

Impact of winter savory extract (*Satureja montana L.*) on biochemical parameters in serum and oxidative status of liver with application of the principal component analysis in extraction solvent selection

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Abstract. – **OBJECTIVE:** *Satureja montana L.* is traditionally used as spice and for treatment various diseases. Many studies have shown antioxidative effect of *Satureja* species. Our thorough study in an animal model was performed through measurement of biochemical parameters in the serum, histology analysis and determination of oxidative status of the liver, coupled with investigation of extraction solvent selection using principal component analysis (PCA).

MATERIALS AND METHODS: Winter savory dry extract (500 mg/kg) dispersion and saline solution were given to Wistar rats for 7 days after exposure to oxidative stress using toxic doses of paracetamol (600 mg/kg). Rats were sacrificed, after which a complete autopsy was performed, the blood obtained was used to determine biochemical parameters, and the liver was sliced for histological analysis and determination of oxidative stress enzymes.

RESULTS: Indicators of hepatic and kidney functions, as well as the concentration of oxidative stress enzymes, were statistically significantly lower in animals treated with *Satureja montana L.* extract compared to the paracetamol group alone before the toxic dose of paracetamol. Liver enzymes were unaltered by pre-treatment with the extract, but the level of lipid peroxidase was decreased, and the level of catalase, glutathione reductase and superoxide dismutase increased proving *in vivo* antioxidant effect. In addition, the number of inflammatory cells is decreased cou-

pled with activity of CYP2E1 enzymes proving hepatoprotective effect.

CONCLUSIONS: *Satureja montana L.* extract in our research has shown hepatoprotective, anti-inflammatory and antioxidative effect. PCA analyses indicated that extraction mediums have a great impact on the antioxidative effect.

Key Words:

Winter savory extract, Rats, Oxidative stress, Solvent extraction, Biochemical parameters, Liver function, Principal component analysis.

Introduction

Oxidative stress participates in the pathology of many diseases, including atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's disease, and cancer. Reactive oxygen and nitrogen species (RONS) are produced by all aerobic cells and play an important role in aging as well as in age-related diseases. There are endogenous and exogenous sources of RONS¹. Oxidative stress results from the imbalance between RONS production and antioxidants defense and is primarily involved in "aging theory". Oxidative stress is also related to several chronic diseases and is increased in the elderly

population. Antioxidant therapy especially using natural remedies decrease the clinical damage induced by oxidative stress^{2,3}. Antioxidant agents are important in enhancing oxidative protection and decreasing damage on biological systems. Various antioxidant agents are used as supplementation to prevent the occurrence or aggravation of a disease or during the treatment with various drugs that could increase oxidative stress (oncology treatment, polypharmacy, diabetes, etc.).

Winter savory (*Satureja montana L.*) belongs to the botanical family Lamiaceae. It is a semi-woody, perennial subshrub, native to warm temperate regions of southern Europe⁴⁻⁶.

Satureja montana L. is widely used as a culinary herb and in traditional medicine for the treatment or relief of various common health symptoms in many parts of the world. However, numerous studies^{5,7-11} have shown that it has pharmacological potential to be used as an antibacterial drug, antidiabetic, anti-HIV and antiproliferative drug.

These pharmacological effects can be attributed to the presence of phytochemicals like flavonoids, sterols, tannins and essential oils^{4,6}. Phenolic compounds are mostly responsible for the anti-oxidative activity of *Satureja montana L.* It has also been confirmed that phenolic acids could inhibit certain enzymatic systems. Perhaps, the most significant effect is on lipid peroxidase – because of the inhibition of this enzyme, phenolic acids can be used as preservatives, anti-inflammatory agents and as antioxidants, which offers numerous possibilities for extending their therapeutic application¹².

In the case of the use of multiple drugs or drugs that damage the liver during prolonged period of use, supplementation with agents that have a hepatoprotective effect is very important. Thus, the aim of our study was to hepatoprotective examine the genus *Satureja montana* since it is widely used as a culinary herb and in traditional medicine. Also, there is increased interest in its cultivation. We have based our investigation on water dispersion of dry extract of *Satureja montana L.* and performed complete biochemical parameters screening of liver status coupled with histopathological analysis and determination of the oxidative status of the liver in an animal model exposed to a toxic dose of paracetamol. Additionally, we investigated the effect of solvent medium on antioxidative capacity based on total phenolic content using principal component analysis.

Materials and Methods

Plant Material and Extract Preparation

Dry extract of winter savory (*Satureja montana L.*, Lamiaceae) used in this study was obtained from the Institute for Studies on Medicinal Plants 'Dr Josif Pančić', Belgrade, Serbia, in 2017. A voucher specimen of the plant (*Satureja montana L.* IPLB 32/16L) was confirmed and deposited in the herbarium of the Institute for Medicinal Plants Research 'Dr Josif Pančić', Belgrade, Serbia.

Air dried aerial parts of *Satureja montana L.*, Lamiaceae were extracted by percolation with 70% ethanol, at room temperature. The extract was concentrated under reduced pressure (bath temp. 50°C) and dried in a vacuum desiccator. The residue was dissolved in distilled water and filtered. The filtrate was evaporated to dryness. The dried mass was suitably diluted with saline solution and used in experiments.

High-Performance Liquid Chromatography

Identification and quantitative analysis of the main components of the *Satureja montana L.* were performed by applying high-performance liquid chromatography (HPLC). For HPLC analysis, the examined extracts were dissolved in 70% ethanol and then filtered through a membrane filter (0.45 µm). HPLC analysis was performed on the Agilent 1200 RR chromatograph with a DAD detector on a Lichrospher 100 RP 250 x 4 mm column, particle size 5 µm. As a mobile phase was used 0.17% formic acid in water (phase A) and acetonitrile (phase B). The injection volume of the sample was 10 µl. Elution was carried out on a gradient basis: 0-100% B, 0-53 min, with a total duration of 55 min. The flow rate was 0.8 mL/min. UV absorbance was measured at 280 and 330 nm^{13,14}.

