

NATURAL DRUGS

BIOLOGICAL ACTIVITIES OF *SANGUISORBA MINOR* L. EXTRACTS – *IN VITRO* AND *IN VIVO* EVALUATIONS

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Abstract: The literature data about *Sanguisorba minor* L. subsp. *muricata* Briq. (fam. Rosaceae) are limited. It has been used in folk medicine for its diuretic and digestive properties, or in the treatment of fever and diarrhea. The aim of the study was to evaluate the antioxidant, antimicrobial and anti-inflammatory potential of *S. minor* subsp. *muricata* root extracts (*in vitro* and *in vivo*). Methanol, ethanol and chloroform extracts were characterized by total phenolic and flavonoid content. Antioxidant activity was estimated by five different assays *in vitro*, and *in vivo* in the animal model of sepsis. Antimicrobial activity was tested against Gram-positive bacteria, Gram-negative bacteria and one fungus by micro-well dilution assay. Cyclooxygenase-1 inhibition assessment of *S. minor* ethanol extract was performed in the presence of Indomethacin. Ethanol and methanol extracts did not differ significantly in the content of phenols and flavonoids, as well as in achieved antioxidant activity. Both of these extracts were superior to the chloroform extract. Ethanol and methanol extracts showed similar antimicrobial activity. Chloroform extract exhibited strong activity against all examined strains of bacteria, but moderate against *Candida albicans*. Ethanol extract induced 76% inhibition of Cyclooxygenase-1 *in vitro*. The same extract decreased the level of the oxidative stress parameters in an animal model of sepsis. All extracts exhibited considerable antimicrobial and antioxidant activities, while ethanol extract showed anti-inflammatory potential. Based on the effects achieved on oxidative stress parameters in sepsis, *S. minor* could be considered as a herbal adjuvant antioxidant therapy in systemic infectious treatment.

Keywords: *Sanguisorba minor* subsp. *muricata*, oxidative stress, antioxidant activity, antimicrobial activity, anti-inflammatory activity

Different species belonging to the genus *Sanguisorba* L., Rosaceae, have natural habitats in Europe and Asia. *Sanguisorba minor* L. subsp. *muricata* Briq. has been found as native in Serbia (1, 2). The research previously done was focused mainly on *Sanguisorba officinalis* L. species, while the information on *S. minor* (small burnet) species is scarce. There is a particular lack of data on *S. minor muricata* subspecies. The intention of this study was to characterize *S. minor* L. subsp. *muricata* Briq. more thoroughly. Studies on different

parts of species *S. minor* confirmed the high content of polyphenols, β -carotene, vitamin E, and vitamin C, which implied the potential for antioxidant activity (3). Several unique phenolic carboxylic acids, triterpenoids, tannins, and new flavonoids were isolated and identified from the aerial parts and root of *S. minor* (4-6). Although the complete information on chemical composition has been limited, the presence of phytoconstituents found in *S. minor* points to the possible anti-inflammatory activity of extracts from different

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parts of the plant – aqueous extract of *S. minor* subsp. *muricata* flowering aerial parts showed dose-dependent anti-inflammatory activity (7, 8). Species *S. minor* has been used in folk medicine, in the form of infusion or tincture, for its diuretic, digestive and appetite-stimulant properties, or as a fever and diarrhea treatment. The aerial parts of this species showed an anti-ulcerogenic effect, which is in accordance with data available for plants from *Sanguisorba* genus – they expressed *in vitro* and *in vivo* hemostatic, antibacterial, neuro-protective, or hypoglycemic effects (9, 10). Besides medicinal activities, young shoots, aerial parts and roots of *S. minor* are edible and usually consumed as mixed vegetable salads (11).

Sepsis is a systemic inflammatory response to severe body infections and may lead to overwhelming shock. If not treated promptly, it may produce finally death, causing multiple organ failure. Mechanisms of inflammation and cellular damage, such as the generation of cytokines and reactive oxygen species, have been involved in the pathogenesis of sepsis. Hypotension and inappropriate oxygen delivery to the tissue may be crucial in the developing pathogenesis of sepsis (12).

Considering the pathophysiological pathways in sepsis, the combination of conventional therapy and antioxidant therapy may decrease the severity of the disease or improve disease outcomes (13, 14). In spite of recent advances in therapy, sepsis remains the primary cause of mortality in hospital conditions (15). Therefore, antioxidant and anti-inflammatory responses of herbal extracts have been elucidated on different animal sepsis models (16, 17, 18).

The aim of this paper was to evaluate the biological activities of *S. minor* subsp. *muricata* root extracts. Antioxidant, antimicrobial and anti-inflammatory effects were studied *in vitro*. Oxidative stress parameters in sepsis were studied after the application of *S. minor* extract on the animal sepsis model.

EXPERIMENTAL

Plant material

The fresh plant was collected from fields of village Kamenica in the Sumadija region of Central Serbia, on sunny days during springtime. The plant material was identified at the Institute for Biology and Ecology at the Faculty of Science, University of Kragujevac, Serbia, by standard botanical keys for plant determination (1, 2). The roots were cleaned from soil and dried at room temperature, in draft and dark place. After drying, the roots of *S. minor* were

ground to powder and extract with reflux of solvent (96% ethanol, methanol and chloroform) at boiling temperature for four hours. The dry extracts were obtained by the rotary vacuum evaporator and stored in the desiccator until the experiment.

Total phenolic and flavonoid content

The total phenolic content of extracts (TPC) was determined by the Folin-Ciocalteu method. The absorbance of the reaction mixture was measured at 750 nm after storing in a dark place for 30 min. All the measurements were done in triplicate. Gallic acid was used as a referent substance and the results were expressed as μg of gallic acid equivalents (GAE) per mg of dry weight (μg GAE per mg dw) (19, 20).

The total flavonoid content of extracts (TFC) was determined by the aluminum chloride colorimetric method. The absorbance of the reaction mixture was measured spectrophotometrically at 510 nm. All the measurements were done in triplicate. The reference substance was rutin and the results were expressed as μg of rutin equivalent per mg dry weight. (21).

Antioxidant activity assays

ABTS radical scavenging activity

Free radical scavenging activity of *S. minor* extracts was determined by 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay. The absorbance of the mixture of each extract and diluted ABTS solution was measured at 734 nm after 6 minutes at room temperature. All the measurements were carried out three times. Trolox was used as a referent substance and the results were expressed as μg of Trolox equivalents (TE) per mg dry extract weight (μg TE per mg dw) (20).

