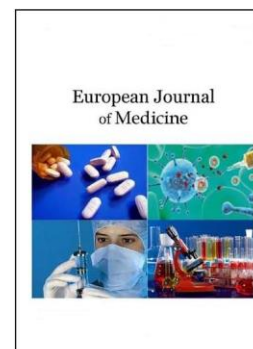


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Articles

Regulation of Transport of Ca²⁺ NMDA-Receptors in Rat Brain Synaptosomes Under the Influence of Polyphenols

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Abstract

It is known that disturbance of Ca²⁺ dynamics in nerve cells is universally involved in all pathologies of Alzheimer's disease (AD). It is likely that the use of chemical agents or small molecules specific for Ca²⁺ channels or membranes of intracellular organelles to correct Ca²⁺ dysregulation of neurons may open up a new approach to the prevention and treatment of AD. The experiments were carried out according to the Weiler method. Synaptosomes were isolated from the brain of rats by two-stage centrifugation at a temperature of 4°C. To measure the amount of cytosolic Ca²⁺ synaptosomes were calculated by the Grinkevich equation. The results of the experiments show that there is a possibility of competition between different polyphenols of Caralinea, Granatum and glutamate for the site of regulation of the opening ion channels of NMDA-receptors.

Keywords: synaptosome, NMDA receptors, glutamate, calcium.

1. Introduction

In recent years, there has been a significant increase in the number of diseases of the nervous system, which, due to their prevalence and consequences, are classified as socially significant neurodegenerative diseases due to a violation of calcium homeostasis of excitable nerve cells in the brain. Alzheimer's disease (AD) is the most common type of neurodegenerative disease and is characterized by memory loss and cognitive decline, which eventually leads to dementia (Alzheimer's Association, 2017). Although the etiology of AD has not been determined, the pathological features associated with AD are mainly the accumulation of extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau (Tiraboschi et al., 2004). More than 30 million people worldwide have developed asthma (Karch et al., 2014). Cellular Ca²⁺ homeostasis plays a key regulatory role in many aspects of neuron physiology, including growth and differentiation, action potential properties, synaptic plasticity, learning, and memory. Impaired cellular Ca²⁺ also contributes to pathophysiological conditions

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such as necrosis, apoptosis, autophagy deficiency, and degeneration (Bezprozvanny, 2009). The hypothesis that Ca^{2+} dysregulation leads to neurodegeneration was first proposed by Dr. Khachaturian in the mid-80s. He suggested that a persistent imbalance of cellular Ca^{2+} could impair normal neuronal function and ultimately lead to neurodegenerative diseases, including AD (Khachaturian, 1987). Although various types of Ca^{2+} disorders have been reported in AD cells for over 30 years (Peterson et al., 1985), both amyloid and tau hypotheses have been described as the main causative factors in AD. Drugs targeting $\text{A}\beta$ have been largely unsuccessful, and Ca^{2+} disruption may have occurred before plaque and tangle deposition. Thus, drugs aimed at correcting the Ca^{2+} abnormality may provide an alternative therapeutic strategy for AD. In this review, we will consider the mechanisms involved in Ca^{2+} disorders in AD and propose an understanding of the correction of Ca^{2+} disorders as a therapeutic strategy for the prevention and treatment of AD.

NMDA-receptors (NMDAR) are Ca^{2+} -favoring glutamate-gated ion channels that are expressed in most central neurons and were initially held responsible for neuronal injury, owing to their high Ca^{2+} permeability and conductance properties (Rothstein, 1996). Continuous activation of large numbers of NMDAR (especially the NR1/NR2B-subtype) leads to increases in intracellular calcium loads and catabolic enzyme activities, which can trigger a cascade of events eventually leading to apoptosis or necrosis (Ndountse, Chan 2009). These downstream effects include mitochondrial membrane depolarization, caspase activation, production of toxic oxygen and nitrogen free radicals, and cellular toxicity (Jung et al., 2009; Fan et al., 2007). The NMDAR are also effective in mediating excitotoxic neuronal injury. For example, neurons expressing high levels of NMDAR are lost early in the striatum of individuals affected with neurodegenerative disease, and injection of NMDAR agonists into the striatum of rodents or non-human primates recapitulates the pattern of neuronal damage in AD (Handley et al., 2006).

