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EXPERIMENTAL PHARMACOLOGICAL STUDY OF EXTRACT FROM *SIDERITIS SCARDICA*, LAMIACEAE

Abstract

For awarding the educational and scientific degree "Doctor of Philosophy"

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"Pharmacology (incl. Pharmacokinetics and Chemotherapy) "

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The dissertation contains 151 standard typewritten pages. It is illustrated with 46 figures, 39 tables, and an appendix. 303 literary sources are cited, all in Latin.
The experimental studies were carried out in the Department of Pharmacology and Clinical Pharmacology, Department of Anatomy, Histology, and Embryology at the Faculty of Medicine of the Medical University of Plovdiv, Department of Medical Microbiology and Immunology "Prof. Dr. Elissay Yanev," Faculty of Pharmacy, MU Plovdiv, Department of "Organic and Inorganic Chemistry" - University of Food Technology, Plovdiv.

The dissertation was approved and referred for official defense by the extended Departmental Council of the Department of Pharmacology and Clinical Pharmacology at the Faculty of Medicine of the Medical University - Plovdiv on 31.08.2022, Protocol No. 97. The dissertation student was granted the right to defense by order P - 2411 /07.10.2022 of the Rector of Medical University Plovdiv.

The dissertation is scheduled for defense before a scientific jury composed of Cor. Member Correspondent Prof. Dr. Mila Vasileva Vlaskovska, Ph.D., dm
Prof. Dr. Stefka Vasileva Valcheva-Kuzmanova, Ph.D., dm
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The public defense of the dissertation work before a scientific jury will take place on 06.07.2023 at 11 a.m. in Auditorium II of the Auditorium Complex of the Medical University - Plovdiv, 15A "Vasil Aprilov" Blvd., Plovdiv. The defense materials are available at the Scientific Department of the University of Plovdiv, 15A "Vasil Aprilov" Blvd., Plovdiv, and are published on the university's website.

CONTENTS

INTRODUCTION

Sideritis scardica, popularly known as Greek mountain tea, Mursalski, or Pirin tea, southern European *Lamiaceae*, has been used for centuries in ethnopharmacology for lung diseases, colds, and gastrointestinal complaints. Already in his "Materia Medica" (1st century), Pedanius Dioscurides describes plants called "Sideritis " The name is derived from the Greek word "*sideros*", "iron," or "he who is or has iron". In the Bulgarian Rhodopes, the herb is also known as "longevity tea" due to its multiple effects.

Since 1989 *Sideritis scardica* has been included in the Red List of Bulgaria, and the IUCN (International Union for Conservation of Nature and Natural Resources) classified the plant as potentially endangered in 2001.

Over 100 different components of its essential oil have been identified so far. Monoterpenes, sesquiterpenes, and diterpenes, as well as fatty acids and their esters, can be found in all studied populations. In addition, triterpenes and more than 40 polyphenols and flavonoids are found in the drug. It also contains benzoic, quinic and cinnamic acid derivatives, ferulic acid, and phenylethanoids glycosides. The exact composition depends on the location and the way the plant is cultivated.

In recent years, there has been a resurgence of interest in the cultivated production of this crop, which, accompanied by the results of scientific works describing the effects of the herb on the CNS, motivated the idea of a scientific work aiming to establish the adaptogenic, neuroprotective and cognition-enhancing properties of this endemic for Bulgaria plant. Research data can be used in choosing the design of clinical trials related to many socially significant diseases in which therapeutic options are limited - neurocognitive disorders, dementia, Alzheimer's disease, depressive disorders, ADHD.

The nature of the research involves the study of acute and chronic toxicity in experimental animals and the preparation of histological preparations from various organs, the study of behavior, and the determination of the levels of pro-inflammatory cytokines in two models of stress and lipopolysaccharide-induced neuroinflammation, a study of learning and memory processes in three models of impaired memory.

Laboratory-bred white rats are the most suitable and most commonly used animal species for conducting relevant experiments, and this is based on data from specialized scientific materials. Data in the available scientific literature proves that stress causes changes in the levels of cytokines (IL -6, IL -1β, IL -10, TNF - α) in the serum of rats and humans. Data from experimental and clinical studies establish a relationship between altered levels of cytokines under the influence of stress and the development of diseases such as depression and neurocognitive degenerative changes. Cytokines can serve as a reliable marker to assess the impact of stress on the body and to evaluate the efficacy of therapy with adaptogenic plant extracts.

The presented dissertation offers a scientific research of fundamental and applied nature built up on assessing biologically active substances from natural origin and with potential for use as adaptogenic agents for prevention and treatment.

MATERIALS AND METHODS

Plant material and extract

Dried plant material from cultivated Mursala tea (*Sideritis scardica*) was purchased from NW Health Ltd, Bulgaria. The extract was produced by hydromethanolic extraction via maceration (70% v/v) and processing using a spray dryer at a temperature below 45 ^{o}C in Vesselino Ltd., Kazanlak.

Phytochemical analysis

The phytochemical studies were conducted in the Department of Organic and Inorganic Chemistry laboratory - University of Food Technology, Plovdiv.

Determination of total phenolics

The amount of total phenols in the obtained extracts was determined by the Folin - Ciocalteu method. 1 ml of Folin - Ciocalteu 's reagent (diluted 5 times) and 0.8 ml of 7.5% Na_2CO_3 were added to the Mursal tea extract (0.2 ml). After 20 minutes, the absorbance was measured at 765 nm against a blank. Results are presented as milligram equivalents of gallic acid per gram (mg GAE / g) dry matter.

Determination of total flavonoids

The content of total flavonoids in the extracts was also determined spectrophotometrically using $Al(NO_3)_3$. Results are presented as milligram quercetin equivalents per gram (mg QE / g) dry matter.

Determination of antioxidant activity

The examined extract (0.15 ml) was mixed with 2.85 ml of freshly prepared DPPH (2,2- diphenyl -1- picrylhydrazyl) solution (0.1 mM in methanol). The reaction mixture was incubated in the dark for 15 min at 37°C. The reduction in absorbance was read spectrophotometrically at 517 nm.

ABTS method

The ABTS radical was prepared by mixing equimolar amounts of ABTS (2,2'-azino - bis (3- ethylbenzothiazoline -6- sulphonic acid) (7 mM in e. H_2O) and potassium persulfate (2.45 mM in water) and staying for 16 h in the dark. Before analysis, 2 ml of the ABTS radical were dissolved in methanol at a ratio of 1:30 to obtain a final absorbance of 1.0÷1.1 at 734 nm. For a, 0.15 ml of the tested extract was mixed with 2.85 ml of a freshly prepared solution of the ABTS radical. The reaction mixture was incubated in the dark for 15 min at 37°C. The reduction in adsorption was read spectrophotometrically at a wavelength of 734 nm.

FRAP method

The reagent was prepared by mixing previously prepared 0.3 M acetate buffer at pH 3.6, 10 mM 2,4,6- tripyridyl - s - triazine (TPTZ), and 20 mM $FeCl_3 \times 6 H_2O$ in a ratio of 10:1:1. The tested extract (0.1 ml) was added to 3 ml FRAP reagent.

The reaction mixture was incubated for 5 min at 37°C in the dark. The absorbance of the colored compound formed was measured at a wavelength of 593 nm. Antioxidant activity results are expressed as mM Trolox equivalents per gram of dry raw material (mM TE / g).

CUPRAC method

Initiation for the reaction to proceed was started by mixing 1 ml $CuCl_2 x 2 H_2O$, 1 ml Neocuproin (7.5 mM in methanol), 1 ml 0.1 M ammonium acetate buffer pH 7; 0.1 ml of the tested extract, and 1 ml of distilled H_2O. The reaction mixture was incubated for 20 min at 50°C in the dark. After cooling the mixture, the absorbance of the resulting compound was read at 450 nm. Results from all four methods are presented as millimoles of Trolox equivalents (mM TE).

Each of the considered methods for the analysis of antioxidant activity is based on a different mechanism and is applied under precisely defined conditions (pH, temperature). Therefore, to determine a given compound's total antioxidant capacity, several parallel methods must be applied.

Determination of sugars

Chromatographic analyses are conducted on high-efficiency liquid chromatography (HPLC) Elite Chrome Hitachi equipped with an LC-20 AD pump, column thermostat, Chromaster 5450 refractometric detector and software program. For the chromatographic separation and the quantitative determination of sugars in Mursala tea extract is used Shodex ® Sugar SP0810 column (300 mm × 8.0 mm id .) with Pb2+ and precolumn Shodex SP-G (5 μm, 6 × 50 mm) and mobile phase H_2O. The mobile phase was vacuum filtered through a 0.2 μm membranous filter (Sartorius AG, Goettingen, Germany). All samples before injection are filtered through filters ISOLab (Germany) with a diameter of 4 mm and pore size of 0.45 μm. The volume of the injected sample was 20 μl. The samples are analyzed with a flow rate 0.5 mL /min, column temperature 80°C, and detector temperature of 35°C. Concentrations are calculated as the areas of the received peaks are substituted into standard equations.

Laboratory preclinical experiments

Determination of acute toxicity

Male sexually mature Wistar albino rats were used, weighing about 180 grams, provided by the Vivarium of the Medical University of Plovdiv. Each experimental group included 6 animals, and received a single dose of the extract. The doses are 500, 1000, 2000, 5000, and 10 000 kg/mg body weight.

Determination of subchronic toxicity.

The experimental animals - 24 male albino Wistar rats were obtained from the Vivarium of the Medical University of Plovdiv. Animals were housed in a ventilated room with a temperature range of 25 ± 2ºC under a 12-h light-dark cycle. Feeding consisted of a standard chow diet and provision of tap water ad libitum. The animals were divided into four groups, each group consisting of 6 rats.

Group 1 was a " sham " group, and groups 2, 3, and 4 received orally an aqueous solution of a hydromethanolic dry extract of *Sideritis scardica* at doses of 100, 200, and 400 mg/kg body weight for 12 weeks. Animals were fasted for 12 h before serum and organ sampling.

Hematological analysis.

Venous blood samples were taken from the jugular vein under human-conditioned inhalation anesthesia. Tests were conducted using the RT-7600 Auto Hematology Analyzer and reagents provided by the manufacturer.

Biochemical analysis

A blood sample was taken from the jugular vein under humane conditioned inhalative anesthesia. Blood samples were collected in tubes and centrifuged at 4000 rpm for 10 minutes. The Serum was immediately frozen at -20ºC for further analysis. A series of biochemical parameters were analyzed using a Rayto automated biochemical analyzer Chemray–120 and kits from Biomaxima, Poland.

Immunological analysis of cytokines in a model of acute cold stress

A venous blood sample was taken from the jugular vein under humane conditioned inhalative anesthesia. Blood samples were collected in tubes and centrifuged at 4000 rpm for 10 minutes. Serum was immediately frozen at -20ºC for further analysis.

Immunological analysis of cytokines in a model of chronic stress

A venous blood sample was taken from the jugular vein under humane conditioned inhalative anesthesia. Blood samples were collected in tubes and centrifuged at 4000 rpm for 10 minutes. Serum was immediately frozen at -20ºC for further analysis.

Cytokine immunoassay in a lipopolysaccharide model of chronic inflammation

The animals of the experimental groups were treated with the extract in doses of 100, 200, and 400 mg/kg two weeks before the start of the experiment. After that, rats from the experimental groups and the positive control were systemically injected intraperitoneally with a lipopolysaccharide solution at a dose of 250 microg/kg for 5 days, and on the last day at a dose of 1 mg/kg - one hour before the collection of serum.

A venous blood sample was taken from the jugular vein under humane conditioned inhalative anesthesia. Blood samples were collected in tubes and centrifuged at 4000 rpm for 10 minutes. Serum was immediately frozen at -20ºC for further analysis.

In Vitro study of pro-inflammatory cytokines

The studies of the levels of the cytokines were performed at the Department of Microbiology and Immunology, Medical Faculty of the MU Plovdiv.

The method used is Enzyme-linked immunosorbent assay ELISA with ready-made commercial kits in strict compliance with the methodical instructions of the manufacturer.

Principle of the method:

For the quantitative study of cytokines, diluted rat sera, internal controls, and assay standards were spotted onto a solid phase with monoclonal antibodies against the respective cytokine. After incubation and washing, peroxidase was applied as conjugate (second anti-species antibody) to form a complex with the cytokine. A second wash follows to remove unbound conjugate. When acquiring a chromogenic substrate for the enzyme, a color reaction occurs, marking the presence of cytokines. The absorbance, which is proportional to the cytokine concentration, was measured colorimetrically by ELISA reader TECAN at 450 and 620 nm. By constructing a standard curve (linear regression), the concentration of each cytokine in pg /ml is determined. Each experimental and control group consisted of 6 animals.

Acute cold stress model

The animals of the experimental group were treated with the extract at a dose of 100, 200, and 400 mg/kg for 30 days. Negative and positive control animals were treated with distilled water. On the last day, 60 minutes after treatment with the extracts, rats from the experimental groups and those from the positive control were placed in plastic troughs and subjected to acute cold stress in a refrigerator at a temperature of 5°C for 1 hour. Behavioral experiments were conducted immediately after that. Each experimental and control group consisted of 6 animals.

Chronic stress model

After a two-week treatment of the test animals from the experimental groups with the extract in a dose of 100, 200, and 400 mg/kg, the chronic stress conditioning began. During 8 weeks, the animals were subjected daily to a different micro stressor. Such can be deprivation of food, deprivation of water, wetting the housing, tilting the cage at an angle of 45 degrees, reversing the light regime for 24 hours - light or dark, and submission to a natural predator noise. The sequence of the stressors was varied weekly to remain unpredictable and to prevent adaptation of the experimental animals. Negative control animals were treated with distilled water and not subjected to stress. The animals of the positive control, as well as the experimental animals treated with the extract in a dose of 100, 200, and 400 mg/kg, were subjected to stressors. Each experimental and control group consisted of 6 animals.

Forced swim test

The forced swim test is used to assess depression in rodents. It consists of two sessions – a pre-test and actual testing. The pre-test is done 24 hours before the actual test. Each animal was placed in a cylindrical tube 20 cm wide and 35 cm high filled with an 18 cm water column (t°= 25°) for 15 min and then removed and returned to its cage. The test session takes place 24 hours later. Each rat was placed in the cylindrical tube for 5 min, and the immobility time (T0) was recorded.

An animal is considered motionless when it is not struggling to escape and is swimming, making only the necessary movements to keep its head above water. A criterion for an antidepressant effect is a significant reduction in T0, respectively an increase in the time of active swimming. Each experimental and control group consisted of 6 animals.

Elevated Plus Maze test (EPM)

In the elevated plus maze (EPM) test, animals are placed in the center of the plus maze and allowed to choose to spend time in the open or closed arms. The maze is an opaque Plexiglas platform raised 40 cm above the ground with two open arms and two closed arms. Rats prefer closed and dark spaces, so they avoid the lit sections of the maze. An increased dwell time in the open arms and an increase in the number of entries into them correlated with an anxiolytic effect. The following parameter was assessed: time spent on indoor and outdoor arms, and

the open/total arm entries ratio. Each experimental and control group consisted of 6 animals.

Social Interaction Test

In the social interaction test, each rat is tested with an unfamiliar partner. Their behavior is observed for 5 minutes. The time of social interaction with the partner is measured. Interactions such as touching the fur or getting on top of the other animal are interpreted as actual contact. Decreased social interaction time (or lack thereof) is an indication of anxious behavior, while increased time speaks of an anxiolytic effect. Each experimental and control group consisted of 6 animals.

Behavioral tests in models of impaired memory

The animals of the experimental groups were treated with the extract in a dose of 100, 200, and 400 mg/kg for 30+ days. Negative control animals were treated with distilled water and positive control animals with distilled water and an additional injection of an amnesic agent on the respective days of experimental activity or training. Each group consists of 8 animals.

