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Abstract. We studied the chemical composition and pharmacological effects of Melilotus officinalis L. after chronic administration to male rats. In Melilotus officinalis L. contains flavonoids, polysarides, triterpene saponins, ascorbic acid, coumarins, tannins. To study the pharmacological action, a biochemical analysis of blood and urine, a hematological blood test, a study of the coagulation system and behavioral properties in the Suok test were carried out. Chronic administration of the Melilotus officinalis L. extract led to a decrease in patterns of orientation-exploratory behavior, an increase in the level of anxiety. The activity of such enzymes as AST and alkaline phosphatase under long-term administration of the Melilotus officinalis L. significantly increased, while the activity of ALT decreased, in addition, there was a significant increase in blood coagulation. Thus, chronic administration of the extract to laboratory animals for 21 days revealed its toxic effect, manifested in liver disorders, changes in the blood coagulation system, and a decrease in pattern-research behavior.

1. Introduction

Currently, for the prevention and treatment of many diseases, medicinal preparations based on herbal raw materials are successfully used, since they have a number of advantages over synthetic drugs: a wide range of therapeutically active natural compounds, the possibility of their combined use, a "smooth" increase in the pharmacological effect, less frequency of adverse reactions [1]. To expand the official raw material base of medicinal plants, they study the chemical composition and pharmacological value of wild plants in various places, which, due to the peculiarity of soil and climatic conditions, differ in the qualitative and quantitative composition of biologically active substances.

Melilotus officinalis L. is a biennial herb of Fabaceae. In the Astrakhan region, it is found everywhere, in wet and dry meadows, along the edges of belt forests.

According to the literature, the stem and leaves of Melilotus officinalis L. contain coumarins, flavonoids (hyperoside, rutin, quercitrin, isoquercitrin, kaempferol and quercetin), triterpene saponins, tannins, resins, carbohydrates (fructose, galactose, arabinose), vitamins (carotene, tocopherol, ascorbic acid, niacin, phylloquinone), alkaloids, phenol carboxylic acids [2, 3]. The seeds contain amino acids (threonine, valine, arginine, isoleucine, glutamine), fatty oil, including linoleic, oleic, palmitic and other acids, in the roots - aromatic substances, melilotic acid and coumarin [3,4].

The Melilotus officinalis L. extract is traditionally used as a mild sedative, showing a cardiotonic effect [2].

Scientific studies provide information on the anti-inflammatory, analgesic, antibacterial, wound-healing, sedative, antispasmodic, immunostimulating effects of Melilotus officinalis L. extract [5, 6, 7]. The use of the extract improves venous and capillary blood flow, it has a pronounced effect on the vascular system. Coumarin and melilotin included in its composition increase capillary resistance. Dikumarin has an anticoagulant and moderate fibrinolytic effect. National and European Herbalistic Commissions recommend Melilotus officinalis L. for chronic venous insufficiency, varicose veins and superficial thrombosis, as an adjunct in the treatment of thrombophlebitis, post-thrombotic syndrome, hemorrhoids and lymphostasis [8].

A pronounced antihypoxic effect of dry water-soluble extract of Melilotus officinalis was noted in models of acute hypobaric and histotoxic hypoxia in rats [9].

However, there is no information in the literature on the pharmacological effects of chronic administration of Melilotus officinalis L. extract to experimental animals, which, in our opinion, requires study.

2. Materials and methods

2.1. Plant material and extract preparation

The air-dried aerial parts of Melilotus officinalis L. were collected and identified in the Herbarium of the Biological Faculty of Astrakhan State University.

Grass (leaves and stems) Melilotus officinalis L. was collected in flood meadows near the village of Novourusovka in the second half of July 2020. Raw materials were collected during the flowering of the plant. Dried by air shadow method.

To prepare an aqueous extract of Melilotus officinalis L., 20 g of plant material crushed to a powdery state was taken and 1 liter of distilled water was poured. The resulting solution was boiled in a water bath for 30 minutes, after which the extract was cooled and filtered. The obtained aqueous extract of Melilotus officinalis L. animals received as a drink.

2.2. Chemical analysis of extracts

Quantitative determination of the sum of flavonoids in the aqueous-alcoholic extract of grass (leaves and stems) of Melilotus officinalis L. was determined by spectrophotometry using the ability of flavonoids of the aqueous-alcoholic extract (60%) of plant raw materials to form a colored complex with an alcoholic solution of aluminum chloride.

