



Basic nutritional investigation

Influence of two cultivars of persimmon on atherosclerosis indices in rats fed cholesterol-containing diets: Investigation in vitro and in vivo

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ABSTRACT

Objective: To assess the influence of two persimmon cultivars on some atherosclerosis indices in rats fed cholesterol (Chol)-containing diets.

Methods: Persimmon cultivars “Fuyu” and “Jiro” as supplementation to rats’ diets were investigated in vitro to compare the contents of their bioactive compounds (polyphenols, flavonoids, flavanols, tannins, carotenoids, and ascorbic acid) and antioxidant potentials. In the in vivo investigation, 36 male Wistar rats were randomly divided into six diet groups, each with six rats: control, control/Fuyu, control/Jiro, Chol, Chol/Fuyu, and Chol/Jiro. During a period of 47 d (42 d of feeding and 5-d adaptation before the experiment) of the trial, rats in the control group were fed a basal diet and two additional control groups (control/Fuyu and control/Jiro) a basal diet plus 5% of lyophilized Fuyu and Jiro, respectively. The Chol, Chol/Fuyu, and Chol/Jiro rat groups were fed a basal diet supplemented with 1% Chol (Chol group) and 1% Chol plus 5% lyophilized Fuyu (Chol/Fuyu group) and plus 5% lyophilized Jiro (Chol/Jiro group), respectively. After completion of the experiment, the rats were anesthetized using Narcotan (halothane) and sacrificed and the atherosclerotic lesions in the aorta were assessed. The obtained results of the investigation of all six groups were compared. Testing of total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triacylglycerols, total cholesterol in the liver, electrophoretic patterns of liver tissue, and three-dimensional fluorescence of serum protein fractions was performed.

Results: The polyphenols and tannins were significantly higher in the Fuyu cultivar ($P < 0.05$). The antioxidant potential of persimmon Fuyu was higher than in the Jiro cultivar, but the difference was significant only according to the 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay ($P < 0.05$). Supplementation of diets with 5% of the lyophilized Fuyu and Jiro hindered the increase in plasma lipids versus the Chol group (total cholesterol 19.4% and 9.5%, low-density lipoprotein cholesterol 25.6% and 13.1%, respectively, $P < 0.05$) and hindered the decrease in plasma antioxidant activity versus the Chol group by 40.0% and 16.8% and by 39.6% and 11.3% for the ABTS and 1,1-diphenyl-2-picrylhydrazyl assays, respectively. The atherosclerotic lesions in the aortas of the Chol/Fuyu and Chol/Jiro groups were significantly less than in the Chol group ($P < 0.05$). Electrophoresis of the proteins from rats’ liver tissue showed changes in 14-kDa bands after persimmon supplementation. A shift in maximum wavelengths in three-dimensional fluorescence of serum protein fractions after persimmon supplementation was found in comparison with the control group and an increase in fluorescence intensity compared with the Chol groups.

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Conclusion: The contents of polyphenols and tannins were significantly higher in the Fuyu cultivar ($P < 0.05$). The antioxidant potentials of Fuyu were higher than those of the Jiro cultivar, but the difference was significant only according to the ABTS assay ($P < 0.05$). Supplementation of 5% lyophilized Fuyu and Jiro to diets of rats fed Chol-containing diets 1) hindered the increase in plasma lipids levels and the decrease in plasma antioxidant activity and 2) significantly decreased the atherosclerotic lesions in the aorta ($P < 0.05$). Electrophoretic patterns of liver tissue and fluorescence spectra can be used as additional biomarkers for determination of atherosclerosis indices.

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Introduction

We previously studied the subtropical fruit persimmon and showed that this fruit contains large quantities of bioactive compounds and possesses high antioxidant potential [1–4]. In the *in vivo* investigation, persimmon had a positive influence on the level of plasma lipids and antioxidant activity in an animal model (rats and mice) fed cholesterol (Chol)-containing diets [1,2,5]. Consumption of fruits, which have synergistic interactions of antioxidant nutrients, also improves other atherosclerosis indices [2,6–8]. The health-protective effects of these natural products are related to their phenolic compounds and to a lesser extent to their dietary fiber [8,9].

The persimmon is a fruit rich in carotenoids, which are important bioactive compounds and have a significant benefit to health [10,11]. The importance of carotenoids in synergy with other bioactive compounds such as ascorbic acid has been investigated [7,11]. Various cultivars of the same fruits have been shown to contain different quantities of bioactive compounds and possess different antioxidant potentials [10,12,13]. In our previous investigations, we did not compare cultivars of persimmon [1–3]. In the present investigation, two persimmon cultivars (“Fuyu” and “Jiro”) were investigated *in vitro* and then *in vivo*, and their influence on laboratory animals fed Chol-containing diets was assessed.

Knowing the amounts of bioactive compounds in natural products does not necessarily indicate their antioxidant potential. Different kinds of teas, aromatic plants, enzymatically modified isoquercitrin, phytosterols, stanols, and oils have shown different antioxidant activities [14,15]. Phytosterols and stanols have received much attention in recent years because of their Chol-lowering properties, and several studies have shown a protective effect against cardiovascular disease [16]. Ingestion of deep frying oil has been reported to cause physiologic and histologic changes in experimental animals’ tissue, increase oxidative stress, and possibly lead to death [17].

The synergetic effect, which could exist between individual bioactive compounds, means that the antioxidant potentials may be higher than their sum [5,8,9,11,14]. It was also decided to compare the total antioxidant potentials of the studied cultivars.

It is known that some antioxidant assays provide different antioxidant activity trends. Therefore, three complementary assays for determining total antioxidant potential were used: 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ferric-reducing/antioxidant power (FRAP), and 1,1-diphenyl-2-picrylhydrazyl (DPPH). The same assays were applied for determination of plasma antioxidant activity.

As far as we know, there are no published comparisons of *in vitro* and *in vivo* investigations of persimmon cultivars Fuyu and Jiro on atherosclerosis indices, including aortic histology and fluorescence studies of serum proteins.

