



Anti-Inflammatory and In Vitro Antioxidant Activities of *Satureja Montana* Dry Extract

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Abstract

Introduction: Many chronic somatic and psychiatric diseases are associated with oxidative stress and inflammation, both of which have detrimental effects on human health.

Aim: To investigate the in vitro antioxidant and in vivo immunomodulatory activities of *Satureja montana* dry extract.

Material and methods: The in vitro antioxidant activity of *Satureja montana* dry extract was assessed using ORAC, HORAC, and electrochemical methods. Immunomodulatory activity was evaluated in acute and chronic stress models by measuring the serum levels of cytokines TNF- α , IL-6, and IL-1 β in a cohort of 112 male 8-week-old Wistar rats. The rats were randomly divided into 7 groups for each of both stress models and then subjected to ELISA analysis (14 groups with 8 rats in each group). The rodents were gavaged with a dry extract of *Satureja montana* (250 mg/kg and 500 mg/kg), rosmarinic acid (15 mg/kg), and carvacrol (500 mg/kg) for 14 days and 60 days, respectively.

Results: We demonstrated that, for all employed in vitro methods, the dried extract of *Satureja montana* exhibited considerable antioxidant activity. *Satureja montana* did not significantly lower serum concentrations of TNF- α , IL-6, or IL-1 β in either stress model as compared to the positive saline control group. On the other hand, in the acute stress model, a dose of 250 mg/kg of *Satureja montana* significantly decreased IL-6 in comparison to carvacrol and significantly reduced TNF- α and IL-6 in comparison to rosmarinic acid.

Conclusion: Although *Satureja montana* dry extract has significant antioxidant activity in vitro, its influence on systemic inflammation is still unknown. Future research will look into how it affects serum levels of pro-inflammatory cytokines.

Keywords

carvacrol, cytokines, oxidative stress, rosmarinic acid, *Satureja montana*

INTRODUCTION

Free radicals play an important role in maintaining homeostasis as they are normally generated through enzymatic and non-enzymatic reactions within the human body.^[1] The levels of free radicals are influenced by both internal and external factors.^[2] Oxidative stress occurs when there is an excessive production of free radicals that overwhelms the endogenous antioxidant systems, leading to the development of various chronic somatic and psychiatric diseases.^[3,4]

There is a close connection between oxidative stress and inflammation.^[5] Inflammatory cells produce free oxygen radicals that can lead to oxidative stress. At the same time, free radicals can stimulate the inflammatory response.^[6,7] One possible mechanism underlying this interaction involves the activation of nuclear factor kappa B (NF- κ B) and the inflammasome, as well as the upregulation of pro-inflammatory cytokines.^[6]

While acute inflammation serves an important purpose in the human body by eliminating harmful agents and promoting tissue repair, uncontrolled chronic low-grade inflammation can contribute to the development of chronic diseases.^[8,9] The prevention and treatment of oxidative stress and inflammation are essential, given their important role in the pathogenesis of socially significant diseases that impact quality of life.

Various mechanisms exist to counteract free radicals and inflammation, including the use of natural/plant or synthetic molecules.^[3,10] Studies have shown that certain medications, such as fluoxetine, sitagliptin, beta blockers, and calcium antagonists, possess in vitro antioxidant activity.^[11-13] Other drugs, including angiotensin receptor blockers (ARBs), metformin, omega 3 fatty acids, probiotics, and vitamin D, have been found to have an impact on inflammation.^[14]

Medicinal plants and their active compounds, such as polyphenols and flavonoids, are also being extensively researched for their antioxidant and anti-inflammatory effects in the treatment of oxidative stress and inflammation.^[1,5] However, despite the growing interest in medicinal plants, only a small part of them have been chemically and pharmacologically researched.^[15]

Satureja montana, commonly known as winter savory, is one of the most pharmacologically active plants within the Lamiaceae family, which consists of over 230 genera and more than 7000 species.^[16,17] This plant is distributed on the Balkan Peninsula and in Bulgaria.^[18] The pharmacological effects of *Satureja montana*, such as antioxidant, antibacterial, and antiviral effects are described in the available scientific data.^[19] These effects are closely related to the type of extract used in the different studies, as well as to the active compounds in its composition.

