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Effects of Bufadienolides from *Bufo viridis* Toad Venom on Blood Biochemical Compositions and Thromboelastographic Parameters

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ABSTRACT: Bufadienolides, cyclopentanophenantrene derivatives that possess similar structures to cardiac glycosides, have been established to reveal cytotoxicity. Their higher concentrations as anticancer means are expected to affect the normal function of the heart, liver, and kidney. In this work, we established the mass ratio of bufadienolides isolated from the venom of *Bufo viridis*. Arenobufagin, gamabufotalin, telocinobufagin, and marinobufagin were found as the major compounds in the sum. Their effects on biological parameters, used as indicators of the normal functioning of organs, were determined in rabbits. The blood samples, taken on the 10th and 30th days following the drug administration, showed these paramaters reaching the control levels for 30 days in all studied doses: 0.15, 0.45, and 0.6 mg/kg. The 0.15 and 0.6 mg/kg doses were established not to cause significant changes in the activities of alanine aminotransferase and aspartate aminotransferase, and the quantities of cholesterol and fasting blood sugar in blood samples. Significant changes were found in quantities of glucose (a reduction) and urea (an increase) on the 10th day of the administration. But their levels reached the control level for the next 20 days. We observed significant differences in thromboelastographic parameters in mice on the 10th day after the drug administration in a 0.15 mg/kg dose. In mice, LD_{50} levels of intravenous, subcutaneous, and oral administrations made 14.5, 110, and 215 mg/kg, respectively. These results enable the suggestion of optimum concentration that does not cause severe damage to the functions of organs.

KEYWORDS: Toad venom; bufadienolides; acute toxicity; chronic toxicity; glucose; urea.

1. INTRODUCTION

Toad venom has a class of compounds named "bufadienolides" of a wide pharmacological spectrum. These compounds have been widely investigated for their cytotoxic effects. For instance, marinobufagin was established to possess higher cytotoxicity than doxorubicin, commercially available anticancer agent [1]. Bufadienolides are expected to reveal high cytotoxic effects on cancer cells. However, they reveal various effects on different cells. Bufalin, another member of this class, for instance showed marked effects on multiple myeloma whereas no significant changes were observed in lymphocytes.

Different mechanisms of action are expected to be a reason for their cytotoxicity. The effect of bufalin under normoxic conditions was shown associated with DNA damage and inhibition of HIF-1α was the reason for its toxicity in hypoxic conditions [2]. In another case, the effect of bufalin on HL-60 cells was linked with the reduction of topoisomerase II α and II β that lowered to undetectable amounts for 18 h. These changes were concluded to lead to DNA fragmentation. Suppression of topoisomerase II α was also determined in ML1 and U937 cells [3]. It was also found to upregulate TNF-α, TNF receptor 1 (TNFR1), and RIPK1 in U-87 and U373 cells when they escape apoptosis [4]. Its loading in PEG was demonstrated to enhance cytotoxicity to U251; PEGylated liposomes were able to increase the drug dose in plasma [5]. Toad venom extract that

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contains bufadienolides has long been used in Chinese medicine for cancer therapy. Arenobufagin, one of the well-studied bufadienolides, was found to show potency against cancer cells. In Hela cells, it was found to change the expression level of proteins leading to pathways. The cytotoxic effect of the compound was linked to differently expressed levels of proteins: proteasome-, calcium ion binding-, oxidative stress-proteins, and metabolism-related enzymes. Molecular docking analysis suggested the blockage of the pathway of Na^+/K^+ cation exchange by arenobufagin [6]. Arenobufagin was also suggested to be potentially useful against estrogen receptor-positive MCF-7 cancer cells [7]. The compound was found to downregulate the expression of Cdc25C (cell division cycle 25C), Cyclin B1, and survivin in U87 glioblastoma cells. It also activated the p38 mitogen-activated protein kinase (MAPK) signaling pathway. In animal experiments carried out on mice, arenobufagin was detected in cerebrospinal fluid [8]. Hepatocellular carcinoma (HCC), one of the deadly forms of cancer, was determined to be affected by arenobufagin as well. The antineoplastic activity was explained by the enhanced level of mitochondria-mediated apoptosis in HCC cells. Arenobufagin resulted in the inhibition of HepG2/ADM tumor xenograft that was linked with the inhibition of mTOR and cleavage of poly (ADP-ribose) polymerase [9].