Animals

The experiment was performed on sexually mature, male, Wistar rats, weighing 200-250 g. The animals were obtained from Military Medical Academy, Belgrade. The rats were kept in transparent polycarbonate cages, under a 12 h light-dark cycle, at a constant ambient temperature (20-25°C) and humidity (55 ± 1.5%). The animals were maintained on standard pellet diet (Veterinary Institute, Subotica, Serbia) and allowed access to tap water *ad libitum* (the food was removed from the animals only 12 hours before and 6 hours after the paracetamol was administered). Animal care

and all experimental procedures were performed in accordance with the ethical principles outlined by the EU Directive 2010/63/EU on animal welfare and under the Law of Animal Welfare of the Republic of Serbia (OG RS 41/09). This study was approved by the Ethical Committee of the University of Novi Sad (Approval No. 01-78/29).

Experimental Design

To evaluate the effects of *Satureja montana* L. extract on paracetamol liver injury, the animals were randomly divided into four experimental groups, each containing six individuals: the control group received a saline solution of 1 mL/kg perorally (p.o.) for seven days; the paracetamol group received 7 days saline solution 1 mL/kg for 7 day and single dose paracetamol 600 mg/kg p.o.; *Satureja montana* L. extract group received dispersed extract in saline solution in dose 500 mg/kg for seven days p.o.; *Satureja montana* L. extract group with pretreatment of a toxic dose of paracetamol received 500 mg/kg for a dispersed dry extract for seven days p.o. and a single dose of paracetamol of 600 mg/kg, p.o.

Following paracetamol toxic dose administration, after 24 hours, the animals were anesthetized with 25% urethane solution (0.75 g/kg, i.p.) and after the loss of all reflexes, the animals were euthanized by cardiopunction, and blood samples and liver tissue samples were collected.

Applied daily doses of *Satureja montana* L. extract were 500 mg/kg⁹. Reconstituted in saline immediately prior to administration. The dose of paracetamol for the experiment (600 mg/kg p.o.), was determined by reviewing the available literature³.

Biochemical and Histological Investigation

Serum biochemical parameters determination

Activities of enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), as well as direct bilirubin were determined in serum samples as a measure of hepatocellular damage. The concentration of creatinine, urea and uric acid as indicators of renal excretory function was also determined in the serum. All analyses were performed on the Olympus AU 400 autoanalyzer (Hamburg, Germany) and by using commercially available kits based on the well-established spectrophotometric methods and according to the manuals supplied.

Determination of Oxidative Status in the Liver

Liver homogenates were made from 1 g of liver tissues which were homogenized in a Potter homogenizer with TRIS–HCl sucrose-buffered solution in a ratio of 1:3 (w/v) at 4°C. The parameters of oxidative stress were analyzed in obtained liver homogenates. The obtained liver homogenates were filtered and used for the spectrophotometric determination of biochemical parameters: the intensity of the lipid peroxidation (LPx); and the activities of antioxidant enzymes including catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD). All measurements were performed in triplicate for every sample.

The intensity of LPx was estimated by measuring the amount of malondialdehyde (MDA), a terminal product of lipid breakdown due to peroxidation damage¹⁵. CAT activity was determined by the method based on monitoring the H₂O₂ decomposition rate at 240 nm¹⁶. The amount of SOD is estimated by the reaction of superoxide with a cytochrome c at 550 nm¹⁷.

Histological Tissue Processing

Liver tissue sections measuring 5x5 cm of the right medial lobe were fixed in 10% neutral buffered formalin for 24 h at 4°C. After fixation, the tissue was dehydrated in increasing concentrations of isopropanol and molded into paraffin (Histowax, Belgium). With the help of tissue diopeter from the molded liver tissue, 2 cylindrical samples, 2 mm in diameter, were taken from each and transferred to a specially prepared paraffin block with 30 holes. In this way, a tissue microarray block (TMA) was formed which contained samples of a total of 15 individuals (two samples from each). Each of the TMA blocks was cut on a rotating microscope (Leica, Germany) at a thickness of 5 µm. In each TMA block, the first section was stained with the periodic acid Schiff (PAS) method and the next two sections were stained immunohistochemically.

Immunohistochemical staining included primary antibodies: rabbit anti-Iba1 (Abcam, UK) at a dilution of 1:500 and rabbit anti-cytochrome P4502E1 protein (CYP2E1) at a dilution of 1:100 (Cusabio, College Park, MD, USA). Visualization was performed with an UltraVision LP detection system using HRP Polymer & DAB chromogen (Thermo Fisher Scientific, Waltham, MA, USA). All antibodies underwent a retrieval reaction that included pretreatment with citrate buffer (at pH 6.0) in a microwave oven at 850W for 20 minutes.

The duration of antibody application was 30 minutes at room temperature. Contrasting was performed with Mayer's hematoxylin and then the plates were mounted with an appropriate medium (Bio-Optica, Italy). The prepared slides were analyzed on a professional biological microscope Leica DMLB, (Leica, Germany) at a magnification of 200 and 400x and the fields of view were photographed with a high-resolution microscope camera Leica MC 190 HD (Leica, Germany). The photos were processed, and the compositions were formed in the CS6 version of Adobe Photoshop (Adobe, USA).

Effect of Solvent on Antioxidative Capacity

Preparation of extracts with different solvent mediums

Extract of *Satureja montana L.* was diluted 20 times using distilled water, methanol 50% and 90% and ethanol 50% and 90% and left to stand for 12 hours in order to mix and dissolve. Centrifugation was then performed at 3000 rpm for 5 minutes. The supernatant was taken as an extract diluted 20 times which was used in all tests. The results were evaluated in relation to the dry extract of *Satureja montana L.*

Oxidative Stress Markers

To measure the total antioxidant capacity of *Satureja montana L.* extracts, we chose the following methods: neutralization of 1,1-diphenyl-2-picrylhydrazyl radical [DPPH·], ferric reducing/antioxidant power assay (FRAP), nitric oxide (NO) scavenging activity and hydroxyl radical (OH·) scavenging activity.

The DPPH radical scavenging capacity method is based on spectrophotometric monitoring of the transformation of a purple-colored, stable, nitrogen-centered DPPH radical (1,1-diphenyl-2-picrylhydrazyl) into a reduced, yellow-colored form of DPPH-H^{18,19}.

The FRAP test is based on a nonspecific response to any system that has a less positive redox potential than the Fe³⁺ + -TPTZ / Fe²⁺ + -TPTZ system will lead to a reduction of Fe³⁺ + -TPTZ, (Tripyridyltriazine, TPTZ). By reduction, the coordination compound Fe (III) gives a dark blue intense color with an absorption maximum at 593 nm²⁰.

