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Prunus avium'dan ekstrakte edilen total antosiyaninlerin genoprotektif etkisi

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ÖZ

Kiraz (Prunus avium) antosiyanin gibi immün sistem için faydalı olan flavonoidler açısından oldukça zengin bir bitkidir. Bu çalışmada amaç Prunus avium'dan ekstrakte edilen total antosiyaninlerin (TAP) in vitro antigenotoksik ve antisitotoksik etkilerini insan lenfositlerinde kromozomal anormallik (CA), tek hücre jel elektroforezi (Comet) ve Mikronükleus testleri (MN) ile değerlendirmektir. Çalışmamızda, insan lenfositlerinde mitomisin-C ve H_2O_2 ile indüklenen DNA hasarına karşı TAP'ın 50, 100, 200 and 400 µg/mL'lik konsantrasyonlarının koruyucu etkisi gözlemlenmiştir. Ayrıca, pozitif, negatif ve çözücü kontrol grupları da dahil edilmiştir. Pozitif kontrole göre TAP; 24 saatlik uygulamanın 200 ve 400 µg/mL'lik, 48 saatlik uygulamanın ise tüm konsantrasyonlarında anormal hücre yüzdesini ve CA/hücre frekansını istatistiksel olarak anlamlı bir şekilde azaltmıştır. Benzer şekilde TAP, mitotik indeksi istatistiksel olarak anlamlı bir şekilde 24 saatlik uygulamanın sadece 200 µg/mL'lik konsantrasyonunda artırırken, 48 saatlik uygulamanın ise tüm dozlarında (200 µg/mL'lik konsantrasyon hariç) artırmıştır. Bu sonuçlara paralel olarak TAP, MN frekansını da pozitif kontrole göre tüm konsantrasyonlarda istatistiksel olarak anlamlı bir şekilde azaltmıştır. Comet testinde yapılan TAP uygulamasıyla tüm dozlarda kuyruk uzunluğu, kuyruk momenti ve kuyruk yoğunluğunda da anlamlı bir azalma olduğu gözlemlenmiştir. Elde edilen bu sonuçlara göre, TAP'ın mitomisin-C ve H_2O_2 gibi genotoksik ajanlara karşı potansiyel antisitotoksik ve antigenotoksik koruyucu bir etkiye sahip olduğu gösterilmiştir.

Anahtar Kelimeler: Antosiyaninler, Prunus avium, Antigenotoksisite, Comet, Kromozomal anormallik, Mikronükleus

Genoprotective Potential of Total Anthocyanin Extracted from *Prunus avium*

ABSTRACT

Sweet cherries (Prunus avium) are rich in flavonoids such as anthocyanins that are beneficial for the immune system. The aim of the present study was to investigate in vitro antigenotoxicity and anticytotoxicity of total antochyanins extracted from Prunus avium (TAP) using by chromosomal aberration (CA), single cell gel electrophoresis (Comet) and micronucleus assay (MN) in human peripheral lymphocytes. 50, 100, 200 and 400 µg/mL concentrations of TAP was screened for its protective activity

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using mytomicin-C and H_2O_2 induced DNA damage in human lymphocytes. Also, positive, negative and solvent controls were used. According to the positive control TAP statistically decreased the abnormal cell percentage and CA/cell in 200 and 400 µg/mL at 24 h treatment and in all concentrations at 48 h. On the other hand, it was significantly increased the mitotic index in only 200 µg/mL at 24h and in all concentrations (except 200µg/mL) at 48h. Similarly, TAP were reduced MN frequency in all concentrations according to the positive control. In the comet assay, significant decreases in comet tail length, tail moment and tail intensity were observed in all concentrations. According to these results, we demonstrate that TAP has potent anticytotoxic and antigenotoxic effect against MMC and H_2O_2- induced genotoxicity.

