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The combined effect of pomegranate extract and tangeretin on the DMBA-induced breast cancer model

Huseyin Fatih Gul[a,*], Necip Ilhan[b], Nevin Ilhan[b], Ibrahim Hanifi Ozercan[c], Tuncay Kuloglu[d]

[a] Department of Medical Biochemistry, Kafkas University, Faculty of Medicine, Kars, Turkey
[b] Department of Medical Biochemistry, Firat University, Faculty of Medicine, Elazıg, Turkey
[c] Department of Medical Pathology, Firat University, Faculty of Medicine, Elazıg, Turkey
[d] Department of Histology and Embryology, Firat University, Faculty of Medicine, Elazıg, Turkey

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Abstract

The aim of this study was to investigate the protective effects of pomegranate extract and tangeretin alone or in combination in DMBA-induced rat breast cancer model.

A total of 68 female rats were randomly divided into 8 groups. The first 4 groups were designed as controls for cancer and treatment groups, and the control groups were composed of only control (C), Pomegranate (P), Tangeretin (T), and Pomegranate+Tangeretin (P+T) groups. The other four groups were designed as cancer and treatment groups and were composed of DMBA (D) and DMBA+Pomegranate (D+P), DMBA+Tangeretin (D+T), DMBA+Pomegranate+Tangeretin (D+P+T) groups. Tumor markers and angiogenesis parameters were studied from plasma samples obtained from rats. Histopathological, immunohistochemical, and TUNEL analyses and expressions of proteins affecting apoptosis and cell cycle were determined in breast tissue samples.

In the DMBA group, plasma CA15-3, CEA, VEGF, MMP-9, and NF-κB levels were significantly increased compared to the controls, but significant decreases were observed in these parameters except MMP-9 in the treatment groups. It was observed that p53 and Bax expressions significantly increased in both D+P and D+P+T groups compared to the DMBA group, and these findings were supported by Tunel and immunohistochemical findings. Cyclin D1 expressions were found to be significantly decreased only in the D+T group and supported by TUNEL and immunohistochemical findings. Immunohistochemical ER-α and Ki-67 immune reactivities were significantly decreased in all treatment groups compared to the DMBA group.

Our results showed that combined application of pomegranate extract and tangeretin may be more beneficial in preventing breast cancer development.

Keywords: Breast cancer model; Tangeretin; Pomegranate extract; Nutraceuticals; Chemoprevention.

1. Introduction

Cancer is defined as a group of diseases that occur as a result of uncontrolled division of cells in different body organs. The clinical appearance and treatment approach for each cancer type is different [1].

Breast cancer is the most common type of cancer among women and accounts for about 23% of all female cancer cases. Among all cancer types, deaths from breast cancer are in fifth highest and ranks first place among women in some undeveloped or underdeveloped countries [2]. Therefore, breast cancer is regarded as one of the most important social health problems around the world [1].

Given the inadequate efficacy of contemporary medicine in the fight against breast cancer due to the resistance to conventional chemotherapeutic drugs and the side effects of long-term use, more effective and less toxic chemotherapeutics (nutraceuticals) that represent natural active phytochemical molecules are gaining importance in the treatment and prevention of breast cancer [3–5].

Polycyclic aromatic hydrocarbons and their derivative 7,12-dimethylbenz [a] anthracene (DMBA) are known as potent carcinogenic and mutagenic agents [6]. DMBA realizes these effects in the organism as a result of damaging the DNA through its metabolites (epoxides) and intermediates (reactive molecules) that occur during degradation [7–9].

As an old, mystical, and quite different fruit, the pomegranate (*Punica granatum*, L.) and its peel are reported to contain many natural bioactive antioxidants and anticarcinogenic phytochemical agents [10,11]. These agents include hydrolyzable tannins such as punicalagin, ellagic acid, and gallic acid and a large number of polyphenolic acid compounds [12].

Pomegranate fruit extract was demonstrated to have antiproliferative, antiangiogenic, anti-invasive, and proapoptotic effects in cancer cells that allow its usage in various cancer types as a ther-

* Corresponding author: Huseyin Fatih Gul, Department of Medical Biochemistry, Kafkas University Faculty of Medicine, 36100 Kars, Turkey. Tel.: +90 (0474) 2251192; fax: (0474) 2251196.
E-mail address: fth_2323@hotmail.com (H.F. Gul).

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apeutic agent and have strong antioxidant and anti-inflammatory properties [13].

It reveals that numerous *in vivo* and *in vitro* studies, many pomegranate products and phytoprotective substances in pomegranate have cytotoxic, antiproliferative, proapoptotic, antiangiogenic, anti-invasive, and antimetastatic effects against breast carcinoma cells [14].

Currently, only two types of nuclear estrogen receptors have been identified: the first of these is the estrogen receptor alpha (ER-α/NR3A1 is more expressed in the breast, ovary, and uterus), the other one is the estrogen receptor beta (ER-β/NR3A2, is expressed from different organs such as the prostate, bladder, bone and ovary, lungs.) [15].

It has been demonstrated that ER-α expressions are higher in neoplastic cells of the breast compared to normal breast epithelial cells. Nowadays, ER-α is seen as a transcription factor responsible for the initiation and progression of breast cancer, and as a result of all of these, it is especially written in studies related to the prevention and treatment of breast cancer [16,17]. The phytoestrogenic molecules present in pomegranate have antagonist effect against ER resulting inhibition of estrogen activity or direct inhibition of ER and estrogen synthetase (aromatase). In addition, these phytochemicals target the release and activation of a large family of Zn^{+2}-dependent endopeptidases (matrix metalloproteinase [MMPs]) in cancers with estrogen receptor positive (ER+) [18]. By selectively inhibiting MMP activity, pomegranate components reduce focal adhesion kinase activation and vascular endothelial growth factor (VEGF) expression. In this way they reduce tumor cell invasions to normal tissue and prevent distant tissue metastases [19]. These effects have led the theory that these molecules might have chemopreventive and chemotherapeutic effects against breast cancer [20].

Various studies have shown that pomegranate or its different fractions downregulate cyclooxygenase-2 (COX-2) expression and consequently inhibit eicosanoid biosynthesis [19,21]. It also emphasized that it synergistically suppresses inflammatory cytokine expression and can inhibit MMPs. The activation of the nuclear factor kappa-βeta (NF-κB) pathway is known to be a strong finding indicating inflammation and cell proliferation [22,23]. Because inflammation can cause permanent oxidative stress in the cancer cell environment, and especially chronic inflammation conditions can lead to the initial stage of cancer by causing DNA damage, and can also give cancer cells a survival advantage by playing a role in other stages of cancer. All these assumptions make phytotherapy agents with high anti-inflammatory effects, such as pomegranate, against inflammatory signaling pathways a potential target for cancer prevention. It is also known that NF-κB causes stimulation of genes involved in the immune response, and regulates cell cycle control and cell death in response to pro-inflammatory cytokines. NF-κB expression is associated with the transcription of genes involved in cell survival, such as the B-cell lymphoma gene-2 (Bcl-2) family and apoptosis inhibitors. *In vivo* and *in vitro* studies revealed the cytotoxic effect of pomegranate fruit extract, by suppressing NF-κB activation in the cell result in increasing the expression of Bcl-2 associated x protein (Bax protein, involved in apoptosis induction) and decreasing the expression of Bcl-2 (the antiapoptocic protein) [20,24,25].

Tangeretin is a polymethoxiflavone compound of 5,6,7,8,4'-pentamethoxy flavon and found in large amounts in the peel of citrus fruits such as tangerine, lemon, orange, and grapefruit. In the literature, bioactivities of citrus polymethoxiflavones have included treatment of metabolic disorders, antiatherosclerotic, antidiabetic, anti-inflammatory, neuroprotective, antimicrobial, and antioxidant regulation [26–28]. Tangeretin is one of the most effective flavonoids in inhibiting human cancer cell proliferation [29]. Tangeretin has been reported to inhibit the proliferation of human cancer cell lines derived from squamous cell carcinoma [30], gliosarcoma [31], leukemia [32], melanoma [33], colorectal cancer [34], stomach, and lung carcinoma [32].

