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POMEGRANATE (*PUNICA GRANATUM*) SEED LINOLENIC ACID ISOMERS: CONCENTRATION-DEPENDENT MODULATION OF ESTROGEN RECEPTOR ACTIVITY

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□ *Pomegranate* (Punica granatum) *seed linolenic acid isomers were evaluated as selective estrogen receptor modulators (SERMs) in vitro. Punicic acid (PA) inhibited (IC_{50}) estrogen receptor (ER) α at 7.2 μM, ERβ at 8.8 μM; α-eleostearic acid (AEA) inhibited ERα/ERβ at 6.5/7.8 μM. PA (not AEA) agonized ERα/ERβ (EC_{50}) at 1.8/2 μM, antagonizing at 101/80 μM. AEA antagonized ERα/ERβ at 150/140 μM. PA and AEA induced ERα and ERβ mRNA expression in MCF-7, but not in MDA-MB-231. Overall, the results show PA and AEA are SERMs.*

Keywords Breast cancer, Conjugated linolenic acid, Phytoestrogen, Pomegranate (*Punica granatum*), Proliferation, Punicic acid, Selective estrogen receptor modulator

INTRODUCTION

Pomegranate (*Punica granatum*) is a valuable medicinal fruit with an extensive literature citing numerous medicinal properties (1). Over 50 compounds with phytoestrogenic and antioxidant activities have been isolated from pomegranate seed, juice, peels, leaf, and flowers. Dried peels of ripe fruits are used to

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treat stomach-ache, whereas the arils (i.e., the juice-encapsulated seeds) with a sweet taste and pleasant odor are commonly eaten (2). Fruit extracts have been shown to be effective against herpes and influenza viruses (3) and to result in suppression of human breast (4) and prostate (5,6) cancer cell proliferation *in vitro* and *in vivo*. Studies have shown that this anticancer effect involves suppression of cell cycle progression, proliferation, inflammation, invasion, and angiogenesis (1,7–9). Moreover, a combination of pomegranate juice concentrate and pomegranate seed extract has been shown to ameliorate bone thinning and experimental depression in ovariectomized mice (10).

Pomegranate components have also been used to relieve symptoms associated with menopause (11). Although women undergoing menopause may appear to benefit from the use of estrogen hormones, such therapy increases the risk of developing breast cancer (12,13). Estrogen replacement therapy, therefore, has an unacceptably high risk/benefit ratio. In contrast to the use of equine or synthetic steroidal estrogen, functional foods containing elevated amounts of "phytoestrogens" have been recommended for the prevention of breast cancer (4,14,15). One such food is pomegranate that contains compounds with hormone-like activity including flavonoids (16).

Although 17β-estradiol (E2) and 17α-estradiol, estrone, estriol, coumesterol, and testosterone have all been reported in pomegranate seeds (16), some studies have failed to find them (17,18). However, linoleic and linolenic acids isomers (Figure 1) are the main ingredients in pomegranate seed oil (19). If these isomers bind to estrogen receptors ERα and ERβ with high affinity, they may account for observed estrogenic activity of pomegranate seed oil *in vivo* (20). Estrogen receptors ERα and ERβ have high affinity for E2, the strongest naturally occurring estrogenic steroid in women, and both receptors occur in normal and malignant breast tissues (16). But ligands possessing low pharmacological activity but specific affinity for ERβ may prevent breast cancer (7,21), providing also benefit for blood pressure control and maintenance of vascular integrity (22), brain development (23), antiaging (24–27), and especially reduction of adverse symptoms in postmenopausal women (28,29).

To assess the putative role of linolenic acid isomers as phytoestrogens, the relative ER binding efficacies of pomegranate-derived compounds were measured in human breast cancer cell lines. Tamoxifen, a selective estrogen receptor modulator (SERM) whose ER binding is well known, was utilized in these studies as a positive control.

MATERIALS AND METHODS

Materials

17α-Estradiol, E2, tamoxifen, linoleic acid, α-linolenic acid, γ-linolenic acid, and resveratrol were purchased from Sigma Chemical Co. (St. Louis,

