Research Article

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Modulatory effect of pomegranate extract on TRPA1, TRPM2 and caspase-3 expressions in colorectal cancer induction of mice

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Abstract

Objectives: This study aimed to evaluate the effects of pomegranate fruit extract (PFE) on levels of transient receptor potential (TRP) channel and caspase-3 (Casp-3) expressions, tumor necrosis factor-α (TNF-α), total sialic acid (TSA), reduced glutathione (GSH), and malondialdehyde (MDA) in mice with induced colorectal cancer (CRC) by investigating effects of PFE on *in vitro* mitotic index (MI).
Methods: Different PFE concentrations on MI against 0.3 µg/mL mitomycin-C (MMC) in cell culture were evaluated by binocular light microscopy. During *in vivo* applications on Balb/c mice, it was given once physiological saline to group I, PFE for ten weeks to group II, a single dose of azoxymethane (AOM) plus dextran sulfate sodium in drinking water (DSS) to group III, and AOM plus DSS plus PFE to group IV. Tissue samples were evaluated by western blotting, spectrophotometric, and histopathological methods.
Results: Expressions of Casp-3, TRP ankyrin 1 (TRPA1), and melastatin 2 (TRPM2) channels and TNF-α, TSA, GSH, and MDA concentrations in evaluated tissues had significantly better levels in PFE-treated groups compared to CRC-induced mice.
Conclusions: Results of the present study indicate that PFE application in mice with induced CRC may be an important modulator of TRPA1 and TRPM2 channels, apoptosis, and inflammatory response by decreasing oxidative stress.

Keywords: caspase; colorectal cancer; oxidative stress; *Punica granatum* L.; TRP channels.

Introduction

Considering the case and death rates for the 10 most common cancer types in 2020, colorectal cancer (CRC) ranks third with 10% and ranks second with 9.4% after lung cancer in mortality [1]. The initiation and development of this type of cancer are generally characterized by mucosal dysplasia after the infiltration of immune cells into colon tissue during inflammation [2]. Azoxymethane (AOM) as a carcinogen and dextran sodium sulfate (DSS) as a proinflammatory among chemical agents are preferred in studies with *in vivo* CRC models associated with inflammation [2, 3].

The range of useful products against health problems, especially cancers, is expanding and demand for natural dietary compounds is increasing daily [4]. Pomegranate (*Punica granatum* L.) fruit always attracts attention due to its rich content of antioxidant and anticarcinogenic compounds such as punicalagin, quercetin, and ellagic acid from phenolic compounds or flavonoids and fatty acids [5–7]. It was stated in a previous research report that pomegranate fruit extract (PFE) suppresses cancer progression and tumor angiogenesis in the colon cancer cell line in the chick chorioallantoic membrane model [8]. However, detailed studies with appropriate parameters

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and animal models are needed to evaluate their therapeutic efficacy against cancer.

Extracts prepared to detect potential protective properties in plants can be evaluated by *in vitro* genetic and cytological toxicity tests. Experimental setups consisting of *in vitro* cell cultures are very useful in reducing time and costs in laboratory environments. Mitotic index (MI) analysis, a classical method of cell cycle and genetic factors in cell culture, may be used initially before moving on to *in vivo* animal models [9, 10].

Organisms have a class of integral membrane proteins called transient receptor potential (TRP) channels that play a key role in interpreting environmental stimuli. These cationic channels are signal transducers by changing intracellular calcium (Ca^{2+}) concentration or membrane potential [11–13]. In humans, the TRP ankyrin 1 channel (TRPA1) is reported to be a temperature-activated stress sensor [11, 12]. TRP melastatin 2 channel (TRPM2) is modulated by oxidative stress and involved in cell death responses under stress [11, 14, 15]. The dual effect of tumor necrosis factor-alpha (TNF-α) cytokine, a tumor suppressor agent in tumorogenesis and detumorogenesis as mainly an inflammatory immune reaction regulator, draws the attention [16]. Sialic acids as terminal carbohydrates in molecules draw attention in tissue damage and cancer-related disease processes [17]. The levels of reduced glutathione (GSH) as an important endogenous element of the antioxidant defense system and malondialdehyde (MDA) as a final product during lipid peroxidation formed due to reactive oxygen substances (ROS) are important oxidative stress biomarkers [18–21]. The expression levels of caspase-3 (Casp-3) from responsible proteases for the apoptosis process usually draw attention as a marker in determining the degree of improvement in the treatment process of cancers [22, 23].