Breast cancer involves a process characterized by unregulated cell proliferation and metastasis. While defining this process, in addition to proteins such as p53, receptor tyrosine-protein kinase/HER-2, or human epidermal growth factor receptor-2 (c-erbB2), human epidermal growth factor receptor-1 (ErbB-1), Proliferating cell nuclear antigen (PCNA), cathepsin D, Bcl-2 many proliferation and mitotic index parameters such as Thymidine marking index, Ki-67 index, ER, and PR are used. These parameters have found clinical use, and they provide information about proliferation and tumor subtype. VEGF, MMPs and markers such as Cancer antigen 15-3 (CA15-3), Carcinoembryonic antigen (CEA) mostly provide information about distant tissue metastases, recurrence, and prognosis [35]. On the other hand, studies reveal that anti-metastatic and anticancer properties of various flavonoids (naringin, hesperidin, naringenin, hesperitin, rutin, nobiletin, tangeretin) can be used in the management of this process. In vivo studies have reported that tangeretin plays a role in metastasis inhibition and apoptosis induction by downregulating MMP-2, MMP-9, and VEGF expressions and upregulating p53/p21 expressions. It further suggests that it inhibits the formation and proliferation of DMBA-induced mammary carcinogenesis in rats by effectively reducing tumor cell proliferation markers such as PCNA, COX-2, and Ki-67 [36,37]. Literature states that Naringenin shows antiestrogenic activity against ER-α + cells and as a result it can suppress cell proliferation, however, there are not enough studies showing the relationship between tangeretin and ERs at the point of prevention of breast carcinomas. With this aspect, it constituted a separate reason for our investigation of ER in our study.

Apart from all these pathways, regarding the WNT/β-catenin signaling pathway and its expression product, Cyclin-D1 gene, which also contribute to healthy mammary gland development, recent studies have shown that they play a role in the development of breast cancer, and it is a separate source of curiosity and inspiration for us has been. Wnt/β-catenin signaling pathway; it has been stated that it is involved in the formation of breast tumors because it is responsible for the signal transduction related to the regulation of cellular adhesion and specific gene expression in the cell [38]. As a result, the displacement of β-catenin, which accumulates in the cytoplasm with various precarcenegenic stimuli, towards the nucleus leads to the transcription of various target genes such as c-myc, Cyclin D1, VEGF, MMPs, which are responsible for breast cancer, and it is also emphasized that it interacts with estrogen receptors [14,35,38].

To summarize, nowadays strategies tendency to specific-targeted has gained importance to destroy breast cancer cells. These targets include that increased antioxidant activity/suppression of oxidative stress, receptor blockade, enzyme blockages, induction of apoptosis, inhibition of cell proliferation, suppression of angiogenetic pathways, induction of cell cycle arrest, inhibition of oncogene expression, inhibition of various of breast adhesion, and cancer invasion. Studies involving therapeutically effective substances on these pathways, especially natural products or derivatives, have been concentrated. Because the high costs, the presence of side effects, the insufficiency of treatment are the major problems in the associated with the contemporary breast cancer treatment methods. Therefore, the aim of this study was to compare the chemopreventive effects of pomegranate extract and tangeretin as natural polyphenolic and flavonoid alternative phytotherapy agents in the breast cancer model created with 7,12-Dimethylbenz [a] anthracene (DMBA) and to investigate the potential anticancer effects of their co-administration.

2. Materials and Methods

2.1. Animals

The study was conducted with a total of 56 female Sprague Dawley rats of 8–10 weeks weighing 205-220 g. The animals were obtained from Firat University Experimental Research Center (FUERC), Elazig, Turkey. All animals were kept at the FUERC at 25±3°C, 50–60% humidity, under a 12 h/12 h light/dark cycle. The rats in all groups were given with the control diet (standard rat food) and water as ad-libitum, and were cared for daily. All studies were performed with the approval of the animal experimental Ethics Review Committee of Firat University (protocol no: 2016/48, date: 06.0.4.2016) and in accordance with the rules of animal welfare.

2.2. Experimental Design

DMBA-induced breast cancer model has been reported as a useful model for evaluating the effects of agents with anticacinogenic potential and for monitoring the metabolism and activation of the tumor at the beginning and progression stages [5]. Breast tumors induced in this way are hormone-dependent adenocarcinomas originating from terminal end-buds in completely undifferentiated glands. In particular, because these tumors are histologically similar to human breast cancer and allow the hormone response relationship to be examined [9], the DMBA-derived breast carcinoma model was used in our study.

The female rats were randomly divided into eight groups. Control group (C group, n=7): A single dose of 1 mL olive oil was administered by gavage on the 15th day of the study. Pomegranate group (P group, n=7): pomegranate extract at a dosage of 5 g/kg in 1 mL Dimethyl sulfoxide (DMSO) was administered by gavage for 30 days and single dose of 1 mL olive oil was administered by gavage on the 15th day of the study following [14]. Tangeretin group (T group, n=7): a 50 mg/kg dosage of tangeretin in 1 mL DMSO was administered by gavage for 30 days and a single dose of 1 mL olive oil was administered by gavage on the 15th day of the study following [39]. Pomegranate extract+Tangeretin group (P+T group, n=6): P and T was administered together at the same doses and in an analogous manner to P group and T group. A single dose of 1 mL olive oil was administered to rats in P+T group by gavage on the 15th day of the study. DMBA Group (D group/Cancer group, n=7): A single dose of 60 mg/kg 7,12-dimethylbenz [a] anthracene (DMBA) in 1mL olive oil was administered on the 15th day of the study by gavage following [14,26]. DMBA+Pomegranate extract group (D+P group, n=8): DMBA was administered together with pomegranate extract. DMBA+Tangeretin group (D+T group, n=7): DMBA was administered together with tangeretin. DMBA+Pomegranate extract+Tangeretin group (D+P+T group, n=7): DMBA was administered together with pomegranate extract and tangeretin. The last 3 groups (D+P, D+T, D+P+T) were designed as phytotherapy group (chemopreventive groups). All phytotherapy agents in these groups were administered in the same doses, same way and on same days. All DMBA agents administered in these groups were administered as explained in the D group (*e.g.*, in the same doses, in the same way, and on the same day).

The study was initiated with 68 animals. Each of the first four groups (C, P, T, P+T) had seven rats, the latter four groups (D, D+P, D+T, D+P+T) was designed to have 10 animals in order not to interrupt the study due to DMBA applications. Some animals died due to gavage and DMBA applications and the study was concluded with a total of 56 animals.

After the administration of DMBA on the 15th day of the study, the period for tumor formation until approximately 115th day was called latent period. During and after this period, the mass formation in the breast was followed by palpation, and the volume of breast tumors detected during the study was evaluated with the aid of electronic calipers by the protocol laid down and recorded [40]. The rats were sacrificed at the end of the 23rd week of the experiment.

Before starting the experiment, the body weights of the animals in the groups were measured once a week during the experiment and before the animals were slaughtered at the end of the experiment. The daily growth rates of animals in the groups were determined by the calculation following Güngör et al. [41].

2.3. Chemical Agents

DMBA: The analytical purity of DMBA (Tokyo Chemical Industry Co., Ltd., Portland, USA) used in the study was >98% (according to Gas Chromatography).

Pomegranate extract: The analytical purity of commercial all pomegranate extract (Pomella, Verdure Sciences, Noblesville, USA) used as a therapeutic agent in the study, was >95% and in powder form, and included the standardized natural polyphenolic content ratio of 37.5% ellagitannin and 2.7% ellagic acid.

Tangeretin: The analytical purity of tangeretin (AvaChem Scientific, San Antonio, USA) used as a therapeutic agent in the study was 98% (by HPLC) and obtained in the powder form.

DMSO: To dissolve pomegranate extract and tangeretin, 0.1% DMSO (Fisher scientific, Leicestershire, UK), a polar solvent, was freshly prepared daily before gavage applications and used (Application doses of therapeutic agents were prepared in 1 mL of 0.1% DMSO).

2.4. Histopathological analyses

The presence of the breast tumor tissue was demonstrated with histopathological examinations. Breast tissues from each group were determined in 10% formol solution for histological studies and then dehydrated by passing through routine histological follow-up series. The tissues were then polished in xylol and embedded in paraffin blocks. Sections with a thickness of 4–6 μm were cut using a hand driven microtome. Sections obtained from paraffin blocks were stained with hematoxylin-eosin (H&E) on an automatic staining device (Ventana Medical System, SN: 712299, REF: 750-700, Arizona, USA) and evaluated under light microscope (Olympus BX50, Olympus Corp., Tokyo, Japan; 200X magnification). The photographs were taken with imaging device (Olympus DP72, Olympus Corp., Tokyo, Japan) and were recorded. In addition, the masses in the breast tissues of the sacrificed animals were photographed and recorded after macroscopic examination. Following microscopic determination of tumor formation in breast tissue by a specialist pathologist, various calculations were executed for determination of tumor incidence rates [26], mean tumor volumes [40], and tumor weights [26] in each group.