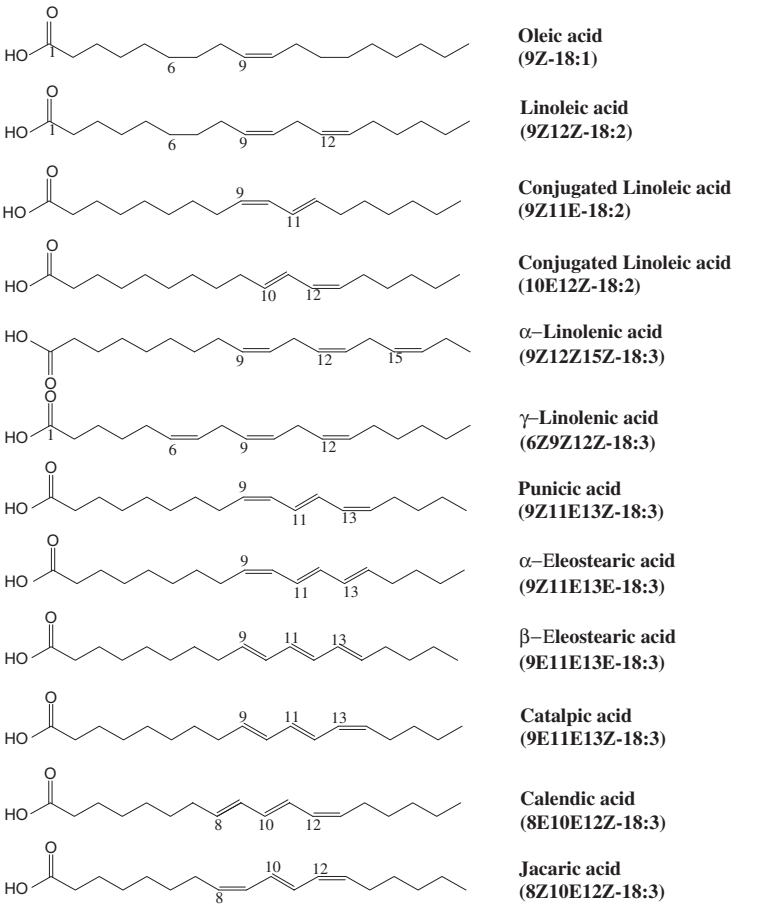

FIGURE 1 Structures of geometric and positional isomers of conjugated fatty acids. Shown above are conjugated linoleic acids (9*Z*, 11*E*-octadecadienoic acid; 10*E*, 12*Z*-octadecadienoic acid) and conjugated linolenic acids as punicic (9*Z*, 11*E*, 13*Z*), α-eleostearic (9*Z*, 11*E*, 13*E*), β-eleostearic (9*E*, 11*E*, 13*E*), catalpic (9*E*, 11*E*, 13*Z*), calendic (8*E*, 10*E*, 12*Z*) and jacaric (8*Z*, 10*E*, 12*Z*) acids.

MO, USA). 8(*E*),10(*E*)-Octadecadienoic acid, 11(*E*),13(*E*)-octadecadienoic acid, punicic acid (PA), α-eleostearic acid (AEA), β-eleostearic acid, α-calendic acid, β-calendic acid, catalpic acid, and jacaric acid were purchased from Larodan Fine Chemicals AB Co. (Malmo, Sweden). Human ERα and ERβ and ERα and ERβ coactivator assay kits were purchased from PanVera (Invitrogen Corp., Carlsbad, CA, USA). The HitHunter™ Estrogen Assay (Fluorescent Detection) Kit was purchased from DiscoveRx Co. (Fremont, CA, USA). Human breast cancer cell lines MCF-7 (ATCC® Number: HTB-22™) and MDA-MB-231 (ATCC® Number: HTB-26™) were obtained from American Type Culture Collection (Manassas, VA, USA). Pomegranate seed oils were

purchased from Ahnam Pharmaceutical Co. (Kyungsan, South Korea) and Punisyn Pharmaceuticals (Haifa, Israel).

Analysis of Fatty Acid Components in Pomegranate Seed Oil

Pure cold-pressed pomegranate seed oil (Punisyn Pharmaceuticals) was dissolved in MeOH and filtered to obtain a stock solution of 100 mg/mL. Conjugated fatty acids (CFAs) of pomegranate seed oil were analyzed with a liquid chromatograph/mass spectrophotometer (LC/MS), model HP1050, with a C_{18}, 250 × 4.6 mm, 5 μm, analytical column (Hewlett Packard, Palo Alto, CA, USA). The mobile phase consisted of MeOH:H_2O (60:40; containing 0.05% triethylamine) flowing at 0.5 mL/min. High-performance liquid chromatographic (HPLC) peaks from analysis of CFAs in pomegranate seed oil were compared to the peaks of authentic standards.

Relative *In Vitro* Binding of Conjugated Linoleic and Linolenic Acids to Human ERα and ERβ

Each compound was dissolved in 100% DMSO to obtain a stock solution of 10 mM and then diluted to various concentrations before performing ER competitive binding assays using HitHunter™ Estrogen Assay kits according to the manufacturer's instructions (DiscoverRx Corp., Freemont, CA, USA). Samples were read on a fluorescence Microtiter plate reader (Victor^{3}™ Multilabel Plate Counter, Perkin-Elmer, Waltham, MA, USA) using a 590 nm emission filter. ERα and ERβ coactivator assays for identifying the ligand as either an agonist or an antagonist for ER were conducted per manufacturer's instructions using the ERα and ERβ Coactivator Assay kit. Samples were read using activation/emission wavelengths of 535/595 nm.

