Asian Journal of Chemistry; Vol. 24, No. 3 (2012), 1010-1014

ASIAN JOURNAL OF CHEMISTRY

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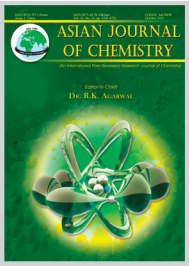

Investigating Inhibitory Effects of *Punica granatum* Fruit Extracts on Lipid Peroxidation in the Fenton Reagent Environment

AYSE DILEK OZSAHIN[1,*], OGUZ AYHAN KIRECCI[2], OKKES YILMAZ[3], YAVUZ ERDEN[3], BURAK BIRCAN[3] and ZEYNEP KARABOGA[3]

[1]Biology Department, Faculty of Science, Bitlis Eren University, 13000 Bitlis, Turkey
[2]Biology Department, Faculty of Science, Inonu University, Malatya, Turkey
[3]Biology Department, Faculty of Science, Firat University, 23169 Elazig, Turkey

*Corresponding author: Fax: +90 434 2285171; Tel: +90 434 2285170; E-mail: molekuler@gmail.com

(*Received*: 12 January 2011; *Accepted*: 1 November 2011) AJC-10582

In this study, it is aimed to find antioxidant activity of methanolic extract of *Punica granatum* fruit, by determining α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging property and also by determining the capacity to prevent the formation of lipid peroxidation which is found in *in-vitro* environment that soybean and corn oils are found. The capacity of preventing lipid peroxidation was analyzed in an *in vitro* environment that oil samples were existed by evaporating methanol phase of extracts. In the experiment, the groups were formed as control group *e.g.*, Fenton reactive, Pomegranate extract + Fenton reactive. It was observed that the lipid peroxidation level formed on the oils had increased at a significant level when compared to Fenton reactive groups according to the control group. When the extract groups are compared (to each other) according to the Fenton reactive group, it was detected that lipid peroxidation level had significantly decreased ($p < 0.0001$). According to the scavenging activity of α,α-diphenyl-β-picrylhydrazyl radical, it was determined that the pomegranate extracts were effective at their highest level at 250-500 µL ($p < 0.0001$). It was concluded that *Punica granatum* effectively decreases the lipid peroxidation level, neutralizes the α,α-diphenyl-β-picrylhydrazyl free radical and has a characteristic of conserving the fatty acid content.

Key Words: Lipid peroxidation, *Punica granatum*, Fatty acid, Antioxidant capacity.

INTRODUCTION

Free radicals are highly reactive molecules, which generally have unpaired electrons in their structure. These are constantly formed in the cell either during activation of some enzymes in the metabolism or in the course of electron leak in mitochondrial respiration. As a result of oxidation reactions, free radicals lead to important deteriorations in the structure of molecules with which they react. Many free radicals are formed during metabolic activities occurring in the cell over the course of vitality. An increase is observed in the formation of free radicals under those circumstances where antioxidant defense systems are insufficient. If the formation of these oxidant molecules dominates antioxidant molecules then cell damage and finally tissue damage occurs. Free radicals may affect any molecular group which makes up the cell. However, polyunsaturated fatty acids (PUFA) in the membrane structure are the first to be affected followed by proteins, DNA and carbohydrates respectively[1,2].

Lipids in the living system are the molecules that are affected by free radicals the most. Lipid peroxidation (LPO) occurs with the attack of $OH^{\bullet}$ radical formed in regions near the membrane to fatty acid side chains of membrane phospholipids[3]. The lipid radical generated ($L^{\bullet}$) reacts with oxygen and forms the lipid peroxyl radical ($LOO^{\bullet}$). Lipid peroxyl radical starts a chain reaction with other lipids and lipid hydroperoxides ($LOOH^{\bullet}$) are formed. These reactions taking place in the cells may cause great damages or lead to necrosis of the cell. Lipid peroxidation rate increases in cellular circumstances where Fe^{2+} and Cu^{2+} ions are present. Molecules sustaining lipid peroxidation become cytotoxic products in the end. The most well-known product among these cytotoxic materials is malondialdehyde (MDA)[4,5]. The amount of malondialdehyde is considered as the criterion for lipid peroxidation formation and it is currently measured as thiobarbituric acid reactive substances (TBARS) products.