DPPH free radical scavenging assay

Total free radical scavenging capacity of the investigated samples was estimated using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, according to Dimitrijevic et al. The mixture of methanolic DPPH solution and tested extract in a total volume of 4 mL was prepared. After shaking and storing in the dark place for 60 min, the absorbance was measured at 515 nm spectrophotometrically. All the measurements were carried out three times. A calibration curve was plotted using percent of DPPH scavenged versus concentration of Trolox as a positive control. The results were expressed as μg of Trolox equivalents (TE) per mg dry extract weight (μg TE per mg dw) (20).

Cupric reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was performed according to Dimitrijevic et al. The absorbance of the reaction mixture was measured at 450 nm spectrophotometrically. The results were expressed as μg of Trolox equivalents per mg of dry weight ($\mu\text{g TE per mg dw}$) (20).

Ferric-reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay of investigated extracts was carried out according to Dimitrijevic et al. The absorbance of the mixture of FRAP reagent and the sample was measured at 595 nm after 5 minutes incubation at 37°C. All the measurements were done in triplicate. The results were obtained using a standard calibration curve and expressed as a $\mu\text{g Fe/mg dw}$ of Fe(II) equivalents per mg of dry weight ($\mu\text{g Fe per mg dw}$) (20).

Total reducing power (TRP) assay

The reducing power of analyzed extracts was determined by the method which was based on the reduction of Fe(III) hexacyanate to Fe(II) hexacyanate. The absorbance of the reaction mixture was measured at 700 nm, spectrophotometrically. All the measurements were done in triplicate. The results were expressed as μg of ascorbic acid equivalents per mg of dry extract weight ($\mu\text{g AAE per mg dw}$) (20).

Antimicrobial activity assay

The *in vitro* antimicrobial activity of the *S. minor* radix extracts was determined using a micro-well dilution assay (CLSI 2009, with some modifications). The initial concentration of the extracts was 100 mg/mL. Antibacterial activity of the extracts was tested against three Gram-positive bacteria (*Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*) and six Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Salmonella enteritidis*). Antifungal activity of the extracts was tested against *Candida albicans*. Bacterial suspensions were prepared from an overnight culture in sterile saline (0.9% NaCl) and their optical density was standardized to 0.5 McFarland. The serial dilutions of extracts were prepared and tested in the range of 0.04 to 100.0 mg/mL in a 96/well microtiter plate with inoculated Mueller–Hinton broth. The final volume was 100 μL and the final bacterial suspension was 10^6 CFU/mL per each well. The procedure was repeated for fungal suspension with appropriate

Sabouraud Dextrose agar. Serial dilutions of DMSO (100%) in the concentration range of 0.02 to 50.0 mg/mL served as a negative control. Doxycycline and nystatin in the concentration range of 0.01–20.0 $\mu\text{g/mL}$ were used as a positive control. Bacterial and fungal growth was detected after adding 20 μL 0.5% triphenyl tetrazolium chloride solution. The results were presented as MIC/MBC in mg/mL. Minimum inhibitory concentration (MIC – concentration of samples without visible growth) and minimal bactericidal/fungicidal concentration (MBC/MFC – concentration of samples that killed 99.9% of bacterial/fungi cells) were done in triplicate.

In vitro anti-inflammatory testing

Cyclooxygenase-1 inhibition assessment

Prior to the *in vivo* inflammation model, an *in vitro* testing was done. Cyclooxygenase-1 (COX-1) inhibition assessment of *S. minor* ethanol extract was performed. The COX-1 assay was conducted in a microtiter scale with purified COX-1 from ram seminal vesicles, according to the previously used method (22). The tested concentration of the extract was 50 $\mu\text{g/ml}$ and the result is presented as the percentage of inhibition. Indomethacin as the COX-1 inhibitor was used as a positive control. The result is a mean value of measurements that were done in triplicate.

In vivo model of sepsis

Animals

The study was approved by the Ethics Committee for Animal Welfare of the Military Medical Academy and the Ministry of Agriculture and Environmental Protection of Republic of Serbia (No. 323-07-7363/2014-05) that respects the rules of the European Parliament's Directive on the protection of animals used for scientific purposes 2010/63/EU. The experiment was conducted on adult, eleven-weeks old, male Wistar rats, weighing between 250 and 300 g. The rats were housed inside climate-controlled conditions with ad libitum access to food and water prior to the experiment. The animals were randomly divided into sepsis and control groups. Within both groups, there were six subgroups, with six animals in each subgroup.

Animal model of sepsis

Sepsis was induced by cecal ligation and puncture (CLP model). Before the procedure, the animals were anesthetized intraperitoneally with sodium-pentobarbital (45 mg/kg body weight – b.w.). The operative procedure included several phases: the middle incision at the venter, cecum ligation one

centimeter from the distal end, puncture of the ligated cecum, and closing of the abdominal wall. The Control group of animals were sham-operated without CLP. All animals had free access to food and water after the operation procedure (23).

Experimental procedure

The effect of ethanol extract of *S. minor* radix was evaluated on a rat model with induced sepsis. The tested extract was administered both orally and intraperitoneally (i.p.). Two different concentrations (100 mg/kg and 300 mg/kg) were used orally, while a single concentration (100 mg/kg) was used i.p. The plasma levels of total sulfhydryl groups (total-SH), thiobarbituric acid reactive species (TBARS), nitrate and nitrite (NOx), superoxide anion radical (O₂⁻) and superoxide dismutase (SOD) were measured. The effect of extract given intraperitoneally in a concentration of 300 mg/kg could not be evaluated, because the deaths were recorded in this animal group.

Animals were randomly assigned to the sepsis (CLP) group or to the sham-operated (control) group. Two hours before surgery, animals in the sham group received 0.5 mL H₂O. The doses of extract for the oral application were prepared as a suspension of extract in the mixture of solvents (water/ethanol 50 : 50). Animals were treated by once-daily oral applications of *S. minor* root extract at a dose of 100 mg/kg (S1 group) or 300 mg/kg (S2) during one week. Animals that have been induced on sepsis (S1+CLP and S2+CLP) and sham-operated animals (S1+sham) received orally the same extract one week before the surgery. The experiment included also i.p. application of the extract at a dose of 100 mg/kg, which was prepared as a suspension of extract in the mixture of solvents (water/ethanol 65 : 35). The experiment was performed along with 6 adequate control groups of animals – sham-operated animals, animals that were treated only by the mixture of solvents (water/ethanol 50 : 50), animals that were treated

only by the mixture of solvents (water/ethanol 65 : 35), animals without treatment by the extract and without induced sepsis, animals without treatment by extract and with induced sepsis, and the animals treated by the extract, but without induced sepsis. Rats were killed 24 h after induced sepsis, sham operation or application of the last dose of the extract.

