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Pomegranate extract demonstrate a selective estrogen receptor modulator profile in human tumor cell lines and in vivo models of estrogen deprivation

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Abstract

Selective estrogen receptor modulators (SERMs) are estrogen receptor (ER) ligands exhibiting tissue-specific agonistic or antagonistic biocharacter and are used in the hormonal therapy for estrogen-dependent breast cancers. Pomegranate fruit has been shown to exert antiproliferative effects on human breast cancer cells in vitro. In this study, we investigated the tissue-specific estrogenic/antiestrogenic activity of methanol extract of pericarp of pomegranate (PME). PME was evaluated for antiproliferative activity at 20–320 μg/ml on human breast (MCF-7, MDA MB-231) endometrial (HEC-1A), cervical (SiHa, HeLa), ovarian (SKOV3) carcinoma and normal breast fibroblast (MCF-10A) cells. Competitive radioactive binding studies were carried out to ascertain whether PME interacts with ER. The reporter gene assay measured the estrogenic/antiestrogenic activity of PME in MCF-7 and MDA MB-231 cells transiently transfected with plasmids coding estrogen response elements with a reporter gene (pG5-ERE-luc) and wild-type ERα (hEG0-ER). PME inhibited the binding of [^{3}H] estradiol to ER and suppressed the growth and proliferation of ER-positive breast cancer cells. PME binds ER and down-regulated the transcription of estrogen-responsive reporter gene transfected into breast cancer cells. The expressions of selected estrogen-responsive genes were down-regulated by PME. Unlike 17β—estradiol [1 mg/kg body weight (BW)] and tamoxifen (10 mg/kg BW), PME (50 and 100 mg/kg BW) did not increase the uterine weight and proliferation in ovariectomized mice and its cardioprotective effects were comparable to that of 17β-estradiol. In conclusion, our findings suggest that PME displays a SERM profile and may have the potential for prevention of estrogen-dependent breast cancers with beneficial effects in other hormone-dependent tissues.

Keywords: Breast cancer; Estrogen; Estrogen receptors; Pomegranate; Selective estrogen receptor modulator (SERM); Uterotrophic assay

1. Introduction

Pomegranate (*Punica granatum*, Punicaceae) is a native to Mediterranean region and has been used extensively in the folk medicine of many cultures [1]. Pomegranate fruit is a rich source of polyphenolic compounds like anthocyanidins (delphinidin, cyanidine and pelargonidin) and hydrolysable tannins (such as punicalgin, pedunculgin, punicalin, gallagic, ellagic acid esters of glucose), which account for 92% of antioxidant activity of whole fruit [2]. Various parts of the pomegranate fruit have been shown to exert antiproliferative, antiangiogenic, antiaromatase and proapoptotic effects on human breast cancer cell lines and chemopreventive properties in mouse mammary organ culture [3–5]. Previous studies have proved the high antioxidant activities of the methanol extract of pomegranate peel in various in vitro and in vivo models [6,7].

Estrogen is a pleiotropic hormone with multiple actions in reproductive tissues (such as breast, uterus and ovary) and in many non-reproductive tissues including bone, the central nervous system and the cardiovascular system [8]. Estrogen is implicated in the development of breast cancer, based on the data from both clinical and animal studies; risk factors associated with breast cancer reflect cumulative exposure of the breast epithelium to estrogen [9–11]. The best strategy for prevention and treatment of estrogen-dependent breast cancer is to selectively block estrogen activity in the affected tissues without compromising its beneficial effects [12]. Unfortunately, the currently available antiestrogen such as tamoxifen (TAM) used in the treatment of estrogen receptor [ER]-positive breast cancer carries side effects and agonism in the uterine endometrium, leading to a questionable connection to endometrial carcinoma [13–15].

Estrogen exerts its effects in target tissues by interacting with two different members of the nuclear receptor super family of hormone-regulated transcription factors, named ERα and ERβ [16–18]. After the binding of hormone to these receptors, the hormone–receptor complexes bind to specific sequences on the DNA [estrogen response elements (EREs)] or interact with other transcription factors without direct ERα or ERβ binding to DNA (*i.e.*, at activator protein 1, Sp1 and other sites) [19–21]. In both cases, liganded ERs recruit coregulator proteins and components of the transcriptional machinery to regulate the transcription of target genes [22–24].

Selective estrogen receptor modulators (SERMs) bind ER and exert estrogen agonist action in some target tissues while acting as estrogen antagonists in others [12]. SERMs may be possibly considered therapeutically for the inhibition of proliferation of breast ductal

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epithelium with maintenance of bone density and reduction in cholesterol levels without uterine endometrial proliferation [25,26]. The genes regulated by SERMs with ERα are distinct from those regulated by ERβ [27]. This shows that drugs targeted selectively to ERα or ERβ will produce more selective clinical effects. Since ERα promotes proliferation of breast cancer cells and ERβ acts as tumor suppressor, we hypothesize that ERα-selective antagonists might be effective in the prevention and treatment of estrogen-dependent breast cancer [28].

In this study, the methanol extract of pomegranate pericarp (PME) was analyzed for its possible SERM like property using human breast (MCF-7, MDA MB-231), endometrial (HEC-1A), cervical (SiHa, HeLa), ovarian (SKOV3) cancer cell lines, normal breast fibroblasts (MCF-10A) and in vivo models (ovariectomized Swiss albino mice) using biochemical markers of SERM activity. Our findings demonstrate that PME binds to ER and down-regulates the ERE-mediated transcription in breast cancer cells without being agonistic in the uterine endometrium and has cardioprotective effects comparable to that of 17β-estradiol.