Materials and methods

Chemicals and reagents

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ABTS, DPPH, Folin-Ciocalteu reagent, butylated hydroxyanisole, lanthanum (III) chloride heptahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, β -carotene, phenolic acids (ferulic, gallic, protocatechuic, vanillic and *p*-coumaric), ascorbic acid, sodium dodecylsulfate (SDS), β -mercaptoethanol, acrylamide, polyacrylamide, Coomassie Brilliant Blue R, and molecular weight marker (14–205 kDa) were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of samples

Persimmon cultivars Fuyu and Jiro were obtained from Muan County, South Korea. The investigated persimmon cultivars were harvested in 2009. The samples were washed, cleaned, peeled, and cut with a plastic knife and then freeze-dried.

Determination of bioactive compounds and antioxidants

All *in vitro* and *in vivo* tests were performed as previously described [1–3,6,12,18,19]. For determination of total, soluble, and insoluble dietary fibers, samples were treated with heat-stable α -amylase, protease, and amyloglucosidase, followed by centrifugation (15 min, $3000 \times g$) to separate the soluble and insoluble fractions, and dialysis against water was done [3].

Determination of minerals (sodium and potassium) and trace elements (iron, copper, zinc, and manganese) was carried out as follows. The samples of persimmon were lyophilized. Then 0.8 g of lyophilized samples was mineralized in a microwave oven with concentrated HNO_3 . The concentrations were estimated by a Perkin-Elmer 5100 ZL atomic absorption spectrometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, UK) using the flame method for sodium, potassium, iron, copper, and zinc and the flameless method for manganese [3].

Determination of contents of bioactive compounds

Polyphenol extraction

Lyophilized persimmon samples were extracted from a 50-mg aliquot with 5 mL of 1.2 M HCl in 50% methanol/water for hydrolyzed polyphenols with heating at 90°C for 3 h and were determined according to the Folin-Ciocalteu method and measured at 765 nm with gallic acid as a standard and previously described in detail [3,6,12,19]. Flavonoids, extracted with 5% NaNO_2 , 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, and 1 M NaOH, were measured at 510 nm. The content of total flavanols was estimated using the *p*-dimethylaminocinnamaldehyde method, and then the absorbance at 640 nm was read. The extracts of condensed tannins (procyanidins) with 4% methanol vanillin solution were measured at 500 nm. (+)-Catechin served as a standard for flavonoids, flavanols, and tannins, and the results were expressed as catechin equivalents. Carotenoids were extracted with acetone and determined by high-performance liquid chromatography [10]. Ascorbic acid was determined by the cupric reducing antioxidant capacity (CUPRAC) method [20].

The total antioxidant potential was determined by the ABTS, FRAP, and DPPH assays.

1. The ABTS diammonium salt (ABTS^{2+}) was generated by the interaction of ABTS (7 mmol/L) and $\text{K}_2\text{S}_2\text{O}_8$ (2.45 mmol/L). This solution was diluted with methanol until the absorbance reached 0.7 at 734 nm.
2. The FRAP assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripyridyltriazine (Fe^{3+} -TPTZ) to a ferrous form (Fe^{2+}), which absorbs light at 593 nm.

3. The DPPH solution (3.9 mL, 25 mg/L) in methanol was mixed with the sample extracts (0.1 mL). The reaction progress was monitored at 515 nm until the absorbance was stable [18,19].

Animals and diets

The animal care committee of the Warsaw University of Life Sciences (Warsaw, Poland) approved this study. Thirty-six male Wistar rats with an average weight of 115 g at the beginning of the experiment were used in this investigation. The animals were divided into six groups (control, control/Fuyu, control/Jiro, Chol, Chol/Fuyu, and Chol/Jiro) of six and housed in plastic cages. Before the beginning of the study, 5 d for adaptation of rats and 1 d for starvation were allocated. The rats in the control, control/Fuyu, and control/Jiro groups were fed a basal diet and a basal diet plus 5% lyophilized Fuyu and 5% lyophilized Jiro, respectively. The basal diet of the Chol group was supplemented with 1% non-oxidized Chol only. Diets of the Chol/Fuyu and Chol/Jiro groups were supplemented with 1% non-oxidized Chol and 5% lyophilized Fuyu and 5% lyophilized Jiro, respectively. The Chol batches were mixed carefully with the basal diet (1:99) just before the diets were offered to the rats. Our prior experiments on laboratory animals have shown that cellulose has no significant hypocholesterolemic effects. Therefore, cellulose was used as a control fiber. All rats were fed once a day at 10:00 h and had unrestricted access to drinking water.

Laboratory tests

After completion of the experiment, the rats were anesthetized using Narcotan (halothane) and sacrificed, and blood samples were taken. Organs were harvested and subsequently dissected for analysis. Plasma was prepared, frozen, and then used for laboratory tests, which included determination of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol, triacylglycerols, and TC in liver. Plasma antioxidant activity by DPPH and ABTS assays was done. The atherosclerotic lesions in the aorta were assessed.

Histologic procedures

Aortas were conserved in a formaldehyde buffered bath in distilled water, cleaned of loose adventitial tissue, and then cut lengthwise. Dye solutions of Sudan III and Sudan IV were used for 10 min. The surface area of aortic atherosclerotic lesions was measured by planimetry using a computer scanning system (Multi Scan Base 14.02) and expressed as a percentage of total intimal surface area.

Liver protein extraction and electrophoresis

Total proteins from lyophilized samples of rat liver (40 mg each) contained 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue. The proteins extracts were left to stand overnight at room temperature. Samples were boiled for 5 min and then centrifuged at $18\,000 \times g$ for 15 min at 15°C. A Hoefer SE-600 apparatus (Hoefer Pharmacia Biotech, Inc., San Francisco, CA, USA) was used for SDS-polyacrylamide gel electrophoresis, and the resolving gel was 12.7% total acrylamide and 1.3% cross linker and the stacking gel was 6% total acrylamide and 1.7% cross linker. The gel size was $140 \times 160 \times 1.5$ mm, and supernatants (10 μ L) were loaded on gels. The electrophoretic run was carried out at a constant current of 45 mA per gel. Gels were stained with 0.25% Coomassie Brilliant Blue G-250 in a methanol/water/glacial acetic solution (5:5:1, v/v) and destained in 1% solution of Brij 35. The gels were scanned in transmission light with an Agfa SNAPSCAN 1236 color image scanner (Agfa-Gevaert N.V., Mortsel, Belgium). Molecular weight markers were 205 to 14 kDa. Generation by densitometric analysis was done with Biogene 11.9 (Vilber Lourmat, France, Martre-La-Vallee).