Gamabufotalin, another bufadienolide, was determined as an active adjuvant therapeutic agent with trivalent sodium arsenite (AsIII) against glioblastoma. U-87 cells were much more susceptible than U-251 cells to gamabufotalin alone and in combination with AsIII. The combination of cytotoxic compounds revealed selectivity in cancer cells. The combined effect led to the downregulation of Cdc25C, Cdc2, survivin, and Cyclin B1 in U-87 cells [10]. In another trial devoted to studying the effects of gamabufotalin on U-87 cells, the bufadienolide derivative was determined in cancer cells in a dose-dependent manner but not detected in primary astrocytes, and major glial cells in CNS. Similarly, gamabufotalin caused the downregulation of Cdc25C, Cdc2, survivin, Cyclin B1, and Cdc25A and Aurora B as well. The treatment with gamabufotalin led to autophagy induction and demonstrated high potency against cancerous glial cells which could be attributed to selectivity by multiple signaling pathways [11]. The tandem of gamabufotalin and arenobufagin showed a dose-dependent effect on U-87 and SW1990 pancreatic cancer cells. Their IC_{50} value in peripheral blood mononuclear cells was 3-5 fold lower compared to those observed in cancer cells. But similar results were not observed with telocinobufagin and bufalin [12]. Another mechanism of selectivity of gamabufotalin was linked with cyclooxygenase-2-mediated cases that could be observed in some diseases such as cancer. In this manner, its biological functions were revealed in apoptosis assays in non-small cell lung carcinoma (NSCLC) cells [13]. Gamabufotalin was found to inhibit VEGF-triggered proliferation and migration of human umbilical vein endothelial cells in vitro. It was concluded to inhibit the VEGFR-2 signaling pathway and be a candidate for angiogenesis-related conditions [14]. Marinobufagin and telocinobufagin showed demonstrated cytotoxic efficiency against several human cancer cell lines: prostate cancer (PC-3), colorectal carcinoma (HCT-116), and glioblastoma (SF-295) cells. These bufadienolides and their acetylated derivatives did not cause any hemolysis of human erythrocytes [15]. In another research, marinobufagin was determined to reveal antiproliferative action on HL-60 leukemia and meristematic cells. The effects of the compound on DNA damage of HL-60 and polymorphic blood cells enabled the conclusion that the antiproliferative effect was not necessarily related to genotoxicity [16].

Besides cancer-related activities, these compounds have been investigated for other pharmacological properties. Telocinobufagin, for instance, was found to show cytotoxicity against chloroquine-resistant Plasmodium falciparum [17]. Bufadienolides are also cardiotonic steroids. Computer simulation analysis demonstrated the affinity of gamabufotalin to ATP-binding sites of VEGFR-2. Inhibited VEGFR-2 kinase activity and suppressed level of VEGFR-2-mediated signaling cascades were in agreement [14]. Their cardiotonic effects were linked with functional selectivity. Telocinobufagin, for example, inhibited the Na^+/K^+-ATPase activity in pig kidney cells. However, a corresponding proliferative response was not observed following the treatment with telocinobufagin. The apoptosis resulting from telocinobufagin was attrributed to an increased level of Bax: Bcl-2 expression ratio and pyknotic nuclei [18].

Our early trials enabled us to isolate the sum of bufadienolides from *Bufo viridis* toad venom, that contained arenobufagin, gamabufotalin, telocinobufagin, marinobufagin, bufarenogin, and bufalin [19]. We further established that the sum of bufadienolides at 0.046-0.093 mg/kg concentration did not reveal embryotoxic and teratogenic effects in rats [20]. In this work, we studied the effects of this sum of bufadienolides on biochemical parameters of blood such as alanine aminotransferase, aspartate aminotransferase, glucose, cholesterol as well as thromboelastographic parameters. Based on obtained results, we interpreted possible effects of the sum of bufadienolides on normal functioning of organs.

