IRSTI 76.03.35 https://doi.org/10.26577/ijbch.2020.v13.i2.01

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Therapeutic effectiveness of *Limonium gmelinii* extract in experimentally – induced ischemic brain damage *in vivo*

Abstract. One of the important directions in the treatment of ischemic stroke (IS) consequences is revascularization of the damaged brain areas including decreasing acute hypoxia and oxidative stress that occur in the ischemic tissue due to reperfusion syndrome after restoration of blood flow. Plant polyphenols are promising candidates capable of exerting a pronounced antioxidant and neuroprotective effects. There are a number of wild plants growing on the territory of Kazakhstan, and one of these plants containing significant number of polyphenols is *Limonium gmelinii* (*L. gmelinii*, genus *Limonium* Mill). In our study we have applied middle cerebral artery occlusion (MCAO) method to induce focal ischemic cerebral stroke in male Wistar rats. The results of assessment of sensorimotor functions in laboratory animals showed that MCAO resulted in sensorimotor deficiency. At the same time, partial recoveries of sensorimotor functions were observed in animals that were treated with extract of *L. gmelinii* after stroke compared to untreated animals. Similarly, histological analysis of the damaged brain regions has revealed focal coagulation necrosis with clearly visualized damaged regions in animals with MCAO, whereas brain tissue of animals exposed to *L. gmelinii* extract after stroke induction was partially restored. Our results demonstrate that extract of *L. gmelinii* possesses neuroprotective properties that require further investigations.

Key words: ischemic stroke, plant polyphenols, ROS, antioxidants.

Introduction

Acute cerebrovascular disease or stroke is one of the three most common cardiovascular diseases in the world [1]. According to WHO (2012), about 6.7 million fatal cases of stroke are recorded every year globally (http://who.int/mediacentre/factsheets/fs310/en/). Among all registered cases, 80% are ischemic strokes. Moreover, 95% of ischemic strokes (IS) are associated with complications of the embolic type arising from plaques located in the extracranial sections of the arterial system.

Given the prevalence of this pathology, as well as the association with high percentage of mortality and primary disability, IS is a global medical and social problem [2]. There is a clear dependence of the increase in the incidence of stroke with an age above 30 years [3; 4], with two thirds of all cases of stroke occurring at the age of 65 years or more [5]. The costs of treating this disease are also of great socio-economic importance. The costs of therapy and maintenance of patients who have undergone IS making up the major expenses of the healthcare industry in many countries around the world [6]. The continuing tendency to "rejuvenate" stroke, a high percentage of mortality and disability also highlights the importance and relevance of the study of stroke in young people. Thus, the development of stroke prevention methods and rehabilitation – based therapy is a very important task for many countries in the world.

Restoration and maintenance of systemic hemodynamics, conducting drug thrombolysis and hemangiocorrection are key circulation recovery methods. In recent decades, the most effective method of treating IS is considered to be medication thrombolysis targeted on restoration of the main blood flow in the affected area in order to prevent

irreversible changes in the brain tissue [7]. Data were obtained that thrombolysis in patients with IS in the acute phase of the disease helps to reduce mortality rate by 17% and reduces the development of disability rate by 25% [8]. However, due to the narrow temporary "therapeutic window" (not more than 3 hours after the development of stroke) and the high risk of developing hemorrhagic complications, the spectrum of therapy with recombinant tissue plasminogen is very limited [9-11]. In most cases, a significant proportion of patients are admitted to specialized hospitals at a later date and the main method for correcting IS is to conduct «standard» basic therapy using drugs aimed at normalizing the rheological properties of blood, antiplatelet, anti-coagulation therapy and maintaining brain tissue metabolism [12-14].

Studies have shown that despite the fact that the restoration of the main blood flow in the area of cerebral ischemia is critical, the subsequent development of reperfusion syndrome causes further damage to nerve tissues [15-17]. As a result of the action of ischemia of brain tissue and the development of reperfusion syndrome, a cascade reaction mechanism is launched, which is accompanied by the accumulation of products of free radical oxidation [18]. Considering the fact that escalation of ROS synthesis is noted both during and after brain ischemia, the products of oxidative reactions are of particular importance in ischemia and secondary cerebral hypoxia [19-23]. In this regard, the antioxidant therapy is justified even in a delayed manner, since one of the main mechanisms of cell death is oxidative stress [24; 25].