The determination of the capacity of the extract to neutralize the NO· radical is based on spectrophotometric measurement of the concentration of generated nitrite ions. This spectrophotometric method is based on mon-

itoring the formation of the purple-pink diazo complex which is the product of the reaction between NO₂⁻ and Griess' reagent at room temperature¹⁸.

The capacity of antioxidants to neutralize the hydroxyl radical is determined by monitoring the degradation reaction of 2-deoxy-D-ribose in the presence of free OH radicals generated in the Fe²⁺/HO₂ system. In this reaction (Fenton's reaction) a formed OH radical takes away the H atom molecule of 2-deoxy-D-ribose and initiates the decomposition of this molecule to the final products. One of the end products is MDA, the concentration of which is determined spectrophotometrically using a thiobarbituric test (TBA test) at 532 nm²¹.

The Folin-Ciocalteu method was used to determine the content of total phenols. The method is based on the oxidation of molecules containing phenolic functional groups²². Tannins, flavonoids and other classes of phenolic compounds that contain phenolic groups and have the ability to reduce the alkaline solution of phosphomolybdic acid from the Folin-Ciocalteu reagent participate in this reaction.

The method for determining the content of total flavonoids in the samples is based on the properties of flavonoids and flavonoid glycosides to give the corresponding metal complexes with metals²³. The aluminium complex is especially important, because it binds to all types of flavonoids, so the total absorption maximum of total flavonoids is easily determined.

Principal Component Analysis

Principal component analysis was conducted using R software packages (version 4.1.1, R Core Team, 2021), and RStudio (version 2021.09.1 Build 372), to characterize and visualize patterns of measured antioxidative capacity in different solvents (distilled water, methanol 50% and 90% and ethanol 50% and 90%) by the first two principal components (PC) vectors. Before analysis variables were centered and scaled to mean 0, and to a standard deviation of 1.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). The intergroup variation between various groups was measured by one-way analyses of variance (ANOVA) followed by Tukey's multiple comparison test. Results were considered statistically significant if $p < 0.05$. Data were analyzed by SPSS software, version 19 (SPSS IBM Corp., Armonk, NY, USA).

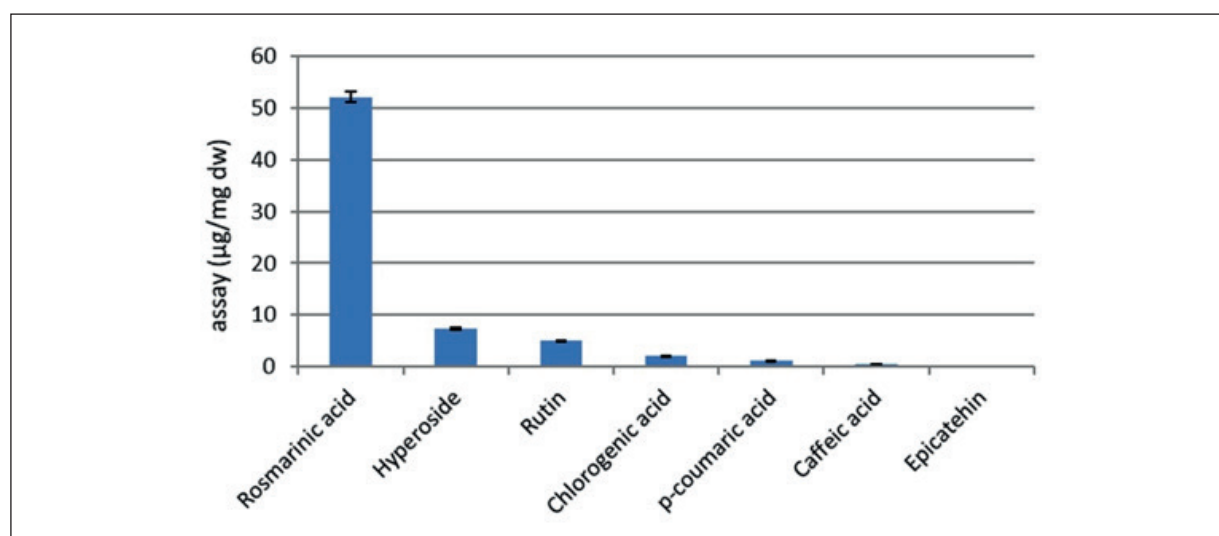


Figure 1. Chemical composition of *Satureja montana L.* extract.

Results

Chemical Composition

The *Satureja montana L.* extract is characterized by a high content of the phenolic compound rosmarinic acid, chlorogenic acid, p-coumaric acid and caffeic acid. Other important compounds were flavonoid compound hyperoside and rutin. Epicatechin was found in traces. Concentrations of the main components are shown in Figure 1.

Influence of *Satureja Montana* Extract on Paracetamol Induced Serum Biochemical Parameters

The application of toxic doses of paracetamol in animals treated with saline solution, significantly increased the activity of ALP, ALT, AST and GGT in serum compared to control. Activities of these enzyme markers of liver injury were not significantly changed in animals treated only with *Satureja montana L.* extract compared to the control group. However, the toxic dose of paracetamol attenuates ALP activity, which was significantly lower in a group of animals treated with *Satureja montana L.* extract and paracetamol compared with the group treated with saline solution and paracetamol. The application of toxic doses of paracetamol significantly increases the level of direct bilirubin in serum compared to control (Table I). Also, the level of direct bilirubin was significantly lower in the group treated with *Satureja montana L.* extract and paracetamol compared to the group treated with saline solution and paracetamol.

Biochemical parameters, indicators of renal function, urea, creatinine and uric acid, were also determined in the serum (Table I). Concentrations of urea, creatinine and uric acid were increased in the group treated with combination of saline solution and paracetamol compared to the control. The intake of paracetamol with *Satureja montana L.* extract significantly restored the concentration of creatinine compared to the group treated with the combination of saline solution and paracetamol.

Influence of *Satureja Montana L.* Extract on Paracetamol-Induced Oxidative Stress Parameters in Liver

Group treated with saline solution and a single dose of paracetamol, when compared with control, significantly increased LPx, and decreased GR. The level of CAT and SOD were decreased in comparison to the control group with saline solution, but the difference was not statistically significant. Group pretreated with *Satureja montana L.* extract and a toxic dose of paracetamol significantly decreased LPx, while there was no effect on GR, CAT and SOD level compared with the group treated with saline solution and paracetamol (Table II).

