



Effects of atorvastatin and artichoke leaf tincture on oxidative stress in hypercholesterolemic rats

Uticaj atorvastatina i tinkture lista artičoke na oksidativni stres kod pacova sa hiperholesterolemijom

Milkica Crevar-Sakač*, Zorica Vujić*, Jelena Kotur-Stevuljević†, Jasmina Ivanišević‡, Zorana Jelić-Ivanović‡, Marina Milenković‡, Milica Markelić§, Zoran Vujčić||

*Department of Pharmaceutical Chemistry, †Department of Medical Biochemistry, ‡Department of Microbiology and Immunology, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia; §Center for Electron Microscopy, Faculty of Biology, University of Belgrade, Belgrade, Serbia; ||Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia

Abstract

Background/Aim. Since combining conventional drugs with herbal medicinal products is in current research focus and possible of great interest as therapy improvement way, the aim of this study was to determine the effects of well-established antiatherosclerotic drug atorvastatin (CAS number 134523-00-5) and commercially available artichoke leaf tincture (ALTINC), used as combined therapy, as well as to compare effects of these two treatments separately. **Methods.** Experimental animals were divided into five groups: the group I (the control group of rats fed with standard diet during 11 weeks), and the remaining 4 groups of rats (II, III, IV and V) fed with standard diet during the first week and then with hypercholesterolemic diet during the next 10 weeks. The group II of rats were left without treatment, while in the groups III, IV and V were rats treated *per os* with atorvastatin (1.15 mg/kg body weight – b.w.), ALTINC (0.1 mL/kg b.w.) and their combination in same doses, respectively, for the last six weeks. **Results.** The cholesterol rich diet led to pronounced hyperlipidemia which

could not be overcome with the therapy. However, the therapy showed positive effects on abdominal aorta wall thickness and parameters of oxidative stress (malondialdehyde – MDA, prooxidative-antioxidative balance – PAB) and antioxidative protection (reduced glutathione – GSH, paraoxanase 1 – PON1, superoxide dismutase – SODA SH groups), especially ALTINC was successful in oxidative status improvement. **Conclusion.** Separate treatments comparison showed that artichoke leaf tincture is very potent antioxidant with beneficial effects in early stages of atherosclerosis. Since atorvastatin and constituents of ALTINC probably have different mechanisms of action, simultaneous use of both therapies could be beneficial but should be further investigated since our results showed that ALTINC is less effective when used in combination with atorvastatin.

Key words:

cynara scolimus; atorvastatin calcium; atherosclerosis; oxidative stress; rats; treatment outcome.

Apstrakt

Uvod/Cilj. Savremena istraživanja sve više se okreću mogućnosti kombinovanja konvencionalne terapije sa biljnim lekovitim proizvodima. Cilj ovog istraživanja bio je praćenje efekata atorvastatina (CAS broj 134523-00-5), poznatog leka koji se koristi u terapiji hiperlipidemije, i tinkture lista artičoke primenjenih pojedinačno, kao i u obliku kombinovane terapije. **Metode.** Eksperimentalne životinje bile su podeljene u pet grupa: grupa I bila je kontrolna i činili su je pacovi

hranjeni standardnom hranom za glodare tokom 11 nedelja, dok su preostale četiri grupe (II–V) činili pacovi koji su hranjeni standardnom hranom tokom prve nedelje, a potom u narednih 10 hranom bogatom holesterolom. Grupa II nije dobijala nikakav tretman, dok su III, IV i V posle 4. nedelje od početka uzimanja hrane bogate holesterolom, tokom narednih šest nedelja tretirane *per os*, redom: atorvastatinom u dozi od 1,15 mg/kg, tinkturom artičoke u dozi od 0,1 mL/kg i njihovom kombinacijom u istim dozama. **Rezultati.** Hrana bogata holesterolom izazvala je uznapredovalu

hiperholesterolemiju na koju terapija nije značajno delovala. Terapija je pokazala povoljan efekat na debljinu zida trbušne aorte, parametre oksidativnog stresa (malondialdehid – MDA, prooksidativni-antioksidativni balans – PAB) i antioksidativne zaštite (redukovani glutation – GSH, SH grupe, paraoksonazu1 – PON1, superoksid dismutazu – SOD). Tinktura lista artičoke pokazala se najboljom u poboljšanju oksidativnog statusa. **Zaključak.** Poređenje pojedinačne terapije pokazalo je da tinktura lista artičoke ima jako antioksidativno dejstvo i pokazuje povoljne efekte u početnim

fazama aterosklerotskog procesa. S obzirom na to da atorvastatin i komponente tinkture lista artičoke verovatno deluju različitim mehanizmima, kombinovana primena može imati povoljne efekte, ali se mora dodatno ispitati jer dobijeni rezultati ukazuju da je tinktura lista artičoke manje efikasna kada se primenjuje zajedno sa atorvastatinom.

Ključne reči:

cynara scolimus; atorvastatin kalcijum; ateroskleroza; stres, oksidativni; pacovi; lečenje, ishod.

Introduction

Hyperlipidemia with plasma-elevated concentrations of cholesterol and low density cholesterol (LDL-C) is considered to be the cause of cardiovascular disease. Treatment of hyperlipidemia needs diet control, exercise and using lipid-lowering compounds such as drugs and diet.

The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.88) inhibitors (statins) have emerged as the most important class of lipid-lowering agents. Through inhibition of HMG-CoA reductase, they restrict the rate-limiting step of cholesterol synthesis resulting in up-regulation of low density lipoprotein (LDL) receptors on the cell membrane and reduction of atherogenic LDL consequences. Several clinical trials have demonstrated the beneficial effects of statins in cardiovascular disorders, extending beyond their effects on cholesterol level, in primary and secondary prevention settings^{1, 2}. As a result, nowadays statins represent one of the most powerful agents for the treatment of cardiovascular events³. Statins also have many cholesterol-independent (pleiotropic) beneficial effects such as their antioxidative activity^{1, 4, 5}.

Although the side effects of statins are relatively low, they can cause rhabdomyolysis in rare cases⁶. Therefore, research to identify natural agents with lipid-lowering properties and with less or no adverse effects, especially medicinal plants, is warranted.

Artichoke (*Cynara scolymus* L.) is a plant which is widely grown in Mediterranean countries. Leaves of this plant are rich in antioxidant constituents. It contains many different polyphenolic compounds such as cynarin, chlorogenic acid, luteolin, apigenin^{7, 8}. Different types of artichoke leaf extract were reported to have a cholesterol-reducing effect on hypercholesterolemic subjects^{9, 10}. In addition, *in vitro* studies show that artichoke extract decreases the production of reactive oxygen species, oxidation of low-density lipoproteins and lipid peroxidation^{11, 12}.

