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Amelioration of subchronic acrylamide toxicity in large intestine of rats by organic dried apricot intake

Mehmet Erman ERDEMLİ[1,*], Zümrüt DOĞAN[2], Yılmaz ÇİĞREMİŞ[3], Müslüm AKGÖZ[4], Eyüp ALTINÖZ[5], Murat GEÇER[4], Yusuf TÜRKÖZ[1]

[1]Department of Medical Biochemistry, Faculty of Medicine, İnönü University, Malatya, Turkey
[2]Department of Anatomy, Faculty of Medicine, Adıyaman University, Adıyaman, Turkey
[3]Department of Medical Biology and Genetics, Faculty of Medicine, İnönü University, Malatya, Turkey
[4]Bioanalysis Laboratory, TÜBİTAK National Metrology Institute, Gebze, Kocaeli, Turkey
[5]Department of Medical Biochemistry, Faculty of Medicine, Karabük University, Karabük, Turkey

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Abstract: Acrylamide (AA) has neurotoxic, mutagenic, and genotoxic effects in humans and experimental animals. Fruit consumption is important for human health, because fruits are the source of many nutrients such as vitamins, minerals, carotenoids, dietary fiber, and phytonutrients. Many agricultural products provide natural melatonin in the diet. At the onset of the study, rats were weighted and randomly divided into four groups each containing 10 rats as follows: group 1: control (fed with normal diet and normal drinking water); group 2: apricot (fed with a daily diet with 5% apricot and normal drinking water); group 3: AA (administered daily acrylamide at 500 µg/kg b.w. via drinking water and fed a normal diet); group 4: apricot-AA (administered daily acrylamide at 500 µg/kg b.w. via drinking water and fed with a diet with 5% apricot). The diet schedule was continued for 12 weeks. At the end of the study, samples of large intestine were collected for biochemical analyses. The highest lipid peroxidation (as malondialdehyde, MDA) levels were observed in the AA groups, but MDA levels decreased significantly ($P < 0.05$) with apricot intake. Glutathione peroxidase activity in the apricot-AA group was higher than in the other three groups ($P < 0.05$). Glutathione S-transferase (GST) enzyme activity increased significantly in the AA group as compared with the other groups ($P < 0.05$). However, GST activity was significantly ($P < 0.05$) decreased by the apricot-supplemented diet. GST-Pi mRNA levels in the AA group increased significantly ($P < 0.05$) as compared with the other groups. In conclusion, the results of the current study demonstrated that AA caused large intestine damage and showed the efficiency of apricot in preventing this damage by inhibiting lipid peroxidation and improving antioxidant enzyme activities.

Key words: Apricot, acrylamide, rat, large intestine, GST-Pi, antioxidant enzymes

1. Introduction

Acrylamide (AA) is a carcinogenic chemical that causes cancer in many experimental animal organs after chronic exposure (Johnson et al., 1986). AA also has neurotoxic, mutagenic, and genotoxic effects in humans and experimental animals (Dearfield et al., 1995; LoPachin et al., 2003). Human nutrients that are prepared by cooking at high temperatures could cause high levels of AA formation. AA is a water soluble chemical and it is absorbed and distributed in animals and humans throughout the whole body. AA has been used in the industrial sector, including clarifying drinking water, treating municipal and industrial wastewaters, and in polyacrylamide gels in biotechnology laboratories since 1958 (IARC, 1995). Exposure to AA in humans may occur via ingestion, dermal contact, and inhalation of AA or AA-containing products such as processed food. AA is especially generated in carbohydrate-rich foods at 120 °C and higher by reacting with asparagines and sugar (Lindsay, 2002). The formation of AA in food involves Maillard reactions of asparagine and reducing sugars (Mottram et al., 2002). This fact is an important reason for acrylamide studies (Tareke et al., 2002)

Apricot (*Prunus armeniaca* L.) has antioxidant properties due to its flavonoid and carotenoid content (Vardi et al., 2008). Apricot is one of the most important dietary sources of carotenoids and one among the Malatya apricot varieties, Kabaası, has the highest total carotenoid content (Akin et al., 2008). Flavonoids are primarily found in vegetables, fruits, red wine, green tea, and onions (Mansuri et al., 2014). Many agriculture products provide natural melatonin in the diet. Melatonin is a direct scavenger

