Experimental Research

The In-Vitro Impact of *Punica Granutum* L. (Pomegranate) Juice on Colorectal Cancer Tumors with *TP53* and *KRAS* Mutation

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ABSTRACT

Objective: Colorectal cancer (CRC) is one of the most common human malignancies. A cure for CRC with *TP53* and *KRAS* mutations remains elusive. Thus, the development of more efficient therapeutic approaches for the treatment of these patients is required. Induction of tumor cell death by certain phytochemicals derived from medicinal herbs has become a new frontier for cancer therapy research. Although the cancer suppressive effect of *Punica granutum* L (pomegranate) juice (PGJ) has been determined in CRC, the effect of PGJ depend on mutation status has not been investigated.

Material and Method: The anti-proliferative activity of PGJ was tested in the SW480 cell line using the WST-1 assay. To determine the effect of PGJ on cell cycle and apoptosis progression in TP53 and KRAS mutated CRC, the expression levels of BIRC5, CCND1 and BCL2 were analyzed in SW480 cells using RT-qPCR.

Results: According to the obtained data, PGJ contains 8,68 ± 0,168 mg/ml ellagic acid. 4% concentration of PGJ inhibited 50% of SW480 cell proliferation in 24h incubation and induced apoptosis though decreasing BCL2 mRNA expression level.

Conclusion: The current study is the first to demonstrate the effect of PGJ on modulation of anti-apoptotic gene expression in a TP53 and KRAS mutated CRC cell line which implies the anti-tumor activity independent from p53 and K-Ras signaling pathways. Further studies and validations are required, we suggest that PGJ may be a strong candidate for studies of therapeutic cancer drugs for patients with TP53 and KRAS mutated CRC.

Keywords: *Punica granutum L. juice, TP53, KRAS, Colorectal Cancer, Apoptosis.*

ÖZET

***Punica Granutum* L. (Nar) Suyunun TP53 ve *KRAS* Mutasyonu Taşıyan Kolorektal Kanser Tümörlerindeki İn-Vitro Etkisi**

Amaç: Kolorektal kanser (KRK) insanlarda en yaygın görülen kanser türlerinden birisidir. *TP53* ve *KRAS* mutasyonları taşıyan KRK hastalarının tedavisinde hala yeterince başarı sağlanamamıştır. Bu nedenle, bu hastaların tedavisi için daha etkin terapötik yaklaşımların geliştirilmesi gerekmektedir. Günümüzde gerçekleştirilmekte olan kanser tedavisi araştırmalarında şifalı bitkilerden elde edilen bazı fitokimyasalların kanser hücrelerinin ölümünü tetiklenme yeteneği üzerinde önemle durulmaktadır. Punica granutum L (nar) suyunun kanser baskılayıcı etkisi KRK'de belirlenmesine rağmen, nar suyunun mutasyon durumuna bağlı etkisi henüz araştırılmamıştır.

Gereç ve Yöntem: WST-1 testi kullanılarak nar suyunun SW480 hücreleri üzerindeki anti-proliferatif etkisi belirlendi. Nar suyu muamelesi sonrası, RT-qPCR yöntemi ile *BIRC5*, *CCND1* ve *BCL2* genlerinin ekspresyon seviyeleri saptanarak *TP53* ve *KRAS* mutasyonlu KRK tümörlerinde nar suyunun apoptoz üzerindeki etkisi değerlendirildi.

Bulgular: İçeriğinde 8,68 ± 0,168 mg / mL ellagik asit içerdiği belirlediğimiz nar suyunun %4'lük konsantrasyonunun 24 saat inkübasyon süresinde SW480 hücre proliferasyonunu %50 oranında azalttığı ve *BCL2* mRNA ekspresyon seviyesini düşürerek apoptozu tetiklediği saptandı.

Sonuç: Mevcut çalışma, nar suyunun TP53 ve KRAS mutasyonlu KRK hücrelerinde anti-apoptotik genlerin ekspresyon seviyelerini değiştirerek TP53 ve KRAS sinyal yolaklarından bağımsız olarak anti-tümör etkisine yol açtığını gösteren ilk çalışmadır. İleri araştırmalara ve doğrulamaya gereksinim olmakla birlikte bulgularımız, nar suyunun *TP53* ve *KRAS* mutasyonlu KRK hastaları için ilaç araştırmalarına güçlü bir aday olabileceğini göstermektedir.

Anahtar Sözcükler: *Punica granutum L. suyu, TP53, KRAS, Kolorektal Kanser, Apoptoz.*

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Colorectal cancer (CRC) is the second most common malignancy in females and the fourth most common malignancy in males in Turkey and remains the second leading cause of cancer death in advanced countries (1).

