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In silico, *in vitro*, *in vivo* Evaluation of Antioxidant Activity and Toxic Effects of Phosphorus-Containing Derivatives of 2,6-Di-*tert*-Butylphenol

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By using PASS online web resource, it was predicted that phosphorus-containing derivatives of 2,6-di-*tert*-butylphenol had high probability to possess antioxidant activity and to act as active oxygen metabolite "traps" and heavy metal antidotes. The values of the acute toxicity index of phosphorus-containing phenols for rats, aquatic organisms, and bioaccumulation factor, calculated by the GUSAR programme, indicate potentially low toxicity. The results of the forecast are comparable with the data of experimental studies *in vitro* and *in vivo* experiments.

Keywords: phosphor substituted derivatives of 2,6-di-*tert*-butylphenol, toxicity, *in silico*, antioxidant, *in vitro*, Russian sturgeon, *in vivo*, fish feeds, lipid peroxidation.

Introduction

Various polyfunctional phenol antioxidants are widely used for the treatment and prevention of pathologies that are caused or accompanied by the development of oxidative stress, because such compounds can efficiently inhibit the chain process of free radical oxidation by various mechanisms (Denisov and Afanas'ev, 2005). Among the bioactive antioxidants, special attention is paid to 2,6-dialkylphenols, which have organic or element-organic groups in *para*-position, possessing an anti-peroxide and antiradical effect (Oxidants and Antioxidants, 2002). Sterically hindered phosphorylated phenols can be considered as hybrid molecules, in which phosphorus-containing groups are responsible for suppression of the degenerate branching of oxidation chains due to the destruction of hydroperoxides, and such suppression enhances the antioxidant activity of the compound. In addition, the antioxidant activity of phosphorylated phenols can also be caused by their capacity to bind metal ions upon metal-induced oxidation as complex-forming agents. Some of bisphosphonates (BPs) derivatives – synthetic structural analogues of inorganic pyrophosphate – besides their well-known anti-bone resorption properties, antimicrobial activity and anti-tumour properties have been proved to possess selective antioxidant properties *in vitro*, presumably, due to their iron chelating ability (Dombrecht et al., 2004; Dombrecht et al., 2006; Kunda et al., 2012; Lahbib et al., 2015; Korkmaz et al., 2018). BPs are known to possess a strong capacity for complexing metal-ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} or Fe^{2+}, which originates from the potential of both phosphoryl groups coordination to the cation. At the same time, nitrogen-bisphosphonates (N-BPs) can have serious side effects; such species are the most widely used drugs for bone fragility disorders, and the effect may partly be explained by its negative influence on the antioxidant status (Kalyan et al., 2014)

At present, antioxidants are created on the basis of alkyl phenol derivatives containing fragments of phosphonates, phosphonic acid esters, which have an antioxidant effect significantly exceeding the analogues used in the industry (Azmukhanova et al., 2016). For example, the calcium salt of bis-[monoethyl(3,5-di-*tert*-butyl-4-hydroxybenzyl)] phosphonate is used as a stabilising additive in materials which contact with food (Federal Register, 1998). It is also known that certain compounds based on phosphonic acid can be toxic (Cao et al 1982; Rott et al., 2018).

Thus, creating phosphorylated phenolic antioxidants possessing minimal danger for men and the environment along with efficient activity is an urgent task. The search for new promising biologically active compounds can be carried out using *in silico* methods, which precedes experimental studies *in vitro* and *in vivo*. Recently, the computer programme PASS (Prediction of Activity Spectra for Substances) has been widely used to predict the bioactivity of various organic compounds, which allows to estimate the probable profile of their biological activity on the basis of structural formula analysis (Kabir et al., 2016; Jamkhande et al., 2014)

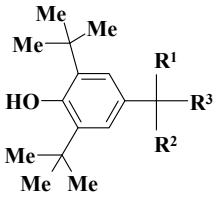
The purpose of this work was to predict the toxicity and antioxidant activity of phosphorylated phenols **1a-j** and **2a,b** (Fig. 1) using *in silico* methods and to compare the results with experimental data *in vitro* and *in vivo*.

