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Red Grapefruit Positively Influences Serum Triglyceride Level in Patients Suffering from Coronary Atherosclerosis: Studies in Vitro and in Humans

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The contents of the bioactive compounds in red and blond grapefruits and their influence on humans suffering from hypertriglyceridemia were studied. It was found that red grapefruit has a higher content of bioactive compounds and a higher antioxidant potential than blond grapefruit, determined by oxygen radical scavenging capacity, 1,1-diphenyl-2-picrylhydrazyl, carotenoid bleaching, and Folin−Ciocalteu assays. Fifty-seven hyperlipidemic patients, ages 39−72 years, after coronary bypass surgery, recruited from the Institute's pool of volunteers, were randomly divided into three equal in number (19) groups: two experimental (red and blond groups) and one control group (CG). During 30 consecutive days of the investigation the diets of the patients of the red and blond dietary groups were daily supplemented with one equal in weight fresh red or blond grapefruit, respectively. Before and after this trial, serum lipid levels of all fractions and serum antioxidant activity were determined. It was found that serum lipid levels in patients of the red and blond groups versus the CG after treatment were decreased: (a) total cholesterol, 6.69 versus 7.92 mmol/L, 15.5%, and 7.32 versus 7.92 mmol/L, 7.6%, respectively; (b) low-density lipoprotein cholesterol, 5.01 versus 6.29 mmol/L, 20.3%, and 5.62 versus 6.29 mmol/L, 10.7%, respectively; (c) triglycerides, 1.69 versus 2.32 mmol/L, 17.2%, and 2.19 versus 2.32 mmol/L, 5.6%, respectively. No changes in the serum lipid levels in patients of the CG were found. In conclusion, fresh red grapefruit contains higher quantities of bioactive compounds and has significantly higher antioxidant potential than blond grapefruit. Diet supplemented with fresh red grapefruit positively influences serum lipid levels of all fractions, especially serum triglycerides and also serum antioxidant activity. The addition of fresh red grapefruit to generally accepted diets could be beneficial for hyperlipidemic, especially hypertriglyceridemic, patients suffering from coronary atherosclerosis.

KEYWORDS: Fresh red and blond grapefruits; polyphenols; radical scavenging capacities; hypertriglyceridemic patients

INTRODUCTION

Consumption of fruits and vegetables has been associated with reduced risk of some chronic diseases including the most dangerous—coronary atherosclerosis (*1*, *2*). The major bioactive compounds of these natural products are phenolics, especially flavonoids, which are responsible for their health benefits (*3*). The antioxidant properties of phenolics are responsible for the inhibition of oxidation of low-density lipoprotein cholesterol (*4*, *5*). As a consequence, consumption of fruits and vegetables is inversely related to coronary atherosclerosis (*1*). Citrus fruits contain high amounts of bioactive compounds, mostly phenolics (*6*, *7*). Addition of citrus juices or citrus fruits (Sweeties and grapefruits) to cholesterol-containing diets leads to hypocholesterolemic effect and to a decrease in the content of total

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cholesterol in the liver in experiments on laboratory animals and in hypercholesterolemic patients (*6*, *8*−*10*).

However, the results of experiments on laboratory animals could not be automatically applied to humans. Therefore, it was decided to investigate the influence of well-known red and blond grapefruits on patients suffering from hyperlipidemia.

There are many methods for total antioxidant potential determination, and each has its limitations (*11*). Some of these antioxidant assays give different antioxidant activity trends (*12*). Therefore, in the red and blond grapefruits were determined bioactive compounds and antioxidant potential by different radical scavenging tests. These studied grapefruits were used as a supplementation to the diet of patients suffering from coronary atherosclerosis and hyperlipidemia.

As far as we know, there are no publications describing studies of different cultivars of grapefruits and their influence on humans suffering from coronary atherosclerosis and hyperlipidemia.

MATERIALS AND METHODS

Chemicals. The chemicals were purchased from the following companies: Trolox (99%) and naringin (95%) from Sigma-Aldrich (Milwaukee, WI); β-carotene, butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), $FeCl_3 \cdot 6H_2O$, and Folin−Ciocalteu reagent from Sigma Chemical Co. (St. Louis, MO); 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); 2,4,6-tripyridyl-*s*-triazine (TPTZ) (Fluka Chemie, Buchs, Switzerland); 2,2′-azobis(2-methylpropanimidamide dihydrochloride) (AAPH) and fluorescein from Merck Eurolab GmbH (Darmstadt, Germany). All reagents were of analytical grade.