Besides environmental factors such as diet, smoking and alcohol consumption, CRC has been associated with variable heritable gene mutations such as *TP53* and *KRAS* mutations (2). *TP53* encodes the tumor suppressor protein p53. p53 regulates the cell cycle to prevent uncontrolled cell growth and proliferation. Mostly, *TP53* mutations in exons 5-8 leads to overexpression of p53 and results in CRC (3). RAS genes, particularly *HRAS*, *NRAS* and *KRAS* are among the

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most commonly mutated and critical cancer driver genes (4). *KRAS* mutations have always been responsible for enhancing malignancy and silencing them is associated with attenuation of tumorigenicity. A downstream effector of *KRAS*, PI3K/Akt signaling leads to reduction of apoptosis, stimulated cell growth and enhanced proliferation (5). *KRAS* gene is mutated in 35–40% of CRC patients, which is that, occur mostly in codons 12 and 13 of exon 2 (6). *KRAS* mutation leads to lack of benefit from anti-EGFR monoclonal antibodies in CRC (7, 8). Considering advances in the molecular biology and genetics of CRC, there is currently no effective treatment or promising molecular targeting therapy for *TP53* and *KRAS* mutated CRC tumors. For this reason, safer and more effective treatments are desperately needed for the treatment of these patients. In this context, there is great interest in dietary plants with their chemopreventive and chemotherapeutic potential (9).

Punica granutum L (pomegranate) (PG), a plant belonging to Punicaceae family, is a distinctive fruit with a medicinal history, a symbol of life, longevity and health. PG contains polyphenols that are potent antioxidants (10). The major food product made from PG fruit is its juice (PGJ), obtained either from its arils or from the whole fruit (11). The major phenolic component of PGJ is ellagic acid (12). In a recent study, the effect of ellagic acid was comperatively evaluated between CRC cell lines with *KRAS* mutation or *TP53* mutation (13). According to their findings ellagic acid demonstrated anti-tumor effect independent from either *TP53* or *KRAS* mutations (13). In this aspect, since ellagic acid the major compound of PGJ, we hypothesis that, PGJ might be a candidate natural theraupetic to overcome from CRC tumors which is that have both *TP53* and *KRAS* mutation. Thus, in the present study, we evaluated the in-vitro effect of PGJ on anti-apoptotic and cell cycle associated gene expressions in *TP53* and *KRAS* mutated CRC cell line SW480.

MATERIAL AND METHOD

Extraction of PGJ

PG was sold from market in XXX, Turkey during June-July 2014. After manual separations of the arils, small pieces of fresh red fruit of PG were cut and PGJ was obtained by squeezing out of the fruits. Next, the PGJ was centrifuged at 5000 rpm for 5 min at room temperature and then filtered by sterile syringe with filters 0.22 µm, afterwards; the juice was stored at -20°C for analysis.

Determination of the active compound in PGJ by HPLC analysis

For determination of phenolic compounds in PGJ, 32 standards of phenolic compounds were studied by HPLC method according to the literature (14). Ellagic asit was monitored at a wavelength of 320 nm. The peak was identified on the basis of a comparison of the retention time and UV spectrum with an ellagic acid standard.

Cell culture

The SW480 human Dukes' type B, colorectal adenocarcinoma cell line was provided by the American Type Culture Collection (ATCC; Rockville, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Utah, USA) supplemented with 10% fetal bovine serum (FBS, BIOCHROME, Berlin, Germany), 100 µg/ml of streptomycin, and 100 U/ml of penicillin and were incubated in a 5% CO_2 humidified incubator at 37 °C.

Determination of the effect of PGJ on cytotoxicity and cell viability of SW480 cell line

Cell proliferation kit (WST-1, Roche Applied Sciences, Mannheim, Germany) was used to evaluate the effect of different PGJ concentrations on viability of SW480 cells. Cells were seeded at 2 X 10^4/well in 96-well plates for cytotoxicity tests. After 24 h of culture in standard medium, the cells were exposed to graded concentrations of PGJ at 1 to 6% for 12 to 72 h incubation in a 5% CO_2 humidified incubator. For controls, 30 mM of H_2O_2, an inhibitor of proliferation was used as a positive control, and untreated SW480 cells were used as negative control. The standard media were used to normalize the data. All analyses were performed in triplicate. The effect of PGJ on SW480 cell viability measured calorimetrically using an ELISA microplate reader (Tecan Sunrise, Austria) at 450 nm with a reference wavelength at 620 nm. The results were expressed as a percentage of the negative (untreated) control. The absorbance of the untreated control cells was set to 100%, and the absorbance of PGJ treated cells was measured as the surviving percentage as described in our previous study (14).

Evaluation of the Effect of PGJ on Cell Cycle and Apoptosis associated mRNA Expression Profiles of SW480 Cells

mRNA expression profiling was performed to evaluate the apoptotic effect of PGJ on SW480 cells. Cells were seeded at 3x10^5/well in 6-well plates. After 24 h of culture in standart medium, the cells were exposed to 4% PGJ. Untreated SW480 cells were used as a negative control over a 24-h incubation in a humidified incubator.

Total RNA was extracted after 24 h of incubation using TRIzol Reagent (Invitrogen, Carlsbad, USA), treated with DNase I and reverse transcribed using a cDNA synthesis kit (New England Biolabs, UK). The samples were then analyzed using RT-qPCR to profile the *BIRC5* (NM_001168), *CCND1* (NM_053056), *TP53* (NM_000546), and *BCL2* (NM_000633) expression levels according to the standard protocol of RT^2 qPCR Primer Assays using Light Cycler 480II real-time PCR system. The expression level of the human *ACTB* (NM_001101) and *GAPDH* (NM_002046) were evaluated as housekeeping genes. mRNA expression analyses were quadruplicated for each sample. Only sam-

ples with Ct values less than 35 were included in further analyses. Genomic DNA contamination was analyzed by performing a no reverse transcription control with RNA samples using an ACTB RT-qPCR primer assay. The initial copy number in the samples and threshold cycle (Ct) for mRNA expression was determined using the Light Cycler 480II software (Roche Diagnostics, Indianapolis, USA). The $2^{-\Delta Ct}$ method was used to calculate the fold change in mRNA expression between the tested samples (15).

