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Isolation of New Ellagitannins from Plants of Euphorbiaceous and Its Effect on Calcium Transport in the Nerve Cell of the Rat Brain

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ABSTRACT:
The chemical properties of tannin of the Euphorbia species (*E. himufusa*.) species have been studied. Has led to the isolation and characterization of a new hydrolyzable tannin together with known compounds. The structure of this tannin has been established based on spectroscopic and chemical data. In addition, the change in the transport of calcium by the nerve cell under the action of isolated polyphenol was investigated.

KEYWORDS*:* ellagitannins, galloyl group, hexahydroxydiphenoyl group, valoneoyl group; ^{13}C NMR spectroscopy; Q-TOF LC-MS, synaptosomes rat brain

INTRODUCTION: The natural compounds are widely distributed in the plant kingdom. Among of plant origin compounds polyphenols are attracted much attention because their therapeutic effects and lower toxicity, different biologically effects.
Therefore, the plant search containing polyphenols, development of methods for their isolation, to establish their chemical structure and biological activity of study depending on the chemical structure in order to create new and effective drugs is an important and urgent problem of modern bioorganic chemistry.
Ellagitannins – are the most abundant plant polyphenols among hydrolysable tannins. They are ellagic acid esters with sugars. To date, isolated and established the structure of more than 700 individual compounds. Ellagitannins possess a wide spectrum of biological activity, the most important of which are antiviral, antitumor and immunomodulatory activity.
Currently, carryinf out researches out on the isolation of plant tannins, obtaining their derivatives, revealing structure activity relationship (SAR), the investigation of the mechanism of antiviral activity, synthesizing gallo- and ellagotannins, which are a priority for research.
Recent advances in the field of NMR spectroscopy and mass spectrometry allowed to identify tannins with different degree of galloyl and their molecular weight in complex mixture[1,2,3,4]. In addition, these methods are ideally match for the characterization of oligomers and considered the best methods for the analysis of heterogeneity structural tannins[5,6,7,8,9,10,11,12,13].
In the central nervous system, Ca^{2+} is a ubiquitous cell messenger that participates in the regulation of neurotransmitter release and nerve excitability, and is closely associated with cellular differentia-tion and migration, synaptic plasticity, neurite growth, and neuro-nal apoptosis[14,15,16,17].

MATERIALS AND METHODS:

Experimental chemical part:

Ellagitannins separated by column chromatography on silica gel (LS 100/40, Czechoslovakia). TLC was performed on precoated on Silufol UV-254 plated, with benzene–acetone (9:4, v/v). The up-ground parts of plant material Euphorbiaceous (Spurge) collected at the end of vegetative period were air-dried and blended. For the extraction of plant materials used solvents produced by «Himreaktivkomplekt» (Uzbekistan) and «Реахим» (Russia). Optical activity was measured on

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Photoelectrocolorimeter FEC-56m. UV spectra of ellagitannins were recorded in ethanol on EPS-3T (Hitachi, Japan). 13C NMR spectra was recorded obtained on a Bruker 400 (Germany) spectrometer (400 MHz), using acetone-d_6 as solvent and tetramethylsilane (TMS) as internal standard, and the chemical shifts reported in δ (ppm) units relative to 0. LC-MS was performed on a Q-TOF Agilent Technologies mass spectrometer (USA) 6520B series, in the negative ion mode. condition: electrospray ionization source (ESI-), gas spray: 5 l/min, temperature of gas 300^0C, the capillary voltage was 20 V, the 125V, mass: MS 100–2000 m/z, Targeted MS/MS 25–2000 m/z, collision energy – 65. Ionization method: negative. Samples fractionated on Agilent Technologies 1200 series, on column Zorbax SB C18, 3μm, 0.5x150 mm. Mobile phase: A - 0.1% solution of formic acid, B - acetonitrile + 0.1% formic acid. A - the negative full or selected ion monitoring (SIM) scan type. Compounds were also monitored simultaneously using the dual-wavelength absorbance detector at 280 nm. Compounds were separated using the Aqua C18 column as for HPLC. Elution was performed on the instrument Agilent Technologies 1260 series Cap Pump at a flow rate of 15 μl/min. The gradient concentration of solution B - in minutes: 0% - 5 min, 25% - 20 min, 50% - 35-40 min, 0% - 43 min. The solutions were degassed on the Agilent Technologies 1260 μ-degasser instrument. Samples were applied on column (1 μl) by Agilent Technologies WPS.

