Medical university "Prof. Dr. Paraskev Stoyanov" – Varna

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NEUROPHARMACOLOGICAL INVESTIGATION OF MYRTENAL CONJUGATES WITH AMINOADAMANTANE

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The defense materials are published on the website of the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna.

CONTENT

List of the most commonly used abbreviations

5-HT – 5-Hydroxytryptamine (serotonin)
6-OHDA – 6-Hydroxy Dopamine
ACh – Acetylcholine
AChE – Acetylcholine esterase
AD – Alzheimer's disease
ALP – Alkaline phosphatase
ALT – Alanine transaminase
AST – Aspartate transaminase
ATP – Adenosine triphosphate
Aβ – Amyloid β
BChE – Butyrylcholinesterase
CAT – Catalase
CD – Cyclodextrin
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
GABA – Gamma-aminobutyric acid
GPx – Glutathione peroxidase
GR – Glutathione reductase
LPO – Lipid peroxidation
M – Myrtenal
MAC – Myrtenal-adamantane conjugate
MDA – Malondialdehyde
MPTP – 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridin
NA – Noradrenaline
NMDA – N-methyl-D-aspartate
PD – Parkinson's disease
SOD – Superoxide dismutase
TBA – Thiobarbituric acid
tGSH – Total glutathione
TTX – Tetrodotoxin

I. INTRODUCTION

Neurodegenerative diseases are complex. They are characterized by a multifactorial pathoetiology (Ferreira et al., 2010). Nowadays, research is focused on the discovery of new therapeutic agents with more than one target of pharmacological action. This is based on the understanding that innovative, multi-targeted ligands can more effectively counteract the complexity of pathological processes in neurodegenerative diseases, in order to overcome the challenges of a polypharmacological approach (Bolognesi, 2008). The search for potential pharmacological agents with a complex mechanism of action involves various substances, most of which are natural compounds (Ji et al., 2008; Li et al., 2015; Dey et al., 2017).

Essential oils have been used in folk medicine as medicine for many years before their scientific research began. Some of their components have biological activity and are used as drugs - taxanes (with antitumor activity), artemisin (with antimalarial action) and others. Some terpenes have potential beneficial effects in Alzheimer's disease (Yoo and Park, 2012). Myrtenal is a bicyclic monoterpenoid of natural origin. Medicinal plants that contain it in their essential oils have a wide range of biological properties. In the 20th century, numerous effects of myrtenal were discovered on experimental animals – bronchodilator, anti-inflammatory, antiaggregant and antihemolytic (*in vitro*), antibacterial (against G (+) pathogens), antihyperglycemic, analgesic.

Myrtenal is relatively poorly studied in the field of neuroscience. Its potential to favorably affect various systems and organs, including CNS functions, is discussed in the review by Dragomanova et al. (2018). Neuromodulatory and antioxidant properties of the monoterpene, as an element of its neuroprotective action, were established for the first time in two experimental models of neurodegeneration in rats – 6-OHDA-induced parkinsonism (Tancheva et al., 2020) and chemically induced dementia (Dragomanova et al., 2022). Myrtenal has a preventive effect on the oxidative damage in the brain induced by the toxic agent scopolamine in an experimental model of dementia, confirmed by behavioral tests in experimental rodents, biochemical and histopathological studies, which show its potential to reduce the severity of symptoms of neurodegeneration and loss of neurons in the cerebral cortex. For the first time, as a result of the research conducted by Dragomanova (2020), its complex mechanism of action was established, demonstrated with neuromodulatory and antioxidant protective effects on neurodegenerative processes in experimental rodents.

The discovery of adamantane in petroleum in 1933 by the Czech chemists Landa, Machacek and Mzourek marked the beginning of a new field in chemistry devoted to the synthesis and properties of polyhedral organic compounds, which have found practical applications in the creation of drugs, polymeric materials and thermally stable lubricants. In pharmaceutical practice, a small number of medicinal substances containing an adamantane radical in their molecule have been approved. These are the antivirals Amantadine and Rimantadine, Memantine for dementia conditions, the hypoglycemic dipeptidyl peptidase IV inhibitors (Saxagliptine, Vildagliptine and others), the antiherpetic Tromantadine, Adapalene for the treatment of acne and the recently approved Arterolane for Plasmodium falciparum infections.

The attention of the scientific community is directed to a thorough study of the mechanisms of action of all adamantane derivatives applied in medical practice. It continues the synthesis of new compounds and the evaluation of their biological properties in preclinical and clinical studies in various therapeutic areas. Adamantane derivatives are an example of polycyclic compounds that represent a basic structure for the design and development of potential drugs in a wide range of diseases, applied not only as monotherapy, but also as an adjunct to improve the stability and lipophilicity of established drugs.

To date, numerous adamantane-based compounds with significant biological activities have been synthesized. The review by Lamoureux G. and Artavia G. (2010) covers a wide range of substances containing an adamantane structure with pronounced biological effects - antiviral, antibacterial, antimycotic and trypanocidal, anti-inflammatory, analgesic, antiulcer, antidepressant, anxiolytic, anticonvulsant, antiparkinsonian, neuroleptic , immunostimulating, antitumor, hypoglycemic, dilating cerebral vessels, antihypertensive, antioxidant and others. Some of them have the potential to positively affect multiple sclerosis, peripheral neuropathy, addictions, schizophrenia, neurological and neurodegenerative diseases, asthma and others. The adamantane core has been used to achieve a specific spatial organization of the functional groups that make up the pharmacophore of the basic chemical structure, with the aim of enhancing the features essential for molecular recognition in the interaction of the ligand with the target under physiological conditions.

It is known that the chemical modification of natural substances by conjugation with various synthetic components is a modern and reliable method for obtaining new biologically active compounds. It has been established that in many cases the medicinal properties of the obtained derivatives are more pronounced than those of the starting substances and may even exceed the effects of the standards used in various therapeutic areas. Widespread monoterpenes as natural products are the object of increased attention in the search for potential medicinal properties for the needs of various branches of medicine and pharmacy. Therefore, the inclusion of available compounds from this group as building blocks in the design and synthesis of new and effective therapeutic agents in various diseases is a promising and progressive direction in experimental pharmacology. Currently, the information in the world scientific literature about established positive effects on models of CNS-damages is scarce. No studies related to the study of the neuroprotective potential of myrtenal conjugates in an experimental model of dementia were also found.

The two compounds of myrtenal with aminoadamantane, the subject of research in this thesis, were designed and synthesized to avoid some disadvantages of the natural monoterpene in order to increase its biological efficacy. Due to the proven complex neuroprotective effect of the substance, not only analogous but also better properties are expected from its aminoadamantane derivatives, which are yet to be studied in vivo in experimental animal models. Conducting various neuropharmacological studies, important for studying the effects of both compounds in intact and demented experimental animals, could contribute to the discovery of specific mechanisms of action in neurodegenerative damages.

II. OBJECTIVE AND TASKS

The objective of the dissertation is to study *in vivo* the potential neuropharmacological effects of synthetic myrtenal conjugates with aminoadamantane and to reveal their main neuroprotective mechanisms on an experimental model of dementia of the Alzheimer type in rats.

The following tasks arise from the goal formulated:

1. Study of the physicochemical parameters of myrtenal conjugates with aminoadamantane; *in silico* analysis to predict their neurobiological effects and the theoretical possibilities for their binding to biological targets.

2. A study of the neuropharmacological effects of myrtenal derivatives in healthy experimental rats.

2.1. Study of the influence of substances on memory, learning and research activity after their repeated application.

2.2. Biochemical mechanisms of influence in memory-related brain structures.

2.2.1. Antioxidant activity – determination of LPO, GSH, CAT, SOD, GPx.

2.2.2. Changes in acetylcholinesterase activity.

2.2.3. Neuromodulatory properties of the compounds (NA and 5-HT content).

3. Study of the potential preventive effects of the myrtenal derivatives on rats with experimental dementia of the Alzheimer type.

3.1. Study of the memory-restoring abilities of the substances in demented rats with scopolamine-induced brain damage.

3.2. Biochemical mechanisms of the neuroprotective action of the myrtenal derivatives in demented rats with scopolamine-induced memory impairment.

3.2.1. Antioxidant activity – determination of LPO, GSH, CAT, SOD, GPx.

3.2.2. Anticholinesterase activity of the substances.

3.2.3. Neuromodulatory properties of the compounds (NA and 5-HT content).

4. Comparing the potency of the neuroprotective effects of synthetic aminoadamantane conjugates with natural myrtenal in healthy and demented experimental rodents.

III. MATERIALS AND METHODS

1. Experimental animals

The experiments were carried out on male mature Wistar rats (180 ÷ 220 g). The animals were raised under standard laboratory conditions in plastic cages – 12-hour light/dark cycle, unlimited access to drinking water and food for rodents, optimal temperature, humidity and ventilation of the premises. The experimental procedures were performed in accordance with the rules for handling experimental animals (Ordinance No. 20 of 01.11.2012 on the minimum requirements for the protection and humane treatment of experimental animals and the requirements for facilities for their use, breeding and/or delivery, effective as of 01.01.2013, issued by the Ministry of Agriculture and foods, Promulgated in the State Gazette No. 87 of 09.11.2012) and in accordance with the rules of the Ethics Committee of the Institute of Neurobiology at the Bulgarian Academy of Sciences and the Ethics Committee of Scientific Research at the Medical University "Prof. Dr. P. Stoyanov" - Varna.

Ninety rats were used for the purpose of the study. The rodents were randomly assigned to groups of 10 each. The research was conducted in two phases. The first involved investigating the effects of myrtenal conjugates in intact rodents (n = 40) and the second – in animals with scopolamine-induced dementia of the Alzheimer's type (n = 50). Healthy animals were divided into the following groups: controls (saline), myrtenal (M) (40 mg/kg), MAC-197 (1 mg/kg), MAC-198 (1 mg/kg). In the second stage of the experiment, the rats were divided as follows: controls (saline), scopolamine (Sc) (1 mg/kg), Sc + M, Sc + MAC-197, Sc + MAC-198.

Solutions of the respective substances were administered intraperitoneally, daily, for 11 days, in separate inoculations. They were prepared according to the rules of good laboratory practice for the preparation of extemporaneous dosage forms, described below (item 2.). The experimental protocol for inducing a chemically induced Alzheimer's type dementia model involved daily intraperitoneal treatment of laboratory rats with scopolamine at the same time of day, for a period of 11 days, at a dose of 1.0 mg/kg body weight. Standardly, in rats, the scopolamine solution is introduced in a volume of 0.5 ml/100 g.

The experiment began with initial training in the behavioral tests used to monitor the condition of the animals described below (item 4.). On the 24th hour after the last treatment, the relevant behavioral tests were conducted – for memory and learning, and for spatial orientation. Rodents were euthanized in compliance with the requirements for ethical treatment of experimental animals. Each rat's brain was prepared as quickly as possible by separating the two brain structures associated with memory processes, the hippocampus and the cortex.

After processing according to the methods described below (item 3.4. Biochemical studies), the samples were prepared to determine the levels of lipid peroxidation products; total glutathione content; the activity of the enzymes catalase, superoxide dismutase and glutathione peroxidase; the activity of brain acetylcholinesterase and for content of the neurotransmitters noradrenaline and serotonin.

2. Pharmacological agents – substances, solutions and emulsions

2.1. Substances

For the purposes of the study, the following were used:

- Scopolamine, ACRÔS Organics, Lot: A0354964
- (-)-Myrtenal 98%, ACRÔS Organics, Lot: A0363097

Myrtenal (M), is a fat-soluble, thermolabile and volatile compound in a liquid aggregate state, with a pleasant aroma. It is stored in well-closed packages in a refrigerator, at a temperature of 2÷8°C.

- Myrtenal-aminoadamantane conjugates MAC-197 и MAC-198

The two compounds MAC-197 and MAC-198, which are the subject of research in the dissertation work, were synthesized in the Department of Medicinal Chemistry of the Novosibirsk Institute of Organic Chemistry of the Russian Academy of Sciences, by reaction of (-)-myrtenal with 2-aminoadamantane (with code no. 197) or 1-aminoadamantane (with code no. 198), followed by $NaBH_4$ reduction of the corresponding amines. The resulting derivatives were transformed into the corresponding hydrochlorides by bubbling gaseous HCl through an ethereal solution of the amines and filtering the resulting precipitate. Spectral analyzes of the conjugates were performed at Keele University, UK. The results were published by Dragomanova et al. (2022).

2.2. Scopolamine solution

Scopolamine solution with a concentration of 20% is prepared in water for injections. Immediately before the treatment of the experimental rats, it was filtered through a membrane filter with a pore size of 0.22 μm, after which it was injected in a volume of 0.5 ml/100 g of body weight.

2.3. Myrtenal emulsion of the oil/water type

The myrtenal emulsion required for the purposes of the study was prepared by placing 1 ml of myrtenal (1.0268 g) in a tube fitted with a stopper, to which 0.05 g of α-lecithin was added, followed by homogenization in an ultrasonic bath. The resulting homogenous mixture was diluted with saline (NaCl 0.9%) to a volume of 5 ml to obtain a 20% emulsion of myrtenal, which was homogenized again to obtain a white milky dispersion. Two milliliters of it were diluted to 50 ml with physiological solution, whereby an emulsion with a concentration of 0.8% myrtenal was obtained, consistent with the used effective dose of the compound in rats (40 mg/kg body weight). According to the experimental protocol, the emulsion was prepared

ex tempore and immediately injected into the experimental rodents intraperitoneally, in a volume of 0.5 ml/100 g b.w.

2.4. Solutions of MAC-197 and MAC-198

Myrtenal conjugates with aminoadamantane MAC-197 and MAC-198 are secondary amines by their chemical structure. In the present study, they were administered in the form of hydrochloride salts, which are characterized by chemical stability in an acidic environment and differ in physicochemical aspects from myrtenal. They were used in the form of solutions in NaCl 0.9%, in which dimethylsulfoxide (DMSO) also participates as a co-solvent. The effective dose of the compounds was 1 mg/kg body weight (Avgustinovich et al., 2014). To prepare the required solutions, 3 mg of each substance was dissolved in 15 ml of saline (NaCl 0.9%), and the amount of DMSO calculated for the preparation of 1-, 3-, and 5-percent solutions was 150, 450, and 750 mg respectively. The solutions thus obtained were filtered through a membrane filter with a pore size of 0.22 μm. In the present work, the content of DMSO in the three solutions (1%, 3% and 5%) is consistent with the literature data for an appropriate co-solvent concentration for intraperitoneal administration (2.16% DMSO solution is isoosmotic with blood serum) (Rowe, Sheskey and Owen, 2006).

3. Methods

3.1. Software and docking studies

3.1.1. Physicochemical parameters of myrtenal conjugates with aminoadamantane

The QikProp 6.2 (2009) package (Schrödinger: New York, NY, USA) was used to calculate the molecular descriptors, and its reliability was verified for the calculated descriptors (Ioakimidis et al., 2008). Known Drug Indices (KDI) were calculated from the molecular descriptor values as described by Eurtivong and Reynisson (2018). The values of each property are presented in a separate column, and for the purposes of Excel processing and to calculate the KDI for each descriptor, the following equations are used: KDI MW: = EXP(−((MW−371.76)ˆ2)/(2*(112.76ˆ2))), KDI Log P: = EXP(−((LogP−2.82)ˆ2)/(2*(2.21ˆ2))), KDI HD: = EXP(−((HD−1.88)ˆ2)/(2*(1.7ˆ2))), KDI HA: = EXP(−((HA−5.72)ˆ2)/(2*(2.86ˆ2))), KDI RB = EXP(−((RB−4.44)ˆ2)/(2*(3.55ˆ2))), KDI PSA: = EXP(−((PSA−79.4)ˆ2)/(2*(54.16ˆ2))). The equations are copied into Excel and the descriptor name (e.g. MW) is replaced with the value in the corresponding column. To derive KDI2A, the equation was used: = (KDI MW + KDI LogP + KDIHD + KDI HA + KDI RB + KDI PSA), a за KDI2B - = (KDI MW ×KDI LogP × KDI HD × KDI HA × KDI RB × KDI PSA).

The research was carried out at Keele University, UK.

3.1.2. *In silico* analysis for predicting the biological effect and theoretical evaluation of the binding possibilities of the myrtenal conjugates with different biological targets

In silico pharmacology (computational pharmacology) uses software products to analyze and integrate biological and medical data from many different sources in order to make predictions using computational approaches. They have an important role in narrowing down the set of potential targets and suggesting secondary targets for known molecules.

For the purposes of the present work, the web-based software SwissADME (http://www.swissadme.ch/) developed by the Swiss Institute of Bioinformatics was used. The SwissTargetPrediction module has been validated and helps to track the possibilities of binding the studied conjugates to different biological targets (enzymes and receptors) (Diana et al., 2019). It is based on the observation that similar bioactive molecules are more likely to share similar biological targets. The most likely macromolecular targets for small molecules with potential biological activity are determined. The prediction is a combination of similarity studies between 3D and 2D structures and their comparison with library data of 376,342 known active substances on 3,068 proteins, with 580,496 possible binding sites.

The obtained probability values (in %) are calculated based on the combined results for the compounds that have the greatest similarity in the 3D and 2D structures to those of the studied molecule (above 0.85 for 3D or above 0.65 for 2D), and for which it is known , that they have an affinity for a given target. It is important to note that this coefficient illustrates the probability that a molecule will bind to a particular target, but not its potential for biological effect. The tabulated "3D/2D" values show how many of the known actives for each listed target are similar to the molecule under study based on structural similarity. Such predictions can be made for different organisms. For the purposes of the present study, theoretical analyzes were performed in human and rat species.

Homology-based predictions are made as follows: the requested molecule is compared to all target-binding molecules that exhibit homology to a protein in the selected organism. Predictions are then made assuming that the ligands of these proteins are the actual ligands of their homologues in the selected organism. If only binding of the ligand closest to the search molecule to a homologous protein is observed, the predictions are listed as "by homology" on the SwissTargetPrediction results page.

The predictions regarding the theoretical targets of the two myrtenal conjugates from the present study were carried out at the Department of Pharmaceutical Chemistry at the Faculty of Pharmacy of the Medical University – Varna.

3.1.3. Affinity of myrtenal conjugates for the acetylcholinesterase enzyme

The program Scigress version FJ 2.6 (Scigress Ultra V. FJ 2.6. 2016, Krakow, Poland) was used to construct the inhibitors, and the MM3 force field (Allinger et al., 1989; Lii and Allinger, 1989a; Lii and Allinger, 1989b) was applied to identify the global minimum using the CONFLEX method followed by structural optimization (Goto and Osawa, 1993). The docking center for the crystal structure of AChE (PDB ID: 5HF9, 2.20 Å resolution, Homo sapiens) is determined by the position of the oxime-substituted pyridine ring nitrogen of the

co-crystallized ligand 4-(aminocarbonyl)-1-[({2-[(E)-(hydroxyimino)methyl]pyridiunum-1-yl}methoxy) methyl]pydidinium (HI6) (x = 12.350, y = -55.749, z = -24.154) (Franklin et al., 2016). Fifty docking cycles were allowed for each ligand using a flexible search efficiency (200%) within a radius of 10 Å. The basic amino acids lysine and arginine are defined as protonated, with aspartic and glutamic acids assumed to be deprotonated. Using the GOLD v5.4.1 software package (Cambridge, UK), the scoring functions GoldScore (GS) (Jones et al., 1997), ChemScore (CS) (Eldridge et al., 1997; Verdonk et al., 2003), PiecewiseLinear Potential (ChemPLP) (Korb et al., 2009) and Astex Statistical Potential (ASP) (Mooij and Verdonk, 2005) were applied to predict the type of bonds and relative energies of the ligands.

The same software was used to prepare the crystal structure for docking, i.e. with the addition of hydrogen atoms, while co-crystallized ligands and crystallographic water molecules were removed. To establish the predictive power of the GOLD docking program, the co-crystallized HI6 ligand was re-docked into the binding sites. The root mean square deviations (RMSDs) were obtained by superimposing the tectonically and docked conformations and measuring the distance of their heavy atoms; the lower the value, the better the prediction, with RMSD < 1.0 Å considered a good indicator. The ChemPLP = 1.896 Å function has the best value, followed by ASP (8.423 Å), CS (6.937 Å) and GS (3.705 Å).

Analyzes were performed at Keele University, UK.

3.2. Physical stability study of myrtenal emulsion and solutions of MAC-197 and MAC-198

Of those obtained by the methods described in item 2.3. and 2.4., the emulsion of myrtenal with α-lecithin (M+L 1%) and the 6 solutions of the synthetic conjugates with the participation of a co-solvent – MAC-197 + DMSO 1%; MAC-197 + DMSO 3%; MAC-197 + DMSO 5%; MAC-198 + DMSO 1%; MAC-198 + DMSO 3%; MAC-198 + DMSO 5%, a total of 7 dispersions are prepared, which are subject to physical stability testing.

