Research Article

The Humic Acids of Peat. Physico-Chemical Properties and Biological Activity in Erythrocytes

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ABSTRACT

Properties of the humic polyelectrolytes (HPE) - both fulvic acids (FA) and mixture of humic (HA) and fulvic acids extracted by water-ethanol solution from peat were studied using IR-, UV-vis-, AA-spectroscopies and by titration. The total content of carboxylic and phenolic groups c^s determined on the base of titration of the HPE by $Ca(OAc)_2$, NaOAc and NaOH solutions was equal to 4,4±0,1 mmol-eq/g. The content of acid groups of the fulvic acids extracted from the HPE by water was equal to 6, 5±0,2 mmol-eq/g. According to IR spectra the FA and the HPE are riched in alcohol and phenolic groups. The hydrophobicity index (HI) calculated as a ratio of absorption at 2328 cm^{-1} (contribution of the nonpolar fragments of the skeleton) to absorption at 1050 cm^{-1} (contribution of polar C-O bonds) was equal to 0,87 for the fulvic acids and 1,11 for the HPE, consequently. The variation of sorption capacity of the HPE in relation to biogenic metals was observed in the following order (mmol-eq/g): Cu (7,6)>Zn (5,6)>Ca (4,3)>Fe (3,1). It may be proposed that not only carboxylic and phenolic groups but quinoid and amide groups participate in the complexation. The improving of energy metabolism catalyzed by lactate dehydrogenase, the decreasing of lipoperoxidation characterized by dose-dependent reduction of malonic dialdehyde level (by 30-70%) and the increasing of SOD activity were estimated *in vitro* by blood of rats.

Keywords: Humic acid, fulvic acid, antioxidant, lactate dehydrogenase, chelation of transition metal.

INTRODUCTION

Humic acids are the class of naturally occurring compounds which are constituents of soil, peat, natural water, coal, mumie and other products of plants, microbes and animals degradation by chemical or biological processes. Humic acids are macromolecules containing different functional groups such as carboxylic, aromatic, phenolic hydroxyl, ketone and quinoid structure.[1]It may be considered as a weak humic polyelectrolytes HPE which are distinguished according to chemical structure, solubility, molecular weight, content of nitrogen and ratio of the C:O.

HA and FA are used in medicine as the prospective compounds. The researches *in vitro*, *in vivo* and in clinical practices are proved their antimicrobial, anti-inflammatory, antitumor, antiulcer, reparative, vulnerary properties and the high efficiency of HPE for the treatment for skin burns.[2-3]

The spectrum of diseases which are effectively treated by FA is continuously expanding. HPE has been used externally to treat haematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis and osteochondrosis. Besides, FA has beentaken orally as a therapy for gastritis, diarrhoea, stomach ulcers, dysentery, colitis and diabetes mellitus.[2] Humic acids as a part of mumie have demonstrated the effectiveness in the treatment of the gerontological diseases in the experiment on rats.[3]

Biochemical conditions that can lead to many of the mentioned diseases are excessive production of there active oxygen species (ROS) and increased production of the free radicals (LPO). The main causes which determine excessive production of the ROS are: 1) the deficiency of the cytochrome oxidase activity in the mitochondria that lead to leakage of electrolytes from the oxidative phosphorylation processes; 2) the unbalanced activity of the superoxide dismutase (SOD) 3) the changes in iron homeostasis. The last factor is the most important in the development of Parkinson's disease which element of therapy is the chelation of Fe^{3+}.[4-6] The presence of reactive groups such as catechol and phenolic hydroxyls in FA and HA determines their antioxidant properties similar to the esters of gallic acids, tannins and ellagic acids effectively protecting the human erythrocytes from hemolysis under the action of ROS.[7]The protective effect of the above-mentioned compounds was also shown in the regulation of the level of lactate dehydrogenase (LDH) in the erythrocytes.[8] The high level of the LDH index is found in the necrosis tissue especially under acute heart failure and damages of kidneys, skeletal muscles, liver, lungs and skin.