Isolation of tannins

Air-dried aerial parts of plants of *E. himufusa*, collected different region Republic of Uzbekistan, were cut into small pieces and extracted with chloroform to remove lipophilic substances. Subsequently, plant materials were extracted with 70% aqueous acetone. Then, aqueous acetone extract was evaporated under vacuum; the aqueous layer was successively partitioned with ethyl acetate. By adding hexane to concentrated ethyl acetate portion (4x1, v/v), obtained sum of polyphenols with yield 2.1%.

Polyphenols were subjected to column chromatography on silica gel, eluted with chloroform-methanol solution (17:3; 17:4; 17:5) and obtained three fractions. From fraction 1 were isolated known phenolic acids.

The fraction 2 and 3 were shown to contain flavonols and tannins by paper chromatography (PC) (n-buthanol-acetic acid-water, 4:1:5; n-buthanol-acetic acid-water, 40:12:28, system -1).

After rechromatography of fraction 3 on silica gel with different solutions were isolated tannins. The structures of tannins were determined by physical-chemical methods. From all studied plants were more than 10 phenolic compounds, 1 of them were fond as new compounds.

1-O-galloyl-6-O-bis-galloyl-2,4-valoneoyl-β-D-glucose – isolated from *Euphorbia himufusa*, 0.45 g of white amorphous powder. $C_{48}O_{31}H_{34}$, $[\alpha]_D$ $+70^0$ (c = 0,5 MeOH), R_f 0.48 (system 1). UV spectrum λ_{max} (EtOH, nm) nm (lgε): 216 (5.22), 270 (4.86) MS *m/z*: 1106 $[M-H]^-$; ^{13}C-NMR (100 MHz, acetone-d_6 + D_2O, ppm): galloyl group: 119.88 (C-1), 110.20 (C-2), 145.82 (C-3), 139.61 (C-4), 145.8 (C-5), 110.20 (C-6), 167.23 (C-7); bisgalloyl group: 120.44 (C-1, C-1 '), 110.15 (C-2, C-2'), 145.54 (C-3, C-3 '), 139.22 (C-4, C-4'), 145.51 (C-5, C-5'), 110.10 (C-6, C-6'), 168.70, 166.40 (C-7, C-7'); valoneoyl group: 114.30 (C-1), 126.44 (C-2), 106.80 (C-3), 145.14 (C-4), 136.10 (C-5), 144.44 (C-6), 169.10 (C- 7), 118.85 (C-1 '), 128.09 (C-2'), 110.58 (C-3 '), 148.84 (C-4'), 135.64 (C-5 '), 147.03 (C-6') , 168.32 (C-7'), 112.22 (C-1"), 138.65 (C-2"), 130.44 (C-3"), 141.17 (C-4"), 143.55 (C-5"), 110.15 (C-6"), 163.36 (C-7"); glucose: 92.37 (C-1), 76.78 (C-2), 76.99 (C-3), 76.99 (C-4), 73.49 (C-5), 63.34 (C-6).

Acid hydrolysis

A solution of compounds (5 mg) in 5% H_2SO_4 (2 ml) was heated in boiling water bath for 9 hours. After cooling, the reaction mixture was extracted with EtOAc. The hydrolysis products were identified by preparative PC and TLC.

Partial hydrolysis of tannins

A solution of compounds (70 mg) in acetone (10 ml) and H_2O (50 ml) was heated at 90 ^{0}C for 24 h. The resulting hydrolysis products were controlled by TLC each hour.

Methylation of tannins, followed by alkaline methanolysis

A mixture of compounds (5 mg), K_2CO_3 (100 mg), $(CH_3)_2SO_4$ (0,1 ml), in acetone (2 ml) was stirred

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overnight at room temperature and then heated at reflux for 3 h. After removal of K_2CO_3 by centrifugation followed evaporation of the solvent, the residue was directly subjected to methanolysis with 1% NaOMe in MeOH (1ml) at room temperature overnight. After acidification with AcOH and evaporation of the solvent, the residue was submitted to preparative TLC.

