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Protective effects of pumpkin (*Cucurbita pepo* L.) seed oil on rat liver damage induced by chronic alcohol consumption

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Abstract: Pumpkin seed oil (PSO) possesses a protective potential against liver injury due to the presence of biologically active ingredients. Adult male albino rats were administrated PSO (*per os*, 2 mL/kg b.w./day) and a 12% ethanol solution in water, *ad libitum*, with an average intake of 8.14 g of ethanol/kg bw/day for 6 weeks. Congestion, hepatic central vein dilation, portal vein branch dilation, Kupffer cell hyperplasia, fatty liver changes, hepatocyte focal necrosis were observed after daily alcohol intake. All observed changes were reduced when PSO was ingested with ethanol. PSO intake itself induced discrete cellular edema, congestion and slight dilatation of the central and portal vain branches. Chronic ethanol intake elevated catalase (CAT) activity and glutathione reductase (GR) protein expression; concomitant PSO intake had no effect on CAT activity or GR protein expression. PSO intake decreased the activities of GR, glutathione-S-transferase (GST) and xanthine oxidase (XOD) in the liver, probably due to the ingestion of antioxidants. Intake of PSO and ethanol significantly decreased cytosolic superoxide dismutase (SOD1) and increased NF-κB protein expression compared to ethanol intake, suggesting that the protective effects of PSO were mediated by the NF-κB signaling pathway. Our results reveal a therapeutic potential of PSO in alcoholic liver disease.

Keywords: chronic alcohol consumption; alcoholic liver disease; pumpkin seed oil; antioxidant enzymes; NF-κB

Abbreviations and acronyms: Alcoholic liver disease (ALD), analysis of variance (ANOVA), catalase (CAT), glutathione (GSH), oxidized form of glutathione (GSSG), glutathione reductase (GR), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), hematoxylin and eosin stain (H&E), high density lipoprotein (HDL), honest significant difference (HSD), lactate dehydrogenase (LDH), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), nitrogen monoxide (NO), pumpkin seed oil (PSO), reactive oxygen species (ROS), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), standard error of the mean (SEM), cytosolic copper-zinc superoxide dismutase (SOD1), mitochondrial manganese superoxide dismutase (SOD2), thiobarbituric acid reactive substances (TBARS), toll-like receptor 4 (TLR4), tumor necrosis factor alpha (TNFα), xanthine oxidase (XOD), body weight (bw).

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INTRODUCTION

Pumpkin (*Cucurbita pepo* L.) seeds possess antioxidant [1], hypolipidemic [2], antidiabetic [3], anti-atherogenic and hepatoprotective [4] activities. Phytochemical analysis has shown that pumpkin seed oil (PSO) is a rich source of unsaturated fatty acids, antioxidants and sterols [5], amino acids, essential fatty acids, β-carotenes, some triterpenes, phytosterols, zinc, selenium [6], tyrosol, vanillin, vanillic acid, ferulic acid, luteolin [7], polysaccharides, para-amino benzoic acids, omega 6 essential fatty acid, linoleic and oleic acid, vitamins A and E [8,9]. Components such as polyphenols and zinc belong to a large group of antioxidants that are involved in the elimination of free radicals through different mechanisms. They can enhance the capacity of antioxidative defense and suggest a strong positive health and therapeutic potential. Open clinical studies of different pumpkin seed preparations are listed by the EMA/HMPC/136022/2010 Committee on Herbal Medicinal Products (HMPC), with no serious adverse effects reported [10]. Although differences come from variations in cultivar or origin, pumpkin seeds yield about 50% fatty oil, (mostly linoleic and oleic acid and tocopherol), and contain putative active constituents, including Δ7-sterols (avenasterol, spinasterol) and Δ5-sterols (sitosterol, stigmasterol) [10,11]. However, the mechanisms of action of PSO seem to be composite and multiple. Due to the previously reported hepatoprotective effects of PSO [4], we tested the hepatoprotective effect of chronic PSO supplementation on alcohol-induced liver damage in adult male Wistar rats that were exposed for 6 weeks to chronic alcohol intake. This treatment was utilized as a model of alcoholic liver disease (ALD) in our previous studies [12,13].

