ORIGINAL ARTICLE

Research of antioxidant properties of theophyllinyl-7-acetic acid derivatives

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Abstract

Objective: Accumulation of reactive oxygen species and nitric oxide (NO) derivatives in the cell exerts damaging effects on its constituents, such as carbohydrates, proteins, lipids, and nucleic acids. These processes has been determined to play a significant role as a nonspecific pathogenetic mechanism in development of multiple disease states, such as atherosclerosis, ischemic stroke, diabetes, *etc*. The search for antioxidant compounds capable of interrupting the pathological biochemical processes at various steps of oxidative and nitrosative stress development, and thus exerting prophylactic and therapeutic effects, is a priority for medical and pharmaceutical sciences. In this article we investigated the effects of newly synthesized esters, hydrazides and hydrazones of theophyllinyl-7-acetic acid on late markers of oxidative and nitrosative stresses.

Methods: Investigation of antioxidant properties of xanthine derivatives was carried out using following *in vitro* methods: inhibition of the NO• radical, inhibition of oxidative modification of proteins, and initiation of lipid peroxidation

Results: Hydrazides and benzylidene-hydrazides of theophyllinyl-7-acetic acid showed more pronounced antioxidant properties than esters. The most active compounds among tested theophyllinyl-7-acetic acid derivatives were hydrazide and (1-phenylethyliden)hydrazide of (8-N-benzylaminotheophyllinyl-7)acetic acid.

Conclusions: *In vitro* studies of 23 derivatives of theophyllinyl-7-acetic acid have been shown that almost all compounds exhibit antioxidant properties. The obtained results also helped us to establish some patterns of structure-activity relationship.

INTRODUCTION

Reactive oxygen species (ROS) are continuously made in a living cell as products of its normal metabolism [1]. They also play role as mediators of important intracellular signaling pathways [2]. During evolution the cell acquired protective mechanisms to limit hyperproduction of ROS, via both enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [3-5], and natural small molecule antioxidants, such as uric acid, vitamins C and E, glutathione and melatonin [6-10]. Increased production of ROS leads to development of oxidative and nitrosative stresses [11-13].

A significant decrease of the total antioxidant status (TAS), resulting from the reduction in enzymatic activities and levels of L-ascorbic acid (vitamin C), alpha (α)-tocopherol (vitamin E) and glutathione, leads to the increase in the ROS concentration, which in turn causes a disruption of balance between pro-oxidant and antioxidant factors.

Accumulation of ROS and nitric oxide (NO) derivatives in the cell exerts damaging effects on its constituents, such as carbohydrates, proteins, lipids, and nucleic acids [13]. The consequences of interactions of ROS and NO derivatives with their targets resulting in formation of oxidative and nitrosative stress products, undesirable for normal cell metabolism. This way, lipid peroxidation results in accumulation of malondialdehyde (MDA), 4-oxo-2-nonenal (ONE), 4-hydroxy-2-nonenal (HNE). Advanced oxidized plasma proteins (especially albumin) are produced during nitrosative stress, as well as advanced glycation ends products (AGE) as result of carbohydrate oxidation.

To date, lipid peroxidation has been determined to play a significant role as a nonspecific pathogenetic mechanism in development of multiple disease states, such as atherosclerosis, ischemic stroke, diabetes, *etc* [14, 15]. The search for antioxidant compounds capable of interrupting the pathological biochemical processes at various steps of oxidative and nitrosative stress development, and thus exerting prophylactic and therapeutic effects, is a priority for medical and pharmaceutical sciences.

Earlier, we demostrated that 3-R-xanthine derivatives containing substitutes in positions 7 and 8, display significant antioxidant activities [16-18]. In continuation of this research we investigated the effects of newly synthesized esters, hydrazides and hydrazones of theophyllinyl-7-acetic acid on late markers of oxidative and nitrosative stresses; these compounds were synthesized by our team earlier [19-21]. Their structures, infrared (IR) and proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy data are represented on Fig.1 and Table 1, respectively.

MATERIALS AND METHODS

Method A: *estimation of antioxidant activity (AOA) by inhibition of NO• radical*

This method is based on photoinduction of sodium nitroprusside, which is accompanied by the accumulation of the NO• radical [22]. The strength of AOA was determined by the rate of ascorbic acid oxidation via the spectroscopic measurement of the absorbance of the sample at 265 nm. As a reference standard we used N-acetylcysteine (NAC) [23].

At first water solutions of ascorbic acid and sodium nitroprusside were prepared. Then, to the 0.01 ml of sodium nitroprusside solution (0.08%), 0.01 ml ascorbic acid solution (0.6%), 0.1 ml solution of examined compounds (in concentrations 10^{-3}, 10^{-5} or 10^{-7} mol/l) and 3 ml distilled water were added. After stirring, reaction was started by immersion of the light source (300 W with $\lambda = 425$ nm) for 30 min. AOAs were estimated by conservation of ascorbic acid concentrations, calculated by following formula (F1):

$$\mathbf{AOA = (Et - Ec) / Ec \times 100\%}$$

-Et; optical density of test sample

-Ec; optical density of control sample

Method B: *estimation of AOA by inhibition of oxidative modification of proteins (OMP) initiated by Fentone's reactive*