To an aliquot of the aqueous alcohol extract (60%) of the leaves and stems of Melilotus officinalis L., according to procedure [10], a 2% solution of aluminum chloride in 96% alcohol was added and left for 20 minutes to form a colored complex of flavonoids of the extract with aluminum chloride. The optical density of the obtained colored solution was measured on a spectrophotometer PE-5400 in a cuvette with a layer thickness of 10 mm, relative to an aqueous-alcoholic extract without aluminum chloride.

A photocolorimetric method based on the color reaction of monosaccharides with phenol in the presence of sulfuric acid [11] was used to quantify polysaccharides in the leaves and stem of Melilotus officinalis L. An aqueous grass extract of the plant Melilotus officinalis L. The extract proteins were precipitated with a solution of lead acetate and the precipitate was separated. Ethyl alcohol was added to the aliquot of the extract and centrifuged. The precipitate was dissolved in water. An aqueous solution of phenol (5%) and concentrated sulfuric acid were then added to the aliquot of the resulting solution according to procedure [11]. After 30 minutes, the optical density of the obtained solution was measured on a spectrophotometer at a wavelength of 490 nm, a solution of 1 ml of water, 5 ml of sulfuric acid (conc.) and 1 ml of phenol [12] was used as a comparison solution.

To calculate the polysaccharide content, data obtained from the glucose calibration curve were used. Glucose solutions of different concentrations were exposed to a solution of phenol and concentrated

sulfuric acid, similar to experimental solutions. After 30 minutes, the optical density of glucose solutions was measured on a spectrophotometer at a wavelength of 490 nm, the comparison solution: 1 ml of water, 5 ml of conc. sulfuric acid and 1 ml phenol [13].

The quantitative content of triterpenesaponins in the grass (leaves and stem) of Melilotus officinalis L. was determined by direct spectrophotometry. The spectophotometric determination of triterpene saponins is based on a reaction with sulfuric acid, as a result of which triterpenoids are protonated by a double bond to form a carboxathione, and in the presence of a carboxyl group in S-28 there is subsequent lactonization. A characteristic absorption maximum is observed at 310 nm.

The optical density of the Melilotus officinalis L. extract was compared with the standard oleanolic acid solution under similar experimental conditions.

Ascorbic acid content was determined in freshly cut stems and leaves of Melilotus officinalis L. The method of extracting ascorbic acid from an aqueous extract with hydrochloric acid and titrating with a solution of 2, 6-dichlorophenolindophenolate sodium [12] was used until pink coloring occurred due to excess indicator in acidic medium.

Spectrophotometry was used to quantify coumarins in the grass (leaves and stems) of Melilotus officinalis L. The basis is a characteristic reaction of azo mixing in an alkaline medium. As a result, cherry staining appears.

To quantify the tannin, a method of depositing tannins of aqueous leaf extract and stems of Melilotus officinalis L. containing tannins with zinc sulfate was used, followed by complexonometric titration.

2.3. Animals and drug treatment

The study of the pharmacological activity of an aqueous extract of *Melilotus officinalis L.* was carried out on 46 male white outbred rats with an average weight of 220 g kept under standard vivarium conditions with free access to water and food. The animals were divided into two groups (table 1).

Table 1. Groups of animals.

№	Group	Solution intragastrically administered to animals	Number of animals
1	Control group	Saline	16
2	Melilotus officinalis L. extract	Melilotus officinalis L. extract	30

An aqueous extract of Melilotus officinalis L.was offered to animals as a freely available drink. The experiment was carried out in the spring (from April to May). The rats received an aqueous extract of Melilotus officinalis L. for 21 days.

To study the pharmacological action of the extract, a biochemical analysis of blood and urine, a hematological blood test, a study of the blood coagulation system and behavioral reactions in the Suok test were performed. Decapitation of animals was carried out after preliminary anesthesia with light diethyl ether. Decapitation was performed on the 22nd day of the experiment.

The animal care procedures were carried out in compliance with the guiding principles, determined by European Community's Council Directives. The procedures, applied during the research, fully meet the European legislation and the guidelines of the National Institute of Health on Laboratory Animal Use and Care, Order No. 199н "On Approval of Laboratory Practice Regulations" of the Ministry of Health of the Russian Federation dated April 1, 2016 and the Protocol No.8 of the Ethic Committee of the Federal State Budgetary Educational Institution of Higher Education "Astrakhan State Medical University" of the Ministry of Health of the Russian Federation dated November 24, 2015.