Long-term alcohol intake inevitably leads to ALD. Important causes for the onset of illness are both continuous daily intake and long-term alcohol consumption. ALD includes a wide spectrum of histopathological changes, from steatosis, alcoholic hepatitis, fibrosis, cirrhosis, to hepatocellular carcinoma. Previous research into ALD pathogenesis showed that oxidative stress was the consequence of alcohol consumption, and that its metabolism was one of the mechanisms of alcohol-induced damage of the liver [14,15]. The generation of free radicals is toxic to cells, causing lipid peroxidation of the cell membrane with production of reactive lipid aldehydes (malondialdehyde and 4-hydroxynonenal). In addition, oxidation of ethanol under the action of alcohol dehydrogenase causes a change in the redox status (a disturbed ratio of NAD/NADH) [16]. Chronic use of ethanol causes increased release of endotoxin originating from gram-negative bacteria of the bowel in the portal circulation and stimulates the inflammatory reaction in the liver. Activated Kupffer cells produce additional free radicals, which then, by activating the NF-κB, cause increased synthesis of numerous cytokines, chemokines, growth factors, eicosanoids, reactive oxygen species (ROS) and nitric oxide (NO) [17-19].

In this work, we examined the potential cytoprotective and regenerative effects of chronic PSO administration on histopathological malformations in rat liver after chronic ethanol ingestion. We analyzed the parameters of oxidative stress, particularly protein expression and activity of cytosolic copper-zinc superoxide dismutase (SOD1), mitochondrial manganese superoxide dismutase (SOD2), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) under the given experimental conditions. In addition, we measured the activities of glutathione-S-transferase (GST) and xanthine oxidase (XOD), the concentrations of total glutathione and thiobarbituric acid reactive substances (TBARS) and the expression of pro-apoptotic p53 protein and transcription factor NF-κB induced by oxidative stress.

MATERIALS AND METHODS

Experimental animals

Twenty-three adult male albino rats, 9-11 weeks old, body mass 200-220 g were used. The animals were kept in standard cages, 3 animals per cage, at constant room temperature of 22±1°C and light regime of 12 h day/night; the rats were fed with standard food for laboratory animals produced by the Veterinary Institute Zemun. The animals were raised in the Vivarium of the Centre for Biomedical Testing, Institute for Research and Development Galenika a.d. Belgrade, Republic of Serbia. All animal procedures were in accordance with the recommendations of the European Parliament on the protection of animals used for scientific purposes according to principles 2010/63/EU. This study was approved by the Ministry of Agriculture and Environmental Protection – Veterinary Administration of the Republic of Serbia, No. 323-07-10690/2015-05, and by the Ethical Committee of Development, Regulatory

Affairs and Quality Division, Galenika a.d., Belgrade, No. 4121/2015.

Experimental design

The animals were divided into 4 groups, each comprising 6 animals except group E, which contained 5 animals (Supplementary Figure S1), as follows: group C – control group; group P – the pumpkin seed oil group (2 mL/kg per day *per os* during 42 days); group E – alcohol group (12% alcohol *ad libitum* during 42 days); group E+P – the group that was treated with both alcohol (12% alcohol *ad libitum*) and pumpkin seed oil (2 mL/kg *per os* during 42 days).

Chemicals

In this study we used "Merck" absolute ethanol and commercially available cold-pressed pumpkin seed oil that was chemically previously analyzed [20]. Details of the PSO composition are provided in Supplementary Table S2.

Doses and treatments

Ethanol solution (12% in distilled water) was administered *per os*, daily *ad libitum* for 6 weeks. The dose was defined in our previous publications [12,13] where it was shown that the applied dose caused an increase of alcohol concentration in the blood to 0.254 g/L on average, which could be considered as moderately toxic [12]. We defined the period of time of 42 days as chronic alcohol intake (1-14 days as acute toxicity, 21-28 days as subacute toxicity, and 28 days and longer as chronic toxicity) [21].

The dose of PSO was extrapolated from many recommended daily intakes of PSO human supplements according to information on posology, the method of administration, duration of use from literature for relevant preparations provided by the EMA/HMPC/136022/2010 Committee on Herbal Medicinal Products (HMPC) [10] to rats through the "metabolic mass" concept [12,22]; taking into account rat metabolic capacity and rate, a PSO dose of 2 mL/kg corresponds to a human toxic dose. PSO (2 mL/kg b. w.) was administered *per os* using a rat gavage tube every day for 6 weeks, once daily, always at 9:00 h.

Organ sampling

Rats were killed after 42 days of treatment by an intraperitoneal injection of ketamine (Ketamidor 10%, Richter Pharma AG, Austria) with a single dose of 0.224 mg/kg bw, causing immediate death. After dissection, one part of the liver was excised for histopathological analyses. The rest of the liver was perfused *in situ* with cold phosphate-buffered saline (PBS), excised, frozen in liquid nitrogen and kept at -70°C for determination of enzymatic activities, GSH, TBARS concentrations and Western blotting.