2.5. Immunohistochemical analyses

After preparing 4–6 μm sections, immunohistochemical stainings were conducted using Avidin Biotin Complex (ABC) based on the protocol reported earlier [42]. Deparaffinized tissue sections were washed in running water for 5 min and were passed through a graded alcohol series. After rinsing, incubation was conducted in 3% H_2O_2 in absolute methanol for 5 min (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA). The slides were incubated with the primary antibodies against Ki-67 (1/100 ratio; Rabbit polyclonal Anti-Ki-67 antibody, PA5-16785, ThermoFisher Scientific, Rockford, USA) and ER-α (1/1000 ratio: Rabbit polyclonal Anti-ER-α antibody, PA1-308, ThermoFisher Scientific, Rockford, USA) at 4°C overnight. After PBS washing, the slides were incubated with biotinylated Goat Anti-Poliyvalent (Anti-mouse/rabbit IgG), and TP-125-BN (Lab Vision Corporation, USA) as secondary antibodies. Streptavidin-peroxidase treatment (TS-125-HR, Lab Vision Corporation, USA) was added for 30 min. Labeling was visualized by development with 3-amino-9-ethylcarbazole (AEC) substrate+AEC chromogen (AEC Substrate, TA-015, and AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA). The slides were washed between steps in PBS for 5 min and distilled water before being counterstained in Harris hematoxylin, dehydrated, and mounted with glycerol for inspection with an Olympus BX 50 photomicroscope. All of the slides were examined independently by two experienced pathologists and the pathologists were blinded to the treatment groups while scoring IHC images. A semiquantitative scoring system was used to describe intensity of staining (No staining:0, very weak staining:0.5, weak staining:1, moderate staining:2, strong staining:3). The overall immunohistochemical score (histoscore) was computed as the percentage of immunopositive tumor cells (immunoreactivity prevalence; 0.1<25%, 0.4=26–50%, 0.6=51–75% and 0.9=76–100%) multiplied by their staining intensity.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis

TUNEL method allows observation of DNA breaks in the cells. After cross sections taken from paraffin blocks at thicknesses of 4–6 μm, they were placed on poly-l-lysine coated lamellae. Apoptotic cell death was detected using TUNEL assay according to the manufacturer's suggestions (ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit, EMD Milipore Corp, Cat no: S7101, Temecula USA). In the assessment of TUNEL staining, the nuclei stained blue with methyl green were considered normal, and the cells with nuclei staining brown were taken as apoptotic. Percent-ages of TUNEL-positive cells were quantified by counting 500 cells from ten random microscopic fields. The percentage of apoptotic cells (apoptotic index [AI]) was calculated as follows:

$$\%\text{apoptotic cells} = \frac{[(\text{total no. of cells conunted}) - (\text{total no. of live cells})] \times 100}{\text{total no. of cells counted}}$$

2.7. Biochemical Analyses

2.7.1. Measurement of plasma CA 15-3, CEA, VEGF, MMP-9, and NF-κB levels by ELISA

CA 15-3, CEA, VEGF, MMP-9, and NF-κB levels in the plasma were determined by enzyme-linked immunosorbent assay (ELISA) method according to manufacturer's recommendations using rat ELISA kits (Ylbiont, Shangai YL Biotech Co Ltd, Shangai, China). Samples were prepared in duplicate and results were read at 450 nm absorbance for all parameters using an automated ELISA reader (ELX 800, Bio-Tek Instruments Inc., Winooski, USA). The assay range of the CA 15-3 kit was 0.3–90 μIU/mL with the sensitivity of 0.15 μIU/mL while that of the CEA kit was 0.05–30 ng/mL with the sensitivity of 0.031 ng/mL. The assay range of the VEGF kit was 10–3000 ng/mL with the sensitivity of 5.01 ng/mL whereas the range of the MMP-9 kit was 0.05–10 ng/mL with the sensitivity of 0.01 ng/mL. The assay range

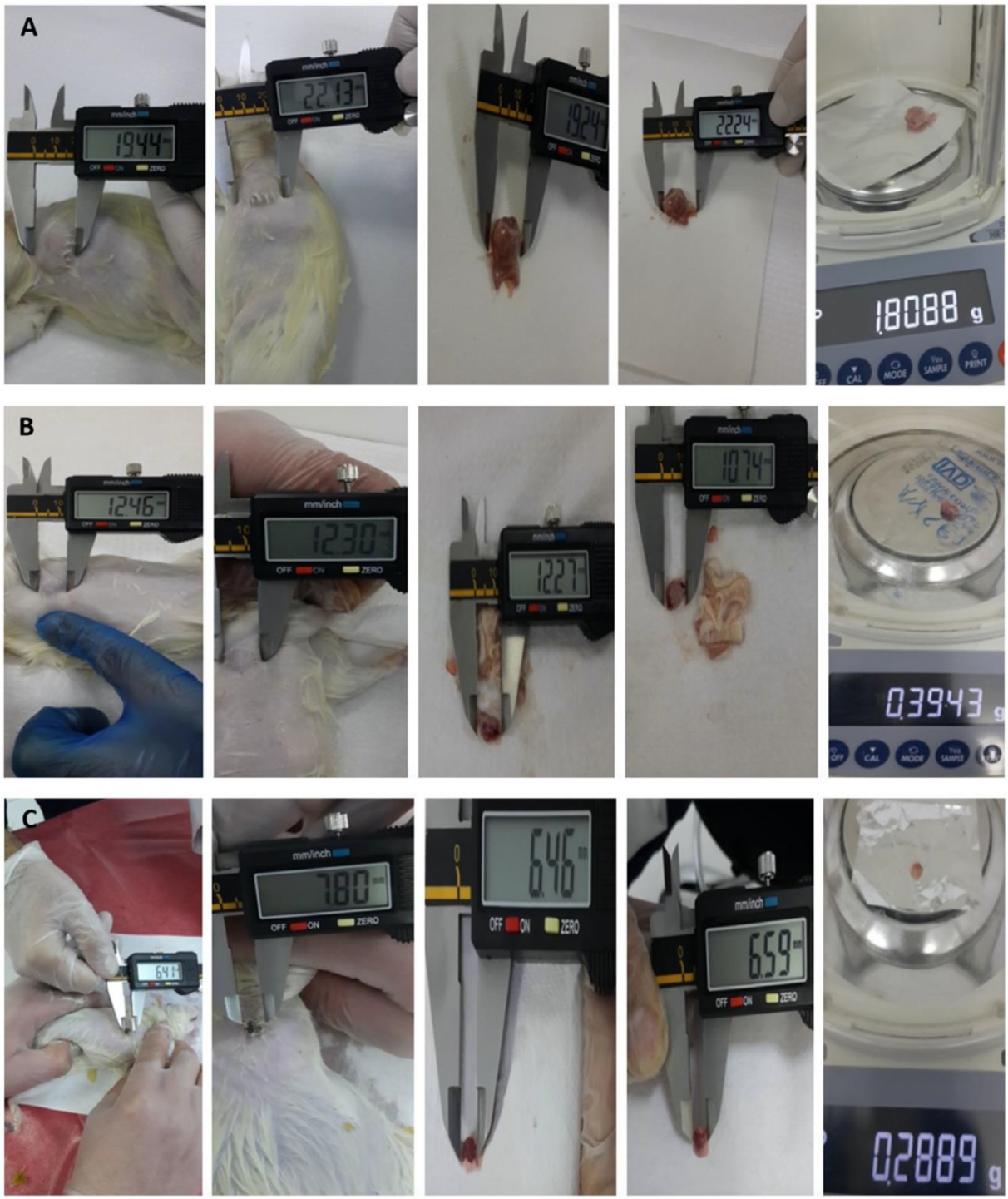

Fig. 1. Macroscopic view of tumor involvement, volume, and weight in the breast A: DMBA group, Cancerous breast tissue group, B: D+P group, Cancerous breast tissue+Pomegranate group, C: D+T group, Cancerous breast tissue+Tangeretin group.

of the NF-κB kit was 0.05–20 ng/mL and its sensitivity was 0.023 ng/mL. The intra-assay coefficient (CV%) and inter-assay CV% values were less than 8% and 10% of all kits as quoted by the manufacturer.

2.7.2. Measurement of tissue protein expression levels by Western blot analysis

Frozen breast tissues were weighed (Sartorius GMBH, Germany) and homogenized with RIPA lysis buffer solution using Cocktail protease inhibitor (1;9,w;v, Santa Cruz Biotechnology, Inc. USA) using a Bullet Blender tissue homogenizer (Next Advanced Inc., Averill Park, NY, USA). All procedures were carried out on ice at +4°C in accordance with the manufacturer's instructions. Homogenized samples were ultracentrifuged at 90.000 rpm for 1 h at +4 °C for the extraction of core protein (for the this process, ultracentrifuge device (BECKMAN COULTER Optima L-100XP Ultracentrifuge, rotor: TYPE 90 Ti, Brea, CA, US) and suitable for device, rotor heads and micro centrifugation tubes were used). Samples were stored at −80°C until analysis. The total protein levels of the obtained supernatants were determined using the Qubit protein measurement kit (Qubit Protein Assay Kit, Invitrogen, Carlsbad, CA, USA) and the Qubit Fluorometer-II instrument (Invitrogen, Carlsbad, CA, USA). Results were recorded in mg/mL.

