Research Results in Pharmacology

Research Results in Pharmacology 8(2): 59–68
UDC: 616.8-085.2/.3
DOI 10.3897/rrpharmacology.8.77172

Research Article

Neuroprotective effects of a 40% ethanol extract of the black walnut bark (*Juglans nigra* L.)

Dmitry I. Pozdnyakov[1], Zhanna V. Dayronas[1], Denis S. Zolotych[1], Anastasya D. Geraschenko[1], Natalya B. Shabanova[1]

1 *Pyatigorsk Medical and Pharmaceutical Institute, branch of Volgograd State Medical University, 11 Kalinina Ave., Pyatigorsk 357532, Russia*

Corresponding author: Dmitry I. Pozdnyakov (pozdniackow.dmitry@yandex.ru)

Academic editor: Mikhail Korokin ♦ **Received** 27 October 2021 ♦ **Accepted** 5 April 2022 ♦ **Published** 14 June 2022

Citation: Pozdnyakov DI, Dayronas ZV, Zolotych DS, Geraschenko AD, Shabanova NB (2022) Neuroprotective effects of a 40% ethanol extract of the black walnut bark (*Juglans nigra* L.). Research Results in Pharmacology 8(2): 59–68. https://doi.org/10.3897/rrpharmacology.8.77172

Abstract

Introduction: Neuroprotection is a promising area of adjuvant therapy of ischemic brain lesions. At the same time, among potentially effective neuroprotectors, herbal remedies are distinguished due to their high efficiency and safety of use. In this work, some aspects of the neuroprotective effect of 40% ethanol extract of black walnut bark were investigated in comparison with its major component juglone.

Materials and methods: The work was performed on male *Wistar* rats, which were simulated with cerebral ischemia by irreversible occlusion of the middle cerebral artery. The acute toxicity of the extract was preliminarily evaluated. During the work, the following parameters were determined: changes in the behavior of animals in the Morris water maze, cerebral blood flow, brain necrosis zone area, the activity of mitochondrial complexes, citrate synthase activity, lactic, pyruvic, and ATP concentrations. The activity of the studied extract was compared with juglone in a concentration of 1 mg/kg (*per os*).

Discussion: The study showed that the use of black walnut bark extract in conditions of cerebral ischemia contributed to an increase in the activity of mitochondrial complexes I-V, citrate synthase, which in turn led to the normalization of aerobic-anaerobic metabolism reactions. The increase in the activity of respiratory complexes is probably mediated by the antioxidant properties of juglone, which is a major component of the test extract of black walnut bark.

Conclusion: Thus, the test extract can be a potentially effective neuroprotective agent and requires further study.

Keywords

Juglans nigra extract, cerebral ischemia, neuroprotection, mitochondrial dysfunction.

Introduction

According to the latest statistics from the World Health Organization, ischemic brain lesions and, in particular, ischemic stroke, remain one of the main causes of death, with a high level of primary disability in the population. More than 50 million cases of ischemic stroke are reported annually. More than half are fatal in the acute phase of the disease (Guzik and Bushnell 2017). Stroke is more common in the elderly, while the incidence rate is higher among women, and the mortality rate, on the contrary, is higher in males. At the same time, ischemic stroke in

the last decade has shown a dramatics tendency to shift to younger age groups and this pathological condition is increasingly observed in the cohort of the population under 55 years of age, among whom the mortality rate is 10–20% higher than in the elderly (Putaala 2020). Ischemic stroke, being a heterogeneous disease, is represented by many clinical variants, among which stroke caused by occlusion of large vessels is the most severe and is registered in 20–40% of cases (Martha et al. 2019). Morphologically, a stroke is represented by a formed area of necrotic tissue – "ischemic nucleus", which occurs almost immediately after vessel occlusion and is resistant to pharmacological intervention. The "ischemic nucleus" is surrounded by a functionally active site with reduced metabolic and bioelectric activity – the "ischemic penumbra" zone (Thirugnanachandran et al. 2019). In the absence of reperfusion, neurons located in the "penumbra" area are also subject to irreversible changes, leading to their death. At the same time, in contrast to the necrotic pyroptotic process of cell destruction that occurs in the "ischemic nucleus", mediated by the activation of caspase-1 (Shi et al. 2017), the death of "penumbra" cells proceeds mainly along the path of apoptosis, as caspase-dependent, and caspase-independent (Radak et al. 2017). Apoptosis is known to be directly related to the disruption of mitochondrial bioenergetic processes, accompanied by a decrease in ATP synthesis. Comelli et al. (2003) found that inhibition of ATP-synthase by oligomycin and subsequent ATP deficiency activates the apoptotic cascade of cell death, with an increase in caspase activity and production of free oxygen radicals. At the same time, the synthesis of high-energy phosphates is directly related to the intensity of electron transport reactions in the mitochondrial respiratory chain and, accordingly, the activity of the respiratory complexes I-V (Fink et al. 2017). In this regard, it can be assumed that a targeted influence on the reactions occurring in the electron transport chain may be a promising direction of neuroprotection in ischemic stroke.

Juglans nigra L. of the *Juglandaceae* family is a widespread deciduous tree that grows mainly in temperate climates. In ethnomedicine, the leaves of *Juglans nigra* L. are used as an antispasmodic and antidiarrheal agent. Recent studies have shown that extracts derived from *Juglans nigra* L. raw materials are characterized by a broad spectrum of pharmacological activities, including antimicrobial, antioxidant, and anti-inflammatory properties (Ho et al. 2019). Most authors associate the high activity of *Juglans nigra* L. extracts with the presence of naphthoquinones and, in particular, juglone in their composition (McCoy et al. 2018), which can be a potentially effective neuroprotector (Sanchez-Cruz et al. 2010). In this regard, it has been hypothesized that an extract obtained from the black walnut bark containing juglone as a major component (Dayronas and Zilfikarov 2015) will have a protective effect on brain neurons in case of ischemic damage.

Aim of the study: To evaluate the neuroprotective potential of an ethanolic extract of black walnut bark on a model of permanent occlusion of the middle cerebral artery in rats.

