Synthesis and Biological Evaluation of new Benzo[*f*]furo[2,3-*h*]- and Benzo[*f*]pyrano[2,3-*h*]coumarin Derivatives.[#]

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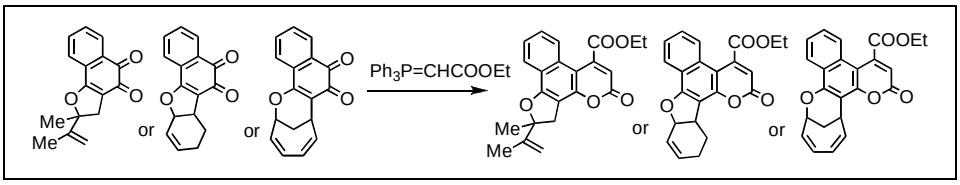

Furocoumarins **3, 5** and pyranocoumarin **7** were synthesized from the reaction of furonaphthalenediones **2, 4** and pyranonaphthalenedione **6** respectively with carbethoxymethylene(triphenyl)phosphorane in refluxing DCM for 3-6 hours or under microwave irradiation in toluene for a few minutes. Compounds **3, 5**, **7** and their precursors were tested as anti-inflammatory/antioxidant agents. They were found to compete significantly high DMSO for ·OH radicals, to scavenge $O_2^{\cdot-}$ and to inhibit lipoxygenase to a high extent.

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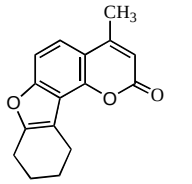
INTRODUCTION

Coumarins are an interesting class of heterocyclic compounds exhibiting many biological activities, while a lot of their derivatives are present in commercial drugs [1-4]. Coumarin derivatives were shown to be capable of reducing tissue swelling due to several stimuli. They reduce edema in rodents caused by thermal damage [5] and they are effective against other lymphoedemas [6], macrophage activation and proteolysis due to released lysosomal enzymes [7,8]. They possess anti-inflammatory and smooth muscle relaxing properties and can be used for edema therapy [5,9]. Many of them have special ability to scavenge reactive oxygen species (ROS)-free radicals, such as hydroxyl radicals, superoxide radicals or hypochlorous acid and to influence processes involving free radical-injury [10,11].



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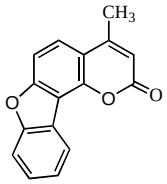

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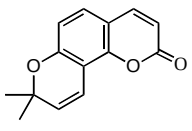

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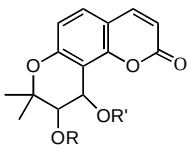


