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The investigation of the possible antigenotoxic in vivo effects of pomegranate (*Punica granatum* L.) peel extract on mitomycin-C genotoxicity

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Abstract: The aim of this study was to investigate the possible antigenotoxic effects of the extract of pomegranate (*Punica granatum* L.) peel, which has high antioxidant activity against the genotoxic effects that mitomycin-C creates in the bone marrow cells of mice by a chromosomal aberration, mitotic activity, and micronucleus test system. There were six groups including a negative control group, positive control group (2 mg/kg mitomycin-C (MMC)), 150 mg/kg *P. granatum* L. (PG) peel extract, 300 mg/kg PG peel extract, 150 mg/kg PG + MMC, and 300 mg/kg PG + MMC. The extract of pomegranate peel was given to mice by oral gavage for 15 days. At the end of day 15, 2 mg/kg MMC was intraperitoneally administered to the mice. Chromosomal aberration, mitotic activity, and micronucleus rates were determined in bone marrow cells of the mice, which were euthanized 24 h after this application. As a result of the observations carried out, it was determined that the chromosomal aberrations and micronucleus ratios in the MMC group were at the maximum level in terms of chromosomal aberration rates and micronucleated polychromatic erythrocytes; this rate decreased with 150 mg/kg PG and 300 mg/kg PG peel extract application with MMC, and PG peel extract applied with MMC significantly increased this decrease due to dose increase ($P < 0.001$). It was observed that MMC application decreased mitotic activity and polychromatic erythrocyte/normochromatic erythrocyte ratios, while statistically these rates significantly increased in the groups administered peel extract with MMC when compared to the MMC group ($P < 0.001$). As a result, it was determined that the peel extract at the stated dose showed antigenotoxic effects against the genotoxicity caused by mitomycin-C in the bone marrow cells of mice.

Key words: *Punica granatum* L., pomegranate, mitomycin-C, chromosomal aberration, micronucleus, genotoxicity

1. Introduction

Pomegranate (*Punica granatum* L.) is an important species of the family Punicaceae used in the treatment of different diseases in Asia, the Americas, and Africa by general medicine [1,2]. In recent years, studies on pomegranate have shown that various chemical components in pomegranate are important in terms of pharmacological effects. Studies conducted with separate extracts of pomegranate fruit and leaf parts have proved its therapeutic properties [3]. The part of the pomegranate that has the highest antioxidant capacity is the peel. Pomegranate peel has 10 times more antioxidant activity when compared to the edible part and 2 times more when compared to its seeds [4,5]. Studies on pomegranate peel have generally focused on antioxidant activity and phenolic content. In recent years, it has been found that pomegranate peel has antidiabetic, antiviral, anticarcinogenic, and antibacterial properties [6]. Pomegranate peels, used for wound healing, blood arresters, diarrhea inhibitors, skin infections, and viral diseases in the field of traditional medicine, are also used in the compositions of recommended drugs for various health disorders such as antimicrobial, antitumoral, antiinflammatory, and antiulcer drugs, as well as for diabetes and genital infections, thanks to the studies carried out in recent years [7–14]. There are important phenolic compounds such as tannins (punicalagin, punicalin, elagic acid, and gallic acid) and flavanoids (catechin, anthocyanin, and other complex flavanoids) in parts of the pomegranate peel [15,16].

Mitomycin-C (MMC) is a drug isolated from *Streptomyces caespitosus* and used for the treatment of adenocarcinoma and transitional tumors in the genitourinary system. MMC, an alkylating agent, inhibits RNA and protein synthesis by breaking the formation of DNA cross-linking and shows antineoplastic properties [17–19].

In addition to cross-linking, it is also activated by quinone reduction followed by C-10 (urethane group) and at the same time C-7 (aziridine group) in order to exhibit alkylating properties [20]. During the damage created by MMC in the DNA as a cross-linking and alkylating agent, it creates new damage to the DNA by creating free

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radicals. MMC also inhibits the activities of enzymes such as phagocyte NADPH-oxidase, NADPH-cytochrome P450, xanthine oxidase, and mitochondrial NADH-dehydrogenase. It produces free radicals in electron reduction reactions with redox cycles that affect most enzymes and forms a semiquinone midproduct [21,22]. It has been supported by studies that it has significant therapeutic effects thanks to the presence of compounds in the pomegranate peel, especially punicalagin and punicalin. However, no studies have been found on the possibility of the protective effects of pomegranate peel extract against genotoxicity caused by MMC. The aim of this study was to investigate the possible antigenotoxic effects of pomegranate peel extract on the genotoxicity created by MMC at determined doses.

