JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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# Aorta and Liver Changes in Rats Fed Cholesterol-Containing and Raw Vegetable-Supplemented Diets: Experiments in Vitro and in Vivo

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**ABSTRACT:** The aim of this investigation was to compare the liver and aorta changes in rats fed cholesterol-containing diets and the possible improvement when diets would be supplemented with frequently used raw vegetables. The phenolic compounds of three vegetables in methanol−water (1:1) fraction were characterized using electrospray ionization mass spectra (ESI-MS). Results showed that the content of polyphenols, flavonoids, quercetin, flavanols, tannins, and ascorbic acid varied for garlic and white and red onions ranging from 6.68 to 18.08 mg GAE/g DW, 490.4−701.0 μg CE/g DW, 281.2−1100.0 μg, 32.40−41.30 μg CE/g DW, 2.88−3.12 mg CE/g DW, 1.87−2.33 mg AA/g DW, 1388.2−1442.3 μg CGE/g DW, respectively. The radical scavenging capacities (μM TE/g DW) for the same investigated vegetables for ABTS, FRAP, CUPRAC, and DPPH assays ranged from 48.78 to 92.42, 9.41−28.56, 3.06−10.41, and 6.49−23.42, respectively. Good correlations were observed between the phenolic contents and the radical scavenging capacities of the vegetables. The interaction between BSA and quercetin, BSA and garlic and onions extracts was measured by 3-dimensional fluorescence (3D-FL) and Fourier transform infrared (FT-IR) spectroscopy. The highest polyphenol content was found in methanol/water fraction of onions and garlic; therefore, for the investigation of in vitro interactions with BSA only polyphenols of this fraction were used. For in vivo studies, 30 male Wistar rats were divided into 5 groups each of 6 and named Control, Chol, Chol/Garlic, Chol/OnionRed, and Chol/OnionWhite. During 6 weeks, the rats of all 5 groups were fed a basal diet (BD). The rats of the Control group were fed the BD only. The BD of the Chol group was supplemented with 10 g/kg of nonoxidized cholesterol (NOC). Each of the other three groups was supplemented with 10 g/kg of NOC and 500 mg of raw fresh garlic, 500 mg of raw fresh red onion, and 500 mg of raw fresh white onion on 1 kg of body weight for Chol/Garlic, Chol/OnionRed, and Chol/OnionWhite diet groups, respectively. In order to detect the changes in the liver and aorta, a histological procedure was applied, and the liver enzymes were determined and compared. It was found that the main changes vs the Control group were in the liver of rats fed the cholesterol-containing diet without vegetable supplementation. Significantly less histological changes in the liver and lower level of liver enzymes vs those of the Chol group were detected in rats of the Chol/Garlic group ($P < 0.05$). The interaction between the polyphenol extract of garlic and BSA in vitro showed its strong ability comparable with that of quercetin to quench the intrinsic fluorescence of BSA. In conclusion, all studied vegetables showed protective effects, but raw garlic supplemented with cholesterol-containing diets significantly prevented the aorta and liver damages of rats.

**KEYWORDS:** Raw vegetables, bioactive compounds, antioxidant potential, rats, cholesterol-containing diets, aorta and liver damages

## INTRODUCTION

Anthocyanins and polyphenols from various vegetables, fruits, and herbal plants have antioxidant activities. Flavonoids of vegetables prevent liver damage which occurs in rats fed cholesterol-containing diets.[1−5] The physiological effects of red Welsh onion were examined and compared with those of white Welsh onion.[2] Nakayama et al.[3] found in young male mice fed a high-fat diet that the numbers of the fatty droplets in the liver cytoplasm were markedly increased as well as the liver weight. The combined modulatory effect of garlic and fish oils on the antioxidant and drug metabolic systems and the effect of onion byproduct on bioactivity and safety markers in healthy rats were studied.[4,5] Some authors claim that herbal medicine and vegetables exercise hepatoprotective

**Received:** April 16, 2011
**Accepted:** May 26, 2011
**Revised:** May 25, 2011
**Published:** May 26, 2011

activities in animals fed a high-fat diet.[3,6] The hepatoprotective activities of flax and pumpkin seeds on 30 hypercholesterolemic male rats were evaluated.[6] The authors found an improvement in histological sections of liver lipid hepatocytes storage only in rats fed flax and pumpkin mixture. The most frequently used raw vegetables are garlic and onions.[7,8] It was shown that these vegetables contain high amounts of bioactive compounds[9,10] and that consumption of these vegetables prevents some diseases.[11−15] It was reported that garlic and onion are effective in preventing of cardiovascular disease because of their hypocholesterolemic, hypolipidemic, antihypertensive, antidiabetic, antithrombotic, and antihyperhomocysteinemia effects.[16] It is also of great interest to investigate if frequently consumed raw vegetables possess liver protective properties for rats fed cholesterol-containing diets. In the investigation of 30 Wistar hypercholesterolemic male rats, the diets were supplemented with raw garlic and white and red onions. For this aim, at the end of the investigation some atherosclerosis indices, liver enzymes, and histopathology of the diet groups were determined and compared. The interaction between drugs and BSA is important in the metabolism of drugs.[17] Such interaction between the extracted polyphenols and BSA can provide knowledge for the use of vegetables in everyday consumption. The functional properties of garlic and onions will be studied by the interaction of quercetin and vegetable polyphenol extracts with a small protein such as BSA, using 3D-FL and FTIR. As was already stated, in order to assess the vegetables' effectiveness a wide range of laboratory tests were applied, and histology examination of liver and aorta was performed. As far as we know, no results of such an investigation were published.

## MATERIALS AND METHODS

**Chemicals.** 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent (FCR), lanthanum(III) chloride heptahydrate, $FeCl_3$ $6H_2O$, $CuCl_2$ $2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), quercetin, BSA, and cholesterol of analytical grade (USP) were purchased from Sigma Chemical Co., St. Louis, MO, USA. 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Ten percent buffered formalin (10 mL of 10% formalin, 10 mL of 10% $CaCl_2$, and 80 mL of $H_2O$) was from POCh (Gliwice, Poland). A 1% alcohol solution of Red Oil in 60% 3-methyl phosphate (Sigma Aldrich) for tissue dye was used. Deionized and distilled water was used throughout.

**Samples and Preparation.** In this investigation, garlic (*Allium sativum* L.) and white (Armstrong) and red (Red Baron) onions (*Allium cepa* L.), harvested in 2008, were used. The bulbs of garlic and white and red onions before the in vitro experiment were washed, cleaned, peeled, and cut with a plastic knife (halves for garlic and pieces for onions). The vegetables were weighed, chopped, and homogenized under liquid nitrogen in a high speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50−100 g) was then 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 the bioactive substances were analyzed.

**Determination of the Studied Bioactive Compounds and Antioxidant Potentials.** The studied bioactive compounds were determined as previously described.[10,18] Polyphenols were extracted from lyophilized vegetables with 50% dimethyl sulfoxide (DMSO) (concentration 25 mg/mL) at room temperature twice during 3 h.