Fruit Samples. Israeli Jaffa red and blond grapefruits harvested in the 2004−2005 season were from the Sharon region and purchased from the same farmer. The fruits were cleaned with tap water and dried. Then the peels were manually separated from the edible parts. The peeled fruits were weighed, chopped, and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. Then a weighed portion (50−100 g) was lyophilized for 48 h (Virtis model 10-324), and the dry weight was determined. The samples were ground to pass through a 0.5-mm sieve and stored at −20 °C until analysis. The extracts were prepared as previously described (*10*).

Analyses of Grapefruits. Dietary fiber in the selected samples was analyzed according to a modified AOAC method (*13*). Samples were treated with heat-stable α-amylase, protease, and amyloglucosidase, followed by centrifugation (15 min, 3000*g*) to separate the soluble and insoluble fractions and dialysis against water.

Total polyphenols and phenolic and ascorbic acids were determined as previously described (*8*).

Anthocyanins were estimated according to a pH differential method from fruit extracts by acetonitrile containing 4% acetic acid (*14*). Absorbance was measured in a Beckman spectrophotometer at 510 and 700 nm in buffers at pH 1.0 and 4.5, using $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$ with a molar extinction coefficient of cyanidin-3-glucoside of 29600. Results are expressed as micrograms of cyanidin-3-glucoside equivalents per 100 g of fresh weight (FW).

Flavonoids were extracted with 5% $NaNO_2$, 10% $AlCl_3 \cdot 6H_2O$, and 1 M NaOH, and their absorbance was measured at 510 nm with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as micrograms per 100 g of FW (*15*).

Determination of the Radical Scavenging Activity. The antioxidant effect of the whole fruit can be low, even in cases when antioxidant potential of individual bioactive compounds is high (*16*). Therefore, in addition to phenolic compounds different antioxidant tests were used.

Radical Scavenging Activity Using DPPH. The results are expressed as inhibition percentage: % radical scavenging activity = (control OD − sample OD/control OD) × 100, where OD is optical density. Changes in the absorbance of the samples were measured at 517 nm (*17*, *18*).

Antioxidant Activity Using β-Carotene Linoleate Model System. The results were evaluated in terms of bleaching of the β-carotene, measuring the absorbance at 470 nm: $AA = 100\,[1 - (A_0 - A_t)/(A°_0 - A°_t)]$, where A_0 and $A°_0$ are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and $A°_t$ are the absorbances measured in the test sample and control, respectively, after incubation for 180 min. The results are expressed in percentage of inhibition. BHA was used for comparison in both methods (*17*, *18*).

Oxygen Radical Absorbance Capacity (ORAC) Assay. Fifty grams of the pulp was weighed and extracted with water and dimethyl sulfoxide (DMSO). The solutions were combined and subjected to ORAC assay (*19*) with minor modifications on a fluorescent plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT). The results are expressed as micromoles of Trolox equivalent per 100 g of FW.

HPLC Method. The naringin contents of the samples were quantified using HPLC performed on a Shimazu HPLC system with a diode array detector. The separation was carried out on a Shimadzu VP-ODS column (25 cm × 4.6 μm). A binary phase solvent system was used with A (0.1% formic acid/water) and B (0.1% formic acid/methanol). Column temperature was set at 25 °C, and flow rate was 0.5 mL/min. The UV−vis detector was set at 285 nm. Solvent gradient was as follows to ensure that most of the phenolic compounds can be detected: 0−10 min, 90% A; 10−28 min, 90−70% A; 28−35 min, 70−55% A; 35−45 min, 55−40% A; 45−50 min, 40−60% A; 50−55 min, 40−90% A; 55−70 min, 90% A.

Subjects, Clinical Investigation, Laboratory Tests, and Dietary Intervention. Ninety-two patients between the ages of 39 and 72 years were examined. All of them underwent bypass surgery due to two- or three-vessel coronary artery disease (CAD). The clinical manifestations of CAD in these patients appeared at least 2 years before the coronary bypass surgery, but following surgery they were free of anginal syndrome. No lipid-lowering and/or antioxidant-increasing drugs were used during the 30 days of the investigation. All patients were at least 12 months after the surgery. From the total number of patients (92) only 57 with hypertriglyceridemia, whose drug treatment with Simvastatin (one of the preparations of the statin group) was not effective, were chosen and randomly divided into three equal in number groups: two experimental (red and blond grapefruits) and one control (CG), each group having of 19 patients.