Keywords: Anthocyahins, *Prunus avium*, Antigenotoxicity, Comet, Chromosomal aberration, Micronucleus

1. INTRODUCTION

T Sweet cherry (Prunus avium L., 2n = 2x = 16) commonly called wild cherry is classified in the Prunus genus and it is one of the most popular temperate fruits. As the main ancestor of the cultivated cherry, this fruit which can conveniently be grown in mild climate zones, has a wide usage areas such as industry of fruit juice, table consumption, jam and cake production. Also, it has a great market value and demand potential [1]. They are an marvelous resource of many substances such as nutrients and phytochemicals. Besides healty diet, they contain various phenolic compounds including hydroxycinnamate, flavonols, procyanidins and anthocyanins [2, 3, 4, 5]. Absorbsion of the sweet cherry substances in the metabolism can be easily reach to the blood [6], they can be joined cellular components, both in the plasma membrane and in the cytoplasmic matrix [7, 8].

To exert their bioactive properties, the chemical position, number, and types of substitutions of anthocyanin molecule has important role [9, 10] and the structure/function correlations also impress the intracellular localization of the pigments [11].

Anthocyanins are water-soluble vacuolar pigments that may appear red, purple, or blue depending on the pH. In addition to their coloring effects in fruits, anthocyanins recently have attracted lots of attention due to their beneficial effects, such as anti-inflammatory [12], antitumor [13] and antioxidative [14; 15] properties. Also, anthocyanin-rich treatments have exhibited favorable potencies on blood pressure and other cardiovascular risk factors [16].

Apart from the above-mentioned studies, researchers reported that feeding with anthocyanin-rich extract in rats protected against tert-butyl hydroperoxide-induced hepatic toxicity [17]. More recently, anthocyanins have been shown to be an effective chemopreventive agent against 1,2-dimethyhydrazine- and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced mammary carcinogenesis in rats [18].

Anthocyanins are well-known antioxidants, but there is so far limited evidence for the protective role of these substances against DNA damage. For this reason, the purpose of this work was to evaluate anti-genotoxic/cytotoxic effect of total anthocyanins extracted from Prunus avium using hydrogen peroxide and mytomycin C as an oxidative mutagen.

2. MATERIALS AND METHODS

2.1. Chemicals

Extraction, purification and measurement of the test substances (TAP) were determined by Demir et al. [19]. Also, researchers detected the inhibitory effect of TAP on polyphenol oxidase (PPO). IC50 value of TAP on PPO was 195.06 µg/mL. For this reason we chose the test concentrations as 400, 200, 100 and 50 µg/mL. Also, we obtained the test substances from Sakarya University, Vocational School of Pamukova.

The other chemicals which were used for genotoxicity tests: Choromosome medium B (Cas no: F 5023), Biocoll (Cas No: L6115), PBS (Cas no: L1825), Trypan Blue (Cas no: L6323) were obtained from Biochrome (Berlin, Germany). Mitomycin C (Cas no: 50-07-7), Colchicine (Cas no: 9754), Cytochalasin B (Cas no: 14930-96-2) were obtained from Sigma (St.Louis, MO, USA.). H_2O_2 (Cas no: 7722-84-1), Low-melting agarose (Cas no: 9012-36-6), Normal-melting agarose (Cas no: 9012-36-6), DMSO (Cas no: 67-68-5), Tris (Cas no: 77-86-1), EDTA (Cas no: 6381-92-6), NaOH (Cas no: 1310-73-2), Triton-x-100 (Cas no: 9002-93-1), Ethidium bromide (Cas no: 1239-48-8) were obtained from Applichem (Darmstadt, Germany).

2.2. Collection of Blood Samples

For all genotoxicity tests, peripheral venous blood was obtained from 4 healthy donors (2 male, 2 female, non-smokers, aged 20-25 years) not exposed to any drug therapy or known mutagenic agent over the past 2 years, not exposed to ionizing radiation with in the previous 6 months and with no history of chromosome fragility or recent viral infection.

2.3. Chromosomal aberration assay

Whole blood treated with an anti-coagulant (heparin) added to culture medium (chromosome medium B) containing a mitogen (phytohemagglutinin). The lymphocytes were treated for 24 and 48 hours with MMC (0.2 µg/mL) plus 50, 100, 200 and 400 µg/mL concentrations of TAP 24 hours after initiation of the cultures. A solvent (DMSO %10 v/v), a negative control and a positive control mytomycin C (MMC; 0.2 µg/mL) were also maintained in all experiments. Cultures were incubated for 72 hours and to arrest the cells in metaphase, colchicine at a final concentration of 0.06 µg/ml was added to all test tubes 2 h before the end of the incubation period. Cells were then harvested by centrifugation (1200 rpm for 10 minutes), and the pellet was treated with 0.075M of KCl for 30 minutes at 37 ^{0}C. They were centrifuged again and fixed in cold methanol/acetic acid (3:1). The fixation process was repeated three times. Slides were stained with 5% Giemsa (pH=6.8) in Sorensen buffer for 20–25 minutes, washed in distilled water, dried at room temperature and mounted with Depex.