It is not unusual that patients on statin therapy concomitantly take herbal medicinal product (e.g. extracts of artichoke leaf products or another) known for its antioxidant properties, therefore it is important to investigate any possible synergistic or opposite action of these treatments. Comparing antioxidant properties of these two therapies can give answer the question if it is reasonable to suggest artichoke leaf product usage in the beginning stages of atherosclerosis.

Methods

Diets, chemicals and herbal product

In this experiment we used normal, commercial rat chow diet and special atherogenic diet prepared in our laboratory by adding cholesterol, sodium cholate and sunflower oil to commercial diet¹³. Cholesterol and sodium cholate hydrate were purchased from Sigma Aldrich (Dorset, United Kingdom). Sunflower oil was purchased from "Dijamant" (Zrenjanin, Serbia).

Concentrated phosphoric acid used for HPLC analysis was bought from J.T. Baker (Deventer, Netherland). Acetonitrile, HPLC grade, methanol, as well as cynarin and chlorogenic acid were purchased from Sigma Aldrich (Steinheim, Germany). Ultra pure water used for chromatography was prepared by means of TKA water purification system (Niederelbert, Germany).

Atorvastatin calcium standard used in the treatment was donated from Nobel Ilaç (Turkey).

Herbal product used in the experiment was commercial artichoke leaf tincture bought from the Institute for Medicinal Plant Research "Dr. Josif Pančić" (Belgrade, Serbia). Plant material used for tincture preparation was dried primary rosette of artichoke plant. The method of extraction carried out by manufacturer was single percolation during 24 hours. After that time extract was collected, left three days and then filtered. One part of plant material gives five parts of extract. Extraction was performed with mixture of ethanol and water (38:62, w/w).

All other chemicals used for biochemical assays during the experiment were of the highest purity available and were obtained from Sigma Aldrich (Dorset, United Kingdom).

Quantification of cynarin and chlorogenic acid in artichoke leaf tincture

Since the manufacturer had not provided information on the chemical composition of the tincture, the content of cynarin and chlorogenic acid was determined by HPLC method described in Ph.Eur. 7.0 monograph for Artichoke Leaf (*Cynarae folium*).

HPLC system used for experiment consisted of Agilent Technologies HP 1200 liquid chromatograph with binary pump and DAD detector (Agilent, Santa Clara, CA, SAD). A ZORBAX Eclipse Plus C18 Analytical (4.6 mm x 250 mm, 5 µm) (Agilent, Santa Clara, CA, SAD) column was used for

chromatographic separation of the compounds. The analytes were eluted by a gradient mobile phase system consisting of mobile phase A (phosphoric acid R : water R (0.5 : 99.5 v/v)) and mobile phase B (phosphoric acid R : acetonitrile R (0.5 : 99.5 v/v)). During the first minute after injection mobile phase was isocratic (92% mobile phase A and 8% mobile phase B). From the first to the twentieth minute linear change from 92% to 75% mobile phase A and from 8% to 25% mobile phase B was performed. For the next 13 minutes the ratio of mobile phases remained 75% mobile phase A and 25% mobile phase B. During the last two minutes of chromatographic run (33–35 minutes) linear change from 75% to 0% mobile phase A and from 25% to 100% mobile phase B was performed. The flow rate was 1.2 mL/min. The column temperature was 40°C and detection was performed on 330 nm.

Reference solution of cynarin was prepared by dissolving 5 mg of standard substance in 50 mL of methanol and further transferring 2.5 mL of prepared solution to 10 mL volumetric flask and diluting to volume with methanol. Reference solution of chlorogenic acid was prepared in the same way. Concentrations of cynarin and chlorogenic acid in prepared standard solutions were 0.025 mg mL⁻¹. A sample of the examined tincture for HPLC analysis was prepared by diluting 1 mL of tincture to 10 mL with the mixture of water and methanol (50:50, v/v). 1 mL of prepared solution was further diluted to 5 mL with the same diluent.

Animals

Male Wistar albino rats (7 weeks old, weighing 150–190 g at the beginning of the experiment) were used for the experiment. Animals were obtained from the Military Medical Academy Farm (Belgrade, Serbia). They were housed in groups of three in a controlled environment with 12 h light and dark cycles and were allowed free access to food and water.

All experimental procedures and protocols conformed to institutional guidelines for the care and use of animals in research No 5/10 (Ethics Committee of the University of Belgrade – Faculty of Pharmacy).

Study protocol

Rats were divided into five groups: the group I included control group rats fed with standard, normal diet (ND), and the remaining 4 groups (II, III, IV and V) were rats fed with normal pellet chow for one week, and then with hypercholesterolemic diet (standard pellet chow supplemented with 2% cholesterol, 3% sunflower oil and 1% sodium cholate) for the next ten weeks. The group II [atherogenic diet (AD) group] was left without treatment, while the groups III, IV and V consisted of atherogenic diet rats treated *per os* after four weeks without therapy, with atorvastatin (1.15 mg/kg b.w.), artichoke leaf tincture (0.1 mL/kg b.w.), and their combination, respectively, for the next six weeks. This therapy was applied to animals with a gastric probe. Atorvastatin was prepared as suspension in saline solution and artichoke leaf tincture was diluted with the same solvent for simplicity of application.

Doses of atorvastatin and ALTINC were chosen and calculated according to doses usually prescribed to humans and available data from experiments previously conducted by other researchers^{14–16}. It was taken that grown up person of average body weight 70 kg takes 80 mg of atorvastatin daily, which means approximately 1.15 mg per kg body weight. In manual published by the manufacturer of commercial tincture is written that grown-up person should take 30 drops of tincture 3 times a day which means 90 drops in total daily. If average body weight of grown up person is 70 kg, that means 1.29 drops of ALTINC per kg body weight. For easier measurement and possible loss during treatment dose of ALTINC was 0.1 mL/kg b.w. These doses were extrapolated to experimental animals' average body weight. Body weights of rats were measured and doses of atorvastatin and ALTINC were recalculated once a week.

At the end of the experiment, animals from each group were fasted overnight, anesthetized by growing concentration of CO₂ and blood samples were collected directly from the heart in test tubes containing heparine and centrifuged 3,000 rpm for 15 min. Plasma samples were stored at -80°C until they were analyzed. One part of the blood samples was hemolysed and erythrocyte hemolysates were kept at -80°C until further analysis.