Histopathological examination

The histopathological examination was performed by fixing liver tissue in 4% neutral buffered formalin solution for 3 weeks, followed by dehydration in increasing concentrations of ethanol and xylene, and embedded in Histowax (Histolab Product AB, Sweden). Sectioning of the tissue block at 5 μm thickness was performed on a rotary microtome and stained with H&E [23]. For histopathological examination, light microscopy (Leica DM LS2, Germany) was used and original microphotographs were made using Canon Power Shot S70.

The degree of congestion, dilatation of the central vein, dilatation of the hepatic portal vein branch, inflammation of the portal area, Kupffer cell hyperplasia, newly formed bile ducts, fatty liver changes and edema of hepatocytes in the liver of each animal were ranked in one of 5 categories as follows: 0 – absence of change; 1 – discrete changes, 2 – moderate changes; 3 – pronounced changes; 4 – very pronounced changes. All histopathology slides were interpreted by 3 independent pathologists blinded to other data, with the final result being the mean of their findings.

Measurement of enzyme activities and total GSH and TBARS concentrations

Procedures used for hepatic tissue sample preparation and measurement of SOD1, SOD2, CAT, GSH-Px, GR, GSTs and XOD activities as well as TBARS and protein concentrations were previously described in detail [24]. Briefly, thawed liver samples were homogenized and sonicated at 4°C in 10 volumes of 0.25 M sucrose, 1 mM K_2EDTA and 0.05 M Tris-HCl buffer (pH 7.4),

and centrifuged for 90 min at 105000 *g* at 4°C. The supernatant was used to determine enzyme activities and total GSH concentration.

Total SOD activity was determined by the adrenaline method [25] and expressed as U/mg protein. One SOD unit was defined as the amount of enzyme required to decrease the rate of adrenalin auto-oxidation by 50% at pH 10.2. Preincubation with 8 mM KCN was used to determine SOD2 activity. SOD1 activity was calculated as the difference between total SOD activity and SOD2 activity. CAT activity was determined by monitoring hydrogen peroxide consumption [26] and expressed as U/mg protein. The activity of GSH-Px was determined using t-butyl hydroperoxide as a substrate [27] and expressed as mU/mg protein. GR activity was determined by NADPH oxidation concomitant with the reduction of the oxidized form of glutathione (GSSG) [28] and expressed as mU/mg protein. Total GST activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate [29] and expressed as U/mg protein. XOD activity was determined by monitoring uric acid production in the presence of xanthine as a substrate [30] and expressed as mU/mg protein. Total GSH concentration was determined by the enzymatic recycling assay in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid), GR and NADPH [31] and expressed as nmol/mg protein.

For TBARS measurement, thawed liver samples were homogenized and sonicated at 4°C in 10 volumes of 1 mM K_2EDTA and 0.05 M Tris-HCl buffer (pH 7.4) and centrifuged for 10 min at 10,000 ×*g*, 4°C. The concentration of TBARS, which reflects the degree of lipid peroxides, was measured in the supernatant [32] and expressed as nmol/mg protein.

Protein concentrations in all samples were measured by the Lowry protein assay [33].

Spectrophotometric measurements were performed using the SAFAS UVmc2 spectrophotometer (SAFAS, Monaco) for SODs; Shimadzu UV-160 spectrophotometer (Shimadzu Scientific Instruments, Shimadzu Corporation, Japan) for CAT, GSTs and XOD; Thermo Scientific Evolution 300 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) for GSH-Px and GR; Thermo Scientific Multiskan Spectrum microplate spectrophotometer (Thermo Fisher Scientific Oy, Finland) for total GSH, TBARS and protein concentrations.

Preparation of whole cell extracts and Western blotting

Homogenization of liver tissue was performed in 20 mM HEPES pH 7.4 buffer (containing protease and phosphatase inhibitors), the homogenate was incubated on ice for 2 h in the same buffer and centrifuged at 10,000 ×*g* for 30 min at 4°C to obtain the supernatant used as a whole cell liver extract [34].

Protein concentrations in samples were analyzed by the method of Markwell et al. [35]. For Western blot analyses, the following primary antibodies were used: anti-CuZnSOD (SOD1) (Stressgen, United States), anti-MnSOD (SOD2) (Stressgen), anti-catalase (Calbiochem, United States), anti-glutathione peroxidase (GSH-Px), anti-glutathione reductase (GR), anti-NF-κB anti-p53 (Santa Cruz Biotechnology, United States). Rabbit polyclonal anti-β-actin (ab8227, Abcam, United Kingdom) antibody was used to detect actin as a loading control and the secondary antibody was detected using chemiluminescence reagent (ECL, Pierce, United States). Analysis of protein bands densitometry was performed using X ray films and Image J software.