**Extraction of Phenolic Compounds for MS.** The lyophilized samples of garlic and onions (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 C, the aqueous solution was extracted with diethyl ether and ethyl acetate, and then the remainder of the aqueous solution was freeze-dried. The organic fractions were dried and redissolved in methanol. These extracts were used for MS, for the determination of bioactive compounds and FTIR analyses.[19]

**MS Analysis.** A mass spectrometer, TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland) was used. Analytes were ionized by electrospray ionization (ESI) in negative mode. Vaporizer temperature was kept at 100 C. Settings for the ion source were as follows: spray voltage, 3000 V; sheath gas pressure, 35 AU; ion sweep gas pressure, 0 AU; auxiliary gas pressure, 30 AU; capillary temperature, 200 C; and skimmer offset, 0 V.

**Determination of the Bioactive Compounds.** To determine the total amount of polyphenols in the extracts of the studied vegetables, the Folin−Ciocalteu reagent (FCR) was used, and the measurement was performed at 765 nm with gallic acid as the standard. Results were expressed as mg of gallic acid equivalent (GAE). Flavonoids, extracted with 5% $NaNO_2$, 10% $AlCl_3$ $6H_2O$, and 1 M NaOH, were measured at 510 nm. The total flavanol amount was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance at 640 nm was read. To ensure the presence of flavanols on the nuclei, subsequent staining with the DMACA reagent resulted in an intense blue coloration in onion.

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 (CE).

The absorbances for total anthocyanins were measured for two pH values (1.0 and 4.5) in a Beckman spectrophotometer at 510 nm, using the pH differential method. Results were expressed as mg of cyanidin-3-glucoside equivalent (CGE).

Total ascorbic acid was determined by CUPRAC assay. The water extract was prepared from 100 mg of lyophilized sample and 5 mL of water, then mixed, stirred for 24 h, and centrifuged. The extract (1 mL) was mixed with 2 mL of 3.0 $10^{-3}$ M of lanthanum(III) chloride heptahydrate. Ethyl acetate (EtAc) was used for extraction in order to avoid the interference of flavonoids. For the determination of ascorbic acid by CUPRAC assay, the aqueous phase was examined. One milliliter of Cu (II)-neocuproine (Nc), in ammonium acetate-containing medium at pH 7, was mixed with 1 mL of the obtained extract where the absorbance of the formed bis-(Nc)-copper(I) chelate was measured at 450 nm.[20]

**Determination of the Antioxidant Potentials.** For the determination of the antioxidant potential,[21] the following 4 tests were used: (a) FRAP assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine ($Fe^{3+}$-TPTZ) to a ferrous form ($Fe^{2+}$), which absorbs light at 593 nm. (b) The $ABTS^{\bullet+}$ radical cation was generated by the interaction of ABTS (7 mmol/L) and $K_2S_2O_8$ (2.45 mmol/L). The mixture was allowed to stand at room temperature for 12 h to give a dark green solution. This solution was diluted with methanol until the absorbance reached 0.7 at 734 nm. One milliliter of the resulting solution was mixed with 10 $\mu$L of extracts from studied natural products. (c) DPPH. Different concentrations (50 and 100 $\mu$L equivalent to 50 and 100 ppm) of extracts of the studied vegetables and BHA (25 and 50 ppm) were taken in different test tubes. The volume was adjusted to 100 $\mu$L by adding MeOH. Five milliliters of a 0.1 mM methanol solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27 C for 20 min. The control was prepared as described above without any extract, and MeOH was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the

Table 1. Polyphenol Compounds in Dimethyl Sulfoxide (DMSO) Extract of the Studied Vegetable Samples[a]

| vegetable samples | polyphenols (mg GAE) | flavonoids (μg CE) | quercetin (μg) | flavanols (μg CE) | tannins (mg CE) | asc acid (mg AA) | anthocyanins (μg CGE) |
|---|---|---|---|---|---|---|---|
| onion white | 18.08 ± 0.9 b | 574.7 ± 27.1 b | 480.1 ± 24.1 b | 35.75 ± 1.6 a | 3.12 ± 0.2 a | 2.11 ± 0.1 a | 1392.3 ± 66.1 a |
| onion red | 12.92 ± 0.6 b | 701.0 ± 34.2 c | 1100 ± 55.2 c | 41.30 ± 1.9 b | 2.88 ± 0.2 a | 1.87 ± 0.09 a | 1388.2 ± 65.9 a |
| garlic | 6.68 ± 0.3 a | 490.4 ± 23.5 a | 281.2 ± 13.9 a | 32.40 ± 1.5 a | 2.88 ± 0.2 a | 2.33 ± 0.1 a | 1442.3 ± 72.7 a |

[a] Values are the means ± SD of 5 measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P < 0.05$); per g dry weight. Abbreviations: CE, catechin equivalent; GAE, gallic acid equivalent; CGE, cyanidin-3-glucoside equivalent.

following formula: % radical scavenging activity = (control OD − sample OD/control OD) 100. (d) Cupric reducing antioxidant capacity (CUPRAC): This assay is based on utilizing the copper(II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. The absorbance at 450 nm was recorded against a reagent blank.[20]

**Fluorimetry and Fourier Transform Infrared (FT-IR) Spectra Studies.** Two dimensional (2D-FL) and three-dimensional (3D-FL) fluorescence measurements were done using a model FP-6500, Jasco Spectrofluorometer, serial N261332, Japan. Fluorescence emission spectra for all vegetable samples at a concentration of 0.25 mg/mL were taken at an emission wavelength (nm) of 330 and recorded from a wavelength of 265 to a wavelength of 310 nm, at emission wavelengths of 685 nm from 300 to 750 nm, and at excitation wavelengths of 350 nm from 370 to 650 nm. Quercetin was used as a standard. 3D-FL spectra of the investigated vegetable extracts were collected with subsequent scanning emission spectra from 250 to 750 at 1.0 nm increments by varying the excitation wavelength from 230 to 350 at 10 nm increments. The scanning speed was set at 1000 nm/min for all measurements. All measurements were performed with emission mode and with an intensity up to 1000. All solutions for protein interaction were prepared in 0.05 mol/L Tris-HCl buffer (pH 7.4), containing 0.1 mol/L NaCl. The final concentration of BSA was 2.0 $10^{-4}$ mol/L. All solutions were kept in the dark at 0−4 C. The BSA was mixed with quercetin. The extracted polyphenol samples were mixed in the properties of BSA/extract at 1:1. The mixtures of the product after the interaction with BSA were lyophilyzed and subjected to FTIR.[22]

The presence of polyphenols in the investigated vegetable samples and the interaction between polyphenols and bovine serum albumin (BSA) were studied by Fourier transform infrared (FT-IR) spectroscopy. A Nicolet iS 10 FT-IR spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR$^{TM\ ATR}$ (Attenuated Total Reflectance) accessory was used to record IR spectra.[22,23]

