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PHARMACOLOGICAL, TOXICOLOGICAL AND NEUROBIOLOGICAL STUDIES OF MYRTENAL – A BICICLIC MONOTERPENOID OF NATURAL ORIGIN

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The defence materials are available at the office of the Institute of Neurobiology of BAS,”Acad. Bonchev” Str., bl. 23, fl. 2.

CONTENT

List of the most commonly used abbreviations

ACh – Acetylcholine
AChE – Acetylcholinesterase
AD – Alzheimer's Disease
BBB – Blood-brain barrier
CAT – Catalase
ChAT – Cholinacetyltransferase
CNS – Central Nervous System
CYP450 – Cytochrom P450
DA – Dopamine
EMA – European Medicines Agency
GABA – Gamma-aminobutyric acid
GPx – Glutathione peroxidase
GR – Glutathione reductase
GSH – Glutathione
HB – Hexobarbital
IT – Initial training
LA – Lipoic acid
LPO – Lipid peroxidation
M – Myrtenal
MDA – Malone dialdehyde
ROS – Ractive Oxigen Species
SOD – Superoxide dismutase
tGSH – Total gluthatione

I. Introduction

Over the centuries, humans have accumulated empirical knowledge of the healing properties of plants. Nowadays, a significant proportion of the Earth's population mostly uses products of natural origin for the primary treatment of various diseases. Medicinal drugs contain a high percentage of biologically active substances. They are rich in various chemical compounds such as alkaloids, glycosides, saponins, polysaccharides, tanning agents, flavonoids, ligans, coumarins, essential oils, terpenes, phenols, vitamins, organic and inorganic acids, trace elements and ballast substances. All of these active components have a powerful influence on human metabolic processes, which is responsible for the ever-increasing interest in them. On the other hand, the aim is to limit synthetic and semi-synthetic medicinal products as a result of proven undesirable effects and higher toxicity. A large proportion of modern medicinal products are synthesised in a laboratory environment analogues of plant biologically active substances. According to data from Newman and Cragg (2007), over a period of 25 years (1981-2006), thanks to efforts to develop natural products as potential therapists, a number of naturally occurring medicines have been put into practice, accounting for 63% of all registered pharmacotherapeutic agents. Investigating the pharmacological profile of little-known and/or inadequately studied biologically active substances is a step towards introducing new and more effective medicinal products into clinical practice.

Since the identification of Alzheimer's disease in 1906, many scientific teams have been working on therapeutic options, but an effective treatment for neurodegenerative diseases has not yet been developed. The most important reason for this is the complexity of the disease process, which involves various pathogenic mechanisms, including those related to the environment and genetic predisposition. These diseases are diverse due to the different factors contributing to their progression or so-called multifactorial pathaetiology. At this stage, available and used medicinal products are somewhat effective in responding symptomatically to neurodegenerative changes. Therefore, the detection of biologically active substances with potential effects for the treatment and prevention of neurodegenerative damage, including Alzheimer's disease is an important aspect of modern experimental science.

The medicinal action of plants in the majority of cases is due to components of their essential oils. Essential oil containing plants are widely used in the pharmaceutical and food industries, in perfumery and cosmetics (lavender, rose, chamomile, mint, geranium and other essential oils) (Strehle et al., 2006), in aromatherapy, as well as in various fields of medicine. Essential oils contain active principles with a variety of pharmacological actions (anti-inflammatory, diuretic, spasmodic, antiseptic, carminative, soothing). The irritant properties of some of them on the lining of the gastrointestinal tract enhance salivary and gastric secretion, thereby improving digestion (cinnamon, cloves, mint, etc.). Essential oils of rosemary and pine, which are included in some medicines, are applied externally in colds.

Terpenes (terpenoids, isoprenoids) are the largest group of natural products represented in all classes of living organisms. Terpenoids that have biological activity are used as medicinal

products – taxanes have anti-tumour activity, artemisine antimalarial, and others have anti-inflammatory, antibacterial and antiviral activity. For example, the commonly used in upper respiratory tract diseases is the medicinal product GeloMyrtol (capsules) containing a standardised mixture of terpenes limonene, zineol and α-pinene. Similarly, Tavipec (capsules) contains lavender oil. Algesal (muscle pain cream) is a dermal medicinal product that includes the local anaesthetic Myrtecaine (conjugate of Lidocaine with the monoterpenoid myrtenal, a metabolite of α-pinene) Therpenoids ginsenosides, gyncolides and cannabinoids have shown promising in vitro and in vivo biological activities, but have not yet been studied (Wang et al., 2010). In addition, other terpenoids, including iridoid glycosides, oleanolic acid, tenoufolin, cryptotaninone and ursolic acid, are at a preclinical stage (in vitro and in vivo in animal models). Their mechanisms of action are different – interference with acetylcholinesterase activity, reduction of α-coagulation, reduction of oxidative stress by various mechanisms and others (Yoo & Park, 2012).

An interesting and prospective subject for more detailed studies is the classic representative of the low-molecular monoterpene – Myrtenal, a secondary metabolite of α-pinene. It is present in essential oils of a number of plants - *Myrtus communis, Artemisia spp., Origanum majorana, Origanum vulgare, Glycirrhyza glabra, Rozmarinus officinalis, Thymus spp., Salvia officinalis, Lavandula spp.,* and in the royal clay (propolis).

Studies on the biological properties of Myrtenal and related effects mainly target influencing experimental models of various diseases – diabetes, tumours, psoriasis, etc. Bronchodilator, anti-inflammatory, anti-aggregative and anti-haemolytic, antibacterial, as well as anti-tumour, anti-acetylcholinesterase and anti-malarial pharmacological activities were detected in experimental conditions. Induction of vasodilatation, decrease in heart rate, hypotensive effects have been observed.

In neuroscience, the monoterpenoid is relatively little studied. Information on the neurobiological properties of Myrtenal is scarce. In recent years, new single reports of positive effects of essential oils containing mirtenal on the memory of experimental rodents have emerged. Myrtenal and newly synthesised CNS analogue effects resulting from interaction with GABA receptors in rodents have been hypothesised. The observed anxiolytic properties of essential myrtle oil comparable to those of the benzodiazepine preparation Diazepam have also been associated with a possible central mechanism of action.

There is growing evidence to support the claim that neurodegenerative disorders, such as AD, are mediated by the destruction of cholinergic neurons and increased oxidative stress. For these reasons, attention is focused on the search for antioxidant natural substances with a combined mechanism of action to prevent and/or treat Alzheimer's disease, capable of strengthening cholinergic function and antioxidant protection capacity. The potential of Myrtenal to affect transmitter mediation in the brain is of particular interest, but the reliable information available in the literature on its effects on the levels of major brain neurotransmitters is very limited. The in vitro inhibitory effects of the monoterpenoid on the activity of AChE, the enzyme responsible for regulating acetylcholine levels in cholinergic synapse, suggest positive effects on the memory of rodents with induced dementia. No data on

the effects of mirtenal on experimental animal models of neurodegeneration have been found at this time.

Conducting a variety of pharmacological, toxicological and neurobiological studies, particularly important for the study of the effects of Myrtenal in healthy rodents and in an experimental model of Alzheimer's dementia, can contribute both to the verification and complementarity of data from existing studies and to the discovery of new mechanisms of action of the monoterpenoid in neurodegenerative impairment.

II. OBJECTIVE AND TASKS

The objective thus formulated results in the following tasks:

I. Study of the effects of Myrtenal in healthy rodents

1. Myrtenal Toxicology Studies

- Determination of Mean Lethal Dose (LD50 i.p.) in Mice
- Prolonged Toxicity Study in Mice, after treatment with Toxic Doses

2. Study of major pharmacological CNS effects of Myrtenal in laboratory rodents (mice and rats)

- Effects of combination with CNS depressant modelling drugs
- Anxiolytic properties of Myrtenal
- Anaesthetic effects of Myrtenal in two pain models

3. Study on the neurobiological effects of Myrtenal with behavioural tests in laboratory rodents (mice and rats)

- Effects on memory and learning abilities, neuromuscular coordination and exploratory activity

4. Biochemical effects of Myrtenal in experimental rodent brain

- Antioxidant activity
- Changes in AChE activity
- Study on neuromodulatory properties of Myrtenal

II. Verification of a scopolamine-induced dementia model

1. Behavioural, biochemical and histopathological verification of the scopolamine-induced dementia model in laboratory mice and rats

III. Study of the effects of Myrtenal in rodents with scopolamine-induced dementia

1. Assessment of the preventive effects of Myrtenal on two modifications of an experimental rodent dementia model

- Neuroprotective action of Myrtenal in mice at increasing doses
- Neuroprotective action of Myrtenal in rats in various experiments

2. Mechanisms of the neuroprotective action of Myrtenal

- Effects of Myrtenal on cholinergic mediation: assay of the activity of AChE and mediator ACh levels in rodent brain
- Antioxidant action of Myrtenal in dementia rodent brain

IV. Comparison of the effects of Myrtenal in healthy and dementia experimental rodents

III. Materials and Methods

1. Experimental animals

The experiments were carried out on male sexually mature ICR mice (18 ÷ 22 g) and male sexually mature Wistar rats (180 ÷ 220 g). The animals were kept under standard laboratory conditions in plastic cells – 12-hour light/dark cycle, unrestricted access to drinking water and food for rodents, provided optimum temperature, humidity and indoor ventilation. The experimental procedures were carried out in accordance with the rules for working with experimental animals of the Committee on Ethics of Bulgarian Food Safety Agency and with national laws and rules (Ordinance No. 20 of 01.11.2012 on the minimum requirements for the protection and welfare of experimental animals and requirements to establishments for their use, rearing and/or delivery, effective from 01.01.2013, issued by the Ministry of Agriculture and Food, Prom. SG issue 87 of 09.11.2012) based on the European Directive and in accordance with the rules for working with laboratory animals of the Ethics Committee of the Institute of Neurobiology at the Bulgarian Academy of Sciences.

228 mice and 154 rats (a total of 382 experimental animals) were used for the study.

2. Pharmacological agents

The following substances and solutions were used in the experimental procedures:

- Alfa-lipoic acid, Thoictic acid – solution for injection 600 mg/50 ml Thiogamma Turbo-Set (WÖRWAG PHARMA), Lot: 16J171
- Barbital Sodium – provided by INB-BAS
- Galantamine – solution for injection 10 mg/ml, 1 ml ampules Nivalin (Sopharma), Lot:11215
- Diazepam – provided by INB-BAS
- (-)-Myrtenal 98 % - ACRÔS Organics, Lot: A0363097
- Acetic acid ≥ 99.8 %, 2.5L, Sigma-Aldrich
- Scopolamine – ACRÔS Organics, Lot: A0354964
- Sodium Chloride 0.9% solution – solution for infusion 0.9 % NaCl, 500 ml, BRAUN
- Flumazenil – solution for infusion/injection 0.1 mg/ml, 5 ml, Anexate, Roche Bulgaria Ltd.
- Hexobarbital Sodium – provided by INB-BAS

3. Experimental model of chemically-induced dementia in experimental rodents

The experimental protocol for inducing an Alzheimer's type of dementia included daily intraperitoneal treatment of laboratory animals with scopolamine at the same time of the day for 11 days at doses of 0.5, 1.0, 1.5 and 3.0 mg/kg bw in mice and 1.0 mg/kg bw in rats, respectively. In order to mimic non-linear progression of the induced lesions, a dosage combination was further developed in rats where scopolamine was administered at 0.1 mg/kg

for 8 days and a single dose of 20.0 mg/kg on the 9th final day. Standard for male mature ICR mice, scopolamine is administered in a volume of 0.1 ml/10 g and for male Wistar rats in a volume of 0.5 ml/100 g with individual syringes at separate inoculations.

The experiment involves behavioural tests used to monitor the condition of animals - for memory and learning abilities, spatial orientation and neuromuscular coordination; biochemical and histopathological (cortex and hippocampus) research. To investigate the effects of the cholinoblocker on rodent body weights, as well as the change in relative weights of important organs (brain and liver), rodents were euthanized after completion of behavioural tests at the end of treatment, subject to animal welfare requirements. Mouse and rat brains were removed rapidly, weighed immediately after decapitation and prepared (according to the described methodologies) for the relevant tests – biochemical and histopathological (bark and hippocampus).

4. Study of Myrtenal effects in healthy mice and rats

4.1. Determination of acute and prolonged Myrtenal toxicity in mice following intraperitoneal administration at a single high dose

Determination of the mean lethal dose (LD_{50} i.p.) of Myrtenal in mice was performed using the Litchfield and Wilcoxon Method (1949). The calculations and construction of logarithmic regression line were based on the ratio of the two variables, the observed effect (mortality, %) of the administered dose or the number of animals killed in the administered dose group (Thompson and Weil, 1952; Alaoui et al., 1998). Logit Analysis for MC Excell was used. Animals (n = 12) were injected once at three high doses (170, 200 and 220 mg/kg) of the test substance, the lowest of which was the estimated mice LD_{50} by intravenous administration. They've been under surveillance for seven days. Exited rodents, after dissection, were examined macroscopically *post mortem* to determine the cause of death.

4.2. Study of Myrtenal effects on body weights and relative organ weights in experimental rodents

- Effects on body mass and relative brain and liver weights in mice following repeated 5 and 11 days administration of Myrtenal at 20 mg/kg dose
- Effects on body weight in rats following repeated 5 days administration of Myrtenal at 30 mg/kg dose
- Effects on body mass in rats following multiple 9 days administration of Myrtenal at 40 mg/kg dose

4.3. Study of Myrtenal effects on CNS

Myrtenal has been studied to potentiate CNS depressant effects of barbiturates and benzodiazepines.

- Potentiation the effects of barbiturates – the rats used (n = 24), were divided into groups of 6. The animals in each group were treated with Hexobarbital (100 mg/kg); combination of Hexobarbital and Myrtenal (20 mg/kg); Barbital (225 mg/kg); combination of Barbital and Myrtenal (20 mg/kg). The assay was performed using the method of Simon et al. (1982)
- Effects in combination with Diazepam - rats (n = 16) were distributed in 2 groups of 8: Diazepam injected controls and a group of animals treated with the combination of Diazepam and Myrtenal (30 mg/kg).

The anxiolytic potential of Myrtenal when administered alone has been evaluated.

- Study of the CNS effects of Myrtenal alone – Study of its anxiolytic activity in mice after acute and repeated (7- and 14-day) administration at 30 mg/kg with *Marble Burying Test* (Njung 'e and Handley, 1991).

4.4. Myrtenal analgesic effects study in mice after acute and repeated 7- and 14-day dosing at 30 mg/kg – pain model with central mechanism (*Hot plate test*) and visceral pain model (*Acetic acid writhing test*)

4.5. Study of the effects of Myrtenal on memory and learning capacity, neuromuscular coordination and research behaviour in healthy animals

- Effects of Myrtenal in ***mice*** following acute and repeated 5 and 11 days of dosing at 20 mg/kg on memory and learning (*Step through test*), neuromuscular coordination (*Rota rod test*) and exploratory activity (*Hole board test*)
- Effects of Myrtenal in ***rats*** after acute and repeated 5 days of dosing at 30 mg/kg on memory and learning (*Step through test*) and neuromuscular coordination (*Rota rod test*)

4.6. Study on the effects of repeated administration of Myrtenal on basic biochemical parameters in the brain – determination of LPO product concentration, brain AChE activity and concentration of ACh mediator in rodent brain

5. Study of the effects of Myrtenal in a model of neurodegenerative impairment

To compare the properties of Myrtenal to correct changes caused by the toxic agent scopolamine, the reference galantamine (anticholinesterase pharmacological agent) and lipoic acid (antioxidant) were used.

To investigate the effects of Myrtenal on memory and learning, the *Step through test*, the *Novel object recognition test* were used, on the neuromuscular coordination in mice and rats with scopolamine-induced dementia – *Rota rod test*, and on exploratory activity - the *Hole board test* and *Open field test* were used.

5.1. Study of the effects of Myrtenal in experimental mice with scopolamine-induced dementia

- Study of changes in body mass, memory and learning ability, coordination and exploratory behaviour of mice following multiple 11-day treatment with scopolamine (1.5 mg/kg); scopolamine (1.5 mg/kg) and Myrtenal in doses of 10, 20 and 30 mg/kg; scopolamine (1.5 mg/kg) and LA (30 mg/kg) (alone or in combination with Myrtenal)

- Study of changes in body mass, memory and learning ability, brain oxidation status in mice with monitoring of levels of LPO products, tGSH and activity of CAT, SOD and GPx enzymes after repeated 14-day treatment with scopolamine (1.0 mg/kg); scopolamine (1.0 mg/kg) and Myrtenal at doses of 30, 40 and 50 mg/kg; scopolamine (1.0 mg/kg) and LA at 30 mg/kg (alone or in combination with Myrtenal)

5.2. Study of the effects of Myrtenal in experimental rats with scopolamine-induced dementia

- Study of memory state, learning ability and exploratory behaviour of rats following multiple 11-day treatment with scopolamine (1.0 mg/kg); scopolamine (1.0 mg/kg) and Myrtenal (40 mg/kg); scopolamine (1.0 mg/kg) and galantamine (1.0 mg/kg); scopolamine (1.0 mg/kg) and LA (30 mg/kg) (alone or in combination with Myrtenal)

- Study of changes in body mass, memory and learning status, motor activity, brain oxidation status in rats with monitoring of levels of LPO, tGSH and activity of CAT, SOD and GPx enzymes; study of neuromodulatory effects of Myrtenal by determining brain neurotransmitter ACh levels in the brain; histopathology studies at brain sections in dement rats following repeated 9 days of treatment with scopolamine in dose variation (0.1 mg/kg for 8 consecutive days and a single dose of 20 mg/kg on the last 9 days); scopolamine (dose variation) and Myrtenal (40 mg/kg); scopolamine (dose variation) and galantamine (1.0 mg/kg) (alone or in combination with Myrtenal); scopolamine (dose variation) and LA (30 mg/kg) (alone or in combination with Myrtenal).

5.3. Study on the effects of repeated administration of Myrtenal on basic parameters of oxidative stress in the brain – determination of LPO product concentration, tGSH content, SOD, CAT and GPx antioxidant enzyme activity

5.4. Cholinergic transmission state study – determination of brain AChE activity and ACh mediator concentration

6. Behavioural experimental protocols in healthy animals

6.1. Methods for testing the CNS effects of Myrtenal

6.1.1. Anxiety test method *(Marble burying test, MBT)* (Njung'e and Handley, 1991)

The model is based on the observation that rats and mice bury objects that are harmful or unknown to them, which is perceived as a sign of anxiety – neophobia. The number of marbles buried before and after administration of pharmacologically active substances is an indication of levels of anxiety. After treatment of experimental rodents with anxiolytic substances (e.g. benzodiazepines), the number of glass balls buried is reduced.

The study of the presence of myrtenal's own CNS effects was conducted in three stages – acute, 7- and 14-day treatment of experimental rodents with Myrtenal at 30 mg/kg bw. The experiment was conducted in male ICR mice.

6.1.2. Method of testing for CNS depressant effects (Simon et al., 1982)

The presence of CNS depressant effects was assessed by measuring sleep onset time (lag time) and sleep duration in male Wistar rats.

After a single acute administration of the hypnotic drug (barbiturate, benzodiazepine), the time taken for each delicately placed animal on its back to lose its reflex to stand up and the time of awakening as demonstrated by the rodent standing up are recorded. Sleep duration (min) is expressed as the time interval between loss of reflex and recovery.

6.2. Pain sensitivity methods

6.2.1. Peripheral pain sensitivity test in mice *(Acetic acid writhing test)* (Koster et al., 1959; Collier et al., 1968)

Acetic acid test is a chemical method used to induce pain of peripheral origin in mice. In this case, the analgesic effect is assessed by inducing visceral pain and, in particular, abdominal spasms (traditionally pain stimulated behaviour). To investigate the effects of Myrtenal on acute and/or repeated 7- and 14-day administration (pre-treatment) 30 min after treatment, pain is induced by intraperitoneal injection of the Acetic acid solution in a volume of 0.1 ml/10 g bw. Mice shall be observed for 20 min, the number of abdominal spasms being recorded at 5, 10, 15 and 20 minutes after administration of the acid. An indicator of analgesic activity of the test compound is a reduction in the incidence of spasms.

6.2.2. Hot plate test (Eddy and Leimbach, 1953)

The test is used to induce thermal pain of narcotic type (pain of central origin) and its interference with various substances in experimental rodents. The nociceptive responses were recorded in male ICR mice placed on a heated dry surface at a constant temperature of 55 °C (±3 °C). Testing starts 60 min after the injection. The number of hind paws licks and jumps of the rodent over a specified period of time shall be recorded. The observed reactions of induced thermal stimulus are considered supraspinal. In terms of the analgesic properties of new substances, both the licking of the paws and the reduction in the number of jumps are indicative, which more often reflects locomotor effects. Jumping is a more complex response and involves the emotional component of the escape (Espejo and Mir, 1993).

7. Behavioural experimental protocols in healthy and demented rodents

7.1. Methods for testing memory capabilities

7.1.1. Memory and learning abilities *(Step through/Passive Avoidance test)* (Jarvik and Kopp, 1967)

In this test, in order to avoid electric current in the feet, the rodent must learn to stay in the brightly lit compartment of the apparatus and not to enter the preferred dark compartment. Male ICR mice and Wistar rats were used for the purpose of the study. The latent time, i.e. the time after which the animal moves into the dark room, is measured. A latent time of 180 seconds is used as a training criterion. The test shall be carried out in two stages, initial training before treatment with the test substances and final testing at 24 hours after the end of their application.

7.1.2. Novel object recognition test (Aggleton, 1985; Ennaceur and Delacour, 1988)

The test examines working memory – based on the recognition of familiar and new objects, and is considered to be slightly influenced by non-specific motor stimuli. It uses the innate curiosity of rodents, relying predominantly on their tendency to explore something new to them. The experiment was conducted in male Wistar rats. The time spent examining the unknown (new) objects over a period of 5 minutes, reflecting the state (extent of use) of the recognition memory, shall be reported. A so-called discriminaton index (DI) is calculated, which is the ratio of the time (t) spent exploring the new site to the sum of the study times of the old and new, or: DI = t (old)/t (old + new). DI values range from 0 to 1. DI > 0.5 means subjects remember and distinguish objects and DI < 0.5 indicates impairment of recognition memory.

7.1.3. Motor activity and exploratory behavior testing methods

7.1.3.1. Open field test (OFT) (Walsh and Cummins, 1976)

The method ensures simultaneous consideration of the test subject's motor and exploratory activity. The high incidence of observed behavioural phenomena (number of crossings of marked lines on the field and of animals standing on their hind paws) indicates increased motor

and exploration activity. The test is performed on male Wistar rats. Initial training shall be carried out. Re-testing carried out at 24 hours after the end of treatment with the test substances makes it possible to assess the baseline activity of the animals and its change in response to their administration.

7.1.3.2. Neuro-muscular coordination test *(Rota rod test)* (Jones and Roberts, 1968)

During testing rodents are subjected to forced motor activity. Male Wistar rats and ICR mice are used. Read the residence time (up to 180 sec) before they fall out of the rotating cylinder. The duration of retention is a measure of balance, endurance, motor coordination and physical condition of rodents. Initial training shall be given to each animal prior to the start of the injection of the test substances. The balance and coordination test for the observation period (up to 180 sec) shall be repeated 24 hours after the last treatment by measuring the retention time on the rotating axis.

7.1.3.3. Spatial orientation test *(Hole board test)* (Boissier and Simon, 1964)

The experimental protocol is based on the innate research behaviour of rodents placed in a new environment – the stereotype of head-to-ear dip in holes located on the floor of the setup. The frequency of this activity and its variations shall be monitored. In repeated experiments, the degree of study of environment and spatial orientation (recognition of familiar surroundings) decreases, indicating knowledge (memory). The test is conducted in male ICR mice and Wistar rats in two stages – initial training and test phase, 24 hours after the last treatment.

8. Biochemical research

The rodents (mice and rats) were euthanized with carbon dioxide after testing and basic biochemical parameters in the brain were investigated.

8.1. Methods for determining brain oxidative status

The studies were carried out in the laboratory "Free Radical Processes“ at INB-BAS, under the direction of Assoc. Prof. A. Alexandrova.

The following have been determined: protein content (Lowry (1951), levels of lipid peroxidation products (Hunter et al., 1963), total glutathione (Tietze, 1969), superoxid dismuthase activity (Beauchamp and Fridovich, 1971), catalase activity (Aebi, 1970) and glutathione peroxidase activity (Gunzler et al., 1972).

8.2. Method for the determination of brain ACh levels (Fellman, 1969; Козлов, 1999)

The studies were carried out at the Faculty of Medicine at Medical University – Sofia, from Assist. Prof. R. Klissurov, Ph.D.

8.3. Method for the determination of brain AChE activity (Ellman et al., 1961)

The studies were carried out in the Scientific and Research Laboratory of Military Toxicology in Military Medical Academy – Sofia, Department of Disasters and Emergencies, under the direction of Prof. Dr. Ivan Sumnalev, PhD, and with the active cooperation of Prof. Dr. Christopher Dishovski, PhD.

8.4. Lipid profile determination

Samples of rat blood serum were processed at the Cybalab Medical Laboratory in Sofia, according to standard methodologies. Total cholesterol (Chol), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were measured.

9. Docking Research

The tests were carried out by the Chief Assistant M. Atanasova, PhD, at the Laboratory of Medicinal Design and Bioinformatics at the Faculty of Pharmacy, Medical University of Sofia.

9.1. Investigation of Myrtenal affinity for AChE enzyme

9.2. Investigation of Myrtenal affinity for $GABA_A$-receptor

10. Histopathological examinations

The studies were carried out in the Histology Laboratory at the Department of Anatomy and Cellular Biology at the Medical University of Varna, with an algorithm developed by Assoc. Prof. Dr. Stoyan Pavlov, PhD, for software processing of images obtained from histological samples from the brain of experimental rats. Rodents with dementia induced by the proposed dose modification of scopolamine treated with Myrtenal (40 mg/kg) for 9 days were used.

For histological evaluation, serial sections of brain hemispheres of 10 μm thickness stained with Cresyl-violet according to Kluwer-Barrera were prepared from the brains of randomly selected animals in each group. Coloured preparations were captured using the Zeiss AxioImager Z2 microscope with Zeiss Neo-Fluar objective (v20x; NA = 0.5) and AxioCam MR rev.3 camera at lateral resolution of the obtained images 0.3225 μm/px.

The histopathological examination of rat brains was conducted in certain areas of the terminal brain, namely: cortex and hippocampal formation. Morphological assessment of signs of degeneration (qualitative analysis) was performed by two investigators. Quantitative analysis of Cresyl-violet stained preparations included: estimation of the mean cortical thickness; determination of the average cell number per unit area – the mean volumetric fraction of Cresyl-violet positive structures and the mean density (number/mm^2) of objects divided into two classes according to their area (greater or less than ~31 μm^2) were measured.

The results of the measurements were analysed using R language and environment for statistical computing.

11. Statistical analysis

11.1. Histopathological results analysis – R Language and environment for statistical computing (version 3.5.2, July 2018)

Quantitative analysis from histopathological studies was performed using R Language and environment for statistical computing (version 3.5.2, July 2018). The mean values of cortex thickness, optical density, volume fraction and profile density were compared between groups of animals using a generalised linear model in which the category independent variable treatment group was coded with fictitious variables (0 and 1) for each category.

11.2. Mathematical models for of result analysis

Two sets of data (concerning healthy and damaged rodents) were statistically processed, each consisting of multiple groups and subgroups of behavioural and biochemical parameters obtained in the rat and mouse study. The non-parametric Mann-Whitney-Wilcoxon method and criterion, not using statistical distribution information and allowing small numbers to be used, was chosen as the most suitable method for processing this type of results ($n \geq 3$).