In the diazepam-impaired memory model, the positive control animals were injected intraperitoneally with a 2.5 mg/kg diazepam solution one hour before the experiments and training sessions.

In the scopolamine-impaired memory model, the positive control animals were injected intraperitoneally with a scopolamine solution of 1 mg/kg one hour before the experiments and training sessions.

In the impaired memory model, the positive control animals were injected intraperitoneally with sodium nitrite solution 25mg/kg one hour before conducting the experiments and training sessions.

Activity cage

Spontaneous locomotor activity was examined using an automatic electronic Activity apparatus cage (Ugo Basile, Italy). The animal is placed in a plastic cage with transparent walls and a transparent lid. The horizontal and vertical activity of the animal is recorded by means of infrared photosensors located on the walls and floor of the cage. Each animal was placed in the center of the apparatus and allowed to freely roam the field for 5 min at a time. Horizontal and vertical activity are reported in relative units as the number of vertical passes must be higher than that of horizontal ones. Data were obtained from the experimental and control groups, each consisting of 8 animals. This test is also a starting point for conducting other behavioral tests.

T-maze test

The T-test is a classic method for studying spatial orientation and working memory. The T -maze is constructed of black plexiglass and consists of three arms at an angle of 90˚. The test is conducted over 3 days. On the first day, the animals are deprived of food and habituated to the setup in sessions of 5 minutes each. The second day continues with habituation, with a small amount of food pellets scattered around the maze in each arm. On the third day, the actual test was performed, with each animal undergoing ten passages. During the first passage,

an animal is forcibly directed to one arm of the maze, and at the end, a reward awaits it in the form of a food pellet. On each subsequent pass, the reward is moved to the opposite arm of the maze. In this paradigm, rats inherently tend to switch arm visits during successive trials, which is called "spontaneous alternation" (Lalonde, 2002). This tendency can be reinforced by baiting with food when the animals are deprived of food. The rat in each subsequent trial must always choose the opposite arm of its previous visit, otherwise, a memory error is recorded. After choosing a non-rewarded arm, a self-correction procedure was applied, with the food pellet left and the arm in which it was located open until visited, allowing the rat to change its choice. The food reward remains in place until it is found and eaten. The following indicators are taken into account:
Correct choice = shoulder visit with reward.
WMI (working memory index) = number of correct choices/number of total trials
The obtained results are based on data from experimental and control groups, each consisting of 8 animals.

Novel Object Recognition Test NORT

The experimental setup consists of a dark Plexiglas structure with dimensions of 60 x 60 x 40 cm (width x length x height). The examination is conducted on two consecutive days. Each group is composed of 8 animals.
On the first day, the animals were placed in the cage for 3 min to explore the territory without the presence of the objects. This allows the habituation of the animals to the experimental field. Two identical objects (A+A) were then placed, and the animals were allowed to explore the objects for 5 min.
On the second day, the actual testing occurs, as the animal is placed in the same environment with two objects, but this time one is the same as the ones from the training day (familiar, A), and the other is different (B, novel). The time spent surveying the new and the old object is counted for a period of 5 minutes. The evaluation is obtained through a discrimination index (DI), which is calculated according to the formula:
DI = t B / (t A + t B), where:
t A - time to explore object A
t B - time to explore object B

U-maze

The U-maze is constructed of black Plexiglas and consists of three arms at an angle of 120˚. Each arm measures 10cm x 50cm x 30cm (width x length x height). The test is performed in the following steps:
1. The three arms are distributed randomly:
1) arm - A; 2) arm - B; 3) arm - C.
2. The rodent is placed in the center of the maze and allowed to move freely until it enters another arm along with the tail. The sequence of entry into the different arms - A, B, or C - is noted.
The total number of entries is counted, excluding the first arm in which the animal is placed (B).

A sequence of three letters is defined as a triad, and when they are three different in sequence, this is called an alternation. The presentation of experimental animals is determined by the formula for the percentage of alternations:
(Total number of alternations / total number of triads)*100
3. The animal is observed once for 5 minutes.
The test is conducted on two consecutive days. Each group is composed of 8 animals.

Active learning method (Two-Way Active Avoidance test, Shuttle-box test)

This is a two-way conditioned active avoidance test in which animals learn that a particular stimulus (sound, light, conditioned stimulus) is a reliable predictor of an upcoming punishing experience (electric shock, unconditioned stimulus) and can take action to avoid it. This task is classified as a conditioned reflex - the animal must remember the relationship between the conditioned and unconditioned stimulus in order to be in anticipation of the conditioned stimulus and avoid it. The test is conducted in a specially designed apparatus manufactured by Ugo Basile, an Italian experimental medical device company. Experiments are carried out in a cage with dimensions suitable for this type of experimental animal. The standard apparatus is an automated cage divided in two by a vertical partition with an opening in the center through which the rats pass. The floor is a grid of parallel metal rods spaced 1 cm apart. The ceiling of the cage is made of a transparent cover, on which a lamp is mounted that emits a light signal at the same time as the sound stimulus (conditional stimuli).
The standard training program consisted of 30 training sessions with the following parameters: simultaneously applied light and sound stimuli (670 Hz, 70 dB) for 6 seconds, followed by 0.4 mA electrical stimulation on the grid floor of the cage for 3 seconds. The break between individual training sessions is 12 seconds. The training session continues for 4 consecutive days.
The short-term memory test is conducted on the 5th day and the long-term memory test on the 12th day (counted from the first day of training), following a program with the same parameters but without electrical stimulation. The number of conditional answers (avoidances) is automatically counted. Each group is composed of 8 animals.

Methods of passive learning (Passive conditioned avoidance with punitive reinforcement of rats (step-through and step-down tests).

Step-through test

An automatic electronic device is used for passive avoidance with penal reinforcement (Automatic set-up for passive avoidance, "step-through", Ugo Basile, Italy). The device has two compartments, light and dark, separated by a sliding door. Each experimental day included 3 training sessions every 60 minutes according to the standard program for the apparatus with the following parameters: a delay of 7 seconds before opening the door, followed by 12 seconds of open door. The test animal is placed in the light room, and after a latency period of 7 s, the door is opened automatically, giving it access to the dark room. After

entering the dark room (latency time), the door is closed behind the animal, and it is subjected to a short-term punishing stimulus (electrical current on the feet, with an intensity of 0.4 mA for 9 sec). If the rat does not pass into the dark room, a time counter (in seconds) is automatically activated, which counts a maximum time of 3 minutes (180 ± 2 seconds).

A two-day training session is held. There are two memory tests: a short-term memory test - 24 hours after the training session (on the 3rd day) and a long-term storage memory test (on the 10th day). Each experimental day includes 3 training sessions every hour. The criterion of successful training is the animal's stay in the bright chamber of the apparatus within the maximum time of 178 seconds during 2 consecutive training sessions. Each group is composed of 8 animals.

Step-down test

An automatic electronic device is used for passive avoidance with penal reinforcement (Automatic set-up for passive avoidance, " step-down ", Ugo Basile, Italy). It is a standard cell with a plastic vibrating platform in the middle, and the program set in the electronic part of the device is used. Each experimental day includes 2 training sessions of 60 minutes. The training session consists of 2 consecutive days. On the 3rd day, the test for short-term memory is conducted, and on the 8th day – the test for long-term memory traces.

The experimental animal is placed on the plastic platform, which, after turning on the apparatus, vibrates vertically. The reaction time (latency) is taken into account - getting off the platform with 3 or 4 paws. A 60-second stay on the platform during 2 consecutive training sessions is taken as a criterion for successful training of the animal. Each group is composed of 8 animals.

Edema of the hind paw induced by administration of carrageenan

The mursala tea extract was administered at doses of 100, 200, and 400 mg/kg for 30 days.Before treatment, the volume of the right hind paw of the animals of all groups was measured. Then, 0.1 ml of a 1% solution of carrageenan in 0.9% sodium chloride was injected into the right hind paw of all animals to induce carrageenan edema. Immediately after the carrageenan injection, the negative control animals were injected intraperitoneally with 0.1 ml of 0.9% sodium chloride solution; the animals from the positive control group were given diclofenac at a dose of 25 mg/kg of body weight, and the animals from the experimental groups were injected intraperitoneally with the amnestic agents. Each group consists of 6 animals. A plethysmometer device (Ugo Basile, Italy) measured the volume of displaced fluid from the right hind paw of the rat 2, 3, 4, and 24 hours after carrageenan treatment, respectively. The percentage of paw edema is calculated using the following formula:

Paw edema (%) = $(Vt - Vo)/ Vo* 100$

Vo – average paw volume before treatment,

Vt – average paw volume per hour

A criterion for anti-inflammatory activity is reduced swelling of the paw compared to the control group.

Statistical data processing

The statistical processing of the obtained results is carried out using an IBM software package SPSS 19.0. To determine the distribution, 1-Sample Kolmogoroff – Smirnoff test is conducted. For each indicator, an arithmetic mean value and a standard error of the arithmetic mean are determined. Comparison of the results between groups was performed with ANOVA test, test for homogeneity of distribution between groups Levine, and significant results were presented by post - hoc test LSD in the presence of homogeneity and Games - Howell in the absence of homogeneity. Results are considered statistically significant at a significance level of $p < 0.05$.

Results

Acute and subchronic toxicity experiments.

The conducted acute and chronic toxicity experiments showed no mortality or gross morphological changes.

Phytochemical analysis and antioxidant activity.

The results of the phytochemical analysis and antioxidant activity of the studied extract are presented in Table 1. The antioxidant activity was determined by four methods: DPPH, ABTS, FRAP, and CUPRAC.

Table 1. Phytochemical analysis.

Parameter	**Value**
Sucrose g/100 g	13.30
Glucose g/100 g	4.61
Fructose g/100 g	4.30
Total sugars g/100 g	22.21
Total polyphenols, mg GAE /g	88. 66±2.57
Totlani flavonoids, mg QE /g	22. 01±1.23
Antioxidant activity, mM TE /g	
DPPH	693.89±3.61
ABTS	1009.60±13.82
FRAP	552.66±5.65
CUPRAC	1418.60±23.39

Results of hematological studies.

The results of hematological studies (Table 2) did not show statistically significant changes in any parameter. A decrease in platelets was observed, but it was within the reference values described for the species (Charles River Laboratories Preclinical Services Montreal Inc., 2008).

Table 2. Hematological analysis after 12 weeks of treatment.

Parameter	K0	100 mg/kg	200 mg/kg	400 mg/kg
WBC 10 $^{-9}$ /L	4±0.70	4 .01±1.2	3.74±0.84	4.11±1.26
RBC 10 $^{-12}$ /L	7.93±0.46	7.44±0 .71	7.33±0. 6	7.57±0. 52
HGB g/L	147.7±8. 87	143.83±8.86	140.5±8.85	140.33±7.87
HCT L/L	0.42±0. 02	0.41±0.02	0.34±0.14	0.4±0.02
MCV fL	52.53±1.04	54. 63±2.35	53. 48±1.65	52. 6±1.17
MCH pg	18.63±0.33	19.37±0.83	18.32±1.3	18.53±0.48
MCHC g /L	354.67±3.27	355±2.68	342.83±25.5	352.33±3.33
PLT 10 $^{-9}$ /L	680.67±186.45	546.17±315.58	520.67±179.29	569.83±241.87

Results of biochemical studies

The results of the biochemical tests (Table 3) showed a statistically significant decrease in triglycerides and calcium. However, the documented values are within the reference range described for the breed (Charles River Laboratories Preclinical Services Montreal Inc., 2008).

Table 3. Biochemical parameters after 12 weeks of treatment

Parameter	K0	100 mg/kg	200 mg/kg	400 mg/kg
Glu mg/dL	65.5±11.43	81.17±19.05	80.33±14.36	83.83±17.94
ASAT U/L	135.83±16.5	133±25.57	117.17±23.18	135.33±18.61
ALAT U/L	5.5±1.05	7.33±2.73	5.67±1.37	6.17±1.94
Cho mg/dL	69.79±13.11	78.33±6.76	72.82±15. 94	66.54±10.31
Ua mg/dL	1.73±0.3	1.82±0.67	1.85±1.50	1.92±0.44
T gc mg/dL	78.5±14.32	62.67±9.54	52.33±20.51 *	47±10.26 *
Ca mg/dL	8.23±0.23	7.97±0.27	7.2±0.74 *	7.7±0.30

Results of histological studies

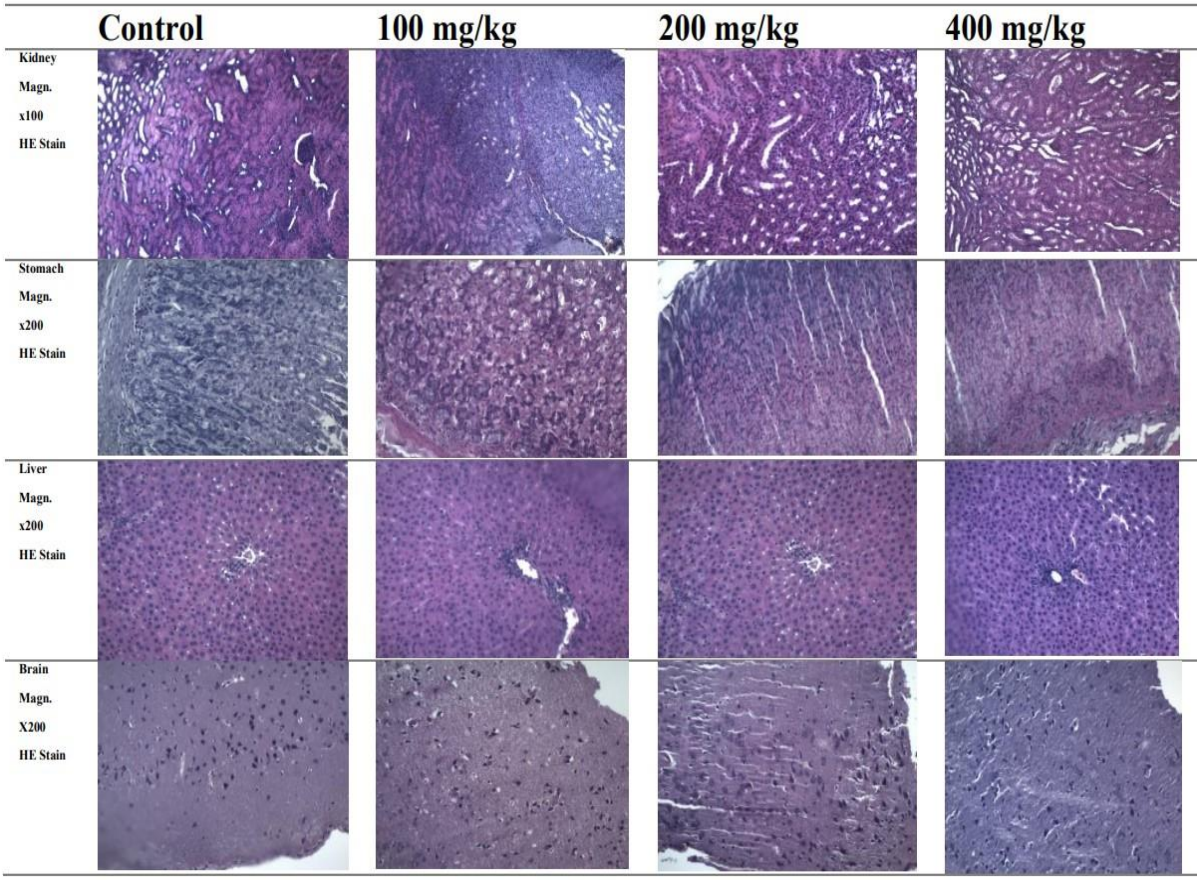

Figure 1. Preparations from histological examinations after 12-week treatment.

The conducted histological study demonstrated that the organs of the animals from all studied groups had normal morphology (Figure 1).

Kidney. No changes were observed in the microstructure of the cortex and medulla of the treated animals. This applies both to the glomeruli and to all duct systems.

