Study of environmental toxicity of the preparation based on humic acids

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Abstract. Preparations based on humic acids are widely used in veterinary practice, as they are based on raw materials of natural origin. The environmental toxicity of the drug based on humic substances “Gumi drinking” was assessed by biotesting using test objects of different taxonomic groups. As a result of the research in the acute and subacute experiment on Tetrahymena pyriformis W. LD$_{50}$=575,1±1,01 mg/ml, in a chronic experiment the maximum inactive dose was 10$^{-1}$ mg/ml. When assessing the impact of “Gumi drinking” on the germination of seeds of crops such as radishes, oats, cucumbers, the effects of inhibition of development were noted: $E_T$ (radish) = 1,35%, $E_T$ (cucumber) = 0,13%, $E_T$ (oat) = 0,66%. Studies using the test object Eisenia foetida for 14 days found that the survival rate was 100%, reducing the columellar weight did not occur. In the study of embryotoxicity on the eggs of Lymnaea stagnalis L. a dose dependent effect of inhibition of shellfish hatching under the action of solutions in different concentrations was revealed. "Gumi drinking” in single-day guppies at exposure of 96 hours at a concentration of 500 mg/d on the 4th day of the experiment caused the death of 3.4% of fry. The effect of the drug on earthworms Nicodrilus caliginosus and coprophages did not have a negative impact. Thus, "Gumi drinking” can be attributed to non-toxic substances according to the degree of danger to the environment.

1. Introduction

To date, the market of veterinary medicines and feed additives is mainly represented by synthetic means, which as a result of application can provoke the development of undesirable effects, such as antibiotic resistance, immunosuppression, etc. Therefore, the search for new means based on raw materials of natural origin is currently relevant. Humic substances are unique natural compounds in the chain of organic residues. Humic acids and their various compounds began to be used in agronomic practice in 1920 as substances necessary to produce artificial soil structures to increase crop yields, and in 1966 were first tested as stimulants of animal productivity [1, 2]. Recently, preparations from humic substances are widely used in animal husbandry and veterinary medicine as feed additives and medicines, as they exhibit high biological activity, are environmentally friendly, contribute to improving the productivity of animals and poultry and improve product quality [3].

Humates are an affordable raw material that can be used in agriculture and animal husbandry in the form of a humate beverage or dry feed as a source of mineral and organic substances to stimulate growth [4]. Humates are humic acid salts in which the cation / anion exchange units of humic acid are Ca$^{2+}$, Na$^+$, Al$^{3+}$, and Fe$^{3+}$. The composition of humates includes humus, humic acid, fulvic acid, ulminic acid and trace elements [1, 5].
In the external environment annually gets several tons of drugs in the form of metabolites, and the drugs themselves. Excreted from the body of animals, drugs enter the environment with feces or urine and can have a negative impact on the entomofauna of manure, thereby disturbing the ecological balance in nature. Therefore, the study of the impact of drugs and their metabolites on the environment is a prerequisite for assessing the safety of drugs and feed additives.

2. Materials and methods

The preparation of humic substances "Gumi drinking" produced by Scientific-innovation enterprise BashInkom, Ltd is an aqueous solution of humic acid salts obtained by alkaline extraction from brown coal enriched with calcium, phosphorus and trace elements in chelated form. The study and evaluation of the Ecotoxicity of the drug "Gumi drinking" was carried out by biotesting using test objects of different taxonomic groups. As test objects used: cucumber seeds, radish seeds, oat seeds, soil oligochaetes Eisenia foetida, populations of infusoria Tetrahymena pyriformis W., clams – pond great Lymnaea stagnalis L., one-day fry guppies Poecilia Reticulata Peters.

Studies on Tetrahymena pyriformis W. were carried out in acute, subacute and chronic experiments. In acute and subacute experiments, "Gumi drinking" was studied on a population in a stationary growth phase. The samples were incubated at 25°C for 3 hours (acute experiment), for 24 hours (subacute experiment). Toxicity was assessed by mortality parameters. The samples were incubated at 25°C for 96 hours. According to the results of the chronic experiment, the indicators characterizing the regularities of population growth (growth rate, generation time, number of generations, population size), as well as the adaptogenicity coefficient by population size, acid resistance, allowing to judge the membranotoxic effect, and mutagenic activity were determined.

The adaptogenicity coefficient reflects adaptive changes in the organism and is determined by the quantitative assessment of population size fluctuations during the first (24-96 hours), seventh (312-384 hours) life cycles and in the logarithmic growth phase (48-336 hours). Adaptogenicity coefficient was calculated as the ratio of the number of organisms in the control. Acid resistance was defined as the amount of 0.02N sulfuric acid solution required to immobilize 1 ml of infusion suspension and expressed as a percentage relative to control.