The statistical analysis carried out through the programming envelope "R" and the statistical functions in Excel 2016 covers the following methods: descriptive statistics (diagrams and tables), non-parametric variation analysis to compare 2 parametric variables according to the Mann-Whitney-Wilcoxon criterion, direct unilateral probability analysis according to the Bernoulli method in binary variables, bilateral comparison of the relative shares of qualitative (non-parametric) indicators. Intergroup and intra-group comparisons of these sets are performed according to the Student criterion (assuming that the signs tested are subject to normal distribution) and correlation analysis, Spirmen ranking criterion.

When comparing the effects of the test substance in healthy and dementia animals, the following approach was used: the magnitude of its effect under control conditions was calculated as a difference between the mean of the test parameters in healthy animals and the single values for Myrtenal-treated rodents. Similarly, for rodents with induced impairment, a sample of the difference in mean between scopolamine controls and single values in dementia animals treated with mirtenal was obtained. The two samples were compared statistically using t-Test for independent samples with different dispersions and bilateral distribution.

In all statistical analyses conducted, an acceptable confidence level $P < 0.05$ divided by three ascending classes is assumed: $P < 0.05$, $P < 0.01$ (high significance) and $P < 0.001$ (very high significance).

IV. RESULTS AND DISCUSSION

1. Biological and toxic effects of Myrtenal on healthy experimental rodents

Preclinical data on the monoterpenoid's metabolism and safety are limited. The toxicological characteristic of Myrtenal is not sufficiently complete and only provides information on the absence of genotoxicity. This, as well as missing acute and subchronic toxicity data from some routes of administration, are the reason for conducting the following experiments.

1.1. Myrtenal toxicological screening. Determination of LD_{50} for mice when adinistered intraperitoneally

LD_{50} is a single dose of a specific substance that causes mortality in 50 % of experimental animals (Gehring, 1973). Mean lethal doses (LD_{50}) of Myrtenal for mice – 170 mg/kg after intravenous administration, for rats – 2300 mg/kg after oral administration and for rabbits > 5000 mg/kg after dermal treatment are currently established.

LD_{50} for Myrtenal has not been determined for mice upon intraperitoneal administration, which provokes this study. Healthy male ICR mice were used for this purpose. Myrtenal was injected intraperitoneally as an *ex tempore* emulsion. Subject to the basic principle of reducing the number of animals in vivo experiments (Up and Down Procedure (UDP), EMEA Guideline 425, 1998), laboratory mice (n = 12), divided into 3 groups of 4, were treated once with high doses of the test substance, 170, 200 and 220 mg/kg body weight, respectively. Dose selection takes into account data from other routes of administration (Food and Chemical Toxicology, 1988) and the information available in the literature. Myrtenal was administered to the acceptable volumes of 0.1 ml/10 g body weight in mice according to standard requirements.

Animals were observed for 7 days and survival in the groups was measured at 1 and 24 hours, at 5, 6 and 7 days after single treatment. At 1 and 24 hours, there was no mortality at all doses administered. The dose of 170 mg/kg Myrtenal did not affect the vitality of the test animals until the end of observation. The dose of 200 mg/kg until day 5 did not cause mortality, but on days 6 and 7 the mice condition changed dynamically – at the end of the observation period the survivors in the group were 25%. The highest dose of the substance (220 mg/kg) caused a sharp decrease in survival as early as day 5 (25%) and all animals died on day 6.

There is no description of symptoms of acute Myrtenal poisoning in mice in the literature. In our experiment, the picture of acute intoxication resulting from a single treatment with various toxic doses of Myrtenal included symptoms (transient at lower doses) such as vocalization, cyanosis, seizures alternating with ataxia and abdominal spasms probably due to local irritation. The cause of death of mice was caused by asphyxiation with seizures. Toxicity symptoms observed with parenteral administration of Myrtenal are similar to the acute toxicity

picture described with oral administration of high doses of α-pinene, of which Myrtenal is the metabolite.

Evaluation of the data from the experiment and determination of the mean lethal dose of Myrtenal following intraperitoneal administration was performed using the Litchfield and Wilcoxon Method (1949). The calculations and construction of the logarithmic regression line are based on the ratio of the two variables - the observed effect (mortality, %) of the administered dose (Fig. 1). (Logit Analysis for MC Excell) (Thompson and Weil, 1952; Alaoui et al., 1998).

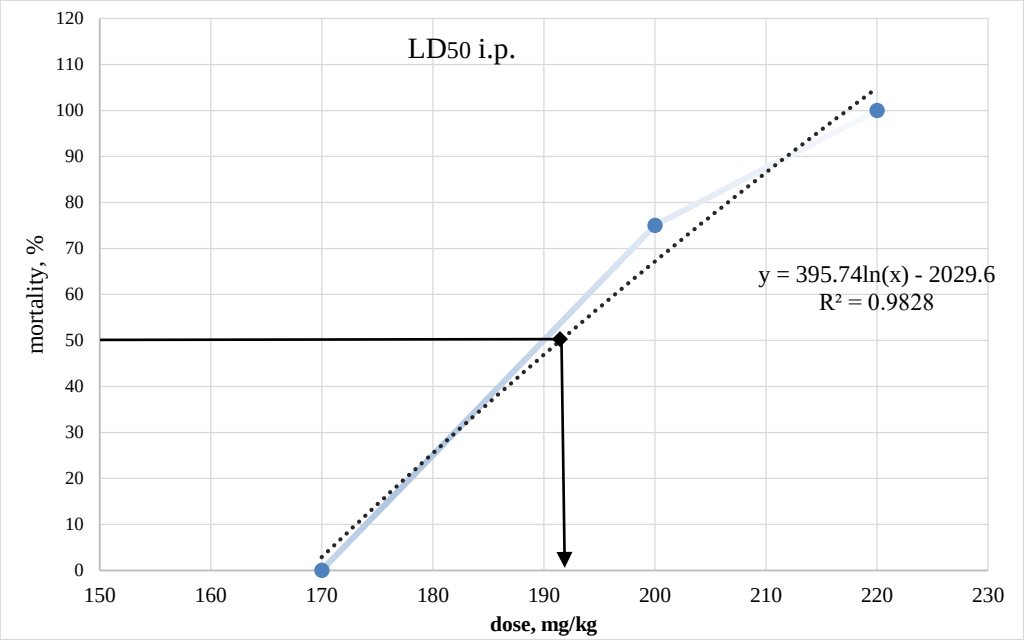


Fig. 1. Calculation of the LD50 i.p. of Myrtenal in mice considering mortality (%) (experimental line – *blue*; logarithmic straight - *black*)

The R^2 (root mean square deviation) value is a statistical indication of how close the experimental data are to the regression line. $R^2 = 1$ (100%) or close values demonstrated that the model explains the overall variability of response data around the mean.

The estimated intraperitoneal LD_{50} of Myrtenal for mice, based on the results of the acute experience and the methodology used, was 191.50 mg/kg. According to the criteria for quantitative toxicity assessment based on the median lethal dose, Myrtenal is classified as toxic according to the Stallard and Whitehead classification (LD50 = 25 ÷ 200 mg/kg) and as moderately toxic according to Hodge and Sterner (LD50 = 50 ÷ 500 mg/kg).

The difference in the mean lethal dose for intravenous (170 mg/kg) and intraperitoneal (190 mg/kg) administration of the same species (mice) revealed a potential interaction with barrier system structures that the molecule must overcome upon absorption. This specificity confirms the available data on Myrtenal's affinity for cell membrane structures and possible location of its action.

1.2. Effects of Myrtenal on body weights in experimental rodents

The change in total body weight, as well as in the relative weights of important organs in experimental animals, is one of the main indicators for assessing the toxicological properties/characteristics of the test substance.

Mice trated with Myrtenal 20 mg/kg for 5 and 11 days were observed. At day 5, there was an increase in the test item and at day 11 rodents significantly increased their body weight (by 10.6%) compared to the control group ($p < 0.05$). Similar to mice and rats treated with Myrtenal at 30 mg/kg over 5 days, they gained normal and even faster weight compared to controls.

Characteristics of body weight dynamics allow to rule out a toxic effect with longer 11-day monoterpenoid administration in mice and point to a low risk of repeated 5-day toxicity in rats. This also indicates a lack of anorexigenic effect in healthy rodents as an undesirable effect of repeated daily administration of the substance.

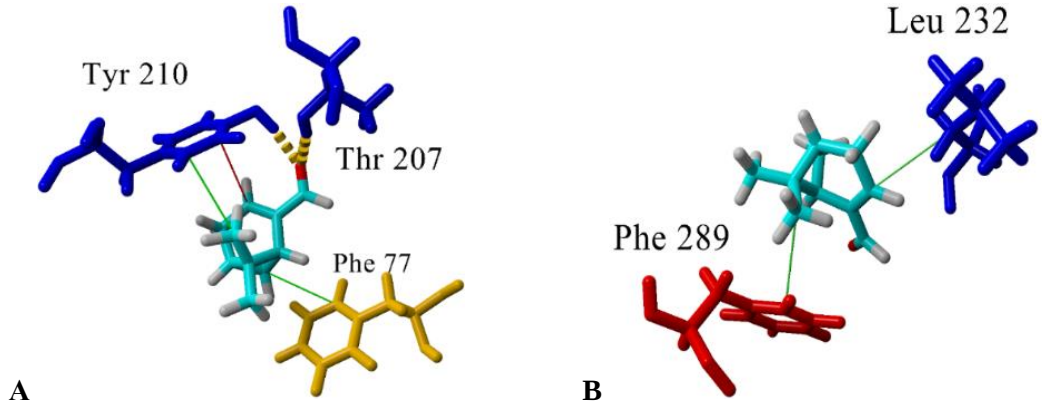
Longer 9-day administration of the monoterpenoid at the higher dose (40 mg/kg) to rats resulted in a statistically significant decrease in the weights of Myrtenal-treated animals compared to the control group ($p < 0.001$). In our opinion, weight loss is due to suppression of appetite in rats, typical of most essential oils. In small doses, they increase salivary and gastric secretions and improve digestion. However, administration at high doses produces opposing effects.

Studies performed for the first time revealed effects of Myrtenal in healthy rodents – mice and rats. To date, studies to clarify the mechanisms of action of this monoterpenoid worldwide have mainly focused on different disease models (listed in the literary overview).

2. CNS effects of Myrtenal in healthy experimental rodents

2.1. Docking studies – human GABAA receptor

Currently, in the absence of a known specific target, the action of monoterpenes is considered to be non-specific. Çiçek (2018) summarizes the potential of natural substances with different chemical structures to affect GABA receptors. According to the author, monoterpenes, including those metabolically bound to Myrtenal, such as α-pinene and Myrtenol, possess the necessary properties for positive or negative GABA receptor modulation. Figure 2 presents the intermolecular interactions of Myrtenal at the two binding sites, the classic benzodiazepine (A) and lower affinity binding sites (B).



Фиг. 2. Intermolecular interactions of Myrtenal at the two binding sites, the classic benzodiazepine (A) and lower affinity binding sites (B)

The amino acid residues γ2-C^-, α1-D^+ and β3-E^+ GABA receptor chains are coloured *yellow, blue and red respectively*; **hydrogen bonds are represented** *by yellow disconnected lines*; **p-π interaction** – *by red line* **and Van der Waals interactions** – *by green lines*

In the classic benzodiazepine binding site, Myrtenal forms two hydrogen bonds to Thr207 and Tyr210 of the α1-D^+ chain. The $^3Csp^2$ carbon atom of the dual bond of the Myrtenal pinene structure is involved in p-π interaction with Tyr210. The position is further stabilized by two weaker Van der Waals interactions with Tyr210 and Phe77 of γ2-C^- chain.

At the lower affinity binding site, Myrtenal forms weak Van der Vaals interactions with Leu232 of the α1-D^+ chain and Phe289 of the β3-E^+ GABA receptor chain.

In conclusion, Myrtenal, like Diazepam, is involved in stronger intermolecular interactions such as hydrogen bonds and p-π contacts at the classic benzodiazepine binding site, which also affects the higher value of the assessment function. This suggests a higher affinity of Myrtenal at the benzodiazepine binding site.

2.2. Interaction of Myrtenal with CNS depressants *in rats*

Central nervous system interference has been identified with many other essential oil components. To study its CNS effects, Myrtenal was administered to experimental rats concomitantly with CNS depressants (barbiturates and benzodiazepines) with different mechanisms of action.

2.2.1. Interaction of Myrtenal with barbiturates

For the purpose of this study, two representatives of the barbiturate group - Hexobarbital Sodium and Barbital Sodium, were used as reference.

They are characterised by a common chemical structure, but their different substitutes determine differences in pharmacokinetics, respectively in their pharmacotherapeutic effects. Due to their sedative properties, the effects of new substances co-administered with them are used as model substrates for pharmacological investigation. This pharmacological approach

makes it possible to determine whether the effect of the test substance is due to central or metabolic mechanisms of interaction with the administered barbiturate.

Hexobarbital undergoes extensive hepatic metabolism and is a typical substrate of hepatic CYP450-dependent monooxygenases. Any abnormality in sleep duration when combined with a new substance will mean that it (last mentioned) is also a substrate of monooxygenases and will therefore alter the biotransformation of HB as measured by differences in induced sleep. Compared to hexobarbital, barbital does not undergo hepatic metabolism and is excreted about 98 % unchanged in the urine (Stolman, 1965). An extension of the duration of narcosis when combined with another substance would mean that it exhibits predominantly central mechanisms of action.

Differences in the biotransformation of the two barbiturates were used in our experiment to study the mechanism of interaction of Myrtenal with the reference species and,in particular, to answer the question: Does Myrtenal change the duration of barbiturate-induced sleep at the level of hepatic metabolism or are central effects behind it?

Following a single acute administration of the two reference subjects, alone and in combination with Myrtenal, the time to sleep onset (lag time) and duration of sleep induction (time to awaken) were followed; for this purpose, rats (n = 24) were divided into 4 groups. Sleep duration was compared in the two barbiturate treatment groups at their effective hypnotic doses (100 mg/kg for HB and 225 mg/kg for Barb). Results of acute reference administration (alone and in combination with Myrtenal in 20 mg/kg single dose) are presented in Figure 3.

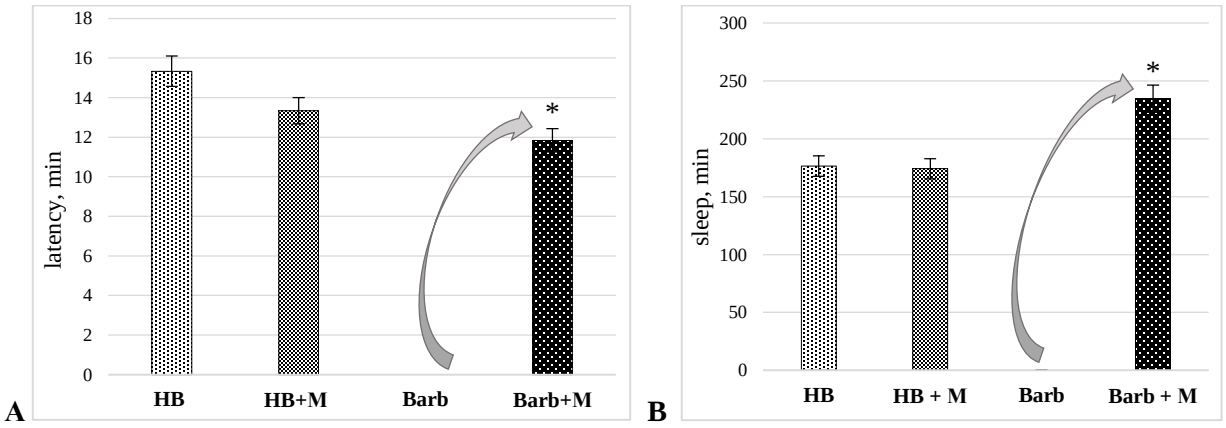


Fig. 3. Interference with hypnotic effects in rats following acute administration of barbiturates (alone and in combination with Myrtenal at 20 mg/kg single dose) – latency (min) (A) and sleep duration (min) (B) (*P < 0.05 vs. Barb) *(t-Test)*

When monitoring latency, Myrtenal in combination with HB was found to reduce the parameter by 13.05%, with no statistical certainty (Fig. 3 A) without affecting sleep duration (Fig. 3 B). Barbital alone caused intense sedation, without narcosis – animals have difficulty and slow movements, but when turned on their backs they restore the position of the body. However, its combination with Myrtenal induced narcosis of longer duration (34.7%) than that

induced by co-administration of Myrtenal with hexobarbital (Fig. 3B). The reported lag time in the group of rats co-treated with Barb + M was 11.25% shorter than in the group injected with HB + M.

The results justify rejection of an existing hypothesis of interaction of hexobarbital with the monoterpenoid at the level of hepatic metabolism.

The absence of significant effects of Myrtenal on hexobarbital sleep and its prolonging effect on barbital sleep in the conditions of our experiment support the assumption that the mechanism of observed effect is central rather than metabolic.

2.2.2. Interaction of Myrtenal with benzodiazepines (Diazepam)

Benzodiazepines are a group of psychoactive substances with anxiolytic, sedative, anticonvulsant and relaxing effects. Diazepam is a positive allosteric modulator (at GABAA level) that potentiates the effects of GABA, resulting in an increase in the frequency of chlorine channel opening and neuropolarisation, respectively. Because this class of medicines alone is difficult to induce hypnosis, the combination of Diazepam with Ketamine Hydrochloride (a general anaesthetic) is recommended for achieving this effect in experimental rodents.

The purpose of the experiment, similar to the previous one conducted with barbiturates, is to clarify the ability of Myrtenal to affect the CNS when combined with pharmacological agents targeting the GABAA receptor. The standard sedative dose of Diazepam is 3.0 ÷ 5.0 mg/kg. To achieve deeper CNS sedation and suppression in our experimental rodents that does not pose a risk to them, a higher than standard dose of Diazepam has been selected. According to the protocol, rats (n = 16) were divided into 2 groups of 8 control rats injected with Diazepam at our chosen dose of 30 mg/kg and a group of animals treated with the combination of Diazepam and Myrtenal (30 mg/kg). The monoterpenoid was administered 60 minutes before the benzodiazepine due to the slower onset of the emulsion action.

As with barbiturate treatment, sleep onset (lag time) and duration of induced hypnosis were taken into account after acute administration of the test substances. Rodents injected with Diazepam alone are heavily sedated but not fully hypnotised, as expected. A hypnosis with a mean sleep duration of 16 minutes was observed in 75% (n = 6) of rats treated concomitantly with Diazepam and Myrtenal.

To monitor the state of the animals when half of the rats in the second group recovered from hypnosis, Flumazenil, a benzodiazepine anatagonist, was administered at a dose of 0.5 mg/kg (Suzuki et al., 1991) upon awakening from sleep. Due to its structural similarity to benzodiazepines, Flumazenil has the potential to compete with them for the binding site on GABAA receptors. This allows to investigate a suspected correlation between GABA ergic mediation and the monoterpenoid. Upon administration of Flumazenil, abrupt exhilaration and a rapidly recovered muscle tone - no paw dragging and no ataxia - were observed at the first signs of awakening.

As a result of these experiments, the effects of Myrtenal can also be assumed to be due to interaction at the GABA receptor level. Similar hypotheses of interference with GABA-eergic neurotransmission in relation to the mechanism of action of newly synthesised Myrtenal

derivatives have been reported in the literature. GABA-modulating activity of α-pinene (whose metabolite is Myrtenal) produced by binding to the benzodiazepine locus is also known (Yang H et al., 2016). Moreira et al. (2014) observed anxiolytic effects of the hydroxylic derivative Myrtenol (another α-pinene metabolite) in rats. Experimental animals recovered with Flumazenil, which the researchers considered indicative of Myrtenol's affinity for GABA receptors.

Results from the acute experiment with Myrtenal and following Flumazenil administration, which are analogous to studies with other oxygen-containing monoterpenes, point to possible anxiolytic properties of the monoterpenoid.

2.3. Investigation of the CNS effects of Myrtenal when administered alone. Study of Myrtenal's anxiolytic activity in mice

The study was conducted in three stages – acute, 7- and 14-day treatment of experimental rodents with Myrtenal at 30 mg/kg. Marble burying test was used to evaluate anxiolytic activity. Upon acute administration, mice (n = 18) were divided into 3 groups – control and 2 injected groups with Diazepam at 1.0 mg/kg (literature data) and Myrtenal at the specified dose, respectively. After 30 minutes, the number of marbles buried was recorded.

Results are presented in Figure 4.

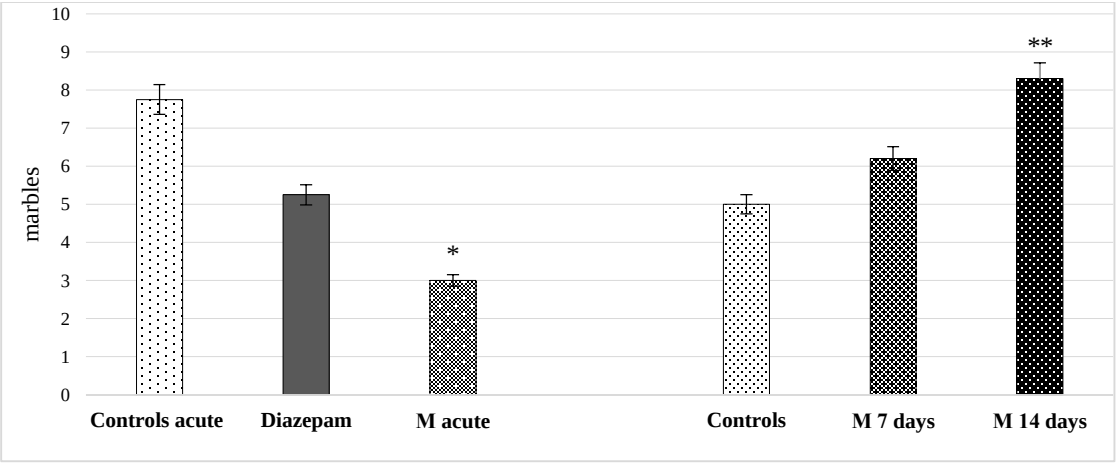


Fig. 4. Anxiolytic activity in mice following acute and repeated 7- and 14-day administration of Myrtenal (30 mg/kg); *P < 0.05, **P < 0.01 vs. Controls

In the acute trial, Diazepam lowered the indicator by 32.3 % compared to controls with borderline statistical certainty, while in Myrtenal the reduction in the number of buried sites (by 61.3%) was statistically plausible ($p < 0.05$). The observed decrease in the Myrtenal-treated group compared to Diazepam-injected animals was 42.8%. The results confirmed the higher affinity of the test substance for the GABAA receptor compared to the reference found in the Docking studies.

No information on possible anxiolytic properties of the monoterpenoid has been found in the literature to date. The results we obtained for the presence of anxiolytic activity of Myrtenal after a single dose in mice were similar to those reported by Kapitsa et al. (2012), which found that Myrtenal derivatives with Aminoadamantan demonstrated anxiolytic activity in male mice.

Upon repeated administration, on day 7, an increase of 24% was observed (Fig. 4). The trend was maintained, with the highest on day 14, when a statistically significant increase in the number of buried pellets (by 66%) compared to control pellets ($p < 0.01$) was observed in animals treated with Myrtenal. This points to increased levels of anxiety as a result of repeated treatment.

At this stage, there are no studies to address anxiety with subchronic administration of the monoterpenoid and/or its analogues. Results obtained in experimental mice indicate that, unlike acute experience, Myrtenal is unlikely to exhibit anxiolytic properties after repeated administration in the Marble burying test.

The experiments performed in laboratory rodents confirmed data from the Docking study on the presence of CNS effects of Myrtenal by interfering with GABA-ergic neurotransmission.

3. Neuropharmacological activity study of Myrtenal in healthy experimental rodents

No data on the effects of Myrtenal on pain sensitivity in experimental rodents were found in the literature.

3.1. Myrtenal (30 mg/kg) analgesic activity study after acute and repeated administration in mice – central mechanism pain model *(Hot plate test)*

During acute administration, mice were injected with Myrtenal at 30 mg/kg. The other two stages of the experiment included daily monoterpenoid pre-treatment at the same dose for 7 and 14 days. The test was carried out 60 minutes after the substance was injected. The number of licks of the hind paws and jumps of each rodent over a period of 3 minutes was recorded.

The observed reactions to the induced thermal stimulus in the hot plate test are considered supraspinal (Parkhouse et al., 1975). Licking the paws is more indicative of the analgesic properties of new substances, while the reduction in the number of jumps often reflects locomotor effects. Jumping, on the other hand, represents a more complex response and involves the emotional component of flight.

Single treatment with Myrtenal did not result in significant changes in the number of jumps compared to control mice – the decrease was 3.12 % in the absence of confidence, while multiple 7- and 14-day dosing resulted in a greater change in parameters (Fig. 5).

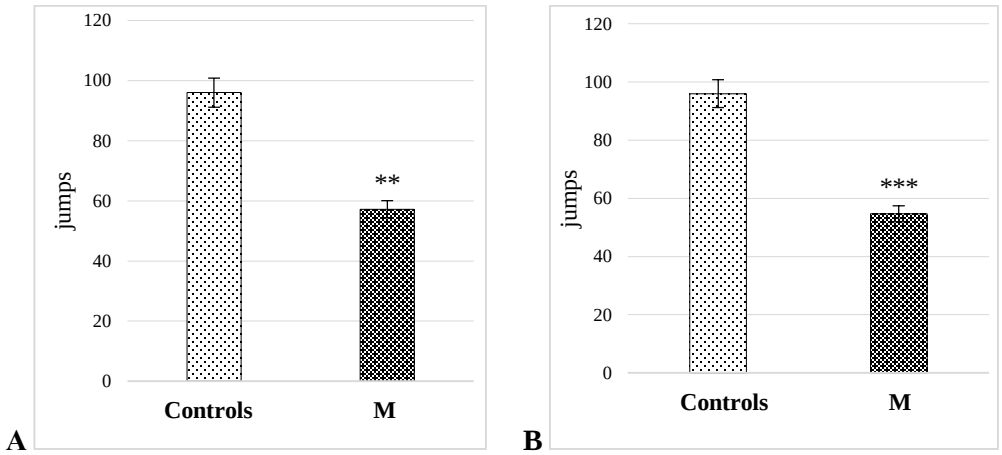


Fig. 5. Effects of Myrtenal (30 mg/kg) in mice after 7-day (A) and 14-day (B) application – number of jumps (P < 0.01 versus Controls, ***P < 0.001 versus Controls)**

Compared to the control group, there was a statistically significant reduction in the number of jumps of 40.4 % with the 7-day treatment ($p < 0.01$) (Fig. 5 A) and 43 % with the 14-day treatment ($p < 0.001$) (Fig. 5 B).

Results for both study periods indicate the presence of an analgesic effect of Myrtenal on the longer term application as a suppressed locomotor reaction to the thermal stimulus.

In conclusion, repeated 7- and 14-day treatment tests with Myrtenal confirmed the effect of locomotor activity on animals to avoid pain irritation. The reduced number of jumps reflects the emotional component of the reaction, which is supported by Myrtenal's anxiolytic activity results. It is also possible that the analgesic activity observed is related to the membranostabilising effects of the monoterpenoid (*literature data*) rather than CNS suppression. No evidence of analgesic potential of terpenes following repeated systemic administration has been found in the literature.

3.2. Myrtenal (30 mg/kg) analgesic activity study after acute and repeated administration in mice – visceral pain model *(Acetic acid writhing test)*

Peripheral pain sensation was investigated by induction of visceral pain with glacial acetic acid solution. In a single administration, mice (n = 12) were divided into two groups – controls and Myrtenal-treated.