2. Materials and methods

2.1. Cell and culture

MCF-7, MDA MB-231, HEC-1A, SiHa, HeLa, SKOV3 and MCF-10A (nontumorigenic) cell lines were obtained from American Type Culture Collection (Manassas, VA). MCF-7, MDA-MB-231, SiHa, HeLa and SKOV3 cells were cultured in phenol red-free Dulbecco's modified Eagles medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml benzyl penicillin and 100 μg/ml streptomycin. HEC-1A was grown in Mc-Coys 5A modified medium from American Type Culture Collection. MCF-10A was grown in MEGM medium supplemented with MEGM Single Quots (Lonza Group, Switzerland). The culture was maintained at 37°C in a humidified atmosphere of 5% CO_2. Estrogen-depleted media contained no PR and were supplemented with charcoal/dextran-treated FBS (FBS DCC, Hyclone; Thermo Fisher Scientific, Switzerland).

2.2. Preparation of pomegranate peel extracts

Pomegranate (*P. granatum*) peels were manually removed, dried and powdered to get 60 mesh sizes. The peel powder (10 g) was extracted with methanol (100 ml each) in a Soxhlet apparatus for 20 h. The extract was concentrated using rotary vacuum to get the solid mass. The yield obtained was 5% (w/v). The concentrate was dissolved in dimethyl sulfoxide (Sigma-Aldrich), referred as PME and was used for further experiments. Qualitative screening of PME was made to analyze the presence of alkaloids, flavonoids, polyuronides, phenols, reducing compounds, saponins and tannins [29,30]. Aluminum chloride colorimetric method was used for flavonoids determination (in terms of quercetin equivalents) and total phenols (in terms of gallic acid equivalents) were estimated by Folin-Ciocalteu reagent as described previously [31]. Phytochemical investigation has revealed the presence of phenolics, flavonoids and alkaloids in PME. The total phenols in terms of gallic acid equivalent (standard curve equation: $y=0.0648x+0.005$, $r^2=.9921$) was 363.97±11.19 mg/g in the extract powder. The flavonoid content of the extracts in terms of quercetin equivalent (standard curve equation: $y=0.0096x+0.0114$, $r^2=0.999$) was 118.83±4.71 mg/g of dry extract powder.

2.3. Cell viability assessment by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay

The 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT; Sigma-Aldrich) assay was used to measure cell viability [32]. Briefly, cells were plated at a density of 5000 cells/well in 96-flat-bottomed-well plates and treated with 20–320 μg/ml of PME, ICI 182–780 (100 nM; Sigma Aldrich) and 17β-estradiol (0 nM, E_2; Sigma Aldrich) with or without PME (80 μg/mL). After 48-h incubation, the medium was replaced with MTT dissolved at a final concentration of 2 mg/ml in serum-free, phenol red-free medium and incubated for 2 h at 37°C. Then, MTT-formazan was solubilized in lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide), and the optical density was measured spectrophotometrically at 570 nm. Cell survival was expressed as percentage over the untreated control. Cell survival (CS) was calculated as (OD drug exposed cells/mean OD control cells)×100.

2.4. BrdU incorporation assay

Cell proliferation was assayed using BrdU cell proliferation assay kit purchased from Calbiochem (San Diego, CA). Cells were seeded in 96-well plate at a density of 2×10^3 cells/well and incubated with 0, 20, 40, 80, 160 and 320 μg/ml of PME, ICI (100 nM) and E_2 (10 nM) with or without PME (80 μg/ml) for 48 h. BrdU (100 μM) was added to each well 3 h before termination of the treatment, and the colorimetric measurement was completed according to the kit manual. Each experimental point was assayed in five different wells, and each study was carried out in triplicates.

2.5. Competitive ER binding assay (hydroxyapatite assay)

Hydroxyapatite (HAP) assay was carried out to confirm the competitive binding of the extract to the ER [33]. Cytosol was prepared from MCF-7 cells grown in estrogen-depleted medium, and the protein content was measured spectrophotometrically at 570 nm using Bradford reagent. About 40 μg of the total protein was incubated overnight at 4°C with the varying concentration of PME (range 20–320 μg/ml) and 20 nM [^{3}H] estradiol ±100-fold molar excess of E_2. A 60% HAP suspension in TEM buffer was added, and the mixture was incubated at 4°C for 15 min. The HAP-bound receptor [^{3}H] E_2 complex was separated by centrifugation at 200×*g* for 15 min. After washing twice with Tris buffer (10 mM), the HAP pellet was extracted with 1 ml absolute ethanol. These extracts were added to 4 ml scintillation cocktail, and the radioactivity was measured in Wallac 1409 liquid scintillation counter. Data were expressed as the ratio of bound [^{3}H] E_2 in the presence of a competitor to the bound [^{3}H] E_2 in control ×100. IC_{50} value was calculated as the concentration of competitor required to reduce the specific radioligand binding by 50%.

2.6. Transient transfection and reporter gene assays

For each transfection experiment at 10^5 cells were plated per well in 12-well dishes in phenol red-free DMEM with 10% FBS DCC treated. After 24 h, MCF-7 and MDA-MB-231 cells were transfected with 2.5 μg ER (pHEG0-ER) and 2.5 μg pG5-ERE-luc expression vectors with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h, cells were washed once with PBS, and 2 ml phenol red-free DMEM with 10% FBS DCC treated was added, containing final concentrations of PME (80 μg/ml) or E_2 (10 nM) or 4-hydroxy TAM (500 nM). Protein was extracted 48 h later and firefly and *Renilla* luciferase activity were measured on a TD20/20 luminometer (Turner Designs, Sunnyvale, CA) using a Dual Luciferase Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase expression. Expression vectors were kindly provided by Dr. Paul Murugan (Molecular Imaging Program, Stanford University, Palo Alto, CA) and Bert W. O'Malley (Baylor College of Medicine, Houston, TX).

