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RESULTS OF THE STUDY ON BENEFICIAL AND TOXIC MATTERS CONTAINED IN TISSUES AND ORGANS OF SOME BLISTER BEETLES*(MELOIDAE)* DISTRIBUTED IN MONGOLIA

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Abstract: -

Blister beetles of 34 species belonging to such genera as Epicauta, Lytta, Mylabris, Meloi, Cerocoma, Zonitis, Apalus, Eusonitis, Megatrachelus, and Stenoria of family Meloidae are distributed in our country [2]. These beetles feed flower organs and leaves of plants belonging to families of Leguminosae and Fabaceae and concentrations of organic matters and toxic substances vary with the organs, where they are contained. Proteins account for 49.3% of dry matters of the organs and tissues of Caragana blister beetle (Lytta caragana) fed leaf of the vetch (Vicia sativa) and 60.0% of matters of the beetle fed Astragalus sp. As well, the beetle body contains large amounts of non-essential amino acids including alanine, aspartic acid, serine, cysteine, glutamic acid, arginine and tyrosine, and essential amino acids such as valine, histidine, lysine, leucine, methionine, proline, threonine and phenylalanine [16]. Tocopherol esters contained in Siberian blister beetle (Epicauta sibirica), Caragana beetle (Lytta caragana) and Frolov blister beetle amount to 214.08 mg/g, 98.83 mg/g and 113.07 mg/g respectively [12]. Chemical nomenclature of cantharidin contained in the organs and tissues of blister beetle is Exo-1,2-cis-den-1-3,6-epoxyhexahydropthalic anhydride and its chemical formula is $C_{10}H_{12}O_4$. Tocopherol, proteins, amino acids, selenium, other macro and micro elements and cantharidin were extracted from dried tissues and organs of beetle of 4 species belonging to family Meloidae, and the effect of tocopherol was tested on sheep, chickens and mice, while cantharidin toxicity or LD_{50} andLD_{100} was determined in mice.

Keywords: **-** *Meloidae,* tocopherol, cantharidin

INTRODUCTION

In medicinal writing of 18th century, it was briefly mentioned that tocopherol esters contained in Caragana blister beetle (*Lytta caragana)* is used in medicine. Particularly, dried extract powder of this beetle was used for preparation of oral solution and treatment of infertility in women. However, it was necessary to be cured under the control of physician. It was written that if signs of intoxications were observed, it can be cured by use of a medicine made of locust of gobi [12]. There are extensive information by a number of overseas researchers on distribution of more than 4000 species of beetles belonging to family *Meloidae*. Beetles of family *Meloidae* are distributed commonly in the rangelands, where plants of such genera *Piptanthus, Thermopsis, Trigonella, Medicago, Melilotus, Vicia, Caragana* and *Astragalus* belonging to family *Leguminodae Juss* in forest steppe, steppe, steppe, gobi and semi-desert steppe regions in Mongolia. Especially 18 species belonging to genus *Mylabris* are most broadly distributed as reported by scientists of our country and Hungary [1,2,4,5,9,10,11,12 and 13].

Various hypotheses on that body of blister beetle contains toxic and medicinal substances were propounded by Olaus Borrichus (1678), Thouvenel (1780), Beaupoil (1803), Robiguet (1812) and Courber (1860). Thouvenel (1780) dried blister beetles and extracted three differently colored compounds by use of organic solvents for separation, and these three colored or yellowish green, oily yellowish and brighter green wax like preparations were obtained. The author noted brighter green wax like preparation is highly toxic. French researcher Spiegel (1894) extracted cantharidin from dried powder of blister beetle *Lytta vericataria* and determined its chemical formula to be $C_{10}H_{12}O_4$ [4]. A number of authors reported about cantharidin is contained in the body of blister beetles, especially beetle of genus *Lytta* contains 2.6-4.06% cantharidin as informed by Beaurgard (1890), Regiun (1847) and Bluhm (1865) and beetle of genus *Lytta*contains 0.56%, *Mylabris*1.03% and*Epicauta*2.02% according to results of the study by Beaurgard (1890),Lepine (1861) and Riu (1930) [2]. Different contents of cantharidin depending on species and genera of beetles are seen to be associated with absence of studies on features of feedstuff, body size and gender of the beetles. Some authors, including Stockard, Papanicolan (1914), Frank (1922), Allen, Doisy (1922-1925) Trendelenburg (1929), Butenand (1931) and Pigulevskii(1970) investigated the pharmacological characteristics of cantharidin and reported the compound is similar to folliculsterone, a hormone of sex gland or gonad. As well, Diels and Alder (1950) received Noble prize for their contribution to develop method and reagents for obtaining cantharidin. Afonskii (1969) noted that tocopherol obtained from natural raw material is more effective by 1000 percent than synthetically produced tocopherol and it can be explained by presence of mixture of substance with certain effect [16, 17,18].