2. Materials and methods

2.1. Animals

In the experiments carried out, 48 *Mus musculus* albino female mice were used. The mice were each 8 weeks old and weighed 25 ± 5 g. Animals were obtained from the Experimental Animals Research and Application Center at Kafkas University. During the period of the study, the use of laboratory animals was carried out in accordance with national ethical rules and was approved by the Animal Experiments Local Ethics Committee at Kafkas University (KAÜ-HADYEK: 2017/46).

The mice were provided distilled water and regular mouse feed ad libitum. Mice, which were checked 3 times daily, were housed in laboratory conditions with 50% relative humidity, 20 ± 2 °C temperatures, and a light period with 12 h of light and 12 h of dark.

2.2. *Punica granatum* peel extract

Pomegranate (*Punica granatum* L.) was obtained from the Antalya region. The peel parts of the fruit were taken and dried in a sun-free area with air circulation and ground with an IKA A11 basic laboratory scale mill (Staufen, Germany). Fifty grams of ground plant sample was taken into a Soxhlet device cartridge, which was washed with extraction solvent and then transferred into a 500-mL Soxhlet extractor. The solvent was extracted (10-15 siphons) for approximately 10 h until it became clear.

After 10 h, the liquid extracts were filtered using filter papers with pore size of <2 µm and 110 mm diameter (Grade 589/3, Whatman, UK) to remove particles. The filtered extract sample was evaporated with a rotary evaporator at 35–45 °C. The pomegranate peel extract remaining in the flask was kept in the desiccator for 12 h. The pomegranate peel extract, which was completely removed from the solvent, was weighed with sensitivity of 0.1 mg, placed in the extract box, and stored at 4 °C for the study [23].

2.3. Experimental design

Mice were divided into 6 equal groups after they were fasted for 24 h (n = 8). Group 1 was the negative control group. Mice in this group were orally given distilled water for 15 days to allow them to experience the same stress as mice in the other group. Group 2 contained mice orally given distilled water for 15 days. The mice were intraperitoneally given 2 mg/kg mitomycin-C on the 15th day. Group 3 contained mice that were given 150 mg/kg PG peel extract by oral gavage for 15 days. Group 4 contained mice that received 300 mg/kg PG peel extract by oral gavage for 15 days. Group 5 contained mice given 150 mg/kg PG peel extract by oral gavage for 15 days. At the end of the 15th day, they were intraperitoneally given MMC at a dose of 2 mg/kg. Group 6 contained mice that were given 300 mg/kg PG peel extract by oral gavage for 15 days. At the end of the 15th day, they were intraperitoneally administered MMC at a dose of 2 mg/kg and the experiment was completed. The substances used in the experiment were administered by preparing the solution depending on the daily weight results of the mice. Twenty-four hours after MMC applications, the femoral bones of the mice were removed by cervical dislocation under ether anesthesia. Test concentrations (150 mg/kg and 300 mg/kg) were determined considering the lower and upper limit ranges used in previous studies [24–26].

2.4. Chromosomal examination and detection of mitotic index

For testing for chromosomal aberration and detection of the mitotic index, the mice were intraperitoneally given 2 mg/kg colchicine dissolved in distilled water 2 h before euthanasia. The bone marrow of the femoral bone was removed with a syringe and taken into centrifuge tubes containing 3 mL of fetal calf serum. Samples of one of the femoral bones were centrifuged at 1100 rpm for 10 min to discard the supernatant. Then 5 mL of 0.075 M KCl solution was heated for 30 min in an oven set at 37 °C. Cells were incubated for 20–30 min in warmed hypotonic solution. After incubation, the cells were centrifuged again at 1100 rpm for 10 min and the supernatant was discarded.

