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Effect of Different Olive Oils on Bile Excretion in Rats Fed Cholesterol-Containing and Cholesterol-Free Diets

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The mechanism of the hypocholesterolemic effect of olive oils was investigated in 60 Wistar rats adapted to cholesterol-containing and cholesterol-free diets. The rats were divided in six diet groups of 10. The control group was fed only basal diet (BD), which contained wheat starch, casein, cellulose, and mineral and vitamin mixtures. For the five other groups, 10 g/100 g virgin (virgin group) or lampante (lampante group) olive oils, 1 g/100 g cholesterol (chol group), or both cholesterol and oil (chol/virgin and chol/lampante groups) were added to the BD. The experiment lasted 4 weeks. Before and after the experiment the bile was collected, and its flow and biliary bile acids and cholesterol concentrations were registered. Plasma lipids, liver cholesterol, plasma antioxidative potential (TRAP), fecal output, fecal bile acids, and fecal cholesterol excretion were measured. Groups did not differ before the experiment. After the experiment significant hypocholesterolemic and antioxidant effects were registered mainly in groups of rats fed cholesterol-containing diets supplemented with both olive oils (chol/virgin and chol/lampante). Significant increases in the bile flow and in the bile cholesterol and bile acids concentrations were observed (19.2% and 16.9%, 30.5% and 18.2%, and 79.6% and 45.6% for the chol/virgin and chol/lampante groups, respectively). Also, significant increases of the fecal output and fecal excretion of bile acids and cholesterol in rats of these groups were found. In conclusion, olive oils positively affect plasma lipid metabolism. The hypocholesterolemic effect of olive oils is genuine and is most likely mediated through increases in bile flow and biliary cholesterol and bile acids concentrations and subsequent increases in their fecal excretion.

KEYWORDS: Olive oils; rats; bile volume, bile cholesterol, bile acids; fecal excretion

INTRODUCTION

Epidemiological and clinical investigations have demonstrated significant decreases in morbidity and mortality from cardiovascular and other diseases among fruit and vegetable consumers (*1*−*4*). In the past decade some authors have recommended adding olive oils to the diet along with fruits and vegetables (*5*, *6*). These authors indicate that olive oils possess hypolipidemic properties, and therefore the use of these oils could be very beneficial for patients suffering from hypercholesterolemia—one of the major risk factors of atherosclerosis (*7*−*10*).

In our recent experiments on laboratory animals (*11*), we have found that the addition of olive oils to cholesterol-containing diets led to hypocholesterolemia and to a decrease in the content of total cholesterol in liver. The primary hypothesis concerning the mechanism of the cholesterol-lowering effect of diets supplemented with dietary fibers of fruits and vegetables is an increased excretion of cholesterol and bile acids (*12*−*15*). It was shown that the cholesterol-lowering effect of dietary fiber of guar gum is due to higher biliary cholesterol and biliary acid flux and enhanced fecal losses of these compounds (*16*). However, the content of dietary fibers in vegetable oils is minimal. Is the cholesterol-lowering effect of olive oils in rats genuine or maybe a redistribution of cholesterol in the animal

