

MEMORY-IMPROVING EFFECTS OF MYRTENAL-ADAMANTANE CONJUGATES

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ABSTRACT

Myrtenal (M) is a natural bicyclic monoterpenoid with poorly studied neuroprotective potential. Our previous results established memory improvement in rats accompanied by significantly increased brain acetylcholine (ACh) levels. Adamantane is used as a pharmacophore for new drugs design based on known pharmacological agents for the development of medicines with stronger effects or with different therapeutic properties.

The aim of this study was to evaluate on male Wistar rats the memory-improving potential of two Myrtenal-Adamantane derivatives MDs (MD-197, MD-198), synthesized in Novosibirsk Institute of Organic Chemistry. Both substances (1 mg kg⁻¹ b.wt.) and the referent Myrtenal (40 mg kg⁻¹ b.wt.), were applied as intraperitoneal (i.p.) emulsions for 11 consecutive days. Memory status was evaluated via a Passive avoidance test (at the beginning and at the end of the experiment). Acetylcholine esterase (AChE) activity, noradrenaline (NA), and serotonin (5-HT) content in brain structures related to memory (cortex and hippocampus) were biochemically measured. Results demonstrated significant memory improvement after multiple applications of both MDs. The significant inhibition of hippocampal AChE activity after MD-197 and MD-198 treatment was accompanied by specific changes in monoamine levels in the cerebral cortex and hippocampus. MD-197 significantly increased brain NA and 5-HT content showing potential antidepressant properties. All established effects of the two Myrtenal-Adamantane conjugates were stronger than those of the natural Myrtenal.

This research revealed for the first time MDs' improving memory capacity related to their AChE inhibiting activity and neuromodulatory ability. These original data provide a reason for further investigation of these new perspective substances on the experimental model of dementia.

Keywords: acetylcholine esterase, Myrtenal-Adamantane derivatives, noradrenaline, serotonin Passive avoidance test, rats.

INTRODUCTION

(-)-Myrtenal ((1R)-2-pinen-10-al, (1R)-6,6-dimethylbicyclo[3.1.1]hept-2-en-2-carbox-aldehyde) is a bicyclic monoterpenoid of natural origin (Fig. 1). Monoterpenes are essential oil components with

established beneficial effects on memory, carried out by different mechanisms of action - affecting acetylcholinesterase activity, reducing A β -aggregation and oxidative stress, etc. [1].

The substance is a component of many plant species essential oils. These medicinal plants possess a

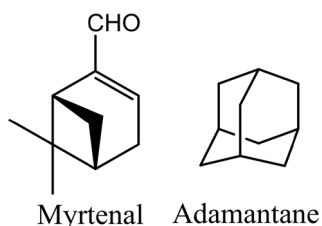


Fig. 1. Chemical structures of Myrtenal and Adamantane.

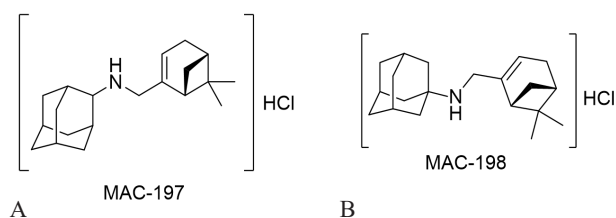


Fig. 2. Chemical structure of MD-197 (A) and MD-198 (B).

wide range of biological activities with the potential to influence various systems and organs, including central nervous system (CNS) functions [2]. Numerous M's effects have been found in experimental animals [3 - 6]. Its analgesic [7] and anxiolytic [8] potentials were proven for the first time in our experiments. Data on the *in vitro* anti-cholinesterase activity of the substance [9] was not confirmed in our studies *in vivo* [10]. On the other hand, M modulates the levels of the brain serotonin and noradrenaline in rats in our earlier studies [11].

Adamantane is an organic compound formed by the fusion of three cyclohexane rings and it is the simplest diamondoid [12] (Fig. 1). The role of the adamantyl moiety as a pharmacophore in biologically active compounds is well known. The inclusion of the adamantyl nucleus in the molecules can significantly affect their lipophilicity, and pharmacological and biological properties [13] as well as increase the permeability of the blood-brain barrier for the modified compound [14, 15].