Thirty micrograms of the total protein was loaded on gels (NuPAGE 4–12% Bis-Tris; Life Technologies Corporation, Carlsbad, CA, USA) for electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with mouse

Table 1
Live weight (LW) gain differences between groups

Groups	First week LW (g)		23rd week LW (g)		Total LW gain (g)		Growth rates	
	Median	(min–max)	Median	(min–max)	Median	(min–max)	Median	(min–max)
C	215	(207–225)	308.5	(298–323.5)	97.1	(87.7–99.5)	0.68	(0.62–0.71)
P	218	(207.5–226)	293.5	(290–325.1)	83.5	(70–99.1)	0.59	(0.50–0.70)
T	213.5	(198–219)	295	(263.6–307.8)	80.2	(55–109.8)	0.56	(0.39–0.78)
T+P	219.7	(202–227)	312.5	(290–325.7)	90.8	(86.1–106.2)	0.64	(0.61–0.75)
D	208.6	(203.8–228)	255.5	(245–260)	44.5*	(32–53.7)	0.31*	(0.23–0.38)
D+P	205.6	(201–217.5)	277	(256.4–287.2)	67.5†	(55.4–81)	0.47†	(0.39–0.57)
D+T	210	(200–214.3)	269.7	(265–280)	66‡	(55–69.4)	0.46‡	(0.39–0.49)
D+T+P	210	(200.7–220)	280	(274.5–310.5)	71.9†	(65.5–90.5)	0.50†	(0.46–0.64)

Values are given as median and (minimum value–maximum value).
Abbreviations: D+P group, cancerous breast tissue+pomegranate group; D+T group, cancerous breast tissue+tangeretin group; D+P+T group: cancerous breast tissue+pomegranate+tangeretin group; P group, pomegranate group; T group, tangeretin group; P+T group, pomegranate+tangeretin group.
* $P<.001$: Compare to the Control (C) group, Healthy breast tissue (One-way ANOVA, Bonferroni test),
† $P<.001$.
‡ $P<.005$ Compare to DMBA (D) group, Cancerous breast tissue group (One-way ANOVA, Bonferroni test).

monoclonal antibody against p-53 (cat no: NB200-103, at 1:200 dilutions; NOVUS BIOLOGICALS, Littleton, USA). So as to reveal the expressions of other target proteins, Mouse monoclonal Anti-Bax primer antibodies (cat no: NBP1-28566, at 1:200 dilutions; NOVUS BIOLOGICALS, Littleton, USA), Mouse monoclonal Anti-Bcl-2 primer antibody (cat no:NB100-78543, at 1:1000 Dilutions; NOVUS BIOLOGICALS, Littleton, USA), Mouse Monoklonal Anti–β-Catenin primer antibody (cat no: NBP1-54467, at 1:1000 Dilutions; NOVUS BIOLOGICALS, Littleton, USA) and Mouse monoclonal Anti-Cyclin D1 primer antibody (cat no: NBP2-32840, at 1:1000 Dilutions; NOVUS BIOLOGICALS, Littleton, USA) were used. Probed mouse monoclonal antibody against β-Actin was used as the reference protein (cat no: NB600-501, at 1:2000 dilutions, NOVUS BIOLOGICALS, Littleton, USA). The blot was incubated with horseradish peroxidase-conjugated secondary antibody, and the protein bands were visualized by chromogenic substrates using WesternBreeze Chromogenic Immunodetection Kit (WesternBreeze Chromogenic Kit–Anti-Mouse, WB7103, Invitrogen, Carlsbad, CA, USA). Finally, membranes containing protein bands were scanned at 600 dpi and transferred to computer. Image-j program (National Institute of Health, Bethesda, USA). Relative protein concentrations were calculated by standardizing with β-Actin protein (housekeeping used in our study as reference) and the expressions levels of p53, Bax, Bcl-2, β-Catenin, and Cylin D1 proteins were compared.

2.8. Statistical analyses

The data for all variables were reported as mean±SD of independent observations for each group. One-way analysis of variance (ANOVA) was performed to compare the means between the different treatments and in the case of significance Dunn's Test or Bonferroni post-hoc comparison methods were utilized to deduce pairwise differences. Mann-Whitney U test was used to determine the occurrence of cancer (control-DMBA) while Kruskal-Wallis test was used to determine the chemopreventive efficacy of the drugs (D/ D+P, D+T, D+P+T). The level of significance was accepted as $P<.05$ throughout the experiments. All the statistical analyses were performed with SPSS 22.0 statistical software package (Firat University License No: 42021148f00141a304ab).

3. Results

3.1. General Macroscopic Evaluations of Groups

During and after the experiment, macroscopic changes in animals were evaluated by inspection and palpation. During this period, no pathology was observed in the general condition of the animals in the control group. Compared to the control group, the hair of the animals in the DMBA group were coarse mixed with occasional rashes. Although some rats in the treatment groups showed similar symptoms, they were not as common as in the DMBA group. After the initial one third of the study, vaginal hemorrhages were observed in several animals in the DMBA group, while blindness was observed in the eyes of three animals in the same group.

In the macroscopic evaluation of the organs and tissues removed after sacrifice, hepatomegaly, and splenomegaly were observed especially in the majority of the animals in the DMBA group, while widespread tumoral involvement was observed in the breast tissue of five animals from the same group (Fig. 1, Fig. S1). Furthermore, as a result of macroscopic organ evaluation of rats in the same group, mass formation in the lung and uterine tissues of some animals and necrotic foci, as well as signs of excessive fat in the abdominal organs were observed (Fig. S2). While these findings were similar in other treatment groups, both the number of tumor involvement in breast tissue and the prevalence of macroscopic lesions in other organs were fewer than those in the DMBA group. Macroscopic and microscopic evaluations for tumor formation are detailed in histopathological findings.

3.2. Body Weights and Growth Rates of Groups

All rats were routinely monitored to determine whether oral administration of pomegranate and tangeretin had any adverse effects on body weight gain or growth rate. In the study, no significant difference was found in the initial and final body weights of the animals in the P, T, and P+T groups given only these treatment agents for a month, whereas it was found that body weights increased at a normal rate in all groups (Table 1). It was observed that body weights and growth rates were significantly lower in rats in all DMBA groups compared to controls ($P<.001$; Table 1). Body weight and growth rates were significantly higher in all treatment groups (D+P; D+T; D+P+T) compared to DMBA ($P<.001$; $P<.005$; $P<.001$, respectively; Table 1). While DMBA was found to cause significantly reduced weight gain and growth rates compared to controls, there was a significant increase in live weight (LW) gain and growth rates with pomegranate extract and tangeretin treatments in rats induced by DMBA.

3.3. Biochemical Findings of Groups

3.3.1. Plasma CA 15-3 and CEA Levels

Plasma CA 15-3 levels in the D group (36.35 μIU/mL) were significantly higher than in the control group (11.35 μIU/mL; $P=.001$). When D group and D+P (17.72 μIU/mL), D+T (18.99 μIU/mL), and D+P+T (18.44 μIU/mL) groups were compared, plasma CA 15-3 levels were found to be statistically significant (for three groups; $P<.05$; Table 2).

Table 2
Plasma tumor markers levels of eight groups

Groups	CA 15-3 (μIU/mL)		CEA (ng/mL)	
	Median	(min–max)	Median	(min–max)
C (n=7)	11.35	(7.49–13.92)	2.80	(2.22–3.41)
P (n=7)	15.08	(10.02–27.65)	3.45	(2.16–4.13)
T (n=7)	16.95	(9.33–19.71)	3.53	(2.60–4.05)
P+T (n=6)	16.02	(9.54–20.38)	3.09	(2.11–4.15)
D (n=7)	36.35*	(18.54–60.58)	4.47*	(4.00–8.70)
D+P (n=8)	17.72†	(12.61–34.93)	3.99†	(2.80–5.24)
D+T (n=7)	18.99†	(14.25–29.05)	3.61†	(2.79–4.25)
D+P+T (n=7)	18.44†	(11.33–20.59)	3.60†	(2.93–4.42)

Abbreviations: D+P group, cancerous breast tissue+pomegranate group; D+T group, cancerous breast tissue+tangeretin group; D+P+T group: cancerous breast tissue+pomegranate+tangeretin group; P group, pomegranate group; T group, tangeretin group; P+T group, pomegranate+tangeretin group.
Values are given as median and (minimum value–maximum value).
* P=.001: Compare to the Control (C) group, Healthy breast tissue (Mann-Withney U test),
† P<.05: Compare to DMBA (D) group, Cancerous breast tissue group (Kruskal-Wallis. Dunn test).

Plasma CEA levels were significantly higher in the D group (4.47 ng/mL) compare to the control group (2.80 ng/mL; P=.01). When the D group and D+P (3.99 ng/mL), D+T (3.61 ng/mL), and D+P+T (3.6 ng/mL) groups were compared, plasma CEA levels were significantly lower in all treatment groups (P<.05; Table 2).

3.3.2. Plasma VEGF, MMP-9, and NF-κB Levels

Plasma VEGF levels were significantly higher in the D group (430.96 ng/mL) compare to the C group (203.66 ng/mL; P=.001). When D group and D+P (265.5 ng/mL), D+T (264.98 ng/mL), and D+P+T (234.88 ng/mL) groups were compared, statistically significant decreases were observed in plasma VEGF levels in all treatment groups were compared (for three groups; P<.05; Table 3).

