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Original Research

Rapana venosa consumption improves the lipid profiles and antioxidant capacities in serum of rats fed an atherogenic diet☆

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ABSTRACT

In the recent years, the consumption of seafood has increased. There are no results on the studies of *Rapana venosa* (Rv) as a supplementation to the diets. We hypothesized that Rv would increase antioxidant capacity and reduce blood lipids, based on the composition of bioactive compounds and fatty acids. Therefore, the aim of this investigation was to evaluate in vitro and in vivo actions of Rv from contaminated (C) and non-C (NC) regions of collection on lipid profiles, antioxidant capacity, and enzyme activities in serum of rats fed an atherogenic diet. Twenty-four male Wistar rats were divided into 4 groups of 6 each and named control, cholesterol (Chol), Chol/RvC and Chol/RvNC. Rats of all 4 groups were fed the basal diet, which included wheat starch, casein, soybean oil, cellulose, vitamin (American Institute of Nutrition for laboratory animals vitamin mixtures), and mineral mixtures (American Institute of Nutrition for laboratory animals mineral mixtures). During 28 days of the experiment, the rats of the control group received the basal diet only, and the diets of

Abbreviations: 3D-FL, 3-dimensional fluorescence; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BD, basal diet; BSA, bovine serum albumin; C, contaminated; Ca, calcium; Chol, cholesterol; Chol/RvC, cholesterol/*Rapana venosa* contaminated; Chol/RvNC, cholesterol/*Rapana venosa* noncontaminated; CUPRAC, cupric reducing antioxidant capacity; DM, dry matter; ESI-MS, electrospray-ionization mass spectrometry; Fe, iron; FER, feed efficiency ratio; FI, fluorescence intensity; FRAP, ferric-reducing/antioxidant power; HDL-C, high-density lipoprotein cholesterol; K, potassium; LDL-C, low-density lipoprotein cholesterol; MS, mass spectrometry; NC, noncontaminated; P, phosphorus; PUFA, polyunsaturated fatty acids; Rv, *Rapana venosa*; TAC, total antioxidant capacity; TC, total cholesterol; TG, triglycerides.

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Fluorescence
Mass spectra

the other 3 groups were supplemented with 1% of Chol, 1% of Chol, and 5% of *Rv* dry matter from C and NC areas. Dry matter from C and NC areas supplemented diets slightly hindered the rise in serum lipids vs Chol group: total Chol, 13.18% and 11.63% and low-density lipoprotein Chol, 13.57% and 15.08%, respectively. Cholesterol significantly decreased the value of total antioxidant capacity. The supplementation of *Rv* to the Chol diet significantly affected the increase of antioxidant capacity in serum of rats, expressed by the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) method. The water extracts of *Rv* exhibited high binding properties with bovine serum albumin in comparison with quercetin. In conclusion, atherogenic diets supplemented with *Rv* from C and NC areas hindered both the rise in serum lipids levels and the decrease in the antioxidant capacity. Based on fluorescence and electrospray-ionization mass spectrometry profiles and in vivo studies, changes in the intensity of the found peaks were estimated in the serum samples after supplemented diets. These findings indicate that the supplementation of *Rv* to the atherogenic diets improve the lipid profiles and the antioxidant status in serum of rats.

1. Introduction

It was already shown that the contents of bioactive compounds and the antioxidant capacity of mussels from contaminated (C) and noncontaminated areas differ and in some cases significantly; therefore, their influence on serum indices of experimental animals as supplementation to the diets varied [1-3]. Liu et al [4] analyzed the concentrations and distribution patterns of 18 polychlorinated biphenyls in wild *Rapana venosa* (*Rv*), collected from Nanpai river coast in Bohai Bay. The pathways of carotenoid metabolic transformation in the *Rv* tissues, studying trophic system mollusk filter mollusk predator on examples of *Mytilus galloprovincialis* and *Rv*, were discussed by Borodina et al [5]. The results of this study might play an important role in protecting consumer health and safety screening of marine products [6]. In recent years, the consumption of seafood has increased [7,8]. It is of great interest to know the influence of various seafood species consumption on plasma indices (serum lipids, total antioxidant capacity [TAC], and enzyme activities). However, the influence of *Rv* consumption on serum animals' indices had not yet been determined. Therefore, it was decided to conduct an investigation in vivo to explore the influence of *Rv* dry matter (DM) from C and non-C (NC) areas on grown parameters and some plasma indices in rats fed diet with 1% cholesterol (Chol). For this purpose, a wide range of rats' serum indices such as lipids, enzymes, glucose, urea, minerals, and antioxidant capacities was determined and compared with each other. The fluorescence properties of serum of rats before and after the atherogenic diet were determined by the changes in the intensity of the found peaks. Rats were dissected, and some internal organs were isolated for somatic indices determination.

Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. It was interesting to investigate in vitro how this protein interacts with polyphenols extracted from *Rv* samples to get useful information of the properties of polyphenol-protein complex of the supplemented material. Therefore, the functional properties of *Rv* were studied by the interaction of water polyphenol extracts with a small protein such as bovine serum albumin (BSA), using 3-dimensional fluorescence (3D-FL). The *Rv* samples were compared by their mass spectrometry (MS) profiles as well. As previously mentioned, the potential benefits of using *Rv* were not previously studied. Herein, we hypothesized that the lipid-lowering properties of *Rv* can be enhanced by its composition combined with bioactive compounds and fatty acids. Therefore, the objective of this study was to determine the effect of *Rv* as a supplementation to the rats fed atherogenic diets. The specific objective to test the hypothesis included in vitro studies of binding properties of polyphenols extracts of *Rv* with BSA.

2. Methods and materials

2.1. Chemicals

6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid, butylated hydroxyanisole, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu reagent, Tris,tris (hydroxymethy1)aminomethane, lanthanum(III) chloride heptahydrate, copper (II) chloride dihydrate, 2,9-dimethyl-1,10-phenan-throline(neocuproine), iron (III) chloride hexahydrate, Chol of analytic grade (United States Pharmacopeia), and BSA were purchased from Sigma Chemical Co, St Louis, MO, USA. 2,4,6-Tripyridyl-s-triazine was from Fluka Chemie, Buchs, Switzerland. All reagents were of analytic grade.

