Pomegranate Extract Protects Endothelial Cells from TNF-α Associated Damage

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Abstract. Pomegranates are known for being rich in polyphenols and are considered to have immense therapeutic potential. The present study investigates the hypothesis that the Methanolic Extract of Pomegranate (PME), a rich source of antioxidants, may reverse the adverse effects of TNF-α in endothelial cells. This was done by pre-treating the endothelial cells EA.hy926 with PME (80 μg/ml) before subjecting them to apoptotic stimuli, which was TNF-α in combination with cyclohexamide. PME was found to rescue a population of cells from apoptosis induced by TNF-α modulating the levels of BCL2 and BAX involved in intrinsic apoptotic pathway. PME was found to increase the BCL-2/BAX ratio and reverse the elevated levels of effector caspase and thus assist cells to escape from apoptotic stimuli. Also, the extract was found to attenuate oxidative stress by reducing the levels of Reactive Oxygen Species (ROS). Supplementing its anti-atherosclerotic potential, PME pre-treatment diminished the elevated levels of adhesion molecules like VCAM upon TNF-α treatment. PME may therefore have therapeutic implications in protecting the endothelium from TNF-α triggered atherosclerosis.

Keywords: Pomegranate · apoptosis · atherosclerosis · Reactive Oxygen Species

1 Introduction

Punica granatum or pomegranate has been known to have immense medicinal properties. Different parts of the plants, and their juices and extracts are understood to be beneficial in cardiovascular health, male infertility, cancer, diabetes and Alzheimer's disease [1]. Our earlier investigations demonstrated that Methanolic Extract of Pomegranate pericarp (PME) could serve as a Selective Estrogen Receptor Modulator (SERM) by reducing estrogen dependent breast cancer proliferation while having favorable spectrum of activity in other hormone-dependent tissues [1, 2]. We also found feeding the extract to ovariectomized mice could reduce Low Density Lipopolysaccharides (LDL) levels and triglycerides with respect to control. PME was additionally found to antagonize the activity of 27-hydroxycholesterol, a cholesterol metabolite and an endogenous

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SERM, could compete with the oxysterol and reduce 27HC-mediated proliferation of estrogen receptor positive breast cancer cells [3].

The decrease in ovarian hormones in the postmenopausal period can result in inflammation in many regions that includes vasculature among others like bone and neural cells. The responses of endothelium to this can often lead to cardiovascular diseases like atherosclerosis [4]. Therapies employing SERMs have been shown to be beneficial in cardiovascular health associated with post menopause [5, 6].

SERMs are a class of compounds that can activate or inhibit estrogen receptors depending on the cofactors that bind, which in turn is tissue dependent. These are employed in treating breast cancer, osteoporosis and other ailments that involve estrogen-based therapy. There have been studies indicating the role of SERMs like raloxifene in improving endothelial function, plasma lipid profile, and their effects have been demonstrated to be comparable to or better than, those of hormone replacement therapy in post menopausal condition, all of which women [7]. The widely used SERMs like Tamoxifen and Raloxifene are reported to improve vascular dysfunction by its anti- inflammatory properties, by maintaining a favorable lipid profile and by modulating endothelial cells especially in post menopausal all of which result in better cardiovascular health [4].

Against this backdrop, we hypothesize that PME, being a potent SERM and a rich source of antioxidants, could have protective effects in endothelial cell function. The initial step in atherogenesis have been proposed to be endothelial dysfunction [6]. Therefore, to understand the potential role of PME, we assessed it against apoptosis, oxidative stress, and other critical events [8] associated with atherosclerosis. In this study, conducted *in vitro* in endothelial cell line EA.hy 926, we demonstrate that PME attenuate TNF-α mediated apoptosis in endothelial cells, reduce cell adhesion and reactive oxygen species thus protecting the cells from oxidative stress and further damage.

