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ABSTRACT

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STUDY OF BIOLOGICAL EFFECTS OF METHANOLIC EXTRACT OF *MICROMERIA FRIVALDSZKYANA* (DEGEN) VELEN. (LAMIACEAE)

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The dissertation paper contains 132 pages and is illustrated with 44 figures and 12 tables. The bibliography includes 257 references.

The doctoral student works as an assistant professor at the Department of Pharmacology, Toxicology and Pharmacotherapy at the Faculty of Pharmacy, Medical university of Plovdiv.

The research was carried out in the Department of Pharmacology, Toxicology and Pharmacotherapy, the Department of Medical Biochemistry at the Faculty of Pharmacy, Medical University of Plovdiv.

The dissertation has been approved and scheduled for public defense by the Council of the Department of Pharmacology, Toxicology, and Pharmacotherapy at the Faculty of Pharmacy, Medical University of Plovdiv, held on October 4, 2024.

The doctoral student has been dismissed with the right to defend, according to Order No. R-899/31.10.2024 of the Vice-Rector for Research and Doctoral Studies at the Medical University of Plovdiv, Prof. M. Tokmakova, MD, PhD.

The dissertation paper has been scheduled for public defense at an open session of a Scientific jury consisting of:

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The public defense of the PhD dissertation will be conducted on 24.04.2025 at 11:00 h in II Auditorium Hall of the Medical University of Plovdiv.

All materials related to the procedure of the defense are available at the Department of Pharmacology, Toxicology and Pharmacotherapy and the Scientific Department and are published on the website of the Medical University of Plovdiv and can be sent if required by e-mail: Kristina.Stavrakeva@mu-plovdiv.bg.

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LIST OF ABBREVIATIONS

DNA - deoxyribonucleic acid
RA - rosmarinic acid
CNS - central nervous system
8-OH dG - 8-hydroxydeoxyguanosine
ABTS - 2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)
ALT – alanine aminotransferase
AST – aspartate aminotransferase
CAT - catalase
CCl_4 – carbon tetrachloride
COX-1 - cyclooxygenase 1
COX-2 - cyclooxygenase 2
DPPH - 2,2-diphenyl-1-picrylhydrazyl
FRAP - ferric reducing antioxidant power
GSH – reduced gluthatione
IL-1α/β – interleukin 1α/β
IL-6 - interleukin 6
IL-8 - interleukin 8
IL-12 - interleukin 12
MAPK - mitogen-activated protein kinases
MDA - malondialdehyde
NAPQI - N-acetyl-p-benzoquinone imine
NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells κB
NO – nitric oxide
Nrf2 - nuclear factor erythroid 2-related factor 2
PPT - pain pressure threshold
SOD – superoxide dismutase
t-BHP – tert-butyl hydroperoxide
TNF-α – tumor necrosis factor alpha

INTRODUCTION

Currently, there has been an increased interest in plant-based medicinal products and nutritional supplements. Approximately 80% of the global population uses herbal products for the prevention and treatment of health issues. Only 5-15% of the higher plants on Earth have been studied and utilized as sources of bioactive compounds. These trends stimulate the research of new plant species in order to uncover their therapeutic potential (Schuster, 2001; Ekor, 2014).

Species from the *Micromeria* genus have demonstrated anti-rheumatic, antiseptic, antimicrobial, antioxidant, gastroprotective, hepatoprotective, anti-inflammatory, anticholinesterase, central nervous system (CNS) stimulating, and general tonic effects. *Micromeria frivaldszkyana* is a species endemic to Bulgaria, and the information referring to it is scarce. The available data contains phytochemical studies on the metabolites of the plant and its antioxidant and antimicrobial activity. *M. frivaldszkyana* shows a very high content of rosmarinic acid and flavonoids, with hesperidin being present in the highest quantity. Research on rosmarinic acid indicates a variety of biological effects, including antioxidant, anti-inflammatory, neuroprotective, antinociceptive, and hepato-protective actions. Hesperidin has been found to exhibit antioxidant, anti-inflammatory, analgesic, and hepatoprotective effects. Based on this data, we can expect that a total extract from the plant will exhibit similar biological effects (Vukelic, 2015; Nikolova et al., 2017; Mladenova et al., 2021).

Enriching the information regarding the phytochemical content of *M. frivaldszkyana* will enhance knowledge about its composition and contribute to the characterization of its biological potential. Revealing unknown properties may serve as a source for the synthesis of new pharmaceutical drugs or dietary supplements containing extracts from the plant. Studying its toxicity will determine its relevance for use and application. The present experimental study consists of examining the qualitative and quantitative composition of the methanolic extract from *M. frivaldszkyana*, acute toxicity, analgesic and anti-inflammatory effects, impact on cognitive functions, and hepatoprotective action.

AIM

The aim of the study is to obtain a methanol extract from the aerial parts of *Micromeria frivaldszkyana* (Lamiaceae), investigate the chemical composition of the extract, and determine some of the biological effects of the obtained extract.

OBJECTIVES

1. Obtaining a methanolic extract from the aerial parts of the studied plant species.
2. Determining the chemical composition of the obtained extract.
3. Determining acute toxicity of the extract through oral administration in rats.
4. Comparative study of the analgesic effect of the extract and standard rosmarinic acid in rats.
5. Comparative study of the anti-inflammatory effect of the extract and standard rosmarinic acid in rats.
6. Comparative study of the effects of the extract on learning and memory processes in rats.
7. Investigation of hepatoprotective effect of the extract in models of hepatotoxicity in rats.

MATERIALS AND METHODS

1. Materials

1.1. Plant material

Aerial parts of *M. frivaldszkyana* were collected in June (during full bloom) during the vegetation period of 2019-2020 within the Bulgarka Nature Park, floristic region of Middle Stara Planina Mountain, in the area of Mount Shipka. Herbarium samples from *M. frivaldszkyana* were deposited in the herbarium of the Agricultural University, Plovdiv (SOA), with designated reference number 062648.

1.1.1. Phytochemical analysis

The primary and secondary metabolites are analyzed from the polar phase, while lipid studies are conducted using the organic phase. A total of 500 µL of a water/methanol solution (3:1 v/v) was used for phase separation. A total of 400 µL of the upper lipophilic phase was dried, re-suspended in an acetonitrile/isopropanol mixture (7:3) and analyzed on an Orbitrap LC-MS system (Exactive, Thermo Scientifc). The primary metabolites were analyzed by GC-MS with an obligatory step of derivatization, performed according to Lisec et al. (Lisec et al., 2006). For the specialized metabolites, the aqueous methanolic extracts were transferred to LC-MS glass vials and analyzed using Thermo Q Exactive Focus (Thermo Scientific, Waltham, MA, USA) with a reverse-phase C18 column chromatography. The annotation of the metabolites analyzed by GC-MS was carried out using the Golm Metabolome Database (Kopka et al., 2005).

The data shown are based on six biological replicates. R version 4.3.0 was applied for the analyses of primary and secondary metabolites (R Core Team, 2023). The _ggplot2_package was used for the preparation of the boxplots (Kassambra, 2022). The top 20 most abundant metabolites were determined and ordered based on their mean relative abundance. The lipidomics data were imported into Microsoft Excel, and each of the detected lipid groups was calculated as a percentage of the total lipid content. The data were presented as the means of six biological replicates.

1.2. Animals used

The experiments are conducted on male Wistar rats with an average weight of 150-270 g. After acquisition from the Vivarium of the Medical University of Plovdiv, the animals are distributed in cages in groups of 8 according to the requirements of the Bulgarian Food Safety Agency (BFSA) with permit No. 352/30 May 2023 and protocol No. 6/05 October 2023 from the Ethics Committee of the Medical University of Plovdiv. They are provided with standard laboratory food and water *ad libitum*, and standard laboratory conditions are maintained in the rooms (temperature 22 ±1°C, air humidity 45%, and a 12-hour light/dark cycle). Before the experiments begin, the rats are allowed to acclimate to the new environment for 24 hours. The experiments are conducted in compliance with the requirements of the Helsinki Convention and Regulation No. 20 of November 1, 2012.

2. Methods

2.1. Preparation of plant material

The freshly cut plants are sorted, dried in a drying oven with active ventilation at 30°C, and stored at room temperature in the dark. The plant material is ground by a mechanical mill to a particle size of less than 400 µm. The ground samples are stored in paper bags until the analysis is performed.

For the extraction process, 10 g of powdered plant material was extracted in 70% methanol (1:10 w/v). The mixture was continuously stirred for 24 h at room temperature in a flask protected from light. In addition, a triplicate ultrasonic extraction was performed, consisting of 3 cycles of 15 min at 30°C. After centrifugation at 6000 g for 15 min, the resulting supernatant was filtered using Whatman No. 1 filter paper. The same extraction procedure was repeated twice on the remaining plant material. The three extracts were combined, and the solvent was evaporated under reduced pressure using a rotary evaporator (Heidoplh, Germany) at 50°C until complete dryness was achieved.

The dried extract was dissolved in water until the corresponding concentrations required for the experiments were obtained.

2.2. Acute toxicity of the extract and determination of LD_{50} through oral administration in rats

Acute toxicity testing was performed as described in the scientific literature with some modifications (Zheleva-Dimitrova et al., 2019). Eight groups of three animals (160–270 g) were treated orally with a water solution of the desiccated methanolic extract in doses of 5000, 2000, 1500, 1000, 800, 600, 400, and 200 mg/kg. Substantial observation was conducted for 24 h, with a focus on lethality and signs of toxicity. At the 24th hour, the mortality in each group was checked.

The LD_{50} value was calculated by the following formula: $LD_{50} = \frac{[M_0 + M_1]}{2}$, where M0 is the highest dose leading to no mortality among treated animals and M1 is the lowest dose at which mortality is detected among treated animals.

The number of the rats was chosen according to the current principle of the 3R for the humane treatment of laboratory animals (Replacement, Reduction and Refinement). In order to decrease the number of animals used in the experiments, we employed groups of 3 in accordance with a recent research paper. After determining the LD_{50}, studies on biological effects continue with doses of 1/10 and 1/20 of the established LD_{50} (Zheleva-Dimitrova et al., 2019; Hanafy et al., 2016).

2.3. Investigation of analgesic activity

2.3.1. "Hot plate" test with thermal stimulus

The "hot plate" test was conducted using the Hot Plate apparatus by Ugo Basile, Italy. The animals (n=8) were treated with the tested substances for 14 days as follows:

Group 1 (control): 0.1 ml/100 g body weight of saline

Group 2 (positive control): Metamizole 150 mg/kg body weight (administered once on the day of testing)

Group 3: Methanolic extract at a dose of 250 mg/kg body weight

Group 4: Methanolic extract at a dose of 400 mg/kg body weight

Group 5: Methanolic extract at a dose of 500 mg/kg body weight

Group 6: Solution of rosmarinic acid in saline 30 mg/kg body weight

Immediately after the last administration of the substances, the test is conducted at a constant plate temperature of 55°C (±5°C), while the time for vocalization, avoidance, or licking of the hind paw recorded in seconds. Three additional tests are performed at 60, 120, and 180 minutes following the injection. The maximum time the animal can remain on the plate is 30 seconds.

2.3.2. Mechanical stimulus test "Analgesimeter"

The animals were treated with the substances listed in section 2.2.1 for 14 days.

Thirty minutes after the last application, testing was conducted using an Analgesimeter (Ugo Basile, Italy). The test involves gradual application of increasing mechanical pressure to the hind paw of the rat. The animal withdraws its limb when the pain threshold is reached. The pressure force (in relative units of PPT on the linear scale of the apparatus) at which the motor response occurs is recorded. Testing is performed immediately after injection and at 60, 120, and 180 minutes following the administration of the substances.

2.4. Investigation of anti-inflammatory activity in a model of hind paw inflammation in rodents

Male Wistar rats (weight 150-180 g) were divided into 6 groups (n=8) and treated orally as follows:

Group 1 (control): 0.1 ml/100 g body weight of saline

Group 2 (positive control): Diclofenac sodium at a dose of 25 mg/kg body weight (administered once on the day of testing)

Group 3: Methanolic extract at a dose of 250 mg/kg body weight

Group 4: Methanolic extract at a dose of 400 mg/kg body weight

Group 5: Methanolic extract at a dose of 500 mg/kg body weight

Group 6: Solution of rosmarinic acid in saline at a dose of 30 mg/kg body weight

The saline, *M. frivaldszkyana* extracts, and RA were applied orally for 14 days using a gastral tube, while diclofenac was given once on the day of the experiment via the same route. One hour after the last treatment a one-percent solution of λ-carrageenan in saline (0.1 mL) was injected subplantarly into the right hind paw. A plethysmometer apparatus (Ugo Basile, Gemonio, Italy) was employed to measure the paw volume before the injection (V_0) and at the 1st, 2nd, 3rd, 4th, and 5th hours after the injection (Vn).

The percentage of the increase in the paw volume was evaluated according to the following formula:

$$(\%) = [(Vn - V_0)/V_0] \times 100$$

Where:

Vn - right paw edema measured after carrageenan injection at the n-th hour

Vo - edama of the same paw of the same animal measured before carrageenan injection

2.5. Investigation of the effect on cognitive functions

Animals (10 per group) were treated orally for 14 days as follows:
Group 1 (control): 2 ml per animal of distilled water daily
Group 2: Methanolic extract at a dose of 250 mg/kg body weight
Group 3: Methanolic extract at a dose of 400 mg/kg body weight
Group 4: Methanolic extract at a dose of 500 mg/kg body weight

2.5.1. Investigation of locomotor activity and exploratory reflex

An automatic cage is used to record the horizontal and vertical activity of rats with the help of photo-sensors (Activity cage). The horizontal activity and vertical rearing are measured for 5 minutes for each animal individually. The study is conducted immediately before the first training session in the Shuttle-box apparatus (on the first day of the experimental session), as well as on the 7th and 14th days of the experimental session.

2.5.2. Two-way active avoidance test

The experiments were conducted by Automatic Reflex Conditioner apparatus from Ugo Basile, Italy.

An initial training session was carried out over 5 days. Each day, the rats underwent 30 trials with the following parameters: 6 seconds of simultaneous activation of a light and sound stimulus (670 Hz, 70 dB), followed by 3 seconds of electrical stimulation (0.4 mA) via the grid floor of the cage. There was a 12-second pause between each trial. The electrical stimulation was terminated if the animal moved to the other side of the cage.

The memory test was performed on the 12th day using the same parameters, without the electrical stimulation.

2.5.3. Step-through passive avoidance test

On the first day, rats were placed in the apparatus for environmental familiarization for 1 minute, then were subjected to 3 training sessions at 60-minute intervals according to the standard program of the apparatus with the following parameters: a 7-second delay before the door opens, followed by 12 seconds with the door open.

If the dark compartment is entered by a rat, the door is closed, and electrical stimulation (0.4 mA) is applied for 3 seconds. If the rat does not enter the dark compartment, a timer automatically starts, recording a maximum of 3 minutes (180 ± 2 seconds).

The criterion for learning is considered when the animal stays in the light compartment of the apparatus for the maximum time of 178 seconds during two consecutive trials.

On the following day, another training session was conducted. The long-term memory test was performed 24 hours after the training session, following the same program, without electrical stimulation.

2.5.4. Step-down passive avoidance test

On the first day, rats were placed in the apparatus for environmental familiarization for 1 minute, then were subjected to 3 training sessions at 60-minute intervals according to the standard program of the apparatus. The animal is being placed on a plastic platform, which vibrates vertically once the apparatus is activated. If the rat steps off the platform with 3 or 4 paws, electrical stimulation (0.4 mA) is applied through the grid floor of the cage for 3 seconds. The following day, another training session was conducted.

The animal is considered trained if it stays on the platform for more than 60 seconds during two consecutive trials.

The long-term memory test was conducted 24 hours after the training session, following the same program, but without the electrical stimulation. The maximum time the animal can stay on the platform is 5 minutes (300 seconds).

2.5.5. Investigation of spatial working memory (Y-maze)

The Y-maze is a maze consisting of three arms, each 50 cm in length, 10 cm in width, and with walls 30 cm high. The arms are arranged at a 120° angle and are randomly labeled as A, B, and C. The rats are being placed in the center of the maze and their movement is tracked for 8 minutes. The sequence of entries into the arms of the maze was recorded. An entry into an arm is considered when the rat passes all four paws into the arm.

Normally, alternating entries into the arms are observed, for example, ABC, CBA, ACB, etc. (alternation). The number of alternations is determined based on overlap, for example, a sequence such as A–C–B–C–A contains 2 alternations.

The percentage of spontaneous alternations (SA%) is calculated using the following formula:

SA% = [number of spontaneous alternations / (total number of entries – 2)] × 100

After testing each rat, the maze is cleaned with 70% ethanol before the next animal is placed inside.

2.5.6. Investigation of anxiety behavior using the Elevated Plus Maze (X-maze)

The elevated plus maze consists of two open arms and two closed arms arranged in a cross shape, positioned 50 cm above the floor. The animals were placed in the center of the maze and observed for 5 minutes. The following parameters were recorded: time spent and number of entries into the open and closed arms, total number of arm entries, and the ratio of entries into the open arms to the total number of entries. The criterion for reduced anxiety in the animals is increased time spent in the open arms, an increase in the number of entries into the open arms, and an increase in the ratio of entries into the open arms to the total number of entries. After testing each rat, the maze is cleaned with 70% ethanol before placing the next animal.