The initiation of OMP is carried out in brain homogenates of nonlinear white rats using Fenton medium [1]. For this reason, to 250 mg of tissue homogenate, 7 ml 0.5 M phosphate buffer (dilution temperature 5°C) was added and centrifuged at 11000g for 30 min (at 10°C). Then, to 0.1 ml of the prepared supernatant 0.1 ml of the test compounds (in concentrations of 10^{-3}, 10^{-5} or 10^{-7} mol/l), 0.1 ml of 2.8% solution of iron(II) sulfate and 0.1 ml of 4% hydrogen peroxide were added, and incubated at 37°C for 2 h. Then, 0.1 ml of 20% trichloroacetic acid was added and centrifuged for 30 min at 3000g (15°C). To the residue, that remained after the centrifugation, 1 ml of 2.2% 2,4-dinitrophenylhydrazine (prepared in 7% solution of hydrochloric acid) was added, incubated for 1 h at 37°C and centrifuged for 10 min at 3000g. The sediment was washed by 3 ml of ethyl acetate. After drying, 3 ml of 8 M urea solution and 1 ml 2 M hydrochloric acid solution were added. Thereafter, we determined the optical density of obtained solution spectrophotometrically at 274 nm and 363 nm (control solution = 0.5 M phosphate buffer). Emoxypine was used as a reference standard [24]. The quantity of aldehyde dinitrophenylhydrazones (APH) and ketone dinitrophenylhydrazones (KPH) was defined. Results were recalculated on total protein and were estimated as units per gram of protein. AOA was calculated by formula 2 (F2):

$$\mathbf{AOA = (Ec - Et) / Ec \times 100\%}$$

-Et; optical density of test sample

-Ec; optical density of control sample

Method C: *estimation of AOA at non-enzymatic initiation of lipid peroxidation*

Suspension of egg lipoproteins was used as substrate [25]. It was prepared by homogenisation of egg yolk on phosphate buffer (pH = 7.4). Then test compounds (in concentrations of 10^{-3}, 10^{-5} or 10^{-7} mol/l) were added to the suspension. Reaction of free radical oxidation was initiated by addition of 0.025 M solution of $FeSO_4 \cdot 7H_2O$ with the next incubation of obtained mixture at 37°C during 60 min. Reaction was stopped by addition of 50% trichloroacetic acid solution with trilon B. After centrifugation (30 min, 3000g) supernatant was added to thiobarbituric acid (TBA) solution. Then mixture was heated on water bath for 60 min. Colored complex of MDA with TBA were extracted by addition of butan-1-ol. The concentration of MDA, which showed the intensivity of free radical oxidation processes, was estimated by the spectroscopic measurement of the absorbance of the sample at 532 nm. AOA was calculated by F2. Dibunol was used as reference drug [26].

Statistical analysis

Statistics were done using the STATISTICA® software [27]. Data is presented as the mean ± SEM. The fidelity of differences between experimental groups was estimated via Student's *t*-test and Fisher's exact test.

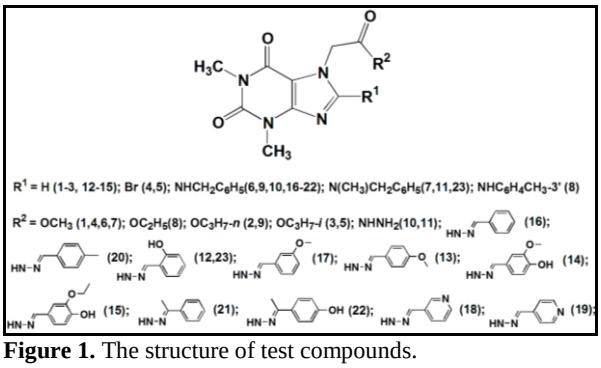

Figure 1. The structure of test compounds.

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Table 1. IR and ^{1}H NMR spectra data of test compounds