2.2. Animals and sites of collection

Samples of *Rv* were collected in 2 regions of Bulgarian Black Sea: (*a*) an ecologically NC (Cape Galata) and in (*b*) C (Port Varna) sites at sea depth of 3 to 4 m. The samples were collected as previously described [9]. A map showing sampling locations is given [10]. The collected *Rv* from both C and NC sites was characterized by a similar maximum length and size of analyzed organisms, which were 75% to 85% of the maximum size reached within each population (4.37 ± 0.5 cm). This approach guaranteed that compared snails had similar metabolic conditions, and the influence of physiologic differences between 2 populations was less pronounced [11]. Whole soft tissue from 30 specimens of each population was rapidly frozen in liquid nitrogen and stored at –80°C. Then the samples were dried in glass flasks on Finn-Aqua,

Lyovac GT-2 equipment for 36 hours. The obtained DM of these 2 populations was used for analysis and also supplements to animals diets.

2.3. Rats and diets

The Animal Care Committee of the Warsaw Agricultural University, Poland, approved this study. The experiment was performed on male Wistar rats; the mean weight of the animals (n = 24) at the beginning of the experiment was 102 ± 5 g. They were divided into 4 groups of 6 and housed in the plastic cages (5 days adaptation and 28 days of feedings with experimental diets). These 4 groups were named control, Chol, Chol/*Rv*C, and Chol/*Rv*NC. During first 5 days, all 4 groups were fed the basal diet (BD), which included wheat starch, casein, soybean oil, cellulose, vitamin (American Institute of Nutrition [AIN] for laboratory animals vitamin mixtures, vitamin mix catalog number 960402), and mineral mixtures (AIN for laboratory animals mineral mixtures, mineral mix catalog number 960400) of the AIN for laboratory animals (Table 1). The rats of the control group during 28 days of the experiment received the BD only, and the diets of the other 3 groups were supplemented with 1% of Chol, 1% of Chol, and 5% of snails DM from C and NC areas for Chol, Chol/*Rv*C, and Chol/*Rv*NC groups, respectively. The cholesterol batches were mixed carefully with the BD (1:99) just before the diets were offered to the rats. The feed intake was monitored daily and body gains every week. At the end of the experiment after 24 hours of starvation, the rats were anesthetized using diethyl ether, and the blood samples were taken from the left atrium of the heart. Serum was prepared in the following way: the blood samples were allowed to clot at room temperature and centrifuged for 15 minutes at 1200*g* at 2°C to 8°C and stored in a –80°C freezer for later analyses.

2.4. Extraction of phenolic compounds

The lyophilized samples of *Rv* (1 g) were extracted with 40 mL of methanol at 40°C during 4 hours. The samples of *Rv* were also extracted with water at room temperature with the same proportion of the sample to solvent (1:40). Ultrasound-assisted extraction was carried out with Ultrasonic Cleaner Delta DC-80H, operating frequency, 40 kHz; output power, 80 W, and heater, 45 W. The extracts were filtered in a Buchner funnel. These extracts were submitted for determination of bioactive compounds [12].

The polyphenols were determined by Folin-Ciocalteu method with measurement at 750 nm with spectrophotometer (model 8452A; Hewlett Packard Corporation, Palo Alto, CA, USA). The results were expressed as milligrams of gallic acid equivalents per gram of dry matter [13].

Table 1 – Ingredient composition of diets fed to rats

Ingredient (g/kg)	Control	Chol	Chol/*Rv*NC	Chol/*Rv*C
Casein	150	150	150	150
Soybean oil	100	100	100	100
Cellulose	10	10	10	10
Mineral mix, AIN-93-MX	36.7	36.7	36.7	36.7
Vitamin mix, AIN-93-VX	10	10	10	10
Choline chloride	2	2	2	2
Cholesterol	–	10	10	10
*Rv*NC	–	–	50	–
*Rv*C	–	–	–	50
Wheat starch	691.3	681.3	631.3	631.3

Abbreviations: AIN-93-MX, AIN for laboratory animals mineral mixtures; AIN-93-VX, AIN for laboratory animals vitamin mixtures.

2.5. Determination of TACs of Rv DM

Total antioxidant capacities of *Rv* DM were determined by the following assays:

Cupric reducing antioxidant capacity (CUPRAC): This assay is based on using the copper (II)–neocuproine reagent as the chromogenic oxidizing agent. To the mixture of 1 mL of copper (II)–neocuproine and ammonium acetate buffer solution, acidified and nonacidified methanol extracts of *Rv* (or standard) solution (x, in milliliters) and H_2O ([1.1 – x] mL) were added to make a final volume of 4.1 mL. The absorbance at 450 nm was recorded against a reagent blank [14].

2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt: $ABTS^{\bullet+}$ was generated by the interaction of ABTS (7 mmol/L) and potassium persulfate (2.45 mmol/L). This solution was diluted with methanol, until the absorbance in the samples reached 0.7 at 734 nm [15] (Fig. 1A and B).

The TACs of serum samples were determined by ABTS method in the same way as described above and by ferric-reducing/antioxidant power (FRAP). This assay measures the ability of the antioxidants in the investigated samples to reduce ferric tripyridyltriazine to a ferrous form, which absorbs light at 593 nm [16].

2.6. Fluorometric measurements

Two-dimensional fluorescence and 3D-FL measurements were done to compare the fluorescence properties of different *Rv* samples, using a model FP-6500, serial N261332; JASCO, Tokyo, Japan. Fluorescence-emission spectra for *Rv* samples at a concentration of 0.25 mg/mL in water and methanol were taken at emission wavelength (nanometer) of 330 and recorded from wavelength of 265 to a wavelength of 310 nm, at emission wavelengths of 685 nm from 250 to 700 nm. Then *Rv* extracts were collected with subsequent scanning emission spectra from 250 to 700 nm at 1.0-nm increments by varying the excitation wavelength from 230 to 500 nm 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 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 sodium chloride. All solutions were kept in dark at 0°C to 4°C. The 2.0×10^{-5} M BSA was mixed with 0.22×10^{-5} M quercetin and extracts of *Rv* samples (40 μg/mL) [17,18].