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2. RESULTS AND DİSCUSSİON

2.1. Isolation of bufadienolides

The sum of bufadienolides from the *Bufo viridis* toad venom was obtained by extraction. For this, a 1g total venom was subjected to extraction with ethanol (200 ml) for seven days in a dark bottle by slowly stirring at room temperature. After concentrating, the extract was analyzed by HPLC following the conditions that were developed by us earlier [19]. The compounds were identified by their retention times. The obtained data showed that the extract consisted of six bufadienolides: arenobufagin, gamabufotalin, telocinobufagin, marinobufagin, bufarenogin, and bufalin. The results in this work were identical to those obtained in our earlier trial [19]. The chemical composition of the obtained extract is shown in Figure 1.

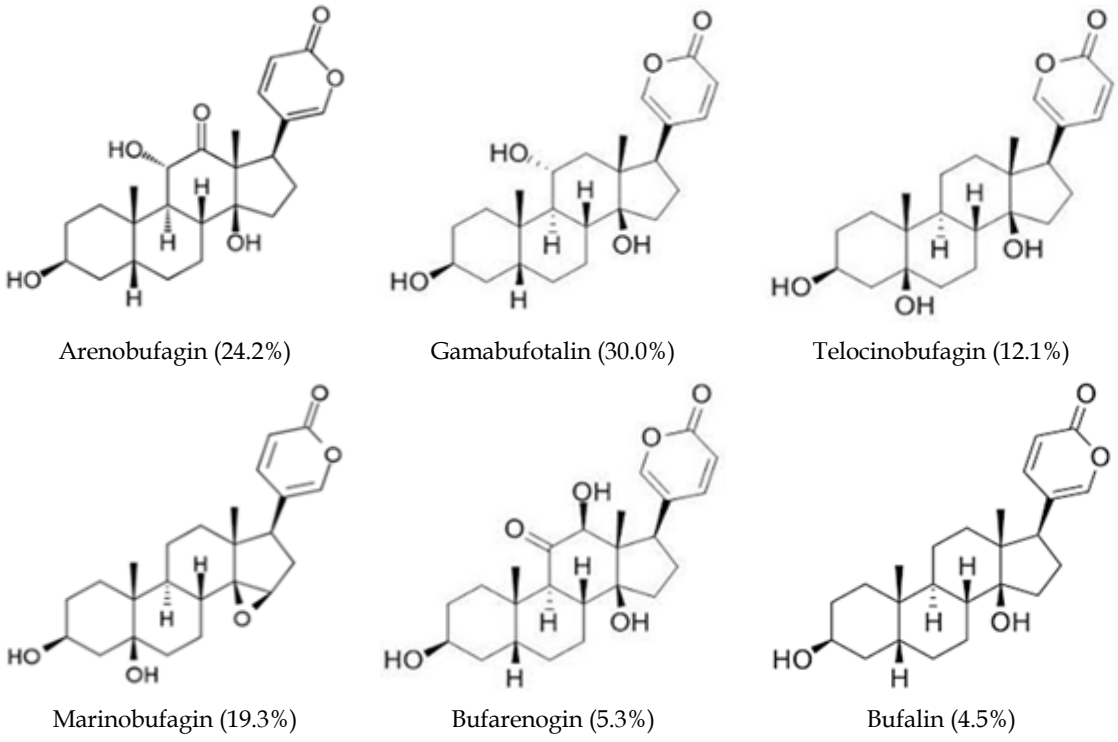

Figure 1. Chemical structure of bufadienolides in the toad venom extract

2.2. Acute toxicity

2.2.1. Intravenous administration

Intravenous administration of the sum of bufadienolides to mice at doses of 5 and 10 mg/kg caused general weakness, rapid and deep breathing, and anxiety in animals. After 5 minutes, most of the mice calmed down. Few animals revealed diaphragmatic breathing in the next 10 minutes and the pain sensitivity reduced. After 30 minutes, the general overall behaviour of the animals returned to normal level. Breathing became even. The death of mice made 20%, one out of five. An increase in the dose of the drug to 20 mg/kg caused a deeper poisoning, and three out of 5 mice died in 26 minutes because of shortness in breathing. In 5 minutes after the administration of 25 mg/kg dose, the animals showed weakness and immobility. By actions they revealed tonic-clonic seizures. The death rate made 80% (four out of five) because of respiratory arrest. The 30 mg/kg concentration led to even more pronounced intoxication, and 100% death of the animals was observed. The LD50 for white mice when administered intravenously was 14.5 mg/kg..

2.2.2. Subcutaneous administration

Subcutaneous administration of the bufadienolides sum at doses of 80, 100, 120 and 140 mg/kg caused muscle weakness, unsteadiness of the "gait", convulsive breathing, tail reaction in mice. In 60 minutes following the subcutaneous administration at a dose of 80 mg/kg, the general condition of the animals returned to normal level. The poisoning was more pronounced at 100 and 120 mg/kg doses, and two and three animals out of five died, respectively. The drug administration at a dose of 140 mg/kg caused the death of all (5 out of 5) mice. LD_{50} after a single subcutaneous injection made 110 mg/kg.

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2.2.3. Oral administration