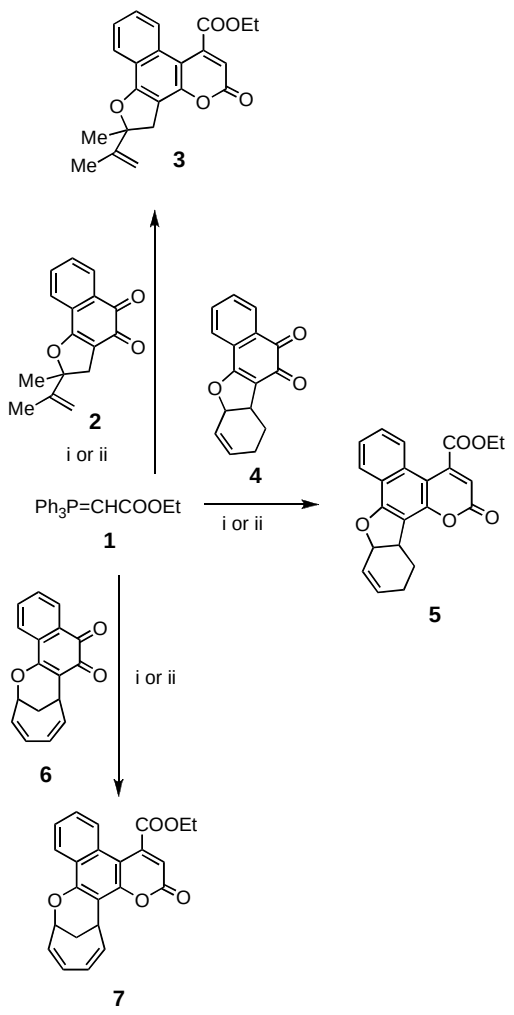
IV

The structural diversity found in this family of compounds led to the division into different categories, from simple coumarins to many other kinds of polycyclic coumarins, such as furocoumarins and pyranocoumarins [3]. The biological importance of furocoumarins [4] mainly focuses on their applications in photochemotherapy, but they also possess important anti-HIV activity. Especially 4-methyl-8,9,10,11-tetrahydro-2*H*-[1]benzofuro-[2,3-*h*]chromen-2-one **I** and 4-methyl-2*H*-[1]benzofuro-[2,3-*h*]chromen-2-one **II** show a considerable interest reacting possibly with DNA [12]. Pyranocoumarins, like 8,8-dimethyl-2*H*,8*H*-pyrano[2,3-*h*]chromen-2-one (seselin) [13] **III** and esters of 9,10-dihydroxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-pyrano[2,3-*h*]chromen-2-one (khellactones) **IV** also possess important anti-HIV activity [14]. Very recently [15] we prepared benzo[*f*]-fused derivatives of **IV,** which were found to possess significant anti-inflammatory and antioxidant activities, as well as inhibitory activity on soybean lipoxygenase.

In continuation to these efforts and in an attempt for new lead with anti-inflammatory/antioxidant activity we report here the preparation of benzo[*f*]-fused derivatives of **I** and **III** and the biological evaluation of the newly synthesized compounds, as well as of their precursors.

RESULTS AND DISCUSSION

Chemistry. The reactions studied and the products obtained are depicted in Scheme 1. For the reactions two methods were used. According to the first method (A),



Scheme 1. Reagents and conditions: i) dry DCM/ reflux [method (A)], ii) dry toluene, MW [method (B)]

used earlier by our group for analogous reactions [16-18], a solution of the easy prepared [19] *o*-quinone **2**, **4, 6** and excess of the ylide **1** (2.2 equivalents) was refluxed in dry dichloromethane (DCM) for 3-6 hours. In the second method (B) a mixture of *o*-quinone and the ylide in dry toluene was heated in a commercial microwave oven (800 W) for 3 min. The progress of the reactions was checked by tlc every minute. The reaction mixtures in both methods were separated by column chromatography.

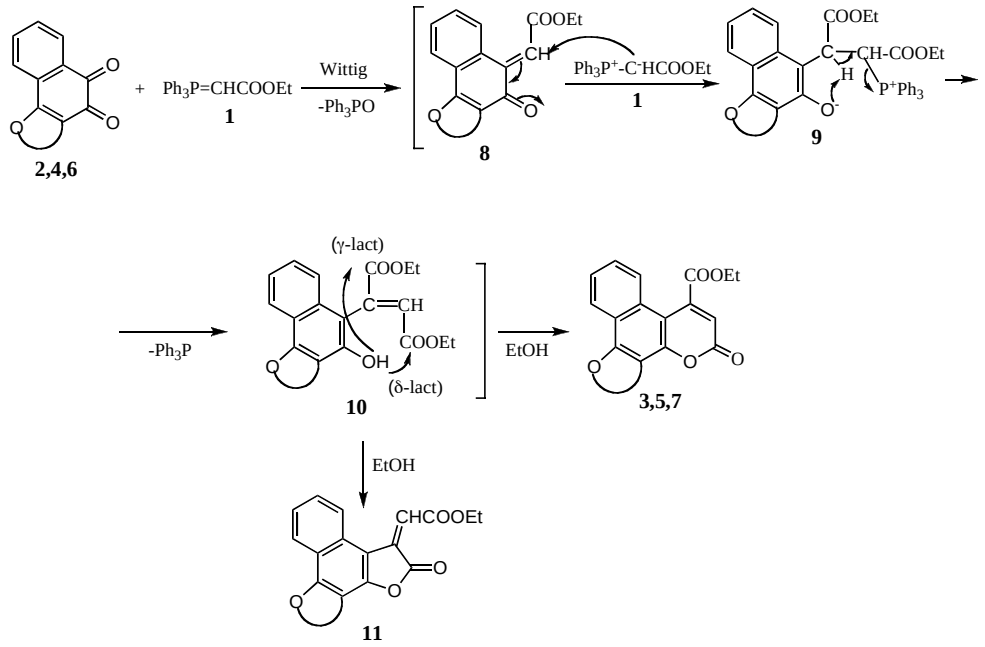
From the reaction of **1** with *o*-quinone **2** only ethyl 2-isopropenyl-2-methyl-5-oxo-2,3-dihydro-5*H*-benzo[*f*]-furo[2,3-*h*]chromene-7-carboxylate **3** was isolated in 66% and in 57% yield by methods (A) and (B) respectively. Similarly from the reaction of **1** with *o*-quinone **4** ethyl 2-oxo-9a,12,13,13a-tetrahydro-2*H*-benzo[*f*][1]benzofuro[2,3-*h*]chromene-4-carboxylate **5** was only obtained in 51% and 45% yield, while from the reaction of *o*-quinone **6** again only ethyl 5-oxo-4,16-dioxapentacyclo[15.4.1.$0^{12,15}$.$0^{3,8}$.$0^{9,14}$]docosa-2,6,8,9,11,13,14,18,20-nonaene-7-carboxylate **7** was isolated in 65% yield by both methods. In all these cases a little better yields were obtained by the method (A), but by method (B) the reactions were completed in just a few minutes.