2.7. Mass spectrometry analysis

The methanol extracts of *Rv* were submitted to MS analysis [12]. A mass spectrometer, a TSQ Quantum Access Max

(ThermoFisher Scientific, Basel, Switzerland) was used. Vaporizer temperature was kept at 100°C. All samples were done by direct infusion in the mass spectrometer by use of electrospray-ionization (ESI) source at negative ion mode, full scan analysis, range of 200 to 400 m/z. For optimization of the acquisition parameters and for identity confirmation, only a part of standards was used, not for all compounds that were found in the investigated samples. 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 at 30 AU; capillary temperature at 200°C; and skimmer offset, 0 V [19-21].

2.8. Serum lipids, enzymes, minerals, and fluorescence measurements of serum

Serum was prepared as previously described and used for a wide range of laboratory tests, which included determination of lipids (TC, LDL-C, high-density lipoprotein Chol [HDL-C], and triglycerides [TG]), enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], alkaline phosphatase [ALP], lipase, and amylase), glucose, urea, albumin, and minerals.

Determination of total Chol (TC), low-density lipoprotein Chol (LDL-C), HDL-C, and TG; liver enzymes activity of AST, ALT, and ALP; glucose, lipase, amylase, urea, and albumin; some minerals potassium (K), chloride (Cl), calcium (Ca) (total), magnesium (Mg), phosphorus (P) (nonorganic), and iron (Fe) in serum was determined with utilization of analyzer Siemens-Advia 1650, according to the principles of the Bayer Chemistry System [22]. All serum samples were investigated also in vitro, using 3D-FL after the in vivo studies. Liver, kidneys, heart, and spleen were dissected; washed with saline solution; frozen in liquid nitrogen; and stored at –80°C for somatic index determination.

2.9. Statistical analyses

The reported values are means ± SD of n = 24, 6 per group. One-way analysis of variance (ANOVA) for statistical evaluation of results in vivo was used, following by Duncan new multiple-range tests to assess differences between groups' means. *P* values of <.05 were considered to be significant.

3. Results

3.1. Polyphenols, antioxidant capacities, and fatty acids

The results of the determination of the contents of bioactive compounds and antioxidant capacities are shown in the Table 2. As can be seen, the contents of polyphenols and antioxidant capacities by ABTS and CUPRAC assays in water extracts were significantly higher than in methanol (*P* in all cases <.05). As can be seen, according to 2 used assays, the significantly highest level of antioxidant capacity was registered by ABTS assay in water extracts of C *Rυ* samples, but all bioactive indexes were slightly higher in C samples but not always significant.

The ESI-MS in negative ion mode of studied extracts slightly differs between the samples (Fig. 1). In all samples, the main peaks were at 309 and 335 m/z (100%) and corresponded to gondolic and docosadienoic acids (Table 3), but small peaks differ from one group to another. The difference in the average size peaks between the samples was the following: in *Rυ*NC was a peak of myristic acid and in *Rυ*C were 2 additional peaks, which are not demonstrated in *Rυ*NC such as palmitoleic and eicosadienoic acids. The amount of fatty acids in *Rυ*C was slightly higher than in *Rυ*NC but not significant (Fig. 1 and Table 3).

Table 2 – Bioactive compounds of *Rυ* from C and NC areas

Samples	POL (mg GAE/g)	ABTS (μmol TE/g)	CUPRAC (μmol TE/g)
*Rυ*NCW	8.23 ± 0.76[c]	53.39 ± 4.12[c]	11.75 ± 1.17[c]
*Rυ*CW	9.37 ± 0.82[d]	54.80 ± 4.61[d]	15.11 ± 1.56[d]
*Rυ*NCMeOH	2.66 ± 0.23[a]	15.53 ± 1.41[a]	5.51 ± 0.55[a]
*Rυ*CMeOH	3.71 ± 0.31[b]	23.42 ± 1.64[b]	6.95 ± 0.62[b]

Results are presented as means ± SD, n = 3. The values with different letters within the columns are significantly different (*P* < .05); per gram dry weight.
Abbreviations: POL, polyphenols; *Rυ*NCW, *Rυ* from NC area, extracted with water; *Rυ*CW, *Rυ* from C area, extracted with water; *Rυ*NCMeOH, *Rυ* from NC area, extracted with methanol; *Rυ*CMeOH, *Rυ* from C area, extracted with methanol.

3.2. Fluorometric data

The binding properties of the *Rυ* samples in comparison with the pure flavonoids such as quercetin are shown in 2-dimensional fluorescence spectra. The following changes appeared when the water extracts of *Rυ* were added to BSA (initially the main peak was at emission 335 nm and fluorescence intensity (FI) of 894 [Fig. 1C, the upper line is BSA]). The interaction of BSA and 40 μg/mL of water extract of *Rυ* during 1 hour at 37°C from NC area (Fig. 1C, second line from the top) showed the position of the main peak at the wavelength of 335 nm and the decrease in FI of 763.22. The reaction with the *Rυ* extracts and quercetin decreased the FI of BSA to 623 (Fig. 1C, the lowest line). The interaction of BSA and 40 μg/mL of water extract of *Rυ* during 1 hour at 37°C from C area (Fig. 1D, second line from the top) showed the same position of the main peak, and the decrease in FI was to 632.29. The reaction with the *Rυ* extracts and quercetin decreased the FI of BSA to 464.54 (Fig. 1D, the lowest line). The water extracts showed the results of the decrease (percentage) of BSA intensity (Fig. 1C): BSA + *Rυ*NC, 14.6%; BSA + *Rυ*NC + quercetin, 30.3%; BSA + *Rυ*C, 29.3%; and BSA + *Rυ*C + quercetin, 48.0% (Fig. 1D). These data were slightly higher than with water extracts than in methanol extract. Such strong binding properties of water extracts are proportional to their amount of polyphenols (Table 2). These results were also in direct relationship with the antioxidant capacities of the extracts (Table 2). The combination of bioactive compounds as total phenols, determined spectroscopically, with antioxidant assays, fluorescence, and mass spectra can be used for characterization of *Rυ* samples.