In recent decades, plant polyphenols have been of increasing interest due to their proven antioxidant properties and the ability to inactivate free radicals [26–31]; therefore, the search for new plant objects rich in bioavailable polyphenols is in high demand. One of the plants abundant in polyphenols is *Limonium gmelinii* (*L. gmelinii*), a representative of the genus *Limonium*, growing in large numbers in Kazakhstan [32; 33]. It was previously shown that the extract of polyphenols isolated from the roots of *Limonium gmelinii* has a wide range of therapeutic properties [31; 34; 35]. In addition, it neutralizes the toxic effect of the pro-inflammatory cytokine TNF-α, has antioxidant properties, inhibits endothelial cell activation, reduces the generation of ROS in endotheliocytes and astrocytes of the brain, inhibits the activation of the NADPH oxidase enzyme and blocks the development of oxidative stress in neurons, thus providing a complex protective effect and inhibiting the development of oxidative stress *in vitro* [36]. However, the question of what effect the extract of *Limonium gmelinii* has in case of ischemic stroke and *in vivo* reperfusion syndrome remained open. In this regard, the aim of the work was to evaluate the effectiveness of the use of the extract of polyphenols isolated from the roots of *Limonium gmelinii*, in conditions of ischemic brain damage *in vivo* in a model of laboratory animals.

Materials and methods

Preparation of polyphenol extract. Preparation of polyphenol extract was conducted at the Department of Chemistry and Chemical Technology, al-Farabi Kazakh National University according to a described previously method [31]. Briefly, the polyphenols from roots of *Limonium gmelinii* will be double extracted with 50% ethanol (1:6) for 5 hrs followed by vacuum drying in 40-60 °C.

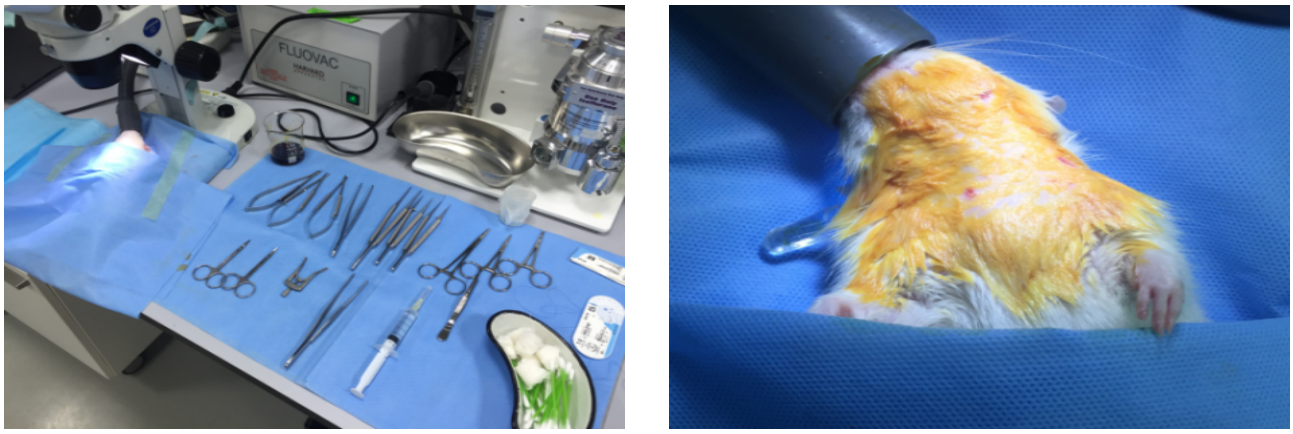
Object of study. Outbred male Wistar rats weighing 280-300 g were used, which were kept in vivarium conditions, including a 12-hour day/night cycle, at a temperature of 22-23 °C. For the experiments, the animals were divided into 4 groups: 1 – control animals, 2 – animals with MCAO, 3 – animals with MCAO, which were treated with *Limonium gmelinii* extract intragastrically at a dosage of 200 mg / kg for 28 days, 4 – animals that received only *Limonium gmelinii* extract in the same dosage. A day before the induction of stroke, the next day, on the 14th and 28th day after induction, the sensorimotor functions of animals were evaluated. On day 29, control and experimental animals were euthanized under isoflurane anesthesia and brain samples were taken.