Beetle *Mylabris speicosa* was collected during July and August, then the material was processed by drying and grinding, followed by storage in 50% ethanol for a week and experiments using this extract demonstrated a drop of the extract caused the appearance of small blisters on the skin [10]. Also there is a report that blister beetles are not toxic if they are eaten by *Daurian hedgehog*, chicken (*Gallus gallus domestica*) and house martin (*Delichon urbicum*), but they are toxic and cause death if the beetle is eaten by other animals [6]. In 1988-1996, Ch.Chuluunjav, D.Donrov, D.Baatar and D.Dashtsereninvestigated tocopherol esters contained in the body of blister beetle and determined its concentrations *Epicauta sibirica*(214.1 mg/g), *Lytta caragana* (98.7 mg/g) and *Mylabris frolovi* (113.0 mg/g).

Materials and methods

1.To catch blister beetle, standard size net used broadly for insect collection was employed.

2.Collected beetles were killed by use of special container heating at 70-80^{0}C or freezing at minus temperatures. Dead beetle bodies are dried by use of fan in shadowy place. Dried mass was then powdered by coffee grinder.

3.To isolate tocopherol esters, 2 to 3 g powdered beetle mass was extracted with ether, washed with ethanol and then filtrated through special sieve. Then 3 ml 50% KOH and 50 mg pyrogallol were added to above extract and the mass was saponized for 15 minutes in rotation refrigerator on water heater with constant temperature 70^0 C. Distilled water equal to volume of this solution was added and both saponized and non-saponized fractions were divided by use of separation funnel. Separated portions were washed consecutively with 50 ml and 25 ml ether twice each, and then the portions were combined and washed with water until neutralization. Ether extract was dehydrated through filtration using funnel with sodium sulfate anhydrous and then the solvent was evaporated by use of vacuum heater with rotation chiller and dry residues were resolved in 20 ml ethanol, followed by pouring into 25 ml flask. This ethanol solution was then filtrated through celite-504 loaded, 5 ml adsorption column with 1.5 ml diameter and remained tocopherol was washed and separated with 18 ml ethanol, dry residue was obtained by evaporation using heater with rotation chiller and collected by solving in fish oil, followed by sterilization in order to prepare oily solution of tocopherol.

4.Content of cantharidin was determined by method of Woodword. Dried and powdered (flour) specimen was extracted in acetone and hydrochloric acid, purified with petroleum ether and finally the cantharidin was separated by re-crystallization with acetone. Cantharidin in chest, belly and head of beetle was isolated as described by Courber (1855), Ferrer (1859) and Berthoud, Leidy (1867). Toxicity of each doses selected in the present study was measured by use of bait, which was prepared by absorbing doses in grain, according to method of Karber, Pershin, Miller and Tainter or probit analysis method, and obtained data were processed by statistical analysis.

5.To perform experiments to investigate the activity of oily preparation obtained from organs and tissues of beetles of 3 species and its effects on female animal reproductive functions, the animals were divided into 5 variants each consisting of 4 animals according to doses of the preparation. The preparation was injected by estimating each mouse of variants 1

to 4 to receive tocopherol in doses from 0.001 to 0.000001 g respectively, mice of variant 5 to were injected 0.06 ml fish oil and control mice remained not treated. At hour 48 after experiment start, all mice were sacrificed by placing inside container with ether, then uteri of mice were accurately removed and weighed on analytic balance. Uterine weight of each variant mice were compared to those in control and they increased from lower to higher doses by 6% to 27.1%.