**Animals and Diets.** The Animal Care Committee of the Warsaw Agricultural University, Warsaw, Poland University, approved this study. The mean weight of the male Wistar rats ($n = 30$) at the beginning of the experiment was 100 ± 2.8 g. Before the experiment, all rats were put on 5 days of adaptation. The rats were housed in metabolic cages (TECNIPLAST S.p.A., 21020, Italy). The rats were divided into 5 groups of 6 and were named Control, Chol, Chol/Garlic, Chol/OnionRed, and Chol/OnionWhite. During 42 days of the experiment, the rats of all 5 groups were fed a basal diet (BD), which included wheat starch, casein, soybean oil, vitamin, cellulose, and mineral mixtures. The rats of the Control group were fed only the BD. The BD of the other 4 groups was supplemented with 10 g/kg of nonoxidized cholesterol (NOC) of analytical grade (Chol group), 10 g/kg of NOC and 500 mg of raw fresh garlic on 1 kg of body weight (Chol/Garlic), 10 g/kg of NOC and 500 mg of raw fresh red onion on 1 kg of body weight (Chol/OnionRed), and 10 g/kg of NOC 50 g/kg and 500 mg of raw fresh white onion on 1 kg of body weight (Chol/OnionWhite). The cholesterol batches were mixed carefully with the BD (1:99) just before the diets were offered to rats. All rats were fed once a day at 10.00 h ad libitum. They had unrestricted access to drinking water. The feed intake was monitored daily and body gains every week. At the end of the experiment, the rats were anaesthetized using Narcotan (Zentiva), and the blood samples were taken from the left atrium of the heart, and plasma was prepared and used for laboratory tests. As is already stated above, the tests include determination of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), and plasma antioxidant activity (PAA) by ABTS and FRAP. AST, ALT, and ALP were determined with utilization of analyzer Siemens-Advia 1650 according to the principles of the Bayer Chemistry System. The liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined as previous described.[18] ABTS and FRAP were adopted for determination of plasma antioxidant activity.

**Histological Procedures.** (a) 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 atheromatous 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. (b) From the liver of each experimental rat left lateral lobe and isolated specimens were dissected. The sections were fixed with buffered 10% formalin. After 10 days liver samples were cut in Criostat at a temperature of −20 C on slicks of 10 μm thickness and placed in distillated water (with few $NH_3$ drops). The slicks, which floated on the water surface, were stained in 10% Red Oil during 10 min and then were placed on fat free basal glass and closed in glycerol-gelatin. Morphology of liver lobules was evaluated by a light microscope. Also, the fat content in hepatocytes was evaluated. For this purpose, 100 cells were selected, and fat's integral density was measured using computer program, Lucia v.4.x. (Nikon), at 100 magnification, and the results were expressed in $\mu g/\mu m^3$. The results of morphometry were analyzed using statistics.

**Statistical Analyses.** The results of the investigation in vitro are the mean ± SD of five measurements. Where it was appropriate, differences between groups were tested by two-way analysis of variance (ANOVA). The $P$-values of <0.05 were considered significant.

## RESULTS

**Bioactive Compounds.** The results of the determination of all studied bioactive compounds in raw garlic and onions are summarized in the Table 1. As can be seen, the contents of polyphenols and tannins are higher in white onion, flavonoids, quercetin, and flavanols in red onion, and ascorbic acid and anthocyanins in garlic but not always significant ($P > 0.05$). The results of the determination of the antioxidant potential in raw garlic and onions are summarized in the Table 2. According to ABTS, CUPRAC, and DPPH values the highest antioxidant potential was in white onion, and only according to FRAP in red onion, but not always significant ($P > 0.05$). The bioactivity of the extracted fractions ranged according to the polyphenol

**Table 2. Antioxidant Activity of All Studied Vegetable Samples ($\mu$M TE/g) in Dimethyl Sulfoxide (DMSO) Extract[a]**

| vegetable samples | ABTS | FRAP | CUPRAC | DPPH |
|---|---|---|---|---|
| onion white | 92.42 ± 4.5 c | 14.91 ± 0.7 b | 10.41 ± 0.5 c | 23.42 ± 1.1 c |
| onion red | 76.13 ± 3.7 b | 28.56 ± 1.3 c | 7.78 ± 0.04 b | 15.47 ± 0.7 b |
| garlic | 48.78 ± 2.3 a | 9.41 ± 0.4 a | 3.06 ± 0.02 a | 6.49 ± 0.3 a |

[a] Values are the means ± SD of 5 measurements. Values in columns for every value of antioxidant activity bearing different letters are significantly different ($P < 0.05$); per g dry weight. Abbreviations: ABTS, 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; CUPRAC, cupric reducing antioxidant capacity; FRAP, ferric-reducing/antioxidant power; DPPH, 1,1-diphenyl-2-picrylhydrazyl method.