Statistical analysis

Since there are 2 independent factors (PSO and ethanol), statistical data-processing examined the significance of differences using two-way ANOVA with (i) PSO (P) and (ii) ethanol (E) as 2 independent factors and post hoc compared by Tukey's HSD t-test according to the described protocols [36]. The level of statistical significance for all tests was $P<0.05$. Statistical analyses of the Western blots were performed on logarithmically transformed data. Results are presented as the mean±SEM. The effects of the treatments on the histopathological changes were tested by Pearson's chi-squared test. The results are given as the percentage of the total sum of cases.

RESULTS

Histopathological analysis

Ethanol intake induced statistically significant ($\chi 2$ test, $P<0.05$) focal necrosis, congestion, dilatation of the central vein and portal vein branches, inflammation

Table 1. Histopathological changes in rat hepatic tissue after a 6-week treatment with pumpkin seed oil alone (P), ethanol alone (E) and their combination (E+P). There was a significant difference among experimental groups for every type of evaluated histopathological change.

		Control (C)					Pumpkin seed oil (P)					Ethanol (E)					Ethanol + Pumpkin seed oil (E+P)						
Severity		**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**χ2**	**P**
Focal necrosis	n	6					6						1	3	1		6					23.000	0.006
	%	100					100						20	60	20		100						
Congestion	n	6						4	2						3	2		3	2	1		41.865	0.001
	%	100						67	33						60	40		50	33	17			
Dilatation of the central vein	n	6							6						2	3		4	2			57.500	0.001
	%	100							100						40	60							
Dilatation of portal vein branches	n	6						3	3						1	4		2	4			46.657	0.001
	%	100						50	50						20	80		33	67				
Portal spaces inflammation	n	6					6						2	3			3	3				22.080	0.001
	%	100					100						40	60			50	50					
Fatty change (steatosis)	n	6					6						2	3			6					23.000	0.001
	%	100					100						40	60			100						
Kupffer cell hyperplasia	n	6					6							2	3			6				46.000	0.001
	%	100					100							40	60			100					
Newly formed bile ductules	n	6					6						1	4					3	3		31.543	0.001
	%	100					100						20	80					50	50			
Cellular edema	n	6					3	2	1			5					5	1				25.635	0.012
	%	100					50	33	17			100					83	17					
		n=6					n=6					n=5					n=6						

Absence of histopathological change is marked by 0, discrete changes are marked by 1, moderate changes by 2, pronounced changes by 3 and very pronounced changes by 5 (the numbers (n) and percentage of rats in each category are presented). The effects of treatments were tested by Pearson's chi-squared test (χ^2 and P value are presented) with the level of statistical significance of 0.05.

of portal spaces, steatosis, Kupffer cell hyperplasia and newly formed bile ductules when compared to the control group (Table 1, Fig. 1). PSO treatment significantly decreased the degree of histopathological changes in the hepatocytes of animals treated with ethanol, (P<0.001, E+P group vs the E group). Also, PSO completely neutralized the focal necrosis of hepatocytes, steatosis, Kupffer cell hyperplasia and cellular edema induced by ethanol. PSO intake also decreased ethanol-induced dilatation of the central vein and portal vain branches, congestion and inflammation of portal spaces, and improved the formation of new bile ducts in the liver. PSO intake produced slight histological changes compared to the controls (slight congestion and dilatation of the central vain and portal vain branches and some cellular edema, P<0.05).

Changes in the activities of antioxidant enzymes (SOD1, SOD2, CAT, GSH-Px and GR), GST, XOD and concentrations of total GSH and TBARS

Consumption of ethanol (E group) caused the elevation of CAT activity (ANOVA significant ethanol (e) effect, P<0.001) that was not prevented by the intake of PSO: CAT activity was higher in the liver of rats that ingested both ethanol and PSO (E + P group) compared to the controls (Fig. 2). Ethanol intake had no effect on the activity of other measured antioxidant enzymes. Consumption of both ethanol and pumpkin oil (E + P group) had no effect on the activities of other antioxidant enzymes (SOD1, SOD2, GSH-Px, GR, GST), XOD activity and the amount of glutathione and TBARS. Consumption of PSO (P group) decreased the activity of GR, GST and XOD (ANOVA significant (p) effect, P<0.01, P<0.001 and P<0.05, respectively) compared to the other two groups (E and E + P).