Results of the study

Tocopherol ester contained in blister beetles of above three species was isolated and its effect was tested in sheep via injection of 0.3 to 0.5 g tocopherol powder or 4.5 to 5 ml oily solution at days 15 or 16 after the experiment start and as result, it caused ovulation at hours 74 to 96 hours. Use of tocopherol ester powder in comparison with vitamin E powder in egg laying hens resulted in 2.4% increase of egg production and 2.02% increase of egg weight. Organs and tissues of Siberian blister beetle fed alfalfa (*Medicago falcata*), Caragana blister beetle fed the vetch (*Vicia sativa*) and Frolov blister beetle fed *Thermopsislancelatum* contain 214.08 mg/g, 98.73 mg/g and 113.07 mg/g tocopherol respectively. Therefore, it is seen that contents of tocopherol in the beetle depend not only on their own species, but also on the species of plants eaten by them. Study results are shown in table 1.

Table 1. Concentrations of tocopherol contained in tissue and organs of blister beetle.

№	Species of beetle	Replicates of analysis	X	S±	C	P
1	*Epicauta sibirica*	5	214,08	±41,74	5,13	0,005
2	*Lytta caragana*	5	98,73	±23,70	4,16	0,010
3	*Mylabris frolovi*	5	113,07	±25,34	4,46	0,005

Results of analyses for comparison of concentrations of tocopherol contained in tissue and organs of blister beetle in various feedstuffs were summarized in table 2.

Table 2. Values of tocopherol contained in blister beetle compared with vitamin supplements and grains.

№	Name of products	Total tocopherol(mg/g)
Feeds of animal origin		
1	*Epicauta sibirica*	214,1
2	*Lytta caragana*	98,7
3	*Mylabris frolovi*	113,0
Vitamin supplements(others)		
1	Alfalfa flour	19,7-25,8
2	Timothy flour	8,9-15,3
3	Green algae	18,0
Grains(others)		
1	Barley germ oil	283,0
2	Maize germ oil	86,0-104,0
3	Wheat germ oil	255,0-268,0

Above table shows tocopherol concentration in maize germ oil is similar to that contained in beetles including *Lytta caragana*and*Mylabris frolovi*, higher than vitamin supplement and lower than barley and wheat germ oil.

Pure fish oil selected as tocopherol solvent was used on the mice of variant 5 in the experiment at the same doses of 0.06 ml as doses in other variants and uterine size or weight of this variant mice remained unchanged as compared to those of control animals. On the basis of above finding, it is led to see fish oil exerts no any effect on reproductive organs of mice. The dose of 0.0001 g used in third variant, where the mouse uterine weights were the highest, is seen to be effective. Uterine weights of mice in this variant were 89.95 mg or increased by 271.1%, while uterine weights of mice in fourth variant treated with oily preparation containing 0.001 g tocopherol were less by 9.2% as compared to those in variant 3. It is determined that the most suitable dose of oily preparation containing tocopherol to exert effects on reproductive organs of mouse is 0.071 mg/kg. Testing of tocopherol effects on ewes demonstrated injection of 4.5 to 6.0 ml oil solution at day 15 or 16 after checking the first estrus resulted in complete ovulation at hours 74 to 96. Addition of tocopherol powders to feedstuff of egg laying hens leads to exert the same action as medicinal tocopherol or vitamin E. Results of the experiment are shown in table 3.

Table 3. Effect of tocopherol obtained from natural source or beetle on egg production by hens

Жор	No. of hens in the experiment	Preparatory period	At the end of experiment	Differences	Preparatory period	At the end of experiment	Differences	Preparatory period	At the end of experiment	Differences
1	2	3	4	5	6	7	8	9	10	11
Natural tocopherol	14	1,534	1,814	+0,101	7,1	15,6	+2,4	46,24	52,17	+2,02
Synthetic tocopherol	14	1,592	1,975	+0,262	6,6	14,7	+1.5	47,07	52,28	+2,13
Control	14	1,517	1,713	-	9,3	13,2	-	45,82	50,15	-

Results of the experiment in egg laying hens show egg production egg weight increased by 2.4% and 2.01% respectively. To determine the toxic doses of tocopherol in the experiment, the preparation from beetles *Lytta caragana* and *Epicauta sibirica* were used, 2, 4, 8 and 10 mg doses were injected in a total of 32 mice of two variants in 4 replicates and toxicity was determined. Toxicity levels of the preparations from two beetles in the experiment were similar to each other and LD_{50} and LD_{100} were 166.6 mg/kgand 307.6 mg/kg respectively.