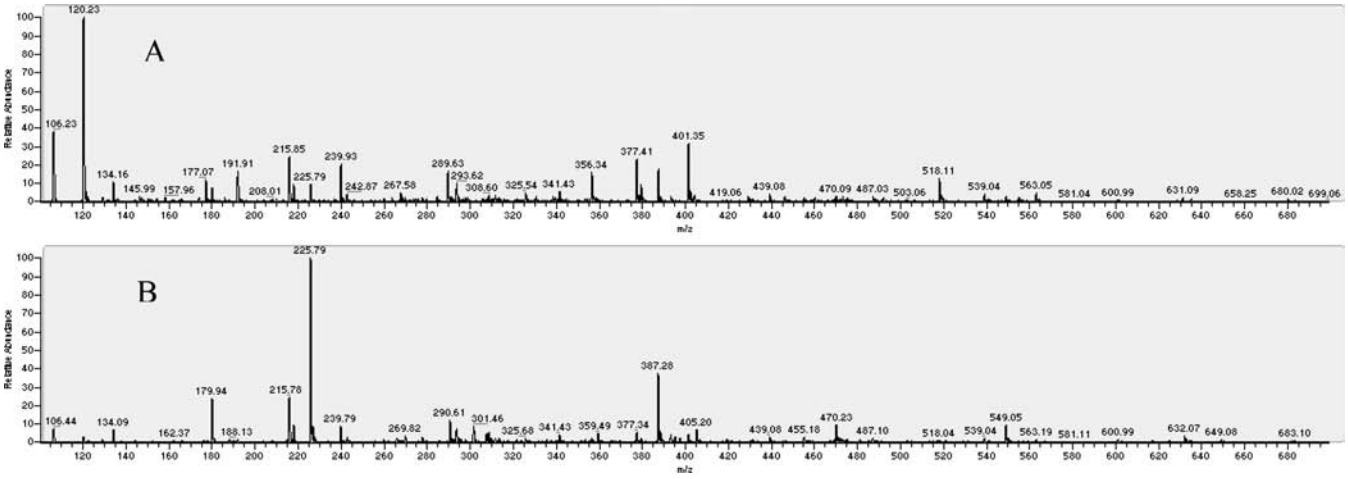


**Figure 1.** ESI-MS spectra of methanol/water fractions of garlic (A) and onions (B) in negative ion mode.

content in the following order: polyphenols (mg GAE/g DW) in methanol/water fraction in onions 2.421 and garlic −0.486; in ethyl acetate fraction for onions −0.532 and garlic 0.091; and diethyl ether for onions −0.281 and garlic 0.049. The MS spectrum shows (Figure 1) the $m/z$ peaks commonly found in garlic at 106 with an abundance for garlic of 40%, onions −10%; at 226 ($A$ = 100%) for onions and for garlic of 10%; at 240 ($A$ = 10%) for onions and 240 ($A$ = 20%) for garlic; 377 ($A$ = 5%) for onion and 377 ($A$ = 20%); at 120 ($A$ = 100%, benzoic acid) for garlic, 177 ($A$ = 15%), 192 ($A$ = 18%, ferulic acid); 289 ($A$ = 17%, catechin); in onions 270 ($A$ = 8%, apigenin), 301 ($A$ = 9%, quercetin); 180 ($A$ = 25%, gallic acid). In the positive mode, some important peaks were shown in onions: 150 ($A$ = 22%), 177 ($A$ = 28%), and 325 ($A$ = 15%) for hematoxylen; 611 ($A$ = 6%) for rutin; 309 ($A$ = 40%) for fisetin; and 325 ($A$ = 14%) for quercetin. On the basis of these results only methanol/water fractions of garlic and onions (Figure 1) were used in in vitro studies of the interaction with BSA and quercetin.

**Fluorimetry and Fourier Transform-Infrared (FT-IR) Spectra Studies.** As was mentioned in the Introduction, two-dimensional (2D-FL) and three- dimensional (3D-FL) fluorescence (FL) was used. 2D-FL showed the change in the peak intensity and the location of the peaks, and 3D-FL spectra illustrated the elliptical shape of the contours of the main peaks. One of the main peaks for BSA (Figure 2A and B) was found at ex/em of 225−230/335 nm (the peak is shown only partly, in Figure 2A, because of the ability of the fluorimeter to evaluate a narrow range of excitation). The second main peak appeared for these samples at ex/em of 280/345 nm. The interaction of BSA and quercetin (Figure 2C,D,G) and BSA and garlic (Figure 2F) showed a shift in the second peak of 10 nm (335 nm) and the decrease in the fluorescence intensity. The fluorescence intensity was initially for BSA of 881.97 (Figure 2E, upper curve) and after quenching during 1 h at 37 C with quercetin reached 748.61 (Figure 2E, lower curve). The decrease was about 15.1%. In the case of garlic from the initial value to the end of the reaction, the intensity decreased to 735 (the decrease was of 16.7%; Figure 2F, lower curve). For white and red onions, the decrease was slightly lower at 12.4% and 11.7%, respectively. The spectra of polyphenols from garlic and white onion showed similar peaks and similar fluorescence intensities (Figure 2G and H).

**FTIR Spectra.** The FTIR spectra of BSA and quercetin (Figure 3A, upper curve) were compared with pure quercetin and BSA (Figure 3A, middle and lower curves). The amide I and amide II peaks of BSA (Figure 3A, lower curve) were shifted from 1544 to 1540 $cm^{-1}$ and from 1654 to 1626 $cm^{-1}$ upon interaction with quercetin (Figure 3A, upper curve) and to 1544 and 1630 $cm^{-1}$ upon interaction with the garlic extract (Figure 3B, middle curve). FTIR of quercetin (Figure 3A, middle curve) shows a broad phenolic OH band centered around 3404 $cm^{-1}$, characteristic −CO stretching at 1663 $cm^{-1}$, aromatic bending and stretching around 1091 and 1663 $cm^{-1}$, and −OH phenolic bending around 1197 and 1374 $cm^{-1}$. FTIR spectrum of quercetin could confirm the relative chemical stability of quercetin. FTIR spectra of garlic (Figure 3C, lower curve) showed a peak characteristic of −CO stretching at 1634 $cm^{-1}$ and aromatic bending. The peaks at 2925 and 2852 $cm^{-1}$ are related to the C−H bond of saturated carbons (Figure 3C, lower curve). The characteristic band of 1663 $cm^{-1}$ of quercetin −CO stretching (Figure 3A, middle curve) is seen as small shoulder due to the overlapping of the dominant −CO stretching of garlic (1744 $cm^{-1}$; Figure 3C, middle curve). The phenolic OH, corresponding to quercetin, is seen around

