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# POTASSIUM PHENOSAN AS AN ADAPTOGEN TO STRESS

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## ABSTRUCT

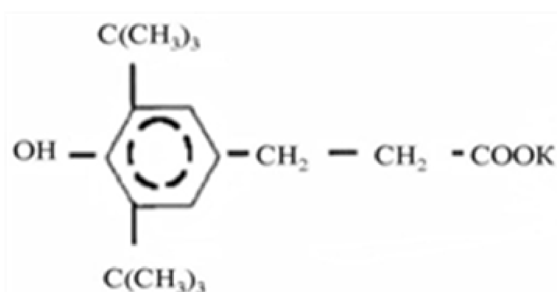
Stress lead to an increase in the generation of ROS by mitochondria. It is known that antioxidants reduce the generation of ROS by these organelles. One from them is an synthetic antioxidant potassium phenosan (3,5-di-tert-butyl-4-hydroxy-phenyl propionate potassium). The aim of this work was to elucidate the effects of acute hypobaric hypoxia (AHH) and injection to animals of the potassium on the fatty acid composition of the lipid fraction membranes and bioenergetical functions of rat liver mitochondria. AHH has caused changes in the fatty acid (FA) composition in lipid fraction of mitochondria and leads to dysfunction of these organelles. Injection to rats of $10^{-14}$mol/kg of potassium phenosan has prevented the changes pool of fatty acids in lipid fraction of mitochondrial membranes and helps to restore bioenergetic characteristics of mitochondria. The drug had caused the induction Bcl-2 in spleen cells of mice and it increased their survival rates under hypoxic conditions and low temperature stress. It is assumed that protective activity of the drug due to its antioxidant activity and influence of potassium phenosan on the activity of antioxidant enzymes.

**KEYWORDS:** ROS, mitochondria, lipid peroxidation? Fatty acids composition.

## INTRODUCTION

Under stress conditions there is activation of the sympathoadrenal system and increase, the level of catecholamines in the blood, which leads to release of $Ca^{2+}$ from vascular bed into cells. The increase in the concentration of intracellular $Ca^{2+}$ causes the accumulation of these ions in mitochondria, which leads to the increase of ROS generation, reactive nitrogen

species and to violation of the bioenergetic functions of these organelles, leading to the development of several pathological conditions.[1, 2] In connection with this rather urgent problem is finding new drugs, adaptogens, that increase resistance to stress. For the first time the term "adaptogens" was introduced by N.V.Lazarev (1959, 1962), who formulated the concept on the state of non-specifically increased resistance of organism. Drugs that cause this condition were named "adaptogens." He and co-workers demonstrated the ability of these drugs to increase the body's resistance to a wide range of damaging effects: hypoxia, sharp fluctuations of temperature, intoxications, infectious agents, etc.[3] The similarity of the effects produced by a completely different pharmacological agents, allowed N.I. Lazarev to make a conclusion that there is a single non-specific mechanism of increasing resistance of the organism. In stress condition is a shift of antioxidant-prooxidant balance in the direction of increasing the content of ROS in the cell. According to the literature one of the main sources of ROS under stress condition are mitochondria[4, 5], which are both the source and target for ROS. Excessive generation of ROS, leads to oxidation of the thiol groups of proteins, activation of lipid peroxidation and to swelling of mitochondria.[6, 7] In addition, excessive generation of ROS can influence on functional state of the redox-sensitive intracellular signalling pathways starting the program cell death.[8] Under these conditions, are formed toxic to cells products: aldehydes and 4-hydroxy-2, 3-nonenal. These toxicants inhibit enzymes involved in basic metabolic pathways, mainly in the citric acid cycle, they affects the electron transport in the respiratory chain of mitochondria due to depletion of the pool of NADH in the mitochondrial matrix.[9] In this regard, it could be assumed that these preparations should primarily to affect the generation of ROS by these organelles. For this role is primarily have pretensions of antioxidants, particularly synthetic phenolic antioxidants, having relatively high coefficients of interaction with peroxyl radicals (k7).[10] As the object of research was selected a drug, that represents spatially hindered phenol -: potassium phenosan (3,5-di-tert-butyl-4-hydroxy-phenyl propionate potassium):