Differences in protein fractions of the serum samples after the different diets were determined by three-dimensional fluorescence (3-D FL). The protein fraction was extracted with phosphate buffer. Fluorescence spectra of serum buffer extracts at a concentration of 0.01 mg/mL were recorded on a model FP-6500 spectrofluorometer (serial no. 261332; Jasco, Tokyo, Japan) equipped with 1.0-cm quartz cells and a thermostat bath. The 3-D FL spectra were collected with subsequent scanning emission spectra from 260 to 750 nm at 1.0-nm increments by varying the excitation wavelength from 250 to 350 nm at 10-nm increments. All measurements were carried out in emission mode and with an intensity of up to 1000 [21].

Statistical analysis

The results of this *in vitro* investigation were the mean \pm standard deviation of five measurements. Differences between groups were tested by two-way analysis of variance. In the assessment of antioxidant potential, Spearman correlation coefficient (*R*) was used. Linear regressions were also calculated. $P < 0.05$ was considered statistically significant.

Results

In vitro

Minerals and trace elements

The essential minerals in both cultivars were 25.1 ± 2.4 and 24.3 ± 2.2 mg of sodium and 991.2 ± 32.2 and 989.1 ± 32.2 mg of potassium per 100 g for Fuyu and Jiro, respectively, and 491.1 ± 28.4 and 487.4 ± 29.8 μ g of iron, 493.2 ± 31.6 and 492.6 ± 31.3 μ g of manganese, 40.7 ± 4.2 and 40.6 ± 4.1 μ g of copper, and 66.1 ± 4.9 and 65.8 ± 4.9 μ g of zinc per 100 g for Fuyu and Jiro, respectively. As is clear, the contents of total, soluble, and insoluble dietary fibers in the studied cultivars were comparable at 6.3 ± 0.3 and 6.2 ± 0.3 , 2.5 ± 0.1 , 2.4 ± 0.1 and 2.3 ± 0.1 , and 3.8 ± 0.2 and 3.8 ± 0.2 in 100 g of dry weight for Fuyu and Jiro, respectively ($P > 0.05$).

The results of the determination of the main bioactive compounds (polyphenols, flavonoids, flavanols, ascorbic acid, tannins, total, and β -carotenoids) and antioxidant potentials of persimmon cultivars Fuyu and Jiro by the three assays used are presented in Table 1. The contents of polyphenols and tannins were significantly higher in the Fuyu cultivar ($P < 0.05$). The contents of phenolic acids (milligrams per 100 g of fresh weight [FW]) were 10.19 ± 0.9 and 9.21 ± 0.5 , 22.19 ± 2.3 and 21.02 ± 1.9 , 7.21 ± 0.5 and 6.65 ± 0.5 , 0.93 ± 0.01 and 0.71 ± 0.5 , and 59.7 ± 6.9 and 57.4 ± 5.4 for ferulic, gallic, protocatechuic, vanillic, and *p*-coumaric acids for Fuyu and Jiro, respectively. Among the phenolic acids, the significantly highest concentration was of *p*-coumaric acid ($P < 0.05$). The antioxidant potentials of the persimmon cultivar Fuyu (Table 1) was higher than in the Jiro cultivar, but the difference was significant only according to ABTS assay ($P < 0.05$). A low correlation was observed between DPPH, FRAP, and ABTS values and the dietary fiber content ($R^2 = 0.4628, 0.4749$, and 0.5111, respectively).

A high degree of correlation was observed between the DPPH, FRAP, and ABTS values and polyphenols ($R^2 = 0.8267-0.9951$). A very good correlation was found between DPPH, FRAP, and ABTS values and the content of individual phenolic acids: the best was between *p*-coumaric and ferulic acids and DPPH values ($R^2 = 0.96-0.92$). A good correlation was also registered between gallic acid and DPPH, FRAP, and ABTS values ($R^2 = 0.84-0.86$).

In vivo

The data of the feed intake, body weight gains, feed efficiency ratio, and antioxidant status of serum of rats of all diet groups are

Table 1

Contents of studied bioactive compounds (grams per dry weight) and their antioxidant potentials (micromoles per liter of trolox equivalents per gram of dry weight) in persimmon cultivars^a

Indices	Fuyu	Jiro
Polyphenols (mg GAE)	20.50 \pm 1.3 ^b	17.05 \pm 0.9 ^a
Flavonoids (mg CE)	1.14 \pm 0.05 ^a	1.04 \pm 0.05 ^a
Flavanols (μ g CE)	91.5 \pm 4.3 ^b	69.2 \pm 3.4 ^a
Ascorbic acid (mg)	1.16 \pm 0.05 ^a	1.18 \pm 0.05 ^a
Tannins (mg CE)	4.30 \pm 0.2 ^b	3.15 \pm 0.14 ^a
β -Carotene (μ g)	1.56 \pm 0.05 ^a	1.23 \pm 0.05 ^a
Total carotenoids (μ g)	2.64 \pm 0.07 ^a	2.95 \pm 0.1 ^a
DPPH	49.84 \pm 2.3 ^a	46.15 \pm 2.2 ^a
FRAP	35.97 \pm 1.8 ^a	29.61 \pm 1.4 ^a
ABTS	79.19 \pm 3.4 ^b	51.46 \pm 2.3 ^a

ABTS, 1,2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; CE, catechin equivalent; DPPH, 1,1-diphenyl-2-picrylhydrazyl method; FRAP, ferric-reducing/antioxidant power; GAE, gallic acid equivalent; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

^a Values are means \pm SDs of five measurements. Means in rows without superscript letters in common differ significantly ($P < 0.05$).