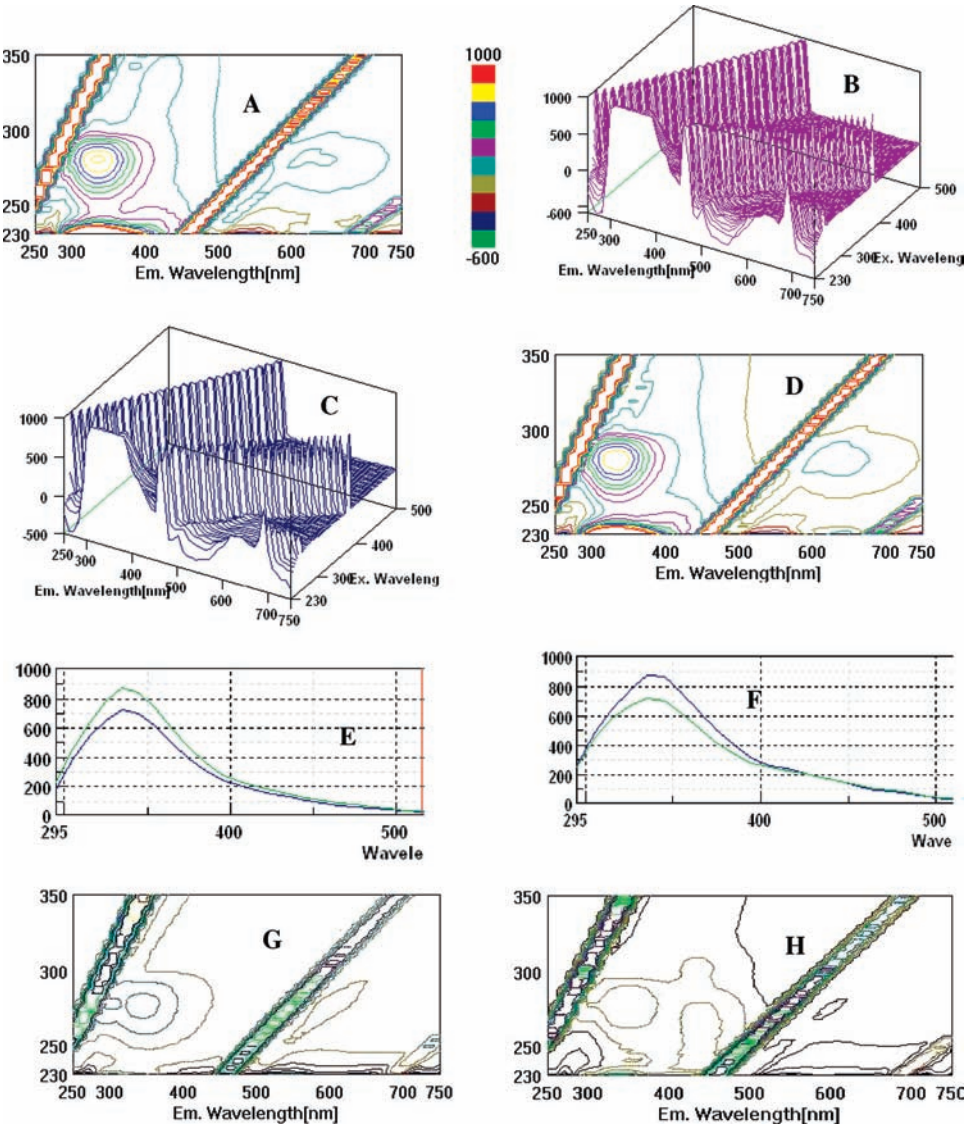


**Figure 2.** Elliptical shape of the contour of 2.0 $10^{-4}$ mol/L BSA (A), three-dimensional fluorescence (3D-FL) spectrum of 2.0 $10^{-4}$ mol/L BSA (B), 3D-FL spectrum of interaction between 2.0 $10^{-4}$ mol/L BSA and 2.0 $10^{-4}$ mol/L quercetin (C), elliptical shape of the contour of the interaction between 2.0 $10^{-4}$ mol/L BSA and 2.0 $10^{-4}$ mol/L quercetin (D), change in the fluorescence intensity (ID) as a result of binding a nity of 2.0 $10^{-4}$ mol/L BSA and 2.0 $10^{-4}$ mol/L quercetin by 2D-FL (E, upper line, BSA; lower line, BSA + quercetin), change in the fluorescence intensity (ID) as a result of binding a nity of 2.0 $10^{-4}$ mol/L BSA and 40 $\mu$g/mL garlic extract (F, upper line, BSA; lower line, BSA + garlic extract), elliptical shape of the contour of garlic (G), and elliptical shape of the contour of white onion (H). The 3D-FL was run in emission mode and fluorescence intensity up to 1000, emission wavelengths from 250 to 750 nm, and excitation wavelengths from 230 to 350 nm; the scanning speed was 1000 nm/min; E and F, emission wavelength is on the *x*-axis and fluorescence intensity on the *y*-axis; A, B, C, D, G, and H, emission wavelengthis on the *x*-axis and excitation wavelength on the *y*-axis.

3405 $cm^{-1}$ for the quercetin-BSA at 3183 $cm^{-1}$ (Figure 3A, upper curve). Matching between the peaks in the range from 4000 to 400 $cm^{-1}$ is (BSA + quercetin):(quercetin) = 12.4%; (BSA + quercetin):(BSA) = 49.1% (Figure 3A and D); and (BSA + garlic):(garlic) = 7.2% (Figure 3B and C). The comparison of the peaks as a result of the interaction between BSA and quercetin and BSA and garlic extract (BSA + quercetin):(BSA + garlic) was about 90.2% (Figure 3B). Other vegetables samples were similar (89.4% and 88.3% for white and red onions). Correlation between the peaks of garlic to onions was about 64.4%. The observation that the protein conformation was not affected with the addition of the flavonoid was also demonstrated by FTIR spectroscopy. If there had been a change of BSA conformation, a shift in the peak of amide I band, or disappearance of the peak corresponding to the N−H residual amide II band would have been observed, neither of which occurred.[23] The changes in the peak location and fluorescence intensity after interaction of polyphenols and BSA found in fluorescence were also detected in FTIR. The calculated percentages of matching after quenching were similar in these two methods.

**Effect on Body Weight.** Animal's body weight did not differ significantly between groups at the end of experiment (data not shown).

**Plasma Atherosclerosis Indices.** Loading of rats during the 6 weeks diet containing 1% of cholesterol (0.20 g/day) influenced significant changes in the plasma lipids profile, expressed

