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Assessment of the Safety of Pine Tree Feed Additives

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Abstract

Background: The research was focused on evaluating the safety of a pine tree energy feed additive, a product developed by Himinvest Scientific and Technical Center LLC, located in Nizhny Novgorod, Russia. The need for this study arose from the growing utilization of such feed additives in the agriculture sector and the necessity to ensure their safe usage for livestock health and welfare.

Methods: The experiments were carried out on white outbred mice and outbred rats of the Wistar line. The authors conducted a study of acute and chronic toxicity and the local irritant effect of the pine tree energy feed additive. In the experiments, the authors did not establish gender-related differences in the sensitivity of animals to the toxic effect of the preparation.

Results: As a result of the conducted studies on white outbred mice, it was found that with a single intragastric injection of the feed additive, it was a relatively harmless substance. Besides, with repeated intragastric administration of the pine tree energy feed additive to outbred rats in subtoxic doses, no significant defects of the functional state of the main organs and systems of the body were observed.

Conclusion: The findings suggest that the pine tree energy feed additive is relatively harmless, thus supporting its safe use. No significant toxicity or irritant effects were observed in the test subjects, making it a viable option for feeding purposes.

Introduction

The Himinvest Scientific and Technical Center (STC) LLC has developed a unique technology for processing wood green shoots based on the extraction of biologically active substances with a new selective extractant. The extractant is non-toxic and allows for improving the properties of the products obtained, and also has antibacterial properties that ensure the preservation of consumer appeal of the products for a long period [1]. The pine tree energy feed additive is a highly effective product for farm animals and poultry [2]. The pine tree energy feed additive is based on biologically active substances of wood greens extracted with a composition of polyatomic alcohols widely used in the food industry, which differ favorably from many similar products in the absence of negative side effects [3]. The excellent taste qualities help to increase the appetite of farm animals and improve the eatability of their diet [4]. The pine tree energy additive is a homogeneous viscous liquid with a characteristic pine tree odor, olive green or dark green color, the water content of not more than 50%, a pH of 8.0-9.0, a density not less than 1.126, and mass fraction of carotene per 100 g of the extract not less than 3 mg% [5].

When conducting studies of the chemical composition of the pine tree energy supplement, the content of vitamins (B1, B2, B3, B5, B6, B9) and carotenoids was established. The energy value equals 250 kcal/100 g of the supplement. The feed additive is successfully used in animal husbandry [6-9].

Methods

Study design

The experiments were carried out on white outbred mice and outbred rats of the Wistar line. Toxicological studies of the additive were carried out on animals following Standard Operating Procedures developed at the Center for Preclinical Research (CPR) of the Institute of Fundamental Medicine of the Privolzhsky Research Medical University (PIMU). The basic rules of maintenance and care corresponded to the standards given in the "Guide for the Care and Use of Laboratory Animals" (ILAR publication, 1996, National Academy Press) and the national standard of the Russian Federation, GOST 33044-2014 "Principles of good laboratory practice", and agreed with the Bioethical Commission of the Central Research Institute.

The study's objectives were to:

- Assess the acute toxicity of the pine tree energy feed additive in mice by administering various dosages and observing for any signs of lethality or physiological changes.
- Examine the chronic toxicity of the same feed additive in rats over 90 days, evaluating body weight gain, food intake, temperature, respiratory rate, behavior indicators, and the condition of various organs.
- Determine the impact of the feed additive on organ mass coefficients in both acute and chronic settings.
- Analyze any gender differences in the manifestation of the feed additive's toxic effects.

Acute toxicity

The experiments were carried out on white outbred mice (36 females and 36 males) weighing 19.5 ±0.7 g, divided into the following groups:

1. Control: 1% starch solution was administrated intragastrically (6 females and 6 males);
2. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 5 g/kg (6 females and 6 males);
3. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 10 g/kg (6 females and 6 males);
4. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 15 g/kg (6 females and 6 males);
5. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 20 g/kg (6 females and 6 males);
6. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 25 g/kg (6 females and 6 males).