Lipid peroxidation constitutes a problem also for production and storage phases in the food industry. Lipid peroxidation not only leads to deterioration of foods with high fat content but also causes degenerative diseases such as cancer and aging if these peroxide foods are consumed. Moreover, peroxide products lead to formation of peroxyl and hydroxyl radicals, which have mutagenic effects[6].

Punica granatum, which is widely grown in Turkey, is a fruit with antioxidant properties. This plant, homeland of which is accepted to be Iran, is rich in terms of vitamins, polysaccharides, polyphenol and minerals[7]. Three different polyphenol compounds such as delphinidin, cyanidin and pelargonidin are present in the arils of the pomegranate[8]. Moreover, existence of important chemicals in the essence and peel of pomegranate arils has drawn attention and therefore it has been claimed to be a natural antioxidant[9]. It has been detected in the recent studies that the importance of this plant has increased and pomegranate juice is antioxidant against free radicals[10]. This fruit has an economic value and it is abundantly consumed especially in winter.

It is aimed in this study to analyze the inhibitory effects of fruit extracts obtained from *Punica granatum* plant on lipid peroxidation formation *in vitro* circumstances where vegetable oils are present and to compare antioxidant characteristics of them by determining their performance of scavenging free radicals. Our main purpose is to prevent lipid peroxidation formation during production and consumption of vegetable oils which are frequently consumed in our daily lives and to develop methods for preserving food products against spoilage by using this research.

EXPERIMENTAL

Oleic acid (18:1, n 9), linoleic acid (18:2, n 6), linolenic acid (18:3, n 3), Twin 20, *Tris*-base and hydrochloride, HPLC grade methanol, acetonitrile, *n*-hexane, isopropyl alcohol, $FeCl_2$, $2H_2O$, H_2O_2, KH_2PO_4, butylated hydroxytoluene (BHT), *n*-butanol, α,α-diphenyl-β-picrylhydrazyl, (DPPH), dimethyl sulphoxide (DMSO), 2-thiobarbituric acid (TBA) and ethyl alcohol were purchased from Sigma-Aldrich.

The samples used in this research with in the boundaries of Elazig, in the coordinates of 38º 43'21" N - 39º13'19" E and at the altitude of 1270-1280 m, in the village of Gümüsbaglar, in August-September in 2008 were collected. In the creation process, maturity of the fruits were considered.

The fruits were immediately washed and frozen, to be further freeze-dried. Freeze-dried samples were maintained at -20 ºC prior to analysis. Only healthy looking fruits (without mechanical damage or bacterial infection) were selected for examination.

Preparation of the vegetable oils: For the samples of fatty acids, which were used in the experiment, soy and corn oil were used. 0.3 mL oil sample was prepared in 15 mL DMSO resolution.

Preparation of exracts: Fruit samples were homogenized at a ratio of 1:10 g/mL with methanol at a rate of 85 %. After filter of homogenates, it was centrifuged at 5000 rpm at 4 ºC for 5 min. After the centrifugation, supernatant which was in the upper part, was removed and the part of rotovapour and methanol were removed at 45 ºC under vacum. The rest were dissolved in DMSO for the usage in the experiment.

Antioxidant Assay by DPPH radical scavenging activity: The free radical scavenging effect in extracts was assessed by the decolouration of a methanolic solution of DPPH according to the method of Brand-Williams *et al.*[11]. A solution of 25 mg/L DPPH in methanol was prepared and 4 mL of this solution was mixed with 25, 50, 100, 250 and 500 µL of extract in DMSO. The reaction mixture was stored in darkness at room temperature for 0.5 h. The absorbance of the mixture was measured spectrophotometrically at 517 nm[12].