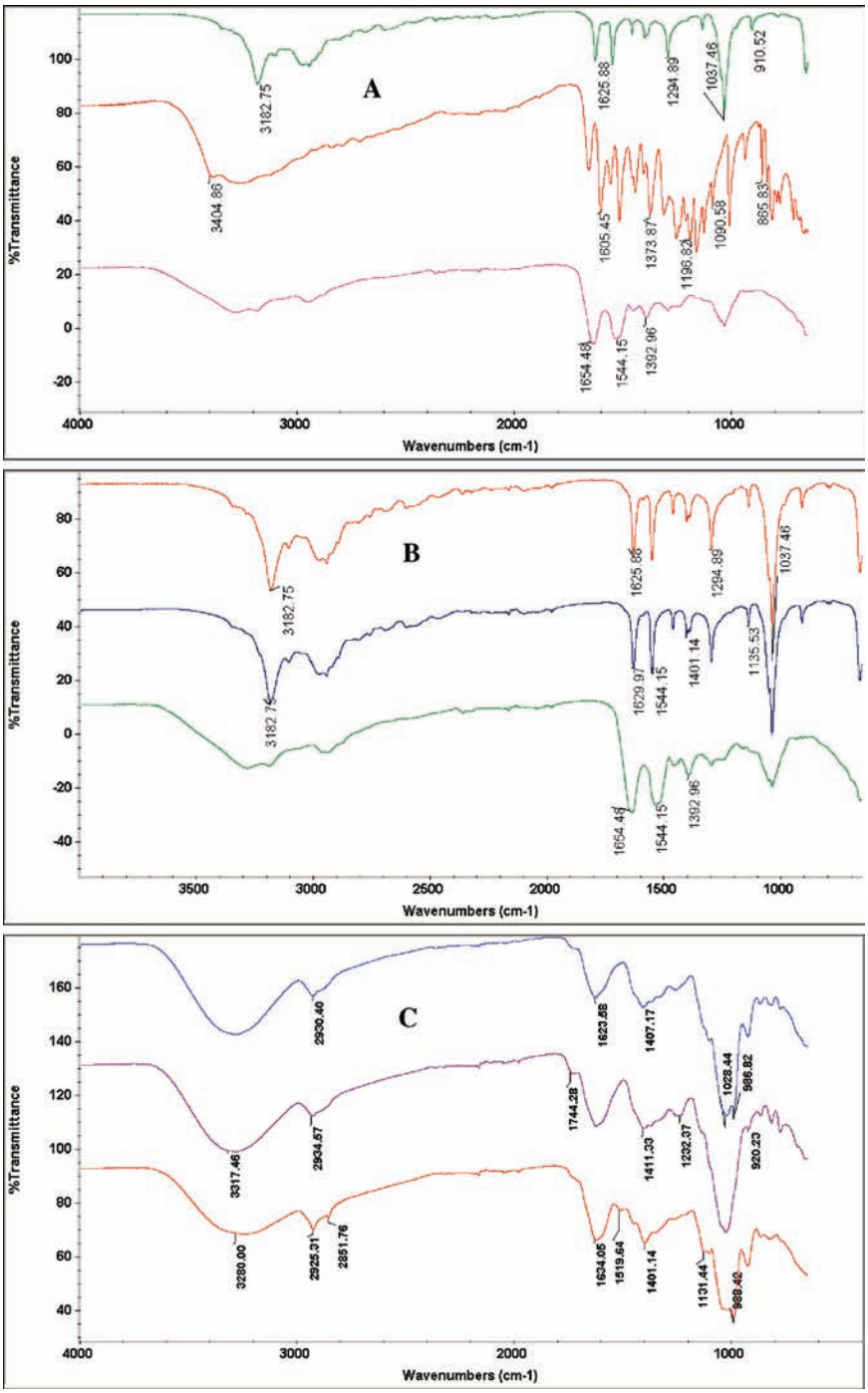


**Figure 3.** Infrared study of FTIR spectra of (A) (upper curve) BSA and quercetin; (middle curve) quercetin; (lower curve) BSA. (B) (Upper curve) BSA and quercetin; (middle curve) BSA + garlic extract; and (lower curve) BSA. (C) (Upper curve) red onion, (middle curve) white onion, and (lower curve) garlic. The interaction of 2.0 $10^{-4}$ mol/L BSA and 2.0 $10^{-4}$ mol/L quercetin and 2.0 $10^{-4}$ mol/L BSA and 40 $\mu$g/mL garlic extract was during 1 h, at 37 C, then after the fluorimetric measurements the sample was lyophilized and subjected to FTIR.

in increase of TC (45.8%) and LDL-C (25.3%). Atherogenic indices (TC/HDL-C) increased from 2.92 (Control) to 4.26 (Chol) ($P < 0.05$). Supplementation of the Chol diet with raw lyophilized vegetables (500 mg of fresh vegetables/1 kg of body weight) improved the plasma lipids profile in different ways: the highest decrease of TC and LDL-C (23.9 and 27.4%, respectively, $P < 0.05$) was found in the Chol/Garlic group vs Chol group. A similar relationship in the case of atherogenic indices (4.10) was found with a decrease of 3.76%. Onions, white and red, supplemented to Chol/OnionWhite and Chol/OnionRed diet groups vs Chol group also hindered an increase of TC and LDL-C in plasma of rats by 14.6 and 14.0 vs 7.5 and 9.5%, respectively. In this case, the increase of atherogenic indices was 4.95 and 4.45 (increase by 16.2 and 4.46%), respectively.

Cholesterol loading in rat diet significantly influenced ($P < 0.05$) the decrease (vs Control group) of plasma antioxidant

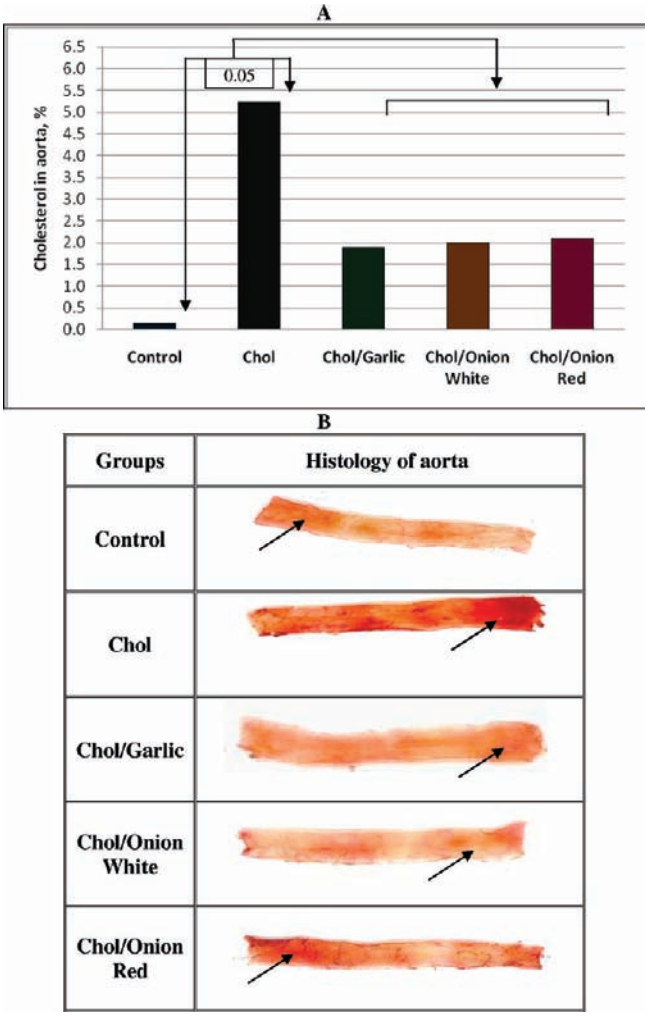


**Figure 4.** (A) Cholesterol in aorta of rats fed different diets supplemented with garlic, white, and red onion (% of area). (B) Changes in the histology of the aorta. The arrows show the most concentrated area with lesions in the aorta. Diet groups: Control, Chol, Cholesterol; Chol/Garlic, Cholesterol/Garlic; Chol/OnionWhite, Cholesterol/Onion White; Chol/Onion Red, Cholesterol/Onion Red.

activity (ABTS) by 15. 61%. An increase of antioxidant activity in rat plasma supplemented with raw vegetables was determined as 15.07, 10.96, and 10.27% for Chol/Garlic, Chol/OnionWhite, and Chol/OnionRed diet groups, respectively (vs Chol group).

The changes in the antioxidant activity of rat plasma determined by the FRAP assay was slightly different. The supplementation of cholesterol in all diet groups decreased the plasma antioxidant activity by 9.47% in comparison with the Control diet group, but the addition of vegetables to the cholesterol diet increased the antioxidant activity by 7.84, 6, 54, and 5.88% in the Chol/Garlic, Chol/OnionWhite, and Chol/OnionRed diet groups, respectively (vs Chol group).

**Liver Enzymes.** Somatic index of the liver was significantly higher ($P < 0.05$) in all rat loading diets with cholesterol (0.19−0.20 g Chol/day/rat) in comparison with the Control group. An increase of liver weight results from the accumulation of lipids by lipogenesis increase in hepatocytes, where the size of this accumulation on the basis of fat integral density (FID) in 100 hepatocytes (from each group) was evaluated.

The determination of serum alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) shows that these liver enzymes correlate with the level of the liver damage. Therefore, the significantly highest fat integral density in liver of the Chol diet group was 349.71 ± 28.13 $\mu g/\mu m^3$, and the levels of ALP, AST, and ALT were 283.0 ± 97.6, 102.2 ± 12.7, and 37.8 ± 6.8 U/L, respectively.

**Histopathology of the Aorta and Liver.** Antiatherogenic influence of raw vegetables on the basis of changes in aorta histology was evaluated. The results of the histology examination of the aorta are shown in Figure 4. The following data were obtained (% of cholesterol): control, 0.15 ± 0.09; Chol, 5.22 ± 1.04; Chol/Garlic, 1.87 ± 0.05; Chol/OnionWhite, 1.99 ± 0.12; and Chol/Onion Red, 2.10 ± 0.21. All vegetables which were used in the experimental diet supplemented with cholesterol significantly ($P < 0.05$) decreased the percentage of cholesterol in aortic arch after 6 weeks of the feeding period. The antiatherogenic influence of raw vegetables from the *Alliaceae* family ranged in the following order: garlic > onion white > onion red. The histopathology of the liver is shown in Figure 5. The fat integral density of 100 hepatocytes ($\mu g/\mu m^3$) was the following: 95.07 ± 6.37; 349.71 ± 28.13; 148.82 ± 10.71; 175.18 ± 12.94; and 206.17 ± 14.99 for the Control, Cholesterol, Chol/Garlic, Chol/OnionWhite, and Chol/OnionRed groups. Raw garlic was more effective than onions because the decrease of the FID value in hepatocytes (vs Chol) amounted to 57.0%, while in onions groups, it reached 49.9% and 41.0% for Chol/OnionWhite and Chol/OnionRed groups, respectively. As can be seen, the raw-garlic-supplemented diet significantly better ($P < 0.05$) protects rat livers from damage.