The elucidation of cellular and metabolic pathways that may be associated with the progression mechanism of cancer in the prevention of cancer by PFE is becoming increasingly important every day in terms of increasing the efficiency of treatments. The present study aimed to reveal effects on TRP (A1 and M2) channels, TNF-α, Casp-3, and oxidative stress to shed light on subcellular biochemical and molecular functioning *in vitro* and *in vivo* of PFE in mice with induced CRC.

Materials and methods

Plant sample and extraction

Pomegranate fruits were collected at the ripening stage (September-October 2020) from a commercial farm located around Kozan and Pozantı districts (between 37°26′39.1″N 34°52′05.2″E and 37°27′22.3″N 35°49′06.3″E) in Adana Province in Turkey's Mediterranean Region. It was then dried in an oven in a closed room. Afterward, fruits were roughly cut into smaller slices each time to be ready for grinding and dried in a dust-free and darkened environment. The dried product was ground by a blade grinder (MUM58K20, Bosch) and kept in the dark and at room temperature. In a clevenger apparatus, distilled water (250 mL) and plant material (30 g) in a glass balloon connected to a cooler for extraction were boiled for 2.5 h. Oil molecules moving with water vapor were condensed in a cooler to obtain a liquid extract. This extract was filtered and stored at 2–8 °C for use in experiments and renewed every two days.

Experimental animals and collection of samples

The applications were performed with the permission of the Kafkas University Local Ethics Committee (KAU-HADYEK 2021/110). The experimental environment had a temperature range of 21 ± 2 °C, a humidity of 49 ± 5%, and a 12 h light/dark cycle. Accordingly, 28 female (11 ± 1 weeks old) Balb/c mice were divided into 4 equal groups and kept in stainless steel cages (26/15/50 cm length/width/height) for seven days without any experimental application and fed water and standard complete pellet mouse chow with 26% crude protein (Bayramoglu Feed and Flour Industry Trade Inc., Erzurum Turkey). The experimental CRC model caused by AOM (Azoxymethane, Sigma Aldrich) and DSS (dextran sulfate sodium salt, Alfa Aesar by Thermo Fisher Scientific) was used based on previous studies [2, 3]. According to this model, *in vivo* experimental applications were performed for ten weeks. Physiological saline was given by intraperitoneal (IP) injection once to group I, which was the control group, orally 200 mg/kg/day PFE for ten weeks to group II, single IP injection of 10 mg/kg AOM plus 2.5% (w/v) DSS in drinking water for a week in 14 days after the first week to group III, and AOM plus DSS plus PFE to group IV.

At the end of experimental applications, blood samples were taken intracardially from mice injected IP pentobarbital, and then experimental animals were sacrificed by decapitation to obtain tissue samples. Blood samples were kept in tubes at 4 °C for 20 min and then centrifuged at 2500 rpm for 10 min. The serum samples were stored at −20 °C. The colon samples from the distal part (to 2, 5–3 cm above the anus) for histopathological evaluation were fixed with a 10% neutral-buffered formalin solution. At the end of 24 h period, each tissue sample was embedded in paraffin so that sections could be taken. Sections (4 µm) were stained with hematoxylin and eosin (H&E) using the standard histological staining method.