Since Myrtenal has the property to affect the CNS and Adamantane is a known pharmacophore that modulates the biological properties of the parent molecule, the synthesis of two Myrtenal-Adamantane conjugates (MD-197, MD-198) was previously described [16] and their structures introduced [17], (Fig. 2).

In the pilot experiments they have shown the

potential to affect CNS functions after a single administration to experimental mice [18].

This study on male Wistar rats aimed to evaluate the improvement of memory potential of two new Myrtenal-Adamantane conjugates and compare their effects with the natural monoterpene Myrtenal.

EXPERIMENTAL

Drugs

(-)-Myrtenal ($\geq 97\%$) used for chemical synthesis was purchased from Sigma Aldrich (St. Louis, MO, USA), while 1- and 2-aminoadamantane hydrochlorides, and (-)-Myrtenal 98% were purchased from ACRÔS Organics (Geel, Belgium). MDs were synthesized in the Department of Medicinal Chemistry of Novosibirsk Institute of Organic Chemistry (Novosibirsk, Russia). The spectral data were consistent with those previously reported [19].

Animals

The experimental male adult Wistar rats (180 - 220 g) were kept under standard laboratory conditions, with a 12-h light/dark cycle, drinking water and food for rodents *ad libitum*. The experimental protocols followed the rules of the Ethics Committee of the Bulgarian Food Safety Agency and were in compliance with national laws and rules (Ordinance No.20/ 01.11.2012 on the minimum requirements for the protection and welfare of experimental animals and requirements for establishments for their use, rearing and/or delivery, effective from 1 January 2013, issued by the Ministry of Agriculture and Food, Prom. SG issue 87 of 9 November 2012), based on the European Directive.

Experimental design

Forty male Wistar rats (180 - 220 g) were used in the experiments. Rodents were randomly assigned to the following groups ($n = 10$): 1) Controls (saline), 2) Myrtenal (40 mg kg^{-1}), 3) MD-197 (1 mg kg^{-1}), 4) MD-198 (1 mg kg^{-1}). The substances were administered intraperitoneally daily for a period of 11 days, in separate inoculations. The solutions were prepared immediately before use (*ex tempore*) according to the rules of good laboratory practice. All animal groups were submitted to a Passive avoidance test for the evaluation of memory ability status. After the last day of testing, the rats were

euthanized via mild CO₂ inhalation. Then, their brains were quickly removed, and two main brain structures related to memory - the cortex and hippocampus, were separated. The biochemical analysis included brain acetylcholinesterase (AChE) activity, noradrenaline, and serotonin levels evaluation.

Behavioral Studies

The Passive avoidance test [20] was used to determine short-term and long-term memory states [21]. For initial latency (IL) evaluation, each animal was placed in the light half of the apparatus, before all treatment and light-dark entrance time was measured. When a rat entered in the dark part of the apparatus (with four paws), the door closed, and a weak electrical shock was released through the floor (0.7 mA for 3 sec). For step-through initial latency evaluation each animal was placed in the light half of the apparatus and the time to enter the dark compartment was measured, without electrical shock given. The indicator was measured on the 1 h and on the 12th day after the first application. The observation period was up to 180 sec. As a memory status indicator, the difference in latency time vs. IL was recorded.

Biochemical Studies

Evaluation of brain AChE activity. It was determined according to the Ellman protocol [22]. Brain supernatants were added to a solution of 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) and incubated at 37°C for 5 min. The appearance of yellow color in the reaction of thiocholine with DTNB was spectrophotometrically detected ($\lambda = 412$ nm). The results are expressed as AChE $\mu\text{mol min/g}$ protein.

Evaluation of noradrenaline and serotonin levels. The concentration of both monoamines in the brain cortex and hippocampus was measured via fluorescence reaction by the methods of Jacobowitz and Richardson [23]. For Noradrenaline extraction a phosphate buffer was used and for 5-HT - 0.1 N HCl. For NA fluorescence, the reaction requires ethylenediaminetetraacetic acid (EDTA), iodide solution, alkaline sulfite, and 5N CH₃COOH, whereas for 5-HT, o-phthalaldehyde must be added. Monoamines were determined at $\lambda = 385/485$ nm for NA and $\lambda = 360/470$ nm for serotonin, a calculation based upon standard solution fluorescence, and expressed as $\mu\text{g/g}$ of fresh tissue.