2.5.7. New object recognition test

The test was conducted in a transparent plastic cage (Activity cage setup). Each rat was placed in the apparatus for 5 minutes and allowed to familiarize itself with the environment. After each animal was removed from the apparatus, it was cleaned with ethanol to remove any scent before placing the next animal. On the second day, testing was carried out by placing two identical objects at opposite ends of the apparatus. The rat was placed in the apparatus for 5 minutes and allowed to explore the objects. After 1.5 hours, a recognition memory test was performed, in which one of the previously familiar objects is replaced with a new one, differing in shape and colour. The time spent exploring each object and the total exploration time are recorded. The indicator of recognition memory is discrimination index (DI). It is calculated using the formula: DI = time spent exploring the new object / (time spent exploring the new object + time spent exploring the familiar object). Higher DI values indicate better recognition memory.

2.6. Investigation of hepatoprotective action

2.6.1. Model of paracetamol-induced hepatotoxicity

Male Wistar rats (n=8) were treated orally for 7 consecutive days as follows:

Group 1 (Control): 0.1 ml/100 g body weight of saline without liver injury (no treatment)

Group 2: Methanolic extract at a dose of 500 mg/kg body weight (no liver injury)

Group 3: 0.1 ml/100 g body weight of saline (liver injury induction)

Group 4: Methanolic extract at a dose of 250 mg/kg body weight

Group 5: Methanolic extract at a dose of 400 mg/kg body weight

Group 6: Methanolic extract at a dose of 500 mg/kg body weight

Group 7: Rosmarinic acid at a dose of 100 mg/kg body weight

Group 8: Silymarin at a dose of 125 mg/kg body weight

In the evening of the 6th day, all animals were fasted for 12 hours. During this period, they had access only to water. No food was provided. On the 7th day, animals from groups 3, 4, 5, 6, 7, and 8 were treated orally with 2000 mg/kg body weight of paracetamol to induce hepatotoxicity. Three hours after, the substances were administered. Forty-eight hours following the paracetamol administration, the animals were decapitated, and blood was collected for biochemical analysis of liver function markers. The entire liver was excised, washed with ice-cold physiological saline, and then homogenized for the determination of oxidative stress markers.

2.6.2. Model of t-BHP-induced hepatotoxicity

Animals were distributed into groups as follows:

Group 1 (control): 0.1 ml/100 g body weight of saline without liver damage

Group 2: Methanolic extract at a dose of 500 mg/kg body weight without liver damage

Group 3: 0.1 ml/100 g body weight of saline (liver injury induction)

Group 4: Methanolic extract at a dose of 250 mg/kg body weight

Group 5: Methanolic extract at a dose of 400 mg/kg body weight

Group 6: Methanolic extract at a dose of 500 mg/kg body weight

Group 7: Rosmarinic acid 100 mg

Group 8: Silymarin 125 mg/kg body weight

The method described by Yang et al. was used (Yang et al., 2013). For 5 days, the animals were treated with the listed substances under investigation. On the 5th day of treatment, 30 minutes after the administration of the substances, animals from groups 3, 4, 5, 6, 7, and 8 were intraperitoneally injected with 0.5 mmol/kg t-BHP. Eighteen hours after the administration of t-BHP, the animals were decapitated, and blood was collected for biochemical analysis of liver function markers, along with the entire liver for the determination of oxidative stress markers.

2.7. Determination of markers for liver function assessment in serum

To assess liver function, the enzymatic activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum was measured using a spectrophotometric method. The same method was applied for determining total and direct bilirubin levels.

2.8. Determination of markers for oxidative stress and antioxidant defence

The antioxidant effect of the studied extract in liver tissue homogenates was determined by the levels of reduced glutathione (GSH) and catalase (CAT) using the ELISA method. The degree of lipid peroxidation was assessed in serum by measuring the level of malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) using the same method described by Ohkawa et al. (Ohkawa et al., 1979).

2.9. Determination of pro-inflammatory cytokine levels in liver tissue homogenate

The levels of pro-inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in liver tissue homogenates of rats with paracetamol-induced hepatotoxicity were determined using the ELISA method.

3. Statistical analysis of the results

The results of the experiments were analyzed using SPSS 19.0 software (SPSS Inc, Chicago, IL, USA). For each parameter in each group, the arithmetic mean and its standard error (mean ± SEM) were calculated, and differences were considered statistically significant at $p<0.05$. The distribution of the obtained results was assessed using the Kolmogorov-Smirnov test. In case of normal distribution, one-way analysis of variance (ANOVA) was used. Comparison of the experimental groups and the control group was performed using the Tukey multiple comparison test.

RESULTS AND DISCUSSION

1. Phytochemical composition

M. frivaldszkyana is a Bulgarian endemic species, whose phytochemical composition and therapeutic activity are poorly studied. Available data are primarily related to the essential oil content in species from the genus (Sarikurkcu et al., 2020). The lack of sufficient information in the scientific literature directs the present study towards exploring the phytochemical composition of the plant. Investigating the metabolome of *M. frivaldszkyana* will contribute to clarifying the pharmacological activity of the plant.

M. frivaldszykyana dry powder was subjected to metabolite extraction by using 70% methanol and a subsequent fractionation and solvent evaporation using a rotary evaporator. The polar phase was used for both the GC-MS and UPLC-MS-MS analyses, and the non-polar fraction was used for the lipid investigation.

- **Analysis of primary metabolites**

Primary metabolites in plants are essential for their growth and development but are not directly related to their biological activity. The study of the primary metabolome is included in the current research due to its connection with secondary biochemical pathways, which will allow for a deeper understanding of the plant's metabolism. Additionally, a lipidomic analysis was performed, which complements the existing knowledge about the activity of certain representatives of this class of compounds (Hussein et al., 2017).

The GC-MS analysis resulted in the identification of 83 coumpounds, classified as amino acids, organic acids, sugars, and sugar alcohols. Sucrose was the most abundant metabolite, followed by different sugars and sugar alcohols such as glucose, mannose, fructose, maltose, galactoinol, myo-inositol, and glycerol (figure 1). In addition, significant amounts of citric and quinic acids were detected. Proline exhibited the highest levels among the amino acids, followed by alanine. Relatively high amounts of valine and pyroglutamic acid were also detected in the samples. Overall, the analysis of the semi-polar phase showed a high presence of sugars and sugar alcohols.

Lipids are primary plant metabolites; therefore, a lipidomic study on the non-polar fraction was also conducted. A total of 163 lipid compounds were identified and distributed in 10 lipid classes: diacylglycerols, digalactosyldiacylglycerols, lisophospholipids, lysomonogalactosyldiacylglycerols, lysodigalactosyldiacyl-glycerols, monogalactosyldiacylglycerols, phosphatidylcholine, phospholipids, sphingolipids, and triacylglycerols (figure 2). In order to better visualize the lipid type distribution, the respective percentages of the total lipid content for each lipid group are presented. The results showed triacylglycerols as the most abundant lipid group.

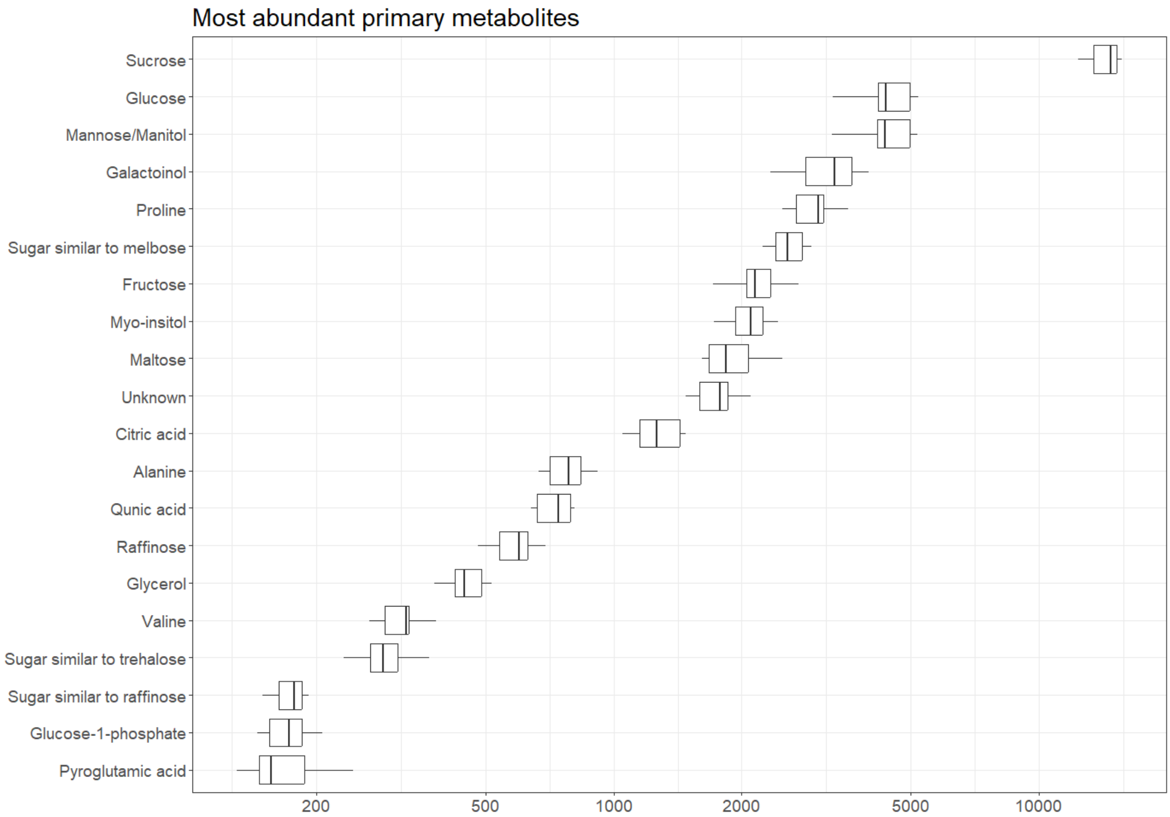

Figure 1. A boxplot representing primary metabolites in *M. frivaldszkyana* ordered according to their relative abundance (RA) (x-axes). The presented data are from six biological replicates measured by GC-MS. Black lines within the boxes indicate medians, and whiskers indicate upper and lower quantiles.

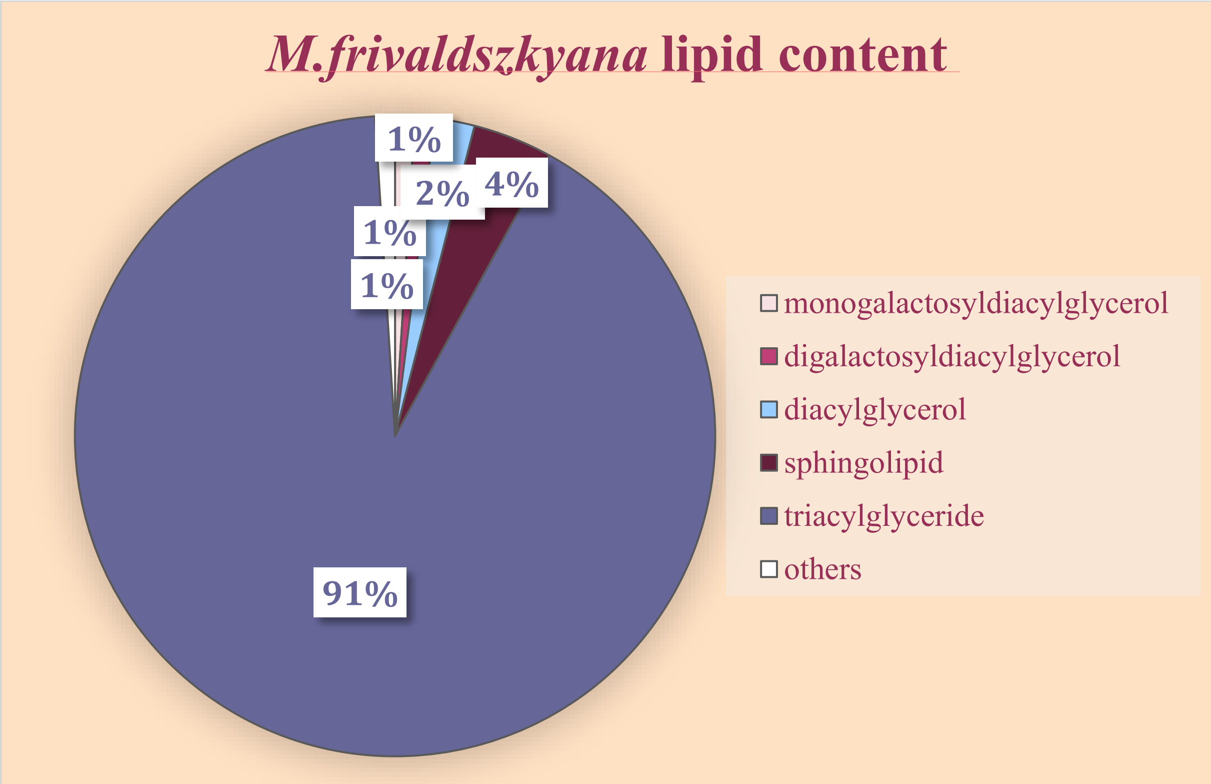

Figure 2. Lipid content of *M. frivaldszkyana*. Lysophospholipids, lysomonogalactosyldiacylglycerols, lysodigalactosylglycerols, phospholipids, phosphatidylcholine, and phosphatisylethanolamine are given as "Others", since they represent less than 1% of the total lipid amount.

- **Analysis of Secondary Metabolites**

Plants synthesize a wide variety of secondary metabolites, among which are compounds with potential biological activity (Guerriero et al., 2018). The main groups of secondary metabolites are analyzed in order to identify the compounds that determine the therapeutic characteristics of *M. frivaldszkyana*.

The untargeted UPLC-MS-MS analysis of the *M. frivaldszkyana* methanolic extract samples resulted in the detection of 192 compounds. The number of the identified compounds was 123, while 69 remain unknown. The secondary metabolites with highest concentrations were flavonoids, predominantly flavonoid glucosides. The highest levels in the samples were registered for linarin and its derivatives, quinic acid, and derivatives of quercetin, kaempferol, naringenin, and apigenin. Rosmarinic acid was among the most prominent substances detected and was further selected as a reference compound in the experimental design (figure 3).

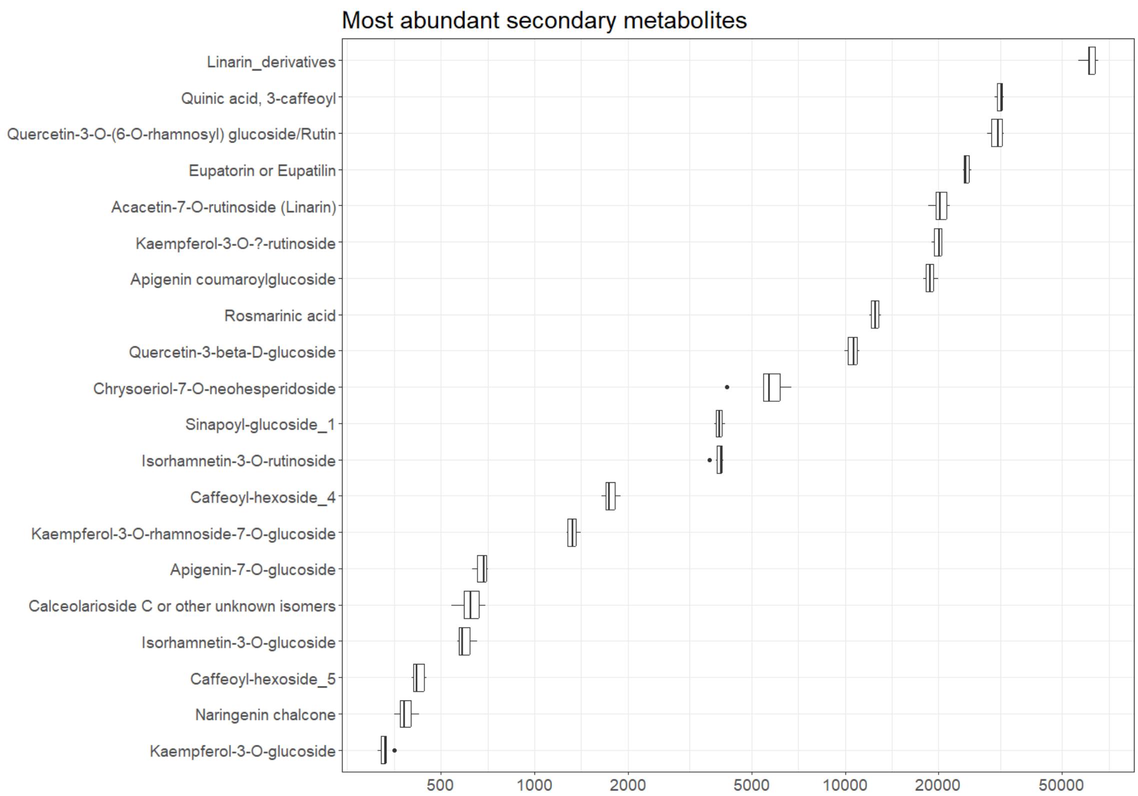

Figure 3. A boxplot of secondary metabolites in *M. frivaldszkyana* measured by UPLC-MS-MS and ordered according to their relative abundance (RA) ($n = 6$). Black lines within the boxes indicate medians, whiskers indicate upper and lower quantiles, and black dots indicate outliers.