The oral introduction of the drug through a tube at doses of 100, 150, 200, 250, and 300 mg/kg led to the appearance of hiccups and chewing movements in 3-5 minutes. Further, weakness and general oppression were observed. The oral drug administration at a dose of 100 mg/kg after 60 minutes, the general overal behaviour of the mice returned to normal levels, and their behaviour did not differ from intact animals after 180 minutes. There were no deaths of mice. The doses of 150 and 200 mg/kg caused acutely developed poisoning and resulted in the death of 1 and 2 animals (out of five), respectively. The drug administration at the 250 mg/kg dose led to the death of 3 mice. Total animal death was observed following the administration at 300 mg/kg. The oral LD_{50} for mice made 215 mg/kg.

Thus, the toxicity levels of the bufadienolides sum varied in different modes of administration to animals. LD_{50} level of intravenous administration (14.5 mg/kg) was several-fold greater compared to subcutaneous (110 mg/kg) and oral (215 mg/kg) provision which could be attributed to their effects on blockage of Na^+/K^+ exchange.

2.3. Chronic toxicity

Chronic toxicity effects of bufadienolides were studied on rabbits following the intravenous administration at doses of 0.15, 0.30 and 0.45 mg/kg. The administered 0.30 and 0.45 mg/kg concentrations caused muscle weakness, immobility, palpitations in 2 and 3 rabbits, respectively. This condition lasted for 15-30 minutes. The observed phenomena mainly depended on the susceptibility of individual animals. After such a short-term "malaise", the animals began to eat well and further their behaviour did not differ from intact animals. By the end of the experiment (1 month), the weight gain of the experimental rabbits did not differ significantly from those in the control group.

2.4. Blood biochemical parameters

Taking the high cardiotropic effects of bufadienolides [21, 22, 23] into consideration, we studied their effects on general toxicology with the purpose of possible implementation into medical practice as a cardiotropic drug. Therefore, our upcoming experiments were dedicated to investigating the influence on some biochemical parameters such as total protein, alanine aminotransferase, aspartate aminotransferase, cholesterol, glucose, and urea in the blood of rabbits. The obtained results are given in Table 1.

2.4.1. Alanine aminotransferase

Alanine aminotransferase is one of the enzymes used in laboratory assays in clinical practice to detect liver disease and general overall health. Alanine aminotransferase activity is influenced by many factors including medications [24]. In the liver, it transfers an amino group from alanine to α-ketoglutarate, thus forming glutamate and pyruvate [25]. The elevated level of alanine aminotransferase caused an increased level of coronary artery risk 1.3- and 2.1-fold in men and women, respectively. If the increase in the alanine aminotransferase continues following the administration of a drug, the medication is to be discontinued [26]. Shortly, alanine aminotransferase is one of the reliable indicators of a healthy condition of an organism. In our study, bufadienolides did not cause any increase in all three different concentrations. The intermediate 0.30 mg/kg concentration significantly lowered its quantity on the 30^{th} day of the treatment (Table 1).