**Potassium phenosan**

The aim of this work was to study the influence of this drug on the functional state of rat liver mitochondria in model experiments and in stress conditions. In addition in the work studied the effect of potassium phenosan in doses $10^{-14}$ -$10^{-4}$ mol/kg on the content of antiapoptotic protein Bcl-2 in the spleen of the mice of line F1(CBA × C57BI.). The choice of this object of study is related to the fact that one of the functions of this protein, which prevents the implementation of programmed cell death through apoptosis, constitutes also an impact on the survival and recognizability of hematopoietic cells in the centers of reproduction.[11]

## MATERIAL AND METHODS

The experiment was performed in the Institute of biochemical physics. N. M. Emanuel Russian Academy of Sciences. Tests was performed on male rats of Wistar line weighing 100-120 g.

### Regulatory standards

The study was performed according to the Rules of laboratory practice in the Russian Federation, in accordance with the rules adopted by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123), Strasburg, 1986). The research was performed according to the approved protocol, in accordance with standard operating procedures of the researcher (SOPR), as well as with the Guidelines at laboratory animals and alternative models in biomedical researches on laboratory animals.[12] An experimental Protocol was developed with the participation and approval of the bioethical Commission of the Institute.

### Isolation of mitochondria

Isolation of mitochondria was performed by differential centrifugation.[13] The first centrifugation at 600 g for 10 minutes, the second at 9000 g, 10 min. The pellet was re-suspended in the medium, containing: 0.25M sucrose, 10 mM HEPES, pH 7,4.

### Rate of mitochondria respiration

Respiration of mitochondria was recorded polarographically (an LP-7 polarograph, Czech Republic) using a Clark oxygen electrode. The incubation medium contained 0.25 M sucrose, 10 mM Tris-HCl, 2 mM $KH_2PO_4$, 2 mM MgCl2, 10 мM KCl (pH 7.5) (28°C).The rate of respiration was expressed in ng- mole $O_2$/(mg protein min).

**Lipid peroxidation (LPO) activity**

LPO activity was assessed by fluorescent method.[14] Lipids were extracted by the mixture of chloroform and methanol (2:1). Lipids of mitochondrial membranes (3–5 mg of protein) were extracted in the glass homogenizer for 1 min at 10°C. Thereafter, equal volume of distilled water was added to the homogenate, and after rapid mixing the homogenate was transferred into 12mL centrifuge tubes. Samples were centrifuged at 600 g for 5 min. The aliquot (3 mL) of the chloroform (lower) layer was taken, 0.3 mL of methanol was added, and fluorescence was recorded in 10mm quartz cuvette with a spectrofluorometer (FluoroMaxHoribaYvon, Germany). Background fluorescence was recorded using a mixture of 3mL chloroform and 0.3 mL methanol. The excitation wavelength was 360 nm, the emission wave length was 420–470 nm. The results were expressed in arbitrary units per mg protein.

**Fatty acid methyl esters (FAMEs)**

FAMEs were produced by acidic methanolysis of mitochondrial membrane lipids.[15,16] Mitochondrial suspension (200 μL) was placed in a special hermetically closed tube, 5 mL of methanol was added, and the sample was placed in the freezer for 1 h. Thereafter, 600 μL of acetyl chloride was added, and the sample was boiled for 1 h with stirring. FAMEs were extracted with hexane, and solutions obtained were analyzed.

**FAME identification**

FAME identification was performed by chromato mass spectrometry (CMS) using a Hewlett-Packard-6890 spectrophotometer with a HP-5972 mass-selective detector and after the retention times.[17] FAME were separated in the HP-5MS capillary column (30 m ×0.25 mm, phase film thickness of 0.25 μm) at programmed temperature increase from 60 to 285°C at the rate of 5°C/min. Evaporator temperature is 250°C, detector temperature is 280°C. Mass spectra were obtained in the regime of electron impact ionization at 70 eV and the scan rate of 1 s/10 mass in the scan mass range of 40–450 a.u.m.