RESULTS AND DISCUSSION. Recently, researches carried out by scientists of Bioorganic Chemistry of the Academy of Sciences of Uzbekistan, have shown prosperity of use of natural phenolic compounds as an antiviral, antioxidant, anti-tumor agents[17,18,19,20,21,22,23]. It was found that these compounds have high biological activity, which manifests itself in suppressing the activity of various viral infections, redox enzymes and etc. Also, it was shown that natural polyphenols have anti-HIV activity[24].

Based on these research results, we studied polyphenols of three plants belonging to Euphorbia family[25,26,27]. More than 30 individual compounds were isolated from these plants, determined their chemical structures and biological activities. It was revealed that some of isolated compounds found as a new, not previously noted compound in the literature. On the basis obtained physic-chemical and spectral date were characterized chemical structures of compounds.

By the known method[25], air-dried up-ground parts of plants extracted with chloroform, aqueous acetone, ether and ethyl acetate. By using column chromatography, from ethyl acetate extraction were isolated one new compounds.

To determine the monomer composition and chemical structure of compound-1 were carried out chemical transformations according to Scheme 1. In products of acid hydrolysis with 5% H_2SO_4 detected glucose (I), gallic (II) and valoneic acid (III). Methylation of compound with dimethyl sulfate and anhydrous K_2CO_3 resulted in the formation permethylate, upon methanolysis with sodium methoxide formed methyl tri-O-methylgallate (IV) (TLC, Rf 0.75, solvent system 1: benzene-acetone 4:1) and trimethyl-octa-O-methylvaloneate (V) (TLC, Rf 0.27, system 1). The partial hydrolysis of compond-1 (heating in water at 90^0C) resulted in formation 1-O-galloyl-β-D-glucose (VI), 6-O-bisgalloyl-β-D-glucose (VII) and 2,4-valoneoyl -β-D-glucose (VIII) (Fig.1).

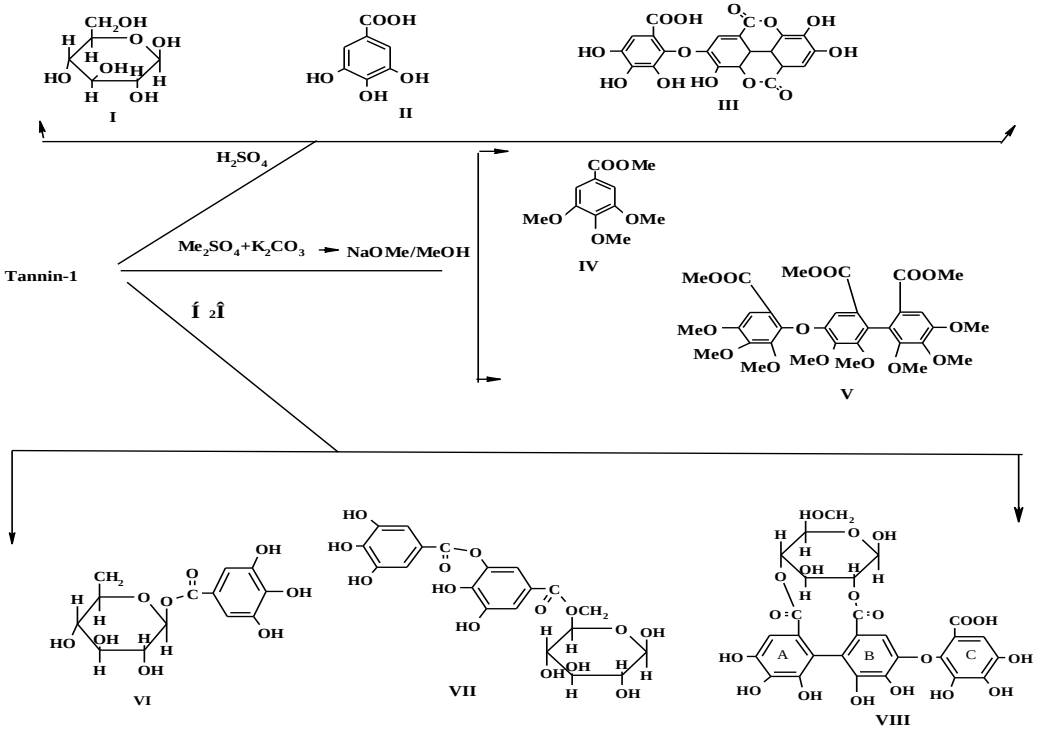

Figure 1. Chemical transformations of compound-1

In the ^{13}C NMR spectrum of the compound-1, in the complete suppression of spin-spin coupling with