Cells were fixed in 5 mL of cold Carnoy's solution (methanol and glacial acetic acid, 3:1) prepared 1 day before the study. After this step, the supernatant was discarded by centrifugation again. Then the fixation was repeated 3 times and the supernatant was discarded, leaving 0.5 mL in the tube. The cells at the bottom were suspended with a Pasteur pipette and spread on moist clean slides by dropping them from 3–4 cm above. After smearing, the preparations were dried at room temperature for 24 h and stained with 10% Giemsa dye for 10 min. The preparation of metaphase preparations for chromosomal aberration and mitotic activity detection was performed according to Preston et al. [27]. For chromosomal aberration and the detection of mitotic activity, mice were intraperitoneally given 2

mg/kg colchicine dissolved in distilled water 2 h before euthanasia.

2.5. Micronucleus frequency test

The femur bone marrow was injected into a centrifuge tube containing 3 mL of fetal calf serum. After the tubes were centrifuged at 2000 rpm for 5 min, the supernatant was removed from it and a drop of fetal calf serum was placed on the part left, and it was suspended. Each drop of the samples taken from tubes was placed on a moist and clean slide and smeared. After drying, the prepared preparations were fixed in methyl alcohol for 10 min. The fixed preparations were first stained with 0.25% May–Grünwald dye for 5 min and then washed in distilled water. In the second stage, they were again stained with May–Grünwald dye of 0.125% for 5 min and washed again in distilled water. In the final stage, the preparations were stained with 20% Giemsa dye for 30 min and then passed through distilled water again and allowed to dry (Figure 1). For the detection of micronuclei in mouse bone marrow preparations, the Schmid method was used as a reference [28].

2.6. Statistical analysis

IBM SPSS Statistics 22.0 (IBM Corp, Armonk, NY, USA) was used for statistical assessments. One-way analysis of variance (ANOVA) was used to compare the differences between the control and experimental groups. Significant differences were evaluated at $P < 0.05$.

3. Results

3.1. Chromosomal aberration rates in the cells of the bone marrow of control and experimental groups

Six groups including 8 mice in each group were formed to determine whether pomegranate peel extract, which has high antioxidant activity due to containing polyphenols, has any effect on chromosomal aberrations and mitotic activity by mitomycin-C, which has genotoxic effects in the bone marrow cells of the female mouse (*Mus musculus* var. *albinos*). Groups were determined as a negative control group, MMC group, 150 mg/kg PG peel extract group, 300 mg/kg PG peel extract group, 150 mg/kg PG peel extract + MMC group, and 300 mg/kg PG peel extract + MMC group.

Five preparations were prepared from each of the dose groups. A total of 100 cells in the metaphase stage were counted from the preparations obtained from each sample. The structural chromosomal aberration rates observed in these 100 cells were determined and are shown in Table 1.

According to the statistical data among the groups, when chromosomal aberration rates were compared among the groups in terms of chromosomal aberration rates, it was observed that the chromosomal aberration rate in the MMC group was at the maximum level. This ratio decreased due to the application of 150 mg/kg and 300 mg/kg PG peel extract together with MMC, and the PG peel extract applied together with MMC significantly increased this decrease due to dose increase ($P < 0.001$) (Figure 2). In microscopic examination, chromosomal aberration types such as chromosome fracture (Figure 3), fragment, sister chromatid association (Figures 4 and 5), and chromatid fracture (Figure 6) were detected.

3.2. Mitotic activity rates in the bone marrow cells of control and experimental groups

Five preparations were prepared from each animal. From the prepared bone marrow preparations, 1000 cells were randomly counted in each animal. Mitotic activity rates were determined by counting the cells in the metaphase stage (Table 2).

