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Antiproliferative effects of Turkish pomegranate (*Punica granatum* L.) extracts on MCF-7 human breast cancer cell lines with focus on antioxidant potential and bioactive compounds analyzed by LC-MS/MS

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Abstract

In this study, eight different pomegranate (*Punica granatum* L.) cultivars from Turkey were evaluated for their antioxidant and cytotoxic effects on the MCF-7 breast cancer cell lines and MCF-10A breast fibrocystic epithelial cell lines with a focus on their chemical compositions by LC-MS/MS. Cell lines were treated with pomegranate juice extracts in different doses at selected time intervals (24th, 48th, and 72nd hour). Afterwards, WST-1 cell proliferation assay was performed to investigate the cytotoxicity of the extracts. Accordingly, all extracts decreased the cell viability of MCF-7 breast cancer cell lines and had no cytotoxic effect on the cell viability of MCF-10A cell lines. Among eight extracts, P7 (Izmir 1513), which was rich in anthocyanins such as cyanidin chloride (69.76 ± 8.02 μg/g extract), cyanidin-3-O-glucoside (903.66 ± 101.89 μg/g extract), and punicalagin (992.09 ± 174.53 μg/g extract), was found to demonstrate the strongest cytotoxic activity on MCF-7 breast cancer cell lines by decreasing the cell viability in half at 24th hour with an IC_{50} value of 49.08 μg/ml.

Practical applications

Eight commercially valuable pomegranate (*Punica granatum*) cultivars from Turkey were examined. Pelargonidin, cyanidin, cyanidin-3-O-gl, callistephin, and delphinidin-3-O-gl were quantified. Two cultivars (P1 and P3) showed comparatively higher antioxidant effects. A cultivar (P7) showed strongest cytotoxic activity against MCF-7 breast cancer cell line. The cultivars have potential to be used as natural antioxidant and anticancer agents.

KEYWORDS

antioxidant, cultivated, cytotoxicity, LC-MS/MS, MCF-7, pomegranate

1 | INTRODUCTION

Being one of the most talismanic plants throughout human history, *Punica granatum* L., (Pomegranate) is a dicotyledonous, perennial plant, belonging to Punicaceae family. The plant has such a long story that it has been considered as one of the oldest foods in the world. Since ancient times, pomegranate has been acting as a symbol of fecundity in both Eastern and Western cultures, and the fruits have always embodied feminine features such as beauty and fertility (Stone, 2017).

Since the red stains of the fruits were believed to symbolize blood drops, presumably based on the well-known principle "Similia Similibus Curantur" meaning "like cure like", the plant has been used to stop the menstrual bleeding traditionally in Greece. Besides in some cultures, pomegranate has been a treatment option for vaginal discharge (Shaygannia et al., 2016). In Turkey, a decoction prepared from the bark of the plant has been used to treat intestinal worms and tenias, and an infusion prepared from the peel of the fruit has been used to treat diarrhea. Furthermore, pomegranate juice has been used for stomach complaints and as a tonic (Baytop, 1999). As is seen, not only the fruits but also the fruit peels, leaves, roots, and/or flowers of the plant have been used traditionally in different cultures (Ge et al., 2021; Ismail et al., 2012).

Iran, where pomegranate was originated, remains of the largest producers of the fruits along with India and Turkey. In the US, California has been a hot spot for the cultivation of the fruit, and Spain is the center of the pomegranate industry in Europe. Being one of the most alluring fruits and being even considered as a source of national pride for many cultures with great economic importance, the interest in pomegranate research has also increased. Only on PubMed, it is possible to reach approximately 1,000 articles about pomegranate and its beneficial effects on health that were published between 2005 and 2019 (Puneeth & Chandra, 2020; Stone, 2017). Thanks to the intensive research into the pharmacological and chemical properties of the plant, scientific evidence for the traditional uses have also been provided.

According to the studies, different types of phytochemicals have been identified from the plant. Ellagitannins and gallotannins (e.g. castalagin, punicalin), ellagic acid and its derivatives (e.g. diellagic acid, eschweilenol C), catechin and procyanidins (e.g. gallocatechin, procyanidin B1), anthocyanins and anthocyanidins (e.g. cyanidin, delphinidin, pelargonidin), flavonols (e.g. luteolin, quercetin), organic acids (e.g. caffeic acid, chlorogenic acid), simple gallyol derivatives (e.g. brevifolin, methyl gallate), fatty acids and triglycerides (e.g. linoleic acid, punicic acid), sterols and terpenoids (e.g. asiatic acid, ursolic acid), alkaloids (e.g. pelletierine, pseudopelletierine), and other compounds (e.g. icariside D1, mannitol) were determined in different parts including seeds, bark, leaves, and juice of pomegranate (Heber et al., 2006). Having an abundance of beneficial phytochemicals, pomegranate has been shown to possess quite positive effects on human health. First of all, its antioxidant properties are well-known (Masci et al., 2016). Moreover, with numerous in vitro and in vivo studies, the plant was found to demonstrate anti-inflammatory, antidiabetic, antimicrobial, and anticancer properties (Fourati et al., 2020). The consumption of a phytochemical-rich diet has been associated with a reduced risk of severe diseases including different types of cancer. Expectedly, the anticancer potential of the plant has attracted the most attention of scientists. Speaking of which, over half of the anticancer medications we use today are derived from natural resources, and that being the case, pomegranate has been put a notch above the rest being effective against breast cancer, prostate cancer, colorectal cancer, leukemia, bladder cancer, glioblastoma, hepatocellular cancer, pancreatic cancer, skin cancer, and lung cancer (Khwairakpam et al., 2018; Lee et al., 2013).

Although there have been a plethora of studies aiming to bring new approaches to the treatment and prevention of breast cancer, it is still the most common cancer among women around the world. In fact, in well-developed countries where it is easier for individuals to access improved treatment opportunities, breast cancer mortality is decreasing. However, the incidence of the disease is still steadily increasing (Britt et al., 2020). Different strategies including surgery, chemotherapy, radiotherapy, and hormonal therapy have been used for the management of breast cancer. In an effort to enhance the life quality and survival rate of the patients, and also to alleviate the possible side effects of those treatments, natural alternatives such as medicinal plants with anticancer potential have also been used in cancer therapy (Akram et al., 2017). Within this framework, as has already been mentioned above, studies show that pomegranate might come out as an eye-catching natural option for the treatment of breast cancer. There have been studies carried out to investigate the cytotoxic potential of the plant on MCF-7 breast cancer cell lines specifically. Reportedly, pomegranate acts in different steps of breast cancer, from tumor cell proliferation to metastasis and angiogenesis, it is also targeting the levels of regulation of cell growth and apoptosis, and consequently, it is considered to have the potential to be an adjuvant in chemotherapy (Vini & Sreeja, 2015).

Bearing all this in mind, this study aimed to investigate the major compounds, antioxidant, and anticancer potentials of fruit juice extracts of eight different pomegranate cultures from Turkey. The qualitative and quantitative analyses of the constituents were performed by LC-MS/MS. Total phenolic and total flavonoid contents of the extracts were determined as gallic acid and catechin equivalents respectively. Antioxidant activities of the species were investigated by using DPPH radical scavenging and FRAP methods. To evaluate the anticancer potential of the extracts, cell proliferation analysis was conducted on MCF-7 and MCF-10 breast cancer cell lines at different time intervals (24th, 48th, and 72nd hour). With the findings of the current study, we hope to bring new insights and contribute a resource for further studies on pomegranate with its significant antioxidant and anticancer properties.