The liver of the rat was immediately removed, washed in ice-cold saline solution and blotted. Accurately-weighed pieces of tissue were minced and homogenized in nine volumes of 0.1 M phosphate buffer (pH 7.4), containing 1.15% KCl, using a polytron homogenizer. A portion of the homogenate was kept at -80°C for the determination of malondialdehyde (MDA) and reduced glutathione (GSH) content.

Biochemical analysis

In plasma samples alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), total proteins, glucose, total cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were assayed by routine enzymatic methods using an ILab 300+ analyzer (Instrumentation Laboratory, Milan, Italy) and Randox Laboratories (Armdore, UK) reagents. LDL-C was calculated using the Friedwald equation.

Oxidative stress parameters

Malondialdehyde (MDA) concentrations were measured using the thiobarbituric acid reactive substances (TBARS) assay employing the molar absorption coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ and spectrophotometry at 535 nm previously described by Girotti et al.¹⁷. Superoxidedismutase (SOD, EC 1.15.1.1) activity was measured according to the previously published method by Misra and Fridovich¹⁸. Reduced glutathione (GSH) content was measured according to the method of Jollow et al.¹⁹. Concentrations of sulfhydryl (SH) groups in plasma and erythrocytes were determined using 0.2 mmol/L 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reported by Ellman²⁰. Plasma pro-oxidant-antioxidant balance (PAB) was measured according to a previously published method²¹. Rates of paraoxonase

(PON1, EC 3.1.8.1) activity towards diazoxon were measured spectrophotometrically in plasma according to the method described by Richter and Furlong²². Diazoxon was purchased from Chem Service (West Chester, PA, USA). The activities are reported as IU/L.

Abdominal aorta wall thickness examination

For light microscopic examination, the lower portions of the abdominal aorta were dissected, formaldehyde-fixed and routinely embedded in paraffin. To distinguish between muscle cells and extracellular components of the aortic wall, 5 μ m thick, the transversal sections of the aorta were rehydrated and stained according to the Azan trichrome staining method. Stained sections were analyzed microscopically (DMLB light microscope, Leica Microsystems, Mannheim, Germany). Wall thickness was measured using Image J software (NIH, Bethesda, Maryland, USA) by random selection of ten positions on the sections from every animal.

Statistical analysis

The characteristics of the study populations are presented as means \pm standard deviations. Studied variables were compared using ANOVA with Tukey-Snedecor test as a *post hoc* analysis. Statistical analyses were performed using PASW[®] Statistic version 18 (Chicago, Illinois, USA) and Microsoft[®] Office Excel 2007. A value of *p* less than 0.05 was considered statistically significant.

Results

Content of cynarin and chlorogenic acid in artichoke leaf tincture

The determined content of both, cynarin and chlorogenic acid in investigated tincture was 0.2%.

Biochemical parameters

Table 1 indicates basic biochemical and lipid parameters, as well as animal body weight after six weeks of treatment. Results showed an increased concentration of liver enzymes (AST and ALT) in the groups on the atherogenic diet and atorvastatin (alone or in combination with ALTINC). The group that received only ALTINC showed the same level of liver enzyme activities as normally fed rats.

Lipid parameter concentrations were significantly higher in all the groups fed with atherogenic diet, except HDL-C concentrations which were similar in all the experimental groups and triglycerides which were lower in the groups on atherogenic diet. The group on atorvastatin treatment showed the lowest concentration, although with no statistical significance.

Abdominal aorta examination

The atherogenic diet led to a significant increase in abdominal aorta wall thickness comparing to rats fed with normal diet (Figure 1). It was found that all types of therapy had beneficial effect, but only the combination of atorvastatin and ALTINC decreased the thickness of the aortic wall with a statistically significant difference comparing to rats fed with atherogenic diet ($p < 0.001$) (Figure 1). Microscopic analysis of abdominal aorta (Figure 2A) revealed proper organization of muscular layers and elastic laminae maintained in most groups. Exceptions were the groups on AD and the group on AD + Ator (Figures 2B and C), where plaques were visible, distorting the proper organization of aortic wall tunica media.

Determination of oxidative stress and antioxidative parameters in liver tissue

To assess tissue oxidative status upon challenge with the atherogenic diet, as well as after antihyperlipidemic statin drug treatment, and under the influence of artichoke leaf

Table 1

Effect of atorvastatin (1.15 mg/kg body weight) and artichoke leaf tincture (ALTINC) (0.1 mL/kg body weight) treatment on general biochemical and lipid parameters after treatment						
Parameter	ND	AD	AD + Ator	AD + ALTINC	AD + Ator + ALTINC	<i>p</i>
Weight gain (% comparing to weight at the beginning of the experiment)	154 \pm 21	196 \pm 31 ^a	187 \pm 18 ^a	175 \pm 27	191 \pm 18 ^a	\leq 0.05
ALT (IU)	39.5 \pm 7.0	51.8 \pm 12.8	56.2 \pm 11.0	47.5 \pm 12.2	64.3 \pm 10.5	\leq 0.01
AST (IU)	122.8 \pm 39.6	167.2 \pm 19.1	205.4 \pm 35.7 ^{aa}	127.5 \pm 52.8 ^c	169.8 \pm 19.3	\leq 0.01
tCHOL (mmol/L)	1.45 \pm 0.19	2.96 \pm 0.49	3.67 \pm 1.75	4.48 \pm 1.39 ^a	3.80 \pm 1.66	\leq 0.01
LDL (mmol/L)	0.64 \pm 0.21	2.28 \pm 0.53	3.06 \pm 1.72	3.83 \pm 1.45 ^a	3.15 \pm 1.67	\leq 0.01
HDL (mmol/L)	0.478 \pm 0.057	0.446 \pm 0.108	0.428 \pm 0.064	0.442 \pm 0.092	0.455 \pm 0.075	0.86
nonHDL (mmol/L)	0.97 \pm 0.14	2.51 \pm 0.49	3.24 \pm 1.74	3.49 \pm 1.64	3.34 \pm 1.68	\leq 0.05
TG (mmol/L)	0.737 \pm 0.205	0.508 \pm 0.114	0.382 \pm 0.065	0.455 \pm 0.126	0.422 \pm 0.137	\leq 0.001
TP (g/L)	70.8 \pm 7.8	71.5 \pm 4.7	72.1 \pm 1.4	69.2 \pm 2.3	69.9 \pm 1.6	0.780
GLU (mmol/L)	7.3 \pm 1.5	7.1 \pm 1.8	6.6 \pm 0.8	7.8 \pm 1.1	8.3 \pm 2.0	0.362

ALT – alanine aminotransferase; AST – aspartate aminotransferase; tCHOL – total cholesterol concentration; LDL – concentration of cholesterol contained in low density lipoproteins; HDL – concentration of cholesterol contained in high density lipoproteins; nonHDL – concentration of cholesterol apart from cholesterol contained in high density lipoproteins; TG – concentration of triglycerides; TP – concentration of total proteins; GLU – concentration of glucose; ND – rats fed with normal diet; AD – rats fed with atherogenic diet, AD + Ator – rats fed with atherogenic diet treated with atorvastatin; AD + Ator + ALTINC – rats fed with atherogenic diet treated with atorvastatin and artichoke leaf tincture combination; AD + ALTINC – rats fed with atherogenic diet treated with artichoke leaf tincture.