* Correspondence: ermanerdemli@hotmail.com

of free radicals and the most powerful known antioxidant (Nazıroğlu et al., 2012). Oxidative stress occurs in cells due to an imbalance between prooxidants and antioxidants (Nazıroğlu, 2007). Oxidative stress plays important roles in atherosclerosis, inflammation, ischemia-reperfusion injury, gene mutation, carcinogenesis, and tissue injury (Halliwell, 2014). The antioxidant defense systems involve both nonenzymatic (such as vitamin E and vitamin C) and enzymatic (such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX)) systems in intracellular and extracellular compartments (Nazıroğlu et al., 2012; Halliwell, 2014). Natural antioxidants could help the endogenous antioxidant defense system against reactive oxygen species (ROS) to protect against oxidative damage.

The aim of the current study was to investigate the protective effects of apricot on subchronic AA-induced toxicity in the large intestine of rats by measuring lipid peroxidation (as malondialdehyde, MDA) levels, glutathione S-transferase Pi (GST-Pi) gene expression, and glutathione S-transferase (GST) and GPX enzyme activities**.**

2. Materials and methods

2.1. Animals and experimental design

In this study, forty female Sprague Dawley rats (Experimental Research Department of İnönü University, Faculty of Medicine, Malatya, Turkey), weighing 200 ±20 g, were kept in individual cages for 12 weeks in a well-ventilated room with a 12-h light/dark inverted cycle (0800 and 2000 hours), under standard temperature and humidity. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals by the İnönü University Animal Ethics Committee (Protocol no: 2010/05). At the onset of the study, rats were weighed and randomly divided into four groups, 10 rats in each, as follows: group 1: control (fed with normal diet and normal drinking water); group 2: apricot (fed a daily diet with 5% apricot and normal drinking water) (Vardi et al., 2008); group 3: AA (administered daily acrylamide at 500 µg/kg of body weight (b.w.) via drinking water and fed a normal diet) (Barber et al., 2001); group 4: apricot-AA (administered daily acrylamide at 500 µg/kg of b.w. via drinking water and fed a diet with 5% apricot). The diet schedule was continued for 12 weeks. At the end of the study, animals were decapitated under xylazine and ketamine anesthesia and samples of large intestine were taken from the four groups.

2.2. Biochemical analysis

The large intestine tissues were homogenized in ice within 0.1 M Tris-HCl buffer (pH 7.5; includes protease inhibitor, phenylmethylsulfonyl fluoride, 1 mM) using a homogenizer (IKA Ultra Turrax T25 basic) at 16,000 rpm and at 4 °C for 3 min. The homogenates were used in the measurement of MDA levels and GPX and GST-Pi enzyme activities.

The MDA level was examined by using the Ohkawa method with minor modifications (Ohkawa et al., 1979). Briefly, 0.5 mL of supernatant was mixed with 3 mL of 1% H_3PO_4 and 1 mL of 0.6% thiobarbituric acid. This mixture was heated in a boiling water bath for 45 min. The mixture was extracted in 4 mL of n-butanol. The n-butanol was used as a blank and tetramethoxypropane was used as a standard. MDA levels were determined at 535 nm in a spectrophotometer and expressed as nmol/g wet tissue.

GPX activity was determined using the Paglia and Valentine method (Paglia and Valentine, 1967). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0, 5 mM ethylene diamine tetraacetic acid), NADPH (1 mM), GSH (5 mM), sodium azide (1 mM), glutathione reductase (1 IU), and a supernatant of tissues. Following a 30-min incubation at 37 °C, the reaction was initiated by the addition of H_2O_2 (1 mM) as the substrate. The absorbance values (at 340 nm) were measured by a spectrophotometer and recorded at the end of a 5-min period. The enzyme activity was given in units per gram of protein.

GST activity was determined in accordance with the Habig method (Habig et al., 1974). GST enzyme activity was determined by measuring the enzyme amount catalyzing 1 µmol of S-(2,4 dinitrophenyl)-glutathione occurring in 1 min by using GSH and 1-chloro-2,4-dinitrobenzene at 340 nm and 37 °C. The supernatants of large intestine tissues were used for determining the level of GST activity. GST (Sigma-Aldrich) was dissolved in normal saline and serial enzyme standards were prepared to have 5, 3.75, 2.5, 1.25, and 0.63 units of enzyme activity per milliliter. Each of these standards was studied as a sample and absorbance measurements were performed spectrophotometrically. Then a GST standard graphic was plotted and GST activity was measured by this graphic as mU/mg of protein. Tissue protein concentrations were then determined using the Lowry method with minor modifications (Lowry et al., 1951).