The formation of coumarins **3, 5, 7** can be explained by the known [16,17] mechanism depicted in Scheme 2. Wittig monoolefination of the *o*-quinone used with ylide **1** gives the *o*-quinonemethide intermediate **8**, which by further Michael addition of a second ylide species **1** affords the betaine **9**. Intramolecular Hoffman elimination of triphenylphosphine from **9** leads to the *o*-hydroxyarylbutenodioate **10**, which through δ-lacto-nization gives the obtained coumarins **2, 3, 7**. It is interesting to mention that no γ-lactonization products **11** were isolated as profoundly [20] the favored confor-mation and configuration of the *o*-hydroxyaryl-butenedioic ester **10** favor the δ-lactonization.

The products **3**, **5**, **7** gave satisfactory analytical and spectral data in full accord with their assigned structures. The assignment of ^{1}H chemical shifts is described in the Experimental part and it is in accordance to the known chemical shifts of the starting quinones [19]. NOE experiments show in all the three cases interaction between methylene protons of the carbethoxy group with the peri-hydrogen of their benzo[*f*]fused ring (4%, 4% and 2.5% for **3**, **5** and **7** respectively).

Biology. The reducing abilities of the examined compounds were determined by their interaction with the free stable radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) [21]. Antioxidants can react with DPPH and produce 1,1-diphenyl-2-picryl-hydrazine. DPPH gives a strong absorption band at 517 nm due to its odd electron. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The change of absorbance produced in this reaction is assessed to evaluate the antioxidant potential of test samples and this assay is useful as a primary screening system. All compounds were compared to nor-dihydroguaeretic acid (NDGA) and butylated hydroxytoluene (BHT), used as standard drugs. Simple coumarin also was tested in the most of the assays.

Compounds **3**, **5**, **7** and their precursors compounds were tested in two different concentrations for their interaction with the stable free radical DPPH (Table I). This interaction indicates their radical scavenging ability in an iron-free system. Compound **4** showed the highest interaction (44.5-60.4 %). The interaction was found to be time and concentration dependent. It expresses the



Scheme 2

Table 1
Interaction % of the examined compounds and reference drugs with the stable free radical DPPH; Effects of the examined compounds on the mediated oxidation of dimethyl sulphoxide (DMSO) HO. *% in vitro*; % Inhibition of soybean Lipoxygenase *in vitro* (LOX%).

Compound	% Int. DPPH 0.1mM		% Int. DPPH 0.2mM		HO· % 0.1mM	LOX IC_{50} or % inh.
	20 min	60 min	20 min	60 min		
2	22	26	25.3	26.2	80	No(0.1mM) / 70 %(1mM)
3	17.2	20.9	22	23	100	71.1%(0.1mM) / 84.5%(1mM)
4	44.5	46.3	60.4	60.6	8	0.1mM
5	20	25.6	24.6	25.6	74.5	0.45mM
6	21.5	23	21.5	23	No	0.38mM
7	21	23	20	24.5	100	63 %(0.1mM) / 85.4 %(1mM)
NDGA	81	82.6	80	80	nt	nt
BHT	31.3	60	52.7	78	nt	nt
Trolox	nt	nt	nt	nt	88.2	nt
Coumarin	4.9	6.7	5.8	21	78	15.1 % (0.1mM)
Querquetin	nt	nt	nt	nt	nt	0.18mM