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protons detected signals specific to glucose, gallic and valoneic acids[28,29,30]. Intensive signal at 92.37 ppm, corresponding to the anomeric center of glucose molecule has β-configuration[31,32,33]. Chemical shifting at 92.37, 76.78, 76.89, 63.34 ppm corresponding to the atoms C-1, C-2, C-4 and C-6 of sugar residue indicates the acylation of the carbohydrate residues at these positions. In the ^{13}C NMR spectrum present the signals of three residues of gallic acid: the signals of carbon atoms of carbonyl groups appear at 167.23, 168.70, 166.40 ppm and signals of carbon atoms of trigalloyl group observed at 119.88, 120.44, 120.44 ppm. Signals of C-2, C-6 and C-3, C-5 carbon atoms give relative signals and intensities at 110.20, 110.15 and 110.20, 110.10 ppm, also at 145.82, 145.54 and 145.80, 145.51 ppm, respectively. The carbon atom C-4 of this residue overlapped and as a result of the diamagnetic shift resonates at 139.61 and 139.22 ppm. In the spectrum also observed signals of 21 carbon atoms valoneoyl group. The resonance signals of C-1, C-1` and C-1`` carbon atoms of valoneoyl group observed at 114.30, 118.85 and 112.22 ppm, respectively. Intense signals at 144-145 ppm refer to the C-4 and C-6 carbon atoms of valoneoyl group. The chemical shifts of C-3, C-3`, and C-3`` carbons observed at 106.80, 110.58 and 130.44 ppm. Analysis of the ^{13}C-NMR spectra shows that the chemical shifts of valoneoyl group coincide with the literature data[29,30,31,32,33]. Signals of C-7, C-7`and C-7`` carbon atoms in carbonyl groups give intensive signals at 169.1, 168.32 and 163.36 ppm.

The UV spectrum of the compound-1 has the absorptions characteristic for phenolic compounds (λ_{max} 216, 270 nm).

These data are confirmed by the mass spectrometry defragmentation of compound-1, obtained on the instrument LC-MS Q-TOF in negative ionization mode. As can be seen from the scheme, the molecular ion compound-1 at *m/z* 1105 split into two fragments at *m/z* 953 and 153. This indicates that the breaking the ester bond between glucose and galloyl group, which is consistent with the literature data. Secondary ion at *m/z* 953 further cleaved into fragments at *m/z* 649 and 305. The presence of intense ion signal at *m/z* 469 in the mass spectrum fragmentation, formed as a result of split of the ion at *m/z* 649 indicates containing valoneoyl group of compound-1 (Fig. 2).