## DISCUSSION

As was stated, raw garlic and onions are the most consumed vegetables with high amounts of bioactive compounds and antioxidants.[2,4,10,15] The ABTS results shown in Table 2 are unusually higher than those reported by other similar antioxidant assays. Such situations are not frequently observed in antioxidant literature. It can be suggested that this arose from possible synergistic polyphenol−protein interactions[24] or from possible solvent effects (e.g., of DMSO) on ABTS findings.[25] Our previous investigations of the same vegetables harvested in different geographic and climatic conditions showed that red onion was preferable.[18,26] Also other authors found that the bioactivity of the same vegetables grown in different geographic and climatic conditions differs significantly.[8−10] On the basis of these findings, we conducted an investigation in vitro to assess the bioactivity of these vegetables and then in vivo studies to investigate their influence on livers and aorta of rats fed cholesterol-containing diets.

The influence of the studied vegetables on plasma atherosclerosis indices shows that the level of plasma lipids were significantly less in groups of rats when a cholesterol-containing diet was supplemented with raw garlic. In this study, it was found that a diet supplemented with raw garlic most effective protects the livers of rats fed cholesterol ($P < 0.05$). The hepatic lipid and cholesterol concentrations were conspicuously decreased in garlic powder fed groups.[27,28] The same relationship was registered in plasma antioxidant activity. Also, the below cited authors showed similar results.[27] They claim that the decrease in the plasma antioxidant activity was predictable: a cholesterol supplemented diet leads to a decrease in plasma antioxidant activity.[27] The decrease of TC and LDL-C in blood without effect on HDL

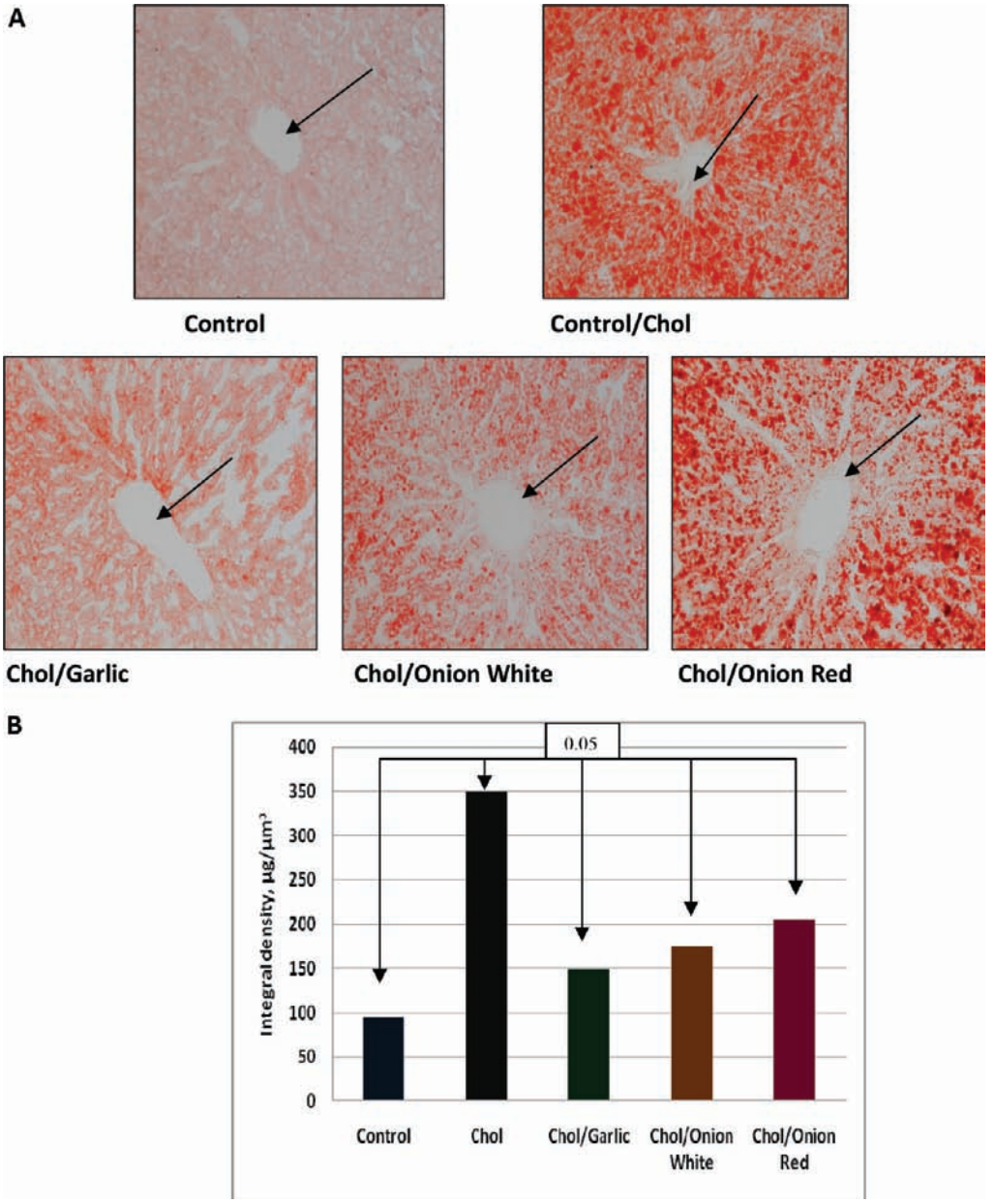


**Figure 5.** (A) Hepatic tissue morphology of Wistar rats. Stain of Red Oil. Magnified 10 . Control: view of normal rat hepatic tissue (without fat). Architecture shows radiated arranged hepatic cords around the central normal rat hepatic tissue (without fat). Architecture shows radiated arranged hepatic cords around the central venule. Control/Chol: around the central venule are the hepatic cell cords. Intensive red coloring is the effect of high contents of fat inside hepatic cells. Chol/Garlic: the intermediate red color on the picture shows that the volume of fat is between the Control/Chol and the Control diet groups. Chol/Onion White: the diffusion of red color is an indicator of the protective influence against the load of cholesterol. Chol/Onion Red: the intensive red color, as in the liver of Control/Chol group, is an effect of high amounts of fat inside hepatic cells. (B) Hepatic tissue of rats fed different diets supplemented with garlic, white and red onion (integral density).