Note: The results are given as mean \pm standard deviation.

> *p* – values in the last column according to ANOVA; *p* – values according to *post-hoc* Tukey-Snedecor test: ^a $p < 0.05$, ^{aa} $p < 0.01$ vs ND; ^c $p < 0.05$ vs AD + Ator.

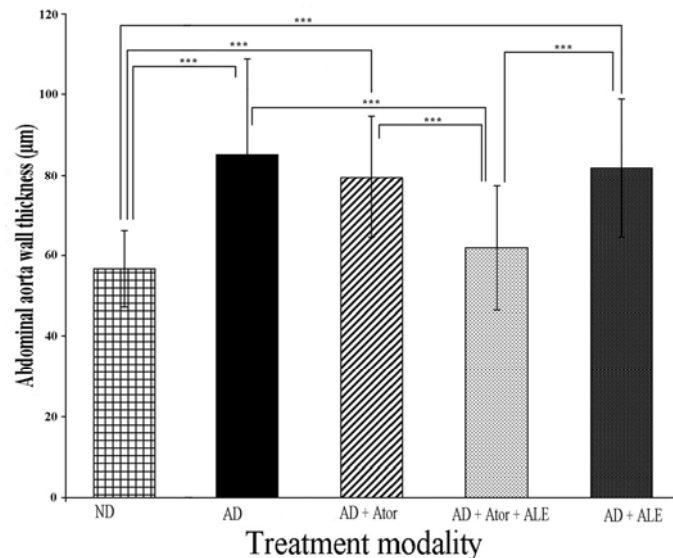


Fig. 1 – Average values of abdominal aorta wall thickness (μm) after six weeks of treatment.

ND – normal diet; AD – atherogenic diet; AD + Ator – atherogenic diet with atorvastatin treatment, AD + Ator + ALTINC – atherogenic diet with atorvastatin and artichoke leaf tincture (ALTINC) treatment; AD + ALTINC – atherogenic diet with artichoke leaf tincture treatment.

Each bar represents the mean \pm SEM ($n = 6$). Statistical comparison was performed using ANOVA with Tukey-Snedecor test as *post hoc* analysis. The stars show a p value between groups connected with lines; $***p < 0.001$.

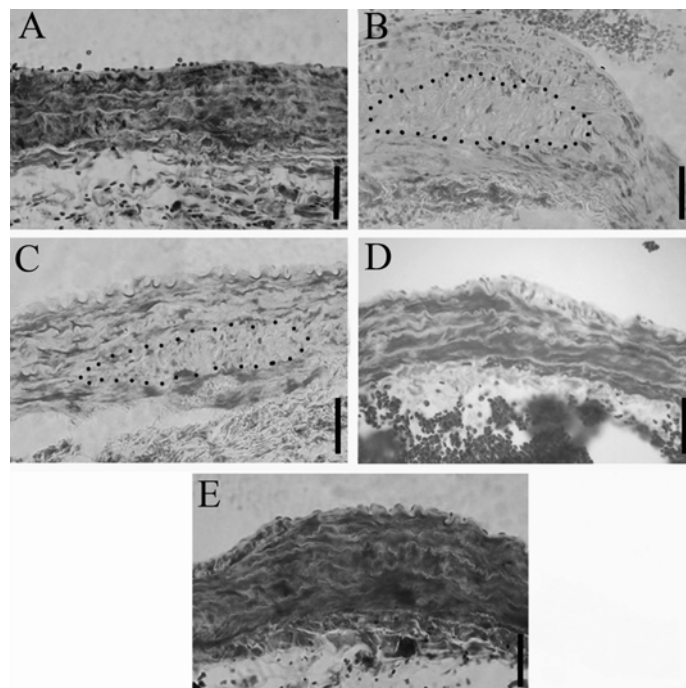


Fig. 2 – Representative photomicrographs of cross-sections through the abdominal aorta.

A – normal diet; B – atherogenic diet; C – atherogenic diet with atorvastatin treatment; D – atherogenic diet with atorvastatin and artichoke leaf tincture treatment; E – atherogenic diet with artichoke leaf tincture treatment.

The differences in *tunica media* thickness among the groups are observed. Also, in *tunica media* of rats on atherogenic diet and rats on atherogenic diet treated with atorvastatin, plaques are visible (spots marked area), leading to the distortion of proper organization of muscle layers and elastic laminae of *tunica media* (Azan trichrome staining, scale bars 50 μm).

tincture treatment we performed liver tissue oxidative stress analysis, i.e. MDA and GSH determination.

Malondialdehyde increased after the atherogenic diet. The therapy slightly lowered MDA, comparing with the group that was on the atherogenic diet, but without statistical significance (Figure 3). Our results showed a decrease

in liver tissue GSH concentration after the atherogenic diet. Treatment with atorvastatin, ALTINC and the combination of both increased the concentration of GSH several fold (Figures 2D and 2E). Reduced glutathione was highest in the group that received ALTINC, far above GSH for animals on the ND ($p < 0.001$).