In this paper we studied physico-chemical characteristics of the humic acids derived from peat by extraction of water-ethanol and estimated their biological activity *in vitro* in rat erythrocytes. For this purpose we studied: 1) the IR- and UV-vis spectra of the FA and the HA; 2) the content of acid groups; 3) the part of water soluble

fraction of the humic acids as the FA; 4) the sorption of the transition metal ions – Fe(III), Zn, Cu; 5)antioxidant properties of the humic acid and their influence on the energy metabolism in therat erythrocytes.

MATERIALS AND METHODS

The fulvic acids and the mixture of fulvic and humic acids (peat of Nizhny Novgorod, Beauty Land Company, Russia), sodium hydroxide, sodium carbonate, hydrochloric acid, ethanol, purified water (resistivity ≥18 M⍰·Cm).

Total nitrogen was assayed by Kjeldahl method (VELP Scientifica). Absorption spectra of the aqueous solutions were recorded by "Bio line Specord S-100" (Analytik Jena). IR spectra were recorded on «IR Prestige-21» (Shimadzu, Japan) in a range of 4000-500 cm^{-1} (tabl. KBr).Potentiometric titration was provided on pH-meters "pH-150M" (Gomel, Belarus). Content of Fe, Zn, Cu, and Ca were determined by the atomic absorption spectrometry (AAS Shimadzu AA 7000, Japan).

Direct Potentiometric titration of HPE

100 ml of the FA solutions and the mixture of FA and HA (10 mg/%) were titrated by 0.1M NaOH and 0,1M Na_2CO_3. The system was stirred until equilibrium was established after each addition of titrant (0.2 ml). The equivalent points were determined from values of pH at the end of the titration of carboxyl (pH 7.0) and hydroxyl (pH 10-11) groups.

Back potentiometric titration of HPE

0,1M NaOH solution was added to the HPE solution up to the values pH 12. The titrant was 0,1M HCl. The system was stirring until the stable pH value was obtained.

Determination of acid groups by an acetate method

The HPE (0.01 g) was treated with 2 ml of ethanol; 10 ml of a 0,1M solution of sodium acetate and 40 ml of water were added. The reaction mixtures were stirred at 100°C in a flask with a reflux condenser for 40 min. The precipitate was filtered off and thoroughly washed with 100 ml of water and the set free acetic acid was titrated by 0,05M KOH in the presence of phenolphthalein. The content of acid groups c^s was calculated by the equation

$$c^s = \frac{V(KOH) \bullet c(KOH) \bullet V_1}{m \bullet V_2} (\text{mmol-eq/g}),$$

where V(KOH) –the volume of KOH used for the titration of the sample, ml; c(KOH) – concentration of the KOH, M; m –the weight of solid, g; V_1 – the total volume, ml; V_2 – the volume of the aliquot, ml.

Elemental analyses on content of Fe, Zn, Cu, Ca were provided by using the atomic absorption spectrometry (AAS Shimadzu AA 7000, Japan) with the reference salt solution on Fe, Zn, Cu, Ca and the hole-cathode lamps at the wavelength λ, nm:248,3; 213,9; 324,8; 422,7,accordingly. Acetylene-air flame was used to analyze all elements with the rate of 2,2 $L \cdot min^{-1}$.

Elemental analysis of sorption capacity of the HPE

The excess of $Ca(OAc)_2$ aqueous solution was added to the suspension (HA + FA) while stirring. The reaction mixture was kept for 24 hours, and then the precipitate was filtered off, washed with water and dried. The calcium concentration was determined by the AAS method.

Biomedical research

The experiment was made *in vitro* by the blood of Wistar rats received from "Stolbovaya" nursery station of Federal State Institution of Science «Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency» (*Russian Federation, Moscow*). All animals were kept under standard conditions with free access to food and water according to the rules of the ET/S 129, 1986 and the directives 86/609 ESC.