It has been proven that the T-type channel can contribute to exocytosis and may be involved in spontaneous synaptic transmission. L-glutamate is the main excitatory neurotransmitter in the brain that stimulates the receptor-gated Ca^{2+} channel. Depending on the mode of operation, L-glutamate activates two classes of receptors, namely the ionotropic receptor (iGluR) and the metabotropic receptor (mGluR). mGluRs are associated with G proteins that generate Ca^{2+} signals by activating phospholipase C or by modulating adenylate cyclase activity. While α -amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid sensitive receptor (AMPA) and NMDAR are the two main types of iGluR. Both AMPA and NMDAR are important for neuronal plasticity by modulating long-term potentiation (LTP) of postsynaptic neurons. NMDAR is permeable to both Na^+ and Ca^{2+} . NMDAR-mediated Na^+ influx promotes postsynaptic depolarization, while Ca^{2+} entering through NMDAR generates Ca^{2+} transients that promote neuronal transcription upon LTP induction (Miyashita et al., 2012). Like iGluRs, ionotropic purinergic receptors mediate extracellular Ca^{2+} influx in response to extracellular ATP. They are the main postsynaptic Ca^{2+} entry channels at the resting potential when NMDARs are blocked by Mg^{2+} , and their activation has multiple modulating effects on synaptic plasticity (Khoshimov et al., 2020; Pankratov et al., 2003).

Several therapeutic agents currently targeting plasma Ca^{2+} channels have shown good efficacy in both *in vitro* and *in vivo* models of AD. Some of them are already approved drugs for the treatment of AD (memantine) or are undergoing clinical trials (nimodipine) (Zhang et al., 2016). However, all of these drugs target plasma membrane channels rather than the most common disrupted intracellular Ca^{2+} signaling pathways, such as the ER and lysosome pathways. Therefore, additional research efforts are needed to elucidate the role of intracellular Ca^{2+} in the pathogenesis of AD. As our knowledge of the molecular mechanisms linking Ca^{2+} disturbances to AD pathology becomes clearer, more specific therapeutic agents targeting channels on the membranes of intracellular organelles such as the ER, mitochondria, and lysosomes will be developed and provide new hope for prevention and treatment. Collected all these literature data shows that, in Alzheimer's disease, there is a hypothesis of excitotoxicity of glutamate (Wen et al., 2016).

It is known that various mechanisms associated with glutamate excitotoxicity are explained by neuronal death characteristic of various neurodegenerative diseases, including an increase in intracellular calcium levels, accumulation of oxidizing free radicals, impaired mitochondrial function, and activation of apoptosis and autophagy programs. The identification of a key post-receptor signaling event that leads to neuronal dysregulation and death has the potential to become a molecular target for therapeutic interventions in various neurodegenerative diseases.

(Khoshimov et al., 2018). Based on this data, the study focuses on the mechanism of action of polyphenolic compounds on glutamate-binding sites of NMDAR in rat brain synaptosomes.

The aim of the study is to characterize the mechanism of action of polyphenolic compounds of *Caralinia* isolated from *Caralinia kaspia* plants and Granatum polyphenol isolated from *Punica granatum* plants *in vitro* on the regulation of Ca^{2+} NMDA glutamate receptor transport in rat brain synaptosomes.