Materials and methods

Animals

The work was performed on 80 male *Wistar* rats weighing 200–220 grams, 3 months old, and 8 sexually mature male *Balb/c* mice weighing 20–22 grams. The animals were obtained from the Rappolovo Nursery (Leningrad region, Russia) and during the experiment were kept under controlled conditions in the Laboratory of Living Systems at Pyatigorsk Medical and Pharmaceutical Institute. The conditions of animal housing: ambient temperature of +22±2 °C, relative humidity of 60±5%, with a 12-hour dar-light cycle. The rats were housed by 5 animals in macrolon cages with granular cellulose bedding with free access to water and food. The study was carried out according to the recommendations of Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, of September 22, 2010, and ARRIVE guidelines (Percie du Sert et al. 2020). The study was approved by the local ethics committee (Minutes No. 20 dated 05.16.2020).

Test-objects

As a test object, we used a standardized extract of black walnut bark obtained by extraction with 40% ethanol in a Soxhlet apparatus for 48 hours, followed by evaporation in a vacuum evaporator. The resulting dry residue was dissolved in purified water. Walnut bark was collected in the autumn in the Caucasian Mineral Waters region (44°3'13"N, 43°5'18"E), herbarium sample No. 217. The test extract was obtained and standardized according to the scheme described in patent *RU 2 608 026 C1* and characterized by the content of berberine, juglone, juglandin, vitamin C, sitosterol, and betulin. As a comparison, juglone (Sigma-Aldrich) at a dose of 1 mg/kg (Peng et al. 2015) was used. The dose of the test extract was selected during the study, based on the data obtained in the assessment of acute toxicity.

Study design

At the first stage of the study, the toxicity of the test extract was evaluated in an acute experiment, according to the protocol of the experimental procedure "Up and Down" in the *Balb/c* mice. Next, the neuroprotective potential of the black walnut bark was assessed on the model of permanent occlusion of the middle cerebral artery in rats. At the same time, the following experimental groups were formed: SO – sham-operated animals (without pathology); NC – negative control group (with cerebral ischemia, but lacking pharmacological support), and the group of rats receiving juglone and test-extract. The number of animals in a group was 20. Black walnut bark extract and juglone were administered *per os* 30 minutes after the simulation of ischemia and then once a day for 3 days. On

the 4th day, the rats were tested in the Morris water maze, and the changes in the cerebral blood flow rate were assessed. After that, the rats were decapitated under chloral hydrate anesthesia (350 mg/kg intraperitoneally), venous blood and brain were taken and used as the analyzed biomaterial. In 10 animals from the group, the size of the brain necrosis zone was determined, in 10 remaining animals, changes in the activity of mitochondrial respiratory complexes, citrate synthase, and ATP level were assessed. The study design is shown in Fig. 1.

Biomaterial preparation

Fresh citrated venous blood was centrifuged at 1000 g for 15 minutes to obtain serum, in which the concentrations of two parameters (lactic and pyruvic acids) were determined. The right hemisphere (ischemic) was cut off from the brain and homogenized in 10 animals from the group in phosphate-buffered saline (pH=7.4) in a ratio of 1:7 and used to assess the change in the size of the necrosis zone. In the remaining 10 animals, the right hemisphere was homogenized in an isolation medium (1 mmol EGTA + 215 mmol mannitol + 75 mmol sucrose + 0.1% BSA solution + 20 mmol HEPES, with pH of 7.2). For mitochondrial isolation, the resulting homogenate was centrifuged at 1100 g for 2 minutes. The resulting supernatant in the amount of 700 µl was transferred into Eppendorf tubes and mixed with 75 µl of 10% percol and centrifuged at 18000 g for 10 minutes. The precipitate was resuspended in 1 ml of the isolation medium and centrifuged for 5 minutes at 10000 g. This supernatant was used to determine changes in the activity of complexes of the mitochondrial respiratory chain, citrate synthase activity, and ATP concentration.

Acute toxicity determination

The study of the toxicity of the extract was carried out using the available method for studying the oral toxicity of chemical substances in an acute experiment – the "Up and down" procedure. According to this protocol, two tests were conducted: limit testing and basic testing. The analysis and the LD_{50} calculation corresponded to the OECD Guidelines for the Testing of Chemicsla No. 425. According to these guidelines, the range of administered doses for the test-compounds was: 1.75; 5.5; 17.5; 55; 175; 550; 1750; 5000 mg/kg with a dose extension coefficient 3.2. The administration of the compounds was carried out fractionally with 1 hour time interval until the required dose was reached, according to the following scheme: the first animal receives the test objects at a dose of 1.75 mg/kg, if the animal survives, the administration of the compound continues in increasing order of doses until the 1st case of death is reported. After registering a fatal outcome, the next animal receives a lower dose of the test compounds than the previous one. The criteria for stopping the experiment were:

- consecutive deaths of 3 animals;
- inverting the administered dose 3 times in 4 consecutive animals;
- no death of 4 consecutive animals when administering one dose, after reaching a fatal outcome.

Cerebral ischemia model

Cerebral ischemia was simulated by the method of irreversible occlusion of the middle cerebral artery. The course of the operation was the following: in anesthetized