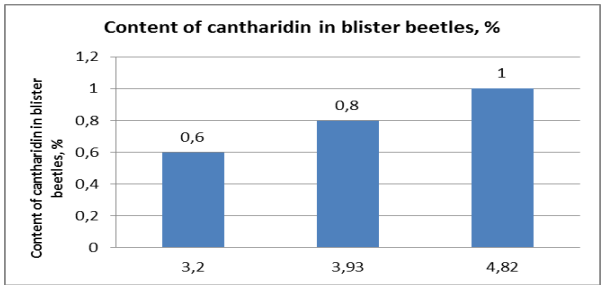
Results of the measurements of amino acid contents in proteins contained in organs and tissues of the beetles *Lytta caragana* and *Mylabris frolovi* show the concentrations of proteins and amino acids differ depending from species of the plants, which were eaten by the beetles. Comparison of protein and amino acid contents in the beetle *Lytta caragana* feeding the vetch in its tiller stage with those in the beetle *Mylabris frolovi* feeding Termompsis and Astragalus spp at their flowering stage revealed protein content is less by 10.9%, contents of aspartic acid, glutamic acid and cystein among non-essential amino acids are greater by 0.83%, 0.65% and 0.58% respectively, whereas alanine, glycine and serine are less by 1.52%, 0.25% and 0.71% respectively. Of essential amino acids, valine, lysine, leucine, methionine, proline and tyrosine contents are greater by 0.17%, 0.51%, 0.45%, 0.15%, 1.76% and 0.02% respectively, while contents of arginine, histidine, threonine and phenylalanine are less by 0.78%, 1.40%, 0.20% and 1.05% respectively. Measurements of proteins and amino acids contained in organs and tissues of the beetles *Mylabris frolovi* fed Termopsis sp at its flowering stage with protein analyzer and color changes of amino acids demonstrated proteins account for 58.5%, alanine, aspartic acid, glycine, glutamic acid, serine and cystein among non-essential amino acids for 1.03%, 1.12%, 2.51%, 2.16%, 1.56% and 1.06% respectively, and arginine, valine, histidine, lysine, leucine, methionine, proline, tyrosine, threonine and phenylalanine among essential amino acids for 1.63%, 0.52%, 0.97%, 0.93%, 1.5%, 0.52%, 2.49%, 0.39%, 1.91% and 1.52% respectively.
Contents of proteins and amino acids contained in organs and tissues of the beetles *Lytta caragana* and *Mylabris frolovi* are shown in table 4.

Table 4. Contents of amino acids in tissues and organs of blister beetles

Amino acids	*Lytta caragana*		*Mylabris frolovi*			
	The vetch(tillering)		Astragalus sp(flowering)		Termompsis(flowering)	
1	2	3	4	5	6	7
Proteins	(%)	(g/kg)	(%)	(g/kg)	(%)	(g/kg)
	48,10		60,08		58,50	
Non-essential amino acids						
Alanine	1,13	0,56	2,65	1,59	1,03	0,95
Aspartic acid	1,82	0,89	0,99	0,59	1,12	0,66
Glycine	2,47	1,21	2,72	1,63	2,51	1,47
Glutamic acid	3,07	1,50	2,42	1,45	2,16	1,26
Serine	1,93	0,95	2,64	1,59	1,56	0,91
Cystein						
Essential amino acids						
Arginine	0,84	0,41	1,62	0,97	1,68	0,95
Valine	0,86	0,42	0,69	0,41	0,52	0,30
Histidine	0,87	0,42	2,27	1,36	0,97	0,57
Lysine	1,28	0,62	0,77	0,46	0,93	0,54
Leucine	1,14	0,56	0,69	0,41	1,50	0,88
Methionine	0,91	0,45	0,76	0,46	0,52	0,32
Proline	2,08	1,02	0,32	0,19	2,49	1,46
Tyrosine	0,37	0,18	0,35	0,21	0,39	0,23
Threonine	2,27	1,12	2,47	1,48	1,91	1,12
Phenylalanine	1,44	0,71	2,49	1,49	1,51	0,88