No = no results under the experimental conditions; nt not tested

reducing activity of the tested compounds and indicates their ability to scavenge free radicals. No changes were observed in the interaction values for compounds **3** and **7** in comparison to their precursors **2** and **6** (Table 1). On the contrary compound **5** has presented lower % interaction in comparison to its precursor compound **4**.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflammed site and this is connected to other oxidizing species such as HO·. Hydroxyl radicals are produced by reactions that depend on transition metals, particularly iron. Thus, the superoxide anion radical and the HO· scavenging abilities of these coumarins were tested. The superoxide anion radical was measured by the reduction of nitroblue tetrazolium (NBT) to formazan. The assay was also adapted to assess the ability of antioxidants to react with $O_2^{\cdot-}$. Compound **2** presented the higher ability (IC_{50} 0.1mM) followed by compound **3**. No result was found for compound **5**, whereas the ability is high for compound **7** (Table 2). It seems that the tested compounds inhibited superoxide radical formation.

The competition of compounds with dimethyl sulfoxide (DMSO) for HO· generated by the Fe^{3+}/ ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. In these experiments (Table

1) the tested compounds markedly inhibited (74.5-100 %) the oxidation of DMSO (33mM) except of compound **6** (no) and of compound **4** (8%).

All compounds were evaluated for inhibition of soybean lipoxygenase (LOX) by the UV absorbance based on enzyme assay [22]. While one may not extrapolate the quantitative results of this assay to the inhibition of mammalian 5-LOX, it has been shown that inhibition of plant LOX activity by non steroidal anti-inflammatory drugs (NSAIDs) is qualitatively similar to their inhibition of the rat mast cell LOX and may be used as a simple qualitative screen for such activity.

Inhibitory activities were measured against soybean lipoxygenase, *in vitro*. All three compounds and their precursors present inhibitory activities on soybean lipoxygenase (LOX) under our experimental conditions. For compound **3** the inhibitory activity is higher compared to its precursor **2**. Compound **4** (precursor of compound **5**) presents the lower IC_{50} value and it is the most potent compound, whereas compound **5** is less potent. The same was observed for compound **7** and its precursor **6**.

Mixing heme proteins with hydrogen peroxide generates powerfully oxidizing activated heme species and radicals on amino acid side chains that can cause lipid peroxidation. As a model of such reactions we used the peroxidation of arachidonic acid by a mixture of heme and hydrogen peroxide [23].

No results were taken from the lipid peroxidation assay, due to dissolution problems under our experimental conditions.

We emphasize that our results are obtained from *in vitro* only and from a small number of derivatives. The value of these tests is that they enable one to investigate the possibility of direct pro-oxidant or antioxidant effects of compounds *in vivo*. The fact that such effects could be feasible *in vitro* does not mean that they actually happens *in vivo* and further studies are underway to examine this question. Another important feature is whether the concentrations at which compounds exert their antioxidant effects are relevant to the concentrations present *in vivo*. The ability of lipophilic compounds to concentrate within lipophilic regions, such as the interior of membranes, must not be ignored.

The tested compounds present potent antioxidant activities in correlation to high inhibitory activity on LOX. Since in most of the cases the biological results lead to the observation that precursors are more potent than their final products, more attempts are needed in the field of design in order to synthesize better molecules in terms of biological response.

Table 2

In vitro IC_{50} values of superoxide radical scavenging activity

Compound	Superoxide scavenging activity IC_{50}
2	0.1mM
3	1mM
4	0.55mM
5	No
6	0.69mM
7	0.1mM
Caffeic acid	0.39mM
Coumarin	88.9 % (1mM)

No = no results under the experimental conditions

EXPERIMENTAL

Chemistry. Mps were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra were obtained with a Perkin-Elmer 1310 spectrophotometer as Nujol mulls. NMR spectra were recorded on a Bruker AM 300 (300 MHz and 75 MHz for ^1H and ^{13}C respectively) using $CDCl_3$ as solvent and TMS as an internal standard. *J* values are reported in Hz. Mass spectra were determined on a VG-250 spectrometer at 70 eV under Electron Impact (EI) conditions. Microanalyses were performed on a Perkin-Elmer 2400-II Element analyzer. Silica gel N° 60, Merck A.G. has been used for column chromatography. A recently reported procedure [19] was used for the preparation of compounds **2, 4, 6**.