2. Materials and methods

2.1. Isolation of synaptosomes

Experiments were carried out on 20 outbred albino male rats weighing (280-300 g) kept in the standard diet of the vivarium. All experiments were carried out in accordance with the requirements of the World Society for the Protection of Animals and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (European Convention..., 1986). Synaptosomes are obtained by two-stage centrifugation Centrifuge K-24 (ELN13893354.Veb MLV Zenrifugenbau Engelsdorf. Germany) (Weiler et al., 1981). The entire isolation procedure is carried out at -4°C . After decapitation, the brain is removed as quickly as possible and crushed on ice. The crushed tissue is homogenized at a ratio of 1:10 in the isolation medium - 0.32 M sucrose solution in 0.01 M Tris-HCl buffer with the addition of 0.5 mM EDTA (pH 7.4). The obtained homogenate is exposed to a 4-stage centrifugation. The supernatant after the first centrifugation (10 min, 4500 rpm) is carefully removed without capturing the myelin layer and exposed to further centrifugation for 20 min at 14000 rpm. The obtained dense precipitate P2 is resuspended in the isolation medium. The obtained suspension is used further in the experiment as a coarse synaptosomal fraction (synaptosomal-mitochondrial). In the case of 4-stage isolation, the second centrifugation is carried out at 11,000 rpm for 20 minutes. The dense pellet of P2 is resuspended in 0.32 M sucrose solution (pH 7.4) and then carefully layered on 0.8 M sucrose solution (pH 8.0), after which it is centrifuged for 25 minutes at 11,000 rpm. As a result of centrifugation in a sucrose gradient, fractions are separated - mitochondria settle tightly at the bottom of the tube, and synaptosomes remain in suspension in a layer of 0.8 M sucrose. This layer is carefully removed, mixed with an equal amount of isolation medium and left for 15 minutes to restore the ultrastructure of synaptosomal particles, after which it is exposed to further centrifugation at 14,000 rpm for 30 minutes. The dense final precipitate P4 is resuspended in the isolation medium and then used in the experiment as a synaptosomal fraction.

2.2. Measuring intracellular Ca^{2+}

To measure the amount of membrane bound Ca^{2+} , 20 μM chlortetracycline (CTC) was added to synaptosomes placed in a medium similar to that used for cell isolation, but without apyrase and MgCl_2 . It is incubated 60 min. to achieve maximum interaction of CTC with membrane-bound Ca^{2+} , both on plasma and intracellular membranes. The CTC excitation wavelength is 405 nm, the registration wavelength is 530 nm. The results were expressed as percentages, taking as 100 % the difference between the maximum fluorescence intensity (fluorescence of the dye saturated with Ca^{2+}) and its minimum value (fluorescence of the indicator in the absence of Ca^{2+}) obtained after the addition of EGTA.

The amount of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{in}}$) was calculated using the Grinkevich equation (Gryniewicz et al., 1985) in synaptosomes isolated from rat brains. To measure free cytosolic Ca^{2+} , synaptosomes (1×10^8 cells/ml) were loaded with 4 μM Fura-2AM acetoxymethyl ester for 40 min at 37°C . At the same time, in the dye molecules that have penetrated into the cytoplasm, under the action of intracellular esterases, the ester group is cleaved off, resulting in the Fura-2 anion that binds Ca^{2+} . After completion of the loading, the dye remaining in the medium was removed by double washing and centrifugation in standard medium. In the experiments, the cell concentration in the cell was 5×10^6 cells/ml. Fluorescence excitation was induced at 337 nm and fluorescence registration at 496 nm. Ca^{2+} saturated dye fluorescence (F_{max}) was determined by adding 50 μM digitonin to cells loaded with Fura-2AM. F_{min} was determined by measuring the fluorescence intensity in a calcium-free medium, $F_{\text{min}} = [(F_{\text{max}} - F_{\text{af}})/3] + F_{\text{af}}$, where F_{af} is cell autofluorescence determined by adding 0.1 mM MnCl_2 to thymocytes loaded with Fura-2AM and processed with digitonin.

2.3. Statistical analysis

The measurements were carried out on a universal spectrometer USB-2000 (USB2E7916.OceanOptics.USA.2010). Statistical significance of differences between control and experimental values, determined for a data series using a paired t-test, where control and experimental values are taken together, and an unpaired t-test, when taken separately. A P value <0.05 indicates a statistically significant difference. The results obtained are statistically processed in Origin 7.5 (Origin Lab Corporation, USA).

