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Research Article
Anti-Tumoral Effects of Pomegranate Fruit Membranes Across Different Phenotypes

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Abstract

Background and Objective: Pomegranate, a fruit from the cinnamon family, contains hundreds of seeds surrounded by membranes, which contribute to its unique taste and growth in temperate climates. This study aimed to investigate the anti-tumoral activities of membrane extracts from pomegranates of different phenotypes on lung cancer cell lines under *in vitro* conditions. **Materials and Methods:** Pomegranate fruits from three different phenotypes (Devedişi, Hicaz and Nuz sour) were harvested and the membranes were collected and dried. Solid-liquid extraction was used to obtain the extracts. The anti-tumoral effects of the membrane extracts were assessed using human healthy lung fibroblast cells and A549, a human Non-Small Cell Lung Cancer (NSCLC) cell line, as control and experimental groups, respectively. Malondialdehyde (MDA) was determined by measuring of lipid peroxidation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was measured for cytotoxic activity conducted on the cell lines. Data are presented as Mean±SEM from triplicate experiments, analyzed by One-way ANOVA with Tukey's *post hoc* test (GraphPad Prism 8.0), with $p<0.05$ considered significant. **Results:** The most effective compound in terms of cell viability was the Devedişi membrane extract. The MDA analysis further revealed that Nuz sour membrane extract exhibited the highest anti-tumoral activity. **Conclusion:** These findings suggest the potential of pomegranate membrane extracts as a source of anti-cancer agents, particularly for lung cancer therapy.

Key words: Cell culture, anti-tumor, malondialdehyde (MDA), phenotypic variation, pomegranate membrane extract, A549 cell line, cytotoxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The pomegranate, belonging to the Punicaceae family, is an interesting and promising edible fruit species for different regions of the World, consisting of hundreds of grains with many small seeds and is very well adapted to semiarid soils and dry weather conditions. It has a slightly sour or sweet taste according to its phenotype, consisting of the peel, seed, grain and the whitish membrane covering the grain. The grains constitute 60-67% of the total pomegranate, while 33-40% is the peel. Approximately 75% of the total fruit weight is water, 1.6% protein, 16 mg/1000 g ascorbic acid, 0.7% ash, 0.58% acidity and it contains bioactive components such as phenolics, flavonoids, ellagitannins and proanthocyanidins and a significant amount of minerals. The chemical components of pomegranates vary depending on the region where they are grown, soil type, planting and harvesting times, storage conditions and climate[1,2].

There are studies in the literature showing that pomegranate and pomegranate-based products not only taste good but also have health benefits, as studies have shown that they lower blood pressure, prevent the oxidation of low and high-density cholesterol and prevent the development of atherosclerosis[3].

The bioactive components found in pomegranates are mainly in the peel and seed and the edible part of the fruit is approximately 52%. Therefore, the reason why the juices sold in the market contain high antioxidants is due to the polyphenols in the peel passing into the juice as the whole fruit is pressed during production[4]. The reason for these effects in pomegranate-based products is the phenolic compounds they contain, which have a strong antioxidant effect[5]. The consumption of pomegranate fruit, which is rich in polyphenols, has been reported to have anti-proliferative, anti-angiogenic, anti-invasive and pro-apoptotic effects on many cancer cells *in vitro* and *in vivo*[6]. Another study showed that polyphenols in fermented pomegranate juice were shown to be 2 times more effective antiproliferative than those in fresh fruit juice in MCF7 and MB-MDA-231 cell lines[7]. There are studies in the literature showing that pomegranate fruit extracts have cytotoxic and apoptotic effects on some types of cancer[8,9]. It was determined that pomegranate juice extract dose-dependently inhibited cell growth/cell viability and induced apoptosis in human prostate cancer (PC3) cells when applied with PFE (10-100 µg/mL; 48 hrs). In a study conducted with human PC3 prostate cancer cells, it was explained that pomegranate juice extract had antiproliferative and proapoptotic properties[10].

The health effects of pomegranate juice and its extracts have been reported in many studies, but no study has been found in the literature regarding the active ingredient and benefits of the membranes covering the grains.

This study aims to evaluate the anti-tumoral effects of pomegranate fruit membranes across different phenotypes. It seeks to determine their potential in inhibiting tumor growth and variation in effectiveness among phenotypic differences.

