antioxidant power

Since antioxidants tend to exert their anti-cancer actions by modulating major cellular processes including programmed cell death (apoptosis) and angiogenesis (Park et al., 2022) we have evaluated the antioxidant power of the various treatments in the plasma of all tested groups before and after treatment using the FRAP assay which depends on the reduction of the ferric cation to ferrous, and the obtained data is presented in Table 6. Despite the 10.79 % recorded increase in the FRAP value after PE treatment in the control group, statistically, this was none significant ($p=0.454$). On the other hand, chemotherapy alone or combined with PE supplementation resulted in a statistically significant ($p\leq 0.001$ and $p=0.04$) mean FRAP values increment of 19.17% and 16.1%, respectively.

DISCUSSION

Breast cancer is a global health problem and the most common cancer amongst women worldwide and in Egypt (Amato et al., 2023). Moreover, In terms of female cancer-associated death rates, BC ranks as the first (Siegel et al., 2023).

In human malignancies, transcription factors control gene expression through epigenetically modulated particular sites known as promoters by DNA-binding actions (Jiang and Li, 2023) and

Table 6. Ferric Ion Reducing Antioxidant Power (FRAP) levels before and after treatment.

		Control + PE (n=25)	Chemotherapy (n = 25)	Chemotherapy + PE (n = 31)	P_1
FRAP (mmol/L)	**Before**				
	Mean ± SD	982.35±198.44	965.78 ± 197.87	935.50 ± 233.79	0.703
	After				
	Mean ± SD	1088.39-688.7	1151.60 ± 185.82	1086.21 ± 265.07	0.822
	P_2	0.454	≤ 0.001***	0.040*	
	Change (%)	↑ 10.79 %	↑ 19.17 %	↑ 16.1 %	

P_1: p-value for comparing between the studied groups (One-way ANOVA
P_2: p-value for comparing between before and after (Paired t-test)
*: Statistically significant at p ≤ 0.05, **: Statistically significant at p ≤ 0.01
***Statistically significant at p ≤ 0.001