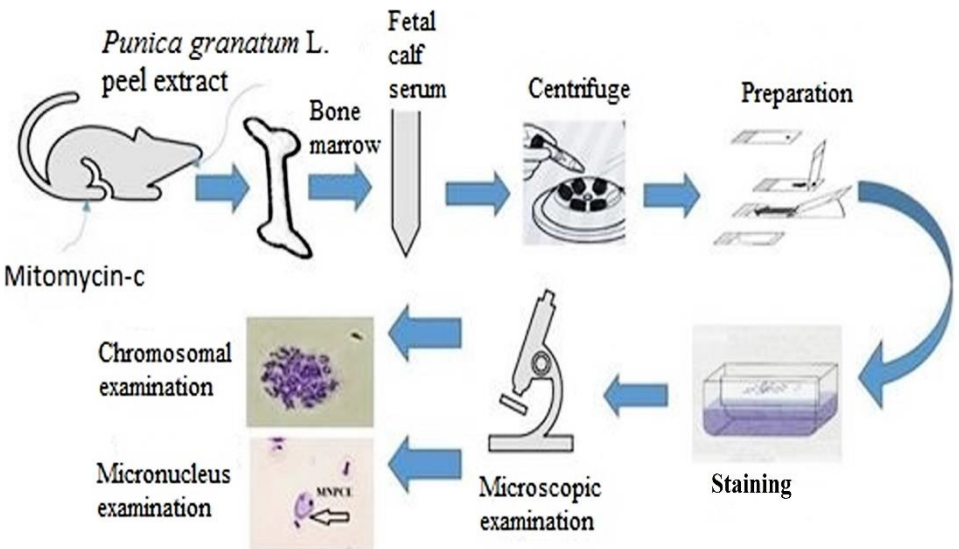

Figure 1. Chromosome and micronucleus methods in mouse bone marrow cells.

Table 1. Chromosomal aberration rates in mouse bone marrow cells in control and experimental groups.

Chromosomal aberration rates of control and experimental groups in mouse bone marrow cells					
Groups	A	B	C	D	Total
NC	-	1	1	1	3
MMC	30	28	40	36	134
150 mg/kg PG	-	2	1	1	4
150 mg/kg PG + MMC	19	17	20	30	86
300 mg/kg PG	-	3	1	-	4
300 mg/kg PG + MMC	14	10	11	28	63

NC: Negative control group, MMC: mitomycin-C, PG: *P. granatum* L., A: chromosome fracture, B: sister chromatid combination, C: fragments, D: chromatid fracture.

SPSS 22 was used for statistical analysis of the data obtained from the study. One-way ANOVA was used to determine the difference between the control and experimental groups and $P < 0.05$ was considered statistically significant.

According to the comparison of the results of mitotic activity in the groups, MMC application decreased mitotic activity, whereas it was found that this ratio increased significantly in the group with PG peel extract together with MMC compared to the MMC group ($P < 0.001$) (Figure 7).

3.3. Micronucleus frequencies of control and experimental groups in mouse bone marrow cells

Five preparations from each animal were prepared from bone marrow preparations for micronucleus tests. Micronucleus frequencies were determined by counting 2000 polychromatic erythrocyte (PCE) cells from just one animal (Table 3).

When micronucleated polychromatic erythrocyte (MNPCE) rates were examined, it was observed that MNPCE rates of animals in the MMC group increased like the increase in other parameters and this increase decreased with the application of the PG peel extract, and this decrease was parallel based on the dose increase of the PG peel extract ($P < 0.001$) (Figure 8).

When PCE/normochromatic erythrocyte (NCE) ratios were examined, it was found that the MMC group had lower levels compared to the other groups. This ratio started to increase in the MMC groups treated with PG peel extract, and this ratio increased in direct proportion with the dose increase of the PG peel extract and was statistically significant ($P < 0.001$) (Figure 9). PCE, NCE, and MNPCE counts were taken and photographed in microscopic examination (Figure 10).

4. Discussion

Our study showed that pomegranate extract has protective effects against MMC-induced genotoxicity. This protective effect is directly proportional to the dose increase. At the same time, the data obtained from the groups treated only with PG peel extract were similar to data from the control group.

It has been determined in various studies so far that pomegranate has antioxidant, antiviral, antibacterial, antidiabetic, anthelmintic, antidiuretic, and antineoplastic properties. It is also known to prevent tumor metastasis and show anticarcinogenic effects due to phenolic compounds found in various parts of pomegranate fruit [29–32].