2 L, 5 L, 10 L of the aqueous solutions of HPE (10 mg/%) were added to the whole blood (1 ml) stabilized by sodium citrate. The erythrocytes were washed two times by 0.9% NaCl solution, then were centrifuged for 10 minutes at 1600 g. The level of malonic dialdehyde as a marker of lipid peroxidationin the plasma and erythrocytes was measured according to the M.Ukiyama, M.Mihara methods. The SOD activity (SOD, EC 1.15.1.1) in the hemolysate of washed red blood cells (1:10)was determined by inhibition of the auto oxidation product of adrenaline.[9] The lactate dehydrogenase activity (LDH, EC 1.1.1.27) was determined in the hemolysate of washed red blood cells (1:40). Catalytic activity of the LDH in direct reactions (LDG_{dir}) was estimated by using the 50 mM sodium lactate as a substrate; but the indirect reaction (LDG_{ind}) –by using the 23 mM sodium pyruvate.[10] Calculation of the specific activity of enzymes was made according to the modified Lowry method.[11]

RESULTS AND DISCUSSION

Physico-chemical properties

The concentration of acid groups of the humic acids c^s calculated according to the data from direct and back potentiometric titration was close to 4,4-4,5 mmol-eq/g and 6,1-6,4 mmol-eq/g for the samples (HA + FA) and the FA, respectively.[12,13]Itisassumedthatthevalue of c^s determines the concentration of carboxyl groups at pH 7and the values of c^s correspond to the sum of phenolic and carboxyl groups at pH 10-11 (Figure 1).

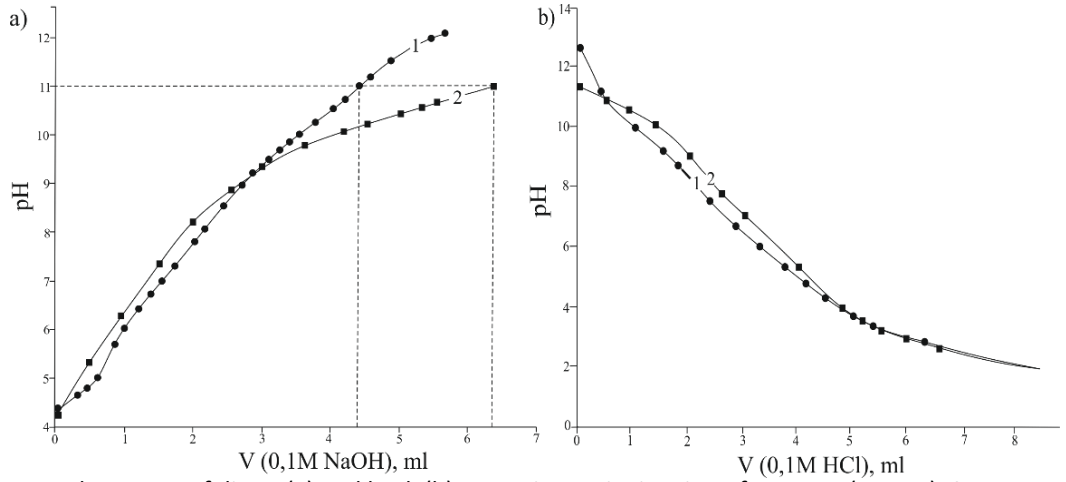


Figure 1: The curves of direct (a) and back (b) Potentiometric titration of HPE: 1 – (HA+FA); 2 – FA.

The results of FA titration by the acetate method are underestimated in comparison with Potentiometric titration of the samples (Table 1).

The concentration c^s of acid groups of the (HA + FA)determined by atomic absorption spectroscopy using salt complexes of calcium as products of reaction of the humic acids with calcium acetate was close to c^s estimated by Potentiometric titration (4,3 mmol-eq/g). These results confirm that Potentiometric titration of the humic acids as weak polyelectrolyte is the valid method.

Table 1: The assay of acid groups in the HPE.