3.3. In vivo studies

The influence of *Rυ* on performance parameters in rats is summarized in the Table 4. As can be seen, the feed intake was significantly higher (*P* < .05) in Chol than in the control

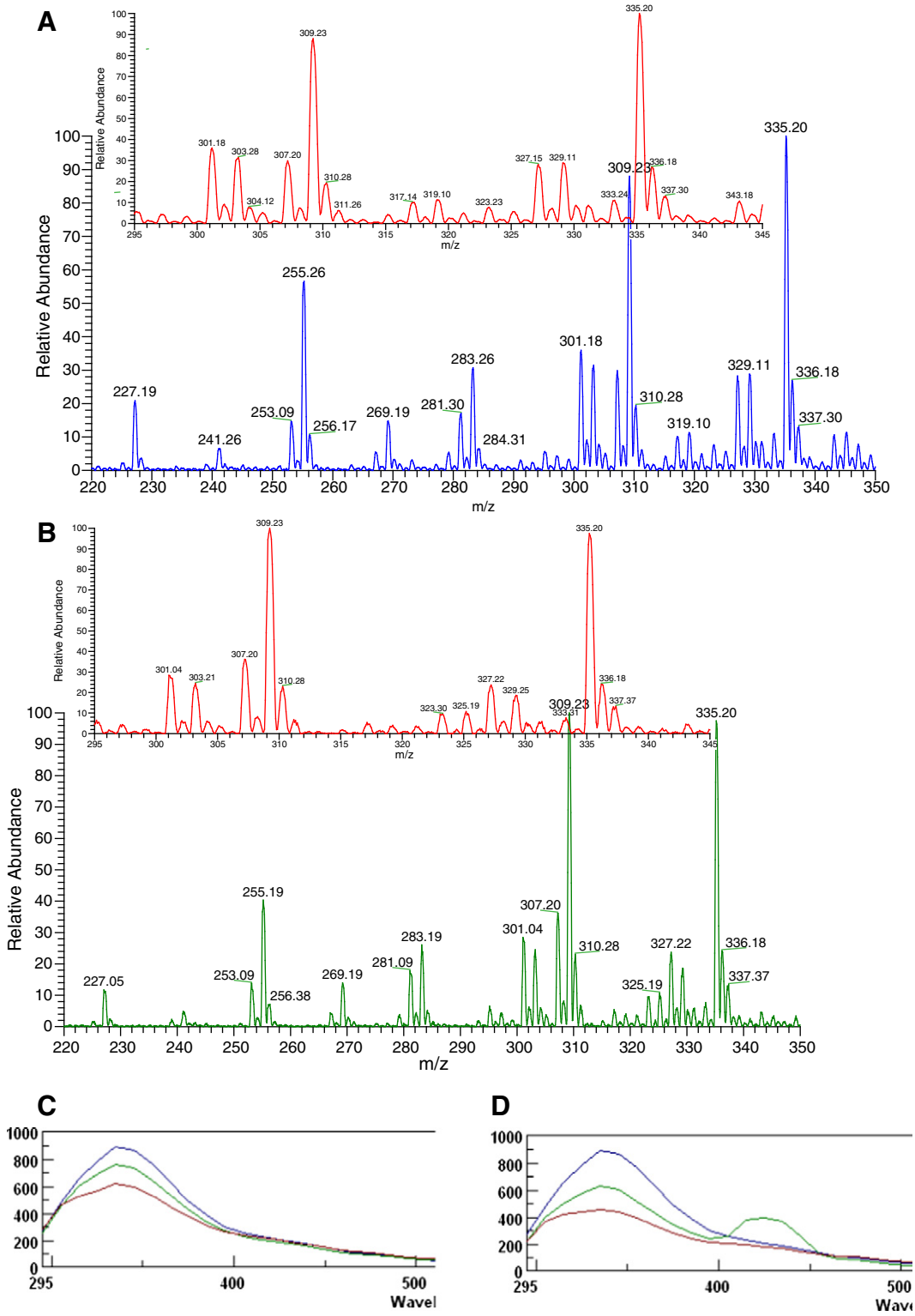

Fig. 1 – Electrospray-ionization MS spectra in negative ion mode of *Rv*NC (A) and *Rv*C (B) methanol extracts. C, Two-dimensional fluorescence spectrum illustrates the interaction between BSA, quercetin, and water extracts of *Rv*NC and *Rv*C samples with BSA: BSA (upper line), BSA + *Rv*NC (second line from the top), and BSA + *Rv*NC + quercetin (the lowest line). D, BSA (upper line), BSA + *Rv*C (second line from the top), and BSA + *Rv*C + quercetin (the lowest line). The change in the FI is a result of binding affinity of *Rv* extracts. In all reactions were used the following conditions: BSA (2.0×10^{-5} mol/L), quercetin (0.22×10^{-5} mol/L), and water extracts in concentration of 40 μg/mL. The binding was during 1 hour at 37°C. Fluorescence intensities are on y-axis and emission wavelengths on x-axis.

Table 3 – Mass spectral data (molecular ion) of fatty acids from *Rv*

Samples	[M-H]⁻(% in MS)	Compound
*Rv*NCMeOH	227 (16)	Myristic acid (14:0)
	255 (44)	Palmitic acid (16:0)
	283 (25)	Stearic acid (18:0)
	301 (35)	Eicosapentaenoic acid (20:5)
	309 (82)	Eicosenoic acid (20:1)
	335 (100)	Docosadienoic acid (22:2)
*Rv*CMeOH	253 (12)	Palmitoleic acid (16:1)
	255 (40)	Palmitic acid (16:0)
	283 (24)	Stearic acid (18:0)
	301 (27)	Eicosapentaenoic acid (20:5)
	307 (36)	Eicosadienoic acid (20:2)
	309 (100)	Eicosenoic acid (20:1)
	335 (96)	Docosadienoic acid (22:2)

The methanol extracts of *Rv* samples done by direct infusion in the mass spectrometer by use of ESI source at negative ion mode, full scan analysis, range of 200 to 400 m/z.

group (611 vs 578 g, respectively). Feed intake in both groups supplemented with *Rv*C and *Rv*NC areas was similar. Body gains in Chol/*Rv*C were the highest (220.8 g) and differed significantly from the other groups. As it was shown, feed efficiency ratio (FER) in Chol/*Rv*C was also the best and amounted to 2.66. This value significantly differs from FER value (Table 4) in the control group (3.80). The contents of minerals (K, Cl, Ca, Mg, K, and Fe, [Table 5]) in rats' serum from the Chol group were similar to their content in the control group. Supplementation of the Chol groups with *Rv*C and *Rv*NC did not influence on the contents of the studied minerals in serum of these rats.