In order to keep the genotoxic effects of physical and chemical substances on DNA to a minimum level, the investigation of antigenotoxic effects of natural herbal products in recent studies has increased the importance of natural antioxidants [33]. Humans resist the negative effects of mutagenic substances they are exposed to thanks to the natural antioxidants found in their bodies. The production of natural antioxidants in the body slows down depending on age. Therefore, people need antioxidant supplements. Increasing the use of various antigenotoxic compounds to minimize the effects of DNA damage caused by genotoxic agents in the living body is known to

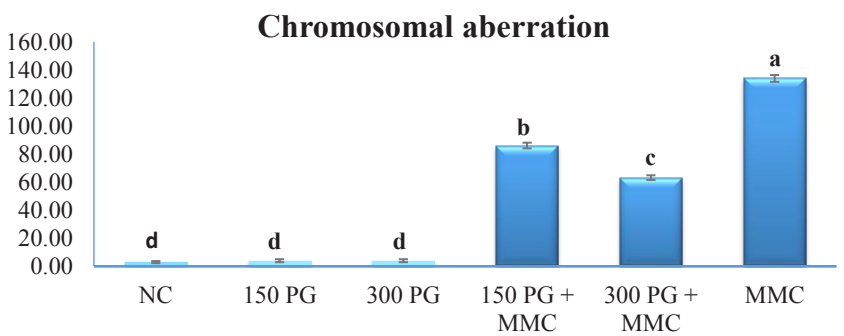

Figure 2. Chromosomal aberration rates of control and application groups.

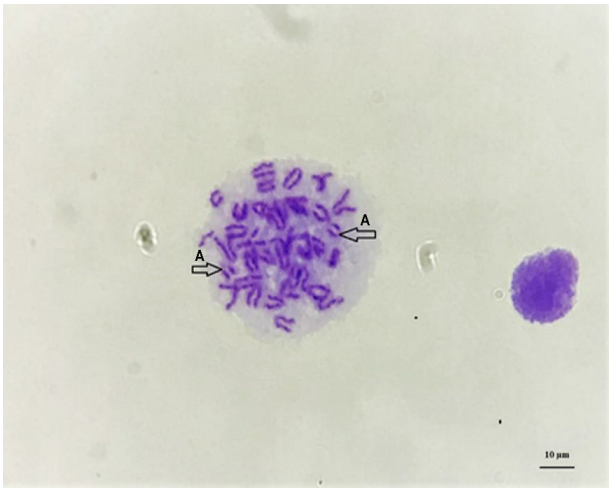

Figure 3. Chromosome fracture image in bone marrow cells of experimental group mice (A) (1000×).

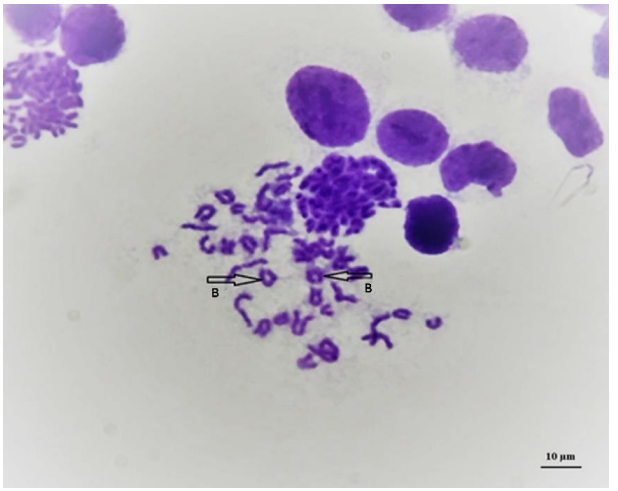

Figure 5. Sister chromatid association (B) image in bone marrow cells of experimental group (1000×).

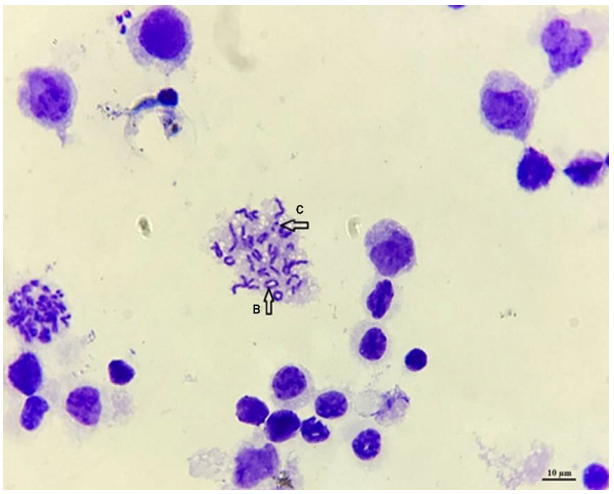

Figure 4. Fragment (C) and sister chromatid association (B) image in bone marrow cells of experimental group (1000×).