The observed results differ from those reported for other species of the same genus. The samples of *M. frivaldszkyana* show significantly higher levels of Ca^{2+} (13 223 mg/kg) and Na^{+} (109 mg/kg), likely under the influence of the specific environmental conditions in the species' habitat. The content of other elements (K, Zn, Mn) also stands out with higher levels compared to *M. croatica* and *M. pseudocroatica* (Kremer et al., 2012).

The results of the metabolomic study confirm a significant portion of the available published data regarding the content of sugars and organic compounds (figure 1) (Mladenova et al., 2021), but provide additional information on the polyphenolic content of the methanolic extract from the plant.

Linarin or acacetin 7-O-rutinoside and its derivatives are among the secondary metabolites in the highest quantities, along with rosmarinic acid and other flavonoid glycosides (figure 3). Flavonoids and their glycosides are described in the literature as compounds with various biological functions and low toxicity (Yang et al., 2018).

Sarikurkcu et al. (2020) established that the main phenols in the water and methanolic extracts of the plant *M. myrtifolia* are: rosmarinic acid, chlorogenic acid, and caffeic acid. In the ethyl acetate extract, the same researchers reported rosmarinic acid and apigenin as the main compounds (Sarikurkkcu et al., 2020).

Al-Hamwi et al. also found the presence of chlorogenic acid in the ethanolic extract of *M. barbata* (Al-Hamwi et al., 2015).

In a study on the qualitative composition of species from *Micromeria*, Nikolova et al. (2017) report that the main components of the acetone extract of *M. cristata* and *M. juliana* are apigenin, luteolin, and their derivatives, while chlorogenic acid is found in the methanolic extracts of *M. dalmatica* and *M. frivaldszkyana* (Nikolova et al., 2017).

The methanolic extract of *M. fruticosa* was studied by Abu-Gharbieh et al. (2016), who found a high content of phenolic acids and flavonoids, with quercetin-3-O-rutinoside and rosmarinic acid being the most abundant, followed by rutin, kaempferol-3-O-rutinoside, and apigenin (Abu-Gharbieh et al., 2016).

Vladimir-Knežević et al. (2011) determine and compare the polyphenolic content of *M. croatica*, *M. juliana*, and *M. thymifolia*. The data they obtain for the ethanolic extracts of the plants correlate with our results. Among the flavonoids, derivatives of acacetin, apigenin, and luteolin are found. Among the phenolic acids, rosmarinic acid and chlorogenic acid dominate, with rosmarinic acid being more prevalent. (Vladimir-Knežević et al., 2011).

The identification of polyphenolic compounds in the ethanol extract of *M. graeca* also reported rosmarinic acid, caffeic acid, chlorogenic acid, and gallic acid, with rosmarinic acid being the predominant one. Regarding the flavonoid content, the main identified compounds are apigenin, apigenin-7-glucoside, and diosmin (Brahmi et al., 2017).

2. Laboratory tests

2.1. Acute toxicity

During the evaluation of the acute toxicity, no toxic effects were registered after a single oral application of the extract in doses: 200, 400, 600, 800, 1000, 1500, 2000 and 5000 mg/kg bw. Based on these results, we estimated the doses for further evaluation of the effects of the extract as 250 and 500 mg/kg bw. According to Hanafy et al. (2016), the doses suitable for further evaluation of the pharmacological effects of extracts of natural origin are 1/10 and 1/20 of the estimated LD_{50} (5000 mg/kg bw in this case) (Hanafy et al., 2016). We added the intermediate dose 400 mg/kg to obtain more detailed data on the potential effect of the extract.

As far as the author is aware, this is the first *in vivo* study on the toxicity of *M. frivaldszkyana* extract. The results show no toxicity after oral administration of the methanolic extract of the plant to male Wistar rats. Additional experiments are needed to extrapolate these findings to other biological species.

2.2. Investigation of analgesic effect

2.2.1. Test with mechanical pressure (Analgesimeter)

No antinociceptive effect of the methanolic extract from *M. frivaldszkyana* was observed at the three tested doses (250 mg/kg, 400 mg/kg, 500 mg/kg body weight) after 14-day administration in the paw pressure test in rats.

2.2.2. "Hot plate" test

Table 1. Antinociceptive effect of methanolic extract of *M. frivaldszkyana* in "hot plate" test in rats

hour / *groups*	*H0*	*H1*	*H2*	*H3*
control	6.45 ± 0.51	8.14 ± 0.68	7.14 ± 0.54 ℣℣℣	6.75 ± 0.47 ℣℣℣
metamizole 150 mg/kg	9.44 ± 1.18	10.48 ± 0.35	10.64 ± 0.54	10.21 ± 0.36
m. extract 250 mg/kg	7.88 ± 0.88	9.19 ± 1.35	8.11 ± 0.73 ℣	7.59 ± 0.45 ℣℣℣
m. extract 400 mg/kg	9.08 ± 0.47	8.64 ± 0.76	7.85 ± 0.35 ℣	7.74 ± 0.49 ℣℣
m. extract 500 mg/kg	7.95 ± 0.48	7.01 ± 0.41 ℣	6.97 ± 0.65 ℣℣℣	6.54 ± 0.49 ℣℣℣
rosmarinic acid 30 mg/kg	6.49 ± 0.43	7.10 ± 0.67 ℣	7.50 ± 0.57 ℣℣	6.53 ± 0.21 ℣℣℣

Note: Values are presented as mean ± SEM.
℣ $p<0.05$ compared to metamizole; ℣℣ $p<0.01$ compared to metamizole;
℣℣℣ $p<0.001$ compared to metamizole

As seen from Table 1, the extract of the plant does not show analgesic effects after 14 days of administration in thermal pain stimulus test. No data regarding analgesic effects of species from the *Micromeria* genus was found in the scientific literature. Rosmarinic acid, which is present in large amounts in the methanolic extract of *M. frivaldszkyana*, exhibits antinociceptive effects after oral administration in mice in abdominal constriction test with acetic acid, "hot plate" test, and formalin test (Lucarini et al., 2013; Boonyarikpunchai et al., 2014). Rahbardar G et al. (2017) report an improvement in the symptoms of neuropathic pain induced by ligation of the sciatic nerve after 14 days of treatment in rats. Reduced levels of several pro-inflammatory cytokines (NO, IL-1β, PGE-2, COX-2, and matrix metalloproteinase 2) were also observed in animals treated with rosmarinic acid (Rahbardar et al., 2017).

Our results (Tables 2 and 3) demonstrate the absence of analgesic effects in "hot plate" and "analgesimeter" tests, which is likely primarily due to the extract's influence on inflammatory processes (Lucarini et al., 2013; Rahbardar et al., 2017). The obtained data is supported by the study of Criscensia et al. (2023), where the examined eupatorin did not demonstrate an analgesic effect in "hot plate" test and "tail-flick" test (Criscensia et al., 2023). It is believed that, similar to the studied eupatorin, which is found in large quantities in *M. frivaldszkyana*, the mechanism is peripheral rather than central (Gupta et al., 2005). The peripheral analgesic activity of eupatorin, as well as the methanolic extract of *M. frivaldszkyana*, may be the result of modulation of pain of an inflammatory origin (Chai et al., 2014).

2.3. Investigation of the anti-inflammatory effect in a hind paw inflammation model in rodents

The carrageenan-induced paw edema in rats is a widely used model for examination of new anti-inflammatory agents. This model was selected for the dissertation due to the opportunity it provides for evaluating the specific phase of inflammation in which the extract is active. Depending on the activity recorded during a particular phase, possible target molecules associated with this activity can be predicted.

According to Figure 4, no statistically significant differences in paw volume are observed 30 minutes after the administration of carrageenan. The standard anti-inflammatory drug diclofenac significantly reduces the swelling of the hind paw at the following time points: 60, 120, 180, 240, 300 minute of the experiment compared to the control group (17.87 ± 3.38 vs. 35.72 ± 2.28, p<0.01; 20.87 ± 2.78 vs. 49.61 ± 1.96, p<0.001; 25.89 ± 4.15 vs. 57.57 ± 2.08, p<0.001; 23.99 ± 3.56 vs. 62.12 ± 2.88, p<0.001; 27.57 ± 3.43 vs. 63.04 ± 2.33, p<0.001), with this trend established 30 minutes after administration. (15.98 ± 2.20 vs. 23.85 ± 2.28).

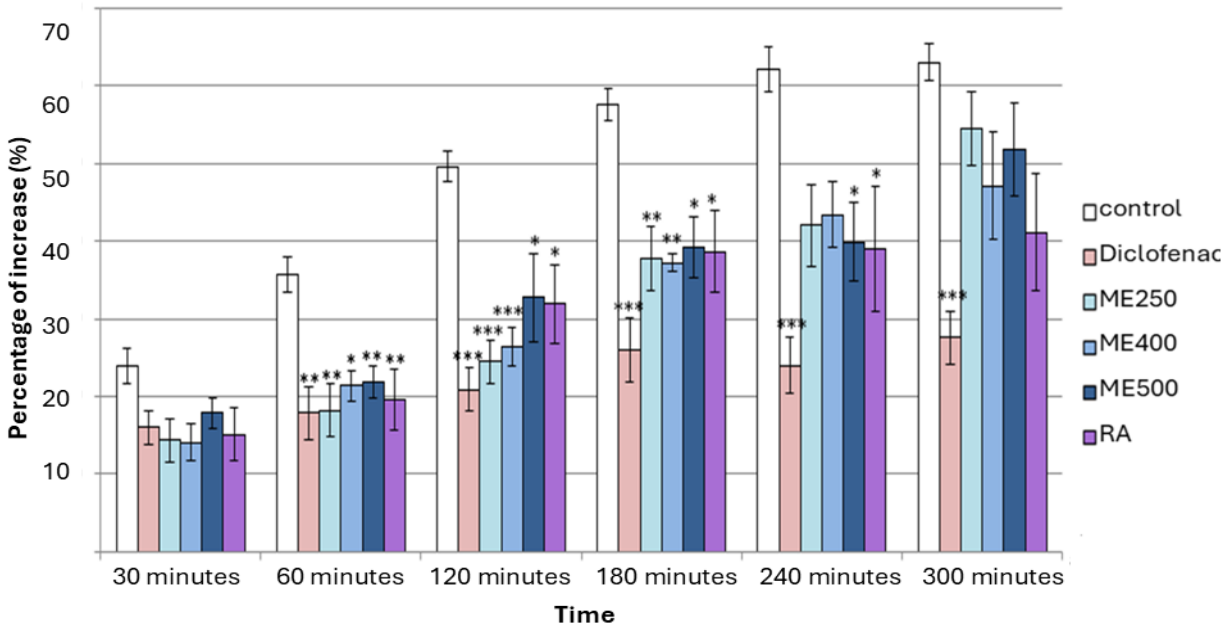

Figure 4. Effects of methanolic extract of *Micromeria frivaldszkyana* in carrageenan-induced hind paw edema in rats

Note: *p<0.05 compared to the control at the same time point; **p<0.01 compared to the control at the same time point; ***p<0.001 compared to the control at the same time point

At the first hour of the study, a significant decrease in the values of the methanolic extract of *M. frivaldszkyana* was observed in all tested doses – 250 mg/kg, 400 mg/kg, and 500 mg/kg, compared to the control group (18.21 ± 3.38 vs. 35.72 ± 2.28, p<0.01; 21.33 ± 1.89 vs. 35.72 ± 2.28, p<0.05; 21.85 ± 1.99 vs. 35.72 ± 2.28, p<0.01).

The same trend is observed at the second hour of the experiment in the ME250, ME400, and ME500 groups compared to the control group (24.46 ± 2.84 vs. 49.61 ± 1.96, p<0.001; 26.42 ± 2.45 vs. 49.61 ± 1.96, p<0.001; 32.70 ± 5.66 vs. 49.61 ± 1.96, p<0.05).

The trend can be seen at the third hour in rats treated with doses of 250 mg/kg, 400 mg/kg, and 500 mg/kg compared to the control group (37.83 ± 4.12 vs. 57.58 ± 2.08, p<0.01; 37.24 ± 1.20 vs. 57.58 ± 2.08, p<0.01; 39.25 ± 3.96 vs. 57.58 ± 2.08, p<0.05).

The effect is also present at the fourth hour, but statistical significance is observed only for the highest tested dose of 500 mg/kg compared to the control group (39.88 ± 5.12 vs. 62.12 ± 2.88, $p<0.05$).

Similar results are observed with rosmarinic acid, which exhibits significant anti-inflammatory activity at the same time points—first, second, third, and fourth hour after injection—compared to the control group of rats (19.54 ± 3.97 vs. 35.72 ± 2.28, $p<0.01$; 31.88 ± 5.01 vs. 49.61 ± 1.97, $p<0.05$; 38.66 ± 5.21 vs. 57.58 ± 2.09, $p<0.05$; 39.05 ± 8.08 vs. 62.12 ± 2.88, $p<0.05$).

The inflammatory response to carrageenan consists of two phases. During the first phase (the first hour after injection), the observed inflammation is associated with increased production of histamine, serotonin, and bradykinin. The second phase is characterized by the production of free radicals, pro-inflammatory cytokines, nitric oxide synthesis, neutrophil infiltration, cyclooxygenase 2 (COX-2) activation, and consequently the production of prostaglandins (Halici et al., 2007; Zhang et al., 2020).

Our results show that the methanolic extract of *M. frivaldszkyana*, administered orally for 14 days, reduces the hind paw edema during the early phase of inflammation (from the first to the third hour after carrageenan injection) (figure 4). This result may be associated with reduced production of free radicals, lower levels of proinflammatory cytokines, nitric oxide, and decreased COX-2 activity. The observed anti-inflammatory activity could be linked to the antioxidant properties of the extract (Nikolova et al., 2017).

Anti-inflammatory activity has been reported for many of the compounds present in high concentrations in the methanolic extract.

Anti-inflammatory properties (reduced COX-2 activity and nitric oxide production) have been demonstrated for luteolin *in vitro* (Tian et al., 2020; Mottaghipisheh et al., 2021), while *in vivo* experiments show reduced levels of TNF-α in the serum of an LPS-induced experimental model (Ye et al., 2022). Our experiment does not include luteolin as a standard due to its poor absorption after oral administration (0.47% bioavailability) (Li et al., 2019).

The second metabolite present in the highest levels in the extract is 3-O-caffeoylquinic acid (chlorogenic acid), which is known for its anti-inflammatory activity. *In vitro* experiments show reduced production of nitric oxide and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in a lipopolysaccharide-induced inflammation model in rodents (Hwang et al., 2014). Oral administration of the compound activates the nuclear erythroid factor-2 (Nrf2) pathway and suppresses inflammation in rats treated with thioacetamide (Hussein et al., 2021; Magaña et al., 2021). Chlorogenic acid also interacts with the NF-κB pathway and induces changes in the levels of pro-inflammatory and anti-inflammatory cytokines (Chen et al., 2018; Murai et al., 2023).

Anti-inflammatory activity has also been reported for rutin and its glycosides *in vitro* (Choi et al., 2021). Possible mechanisms include reduced levels of IL-6, IL-1β, TNF-α, and inhibition of the nuclear factor-kappa B (NF-κB)/mitogen-activated protein kinase (MAPK) pathway (Muvhulawa et al., 2022). In addition, this compound reduces edema in the same inflammation model of the hind paw in rats (Selloum et al., 2003).

Eupatorin also exhibits anti-inflammatory effects in carrageenan-induced hind paw edema model in rodents and the ear inflammation model induced by 12-O-tetradecanoylphorbol-13-acetate. The possible mechanism is associated with decreased synthesis of TNF-α and COX-2 (Laavola et al., 2012; Gonzalez-Cortazar et al., 2022; Chriscensia et al., 2023).

There are studies on the anti-inflammatory activity of kaempferol-3-O-rutinoside and apigenin — compounds that are also present in high levels in the methanolic extract of *M. frivaldszkyana*. Their activity, both *in vitro* and *in vivo*, has been described in numerous review articles, some of which also include results from clinical trials (Ali et al., 2017; Ginwala et al., 2019; Alam et al., 2020; Yoon et al., 2023).

The compound with the best-known anti-inflammatory activity, found in large quantities in the extract, is rosmarinic acid (RA). A significant reduction in the hind paw edema in mice induced by carrageenan injection has been observed (Boonyarikpunchai et al., 2014). Similar results have been reported by other authors using this inflammation model in rats. In addition to reducing edema, they also reported decreased levels of pro-inflammatory cytokines IL-1β and TNF-α in the serum of rodents treated with rosmarinic acid (Usha et al., 2014). *In vitro* studies also show decreased levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Yoou et al., 2016). Other inflammatory diseases in which the anti-inflammatory effect of rosmarinic acid (RK) has been established include Freund's adjuvant-induced arthritis and collagen-induced arthritis (Youn et al., 2003; Gautam R et al., 2019), colitis (Zhao et al., 2018), periodontitis (Zdarilova et al., 2009; Luo C et al., 2020).

It can be concluded that the observed anti-inflammatory activity of the extract is likely a result of the high content of flavonoids, for which such characteristics have been reported (chlorogenic acid, rutin, eupatorin, kaempferol-3-O-rutinoside, apigenin, etc.).