When the plasma MMP-9 levels were compared in terms of median values, a statistically significant increase was observed in the D group (1.5 ng/mL) compared to the C group (0.96 ng/mL; P<.005). In comparison of D group, D+P (1.22 ng/mL), D+T (1.34 ng/mL), D+P+T (1.15 ng/mL) groups did not demonstrated a significant difference in MMP-9 in all treatment groups (Table 3).

Median values of NF-κB levels were significantly higher in D group (3.46 ng/mL) compared to control group (1.91 ng/mL; P<.005). When D and treatment groups were compared, NF in both D+P (2.35 ng/mL) group (P<.05), and D+P+T (2.28 ng/mL) group were significantly lower (P<.05) while the decrease in D+T (2.36 ng/mL) group was found to be statistically insignificant (Table 3).

3.3.3. Tissue p53 Protein Expression Levels

According to Western blot analysis, p53 protein expression levels in breast tissue were found to be 22% lower in D group (78%) compared to control (100%) group (P<.01). When the treatment groups were compared with DMBA, tissue p53 protein expression levels showed significant increases in D+P (136%) and D+P+T (146%) groups, whereas the increase in D+T (109%) was not significant (Fig. 2B).

3.3.4. Tissue Bax Protein Expression Levels

According to the Western blot histogram shown in Fig. 2C, Bax protein expression levels in breast tissue were significantly reduced in the D group (53%) compared to the control group (100%) by approximately 50% (P≤.001). When the treatment groups were compared with D group, tissue Bax protein expression levels were significantly increased in D+P (131%) and D+P+T (140%) groups, whereas the increase in D+T (117%) group was not significant (Fig. 2C).

3.3.5. Tissue Bcl-2 Protein Expression Levels

When the histogram in Fig. 2D was examined, it was evident that Bcl-2 protein expression levels in breast tissue increased significantly in the D group (138%) compared to the C group (100%) by 38% (P≤.001). In the D+P (121%), D+T (127%), and D+P+T (111%) treatment groups, Bcl-2 protein expression levels did not significantly differ from the D group.

3.3.6. Tissue β-Catenin Protein Expression Levels

As indicated in Fig. 2E, β-actin-standardized tissue β-Catenin protein expression levels were significantly increased by 40% in the D group (140%) compared to the C group (100%;P<.005). Tissue β-Catenin protein expression levels in the D+P (118%), D+T (122%), and D+P+T (113%) treatment groups were found not to significantly different from D group.

Table 3
Plasma angiogenesis levels of eight groups

Groups	VEGF (ng/mL protein)		MMP-9 (ng/mL protein)		NF-κB (ng/mL protein)	
	Median	(min–max)	Median	(min–max)	Median	(min–max)
C	203.66	(187.09–244.42)	0.96	(0.86–1.23)	1.91	(1.55–2.63)
P	252.58	(193.11–269.20)	1.01	(0.85–1.67)	1.84	(1.78–2.11)
T	228.26	(164.61–270.10)	1.12	(0.78–1.35)	2.01	(1.61–2.26)
P+T	215.35	(200.16–262.02)	1.03	(0.80–1.41)	1.75	(1.54–2.13)
D	430.96*	(271.16–568.48)	1.50†	(1.07–2.12)	3.46†	(2.34–5.06)
D+P	265.50‡	(165.43–302.52)	1.22	(1.11–1.97)	2.35‡	(1.97–3.55)
D+T	264.98‡	(230.93–286.47)	1.34	(0.80–1.90)	2.36	(2.27–3.92)
D+P+T	234.88‡	(217.71–327.73)	1.15	(1.04–1.68)	2.28‡	(2.01–2.68)

Abbreviations: D+P group, cancerous breast tissue+pomegranate group; D+T group, cancerous breast tissue+tangeretin group; D+P+T group: cancerous breast tissue+pomegranate+tangeretin group; P group, pomegranate group; T group, tangeretin group; P+T group, pomegranate+tangeretin group.
Values are given as median and (minimum value- maximum value),
* P=.001.
† P<.005 Compare to the Control (C) group, Healthy breast tissue (Mann-Withney U test),
‡ P<.05: Compare to DMBA (D) group, Cancerous breast tissue group (Kruskal-Wallis. Dunn test).

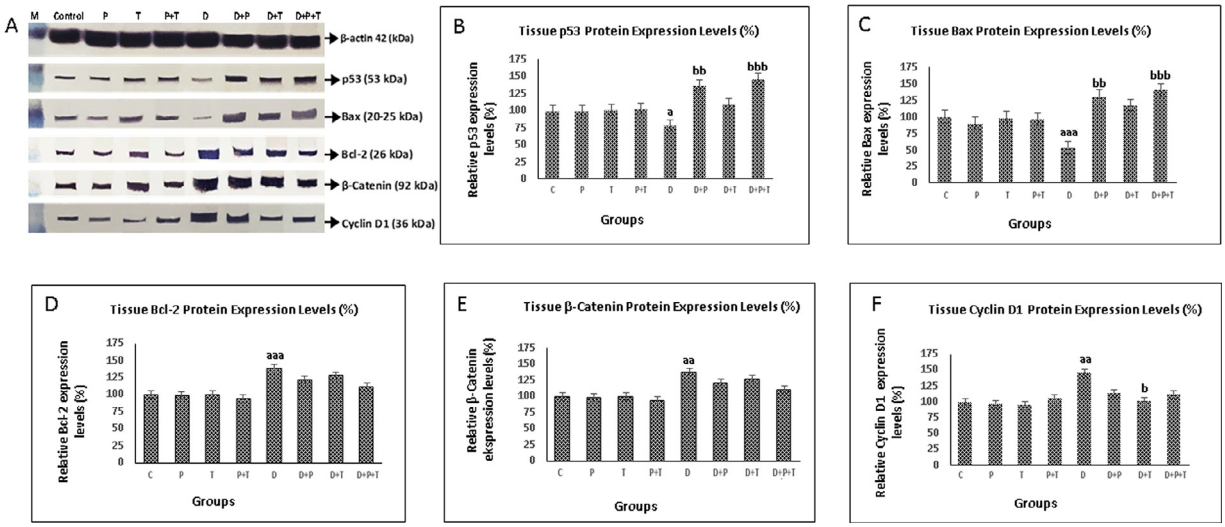

Fig. 2. Protein expression levels and patterns of p53, Bax, Bcl-2, β-Catenin, Cyclin D1 in mammary tissue of rats A: Western blotting results of the protein expressions. Marker: Molecular weights of the protein bands in the SeeBlue Plus2Pre-Stained Standard. Western blotting was normalized to β-actin expression and expressed relative to the control sample. The histograms of protein expression levels of groups for B: p53 Protein, C: Bax Protein, D: Bcl-2 Protein, E: β-Catenin Protein, and F: Cyclin D1 Protein, P group: Pomegranate group, T group: Tangeretin group, P+T group: Pomegranate+Tangeretin group, D+P group: Cancerous breast tissue+Pomegranate group, D+T group: Cancerous breast tissue+Tangeretin group, D+P+T group: Cancerous breast tissue+Pomegranate+Tangeretin group, ($^{a}P<.01$; $^{aa}P<.005$; $^{aaa}P\leq.001$: Compared to the Control [C] group, Healthy breast tissue). ($^{b}P<.01$; $^{bb}P<.005$; $^{bbb}P<.001$: Compared to the DMBA [D] group, Cancerous breast tissue group).

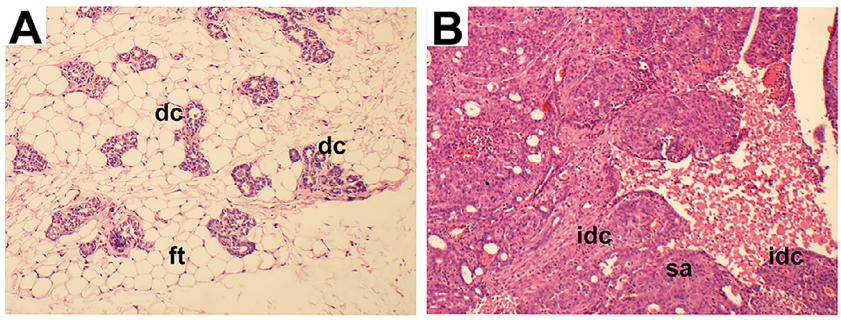

Fig. 3. Histopathological view of breast tissue A: Control group, Healthy breast tissue, ft: fatty tissue, dc: ducts, B: DMBA group, Cancerous breast tissue, sa: solid area, idc: invasive ducts. H&E, 200X.