The influence of *Rv* on the lipids profile of rats loaded with Chol is presented in Table 6. As was shown, an increase of TC and its fraction LDL in rats' serum from Chol group vs control amounted to 40.2 and 55.5%, respectively ($P < .05$). No significant changes in the levels of HDL-C and TG between control and Chol groups were noted. As presented, supplementation of the atherogenic diets with 5% DM of *Rv* from C and NC areas hindered the rise in serum lipids vs Chol group by 12.4% (TC), 14.8% (LDL-C), and 42.6% (TG) (average values for both groups with *Rv* [Table 6]). The atherogenic index TC/HDL-C increased from 3.29 (control) to 4.37 (Chol) ($P < .05$). Cholesterol diets supplemented with 5% DM of *Rv* improved the atherogenic indices: this effect in Chol/*Rv*NC was higher than in Chol/*Rv*C.

Table 5 – The influence of *Rv* from C and NC areas on the contents of minerals (mmol/L) in serum of rats fed atherogenic diets

Minerals	Control	Chol	Chol/*Rv*C	Chol/*Rv*NC
K	3.38 ± 0.23[a]	3.66 ± 0.19[a]	3.42 ± 0.18[a]	3.58 ± 0.45[a]
Cl	95.40 ± 1.34[a]	94.60 ± 1.95[a]	95.50 ± 2.59[a]	96.60 ± 1.95[a]
Ca	2.72 ± 0.02[a]	2.69 ± 0.08[a]	2.74 ± 0.11[a]	2.69 ± 0.19[a]
Mg	1.00 ± 0.09[a]	1.06 ± 0.10[a]	1.12 ± 0.09[a]	1.09 ± 0.24[a]
P	2.74 ± 0.40[a]	2.49 ± 0.34[a]	3.12 ± 0.46[a]	3.02 ± 0.91[a]
Fe	20.51 ± 10.62[a]	14.72 ± 2.68[a]	19.28 ± 6.81[a]	18.27 ± 4.77[a]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$).

A significant decrease ($P < .05$) of serum TAC (21.4% for FRAP and 14.6% for ABTS assays) was registered in the Chol group vs the control group (Table 7). Cholesterol diet, which contained 5% of *Rv*C and *Rv*NC, influenced the increase ($P < .05$) of the plasma TAC, which was evaluated by ABTS assay (21.4 and 35.9%, respectively). In the case of FRAP values of antioxidant capacity, similar effect was obtained only for *Rv*NC (27.3%).

No significant changes in the serum of rats in the level of glucose (3.25-4.69 mmol/L) and urea (3.74-4.69 mmol/L) were found. Addition of *Rv*C for the diet Chol group caused an increase ($P < .05$) in albumin content in rats' serum (9.2% an average, Table 7).

For the first time, we compared by fluorescence studies the obtained serum samples (Fig. 2 and Table 8). In all samples were found 2 peaks (A and B). Peak A was with the maximum wavelength of 280/330 nm in all diets but with the lowest intensity in Chol/*Rv*C group (Fig. 2C and CC). Peak B was similar in control and Chol groups and with a very small increase in intensity in Chol group. Peak B in supplemented diets slightly shifted in Chol/*Rv*NC (Fig. 2 D and DD and Table 8), and the intensity was similar.

The influence of *Rv* from C and NC areas on serum enzymes activity is presented in Table 9. Aspartate aminotransferase activity in rats' serum from the Chol group was higher than in the control (145.8 vs 104.0 IU/L) ($P > .05$). *Rapana venosa* C and NC lower the AST activities, but the results were also not significant. No significant changes for ALT, ALP, lipase, and amylase activities in serum rats fed during 28 days Chol/*Rv*C and Chol/*Rv*NC were found (Table 9).

Table 4 – The influence of *Rv* from C and NC areas on performance in rats fed atherogenic diets

Measurements	Control	Chol	Chol/*Rv*C	Chol/*Rv*NC
Feed intake (g)	570.12 ± 28.11[a]	611.00 ± 15.61[b]	587.22 ± 16.10[ab]	577.00 ± 25.97[ab]
Body gains (g)	153.14 ± 24.80[a]	171.08 ± 18.84[a]	220.80 ± 9.22[b]	171.35 ± 49.87[a]
Feed intake (g/d)	17.28 ± 0.85[a]	18.52 ± 0.47 [b]	17.79 ± 0.49[ab]	17.48 ± 0.79[ab]
Body gains (g/d)	4.64 ± 0.75[a]	5.18 ± 0.57[a]	6.69 ± 0.28[b]	5.19 ± 1.51[a]
FER	3.80 ± 0.63[b]	3.60 ± 0.33[bc]	2.66 ± 0.09[ac]	3.65 ± 1.29[bc]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$).

Table 6 – The influence of *Rv* in vivo on lipids profile (millimolar) in serum of rats fed atherogenic diets

Measurements	Control	Chol	Chol/*Rv*C	Chol/*Rv*NC
TC	1.84 ± 0.23[a]	2.58 ± 0.32[b]	2.24 ± 0.22[b]	2.28 ± 0.42[b]
HDL-C	0.56 ± 0.04[a]	0.59 ± 0.03[a]	0.52 ± 0.08[a]	0.61 ± 0.15[a]
LDL-C	1.28 ± 0.21[a]	1.99 ± 0.32[b]	1.72 ± 0.22[b]	1.67 ± 0.38[b]
TC/HDL-C	3.29 ± 0.36[a]	4.37 ± 0.55[bc]	4.31 ± 0.69[bc]	3.74 ± 1.08[ac]
AI	2.29 ± 0.36[a]	3.37 ± 0.55[bc]	3.31 ± 0.69[bc]	2.74 ± 1.08[ac]
TG	1.36 ± 0.90[a]	1.55 ± 0.49[a]	1.04 ± 0.60[a]	0.74 ± 0.36[a]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$).
Abbreviation: AI, atherogenic index.

As it was previously mentioned, some organs (liver, heart, kidneys, and spleen) were isolated to determine their participation in body weight (somatic index). The somatic index of the liver (Table 10) was significantly higher in rats fed diets with Chol and snails *Rv*C and *Rv*NC in comparison with rats, which obtained the control diet (3.75 and 3.71 vs 3.12, respectively). The somatic index values for heart, kidneys, and spleen did not differ among groups of rats.