All patients consumed a generally accepted diet for coronary atherosclerosis (vegetables, fruits, and limited quantities of fats). The diet contains ∼1700 kcal, and the percentage of energy was 66% of carbohydrates, 25% of protein, and 9% of fat. For 30 consecutive days this diet was supplemented once a day for patients of the red and blond groups by one Israeli red or blond grapefruit, respectively. An assigned member of the investigation team checked the consumption of diets, lifestyle, and physical activity of all 57 patients. Before and after completion of the study all patients were examined. Systolic and diastolic blood pressure, heart rate, and weight were registered. During the trial period there were no complications or drop out of participants. After an overnight fast, the blood samples were collected a day before and a day after completion of the investigation. Serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), plasma circulation fibrinogen (PCF), and prothrombin time (PT) were determined as previously described (*20*).

Serum antioxidant activity was determined (*21*) by ABTS, and Trolox [millimoles of Trolox equivalents (TE) per liter] equivalent antioxidant capacity (TEAC) was calculated. The ferric reducing antioxidant power (FRAP) measured the intensity of blue color complex at absorption maximum (593 nm), which developed when a ferric [Fe^{III}-2,4,6-tripyridyl-*s*-triazine (TPTZ)] complex was reduced to ferrous (Fe^{II}) form. The antioxidant activity was measured in millimoles per liter (*22*). The Folin−Ciocalteu assay was used as well (*15*).

Statistical Analysis. Values of the indices investigated in vitro are given as means ± standard deviation (SD) of five times analyzed fruit samples. When appropriate, the data in the in vivo part were tested by two-way ANOVA using GraphPad Prism, version 2.0 (GraphPad Software, San Diego, CA) followed by Duncan's new multiple-range

Table 1. Contents of Dietary Fibers, Anthocyanins, and Flavonoids in Fresh Peeled Red and Blond Grapefruits[a]

fruit	total fibers[b]	insoluble fibers[b]	soluble fibers[b]	flavonoids[c]	anthocyanins[d]
red peeled grapefruits	1.39 ± 0.1a	0.87 ± 0.08a	0.52 ± 0.05a	21.61 ± 1.3a	51.5 ± 4.6a
blond peeled grapefruits	1.37 ± 0.1a	0.86 ± 0.08a	0.51 ± 0.05a	19.53 ± 1.2a	49.3 ± 4.5a

[a] Values are means ± SD of five measurements. Means in columns with different letters differ significantly ($P < 0.05$). [b] Grams per 100 g of fresh weight. [c] Milligrams per 100 g of fresh weight. [d] Micrograms per 100 g of fresh weight.

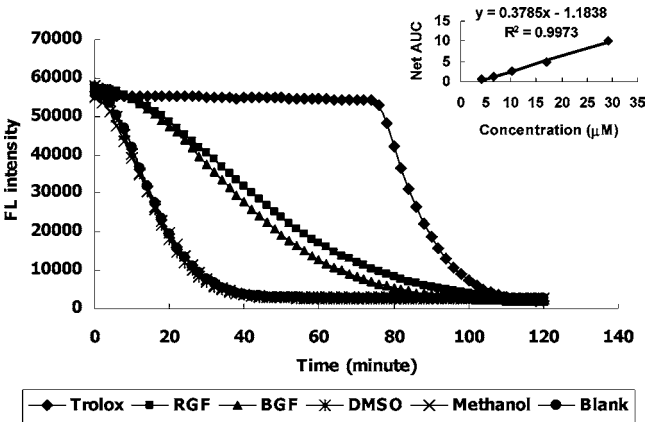

Figure 1. Typical kinetic curves of Trolox, DMSO extracts from red and blond grapefruits, and used solvents for extraction and dissolving during reaction with peroxyl radicals under the ORAC assay conditions. (Inset) Trolox calibration curve and equation. Abbreviations: ORAC, oxygen radical absorbance capacity; DMSO, dimethyl sulfoxide; BGF, blond grapefruit, diluted 640 times; RGF, red grapefruit, diluted 640 times; blank, PBS buffer; Trolox, standard; T, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Solvents: methanol, diluted 160 times; DMSO, diluted 160 times,

test to assess differences between group means. Differences of $P < 0.05$ were considered to be significant.