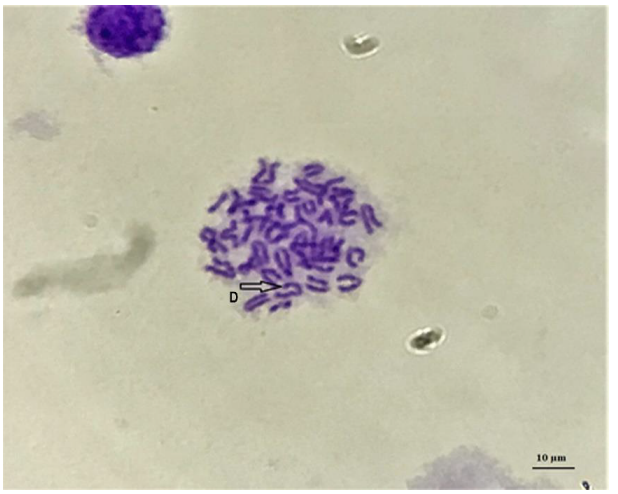

Figure 6. Chromatid fracture (D) image in bone marrow cells of experimental group (1000×).

be very important in preventing diseases such as cancer caused by mutations. Various studies have revealed the presence of compounds with high antioxidant activity in pomegranate. These useful antioxidants are known as polyphenols and they are powerful defenders against free radicals in the body [34–37].

Abdou et al. showed that the PG peel and seed extract inhibited genotoxic effects induced by CCl_4. Chromosomal aberrations, sperm anomalies, molecular analysis, and nucleic acid determination were used in that study. The results documented that CCl_4 increases chromosomal disorders, causes sperm anomalies, and produces genotoxicity as seen by the number of amplified fragments of DNA as well as reducing the total soluble protein content. However, it was observed that the PG significantly reduced the CCl_4 genotoxicity in the groups receiving PG seed extract. As a result, it is thought that this protective effect of the PG peel and seed extract with antigenotoxic effects against CCl_4 genotoxicity are thanks to its defensive ability against antioxidants and free radicals [38].

Hussien et al. investigated the antigenotoxic effects of PG peel extract against genotoxicity caused by malathion and atrazine pesticides in a study conducted on male albino mice. In this study, researchers using the comet assay analysis method gave the substances they used by oral gavage for 30 days. The results of this study showed that

Table 2. Control and experimental groups' mitotic activity rates in mouse bone marrow cells.

Groups	Number of subjects	Total cell count	Number of interphase cells	Number of metaphase cells	Group average ± SD	Metaphase cell ratio average (%)
NC	8	8000	7686	314	39.25 ± 1.83[a]	3.925
MMC	8	8000	7870	130	16.25 ± 1.28[c]	1.625
150 mg/kg PG	8	8000	7688	312	39.00 ± 2.00[a]	3.900
150 mg/kg PG + MMC	8	8000	7831	169	21.12 ± 1.13[b]	2.110
300 mg/kg PG	8	8000	7691	309	38.62 ± 2.00[a]	3.862
300 mg/kg PG + MMC	8	8000	7835	165	20.62 ± 1.41[b]	2.062

Different letters in the same column represent statistical significance.