Compound	IR, cm^{-1}	^{1}H NMR ($CDCl_3$), δ, pm
1	3050 (Ar-H); 1701, 1698 (C=O); 1667 (C=N); 1602 (C=C)	8.04 (s, 1H, C^8H); 5.18 (s, 2H, N^7-CH_2-); 3.69 (s, 3H, -OCH_3); 3.42 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3)
2	3030 (Ar-H); 1741, 1695 (C=O); 1663 (C=N); 1602 (C=C)	8.06 (s, 1H, C^8H); 5.16 (s, 2H, N^7-CH_2-); 4.08 (tr, 2H, -OCH_2-); 3.42 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3); 1.58 (m, 2H, -CH_2-); 0.88 (tr, 3H, CH_3)
3	3015 (Ar-H); 1708, 1696 (C=O); 1666 (C=N); 1600 (C=C)	8.04 (s, 1H, C^8H); 5.07 (s, 2H, N^7-CH_2-); 4.96 (m, 1H, -OCH=) 3.41 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3); 1.18 (d, 6H, $2CH_3$)
4	1703, 1698 (C=O); 1659 (C=N); 1620 (C=C)	5.18 (s, 2H, N^7-CH_2-); 3.75 (s, 3H, -OCH_3); 3.43 (s, 3H, N^3-CH_3); 3.21 (s, 3H, N^1-CH_3)
5	1703, 1698 (C=O); 1659 (C=N); 1610 (C=C)	5.14 (s, 2H, N^7-CH_2); 4.96 (m, 1H, -OCH-); 3.41 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3); 1.21 (d, 6H, $2CH_3$)
6	3327 (NH); 3030 (Ar-H); 1710, 1692 (C=O); 1649 (C=N); 1618 (C=C)	7.79 (t, 1H, C^8-NH-); 7.40-7.23 (m, 5H, Ar-H); 4.94 (s, 2H, N^7-CH_2-); 4.57 (d, 2H, -CH_2-); 3.71 (s, 3H, -OCH_3); 3.35 (s, 3H, N^3-CH_3); 3.14 (s, 3H, N^1-CH_3)
7	3030 (Ar-H); 1708. 1690 (C=O); 1660 (C=N); 1624 (C=C)	7.54-7.12 (m. 5H. Ar-H); 5.01 (s, 2H, N^7-CH_2-); 4.49 (s, 2H, N-CH_2-); 3.99 (s, 3H, -OCH_3) 3.47 (s, 3H, N^3-CH_3); 3.23 (s, 3H, N^1-CH_3); 3.08 (s, 3H, N-CH_3)
8	3361 (NH); 3050 (Ar-H); 1715, 1695 (C=O); 1672 (C=N); 1629 (C=C)	9.19 (s, 1H, C^8-NH-); 7.54 (d, 1H, Ar-H), 7.46 (s. 1H, Ar-H), 7.23 (t. 1H, Ar-H), 6.84 (d, 1H, Ar-H); 5.14 (s, 2H N^7-CH_2-); 4.19 (q, 2H, -OCH_2-) 3.45 (s, 3H, N^3-CH_3); 3.20 (s, 3H, N^1-CH_3); 2.31 (s, 3H, Ar-CH_3); 1.24 (t, 3H, CH_3)
9	3330 (NH); 3037 (Ar-H); 1705, 1688 (C=O); 1642 (C=N); 1619 (C=C)	7.82 (t, 1H, C^8-NH-); 7.40-7.18 (m, 5H, Ar-H); 4.96 (s, 2H, N^7-CH_2-); 4.54 (d, 2H, N-CH_2-); 4.04 (t, 2H, -OCH_2-); 3.31 (s, 3H, N^3-CH_3); 3.12 (s, 3H, N^1-CH_3); 1.58 (m, 2H, -CH_2-); 0.97 (t, 3H, CH_3)
10	3320, 3233 (NH); 3025 (Ar-H); 1690 (C=O); 1651 (C=N); 1621 (C=C)	9.26 (s, 1H, CONH); 7.54 (t, 1H, C^8-NH-); 7.35-7.17 (m, 5H, Ar-H); 4.73 (s, 2H, N^7-CH_2-); 4.55 (d, 2H, NCH_2-); 4.28 (br s, 2H, -NH_2); 3.38 (s, 3H, N^3-CH_3); 3.16 (s, 3H, N^1-CH_3)
11	3340, 3230 (NH); 3070 (Ar-H); 1691 (C=O); 1651 (C=N); 1614 (C=C)	9.30 (s, 1H, CONH); 7.60 (m, 5H, Ar-H); 4.78 (s, 2H, N^7-CH_2-); 4.48 (s, 2H, N-CH_2-); 4.24 (br s, 2H, -NH_2); 3.36 (s, 3H, N^3-CH_3); 3.16 (s, 3H, N^1-CH_3); 2.82 (s, 3H, CH_3)
12	3610 (OH); 3269 (NH); 3050 (Ar-H); 1693 (C=O); 1641 (C=N); 1600 (C=C)	11.67 (s, 1H, CONH); 10.07 (s, 1H, -OH); 8.37 (s, 1H, N=CH); 8.05 (s, 1H, C^8H); 7.27 (m, 1H, Ar-H), 6.87 (m, 3H, Ar-H); 5.52 (s, 2H, N^7-CH_2-); 3.44 (s, 3H, N^3-CH_3); 3.19 (s, 3H, N^1-CH_3)
13	3346 (NH); 3066 (Ar-H); 1702 (C=O); 1666 (C=N); 1598 (C=C)	11.60 (s, 1H, CONH); 8.02 (s, 1H, C^8H); 8.00 (s, 1H, N=CH); 7.78 (d. 2H, Ar-H), 7.00 (d, 2H, Ar-H); 5.53 (s, 2H, N^7-CH_2-); 3.82 (s, 3H, -OCH_3); 3.42 (s, 3H, N^3-CH_3); 3.19 (s, 3H, N^1-CH_3)
14	3600 (OH); 3320 (NH); 3070 (Ar-H); 1694 (C=O); 1641 (C=N); 1601 (C=C)	11.57 (s, 1H, CONH); 9.52 (s, 1H, OH); 8.08 (s, 1H, N=CH); 7.92 (s, 1H, C^8-CH); 7.29 (s, 1H, Ar-H), 7.10 (d, 1H, Ar-H), 6.82 (d, 1H, Ar-H); 5.52 (s, 2H, N^7-CH_2-); 3.80 (s, 3H, -OCH_3); 3.43 (s, 3H, N^3-CH_3); 3.19 (s, 3H, N^1-CH_3)
15	3620 (OH); 3210 (NH); 3090 (Ar-H); 1693 (C=O); 1640 (C=N); 1600 (C=C)	11.55 (s, 1H, CONH); 9.44 (s, 1H, OH); 8.06 (s, 1H, N=CH); 7.90 (s, 1H, C^8H); 7.30 (s, 1H, Ar-H), 7.08 (d, 1H, Ar-H), 6.82 (d, 1H, Ar-H); 5.51 (s, 2H, N^7-CH_2-); 4.04 (q, 2H, -OCH_2-); 3.40 (s, 3H, N^3-CH_3); 3.20 (s, 3H, N^1-CH_3); 1.31 (t, 3H, -CH_3)
16	3308, 3130 (NH); 3075 (Ar-H); 1700 (C=O); 1675 (C=N); 1620 (C=C)	11.56 (s, 1H, CONH); 8.00 (s, 1H, N=CH); 7.61 (t, 1H, C^8-NH); 7.76-7.19 (m, 10H, Ar-H); 5.31 (s, 2H, N^7-CH_2-); 4.58 (d, 2H, NCH_2-); 3.40 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3)
17	3320, 3140 (NH); 3030 (Ar-H) 1698 (C=O); 1679 (C=N); 1619 (C=C)	11.62 (s, 1H, CONH); 7.91 (s, 1H, N=CH); 7.60 (t, 1H, C^8-NH-); 7.40-6.90 (m, 9H, Ar-H); 5.30 (s, 2H, N^7-CH_2-); 4.57 (d, 2H, NCH_2-); 3.82 (s, 3H, OCH_3); 3.40 (s, 3H, N^3-CH_3); 3.17 (s, 3H, N^1-CH_3)
18	3309, 3132 (NH); 3050 (Ar-H); 1700 (C=O); 1645 (C=N); 1615 (C=C)	11.70 (s, 1H, CONH); 8.83 (s, 1H, Ar-H); 8.55 (d, 1H, Ar-H); 8.10 (d, 1H, Ar-H); 8.05 (s, 1H, N=CH); 7.55 (t, 1H, C^8NH); 7.44-7.20 (m, 6H, Ar-H); 5.31 (s, 2H, N^7CH_2-); 4.58 (d, 2H, NCH_2-); 3.41 (s, 3H, N^3-CH_3); 3.19 (s, 3H, N^1-CH_3).
19	3310. 3200 (NH); 3040 (Ar-H); 1700 (C=O); 1645 (C=N); 1618 (C=C)	11.89 (s, 1H, CONH); 8.59 (d, 2H, Ar-H); 7.97 (s, 1H, N=CH-); 7.62 (m, 1H, C^8-NH-); 7.59-7.20 (m, 6H, Ar-H); 5.33 (s, 2H, N^7-CH_2-); 4.57 (d, 2H, -NCH_2-); 3.40 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3)
20	3313, 3130 (NH); 3030 (Ar-H); 1694 (C=O); 1680 (C=N); 1617 (C=C)	11.54 (s, 1H, CONH); 7.88 (s, 1H, N=CH-); 7.60 (d, 1H, C^8-NH-); 7.58-7.18 (m, 9H, Ar-H); 5.30 (s, 2H, N^7-CH_2-); 4.57 (d, 2H, -NCH_2-); 3.40 (s, 3H, N^3-CH_3); 3.19 (s, 3H, N^1-CH_3); 2.34 (s. 3H, CH_3)
21	3340, 3110 (NH); 3035 (Ar-H); 1692 (C=O); 1680 (C=N); 1619 (C=C)	10.94 (s, 1H, CONH); 7.65 (t, 1H, C^8-NH-); 7.84-7.19 (m, 10H, Ar-H); 5.36 (s, 2H, N^7-CH_2-); 4.59 (d, 2H, -NCH_2-); 3.41 (s, 3H, N^3-CH_3); 3.18 (s, 3H, N^1-CH_3); 2.27 (s. 3H, CH_3)
22	3620 (OH); 3390. 3320 (NH); 3080 (Ar-H); 1687 (C=O); 1644 (C=N); 1614 (C=C)	10.74 (s, 1H, CONH); 9.53 (s, 1H, -OH); 7.60 (t, 1H, C^8-NH-); 7.64-6.74 (m, 9H, Ar-H); 5.32 (s, 2H, N^7-CH_2-); 4.57 (d, 2H, -NCH_2-); 3.41 (s, 3H, N^3-CH_3); 3.19 (s, 3H, N^1-CH_3); 2.21 (s, 3H, -CH_3)
23	3610 (OH); 3230 (NH); 3050 (Ar-H); 1697 (C=O); 1647 (C=N); 1613 (C=C)	11.62 (s, 1H, CONH); 10.00 (s, 1H, OH); 8.30 (s, 1H, N=CH-); 7.30 (m, 6H, Ar-H); 6.87 (m, 3H, Ar-H); 5.38 (s, 2H, N^7-CH_2-); 4.50 (d, 2H, -NCH_2-); 3.38 (s, 3H, N^3-CH_3); 3.16 (s, 3H, N^1-CH_3); 2.82 (s, 3H, CH_3)