Histological staining for PAS in the control group and the group that received only *Satureja montana L.* extract showed complete preservation of cytoarchitectonics of liver tissue. The hepatocytes were properly arranged in the form of Remack's trabeculae extending radially from the central vein while sinusoidal spaces of proper appearance were not dilated. Portal spaces were correctly

Table I. Effects of *Satureja montana L.* extract on biochemical parameters in serum expressed as mean values \pm SEM.

	Saline solution (C)	Saline solution + paracetamol (ConP)	<i>Satureja montana</i> extract (S)	<i>Satureja montana</i> extract + paracetamol (S+P)
ALP (U/l)	293.50 \pm 27.83	373.17 \pm 42.14*	293.67 \pm 49.77 ^y	337.17 \pm 22.84 ^{*,x,y}
ALT (U/l)	33.83 \pm 2.04	39.67 \pm 0.82*	34.67 \pm 5.47	39.17 \pm 1.83 ^{*,x}
AST (U/l)	97.00 \pm 8.22	105.83 \pm 6.65*	100.17 \pm 5.08	109.17 \pm 2.79 ^{*,x}
GGT (U/l)	0.83 \pm 0.41	1.83 \pm 0.41 ^z	1.08 \pm 0.49 ^y	1.67 \pm 0.52 ^{*,x}
Direct bilirubin (μ mol/L)	0.17 \pm 0.05	0.37 \pm 0.05 ^z	0.27 \pm 0.05 ^{*,y}	0.28 \pm 0.04 ^{*,y}
Urea (mmol/L)	7.20 \pm 0.15	7.42 \pm 0.33*	7.25 \pm 0.29	7.35 \pm 0.59
Creatinine (μ mol/L)	38.17 \pm 1.17	39.67 \pm 0.82*	36.50 \pm 0.55 ^{*,y}	36.00 \pm 2.10 ^{*,y}
Uric acid (μ mol/L)	67.00 \pm 4.94	80.00 \pm 3.52*	79.00 \pm 2.61*	79.33 \pm 10.33*

ALP: alkaline phosphatase; ALT: aspartate aminotransferase; AST: alanine aminotransferase; GGT: gamma-glutamyl transferase; * p <0.05 compared with C group, ^x p <0.05 compared with S group, ^y p <0.05 compared with ConP group, ^z p <0.01 compared with C group.

positioned without visible inflammatory elements. The cytoplasm of hepatocytes of these groups was generally lighter in color with a smaller amount of PAS positive glycogen granules in it (Figure 2 liver (PAS) A and C). In the group of paracetamol, in addition to the preserved cytoarchitectonics, a pronounced inflammatory reaction was observed, which is predominantly present in the port spaces, probably caused as a consequence of the hepatotoxic effect of paracetamol. In hepatocytes of the central zone, a slightly higher positivity for glycogen granules was observed (Figure 2 liver (PAS) B). In the group with pretreatment with *Satureja montana L.* extract and then with the use of paracetamol, a difference was noticed, primarily in terms of reducing the number of inflammatory cells. A slightly higher accumulation of PAS + glycogen within hepatocytes was also observed in this group (Figure 2 liver (PAS) D).

By applying an anti-Iba1 immunohistochemical marker that stains liver macrophages, the so-called Kupffer cells in the control and *Satureja montana L.* extract groups we could see moderate cytoplasmic positivity of the mentioned cells

located in sinusoidal spaces (Figure 2 liver (anti-Iba1) A and C). On the other hand, in the paracetamol group, a pronounced periportal and port mixed inflammatory infiltrate of lymphocytes, plasma cells, but also clearly visible Iba1 positive Kupffer cells (Figure 2 liver (anti-Iba1) B) was observed. In the group of paracetamol with pretreatment with *Satureja montana L.* extract, an increased number of Kupffer cells was observed in relation to the control group of individuals, but in a significantly smaller number in relation to the group where paracetamol (Figure 2 liver (anti-Iba1) D) was used.

The metabolic activity of cytochrome P450 identified by the CYP2E1 antibody was, as expected, scarcest in the control group of individuals (Figure 2 liver (anti-CYP450-2E1) A) and somewhat more pronounced in the group that received *Satureja montana L.* extract (Figure 2 liver (anti-CYP450-2E1) C). The use of paracetamol led to the diffuse activity of this enzyme in the form of cytoplasmic positivity in immunohistochemical staining (Figure 2 liver (anti-CYP450-2E1) B), while on the other hand in the group *Satureja*

Table II. Effects of *Satureja montana L.* extract on lipid peroxidation (LPx) and enzymes catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) in liver homogenates expressed as mean values \pm SEM.

	Saline solution (C)	Saline solution + paracetamol (ConP)	<i>Satureja montana</i> extract (S)	<i>Satureja montana</i> extract + paracetamol (S+P)
LPx (nmolMDA/mg proteins)	0.24 \pm 0.020	0.45 \pm 0.060 *	0.27 \pm 0.020	0.27 \pm 0.020 ^{x,y}
CAT (nmol/mg proteins/min)	152.11 \pm 30.71	97.65 \pm 41.26	133.50 \pm 67.49	125.45 \pm 44.21
GR (nmol/mg proteins)	0.16 \pm 0.019	0.10 \pm 0.013 *	0.13 \pm 0.013 ^y	0.10 \pm 0.033
SOD (U/mL)	70.68 \pm 3.56	64.85 \pm 4.76	68.91 \pm 3.03	66.68 \pm 5.10

LPx: lipid peroxidation; CAT: catalase; GR: glutathione reductase, SOD; superoxide dismutase. * p <0.05 compared with C group, ^x p <0.05 compared with S group, ^y p <0.05 compared with ConP group.