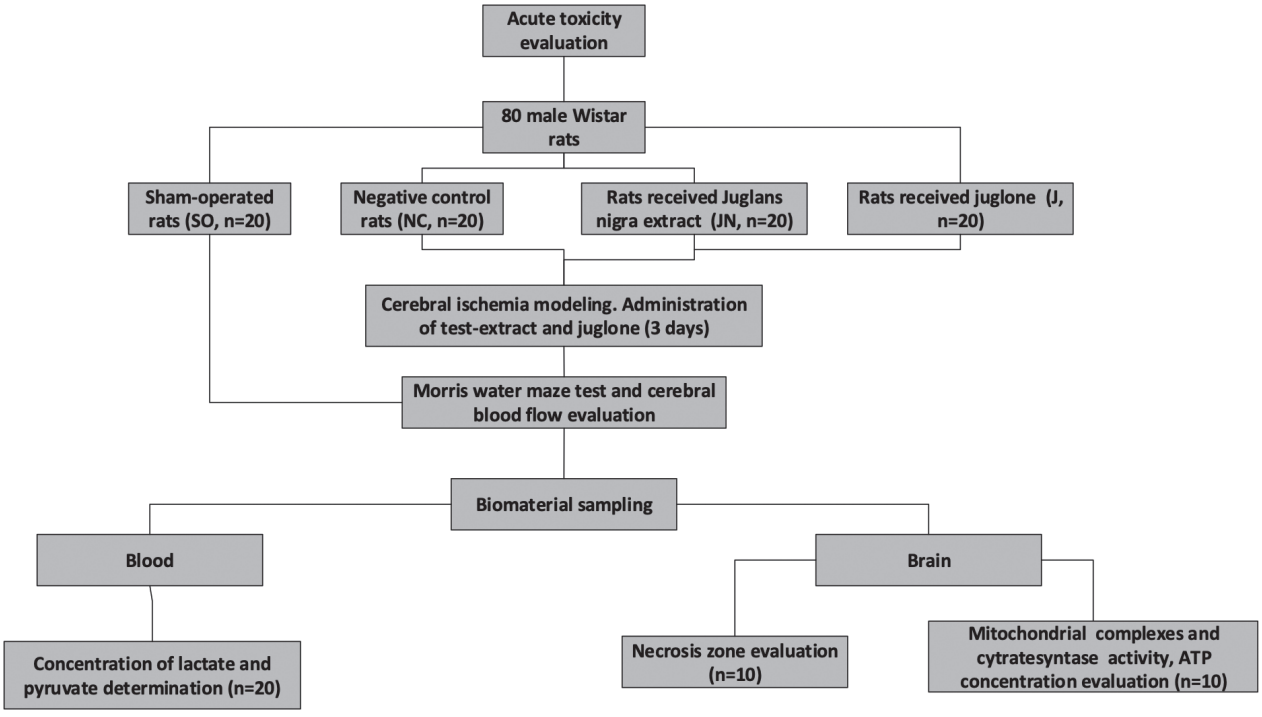

Figure 1. Study design.

animals (chloral hydrate 350 mg/kg, intraperitoneally), on the depilated area below and to the right of the eye, the skin was dissected and the muscles were moved apart. Then the process of the zygomatic bone was removed and the skull was exposed. Next, a burr was used to make a trepanation hole above the intersection of the middle cerebral artery and the olfactory tract; the dura mater was removed, and the artery was electrocoagulated and then cut to avoid vessel recanalization, and the wound was sutured in layers. The suture was treated with an antiseptic solution Benzyldimethyl [3-myristoilamine propyl] ammonium chloride monohydrate (0.01% solution). Animals were left under a heating lamp until emergence from anasthesia (Tamura et al. 1981).

Morris water maze test

The Morris water maze device is a water arena with a diameter of 150 cm with a wall height of 60 cm and a movable platform with a diameter of 10 cm (Open science, Russia). During the study, the unit was filled with water to the level of 50 cm, after which the water was stained. Before cerebral ischemia simulation, the rats were trained through the testing procedure: within 2 minutes, the animals were allowed to find a platform, provided no complete of the task; after that, the rats were moved to the platform for 10 sec., and the testing was repeated. The training lasted for 5 days. After simulating ischemia in similar conditions, the test was repeated. The latency period of reaching the platform in seconds and the distance that the animal covered until finding the platform in meters were recorded. The experiment was recorded and processed using the Minotaur software (Neurobiotics, Russia) with infrared monitoring of activity (Vorhees and Williams 2006).

Assessment of changes in the rate of cerebral blood flow

The cerebral blood flow rate was assessed in the middle cerebral artery basin in animals anesthetized with chloral hydrate (350 mg/kg, intraperitoneally). In rats, in the parietal region, the cranium was opened with the removal of the dura mater and arachnoid meninges, after which the ultrasonic Doppler sensor UZOP-010-01 with an operating frequency of 25 MHz was installed. A contact gel was used as a sound-conducting medium. The change in the rate of cerebral blood flow was determined in cm/sec using the MM-D-K-Minimax Doppler v.1.7 software. (Saint-Petersburg, Russia).

Necrosis zone evaluation

The size of the necrosis zone was determined by the triphenyltetrazolium method. The brain was removed, with the cerebellum cut off, and divided into the hemispheres. Both hemispheres were weighed, then separately homogenized and placed in cups with 10 ml of a 1% solution of triphenyltetrazolium chloride in phosphate buffer (pH=7.4). The sample was placed in a water bath for 20 minutes at 37 °C. Tne samples were centrifuged at 5000 rpm/10 min. Next, 3 ml of cooled chloroform was added to the supernatant and incubated for 15 minutes at 40 °C with periodic stirring. The resulting mixture was re-centrifuged, and the optical density of the chloroform extract of formazan was measured at 492 nm against pure chloroform. The calculation of the necrosis zone was expressed as a percentage of the total mass of the hemispheres by formula (1) (Pozdnyakov et al. 2019):

$$x = 100 - \frac{\varepsilon_1 M_1 + \varepsilon_2 M_2}{\varepsilon_1 (M_1 + M_2)} \times 100 \quad (1),$$

where x is the size of the zone of necrosis as a percentage of the total mass of the brain; ε_1 is the optical density of the sample with an intact hemisphere; ε_2 is the optical density of the sample with a damaged hemisphere; M_1 is the mass of the intact hemisphere; M_2 is the mass of the damaged hemisphere.

Assessment of the activity of mitochondrial complexes

The activity of the mitochondrial respiratory chain complexes I, II, IV, and V was studied by adding the corresponding oxidation substrates to the analyzed medium: pyruvate – 10 mmol/ml; malic acid – 1 mmol/ml; succinic acid – 10 mmol/ml; ascorbate – 2 mmol/ml; adenosinediphosphate – 1 mmol/ml; and N, N, N ‘, N’ tetramethyl-1,4-phenylenediamine (TMPD) – 0.5 mmol/ml (Connolly et al. 2018). The complex I activity was investigated by the difference in oxygen consumption when malate/pyruvate was used as an oxidation substrate. The complex II activity was investigated by the difference in oxygen consumption rate (OCR) when succinate was used as an oxidation substrate and oligomycin was added to the medium. The activity of complex IV was defined by the difference in OCR after adding the mixture of rotenone/TMPD/ascorbate as an oxidation substrate. The activity of complex V was defined by the difference in OCR after the introduction of rotenone and ADP to analyze the medium. The activity of complex III was assessed spectrophotometrically based on an increase in the optical density of the incubation medium with 1 M succinate solution + 0.5 M cytochrome C solution + 0.2 M KCN solution + 10 mM rotenone solution and 10 µl of the analyzed sample at 550 nm (Spinazzi et al. 2012). Oxygen consumption was determined in ppm and expressed in terms of protein concentration in the analyzed sample. Protein content was estimated using the Bradford method (Carlsson et al. 2011).