3.2.1. Analytical centrifugation (Seifert et al., 1991)

The myrtenal emulsion and the solutions of the two conjugates (in a volume of 1.5 ml of each sample) were centrifuged at room temperature - 5 cycles x 3000 rpm for 1 minute and 3 cycles x 15000 rpm for 1 minute. At the end of each cycle, a visual assessment was made for the presence of phase separation, after which the samples were cooled for one day, at a temperature of 2÷8°C. At hour 24, it was again monitored for detectable changes and the centrifugation protocol described was repeated. This was followed by rehomogenization of the preparations in an ultrasonic bath for 30 minutes, heating to 40°C in a water bath and repeating the analytical centrifugation, according to the protocol. At each stage of the work, the presence of changes in preparations was monitored and documented.

3.2.2. Study in a climatic chamber

From the 7 dispersions prepared in this way, 5 milliliters each were placed in a climatic chamber (Climatic chamber ARGO LAB, CH 150) at a temperature of 25°C (+/– 2°C) and a humidity of 60% (+/– 5%).

At 72 hours, the samples were monitored for detectable changes. The duration of the test protocol is 21 days, with observations taking place every 7 days.

The stability studies were carried out in the Scientific Laboratory "Technology of Pharmaceutical Forms" at the Department of "Pharmaceutical Technologies" at the Faculty of Pharmacy of the Medical University – Varna.

3.3. Behavioral tests

3.3.1. Mwmory and learnig test *(Step through/Passive Avoidance test)* (Jarvik and Kopp, 1967)

The test developed in 1986 by Venault et al., was used to determine the state of long-term memory. The experimental set-up consists of two parts - light and dark, separated by a partition, which must be initially open and closed when the animal enters the dark part. The goal is for the rats to learn to stay in the brightly lit compartment of the device, despite their instinctive preference for the dark chamber, to avoid the weak electric current flowing across its floor (0.1 A for 3 sec) that they feel with their paws. The latent time or the period after which the animal enters the dark chamber is calculated. A latency time of 180 seconds is used as a learning criterion.

The test is conducted in two stages – initial training before the start of treatment with the test compounds and a final examination 24 hours after the end of their application.

3.3.2. Exploratory behavior test *(Hole board test)* (Boissier and Simon, 1964)

The experimental protocol is based on the innate exploratory behavior of rodents placed in a novel environment. A characteristic phenomenon is the stereotypical behavior of "dipping" head to ear into the holes located on the floor of the stage. The frequency of this activity is an indicator of exploratory behavior. The lack of activity is considered to be a manifestation of anxiogenesis (a state of anxiety), and the increased number of activities is explained by a reduced level of anxiety and a manifestation of normal exploratory activity. The performance is an open platform (60 x 60 cm) surrounded by transparent walls. On the experimental field, 16 holes with a diameter of 3 cm were made, located at a distance of 10 cm (from center to center). The presence of a change in the behavior of the animals caused by the application of the investigated substances was monitored.

The test was conducted 24 hours after the last treatment, with each animal placed in silence on the uniformly illuminated field for a period of 3 minutes.

3.4. Biochemical tests

After conducting the behavioral tests, the rats were euthanized with carbon dioxide. Basic biochemical parameters in the brain were measured - levels of MDA and tGSH; activity of CAT, SOD, GPx; brain AChE activity; levels of the mediators NA and 5-HT.

3.4.1. Oxidative status in the cerebral cortex

The researches were carried out in the "Free Radical Processes" laboratory at the Institute of Neurobiology – Bulgarian Academy of Sciences.

3.4.1.1. Preparation of brain homogenate

After decapitation, the brain of each experimental animal was carefully removed, followed by washing with chilled 0.15 M KCl and separation of the cerebral cortex. Each of the thus obtained preparations was homogenized separately in chilled 0.15 M KCl – 10 mM potassium phosphate buffer (pH 7.4) and was centrifuged for 10 minutes at 3000 rpm at a temperature of 4°C. Determination of tGSH and MDA content in a portion of this postnuclear brain homogenate was performed. Another part of the homogenate was used to determine the activity of antioxidant enzymes (CAT, SOD, GPx). For this purpose, a supernatant was prepared by centrifugation at 12,000 rpm and a temperature of 4°C for 20 min.

3.4.1.2. Determination of protein content (Lowry, 1951)

The method is based on the known ability of copper ions in an alkaline environment to form complexes with the aromatic amino acids, such as tyrosine and tryptophan, with which they interact with high affinity. Folin's reagent was added to the brain homogenate samples, aiming to form a blue colored complex with an absorption peak at $\lambda = 700$ nm, the intensity of which was proportional to the amount of protein in the sample. A calibration curve obtained with bovine serum albumin (Pentex USA) was constructed to calculate the protein concentration (in mg/mL).

3.4.1.3. Determination of lipid peroxidation products levels (Hunter et al., 1963)

The method is based on the analysis of thiobarbituric acid reactive substances (TBARs), which are obtained as a by-product of lipid peroxidation, using TBA as a reagent. Malondialdehyde (MDA) present in the sample, which is one of several low molecular weight end products resulting from the decomposition of some primary and secondary products of lipid peroxidation, as well as generated from lipid hydroperoxides under the hydrolytic reaction conditions, was determined. In an acidic environment, at high temperature, a colored complex is formed with an absorption maximum at $\lambda = 532$ nm.

To prepare a solution with a protein concentration of 1 mg/mL, the separated postnuclear brain homogenate was diluted and the samples were incubated for 1 hour at a temperature of 37°C. A mixture of acids (2.8% TCA + 5N HCl + 2% TBA) was added to them in a ratio of 2:1:2, and then heated in a water bath for 15 minutes at the boiling temperature. This was followed by cooling the samples, centrifugation for 5 min at 3000 rpm and measurement at $\lambda = 532$ nm.

To calculate the content of lipid peroxidation products resulting from the reaction with TBA, a calibration curve was constructed using MDA as a standard. Results are reported as nmol malondialdehyde (MDA) per mg protein, using a molar absorption coefficient of 1.56 x 10^5 M^{-1} $см^{-1}$.

3.4.1.4. Determination of total glutathione content (tGSH) (Tietze, 1969)

The method is based on the conversion of oxidized glutathione to reduced glutathione upon interaction with NADPH, the reaction being catalyzed by GR:

$$\mathbf{GSSG + NADPH \rightarrow 2GSH + NADP}$$

The reaction of reduced glutathione with dithio-nitro benzoate (DTNB) gives a colored yellow compound with an absorption peak at $\lambda = 412$ nm. To determine the concentration of glutathione in the sample (in ng/mg protein) oxidized glutathione is used as a reference standard.

3.4.1.5. Determination of superoxide dismutase activity (Beauchamp and Fridovich, 1971)

Determination of Cu, Zn-superoxide dismutase (SOD) activity is based on the ability of the enzyme to catalyze the dismutation reaction of superoxide anion radicals to hydrogen peroxide:

$$\mathbf{2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2}$$

Reduction of nitroblue-tetrazolium (NBT) by superoxide anion radicals photochemically generated by riboflavin leads to the formation of an insoluble formazan, which stains the reaction medium blue. Inhibition of NBT reduction in the presence of the enzyme preparation was measured spectrophotometrically at $\lambda = 560$ nm. It was expressed in U/mg protein, taking as a unit of SOD activity the amount of enzyme that provides 50% inhibition of nitroblue-tetrazolium reduction.

A mixture of the necessary reagents (0.05 M potassium phosphate buffer (pH 7.8), 0.1 M methionine, 0.000044 M riboflavin, 0.001 M NBT and 0.03 M KCN) was prepared, which was placed in two set of cuvettes. In one group, increasing amounts of SOD were added. The solution in these cuvettes was illuminated at $\lambda = 560$ nm for 6 min, and the solution in the

other set of cuvettes, used as a control that did not contain enzyme preparation, was left in the dark for the same time of 6 min. The decrease in NBT reduction relative to that of the control was measured at λ = 560 nm.

3.4.1.6. Determination of catalase activity (Aebi, 1970)

Catalase is known to have the property of catalyzing the breakdown of hydrogen peroxide to water and molecular oxygen:

$$\mathbf{2H_2O_2 \rightarrow 2\ H_2O + O_2}$$

To measure catalase activity from the brain supernatant, a solution of H_2O_2 (10 mM) in 50 mM potassium phosphate buffer (pH 7.0) was prepared to which the enzyme was added. Against a blank sample at λ = 240 nm, the decrease in absorbance was reported, which was proportional to the degradation of H2O2 and is an indicator of the catalase activity, expressed as ΔA240/min/mg protein.

3.4.1.7. Determination of glutathione peroxidase activity (Günzler et al., 1972)

Glutathione peroxidase catalyzes the reduction of organic peroxides, which is carried out with the participation of reduced glutathione as a co-substrate:

$$\mathbf{ROOH + 2GSH \rightarrow ROH + 2GSSG + H_2O}$$

Oxidized glutathione is reduced in the presence of GR and NADPH by the reaction:

$$\mathbf{GSSG + NADPH \rightarrow GSH + NADP^+}$$

The oxidation of NADPH, which has an absorption peak at λ = 340 nm, is an indicator of glutathione peroxidase (GPx) activity, which is estimated by the decrease in absorbance at the same wavelength.

Brain supernatant samples were incubated in a reaction medium containing 0.05 M potassium phosphate buffer (pH 7.0), 1 mM EDTA; 1 mM NaN_3 ; 0.2 mM NADPH; 1 mM GSH and 1 U/mL GR, for 5 min, at room temperature, with the reaction starting after the addition of 0.0015 M t-butyl peroxide. The decrease in absorbance was reported at λ = 340 nm. Glutathione peroxidase activity was expressed as nmol NADPH oxidized per minute, per mg protein, with a molar extinction coefficient of 6.22 × 106 $M^{-1}cm^{-1}$.

3.4.2. Determination of acetylcholinesterase activity in the brain (cortex and hippocampus) (Ellman, 1961)

The studies were carried out in the Research Laboratory of the Institute of Neurobiology at the BAS, Department "Biological Effects of Natural and Synthetic Substances".

Cortical and hippocampal AChE activity was determined according to the protocol of Ellman et al. (1961). Brain supernatants were added to a solution containing 1.0 mM acetyl thiocholine (AcSCh), 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 100 mM phosphate buffer (pH 8.0) , then the samples were incubated for 5 min at 37°C. The intensity of the yellow coloration that results from the reaction of thiocholine with DTNB was measured spectrophotometrically at a wavelength of λ = 412 nm. Results were presented as AChE in μmol min/g protein.

3.4.3. Determination of monoamine content in brain (cortex and hippocampus)

The concentration of norepinephrine (NA) and serotonin (5-HT) in the cerebral cortex and hippocampus was measured by fluorescence reaction according to the method of Jacobowitz and Richardson (1978). Norepinephrine was extracted in phosphate buffer and 5-HT in 0.1 N HCl. For the required fluorescence of NA, the reaction requires ethylenediamine tetraacetic acid (EDTA), iodide solution, alkali sulfite, and 5N CH_3COOH, while for that of 5-HT, o-phthalaldehyde must be added. Monoamine concentration (in μg/g fresh tissue) was determined at λ = 385/485 nm for NA and λ = 360/470 nm for serotonin, calculated on the basis of fluorescence of a standard solution.

3.5. Statistical analysis of the results

Two sets of data, each of which consists of separate groups and subgroups of behavioral and biochemical indicators obtained in the course of the experimental study on rats, were subjected to statistical processing.

Results are expressed as means (±) standard error of the mean (SEM). Statistical analysis of data was performed by one-way analysis of variance (ANOVA) using GraphPadPrism 7.0 software (San Diego, CA 92108, USA).

When comparing the effects of the investigated substance in healthy and demented animals, the following approach was used: the magnitude of its effect under control conditions was calculated as the difference between the average value of the investigated parameters in healthy animals and the single values for rodents treated with myrtenal; similarly, for the lesion-induced rodents, a sample was obtained from the mean difference in the scopolamine controls and the single values in the demented animals treated with myrtenal. The two samples were compared statistically by t-Test for independent samples with different variance and two-tailed distribution.

The evaluation of the neuroprotective properties of myrtenal derivatives on a scopolamine-induced dementia model in rats was performed by means of an exploratory Principal Component Analysis (PCA) on standardized (z-transformed) values of the measured behavioral and neurochemical parameters. Animals from the following groups were used: control, scopolamine (Sc), Sk+M, Sk+MAC-197 and Sk+MAC-198. Missing values were replaced by group mean before standardization. All calculations and graphs required for the purposes of the PCA study were performed with the IBM SPSS 19.0 program. In addition, to confirm the clustering observed in PCA clustering, hierarchical cluster analysis (centroid clustering method, squared Euclidean distance) was applied.

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

IV. RESULTS AND DISCUSSION

1. Physicochemical characteristics of myrtenal conjugates. Investigation of the putative ability of the two myrtenal derivatives to cross the blood-brain barrier and their ability to affect the CNS based on their physicochemical properties

The calculated molecular descriptors – MW (molecular weight, g/mol), log P (water-octanol partition coefficient), HD (hydrogen bond donors), HA (hydrogen bond acceptors), PSA (polar surface area, Å2) and RB (rotating bonds) of the ligands are given in Table 1.

Table 1. Molecular descriptors of known drugs 2a and 2b (KDI2a/2b) with their respective indices for myrtenal and its derivatives

	RB	MW (g/mol)	HD	HA	Log *P*	PSA (Å²)	KDI_{2A}	KDI_{2B}
MAC-197	3	285.5	1	1.5	4.5	9.6	4.05	0.07
MAC-198	3	285.5	1	1	4.9	10.7	3.89	0.04
Myrtenal	1	150.2	0	2	1.8	36.6	3.38	0.01

All molecular descriptor values were in lead-like chemical space, except for log *P*. The two conjugates MAC-197 and MAC-198 have relatively high log *P* values, in the upper range limit for drug molecules (drug-like chemical space) due to their adamantine moiety. The range of these metrics as well as known drug space (KD) was published by Zhu et al. (2012).

The log *P* values indicate the increased lipophilicity of the conjugates compared to the natural product myrtenal. Relatively high values of log *P* and low values of HD, HA and PSA determine the optimal ability of the test substances to pass through the BBB.

For a more in-depth study of the effects of the molecular descriptors used, 208 compounds with experimentally established permeability of the blood-brain barrier were collected (Usansky et al., 2003; Garg and Verma, 2005; Guerra et al., 2008; Muehlbacher et al., 2011). These values are defined as the logarithmic ratio between their concentrations in the brain (c_{Brain}) and in the blood (c_{Blood}):

$$\log BB = log\left(\frac{c_{Brain}}{c_{Blood}}\right)$$

The values were correlated with the molecular descriptors, and the results are illustrated in Figure 1.

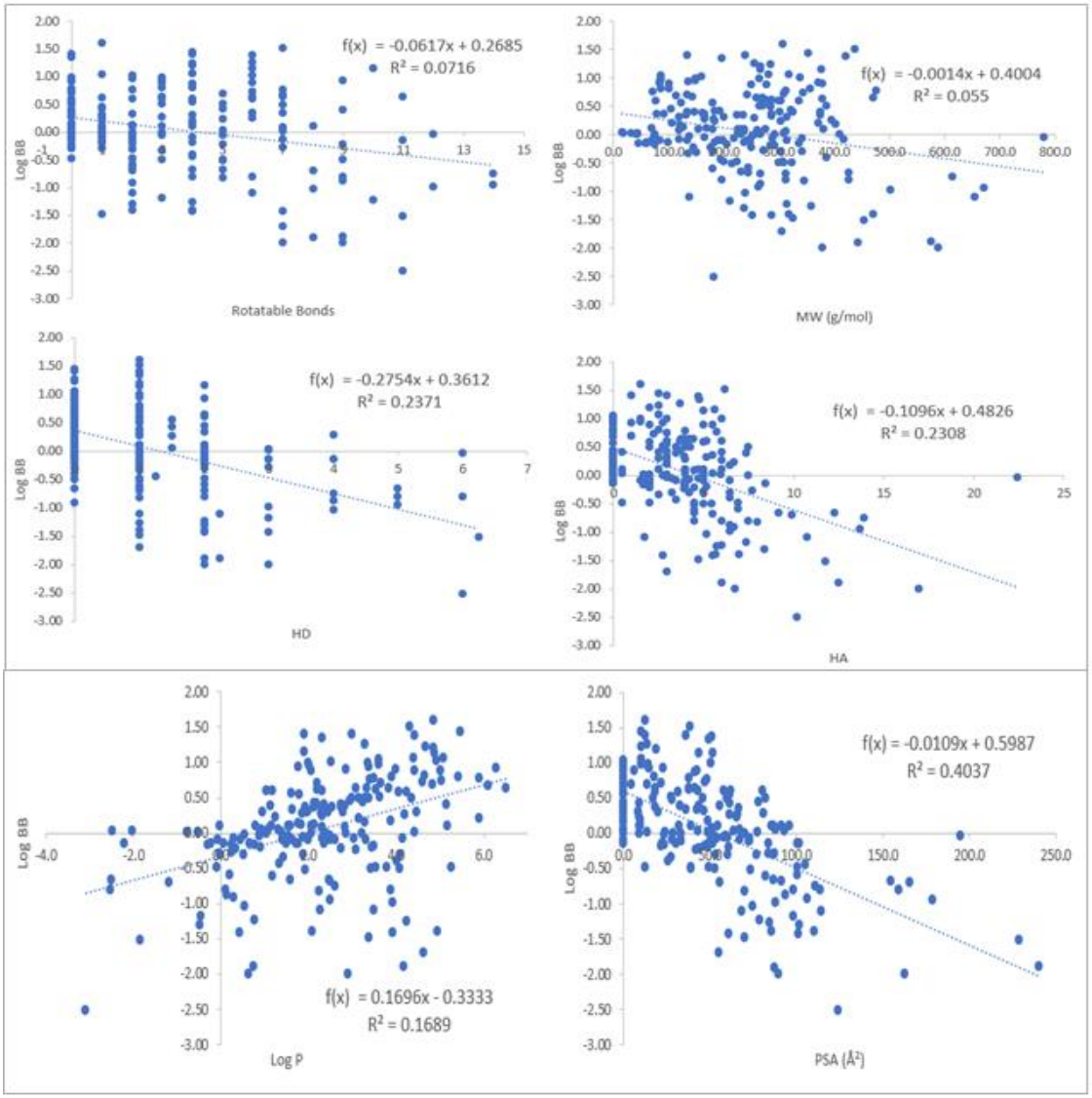


Figure 1. Correlations of molecular descriptors with experimentally obtained values of log BB (n = 208).

** Higher values of HD, HA and PSA make permeability more difficult, while higher values of log P facilitate it.*

When comparing the correlations and trends with the MAC-197 and MAC-198 values from the figure, it can be seen that the ligands are well localized in the property space. The results show that both compounds have relatively high values of log *P* and low values of HD, HA and PSA, which is estimated to be an optimal ability to pass through the blood-brain barrier.

The balance of molecular descriptors (MW, log *P*, HD, HA, PSA and RB) was assessed by calculating known drug indices (KDI) for the ligands. This method is based on the analysis of drugs in clinical use, by which the statistical distribution of each descriptor is fitted to a Gaussian function and normalized to unity, allowing the index to be calculated.

Both index summation (KDI2a) and multiplication (KDI2b) methods were used (Eurtivong and Reynisson, 2018), which is shown for KDI2a in Equation 1 and for KDI2b in Equation 2, and the numerical results are given in Table 1 (above):

$$KDI_{2a} = I_{MW} + I_{\log P} + I_{HD} + I_{HA} + I_{RB} + I_{PSA} \quad (1)$$

$$KDI_{2b} = I_{MW} \times I_{\log P} \times I_{HD} \times I_{HA} \times I_{RB} \times I_{PSA} \quad (2)$$

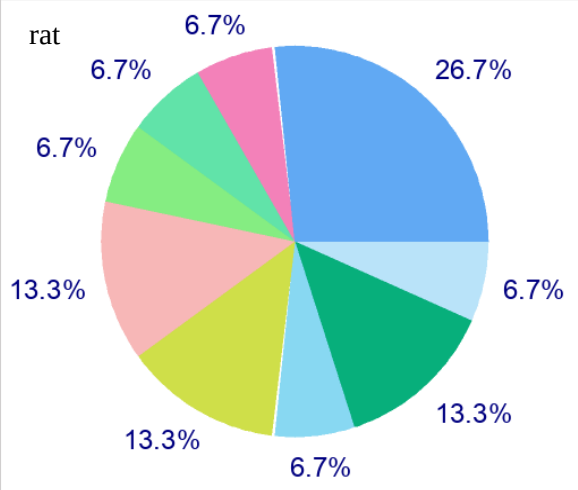
The KDI2a values for the ligands were, respectively, 4.05 (MAC-197), 3.89 (MAC-198), and 3.38 for M, with a theoretical maximum of 6, with an average for known drugs of 4.08 (±1 ,27). The collection of 1880 drug records of Eurtivong and Reynisson (2018) was analyzed. Of these, 245 have an indication for affecting CNS diseases.

The mean for CNS pharmaceuticals was 4.54 (±0.67), slightly higher than that for MAC-197 and MAC-198. KDI2b had values of 0.07 (MAC-197), 0.04 (MAC-198) and 0.01 for myrtenal, respectively, with a theoretical maximum of 1 and with a KDS mean of 0.18 (±0.20). CNS-active drugs had a mean of 0.21 (±0.19). The KDI2b metric is categorical because the parameters are multiplied, with a low number resulting in a low total value. The data allow to conclude that myrtenal derivatives have favorable physicochemical parameters for entering the CNS.