Statistical Analysis

Results are expressed as means \pm the standard error of the mean (SEM). Data statistical analyses were performed by one-way analysis of variance (ANOVA) using the GraphPad Prism 7.0 software (San Diego, CA 92108, USA). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of MDs on memory performance

A significant memory improvement after single (Fig. 3(A)) and multiple (Fig. 3(B)) applications of both MDs was detected.

The control group had preserved memory in the acute experiment, as expected - the latency time was significantly increased compared to IL ($P < 0.0001$) (Fig. 4(A)). In a single injection, M demonstrated hypomnesic properties as established in our previous studies [8], lowering the indicator values by 72.8 % as compared to controls ($P < 0.0001$). The acute administration of both MDs caused an increase in the latency time. MD-198 application caused an effect, comparable to that of the controls, and differed to the M-group with high level of significance ($P < 0.001$).

After repeated treatment, the control group again showed preserved memory ability. It was demonstrated by a significantly increased index value over that of IL ($P < 0.0001$) (Fig. 3(B)). M-treated rats again demonstrated reduced memory capacity in compare to the controls (by 65 %, $P < 0.05$). Repeated application of the two M derivatives for 11 days preserved rat memory, close to those of the controls and with higher values than M group ($P < 0.05$).

Brain AChE activity

Both M derivatives showed an affinity towards the AChE active site in docking analysis from our pilot studies. The most *in vivo* protocols have tracked the effects of compounds only in the hippocampus of experimental rats. In our current experiments, we found that the two substances affected differently the enzyme activity in both structures responsible for memory processes, namely the cortex and the hippocampus (Fig. 4).

In the cerebral cortex, both M and MD-198 increased AChE activity - by 52.5 % ($P < 0.05$) and 114.06 % ($P < 0.001$), respectively (Fig. 4(A)). MD-197 did not change

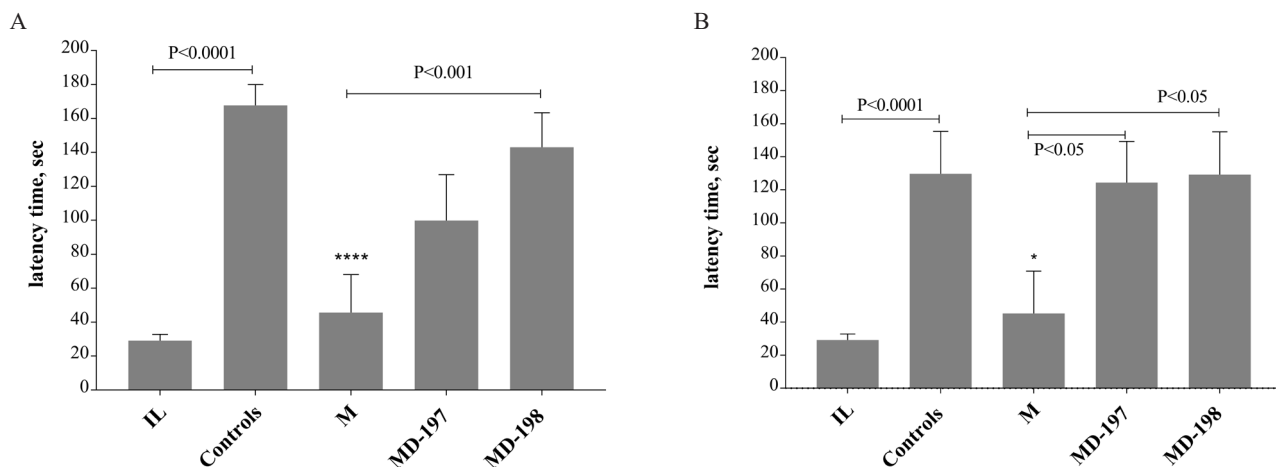


Fig. 3. Effects of MD-197 and MD-198 on memory after acute (A) and multiple treatment (B) in rats; M was used as a referent. Data are expressed as the mean \pm SEM (n = 10). **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05 vs. IL.