Determination of oxidative stress parameters

The level of total sulfhydryl groups (total SH) was measured spectrophotometrically at 412 nm, according to the method described by Sedlak and Lindsay. The principle of the method is based on the reduction of DTNB reagent by thiol groups to yellow color mercaptobenzoic acid (24). The results were expressed in mM/L.

The level of thiobarbituric acid reactive species (TBARS) was measured spectrophotometrically at 532 nm according to the method described by Girotti (25). The results were expressed in uM/L.

Nitrate and nitrite concentration (NOx) was determined by a spectrophotometric assay based on the Griess reagent. The absorbance was measured at 492 nm. The results were expressed as nmol/ml (26).

Superoxide anion was detected with nitroblue tetrazolium (NBT). The method was based on the reduction of NBT by the investigated sample and the extinction change was monitored for five minutes at 515 nm. The results were expressed as nM NBT/min/mL (27).

Superoxide dismutase activity was determined as a percentage of adrenaline auto-oxidation in an alkaline environment. The auto-oxidation was monitored spectrophotometrically at 480 nm for ten minutes. The results were expressed as U/ml (28).

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, US). Descriptive data are expressed as the mean

Table 1. Antioxidant effect of *S. minor* extracts.

<i>S. minor</i> extracts	ABTS µg TE/mg	DPPH µg TE/mg	CUPRAC µg TE/mg	FRAP µg Fe/mg	TRP µg AAE/mg	TPC µg GAE/mg	TFC µg RE/mg
Ethanol	77.54a** ± 0.16	96.51 a** ± 0.07	346.49 a** ± 0.14	188.22 a** ± 0.5	1.16 ± 0.01	457.45 a** ± 4.59	936.50 ± 17.50
Methanol	76.97a** ± 10.23	97.29 a** ± 0.04	343.35 a** ± 0.13	214.02 a** ± 0.32	1.19 ± 0.02	497.47 a** ± 3.80	836.50 ± 18.92
Chloroform	46.11b** ± 1.03	40.51 b** ± 0.12	96.80 b** ± 0.14	48.02 b** ± 0.63	0.11 ± 0.02	41.45 b** ± 0.54	939.00 ± 13.92

Different letters in superscript in the same column indicate significant difference at $p < 0.01$ (**).

Table 2. Antimicrobial activity of *S. minor* extracts (MIC/MBC in mg/mL) and positive controls (MIC/MBC(MFC) in µg/mL) against pathogenic microbial strains.

Microorganisms	ATCC	<i>S. minor</i> extracts			Positive controls
		Ethanol	Methanol	Chloroform	Doxycyclin/Nystatin
Gram-negative bacteria					
<i>E. coli</i>	25922	12.50/50.0	6.25/12.50	0.20/6.25	15.61/15.61
<i>P. aeruginosa</i>	9027	12.5/25.0	6.25/25.0	0.39/1.56	15.61/15.61
<i>S. enteritidis</i>	13076	12.50/25.0	6.25/12.50	0.78/0.78	0.90/1.90
<i>P. mirabilis</i>	12453	12.5/25.0	12.5/25.0	0.39/6.25	7.81/15.61
<i>K. pneumoniae</i>	10031	12.5/25.0	12.5/25.0	0.39/6.25	15.61/15.61
<i>E. aerogenes</i>	13048	12.5/25.0	12.5/25.0	1.56/6.25	7.81/15.61
Gram-positive bacteria					
<i>B. cereus</i>	11778	12.50/50.0	6.25/12.5	0.10/0.39	0.90/15.61
<i>S. aureus</i>	25923	25.0/25.0	6.25/12.50	0.10/0.39	7.81/15.61
<i>E. faecalis</i>	19433	6.25/12.50	6.25/6.25	0.39/0.39	0.90/1.90
Fungi					
<i>C. albicans</i>	14053	12.50/25.0	12.50/50.0	12.50/25.0	7.81/15.61

value \pm standard deviation (SD). Student's t-test, one-way analysis of variance (ANOVA) and appropriate nonparametric test (Mann-Whitney) were used to identify significant differences in the data. Differences were considered statistically significant if $p < 0.05$ and highly significant if $p < 0.01$.

RESULTS

Antioxidant activity assays

Antioxidant capacity of *S. minor* extracts, evaluated by five different methods, as well as total phenolic (TPC) and flavonoid content (TFC) are presented in Table 1. These results represent the mean value \pm standard deviation of three replicate experiments (Table 1).

Methanol and ethanol extracts presented high phenol content; in contrast, the chloroform extract showed more than ten times lower phenol content. Methanol extract contained the highest level of phenols in comparison to the other two investigated extracts. The lowest level of TPC was present in chloroform extract. There was a highly significant difference between TPC from methanol and chloroform extract and between ethanol and chloroform extract. However, there was no significant difference between the flavonoid content of all the investigated extracts.

Antioxidant activity of three different *S. minor* extract was evaluated using two free radical scavenging assays, DPPH and ABTS. The DPPH and ABTS radical scavenging potential of the methanol

and ethanol extract was found to be significantly higher than chloroform extract ($p < 0.01$). The activity of methanol and ethanol extract was lower in the ABTS over the DPPH assay.

The highest CUPRAC capacity was observed for ethanol extract followed by methanol extract and the lowest activity was shown by chloroform extract. Methanol and ethanol extract of *S. minor* showed significantly higher antioxidant activity than chloroform extract, measured by the CUPRAC assay ($p < 0.01$). Considering the results of the ferric reducing antioxidant power assay, the highest antioxidant activity was observed for methanol and ethanol extract. The FRAP value for chloroform extract was nearly five times lower than the FRAP value for methanol extract ($p < 0.01$).

The results of total reducing power for ethanol and methanol extracts are similar. Among the tested samples, methanol extract showed the highest total reducing power, followed by ethanol extract, while chloroform extract showed ten times weaker reducing power than methanol and ethanol extract.

Antimicrobial activity assay

The results of *in vitro* antimicrobial activity of *S. minor* subsp. *muricata* extracts against nine bacterial and one fungus strain are presented in Table 2. The MIC and MBC values of extracts varied from 0.10 to 25.0 mg/mL and 0.39 to 50.0 mg/mL, respectively.

The chloroform extract of *S. minor* radix showed the strongest antibacterial activity among

investigated extracts, both on Gram-positive and Gram-negative bacteria (Table 2). Chloroform extract exhibited bacteriostatic activity with MIC = 0.1-1.56 mg/mL, while bactericidal effect was ranged 0.39-6.25 mg/mL. *B. cereus* and *S. aureus* showed the highest sensitivity to the chloroform extract with MIC and MBC values 0.10 mg/mL and 0.39 mg/mL, respectively.