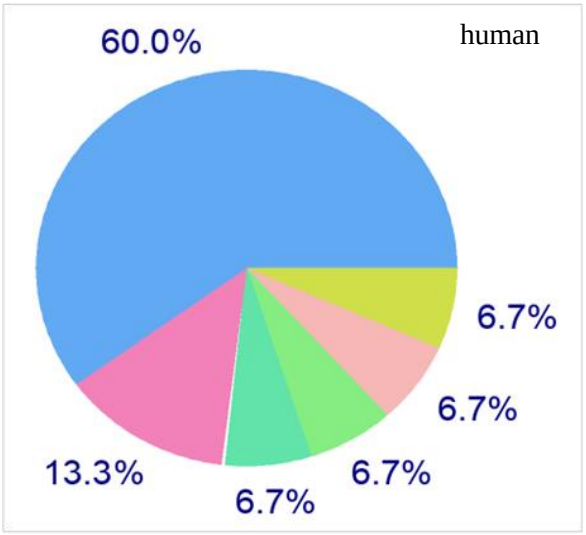
2. Prediction of potential targets of myrtenal aminoadamantane conjugates using SwissADME software

2.1. Predicting the potential targets of MAC-197 in both biological species – rat and human

Upon analysis of the results of the software study, it is striking that the main predicted binding targets of MAC-197 in rats and humans are of the G-protein-coupled receptor family, with the observed difference being associated with species (Figure 2).



26.7%	G-protein bound receptors
13.3%	Enzymes
13.3%	Kinases
13.3%	Voltage-gated ion channels
6.7%	Electrochemical transporters
6.7%	Isomerases
6.7%	Other cytosol proteins
6.7%	Ligand-operated ion channels
6.7%	Nuclear receptors



60.0%	G-protein bound receptors
13.3%	Hydrolases
6.7%	Electrochemical transporters
6.7%	Voltage-gated ion channels
6.7%	Enzymes
6.7%	Membrane receptors

Figure 2. Affinity of MAC-197 for predicted targets in rat and human

In experimental rats, the potential targets of the studied compound refer to various groups of biological structures such as G-protein coupled receptors, enzymes, voltage-gated ion channels and others, while in humans a greater affinity of MAC-197 to metabotropic receptors is predicted (60 %).

Table 2 provides a detailed list of targets and binding probability in laboratory rats to which MAC-197 exhibits affinity.

Table 2. Targets and predicted probability of binding to MAC-197 in rats

Target	Code (short name)	Target class	Probability,%
Muscarinic acetylcholine receptor M1 (by homology)	CHRM1	Family A G protein-coupled receptor	13.07919551770
Muscarinic acetylcholine receptor M2	CHRM2	Electrochemical transporter	13.07919551770
Alpha-2c adrenergic receptor (by homology)	ADRA2C	Nuclear receptor	9.78745343258
Ionotropic glutamate receptor NMDA 1	GRIN1	Isomerase	9.78745343258
Serotonin 1a (5-HT1a) receptor	HTR1A	Family A G protein-coupled receptor	9.78745343258
Serotonin 2a (5-HT2a) receptor (by homology)	HTR2A	Kinase	9.78745343258
Lanosterol synthase	LSS	Voltage-gated ion channel	9.78745343258
Sigma opioid receptor (by homology)	SIGMAR1	Other cytosolic protein	9.78745343258
Serotonin transporter (by homology)	SLC6A4	Enzyme	9.78745343258
Norepinephrine transporter	SLC6A2	Kinase	9.78745343258

Alpha-1a adrenergic receptor (by homology)	ADRA1A	Ligand-gated ion channel	9.78745343258
Alpha-1b adrenergic receptor	ADRA1B	Family A G protein-coupled receptor	9.78745343258
Squalene synthetase	FDFT1	Enzyme	9.78745343258
C-X-C chemokine receptor type 3 (by homology)	CXCR3	Voltage-gated ion channel	9.78745343258
Cyclooxygenase-2 (by homology)	PTGS2	Family A G protein-coupled receptor	9.78745343258
Muscarinic acetylcholine receptor M5 (by homology)	CHRM5	Lyase	9.78745343258
Muscarinic acetylcholine receptor M3 (by homology)	CHRM3	Family A G protein-coupled receptor	9.78745343258
Delta opioid receptor (by homology)	OPRD1	Family A G protein-coupled receptor	9.78745343258
Serotonin 1b (5-HT1b) receptor (by homology)	HTR1B	Family A G protein-coupled receptor	9.78745343258
Serotonin 1d (5-HT1d) receptor (by homology)	HTR1D	Electrochemical transporter	9.78745343258
Serotonin 7 (5-HT7) receptor	HTR7	Enzyme	9.78745343258
Transient receptor potential cation channel subfamily M member 8	TRPM8	Unclassified protein	9.78745343258
Neuronal acetylcholine receptor; alpha3/beta4	CHRNA3/CHRNB4	Voltage-gated ion channel	9.78745343258
Neuronal acetylcholine receptor; alpha2/beta4	CHRNB4	Ligand-gated ion channel	9.78745343258
Neuronal acetylcholine receptor protein alpha-7 subunit	CHRNA7	Hydrolase	9.78745343258
Mas-related G-protein coupled receptor member X1 (by homology)	MRGPRX1	Enzyme	9.78745343258
Histamine H3 receptor	HRH3	Family A G protein-coupled receptor	9.78745343258

The structure of MAC-197 has the highest binding potential to M1- and M2-cholinergic receptors, as well as to adrenergic, serotonergic, glutamatergic and opioid receptors, as well as to norepinephrine and serotonin transporters, in expression of its ability to influence CNS in rats.

The study of theoretical targets and the probability of binding in humans are presented in Table 3.

Table 3. Targets and predicted probability of binding to MAC-197 in humans

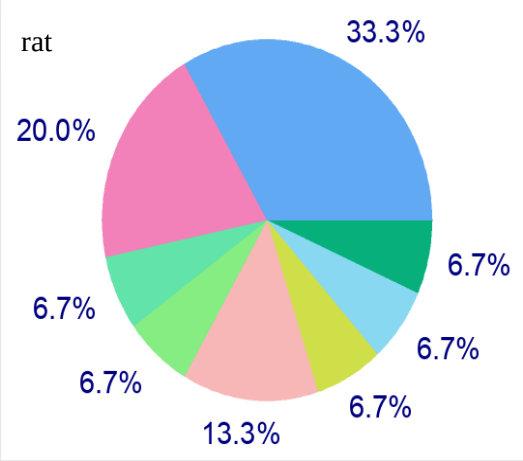
Target	Code (short name)	Target class	Probability,%
Muscarinic acetylcholine receptor M1	CHRM1	Family A G protein-coupled receptor	13.07919551770
Muscarinic acetylcholine receptor M2	CHRM2	Family A G protein-coupled receptor	13.07919551770
Butyrylcholinesterase	BCHE	Hydrolase	9.78745343258
Acetylcholinesterase	ACHE	Hydrolase	9.78745343258
Adrenergic receptor alpha-2	ADRA2C	Family A G protein-coupled receptor	9.78745343258
Serotonin 2a (5-HT2a) receptor	HTR2A	Family A G protein-coupled receptor	9.78745343258
Intermediate conductance calcium-activated potassium channel protein 4	KCNN4	Voltage-gated ion channel	9.78745343258
Lanosterol synthase	LSS	Enzyme	9.78745343258
Serotonin 2b (5-HT2b) receptor	HTR2B	Family A G protein-coupled receptor	9.78745343258
Serotonin 7 (5-HT7) receptor	HTR7	Family A G protein-coupled receptor	9.78745343258
Sigma opioid receptor	SIGMAR1	Membrane receptor	9.78745343258
Serotonin transporter	SLC6A4	Electrochemical transporter	9.78745343258
Alpha-1a adrenergic receptor	ADRA1A	Family A G protein-coupled receptor	9.78745343258
Dopamine D5 receptor	DRD5	Family A G protein-coupled receptor	9.78745343258
Dopamine D1 receptor	DRD1	Family A G protein-coupled receptor	9.78745343258
Alpha-1d adrenergic receptor	ADRA1D	Family A G protein-coupled receptor	9.78745343258
Serotonin 2c (5-HT2c) receptor	HTR2C	Family A G protein-coupled receptor	9.78745343258
Serotonin 6 (5-HT6) receptor	HTR6	Family A G protein-coupled receptor	9.78745343258
Cytochrome P450 3A4	CYP3A4	Cytochrome P450	9.78745343258
Alpha-2a adrenergic receptor	ADRA2A	Family A G protein-coupled receptor	9.78745343258
Geranylgeranyl pyrophosphate synthetase	GGPS1	Enzyme	9.78745343258
Neuronal acetylcholine receptor; alpha4/beta2	CHRNA4 CHRNB2	Ligand-gated ion channel	9.78745343258
Lysosomal Pro-X carboxypeptidase	PRCP	Protease	9.78745343258
HERG	KCNH2	Voltage-gated ion channel	9.78745343258
Serotonin 1a (5-HT1a) receptor	HTR1A	Family A G protein-coupled receptor	9.78745343258
Squalene synthetase (by homology)	FDFT1	Enzyme	9.78745343258

C-X-C chemokine receptor type 3	CXCR3	Family A G protein-coupled receptor	9.78745343258
Neuronal acetylcholine receptor; alpha3/beta4	CHRNA3 CHRNB4	Ligand-gated ion channel	9.78745343258
Cyclooxygenase-2 (by homology)	PTGS2	Oxidoreductase	9.78745343258
Muscarinic acetylcholine receptor M4	CHRM4	Family A G protein-coupled receptor	9.78745343258
Muscarinic acetylcholine receptor M5	CHRM5	Family A G protein-coupled receptor	9.7874534325
Muscarinic acetylcholine receptor M3	CHRM3	Family A G protein-coupled receptor	9.78745343258
Glutamate NMDA receptor; GRIN1/GRIN2B	GRIN1 GRIN2B	Ligand-gated ion channel	9.78745343258
Glutamate NMDA receptor; GRIN1/GRIN2A	GRIN2A GRIN1	Ligand-gated ion channel	9.78745343258
Delta opioid receptor	OPRD1	Family A G protein-coupled receptor	9.78745343258
Serotonin 1b (5-HT1b) receptor	HTR1B	Family A G protein-coupled receptor	9.78745343258
Serotonin 1d (5-HT1d) receptor	HTR1D	Family A G protein-coupled receptor	9.78745343258
Transient receptor potential cation channel subfamily M member 8 (by homology)	TRPM8	Voltage-gated ion channel	9.78745343258
Mas-related G-protein coupled receptor member X1	MRGPRX1	Unclassified protein	9.78745343258
Histamine H3 receptor	HRH3	Family A G protein-coupled receptor	9.78745343258
Glutamate [NMDA] receptor subunit epsilon 2	GRIN2B	Ligand-gated ion channel	9.78745343258

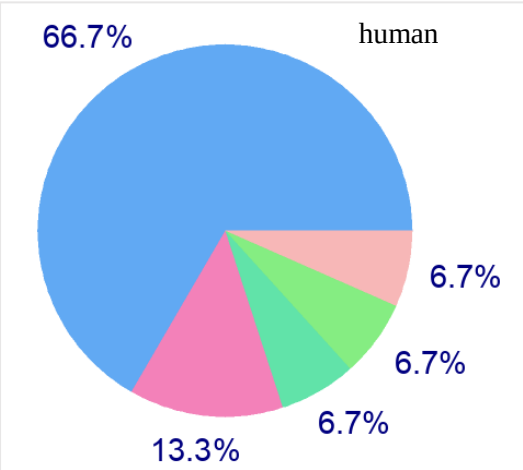
In humans, MAC-197 has additional potential to bind to acetylcholinesterase and pseudocholinesterase, subtype D1 and D5 dopamine receptors, and CYP 3A4, in contrast to rats. In comparison, MAS-197 has 27 potential targets in the rat, while 41 in the human.

2.2. Predicting the potential targets of MAC-198 in both biological species – rat and human

Analysis of the results of the software study revealed that both MAS-197 and MAS-198 showed a species-specific difference in the main binding targets in rats and humans (Figure 3).



33.3%	Enzymes
20.0%	G-protein-coupled receptors
13.3%	Other cytosolic proteins
6.7%	Electrochemical transporters
6.7%	Voltage-gated ion channels
6.7%	Nuclear receptors
6.7%	Fatty acid binding proteins
6.7%	Proteases



66.7%	G-protein-coupled receptors
13.3%	Hydrolases
6.7%	Membrane receptors
6.7%	Voltage-gated ion channels
6.7%	Electrochemical transporters

Figure 3. Affinity of MAC-187 for predicted targets in rat and human

Here again, in the human species, the prediction showed as the main group of potential targets the family of G-protein-coupled receptors (66.67% probability), as with the other myrtenal derivative. On the other hand, in rats, enzymes have the highest binding affinity for MAC-198 (33%), which is distinct from the main group of theoretical targets for MAC-197 in rats – G-protein-coupled receptors (23%).

Table 4 provides a detailed list of the predicted targets and binding probability in rats to which MAC-198 exhibits affinity.

Table 4. Targets and predicted probability of binding to MAC-198 in rats

Target	Code (short name)	Target class	Probability,%
Muscarinic acetylcholine receptor M1 (by homology)	CHRM1	Family A G protein-coupled receptor	13.0791955177
Muscarinic acetylcholine receptor M2	CHRM2	Electrochemical transporter	12.2581769115
Alpha-2c adrenergic receptor (by homology)	ADRA2C	Nuclear receptor	9.78745343258
Serotonin 1a (5-HT1a) receptor	HTR1A	Family A G protein-coupled receptor	9.78745343258

Ionotropic glutamate receptor NMDA 1	GRIN1	Isomerase	9.78745343258
Serotonin 2a (5-HT2a) receptor (by homology)	HTR2A	Kinase	9.78745343258
Lanosterol synthase	LSS	Voltage-gated ion channel	9.78745343258
Sigma opioid receptor (by homology)	SIGMAR1	Other cytosolic protein	9.78745343258
Norepinephrine transporter	SLC6A2	Kinase	9.78745343258
Serotonin transporter	SLC6A4	Enzyme	9.78745343258
Alpha-1a adrenergic receptor (by homology)	ADRA1A	Ligand-gated ion channel	9.78745343258
Alpha-1b adrenergic receptor	ADRA1B	Family A G protein-coupled receptor	9.78745343258
Histamine H3 receptor	HRH3	Family A G protein-coupled receptor	9.78745343258
C-X-C chemokine receptor type 3 (by homology)	CXCR3	Voltage-gated ion channel	9.78745343258
Squalene synthetase	FDFT1	Enzyme	9.78745343258
Muscarinic acetylcholine receptor M5 (by homology)	CHRM5	Lyase	9.78745343258
Muscarinic acetylcholine receptor M3 (by homology)	CHRM3	Family A G protein-coupled receptor	9.78745343258
Serotonin 1d (5-HT1d) receptor (by homology)	HTR1D	Electrochemical transporter	9.78745343258
Delta opioid receptor (by homology)	OPRD1	Family A G protein-coupled receptor	9.78745343258
Cyclooxygenase-2 (by homology)	PTGS2	Family A G protein-coupled receptor	9.78745343258
Serotonin 1b (5-HT1b) receptor (by homology)	HTR1B	Family A G protein-coupled receptor	9.78745343258
Mas-related G-protein coupled receptor member X1 (by homology)	MRGPRX1	Enzyme	9.78745343258
Transient receptor potential cation channel subfamily M member 8	TRPM8	Unclassified protein	9.78745343258
Serotonin 7 (5-HT7) receptor	HTR7	Enzyme	9.78745343258
Neuronal acetylcholine receptor; alpha3/beta4	CHRNA3/CHRNB4	Voltage-gated ion channel	9.78745343258
Neuronal acetylcholine receptor; alpha2/beta4	CHRNB4	Ligand-gated ion channel	9.78745343258
Neuronal acetylcholine receptor protein alpha-7 subunit	CHRNA7	Hydrolase	9.78745343258
Serotonin 2c (5-HT2c) receptor	HTR2C	Family A G protein-coupled receptor	9.78745343258
Dopamine D3 receptor (by homology)	DRD3	Enzyme	9.78745343258

It is important to note that in rats, MAC-198 does not have the affinity for binding to neurotransmitter transporters as seen with the other derivative MAC-197.

Investigation of the theoretical targets of MAC-198 in human species are presented in Table 5.

Table 5. Targets and predicted probability of binding to MAC-198 in humans

Target	Code (short name)	Target class	Probability,%
Muscarinic acetylcholine receptor M1	CHRM1	Family A G protein-coupled receptor	13.07919551770
Muscarinic acetylcholine receptor M2	CHRM2	Family A G protein-coupled receptor	12.25817691150
Butyrylcholinesterase	BCHE	Hydrolase	10.60999491330
Acetylcholinesterase	ACHE	Hydrolase	9.78745343258
Adrenergic receptor alpha-2	ADRA2C	Family A G protein-coupled receptor	9.78745343258
Serotonin 2a (5-HT2a) receptor	HTR2A	Family A G protein-coupled receptor	9.78745343258
Serotonin 2b (5-HT2b) receptor	HTR2B	Family A G protein-coupled receptor	9.78745343258
Serotonin 7 (5-HT7) receptor	HTR7	Family A G protein-coupled receptor	9.78745343258
Sigma opioid receptor	SIGMAR1	Membrane receptor	9.78745343258
Serotonin transporter (by homology)	SLC6A4	Electrochemical transporter	9.78745343258
Intermediate conductance calcium-activated potassium channel protein 4	KCNN4	Voltage-gated ion channel	9.78745343258
Alpha-1a adrenergic receptor	ADRA1A	Family A G protein-coupled receptor	9.78745343258
Dopamine D5 receptor	DRD5	Family A G protein-coupled receptor	9.78745343258
Dopamine D1 receptor	DRD1	Family A G protein-coupled receptor	9.78745343258
Alpha-1d adrenergic receptor	ADRA1D	Family A G protein-coupled receptor	9.78745343258
Serotonin 2c (5-HT2c) receptor	HTR2C	Family A G protein-coupled receptor	9.78745343258
Serotonin 6 (5-HT6) receptor	HTR6	Family A G protein-coupled receptor	9.78745343258
Cytochrome P450 3A4	CYP3A4	Cytochrome P450	9.78745343258
Alpha-2a adrenergic receptor	ADRA2A	Family A G protein-coupled receptor	9.78745343258
Geranylgeranyl pyrophosphate synthetase	GGPS1	Enzyme	9.78745343258
Histamine H3 receptor	HRH3	Family A G protein-coupled receptor	9.78745343258
Neuronal acetylcholine receptor; alpha4/beta2	CHRNA4 CHRNB2	Ligand-gated ion channel	9.78745343258
Lysosomal Pro-X carboxypeptidase	PRCP	Protease	9.78745343258
Serotonin 1a (5-HT1a) receptor	HTR1A	Family A G protein-coupled receptor	9.78745343258
HERG	KCNH2	Voltage-gated ion channel	9.78745343258

C-X-C chemokine receptor type 3	CXCR3	Family A G protein-coupled receptor	9.78745343258
Squalene synthetase (by homology)	FDFT1	Enzyme	9.78745343258
Glutamate NMDA receptor; GRIN1/GRIN2B	GRIN1 GRIN2B	Ligand-gated ion channel	9.78745343258
Glutamate NMDA receptor; GRIN1/GRIN2A	GRIN2A GRIN1	Ligand-gated ion channel	9.78745343258
Neuronal acetylcholine receptor; alpha3/beta4	CHRNA3 CHRNB4	Ligand-gated ion channel	9.78745343258
Muscarinic acetylcholine receptor M4	CHRM4	Family A G protein-coupled receptor	9.78745343258
Muscarinic acetylcholine receptor M5	CHRM5	Family A G protein-coupled receptor	9.78745343258
Muscarinic acetylcholine receptor M3	CHRM3	Family A G protein-coupled receptor	9.78745343258
Glutamate [NMDA] receptor subunit epsilon 2	GRIN2B	Ligand-gated ion channel	9.78745343258
Serotonin 1d (5-HT1d) receptor	HTR1D	Family A G protein-coupled receptor	9.78745343258
Delta opioid receptor	OPRD1	Family A G protein-coupled receptor	9.78745343258
Cyclooxygenase-2 (by homology)	PTGS2	Oxidoreductase	9.78745343258
Serotonin 1b (5-HT1b) receptor	HTR1B	Family A G protein-coupled receptor	9.78745343258
Mas-related G-protein coupled receptor member X1	MRGPRX1	Unclassified protein	9.78745343258
Transient receptor potential cation channel subfamily M member 8 (by homology)	TRPM8	Voltage-gated ion channel	9.78745343258
Dopamine D3 receptor	DRD3	Family A G protein-coupled receptor	9.78745343258

The potential targets of MAC-198 in rats are 29, and in humans – 41. The differences are related to the additional theoretical possibility of interaction of the compound with CYP 3A4, with acetylcholinesterase, butyrylcholinesterase, as well as with D_1-, D_5- and M_4-receptors in the human body.