presented in Table 2. There was a significant decrease in feed intake and body weight gains in the Chol versus the other five diet groups ($P < 0.05$). Supplementation of diets with 5% lyophilized Fuyu and Jiro hindered the decrease in the plasma antioxidant DPPH assay (Table 2). Supplementation of the Chol diet groups with persimmon fruits increased feed intake in the Chol/Jiro and Chol/Fuyu groups by 12.8% and 15.4%, respectively. Supplementation of diets with 5% lyophilized Fuyu and Jiro hindered the increase in plasma lipids versus the Chol group (TC 19.4% and 9.5%, LDL-C 25.6% and 13.1%, respectively, $P < 0.05$). An increase of TC in plasma of rats fed the Chol versus the control diet was 30.2% ($P < 0.05$). An increase in the level of LDL-C was also registered in the Chol versus the control group by 45.5%. The patterns of changes in LDL-C level were similar to those of TC. In contrast, in rats of the Chol/Fuyu group, whose diet was supplemented with the persimmon cultivar Fuyu, the increase was not significant ($P > 0.05$). Any significant changes were registered in levels of high-density lipoprotein cholesterol and triacylglycerols ($P > 0.05$). As expected, after completion of this experiment, the changes in plasma lipid levels and antioxidant activity of the two additional control groups (control/Fuyu, control/Jiro) were not significant. In our previous investigation, we found significant changes in these indices only in groups of rats fed Chol-containing diets [1,2]. Liver TC concentration in rats of the Chol, Chol/Fuyu, and Chol/Jiro groups were 8.1, 4.9, and 5.7 times higher than in the three control groups ($P < 0.05$ in all three cases). The Fuyu- and Jiro-supplemented diets significantly hindered the increase of liver TC (39.5% and 29.6% versus Chol group, respectively, $P < 0.05$).

The electrophoretic patterns of buffer sample-extracted proteins of rat livers after persimmon-supplemented, control, and Chol diets consisted of 32 distinct bands. Most of these bands were in the range of 24 to 66 kDa. Differences in the intensity of 14-kDa bands between the sera of the control and other diet groups are visible (arrow indicates 14-kDa bands of control negative, Fig. 1A,B), indicating larger amounts of proteins than in the other samples. Profiles of band volumes presented by overlaying the four curves (Fig. 1C) show that curve 13 (control negative) had a larger volume than the other serum samples. Any polymorphism between the serum proteins was observed in all diet groups. The control/Fuyu and control/Jiro groups revealed exactly the same protein patterns as the control group (data not shown).

The histologic investigation of aortas showed that the Chol-containing diet led to changes in the aorta. The highest concentration of lesions was in the arch of aorta (Fig. 2A). The Chol diet group showed the greatest aortic changes compared with the three control diets and the groups supplemented with persimmon (Fig. 2B).

The 3-D FL spectra (Fig. 3) illustrated the elliptical shape of the contours. The x-axis represents the emission spectra from

260 to 750 nm, and the y-axis represents the excitation spectra from 250 to 350 nm (0.01 mg/mL of buffer serum extracts after different persimmon-supplemented diets: Fig. 3A, control; Fig. 3B, control/Fuyu; Fig. 3C, control/Jiro; Fig. 3D, Chol; Fig. 3E, Chol/Fuyu; Fig. 3F, Chol/Jiro; Fig. 3G, bovine serum albumin; Fig. 3H, α - β -globulin human). In 3-D FL spectra, the excitation and emission wavelengths and the fluorescence intensity were used as the axes to investigate the extracted bioactive compounds in the serum samples, and the contour spectra provided more information.

The contour map (Fig. 3A, peaks at excitation/emission 275/330, 1000; excitation/emission 275/640, 174; Fig. 3B, peaks at excitation/emission 275/330, 1000, excitation/emission 275/640, 207; Fig. 3C, peaks at excitation/emission, 275/330, 911, excitation/emission 275/640, 150; Fig. 3D, peaks at excitation/emission, 275/340, 491, excitation/emission 275/650, 74; Fig. 3E, peaks at excitation/emission 275/340, 666, excitation/emission 275/640, 117; Fig. 3F, peaks at excitation/emission 275/340, 504, excitation/emission 275/640, 26; Fig. 3G, peaks at excitation/emission 275/340, 562, excitation/emission 275/650, 104; Fig. 3H, peaks at excitation/emission 275/330, 295, excitation/emission 275/640, 38) displayed a view of the FL spectra. A comparison of the presented contours showed that serum samples had exactly the same maximum wavelength of the peak, including the standard proteins with a slight shift for the Chol groups. The difference was in the intensity of the peaks. The serum of the Chol group showed the lowest intensity compared with the control groups and the two persimmon-supplemented diet groups.

Discussion

In our previous investigations, we found that persimmon contains large quantities of nutraceuticals, and its antioxidant potential is high [1–6]. However, we did not investigate different persimmon cultivars. Even fruit cultivars grown in the same geographic and climatic conditions have been shown to differ significantly [10–12,22]. Therefore, in this investigation, the two persimmon cultivars Fuyu and Jiro were investigated in vitro and in vivo to compare their bioactivities and their influence as supplements on known diets on atherosclerotic indices in rats fed Chol. Among the phenolic compounds in the two persimmon varieties, gallic and *p*-coumaric acids were predominant. A high content of gallic acid is important because of its strong antioxidant properties. These results are in accordance with other reports [10,13]. Total polyphenols were in the range of 17 to 21 mg of GAE/g of dry weight for the two cultivars. These data were higher than those reported by others [10], where the total polyphenols were 127 to 295 mg of GAE/kg of FW. In another report [13], the polyphenols were estimated as 16 to 42 mg of GAE/g of dry weight. These results were in accordance with our

Table 2

FI, BWG, FER, and plasma antioxidant activity (millimoles of trolox equivalents per liter)*

Indices	Control	Control/Jiro	Control/Fuyu	Control/Chol	Chol/Jiro	Chol/Fuyu
FI (g)	963.7 ± 50.9 ^b	963.9 ± 50.9 ^b	963.8 ± 50.9 ^b	848.5 ± 83.1 ^a	957.3 ± 71.8 ^b	979.4 ± 44.9 ^a
BWG (g)	254.1 ± 54.4 ^{ab}	254.3 ± 54.4 ^{ab}	254.2 ± 54.4 ^{ab}	228.7 ± 26.5 ^a	266.6 ± 46.3 ^{ab}	292.1 ± 49.6 ^b
FI (d/g)	20.1 ± 1.1 ^b	20.1 ± 1.1 ^b	20.1 ± 1.1 ^b	17.7 ± 1.70 ^a	19.9 ± 1.5 ^b	20.4 ± 0.9 ^b
BWG (d/g)	5.3 ± 1.7 ^{ab}	5.3 ± 1.7 ^{ab}	5.3 ± 1.7 ^{ab}	4.8 ± 1.0 ^a	5.6 ± 1.0 ^{ab}	6.1 ± 1.1 ^b
FER	3.9 ± 0.7 ^a	3.9 ± 0.7 ^a	3.9 ± 0.7 ^a	3.7 ± 0.3 ^a	3.7 ± 0.6 ^a	3.4 ± 0.5 ^a
ABTS	1.49 ± 0.08 ^a	1.47 ± 0.08 ^a	1.48 ± 0.08 ^a	0.95 ± 0.07 ^b	1.33 ± 0.07 ^a	1.11 ± 0.06 ^b
DPPH	0.83 ± 0.05 ^a	0.81 ± 0.05 ^a	0.82 ± 0.05 ^a	0.53 ± 0.04 ^b	0.74 ± 0.05 ^a	0.59 ± 0.04 ^b

ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); BWG, body weight gain; Chol, cholesterol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FER, feed efficiency ratio; FI, feed intake; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

* Values are means ± SDs ($n = 6$). Means in rows with different superscript letters are significantly different ($P < 0.05$).

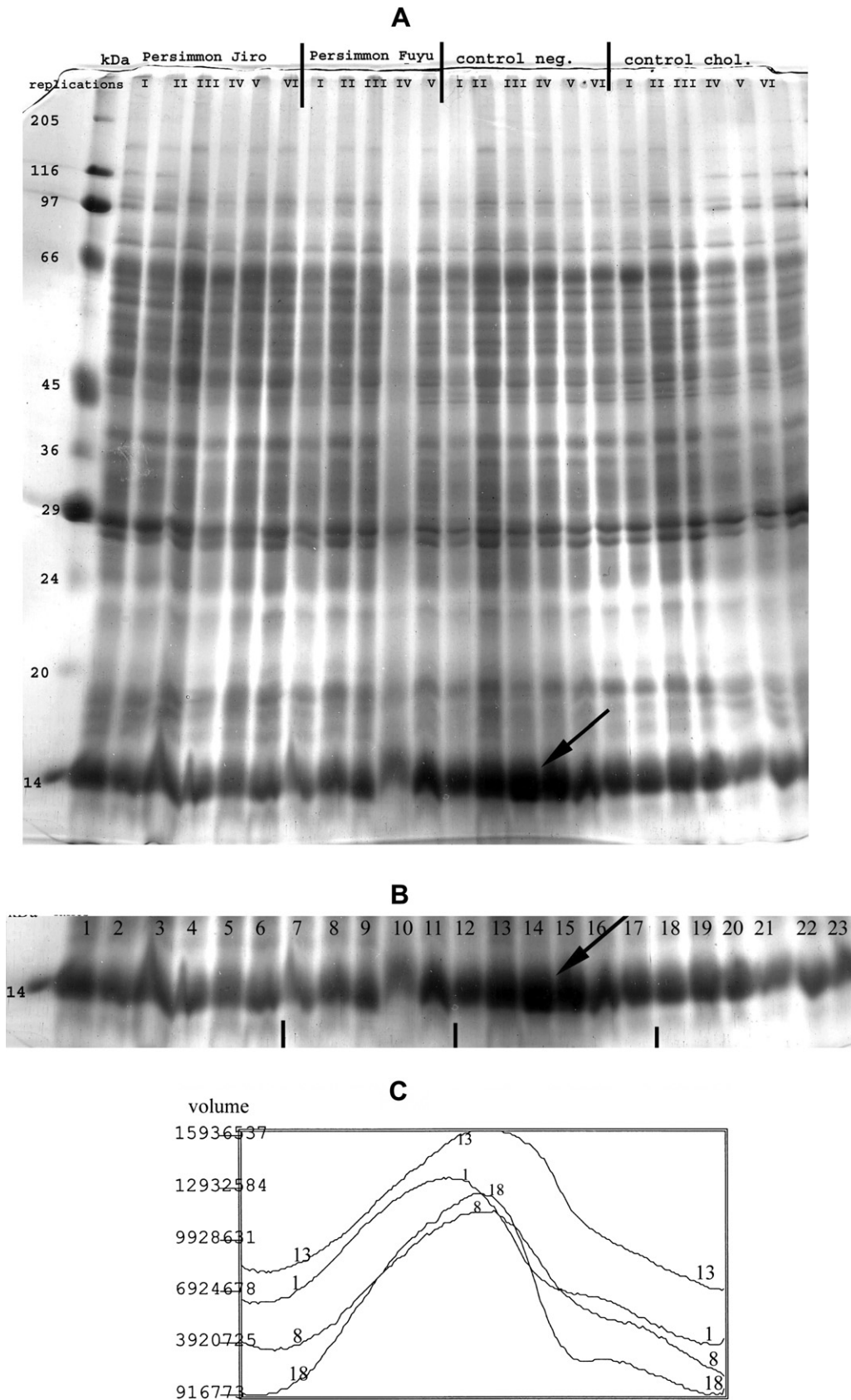


Fig. 1. Comparison of the band intensity of liver tissue proteins after persimmon-supplemented diets extracted with sample buffer containing sodium dodecylsulfate and mercaptoethanol and separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis. (A) Molecular markers (kDa): 205, myosin; 116, β -galactosidase; 97, phosphorylase b; 66, albumin; 45, ovalbumin; 36, glyceraldehyde-3-phosphate dehydrogenase; 29, carbonic anhydrase; 24, trypsinogen, treated with phenyl methyl sulfonyl