2.4. Micronucleus assay

Heparinized whole blood samples from healthy donors were added to 2.5 mL of culture medium for the experiment. Lymphocytes incubated at 37 ^{0}C for 72 hours were treated with concentrations of TAP as the above mentioned after 24 hour from the beginning of the culture. Cytochalasin B (5.2 µg/ml) was added 28 h before the collection of the cultures to arrest cytokinesis and obtain binucleated (BN) cells. Then, cells were harvested by centrifugation (1000 rpm for 10 minutes), and the pellet was treated with hypotonic solution (0.075 M KCl) at 4 °C for 5 min and then fixed twice with cold methanol:acetic acid (3:1, v/v). In the third fixative, formaldehyde (%1) was added to preserve the cytoplasm. Slides were prepared by dropping and air-drying. Slides were stained with 5% Giemsa (pH=6.8) in Sorensen buffer for 13–15 minutes, washed in distilled water, dried at room temperature and mounted with Depex.

2.5. Comet assay in isolated human lymphocytes

The comet assay was conducted under alkaline conditions according to Singh et al. [20]. Heparinized whole blood was obtained and lymphocytes were isolated using Biocoll separating solution before the performance of the test. The trypan blue test was conducted for detenting the cell viability. The lymphocytes were incubated with H_2O_2 (3.4 µg/mL) plus 50, 100, 200 and 400 µg/mL concentrations of TAP for 1 hour at 37 ^{0}C. A Negative, solvent (DMSO %10 v/v) and positive controls (H_2O_2; 3.4 µg/mL) were also included. Following incubation, the lymphocytes were centrifuged at 3000 rpm for 5 min, and the supernatant was then drawn, and they were resuspended in PBS. 100 µl cell suspension were mixed with 150 µl low melting point agarose (0.65% in PBS) and added to microscope slides, which had been covered with a bottom layer of 0.65% agarose and immediately covered with a cover slip. Slides were lysed (pH 10; 4 °C) for at least one hour. Slides were processed using a time of alkali denaturation (300 mM NaOH, 1 mM EDTA, pH=13) of 25 min and electrophoresis (1 V/cm, 300 mA) of 20 min. After the process slides were rinsed with neutralization buffer (0.4 M Tris, pH=7.5) and 50 µL ethidium bromide (20 µg/mL) was added to stain the lymphocytes (20 µg/mL). 100 randomly selected cells stained with ethidium bromide were analyzed from each slide using a

fluorescent microscope (BAB research microscope). The tail length, tail moment and tail intensity were determined, using specialized Image Analysis System (BS 200 ProP; BAB Imaging System, Ankara, Turkey).

2.6. Slide evaluation

In CA assay, 100 metaphase plaques per donor (total, 400 metaphases per concentration) were analyzed. MI was also determined by scoring 3000 cells from each donor (total, 12 000 cells per concentration). MN were scored from total, 4000 binucleated cells per concentration. In the comet assay, slides were examined using a fluorescent microscope. Slides were prepared for each concentration of TAP. The comet parameters used for the evaluation are tail length, tail DNA% and tail moment. For this reason, 100 comets on each slide were analyzed with a specialized image analyses system to determine these parameters.

The percent reduction in MMC and H_2O_2-induced damage by TAP was calculated according to Waters et al. [21] using the following formula: % Reduction = (A–B)/(A–C)*100 where A corresponds to the damage obtained for the treatment with MMC or H_2O_2 (positive control), B is a group treated with TAP plus MMC or H_2O_2, and C is negative control.