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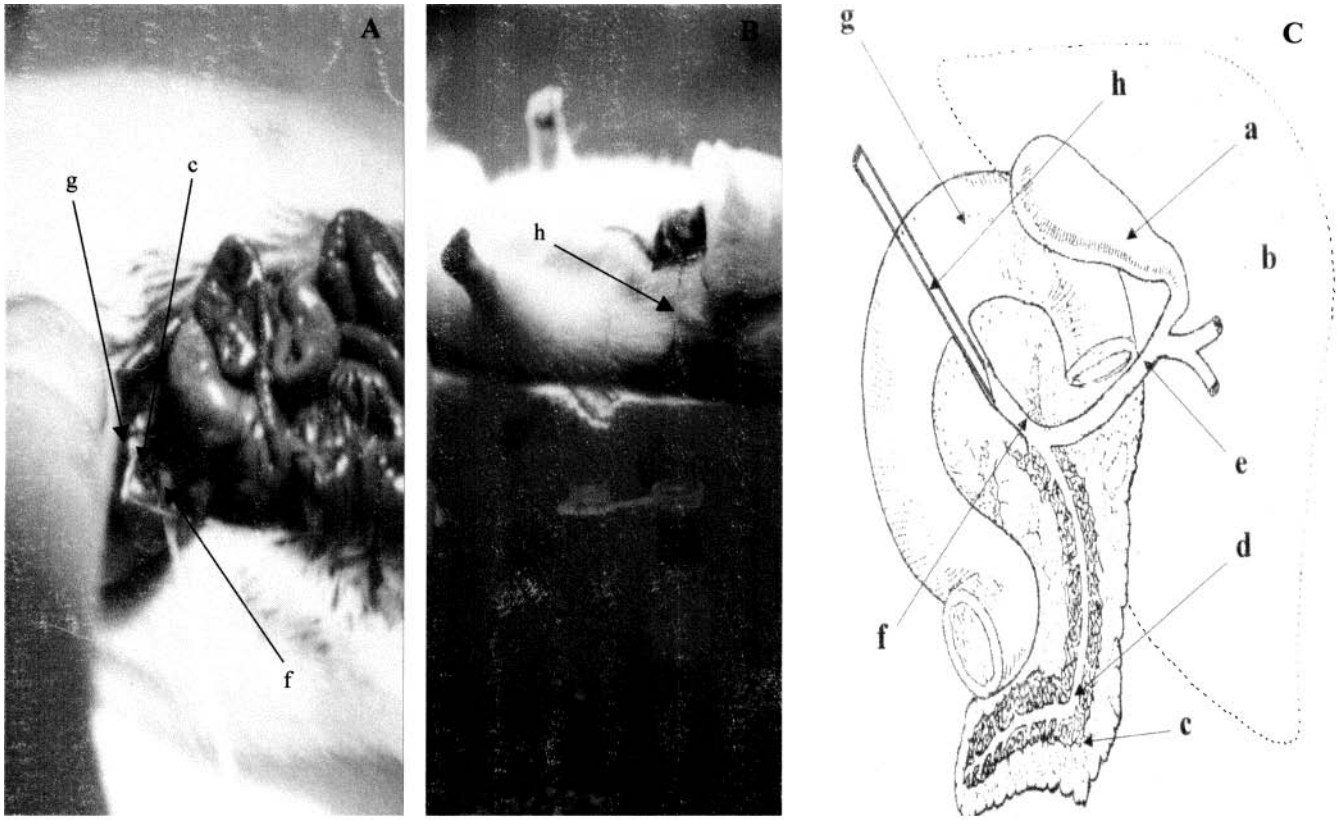

Figure 1. (A) Experimental picture: c, common bile duct; f, catheter for bile and pancreatic juice collection; g, duodenum. (B) Collection of pancreatic juice and bile: h, the place of inserted catheter for bile and pancreatic juice collection. (C) Schematic presentation of the pancreatic and bile duct systems in rat: a, gall blader; b, liver; c, pancreas; d, pancreatic duct; e, bile duct; f, common bile duct; h, catheter for bile and pancreatic juice collection.

body? To answer this question, we decided to study the effect of olive oils on bile flow, bile cholesterol and bile acids concentrations, and fecal losses of these compounds in rats fed cholesterol-containing and cholesterol-free diets.

We also wanted to verify the connection between the antioxidant potential of olive oils and the above-mentioned variables. Therefore, two olive oils with different antioxidant potentials were used.

As far as we know, there are no similar investigations concerning olive oils.

MATERIALS AND METHODS

Oil Samples. In our previous investigation (*11*) we found that virgin olive oil possesses the highest and lampante olive oil the lowest contents of antioxidant compounds and, as a consequence, antioxidant potential (*11*). Therefore, in this study were used these olive oils. The studied antioxidant compounds were determined as follows: phenols were extracted from the oils as described by Espin et al. (*17*) and Pellegrini et al. (*18*), and the content was determined according to the Folin−Ciocalteu method (*19*), using gallic acid as a standard for the calibration curve. Tocopherols were evaluated using normal phase HPLC.

Bile Collection. The collection of the bile was performed according to the method of Zabielski et al. (*20*).

A catheter (0.50 mm i.d., 0.63 mm o.d., of SIMS Portex Ltd.) was inserted into the common bile duct as is shown in **Figure 1**. The bile was collected under general urethane narcosis (1.8 g of urethane/kg of the animal's body weight) during 1 h into a preweighed tube that had been cooled on ice for 30 min.