2 | MATERIAL AND METHODS

2.1 | Plant material and extraction

Eight different pomegranate cultivar fruits were obtained from Aegean Agricultural Research Institute (Menemen, Izmir, Turkey), Republic of

Turkey Ministry of Agriculture and Forestry. The samples namely P1-3, P2- 12, P3- 23, P4- 38, P5-11-2-3, P6- Eksilik Nar, P7- Izmir 1513, and P8- Katirbasi Eksi were encoded as P1 to P8 respectively.

The fruits were washed and peeled, then the arils were squeezed by using a juice extractor. The obtained juice was centrifuged at 8,806 *g*, 4°C, for 10 min (Thermo Scientific, Heraeus Biofuge Stratos, Germany) and then the supernatant was lyophilized (Labconco FreeZone Freeze dry system, Kansas City, MO, USA) to dry powder. The dried powder extracts were stored at −80°C till the bioactivity and LC-MS/MS analyses.

2.2 | Chemicals

2.2.1 | For LC-MS/MS analysis

The methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Standard compounds given in Table 1 were obtained from Sigma-Aldrich (St. Louis, MO) and ExtraSynthese (Genay-France).

2.2.2 | For antioxidant activity

DPPH (2,2′-diphenyl-1-picrylhydrazyl) (Sigma Chemical Co., St. Louis, MO, USA), gallic acid (Sigma Chemical Co., St. Louis, MO, USA), quercetin (Fluka Chemical Co., Buchs, Switzerland), catechin (Fluka Chemical Co., Buchs, Switzerland), TPTZ (2,4,6-tripyridyls-triazine) (Merck Chemical Co., Darmstadt, Germany) were purchased and all other chemicals or reagents were of analytical grade.

2.2.3 | For cell proliferation analysis

The cell culture materials (Biochrome, Berlin, Germany), the MCF-7 human breast cancer cell line and fibrocystic breast tissue MCF-10A (American Type Culture Collection, ATCC, Rockville, MD), the mediums (Biochrome, Berlin, Germany), 10% fetal bovine serum (FBS) (Biochrome, Berlin, Germany), 1% glutamine (Biochrome, Berlin, Germany), 1% penicillin (Biochrome, Berlin, Germany) were purchased.

2.3 | LC-MS/MS analyses of the extracts

2.3.1 | Preparation of the samples

Stock mixture (10 mg/L in methanol) was prepared by using 22 standard compounds, the concentrations of the mixture were arranged as 0.1 mg/L and 5 mg/L, respectively. Curcumin (100 mg/L) was added 50 µl to the stock mixture as internal standard (IS). The pomegranate extract (100 mg) was dissolved in 25 ml ethanol-water (60:40; v/v) to prepare the test solutions, then the concentration of the extracts was arranged as 1 mg/L (Seyhan et al., 2019).

All measurements were repeated three times. Differences were considered significant at $p < .05$.

2.3.2 | Instruments and chromatographic conditions

LC-MS/MS apparatus consisted of Zivak® HPLC and Zivak® Tandem Gold Triple Quadrupole mass detector to analyze the pomegranate extracts. C18 column (Synergy Max, 250 × 2 mmi. d., 5 µm) and a gradient method were used in the LC analyses. The injection volume was 10 µL and the mobile phase used was 0.1% formic acid in water (A) and 0.1% formic acid in methanol at a flow rate of 0.25 ml/min. The gradient elution was 0–1 min 45% B, 1.01–20 min 100% B, and 20.01–23 min 45% B. The column temperature was adjusted to 30°C.

Optimization of the LC/MS/MS procedure, validation of experiments, and uncertainty evaluation parameters were expressed by Seyhan et al. (2019) and Kalın et al. (2015) in detail.

2.4 | Total phenolic content (TPC) and total flavonoid content (TFC)

The total phenolic content determination was performed using the modified Folin-Ciocalteu method described by Slinkard and Singleton (1977). The equation obtained from the gallic acid regression curve ($y = 0.7459x + 0.0004$) was used to calculate the results and the results were expressed in mg of gallic acid equivalents (GAE)/g extract.

For the determination of the total flavonoid content, the aluminum chloride method developed by Kim et al. (2003) was used. The results were calculated using the equation obtained from the catechin regression curve ($y = 1.8523x + 0.0081$) and expressed in mg of catechin equivalents (CE)/g extract.

2.5 | Antioxidant activity

2.5.1 | DPPH radical scavenging activity

Eight different pomegranate extracts were tested using the DPPH radical scavenging activity assay modified by Brand-Williams et al. (1995). The absorbance values of the extracts and the standard (quercetin) were measured at 517 nm (BioTek Eon, Winooski, VT, USA). The methanol was used as a control. The following formula was used to calculate the percentage of DPPH scavenging activity of the extracts.

$$\text{DPPH scavenging activity\%} = (1 - (\text{Absorbance of sample at 517 nm}) / (\text{Absorbance of control at 517 nm})) \times 100$$

TABLE 1 LC-MS/MS analysis results of the pomegranate extracts

	Compounds	3 (P1)[a]	12 (P2)[a]	23 (P3)[a]	38 (P4)[a]	11-2-3 (P5)[a]	Ekşilik Nar (P6)[a]	İzmir 1513 (P7)[a]	Katırbaşı Ekşi (P8)[a]
1	Fumaric acid	738.69 ± 68.48	854.14 ± 79.18	363.68 ± 33.71	259.55 ± 24.06	492.96 ± 45.70	168.03 ± 15.58	669.10 ± 62.02	151.54 ± 4.16
2	Pyrogallol	17.88 ± 2.20	16.45 ± 2.02	12.05 ± 1.48	17.63 ± 2.17	10.83 ± 1.32	14.69 ± 1.81	15.40 ± 1.89	13.12 ± 1.61
3	p-Coumaric acid	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
4	Gallic acid	4.44 ± 0.71	11.42 ± 1.82	5.34 ± 0.85	3.29 ± 0.52	3.64 ± 0.58	4.59 ± 0.73	4.15 ± 0.66	3.94 ± 0.63
5	1.3-Cynarin	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
6	Ascorbic acid	50.96 ± 5.86	35.62 ± 4.09	29.54 ± 3.39	1.42 ± 0.16	1.25 ± 0.14	6.91 ± 0.79	69.76 ± 8.02	15.31 ± 1.76
7	Chlorogenic acid	5.62 ± 0.79	23.76 ± 3.35	5.97 ± 0.84	4.78 ± 0.67	4.96 ± 0.70	9.64 ± 1.36	7.96 ± 1.12	8.21 ± 1.16
8	Quercetin	Nd	Nd	Nd	0.41 ± 0.07	Nd	Nd	Nd	Nd
9	Isoquercetin	0.87 ± 0.19	Nd	Nd	0.12 ± 0.03	Nd	Nd	Nd	Nd
10	Epicatechin	9.98 ± 0.79	9.43 ± 0.75	12.60 ± 1.00	20.41 ± 1.62	7.36 ± 0.11	9.96 ± 0.79	13.56 ± 1.07	12.09 ± 0.95
11	Epigallocatechin	13.89 ± 1.57	5.75 ± 0.65	36.79 ± 4.16	9.85 ± 1.11	1.05 ± 0.12	80.77 ± 9.15	81.59 ± 9.24	80.31 ± 9.09
12	Epigallocatechin gallate	5.28 ± 0.48	Nd	Nd	Nd	Nd	5.03 ± 0.45	5.08 ± 0.46	4.94 ± 0.45
13	Luteolin-7-Glu	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
14	Kaempherol-3-Rutinoside	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
15	Pelargonin chloride	71.38 ± 8.08	84.92 ± 9.61	135.44 ± 15.32	27.92 ± 3.16	9.01 ± 1.02	Nd	56.56 ± 6.40	82.21 ± 3.31
16	Pelargonidin chloride	380.33 ± 37.12	102.31 ± 9.98	274.35 ± 26.78	1.27 ± 0.12	5.95 ± 0.58	37.31 ± 3.64	302.89 ± 59.56	38.09 ± 3.71
17	Cyanidin chloride	124.44 ± 14.57	106.52 ± 12.47	77.04 ± 9.02	21.24 ± 2.49	11.70 ± 1.37	45.87 ± 5.37	168.00 ± 19.67	45.05 ± 5.27
18	Cyanidin-3-O-glucoside	893.93 ± 100.79	417.64 ± 47.09	774.53 ± 87.33	586.54 ± 66.13	289.67 ± 32.66	211.02 ± 23.79	903.66 ± 101.89	173.44 ± 19.56
19	Callistephin	588.44 ± 59.26	156.55 ± 15.76	489.22 ± 49.26	113.74 ± 11.45	74.76 ± 7.52	48.21 ± 4.78	505.38 ± 50.89	45.16 ± 4.55
20	Procyanidin B2	16.03 ± 2.54	3.43 ± 0.55	0.62 ± 0.10	1.99 ± 0.32	Nd	0.78 ± 0.12	8.47 ± 1.34	0.68 ± 0.11
21	Punicalagin	509.93 ± 89.71	427.68 ± 75.24	706.79 ± 124.34	2.01 ± 0.35	Nd	855.88 ± 150.57	992.09 ± 174.53	21.33 ± 3.75
22	Delphinidin-3-O-glucoside	439.93 ± 64.72	93.48 ± 13.73	1007.75 ± 148.05	178.40 ± 26.24	433.51 ± 63.77	185.22 ± 27.25	694.02 ± 101.90	138.65 ± 20.36