AIM

However, there is insufficient information on other pharmacological effects of *Satureja montana*, prompting further examination of its in vitro antioxidant activity and its potential to reduce inflammation in the present study.

MATERIALS AND METHODS

In vitro antioxidant activity

Oxygen radical absorbance capacity

The Oxygen Radical Absorbance Capacity (ORAC) technique developed by Ou et al. was used, with a few modifications detailed by Denev et al.^[20,21] This method assesses an in vitro antioxidants ability to neutralize peroxide radicals. The method is based on inhibiting fluorescein fluorescence decline during oxidation in the presence of an in vitro antioxidant. As a peroxide radical generator, the thermal breakdown of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) was used. The results are given in terms of mol Trolox equivalents per gram of extract (TE/g). FLUOstar OPTIMA fluorimeter (BMG LABTECH, Offenburg, Germany) was used for the measurements. The excitation wavelength was 485 nm, and the emission wavelength was 520 nm.

Hydroxyl radical averting capacity

The Hydroxyl Radical Averting Capacity (HORAC) method developed by Ou et al.^[22] assesses an in vitro antioxidant's ability to form complexes in Fenton-like reaction caused by the interaction of Co (II) with H₂O₂. The results are given in terms of mol gallic acid equivalents per gram of extract (GAE/g). FLUOstar OPTIMA fluorimeter (BMG LABTECH, Offenburg, Germany) was used for the measurements. The excitation wavelength was 485 nm, and the emission wavelength was 520 nm.

Electrochemical method

The electrochemical technique was used for evaluating the antioxidant activity in vitro.^[23] The methodology of the experiment consists in recording voltamperograms of cathodic electroreduction of oxygen with the "Analyst AOA" (RU.C.31.113.A N28715) linked to a PC. The in vitro antioxidant activity of the investigated samples was calculated using the kinetic criteria K (in micromoles per liter/minute), which indicates the quantity of reactive oxygen species in time and is represented as a percentage of the Trolox kinetic criterion via the formula:

$$AOA = K_{\text{sample}} / K_{\text{Trolox}}$$

Laboratory experiments

Animals

All experiments are in agreement with the approval of the Ethics Committee of Medical University of Plovdiv (as of protocol No. 01-2/10.04.2020) and the approval of the Bulgarian Food Safety Agency (as of protocol No. 258), based on the position of the Ethic Committee, Bulgarian Food Safety Agency No. 174 from October 8, 2019.

Rats were raised in the vivarium of the Medical University of Plovdiv and housed under standard conditions: 20°–22°C, 12-hour light/dark cycle, with free access to food and water.

Substance preparation

Carvacrol and rosmarinic acid (RA) were bought from Sigma-Aldrich (St. Louis, Missouri, USA). Carvacrol was dissolved in olive oil and RA was dissolved in saline. Both solutions were administered orally via stomach gavage (1 ml/100 g b.w.).

The dry extract of *Satureja montana* (SME) was prepared by methodology of Vesselino EOOD, Kazanlak, Bulgaria via methanol-aqueous (70:30) extraction, followed by spray drying at 40°C until complete evaporation of both solvents. The extract was dissolved in saline and applied orally via stomach gavage in the volume of 0.5 ml/100 g b.w. (for a dose of 250 mg/kg b.w.) and 1 ml/100 g b.w. (for a dose of 500 mg/kg b.w.).

Investigation of pro-inflammatory cytokines levels in acute and chronic stress models

Acute stress model

For investigation of the impact of dry SME on the serum concentration of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in acute cold stress model, 56 male 8-week-old Wistar rats with average bodyweight 110 g (range 100–120 g) were used. The rodents were randomly divided into 7 groups (n=8). One of the groups was sham control, which was only gavaged without administration of substances. The rest groups were treated daily for 14 days orally via stomach gavage with saline and olive oil – 1 ml/100 g b.w. (positive controls), dry SME at doses of 250 mg/kg b.w. and 500 mg/kg b.w., carvacrol 500 mg/kg b.w., and RA 15 mg/kg b.w. – experimental groups.