RESULTS

In Vitro. The contents of total, soluble, and insoluble dietary fibers in red and blond peeled grapefruits were comparable. The contents of flavonoids and anthocyanins were higher in red grapefruit, but the differences were not significant (**Table 1**).

Typical kinetic curves of DMSO extract from red and blond grapefruits during the reaction with peroxyl radicals under the ORAC assay conditions are presented in **Figure 1**. DMSO under dilution does not have any antioxidant capacity, nor do any other solvents used for ORAC measurements.

The contents of phenolic and ascorbic acids and naringin in red and blond peeled grapefruits (**Figure 2A**) were comparable ($P > 0.05$). Among the phenolic acids, the highest concentration was of ferulic and the lowest was of caffeic acid. The differences in the contents of phenolic acids were significant ($P < 0.05$). The content of ascorbic acid was significantly higher than that of each phenolic acid ($P < 0.05$). The used antioxidant assays (**Figure 2B**) showed that the radical scavenging activity of the red peeled grapefruit and the total polyphenol contents were significantly higher than those of the blond ($P < 0.05$). The contents of the bioactive substances in the red and blond peeled grapefruits and their antioxidant potentials were comparable with the same variables in fruits harvested in 2003−2004 (*20*). Apparently there are other antioxidants besides naringin in DMSO fractions. Indeed, the HPLC chromatographs of the DMSO fraction show complex peaks detected at 280 nm, because most phenolic compounds absorb at this wavelength. It is likely that other phenolic compounds contribute collectively to the radical scavenging capacity (**Figure 3**).

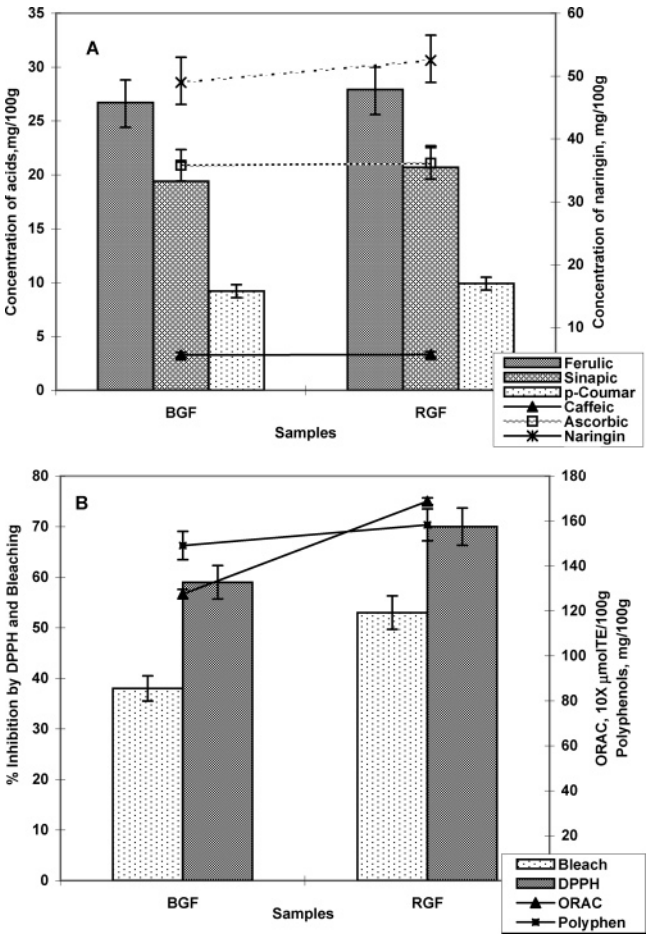

Figure 2. (**A**) Contents of phenolic and ascorbic acids and naringin (mg/100 g of FW) in the red and blond peeled grapefruits. (**B**) Antioxidant capacities by bleaching with β-carotene and DPPH (percent inhibition) and ORAC (10 × μmol of TE/100 g of FW), polyphenols (mg/100 g of FW) in red and blond Israeli grapefruits. Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging test; ORAC, oxygen radical absorbance capacity; β-carotene, β-carotene bleaching; BGF, blond grapefruit; RGF, red grapefruit; TE, Trolox equivalent.