Each sample was run in triplicate and results were expressed as either an IC_{50} value in terms of mean value of relative fluorescence units (RFUs) or millipolarization units (mP, fluorescence polarization detection) expressed as EC_{50} value of the ligands.

Cell Culture

Cells of the MCF-7 (ER-positive human breast cancer cells) and MDA-MB-231 (ER-negative human breast cancer cells) lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-glutamine, 1.5 g/L $NaHCO_3$, 4.5 g/L glucose, and 10% fetal calf serum. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Stock cultures were seeded at a density of 1×10^5 cells/mL and permitted to grow for 48–72 h before use.

Cellular Proliferation

In this assay, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was used as a means of measuring the activity of living cells through mitochondrial dehydrogenases, yielding a purple formazan product. The intensity of the purple color that developed was a measure of active dehydrogenase activity in viable cells and, hence, a measure of relative live cell numbers. MCF-7 and MDA-MB-231 cells (1×10^4 cells/well) were seeded and incubated for 24 h. Various concentrations of E2 and individual CFAs were then added to 96-well culture plates in a total volume of 100 μL/well of fresh DMEM. These were incubated at 37°C for 1–5 days. At the end of the incubation period, 50 μL of -3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium (2 mg/mL in phosphate-buffered saline) was added to each well. After 4 h, the formazan product was dissolved in DMSO and the resulting color measured at 540 nm on a microtiter plate reader.

RT-PCR Analysis of ER mRNA Expression in MCF-7 and MDA-MB-231 Cell Lines Induced by Linolenic Acid Isomers

For RNA extraction, MCF-7 and MDA-MB-231 cells (1×10^6 cells/mL) were grown in 50 mm diameter culture dishes and preincubated in phenol red-free and FBS-free DMEM at 37°C for 24 h. Then individual compounds, at various final concentrations, were added to each dish and incubated for 3 additional days. Controls consisted of cell samples treated with MeOH or without any compound. Immediately after treatment, total RNA was extracted directly with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNA were quantified spectrophotometrically and checked by electrophoresis.

Two micrograms of total RNA was reverse transcribed using the commercially available first-strand cDNA synthesis kit for reverse transcriptase-polymerase chain reaction (RT-PCR) (Reverse Transcription System; Promega, Fitchburg, MA, USA) after incubation at 50°C for 45 min to remove secondary RNA structures, according to the manufacturer's instructions.

Amplification of cDNA was carried out using the following primers (30): for ERα, sense 5′-CAG GGGTGA AGT GGG GTC TGC TG -3′ and antisense 5′-ATG CGG AAC CGA GAT GAT GTA GC -3′, giving an amplification product of 483 bp; for ERβ, sense 5′-GTC CAT CGC CAG TTA ATC ACA TC-3′ and antisense 5′-GCC TTA CAT CCT TCA CAC GA -3′, giving an amplification product of 242 bp. For the ubiquitously expressed β-actin, the following primers were used: sense 5′-ACA CTG TGC CCA TCT ACG AGG -3′ and antisense 5′-AGG GGC CGG ACT CGT CAT ACT 3′, giving an amplification product of 600 bp. The PCRs were carried out by using the PCR Master Mix kit (Promega) per manufacturer's instructions.

The amplification conditions for ERα, ERβ, and β-actin were as follows: a single step of 10 min at 95°C to activate the enzyme, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 2 min, and a final extension of 5 min at 72°C. Each PCR amplification was performed in triplicate. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

RESULTS

Analysis of Fatty Acid Components in Pomegranate Seed Oil

As shown in Figure 2, Israeli pomegranate seed oil was found to contain linoleic acid (~20%), α-linolenic acid (~3%), and some conjugated linolenic acids like PA (9*Z*, 11*E*, 13*Z*, ~65%) and AEA (9*Z*, 11*E*, 13*E*, ~7%). These were determined by using liquid chromatography/mass spectrometer in comparison with the specific m/z (mass to charge ratio) and retention times of authentic standard linolenic acid isomers. For confirmation of the AEA, the liquid chromatogram of an unspiked and a spiked AEA and calendic acid with pomegranate seed oil was performed (data not shown). Standard AEA corresponded to the deduced peak of AEA of pomegranate seed oil. However, the calendic acid mixture was shifted to a mixture of two peaks, the second main band shifted slightly toward a longer retention time. The weak subband was supposed to be AEA. This result confirms that the band between PA and linoleic acid is AEA.