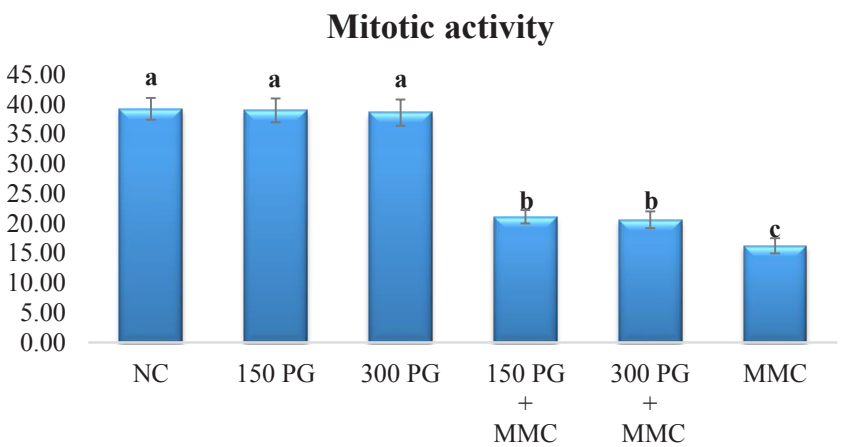

Figure 7. Mitotic activity rates of control and application groups.

Table 3. Micronucleus test results of control and experimental groups

Groups	Total PCE	MNPCE	MNPCE (%)	Group average ± SD	Total erythrocytes (PCE + NCE)	Number of PCEs	Number of NCEs	PCE/NCE ratio
NC	16000	318	1.987	39.75 ± 1.67[d]	8000	5517	2483	2.21
MMC	16000	757	4.730	94.62 ± 1.41[a]	8000	3895	4105	0.94
150 mg/kg PG	16000	329	2.056	41.12 ± 2.42[d]	8000	5374	2626	2.04
150 mg/kg PG + MMC	16000	565	3.531	70.62 ± 1.06[b]	8000	4121	3879	1.05
300 mg/kg PG	16000	331	2.060	41.37 ± 1.69[d]	8000	5437	2563	2.11
300 mg/kg PG + MMC	16000	482	3.012	60.25 ± 1.49[c]	8000	4731	3269	1.44

Different letters in the same column represent statistical significance.

PG peel extract alone showed no significant antigenotoxic effect against the genotoxicity of malathion and atrazine pesticides [39].

Malviya et al. investigated the antioxidant and antibacterial properties of PG peel. The antioxidant activity of the PG peel extracts made with different solvents was obtained from ethanolic extract at the highest levels; on the other hand, the antibacterial activity was tested against four bacterial strains. They reported that the PG peel showed antibacterial effects against all tested bacteria and had significant antioxidant activity in terms of high phenolic content [40].

Avila et al. investigated the antigenotoxic effects of PG fruit extract against the genotoxicity caused by chromium in mouse bone marrow. In this study, carried out for 10 days, doses of the PG fruit extract were given by oral

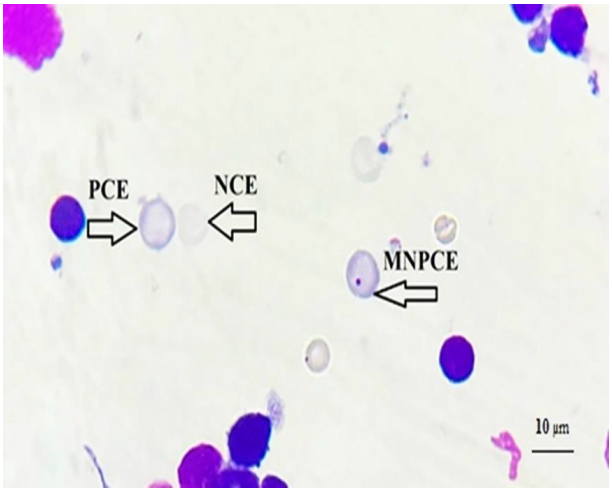

Figure 8. MNPCE rates of control and application groups.

gavage at 25, 50, and 75 mg/kg, while chromium was given intraperitoneally at 30 mg/kg. It was observed that the dose of the PG fruit extract having the most antigenotoxic effect was 50 mg/kg. According to the results of the study, the PG fruit extract showed significant antigenotoxic effects on chromium genotoxicity [41].

In an in vivo study on rats, Zahin et al. investigated the possible antioxidant effects of the punicalagin and ellagic acid compounds that exist in PG peel by Ames test. In that study, benzo(a)pyrene ($C_{20}H_{12}$) was used for mutagenicity. As a result, it was observed that punicalagin and ellagic found in PG peel have significant antimutagenic effects against benzo(a)pyrene-induced mutagenicity in parallel with the dose [42].

