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INFLUENCE OF SELECTED *STACHYS* EXTRACTS ON CARBON TETRACHLORIDE-INDUCED LIVER DAMAGE IN RATS

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The influence of methanol extracts of four *Stachys* species from Balkan on carbon tetrachloride induced hepatotoxicity in rats was investigated. The extracts were obtained from aerial parts of *S. beckeana* Dörfler & Hayek, *S. anisochila* Vis. et Pančić, *S. plumosa* Griseb. and *S. alpina* L. subsp. *dinarica* Murb. The liver damage was induced by *s.c.* injections of carbon tetrachloride (2.5 ml/kg b.w.) and the extracts were then consecutively applied for five days. Their effects were evaluated through alteration of biochemical parameters (liver enzymes in the serum), as well as through histopathological changes in the liver (liver damage score, LDS). Treatment of CCl_4-intoxicated rats with methanol extracts of investigated *Stachys* taxa (200 and 100 mg/kg b.w. *p.o.*) significantly reduced increased level of marker enzymes aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) in the serum (with the exception of AST and ALT after 100 mg/kg of *S. anisochila* extract was administered). Vast degenerative and vascular changes in CCl_4-treated rats were also notably reduced after the treatment with investigated extracts, corroborating the biochemical observations and confirming their hepatoprotective effects. The best dose-dependant activity was achieved by the methanol extract of *S. alpina* subsp. *dinarica*.

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1. Introduction

Liver damage mainly occurs due to excessive alcohol consumption, viral infections, and as a consequence of drug adverse effects. Nowadays liver diseases constitute a major medical problem of worldwide proportions [1] and there is a general agreement that the number of useful liver drugs currently available is far from sufficient. Thus, there is an ongoing need for finding new substances that can effectively prevent and cure hepatic damage, minimizing adverse effects. Herbal drugs as well as compounds of herbal origin have been intensively studied for their possible beneficial effects in liver injuries [2]. Many plant isolates were shown to possess hepatoprotective activity and in many cases it was also concluded that such an activity is strongly

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influenced by their antioxidant and free radical scavenging effects [3]. Carbon tetrachloride-induced liver damage is one of the most often used animal models for assessment of hepatoprotective effect of various substances. Hepatotoxic effects of CCl_4 result in hepatocelular necrosis in mice and rats, the release of AST and ALT, decreased levels of antioxidative enzymes and increased lipid peroxidation products [4].

The genus *Stachys* L. (Lamiaceae) comprises over 300 species widespread throughout Eurasia and America. Phytochemical studies on species of this genus revealed the presence of many secondary metabolites: different polyphenols (flavonoids, tannins, phenolic acids, phenylethanoid glycosides), iridoids, terpenoids, and sterols [5, 6]. Presence of a variety of secondary metabolites and a long traditional use implicated numerous pharmacological investigations on these plants. Many of the effects were demonstrated: antioxidant [7, 8], anti-inflammatory [9-11], anti-nephritic [12], cytotoxic [13, 7], and anxiolytic [14]. Antimicrobial activity was evaluated on different *Stachys* essential oils and extracts as well [15, 16].

It was previously shown that methanol extracts of Balkan endemics: *S. anisochila* Vis. et Pančić, *S. beckeana* Dörfler & Hayek, *S. plumosa* Griseb., and *S. alpina* L. subsp. *dinarica* Murb. exhibit antoxidant and free radical scavenging effects [17], as well as anti-inflammatory [18], and behavioral activities [19]. The goal of this work was preliminary investigation of the influence of these four before mentioned *Stachys* extracts on carbon tetrachloride-induced liver damage in rats.

2. Materials and methods

Plant material and extraction

The aerial parts of the plants were collected during their flowering period from natural populations: *S. anisochila* in gorge of Beli Rzav (W Serbia), June 2003; *S. beckeana* from Mt. Durmitor (Montenegro), August 2003; *S. plumosa* in Jelašnička klisura gorge (SE Serbia), June 2002; *S. alpina* subsp. *dinarica* on Mt. Jahorina (Bosnia and Herzegovina), July 2004. Voucher specimens were deposited in Herbarium collection of Natural History Museum in Belgrade (BEO - ko820033/6, ko620041/4, ko320025/6, and ko720049/83, respectively). Plant material was air dried at room temperature and finely grounded. Each sample was bimacerated with chloroform (3 and 2 days; plant material/solvent ratio=1:7) and further extracted in the same way with methanol. Solvents were completely evaporated under reduced pressure. Obtained dry methanol extracts of *S. anisochila* (SA), *S. beckeana* (SB), *S. plumosa* (SP) and *S. alpina* subsp. *dinarica* (SAD) were used for the experiments.