RESULTS

Method A

All tested compounds (**1-23**) showed relatively high antioxidant properties and their values, in some cases, exceed the standard drug used (NAC) (Table 2).

Among esters of theophyllinyl-7-acetic acid (**1-9**), AOA was within 77.21-243.6% (at concentration of 10^{-3} mol/l). The most active compound in this group was methyl 8-bromotheophyillinyl-7-acetate (**4**), which exceeded the NAC standard by an AOA of 241.1%. At the concentration of 10^{-5} mol/l, activity of all esters decreased in 5-7 times, but most of the compounds already exceeded the standard. At the concentration of 10^{-7} mol/l, more than 40% of substances showed activity less then NAC.

On the other hand, hydrazides and benzylidene-hydrazides of theophyllinyl-7-acetic acid (**10-23**) showed more pronounced antioxidant properties than esters. Their AOA was within 64-277.9% (10^{-3} mol/l). Hydrazide of 8-N-benzylamino-1,3-dimethylxanthinyl-7-acetic acid (**10**) and (1-phenylethyliden)hydrazide of 8-N-benzylamino-1,3-dimethylxanthinyl-7-acetic acid (**21**) presented the best values. Activity of these compounds exceeded the result of NAC at all concentrations (264.87% and 275.44% at 10^{-3}, 45.15 % and 47.24% at 10^{-5}, 17.42% and 27.61% at 10^{-7} mol/l, respectively).