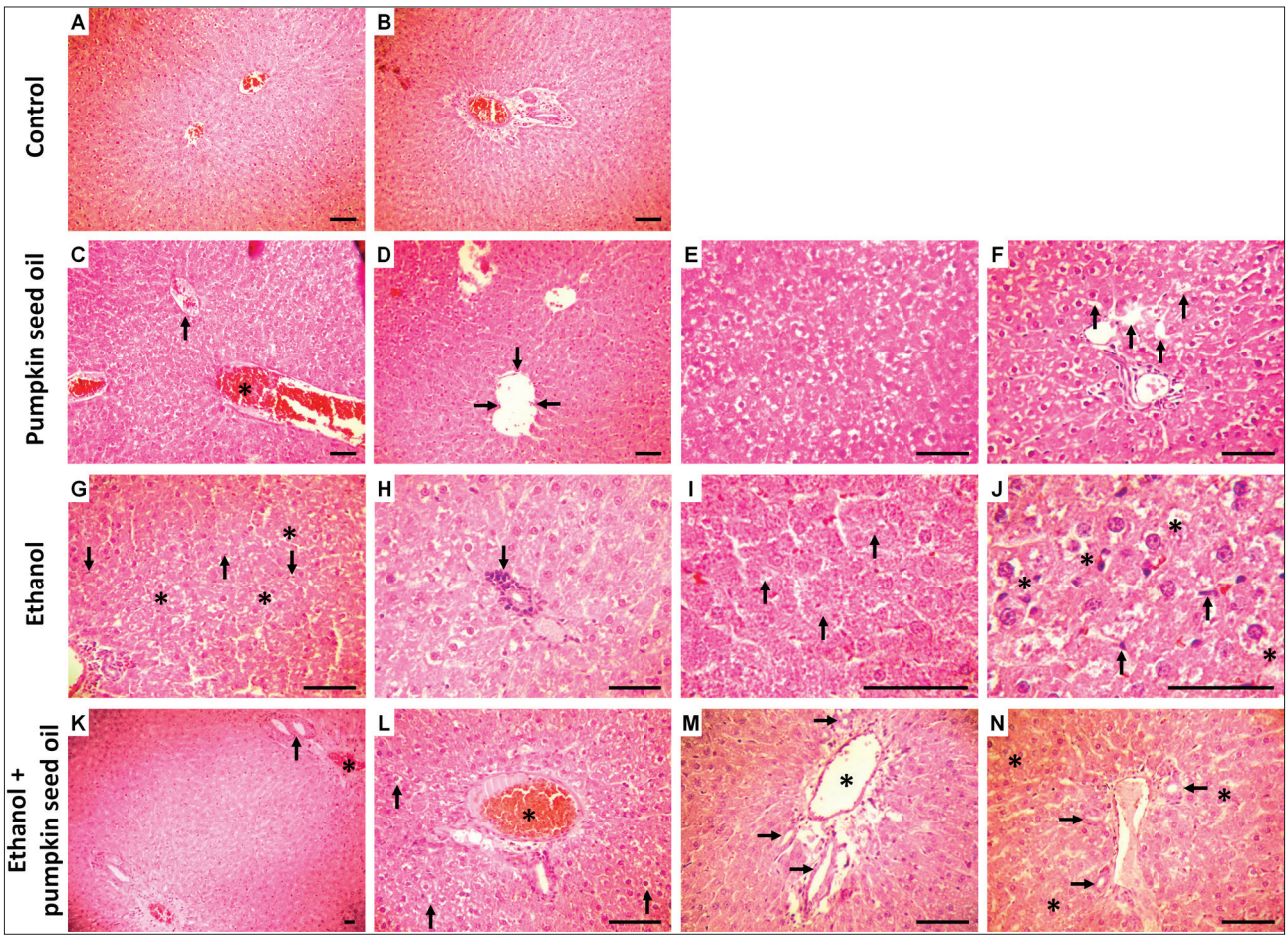

Fig. 1. Histopathological analysis of rat liver. Photomicrographs of tissue sections stained with H&E. Control group: normal structure of the liver with hexagonal shaped lobules: **A** – with a central vein in the middle and trabecular arrangement of hepatocytes and **B** – portal triads; (A and B ×100). Pumpkin seed oil-treated group: **C** – very pronounced congestion of the central vein (arrow) and portal vein branch (asterisk); **D** – moderate dilatation of the central vein (arrows); **E** – pronounced edema of hepatocytes; **F** – very pronounced edema of hepatocytes (arrows); (C and D ×100; E and F ×200). Ethanol-treated group: **G** – moderate fatty changes (asterisk) and moderate hyperplasia of Kupffer cells (arrow); **H** – moderate lymphocyte and plasma cell infiltration of the portal triad (arrow); **I** – focal necrosis of hepatocytes (arrow); **J** – pronounced fatty changes (asterisk) and pronounced hyperplasia of Kupffer cells (arrow); (G and H ×200; I and J ×400). Ethanol + pumpkin seed oil-treated group: **K** – moderate dilatation of the portal vein branch (asterisk) and pronounced newly formed bile ductules (arrow); **L** – moderate dilatation and pronounced congestion of the portal vein branch (asterisk), and focal discrete edema of hepatocytes (arrow); **M** – moderate newly formed bile ductules (arrow) and moderate dilatation of the portal vein branch (asterisk); **N** – moderate newly-formed bile ductules (arrow) and focal discrete edema of hepatocytes (asterisk); (K ×40; L, M and N ×200). The size of the scale bar on every individual image is 100 μm.