4. Discussion

Seafood (oysters, mussels, and other molluscs) is an important source of protein, vitamin, polyunsaturated fatty acids (PUFA), minerals, and also bioactive compounds; and, nowadays, their consumption is increasing [17]. The composition of the seafood depends on environment and its pollution and was monitored in our recent studies [10,17,23]. *Rapana venosa* and mussels can be used as bioindicators, representing an important tool for the biomonitoring of environmental pollution in coastal areas. Moncheva et al [10] have shown that *Rv* collected from Black Sea, especially from polluted sites, is a valuable source of bioactive compounds (polyphenols, tannins, flavonoids, and flavanols), and their antioxidant activity was higher than in nonpolluted samples. Other investigators reported similar results [17,23].

In our previous study, it was shown that supplementation of the diet for rats, which contained 1% Chol and mussels from different sites of South Korea, polluted (Mokpo port) and nonpolluted (Mokpo bay) may influence the plasma parameters of these animals [1,2]. Such supplementation improves animals' protein metabolism and positively affects plasma lipid profile and plasma antioxidant activity. Antioxidant tests can be used as an additional index for the quality of mussels [1,2].

Table 7 – The influence of *Rv* on total antioxidant capacities, glucose urea, and albumin in serum of rats from rats fed atherogenic diets

Measurements	Control	Chol	Chol/*Rv*C	Chol/*Rv*NC
FRAP (mmol TE/L)	0.14 ± 0.01[b]	0.11 ± 0.01[a]	0.11 ± 0.01[a]	0.14 ± 0.02[b]
ABTS (mmol TE/L)	1.37 ± 0.23[b]	1.17 ± 0.20[a]	1.42 ± 0.05[b]	1.59 ± 0.25[b]
Glucose (mmol/L)	3.25 ± 1.13[a]	3.62 ± 0.73[a]	3.23 ± 1.27[a]	4.69 ± 1.20[a]
Urea (mmol/L)	3.74 ± 0.79[a]	4.69 ± 0.65[a]	4.55 ± 1.05[a]	3.74 ± 0.42[a]
Albumin (g/dL)	3.15 ± 0.13[a]	3.38 ± 0.10[a]	3.78 ± 0.12[b]	3.60 ± 0.22[b]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$).

However, the influence of *Rv* consumption on plasma animals' indices was not studied. Therefore, we have decided to evaluate the influence of *Rv* (5% in the diet) from C and NC areas of the Black Sea on growth, plasma, and serum parameters in rats fed diets containing 1% Chol for 28 days.

The fatty acid composition in *Rv* samples was similar to the cited reports. The most abundant fatty acid esters in the fractionated samples were in order as 16:0, 22:6, 14:0, 16:1, 18:4, and 20:5 fatty acids [19-21]. The major contributing factor to the seasonal variation of PUFA was n-3 fatty acids. These results led to the lowest levels of P/sulfur and n-3/n-6 in summer. The data suggest that bivalve shellfish would be excellent sources of n-3 fatty acids, especially eicosapentaenoic and docosahexaenoic acids [19], and this is in line with our findings. We found that the food intake was significantly higher in Chol (vs control) and the body gains as well in Chol/*Rv*NC (vs other groups) (P in both cases <.05). As shown, the FER was significantly lower in Chol/*Rv*NC group vs control (2.66 and 3.80, respectively) ($P < .05$) and pointed the best feed utilization. The increase in body weight gain in rats fed for 4 weeks diet with Chol and 5% share of mussels from polluted waters (compared with mussels from nonpolluted) was found. Feed efficiency ratio values in this study were similar in all rats [2].

As was shown, *Rv* did not influence on some minerals contents in serum K, Cl, Ca, Mg, P, and Fe. By contrast, other results were obtained in rats fed diets with Chol and mussels from polluted and unpolluted areas [1]. In this study, the contents of Mg and P were increased, and amount of K decreased in groups with Chol and mussels. Oppositely, the content of Fe increased only in rats fed diet with mussels of nonpolluted area; however, amounts of Cl and Ca were without changes. It seems that the differences in their contents in the serum of rats were dependent on the mineral composition of mussels and snails and their different aquatic environment (Korea Mokpo Coast and Black Sea in Bulgaria).

Lipid profile in serum of rats was lower than expected after supplementation of snails to the diet of Chol. The resulting decrease in the contents of TC and LDL-C in the groups receiving *Rv* (vs Chol) was not significant. A significant decrease in TC and LDL-C was obtained in rats fed a diet with Chol and mussels [1], but there was no significant difference between the addition of bivalve molluscs from waters C and NC. The present study and the references cited above have not been reported changes in HDL-C and TG contents.

It has been shown [24] that protein hydrolysates of sardines (5%) administered in the diet of rats with Chol (1%) affected hypolipidemic status: decreased content of TC, LDL-C, and TG. This effect was related to their antioxidant activity to protect lipids from oxidation. In this study, a significant increase of antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase, and catalase) in the liver was obtained. Mahfouz and Kummerow [25] found in experiment on rabbits that Chol-rich diets have different effects on lipid peroxidation, Chol oxides, and antioxidant enzymes.