On day 15 from the beginning of the experiment, rodents from both positive controls and all experimental groups were exposed to the stress factor, –4°C for 60 minutes in the refrigerator. Animals were placed in plastic boxes 20×20 cm, and allowed to move freely into the box. At the same time, there were four animals in the refrigerator, each of it in a single box. Boxes were cleaned with 70% alcohol after each animal. The sham group was not exposed to the stress factors.

Fifteen minutes after removing the rodents from the refrigerator, they were decapitated under narcosis with ether and 2 ml blood was collected. The blood was centrifuged and 1 ml of serum was separated. The investigation of serum levels of IL-1 β , IL-6, and TNF- α was performed with ELISA method and Ebioscience kits (ThermoFisher Scientific, USA).

Chronic unpredictable mild stress model

To investigate the effect of dry SME on the serum pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in a chronic unpredictable mild stress model, 56 male, 8-week-old Wistar rats with average bodyweight of 110 g (100–120 g) were used. They were randomly divided in 7 groups (n=8), which were similar to these, described above in acute stress model. From the first day of the experiment, animals from both positive controls and all experimental groups were exposed to mild stress stimuli – food or water deprivation for 24 hours, placing an empty water bottle for 1 hour, tilting of home cage (45° for 3 hours), leaving of light for 24 hours, soiling the bedding (200 ml, 25°C water per 100 g bedding material), predator sounds (two rounds of 20 minutes). Stress factors were applied 60 minutes after the daily treatment. Each stressor was used once a week and the order was changed every week of the experiment to avoid habituation to them. The sham group was not exposed to the stress stimuli.

On day 60, one hour after the exposure to the stressor, the rodents were decapitated under narcosis with ether and 2 ml of blood was collected. This blood was centrifuged and 1 ml of serum was separated. The investigation of serum levels of IL-1 β , IL-6, and TNF- α was performed as described above in the section of acute stress model.

Serum cytokine measurement in treated rat groups by ELISA

Diluted rat serums (1:2) for IL-1, IL-6, and TNF, as well as internal controls and test standards, were dripped on solid phase with monoclonal antibodies against the respective cytokine for quantitative cytokine testing. A peroxidase conjugate (second anti-species antibody) is added after incubation and washing to generate a cytokine complex. A second wash is used to eliminate any unbound conjugate. A color response occurs when a chromogenic substrate is introduced to the enzyme, indicating the presence of cytokines. Absorption is evaluated colorimetrically on a TECAN ELISA reader at 450 nm and 620 nm and is proportional to cytokine concentration. A standard curve is used to determine the concentration of each cytokine in pg/ml.

Statistical analysis

The results were analyzed statistically using one-way ANOVA and LSD post hoc tests with IBM SPSS 19.0 software. Results were expressed as arithmetic means (\bar{X}), standard error of the mean (\pm SEM for cytokine levels), and stan-

ard deviation (\pm SD for the antioxidant activity). A p -value ≤ 0.05 was considered statistically significant. For each one-way ANOVA test F statistics and p value is given. Statistical significance between groups, which is found with post hoc analyses, is presented as a p -value.

RESULTS

In vitro antioxidant activity

The dry SME showed in vitro antioxidant activity in all used methods. The results are presented in **Table 1**.

Table 1. In vitro antioxidant activity of dry SME

ORAC, μ mol TE/g $\bar{X} \pm$ SD	HORAC, μ mol GAE/g $\bar{X} \pm$ SD	AOA = $K_{\text{SAMPLE}} / K_{\text{TROLOX}}$ 16.236/0.634
8529.3 \pm 159.0	2114.4 \pm 17.6	25.6

Measurement of serum cytokines levels in models of acute and chronic stress

Acute stress model

Acute cold stress significantly increased the serum concentrations of TNF- α and IL-6 in the positive controls, treated with olive oil ($p=0.041$ and $p<0.001$, respectively). In the positive saline control, a statistically significant increase was found only in IL-6 serum concentrations ($p=0.003$). The same cytokine was found to be statistically higher in the groups treated with dry SME 500 mg/kg b.w., RA, and carvacrol compared with sham control ($p=0.028$, $p<0.001$, and $p<0.001$, respectively). TNF- α was statistically increased in the groups that received RA and carvacrol when compared to the sham control ($p=0.003$, $p=0.015$, respectively).