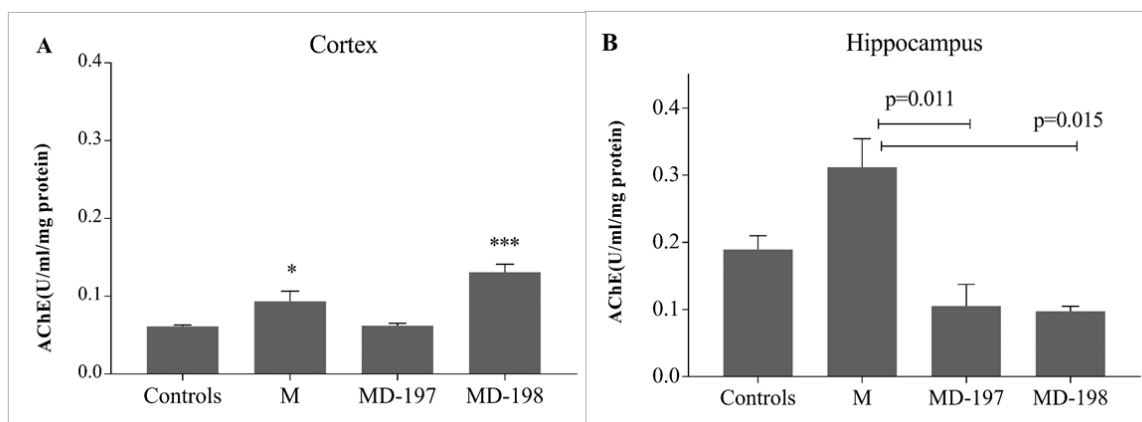


Fig. 4. Effects of MD-19 and MD-198 on AChE activity in rats' brain cortex (A) and hippocampus (B). Data are expressed as the mean \pm SEM (n = 5); *** P < 0.001, * P < 0.05 vs. Controls.

this indicator compared to the controls, which allows us to conclude that there are no effects of MD-197 on AChE enzyme activity in the cortex.

Hippocampal AChE activity was significantly inhibited after MD-197 and MD-198 treatment in comparison to M (Fig. 4(B)). Like in the cortex, in the hippocampus M also caused an activation of the enzyme, as was established in our previous studies [8]. On the contrary, Myrtenal's conjugates suppress it. Compared to M, MD-197 lowers AChE activity by 66.3 %, and MD-198 - by 68.8 %, with a level of significance P < 0.01. Compared to the control group hippocampal AChE

activity was decreased by both MDs – MD-197 by 44.4 % and MD-198 by 48.6 %. The results are summarized in Table 1.

Norepinephrine (NA) content

In the cerebral cortex, M decreased the mediator level by 26.8 % and MD-198 – by 59.6 % as compared to controls (Fig. 5(A)). On the contrary the other M derivative MD-197 increased NA content by 47.5 % as compared to the controls and was three times higher than the effect of MD-198 (P < 0.05).

In the hippocampus, M also caused a decrease of

Table 1. AChE activity in two brain structures related to memory processes: cortex and hippocampus.

%	Controls	M	MD-197	MD-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)
Total effect		+ 117.34	- 43.23	+ 65.5

