



Hypolipidemic Effect of Beer Proteins in Experiment on Rats

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Amino acid analysis, electrophoretic separation and Fourier transform-infrared spectra (FT-IR) were used to determine and characterize proteins and amino acids in two types of beer: with a high content of proteins (Beer 1) and with a low content of proteins (Beer 2). The concentration of total proteins, albumin and of most studied amino acids in Beer 1 were significantly higher than in Beer 2 (P<0.05–0.0005). Thirty-six rats were divided in three groups, each 12. The rats of the control group were fed basal diet (BD) only and the BD of the two experimental groups (Beer 1 and Beer 2) was supplemented with lyophilized, polyphenol-free Beer 1 and Beer 2, respectively. Before and after completion of the 4 w feeding period the total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol, triglycerides (TG), lipid peroxides (LP) and total radical-trapping antioxidative potential (TRAP) in all three groups were examined. In Beer1 group a significant decrease in the level of TC, LDL-C and TG was registered (P<0.05, 0.05 and 0.005, respectively). No differences in the level of LP and TRAP in all three groups were found. This investigation proves that beer with high protein and essential amino acid concentrations does affect the plasma lipids level in rats.

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Introduction

In most Western countries alcohol beverages are an integral part of diets (Renaud and Lorgeril, 1992). Epidemiological, experimental and clinical investigations show that various alcoholic drinks have a cardio protective effect when consumed in such diets (Thun *et al.*, 1997). Moderate consumption of alcoholic beverages leads to improved lipid metabolism and

to increased antioxidant and anticoagulant activity (Renaud and Lorgeril, 1992; Gorinstein *et al.*, 1997a, b). Until now, red wine has been regarded as the main cardio protective beverage (Klatsky and Armstrong, 1993). But recently published results by Innes (1998) suggest that beer may also have beneficial effects on risk factors of the heart diseases. Participants in his investigation consumed red wine or beer and he found that effectiveness in both groups were equal. According to Innes, effectiveness, low cost, acceptable adverse-event profile and single-dose dispensers of beer are the basis for replacing red wine as the cardioprotective beverage. Thus, it was decided to use beer in this

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investigation. However, it must be emphasized that extensive consumption of energy as alcohol may predispose to obesity and other problems and that cigarette smoking can negate the benefits of moderate beer consumption (Williamson *et al.*, 1987). Some authors have shown that the positive biochemical changes in the moderate alcoholic beverages consumers are also connected to polyphenols in dry matter (Cestaro *et al.*, 1996; Gorinstein *et al.*, 1998a,b; Carbonneau *et al.*, 1998). However, the dry matter of alcoholic beverages contains not only polyphenols, but also proteins and amino acids. It is common knowledge that proteins and essential amino acids of food are supplying the required building blocks for protein biosynthesis of humans (Belitz and Grosch, 1999). Now some authors suggest that these substances have much wider biological functions (Niazi *et al.*, 1979; Morgan, 1986). A well-documented experiment shows that casein or whey protein fed to piglets during the suckling period affects blood lipid levels, HMG CoA reductase activity, glucagon, cortisol, and weight gain (Larson *et al.*, 1996). It must also be mentioned that dietary plant proteins (especially soy) play a positive role in plasma cholesterol control (Anderson *et al.*, 1995).

It could not be excluded that proteins and essential amino acids of alcoholic beverages are biologically active. In our early experiments on rats with beer and white wine, we did not take into consideration the proteins of these beverages (Gorinstein *et al.*, 1998a,b). Are they playing a certain role in improving lipid metabolism and antioxidant activity?

In order to answer this question two trade beers, with a high content of proteins (Beer 1) and with a low content of proteins (Beer 2) were chosen. It was decided to study the quantitative content and qualitative characteristics of proteins and essential amino acids in Beer 1 and Beer 2 and to compare their influence on the plasma lipids and the total antioxidant activity in rats. In order to exclude the influence of alcohol and phenolics, lyophilized polyphenol-free Beer 1 and Beer 2 were used. To the best of our knowledge there are no such studies.

Materials and Methods

Chemical materials

All reagents were of analytical grade. Deionized and distilled water was used throughout. All the chemicals used were purchased from Sigma Chemical Co.