The other two stages of the experiment involved daily pre-treatment with the monoterpenoid at the same dose. Mice (n = 24) were divided into 4 groups – 2 control groups and 2 injected with Myrtenal over a period of 7 and 14 days, respectively. 30 minutes after the last administration of the monoterpenoid, was induced pain and the animals were observed over 20 minutes period, with the number of spasms counted every 5 minutes.

The results of the single treatment of mice with the test substance are presented in Fig. 6.

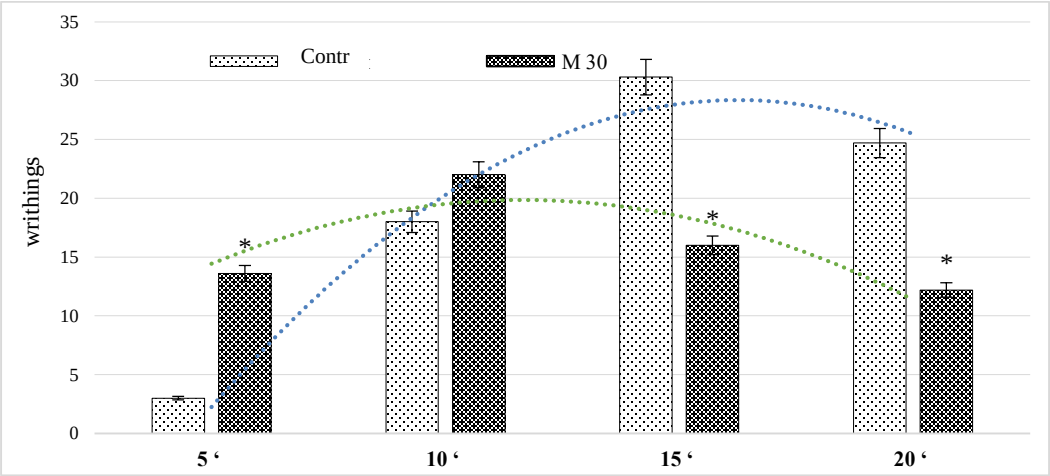


Fig. 6. Analgesic effect of Myrtenal in mice after a single 30 mg/kg dose (*P < 0.05 vs. controls)

At the first reading (at 5 minutes), a 3.5 fold higher incidence of spasms compared to the control group was observed, whereas at 15 and 20 minutes, a significant decrease in the number of writhings was observed by 47.2 % and 50.7 %, respectively ($p < 0.05$).

Our data are consistent with those of Silva et al. (2014), which obtained similar results in the analgesic activity study of the Myrtenal's hydroxyl derivative Myrtenol in mice. Acute administration of Menthol (another hydroxyl derivative, representative of monoterpenes) also showed analgesic activity when conducting the Acetic acid test (Galeotti et al., 2002).

It is important to note that after repeated administration of Myrtenal, contrary to acute treatment results, at all reported time intervals of the experiment, the incidence of spasms compared to the control group was increased. In the 7-day trial, the increase in observed parameter from controls at 5 min was 150 % ($p < 0.05$), at 10 min – 14.4 %, at 15 min – 32.9 % and at 20 min – 27.9 %. At 14 days, at 5 minutes, there was an increase in the number of spasms of 137.5 % compared to controls ($p < 0.05$) and in the remaining time intervals the indicator did not change significantly.

Summary results of studies performed to evaluate the analgesic effect of Myrtenal administered at 30 mg/kg to mice by responding to peripheral (visceral) pain, in particular induction of abdominal spasms, showed:

1. For both acute and prolonged treatment, an increase in writhings was observed at 5 min, possibly due to local irritation from the intraperitoneally administered emulsion;

2. Myrtenal significantly reduces spasms in a single application at 15 and 20 minutes, similar to other oxygen-containing monoterpenes;

3. During continuous application (7 and 14 days), Myrtenal showed no analgesic activity in visceral pain testing.

Results of the peripheral pain sensory test obtained following single administration of Myrtenal confirm evidence of analgesic properties (membranostabilising effects) of the monoterpenoid, while repeated treatment does not produce similar effects.

3.3. Myrtenal's effects on behaviour in experimental rodents

3.3.1. Effects of Myrtenal on memory capabilities of mice and rats after acute and repeated administration (*Step through test*)

▪ ***Acute administration***

The latent time to dark field entry was reported in mice treated with Myrtenal at 20 mg/kg dose (Fig. 7 A) and in rats treated with the test substance (30 mg/kg) (Fig. 7 B).

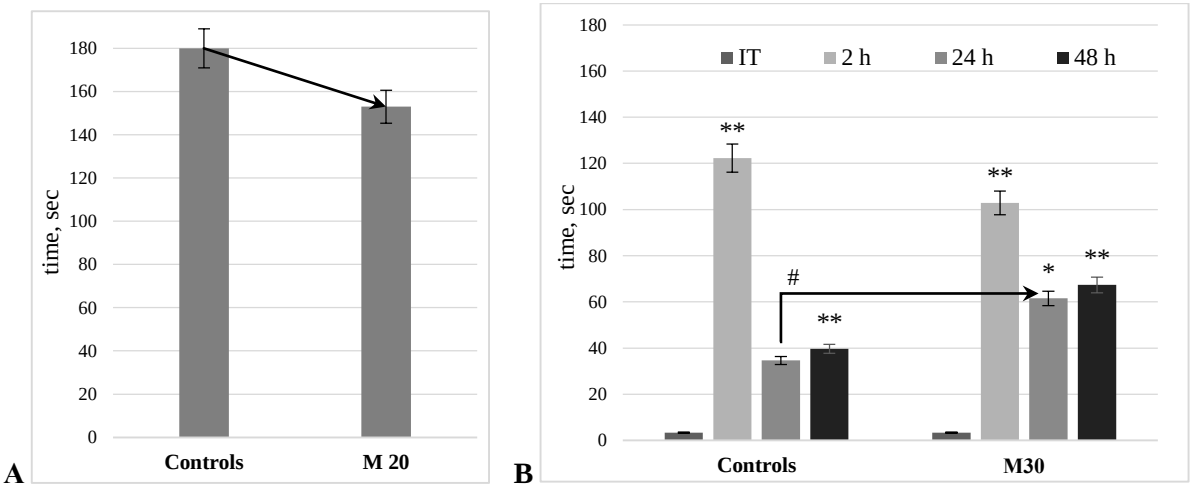


Fig. 7. Effects on memory capabilities in rodents after single administration of Myrtenal – lag time recording (sec); A – Mice, B – Rats (*P < 0.05, **P < 0.01 vs. Initial training (IT), $^{\#}$P < 0.05 vs. Controls); Wilcoxon Rank Test - "R"

Results showed that all mice in the control group remembered the situation and did not enter the dark part of the field, which was interpreted as good short-term memory (Fig. 7A). Lag time in animals injected with Myrtenal was reduced by 15% compared to controls.

In rats, values of memory status indicator (lag time) were recorded at 2, 24 and 48 hours after single treatment (Fig. 7B). Results revealed some particularities in the observed effect of the monoterpenoid over time, most likely related to its pharmacokinetic characteristics. Myrtenal, as a lipophilic substance, exhibits slower but longer term effects on memory capabilities. At 24 and 48 hours, the test substance showed better results in terms of lag time compared to controls. At 24 hours, the values of the indicator were statistically significantly increased (by 35.86%) compared to the control group ($p < 0.05$). At the last observation interval (48 hours), Myrtenal treated animals demonstrated superior educational abilities – the difference in lag time compared to controls was 74.7% and at 48 hours compared to 2, the decrease in values was 34.52%.

Experimental results indicate that with single administration, Myrtenal improves rat learning abilities over a period of more than 24 hours. The 30 mg/kg dose administered to rats affected memory capacity in a positive direction, while the 20 mg/kg dose did not produce similar effects in mice.

- ***Repeated administration***

This also takes into account latent time to enter the dark part of the field: in mice treated with Myrtenal at 20 mg/kg for 11 days (Fig. 8 A) and in rats treated with the test substance at 30 mg/kg for 5 days (Fig. 8 B). Results are presented in Fig. 8.

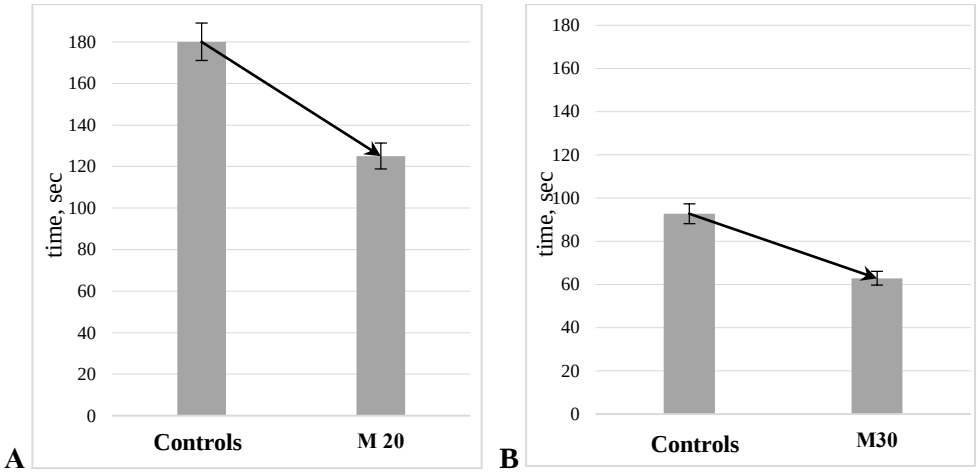


Fig. 8. Effects on memory capabilities in rodents after repeated administration of Myrtenal – lag time recording (sec); A – Mice, B – Rats

All control mice did not enter the dark part of the setup (remember), while 50 % of Myrtenal-treated mice entered, i. e. did not show good long-term memory status (Fig. 8 A). Latency time in Myrtenal group was reduced by 30.6 % compared to controls.

In rats, 5-day dosing of Myrtenal at 30 mg/kg showed no superior effects on memory compared to the control group – the value of the indicator was reduced by 32.2 % with no statistical significance (Fig. 8 B).

Both acute and repeated administration of Myrtenal did not have a positive memory impact in the experimental mice relative to the control group. In rats, repeated treatment with Myrtenal also did not improve memory capabilities, while single administration influenced memory possibilities in a positive direction.

The results we have obtained confirm the established CNS depression by acting on the GABAA receptor as a possible mechanism of action. Similar hypomnesic effects are also characteristic of the acute administration of the classical sedative anxiolytic agent Diazepam.

3.3.2. Effects of Myrtenal on neuromuscular coordination in mice and rats after acute and repeated administration *(Rota rod test)*

The neuromuscular coordination test is considered to be indicative (screening) of the potential of various substances to affect endurance, balance, motor coordination and physical condition in experimental rodents and, in the case of repeated testing, of their educational abilities.

During testing rodents are subjected to forced motor activity on the arm of a horizontal, constant speed, cylindrical axis. Their retention time (up to 180 sec) was measured before they fell out of the rotating cylinder.

The test results in the experimental mice are presented in Fig. 9.

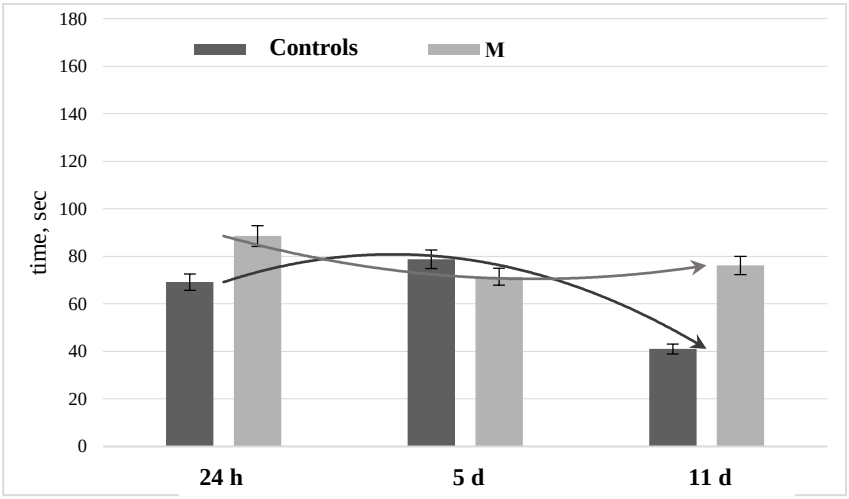


Fig. 9. Effects on neuromuscular coordination in mice following single and repeated (5 and 11 days) administration of Myrtenal at a dose of 20 mg/kg – counting retention time (sec)

At 24 hours, once-treated with Myrtenal animals showed an unreliable increase in retention time on the rotating arm of the production (by 19.4%) compared to the control group. After 5 days of administration of the test substance, mice are very active "acrobats“ – they show curiosity, with no worsening of neuromuscular coordination, with a slight reduction in residence time compared to controls (by 9.38%). Repeated 11-day treatment resulted in a more sensitive improvement in balance and coordination in animals – their residence time on the rotating axis was increased by 85.8%, but is nonetheless statistically unsignificant compared to controls. According to Galeotti et al. (2002), a single systemic administration of monoterpene Menthol to mice did not cause any change in coordination when conducting the Rota rod test.

The results of the test performed in experimental rats are presented in Fig. 10.

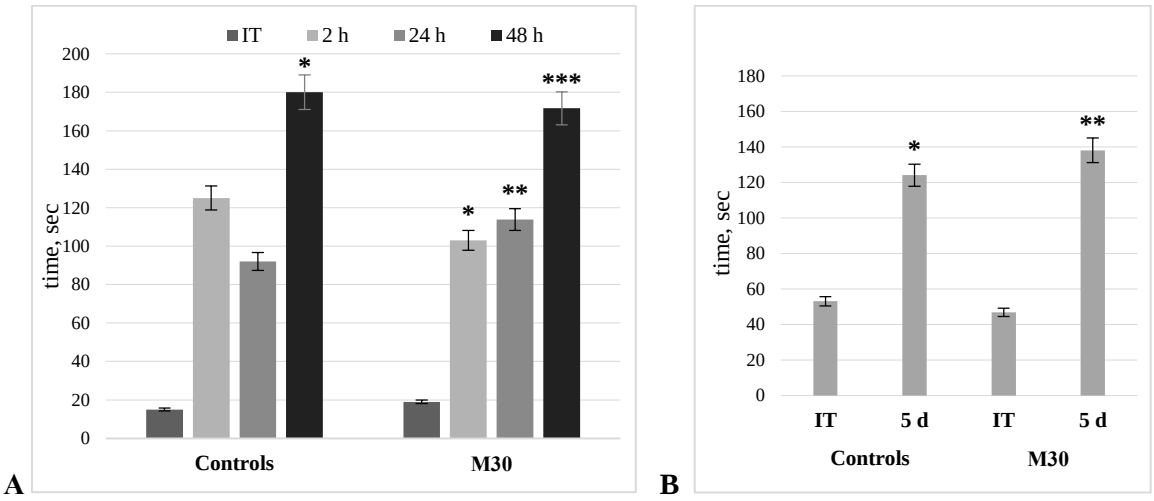


Fig. 10. Effects on neuromuscular coordination in rats following acute (A) and 5-day (B) administration of Myrtenal (30 mg/kg); *P < 0.05, **P < 0.01, ***P < 0.001vs. IT; Wilcoxon Rank Test - "R"

In the acute experiment, the time of rats in both groups spent on the rotating arm of the axis prior to falling relative to baseline changed dynamically (Fig. 10 A).

After single administration in the control group relative to the initial training, the indicator was reliably increased at 48 hours ($p < 0.05$). Significant changes in residence time from baseline were observed in Myrtenal-treated animals, with values close to those of controls at 48 hours, with confidence levels $p < 0.001$ relative to IT. Changes in indicator (%) across groups from baseline (autocontrol), as expressed by increases in values, showed improved neuromuscular coordination. The results in the Myrtenal group were similar to those in the control rats at 24 hours, while the observed effects were less apparent at 2 and 48 hours.

Apart from being a tool to evaluate coordination and balance over time of observation, in repeated testing, the experiment is indicative of both stimulated neuromuscular transmission and stimulation of the CNS as a whole.

При животните, третирани с миртенал за период от 5 дни, в края на теста се наблюдава тенденция към по-продължително задържане върху постановката (с 11.3 %) в сравнение с контролната група, но разликите не са статистически достоверни (Фиг. 10 Б).

Animals treated with Myrtenal for 5 days tended to prolong the retention time (11.3%) compared to the control group at the end of the test, but the differences were not statistically signifficant (Fig. 10 B). In a study of the mechanism of action of (-) -Myrtenol (the hydroxyl derivative of Myrtenal) in a rat experiment, Moreira et al. (2014) also did not detect changes in retention time with Rota rod test, respectively did not detect any anxiolytic properties. In conclusion, neither acute nor repeated administration showed any impairment of Myrtenal on neuromuscular coordination in mice and rats. The results obtained are consistent with studies of Galeotti et al. (2002) and Moreira et al. (2014) on the properties and action of monoterpenes in

experimental rodents that do not indicate effects of Myrtenal on the motor performance of laboratory animals.

In our view, the slower dynamics in reducing the retention time on the axis of the apparatus in Myrtenal-treated rodents relative to the controls are due to the lipophilicity of the test substance, which is a prerequisite for its kinetics.

3.3.3. Effects of Myrtenal on exploratory behaviour in mice after acute and repeated administration (*Hole board test*)

The experimental protocol is based on the innate research behaviour of rodents placed in a new environment – the head-to-ear stereotype of "dipping“ of holes in the floor of the setup (Rogers et al., 1999). The exploratory behaviour of mice and rats in this test differs from the total locomotor activity – the number of attempts by the animal to enter the holes is an indicator not only of exploratory activity but also of spatial orientation (File, 2001). To study behavior in the educational phase, the experiment is repeated. With each subsequent test, the number of head dips decreases due to familiarity with the situation.

The mouse experiment was conducted in three stages: acute, 5-day and 11-day administration of Myrtenal at a dose of 20 mg/kg (Fig. 11). The number of head dips is recorded over a period of 3 minutes.

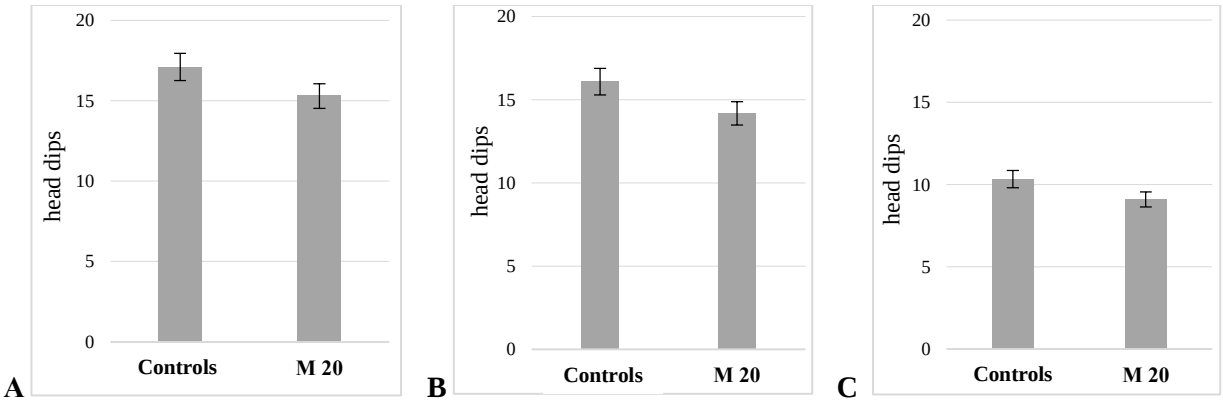


Fig. 11. Effects of Myrtenal (20 mg/kg) on exploratory activity in mice after acute (A) and repeated 5- and 11-day treatment (B and C)

Monitoring of the total number of head dips over the entire period of time showed that acute (single) administration of Myrtenal showed no significant changes in study behaviour compared to the control group. Similar results were obtained with repeated 5- and 11-day treatments, where the reduction in the activity of 11.5 % and 11.91%, respectively, were not statistically significant.

The dynamics of the test parameter over the entire time interval of the experiment are expressed by the number of "dips“ per minute (1st, 2nd and 3rd minutes).

In acute trial, Myrtenal application resulted in statistically unsignificant decreases in the number of „dips“ compared to control animals. During multiple 5-day treatment, the 1-minute study activity was identical to that of the control group, while at 2 and 3 minutes there was a decrease of 13.46 % and 17.7 %, respectively.

Results showed that the exploratory activity of mice at 1st minute of single and repeated Myrtenal treatment was significantly reduced. Compared to acute administration at the first point (1 min), the number of „dips“ was significantly reduced in both the 5-day ($P < 0.05$) and the 11-day experience ($P < 0.01$). At 2 and 3 minutes in the Myrtenal group, the differences between the acute and the 5-day experiments were insignificant, while in the 11-day administration, the reduction in parameter values was clearly marked by 34.5 % at 2 and 33.1 % at 3 minutes.

We believe that the results obtained can be seen as confirmation of the hypothesis of spatial orientation improving effects of Myrtenal with longer treatment.

Exploratory activity of the test animals in all three phases of the experiment (single, 5 and 11 days of administration), at the three time points (1, 2 and 3 minutes) followed a trend identical in both the controls and the monoterpenoid treated mice in the absence of significant differences. This means that the test substance does not alter the normal habitation of animals observed in the controls.

From the results obtained it can be concluded that repeated administration of Myrtenal lowers the research interest in rodents, which is associated with stimulated spatial orientation (Table 1).

Table 1. Change in exploratory activity in mice following acute 5 and 11 days of Myrtenal (20 mg/kg) compared to control group

	24 hours	5th day	11th day
Change compared to Controls (%)	↓ **10.6**	↓ **11.5**	↓ **11.91**

Протоколът за изследване на проучвателната активност и пространствената памет е използван в експерименталната фармакология за определяне наличието на анксиолитична активност при мишки (Takeda et al., 1998). Тази особеност на наблюдаваното поведение при многократното приложение на миртенала потвърждава наличието на анксиолитичен потенциал, установен при другите опитни постановки.

This exploratory activity and spatial memory test protocol was also used in experimental pharmacology to determine the presence of anxiolytic activity in mice (Takeda et al., 1998). This pattern of behaviour observed with repeated administration of Myrtenal confirms the presence of anxiolytic potential identified in other experimental settings.

4. Brain biochemistry studies in healthy rodents following Myrtenal administration

The objective of the study was to clarify the potential of the monoterpenoid to improve memory in intact laboratory rodents by investigating its anti-cholinesterase and antioxidant properties. This was based on the information available in the literature on the ability of Myrtenal to inhibit AChE activity in *in vitro* studies (Kaufmann et al., 2011), as well as on the manifestation of antioxidant properties in colorectal cancer and diabetes mellitus models listed in the review.

Lipid peroxidation levels and brain AChE activity were determined in mice treated with Myrtenal at a dose of 20 mg/kg. The neuromodulatory effects of the monoterpenoid on the ACh content in the brain of rats were investigated after 11 days of administration at a dose of 40 mg/kg.

4.1. Determination of brain lipid peroxidation product levels in mice following repeated 5 and 11 days administration of Myrtenal (20 mg/kg)

Changes in the levels of lipid peroxidation products in the brain of mice were followed at two time intervals – 5 and 11 days. The results are presented in Fig. 12.

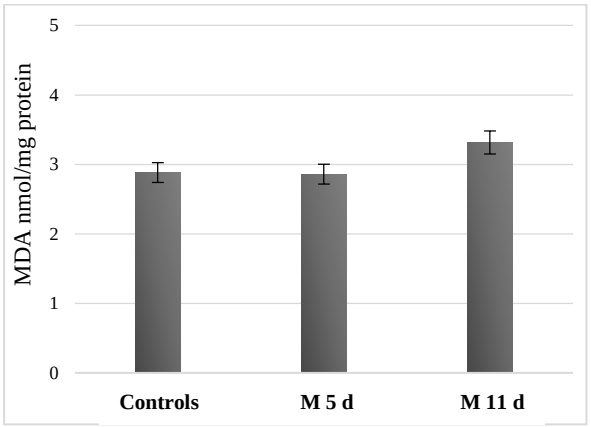


Fig. 12. Effects on brain lipid peroxidation product levels in mice after 5 and 11 days application of Myrtenal (20 mg/kg)

At day 5, there were no differences in the test parameter between the Myrtenal-treated group and the control group. At day 11, there was an increase (14.95 %) in LPO product levels compared to controls, which was borderline reliable.

The observed effects of Myrtenal on LPO product levels are similar to those observed with a number of antioxidates, which under certain conditions acquire pro-oxidant activity. Vitamins which, by exercising their antioxidant action through the reduction of free radicals, may exhibit opposing properties may serve as an example. Vitamin C, which has been shown to have antioxidant activity, reduces levels of oxygen-containing free radicals such as hydrogen peroxide (Duarte and Lunec, 2005). At the same time, it reduces concentrations of metal ions,

leading to the formation of free radicals (through Fenton's reaction). For these reasons, vitamin is assumed to exert predominantly antioxidant activity in the body (Carr and Frei, 1999). Limited data are available for other antioxidants obtained exogenously, such as some polyphenols (Halliwell, 2007), zinc (Hao and Maret, 2005) and vitamin E (Schneider, 2005).

4.2. Effects of Myrtenal on brain acetylcholinesterase activity

Molecular docking studies have predicted that one of the main targets for the action of Myrtenal and other monoterpenes is AChE (Taktak and Badawy, 2019).

4.2.1. Docking studies with acetylcholinesterase

Docking studies on Myrtenal's ability to affect AChE activity were performed by comparison with Galantamine as standard for anticholinesterase activity. The results are presented in Fig. 13.

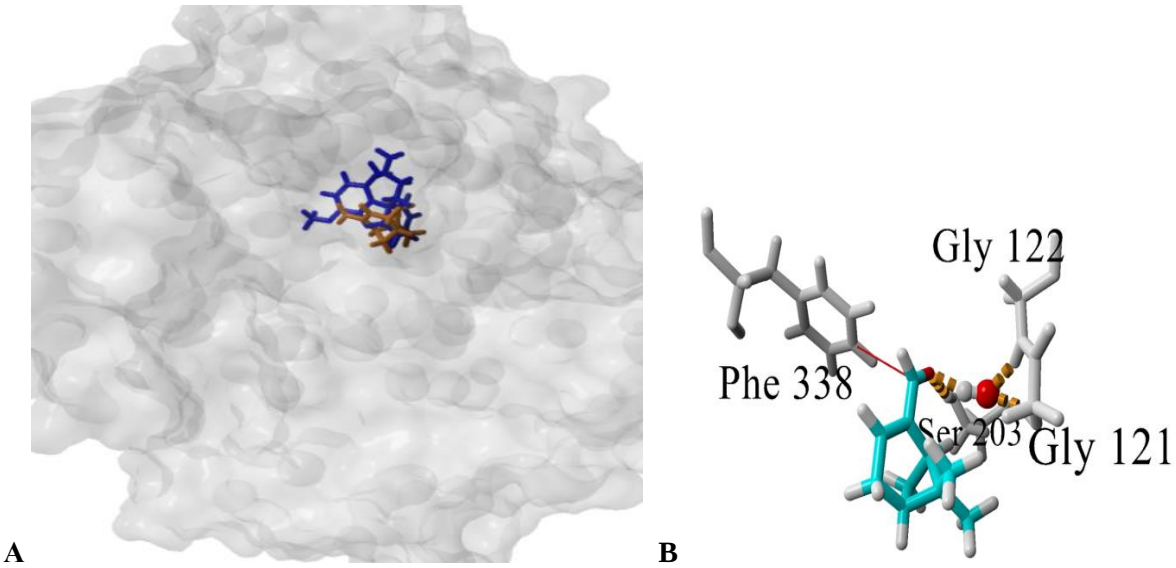


Fig. 13. Superposed galantamine (*dark blue***) and Myrtenal (***orange***) in the active centre of AChE (A); Myrtenal intermolecular interactions in the active centre (B): hydrogen bonds** *are represented by orange disconnected lines***; p-π interaction** – *by a red line***, structural water molecule in the active centre** – *by spheres and rods*

The figure shows that Myrtenal forms a hydrogen bond to Ser203 from the catalytic centre at the binding point, as well as a hydrogen bond to the structural water, which serves as a bridge between the enzyme and the ligand, through a network of hydrogen bonds. The Csp^2 carbon atom in the aldehyde group of the test substance is involved in p-π interaction with Phe338 amino acid residue from the anion pocket at the binding site.