Cell culture and preparation

For obtaining cell culture and preparations, the method detailed in previous studies was used with minor modifications [10, 24]. Briefly, eleven drops (approximately 0.4 mL) of blood sample were infiltrated into cell mediums (Chromosome Synchro P Complete Medium, Ref: EKAMTB100, Italy) under sterile conditions. These mediums were kept in an oven at 37 ± 1 °C for 72 h. Then, 100 µL of solution A and 100 µL of solution B (Euroclone SynchroSet, Ref: EKAMTS008, Italy) were added after 48 and 65 h of incubation, respectively. The procedures in this section were applied in the same way for other groups: 0.3 µg/mL mitomycin-C (MMC) was administered to group II (positive control), and 0.3 µg/mL MMC plus 100, 200, 400, and 800 mg/kg PFE was

administered to groups III, IV, V, and VI, respectively. After, 40 μL of colchicine solution (Cat no: C9754, Sigma, USA) 2 h before incubation (72 h) was mixed, and the supernatant was removed following centrifugation at 2000 rpm for 10 min. Next, 5 mL of hypotonic solution at 37 °C was dropped on the bottom, incubated for 30 min, and the supernatant was removed. Cold fixative (a mixture of glacial acetic acid and absolute methanol, 1/3) was added, and supernatants were discarded. Fixative was added again, and this process was repeated 3 times. After dropping the dip suspension onto the slide, preparations were dried at approximately 25 °C for 24 h. The dry preparations were kept for about 10 min in 10% Giemsa dye prepared with Sorensen Buffer Solution about 15 min before. The dry preparations post-process were covered with Entellan, and the microscopic examination process was started after drying.

Determination of mitotic index (MI)

To detect effects of PFE on mitosis, a total of 2,000 cells were observed from permanent preparations of each group, and those at the metaphase stage were recorded. MI was analyzed by evaluating the percentage of metaphase as one of the stages of cell division. MI level of different areas on permanent preparations was recorded after standardization with repeated observations [10, 24].

Western blot analysis in colon tissue samples

The Western blotting analysis of TRPA1, TRPM2, and Casp-3 in colon samples was performed according to details of standard western blotting methods indicated previously [11, 15]. Briefly, the protein concentration was measured using Bradford's solution. To identify TRP cation channels, Casp-3 expression levels in samples were homogenized in lysis buffer and centrifuged at 16,000×*g* for 20 min; following that, supernatants were removed and preserved for analysis. The primary and secondary antibodies of mouse TRPA1, TRPM2, and Casp-3 were purchased from Cell Signaling Technology (EKA Biolab Technology, Ankara, Turkey). The Gel Imagination System (G: Box, Syngene Inc., Cambridge, UK) was used to display bands. The band density obtained from β-actin antibody was used as an internal control for comparison. The band densities of TRPA1, TRPM2, and Casp-3 in colon samples were normalized to relative β-actin protein values.

Serum MDA, GSH, TSA, and TNF-α analysis

The levels of MDA were measured as described by Yoshioka et al. [19], and levels of GSH according to the method reported by Beutler et al. [20] using a spectrophotometer (T60 UV/VIS Spectrophotometer, PG Instruments Ltd, UK). The levels of TSA were measured as described by Sydow [25]. The results were calculated from a standard curve obtained according to absorbances of standard solutions prepared with different concentrations (1,1,3,3-tetramethoxypropane for MDA, L-glutathione reduced for GSH, N-acetylneuraminic acid for TSA, all three Sigma-Aldrich). TNF-α analysis was performed spectrophotometrically in a microplate reader (BioTek Epoch, USA) with a commercially produced assay based on an enzyme-linked immunosorbent assay for antigen detection (Cloud Clone Corp., Wuhan, PRC). The results were expressed as relative values using a standard curve.

Statistical analysis

Statistical calculations of data were conducted using a software package (SPSS 20.0 for Windows, IBM) on a computer. One-way analysis of variance (ANOVA) test was used to determine whether there was a significant difference between group means. Whether there was a significant difference in the mean value of each group compared to others was evaluated using the Tukey test, one of the multiple comparison tests. p-Values less than 0.05 or 0.01 obtained from tests were regarded as significant. All results were expressed as mean ± standard deviation ($\chi \pm SD$).

Results

When the toxicological potential of PFE was evaluated, no histopathological findings or irritations were found in any parts such as mucosa or epithelial cells belonging to the oral tract, pharynx, and trachea of mice. When experimental animals were evaluated in terms of weight, weight losses were encountered from the first DSS application compared to the control group. By last week, the mean weight of group-administered AOM was lower than that of the control group, and this change approached normal in AOM plus PFE group ($p<0.01$) (Figure 1A).