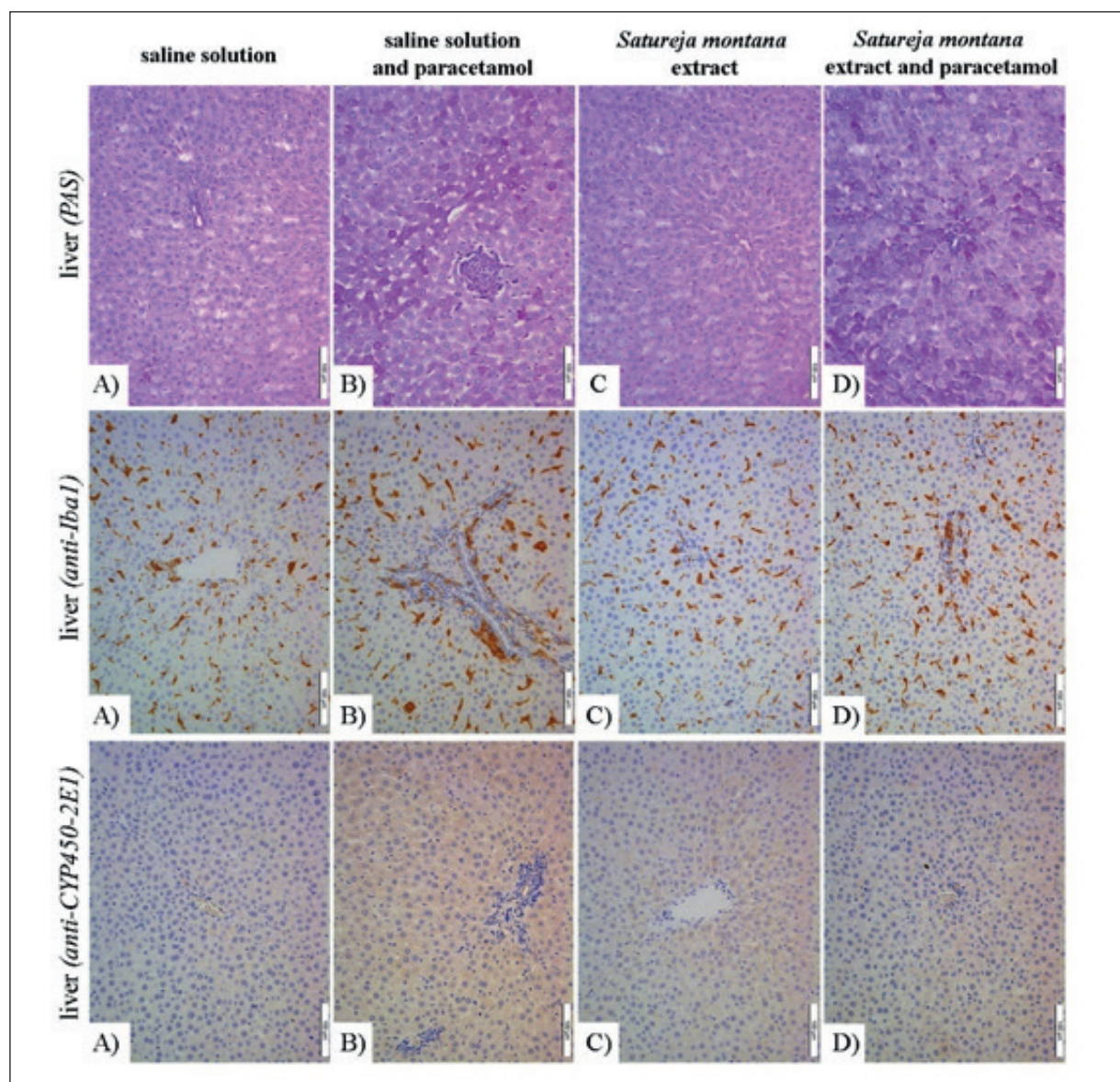


Figure 2. Liver tissue of animals treated with saline solution, saline solution+paracetamol, *Satureja montana L.* extract and *Satureja montana L.* extract and paracetamol staining PAS, rabbit anti-Iba1 and rabbit anti-cytochrome P4502E1 protein.

montana L. extract + paracetamol this positivity was predominantly visible in predominantly pericentral hepatocytes (Figure 2 liver (anti-CYP450-2E1) D).

***In Vitro* Antioxidant Activity of *Satureja Montana L.* Extract**

The obtained results indicate that examined extracts have significant and considerable antioxidative potential and antiradical effects (Table III).

The antioxidant activity of winter savory extract was evaluated by the DPPH free radical scavenging test. The results for examined extract

were in range 53.6-80.7%. The lowest antioxidant activity was obtained in extract prepared with water, while the highest activity was recorded in extract prepared with 50% ethanol.

Results of FRAP, nitric oxide (NO) scavenging activity and hydroxyl radical (OH \cdot) scavenging activity indicated that the highest activity is expressed in extract prepared with 50% methanol.

Total Phenolic Content and Flavonoid Content

Antioxidant activity of winter savory was reported to be derived mostly from the presence of

Table III. Antioxidant capacity tests in different solvent extracts.

	Water	Me 50%	Me 90%	Et 50%	Et 90%
DPPH (%)	53.64	73.95*	73.54	80.66*	70.71
FRAP (FRAP units)	40.77	43.12 ^{z,q}	29.59	39.3	27.75
Nitric oxide (NO) scavenging activity (%)	58.99 ^{y,q}	60.37 ^{y,q}	54.81 ^{y,q}	37.66	44.33 ^y
Hydroxyl radical (OH·) scavenging activity (%)	52.73 ^{y,q}	90.4 ^{x,y,z,q}	55.61 ^{y,q}	7.73	7.25

DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing/antioxidant power assay; Me 50%: methanol 50%; Me 90%: methanol 90%; Et 50%: ethanol 50%; Et 90%: ethanol 90%. * $p < 0.05$ compared to the water, ^y $p < 0.05$ compared to the Et 50%, ^q $p < 0.05$ compared to the Et 90%, ^z $p < 0.05$ compared to the Me 90%.

phenolic compounds. We measured total phenolic content (TPC) in order to determine the extent of contribution of these chemical compounds to the free radical scavenging capacity of *Satureja montana L.* extract. The results obtained by the Folin-Ciocalteu method showed that the quantity of TPC in 90% methanol extract was significantly higher than in other investigated extracts. The quantity of TPC in 50% ethanol extract was significantly higher than in water and 90% ethanol extracts (Table IV).

The flavonoid content (FLV) of investigated extract ranged from 122.85 to 224.02 mg QE/g of a dry extract. The quantity of FLV in 50% methanol extract was significantly higher than in water extract, there is no statistically significant difference in comparison to other extraction mediums (Table IV).