None of all tested substances had a significant impact on lowering the serum cytokine levels compared to corresponding positive control ($p>0.05$ for all measurements). The results are presented on **Figs 1A-B**.

The dry SME at a dose at 250 mg/kg b.w. decreased significantly the serum concentration of IL-6 and TNF- α compared to RA ($p=0.011$ and $p=0.036$). When the same dose of dry SME was compared with the carvacrol treated group, a significant impact was found only on the IL-6 levels ($p=0.019$). The higher dose of *Satureja montana* dry extract did not show significant anti-inflammatory effect compared to RA and carvacrol. The results are presented in **Fig. 2**.

Chronic unpredictable mild stress model

Chronic stress significantly increased serum concentrations of IL-6 and TNF- α in the positive olive oil control group ($p=0.001$ and $p=0.006$, respectively) as well as IL-6 in the positive saline control group ($p=0.015$). The chronic

stress had no impact on serum levels of IL-1 β .

The dry SME decreased non-significantly the serum concentrations of IL-6 and TNF- α compared to the positive saline control ($p>0.05$ for all measurements). RA increased statistically significantly IL-6 compared to the sham control ($p=0.013$) and showed non-significant anti-inflammatory effect compared to the positive saline control ($p>0.05$). Carvacrol decreased significantly serum concentrations of IL-6 and TNF- α compared to the olive oil control ($p=0.011$ and $p=0.004$, respectively). The results are presented in **Fig. 3**.

Both doses of the dry SME 250 mg/kg b.w. and 500 mg/kg b.w. did not show significant effect on serum concentrations of IL-6 and TNF- α when compared with the RA or the carvacrol treated groups ($p>0.05$ for all measurements). Visualization of results is not shown.

DISCUSSION

The presence of multiple methods for determining in vitro antioxidant activity leads to results that are incomparable across different studies. To ensure the accuracy of these findings, it is necessary to employ more than one in vitro method for verification.^[24] In the present study, three specific assays were chosen for their precision.^[25]

The in vitro antioxidant capacity of the dry *Satureja montana* extract (dry SME) analyzed in this study was found to be lower than that reported by Moreira SA.^[26] These variations can be attributed to differences in the quantitative and qualitative composition of the extracts used in both studies. External factors such as geographic region, weather conditions, and vegetative period contribute to differences in the composition of medicinal plants.^[27] It should be noted that, to the best of our knowledge, no other research team has used the electrochemical and HORAC methodologies to assess *Satureja montana*'s in vitro antioxidant activity.

The in vitro antioxidant activity of the dry SME is attributed to the presence of phenolic compounds in the medicinal plant's composition.^[28] Our previous study identified rosmarinic acid (RA) and carvacrol as active ingredients in the dry SME with an average amount of 45 mg/g and 0.02 mg/g, respectively.^[29] Carvacrol is known to have a lower capacity to neutralize reactive oxygen species compared to other phenolic derivatives.^[30] For that reason, we suppose that the observed in vitro antioxidant activity of the dry SME is mainly due to the presence of RA in its composition. This hypothesis is supported by several other researchers.^[31]

Based on the results obtained for the antioxidant activity of *Satureja montana*, an investigation of serum concentrations of TNF- α , IL-6, and IL-1 β was conducted. It is well-known that both acute and chronic stress lead to increase in the serum concentrations of pro-inflammatory cytokines.^[32,33] The results obtained in both positive control groups when compared to the sham control are consistent with the existing literature data.^[32,33]