2.4.2. Aspartate aminotransferase

Aspartate aminotransferase is another transaminase enzyme that plays a significant role in the normal functioning of the liver. It catalyzes the transamination between the tandems of aspartate–alpha-ketoglutarate and oxaloacetate–glutamate [27]. Aspartate aminotransferase is found in cerebrospinal fluid, exudates, and transudates related to cellular damage [28]. By providing the transfer of amino groups, alanine aminotransferase and aspartate aminotransferase contribute to glucose and amino acid metabolism. Abnormally higher levels of these two enzymes in the liver might indicate an enhanced level of hepatic transamination [29]. Similarly, the treatment did not lead to increases in aspartate aminotransferase levels, but a significant reduction was observed in the II and III groups compared to the control (Table 1).

2.4.3. Cholesterol

In this work, we referred to cholesterol as one of the indicators of the normal functioning of the heart. A number of researches have indicated interactions between hyperlipidemia and cardioprotective mechanisms. In one work, a cholesterol diet in rats was established to decrease cardiac ATP and enhance myocardial oxidative stress [30]. Digoxin and ouabain, cardiac glycosides that possess cyclopentanophenantrene skeleton, were reported to increase the synthesis of cholesterol in cardiomyocytes

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and reveal no Na^+/K^+-ATPase independent effects [31]. In our work, we determined that bufadienolides, cyclopentanophenantrene derivatives, did not cause any significant changes in blood cholesterol levels following the administration of drugs in three different concentrations (Table 1). Thus, we suggest bufadienolides do not reveal the effects resulting from cardiac glycosides.

2.4.4. Glucose

We determined significantly lowered blood glucose levels on the 10th day of the treatment in all three groups. On the 30th day of the treatment, its level reached control. Fasting blood sugar level was mild enough that the increase was observed on the 30th day in group IV animals, treated with 0.45 mg/per dose. These results indicate that the sum of bufadienolides used in this work did not seriously impact blood parameters that are associated with the normal functioning of the liver, kidney, and heart (Table 1).

2.4.5. Urea

Blood urea nitrogen (BUN) is considered one of indicators of damaged liver functioning. Enhanced levels of BUN were associated with adverse renal outcomes and suggested as an indicator for kidney disease progression [32]. Besides, BUN was reported as predictive indicator for cardio-vascular diseases [33]. In our work, significant increases by the administration of bufadienolides were observed only in blood urea. Concentration-dependent changes explain their possible negative effects on liver functioning by the 10th day of the drug administration. However, by the 30th day of the treatment, blood urea level lowered to a control level (Table 1).

Table 1. Effects of the sum of bufadienolides on the biochemical parameters of rabbits (Confidence interval means standard deviation, calculated from four replicates)

	Days of treatment	Groups/Doses mg/kg			
		I-Control	II-0.15	III-0.30	IV-0.45
Total protein (g/L)	10th day	61±5.4	54±6.0	52±5.6	53±5.3
	30th day	62±5.6	56±6.0	60±0.1	56.0±0.2
Alanine amino-transferase (mmol/L)	10th day	0.45±0.04	0.40±0.04	0.50±0.04	0.46±0.04
	30th day	0.42±0.04	0.55±0.03	*0.25± 0.02*	0.45±0.02
Aspartate amino-transferase (mmol/L)	10th day	0.40±0.04	*0.30± 0.03*	0.40±0.04	0.41±0.04
	30th day	0.35±0.03	0.35±0.03	*0.20± 0.02*	0.40±0.04
Cholesterol (mmol/L)	10th day	4.0±0.4	3.7±0.4	3.8±0.4	4.2±0.4
	30th day	3.7±0.4	3.8±0.4	3.9±0.4	4.0±0.3
Glucose (mmol/L)	10th day	6.1±0.6	*3.5± 0.3*	*3.0± 0.3*	*4.0± 0.4*
	30th day	6.0±0.6	5.1±0.5	4.9±0.5	6.0±0.4
Urea (mmol/L)	10th day	4.5±0.2	**5.8±0.5**	**6.2±0.7**	**6.3±0.6**
	30th day	4.0±0.4	4.6±0.4	4.4±0.5	4.8±0.5

* Significant increases are shown in bold, and significant reductions are italicized ($P<0.01$ in all cases).