Abbreviation: Nd, not detected.

[a]Values are given as μg/ml.

2.5.2 | Ferric-reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was established to identify the reducing activities of eight different pomegranate extracts (Benzie & Strain, 1996). The absorbance values of the extracts and the standard (quercetin) were measured at 593 nm. $FeSO_4.7H_2O$ solution was used to create a standard regression curve and the reducing power of the extracts was expressed as FRAP value (mM Fe^{2+} equivalents) using the equation ($y = 0.0153x + 0.0001$).

2.6 | Cell proliferation analysis

The cell proliferation potential of the eight different pomegranate extracts was determined using WST-1 Cell Proliferation Reagent (Roche, Manheim, Germany). Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA) was used to count the cells. The seven different concentrations (750, 250, 100, 50, 5, 2.5, 1 μg/ml) of the extracts were carried out to the cells at 24th, 48th, and 72nd h time intervals. The detailed information about the experiment was given by Seyhan and et al. (2019). The absorbance of the cells was measured using a Multiscan ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The viability of untreated -control-cells was accepted as 100%. Data are signified as percentages of absorbance readings compared to control wells on a relative proliferation index scale (Mean ± Standard deviation). The IC_{50} values were calculated using an approximated sigmoidal curve. All tests were performed in triplicate.

2.7 | Annexin V Apoptosis Detection Assay

Cells were seeded into 6 well plates (Biochrome, Berlin, Germany) at the density of 2×10^5 per well. After the cells adhered the plates, used medium was changed with fresh medium supplemented with 3% FBS. Afterwards, selected doses of pomegranate extract according to WST-1 assay, were applied to the cells at selected time intervals (24th and 48th hour). Muse™ Annexin V Dead Cell Assay (EMD Millipore Corporation, Hayward, CA, USA) was used to determine the apoptosis due to exposure to pomegranate extracts. Musetm Annexin V Dead Cell Assay was performed according to manufacturer's protocol and apoptotic cells were measured using Muse Cell Analyzer (EMD Millipore, Billerica, MA, USA).

2.8 | Statistical analysis

One-Way ANOVA (And Dunnet test for the post hoc analysis) and Multiple T-tests were used to perform for statistical evaluations. Values of $p < .05$ were considered as significant. All values are expressed shown as mean ± standard deviation. GraphPad Prism 6 Software (GraphPad Prism Soft-ware, San Diego, CA) was used for the statistical analysis and IC_{50} value calculations.

3 | RESULTS AND DISCUSSION

3.1 | LC-MS/MS analyses of the extracts

In the current study, chemical compositions of the extracts prepared from fruit juices of eight different pomegranate cultures from Turkey were determined by LC-MS/MS and the results were given in Table 1. According to the results, fumaric acid, pyrogallol, gallic acid, ascorbic acid, chlorogenic acid, epicatechin, epigallocatechin, pelargonidin chloride, cyanidin chloride, cyanidin-3-O-glucoside, callistephin, and delphinidin-3-O-glucoside were detected in all eight extracts. Pelargonin chloride was present in seven extracts except for P6, whereas procyanidin B2 and punicalagin were found in all extracts except P5. P1 was found to be richest in terms of pyrogallol, epigallocatechin gallate, pelargonidin chloride, and callistephin. P2 is remarkably richer than other extracts in fumaric acid, besides its gallic acid, and chlorogenic acid contents are the highest among the eight extracts. P3 is the extract with an abundance of delphinidin-3-O-glucoside, also it contains a high amount of pelargonin chloride. P7, is another extract with rich phytochemical constituents in it, speaking of which, it is the richest extract in terms of ascorbic acid, epigallocatechin, cyanidin chloride, cyanidin-3-O-glucoside, and punicalagin. Worth taking a look at, P7 extract is considered to require special attention since it has the highest polyphenolic content and the highest anthocyanin content in all studies extracts. It also has significant amounts of fumaric acid, pelargonidin chloride, callistephin, delphinidin-3-O-glucoside.

In a previous study that was designed to investigate the organic acids and some phenolic compounds of 13 different pomegranate varieties from Turkey, the determined major phenolic compounds in the fruit juice extracts were catechin, quercetin, phloridzin, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, *o*- and *p*-coumaric acids (Poyrazoglu et al., 2002). In another study, 11 standard pomegranate cultivars from Turkey were selected and their phenolic compounds were identified by HPLC. Accordingly, gallic acid, catechin, chlorogenic acid, caffeic acid, ferulic acid, and *o*- and *p*-coumaric acids were found to be present in the extracts. Same as our study, gallic acid and chlorogenic acid were detected in the three cultivars that were studied in both studies (23, Izmir 1513, and Katırbaşı Ekşi) (Gundogdu & Yilmaz, 2012). The first report about the analysis of anthocyanin profiles of nine different fruit juice extracts from Turkey by HPLC was published in 2013. 3-glucoside and 3,5-diglucoside of delphinidin, cyanidin, and pelargonidin were found to be present in the extracts (Turkyilmaz, 2013). Similar to the results of the current study, Izmir 1513 extract was found to be the richest extract in terms of anthocyanins.

In a nutshell, anthocyanins are the most important compounds that originate mostly from the arils of pomegranate and contribute to the red color directly. Since customers tend to choose the fruits with brighter red color, mainly the red-color rich, and therefore the anthocyanin-rich pomegranate is stipulated by the industry. The concentrations of anthocyanin in different pomegranate cultivars

are dependent on environmental conditions, incidentally, low temperatures enhance anthocyanin accumulation and high temperatures simply reduce the concentration (Borochov-Neori et al., 2011). The current study provides promising data about anthocyanin-rich pomegranate cultivars from Turkey that may have economic value in today's industry.