Principal Component Analysis

Obtained eigenvalues pointed to the importance of the first three principal components in the clustering of data (Figure 3). Since their values exhibited 1.0, we have focused on the interpretation of the multivariate dataset through the interaction among all three components.

The first and second principal components (PC) accounted for 70.2% of the overall variation in a dataset (Figure 3 B). All tested groups were separated across the biplot, either by the first or second dimension. Significant vectors for the separation of groups across the first dimension were radical scavenging capacity assays like DPPH, NO, and OH. Since some of the vectors clearly have not contributed significantly to the group separation, we have decided to plot the third and fourth PC (Figure 3 D). Thus, it can be clearly seen that flavonoids' major contribution is attributed to the second PC, which separates the solvents like ethanol and water from both methanolic concentrations (Figure 3 C). When it comes to the third PC, the FRAP value is the most dominant vector

in a separation of groups across the biplot, together with total phenols content (Figure 3 D).

Discussion

Chemical Composition

Examined *Satureja montana L.* extracts were rich source of polyphenolic compounds: rosmarinic acid ($52.1 \pm 1.04 \mu\text{g}/\text{mg}$) was the most abundant and it was accompanied by lower amounts of chlorogenic acid, p-cumarinic acid and caffeic acid. Other important compounds were flavonoid compound hyperoside and rutin.

Previously analyzed extracts of *Satureja* species were also rich in phenolic constituents^{12,24-28}. Rosmarinic acid was among the major compounds, present in large quantities^{26,29}. Phytochemical research of ethyl acetate and n-butanol extract of *Satureja montana L.*, collected in the region of Zlatibor, Serbia, showed the presence of the following phenolic compounds: hydroxybenzoic acid derivatives [protocatechin, syringic and vanillin), hydroxycinnamic acid derivatives (caffeine, p-coumarin and ferulic acids) and flavanol (+) catechin and 2-epicatechin]¹³.

Kremer et al²⁸ investigated the composition of *Satureja montana L.* and *Satureja subspicata* growing in Croatia and found that rosmarinic acid was the most abundant compound in both species and its content (w/w in dry mater) ranged from 1.11% to 3.31% respectively. Also, rosmarinic acid was identified among four major compounds in *Satureja montana L.* growing in Portugal with a content of 21.19%²⁵. Ethanol extract of aerial parts of *Satureja Kitabelii* growing in Serbia was rich in total phenolics and flavonoid contents, while rosmarinic acid was the dominant compound (18.30-29.52 mg/g)²⁶.

EX Vivo

Investigation of antioxidant activity of *Satureja montana L.* extract was performed on rats,

Table IV. Antioxidant capacity tests in different solvent extracts.

	Water	Me 50%	Me 90%	Et 50%	Et 90%
TPC (mg GAE/g SE)	1312.23	1418.4	1664.78 ^{*x,y,z}	1485.57 [*]	1403.03
FLV (mg QE/g SE)	122.85	224.02 [*]	181.07	205.38	167.15

TPC: total phenolic content; FLV: flavonoid content; Me 50%: methanol 50%; Me 90%: methanol 90%; Et 50%: ethanol 50%; Et 90%: ethanol 90%. ^{*}*p* <0.05 compared to the water, ^y*p*<0.05 compared to the Me 50%, ^z*p*<0.05 compared to the Et 50%, ^x*p*<0.05 compared to the Et 90%.

using paracetamol as a hepatotoxic agent. Toxic doses of paracetamol produce high concentrations of N-acetyl-p-benzoquinone imine, resulting in a significant reduction of reduced glutathione in the liver, a major endogenous peptide significant for the prevention of tissue damage by reactive, prooxidative compounds. In such conditions, severe forms of centrilobular liver necrosis occur, primarily due to irreversible, covalent binding of prooxidant N-acetyl-p-benzoquinone imine to the protein structure of hepatocyte³⁰. Results that we obtained are not in correlation with previous research on where pretreatment with methanol extract of *Satureja macrostema*, Lamiaceae (200, 400 and 600 mg/kg), before toxic dose of CCl₄ reduced the elevated levels of AST, ALT, ALP and increased the level of HDL³¹. Also, in one study pretreatment with *Satureja montana L.* extract prior to a toxic dose of CCL₄ exhibited significantly lower levels of AST, ALT and bilirubin³².

In our study pretreatment with *Satureja montana L.* extract showed a decrease in the concentration of ALP in comparison to the paracetamol group, however ALT, AST and GGT were not altered and were statistically increased in comparison to the control group (saline solution), indicating cell leakage. Though cytoarchitectonic in histological samples were intact in all groups, elevated accumulation of PAS and glycogen was observed in groups given toxic dose of paracetamol, indicating damage to the cell membrane. This result correlated with previously elevated level of liver enzymes which were due to damage of hepatocyte cell membranes. In addition, the level of LPx was increased and GR decreased in the paracetamol group indicating the reduced antioxidant capacity of liver tissue, but pretreatment with *Satureja montana* prevented this effect of a toxic dose of paracetamol returning level of antioxidative enzymes on the level as in a group treated only with saline solution. Since *Satureja montana L.* extract significantly reduces the intensity of lipid peroxidation and potentiates the activity of enzymes of antioxidant protection. We can conclude

that the use of these extract protects animals from reactive oxygen species and reduces the effects of exposure to oxidative stress.

Influence on oxidative damage of lipids, i.e., lipid peroxidation intensity was evaluated indirectly through the concentration of MDA, which is a secondary product in this process. MDA is the major reactive aldehyde that occurs as a result of the peroxidation of biological membranes containing polyunsaturated fatty acids^{33,34}. Catalase, as one of the most important enzymes in the protection of the organism from oxidative stress, is predominantly found in erythrocytes and in the liver. The CAT function is the protection of cellular organelles, especially mitochondria, which are the most endangered organelles due to cellular respiration^{34,35}. In our results concentration of catalase in the group of animals treated with *Satureja montana L.* extract prior to the toxic dose of paracetamol is higher compared with the group that was treated with a saline prior to the toxic dose of paracetamol, but there was no statistical significance between groups. This result can be explained by a short duration of sight for a period of 7 days.