MATERIALS AND METHODS

Study area: In this study, pomegranates of three different phenotypes (Devedisi, Hicaz pomegranate, Nuz Sour), which are widely grown in Oğuzeli district of Gaziantep Province, were collected at harvest time of between October-December at 2023 and after being identified by the Agricultural Engineer in the District Directorate of Agriculture, the membrane parts were removed and dried in the shade. Extractions were obtained from suitable solvents.

Membrane materials: The membrane parts on the pomegranate seeds (the whitish parts covering the seeds) were separated and dried in a cool environment with good air flow, away from direct sunlight, in all three different phenotypes. Solid-liquid extractions were prepared.

Obtaining membrane extractions: Liquid membrane extracts were prepared for all three phenotypes. Extracts were obtained by solid-liquid extraction. The extraction process was carried out by taking the soluble substances contained in the pomegranate membrane from the solid phase to the liquid phase with the application of a solvent. The shade-dried membranes were ground into powder using a grinder. There are traditional and modern methods in extraction processes. Within the scope of the project, extraction experiments were carried out in different ways. After the extraction process was completed, the samples were filtered and liquid extracts were obtained. In this way, 6 different extraction samples from each phenotype were prepared as shown in the table below, as shown in Table 1.

Cytotoxic activity measurement: The MTT is one of the enzymatic test methods used to determine the cytotoxic effect of pomegranate extracts on A549 and human healthy lung fibroblast cell lines. Cells were grown in a 96-well microplate containing growth media. The cells were treated with various doses of pomegranate extracts (6.25-100 mg/L)

Table 1: Detail of extraction methods and times

Name of samples	Solvents used			Duration and extraction method		Ambient conditions
	Methanol	Ethanol	DimethylSulfoxide (DMSO)	Solid-liquid extraction with the help of a magnetic stirrer	Ultrasonic assisted extraction	
Hicaz pomegranate membrane (HPM)	1:10	1:10	1:10	16 hrs	4 hrs	Room conditions
Deve Disi pomegranate membrane (DDPM)	1:10	1:10	1:10	16 hrs	4 hrs	Room conditions
Nuz Sour pomegranate membrane (NSPM)	1:10	1:10	1:10	16 hrs	4 hrs	Room conditions

Table 2: Extraction conditions

1NSPM	Methanol 4 hrs ultrasonic	7HPM	Methanol 4 hrs ultrasonic	13DDPM	Methanol 4 hrs ultrasonic
2NSPM	Ethanol 16 hrs	8HPM	Ethanol 16 hrs	14DDPM	Ethanol 16 hrs
3NSPM	Methanol 16 hrs	9HPM	Methanol 16 hrs	15DDPM	Methanol 16 hrs
4NSPM	DMSO ultrasonic 4 hrs	10HPM	DMSO ultrasonic 4 hrs	16DDPM	DMSO ultrasonic 4 hrs
5NSPM	DMSO 16 hrs	11HPM	DMSO 16 hrs	17DDPM	DMSO 16 hrs
6NSPM	Ethanol ultrasonic 4 hrs	12HPM	Ethanol ultrasonic 4 hrs	18DDPM	Ethanol ultrasonic 4 hrs

for 48 hrs. The MTT solution (5 mg/L MTT in PBS) was added to each well at a 1:10 ratio following the incubation period. After 3 hrs, the microplates were centrifuged at 800 g for 5 min and 150 µL of DMSO was introduced to dissolve the formazan crystals. Absorbance was then measured at 570 nm using a microplate spectrophotometer (Synergy-HT; Biotech Winooski, VT, USA). The results were expressed as the percentage of viable cells, with values calculated as the average of 4-6 repetitions. Untreated cells served as the negative control (NC) and cells treated with 1% Triton X-100 were used as the positive control (PC). The concentrations to be used were determined according to previous studies in the literature[11].

Lipid peroxidation measurement: To evaluate lipid peroxidation activities, A549 and human healthy lung fibroblast cells were seeded in 24-well plates at 5×10^4 cells/well. It was incubated with the IC50 dose of the extract with the highest anti-tumoral activity for 24, 48 and 72 hrs. Cells to which the solvent used in extract preparation was applied were used as a negative control. The solution was heated in a water bath at 95°C for 60 min and the absorbance was measured at 532 nm. MDA concentration was calculated using an extinction coefficient of 1.56 105 M^{-1} cm^{-1} and expressed as nmol MDA/mg protein[12].