Sample	Methods		c^s, concentration of acid groups, mmol-eq/g
	Reagent	Assay	
FA	0,1M NaOH	Direct potentiometry	6,4±0,03
	0,1M NaOH 0,1M HCl	Back potentiometry	6,1±0,02
	0,M CH_3COONa 0,05M KOH	Acetate method, back titration	4,6±0,05
HA+FA	0,1M NaOH	Direct potentiometry	4,5±0,05
	0,1M NaOH 0,1M HCl	Back potentiometry	4,4±0,07
	0,1M CH_3COONa 0,05M KOH	Acetate method, back titration	3,8±0,04
	$Ca(OAc)_2$	AAS	4,3±0,01

The total concentration of acid groups of HPE reflects the ion exchange and chemisorptions properties that corresponds to the sorption capacity or capacity of absorption.[12,13]When the biogenic transition metal ions have been used, the sorption capacity of the HA + FA in the form of soluble salts was changed in the order (mmol-eq/g): Fe^{3+} (3,1) <Zn^{2+} (5,6) < Cu^{2+} (7,6). The high chelating ability of the HA + FA by transition metals ions may be explained by the presence of not only carboxyl, phenolic, catechol groups, but also keto-, amido- and quinoid structures.

The data of IR-spectra confirm and supplement the information about chemical nature of functional groups of the HPE (Figure 2, Table 2). The IR spectra were analyzed by using the literature data of IR-spectra of different fulvic acids samples of peat (Table 2).[14-18]

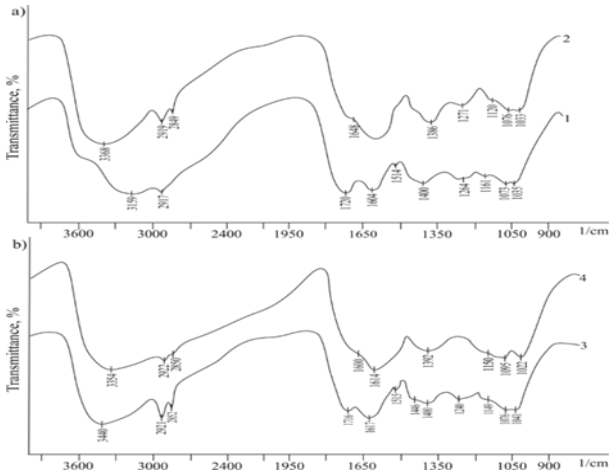
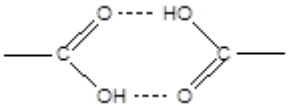


Figure 2: IR-spectra: 1 – fulvic acid; 2 – Na-FA; 3-HPE; 4 – ferric humate.

The main functional groups which humic acids may contain in composition of its heterogeneous structure are presented in the Table 2.



The presence of un dissociated carboxylic groups are confirmed by stretches of the $(C = O)_{st}$ groups in 1720-1680 cm^{-1} range, stretches of the $(C-O)_{st}$ bonds between 1315 and 1200 cm^{-1}.The absorption bands in 1400-1200 cm^{-1} range are due to the interaction between the planar deformation of the O-H bonds and the stretches of the C-O dimers. The dimers have a broader absorption bands of stretches of the O-H bonds in 3500-3000 cm^{-1} range (Figure 2, Table 2, data on the SRFA samples, the PPFA, referred to in the article, and the FA sample).[8]

Table 2: Data of IR-spectra of the humic acids.

Samples		Wave number, cm^{-1}						
		OH_{st}, $NH_{st.}$ alcohols, phenols, acids, amines	CH_{3st} CH_{2st} CH_{st} alkyl groups	$C=O_{st}$ ketons, acids, quinones. $C=O_{st}$ (amide I)	$C=C_{st}$ $C=O_{st}$ amide II, aromatic cycles $(COO^-)_{st, as}$	$C-OH_{st}$ $C-OH_{st}$ $(COO^-)_{st, s}$ acids, phenols	$C-O_{st}$ alcohols, phenols, esters	HI
FA	SRFA[8] (IHSS)	–	2942 wk	1722, 1623 shoulder	–	1393, 1206	–	1,10
	PPFA[8] (IHSS)	–	2942 wk	1722 str, 1623 shoulder	–	1400, 1233	–	1,13
	SAFA[8] (Shanghai Aladdin Reagent Company)	3393 str, brd	2850 wk	1593	–	1398	1041	0,87
	FA[16]	3600-3100 wk, brd	2850 wk	1670-1635 wk	1550-1540 wk	1410 str	1130-1110	–
	FA (Beauty Land Company, Nizhny Novgorod)	3300-3100 str, brd	2920 wk 2850 wk	1720 str	1604 str 1514 wk	1400 1264	1073 1035	0,89
	FA[18]	3400	2920	1720 1650	1540	1400 1200	1050	–
FA+HA		3416 str	2921 wk 2850 wk	1714 1623	1516	1327 1267	1076 1041	1,11
Ca-(FA+HA)		3388-3380 str	2922 wk 2850 wk	–	1600-1560	1411		–
Fe-(FA+HA)		3364-3342 str	2922 wk 2852 wk	–	1622	1392	1096 1038	–
Cu-(FA+HA)		3381-3203 str	2929 wk 2884	–	1600 1573	1392	1111 1061	–
Zn-(FA+HA)		3500-3300	2922 2850	–	1616 1514	1400	1141 1065	–