2.7. Reverse Transcriptase PCR

MCF-7 cells were incubated with phenol red-free DMEM supplemented with 10% charcoal-treated FBS for 48 h before using in the assay. After 48 h, test compounds [PME (40, 80, 160 μg/ml), E_2 (10 nM) and ICI (1 μM)] were added to this medium. After incubating for 24 h, total RNA was purified, cDNA was synthesized and RT-PCR was done according to the protocol that we previously described [34]. The primer pairs were ERα forward 5′-CCACCAACCAGTGCACCATT-3′, ERα reverse 5′-GGTCTTTTCGTATC CCACCTTTC-3′, pS2 forward 5′TTTGGAGCAGAGAGGA GGCAATGG3′, pS2 reverse 5′ TGGTATTAGGATAGAAGCACCAGGG3′, PR forward 5′-CGCGCTCTACCCTGCACTC-3′ and PR reverse 5′-TGAATCCGGCCTCAGGTA GTT-3′. The transcripts were normalized with GAPDH expression level.

2.8. Animals

The animal experiment protocol was approved by Institutional Animal Ethics Committee, Rajiv Gandhi Centre for Biotechnology, India, and was performed in accordance with guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. Thirty female Swiss albino mice (8 weeks old, 17–25 g) were randomized in treatment six groups of five animals each. Animals were housed at 23°C±5°C and relative humidity 60%±5% with 12-h light–dark cycle. Food (standard rodent pellet diet) and water was provided *ad libitum*. Four groups were bilaterally ovariectomized while one group was subjected to sham operation. Ovariectomy was performed under ketamine and xylazine anesthesia (80 mg/kg BW+10 mg/kg BW, ip) [35]. Animals were housed individually for a week after the surgery and later on in a group of five. Body weight of the animals was recorded daily.

2.9. Treatment and experiment design

Sham-operated (SH) and ovariectomized (OVX) control animals were administered orally with 0.1% ethanol, which was used as vehicle. The other four OVX groups were administered with E_2 (1 mg/kg BW), PME (50 and 100 mg/kg BW) and TAM (10 mg/kg BW). Doses of E_2, TAM and PME were selected on the basis of doses used by earlier researchers for the same activity and on the basis of LD_{50} value of polyphenols [36–38]. Drug treatments were started 14 days after ovariectomy and continued for 7 days. At necropsy, on day 8, blood was collected from dorsal aorta under anesthesia. After centrifugation, serum was harvested and kept at −20°C until analysis. Uterine horns were dissected free of adhering fat and mesentery, and absolute weight of uterine tissue was recorded and normalized with BW (relative weight of uterus, *i.e.*, weight of uterus per 100 g of BW) of animals.

2.10. Morphometric analyses

Sections of uterus were stained with hematoxylin and eosin and prepared for light microscopy. The radius of the uterine endometrium (×10 objective) occupied by the mucosa (luminal epithelium, glandular epithelium and lamina propria) and myometrium, and the height of the luminal epithelium (×20 objective) were determined using optical micrometry. Measurements were standardized using the image of a stage micrometer at the same magnification. Four areas in each of three transverse sections of uterus were analyzed per animal. For the parameter of epithelial cell height, four measurements were made within four areas of the three transverse sections of each uterus per animal.

2.11. Serum estradiol and other biochemical analysis

Blood samples were allowed to clot at 4°C for 2 h and then centrifuged at 2000×*g* for 10 min. This serum was transferred to new tubes and was used for performing the analysis. Serum E_2 levels were determined by chemiluminescence immunoassay using Bayer ADVIA Centaur (Siemens Medical Solutions Diagnostics). Serum lipid profile, alkaline phosphatase (ALP), calcium and phosphorus were analyzed using Olympus AU400 clinical chemistry analyzer (Olympus).

2.12. Statistical analysis

The experiments were performed in triplicates. All values were expressed as mean ±S.E., and Tukey's post hoc test was done to analyze significance of difference between different groups using the Statistical Analysis Software Package (version 16.0; SPSS, Chicago, IL). Values with $P \leq .05$ were considered significant.

3. Results

3.1. Cell growth inhibition in ER-positive breast cancer cells

Our primary aim was to investigate whether PME imparts inhibitory effects in breast cancer cells. MCF-7 (ER-positive), MDA-MB-231 (ER-negative) and MCF-10A (nontumorigenic) cells were treated with PME (20–320 μg/ml), ICI (100 nM) and E_2 (10 nM)±PME (100 μg/ml) for 48 h and the inhibitory and antiproliferative effects were studied using MTT assay and BrdU cell proliferation assay. As shown in Fig. 1A, PME treatment resulted in significant dose-dependent inhibition of cell growth in MCF-7 cell lines but not in MDA MB-231 and MCF-10A cell lines. PME also inhibited the E_2-induced proliferation in MCF-7 cell lines. PME decreased the incorporation of BrdU in MCF-7 cell lines proposing its antiproliferative and potential antiestrogenic properties (Fig. 1B).

3.2. Inhibition of the binding of labeled E_2 to ER

In an attempt to ascertain whether PME interacts with ER, competitive binding studies were carried out. MCF-7 cytosolic lysate was used as a source of ER. Displacement curves for E_2 and PME are shown

