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ISOLATION OF HISPIDIN FROM A WALNUT-TREE FUNGUS AND ITS ANTIOXIDANT ACTIVITY

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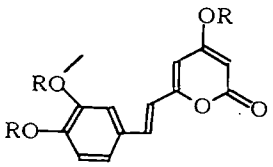
An α-styrylpyrone derivative — hispidin — has been isolated from a walnut-tree fungus. Its antioxidant activity has been studied in in vitro *and* in vivo *experiments on animals of different ages.*

Compounds with pronounced antioxidant properties are being used successfully at the present time in geriatric practice and in the treatment of many diseases resulting from an extreme activation of the peroxide oxidation of lipids [1, 2]. They are also widely used as stabilizers of food products and household materials [3]. It is most promising to search for antioxidants among polyphenolic compounds such as flavonoids, sesquiterpene esters, and terpenoid coumarins, and other substances [4]. Continuing work in this direction, from a fungus of the Persian walnut tree (*Juglans regia* L.) we have isolated substance (**1**) with the composition $C_{13}H_{10}O_5$, an α-styrylpyrone derivative according to its UV and IR spectra.

The compound isolated contained three hydroxy groups and formed a triacetyl derivative (**2**) and a trimethyl ether (**3**). On the basis of spectral characteristics and chemical transformations, substance (**1**) was identified as hispidin (3′,4,4′-trihydroxy-6-styryl-2-pyrone), which has been isolated previously from *Polyporus hispidulus* (Bull.) [5]. The structural closeness of this compound to substances possessing antioxidant activity impelled us to study in *in vitro* and *in vivo* experiments the influence of hispidin on the peroxide oxidation of lipids and the phospholipid spectrum of the rat cardiac muscle.



1. R=H
2. R=$COCH_3$
3. R=CH_3

The results of the experiments showed that in concentrations of 5×10^{-7} to 1.5×10^{-6} M hispidin exerted an inhibiting influence on the kinetics of the peroxide oxidation of lipids (POL) in a system containing a suspension of egg yolk in the presence of Fe^{2+}. In a concentration of 1.1×10^{-6} M, the preparation caused a delay in the development of chemiluminescence (ChL) and a decrease in the amplitudes of the rapid and slow flashes, and also a decrease in stationary luminescence.

Since hispidin, like other phenolic antioxidants [3, 4], is characterized by the presence of an aromatic ring with phenolic groups and contains a number of conjugated double bonds, the action of this substance, like that of α-tocopherol, is apparently due to its direct interaction with the radicals being formed. This was confirmed by a mathematical analysis of the kinetics of the POL. The addition of hispidin and of α-tocopherol to the test system caused a unidirectional change in the linear dependence of the intensity of luminescence in the initial stage of the slow flash and a decrease in the angle of slope of the straight lines reflecting the dependence of δ on t.

It was shown in a separate series of *in vitro* experiments that hispidin in concentrations of 6.25×10^{-7} to 2.5×10^{-5} M blocks the Fe^{2+}-induced enzymatic peroxide oxidation of lipids in a rat liver homogenate (Table 1). This effect was most pronounced at a concentration of 2.5×10^{-5} M and was fully comparable with the effects of quercetin and Ionol.

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TABLE 1. Influence of Various Concentrations of Hispidin, Quercetin, and Ionol (%) on the Fe^{2+}-induced Peroxide Oxidation of Lipids in a Rat Liver Homogenate

Substance	Concentration				
	6.25×10^{-7}M	1.87×10^{-6}M	3.75×10^{-5}M	7.5×10^{-6}M	2.5×10^{-5}M
Hispidin	13.62±3.1	25.0±2.7	26.6±2.1	46.9±2.8	67.3±3.1
Quercetin	49.2±3.2	63.0±4.7	64.3±4.2	74.4±5.7	78.1±6.1
Ionol	64.4±4.8	78.3±4.9	81.0±5.7	82.40±5.1	83.1±6.6

An investigation of the membranotropic activity of hispidin performed on erythrocytes in *in vitro* experiments showed that in concentrations of 1×10^{-6} to 1×10^{-5} g/ml it caused no hemolysis, and in the concentration showing the greatest antioxidant activity it caused slight hemolysis (12-15%), which indirectly characterizes this substance as of low toxicity.

The antioxidant activity revealed in the *in vitro* experiments served as the basis for performing experiments directly on animals. We studied the cardiac muscles of male white rats aged 20 months and weighing 340-380 g, since an accumulation of the products of POL is observed in various organs and tissues of old animals [1]. In our experiments this was shown by a 1.7-fold increase in the amount of dienic conjugates (DCs) and a 1.6-fold increase in the level of malondialdehdye (MDA) in the myocardium of old rats as compared with young ones. In addition to this, in the old rats certain changes were observed in the phospholipid spectrum. Thus, the levels of phosphatidylserine and of lyso-phosphatidylethanolamine had each increased 1.4-fold, while the level of cholesterol in the hearts of the experimental animals had increased 1.8-fold. All of this showed that on ageing changes take place in the POL process and in the phospholipid composition of the cardiac muscle.

On the regular administration of hispidin to old animals, positive shifts were observed in the POL indices and in the phospholipid spectrum. The most pronounced effect was observed on the administration of hispidin for 15 days. The levels of DCs and MDA in a heart homogenate of the experimental animals had then fallen by a factor of 1.9. In addition to this, substantial decreases were also found in the levels of lyso-phosphatidylcholine (1.4-fold), lyso-phosphatidylethanolamine (1.2-fold) and total cholesterol (1.4-fold). The level of phosphatidylethanolamine approximated to, and those of phosphatidylcholine and sphingomyelin even exceeded, their levels in young rats (Table 2). These shifts indicate a normalization of the POL and of the phospholipid composition in the myocardium under the action of hispidin.