Ethical approval. The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals (Protocol №2 from 06.18.2015, the Local Ethical Commission of the Center for Life Sciences of Nazarbayev University, Astana).

Creating a model of ischemic stroke by middle cerebral artery occlusion (MCAO). For an experimental study of the effects of plant polyphenols on the brain, an IS model caused by occlusion of the middle cerebral artery (MCAO) was used. This model is characterized by the stability of damage to brain structures and is convenient for assessing the functional changes. The MCAO model differs from other experimental models in the ability to cause significant sizes of IS [37] and is similar to the development of IS in humans. When modeling a stroke, special material and equipment were used: an Olympus optical binocular microscope (Olympus,

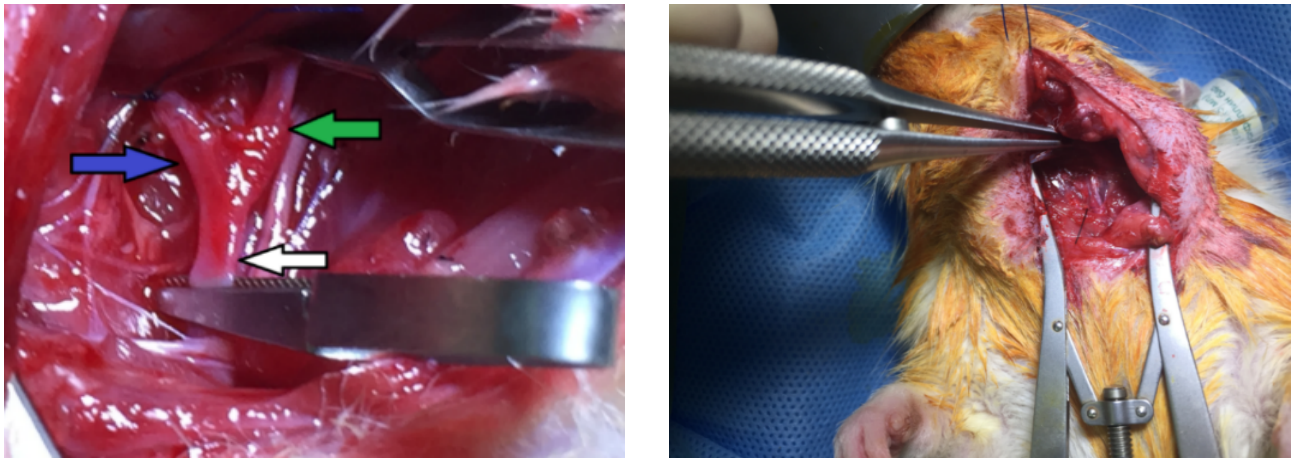
Japan), a coagulator, a gas anesthesia system (Harvard Apparatus, USA), an Isoflurane inhalation anesthetic (5% solution), Lawton microsurgical instruments (Lawton, Germany), suture material: prolen 6/0, silk 6/0, vicryl 6/0 (Ethicon, USA) (Figure 1).

IS model has been reproduced by MCAO in rats according to a previously described protocol [38]. As a temporary obturator of the lumen of the middle cerebral artery, a 4/0 nylon monofilament with a thickened silicone tip (Doccol Corp. USA) was used, which was introduced under visual control into a.carotis interna to a level of 17-20 mm. In this position, the monofilament was left for 2 hours to create a focal zone of acute cerebral ischemia (Figure 2). To confirm the presence of focal ischemic stroke of the brain 24 hours after occlusion of the middle cerebral artery in laboratory animals, brain tissue was taken under isoflurane anesthesia.



A B

Figure 1 – General view of the preoperative preparation.
Note: A – preoperative preparation; B – laying the animal on its back with the processing of the surgical field.



A B

Figure 2 – Model of middle cerebral artery occlusion (MCAO).
Note: A – mobilization of the common, external and internal carotid arteries: blue arrow – external, green arrow – internal, white arrow – common carotid artery; B – general view of the middle cerebral artery occlusion model (MCAO).