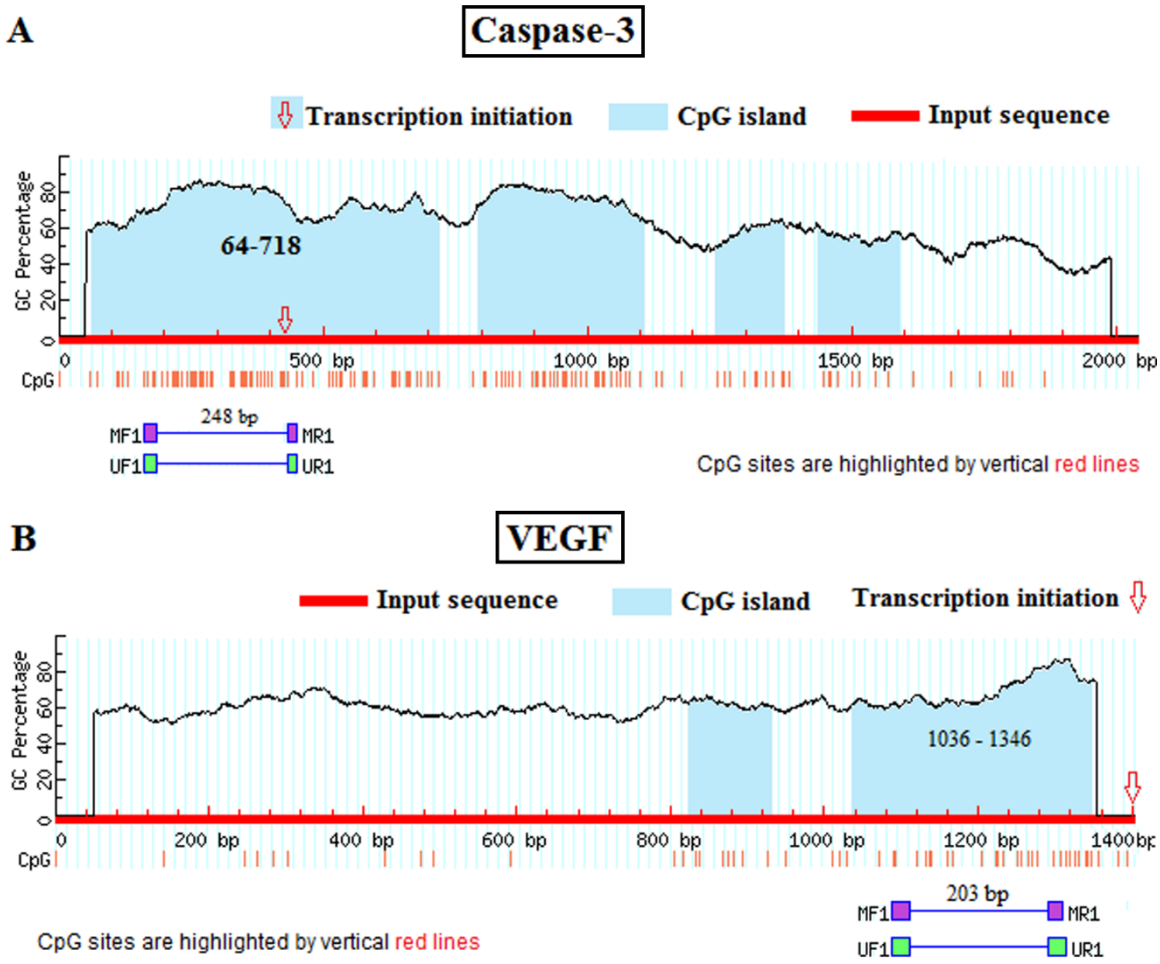

Figure 1. Graphic view indicating promoter CpG rich islands for **(A)** *Caspase-3*; and **(B)** *VEGF* genes as predicted by the MethPrimer software. The promoter CpG islands are shown in sky blue; CpG sites are indicated by vertical red lines; the transcription initiation site is shown by vertical arrows; the position of the methylated (violet) and unmethylated (green) MS-PCR primers and amplicon size (bp) is shown (www.urogene.org/cgi-bin/methprimer/methprimer.cgi) was used to submit the promoter nucleotide sequences for both genes to the Methprimer software tool. The input parameter for primer selection was the CpG island prediction. The default settings were applied to every additional setting.