Beer samples

As was mentioned, in this experiment two trade beer samples, were used with a high content of protein (Beer 1) and with a low content of protein (Beer 2). The contents of the most important components in Beer 1 and Beer 2 are summarized **Table 1**. As can be seen, the two beers significantly differ in their protein concentration. It was found by the method of Prosky *et al.* (1992), that the content of dietary fiber in both beers was minimal.

Protein extraction

The samples of high molecular weight fraction of Beer 1 and Beer 2 were prepared according to Dale and Young (1988). Polyphenol substances were removed from beer samples by extraction with ethyl alcohol. Then proteins were extracted using solid ammonium sulfate, which was added to beer to obtain 80% relative saturation of ammonium sulfate. The precipitate formed during 48 h at room temperature was collected by centrifugation (20 min at $7,000 \times g$). Samples were placed in dialysis tubing, using 2000 Da tubes, and dialysed against several changes of distilled water for 72 h. The material retained by the dialysis membrane was freeze-dried and storage at 4°C until use. This fraction was designated as Ammonium Sulfate Protein Precipitate (ASPP).

The protein content

Proteins were determined according to Lowry *et al.* (1951) and Bradford (1976), using Uvikon 930 spectrophotometer as nitrogen and calculated using a factor of 6.25. Albumin concentrations were measured colorimetrically at 628 or 600 nm using Sigma diagnostic kits 631-2 and 625-2, respectively.

Fourier transform-infrared spectra (FT-IR)

A Perkin-Elmer 2000 FT-IR spectrometer was used to record IR spectra. Lyophilized material was mixed with KBr and the pellet was pressed at $10,000 \text{ kg/cm}^2$ for 15 s.

Amino acid content

Amino acid composition in bound form was done by the procedure of Spackman *et al.* (1958). Two separate assays were conducted for amino acid determination: one for all amino acids based on acid hydrolysis. During this procedure, tryptophan was degraded by acid hydrolysis. In order to prevent the degradation of tryptophan, second separate assay was conducted using hydrolysis with LiOH.

Therefore in the first assay, lyophilized samples were hydrolysed for 22–44–66 h at 110°C with 6 mol/L HCl in evacuated tubes with and without previous oxidation by performic acid. The vacuum-dried hydrolyzate was analysed and applied on a Beckman 120C automatic amino acid analyzer. In the second assay, lyophilized samples were hydrolysed with 4 mol/L LiOH for 20–24–28–36–40 h at 110°C followed by treatment with 6 mol/L HCl for 22 h at the same temperature for tryptophan determination.

Electrophoretic separation

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli (1970), on homemade acrylamide mini gels with gradient of 10–20%. Equal amounts of protein samples ($20 \mu\text{g}/\mu\text{L}$) were loaded on the gels. M_r markers (Sigma) were from 14 to 90 kDa. The sample buffer contained 10 mmol/L Tris-HCl, pH 8.0, 25 g/L SDS, 1 mmol/L

EDTA, 0.1 g/L bromophenolblue, and in the case of reduced peptides, 50 g/L 2-mercaptoethanol (2-ME). Extracted proteins from beer samples were dissolved in this sample buffer. Then all the prepared samples were put on gel. The run was carried out for 2 h. The gel was fixed and stained with Coomassie Brilliant Blue R-250 or silver stained and destained, as reported by Van-Seuningen and Davril (1992).

Animals

The Animal Care Committee approved and supervised this study. Thirty-six male Wistar rats with initial standard weight of 120 g each were used in this experiment. All rats were divided into three groups: two experimental (Beer 1 and Beer 2) and one control (CG), each of 12. The rats were housed individually in stainless-steel metabolic cages.

Diets

The duration of the experiment was 4 wk (Gorinstein *et al.*, 1998a–d, 2000). During this period, all three groups of rats were fed basal diet (BD), which included (g/kg diet): wheat starch (690), casein (180), soybean oil (60), mineral (60) and vitamin (10) mixtures. The BD contained, as percentage of energy 62% carbohydrates, 24% fats and 14% of protein. The BD of all groups of rats was equal in protein content and in amino acids composition, which included valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine, tryptophan and threonine. The rats of CG were only fed BD. The dry matter equal to 6.0 mL of original beer was properly prepared and was given separately daily to the rats of Beer 1 and Beer 2 groups. In our early investigations, we had experimented with the same quantity of beer and proved that whole beer positively affects lipid metabolism and antioxidant activity in rats (Gorinstein *et al.*, 1998a,b). As was stated, in order to test the influence of proteins and amino acids only lyophilized polyphenol-free Beer 1 and Beer 2 were used. It was verified that after extraction of polyphenols the two kinds of beer have negligible total radical-trapping antioxidative potential (TRAP).