Measurement of citrate synthase activity

Citrate synthase activity was determined according to the method described by Shepherd and Garland (1969). The method is based on the determination of the colored degradation products of 5.5’-di-thiobis- (2-nitrobenzoic acid) in the presence of acetyl-CoA and oxaloacetate. The reaction mixture contained: 100 mM solution,

5.5'-di-thiobis- (2-nitrobenzoic acid); 100 mM acetyl CoA solution; 0.1% Triton-X solution; 4 µl of the analyzed supernatant and Tris-HCl buffer solution with pH = 7.8 to 100 µl. The optical density of the mixture was recorded at 412 nm for 3 minutes. The citrate synthase activity was determined by the change in the optical density of the medium and was expressed in U/mg of protein. Protein concentration was determined according to the Bradford method.

Determination of lactic acid concentration

The concentration of lactate in blood serum was determined by the enzymatic reaction with the formation of quininomine, the concentration of which is proportional to the content of lactic acid in the sample. Incubation medium: phosphate buffer (pH 6.8), Pipes 50 mmol/L, 4-chlorophenol 6 mmol/L, AAP4 0.4 mmol/l, 2000 U/L lactoxydase, and U/L peroxidase. The volume of the test sample was 10 µl. Sampling was carried out at 500 nm. Calculation of the lactic acid content was carried out according to the formula (2) (Pozdnyakov et al. 2019):

$$C = \frac{E_x}{E_0} \times 3{,}34 \mu mol / L \quad (2),$$

where Ex is absorbance of the test sample; E_0 is absorbance calibration sample.

Determination of pyruvic acid concentration

The content of pyruvic acid in blood serum was determined by a decrease in NADH in the lactate dehydrogenase reaction. Incubation medium: Good's buffer 1000 µL, NADH 200 µL, and LDH (2000 U/L) 20 µl. The volume of the sample was 600 µl. Samples were measured at 340 nm. Calculation of the pyruvic acid concentration was carried out according to the formula (3) (Pozdnyakov et al. 2019):

$$C = \frac{E_x}{E_0} \times 1{,}25 \mu\mu \text{ o}\lambda / L \quad (3),$$

where Ex is absorbance of the test sample; E_0 is absorbance calibration sample.

Determination of ATP concentration

The ATP content in the brain supernatant of animals was assessed by enzyme-linked immunosorbent assay using species-specific reagent kits. Reagents were provided by Cloud Clone Corp. (USA). The assay progress was consistent with the kit manufacturer's recommendations.

Statistical analysis

Statistical processing of the obtained results was performed using the STATISTICA 6.0 application software package (StatSoft, USA). Data were expressed as M (mean)±SEM. Comparison of means groups was carried out by the method of one-way analysis of variance with post-processing Newman-Keuls test.

Results

Assessment of acute toxicity of the test extract

While testing the maximum permissible dose, it was found that when the extract of black walnut bark at a dose of 5000 mg/kg was administered to the animals, there was no death of mice. At the same time, no significant deviations of the general condition of the animals from the control group (n=4) were found. In this regard, based on the OECD Guidelines No. 425, the main testing was not started and the LD_{50} value of the test extract was accepted as a value of 5000 mg/kg (orally). At the same time, based on the literature data, it can be assumed that the effective dose for the test extract will be a dose of 1/100 of the LD_{50}, which is 50 mg/kg (Pozdnyakov et al. 2019).

Evaluation of the effect of the test extract on changes in the behavior of animals in the Morris water maze test

While assessing the effect of the test extract on the change in the behavior of animals in the Morris water maze, it was found that in the NC group of rats, compared with the SO animals, there was an increase in the latency time of platform search (Fig. 2B) by 2.6 times ($p<0.05$) and in the distance covered (Fig. 2A) by 2.3 times ($p<0.05$). The use of juglone contributed to a decrease in the time for completing the task in the research maze concerning the NC group of rats by 35.7% ($p<0.05$) and in the distance covered by 39.1% ($p<0.05$), whereas when administering the investigated extract, these indicators decreased by 40.1% ($p<0.05$) and 49.2% ($p<0.05$), respectively. At the same time, no statistically significant differences were found between the groups of animals that received the studied extract and juglone.

Evaluation of the effect of the test extract on the change in the cerebral blood flow rate, the size of the brain necrosis zone, and the concentrations of lactic and pyruvic acids in the blood serum of rats

While carrying out this block of experimental work, it was found that in the NC group of rats, the rate of cerebral blood flow (Fig. 3) was lower than that in the SO of animals by 84.1% ($p<0.05$). At the same time, against the background of administering juglone and the test extract, there was an increase in the level of cerebral blood flow in comparison with the NC group of rats by 2.8 times and 3.4 times (both $p<0.05$), respectively.

At the same time, there was an increase in the concentration of lactate in the blood serum of NC animals and a decrease in the content of pyruvate (Table 1) when compare to those in SO rats by 5.7 times ($p<0.05$) and by 47.5% ($p<0.05$), respectively. Against the background of the use of juglone, there was a decrease in the lactate concentration in the blood serum of animals by 38.2% ($p<0.05$), accompanied by an increase in the concentration of pyruvate by 32.3% ($p<0.05$). It should be noted that when rats were treated with the black walnut bark extract,