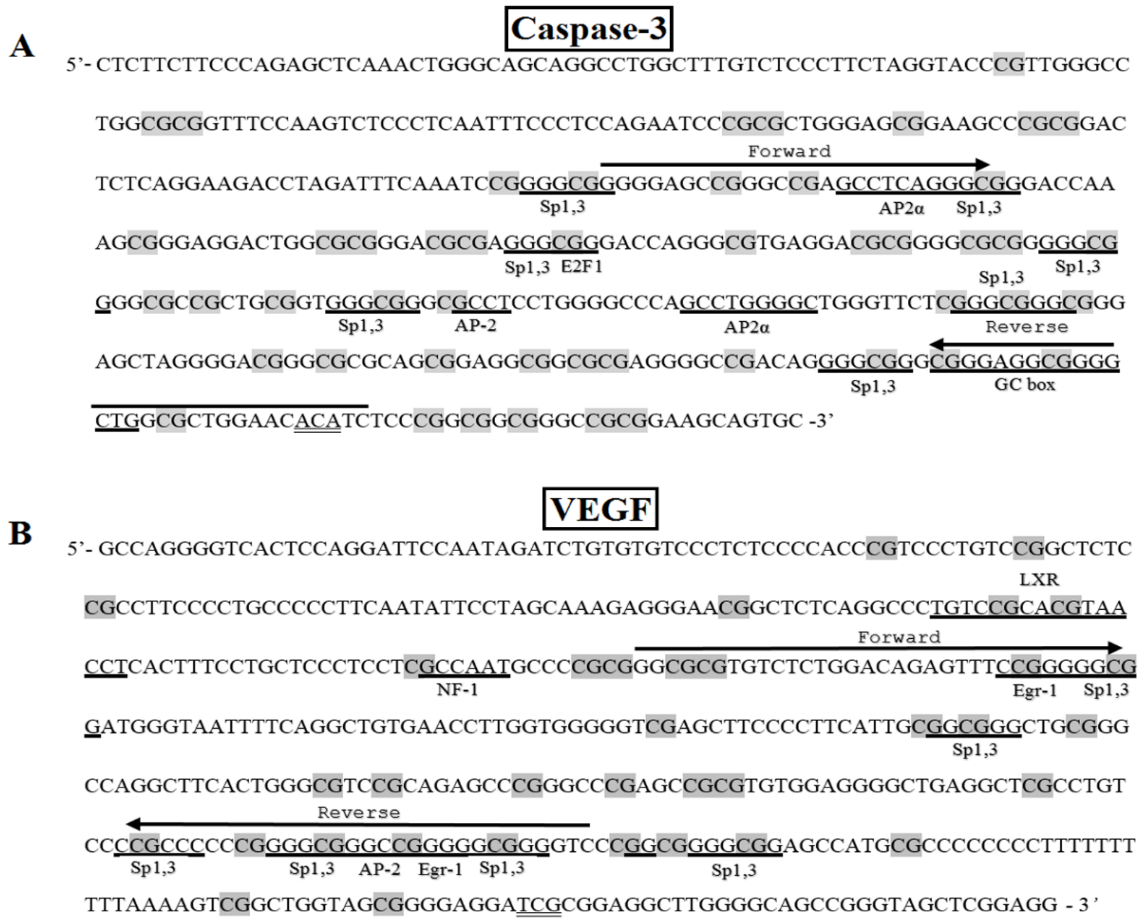

Figure 2. Nucleotide sequence of the promoter region for (A) *Caspase-3* (GeneBank Accession: AY219866.1) and; (B) *VEGF* (Gene Bank Accession: AH001553.2) genes showing the potentially methylated CpG nucleotides (highlighted in gray), putative transcription factor binding sites (bold underlined), transcription initiation locus (double underlined), location of the forward and reverse MS-PCR primers (arrows).