The reaction to mutagenicity was determined by the presence of surviving organisms after incubation in allyl alcohol, with the addition of which the original strain Tetrahymena pyriformis W. dies in 12 hours.

In the study of phytotoxicity, a native extract was obtained from the drug at a ratio of sample weight and distilled water volume of 1 g: 10 ml, exposure for 3 days at room temperature of 20±5°C. Seeds were sown 25 pieces in Petri dishes, adding native extract in the volume of 15 ml. Petri dishes were incubated for 7 days at a temperature of +24°C, relative humidity 50-70%, in the absence of light. The length of the roots of the seedlings was measured along the root of the maximum length. The average value of root length (L) was determined from three repetitions on each seed culture in the experiment and control. Compared them with each other and determined the braking coefficient by the formula:

\[ E_T = \frac{(L_C - L_{EXP})}{L_C} \times 100\% \]  

where:
- \( E_T \) – growth inhibition effect expressed as a percentage;
- \( L_C \) – root length in control;
- \( L_{EXP} \) – length of roots in experiment.

When assessing it is believed that the substance has a phytotoxic effect at \( E_T \geqslant 20\% \) in at least one seed culture.

The study of toxicity in the test model of Eisenia foetida was carried out during an acute experiment. Test object-genetically homogeneous laboratory population of earthworm species Eisenia foetida. 7 individuals of Eisenia foetida weighing 200-400 mg per 600 g of model medium containing samples of the drug "Gumi drinking" in concentrations: 100 mg/kg, 200 mg/kg, 500 mg/kg were used. Experimental containers were incubated at room temperature for 14 days. Toxicity criteria were: death of animals, statistically significant decrease in columellar weight gain (mg/individual) in the
experimental group compared with the control. Changes in behavioral responses of animals compared to the control group and visible morphological changes of the body were also taken into account.

In the study of embryotoxic action, synchronized egg laying of Lymnaea stagnalis L. in the gastrula stage was used. Used a negative control of tap water.

Each ovipositor was divided into five approximately equal parts, which were randomly disbanded into one control and four experimental groups, and placed in an experimental dish. The initial number of germ capsules in each Petri dish was counted and 10 ml of the test solutions were poured. 17-day incubation of Petri dishes was carried out at room temperature, natural photoperiod, until complete hatching. At the end of the experiment counted the number of dead embryos and hatchlings. The indicator of successful hatching, expressed as the ratio of the number of hatched individuals to the initial number of germ capsules, was calculated. As a result of the experiment in the test model Lymnaea stagnalis L. evaluated the effect of inhibition of hatching, which was calculated on the basis of an average of three repetitions of the indicator of successful hatching for each concentration relative to the control formula:

$$Inhibition \ of \ hatching = \left( \frac{K - O}{K} \right) \times 100\%$$

where

- **K** - % of successful hatching in the control;
- **O** - % of successful hatching in the experiment.

When calculating the average indicator of successful hatching from three repetitions, the value of the coefficient of variation – the percentage of the standard deviation from the arithmetic mean – which should not exceed 30 was taken into account.

Inhibition of hatching is an embryotoxic effect characterizing the degree of reduction of hatching of juveniles in the experiment with respect to control. The risk of embryotoxicity on the eggs of Lymnaea stagnalis L. was assessed by the following indicators: average effective concentration (EC\(_{50}\)); threshold concentration (EC\(_{15}\)); EC\(_{50}/EC_{15}\) – an indicator characterizing the zone of acute action.

In addition, biotesting of the drug was carried out using single day fry guppies Poecilia Reticulata Peters. The study was conducted in an aquarium environment, using ambient light and natural light period. The duration of the biotesting was 4 days (96 hours). During biotesting, the fish were not fed. The temperature of the analyzed sample is 20-22°C, the concentration of dissolved oxygen is 8.6 dm/dm\(^3\). The ratio of water and ichthyomass was 1.5 g per liter, for each concentration of 10 specimens of fish in experiments and control.

Physiological activity of fish tested at a standard substance, potassium bichromate, LC\(_{50}\) K\(_2\)Cr\(_2\)O\(_7\) for 24 hours was 127 mg/dm\(^3\) (which fell within the range of required concentrations of 106-175 mg/dm\(^3\)). The experiments were carried out in 3-fold repetition.

3. Research result

In acute and subacute experiments on Tetrahymena pyriformis W. at a drug concentration of 100-700 mg/ml, changes in body shape and the nature of the movement of the infusoria were observed. LD\(_{50}\) values of the drug samples amounted to 575,1±1,01 mg/ml, which corresponds to low-hazard compounds (class 4).