2.3. Total RNA extraction and RT-qPCR

One hundred milligrams of large intestine tissue was homogenized in 2 mL of RNA solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarkosyl, and 1% beta-mercaptoethanol) for 40 s at 13,500 rpm. The samples were stored at –80 °C until the analyses were initiated. Prior to RNA extraction, the homogenate was melted on ice and 600 µL of homogenate was transferred to another tube and centrifuged for 10 min at 18,000 rpm. The supernatant was transferred to a total RNA isolation kit filter (74104, RNeasy Mini Kit, QIAGEN, Hilden, Germany) and the manufacturer's protocol was observed.

The total extracted RNA was run on 1% agarose gel and the 18S and 28S ribosomal bands were visualized following ethidium bromide staining over a UV transilluminator. The concentration of purified total RNA was measured by a NanoDrop spectrophotometer. GST-Pi and GAPDH mRNA sequences were reverse transcribed by using the SuperScript III reverse transcriptase kit (11732-088, Invitrogen, Carlsbad, CA, USA). The manufacturer's suggested protocol was applied with minor modifications. Then 1 µg of starting total RNA and oligo dT-18 primers were used to extend all mRNAs in the first strand cDNA synthesis. GAPDH and GST-Pi primer sequences were obtained by Wang et al. and Fatemi et al. respectively (Table) (Wang et al., 2006; Fatemi et al., 2006).

Quantitative PCR was carried out with real-time PCR (Roche LC480). The PCR mixture contained 0.2 µL of cDNA, 0.2 µL of forward (100 pmol/µL) and 0.2 µL of reverse primers (100 pmol/µL), and 10 µL of SYBR Green I Master Mix solution (Roche 04707516001). Amplification was performed at 95 °C for 5 min, followed by 50 cycles of 95 °C for 30 s denaturation, 55 °C for 40 s annealing, and 72 °C for 40 s extension. All qPCRs were performed in three wells in the same plate together with the housekeeping gene. Relative mRNA expression levels of GST-Pi were calculated according to the GAPDH housekeeping gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Statistical analysis

Statistical analysis was conducted using SPSS 15 (Chicago, IL, USA). The Shapiro–Wilk test was used for the normality tests applied prior to analysis. The findings demonstrated that the data for biochemical parameters were not normally distributed ($P < 0.05$). Thus, the Kruskal–Wallis analysis of variance and the Mann–Whitney U test were performed on the biochemical parameters. The data for gene expressions were normally distributed ($P < 0.05$), and thus the differences among groups based on individual parameters were tested by an analysis of variance (ANOVA), which was followed by Tukey's post hoc multiple comparison test. Results for the biochemical parameters were expressed in terms of medians ± SEM, while results for the gene expression were expressed as mean ± SD. The significance level was set at $P < 0.05$.

3. Results

MDA level findings are presented in Figure 1. The highest MDA levels were observed in the AA groups. When an AA group was compared to the apricot and apricot-AA groups, it was observed that the MDA levels were decreased significantly by apricot ($P < 0.05$). GPX enzyme activities are presented in Figure 2. The GPX enzyme activity in the apricot-AA group was observed as higher than that in the other groups ($P < 0.05$).

Table. Primer sequences of GAPDH and GST-Pi.

Primer	Primer sequences	Amplicon size, bp
GAPDH-F	CAAATTCAACGGCACAGTCA	540
GAPDH-R	ACACATTGGGGGTAGGAACA	
GST-Pi-F	CCTCACCCTTTACCAATCTA	462
GST-Pi-R	TTCGTCCACTACTGTTTACC	