The bile flow and the biliary bile cholesterol and bile acids contents were determined according to the method of Zabielski et al. (*20*).

Plasma Lipids and Liver Cholesterol Determination. It is generally accepted that the most reliable data on blood lipid metabolism can be obtained from fasting animals, 14−16 h after their last feeding. Therefore, food was removed from the cages at 6:00 p.m. the day before, and samples were collected at 9:00 a.m. of the next day. Total plasma cholesterol (TC) was determined with Randox kit reagents, catalog no. CH 280, appl. no 7 (*21*), high-density lipoprotein cholesterol (HDL-C) was determined according to the method of Izawa et al. (*22*), low-density lipoprotein cholesterol (LDL-C) was determined using the Friedewald et al. method (*23*), triglycerides (TG) were determined with Randox kit reagentss, catalog no. 1697, appl. no. 8 (*24*), and total phospholipids (TPH) were determined with ANALCO kit reagents catalog no. A-161 (*25*). For the determination of liver cholesterol 0.5 g of liver tissue was homogenized in 2 mL of 0.9% NaCl. Homogenized liver was centrifuged two times for 10 min at 3000/min. Then the total cholesterol was determined (*21*), with Randox kits reagent catalog no. CH 280, appl. no. 7 (International Headquarters Randox Laboratories, Distributor Hand − Prod, Warsaw, Poland).

To be able to compare the present results with the results of our previous investigations (*11*, *26*−*28*), coefficients of correction were used (for TC, −1.55; LDL-C, −2.19; HDL-C, −1.92; TG, −0.7; and TC in liver, −2.55).

Total Radical-Trapping Antioxidative Potential (TRAP) Determination. The determination of the plasma radical-trapping antioxidative potential was done as previously described (*11*).

Determination of Fecal Cholesterol and Fecal Bile Acid Excretion. Feces were collected 3 days before and on the three final days of the experiment. They were freeze-dried, weighed, and milled using a standard laboratory mill, and then the fecal cholesterol and bile acids were determined (*29*−*31*).

Rats. The Animal Care Committee of Warsaw Agriculture University approved this study.

Sixty male Wistar rats with the initial weight of 120 g were used in this experiment. They were provided by the Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences (Jablonna, Poland). All rats were housed individually in stainless steel metabolic cages and were divided into six groups of 10.

Diets. All groups of rats were fed a basal diet (BD) that included wheat starch (68.5%), casein (17.5%), cellulose (8%), and mineral (5%)

Table 1. Some Antioxidant Compounds and TRAP of the Two Used Olive Oils[a]

sample	tocotrienols (mg/kg)	tocopherols (mg/kg)	total polyphenols (mg/kg)	*o*-diphenols (mg/kg)	TRAP (nmol/mL)
extra virgin	329 ± 31.1a	353 ± 23.7a	4.6 ± 0.4a	2.7 ± 0.2a	668 ± 49.2a
lampante	148 ± 15.1b	169 ± 15.8b	2.1 ± 0.2b	1.3 ± 0.2b	225 ± 42.8b

[a] Data are means ± SD of five measurements. Means in columns without letters in common differ significantly ($P < 0.05$).

Table 2. Plasma Lipids and Total Cholesterol Concentration in Liver of Rats Fed Diets with and without 1% Cholesterol and with and without Virgin and Lampante Oils[a]

	plasma lipids (mmol/L)					liver (μmol/g)
diet	TC	LDL-C	HDL-C	TG	TPH	TC
control	2.84 ± 0.15c	1.21 ± 0.05c	1.63 ± 0.07a	0.70 ± 0.04b	1.75 ± 0.08a	5.88 ± 0.24c
lampante	2.83 ± 0.15c	1.19 ± 0.05c	1.64 ± 0.07a	0.70 ± 0.05b	1.71 ± 0.08a	5.81 ± 0.24c
virgin	2.79 ± 0.15c	1.17 ± 0.05c	1.61 ± 0.07a	0.71 ± 0.05b	1.70 ± 0.08a	5.77 ± 0.24c
chol	4.80 ± 0.21a	3.18 ± 0.12a	1.62 ± 0.07a	0.86 ± 0.05a	1.73 ± 0.08a	48.1 ± 0.31a
chol/lampante	3.71 ± 0.18b	2.07 ± 0.05b	1.63 ± 0.07a	0.74 ± 0.05b	1.36 ± 0.06b	31.7 ± 0.25b
chol/virgin	3.59 ± 0.18b	1.91 ± 0.05b	1.65 ± 0.07a	0.72 ± 0.05b	1.30 ± 0.06b	30.6 ± 0.25b
two-way ANOVA (*P* value)						
lampante	NS	NS	NS	NS	NS	NS
virgin	NS	NS	NS	NS	NS	NS
chol	<0.001	<0.001	NS	<0.001	NS	<0.001
chol/lampante	<0.050	<0.050	NS	<0.050	<0.010	<0.050
chol/virgin	<0.050	<0.050	NS	<0.050	<0.010	<0.050