2 Materials and Methods

2.1 Materials

Fetal Bovine serum (FBS) (PAN: Cat: 3302), Dulbecco's modified Eagles medium (DMEM), Endothelial Cell Growth Basal Medium-2 (Lonza, CC-3156), Antibiotic-Antimycotic (100X) (High Media: Cat: A002), Charcoal treated Serum (Gibco: A33821–01), MTT (Calbiochem Cat: 475989), TRI reagent (Sigma, Cat: 93289), Bradfords reagent (Sigma), dimethyl sulfoxide (DMSO) (Sigma), Superscript III (Invitrogen, 18080044), Human TNF-α and cyclohexamide (PAN: P60–36720100), OXy DNA assay kit (Calbiochem), DCFDA and from PI (Sigma) and Cell ELISA colorimetric detection kit (Thermo scientific). The cell line EA.hy926 was acquired from American Type Culture Collection (ATCC).

2.2 Preparation of the Extract

Pomegranate (*P. granatum*) was peeled and pericarp was powdered after drying to obtain 60 mesh sizes. The powder was then extracted with methanol at dilution of 10 g powder to 100 ml methanol, using Soxhlet apparatus for a period of 20 h. It was further concentrated

using rotary vacuum drier to obtain a yield of 5% (weight/volume). This was dissolved in dimethyl sulfoxide (Sigma-Aldrich) and is termed as Pomegranate Methanoloic Extract or PME and was used for further experiments.

2.3 Cell Culture

Endothelial cell lines EA.hy926 were cultured in EBM-TM-2 Endothelial Cell Growth Basal Medium-2 supplemented with growth factors and 10% heat-inactivated fetal bovine serum (FBS). Antibiotic-Antimycotic (100X) was used at a concentration of 10 ml/L. The experiments were done in phenol red less medium. The cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO2. Before every treatment the cells were treated with 10% charcoal-treated serum to remove the effects of endogenous growth factors and hormones.

2.4 Cell Viability Assessment by MTT Assay

The cell viability after drug treatment was measured using 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide assay. Cells were seeded at a density of 8,000 cells/well in 96 well plates. After 24 h, the cells were treated with TNF-α (20 ng/ml); PME (80 μg/ml); TNF-α (20 ng/ml) and cyclohexamide (10 μg/ml) combined; and PME (80 μg/ml), TNF-α (20 ng/ml) and cyclohexamide (10 μg/ml), all three combined, for 24 h following which the medium was removed and 100 μl of MTT solution dissolved in phenol red less medium was added making the final concentration as 0.5 mg/ml and was incubated for 4 h at 37 °C. Following this, formazan crystals were solubilized using DMSO, and the absorbance was measured at 570 nm using multi-well spectrophotometer. Cell viability was calculated as ($OD_{Average}$ drug treated cells- $OD_{Average}$ Blank/Average OD vehicle control treated cells - $OD_{Average}$ Blank) × 100 and expressed as percentage.

2.5 RNA Extraction and Reverse Transcriptase PCR

EA.hy926 cells were stimulated with TNF-α (20 ng/ml) and cyclohexamide (CHX, 10 μg/ml) with and without 12-h pre-treatment of PME (80 μg/ml), and mRNA of treated and untreated cells were extracted after 6 h. RNA was isolated by TRiZol method. The cells were incubated with TRIzol for 5 min at room temperature followed by addition of chloroform (0.2 ml/1 ml of Trizol). The samples were centrifuged and aqueous phase was transferred and precipitated by isopropanol and further washed with 75% ethanol and then air dried. The RNA pellets were dissolved in nuclease free water and quantified. One microgram of total RNA use to reverse transcribe using Superscript III. Reverse-Transcriptase PCR was done for, BCL2, BAX, ICAM1, VCAM1 and CASP 9. The PCR cycle was as follows: 94 °C for 10 min, 55–60 °C for 2 min, and 72 °C for 3min for 35 cycles using the PCR mix. The primers were obtained from sigma (Table 1). The PCR products were electrophoresed on a 2% agarose gel and visualized using UV illumination after staning with ethidium bromide. The gene expression was calculated relative to endogenous control β-actin.