In conclusion, Myrtenal could bind to AChE to a lesser extent than galantamine. It enters deep into the active center, reaching the catalytic region, forming two hydrogen bonds and one p-π interaction.

4.2.2. Brain acetylcholinesterase activity study in mice after repeated treatment with Myrtenal

Changes in AChE activity in the brain of mice following administration of Myrtenal at a dose of 20 mg/kg were followed in two time intervals of 5 and 11 days (Fig. 14).

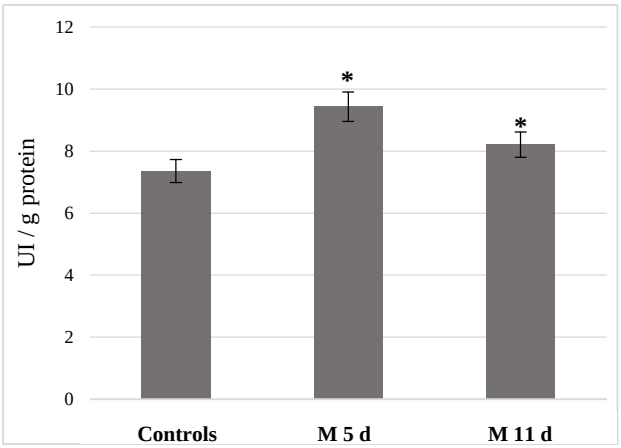


Fig. 14. Effects on brain AChE activity in mice after 5 and 11 days application of Myrtenal (20 mg/kg); *P < 0.05 Vs. Controls (Wilcoxon Rank Test – "R")

Statistically significantly increased enzymatic activity was observed in Myrtenal-treated mice versus controls in both ranges by 28.2 % at 5 and 11.53 % at 11 days ($p < 0.05$).

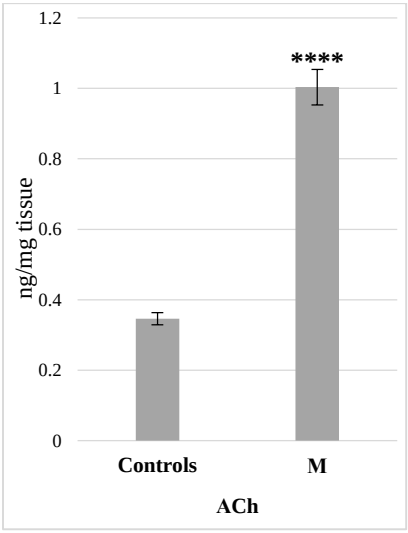
The potential for modulating cholinergic mediation of various naturally active substances, including monoterpenes that have affinity for AChE, has been brought to the attention of a number of researchers. The anti-cholinesterase activity of α-pinene, the metabolite of which is Myrtenal (Russo, 2011) is known. The in vitro studies of Kaufmann et al. (2011) on the anti-cholinesterase properties of Myrtenal cited above are also in this direction.

Our results from the study of brain AChE activity in *in vivo* experiments in mice did not confirm the presence of anti-cholinesterase properties but, on the contrary, showed significantly increased enzyme activity as a result of Myrtenal administration.

The observed increased activity of brain AChE in mice following treatment with the monoterpenoid studied is responsible for monitoring the levels of major mediators in the brain of experimental rodents, in particular acetylcholine (ACh), on the one hand, because of its relationship to AChE and, on the other hand, because of the mediator's function as a neurotransmitter and neuromodulator in CNS, with a leading role in memory capabilities.

4.3. Effects on brain acetylcholine levels in rats after repeated administration of Myrtenal

For the purpose of the study, rats (n = 12) were devided to 2 groups: controls and Myrtenal-treated with a dose of 40 mg/kg daily for 11 days. At the end of the trial, the animals were euthanized and their brains were separated to determine ACh levels. The results are presented in Fig. 15.



ACh	Mean ± SEM
Controls	0.3467 ± 0.01054
M	1.003 **** ± 0.008028

Fig. 15. Effects on brain ACh levels in rats after 11 days treatment of Myrtendl (40 mg/kg); ****P < 0.0001 Vs. Controls (*t*-Test)

There was a significant increase in ACh content in the rat brain following repeated administration of Myrtenal compared to the control group. The 189.4 % change has a high level of statistical confidence (p P 0.0001). Acetylcholine is one of the mediators with a leading role, not only in memory processes but also in pathophysiological processes associated with neurodegenerative diseases.

Severely elevated levels of the neurotransmitter are associated with a mechanism to stimulate its exocytosis in the synapses. Another possible cause of increased brain ACh concentration is stimulation of activity and/or elevation of cholinacetyltransferase (ChAT), an enzyme responsible for synthesis of ACh in neurons. In their studies, Lee et al. (2017) found that the effects of α-pinene on memory functions in rats were due to increased expression of mRNA (matrix RNA) of ChAT in the cerebral cortex.

We believe that high levels of the ACh neurotransmitter can explain the increased brain AChE activity we found in combination with the observed effects on memory and behaviour of experimental mice.

Behavioural experiments and biochemistry studies conducted in healthy laboratory mice and rats, as well as docking studies, demonstrated Myrtenal's affinity for the active centre of the brain AChE and for the GABAA receptor.

The CNS potentiating effects of Myrtenal observed in combination with classical sedative and hypnotic pharmacological agents (Hexobarbital, Barbital and Diazepam) confirm its ability to affect brain function. Experimental CNS suppressive activity when administered on its own to mice was consistent with the greater affinity of the monoterpenoid for the benzodiazepine locus at the GABAA receptor compared to Diazepam indicated in the theoretical study.

In conclusion, Myrtenal in healthy experimental rodents did not show damaging effects on memory and learning ability, neuromuscular coordination and spatial orientation. The test substance induces CNS suppression, possibly by interfering with GABA-ergic neurotransmission, and demonstrates neuromodulative properties manifested by elevating levels of the basic brain mediator acetylcholine.

In our view, all this is a strong reason to extend the study to investigate the effects of the monoterpenoid on models of memory impairment and experimental dementia.

5. Myrtenal effects in a model of neurodegenerative impairment

5.1. Verification of the scopolamin-induced dementia model

The verification of the model of chemically-induced dementia of Alzheimer's type was performed according to the experimental protocol (Chapter III, item 3), which included daily 11-day treatment of laboratory animals with scopolamine, at the same time of day, in doses, respectively for mice 0.5, 1.0, 1.5 and 3.0 mg / kg and for rats - 1.0 mg / kg. The effective doses were selected after analysis of the literature data. Behavioural and biochemical studies have been conducted. Additionally, to mimic non-linear progression in the induced impairment, the process of inducing memory impairment was modified by chosen dose combination for rats, in which rodents were repeatedly treated with scopolamine at a dose of 0.1 mg/kg for 8 days and on the 9 final day with a single dose of 20.0 mg/kg. Behavioural, biochemical and histological studies have been conducted in the brain of experimental animals.

5.1.1. Effects of scopolamine on memory and learning abilities in experimental rodents *(Step through test)*

The experimental apparatus is the same as that used in healthy rodent testing. The results are presented in Fig. 16.

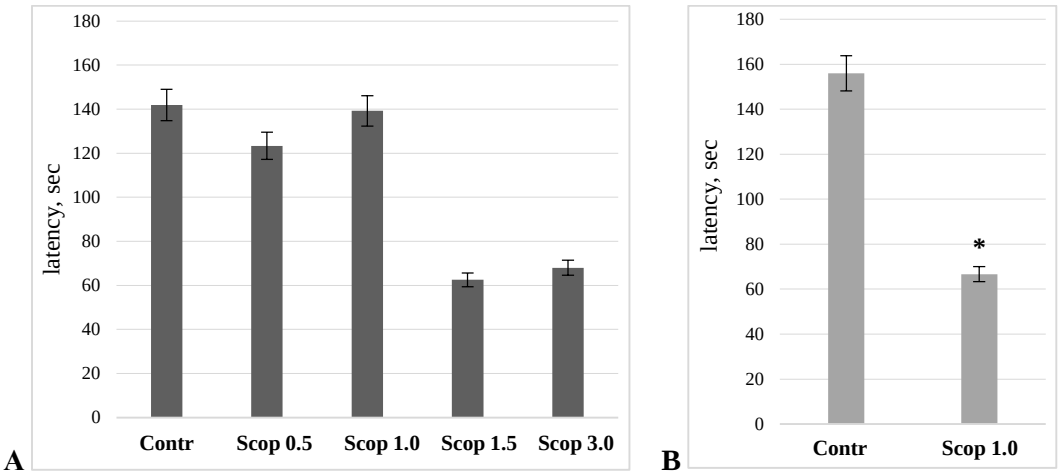


Fig. 16. Memory effects in mice treated with scopolamine at increasing doses (0.5, 1.0, 1.5 and 3.0 mg/kg) (A) and in rats treated with scopolamine at 1.0 mg/kg (B); *P < 0.05 Vs. Controls (*t*-Test)

Compared to the control group, lag time in mice repeatedly treated with scopolamine at the selected effective doses was shown to vary in magnitude (Fig. 16 A). The 0.5 mg/kg dose caused a reduction in latency compared to controls of 13% and 1.0 mg/kg of 1.9%. The results differed between the high dose groups (1.5 and 3.0 mg/kg) where significant but statistically unreliable reductions in latency time were reported compared to controls of 55.9 % and 52.1%, respectively.

According to Golechha et al. (2012), a single intraperitoneal administration of scopolamine at 1.0 mg/kg in mice produced a significant reduction in latency time of 75%. On the other hand, Kumar et al. (2012) found that a single treatment of mice with scopolamine at 2.0 mg/kg did not result in noticeable changes in the indicator.

Rats treated with scopolamine at 1.0 mg/kg had a significant decrease in lag time compared to control group ($p < 0.05$) (Fig. 16 B). This showed that the toxic agent at the administered dose, after repeated treatment, caused memory impairment in this rodent species, while in mice it did not significantly affect memory capabilities, possibly as a result of their faster metabolism.

Many studies have identified the damaging effect of the non-selective M-cholinoblocker on memory in rats undergoing the Passive avoidance test (Step through) (Doyle and Regan, 1993; Foley et al., 2004; Popović et al., 2015). We believe that our results, which also correspond to data available in the literature, confirm the opinion on the time- and dose-dependent damaging effects of the toxic agent on attention and memory in experimental animals (Ebert et al., 1998).

5.1.2. Scopolamine effects on neuromuscular coordination *(Rota rod test)*

The experimental setup is the same as that used in the testing of healthy animals. The results are presented in Fig. 17.

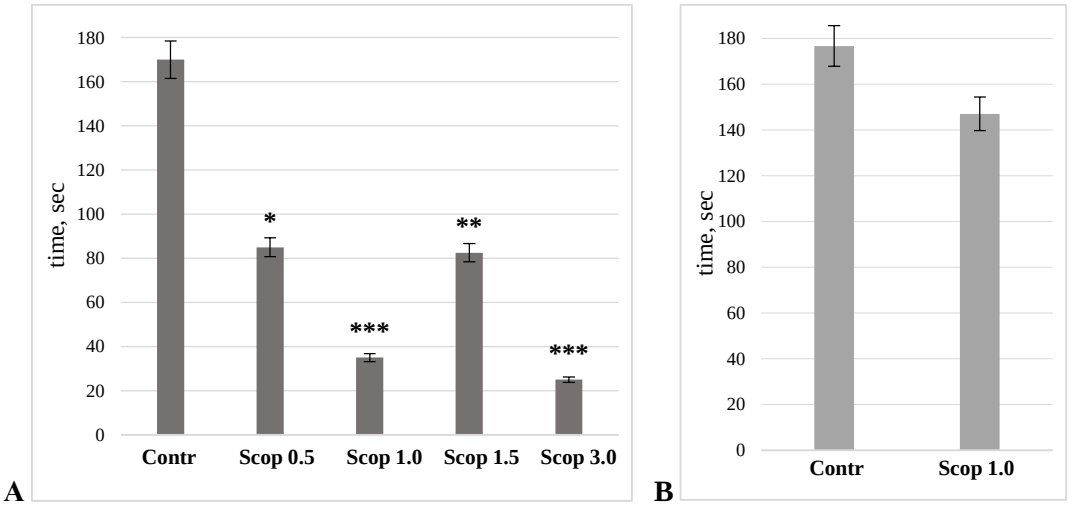


Fig. 17. Effects on neuromuscular coordination in mice treated with scopolamine at increasing doses (0.5, 1.0, 1.5 and 3.0 mg/kg) (A) and in rats treated with scopolamine at 1.0 mg/kg (B); *P < 0.05, **P < 0.01, ***P < 0.001 Vs. Controls

In mice, scopolamine at all administered doses produced a statistically significant reduction in the time it remained on the rotating axis versus controls (Fig. 17 A.). These results were consistent with Goverdhan et al. (2012) inhibition of motor activity in mice injected intraperitoneally with scopolamine at 1.4 mg/kg for 9 days.

In the rats in the scopolamine group, an unreliable decrease in retention time (16.8%) in the experimental setting compared to the control group indicated that the toxin agent did not significantly affect the neuromuscular coordination of animals during 11 days of treatment (Fig. 17 B). Significant impairment of motor coordination in rats, as demonstrated by a decrease in retention time with Rota rod test versus controls, established Asgharzade et al. (2015) using scopolamine at the same dose of 1.0 mg/kg i.p., but for a longer time - 20 days. According to studies of Capacio et al. (1992), scopolamine administered once at different doses did not affect motor coordination in rats. Analysis of literature data and the results obtained lead to the conclusion that longer administration of scopolamine is required to induce neuromuscular coordination damage in rats.

In the context of our experiment, the different effects of scopolamine administered at the same dose in mice and rats are probably due to species differences.

5.1.3. Effects of scopolamine on spatial orientation / exploratory behaviour *(Hole board* and *Open field test)*

The setup and methodology for conducting the Hole board test are the same as those used in the healthy animal experiment. The results are presented in Fig. 18.

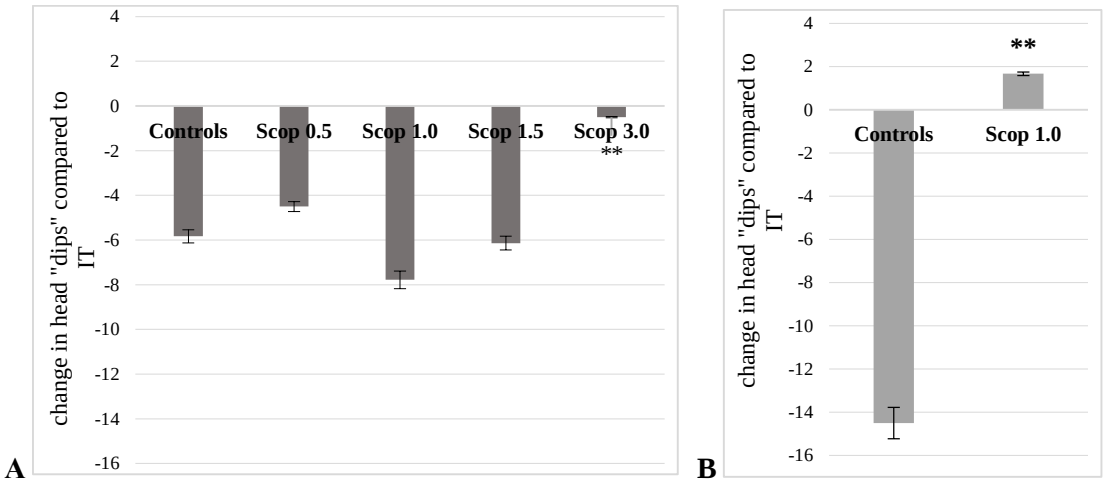


Fig. 18. Effects on spatial orientation and exploratory behaviour in mice treated with scopolamine at increasing doses (0.5, 1.0, 1.5 and 3.0 mg/kg) (A) and rats treated with scopolamine at 1.0 mg/kg (B) – considering the change in total head dips compared to IT; **P < 0.01 Vs. Controls

All groups of mice, with the exception of those receiving the highest dose of the toxic agent (3.0 mg/kg), had a significant decrease in the research activity compared to the initial training, which meant that the toxic agent at lower doses caused minor impairment of orientation (Fig. 18 A). At the high dose of scopolamine (3.0 mg/kg), the difference in spatial orientation from baseline was reduced, which was interpreted as lack of memory with respect to the environment/ memory of the situation and disorientation in animals. Our results confirm those of Shannon and Peters (1990), which found that the cholinoblocker administered at doses of 0.3 to 10.0 mg/kg in mice produced a dose-related increase in exploratory activity, with a maximum effect at 3.0 mg/kg.

In rats, following repeated administration of the choline-blocker, there was a statistically significant increase in the number of „dips“ compared to controls ($p < 0.01$) and no change from initial training. Such behaviour is considered to be a disorientation in animals. The behavioural responses we observed were similar to those obtained by other researchers. In a 1972 publication, Swonger and Rech reported the properties of scopolamine to inhibit rat habitation processes.

Comparison between the two rodent species showed that scopolamine induced changes in exploratory behaviour in rats following administration at 1.0 mg/kg, while similar effects in mice were observed at 3.0 mg/kg.

The Open-field test provides simultaneous detection of rodent motor and research activity. The experimental setup is the same as the new object recognition test. The effects of scopolamine on study activity in rats treated with a dose of 0.1 mg/kg for 8 days were investigated (Fig. 19).

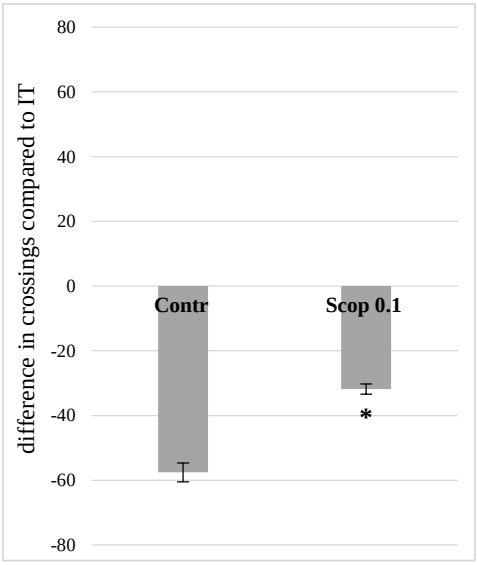


Fig. 19. Effects on exploratory activity in rats after 8 days of administration of scopolamine (0.1 mg/kg) – difference in activity compared to IT; *P < 0.05 Vs. Controls *(t-Test)*

In control rats, repeated testing on day 8 resulted in a decrease in motor activity (number of quadrant crossings), which was interpreted as normal habituation and preserved spatial orientation (Cerbone and Sadile, 1994). A statistically significant reduction in the number of crossings of the quadrants in the Scopolamine group versus the control group ($p < 0.05$) may be considered as a manifestation of the harmful action of the toxic agent.

5.1.4. Effects of scopolamine on basic brain biochemistry metrics in experimental rodents

- *Oxidative status in the brain of mice and rats following scopolamine administration*

The content of lipid peroxidation products and total glutathione have been evaluated (Fig.20).

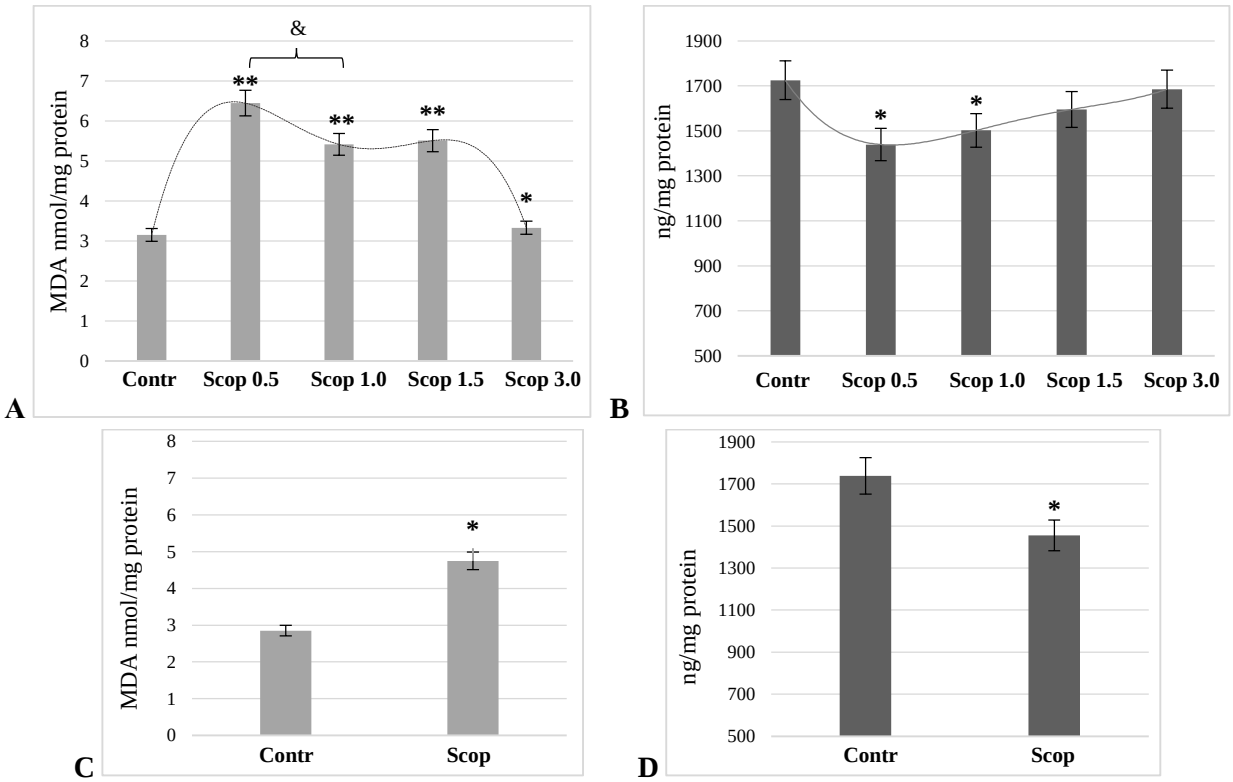


Fig. 20. Effects on oxidative status in rodents brain
***A and B* – mice treated with scopolamine in increasing doses (0.5, 1.0, 1.5 and 3.0 mg / kg) - determination of the levels of LPO products (A) and tGSH (B); *P < 0.05, **P < 0.01 Vs. Controls**
***C and D* – rats treated with scopolamine in dose combination (0.1 mg/kg for 8 days + 20 mg/kg on day 9) – determination of the levels of LPO products (C) and tGSH (D); *P < 0.05 Vs. Controls**

Scopolamine has been shown to cause oxidative stress in the brain in all animals, manifested by statistically significant increases in LPO product levels compared to controls ($p < 0.01$) (Fig. 20 A). The lowest dose (0.5 mg/kg) increased the indicator to the greatest extent ($p < 0.01$), significantly higher than 1.0 mg/kg ($p < 0.05$). At the highest dose (3.0 mg/kg), the changes were of lower confidence level ($p < 0.05$). The effects of the 1.5 mg/kg dose on the oxidation of lipids were similar to those reported by Goverdhan et al. (2012), which after treatment of experimental mice with scopolamine at 1.4 mg/kg i.p. showed significant increase in LPO levels compared to controls.

Figure 20 (B) shows that lower doses of scopolamine (0.5 and 1.0 mg/kg) produced a significant decrease in tGSH levels ($p < 0.05$), as opposed to higher (1.5 and 3.0 mg/kg). A 'dose-effect' relationship ($R^2 = 0.9878$, R-squared value on chart) was observed – as the dose of the toxic agent increased, the change in tGSH concentration compared to controls was reduced. Our statistically significant decrease in tGSH levels in rodents treated with scopolamine at 0.5 and 1.0 mg/kg compared to the control group ($p < 0.05$) confirmed scopolamin-induced oxidative stress following repeated administration in mice, which is related to increased levels of LPO products.

In conclusion, brain oxidative status studies conducted with repeated administration of scopolamine at various doses showed the most significant increase in oxidation of lipids at low doses of scopolamine and decreased with increasing doses. On the other hand, as expected, tGSH concentrations were most markedly decreased with low doses of scopolamine, which is associated with high levels of oxidation products.

Compared to the control group, in rats treated with scopolamine, levels of LPO in the brain associated with the presence of oxidative stress were statistically significantly increased by 66.67 % ($p < 0.05$) (Fig. 20 B). We believe that the results obtained, corresponding to those of other teams, confirm the harmful effect of the toxic substance on the ability to remember and learn. Similarly, data from the Rahimzadegan and Soodi study (2018) compare memory impairment and levels of oxidative stress in the brain of rats following single and repeated doses of scopolamine. According to the authors, compared to a single dose, multiple treatment disrupts memory function more intensively and for longer, with chronic oxidative stress being the likely damaging mechanism.

The applied dose variation of scopolamine caused a consistent decrease in tGSH concentrations, directly related to the levels of LPO products in the same animals. The parameter was statistically significantly reduced by 16.3 % compared to control rats ($p < 0.05$) (Fig. 20 D).

Experiments conducted confirmed the leading role of induced oxidative stress in the damaging action of the toxic agent in experimental rodents.

▪ *Brain acetylcholinesterase activity in mice after administration of scopolamine at increasing doses*

Acetylcholinesterase is an enzyme responsible for the metabolic degradation of the mediator ACh released in the cholinergic synapse, and in experimental practice is an indicator of the state of cholinergic neurotransmission. Results of the conducted study of brain AChE activity in mice are presented in Figure 21.

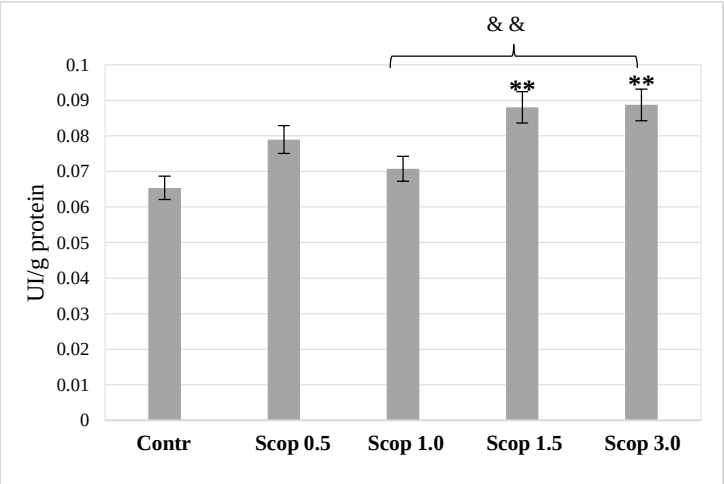


Fig. 21. Effects on brain AChE activity in mice after 11 days of treatment with scopolamine at increasing doses (0.5, 1.0, 1.5 and 3.0 mg/kg); **P < 0.01 Vs. Controls

The statistically significantly higher activity of AChE in the brain of mice after 11 days of treatment with higher doses of the toxic agent (1.5 and 3.0 mg/kg) compared to controls by 35.38 % ($p < 0.01$) and 36.09 % ($p < 0.01$), respectively, was fully consistent with the significant increase in enzyme activity reported by Goverdhan et al. (2012) following repeated (9-day) administration of scopolamine at a dose of 1.4 mg/kg i.p. in mice.