**FAME quantification**

FAME quantification was performed using a Kristall 2000M chromatograph (Russia) with flame-ionization detector and quarts capillary column SPB-1 (50 m×0.32 mm, phase film thickness of 0.25 μm). FAME analysis was performed at programmed temperature increase from 120 to 270°C at the rate of 4°C/min. Temperature of injector and detector – 270°C; the helium carrier gas rate was 1.5 mL/min. Each sample contained 2 μL of the hexane extract.

The FAME content in samples was calculated as the ratio of peak area of a corresponding acid to the sum of peak areas of all found FAMEs.

**The index of double bond (DB)**

The index of double bond (IDS), which characterizes the degree of unsaturation of lipids, was calculated according to the formula: IDS=ΣPjnj/100, where Pj is the contents of the fatty acids (FA) (in %), nj is the number of double bonds in each acid. Also we used the unsaturation coefficient (K) as the ratio of the sum of unsaturated FA to the sum of saturated FA.

**Determination of Bcl-2 protein**

Determination of Bcl-2 protein in cells of the spleen tissue was performed on mice line F1 (CBA × C57BI) weighing 19-20g. Potassium phenosan was administered at a dose of $10^{-4}$ M/kg. Determination of the protein content of Bcl-2 in the cells of spleen tissue was performed by immunoblotting. In the experiment used monoclonal antibodies "Monoclonal Anti-Bcl-2 clone10C4", and immunoglobulin anti-rabbit IgG (Sigma), labeled with horseradish peroxidase.

Determination of protein concentrations was carried out using a set of AES Stainig Kit ("Sigma Aldrich").

**Statistics**

Tables and figures present means values and their standard deviations. The number of experiments was 10.

**Protective activity of the drug was investigated using the model of acute hypobaric hypoxia and model muscle load.**

**Model of acute hypobaric hypoxia**

Modeling of acute hypobaric hypoxia in rats was carried out in the a hyperbaric chamber in low-pressure atmosphere (230.40 mm Hg. tbsp.), which corresponds to the height of 9000 m above sea level. In the first minute in the chamber created the rarefaction corresponding to the 5 thousand meters (corresponding to the atmospheric pressure of 405 mm Hg. tbsp.) above sea level. In each subsequent minute "ascent" carried out on the further one thousand meters. Time staying of rats "at a height" of 9.0 thousand meters above sea level - 5.0 minutes).

**Model of muscular load with the low-temperature stress:** We measured swimming time of mice in the tub with a load of 500 mg at a temperature of 4°C.

To all test animals the preparation was introduced intraperitoneally at a chosen dose 45 minutes before the event.

**The following reagents were used:** potassium carbonate, methanol, chloroform (Merck, Germany), hexane (Panreac, Spain), acetyl chloride (Acros, Belgium), sucrose, Tris, EDTA (Ethylenediaminetetraaceticacid), FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone), malate, glutamate, succinate, ADP, (Sigma, Aldrich, USA), Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Biochemica Ultra, for molecular biology) (MB Biomedicals, Germany).

## RESULTS AND DISCUSSION

Since under stress the main source of ROS are mitochondria, it was necessary to develop a model simulating stress, i.e., to find conditions under which increases ROS production by mitochondria and, consequently, will be activated LPO.[18] We solved this problem by having developed a model of "aging" (the mitochondria isolated from rat liver was incubated in a hypotonic medium at room temperature). To activate LPO mitochondria were placed on 15 minutes in 0.5 ml of medium containing 70 mm KCl, 10 mM HEPES and 1 mM $KH_2PO_4$, pH 7,4. The incubation of mitochondria in a hypotonic saline solution of caused a weak swelling of mitochondria and growth of the ROS generation that resulted in a 3 to 4-fold increase in the intensity of fluorescence of LPO products (fig.1).