Methods

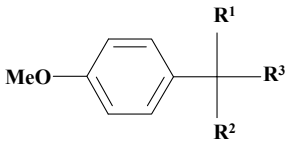
Compounds of **1a-j** and **2a,b** were synthesised as described in (Prishchenko et al., 2008). BHT (2,6-di-*tert*-butyl-4-methylphenol, 99%), trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%) and all other reagents were purchased from Sigma–Aldrich. The spectrum of biological activity of compounds **1a-j** and **2a,b** was predicted *in silico* using PASS (PharmaExpert.ru ©2011–2017 • Version 2.0) and GUSAR (General Unrestricted Structure-Activity Relationships) (Way2Drug.com, 2011–2016) (Lagunin et al., 2011). The structures were drawn using the CHEM Sketch Package 11.0 from the ACD Chem Laboratory. The spectrum of biological activity for substances is presented in the form of a list of types of biological activity, for which the probability of

Fig 1.

Structural formulas of the studied compounds **1a-j** and **2a,b**



R^1=H, R^2=OSiMe$_3$, R^3=PO(OEt)$_2$ (**1a**)
R^1=H, R^2=R^3=PO(OEt)$_2$ (**1b**)
R^1=H, R^2=R^3=PO(OH)$_2$ (**1c**)
R^1=H, R^2=OH, R^3=PO(OH)$_2$ (**1d**)
R^1=OH, R^2=R^3=PO(OH)$_2$ (**1e**)
R^1=OSiMe$_3$, R^2= R^3=PO(OEt)$_2$ (**1f**)
R^1=OH, R^2=R^3=PO(OH)(CH$_2$)$_2$ NC$_4$H$_6$O (**1g**)
R^1=H, R^2=R^3=PO(OH)CH$_2$NC$_4$H$_6$O (**1h**)
R^1=OH, R^2=R^3=PO(OH)(CH$_2$)$_2$ Ph (**1i**)
R^1=H, R^2=R^3=PO(OH)(CH$_2$)$_2$ Ph (**1j**)



R^1=H, R^2=R^3=PO(OEt)$_2$ (**2a**)
R^1=H, R^2=R^3=PO(OH)$_2$ (**2b**)

presence (P_a) and the probability of lack of activity (P_i) are calculated. P_a and P_i values are independent and their values vary from 0 to 1. In this paper, the types of biological activity for which P_a is more than P_i were evaluated.

Quantitative prediction of the ecotoxicity of chemical compounds was carried out using the GUSAR software. Quantitative structure-activity relationship (QSAR) was established in the model of oral application of phenolic compounds to rats (endotoxicity). The acute toxicity of phenols for rats was evaluated by a dose that kills 50% of the experimental organisms LD_{50} (mg/kg). The QSAR models were developed for the following endpoints: bioaccumulation factor Log10(BCF), 96-hour fathead minnow 50% lethal concentration (Fathead Minnow LC_{50} Log10(mmol/L)), 48-hour daphnia magna 50% lethal concentration (Daphnia magna LC_{50} -Log10(mol/L)) and Tetrahymena pyriformis 50% growth inhibition concentration (Tetrahymena pyriformis IGC_{50} -Log10(mol/L)). It is known that infusoria Tetrahymena pyriformis have similarities with higher animals for a number of metabolism basic parameters, which allows performing interspecies extrapolation of the results of the analysis (Игнатьев and Шаблий, 1978). All compounds have fallen into the field of application of models (Poroikov et al., 2000; Poroikov et al., 2003).