2.4. Methods for investigation of the effect on cognitive function

2.4.1. Activity cage

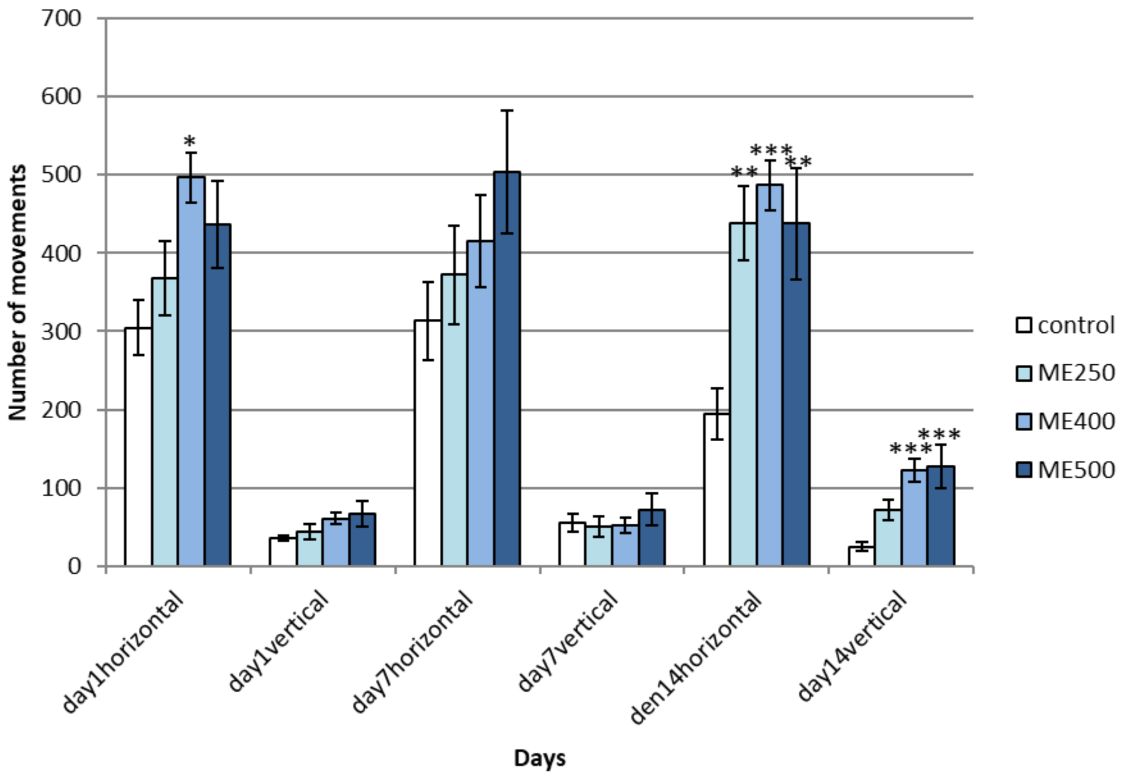

Figure 5. Effect of the methanolic extract of *M. frivaldszkyana* on the number of horizontal and vertical movements in rats, examined using the Activity Cage apparatus.

Note: *p<0.05 compared to the control; **p<0.01 compared to the control; ***p<0.001 compared to the control

The results of the study on motor activity in the groups treated with *M. frivaldszkyana* extract (figure 5) show a trend of increase compared to the control group. The group treated with the methanolic extract at a dose of 250 mg/kg body weight showed a significant increase in horizontal movements on the first day of the study. (368.1 ± 47.02 vs. 304.7 ± 35.0, p=0.02). This trend is also observed in the horizontal movements on the 14th day, with the groups treated with the methanolic extract at doses of 250, 400, and 500 mg/kg showing an increase compared to the control group (438.3 ± 47.85 vs. 194.7 ± 33.17, p=0.006; 486.8 ± 31.65 vs. 194.7 ± 33.17, p=0.001; 437.7 ± 70.85 vs. 194.7 ± 33.17, p=0.006). A significant increase in vertical activity is also observed in the groups treated with the methanolic extract at doses of 400 mg/kg and 500 mg/kg on the 14th day (122.6 ± 14.47 vs. 24.9 ± 6.18, p=0.001; 127.6 ± 27.58 vs. 24.9 ± 6.18, p=0.001).

2.4.2. Two-way active avoidance test (two-way active avoidance test with punitive support)

The study on the effects of the methanolic extract from *M. frivaldszkyana* at three doses (250 mg/kg, 400 mg/kg, and 500 mg/kg) on the number of conditioned and unconditioned responses, intertrial crossings, and the latent period in rats, tested using the Shuttle box apparatus, does not show any statistically significant difference between the treatment groups and the control group.

2.4.3. Step-through passive avoidance test

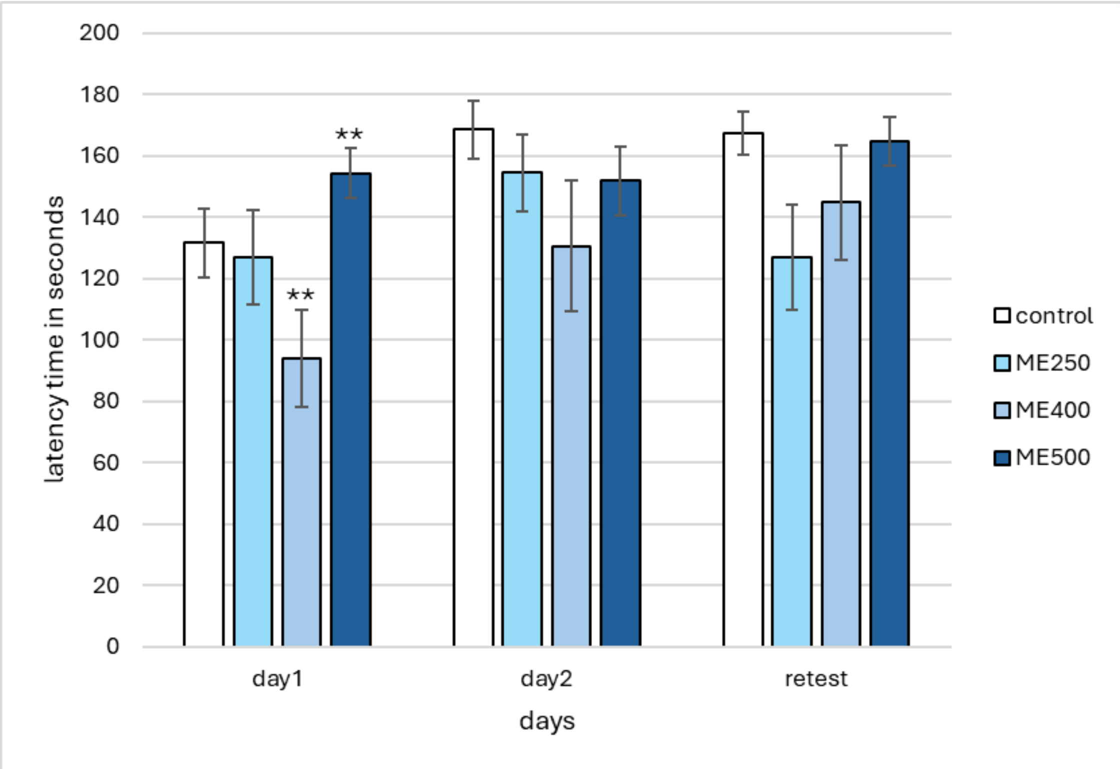

Figure 6. Effect of the methanolic extract from *M. frivaldszkyana* on the latency time in seconds in rats tested with the Step-through apparatus

Note: ** p=0.01 compared to the control group

The passive avoidance test (figure 6) shows a shortening of the latency time in the group treated with the methanolic extract at a dose of 400 mg/kg compared to the control group (93.96 ± 15.82 vs. 131.62 ± 11.12, p=0.01) and an increase at 500 mg/kg (154.32 ± 8.14 vs. 131.62 ± 11.12, p=0.01) on the first day of the training session.

2.4.4. Step-down passive avoidance test

Table 2. Effect of methanolic extract of *M. frivaldszkyana* on the latency time in seconds in rats, examined with the Step-down apparatus.

day / *groups*	*1*	*2*	*Retest*
control	17.96±3.31	25.77±5.76	40.57±4.91
m. extract 250 mg/kg	23.12±3.81	36.73±6.71	46.64±3.81
m. extract 400 mg/kg	23.42±3.99	38.71±5.26	43.28±4.69
m. extract 500 mg/kg	24.61±5.41	41.63±4.74	53.32±3.10

Note: Mean ± SEM are presented.

The Step-down passive avoidance test (table 2) shows no statistically significant difference in the latency time between the treated groups and the control group.

2.4.5. Y-maze

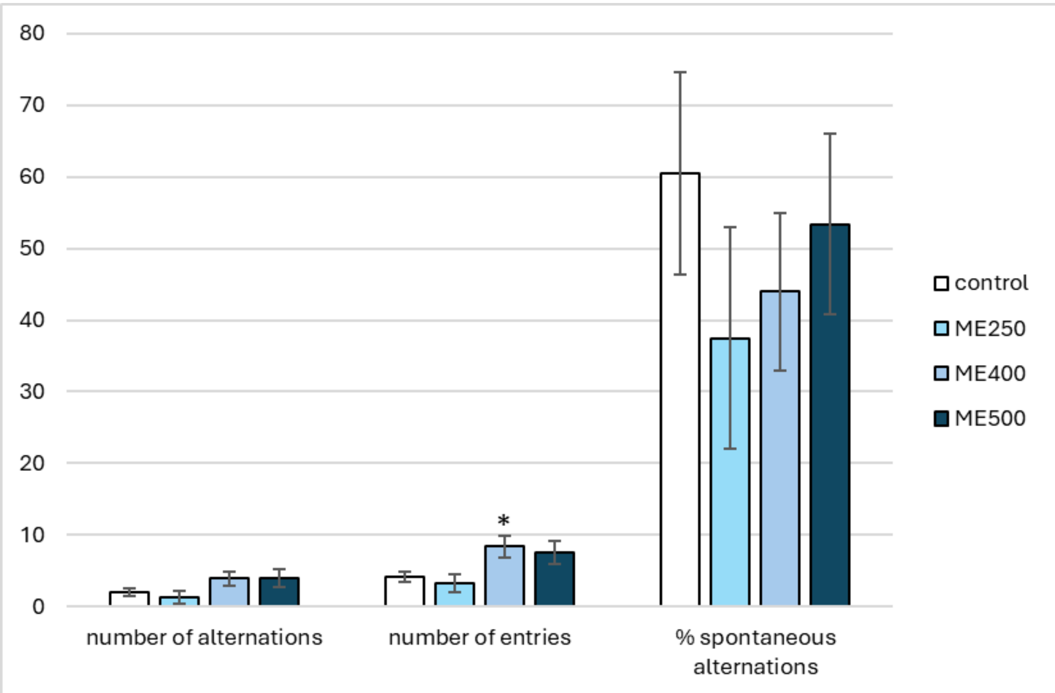

Figure 7. Effect of methanolic extract of *M. frivaldszkyana* in the Y-maze setup

Note: * $p<0.05$ compared to the control group

The results of the spatial memory experiment (figure 7) show a significantly higher number of entries in the group treated with methanolic extract at a dose of 400 mg/kg compared to the control group (8.4 ± 1.52 vs. 2.00 ± 0.61, p=0.046). This result is likely due to the increased motor activity of the animals treated with *M. frivaldszkyana* extract. At the same time, no significant differences in the number of alternations were observed, which supports the hypothesis that the extract stimulates the motor functions of the animals. Increased motor activity is also observed in the Activity cage test (section 2.4.1.).

2.4.6. Elevated plus maze (X-maze)

Table 3. Effect of methanolic extract of *M. frivaldszkyana* in the X-maze setup

groups	*seconds spent in the open space*	*seconds spent in the closed space*	*number of entries into the open arms*	*total number of entries*	*ratio of the number of entries into the open arms to the total number of entries*
control	142.0±30.16	158.0±30.16	1.7±0.52	3.9±0.98	0.32±0.06
m. extract 250 mg/kg	106.7±23.07	193.3±23.07	2.0±0.54	4.7±1.17	0.41±0.07
m. extract 400 mg/kg	104.7±25.89	195.3±25.89	2.7±0.47	5.9±1.06	0.45±0.07
m. extract 500 mg/kg	67.30±22.67	232.7±22.67	3.3±0.86	6.4±1.72	0.63±0.08 *

Note: The mean values for the group are presented ± SEM. * $p<0.05$

There is a trend of increasing the ratio of the number of entries into the open arms to the total number of entries in the groups treated with all three doses of the extract. This trend is dose-dependent, but a statistically significant difference compared to the control group is observed only in the group treated with the methanolic extract at 500 mg/kg (0.63±0.08 vs. 0.32±0.06; p=0.032) (table 3). The results suggest a potential anxiolytic effect of the extract after 14 days of administration in rats.

2.4.7. New object recognition test

Table 4. Effect of methanolic extract of *M. frivaldszkyana* in a new object recognition test

groups	*seconds spent studying the new object*	*discrimination index*
control	3.40 ± 0.62	0.55±0.08
m. extract 250 mg/kg	11.3±4.29	0.58±0.08
m. extract 400 mg/kg	18.3±7.78	0.56±0.07
m. extract 500 mg/kg	16.7±4.06	0.73±0.06

Note: The mean values for the group ± SEM are presented.

The study did not show a statistically significant difference in the discrimination indices between the control and experimental groups (table 4).

No scientific data was found regarding the effects of species from the *Micromeria* genus on cognition. Linarin, the main secondary metabolite in the extract, exhibits inhibitory activity against the enzyme AChE. Inhibition of AChE is considered a promising strategy in the treatment of diseases characterized by low enzyme levels, such as glaucoma, myasthenia gravis, and Alzheimer's disease. (Feng et al., 2015).

Mirza FJ et al. (2021) investigated the effect of RK on cognitive impairment in an amyloid β_{1-42} experimental model of Alzheimer's disease in rodents. Alzheimer's disease is a neurodegenerative disorder characterized by the accumulation of amyloid β plaques and neurofibrillary tangles. Amyloid β_{1-42} induces neurotoxicity by increasing intracellular calcium levels, oxidative stress, and destructive effects on synaptic transmission. Learning behavior and anxiety were assessed using the elevated plus maze paradigm, which demonstrated the antidepressant and anxiolytic effects of RK by increasing the time spent in the open arms. RK is believed to exert its neuroprotective effect by reducing neuronal damage due to oxidative stress (Mirza et al., 2021).

Hasanein and Mahtaj (2015) investigated the memory-enhancing effect of RK on scopolamine-induced memory impairment. Cholinergic deficiency is associated with cognitive dysfunction and memory loss in animal models of Alzheimer's disease. Scopolamine, an anticholinergic agent, also inhibits cholinergic activity and disrupts learning and memory processes. Results from the step-through passive avoidance test model indicate that RK administration has a positive effect on memory and mitigates the negative impact of scopolamine. It is known that rosmarinic acid increases acetylcholine levels and reduces cholinesterase activity in the brains of rodents, which could be a potential mechanism for memory improvement. Given the significant role of oxidative stress in the development of memory impairments, the demonstrated effects may be attributed to the antioxidant and neuroprotective properties of RK (Hasanein and Mahtaj, 2015).

Another model of memory impairment induced by lipopolysaccharide (LPS) investigated the effect of RA using the Y-maze test. The results show a statistically significant decrease in the percentage of spontaneous alternations in the LPS-treated group compared to the control group. Administration of RA led to an increase in the percentage of spontaneous alternations, indicating a protective effect of RA, which may be attributed to reduced acetylcholinesterase (AChE) activity. LPS administration generates high levels of oxidative stress, reflected by decreased GSH levels and increased MDA levels. RA restores the reduced GSH levels and elevated MDA levels. LPS exposure also causes elevated levels of IL-6 and TNF-α. Post-hoc Tukey analysis confirms the reduction in IL-6 and TNF-α levels with RA treatment compared to the LPS-treated group (Thingore et al., 2021).

These data correlate with the results obtained in our study using the step-through passive avoidance test, where on the first day of the training session, a significant increase in the latency time was observed in the groups treated with the methanolic extract at doses of 400 and 500 mg/kg (figure 6). The results obtained in the Y-maze test, showing a significantly higher number of entries in the group treated with 400 mg/kg of methanolic extract (figure 7), are also consistent with data established in the literature. A statistically significant increase in motor activity was observed in the Activity cage test (figure 5). The observed effects are likely due to the antidepressant potential of rosmarinic acid, but further research is needed in this area to clarify the potential mechanism.

2.5. Investigation on the hepatoprotective effect of the extract

The following experimental group designations were used for the figures:

Group 1 - Control: 0.1 ml/100 g body weight of saline solution (without liver damage)

Group 2 - ME500: Methanolic extract at a dose of 500 mg/kg body weight (without liver damage)

Group 3 - P/t-BHP: 0.1 ml/100 g body weight of saline solution + Paracetamol/t-BHP

Group 4 - ME250-P/ME250-t-BHP: Methanolic extract at a dose of 250 mg/kg body weight + Paracetamol/t-BHP
Group 5 - ME400-P/ME400-t-BHP: Methanolic extract at a dose of 400 mg/kg body weight + Paracetamol/t-BHP
Group 6 - ME500-P/ME500-t-BHP: Methanolic extract at a dose of 500 mg/kg body weight + Paracetamol/t-BHP
Group 7 - RA-P/RA-t-BHP: Rosmarinic acid at 100 mg/kg body weight + Paracetamol/t-BHP
Group 8 - Sil-P/Sil-t-BHP: Silymarin at 125 mg/kg body weight + Paracetamol/t-BHP

Paracetamol, also known as acetaminophen, is a widely used drug with antipyretic and analgesic properties. The intake of high doses of paracetamol leads to severe hepatotoxicity in humans and experimental animals (Brune et al., 2015). During metabolism, 60% of paracetamol is converted into glucuronic acid via the enzyme glucuronyl transferase. Thirty-five percent undergoes sulfonation, and about 2% of paracetamol is excreted unchanged in the urine. The remaining small amount of paracetamol is converted to N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P-450 enzymes. NAPQI is a reactive electrophilic molecule that covalently binds to cysteine residues in intracellular proteins, forming 3-(cysteine-S-yl) chelates. These chelates cause tissue damage. When taken in therapeutic doses, NAPQI interacts with glutathione and is eliminated. Paracetamol overdose increases the amount of NAPQI and depletes glutathione stores in the liver. It is supposed that these metabolites bind to sulfhydryl groups and glutathione, forming conjugates that increase oxidative stress in the liver. This potential mechanism outlines the main pathway for paracetamol-induced hepatotoxicity (Bessems and Vermeulen, 2001).