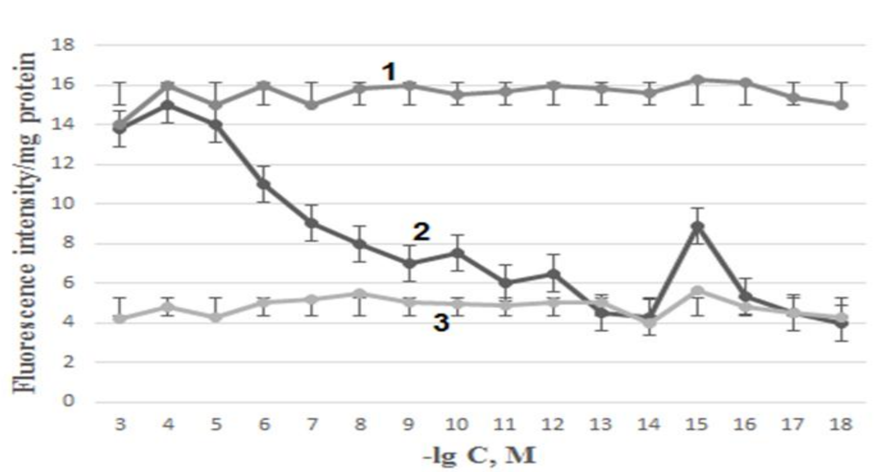



**Fig. 1. The effect of different concentrations of potassium phenosan and "aging" on the intensity of fluorescence of LPO products. Legend: 1- «aging»; 2- «aging» +-potassium phenosan; 3- control.**

The introduction of potassium phenosan into the incubation medium of mitochondria caused the reduction of LPO intensity, which shows dependence from dose. The drug concentration of $10^{-5}$; $10^{-6}$ and $10^{-7}$ M had little effect on the intensity of lipid peroxidation. In the concentrations of $10^{-8}$-- $10^{-14}$ and $10^{-18}$-$10^{-22}$M potassium phenosan was reduced the fluorescence intensity of LPO products to the control level. These data suggest that the most effective use of the drug when administered to animal in concentration range $10^{-9}$-$10^{-14}$M.

Since the drug at the model of "aging" had reduced the intensity of the LPO, it could be assumed that it will exhibit protective activity in stress conditions. As exposure to stress used the model of acute hypobaric hypoxia (AHH), as the choice of this model is due to activation of lipid peroxidation (LPO) and mitochondrial dysfunction under conditions of AHH.[19] AHH led to LPO activation in the mitochondrial membranes of pea seedlings. In this case, the fluorescence intensity of LPO products increased 1.5- 3 times (Fig. 2).

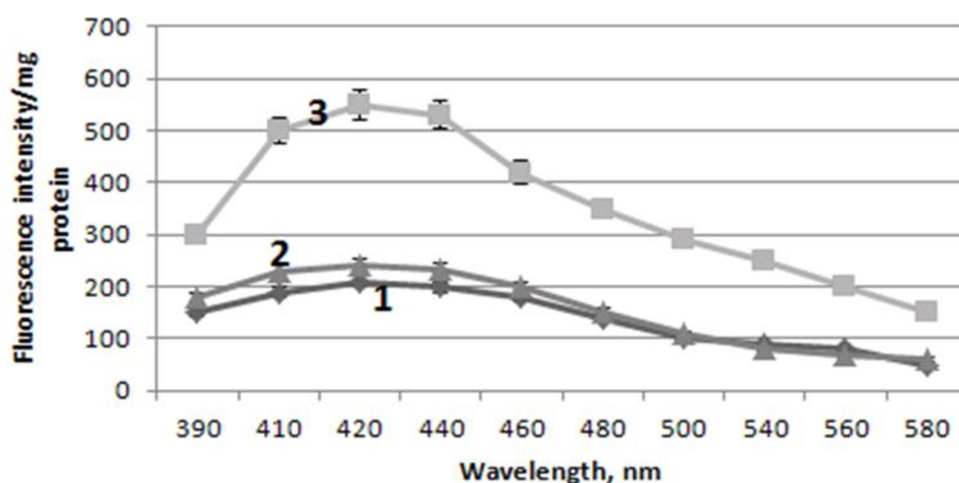



**Fig 2. Effect of acute hypobaric hypoxia (AHH) and potassium phenosan (Phen) on the spectra of fluorescence of LPO products. X-axis: the wavelength in nm; Y-axis: fluorescence intensity in arbitrary units/mg protein.1- Control; 2- AHH+Phen; 3- AHH.**