The intensity of fish sperm lipid peroxidation (LPO) has been assessed by the accumulation of carbonyl oxidation by-products, which react with thiobarbituric acid (TBARS), using the traditional method as described previously (Osipova et al., 2017). The fishery-biological characteristics of juvenile Russian sturgeon (*Acipenser gueldenstaedtii*) were determined according to the procedure as described previously (Пономарев et al., 2002). Russian sturgeon juveniles were fed with an Aller Sturgeon dry granular mixed fodder (grain size 2 mm) without additives and with the addition of compounds **1a-j**, **2a,b** to a concentration of 150 mg/kg. The juveniles were grown for 35 days. Russian sturgeon juveniles with an initial weight of 165 ± 5 g were placed in 250-L aquariums (10–12 individuals per aquarium) equipped with filtration and aeration systems. The amount of feed was calculated using specially developed feed tables on the basis of the body weight and water temperature. The juveniles were fed twice a day manually, and the groats size was consistent with the fish weight. All manipulations were carried out in accordance with Good Laboratory Practice (GLP) standards. The hydrochemical parameters of water remained within the normal ranges during experiments. The results were estimated by the growth rate and survivability of the hydrobionts.

The rate of LPO in the liver homogenate of juvenile Russian sturgeon was evaluated by TBARS according to the standard procedure as described previously (Osipova et al., 2017). The concentrations of TBARS in the enzymatic ($TBARS_e$) and non-enzymatic Fe^{2+}-ascorbate induced LPO ($TBARS_n$), as well as the initial concentration of TBARS, were determined as the kinetic parameters of lipid peroxidation. Compounds **1a-j**, **2a,b** were dissolved in chloroform and added to the liver homogenate of sturgeons to attain an initial concentration of 0.1 mM in the incubation medium. By

special experiment we showed that chloroform did not affect the rate of accumulation of TBARS in the liver homogenate.

Hemolysate of fish erythrocytes was prepared according to the procedure (Справочник, 1975). The assay for the determination of the H_2O_2 decomposition rate was determined spectrophotometrically by monitoring disappearance of H_2O_2 at 240 nm (Katsuwon and Anderson, 1992).

The received data were processed on a PC using MS Excel. The results are presented in the form $M \pm SD$. Statistical significance was assessed using the Student *t* test.

Results and Discussion

In silico studies

It is known that antioxidants are considered as first-line therapy that protects organisms from highly reactive free radicals, especially reactive oxygen species (ROS), which are capable to oxidise biomolecules causing human diseases. Therefore, the search for novel antioxidant agents that prevent or reduce the impact of oxidative stress on cells is a contemporary field. Computer forecasting of pharmacological effects and toxicity of new synthetic compounds is an important direction in search and development of new medicines. In this study, the forecast of toxicity and biological activity of phosphorylated phenols **1a-j** and their esters **2a,b** in comparison with 2,6-di-*tert*-butylphenol and BHT was performed with the help of the PASS software. The compounds studied in the work contain the phosphonic acid group; they have a direct carbon to the phosphorus (C–P) bond, which is very stable and extremely resistant to chemical hydrolysis, thermal decomposition and photolysis. The chemical structures of the compounds differ in the content of phosphonic groups, and most of the compounds belong to BPs, which are resistant to enzymatic and chemical breakdown. BPs are widely used for therapy of numerous metabolic disorders (Drake et al., 2008). In contrast to monophosphonate moiety, BPs are essential for hydroxyapatite affinity. Compounds **1g** and **1h** are the heterocyclic NBPs, which have the nitrogen atom of the pyrrolidone fragment. It was shown that phosphorus- and nitrogen-containing derivatives of 2,6-di-*tert*-butylphenol possess a wide spectrum of biological activity with high inhibition ability ((Dyubchenko et al., 2010; Tyurin et al., 2011; Antonova et al., 2010; Kunda et al., 2012). In order to distinguish functional groups in the molecules which are responsible for antioxidant activity, compounds **2a,b** were also studied in this work. These compounds are derivatives of anisole and in contrast to the compounds **1a-j** they do not contain a sterically hindered phenol fragment.