Changes in relative protein levels of antioxidant enzymes (SOD1, SOD2, CAT, GSH-Px and GR) and transcription factors (NF-κB and p53)

Ethanol ingestion elevated the GR protein level and had no effects on other antioxidant enzyme (SOD1, SOD2, CAT, GSH-Px) protein levels, on XOD activity and the amount of NF-κB and p53 compared to the controls (E vs the C group) (Fig. 3). Intake of both PSO and alcohol also only led to an increase in GR protein level (no changes for other protein levels were measured) compared to the control group (significant ANOVA e and p effects, $P<0.001$ and $P<0.05$, respectively), but the effect was more pronounced for alcohol ingestion (significant ANOVA interaction of factors e and p, $P<0.001$). Intake of PSO increased the GR protein level and had no effects on other antioxidant enzyme (SOD1, SOD2, CAT, GSH-Px) protein

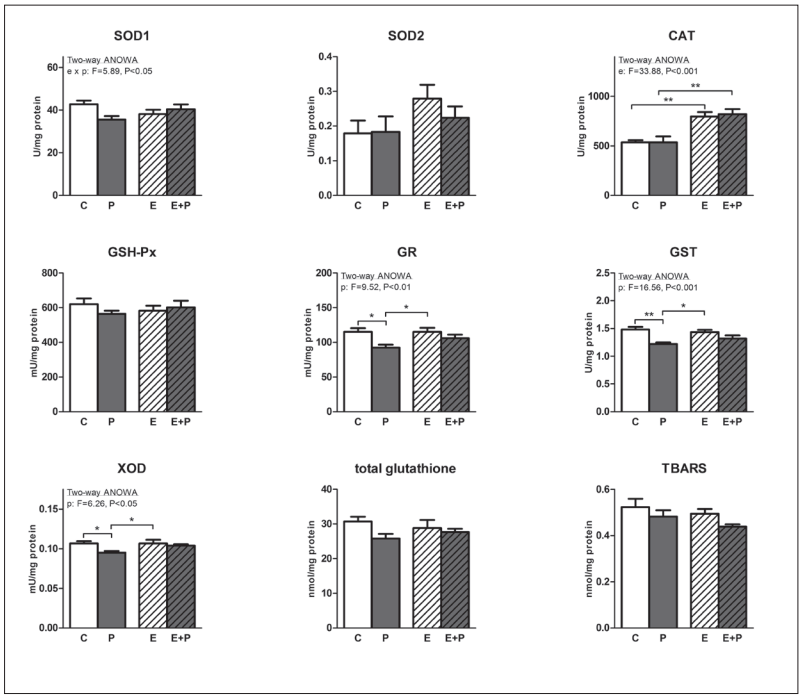

Fig. 2. Activities of antioxidant enzymes (SOD1, SOD2, CAT, GSH-Px and GR), GST, XOD and concentrations of total GSH and TBARS in rat liver after treatment with ethanol and PSO. Data are expressed as the mean±SEM (n=6). Statistical significance was tested by two-way ANOVA (factors: consumption of ethanol – e and consumption of PSO – p), P<0.05 and post hoc compared by Tukey's HSD test. C – control group; P – group treated with PSO; E – group treated with ethanol; E+P – group treated with ethanol and PSO. * P<0.05, ** P<0.01.

levels, on XOD activity and the amount of NF-κB and p53 compared to the controls (P vs the C group). However, differences appeared in the levels of SOD1 and NF-κB when the group that consumed both ethanol and PSO (E+P group) was compared with groups that consumed either ethanol (E group) or PSO (P group). Tukey's HSD post hoc test showed that the protein level of SOD1 in the group that was fed with both ethanol and pumpkin oil was significantly lower than in groups that consumed either ethanol or PSO (P<0.01, both). Consumption of both ethanol and PSO led to a significant elevation of NF-κB protein compared to other groups (significant ANOVA interaction of factors e and p, p<0.05); Tukey's HSD post hoc t-test showed that the protein level of NF-κB in animals that consumed both ethanol and PSO (E+P group) was significantly higher than in groups E (ethanol intake) and P (PSO intake) (P<0.05, both). Although ANOVA showed slight differences in p53 content (ANOVA significant interaction of factors e and p, P<0.05),