2.4. Suok-test

The Suok test, representing a "hybrid" of several traditional behavioural models at once, allows to record a wide range of behavioural reactions, from locomotion and research activity to vegetative markers of behaviour. As a completely new behavioural model, it is recommended for behavioural phenotyping of

various animal lines, for screening the psychotropic effects of drugs, for analyzing the physiological activity of the brain in neurophysiology. The light-dark Suok test (ST) was designed in accordance with the specifications published in the protocols [14, 15]. It consisted of a wooden rod 2.4 m long and 2 mm in diameter. It was illuminated by 4 60-Watt light bulbs hanging at a height of 40 cm (directional light) to illuminate the light part of the test and providing the only illumination in the experimental room. The rod was divided into two parts (dark and light zones) by several segments (15 cm long) on both sides from the central zone (30 cm long) and raised to a height of 20 cm from the floor with two vertical pillars. The experimental room was faintly illuminated during all tests. In all experiments, rats were placed individually in the central part of the test and their behaviour was observed for five minutes by an experienced observer, evaluating the frequency of anxiety and motor behaviour. The Suok test measures orientation, risk assessment behaviour and sensorimotor integration; it was designed to detect behavioural disorders resulting from pathways that mediate anxiety and vestibular function.

Registered: latency period (LP) of going off from the center; time spent in the dark and light compartments of the ST; duration of short-term grooming acts; number of falls down; number of hind paws slipping, number of segments visited; pointing; number and duration of stops; number of cases of peeping down; number of head movements directed to the sides when the body is extended in the black and white compartments. Each group of animals was tested in ST once 30 minutes after the last administration of the solutions. Observation time: 5 minutes.

2.5 Biochemical blood test

Biochemical blood analysis was carried out using a semiautomatic biochemical analyzer BS-3000R. The following parameters were determined: the level of glucose, urea, creatinine, total bilirubin, total protein, the level of AST (aspartate aminotransferase), ALT (alanine aminotransferase), amylase.

The level of urea in the blood serum was determined by a diagnostic kit for determining the concentration of urea in biological fluids by the deacetyl monooxime method (VITAL).

To determine the level of creatinine in the blood serum, we used diagnostic for the quantitative determination of the content of creatinine in serum, plasma and urine by the Jaffa method according to the end point with deproteinization (VITAL).

The total protein in blood serum was determined using a diagnostic kit for the quantitative content of total protein by the biuret method (VITAL).

The activity of alkaline phosphatase in the blood serum was determined by a diagnostic kit for the quantitative determination of the activity of alkaline phosphatase in the serum or blood plasma by the unified end-point method with p-nitrophenyl phosphate (VITAL).

The level of total bilirubin in the blood serum was determined using a diagnostic kit by the Jendrashik method (VITAL).

The activity of α-amylase was determined by a diagnostic kit for determining the activity of alpha-amylase in serum (plasma) of blood and urine by an optimized enzymatic kinetic method (VITAL).

The activity of alanine aminotransferase (ALT) was determined using a diagnostic kit for determining the activity of alanine aminotransferase (ALT) in serum or blood plasma by the unified dinitrophenylhydrazine method of Reitman-Frenkel (VITAL).

The activity of aspartate aminotransferase in blood serum was determined by a diagnostic kit for determining the activity of aspartate aminotransferase (AST) in serum or plasma using the unified Reitman-Frenkel method (VITAL).

The level of glucose in the blood serum was determined using a diagnostic kit for the quantitative determination of glucose in serum, plasma or urine by the glucose oxidase method without deproteinization (VITAL).

The level of cholesterol in blood serum was determined by a diagnostic kit using an enzymatic colorimetric method (VITAL).

2.6 Study of the blood coagulation system
The time from the moment of addition of thromboplastin with calcium to the plasma until the moment of fibrin clot formation was measured. The prothrombin ratio (PO) was calculated using the following formula PO = PVB / (PV100%), where PVB is the patient's plasma prothrombin time in seconds. PV100% - prothrombin time of 100% fresh pool of donor plasma in seconds.