MIC and MBC values of methanol extracts varied from 6.25 to 12.5 mg/mL and 6.25 to 25.0

mg/mL, respectively. MIC and MBC values of ethanol extracts varied from 6.25 to 50.0 mg/mL and 12.50 to 50.0 mg/mL, respectively. *S. enteritidis*, *E. coli*, *P. aeruginosa*, *B. cereus*, *S. aureus* and *E. faecalis* were more susceptible to methanol extract than ethanol extract of *S. minor*.

All the extracts revealed moderate antifungal activity against *C. albicans*, with extracts showing the MIC 12.50 mg/mL and MFC values, ranging between 25.0 and 50.0 mg/mL. Nystatin was applied as a positive control and presented MIC 7.81 µg/mL and MFC 15.61 µg/mL.

Inhibition of COX-1 isoenzyme by *S. minor* radix extract

Ethanol extract of *S. minor* radix was tested for inhibition of COX-1 isoenzyme. The inhibition of COX-1 isoenzyme by *S. minor* radix extract was evaluated in the concentration of 50 µg/mL. The tested extract showed $76 \pm 3.76\%$ of inhibition of COX-1 isoenzyme in the presence of reversible COX inhibitor, Indomethacin, as a positive control (Fig. 1). Indomethacin exhibited $32 \pm 4.76\%$ of COX-1 inhibition, which is more than two times weaker than the effect of the *S. minor* ethanol extract.

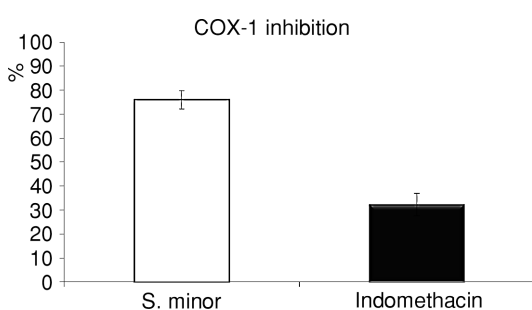


Figure 1. The percentage of inhibition of COX-1 isoenzyme caused by *S. minor* radix ethanol extract. i.p. – intraperitoneally

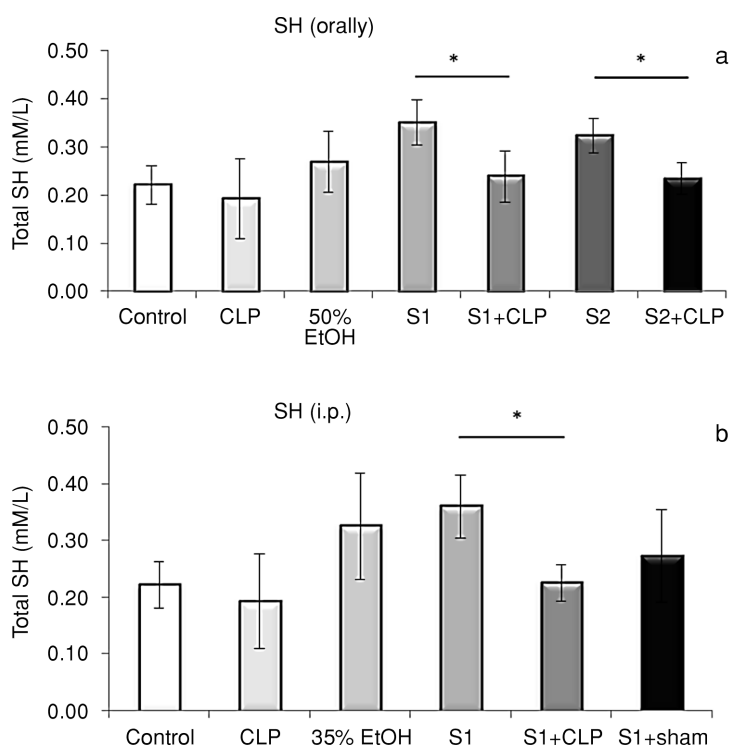


Figure 2. Total-SH levels in the groups treated with *S. minor* radix ethanol extract. Data are presented as following: a – orally administration of extract; b – intraperitoneal administration of the extract. The results are expressed as mean \pm SD (* $p < 0.05$; ** $p < 0.01$). i.p. – intraperitoneally

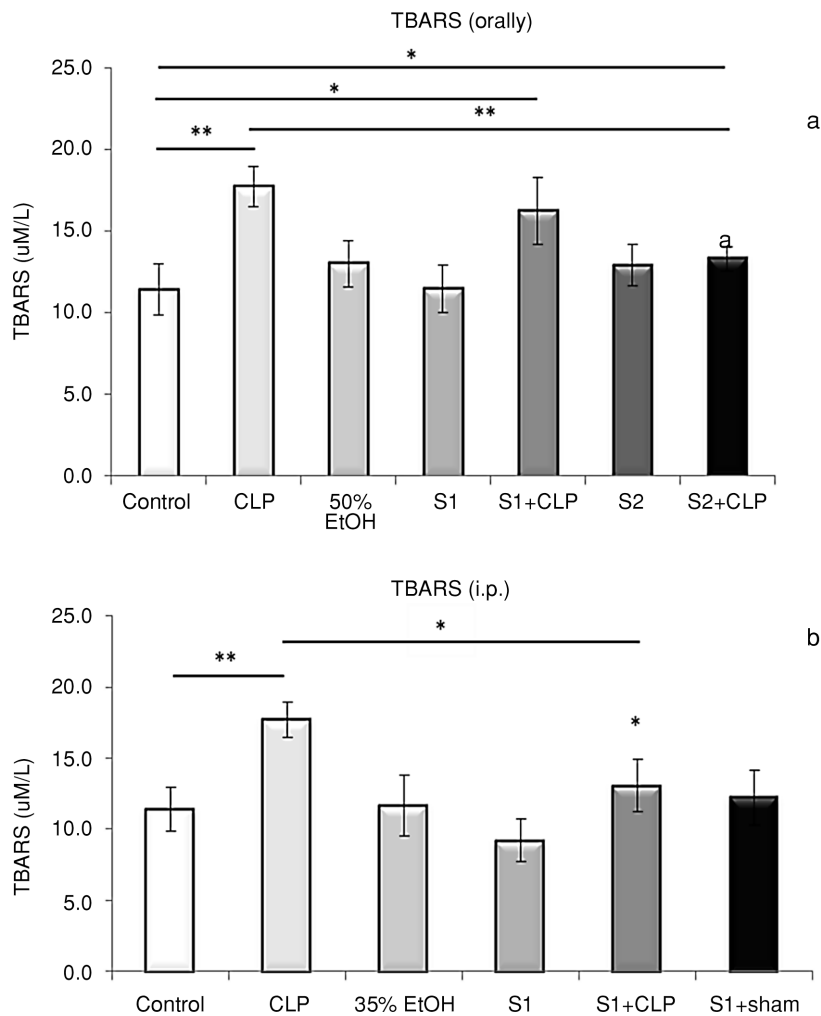


Figure 3. TBARS levels in the groups treated with *S. minor* radix ethanol extract. Data are presented as following: a – orally administration of extract; b – intraperitoneal administration of the extract. The results are expressed as mean \pm SD (* p < 0.05; ** p < 0.01). i.p. – intraperitoneally

Effects of *S. minor* radix extract on sepsis animal model

The effect of ethanol extract of *S. minor* radix on oxidative stress parameters in the animal model of sepsis was evaluated. The tested extract was administered orally in 2 different concentrations (100 mg/kg and 300 mg/kg) and i.p. in one concentration (100 mg/kg). There were no significant differences in values of total-SH between the control group of animals without any treatment and groups of animals treated orally and i.p. by *S. minor* radix extract in all tested concentrations.