It was found using PASS software that the most probable phosphorus-substituted phenols had the potential to treat bone diseases (P_a=0.657÷0.996). According to the information from the literature, the similar activity was found in BPs (clodronate, pamidronate, zoledronic acid and ibandronate), which slowed down the development of pathological processes in the bones and provided an antitumor effect (Morgan and Kaiser, 2012). A high P_a value for a given bioactivity could be caused by the similarity of the structure of the molecules of the tested compounds to the structures of known pharmacologically active substances. Among the large number of predicted types of bioactivity of phosphorus-containing phenols **1a-j** and their esters **2a,b**, we have identified those that were closely related to antioxidant properties, namely, the ability to act as antioxidants and "traps" of ROS initiating oxidative processes (oxygen scavenger), and antidotes, including antidotes binding heavy metals.

According to the computer forecast for compounds **1a**, **1c** and **1f**, the probability of antioxidant activity was revealed (Table 1).

For all compounds except **1a**, the probability to act as "traps" of ROS was rather high, which also predetermines the manifestation of antioxidant activity. Compound **1e** – the derivative of diphosphonic acid, containing a hydroxylic group, had the highest probability to act as an antidote. It is known that introduction of the hydroxyl group at the geminal carbon atom of the P-C-P group (as in etidronate) can increase the affinity for metal (Martin and Grill, 2000).

Table 1

Prediction of biological activity of compounds **1a-j** and **2a,b**

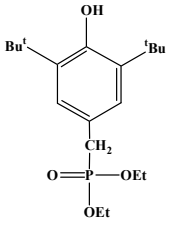
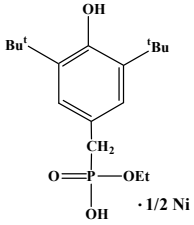
Compounds	Antioxidant		Oxygen scavenger		Antidote	
	P_a	P_i	P_a	P_i	P_a	P_i
1	2	3	4	5	6	7
2,6-di-*tert*-butylphenol	0.613	0.004	0.669	0.010	-	-
BHT	0.587	0.005	0.669	0.010	-	-
1a	0.279	0.027	-	-	-	-
1b	-	-	0.350	0.129	-	-
1c	0.273	0.029	0.535	0.039	0.373	0.013
1d	-	-	0.505	0.049	0.357	0.016
1e	-	-	0.541	0.038	0.385	0.011
1f	0.318	0.020	0.269	0.221	0.197	0.111
1g	-	-	0.315	0.163	-	-
1h	-	-	0.370	0.114	0.301	0.033
1i	-	-	0.393	0.099	-	-
1j	-	-	0.388	0.103	-	-
2a	-	-	0.366	0.117	-	-
2b	-	-	0.546	0.036	-	-

Toxicity of chemical compounds should be taken into account at evaluation of possible use as pharmacological preparations. It is well known that certain phenolic AOs are toxic: butylated hydroxyanisole is carcinogenic (Ito et al., 1983), and enzyme antioxidant system is suppressed by the products of oxidative destruction of BHT (Rice-Evans and Diplock, 1993). Despite the fact that BPs are characterised by a high affinity for bone, and their overall tolerability is generally good, BPs treatment involves some risk of serious adverse drug reactions, e.g., oral and gastric carcinomas (Varenna et al., 2013) or a decline of the antioxidant status in postmenopausal women with osteoporosis (Kalyan et al., 2014). Phenols with two sterically hindered ***tert***-butyl substitutes in ***o***-position have the lowest toxicity (Belostotskaja et al., 2013).

However, certain compounds based on the basis of phosphonic acid have a greater toxicity in contrast to 2,6-di-*tert*-butylphenol and BHT (Table 2) (Cao et al., 1982).

Table 2

The LD_{50} values of 2,6-Di-*tert*-butylphenol derivatives for mice when administered orally

Compounds	2,6-di-*tert*-butylphenol	BHT		
LD_{50}, mg·kg^{-1}	2955	2000	1905	888

In this regard, computer forecast should be performed not only in terms of the biological activity of the substances, but their possible toxic effects also have to be evaluated (Salgueiro et al., 2016).