The ionized form is characterized by asymmetric stretch of the C-O groups in the form of a strong band between 1650 and 1540 cm^{-1} while a less intense band between 1450 and 1380 cm^{-1} belongs to symmetric stretches (Table 2, data of the SAFA samples referred to in the article, and the FA samples).[8,16]

Alcohols and phenols demonstrate both strong absorption bands of stretches of the O-H groups in 3600 to 2400 cm^{-1} range and deformation of the O-H group in 1410 to 1260 cm^{-1}range. The intense bands of stretches of the C-O bonds are in 1230-1000 cm^{-1} range: primary alcohols (1080-1000 cm^{-1}), secondary alcohols (1150-1030 cm^{-1}) and phenols (1230-1140 cm^{-1}). According to the works on the SAFA, the FA and the FA studied samples the fulvic acids from peat are riched in alcohol and phenolic groups.[8,18] Considering the analysis of acidic groups by the Potentiometric method and the IR spectra data the fulvic acids can be considered as inhomogeneous macromolecules which phenolic acids make a sufficient contribution to.

Hydrophobic aliphatic fragments ofhumic acid macromolecules can be estimated from absorption bands between 2960 and 2850 cm^{-1} related to symmetric and antisymmetric stretches (ν_s and ν_{as}) of CH_3 and CH_2-groups.

The hydrophobicity index (HI) in the fulvic acids calculated as relation of the absorption at 2928 cm^{-1} to the absorption in the 1050 cm^{-1} regionwas reflected the ratio of nonpolar fragments to polar groups and is usually close

to 1 (Table 2). The HI of the FA studied samples was equal to 0.89, content of carbon and nitrogen –49,6% and 2,1%, respectively.

The IR spectrum of the mixture of HA and FA is close to the spectrum of the humic acids of the III FA typewhich was isolated by Stevenson from peat.[18]The IR spectra of the products of interaction between the humic acids and calcium, iron, zinc, and copper salts have intense bands in the 1600-1630 cm^{-1} range, specific for stretches of the carboxylate ions, however the band at 1254 $cm^{-1}$is absent (Table 2). Probably, the complexation is largely due to the reactive COOH groups and has a salt character.

High sorption capacity of the HPE in relation to Zn^{2+} and Cu^{2+} ions, almost 1,5 times higher than concentration of acid groups (COOH and Ph-OH),indicates participation of quinoid, amide and other groups in complexation. An approximate estimationof content of the fulvic acid in the HPE sampleswere analyzed by UV spectra of the solutions (HA + FA) in water and in 0,1M NaOH solution at 280 nm (Figure 3). It can be assumed that the system is represented only by two types of macromolecules and the which concentration follows the Lambert-Bouguer-Ber law. Considering the fact thatHA andFA are highly soluble in 0,1M NaOH and, accordingly, the absorption of A_{NaOH} reflects the total concentration ofHA and FA and only fulvic acid is soluble in water, the relative proportion of humic acid can be expressed as:

$$\alpha = \frac{A_{NaOH} - A_{H_2O}}{A_{NaOH}},$$

where A_{NaOH} and A_{H_2O} - absorption of humic and fulvic acids in 0,1M NaOH solution at=280 nm.