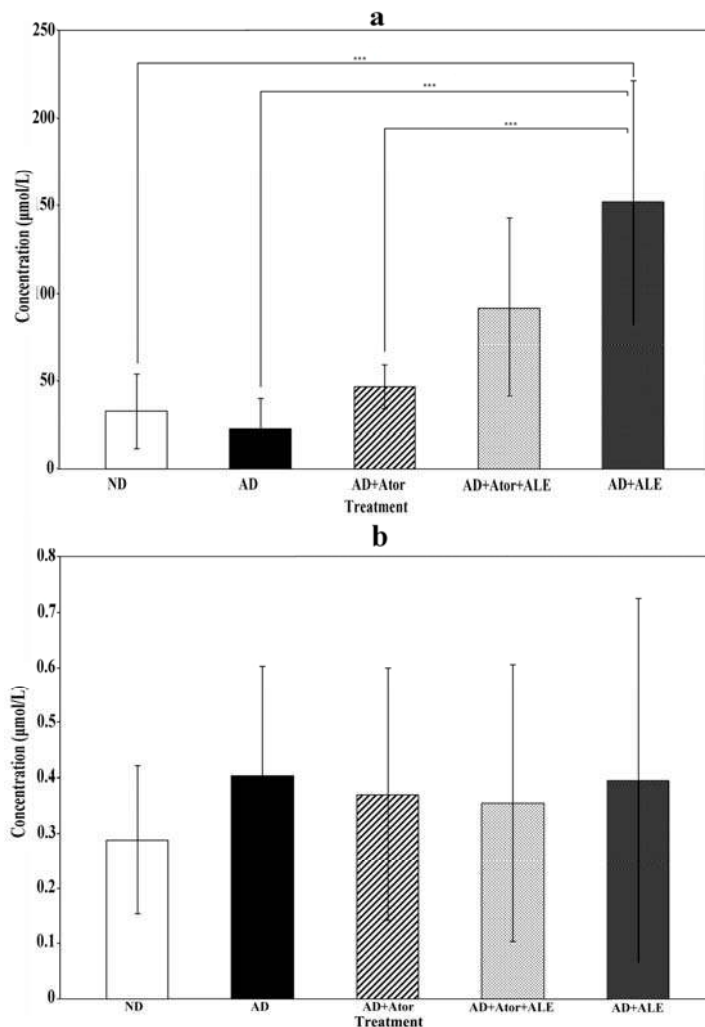


Fig. 3 – Oxidative stress status in liver tissue after six weeks of treatment.

a – glutathione (GSH) concentration; b – malondialdehyde (MDA) concentration; ND – normal diet; AD – atherogenic diet; AD + Ator – atherogenic diet with atorvastatin treatment; AD + Ator + ALTINC – atherogenic diet with atorvastatin and artichoke leaf tinctura treatment; AD + ALTINC – atherogenic diet with artichoke leaf tinctura treatment.

Each bar represents the mean \pm SEM (n = 6). Statistical comparison was performed using ANOVA with Tukey-Snedecor test as *post hoc* analysis. The stars show a *p* value between groups connected with lines. **p* < 0.001.**

Determination of oxidative stress and antioxidative parameters in plasma

Oxidative stress and antioxidative parameters were determined in plasma to estimate the oxidative stress changes in circulation (Table 2).

Malondialdehyde increased in all the treated groups ($p \leq 0.05$ compared with the ND group).

Prooxidative – antioxidative balance (PAB) value was higher in the groups on the AD and atorvastatin treatment regardless of whether it was administered alone or in combination with ALTINC ($p < 0.01$) compared with the ND group. The group that received only ALTINC showed values comparable with the group on the ND. Superoxide dismutase (SOD) activity was slightly lower in the group that was on the atherogenic diet. The therapy increased SOD activity (statistically significant effect of ALTINC, comparing with the groups on normal and atherogenic diets).

We found increased concentrations of total SH groups in all experimental groups compared with the ND group, although this increase was not statistically significant. The concentration of GSH significantly fell under the influence of AD. Atorvastatin and the combined treatment failed to increase GSH, but the ALTINC increased GSH significantly.

PON1 activity significantly decreased in the group given atherogenic diet. The therapy with either atorvastatin or ALTINC alone has no effect on the activity of paraoxonase. Only the combination of atorvastatin and ALTINC was able to maintain PON1 activity at baseline levels.

Determination of oxidative stress and antioxidative parameters in erythrocyte hemolysate

Oxidative stress and antioxidative parameters were measured in erythrocyte hemolysate to determine the oxidative-stress changes in blood cells (Table 3). There was no difference

Table 2
Effect of atorvastatin (1.15 mg/kg body weight) and artichoke leaf tincture (0.1 mL/kg body weight) treatment on parameters of oxidative stress and antioxidative protection in plasma after six weeks of the treatment

Parameters	ND	AD	AD + Ator	AD + ALE	AD + Ator + ALE	<i>p</i>
MDA ($\mu\text{mol/L}$)	0.472 \pm 0.101	0.850 \pm 0.762	0.610 \pm 0.067 ^{as}	0.962 \pm 0.493 ^a	1.223 \pm 0.874 ^a	0.172
PAB (HK units)	186 \pm 57	368 \pm 139 ^{aa}	364 \pm 115 ^{aa}	266 \pm 39 ^{a,c}	367 \pm 135 ^{aa}	\leq 0.05
SOD (IU)	112 \pm 4	109 \pm 10	117 \pm 5	119 \pm 4 ^b	115 \pm 6	0.106
SH groups (mmol/L)	0.205 \pm 0.085	0.248 \pm 0.011	0.482 \pm 0.316	0.553 \pm 0.590	0.347 \pm 0.023	0.215
GSH	32.7 \pm 21.1	9.6 \pm 2.7	10.2 \pm 2.8 ^a	33.7 \pm 14.7 ^{bb,cc}	20.0 \pm 10.8 ^{b,c}	\leq 0.01
PONI (IU/L)	4785 \pm 327	2785 \pm 417 ^a	1991 \pm 473 ^{aaa,b}	2441 \pm 497 ^a	3072 \pm 662	\leq 0.01

MDA – concentration of malondialdehyde; PAB – prooxidative – antioxidative balance; SOD – activity of superoxide dismutase; GSH – concentration of reduced glutathione; PONI – activity of paraoxonase1.

ND – rats fed with normal diet, AD – rats fed with atherogenic diet, AD + Ator – rats fed with atherogenic diet treated with atorvastatin, AD + Ator + ALTINC – rats fed with atherogenic diet treated with atorvastatin and artichoke leaf tincture combination, AD + ALTINC – rats fed with atherogenic diet treated with artichoke leaf tincture.

Note: The results are given as mean \pm standard deviation.

p values in the last column according to ANOVA; *p* values according to *post-hoc* Tukey-Snedecor test: ^a*p* < 0.05, ^{aa} *p* < 0.01, ^{aaa} *p* < 0.001 vs. ND; ^b *p* < 0.05, ^{bb} *p* < 0.01 vs. AD, ^c *p* < 0.05, ^{cc} *p* < 0.01 vs. AD + Ator.