[a] Values are means ± SD, $n = 10$. Means in columns without letters in common differ significantly ($P < 0.05$). Abbreviations: chol, nonoxidized cholesterol; HDL-C, HDL cholesterol; HDL-PH, HDL phospholipids; LDL-C, LDL cholesterol; NS, not significant; TC, total cholesterol; TG, triglycerides; TPH, total phospholipids.

and vitamin (1%) mixtures. The mineral mixture included $CaHPO_4$, KCl, NaCl, K_2HPO_4, $MgCl_2$, Fe_2O_3, $MnSO_4$, $ZnSO_4 \cdot 7H_2O$, KO_3, and $CuSO_4 \cdot 7H_2O$ and the vitamin mixture nicotinamide, calcium pantothenate, thiamin, riboflavin, pyridoxine, and folic acid. The control group was fed only the BD. The other five groups were named virgin, lampante, chol, chol/virgin, and chol/lampante. To the BD of these groups were added 10 g/100 g virgin or lampante oils, 1 g/100 g nonoxidized cholesterol (chol), or both for the chol/virgin and chol/lampante groups, respectively.

Peanut oil (Salvadori Factory, Florence, Italy) with minimal antioxidant capacity according to the TRAP test was used as control oil in the diets for the control and chol groups at a concentration of 10 g/100 g. Cholesterol of analytical grade (USP) was obtained from Sigma Chemical Co., St. Louis, MO. The dietary cholesterol was checked by the HPLC method of Ansari and Smith (*32*) and did not contain cholesterol oxides. The cholesterol batches were mixed carefully with the BD (1:99) just before the diets were offered to the rats. The diets have contained as percentage of energy 67% of carbohydrates, 24% of protein, and 9% of fat. The calculated energy of the used diets was from 394.9 to 400.1 kcal/100 g, and according to ANOVA these differences were statistically not significant ($P < 0.35$).

All rats were fed once a day at 10:00 a.m. ad libitum. They had unrestricted access to drinking water. Food intake and body weight gains were monitored daily.

The experiment lasted 4 weeks. Before the experiment, blood samples were taken from the tail vein. At the end of the trial the rats were anesthetized using diethyl ether and blood samples were taken from the left atrium of the heart. Plasma was prepared and used for laboratory tests. After anesthesia, the abdomen was opened to take samples of the liver for TC determination.

Two time points were used in this experiment: before and after 28 days of feeding period. At these time points the above-mentioned laboratory tests were performed.

Statistics. Values are given as the means ± standard deviation (SD) of five measurements for all studied antioxidant compounds and antioxidant potential. For the in vivo studied parameters the means ± SD are for 10 animals of every group. When appropriate, data were tested by two-way ANOVA, using GraphPad Prism, version 2.0 (GraphPad Software, San Diego, CA), followed by Duncan's multiple-range tests to assess differences between group means. Differences were considered to be significant at $P < 0.05$.

RESULTS

The contents of the antioxidant compounds of the used olive oils and the oils' antioxidant potential are shown in **Table 1**. As can be seen, the contents of all studied antioxidant compounds and the antioxidant potential of the virgin olive oil were significantly higher than those of the lampante olive oil.

Addition of oils or/and cholesterol to the diets did not significantly affect food intake, body weight gain, or feed efficiency (*11*).

According to statistical evaluation (ANOVA), at baseline, the five experimental groups did not differ from the control group in plasma lipid concentrations (P in all cases was >0.05).