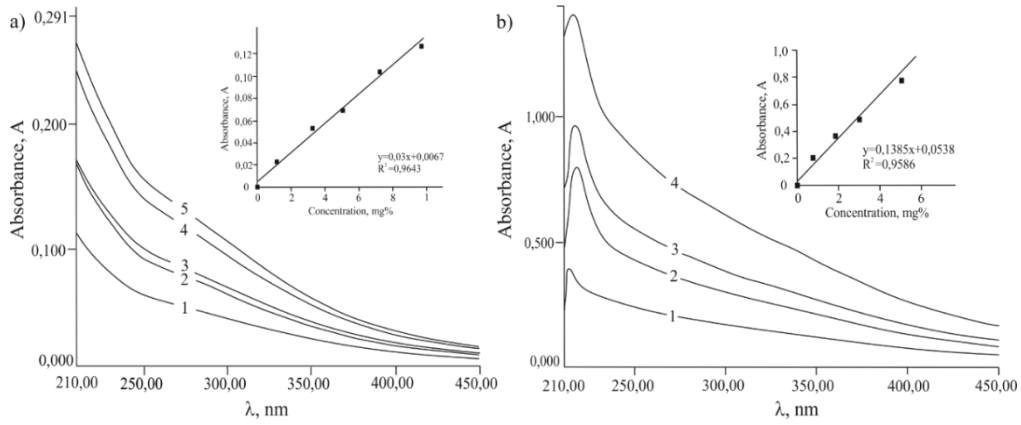


Figure 3: UV-vis spectra of the mixture (HA+FA) in water (a) and in 0.1MNaOH solution (b) at =280 nm. The concentration of the (HA+FA), mg/%: a)1,6; 3,8; 5,3; 7,5; 9,8, respectively; b) 0,98; 2; 3,1; 5,2, respectively. The inserts are represented by the calibration curves $A_{280}=f(c,mg/\%)$.

The content of the fulvic acid calculated as100%-α in the sample corresponded to 35±5%.In general, it can be suggested that the studied fulvic acids, like HPE, containing fragments of phenolic acids are able not only to inactivate ROS but also to exhibit antioxidant activity due to chelation of the iron ions and other metals. Moreover, taking into account the hydrophobicity index HI for the FA and the (HA + FA)equal to 0,87 and 1,11, respectively, the solubility of the fulvic acids in water, the HPE and the FA can be considered as macromolecules with optimal lipophilicity for biological activity.

Biological properties of humic acids

The effect of the humic acids and their sodium salts (Na-FA and Na-(HA+FA)) at non-enzyme antioxidant activity in ROS-processes in blood plasma is non-unique and insignificant (Table 3). The malonic dialdehyde (MDA) level in blood plasma was decreased by 2-12%.

Table 3: Characterization of LPO under the action of the humic acids

№	Colloidal systems of the humic acids	MDA*(plasma)		MDA(erythrocytes)					
		2µg		2µg		5µg		10µg	
		% of control	s^2	% of control	s^2	% of control	s^2	% of control	s^2
1	FA	97,8	0,01	36,2	3,48	67,7	0,04	34,9	0,01
2	Na-FA	86,7	0,02	54,6	0,02	34,7	0,02	19	0,04
3	HA+FA	87,1	0,01	79,9	0,05	26,3	0,02	74,6	0,02
4	Na-(HA+FA)	110,7	0,01	71,6	0,01	29,3	0,02	22,2	0,02
5	Control	100,0	0,02	100,0	0,20	100,0	0,02	100,0	0,25

MDA* - malonicdialdehyde as the main product of LPO

It was registered the strong effect of the HPE at non-enzymatic process of ROS and the dose-dependent effect at the MDA as a product of ROS in erythrocytes (Figure 4).

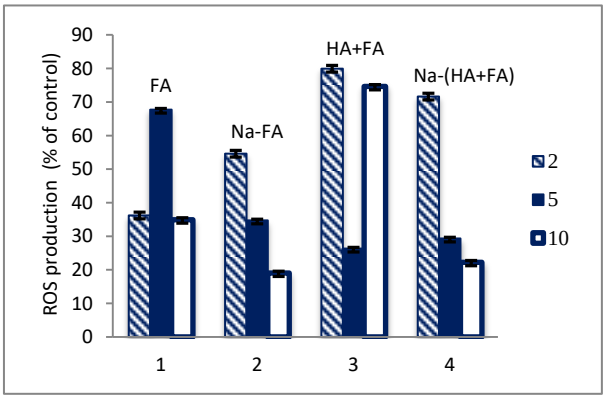


Figure 4: The dose-dependent effect of the humic acids and their salts on LPO (% of control) *invitro*.