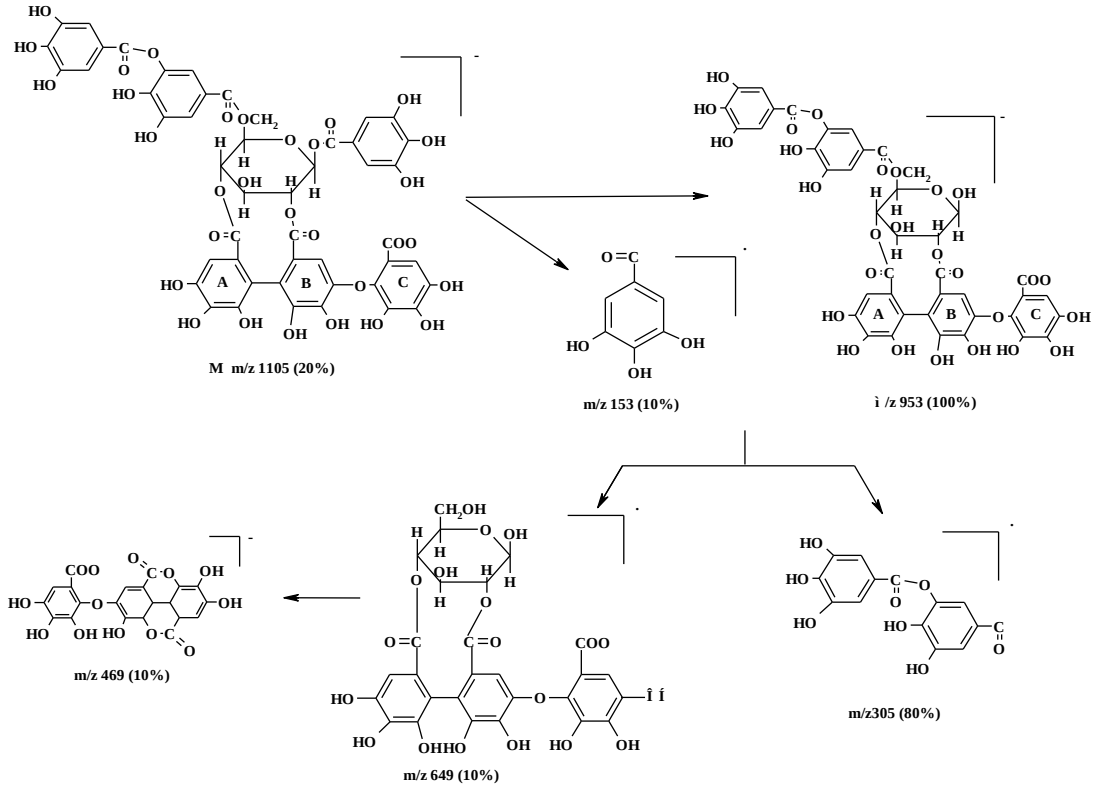

Figure 2. Possible ways of fragmentation compound-1

On the base of analyzing the chemicals products and spectral dates and their comparison with the literature data the structure of compond-1 established (IX) as 1-O-galloyl-6-O-bis-galloyl-2,4-valoneoyl-β-D-glucose (Fig.3).

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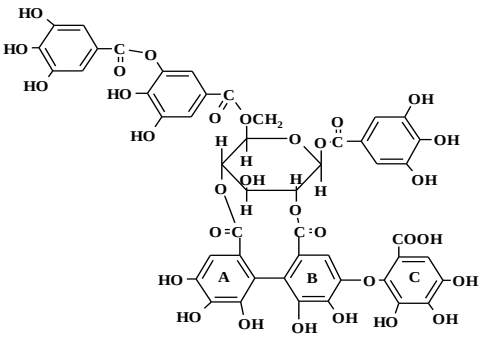

IX

Figure 3.1-O-galloyl-6-O-bis-galloyl-2,4-valoneoyl-β-D-glucose

Experimental biological part

Material and methods. Experiments were conducted on 20 outbred male albino rats weighing (200-250 g) contained in a standard vivarium ration. All experiments were performed in accordance with the requirements of "the World Society for the Protection of Animals" and "European Convention for the protection of experimental animals"[34]. Synaptosomes isolated from rat brain by a two-step centrifugation[35]. The whole procedure of selection was carried out at 4°C. To measure the amount of cytosolic Ca^{2+} was calculated from the equation of Grinkevich[36] in synaptosomes isolated from brain of rats placed in an environment similar to, the one that was used to isolate cells were added 20 μM of chlortetracycline (CTC). Incubated for 60 min to achieve maximal interaction with the membrane - CTC Ca^{2+} as in plasma, and intracellular membranes. CTC excitation wavelength - 405 nm, recording - 530 nm. Results are expressed as a percentage, taking 100% of the difference between the maximum value of fluorescence intensity (fluorescence dye, a saturated Ca^{2+}) and its minimum value (in the absence of fluorescence of the indicator of Ca^{2+}) obtained after adding ethylene-glycol-bis-amino-ethyl-tetra-acetate EGTA. Registration of changes in the dynamics of calcium in cells used USB-2000 spectrometer (Ocean optics, USA_2010)

STATISTICAL ANALYSIS:

The measurements were made using a universal spectrometer (USB-2000). Statistical significance of differences between control and experimental values determined for a number of data using a paired t-test, where the control and the experimental values are taken together, and unpaired t-test, if they are taken separately. The value of $P < 0.05$ indicated a statistically significant differences.
The results obtained are statistically processed to Origin 7,5 (Origin Lab Corporation, USA).