was identified after the action of saponin which presents in garlic.[28] The obtained results can be explained by the cited report,[13] where the experiment with mice fed a diet containing 0.5% cholesterol for 10 weeks resulted in cholesterol supersaturation in gall bladder bile, which promoted the formation of cholesterol gallstones [CGS]. It was found that garlic and onion reduced the CGS incidence by 15−39% and markedly reduced biliary cholesterol. Lipid content was decreased in the liver[29] in experiments on rats fed a high cholesterolemic diet supplemented with garlic oil for 8 weeks. Our results are in agreement with those of others, who studied the influence of garlic and fish oil supplementation to rat diets.[4] The rats received different doses of garlic oil (0−200 mg/kg body weight) three times per week for 6 weeks. Garlic oil increased hepatic glutathione *S*-transferase, glutathione reductase, superoxide dismutase, and ethoxyresorufin *O*-deethylase (EROD) activities, but decreased glutathione peroxidase and *N*-nitrosodimethylamine demethylase activities ($P < 0.05$). Our results were in agreement with those of others, where the evaluation of the effect of garlic extract on lipid profiles and oxidative stress in male albino rats fed a high cholesterol diet was discussed.[30] The garlic extract significantly increased ($p < 0.05$) plasma HDL-cholesterol and decreased plasma TC,

LDL-cholesterol, and TG as well as liver TC and TG as compared with the positive control.[30] No significant difference was observed in plasma LDL-cholesterol and HDL-cholesterol as well as plasma and liver TG between the rats that had ingested high or low doses of garlic extracts. Our results were in agreement with those of others where the effect of individual garlic and flaxseed was studied on hyperlipidemic rats.[31] The histological results of kidney and liver tissues respond to garlic alone and a combined flaxseed and garlic diet, but a slight histopathological change was noticed in flaxseed diet group.[31] Garlic results showed no histopathological changes in the aorta, kidneys, and liver that may illustrate the healing effect of fresh garlic on tissues. Biochemical results indicated that the mean of blood total cholesterol and triacylglycerol was reduced, as an effect of fresh garlic, flaxseed, and the combined fresh garlic and flaxseed diet, but HDL-C was increased in the fresh garlic diet only. These results may support the Mediterranean diet consumption that is rich in fresh food such as fresh garlic and seeds that may protect from heart disease.[31] Our results can be compared with others, where the studies were designed to investigate the effect on treatments of garlic and the improvement of lipids in dietary-induced hyperlipidemic rats.[32] There was no effect of garlic which was given during 30 days as a diet supplement (standardized commercial tablets Cirkulin) for healthy volunteers as tablets (4/day = 264 mg of garlic) on plasma lipids (TC, LDL-C, HDL-C, and TG), but subacute garlic doses significantly influenced the total antioxidant capacity of the body.[33]

An important effect on lipid regulation also has quercetin. Quercetin is responsible the component of onions for the elimination of a reactive form of oxygen and increase of antioxidant activity in the organism.[1] Similar effect for onion and quercetin in rats fed a diet with cholesterol was shown, through an increase of peroxidase glutation activity.[34] Hipolipidemic effect of vegetables from the *Alliacea* family can be related with diminishing of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) activity. The antioxidant activity of onions depends on the dominant onion flavonoids quercetin (Q), which is found mostly in all extracts.[35−37] Our results of the significantly highest fat integral density in the liver of the Chol diet group and the levels of enzymes are in accordance with others, where the male 6-weeks-old spontaneously hypertensive rats (SHR) were fed a control diet or diets high in fat with or without 5% Welsh onion (red or white variety) for 4 weeks.[2] Red and white Welsh onions were effective in suppressing the increase in the lipid peroxides in plasma and in the liver in the case of white Welsh onion. The results of the influence of garlic which has lower antioxidant activity than onions is in accordance with the observation that although the antioxidant activity of red Welsh onion is weaker than that of the white one, antioxidant activity together with the hypolipidemic effect of red Welsh onion might work favorably to suppress the increase in blood pressure.[2] In our case, the garlic diet is more favorable than the onion diets which can be explained by the synergetic effect of other components as in case of Welsh onion of the detection of quercetin and anthocyanins. The interaction of garlic extract with quercetin and BSA was stronger than with onions. Our results are in correspondence with others, where rats were fed the onion byproduct, and the extracts caused anemia as expected in rodents for Allium products.[5] Glutathione reductase and glutathione peroxidase activities in erythrocytes increased when rats were fed with the onion extracts. The onion byproducts which have no genotoxicity in experiments in vitro may support the antioxidative defense in vivo. Our results can be compared with another experiment on rats where dietary quercetin for exerting its antioxidative effect was evaluated in high cholesterol-fed rats, using quercetin-containing diets (31−1260 mg quercetin/kg body wt/day) and onion diets (19−94 mg quercetin aglycon equivalent/kg body weight/day).[7] Plasma antioxidative activity was elevated depending on the amounts of quercetin or onion diet intake.

The supplementation of garlic and onions improved the level of liver enzymes in rats fed cholesterol.[18] Also, other authors found similar correlations with other vegetables and plants: inclusion of propolis in diets prevents aluminum-induced genetic and hepatic damage in the rats' liver.[38] Interesting results were obtained by Ushida et al.:[39] rabbits were fed during 8 weeks with a 0.5% cholesterol diet. Surface area of the atherosclerotic lesions of the aortic arch was high (76.4%), but the total area of lesions was significantly lower (48.2%) in rabbits also obtaining kefiran (exopolysaccharide produced by *Lactobacillus kefiranofaciens*). The cited authors did not find a significant effect of cholesterol and kefiran on the lipid profile in blood, but the lipid level was very high. Such an effect of kefiran was connected[39] with antiinflamatory and antioxidant actions. The effect of kefiran action on the reduction of the area of atherosclerotic lesions was shown by suppression of inflammation, lesser cholesterol accumulation in macrophagas, and lower serum lipids. Therefore, oligosasharides from kefiran may also influence the reduction of cholesterol in the liver tissue as well. Our results that garlic improves hepatic structure and function of the liver can be explained on the basis of experiments with different vegetables. The influence of purple carrot on a high-fat diet-fed rat model of the metabolic syndrome was higher in comparison with that of -carotene. As the juice itself contained low concentrations of carotenoids, it is likely that the anthocyanins are responsible for the antioxidant and anti-inflammatory properties of purple carrot juice for improving glucose tolerance as well as cardiovascular and hepatic structure and function.[40] In summary, the contents of bioactive compounds and the antioxidant potentials of the studied vegetables are relatively high. Diet supplementation with raw garlic compared to that with onions provides better protection to the aorta and liver of cholesterol-fed rats.

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## ACKNOWLEDGMENT

We are thankful to Dr. Elena Katrich (The Institute for Drug Research, School of Pharmacy, The Hebrew University, Hadassah Medical School, Jerusalem, Israel) and to Rakhel Abitbol and to Oshra Gindy (Department of Chemical Engineering, Hadassah College, Jerusalem, Israel) for their assistance in the determination of antioxidant activity, 3D fluorescence, and FTIR in the investigated vegetables.

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