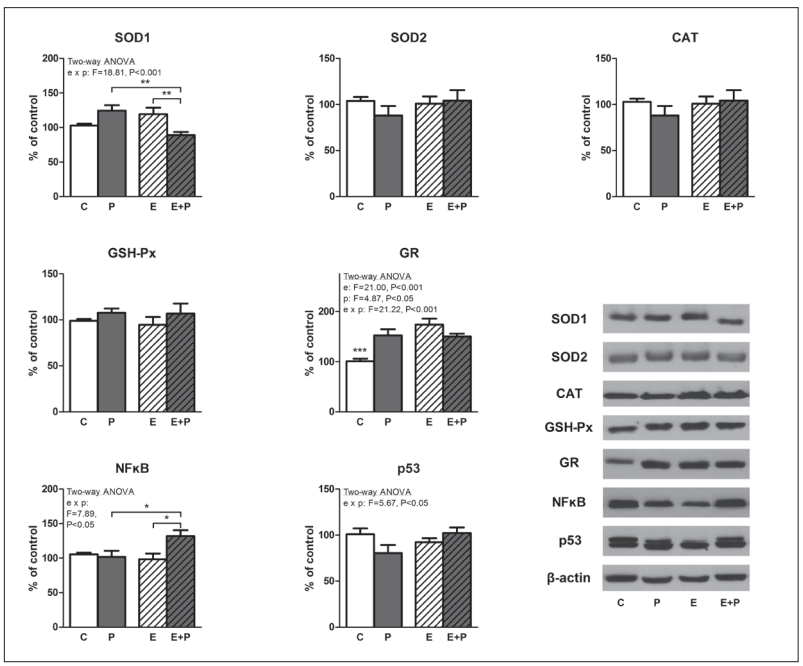

Fig. 3. Western blot analysis of antioxidant enzymes (SOD1, SOD2, CAT, GSH-Px and GR) and transcription factors (NF-κB and p53) in rat liver after treatment with ethanol and PSO (representative Western blots are shown). Whole cell extracts from liver (50 μg protein) were subjected to SDS-PAGE and Western blotting. β-Actin was used as loading control. Representative Western blots and relative quantification of corresponding protein levels in the control group. Values are means±SEM (n=6) and are presented as the percentage of the control. Statistical significance was tested by two-way ANOVA (factors: consumption of ethanol – e and consumption of PSO – p), P<0.05 and post hoc compared by Tukey's HSD test. C – control group; P – group treated with PSO; E – group treated with ethanol; E+P – group treated with ethanol and PSO. * P<0.05, ** P<0.01, *** P<0.001.

the post hoc Tukey's HSD t-test showed that there were no differences between groups.

DISCUSSION

Our study revealed the hepatoprotective effect of PSO when administered with ethanol. The protective effect was evident at the level of histopathological changes. Significant and severe focal necrosis was completely absent in rats that received 2 mL/kg bw of PSO along with *ad libitum* ethanol ingestion. All other observed histopathological changes were also attenuated by PSO, including congestion, dilatation of portal and central veins, portal spaces inflammation, steatosis and Kupffer cell hyperplasia, and moderate and pronounced newly formed bile ducts that appeared in the liver. As newly formed bile ducts represent a compensatory mechanism during regeneration of damaged hepatocytes, we suggest that the administration of PSO at a dose of 2 mL/kg bw for 6 weeks provided a regenerative effect. PSO also reduced the number of inflammatory cells in the port area and sinusoids. In addition to these protective effects, discrete changes in cellular edema in both experimental groups of animals treated with PSO were observed. While this effect could be defined as an adverse effect, it was negligible in relation to the protective effects.