Table 1. List of primers

GENE	Sequence	Tm	Amplicon
ICAM1	F:CCGAGCTCAAGTGTCTAAAG	59.3	272
	R: GGCCTGTTGTAGTCTGTATTT	57.3	
VCAM1	F:GGATGCGGGAGTATATGAATG	62.9	311
	R:TCAAGGAATGATGAGCAGTATC	60.7	
BCL2(normal)	F:TTTGAGTTCGGTGGGGTCAT	58.4	273
	R:TGACTTCACTTGTGGCCCAG		
BAX	F:GCTGTTGGGCTGGATCCAAG	57.4	170
	R:TCAGCCCATCTTCTTCCAGA		
Caspase 9	F:AGGCAGGTTAGGTCTCTTG	57.3	317
	R:GAGGTCGAGACTGCAATAAG	59.1	

2.6 Measurement of Cell Adhesion Molecules by Cell-ELISA

Cell-based ELISA was used to evaluate whether PME regulates expression of TNF-α induced cell adhesion molecules intercellular adhesion molecule-1 (ICAM1) and vascular adhesion molecule-1 (VCAM1) in EA.hy926 by using cell ELISA colorimetric detection kit with modification [9]. 1×10^4 cells were suspended in 100 μl of phenol red-free plated in 96 well plate and incubated overnight. The cells with or without pre-treatment of PME were treated with TNF-α (20 ng/ml) to enhance expression of adhesion molecules. Next, the cells were fixed with 4% formaldehyde and blocked with blocking buffer. The fixed cells were treated with primary antibody ICAM-1 or VCAM-1 overnight at 4 °C. After wash with wash buffer, the monolayer was incubated with HRP conjugate at room temperature for 30 min. The peroxidase substrate TMB, tetramethylbenzidine was added and incubated in the dark for 15 min. The reaction was halted by adding TMB stop solution and the optical density was measured at 450 nm.

2.7 Assessment of Oxidative DNA Damage by Flow Cytometry

OXy DNA assay kit, which is based on direct binding of fluorescein isothiocyanate (FITC-conjugate) probe to DNA adduct 8-oxyguanine, was used for assessing oxidative damage induced. Exponentially growing EA.hy926 cells were treated with 20, 40, 80, 160 and 320 μg/ml. Cells treated with H_2O_2 (200 MM) served as positive control. Treated and untreated cells were then trypsinised and washed with PBS. The cell pellets were resuspended in ice-cold 70% ethanol followed by wash with PBS and washing solution (Tris-buffered saline/Tween 20, containing thimerosal). After the wash, 100 μl of FITC-Conjugate was added to the cells and incubated for 1 h at room temperature in the dark. This was followed by two washes one with the washing solution and second with PBS. The cells were then resuspended in PBS and filtered using 40 μm nylon cell strainer and analyzed using the Becton-Dickinson FACSaria (excitation 495 nm, emission 515 nm). The data was analyzed using DIVA software.

2.8 Measurement of ROS Level

Generation of Reactive Oxygen Species (ROS) was measured by the oxidative-sensitive fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) [10]. Cells were seeded at cell density of 10^5 and treated with PME (80 μg/ml). Following the treatment, the medium was discarded by aspiration. To this 5 μM DCFH-DA was added and incubated for 15 min at 37 °C. The cells were, washed trypsinized and re-suspended in phenol red-less medium. The cells were filtered using strainer and immediately analyzed by flow cytometry (FACS Aria 1 Becton Dickinson, San Jose, CA).

2.9 Statistical Analysis

All data are presented as mean ± standard deviation. Unpaired t-test was used difference between group means.