In the studies conducted by Abdou et al. [38], Malviya et al. [40], Avila et al. [41], and Zahin et al. [42], different parts of pomegranate plants were extracted with various solvents using different methods. They reported that all of these extracts have antigenotoxic, antioxidant, and antibacterial effects. It is known that the mentioned parameters (such as method, solvent, part of the plant) play important roles in the biological effects of plants [43]. In particular, the solvent plays an important role. Unlike these results, Hussien et al. directly used pomegranate juice, and they reported that it was not antigenotoxic. However, in our study, the Soxhlet method was used for pomegranate extract and only the peel part of the pomegranate was processed.

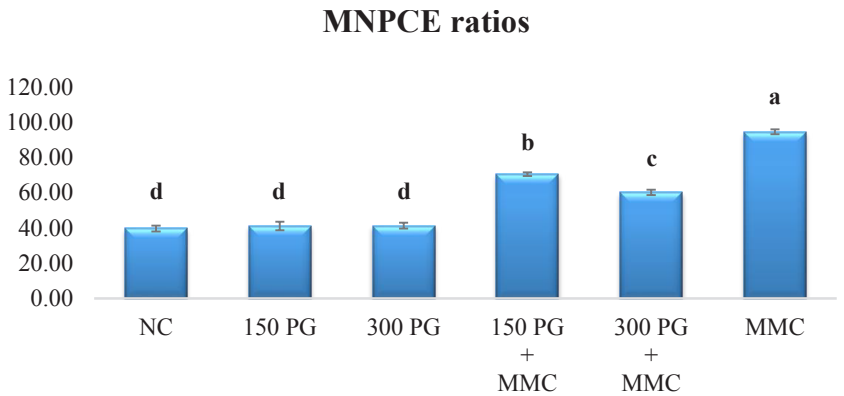

Figure 9. PCE/NCE ratios of control and application groups.

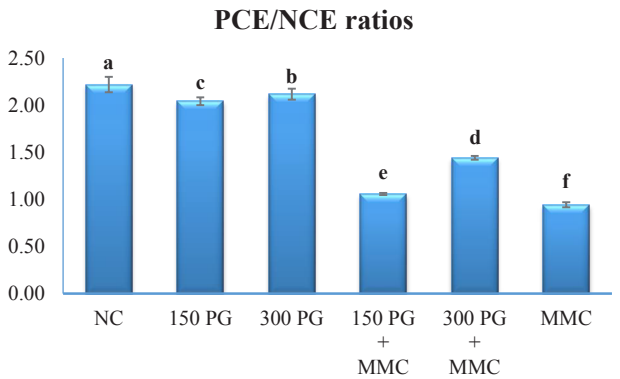

Figure 10. Microscopic image of PCE, NCE, and MNPCEs in bone marrow cells of experimental group (1000×).

According to chromosomal aberration, mitotic activity, and micronucleus analysis results of the present study, the determined doses of the PG peel extracts did not show any genotoxic effects. It was observed that the parameters examined for PG peel extracts were very close to those of the control group. Assessing chromosomal aberration rates, it was found that aberration rates were highest in the MMC group and 150 mg/kg and 300 mg/kg of PG peel extracts together with MMC decreased these levels based on dose increase ($P < 0.001$).

In terms of the mitotic activity rates, the mitotic activity level was low in the MMC group, while it was found that in the groups with PG peel extract together with MMC, the mitotic activity rate significantly increased compared to the MMC group ($P < 0.001$).

According to the results of the micronucleus analysis, PCE/NCE ratios were observed at low levels in the MMC group, while these ratios increased in the groups with PG peel extract and were directly proportional to the dose increase. In addition, this was found to be significant in terms of statistical results ($P < 0.001$).

When MNPCE ratios were examined, it was observed that they showed high values similar to other parameters in the groups with MMC, and the inhibitory effect of PG peel extract showed inhibitory effects on these high values based on the dose ($P < 0.001$).

In conclusion, it has been revealed by many studies that *P. granatum* is rich in phenolic compounds. It can be said that *P. granatum* peel extract used in the dosages indicated in this study showed antimutagenic-antigenotoxic effects as a result of micronucleus, mitotic index, and chromosomal aberration tests.

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