Stomach. The observed images showed no differences in the gastric mucosa or gland of the treated and control groups of animals.

Liver. The liver lobules are well-defined, with an intact border and structure of v. centralis, triads, and cells. Hepatocytes did not show any changes from normal morphology.

Brain. Exemplary images of experimental animals show normal structure and lack of cortical remodeling.

Results of acute stress tests.

A test of social interaction in an acute stress model

Experimental data from the test are presented in Figure 2. Comparing the two controls shows that the K0 group had a significantly higher value of the social interaction time parameter compared to K+. This indicates that the selected social interaction test is suitable for assessing behavioral changes in an acute stress

model. It is noteworthy that the value of the reported time for social interaction at a dose of 400 mg/kg (11.8333) approaches that of K0 (13.0000). In all groups treated with the extract, a dose-dependent improvement of the parameter was observed, being statistically significant at a dose of 200 mg/kg, p=0.023 and 400 mg/kg p<0.001.

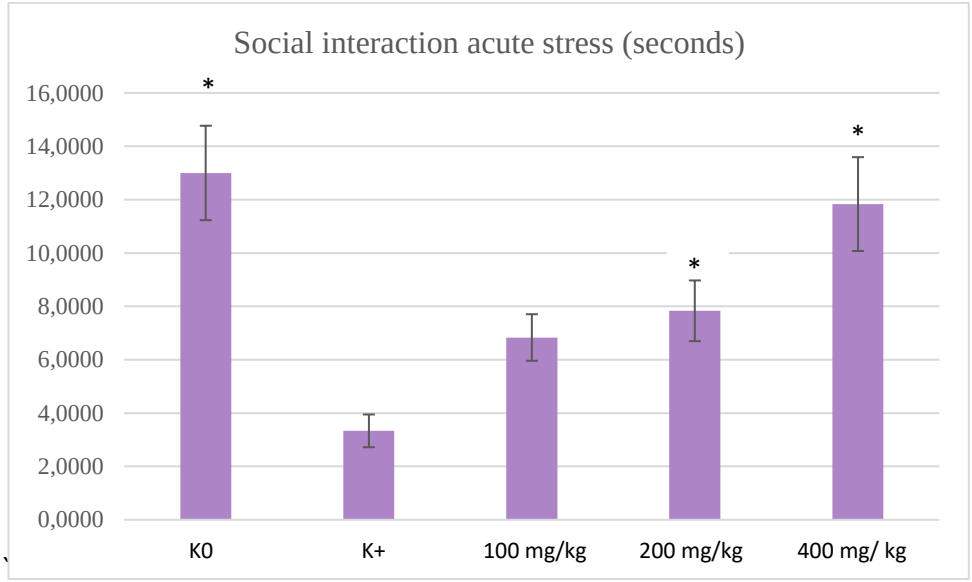

Figure 2. Results of a social interaction test in an acute stress model.

Elevated Plus Maze results in an acute stress model

When comparing the two controls, a significantly higher value of the parameter time spent in the open arms of the maze was observed for the K0 group. This indicates that the selected elevated plus maze test is suitable for assessing behavioral changes in an acute stress model. All groups treated with the extract showed an improvement in the parameter, but it was not statistically significant, therefore, the data are not presented.

Figure 3. presents the results of the elevated cross-maze test under an acute stress model for the parameter number of outdoor entries/total number of maze arm entries. All groups treated with the extract showed an improvement in the parameter, which was statistically significant at a dose of 200 mg/kg, p=0.024, and 400 mg/kg, p=0.022.

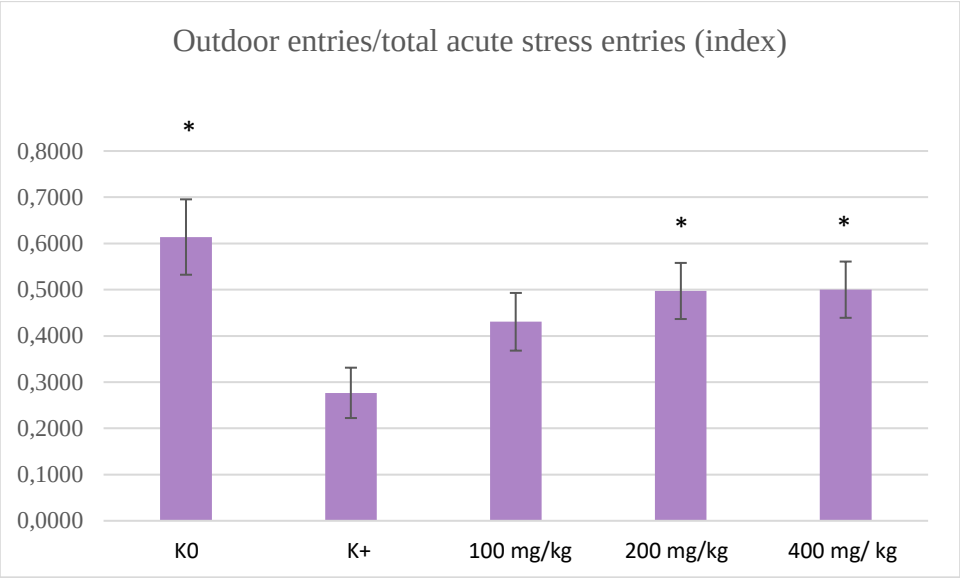

Figure 3. Results of an elevated plus maze test in an acute stress model

FST results in an acute stress model

When comparing the two controls, a significantly higher value of the active swimming time parameter was found in the K0 group (Figure 4). This indicates that the selected forced swim test is suitable for assessing behavioral changes in an acute stress model. In all groups treated with the extract, a dose-dependent improvement of the parameter was observed, and it was statistically significant at a dose of 400 mg/kg p=0.002.

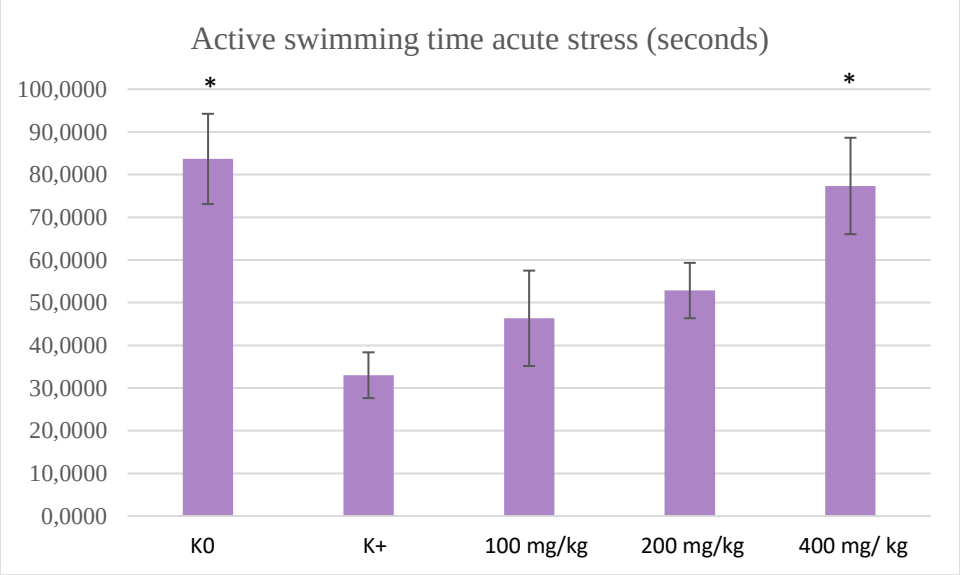

Figure 4. Results of a forced swim test in an acute stress model

Results of chronic stress tests.

Results of a social interaction test in a model of chronic stress

When comparing the control groups, a significant increase in the social interaction time parameter was observed in the K0 group compared to K+ (Figure 5). This indicates that the selected social interaction test is suitable for assessing behavioral changes in a chronic stress model. Except for K0, all groups treated with the extract showed an improvement in the parameter, which was statistically significant at a dose of 200 mg/kg, p=0.017.

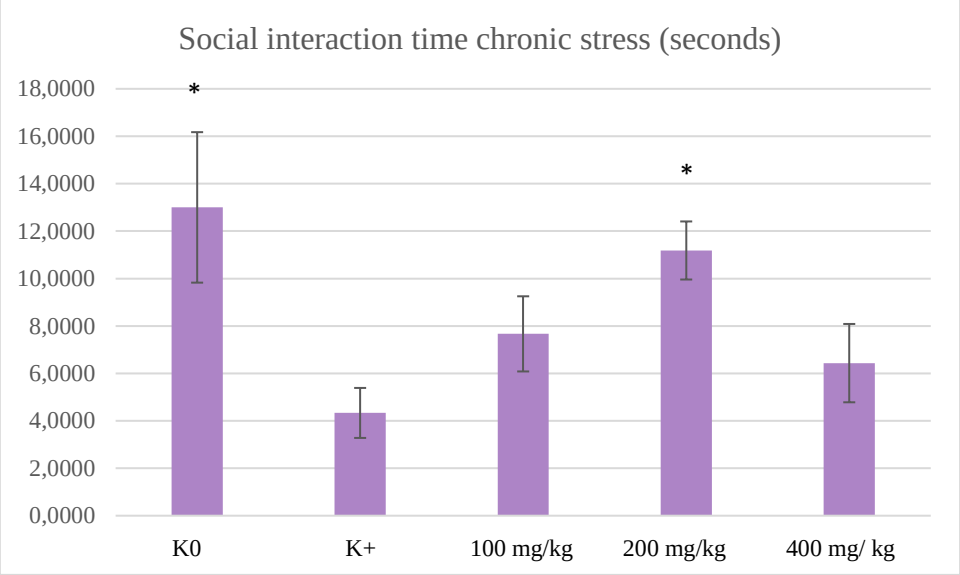

Figure 5. Results ofthe social interaction test in a model of chronic stress.

EPM results in a chronic stress model

When comparing the two controls, a significant increase in the parameter time spent in the open arms of the maze was observed for the K0 group, which proves that the selected elevated cross-maze test is suitable for evaluating behavioral changes in a chronic stress model. In all groups treated with the extract, an improvement in the parameter was observed, but it was not statistically significant, and therefore these data are not summarized in a figure.

For the parameter number of entries into the open air/total number of entries into the arms of the maze (Figure 6), all groups treated with the extract showed an improvement in the parameter, which was statistically significant at a dose of 200 mg/kg, p=0.004 and 400 mg/kg p=0.001.

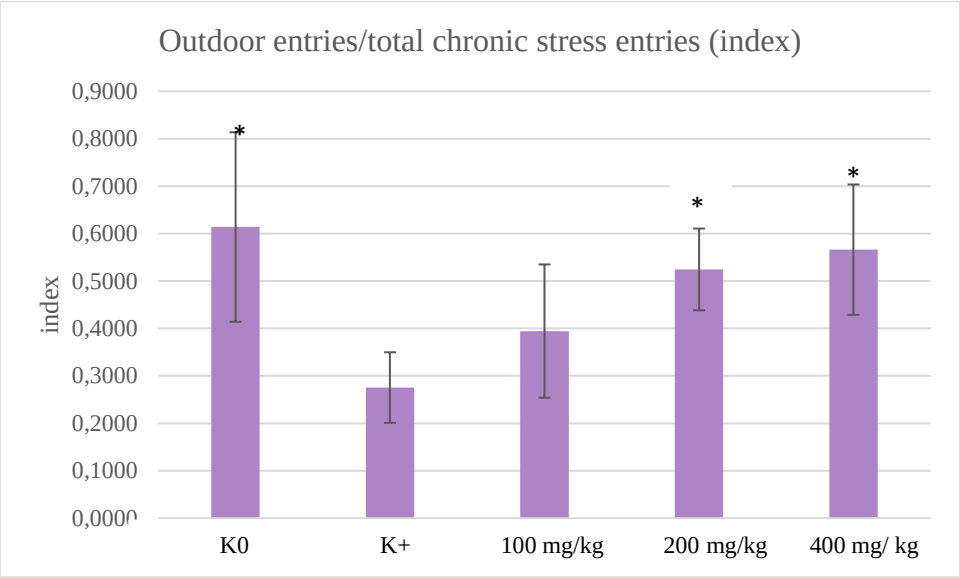

Figure 6. Results of the elevated plus maze test in a model of chronic stress

Results of a forced swim test in a chronic stress model

The data from the conducted experiment (Figure 7) show that the maximum value (83.6667) of the parameter of active swimming time compared to K+ (36.1667) is reported for the K0 group. This indicates that the selected forced swim test is suitable for assessing behavioral changes in a chronic stress model. Except for K0, all groups treated with the extract showed an improvement in the parameter, and it is important to note that it is statistically significant only at a dose of 200 mg/kg (70.6667) p=0.028.

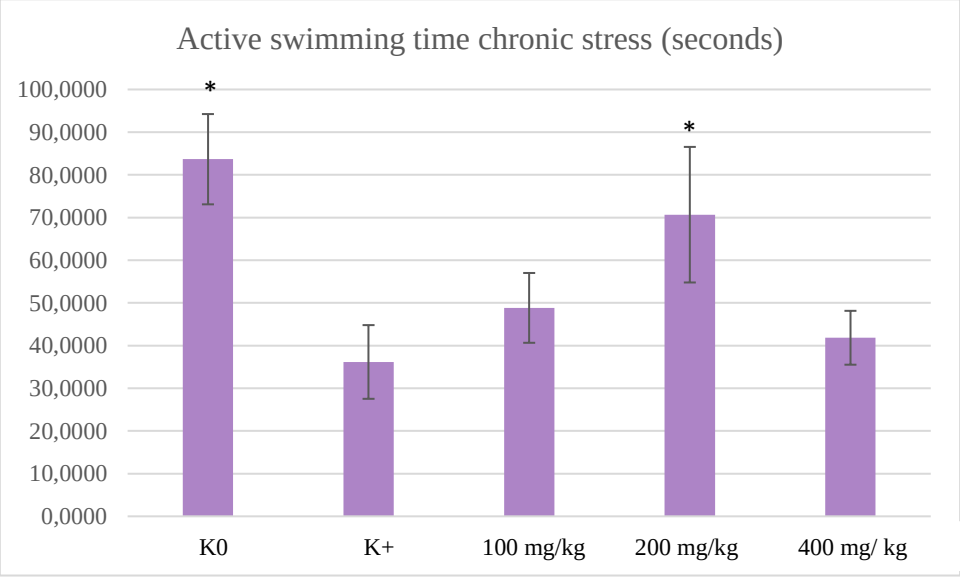

Figure 7. Results of an elevated forced swim test in a chronic stress model

Pro-inflammatory cytokines in an acute stress model

IL 6 levels in an acute stress model

The cold stress model successfully statistically significantly demonstrated an increase in IL 6 levels relative to K0 (Figure 8). In the extract-treated groups, a statistically relevant decrease in the concentration relative to K+ at all doses of the extract was observed at $p < 0.001$.
It is important to note that the reported values of IL 6 for all extract-treated groups were lower compared to K0, and this can be interpreted as evidence of its effectiveness.