In this case, there was a decrease of the relative percentage content of unsaturated fatty acids (FA) with 18 carbon atoms and an increase in stearic acid content in the lipid fraction of mitochondrial membranes, leading to a reduction ΣSjn/C18:0, where Cj - is the content of unsaturated fatty acids (relative percent), n- is number double bonds. In the control group the ratio was 2.70 ± 0.12, but at the AHH it decreased to 1.18 ± 0.20. It should be noted that in terms of AHH most significant changes were observed in the content of linoleic and linolenic acids. The Injection to animals $10^{-14}$ mol / kg of potassium phenosan prevents

changes in the FA composition, containing 18 carbon atoms in the mitochondrial membranes even in conditions of AHH (Fig. 3).

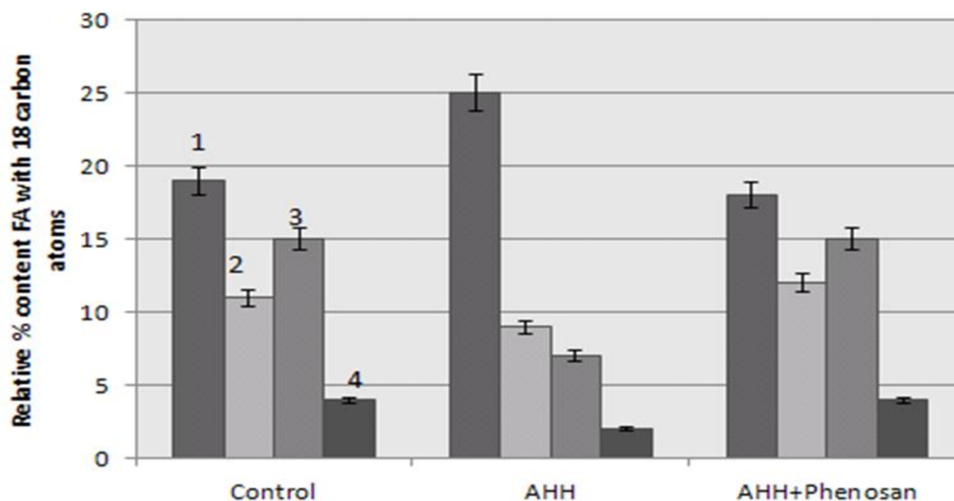



**Fig.3. The influence of the AHH and potassium phenosan on the relative content FA with 18 carbon atoms in %. 1-18:0; 2-18:1 ω9; 3-18:2 ω6; 4-18:3 ω3**

Changes in the lipid composition of mitochondrial membranes, probably entail changing and lipid-protein interactions and, consequently, and of the activity of membrane-bound enzymes, in particular, enzymes of the mitochondrial respiratory chain. Indeed, AHH resulted in a reduction in the maximum rates of oxidation of NAD-dependent substrates (table 1). At the same to 35% decreased, and the efficiency of oxidative phosphorylation.

**Table1.The effect of acute hypobaric hypoxia and potassium phenosan on the rates of oxidation of NAD-dependent substrates (oxidation rate are presented in ng. mol $O_2$/mg protein × min). (The number of experiments is 10).**

| Goup | Vo | $V_3$ | $V_4$ | $V_3$ /$V_4$ | FCCP |
|---|---|---|---|---|---|
| Control | 6.5 ±1.4 | 28.1±1.1 | 8.0±0.4 | 3.51±0.04 | 27.5±1.0 |
| AHH | 7.3±1.2 | 21.2±1.6 | 9.3±0.2 | 2.27±0.03 | 19.4±2.0 |
| AHH+Phenn | 8.3±1.6 | 28.4±1.3 | 7.8±0.7 | 3.62±0.07 | 32.4±2.5 |

*The incubation medium contained: 0.25 M sucrose, 10 mM Tris-HCl, 2 mM $KH_2PO_4$, 5mM $MgSO_4$, 10 mM KCl, pH 7.5. Further additives: 200µM ADP, $10^{-6}$M FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), 4 mM glutamate, 1 mM malate. Legend: AHH- acute hypobaric hypoxia; Phen- potassium phenosan, Vo - substrate oxidation rate; $V_3$ - substrate oxidation rate in the presence of ADP; $V_4$ - the rate of oxidation at rest (substrate oxidation rate at exhaustion ADP).*