Statistical analysis

Depending on PGJ exposure, one-way ANOVA and Tukey's analyses were performed to analyze the viability of SW480 cells. Independent sample T test was used to determine the statistical significance of the changes in *BIRC5*, *CCND1* and *BCL2* expressions. SPSS 16.00 software was used for calculation of one-way ANOVA. A two-tailed p value of ≤ 0.05 in 95% CI was considered as significant. RT^2 Profiler PCR Array Data Analysis software was used for evaluation of mRNA expression levels. *P* values of < 0.05 and/or Fold change values of >2 and <-2 were determined to be statistically significant (15).

PGJ Inhibits SW480 Cell Proliferation

The amount of ellagic acid was calculated as 8,68 ± 0,168 mg/ml (n =2) in standardized PGL according to HPLC/DAD analyses. In addition; 32 standards of phenolic compounds were studied for determination of the other phenolic compounds in PGJ. According to HPLC analysis, although the similar UV-VIS spectrum of ellagic acid was shown for some peaks, due to there was no standard of phenolic compound such as ellagitannin unfortunately, the peak could not identify.

SW480 cells were seeded at a density of $2x10^4$cells/well in 96-well plates. Cell proliferation was assessed using the WST-1 assay after 12–72 h of exposure to PGL doses ranging from 1 to 6%. SW480 cells exhibited reduced cell numbers in a dose- and time-dependent manner (Table 1, Table 2, Figure 1).The inhibitory concentration at which 50% of the cells died within 12 and 24 h were identified (IC50). The percentage decrease in the proliferation of SW480 was 51.74% at 5% PGL concentration in 12 h (p <0.0001) and 50.24% at 4% PGL concentration in 24 h (p <0.0001, Table 1, Figure 1).

RESULTS

Table 1. *Dose dependent inhibitor effect of PGJ on cell viability.*

		Compare to (-) control (Untreated sample)					Compare to (+) control (H_2O_2)				
					95% CI					95% CI	
Time	PGJ dose	Mean Difference	Std. Error	P value	Lower Bound	Upper Bound	Mean Difference	Std. Error	P value	Lower Bound	Upper Bound
12h	1%	0.57	0.02	<0.0001	0.51019	0.63696	-1.51	0.02	<0.0001	-1.57664	-1.44986
	2%	0.72	0.02	<0.0001	0.65326	0.78004	-0.94	0.02	<0.0001	-1.00306	-0.87629
	3%	0.75	0.02	<0.0001	0.69121	0.81799	-0.80	0.02	<0.0001	-0.85999	-0.73321
	4%	0.83	0.02	<0.0001	0.76371	0.89049	-0.76	0.02	<0.0001	-0.82204	-0.69526
	5%	0.92	0.02	<0.0001	0.85396	0.98074	-0.69	0.02	<0.0001	-0.74954	-0.62276
	6%	0.94	0.02	<0.0001	0.88091	1.00769	-0.60	0.02	<0.0001	-0.65929	-0.53251
24h	1%	0.70	0.01	<0.0001	0.65807	0.73498	-1.55	0.01	<0.0001	-1.58375	-1.50685
	2%	0.77	0.01	<0.0001	0.72855	0.80545	-0.85	0.01	<0.0001	-0.88723	-0.81032
	3%	0.83	0.01	<0.0001	0.79230	0.86920	-0.78	0.01	<0.0001	-0.81675	-0.73985
	4%	0.91	0.01	<0.0001	0.87642	0.95333	-0.71	0.01	<0.0001	-0.75300	-0.67610
	5%	1.03	0.01	<0.0001	0.99315	1.07005	-0.63	0.01	<0.0001	-0.66888	-0.59197
	6%	1.10	0.01	<0.0001	1.06162	1.13853	-0.51	0.01	<0.0001	-0.55215	-0.47525
48h	1%	1.17	0.03	<0.0001	1.07985	1.25115	-1.55	0.03	<0.0001	-1.63660	-1.46530
	2%	1.18	0.03	<0.0001	1.09027	1.26158	-0.39	0.03	<0.0001	-0.47110	-0.29980
	3%	1.22	0.03	<0.0001	1.13760	1.30890	-0.38	0.03	<0.0001	-0.46068	-0.28937
	4%	1.27	0.03	<0.0001	1.18397	1.35528	-0.33	0.03	<0.0001	-0.41335	-0.24205
	5%	1.30	0.03	<0.0001	1.21862	1.38993	-0.28	0.03	<0.0001	-0.36698	-0.19567
	6%	1.34	0.03	<0.0001	1.25292	1.42423	-0.25	0.03	<0.0001	-0.33233	-0.16102
72h	1%	1.26	0.02	<0.0001	1.20304	1.32486	-1.60	0.02	<0.0001	-1.66328	-1.54147
	2%	1.39	0.02	<0.0001	1.33307	1.45488	-0.34	0.02	<0.0001	-0.39933	-0.27752
	3%	1.41	0.02	<0.0001	1.34854	1.47036	-0.21	0.02	<0.0001	-0.26931	-0.14749
	4%	1.44	0.02	<0.0001	1.38377	1.50558	-0.19	0.02	<0.0001	-0.25383	-0.13202
	5%	1.45	0.02	<0.0001	1.39169	1.51351	-0.16	0.02	<0.0001	-0.21861	-0.09679
	6%	1.46	0.02	<0.0001	1.39537	1.51718	-0.15	0.02	<0.0001	-0.21068	-0.08887