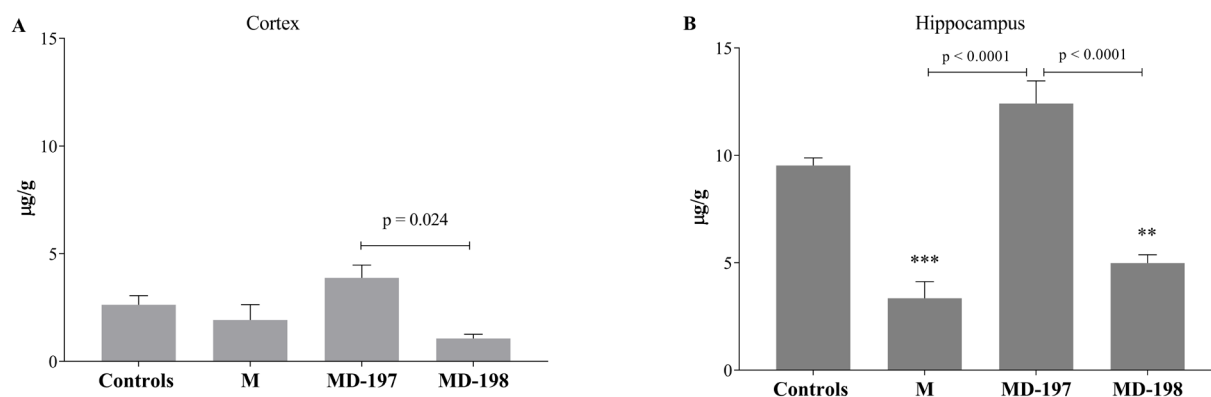


Fig. 5. Effect of MD-197 and MD-198 on brain NA content in rats' cortex (A) and hippocampus (B); M was used as a referent. Data are expressed as the mean \pm SEM (n = 5). *** $P < 0.001$, ** $P < 0.01$ vs. Controls.

64.9 % of NA as compared to controls ($P < 0.001$) (Fig. 5(B)). The influence on NA content by M derivatives maintains the same trends as in the cortex. MD-197 increased NA levels by 30.2 % ($P < 0.06$), while MD-198 reduced the mediator content by 47.7 % in comparison to the control group ($P < 0.01$). The difference between MD-197 and MD-198 effect was at $P < 0.0001$ level of significance.

Serotonin (5-HT) content

In the cortex, M lowered not only NA level but also level of serotonin - by 53.2 % compared to the controls (Fig. 6(A)). But the effect of M derivatives was the opposite. MD-197 increased 5-HT content threefold as compared to myrtenal ($P = 0.0237$) and by 30.2 % as compared to controls. Similarly, MD-198 increased the mediator level even more than MD-197 – it was four fold as compared to the M group ($P = 0.0015$) and by 72.7 % as compared to controls ($P < 0.05$).

In the hippocampus, M caused a decrease in 5-HT content with a significance level of $P < 0.001$ compared to controls (Fig. 6(B)). Again, M derivatives affected the mediator content in the opposite direction. As in the

cortex, MD-197 increased the level of 5-HT by 31.9 % as compared to the controls ($P = n.s.$). The reduction after the MD-198 application was by 83.1 % as compared to the control group ($P < 0.0001$), and its difference with MD-197 group was at a $P < 0.0001$ level of confidence.

Memory impairment is a complex process with not fully understood etiology and pathogenesis. Numerous studies are aimed at prevention and therapy of diseases related to memory decline, including neurodegenerative processes. Because of the multifactorial and complex nature of these disorders, the goal of many researches is focused on discovering therapeutic candidates that possess multitarget mechanisms of action. Such compound is the monoterpene Myrtenal, which in our previous studies showed the potential to affect neurodegenerative processes in experimental models of Parkinson's disease and Alzheimer's type dementia [10, 24]. On the other hand, adamantane is a powerful pharmacophore often used in the modulation of various molecules. The conjugates that are the subject of the present study were obtained by reaction of (-)-myrtenal with 1- or 2-aminoadamantane, followed by NaBH_4 reduction of the corresponding imines as it was described

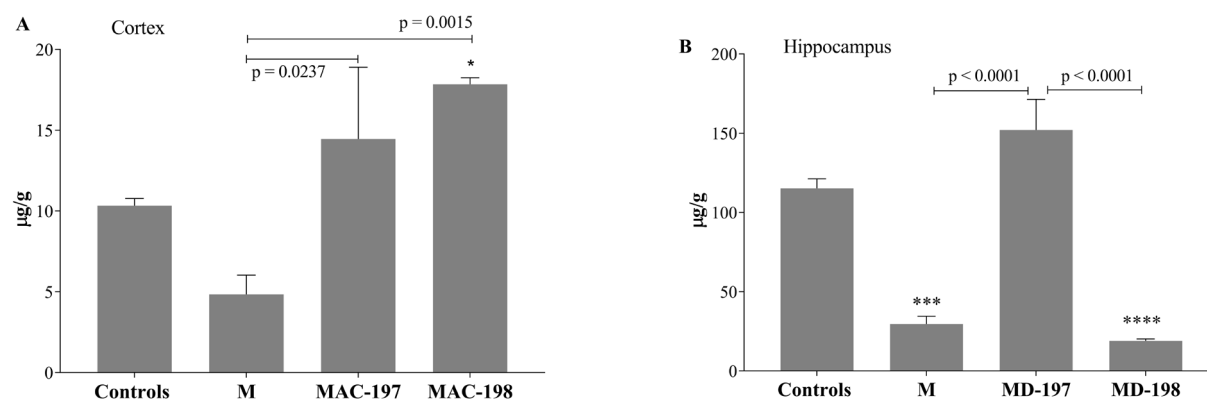


Fig. 6. Effect of MD-197 and MD-198 on brain 5-HT content in rats' cortex (A) and hippocampus (B); M was used as a referent. Data are expressed as the mean \pm SEM (n = 5). **** P < 0.0001, *** P < 0.001 vs. Controls.