Evaluation of sensorimotor activity in laboratory animals. Analysis of locomotor functions of the front and hind legs of laboratory animals «Beam walk» balance test was performed. The rat was placed at the beginning of the board (wide part) and bright light was turned on, forcing the animal to move along a

narrowing path to the shelter (dark chamber). The entire testing process was recorded on a video camera installed at a sufficient distance from the test site, so the whole track got into the frame.

To assess the locomotor function of laboratory animals, the number of settings of the limb on the lower board (error), the amount of limb slipping from the upper board to the lower board (when the foot is placed on both boards) and the total number of steps taken from the start line to the animal's entrance into dark camera were calculated. Errors and slippage accounting was carried out for the front and hind legs separately. The videos were analyzed frame by frame using RealTimer software. The data obtained for three attempts were averaged. The severity of sensorimotor deficiency was calculated by the formula in percent:

$$\frac{\text{Error} + 0.5 * 100 * \text{Slipping}}{\text{Total number of steps}}$$

Preparation and analysis of histological slides of the brain. The rat brain was fixed in a 10% solution of neutral formalin. After fixation, the samples were washed from formalin, then brain samples were gradually dehydrated in 70%, 95%, 95%, 100%, 100% ethanol, followed by immersion in xylene. Thereafter samples embedded in paraffin blocks. Using a microtome (Leica, Germany), 5 μm thick sections were obtained, mounted on glass slides and spread on a warm table, then treated with xylene to exclude paraffin. Histological sections were rehydrated according to the reverse procedure in 100%, 100%, 95%, 95%, 70% ethanol, and in distilled water. After that, the sections were stained with hematoxylin-eosin. After staining, the slides were coated with Canadian balsam and analyzed under a microscope. Stained samples were analyzed using a Carl Zeiss Axio Vert light microscope (Carl Zeiss, Germany).

Statistical analysis. The data obtained are presented as mean ± standard error of the mean (Mean ± SEM). Standard deviations between experimental groups were evaluated using Student's t-test. Values were considered significantly different at $p \leq 0.05$.

Results and discussion

Induction of ischemic stroke by occlusion of the middle cerebral artery and administration of polyphenol extract. For the purpose of preoperative preparation, the animal was not given food and water on the day of surgery. To initiate inhalation anesthesia, the animal was placed in an anesthetizer chamber using a 5% Isoflurane solution under conditions of 1.0 L / min oxygen (O_2). The choice of anesthesia in favor of the inhaled anesthetic Isoflurane was determined due to the good controllability of anesthesia, the presence of interspecific universality of anesthesia, and the absence of toxic effects on the animal's body. The drug has a moderate irritant effect, pharyngeal and laryngeal reflexes become dull quickly, but there is some depression of the respiratory system. Heart rate and release practically do not change. Moreover, a decrease in stroke volume is compensated by an increase in heart rate. After immersing the animal in the surgical stage of anesthesia, a 1.5% level of Isoflurane was used to maintain anesthesia.

After recovery of the animal from anesthesia, signs of the development of ischemic lesion of a part of the brain in the basin of the middle cerebral artery were visually observed, which manifested as ptosis of the right eye and the development of paresis of the right upper limb. To confirm the development of focal ischemic stroke, a pathomorphological study of the brain was performed in some of the operated animals 24 hours after the operation. The remaining animals were slaughtered on the 29th day after the sensorimotor functions of the animals were evaluated.

It was found that in animals that were subjected to MCAO procedure, 24 hours after surgery, the presence of ischemic cerebral infarction was observed (Figure 3).

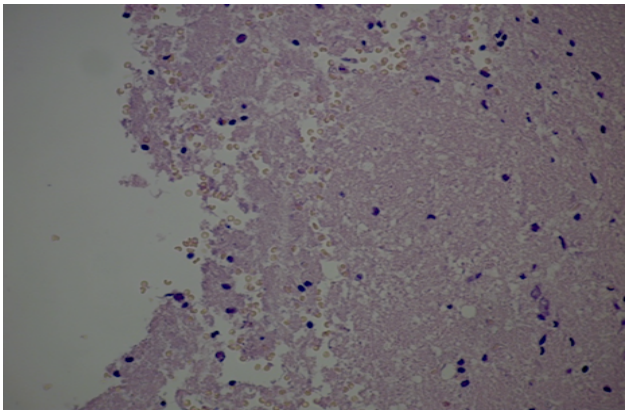


Figure 3 – Histostructure of the brain of rats with induced stroke. The center of ischemic infarction. Hematoxylin-eosin stain, x200.