3.3.7. Tissue Cyclin D1 Protein Expression Levels

In the histogram showing the expression of cyclin D1 in breast tissue (Fig. 2F), normalized tissue Cyclin D1 protein expression levels increased by 45% in D group (145%) compared to the C group (100%) and this difference was found to be significant ($P<.005$). In addition, tissue Cyclin D1 expression levels was not significantly different in D+P (138%), D+T (100.5%), and D+P+T (116%) treatment groups when compared to the D group while a statistically significant difference was observed only in the D+T group ($P<.01$).

3.4. Histopathological, Immunohistochemical Evaluation, and TUNEL Analysis

3.4.1. Histopathological Evaluation

The result of the examination of hemotoxylin-eosin staining under light microscope for the detection of cancer cells revealed that solid foci were observed in the cross-sectional areas of breast tissue belonging to D group consisting of cancer rats (Fig. 3) and the appearance of invasive ductal carcinoma (idc) in ductal structures are indicated in Fig. 3B. In addition, the incidence of tumor involvement, tumor volumes and tumor weights of all groups as a result of histopathological evaluations are indicated in Table 4 (Fig. 1).

3.4.2. Immunohistochemical Evaluation

3.4.2.1. Evaluation of ER-α immunoreactivity. The examination of immunohistochemical staining for ER-α immune reactivity under light microscope (Fig. 4) indicated that ER-α immunoreactivity was similar in C, P, T, P+T groups (Fig. 4A–D). Compared with the control group, ER-α immunoreactivity was significantly increased in D and treatment groups (D+T, D+P, D+P+T;$P<.05$). When compared with the D group, ER-α immunoreactivity was significantly decreased (Fig. 4E–H) in all treatment (D+T, D+P, D+P+T) groups ($P<.05$; Table 5).

3.4.2.2. Evaluation of Ki-67 immunoreactivity. The immunohistochemical staining for Ki-67 immune reactivity under light microscope revealed that Ki-67 immunoreactivity was similar in C, P, T, P+T groups (Fig. 5A–D). Ki-67 immunoreactivity was significantly increased in cancer and treatment groups compared to the control group ($P<.05$). When compared with D group, Ki-67 immuneoreactivity was significantly decreased in all treatment D+T, D+P, D+P+T groups (Fig. 5E–H;$P<.05$; Table 5).

3.4.3. TUNEL Analysis

The examination of TUNEL staining under light microscope for the detection of apoptotic cells (Fig. 6) indicated that TUNEL positivity was similar in C, P, T, P+T groups (Fig. 6A–D). TUNEL

Table 4
Tumor involvement, incidence, mean tumor volumes, and the distribution of tumor weights by groups

Groups	Rat no	Tumor involvement	Tumor incidence (%)	Mean tumor volumes (cm^3)	Mean tumor weights (g)
C	7	-	-	-	-
P	7	-	-	-	-
T	7	-	-	-	-
P+T	6	-	-	-	-
D	7	5*	71.42	5.1	1.49
D+P	8	1*	12.50	1.42	0.39
D+T	7	1*	14.28	0.28	0.29
D+P+T	7	-	-	-	-

Abbreviations: D+P group, cancerous breast tissue+pomegranate group; D+T group, cancerous breast tissue+tangeretin group; D+P+T group: cancerous breast tissue+pomegranate+tangeretin group; P group, pomegranate group; T group, tangeretin group; P+T group, pomegranate+tangeretin group.

* Indicates the histopathological type of tumor; 95% of tumor involvement was diagnosed as invasive ductal carcinoma (idc).

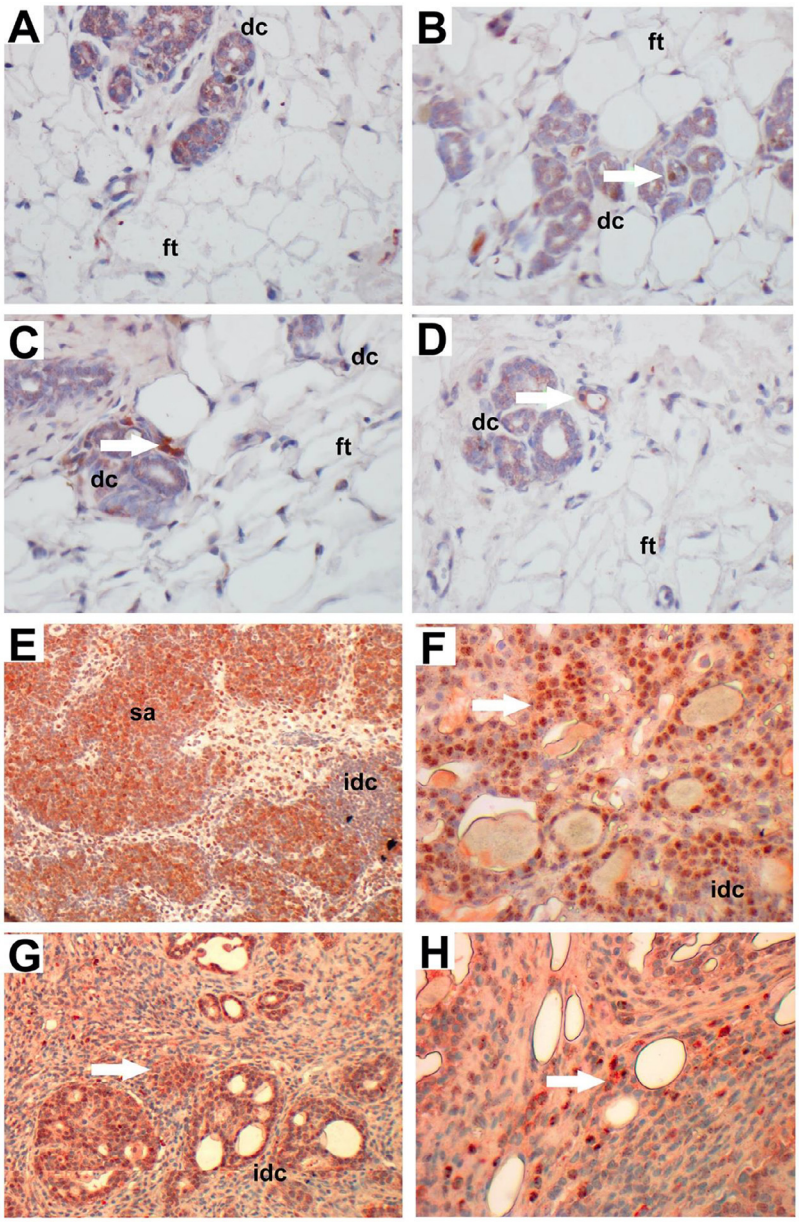

Fig. 4. Immunohistochemical analysis of estrogen receptor-alpha (ER-α) status in the breast tissue of control and experimental animals A: Control group, Healthy breast tissue, B: P group, Pomegranate group, C: T group, Tangeretin group, D: P+T group, Pomegranate+Tangeretin group, E: DMBA group, Cancerous breast tissue group, F: D+P group, Cancerous breast tissue+Pomegranate group, G: D+T group, Cancerous breast tissue+Tangeretin group, H: D+P+T group. Cancerous breast tissue+Pomegranate+Tangeretin group, ft: fatty tissue, dc: ducts, white arrow: ER-α positivity cells in healthy or cancerous breast tissue. sa: solid area, idc: invasive ducts, 200X.

Table 5
Immunohistochemistry histoscores and apoptotic index (%) of the eight experimental groups

	ER-α histoscore		Ki-67 histoscore		Apoptotic index (%)	
	Normal tissue	Malign tissue	Normal tissue	Malign tissue	Normal tissue	Malign tissue
C	0.26±0.12	-	0.23±0.12	-	4.14±1.77	-
P	0.29±0.18	-	0.22±0.11	-	2.57±1.27	-
T	0.35±0.18	-	0.27±0.14	-	3.42±0.97	-
P+T	0.25±014		0.29±0.16		3.33±1.96	
D	-	2.57±0.34*	-	2.57±0.34*	-	0.71±0.48*
D+P	-	0.92±0.19*,†	-	0.88±0.13*,†	-	4.25±1.83†
D+T	-	0.81±0.32*,†	-	0.90±0.32*,†	-	3.57±1.27
D+P+T	0.52±0.25*,†	-	0.6 ±0.2*,†	-	4.28±1.70*,†	-

Since no tumor formation was found in D+P+T group (Cancerous breast tissue+Pomegranate+Tangeretin group) falls under normal tissue in table.
Abbreviations: D+P group, cancerous breast tissue+pomegranate group; D+T group, cancerous breast tissue+tangeretin group; P group, pomegranate group; T group, tangeretin group; P+T group, pomegranate+tangeretin group.
Values are given as mean ± standard deviation,
* $P<.05$: compare to Control (C) group, Healthy breast tissue (One-way ANOVA, post-hoc Tukey HSD),
† $P<.05$: Compare to DMBA (D) group, Cancerous breast tissue group (One-way ANOVA, post-hoc Tukey HSD).

positivity was significantly decreased in D group when compared with control group. TUNEL positivity was significantly increased in pomegranate extract and D+P+T treated groups compared to D group ($P<.05$) and higher than C group. This difference was not statistically significant in the D+T group (Table 5; Fig. 6E–H).