Method B

Esters of theophyllinyl-7-acetic acid (**1-9**) at concentrations of 10^{-3} and 10^{-5} mol/l showed lower AOA than emoxypine, but at 10^{-7} mol/l almost all compounds were more active than the reference drug (Tables 3&4).

More active were hydrazides and benzylidene-hydrazides of theophyllinyl-7-acetic acid (**10-23**). AOA of these compounds at determining of APH was within 14.18-53.47% at 10^{-3}, 12.46-51.06% at 10^{-5} and 3.01-56.66% at 10^{-7} mol/l; results for emoxypine were 34.5%, 23% and 18% at the same concentrations, respectively. In determining of KPH, AOA of compounds (**10-23**) was within 17.66-66.03%, 15.57-64.92% and 0.87-69.29% in coincident concentrations

Table 2. Antioxidant activity of test compounds (n = 5) by inhibition of NO• radical (mean ± SEM).

Compound	10^{-3} mol/l		10^{-5} mol/l		10^{-7} mol/l	
	E	%	E	%	E	%
1	3.294 ± 0.065^{2}	210.17	1.361 ± 0.093^{1}	28.15	1.207 ± 0.068	13.65
2	3.435 ± 0.064^{2}	223.45	1.377 ± 0.053^{2}	29.66	1.248 ± 0.081^{1}	17.51
3	2.848 ± 0.066^{2}	168.17	1.328 ± 0.07^{2}	25.05	1.141 ± 0.043	7.44
4	3.649 ± 0.024^{2}	243.6	1.404 ± 0.113^{1}	32.20	1.145 ± 0.042	7.82
5	2.885 ± 0.052^{2}	171.66	1.338 ± 0.077^{2}	25.99	1.426 ± 0.268	34.27
6	1.882 ± 0.070^{2}	77.21	1.246 ± 0.03^{2}	17.33	1.233 ± 0.071^{1}	16.10
7	1.922 ± 0.085^{2}	80.98	1.355 ± 0.096^{1}	27.59	1.124 ± 0.035	5.84
8	2.633 ± 0.054^{2}	147.93	1.259 ± 0.077^{1}	18.55	1.217 ± 0.083	14.59
9	2.222 ± 0.054^{2}	109.23	1.272 ± 0.091^{1}	19.77	1.162 ± 0.062	9.42
Control			**1.062 ± 0.025**			
10	3.857 ± 0.015^{2}	267.33	1.718 ± 0.004^{2}	63.62	1.354 ± 0.004^{2}	28.95
11	1.868 ± 0.003^{2}	77.9	1.456 ± 0.002^{2}	38.67	1.217 ± 0.002^{2}	15.9
12	2.117 ± 0.002^{2}	101.62	1.466 ± 0.001^{2}	39.62	1.131 ± 0.002^{2}	7.71
13	2.788 ± 0.001^{2}	165.52	1.662 ± 0.002^{2}	58.29	1.359 ± 0.001^{2}	29.43
14	2.966 ± 0.001^{2}	182.48	1.74 ± 0.001^{2}	65.71	1.262 ± 0.003^{2}	20.19
15	2.158 ± 0.002^{2}	105.52	1.402 ± 0.001^{2}	33.52	1.179 ± 0.001^{2}	12.29
16	1.957 ± 0.001^{2}	86.38	1.534 ± 0.001^{2}	46.10	1.234 ± 0.001^{2}	17.52
17	2.56 ± 0.001^{2}	143.81	1.42 ± 0.001^{2}	35.24	1.111 ± 0.001^{2}	5.81
18	1.863 ± 0.001^{2}	77.43	1.191 ± 0.002^{2}	13.43	1.102 ± 0.010^{2}	4.95
19	2.629 ± 0.001^{2}	150.38	1.423 ± 0.001^{2}	35.52	1.129 ± 0.001^{2}	7.52
20	3.125 ± 0.001^{2}	197.62	1.377 ± 0.002^{2}	31.14	1.129 ± 0.001^{2}	7.52
21	3.968 ± 0.001^{2}	277.9	1.74 ± 0.001^{2}	65.71	1.461 ± 0.001^{2}	39.14
22	1.75 ± 0.001^{2}	66.67	1.21 ± 0.001^{2}	15.24	1.113 ± 0.012^{2}	6
23	1.722 ± 0.001^{2}	64	1.192 ± 0.001^{2}	13.52	1.106 ± 0.017^{2}	5.33
Control			**1.05 ± 0.002**			
NAC	1.209 ± 0.001	2.46	1.398 ± 0.002	18.47	1.316 ± 0.001	11.53
Control			**1.18 ± 0.007**			

Compared to control: $^{1}P < 0.05$, $^{2}P < 0.01$.

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Table 3. Antioxidant activity of test compounds (n = 5) by inhibition of oxidative modification of protein (APH; mean ± SEM)