Experimental design

Experiments were performed on male Wistar rats, 6-8 weeks old (180 to 220 g) bred at the Farm for Experimental Animals, Military Medical Academy, Belgrade, Republic of Serbia. They were housed in plastic cages, under standard laboratory conditions (21-22 °C, 12 h light/dark cycle, 30-70% relative humidity) with commercial food and tap water *ad libitum.* All procedures in the study conformed to EEC Directive 86/609 and are based on the Guidelines for Animal Study № 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Republic of Serbia).

The animals were divided into fifteen groups of five animals each. Group C0 served as the intact control and was without the treatment (0.1% saline solution, 1 ml/kg *p.o.*). Group C1 (the positive control) was given only CCl_4 dissolved in olive oil (1:1) (2.5 ml/kg b.w. *s.c.*) on the first day of the experiment. In groups T1 and T2 hepatitis was induced by giving *s.c.* injections of CCl_4 in olive oil (1:1, 2.5 ml/kg b.w.) and after 30 minutes they were given extracts in doses of 200 and 100 mg/kg b.w. *p.o.* In these groups the treatment was continued for the following four days. At fifth day, 6 hours after the last dose, the rats were anesthetized and sacrificed by cervical dislocation.

The blood was collected and the serum separated (10 minutes at 2000 rpm) and used for biochemical assays. The activities of serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were spectrophotometrically determined using diagnostic kits (Randox Laboratories, UK) and enzyme activity was expressed in U/L.

Liver was dissected out and samples were carefully spread over a metal tray coated with wax and fixed with 10% neutral buffered formalin solution. Five days after fixation all tissue samples were divided into 10 portions in order to be prepared for making sections. After process of fixation, all tissue samples were dehydrated in graded alcohol (100%, 96% and 70%), xylol and embedded in paraffin blocks. Finally, 2 μm thick paraffin sections were stained by haematoxylin and eosin (HE) method. From each specimen, whole visual fields magnified by 200× were analyzed using light microscope according to the 5-point semiquantitative scale, i.e. liver damage score (LDS) for degenerative and vascular changes (Table 1).

Table 1. The frequency and severity of the hepatic lesions - liver damage score (LDS).

Grade	Definition
0	Normal findings.
1	Single hepatocytes with small cytoplasmatic granules, slightly enlarged, and with normal nuclei. Mild dilatation of blood vessels.
2	> 50% hepatocytes with mild vacuolisation of cytoplasm and nucleoplasm. Strong vasodilatation with erythrocytes accumulation (*stasis*) associated with hyperemia and edema.
3	All hepatocytes with pronounced vacuolisation of cytoplasm and nucleoplasm, and pycnotic nuclei. Transmural rupture of small number (up to 50%) of blood vessels (moderate focal *haemorrhagiae diapedesis*) and perivascular accumulation of polymorphonuclear cells (PMNCs).
4	Pronounced plasmalysis and caryolysis. Massive hemorrhagic foci and PMNCs infiltration.
5	*Necrosis hepatis.* Diffuse hemorrhagic and PMNCs infiltration of hepatic tissue.

Statistical analysis

Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference test. A value of $p<0.05$ was considered to indicate statistical significance. All the results were expressed as mean ± S.D. for five animals in each group.

3. Results

Rats intoxicated with CCl_4 alone (the positive control, C1) have developed hepatocellular damage as evident from a significant elevation ($p<0.001$) in the serum activities of AST, ALT and AP (Table 2). Treatment with investigated extracts, except with that of *S. anisochila*, significantly decreased serum activities of AST and ALT in CCl_4-intoxicated rats. Similarly, serum activity of AP was considerably decreased by all extracts in all applied doses ($p<0.001$ vs. C1) (Table 2).

Table 2. Effects of Stachys extracts on CCl_4-induced liver injury: serum activity of liver enzymes.

Experimental groups	AST (U/L)*	ALT (U/L)*	AP (U/L)*
Intact control (C0)	199.04 ± 21.91	62.86 ± 10.97	552.80 ± 47.79
CCl_4 treated group (C1)	522.05 ± 59.22[a]	134.90 ± 19.77[a]	928.80 ± 64.70[a]
200 mg/kg SA (T1)	428.64 ± 11.11[b]	111.70 ± 10.76[b]	447.60 ± 35.88[c]
100 mg/kg SA (T2)	531.13 ± 20.79	262.30 ± 21.73[c]	557.60 ± 92.73[c]
200 mg/kg SB (T1)	303.80 ± 5.09[c]	74.03 ± 13.61[c]	750.20 ± 55.46[c]
100 mg/kg SB (T2)	222.09 ± 12.68[c]	46.09 ± 4.20[c]	510.80 ± 40.69[c]
200 mg/kg SP (T1)	277.60 ± 22.69[c]	69.40 ± 21.87[c]	256.40 ± 43.85[c]
100 mg/kg SP (T2)	200.80 ± 3.49[c]	32.48 ± 6.37[c]	138.20 ± 11.53[c]
200 mg/kg SAD (T1)	240.60 ± 30.18[c]	41.90 ± 9.72[c]	328.40 ± 26.56[c]
100 mg/kg SAD (T2)	275.87 ± 16.69[c]	37.01 ± 5.58[c]	221.70 ± 21.88[c]