Our measurement results were compared to contents of amino acids in meat and bone flour containing 45 to 50% proteins, measured in Russia. Meat and bone flour contains 0.7% cystein, 6.5% arginine, 4.8% valine, 1.6% histidine, 5.4% lysine, 5.8% leucine, 1.5% methionine, 3.3% threonine and 3.6% phenylalanine, and as compared to above contents, both non-essential and essential amino acids in the tissues and organs of blister beetles were quantitatively complete, but the contents were less. The beetles *Lytta caragana*were collected from the rangelands, where Astragalus sp plants grow, dried and grinded and then measurement in the Geological central laboratory revealed selenium concentration is 0.072 mg/kg. Measurements of minerals in organs and tissues of the beetles *Lytta caragana* demonstrated aluminum, magnesium, sodium, phosphorus, silica, zinc and copper concentrations were 0.01%, 0.50%, 0.03%, more than 1.0%, 0.003%, 0.003% and 0.001% respectively.
A number of scientists, especially Beauregard (1890), Beguin (1847) and Bluhm (1930) demonstrated blister beetle contains cantharidin, the genus *Lytta*2.6 to 4.06%, the genus *Mylabris*1,03% and the genus*Epicauta*2,02% […]. However,

our measurements of cantharidin in the beetles *Lytta caragana*, *Epicauta sibirica* and*Mylabris speciosa* according to method of Woodword proved its contents in above three beetles are 0.03%, 0.09% and
0.1% respectively, as well as its contents in the beetle belly, chest and head regions are 46.0%, 36.0% and 18.0% respectively. It can be predicted that cantharidin content in the body of beetle varies with species, structure, compositions and growth stages of the plants, which are eaten by the beetle, and season, during which they were collected. Graph 1. Content of cantharidin in blister beetles, %



Toxicity of cantharidin was determined by dividing animals into 5 groups using the doses of 0.6, 0.8, 1.0, 1.2 and 1.4 mg/kg respectively as described by Pershin, Miller and Tainter and obtained data were analyzed by two statistical methods to determine LD_{50} and LD_{100}.

$$LD_{50}\ LD_{100}\text{-}\frac{\sum(Z*D)}{m}$$

Z-arithmetic average of sums for dead mice at 2 consecutive doses
D-difference of two consecutive doses m- number of mice
in a group

$$LD_{50}LD_{100}\text{-}\frac{\sum(0,5*0,2)+(2*0,2)+(4*0,2)+(6*0,2)}{7}\ mg/kg=1,4\text{-}\frac{2,5}{7}=1,4\text{-}0,357=1,04\text{mg/kg}$$

Table 5. Results of calculation of toxicity of cantharidin by methods of Pershin, Miller and Tainter

Group	Dose, mg/kg	No. of animals		Mortality rate (%)	Probit
		Survived	Dead		
I	0,6	7	0	0	3,2
II	0,8	6	1	14,2	3,93
III	1,0	4	3	42,8	4,82
IV	1,2	2	5	71,4	5,57
V	1,4	0	7	100	6,8

$$LD_{50}\text{-}\frac{\sum(a+в)*(m-n)}{200}$$

a+в-sum of doses
m-n-difference of mortality rates

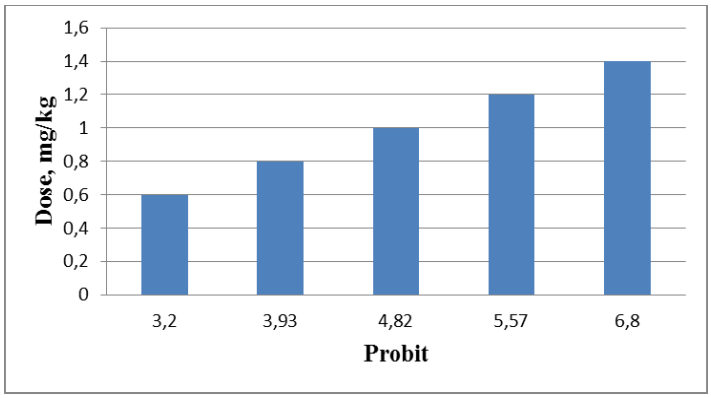
$$LD_{50}\text{-}\frac{(1,4*14,2)+(1,8*28,6)+(2,2*28,6)+(2,6*28,4)}{200}=\frac{208,12}{200}=1,04\ \text{mg/kg}$$

Thus, statistical analysis of the experimental data by above two methods demonstrated LD_{50}or amount of cantharidin per 1 kg body weight of animal was 1.04 mg. Based on the results of measurement of cantharidin toxicity, graphic construction and LD_{16} andLD_{84} were determined by probit analysis method, square waves and errors of median lethal doses were estimated (Graph 1).
Graph reveals it wasLD_{16}=0.7 mg/kgLD_{84}=1.37 mg/kg.

$$S_2LD_{50}=\pm\frac{1,37-0,7}{\sqrt{2*21}}=\frac{0,67}{6,4}=\pm0.1\ \text{mg/kg}$$

$$SLD_{50}=\pm t*SLD_{50}=1.04\pm2.04*0.1=1.04\pm0.204\text{mg/kg}$$

Graph 2.