The software analysis performed on the potential targets of the aminoadamantane myrtenal conjugates recorded interspecies differences (rat vs. human) as well as those determined by the chemical structure of the compounds. The affinities of the two derivatives in rats for the theoretical binding targets were similar, with MAC-198 reporting an additional ability to interact with the $5HT_{2C}$- and D_3-receptors compared to MAC-197. Regarding the potential targets in man, the two derivatives again showed similar binding potential. They differ in that MAC-197 has a characteristic affinity for H_3-histamine receptors, and for MAC-198 – for D_3-receptors. The compounds have the theoretical potential to bind to many structures in the CNS, thereby influencing various neurotransmitter systems and regulatory processes.

3. Physical stability of myrtenal emulsion and MAC-197 and MAC-198 solutions for intraperitoneal administration

The ***myrtenal emulsion*** showed first signs of physical instability at the 72nd hour from the start of the analytical centrifugation cycle (a decrease in the degree of milky opalescence in the upper layers of the tube), which deepened over time. After rehomogenization in an ultrasonic bath and centrifugation, a few oil droplets were observed and resuspended after shaking. Macroscopically, it is reported that the emulsion opalescents less.

On the 14th day of the 21-day study at a temperature of 25°C (+/– 2°C) and a humidity of 60% (+/– 5%) in the test tube with the myrtenal emulsion, larger oil drops were noticed at the bottom, which are retained until the end of the observation.

The ***solutions of the myrtenal conjugates with aminoadamantane*** in the three concentrations of the co-solvent DMSO used (1, 3 and 5%) remained unchanged until the end of the experiment.

The obtained results allowed to choose the lowest concentration of the co-solvent dimethylsulfoxide in the preparation of the working solutions for intraperitoneal administration, which in this case is 1%.

4. Experiments on intact rats

4.1. Effects of myrtenal conjugates on the general condition of the rodents

4.1.1. Change in body mass

The change in the body weights of the rats during the period of the experiment is an important indicator of the effect of the investigated substances on their general physiological status. The results are presented in Figure 4.

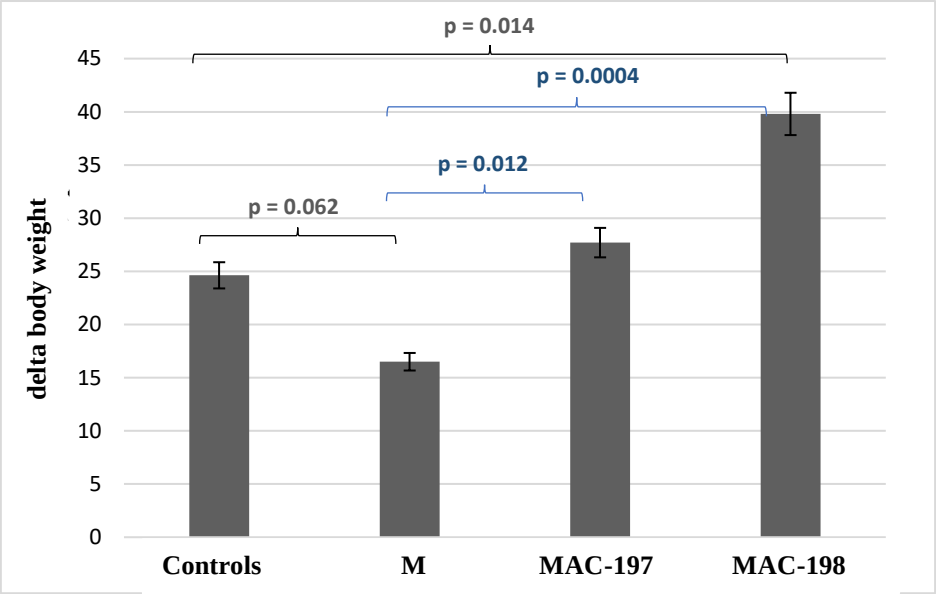


Figure 4. Change in body weights of rats after 11 days of administration of M (40 mg/kg), MAC-197 (1 mg/kg) u MAC-198 (1 mg/kg)

As expected, myrtenal led to a decrease in the weight of rodents compared to controls (with borderline significance), also found in previous studies by Dragomanova (2020). This, in our opinion, is the result of appetite suppression characteristic of all essential oils containing the natural monoterpene. It was not observed with its newly synthesized derivatives.

MAC-197 administration caused a minor change in body weight comparable to that of control animals, while the increase in weight relative to the myrtenal group was 67.9% (P = 0.012). After the application of MAC-198, the indicator was significantly increased, both compared to the control group – by 61.6% (P = 0.014), and compared to the myrtenal-treated rats - by 141.2% (P = 0.0004). This is probably due to the altered physicochemical properties of the derivatives, differing from those of the parent product in the absence of aroma and volatility, the main causes of appetite suppression by essential oils.

The results of this pilot observation represent a reference for the lack of general toxic effect of the two myrtenal derivatives after repeated intraperitoneal administration.

4.1.2. Macroscopic observation

After the test animals were euthanized, as part of the pilot studies, macroscopic observation was performed to detect noticeable damage to the main vital organs. No damage was detected in the heart, liver, kidneys, stomach, bile and intestines during the dissection of the rats from the individual groups.

4.2. Influence of the myrtenal-aminoadamantane derivatives MAC-197 and MAC-198 on the memory abilities of laboratory rodents

4.2.1. Short-term and long-term memory status *(Passive avoidance test)*

4.2.1.1. Latency time

Memory improvement was observed after single (Fig. 5 A) and repeated (Fig. 5 B) administration of the two myrtenal derivatives MAC-197 and MAC-198 in rats.

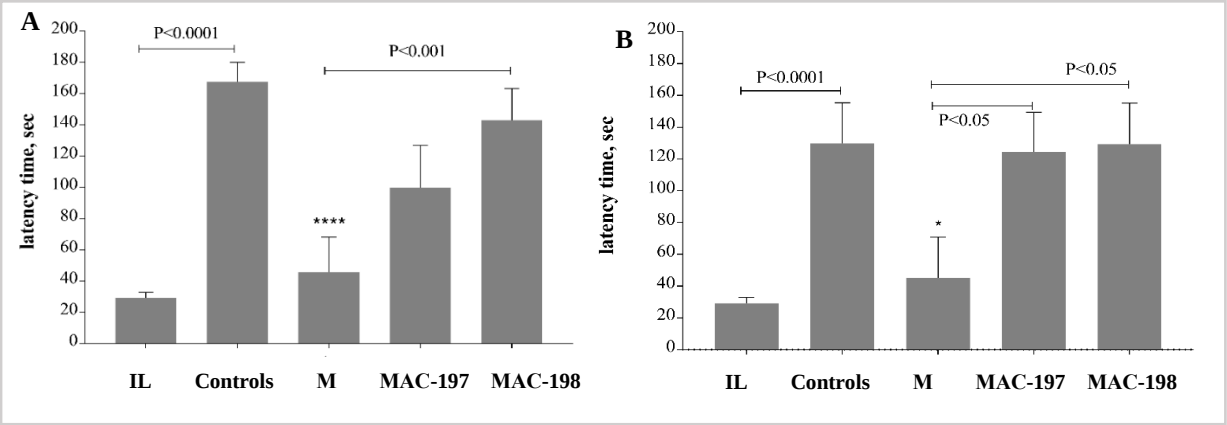


Figure 5. Effects of MAC-197 and MAC-198 on memory in rats after acute (A) and repeated treatment (B) – latency reading (sec); M (40 mg/kg) was used as reference; ****P < 0.0001, *P < 0.05 vs. controls

The control group had preserved memory in the acute experiment and, as expected, latency was significantly increased compared with IL ($P < 0.0001$) (Fig. 5 A). The results of our previous studies showing that myrtenal causes impairment of short-term and long-term memory in intact laboratory rodents, which we attribute to its effect on the GABA receptor and benzodiazepine-like hypomnesic properties, were confirmed (Dragomanova, 2020).

In the single administration, the monoterpene decreased the latency values by 72.8% compared to the control group ($P < 0.0001$). Acute treatment of rats with both adamantane conjugates of myrtenal resulted in an increase in latency. Administration of MAS-198 produced an effect comparable to that of controls, which differed from the myrtenal group at a high level of significance ($P < 0.001$).

After the repeated administration, the control rodents again showed preserved memory abilities, demonstrated by a significantly increased value of the indicator as compared to IL ($P < 0.0001$) (Fig. 5 B). Myrtenal-treated rats showed a reduced memory capacity (by 65%) compared to controls ($P < 0.05$) in repeated administration. Repeated administration of both myrtenal derivatives for 11 days preserved the memory of the rats close to that of the controls, while the values of the indicator in the animals of the myrtenal group were statistically significantly lower ($P < 0.05$).

4.2.1.2. Delta latency time

The effect of myrtenal derivatives on memory in experimental rats with both regimens of their administration, accounted for by the observed changes in latency time, is shown in Figure 6.

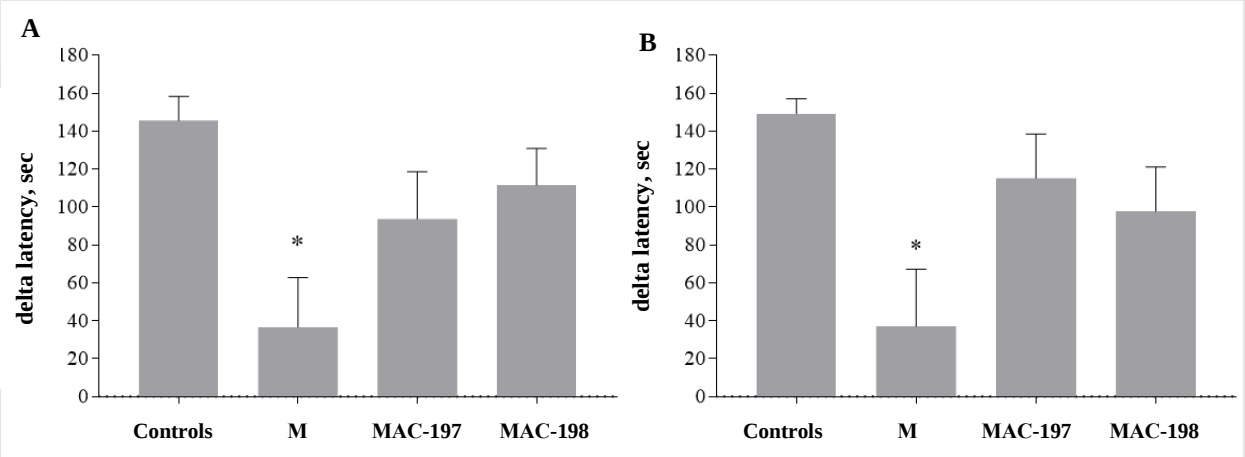


Figure 6. Effects of MAC-197 and MAC-198 on memory after acute (A) and repeated treatment (B) in rats - reporting of delta latency time (sec); M (40 mg/kg) was used as reference; *P < 0.05 vs. control group

As expected, myrtenal significantly reduced the change in latency compared to the control group of rats ($P < 0.05$), both in acute (single) by 49.7% (Fig. 6 A) and repeated application – with 75.2% (Fig. 6 B).

In contrast, its conjugates with aminoadamantane in both administration schemes did not noticeably affect the indicator, which confirms the absence of the hypomnesic effect characteristic of the natural reference in rats.

4.2.1.3. Number of avoidances of the dark part of the staging

Another indicator for determining the state of memory is the number of avoidances reported with the *Passive avoidance* test. The results are presented in Figure 7.

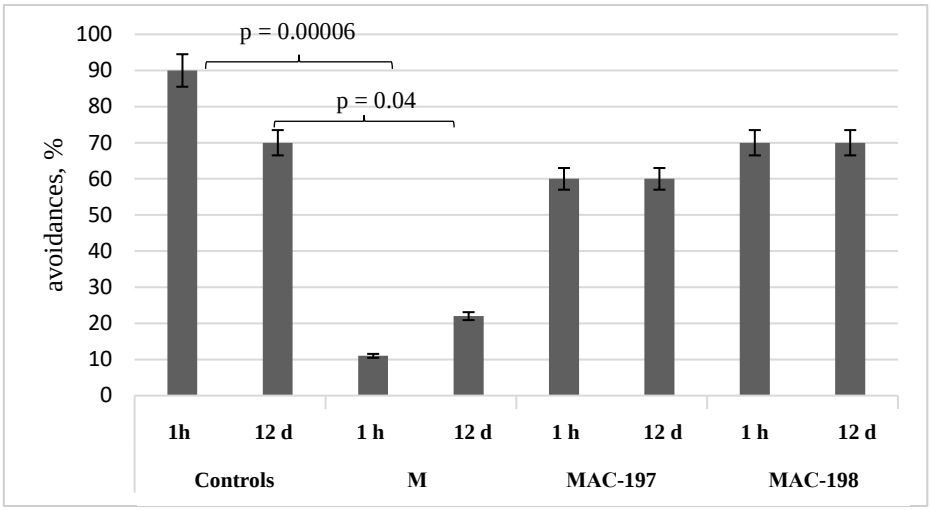


Figure 7. Effects of MAC-197 and MAC-198 on memory after acute and repeated treatment in rats - reporting of the number of avoidances per group, %

In this study as well, myrtenal caused a deterioration in memory, as measured by a significant decrease in the number of avoidances of the dark compartment compared to control rats. The effect was present both one hour after the single administration (P = 0.00006) and after the 11-day treatment (P = 0.04). Its aminoadamantane derivatives did not lead to significant changes in the indicator, which supports our statement about the absence of a damaging effect on memory processes in intact rats.

In the present study, the potential of the two newly synthesized conjugates of myrtenal with aminoadamantane to improve memory in rodents was demonstrated for the first time. Behavioral tests showed preserved memory function in rats treated with both compounds. After their single and repeated application, the values of the indicators are close to those of the control group.

4.3. Effect of myrtenal conjugates on brain acetylcholinesterase activity

4.3.1. Docking studies on the potential of myrtenal derivatives to influence brain AChE activity

The predicted affinity of MACs for the enzymatic catalytic site of AChE is shown in Table 6.

Table 6. AChE catalytic site binding affinity as predicted by the GoldScore (GS), ChemScore (CS), ChemPiecewise Linear Potential (ChemPLP) and Astex Statistical Potential (ASP) point functions using GOLD (v5.4.1); HI6 *is the co-crystallized ligand (4-(aminobarbonyl)-1-[({2-[(E)-(hydroxyimino)methyl]pyridiunum-1-yl}methoxy) me-thyl]pydidinium)*

	ASP	ChemPLP	CS	GS
MAC-197	34.9	68.4	40.9	47.3
MAC-198	35.0	64.2	40.3	45.1
Myrtenal	23.8	44.0	28.5	35.1
HI6	48.1	85.6	29.1	62.4
Acetylcholine	**24.1**	**43.1**	**18.2**	**36.6**
Donepezil	59.1	83.2	43.3	56.9
Tacrine	42.1	66.0	35.1	57.1

For further comparison, the neurotransmitter ACh and the drugs Tacrine and Donepezil were attached to the binding site. The latter two compounds are acetocholinesterase inhibitors that find clinical use in AD therapy. According to all four point functions, both MAC-197 and MAC-198, compared to myrtenal and ACh, interact more strongly with the active site of AChE (Table 6). Myrtenal-adamantane conjugates were found to have a slightly lower predicted affinity than the drugs Donepezil and Tacrine.

According to the ChemPLP software prediction, MAC-197 forms one hydrogen bond with the hydroxyl oxygen atom in Tyr124 for AChE (Fig. 8 A). The myrtenal radical is pushed deep into the binding pocket of the active site with adamantane at the entrance (Fig. 8 B).

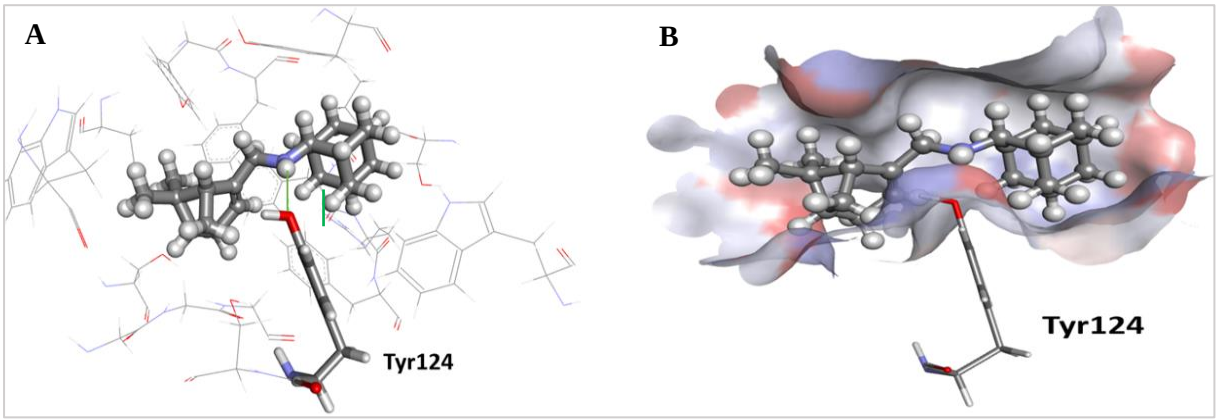


Figure 8. Hyperposition of MAC-197 in the AChE binding site as predicted by the ChemPLP scoring function

A. *The predicted configuration is shown in ball-and-stick format, and hydrogen bonding to Tyr124 (stick format) is shown as a green line.*

B. *The amino acids forming the lipophilic binding pocket shown as lines are Trp86, Gly120, Gly121, Gly122, Trp286, Val294, Phe295, Phe297, Tyr337, Phe338, Tyr341, and His447. The surface of the protein is depicted in different colors: blue – areas with a partial positive charge on the surface; red – regions with a partial negative charge; gray – neutral areas. The ligand occupies the deep binding pocket.*

The pocket is made up of many lipophilic amino acid residues accommodating the two aliphatic components of the ligand. Interestingly, MAC-198 is predicted to have a reverse binding conformation, i.e., the myrtenal moiety is located at the entrance of the binding pocket. Finally, myrtenal is predicted to occupy the bottom of the binding site, i.e., the site of the myrtenal residue for MAC-197.

Molecular modeling shows that AChE is a plausible target for the tested substances because the ligands form hydrogen bonds and fit well into the binding pocket without straining the molecular conformation into energetically unfavorable interactions.

4.3.2. Effects of myrtenal conjugates on brain AChE activity *(in vivo)*

Both myrtenal derivatives MAC-197 and MAC-198 showed affinity for the active site of AChE according to docking analysis. Most in vivo protocols usually monitor the effects of compounds on enzyme activity only in the hippocampus of experimental rats. In our experiments, it was found that the studied derivatives affect in a different way the enzyme activity in the two main structures responsible for memory processes, namely the cortex and the hippocampus. The results are presented in Figure 9.

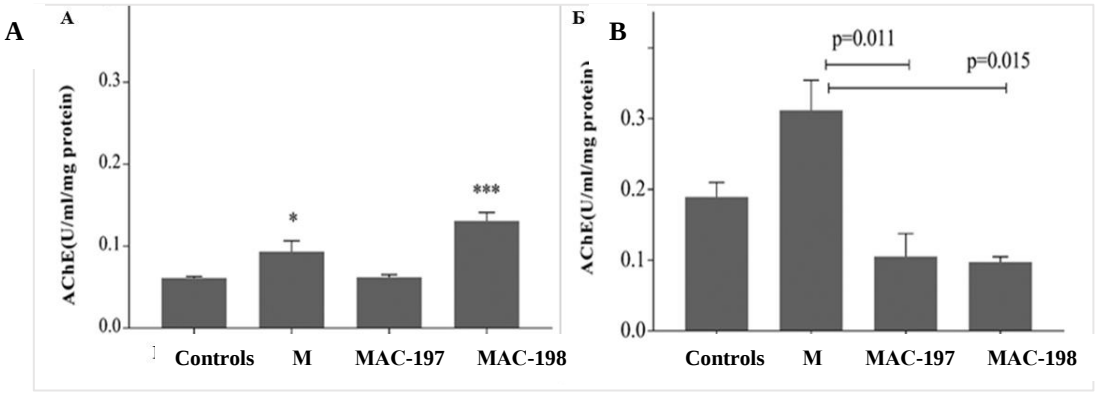


Figure 9. Effects of MAC-197 and MAC-198 on AChE activity in rat cerebral cortex (A) and hippocampus (B). Data are expressed as mean ± SEM (n = 5); ***P <0.001, *P <0.05 vs. control group

In the cerebral cortex, both myrtenal and MAC-198 increased AChE activity by 52.5% ($P < 0.05$) and 114.06% ($P < 0.001$), respectively (Fig. 9 A). MAC-197 did not change the studied indicator compared to the controls, which allows us to conclude that MAC-197 has no registered effects on acetylcholinesterase activity in the cortex.

Hippocampal AChE activity was significantly inhibited by the administration of MAC-197 and MAC-198 in contrast to myrtenal-treated rats (Fig. 9 B), indicating that in the hippocampus it again causes activation of the enzyme found by Dragomanova (2020). On the other hand, conjugates inhibited the enzyme activity. Compared with myrtenal, MAC-197 decreased AChE activity by 66.3% and MAC-198 by 68.8%, with a significance level of $P < 0.01$. Hippocampal AChE activity was reduced by both investigated substances, respectively in MAC-197 by 44.4% and in MAC-198 by 48.6%, compared to the control group. The results are summarized in Table 7.