3. Results and discussion

Many neurodegenerative diseases are accompanied by an increase in glutamatergic transmission, which occurs due to an increase in the release of L-glutamate. The excitatory neurotransmitter L-glutamate causes damage and death of neurons, and therefore the damaging effect of glutamate on neurons is referred to as "excitatory amino acid toxicity" or "excitotoxicity" (Duchen, 1999; Khoshimov et al., 2021; Rakhimov et al., 2021, Khoshimov et al., 2021).

The experiments used synaptosomes, which are an adequate and convenient model for studying presynaptic processes. The activity of L-glutamate was judged by the change in the intensity of the fluorescent signal, by the change in $[Ca^{2+}]_{in}$.

Polyphenols are known to be a group of naturally occurring phytochemicals that are present in high amounts in fruits, vegetables and natural foods and are characterized by the presence of several hydroxyl groups on aromatic rings. These compounds are divided into two main categories: the flavonoids and non flavonoids, based on the number of phenol rings and the way in which these rings interact.

The experiments were performed in two stages both in the presence and in the absence of physiological Ca^{2+} concentrations. In experiments, the effect of L-glutamate on the level of $[Ca^{2+}]_{in}$ synaptosomes from the rat brain was investigated. The fluorescence ratio was determined using a Ca^{2+} -sensitive chlortetracycline (CTC) probe. Ethylene glycol bis[2-ethylamino] tetraacetate (EGTA) was used to determine the removal of Ca^{2+} from the extracellular medium.

The chelating ability of L-glutamate with respect to calcium ions was studied in intact synaptosomes both in the presence and in the absence of EGTA. As a result, it was shown that L-glutamate in solution, as in EGTA, has the ability to bind calcium ions in intact synaptosomes, which suggests that the L-glutamate is a calcium ion chelator. Pre-incubation with EGTA led to a decrease in fluorescence by 5 %. In the presence of EGTA in the incubation medium, glutamate at concentrations of 10-100 μM dose-dependently increases the fluorescence level by 25-48 %, which is explained by an increase in the concentration of $[Ca^{2+}]_{in}$, caused by glutamate, primarily due to activation of membrane permeability, Ca^{2+} movement into the cell and release of Ca^{2+} from intracellular depots of the cell (Figure 1).

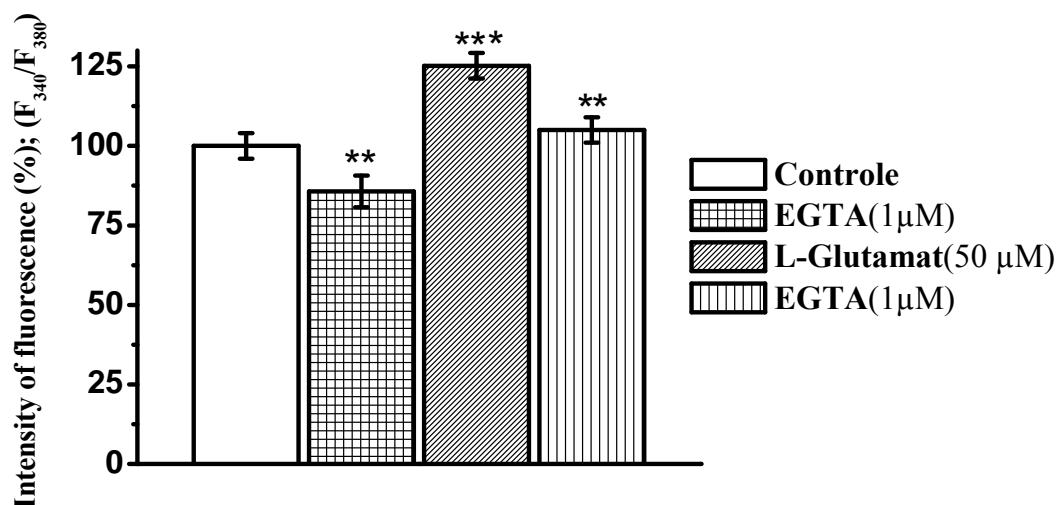


Fig. 1. Effect of L-glutamate (50 μM) on the fluorescence intensity in a suspension of rat brain synaptosomes upon incubation with EGTA (1 μM)
Reliability indicator: * – P < 0.05; ** – P < 0.01; *** – P < 0.001. (n = 6)