The codes of the membrane samples extracted in 3 different solvents: Methanol, Ethanol and DMSO for 4 hrs with ultrasonic support and for 16 hrs using the magnetic stirrer extraction method are NSPM, HPM, DDPM, pomegranate membrane extraction conditions were as in the Table 2.

Statistical analysis: The data are expressed as the Mean±standard error of the mean (SEM), derived from experiments conducted in triplicate. Statistical analysis of variance was performed using a One-way Analysis of Variance (ANOVA) with GraphPad Prism 8.0 software (GraphPad, La Jolla, CA). The Tukey's test was applied for *post hoc* comparisons. Differences were deemed statistically significant when $p<0.05$.

RESULTS

A total of 17 compounds were evaluated for their anticancer activity through the MTT assay as shown in Table 3. The IC_{50} values for each compound were determined based on the assay results, which reflect the concentration of each compound required to inhibit cell growth by 50%. Among the tested compounds, 7 demonstrated IC_{50} values lower than 45, indicating a potent anticancer effect at relatively low concentrations. These compounds showed significant activity compared to others in the series. The full ranking of the compounds based on their IC_{50} values and corresponding anticancer activity is as follows: 15DDPM> 10HPM> 5NSPM> 9HPM> 13DDPM> 3NSPM> 4NSPM> 7HPM> 12HPM> 8HPM> 6NSPM> 16DDPM >1NSPM >17DDPM> 11HPM> 2NSPM> 18DDPM. This ranking highlights the most effective compounds, suggesting their potential for further development and investigation in anticancer therapies.

The compounds with IC_{50} values below 45 were further tested for MDA (malondialdehyde) levels, as seen in Table 4. Based on the MDA analysis, the compounds were ranked from the most effective to the least effective as follows: 5NSPM > 13DDPM > 10HPM > 3NSPM > 15DDPM > 4NSPM > 9HPM. This ranking reflects the compounds' effectiveness in reducing MDA levels, with 5NSPM showing the highest efficacy and 9HPM showing the lowest among the tested compounds.

Table 3: Cell viability (%) of compounds

Compounds			Compounds		
Control (-)	MTT analysis (% cell viability)		Control (-)	MTT analysis (% cell viability)	
Control (+)			Control (+)		
	100	47.2±5.65*		100	52.9±4.32*
	50	54.3±5.2*	2	50	60.1±7.89*
	25	63.01±8.43*		25	65.2±7.04*
1	12.5	73.9±4.65		12.5	79.32±5.76
	6.25	75.4±6.12		6.25	85.4±3.24
	100	38.9±2.67*		100	43.2±3.21*
	50	46.3±4.54*	4	50	47.8±3.55*
3	25	55.3±2.56*		25	57.4±4.52*
	12.5	70.2±7.43		12.5	69.09±1.78*
	6.25	75.3±3.56		6.25	72.1±2.98
	100	35.4±2.31*		100	32.1±2.79*
	50	47.8±4.53*		50	53.2±3.21*
5	25	56.3±6.76*	6	25	61.09±5.21*
	12.5	60.4±5.68*		12.5	65.4±3.58*
	6.25	65.3±6.90*		6.25	72.1±3.52
	100	38.9±3.07*		100	43.3±1.99*
	50	46.7±4.61*	8	50	49.7±2.87*
	25	67.3±4.92*		25	58.3±3.67*
7	12.5	75.4±3.56		12.5	66.7±5.78*
	6.25	82.1±6.43		6.25	79.08±5.23
	100	42.9±3.99*		100	24.3±2.34*
	50	45.3±3.78*	10	50	45.2±3.56*
9	25	56.8±2.90*		25	48.5±2.87*
	12.5	65.4±4.56*		12.5	58.2±5.43*
	6.25	66.7±3.56*		6.25	69.6±4.78*
	100	54.2±4.33*		100	34.12±3.45*
11	50	58.9±4.21*		50	48.5±4.32*
	25	63.4±6.78*	12	25	58.5±4.58*
	12.5	75.5±7.98		12.5	64.2±4.09*
	6.25	79.5±8.01		6.25	74.3±3.89
	100	45.8±4.30*		100	42.9±2.30*
13	50	46.7±2.78*		50	41.7±1.82*
	25	57.9±4.53*	14	25	52.9±0.23*
	12.5	63.2±3.21*		12.5	59.8±2.21*
	6.25	76.4±4.56		6.25	73.2±2.56
	100	32.7±2.01*		100	58.9±3.78*
15	50	38.9±2.45*		50	64.3±3.85*
	25	46.9±2.98*	16	25	69.2±4.34*
	12.5	56.7±2.45*		12.5	75.4±3.23
	6.25	74.3±3.26		6.25	80.01±7.54
	100	47.6±3.21*		100	51.2±4.37*
17	50	54.3±2.68*		50	58.9±3.29*
	25	57.8±3.27*	18	25	64.3±3.44*
	12.5	65.3±3.49*		12.5	69.3±2.89*
	6.25	71.23±5.64		6.25	69.02±3.57*