4. Discussions

Current treatment options for breast cancer include surgical resections, radiotherapy and neo-adjuvant/adjuvant chemotherapy, and hormonal and biological treatments. Although there are many chemotherapeutic drugs for the treatment of breast cancer, resistance to these drugs and serious side effects are two drawbacks. Thus, there is an urgent need for research and development of new alternative drugs that are more effective and less toxic.

In order to test such alternative drugs, experimental animals with the induced cancer models serve as an invaluable tool. DMBA induced breast cancer model was utilized in the present study. It was reported earlier that breast cancer is induced after approximately 13, 16, and 12 weeks following administration to rats [14,26,33]. In our study, as a result of DMBA application at a dose of 60 mg/kg, the first tumor was formed after 15 weeks and this latent time was found to be consistent with the dose-dependent tumor formation times used in earlier cancer models.

When we compared the initial and final body weights of the animals, there was no statistically significant difference between the control group and the P, T, and P+T groups, whereas the body weights of the rats in these four groups designed as sham groups were found that they increased at a normal rate (Table 1). In the study, significant decreases in weight gain and growth rates compared to controls were determined in DMBA-induced breast cancer rats and significant increases in live weight growth and growth rates as a result of oral chemopreventive administration of pomegranate and tangeretin were consistent with the literature [26,43,44].

Breast cancer involves a process characterized by unregulated cell growth and metastasis. Anti-cancer properties of various flavonoids (naringin, hesperidin, naringenin, hesperitin, routine, nobiletin, tangeretin) have been demonstrated in the management of this process. For example, in the DMBA-induced breast cancer model in female Sprague Dawley rats, these flavonoids were demonstrated to suppress breast cancer by inducing apoptosis and inhibiting cell proliferation [45]. In other *in vivo* studies, tangeretin was indicated to play a role in tumor cell proliferation markers such as PCNA, COX-2, and Ki-67 and to inhibit the formation and proliferation of DMBA-induced breast carcinogenesis in rats [37,43].

A marginal and insignificant increase in p53 and Bax protein expression levels in the phytotherapy group treated with tangeretin compare to DMBA group makes the apoptotic efficacy of tangeretin controversial. In all treatment groups compared to the DMBA group, tissue Cyclin D1 expression levels were significantly decreased in tangeretin treated group, whereas β-Catenin expression levels were found to be similar. These findings suggest that Tangeretin inhibits proliferation of cancerous cells using cyclin D1 suppression not through β-Catenin expression and performing cell cycle independent of p53 (with p21 or 27) in G1 phase.

The statistically insignificant increase in the number of cells entering the apoptosis pathway in the breast tissues of the rats in the tangeretin supplied treatment group compared to the cancer group at TUNEL and immunohistochemical levels supports the inability of tangeretin to use the apoptosis pathway effectively. In addition, significant and effective decreases in the immune reactivity of the mitotic index parameters ER-α and Ki-67 in the tangeretin group as a treatment agent compared to the DMBA group support the other findings suggesting that tangeretin acts as a tumor suppressor gene against cancer cells. Hence, we think that tangeretin shows its chemopreventive activity against DMBA-induced breast cancer with cytostatic effect rather than cytotoxic effect.

There are numerous studies linking the chemopreventive and therapeutic properties of tangeretin and similar flavones with various cytotoxic effects on various cancers [34,37,46–48]. However, tangeretin's anti-cancer properties of cytostatic nature were also reported [49] and our study results are more congruent with the conclusion of the cytostatic nature. Furthermore, in order to clarify these different interpretations in the literature, we believe that more conclusive studies targeting the effects of flavones on cancer are needed.

Some researchers have described the cancer-inhibiting effects of tangeretin and various flavon derivatives with their cytotoxic properties while reducing their proliferation and inducing apoptosis in certain cancer cells [28,46–48]. For example, according to the reports of *in vitro* gastric cancer study Dong et al. [46] tangeretin has a dose-dependent cytotoxic effect. This effect of tangeretin in cancer cells is attributed to the mitochondrial membrane polarity in a p53-dependency and disrupting the expression of proapoptotic

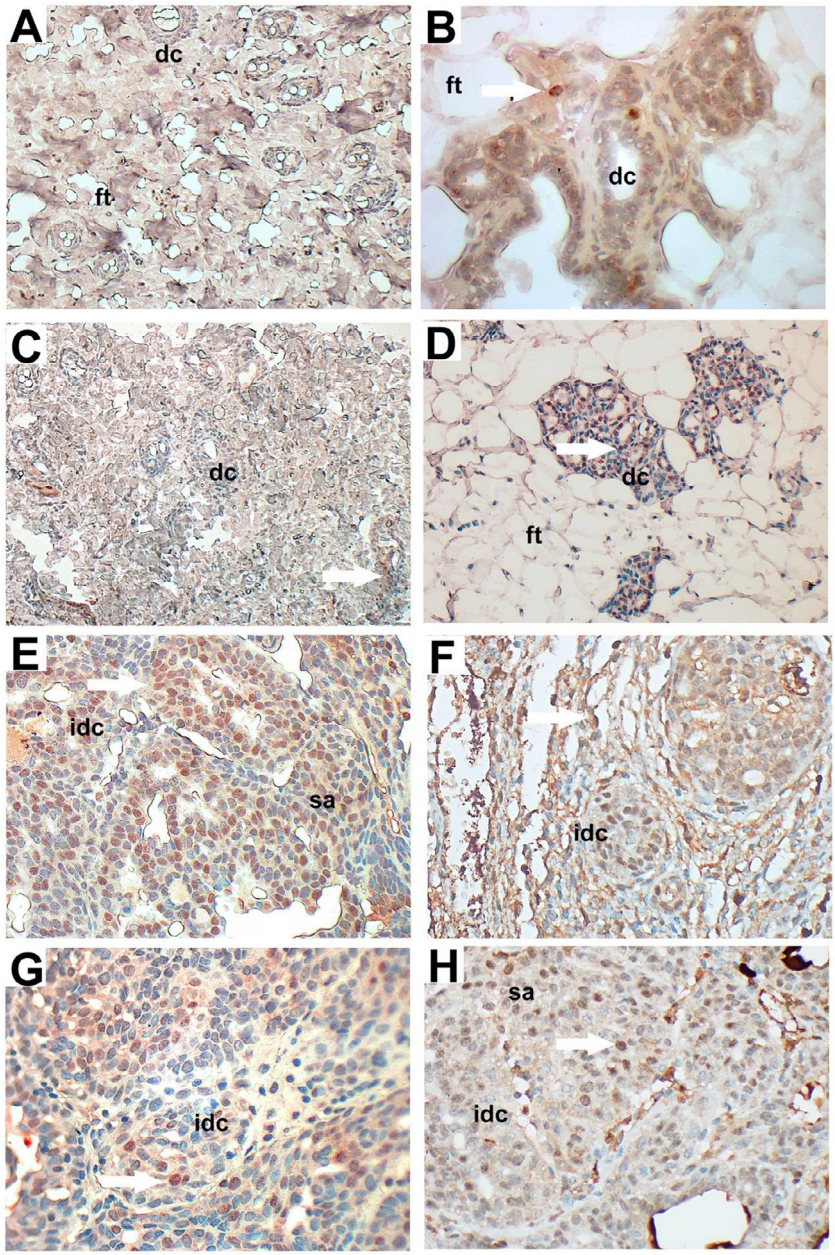

Fig. 5. Immunohistochemical analysis of Ki-67 status in the breast tissue of control and experimental animals A: Control group, Healthy breast tissue, B: P group, Pomegranate group, C: T group, Tangeretin group, D: P+T group, Pomegranate+Tangeretin group, E: DMBA group, Cancerous breast tissue group, F: D+P group, Cancerous breast tissue+Pomegranate group, G: D+T group, Cancerous breast tissue+Tangeretin group, H: D+P+T group. Cancerous breast tissue+Pomegranate+Tangeretin group, ft: fatty tissue, dc: ducts, white arrow: Ki-67 positivity cells in healthy or cancerous breast tissue. sa: solid area, idc: invasive ducts, 200X.

markers in these cells with the condition to up-regulate the intrinsic and extrinsic apoptotic pathway and thus leading the suppression of the proliferation of tumor cells [47].