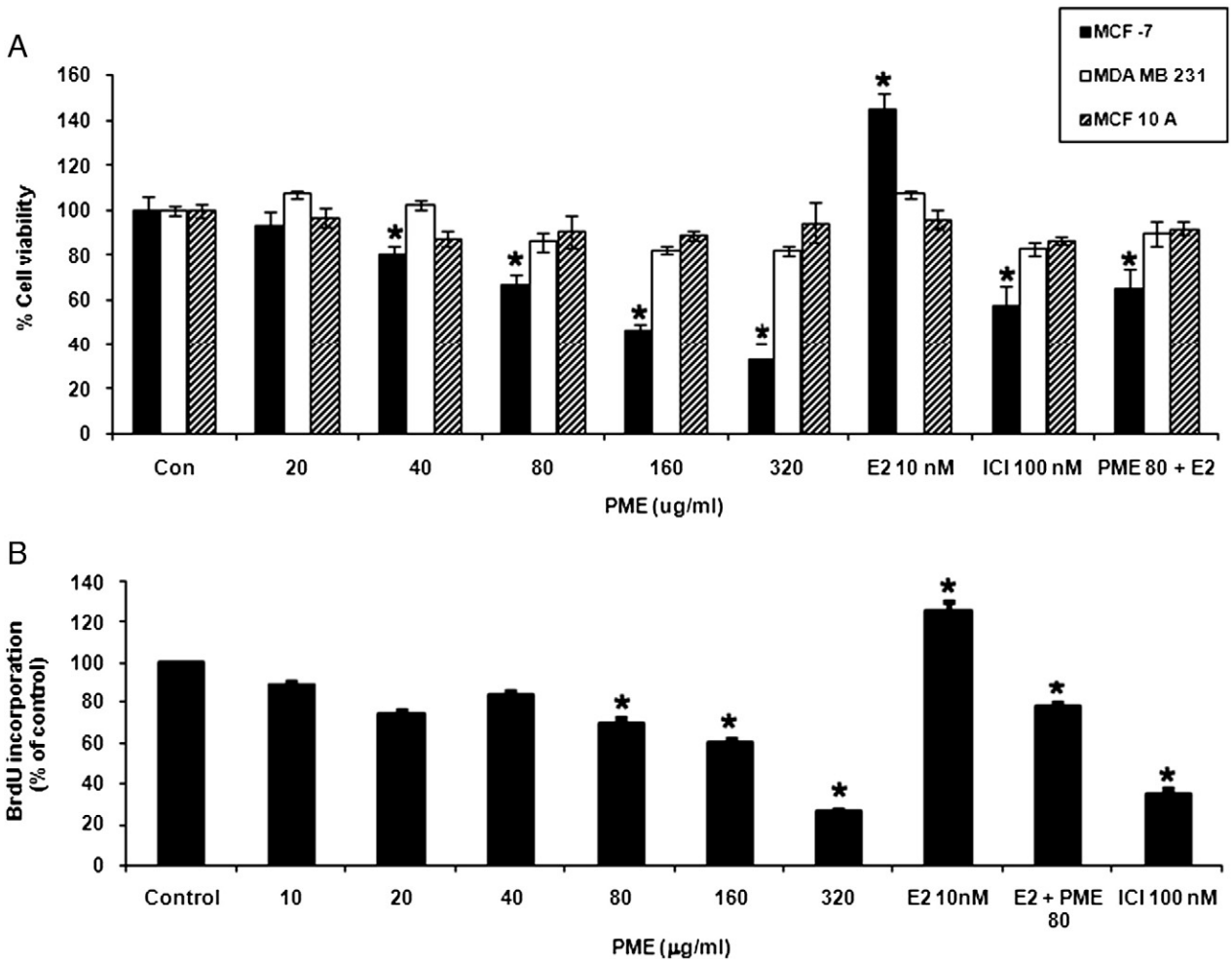

Fig. 1. Effect of PME on cell viability/proliferation. MCF-7, MDA MB-231 and MCF-10A cell lines were treated with 0, 20, 40, 80, 160 and 320 μg/ml of PME, ICI 182–780 (100 nM) and E_2 (10 nM) with or without PME (100 μg/ml) for 48 h, and the cell viability was determined by MTT assay. The cell survival was expressed as percentage over the untreated control. A dose-dependent growth inhibition of MCF-7 cells was observed after treatment with PME. Results are mean values±S.E. of five replicates. *P<.05 when compared with untreated control. (B) MCF-7 cells were treated with 0, 20, 40, 80, 160 and 320 μg/ml of PME, ICI 182–780 (100 nM) and E_2 (10 nM) with or without PME (100 μg/ml) for 48 h. Cell proliferation was determined by BrdU incorporation.

in Fig. 2. PME (20–320 μg/ml) displaced specifically bound [^{3}H] E_2 in a concentration-dependent manner with an IC_{50} value (the concentration of the extract required to reduce the specific radioligand binding by 50%) of 171.31 μg/ml.

3.3. Down-regulation of the ER-mediated transcription via ERE

We examined the effect of PME on transcriptional activity of ERα in MCF-7 (ER-positive) and MDA MB-231 (ER-negative) human breast cancer cell lines. The cells were cotransfected with ERE containing luciferase reporter plasmid and an ER expression plasmid. ERα expression plasmid was provided to ensure a high level expression of ERα under all assay conditions. Fig. 3 demonstrates the effect of control (absence of compounds), E_2 (10 nM), PME (80 μg/ml) and TAM (500 nM) on luciferase activity in MCF-7 and MDA MB- 231 cells transfected with ERE and ERα. E_2 and TAM were used as controls for agonistic and antagonistic activity respectively. In MCF-7 and MDA MB-231 cells transfected with ERα, incubation with E_2 significantly increased (P<.05) the luciferase activity at a concentration of 10 nM compared with control. Tamoxifen reduced the luciferase activity significantly (P<.05) at a concentration of 500 nM. PME decreased the basal luciferase activity at a concentration of 80 μg/ml in MCF-7 cells and MDA MB-231 cells transfected with ERα. The basal luciferase activity was not altered by any of the treatments in MDA MB-231 cells without ER transfection.

3.4. Down-regulation of estrogen responsive genes by PME

The effect of PME on ERα, pS2 and PR gene expression was studied as a model for endogenous estrogen responsive gene expressed by MCF-7 cells. E_2 and ICI were used as agonistic and antagonistic controls respectively. MCF-7 cells were incubated with PME (40–160 μg/ml) or E_2 (10 nM) or ICI (1 μM) for 24 h and RT PCR was performed to amplify the ERα, pS2 and PR messages using GAPDH as endogenous control. As shown in Fig. 4, PME decreased the expression of ERα, pS2 and PR gene to levels approximately as great as those produced by ICI. E_2 up-regulated the expression of these genes except ERα PME suppressed this estrogen-enhanced gene expression.