Compound	10^{-3} mol/l		10^{-5} mol/l		10^{-7} mol/l	
	U /g of protein	%	U /g of protein	%	U /g of protein	%
1	40.359 ± 1.972[1]	11.08	38.613 ± 3.682	14.92	42.211 ± 2.052	6.99
2	39.873 ± 0.838[2]	12.15	37.269 ± 1.902[2]	17.88	36.899 ± 1.803[2]	18.7
3	38.169 ± 3.323[1]	15.9	46.656 ± 2.219	-2.8	39.947 ± 2.456[1]	11.98
4	38.846 ± 2.355[1]	14.41	42.021 ± 2.312	7.41	40.158 ± 2.992	11.52
5	33.936 ± 5.036[1]	25.23	42.306 ± 1.669	6.79	37.555 ± 1.483[2]	17.25
6	35.629 ± 2.476[2]	21.5	39.587 ± 2.099[1]	12.78	38.603 ± 4.022	14.95
7	41.555 ± 2.907	8.44	37.597 ± 1.93[2]	17.16	39.079 ± 1.588[2]	13.9
8	39.015 ± 5.691	14.04	36.338 ± 4.181[1]	19.94	39.375 ± 1.26[2]	13.24
9	40.888 ± 1.368[1]	9.91	38.91 ± 2.393[1]	14.27	38.105 ± 2.383[1]	16.04
Intact	14.07 ± 1.103	-	14.07 ± 1.103	-	14.07 ± 1.103	-
Control			**45.386 ± 0.672**			
10	5.109 ± 0.003[2]	48.65	4.87 ± 0.01[1,3]	51.06	4.312 ± 0.025[2]	56.66
11	7.472 ± 0.029[2]	24.9	7.54 ± 0.05[1]	24.22	6.526 ± 0.029[2]	34.41
12	7.489 ± 0.002[2]	24.73	7.6 ± 0.01[1]	23.62	8.65 ± 0.002[2]	13.07
13	4.679 ± 0.003[2]	52.97	5 ± 0.04[1,3]	49.75	8.006 ± 0.002[2]	19.54
14	5.23 ± 0.002[2]	47.44	5.8 ± 0.03[1,3]	41.71	7.852 ± 0.005[2]	21.09
15	6.22 ± 0.002[2]	37.49	6.5 ± 0.02[1]	34.67	8.561 ± 0.003[2]	13.96
16	7.529 ± 0.002[2]	24.33	7.87 ± 0.04	20.9	9.146 ± 0.03[2]	8.08
17	7.05 ± 0.002[2]	29.15	7.39 ± 0.02[1]	25.73	8.132 ± 0.016[2]	18.27
18	4.889 ± 0.002[2]	50.86	5.11 ± 0.02[1,3]	48.64	7.268 ± 0.022[2]	26.95
19	8.539 ± 0.003[2]	14.18	8.71 ± 0.04	12.46	9.650 ± 0.002[2]	3.02
20	6.31 ± 0.002[2]	36.58	6.55 ± 0.01[1]	34.17	7.859 ± 0.003[2]	21.02
21	4.63 ± 0.002[2]	53.47	5 ± 0.04[1,3]	49.75	7.56 ± 0.002[2]	24.02
22	4.859 ± 0.002[2]	51.17	5.2 ± 0.09[1,3]	47.74	8.584 ± 0.025[2]	13.73
23	5.03 ± 0.003[2]	49.45	5.2 ± 0.07[1,3]	47.74	8.559 ± 0.002[2]	13.98
Intact	1.07 ± 0.002[1]	-	1.07 ± 0.002[1]	-	1.07 ± 0.002[1]	-
Control			**9.95 ± 0.07**			
Emoxypine	10.297 ± 0.064	34.5	12.104 ± 0.152	23	12.891 ± 0.049	18
Control			**15.72 ± 0.126**			

[1]P < 0.05 and [2]P < 0.01 compared to control; [3]P < 0.05 compared to emoxypine.

(emoxypine: 35.4%, 21.8%, 12.5%). The most active compounds were **10** and **21**. Activity of hydrazide of 8-N-benzylaminotheophyllinyl-7-acetic acid (**10**) increased depending on concentration and was maximal at 10^{-7} mol/l (56.66% in APH and 69.29% in KPH assay). (1-Phenylethyliden)hydrazide of 8-N-benzyl-aminotheophyllinyl-7-acetic acid **(21**) was more active than reference drug on 18.97% (APH) and on 30.63% (KPH) at 10^{-3} mol/l. The lowest activity was recorded for pyridyl-4'-methylidenhydrazide of 8-N-benzyl-aminotheophyllinyl-7-acetic acid (**19**), that was much inferior than the activity of the reference drug.

Method C

On this model almost all synthesized compounds showed antioxidant properties, which approximated to the reference drug or exceeded it (Table 5).

Among esters of theophyllinyl-7-acetic acid (**1-9**), methyl 8-N-methyl-N-benzylaminotheophyllinyl-7-acetate (**7**) was the most active compound; its AOA exceeded dibunol for 56.87% at 10^{-3}, 2.57% at 10^{-5} and 13.73% at 10^{-7} mol/l concentration.

As in previous two methods, hydrazides (**10-11**) and benzylidene-hydrazides (**12-23**) of theophyllinyl-7-acetic acid were more active than esters. Highest antioxidant properties were shown by compounds **10**, **13** and **21**, which exceeded result of the reference drug at all concentrations.

DISCUSSION

The *in vitro* studies of 23 derivatives of theophyllinyl-7-acetic acid have been shown that almost all compounds exhibit antioxidant properties. Obtained results also helped us to establish some patterns of structure-activity relationship. As a basic structure for further chemical modification, we chosed methyl ester of theophylline-7-acetic acid (compound **1**), which showed antioxidant action *in vitro* on the model of

photoinduced nitroprusside oxidation (method A), due to its NO• scavenger properties. At the same time ester **1** exhibited moderate antioxidative properties *in vitro* in the model of OMP (on the markers of OMP; APH and KPH) (method B) and on the model of lipid peroxidation (MDA reduction) (method C). Compound **1** modification by elongation of the ester residue (propyl ester **2**) caused increase in AOA in all three *in vitro* models: the inhibition of OMP markers (APH and KPH) formation and lipid peroxidation (MDA), and inhibition of NO• formation in the reaction of the photoinduced nitroprusside oxidation. Branching of ester radical (isopropyl ester **3**) decreased the NO• scavenger properties, but increased antioxidant effect *in vitro* in the oxidation of proteins (by Fenton's reagent), and lipids (by iron(II) salts). Thus, our studies showed that the NO• scavenger properties of the esters **1-3** dominate over properties of inhibitors of OMP and lipid peroxidation *in vitro*. We assume that this is connected with the presence of free position 8 of xanthine heterocycle. Molecules of esters **1-3** linked to NO• radical due to the formation of stable 8-nitroso-xanthine complexes, that can predict their cyto-protective properties under nitrosative stress.