In recent years, there has been an increasing popularity of the usage of natural products for the prevention and treatment of paracetamol-induced hepatotoxicity in *in vivo* animal models (Ahmad et al., 2019).

Tert-butyl hydroperoxide (t-BHP) is an organic hydroperoxide that can be metabolized to form t-butoxyl and methyl radicals. These free radicals can induce lipid peroxidation, glutathione depletion, increased cell membrane permeability, and DNA damage. Numerous researchers use t-BHP to assess the antioxidant activity of various compounds by monitoring the activity of AST, ALT, and the levels of MDA, GSH, and CAT (Oh et al., 2012; Zhu et al., 2022).

The reference substance for antioxidant activity that we used to monitor the hepatoprotective effect is silymarin. Silymarin is a flavonoid found in the plant *Silybum marianum*, which also exhibits significant neuroprotective activity, likely due to its ability to inhibit oxidative stress, apoptosis, and inflammation (Onaolapo et al., 2016).

2.5.1. Changes in serum levels of biomarkers in paracetamol-induced hepatoxicity

2.5.1.1. Total and direct biliribun

Table 5. Effect of methanolic extract from *M. frivaldszkyana* on serum levels of total and direct bilirubin in a paracetamol-induced hepatotoxicity model

groups	*total bilirubin*	*direct bilirubin*
control 0.1 ml/1000 g bw saline	6.54±1.27	1.33±0.63
m. extract 500 mg/kg without liver damage	4.57±0.83	0.41±0.91
0.1 ml/100 g bw saline + paracetamol	6.12±0.57	1.70±0.53
m. extract 250 mg/kg + paracetamol	5.47±0.33	1.77±0.48
m. extract 400 mg/kg + paracetamol	6.58±0.58	2.81±1.21
m. extract 500 mg/kg + paracetamol	5.97±0.37	1.94±0.63
rosmarinic acid 100 mg/kg bw + paracetamol	8.08±0.21 δδ	2.22±0.40
silimarin 125 mg/kg bw + paracetamol	8.25±0.72 δδ	2.22±0.59

Note: Mean ± SEM are presented.
δδ $p<0.01$ compared to methanolic extract 500 mg/kg;

The results (table 5) show a statistically significant increase in total bilirubin levels in the group treated with rosmarinic acid (p=0.009) and the group treated with silymarin (p=0.005) compared to the group treated with methanolic extract at a dose of 500 mg/kg without liver damage. No statistically significant deviation was observed in direct bilirubin levels.

2.5.1.2. AST

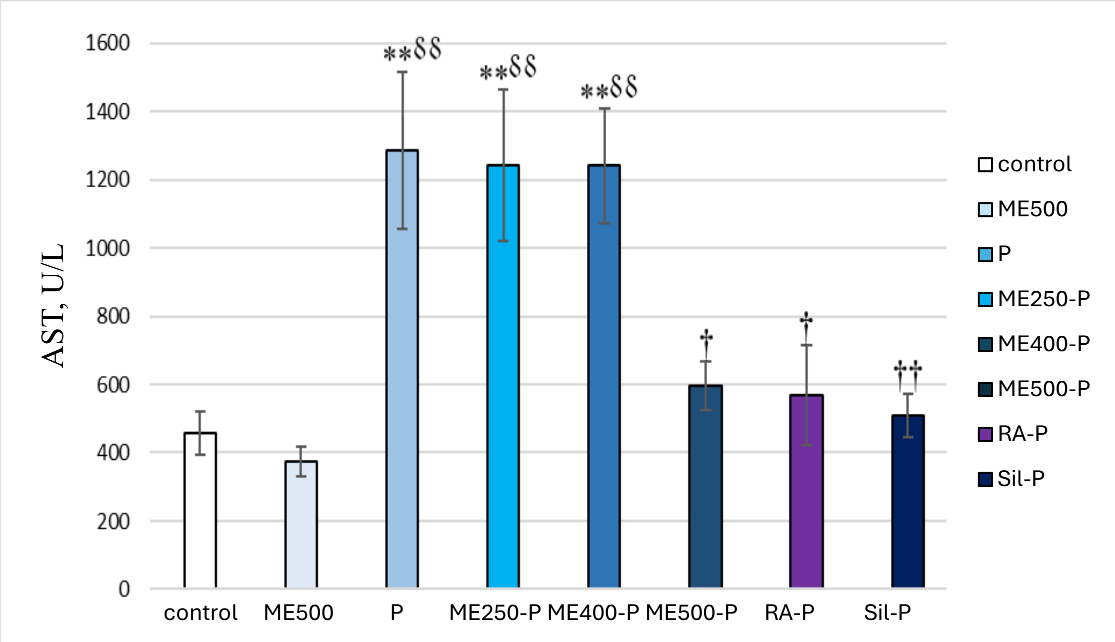

Figure 8. Effect of methanolic extract from *M. frivaldszkyana* on serum levels of aspartate aminotransferase in a paracetamol-induced hepatotoxicity model

Note: ** p< 0.01 compared to control group; §§ p<0.01 compared to methanolic extract 500 mg/kg; † p<0.05 compared to paracetamol; †† p<0.01 compared to paracetamol

According to the conducted analysis (Fig. 8), a significant increase in serum AST levels was observed in rats from the P, ME250-P, and ME400-P groups compared to rats without liver damage treated with saline solution (1284.94 ± 229.22 vs. 457.46 ± 63.68, p=0.004; 1241.21 ± 222.14 vs. 457.46 ± 63.68, p=0.008; 1240.38 ± 169.8, vs. 457.46 ± 63.68, p=0.008). Similar results were observed in comparison to the ME500 group (1284.94 ± 229.22 vs. 373.84 ± 44.15, p=0.002; 1241.21 ± 222.14 vs. 373.84 ± 44.15, p=0.004; 1240.38 ± 169.8 vs. 373.84 ± 44.15, p=0.004).

The comparison between the groups in the paracetamol-induced hepatotoxicity model shows a significant decrease in serum AST levels in the groups treated with rosmarinic acid and silymarin when compared to the levels in rats treated with paracetamol alone (Group P) (568.43 ± 148.36 vs. 1284.94 ± 229.22, p=0.02; 509.03 ± 62.36 vs. 1284.94 ± 229.22, p=0.025). A tendency toward a decrease was also observed in the animals from the ME500-P group (568.43 ± 148.36 vs. 1241.21 ± 222.14, p=0.009) compared to Group P.

2.5.1.3. ALT

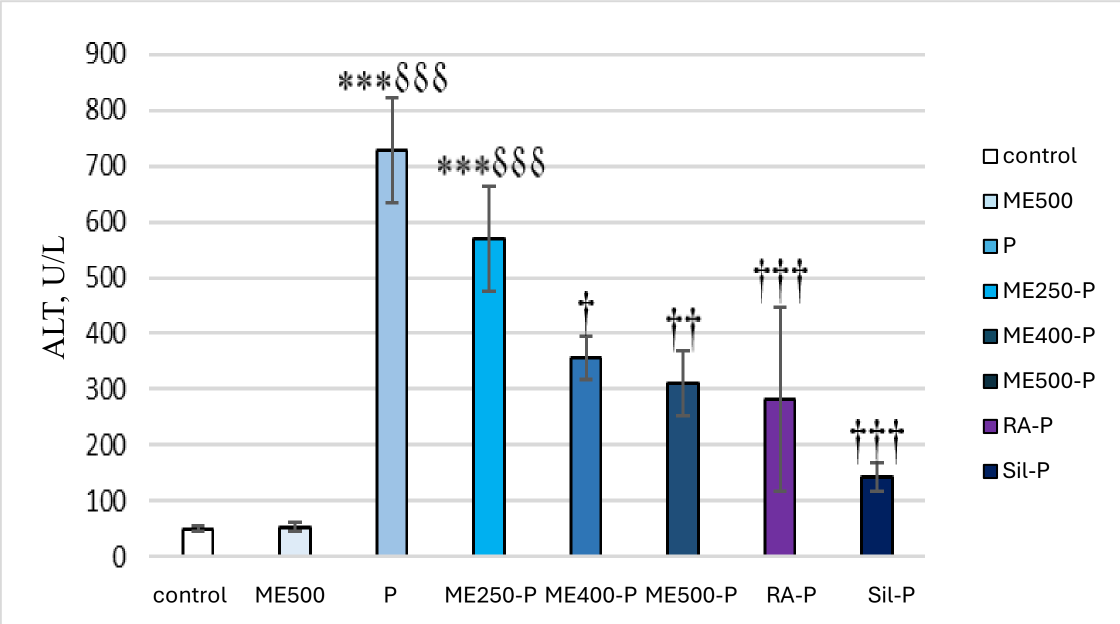

Figure 9. Effect of methanolic extract from *M. frivaldszkyana* on serum levels of alanine aminotransferase in a paracetamol-induced hepatotoxicity model

Note: *** p<0.001 compared to control group; §§§ p<0.001 compared to methanolic extract 500 mg/kg; † p<0.05 compared to paracetamol; †† p<0.01 compared to paracetamol; ††† p<0.001 compared to paracetamol

Figure 9 shows that the administration of *M. frivaldszkyana* extract induces a dose-dependent decrease in serum ALT levels compared to Group P. Statistical analysis demonstrates a significant reduction in the control, ME500, ME400-P, ME500-P, RA-P, and Sil-P groups compared to Group P (50.63 ± 5.07 vs. 728.9 ± 93.94, p<0.001; 53.61 ± 8.32 vs. 728.9 ± 93.94, p<0.001; 356.34 ± 39.09 vs. 728.9 ± 93.94, p=0.04; 311.88 ± 58.31 vs. 728.9 ± 93.94, p=0.01; 281.81 ± 165.88 vs.728.9 ± 93.94, p= 0.005; 142.76 ± 25.59, p<0.001).

2.5.2. Determination of markers for assessing liver function in paracetamol-induced hepatotoxicity in liver homogenate

2.5.2.1. Catalase (CAT)

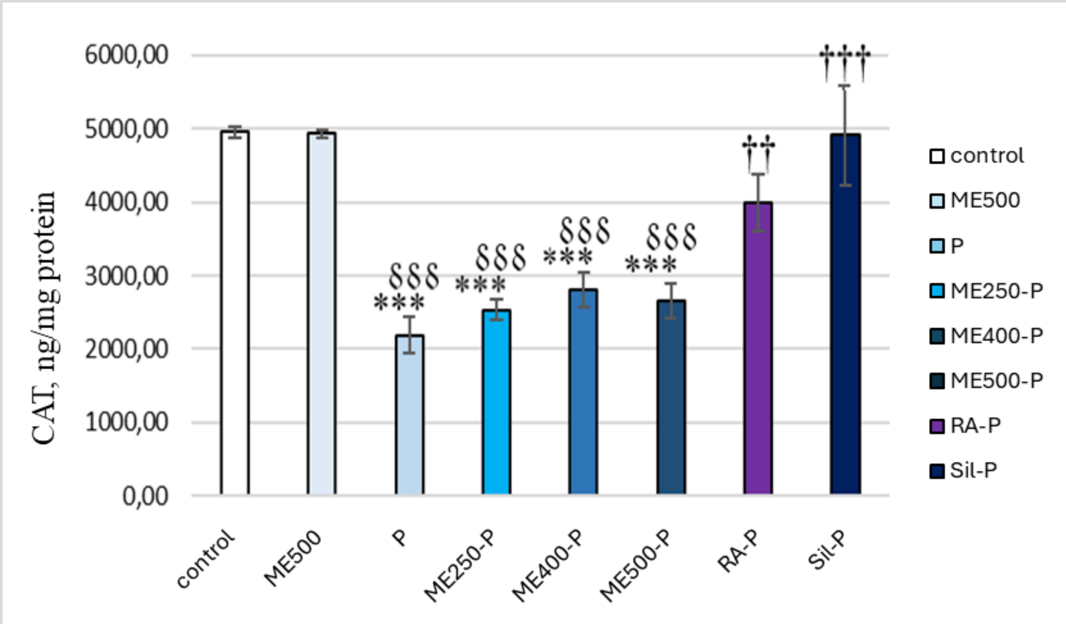

Figure 10. Effect of methanolic extract from *M. frivaldszkyana* on catalase levels in a paracetamol-induced hepatotoxicity model

Note: *** p< 0.001 compared to control; §§§ p<0.001 compared to methanolic extract 500 mg/kg; †† p<0.01 compared to paracetamol; ††† p<0.001 compared to paracetamol

The conducted analysis (figure 10) demonstrates a significant increase in catalase enzyme levels in the groups of rats treated with silymarin and rosmarinic acid compared to the P group (4907.14 ± 683.10 vs. 2212.23 ± 254.01, p<0.001) and (3986.52 ± 387.38 vs. 2212.23 ± 254.01, p=0.07).

Significantly higher levels of CAT are also observed in the control group compared to groups P (4951.56 ± 71.55 vs. 2212.23 ± 254.01 p<0.001), ME250-P (4951.56 ± 71.55 vs. 2529.66 ± 142.52, p<0.001), ME400-P (4951.56 ± 71.55 vs. 2807.60 ± 243.82 p<0.001), ME500-P (4951.56 ± 71.5510 vs. 2653.84 ± 245.31, p<0.001).

2.5.2.2. Reduced gluthatione (GSH)

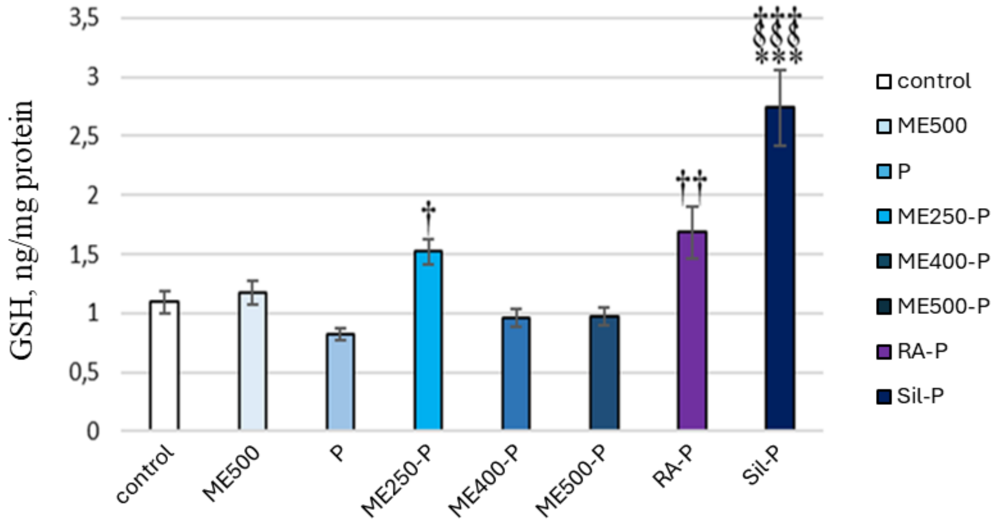

Figure 11. Effect of methanolic extract from *M. frivaldszkyana* on reduced glutathione levels in a paracetamol-induced hepatotoxicity model

Note: *** $p<0.001$ compared to control; §§§ $p<0.001$ compared to methanolic extract 500 mg/kg; † $p<0.05$ compared to paracetamol; †† $p<0.01$ compared to paracetamol; ††† $p<0.001$ compared to paracetamol

The statistical analysis conducted (figure 11) demonstrates a significant increase in GSH levels in the group of rats treated with silymarin compared to all other groups included in the study (Sil-P vs. control, $p<0.001$; vs. ME500, $p<0.001$; vs. P, $p<0.001$; vs. ME250-P, $p<0.001$; vs. ME400-P, $p<0.001$; vs. ME500-P, $p<0.001$; vs. RA-P, $p<0.001$). Increased levels of reduced glutathione are also observed in the RA-P and ME250-P groups compared to the P group (1.68 ± 0.22 vs. 0.82 ±0.49 p=0.007 and 1.52 ± 0.11 vs. 0.82 ± 0.05, p=0.049).