Table 3
Effect of atorvastatin (1.15 mg/kg body weight) and artichoke leaf tincture (0.1 mL/kg body weight) treatment on parameters of oxidative stress and antioxidative protection in erythrocyte hemolysate after six weeks of the treatment

Parameters	ND	AD	AD + Ator	AD + ALE	AD + Ator + ALE	<i>p</i>
MDA ($\mu\text{mol/L}$)	0.050 \pm 0.006	0.030 \pm 0.020	0.076 \pm 0.031	0.188 \pm 0.069	0.098 \pm 0.056	\leq 0.01
SOD (IU/L)	3.08 \pm 3.08	6.36 \pm 4.05	6.57 \pm 4.15	11.84 \pm 3.44 ^a	10.97 \pm 1.64	\leq 0.01
SH groups (mmol/L)	0.171 \pm 0.105	0.283 \pm 0.162	0.254 \pm 0.173	0.273 \pm 0.126	0.324 \pm 0.046	0.36
GSH (mmol/L)	0.91 \pm 0.23	1.88 \pm 0.83	2.54 \pm 1.25	10.25 \pm 10.39 ^{ab,b,c}	4.24 \pm 0.83	\leq 0.01

MDA – concentration of malondialdehyde; SOD – activity of superoxide dismutase; GSH – concentration of reduced glutathione;

ND – rats fed with normal diet; AD – rats fed with atherogenic diet; AD + Ator – rats fed with atherogenic diet treated with

atorvastatin; AD + Ator + ALE – with atherogenic diet treated with atorvastatin and artichoke leaf extract combination; AD + ALE – rats fed with atherogenic diet treated with artichoke leaf extract.

Note: The results are given as mean \pm standard deviation.

p – values in last column according to ANOVA; *p* – values according to *post-hoc* Tukey-Snedecor test: ^a*p* < 0.05, ^{aa} *p* < 0.01 vs. ND; ^b *p* < 0.05 vs. AD; ^c *p* < 0.05 vs. AD + Ator.

in MDA levels and concentration of SH groups between all examined groups ($p > 0.05$).

SOD activity was significantly higher ($p < 0.05$) in the groups on the atherogenic diet regardless of the therapy. SOD activity was highest in the groups that had received ALTINC as treatment (alone or in combination with atorvastatin). All the groups receiving therapy showed higher GSH concentrations. The treatment with ALTINC led to the highest level of GSH ($p < 0.01$ comparing with the normal diet; $p < 0.05$ comparing with the atherogenic diet).

Discussion

Cholesterol feeding has often been used in experimental animals to study the pathogenesis of atherosclerosis. We supplemented a rat commercial diet with cholesterol, sodium cholate and sunflower oil as atherogenic compounds. Sodium cholate, which has an inhibitory action on hepatic *cholesterol 7- α hydroxylase* (EC 1.14.13.17) activity, is known to improve cholesterol absorption because of its emulsifying properties²³.

It is well-known that oxidative stress is a mechanism underlying pathogenesis of atherosclerosis. Our findings, which are in accordance with previous studies^{23, 24}, show that hypercholesterolemia disturbs PAB in favor of prooxidation in liver tissue (Figure 2).

Atorvastatin is the first choice drug for reducing lipids in hypercholesterolemic patients. Several studies have demonstrated that atorvastatin has no influence in high HDL-C concentration⁵. Atorvastatin has been found to exert beneficial cardiovascular effects independent of its ability to lower lipid amounts, possibly due to its antioxidant properties^{1, 4, 13}. The mechanism involved in this phenomenon may be the ability of atorvastatin to reduce the production of reactive oxygen species¹. Also, atorvastatin may play a role in protecting low density lipoprotein (LDL) and HDL from oxidation by increasing antioxidant activity of the HDL-associated enzyme PON1^{4, 5}.

Different types of extracts of artichoke leaf have been considered to show antiatherogenic properties. It is reported that aqueous extract of artichoke leaf inhibits cholesterol biosynthesis in hepatocytes, decreases the oxidation of LDL, modulate endothelial functions and has choleric activity¹⁶. Studies clearly show that cynarin is the main dicaffeoylquinic acid and chlorogenic acid is the major monocaffeoylquinic acid, whereas luteolin-7-O-glucoside is the major flavonoid. Both caffeoylquinic acids and flavonoids present in artichoke extracts are considered to be responsible for its antiatherogenic actions. Llorach et al.⁷ investigated different artichoke byproducts (raw artichoke, blanched artichoke and artichoke blanching waters). They extracted phenolic constituents (expressed as caffeic acid derivatives) with methanol and water. Both, methanol and water extracts from artichoke byproducts showed a high free radical scavenging activity as well as capacity to inhibit lipid peroxidation. Gebhardt and Fausel² performed *in vitro* tests and exposed cultured rat hepatocytes to oxidation and cytotoxicity with tert-butyl hydroperoxide in order to characterize the antioxidative and hepatoprotective properties of aqueous artichoke extracts.

They added aqueous artichoke extracts to rat hepatocytes prior or simultaneously with tert-butylhydroperoxide. *In vitro* studies of Gebhardt and Fausel¹² have provided evidence that the antioxidant potential of aqueous extract of artichoke leaves is dependent on radical scavenging and metal ion chelating effects of its constituents such as cynarin, chlorogenic acid and flavonoids. However, pure constituents of artichoke leaf have been shown to produce less inhibitory activity on free radical production than any type of extract^{11, 12}.

In the present study the cholesterol rich diet led to an increase in the concentration of several lipid parameters (total cholesterol, LDL-C, nonHDL-C), though not all: triglycerides and HDL-C were unaffected. The therapy with atorvastatin and ALTINC did not reduce the concentration of lipid parameters, which was also noticed by other researchers²⁵ and which could be explained, at least in part, with supremacy of diet-induced lipids. Also, this therapy did not influence HDL-C which is consistent with the published data^{4, 5}.

Measurements of the abdominal aorta wall thickness have proven useful in predicting cardiovascular disease risk²⁶. Increase in the thickness of the abdominal aorta wall in this study indicates the damage of the wall due to plaque formation in atherosclerosis. Intake of AD led to the thickening of the aortic wall (Figure 1), as well as to plaque formation (Figure 2). Relatively short atorvastatin treatment failed to restore the aortic wall as shown in Figures 1 and 2. Treatment with ALTINC (alone and especially in combination with atorvastatin) managed to reduce abdominal aorta wall thickness and reversed plaque formed during atherosclerosis. More favorable effect of ALTINC comparing to atorvastatin prove that ALTINC is better antioxidant. The effect of combination of atorvastatin and ALTINC shows that these two treatments have an additive effect.