The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs control-Abs sample)]/(Abs control)] × 100
where, Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract /standard.

Antioxidative activity testing in the fatty acid environment: Antioxidative activities of the *Punica granatum* extracts were determined by the method of Shimoi *et al.*[13] with the following modifications: 1 mM $FeCl_2$ ($FeCl_2 \cdot 2H_2O$) and 3 µM hydrogen peroxide (H_2O_2) solutions were prepared freshly for every treatment, using doubly deionized water. Extracts of *Punica granatum* were also prepared freshly using DMSO. Buffer solutions were prepared with 0.2 % Twin 20, 0.05 M *Tris*-HCl-BASE and 0.15 M KCl (pH = 7.4).

In vitro experiment for both corn and soybean oil; the first group was used as a control and 0.4 mL fatty acid mixture (for corn oil; linolenic acid (18:3, LNA): 0.83 %/1 mL; 58.72 %/1 mL linoleic acid (18:2, LA) and 26.97 %/1 mL oleic acid (18:1) for soybean oil: linolenic acid (18:3, LNA): 4.9 %/1 mL; 54.85 %/1 mL linoleic acid (18:2, LA) and 22.71 %/1 mL oleic acid (18:1) was suspended in 5 mL buffer solution. The second group was a Fenton reagent group ($FeCl_2$+H_2O_2, Fenton R) and 0.4 mL fatty acid mixture 40 µM $FeCl_2$ and 60 µM H_2O_2 were suspended in 5 mL buffer solution. The third groups were Fenton's R plus *Punica granatum* (PG) extract and 0.4 mL fatty acid mixture 40 µM $FeCl_2$ and 60 µM H_2O_2 and 1 mL fruit extracts were suspended in 5 mL buffer solution. All of the mixtures were incubated at 37 ºC for 24 h. After incubation, 100 µL of 4 % (w/v) butylated hydroxytoluene (BHT) DMSO solution was added to prevent further oxidation. Then, 1 mL of each mixture was taken and 1 mL 0.6 % of 2-thiobarbituric acid (TBA) was added to the reaction mixture and incubated at 90 ºC for 40 min. Samples were allowed to cool to room temperature and the pigment produced was extracted with 3 mL of *n*-butanol. Samples were then centrifuged at 6,000 rpm for 5 min and the concentration of the upper butanol layer was measured using a HPLC-fluorescence detector.

Quantitation of oxidized products: The extent of oxidation of unsaturated fatty acids was determined by reading the fluorescence detector, which was set at (excitation) = 515 nm and (emission) = 543 nm and the amount of malondialdehyde expressed as thiobarbituric acid-reactive substances (TBARS) was calculated from a calibration curve using 1,1,3,3-tetraethoxypropane (TEP) as the standard. The MDA-TBA complex was analyzed using the HPLC equipment. The equipment consisted of a pump (LC-10 ADVP), a UV-visible detector (SPD-10AVP), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and VP software (Shimadzu, Kyoto Japan). An Inertsil ODS 3 column (15 × 4.6 mm, 5 µm) was used as the HPLC column. The column was eluted isocratically at 20 ºC with a 5 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (85:15, v/v) at a rate of 1 mL/min[14].

Stability of fatty acids: The remaining mixtures of oleate, linoleate and linolenate in the test tube were converted to methyl esters by using 2 % sulfuric acid (v/v) in methanol[15]. Fatty acid methyl ester forms were extracted with *n*-hexane. Analysis was performed in a Shimadzu GC-17A V3 instrument gas chromatograph equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm i.d. Permabond fused-silica capillary column (Macherey-Nagel, Germany). The oven temperature was programmed- 215 ºC, with an increment of 4 ºC/min. Injector and FID temperatures were 240 and 280 ºC, respectively. The nitrogen carrier gas flow was 1 mL/min. The methyl esters of oleate, linoleate and linolenate were identified by comparison with authentic external standard mixtures analyzed under the same conditions. Class GC 10 software (version 2.01) was used to process the data. The results were expressed as µmol/mL.