It is known that the mechanism of l-glutamate is associated with short-term presynaptic plasticity associated with the release of neurotransmitters and instead determines the form of the response of the postsynaptic neuron and plays a key role in encoding information in the nervous system. This allows the regulation of presynaptic Ca^{2+} channels to either promote or inactivate incoming Ca^{2+} currents. This strong dependence of neurotransmitter release on presynaptic Ca^{2+} current may predict regulatory mechanisms that will affect short-term presynaptic plasticity.

Re-incubation of Caralinia and Granatum (10-100 μ M) with the CTC-synaptosom complex increases the level of $[Ca^{2+}]_{in}$ contrast to glutamate (Figure 2).

A complex effect of Caralinia and Granatum polyphenols together with L-glutamate on the $[Ca^{2+}]_{in}$ level in synaptosomes was carried out.

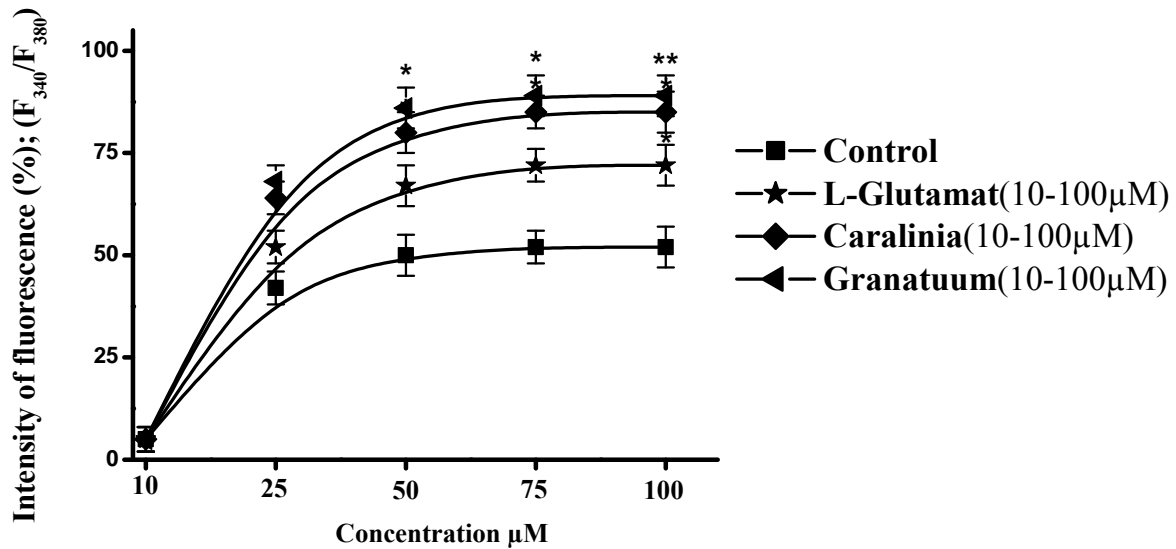


Fig. 2. Effect of Caralinia and Granatum and L-glutamate at concentrations (10–100 μ M) on the intensity of CTC-fluorescence of a rat brain synaptosome suspension. Reliability indicator: * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$. (n = 6)