Wittig Reactions of o-Quinones 2, 4, 6 with phosphorus ylide 1.

General Method A. A solution of *o*-quinone **2, 4, 6** (1 mmol) and ylide **1** (2.2 mmol) in DCM (50 mL) was heated under reflux for 3-6 h. The solvent was evaporated in a rotary evaporator and the residue was subjected to a column chromatography (silica gel, hexane/ethyl acetate 4:1) to give products **3, 5, 7**.

General Method B. A mixture of *o*-quinone **2, 4, 6** (1 mmol) and ylide **1** (2.2 mmol) in dry toluene (2 mL) was placed in a conical flask in the microwave oven and it was irradiated at 800 W for 3 min. DCM (3 mL) was added to the crude reaction mixture, the solvents were evaporated in a rotary evaporator and the residue was subjected to a column chromatography as above in method A.

Ethyl 2-Isopropenyl-2-methyl-5-oxo-2,3-dihydro-5*H*-benzo-[*f*]furo[2,3-*h*]chromene-7-carboxylate (3). This compound was obtained from the reaction of **2** with **1** in 66% yield by method A and in 57% yield by method B as yellow crystals, mp 85-86°C (DCM-hexane); IR (cm^{-1}) 3050, 1720, 1710, 1578; ^1H NMR: δ 1.41 (t, 3H, *J*=6.9, $COOCH_2CH_3$), 1.72 (s, 3H), 1.88 (s, 3H), 3.35 (d, 1H, *J*=15.7, C-CH*H*), 3.56 (d, 1H, *J*=15.7, C-C*H*H), 4.53 (q, 2H, *J*=6.9, $COOCH_2CH_3$), 4.93 (s, 1H, C=CH*H*), 5.18 (s, 1H, C=C*H*H), 6.32 (s, 1H, 6-H), 7.49-7.59 (m, 2H), 7.81 (d, 1H, *J*=7.9, 11-H), 8.12 (d, 1H, *J*=7.9, 8-H); ^{13}C NMR: δ 13.9, 18.5, 26.3, 39.1, 62.8, 94.0, 104.1, 109.4, 110.1, 110.7, 119.0, 123.0, 123.8, 125.4, 128.1, 129.3, 146.5, 146.8, 153.7, 160.0, 160.3, 167.8; EIMS: m/z 365 (M^++H, 56%), 364 (M^+, 84), 350 (71), 349 (100), 335 (10), 321 (58), 293 (17), 275 (44). *Anal.* Calcd. for $C_{22}H_{20}O_5$: C, 72.50; H, 5.54. Found: C, 72.13; H, 5.46.

Ethyl 2-Oxo-9a,12,13,13a-tetrahydro-2*H*-benzo[*f*][1]benzofuro[2,3-*h*]chromene-4-carboxylate (5). This compound was obtained from the reaction between **4** and **1** in 51% yield by method A and in 45% yield by method B as yellow crystals, mp 148-150°C (DCM-hexane); IR (cm^{-1}) 3060, 1715, 1705; ^{1}H NMR: δ 1.41 (t, 3H, *J*=6.9, $COOCH_2CH_3$), 2.01-2.28 (m, 3H, 12-H, 13-2H), 2.29-2.41 (m, 1H, 12-H), 3.84 (ddd, 1H, J_1=4.9, J_2=7.9, J_3=11.8, 13a-H), 4.54 (q, 2H, *J*=6.9, $COOCH_2CH_3$), 5.29 (d, 1H, *J*=7.9, 9a-H), 6.18 (d, 1H, *J*=9.8, 10-H), 6.31 (ddd, 1H, J_1=2.9, J_2=5.9, J_3=9.8, 11-H), 6.34 (s, 1H, 3-H), 7.43-7.60 (m, 2H), 7.81 (d, 1H, *J*=7.9, 8-H), 8.09 (d, 1H, *J*=6.9, 5-H); ^{13}C NMR: δ 13.9, 23.1, 24.3, 39.1, 62.8, 81.4, 110.3, 114.7, 119.2, 123.1, 123.7, 125.3, 127.2, 128.2, 129.2, 134.9, 146.9, 147.5, 156.5, 160.0, 160.9, 167.8; EIMS: m/z 363 (M^++H, 76%), 362 (M^+, 100), 334 (25), 315 (37), 305 (68), 297 (25), 288 (75), 268 (40), 260 (89), 236 (63), 215 (50), 202 (69). *Anal.* Calcd. for $C_{22}H_{18}O_5$: C, 72.92; H, 5.01. Found: C, 72.72; H, 5.15.