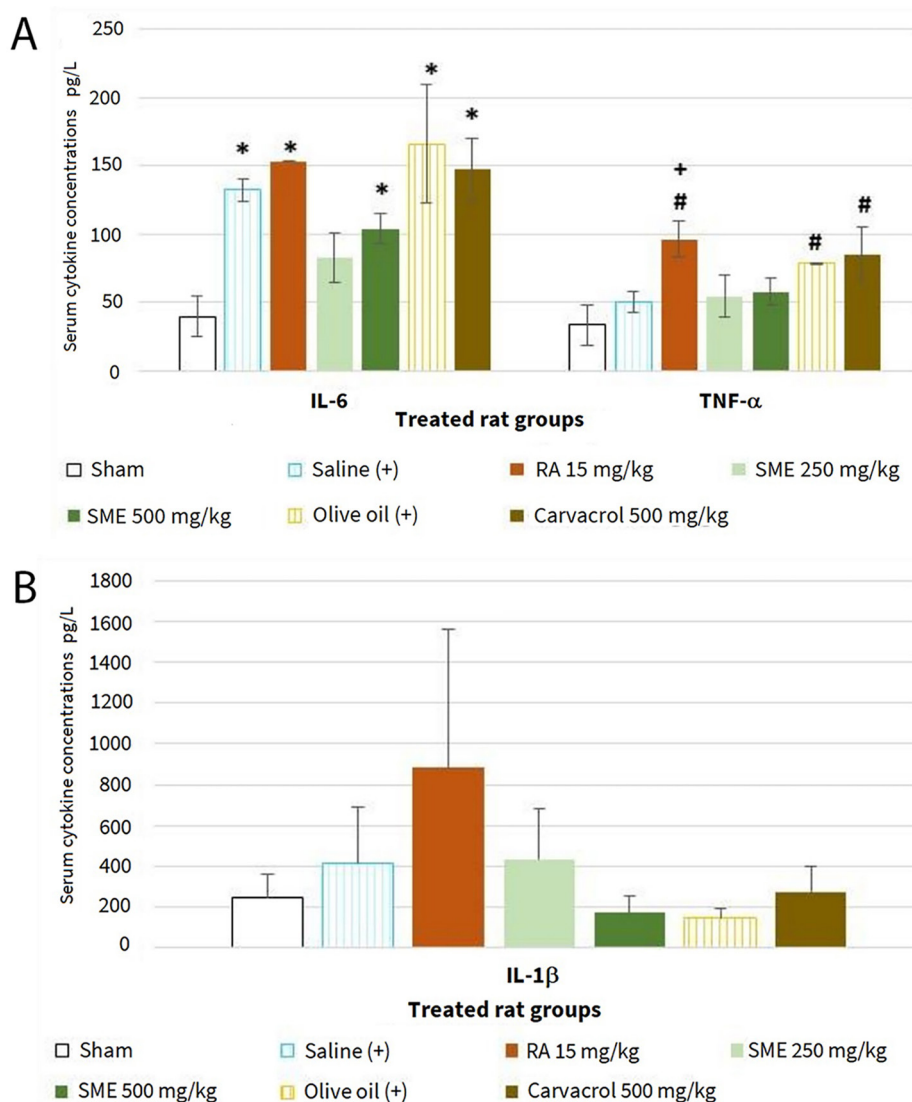


Figure 1.

A. Effect of dry extract of *Satureja montana*, rosmarinic acid, and carvacrol on serum concentration of IL-6 and TNF-α in acute stress experimental model. Comparison of the results with one-way ANOVA test yielded the following results: IL-6: $F=3.605$; $p<0.0001$; TNF-α: $F=2.642$; $p=0.007$. The further comparison of the groups with LSD post hoc test found the following: * A significant increase of serum concentration of IL-6 when compared with sham control; # A significant increase of serum concentration of TNF-α when compared with sham control; + A significant increase of serum concentration of TNF-α when compared with positive saline control. Comparison of the results with one-way ANOVA test found following results IL-1β: $F=1.055$. **B.** Effect of dry extract of *Satureja montana*, rosmarinic acid, and carvacrol on serum concentration of IL-1β in acute stress experimental model.

RA and carvacrol are known to decrease pro-inflammatory cytokines.^[34,35] However, in the present study, these two phenolic compounds exhibited contradictory effects, likely due to the experimental method used to induce systemic inflammation. To the best of our knowledge, no other study has investigated the effects of both phenolic compounds on pro-inflammatory cytokines in stress models. It is possible that the anti-inflammatory activity of RA and carvacrol is insufficient to counteract the pathogenic changes involved in inflammation.

No data were found regarding the effect of *Satureja montana* on pro-inflammatory cytokines, making it challenging

to compare the results obtained in this study. It is possible that the lack of significance in the results is due to the extract's inability to counteract all activated inflammatory mechanisms. Additionally, there may be potentiation or synergism between RA and carvacrol in the composition of the dry SME, as well as the development of tolerance with long-term use. Furthermore, carvacrol demonstrated a significant anti-inflammatory effect in the chronic stress model, which was not observed in the *Satureja montana*-treated groups. This discrepancy may be attributed to the small amount of carvacrol present in the extract's composition (0.020 mg/g).