The diets were served once a day at 10 a.m. ad libitum together with the dry matter of the beverages introduced with distilled water by stomach intubations. The food intake was monitored daily and body gains on a weekly basis. The energy of the BD supplemented with beverages for the rats of Beer 1 and Beer 2 groups (397.3–401.7 kcal/100 g of diet) and the energy of the BD for rats of CG (393.7 kcal/100 g of diet) did not differ.

Laboratory tests

Before and after completion of 4 wk feeding period, blood samples from the tail vein were drawn. After the centrifugation plasma was removed, a wide range of laboratory tests was performed. These tests included total cholesterol (TC), low-density lipoprotein chole-

sterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), lipid peroxides (LP) and total radical-trapping antioxidative potential (TRAP). TC, HDL-C and TG were determined enzymatically. TC and TG were measured as described by Trinder and Webster (1984) with kits (PAP 100, # 6.122.4 and 6.123.6, respectively). HDL-C was determined by enzymatic methods after the precipitation of LDL-C and VLDL-cholesterol fractions with phosphotungstic acid in the presence of magnesium ions with kit (cat. number 6.159.1; Bio Merieux). LDL-C was calculated by using the Friedewald equation (Friedewald *et al.*, 1972). Lipid peroxides were determined colorimetrically (Tateishi *et al.*, 1987) in direct reaction between methylene blue derivative (MCDP, 10-*N*-Methylscarbamoyl-3, 7-dimethylamino-10H-phenothiazine) catalysed by hemoglobin using kit 9#CC-004 from Kamiya Biomedical Company (Seattle, WA, U.S.A.). In our previous experiments with laboratory animals, we used only the measurements of lipid peroxides for evaluation of the plasma antioxidant status of rats (Gorinstein *et al.*, 1998a–d, 2000, 2001). Body's antioxidant system includes antioxidant enzymes as well as small antioxidant molecules. In the plasma of the laboratory animals the nonenzymatic antioxidants are of major importance (Slavikova *et al.*, 1998). Therefore, this time we also decided to also measure the total radical-trapping antioxidative potential (TRAP). The TRAP measurement was performed as previously described (Slavikova *et al.*, 1998).

Statistical analysis

To verify the statistical significance of all parameters the values of means, standard deviation and 95% CI of means were calculated. Where it was appropriate, the data by 2-way ANOVA were tested. The *P* values of less than 0.05 were adopted as statistically significant. All following data are means of five measurements.

Results and Discussion

Proteins and amino acids

Table 1 summarizes the analytical indices in Beer 1 and Beer 2. As can be seen, the total protein and albumin contents were significantly higher in Beer 1 than in Beer 2 ($P < 0.005$ in both cases).

The content of all studied amino acids except lysine, arginine, tryptophan and threonine were significantly higher in Beer 1 than in Beer 2 (**Table 2**).

The results of the electrophoretic separation of the proteins of Beer 1 and Beer 2 are shown in **Fig. 1**. Beer 1, (**Fig. 1**, lane 2) and Beer 2 (**Fig. 1**, lane 3) demonstrate similar bands. The samples show that the main protein material remains in one band of 14 and at 43–45 kDa. All the patterns have two bands in common of 14 and 43 kDa. It is a complete similarity in the bands of Beer 1 and Beer 2. These results are in accordance with the results of our previous investigation (Gorinstein *et al.*,

Table 1 Analytical indices in Beer 1 and Beer 2

	Beer 1	Beer 2	<i>P</i>
Total proteins (g/L)	5.7±0.5	4.3±0.4	<0.05
Albumin (g/L)	2.5±0.2	2.0±0.2	<0.05
Sugars (g/100 g dry matter)			
Glucose	0.9±0.1	0.8±0.1	NS ^a
Maltose	38.5±3.2	38.6±3.3	NS
Maltotriose	30.5±3.1	31.1±3.0	NS
Dextrins	30.1±3.1	29.5±3.0	NS
Total polyphenols (mg/L)	346.1±31.2	338.9±30.8	NS
Alcohol (g/100 mL)	5.3±0.5	5.1±0.5	NS

Values are means±SD of 5 measurements.