Binding Activity of Conjugated Linoleic and Linolenic Acids with Human Estrogen Receptors α and β *In Vitro*

Figure 3 reveals the ERα and ERβ binding affinity of three phytoestrogenic groups: linoleic acid isomers, linolenic acid isomers, and others, including resveratrol and 17α-estradiol, relative to E2. The relative potency of the phytoestrogenic compounds was assessed by a competitive ER binding assay and shown in Table 1. To identify the binding activity of conjugated linoleic and linolenic acids with ERs, EFC fluorescent detection kit by HitHunter^{TM} was employed, measuring fluorescent intensity in RFUs to obtain IC_{50} values. The competitive binding of the compound of interest at each concentration was obtained by subtracting the RFU with no enzyme donor (ED) reagent and expressed as percentage of the specific binding for the E2. Concentrations of ligands that reduced the specific binding of E2 by 50% were determined as IC_{50} values. Relative potency of ER binding affinity for each ligand was calculated by dividing the IC_{50} of the ligand by the IC_{50}

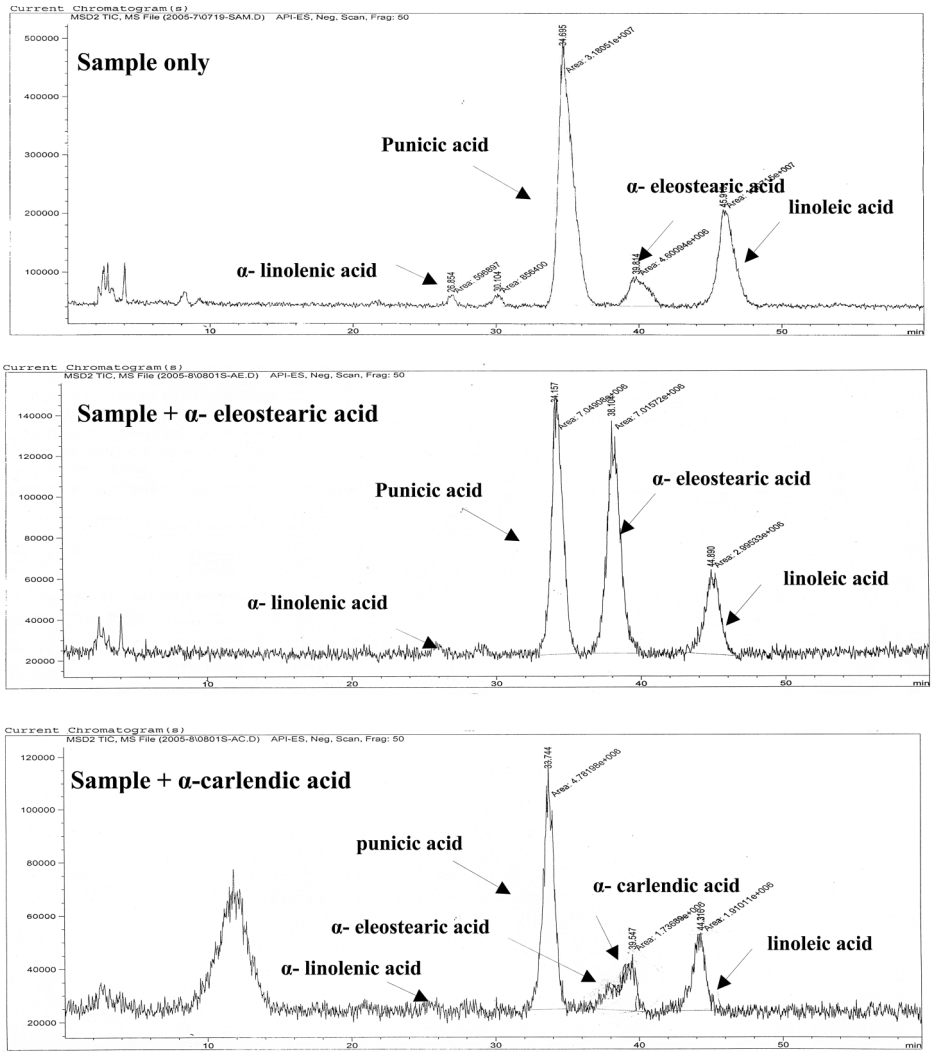

FIGURE 2 Identification of punicic acid and α-eleostearic acid in Israel pomegranate seed oil by LC/MS. The unknown peaks of seed oil were identified by relative retention times in comparison to peaks of authentic linolenic acid analogue standards and by molecular size (m/z). Punicic acid (retention time 35.5 min) and linoleic acid (47.3 min) were identified using mass spectrometry transitions of m/z 277.2 and 280.0, respectively.

and expressed as percent. Detections for each competitor and E2 were performed in triplicate detection and shown in Table 1.