Studies in Humans. The heart rate, systolic and diastolic blood pressure, and weight of the patients after completion of the investigation were without significant changes (data not shown).

A significant decrease in the level of TC and LDL-C was found in both experimental groups ($P < 0.05$). The increase in the HDL-C in the red and blond groups versus the CG (**Table 2**) was not significant ($P > 0.05$). The decrease in the concentration of triglycerides was significant ($P < 0.05$) only in the patients of the red group, whose diet was supplemented with peeled red grapefruits.

After completion of the investigation, the serum antioxidant activity in patients of the red and blond groups versus CG was significantly increased (**Figure 4**): 1.91 versus 1.40 mmol/L, +36.4%, and 1.65 versus 1.40 mmol/L, +17.8%, respectively.

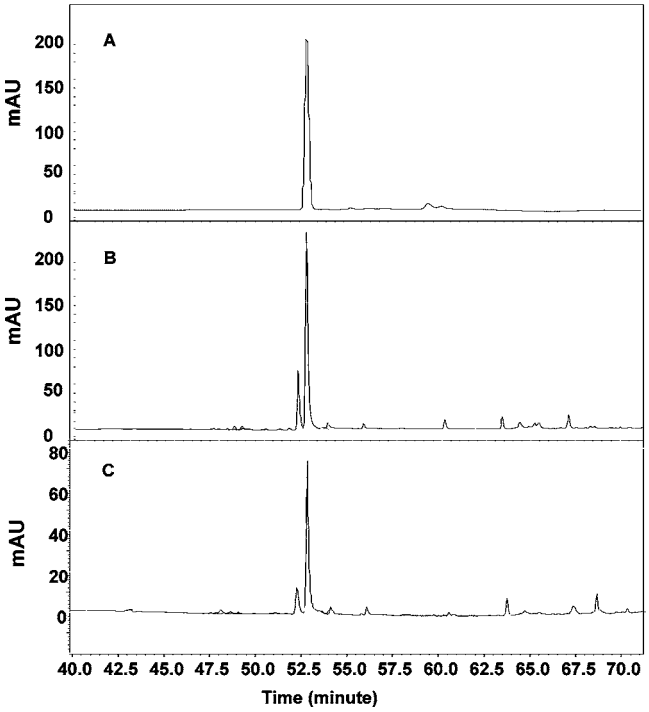

Figure 3. HPLC chromatograms of the blond and red grapefruits in dimethyl sulfoxide (DMSO) fractions: (**A**) naringin, 52.75 min; (**B**) blond grapefruit, 52.75 min; (**C**) red grapefruit, blond grapefruit, 52.74 min. The major peak is due to naringin, the dominant phenolic compound in grapefruits. See text for detailed HPLC conditions.

Table 2. Changes in Serum Lipid Concentration (Millimoles per Liter) in the Control, Red, and Blond Groups after Completion of the Investigation[a,b]

diet	TC	LDL-C	HDL-C	TG
control	7.92 ± 0.4a	6.29 ± 0.2a	1.20 ± 0.1a	2.32 ± 0.1a
red	6.69 ± 0.3b	5.01 ± 0.2c	1.36 ± 0.1a	1.69 ± 0.1b
blond	7.32 ± 0.3a	5.62 ± 0.2b	1.30 ± 0.1a	2.19 ± 0.1a
two-way ANOVA (*P* value)				
red	<0.0125	<0.005	NS	<0.005
blond	NS	<0.01	NS	NS

[a] Values are means ± SD; $n = 19$. Means in columns without letters in common differ significantly ($P < 0.05$). [b] Abbreviations: red, experimental group, diet supplemented with one red grapefruit; blond, experimental group, diet supplemented with one blond grapefruit; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

Similar relationships were obtained using the Folin−Ciocalteu reagent; the red and blond groups versus CG significantly increased: 3.52 versus 3.15 mg of gallic acid equivalents (GAE)/mL, +11.7%, and 3.24 versus 3.09 mg of GAE/mL, +4.9%. The FRAP assay results were as follows: 1.1 versus 0.85 mmol/L +29.4%, and 0.92 versus 0.81 mmol/L, +13.6%.