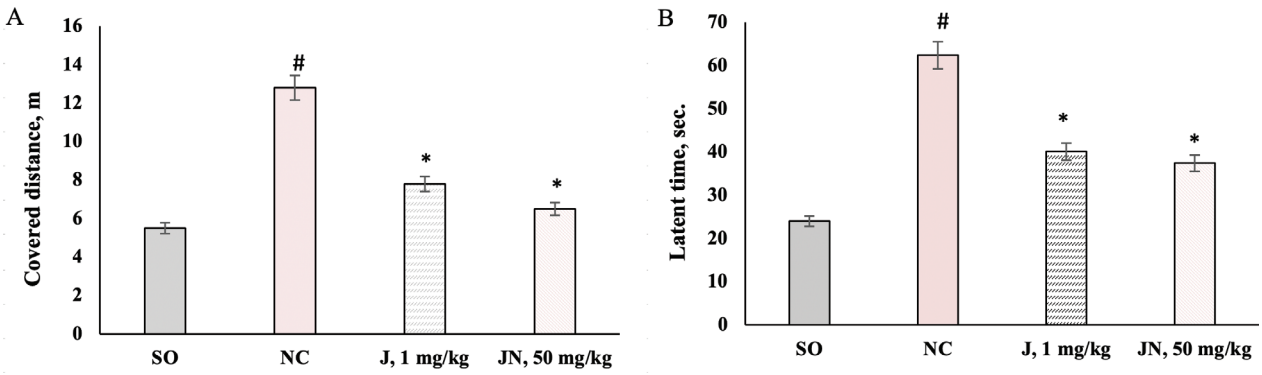

Figure 2. Influence of the test extract of black walnut bark and juglone on changes in animal behavior in the Morris water maze test. **Note:** SO – sham-operated animals, NC – negative control, J – a group of animals that received juglone, JN – a group of animals that received the studied extract of black walnut bark, # – statistically significant relative to SO rats (Newman-Keuls test, $p<0.05$); * – statistically significant relative to NC rats (Newman-Keuls test, $p<0.05$).

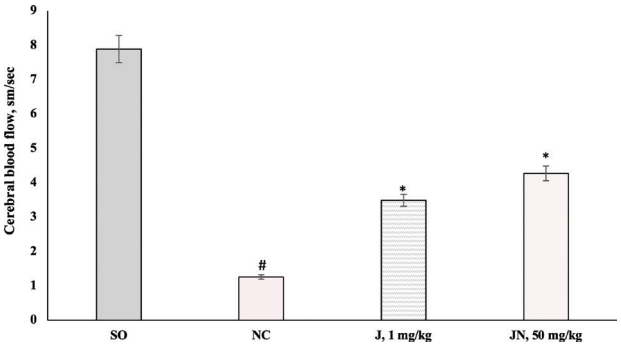

Figure 3. Influence of the test extract and juglone on changes in cerebral blood flow rate in rats. **Note:** SO – sham-operated animals, NC – negative control, J – a group of animals that received juglone, JN – a group of animals that received the studied extract of black walnut bark, # – statistically significant relative to SO rats (Newman-Keuls test, $p<0.05$); * – statistically significant relative to NC rats (Newman-Keuls test, $p<0.05$).

where as the content of lactic acid compared with the same indicator of the NC group of animals decreased by 47.1% ($p<0.05$), the content of pyruvic acid, on the contrary, increased by 40.7% ($p<0.05$). At the same time, the area of brain necrosis in the animals that were treated by juglone and the test extract decreased in comparison with the NC group of animals by 19.4% ($p<0.05$) and 30.9% ($p<0.05$), respectively (Table 1).

Table 1. Influence of the test extract of black walnut bark and juglone on the change in the concentrations of lactic and pyruvic acids in the blood serum of rats and brain necrosis zone area

Group	Lactic acid, mmoles/L	Pyruvic acid, μmoles/L	Necrosis zone area, %
SO	1.2±0.04	125.6±8.27	0
NC	6.8±0.05#	65.9±13.29#	35.9±2.469
J, 1 mg/kg	4.2±0.06*	87.2±14.88*	28.7±2.971*
JN, 50 mg/kg	3.6±0.05*	92.7±13.92*	24.6±3.225*

Note: SO – sham-operated animals, NC – negative control, J – a group of animals that received juglone, JN – a group of animals that received the studied extract of black walnut bark, # – statistically significant relative to SO rats (Newman-Keuls test, $p<0.05$); * – statistically significant relative to NC rats (Newman-Keuls test, $p<0.05$).

Evaluation of the effect of the test extract on the change in the activity of the complexes of the mitochondrial respiratory chain, citrate synthase, and the concentration of ATP in the brain of rats

Evaluating the change in the activity of mitochondrial complexes (Fig. 4) in the NC group of rats, it was found that in this group of animals relative to the SO rats, the activity of complexes I-V decreased by 67.6%; 74.1%; 61.2%; 60.5% and 43.8%, respectively (all indicators $p<0.05$). In the animals that were treated by juglone, an increase in the activity of mitochondrial complexes was observed, while the activity of complex I increased by 92.4% ($p<0.05$); complex II – by 165.9% ($p<0.05$); complex III – by 43.3% ($p<0.05$); complex IV – by 34.6% ($p<0.05$) and complex V – by 87.5% ($p<0.05$). When using the test extract of black walnut bark, there was an increase in the activity of respiratory complexes I-V concerning the NC group of rats by 57.8%; 126.7%; 49.1%; 54.4%, and 67.3% (all indicators $p<0.05$).

The activity of citrate synthase and the concentration of ATP (Table 2) in the NC group of animals were lower than those in the SO of rats by 56.1% ($p<0.05$) and 56.6% ($p<0.05$), respectively. At the same time, in animals receiving juglone, the activity of citrate synthase increased by 38.9% ($p<0.05$) in comparison with the NC group of rats, with an increase in ATP content by 38.3% ($p<0.05$). The

Table 2. Influence of the test extract of black walnut bark and juglone on changes in the citrate synthase activity and ATP concentration in the brain in rats

Group	ATP concentration, pg/ml	Citrate synthase activity, U/mg of protein
SO	1594.2±78.645	4.1±0.659
NC	691.4±90.622#	1.8±0.142#
J, 1 mg/kg	956.4±54.005*	2.5±0.821*
JN, 50 mg/kg	1002.4±93.285*	2.9±0.287*

Note: SO – sham-operated animals, NC – negative control, J – a group of animals that received juglone, JN – a group of animals that received the studied extract of black walnut bark, # – statistically significant relative to SO rats (Newman-Keuls test, $p<0.05$); * – statistically significant relative to NC rats (Newman-Keuls test, $p<0.05$).

