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DETERMINATION OF TOXICITY INDICATORS AND ASSESSMENT OF THE SENSIBILIZING ACTION OF THE PREPARATION FOR THE EXTERNAL USE 'OINTMENT FOR WOUNDS'

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Summary. The purpose of the work is a determination of toxicity and sensitizing effects of a new external preparation 'Ointment for wounds', based on the essential oils of Siberian pine, eucalypt, tea-tree, cedar, clove, and oil solution of chlorophyllite. The experimental study was performed on rats weighing 160–190 g, 2–3 months of age and mice weighing 18–21 g, 3 months of age. At the stages of preclinical study, it was determined acute and subacute effects of the drug, when administrated on the skin and directly into the stomach of experimental animals. The skin-irritant effect of the phytopreparation was investigated when applied to the skin and buccal mucous membrane. The sensitizing effect of the ointment was evaluated by reproducing local reactions. The acute toxicity assessment was performed on the survival rate of laboratory animals after oral administration of the preparation at doses from 5,000 to 25,000 mg/kg of body weight, with 5,000 units increments. Subacute toxicity in the experiment was evaluated by the dynamics of morphological and biochemical parameters of blood and the study of the coefficients of laboratory animals' internal organs mass. It was found that the introduction of the drug 'Ointment for wounds' in the stomach did not cause significant changes in the behavior of rats, all animals remained alive. Studies have shown the absence of skin-irritant effect of the preparation, as well as the absence of irritant effect on the buccal mucous membrane. Even in a long-term experiment to study subacute toxicity when applied to the skin, no toxic effects of the preparation on the basis of essential oils and oil solution chlorophyllite of were found. The index sensitizing effect of the preparation was less than one, indicating the absence of sensitizing effect. According to the classification of substances by toxicity and danger (requirements of SOU 85.2-37-736:2011 and GOST 12.1.007-76) belong to hazard class IV. In general, 'Ointment for wounds' does not have a toxic effect on the functions of vital organs, and at repeated administration is almost harmless.

Keywords: essential oil, chlorophyllite, toxicity, rats, mice, skin-irritant effect, sensitizing action, morphological composition of blood, transaminase activity

Introduction. The market of veterinary dermatological medicines in Ukraine is presented mainly by expensive foreign drugs. It became necessary to expand the range of drugs with the latest developments (Bushuieva, 2013; Gerecke, 2005).

Popular multicomponent drugs — derivatives of antibiotics and corticosteroids, do not always act adequately at all levels of the pathological process. Considerable attention of domestic and foreign researchers is focused on the search for highly effective and environmentally safe therapeutic and preventive agents used in skin diseases in animals (Horiuk, 2018; Shaheen, Tantary and Nabi, 2016).

Recent trend is the use of biologically active components of plant origin as an alternative to antibiotic therapy (Tamminen, Emanuelson and Blanco-Penedo, 2018; Vorobets et al., 2018). Successfully selected combinations of essential oils are often no less effective than synthetic biocides, and the risk of the emergence of resistant strains of microorganisms is reduced (Sobrinho Santos et al., 2019; Queiroga et al., 2018).

The cost of veterinary drugs and medical measures with the use of raw materials of natural origin is more attractive. In dermatology, the combination of antimicrobial, anti-inflammatory, immunomodulatory and reparative properties is the main criterion for the selection of biologically active substances of plant origin (Wolski et al., 2017).

Among the herbal preparations, the essential oils of Siberian pine (Carrión-Prieto et al., 2018; Shpatov et al., 2017), eucalyptus (Adnan, 2019; Harkat-Madouri et al., 2015), clove (Packyanathan and Prakasam, 2017), cedar (Bennouna et al., 2018), tea-tree (Li et al., 2016; Smith et al., 2014) and oil solution of chlorophyllite have the above mentioned pharmacological characteristics. The possibility to combine the diverse properties of plants in a single therapeutic agent based on the listed above essential oils and oil solution of chlorophyllite became the basis for the creation of an experimental drug in the form of ointment.