The ionized fulvic acids and their mixture with the humic acids Na-FA and the Na-(HA + FA) demonstrate a better effect at 5-10 µg per 1 ml compared to the non-ionized forms. The non-ionized forms of the FA and the (HA + FA) are less sensitive to the concentration acting on erythrocytes. The MDA level was significantly reduced by action of the ionized and the non-ionized humic acids from 30 to 60% (Table 3).

Reducing the intensity of lipo peroxidation in the erythrocytes in terms of the MDA was most effective in the case of use of the fulvic acids and their salts.

The increase of the superoxide dismutase (SOD) activity in the erythrocytes was noted when 5 µg per 1 ml of all the humic acids were added (Table 4, Figure 5).

Table 4: The activity of SOD (%, from control) under action of the humic acids and their salts.

Dose, µg/ml	FA		Na-FA		HA+FA		Na-(HA+FA)	
	%	s^2	%	s^2	%	s^2	%	s^2
2	112,4	3,28	136,0	16,97	100,5	10,71	109,6	6,81
5	136,1	1,68	181,6	9,58	159,7	7,75	134,9	8,69
10	182,7	15,06	105,9	8,33	123,1	4,49	142,8	3,49

The effect of the fulvic acids and their salts (FA and Na-FA) at all doses on the activity of SOD was more significant than the mixtures of fulvic and humic acids (HA + FA) except for the ionized form of the Na-FA in the large dose (10 µg per 1 ml).

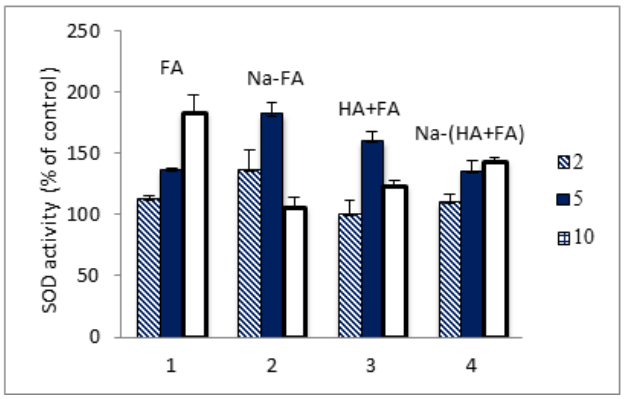


Figure 5: Dose-depend effect of the humic acids and their salts on enzyme activity of SOD (% from control) *invitro*.

Probably, the increase of antioxidant reserves of erythrocytes contributed to breakdown of LPO chain reactions as evidenced by both the decrease in MDA level and the increase in SOD activity. At the same time, the predominance of the enzymatic and non-enzymatic links of antioxidant defense system over the processes of lipoperoxidation may indicate the inhibition of system of biological oxidation under the influence of humic acids.

The enzyme lactate dehydrogenase (LDH) played a key role in the energy metabolism of cells, and the ratio of lactate/pyruvate and NAD/NADH$^+$ in cells depended on it. In the direct reaction pyruvate is formed from lactate which can be used in the Krebs cycle under aerobic conditions. The indirect reaction of the LDH led to formation of lactate from pyruvate and characterizes the degree of expression of anaerobic process in the cell.

The LDH activity was increased by 26-55% compared to the control when all humic acids and their salts were added in the direct reaction at all doses: 2, 5 and 10 µg (Figure 6, Table 5).