3 Results

3.1 Effect of PME on TNF-A Induced Cell Apoptosis

Atherosclerosis is often seen to begin with endothelial dysfunction apoptosis. TNF- α is one such pro-apoptotic factor which reduces the adhesion and survival of endothelial cells [11]. Hence, using TNF-α as an inducer of apoptosis, the potential of PME to rescue cells from undergoing apoptosis was investigated. MTT assay demonstrated that TNF-α alone did not cause remarkable loss of cell viability, while concomitant treatment with

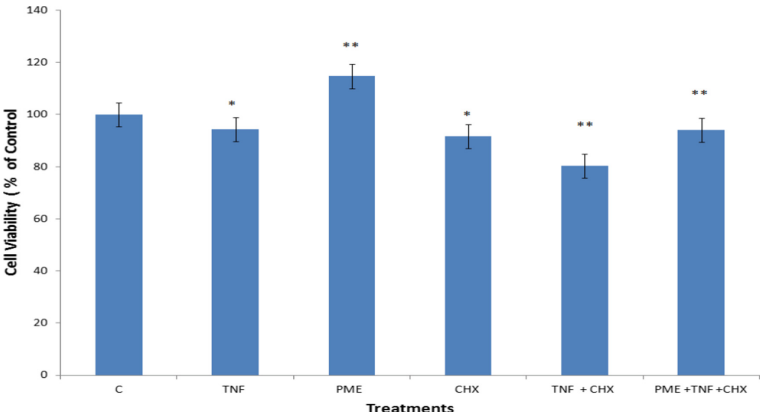

Fig. 1. Effect of PME pre-treatment on TNF-α and cyclohexamide induced apoptosis: Cells were treated with TNF-α (TNF, 20 ng/ml), cyclohexamide (CHX, 10 μg/ml), PME alone (80 μg/ml), combination of TNF-α(20 ng/ml), and cyclohexamide (CHX, 10 μg/ml) with and without PME pre-treatment (80 μg/ml for 12 h). The viability of cells was analyzed after 24-h treatment. The cell viability was determined by MTT assay. Cell survival was expressed as percentage over the untreated control. Results are expressed as mean ± S.E. of the five replicates over the untreated control. *$P < 0.05$, ** $P < 0.01$ vs control.

TNF-α (20 ng/ml) and cyclohexamide (CHX) (10 μg/ml) induced apoptosis in these cells (Fig. 1). PME (80 μg/ml) could rescue cells from TNF-α (20 ng/ml) and cyclohexamide (CHX) (10 μg/ml) induced loss of cell viability.

3.2 Effects of PME on TNF-A-Induced Pro-apoptotic and Anti-apoptotic Genes

We also examined the transcript levels of pro-apoptotic proteins like Bax, caspase 9 and anti-apoptotic gene BCL2 by concomitant treatment with TNF-α and cyclohexamide with and without pre-treatment with PME. It was observed that the increased expression of caspase 9 was reversed upon treatment of PME at transcriptional levels while BCL2 was found to be elevated upon PME pre-treatment (Fig. 2), although Bax expression did not show a significant downregulation. Hence, the ratio of BCL2 to Bax was evaluated, since it is one of the factors that decide the fate of cells in undergoing apoptosis. It was observed that PME could increase the BCL2/Bax compared to TNF + CHX treatment, essentially demonstrating that PME pre-treatment can reduce apoptosis of endothelial cells from TNF + CHX treatment.