The values of total SH-levels in groups treated orally by extract are presented in Figure 2a. The orally applied extract did not affect significantly the total-SH concentration in sepsis. However, there was a decrease of total-SH in the CLP group treated

by 100 mg/kg *S. minor* radix extract, in relation to the S1 group ($p = 0.007$). The same pattern was observed between analog treated and control groups – 300 mg/kg applied orally ($p = 0.034$). The difference in the concentrations of extracts used in the experiment could not significantly influence the total-SH level ($p > 0.05$). Intraperitoneal administration of *S. minor* radix extract had no significant influence on the value of total-SH in the CLP group. The total-SH level in groups treated by i.p. administration of the extract is presented in Figure 2b. The same pattern, like in the case of orally administered extracts was observed – there was a significant decrease of total-SH in the CLP group treated i.p. by the extract, in relation to the S1 group ($p = 0.027$).

The mean values of TBARS measured in the groups of animals treated orally by *S. minor* radix

extract are presented in Figure 3a. There was a significant decrease in the TBARS level in the sepsis group treated by the extract (S2+CLP) related to the CLP group ($p < 0.01$). This correlation however was not observed between the group treated by lower extract concentration (S1+CLP) and the CLP group. Level of TBARS in sham-operated (control) group is different from level of TBARS in CLP groups treated orally by both concentrations – 100 mg/kg and 300 mg/kg ($p = 0.008$, $p = 0.04$ respectively). The mean TBARS values in the experiment after i.p. administration of *S. minor* radix extract are presented in Figure 3b. TBARS level in the CLP group after i.p. treatment by the tested extract was significantly lower compared to the control CLP group ($p = 0.004$).

The mean values of total nitrate and nitrite plasma level in the groups treated orally by the

S. minor extract are presented in Figure 4a, while the results obtained after i.p. administration are shown in Figure 4b. As it is expected, the NO_x value in the control sepsis group (CLP) was increased related to the control, sham-operated, group ($p < 0.01$). The level of NO_x in the CLP group treated orally by the extract in the concentration 100 mg/kg (S1+CLP) was significantly lower compared to the control and CLP group ($p < 0.05$, $p < 0.01$ respectively). The higher concentration of the extract in S2+CLP group caused a significant decrease in NO_x related to the CLP group ($p < 0.01$). Intraperitoneal administration of *S. minor* extract did not affect the NO_x level in S1+CLP group related to CLP or control groups.

The obtained mean values of superoxide anion radical plasma levels are presented in Figure 5 (5a after orally applied and 5b after i.p. applied extract). The level of O_2^- was significantly higher in the CLP

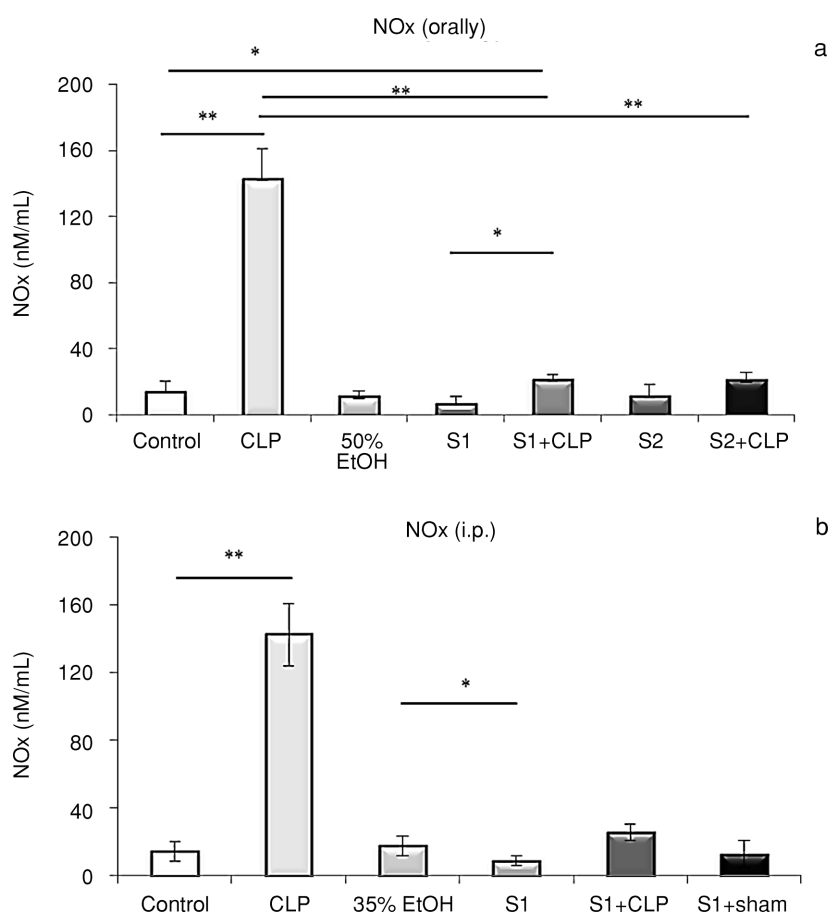


Figure 4. Nitrate and nitrite concentration (NO_x) in the groups treated with *S. minor* radix ethanol extract. Data are presented as following: a – orally administration of extract; b – intraperitoneal administration of the extract. The results are expressed as mean \pm SD (* $p < 0.05$; ** $p < 0.01$).