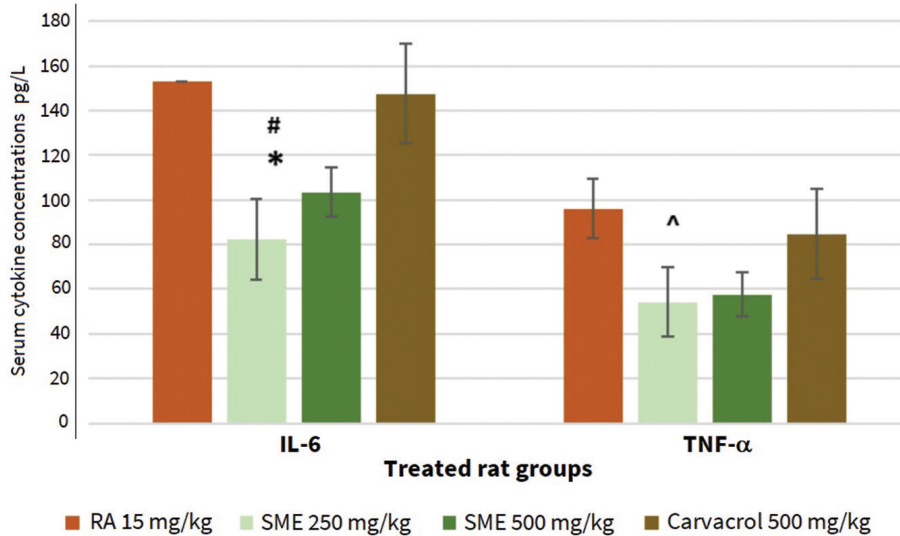


Figure 2. Comparative effect of dry extract of *Satureja montana*, rosmarinic acid, and carvacrol on serum cytokines levels in acute cold stress model. Comparison of the results with one-way ANOVA gave the following results: IL-6: $F=3.605$; $p<0.0001$; IL-1 β : $F=1.055$; $p=0.413$; TNF- α : $F=2.642$; $p=0.007$. Further comparison of the groups with LSD post hoc test found the following: * A significant decrease of IL-6 serum concentration in the group treated with *Satureja montana* 250 mg/kg b.w. compared with RA treated group ($p=0.011$); # A significant decrease of IL-6 serum concentration in the group treated with *Satureja montana* 250 mg/kg b.w. compared with carvacrol treated group ($p=0.019$); ^ A significant decrease of TNF- α serum concentration in the group treated with *Satureja montana* 250 mg/kg b.w. compared with RA treated group ($p=0.036$).