**P vaues evaluated using using one-way ANOVA and Tukey's test.*

Table 2. *Time dependent inhibitor effect of PGJ on cell viability.*

		(-) Control (Untreated)			(+) Control (H_2O_2)		
Time comparison		12h-24h	12h-48h	12h-72h	12h-24h	12h-48h	12h-72h
Mean Difference		-0.05	-0.09	-0.16	-0.02	-0.05	-0.07
Std. Error		0.02	0.02	0.02	0.01	0.01	0.01
P Value		0.049	0.001	<0.0001	0.691	0.016	0.001
95% CI	Lower Bound	-0.09580	-0.13780	-0.20980	-0.05865	-0.09500	-0.11558
	Upper Bound	-0.00020	-0.04220	-0.11420	0.02675	-0.00960	-0.03017
			1% PGJ			2% PGJ	
Time comparison		12h-24h	12h-48h	12h-72h	12h-24h	12h-48h	12h-72h
Mean Difference		0.07	0.50	0.53	0.00	0.37	0.52
Std. Error		0.02	0.02	0.02	0.03	0.03	0.03
P Value		1.000	0.020	<0.0001	0.060	0.052	0.002
95% CI	Lower Bound	0.02183	0.44881	0.47526	-0.09854	0.26838	0.41443
	Upper Bound	0.12807	0.55504	0.58149	0.10324	0.47017	0.61622
			3% PGJ			4% PGJ	
Time comparison		12h-24h	12h-48h	12h-72h	12h-24h	12h-48h	12h-72h
Mean Difference		0.03	0.38	0.49	0.04	0.35	0.46
Std. Error		0.02	0.02	0.02	0.01	0.01	0.01
P Value		0.344	<0.0001	<0.0001	0.048	<0.0001	<0.0001
95% CI	Lower Bound	-0.01974	0.33076	0.44496	-0.00024	0.31251	0.41556
	Upper Bound	0.07604	0.42654	0.54074	0.07979	0.39254	0.49559
			5% PGJ			6% PGJ	
Time comparison		12h-24h	12h-48h	12h-72h	12h-24h	12h-48h	12h-72h
Mean Difference		0.07	0.30	0.37	0.11	0.30	0.35
Std. Error		0.02	0.02	0.02	0.00	0.00	0.00
P Value		0.042	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
95% CI	Lower Bound	-0.00716	0.22352	0.29984	0.09385	0.29035	0.33605
	Upper Bound	0.13966	0.37033	0.44666	0.12170	0.31820	0.36390

**P vacues evaluated using using one-way ANOVA and Tukey's test.*

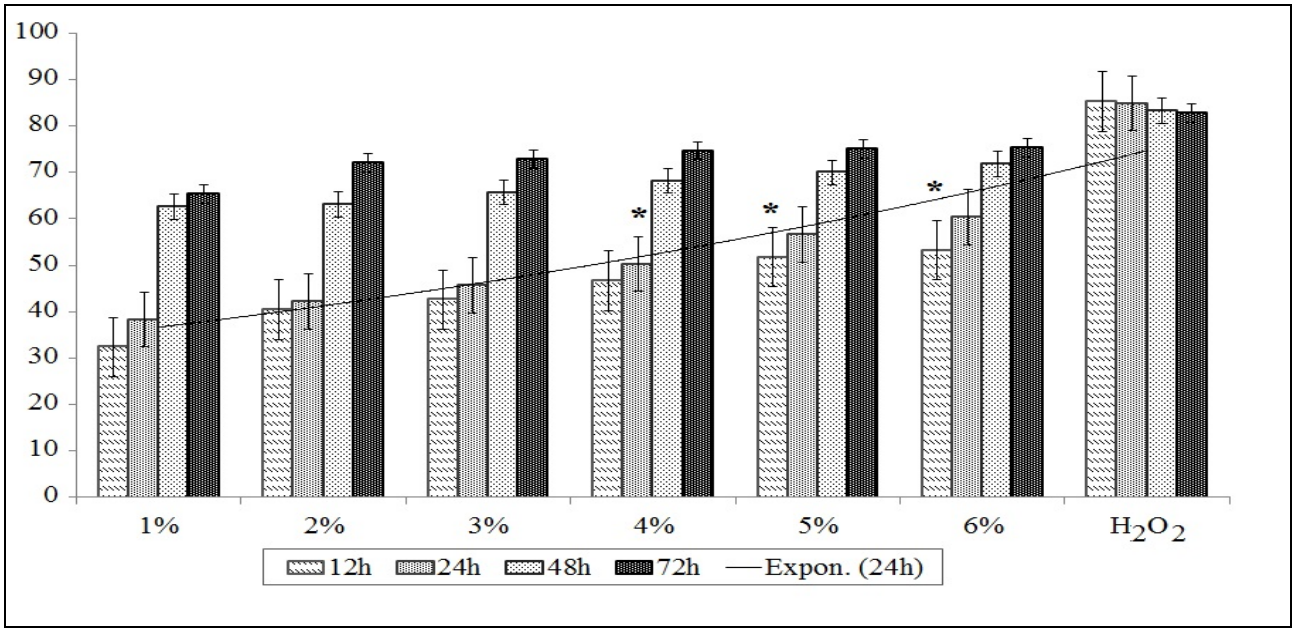


Figure 1. *Inhibition of cell viability at different concentrations of PGJ. *P < 0.05; Evaluated using one-way ANOVA and Tukey's tests using SPSS 16.00 software for Windows (IBM, Chicago, IL).*