Effects of different PFE concentrations on mitotic index

A significant difference in the effects of different ratios of PFE on MI in human peripheral lymphocyte cells compared to MMC was found; the 200 mg/kg concentration of PFE was better than other concentrations, and MI levels are shown in Figure 1B.

Expression levels of TRPA1, TRPM2, and Casp-3 were modulated in colon samples by PFE treatment

The increased TRPA1 and TRPM2 expression levels were considered in mice with induced CRC. A direct relationship was determined between an increase in TRPA1 and TRPM2 expression levels and expression levels of Casp-3 in CRC. PFE reduced the expression of TRP channels and Casp-3, with important roles in apoptosis in CRC model mice. In current data, protein expression values of TRPA1 (Figure 2A and B), TRPM2 (Figure 2A and C), and Casp-3 (Figure 2A and D) were higher in the CRC group in comparison with the control group and PFE group ($p<0.01$). However, protein expression levels of TRPA1, TRPM2, and Casp −3 were downregulated in colon samples by PFE treatment ($p<0.05$).

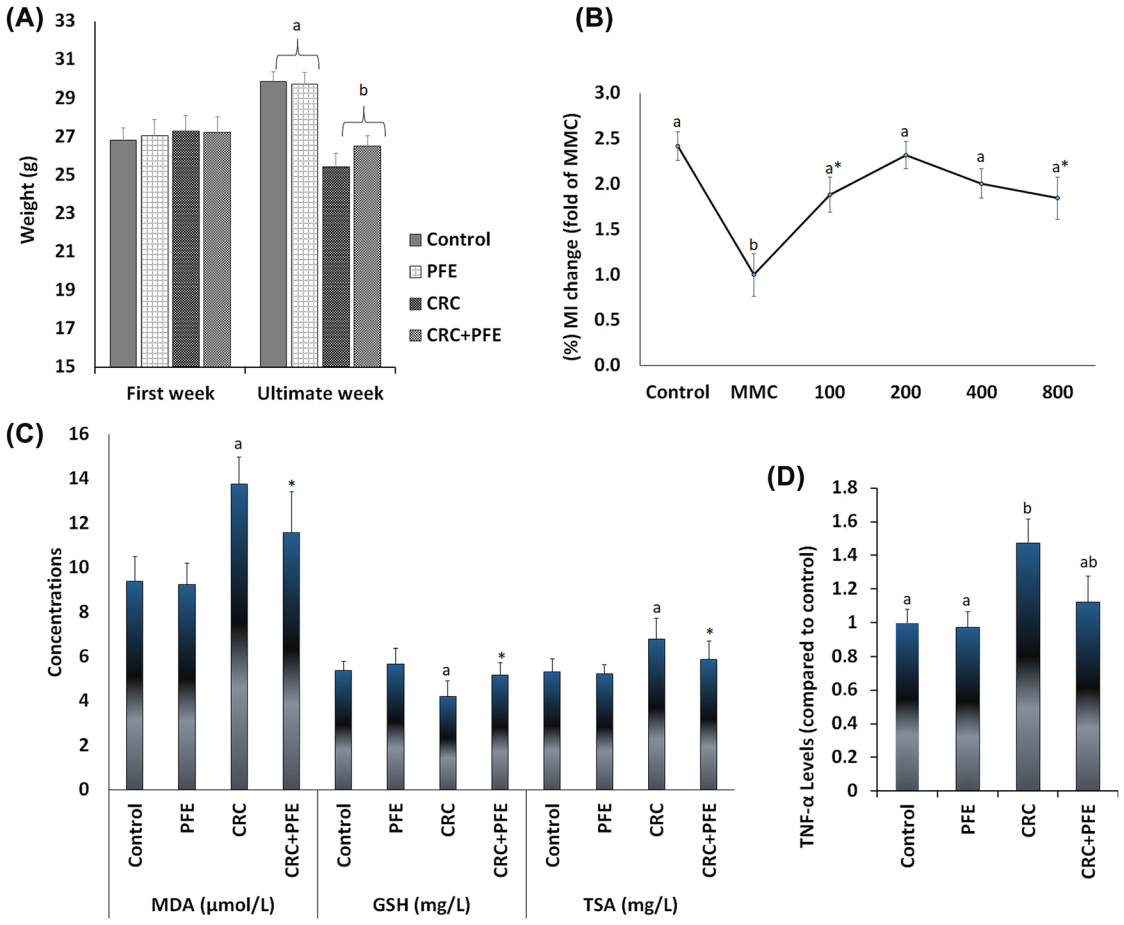

Figure 1: The weight measurements of experimental animals (A), the effects of different PFE concentrations (mg/kg) on the level of MI in lymphocyte cell culture (B) and the levels of MDA, GSH and TSA of experimental animals (C) and the levels of TNF-α in colon tissue of mice (D). Dissimilar letters on columns show significantly distinct means, $p<0.01$ for Figure 1A and $p<0.05$ for Figure 1D. $X^{a,b}$: significant difference compared to the MMC group, $p<0.01$ for Figure 1B. $\chi^{a,*}$: significant difference compared to the control group and CRC group, respectively, $p<0.05$ for Figure 1C.

Oxidative stress parameters in serum samples by PFE treatment

The serum MDA level increased when the GSH level decreased in mice with CRC induction. TSA levels decreased in the PFE treatment group compared with the CRC induction group. PFE administration supported the antioxidant defense system by decreasing oxidative stress and sialic acid levels in the CRC induction group (Figure 1C).

The level of TNF-α was changed in colon samples by PFE treatment

The colon TNF-α concentrations increased in the CRC induction group compared with the control and PFE groups. PFE treatment acted as a normalizer on colon TNF-α concentrations in CRC induction (Figure 1D).

Histopathological examination results

It was observed that tissue samples taken from the colon in control and PFE group mice exhibited a normal histological structure (Figure 3A and B). In colon tissue samples of mice in the CRC induction group, local disorganization in the columnar epithelium in the lamina epithelial layer and degenerative and necrotic changes in Lieberkühn crypts and goblet cells were detected (Figure 3C). In addition, focal mononuclear and histiocytic cell infiltrations were observed in some areas of lamina propria. While there was a regression in lesions in PFE treatment group animals compared to the CRC induction group animals, it was