The studied feed additive was administered once intragastrically using a non-traumatic olive-tipped metal probe. In the control series, 1% starch solution was intragastrically injected in an appropriate volume. The indicators of toxic effect in the acute experiment were lethality, time of death, symptoms of poisoning (within 14 days), general condition and behavior of animals (within 14 days), body weight assessment (once a week), macroscopic description of internal organs and external integuments (after euthanasia) and determination of mass coefficients of internal organs (after euthanasia).

Chronic toxicity

The experiments were carried out on 160 outbred Wistar rats (80 females and 80 males) weighing 221.06±0.84 g, divided into the following groups:

1. Control: 1% starch solution was administrated intragastrically (20 females and 20 males);

2. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 2.5 g/kg (20 females and 20 males);
3. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 5 g/kg (20 females and 20 males);
4. The pine tree energy additive (Himinvest STC LLC, Russia) was administrated in a dose of 7.5 g/kg (20 females and 20 males).

The studied feed additive was administered daily intragastrically to adult rats using a non-traumatic olive-tipped metal probe. In the control series, 1% starch solution was intragastrically injected in an appropriate volume of 1.5 ml. The calculation of doses was based on the body weight of animals, instructions for the use of the preparation, and the maximum possible single-dose volume for intragastric administration (5 ml) [10]. The doses in the study of chronic toxicity with intragastric administration to mature rats were the following: the therapeutic dose was 2.5 g/kg and 5 g/kg, and the subtoxic dose was 7.5 g/kg.

Study procedure

During the experiment (in the initial state, 30, 60, and 90 days after the administration, and 30 days after the withdrawal of the feed additive), changes in integral indicators were recorded. The general condition of the animals was assessed by indicators of body weight dynamics, feed and water consumption, rectal temperature, respiratory rate, and rat behavior in the open field test [11]. In addition, we evaluated the hematological parameters (the number of erythrocytes, leukocytes, platelets, hemoglobin level, leukocyte formula, and erythrocyte sedimentation rate (ESR)), biochemical parameters, and activity of serum enzymes (total protein, albumin, creatinine, urea, glucose, triglycerides, cholesterol, total bilirubin, alkaline phosphatase activity, aspartate and alanine aminotransferases, calcium, potassium, sodium) and functional activity of rat kidneys by urine examination in the initial state and once a month after that [12-14]. After the administration of the preparations, a pathomorphological examination was performed, which included a necropsy, macroscopic examination, weighing, and histological examination of internal organs. The local irritant effect was evaluated in pathomorphological studies of the site of repeated administration of the preparation (macro and microscopic description of the stomach) [15, 16].

Methods of statistical data analysis

The obtained results were processed using Statistica 5.5 application software packages [17]. The data were checked for the normality of the distribution using the Shapiro-Wilk W-test [18]. The data set had a normal distribution, and therefore the group arithmetic mean (M) and the standard error of the mean (SEM) were calculated, which together with the value of the number of animals (n) are presented in the final tables.

To compare the samples (n=20) with a normal distribution of populations, the parametric Student's t-test was used for paired samples (for dependent samples) or unpaired samples (the independent ones). In the case of significant deviations of the distribution of the trait from the normal law, as well as with small sample sizes ($n \leq 10$), nonparametric criteria were used: for two dependent samples, the Wilcoxon criterion, for two independent samples, the Mann-Whitney criterion, in the case of comparing more than two groups, the Kraskel-Wallis criterion. The differences between the groups were considered statistically significant at a significance level of $p<0.05$ [19].

Results

Acute toxicity

The studies of acute doses of feed additives in mice are shown in Table 1. There were no cases of lethality in any of the groups. After intragastric administration of maximum doses of the feed additive during the first 4 hours of observation, inhibition of motor activity, reflex functions, and responses to external stimuli was noted. By the end of the daily observation, there were no visible differences in the motor and eating behavior of animals, in the state of external integuments and visible mucous membranes, or reactions to external stimuli in comparison with the control group. During 14 days of observation, all the animals looked healthy. The amount and consistency of fecal masses, the frequency of urination, and the color of urine corresponded to the physiological norm.