A prerequisite for the registration of new medicines is a preclinical testing in laboratory animals to determine the

nature and severity of the possible harmful effects of the drug on the body, and in particular to determine the toxicity and sensitizing effects (Kosenko et al., 1997; Kotsiumbas et al., 2006).

The purpose of the work is to determine toxicity indicators and to evaluate the sensitizing effect of a new drug for external use 'Ointment for wounds'.

Materials and methods. For experimental evaluation of the toxic-hygienic parameters of the medicinal product 'Ointment for wounds' sexually mature animals were used: rats weighing 160–190 g, aged 2–3 months, and mice, weighing 18–21 g at the age of 3 months, which were maintained in the vivarium of the Research Epizootology Station of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine (Rivne). The animals were kept on a standard diet according to the requirements of sanitary and hygienic standards, and received food and water *ad libitum*. All experiments were conducted in accordance with the guidelines 'Preclinical Studies of Veterinary Medicines' (Kotsiumbas et al., 2006) and 'Methodological Guidelines for Toxicological Evaluation of Chemicals and Pharmacological Preparations Used in Veterinary Medicine' (Vysotskiy et al., 2007). The animals were housed in a test room, in standard cages, under natural day-night light mode, at temperature 20–25°C, humidity not more than 55%. The basis of the diet was pelleted feed that has been manufactured according to the 'Scientific and Practical Recommendations for Laboratory Animals Keeping and Work with Them' (Kozhemiakin et al., 2002). All manipulations with experimental animals were conducted in accordance with the rules of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 86/609/EEC (CEC, 1986). The study was conducted in four stages.

In the first stage, study of ointment acute toxicity was performed on 40 white outbred rats of both sexes, weighing 160–190 g. The drug was administered to the animals once, using an oral probe, at doses from 5,000 to 25,000 mg/kg, with an interval of 5,000 units (by the absolute weight of the preparation). Each dose was examined in 8 animals. The control group (5 animals) was also injected intragastrically with water. Rats did not receive food for the night preceding the test and for 3 hours after administration of the drug. Observations on the animals were carried out for the next 14 days.

In the second stage, subacute toxicity was studied. The study was performed on 20 white rats (2–2.5 months of age, both sexes equally). The drug 'Ointment for wounds' was administered to animals daily intragastrically on an empty stomach, in the form of an aqueous emulsion at a dose of 0.4 ml of the drug per animal. The control group of rats received water intragastrically. The duration of the experiment was 21 days. Then, animals under brief ether

anesthesia were euthanized with instantaneous one-stage decapitation, organs (liver, kidney, heart, lungs, spleen, pancreas) were removed, and weighed on torsional scales and body mass coefficients were calculated (Kosenko et al., 1997).

In addition, blood samples were taken to assess subacute toxicity of the drug. The blood was tested by conventional methods for the concentration of hemoglobin, the number of red and white blood cells, the hematocrit volume. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), as well as the content of total protein, urea and glucose in the serum were determined using the biochemical analyzer 'Cobas c 311' (Roche Diagnostics, Switzerland).

The third stage of the study involved the study of skin irritation: it was determined on 12 white rats by rubbing of the test sample of the ointment, emulsified with water in a ratio of 1:10, in the shaved areas for 30 days. Changes in the functional state of the skin of experimental animals were determined by the degree of inflammatory reaction. Assessment of the skin was performed on the 10th, 20th, and 30th days of the study.

In the fourth stage, sensitizing effect of the ointment according to the local reactions was examined. The study was conducted in 6 white rats, aged 2.5 months. Ointment, emulsified with sterile water in the ratio of 1:10, was introduced once, at a dose of 0.2 ml, in the area of the transitional fold of the oral cavity, on the submucosal layer of the mucous membrane.