*It represents statistical differences compared to the control group, $p<0.05$

DISCUSSION

Pomegranate cultivation is generally carried out to consume pomegranate juice and fruit and in pomegranate processing facilities, farmers evaluate the remaining parts, such as peel, membrane and seeds after extracting the juice of the fruit as waste due to limited knowledge and opportunities.

Data in the literature suggest that pomegranate fruit contains phytochemicals such as ellagitannins and punicalagin and these high molecular weight polyphenols are thought to inhibit the proliferation of cancer cells and mediate protective effects against inflammatory disorders[13,14].

The pharmacological effects of the components contained in pomegranate and pomegranate-based

Table 4: MDA levels of compounds

Compound	MDA level (nmol/mg protein)
Control	0.73±0.12
3NSPM	
100	1.2±0.34*
50	0.97±0.25*
25	0.89±0.24*
12.5	0.86±0.19
6.25	0.78±0.37
4NSPM	
100	0.89±0.28*
50	0.84±0.22
25	0.84±0.21
12.5	0.81±0.17
6.25	0.74±0.19
5NSPM	
100	1.69±0.41*
50	1.36±0.33*
25	1.23±0.26*
12.5	0.96±0.18*
6.25	0.81±0.17
9HPM	
100	0.87±0.25
50	0.83±0.31
25	0.75±0.22
12.5	0.76±0.20
6.25	0.75±0.14
10HPM	
100	1.35±0.32*
50	1.11±0.28*
25	0.92±0.12*
12.5	0.89±0.22*
6.25	0.79±0.16
13DDPM	
100	1.57±0.27*
50	1.34±0.31*
25	0.96±0.22*
12.5	0.9±0.18*
6.25	0.85±0.18
15DDPM	
100	0.99±0.23*
50	0.97±0.33*
25	0.82±0.31
12.5	0.79±0.21
6.25	0.76±0.19

* It represents the statistical differences between them and the control group, $p<0.05$

products have a wide range of clinical uses, from the prevention of various cancers to the treatment of chronic inflammatory diseases[15].

Although the consumption of fruit juices such as grape juice and blueberry, which have high phenolic antioxidant content, has increased in recent years, pomegranate is the fruit juice with much higher antioxidant levels compared to other fruit juices and even compared to red wine[16,17].

The results of epidemiological studies on fruits with high phenolic content, such as pomegranate, have shown that regular consumption of these fruits reduces mortality rates from cardiovascular, cerebrovascular and cancer-related diseases[18].

It has been reported that consumption of pomegranate fruit, which has a high content of polyphenolic compounds, has anti-proliferative, anti-angiogenic, anti-invasive and pro-apoptotic effects on cancer cells in *in vivo* and *in vitro* studies[19,20].

It has been reported that pomegranate fruit extracts arrest cell growth in the G1 phase of the cell cycle in the A 549 cell line depending on concentration and that they exert this effect by regulating the CKI cyclin-CDK system and inhibiting MAPK, NFκB and PI3K/Akt signaling.

It has also been determined that pomegranate fruit extract has an inhibitory effect on cell growth in A549 lung cancer cells[21].

This current study experimentally assessed the anti-tumoral activities of various pomegranate peel extracts by performing MTT assays on the human Non-Small Cell Lung Cancer (NSCLC) cell line A549. The extracts were carefully prepared using appropriate methods to ensure optimal extraction of bioactive compounds from the pomegranate membranes. Our findings indicated that the camel tooth membrane extract was the most effective compound in reducing cell viability among the tested samples, demonstrating significant anti-cancer potential.

When compared with current results with existing literature, found that similar studies have reported a marked reduction in cell viability following treatment with pomegranate fruit extract, particularly when incubated for 72 hrs at concentrations between 50-150 µg/mL. These studies utilized MTT and trypan blue assays to measure cell viability and cytotoxicity, confirming that pomegranate extracts exhibit significant cytotoxic effects on various cell lines. Our study supports these findings, highlighting the potential of pomegranate-derived compounds, particularly those from the membrane, as effective agents in reducing cell viability and exerting cytotoxic effects on cancer cells.