As can be seen, the relative potencies of linoleic acid isomers were stronger than those of linolenic acid isomers and resveratrol for both ERα and ERβ binding, respectively. The relative differences in binding affinity of each CFA for ERα or ERβ receptors, however, was modest, thus PA inhibited

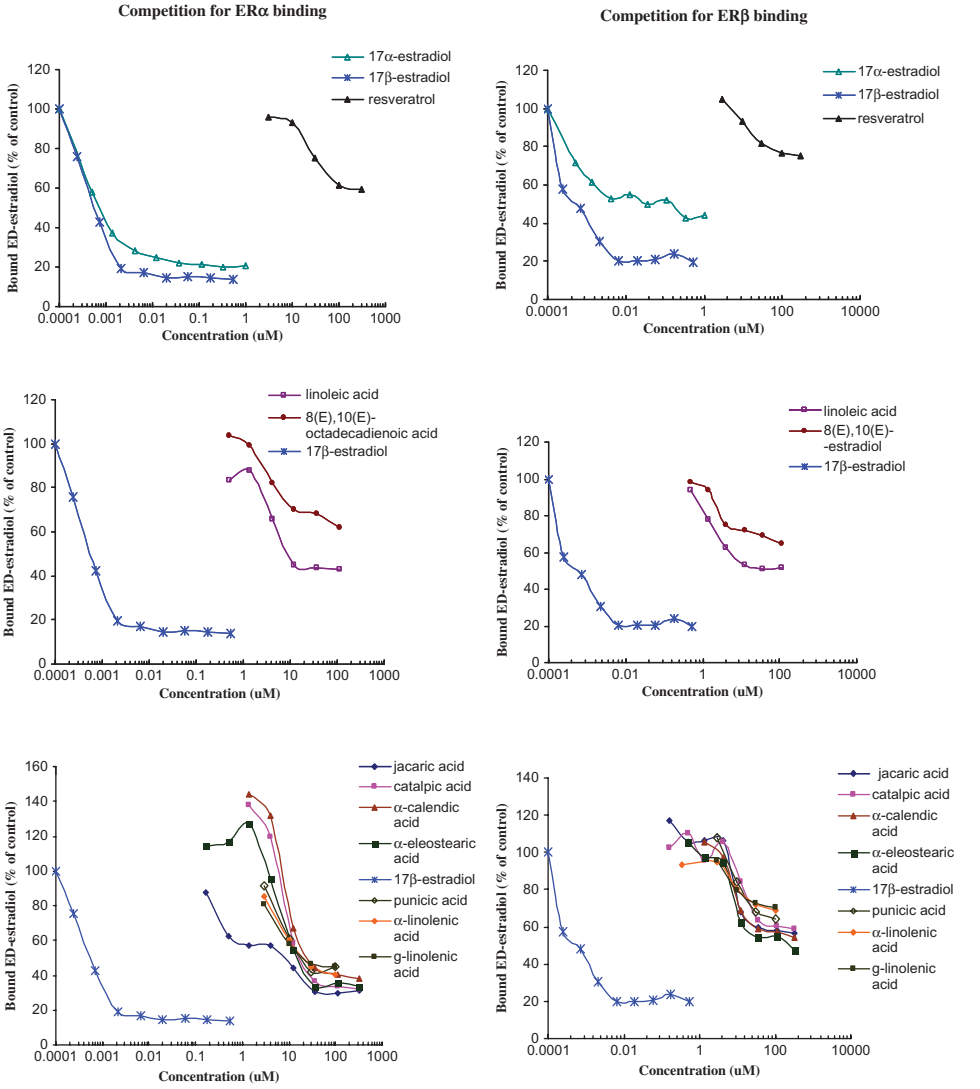

FIGURE 3 Relative ERα and ERβ binding activity of linoleic and linolenic acids group (linoleic acid and 8(*E*),10(*E*)-octadienoic acid) compared to 17-β-estradiol in a competitive ER binding assay. ED represents enzyme donor, a small peptide fragment of β-galactosidase. (a) ERα binding activity of control compounds (17α-estradiol, 17β-estradiol, and resveratrol). (b) Relative ERα binding activity of linoleic acids group (linoleic acid and 8(*E*),10(*E*)-octadienoic acid). (c) Relative ERα binding activity of linolenic acids group (jacanic acid, catalpic acid, α-calendic acid, α-eleostearic acid, punicic acid, α-linolenic acid, γ-linolenic acid) in comparison to 17-β-estradiol. (d) Relative ERβ binding activity of a control group of ER agonists (17α-estradiol, 17β-estradiol, and resveratrol). (e) Relative ERβ binding activity of linoleic acids group (linoleic acid, 8(*E*),10(*E*)-octadecadienoic acid). (f) Relative ERβ binding activity of linolenic acids group (jacaric acid, catalpic acid, α-calendic acid, α-eleostearic acid, punicic acid, α-linolenic acid, γ-linoleic acid).