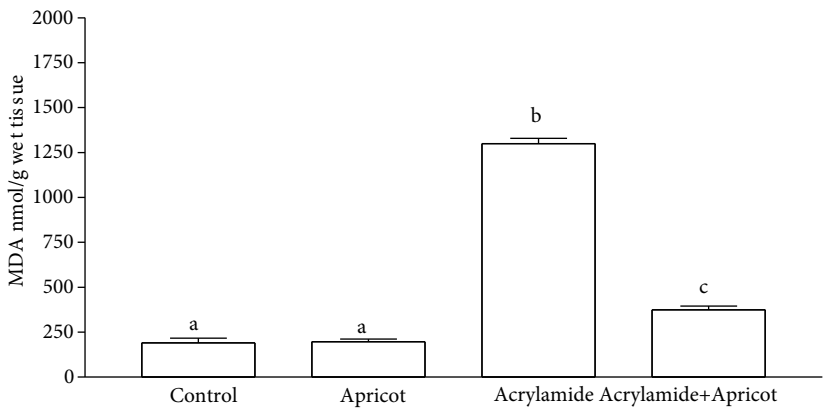

Figure 1. MDA levels in large intestine tissues. The values are shown as median ± SEM. Different lettering indicates statistically significant differences among the groups ($P < 0.05$).

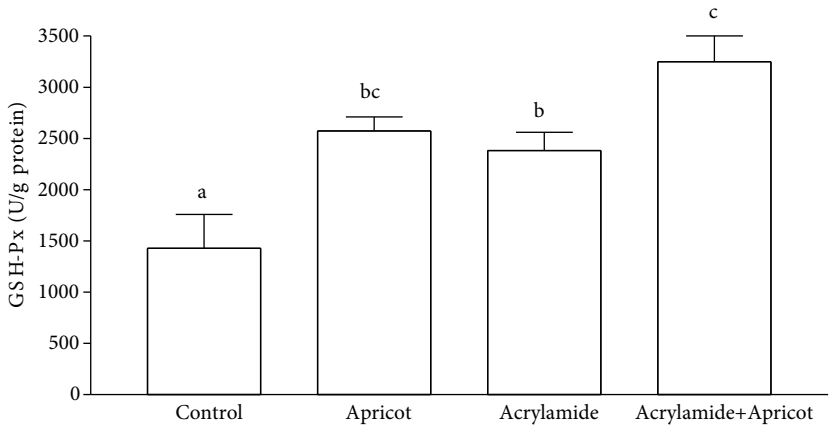

Figure 2. GSH-Px levels in large intestine tissues. The values are shown as median ± SEM. Different lettering indicates statistically significant differences among the groups ($P < 0.05$).

In addition, we observed that GST enzyme activity increased significantly in the AA group as compared with the other groups ($P < 0.05$). However, the apricot-supplemented diet decreased GST activity significantly (Figure 3).

The ratio of GST-Pi /GAPDH mRNA is shown in Figure 4 and the appearance of GAPDH and GST-Pi cDNAs in real-time PCR is shown in Figure 5. The ratio of GST-Pi / GAPDH mRNA was 0.71, 0.73, 3, and 1.5 in the control, apricot, AA, and apricot-AA groups, respectively. GST-Pi mRNA levels in the AA group increased significantly when compared with the other groups ($P < 0.05$).

4. Discussion

The aim of this study was to investigate the protective effects of apricot on subchronic acrylamide toxicity in large intestine tissues of rats by measuring the MDA, GST-Pi gene expression, and GST and GPX enzyme activities**.** The results of this study demonstrated that the administration of a 5% apricot diet prevented the oxidative damage caused by AA. The mechanism of this prevention might be associated with a decrease in oxidative stress.

Apricot is an important dietary antioxidant due to its flavonoid and carotenoid content. Flavonoids have a wide range of biological activities as polyphenolic antioxidants such as inhibition of lipid peroxidation, platelet aggregation, and capillary permeability (Ramos et al, 2005). They also inhibit the enzymes that produce superoxide anions (xanthine oxidase and protein kinase C) and ROS (cyclooxygenase, lipoxygenase) (Pietta, 2000). Carotenoids are strong antioxidants due to their lipophilicity and remarkable radical scavenging activities (El-Demerdash et al., 2004). Thus, apricot could protect cell membranes due to its ingredients that act against