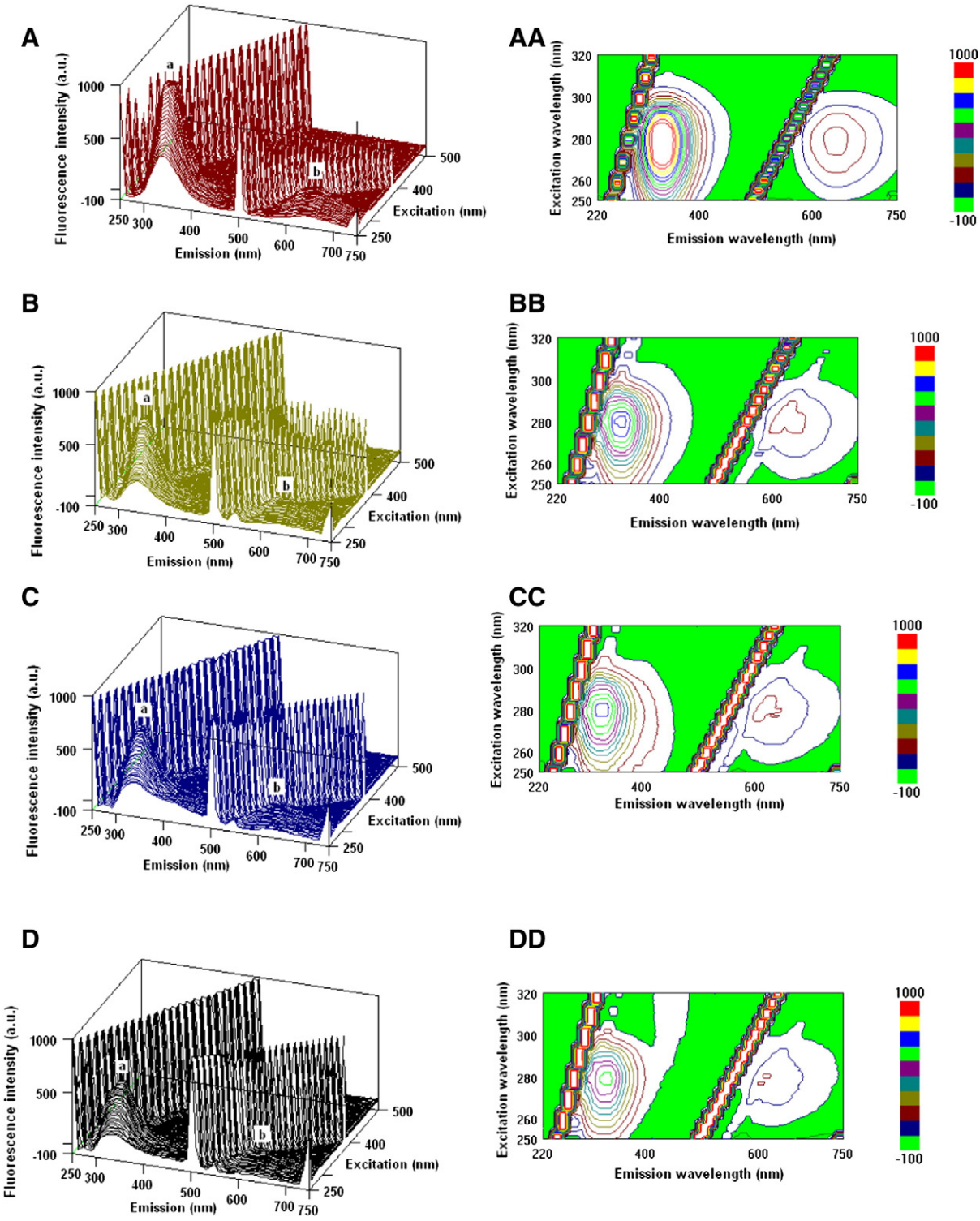

Fig. 2 – Three-dimensional fluorescence spectra and contour maps of rat's serum samples. A, AA, Three-dimensional fluorescence spectra and its contour serum from control group; B, BB, Three-dimensional fluorescence spectra and its contour serum from Chol group; C, CC, Three-dimensional fluorescence spectra and its contour serum from Chol/RvC group; D, DD, Three-dimensional fluorescence spectra and its contour serum from Chol/RvNC group. A and B, 2 peaks in the serum samples.

It has been shown that Chol significantly decreased TAC in rat's plasma, which was evaluated by ABTS and FRAP assays, which are consistent with the results of other authors [26]. Co-administration of gastropods *Rv* had affected the antioxidant activity, and a significant increase in the TAC values, which were evaluated using ABTS, occurred in both groups with the participation of snails, but for FRAP assay, only in rats from group Chol/RvNC were found. Furthermore,

Table 8 – The influence of *Rv* from C and NC areas on serum fluorescence properties in rats fed atherogenic diets

Measurements	Control	Chol	Chol/RvC	Chol/RvNC
Peak A, $\lambda_{ex}/\lambda_{em}$, nm/nm	280/330	280/330	280/330	280/330
Intensity, F_0	1000 ± 45[b]	1000 ± 53[b]	570 ± 24[a]	1000 ± 42[b]
Peak B, $\lambda_{ex}/\lambda_{em}$, nm/nm	279/640	279/640	280/630	280/620
Intensity, F_0	189 ± 16[b]	197 ± 18[b]	123 ± 14[a]	125 ± 14[a]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$). Fluorescence measurements of peaks A and B maxima (Fig. 2) and their intensities in serum were measured by 3D-FL.

Table 10 – The influence of *Rv* from C and NC areas on somatic index of some organs (%) isolated from rats fed atherogenic diets

Organs	Control	Chol	Chol/RvC	Chol/RvNC
Liver	3.12 ± 0.22[a]	3.47 ± 0.32[ac]	3.75 ± 0.22[bc]	3.71 ± 0.66[bc]
Heart	0.35 ± 0.02[a]	0.36 ± 0.03[a]	0.33 ± 0.02[a]	0.35 ± 0.03[a]
Kidneys	0.64 ± 0.08[a]	0.63 ± 0.05[a]	0.65 ± 0.04[a]	0.70 ± 0.09[a]
Spleen	0.21 ± 0.08[a]	0.18 ± 0.02[a]	0.19 ± 0.02[a]	0.19 ± 0.01[a]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$).

other investigators have obtained similar results concerning effect of mussels on TAC in plasma of rats fed diets with Chol [1]. Antioxidant activity of methanol extract from *Rv* depends on polyphenols content and was higher for snails from the polluted area than from nonpolluted [17].

According to Moncheva et al [10], methanol extract from DM of *Rv* polluted and to a lesser extent *Rv* nonpolluted showed high amounts of total polyphenols (23.22 vs 15.60 mg GAE/g DW) and exhibited high levels of antioxidant activities in some radical scavenging assays. Obtained results of antioxidant capacity from *Rv* polluted and nonpolluted [10] areas were 56.38 and 33.79 (for ABTS), 54.79 and 33.7 (for CUPRAC), and 62.34 and 30.31 μmol/L TE/g DW (for 1, 1-diphenyl-2-picrylhydrazyl). Our conclusions are in the same line with Mousumi et al [27], where it was shown that the dietary antioxidants of fresh water mussels were found to be effective in the prevention of oxidative stress in inflammatory arthritis. The cash-crop by-product gives a rare opportunity for income and nutrition, not only by supplying cheap and available energy (protein and dietary factors) but also by providing antioxidants effective against chronic inflammatory disease. Phenolic compounds from vegetables [28], oil palm [29], and polyphenol-rich extract from blackcurrant pomace [30] may possibly attenuate atherosclerosis and other forms of cardiovascular disease and serum lipid changes induced by a high-fat diet.