During the entire observation period, three control weighing was performed (Table 2). The animals from the experimental groups gained weight during the entire observation period as well as in the control group. There was no difference in body weight gain between the groups. After the end of the experiment, all the animals were euthanized to determine the mass coefficients and macroscopic description of the organs. The data is presented in Table 3. There were no differences in the mass coefficients of organs between the group with a single intragastric administration of the pine tree energy feed additive in acute doses and the control group of animals.

There were no differences in the mass coefficients of organs between the group with a single intragastric administration in acute doses of the feed additive and a control group of animals. There were no gender differences in the manifestation of the toxic effect of the feed additive.

Group of animals	Dose, ml/kg				
	5.0	10.0	15.0	20.0	25.0
Male mice	0/6	0/6	0/6	0/6	0/6
Female mice	0/6	0/6	0/6	0/6	0/6

Table 1: Acute toxicity (died/survived) of the pine

Observation time	Control		Pine tree energy additive (Himinvest STC LLC, Russia)									
			5.0		10.0		15.0		20.0		25.0	
	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6
Background	20.00±0.37	19.75±0.28	19.67±0.21	19.58±0.30	19.25±0.25	19.58±0.37	19.92±0.33	20.25±0.21	19.92±0.35	19.75±0.38	19.42±0.54	19.58±0.57
7 days	21.42±0.30	20.92±0.33	21.00±0.29	21.08±0.30	20.58±0.24	21.08±0.27	20.75±0.25	21.33±0.28	20.75±0.42	20.83±0.33	20.67±0.51	21.25±0.50
14 days	22.42±0.30	22.25±0.38	22.50±0.37	22.25±0.31	22.17±0.21	22.08±0.40	22.00±0.29	22.50±0.37	21.92±0.51	22.00±0.39	21.83±0.59	22.33±0.44

Table 2: The effect of a feed additive of the pine tree energy feed additive on body weight with a single intragastric administration to white outbred mice (M± SEM)

Observation time	Control		Pine tree energy additive (Himinvest STC LLC, Russia)									
			5.0		10.0		15.0		20.0		25.0	
	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6	m n=6	f n=6
Heart	4.79±0.07	4.77±0.09	4.84±0.09	4.89±0.10	4.78±0.08	4.71±0.07	4.80±0.08	4.79±0.05	4.91±0.13	4.70±0.10	4.70±0.15	4.84±0.11
Lungs	10.70±0.56	9.85±0.33	10.21±0.33	9.96±0.17	9.93±0.25	10.10±0.23	10.03±0.10	10.18±0.14	10.18±0.17	10.07±0.37	10.79±0.35	9.79±0.33
Thymus	1.78±0.09	1.83±0.04	1.80±0.09	1.90±0.07	1.83±0.07	1.88±0.05	1.82±0.06	1.83±0.06	1.82±0.02	1.83±0.06	1.77±0.03	1.80±0.07
Liver	51.57±1.34	52.03±1.31	50.15±1.47	52.45±2.81	50.72±1.94	51.89±0.98	51.88±0.82	51.39±1.30	51.99±1.55	50.91±1.66	52.74±1.30	52.36±1.80
Spleen	3.77±0.07	3.78±0.08	3.78±0.13	3.70±0.14	3.74±0.15	3.74±0.10	3.65±0.09	3.71±0.10	3.70±0.10	3.60±0.16	3.81±0.04	3.70±0.08
Kidneys	13.76±0.32	13.97±0.29	13.73±0.24	13.81±0.30	13.930.28±	13.51±0.36	13.74±0.42	13.62±0.15	13.80±0.28	13.37±0.45	13.83±0.39	13.42±0.47
Brain	16.98±0.29	16.75±0.40	17.15±0.30	16.55±0.71	16.95±0.17	16.88±0.26	17.21±0.15	16.87±0.16	17.35±0.10	17.07±0.16	16.92±0.31	16.86±0.24

Table 3: The effect of the pine tree energy feed additive on the mass coefficients of organs with a single intragastric administration to white outbred mice (M± SEM)