Then, we studied the effect of electron donating group at position 8 of xanthine molecules on AOA. So, the insertion of bromine atom of the molecules of esters **1** and **2** had a positive effect on antioxidant properties *in vitro*. The obtained 8-bromoxanthines **4** and **5** significantly reduced concentration of products of OMP and lipid peroxidation. Compounds **4** and **5** had a more pronounced NO• scavenger properties than the starting compounds **1** and **2**. Substitution of bromine atom on weaker electron donating groups such as amine residues, primary (benzylamine, compound **6**) or secondary (N-methylbenzylamine, compound **7**), led to a change in direction of the antioxidant effect; decreasing NO• scavenger properties and increasing of the inhibitory properties on OMP and lipid peroxidation markeres.

Table 4. Antioxidant activity of test compounds (n = 5) by inhibition of oxidative modification of protein (KPH; mean ± SEM)

Compound	10^{-3} mol/l		10^{-5} mol/l		10^{-7} mol/l	
	U/g protein	%	U/g protein	%	U/g protein	%
1	26.56 ± 1.387[1]	11.5	26.582 ± 1.327[1]	11.42	26.571 ± 0.722[2]	11.46
2	26.433 ± 1.721	11.92	25.502 ± 1.481[1]	15.02	21.375 ± 1.2[2]	28.77
3	24.031 ± 1.881[2]	19.92	32.423 ± 0.836[1]	-8.04	24.285 ± 1.128[2]	19.08
4	24.73 ± 1.581[2]	17.59	27.439 ± 1.199	8.57	23.185 ± 1.51[2]	22.74
5	22.73 ± 3.092[1]	24.26	28.211 ± 1.218	5.99	23.185 ± 0.721[2]	22.74
6	21.682 ± 0.883[2]	27.75	25.64 ± 1.237[2]	15.56	23.947 ± 1.444[2]	20.2
7	25.365 ± 1.049[2]	15.48	24,931 ± 0.444[2]	16.92	23.608 ± 0.884[2]	21.33
8	27.015 ± 1.968	9.98	25.153 ± 1.624[1]	16.18	24.857 ± 0.889[2]	17.17
9	28.158 ± 0.78	6.17	26.486 ± 0.94[2]	11.74	22.603 ± 0.933[2]	24.68
Intact	11.728 ± 0.911	-	11.728 ± 0.911	-	11.728 ± 0.911	-
Control			**30.01 ± 0.556**			
10	3.14 ± 0.003[2]	54.29	2.41 ± 0.02[1,3]	64.92	2.11 ± 0.002[1]	69.29
11	5.569 ± 0.032[2]	18.94	5.68 ± 0.01	17.32	4.871 ± 0.002[1]	29.1
12	4.208 ± 0.003[2]	38.75	4.32 ± 0.01[1,3]	37.12	6.516 ± 0.002[2]	5.15
13	2.559 ± 0.002[2]	62.75	2.87 ± 0.02[1,3]	58.22	5.658 ± 0.003[1]	17.64
14	3.109 ± 0.003[2]	54.75	3.34 ± 0.02[1,3]	51.38	4.981 ± 0.003[1]	27.5
15	3.65 ± 0.002[2]	46.87	4 ± 0.02[1]	41.78	6.228 ± 0.003[1]	9.34
16	5.103 ± 0.004[2]	25.72	5.26 ± 0.01	23.44	6.087 ± 0.002[1]	11.4
17	4.31 ± 0.002[2]	37.26	4.6 ± 0.01[1]	33.04	5.107 ± 0.003[1]	25.66
18	3.649 ± 0.003[2]	46.88	4 ± 0.01[1,3]	41.78	5.231 ± 0.002[1]	23.86
19	5.657 ± 0.003[2]	17.66	5.8 ± 0.02	15.57	6.81 ± 0.002[1]	0.87
20	4.658 ± 0.003[2]	32.2	4.97 ± 0.07[1]	27.66	5.459 ± 0.003[1]	20.54
21	2.334 ± 0.001[1]	66.03	2.87 ± 0.01[1,3]	58.22	4.901 ± 0.002[1]	28.66
22	2.609 ± 0.002[1]	62.02	2.71 ± 0.03[1,3]	60.55	5.9 ± 0.003[1]	14.12
23	3.449 ± 0.002[1]	49.8	3.71 ± 0.05[1,3]	46	5.801 ± 0.004[1]	15.56
Intact	1.37 ± 0.02[1]	-	1.37 ± 0.02[1]	-	1.37 ± 0.02[1]	-
Control			**6.870±0.002**			
Emoxypine	7.356 ± 0.196	35.4	8.905 ± 0.221	21.8	9.964 ± 0.168	12.5
Control			**11.387±0.239**			

[1]P < 0.05 and [2]P < 0.01 compared to control; [3]P < 0.05 compared to emoxypine.