When SW480 cells were treated with H_2O_2, we observed 85.35% and 84.86% reduction in proliferation in 12 and 24 h respectively.

The role of PGJ in the modulation of mRNA expressions in SW480 cells

The optimal activity of PGJ was determined to occur at concentration of 4% on 24 h of incubation. Therefore, we evaluated the effect of 4% PGJ on cell cycle and apoptosis associated mRNA expressions in SW480 cells. The expression levels of *BIRC5*, *CCND1* and *BCL2* were evaluated in SW480 line treated with 4% PGJ. The results from untreated SW480 samples were compared to those obtained from cells treated with 4% PGJ. According to independent sample T test; there were no statistically significant *P* value. However, the expression of BCL2 was down regulated (3.8 fold) after treatment with 4% PGJ (p =0.736; Table 3, Figure 2).

Table 3. *Differential expression of mRNAs in SW480 cells in the presence or absence of 4% PGJ.*

	BIRC5	*CCND1*	*BCL2*
Untreated SW480			
2^(-Avg.(Delta(Ct))	0.10083	0.277392	0.449845
SW480 + 4% PG			
2^(-Avg.(Delta(Ct))	0.0856	0.1505	0.116933
Fold Change	0.85	0.54	0.26
95% CI	0.00001; 2.83	0.00001; 1.03	0.00001; 1.08
**p*	0.718	0.542	0.736

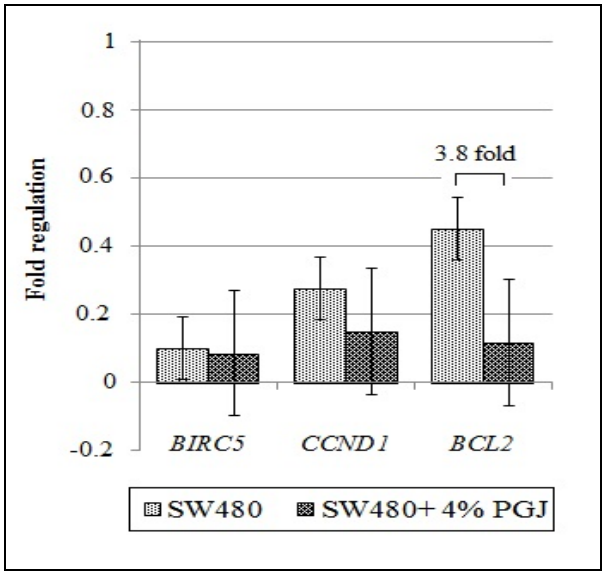


Figure 2. *Changes in mRNA expression levels in SW480 cells after 24 h 4% PGJ treatment. Evaluated with independent sample T-tests using RT² Profiler PCR Array Data Analysis.*

There were no significant fold differences in *BIRC5* (1.17 fold; p =0.718) and *CCND1* (1.84 fold; p =0.542) expression between untreated and 4% PGK treated SW480 cells.

DISCUSSION

The pomegranate fruit possesses therapeutically important constituents. Almost all parts of pomegranate serve as repository for biologically active constituents, which can cure wide variety of disease such as tissue inflammation, cancer, diabetes, skin diseases, bleeding disorders and cardiovascular diseases (16-24). The major food product made from pomegranate fruit is its juice, obtained either from its arils or from the whole fruit (25). Pomegranate fruit is a rich source of polyphenols such as the flavonoid and gallo- and ellagitannin classes. The ellagitannins represent a significant portion of PGJ polyphenols and coexist with the major product of hydrolysis of this class of tannins, ellagic acid. A number of health-beneficial effects manifested by PGJ consumption are attributed to the presence of ellagic acid (26, 27). Yousef et al. (13) evaluated the inhibitor effect of ellagic acid in a concentration a range of 0 to 200% on cell proliferation of CaCo-2 and HCT-116 colorectal cancer cell lines and they demonstrated the IC50 of ellagic acid was 200 µg/ ml at 24h and 100 µg/ ml at 48 h for both of CaCo-2 and HCT-116 cells similarly. In the present study we determined 8,68 ± 0,168 mg/ml ellagic acid as the major component of PGJ and we evaluated the anti-proliferative effect of PGJ between a concentration range of 1 to 6% for 12 to 72h in SW480 cells. SW480 cells differ from Caco-2 and HCT-116 with their genetic background regarding *TP53* and *K-RAS* mutation status (28). The p53 transcription factor regulates the expression of genes with central roles in cellular processes including DNA repair, cell cycle, and apoptosis. Thus, mutations in *TP53* confer significant oncogenic functions and promote metastasis and resistance to anticancer therapy (29). In addition, *KRAS* activating mutations in exon 2 and exon 3 avoid the sufficient therapy with EGFR inhibitors (30−32). CaCo-2 cells have mutation in *TP53* gene (E204X) and HCT-116 cells have mutations in *K-RAS* gene (G13D). SW480 cells have mutations in both *TP53* (R273H; P309S) and *KRAS* (G12V) genes (28). Thus, SW480 cells are types of colorectal tumors which are more resistant to current medical therapies in compare to CaCo-2 and HCT-116 cells. According to present findings, we defied the IC50 of PGJ in concentration of 4% for SW480 cells in 24h incubation. 4% concentration of PGJ (~217 µg/mL ellagic acid) is similar to IC50 concentration of ellagic acid for CaCo-2 and HCT-116 in the study of Yousef et al. (13). In this aspect, similar to the effect of ellagic acid on CaCo-2 and HCT-116 cells, PGJ showed an anti-proliferative effect on SW480 cells independent from the *TP53* or *K-RAS* mutation status.