3.2 | Total phenolic content (TPC) and total flavonoid content (TFC)

Total phenolic and flavonoid contents of the juice extracts prepared from eight different pomegranate cultivars from Turkey were determined as gallic acid (GAE), and catechin (CE) equivalents respectively. The results were given in Table 2. According to the results, the P3 (23) extract was found to be the richest in terms of TPC. As well as having the highest TPC, P3 also had the highest TFC among eight extracts. TPC and TFC of P1 (3) extract were also found to be noteworthy. Moreover, as shown in Table 2, the ranking of pomegranate extracts based on their TPCs were detected as P3 > P1 > P2 > P7 > P4 > P5 > P6 > P8, and the ranking of the extracts based on their TFCs were found as P3 > P1 > P7 > P2 > P4>P5 > P6 > P8.

In order to appraise the characterization of pomegranate cultivars, TPC is undoubtedly a critical parameter that is considered as a hallmark of their antioxidant capacities that were linked to their health-promoting activities (Djeridane et al., 2006). Up to date, a wide range of research about the TPCs and TFCs of different pomegranate cultivars from Turkey and other countries has been launched. Incidentally, the studies were conducted on different parts of the pomegranate including the peel, the seed, juice, and the whole fruit. The TPCs and TFCs of those parts of the plant were found to be remarkably different from each other, and amongst those parts, the peels were shown to possess significant superior TPCs and TFCs compared to other parts of pomegranate (Bassiri-Jahromi & Doostkam, 2019). Since the current study is a comparative evaluation of TPC and TFC values of only the juice extracts prepared from different cultivars, it would be more appropriate to compare the obtained results to other studies carried out with the juice extracts, as well. To give an example, Cam et al. investigated the antioxidant capacities of juices prepared from eight different cultivars from Turkey, and the TPCs were determined to vary between the range of 208.3–343.6 mg CEs/100 ml juice respectively (Cam et al., 2009). Therewithal, six other cultivars from Turkey were evaluated for their TPCs and the average TPC was calculated as 1507 mg GAE/L (Ozgen et al., 2008). As there is an increasing demand for commercial pomegranate juices because of their purported benefits for human health, many different brands have appeared in the market recently, and to investigate their phytochemical contents and antioxidant capacities, a study was carried out in Turkey. Reportedly, six of the seven juices studied were found to exhibit high TPCs, which ranged from 2,602 to 10,086 mg GAE/L (Tezcan et al., 2009). As mentioned before, higher total phenolic concentrates were correlated with the taste of the cultivars, and the growing area is the main factor affecting the TPC of the plant (Middha et al., 2013).

3.3 | Antioxidant activity

Since the oxidation aspects can vary in the systems, it has become crucial to implement different methods for the evaluation of the antioxidant capacity of a plant (Ersoy et al., 2020). Therefore, the antioxidant activities of the juice extracts prepared from eight different pomegranate cultivars were assessed using two complementary methods, which are DPPH free radical scavenging, and FRAP assays. The results are present in Table 2. Based on the EC_{50} values, P3 extract exhibited the strongest DPPH radical scavenging activity with 0.21 ± 0.01 mg/ml. P1 extract showed appeared the most highest reducing active in FRAP activity with 127.52 ± 4.19 μM Fe^{+2} FRAP value. The ranking of pomegranate extracts based on their DPPH assay results were determined as P3 > P1 > P7 > P5 > P4>P2 > P6 > P8, and the ranking of the extracts due to their FRAP assay results were found as P1 > P3 > P7 > P2>P5 > P4 > P6 > P8.

TABLE 2 Total phenolic (TPC) and flavonoid (TFC) contents, and antioxidant activity results of the pomegranate extracts

Extracts	TPC (mg GAE/g)	TFC (mg CE/g)	EC_{50} (DPPH) (mg/ml)	FRAP[a] (μM Fe^{+2})
P1	9.20 ± 0.68^{f}	$3.75 \pm 0.36^{c,d}$	0.24 ± 0.03^{d}	$127.52 \pm 4.19^{e,g}$
P2	7.20 ± 0.48^{d}	$3.16 \pm 0.12^{b,d}$	$0.35 \pm 0.003^{c,e}$	91.37 ± 4.94^{b}
P3	11.87 ± 0.75^{e}	3.97 ± 0.11^{c}	0.21 ± 0.01^{d}	121.07 ± 2.24^{e}
P4	$6.45 \pm 0.43^{c,d}$	2.88 ± 0.28^{b}	$0.34 \pm 0.03^{b,e}$	$78.59 \pm 5.08^{c,d}$
P5	6.16 ± 0.31^{c}	2.84 ± 0.31^{b}	$0.33 \pm 0.03^{b,e}$	$86.18 \pm 5.78^{b,c}$
P6	5.20 ± 0.35^{b}	2.22 ± 0.19^{a}	0.38 ± 0.02^{b}	63.99 ± 4.29^{a}
P7	7.10 ± 0.35^{d}	$3.19 \pm 0.20^{b,d}$	0.32 ± 0.03^{e}	103.84 ± 6.00^{f}
P8	4.22 ± 0.38^{a}	1.97 ± 0.15^{a}	0.56 ± 0.05^{a}	57.97 ± 2.99^{a}
Quercetin	—	—	0.0025 ± 0.000023^{f}	129.14 ± 1.45^{g}

Note: Data are presented as the mean of three replicates ± standard deviation.

Different superscript letters in the same column indicate a significant difference ($p < .05$).

EC_{50}; the effective concentration which the antioxidant activity was 50%.

[a]FRAP values for extracts at 0.67 mg/ml, for quercetin at 0.006 mg/ml concentration.

It can be observed that the hierarchy for TPC and antioxidant capacity of the samples were found to be quite similar. It has been reported by previous studies on the antioxidant properties of pomegranate extracts that TPC values had indicated high correlation coefficients with antioxidant activity results (Cam et al., 2009). In this context, it was expected that the extracts with higher TPCs, would exert a high antioxidant activity, and the results were found to be consistent with this supposition.

Pomegranate has always been propagated as a superfood full of antioxidants. Being rich in phenolic compounds and particularly anthocyanins, it has always been associated with health-promoting benefits for the treatment of diseases related to oxidative stress, consequently, there has been mounting evidence of its antioxidant properties provided by a plethora of studies. Over and above that, pomegranate and pomegranate derived products such as pomegranate sauce have been ingredients in various foods not only to enhance the taste but also as a preservative, aiming to avoid loss of bioactive compounds (Karabiyikli & Kisla, 2012). Widely acclaimed natural antioxidants like green tea and wine were shown to possess less antioxidant properties in comparison to pomegranate, to be specific, pomegranate juices had exhibited three times stronger activity than both (Gil et al., 2000). Similar results were obtained from a study that had compared pomegranate juice to apple juice regarding their antioxidant potentials (Guo et al., 2008). Interestingly, commercial pomegranate juices exerted higher antioxidant activity compared to homemade juices, and the possible explanation for that was, the whole fruit with parts that contain antioxidant constituents such as the skin and arils were also used in the industry, but not while making homemade juices (Dżugan et al., 2018).

Even supposing the previous studies have established the antioxidant properties of pomegranate juices very well, the results of the current study have indicated that the studied cultivars, especially P1 and P3 demonstrated stronger antioxidant activities compared to other previously studied juices (Derakhshan et al., 2018; Ozgen et al., 2008; Tehranifar et al., 2010).

3.4 | Cell proliferation analysis

Cell proliferation experiments were performed using WST-1 assay. The MCF-7 and MCF-10A cell lines were treated with different concentrations (10, 25, 50, 100, and 200 μg/ml) of pomegranate juice extracts at 24th, 48th, and 72nd hours. Cell viability is represented as the percentage absorbance by comparison with untreated cells (% of the control). The effects of pomegranate juice extracts on MCF-7 and MCF-10A cell lines were given in Figure 1 and IC_{50} values of the extracts were presented in Table 3.