2.5.2.3. Malondialdehyde (MDA)

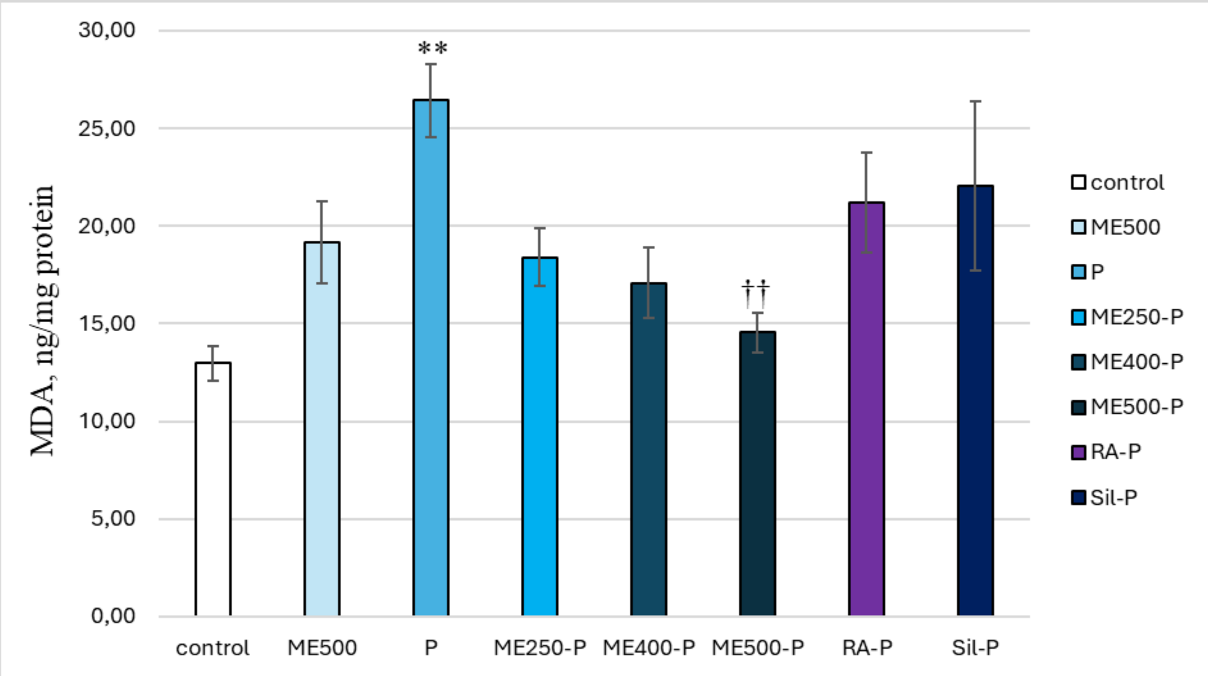

Figure 12. Effect of methanolic extract from *M. frivaldszkyana* on malondialdehyde levels in a paracetamol-induced hepatotoxicity model

Note: ** p<0.01 compared to control; †† p<0.01 compared to paracetamol

Figure 12 shows a significant increase in serum MDA levels in the P group compared to the control group (26.42 ± 1.89 vs. 12.97 ± 0.89, p=0.003) and in ME500-P compared (26.42 ± 1.89 vs. 14.54 ± 1.02, p=0.011).

2.5.2.4. 8-OH-dG

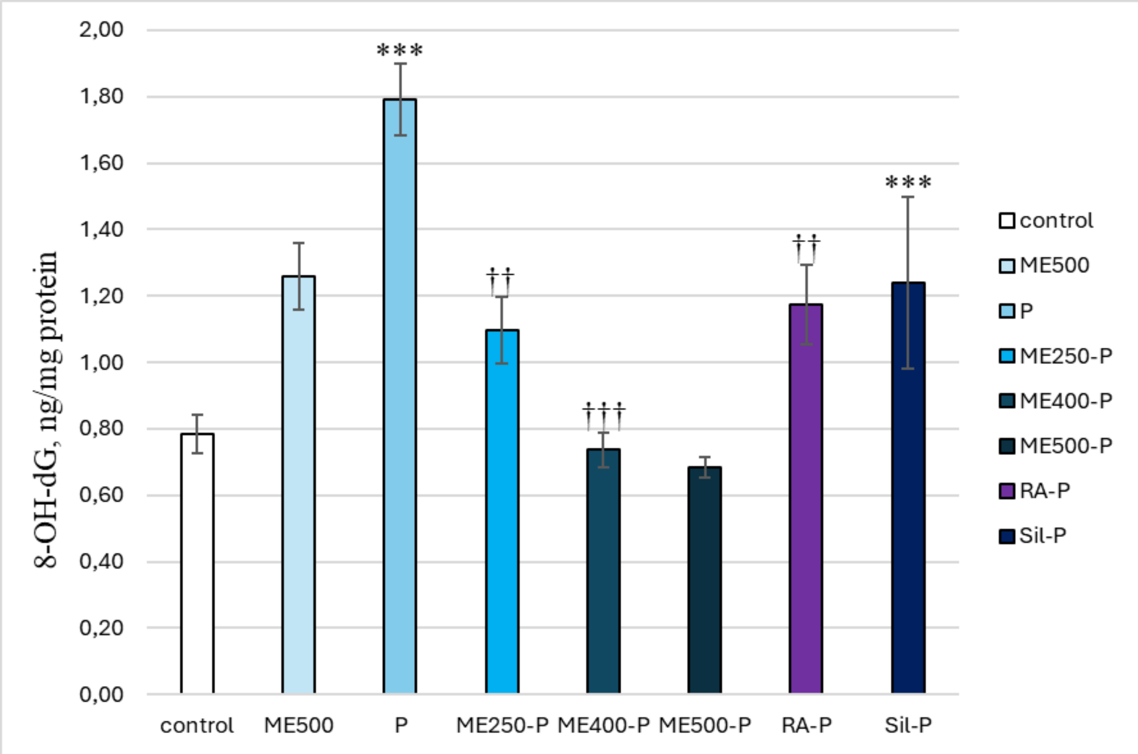

Figure 13. Effect of methanolic extract from *M. frivaldszkyana* on 8-hydroxy-deoxyguanosine levels in a paracetamol-induced hepatotoxicity model

Note: *** p<0.001 compared to control; †† p<0.01 compared to paracetamol; ††† p<0.001 compared to paracetamol

The statistical analysis of the obtained results (figure 13) shows a significant increase in 8-OH-dG levels in the P group compared to the control group (1.79 ± 0.11 vs. 0.78 ± 0.06, p<0.001) and compared to ME250-P (1.10 ± 0.10 vs. 1.79 ± 0.11, p=0.003), ME400-P (1.79 ± 0.11 vs. 0.74 ± 0.52, p<0.001) and ME500 (1.79 ± 0.11 vs. 1.26 ± 0.10, p<0.001).

A significant increase in the values of the marker is also observed in the RA-P group compared to the P group (1.17 ± 0.12 vs. 1.79 ± 0.11, p=0.01), as well as in the Sil-P group compared to the control group (1.95 ± 0.24 vs. 0.78 ± 0.06, p<0.001), ME500 (1.95 ± 0.24 vs. 1.79 ± 0.11, p=0.002), ME400-P (1.95 ± 0.24 vs. 0.74 ± 0.05); ME500-P (1.95 ± 0.24 vs. 0.68 ± 0.31, p<0.001), RA-P (1.95 ± 0.24 vs.1.17 ± 0.12, p<0.001).

2.5.2.5. Interleukin 6

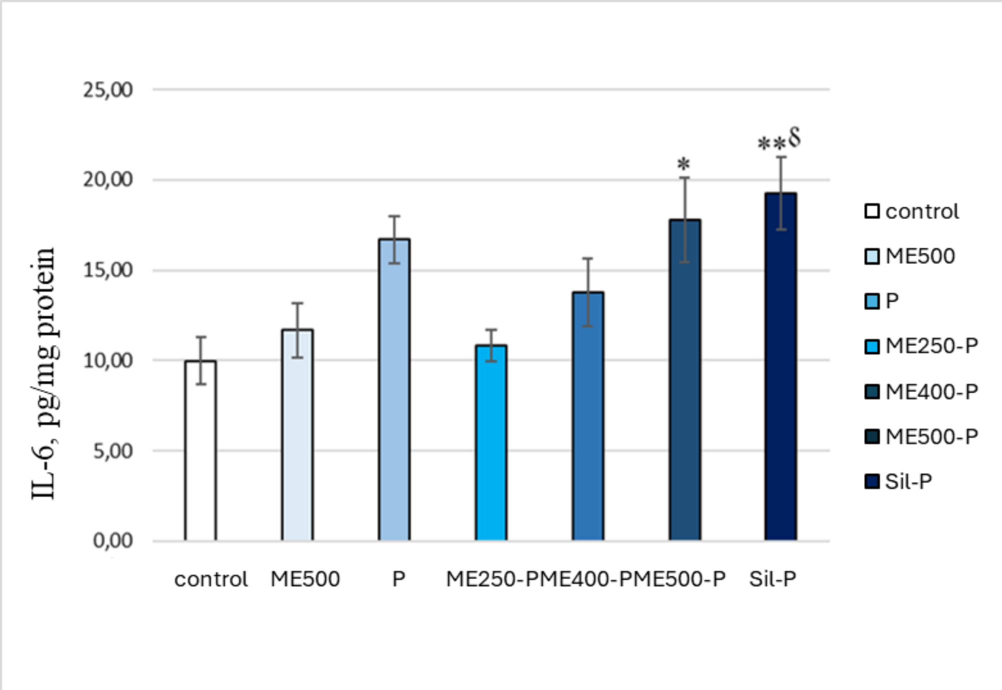

Figure 14. Effect of methanolic extract from *M. frivaldszkyana* on interleukin 6 levels in a paracetamol-induced hepatotoxicity model

Note: * p< 0.05 compared to control; ** p< 0.01 compared to control;
δ p<0.05 compared to methanolic extract 500 mg/kg

According to the conducted analysis (figure 14), significantly lower IL-6 values are observed in the control, ME500, and ME250-P groups compared to Sil-P (9.99 ± 1.29 vs. 19.25 ± 2.01, p=0.007; 11.67 ± 1.53 vs. 19.25 ± 2.01, p=0.042; 10.81 ± 0.86 vs. 19.25 ± 2.01, p=0.017). Significantly lower levels of the marker are observed in the control group compared to the ME500-P group (9.99 ± 1.29 vs. 17.79 ± 2.37, p=0.034), while a trend towards a significant decrease is seen in the ME250-P group compared to the ME500-P group (10.81 ± 0.86 vs. 17.79 ± 2.37, p=0.076).

2.5.2.6. Tumor necrosis factor alpha (TNF-α)

The conducted analysis (Fig. 15) demonstrates a significant decrease in TNF-α levels in the group with liver damage treated with an extract at a dose of 400 mg/kg body weight, compared to the groups ME500 (45.86 ± 3.78 vs. 115.92 ± 11.16, p=0.037), P (45.86 ± 3.78 vs. 126.55 ± 22.67, p=0.011), ME250-P (45.86

± 3.78 vs. 125.97 ± 21.93, p=0.011), and Sil-P (45.86 ± 3.78 vs. 115.74 ± 17.69, p=0.038). Similar results are observed in the ME500-P group (48.42 ± 3.60 vs. 115.92 ± 11.16, p=0.05; 48.42 ± 3.60 vs. 126.55 ± 22.67, p=0.015; 48.42 ± 3.60 vs. 125.97 ± 21.93, p=0.016; 48.42 ± 3.60 vs. 115.74 ± 17.69, p=0.051, respectively).

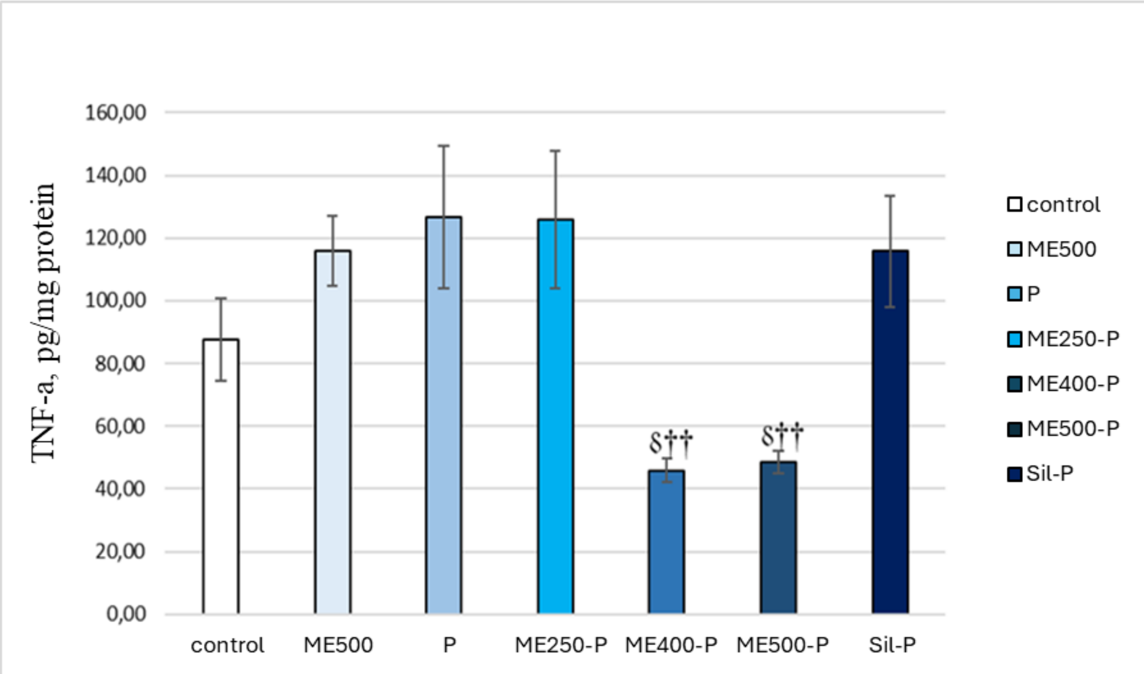

Figure 15. Effect of methanolic extract from *M. frivaldszkyana* on tumor necrosis factor-alpha (TNF-α) levels in a paracetamol-induced hepatotoxicity model

Note: δ p<0.05 compared to methanolic extract 500 mg/kg;
†† p<0.01 compared to paracetamol

2.5.3. Changes in serum biomarker levels in t-BHP-induced hepatotoxicity

2.5.3.1. Total and direct bilirubin

In the study of the effects of the methanolic extract of *M. frivaldszkyana* at doses of 250 mg/kg, 400 mg/kg, and 500 mg/kg body weight on total and direct bilirubin levels, no statistically significant differences were observed between the groups and the control group.

2.5.3.2. AST

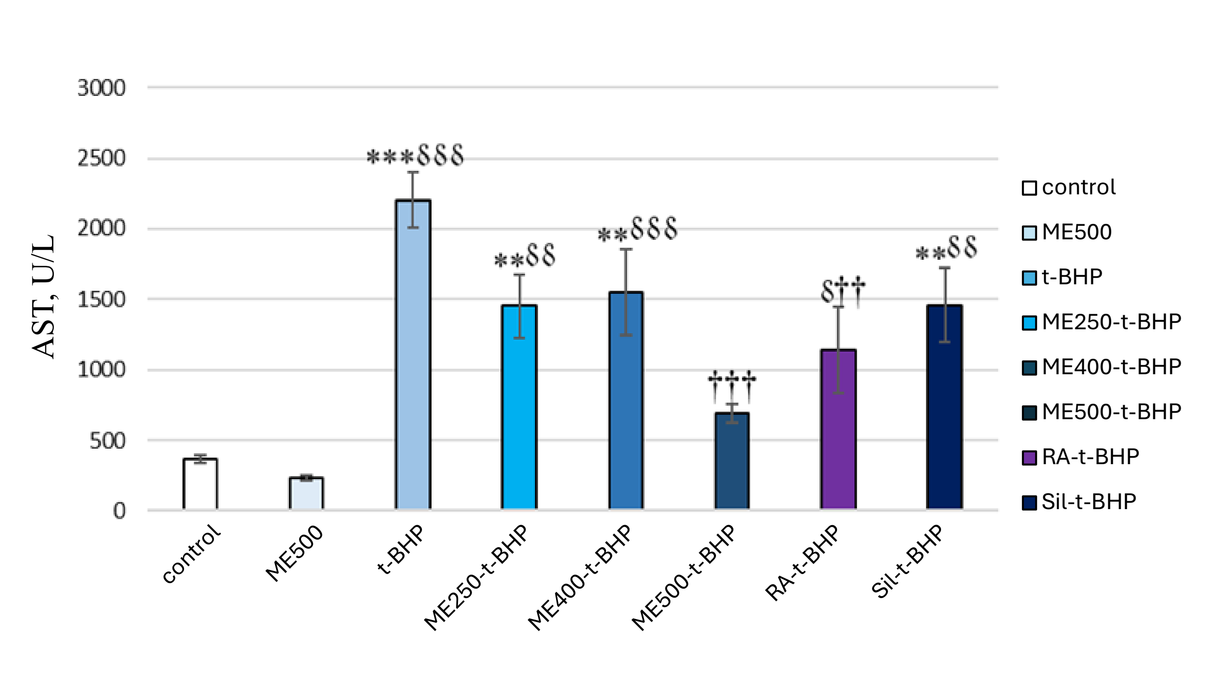

Figure 16. Effect of methanolic extract from *M. frivaldszkyana* on serum aspartate aminotransferase levels in a butyl hydroperoxide-induced hepatotoxicity model

Note: ** $p<0.01$ compared to control; *** $p<0.001$ compared to control; § $p<0.05$ compared to methanolic extract 500 mg/kg; §§ $p<0.01$ compared to methanolic extract 500 mg/kg; §§§ $p<0.001$ compared to methanolic extract 500 mg/kg; †† $p<0.01$ compared to t-BHP; ††† $p<0.001$ compared to t-BHP

The conducted analysis (figure 16) demonstrates a significant increase in serum AST levels in the t-BHP, ME250-t-BHP, ME400-t-BHP, and Sil-t-BHP groups compared to the control group (2203.88 ± 194.85 vs. 368.89 ± 29.87, $p<0.001$; 1449.9 ± 224.7 vs. 368.89 ± 29.87, p=0.012; 1549.13 ± 303.21 vs. 368.89 ± 29.87, p=0.003; 1457.66 ± 263.73 vs. 368.89 ± 29.87, p=0.009) and group ME500 (2203.88 ± 194.85 vs. 233.55 ± 18.7, $p<0.001$; 1449.9 ± 224.7 vs. 233.55 ± 18.7, p=0.003; 1549.13 ± 303.21 vs. 233.55 ± 18.7, p=0.001; 1457.66 ± 263.73 vs. 233.55 ± 18.7, p=0.002). A trend toward a significant increase in the values of the marker is observed when comparing the ME500 and RA-t-BHP groups (233.55 ± 18.7 vs. 1142.44 ± 304.4, p=0.052).