RESULTS AND DISCUSSION:

Previous studies have shown that in the experiments carried out, the effect of various polyphenols on the change in the dynamics of calcium in the synaptosomes of the rat brain, polyphenols act as antioxidant and neuroprotective properties[37,38,39,40,41,42,43,44,45].
Based on these data, the effect of polyphenol; *1-O-galloyl-6-bisgalloyl-2,4-valoneoyl-β-D-glucose* isolated from the plant (*EUPHORBIA HIMUFUSA*) on calcium transport in rat brain synaptosomes was studied.
Preincubation of Euphorbin (10-100 μM) with the complex of the CTC-synaptosomes increases the fluorescence and accordingly, the level of $[Ca^{2+}]_{in}$ difference from L glutamate (Fig. 4).

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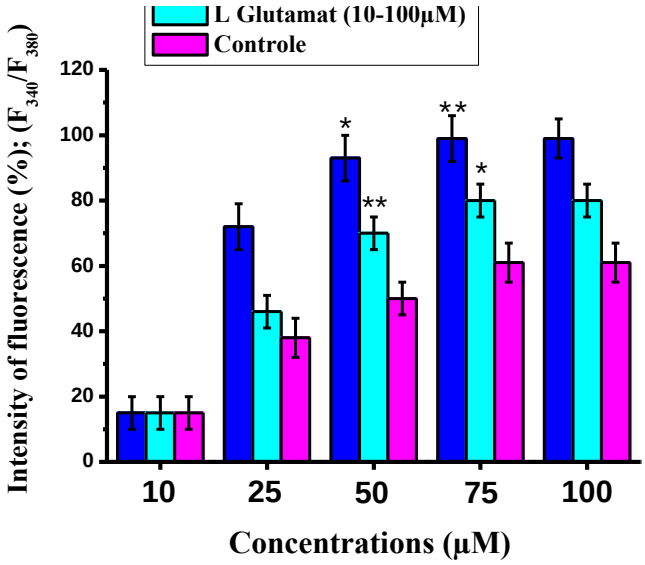

Figure 4. Effects of Euphorbine and L glutamate in concentrations (10-100 µM) on the intensity of CTC fluorescence of brain synaptosomes suspension in rats. Reliability level * - P <0,05; ** - P <0.01; *** - P <0.001. (n = 6).

Euphorbin (10-100 µM) reduced the fluorescence and accordingly the level of $[Ca^{2+}]_{in}$ against the background of L glutamate (50 µM) on the complex of CTC-synaptosomes (Fig.5).

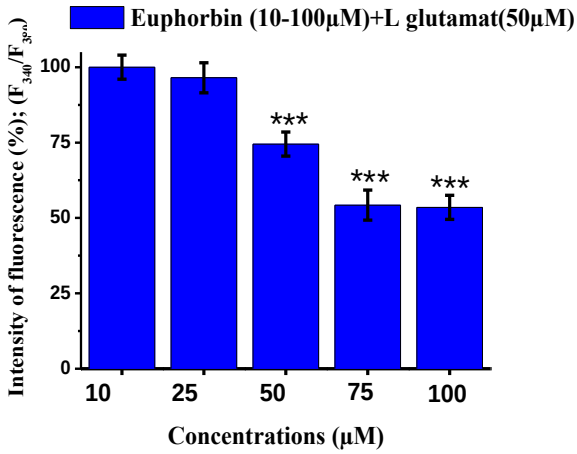

Figure 5. Effect on fluorescence intensity in synaptosomes suspension in conditions of incubation with euphorbin (10-100 µM) L glutamate (50 µM). Reliability level * - P <0,05; ** - P <0.01; *** - P <0.001. (n = 6).

The preliminary preincubation of euphorbin (10 µM) with synaptic membranes, then the addition of CTC- L glutamate resulted in a decrease in fluorescence and a level of $[Ca^{2+}]_{in}$, respectively. A dose-dependent increase in euphorbin concentration to (10-100 µM), respectively, resulted in a dose-dependent decrease in the effect of L glutamate (Fig. 5).
The effect of L glutamate was observed depolarization of the synaptic membrane and an increase in intracellular calcium without an appreciable change in the concentration of internal sodium ions. Increase in synaptosomal calcium was inhibited by the addition of L glutamate. Activation of L

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glutamate receptors causes the opening of calcium channels ionotropic receptors, calcium influx into synaptosomes and depolarization of the synaptosomal plasma membrane, followed by the release of amino acid neurotransmitters.