2.7 Urine analysis
Urine analysis was performed using reagent test strips. Dirui A series are available for qualitative and semi-quantitative urine analysis. At the same time, the density, acidity (pH), protein, leukocytes, erythrocytes, and bilirubin were determined in the urine. Collected fresh urine of animals in a clean and dry container. The urine was not centrifuged. The sample was thoroughly mixed before testing. Urine testing was performed within an hour after collection. Immerse the reagent region of the strip in the urine sample for 2-3 seconds. The edge of the strip was passed along the edge of the vessel to remove excess urine. Holding the strip horizontally, compare the test result on the strip to the color chart on the label of the pencil case. The strip results were read visually.

2.8 Hematological blood test
The analysis was performed using an XN-1000 automatic hematology analyzer. The following indicators were determined: hemoglobin level, hematocrit, total red blood cells, total leukocytes, total platelets, lymphocytes, monocytes, granulocytes.

2.9. Statistics
All results are expressed as mean ± SEM. Data are analyzed by Student test for comparisons between experimental groups. A probability of less than 0.05 is considered statistically significant.

3. Results and discussion
Studies of grass extract (leaves and stems) *Melilotus officinalis L*., showed the presence of flavonoids, saponins, polysaccharides, tannins, coumarins, ascorbic acid (table 2).

Table 2. Content of biologically active substances in liquid herb extracts.

Biologically active substances to be determined	existence	Qualitative tests	Observed phenomena
flavonoids	+	Concentr. HCl, Znmetal	orange-red coloring
saponins	+	0.1 mol/l HCl, 0.1 mol/l NaOH	foaming reaction - stable foam is formed; precipitation reaction – precipitate formed
polysaccharides	+	Feling'sReagent	Bulk precipitate
tannins	+	1% gelatinsolution	deposit
coumarins	+	10% KOH, 10% HCl	Haze solution
Ascorbic acid	+	C 10%$KMnO_4$	discoloration of the added reagent

The quantitative content of tannin in the aqueous extract of the Melilotus officinalis L. was 1.23% (table 3).

Table 3. Quantitative content of biologically active substances in herb extract.

Tested samples	Content of biologically active substances, %					
	Ascorbic acid	tannin	saponin	coumarin	flavonoid	polysaccharide
Grass extract of Melilotus officinalis L.	0.31	1.23	0.35	2.57	1.7	0.9

An experimental study showed that the total content of flavonoids in the grass Melilotus officinalis L. was 1.7% in terms of luteolin-7-glycoside (table 3) (the maximum optical density of the solution after complexation was 0.739 at a wavelength of 400 nm).

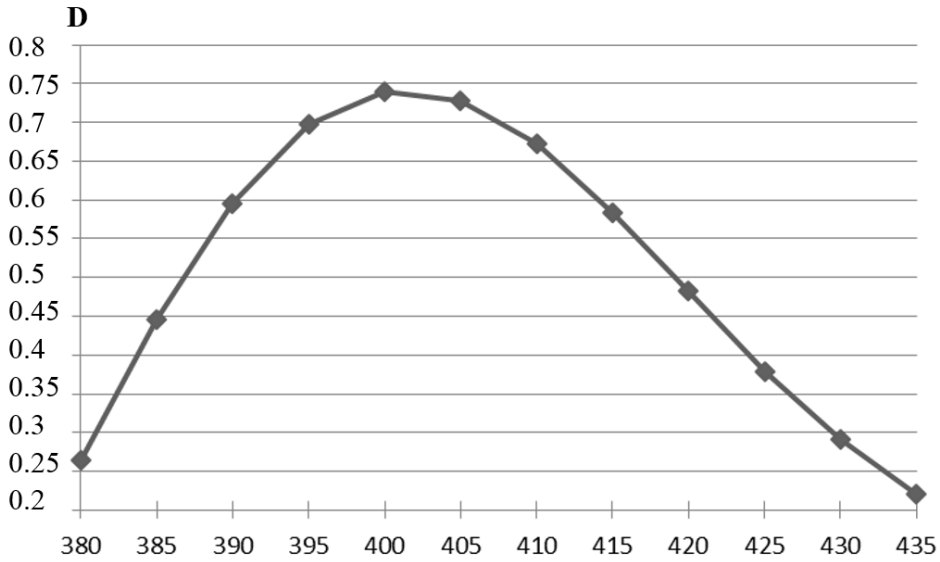

Figure 1. UF spectra of the aqueous alcohol solution of the terrestrial portion of Melilotus officinalis L. collected on the territory of the Astrakhan region.

The content of polysaccharides (table 3) in the grass (leaves and stem) of Melilotus officinalis L. was 0.9%.