The main purpose of cancer therapy is to target proliferating cells to induce cellular death pathways. The p53 protein is a transcription factor, which can induce apoptosis by regulating the pro-apoptotic and anti-apoptotic genes. The ability of p53 to promote cell death could be directly linked to its tumor suppressive

function. Development of certain tumors in p53 null mice was associated with decreased cell death rather than increased cell cycle progression (33, 34). Bcl-2, an anti-apoptotic protein, is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins (35). Bcl-2 promotor contains a p53-negative response element, raising the possibility that Bcl-2 may be a direct target of p53-mediated transrepression (36). p53 may also directly impact Bcl-2 activity as part of a transcription-independent program of cell death. In this process, cytoplasmic p53 binds to proapoptotic Bcl-2-family proteins, leading to permeabilization of mitochondria and apoptosis (37-39). Structural studies have demonstrated that the DNA-binding domain of p53 is required for direct p53–Bcl-2 interaction (40, 41). Thus, TP53 mutations causes' impaired Bcl-2 interaction though impaired DNA binding (42). In the study of Bishayee et al. (43), PE dose-dependently suppressed cell proliferation and induced apoptosis in mammary tumors though increasing Bax and decreasing Bcl2 protein expressions. However, they did not evaluate the p53 mutation status of mammary tumors. In the present study, PGJ caused 3.8 fold decreases in the regulation of *BCL2* mRNA expression in SW480 cells (p =0.736). Similar to SW480 cell line, HT-29 cell line is a colorectal cancer cell line with a mutation in *TP53* and in an EGFR pathway gene, *BRAF* mutation (28). In the preliminary studies of Banerjee et al. (44) in HT-29 cells, PG induced apoptosis and increased the expression of microRNA-126. microRNAs (miRNAs) are small non-coding RNAs of 18–25 nucleotides in length that bind to complementary UTR regions of target mRNAs, regulating the transcriptional activity of the target gene (45). Variable dietary factors, including micronutrients and non-nutrient dietary components, have been shown to alter gene expression via modulating miRNA (46). According to findings of Bishayee et al. (43), PG involve in regulation of VCAM-1 and PI3K/AKT-mTOR pathways via modulating miR-126 in HT-29 colon cancer cells. Besides, miR-126 not only targets these signaling pathways. According to microRNA and mirSNP databases, while *BCL2* is also a directly target of miR-126, *TP53* is not targeting by this miRNA (Figure 3).

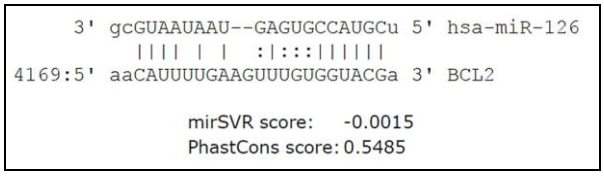


Figure 3. *miR-126 binding sites in 3'UTR of BCL2 gene (www.microRNA.org).*

This data imply that, PGJ may cause reduction in *BCL2* mRNA level though modulating miR-126 level, independent from *TP53* status.

Survivin, which encoded by *BIRC5* gene, is also a target of p53 for its action and downregulation and p53 may induce apoptosis by antagonizing the anti-apoptotic activity of survivin. Survivin inhibits caspases and blocks apoptosis and is expressed highly at G2/M phase and declines rapidly in G1 phase of cell cycle (47, 48). G1 phase of cell cycle requires Cyclin D1 protein which encoded by *CCND1* gene to dimerize with CDK4/6 and regulate the G1/S phase transition (49). Rocha et al. (50) demonstrated that, p53 indirectly involve in regulation of Cyclin D1. Kasimsetty et al. (51) demonstrated the inhibitor effect of PG on HT-29 cell line, mediated through cell cycle arrest in the G0/G1 and G2/M stages of the cell cycle followed by induction of apoptosis. However, in the present study, although we determined a reduction in *CCND1* and *BIRC5* expression level after PGJ treatment, the fold differences were negligible (1.84 fold; p =0.542 and 1.17 fold; p =0.718; respectively). HT-29 cells consist of a *TP53* mutation (R273H) and a *BRAF* (V600E) mutation instead of a *KRAS* mutation, which is another down-stream gene of EGFR signaling pathway (28). According to study of Pek et al. (52), in CRC tumors with *KRAS* or *BRAF* mutations, CDK4/6 and MAPK co-regulated gene set is highly enriched and targeting this KRAS-associated gene signature with Cdk4/6 and MEK inhibitors efficiently inhibited CRC growth and elicited apoptosis in *KRAS*-dependent and *BRAF*-mutant CRC. Kasimsetty et al. (51), demonstrated the cell cycle arrest in the G0/G1 and G2/M stages after PG treatment using a flow cytometric cell cycle analysis, but they didn't evaluated the molecular background of this effect. Whereby we did not demonstrate any significant alteration in the expression level of *CCND1* and *BIRC5* genes, PGJ may target Cdk4 or Cdk6 but not Cyclin D1. In addition, PGJ may not be directly effect on *BIRC5* expression since it is a direct target of *TP53*, which is mutated in these cell lines.