P7 extract was found to demonstrate the strongest cytotoxic activity on MCF-7 breast cancer cell lines by decreasing the cell viability in half at the 24th hour ($p < .05$, and IC_{50} value of P7 at 24th hour is 49.08 μg/ml). P2 was found as the second most effective extract at the 24th hour on MCF-7 cell lines, and it decreased the cell viability statistically significantly on MCF-7 cell lines from lower to higher concentrations at the 24th hour ($p < .05$). Also, P4 extract inhibited the cell proliferation at all concentrations on MCF-7 cell lines at the 24th hour ($p < .05$). P5, P6, and P8 led to MCF-7 decrease at higher doses (50–200 μg/ml) at the 24th hour. P3 extract decreased the cell viability statistically significantly of MCF-7 only at 200 μg/ml ($p < .01$). None of the studied extracts showed any statistically significant cytotoxic effect on the MCF-10A cell line at the 24th hour.

At the 48th hour, P4 exerted the strongest cytotoxic activity on MCF-7 cell lines in all concentrations ($p < .05$). P2 also decreased the cell viability of MCF-7 cell lines remarkably, especially in higher doses ($p < .0001$), and IC_{50} value of P2 extracts was calculated as 31.22 μg/ml at 48th hour. P5 extract showed statistically significant inhibition on the cell proliferation of MCF-7 cell lines from 25 μg/ml to 200 μg/ml concentrations ($p < .05$). Moreover, P1, P7, and P8 extracts decreased the cell viability of MCF-7 cell lines statistically significantly from 50 μg/ml to 200 μg/ml concentrations. Correspondingly with WST-1 data of pomegranate extracts at the 24th hour, no cytotoxic effect was detected on MCF-10A cell lines at the 48th hour.

P2 showed a statistically significant decrease in the cell viability at 200 μg/ml concentration on MCF-7 cell lines at the 72nd hour ($p < .05$). P5, P6, P7, and P8 extracts inhibited the cell viability of MCF-7 to 60% at 200 μg/ml ($p < .05$). According to the cell proliferation experiment of all extracts, no cytotoxic effect was observed on MCF-10A cell lines at the 72nd hour.

Fighting with cancer by using chemotherapeutic drugs has its limitations, such as inconsistent clinical responses, serious side effects, and drug resistance possibility. On that account, medicinal plants with multi-targeted actions and comparatively fewer side effects are ideal candidates for cancer therapeutics; and pomegranate is one of the most promising antitumorigenic plants with its scientifically proven antiproliferative and proapoptotic effects on different cancer cells. The extensive research on the anticancer potential of pomegranate has been enlightening by suggesting that it should not be seen as a chemopreventive only, but also as a chemotherapeutic agent. (Khwairakpam et al., 2018; Vlachojannis et al., 2015).

Pomegranate extracts were shown to be effective against breast, prostate, colon, liver, skin, lung, bladder, brain cancers, and also leukemia. The antioxidant properties of the plant, which have been emphasized before, have a crucial role in cancer prevention. The extracts were shown to be able to scavenge ROS and RNS, phenolic components of the extracts were also able to possess antioxidant and antigenotoxic effects. For instance, isolated punicalagin could inhibit oxidative DNA products, delphinidin, cyanidin, and pelargonidin could inhibit hydrogen peroxide-induced lipid peroxidation. In addition, punicalagin and ellagic acid demonstrated remarkable antimutagenic activities. Cyanidin was shown to be capable of reducing ROS levels in MCF-7 cell lines and inhibiting the proliferation of MCF-7 cell lines. Cyanidin-3-glucoside was found to be responsible for the repression of angiogenesis in breast cancer cell lines, by attenuating STAT3 expression via inducing miR-124, and therefore inhibiting the cytokine VEGF expression and secretion. It should be underlined that the chemopreventive effects of the juice are markedly higher than the single purified constituents,

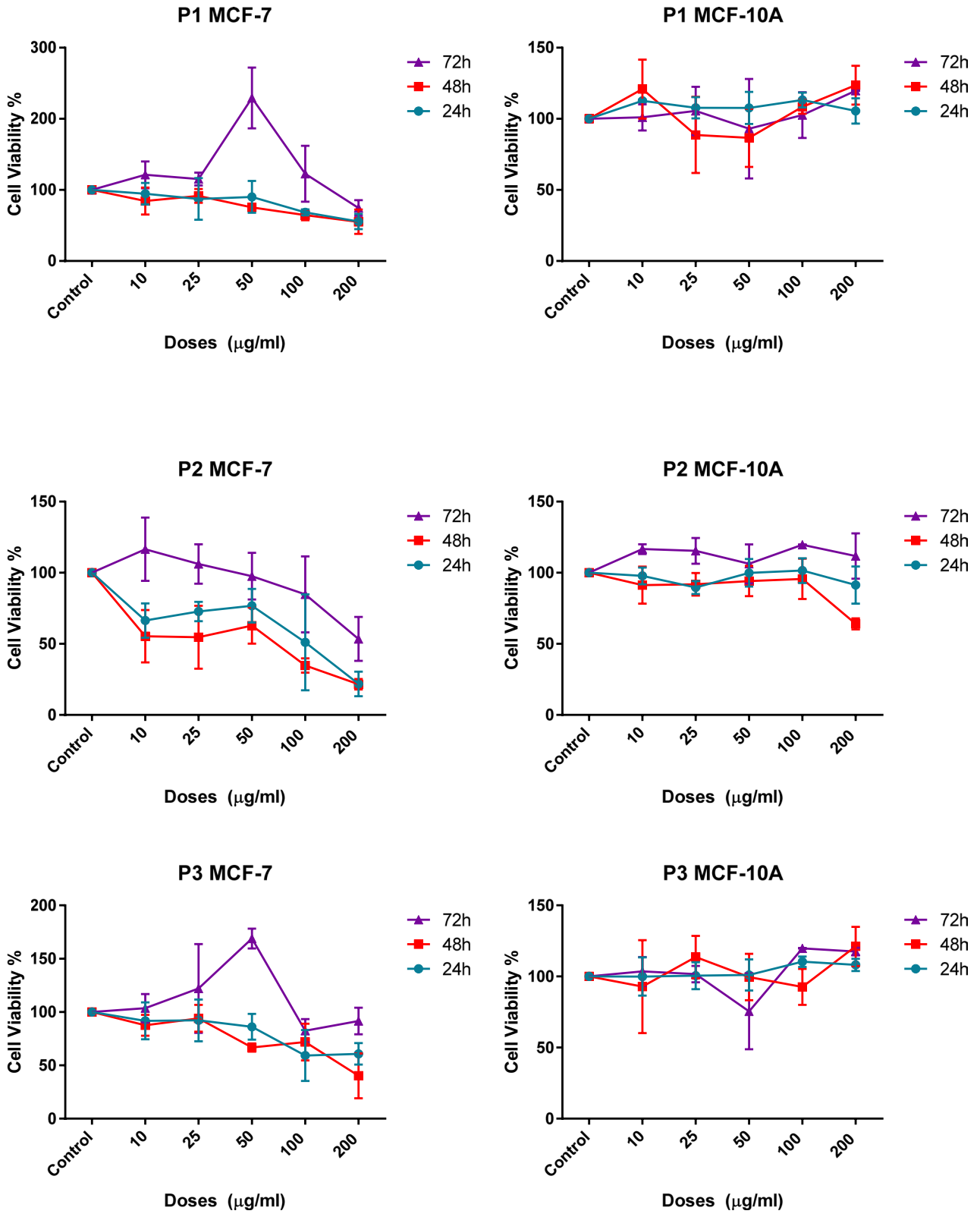


FIGURE 1 Cell viability % of the pomegranate extracts on MCF-7 and MCF-10A cell lines

probably due to the synergism between the multiple compounds in the extract (Ma & Ning, 2019; Takeuchi et al., 2011; Turrini et al., 2015). As mentioned above, P7 extract, which was rich in terms of cyanidin, cyanidin-3-O-glucoside, and punicalagin, had appeared as the strongest cytotoxic at 24 hr, and this high activity is considered to be due to these components.