The results were evaluated in points given in Table 1.

Table 1 — Evaluation of the sensitizing effect of the preparation in points

0 points	No inflammation (no sensitizing effect) (1s = 0)
1 point	Slightly noticeable inflammation (mild sensitizing action) (1s = 1)
2 points	Well-marked inflammation (moderate sensitizing action) (1s = 2)
3 and 4 points	Brightly marked inflammation (expressed sensitizing action) (1s = 3)

Statistical processing of the results was performed by variation statistics using Statistica 6.0 (StatSoft, USA). Nonparametric research methods were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (\bar{x}), standard error of the mean (SE) was determined. The difference between the two averages was considered statistically significant when: * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$.

Results and discussion. According to the results of the first stage it was found that after intragastric administration of the drug in doses of 5,000, 10,000,

15,000, 20,000, and 25,000 mg/kg the signs of intoxication in rats were not observed: animals were clean, active, had a satisfactory appetite, responded to sound and light stimuli. The processes of urination and bowel movements were normal. Respiratory disorders and convulsions were not noted. The condition of hair, skin, mucous membranes remained physiologically normal throughout the observation period. Reflex excitability in all animals was maintained. During the experiment, no lethal outcomes were reported in any group (Table 2).

Table 2 — Results of the study of acute toxicity of 'Ointment for wounds', when administered intragastrically

Group No.	Sex	Dose, mg/kg	Died rats/surviving rats
1	males	5,000	0/4
	females		0/4
2	males	10,000	0/4
	females		0/4
3	males	15,000	0/4
	females		0/4
4	males	20,000	0/4
	females		0/4
5	males	25,000	0/4
	females		0/4

In the second stage, the composition of the peripheral blood, biochemical parameters were investigated and the coefficients of the mass of the internal organs were determined under the conditions of the subacute action of the preparation for external application 'Ointment for wounds'. Studies of rat blood found that in rodents of the experimental group, hemoglobin and red blood cells significantly exceeded control group, by 13.9 and 7.6%, respectively, whereas hematocrit and white blood cells decreased relative to controls ($p < 0.001$) by 4.6 and 10.4%, respectively (Table 3).

Table 3 — Effect of administration of 'Ointment for wounds' on blood hematological parameters of rats ($M \pm m$, $n = 10$)

Parameters	Group of animals		Reference value
	Control	Experimental	
Hemoglobin, g/l	129.2 \pm 1.49	147.2 \pm 1.12***	120.00–150.00
Red blood cells, T/l	6.03 \pm 0.05	6.49 \pm 0.1**	6.00–7.80
Hematocrit, %	37.2 \pm 0.11	35.5 \pm 0.17***	35.00–47.00
White blood cells, g/l	6.82 \pm 0.03	6.11 \pm 0.12***	5.9–13.2

Notes: ** — $p < 0.01$, *** — $p < 0.001$.

It should be noted that fluctuations in peripheral blood composition were within the reference levels (Trakhtenberg et al., 1991; Abrashova et al., 2013), and the obtained data indicate a possible stimulatory effect of the drug on the organs of hematopoiesis, and the absence of toxic effect when the drug was administered intragastrically.

In the study of biochemical parameters of white rats' blood, a significant excess of all investigated parameters relative to the control group was established: total protein — by 16.5%, ALP — by 10.1%, ALT — by 12.2%, and AST — by 12.3% (Table 4), however, these indicators were also within the reference levels (Trakhtenberg et al., 1991; Abrashova et al., 2013), which on the one hand indicates the absence of toxic actions for the introduction of the drug 'Ointment for wounds', and on the other — it stimulates the hepatobiliary system.