3.5. Cell growth inhibition in endometrial, cervical and ovarian cancer cells

HEC-1A, SiHa, HeLa and SKOV3 cells were treated with PME (20–320 μg/ml), ICI (100 nM) and E_2 (10 nM)±PME (80 μg/ml) for 48 h, and the inhibitory effects were studied using MTT assay. In Fig. 5, the dose–response curve concerning the effect of PME on cell viability of HEC-1A, HeLa and SKOV3 cells revealed that, at low concentrations, there was no stimulation above control and no inhibitory effect on cell proliferation. However, PME showed a growth inhibitory effect, statistically signifant in SiHa cells at very high concentrations (320 μg/ml).

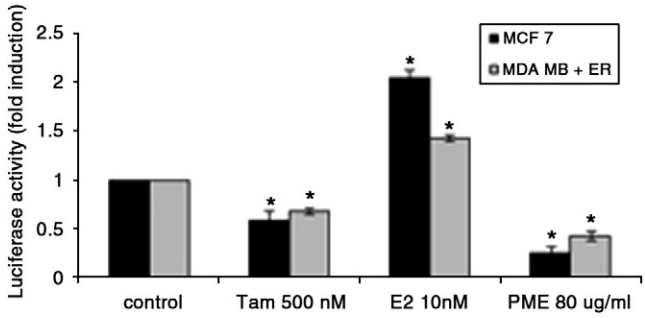

Fig. 3. PME down-regulates the ER-mediated transcription via ERE. MCF-7 and MDA-MB-231 cells transfected with ER (pHEG0-ER) and *pG5* ERE-luc expression vectors were incubated with PME (80 μg/ml), E_2 (10 nM) or TAM (500 nM) and assayed for luciferase activity. Results are shown as fold induction compared with control cells.

3.6. Uterotropic effect in the uterus of ovariectomized mice

The uterine wet weights and epithelial heights were the main physiological endpoints utilized for the assessment of estrogenecity. The positive control E_2 increased absolute and normalized uterine wet weight in OVX animals by approximately two times at a dose of 1 mg/kg BW compared with the vehicle-treated OVX control (Fig. 7; P<.05). The uterine weight did not differ significantly between the groups that received PME (50 and 100 mg/kg BW) and the vehicle-treated OVX control group, indicating the lack of estrogenecity of PME on uterine endometrium in the doses tested in our study. Tamoxifen produced positive uterotrophic effect at a dose of 10 mg/kg BW in comparison to the OVX control (Fig. 7, P<.05).The gross morphology of uterus appeared normal in all the treatments, and E_2 treatment exhibited a substantial increase in the radius of uterine horns in comparison with the SH control and OVX control mice (Figs. 6 and 7; P<.05). Based on histology, a proliferative response of the uterine epithelium was observed in E_2- and TAM-treated mice in comparison to the SH control and OVX control mice. There was no evidence of

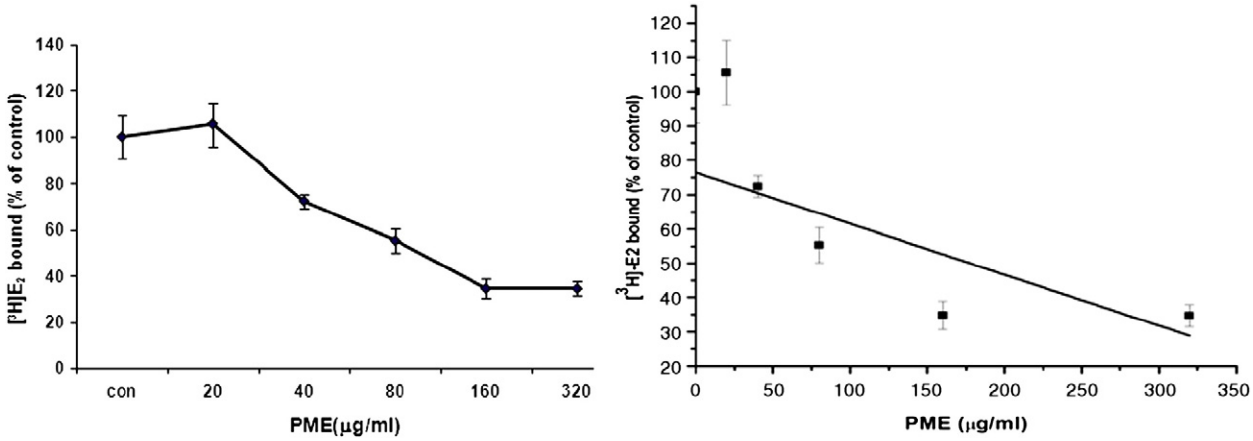

Fig. 2. PME inhibits the binding of labeled E_2 to ER. Binding of 20 nM [^{3}H] E_2 to cytosolic ER in the presence of varying concentrations (20–100 μg/mL) of PME. Specific bound radioligand was calculated by subtracting nonspecific bound counts from total bound counts. All results are shown as percentage of binding in the absence of competitor. Data presented as mean±S.E. from three separate experiments for each data point. *P<.05 when compared with untreated control.