L Glutamate partially reduces the action of euphorbin, which may indicate that part of the external calcium comes under the influence of euphorbine also through the open glutamine site and in place of calcium channels NMDA-receptors.

Even the preliminary addition of L glutamate does not completely abolish the action of euphorbin, which may indicate that euphorbin has several mechanisms of action on rat brain neurons, the result of which is an increase in $[Ca^{2+}]_{in}$.

From the literature data it is known that, Mg^{2+} ions selectively block the activity of NMDA-receptors. Glycine enhances NMDA-receptor responses by increasing the frequency of channel opening. In the complete absence of glycine, the receptor is not activated by L glutamate.

Indeed, the addition of glycine to the incubation medium (5 μM) enhanced the L glutamate-dependent increase in fluorescence by 15-22%. At the same time, Mg^{2+} ions (50 μM) inhibited L glutamate-induced Ca^{2+} release from intracellular depots (Fig.6).

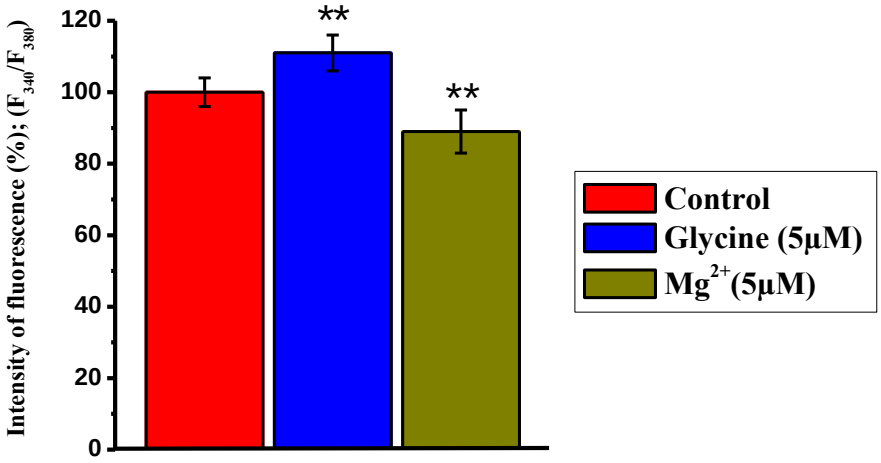

Figure 6. Effect of glycine and Mg^{2+} ions on L glutamate-inducible Ca^{2+} intracellular depot. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 5).**

It is known that glycine stimulating effects of L glutamate and competitive receptor antagonists such as AP_5, AV-2-1 toxin can prevent activation of L glutamate. Other drugs and Mg^{2+} ions may block the open channel through the non-competitive antagonism. These medications include experimental neuroprotective drug MK-801 and argiolobatin.

In order to identify, possible interaction with polyphenol euphorbin areas over stimulation NMDA-receptor responsible for the opening of calcium channels, investigated its effect on the background of the non-competitive antagonists such as magnesium ions, argiolobatin and calcium channel blockers - nifedipine

It is shown that magnesium ions in millimolar concentrations significantly inhibit the fluorescence of the L glutamate-CTC-synaptosomes complex. The inhibitory effect of magnesium ions against the background of euphorbin (50 μM) of the fluorescence of the CTC-synaptosomes complex did not change.

In these studies, it was shown that in the presence of euphorbin, the inhibitory effect of magnesium ions (50 μM) was not observed. This is probably due to the fact that there is no competition between Mg^{2+} and euphorbin over sites that stimulate the opening of ion channels. It has also been shown that the action of argiolobatin (10 μM) on the calcium channels

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of the NMDA-receptor in the presence of euphorbin (50 μM) does not change (Figure 7).