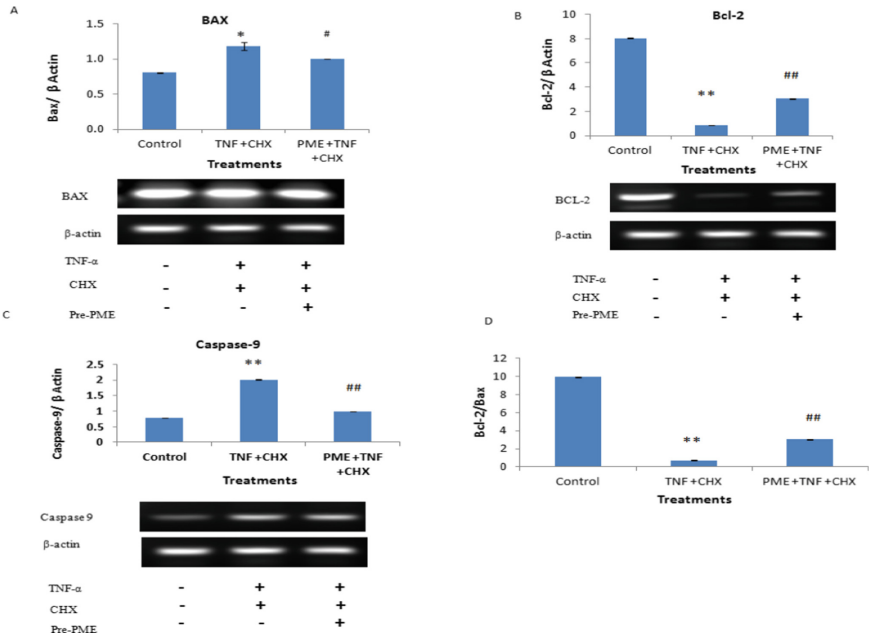

Fig. 2. Effect of PME pre-treatment on TNF-α and cyclohexamide induced changes in BAX, BCL2, CASP 9 expression levels in endothelial cells. EA.hy926 were stimulated with TNF-α (20 ng/ml) and cyclohexamide (CHX, 10 μg/ml) with and without 12-h pre-treatment of PME (80 μg/ml) and mRNA was extracted after 6 h and transcript levels and relative expression of BAX (A), BCL2(B), CASP 9 (C) were assessed as described in the materials and methods. Data shown are means ± SEM (n = 3).D) The relative expression of BCL2 with BAX after normalization with endogenous control β actin was calculated and plotted for treated and untreated endothelial cells. *P < 0.05, ** P < *0.01 vs control,#P < 0.05 Vs TNF + CHX.## P < 0.01 Vs TNF + CHX.*

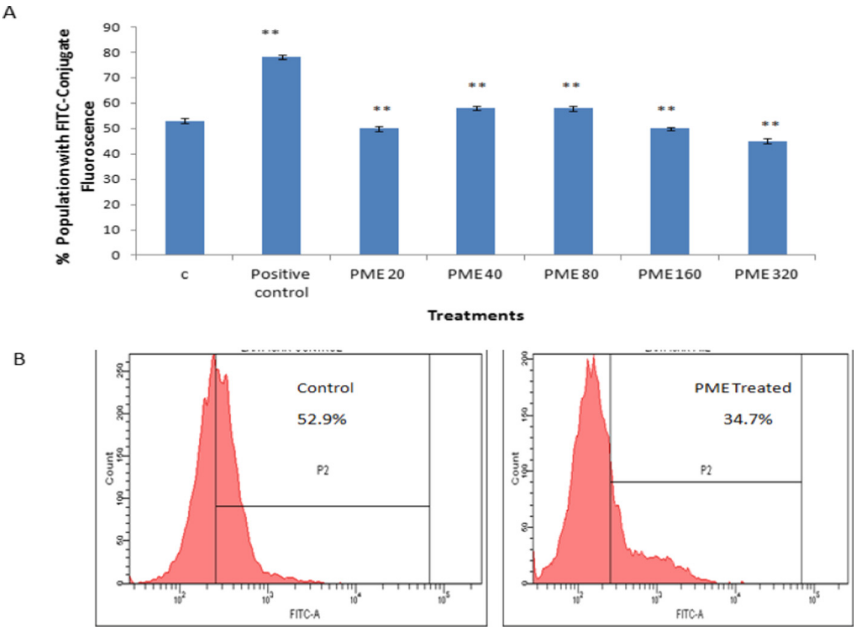

Fig. 3. Assessing oxidative damage and Redox upon PME treatment: a) DNA adduct formation which was measured by fluorometric oxy DNA assay in PME treated (20, 40, 80, 160 and 320 μg/ml) and a positive control H_2O_2. Even higher doses (160–320 μg/ml) did not induce oxidative damage when compared to the augmented fluorescence in positive control treated with H_2O_2 (54.1% to 77.8%). DNA adduct formation was calculated and expressed as mean ± S.E. of the 3 replicates over the untreated control. b) Intracellular ROS level estimated with DCFH-DA demonstrated that PME could bring down ROS levels from 52.9% to 34.7%. *P < 0.05, **P < 0.01 vs control.