Thus, from a walnut-tree fungus we have isolated a polyphenolic compound and have identified it as hispidin. In *in vitro* and *in vivo* experiments the substance under study has shown a pronounced inhibiting action on the peroxide oxidation of lipids. This effect appeared particularly clearly in old rats. The results obtained indicate the desirability of a more profound investigation of hispidin in the geriatric aspect.

EXPERIMENTAL

Isolation of Hispidin. The dried and comminuted fungus of the Persian walnut tree (*Juglans regias*) (1 kg) collected in Tashkent in April, 1983, was extracted three times at room temperature. The combined ethyl acetate extracts were evaporated in vacuum to a volume of 0.6 liter. The resulting yellow precipitate was filtered off, dried, and recrystallized twice from acetone. This gave 21.0 g (2.1% of the air-dry weight of the fungus) of a substance with the composition $C_{13}H_{10}O_5$ and mp 253-255°C. IR spectrum (KBr, ν_{max}, cm^{-1}): 3360-3020 (OH groups), 1670 (α-pyrone C−O), 1603, 1560, 1552 (C—C bonds). UV spectrum (ethanol, λ_{max}, nm): 223, 255, 370 (log ε 4.26, 4.01, 4.12), which are characteristic for α-styrylpyrone derivatives. PMR spectrum (Py-d_5): 5.56 and 6.06 (1H, d, 2.0 Hz, each, H-3 and H-5), 6.61 (1H, d, 16.0 Hz, H-α), 6.91 (1H, dd, 8.0 and 2.0 Hz, H-6′), 7.02 (1H, d, 8.0 Hz, H-5′), 7.32 (1H, br.s, H-2′), 7.40 ppm (1H, d, 16.0 Hz, H-β).

Mass spectrum, *m/z* (%): M^+ 246 (28), 163 (10), 111 (12), 110 (100), 81 (17), 69 (12), 64 (26), 63 (15).

The hispidin formed a triacetyl derivative with mp 161-163°C (δ 2.21 ppm, 9H, singlet, in the PMR spectrum) and a trimethyl ether with mp 165-166°C (M^+ 288).

The influence of the compounds on POL was studied in a system containing a suspension of egg yolk from the change in the parameters of chemiluminescence [6] accompanying this process in the presence of Fe^{2+} (1 ml of a 10^{-2} M solution of $FeSO_4$ in 2.9 ml of phosphate buffer), and the recording of the stages of ChL (rapid flash, latent period, slow flash) was begun. The antioxidant activities of the substances were calculated by using the index δ (tangent of the angle of slope of the exponential stage of the slow flash of the ChL) from the formula given in [2]. In an individual series of experiments we determined the antioxidant activity of hispidin in a liver homogenate *in vitro*. The peroxide oxidation of lipids was initiated

TABLE 2. Influence of Hispidin on the Indices of the Products of the Peroxide Oxidation of Lipids (μM), and the Phospholipid Composition (% of the sum of all the phosphatides and the total lipids) of the Rat Cardiac Muscle ($M \pm m$)

Index	Intact (3 months)	Control (20 months)	Administration for 7 days	Administration for 15 days
Dienic conjugates	0.34±0.042	0.61±0.024	–	0.33±0.0180*
Malondialdehyde	0.45±0.044	0.70±0.067	–	0.37±0.028*
Lyso-phosphatidylcholine	7.29±0.34	8.41±0.62	8.25±0.52	4.97±0.5*
Phosphatidylserine	5.90±0.39	8.40±0.23*	8.20±0.72	9.79±0.94
Lyso-phosphatidylethanolamine	5.22±0.21	7.54±0.23	7.32±0.53	5.77±0.49
Sphingomyelin	7.44±0.62	7.74±0.59	7.08±0.91	8.00±0.88
Phosphatidylcholine	29.31±1.02	28.89±0.73	28.54±1.02	32.76±1.64
Phosphatidylethanolamine	37.88±0.71	34.81±2.01	35.74±2.63	36.73±2.47
Cholesterol	6.15±0.41	11.23±0.11*	11.44±0.51	6.70±0.51*

*Changes significant in relation to the corresponding control at $P < 0.05$ are marked with asterisks.

by 10 μM $FeSO_4$ in the presence of 200 mM aspartate in a medium containing 145 mM KCl, 25 mM Tris-HCl, pH 7.4. The accumulation of LOP products was determined by means of the color reaction [7] with thiobarbituric acid (TBA).

The hemolytic activity of the preparation was evaluated from the induction of the release of hemoglobin from a 2% suspension of rat erythrocytes that had been incubated in a medium containing 140 mM NaCl, 10 mM Tris citrate buffer, pH 7.5, and 1 mM EDTA. The percentage hemolysis was calculated relative to lysis of the cells of control samples in distilled water, taken as 100%.

In the *in vivo* experiments, hispidin was administered orally in a dose of 10 mg/kg to old (20-month) rats once a day for 7 and 15 days. We used young (3-month rats) for comparison. After the lapse of the indicated periods, the animals were sacrificed by decapitation. The levels of CDs and MDA in the cardiac muscles were determined by a standard method [7]. Lipids were extracted and fractionated by horizontal flow chromatography [8]. The amounts of the individual lipid fractions were determined spectrophotometrically [9]. The results obtained were treated statistically as in [10]

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