by Suslov et al. [16]. After that, they were chemically transformed into corresponding hydrochlorides by bubbling gaseous HCl through an ethereal solution, followed by filtration of the precipitate formed. The myrtenal moiety, conjugated with an adamantane fragment, demonstrated enhanced biological activity, and increased metabolic stability.

In the present study, for the first time we demonstrated memory improving capacity of the two newly synthesized conjugates of myrtenal with adamantane in intact rodents. Behavioral tests pointed out preserved memory function in the rats by both compounds. After their single and repeated administration, the values were close to those of the controls. These data correlated well with the results of AChE activity evaluation in the cortex and hippocampus. Both MDs inhibited the hippocampal enzyme activity but did not possess significant anti-cholinesterase properties in the cortex. Their multidirectional effects in the two brain structures mostly related to memory were presented in Table 1. We can assume anticholinesterase potential of both compounds, mostly pronounced of compound MD-197. The same derivative decreased mostly hippocampal AChE activity. It is known that AChE activity is physiologically higher in the hippocampus. Probably this is the reason for the observed beneficial effects of both compounds on the memory of experimental rats.

Brain acetylcholinesterase activity directly determines levels of memory mediator acetylcholine, but other brain neurotransmitters are also implicated

in memory status. The contents of norepinephrine and serotonin are relevant to the degree of manifestation of both cognitive and non-cognitive symptoms in dementia, such as depression, anxiety, agitation, eating and sleeping disorders, and aggression [25]. Cognitive and non-cognitive symptoms of Alzheimer's disease are related to modulated levels of biogenic amines in patients' brains. Based on the role of norepinephrine (known as noradrenaline) levels in cognitive dysfunction and the progression of neurodegenerative processes, Gutierrez et al. proposed it as a potential therapeutic target [26]. Serotonin also is a leading mediator not only to the occurrence of depression but together with acetylcholine it is involved in cognitive problems [27]. Cholinergic/serotonergic imbalance contributes to cognitive impairment [28].

Some specific changes in monoamine levels in the cerebral cortex and hippocampus were observed upon administration of the two synthesized substances. Interestingly, the effects of the two conjugates are opposite. While MD-198 lowers the content of norepinephrine and serotonin in brain structures, MD-197 significantly increased them.

The effects of both compounds were compared with those of myrtenal. The natural monoterpene has hypomnesic properties, which we found in our previous research [8]. In our opinion, this is related to its anxiolytic potential. In contrast, adamantane conjugates did not negatively affect the memory processes of experimental rats. These results were

confirmed by the anti-cholinesterase properties of the two newly synthesized compounds, while for M an AChE-inhibition was absent. Regarding the effect on the levels of biogenic amines in the brain, while M lowers NA and 5-HT, MDs have different effects. MD-198 exhibited monoterpene-like properties, demonstrated by a decrease in NA and 5-HT levels, while MD-197 significantly increased them. These results revealed the broad spectrum of their therapeutic potential. In addition to neuroprotection involving anticholinesterase and neuromodulatory mechanisms of action, MD-197 has also the potential to affect depressive symptoms in neurodegenerative processes. These results revealed the broad spectrum of the therapeutic potential of the two new conjugates, which deserves further investigation.

CONCLUSIONS

Present research revealed for the first time memory improving capacity of the two newly synthesized myrtenal-adamantane conjugates which was related to their AChE-inhibiting effects and neuromodulatory activity in cortex and hippocampus of rats. All established behavioral and biochemical effects of the two MDs were stronger than those of the natural Myrtenal. The established original data provide a reason for further investigation of these new perspective substances on experimental models of dementia.

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