The results obtained and the data from the literature review support the conclusion that a high dose of scopolamine causes an increase in AChE brain activity accompanied by impaired memory and learning abilities of animals.

- *Effects of scopolamine on brain acetylcholine levels in rats*

The change in brain ACh concentrations in rats is presented on Figure 22.

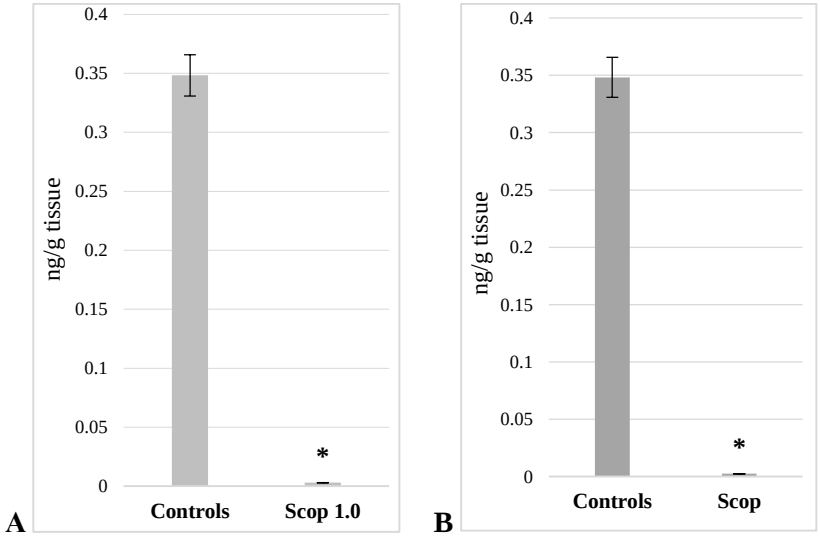


Fig. 22. Effects on brain ACh levels in rats after 11 days of treatment with scopolamine at 1.0 mg/kg (A) and after treatment with the dose combination (B); *P < 0.05 Vs. Controls

Scopolamine administered at 1.0 mg/kg for 11 days produced a significant decrease in ACh levels (p < 0.05) (Fig. 80 A), which is directly related to the activation of the AChE enzyme in the brain of healthy mice responsible for its degradation. Stimuli causing release of ACh are assumed to initiate overexpression of AChE on a feedback basis to prevent excessive activation of neurotransmission (Kaufer et al., 1998). The results obtained are similar to those of Haider et al. (2016), which administered intraperitoneal scopolamine at 1.0 mg/kg for 14 days in rats and revealed a decrease in ACh concentration similar to the changes in mediator levels

we observed. Also, according to studies of Bhuvanendran et al. (2018), the 9-day intraperitoneal injection of scopolamine at 1.0 mg/kg in rats caused a decrease in acetylcholine levels.

The M-cholinoblocker in the dose combination, subtoxic (0.1 mg/kg) and final single dose (20 mg/kg), produced a significant change in the levels of the brain mediator studied, similar to the 1.0 mg/kg dose administered over 11 days (Fig. 22 B).

Similar changes observed in both protocols suggest that the effects of repeated administration of scopolamine on ACh mediator levels are not directly dependent on the dose administered.

1.1.2.4. Histological studies of brain tissue in experimental rats with scopolamine-induced alterations

The studies were conducted according to the described methodology (Gr. III, pt. 10) with the brains of rats used in the study of the effects of scopolamine administered in the dose combination.

In the control rats in both areas of interest (cortex and hippocampus), tissue had structure, cell composition and morphology characteristic of healthy animals.

In the cerebral cortex of animals treated with scopolamine, signs of increased neuronal degeneration (Fig. 23) are evident. In the cortex, gliosis and numerous neurons are observed with fine vacuolation of cytoplasm and marked loss of Nislovae granulation (chromatolysis). These changes are mainly located in the third and fifth layers of the cortex, but also affect the others. Neural shadows, as well as areas of aggregation of microglia, indirect signs of neuronal death, as well as neurons with picnosis or perineuronal microgliosis (close apposition between the body of a neuron and several small nuclei of neuroglia) are also present.

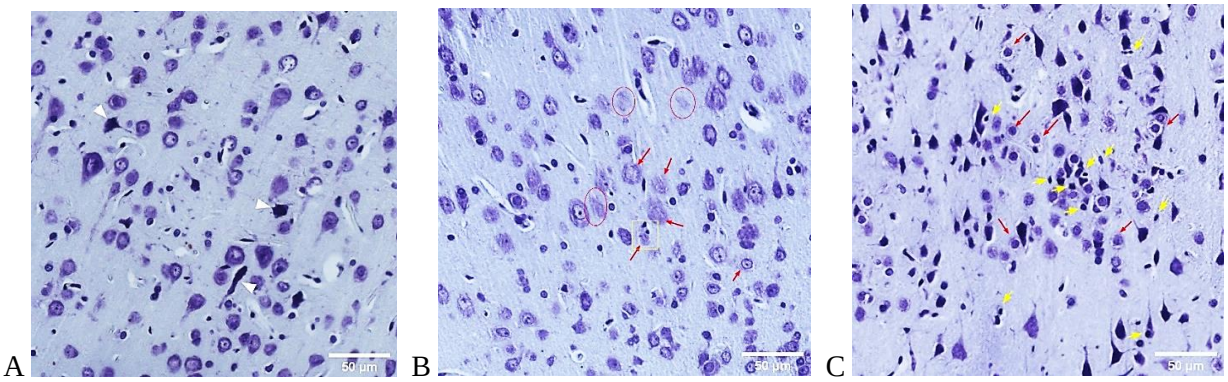


Fig. 23. Cerebral cortical abnormalities in rats treated with scopolamine in the dose combination

A – Controls. Normal morphology of neurons in layer V of motor cortex (*white arrows* – artificial changes occurring *post mortem*, often mistaken for pathological)**;**
B – Scopolamine group. Chromatolysis (*red arrows***), "shadows" of neurons (***red ellipses***) and gliosis in layer V of the retrosplenic cortex* (***yellow rectangle-perineuronal microglycosis***);**

C – Scopolamine group. Vacuolization and chromatolysis (*red arrows***) and solid gliosis (***yellow arrows – glial cells***) in layer III-IV of the sensory cortex (S1)**

Scale – 50 µm

***** *This part of the cortex is probably involved in visual spatial association and integration and may be related to episodic memory in rats.*

In the rats of the scopolamine group, changes in hippocampal formation were significantly less pronounced than in the cortex, where only single neurons with chromatolysis and nucleus picnosis were observed.

After testing, the density (number) of all cortical profiles absorbing more than 20 % of light was demonstrated in scopolamin-treated rats compared to healthy controls ($p = 0.0182$) (Fig. 24 A).

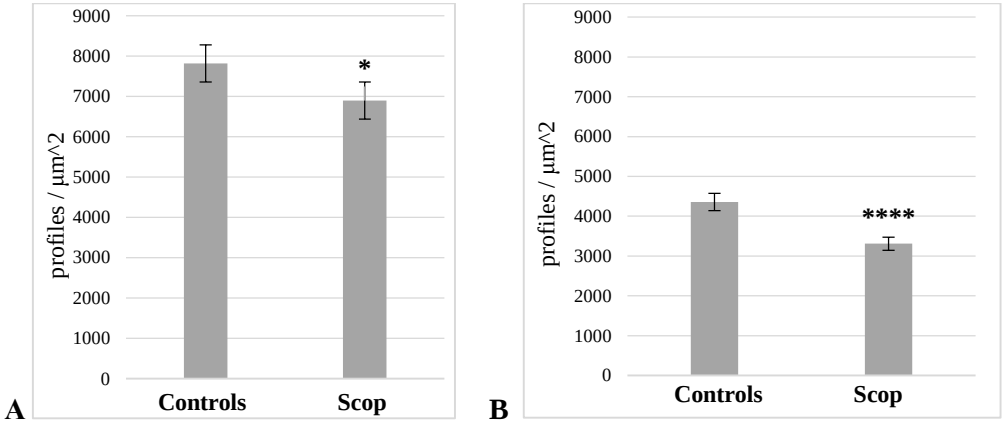


Fig. 24. Effects of scopolamine on the total number of profiles (density) (A) and on the number of profiles (density) with an area greater than 31 µm^2 (B) in the cortex of scopolamine treated rats (dose madification); *P < 0.05, ****P < 0.0001 Vs. Controls

Examination of profiles with an area of less than 31 µm^2 belonging to fragments and/or neuroglia revealed no significant differences compared to controls. On the other hand, larger area profiles, mainly corresponding to neurons, showed a significant decrease in mean density as a result of scopolamine administration ($p = 3.713 * 10^{-5}; R^2 = \eta^2 = 0.30$) (Fig. 24 B).

In a study of the total number of degenerating neurons with picnotic and condensed nuclear morphology, Palle and Neerati (2017) observed damaging effects of scopolamine in the brain of rats after a single i.p. dose of 20 mg/kg.

Scopolamine, as a muscarinic antagonist of the tropane alkaloid group, suppresses a number of CNS functions, including memory, learning ability and others, and is often used in experimental studies to induce model cognitive damage (Klinkenberg and Blokland, 2010). We believe that our experiments complement the many studies that validate this chemically-induced dementia in animals as a reliable experimental model causing traceable behavioural and biochemical changes.

Based on the results obtained in the induction of modelling damage, both in the memory capabilities of animals and in biochemical parameters, our further studies were performed with scopolamine at doses of 1.0 and 1.5 mg/kg, and the variability of repeated administration of a subcotoxic dose (0.1 mg/kg) in combination with subsequent single treatment with a high dose of the toxic substance (20 mg/kg).

5.1. Effects of Myrtenal on rodents with experimental Alzheimer's type of dementia

A number of studies provide evidence to support the hypothesis of the role of cholinergic mediation in learning and memory processes (Contestabile, 2011). In addition, according to many clinical and preclinical studies, the main factor for neurological disorders and related neurodegenerative diseases, including AD is the increased oxidative stress confirmed by the conclusions that protein side chains have been modified either directly by reactive oxygen types (ROS) or reactive nitrogen types (RNS), or indirectly by LPO products (Jomova et al., 2010).

Based on this information, an experimental study was conducted to investigate the preventive effects of the monoterpenoid Myrtenal in a scopolamine-induced model of Alzheimer's dementia in laboratory rodents. Memory and learning abilities, neuromuscular coordination and spatial orientation were investigated in mice and rats through behavioural tests. The levels of oxidative stress and their response to Myrtenal as well as the neuromodulatory capabilities of the test substance have been determined by measuring the levels of the brain neurotransmitter ACh in experimental animals. Histopathological analysis of rat brain tissue sections was performed.

Two experiments were conducted with mice and two with rats.

Mice

Experiment I – Monitoring of changes in body weights, state of memory and learning capabilities, coordination and research behaviour of animals after 11 days of treatment with the following substances:

- Scopolamine (Scop) – 1.5 mg/kg
- Scopolamine (1.5 mg/kg) and Myrtenal (M) at doses of 10, 20 and 30 mg/kg
- Scopolamine (1.5 mg/kg) and Lipoic acid (reference with antioxidant activity) (LA) at a dose of 30 mg/kg (alone or in combination with Myrtenal)

Experiment II – проследяване на измененията в телесните маси, състоянието на паметовите и обучителните способности, оксидативния статус в мозъка на мишките с проследяване нивата на продуктите на ЛПО, tGSH и активността на ензимите CAT, SOD и GPx, след 14-дневно третиране със: Monitoring of body mass changes, memory and learning abilities, oxidative status in the brain of mice with evaluation of LPO, tGSH and CAT, SOD and GPx enzyme activity after 14 days of treatment with:

- Scopolamine – 1.0 mg/kg
- Scopolamine (1.0 mg/kg) and Myrtenal at doses of 30, 40 and 50 mg/kg

- Scopolamine (1.0 mg/kg) and Lipoic acid at a dose of 30 mg/kg (alone or in combination with Myrtenal)

Rats

Experiment I – Monitoring state of memory and learning capabilities and research behaviour of animals after 11 days of treatment with the following substances:

- Scopolamine – 1.0 mg/kg
- Scopolamine (1.0 mg/kg) and Myrtenal at a dose of 40 mg/kg
- Scopolamine (1.0 mg/kg) and Galantamin (reference with anticholinesterase activity) (Gal) at a dose of 1.0 mg/kg
- Scopolamine (1.0 mg/kg) and Lipoic acid at a dose of 30 mg/kg (alone or in combination with Myrtenal)

Experiment II – Monitoring of body mass changes, memory and learning abilities, motor activity, brain oxidation status in rats with evaluation of LPO, tGSH levels and CAT, SOD and GPx enzyme activity; testing of Myrtenal neuromodulatory effects by determining brain ACh concentration; histopathological studies in brain sections in dementia rats after 9 days of treatment with:

- Scopolamine – dose variation 0.1 mg/kg on 8 consecutive days and a single dose of 20 mg/kg on the last day
- Scopolamine (dose variation) and Myrtenal at a dose of 40 mg/kg
- Scopolamine (dose variation) and Galantamin at a dose of 1.0 mg/kg (alone or in combination with Myrtenal)
- Scopolamine (dose variation) and Lipoic acid at a dose of 30 mg/kg (alone or in combination with Myrtenal)

5.2.1. Study on the effects of Myrtenal on body weight in rodents with scopolamine-induced dementia

In mice with scopolamine-induced impairment, when administered at 1.5 mg/kg for 11 days, changes in body mass of all groups of animals were similar to those of the scopolamine alone group. Monoterpenoid at the selected doses (10, 20 and 30 mg/kg) did not improve the condition in rodents with dementia as measured by weight gain. The results were in contrast to healthy mice treated with Myrtenal at the same dose and duration, which showed significant increase in body weight relative to both baseline ($p < 0.01$) and controls ($p < 0.05$).

Results from the second – longer 14-day study, in mice treated with scopolamine at 1.0 mg/kg showed that Myrtenal at higher doses (40 and 50 mg/kg) exhibited protective effects against the M-choline receptor blocker, with a significantly greater increase in body mass compared to the scopolamine group with confidence levels of $p < 0.05$ and $p < 0.01$, respectively.

These results suggest that only high doses of Myrtenal (40 and 50 mg/kg) antagonise the anorexigenic properties of scopolamine in dement mice, while in healthy animals the lower dose (20 mg/kg) is effective enough to cause a significant increase in the indicator over controls.

It is important to underline that rats treated only with the 9-day dose combination of scopolamine proposed by us did not experience any change in body mass compared to controls. On the other hand, administration of Myrtenal at 40 mg/kg resulted in a credible decrease in weights, unlike in dementia mice, where the same dose of Myrtenal resulted in an increase in body weights of 58.2 % compared to the scopolamine group ($p < 0.05$). For unknown to us reasons, concomitant administration of the reference Galantamine with Myrtenal produced an increased anorexigenic effect with a decrease in body weight, the difference being statistically plausible both compared to the scopolamine group ($p < 0.01$) and to the control group, which showed very high significance ($p < 0.001$).

Reductions in body weights were also characteristic of healthy rats treated with Myrtenal at the same dose and duration (40 mg/kg for 9 days), while a lower dose of 30 mg/kg of the test substance administered for a shorter period of 5 days produced no anorexigenic effects.

We believe that the adverse effects of Myrtenal in rats observed at higher doses may be due to their slower metabolism than in mice.

5.2.2. Effects of Myrtenal on memory and learning abilities in rodents with scopolamine-induced dementia

The experimental protocol is the same as the one used in healthy rodents. Laboratory animals shall undergo pre-treatment training. The test was performed 24 hours after the last injection of the test substances. The lag time associated with the learning abilities of the experimental animals has been evaluated.

5.2.2.1. Effects of Myrtenal on memory and learning capacity in mice with scopolamine-induced dementia (*Step through test*)

Results of the tests conducted after 11 and 14 days of treatment with Scopolamine at doses 1.5 mg/kg and 1.0 mg/kg, with Myrtenal at various doses and with the reference lipoic acid at the selected dose are presented in Figure 25.

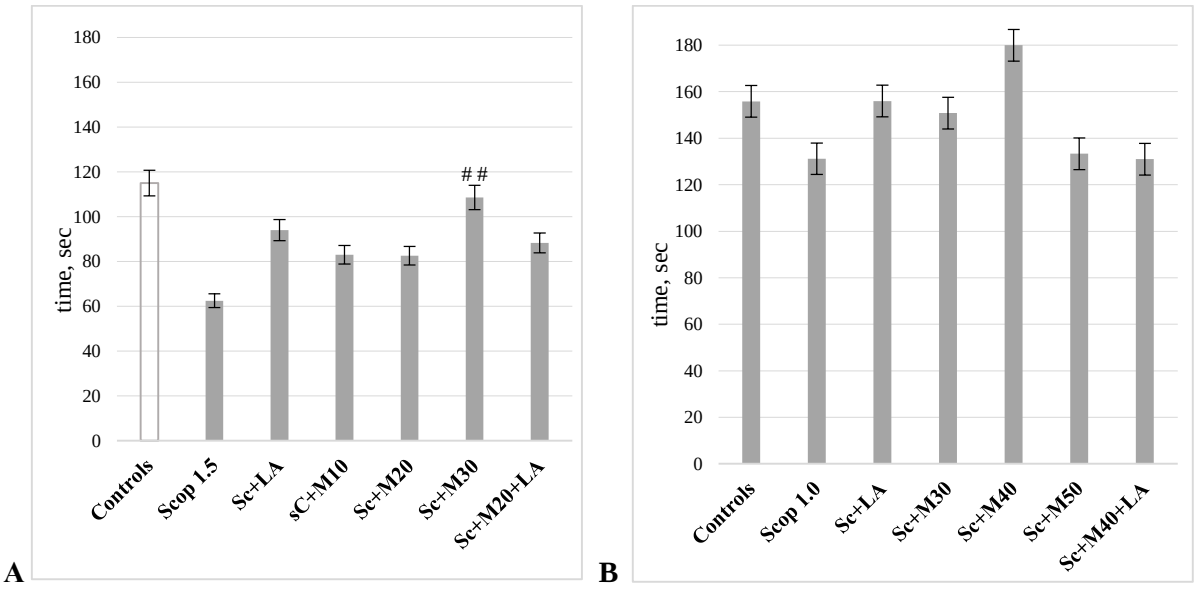


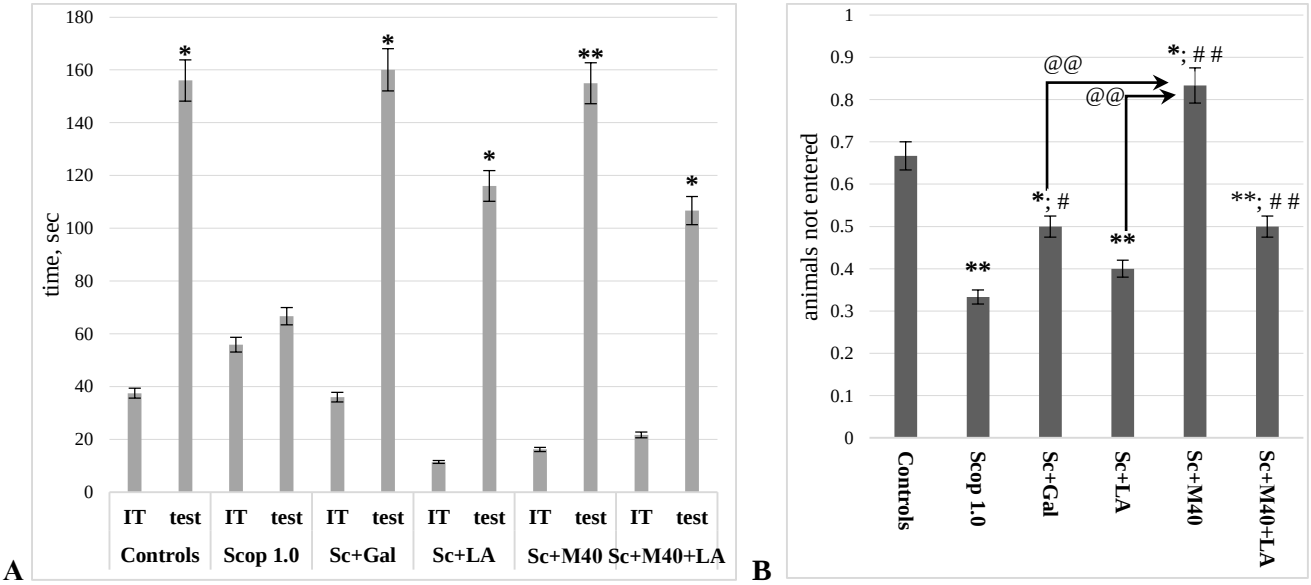
Fig. 25. Effects on memory in mice with scopolamine-induced dementia after 11 days of treatment with scopolamine (1.5 mg/kg) and Myrtenal at doses of 10, 20 and 30 mg/kg (A) and after 14 days of treatment with scopolamine (1.0 mg/kg) and Myrtenal at doses of 30, 40 and 50 mg/kg (B) and comparing them to those of the reference LA administration alone or in combination with Myrtenal; $^{\#\#}P < 0.01$ Vs. Scop. group

In the 11-day treatment with test substances in mice with dementia induced by 1.5 mg/kg scopolamine, Myrtenal administered at 30 mg/kg resulted in the highest and statistically reliable increase in latency by 73.76% compared to the group of animals treated with Scopolamine alone ($p < 0.01$) (Fig. 25 A). Compared to this group, the increase in latency time in mice using the reference LA was 50.4% and in the groups treated with Myrtenal at 10 and 20 mg/kg was 32.8 and 32.16% respectively. When Myrtenal (20 mg/kg) was co-administered with LA, the latency time was increased by 41.33%, with no statistical certainty indicating no additive effect.

Results of the 14-day experiment in mice treated with the M-choline receptor blocker at 1.0 mg/kg indicate that longer duration of administration causes disorientation in animals (Fig. 25 B). An inadequate decrease in latency time compared to active controls is a manifestation of the damaging effect of the toxic agent. The effects on memory capabilities observed were similar to those demonstrated in a study of the influence of scopolamine on neuromuscular co-ordination, where the dose of 1.0 mg/kg produced a credible reduction time spent on the rotating lever ($p < 0.01$). Best results were observed in animals treated with the median dose of Myrtenal (40 mg/kg) and all mice in the group demonstrated preserved memory and learning abilities with normal activity. In these animals, the lag time was increased compared to the dementia group by 37.2 % (with a borderline statistical plausibility). Co-administration of LA and Myrtenal at 40 mg/kg did not improve memory capacity in mice with scopolamine-induced dementia.

5.2.2.2. Effects of Myrtenal on memory and learning capacity in rats with scopolamine-induced dementia (*Step through test*)

The results of the test for memory and learning status conducted after 11 days of treatment with scopolamine at 1.0 mg/kg, Myrtenal at 40 mg/kg and the reference LA and Gal at the selected doses are presented in Figure 26.



***P < 0.05, **P < 0.01 Vs. IT**

Fig. 26. Effects on learning (A) and memory (B) capabilities in rats with scopolamine-induced dementia (1.0 mg/kg) after 11 days of treatment with Myrtenal (40 mg/kg) and comparing them to those of the references LA and Gal (*P < 0.05, **P < 0.01 Vs. Controls, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 Vs. Scop. group; $^{@@}$P < 0.01 Vs. Sc+M group)

Compared to initial training, except for dementia rats (treated with scopolamine alone), there was a statistically significant increase in lag time in all other groups, which was a manifestation of retained memory / learning abilities (Fig. 26A). The increase in indicator was greatest in the groups treated with the reference LA ($p < 0.05$) and Myrtenal ($p < 0.01$), with values close to those of controls. The combination of the two substances in dementia rats did not result in an improvement in memory capacity compared to the effects observed with their administration alone. These differences point to a similar antioxidant mechanism of response, which can be considered essential for the protective effects of Myrtenal.

Като допълнителен критерий за оценка на паметовите способности при изследваните плъхове, е определен броя на невлезлите в тъмната част на постановката плъхове. И тук най-добри са резултатите при групата животни, третирани с миртенал, което говори за запазена памет (Фиг. 26 Б). Показателят е статистически достоверно повишен в сравнение със скополаминовата група ($p < 0.01$) и в сравнение с групите,

третирани с двата референта ($p < 0.01$), а така също и спрямо контролната – с 24.9 % ($p < 0.05$).

The number of rats not entering the dark part of the setup was determined as an additional criterion for assessing memory capabilities in the test rats. The best results were in the Myrtenal group of animals, indicating memory protection (Fig. 26 B). The indicator was significantly increased compared to the scopolamine group ($p < 0.01$) and compared to the groups treated with the two referents ($p < 0.01$) as well as the control group by 24.9 % ($p < 0.05$).

In dementia mice from the 11-day study treated with Myrtenal at the highest dose of 30 mg/kg, there was a significant increase in latency time compared to the group of rodents treated with scopolamine alone (1.5 mg/kg) to similar control values ($p < 0.01$). Similar effects were seen with longer 14-day treatment with the toxic agent at the lower dose of 1.0 mg/kg, where Myrtenal administered at 40 mg/kg increased latency time compared to the scopolamine group of mice. In rats, the same dose of Myrtenal (40 mg/kg) produced similar results in terms of memory capacity of the animals, with a confidence level $p < 0.01$ compared to the references (lipoic acid and galantamine).

In conclusion, upon repeated administration, Myrtenal exerts protective effects on the memory capabilities of rodents with scopolamine-induced dementia in *Step through test*. Dementia-enhancing properties in rodents identified Lee GY et al. (2017) by examining the protective effects of α-pinene, of which Myrtenal is a metabolite, on memory capabilities in mice after a single 1.0 mg/kg dose of scopolamine. According to the authors, higher doses of α-pinene produce a credible increase in lag time with the *Passive avoidance test* ($p < 0.01$).

5.2.2.3. Effects of Myrtenal on memory capacity in rats with scopolamine-induced dementia *(Novel object recognition test)*

The novel object recognition test was conducted after 9 days of treatment with the dose combination of scopolamine, concomitantly with Myrtenal 40 mg/kg and the references LA and Gal. The effects of Myrtenal on the state of recognition memory were assessed by calculating a discrimination index (DI). The results of the experimental rat testing are presented in Figure 27.