The calculation of the acute toxicity of test compounds was carried out with the help of the GUSAR software using rats as model organisms (Table 3).

Such calculations predict a low toxicity of phosphorus-containing phenols (4 and 5 category of danger). The predicted LD_{50} value is in the range of 741–2893 mg/kg, while the lowest toxicity category 5 is predicted for the **1c-e** and 2,6-di-*tert*-butylphenol compounds.

The calculated *in silico* values of BCF for all phosphorus-substituted derivatives of 2,6-di-***tert***-butylphenol lie in the range from –0.093 to 2.298.

According to the recommendations of the application D to the Stockholm Convention on Persistent Organic Pollutants (http://chm.pops.int), in which the criteria for estimation of the ecotoxicant bioaccumulation are determined, the BCF values indicate low ecological toxicity of compounds **1a-j** and **2a,b**. It should be noted that these values for all the investigated phosphorus-containing phenols are lower than the values for the BHT and 2,6-di-*tert*-butylphenol. These data on

Table 3

Predicted acute toxicity for rats as model organisms using oral administration (LD_{50}) and environmental toxicity of compounds

Compounds	LD_{50}, mg/kg	Bioaccumulation factor Log10(BCF)	Daphnia magna LC_{50} -Log10(mol/L)	Fathead Minnow LC_{50} Log10(mmol/L)	Tetrahymena pyriformis IGC_{50} -Log10(mol/L)
1	2	3	4	5	6
2,6-di-*tert*-butylphenol	2136	2.515	4.962	–2.245	1.564
BHT	1821	2.833	5.046	–2.707	1.850
1a	1295	2.161	8.158	–4.775	1.395
1b	1115	1.691	7.344	–4.264	1.223
1c	2893	2.156	7.144	–3.363	1.951
1d	2328	2.046	6.943	–2.592	1.819
1e	2850	1.929	6.984	–3.238	1.745
1f	941	0.802	7.812	–4.550	0.878
1g	1212	–0.093	7.367	–3.515	1.839
1h	1210	2.298	7.502	–3.369	1.401
1i	1162	1.084	7.942	–5.418	1.890
1j	1343	1.267	8.187	–5.653	2.153
2a	741	1.417	7.323	–2.543	0.989
2b	1687	1.219	6.596	–1.230	0.809

acute toxicity of compounds for aquatic organisms (Table 3) also can indicate low ecological toxicity of the studied phosphorus-substituted phenols.

The effect of the studied compounds on the level of TBARS accumulation at different stages of oxidation of Russian sturgeon sperm in vitro

Previously, we determined that phosphorus-containing sterically hindered phenols possessed inhibiting activity in reactions of Russian sturgeon liver lipid peroxidation (Antonova et al., 2008.). Novel antioxidative agents **1a-j** and **2a,b** at a concentration of 0.1 mM were studied in comparison with BHT and 2,6-di-*tert*-butylphenol on the process of LPO of the Russian sturgeon sperm under conditions of long-term oxidation process. Fish spermatozoa contain a high level of polyunsaturated fatty acids, which are sensitive to ROS-induced damage mediated by peroxidation (Trenzado et al., 2006). Table 4 presents the data on the change in the concentration of TBARS in

the sturgeon sperm in the presence of the studied compounds during 48-h incubation. In the control experiment, the content of TBARS increased with time. Addition of all compounds led to a decrease of the LPO level in the Russian sturgeon sperm at all studied time intervals.