The quantitative content of triterpene saponins in the extract of grass (leaves and stems) of Melilotus officinalis L. was 0.35%, based on oleanolic acid (the maximum optical density of the test solution of Melilotus officinalis L. was 0.850 at a --wavelength of 310 nm).

The study revealed the content of coumarins in the extract of grass (leaves and stems) Melilotus officinalis L. It amounted to 2.57%.

The amount of ascorbic acid in the leaves and stem of Melilotus officinalis L. was 0.31%.

In the course of testing animals in the Suok test, we obtained the following results. With chronic administration of Melilotus officinalis L., behavioral responses changed. Thus, in the light compartment of the Suok test, there was a significant decrease in the number of compartments crossed by animals, the number of peeking downward, while the number of stops and the time spent by rats on stops increased in comparison with control animals. The time spent in the light compartment and the number of directed head movements tended to decrease, with a simultaneous slight increase in paw slippage relative to the same indicators in the control group.

In the dark compartment of the Suok-test, only the time spent on stops changed significantly, while under the influence of Melilotus officinalis L., the indicator increased in comparison with the control

group. Thus, chronic administration of Melilotus officinalis extract led to a decrease in patterns of orientation-exploratory behavior, an increase in the level of anxiety.

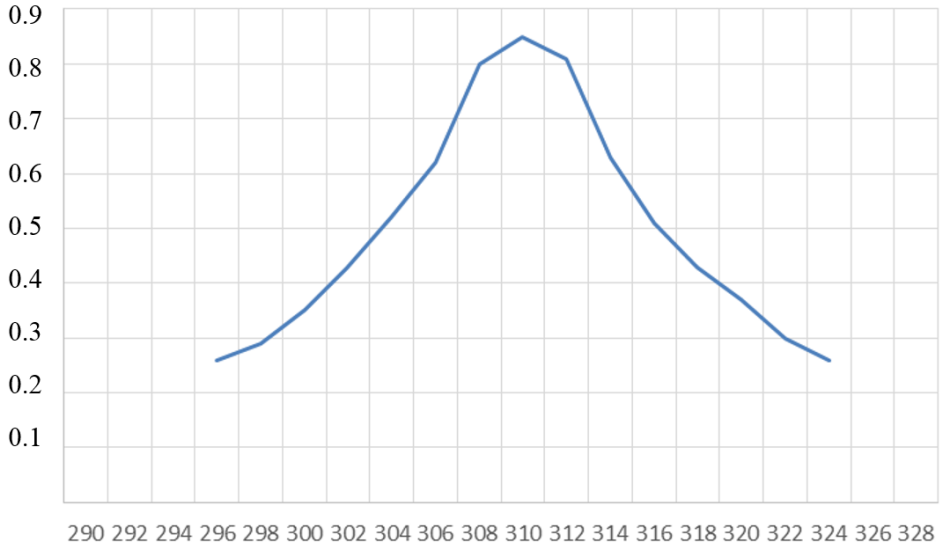

Figure. 2. Electron absorption spectra of alcohol solution of grass Melilotus officinalis L.

Biochemical parameters of blood with the introduction of Melilotus officinalis L. changed as follows (table 5). The levels of glucose and cholesterol in blood serum under the influence of sweet clover extract slightly increased relative to the values of the control group. The activity of such enzymes as AST and alkaline phosphatase under the influence of long-term administration of Melilotus officinalis L. increased significantly, while the activity of ALT significantly decreased in comparison with similar indicators in control animals.

Table 4. Study of behavior in the Suok-test at the administration of the extract.

Experimental groups	light part		dark part	
	Control	Melilotus extract	Control	Melilotus extract
Orientation (side-directed exploration)	7.9±0.64	6.9±0.62*[a]	6.1±1.20	6.2±1.34
Horizontal activity (number of sectors visited)	28.3±2.35	19.9±2.23*	30.2±4.10	33.3±3.53
Stopping activity (timer of stops)	28.3±4.52	96.0±8.46*	29.4±5.95	57.7±4.56*
Stopping activity (number of stops)	3.3±0.74	5.0±0.96 *	4.5±0.70	3.8±0.22
Head dips (number of exploratory looks down)	3.8±0.98	3.4±0.84	5.1±1.23	4.3±1.17
Time spent, sec	156.6±28.18	205.3±39.61	153.3±28.03	226.8±28.03
Number of missteps	1.0±0.41	2.5±0.67	1.1±0.48	1.8±0.42

[a].*P < 0.05 vs. experimental groups.