Subcortical in the white matter and subcortical nodes, a focus of complete coagulation necrosis is found, in which the shadows of neurocytes, accumulations of red blood cells are visible. On the periphery of the focus of necrosis, widespread ischemic injuries of neurocytes were observed,

which lost most of their processes, extended and acquired an angular shape. Large focal hemorrhages were also observed in the cerebellum. In pear-shaped cells (Purkinje cells), homogenizing changes were observed in the form of a pale colored cell body, wrinkling of nuclei. The cells of the granular layer were in a state of dystrophy, the cytoplasm of part of the cells was vacuolated (Figure 4).

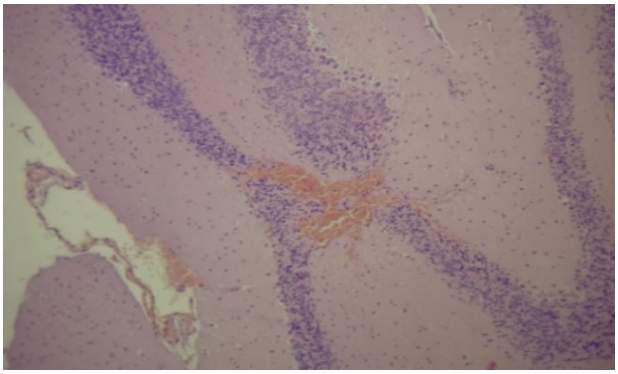


Figure 4 – Homogenizing changes and large-focal hemorrhages in the tissues of the cerebellum. Stained with hematoxylin and eosin, x100.

The presence of foci of ischemic infarction of the substance of the brain, as well as common ischemic damage to neurocytes of the cortex and white matter of the cerebral hemispheres, indicated the presence of ischemic stroke in the operated animals.

Assessment of sensorimotor functions in animals with induced stroke and exposed to Limonium gmelinii. The results of the analysis of motor function by the method of narrowing lane revealed a pronounced sensorimotor deficiency in all animals with induced stroke on the first day after MCAO (Figure 5).

After 2 and 4 weeks, a slight improvement in sensorimotor function was detected in animals with MCAO with no treatment. However, in animals that received extract of *Limonium gmelinii* after MCAO, a statistically significant improvement in the motor functions of the fore and hind limbs was observed after two weeks with a further decrease in sensorimotor deficiency compared to untreated animals.

Histological analysis of the brain of animals with induced stroke and exposed to extract of Limonium gmelinii. A histological examination of brain sections in animals without MCAO that received extract of *Limonium gmelinii*, as well as in animals of the control group, did not reveal pathological changes in the brain histostructure (Figure 6). Necrosis of neurons and glial cells was not observed, circulatory disorders were not detected. The nuclei of cells with clear boundaries were well detected in a light microscope.