Table 4

TBARS level (nmol/10^9 sperm cells) in the Russian sturgeon sperm lipids in the presence of the studied compounds during long-term *in vitro* oxidation

Compounds	TBARS level (nmol/10^9 sperm cells)		
	3 h	24 h	48 h
1	2	3	4
control	1.65 ± 0.06	1.78 ± 0.16*	2.03 ± 0.10*
2,6-di-*tert*-butylphenol	1.34 ± 0.04	0.98 ± 0.09	1.48 ± 0.09
BHT	1.14 ± 0.11	1.57 ± 0.11	1.93 ± 0.05
1a	0.94 ± 0.09	1.07 ± 0.06	0.99 ± 0.14
1b	1.16 ± 0.06	1.19 ± 0.02	0.91 ± 0.06
1c	0.73 ± 0.05	0.84 ± 0.15	0.87 ± 0.06
1d	1.27 ± 0.02	1.42 ± 0.07	0.99 ± 0.03
1e	0.56 ± 0.05	0.73 ± 0.03	0.93 ± 0.07
1f	0.97 ± 0.08	0.84 ± 0.03	0.91 ± 0.16
2a	0.79 ± 0.07	1.00 ± 0.08	1.12 ± 0.01
2b	0.59 ± 0.03	0.61 ± 0.01	0.81 ± 0.11

The average values for a series of experiments are given: differences from the control experimental group ($p < 0.01$);
* differences from the control experimental group (3 h) ($p < 0.05$). The values are expressed as mean ± SD.

The inhibitory activity of BHT significantly decreases with time, and in the remote oxidation stages of LPO the antioxidant activity is only 12% and 5%. *In vitro* studies evidence for higher efficiency of antioxidant activity of phosphorylated phenols compared with the activity of the 2,6-di-*tert*-butylphenol and BHT. This is probably related to the presence of several reactive sites in the molecules of phosphorus-containing phenols considered herein, where such sites can act as inhibitors of oxidative processes acting by different mechanisms and can show intramolecular synergism.

In this model system, as in the oxidation of liver lipids (Patent RU, 2010), it was shown that compounds **1c**, **1e** and **2b** containing two residues of unesterified phosphonic acid, were the most efficient antioxidants with prolonged inhibitory activity. It was forecasted *in silico* that these compounds **1c**, **1e** and **2b** had the highest probability to act as an oxygen scavenger. Antioxidant activity of **2b**, which does not contain a sterically hindered phenolic fragment, is probably caused by the presence of two phosphorus-containing groups.

Higher efficiency of an antioxidant action of the compounds in the model system of Russian sturgeon sperm oxidation, in contrast to Russian sturgeon liver (Antonova et al., 2008), may probably be explained by the specific characteristics of fatty acid composition of fish sperm cells (high level of polyunsaturated fatty acids), as well as by low concentration of the enzymes of the antioxidant protecting system, which could act as inhibitors of free-radical processes.

Thus, in this study, the capacity of phosphonates containing a sterically hindered phenolic fragment to act as antioxidant agents, which inhibit sperm lipid peroxidation, was found *in vitro*, and it was shown that this effect exceeded the effect of known antioxidants. It should be noted that they are able to increase

resistivity of biomembranes to oxidative stress, which is an important feature.

Experiments in vivo

Experiment studies of phosphorus-substituted derivatives of 2,6-di-*tert*-butylphenol have shown the outlooks of their application as cryoprotectant additives in basic protective environments during low-temperature conservation of sturgeon sperm (Pimenov et al., 2012; Osipova et al., 2014). The influence of additives of sterically hindered phenol derivatives to the feed of juvenile Russian sturgeon on growth, development and biochemical indicators of hydrobyons compared with BHT and trolox was studied in this work. It was shown that addition of the compounds **1a-c**, **1e-h** at the concentration of 150 mg per 1 kg of fish feed did not lead to the change in the behaviour of sturgeon fish, and the survival rate was 100%. In the groups of fish, fed with additives of compounds **1a** and **1b**, absolute growth decreased by 1.2 and 2 times, respectively, in comparison with the control variant (Table 5).