No significant changes in serum circulation fibrinogen level, prothrombin time (PT), and other anticoagulation tests were registered in patients of all groups (data not shown).

The calculated correlations between the decrease of triglycerides and the increase of antioxidant capacity of serum and citrus diet contribution to the antioxidant potential of both experimental groups (**Figure 5**) were decisive for red grapefruit ($R^2 = 0.98$) and slightly lower for blond ($R^2 = 0.96$).

DISCUSSION

It is common knowledge that one of the major risk factors of atherosclerosis is hyperlipidemia (*23*). It has been shown over and over again that LDL-C is the most dangerous among serum lipids and that its oxidation leads to increased penetration into arterial walls (*24*, *25*). In the past the role of serum triglycerides as a risk factor for atherosclerosis was considered to be controversial (*26*). However, the very recent data indicate that the association between the serum triglycerides level and coronary atherosclerosis is strong, graded, and independent (*26*, *27*). Now some authors claim that the apolipoprotein content of triglyceride-rich lipoproteins independently predicts early atherosclerosis in healthy middle-aged men (*28*). The most acceptable method of treatment of this condition is a combination of a hypolipidemic agent—3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins: Crestor, Lescol, Lipitor, Simovil, Simvacor, Simvastatin, Torid)—together with proper diet (*29*, *30*). Therefore, a modified Mediterranean-type diet rich in omega-3 fatty acids efficiently potentiated the cholesterol-lowering effect of Simvastatin (*30*). However, in some patients the above-mentioned hypolipidemic drugs are not effective, especially in the cases of hypertriglyceridemia.

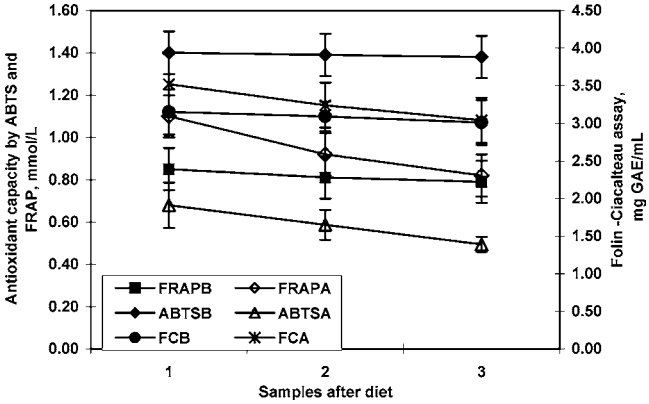

Figure 4. Changes of the serum antioxidant activity after completion of the investigation in one, two, and three samples of serum from red, blond, and control patient groups after diet with corresponding fruits. Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FRAP, ferric reducing antioxidant power; FC, Folin–Ciocalteu assay; FRAPB, antioxidant activity measured by FRAP before the experiment; FRAPA, antioxidant activity measured by FRAP after the experiment; ABTSB, antioxidant activity measured by ABTS scavenging cation before the experiment; ABTSA, antioxidant activity measured by ABTS scavenging cation after the experiment; FCB, antioxidant activity measured by Folin–Ciocalteu assay before the experiment; FCA, antioxidant activity measured by Folin–Ciocalteu assay after the experiment.

It was shown that supplementation of proper diets with citrus fruits or their juices could be helpful in the treatment of hyperlipidemia (*10*, *31*, *32*).

Citrus fruits are characterized by high concentrations of bioactive compounds: dietary fibers and antioxidants, especially polyphenols (*3*, *8*). However, the differences between cultivars of the same citrus fruit are less known (*20*).

The ORAC, bleaching, and DPPH values of red grapefruit are significantly higher than that of blond grapefruit in both water and DMSO fractions. In addition, it was found that the naringin content is higher in red grapefruit than in blond. Naringin is the major phenolic compound found in grapefruits; however, it is poorly soluble in water. In fact, there is no detectable amount (HPLC, 280 nm) of naringin in the water fraction of the juice. Therefore, other compounds may be more important for health benefits and do not act as peroxyl radical scavengers. It was found that the antioxidant potential of red grapefruits was higher than that of blond grapefruits; therefore,