Since the current study is focused on the cytotoxic effects of pomegranate on breast cancer cell lines, it is necessary to shed more light

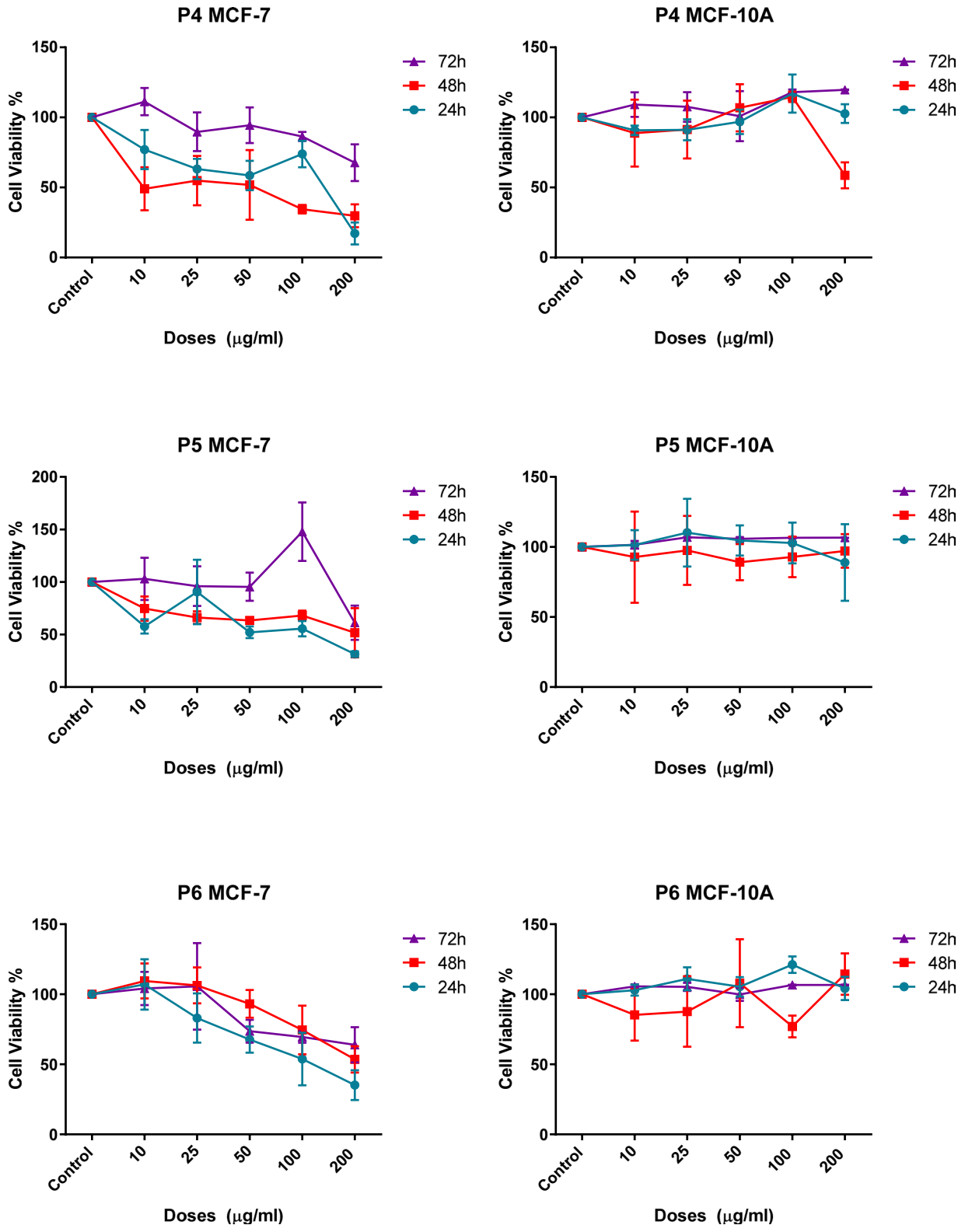


FIGURE 1 (Continued)

on the appreciable antitumorigenic activity of the plant. Pomegranate extracts were shown to target some key proteins and genes that are involved in breast cancer by modulating them. Tamoxifen is a prominent drug used for the treatment of breast cancer. Reportedly, pomegranate extracts have an affinity to bind the estrogen receptor like this drug, besides the extracts are superior to it by not affecting uterine weight (Vini & Sreeja, 2015). Pomegranate juice extracts were shown to possess the most pronounced cytotoxic effect on estrogen-responsive MCF-7 cell lines, but they were found to be not effective against MCF-10A cell lines, the results of this study are consistent in this context (Kim