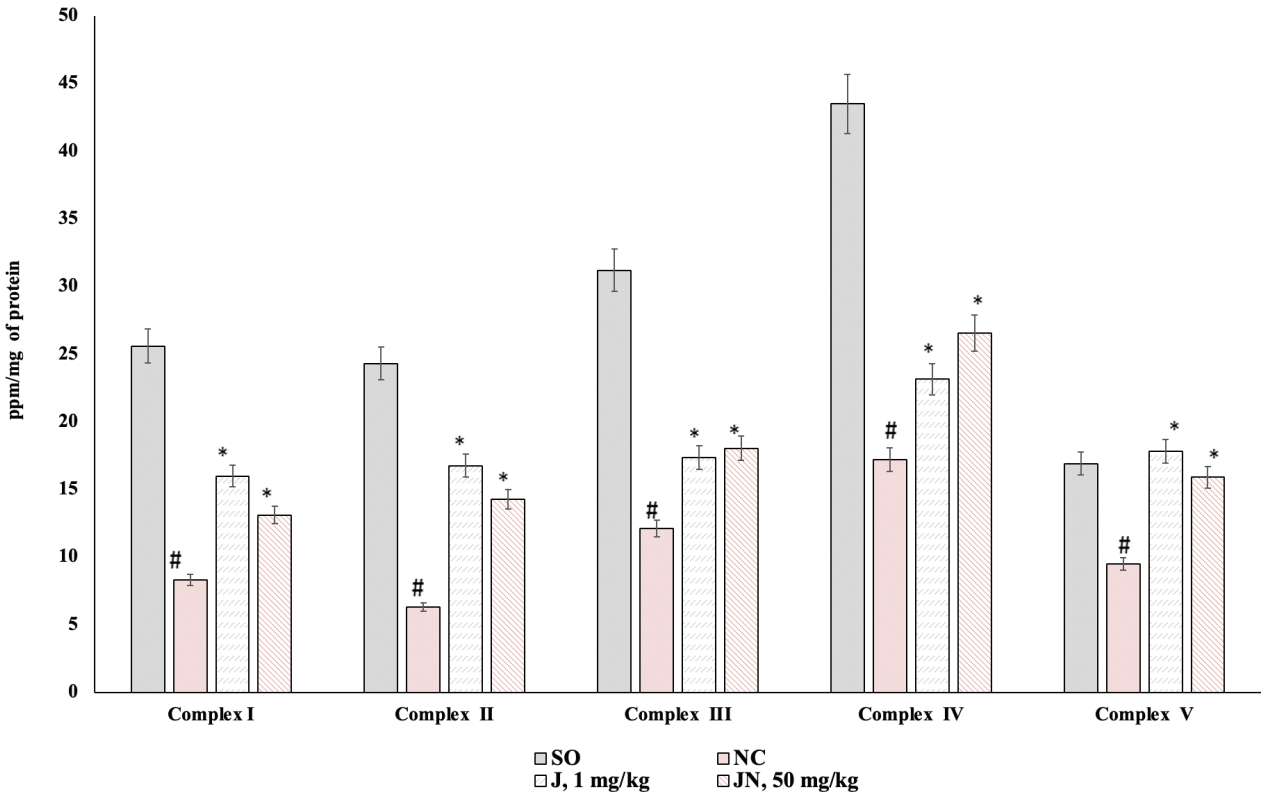

Figure 4. Influence of the test extract of black walnut bark and juglone on changes in the mitochondrial respiratory complexes activity in the brain of rats. **Note:** SO – sham-operated animals, NC – negative control, J – a group of animals that received juglone, JN – a group of animals that received the studied extract of black walnut bark, # – statistically significant relative to SO rats (Newman-Keuls test, $p<0.05$); * – statistically significant relative to NC rats (Newman-Keuls test, $p<0.05$).

use of the test extract of black walnut bark promoted an increase in the concentration of ATP and the activity of citrate synthase by 45.0% ($p<0.05$) and 61.1% ($p<0.05$), respectively, relative to the NC of the group of animals.

Discussion

To date, ischemic stroke remains one of the main problems of modern healthcare with a high level of disability and mortality. It has been found that it is possible to reduce the negative consequences of stroke through the use of neuroprotective agents (Rajah and Ding 2017). At the same time, one of the promising approaches to neuroprotective therapy may be the use of metabolic agents that restore metabolism in the brain tissue by normalizing mitochondrial function (Yang et al. 2016). The present study investigated the neuroprotective properties of a standardized black walnut bark extract compared to juglone, which is the major component of this extract. The study showed that the use of black walnut bark extract in conditions of cerebral ischemia contributed to an increase in the activity of mitochondrial complexes I-V, citrate synthase, which in turn led to the normalization of aerobic-anaerobic metabolism reactions. The increase in the activity of respiratory complexes is probably mediated by the antioxidant properties of juglone, which is the major component of the extract of black walnut bark. It has been established that juglone has chelating properties relative to bivalent ions, and in particular, against Fe^{2+} and thereby suppresses the production of reactive oxygen species (ROS), mediated by an increase in the intensity of lipid peroxidation by Fenton reaction type (Tamafo Fouegue et al. 2016). In turn, a decrease in the generation of ROS leads to the stabilization of electron transport at the level of complexes I-III, increasing their activity and restoring the course of the reaction of aerobic metabolism. The intensity of aerobic metabolic reactions can be judged by the number of formed intermediates, for example, lactic and pyruvic acids, as well as the end products of metabolism in the form of ATP (Tóth et al. 2020). During this study, it was found that the administration of the test extract of black walnut to animals contributed to the elimination of hyperlactatemia, accompanied by an increase in the content of pyruvate in the blood, as well as an increase in the concentration of ATP in the brain, which may indicate the restoration of the optimal course of reactions of aerobic metabolism. At the same time, an increase in citrate synthase activity observed with the use of black walnut bark extract suggests an increase in mitochondrial biogenesis *de novo*, since NADH dehydrogenase is a specific marker of changes in the number of mitochondria (Kelley et al. 2002). An effect on the level of cerebral blood flow is important for achieving the neuroprotective effect of pharmacologically active substances (Sutherland et al. 2011). It

has been shown that juglone has vasodilating properties. Ahmad et al. (2020a) found that juglone practically did not cause vasorelaxation in vessels devoid of the endothelial lining, nor in cells pretreated with a non-selective blocker of NO synthase, nitro-L-arginine methyl ester, which suggests an endothelial mechanism of vasodilation induced by juglone. It is also assumed that the activation of potassium channels plays a significant role in the vasoactive potential of juglone, as indicated by Ahmad et al. (2003b). In addition to vasodilation, in the implementation of the neuroprotective effect of juglone and, accordingly, the extract of black walnut bark, an improvement in the rheological properties of blood can play a significant role. Kao et al. (2020) found that juglone suppress platelet aggregation caused by the activation of glycoproteins IIb/IIIa, and also reduced the level of intracellular calcium and the activity of protein kinase C.