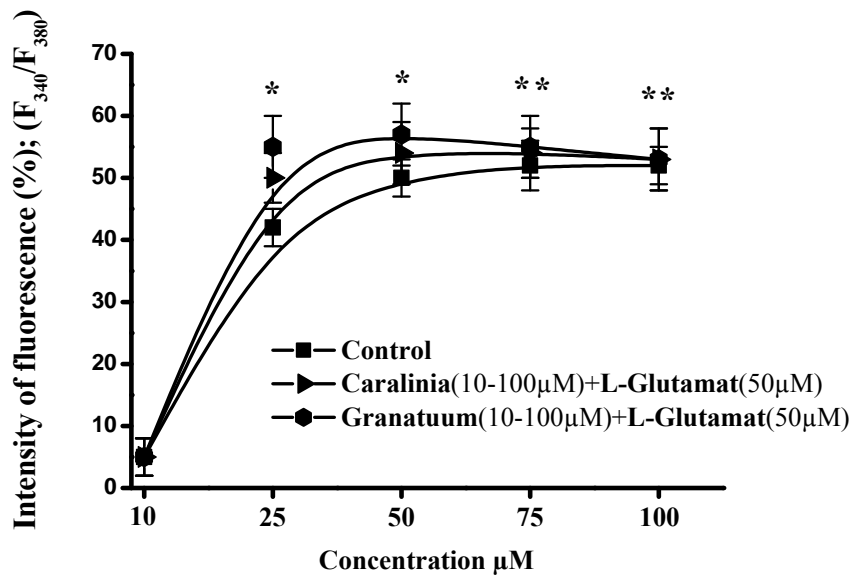


Fig. 3. Effect of Caralinia and Granatum (10-100 μ M) on the fluorescence intensity of a suspension of rat brain synaptosomes incubated with glutamate (50 μ M) Reliability indicator: * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$. (n = 6).

Preliminary re-incubation of Caralinia and Granatum (10 μM) with CTC-synaptosomes and then the addition of L-glutamate led to a decrease in $[\text{Ca}^{2+}]_{\text{in}}$. A dose-dependent and simultaneous increase in the concentration of Caralinia and Granatum (10-100 μM) led to a dose-dependent decrease in the effect of L-glutamate (Figure 3).

It is known in the literature that activation of the L-glutamate receptor, in turn, causes the opening of calcium channels of ionotropic receptors, the entry of calcium into synaptosomes through membranes and depolarization of membranes, followed by the release of amino acid neurotransmitters (Khoshimov et al., 2018).

The results obtained showed that Caralinia and Granatum partially reduce the action of L-glutamate, and this gives information that calcium from the outside comes under the influence of caralinin also through the glutamine site and, as a result, open calcium channels in NMDAR.

Further, pre-supplementation with 100 μM glutamate does not completely abolish the effects of Caralinia and Granatum, suggesting that Caralinia and Granatum have several mechanisms of action on rat brain neurons leading to increased $[\text{Ca}^{2+}]_{\text{in}}$.

It was found that the incubation of Caralinia and Granatum (10–50 μM) in a suspension of synaptosomes significantly increases the intensity of CTC fluorescence. And upon preincubation with L-glutamate (50 μM), Caralinia and Granatum (10–100 μM) significantly reduces the fluorescence intensity.

The addition of glycine (5 μM) to the incubation medium enhanced the glutamate-dependent enhancement of fluorescence by 15-25 %. Mg^{2+} (50 μM) inhibits L-glutamate-induced release of $[\text{Ca}^{2+}]_{\text{in}}$ synaptosomal depots. (Figure 4).