Ethyl 5-Oxo-4,16-dioxapentacyclo[15.4.1.02,15.03,8.09,14]-docosa-2,6,8,9,11,13,14,18,20-nonaene-7-carboxylate (7). This compound was obtained from the reaction between **6** and **1** in 65% yield by both methods A, B as yellow crystals mp 144-146°C (DCM-hexane); IR (cm^{-1}) 3040, 1725, 1715, 1570; ^{1}H NMR: δ 1.39 (t, 3H, *J*=6.9, $COOCH_2CH_3$), 2.26 (d, 1H, *J*=13.8, CC*H*HC), 2.59-2.72 (m, 1H, CCH*HC*), 4.16 (t, 1H, *J*=8.9, 1-H), 4.51 (q, 2H, *J*=6.9, $COOCH_2CH_3$), 5.37 (t, 1H, *J*= 2.0, 10-H), 5.85-5.96 (m, 1H, 20-H), 6.03 (d, 2H, *J*=3.9,19-H), 6.04 (d, J=2.0, 18-H), 6.37 (s, 1H, 6-H), 6.81 (t, 1H, *J*=8.9, 21-H), 7.47-7.58 (m, 2H), 7.74 (d, 1H, *J*=9.8, 13-H), 8.32 (d, 1H, *J*=9.8, 10-H); ^{13}C NMR: δ 13.9, 27.7, 29.3, 62.8, 72.1, 104.0, 109.7, 111.1, 122.2, 123.2, 123.7, 124.9, 125.4, 127.1, 127.9, 129.6, 130.1, 137.4, 146.6, 153.6, 154.9, 160.1, 167.8; EIMS: m/z 375 (M^++H, 25%), 374 (M^+, 100), 359 (6), 346 (20), 318 (7), 302 (11), 273 (12), 215 (48), 202 (37). *Anal.* Calcd. for . $C_{23}H_{18}O_5$: C, 73.77; H, 4.85. Found: C, 73.89; H, 4.78.

Biology

Interaction of the tested compounds with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) [24]. The examined compounds (0.1 mM and 0.2 mM in DMSO) were added to an equal volume of a solution of DPPH (0.1 mM) in absolute ethanol. Ethanol was used as a control solution. After 20 min and 60 min at room temperature the absorbance was recorded at λ 517 nm. Nordihydroguaretic acid and BHT were used as the appropriate standards. The results are summarized in Table 1.

Effect of the tested compounds on the $^{\cdot}$OH radical-mediated oxidation of DMSO [21]. The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system, were detected by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture was prepared from ethylene diamine tetraacetic acid (EDTA) (0.1 mM), Fe^{3+} (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (0.1 mM) and ascorbic acid (10 mM). After 30 min of incubation (37° C) the reaction was stopped with CCl_3COOH (17 % w/v) and the absorbance was recorded at λ 425 nm. The results are shown in Table1.

Non enzymatic assay of superoxide Radicals. Measurement of superoxide radical scavenging activity [25]. The superoxide producing system was set up by mixing phenazine methosulfate (PMS), nicotinamide adenine nucleotide (NADH) and air –oxygen. The production of superoxide was estimated by the nitroblue tetrazolium method. The reaction mixture containing 3 μM PMS, 78 μM NADH, and 25 μM NBT in 19 μM phosphate buffer pH 7.4 was incubated for 2 min at room temperature and the absorption measured at 600 nm against a blank containing PMS. The tested compounds were preincubated for 2 min before adding NADH.(Table 2)

Soybean lipoxygenase inhibition [21]. The solution of the tested compounds in DMSO was incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of enzyme solution ($1/3x10^4$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at λ 234 nm was recorded and compared with querquetin as an appropriate standard inhibitor. The results are summarized in Table 1.

Heme protein-dependent lipid degradation [25]. 50 μM Heme, arachidonic acid (0.4mM), the compounds at the various concentrations tested, H_2O_2 (0.5mM) were incubated together for 10 min at 37°C in KH_2PO_4-KOH buffer (50mM, pH 7.4). The product of peroxidation was detected using the thiobarbituric acid (TBA) test [26]. The compounds were added in DMSO solution, which has no effect on the assay.

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