Injection to rats $10^{-14}$M potassium phenosan for 45 minutes before impact has prevented changes in the functional characteristics of liver mitochondria: the rate of oxidation of NAD-dependent substrates in the presence of ADP and in the presence of FCCP did not differ from control values. At that the respiratory control rate (RCR) was comparable with the corresponding rates in the control group of animals. The drug prevented the activation of LPO and changing the fatty acid composition of mitochondrial membranes, indicating a correction of functional characteristics of mitochondria using this drug.

Moreover, the injection of the drug to mice line F1 (CBA × C57BI) led to induction of anti apoptotic protein Bcl-2 in the cells of the spleen (Fig.4).

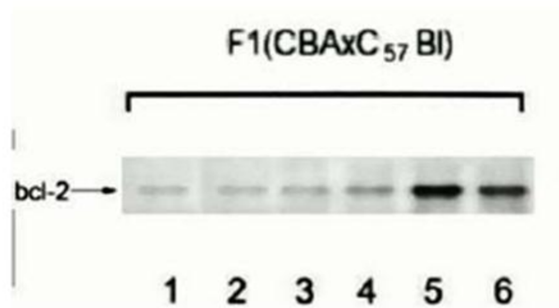



**Fig. 4. Immunoblotting of proteins Bcl-2 in the extract of F1 mice spleen (on the 4th day after injection of potassium phenosan): (1,2) - control; (3.4) – $10^{-14}$mol / kg; (5, 6)) – $10^{-4}$ mol /kg.**

At that potassium phenosan causes an increase in the content of anti-apoptotic protein Bcl-2 in the serum of the mice of AKR line.[20] It is significant that the influence of potassium phenosan on the induction of Bcl-2 had a direct dependence on concentration: with increasing concentration the effect was amplified. We can assume that potassium phenosan, which affecting the induction of anti-apoptotic proteins Bcl-2, may have a number of positive effects, such as reparation, adaptation, anti-inflammatory action. Indeed, by path changing the structural-functional characteristics of mitochondria, the drug had provided a protective effect under stress. Intraperitoneal injection of $10^{-14}$M potassium phenosan at 3.5-4.5 times increased the lifespan of mice in conditions of hypoxia and low temperature stress.

## CONCLUSION

We can assume that the protective activity of the drug, probably due to its antioxidant activity. Indeed, the effective coefficient of interaction of potassium phenosan with peroxyl radicals during the oxidation of methyloleate ($60^o$) $k_7$ is $2.2 \times 10^4$ $(MS)^{-1}$.[21] In addition,

potassium phenosan in ultra-low concentrations ($10^{-9}$-$10^{-10}$ and $10^{-14}$-$10^{-16}$M) caused the modify in the microviscosity of the deep-lying lipid bilayers (~20 A) of the membranes of the endoplasmic reticulum (ER). These changes correlated with the inhibition of LPO, in the presence of of potassium phenosan - (r=0,551; p=0.033).[22] By reducing the intensity of lipid peroxidation in the membranes of mitochondria, the drug prevents the reduction of $C_{18}$ unsaturated fatty acids in lipids of mitochondrial membranes, mainly the reduction of linoleic acid, which is one of the main acids included in the composition of cardiolipin - phospholipid, which provides high functional activity of mitochondria. The injection of potassium phenosan to animals (mice) influences the activity of antioxidant enzymes: the drug reduces the activity of Mn-SOD at 1.5 times and increases the activity of Cu,Zn-SOD is 1.5 times, at that occurs the changed activity of glutathione peroxidase (GP) and glutathione reductase (GR), However, the ratio of the activities of GP/GR remained constant.[23] This is accompanied by the induction of the anti-apoptotic protein Bcl-2, which prevents the programmed cell death. By reducing the excess generation of ROS by mitochondria, the drug effected on aging processes[24], significantly increasing the lifespan of mice leukemic line AKR. Thus, the protective effect of potassium phenosan, is probably associated with its antioxidant activity and influence of potassium phenosan on the activity of antioxidant enzymes.