Second graph shows cantharidin toxicity (LD_{50}) was calculated by method of Miller and Tainter. Use of 0.5% cantharidin in dose of 0.6 g/ha as a bait under pasture condition, where Brandt's voles (*Lasiopodomys brandtti Padde.1861*) are greater, has 83.5% technical effectiveness and it is seen the toxicity is higher. Statistical analysis was used to calculate outcome variables with percentages, and total variable was п-4, average variable x-83.5, standard deviation s-2.6, standard error s_x-1.3, relative error s_x-%-1.5, coefficient interval $x \pm t_{0,05} * s_x$- 83.5±4.0 percent.

Discussion

Pigulevskii (1906) determined cantharidin toxicity in laboratory animals and its general conditions. Ointment was made by using this compound, testing of its effects on animal lips and buccal mucosa revealed blisters resembling the burnt wound on the site of ointment use appeared and oral use of 0.1 to 0.6 mg/kg cantharidin in mature rabbit caused toxicity signs and some of the animals died. [9, 10].Kosorotov(1907) studied toxicity of compounds (tocopherol, cantharidin, etc.) contained in tissues and organs of blister beetles and reported 0.02 g pure cantharidin or 20 ml ethanol extract becomes lethal dose for human. Results of the present study on tocopherol contents in the beetles were compared to the contents of tocopherol in vitamin supplement and wheat germ oil measured in Russia. Valushkin and Shubin performed experiment by use of 10 to 50 mg tocopherol per kg weight of cow in order to induce estrus and enhance conception rate. According to our estimation, tocopherol prepared by us can be used in 35.5 mg/kg dose in cows with 500 kg live weight. Above shows cantharidin concentration is higher in soft tissues or belly region as reported by Boreger and Ulskii (1855), whereas cantharidin is contained in all regions of the beetles, and it is in agreement with the conclusions of Courber, Ferrer, Berthoud and Leidy (1860). Chemical and physical properties of cantharidin obtained by us were compared with those studies performed by overseas researchers, and its chemical formula is $C10H_{12}O_4$, its molecular weight 196 Da, melting temperature 272^0C, and it is discolored, clear crystal, not soluble in water and soluble in water, acetone, chloroform, dimethylsulfoxide, methyl, ethanol, dichloroethylene, methylcyclohexane and methanol as the similar properties. The solvents of cantharidin including 96% ethanol and dimethylsulfoxide, in which solubility represents 87.3% were selected as the best ones and they were consistent with those obtained by other researchers, and correct solvents were chosen in the present study.

Conclusions

1.Tocopherol ester concentrations in tissues and organs of the beetles *Lytta caragana, Mylabris frolovi* and *Epicauta sibirica* belonging to family *Meloidae* were 98.73 mg/g, 113.07 mg/g and 214.08 mg/g and its contents are explained in association with the plants eaten mostly by the beetles.

2.Our experiment on mice for activity of tocopherol prepared by us demonstrates that the dose effective for reproductive organs is seen to be 0.071 mg/kg, as well as comparison of the mice uterine weights with that of control animals reveals the uterine weight increased by 89.05 mg or 271.1 percent.

3. Toxicity of tocopherol isolated from beetles was measured to be 166.6 mg/kg and 307.6 mg/kg for LD_{50} andLD_{100} respectively.

4.Study on tocopherol use in egg laying hens in comparison with natural and medicinal vitamin E demonstrates egg production and egg weight increased by 2.4% and 2.02% respectively.

5.Percentage of proteins contained in the beetles *Lytta caragana* and *Mylabris frolovi*is 48.10% and 58.50 to 60.08% respectively, as well as concentrations of both non-essential and essential amino acids were measured.
6.Cantharidin contents in blister beetles *Epicauta sibirica, Lytta caragana* and *Mylabris speciosa* are 0.09%, 0.03% and 0.1% respectively and its contents in belly, chest and head regions are 46%, 36% and 18% respectively.
7.Toxicity of cantharidin was determined by the method of Pershin, Miller and tainter and LD_{50} was 1.04±0.204 mg/kg.

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