Superoxide dismutase has also shown its antioxidant activity, because the significantly higher activity of this enzyme was demonstrated by a group treated with a saline prior to the toxic dose of paracetamol compared to the group that received a *Satureja montana L.* extract prior to the toxic dose of paracetamol. The mechanism of these effects is explained by the fact that active principles from *Satureja montana L.* extract prevent the development of all the harmful effects of oxidative stress, in different ways and in different tissues. Because extract of *Satureja montana L.* is rich in phenolic compounds and flavonoids, which are highly efficient free radical catchers, we can attribute general antioxidant activity³⁶. However, this fact does not fully explain the results of our research since we did not get statistically significant results regarding the activity of catalase.

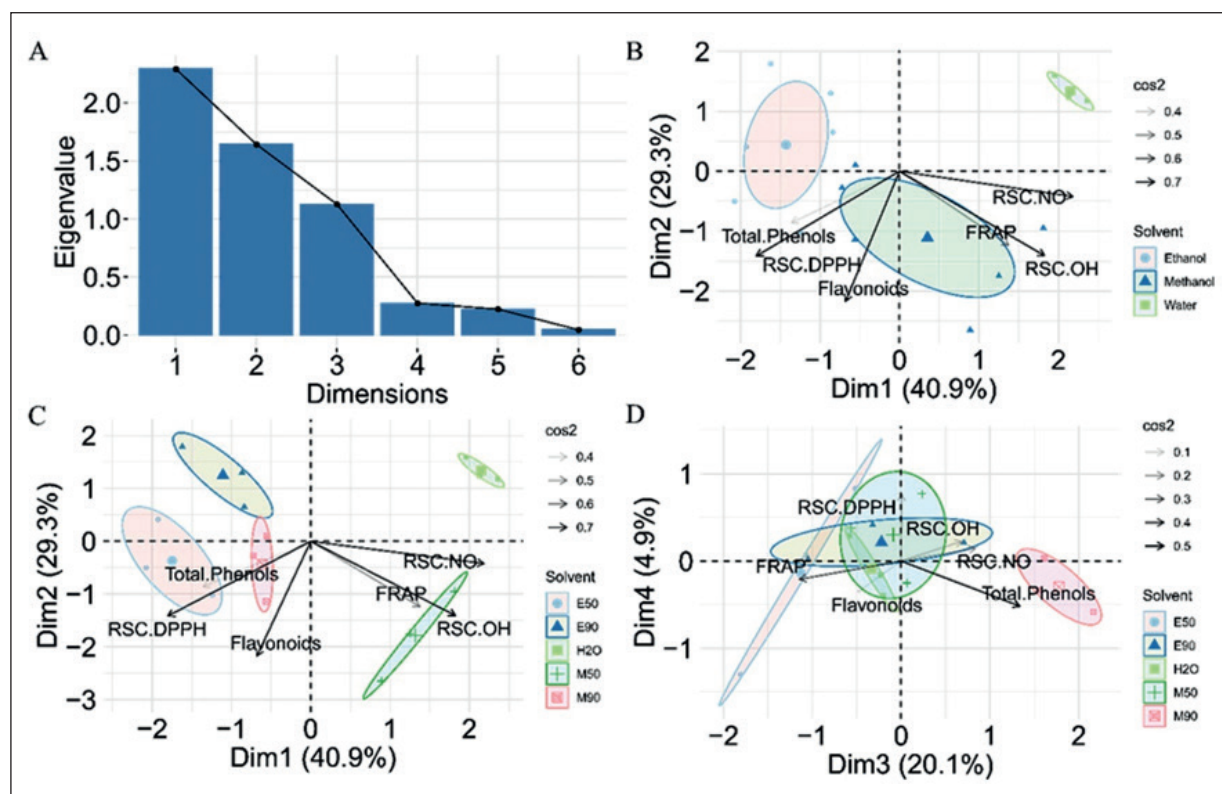


Figure 3. Multivariate analysis of selected solvent types. **A**, Eigenvalues correspond to the amount of variance retained by each component. **B**, PCA plot based on the type of solvent used for the preparation of extracts. **C-D**, PCA plots of different solvent concentrations were used for extraction. PCA was done on the correlation matrix. Group centroids are presented with larger points; ellipse denotes the confidence interval around the group centroid. Dim 1, 2, 3, and 4 are retained principal components. \cos^2 shows variables' contribution in the determination of a given principal component.

Direct bilirubin in the serum was statistically elevated in groups treated with a toxic dose of paracetamol compared to the control group, however the level of direct bilirubin was statistically lower in the group pretreated with *Satureja montana L.* extract. This finding adds to the antioxidative potential of the extract. Serum concentration of urea, creatinine and uric acid as renal function biomarkers were also examined in our study. These biochemical parameters were increased in the paracetamol-treated group, but the repeated use of *Satureja montana L.* extract prevented their concentration from rising.

This is in correlation with a high level of rosmarinic acid that was measured in our extracts which have been showing hepatoprotective effects against carbon tetrachloride-induced liver damage in rats³⁷.

In Vitro Antioxidant Activity

DPPH values which were in a range of 53.64-80.66% show that our extracts exhibit consider-

able antioxidative potential. The highest level of DPPH was detected in 50% ethanol extracts. The study conducted by Četojević-Simin et al³⁸ showed that n-butanol, methanol and water extract of *Satureja montana L.* exerted an excellent antioxidative effect on DPPH radical (at the concentrations 0.20 and 0.30 mg/mL, antioxidative activity = 100%). Also, other studies confirmed the antioxidative effect of *Satureja montana L.* ethanol extract with DPPH activity 108.79 $\mu\text{g/mL}$ and *Satureja Kitaibelii* 71.20-125.65 $\mu\text{g/mL}$ ^{12,26}.

FRAP of the investigated extract was in the range 27.75-43.12 FRAP units, the highest value was detected in 50% methanol extracts. FRAP values of *Satureja montana L.* obtained by Serrano et al¹², were 221.74 and 271.88 $\mu\text{mol Fe/g}$ for cold and hot aqueous extracts respectively and for an ethanol extract, 93.60 $\mu\text{mol Fe/g}$. In a study conducted by Gopčević et al²⁶ for *Satureja Kitaibelii* FRAP values for ethanol extract were in the range 0.74-1.94 $\mu\text{mol Fe/mg}$, with the highest activity for extract obtained by ultrasound extraction.