After 4 weeks of the feeding period, the oil-supplemented diets significantly prevented the rise in plasma TC, LDL-C, TG, and TC in liver (chol/virgin and chol/lampante groups versus the chol group, $P < 0.05$, respectively), which were increased due to dietary cholesterol in diets (**Table 2**). A significant decrease was also found in the level of TPH in the chol/virgin and chol/lampante groups versus the chol group (−24.9% and −21.4%, $P < 0.05$ in both cases).

Virgin and lampante oils in rats fed the BD without cholesterol did not affect the lipid variables measured.

The bile flow and biliary bile acids and biliary cholesterol concentrations in all groups of rats before the experiment were without significant differences.

The results of the changes in these indices after completion of the experiment are summarized in the **Figures 2**, **3**, and **4**, respectively.

As can be seen, the addition of virgin oil and, to a lesser degree, lampante olive oil to rats fed cholesterol-containing diet (chol/virgin and chol/lampante) has significantly increased the bile flow and the concentration of biliary bile cholesterol and biliary bile acids compared with the chol group ($P < 0.05$). As calculated in percentages, the increases in bile flow and biliary bile cholesterol and biliary bile acids concentrations were 19.2% and 16.9%, 30.5% and 7.5%, and 63.7% and 32.7% for the chol/

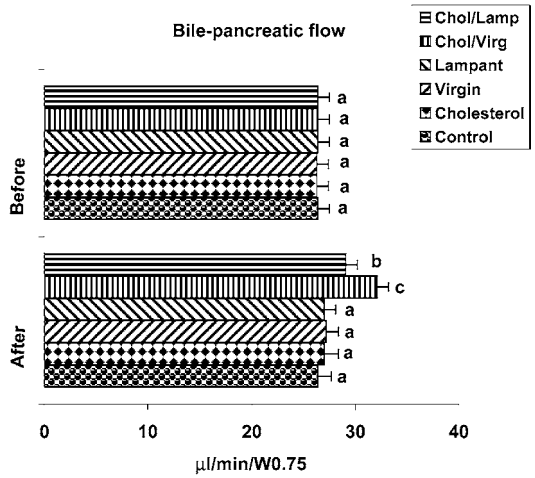

Figure 2. Changes in bile volume after the experiment [means ± SD (horizontal lines)]. Bars with different letters are significantly different ($P < 0.05$).

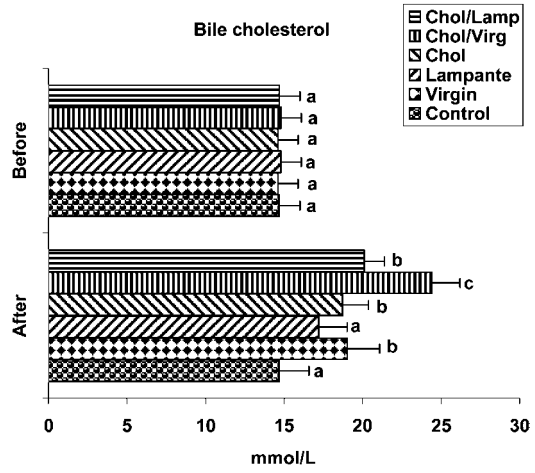

Figure 3. Changes in biliary cholesterol concentration after the experiment [means ± SD (horizontal lines)]. Bars with different letters are significantly different ($P < 0.05$).

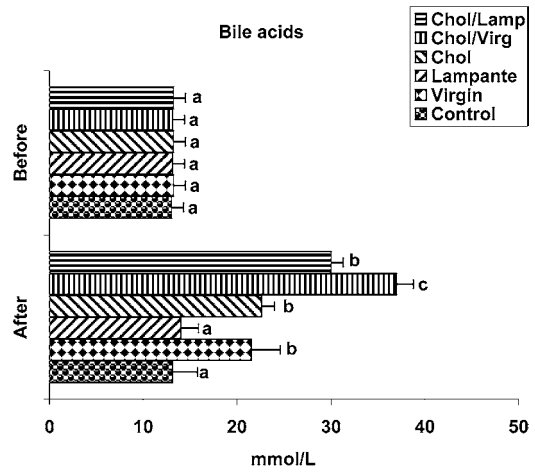

Figure 4. Changes in biliary bile acid concentration after the experiment [means ± SD (horizontal lines)]. Bars with different letters are significantly different ($P < 0.05$).

virgin and chol/lampante groups versus the chol group, respectively ($P < 0.05$ in all cases).