3.3 Effect of PME on Oxidative Damage

8-oxo-20-deoxyguanosine (8-oxo-G) is the first product of oxidative attack to DNA [12], which marks an index of DNA adduct formation which was measured by fluorometric oxyDNA assay. The assay utilizes oxyDNA-FITC conjugate to probe 8-oxoguanine residues on oxidatively damaged DNA. It was found that PME, even at higher doses (160–320 μg/ml), did not induce biologically relevant oxidative damage (Fig. 3.A) when compared to the augmented fluorescence in positive control treated with H_2O_2 (54.1% to 77.8%). PME at doses of, 160 and 320 μg/ml showed protective effects. Additionally, the estimation of intracellullar reactive oxygen species (ROS) levels with DCFH-DA dye using FACS analysis (Fig. 3.B) showed that PME could bring down the levels of ROS. This clearly suggested that PME can prevent oxidative stress in endothelial cells.

3.4 Effect of PME on Expression of Adhesion Molecule in the Presence of TNF-α

TNF-α is known to induce cell adhesion by inducing expression of adhesion molecules like ICAM1and VCAM1 [13], which are known to mediate monocyte adhesion to

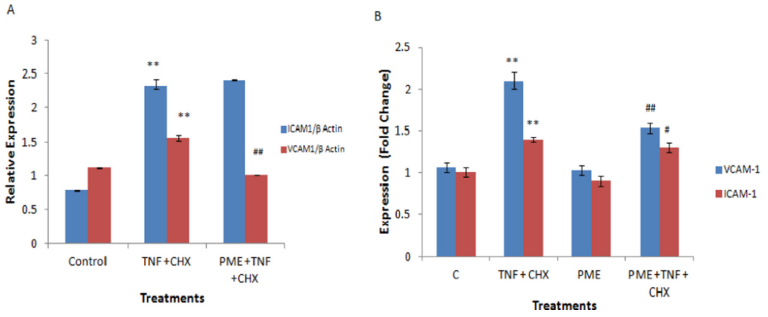

Fig. 4. Effect of PME pre-treatment on TNF-α and cyclohexamide induced changes in VCAM1 and ICAM1 expression in endothelial cells. EA.hy926 were stimulated with TNF-α (20 ng/ml) and with and without 12-h pre-treatment of PME 80 (μg/ml) for 6 h at 37 °C. Measurements of expression of ICAM-1 and VCAM-1 was done with A) ELISA and B) RT-PCR as described in the materials and methods. The relative expression of B.1) ICAM and B.2) VCAM was calculated are expressed as mean ± S.E. of the three replicates over the untreated control. *P < 0.05, **P < 0.01 vs control, #P < 0.05 Vs TNF + CHX..## P < 0.01 Vs TNF + CHX.

endothelial cells. Hence, the next step was to check their gene expression levels with and without pre-treatment with PME. Cell ELISA assay demonstrated that TNF-α could augment the expression of VCAM-1 significantly, but not ICAM-1. (Fig. 4.A). This was further confirmed with RT-PCR (Fig. 4.B). Pre-treatment of these cells with PME in TNF-α treated cells reduced the TNF-α mediated expression of VCAM-1 significantly and not ICAM-1, in concordance with the ELISA results. PME alone did not produce significant change in the expression of these molecules, indicating absence of any adverse effect of PME.