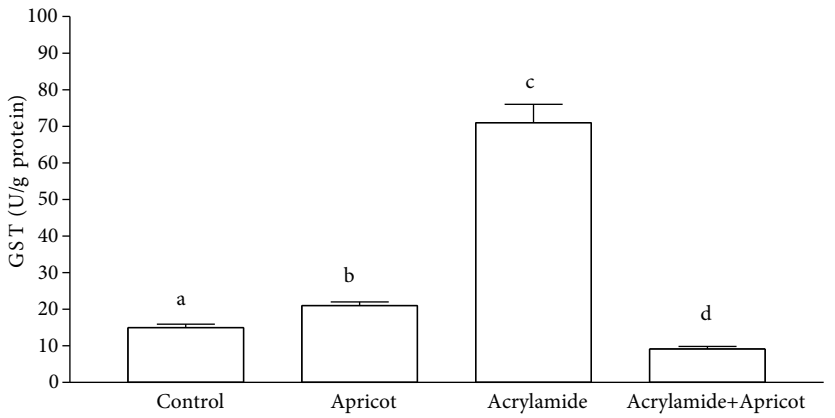

Figure 3. GST levels in large intestine tissues. The values are shown as median ± SEM. Different lettering indicates statistically significant differences among the groups ($P < 0.05$).

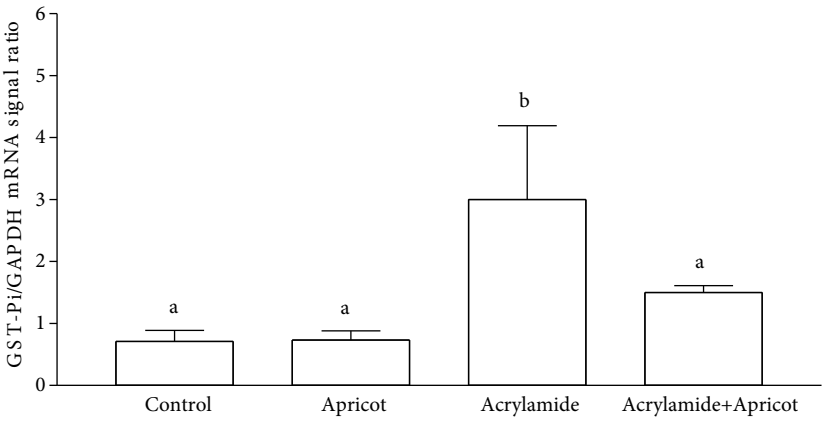

Figure 4. The GST-Pi/GAPDH mRNA ratio in large intestine tissues. The values are shown as mean ± SD. Different letters indicate statistically significant differences between the groups ($P < 0.05$).

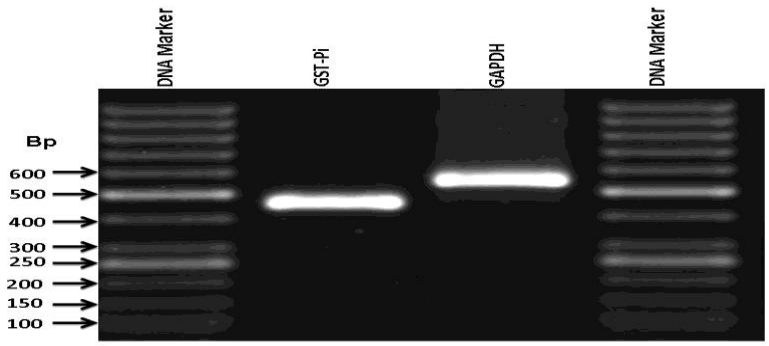

Figure 5. Agarose gel electrophoresis of the RT-PCR results of GAPDH and GST-Pi mRNA. Total RNA was extracted from rat large intestine tissues of the control group animals. RT-PCR was performed to test for the correct size of the GAPDH and GST-Pi genes. The products were loaded on a 1.5% DNA agarose gel and the size of each mRNA was determined by using a DNA marker. PCR conditions and expected sizes of PCR products were given in Section 2.

lipid peroxidation induced by AA. It was reported that a 10% apricot diet and/or β-carotene intake might prevent the deficiencies caused by oxidative stress and ameliorate methotrexate-induced intestinal oxidative damage at biochemical and histological levels in rats (Vardi et al., 2008). Our findings demonstrated that 5% apricot in the diet was capable of protecting the large intestine tissues.