Here, we demonstrated that pomegranate has a chemopreventive effect on breast cancer. Similarly, Khan et al. [50] indicated that polyphenols contained in pomegranate fruit extracts have antiproliferating, anti-invasive effect on breast cancer cells. Researchers have suggested that pomegranate has these phytotherapeutic effects due to inhibition of the NF-κB pathway. In addition, Dikmen et al. [25] a dose-dependent proapoptotic effect on human breast cancer cell lines. In subsequent years, Banerjee et al. [51] conducted a series of *in vivo* and *in vitro* studies that supported the aforementioned studies. Researchers have suggested that pomegranate polyphenols suppress breast cancer in their studies. They concluded that the pomegranate expressed cytotoxic effects by increasing caspase-3 expression and suppressing NF-κB expressions that are associated with inflammation, angiogenesis, and proliferation in cancer cells and stimulating associated apoptotic pathways. Shirode et al. [52] reported that cytotoxic effect was observed on human breast cancer cells the use of pomegranate extract with standardized polyphenol content depending on dose and time. These antiproliferative effects of pomegranate were attributed to inhibition of cell growth by blocking the cell cycle in the G2/M-M phase rather than merely by its consolidated anti-oxidant properties. Thus, the above studies are in agreement with the findings of our study.

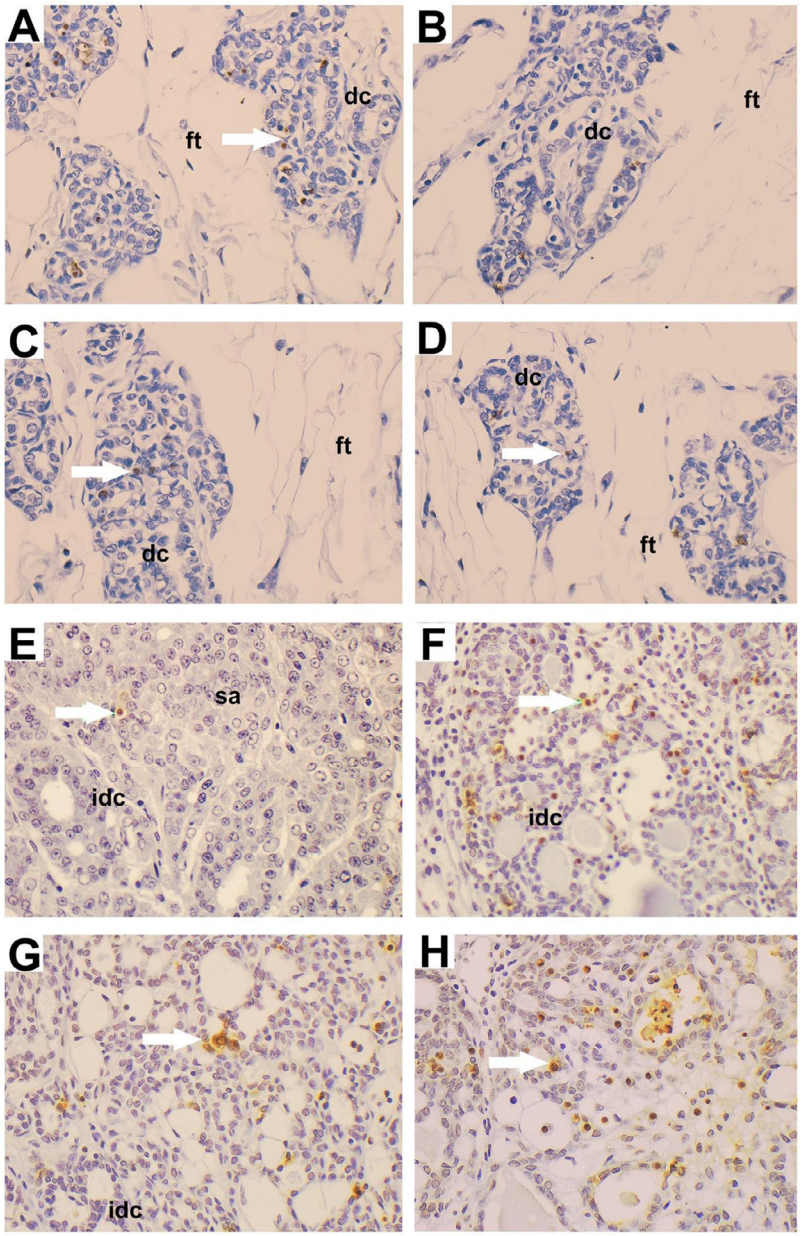

Fig. 6. Immunohistochemical analysis of TUNNEL status in the breast tissue of control and experimental animals A: Control group, Healthy breast tissue, B: P group, Pomegranate group, C: T group, Tangeretin group, D: P+T group, Pomegranate+Tangeretin group, E: DMBA group, Cancerous breast tissue group, F: D+P group, Cancerous breast tissue+Pomegranate group, G: D+T group, Cancerous breast tissue+Tangeretin group, H: D+P+T group. Cancerous breast tissue+Pomegranate+Tangeretin group, ft: fatty tissue, dc: ducts, white arrow: TUNNEL positivity cells in healthy or cancerous breast tissue. sa: solid area, idc: invasive ducts, 400X.

The chemopreventive agent's pomegranate extract and tangeretin alone and combined were found to be effective antiangiogenic /metastasis agents in suppressing DMBA-induced breast carcinogenesis. Some studies investigating the effects of therapeutic agents on the carcinogenesis process have shown that these agents inhibit NF-B activation, leading to downregulation of gene products involved in inflammation (COX-2), proliferation (cyclin D1 and c-myc), invasion (MMP-9), angiogenesis (VEGF), thereby showing that these therapeutics suppress the carcinogenesis process [53–56]. The findings of our study suggested that these effects were achieved by blocking NF-κB instead of MMP-9 inhibition at increased VEGF levels in cancer, and in this respect were consistent with other studies. However, it was concluded that the non-statistical decreases in MMP-9 levels in the treatment groups were not caused by NF-κB inhibition, unlike other studies, and that the epigenetic changes and expression of MMP-9 tissue inhibitors may be excessive. CA 15-3 and CEA are tumor markers used to monitor certain cancers, particularly metastatic breast cancer. In the present study, significant decreases in the levels of CA15-3 and CEA tumor markers with the use of phytotherapy agents is congruent with the earlier studies [26,43]. The effective reductions in plasma levels of tumor markers compared to the cancer group, alone and as a result of combined therapy, shows that these agents can be good chemotherapeutic agents, these effects can be interpreted as a reflection of VEGF blockade. Although, it seems that beneficial results have been obtained in terms of prognosis and metastatic recurrences at the point of protection from breast cancer, a clear interpretation was not considered correct because the presence of

metastasis could not be revealed by histopathological or immunohistochemistry. More comprehensive research is needed with this aspect.

When DMBA group and all treatment groups were compared, the most significant increase in tissue p53 and Bax protein expression levels was found in the treatment group where pomegranate and tangeretin were administered together. We think that the cytotoxic effect observed by the co-administration of these agents is caused by the stimulation of p53 and Bax-dependent apoptotic pathway and this anticancer feature is caused by the cytotoxic effects of pomegranate. The decrease in tissue cyclin D1 expression levels of rats treated with the combination of two treatment agents compared to the DMBA group is higher than the group treated with pomegranate alone, which can be explained by the cytostatic properties of tangeretin. Furthermore, it is thought that the combined use of two therapeutic agents compared to the DMBA group according to TUNEL results significantly increased the number of cells entering the apoptosis pathway on cancer cells and this increase was due to the pro-apoptotic properties of pomegranate. In the immunohistochemical analysis of the study, it was found that the use of both therapeutic agents together decreased the levels of proliferation markers significantly when compared with DMBA group. This was interpreted as the cytotoxic effect of the combined treatment agents in which pomegranate contributes to the cytostatic effect of tangeretin and provides a strong antiproliferative activity.

While unavailability of other studies addressing chemopreventive or therapeutic studies involving pomegranate and tangeretin in the literature restricts the discussion of our study results the earlier studies [57,58] reporting individual effect of each agent alone have indicated similar conclusions.

The histopathological macroscopic findings of the study showed that the incidence rates and tumor volumes of tumor involvement in DMBA-induced animals decreased in the treatment groups and no involvement was observed in the rats in which the two treatment agents were co-administered (Table 4). These results were in agreement with earlier in vivo reports [14,26,43].

5. Conclusions

According to the results of the present study, the combined use of pomegranate extract and tangeretin have chemopreventive efficacy in the rat breast cancer model. It is concluded that pomegranate extract and tangeretin might be perceived as beneficial in preventing the development of breast cancer.

Declarations of interest

The authors declare that there are no conflicts of interest.

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Authors Contribution

Huseyin Fatih Gul, Necip Ilhan, Nevin Ilhan, Ibrahim Hanifi Ozercan: designed the research, conducted experimental breast cancer model, data collection and biochemical analysis; Ibrahim Hanifi Ozercan, Tuncay Kuloglu: conducted pathological and histological analysis; Huseyin Fatih Gul, Necip Ilhan, Nevin Ilhan: performed statistical analysis of data; Huseyin Fatih Gul: wrote the paper; Huseyin Fatih Gul, Necip Ilhan, Nevin Ilhan: final content; All the Authors: read and approved the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2020.108566.

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