As a result, elimination of energy deficit and restoration of cerebral hemodynamics contributed to a decrease in the area of brain necrosis and preservation of cognitive functions in rats, which indicates a high neuroprotective potential of the black walnut bark extract, comparable to juglone. In addition, the test extract was characterized by low systemic toxicity with LD_{50}>5000 mg/kg, while juglone (as an individual compound) is a rather toxic compound. It has been found that juglone promotes the activation of apoptosis, both by caspase-dependent and caspase-independent mechanisms (Wu et al. 2017). Also, juglone is characterized by cytotoxic properties, realized by the mechanism of ferroptosis (Karki et al. 2020).

Conclusion

The study showed that a 40% ethanol extract of black walnut bark has a neuroprotective effect in a model of permanent right-sided middle cerebral occlusion in rats. Administration of the studied extract to animals at a dose of 50 mg/kg (1/100 of the LD_{50}) promoted the restoration of the activity of mitochondrial complexes I-V, citrate synthase, increased the level of cerebral blood flow and the intensity of aerobic metabolic reactions, and reduced the size of the brain necrosis zone comparable to that of juglone. Thus, we can assume the relevance of the further study of 40% ethanol extract of black walnut bark as a means of neuroprotective action.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

The study received no sponsorship.

References

- Ahmad T, Shah AJ, Roberts R (2020a) Mechanisms mediating the vasodilatory effects of juglone in porcine isolated coronary arteries. European Journal of Pharmacology 866: e172815. https://doi.org/10.1016/j.ejphar.2019.172815 [PubMed]
- Ahmad T, Khan T, Alamgeer Shah AJ (2020b) Juglone as antihypertensive agent acts through multiple vascular mechanisms. Clinical and Experimental Hypertension 42(4): 335–344. https://doi.org/10.1080/10641963.2019.1665674 [PubMed]
- Carlsson N, Borde A, Wölfel S, Kerman B, Larsson A (2011) Quantification of protein concentration by the Bradford method in the presence of pharmaceutical polymers. Analytical Biochemistry 411(1): 116–121. https://doi.org/10.1016/j.ab.2010.12.026 [PubMed]
- Comelli M, Di Pancrazio F, Mavelli I (2003) Apoptosis is induced by the decline of mitochondrial ATP synthesis in erythroleukemia cells. Free Radical Biology & Medicine 34(9): 1190–1199. https://doi.org/10.1016/s0891-5849(03)00107-2 [PubMed]
- Connolly N, Theurey P, Adam-Vizi V, Bazan NG, Bernardi P, Bolaños JP, Culmsee C, Dawson VL, Deshmukh M, Duchen MR, Düssmann H, Fiskum G, Galindo MF, Hardingham GE, Hardwick JM, Jekabsons MB, Jonas EA, Jordán J, Lipton SA, Manfredi G, Prehn J (2018) Guidelines on experimental methods to assess mitochondrial dysfunction in cellular models of neurodegenerative diseases. Cell Death and Differentiation 25(3): 542–572. https://doi.org/10.1038/s41418-017-0020-4 [PubMed] [PMC]
- Dayronas ZhV, Vernikovsky VV, Zilfikarov IN (2017) Method of obtaining dietary supplements from nut bark. Patent for the invention RU 2608026 C1, 11.01.2017. [GooglePatents]
- Dayronas ZhV, Zilfikarov IN (2015) Prospective directions of studying representatives of the genus walnut as sources of naphthoquinones. Modern Problems of Science and Education 2: 2. [in Russian]
- Fink BD, Bai F, Yu L, Sivitz WI (2017) Regulation of ATP production: dependence on calcium concentration and respiratory state. American Journal of Physiology. Cell Physiology 313(2): 146–153. https://doi.org/10.1152/ajpcell.00086.2017 [PubMed]
- Guzik A, Bushnell C (2017) Stroke epidemiology and risk factor management. Continuum: Lifelong Learning in Neurology 23(1, Cerebrovascular Disease): 15–39. https://doi.org/10.1212/CON.0000000000000416 [PubMed]
- Ho KV, Schreiber KL, Vu DC, Rottinghaus SM, Jackson DE, Brown CR, Lei Z, Sumner LW, Coggeshall MV, Lin CH (2019) Black walnut (Juglans nigra) extracts Inhibit Proinflammatory cytokine production drom lipopolysaccharide-stimulated human promonocytic cell line U-937. Frontiers in Pharmacology 10: e1059. https://doi.org/10.3389/fphar.2019.01059 [PubMed] [PMC]
- Kao CC, Kung PH, Tai CJ, Tsai MC, Cheng YB, Wu CC (2020) Juglone prevents human platelet aggregation through inhibiting Akt and protein disulfide isomerase. Phytomedicine: International Journal of Phytotherapy and Phytopharmacology 82: e153449. https://doi.org/10.1016/j.phymed.2020.153449 [PubMed]