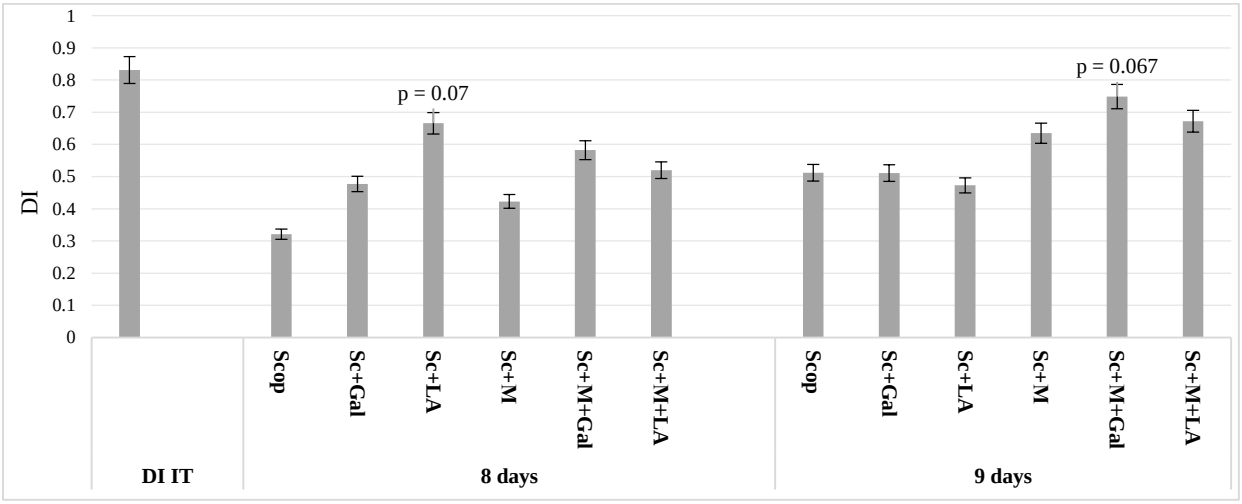


Fig. 27. Effects on recognition memory in rats with scopolamine-induced dementia (dose modification) after 9 days of treatment with Myrtenal (40 mg/kg) and comparing them to those of the references LA and Gal

Dementia (scopolamine) controls lack pronounced DI dynamics at Day 9 versus Day 8, suggesting disorientation and impaired recognition memory as a result of a single injection of the high dose of scopolamine. Our results are similar to those of El-Marasy et al. (2012), according to which single administration of 16 mg/kg scopolamine to male rats significantly inhibited non-spatial functional memory in the object recognition test.

Some increase in DI values compared to the scopolamine group was observed in rats treated with Galantamine at day 8. However, at day 9, the reference did not exert an effect sufficient to antagonise the impairment of recognition memory caused by the high dose of scopolamine. Administration of the other reference, the antioxidant LA, also increases the DI (borderline significance) relative to the scopolamine group. Here, too, the best results were on day 8, but at the high dose of the toxic agent on day 9, the reference was not effective.

Myrtenal on day 8 did not affect scopolamine-impaired recognition memory, but at day 9 there was an increase in DI values of 12 points compared to dementia rats. The most pronounced (borderline plausible) increase in DI on the last day was recorded when Myrtenal was combined with Galantamine. This, on the one hand, demonstrates their ability to antagonise the damaging effects of high dose scopolamine and, on the other hand, suggests that the two substances possess a different mechanism to affect recognition memory.

Results obtained at the low (subtoxic) dose of the toxic agent indicate that the resulting alterations in recognition memory are recovered from the antioxidant reference (LA). This means that the damaging effects of repeated administration of the subcotoxic dose of scopolamine on the state and extent of recognizable memory use in rats is due to induced oxidative stress. It was found that both the reference (Gal and LA) and Myrtenal alone did not have the potential to correct the changes resulting from a single high dose of the M-

cholinoblocker. In this case, only the combination of the monoterpenoid with the galantamine reference with anticholinesterase activity is effective.

Our experiments confirm the hypothesis of the multifactorial etiology and pathogenesis of neurodegenerative damage, thereby underlining the importance of multi-targeted therapy as the most appropriate approach in seeking and finding a tool with a complex mechanism of action to influence the pathological process.

5.2.3. Effects of Myrtenal on neuromuscular coordination in experimental rodents with scopolamine-induced dementia

Rodents with experimentally induced CNS disorders and those receiving pharmacological or other agents could be tested with the Rota rod test for neuromuscular coordination and motor deficiencies. The test setup is the same as that used in healthy animals treated with Myrtenal.

Changes in retention time on the rotating arm of the apparatus in mice with scopolamine-induced dementia (1.5 mg/kg) following multiple 11 days of treatment with Myrtenal at different doses and with the reference (LA and Gal) are presented in Figure 28.

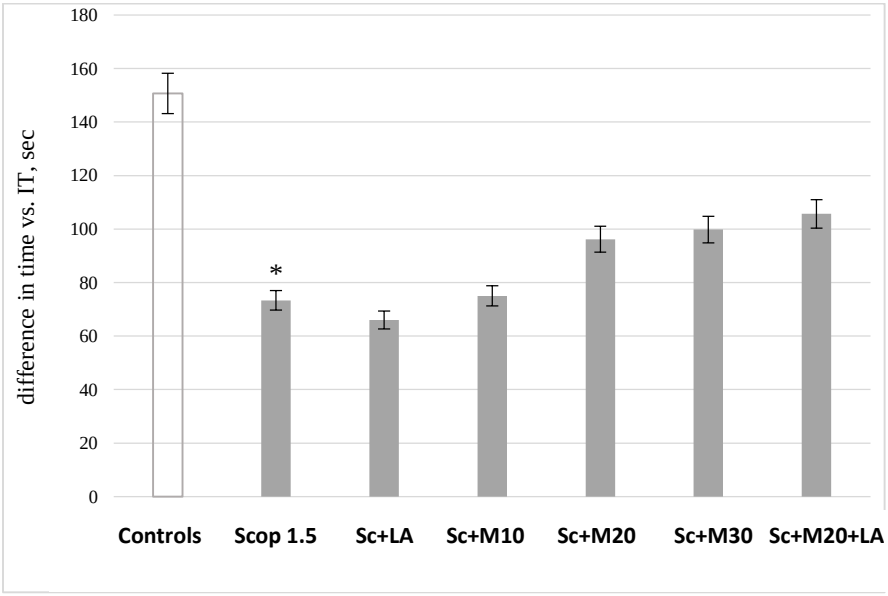


Fig. 28. Effects on neuromuscular coordination in mice with scopolamine-induced dementia (1.5 mg/kg) after 11 days of treatment with Myrtenal at increasing doses (10, 20 and 30 mg/kg) and comparison with those of the reference LA alone or in combination with Myrtenal; *P < 0.05 Vs. Controls

The time differences on the rotating axis in mice are indicative of the dynamics of change in each group. Compared to baseline, retention times in all groups, except those treated with LA alone and Myrtenal at the 10 mg/kg dose, were unSignificantly increased compared to scopolamine by 31.2 and 36.1% in the Myrtenal 20 and 30 mg/kg groups, respectively, and 44.1

% in the animals given Myrtenal in combination with LA. These results showed that both the LA antioxidant and Myrtenal (at all doses) were not effective enough to counteract the damaging action of scopolamine in dement mice.

The test was also performed in rats following multiple 11-day treatment with scopolamine (1.0 mg/kg), Myrtenal 40 mg/kg and the two referents lipoic acid and galantamine. Only the anticholinesterase agent improves coordination and durability in dement rats. And here the Myrtenal has no influence on the motor skills of experimental animals.

In conclusion, in our experiments with *Rota rod test* Myrtenal did not cause any improvement in neuromuscular coordination and durability in dement mice and rats.

5.2.4. Effects of Myrtenal on exploratory activity / orientation in experimental rodents with scopolamine-induced dementia

- *Hole board test*

The test was carried out before the start of the experiment (initial training) and 24 hours after the last treatment of the animals. The number of head dips in the holes located on the field floor was recorded over a period of 3 minutes. Exploratoty activity in rodents following multiple 11 days of monoterpenoid administration in mice with scopolamine-induced dementia (1.5 mg/kg) treated with Myrtenal at increasing doses with the reference LA and in rats with scopolamine induced dementia (1.0 mg/kg) treated with Myrtenal (40 mg/kg) and the TWO referenceS lipoic acid and galantamine was observed. The change in the number of head dips of the test animals compared to IT is presented in Figure 29.

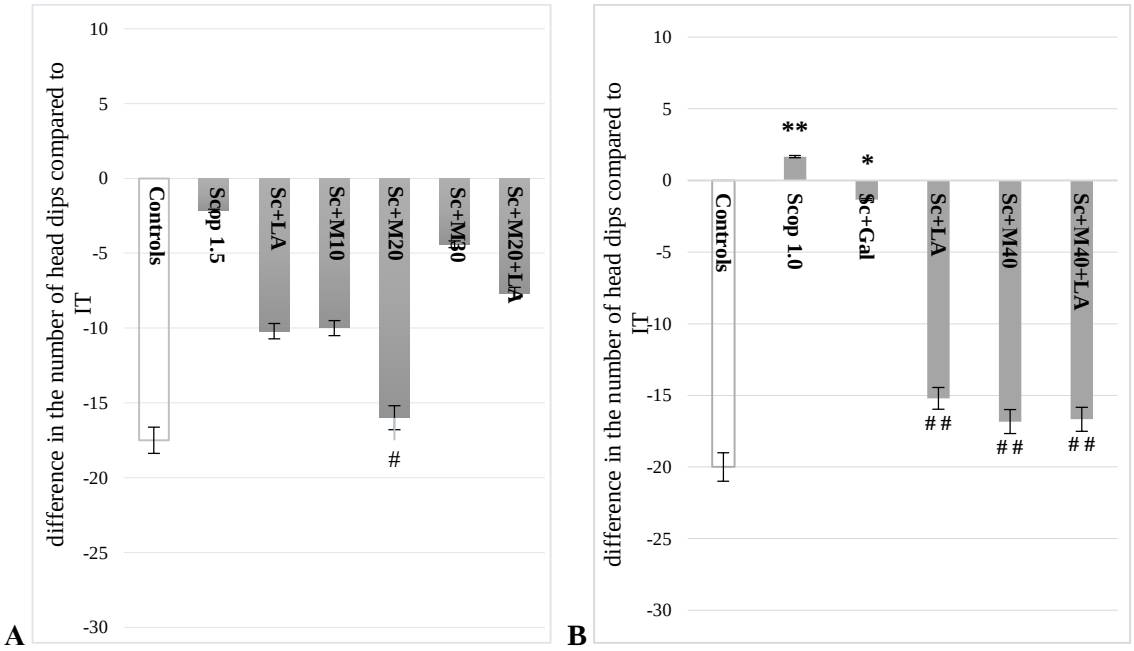


Fig. 29. Effects on exploratory activity in rodents with scopolamine-induced dementia after repeated administration of Myrtenal

***A – Mice* – effects after 11 days of treatment with Myrtenal at increasing doses (10, 20 and 30 mg/kg) and comparing them to those of the reference LA ($^{\#}P < 0.05$ Vs. Scop. group)**

***B – Rats* – effects after 11 days of treatment with Myrtenal (40 mg/kg) and comparing them to those of the references LA and Gal (*P < 0.05, **P < 0.01 Vs. Controls; $^{\#\#}P < 0.01$ Vs. Scop. group)**

Scopolamine impaired the habitation of experimental mice (Fig. 29 A). The best results were achieved in the Myrtenal group (20 mg/kg) with a statistical confidence level $p < 0.05$ compared to dement animals and the change (decrease) in the activity parameter was similar to controls. Based on the studies performed, it can be concluded that Myrtenal at a dose of 20 mg/kg restores the scopolamine-impaired habitation of the test mice as expressed by a decrease in their research activity.

Control rats reduced their exploratory activity similar to mice (Fig. 29 B). Administration of scopolamine resulted in statistically significant increases in the number of head dips compared to controls, indicating impaired habitation ($p < 0.01$). The anticholinesterase reference galantamine did not sufficiently influence the activity of dement animals. On the other hand, the second reference, the antioxidant LA, as well as Myrtenal restored the indicator to levels similar to those of the controls – the change compared to the scopolamine group has a confidence level $p < 0.01$.

In conclusion, it can be summarised that Myrtenal in mice and rats with induced dementia, after repeated administration, reliably improves the exploratory behaviour at *Hole board test*. The observed better effects of LA on behaviour in experimental rodents compared to those of galantamine suggest that oxidative stress caused by the toxic agent is the main damaging mechanism of the exploratory activity, respectively orientation.

- *Open-field test*

The Open field method was used to estimate the baseline exploratory activity in rats with experimental dementia induced by scopolamine at a subcotoxic dose (0.1 mg/kg) and its change in response to the effect induced by the test substances. The experimental setup is the same as the Novel object recognition test. The behavioural modification test was performed at 24 hours after the end of treatment.

Results following repeated 8-day administration of Myrtenal at a dose of 40 mg/kg and the references lipoic acid and galantamine are presented in Figure 30.

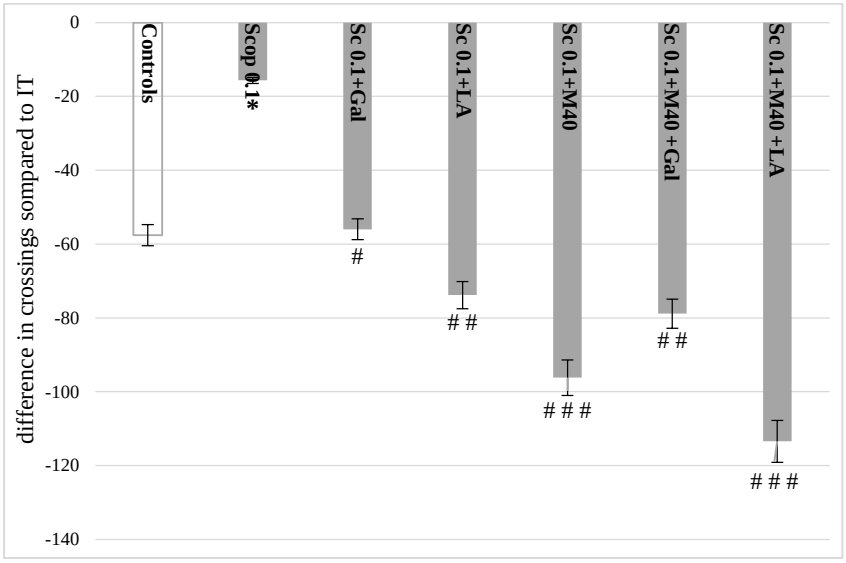


Fig. 30. Effects on exploratory activity in rats with scopolamine-induced dementia (0.1 mg/kg) after 8 days of treatment with Myrtenal (40 mg/kg) and comparing them to those of the references LA and Gal (*P < 0.05 Vs. Controls, $^{\#}$P < 0.05, $^{\#\#}$ P < 0.01, $^{\#\#\#}$P < 0.001 Vs. Scop. group) *(t-Test)*

In the control group, expected normal behaviour was observed as measured by a decrease in exploratory activity. Rats in the scopolamine group demonstrated anxiety and confusion, indicating impaired habitation, with changes in behaviour being significantly reduced compared to controls ($p < 0.05$). Both Myrtenal and the references antagonised but to varying degrees the damaging action of the M-cholinoblocker. The highest level of confidence compared to the scopolamine group was the difference in rodents treated with Myrtenal as well as the combination of the monoterpenoid with LA ($p < 0.001$), with those in the second group showing the highest but statistically unreliable decrease in exploratory activity (by 97%) compared to controls. The results suggest some potentiation of the effects of the administered substances on the investigational behaviour in dement rodents.

In both *Hole board* and *Open field tests* to investigate the state of exploratory activity in rats with scopolamine-induced dementia, the toxic agent impaired their habitation regardless of the dose used (1.0 mg/kg or 0.1 mg/kg). Myrtenal administered at 40 mg/kg antagonised the negative effects of scopolamine on rats behaviour in both experimental settings. In mice, the median dose of Myrtenal (20 mg/kg) over the 11-day treatment restored scopolamine-impaired animal habitation in the *Hole board test*, with the test parameter close to the control values and the change being significantly relative to the scopolamine group ($p < 0.05$).

In conclusion, Myrtenal restores the scopolamine-damaged habitation of experimental animals.

5.2.5. Effects of Myrtenal on brain oxidative status in rodents with scopolamine-induced dementia

Scopolamine is known to generate ROS and induce oxidative changes. This represents an opportunity to investigate the antioxidant capacity of various substances in a model of scopolamin-induced impairment.

5.2.5.1. Effects of Myrtenal on brain oxidative status in *mice* with scopolamine-induced dementia

The effects of Myrtenal on oxidative status in mice with scopolamine-induced dementia (1.0 mg/kg) were studied after 14 days of treatment with the test substance at increasing doses and with the reference LA. The assessment was performed by determining the levels of LPO and tGSH products in the brain of the test mice (Fig. 31) and by determining the activity of the antioxidant enzymes SOD, CAT and GPx (Fig. 32).

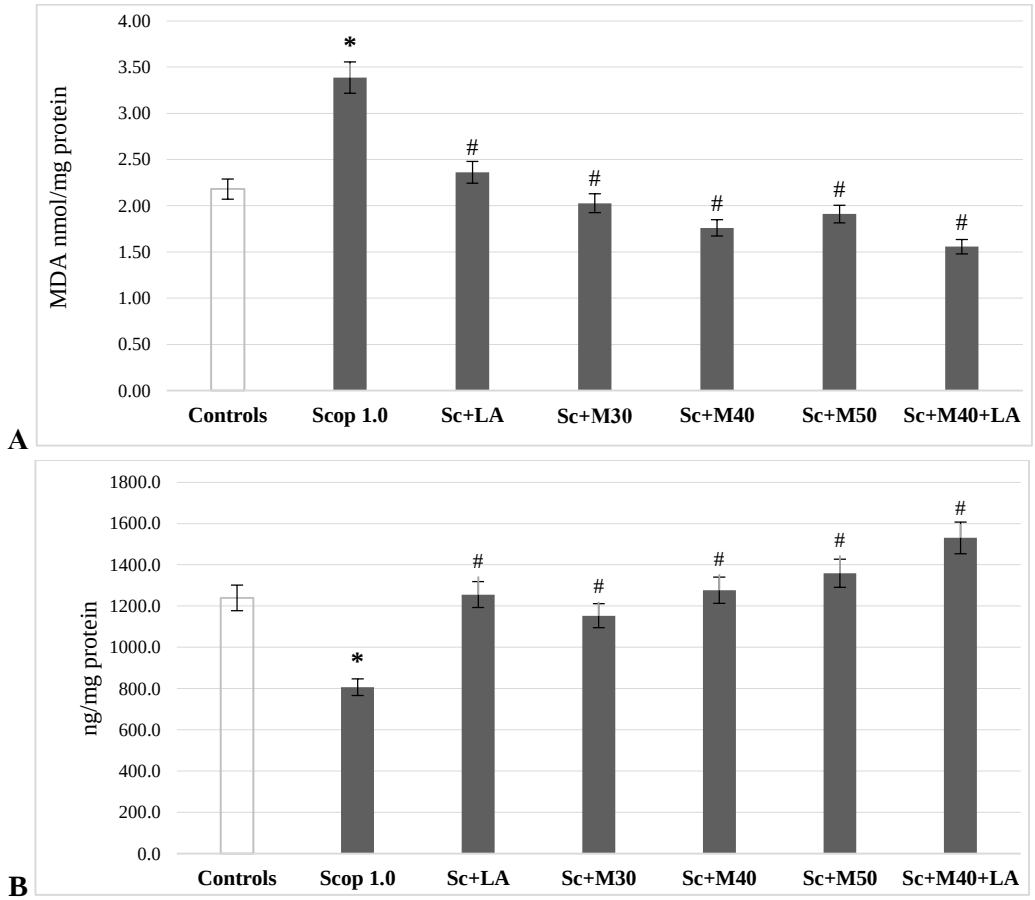


Fig. 31. Effects on brain oxidative status in mice with scopolamine-induced dementia (1.0 mg/kg) after 14 days of treatment with Myrtenal at increasing doses (30, 40 and 50 mg/kg) and comparing them to those of the reference LA – Determination of LPO product (A) and tGSH levels (B) (*P < 0.05 Vs. Controls; #P < 0.05 Vs. Scop. group)

In mice treated with scopolamine alone at the administered dose, there was an increase in LPO product levels of 55.5 % and a decrease in tGSH concentration of 35 % relative to

controls, respectively, with confidence levels p < 0.05. Compared to the reference LA, Myrtenal administered at 40 mg/kg reduced LPO product levels more (with 25.5%) (Fig. 31 A).

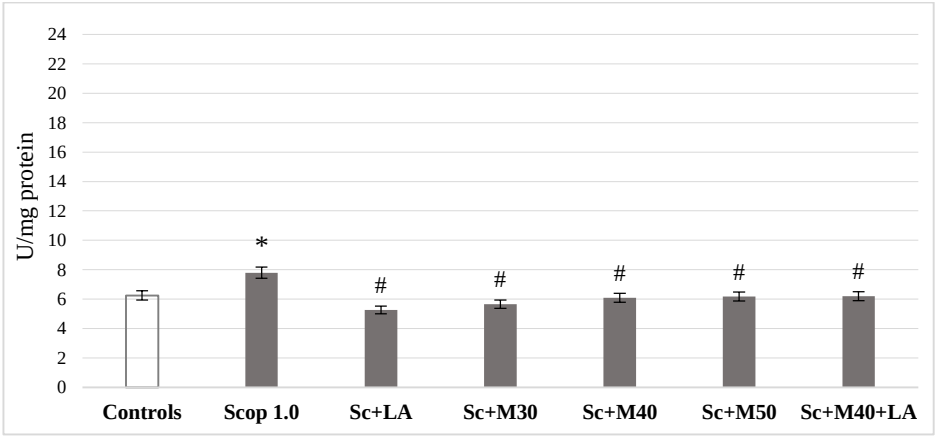
In tGSH, a straight dose proportional relationship was established – with increasing dose, Myrtenal reliably increased brain levels in mice compared to the Scopolamine group values (p < 0.05) (Fig. 100 B). At 30 mg/kg treatment, tGSH concentration rised by 43%, at 40 mg/kg – by 58.3% and at 50 mg/kg – by 68.4%.

The best response to oxidative stress in dementia mice was shown by the group of rodents treated with the combination of Myrtenal and LA. There was a decrease in LPO product levels of 54 % compared to the scopolamine group, 34 % compared to mice treated with LA and 11.4 % compared to those treated with Myrtenal (40 mg/kg) with a confidence level $p < 0.05$. In terms of tGSH concentration, results were similar – Myrtenal's combination with LA achieved the most significant increase in the indicator of 89.7 % compared to the scopolamine group, 21.9 % compared to the LA group and 19.9 % compared to those treated with Myrtenal at 40 mg/kg with a confidence level $p < 0.05$.

Natural antioxidant protection of neuronal and glial cells from oxidative stress includes glutathione and the enzymes SOD, CAT, GPx (Gandhi and Abramov, 2012). Progression of neurodegenerative diseases is accompanied by change in activity and expression of antioxidant enzymes (Pocernich et al., 2011).

The effects of Myrtenal on the activity of the antioxidant enzymes SOD, CAT and GPx in mice with scopolamin-induced dementia are presented in Figure 32.

A



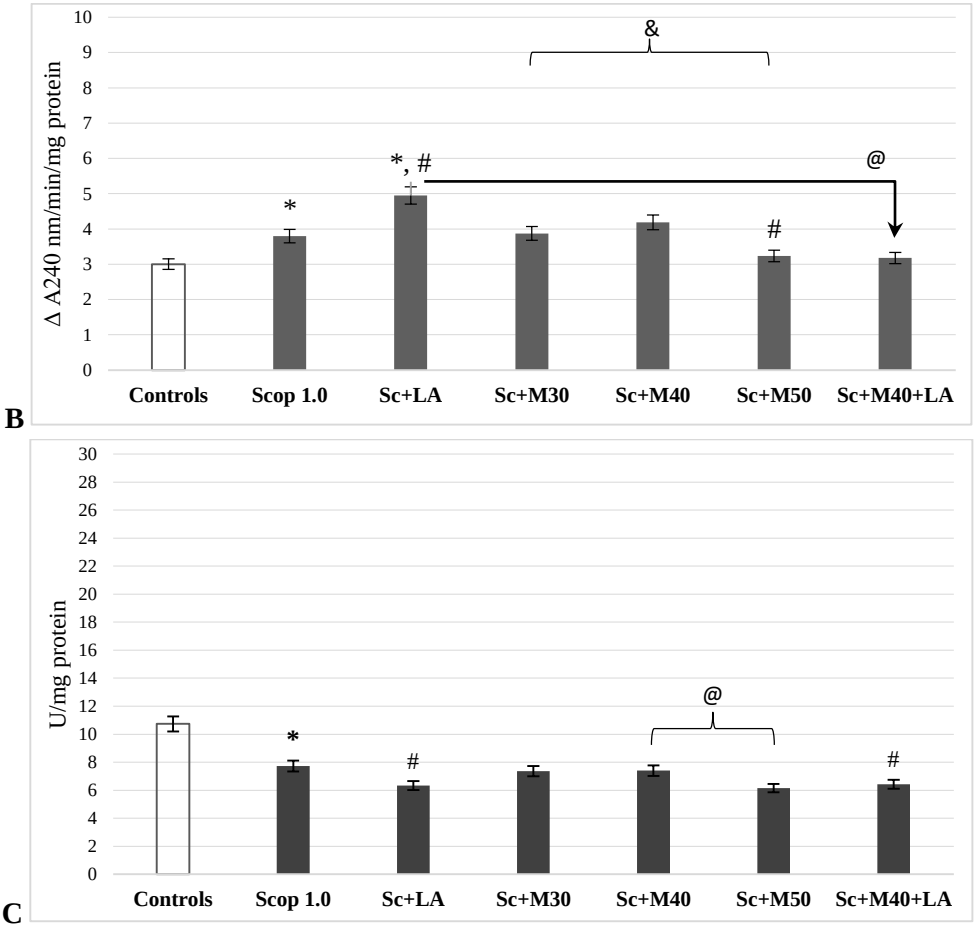


Fig. 32. Effects on the antioxidant enzymes activity – SOD (A), CAT (B) and GPx (C), in the brain in mice with scopolamine-induced (1.0 mg/kg) dementia after 14 days of treatment with Myrtenal at increasing doses (30, 40 and 50 mg/kg) and comparing them to those of the reference LA (*P < 0.05 Vs. Controls, $^{\#}$P < 0.05 Vs. Scop. group)

As a result of scopolamine-induced oxidative stress in rodent brains, enzyme activity compared to the control group was increased to SOD by 24.8%, CAT by 26.3% and GPx activity was statistically significantly reduced by 29.9 % ($p < 0.05$). It has been shown that the various components of the body's antioxidant protection can work together, such as SOD and CAT, which can explain the one-way change in these two enzymes (Fridovich, 1978).

Lipoic acid most significantly affects the SOD enzyme activity, resulting in a 32.4 % decrease compared to the dementia group (Fig. 32 A. Myrtenal alters the indicator in a dose-dependent manner – as the administered dose increases the effect on enzymatic activity. At the 30 mg/kg dose, the reduction in enzymatic activity compared to the scopolamine group was 27.4%, at 40 mg/kg 21.9% and at 50 mg/kg 20.8%, with a confidence level $p < 0.05$. At all three doses of Myrtenal, SOD activity was adjusted to similar values to those of the control animals. Similar to the results of our study with Myrtenal, when examining the neuroprotective effects of

α-pinene on a model of scopolamine-induced dementia, Lee GY et al. (2017) observed increases in SOD antioxidant enzyme levels in a rat hippocampus.