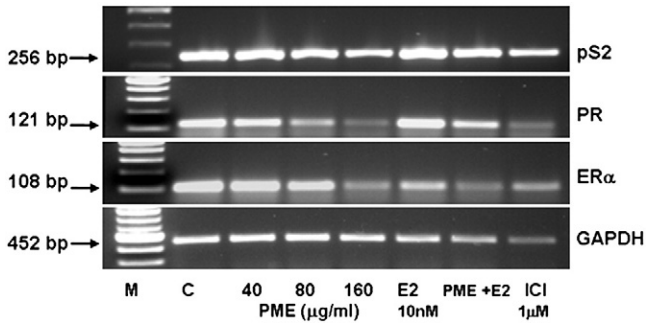

Fig. 4. PME down-regulates pS2, PR and ERα gene expression in MCF-7 cells. MCF-7 cells were incubated with the PME (40, 80 and 160 μg/mL), E_2 (10 nM) or ICI (1 μM) for 24 h [4].

luminal epithelial proliferation in response to PME treatment in comparison to the OVX control (Figs. 6 and 7).

3.7. Effect on serum lipid profile

The effect of PME, E_2 and TAM on serum lipid profile is illustrated in Table 1. Fourteen days after ovariectomy, a significant increase in serum total cholesterol and triglyceride levels was observed in OVX controls, compared with SH control ($P<.05$). In OVX mice, E_2 (1 mg/kg BW) and PME (100 mg/kg BW) significantly decreased serum cholesterol and triglyceride levels compared with both SH and OVX controls (Table 1; $P<.05$). Administration of 100 mg/kg BW PME and 1 mg/kg BW E_2 reduced LDL cholesterol levels in OVX mice compared with those of OVX controls (Table 1; $P<.05$).

4. Discussion

Pomegranate fruit is very rich in constituents such as flavonoids, polyphenols and phytoestrogens [1]. Many studies in vitro and in vivo concerning pomegranate extracts and its individual constituent's support that they are potent antioxidants and demonstrate antimetastatic and antitumor activity [3–5]. The main concerns of food and nutrition research, however, are the properties of plant extracts as a whole, since these may form the basis for the development of "functional foods."

The objective of our study was to determine the SERM activity of PME in breast and uterine cells and lipid metabolism. We used in vitro assays to reflect the estrogenecity/antiestrogenecity of compounds, *i.e.*, cell viability assays, competitive binding studies, reporter gene analysis as well as analysis of endogenous estrogen sensitive markers [39–42]. In vivo assay for estrogenecity, *i.e.*, rodent uterotrophic assay, in ovariectomized mice models was used to check the ability of PME to stimulate uterine growth [43–45]. Markers of lipid metabolism were analyzed to study the beneficial effects of PME in comparison to E_2 and the chemopreventive agent for breast cancer in use, TAM.

Estrogens stimulate the growth of breast cancer cells, whereas antiestrogens arrest its growth [46]. To assess the growth promoting/inhibitory effects of PME, we applied MTT cell viability assay. Since the results depend on the number of cells present and on the mitochondrial activity per cell, it is a very useful assay for cell proliferation and survival [39]. The measurement of cell proliferation or DNA synthesis was done by determining the incorporation of BrdU into cellular DNA. PME inhibited MCF-7 cellular proliferation in a way similar to that of ICI. PME also inhibited E_2-induced proliferation in MCF-7 cells. The extract failed to elicit a significant inhibitory effect on ER-negative tumorigenic as well as nontumorigenic cell lines, suggesting a possible involvement of ER in the inhibitory role of PME. This was further confirmed by competitive radioactive binding studies, which showed that PME binds ER and inhibited the binding of labeled E_2 to ER in a dose-dependent manner.

The competitive binding assay measured the specific binding of the PME to ER but provides little information on whether it activates or inhibits the downstream signaling cascade. The reporter gene expression assay measures estrogenic activity through binding of ERα in MCF-7/MDA MB-231 cells transiently transfected with a plasmid coding EREs with a reporter gene (ERE-luc) and a plasmid coding for ERα. E_2 induced ERE expression via ERα, whereas PME down-regulated the ERα-mediated transcription via ERE in both MCF-7 and MDA MB-231 cells transiently transfected with ERα, suggesting its antiestrogenic effect.

In this study, we have demonstrated that PME inhibited the E_2-dependent transcriptional activity of ERα in a dose-dependent manner. This suppression was associated with an inhibition of expression of ER target genes, PR and pS2. Progesterone plays an important role in mammary gland physiopathology, and PR as well as pS2 has been used as an indicator of breast cancer progression