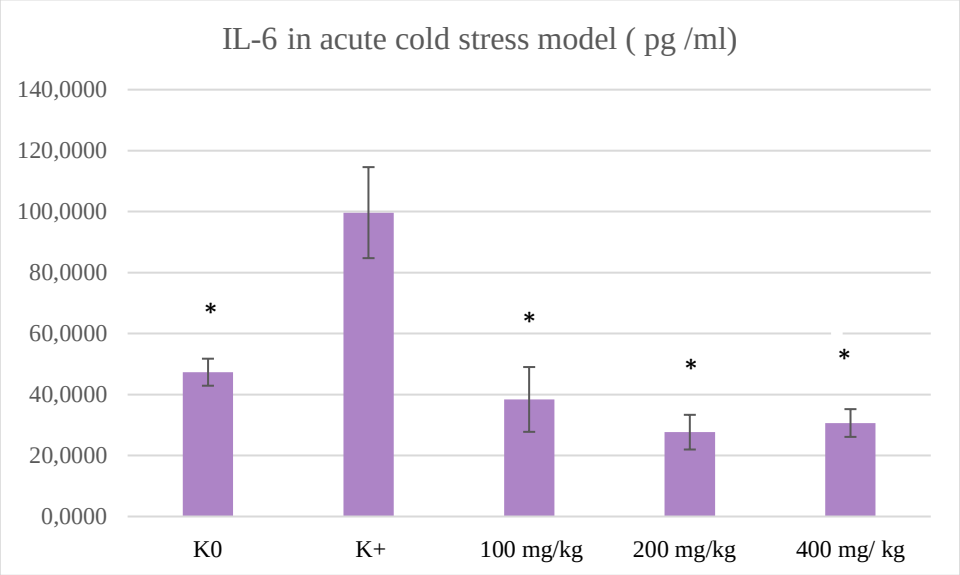

Figure 8 . IL -6 levels in an acute stress model.

IL 6 levels in a chronic stress model

The chronic stress model successfully statistically significantly increased IL 6 levels relative to K0 (Figure 9). A statistically relevant decrease in IL 6 concentration relative to K+ was observed in the extract-treated groups at all doses, $p=0.023$, $p=0.014$, and $p=0.015$, respectively. In addition, IL 6 values found for all extract-treated groups were lower, but not statistically significant compared to K0.

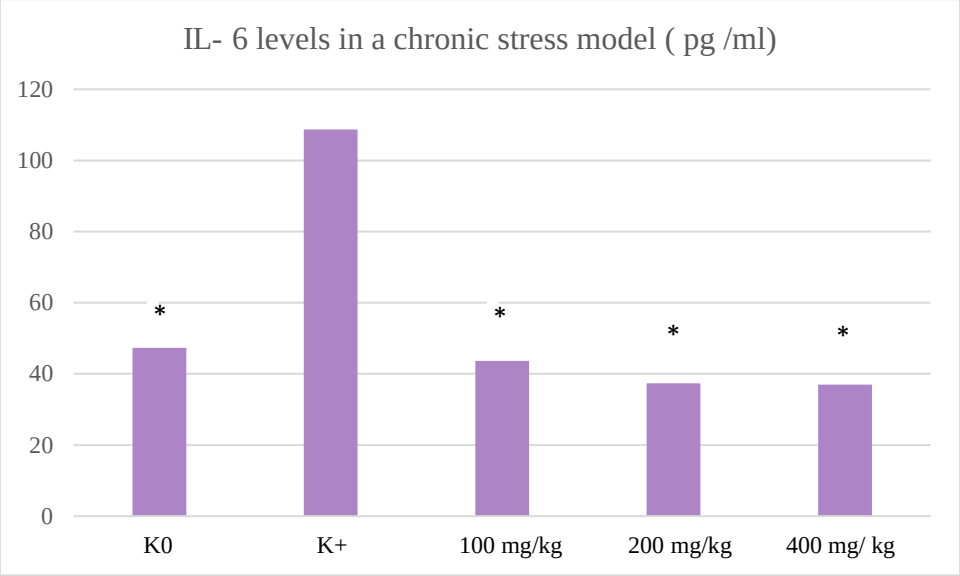

Figure 9 . IL -6 levels in a chronic stress model.

Pro-inflammatory cytokines in a chronic stress model
TNF levels alpha in a chronic stress model

The chronic stress model successfully statistically significantly increased TNF levels alpha versus K0 (Figure 10). A statistically relevant decrease in TNF concentration relative to K+ was observed in the extract-treated groups at a dose of 200 mg/kg p=0.039* L and 400 mg/kg p=0.007* L.

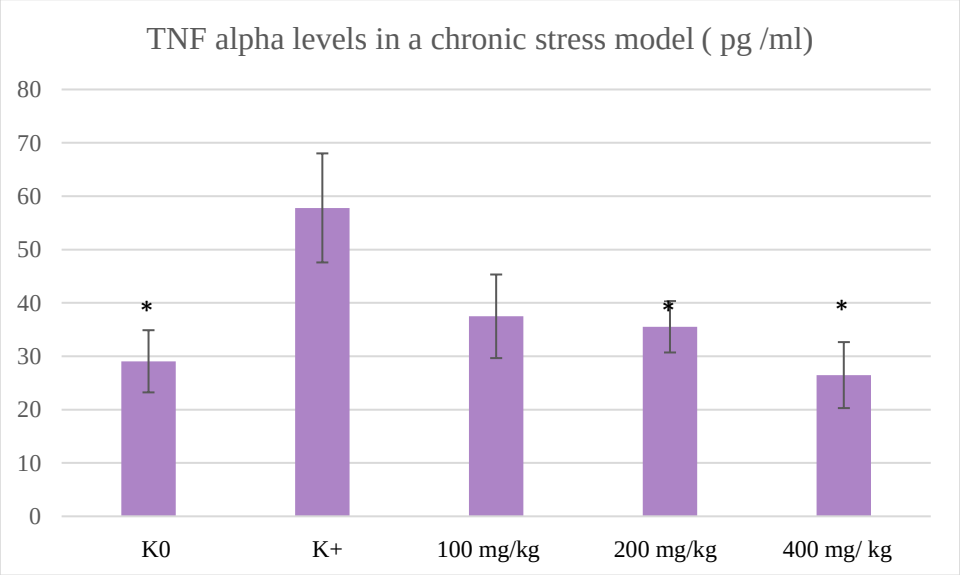

Figure 10. TNF alpha levels in a chronic stress model.

Results in a lipopolysaccharide model of chronic inflammation

Results for Interleukin 1 beta in inflammation through LPS

Lipopolysaccharide successfully statistically significantly increased interleukin one beta levels relative to K0. The groups treated with the extract showed a decrease in levels compared to the positive control, but it was not statistically significant (data not shown).

Results for Interleukin 10 in LPS-induced inflammation model.

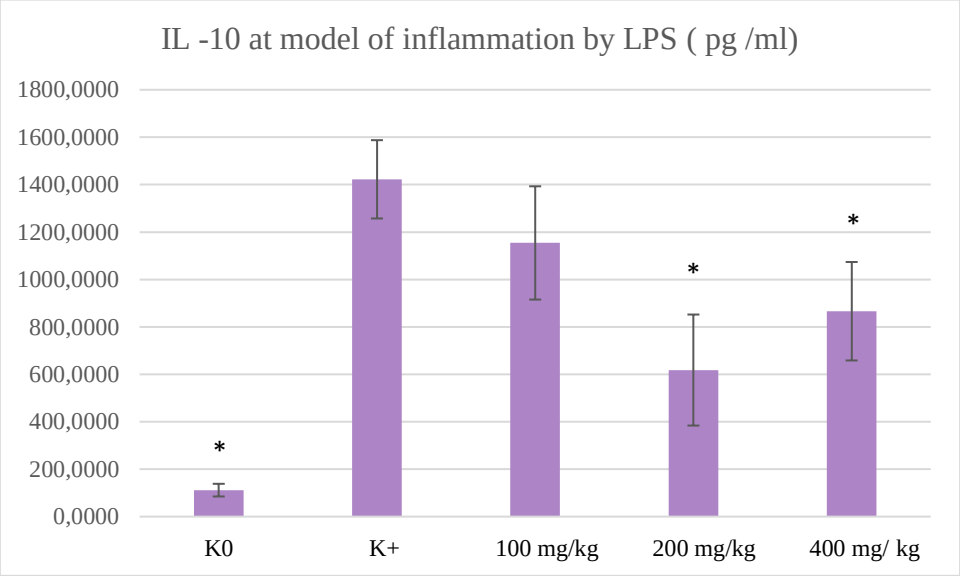

Figure 11. IL -10 levels in a model of LPS inflammation.

Systemic injection of lipopolysaccharide successfully statistically significantly increased serum interleukin-10 levels. From the results presented in Figure 11, it is evident that in all groups treated with the extract, a decrease in the concentration of interleukin 10, relative to K+ was observed, which was statistically significant at a dose of 200 mg/kg p=0.007 and 400 µg/kg p =0,0045

Results for Interleukin 6 in a model of inflammation by LPS

Increased levels of the interleukin interleukin-6 in the serum after systemic injection of lipopolysaccharide have been shown to be statistically significant (Figure 12). In all groups treated with the extract, a decrease in the concentration of IL 6 was observed compared to the control with lipopolysaccharide, with a confidence level of $p < 0.05$ at a dose of 200 mg/kg p=0.024 and 400 µg/kg p=0.029.

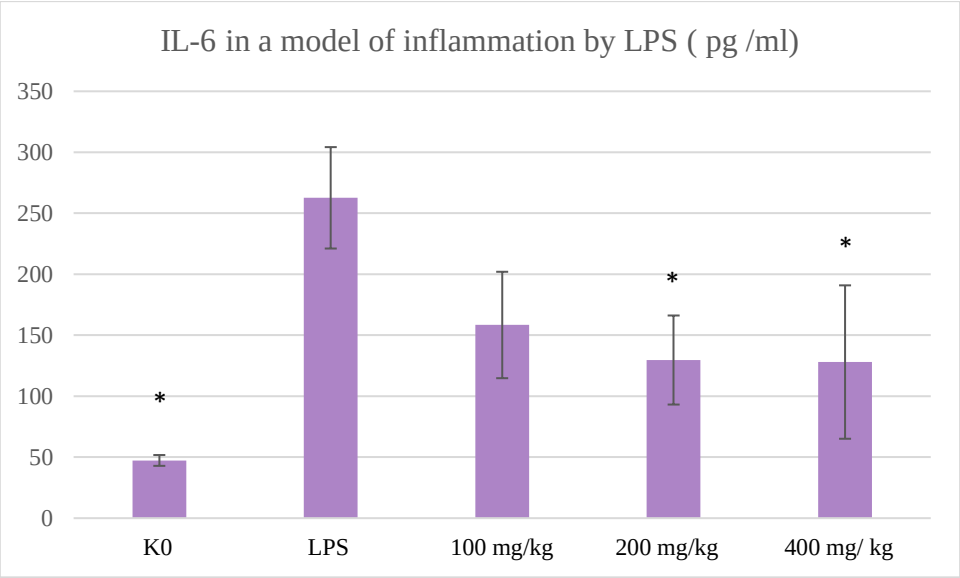

Figure 12. IL -6 levels in a model of inflammation by LPS

Results for Tumor necrosis factor-alpha in a model of inflammation by LPS
Systemic injection of lipopolysaccharide demonstrated increased levels of tumor necrosis factor-alpha in serum relative to the negative control, with the difference being statistically significant (Figure 13). In all groups treated with the extract, there was a decrease in the concentration of tumor necrosis factor-alpha compared to K+, and it was statistically significant at a dose of 400 mg/kg p=0.0017.

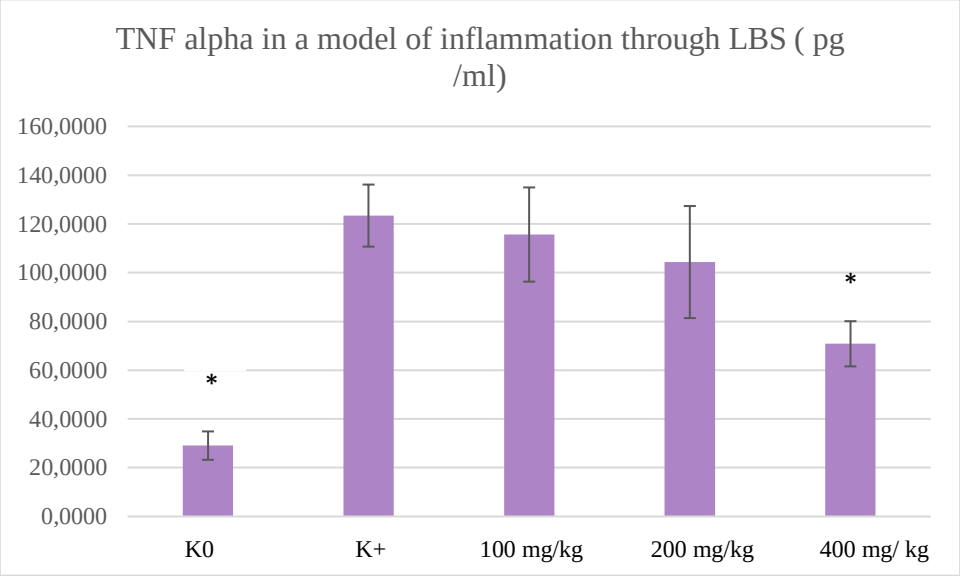

Figure 13. TNF alpha levels in LPS.

Results of an experiment with carrageenan-induced hind paw edema
Carrageenan injection showed a statistically significant increase in hind paw volume in the K0 group at each time point compared to the diclofenac-treated control (Figure 14). A statistically significant reduction in paw volume relative to

K0 was documented at the 400 mg/kg dose at each reporting hour, as well as at all doses at the 4th and 24th hours.

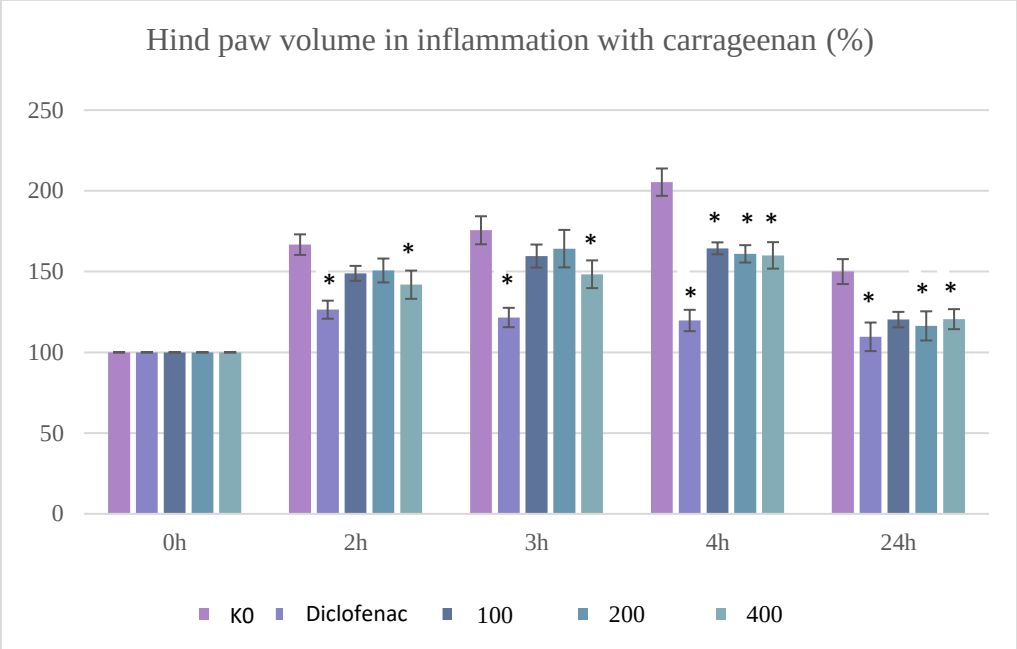

Figure 14. Results in a carrageenan model of inflammation

Results of learning and memory experiments in three models of impaired memory

Results from Activity Cage experiment

Within the scopolamine-impaired memory model, a statistically significant reduction in horizontal and vertical movements was observed in scopolamine-treated control animals relative to K0. In the groups treated with the extract, an increase in the parameters was noted compared to the positive control in all doses, and it was statistically significant at a dose of 400 mg/kg p=0.004 in the parameter for horizontal and vertical movements (Figures 15, 16). In addition, the horizontal movements in all groups are more than the vertical ones, which is a requirement for normal locomotor activity and a prerequisite for conducting further experiments.