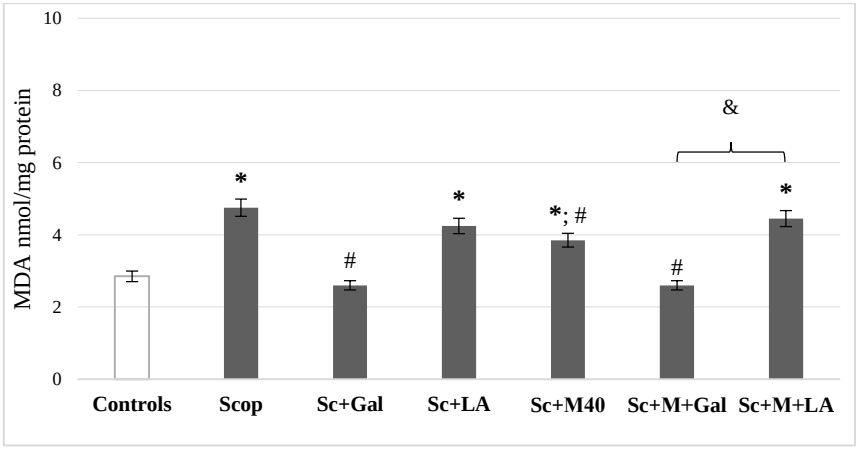
Myrtenal at all three doses administered failed to restore CAT and GPx enzyme activity to close to controls levels. Similar results were seen in the reference mouse group, with the CAT and GPx antioxidant manifested as a pro-oxidant (Fig. 32 B and C). These properties of lipoic acid are known. Pro-oxidant activity as well as antioxidant properties of lipoic acid were already reported in 2002 by Moini et al. and Cakatay in 2006. It is assumed that the ability of LA to function as an anti- or pro-oxidant, at least in part, is determined by the type of oxidative stress and physiological state.

In conclusion, Myrtenal improved oxidative status in mice treated with scopolamine, influencing some parameters in a dose-dependent manner. Increased tGSH concentrations were observed with increasing the dose, while the scopolamine-stimulated SOD activity was restored. The monoterpenoid reduces the content of lipid peroxidation products in the brain of dement rodents to a greater extent than the reference LA, with best results reaching the dose of 40 mg/kg.

5.2.5.2. Effects of Myrtenal on brain oxidative status in *rats* with scopolamine-induced dementia

The study was conducted in brains of rats with dementia induced by administration of scopolamine at the dose modification (0.1 mg/kg for 8 days + 20.0 mg/kg on Day 9) treated with Myrtenal 40 mg/kg and the reference lipoic acid and galantamine for 9 days. Levels of LPO products and tGSH (Fig. 33) as well as antioxidant enzyme activities were investigated (Fig. 34).

A



B

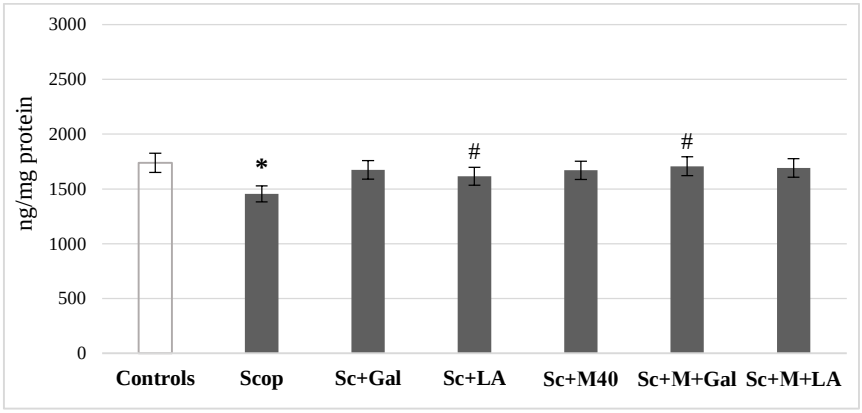


Fig. 33. Effects on brain oxidative status in rats with scopolamine-induced dementia (dose modification) after 9 days of treatment with Myrtenal (40 mg/kg) and comparing them to those of the references LA and Gal – Determination of LPO product (A) and tGSH levels (B) (*P < 0.05 Vs. Controls; #P < 0.05 Vs. Scop. group)

Like other brain oxidative status studies in dementia rats, scopolamine increased the amount of LPO products by 6.67 % relative to controls and decreased tGSH concentration by 16.3%, with a confidence level $p < 0.05$. Myrtenal statistically significantly decreased LPO product levels by 19 % compared to those in the scopolamine group ($p < 0.05$) and increased tGSH by 14.8%.

Anticholinesterase standard galantamine exhibits antioxidant properties which have also been reported by other investigators. In vitro studies have established its activity against ROS (Traykova et al., 2003). Other mechanisms of protective action have also been reported – protection of mitochondrial membrane potential; prevention of membrane fluidity disturbances; reduction of overproduction of ROS resulting from ACh elevation due to inhibition of AChE and allosterical potentiation of the α7-subtype of N-cholinergic receptors (Tsvetkova et al., 2013). Our studies have revealed the antioxidant properties of galantamine in rats with an experimental model of dementiation.

As a confirmation of the pro-oxidant properties of LA identified by some authors, in our studies, the reference exhibits pro-oxidant activity, expressed by raising LPO product levels to similar values to those of scopolamine. Unlike the toxic agent, LA significantly increased tGSH concentrations ($p < 0.05$).

In our opinion, the less pronounced antioxidant properties of the monoterpenoid in this trial are related to the high final dose of the toxic agent and the oxidative stress it caused.

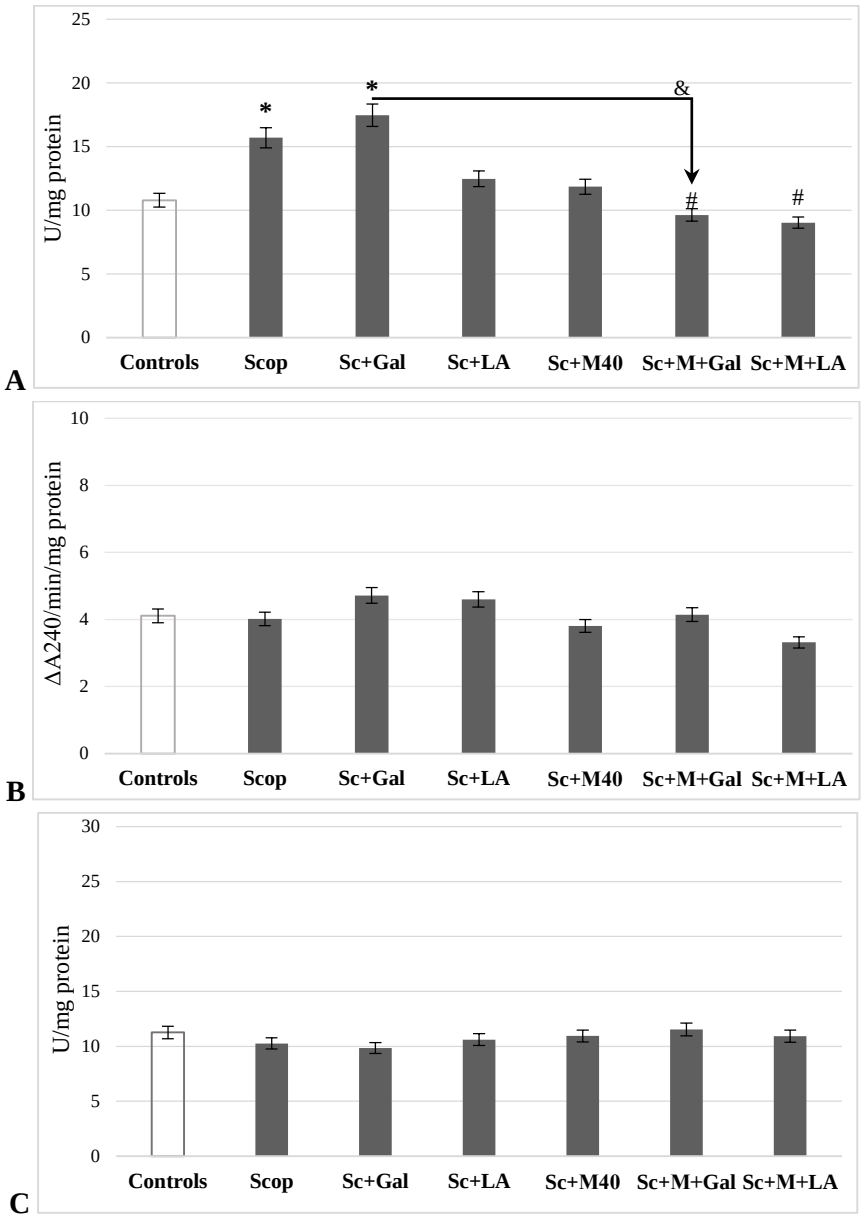


Fig. 34. Effects on the activity of SOD (A), CAT (B) and GPx (C) in the brain in rats with scopolamine-induced dementia (dose combination) following treatment with Myrtenal (40 mg/kg) and comparing them to those of references LA and Gal administered alone or in combination with Myrtenal; *P < 0.05 Vs. Controls; $^{\#}$P < 0.05 Vs. Scop. group; $^{\&}$P < 0.05 Vs. Gal group)

Scopolamine significantly increased SOD enzyme activity (45.5%) compared to controls (p P 0.05) (Fig. 34 A.). Myrtenal restored SOD activity to similar to control values and compared to the scopolamine group the decrease was 24.5 % in the absence of confidence. The reference galantamine did not affect the parameter, but when combined with Myrtenal, there was a significant reduction in activity compared to the group of animals treated with galantamine alone (p < 0.05). Concomitant treatment with the other reference, LA, significantly

reduced enzymatic activity compared to the scopolamine group ($p < 0.05$), similar to the combination with the anticholinesterase standard.

Our results confirm those of Kumar et al. (2014) according to which administration of scopolamine at 2.0 mg/kg causes oxidative stress in the brain of test mice manifested by increases in LPO product levels and SOD enzyme activity ($p < 0.001$).

In the rats with experimental dementia, scopolamine did not alter CAT activity (Fig. 34 B). Myrtenal, both alone and in combination with the reference, also did not change the indicator and galantamine increased catalase activity unreliable to the scopolamine group (by 17.3%).

The substances we tested did not affect the activity of GPx (Fig. 34 C). Ansari and Scheff (2010), conducting post mortem examinations of cortical supernatant in AD patients, also found no significant change in GPx activity.

Many terpenes of plant origin exhibit antioxidant properties by interfering with the various non-enzymatic and enzymatic components of the antioxidant protection of organisms. Our research has revealed Myrtenal's ability to affect the oxidative brain status of dement rats. In these studies, the test substance lowers the amount of LPO products, increases tGSH concentration unsignificantly and restores increased by scopolamine SOD activity (similar to its effects in mice) without affecting CAT and GPx activities. In dement mice, Myrtenal also affected brain oxidative status as evidenced by decrease in LPO product levels, significant increase in tGSH concentration, and recovery of scopolamine-altered SOD and CAT enzyme activities to those of controls, and no effects on GPx activity were observed here either.

5.2.6. Effects of Myrtenal on cholinergic transmission in brain of rodents with scopolamine-induced dementia

5.2.6.1. Effects of Myrtenal on brain AChE activity in mice with scopolamine-induced dementia

Brain AChE activity in mice with experimental dementia induced by scopolamine at 3.0 mg/kg was investigated after 11 days of treatment with Myrtenal (20 mg/kg) and the references lipoic acid and galantamine (Fig. 35).

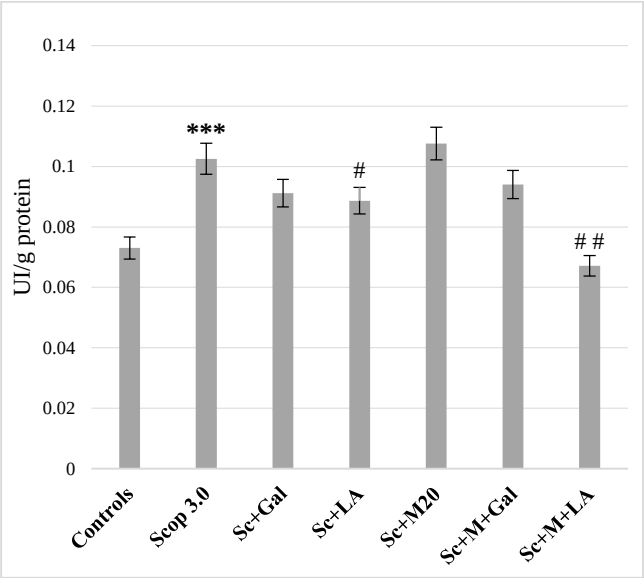


Fig. 35. Effects on brain AChE activity in mice with scopolamine-induced dementia (3.0 mg/kg) after 11 days of treatment with Myrtenal (20 mg/kg); ***P < 0.001 Vs. Controls; #P < 0.05, ##P < 0.01 Vs. Scop. group) *(t-Test)*

In mice treated with scopolamine alone, brain AChE activity compared to the control group was increased by 35.6 % at very high significance ($p < 0.001$). Myrtenal did not alter the effect of scopolamine on the etzyme activity. The reference galantamine inhibited AChE activity by 11.1% without significance. Lipoic acid significantly reduced the indicator compared to the scopolamine group ($p < 0.05$) and its combination with Myrtenal reduced AChE activity to the greatest extent ($p < 0.01$), bringing it closer to that of controls.

Arivazhagan et al. (2006), found that LA inhibits AChE activity in various brain regions after 7 and 14 days of intraperitoneal administration at 100 mg/kg in young and adult rats (4 and 24 months of age). The results of our studies showed similar effects of LA administered at 30 mg/kg in a scopolamin-induced mouse dementia model, as demonstrated by correction of the toxic agent increased AChE activity. No other evidence of anticholinesterase properties of lipoic acid in experimental models of dementia is currently available.

5.2.6.2. Effects of Myrtenal on brain ACh levels in rats with scopolamine-induced dementia

Brain ACh levels in rats with induced dementia were evaluated after 11 days of treatment with scopolamine at 1.0 mg/kg and after 9 days of treatment with the dose combination (0.1 mg/kg for 8 days + 20.0 mg/kg on day 9). Simultaneously, Myrtenal at a dose of 40 mg/kg and the two references lipoic acid and Galantamine, at the selected doses, were administered to animals (Fig. 36).

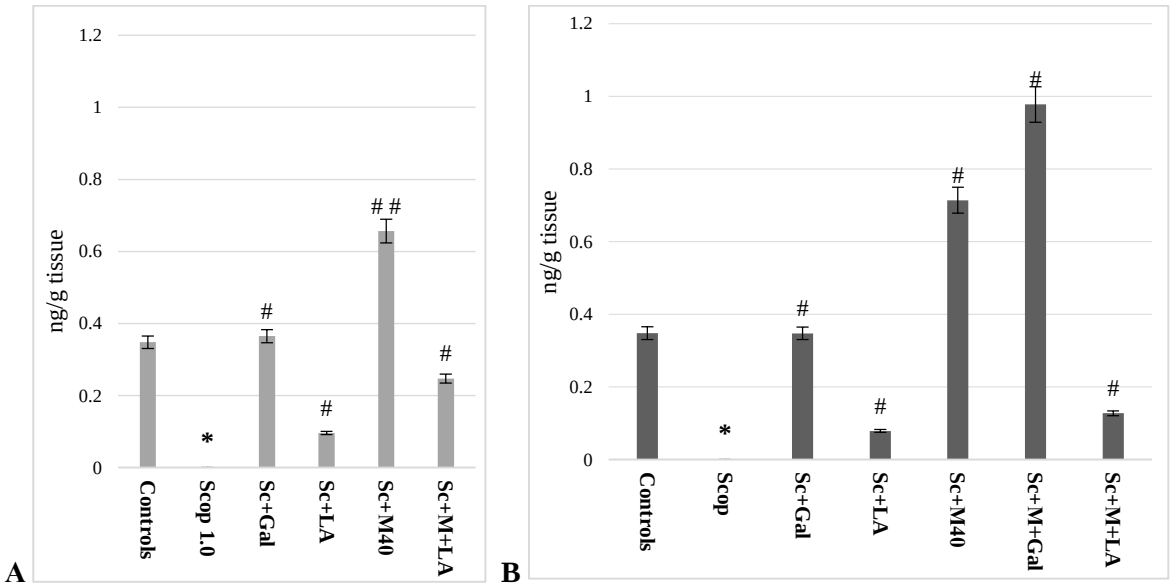


Fig. 36. Effects on brain ACh levels in rats with scopolamine-induced dementia treated with Myrtenal at 40 mg/kg and compared to the reference LA and Gal; *P < 0.05 Vs. Controls; #P < 0.05 Vs. Scop. group)

A – scopolamine administered at a dose of 1.0 mg/kg for 11 days

B – scopolamine administered in the dose combination for 9 days

At both doses, the toxic agent produced a statistically significant decrease in the concentration of the brain mediator compared to the control group ($p < 0.05$). Results obtained in both trials confirmed those of Lee S. et al. (2014), which revealed decreased ACh concentration and increased AChE activity in the brain of mice injected intraperitoneally with scopolamine at 1.0 mg/kg for 7 days.

The decreased concentrations of the ACh mediator in the brain of experimental rodents by scopolamine are not solely due to AChE activation. According to literature, the damaging effects of the toxic agent on cholinergic mediation in the brain of experimental animals are also associated with inhibition of the activity of the enzyme cholinacetyltransferase (ChAT) responsible for the synthesis of the mediator (Heo et al., 2006).

LA is known to increase ACh production by activating ChAT (Holmquist et al., 2007). Our studies indicate that compared to gallantamine, the antioxidant has less influence on mediator concentration changes. Combining LA with the monoterpenoid does not cause an increase in activity.

Myrtenal at 40 mg/kg reliably increased the brain ACh content of dementia rats in both trials and did not inhibit AChE activity in dementia mice. The results suggest that stimulation of ChAT enzyme activity is a possible mechanism to increase mediator levels. Similar properties of α-pinene, the metabolite of which is myrtenal (Lee GY et al., 2017), are identified to support this hypothesis. Combination of the monoterpenoid with the anticholinesterase agent

galantamine most significantly increases the ACh content relative to the dementia group of animals ($p < 0.05$), suggesting synergistic dependence on the effect of both substances.

In conclusion, Myrtenal stimulates ChAT activity and galantamine reduces ACh degradation by inhibiting AChE. The outcome was a significant increase in brain ACh content in dementia rats, indicating that the experiments conducted may contribute to clarifying the mechanism of action of Myrtenal on the mediator levels.

5.2.7. Histopathological examinations

The brain tissue of animals treated with scopolamine exhibits signs of enhanced neuronal degeneration described in the section on verification of the scopolamine model for causing dementia (Fig. 23). Administration of Myrtenal results in a reduction in the signs of damage caused by the toxic agent (Fig. 37).

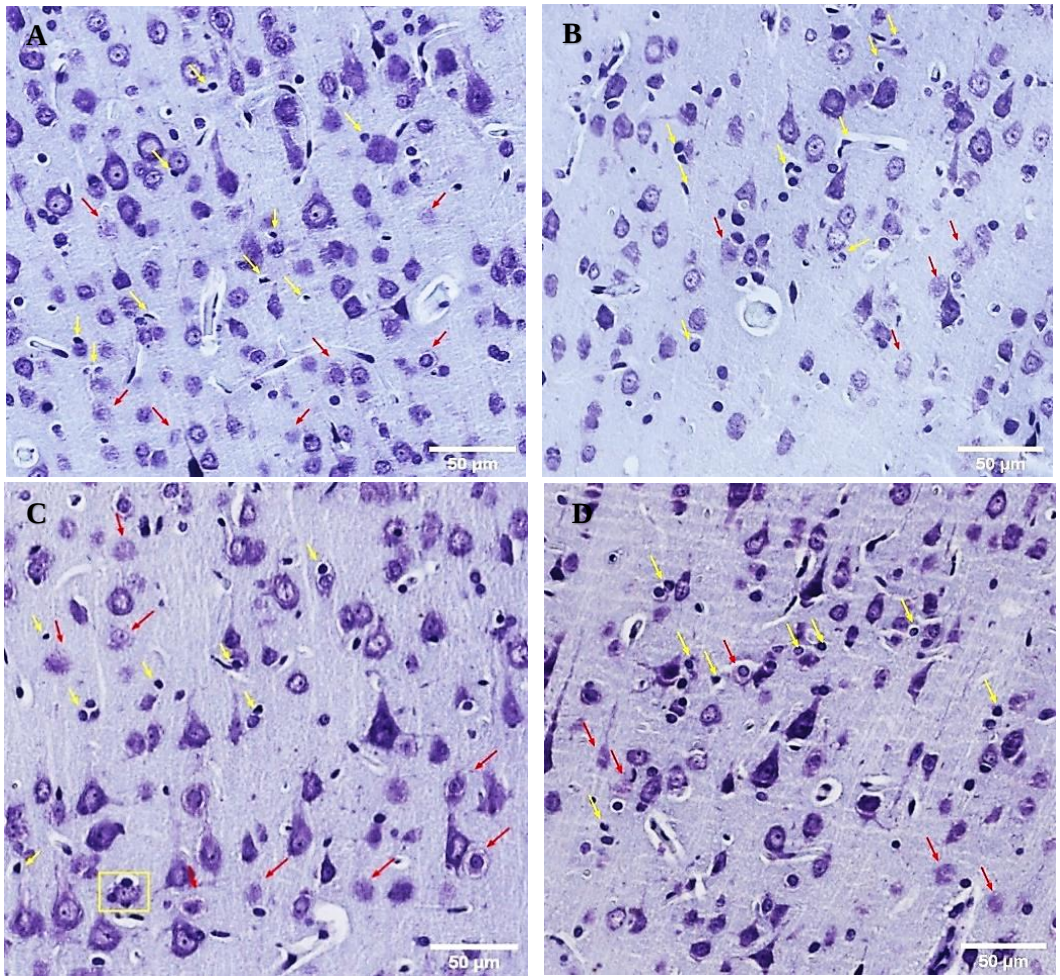


Fig. 37. Qualitative cortex analysis in rats with scopolamine-induced dementia (dose modification) treated with Myrtenal (40 mg/kg) for 9 days – comparison of a result in the dementia group with that treated concomitantly with Myrtenal

A – Motor cortex layer V (M1) of a rat brain from the scopolamine group;
B – Motor cortex layer V (M1) of a rat brain from Scop + M group;
C – Sensory cortex layer V of a rat brain from Scop + M group;
D – Sensory cortex layer V of a rat brain from the scopolamine group
(Red arrows – neurons with signs of chromatolysis and/or vacuolisation*; yellow arrows* – glial cells*; yellow rectangle* – perineuronal gliosis)

In the cortex of dementia rats (scopolamine group), visible gliosis and numerous neurons with fine vacuolation of cytoplasm and marked loss of Nislovae granulation (chromatolysis) were observed. These changes were mainly located in the third and fifth layers of the cortex, but also affected the other layers (Fig. 37 A and B). Neural shadows as well as areas of aggregation of microglia, indirect signs of neuronal death, as well as neurons with picnosis or perineuronal microgliosis (close apoesis between the body of a neuron and several small nuclei of neuroglia) were also observed.

In the group of rats treated with the combination of scopolamine and Myrtenal, the magnitude of glial reaction appeared similar, but the observed signs of cortical degeneration were more moderate. Primary cytoplasmic vacuolisation and chromatolysis were observed (Fig. 37 B and D). The main difference with dementia rats was that cariolysis, cariopiknosis and perineuronal microglycosis in brain tissue were relatively rare.

After examining the density (number of profiles per 1 mm^2) of all cortical profiles absorbing more than 20% of light, a significant decrease in the indicator compared to healthy controls was observed in the rats treated with scopolamine (p = 0.0182) (Fig. 38 A).

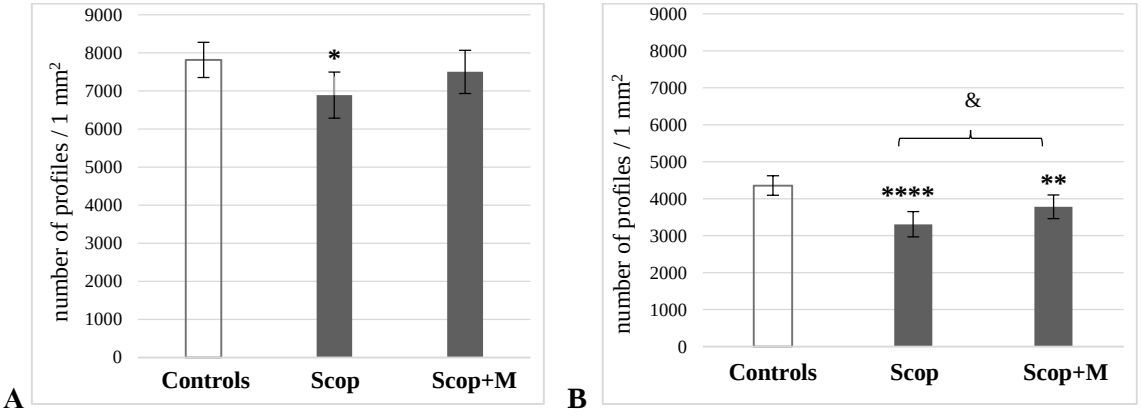


Fig. 38. Effects on the total number of profiles (density) (A) and on the number of profiles (density) with area greater than 31 µm² (B) in brain cortex in rats with scopolamine-induced dementia treated with Myrtenal (40 mg/kg); *P < 0.05, **P < 0.01, ****P < 0.0001 Vs. Controls

Here again, animals receiving a combination of scopolamine and Myrtenal occupy an intermediate position with a slight reduction from controls, with no statistical significance in the change of total number of profiles compared to the scopolamine group.

The cortical cell density was extended by sizing (Fig. 38 B). Profiles with a larger area belong mainly to neurons. They had a significant effect on the mean density of the profiles per unit area (1 mm^2) as a result of the administration of the toxic agent – a significant decrease compared to controls (($p = 9.67 * 10^{-6}$). Rats treated with scopolamine and Myrtenal were in an intermediate position – decreased profile density was again observed, but there was an increase compared to the scopolamine alone group (statistically significant at confidence level p = 0.05, according to Dunett-Tukey Kramer test for unbalanced data).

The results lead to the conclusion that although the density of profiles in animals treated with scopolamine and Myrtenal was reduced compared to healthy controls, there was a significant increase compared to the scopolamine group.

No significant differences similar to those found in the cortex were detected in the hippocampus.

Alongside the identified neuromodulatory properties and the observed positive effects of the test substance on the memory and learning of experimental animals, histopathological studies in the brain of dementia rodents indicate that Myrtenal has the ability to antagonise the damaging action of scopolamine to some extent. The M-cholinoblocker demonstrates its toxic effects by significantly (p = 0.018) decreasing the density of Cresyl-violet-positive fraction of brain cells (number of all profiles absorbing more than 20% of light). Scopolamine significantly reduced the mean density of vital neurons in the cerebral cortex (p = 9.7 x 10-6). In rodents treated with Myrtenal concomitantly with scopolamine, impairment was less pronounced (p = 0.007) and the number of viable cells in this group was markedly increased compared to the group of rats injected with scopolamine alone (p = 0.05). Similar effects on neuroglia were not observed. Such properties of the substance were not manifested in the hippocampus.

Finally, cortical changes show a decreasing trend in the volume of the Nisl substance and/or the number of neurons per unit area in animals with scopolamine-induced dementia. Rats treated concomitantly with scopolamine and Myrtenal tended to reduce these negative effects. Histopathology studies point to the location of Myrtenal's neuroprotective effect in the cortex.

6. Comparison of Myrtenal effects on important parameters in healthy and in dement experimental rodents

To perform the task, the effects of Myrtenal on basic behavioural and biochemical parameters in healthy and dementia experimental rodents were compared. Parallels were drawn between changes in healthy animals treated with Myrtenal compared to controls, and between animals treated with scopolamine alone compared to those treated concomitantly with scopolamine and Myrtenal.

6.1. Comparison of effects on body weights

In healthy rodents, Myrtenal was not generally toxic and did not affect normal body weight gain. Exceptionally, the highest dose used caused a decrease in body weights in rats. In

animals with experimental dementia, the change in this parameter is lower. The toxic effect of scopolamine on body mass in mice is counteracted by higher doses of Myrtenal.