Terms of the study	Control		Pine tree energy supplement, ml/kg					
			2.5 ml/kg		5 ml/kg		7.5 ml/kg	
	m	f	m	f	m	f	m	f
Body weight, g								
Background(n=20)	221.70±0.84	220.25±0.70	221.70±0.52	221.40±0.76	222.95±0.55	221.40±0.63	222.35±0.74	220.35±0.75
7 days (n=20)	232.90±0.75	230.25±0.66	233.05±0.49	231.10±0.71	232.00±0.72	231.75±0.73	231.95±0.69	230.40±0.60
14 days (n=20)	242.15±0.88	240.85±0.77	241.95±0.75	241.55±0.67	241.90±0.88	240.85±0.88	242.20±0.78	240.95±0.63
21 days (n=20)	252.30±0.73	250.45±0.78	252.20±0.79	251.30±0.79	251.45±0.77	250.70±0.71	252.05±0.90	250.40±0.65
30 days (n=20)	261.50±1.16	260.50±1.40	261.90±1.35	259.55±1.11	262.10±1.27	260.10±1.00	262.45±1.34	260.05±1.18
60 days (n=20)	292.50±1.45	285.55±1.63	293.55±1.84	286.50±1.82	295.65±1.74	283.40±1.72	293.40±1.70	284.95±1.78
90 days (n=20)	321.05±2.60	305.55±2.54	322.50±2.33	302.50±2.40	323.15±2.61	303.25±2.62	320.55±2.26	304.45±2.59
30 days of cancellation (n=10)	353.40±3.21	325.50±3.45	350.80±3.65	322.40±3.70	351.05±3.32	321.25±3.68	353.65±3.87	324.55±3.67

Table 4: The effect of intragastric administration of the pine tree energy feed additive on body weight (g) (M ±SEM).

Terms of the study	Control		Pine tree energy supplement, ml/kg					
			2.5 ml/kg		5 ml/kg		7.5 ml/kg	
	m	f	m	f	m	f	m	f
Heart								
90 days (n=10)	3.12±0.09	3.14±0.06	3.10±0.05	3.11±0.05	3.14±0.07	3.22±0.10	3.17±0.12	3.26±0.13
30 days of cancellation (n=10)	3.09±0.07	3.06±0.06	3.13±0.07	3.13±0.09	3.12±0.09	3.09±0.09	3.13±0.09	3.16±0.06
Lungs (two)								
90 days (n=10)	6.26±0.22	6.51±0.16	6.50±0.15	6.60±0.15	6.56±0.14	6.68±0.17	6.56±0.10	6.68±0.18
30 days of cancellation (n=10)	6.09±0.12	6.14±0.08	6.11±0.09	6.18±0.12	6.25±0.11	6.13±0.13	6.31±0.22	6.29±0.16
Thymus								
90 days (n=10)	1.01±0.02	1.00±0.02	1.00±0.01	1.01±0.01	1.02±0.01	1.02±0.01	1.02±0.01	1.02±0.01
30 days of cancellation (n=10)	1.02±0.01	1.00±0.01	1.02±0.01	1.02±0.01	1.00±0.02	1.02±0.02	1.01±0.01	1.02±0.01
Liver								
90 days (n=10)	31.94±0.81	31.08±0.94	32.08±0.52	31.96±0.78	32.04±0.53	31.65±0.81	32.74±1.10	32.44±0.89
30 days of cancellation (n=10)	30.88±1.13	31.07±0.67	31.54±0.66	30.76±0.86	31.91±0.71	30.77±0.62	30.98±0.63	31.27±0.65
Spleen								
90 days (n=10)	3.18±0.14	3.15±0.09	3.04±0.09	3.20±0.09	3.25±0.05	3.12±0.10	3.30±0.13	3.34±0.14
30 days of cancellation (n=10)	3.20±0.10	3.23±0.08	3.27±0.11	3.19±0.08	3.16±0.07	3.15±0.11	3.21±0.11	3.19±0.10
Kidneys (two)								
90 days (n=10)	5.42±0.09	5.49±0.09	5.57±0.11	5.44±0.11	5.61±0.12	5.50±0.12	5.61±0.13	5.69±0.15
30 days of cancellation (n=10)	5.27±0.20	5.37±0.10	5.28±0.13	5.43±0.13	5.29±0.16	5.32±0.20	5.41±0.23	5.46±0.17
Adrenal glands (two)								
90 days (n=10)	0.20±0.01	0.21±0.01	0.20±0.01	0.21±0.01	0.21±0.01	0.20±0.01	0.21±0.01	0.21±0.01
30 days of cancellation (n=10)	0.19±0.01	0.19±0.01	0.20±0.01	0.20±0.01	0.21±0.01	0.22±0.02	0.21±0.01	0.21±0.01
Brain								
90 days (n=10)	6.80±0.14	6.80±0.12	6.65±0.09	6.81±0.14	6.57±0.12	6.72±0.13	6.75±0.14	6.85±0.09
30 days of cancellation (n=10)	6.56±0.07	6.58±0.07	6.52±0.08	6.65±0.06	6.53±0.08	6.55±0.06	6.55±0.13	6.49±0.09
Testes/ovaries (two)								
90 days (n=10)	10.04±0.29	0.52±0.01	10.12±0.26	0.53±0.01	10.23±0.20	0.52±0.01	10.21±0.23	0.52±0.01
30 days of cancellation (n=10)	9.73±0.27		10.07±0.14	0.50±0.01	10.0	0.52±0.02	9.77±0.18	0.52±0.01