^aNS, not significant.

Table 2 Essential amino acids in Beer 1 and Beer 2^a

Amino acids	Beer 1	Beer 2	<i>P</i>
Valine	192.8±20.5 [142.7–242.9]	136.5±13.1 ^b [102.9–170.1]	<0.0500
Methionine	56.1±5.6 [41.7–70.5]	26.7±2.4 [20.5–32.9]	<0.0025
Isoleucine	269.7±21.7 [213.9–325.5]	198.1±17.1 [104.2–192.0]	<0.0050
Leucine	264.8±26.6 [206.7–322.9]	152.5±15.2 [113.4–191.6]	<0.01000
Phenylalanine	114.9±11.6 [106.9–44.7]	66.6±6.7 [49.4–83.8]	<0.01000
Histidine	115.7±9.6 [91.0–140.4]	85.7±8.8 [63.1–108.3]	<0.05000
Lysine	247.9±20.9 [194.2–301.6]	198.4±18.1 [151.9–244.9]	<0.10000
Arginine	231.6±22.1 [174.8–288.4]	217.9±20.9 [164.2–271.6]	<0.35000
Tryptophan	5.2±1.4 [1.6–8.8]	5.1±1.4 [1.5–8.7]	<0.48750
Threonine	261.1±26.7 [192.5–329.7]	257.3±26.6 [188.9–325.7]	<0.47500

^aIn mg/L.

^bMean values, 95% CI of means ± SD of five measurements.

1990) and with those of other authors (Dale and Young, 1988; Yokoi and Tsugita, 1988).

Infrared spectra of proteins of the two beers showed similar bands at 3373 cm⁻¹ (amino acid peak) and at 2936 cm⁻¹ (–CH₂ stretching vibrations). Peaks at 3373 and 3320 cm⁻¹ were similar in the two samples.

Amide I (A I), Amide II (A II) and Amide III (A III) bands (in the range of 1650, 1530 and 1300–1250 cm⁻¹) were typical for a protein spectrum (Kaiden *et al.*, 1987).

Experiment on rats

Addition of lyophilized polyphenol-free beer to the BD of Beer 1 and Beer 2 groups of rats did not cause significant changes in diet intake, body gains or feed conversion efficiency (**Table 3**).

At baseline the rats of Beer 1 and Beer 2 groups did not differ from one another in the plasma lipid concentration and the level of LP (data not shown).

After the trial a significant decrease in the level of TC, LDL-C and TG in the Beer 1 diet group was registered

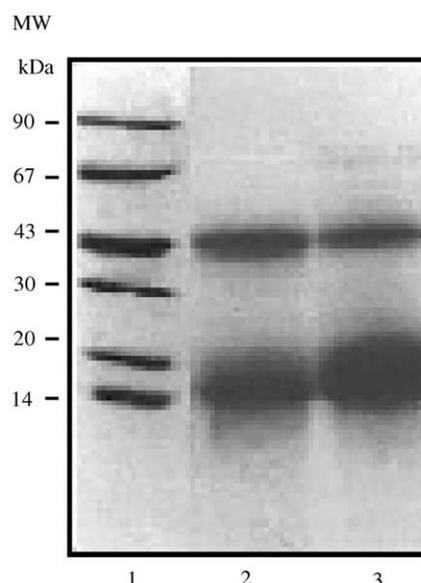


Fig. 1 SDS-PAGE analysis of beer samples. Ammonium sulfate protein precipitate (ASPP) separated on precast commercial gradient gels of 10–20%: lane 1—molecular marker; lane 2 — beer 1; lane 3 — beer 2; Mr markers had the following sizes: 14; 20; 30; 43; 67; and 90 kDa; proteins were detected by silver staining

(*P*<0.05, 0.05 and 0.005, respectively). No significant changes in the level of lipids in Beer 2 and control groups of rats were observed. The level of lipid peroxides in all three groups remained unchanged (**Table 4**). As was mentioned, this time we also decided to also measure the total radical-trapping antioxidative potential (TRAP), which more precisely reflects the plasma antioxidant capacity.