**REFERENCES**

1. Bachurin S O., Shevtsova E., Kireeva E.G., Oxenkrug G.F., Sablin S.O. Ann. N.Y. Acad. Sci., 2003; 993: 334-344.
2. Robinson A.D., Ramanathan K.B., McGee J.E., Newman KP, Weber KT. Am. J. Med. Sci., 2011; 342: 129-134.
3. Yaremenko K. V. Psychopharmacology and biological narcology, 2005; 5(4): 1086 -1092 (in Russian).
4. Todorov I. N. Russian chem. Journal. 2007; 51: 93-106 (in Russian).
5. Kirkinezos I. G., Moraes C. T (2001). Cell & Develomental Biology, 2001; 12: 449–457.
6. Vladimirov Yu. A., Olenev V. I., Suslova T. V., Cheremisina Z. V. Adv. Lipid Res., 1980; 17(1): 173–249.
7. Munoz-Pinedo C., A. Guıo-Carrion, J. C. Goldstein, P. Fitzgerald, D. D. Newmeyer, D. R. Green. PNAS, 2006; 103(31): 11573–81.
8. Ryazantseva N.V., Novitsky V. V., Chasovskikh N. Yu., Kaygorodova N.V., Starikova E. G., Starikov Yu.V., Radzivil T. T., Krat I.V. Cytology, 2009; 51(4): 329-334.(in Russian)

9. Tailor N.L., Day D.A., Millar A.H. J. of Exp. Botany, 2003; 55(394): 1-10.
10. Kovtun G.A. Catalysis and Petrochemicals, 2000; 4: 1-9. (in Russian)
11. Kopnin B.P. Biochemistry, 2000; 65(1): 5-33.(in Russian).
12. Karkishchenko N. N, S. V. Grachev. Guidance on laboratory animals and alternative models in biomedical research. 2nd ed. Moscow; Profile: 2010, pp. 358.
13. Mokhova E.N., Skulachev V.P., Zhigacheva I.V.. BBA, 1977; 501: 415-423.
14. Fletcher B.I., Dillard C.D., Tappel A.L. Ann Biochem., 1973; 52: 1–9.
15. Carreau J.P., Dubacq J.P.. J Chromatogr., 1979; 151: 384–90.
16. Wang J, Sunwoo H, Cherian G, Sim IS. Methods Poultry. Sci., 2000; 79: 1168–71.
17. Golovina R.V., Kuzmenko T.E. Chromatogr., 1977; 10: 545–546.
18. Pryor W.A., Porter N.A. Free Rad. Biol.Med., 1990; 8: 541–543.
19. Lukyanova L. D. Bulletin of experimental biology and medicine.1997; 124 (9): 244-254. (in Russian)
20. Mil', E. M., Albanova A. A., Burlakova E. B. Radiation Biology. Radioecology. 2010; 50 (1): 58-64. (in Russian).
21. Perevozkina M.G. Kinetics and mechanism of inhibitory action of derivatives of phenosan, salicylic acid and their synthetic mixtures with α-tocoferol and phospholipids. Abstract of dissertation for the degree of Candidate of Chemical Sciences, Tyumen, Tyumen State Medical Academy: 2003, pp.24. (in Russian).
22. Palmina N. P. Chasovskaya E., Belov V. V., Mal'tseva E. L. Reports of the Academy of Sciences, 2012; 443(4): 511-515. (in Russian).
23. Gurevich S. M., S. M. Kozachenko, L. G. Nagler. Changes in the activity of antioxidant enzymes under the influence of potassium phenosan in small doses. In: VII international conference Bioantioxidant. 25-26 October 2006, Moscow, Moscow, Publishing house of Russian Peoples Friendship University: 2006, pp.100-102. (in Russian).
24. Erokhin V. N., Semenov V. A., Krementsova A.V., Burlakova. Vegetable and synthetic antioxidants in the prevention of cancer. In: Materials of the IX International symposium "Phenolic compounds: fundamental and applied aspects, 20-25 April 2015, Moscow, Moscow, Publishing house of Institute of plant physiology: 2015, pp. 540-544. (in Russian).