A summary of the effects of Myrtenal on body weights in healthy and dement rodents is presented in Figure 39.

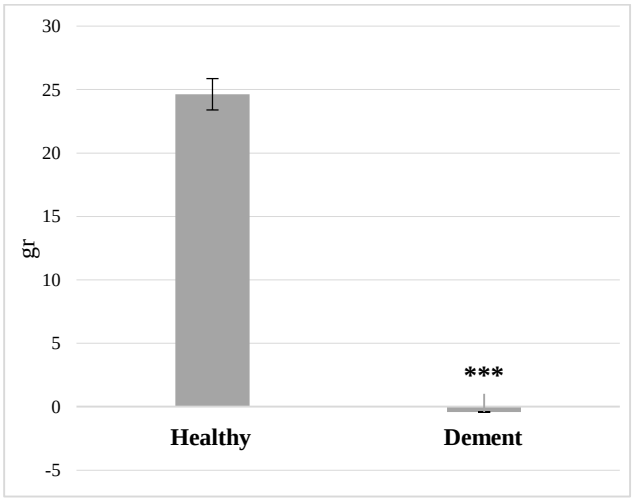


Fig. 39. Effects on body weight in healthy and dementia rodents following administration of Myrtenal; ***P < 0.001 Vs. Controls (*t*-Test)

Therefore, administration of Myrtenal is safe in healthy rodents and anorexigenic in dement ones. Similar properties are also characteristic of the anticholinesterase pharmacological agent galantamine applied for treatment of neurodegenerative diseases and used as a reference in this study. It is very likely that this effect is due to a pharmacokinetic interaction between scopolamine and Myrtenal, manifested in influencing the elimination of the two substances. Another possible explanation of the results is the enhancement, for an unclear reason, of the anorexigenic properties of Myrtenal, characteristic of many essential oil components, when combined with the M-cholinoblocker.

6.2. Comparison of effects on memory capabilities

Myrtenal improved rodent memory capabilities with scopolamine-induced dementia in Step through test demonstrated by increasing latency time, while in healthy animals it reduced latency time, which is a criterion for the educational ability of animals to account for the effects of administered substances (Fig. 40).

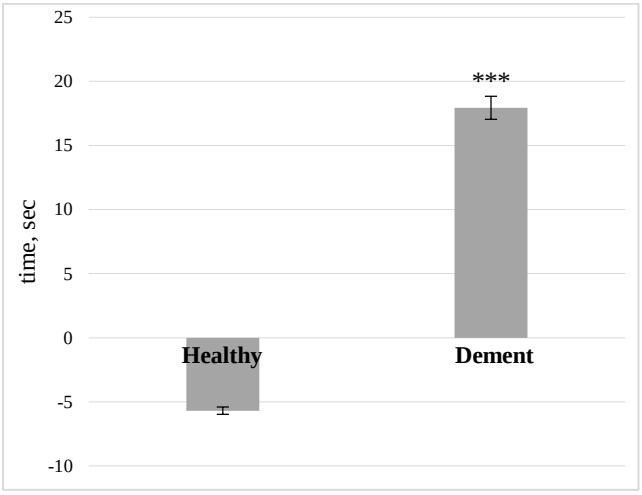


Fig. 40. Effects on memory capabilities in healthy and dementia rodents following Myrtenal administration; ***P < 0.001 Vs. Controls

A possible explanation for the differences observed is the membranostabilising properties of Myrtenal manifested in healthy animals as a response to pain stimili after prolonged treatment with the monoterpenoid in a central mechanism induced pain model.

Another reason for reducing latency time in healthy rodents following administration of Myrtenal is the demonstrated anxiolytic potential. In *Step through test* animals instinctively enter the dark half of the apparatus. However, after training, they shall avoid entering their preferred dark compartment on the basis of the negative experience of a slight electric shock in the feet. It is possible that the shortened lag time or shorter stay in the illuminated compartment in healthy rodents is related to the decreased anxiety observed in investigating the anxiety properties of the monoterpenoid.

The identified effects of Myrtenal on memory capabilities in healthy rodents can also be explained by the difference in results between mice and rats. Compared to control rodents, a reduction in latency time was observed in mice treated with the test substance, while no change in indicator was observed in rats. Interspecies differences in metabolic processes suggesting a faster biotransformation in mice than in rats may be responsible for the shorter or absent effect of Myrtenal on mouse memory capabilities.

6.3. Comparison of effects on neuromuscular coordination

In healthy mice and rats, Myrtenal unsignificantly increased the retention time on the rotation arm in *Rota rod test*. In mice with scopolamin-induced impairment, the monoterpenoid produced minor improvements in neuromuscular coordination, while no effects on rodent balance and motor ability were observed in rats. The results of the experiment showed that Myrtenal had no noticeable effects on neuromuscular coordination in experimental animals.

Many essential oils and their components have activating and energising activity. Terpenes exhibit affinity for different receptors and other body structures and have the ability to

influence life processes. Some have CNS stimulating effects, others have calming and relaxing properties, and some have the ability to correct neuromuscular problems. For example, those extracted from cannabis oil effectively affect tremors in advanced Parkinson's disease that are extremely difficult to overcome. In contrast, Myrtenal achieved insignificant improvement in coordination in healthy rodents, and in the model of neurodegenerative damage we used, it was ineffective.

6.4. Comparison of effects on exploratory behaviour

The comparative summary of the effects of the test substance on exploratory activity in healthy and dement rodents is presented in Figure 41.

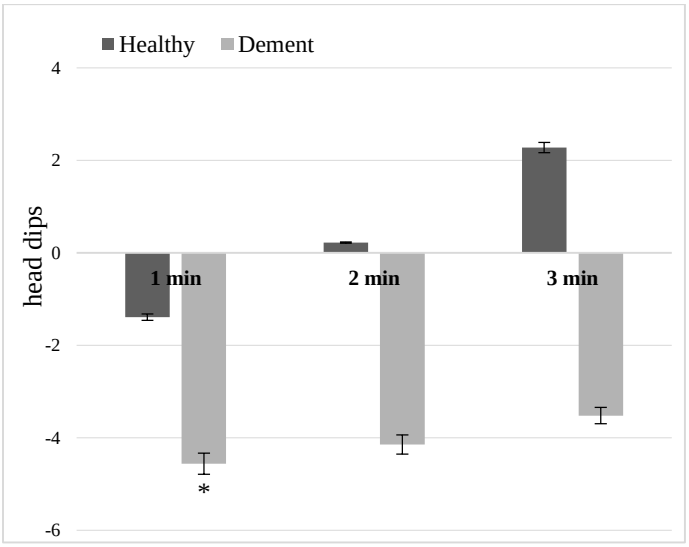


Fig. 41. Effects on exploratory behaviour in healthy and dement rodents following Myrtenal administration; *P < 0.05 Vs. Healthy

Myrtenal in healthy rodents did not affect the activity in the *Hole board test*. Some increase in the number of head dips that were most evident at 3 minutes, according to some authors, were indicative of both the level of exploratory activity and total locomotion. These results are correlated with poor effect of neuromuscular coordination in *Rota rod test* in healthy rodents.

Other terpenes showed similar results to Myrtenal in tests investigating the state of exploration activity and coordination. For example, cineol, a representative of plant monoterpenes, causes a slight increase in activity in the *Hole board test* and no effects in the *Rota rod test* in rodents.

In dement rodents, Myrtenal exhibits protective effects. The reduced incidence of activity in rodents with experimental dementia, which is indicative for normal habitation of animals in a

known enviroment, means that Myrtenal counteracts changes in exploratory behaviour caused by scopolamine.

6.5. Comparison of antioxidant effects (concentration of lipid peroxidation products in the brain)

Myrtenal exhibits antioxidant properties only in animals with scopolamin-induced impairment, while healthy rodents have no effect (Fig. 42).

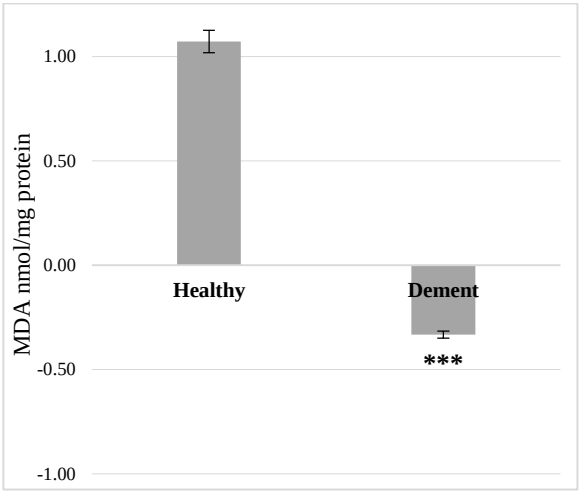


Fig. 42. Effects on lipid peroxidation products levels in the brain in healthy and dement rodents following Myrtenal administrationм ***P < 0.001 Vs. Healthy

In dement mice and rats, Myrtenal reduced the content of brain LPO products compared to healthy rodents with a level of confidence $p < 0.001$.

The antoxidant properties of a number of substances of plant origin with a diverse structure are known. Terpenes are characterised by a protective effect against oxidative stress, usually combined with other effects with known therapeutic benefit in multiple diseases. Different mechanisms of action make them suitable for exploring their options for multitargeted therapy as an innovative strategy against neurodegenerative diseases. Like many other compounds in this class, Myrtenal exhibits antioxidant properties, which are the leading mechanism for its neuroprotective effects.

6.6. Comparison of effects on brain acetylcholinesterase activity

In healthy animals, Myrtenal increased the activity of the brain AChE, while in dement animals no effect on the enzyme stimulated by scopolamine has been observed. A comparison of the effects of the monoterpenoid on brain AChE activity in healthy and dement rodents is presented in Figure 43.

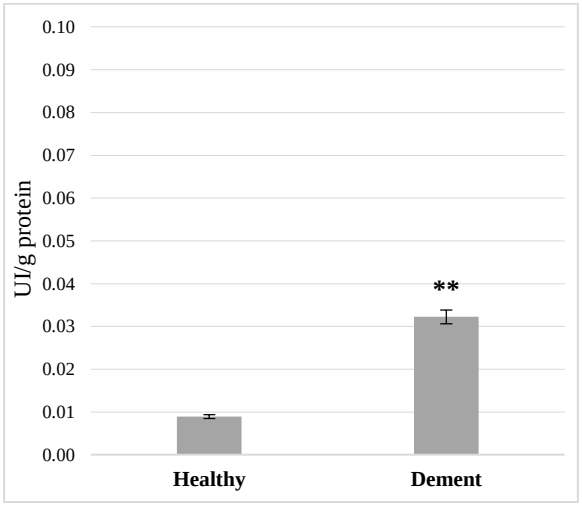


Fig. 43. Effects on brain AChE activity in healthy and dement rodents following Myrtenal administration; **P < 0.01 Vs. Healthy

According to docking-studies, the monoterpenoid has affinity for binding to the active centre of the enzyme, but does not inhibit its activity. Myrtenal does not exhibit anti-cholinesterase properties in experimental animals despite the *in vitro* data on such properties of the substance.

Many active components of plant origin, including terpenes, have anticholinesterase properties manifested to varying degrees. A study of the presence of anticholinesterase effects of Myrtenal in animals (healthy and dement) was conducted for the first time. The monoterpenoid affinity for AChE has been confirmed, but not its ability to inhibit enzyme activity.

6.7. Comparison of effects on brain acetylcholine levels

Myrtenal increased the concentration of the ACh brain neurotransmitter in healthy and demented animals. The test substance showed its neuromodulatory effects more strongly on brain acetylcholine levels in healthy animals (Fig. 44).

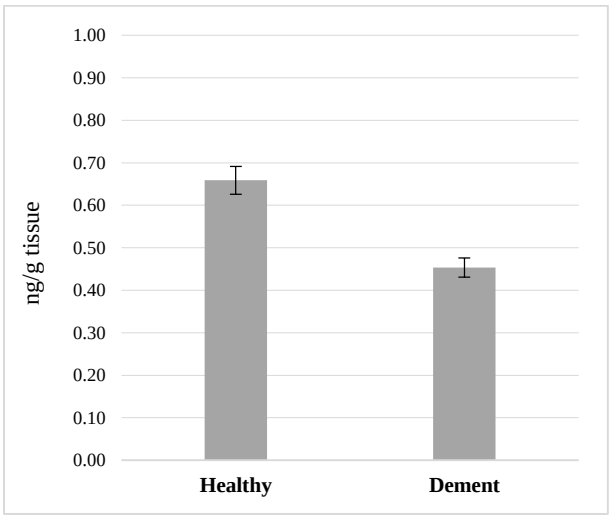


Fig. 44. Effects on brain ACh content in healthy and dement rodents following Myrtenal administration

Increased concentrations of ACh in the brain in both healthy and dement animals were accompanied by increased activity of the enzyme responsible for its degradation in the synapse. The results point to neuromodulatory activity not due to anticholinesterase properties. A possible mechanism is stimulation of mediator synthesis by activation of the ChAT enzyme, which is characteristic of other terpenes in the group of the test substance. The less pronounced neuromodulatory properties of Myrtenal in dement rats are also likely due to antagonisation of the effects of scopolamine, the damaging mechanism of which also involves inhibiting ChAT activity, which contributes to greater inhibition of cholinergic neurotransmission.

Neurodegenerative impairment is characterised by a complex pathogenic mechanism involving mitochondrial dysfunction, neuroinflation and oxidative stress. Multitarget strategies aimed at influencing mitochondrial dysfunction through substances that increase mitochondrial bioenergy and inhibit oxidative stress have potential therapeutic benefits in neurodegenerative diseases.

This thesis explores for the first time the potential of Myrtenal to influence changes in model neurodegeneration. The likely mechanism of neuroprotective action of the monoterpenoid in dement rodents is related to its antioxidant and neuromodulatory properties, manifested to a greater extent in dementia compared to healthy experimental rodents and located in the brain cortex.

VII. CONCLUSIONS

A. Pharmacological and toxicological effects of Myrtenal in healthy rodents

1. The mean lethal dose of Myrtlenal to mice for intraperitoneal administration was determined (LD_{50} i.p. = 191.5 mg/kg).

2. Myrtenal does not cause a general toxic effect on body weight after repeated administration to mice and rats. Prolonged treatment in rats resulted in an anorexigenic effect accompanied by positive changes in the lipid profile.

3. The central effects of Myrtlenal observed by us - potentiation of the action of CNS depressant drugs with different mechanisms of action and the presence of anxiolytic properties, confirm the results of docking studies showing its affinity for the GABAA receptor.

4. Myrtenal, administered for different durations, exhibits analgesic activity, probably related to the CNS depressant effects and membrane stabilizing properties we have identified.

B. Protective effects of Myrtenal in rodents with a model of neurodegeneration

1. After repeated administration, Myrthenal did not show a general toxic effect on body weight in dementia mice, whereas in rats the dose of 40 mg / kg caused significant weight loss.

2. Myrtlenal significantly improved memory and learning abilities in dementia rodents, more pronounced in rats, but without significantly affecting their recognition memory.

3. Myrtenal restored scopolamine-induced damage of rodent exploratory activity in two behavioral tests. There were no positive effects on neuromuscular coordination.

4. Myrtenal demonstrated antioxidant properties, manifested by correcting the oxidative stress caused by scopolamine in the brain. In dementia mice, it decreased the levels of LPO products, dose-dependently increased the content of tGSH and significantly reduced the increased activity of SOD by scopolamine to levels close to those of the reference LA. Its effects in the brains of dementia rats were similar.

5. Myrtenal did not exhibit anti-cholinesterase properties *in vivo*, but increased ACh levels in the brains of dementia rats, possibly by stimulating ChAT enzyme activity.

6. The histopathological examinations localized the neuroprotective effects of Myrthenal in the cerebral cortex.

VIII. CONTRIBUTIONS OF THE THESIS WORK

1. The effects of Myrtenal on healthy rodents (mice and rats) were first studied. To date, studies to clarify the mechanisms of action of the monoterpenoid worldwide are mainly conducted on experimental models of various diseases.
2. New data have been obtained to supplement the toxicological characteristic of Myrtenal in experimental rodents. The mean lethal dose (LD50) for mice following intraperitoneal administration was first determined.
3. CNS activity expressed as potentiation of barbiturates and benzodiazepines effect, as well as anxiolytic potential with administration alone, possibly related to interference with GABA-ergic neurotransmission, have been identified, which are consistent with docking studies indicating Myrtenal's greater affinity for the GABAA receptor compared to Diazepam.
4. New data on the pharmacological effects of Myrtenal in intact rodents have been obtained. Anaesthetic properties have been found to be present for different duration of application. First-time studies on the neuropharmacological activity of Myrtenal did not reveal any harmful effects on the memory, coordination or exploratory behaviour of animals.
5. First-time studies on the anticholinesterase properties of Myrtenal in an *in vivo* model rule out the possibility of blocking enzyme activity, as opposed to available *in vitro* data.
6. The protective potential of the monoterpenoid in rodents with an experimental dementia model associated with its antioxidant and neuromodulatory properties has been first identified. Original histopathological data suggest localization of the neuroprotective effect of Myrtenal in the cortex.
7. A novel dose modification of scopolamine-induced dementia is proposed to recreate the non-linear progression of impairment, which has been verified behaviourally, biochemically and histopathologically.
8. A comparative analysis of the effects of Myrtenal in healthy and dement rodents was performed for the first time, demonstrating a more pronounced effect in animals with induced impairment.

IX. APPENDICES

1. Publications on thesis work

1. Dragomanova S., Tancheva L., Georgieva M. A review: Biological activity of myrtenal and some myrtenal-containing medicinal plant essential oils. *Scripta Scientifica Pharmaceutica***,** [S.l.], 5(2): 22 - 33, Dec. **2018**, ISSN 2367-5500. doi: http://dx.doi.org/10.14748/ssp.v5i2.5614.

2. Tzvetanova E., Georgieva A., Alexandrova A., Tancheva L., Lazarova M., **Dragomanova S.**, Alova L., Stefanova M., Kalfin R. Antioxidant mechanisms in neuroprotective action of lipoic acid on learning and memory of rats with experimental dementia. *Bulgarian Chemical Communications*, 50, Special Issue C: 52 – 57, **2018.**

3. Dragomanova S., Tancheva L., Georgieva M., Klisurov R. Analgesic and anti-inflammatory activity of monoterpenoid myrtenal in rodents. *J of IMAB*, 25(1):2406 - 2413, Jan-Mar **2019,** SJIF (Scientific Journal Impact Factor): 2018 – 7.774; Scopus Journal Metrics: CiteScore 2017 – 0.07, SJR 2017 – 0.103, SNIP 2017 – 0.025.

2. Scientific Communications on thesis work

1. Dragomanova S., Tancheva L., Dishovsky C., Georgieva A., Kalfin R., Georgieva M., Stoeva S. Pilot study of preventive effect of myrtenal on memory and ache activity in ad experimental mice. *Scientiffic Conference "Neuroscience – from theory to experiment"*, INB – BAS, Sofia, Bulgaria, Dec 4, **2014**.

2. Dragomanova S., Tancheva L., Georgieva M., Georgieva A., Dishovsky C., Kalfin R., Atanasova D., Lazarov N. Preventive effect of the natural monoterpene myrtenal on cognitive disorders in dement mice. ***Abstract Book*** *28th ECNP Congress*, Amsterdam, 29 August - 1 September **2015**, *The Netherlands*, *European Neuropsychopharmacology*, 25 (2): S578 - S579, September (**2015**), doi: http://dx.doi.org/10.1016/S0924-977X(15)30812-9 (**Grant)**

3. Dragomanova S., Tancheva L., Georgieva M., Dishovsky C., Georgieva A., Kalfin R., Hodzhev Y., Atanassova D., Lazarov N. Preventive effects of the monoterpene Myrtenal on Alzheimer's disease progression on experimental mouse model. ***Abstract Book***, *14th International Congress on Amino Acids, Peptides and Proteins*, Vienna, Austria, August 3 - 7, **2015,** *Amino Acids* (**2015**), 47: 1652, doi: 10.1007/s00726-015-2016-z.

4. Dragomanova S., Tancheva L., Georgieva M., Georgieva A., Dishovsky C., Stoeva S., Pavlov S., Kalfin R. Preventive effect of myrtenal and lipoic acid in combination on progression of Alzheimer's disease. ***Abstracts*** *of the 29th ECNP Congress*, Vienna, Austria, 17 - 20 Sept **2016**, *European Neuropsychopharmacology*, 26(2):636, doi: http://dx.doi.org/10.1016/S0924-977X(16)31732-1.

5. Dragomanova S., Tancheva L., Georgieva M., Georgieva A., Dishovsky C., Stoeva S., Kalfin R. Effect of monoterpene Myrtenal on experimental dementia in mice. ***Abstract Book*** of *31st International Conference of Alzheimer's Disease International*, Budapest, Hungary, 21 - 24 April, p 210, **2016**.

6. Dragomanova S., Tancheva L., Georgieva M., Klisurov R., Eftimov M., Kalfin R. Study on the mechanism of neuroprotective effect of Myrtenal on rats with experimental dementia. ***Abstract Book*** *AAIC* (*Alzheimer's Association) Satellite Symposia*, Varna, Bulgaria, 19 - 20 Oct., p. 3, **2017**.

7. Klisurov R., **Dragomanova S.**, Tancheva L., Kalfin R. Study on the neuroprotective activity of Myrtenal on rats. ***Abstract Book*** *of 2nd International Biomedical Congress*, Sofia, Bulgaria, 17 - 19 Nov, p. 40, **2017**.

8. Tzvetanova E., Alexandrova A., Tancheva L., Lazarova M., **Dragomanova S.**, Alova L., Stefanova M., Kalfin R. Antioxidant mechanisms in neuroprotective action of Lipoic acid on learning and memory of rats with experimental dementia. ***Abstract Book ICBA 2017***, *1st International Congress of Bio-Antioxidants*, Sofia, Bulgaria; 25 - 27 June, p. 54, **2017**, http://icba2017.orgchm.bas.bg.

9. Dragomanova S., Tancheva L., Georgieva M., Georgieva A., Kalfin R. Neuroprotective mechanisms of combination Myrtenal and Lipoic Acid on experimental Alzheimer's disease model. ***Abstract Book ICBA 2017***, *1st International Congress of Bio-Antioxidants*, Sofia, Bulgaria, 25 - 27 June, p. 84, **2017**, http://icba2017.orgchm.bas.bg.

10. Dragomanova S., Tancheva L., Georgieva M., Kalfin R. Myrtenal effects in pain experimental mice models. *IV Pharmaceutical Business Forum with Scientiffic Conference, Varna, Bulgariaя*, Oct 27 - 29, **2017**.

11. Stoeva S., Georgieva M., **Dragomanova S.**, Tancheva L. Anxiolytic and sedative properties of Myrtenal in experimental rodents. *IV Pharmaceutical Business Forum with Scientiffic Conference, Varna, Bulgariaя*, Oct 27 - 29, **2017**.

12. Tancheva L., Lazarova M., Alexandrova A., Tzvetanova E., **Dragomanova S.**, Alova L., Stefanova M., Kalfin R. Reversibility of the oxidative brain damages and cognitive deficit after scopolamine treatment of rats. *2nd International Conference on Bio-antioxidants (BIO-ANTIOXIDANTS 2018)*, Varna, Bulgaria, 07 - 10 September **2018**.

13. Klisurov R., **Dragomanova S.**, Tancheva L., Kalfin R. Myrtenal changes the oxidative status and the lipid profile in experimental rats. 2nd International *Conference on Bio-antioxidants (BIO-ANTIOXIDANTS 2018)*, Varna, Bulgaria, 07 - 10 September **2018**.

14. Dragomanova S., Tancheva L., Alexandrova A., Tzvetanova E., Lazarova M., Pavlova A., Alova L., Stefanova M., Georgieva M., Kalfin R. Effects of Myrtenal on brain oxidative status in rats with experimental dementia. *2nd International Conference on Bio-antioxidants (BIO-ANTIOXIDANTS 2018)*, Varna, Bulgaria, 07 - 10 September **2018**.

15. Dragomanova S., Klisurov R., Georgieva M., Lazarova M., Dishovsky C., Kalfin R., Tancheva L. Effect of Myrtenal on social behavior and memory of rats. ***Abstract Book*** *of 10th Congress of Toxicology in Developing Countries (CTDC10) and 12th Congress of the Serbian Society of Toxicology (12th SCT)*, Belgrade, Serbia, April 18 - 21, p. 116, **2018**.

16. Dragomanova S., Tancheva L., Lazarova M., Alexandrova A., Tzvetanova E., Klisurov R., Kalfin R. Complex mechanisms of neuroprotective action of myrtenal in an experimental model of dementia. *Scientific Conference with International Participation "Neuroscience, Bioinformatics, Microbiome and Beyond"*, Dedicated to the 150th Anniversary of the Bulgarian Academy of Sciences, Bachinovo, Bulgaria, 17 - 19 September **2019**.

17. Dragomanova S., Tancheva L., Alexandrova A., Lazarova M., Tzvetanova E., Kalfin R. Antioxidant effect of Myrtenal in rodents with experimental dementia. *3rd International Conference on Bio-antioxidants (BIO-ANTIOXIDANTS 2019)*, Nessebar, Bulgaria, 17 - 21 September **2019**, https://www.bio-antioxidants2019.com.

3. Participation in scientific projects on thesis work

Coordinator of a project under the Science Fund at Medical University of Varna "Prof. Dr. P. Stoyanov", on the topic "Preventive effects of natural monoterpenes on memory disorders of experimental rodents", 2014 – 2018, № 14014.

4. Citations

A. Tzvetanova E., Georgieva A., Alexandrova A., Tancheva L., Lazarova M., **Dragomanova S.**, Alova L., Stefanova M., Kalfin R. Antioxidant mechanisms in neuroprotective action of lipoic acid on learning and memory of rats with experimental dementia. *Bulgarian Chemical Communications*, 50, Special Issue C: 52 – 57, **2018.**

1. Lazarova M., Popatanasov A., Klissurov R., Stoeva S., Pajpanova T., Kalfin., Tancheva L. Preventive Effect of Two New Neurotensin Analogues on Parkinson's Disease Rat Model. Journal of Molecular Neuroscience. **2018**, 66. 10.1007/s12031-018-1171-6.
2. Monteiro S., Santos, Fernanda C., Romeiro R., Azulay C., Cerqueira G., Monteiro M., Barreira J. Review Article Mitochondrial Dysfunction and Alpha-Lipoic Acid: Beneficial or Harmful in Alzheimer's Disease?. Oxidative Medicine and Cellular Longevity **2019**, Article ID 8409329, 14 pages, https://doi.org/10.1155/2019/8409329.

B. Dragomanova S., Tancheva L., Georgieva M. A review: Biological activity of Myrtenal and some Myrtenal-containing medicinal plant essential oils. *Scripta Scientifica Pharmaceutica***,** [S.l.], 5(2): 22 - 33, Dec. **2018**, ISSN 2367-5500. doi: http://dx.doi.org/10.14748/ssp.v5i2.5614.

3. Lazarova M., Popatanasov A., Klissurov R., Stoeva S., Pajpanova T., Kalfin R., Tancheva L. Preventive Effect of Two New Neurotensin Analogues on Parkinson's Disease Rat Model. Journal of Molecular Neuroscience, **2018**, 66. 10.1007/s12031-018-1171-6.
4. Il'ina I., Morozova E., Pavlova A., Korchagina D., Tolstikova T., Volcho K., Salakhutdinov N. Synthesis and analgesic activity of aliphatic ketones-derived chiral

hexahydro-2*H*-chromenes. *Med Chem Res*. **2020**, https://doi.org/10.1007/s00044-020-02518-3.

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