Table 7. AChE activity in two brain structures related to memory processes - cortex and hippocampus

%	Controls	M	MAC-197	MAC-198
cortex	100 (0.06121 U/ml/mg protein)	+ 52.51 *	+ 1.17	+ 114.1 ***
hippocampus	100 (0.189075 U/ml/mg protein)	+ 64.83	- 44.4 ($^{\#}$ vs. M)	- 48.6 ($^{\#}$ vs. M)
Summ. effect		+ 117.34	**- 43.23**	+ 65.5

It is apparent that MAC-197 suppressed the activity of the AChE enzyme as a cumulative effect in both brain regions, indicating the potential of this new compound to positively affect memory impairments.

The data from the behavioral tests correlated with the results of the assessment of AChE activity in the cortex and hippocampus. Synthetic derivatives inhibited the enzyme activity in the hippocampus but do not possess significant anticholinesterase properties in the cortex. From the tabulated divergent effects in the two brain structures, the presence of an anticholinesterase potential of the two compounds can be assumed, which is strongly expressed in MAC-197. The same derivative primarily reduced hippocampal AChE activity. AChE activity is known to be physiologically higher in this brain structure. This is probably the reason for the observed beneficial effects of both compounds on the memory of experimental rats.

4.4. Effects of myrtenal conjugates on exploratory behavior

The effects of the newly synthesized myrtenal derivatives MAS-197 and MAS-198 on exploratory behavior examined by Hole board test are presented in Figure 10.

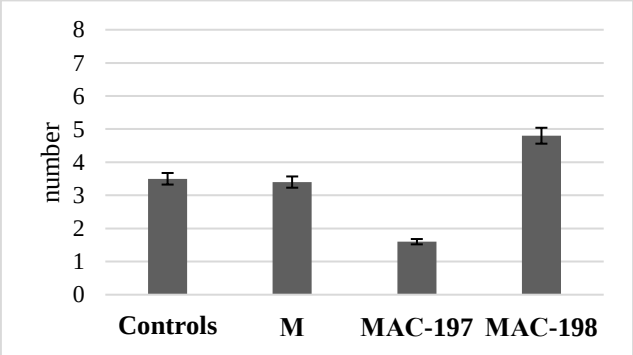


Figure 10. Effects of MAC-197 and MAC-198 on exploratory behavior in rats after 11 days of treatment - counting the number of "head dips"

Myrtenal used as a reference did not change the exploratory behavior in rats after 11 days of administration, while its derivatives caused different changes in the indicator without statistical significance. MAC-197 reduced the number of "head dips" into staging holes by 56.8% compared to controls, and MAC-198 increased them by 29.7%.

The lack of negative effects of the conjugates on memory processes, in combination with the results of the Hole board test, direct attention to the search for another possible reason for influencing the exploratory activity of the experienced rats. This behavioral protocol has been used to examine exploratory activity as an indication of anxiety (d'Isa et al., 2021; Moy et al., 2008; Brown et al., 2008; Takeda et al., 1998). The total number of head-to-ear dips is an indicator of rodent exploratory activity (d'Isa et al., 2021). The use of the setting for the purpose of the study is based on the hypothesis that the behavior of animals placed in a novel situation is the result of a competition between two activities – exploratory and defensive. The three main activities are dipping the head, looking around and moving. The indicated behavioral responses are exploratory, therefore a greater frequency of their manifestation is an indication of a lower degree of anxiety in the tested animals (Brown et al., 2008). A high level of anxiety leads to reduced exploratory activity, and conversely, a low level of anxiety is demonstrated by an increased number of "head dips ". This is the predominant behavior for which there is evidence that it is directly dependent on the effects of various substances.

File and co-authors developed the setup that is widely used in pharmacological manipulations (File and Wardill, 1975a,b; File and Pellow, 1985). The results of the test show that rats treated with MAC-198 induced a slight increase in activity compared to controls (without statistical significance), which was manifested by a decrease in anxiety associated with the unfamiliar environment.

4.5. Effect of myrtenal conjugates on brain oxidative status

Another pathogenetic factor in neurodegenerative disorders is induced brain oxidative stress. The registered effects of the investigated derivatives on basic parameters of the oxidative status in the cerebral cortex were compared with the antioxidant potential of the natural monoterpene.

In our previous studies, it was found that in the cerebral cortex of intact rats, myrtenal exhibited pro-oxidant properties (Dragomanova, 2020).

The results of monitoring the influence of the conjugates - MAS-197 and MAS-198 on the content of malondialdehyde and total glutathione in the cerebral cortex of rats, presented in Figure 11 A and B, show the presence of similar properties for both compounds.

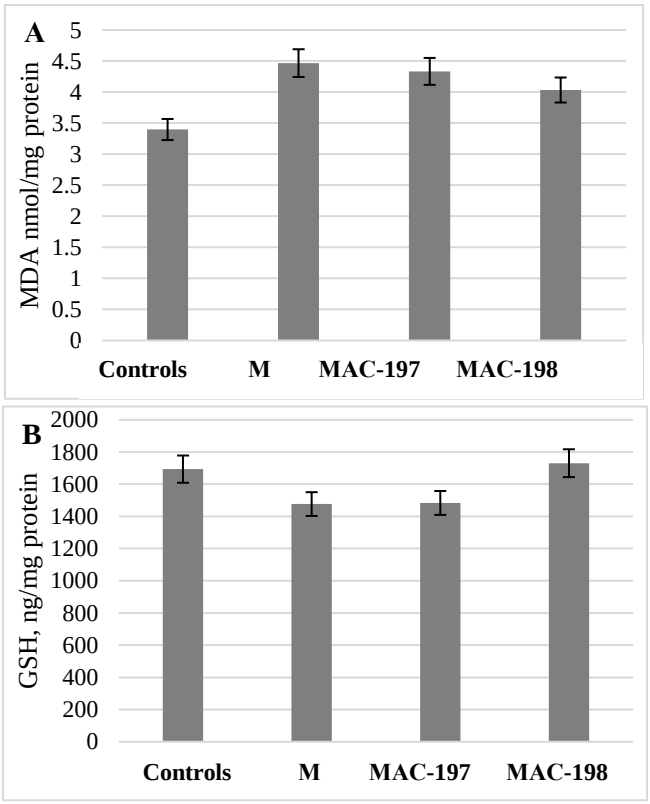


Figure 11. Effects of MAC-197 and MAC-198 on MDA (A) and tGSH (B) levels in rat cerebral cortex

It can be seen that, compared to the control group, the monoterpene increased the levels of MDA, the main product of LPO, by 31.5%, while its derivatives had a weaker effect, respectively, with MAC-197 – by 27.47% and with MAC-198 – by 18.6% (Fig. 11 A). The found reduced content of tGSH - by 12.8%, although without statistical significance, confirms the pro-oxidant potential of myrtenal in intact rodents. Its compounds with aminoadamantane demonstrate similar effects. MAC-197 insignificantly decreased the level by 12.4%, and MAC-198 increased its value by 2.2% (Fig. 11 B).

Next, the effects of the substances on the activity of the endogenous antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase in the cerebral cortex of rats were investigated. The obtained results are illustrated in Figure 12 A, B and C.

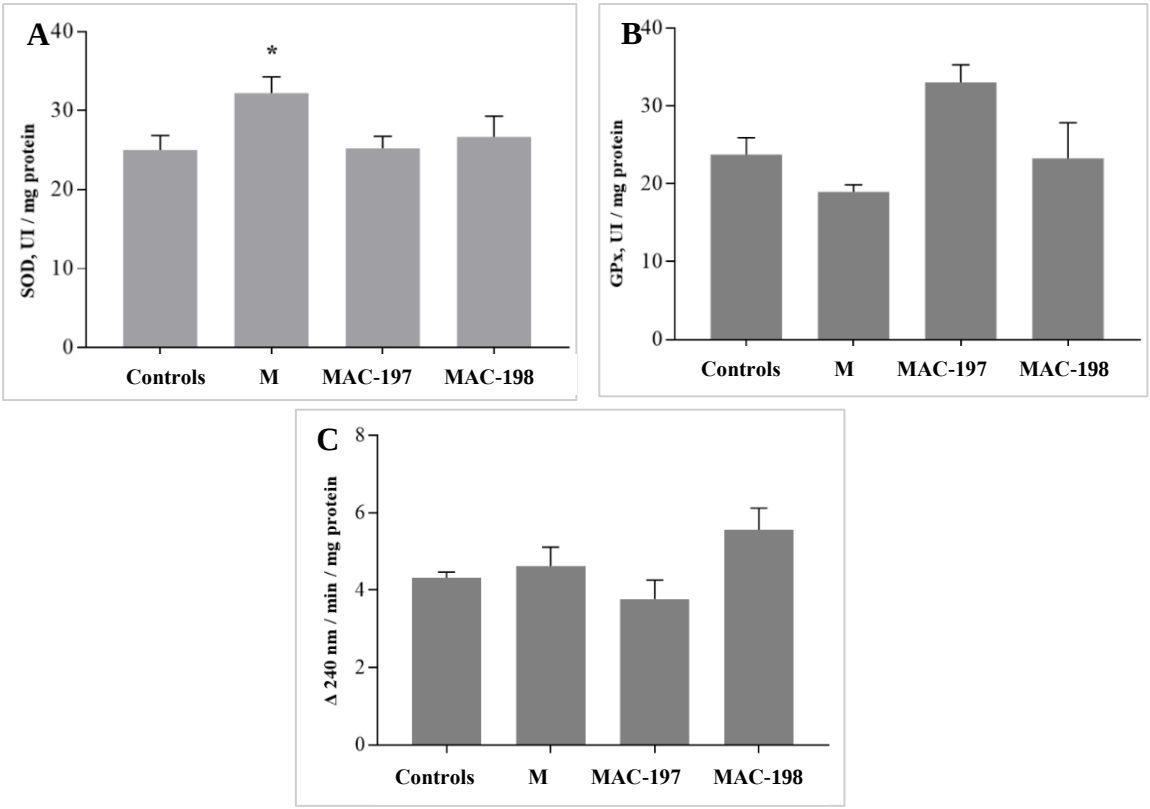


Figure 12. Effects of MAC-197 and MAC-198 on SOD (A), GPx (B) and CAT (B) enzyme activities in rat cerebral cortex; * P < 0.05 vs. control group

Superoxide dismutase (SOD) catalyzes the inactivation of free radicals. The enzyme transforms superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2), which further forms a number of other reactive oxygen-containing radicals, such as hydroxyl radical (·OH) and hydroxyl anions (OH^-) in the Fenton reaction (Fe^{2+} – mediated decomposition of H_2O_2) (Zorov et al., 2014). Myrtenal significantly increased the enzyme activity by 29.1% ($P < 0.05$) (Fig. 12 A) without affecting the other enzymes. Its derivatives do not affect the indicator, keeping its values close to those of the controls.

Glutathione peroxidase (GPx) is a major antioxidant enzyme in brain tissue and therefore modulation of its activity is considered as a possibility to counteract neuronal damage due to oxidative stress (Power and Blumbergs, 2009). GPx is known to be inactivated by its substrates, therefore it is expected that in the presence of oxidative stress, the activity of the enzyme is decreased (Cho et al., 2010). Sabit (2021) published experimental data showing that administration of antioxidants in a neurodegeneration model increased GPx activity.

In our experiments, MAC-197 was found to increase GPx activity (typical of antioxidants) by 39% compared to controls, although not statistically significant, and the 20% decrease in the indicator in M-treated rats (Fig. 12 B) correlated with its pro-oxidant potential, demonstrated when determining MDA and tGSH levels (Fig. 11). These results support the proposition that, like myrtenal, its synthetic adamantane conjugates possess properties modulating the activity of endogenous antioxidant enzymes.

Catalase (CAT) is responsible for deactivating hydrogen peroxide to water and oxygen. According to Gandhi and Abramov (2012), the enzyme has a major role at high H_2O_2 levels.

Here again, myrtenal and its derivatives do not show pronounced modulating properties regarding the enzyme activity, as the monoterpene and MAC-198 increase it, respectively, by 6.8% and 28.7%, without statistical significance, while MAC-197 lowers the indicator by 13.1% (Fig. 12 B).

The results allow to conclude that in intact rats, myrtenal, as well as its conjugates with aminoadamantane, did not demonstrate noticeable antioxidant properties. Myrtenal has known pro-oxidant effects, which, however, not exhibited by its analogs MAC-197 and MAC-198, which revealed their better safety profile.

4.6. Effect of myrtenal conjugates on the monoamines content in memory-related brain structures

Although brain acetylcholinesterase activity has a direct effect on acetylcholine levels, there are other neurotransmitters responsible for the state of cognitive ability. Glutathione peroxidase activity and serotonin levels are relevant to the degree of manifestation of both cognitive and non-cognitive symptoms in dementia, such as depression, anxiety, agitation, eating and sleep disturbances, and aggression (Selles et al., 2018). Cognitive and non-cognitive symptoms of Alzheimer's disease are associated with modulated levels of biogenic amines in the brain of patients. Based on the role of norepinephrine levels in cognitive dysfunction and the progression of neurodegenerative processes, Gutierrez et al. (2022) suggested the neurotransmitter as a potential therapeutic target. According to Meltzer et al. (1998) serotonin is also a leading mediator not only for the onset of depression, but together with acetylcholine has a certain role in the onset of cognitive problems. And Garcia-Alloza et al. (2005) concluded that an imbalance between cholinergic and serotonergic neurotransmission contributes to cognitive impairment.

4.6.1. Noradrenaline content

The changes in the content of norepinephrine in the cerebral cortex and hippocampus of rats after the repeated administration of the studied myrtenal conjugates are shown graphically in Figure 13 A and B.

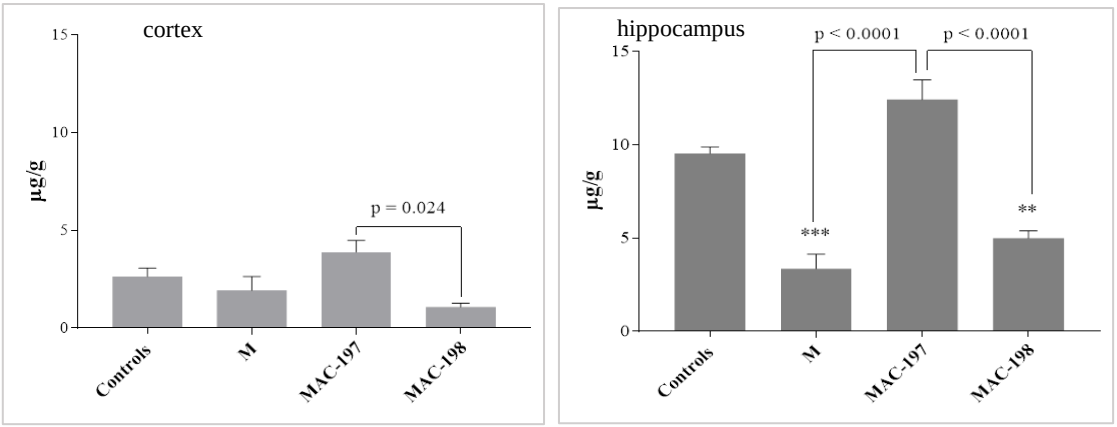


Figure 13. Effects of MAC-197 and MAC-198 on NA content in rat brain cortex (A) and hippocampus (B) after 11-day administration; ** P < 0.01; *** P < 0.001 vs. control group

Compared with the control group, myrtenal and MAC-198 decreased the level of the mediator in the cerebral cortex of rats by 26.8 and 59.6 percent, respectively (Fig. 13 A), while MAC-197 increased it by 47.5% and at this with a value three times higher than that of MAC-198 ($P < 0.05$).

In the hippocampus, myrtenal also decreased (by 64.9%) the level of NA compared with control rodents with high statistical significance ($P < 0.001$) (Fig. 13 B). The influence of myrtenal conjugates on the content of the mediator preserves the trends recorded in the cerebral cortex. MAC-197 significantly increased NA content by 30.2% ($P < 0.05$) relative to controls, while MAC-198 decreased it by 47.7% ($P < 0.01$). The difference between the MAC-197- and MAC-198-induced effect was at a significance level of $P < 0.0001$.

4.6.2. Serotonin content

The registered changes in the content of serotonin in the cerebral cortex and hippocampus of the rats after the repeated administration of the myrtenal conjugates are shown in Figure 14 A and B.

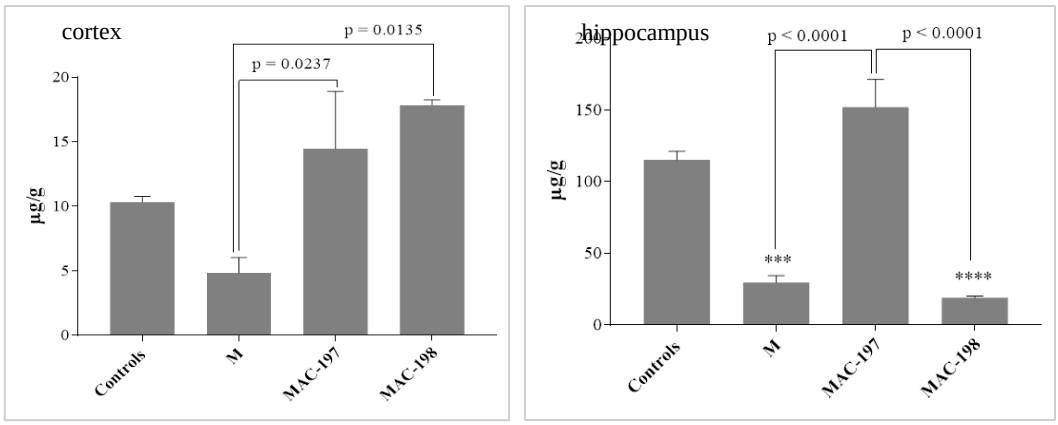


Figure 14. Effects of MAC-197 and MAC-198 on 5-HT content in the brain cortex (A) and hippocampus (B) of rats after 11-day administration; *** P < 0.001, **** P < 0.0001 vs. control group

In the cortex, myrtenal decreased not only the level of NA but also that of 5-HT – by 53.2% compared to control animals (Fig. 14 A). The effect of treating the experimental rats with the monoterpene derivatives was of opposite sign. MAC-197 increased 5-HT content 3-fold compared to the reference group (P = 0.0237) and by 30.2% compared to controls. The other conjugate, MAC-198, increased the level of the mediator even more than MAC-197, with values fourfold higher than those in the M-group rodents (P = 0.0015) and by 72.7% compared to the control (P < 0.05).

Also in the hippocampus, myrtenal decreased 5-HT content with a significance level of P < 0.001 compared to controls (Fig. 14 B). Here, too, the influence of the derivatives on the level of the mediator is in the opposite direction. As in the cortex, MAC-197 increased 5-HT values by 31.9% compared with control animals (P = n.s.). Unexpectedly, treatment with MAC-198 caused an 83.1% reduction in the indicator compared to the control group (P < 0.0001), and the difference in 5-HT content from that of the MAC-197 group was at the level of confidence P < 0.0001.

The results show that both synthesized substances cause specific changes in the levels of biogenic amines, both in the cerebral cortex and in the hippocampus. Of interest are the established completely opposite effects of the two myrtenal conjugates – while MAC-198 lowers the content of noradrenaline and serotonin in brain structures, MAC-197 significantly increases them.

The attempt to compare the established effects of the two compounds with those of myrtenal as a reference provides valuable information about the wide spectrum of therapeutic potential of the two new myrtenal conjugates, which should be the subject of further studies. The natural monoterpene exhibits hypomnesic abilities, also established in our previous studies, which we associate with its anxiolytic potential (Dragomanova, 2020). In contrast, aminoadamantane conjugates did not negatively affect the memory processes of experimental rats. These results are confirmed by the anticholinesterase properties of the two newly synthesized compounds, in contrast to myrtenal, which does not exhibit the ability to suppress the activity of the AChE enzyme in the brain. Regarding the effect on the levels of biogenic amines, myrtenal lowers the NA and 5-HT content, while its derivatives show different and

completely opposite effects. MAC-198 exhibits monoterpene-like properties, demonstrated by decreasing NA and 5-HT levels, and MAC-197 significantly increases them. In addition to neuroprotection involving anticholinesterase and neuromodulatory mechanisms of action, MAC-197's abilities allow it to influence depressive symptoms in neurodegenerative processes.

In conclusion, the conducted studies reveal for the first time the potential of the two newly synthesized myrtenal-adamantane conjugates to improve memory, which is related to their AChE-inhibitory effects and neuromodulatory activity in the cerebral cortex and hippocampus of experimental rats. The behavioral and biochemical effects of both compounds that we found were more pronounced than those of natural myrtenal. The original data obtained provide the basis for further investigation of these new promising substances in experimental models of dementia.