In a chronic experiment on Tetrahymena pyriformis W., the drug had a depressing effect on the growth and vital functions of the population throughout the life cycle, resulting in a decrease in population size relative to control. According to the results of the chronic experiment on infusoria, the maximum inactive dose was 10\(^1\) mg/ml, which indicates low toxicity for infusoria.

The biological effect on the population of the test object expressed in a slight increase in the adaptive capacity of the population compared to the control in samples containing the drug in concentrations of 10\(^2\) mg/ml and 10\(^1\) mg/ml. In samples containing samples of the drug at a concentration of 100 mg/ml, there was a decrease in adaptive capacity of population.

Samples of the drug present in the culture medium Tetrahymena pyriformis W., did not show mutagenic activity, while reducing the resistance of cell membranes of infusoria to adverse environmental effects compared with the control by 20% (table 1).
Table 1. Biological effect on the population Tetrahymena pyriformis W.

| Concentration, mg/ml | Coefficient of adaptogenicity, М±m | Acid resistance, % | Reaction of mutagenicity |
|----------------------|-----------------------------------|--------------------|-------------------------|
| Control              | 1,00±0,04                         | 100                | Negative                |
| 10^-5                | 1,10±0,01                         | 90                 | Negative                |
| 10^-4                | 1,08±0,02                         | 90                 | Negative                |
| 10^-3                | 1,05±0,01                         | 90                 | Negative                |
| 10^-2                | 1,01±0,01                         | 90                 | Negative                |
| 10^-1                | 1,03±0,01                         | 90                 | Negative                |
| 10                   | 1,02±0,01                         | 90                 | Negative                |
| 100                  | 1,02±0,01                         | 90                 | Negative                |

* - reliability of differences with control at p≤0,05

The results of studies of the drug "Gumi drinking" on phytotoxicity are presented in table 2.

Table 2. The results of the study of phytotoxicity.

| Sample          | Test culture (seeds) | Average root length of seedlings, Lср. (mm) | Test reaction | Effect of growth inhibition, Ет (%) |
|-----------------|----------------------|---------------------------------------------|---------------|-------------------------------------|
| Control         | Radish               | 5,19                                        | norm          | -                                   |
|                 | Cucumber             | 7,87                                        | norm          | -                                   |
|                 | Oat                  | 7,56                                        | norm          | -                                   |
| Gumi drinking   | Radish               | 5,12                                        | norm          | 1,35                                |
|                 | Cucumber             | 7,86                                        | norm          | 0,13                                |
|                 | Oat                  | 7,51                                        | norm          | 0,66                                |

Evaluation of the effect of "Gumi drinking" on seed germination showed no toxicity. When acting on the seeds, the effects of inhibition of the development of the roots of radish, cucumber and oat seedlings, which do not reach the phytotoxicity threshold of 20%, are noted. Thus, "Gumi drinking" can be attributed to non-hazardous substances according to the degree of danger to the environment.

The results of the toxicity study "Gumi drinking" on Eisenia foetida showed no death of animals for 14 days under the action of the test drug in concentrations: 100 mg/kg, 200 mg/kg, and 500 mg/kg. Survival in the control was 100%. Throughout the experiment, under the action of the maximum saturated concentration (500 mg/kg), there were changes in behavioral reactions, which manifested themselves in a slight decrease in mobility. Reducing the increase in columella weight (table 3) and visible morphological changes of the animal organism were not observed.

Table 3. Dynamics of columella weight Eisenia foetida at 14 days exposure.

| № repetitions | Columella weight change |
|---------------|-------------------------|
|               | Control                 | Gumi drinking          |
| 1             | 14                      | 12                      |
| 2             | 19                      | 17                      |
| 3             | 14                      | 15                      |
| 4             | 20                      | 18                      |

According to the results of toxicity assessment on Eisenia foetida of "Gumi drinking" can be classified as non-hazardous substances in terms of environmental hazard.

As a result of embryotoxicity studies on egg-laying Lymnaea stagnalis L., a dose-dependent effect of inhibition of shellfish hatching under the action of solutions of 500 mg/ml, 300 mg/ml, and 100 mg/ml was revealed (table 4).
On the basis of the obtained data, the parameters of embryotoxicity were established, the analysis of which allows to refer "Gumi drinking" to the 4th class of danger (low-risk).