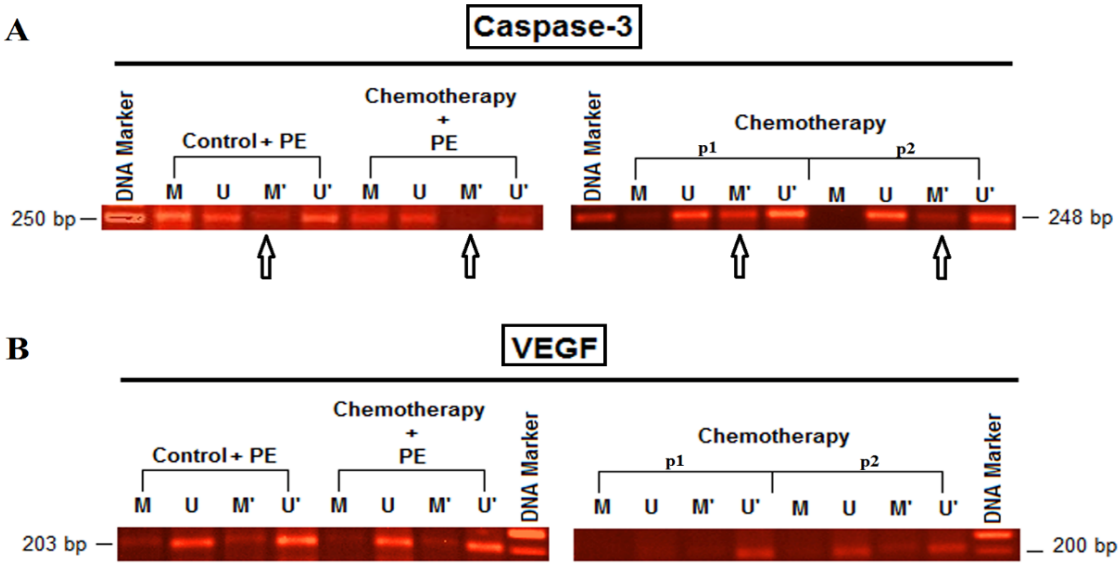

Figure 3. Images of representative gels showing the methylation-specific PCR (MS-PCR) assay results for the *Caspase-3* and *VEGF* genes. M and M' indicate the presence of methylated genes before and after treatment, respectively. The presence of a band in the U and U' lanes indicate unmethylated amplicon before and after treatment, respectively. The MS-PCR products were separated on 2% agarose gel and visualized under UV light, the AccuBand 50 bp DNA marker was used to ascertain the size of PCR products. One control + PE; one chemotherapy + PE; and two patients (p1 and p2) treated with chemotherapy alone are shown. Arrows indicate the bands with altered intensity before and after treatment.