5. Experiments with rats with induced dementia

Scopolamine is a muscarinic antagonist that is widely used as a standard for the induction of dementia in experimental animals (Lagalwar et al., 1999; Lott, 2012; Qin et al., 2015; More et al., 2016). The toxic agent negatively affects short-term memory, memory trace formation, learning, visual recognition memory, visuospatial memory, visuoperceptual function, verbal stimulus response, and psychomotor speed (Flicker et al., 1990; Hasselmo and Wyble, 1997; More et al., 2016). Intraperitoneal administration of scopolamine in laboratory dats causes dysregulation of the cholinergic system of the brain with corresponding consequences: increased AChE activity, oxidative stress and decreased ACh levels (Tang, 2019; Dragomanova et al., 2022; Tancheva et al., 2022). At the behavioral level, these biochemical changes manifest as a marked cognitive deficit in experimental animals, as confirmed by our studies.

In the model we used, experimental rats were treated with scopolamine intraperitoneally at a dose of 1 mg/kg for 11 days. Simultaneously, for the purposes of the study, the reference myrtenal at a dose of 40 mg/kg and its derivatives MAC-197 and MAC-198 at a dose of 1 mg/kg were administered in the same way.

5.1.Effects of myrtenal conjugates on body mass in rats with scopolamine-induced dementia

The results are presented in Figure 15.

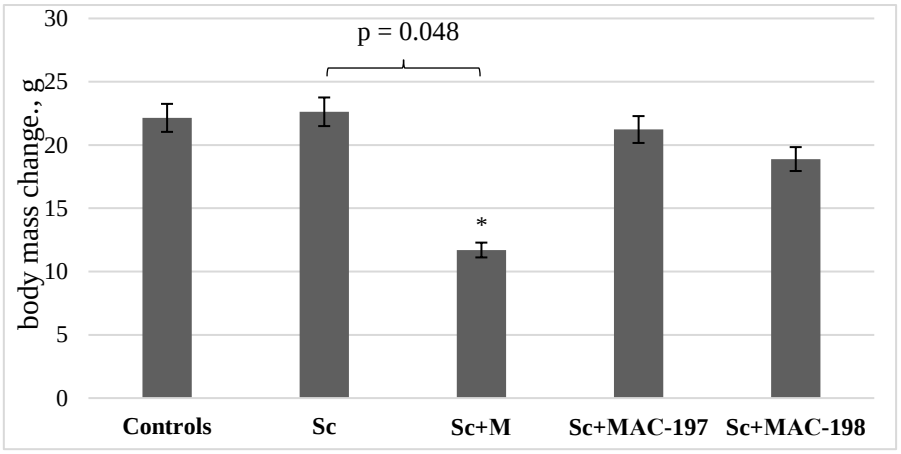


Figure 15. Change in body weights in rats with scopolamine-induced dementia after 11-day administration of myrtenal (40 mg/kg), MAC-197 (1 mg/kg) and MAC-198 (1 mg/kg); *P < 0.05 vs. control group

Scopolamine as a toxic agent did not affect the change in body weights (normal gain) compared to the control group, while myrtenal significantly reduced the index in demented animals ($P < 0.05$). Similar effects were also observed in previous studies by Dragomanova (2020) conducted in rats with experimental dementia treated with myrtenal at the same effective dose of 40 mg/kg. The natural monoterpene replicated the effects found in intact rats, where the change in body mass was less compared to control rodents – by 14.1%, without statistical significance, which we explain by the essential oil's characteristic appetite suppression in experimental animals. As in intact rats, the two myrtenal compounds (MAC-197 and MAC-198) did not demonstrate the property to influence the change in body weight observed in the reference.

5.2. Effects of myrtenal conjugates on memory processes in rats with scopolamine-induced dementia

It is well known that the first clinical symptoms in AD patients are associated with memory deficits (Braak and Braak, 1994). The effects of synthetic myrtenal conjugates on short-term and long-term memory in rats, compared to those of natural myrtenal, were assessed by the Passive avoidance test. The goal is to induce a fearful experience, with the difference in the rodent's latency to respond to baseline training taken as an indicator of memory status.

5.2.1. Behavioral research *(Passive avoidance test)*

The effect of both substances on short-term memory (1 h after the single administration) and long-term memory (11-day treatment) was followed by calculating the change in latency. The results are presented in Figure 16.

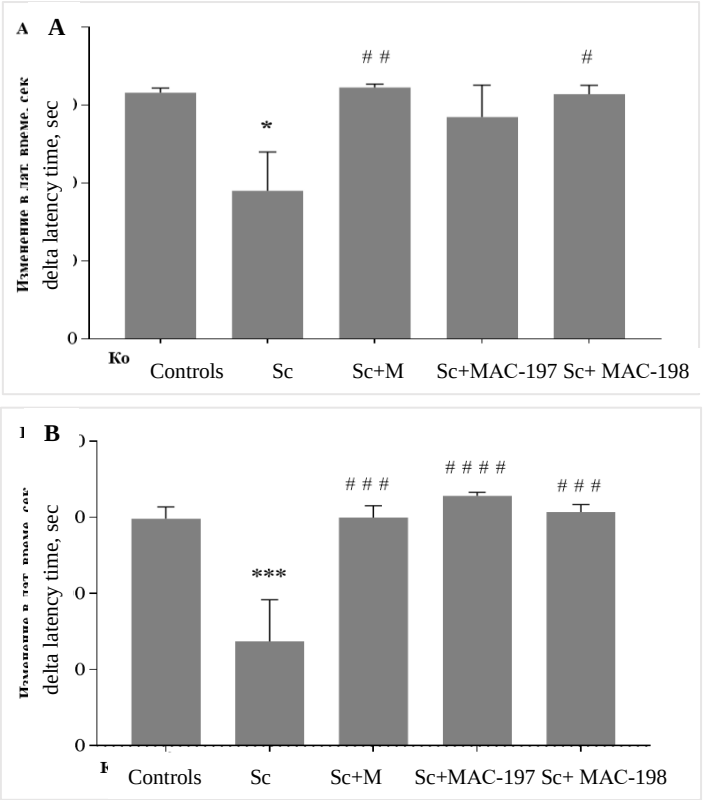


Figure 16. Effects of myretnal conjugates on short-term (A) and long-term (B) memory in rats with scopolamine-induced dementia – reporting the change in latency time; * P < 0.05, *** P < 0.001 vs. control group; # P < 0.05, ## P < 0.01, ### P < 0.001, #### P < 0.0001 vs. scopolamine group

As expected, scopolamine caused significant impairment in both short-term and long-term memory. In the acute treatment, compared with the control group, the toxic agent reduced the change in latency by 39.9% (P < 0.05) (Fig. 16 A), and after repeated administration by 54% (P < 0.001) (Fig. 16 B).

Myrtenal restored memory in rats to levels close to those of controls. Compared with animals in the scopolamine group, the index values were increased by 69.7% (P < 0.01) after the acute administration (Fig. 16 A) and by 118.4% (P < 0.001) on day 12 after the repeated treatment (Fig. 16 B). The results confirmed the data from our previous studies (Dragomanova et al., 2022).

The conjugates we studied produced myrtenal-like effects in rats with scopolamine-induced dementia. MAC-197 showed a better effect on Sc-impaired long-term memory – the increase of the indicator was 139.3% with statistical significance of P < 0.0001 (Fig. 16 B), while MAC-198 restored both types of memory. The change in latency in short-term memory was increased by 65.3% (P < 0.05) (Fig. 16 A) and by 123.8% in long-term memory (P < 0.001) as compared to dement rodents (Fig. 16 B).

In conclusion, myrtenal and MAC-198 counteracted the deleterious effect of scopolamine on both short-term and long-term memory in demented rodents. Compared to these, MAC-197 induced a less pronounced positive effect on memory in rats after a single treatment and a distinctly more pronounced one after repeated administration.

5.2.2. Brain AChE activity *(in vivo)*

Normal functioning of central cholinergic transmission is essential for the regulation of memory and mood (Kolar, 2021). AD is characterized by cholinergic dysfunction manifested by increased AChE activity and decreased brain ACh content (Drinenberg, 2000).

The docking modeling indicated AChE for a potential target for the aminoadamantane conjugates of myrtenal. The activity of the enzyme, as in intact rats, was determined in two brain structures related to memory processes – cortex and hippocampus. The results are presented in Figure 17.

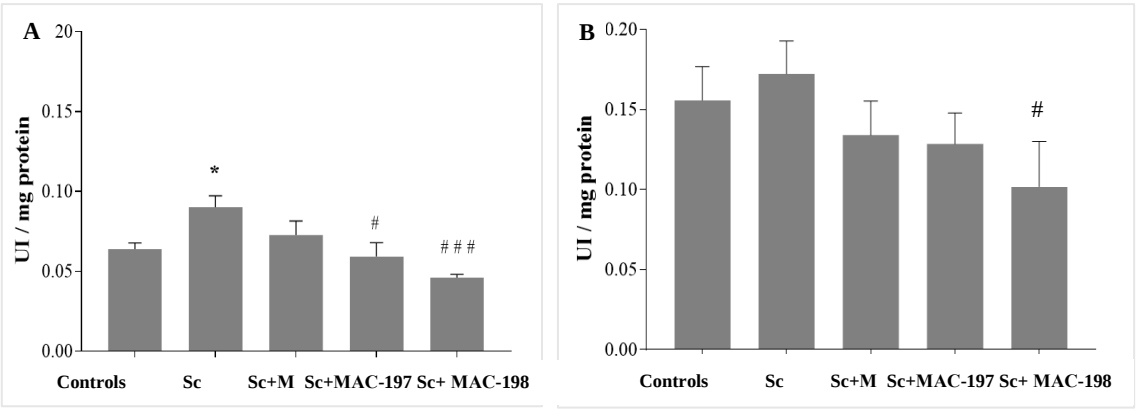


Figure 17. Effects of MAC-197 and MAC-198 on AChE activity in the cerebral cortex (A) and hippocampus (B) of rats with scopolamine-induced dementia; * P < 0.05 vs. control group; # P < 0.05, ### P < 0.001 vs. scopolamine group

Compared with the control group of rats, scopolamine caused an increase in AChE activity in the cortex by 41% (P < 0.05) (Fig. 17 A) and in the hippocampus by 10.6% (P = n.s.) (Fig. 17 B).

In the cerebral cortex, both myrtenal derivatives demonstrated significant anticholinesterase activity (Fig. 17 A), compared to the natural monoterpene, which did not significantly affect the indicator. The enzyme activity was decreased by 34.2% (P < 0.05) by MAC-197 and by 48.7% (P < 0.001) by MAC-198 compared with the Sc-treated group of animals.

In the hippocampus, both myrtenal and MAC-197 showed no distinct trend toward inhibition of acetylcholinesterase activity. The most pronounced decrease of 41% was induced by MAC-198 (P < 0.05 vs. scopolamine group) (Fig. 17 B).

In conclusion, unlike myrtenal, both adamantane conjugates demonstrated AChE-inhibitory properties, which was more pronounced with MAC-198. This effect was observed both in the cortex and in the hippocampus and was supported by molecular modeling results showing a stronger affinity of the derivatives for the active site of the enzyme compared to the monoterpene.

5.3. Effects of myrtenal conjugates on brain oxidative status in rats with scopolamine-induced dementia

Elevated values of parameters assessing the cerebral oxidative status of patients with Alzheimer type dementia have been well documented by many researchers (Markesbery, 1997; Markesbery and Carney, 1999; Markesbery, 1999; Pratico and Delanty, 2000; Floyd and Hensley, 2002). This is one of the most popular hypotheses for the pathogenesis of neurodegenerative disease (Kamat et al., 2016).

A number of studies have shown that oxidative stress is associated with memory dysfunction in the scopolamine-induced animal model of dementia (Giridharan et al., 2011; Nade et al., 2011; Budzynska et al., 2014; Zhao et al., 2016). The damage is characterized by a significant decrease in GSH content, increased CAT activity, increased MDA content, a major product of lipid peroxidation, and a tendency to decrease GPx activity.

Myrtenal, like most natural terpenoids, exhibits antioxidant activity, which was found in a rat model of hepatocellular carcinoma (Babu et al., 2012). In our previous studies, myrtenal demonstrated enhanced neuroprotective effects in the cerebral cortex of rats with a scopolamine model of neurodegeneration wich were manifestated with antioxidant activity among other mechanisms determined (Dragomanova et al., 2022).

The study of the antioxidant properties of the newly synthesized substances was carried out in the cerebral cortex, where their properties to influence acetylcholinesterase activity are more pronounced.

5.3.1. Malondialdehyde and glutathione levels

The results are presented in Figure 18.

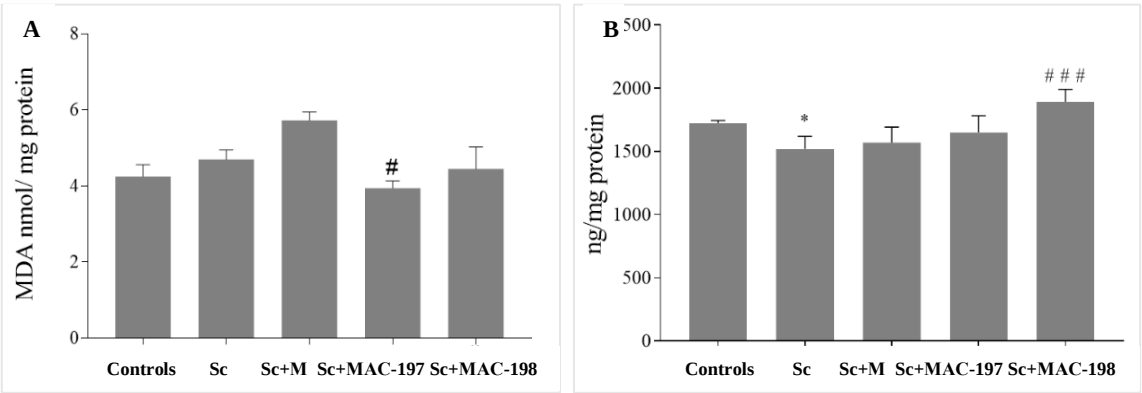


Figure 18. Effects of MAC-197 and MAC-198 on MDA (A) and GSH (B) levels in the cerebral cortex of rats with scopolamine-induced dementia; * P < 0.05, ** P < 0.01 vs. control group; $^{\#}$ P < 0.05, $^{\#\#\#}$ P < 0.001 vs. scopolamine group

In demented rats, the damaging effect of scopolamine was manifested by an increase in MDA levels by 10.6% (P = n.s.) (Fig. 18 A) and a significant decrease in GSH content by

11.9% (P < 0.05) compared to the controls (Fig. 18 B). The natural monoterpene did not exhibit antioxidant effects in the cerebral cortex of demented rats when considering its effects on the two parameters indicative of oxidative stress. The latter correlates with Dragomanova's 2020 data showing that the antioxidant properties of myrtenal are more pronounced in the brain of mice than in rats.

Both conjugates of myrtenal with aminoadamantane showed antioxidant capacity by distinctly changing the levels of the investigated parameters evaluating the oxidative status compared to those in the rats of the scopolamine group. MAC-197 significantly decreased the level of lipid peroxidation products by 16.1% (P < 0.05) (Fig. 18 A), and MAC-198 significantly increased the GSH content by 24.6% (P < 0.001) (Fig. 18 B). Compared to the natural reference M, both tested substances showed better antioxidant properties in the cerebral cortex of rats with induced dementia.

5.3.2. Activity of endogenous antioxidant enzymes

The study of the antioxidant potential of the two conjugates in the cerebral cortex of experimental rats and its comparison with that of myrtenal was extended by studying their effects on the activity of antioxidant enzymes as well (Figure 19).

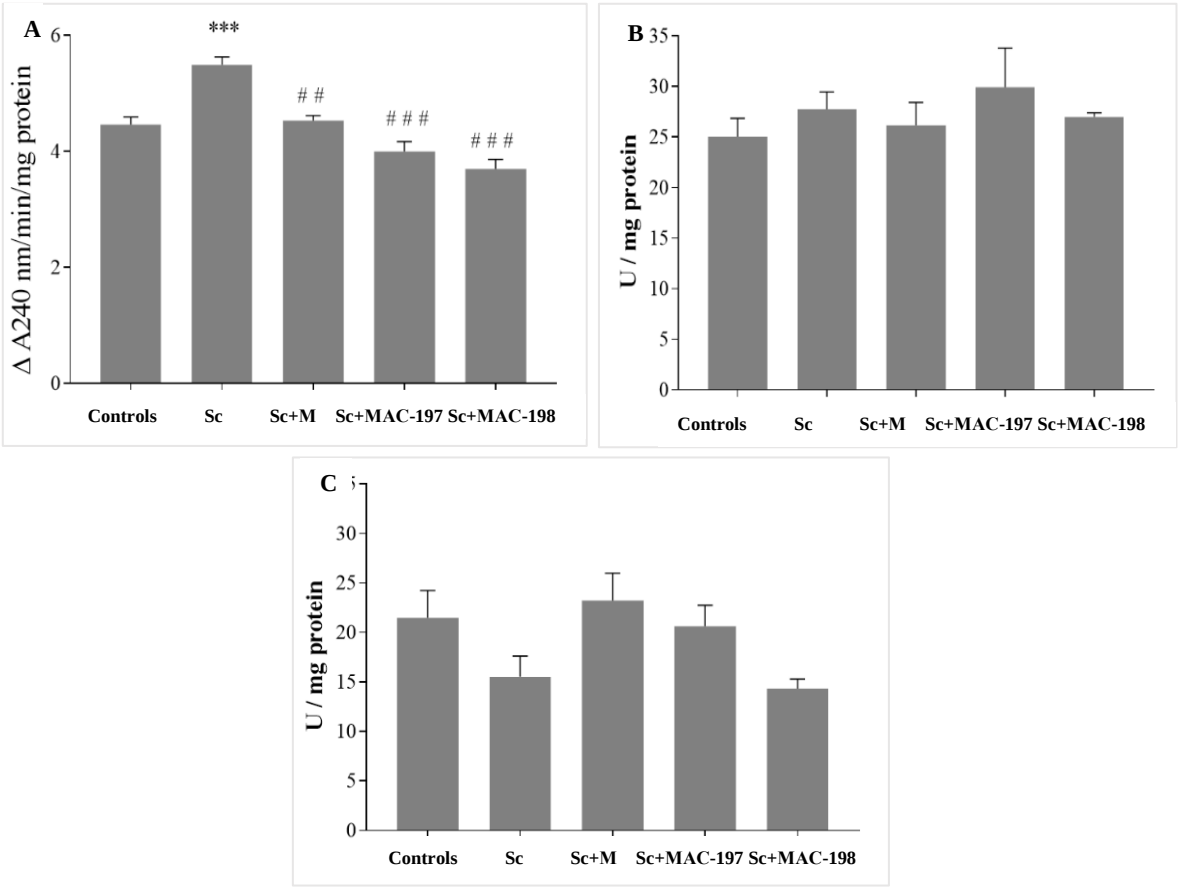


Figure 19. Effects of MAC-197 and MAC-198 on activity of CAT (A), SOD (B) and GPx (B) in the cerebral cortex of rats with scopolamine-induced dementia; *** P < 0.001 vs. control group; ## P < 0,01, ; ### P < 0,01 vs. scopolamine group

Scopolamine-induced oxidative stress was confirmed by significantly increased catalase activity in the cerebral cortex of treated rats by 23.1% compared to controls ($P < 0.001$) (Fig. 19 A). It was seen that glutathione peroxidase activity, which in the presence of oxidative stress was decreased, was also inhibited (by 27.7%) as compared to controls (P = n.s.) (Fig. 19B). Regarding superoxide dismutase, no modulatory properties were observed, both of the toxic agent and of the other substances (Fig. 19 B).

Myrtenal significantly reduced the activity of scopolamine-stimulated CAT (by 17.5%) to values close to those of controls ($P < 0.01$), which was attributed to its specific antioxidant properties (Fig. 19 A). Regarding GPx activity, the monoterpene also antagonized the damaging effect of scopolamine, restoring the index to values close to those of healthy rats and simultaneously increasing the activity by 49.5% compared to the scopolamine group (Fig. 19B). No effects on superoxide dismutase were detected (Fig. 19B).

Myrtenal derivatives exhibited similar to it properties in terms of affecting the activity of antioxidant enzymes. MAC-197 and MAC-198 inhibited scopolamine-activated catalase activity by 27.2% and 32.6%, respectively, restoring it to values close to those of control rats ($P < 0.001$) (Fig. 19 A). The conjugates had no effect on SOD activity in the rat cerebral cortex (Fig. 19B) and less modulated the GPx activity compared to M (Fig. 19B).

The available information on the therapeutic potential of myrtenal, related to its specific antioxidant properties, in models of carcinogenesis and diabetes mellitus are summarized in the dissertation work of Dragomanova (2020). On the basis of the obtained data, as well as the results of our other studies, for the first time the possibility of monoterpene positively affecting neurodegenerative processes was discussed, with antioxidant properties being one of the components of its complex neuroprotective action, found in the brains of rodents with experimental dementia (Dragomanova, 2020; Dragomanova et al., 2022).

Under the conditions of this experiment, the specific antioxidant activity of myrtenal in scopolamine-treated rats was expressed in a significant reduction of the toxic agent-increased CAT activity approaching the control level ($P < 0.01$) and a restoration of GPx activity to values close to those of the controls, although without statistical significance. Both compounds demonstrate antioxidant capacity. Compared to the scopolamine group of rats, MAC-197 significantly reduced the content of MDA by 16.1%, and the most pronounced effect of MAC-198 on increasing the level of GSH was by 24.6% ($P < 0.001$).