Table 5: The effect of intragastric administration of the pine tree energy feed additive on the mass coefficients of rat organs.

Chronic toxicity

The conducted studies of chronic toxicity on white outbred rats did not show the death of animals with intragastric administration of the pine tree energy feed additive for 90 days. During the entire follow-up period for 90 days in the groups receiving intragastric feed additive, there were no differences in body weight gain and food intake, rectal temperature, respiratory rate, and behavior indicators in the open field test compared with the control group of experimental animals (Table 4). There were also no abnormalities in the hematological and biochemical parameters of blood, the activity of serum enzymes, the functional activity of kidneys, and the mass coefficients of organs (Table 5).

Local irritating effect

The study of the injection site revealed no changes in the groups receiving the feed additive compared to the control group. The mucous membrane of the gelatinous part of the stomach was lined with a multi-layered squamous epithelium. The integumentary epithelium of the mucous membrane of the glandular part of the stomach was formed by mucous cylindrical cells, and there were no defects in the epithelial lining. The main and lining cells in the area of the body of the glands had not changed. There were no differences compared to the control group (Figure 1).

Figure 1: A section of the gastric mucosa of (A) a control group rat; (B) a rat receiving the feed additive at a dose of 2.5 ml/kg; (C) a rat receiving the feed additive at a dose of 7.5 ml/kg. Magnification:100 Stained with hematoxylin and eosin.

Discussion

The obtained data on the properties of the coniferous energy feed additive are consistent with the opinions of the authors [20-23]. The additive contains distilled medical glycerin and a natural carrier – coniferous shoots complementing each other. Glycerol absorbed in the rumen of ruminants is used for glucose synthesis and energy production and is considered preferable to the more aggressive propylene glycol. Needles as plant components have a unique composition, rich in ash elements and water- and fat-soluble vitamins of organic origin with higher digestibility [24, 25]. As a result of the conducted studies on white outbred mice, it was found that with a single intragastric injection of the feed additive, it was a relatively harmless substance. Mortality in mice was not detected when the maximum possible single dose of 25 ml/kg was administered. The results of the study of chronic toxicity showed that pine tree energy feed additive with daily (for 90 days) intragastric administration to rats did not cause violations of the functional state of the main organs and systems of the body [26, 27]. There was no locally irritating effect of the feed additive with its repeated intragastric administration. The conducted studies confirm [28, 29] the safety of the additive both in production and in animal husbandry. It reduces the negative impact on the environment and positively affects the health of farm animals ultimately leading to an increase in their productivity and obtaining environmentally friendly livestock products [30]. The results of the study of general toxicity and local irritant action demonstrated the safety of the pine tree energy feed additive when used in both therapeutic and subtoxic doses, produced by the Himinvest Scientific and Technical Center LLC (Nizhny Novgorod, Russia).

The inclusion of the additive in the diet of cows at the beginning of lactation led to an increase of 11.9-12.2% in the average daily milk yield of natural fat milk while reducing the cost of feed per unit of product received.

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Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

All authors contributed equally to this study.

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