Statistical analysis: Statistical analysis was performed using SPSS software (version 15.0). The experimental results were reported as mean ± SEM (standard error of means). Analysis of variance (ANOVA) and an seast significant difference (LSD) test were used to compare the experimental groups with the controls.

RESULTS AND DISCUSSION

Lipid peroxidation: When the level of lipid peroxidation was analyzed in corn oil, it was detected that lipid peroxidation level significantly increased in the Fenton reagent group with respect to control group ($p < 0.0001$) (Fig. 1). When pomegranate extract groups are compared to the Fenton reagent group, it was observed that lipid peroxidation level highly decreased in extract groups ($p < 0.0001$). When control group and *Punica granatum* group were compared, a statistically significant difference was not found ($p > 0.05$).

When the level of lipid peroxidation in soybean oil was compared with respect to control group, it was observed that it increased at a very significant level ($p < 0.0001$); on the other hand, a partial increase was detected in the *Punica granatum* group ($p < 0.05$). When Fenton reagent group and *Punica granatum* group were compared, it was observed that lipid peroxidation levels in extract groups highly decreased ($p < 0.0001$) (Fig. 1).

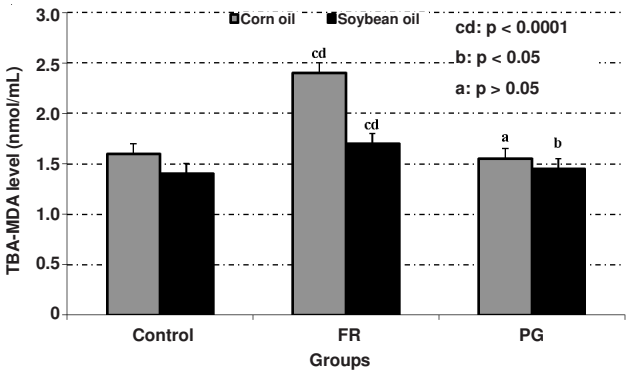

Fig. 1. Levels of MDA-TBARS in the environments of Fenton reagent with *Punica granatum* extracts

The interest in natural antioxidants in plants has increased in the recent years. Data obtained from empirical studies have asserted that plants have a great variety of antioxidant materials. Some of the phytochemicals that are antioxidant are substances such as polyphenols, monoterpenes, flavonoids, diterpenes and tannins[16].

Alkaloids, flavonoids, polyphenolic compounds and tannins present in pomegranate arils are reported to be strong antioxidants[17]. It has been detected that pomegranate fruit and all parts of it are therapeutic and in addition, its rind, roots and leaves are also medicinally important[18]. It has been suggested in general that this impact of pomegranate is originated from the ellagic acid, ellagitannins, punninic acid, flavonoids, anthocyanins, estrogenic flavonols, flavens and polyphenolic compounds it contains[19]. Poyrazoglu *et al*. have indicated in their study on pomegranate arils grown in Turkey that basic phenols of this fruit are gallic acid, chlorogenic acid, caffeic acid, coumaric acid and catechin[20]. In addition to this study, it has also been determined in other studies, which investigate phenolic content of pomegranate that phenolic compounds of this fruit have strong antioxidant effects[21,22].

Research conducted on experimental animals also concluded that this fruit has antioxidant effects. In a study conducted on rats, it was suggested that fruit extract given orally decreased the malondialdehyde level especially in the liver tissue at a significant level; however, the level of antioxidant enzymes was decreased[23]. Moreover, it has been presented in several studies that arils and outer rind of pomegranate has antioxidant, antiinflammatory and antiatheroschlorotic effects against some diseases such as prostate cancer or heart disease[24,25].

Fatty acid methyl ester: According to the fatty acids analysis results, when control group and Fenton reagent groups were compared in corn oil, it was determined that oleic acid, linoleic acid and linolenic acid levels significantly decreased in Fenton reagent group ($p < 0.0001$); however, there were not any statistically significant difference between fruit groups and control group ($p > 0.05$) (Fig. 2).