results. The total carotenoids and β -carotene were slightly lower than those reported by others [10,11]. The amount of total carotenoid content varied depending on different cultivars and their treatment and was 15.2 to 22.1 $\mu\text{g/g}$ [23]. Our results were in accordance with those of Chen et al. [24], where individual phenolics were determined. As discussed above, the gallic acid exhibited the strongest antioxidant activity, and its content was found to be the highest in another cultivar of persimmon, known as Mopan. The gallic acid highly correlated with polyphenols and antioxidant activity as assessed by DPPH [24], and it was found also in the present study. The antioxidant potential of the cultivar Mopan was estimated by ABTS and DPPH as 23.575 and 22.597 μM of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid equivalent/g of FW, respectively, which corresponded with our results concerning Fuyu and Jiro. As expected, the content of bioactive compounds in the cultivars differed: polyphenols and tannins were significantly higher in the Fuyu cultivar ($P < 0.05$). Other researchers have also reported that the bioactivity of fruit cultivars differs significantly [10–12]. The bioactivity of persimmon fruits can be explained by the synergistic interactions between carotenoids, especially between the water-soluble antioxidant ascorbic acid and β -carotene, which is located in the core of the lipophilic compartment [7]. Our data corresponded with other cited reports in which a synergistic effect was found in different natural products. An experiment with infusions of green tea, white tea, or the aromatic plant *Pelargonium purpur-eum* showed increases of total antioxidant capacity of plasma and organs [14]. Phytosterols exert an inhibitory effect against copper-induced lipid peroxidation of LDLs, as shown by the lowered levels of conjugated dienes in oxidized lipoproteins incubated with different concentrations of plant sterols [16]. The effect exerted by β -sitosterol, stigmasterol, and campesterol against lipid peroxidation of LDL, possibly related to phytosterol-lipoprotein interactions, could be of physiologic relevance [16]. Supplementation of the Chol-containing diet with the studied persimmon cultivars was found to hinder the increase of plasma lipid levels and prevented a decrease in plasma antioxidant activity; these changes were expected. Mahfouz and Kummerow [25] found that Chol-rich diets have different effects on lipid peroxidation, cholesterol oxides, and antioxidant enzymes in rats and rabbits. Our results are supported by those of others because the administration of proanthocyanidins from persimmon skin had a strong effect on hyperlipidemia by lowering levels of triacylglycerol, TC, and non-esterified fatty acids [26]. In our previous short-term investigation of fruits and vegetables, no changes were seen in the atherosclerosis indices in rats fed Chol [27]. Other investigators have also reported that short-term experiments on animals fed Chol did not lead to such changes [28,29]. These investigators found that a 30-d experiment of feeding rats with 4% Chol was not enough to induce morphologic alterations [28]. Therefore, proper diets prevented the atherosclerotic process [30,31].

In our present investigation, which was conducted for a longer period (42 d), atherosclerotic changes were detected in the aortas of all three Chol-fed groups.

The hypolipidemic effects and bile acid-binding properties of young persimmon (*Diospyros kaki*) fruit were recently examined

by others [5]. Our results differ from that recently published research, because in our experiment, rats were used as an animal model and the experiment lasted 42 d instead of 70 d in the cited report. Longer periods can produce greater changes in atherosclerotic lesions in the aorta; therefore, the duration of the present experiment was increased to 42 d compared with our previous data of 4 wk [1,2]. Moreover, other investigators [32] have reported aortic changes in Wisteria male rats loading with Chol (3% in the diet) for 4 and 6 wk. After 4 wk atherosclerotic lesions were seen in the aorta, but after 6 wk atherosclerotic lesions in coronary arteries had formed. In previous experiments, 7% persimmon supplementation was used [1,2] compared with the 5% supplementation in the present experiment. The number of rats in each group was six; in another cited experiment, it was only five [5]. These numbers could be increased in both experiments in the future. Our results correspond with another investigation [33], where 10% of two different cultivars of dried persimmon (Fuyu-kaki and Hachiya-kaki) were added to the basal diet of mice. The supplementation of the diet and the duration of the experiment were up to twice those in our experiment. The obtained results were similar to ours: a decrease in the increase in blood plasma lipids, including TC and LDL-C, without changes in triacylglycerols. The effect was almost equal to the two persimmon cultivars [33], as in our obtained results. Thus, persimmon fruits may be beneficial in the development of preventive and therapeutic agents against dyslipidemia [33,34].

The intake of persimmon significantly lowered the concentration of plasma Chol in the present study and corresponded to the data of others [5]. Our results were in agreement with recently published research with another special fruit [8], where the test diet was supplemented only with 2% acai pulp for the control and hypercholesterolemic rats for 6 wk. Supplementing the diet of this group with acai caused a hypocholesterolemic effect by decreasing TC and non-high-density lipoprotein cholesterol. Acai supplementation induced a significant decrease in superoxide dismutase activity only in the hypercholesterolemic rats, indicating an association between diet and acai treatment. In our case, when the duration of the experiment was longer, the results were similar to the cited ones [8]. However, the endpoints appear less suitable for studying the efficacy of bioactive substances, i.e., for prevention. As was discussed in another report [9], for cardiovascular disease the main biomarker is serum cholesterol, blood pressure, and others. It is important to check the concept of biomarkers and their role in demonstrating the efficacy of bioactive substances [9]. Atherosclerotic plaque lesions in the aortic sinus were significantly decreased by feeding male apolipoprotein E-deficient mice a high-fat diet alone or a diet containing enzymatically modified isoquercitrin for 14 wk. Enzymatically modified isoquercitrin has atheroprotective and plaque-stabilizing effects [15]. Male spontaneously hypertensive and Wistar Kyoto rats were fed diets containing 15% fresh soybean oil or deep frying oil for 10 wk [17]. Diets containing deep frying oil resulted in increased plasma thiobarbituric acid-reactive substances and nitric oxide contents and decreased plasma total antioxidant capacity in spontaneously hypertensive and Wistar Kyoto rats. In the present experiment, the entire persimmon was used for supplementation, as it is

fluoride; 20, trypsin inhibitor, 14, α -lactalbumin. Loading, 2 μL : lanes 1–6, persimmon Jiro (diet rat groups cholesterol/Jiro); lanes 7–11, persimmon Fuyu (diet rat groups cholesterol/Fuyu); lanes 12–17, control negative (diet rat groups without cholesterol); lanes 18–23, control cholesterol (diet rat groups control/cholesterol). The difference between bands of liver proteins in the range of 14 kDa of the control group and three other groups is marked by an arrow. (B) Protein patterns of electrophoresis with low molecular weight of 14 kDa. The lanes are the same as in A. (C) Volume densitometric analysis of four lanes in 14-kDa bands. Lane 1, persimmon Jiro; lane 8, persimmon Fuyu; lane 13, control negative; and lane 18, cholesterol. Volume was calculated as the sum of all peak volume intensities included in the defined area under the peaks of the 14-kDa bands.