Liver transaminases (ALT and AST) in the hypercholesterolemic rats were higher than in normal animals, which may be attributed to the injury of liver tissue²⁷. We have noticed that treatment with atorvastatin in current work led to further increase in plasma transaminase levels. Previous research has shown that atorvastatin can induce a mild increase in ALT and AST activity. Long-term therapy in most cases does not lead to liver damage, although there are rare cases of acute hepatotoxicity induced by atorvastatin²⁷. The therapy with ALTINC decreased plasma transaminase activity demonstrating its hepatoprotective effect, which is in accordance with previous studies¹².

Lipid peroxidation is a well-established mechanism of cellular injury and measurement of MDA is widely used as an indicator of oxidative stress in cells and tissues. Rats fed with atherogenic diet in this study showed increase in MDA concentration because of oxidative stress that occurred in atherosclerosis²³. This therapy did not reduce plasma MDA level as expected, presumably because the very concentrated cholesterol diet led to advanced lipid peroxidation, which probably could not be prevented by the antioxidant activity of atorvastatin and ALTINC during the relatively short time of the experiment. On the other side, all types of therapy led to a significant reduction in the concentration of MDA in liver tissue comparing to the group on atherogenic diet, indicating that atorvastatin and aqueous-ethanolic artichoke leaf

extract have antioxidant effects, as shown by previous researchers^{1, 4, 12}. The combination of ALTINC and atorvastatin showed an even stronger effect in preventing lipoperoxidation (Table 2). MDA levels in erythrocyte hemolysate did not differ significantly among the groups, but positive effects of every type of therapy was noticed. The therapy with ALTINC had especially beneficial effects as in liver tissue.

Prooxidative-antioxidative balance was determined only in plasma and it increased upon atherogenic diet due to oxidative stress that occurred. Atorvastatin therapy (alone or in combination with ALTINC) did not lead to a decrease of PAB as expected. Only therapy with ALTINC decreased PAB, which demonstrated antioxidative action of the plant extract (Table 3).

Our results show that any form of therapy increased the concentration of SH groups that play a role in neutralizing free radicals in plasma, as expected. The most effective treatment was with ALTINC. Changes in reduced glutathione levels were the same in all sample types (Tables 2 and 3). Atherogenic diet decreased the level of GSH, which was presumably used in neutralizing free radicals formed during the process of atherogenesis. The treatments in our experiment regenerated spent glutathione. ALTINC showed the strongest effect (Table 2) indicating strong antioxidant activity of some constituents, such as flavonoids and hydroxycinnamic acids to be effective hydrogen donors and metal chelators as shown by Zapolska-Downar et al.²⁸ who tested aqueous and ethanolic extracts of artichoke plant. Caffeic and chlorogenic acid have also been reported to stimulate hydroxyl radical formation and to have pro-oxidant activity on Cu²⁺-induced LDL oxidation²⁸. It has been demonstrated that aqueous and ethanolic extracts of artichoke plant protect LDL against oxidative modification *in vitro*¹¹. Gebhardt and Fausel¹² have reported that aqueous extract of artichoke leaf inhibits the production of MDA induced by exposure of cultured hepatocytes to t-butylhydroperoxide (t-BHP). According to them, the observed antioxidative potential can be attributed to some well-known components of artichoke leaf extract, although their action cannot account for the full potency of the extract. In our study, antioxidant effect of ALTINC was obvious, especially when used alone in the treatment. Atorvastatin is extensively metabolized in the liver under the influence of the enzyme CP3A4 and therefore combinations in which it is used must be carefully selected since a lot of drugs is metabolized by the same enzymes²⁹. Constituents of artichoke leaf tincture (cynarin and chlorogenic acid) are metabolized in the same manner. So, a possible interaction between ALTINC and atorvastatin could be on the metabolism level.

Paraoxonase has emerged as the component of HDL most likely to explain its ability to metabolize lipid peroxides and to prevent their accumulation in LDL. Paraoxonase immunoreactivity is known to be increasingly present in the arterial wall as atheroma advances³⁰. Protection of LDL and HDL from oxidation is probably related to PON's ability to hydrolyze some oxidized phospholipids and/or cholesteryl linoleate hydroperoxides which are present in oxidized LDL. Other studies show that during LDL oxidation in the presence of PON1, the enzyme is partially inactivated³¹. This effect could be related to displacement of calcium ions (which are required for PON arylesterase/paraoxonase activities) by copper ion (in the copper ion oxidative system). In the free radical oxidation system, as well as in the copper ion system, free radicals formed during lipid peroxidation can also inactivate PON³². Our results in plasma samples confirm that PON1 is consumed during atherogenesis so its levels are lowest in the group on an atherogenic diet. Antioxidants in the treatment of atherosclerosis prevent oxidation of LDL cholesterol and thus preserve PON1 activity³². In our study, only ALTINC show this effect. Atorvastatin treatment has no significant effect on this enzyme activity which may indicate lower antioxidant potential of atorvastatin.

An increased antioxidant activity of SOD in erythrocytes was also found by Küçükgergin et al.³³ after treatment with artichoke dry extract dissolved in water which confirmed antioxidant properties of artichoke plant.

Conclusion

Treatment with artichoke leaf tincture and atorvastatin shows beneficial effects on oxidative stress parameters, but has little influence on basic lipid parameters. Investigated artichoke leaf tincture shows even better results in oxidative stress reduction than low-dose atorvastatin which is a widely-prescribed drug in the treatment of atherosclerosis. All the determined parameters show that artichoke leaf tincture (alone or in combination with atorvastatin) is very potent antioxidant in applied doses. Effects of specific constituents of ALTINC on oxidative damage should be further investigated. Since atorvastatin and constituents of ALTINC probably have different mechanisms of action, simultaneous use of both therapies could be beneficial but should be further investigated since our results show that ALTINC is less effective when used in combination with atorvastatin.

Acknowledgements

This work was financially supported by a grant from the Ministry of Education, Science and Technological Development, the Republic of Serbia (Project numbers OI 175035 and 172041).