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2.5. Blood coagulation

In our work, we investigated the effects of the sum of bufadienolides on the blood coagulation process. Many indicators attributing to blood coagulation such as reaction time, clot formation time, and total clotting time made significantly longer mean values at the administered lowest concentration (0.15 mg/kg) compared to control (Table 2). The higher prothrombin consumption level in these blood samples that is not consistent with delayed clotting time can be explained by the thrombin, the main blood coagulation factor, that possibly was inhibited by bufadienolides. However, no remarkable changes in these indicators were determined at higher doses (Table 2). These results explain that bufadienolides, used in this work, might cause concentration-dependent slight hypocoagulation effects. Changes in blood coagulability, namely hypercoagulability, were reported to result from alternating concentrations of platelets, procoagulants, antithrombin, or other inhibitors of thrombosis [34]. Based on these results, we suggest no changes in these parameters by the sum of bufadienolides.

Table 2. Effects of the sum of bufadienolides on blood coagulation and the resistance of rabbit (Confidence interval means standard deviation, calculated from four replicates)

Thromboelastographic parameters	Days of treatment	Groups/Doses mg/kg			
		I-Control	II- 0.15	III-0.45	IV-0.6
Reaction time, R, mm	10th day	22 ± 2.0	**60 ± 6.0**	22 ± 2.0	19 ± 2.0
	30th day	22 ± 2.0	24 ± 2.5	24 ± 2.5	25 ± 2.5
Clot formation time, K, mm	10th day	12 ± 1.0	**25 ± 2.0**	14 ± 1.5	15 ± 1.6
	30th day	12 ± 1.0	13 ± 1.0	12.5 ± 1.0	13 ± 1.5
Coagulation constant (R+K), mm	10th day	34 ± 3.0	**85 ± 6.0**	36 ± 3.0	34 ± 2.0
	30th day	34 ± 3.0	36 ± 4.0	36 ± 4.0	38 ± 4.0
Prothrombin consumption index, R/K	10th day	1.8 ± 0.14	**2.4 ± 0.2**	1.6 ± 0.15	*1.3± 0.14*
	30th day	1.8 ± 0.14	1.8 ± 0.2	1.9 ± 0.18	1.9 ± 0.2
Maximum amplitude MA, mm	10th day	52 ± 5.0	*32 ± 3.0*	44 ± 4.0	39 ± 3.0
	30th day	52 ± 5.0	49 ± 5.0	48 ± 5.0	51 ± 5.0
Blood clotting constant t, mm	10th day	110 ± 11	**142 ± 12**	110 ± 11	110 ± 11
	30th day	110 ± 11	100 ± 10	100 ± 10	102 ± 10
Syneresis constant S, мм	10th day	120 ± 12	**188 ± 16**	123 ± 12	123 ± 12
	30th day	120 ± 12	113 ± 12	112 ± 11	115 ± 12
Total clotting time T, mm	10th day	141 ± 14	**227 ± 20**	146 ± 13	142 ± 13
	30th day	141 ± 14	140 ± 15	133 ± 14	140 ± 14
Clot elasticity coeff. E, (Max100/100-MA) E	10th day	108 ± 10	*47 ± 4.0*	79 ± 6.0	65 ± 6.0
	30th day	108 ± 11	96 ± 10	92 ± 10	104 ± 10
Coagulation index Ci (MA/R+K)	10th day	1.5 ± 0.12	*0.38 ± 0.02*	*1.2 ± 0.1*	*1.0 ± 0.1*
	30th day	1.5 ± 0.2	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.2

* Significant increases are shown in bold, and significant reductions are italicized ($P<0.01$ in all cases).