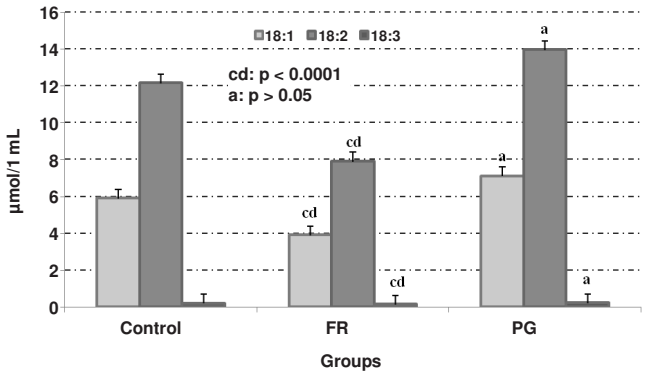

Fig. 2. Levels of linolenic acid (18:3, n-3), linoleic acid (18:2, n-6) and oleic acid (18:1,n-9) in the crop oil reaction environment (µmol/1 mL)

When the oleic acid level in soybean oil was compared with respect to control group, a very high level of decrease was detected in the Fenton reagent group ($p < 0.0001$). On the other hand, a partial decrease was observed in the level of the same fatty acid in *Punica granatum* group ($p < 0.05$) (Fig. 3).

In the present study, when fatty acid quantity was examined each of the three fatty acids in *Punica granatum* including

extracts are high compared to Fenton reagent group. So it is presumed that the flavonoids of extracts may protect unsaturated fatty acids from radical sourced oxidations. All favonoids acted as antioxidants on oxidation of methyl linoleate although the antioxidant response of kaemferol and rutin is weak. Quercetin and myricetin inhibits the hydro peroxide formation in methyl linoleate environment[26,27].

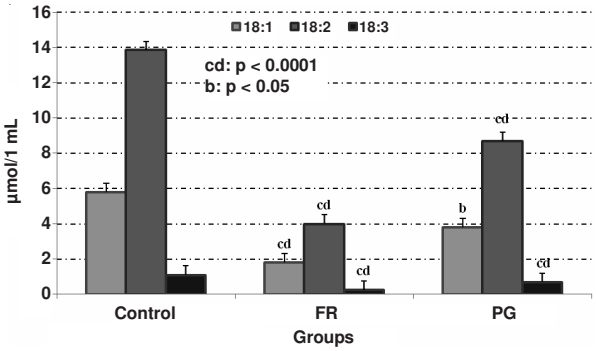

Fig. 3. Levels of linolenic acid (18:3), linoleic acid (18:2, n-6) and oleic acid (18:1, n-9) in the soybean oil reaction environment (µmol/ 1 mL)

Antioxidant activity determination: According to DPPH free radical scavenging results, it was determined that starting from 25 µL concentration of pomegranate extract, its impact increased in parallel with increasing concentration levels ($p < 0.0001$) (Fig. 4). When the radical scavenging activity of fruit extract was compared at the interval of 250-500 µL concentration, it was detected that the fruit was slightly more effective than other concentrations ($p < 0.05$).

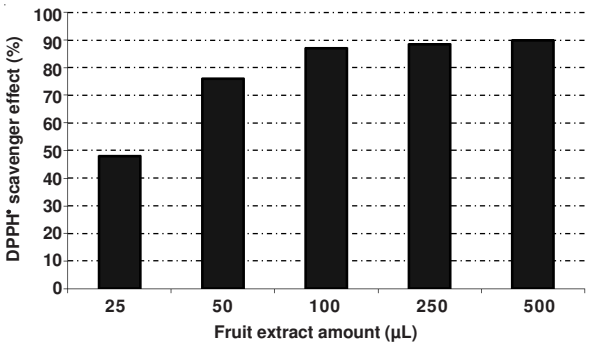

Fig. 4. DPPH• scavenger effect of *Punica granatum* extracts (%)

DPPH free radical scavenging results in soybean oil and corn oil showed that it was antioxidant in both two oil groups starting from 25 µL concentration and its antioxidant activities increased in parallel with increasing concentration levels ($p < 0.0001$). It was found that beyond 500 µL concentration, it showed antagonistic effects (Fig. 5).