i.p. – intraperitoneally

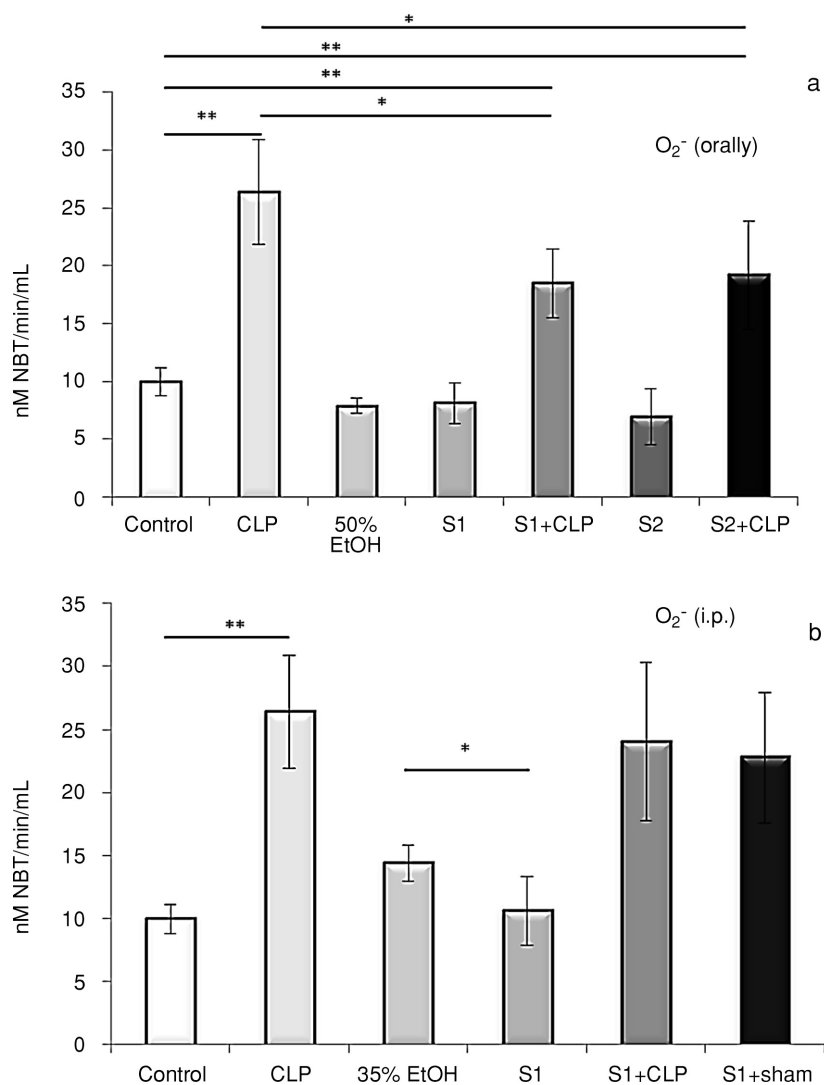


Figure 5. Superoxide anion radical (O_2^-) level in the groups treated with *S. minor* radix ethanol extract. Data are presented as following: a – orally administration of extract; b – intraperitoneal administration of the extract. The results are expressed as mean \pm SD (* p < 0.05; ** p < 0.01).

i.p. – intraperitoneally

group compared to the control group regardless of the way of administration (p < 0.01). Orally applied *S. minor* radix extract in sepsis (S1+CLP and S2+CLP groups) caused a significant decrease in O_2^- level related to the CLP group. However, the level of O_2^- was significantly lower in the control group compared to S1+CLP and S2+CLP groups. Intraperitoneally applied extract in the sepsis group did not affect the superoxide anion level in comparison to the control and CLP groups.

Superoxide dismutase (SOD) activity in the sepsis groups treated orally with *S. minor* radix ethanol extract in both concentrations was signifi-

cantly higher compared to the CLP group (Fig. 6a). There was a significant difference in SOD activity between the control group and S1+CLP group after i.p. administration of the extract (Fig. 6b).

A significant decrease of TBARS level was obtained in the CLP group after i.p. treatment by *S. minor* extract (100 mg/kg) in comparison to the orally applied extract of the same concentration (p < 0.05). There were no significant differences in the value of total-SH, NOx, O_2^- and SOD levels between CLP groups after oral and i.p. treatment by the extract in the same concentration (p > 0.05) (Fig. 7).

DISCUSSION AND CONCLUSION

In order to complete existing data on the biological activities of *S. minor*, we have studied different root extracts of *S. minor* subsp. *muricata* for its antioxidant, antimicrobial and anti-inflammatory (*in vitro* and *in vivo*) effects.

The antioxidant potential of three *S. minor* radix extracts was compared (methanol, ethanol and chloroform). The content of main antioxidant-contributing compounds was determined (phenols and flavonoids). Methanol extract contained the highest level of phenols in comparison to the other two investigated extracts ($497.47 \pm 3.80 \mu\text{g GAE/mg dw}$). The lowest level of TPC was present in chloroform, as a nonpolar solvent ($41.45 \pm 0.54 \mu\text{g GAE/mg dw}$). Considering that the data about

S. minor subsp. *muricata* are scarce, we may look upon these results in the light of the data available on *S. minor* L. Romojaro et al. reported the total phenolic content in *S. minor* of $530.51 \pm 21.12 \text{ mg GAE/100 mg}$, but it was expressed on fresh plant weight (11). Also, the other source claims that *S. minor* water/ethanol extract contains high levels of polyphenols (29). The most investigated species of *Sanguisorba* genus seems to be *S. officinalis*. Cai et al. reported the TPC of methanol extract of *S. officinalis* roots to be $15.87 \text{ g GAE/100g dw}$, which is considerably lower than results for phenols in our methanol extract of *S. minor* roots ($49.74 \text{ g GAE/100g dw}$) (30). The same pattern was observed also in the case of phenols in ethanol extracts – TPC value for *S. officinalis* extract was $121.42 \pm 0.7 \text{ mg GAE/g}$, while our result for *S. minor* is almost four