TABLE 1 ERα and ERβ Estrogen Receptor Binding and Relative Potency of Selected Natural Ligands

	ERα binding		ERβ binding	
Compounds	IC_{50}, nM	Relative potency	IC_{50}, nM	Relative potency
17β-Estradiol	0.48	100	0.35	100
17α-Estradiol	0.62	77.42	0.70	50.00
Linoleic acid	4,000	0.0120	2,000	0.0175
8(*E*),10(*E*)-Octadecadienoic acid	3,200	0.0150	2,500	0.0140
11(*E*),13(*E*)-Octadecadienoic acid	ND	ND	ND	ND
α-Linolenic acid	8,500	0.0056	8,000	0.0044
γ-Linolenic acid	7,000	0.0069	7,000	0.0050
Punicic acid	7,200	0.0067	8,800	0.0040
α-Eleostearic acid	6,500	0.0074	7,800	0.0045
β-Eleostearic acid	ND	ND	ND	ND
α-Calendic acid	8,000	0.0060	7,500	0.0047
β-Calendic acid	ND	ND	ND	ND
Catalpic acid	7,500	0.0064	12,400	0.0028
Jacaric acid	12,000	0.0040	8,200	0.0043
Resveratrol	20,000	0.0024	12,000	0.0029

ND, not determined.

binding to ERα and ERβ (IC_{50}) at 7.2 and 8.8 μM, respectively, while AEA did so at 6.5 and 7.8 μM.

Linolenic Acid Isomers as SERMs

Table 2 reveals ERβ binding of linoleic and linolenic acid isomers and related CFA ligands identified as SERMs by an ER coactivator binding assay.

To assess the ligand dose dependency for the recruitment of coactivator peptides to human ERβ, the ERβ coactivator assay kit was introduced. Under the process of formation of the ERβ/D22 complex, either the formation or the disruption of the complex was determined by addition of increasing concentrations of ligand. The D22 is a fluorescent coactivator-like peptide containing LXXLL motif and flanking sequences that resemble known coactivators. Agonist- and antagonist-bound ERs have greater or lesser polarization values according to their affinity for recruiting D22. Classification of ligands as agonist, antagonist, and selective modulator was according to the EC_{50} value of the ligands to either promote or disrupt the ERβ/D22 interaction. E2 was used as an ER control agonist whereas tamoxifen was used as an ER control antagonist.

The resultant agonists for both ERα and ERβ were α-linolenic acid, γ-linolenic acid, and PA. However, depending on the concentration, α-linolenic acid, PA, AEA, resveratrol, and 17α-estradiol may also antagonize E2 binding. PA acted as agonist for ERα and ERβ at EC_{50} values of 1.8 and 2 μM,

TABLE 2 SERM Activity of Linolenic Acid Isomers and Other Various Ligands in an ER Coactivator Binding Assay

	ERα (ER_{50}, μM)		ERβ (ER_{50}, μM)	
Compounds	Agonist	Antagonist	Agonist	Antagonist
17β-Estradiol	0.3	–	0.37	–
Tamoxifen	–	3.8	–	1.0
Linoleic acid	ND	ND	ND	ND
8(*E*),11(*E*)-Octadecadienoic acid	ND	ND	ND	ND
α-Linolenic acid	7.9	1.3	8.0	1.1
γ-Linolenic acid	60	–	51	–
Punicic acid	1.8	101	2	80
α-Eleostearic acid	–	150	–	140
α-Calendic acid	ND	ND	–	15
Jacaric acid	ND	ND	ND	ND
Catalpic acid	–	180	ND	ND
Resveratrol	–	140	–	110
17α-Estradiol	–	0.21	–	0.20

ND, not determined.

respectively, and as an antagonist at higher concentrations (101 and 80 μM, respectively). AEA acted as an antagonist only against ERα and ERβ at EC_{50} values of 150 and 140 μM, respectively. Finally, other conjugated linolenic acids were observed to act as antagonists for either ERα or ERβ; for example, α-calendic acid was found to act as an antagonist against ERβ, and catalpic acid acted as antagonist against ERα.

Effect of Pure Linoleic and Linolenic Acid Isomers on Proliferation of MCF-7 and MDA-MB-231 Cells

Table 3 contains data derived from growth inhibition studies of pomegranate CFAs on proliferation of ER-positive and ER-negative human breast cancer cell lines. The results of 5-day incubation of cells with PA (0–100 μM) and of AEA (0–50 μM) demonstrated that both compounds were effective in producing effective inhibition of cancer cell proliferation. A 75% growth inhibition was achieved with 100 μM of PA after 5 days of exposure of MCF-7 cells to this CFA although this same concentration of AEA was even more effective. In addition, 70% of MCF-7 and 30% of MDA-MB-231 cells were inhibited by 100 μM of γ-linolenic acid at 5 days. Furthermore, 35% of MCF-7 cells were killed by 100 μM of α-linolenic acid for 1 day. In contrast, this same concentration appeared to slightly increase proliferation of MDA-MB-231 cells (data not shown). Additionally, linoleic acid promoted cell growth in both cancer cell lines (data not shown); whereas both growth stimulation and inhibition in both cell lines were observed with 17β-E2.