Moreover, it is important to note that the extracts did not show any toxic effects on healthy lung cells. This suggests that the pomegranate membrane extracts have a selective cytotoxic effect, primarily targeting cancer cells while leaving healthy cells unharmed. These results emphasize the promising therapeutic applications of pomegranate extracts in cancer treatment, particularly for lung cancer and underscore the importance of extracting bioactive compounds from different parts of the fruit[22].

The oxidation of Polyunsaturated Fatty Acids (PUFA) by free radicals in biological systems is known as lipid peroxidation[23]. Lipid peroxidation is a degenerative process that can cause cytopathological consequences and has been

associated with many pathological processes such as atherogenesis, ischemia-reperfusion injury and ultraviolet-induced carcinogenesis. Additionally, it may play a role in the cytotoxic effects of oxidant-based chemotherapeutic and phototherapeutic drugs[24].

Peroxidation events that occur in biological membranes negatively affect membrane fluidity, potential and permeability to ions, causing organelle contents to be released into the cytoplasm and, therefore, cell damage or cell death[25]. As can be seen, oxidative stress, which occurs as a result of the accumulation of reactive oxygen species in cells, is extremely dangerous and can be the trigger of many pathological processes.

For this reason, MDA, which is the most studied agent that shows lipid peroxidation levels, was evaluated in this study. It is known that pomegranate has a reducing effect on lipid peroxidation[26]. Different studies investigating the effects of pomegranate on serum MDA concentration in different periods have found that using pomegranate for two weeks or longer reduces serum MDA levels at a statistically significant rate[27].

It was determined that MDA levels, which are considered an indicator of oxidative stress, decreased in 1692 people who consumed pomegranate juice regularly. While studies on the use of pomegranate juice and MDA levels are included in the literature, the effect of pomegranate peel was first evaluated in this study[28].

In the present study, the results revealed that the Nuz sour membrane extract exhibited the highest effectiveness in reducing MDA (malondialdehyde) levels in the A549 cells, indicating a potent anti-tumoral activity through lipid peroxidation inhibition. On the other hand, the Hicaz pomegranate membrane extract showed the lowest effect on MDA levels, suggesting a weaker impact on oxidative stress and lipid peroxidation compared to the other phenotypes.

These findings highlight the potential variation in bioactive compounds present in pomegranates of different phenotypes, which may influence their anti-cancer properties. The Nuz sour extract's strong effect on MDA levels suggests that it may possess compounds that effectively target oxidative stress pathways, which are known to contribute to cancer cell survival and progression. Conversely, the Hicaz extract's lower impact could be due to differences in the concentration or types of active compounds present in the membranes or a less pronounced ability to modulate oxidative damage.

The role of oxidative stress in cancer progression is well-documented and compounds that can reduce lipid peroxidation, such as those found in the Nuz sour pomegranate membranes, are particularly valuable for their potential to inhibit cancer cell growth. The results also suggest that the membrane extracts of different pomegranate phenotypes may vary in their therapeutic efficacy, which underscores the importance of phenotype selection when considering natural products for cancer treatment.

CONCLUSION

According to the study, membranes, which are classified as waste in pomegranate juice enterprises, are important components in terms of anti-tumoral activity. As a result, the anticancer activity levels of pomegranate fruit membrane extracts obtained with different extraction methods were also determined in lung cancer cell lines. The different activities of these extracts of different phenotypes may be due to the varying bioactive component contents that they contain. These studies should be expanded because the results of combinations may not only be additive but also synergistic or even antagonistic. It is envisaged that in-depth studies on the anticancer activities of naturally occurring compounds may later lead to the development of an effective cocktail for cancer treatment. Further *in vitro* and *in vivo* studies are needed to evaluate the combinatorial effect of pomegranate membranes with other compounds and to determine whether pharmacological or even antagonistic effects are observed.

SIGNIFICANCE STATEMENT

This study has enabled the determination of the anticancer activities of pomegranate fruit membranes of different phenotypes and the possibility of transforming these waste materials into value-added products. The results of the study have determined compounds that may be effective in terms of anticancer activity and the results are important in determining which extraction methods should be used and which compounds should be studied in further studies. The study provides important basic data in terms of the usability of these waste materials as pharmacological agents in further research studies.

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