The results of the present study show that the newly synthesized myrtenal conjugates MAC-197 and MAC-198 possess similar, yet more clearly manifested antioxidant properties than those of the natural reference.

5.4. Effects of myrtenal conjugates on main brain neurotransmitter levels in rats with scopolamine-induced dementia

Alterations in the noradrenergic system are observed in Alzheimer's disease. Braak and Del Tredici (2012) found that pathological changes of noradrenergic neurons in the locus coeruleus, including the accumulation of τ-protein begin early in the course of the disease, and according to Gannon et al. (2015) later observed enhanced loss of noradrenergic neurons,

along with compensatory changes such as modifications in neuronal structure, neurotransmitter and noradrenergic receptors. The loss of noradrenergic innervation, which significantly exacerbates the pathogenesis and progression of the pathological process, has been confirmed by clinical and preclinical studies (Marien et al., 2004; Heneka et al., 2006; Grudzien et al., 2007; Kalinin et al., 2007; Pugh et al., 2007). Given the well-established functions of the noradrenergic system in cognition, neuroinflammation, and metabolism (Chamberlain and Robbins, 2013), one would predict that such dysfunction would promote AD progression.

Serotonin levels are essential not only for the onset of various forms of depression but also for behavioral changes in AD. Its role together with acetylcholine for cognitive problems in neurodegenerative processes has also been elucidated (Meltzer et al., 1998). An imbalance between the cholinergic and serotonergic neurotransmitter systems contributes to cognitive impairment in Alzheimer's disease (Garcia-Alloza et al., 2005). The disease is associated with a loss of serotonergic neurons and a decrease in 5-hydroxytryptamine levels, which is hydrolyzed in the brains of patients *post mortem* (Kovacs et al., 2003; Mesulam, 2004).

Accordingly, the present work attempted to investigate the effects of myrtenal conjugates on baseline brain neurotransmitter levels in rats with scopolamine-induced dementia.

5.4.1. Noradrenaline content

Norepinephrine, in addition to its role as a neurotransmitter, suppresses brain neuroinflammation. Thus, it acts as an endogenous anti-inflammatory agent in the microenvironment surrounding neurons, glial cells, and blood vessels in the neocortex and hippocampus (Heneka et al., 2010). Experimentally induced degeneration in *locus ceruleus* increases the expression of inflammatory mediators in APP-transgenic mice and leads to increased Aβ deposition. Up to 70% of NA-synthesizing cells are lost in Alzheimer's disease. The mediator has been shown to stimulate microglia in experimental mice, suppressing A*β*-induced production of cytokines and chemokines, leading to enhanced phagocytosis of A*β* deposits.

The effect of myrtenal conjugates on the norepinephrine content in the cortex and hippocampus of demented rats after repeated administration is shown graphically in Figure 20 A and B.

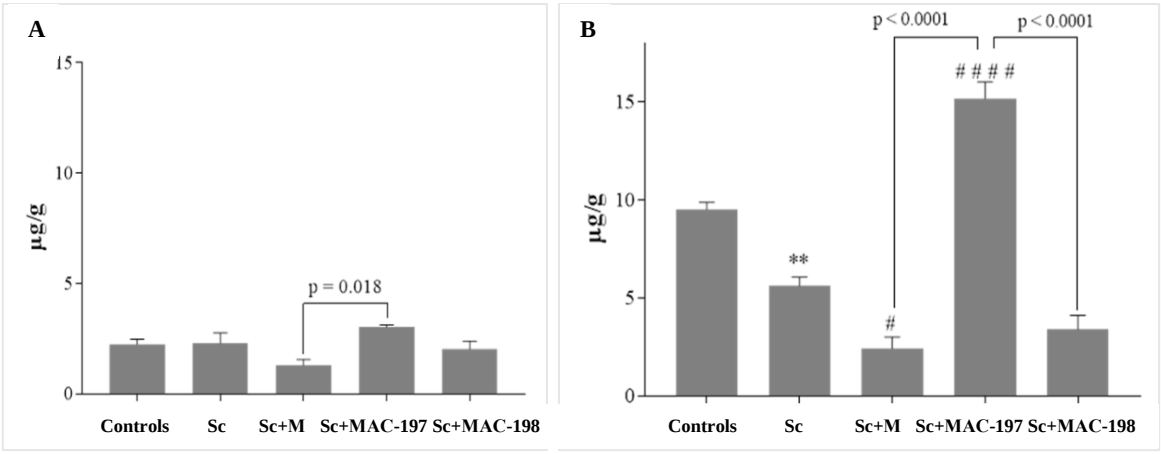


Figure 20. Effects of MAC-197 and MAC-198 on NA content in the cortex (A) and hippocampus (B) of rats with scopolamine-induced dementia after 11-day administration; ** P < 0.01 vs. control group; # P < 0.05, #### P < 0.0001 vs. scopolamine group

Scopolamine did not affect cortical NA content (Fig. 20 A), but induced a significant decrease in the level of the mediator in the hippocampus by 41% (P < 0.01) (Fig. 20 B).

In the cortex of myrtenal-treated rats, NA content was reduced by 44% compared with levels in the scopolamine group (P = n.s.). As shown in the graph, MAC-197 increased the indicator by 134.8% compared to the M group (P = 0.018) and by 35% compared to Controls, while for MAC-198 the effect was less pronounced, with an indicator value, close to that of control rodents (Fig. 20 A).

It was found that in the hippocampus, both the reference and MAC-198 caused an even more pronounced decrease in the content of the mediator, which compared to the scopolamine group reached 57% for myrtenal (P < 0.05), and 39.4% for MAC-198 (P = n.s.) (Fig. 20 B). In contrast, MAC-197 significantly increased NA content by 170% compared to dement rats (P < 0.0001) and by 59.4% in comparison to controls.

In the present study, treatment of experimental rats with Sc resulted in a significant decrease in hippocampal NA content (P < 0.01), which is consistent with data published by Garcia-Alloza et al. (2005) and Falsafi et al. (2012). The results showed that myrtenal and MAC-198 did not affect the decreased NA content in both cortex and hippocampus, whereas MAC-197 increased the neurotransmitter content in the cortex (P = n.s.) and hippocampus (P < 0.01) vs. rodents with scopolamine-induced dementia, revealing its neuroprotective and antidepressant potential. In our opinion, this difference in the effects of the new compounds studied is probably due to the chemical structures of the two derivatives, since the myrtenal radical is connected through an amino group to the first and second positions, respectively, in the adamantane molecule.

Of note is the established divergent action of the two new compounds on norepinephrine content, particularly in the hippocampus of rats with scopolamine-induced dementia. This is probably due to the differences in the structure of their molecules. Of the two myrtenal derivatives studied, MAC-197 increased the content of norepinephrine in brain structures of rats with experimental dementia, which could be interpreted as an additional mechanism of its neuroprotective potential.

5.4.2. Serotonin content

The effect of the studied myrtenal conjugates on the serotonin content in the rat cortex and hippocampus after repeated administration is presented in Figure 21 A and B.

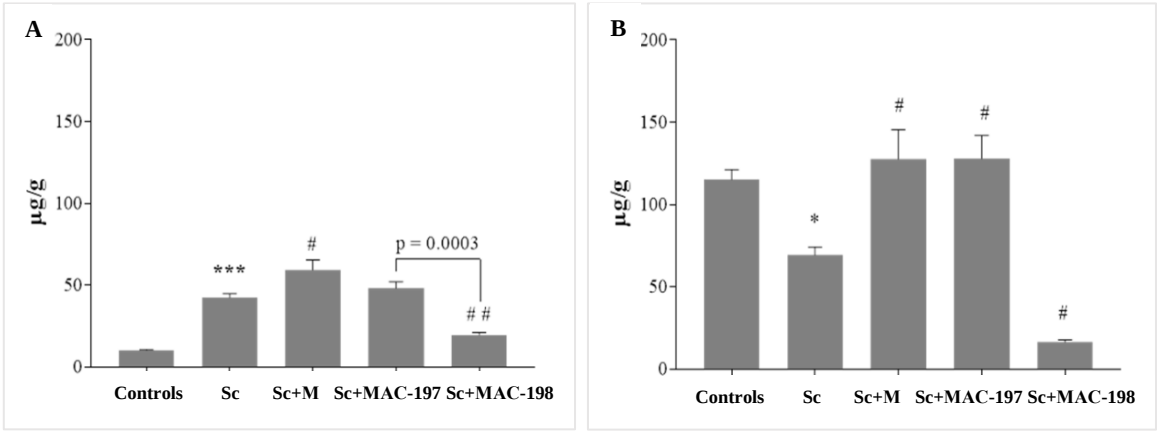


Figure 21. Effects of MAC-197 and MAC-198 on 5-HT content in the cortex (A) and hippocampus (B) of rats with scopolamine-induced dementia after 11-day administration; * P < 0.05, *** P < 0.001 vs. control group; # P < 0.05, ## P < 0.01 vs. scopolamine group

The measured in the hippocampus 5-HT content of the control rats was 10 times higher than in the cortex. Treatment of rodents with scopolamine produced a statistically significant threefold increase in mediator levels in the cortex ($P < 0.001$) (Fig. 21 A), while in the hippocampus the indicator was decreased by 39.8% compared with control animals ($P < 0.05$) (Fig. 21 B).

Myrtenal significantly increased 5-HT content in the cerebral cortex compared to that in the scopolamine group by 62% ($P < 0.05$) (Fig. 21 A). The effect of MAC-197 on the serotonin levels measured in the cortex approached that of the rats treated with the combination of Sk + M. The opposite effect was observed with MAC-198 – the content of the mediator was reduced by 53.4% versus that in Sc group ($P < 0.01$), with a mean value close to that of Controls.

In the hippocampus as well as in the cortex, myrtenal and MAC-197 significantly increased serotonin levels ($P < 0.05$) compared to those in dement rats by 83.8% and 84.4%, respectively, reaching the control level (Fig 21 B). In this study, MAC-198 also exhibited the opposite effect, demonstrated by a significant decrease in 5-HT content by 76.2% compared to that in scopolamine-treated animals ($P < 0.05$).

The results of the study showed that treatment of rats with scopolamine, induced a significant increase in 5-HT content in the cortex ($P < 0.001$) and a decrease in the hippocampus ($P < 0.05$), with control concentrations of the mediator 10 times higher in the hippocampus. This correlates with data from a study by Bert et al. (2001) who found that serotonin levels were different in some brain regions and depended on the age of experimental rats. All tested compounds alter 5-HT levels in the two memory-related brain structures, the cortex and hippocampus. MAC-198 decreases its content, and myrtenal and MAC-197 increase it. An interesting feature in the behavior of myrtenal and MAC-197 was observed – in the cortex, both substances potentiated the effect of scopolamine, and in the hippocampus

they counteracted it. And in these cases, the opposite effects of MAC-197 and MAC-198 were most likely due to differences in the chemical structure of the two derivatives.

In conclusion, MAC-197, as well as myrtenal, stimulated serotonin transmission, which points to a potential neuroprotective effect in an experimental model of dementia. Preclinical studies in APP/PS1 (familial form of AD) transgenic mouse models with 5-HT receptor modulating agents have shown neuroprotective effects (Vakalopoulos, 2017). The author's published theory of serotonergic modulation of memory trace formation by direct enhancement of synaptic signaling may change the view of the role of these brain structures in the pathogenesis of AD and lead to the introduction of effective treatment.

6. Myrtenal derivatives effects comparison

To fulfill the task, the effects of myrtenal derivatives on basic behavioral and biochemical parameters in healthy and demented experimental rodents were compared. A parallel was drawn between the changes in healthy animals treated with both compounds versus those treated simultaneously with scopolamine and the novel MACs.

6.1. Comparative analysis of results in intact and demented rats after administration of myrtenal-aminoadamantane conjugates

6.1.1. Memory

The behavioral changes observed in the Passive avoidance test are presented in Figure 22.

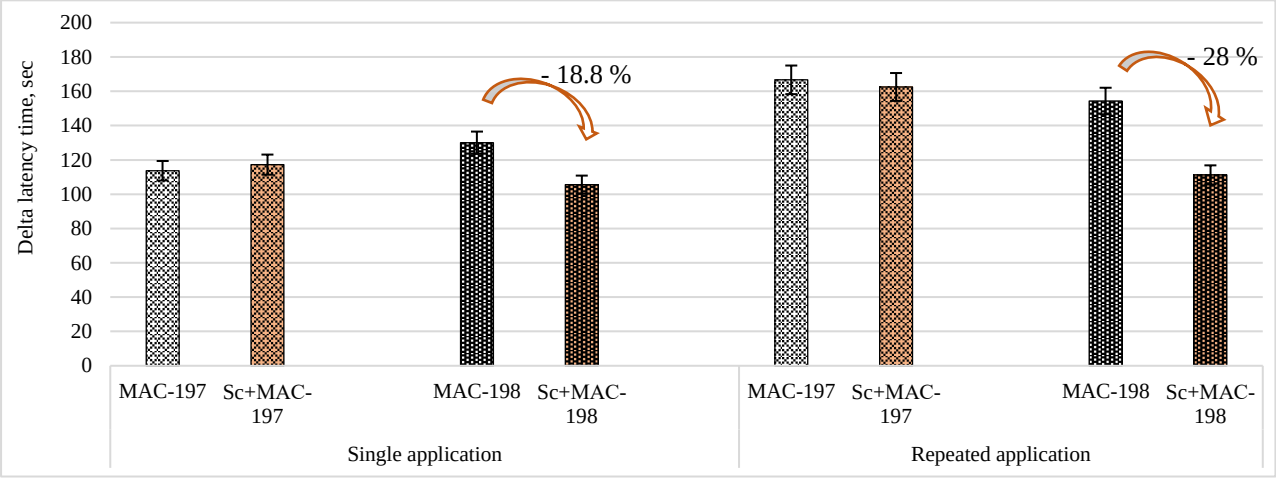


Figure 22. Effects on memory processes in intact and demented rats after single and repeated (11-day) administration of MAC-197 and MAC-198

The study showed that myrtenal conjugates did not impair the short- and long-term memory of intact rodents, unlike the natural monoterpene used as a reference. MAC-197 produced a similar change in latency in intact and demented rats in both single and multiple

treatments, whereas MAC-198 showed a weaker protective effect in the passive avoidance test, as measured by rats with induced damage was implausibly decreased compared to intact rats. In the single administration, MAC-198 caused 18.8% less pronounced change in latency, and after 11 days the indicator was 28% less pronounced compared to the change in intact rodents. The results suggest that the protective effects of MAC-197 were not affected by the toxic agent, in contrast to the effects of MAC-198, which were less pronounced in rats with scopolamine-induced dementia.

Acetylcholinesterase is a key enzyme regulating the levels of acetylcholine, a major mediator of memory, so its increased activity leads to accelerated degradation of ACh. The effect of the test compounds on brain AChE activity is illustrated in Figure 23.

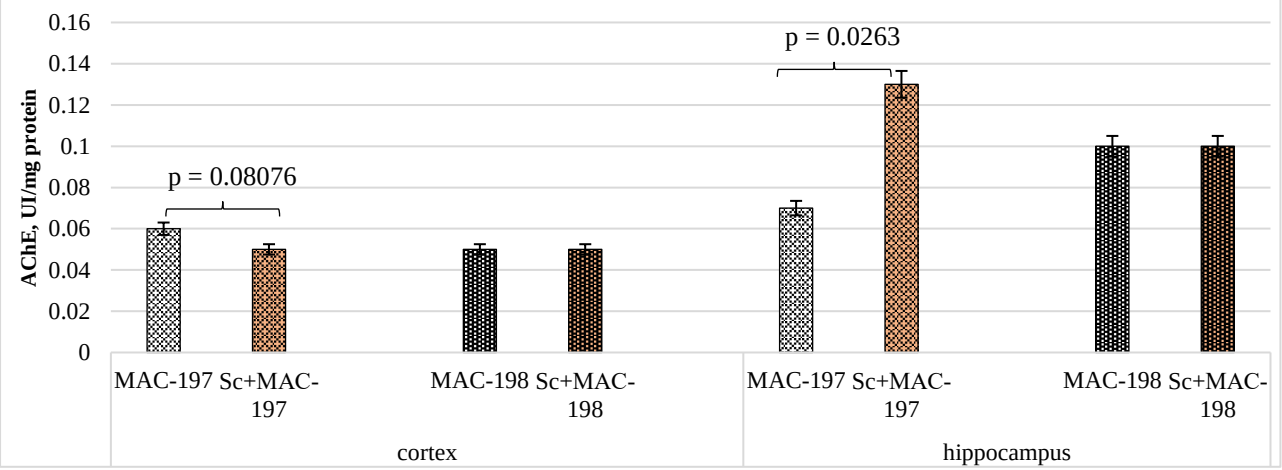


Figure 23. AChE activity in the cerebral cortex and hippocampus of intact and demented rats after 11-day administration of MAC-197 and MAC-198

The two myrtenal compounds have different effects on AChE activity. In contrast to MAC-197, whose effects were affected by scopolamine administration, MAC-198 did not change the values of the indicator in both intact and demented rats in both brain regions examined. The results show that in the cerebral cortex of demented rodents, MAC-197 reduces AChE activity by 17%, with borderline confidence, compared to intact ones, while in the hippocampus it causes opposite effects, which is manifested by a significant increase in enzyme activity – by 85% compared to animals without impairment (P = 0.0263).

It is evident that this derivative possesses properties similar to the parent natural monoterpene, namely the absence of significant anticholinesterase effects in a scopolamine-induced model of dementia (Dragomanova, 2020). The two myrtenal compounds affect AChE activity differently. MAC-198 was found to exhibit significant anticholinesterase properties in rats with chemically induced dementia (Section 5. of Ch. Results and Discussion), and MAC-197 reliably decreased enzyme activity in intact rats (Section 4. of Ch. Results and Discussion).

6.1.2. Brain oxidative status

The changes in basic indicators characterizing the oxidative status in the cerebral cortex of the experimental rats were monitored (Figure 24 A and B).

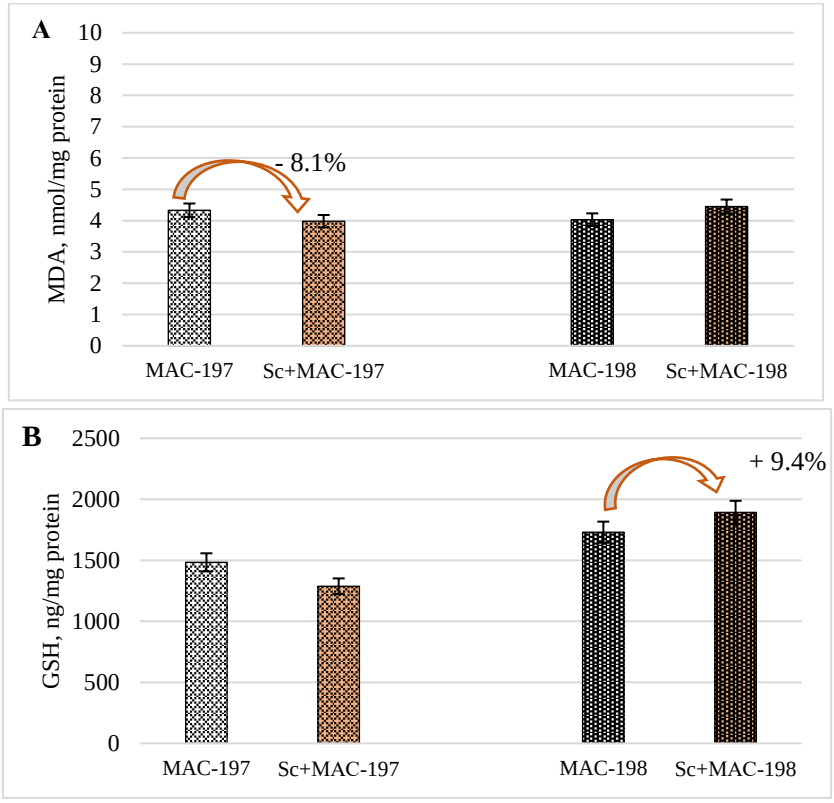


Figure 24. Content of MDA (A) and GSH (B) in the brain of intact and demented rats after 11-day administration of MAC-197 and MAC-198

Dragomanova (2020) found that myrtenal exhibited pro-oxidant properties in intact laboratory rodents and antioxidant potential in rodents with scopolamine-induced damage. Its conjugates, the subject of the present work, modulated the content of MDA and GSH with insignificant difference in intact and demented rodents, which is illustrated in Figure 24. Their effects were little changed in experimental dementia, which is characterized by the presence of oxidative stress. Compared to intact rats, MAC-197 decreased malondialdehyde by 8.1% and MAC-198 increased glutathione by 9.4%.

Antioxidant enzymes are an endogenous defense against oxidative stress, and their levels change with the generation of free reactive species. Studies in intact rats showed no significant changes in their activity as a result of the administration of MAC-197 and MAC-198 (Figure 25 A, B and C).