A significant decrease in the serum concentration of AST is observed in the ME500-t-BHP group compared to the t-BHP group (688.84 ± 67.87 vs. 2203.88 ± 194.85, $p<0.001$), while a trend is observed when comparing the ME500-t-BHP and ME400-t-BHP groups (688.84 ± 67.87 vs. 1549.13 ± 303.21, p=0.078).

A significant decrease in the values of the parameter is also observed in the RA-t-BHP group compared to the t-BHP group (1142.44 ± 304.4 vs. 2203.88 ± 194.85, p=0.012).

2.5.3.3. ALT

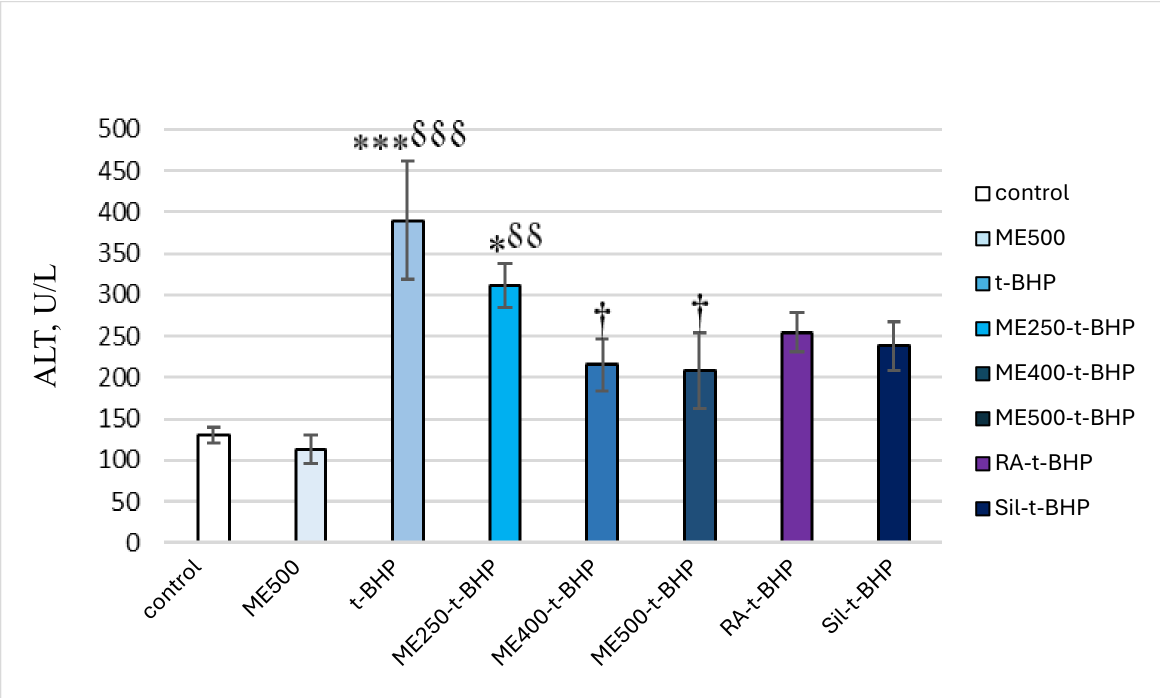

Figure 17. Effect of methanolic extract of *M. frivaldszkyana* on serum levels of alanine aminotransferase (ALT) in the t-BHP-induced hepatotoxicity model

Note: * p<0.05 compared to control; *** p<0.001 compared to control; §§ p<0.01 compared to methanolic extract 500 mg/kg; §§§ p<0.001 compared to methanolic extract 500 mg/kg; † p<0.05 compared to t-BHP

According to the analysis (figure 17), there is a significant decrease in serum ALT levels in the control group compared to the ME250-t-BHP and t-BHP groups (129.94 ± 10.15 vs. 310.5 ± 26.86, p=0.02; 129.94 ± 10.15 vs. 390.15 ± 71.93, p<0.001). Similar results are observed in the ME500 group (113.38 ± 16.91 vs. 310.5 ± 26.86, p=0.007; 113.38 ± 16.91 vs. 390.15 ± 71.93, p<0.001, respectively).

A significant decrease in serum ALT levels is observed in the ME400-t-BHP and ME500-t-BHP groups compared to the t-BHP group (215.33 ± 31.54 vs. 390.15 ± 71.93, p=0.027; 207.56 ± 46.03 vs. 390.15 ± 71.93, p=0.018).

2.5.4. Determination of markers for assessment of liver function in t-BHP-induced hepatotoxicity in liver homogenate

2.5.4.1. Catalase

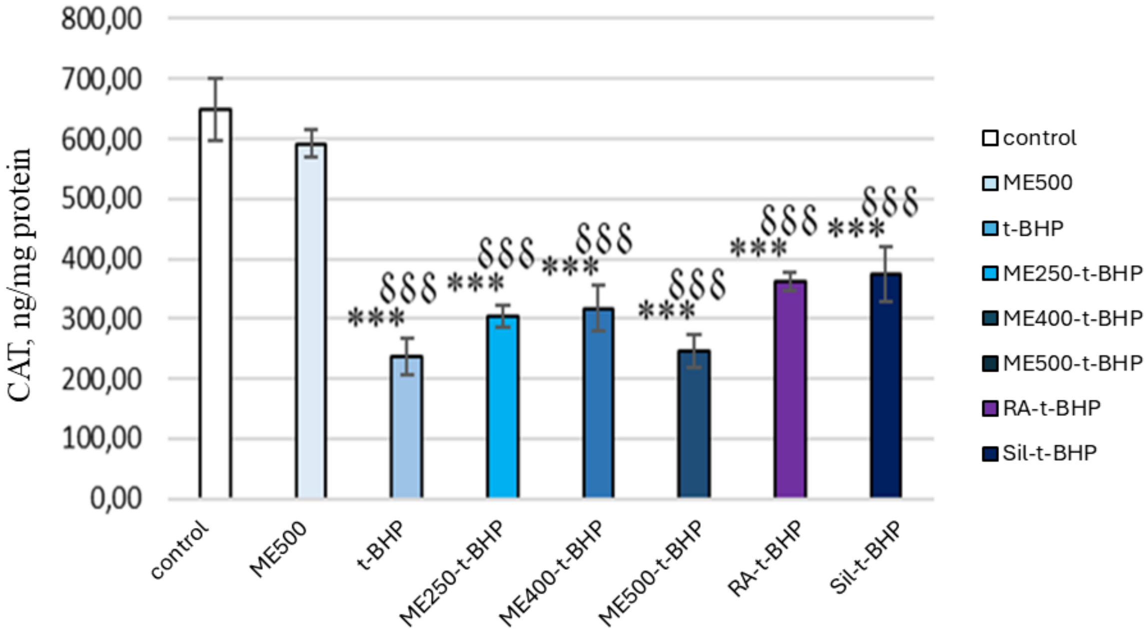

Figure 18. Effect of methanolic extract from *M.frivaldszkyana* on catalase levels in t-BHP-induced hepatotoxicity model

Note: *** p<0.001 compared to control; §§§ p<0.001 compared to methanolic extract 500 mg/kg

Figure 18 demonstrates significantly lower levels in the t-BHP group compared to the control group (237.36 ± 29.89 vs. 649.97 ± 51.64, p<0.001). The analysis shows a significant decrease in catalase activity in the ME250-t-BHP, ME400-t-BHP, and ME500-t-BHP groups compared to the control group (302.86 ± 18.53 vs. 649.97 ± 51.64, p<0.001; 316.8 ± 37.69 vs. 649.97 ± 51.64, p<0.001; 244.95 ± 27.69 vs. 649.97 ± 51.64, p<0.001).

The same trend is observed in the RA-t-BHP and Sil-t-BHP groups compared to the control (360.91 ± 15.36 vs. 649.97 ± 51.64, p<0.001; 374.84 ± 45.86 vs. 649.97 ± 51.64, p<0.001). Similar results are found in the t-BHP group compared to the ME500 group (237.36 ± 29.89 vs. 592.18 ± 21.66, p<0.001).

Significance is also found in the ME250-t-BHP, ME400-t-BHP, and ME500-t-BHP groups compared to the ME500 group (302.86 ± 18.53 vs. 592.18 ± 21.66, p<0.001; 316.8 ± 37.69 vs. 592.18 ± 21.66, p<0.001; 244.95 ± 27.69 vs. 592.18 ± 21.66, p<0.001). The RA-t-BHP and Sil-t-BHP groups also show lower levels compared to the ME500 group (360.91 ± 15.36 vs. 592.18 ± 21.66, p<0.001; 374.84 ± 45.86 vs. 592.18 ± 21.66, p<0.001).

2.5.4.2. Reduced glutathione

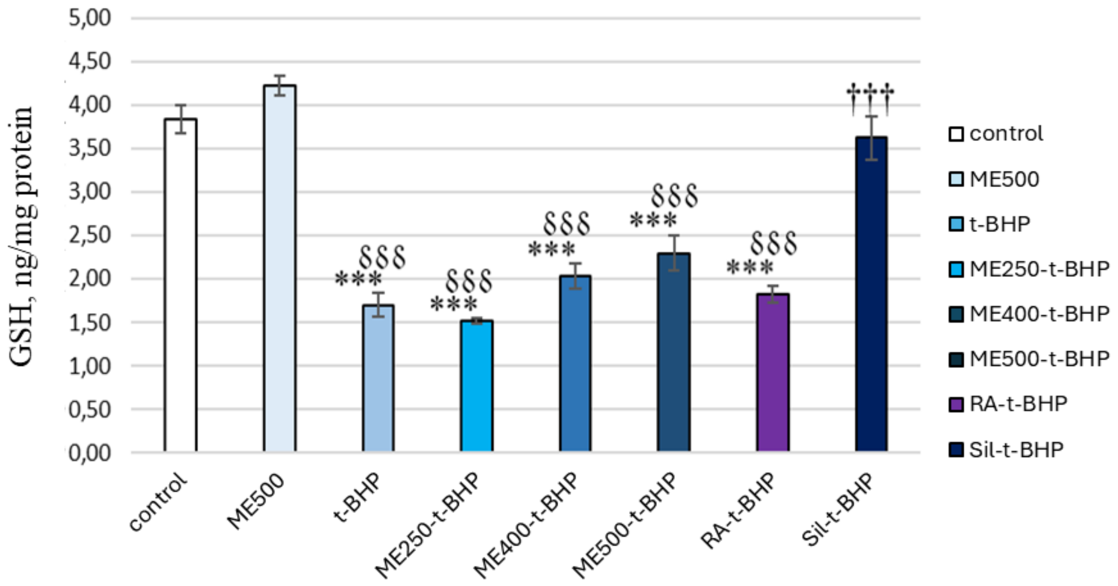

Figure 19. Effect of methanolic extract of *M. frivaldszkyana* on reduced glutathione levels in a butyl hydroperoxide-induced hepatotoxicity model

Note: *** p<0.001 compared to control; §§§ p<0.001 compared to methanolic extract 500 mg/kg; ††† p<0.001 compared to t-BHP

The analysis (figure 19) for determining glutathione levels reveals significantly lower values in the t-BHP group compared to the control group (1.70 ± 0.14 vs. 3.84 ± 0.16, p<0.001). Similar results are observed in the t-BHP group compared to the ME500 and Sil-t-BHP groups, respectively (1.70 ± 0.14 vs. 4.22 ± 0.11, p<0.001; 1.70 ± 0.14 vs. 3.62 ± 0.25, p<0.001).

Significantly lower values are also observed in the groups ME250-t-BHP, ME400-t-BHP, and ME500-t-BHP compared to the Sil-P group (1.52 ± 0.04 vs. 3.62 ± 0.25, p<0.001; 2.03 ± 0.15 vs. 3.62 ± 0.25, p<0.001; 2.29 ± 0.2 vs. 3.62 ± 0.25, p<0.001).

A significant increase is observed in the ME500-t-BHP group compared to the group treated with half the dose, ME250-t-BHP (2.29 ± 0.20 vs 1.52 ± 0.04, p=0.02).

Significantly higher levels of GSH were observed in the control group compared to the groups ME250-t-BHP, ME400-t-BHP, ME500-t-BHP, RA-t-BHP (3.84 ± 0.16 vs. 1.52 ± 0.04, p<0.001; 3.84 ± 0.16 vs. 2.03 ± 0.15, p<0.001; 3.84 ± 0.16 vs. 2.29 ± 0.2, p<0.001; 3.84 ± 0.16 vs. 1.82 ± 0.10, p<0.001). Similar results were observed in the ME500 group compared to groups ME250-t-BHP, ME400-t-BHP, ME500-t-BHP, RA-t-BHP (4.22 ± 0.11 vs. 1.52 ± 0.04, p<0.001; 4.22 ± 0.11 vs. 2.03 ± 0.15, p<0.001; 4.22 ± 0.11 vs. 2.29 ± 0.2, p<0.001; 4.22 ± 0.11 vs. 1.82 ± 0.10, p<0.001).

2.5.4.3. Malondialdehyde

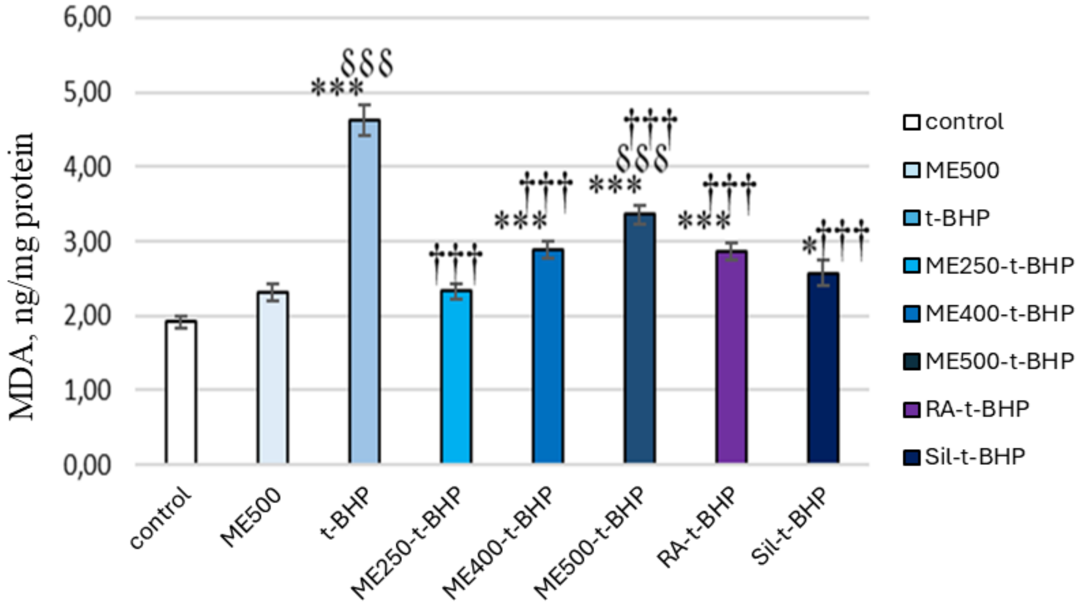

Figure 20. Effect of methanolic extract of *M. frivaldszkyana* on the levels of malondialdehyde (MDA) in a butyl hydroperoxide (t-BHP)-induced hepatotoxicity model

Note: * p<0.05 compared to control, *** p<0.001 compared to control;
§§§ p<0.001 compared to methanolic extract 500 mg/kg;
††† p<0.001 compared to t-BHP

The analysis (Fig. 20) demonstrates a significant increase in MDA levels in the group of rats treated with t-BHP compared to all other groups included in the study (p<0.001). Similar results were found in the ME500-t-BHP group compared to the control group (3.36 ± 0.12 vs. 1.92 ± 0.07, p<0.001), ME500 (3.36 ± 0.12

vs. 2.31 ± 0.11, p<0.001), PS-t-BHP (3.36 ± 0.12 vs. 2.57 ± 0.17, p=0.003), and ME250-t-BHP (3.36 ± 0.12 vs. 2.33 ± 0.10, p<0.001).

A statistically significant decrease in MDA levels or a tendency toward such was observed in the control, ME500, and ME250-t-BHP groups compared to the ME400-t-BHP group (1.92 ± 0.07 vs. 2.89 ± 0.12, p<0.001; 2.31 ± 0.11 vs. 2.89 ± 0.12, p=0.057; 2.33 ± 0.10 vs. 2.89 ± 0.12, p=0.08, respectively). Similar results were observed when comparing the control and ME500 groups with the RA-t-BHP group (1.92 ± 0.07 vs. 2.87 ± 0.11, p<0.001; 2.31 ± 0.11 vs. 2.87 ± 0.11, p=0.075, respectively).