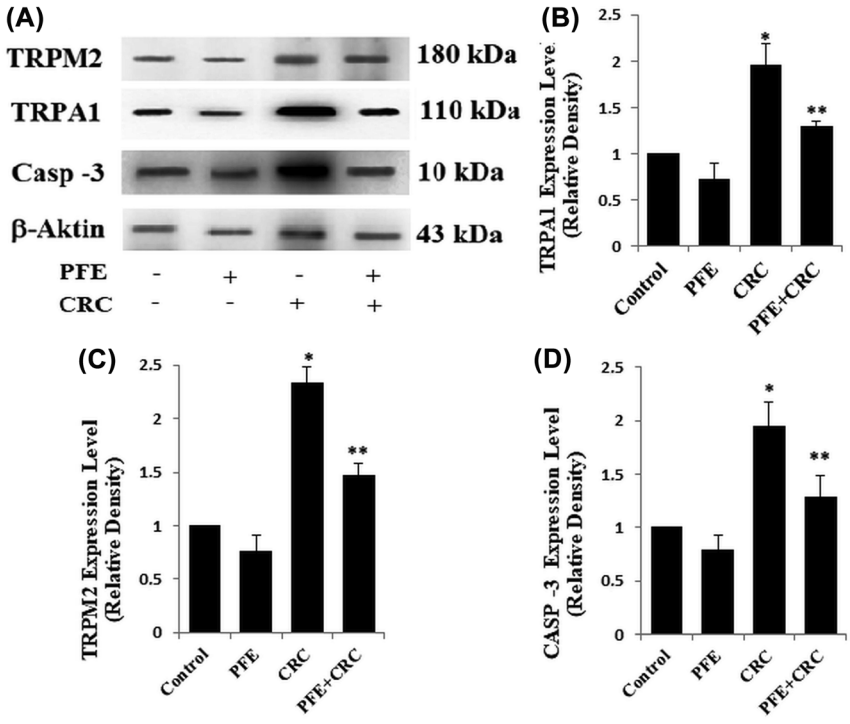

Figure 2: The protein expression levels of TRPA1, TRPM2, and Casp-3 in colon samples of mice.
Different symbols on columns show significant difference compared to the control group and CRC group, $p<0.01$ and $p<0.05$, respectively.

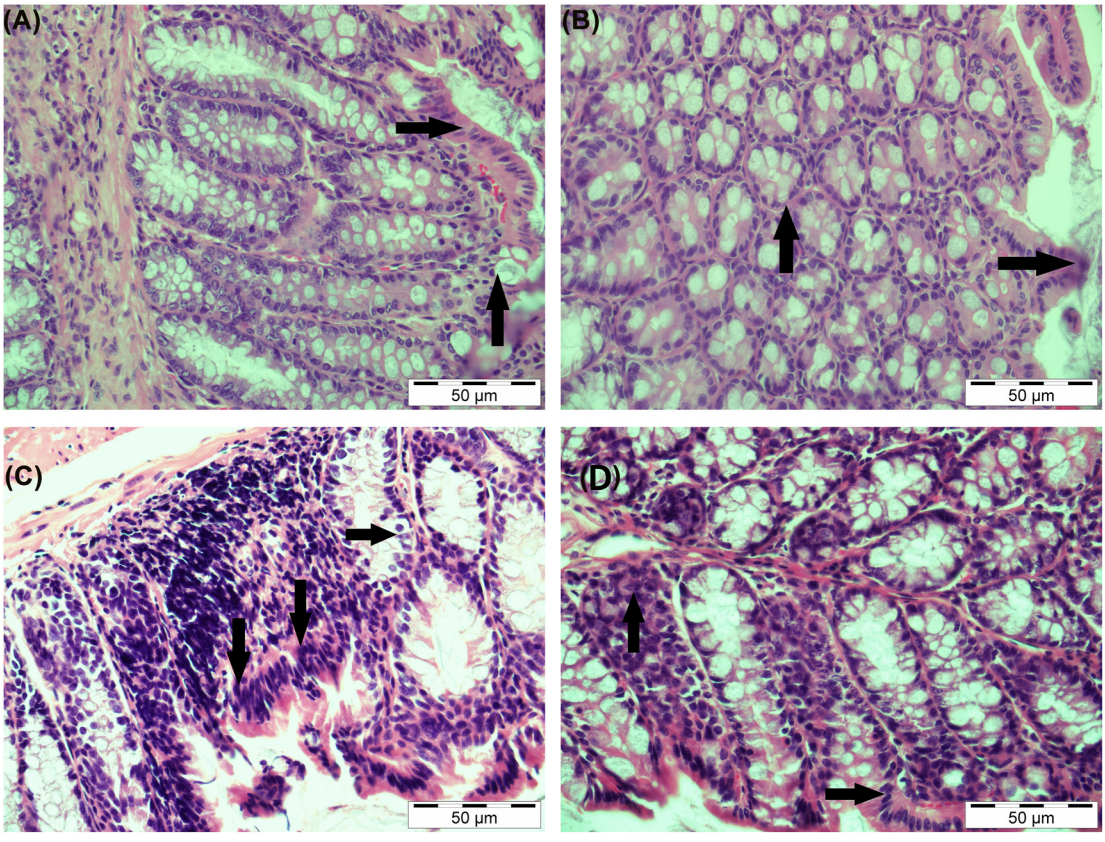

Figure 3: Control group (A); normal colon histological section, *L. epithelialis* (horizontal arrow), goblet cell (vertical arrow), HE, 50 µm. PFE group (B); normal colon histological section, *L. epithelialis* (horizontal arrow), Lieberkühn crypts (vertical arrow), HE, 50 µm. CRC induction group (C); disorganization in *L. epithelialis* epithelial cells (vertical arrow), degenerative changes in Lieberkühn crypts (horizontal arrow), HE, 50 µm. PFE treatment group (D); *L. epithelialis* (horizontal arrow), regeneration attempt in Lieberkühn crypts (vertical arrow), HE, 50 µm.

found that cells in the lamina epithelial layer had normal histological structure and alignment similar to the control and PFE group animals. Among findings, there were regeneration attempts in Lieberkühn crypts (Figure 3D).