Addition of virgin but not of lampante olive oil to rats fed cholesterol-free diet significantly increased the concentration of biliary bile cholesterol and biliary bile acids compared with the control group ($P < 0.05$).

Addition of lampante olive oil to the group of rats fed cholesterol-free diet significantly changed only the bile volume, compared with the control group ($P < 0.05$).

Table 3. Changes in Fecal Output and Bile Acids and Cholesterol Concentrations in All Groups of Rats after Completion of the Experiment[a]

diet group	dry fecal output (g/day)	bile acids (µmol/day)	cholesterol (µmol/day)
control	0.70 ± 0.1a	14.1 ± 1.1a	13.8 ± 12a
virgin	0.72 ± 0.1a	14.5 ± 1.1a	14.1 ± 1.2a
lampante	0.71 ± 0.1a	14.3 ± 1.1a	13.9 ± 1.2a
chol	0.81 ± 0.1a	17.1 ± 1.6a	15.2 ± 1.3a
chol/virgin	1.32 ± 0.1b	26.3 ± 2.5b	19.3 ± 1.5b
chol/lampante	1.13 ± 0.1b	22.1 ± 2.1b	18.6 ± 1.4b

[a] Data are means ± SD ($n = 10$ for every group). Means in columns without letters in common differ significantly ($P < 0.05$).

Changes in fecal output and bile acids and cholesterol concentrations in all groups of rats after completion of the experiment are summarized in **Table 3**. As can be seen, the dry weight of the fecal output was significantly increased in the chol/virgin and chol/lampante versus chol group (+63% and +39.5%, respectively).

The fecal bile acids excretion was significantly greater in the chol/virgin and chol/lampante groups than in chol group (+53.8% and +29.2%, respectively).

Also, fecal cholesterol excretion was significantly increased in the chol/virgin and chol/lampante groups versus the chol group (+27.0% and +22.4%, respectively).

Correlations were calculated between the total antioxidant potential of the used oils and the differences in plasma cholesterol levels in the chol/virgin and chol/lampante groups versts the chol group (**Figure 5**, parts A and B, respectively). As can be seen, the correlations in both cases were very high ($R^2 = 0.9749$ and 0.9045 for virgin and lampante olive oils, respectively). However, the correlation for the virgin oil, which possesses the highest antioxidant potential, was higher ($R^2 = 0.9749$).

DISCUSSION

Some authors claim that olive oils possess hypolipidemic properties (*33*−*35*). In our previous investigation we also found that olive oils positively influenced plasma lipid metabolism and plasma antioxidant capacity in rats (*11*). It was more evident in groups of rats fed cholesterol-containing diets. However, we could not prove if this effect is genuine or if it is a redistribution of cholesterol in the animal body. Therefore, we decided to conduct an investigation on laboratory animals to find out the possible changes in bile flow, biliary cholesterol and biliary bile acids concentrations, and their fecal excretion. If addition of oils would lead to an increase in the above-mentioned parameters, then the hypolipidemic effect of the studied oils is genuine.

As in our previous experiments (*11*, *36*), we have registered the same tendencies: addition of virgin or lampante olive oils to rats fed cholesterol-containing diet has a positive influence the plasma lipid metabolism and the plasma antioxidant capacity.

A significant increase in the bile flow and in the biliary cholesterol and bile acids concentrations in the chol/virgin and chol/lampante groups of rats compared with the chol group has been registered. Also, the dry weights of the fecal output, the