*Values are given as mean ± S.D.; [a]$p<0.001$ vs. C0; [b]$p<0.05$, [c]$p<0.001$ vs. C1. SA – *S. anisochila* extract; SB – *S. beckeana* extract; SP – *S. plumosa* extract; SAD – *S. alpina* subsp. *dinarica* extract.

High values of liver damage score (LDS) in animals treated with CCl_4 (group C1) indicate vast degenerative and vascular changes in the liver tissue (Table 3).

Histological architecture of liver parenchyma was completely altered with massive fatty changes and necrosis. Blood vessels were mostly disrupted, with numerous focal haemorrhages and broad infiltration of the polymorphonuclear cells (PMNCs) (Fig. 1-II). The severity of changes was more prominent in the centrilobular parts than in periportal area. After the extracts were applied, the best and dose-related hepatoprotective activity was achieved by extract of *S. alpina* subsp. *dinarica* (Fig. 1-VI). At the dose of 200 mg/kg b.w. (T1 SAD) it reduced hepatic CCl_4-induced damage by nearly 50% (LDS = 2.23). Hepatoprotective effect of other investigated *Stachys* extracts was also evident, though differences between applied doses (200 and 100 mg/kg b.w. *p.o.*) were not statistically significant (Table 3). Effects of *S. anisochila* and *S. beckeana* extracts were similar with each other, and better than that of *S. plumosa* extract (Figs. 1-III, IV, and V). Although CCl_4-induced liver damage was significantly less pronounced when *Stachys* extracts were applied ($p<0.001$ vs. C1), overall hepatoprotective effect could be considered as moderate, because values of LDS in the rats treated with investigated extracts were still significantly higher that that of the control group ($p<0.001$ vs. C0).

Table 3. Effects of Stachys extracts on CCl_4-induced liver injury: liver damage score (LDS)

Group	**Liver damage score (LDS)***
Intact control (C0)	0.20 ± 0.04
CCl_4 treated group (C1)	4.29 ± 0.17[a]
200 mg/kg SA (T1)	3.14 ± 0.11[b]
100 mg/kg SA (T2)	3.48 ± 0.04[b]
200 mg/kg SB (T1)	3.09 ± 0.07[b]
100 mg/kg SB (T2)	3.41 ± 0.13[b]
200 mg/kg SP (T1)	3.49 ± 0.30[b]
100 mg/kg SP (T2)	3.80 ± 0.29[b]
200 mg/kg SAD (T1)	2.23 ± 0.14[b, c]
100 mg/kg SAD (T2)	2.96 ± 0.20[b]

* Values are given mean ± S.D.; [a] $p<0.001$ vs. C0; [b] $p<0.001$, vs. C1; [c] $p<0.001$, T1 vs. T2. SA – *S. anisochila* extract; SB – *S. beckeana* extract; SP – *S. plumosa* extract;
SAD – *S. alpina* subsp. *dinarica* extract.