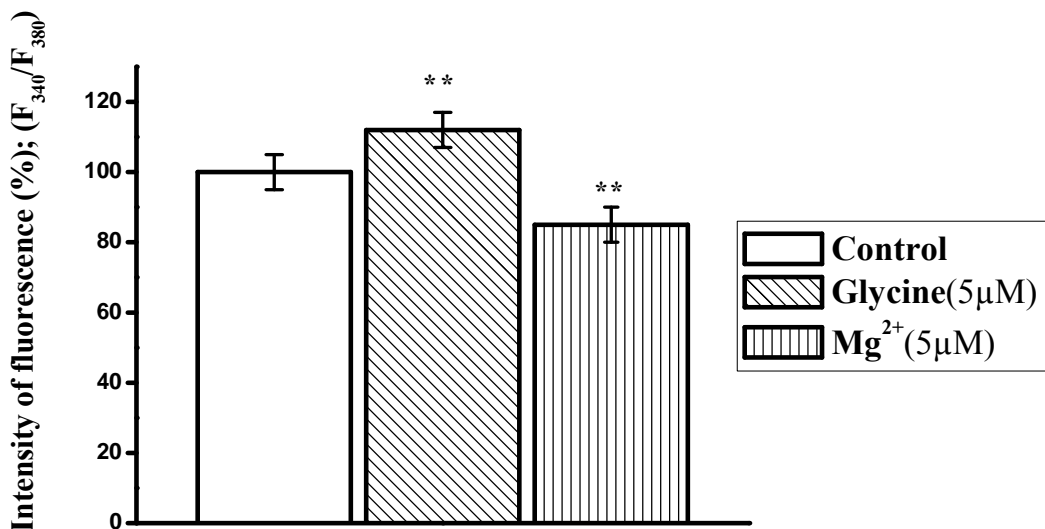


Fig. 4. Effect of glycine and Mg^{2+} ions on glutamate-induced Ca^{2+} intracellular depots. Reliability indicator: * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$. ($n = 6$)

In the following experiments, to identify the possible interaction of Caralinia and Granatum with NMDAR over excitation sites responsible for opening calcium channels, the effects of Mg^{2+} , argiobatin and the calcium channel blocker nifedipine were studied.

Experiments have shown that Mg^{2+} at concentrations of 5 mM significantly inhibits NMDAR activity. The inhibitory effect of Mg^{2+} against the background of Caralinia and Granatum (50 μM) by NMDAR activity did not change.

In the presence of Caralinia and Granatum, the inhibitory effect of Mg^{2+} (5 μM) did not change. This is probably due to the lack of competition between Mg^{2+} and Caralinia and Granatum for sites stimulating the opening of Mg^{2+} ion channels.

In place of the action of argiobatin (1 μM) on calcium channels, NMDAR does not change in the presence of Caralinia and Granatum (5 μM) (Figure 5).

Incubation of nifedipine (0.01 μM) with the CTC-synaptosome complex resulted in a decrease in fluorescence.

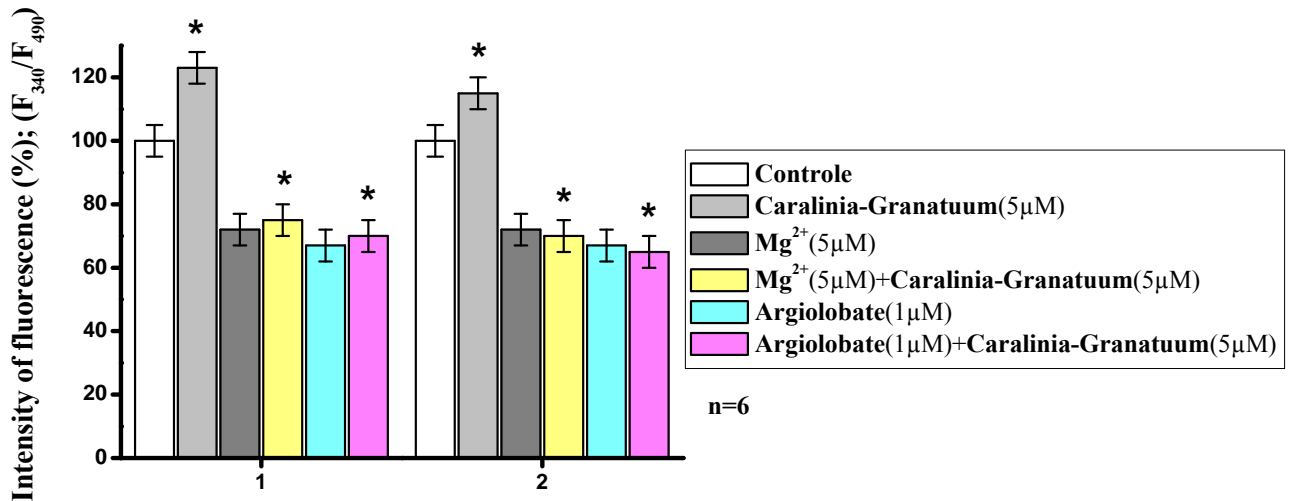


Fig. 5. Effect of non-competitive NMDAR antagonists Mg²⁺ and argiolobatin against the background of 1) Caralinia 2) Granatuum on fluorescence intensity and [Ca²⁺]_{in} level in rat brain synaptosomes.