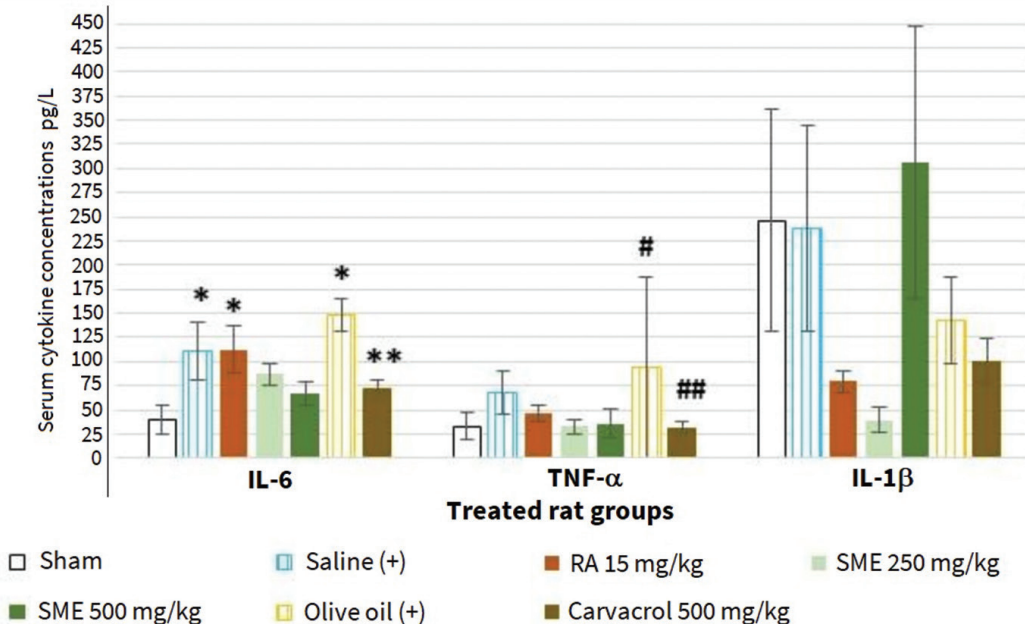


Figure 3. Effect of dry extract of *Satureja montana*, rosmarinic acid, and carvacrol on serum concentration of IL-6, IL-1 β , and TNF- α in a chronic unpredictable mild stress experimental model. Comparison of the results with one-way ANOVA found the following results: IL-6: $F=3.605$; $p<0.0001$; IL-1 β : $F=1.055$; $p=0.413$; TNF- α : $F=2.642$; $p=0.007$. Further comparison of the groups with LSD post hoc test found the following: * A significant increase of serum concentration of IL-6 compared to the sham control; ** A significant decrease of serum concentrations of IL-6 in carvacrol treated group compared; to positive olive oil control; # A significant increase of serum concentration of TNF- α compared to the sham control; ## A significant decrease of serum concentrations of TNF- α in carvacrol treated group compared to positive olive oil control.

CONCLUSION

The results of our study indicate that *Satureja montana* dry extract demonstrates in vitro antioxidant activity across all methods used, confirming the potential of wild-growing Bulgarian *Satureja montana* to decrease the oxidants levels. However, the impact of *Satureja montana* dry extract on systemic inflammation remains inconclusive. Confirming or rejecting the effect of that medicinal plant on the serum concentrations of pro-inflammatory cytokines will be the subject to further studies.

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Conflict of Interests

The authors of this manuscript have declared that no conflict of interests exists.

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Противовоспалительное и антиоксидантное действие сухого экстракта *Satureja Montana* in vitro

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Резюме

Введение: Многие хронические соматические и психические заболевания связаны с окислительным стрессом и воспалением, оба из которых оказывают пагубное воздействие на здоровье человека.

Цель: Изучить in vitro антиоксидантную и in vivo иммуномодулирующую активность сухого экстракта *Satureja montana*.

Материал и методы: Антиоксидантную активность сухого экстракта *Satureja montana* in vitro оценивали с использованием ORAC, HORAC и электрохимических методов. Иммуномодулирующую активность оценивали на моделях острого и хронического стресса путём измерения сывороточных уровней цитокинов TNF-α, IL-6 и IL-1β в группе из 112 самцов 8-недельных крыс Wistar. Крысы были случайным образом разделены на 7 групп для каждой из обеих моделей стресса, а затем подвергнуты ELISA -анализу (14 групп по 8 крыс в каждой). Грызунам вводили через зонд сухой экстракт *Satureja montana* (250 mg/kg и 500 mg/kg), розмариновую кислоту (15 mg/kg) и карвакрол (500 mg/kg) в течение 14 и 60 дней соответственно.

Результаты: Мы продемонстрировали, что для всех использованных методов in vitro высушенный экстракт *Satureja montana* проявлял значительную антиоксидантную активность. *Satureja montana* не приводила к значительному снижению концентрации TNF-α, IL-6 или IL-1β в сыворотке ни в одной из моделей стресса по сравнению с контрольной группой с положительным физиологическим раствором. С другой стороны, в модели острого стресса доза *Satureja montana* 250 mg/kg значительно снижала IL-6 по сравнению с карвакролом и значительно снижала TNF-α и IL-6 по сравнению с розмариновой кислотой.

Заключение: Хотя сухой экстракт *Satureja montana* обладает значительной антиоксидантной активностью in vitro, его влияние на системное воспаление до сих пор неизвестно. В будущих исследованиях предстоит изучать, как это влияет на уровень провоспалительных цитокинов в сыворотке крови.

Ключевые слова

карвакрол, цитокины, окислительный стресс, розмариновая кислота, *Satureja montana*