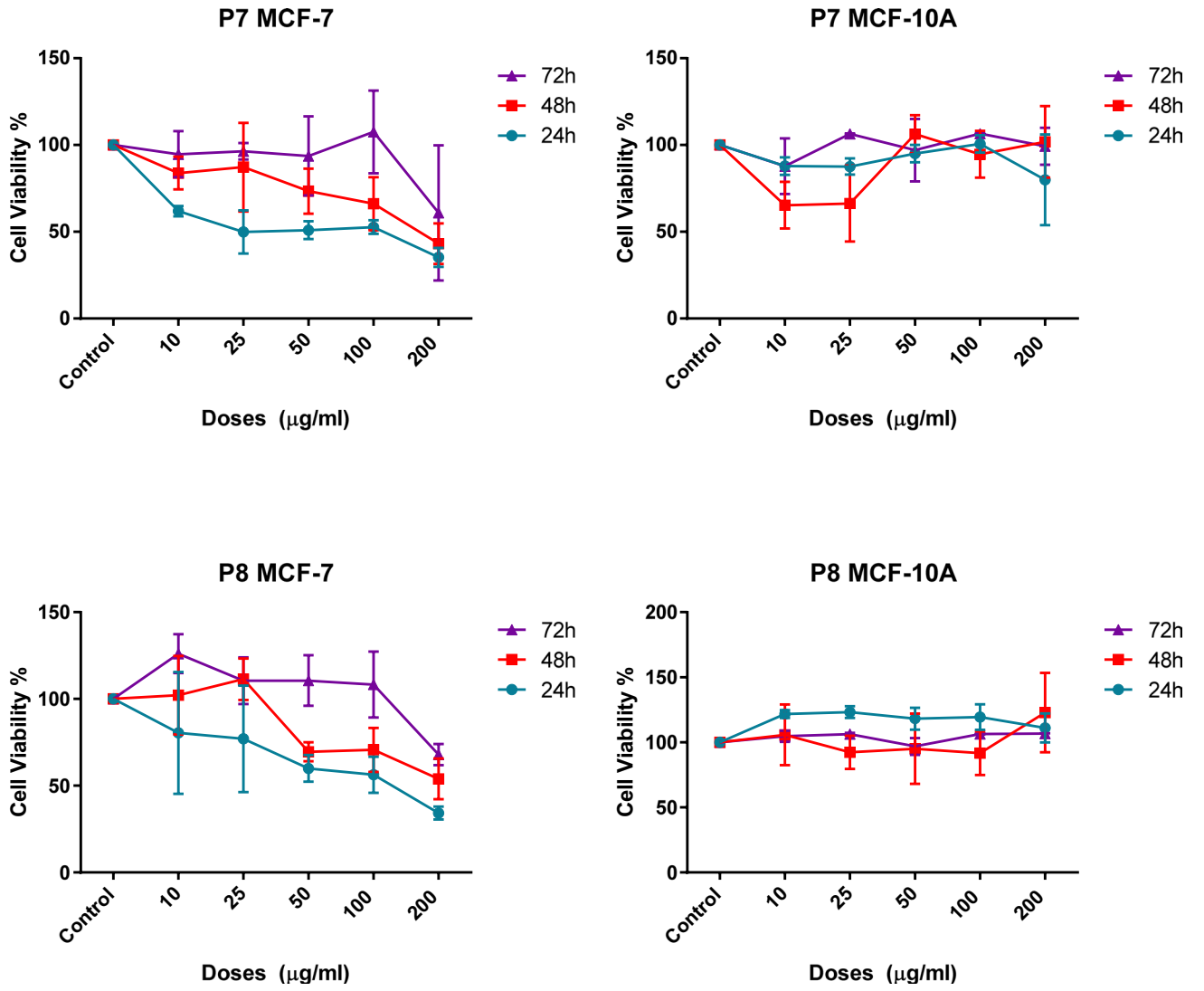


FIGURE 1 (Continued)

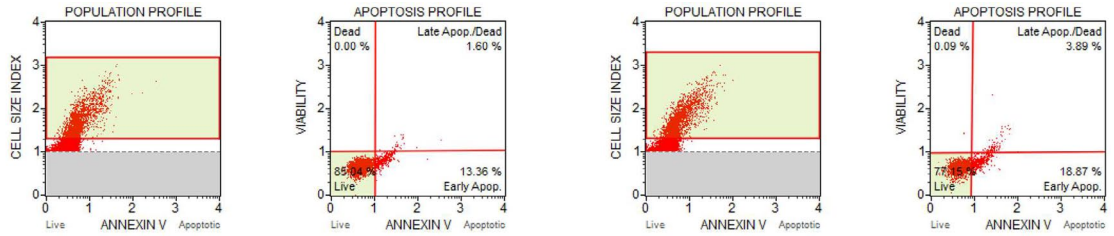
et al., 2002; Russo & Russo, 2006; Shirode et al., 2014; Toi et al., 2003). To set more examples, Rocha et al. indicated that pomegranate juice inhibited the cell growth and metastasis of MDA-MB-231 and MCF-7 breast cancer cells (Rocha et al., 2012). 13 commonly consumed fruits were evaluated for their inhibitory on nitric oxide-induced proliferation in MCF-7 cell lines, and pomegranate was found to be the richest in terms of polyphenolic compounds and able to inhibit of cell growth in MCF-7 cells (Jayakumar & Kanthimathi, 2011). Another study investigating the chemopreventive efficacy of the purified chromatographic peak of pomegranate juice and seed oil established that pomegranate juice (42%) and seed oil (87%) decreased the number of tumor lesions induced by the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) (Mehta & Lansky, 2004).

Despite the fact that there are many studies about the anticancer activity of pomegranate juice extracts on MCF-7 breast cancer cell lines, the current study provides promising results since the extracts showed significant cytotoxicity at different time intervals. The reason of the low cytotoxic effect of the extracts at the 72nd h may be attributed to the low stability of the anthocyanin compounds. In consequence, the fruits of the pomegranate or the pomegranate juice should be consumed as fresh to protect the body from the free radicals and the related diseases.

There is an evidence that pomegranate juice may alter estrogen synthesis by inhibiting the aromatase enzyme (Adams et al., 2010). Therefore, being rich in antioxidant phenolics, and being able to decrease the cell viability of MCF-7 cell lines significantly at different time intervals, these cultivars from Turkey could be beneficial for the prevention and treatment of breast cancer.

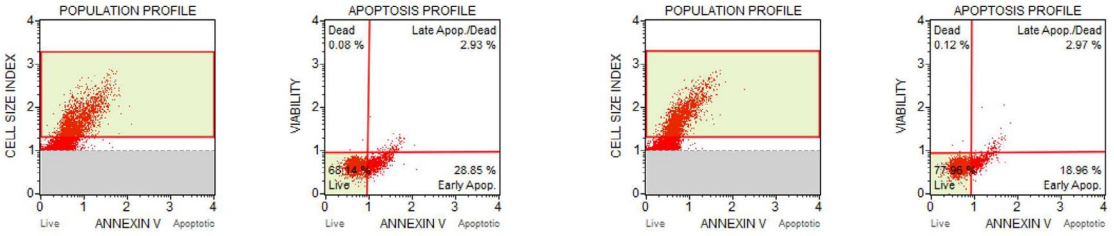
3.5 | Annexin V Apoptosis Detection Assay

The results of the apoptotic potential of the extracts were presented in Table 4. According to the results of the Annexin V assay (Figure 2), P3, P5, and P6 extracts induced cell death with 200 µg/ml and higher than 200 µg/ml doses on MCF-7 cell lines at the 24th and 48th hour. The highest levels of apoptotic death were observed at the 24th and 48th hour with about 30%. Following the P3 extract treatment of 200 µg/ml on MCF-7 cell line, 31.78% total apoptotic cells were detected at 48th hour. Application of more than 200 µg/



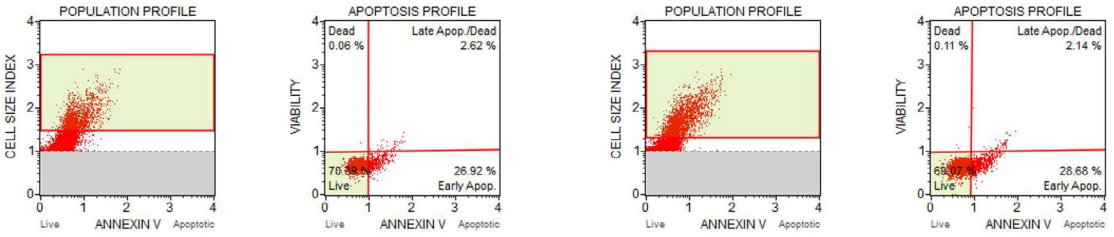
P1	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.09E+05	85.04 %
Early Apoptotic (LR) :	3.29E+04	13.36 %
Late Apop./ Dead (UR) :	3.94E+03	1.60 %
Debris (UL) :	0.00E+00	0.00 %
Total Apoptotic :	3.69E+04	14.96 %

P2	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.24E+05	77.15 %
Early Apoptotic (LR) :	5.47E+04	18.87 %
Late Apop./ Dead (UR) :	1.13E+04	3.89 %
Debris (UL) :	2.48E+02	0.09 %
Total Apoptotic :	6.60E+04	22.76 %



P3	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.75E+05	68.14 %
Early Apoptotic (LR) :	7.40E+04	28.85 %
Late Apop./ Dead (UR) :	7.51E+03	2.93 %
Debris (UL) :	2.09E+02	0.08 %
Total Apoptotic :	8.15E+04	31.78 %

P4	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.55E+05	77.96 %
Early Apoptotic (LR) :	6.21E+04	18.96 %
Late Apop./ Dead (UR) :	9.70E+03	2.97 %
Debris (UL) :	3.83E+02	0.12 %
Total Apoptotic :	7.18E+04	21.93 %