- Karki N, Aggarwal S, Laine RA, Greenway F, Losso JN (2020) Cytotoxicity of juglone and thymoquinone against pancreatic cancer cells. Chemico-Biological Interactions 327: e109142. https://doi.org/10.1016/j.cbi.2020.109142 [PubMed]
- Kelley DE, He J, Menshikova EV, Ritov VB (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51(10): 2944–2950. https://doi.org/10.2337/diabetes.51.10.2944 [PubMed]
- Martha SR, Fraser JF, Pennypacker KR (2019) Acid-base and electrolyte changes drive early pathology in ischemic stroke. Neuromolecular Medicine 21(4): 540–545. https://doi.org/10.1007/s12017-019-08555-5 [PubMed]
- McCoy RM, Utturkar SM, Crook JW, Thimmapuram J, Widhalm JR (2018) The origin and biosynthesis of the naphthalenoid moiety of juglone in black walnut. Horticulture Research 5: e67. https://doi.org/10.1038/s41438-018-0067-5 [PubMed] [PMC]
- Peng X, Nie Y, Wu J, Huang Q, Cheng Y (2015) Juglone prevents metabolic endotoxemia-induced hepatitis and neuroinflammation via suppressing TLR4/NF-κB signaling pathway in high-fat diet rats. Biochemical and Biophysical Research Communications 462(3): 245–250. https://doi.org/10.1016/j.bbrc.2015.04.124 [PubMed]
- Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Würbel H (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biology 18(7): e3000410. https://doi.org/10.1371/journal.pbio.3000410 [PubMed] [PMC]
- Pozdnyakov DI, Pozdnyakova AE, Adzhiahmetova SL, Chervonnaya NM, Zolotych DS, Lyakhova NS, Miroshnichenko KA (2019) Antihypoxic and anti-ischemic properties of the North Caucasus flora plant extracts. Boletín Latinoamericano Y Del Caribe De Plantas Medicinales Y Aromáticas 18(5): 504–517. https://doi.org/10.35588/blacpma.19.18.5.33
- Putaala J (2020) Ischemic stroke in young adults. Continuum: Lifelong Learning in Neurology 26(2): 386–414. https://doi.org/10.1212/CON.0000000000000833 [PubMed]
- Radak D, Katsiki N, Resanovic I, Jovanovic A, Sudar-Milovanovic E, Zafirovic S, Mousad SA, Isenovic ER (2017) Apoptosis and acute brain ischemia in ischemic stroke. Current Vascular Pharmacology 15(2): 115–122. https://doi.org/10.2174/1570161115666161104095522 [PubMed]
- Rajah GB, Ding Y (2017) Experimental neuroprotection in ischemic stroke: a concise review. Neurosurgical Focus 42(4): E2. https://doi.org/10.3171/2017.1.FOCUS16497 [PubMed]
- Sanchez-Cruz P, Garcia C, Alegria AE (2010) Role of quinones in the ascorbate reduction rates of S-nitrosoglutathione. Free Radical Biology & Medicine 49(9): 1387–1394. https://doi.org/10.1016/j.freeradbiomed.2010.07.022 [PubMed] [PMC]
- Shepherd D, Garland PB (1969) The kinetic properties of citrate synthase from rat liver mitochondria. The Biochemical Journal 114(3): 597–610. https://doi.org/10.1042/bj1140597 [PubMed] [PMC]
- Shi J, Gao W, Shao F (2017) Pyroptosis: gasdermin-mediated programmed necrotic cell death. Trends in Biochemical Sciences 42(4): 245–254. https://doi.org/10.1016/j.tibs.2016.10.004 [PubMed]
- Spinazzi M, Casarin A, Pertegato V, Salviati L, Angelini C (2012) Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. Nature Protocols 7(6): 1235–1246. https://doi.org/10.1038/nprot.2012.058 [PubMed]
- Sutherland BA, Papadakis M, Chen RL, Buchan AM (2011) Cerebral blood flow alteration in neuroprotection following cerebral ischaemia. The Journal of Physiology 589(17): 4105–4114. https://doi.org/10.1113/jphysiol.2011.209601 [PubMed] [PMC]
- Tamafo Fouegue AD, Ghogomu JN, Bikélé Mama D, Nkungli NK, Younang E (2016) Structural and antioxidant properties of compounds obtained from Fe2+ chelation by Juglone and two of its derivatives: DFT, QTAIM, and NBO studies. Bioinorganic Chemistry and Applications 2016: e8636409. https://doi.org/10.1155/2016/8636409 [PubMed] [PMC]
- Tamura A, Graham DI, McCulloch J, Teasdale GM (1981) Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. Journal of Cerebral Blood Flow and Metabolism 1(1): 53–60. [PubMed]
- Thirugnanachandran T, Ma H, Singhal S, Slater LA, Davis SM, Donnan GA, Phan T (2018) Refining the ischemic penumbra with topography. International Journal of Stroke 13(3): 277–284. https://doi.org/10.1177/1747493017743056 [PubMed]
- Tóth O, Menyhárt Á, Frank R, Hantosi D, Farkas E, Bari F (2020) Tissue acidosis associated with ischemic stroke to guide neuroprotective drug delivery. Biology 9(12): e460. https://doi.org/10.3390/biology9120460 [PubMed] [PMC]
- Vorhees CV, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature Protocols 1(2): 848–858. https://doi.org/10.1038/nprot.2006.116 [PubMed] [PMC]
- Wu J, Zhang H, Xu Y, Zhang J, Zhu W, Zhang Y, Chen L, Hua W, Mao Y (2017) Juglone induces apoptosis of tumor stem-like cells through ROS-p38 pathway in glioblastoma. BMC Neurology 17(1): 70. https://doi.org/10.1186/s12883-017-0843-0 [PubMed] [PMC]
- Yang X, Zhang Y, Xu H, Luo X, Yu J, Liu J, Chang RC (2016) Neuroprotection of coenzyme Q10 in neurodegenerative diseases. Current Topics in Medicinal Chemistry 16(8): 858–866. https://doi.org/10.2174/1568026615666150827095252 [PubMed]

Author Contributions

- **Dmitry I. Pozdnyakov,** PhD in Pharmacy, Associate Professor of Department of Pharmacology with Course of Clinical Pharmacology, e-mail: pozdniackow.dmitry@yandex.ru, **ORCID ID** https://orcid.org/0000-0002-5595-8182. Developing research design, conducting an experiment, and preparing the final draft of the manuscript.

- **Zhanna V. Dayronas,** Doctor Habil of Pharmacy, Professor of Department of Pharmacognosy, Botany and Technology of Phytopreparations, e-mail: dayronas@mail.ru, **ORCID ID** https://orcid.org/0000-0002-1274-4512. Preparing the studied extract and preparing the final draft of the manuscript

- **Denis S. Zolotych,** PhD in Pharmacy, Associate Professor of Department of Pharmaceutical and Analytical Chemistry, e-mail: metranidazol@mail.ru, **ORCID ID** https://orcid.org/0000-0001-6314-080X. Statistical analysis and preparing the final draft of the manuscript.

- **Anastasya D. Geraschenko,** PhD in Pharmacy, Associate Professor of Department of Pharmacology with Course of Clinical Pharmacology, e-mail: anastasiya_geraschenko@mail.ru, **ORCID ID** https://orcid.org/0000-0003-0294-2926. Experimenting and preparing the final draft of the manuscript.

- **Natalya B. Shabanova,** PhD in Pharmacy, Associate Professor of Department of Pharmacology with Course of Clinical Pharmacology, e-mail: vahlushina05@yandex.ru, **ORCID ID** https://orcid.org/0000-0001-6281-1114. Experimenting and preparing the final draft of the manuscript.