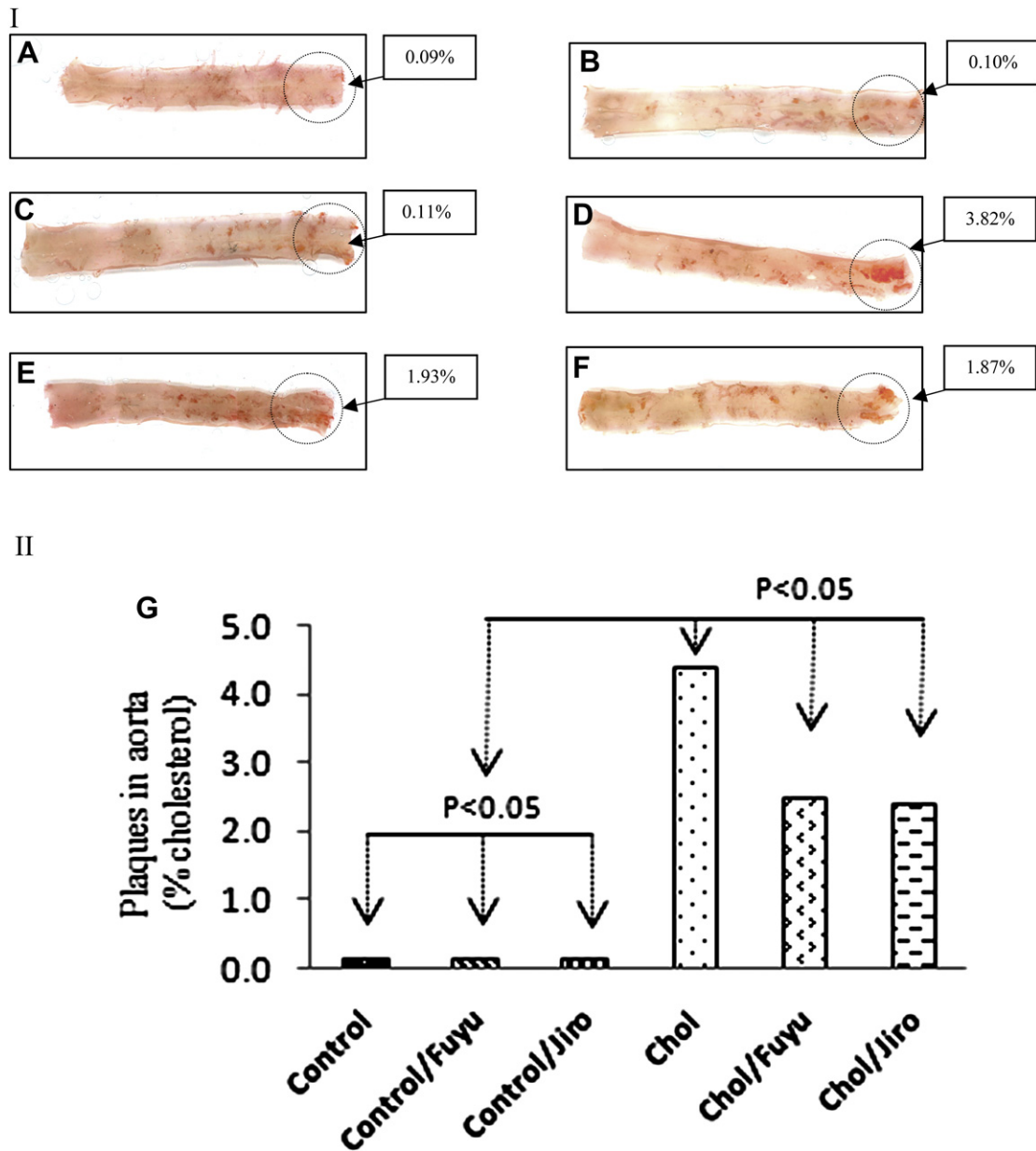


Fig. 2. (I) Aortic changes in the (A) control, (B) control/Fuyu, (C) control/Jiro, (D) Chol, (E) Chol/Fuyu, and (F) Chol/Jiro groups. The positions marked with arrows show the most concentrated area and the percentages of lesions in the arch of the aorta from the total amount. (II) Changes in lesions in the total aorta (percentage of Chol). Chol, cholesterol.

eaten normally. The palatability of persimmon, especially Fuyu, influenced higher feed consumption in the Chol/Fuyu and Chol/Jiro groups than in the Chol group. Such a reaction was observed also as with increase of Chol loading during 42 d. The Chol consumption was 9.8 and 9.6 versus 8.5 g, respectively. As discussed previously, the bioactivity of persimmon fruits modified the effect of Chol on plasma lipids and lesions in the aorta. These effects proportionally depended on the contents of bioactive compounds in the persimmon cultivars (Table 1).

The protein profile of liver tissue samples showed that in two persimmon diets, slightly sharper protein bands were detected in the range of 14 kDa than in the Chol group, but the highest concentration of proteins was in the control group diet. This difference can be explained by the changes in the rats' metabolism, and the decreasing thickness of fat ("cover") around the

liver in rats was noticed during the diet period from 4 to 6 wk. The Chol diet with or without persimmon led to a slight decrease of low-molecular protein synthesis. These changes can be explained by a considerable increase of protein synthesis, with low-molecular proteins changing the balance of metabolism between fats and proteins. Our results correspond with those of others [11] confirming that the effect of supplementing diets with carotenoid, ascorbic acid, and persimmon powders on the antioxidative ability of Osteogenic Disorder–Shionogi rats increases antioxidative ability in the skin and liver. Others have explained this observation by the results that appeared in the liver: sterol regulatory element binding protein-2 gene expression was significantly higher in mice fed persimmon, whereas the mRNA and protein levels of the LDL receptor were unchanged. These results indicate that acceleration of fecal bile