Discussion

The use of an AOM and DSS-induced rodent CRC model for *in vivo* studies in the treatment and molecular biology of colon cancer is common because it has similar characteristics to laboratory findings in humans [2, 3]. In the present study, protective metabolic effects of PFE by stimulating TRP channel expression were determined as the main findings regarding oxidative stress, cytokine release, and apoptosis in experimental CRC induction.

The MI test, as one of the genotoxicity tests is among *in vitro* methods commonly used to investigate carcinogenic and mutagenic effects of factors with unknown genotoxicity or cytotoxicity as an indicator in which chromosomes in the centre of cell proliferation and apoptosis are affected [9, 10]. The *in vitro* antiproliferative, apoptotic, and antioxidant activities of tannin and ellagic acid concentrated in pomegranate extract, were demonstrated in colon cancer cell lines by Seeram et al. [5]. In the present study, PFE significantly increased MI levels compared to the MMC group. PFE with 200 mg/kg concentration among extracts examined with different densities (100, 200, 400, and 800 mg/kg) was more effective than others on MI level. After this determination *in vivo* stage was started in experimental mice with induced CRC (Figure 1B).

Pathological problems occur in cells and tissues due to negative side effects of ROS in metabolism [18, 21, 25]. It is known that increased ROS or oxidative stress in colorectal carcinogenesis is a very important risk factor, and MDA levels in tissue with CRC increase with clinical staging of disease [21]. High MDA levels have been suggested as an additional marker for diagnosing CRC and other toxicity-related symptoms [26, 27]. Concentrations of GSH as a molecular marker for the CRC vary significantly depending on the type of treatment and metastasis site [28]. Increased MDA levels and decreased levels of GSH during CRC can be considered directly related to cell damage, channel disorders in the cell membrane, and lipid peroxidation. Pomegranate extract, due to rich phenolic and flavonoid compounds in its content can shows significant antigenotoxic effects when applied appropriately in the protection of DNA that determines the course of cancer. It has been known that these effects is necessary to evaluate with more parameters in appropriate animal model [7, 8, 29]. In the present study, it was concluded that PFE in extract treatment of mice with induced CRC responded with decreased MDA levels and increased GSH levels in protecting cell integrity by regulating the expression of TRP channels in cell membranes, and findings on oxidative stress are consistent with those of other studies [7, 26] (Figure 1C).

TRP channels directly provide control of Ca^{2+}, Mg^{2+}, and Na^{2+} entry as cations in cells of living organisms. TRPM channels from these channels are considered important in treating proinflammatory diseases and cancers [30]. TRPM2 channel, which is affected by organic, inorganic, or physical stimuli such as temperature and voltage difference, is affected by oxidative stress. Its expression has been reported to cause cell death responses during stress [14]. It is claimed that TRPA1 as an O_2 sensor and a nociceptive receptor and TRPM2 as a host's defence responder against infections and their blockades may be the main targets directly related to reactive oxygen load in management and alleviation of oxidative stress process in various diseases with oxidative stress in pathogenesis [31]. It has been noted that activation of TRPM2 and TRPA1 can be induced with H_2O_2, which is considered a very important source of ROS [32]. Hydroxyl radical scavengers effectively alleviate ROS or oxidative stress-related diseases through the blockade of TRPM2 and TRPA1 [31]. The importance of antagonist applications to TRPA1 was also emphasized for the treatment of diseases associated with colitis or colon damage, in which TRPA1 of the gastrointestinal tract is expressed in a positive correlation to other TRP channel expression, mainly in dorsal root ganglion primary sensory nerve cells in extrovert neuronal integrity that is generally responsive to capsaicin [33]. It has been suggested that the nociceptive effect of corilagin tannin in pomegranate may play a role in the antagonism of the TRP channel in the stimulation of capsaicin [34]. The current study revealed that PFE prepared from pomegranate fruit rich in phenolic and flavonoid components has a directly alleviating effect on expression levels of TRPA1 and TRPM2 channels (Figure 2B and C).