In conclusion, our observations and previous studies suggest that modulation of gene expressions may be an important mechanism underlying the biological effects of PGJ. PGJ may target specific genes though modulating miRNA expressions. Advance studies are required to define the effect of PGJ on these miRNAs. Moreover; to elucidate the molecular mechanism of this effect; beside ellagic acid; the role of other phenolic compounds of PGJ on regulation on these miRNA expressions need to be analyzed. Our results provide evidence that PGJ can induce apoptosis with reducing *BCL2* gene expression independently from *TP53* mutation status, suggesting a new mechanism of action for this extract. To the best of our knowledge, this is the first time that the pro-apoptotic capability of PGJ has been demonstrated in a both *KRAS* and *TP53* mutated CRC cell line which may contribute to the development of a treatment for drug resistant CRC due to *KRAS* and *TP53* mutations.

Conflicts of Interest: The authors declare that they have no conflict of interest.

REFERENCES

1. Turkish Ministry of Health. 1999; http://www.saglik.gov.tr
2. Yan WF, Wu G, Sun PC, Qiu D. P53 mutations occur more commonly than KRAS mutations in colorectal adenoma. Int J Clin Exp Med 2015; 15: 1370-5.
3. Iacopetta B. TP53 mutation in colorectal cancer. Hum Mutat 2003; 21: 271-6.
4. Neumann J, Zeindl-Eberhart E, Kirchner T, Jung A. Frequency and type of KRAS mutations in routine diagnostic analysis of metastatic colorectal cancer. Pathol Res Pract 2009; 205: 858-62.
5. Danielsen SA, Eide PW, Nesbakken A, Guren T, Leithe E, Lothe RA. Portrait of the PI3K/AKT pathway in colorectal cancer. Biochim Biophys Acta 2015; 1855: 104-21.
6. Bos JL, Fearon ER, Hamilton SR, et al. Prevalence of ras gene mutations in human colorectal cancers. Nature 1987; 3: 293-7.
7. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008; 23: 1757-65.
8. De Roock W, De Vriendt V, Normanno N, Ciardiello F, Tejpar S. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. Lancet Oncol 2011; 12: 594-603.
9. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer. 2003; 3: 768-80.
10. Shirode AB, Bharali DJ, Nallanthighal S, Coon JK, Mousa SA, Reliene R. Nanoencapsulation of pomegranate bioactive compounds for breast cancer chemoprevention. Int J Nanomedicine 2015; 10: 475-84.
11. Kalaycıoğlu Z, Erim FB. Total phenolic contents, antioxidant activities, and bioactive ingredients of juices from pomegranate cultivars worldwide. Food Chem 2017; 15: 496-507.
12. Lansky EP. Beware of pomegranates bearing 40% ellagic acid. J Med Food 2006; 9: 119-22.
13. Yousef AI, El-Masry OS, Abdel Mohsen MA. Impact of cellular genetic make-up on colorectal cancer cell lines response to ellagic acid: implications of small ınterfering RNA. Asian Pac J Cancer Prev 2016; 17: 743-8.
14. Tezcan G, Tunca B, Bekar A, et al. Olea europaea leaf extract improves the treatment response of GBM stem cells by modulating miRNA expression. Am J Cancer Res 2014; 6: 572-90.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta DeltaC (T)) Method. Methods 2001; 25: 402-8.
16. Forouzanfar F, Afkhami Goli A, Asadpour E, Ghorbani A, Sadeghnia HR. Protective effect of Punica granatum L. against serum/glucose deprivation-induced PC12 cells injury. Evid Based Complement Alternat Med 2013; 2013: 716730. doi: 10.1155/2013/716730.
17. Middha SK, Usha T, Pande V. A review on antihyperglycemic and antihepatoprotective activity of ecofriendly Punica granatum peel waste. Evid Based Complement Alternat Med 2013; 2013: 656172. doi: 10.1155/2013/656172.
18. Raafat K, Samy W. Amelioration of diabetes and painful diabetic neuropathy by Punica granatum L. extract and its spray dried biopolymeric dispersions. Evid Based Complement Alternat Med 2014; 2014: 180495. doi: 10.1155/2014/180495.
19. Howell AB, Souza DHD. The pomegranate: effects on bacteria and viruses that influence human health. Evid Based Complement Alternat Med 2013; 2013: 606212. doi: 10.1155/2013/606212.
20. Colombo E, Sangiovanni E, DellAgli M. A review on the antiinflammatory activity of pomegranate in the gastrointestinal tract. Evid Based Complement Alternat Med 2013; 2013: 247145. doi: 10.1155/2013/247145.
21. Aviram M, Rosenblat M. Pomegranate protection against cardiovascular diseases. Evid Based Complement Alternat Med 2012; 2012: 382763. doi: 10.1155/2012/382763.
22. Bhatia D, Thoppil RJ, Mandal A, Samtani KA, Darvesh AS, Bishayee A. Pomegranate bioactive constituents suppress cell proliferation and induce apoptosis in an experimental model of hepatocellular carcinoma: role of Wnt/β-catenin signaling pathway. Evid Based Complement Alternat Med 2013; 2013: 371813. doi: 10.1155/2013/371813.
23. Vlachojannis C, Zimmermann BF, Chrubasik-Hausmann S. Efficacy and safety of pomegranate medicinal products for cancer. Evid Based Complement Alternat Med 2015; 2015: 258598. doi: 10.1155/2015/258598.
24. Panth N, Manandhar B, Paudel KR. Anticancer activity of punica granatum (Pomegranate): A review. Phytother Res 2017; 31: 568-78.
25. Lei F, Zhang XN, Wang W, et al. Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice. Int J Obes 2007; 31: 1023-9.
26. Larrosa M, Tomás-Barberán FA, Espín JC. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using mitochondrial pathway. J Nutr Biochem 2006; 17: 611-25.
27. Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D. Antioxidant, antimalarial, and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids of Punica granatum L. Planta Med 2007; 73: 461-67.