**Table 4. Results of embryotoxic action on the ovipositor of Lymnaea stagnalis L. (analysis of averaged data from three repetitions).**

| Concentration, mg/ml | Successful hatching, % | Inhibition of hatching, % |
|----------------------|------------------------|--------------------------|
| Control              | 85.55                  | -                        |
| 100                  | 83.41                  | 2.5                      |
| 300                  | 77.09                  | 9.9                      |
| 500                  | 68.41                  | 20.0                     |

The results of the study of toxicity of the drug "Gumi drinking" on one-day guppies in acute experience with a duration of 96 hours are presented in table 5.

**Table 5. Dynamics of survival of one-day guppies in acute experience, 96 hours, n=30.**

| Concentration, mg/l | 1 Experience day | 2 Experience day | 3 Experience day | 4 Experience day |
|---------------------|------------------|------------------|------------------|------------------|
| Control             | 30               | 30               | 30               | 30               |
| Gumi drinking, 100 mg/l | 30       | 30               | 30               | 30               |
| Gumi drinking, 300 mg/l | 30       | 30               | 30               | 30               |
| Gumi drinking, 500 mg/l | 30       | 30               | 30               | 29               |

| % from the control |
|-------------------|
| Control           | 100              | 100              | 100              | 100              |
| Gumi drinking, 100 mg/l | 100       | 100              | 100              | 100              |
| Gumi drinking, 300 mg/l | 100       | 100              | 100              | 100              |
| Gumi drinking, 500 mg/l | 100       | 100              | 100              | 96.6             |

These tables show that the drug "Gumi drinking" in concentrations of 100, 300 mg/l does not cause the death of one-day fry throughout the exposure of the experiment. At a concentration of 500 mg/l on the 4th day of the experiment, the death of fry 3.4% was noted. Thus, according to the degree of acute toxicity of "Gumi drinking" refers to low-toxic compounds for aquatic organisms.

The effect of the drug "Gumi drinking" on earthworms Nicodrilus caliginosus was manifested as follows. In earthworms, there was no decrease in mobility and reactivity. There was no death of worms. There are no signs of toxicosis.

The effect of the drug "Gumi drinking" on coprophages has been studied on dung beetles. The results suggest that "Gumi drinking" does not have a negative impact on the number of coprophagous imago beetles. Thus, the number of imago beetles in the feces of the control group on the 3rd day of sampling was 35.4±1.2 examples of beetles, and in samples from animals treated with the drug "Gumi drinking", 35.2±1.4 examples of beetles. In the following days of fecal sampling, the number of imago beetles decreased in all groups, including the control group, and on the 28th day no adult beetles were found. A similar pattern was observed in the feces, the bookmark which was carried out on the 3rd and 5th day after the introduction of the drug "Gumi drinking". No negative effect of the drug excreted in feces on beetles larvae has been established. The number of them on the 3rd day of sampling from animals of the experimental group (bookmark samples a day after administration of the drug "Gumi
drinking" was respectively 25,9±8,7 copies, and in the feces of the control group-26,0±8,2 copies. In the next day of sampling, the number of beetle larvae increased in the feces of all groups. There was a decrease in the number of beetle larvae during 14 days. After sampling feces, laid 1 day after the introduction of the animal drug "Gumi drinking".

**Table 6. Effects on coprophages.**

| Groups             | Timing bookmark faeces after giving the drug "Gumi drinking", day. |
|--------------------|-----------------------------------------------------------------|
|                    | 1                  | 7      | 14     | 28     | 3      | 14     | 28     |
| Gumi drinking      | 35,2±1,4           | 22,8±7,6| 4,0±0,4| 0      | 25,9±9,4| 17,4±7,2| 3,2±1,2| 0      |
| Control            | 35,4±1,2           | 21,7±7,0| 4,0±1,6| 0      | 26,0±8,2| 17,0±8,4| 3,6±1,4| 0      |

The use of "Gumi drinking" did not have a negative impact on the number of both imagos and larvae of coprobiont beetles. Also, found a significant effect on the species composition of adult beetles. From the feces of all groups, 6 species of beetles of the family Scarabaeidae, 6 species of the family Hydrophilidae and 3 species of the family Histeridae were isolated. The most numerous species in fecal tortillas of all groups were species Aphodius erraticus and A. haemorrhoidalis of the family Scarabaeidae and species Criptophleurum minitum and Cercyon quisquilius of the family Hydrophilidae. Thus, the drug "Gumi drinking" does not have a negative impact on the number of larvae of coprophage beetles in the first 1-3 days after application of the drug to animals. The drug does not have a negative impact on the qualitative and quantitative composition of imago and larvae of coprobiont beetles.

**4. Conclusion**

As a result of studies on the Ecotoxicity of the drug "Gumi drinking" with the participation of models from different taxonomic groups, it was found that "Gumi drinking" refers to low-toxic compounds and does not pose a danger to the environment.

**Reference**

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