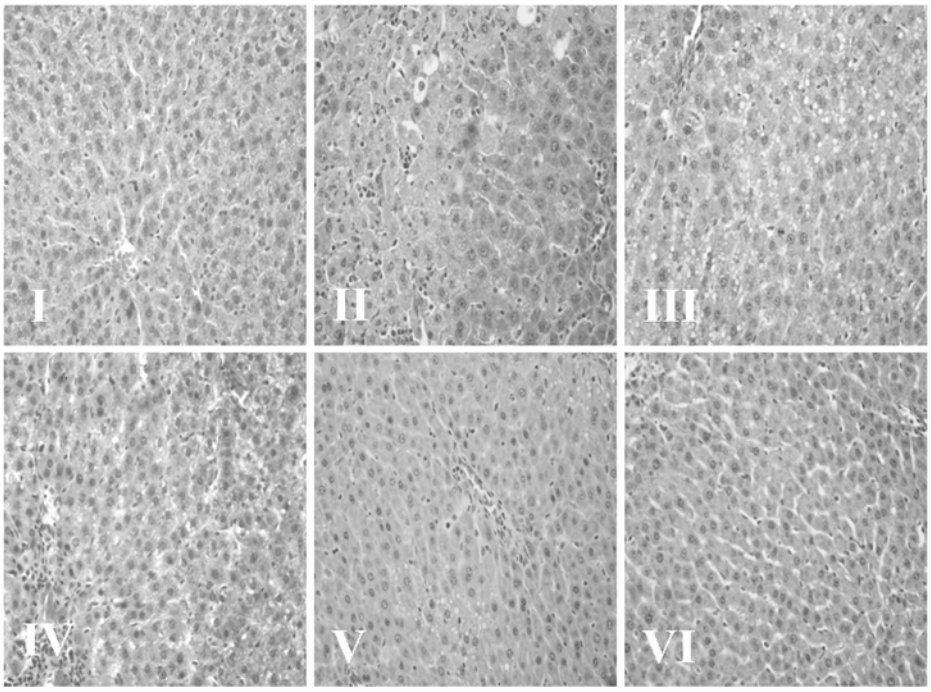

Fig. 1. Hepatoprotective effects of Stachys extracts on CCl_4-induced liver injury (HE staining, magnifcation ×200). I - group C0 (LDS = 0.2); II - group C1 (LDS = 4.29); III – S. anisochila extract, 200 mg/kg b.w. (LDS = 3.14); IV – S. beckeana extract, 200 mg/kg b.w. (LDS = 3.09); V – S. plumosa extract, 200 mg/kg b.w. (LDS = 3.49); VI – S. alpina subsp. dinarica extract, 200 mg/kg b.w. (LDS = 2.23)

4. Discussion

Carbon-tetrachloride is extensively being used as a model substance for producing hepatotoxic effects such as fatty degeneration, fibrosis, hepatocellular death, and carcinogenicity. The mechanism of its hepatotoxic action is very complex and based upon its activation by cytochrome P450 enzymes, which results in producing highly reactive metabolites tricloromethyl radical ($\cdot CC1_3$) and trichloromethyl peroxy radical ($\cdot CC1_3O_2$). These reactive species are capable for covalently binding locally to cellular macromolecules, with preference for fatty acids from membrane phospholipids. Attacking polyunsaturated fatty acids (PUFA) in membranes free radicals initiate lipid peroxidation (LP), setting off a free radical chain reaction sequence [20]. In the response to hepatotoxicity a sustained regenerative and proliferative changes in liver occur and final outcome depends on dose, exposure time, presence of potentiating agents, or age and state of the affected organism. Antioxidants and free radical scavengers (FRS) thus can beneficially influence to final outcome in CCl_4 model of liver damage and toxicity [21].

In this work results of preliminary investigation of influence of selected *Stachys* extracts on CCl_4-induced liver damage in rats were presented. Our results suggest that four investigated *Stachys* extracts possess certain hepatoprotective effect. Restoration of markers of liver damage (enzyme levels and LDS) was observed after administration of all investigated *Stachys* extracts in our experiment (except for AST and ALT after 100 mg/kg SA). Since the free radical formation and LP have the predominant influence in CCl_4-induced liver damage, the exhibited effect of selected *Stachys* species is probably caused by the presence of FRS and antioxidant substances in their methanol extracts. This is supported with our previous findings on the free radical scavenging and antioxidant activity of these extracts evidenced *in vitro*, through the experiments on LP inhibition, scavenging of hydroxyl and DPPH radicals and determination of redox potential [17]. These previously published data have shown that the lowest overall activity and the lowest phenolics content was exhibited by *S. plumosa* extract, which expressed the lowest hepatoprotective activity in the present experiment. Extracts of *S. beckeana* and *S. alpina* subsp. *dinarica* were the most active as inhibitors of LP. Moreover, *S. alpina* subsp. *dinarica* extract

expressed much better overall inhibitory effect on LP (60-70% of LP inhibition at a range 62.5-125 μg/ml) [17], which is strongly correlated with the expressed hepatoprotective activity in the present experiment. As it was shown by liver enzymes, investigated extracts were slightly more effective in lower dose applied (100 mg/kg *p.o.*). This could be explained by prooxidant effect of extract constituents, which is not unusual. Namely, it is known that many of active substances from medicinal plants have dual nature, i.e. depending of dose applied they may produce both an antioxidant and a prooxidant action. The last one, as a rule, occurs at higher doses used. Also, our previous study showed that methanol *Stachys* extracts at higher concentrations had weaker scavenging effect on hydroxyl and DPPH radicals than in lower concentrations tested [17].

5. Conclusion

In conclusion, we observed beneficial influence of investigated *Stachys* extracts in CCl_4-intoxicated rats. We could assume that these extracts possess hepatoprotective property due to their proven antioxidant and free radical properties. However, other possible mechanisms, such as inhibition of cytochrome P450 enzyme and influence on different signal pathways in liver cells, should not be neglected. Further investigations on these matters are warranted, particularly that of *S. alpina* subsp. *dinarica*, as well as elucidation of compounds responsible for such activities.

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