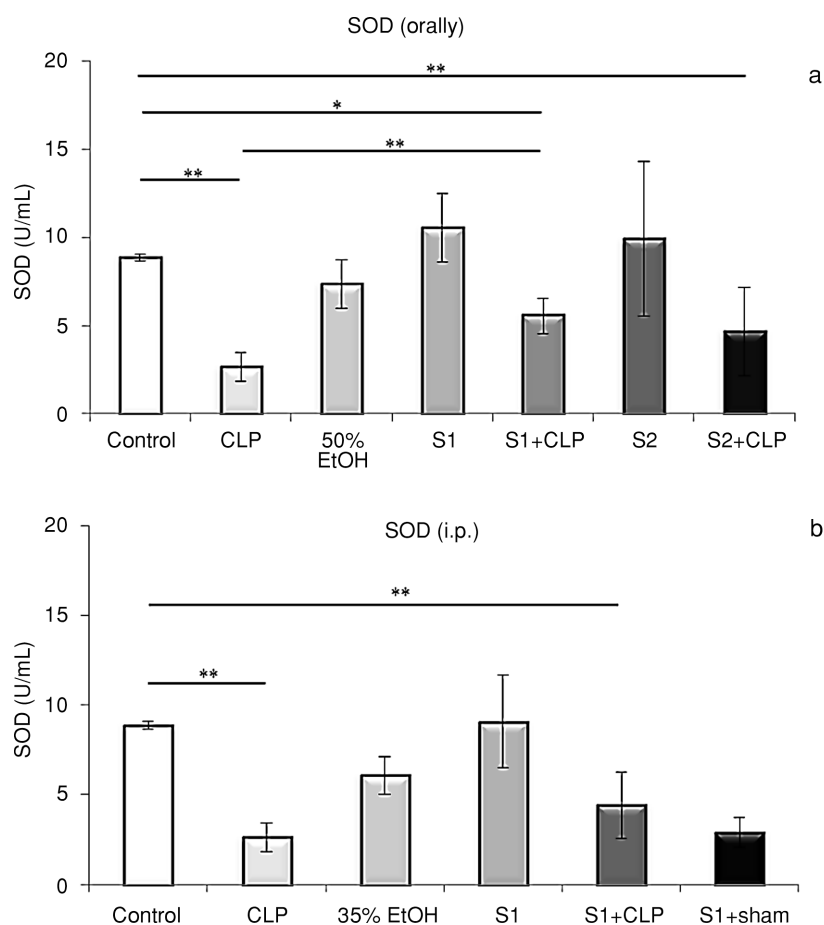


Figure 6. Superoxide dismutase (SOD) activity in the groups treated with *S. minor* radix ethanol extract. Data are presented as following: a – orally administration of extract; b – intraperitoneal administration of the extract. The results are expressed as mean \pm SD (* $p < 0.05$; ** $p < 0.01$).

i.p. – intraperitoneally

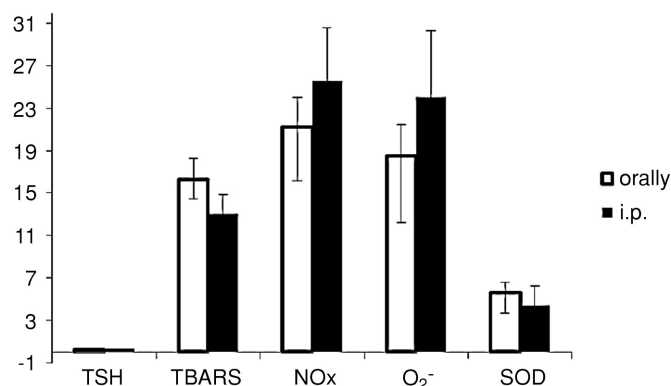


Figure 7. Comparison of oxidative stress parameters and SOD activity in the groups treated orally and intraperitoneally by *S. minor* radix ethanol extract (mean \pm SD).

times higher (457.45 ± 4.59 mg GAE/g) (10). However, this simple and direct comparison of flavonoid content from our research and previous data is not feasible, due to different equivalents for result expression (unless rutin/querceetin relation is matched using their molecular weights). The striking difference between previously published results and ours seems to be the correlation of antioxidant activity and TFC. The positive correlation between antioxidant activity and total flavonoid content was noted in aerial parts extracts of *S. minor* (31). We observed an inverse correlation between the antioxidant activity and TFC in root extracts. The chloroform extract showed the highest TFC, but the lowest antioxidant activity. Therefore, flavonoids in investigated *S. minor* extracts do not contribute significantly to the antioxidant activity. It seems that total phenols are mainly responsible for the antioxidant activity since a positive correlation was observed between antioxidant activity of ethanolic and methanolic *S. minor* extracts and the content of phenols in our research.

The antioxidant potential of *S. minor* extracts was evaluated by several currently approved methods (ABTS, DPPH, CUPRAC, FRAP, and TRP assays). The extract prepared in chloroform, as a less polar solvent, acted against DPPH and ABTS radical with comparable power but methanol and ethanol extracts exhibited higher scavenging potential, which may be attributed to the high level of antioxidant components in these extracts (32). The antioxidant activity of our sample measured by the ABTS assay was lower than the antioxidant activity of *S. officinalis*, according to Li et al. (33). The ethanol extract of *S. minor* showed slightly higher antioxidant activity than methanol extract, deter-

mined by CUPRAC and FRAP method (Table 1). The lowest CUPRAC and FRAP activity was shown by chloroform extract, just as expected according to the total phenolic content. The results from this study of total reducing power for ethanol and methanol extracts are similar (Table 1). These two extracts have the highest amount of phenolics and therefore show a high reducing power (32).

Methanol and ethanol extracts did not differ significantly in the content of phenols, as well as in achieved antioxidant activity measured by five different methods, which may be due to no high differentiation in the polarity of these solvents. On the contrary, both of these extracts were superior to chloroform extract in regards to the aforementioned parameters. We assume that chloroform, due to the low polarity, could not efficiently extract all compounds that contribute to antioxidant activity (34). We have presented the comparison of antioxidant activity among our investigated extracts since we have not encountered any similar research data that could serve as a good match for comparison of antioxidant activity to investigated species *S. minor* subsp. *muricata*.

Antimicrobial activity of investigated *S. minor* subsp. *muricata* extracts varied depending on the group of microorganism, and the type of extracts. The chloroform extract of *S. minor* radix showed the strongest antimicrobial activity (Table 2). It exhibited strong activity against all examined strains of bacteria: *B. cereus*, *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *E. aerogenes*, *P. mirabilis*, *K. pneumoniae*, *S. enteritidis*. *B. cereus* and *S. aureus* showed the highest sensitivity to the chloroform extract.

A recently published study by Finimundy et al. also reported the antibacterial efficacy of the

S. minor root extracts against *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli* using the microdilution method (35). Antibacterial activity of *S. minor* root extracts against *S. aureus*, *B. cereus*, *E. coli* had also been confirmed in the study of Karkanis et al. (36). The MIC and MBC values of investigated extracts in both of the studies were slightly lower than in our study for the same bacteria. These differences may be attributed to different growing conditions that can affect the bioactive potential of plant extract (35). *S. minor* root extracts also exhibited activity against *Enterobacter cloacae*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Micrococcus flavus* (35, 36).

In our study, all the extracts revealed similar and moderate antifungal activity against *C. albicans*. The antifungal properties of *S. minor* have been previously reported by Finimundy et al. against six fungi, namely *Aspergillus fumigatus*, *A. Niger*, *A. ochraceus*, *Penicillium funiculosum*, *P. ochrochloron*, and *P. verrucosum* var. *cyclopium* (35). Karkanis et al. recorded similar effects of the *S. minor* root extracts against the same fungi strains (36).