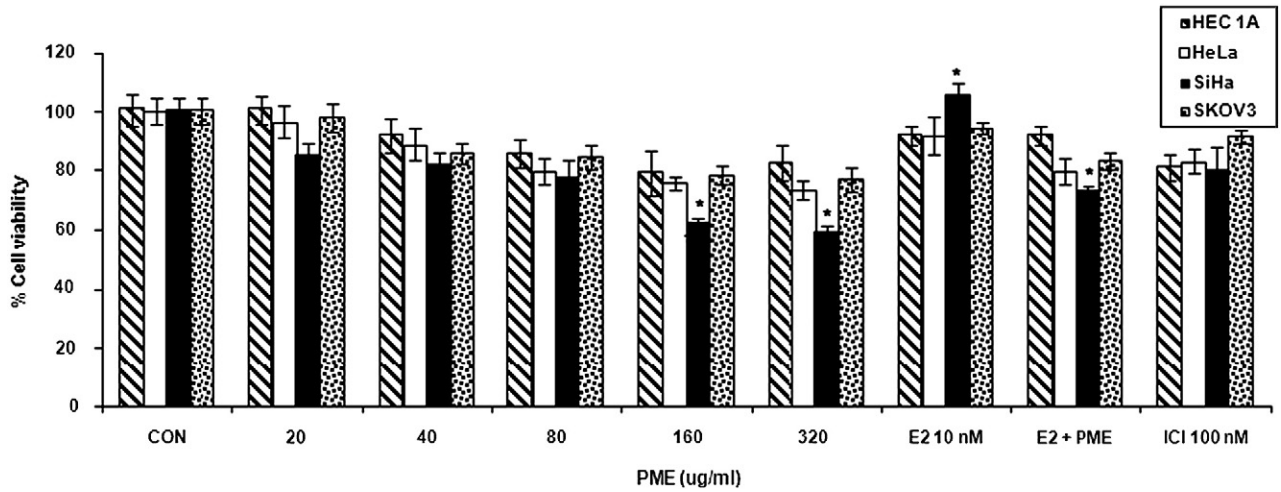

Fig. 5. Effect of PME on cell viability/proliferation. HEC 1A, HeLa, SiHa and SKOV3 cell lines were treated with 0, 20, 40, 80, 160 and 320 μg/ml of PME, ICI 182–780 (100 nM) and E_2 (10 nM) with or without PME (100 μg/ml) for 48 h, and the cell viability was determined by MTT assay. The cell survival was expressed as percentage over the untreated control. A dose-dependent growth inhibition of MCF-7 cells was observed after treatment with PME. Results are mean values±S.E. of five replicates. *$P<.05$ when compared with untreated control.

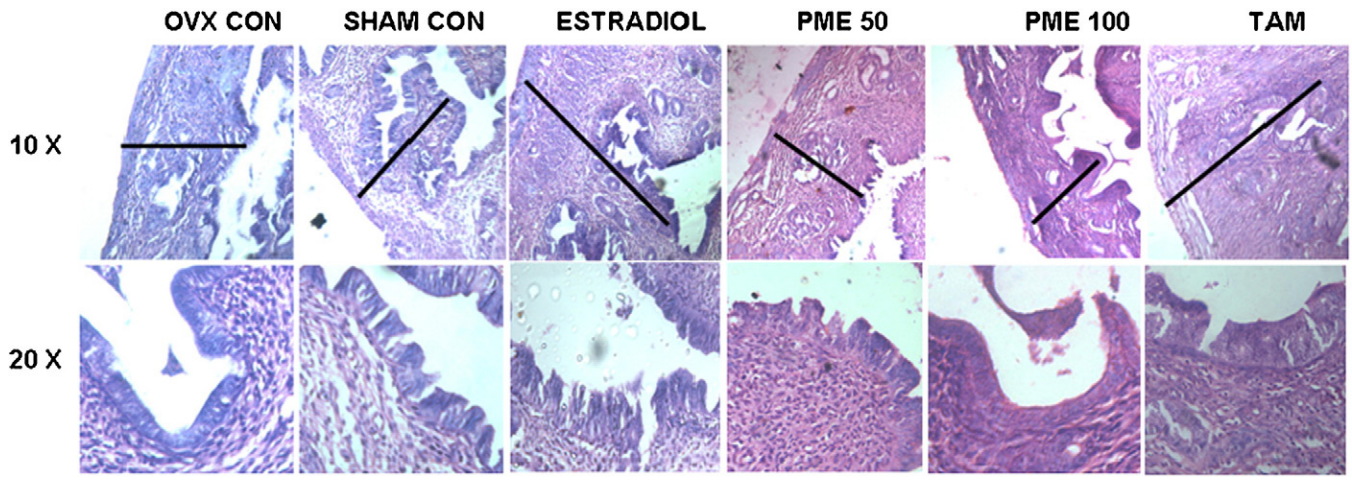

Fig. 6. Effect of the test compounds in uterine histology. Photomicrographs showing the uterine radius (×10) and luminal epithelium (×20) from mice exposed for 7 days to vehicle (0.1% ethanol), E_2 (1 mg/kg BW), PME (50 and 100 mg/kg BW) and TAM (10 mg/kg BW). Representative longitudinal sections stained with hematoxylin and eosin are shown.