2.5.4.4. 8-OH-dG

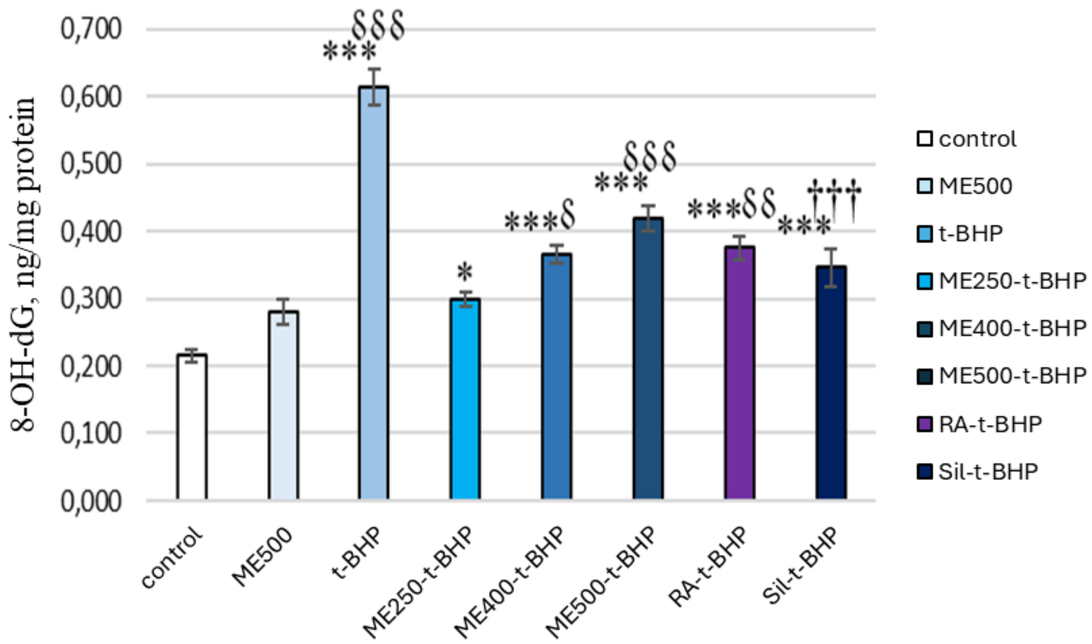

Figure 21. The effect of methanolic extract from *M. frivaldszkyana* on the levels of 8-hydroxy-deoxyguanosine in a butyl hydroperoxide-induced hepatotoxicity model

Note: * p<0.05 compared to control; *** p<0.001 compared to control; § p<0.05 compared to methanolic extract 500 mg/kg; §§ p<0.01 compared to methanolic extract 500 mg/kg; §§§ p<0.001 compared to methanolic extract; ††† p<0.001 compared to t-BHP

The analysis (figure 21) demonstrates a significant increase in 8-OH-dG levels in the rats treated with t-BHP compared to all other groups included in the experiment ($p<0.001$). Similar results are observed in the ME500-t-BHP group when compared to the control group (0.419 ± 0.018 vs. 0.215 ± 0.009, $p<0.001$), ME500 group (0.419 ± 0.018 vs. 0.279 ± 0.018, $p<0.001$), and ME250-t-BHP group (0.419 ± 0.018 vs. 0.299 ± 0.010, $p=0.001$).

A significant increase in 8-OH-dG levels is observed in the negative control group compared to the healthy rats treated with saline (0.614 ± 0.027 vs. 0.215 ± 0.009, $p<0.001$), while a trend is found in the ME250-t-BHP group compared to the healthy rats treated with saline (0.299 ± 0.010 vs. 0.215 ± 0.009, $p=0.053$).

A statistically significant decrease in 8-OH-dG levels is observed in the control and ME500 groups compared to the ME400-t-BHP group (0.215 ± 0.009 vs. 0.366 ± 0.014, $p<0.001$; 0.279 ± 0.018 vs. 0.366 ± 0.014, $p=0.041$, respectively). Similar results are found when comparing the control and ME500 groups with the RA-t-BHP group (0.215 ± 0.009 vs. 0.375 ± 0.017, $p<0.001$; 0.279 ± 0.018 vs. 0.375 ± 0.017, $p=0.016$, respectively).

Oxidative stress is considered a key pathway through which hepatocellular damage occurs and plays a role in the progression of this damage. The relationship between oxidative processes and pathological alterations is confirmed by the significantly reduced levels of glutathione peroxidase, which negatively correlate with serum ALT levels in cirrhosis and hepatitis (Osman et al., 2007). In chronic hepatitis, significantly higher levels of MDA and 8-OHdG are observed (Zhu et al., 2012).

There is data about hepatoprotective effect of luteolin in a carbon tetrachloride (CCl_4)-induced liver damage model. The administration of carbon tetrachloride leads to an increase in the enzymes AST, ALT, and total bilirubin levels. Treatment with luteolin demonstrates a significant dose-dependent protective effect, with approximately a 60% reduction in serum levels of AST and ALT and a 50% reduction in total bilirubin levels. The same study also shows a significant decrease in MDA levels, which is associated with reduced oxidative stress (Li et al., 2023).

It is well-known that chlorogenic acid reduces hepatic oxidative stress in a paracetamol-induced hepatotoxicity model by activating the nuclear factor Nrf2 (nuclear factor erythroid 2-related factor 2) (Wei et al., 2018). There is also evidence of normalization of elevated serum AST and ALT levels with chlorogenic acid intake in non-alcoholic steatohepatitis induced by a methionine and choline-deficient diet (Miao et al., 2022).

It has been established that quercetin-3-O-rutinoside has a beneficial effect on liver damage induced by paracetamol by restoring GSH levels and hepatic enzymes in rats. It is also known to affect hepatorenal toxicity in rats by influencing oxidative stress and stimulating mitochondrial energy production (Subramanya et al., 2018).

There is data showing the hepatoprotective action of eupatorin *in vivo* (Chriscensia et al., 2023).

Goudarzi et al. (2021) report normalization of AST and ALT enzyme levels following the administration of apigenin in a model of hepatotoxicity induced by methotrexate. The same researchers also report elevated MDA levels following methotrexate treatment, with subsequent restoration of levels after apigenin administration (Goudarzi et al., 2021). GSH plays a crucial role in cellular defense against oxidative stress and chemically reactive toxic compounds (Meeran et al., 2018). Goudarzi and colleagues observed a depletion of GSH in the group treated with methotrexate compared to the control group, along with an increase in levels in the group treated with apigenin. They also found reduced CAT enzyme activity in rats treated with methotrexate, which was improved upon administration of apigenin (Goudarzi et al., 2021). Apigenin, which exhibits pronounced anti-inflammatory properties, inhibits the formation and release of pro-inflammatory cytokines (Thangaiyan et al., 2018). These findings are supported by the results of Goudarzi et al., who report reduced levels of TNF-α and IL-1-β in comparison to the methotrexate-treated group. Other studies suggest that apigenin inhibits liver damage in experimental models of hepatotoxicity induced by N-nitrosodiethylamine, lipopolysaccharide, and paracetamol by suppressing inflammatory processes and oxidative damage in rats (Yang et al., 2013; Ali et al., 2014).

Several authors report the antioxidant activity of rosmarinic acid (RA), which significantly reduces plasma ALT and GSH in experimental models of hepatotoxicity. Inflammation is closely linked to oxidative stress due to the ability of free radicals to affect intracellular signal transduction and gene regulation. In alcoholic liver disease, lipid peroxidation causes inflammation and fibrosis, while in chronic hepatitis, elevated levels of TNF-α and β (Poli, 2000). Rosmarinic acid is likely to demonstrate therapeutic potential through the inhibition of the NF-κB pathway and downregulation of TNF-α and COX-2 (Moon et al., 2010).

Ahmed et al. (2019) found that the administration of naringenin in rats treated with paracetamol reduced the elevated serum levels of AST, ALT, and TNF-α (Ahmed et al., 2019).

Hassan et al. (2021) investigated the effect of naringenin, hesperidin, and their combination in rats with hepatotoxicity induced by diclofenac. Daily intraperitoneal injection of diclofenac for 4 weeks resulted in significantly higher serum levels of the proinflammatory cytokine TNF-α, liver enzymes AST, ALT, total bilirubin, and liver GSH. These changes reflect liver damage and necrosis, oxidative stress, suppression of the antioxidant defense system, inflammation, and apoptosis. Co-administration of naringenin and hesperidin led to a significant reduction in the activity of the studied markers (Hassan et al., 2021).

2.6. Antioxidant action

Lipid peroxidation is a process in which free radicals attack lipids containing carbon-carbon double bonds. Significant substrates of lipid peroxidation are polyunsaturated fatty acids, which are lipids with two or more double bonds. They are classified as omega-3 (n=3) and omega-6 (n=6) fatty acids, depending on the location of the last double bond relative to the terminal methyl end of the molecule. The main omega-6 fatty acid is arachidonic acid, which can undergo enzymatic oxidation to form prostaglandins, leukotrienes, thromboxanes, and other cyclooxygenase, lipoxygenase, or cytochrome P-450 products. Arachidonic acid can also be metabolized through a non-enzymatic pathway to MDA and other end products of lipid oxidation. The formed aldehydes lead to the loss of membrane integrity by altering fluidity and subsequent inactivation of membrane proteins. In cases of enhanced lipid peroxidation, the degree of oxidative stress exceeds the capacity for repair, leading to apoptosis or necrosis. The resulting cellular damage facilitates the development of various pathological conditions (Ayala et al., 2014).

According to a study by Stojanovic G and Palic I (2008), the methanolic extract of *M. fruticosa* exhibits slightly lower activity compared to the commercial antioxidant BHT (butylated hydroxytoluene). Methanolic extracts from *M. graeca* and *M. juliana* show antioxidant activity similar to that of alpha-tocopherol. The observed activity of these extracts is likely attributed to their high phenolic content (Stojanovic and Palic, 2008).

Vladimir-Knežević et al. (2011) investigated the antioxidant activity of *M. croatica*, *M. juliana*, and *M. thymifolia*. The three extracts demonstrated antioxidant characteristics, which are believed to be primarily associated with their high content of polyphenolic compounds (Vladimir-Knežević et al., 2011).

Nikolova et al. (2017) used the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method to determine the strongest antioxidant activity in the methanolic extracts of *M. frivaldszkyana* and *M. dalmatica* compared to the other studied species, *M. juliana* and *M. cristata* (Nikolova et al., 2017).

Mladenova et al. (2021) studied the antioxidant activity of *M. frivaldszkyana* using several methods: DPPH, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), copper (CUPRAC) ions, and the ability to neutralize peroxide radicals (Oxygen Radical Absorbance Capacity- ORAC) (Mladenova et al., 2021). The results indicate that the ORAC value for *M. frivaldszkyana* is higher compared to many other Bulgarian medicinal plants (Kratchanova et al., 2010).

The presented data confirm our results regarding GSH, CAT, and the toxic molecules MDA and 8-OH-dG in the t-BHP and paracetamol-induced inflammation model.

CONCLUSION

The comprehensive analysis of the methanolic extract from *M. frivaldszkyana* identifies sucrose, glucose, mannose, fructose, and sugar alcohols as the primary metabolites in the highest quantities. Triacylglycerols are the lipid group present in the most abundant amount. Inorganic elements such as K, Mg, Zn, and Ca are also present in significant quantities. The predominant secondary metabolites are flavonoids and polyphenols. The extract is rich in luteolin, chlorogenic acid, rosmarinic acid, rutin, eupatorin, kaempferol-3-O-rutinoside, and apigenin. *In vivo* evaluation of acute toxicity shows no mortality or toxic effects in male Wistar rats when administered orally at doses up to 5000 mg/kg body weight. Furthermore, 14-day oral administration of 250, 400, and 500 mg/kg body weight of *M. frivaldszkyana* extract reveals its anti-inflammatory potential in the carrageenan-induced hind paw edema model in rats. This activity can be attributed to the high concentrations of flavonoids in the extract. The high content of phenolic compounds (chlorogenic acid, rosmarinic acid, rutin, eupatorin, kaempferol-3-O-rutinoside, and apigenin) is likely to be the primary factor associated with the observed anti-inflammatory activity of the extract. The extract does not exhibit analgesic effects in the "analgesimeter" and "hot plate" models. The evaluation of hepatotoxicity models induced by paracetamol and t-BHP demonstrates potential hepatoprotective characteristics of the methanolic extract from *M. frivaldszkyana*, which are likely due to the antioxidant activity of the flavonoids in its composition. The extract does not exhibit analgesic effects in the "analgesimeter" and "hot plate" models. The evaluation of hepatotoxicity models induced by paracetamol and t-BHP demonstrates potential hepatoprotective characteristics of the methanolic extract from *M. frivaldszkyana*, which are likely due to the antioxidant activity of the flavonoids in its composition.

CONCLUSIONS

Regarding the phytochemical composition of the plant:

1. A total of 83 compounds were identified through GC-MS analysis, classified as amino acids, organic acids, sugars, and sugar alcohols.
2. A total of 163 lipid compounds were identified, distributed into 10 classes, through lipidomic analysis of the non-polar fraction.
3. A total of 192 compounds were discovered – 123 identified and 69 unknown compounds, through UPLC-MS-MS analysis of methanolic extracts of *M. frivaldszkyana*. The secondary metabolites with the highest concentrations were flavonoids, primarily flavonoid glycosides. Rosmarinic acid is one of the most significant substances identified.

Regarding the studied biological activities:

1. The methanolic extract of *M. frivaldszkyana* does not induce toxic effects when administered orally to rats at doses up to 5000 mg/kg body weight.
2. The plant extract did not show analgesic activity in tests with mechanical and thermal pain stimuli.
3. The methanolic extract of *M. frivaldszkyana* at all three tested doses exhibits an anti-inflammatory effect in an acute exudative inflammation model induced by carrageenan.
4. The administration of the methanolic extract of *M. frivaldszkyana* does not improve spatial working and episodic memory in naïve rats, but based on its phytochemical composition, such an effect could be expected in models of impaired memory.
5. The methanolic extract of *M. frivaldszkyana* influences oxidative stress in experimental models of hepatotoxicity primarily by suppressing free radical production (reduced oxidative stress markers), rather than by enhancing antioxidant enzyme activity. This suggests its mainly preventive role in liver damage.

CONTRIBUTIONS

CONTRIBUTIONS WITH SCIENTIFIC AND THEORETICAL SIGNIFICANCE

1. For the first time, the acute toxicity of the methanolic extract of *M. frivaldszkyana* was investigated following oral administration in rats.
2. The comprehensive metabolomic analysis of the extract reveals a high content of phenolic acids and flavonoids, which likely determine the observed biological activities of the extract.

CONTRIBUTIONS WITH SCIENTIFIC AND PRACTICAL SIGNIFICANCE

1. For the first time, a potential hepatoprotective effect of the methanolic extract of *M. frivaldszkyana* was established in a rat model of liver toxicity.
2. For the first time, an anti-inflammatory effect of the extract was established in a rat model of hind paw inflammation.

SCIENTIFIC PUBLICATIONS AND PARTICIPATION IN SCIENTIFIC CONFERENCES, RELATED TO THE DISSERTATION

I. Scientific publications

1. **Stavrakeva K**, Metodieva K, Benina M, Bivolarska A, Dimov I, Choneva M, Kokova V, Alseekh S, Ivanova V, Vatov E, Gechev T, Mladenova T, Mladenov R, Todorov K, Stoyanov P, Gyuzeleva D, Popova M, Apostolova E. Metabolic Composition of methanolic extract of the Balkan endemic species *Micromeria frivaldszkyana* (*Degen*) Velen and its anti-inflammatory effect on male Wistar rats. *International Journal of Molecular Sciences,* 2024
2. **Stavrakeva K**, Popova M, Esad M, Apostolova E, Kokova V, Bacelova M, Alakidi A& Bivolarska A. Drug-induced liver toxicity. *Acta Medica Bulgarica, 2024*
3. **Stavrakeva K.** Biological potential of the Balkan endemic species *Micromeria Frivaldszkyana* (Degen) Velen. (Lamiaceae). *Scientific Works of the Union of Scientists Plovdiv*, 2024

II. Participation in congresses, conferences and other scientific forums

1. Metodieva K, **Stavrakeva K**, Dimov I, Choneva M, Kokova V, Alseekh S, Ivanova V, Vatov E, Gechev T, Mladenova T, Mladenov R, Todorov K, Stoyanov P, Gyuzeleva D, Popova M, Benina M, Bivolarska A, Apostolova E. Phytochemical characterisation of water and ethanol extract from *Micromeria friwaldszkyana.* - Poster presentation. 48th Federation of European Biochemical Societies Congress 2024, 29.06-03.07.2024, Milan, Italy.
2. **Stavrakeva K.** Biological potential of the Balkan endemic species *Micromeria Frivaldszkyana* (Degen) Velen. (Lamiaceae) – a review. X international scientific conference of young scientists –Plovdiv, 20-23 June 2024.
3. **Stavrakeva K,** Kokova V, Apostolova E. Experimental study on the anti-inflammatory and analgesic effect of *Micromeria frivaldszkyana (Degen) Velen* – a review. VIII National congress of pharmacology, clinical pharmacology and therapy, Pleven, 15-17 November 2024.

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