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Table 5. Antioxidant activity of test compounds (n = 5) at non enzymatic initiation of free radical lipid peroxidation (M±m)

Compound	10^{-3} mol/l		10^{-5} mol/l		10^{-7} mol/l	
	E, M±m	%	E, M±m	%	E, M±m	%
1	0.182 ± 0.018	6.67	0.18 ± 0.023	7.69	0.164 ± 0.017	15.9
2	0.176 ± 0.018	9.74	0.143 ± 0.012[1]	26.67	0.166 ± 0.016	14.87
3	0.154 ± 0.014[1]	21.03	0.163 ± 0.014	16.41	0.176 ± 0.020	9.74
4	0.134 ± 0.012[2]	31.28	0.169 ± 0.011	13.33	0.156 ± 0.022	20
5	0.241 ± 0.022	-23.59	0.205 ± 0.032	-5.13	0.21 ± 0.026	-7.69
6	0.16 ± 0.018	17.95	0.154 ± 0.013[1]	21.03	0.178 ± 0.018[2]	8.72
7	0.058 ± 0.009[2]	70.26	0.137 ± 0.016[1]	29.74	0.126 ± 0.015	35.38
8	0.111 ± 0.011[2]	43.08	0.152 ± 0.013[1]	22.05	0.152 ± 0.019	22.05
9	0.109 ± 0.011[2]	44.1	0.153 ± 0.013[1]	21.54	0.164 ± 0.017	15.9
Control			**0.195±0.009**			
10	0.041 ± 0.001[2]	85.46	0.093 ± 0.003[2]	67.02	0.157 ± 0.001[2]	44.33
11	0.069 ± 0.005[2]	75.53	0.098 ± 0.004[2]	65.25	0.214 ± 0.005[2]	24.11
12	0.088 ± 0.003[2]	68.79	0.13 ± 0.001[2]	53.9	0.201 ± 0.004[2]	28.72
13	0.048 ± 0.004[2]	82.98	0.093 ± 0.001[2]	67.02	0.194 ± 0.003[2]	31.21
14	0.077 ± 0.004[2]	72.7	0.105 ± 0.009[2]	62.77	0.246 ± 0.004[2]	12.77
15	0.092 ± 0.004[2]	67.38	0.139 ± 0.003[2]	50.71	0.201 ± 0.004[2]	28.72
16	0.112 ± 0.004[2]	60.28	0.147 ± 0.003[2]	47.87	0.242 ± 0.003[2]	14.18
17	0.155 ± 0.004[2]	45.03	0.171 ± 0.002[2]	39.36	0.264 ± 0.006[1]	6.38
18	0.144 ± 0.004[2]	48.94	0.168 ± 0.004[2]	40.43	0.267 ± 0.004[2]	5.32
19	0.158 ± 0.004[2]	43.97	0.176 ± 0.004[2]	37.59	0.253 ± 0.003[2]	10.28
20	0.075 ± 0.003[2]	73.4	0.14 ± 0.005[2]	50.35	0.242 ± 0.002[2]	14.18
21	0.048 ± 0.003[2]	82.98	0.131 ± 0.002[2]	53.55	0.188 ± 0.003[2]	33.33
22	0.137 ± 0.004[2]	51.42	0.159 ± 0.004[2]	43.62	0.225 ± 0.006[2]	20.21
23	0.13 ± 0.013[2]	53.90	0.151 ± 0.001[2]	46.45	0.244 ± 0.002[2]	13.47
Control			**0.282 ± 0.001**			
Dibunol	0.22 ± 0.033[1]	13.39	0.185 ± 0.02[1]	27.17	0.199 ± 0.018[1]	21.65
Control			**0.254 ± 0.001**			

Compared to control: [1]P < 0.05, [2]P < 0.01.

In order to obtain a more effective NO• scavenger, we carried out further modification on the theophylline-7-acetic acid derivatives at the carboxyl group. However, the substitution of the alkoxy radicals of esters **1-3** on benzylidene fragment led to a decrease in the NO• scavenger properties and to enhance the ability of inhibiting the OMP and lipid peroxidation *in vitro*. This, in our view, may be due to the steric factor; the presence of a massive substituent at the position 7 hinders access to the NO• radical to position 8 of the xanthine molecule. This assumption is indirectly confirmed by the results of AOA data of ester of 8-benzylaminotheophyllinyl-7-acetic acid **6** with its benzylidene-hydrazides **16**, **17**, **20-22**, which showed that benzylidene-hydrazides not only significantly reduce the formation of markers of lipid peroxidation and OMP, but inhibit also the formation of NO• radical.

The most optimal way to increase antioxidant properties was substitution of ester residue to a hydrazide group. Thus, the hydrazide **10** showed the most pronounced antioxidant properties in all models of AOA estimation *in vitro*.

In the studies for AOA of benzylidene-hydrazides **16-23**, it was found that the presence in the benzylidene fragment of electron donating groups such as -OH, $-OCH_3$, $-CH_3$, in most cases, increased the activity, but the combination of these two groups did not lead to the summation of AOA. This tendency can clearly be seen during comparison of results of benzylidene-hydrazides (**12-17** and **20**) and is common for all three experimental models (methods A-C).

Summarizing the obtained results, it should be noted that the most active compounds among the tested theophyllinyl-7-acetic acid derivatives were hydrazide **10** and (1-phenylethyliden)hydrazide **21** of 8-N-benzyl-aminotheophyllinyl-7-acetic acid. We assume that their expressed NO• scavenging properties are caused by the presence of hydrazide fragment in the structures of the compounds. Thus, the obtained results of the estimation of AOA of theophylline-7-acetic acid derivatives *in vitro* gave experimental substantiation of search for NO• scavengers among their hydrazides.

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