Reliability indicator: * – P < 0.05; ** – P < 0.01; *** – P < 0.001 (n = 6).

Pre-incubation of Caralinia and Granatuum (5 µM) against the background of nifedipine (0.01 µM) with the CTC-synaptosomal complex did not lead to a change in fluorescence (Figure 6), which indicates the absence of competition between Caralinia and Granatuum and nifedipine for the site of regulation of dihydropyridine-sensitive calcium channels in synaptosomal membranes.

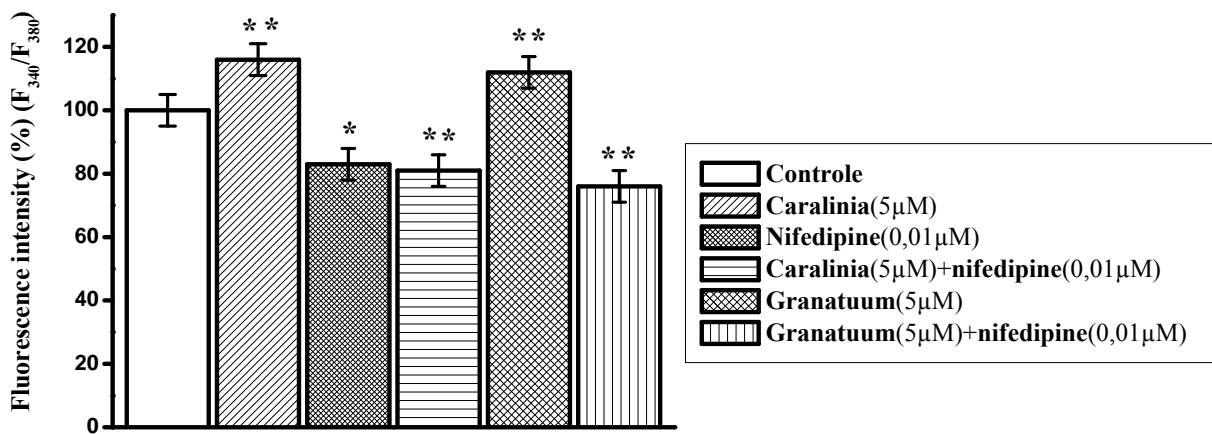


Fig. 6. Effect of Caralinia and Granatuum on calcium-dependent processes of the NMDAR in the presence of nifedipine.

Reliability indicator: * – P < 0.05; ** – P < 0.01; *** – P < 0.001. (n = 6)

Caralinia and Granatuum do not act on dihydropyridine-sensitive calcium channels in the membrane of rat brain synaptosomes.

4. Conclusion

The results obtained show that the Caralinia and Granatuum polyphenols used significantly increased [Ca²⁺]_{in} levels in synaptic membranes compared to controls. Preliminary results indicate there is a possibility of competition between the polyphenols Caralinia, Granatuum and the L-glutamate antagonist for the NMDAR ion channel opening regulation site.

Under the action of Caralinia and Granatuum polyphenols responsible for opening calcium channels with other NMDAR sites, no changes in the [Ca²⁺]_{in} level in synaptosomes were observed

against the background of Glycine, Mg²⁺, argiobatin and nifedipine. The conclusion of this work makes it possible to create a neuroprojective drug with a therapeutic effect for the treatment of AD based on these polyphenols in pharmacology has good prospects.

5. Acknowledgements

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6. Conflict of interest

No conflict of interest was declared.

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