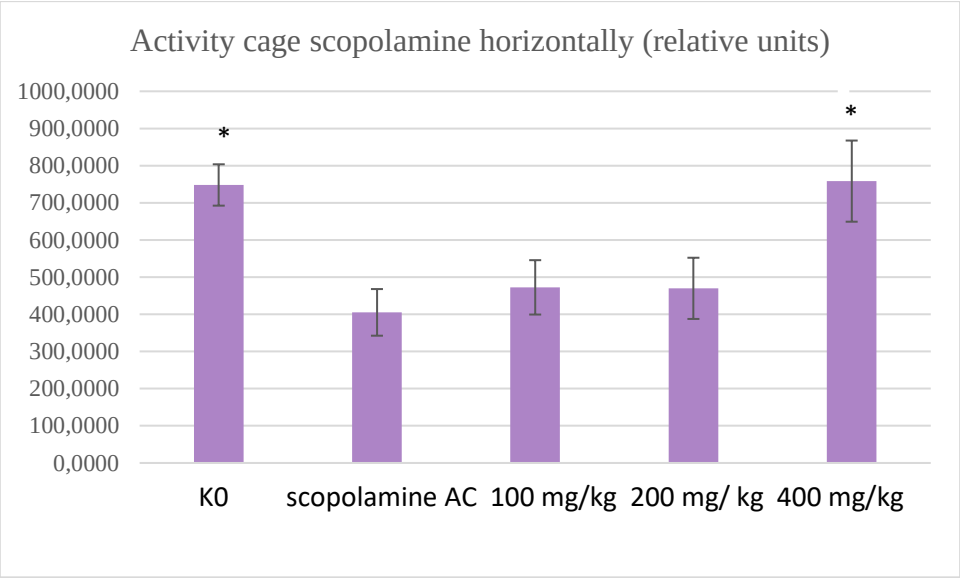

Figure 15. Results from Activity Cage experiment in a scopolamine-impaired memory model horizontally.

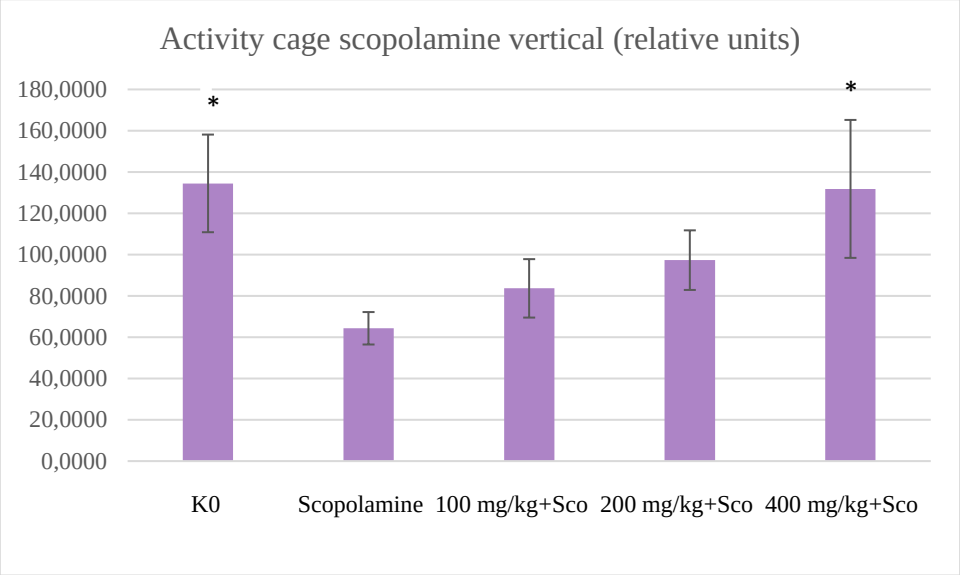

Figure 16. Results from Activity Cage experiment in a scopolamine-impaired memory model vertically.

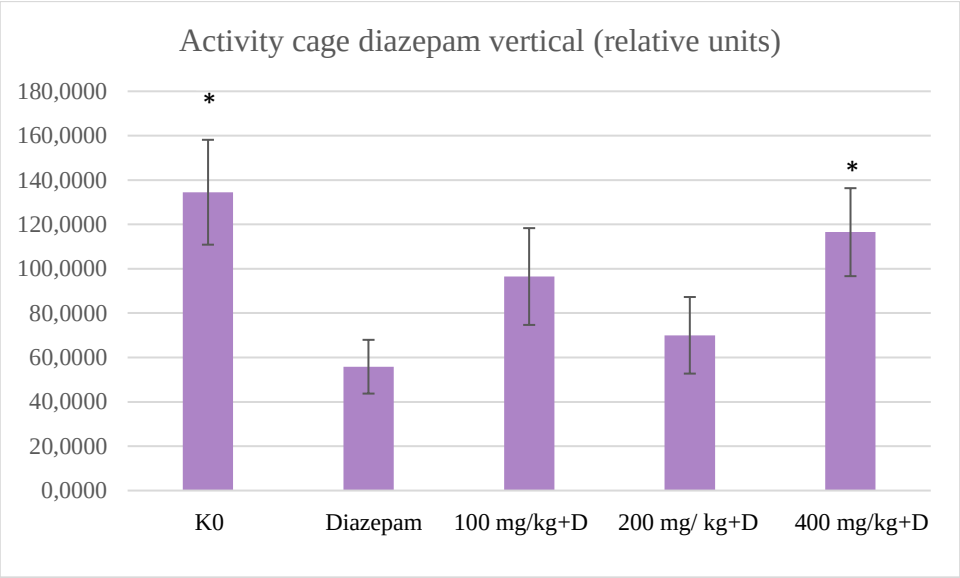

Figure 17. Results from Activity Cage experiment in a diazepam-impaired memory model vertically.

Within the diazepam-impaired memory model, a statistically significant reduction in horizontal and vertical movements was observed in scopolamine-treated control animals relative to K0. The groups treated with the extract showed an increase in the parameters compared to the positive control in all doses, being statistically significant at a dose of 400 mg/kg p=0.036 in the parameter for vertical movements (Figure 17). In addition, the horizontal movements in all groups are more than the vertical ones, which is a requirement for normal locomotor activity and a prerequisite for conducting further experiments.

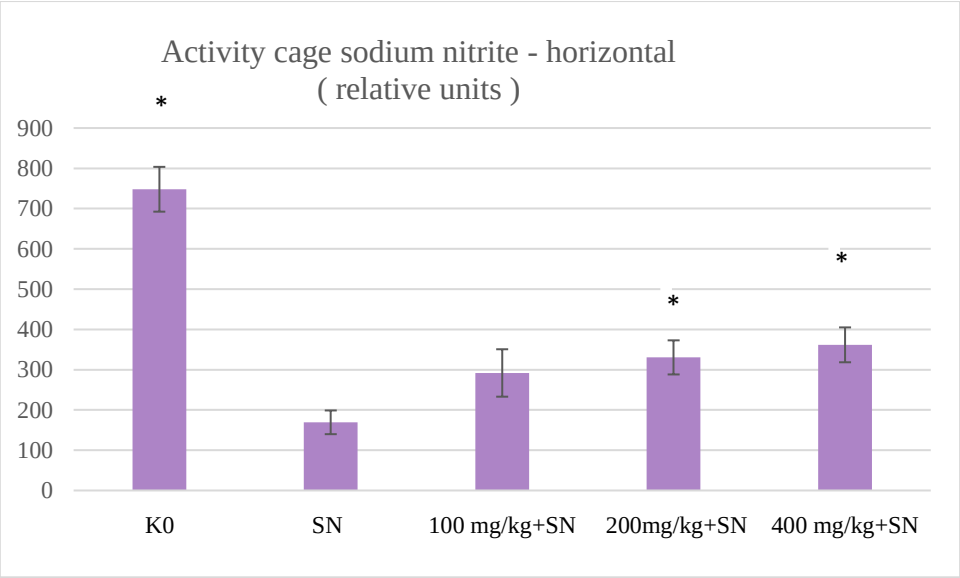

Figure 18. Results from Activity Cage experiment in a model of sodium nitrite memory impairment horizontally.

In the sodium nitrite memory impairment model, there was a statistically significant reduction in horizontal and vertical movements of animals in the sodium nitrite control group relative to K0. In the groups treated with the extract, an increase in the parameters compared to the positive control was noted in all doses, being statistically significant at a dose of 200 mg/kg p=0.023 and 400 mg/kg p=0.008 in the parameter for horizontal movements (Figure 18). In addition, horizontal movements in all groups are more than vertical ones, which is a requirement for normal locomotor activity and a prerequisite for conducting subsequent experiments.

Results of a Y-Maze experiment

In the Y-Maze experiment in a scopolamine-impaired memory model (Figure 19), a statistically significant improvement in the performance index was observed at K0 compared to the positive control. This indicates that the selected test is suitable for assessing working and spatial memory. Except for K0, a statistically relevant improvement of the studied parameter was observed in the groups treated with the extract at a dose of 100 mg/kg p=0.018, 200 mg/kg p=0.034, and 400 mg/kg p=0.012.

In the Y-Maze experiment in a diazepam-impaired memory model (data not presented), a statistically significant improvement of the working index at K0 was found compared to the positive control. This indicates that the selected test is suitable for assessing working and spatial memory. Except for K0, an improvement of the studied parameter was observed in the groups treated with the extract, but the results were not statistically significant.

In the Y-Maze experiment in a sodium nitrite-impaired memory model (data not shown), a statistically significant improvement in performance index was reported for K0 compared to the positive control. This indicates that the selected test is suitable for assessing working and spatial memory. Except for K0, an improvement of the studied parameter was observed in the groups treated with the extract, but the results were not statistically significant.

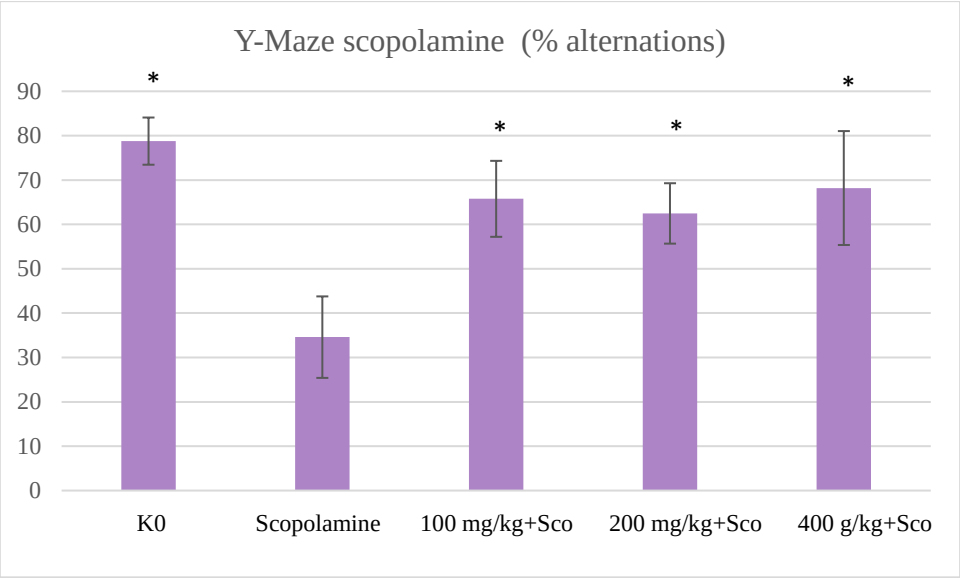

Figure 19. Results of Y-maze experiment in a scopolamine-impaired memory model.

Results of a T-maze experiment

In the scopolamine-impaired memory model, a statistically significant improvement in performance index was observed at K0 compared to the positive control (Figure 20). This indicates that the selected test is suitable for assessing working memory. Except for K0, improvement of the studied parameter was observed in the groups treated with extract at a dose of 400 mg/kg p=0.027.

The T-Maze experiment in a diazepam-impaired memory model (data not shown) demonstrated a statistically significant improvement in the performance index at K0 relative to the positive control. This indicates that the selected test is suitable for assessing working and spatial memory. Except for K0, an improvement of the investigated parameter was observed in the extract-treated groups, but the results were not statistically significant.

In the T-Maze experiment in a sodium nitrite-impaired memory model (data not shown), there was also a statistically significant improvement in the performance index at K0 relative to the positive control. This indicates that the selected test is suitable for assessing working and spatial memory. Except for K0, an improvement of the studied parameter was observed in the groups treated with the extract, but the results were not statistically significant.

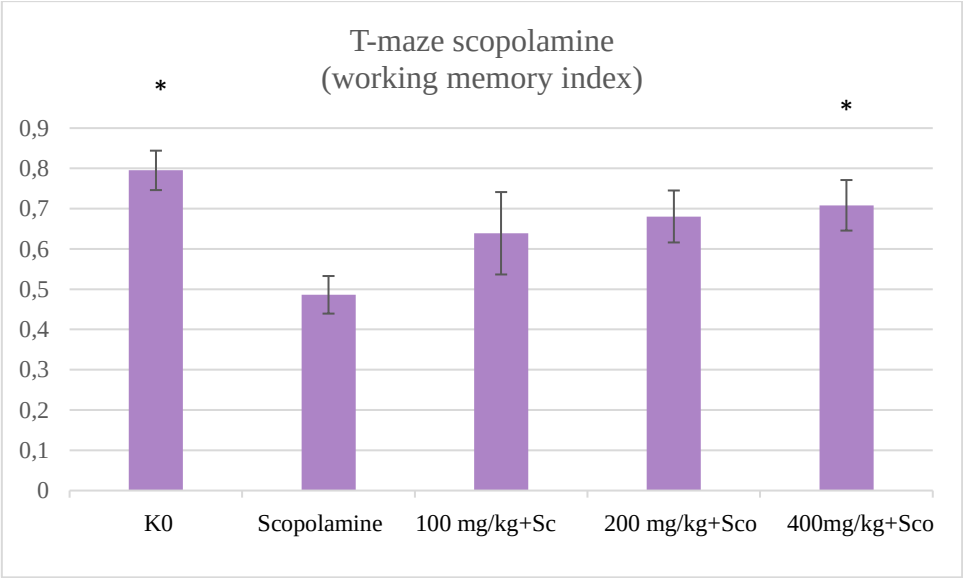

Figure 20. Results of a T-maze experiment in a scopolamine-impaired memory model.

Results of NORT experiment

The novel object recognition experiment - NORT in a scopolamine-impaired memory model (Figure 21) demonstrated a statistically significant increase in the measured working memory index between K0 and the positive control. In addition, an improvement of the parameter was noted in all groups treated with the extract relative to K+, being statistically significant at a dose of 400 mg/kg p=0.005.

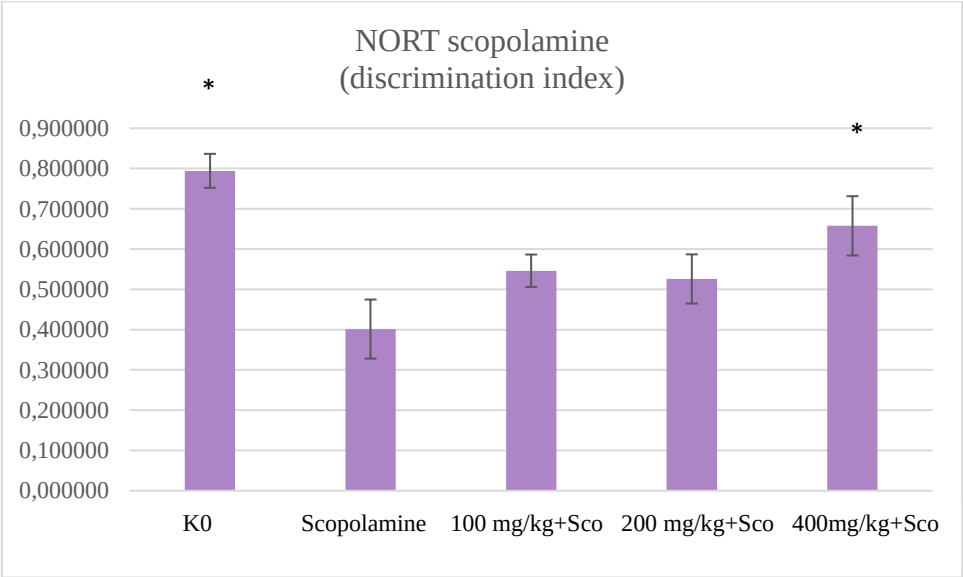

Figure 21. Results of NORT experiment in a scopolamine-impaired memory model.