TABLE 3 Relative Ability of Pomegranate-Derived Conjugated Fatty Acids to Inhibit Proliferation of ER-Positive MCF-7 and ER-Negative MDA-MB-231 Breast Cancer Cells

Compound	Concentration (μM)	MCF-7 (% inhibition)	MDA-MB-231 (% inhibition)
17β-Estradiol	10[a]	29	0
α-Linolenic acid	50	0	0
α-Linolenic acid[b]	100	35	0
γ-Linolenic acid	100	70	30
Punicic acid	50	75	70
α-Eleostearic acid	50	100	100

[a]Percent inhibition of control cell growth after 5-day continuous exposure to the test compound at the stated concentration.

[b]At 1 day and continuous exposure, 100 μM α-linolenic acid killed 35% of MCF-7 cells although slightly increasing proliferation of MDA-MB-231 cells (data not shown).

Induction of ER mRNA Expression in MCF-7 and MDA-MB-231 Cell Lines by Linolenic Acid Isomers

Figure 4 shows that AEA, PA, and a MeOH extract of pomegranate seed oil promote ER mRNA expression in a manner analogous to that produced by E2 for both ERα and ERβ receptors in MCF-7 but not in MDA-MB-231 cells (data not shown).

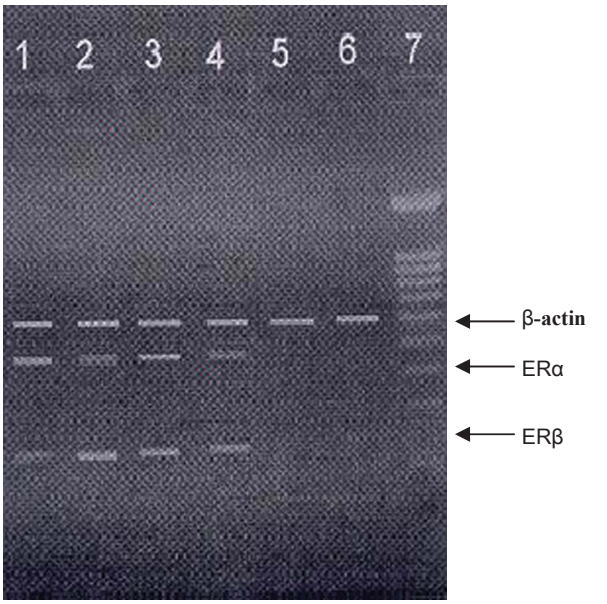

FIGURE 4 Agarose gel visualizing RT-PCR products from MCF-7 cells for induction of ERα and ERβ mRNA expression. Lane (1), pomegranate seed oil MeOH extract (30 μg/mL); (2) α-eleostearic acid (8 μM); (3) punicic acid (8 μM); (4) 17β-estradiol (1 nM); (5) MeOH; (6) control; (7) 100 bp DNA ladder [1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200 bp].

DISCUSSION

This report examined the potential ability of phytoestrogenic and antioxidative components of pomegranate to act as SERMs in human breast cancer cell lines. As expected PA was determined to be the main component of seed oil, whereas AEA was observed as a minor component that might, nonetheless, contribute phytoestrogenic activity. These two linolenic acid isomers were identified as principal components of pomegranate with the potential to act as SERMs. Using a HitHunter^{TM} estrogen assay kit, AEA and PA were observed to bind to ER with an affinity that was about 1/10,000 compared with that of E2 *in vitro*. In this study, antiestrogenic activity of linolenic acid isomers was detected in which α-linolenic acid and PA were found to act as mixed agonist–antagonist compounds whereas, in contrast, AEA was a selective antagonist for both ERα and ERβ. In addition, other conjugated linolenic acids acted as selective antagonists for ERα or ERβ. For example, α-calendic acid was found to be an antagonist for ERβ, whereas catalpic acid acted as an antagonist for ERα. Therefore, PA as well as other linolenic acid isomers, except γ-linolenic acid, act as SERMs through competitive binding to the ERs, in a manner analogous to that of tamoxifen.