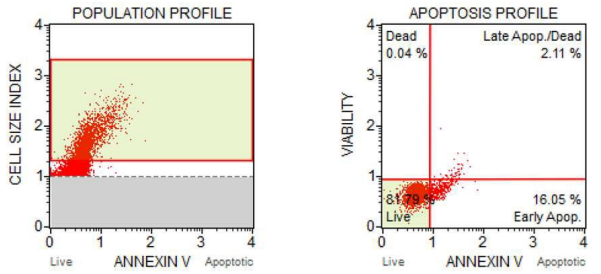


P5	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.23E+05	70.39 %
Early Apoptotic (LR) :	4.72E+04	26.92 %
Late Apop./ Dead (UR) :	4.60E+03	2.62 %
Debris (UL) :	1.09E+02	0.06 %
Total Apoptotic :	5.18E+04	29.54 %

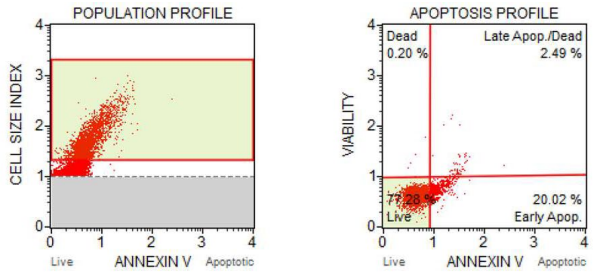
P6	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.33E+05	69.07 %
Early Apoptotic (LR) :	9.67E+04	28.68 %
Late Apop./ Dead (UR) :	7.20E+03	2.14 %
Debris (UL) :	3.79E+02	0.11 %
Total Apoptotic :	1.04E+05	30.82 %

FIGURE 2 Annexin V results of the pomegranate extracts on MCF-7 and MCF-10A cell lines

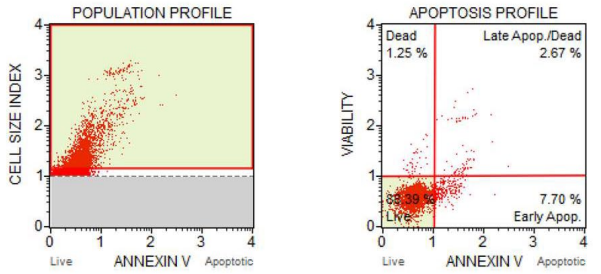
ml of P5 and P6 extracts on MCF-7 cell line showed that the extracts induced cell death with 29.54% and 30.82% at the 24th hour, respectively. Considering the results of the other extracts, around 20% total apoptotic cells were detected with treatment different doses at 24th and 48th hours. Despite P7 is the strongest cytotoxic extract, the apoptotic potential of the extract is poor. When the result is considered the other cell death mechanisms might be responsible for the cytotoxic effect of the P7 extract.



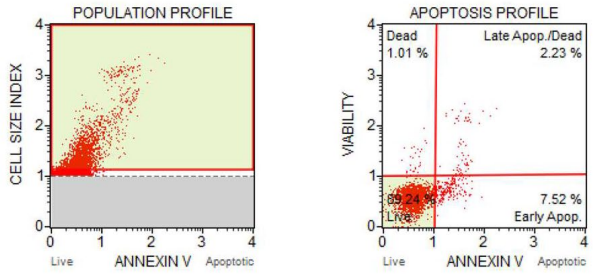
P7	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.53E+05	81.79 %
Early Apoptotic (LR) :	4.96E+04	16.05 %
Late Apop./ Dead (UR) :	6.53E+03	2.11 %
Debris (UL) :	1.33E+02	0.04 %
Total Apoptotic :	5.61E+04	18.16 %



P8	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.89E+05	77.28 %
Early Apoptotic (LR) :	7.48E+04	20.02 %
Late Apop./ Dead (UR) :	9.31E+03	2.49 %
Debris (UL) :	7.63E+02	0.20 %
Total Apoptotic :	8.41E+04	22.52 %



Control 1	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.52E+05	88.39 %
Early Apoptotic (LR) :	1.32E+04	7.70 %
Late Apop./ Dead (UR) :	4.60E+03	2.67 %
Debris (UL) :	2.15E+03	1.25 %
Total Apoptotic :	1.78E+04	10.37 %



Control 2	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.93E+05	89.24 %
Early Apoptotic (LR) :	1.63E+04	7.52 %
Late Apop./ Dead (UR) :	4.82E+03	2.23 %
Debris (UL) :	2.18E+03	1.01 %
Total Apoptotic :	2.11E+04	9.75 %

FIGURE 2 (Continued)

4 | CONCLUSION

Being one of the oldest and very commonly consumed foods in the world, pomegranate is an alluring fruit that is considered a source of national pride for many cultures, and Turkey is one of the hotspots of the pomegranate industry. Many new cultivars are being developed and extensive research is being made to determine the cultivars with richer phytochemicals, superior physical properties, and more beneficial effects for human health. Aiming to contribute to the research in this context, this study pointed out all studied cultivars were able to exert important biological activities that are attributed to their constituents. Izmir 1513 (P7) cultivar, containing biologically important compounds such as cyanidin, cyanidin-3-O-glucoside, and punicalagin, was found to attenuate the MCF-7 breast cancer cell growth at 24 hr significantly. Cultivars 3 (P1) and 23 (P3) were shown to possess comparatively higher antioxidant effects with higher TPCs and TFCs. As the search for natural antioxidant and anticancer agents from abundant, sustainable, low-cost, and environmentally friendly resources continues, pomegranate and pomegranate derived products will remain top-notch not only for the consumers but also for scientists worldwide.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

TABLE 3 IC_{50} values of the pomegranate extracts on cell viability

	MCF–7[a]			MCF–10A[a]		
Extracts	24th hr	48th hr	72nd h	24th h	48th h	72nd h
P1	245.5	263.5	Nc	Nc	Nc	Nc
P2	86.12	31.22	210.1	Nc	241.2	Nc
P3	266.3	162.5	407.3	Nc	Nc	Nc
P4	87.18	22.02	339.5	Nc	203.5	Nc
P5	93.22	484.0	Nc	Nc	Nc	Nc
P6	111.1	207.0	285.6	Nc	Nc	Nc
P7	49.08	174.5	203.2	Nc	Amb	Amb
P8	103.2	206.4	205.7	Nc	Nc	Nc

Abbreviations: Amb, ambiguous; Nc, not calculated.

[a]Values are given as μg/mL

TABLE 4 Annexin V results of the pomegranate extracts on MCF-7 cell lines

ExtractsW	Doses (μg/ml)	Time (h)	Early apoptosis (%)	Late apoptosis (%)	Total apoptosis (%)
P1	200	48th	13.36	1.60	14.96
P2	100	48th	18.87	3.89	22.76
P3	200	48th	28.85	2.93	31.78
P4	100	48th	18.96	2.97	21.93
P5	>200	24th	26.92	2.62	29.54
P6	>200	24th	28.68	2.14	30.82
P7	>200	24th	16.05	2.11	18.16
P8	>200	24th	20.02	2.49	22.51

AUTHOR CONTRIBUTIONS

Esra Eroğlu Özkan: Conceptualization; Data curation; Investigation; Methodology; Project administration; Supervision; Validation; Writing-original draft; Writing-review & editing. **Mehmet Fatih Seyhan:** Conceptualization; Data curation; Investigation; Methodology; Validation. **Ozlem Kurt Sirin:** Data curation; Investigation; Methodology; Project administration; Visualization. **Tugba Yilmaz Ozden:** Data curation; Investigation; Methodology; Validation; Writing-review & editing. **Ezgi Ersoy:** Visualization; Writing-original draft; Writing-review & editing. **Seda Damla Hatipoglu Cakmar:** Investigation; Methodology; Validation. **Ahmet Ceyhan Goren:** Investigation; Methodology; Validation. **Hulya Yilmaz Aydogan:** Investigation; Methodology; Supervision. **Oguz Ozturk:** Investigation; Methodology; Supervision.

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