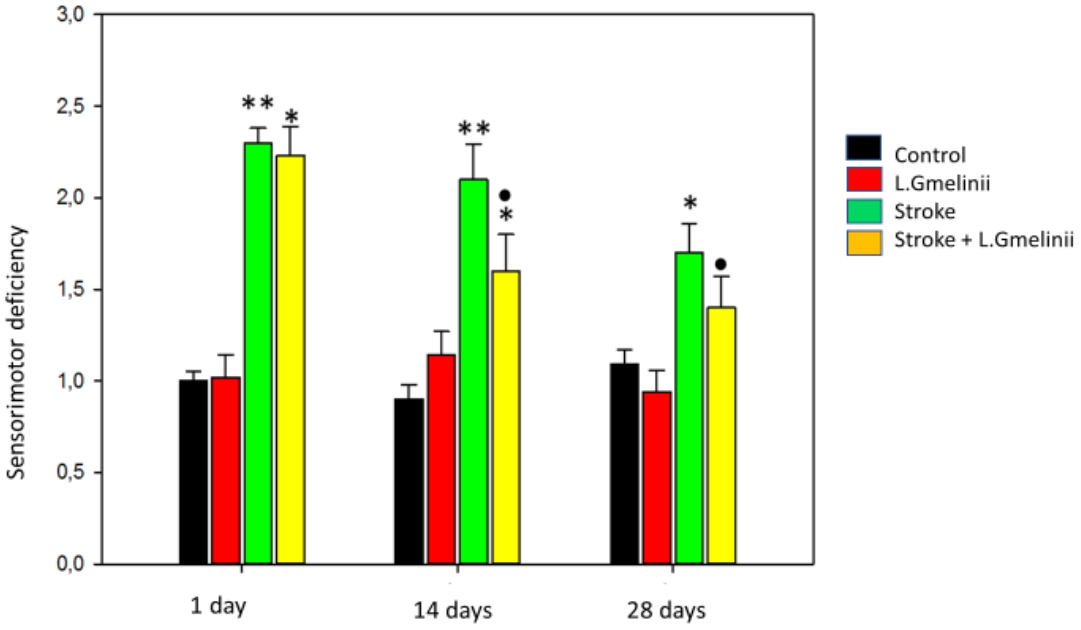
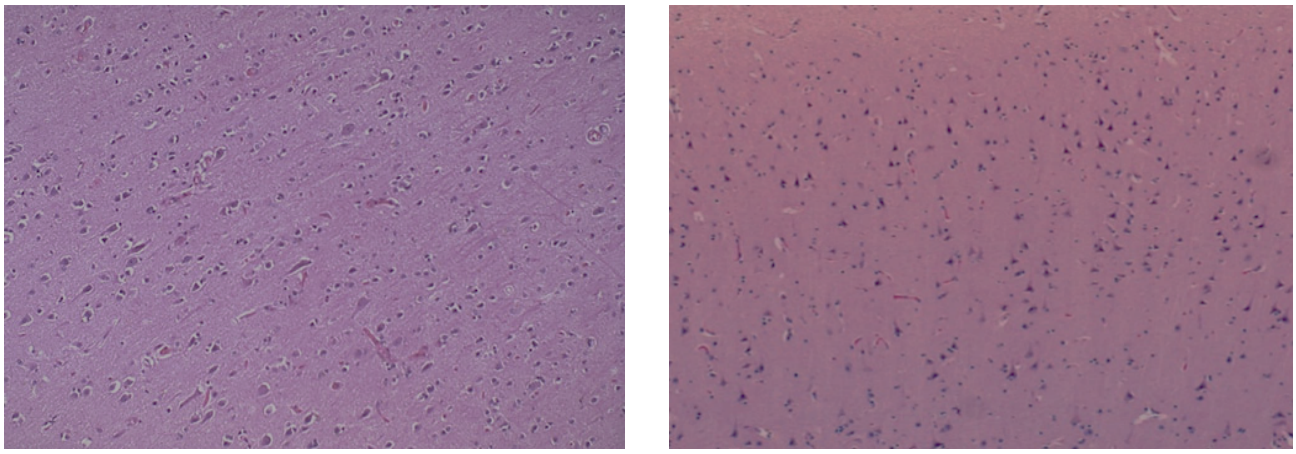