After the experiment the TRAP remained unchanged in all three groups of rats (**Table 5**).

Epidemiological, experimental and clinical investigations have shown that moderate alcoholic beverages consumption positively influences morbidity and mortality of patients with coronary atherosclerosis (Leger *et al.*, 1979; Moor and Pearson, 1986; Friedman and Kinball, 1986). This positive influence is due to a significant improvement in lipid metabolism, antioxidant and anticoagulant activities (Frimpong and Lapp, 1989; Renaud and Lorgeril, 1992). Some authors claim that the biological activity of beverages is mainly connected to polyphenols than to alcohol content (Mosinger, 1994; Fuhrman *et al.*, 1995; Cestaro *et al.*, 1996). However, Brenner *et al.* (2001) indicate that this activity is due to both components. Amino acids, peptides and proteins are important constituents of food in general and of alcoholic beverages in particular. It is common knowledge that amino acids of food supply the required building blocks for protein biosynthesis of humans (Belitz and Grosch, 1999). However, food proteins possess much wider biological functions: they affect blood lipid levels and plasma antioxidant activity (Niazi *et al.*, 1979; Morgan, 1986; Larson *et al.*, 1996). In our early experiments, we did not investigate the role of proteins in alcoholic beverages (Gorinstein *et al.*, 1998a–d). In this study total proteins,

Table 3 Diet intake, body gains and efficiency of diets*

Groups	Av. intake of diet (g/4 weeks)	Av. body gain (g/4 weeks)	Diet efficiency ratio
Beer 1	373.1 ± 49.5 ^a [265.2–481.0]	92.8 ± 29.1 ^a [29.4–156.2]	0.248 ± 0.05 ^a [0.138–0.358]
Beer 2	370.2 ± 52.4 ^a [256.0–484.4]	91.4 ± 28.9 ^a [28.4–154.4]	0.247 ± 0.05 ^a [0.137–0.357]
CG	369.3 ± 50.9 ^a [258.9–479.7]	90.9 ± 29.2 ^a [27.3–154.5]	0.246 ± 0.05 ^a [0.136–0.356]

*All results show mean values, 95% CI of means and ± SD.

Means in columns not sharing the same superscripts are significantly different ($P \leq 0.05$).

Table 4 Mean values, 95% CI of means ± SD ($n = 12$) of the plasma levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides (mmol/L) and lipid peroxides ($\mu\text{mol/L}$)

	TC	LDL-C	HDL-C	TG	LP
Control	2.79 ± 0.11 ^a [2.55–3.03]	1.20 ± 0.08 ^a [1.03–1.37]	1.55 ± 0.09 ^a [1.35–1.75]	0.65 ± 0.04 ^a [0.56–0.74]	1.21 ± 0.09 ^a [1.01–1.41]
B ¹	2.53 ± 0.10 ^b [2.31–2.75]	0.97 ± 0.08 ^b [0.80–1.14]	1.55 ± 0.09 ^a [1.35–1.75]	0.45 ± 0.04 ^b [0.36–0.54]	1.19 ± 0.08 ^a [1.02–1.37]
B ²	2.79 ± 0.11 ^a [2.55–3.03]	1.19 ± 0.09 ^a [0.99–1.39]	1.56 ± 0.09 ^a [1.36–1.76]	0.64 ± 0.04 ^a [0.55–0.73]	1.20 ± 0.09 ^a [1.00–1.40]
ANOVA (P value)					
B ¹	<0.05	<0.05	NS	<0.05	NS
B ²	NS	NS	NS	NS	NS

*Abbreviations used: B¹, experimental group fed Beer 1 supplemented diet; B², experimental group fed Beer 2 supplemented diet; CG, control group; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; LP, lipid peroxides; NS, not significant; TC, total cholesterol; TG, triglycerides. Means in a column not sharing the same superscripts are significantly different ($P \leq 0.05$).