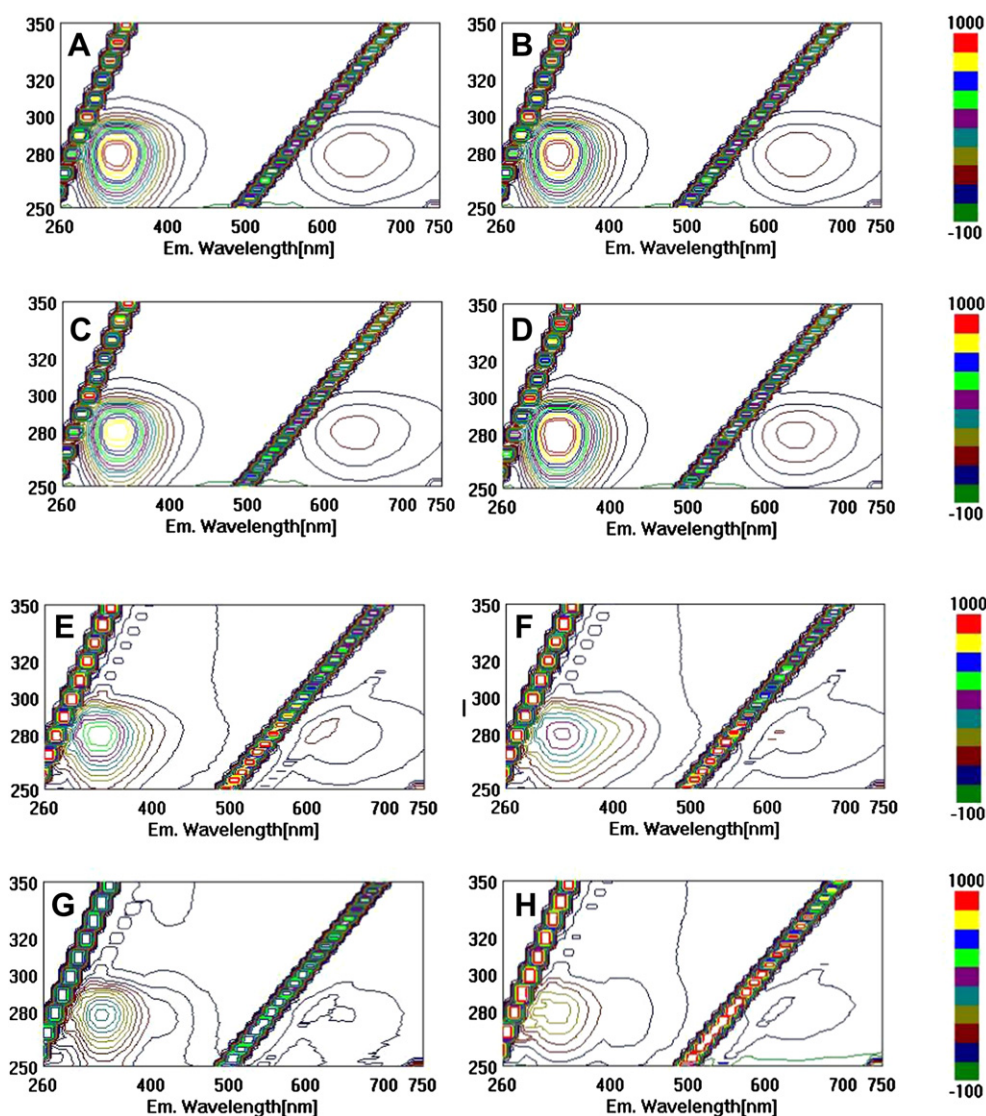


Fig. 3. Contour maps display corresponding three-dimensional fluorescence spectra. Three-dimensional fluorescence spectra were produced with emissions from 260 to 750 nm (x-axis), excitation wavelengths from 250 to 350 nm (y-axis), scanning speed of 1000 nm/min, emission mode, and fluorescence intensity values were from –100 to 1000. (A) Control, (B) control/Fuyu, (C) control/Jiro, (D) cholesterol, (E) cholesterol/Fuyu, (F) cholesterol/Jiro, (G) bovine serum albumin, (H) Human α - β -globulin. Em., emission.

acid excretion in mice is a major mechanism of the hypolipidemic effect induced by persimmon [6,33,34]. In our previous study an increase of bile flow, excretion of bile acids, and Chol bile in rats loading with Chol and supplemented with diets of grapefruits or olive oils was also obtained [35,36]. The registered results show that Chol-containing diets in the Chol, Chol/Fuyu, and Chol/Jiro groups decreased plasma antioxidant activity, and only the addition of the studied cultivars to the diets hindered this process. It was demonstrated by plasma lipid changes and TC concentration in the liver. The supplementation of cultivars to the diets led to a significant decrease of live TC concentration in the Chol/Fuyu and Chol/Jiro groups versus the Chol group.

One main peak was observed in 3-D FL spectra of serum buffer (albumin and globulin) extracts of the Chol/Fuyo, Chol/Jiro, Chol, and three control diet groups at λ excitation/emission 275/340–330 nm and a minor one at 275/650–640 nm with the highest fluorescence intensity in the Chol/Fuyo group, which was similar to that in the control groups [21]. The difference between the two groups of persimmon supplementation was minor. The

standards showed similar maximum peaks at the same wavelength as the investigated serum samples.

Changes in albumin and globulin fractions in the serum of rats receiving diets supplemented with persimmon compared with Chol were found in the decrease of fluorescence intensity and small shift of the major peak in 3-D FL.

Most scientists currently agree that atherosclerosis is a complex process, characterized by an inflammatory, fibrofatty, proliferative response to damage of the artery wall involving smooth muscle cells, monocyte-derived macrophages, T-lymphocytes, and platelets and that hyperlipidemia constitutes a major etiologic-pathologic factor for this disease. In our present investigation, we found that not only garlic but also fruits (two persimmon cultivars) positively influence the well-known atherosclerosis indices: plasma lipid levels and plasma antioxidant activity [37]. The non-classic risk factors (albumin and globulin serum fractions) fully correlated with the classic risk factors for atherosclerosis (lipid profile and atherosclerotic changes in the aorta).

Conclusion

The contents of polyphenols, flavonoids, and flavanols were significantly higher in the Fuyu cultivar. Both cultivars possess comparable high antioxidant potential. Supplementation of 5% lyophilized Fuyu and Jiro to diets of rats fed Chol-containing diets hindered the increase in plasma lipids levels and the decrease in plasma antioxidant activity and significantly decreased atherosclerotic lesions in the aorta. Electrophoresis and fluorescence were applied to see the differences in the proteins, liver tissue, and serum. 3-D FL and radical scavenging assays in the serum of rats whose diets were supplemented with persimmon showed minor structural changes in albumin and globulin fractions.

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