The main pathway of AA detoxification is catalyzed by being conjugated with the GST/GSH system in the gastrointestinal tract including the esophagus, stomach, and the small and large intestine. Thus, the tissues of the digestive system could be protected from the toxic effects of AA (Odland et al., 1994). However, detoxification of AA in the gastrointestinal system is usually dependent on the GST activity and GSH levels in the system. After AA is taken into the body, some of it is detoxified by the GST/GSH system in the liver, and the remaining AA is oxidized by cytochrome P-450 enzymes and transformed into glycidamide, which is a genotoxic metabolite (Calleman et al., 1990). The mutagenic effects of AA on humans are less than those on animals since the formation of glycidamide is lower in the human body (Favor and Shelby, 2005). AA could interact with important cellular nucleophiles involving –SH, $–NH_2$, or –OH. Thus, it reacts with GSH to form glutathione-S-conjugates. An increased generation of S-conjugates between AA and GSH might result in decreasing GSH levels in tissues with an increase of AA concentration (Yousef and El-Demerdash, 2006). GSH and nicotinamide-adenine dinucleotide phosphate (NADPH) oxidation was coupled in the presence of the glutathione reductase enzyme (Tietze, 1969). GSH depletion is an important biomarker of oxidative stress in neuronal cells

(Nazıroğlu et al., 2013). MDA is one of the end products of lipid peroxidation that reacts with thiobarbituric acid to give a specific color absorbing at 535 nm (Ohkawa et al., 1979). In the current study, subchronic AA administration at a dose of 500 µg/kg via drinking water significantly increased the MDA levels and GST activity in the large intestine as compared with the control and apricot groups. In addition, GPX activity in the AA group was decreased as a result of AA administration as compared with the control and apricot groups. Similarly, Altinoz and Turkoz (2014) demonstrated that acute AA toxicity caused DNA damage in lymphocytes and increased MDA levels and decreased GSH levels in plasma. It was demonstrated that AA administration increased the MDA levels in different AA-treated rat tissues, corroborating the results of the current study.

Zödl et al. (2007) studied the intestinal mechanisms of acrylamide absorption and its effects on biotransformation enzymes (CYP2E1 and GST) and GSH levels. They treated cell cultures with acrylamide at different doses (0.1, 0.5, and 1 mM). They demonstrated that the transport of acrylamide in the Caco-2 cells of the intestine usually occurred via passive processes. Acrylamide caused a decrease in GSH levels but an increase in the CYP2E1 mRNA gene expression on the 14th and 21st days in parallel to the increasing acrylamide doses. In contrast, increasing acrylamide caused no effects on GSH activity. They also did not observe any changes in the *CYP2E1* gene expression at 0.1 mM acrylamide. The study argued that increased *CYP2E1* gene expression led to an increase in the generation of glycidamide and a decrease in GSH levels.

Studies have shown that GST-Pi plays a crucial role in detoxification from chemicals, and it is present in several tissues, especially in the epithelium of the urinary and gastrointestinal tracts and the respiratory system (Terrier et al., 1990). The GST-Pi expression level is important in detecting cell susceptibility to a broad spectrum of toxic chemicals (Hayes and Pulford, 1995). Previous studies have demonstrated that the GST-Pi mRNA gene expression increased significantly in hypophysis when rats were exposed to acrylamide at doses of 2.5 mg, 10 mg, and 50 mg/kg b.w. daily via drinking water (Bowyer et al., 2008). It could be stated that an increased GST-Pi mRNA gene expression in the AA group might be the reason for oxidative stress. Acrylamide-GSH complexes in particular could have led to an increase in oxidative stress by decreasing GSH storage. In addition, glycidamide, an epoxide metabolite of AA, is another mutagenic, genotoxic, and oxidant chemical. Moreover, the increase of both oxidative stress and *NF-kB* gene transcription might be the mechanism explaining how differential the gene regulation was. The NF-kB transcription factor, induced by hydrogen peroxide, cytokines, and UV radiation, is increased via oxidative stress. The results of this study are parallel to the findings of a study by Piacentini and Karliner (1999). It was determined that the administration of apricot decreased the GST-Pi mRNA gene expression, which was increased by the administration of AA. This could be due to the strong antioxidant properties of apricot, since antioxidants cause a decrease in the NF-kB transcription factor and gene expression (Wang et al., 2006).

In conclusion, this study shows that AA causes damage in the large intestines, but apricot intake is efficient in preventing this damage by inhibiting lipid peroxidation and supporting antioxidant enzyme activities. Studies on humans and animals show that the increase of the GST-Pi gene expression in tissues is related to precarcinogenic changes. In this study, the GST-Pi gene expression, GST activity, and MDA levels increased significantly in the AA group. These values were improved to levels close to the control group as a result of apricot intake. These findings demonstrated that AA caused precarcinogenic and toxic changes in large intestine tissues, which could be prevented by organic dried apricot intake.

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