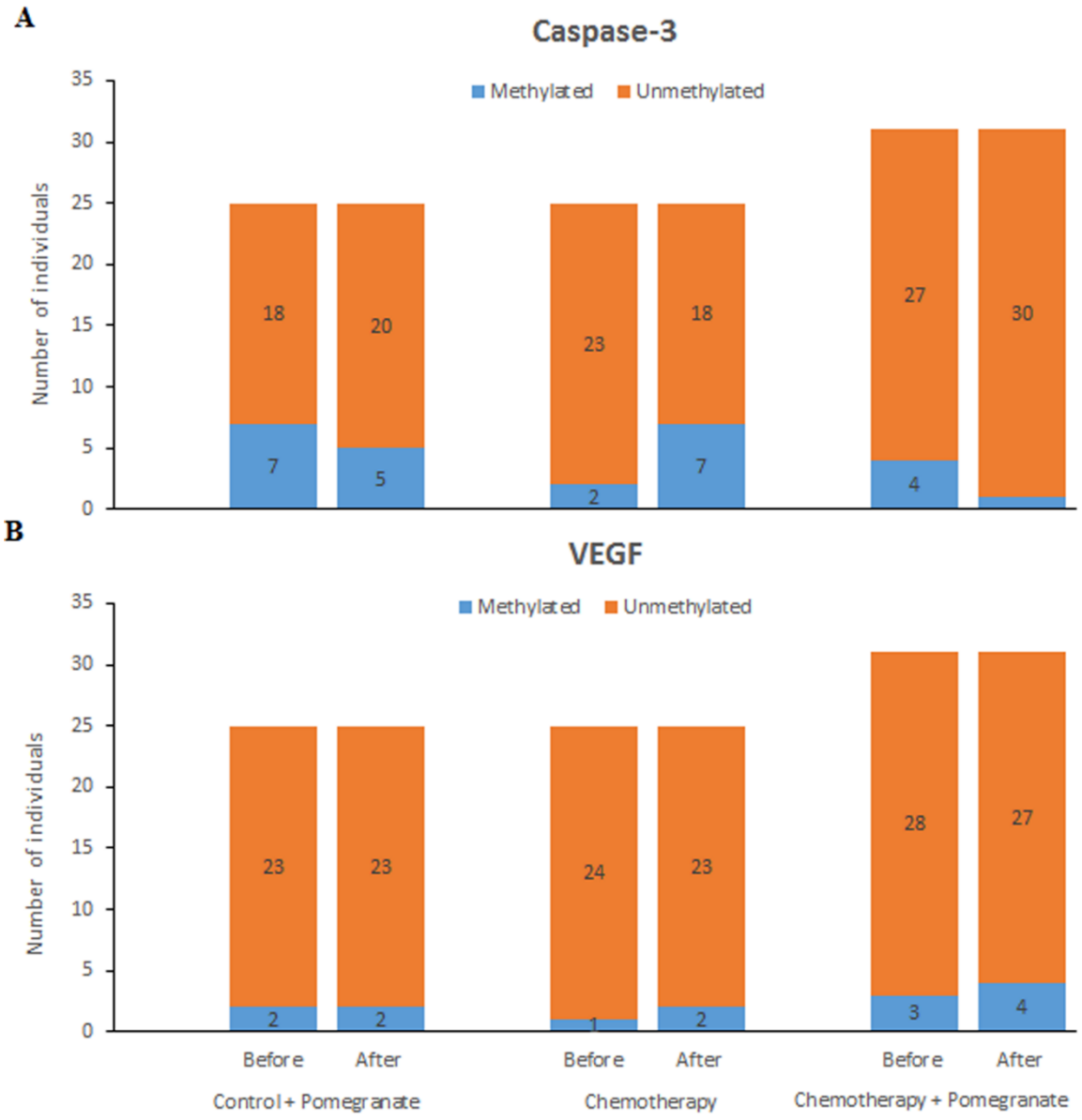

Figure 4. Bar graph representation indicating the number of individuals with methylated and unmethylated (A) *Caspas-3,* and (B) *VEGF* gene promoter regions before and after different treatments as measured by Methylation-specific PCR.

transcription factors being used for the diagnosis or prognosis of breast cancer have been previously described (Liu et al., 2021; Salama et al., 2021). Evading apoptosis and enhanced angiogenesis are two core hallmarks of cancer (Ravi et al., 2022).

Since caspase-3 catalyzes most of the protein breakdown occurring during apoptosis, cleaved caspase-3 is thought to be an accurate predictor of dying apoptotic cells, Cleaved Caspase 3 has been associated with the prognosis of various types of cancer (Liu et al., 2017; Silva et al., 2022).

On the other hand, the prognostic significance for the pro-angiogenic VEGF gene expression has been previously reported in several cancers (Abbink et al., 2020; Trifanescu et al., 2023). The use of anti-angiogenic drugs through targeting VEGF for the treatment of different malignancies has been also reported before (Agrafiotis et al., 2023; Bejari et al., 2023). The green treatment approach for cancers is on a continuous rise hoping to eliminate human tumors without harming normal cells. For this reason, multiple natural products were used in clinical trials to prove their efficacy as therapeutic agents for human malignancies by

investigating their effect on the biochemical and molecular mechanisms of cancer cells.

To the best of our knowledge, none of the studies that have suggested various anti-cancer mechanisms for pomegranate have examined the possible impact of PE supplementation on the methylation state of genes relevant to cancer, such as the *Caspase-3* gene that encodes the executioner Caspase-3 protein that promotes cell death via apoptosis, or the *VEGF* gene which is thought to be the main promoter for tumor angiogenesis and therefore, enhancing the survival and proliferation of cancer cells (Zhang et al., 2009; Wang et al., 2017).

This study aimed to examine the potential of PE to modulate the promoter methylation state of the *caspase-3* and *VEGF* genes in BC patients. The influence of PE and chemotherapy on caspase-3 and VEGF protein levels was also determined to investigate if methylation was the main mechanism of action affecting the expression status for these two cancer-modulating genes. Finally, to shed a little insight into how treatment affected the plasma antioxidant power of participants, the FRAP levels were also examined.

The current study indicated a remarkable alteration in the methylation status of the Caspase-3 and to a lesser extent the VEGF promoter after PE treatment. A tendency whereby the methylation of the *VEGF* promoter increased and that of the *caspase-3* decreased after PE treatment. Most interestingly, a significant reduction in the intensities of the Caspase-3 methylated amplicons in both control and combined chemotherapy treatments was observed, however weak methylation bands still existed (Figure 3A). This explains the small change in the overall number and percentage of patients carrying the methylated *Caspase-3* gene despite the remarkable reduction of methylation of its promoter since the promoter was considered methylated regardless of the intensity of the methylated amplicon obtained. The methylation data for the *Caspase-3* gene was further supported by the ELISA assay that showed a significant increase (+74.2%) in Caspase-3 protein levels after combination treatment of PE with chemotherapy which clearly could be attributed to the reduced *Caspase-3* promoter methylation observed.