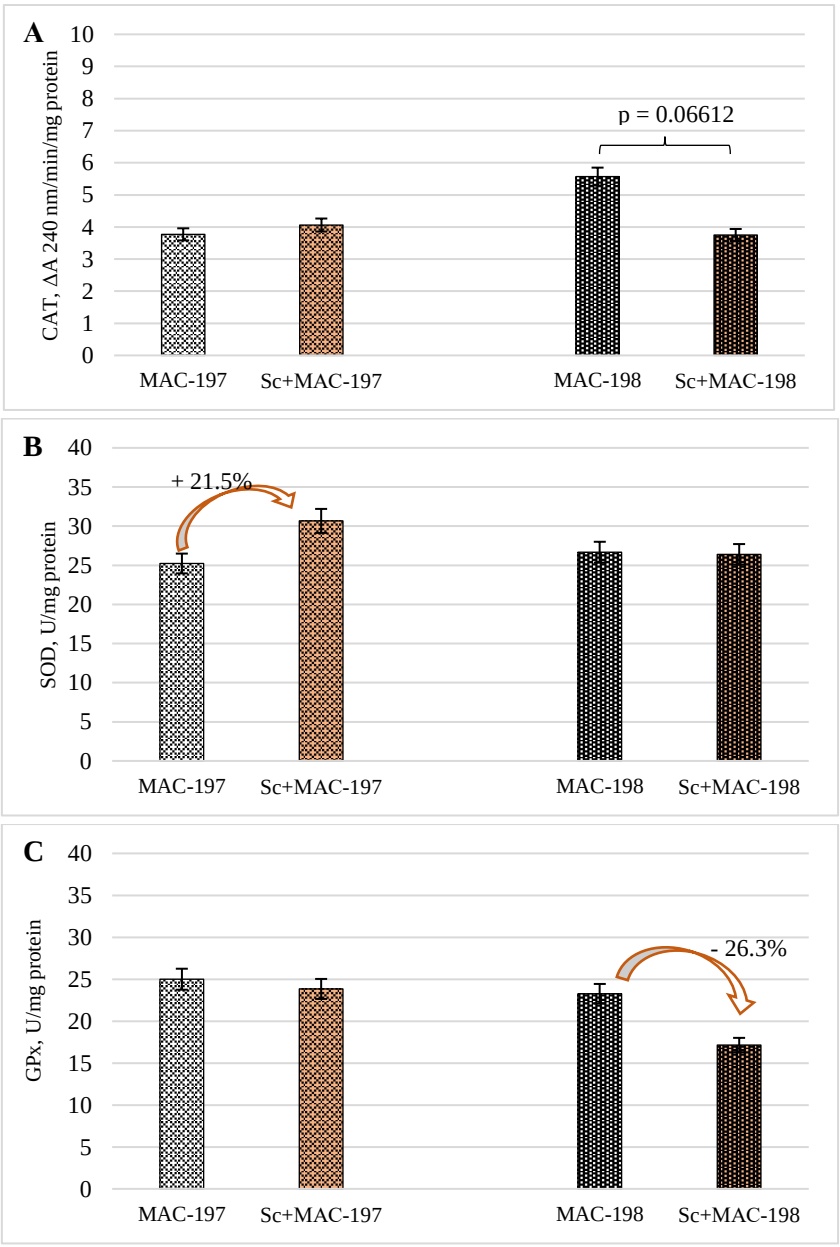


Figure 25. Activity of CAT (A), SOD (B) and GPx (B) in the brain of intact and demented rats after 11-day administration of MAC-197 and MAC-198

MAC-198 decreased CAT activity in intact rats by more than 20% without statistical significance (Section 4. of Ch. Results and Discussion), and in demented rodents it suppressed toxin-stimulated catalase function by 32.6% ($P < 0.001$) (Section 5. of Ch. Results and Discussion). The difference in the effects of this compound on CAT in intact and demented rodents was expressed in a weaker activity of the enzyme in scopolamine-lesioned rats (by 32.7%, borderline significance), compared to the intact group (Fig. 25 A). This result is indicative of the presence of distinct antioxidant properties of MAC-198 in scopolamine-induced dementia.

In both intact and demented rats, the derivatives did not alter SOD activity in the cerebral cortex (Sections 4. and 5. of Ch. Results and Discussion). MAC-197 increased by 21.5% the activity of the enzyme catalyzing the inactivation of free radicals in demented rodents

compared with intact ones (P = n.s.) in response to scopolamine-induced oxidative stress (Fig. 25 B).

Regarding the influence on glutathione peroxidase activity, only MAC-197 has been found to have antioxidant properties, manifested in intact rats by increasing its activity by 39% compared to controls (Section 4. of Ch. Results and discussion). Our results correspond with the data of Sabit (2021), who proved in his dissertation that GPx activity increases when antioxidants were included. In demented rats, MAC-197 maintained GPx activity to values close to those of controls, counteracting the decrease induced by the toxic agent (Section 4. of Ch. Results and Discussion), while MAC-198 did not change the indicator as compared to the demented group.

From the comparison made, illustrated in Figure 25 B, it can be seen that MAC-198 decreased the enzyme activity in demented rats by 26.3% compared to that in intact animals, which was due to its weak potential to counteract the decreased GPx activity, as a result of toxin-induced oxidative stress.

Based on the comparative analysis, it can be concluded that myrtenal conjugates with adamantane have specific antioxidant properties to counteract the generated by scopolamine oxidative stress.

6.1.3. Brain neurotransmitter monoamine levels

The effects of the studied myrtenal conjugates on the content of norepinephrine in the cortex and hippocampus of intact and demented rats after repeated administration are shown in Figure 26.

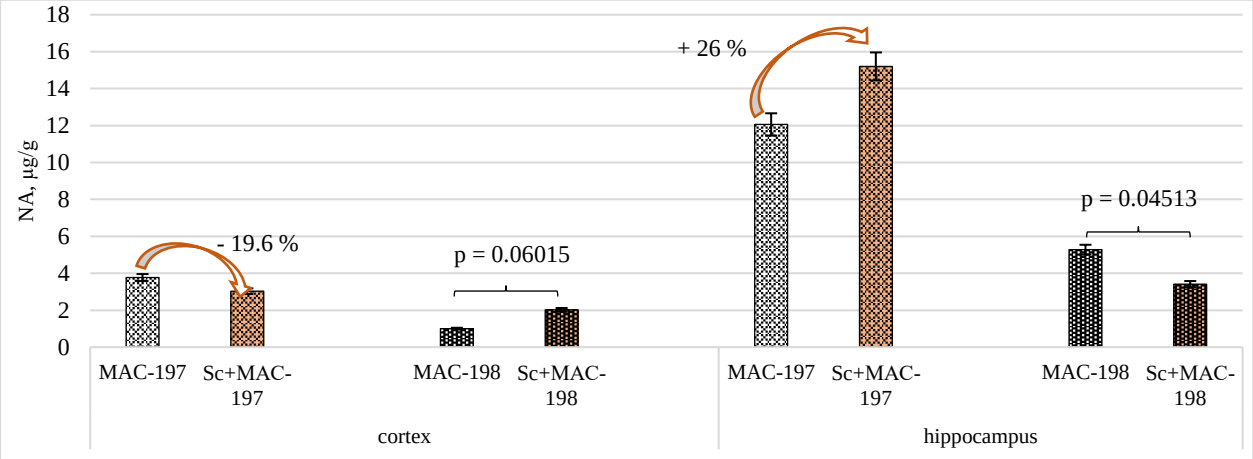


Figure 26. NA content in the cerebral cortex and hippocampus of intact and demented rats after 11-day administration of MAC-197 and MAC-198

In intact rats, MAC-197 increased the content of norepinephrine in the cortex and hippocampus compared to controls, and MAC-198 decreased it (Section 4. of Ch. Results and discussion). These properties were also manifested in rodents from the scopolamine group (Section 5. from Ch. Results and discussion). A unidirectional effect in NA values was also

observed when comparing the effects of the two substances in intact rats and those with experimental dementia (Fig. 26). MAC-197 increased the level of NA in the cortex of the demented group less strongly (by 19.6%, non-significant), while the content of the mediator in the hippocampus of this group was 26% higher than that of the intact animals. Surprisingly, the results were similar for the other myrtenal derivative. MAC-198 produced a smaller decrease in cortical norepinephrine content in demented rats compared to intact rats ($p = 0.06015$) and enhanced the effect of the mediator in the hippocampus, where the difference between the two groups was at a significance level of $P < 0.05$. The effects of myrtenal-adamantane conjugates on norepinephrine levels differed in the two brain regions studied, which is in confirmation of data published by Míguez et al. (1999).

The effect of the studied myrtenal conjugates on serotonin content in the cortex and hippocampus of intact and demented rats after repeated administration is illustrated in Figure 27.

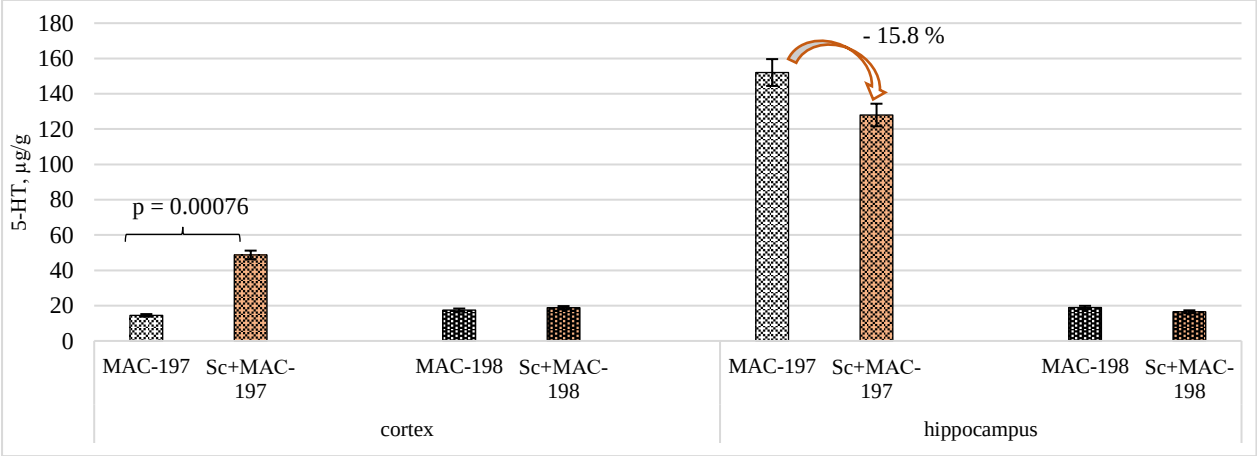


Figure 27. 5-HT content in the cerebral cortex and hippocampus of intact and demented rats after 11-day administration of MAC-197 and MAC-198

And here, as in the study of norepinephrine levels, MAC-197 affected the serotonin content in the two brain areas, that are the subject of the study, in a different direction. The compound increased the level of 5-HT in the cortex and hippocampus in both intact and demented rodents. In the cortex of rats with experimental dementia, MAC-197 led to a stronger increase in 5-HT content than in intact animals, with a high level of significance ($P < 0.001$), while in the hippocampus the change was less pronounced, with an insignificant decrease by 15.8%. MAC-198 did not alter its effect on 5-HT content in either brain region as a result of treating experimental rats with the toxic agent.

In conclusion, the effects of myrtenal conjugates on the levels of the brain monoamine mediators NA and 5-HT in the cortex and hippocampus were multidirectional and also varied with scopolamine administration. We believe that the potential of newly synthesized substances to influence diseases associated with an imbalance of noradrenergic and serotonergic neurotransmission is a promising direction for future research.

6.2. Analysis of the results of behavioral and biochemical studies in demented rats after administration of myrtenal derivatives. Evaluation of the effectiveness of the two molecules in experimental neurodegeneration of the Alzheimer type

A summary analysis of the effects of myrtenal and its aminoadamantane conjugates on experimental animals, according to the linear combination of biochemical and behavioral data, in the experimental model of dementia, is illustrated in Figure 28.

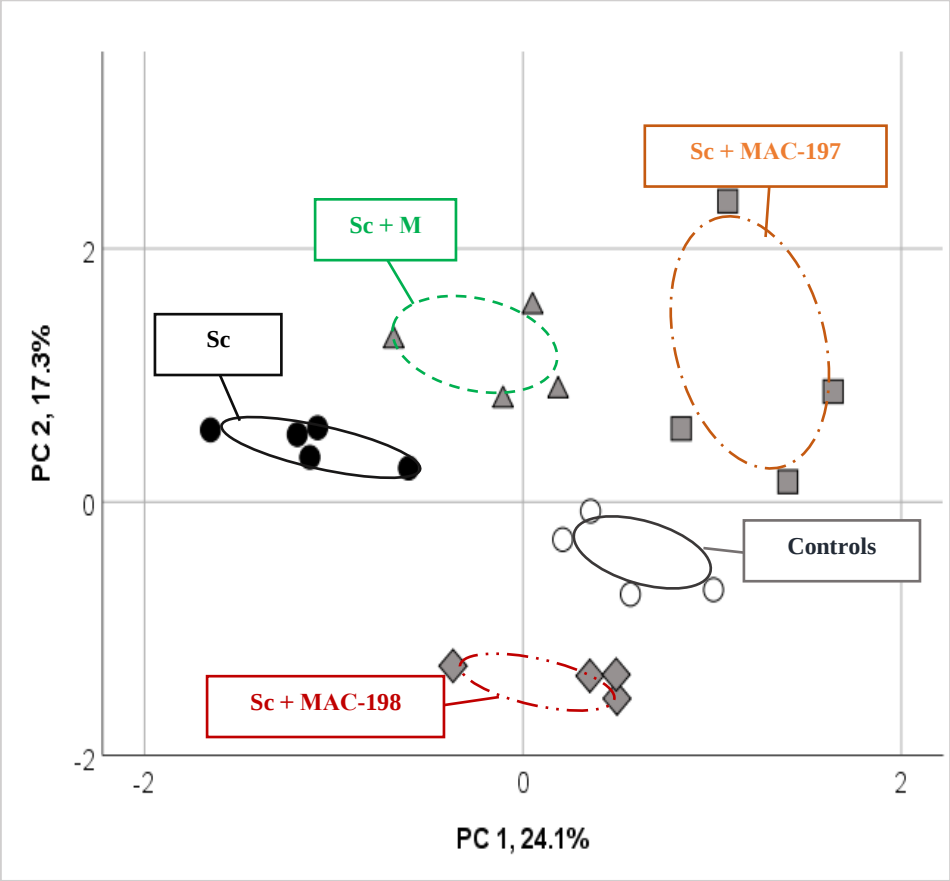


Figure 28. Graphical representation of the principal component analysis result

The values of the first two principal components (PC1 and PC2) plotted on a two-dimensional plane were used as coordinates. Ellipses indicate the grouping found in the hierarchical cluster analysis

Each point in the figure represents a separate experimental animal. Principal component analysis showed that rats formed distinct groups depending on the administered substance, which is an evidence of a systemic effect.

The statistical method used allows to integrate the obtained biochemical and behavioral data in their totality and thus to evaluate the overall neuroprotective effect of myrtenal-adamantane derivatives on experimental dementia in rats. The two compounds MAC-197 and MAC-198 are clearly distinguished from the scopolamine group and are in relative proximity to the Controls. On the other hand, myrtenal-treated demented rats also formed an independent cluster.

These results confirm the assumption that the natural monoterpene myrtenal and its synthetic derivatives, the subject of the study, exhibit specific neuroprotective properties.

V. CONCLUSIONS

1. The physicochemical properties of synthetic myrtenal conjugates with aminoadamantane (MAC-197 and MAC-198) favor their passage through the blood-brain barrier, and software exploration of potential targets reveals their ability to bind to structures in the CNS and thereby affect various neurotransmitter systems and regulatory processes.

2. Repeated intraperitoneal administration of the two compounds (11 days, at a dose of 1 mg/kg) did not cause negative effects on memory processes in intact rats, no general toxic effect was detected and no macroscopic damage to the internal organs (heart, liver, kidneys, stomach, bile and intestine) after dissection.

3. Molecular modeling indicated that brain AChE is a plausible target for the aminoadamantane conjugates of myrtenal. Their anticholinesterase potential, established in docking studies, was confirmed *in vivo* on intact rats, and it was most strongly expressed with MAS-197 in the hippocampal region. Synthetic derivatives significantly improved impaired short-term and long-term memory in demented rodents, which was related to their anticholinesterase activity in the cerebral cortex, and in the case of MAS-198 – in the hippocampus.

4. Myrtenal analogues demonstrated pronounced neuromodulatory properties in both memory-related brain structures of intact rats (hippocampus and cortex), inducing specific changes in biogenic amine levels – Mac-198 decreases NA and 5-HT levels, and MAC-197 significantly increased them. These differences were also preserved in demented rodents – MAC-197 increased the content of NA in the cerebral cortex and hippocampus, and of 5-HT in the hippocampus, while MAC-198 decreases the concentration of 5-HT in the cortex and hippocampus, and of NA in the hippocampus.

5. The antioxidant properties of myrtenal analogs, more pronounced than those of the natural reference, were able to correct scopolamine-induced oxidative stress in the cerebral cortex of demented rats. The two new substances suppressed the increased catalase activity, MAC-197 lowered MDA content and MAC-198 increased total glutathione levels.

6. The two conjugates of myrtenal with aminoadamantane MAC-197 and MAC-198 exhibited more pronounced effects on scopolamine-impaired memory in rodents, as well as more distinct anticholinesterase, antioxidant and neuromodulatory properties, compared to the natural reference.

7. The complex neuroprotective mechanisms of the two investigated substances (anticholinesterase, neuromodulatory and antioxidant) were strongly manifested in rodents with scopolamine-induced dementia, compared to intact rats.

VI. CONTRIBUTIONS

1. For the first time, the neuropharmacological effects of newly synthesized conjugates of myrtenal with aminoadamantane have been investigated. In healthy laboratory rats, the potential of both compounds (MAC-197 and MAC-198) to improve memory has been demonstrated without damaging effects on exploratory behavior.

2. The neuroprotective potential of Myrtenal-aminoadamantane derivatives was established for the first time in rats with an experimental model of dementia, due to their complex mechanisms of action – anticholinesterase, antioxidant and neuromodulatory.

3. The potential of the newly synthesized substances to influence diseases associated with an imbalance of noradrenergic and serotonergic neurotransmission by differentially altering the levels of the brain mediators NA and 5-HT in the cortex and hippocampus of both intact and demented rats has been revealed, which is promising direction for future research.

4. A comparative analysis of the effects of myrtenal derivatives in healthy and demented rats was performed by integrating behavioral and biochemical data. The synthetic conjugates were found to possess specific neuroprotective properties that were more pronounced in memory-impaired rodents than healthy ones.

5. The obtained for the first time in this study original data revealed a greater efficacy of synthetic conjugates of myrtenal with aminoadamantane, compared to the starting natural product, which is a basis for their future development as neuroprotective agents in memory disorders.

VII. LIST OF SCIENTIFIC PUBLICATIONS AND REPORTS RELATED TO THE DISSERTATION

Publications

1. Dragomanova S., Andonova V. Adamantane-containing drug delivery systems. *Pharmacia* **2023**; 70(4): 1057-1066. https://doi.org/10.3897/pharmacia.70.e111593

2. Dragomanova S., Andonova V., Lazarova M., Munkuev A., Suslov E., Volcho K., Salakhutdinov N., Stefanova M., Gavrilova P., Uzunova D., Kalfin R., Tancheva L. Memory-improving effects of myrtenal-adamantane conjugates. *Journal of Chemical Technology and Metallurgy*. **2023**; 58, 3, 627-634.

Scientific reports

1. Dragomanova S., Lazarova M., Munkuev A., Suslov E., Volcho K., Salakhutdinov N., Stefanova M., Gavrilova P., Uzunova D., Kalfin R., Tancheva L. Mechanisms of memory-improving effects of Myrtenal-Adamantane Conjugates in Wistar Rats. Научна конференция с международно участие „Innovations in Drug molecules“, 19 - 22 юли, 2022 г., Хисаря.

2. Dragomanova S., Lazarova M., Munkuev A., Suslov E., Volcho K., Salakhutdinov N., Bibi A., Reynisson J., Tzvetanova E., Alexandrova A., Georgieva A., Uzunova D., Stefanova M., Kalfin R., Tancheva L. Effects of myrtenal-adamantane conjugates on experimental model of dementia. III National Scientific Conference "Courage and youth in pharmacology 2022", September 30 - October 2, 2022, Tsigov chark

3. Dragomanova S., Andonova V., Lazarova M., Stefanova M., Tsvetanova E., Alexandrova A., Uzunova D., Volcho K., Munkuev A., Suslov E., Salakhutdinov N., Kalfin R., Tancheva L. Myrtenal-Aminoadamantane Derivatives in Experimental Dementia. Ninth Pharmaceutical Business Forum and Scientific and Practical Conference, Varna, Oct 20-21, 2023.

4. Dragomanova S., Andonova V., Kalfin R., Tzvetanova E., Alexandrova A., Munkuev A., Suslov E., Volcho K., Salakhutdinov N., Tancheva L. Antioxidant Potential of Myrtenal and Its Adamantane Conjugates in Dementia Rat Model. The 23rd European Meeting on Environmental Chemistry (EMEC 23), 03-06 Dec 2023, Budva, Montenegro.

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Thank you!