ALT and AST are enzymes that show the state of liver function, therefore, their change under the influence of sweet clover may indicate a violation of the liver. Alkaline phosphatase is an enzyme that also wards off liver disease. In our study, the activity of the enzyme increased upon chronic administration of Melilotus officinalis L.

Table 5. Biochemical parameters of the blood of male rats treated with the extract.

exposure	Level of glucose mmol / l	Level of cholesterol mmol / l	Level of total protein mg / l	Activity of ALT nmol / l	Activity of AST nmol / l	Level of total bilirubin mmol/ l	Level of creatinine mmol/ l	Level of urea mmo / l	Activity of α-amylase mg / s l	Activity of alkaline phosphatase nmol / l
Control	5.3± 0.24	1.9± 0.20	56.6± 2.39	62.2± 3.53 *	118.5± 38.44 *	7.7± 1.72	34.7± 1.98	8.1± 0.63	633.0± 36.65 *	731.8± 26.45 *
Melilotus extract	4.5± 0.38	1.4± 0.22	50.9± 2.49	74.2± 2.92	8.4± 2.48	7.6± 2.13	33.5± 2.08	9.2± 0.76	200.0± 21.42	579.5± 18.50

[a.] *P < 0.05 vs. experimental groups.

Indicators such as the level of total bilirubin, creatinine and urea practically did not change, while the activity of α-amylase significantly increased in comparison with the control group. This enzyme reflects the work of the pancreas, and, therefore, its change under the action of the Melilotus officinalis L. may indicate a violation of the organ.

The blood coagulation system during chronic administration of the sweet clover extract was changed as follows (table 6). In animals with chronic administration of Melilotus officinalis extract, there was a significant increase in MNO and prothrombin time, with a slight decrease in the prothrombin index relative to similar indicators in the control.

Table 6. Changes in the anticoagulation system of rat blood after administration of the extract.

	MNO.	Prothrombin time, sec	Prothrombin index, %
Control	2.2±0.30 *	23.5±1.66 *	62.9±8.30
Melilotus extract	1.4±0.09	17.2±0.90	73.4±3.57

Strengthening of blood clotting under the influence of Melilotus officinalis L. extract may be due to abnormal liver function or the blood-thinning effect of biologically active substances of the extract itself.

Under the influence of the Melilotus officinalis L. extract, the urine indices practically did not differ from those in the control animals (table 7).

Table 7. Changes in urine indicators with the introduction of sweet clover extract.

exposure	þ	Ph	leukocytes leu/mkl	Level of protein g/l	Level of bilirubin mkmol/l
Control	1.019	6.83	отр	0.033	1
Melilotus extract	1.015	6.33	отр	0.033	2

Hematological blood parameters under the influence of Melilotus officinalis L. extract changed as follows (table 8). Chronic administration of Melilotus officinalis L. extract led to a significant decrease in hemoglobin level and monocyte content relative to the control values. The rest of the indicators differed from the control group less significantly.

Table 8. Hematological parameters of the blood of male rats treated with the extract.

exposure	Level of hemoglobing/l	quantity of erythrocytes x 10^{12}/l	quantity of leukocytes, 10^4/l	Level of hematocrit%	quantity of platelets x10^9/l	lymphocytes, %	monocytes, %	granulocytes %
Control	113.5± 4.60 *[a]	5.5± 0.26	8.2± 0.24	31.95± 3.89	390.8± 19.37	51.9± 2.60	11.6± 1.30 *	36.5± 3.66
Melilotus extract	130.8± 4.71	5.8± 0.75	13.0± 2.05	30.6± 0.76	476.2± 39.20	55.8± 6.39	18.3± 2.60	25.8± 4.41

[a.] *P < 0.05 vs. experimental groups.

Thus, the work studied the chemical composition of the sweet clover extract.

Chronic administration of the extract to laboratory animals for 21 days revealed its toxic effect, which manifested itself in impaired liver function, changes in the blood coagulation system, and a decrease in patterns of orientation-exploratory behavior. There was also a change in the enzymatic activity of the pancreas. In connection with the identified changes, it is necessary to conduct a more detailed analysis of liver and pancreatic dysfunctions in chronic use of sweet clover.

Studies have shown that the aboveground part (stems and leaves) of Melilotus officinalis L. growing in the soil and climatic conditions of the Astrakhan region is characterized by a high content of biologically active substances, which can be traced when comparing the results of studies given in the literature.

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