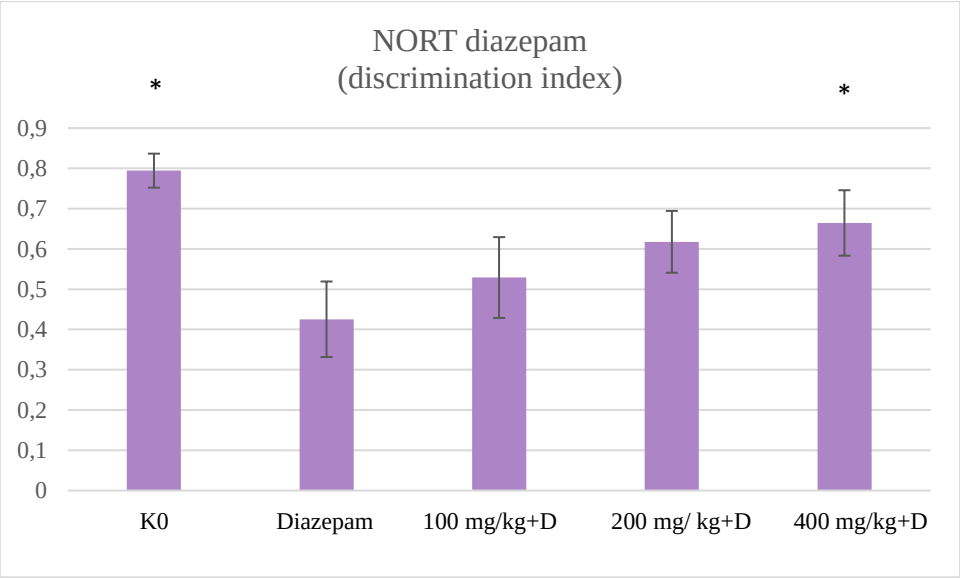

Figure 22. Results of a NORT experiment in a diazepam-impaired memory model.

NORT novel object recognition experiment in a diazepam-impaired memory model (Figure 22) demonstrated a statistically significant increase in the measured working memory index between K0 and the positive control. In addition, an improvement of the parameter was noted in all groups treated with the extract in relation to K+, and it was statistically significant at a dose of 400 mg/kg p=0.045. The NORT experiment did not demonstrate statistical significance of the results in the sodium nitrite-impaired memory model (data not shown).

Stepdown experiment results

The results of the passive avoidance step-down experiment in the scopolamine-impaired memory model demonstrated a statistically significant difference in the latency parameter between K0 and the positive control on day 3 (Figure 23) and day 8 (Figure 24). The obtained data show that the experiment is suitable for studying short-term and long-term memory. Except for K0, an improvement in the parameter was documented at all doses, being statistically relevant on day 3 at a dose of 400 mg/kg p = 0.003, as well as on day 8 at a dose of 200 mg/kg p = 0.025 and 400 mg /kg p = 0.002.

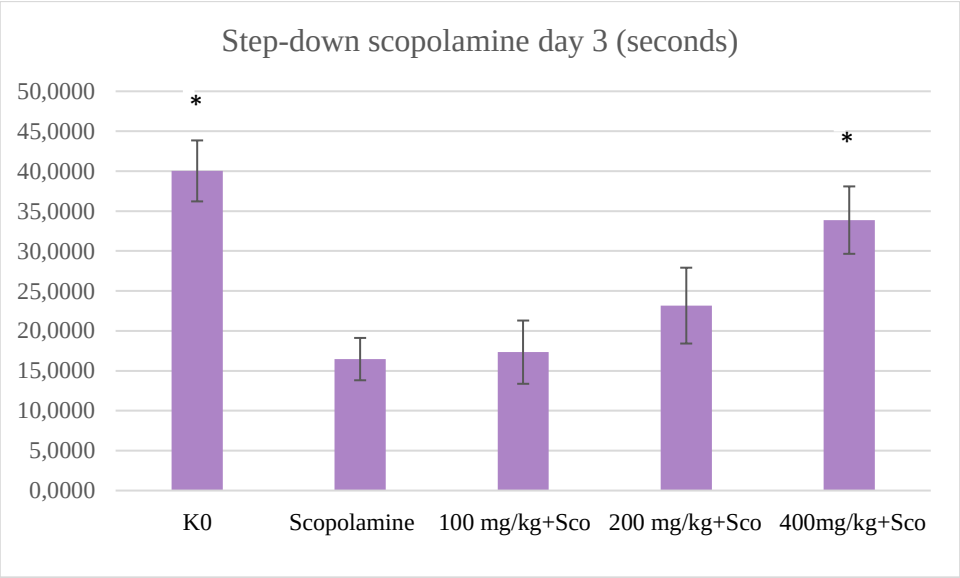

Figure 23. Results of a step-down experiment in a scopolamine-impaired memory model - short-term memory.

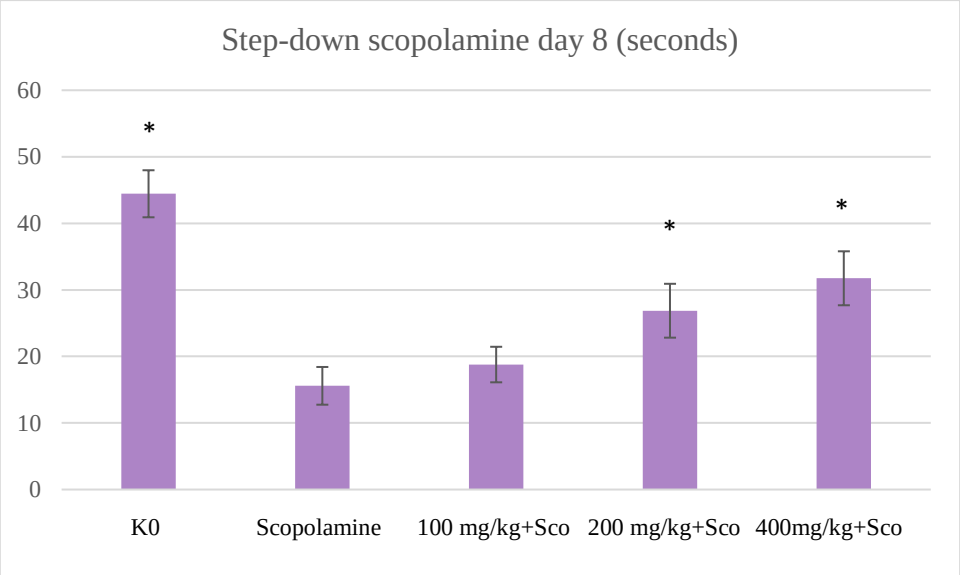

Figure 24. Results of a step-down experiment in a scopolamine-impaired memory model - long-term memory.

The results of the passive avoidance step-down experiment in a diazepam-impaired memory model demonstrated a statistically significant difference in latency as a parameter between K0 and the positive control on days 3 (Figure 25) and 8 (data not shown). This indicates that the experiment is suitable for studying short-term and long-term memory. Except for K0, an improvement in the parameter was documented at all doses, being statistically relevant on day 3 at doses of 200 mg/kg $p = 0.003$ and 400 mg/kg $p = 0.033$.

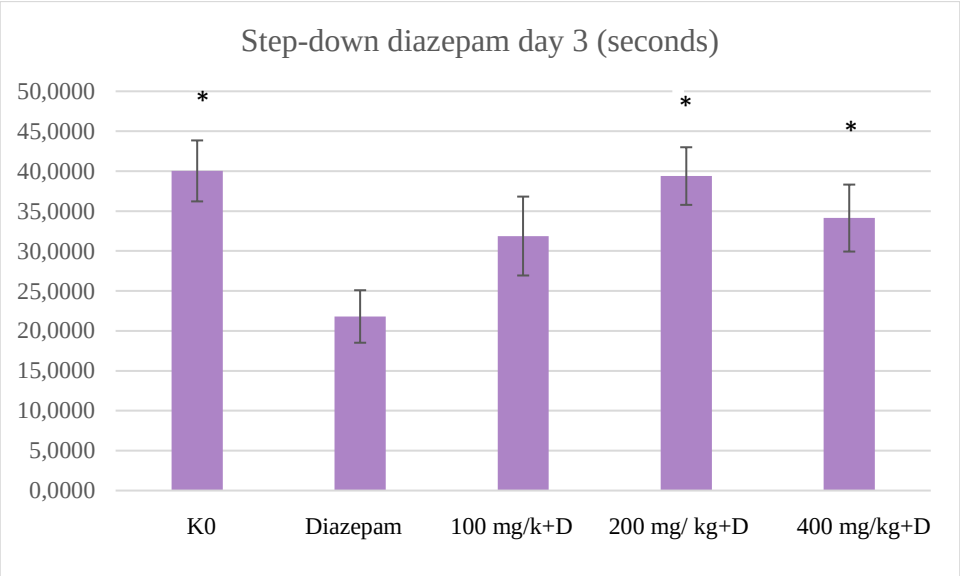

Figure 25. Results of a step-down experiment in a diazepam-impaired memory model - short-term memory

The results of a step-down experiment in a sodium nitrite-impaired memory model showed no statistically significant change between K0 and the positive control (data not shown), possibly due to the achievement of severe hypoxia and inertness in the experimental animals treated systemically with sodium nitrite, which brings the results of the control group closer to those of the trained animals and masks them.

Results of the Step through experiment

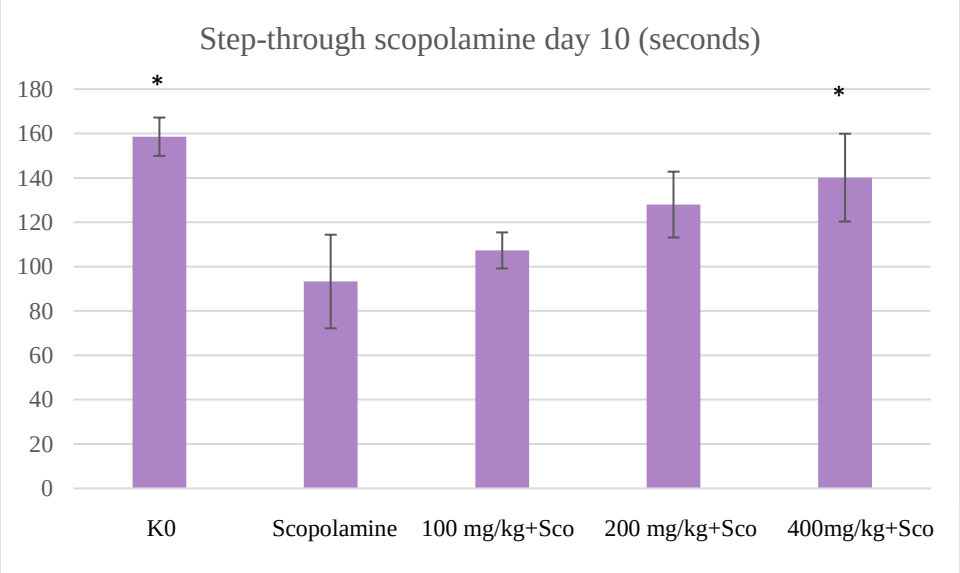

Figure 26. Results of a step-through experiment in a scopolamine-impaired memory model - long-term memory

The Step-through passive avoidance experiment in a scopolamine-impaired memory model demonstrated a statistically significant difference in latency as a parameter between K0 and the positive control on days 3 (data not shown) and 10 days (Figure 26). This shows that the experiment is suitable for studying short-term and long-term memory. Except for K0, an improvement in the parameter was documented at all doses, being statistically relevant on day 10 at a dose of 400 mg/kg p = 0.039.

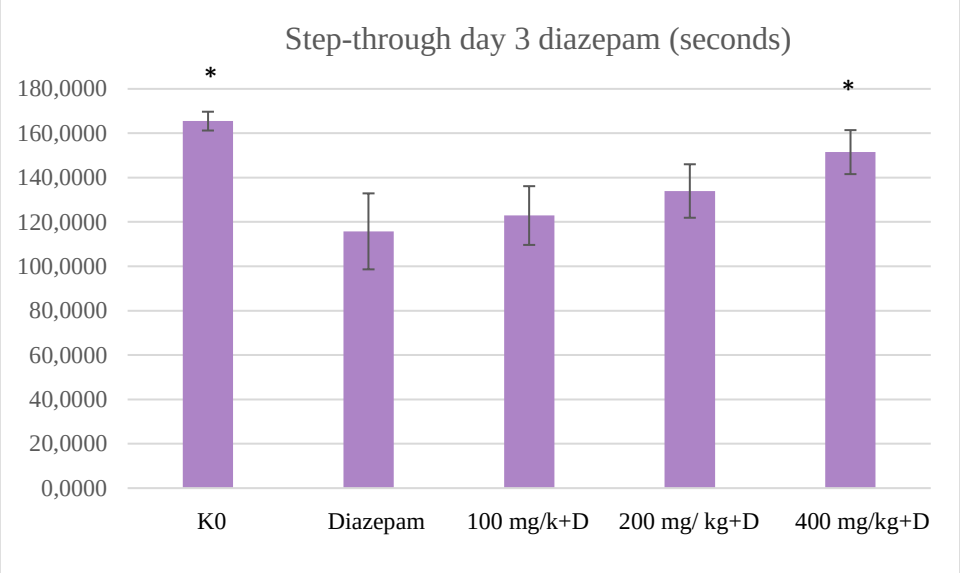

Figure 27. Results of a step-through experiment in a diazepam-impaired memory model - long-term memory

The Step-through passive avoidance experiment in a diazepam-impaired memory model demonstrated a statistically significant difference in latency as a parameter between K0 and the positive control on day 3 (Figure 27) and day 10 (data not shown). This shows that the experiment is suitable for studying short-term and long-term memory. Except for K0, an improvement in the parameter was documented at all doses, being statistically relevant on day 3 at a dose of 400 mg/kg p = 0.044.

Results of Shuttle box experiment

The results of the experiment on active avoidance of a punitive stimulus - shuttle box in a model of impaired memory by scopolamine demonstrate a statistically significant difference in the number of conditioned responses (avoidances) between K0 and the positive control on the 5th (Figure 28) and 12th day (Figure 29).