Table 5

The effect of additives of the test compounds in fish feed on the growth indices, the level of accumulation of secondary products of TBARS in the liver of the Russian sturgeon in relation to control and the rate of decomposition of H_2O_2 by erythrocytes hemolysate *in vivo*

Compounds	Absolute growth, g	Average daily growth rate, %	TBARS, % of control			% inhibition rate of H_2O_2 decomposition by erythrocytes hemolysate
			$TBARS_e$	$TBARS_n$	TBARS	
1	2	3	4	5	6	7
control	28.11 ± 0.22	0.47 ± 0.01	100 ± 4.3	100 ± 5.2	100 ± 3.1	100 ± 5.2
1a	23.09 ± 0.15	0.38 ± 0.01	109 ± 3.3	104 ± 3.5*	106 ± 4.5	91 ± 3.1
1b	14.16 ± 0.05	0.24 ± 0.02	118 ± 5.1	109 ± 2.5	116 ±1.5	84 ± 2.3
1c	47.31 ± 0.18	0.69 ± 0.03	68 ± 2.4	85 ± 4.1	58 ± 3.2	140 ± 4.0
1e	38.50 ± 0.95	0.61 ± 0.03	66 ± 3.3	72 ± 2.3	67 ± 2.4	108 ± 2.5
1g	27.86 ± 0.86**	0.44 ± 0.01	90 ± 2.3	95 ± 1.4	98 ± 4.3*	90 ± 2.1
1h	34.12 ± 0.49	0.55 ± 0.02	86 ± 5.2	89 ± 4.1	97 ± 4.1*	90 ± 3.3
BHT	32.23 ± 0.09	0.50 ± 0.01	88 ±3.1	88 ± 3.2	86 ± 2.1	105 ±3.5
trolox	30.19 ± 0.65**	0.50 ± 0.01	75 ± 3.1	89 ± 5.5	80 ± 3.5	126 ± 4.2

The average values for a series of experiments are given: differences from the control experimental group ($p < 0.001$);
* differences from the control experimental group ($p < 0.05$);
** differences from the control experimental group ($p > 0.05$). The values are expressed as mean ± SD.

It was noted that the juveniles that received the feed with the addition of compound **1b** consumed the mixed fodder reluctantly and reacted to external stimuli weakly, unlike the other groups of sturgeon. When compound **1g** was added to fish feed, the weight gain was comparable with the control values. The addition of the compounds **1c**, **1e**, **1h**, BHT and trolox to the feed promoted more intense fish growth in comparison with the control. The highest values of absolute increase and the average daily growth rate were found when compounds **1c** and **1e** were added to the fish feed.

As the biochemical indicators of hydrobionts, the rate of H_2O_2 decomposition by hemolysate of fish blood erythrocytes and the level of lipid peroxidation of liver homogenate of juvenile Russian sturgeon were determined. The addition of phosphorus-containing derivatives BHT **1a** and **1b** to the feed led to a slight increase in the level of enzymatic lipid peroxidation ($TBARS_e$) and non-enzymatic lipid peroxidation ($TBARS_n$), and the initial level of TBARS in the liver homogenates, which, probably, was the reason for the decrease in the growth of juvenile fish. The kinetic parameters of liver lipid oxidation with

the addition of the other compounds were significantly reduced; it was found that compounds **1c** and **1e** had most efficient antioxidant activity.

It is known that H_2O_2 is the most stable form of ROS. It has several useful functions for a cell, but at high concentrations has toxic effects (Martínez-Álvarez et al., 2005). When the compounds **1c**, **1e**, BHT and trolox were added to the feed, the activity of the hemolysate of blood erythrocytes in juvenile sturgeon increased in comparison with the control group (Table 5). The largest increase was observed upon the addition of **1c**, which may indicate the activation of the antioxidant protection system of the juvenile Russian sturgeon. This, in turn, contributes to an increase of the hydrobionts resistance in stressful situations, when the process of peroxide oxidation of lipids increases, for example, in the conditions of aquatic environment contamination with hazardous pollutants.