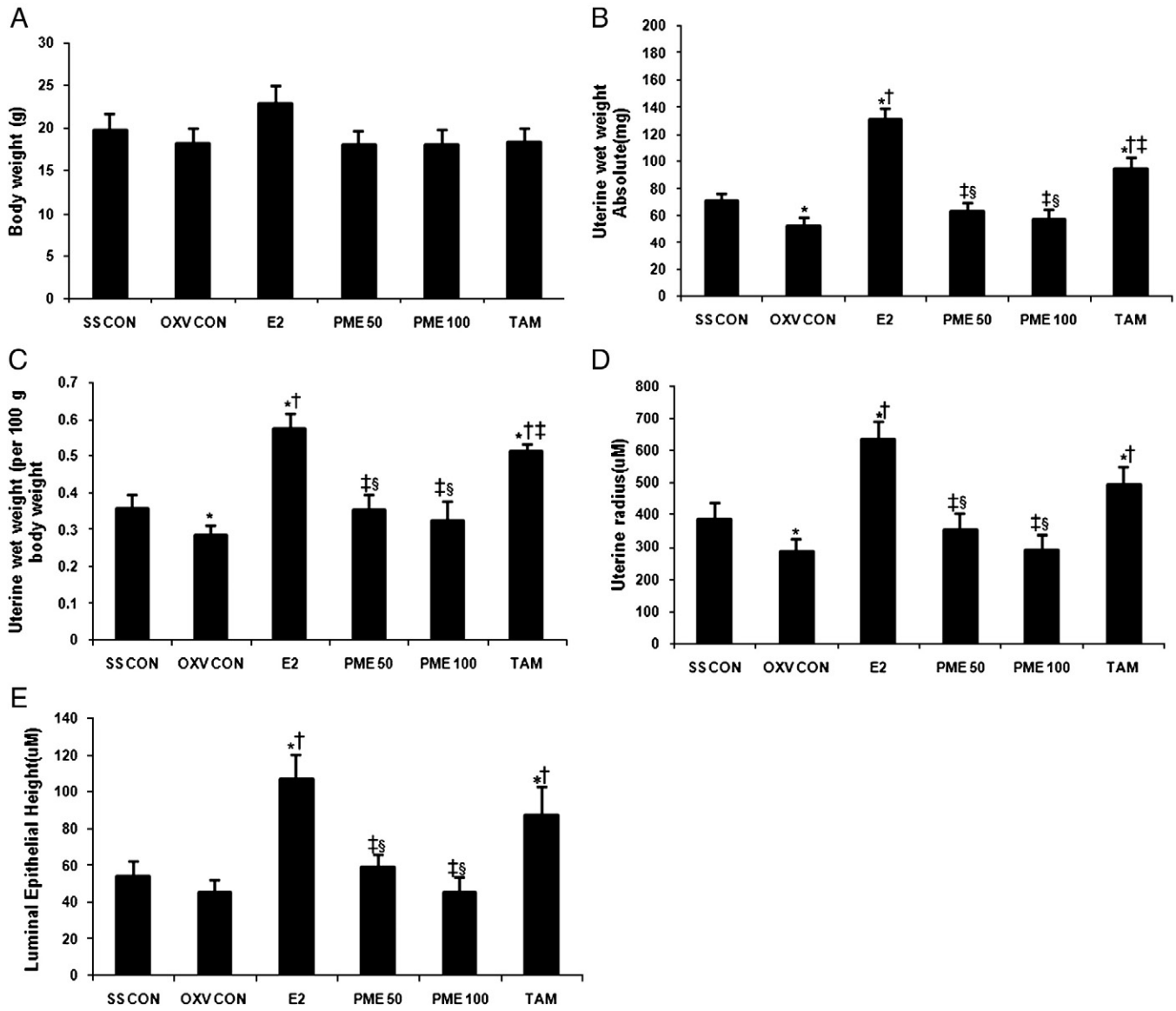

Fig. 7. Body weight, uterine wet weight (expressed as absolute values and as percentage of BW), uterine radius and uterine luminal epithelial height, of SH control and OVX mice exposed to 0.1% ethanol, E_2 (1 mg/kg BW), PME (50 and 100 mg/kg BW) and TAM (10 mg/kg BW) for 7 days. Data expressed as mean±S.E. (n= 5). *P<.05 *vs.* SH control, †P<.05 *vs.* OVX control, ‡P<.05 *vs.* E_2, §P<.05 *vs.* TAM.

Table 1
Effect of E_2, PME and TAM on serum lipid profile of sham-operated and ovariectomized mice

	SH control	OVX control	E_2	PME 50	PME 100	TAM
Total cholesterol (mg/dl)	60.2±12.31	85.8±4.32[a]	63.2±8.64[b]	69±10.60	64.8±7.01[b]	67.8±10.84
HDL cholesterol (mg/dl)	34.8±8.89	30±3.16	37±3.74	32.4±3.78	30.8±1.92	34.6±5.31
LDL cholesterol (mg/dl)	16.8±3.70	22.2±1.92	11.6±1.67[b]	15.8±4.71	13.6±1.94[b]	14.2±0.84[b]
Triglycerides (mg/dl)	57±5.43	101±8.94[a]	75.4±7.3[a]	75±3.6[b]	73±4.47[b]	96.4±4.03

Serum lipid profile of SH control and OVX mice exposed to 0.1% ethanol, E_2 (1 mg/kg BW), PME (50 and 100 mg/kg BW) and TAM (10 mg/kg BW) for 7 days.
Data expressed as mean±S.E. (n= 5).
HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a] P<.05 *vs.* SH control.
[b] P<.05 *vs.* OVX control.

and a predictor for TAM resistance of breast tumors [47–50]. ERα transcripts were also strongly reduced in cells incubated with PME. These results clearly show that PME is a negative regulator of ERα transcription signaling. Since ERα, and not ERβ, promotes proliferation of breast cancer cells, PME as an ERα antagonist could be a promising alternative in breast cancer therapy [28].

Antiestrogen-based drug therapy in breast cancer chemoprevention has been difficult because of the effect of the drugs to the uterine endometrium [51]. A troublesome side effect of TAM in women has been its hypertrophic effect on the uterus [52]. It is likewise an estrogen agonist in the murine uterus. The uterine growth studies reaffirms that TAM act as an estrogen agonist on the murine uterus, even though it antagonizes the effect of estrogen on cancer of the breast. PME, on the other hand, inhibited the proliferation of endometrial, cervical and ovarian carcinoma cell lines and had no uterotrophic effect whatsoever, even at a higher dose, suggesting that PME will target breast cancer without causing estrogenic effects in the uterine endometrium.

SERMs such as TAM and raloxifene are found to be cardioprotective [53,54]. The present study reveals that PME shares some actions on lipid metabolism with other antiestrogens. OVX animals given PME and E_2 had a marked decrease in total cholesterol and triglyceride levels compared with OVX controls. Both total cholesterol and triglyceride levels were increased in OVX mice. These were attenuated considerably by treatment with PME and that was comparable with that of E_2 and TAM. This lipid lowering partially explains the ER agonistic effects of PME on cardiovascular system in ovariectomized mice models, which mimic estrogen deprivation in postmenopausal women.

Our findings demonstrate that PME binds to ER, translocates the ligand receptor complex to the nucleus and down-regulates the ERE-mediated transcription in breast cancer cells without being agonistic in the uterine endometrium and has cardioprotective effects comparable to that of E_2. In our experiments, we cannot attribute the biological effects observed to particular constituents because many other compounds are present in PME. Our data support that PME show greater effect than TAM alone, implicating those combinations of constituents present in PME may be highly important in the final biological activity.

The findings provided evidence for SERM activity of *P.granatum* pericarp and its possible potential application as a hormonal therapy for estrogen-dependent breast cancers and as an alternative to hormone replacement therapy. Further in vitro and in vivo studies and characterization of the active components of PME will shed light on its health-promoting effects and suitability in hormone-dependent breast cancer therapeutics.

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