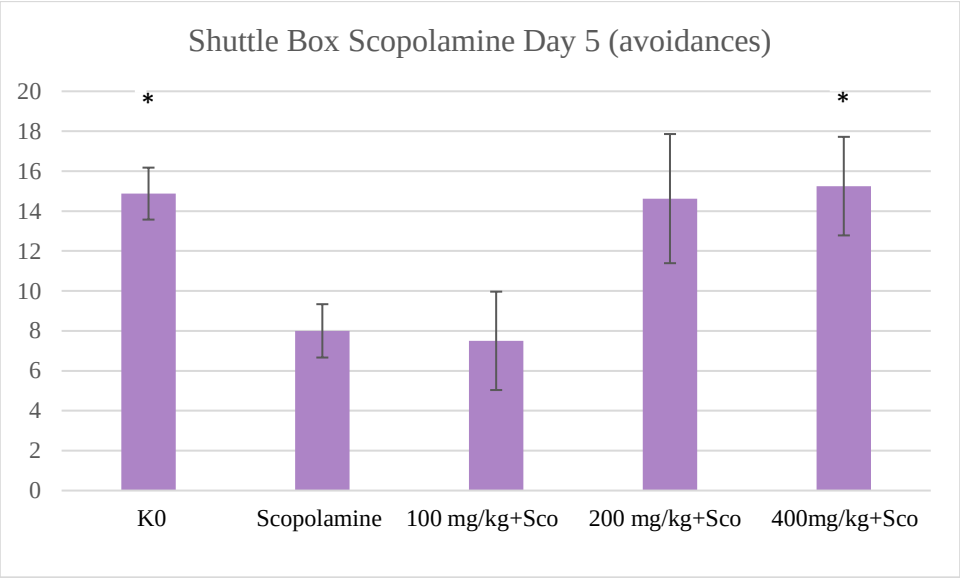

Figure 28. Results of a shuttle box experiment in a scopolamine-impaired memory model - short-term memory

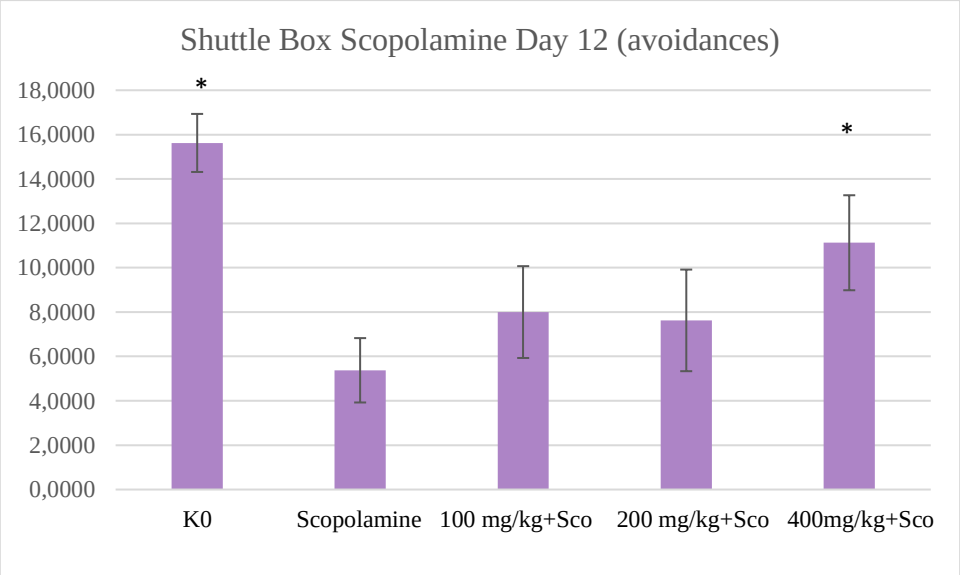

Figure 29. Results of a shuttle box experiment in a scopolamine-impaired memory model - long-term memory

The results of the experiment on active avoidance of a punitive stimulus - shuttle box in a model of memory impaired by diazepam demonstrated a statistically significant difference in the number of conditioned responses (avoidances) between K0 and the positive control on the 5th (Figure 30) and 12th day (Figure 31). The data obtained show that the experiment is suitable for studying short-term and long-term memory. Apart from K0, a statistically significant improvement of the parameter was documented in a dose of 400 mg/kg on day 5 p=0.010, as well as on day 12 in a dose of 400 mg/kg p=0.008.

The results of the shuttle box experiment in a sodium nitrite-impaired memory model did not provide a statistically significant change compared to the positive control (data not shown).

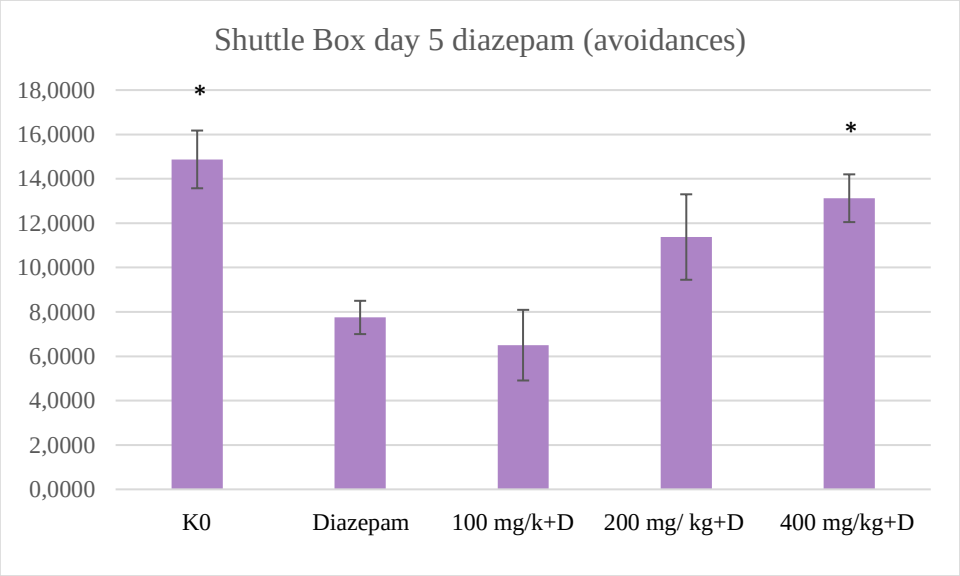

Figure 30. Results of a shuttle box experiment in a model of diazepam-impaired short-term memory

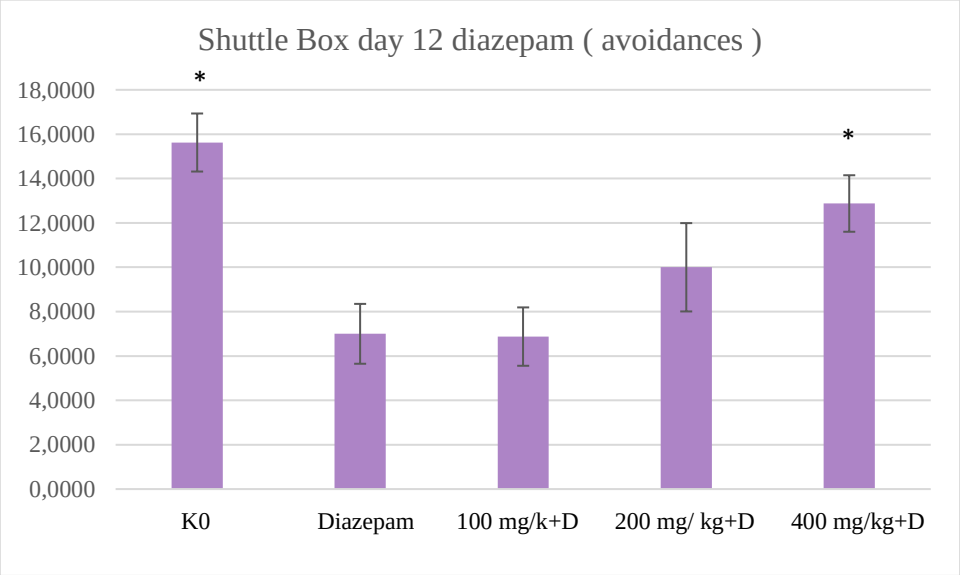

Figure 31. Results of a Shuttle Box experiment in a model of diazepam-impaired long-term memory

Summary of results

Tables 4, 5, and 6 present the aggregated statistically significant results by group of experiments regarding the effect of *Sideritis scardica* extract studies in the administered doses.

Table 4. Summary of statistically significant results of inflammation experiments.

Statistically significant results compared to K+ control group				
Inflammation				
Sideritis scardica extract		Dose mg/kg		
		100	200	400
Carrageenan	2:00			X
	3 hours			X
	4 hours	X	X	X
	24 hours	X	X	X
LPS	IL1beta			
	IL10		X	X
	IL6		X	X
	TNFα			X

Table 5. Summary of statistically significant results from acute and chronic stress experiments.

Statistically significant results compared to K+ control group					
Stress					
Sideritis scardica extract			Dose mg/kg		
			100	200	400
Acute	FST	T acitve			X
	SI	T social		X	X
	EPM	T open			
		Open Index		X	X
	IL1beta				
	IL10				
	IL6		X	X	X
	TNFα				
Chronic	FST	Active		X	
	SI	T social		X	
	EPM	T open			
		Open Index		X	X
	IL1beta				
	IL10				
	IL6		X	X	X
	TNFα			X	X

Table 6. Summary of statistically significant results of experiments for impaired memory models.

Statistically significant results compared to K+ control group						
Learning and memory						
Sideritis scardica extract				Dose mg/kg		
				100	200	400
Activity cage	X	Diazepam				
		Scopolamine				X
		NaNO2			X	X
	Y	Diazepam				
		Scopolamine				X
		NaNO2				
T-Maze	Diazepam					
	Scopolamine					X
	NaNO2					
NORT	Diazepam					X
	Scopolamine					X
	NaNO2					
Y-Maze	Diazepam					
	Scopolamine			X	X	X
	NaNO2					
Step-down	Short-term memory		Diazepam		X	X
			Scopolamine			X
			NaNO2			
	Long-term memory		Diazepam			
			Scopolamine		X	X
			NaNO2			
Step-through	Short-term memory		Diazepam			X
			Scopolamine			
			NaNO2			
	Long-term memory		Diazepam			
			Scopolamine			X
			NaNO2			
Shuttle Box	Avoidance	Short-term memory	Diazepam			X
			Scopolamine		X	X
			NaNO2			
		Long-term memory	Diazepam			X
			Scopolamine			X
			NaNO2			

Discussion

In vitro studies by Tadic et al. involving B16 cells and HL-60 cells showed that the highly lipophilic diethyl ether extract of *S. scardica* can exhibit moderate cytotoxic effects (Tadić, 2012). Toxicity evaluation of four *Sideritis scardica* extracts, obtained by various solvents from Feistel et al., reveals no toxicity nor concerns about mutagenic effects (Feistel, 2018). In the same study, the authors performed the Ames test on bacterial cultures and observed no mutagenic effect, as measured by an increase in the number of revertant colonies compared to the control number, for any of the tested extracts up to concentrations of 5000 µg/plate12. The current 12-week subchronic toxicity study revealed no mortality or gross pathology. In general, the significant toxicity of plants of the Lamiaceae family is more associated with volatile components such as terpenes found in the oils of various plants, which do not accumulate significantly in an alcoholic aqueous extract (Rozman, 2007).

Neuroinflammation is part of the immune response to noxious stimuli in the CNS. Its function is to remove necrotic cells and tissues caused by pathogens. Excessive neuroinflammation contributes to the progression of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS)(Jacobs, 2012). Carrageenan acts on the Bcl10, NF-κB, IκBα pathway to activate inflammatory mediators. This pathway initially involves phosphorylation steps, followed by nuclear translocation of phospho-NFκB. This triggers the transcription and translation of inflammatory biomarkers such as COX, NOS, IL-6, etc. Inflammation causes many effects. One of the effects is vasodilation of the capillaries/blood vessels. Beneath the surface, where carrageenan is applied, the inflammation causes dilation of the capillaries below the surface of the skin. This increases blood flow to the area and appears as swelling/redness of the affected area. (Necas, 2013) CGN-activated inflammatory cascades related to innate immunity and ROS generation can be integrated at the level of IKK, as well as the IKK signalosome (Bhattacharyya, 2008). This allows the interpretation of a possible mechanism of action, which, apart from the involvement of pro-inflammatory cytokines, also speaks of possible interaction with COX, as well as direct isolation of ROS through antioxidant activity.

In addition, the experiment with systemic inflammation via lipopolysaccharide demonstrated a significant reduction of investigated pro-inflammatory cytokines (IL 6, IL 10, TNF alpha) and reinforces the idea of an anti-inflammatory effect of the herb, known for centuries and described for many plants from the genus. Lipopolysaccharides (LPS) are endotoxins composed of O-antigen found in the outer membrane of Gram-negative bacteria and have been reported to be the most potent stimuli for microglial activation (Henry, 2009). Toll-like receptor 4 (TLR4) is expressed by microglial cells (Okun, 2009) and is responsible for the inflammatory cascade in microglia upon LPS binding.

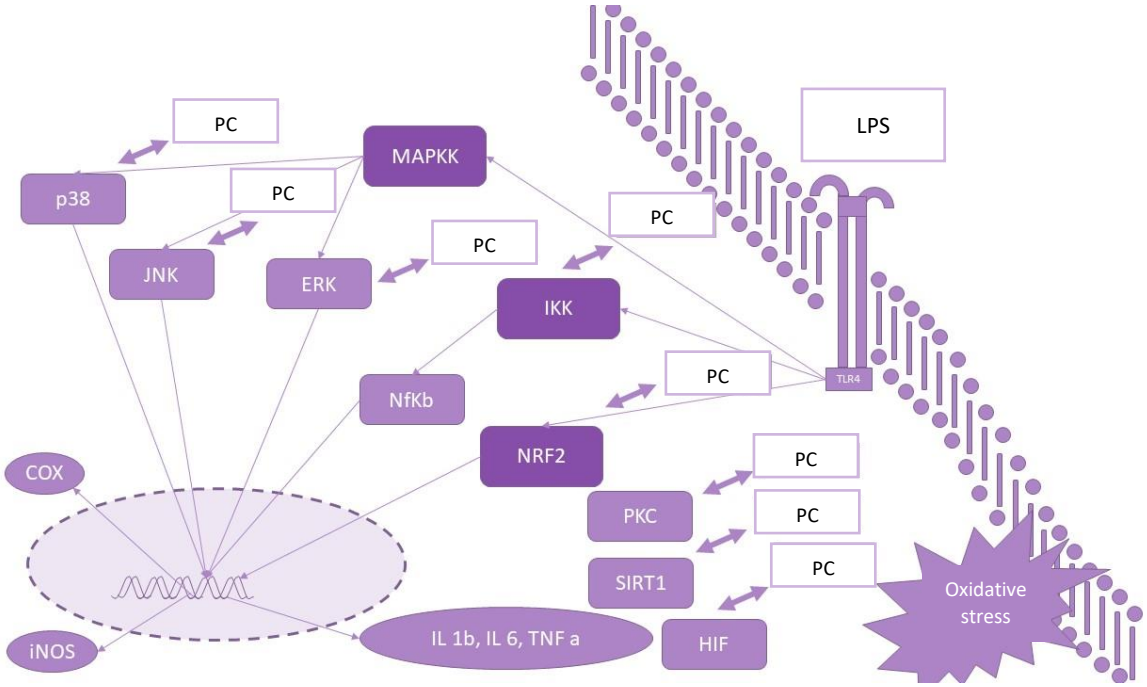

Figure 32. Cascades in inflammation and oxidative stress

Microglia appear to be the main cells in the brain that express the IL-6 receptor and potently secrete IL-6 during peripheral immune stimulation (Burton, 2013). IL-6 knockout (IL-6-/-) mice showed a general reduction in the number of activated brain macrophages associated with cortical lesions, suggesting a role for IL-6 in the orchestration of central nervous system inflammation (Penkowa, 1999). In pathological conditions, microglia release large amounts of TNF-α, and this de novo production of TNF-α is an important component of the neuroinflammatory response that is associated with several neurological disorders (Montgomery, 2012). Interleukin-1 (IL-1) is one of the most well-known pro-inflammatory cytokines that act in the brain during various strokes and neurodegenerative diseases.