Table 5 Mean values ± SD of the total radical-trapping antioxidative potential (TRAP) in all 3 groups of rats before and after the experiment ($\mu\text{mol/L}$)

	Beer 1	Beer 2	Control
Before	278.1 ± 26.1 ^a	277.9 ± 25.9 ^a	278.0 ± 26.0 ^a
After	277.9 ± 26.0 ^a	278.1 ± 26.1 ^a	278.2 ± 26.0 ^a

Means in columns and rows not sharing the same superscripts are significantly different ($P \leq 0.05$).

and its water-soluble fraction – albumin and essential amino acids were investigated. To exclude the influence of alcohol and phenolics lyophilized, polyphenol-free beer was used. The contents of the studied compounds are influenced by some conditions, which include climatic conditions, ripeness of the used raw materials and the technological process. Therefore, it was predictable that the results of our investigation would be slightly different from the results of other authors.

The qualitative characteristics of the studied proteins in Beer 1 and Beer 2 were identical. The contents of total proteins and albumin in Beer 1 were significantly higher than in Beer 2. The contents of most essential amino acids (except lysine, arginine, tryptophan and threonine) in Beer 1 were significantly higher than in Beer 2. As was mentioned, alcohol and polyphenols from both beers were removed. Therefore, the influence of the above-mentioned substances on lipid metabolism and antioxidant activity was excluded. After completion of the 4 wk feeding period the plasma lipid spectrum and

the antioxidant activity were examined. A significant decrease in the level of TC, LDL-C and TG was registered only in the Beer 1 diet group ($P < 0.05$, 0.05 and 0.005, respectively). No significant differences in the contents of lipid peroxides and TRAP in both experimental and in the control groups were registered. As was mentioned, some authors claim that proteins affect lipid metabolism in laboratory animals (Larson *et al.*, 1996). In our previous investigation we had compared the effect of beer *vs.* white wine on lipid metabolism and plasma antioxidant activity in rats (Gorinstein *et al.*, 2001). The two above-mentioned alcoholic beverages have different raw materials: barley and grapes, respectively. For the present investigation two types of beer with relatively high and low protein concentration (Beer 1 and Beer 2, respectively) were chosen. We tried to verify two points: (1) Could beer proteins as a supplement to the basal diet influence plasma lipid levels of rats? (2) Is this influence connected to the protein concentration? Our investigation has shown that the qualitative characteristics of the proteins of the two beers were identical. The trial has demonstrated that only diet, supplemented with Beer 1 positively influences lipid levels in rats. The difference between these two beers was the concentration of proteins. So, the answers to both questions are positive. The natural conclusion of this investigation is that only beer with high protein concentration can positively influence plasma lipid levels of rats.

It is common knowledge that dietary fiber positively influences the plasma lipid levels (Bartnikowska, 1999;

Gorinstein *et al.*, 2000). However Glore *et al.* (1994) claim that the independent ability of dietary fiber to lower plasma lipid level is controversial. Some authors even deny the hypolipidemic properties of dietary fiber (Bobek *et al.*, 1998). This controversy may be connected with low quantities of dietary fiber used by Bobek *et al.* (1998). According to Leontowicz *et al.* (2001), a hypolipidemic effect can be reached only when the BD for rats is supplemented with 50 and more grams of dietary fiber on kilograms of diet. In our experiment the dietary fiber content of Beer 1 and Beer 2 was minimal. Therefore, the positive influence on the plasma lipids of Beer 1 is due to its high content of proteins.

Some authors have shown that proteins possess antioxidant properties (Huang and Fwu, 1992; Heinonen *et al.*, 1998). Huang and Fwu (1992) have demonstrated that protein insufficiency enhances lipid peroxidation and reduces the activities of antioxidative enzymes in rats fed diets high in polyunsaturated fat. Heinonen *et al.* (1998) have reported that protein affects the antioxidant activity of phenolic compounds in a lecithin–liposome oxidation system. We did not find that proteins of beer possess antioxidant properties. Therefore, there is a need for further investigations.

Conclusions

Lyophilized, polyphenol-free beer with high concentration of proteins and essential amino acids positively affect plasma lipid level of rats: a significant decrease in TC, LDL-C and TG. Proteins of beer do not affect the plasmas antioxidant activity. It can be suggested that only polyphenols of alcoholic beverages are able to improve the plasma antioxidant status. Nevertheless, further studies are required to verify the possible antioxidant properties of the proteins of alcoholic beverages.

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