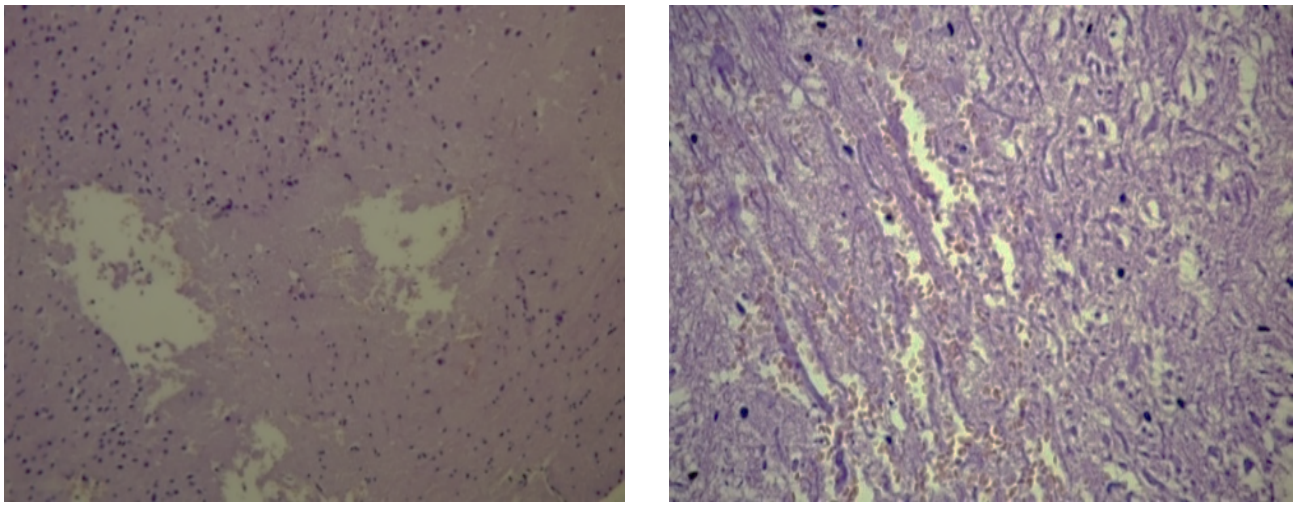
Figure 5 – Test results of sensorimotor functions in rats. ** – $p \leq 0.01$, * – $p \leq 0.05$ compared to the control, ● – $p \leq 0.05$, compared to animals with a stroke (Student's t-test).



A B

Figure 6 – Normal histostructure of brain tissue of intact animals (A) and exposed to *Limonium gmelinii* without MCAO (B). Stained with hematoxylin and eosin, x100.

In animals with induced stroke, on the 29th day after MCAO, pathological changes in the histological structure of the brain were still observed (Figure 7). Shadows of neurons were observed in the foci of necrosis, hemolyzed erythrocytes were present in the areas of lysed cells. On the periphery of the focus of necrosis was determined ischemic damage to neurocytes. Outside the focus of necrosis in the cortex and extracortical areas of the cerebral hemispheres, medulla oblongata, and cerebellum, neurocytes were observed in the state of protein dystrophy. Foci of dystrophy, an increase in the number of microgliocytes were observed in glia. Foci of fibrosis were determined in the meninges.



A B

Figure 7 – Foci of ischemic infarction. Note: A – In the histostructure of the brain; B – In the meninges. Stained with hematoxylin and eosin, x100.

Rats that received extract of *Limonium gmelinii* after stroke induction showed a slight improvement in the brain tissues (Figure 8). No extensive areas of necrosis were observed, although there were signs of frustration in the small foci. Along the periphery of small foci of necrosis, proliferation of astrocytic glia, isolated capillaries with red blood cell stasis were visible. Glia with foci of dystrophy,

an increase in the number of microgliocytes were also detected.

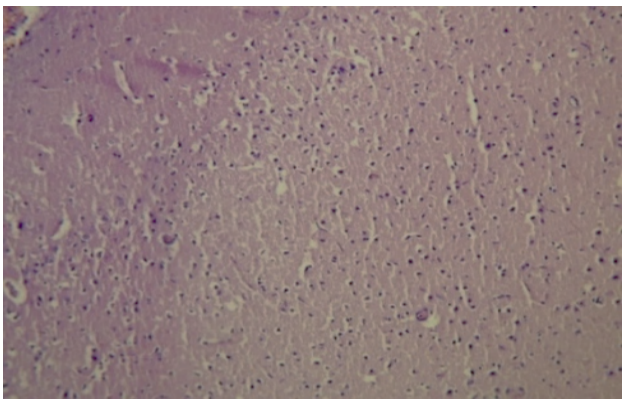


Figure 8 – Small focus of ischemic infarction. Stained with hematoxylin and eosin, x100.