The literature also points out that *S. officinalis* is active against *S. aureus*, *P. aeruginosa*, *C. albicans* and *Candida guilliermondii* (37, 38). The investigated ethanol extract of *S. minor* radix showed stronger bacteriostatic activity against *S. aureus*, *P. aeruginosa*, *E. coli*, *B. subtilis* and *C. albicans* in comparison to the previously published data for ethanol extract of *S. officinalis* underground parts (MIC = 15.63-250 mg/mL) (39). The results of the bacteriostatic activity of investigated chloroform extract of *S. minor* radix against *S. aureus*, *P. aeruginosa*, *E. coli* and *C. albicans* were similar to the chloroform extract of *S. officinalis* herbs (MIC = 0.128-1.024 mg/mL) (38).

The design of conducted *in vitro* research included the experience of traditional medicine in Serbia, which utilizes *S. minor* in the form of ethanol extract (tincture). Since our results of the antioxidant activity showed similar activity of ethanol and methanol extract, we preferred to include ethanol extract of *S. minor* radix, in preliminary *in vitro*, and the following testing. In the preliminary anti-inflammatory *in vitro* assay, we obtained 76% of COX-1 inhibition by ethanol extract of *S. minor*, which led us to the thinking of its anti-inflammatory properties (40).

Proceeding to an animal model of sepsis, we followed oxidative stress parameters, since they are nonspecific but crucial indicators of inflammation and energy disturbances during sepsis (41, 42). Sepsis is still a leading problem, causing significant

morbidity and mortality even in the era of modern technologies used in health care management. Nowadays, the focus of investigations for efficient therapy in sepsis has been slightly shifted toward the antioxidant therapies that could successfully accompany conventional therapy. Herbal extracts with antioxidative and anti-inflammatory attributes may offer arguments for playing such a role in the treatment of sepsis (14, 43). The effect of ethanol *S. minor* extract on the systemic tissue oxidative status after oral and intraperitoneal administration in rats with sepsis could be promising.

These results present the first report of the antioxidative activity of *S. minor* extract in sepsis. No change of values of total-SH, TBARS, NO_x, O₂⁻ and SOD between control group without any treatment and control groups treated orally/intraperitoneally by *S. minor* extract indicates that administration of *S. minor* extract does not affect the physiological environment in which the cells signaling pathways are performed (44, 45). The tissue thiols availability is crucial for a metabolic challenge in systemic inflammation such as sepsis since they represent significant contributors to the body defense system against reactive oxygen species (45). Besides the antioxidant role, thiols contribute to other processes such as signal transduction, detoxification and apoptosis (46). A decreased level of thiols has been associated with various disorders, including sepsis (46). However, the administration of ethanol extract of *S. minor* to animals with sepsis did not affect the level of total thiols, neither orally nor intraperitoneally applied (Figs. 2 and 3) (47). Though basically lipid peroxidation is a measure of membrane damage, it also affects protein synthesis in all tissues, especially during aging. Furthermore, cellular dysfunctions induced by oxidative stress are mediated by lipid peroxidation end products (44, 48). Malondialdehyde generated during lipid peroxidation is considered the most important mediator of toxic effects caused by oxidative stress occurring in the cell. Excess accumulation of malondialdehyde is associated with complications in sepsis (44, 48). Our results indicate that *S. minor* extracts applied both orally and intraperitoneally produce a positive effect on TBARS level. Orally applied extract in the concentration of 300 mg/kg significantly decreases TBARS level compared to the CLP group (Fig. 4). Investigated extract expressed a significant decrease in values of TBARS in both tested concentrations when compared to the control group, which suggests its antioxidant effect. These results showed also that the intraperitoneal administration of *S. minor* extract was superior to oral administration, concerning the

reduced TBARS level in sepsis. Such a positive effect of investigated extract seems to be due to high phenol content (19, 21, 30). In particular, the effect of increased total SH content in healthy animals could be the reflection of a significant antioxidant potential of the tested extract. Literature data point to a decrease of TBARS level when vitamin C, as a strong antioxidant, has been applied intraperitoneally. Since the investigated extract of *S. minor* also induced a decrease of TBARS level, we conclude that *S. minor* extract may have a promising role in sepsis (42). The plasma levels of nitrite and nitrate, as the final products of nitric oxide oxidation pathways, are increased in sepsis (49). Superoxide anion induces lipid peroxidation and damages the cell membranes and its level in sepsis is increased, too. This anion is involved in several reactions in which products are a variety of reactive oxygen and nitrogen species. Superoxide dismutase controls the O_2^- level by converting it into less active oxygen and hydrogen peroxide (50). Orally applied *S. minor* extract in both concentrations significantly decreased the NOx and O_2^- plasma levels compared to the CLP group (Figs. 4a and 5a). The level of SOD was significantly decreased after oral administration of extract in both concentrations compared to the control group. *S. minor* extract in lower concentration gave increased SOD activity compared to CLP group. Intraperitoneal administration of *S. minor* extract did not show a positive effect on reduction of NOx and O_2^- levels, while SOD activity was decreased compared to control group (Figs. 4b, 5b and 6b). It can be assumed that the route of administration of *S. minor* extract did not affect the achieved results in oxidative stress parameters reduction.

Considering the exhibited antioxidant activity and high phenolic content, *S. minor* subsp. *muricata* could be used as a natural antioxidant and as a nutritional supplement with high antioxidant potential. The chloroform extract of *S. minor* showed strong antibacterial activity and therefore has the potential for further more comprehensive studies. The synergistic effect of this plant extract with commonly used antibiotics could be also interesting for further investigation. Based on the achieved effects on oxidative stress parameters in an animal model of sepsis, *S. minor* subsp. *muricata* could be considered as a herbal adjuvant antioxidant therapy in systemic infectious treatment. *S. minor* ethanol extract induced inhibition of COX-1 *in vitro* which justifies the use of *S. minor* in traditional medicine as an anti-inflammatory agent.

The results presented in this study indicate a wide spectrum of biological properties of *S. minor* which could be used as a potential source of natural

bioactive compounds in pharmaceutical and medicinal treatments. Hence, further investigations oriented on isolation and characterization of the present antioxidants, antimicrobial and anti-inflammatory substances are necessary in order to determine the effects of this plant species and the underlying mechanism.

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Ethics approval and consent to participate

The study was approved by the Ethics Committee for Animal Welfare of the Military Medical Academy and the Ministry of Agriculture and Environmental Protection of Republic of Serbia (No. 323-07-7363/2014-05) that respects the rules of the European Parliament's Directive on the protection of animals used for scientific purposes 2010/63 / EU.

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Declaration of interest statement

The authors report no declarations of interest.

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