Table 4 — Biochemical parameters of white rats' blood on the 21st day of the experiment ($M \pm m$, $n = 10$)

Parameters	Group of animals		Reference value
	Control	Experimental	
Total protein, g/l	51.6 \pm 0.29	60.1 \pm 0.15***	50.0–70.0
ALP, U/l	231.0 \pm 3.0	254.4 \pm 4.2***	220.0–330.00
ALT, U/l	52.6 \pm 0.7	59.0 \pm 0.51***	50.0–70.0
AST, U/l	119.0 \pm 0.62	133.6 \pm 2.07***	100.0–140.0

Note: *** — $p < 0.001$.

After calculating the coefficients of the mass of the internal organs, it was found that the coefficient of lung mass was higher than the control by 9.3% ($p < 0.05$), and the kidney — lower by 10.1%, whereas the values of the coefficients of weight of liver, heart and spleen did not differ from those in the animals of the control group (Table 5), which confirms the absence of toxic action of the drug 'Ointment for wounds', and indicates the development of adaptogenic processes in the rats of the experimental group.

Table 5 — Mass ratios of white nonlinear rats' internal organs on day 21st of the study of subacute toxicity of the preparation 'Ointment for wounds' ($M \pm m$, $n = 10$)

Internal organs	Group of animals	
	Control	Experimental
Lungs	1.08 \pm 0.03	1.18 \pm 0.02*
Liver	5.30 \pm 0.19	5.06 \pm 0.1
Kidneys	0.99 \pm 0.01	0.89 \pm 0.01**
Heart	0.6 \pm 0.01	0.56 \pm 0.01
Spleen	0.45 \pm 0.01	0.48 \pm 0.02

Notes: * — $p < 0.05$, ** — $p < 0.01$ relative to control.

According to the results of the third stage of the research, it was found that the index of skin-irritant action of the medicinal product 'Ointment for wounds' was 0 points, i. e. no skin-irritant action was established.

The results of the study of the sensitizing effect of the preparation 'Ointment for wounds' (the fourth stage of research) are shown in Table 6.

Table 6 — Evaluation of the sensitizing effect of 'Ointment for wounds' ($M \pm m$, $n = 3$)

Animal group	Sensitizing effect index
Control	0.33 ± 0.04
Experimental	$0.67 \pm 0.05^*$

Note: * — $p < 0.05$.

In the experiment, the index of sensitizing action of the medicinal product 'Ointment for wounds' was less than one, although it probably two times exceeded the control, which on the one hand indicates the absence of sensitizing effect of this drug, and on the other — stimulating effect on the immune system of rats.

Thus, according to the results of toxicological studies, analysis of possible changes in the structure and functions of vital organs, the study of experimental animals' blood parameters, we can conclude that there is no toxic effect, locally irritating and sensitizing effect of the drug «Ointment for wound», both after oral administration and after dermal application, i. e. it is harmless.

Conclusions. 1. The results of acute toxicity studies indicate that the LD_{50} for the drug 'Ointment for wounds', when administered intragastrically, is greater than 25,000 mg/kg (the absolute mass of the drug). Intragastric administration of the maximum dose of the preparation did not have any negative effects.

2. Under the conditions of subacute action study of the drug 'Ointment for wounds' after external application in the experimental group of rats, significant changes in hematological, biochemical parameters of blood were found, but they were within the reference levels and weight coefficients of internal organs, indicating the absence of toxic effects of the drug and the development of adaptogenic processes in rats of the experimental group.

3. The index of skin-irritant effect of the medicinal product 'Ointment for wounds' was 0 points, and the index of sensitizing effect of the drug was less than one for the experimental group (although it probably two times exceeded the control), indicating the absence of skin-irritant and sensitizing effect, and the stimulating effect on the immune system of rats.

4. According to the classification of substances by toxicity and danger (requirements of SOU 85.2-37-736:2011 and GOST 12.1.007-76) the preparation 'Ointment for wounds' refers to the IV class of toxicity — low toxic substances and the IV class of danger — low-hazard substances.

5. The obtained results indicate the feasibility of further clinical studies on target animals due to safety of the preparation.

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