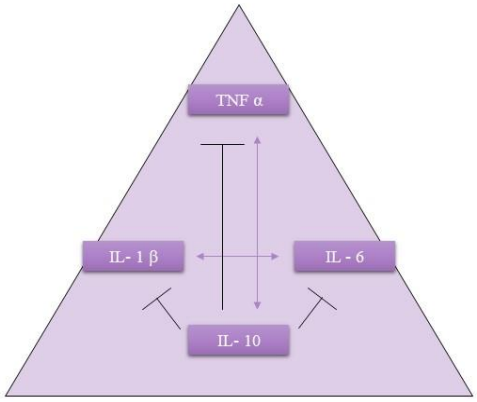

Figure. 33 Cytokine interaction

The standard model of synaptic consolidation assumes that changes in synaptic protein synthesis and changes in membrane potential are achieved by activation of intracellular transduction cascades. These molecular cascades trigger transcription factors that lead to changes in gene expression. The result of gene expression is the permanent change of synaptic proteins as well as synaptic remodeling and growth. In the short time period immediately following training, the molecular cascade, the expression, and processing of both transcription factors and immediate early genes, is susceptible to perturbations. Disturbances caused by specific drugs, antibodies, and gross physical trauma can block the effects of synaptic consolidation. (Roediger, 2007, Squire, 1995)

System consolidation is the second form of memory consolidation. This is a reorganization process where memories from the hippocampus area, where memories were first encoded, are moved to the neo-cortex in a more permanent form of storage. (Dudai, 2015)

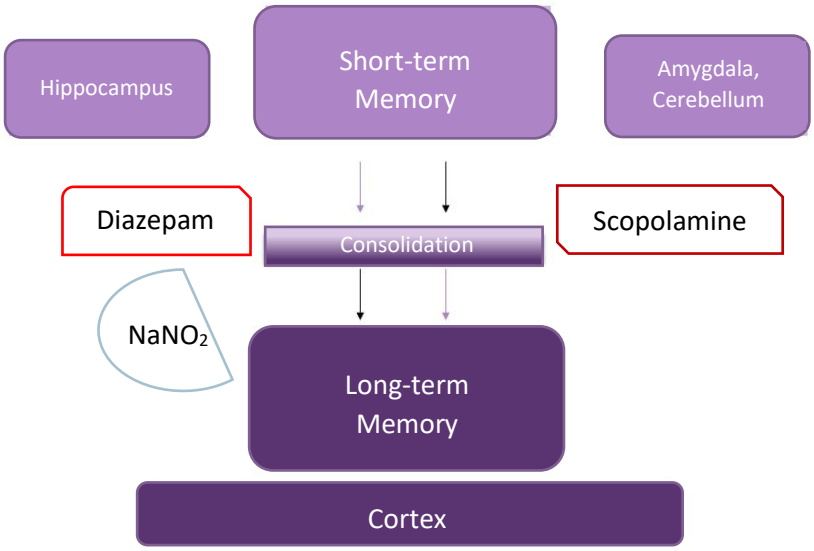

Figure 34. Memory consolidation

In addition to the pronounced antioxidant activity of the preparation as a postulate for a protective effect in the pathophysiology of neurodegenerative diseases and the ability to counteract the load of ROS, systematic research using different models allows us to speculate on possible other mechanisms of action - GABA modulation, anticholinesterase activity, antihypoxic effect.

ACh is important for the transmission of nerve impulses, and its pharmacological action is terminated mainly by AChE and to some extent by BChE (Kamal, 2015; Khan, 2018). Overall, the genus *Sideritis* is a significant source of natural enzyme inhibitors (Sarikurkcu, 2020). Inhibition of AChE and tyrosinase has been documented for extracts of *S. ozturkii* (Zengin et al., 2019). Also, Celep et al . (2019) reported that more than 20% of *S. trojana* extracts had an inhibitory effect on α- amylase and α- glucosidase. In another study (Zengin et al., 2014), the inhibitory effects of *S. galatica* extracts are reviewed, with ethyl acetate and methanol extracts being more active than aqueous ones. In addition to these studies, several researchers have focused on the enzyme-inhibitory properties of some *Sideritis* essential oils (Zengin et al., 2016; Tadić et al., 2017; Deveci et al., 2019). Szwajgier et al. documented anticholinesterase activity for a number of phenolic acids, highlighting pronounced activity for ferulic, para coumaric, and cinnamic acids (Szwajgier, 2018). Verbascoside, isolated from *Verbascum mucronatum* demonstrates moderate acetylcholinesterase activity (Karhraman, 2010).

The current study documents repeated effects of the extract in the scopolamine- and diazepam-induced memory impairment model, with significant values recorded in various parameters at doses of 200 and 400 mg/kg. The results were recorded in tests for short-term and working memory, as well as for long-term

memory**.** The available literature documents a neuroprotective effect for many of the biologically active substances found in *Sideritis scardica*. In a model of bilateral vascular dementia caused by permanent bilateral occlusion of the carotid artery, Cao et al. (2016) showed a significant increase in target quadrant search time and distance while decreasing avoidance time in rats treated with *Scutellaria baicalensis* extract, rich in scutellarein and isoscutellarein derives. Another team turned their attention to the ability of isoscutellarein glucopyranosides isolated from *Stachys japonica* to inhibit acetylcholinesterase and butyrylcholine esterase in an immunological study, postulating a neuroprotective effect (Nughoro, 2018). Coumaric acid dose-dependently increases the general activity of the field-evoked postsynaptic potential (field excitatory postsynaptic potential - fEPSP) after high-frequency stimulation and attenuates the scopolamine-induced blockade of fEPSP in the CA1 region of the hippocampus. Paracoumaric acid-treated rats showed improvement in passive avoidance parameters and prolongation of total latency and latency to the target quadrant in the Morris water maze test of spatial memory (Kim, 2017). Wang et al. document that verbascoside reduced apoptosis in the cortex and hippocampus of APP / PS 1 mice (Wang et al., 2020). A significant improvement in open field test behavior was noted, as well as in spatial recognition, learning and memory ability, demonstrated by a reduction in the latency time of APP / PS1 mice without a significant change of the swimming speed in the MWM test (Wang et al., 2020). Another study investigated the inhibitory acetylcholinesterase activity of forsytoside A at the chemical and biological level. Forsytoside A inhibits acetylcholinesterase in a mixed type of inhibition (Yan, 2017). Jang et al. (2010) reported a positive effect of luteolin therapy before lipopolysaccharide intoxication in Neuro.2a cells and postulate a neuroprotective effect. In addition, a four-week diet of 20 μg / kg improved spatial memory and levels of inflammatory markers in mice, with the authors commenting on modulation of microglial-induced inflammation (Jang, 2010). In a mouse model, acteoside significantly attenuated cognitive deficits in the Y-Maze test and reduced glutamate-induced neuronal damage in the CA1 regions of the hippocampus (Ji, 2020). Modulation of GABA receptors offers an advantageous hypothesis in the interpretation of the results for the mechanism of action for both stress models, as well as in the diazepam-impaired memory model (Hanrahan, 2011). Luteolin displaces flunitrazepam from the benzodiazepine binding site in vitro with low affinity and has demonstrated anxiolytic-like effects administered orally in mice.

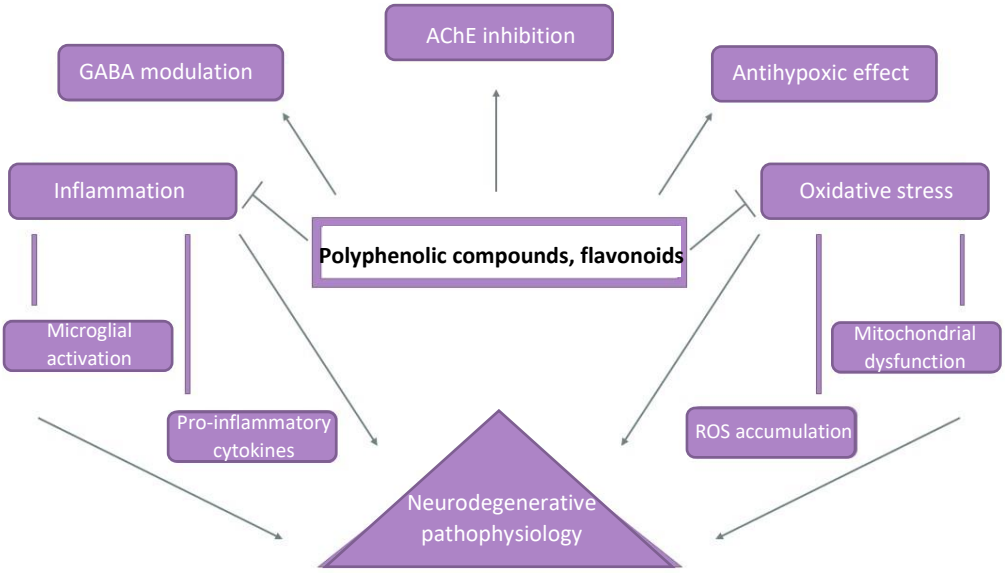

Figure 35. Discussed mechanisms of action.

Experiments show the anxiolytic and antioxidant properties of chlorogenic acid in a dose of 20 mg/kg, as the effect is reduced by flumazenil, suggesting the involvement of GABA receptors in the mechanism of action (Bouayed, 2007). Anticholinesterase properties of the substance have also been described in a scopolamine-induced amnesia model in mice (Kwon et al., 2010). An antihypoxic effect of the *Sideritis scardica* extract is also not excluded, although the sodium nitrite impaired memory model appears to be too aggressive.

CONCLUSION

The studied extract of *Sideritis scardica* leads to 100% survival of experimental animals 24 hours after a single administration at a dose of up to 10,000 mg/kg. No morphological changes were registered in the histological examination of the kidney, liver, stomach, and brain of rats treated for 12 weeks. Based on the results obtained by us and comparing them with data from other authors, we can conclude that the extract is practically non-toxic.

Hematological and biochemical analyses showed values of various parameters within the reference range for the respective species. We note statistically significantly reduced calcium values, which could find an interpretation in the results of the impaired memory experiments.

The present work confirms the data available in the scientific literature about the pronounced anti-inflammatory properties of *Sideritis scardica*. A reduction in hind paw edema was observed at all doses in the late phase of the inflammation. The extract effectively counteracted lipopolysaccharide-induced systemic inflammation and statistically significantly reduced the levels of IL -6, IL -10, and TNF alpha. Along with the pronounced antioxidant activity, these findings support the results of experiments on stress and impaired memory, such as the pro-inflammatory cytokines play a key role in the processes of neuroinflammation. IL -6 and TNF alpha increase statistically significantly in a chronic stress model, this finding mediating their possible future application as stress markers. The acute cold stress model was able to statistically significantly increase only IL -6 levels. Meanwhile, the extract showed an effect in acute and chronic stress behavioral experiments at doses of 200 and 400 mg/kg.

A systematic experimental approach to impaired memory trials demonstrated improvement in both short-term and long-term memory at doses of 200 and 400 mg/kg. It also highlights possible additional mechanisms of action, such as GABA modulation and acetylcholinesterase inhibition, which is consistent with the available literature. An antihypoxic effect is also not excluded, although the sodium nitrite-induced model stands out as too aggressive.

CONCLUSIONS

1. When determining acute toxicity, no lethality was observed for the administered *Sideritis scardica* extract in doses up to 5000 mg/kg, and in the study of subchronic toxicity, after treating the experimental animals with the extracts for 12 weeks, no pathological changes were found in histological studies.
2. In the assessment of hematological and biochemical indicators in the subchronic toxicity experiment, all indicators were within the reference values for the species. A statistically significant reduction in calcium and triglycerides was documented.
3. The cold stress model reliably increased TNF levels alpha in serum relative to the negative control, the extract reduced anxiety in behavioral tests and decreased IL 6 alpha levels statistically significantly relative to the positive control and vice versa
4. The applied model of chronic stress reliably increased the levels of IL 6 and TNF alpha compared to the negative control, and the extract reduced anxiety in behavioral tests in a chronic stress model and reduced IL 6 and TNF levels alpha statistically significant relative to the positive control.
5. The extract improves short-term and long-term memory in behavioral tests in a model of amnesia induced by scopolamine and diazepam.
6. The extract exhibits marked anti-inflammatory activity in a local inflammation model by carrageenan and by systemic injection of lipopolysaccharide.

CONTRIBUTIONS

With scientific and theoretical significance

1. A systematic analysis of acute and chronic stress experiments was performed to establish anxiolytic effect of substances of plant and synthetic origin.
2. A systematic analysis of learning and memory experiments in different memory impairment models was conducted to establish a neuroprotective effect of substances of plant and synthetic origin.
3. A relation between the development of anxiety and increased levels of pro-inflammatory cytokines has been established in preclinical studies.

With scientific and practical significance

1. For the first time in Bulgaria, a systematic pharmacological study of the anti-inflammatory, anxiolytic, and neuroprotective effect of *Sideritis scardica* was conducted.
2. Markers have been determined for the study of anxiolytic and anti-inflammatory effects.
3. A relation between dose and effect of the Sideritis scardica extract has been established.

List of scientific publications

1. Yanchev N, Petkova N, Delev D. Chemical composition and acute toxicity of *Sideritis scardica* extract, Scientific works of the Union of Scientists in Bulgaria-Plovdiv. Series D. Medicine, pharmacy and dental medicine, item XXVI. ISSN 1311-9427 (Print), ISSN 2534-9392 (On-line). 2021. Scientific works of the Union of Scientists in Bulgaria Plovdiv, series G. Medicine, Pharmacy and Dental medicine, Vol. XXVI. ISSN 1311-9427 (Print), ISSN 2534-9392 (Online). 2021. 177-181
2. Yanchev N, Delev D. Anti-inflammatory effect of plants from the genus *Sideritis*. Overview Scientific works of the Union of Scientists in Bulgaria - Plovdiv. Series D. Medicine, pharmacy, and dental medicine, item XXV. ISSN 1311-9427 (Print), ISSN 2534-9392 (Online). 2020. Scientific works of the Union of Scientists in Bulgaria Plovdiv, series G. Medicine, Pharmacy and Dental medicine, Vol. XXV. ISSN 1311-9427 (Print), ISSN 2534-9392 (Online). 2020. 245-250
3. Yanchev N, Delev D, Vilmosh N. Sideritis scardica reduces carrageenan-induced hind paw edema in Wistar rats . Scientific works of the Union of Scientists in Bulgaria–Plovdiv, 2022, in print
4. Yanchev N, Delev D, Potential on the Mursala tea (*Sideritis scardica*, Lamiaceae) in therapy of CNS diseases, Nauka pharmakologia 1/2021
5. Yanchev N, Delev D, Vilmosh N, Atanasova P, Hrischev P. Subchronic toxicity of *Sideritis scardica*, Lamiaceae on male Wistar rats, Folia medica (in copy editing)
6. Yanchev N, Petkova N, Ivanov I, Delev D. Total Polyphenolic Content and Antioxidant Activity of Different Extracts from *Sideritis scardica*. Trop J Nat Prod Res. 2022; 6(7):1113-1118.

Participation in scientific conferences and forums

1. 8th International Scientific Conference of Young Scientists - Plovdiv 2020 ICYS 2020, Anti-inflammatory effects of plants from the genus *Sideritis*. A review, Yanchev N, Delev D - poster and publication
2. 34th ECNP Congress, 2-5 October 2021, Adaptogenic effect of *Sideritis scardica*, Lamiaceae on male Wistar rats in a chronic stress model, Yanchev N, Delev D, Vilmosh N - poster
3. Conference "Science and Youth 2021" MND Asclepius, Adaptogenic effect of *Sideritis scardica*, Lamiaceae in an acute stress model on male Wistar rats, Yanchev N, Delev D, Vilmosh N - poster
4. "Medicine of the future" Scientific conference on the occasion of the 75th anniversary of Medical University, Antioxidant activity of *Sideritis scardica* extracts, Lamiaceae, Yanchev N, Petkova N, Delev D - poster
5. IX International Scientific Conference of Young Scientists - Plovdiv ICYS 2022, Sideritis scardica reduces carrageenan-induced hind paw edema in Wistar rats, Yanchev N, Delev D, Vilmosh N - poster and publication
6. National Conference of SUB Plovdiv, 2021 Chemical composition and acute toxicity of *Sideritis scardica* extract, Yanchev N, Petkova N, Delev D - poster and publication