In line with these findings, it was recently reported that PE extract increased caspase-3 expression in an animal model of colorectal cancer, which promoted apoptosis machinery (Kusmardi et al., 2021). A similar influence of pomegranate peel extract on caspase-3 was also observed in the hepatocellular carcinoma cell model in which the extract was able to induce elevation of caspase-3 and enhance apoptosis (Elbakry et al., 2023). In addition, PE extract inhibited caspase-3 activity in an *in vitro* model of mammary cancer stem cells, which is in harmony with the results of this study(Dai et al., 2010). Likewise, pomegranate seeds and peel extract improved gene expression of apoptotic markers including caspase-3 in liver cancer cells (Nasr et al., 2023).

Enhancing the caspase-3 expression and apoptosis by altering the DNA methylation status of the *Caspase-3* gene was reported using different treatments against different types of cancers (Antwih et al., 2013; Napso and Fares, 2014; Metin and Mustafa, 2018).

Since cancer cells need angiogenesis to spread, suppressing VEGF signaling is a tempting strategy for inhibiting tumor metastasis. Despite our data that showed that PE supplementation had a less evident change on the methylation status of VEGF promoter status in the context of both the methylation percentage or methylation amplicon intensity, the current study revealed that the supplementation of PE with chemotherapy resulted in a significant reduction (- 36.99 %) of VEGF protein levels after treatment. These findings suggest that alteration of the methylation status of the VEGF promoter might not be the major mechanism by which PE has reduced the VEGF protein level. PE was previously reported to downregulate VEGF expression in MCF-7 cells (Moradi-Gharibvand et al., 2022).

Moreover, it was also reported that PE resulted in attenuation of VEGF expression in triple-negative BC cell lines, which is in agreement with our findings (Ahmadiankia et al., 2018). In the same context, the downregulation of mRNA

of the VEGF by pomegranate peel extract was also reported by another study (Dana et al., 2015). Dietary antioxidants alter the pattern of DNA methylation by several methods, such as controlling epigenetic enzymes (Beetch et al., 2020). The current study showed that PE supplementation has resulted in the elevation of the ferric-reducing antioxidant potential in patients treated with chemotherapy and PE suggesting improvement of antioxidant power in this group of patients.

In this respect, and in agreement with our results, the beneficial effects of the antioxidant power of pomegranate have been reported in different human cancers(Cortez-Trejo et al., 2022). This information is in line with the anti-genotoxic effect of pomegranate extract that was attributed, at least in part, to its antioxidant capacity which was reported in another study (Turrini et al., 2015).

The effect of several antioxidant natural compounds or vitamins such as Vitamin E as a prophylactic compound should be investigated for its anti-cancer properties through the regulation of epigenetics of key cancer-related genes regulating key cellular processes such as apoptosis, autophagy, and Epithelial-Mesenchymal Transition (EMT) (Hamdy et al., 2009).

CONCLUSION

To the best of our knowledge, the effect of PE on the epigenetic regulation of the DNA methylation status of the *Caspase-3* or the *VEGF* genes was never been reported before, the current study has confirmed the pro-apoptotic and anti-angiogenic influences of PE in randomized BC control study and suggests for the first time the selective modulation of DNA methylation status of the Caspase-3 promoter region as a major and novel mechanism of action for such effect. Our findings consolidate the in-clinic use of pomegranate extract in combination with chemotherapy for the treatment of BC. The MS-PCR analysis was confirmed by ELISA assays showing that the administration of PE with chemotherapy significantly enhanced the expression of Caspase-3 and reduced VEGF protein levels compared to chemotherapy alone. Altogether, this current study confirms PE pro-apoptotic and anti-angiogenic influences in BC patients by the modulation of promoter epigenetics. Despite the novel data that might be displayed in this report, the major study limitations might be that further studies including larger numbers of patients, and different doses of PE with alternative chemotherapeutic regimens against different types of cancer might be required for widespread validation of these findings.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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