Hydroxyl radical (OH \cdot) scavenging activity (%) of the investigated extract was in the range 7.25-90.4%, and the highest activity was shown in an extract prepared with methanol 50%. Our results could be compared with the results obtained by Ćetković et al³⁹ for *Satureja montana* L. *subsp. Kitaibelii* extract with reported values from 42.67 to 100%, they have shown that the abilities of examined samples to scavenge hydroxyl radicals decreased in the following order: n-butanol>ethyl acetate> water>chloroform.

Since our results are the first related to the nitric oxide (NO) scavenging activity (%) in this plant, there is no available literature data for comparison.

The TPC in the investigated extracts ranged between 1312.1378 mg GAE/g and 1664.78 mg GAE/g, whereas FLV in the extracts varied from 122.85 to 224.02 mg QE/g. Our measured TPC values were higher than obtained by López-Cobo et al¹⁴ for *Satureja montana subsp. Kitaibelii*, (25.82 mg GAE/g), Hajdari et al²⁷ for methanol extracts of *Satureja montana* L. (68.1–102.6 mg GAE/g), Ćavar Zeljković et al⁴⁰ for methanol extracts of *Satureja montana* L. (614.7 mg GAE/g) and Serrano et al¹² for *Satureja montana* ethanolic extract (111.18 mg GAE/g), and higher than reported by Hudz et al⁶ that stated the concentration of TPC for *Satureja montana* tincture ranged from 13.0-22.7 mg GAE/g, and 26.16-45.63 mg rutin/g.

Ćetković et al³⁹ reported total content of phenolic compounds in ethyl acetate (969.43 μ g CAE/g) and n-butanol (1358.14 μ g CAE/g) extracts of *Satureja montana subsp. Kitaibelii* were significantly higher than in water extract (96.36 μ g/g) for the extraction of phenolics from the methanol extract, also Gopcevic et al²⁶ reported TPC values in the range 105.06-195.95 GAE/g for *Satureja Kitaibelii*. The flavonoid contents of our investigated extracts were higher than the reported contents of *Satureja montana* L. methanolic extract (38.3-67.0 mg CA/g) and ethanolic extract of *Satureja Kitaibelii* 29.18-44.0 mg Qu/g^{26,27}.

Multivariate analysis showed a distinct property of tested solvents. Evidently, the solvent concentration showed that different metabolites and the scavenging capacity are also modified depending on whether 50 or 90% of alcohol was used for extraction. Overall, water extracts showed the lowest antioxidant capacity. Base on the type of solvent in PCA plot analysis liquid selection used for extraction medium have great impact on antioxidative potential.

Interestingly, 90% of methanolic extracts show some similarity with 50% ethanolic extracts, and they tend to cluster close by as seen in Figure 2 C. When it comes to the 50% methanolic extracts, they have completely different properties in comparison with others. This concentration is characterized by high radical scavenging capacity, particularly NO- and OH- radicals, and total phenolic content. This might mean that the threshold for effective extraction of antioxidative compounds might be lower than it is typically thought. To further confirm this claim additional experiments might be necessary, with an aim to pinpoint the concentration, and solvent as the most effective. Better antioxidative effect relates to better solubility of rosmarinic acid in methanol and ethanol than in water⁴¹. Methanolic extracts of *Satureja hortensis* L. have been shown to have pharmacologically important phenolic compounds, especially rosmarinic acid same as *Satureja montana* in our study, and thus it is valuable for pharmaceutical and functional food formulations in order to maintain normal health conditions or as a remedy in various diseases caused by oxidative damage⁴².

Histology Discussion

The use of *Satureja montana* L. extract alone does not interfere with the activity of glycogen or the number of tissue macrophages, i.e., Kupffer cells. On the other hand, *Satureja montana* L. extract itself causes CYP2E1 activation to a lesser extent. With the use of paracetamol and *Satureja montana* L. extract, hepatocytes are with significantly preserved cytoarchitectonics and with a somewhat pronounced accumulation of glycogen, which is also found in the paracetamol control group. The use of *Satureja montana* L. extract indicates an anti-inflammatory effect with a reduction in the number of Kupffer cells by observing the paracetamol group. Also compared to the paracetamol group, it caused a smaller inductive effect in terms of CYP2E1. These results suggest that *Satureja montana* L. extract has an anti-inflammatory effect and since it decreases activation of CYP2E1 it prevents induction of the state of oxidative stress.

Conclusions

Satureja montana L. extract in our research has shown potential of hepatoprotective, an-

ti-inflammatory and antioxidative effect. The level of alkaline phosphatase (ALP) was decreased and other liver enzymes (ALT, AST, GGT) were unaltered by pre-treatment of *Satureja montana L.* extract. Lipid peroxidase levels were also statistically significantly reduced, and increased levels of CAT, GR and SOD (statistically insignificant) showed *in vivo* antioxidant activity. In addition, the number of inflammatory cells is decreased coupled with activity of CYP2E1 enzymes thus pre-treatment *Satureja montana L.* extract has a hepatoprotective effect. It is important to note that our findings using principal component analysis indicated that solvent selection has a great impact on the antioxidative effect of dry extract of Winter savory. Current results indicate that dry extract of *Satureja montana L.* has potential for antioxidant supplementation with diminishing effect on oxidative damage on the liver in an animal model, suggesting a potential use in antioxidant-rich natural products. Further investigation should be conducted taking into consideration the selection of formulation medium or novel carrier for the *Satureja montana L.* dry extract (like solid lipid nanoparticle).

Institutional Review Board Statement

Animal care and all experimental procedures were performed in accordance with the ethical principles outlined by the EU Directive 2010/63/EU on animal welfare and under the Law of Animal Welfare of the Republic of Serbia (OG RS 41/09). This study was approved by the Ethical Committee of the University of Novi Sad (Approval No. 01-78/29).

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Conflicts of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

The datasets related to this study are available from the corresponding author on reasonable inquiry.

Authors' Contributions

B.M. and M.S. designed the research and drafted the article. J.Ž. prepared extracts, I.Č. was responsible for histological processing while I.B. did principal component analysis. J.Č.P., B.T. and M.V. analyzed and interpreted the data. A.R., M.M. and M.L.P. elaborated the manuscript. They are also responsible for the intellectual content of the work. All authors contributed to the revision of the manuscript, read and approved the submitted version.

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