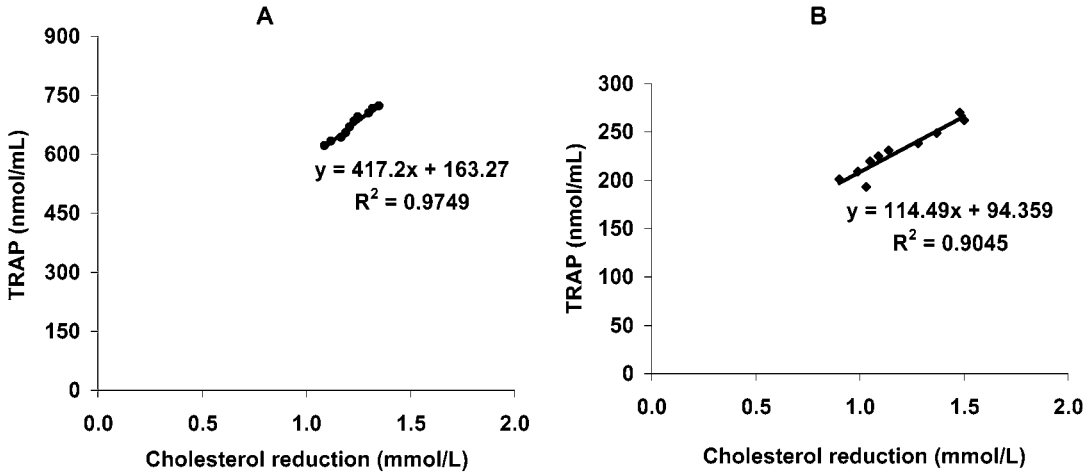

Figure 5. Correlation between plasma hypocholesterolemic effect and antioxidant potential of virgin (A) and lampante (B) oils.

fecal bile acid, and fecal cholesterol excretion were significantly increased in these groups of rats.

Therefore, the higher biliary flux led to greater fecal elimination of bile acids and bile cholesterol. The highest biliary flux and the greatest fecal elimination of bile acids and cholesterol were observed in rats of the chol/virgin dietary group.

We have expected these results: we used diets supplemented with 1% of dietary cholesterol and have added highly effective olive oils. These results are in accordance with the data of others, who have found that supplementation of the diet with cholesterol and cholesterol-lowering compounds significantly increases biliary flux and consequently the fecal elimination of bile acids (*16*).

On the other hand, addition of virgin or lampante olive oils to groups of rats fed cholesterol-free diet did not significantly affect the biliary cholesterol and bile acids concentrations or their fecal elimination.

According to our data, the plasma hypocholesterolemic effect of olive oils is genuine: (a) we have found a significant decrease in the liver cholesterol in groups of rats fed added olive oils, and therefore the liver did not become a depot for the dietary cholesterol; (b) the hypocholesterolemic effect was mediated by the increases in bile flow and biliary cholesterol and bile acids concentrations and subsequent increases in fecal output and fecal bile acids and fecal cholesterol excretion in cholesterol-fed rats (chol/virgin and chol/lampante).

Also, others have suggested that the cholesterol-lowering effect is mediated by an increase in the intestinal pool of bile acids (*16*, *31*, *37*).

The calculated correlations between the antioxidant potential of olive oils and their plasma hypocholesterolemic effect have shown a direct relationship: the higher the antioxidant potential, the greater the hypocholesterolemic effect. These findings are very relevant: they explain the mechanism of higher bioactivity of oils with a high antioxidant potential. Therefore, from a practical point of view, to obtain the best results, it is preferable to use an olive oil with high antioxidant potential.

The mechanism of the hypocholesterolemic and increased bile flow effects of olive oils cannot be explained in the same way as the effects of dietary fibers: the content of dietary fibers is olive oils is minimal. However, it can be explained in another way. We have found in this experiment a direct correlation between the contents of antioxidant compounds (antioxidant activity) of olive oils and their hypocholesterolemic effect. Therefore, we supposed that antioxidants of olive oils are their main bioactive compounds, which leads to this effect. To add further support to this hypothesis, we are planning to conduct a similar experiment using extracted antioxidant compounds instead of whole olive oils. We intend to use in the future experiment rabbits or hamsters, which are much closer to humans with regard to lipid metabolism than rats. To obtain more data about cholesterol catabolism, we will also measure cholesterol in liver and in plasma as free and esterified cholesterol.

CONCLUSIONS

1. Olive oils positively affect lipid metabolism. The hypocholesterolemic effect of olive oils is genuine and is most likely mediated through an increase in the bile flow, an increase in biliary cholesterol and bile acids concentrations, and subsequent increases in the fecal excretion of these compounds.

2. The antioxidants of olive oils are their main bioactive compounds and play a leading role in the mechanism of the hypocholesterolemic effect.

3. To obtain the best hypocholesterolemic effect, it is necessary to use olive oils with high antioxidant potential.

4. Olive oil producers should label the antioxidant potential of their products.

ABBREVIATIONS USED

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NOC, nonoxidized cholesterol; TC, total cholesterol; TG, triglycerides; TPH, total phospholipids; TRAP, total radical-trapping antioxidative potential.

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