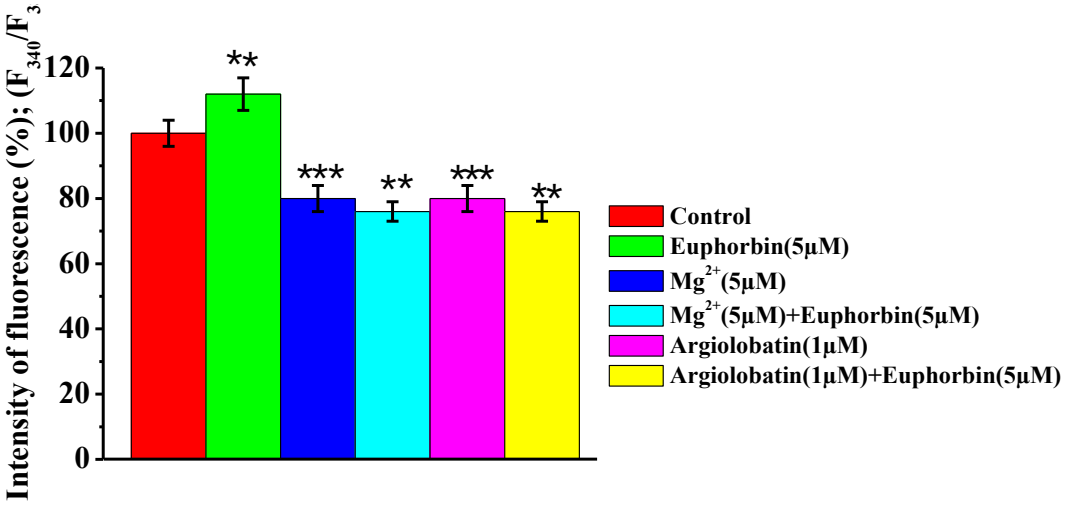

Figure 7. Effect of non-competitive NMDA-receptor antagonists Mg^{2+} and argiolobatin on the background of euphorbin on fluorescence intensity and the level of $[Ca^{2+}]_{in}$ in the brain synaptosomes of rats. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

When investigating the effect of euphorbin on calcium-dependent NMDA-receptor processes were studied against the background of the blocker of the L-type Ca^{2+} channels of nifedipine in the brain synaptosomes of rats.

Preincubation of nifedipine (0.01 μM) with the suspension complex of the CTC-synaptosomes resulted in a decrease in fluorescence. Preincubation of euphorbin (50 μM) with the suspension complex of the CTC-synaptosomes, no decrease in fluorescence. Preincubation of euphorbin (50 μM) against a background of nifedipine (0.01 μM) with a complex of CTC-synaptosomes did not result in a change in fluorescence (Fig.8), indicating that there is no competition between euphorbin and nifedipine for the site of regulation of dihydropyridine-sensitive calcium channels.

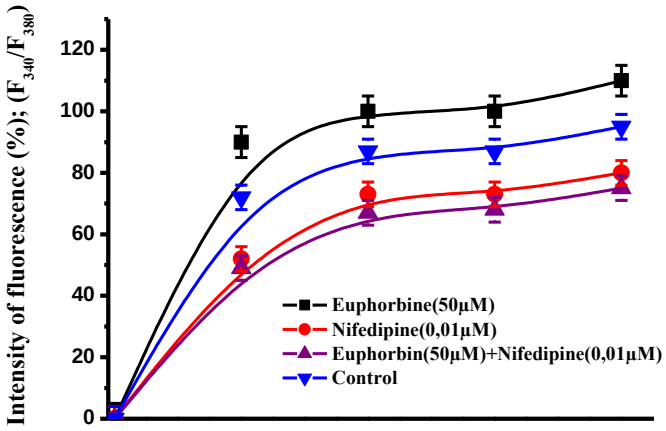

Figure 8. Effect of euphorbin on calcium-dependent NMDA-receptor processes on the background of nifedipine. Reliability level * - P <0,05; ** - P <0.01; * - P <0.001. (n = 6).**

This is explained by the fact that, euphorbin does not work for the site of regulation of the dihydropyridine-sensitive calcium channels of the rat brain synaptosomes membrane.

CONCLUSIONS:

The chemical properties of tannins of the Euphorbia species (*E. himufusa*.) Have been investigated and studied. and a new hydrolyzable tannin was isolated. The structure of this tannin is established. The isolated polyphenol euphorbin was found to slightly increase fluorescence and $[Ca^{2+}]_{in}$ levels, respectively, in synaptic membranes compared to controls. The results indicate that there is possible competition between euphorbin and L-glutamate for the site of regulation of NMDA receptor ion channel opening. In addition, it was found that the action of euphorbin polyphenol, responsible for the opening of calcium channels with other sites of NMDA-receptors, against the background of magnesium ions, argiolobatin and nifedipine, changes in the level of $[Ca^{2+}]_{in}$ synaptosomes were not observed.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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