28. Ahmed D, Eide PW, Eilertsen IA, et al. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2013; 16; 2:e71. doi: 10.1038/oncsis.2013.35.
29. Violette S, Poulain L, Dussaulx E, et al. Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. Int J Cancer 2002; 98: 498-504.
30. Allegra CJ, Jessup JM, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 2009; 27: 2091-6.
31. Therkildsen C, Bergmann TK, Henrichsen-Schnack T, Ladelund S, Nilbert M. The predictive value of KRAS, NRAS, BRAF, PIK3CA and PTEN for anti-EGFR treatment in metastatic colorectal cancer: A systematic review and meta-analysis. Acta Oncol 2014; 53: 852-64.
32. Knickelbein K, Zhang L. Mutant KRAS as a critical determinant of the therapeutic response of colorectal cancer. Genes Dis 2015; 2: 4-12.
33. Symonds H, Krall L, Remington L, et al. p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 1994; 26; 78: 703-11.
34. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions in vivo. Cancer Cell 2002; 1: 289-98.
35. Hardwick JM, Soane L. Multiple functions of BCL-2 family proteins. Cold Spring Harb Perspect Biol 2013; 1: 5. Pii: a008722
36. Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res 1994; 15: 3131-5.
37. Moll UM, Wolff S, Speidel D, Deppert W. Transcription-independent pro-apoptotic functions of p53. Curr Opin Cell Biol 2005; 17: 631-6.
38. Erster S, Moll UM. Stress-induced p53 runs a transcription-independent death program. Biochem Biophys Res Commun 2005; 10: 843-50.
39. Talos F, Petrenko O, Mena P, Moll UM. Mitochondrially targeted p53 has tumor suppressor activities in vivo. Cancer Res 2005; 1: 9971-81.
40. Petros AM, Gunasekera A, Xu N, Olejniczak ET, Fesik SW. Defining the p53 DNA-binding domain/Bcl-x(L)-binding interface using NMR. FEBS Lett 2004; 13: 171-4.
41. Tomita Y, Marchenko N, Erster S, et al. WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. J Biol Chem 2006; 31: 8600-6.
42. Hemann MT, Lowe SW. The p53-Bcl-2 connection. Cell Death Differ 2006; 13: 1256-9.
43. Bishayee A, Mandal A, Bhattacharyya P, Bhatia D. Pomegranate exerts chemoprevention of experimentally induced mammary tumorigenesis by suppression of cell proliferation and induction of apoptosis. Nutr Cancer 2016; 68: 120-30.
44. Banerjee N, Kim H, Talcott S, Mertens-Talcott S. Pomegranate polyphenolics suppressed azoxymethane-induced colorectal aberrant crypt foci and inflammation: possible role of miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR. Carcinogenesis 2013; 34: 2814-22.
45. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-33.
46. Chen J, Xu X. Diet, epigenetic, and cancer prevention. Adv Genet 2010; 71: 237-55
47. Sah NK, Khan Z, Khan GJ, Bisen P S. Structural, functional and therapeutic biology of survivin. Cancer Lett 2006; 244: 164-71.
48. Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 2003; 22: 8581-9.
49. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev 1993; 7: 812-21.
50. Rocha S, Martin AM, Meek DW, Perkins ND. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. Mol Cell Biol 2003; 23: 4713-27.
51. Kasimsetty SG, Bialonska D, Reddy MK, Ma G, Khan SI, Ferreira D. Colon cancer chemopreventive activities of pomegranate ellagitannins and urolithins. J Agric Food Chem. 2010; 24: 2180-7.
52. Pek M, Yatim SMJM, Chen Y, et al. Oncogenic KRAS-associated gene signature defines co-targeting of CDK4/6 and MEK as a viable therapeutic strategy in colorectal cancer. Oncogene 2017; 31: 4975-86.