3. RESULTS

To evaluate the anti-genotoxic/cytotoxic effect of TAP, three parameters (CA, MN and comet) were analyzed. The results reported in Table 1 show the inhibition of CAs after treatment with increasing concentrations of TAP on human lymphocytes treated with MMC. This table also presents the mitotic index (MI) obtained for each treatment, which varied 1.78–5.15%. As a positive control, MMC induced 6 types of CAs; chromatid and chromosome breaks; fragments; sister-chromatid union; dicentric chromosomes and chromatid exchange. Chromatid breaks were observed as the most common aberrations, whereas chromosome breaks and fragments were also observed respectively. Dicentric chromosome abnormality was only observed in the 48-hour MMC treatment period. Unlike, sister-chromatid union and dicentric chromosome abnormality were seen neither 24 hour nor 48 hour harvest period with TAP + MMC treatments. On the other hand, with TAP + MMC application, the abnormal cell percentage and the number of CA/Cell number were significantly lessened compared to the positive control in all concentrations and both harvest period (except 50 and 100 µg/mL in 24-hour treatment).

The simultaneous treatment of cells with TAP plus MMC showed a significant reduction of DNA damage when compared to treatment with MMC only. The reduction ranged from -0.81% to 27.67% in 24 hour treatment and from 33.68% to 56.60% in 48 hour treatment for the frequency of cells with chromosomal aberrations. However, no statistically significant reduction was observed for treatments combining lower concentrations of TAP (50 and 100 µg/mL) and MMC in 24 hour treatment.

To evaluate possible anti-clastogenic and/or aneugenic effects of the total anthocyanin extracted from *Prunus avium*, the cytokinesis-block MN assay was conducted. After the treatments with TAP+MMC, binucleated (BN) cells were counted. The results of micronuclei frequency, number of micronucleus in BN cells, standard deviation and the percentage of reduction are presented in Table 2. According to the results, TAP significantly reduced the frequency of lymphocytes with micronuclei in all concentrations. This significant reduction varied from 52.21% to 71.72% when compared to treatment with MMC only.

Table1. Anti-chromosomal aberration effect of TAP in human lymphocytes induced with MMC

Test substance	Treatment		Aberrations									
	Period	Doses	Structral									
	(h)	(µg/ml)	ctb	csb	f	cte	scu	dc	AC±S.E. (%)	CA/Cell±S.E.	Red%	MI±S.E
Positive Control	24	0,20	104	35	18	13	3	-	36.50±2.41	0.433±0.33	-	1.78±0,12
Control	24	0	15	5	5	-	-	-	5.75±1.16	0.063±0.125	-	3.68±0.17
Solvent Control	24	10%v/v	13	6	3	-	1	-	5.50±1.14	0.050±0.111	-	3.92±0.18
TAP	24	50	98	45	21	4	-	-	36.75±2.41	0.420±0.323	-0.81	1.94±0.13
		100	88	33	21	5	-	-	31.75±2.33	0.368±0.302	15.45	2.04±0.13
		200	76	27	13	4	-	-	28.75±2.26*	0.300±0.273***	26.83	2.16±0.13*
		400	73	29	13	6	-	-	28.00±2.24**	0.303±0.275***	27.67	2.08±0.13
Positive Control	48	0,20	292	133	47	54	2	1	72,50±2,23	1,330±0,033	-	1,79±0,001
Control	48	0	1	-	1	-	-	-	0,50±0,35	0,005±0,004	-	4,78±0,200
Solvent Control	48	10%v/v	3	-	-	1	-	-	1,00±0,50	0,010±0,005	-	5,15±0,200
TAP	48	50	195	59	20	48	-	-	48,25±2,50***	0,483±0,020***	33.68	2,17±0,001*
		100	171	37	21	28	-	-	42,00±2,47***	0,420±0,024***	42.36	2,14±0,001*
		200	136	27	10	28	-	-	31,75±2,33***	0,318±0,025***	56.60	2,10±0,001
		400	114	19	16	39	-	-	32,00±2,33***	0,320±0,025***	56.25	2,21±0,001*

ctb, chromatid break; csb, chromosome break; f, fragment; cte, chromatid exchange; scu, sister chromatid union; dc, dicentric, AC; abnormal cell, CA; Chromosomal aberration, Red; reduction, MI; mitotix index. 400 metaphases were scored for each treatment for CA, 12.000 metaphases were scored for each dose level for the MI.