Singh *et al.*[22] have determined for the first time that this fruit, especially its rind and seeds have antioxidant effects. Antioxidant activity of *Punica granatum* has been investigated in many research studies and it has been determined just as we have done in present study that this fruit has good antioxidant effect in terms of DPPH radical scavenging activity. It has been detected that this antioxidant effect originates from hydrophilic and lipophilic compounds present in the fruit[28,29].

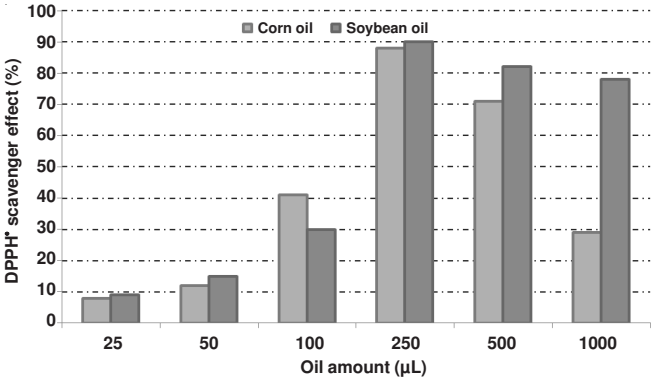

Fig. 5. DPPH• scavenger effect of corn and soybean oil (%)

In addition to the above mentioned research, it has been determined in some studies conducted on antioxidant effect of this fruit that antioxidant activity is affected by environmental conditions. Kulkarni *et al.*[9] detected that antioxidant activity and chemical content of pomegranate arils changed along the course of ripening. Mirdehghan and Rahami[30] studied the impact of seasonal changes on the phenolics and minerals in the pomegranate fruit and reported that total phenolics and minerals significantly changed along the development and ripening of the fruit; moreover, the fruit was rich in terms of bioactive compounds.

Our findings confirm that antioxidant characteristic of pomegranate fruit may change according to the environmental conditions during its growth because the fruit sample used in our study was from Elazig and it was observed that there were differences in its performance of radical scavenging and lipid peroxidation formation preventing when compared to the findings of previous studies. It was concluded that these differences might have emanated from the amount and variety of phenolic compounds present in the fruit since the ratios of vitamins, minerals and phenolics available in the fruit might differ from one region to another because of the amounts of elements present in the soil[31,32]. It can be suggested that the reason for these differences is not dependent on just one factor but related to factors such as sunlight, soil structure, seasonal differences, the area where the fruit is grown, the type of fruit and the ripening period of the fruit[33].

Conclusion

In this study, antioxidant characteristics of extracts obtained from *Punica granatum* plant, which is widely used in Turkey, on lipid peroxidation *in vitro* conditions with the presence of vegetable oils (soybean oil and corn oil) was also investigated in our study. As a result of present study, it was detected that this fruit had a significant preventive effect on lipid peroxidation *in vitro* conditions with Fenton reagent for both corn oil and soybean oil. Moreover, it was determined as a result of our study that this fruit had preventive effect on oleic, linoleic and linolenic fatty acids in soybean and corn oils that we used.

In conclusion, it has been determined that radical scavenging activity of pomegranate fruit is considerably high and it decreases lipid peroxidation formation in vegetable oils which are frequently consumed in our day to day lives. We assume that these results confirm the idea of using natural antioxidant

compounds in a safer way instead of artificial antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are used to preserve food products from deterioration, which may occur especially in vegetable oils.

ACKNOWLEDGEMENTS

This study is supported by TUBITAK's (The Scientific and Technological Research Council of Turkey) 2209-Domestic/ International Research Projects Support Program for University Students.

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