The present investigation has shown that there was no significant effect of *Rv* on levels of glucose and urea in rat's serum and also on AST, ALT, ALP, lipase, and amylase enzymes activities. In available literature, there were no found studies on the effect of snails on serum enzyme profile in rats fed a diet supplemented with Chol. Serum enzymes AST, ALT, and ALP are determined in the evaluation of hepatic disorders [31], and an increase of transaminase reflects active liver damage. A significant increase of these enzymes activity in rat's serum fed Chol diet (vs control) and decrease after feeding diets contained sardinelle muscle protein hydrolysates (vs Chol diet) was presented by Khaled et al [24]. The decreasing of the AST and ALT levels helps to correct the alteration of liver functions.

The liver weight was higher in the rats of the Chol/RvC group; however, the results were not significant (data not shown). The somatic index of the liver was significantly higher in groups of rats fed diets with Chol and snails RvC and RvNC in comparison with rats fed the control diet (20.2 and 18.9%), respectively. An increase in somatic index of liver in Chol group was smaller than in groups of rats fed diet with snails and amounted of 11.2%. The increase of liver somatic index depends on the lipids accumulation involved in lipogenesis of liver hepatocytes. The concentration of the TC in rats liver amounted to 48.1, 31.7, and 33.3 μmol/g in groups: Chol, Chol/mussels polluted, and Chol/mussels nonpolluted, respectively [1]. In cited study, somatic index in the liver amounted to 3.53, 3.22, and 3.25, respectively ($P > .05$). These results indicate that mussel's addition to a diet containing 1% Chol influence greater decrease in the liver somatic index than addition of snails. Our very recent results showed that the fluorescence is significantly quenched because of the conformation of proteins, phenolic acids, and flavonoids [18]. This interaction was investigated using tryptophan fluorescence

Table 9 – The influence of *Rv* from C and NC areas on serum enzymes activities (IU/L) in rats fed atherogenic diets

Measurements	Control	Chol	Chol/RvC	Chol/RvNC
AST	104.0 ± 17.1[a]	145.8 ± .36.8[a]	116.3 ± 44.6[a]	138.2 ± 61.1[a]
ALT	33.3 ± 2.1[a]	30.0 ± 5.4[a]	32.2 ± 8.1[a]	32.0 ± 3.4[a]
ALP	287.0 ± 116.7[a]	279.5 ± 60.8[a]	301.3 ± 96.6[a]	398.4 ± 166.9[a]
Lipase	12.75 ± 2.06[a]	14.00 ± 1.41[a]	13.50 ± 1.30[a]	13.44 ± 1.27[a]
Amylase	1968 ± 502[a]	1792 ± 217[a]	2224 ± 365[a]	2080 ± 645[a]

Results are presented as means ± SD, (n = 24, 6 per group). Data were analyzed by 1-way ANOVA and statistical significance using the Duncan test for group comparisons. The values with different letters within the rows are significantly different ($P < .05$).

quenching. Our results are in agreement with others that quercetin as an aglycon is more hydrophobic and demonstrates strong affinity toward human serum albumin. Other results [32] differ from that reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning ranges used in a similar study. The strong binding properties of phenolics show that they may be effective in prevention of atherosclerosis under physiologic conditions. Our results are in accordance with Sekhon-Loodu et al [33], where it was found that both quercetin glycosides and omega-3 PUFA (n-3 PUFA) are well established for their individual health benefits in ameliorating metabolic disease. The *Rv* is rich not only in polyphenols but also in the fatty acids. Such combination was not studied previously; therefore, the obtained results in improving the lipid spectra and antioxidant capacities are found for the first time in this research. Our conclusions are in line with others, where it was reported that watermelon, the ethanol extract of lotus root, and apple flavonols, rich in antioxidants and other bioactive components, may be a viable method to improve cardiovascular disease risk factors through reduced oxidative stress [33-35].

It is assumed that snails, which are a source of polyphenolic compounds and essential nonstaturated fatty acids, given to rats involving Chol diets can interact like other seafood (clams), and this effect was determined in the previous work. The increasing consumption of seafood led the authors to identify a specific metabolic reaction of rats fed atherogenic diet with snail's supplementation. We accept the proposed hypothesis because the present study demonstrated that the DM of *Rv* supplementation improved the lipid spectrum and antioxidant status in rats fed atherogenic diets. One of the limitations of the present study in rats is the period of treatment after the supplementation of the diet. The period of 28 days is not sufficient to determine the changes in the rat's serum. If a longer time had been given for this treatment, an increase of serum antioxidants and significant treatment effects might have been achieved in rats. Finally, future research into lipid and antioxidant metabolism and related enzymes would be helpful to understand the changes in the metabolism of rats. Contamination of the environment can affect the contents of various substances in the bodies of *Rv*, and, therefore, the collected samples for testing were from polluted and nonpolluted water. Restrictions may result from the number of animals in groups depending on the number of snails harvested from clean and polluted water. Such limitations of the amount of rats and marine animals and the collection from different areas can affect the results of the experiment. Only 6 rats in 1 group are not sufficient, better to increase this amount to 10 rats per group.

In conclusion, DM of *Rv* from C and NC areas supplemented diets with Chol hindered both the rise in serum lipids levels and the decrease in the plasma antioxidant activity. Such supplementation does not affect the enzyme activities and mineral content in serum of rats. Exactly the same very small changes were obtained by in vitro fluorescence studies of the serum samples during supplementation with *Rv*.

We plan to verify the increase of such indexes by longer duration of this experiment. Furthermore, studies are needed to identify and to determine separately the effects of the bioactive ingredients and also fatty acids of *Rv* in prevention of atherosclerosis. These pioneering studies on rats may in the future be extended for further analysis on different marine animals.

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