* Significantly different from the positive control $P < 0.05$ (*z*-test).

** Significantly different from the positive control $P < 0.01$ (*z*-test).

***Significantly different from the positive control $P < 0.001$ (*z*-test).

Table 2: Frequency of micronucleus and reduction percentage in human peripheral lymphocytes treated with different concentrations of TAP + MMC

	Treatment			Distribution of BN cells according to the no. of MN				
Test substance	Peiod (h)	Doses (µg/mL)	BN cell scored	(1)	(2)	(3+)	MN/Cell (%) ± SH	Reduction (%)
Negative control	48	0.00	4000	10	-	2	0.40±0.1	-
MMC	48	0.20	4000	466	88	20	19.60±0.63	-
Solvent control	48	%10 v/v	4000	17	4	1	0.70±0.13	-
MMC+TAP	48	50	4000	261	39	7	9.00±0.45*	52.21
		100	4000	267	30	3	8.40±0.44*	58.33
		200	4000	179	18	6	5.83±0.37*	71.72
		400	4000	265	35	4	8.67±0.45*	56.93

BN; binucleated cell. * Significantly different from the positive control (MMC) P< 0.001 (z-test)

Prior the comet assay, the trypan blue exclusion test was used and cell viability numbers were higher than 98%. The results showed that the cells presented the required viability to perform the comet assay. In the comet assay, three parameters; tail length, tail intensity and tail moment were evaluated to detect anti-genotoxic effect of TAP. Based on the results presented in Table 3, cells treated by 3.4 µg/mL H2O2 during 1 hour at 37 ^{0}C, exhibited a significant increase of DNA damage as compared to the control group. Whereas co-treatment with TAP significantly reduced ($p < 0.001$) DNA fragmentation induced by H2O2 in all concentrations of three test parameters. The results also showed that TAP supported the reduction of tail length. This reduction ranged from 44.08 to 85.80% to the cultures treated with TAP and H2O2.

Table 3. Assessment of TAP on DNA damage by comet assay after *in vitro* exposure of human lymphocytes induced H_2O_2.

Test Substances	Period (h)	Doses (µg/mL)	Tail Intensity (%)	Tail Moment	Tail Length (µ)	Reduction% of Tail Length
Control	1	0	207,05±0,38	22.40±4.21	30.14±4.17	-
Solvent control	1	%10 (v/v)	210,90±0,20	16.97±0.38	27.00± 0.45	-
H_2O_2	1	3.4	235,84±0,68	502.18±18.70	520.53±18.44	-
TAP+H_2O_2	1	50	227,25±1,15*	282.55±29.32*	304.38±30.42*	44.08
		100	222,19±1,01*	105.40±12.71*	119.81±12.95*	81.71
		200	226,10±0,92*	194.07±15.06*	214.07±15.50*	62.49
		400	219,69±1,05*	82.76±12.04*	99.78±12.77*	85.80

200 comet cells were scored for each treatment. * Significantly different from positive control, $p< 0.001$ (t test)

4. DISCUSSION

Flavonoids and anthocyanins are of great interest for their radical scavenging activity. In clinical trials, anthocyanins has been found to have an anti-inflammatory and anticarcinogenic action. Due to antioxidizing capacity of anthocyanins, they prevent cardiovascular diseases, and may control obesity and diminish the action of diabetes [22]. Intake of dietary antioxidants that act as radical-scavengers is expected to be effective in preventing many diseases [23].

The antigenotoxic and anticytotoxic potentials of total anthocyanin extracted from *Prunus avium* were assessed in the present work due to their specifications aforementioned. To evaluate these effects, human peripheral lymphocytes were used as a bioindicator to perform CA, MN and the comet assay. This work is the first anti-genotoxic/cytotoxic evaluation of *Prunus avium* anthocyanins.

Ghosh et al. [24] has showed the protective effects of the anthocyanin and non-anthocyanin fractions extracted from boysenberry and blackcurrant. In this study human neuroblastoma and promyelocyte cells were exposed by hydrogen peroxide and alkaline single cell gel electrophoresis was conducted to determine the oxidatif challenge. Researchers showed that the fractions were able to preserve the cells against H_2O_2-induced toxicity effects and DNA damage. Similar to this study, Lazze et al. [11] also reported that anthocyanin (delphinidin, cyanidin) and their glycoside and rutinoside derivatives reduced DNA damage induced by tert-butyl-hydroperoxide (TBHP) in rat smooth muscle and in rat hepatoma cell lines using Comet assay. Both works show the anthocyanins are able to protect the cells against mutagenic agents such as H_2O_2 and TBHP.