Today plant polyphenols occupy a unique place in science as they potentially are capable of reducing the risk of the disease and might be used in treatment of such conditions as diabetes, cardiovascular disorders, atherosclerosis, neurodegenerative disorders and inflammation. Polyphenols are a structural class of mainly natural organic chemicals characterized by the presence of large multiples of phenol structural units. The number and characteristics of these phenol structures underlie the unique physical, chemical, and biological (metabolic, toxic, therapeutic, etc.) properties of particular members of the class. Polyphenols are rich in vegetables, fruits, grains, bark, roots, tea, and wine. Most polyphenols are generally known to possess potent antioxidant, anti-inflammatory, and anti-apoptotic properties, and their protective effects in ischemic injury have been demonstrated in a number of *in vitro* and *in vivo* studies [27; 28; 39; 40]. For example, magnolia polyphenols have been shown to attenuate oxidative and inflammatory responses in neurons and microglia cells [39]. Green tea polyphenols have also been proven to exhibit multiple neuro protective actions [41; 42]. However, the health effects of polyphenols depend on the chemical structure (eg, glycosylation, esterification, and polymerization) and bioavailability. Bioavailability appears to differ greatly between the various polyphenols, and the most abundant polyphenols in our diet are not those that have the best bioavailability profile [43]. Thus, new plant sources rich with bioavailable polyphenols are still in demand.

We have reported previously that root extract of *Limonium gmelinii* possesses significant hepatoprotective activity in CCl_4-induced liver damage, exceeding those of control flavonoids (silymarin and silibinin) [31]. The roots and rhizomes of the *Limonium gmelinii* (*Plumbaginaceae*) have been used in a traditional herbal medicine in Central Asia for hundreds of years. *Limonium gmelinii* contains a rich source of polyphenols, which is presented by flavonoids of oxidated type (7-14%), hydrolysable tannins and mono-, di and oligo forms of flavan-3-ols (40-60 %). The main monomeric flavane is (-)-epigallocatechin-gallate. Flavonoids of oxidated type are represented by 3,5,7,3',4',6' – hexahydroxy-flavane, isorhamnetin, quercetin, myricetin, their mono- and diglucosides (myricitrin, galactopyranosides of quercetin and myricetin, rhamnoglucoside of myricetin, rutin, etc.). Also, in extract composition was identified new flavone glycoside – Gmelinoside I [44]. 2-o-β-D-galloil and 2,3-o-β-D-digalloilglucose were isolated from hydrolized tannins. *Limonium gmelinii* extract also contains all known 20 natural α-aminoacids, 34 microelements, vitamins (C, E and ß-carotene) and xanthophylls.

Since both CCl_4-induced liver damage and ischemic brain damage are associated with oxidative cell injury and sterile immune response, in the present work we have evaluated therapeutic potential of *Limonium gmelinii* root extract on animal model of ischemic brain injury *in vivo*. Our results have demonstrated that the extract of *Limonium gmelinii* partially restored the motor functions and normalizes the morphology of the brain tissues damaged by MCAO, which allowed us to conclude that the *Limonium gmelinii* extract at a dose of 200 mg/kg per day for 28 days, has neuroprotective properties. However, further investigations are required in order to establish effective doses and active components of the extract.

Conclusion

Consequently, in the course of the work it was shown that in animals with MCAO and subsequent reperfusion in the basin of the middle cerebral artery of the brain, a focus of coagulation necrosis is determined, where dead cells of the nervous tissue are visualized, which indicates the development of focal cerebral infarction of ischemic origin. The histostructure of the brain tissue of animals that were exposed to *Limonium gmelinii* extract after stroke induction was partially restored. Moreover, in rats without MCAO, which were exposed to the studied extract, the histological picture of the brain did not differ from that in control animals. Hence, the

extract of *Limonium gmelinii* partially normalizes the histostructure of the damaged brain of animals with induced stroke, which indicates its neuroprotective effect in vivo; evaluation of the sensorimotor activity of laboratory animals with MCAO and administration of a polyphenol extract showed that the extract of *Limonium gmelinii* partially restores the musculoskeletal functions in rats with induced ischemic brain stroke.

Based on the above, the results obtained allow us to conclude that the *Limonium gmelinii* extract at a dose of 200 mg/kg per day for 28 days, has neuroprotective properties.

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