4 Discussion

Pomegranate mediates its anti-atherosclerotic effects by various mechanisms [14]. Invivo studies have shown that pomegranate juice reduces macrophage total cholesterol and triglyceride, and low density lipoprotein (LDL) and oxidized (ox) LDL uptake [15]. Pomegranate juice or its liquid extract is shown to attenuate development of atherosclerosis on aorta in mouse models [14, 16]. Pomegranate extract consumption on coronary artery disease models have demonstrated that through the reduction of inflammation and oxidative stress at the blood vessels [17] it can reduce aortic sinus and coronary artery atherosclerosis. At clinical levels, pomegranate extract along with simvastatin has been reported to reduce oxidative stress and lipid levels, cholesterol biosynthesis and triglycerides in hypercholesterolemic patients [18]. Earlier studies from our laboratory also showed reduced LDL and tricglyceride levels upon PME treatment in ovariectomized mice [2]. Also, there have reports indicating that SERMs can have protective role in endothelial dysfunction.Notably plant extract has also been proposed as prospective SERMs [19]. With this background, we have attempted here to evaluate the molecular mechanisms of pomegranate methanolic extract (PME), a reported SERM [2], involved invitro in TNF-α mediated deleterious effects on endothelial cells.

This was aimed to understand the potential of PME in rescuing cells from undergoing apoptosis, alteration in cell adhesion, and oxidative status of endothelial cells upon TNF-α induction, given the importance of these events in atherosclerosis.

Studies have shown that TNF-α mediated apoptosis can be attenuated by estrogen and estrogen agonists in different cells [20, 21]. Hence, the first attempt was to evaluate the ability of PME in protecting endothelial cells from induced apoptosis. However, the estrogen receptor mediated action of PME has not been investigated in the study. TNF-α is well studied risk factor for progression of atherosclerotic disease since it is understood to play a critical role in vascular injury, apoptosis and inflammation [22] all of which can possibly lead to atherosclerosis [23]. Also, TNF-α mediated inflammatory response is reported to be estrogen receptor mediated [24]. TNF-α initiates inflammatory reaction in mammalian cells which can be transformed to programmed cell death by two ways, that is, one by co-treatment with a protein synthesis inhibitor cycloheximide, or by employing Smac mimetic [25]. In this study, cyclohexamide was used to direct the cells to undergo apoptosis. We found by MTT assay that PME could rescue a population of cells from TNF-α induced apoptosis (Fig. 1). We went on to check the mRNA level expression of pro-apoptotic and anti-apoptotic genes. Transcriptional level ratio of BCL2 to BAX was markedly downregulated upon TNF-α + CHX treatment and this was reversed upon pre-treatment with PME indicating that PME reduced TNF-α mediated apoptosis, which is concordant with studies that it is not the individual expression of these proteins but their the relative ratios of that determines survival or death to the apoptotic stimuli [26]. In addition, PME downregulated mRNA levels of caspase 9, an initiator caspase (Fig. 2.C) which was otherwise augmented upon TNF-α + CHX treatment. Notably, estrogen by modulating BCL2 and BCL-xL expression is understood to inhibit apoptosis induced by TNF-α and oxidized LDL induced in endothelial cells [21]. Probably, PME being a SERM [2] has a similar mechanism. Our study clearly throws light on the role of PME in regulating levels of BCL2 and BAX and their downstream effectors like caspase 9 in attenuating the undesirable effects of TNF-α.

Another interesting property reported of certain selective estrogen modulators is their ability to activate antioxidant signaling pathway [27]. PME could reduce the levels of oxo-20-deoxyguanosine levels which is an important marker of oxidative tissue injury (Fig. 3.A). This clearly indicated absence of DNA adducts formation in endothelial cells. Additionally, the extract reduced the ROS levels in endothelial cells (Fig. 3.B). Interestingly, pomegranate juice has been found to reduce oxidative stress at clinical levels as well [28].One of the TNF-α induced apoptosis pathway is reported to be via mitochondrial ROS [29]. *Invivo* studies on ovariectomized spontaneously hypertensive female rats showed that pomegranate extract improves the coronary relaxation which is endothelium-dependent by reducing oxidative stress and enhancing cardiovascular parameters [30]. Furthermore, oxidative stress or ROS by various pathways is known to lead to the progression of atherosclerosis by decreasing nitric oxide levels or by increasing adhesion molecules like VCAM-I and ICAM-I [31, 32]which promote atherosclerosis [33]. PME was found to reduce the expression of TNF-α induced VCAM-1 (Fig. 4), probably by reducing ROS levels.