Casp-3 is the main mediator of apoptosis activated in response to stress during treatments [22]. In a study examining proapoptotic and antiproliferative characteristics belonging to pomegranate extract to its prooxidant activity, Weisburg et al. [35] recorded that pomegranate extract showed more antiproliferative specifications in cancer cells than in healthy cells isolated from the human oral cavity. It was also found that Casp-3 activation was reduced in cells treated with pomegranate extract, and apoptosis was associated with the prooxidant nature of pomegranate [35]. The importance of Casp-3 during angiogenesis and recurrence of tumors was emphasized,

and loss of Casp-3 expression, which supports metastasis in resistance to colon cancer therapy, was presented as a promising treatment approach [22]. In the current study, PFE effectively reduced Casp-3 expression during CRC induction and these data are also consistent with a previous study containing altered Casp-3 transcript ratios of patients with CRC [36] (Figure 2D).

TNF-α, which was initially identified as a factor causing necrosis of tumors and had a broad spectrum for biological activity, is indicated as mainly an inflammatory immune reaction regulator [16, 37]. It was reported that colorectal carcinogenesis may develop as related with TNF-α during both persistence of ulcerative colitis due to intense inflammation after relapses and regenerating mucosal epithelial dysplasia despite treatments [23]. The present study indicated that PFE had a lowering effect on TNF-α levels in colitis-associated CRC induction and could be evaluated for the TNF-α signalling pathway (Figure 1D).

Supplementations reducing oxidative stress may also decrease high TSA levels in colon cancer and other diseases [18, 38]. Increased TSA levels in CRC may be related to the sialidase activities of parts such as lysosome, plasma membrane, or cytoplasm of damaged cells, and that cells with abnormal sialylation in carcinogenesis are far from the control of the immune system [17, 39]. According to the current study, it is thought that the decrease TSA levels in the PFE-administered group compared to CRC group may have been related to suppressed sialidase activity and total phenolic and flavonoid components of extract (Figure 1C).

In CRC induction model studies, cellular infiltration and disorganization, degenerative and necrotic changes are generally used to determine the severity of toxic effect and recovery [2, 3, 26, 36]. As stated in the findings section, these changes were generally encountered in the columnar epithelium in *L. epithelialis* layer, Lieberkühn crypts, goblet cells, and *L. propria* in mouse colon tissue samples of the CRC induction group (Figure 3C). There was integrity and homogeneity in cellular areas of colon tissue of mice in control and only PFE groups (Figure 3A and B). Signs of regeneration and healing in colon tissue and cells were evident in the PFE treatment group (Figure 3D). TNF-α and TSA concentrations which are known to increase significantly in cancer and tissue damages, support histopathological findings. General histopathological findings indicate that PFE application may be beneficial against AOM and DSS toxicity used in CRC induction.

Finally, it was concluded that TRPA1, TRPM2, TNF-α, and Casp-3 expression levels were modulated by PFE in colitis-associated CRC model mice and oxidative stress was important in this modulation. It can be suggested that PFE may contribute significantly to changes in inflammation and apoptosis, which are important in the CRC treatment process, depending on induced TNF-α and Casp-3 concentrations. In addition, there is a need for further comparative studies on the source and content of PFE.

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Author contributions: İK formulated the current hypothesis. He is responsible for writing the report and the experimental implementations with SD, MMK, EAT, HB and GA. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was approved by Kafkas University Local Ethics Committee (KAU-HADYEK 2021/06) and experimental applications were made in Kafkas University Experimental Animals Application and Research Center.

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