The present study clearly demonstrates that tail length, tail moment and tail intensity showed significant decreases ($p<0.001$) in all concentrations when we applied TAP against to isolated human lymphocytes induced with H_2O_2. However, Heo et al. [25] studied the ethanol extract of the *Prunus persica* flowers (50–200 µg/ml) and researchers reported that the extracts showed the protection against DNA damage in the skin fibroblast cell exposed to UVB and UVC. Similarly, UVB- or UVC-induced lipid peroxidation was hindered by the extracts. Also, researchers evaluated the protective effect of the extracts against UVB-induced non-melanoma skin cancer in mice. Results showed a delay of tumor development according to the control. Similar to our datas, Lee et al. [26] demonstrated that *Prunus serrulata* var. *spontanea* blossom extracts statistically diminished DNA damage induced by H_2O_2. Additionally, 500 µg/ml concentration of *Prunus* extracts showed 38.8% inhibition in growth of human colon cancer cell line. These results are agreement with our comet assay outputs and indicated that *Prunus* extracts could provide a DNA protection as valuable bioactive materials.

Considering the cytotoxic and genotoxic aspect of antochyanins, they may be regarded as cell-friendly and many scientific studies including present study conducted different organisms and test systems to confirm their benefit. For example, anthocyanins and anthocyanidins extracted from blueberry disclosed the DNA damage protection in hepatocarcinoma HepG2 cells [27].

Several investigations have compared the antiproliferative effects of anthocyanins on normal vs. cancer cells and found that they selectively inhibit the growth of cancer cells with relatively little or no effect on the growth of normal cells [28, 29]. The mechanisms for the selective effect of the anthocyanins on the growth of cancer cells vs. normal cells is/are not known. In our study, cytotoxicity evaluated by MI and the results showed that TAP protects the cells against MMC and increased MI compared to positive control.

Schantz et al. [30] reported that anthocyanin rich bilberry extract showed significant decrease of induced DNA damage in the human colon tumor cell lines. Rocco et al. [31] evaluated the antigenotoxic activity of anthocyanin against to artificial musk and suggest that anthocyanin showed antigenotoxic effects in both *in vivo* and *in vitro* studies using *Danio rerio* and *Dicentrarchus labrax* embryonic cell line. In this work we also evaluated the protection of DNA damage against MMC in the *in vitro* chromosomal aberration test which has long been part of the test battery for genotoxicity testing. The results obtained, total anthocyanins extracted from *Prunus avium* demonstrated to reduce clastogenicity in CA.

DNA damage has a key role in cancer development. The initiating step in cancer development involves exposure to, or uptake of, carcinogens, resulting in permanent DNA damage. Micronuclei are extra nuclear fraction that come about DNA damage. Therefore, e efficient DNA damage detection and repair mechanisms are present to prevent damage becoming a permanent sequence change. Chemopreventive activities of anthocyanins on cancer revealed in *in vitro* studies included radical scavenging activity, stimulation of phase II detoxifying enzymes, reduced cell proliferation, inflammation, angiogenesis, invasiveness and induction of apoptosis and differentiation [32, 33]. Anthocyanins were shown to exhibit anti-carcinogenic effect against cancer cell and tumor types [29, 34]. In the present study, to detect the protection effects of TAP we also conducted MN test. Obtained from MN, the outputs, similar to the above mentioned studies showed that TAP cause to fall the

micronucleus frequencies in all concentrations compared to the positive control.

In conclusion, the present study demonstrates that total anthocyanin extracted from *Prunus avium* exhibit potent genoprotective activities. The observed antigenotoxicity activity of extracts could results at least in part, from their antioxidant properties. However, we believe that further studies will be required to fully investigate the *in vitro* and *in vivo* antigenotoxic activities of *Prunus avium* anthocyanin extracts in mammalian cells and animal experiments.

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