Additionally, it has been reported that estrogen pre-treatment is noted to reduce TNF-α induced ICAM and VCAM expression [34] and reduce ROS levels in endothelial cells

[35]. Pomegranate is rich in ellagitannins, which are protective in cardiovascular diseases by various mechanisms. Within the body ellagitannin is broken down to ellagic acid and further to urolithins by specific gut microbiota [1]. Interestingly, the constituents of pomegranate and its colonic microflora metabolites urolithins have been also tested for its ability to rescue from TNF-α induced damage to endothelial cells and its adhesion to monocytes [36, 37]. It is plausible that ellagic acid, its metabolites or other components in combination might be responsible for these effects of the extract.However since urolithins are synthesized by the microflora, the results here implies that the extract with its components in combination or individually are protective in cardiovascular health and might not require the presence of the bacteria which is an advantage for those who lack the microflora. Although the benefits of pomegranate extract cannot be directly extrapolated to clinical level, this might definitely throw light on the potential of natural extracts in cardiovascular health which is worthwhile to investigate at clinical levels, with emphasis on its molecular mechanism and its active components. Pomegranate has been used in different ayurvedic formulations like rasayanam which is given in indication of digestive and respiratory diseases making a truly functional, therapeutic and a potent nutraceutical. Our laboratory looking into the properties of urolithins, the colon microbiota metabolites of pomegranate and its possibility as a SERM in various tissues.

Possible mechanism of action of PME from the results obtained and previous literature has been summed up in Fig. 5 which can possibly reduce the vasculature damage that might lead to atherosclerosis.

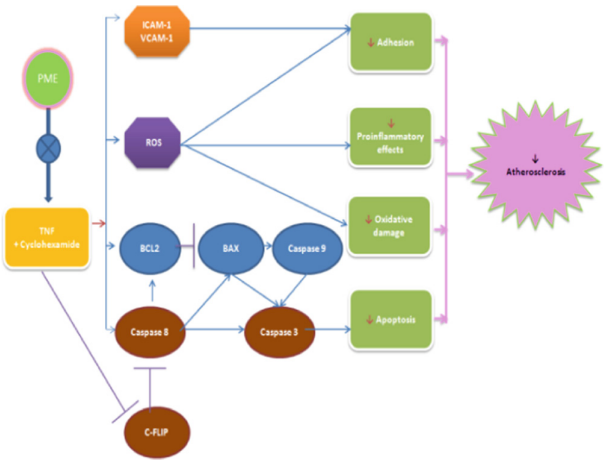

Fig. 5. Proposed Mechanism of PME in preventing TNF-α induced apoptosis: PME was found to reduce the levels of VCAM-1 gene expression and possibly reduce adhesion to monocytes. It could also alter BCL2/BAX ratio, pro-apoptotic genes like caspase which are elevated by TNF-α and diminish the ROS levels. The consequence of these events favors attenuation of TNF-α associated cardiovascular disorders like atherosclerosis. The brown colored boxes are molecules that have been previously reported to be involved in the pathway.

5 Conclusion

In summary, PME could reduce oxidative stress and associated elevated expression of adhesion molecules like VCAM, which has a prominent role in atherosclerosis by aiding leukocytes in migrating and adhering to the neighboring tissues, which is critical in inflammatory response and atherogenesis. PME could also rescue endothelial cells from TNF-α induced apoptosis by acting via various apoptotic and anti-apoptotic molecules and effectors that include BAX, BCL2 and caspases. These results are indicative of the pomegranate extract to have protective effects on atherosclerosis. Our earlier observation that the extract could reduce the overiectomy induced cholesterol rise also adds to proof of these properties.

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Authors' Contributions. VR, SSR.JMA and SS conceived the idea. VR, SSR, JMA performed the experiments. VR drafted the manuscript. SS reviewed the manuscript.

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