The presence of edema can be explained as the consequence of protein accumulation in hepatocytes and the increase in osmotic pressure that led to the accumulation of water and the formation of edema. Linoleic acid, which is found in PSO, has a significant effect on membrane fluidity, which improves osmosis and facilitates the intra- and extracellular exchange of gases [37]. In our previous study [13], we reported that PSO in rats that consumed alcohol lowered the total high-density lipoprotein (HDL) cholesterol ratio but elevated low-density lipoprotein (HDL) cholesterol. More importantly, this study showed that daily ingestion of PSO did not significantly change the level of ethanol consumption (estimated by the total liquid intake) and its concentration in the blood. Therefore, PSO appeared to possess an intrinsic protective property that operated at the level of liver tissue homeostasis and/or at a systemic level as well. The systemic mediator is likely NF-κB. Alcohol intake induces elevated expression of NF-κB [38] by endotoxin release, but therein the concentration of the consumed alcohol was far higher than in our experiment. The absence of changes in NF-κB expression in rats that consumed alcohol in our experiment could have been the consequence of a relatively lower alcohol intake when compared to the referenced work. Also, PSO intake did not induce elevated expression of NF-κB; however, in the group that consumed both alcohol and PSO there was a significant elevation of NF-κB. It appears that the components of PSO, together with alcohol, provoked NF-κB expression. NF-κB in ALD regulates the expression of different proinflammatory mediators in liver macrophages, which depended on ethanol consumption. Chronic alcohol intoxication led to liver damage accompanied by TLR4 activation on macrophages by circulating lipopolysaccharides, which was followed by NF-κB activation that promotes the generation of proinflammatory cytokines [39]. Monocytes also show increased activation of NF-κB after chronic intoxication with ethanol [40], and chronic alcohol ingestion leads to the activation of NF-κB and the induction of TNFα [38], which in turn stimulates the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in liver damage due to oxidative stress [11,12]. There are different potential PSO ingredients that can additionally activate NF-κB. Zn acts upstream, affecting the signals that regulate NF-κB activation [41], and induces liver regeneration through upregulation of cell proliferation-related proteins [42]. At this point we can only speculate that the signs of liver regeneration (e.g. newly formed ducts) and cell proliferation that were observed by histopathological analyses, as well as the detected increased expression of NF-κB, which is a mediator of tissue regeneration [43], imply that NF-κB mediated the protective effects of PSO. The expected increased production of hydrogen peroxide during chronic ethanol consumption also induced NF-κB. Ethanol consumption led to elevated CAT activity, increased cytosolic H_2O_2 concentrations and oxidative stress, but the consumption of PSO (with alcohol) had no effect on the alcohol-induced elevation of CAT activity. This observation suggested that the protective effect of PSO was not at the level of H_2O_2 metabolism. Parallel with the increase in NF-κB and CAT, our results showed decreased expression of cytosolic SOD1. SOD1 activity can be inhibited by high concentrations of hydrogen peroxide [44], thus its decreased expression can be the consequence of the increase in hydrogen peroxide.

PSO is a rich source of lipophilic antioxidants such as tocopherols whose antioxidant activity is manifested as a reduction in lipid peroxidation [45]. The decreased activity of GST that catalyzes GSH conjugation can be associated with the inhibitory effects of α-tocopherol from PSO on GST [46]. Vitamin E activity toward lipid peroxides can indirectly lower the influence of GST, which also metabolizes lipid peroxides; since PSO is rich in lipophilic antioxidants, antioxidant defense homeostasis can be shifted towards lipid cellular compartments that attenuate hydrophilic antioxidant protection. GR activity was decreased in all groups of animals treated with PSO, but Western blot analysis revealed an increased content of GR protein in all experimental groups, but which was accompanied by inhibition of GR activity. The elevated level of GR protein and its low activity suggested that a state of inhibition accompanied a low oxidative load (high cellular concentration of NADPH and/or a low concentration of oxidized GSH). The decreased GST activity also suggests a lack of available GSH as antioxidant protection. Reduced GR activity along with the higher content of GR protein points to a lower turnover of GSH, an essential regulator of the cellular redox state, in the PSO-treated group. However, elevated CAT activity suggested that the liver was exposed to increased concentrations of hydrogen peroxide in rats that consumed alcohol and the inability of PSO to reduce the high influx of hydrogen peroxide. Furthermore, chronic ethanol consumption nullified the protective antioxidant effects of PSO that were expressed as lower levels of GR, GST and XOD as compared to the controls.

The observed decreased activity of XOD could be associated with the protective effects of some components of the PSO. XOD is a multi-substrate enzyme that generates superoxide. A previous study showed that cucurbitane-type triterpene glycosides such as 23,24,25,26,27-pentanorcucurbitane taiwacin B and a known steroid glycoside isolated from *Momordica charantia* significantly inhibited XOD [47]. Our results point to a composite effect of pumpkin oil that operated at different cellular levels and signaling pathways. Since p53 protein levels in the experimental groups were not different from the controls, it can be assumed that chronic ethanol intake-induced stress was not extensive enough to lead to DNA damage, growth arrest and/or apoptosis in the liver. Taken together, our results suggest that PSO functioned at different cellular and systemic levels in ALD through multiple, overall protective molecular mechanisms.

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Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Radic%20et%20al_6170_Supplementary%20Material.pdf