R E F E R E N C E S

- Rosenson RS. Statins in atherosclerosis: lipid-lowering agents with antioxidant capabilities. *Atherosclerosis* 2004; 173(1): 1–12.
- Wang C, Liu P, Liao JK. Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. *Trends Mol Med* 2008; 14(1): 37–44.
- Jasińska M, Owczarek J, Orszulak-Michalak D. Statins: a new insight into their mechanisms of action and consequent pleiotropic effects. *Pharmacol Rep* 2007; 59(5): 483–99.
- Nagila A, Permpongpaiboon T, Tantrarongroj S, Porapakkebam P, Chinnwattana K, Deakin S, et al. Effect of atorvastatin on paraoxonase1 (PON1) and oxidative status. *Pharmacol Rep* 2009; 61(5): 892–8.
- Paragh G, Töröcsik D, Seres I, Harangi M, Illyés L, Balogh Z, et al. Effect of short term treatment with simvastatin and atorvastatin on lipids and paraoxonase activity in patients with hyperlipoproteinaemia. *Curr Med Res Opin* 2004; 20(8): 1321–7.
- Maron DJ, Fazío S, Linton MF. Current perspectives on statins. *Circulation* 2000; 101(2): 207–13.
- Llorach R, Espín JC, Tomás-Barberán FA, Ferreres F. Artichoke (*Cynara scolymus* L.) byproducts as a potential source of health-promoting antioxidant phenolics. *J Agric Food Chem* 2002; 50(12): 3458–64.
- Wang M, Simon JE, Aviles IF, He K, Zheng Q, Tadmor Y. Analysis of Antioxidative Phenolic Compounds in Artichoke (*Cynara scolymus* L.). *J Agric Food Chem* 2003; 51(3): 601–8.
- Ogier N, Amiot M, Georgé S, Maillot M, Mallmann C, Maraninchi M, et al. LDL-cholesterol-lowering effect of a dietary supplement with plant extracts in subjects with moderate hypercholesterolemia. *Eur J Nutr* 2013; 52(2): 547–57.
- Qinna NA, Kamona BS, Albussainy TM, Taha H, Badwan AA, Matalaka KZ. Effects of Prickly Pear Dried Leaves, Artichoke Leaves, Turmeric and Garlic Extracts, and Their Combinations on Preventing Dyslipidemia in Rats. *ISRN Pharmacology* 2012; 2012: 1–7.
- Brown JE, Rice-Evans CA. Luteolin-rich artichoke extract protects low density lipoprotein from oxidation in vitro. *Free Radical Res* 1998; 29(3): 247–55.
- Gebhardt R, Fausel M. Antioxidant and hepatoprotective effects of artichoke extracts and constituents in cultured rat hepatocytes. *Toxicol In Vitro* 1997; 11(5): 669–72.
- Vergnes L, Phan J, Strauss M, Tafuri S, Reue K. Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression. *J Biol Chem* 2003; 278(44): 42774–84.
- Amin KA, Abd El-Twab TM. Oxidative markers, nitric oxide and homocysteine alteration in hypercholesterolemia rats: role of atorvastatin and cinnamon. *Int J Clin Exp Med* 2009; 2(3): 254–65.
- Lunder M, Drevenšek G, Černe D, Marc J, Janič M, Šabovič M. Treatment with low-dose atorvastatin, losartan, and their combination increases expression of vasoactive-related genes in rat aortas. *J Cardiovasc Pharmacol Ther* 2013; 18(2): 177–83.
- Rodríguez TS, Giménez DG, de la Puerta VR. Choleric activity and biliary elimination of lipids and bile acids induced by an artichoke leaf extract in rats. *Phytomedicine* 2002; 9(8): 687–93.
- Girotti MJ, Khan N, McLellan BA. Early measurement of systemic lipid peroxidation products in the plasma of major blunt trauma patients. *J Trauma* 1991; 31(1): 32–5.
- Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972; 247(10): 3170–5.
- Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology* 1974; 11(3): 151–69.
- Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1952; 82(1): 70–7.
- Alamdari DH, Ghayour-Mobarhan M, Tavallaie S, Parizadeh MR, Moobeati M, Ghajouri F, et al. Prooxidant-antioxidant balance as a new risk factor in patients with angiographically defined coronary artery disease. *Clin Biochem* 2008; 41(6): 375–80.
- Richter RJ, Furlong CE. Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics* 1999; 9(6): 745–53.
- Balkan J, Doğru-Abbasoğlu S, Aykaç-Toker G, Uysal M. The effect of a high cholesterol diet on lipids and oxidative stress in plasma, liver and aorta of rabbits and rats. *Nutr Res* 2004; 24(3): 229–34.
- Kotur-Stevuljević J, Memon L, Stefanović A, Spasić S, Spasojević-Kalimanovska V, Bogavac-Stanojević N, et al. Correlation of oxidative stress parameters and inflammatory markers in coronary artery disease patients. *Clin Biochem* 2007; 40(3–4): 181–7.
- Dalal I, Sengupta M, Paul S, Mishra A. Comparative study of the effect of atorvastatin and garlic extract in experimentally induced hypercholesterolemia in rabbits. *Int J Basic Clin Pharmacol* 2013; 2(4): 397–402.
- Järvisalo MJ, Jartti L, Näntö-Salonen K, Irjala K, Rönnemaa T, Harttala JJ, et al. Increased aortic intima-media thickness: a marker of preclinical atherosclerosis in high-risk children. *Circulation* 2001; 104(24): 2943–7.
- Clarke AT, Mills PR. Atorvastatin associated liver disease. *Digest Liver Dis* 2006; 38(10): 772–7.
- Zapolska-Downar D, Zapolski-Downar A, Naruszewicz M, Siennicka A, Krasnodebska B, Kolodziej B. Protective properties of artichoke (*Cynara scolymus*) against oxidative stress induced in cultured endothelial cells and monocytes. *Life Sci* 2002; 71(24): 2897–908.
- Fujino H, Saito T, Tsumenari Y, Kojima J, Sakaeda T. Metabolic properties of the acid and lactone forms of HMG-CoA reductase inhibitors. *Xenobiotica* 2004; 34(11–12): 961–71.
- Durak I, Özbeke H, Devrim E, Karagenc N, Ergüder IB. Effects of cholesterol supplementation on antioxidant enzyme activities in rat hepatic tissues: possible implications of hepatic paraoxonase in atherogenesis. *Nutr Metab Cardiovasc Dis* 2004; 14(4): 211–4.
- Aviram M, Billecke S, Sorenson R, Bisgaier C, Newton R, Rosenblat M, et al. Paraoxonase active site required for protection against LDL oxidation involves its free sulfhydryl group and is different from that required for its arylesterase/paraoxonase activities: selective action of human paraoxonase allozymes Q and R. *Arterioscler Thromb Vasc Biol* 1998; 18(10): 1617–24.
- Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, et al. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radical Bio Med* 1999; 26(7–8): 892–904.
- Küçükgergin C, Aydın A, Özdemirler-Erata G, Mehmetçik G, Koçak-Toker N, Uysal M. Effect of artichoke leaf extract on hepatic and cardiac oxidative stress in rats fed on high cholesterol diet. *Biol Trace Elem Res* 2010; 135(1–3): 264–74.

Received on September 17, 2014.

Revised on November 4, 2014.

Accepted on January 13, 2015.

Online First December, 2015.