Obtained results indicate slight hypocoagulative effects of bufadienolides on blood coagulation only in group II animals on the 10th day. However, no significant changes in the values of thromboelastographic parameters on the 30th day were observed following the administration of a 0.15 mg/kg dose. The results enable us to conclude minor hypocoagulative effects following a single intravenous drug administration at a dose of 0.15 mg/kg. Further administration of the drug by the 10th day of the experiment led to hypocoagulation that gradually returned to normal condition by the 30th day of the experiment. The drug administration at 0.45 and 0.6 mg/kg doses did not reveal significant changes in the blood coagulation process.

3. CONCLUSİON

Intravenous injection of the sum of bufadienolides to rabbits (every other day 15 times) at doses of 0.15, 0.30, and 0.45 mg/kg did not cause significant changes in the morphological composition of the blood, after 10 injections. The glucose level decreased by 34-25% and urea in the blood serum increased by 29-40%. After one month of the experiment, these mean values returned to the control level. No significant changes were observed in other parameters of blood biochemical indicators including alanine aminotransferase, aspartate

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aminotransferase, total protein, and cholesterol. A single intravenous injection of the sum of bufadienolides in the studied doses after 30-60 minutes and with repeated injections at doses of 0.30 and 0.45 mg/kg did not affect the blood coagulation in mice. The injection of 0.15 mg/kg dose led to hypocoagulation by the 10th day that further attenuated and returned to the control level by the 30th day of the experiment. Pathological macro - and microscopic screening of organs and tissue of rabbits did not reveal any specific changes due to the action of the tested substances.

4. MATERİALS AND METHODS

4.1. Chemicals

All the chemicals used in this work were of analytical grade.

4.2. Acute toxicity

General and acute toxicities of the sum of bufadienolides were carried out in 74 white mice weighing 18-20g. The drug was administered once intravenously at doses 5, 10, 15, 20, 25, and 30 mg/kg, and subcutaneously at doses 80, 100, 120, and 140 mg/kg, and orally through a tube at doses 100, 150, 200, 250 and 300 mg/kg. Five animals were used for each dose.

4.3. Chronic toxicity

The chronic toxicity study was carried out on 16 gray rabbits weighing 2.5-3 kg in four groups. The drug was administered intravenously, many times (every other day 15 times) in doses of 0.15, 0.45, and 0.6 mg/kg. The control group of animals was intravenously injected with saline in an equivalent volume. All manipulations with animals were carried out according to the International Agreement on the Humane Treatment of Animals (The European Communities Council Directives of 24 November 1986-806/609/EEC).

4.4. Blood sampling

Blood samples for biochemical and hematological analyzes were obtained from the vein of the ear of a rabbit on the 10th and 30th days of the experiment; for counting the blood cells, samples were taken with the anticoagulant EDTA (25 µl), biochemical parameters in the blood serum (10 min at 3000 rpm).

4.5. Enzymic activity of alanine aminotransferase and aspartate aminotransferase

The alanine aminotransferase and aspartate aminotransferase activities in obtained blood samples were determined following the Cypress protocol. The colour intensities of the formed solutions were read in a Semi-auto chemistry analyzer at 340 nm.

4.6. Quantification of blood biochemicals

4.6.1. Proteins

The quantities of of soluble proteins in blood samples were defined according to Cypress protocol by using Biuret reagent. The formed colour intensities were read at 546 nm in a Semi-auto chemistry analyzer.

4.6.2. Glucose

The quantities of glucose in samples were studied according to the Cypress protocol at 505 nm in a Semi-auto chemistry analyzer.

4.6.3. Cholesterol

Cholesterol in blood samples was quantified according to the Cypress diagnostics protocol. The colour intensity was determined at 510 nm in a Semi-auto chemistry analyzer.

4.6.4 Urea

The amount of urea in samples were determined according to the Cypress diagnostics protocol at 340 nm in a Semi-auto chemistry analyzer.

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