Useful phytoestrogenic efficacy of CFA isomers against ER-positive and ER-negative cell lines, MCF-7 and MDA-MB-231, respectively, might involve inhibition of cell proliferation through cytotoxic effects. Both of the cell lines were more sensitive in these respects to exposure to AEA than to PA. MCF-7 was more sensitive to γ-linolenic acid than was MDA-MB-231 whereas α-linolenic acid inhibited the proliferation of MCF-7, but stimulated the proliferation of MDA-MB-231. Our studies have shown that pomegranate CFAs can act as phytoestrogens and compete for binding to ERs. The differential effect of these compounds is highlighted by the fact that some fatty acids, present as minor components in pomegranate seed oil, result in stimulation of breast cancer cell proliferation whereas others, notably PA and AEA, result in marked inhibition of cell proliferation. Our work demonstrating that linoleic acid can stimulate breast cancer cell proliferation is in agreement with previously reported findings (31).

Although the observation of induction of ER mRNA expression for both types of ERs by linolenic acid isomers and pomegranate seed oil in the human breast cancer cells is qualitative, it shows that these compounds promote expression of both ERα and ERβ mRNA in MCF-7 cells. Induction of expression of ER mRNA synthesis was clearly evident upon incubation of cells with AEA, PA, or pomegranate seed oil, which contains the conjugated linolenic acids. Of interest, none of these compounds altered ER mRNA expression in MDA-MB-231 cells implying ER-positive status is important (Figure 4).

Collectively, these results suggest a novel role as SERMs for specific pomegranate-derived linolenic acid isomers. This may require a re-thinking

of the pharmacology underlying estrogenic activity of pomegranate seed oil. Considering that linoleic acid in *Vitex agnus-castus* L. induced the ERβ-specific mRNA expression in T47D:A18 cells (32), we suggest that linoleic acid should now be considered as an estrogenic compound. Other results like pomegranate seed oil's previously reported estrogenic actions and the suppression of human breast cancer by α-linolenic acid (33) and γ-linolenic acid (34) reinforce our understanding that linolenic acid isomers including PA, as principal components of pomegranate seed oils (35), act as SERMs and, potentially, will also act in the future as effective breast cancer chemopreventive agents.

To identify additional phytoestrogenic components from pomegranate, both the methanolic extract of fruit juice and the pomegranate seed oils were assayed. CFAs isolated and characterized from the pomegranate seed oils included AEA (9*Z*,11*E*,13*E*) and PA (9*Z*,11*E*,13*Z*) acids. Also identified were linoleic acids and linolenic acids, but conjugated linoleic acids like 8*E*,10*E* (9*Z*,11*E*)-octadecadienoic acid and 11*E*,13*E* (10*E*,12*Z*)-octadecadienoic acid, and conjugated linolenic acids like calendic (8*E*,10*E*,12*Z*), jacaric (8*Z*,10*E*,12*Z*), and catalpic (9*E*,11*E*,13*Z*) acids were not detected. These polyunsaturated fatty acids are the geometrical and positional isomers of conjugated double bonds in their structures (shown in Figure 1). Although PA is a well-established component of pomegranate seed oil and is found in concentrations as high as 65% of seed fatty acids (35), the presence of AEA is an original observation.

CFAs are known to have beneficial pharmacologic activity of relevance for the prevention and treatment of atherosclerosis (36), obesity (37–39), cancer (40–42), and hypertension (43). Lipid peroxidation is a central mechanism of tumor growth suppression by AEA in nude mice (44). Peroxisome proliferator-activated receptor (PPAR) upregulation and alteration of lipid composition modulate colon cancer prevention in rats (45). EA was cytotoxic to colorectal DLD-1, hepatoma HepG2, lung A549, MCF-7 breast, and stomach MKN-7 cancer cell lines (46), but ER-binding potencies and antiproliferative effects on human breast cancer cells of purified PA and AEA were not previously examined. PA reduced apolipoprotein B100 secretion and triacylglycerol synthesis in HepG2 cells (38), and PA also reduced monounsaturated fatty acid levels and chemically induced colon carcinogenesis in rats (40). Although these data confirm interaction of both EA and PA with ERs, the preceding discussion suggests that these compounds also modulate signal transduction pathways independently of their modulation of ERs. Indeed, such dual actions of SERMs, one through effects on ERs and one independent of ERs, are increasingly appreciated even with tamoxifen and raloxifen (47).

To apply the functional components of pomegranate to postmenopausal woman with estrogen deficiency symptoms, the phytoestrogenic/antioxidant

components were detected, and their activity as SERMs analyzed. Among seed oil components, the isolated PA and AEA from pomegranate seed oils would appear to satisfy criteria to be considered as SERMs.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. Drs. Lansky and Newman are co-developing a complex anticancer pharmaceutical from pomegranate fruit under the auspices of Punisyn Pharmaceuticals, Ltd. The authors alone are responsible for the content and writing of the paper.

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