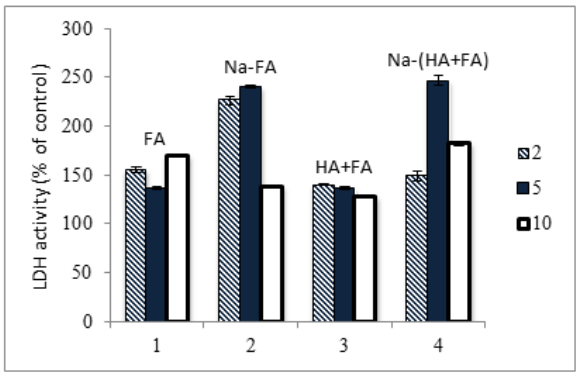


Figure 6: The dose-depend effect of the humic acids and their salts on activity of LDH in direct reaction (% from control) *in vitro*.

It should be noted that the Na-salts of the humic acids in the dose at 2 and 5 µg per ml are more effective than the corresponding non-ionized forms.

Table 5: The activity of the LDH in direct and indirect reaction.

Dose µg/ml	FA		Na-FA		HA+FA		Na-(HA+FA)	
	LDH_d	LDH_{ind}	LDH_d	LDH_{ind}	LDH_d	LDH_{ind}	LDH_d	LDH_{ind}
2	155,2±3,17	110,1±3,71	226,6±4,20	121,2±3,31	139,7±0,66	191,1±2,37	148,9±5,2	123,6±3,10
5	136,8±1,00	97,4±4,17	240,5±1,29	98,2±1,38	136,3±1,13	103,6±2,35	246,6±4,69	107,1±0,97
10	169,4±1,38	177,1±2,06	137,9±1,25	111,6±1,05	127,4±0,42	188,3±1,23	181,9±1,38	170,2±1,50

The activity of the LDHwas increased from 10 to 60% compared to the control in the doses at 2 and 10 µg per ml whereas at 5 µg/ml there was no change in LDH_d activity (Figure 7, Table 5).

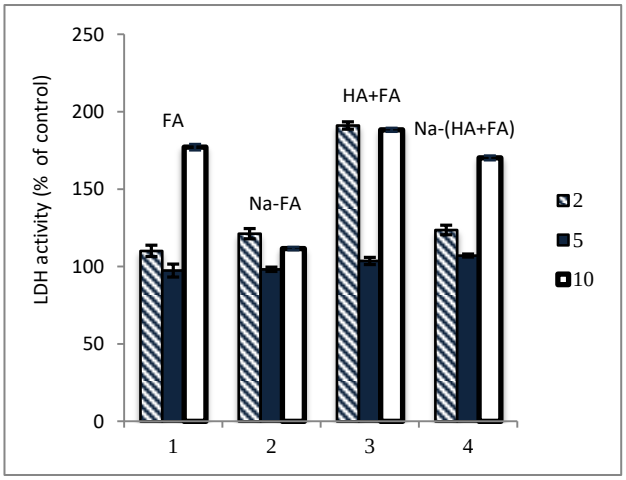


Figure 7: The dose-depend effect of the humic acids and their salts on the activity of LDH in indirect reaction (% from control) *in vitro*.

The increase of the LDH activity in the direct reaction compared to the indirect reaction may lead to increase in the content of pyruvic acid which is formed predominantly by the H-LDH form (LDH_d). The pyruvate under aerobic conditions is rapidly utilized in biochemical reactions in various tissues. The important role of pyruvic acid is in the conversion of pyruvate to acetyl-coenzyme-A in the mitochondria which then is metabolized in the Krebs cycle followed by oxidative phosphorylation to form the main universal energy source, adenosine triphosphate.

CONCLUSION

Thus, the humic acids increase energy metabolism of the cells and have antioxidant properties which are revealed by the increase of the enzymatic activity (SOD) and the non-enzymatic antioxidant protection leading to the decrease of the intensity of free radical oxidation. The dose-dependent effects of action of the humic acids on the lipid peroxidation system and lactatedehydrogenase activity were revealed. The influence of the fulvic acids on both the energy metabolism and on the SOD activity is stronger than mixtures of the HA + FA.

It is very important that the ionized forms of humic acids exert a greater influence on energy metabolism than the non-ionized ones. The explanation for these results is the possibility of the sodium salts of humic acids to participate in metabolism (oxidative and energetic) by filling the "shadows" of red blood cells.[19]

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