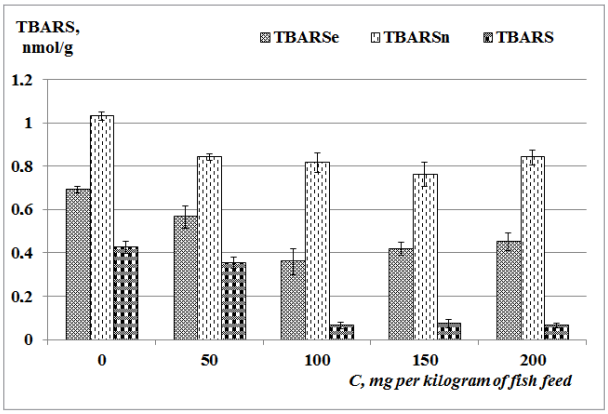
Upon feeding with the addition of other compounds, a slight decrease of the rate of H_2O_2 decomposition by hemolysate of fish blood erythrocytes was observed, which led to a weakening of the activity of the antioxidant protection system in the juvenile Russian sturgeon. Thus, it was shown that the addition of the phosphorus-containing compound **1c** to the fish feed was more efficient than the addition of the widely used antioxidants (trolox, BHT).

The effect of (4-hydroxy-3,5-di-*tert*-butylphenyl) methylenediphosphonic acid (**1c**) additives in different concentrations (50, 100, 150 and 200 mg/kg of fish feed) on the growth of the juvenile Russian sturgeon and the kinetic parameters of the LPO of liver (Fig. 2) was studied *in vivo* experiments.

It was shown that the greatest absolute increase in sturgeon mass was observed when the concentration of **1c** was at the level of 100 mg/kg of feed. The addition of **1c** in concentrations from 50 to 200 mg per 1 kg of fish feed led to a decrease in the kinetic parameters of LPO of liver *in vivo* experiments, which confirms that (4-hydroxy-3,5-di-*tert*-butylphenyl) methylenediphosphonic acid had antioxidant activity. The greatest decrease in the level of LPO was found upon the addition of **1c** in the concentration of 100 mg/kg of feed, which was two times lower than the generally accepted level of antioxidant supplements in feed (200 mg/kg).

Fig 2.

Effect of compound **1c** on the kinetic parameters of lipid peroxidation in the liver homogenate of juvenile Russian sturgeon. Control: concentration of TBARS in the liver homogenate of juvenile fish fed without additives. Average values from a series of measurements are given; differences from the control group *in vivo* ($p < 0.01$).



Thus, the addition of (4-hydroxy-3,5-di-*tert*-butylphenyl)methylenediphosphonic acid in a concentration of 100 mg/kg of feed was optimal for normal growth and development of the sturgeon juvenile. The reduction of the LPO level in the hydrobiont tissues in comparison with the control variant and the increase of catalase activity upon the addition of a phosphorus-containing compound indicated the activation of the antioxidant system of the fish organism, which in turn had positive influence on the growth and development of sturgeons.

The results of the study of the antioxidant activity of phosphorus-containing phenol derivatives in model systems of lipid fragments peroxidation in various bio-objects *in vivo* and *in vitro* experiments indicated a greater efficiency of the antioxidant effect of phosphorus-containing phenols compared with the known antioxidants (2,6-di-*tert*-butylphenol, BHT) and also indicated that these compounds did not have acute toxicity.

Conclusions

In this study, it was shown by QSAR analysis of endo- and ecotoxicity that the phosphorus-substituted derivatives of 2,6-di-*tert*-butylphenol had low toxicity, minimal side effects. The results of computer screening

were confirmed by the data of the experimental *in vitro* and *in vivo* studies. It was shown that these compounds could have antioxidant activity which was predicted *in silico*. The results of the prediction of the biological activity can be used in planning further experimental studies of biological and pharmaceutical activity *in vitro* and *in vivo* systems.

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