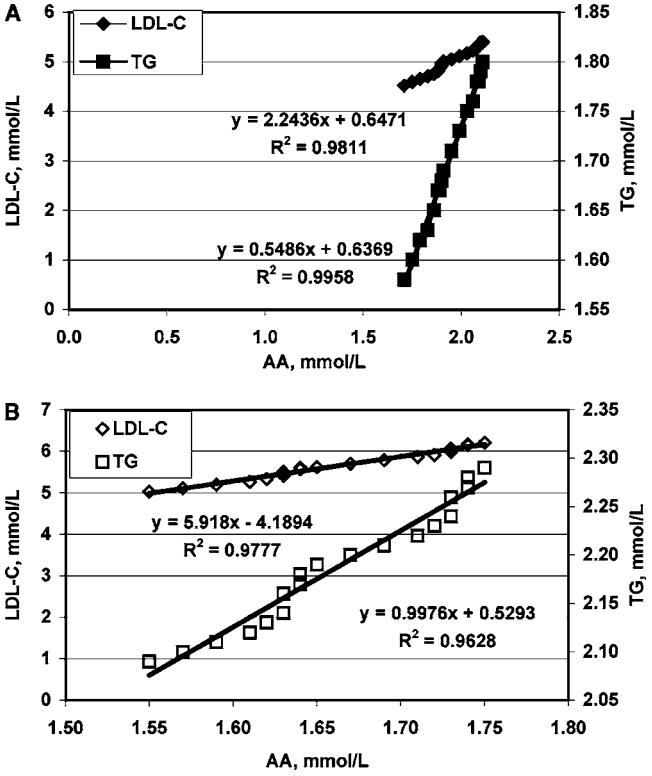

Figure 5. Relationship, calculated by linear regression analysis, for red (**A**) and blond (**B**) grapefruit supplemented diets: (**A**) between (◆) AA by ABTS scavenging radical (mmol/L, *X*) to reduction of cholesterol (mmol/L, Y_1) and (■) antioxidant activity by ABTS scavenging radical (mmol/L, *X*) to reduction of triglycerides (mmol/L, Y_2) and (**B**) between (◇) ABTS (mmol/L, *X*) to reduction of cholesterol (mmol/L, Y_1) and (□) ABTS (mmol/L, *X*) to reduction of triglycerides (mmol/L, Y_2). Abbreviations: AA, antioxidant activity, mmol/L; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides.

it was expected that the influence of the supplementation of diets with red grapefruits could be more effective.

The results of the investigation in humans have shown that a generally accepted antiatherosclerosis diet supplemented with fresh red or blond grapefruits positively influences the serum levels of TC and LDL-C. However, only a diet supplemented with red grapefruits was effective in significantly lowering the level of serum triglycerides.

Both cultivars of the used grapefruits significantly improved the serum antioxidant activity in the investigated patients. These results could be predicted: fruits containing high quantities of dietary fibers and antioxidant compounds, especially phenolics, positively influence the serum antioxidant activity in experiments on laboratory animals and in investigations of humans (*8*, *10*, *31*). The results of this investigation show undoubtedly that proper diet supplemented with fresh red grapefruit significantly decreases the serum levels of lipids, especially of triglycerides. It has to be emphasized that treatment of these patients with statins was not effective. We cannot explain this finding only by the higher contents of the studied bioactive compounds and higher antioxidant potential in red cultivars of grapefruit versus blond cultivars (*20*). We did not find such a significant decrease in the serum triglycerides (*10*) in our previous investigation with other citrus fruits. The concentrations of the bioactive compounds and antioxidant potential were comparable with those of red grapefruits. All results of the in vitro investigation are in agreement with the clinical trails. Therefore, it is likely that the antioxidants in the grapefruits are responsible for the health benefits. However, on the basis of the individual phenolic compounds, the two samples are rather close to each other, but they account for only a small fraction of the antioxidant activity. The remaining antioxidant capacity may be from unknown compounds or the synergistic effects of the compounds. We cannot exclude that only red grapefruit cultivars contain some special bioactive compounds which are responsible for the triglyceride-lowering effect. Therefore, in our opinion, further investigations of the red grapefruit in vitro are necessary.

In conclusion, diet supplemented with fresh red grapefruit positively influences serum lipid levels, especially serum triglycerides and serum antioxidant activity. Addition of fresh red grapefruit to generally accepted diets may be beneficial for hyperlipidemic patients, especially those with high levels of triglycerides.

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