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Research paper

Structure–activity relationship of dihydroxy-4-methylcoumarins as powerful antioxidants: Correlation between experimental & theoretical data and synergistic effect

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ABSTRACT

The chain-breaking antioxidant activities of eight coumarins [7-hydroxy-4-methylcoumarin (**1**), 5,7-dihydroxy-4-methylcoumarin (**2**), 6,7-dihydroxy-4-methylcoumarin (**3**), 6,7-dihydroxycoumarin (**4**), 7,8-dihydroxy-4-methylcoumarin (**5**), ethyl 2-(7,8-dihydroxy-4-methylcoumar-3-yl)-acetate (**6**), 7,8-diacetoxy-4-methylcoumarin (**7**) and ethyl 2-(7,8-diacetoxy-4-methylcoumar-3-yl)-acetate (**8**)] during bulk lipid autoxidation at 37 °C and 80 °C in concentrations of 0.01–1.0 mM and their radical scavenging activities at 25 °C using TLC–DPPH test have been studied and compared. It has been found that the *o*-dihydroxycoumarins **3**–**6** demonstrated excellent activity as antioxidants and radical scavengers, much better than the *m*-dihydroxy analogue **2** and the monohydroxycoumarin **1**. The substitution at the C-3 position did not have any effect either on the chain-breaking antioxidant activity or on the radical scavenging activity of the 7,8-dihydroxy- and 7,8-diacetoxy-4-methylcoumarins **6** and **8**. The comparison with DL-α-tocopherol (TOH), caffeic acid (CA) and *p*-coumaric acid (*p*-CumA) showed that antioxidant efficiency decreases in the following sequence:

TOH > CA > **3** > **4** > **6** > **5** > **2** > **1** = **7** = **8** = *p*-CumA.

Theoretical calculations and the "Lipinski's Rule of Five" were used for explaining the structure–activity relationships and pharmacokinetic behavior. A higher TGSO oxidation stability was observed in the presence of equimolar (1:1) binary mixtures of coumarins with TOH (**1** + TOH, **3** + TOH and **5** + TOH). However, the synergism (14%) was observed only for the binary mixture of **5** + TOH.

1. Introduction

Oxygen is essential for all living organisms, but at the same time it is a source of constant aggression for them. In its ground triplet state, oxygen has weak reactivity, but it can produce strongly aggressive and reactive particles which lead to an oxidative degradation of biological macromolecules, changing their properties and thus the cell structure and functionality. Free radicals are responsible for the pathogenesis of a wide range of diseases – on the one hand, the most serious and difficult to treat health problems such as cancer and cardiovascular diseases, on the other hand they also cause asthma, arthritis, inflammations, neurodegenerative disorders, Parkinson's disease and dementia [1].

Coumarins are an important class of oxygen heterocycles, widespread in the plant kingdom [2]. They have attracted intense interest recently due to their presence in natural sources, and to their possession of diverse pharmacological properties [3]. 4-Methylcoumarins have been found to possess choleretic, analgesic, anti-spermatogenic, anti-tubercular and diuretic properties [4,5]. Polyhydroxy (phenolic) coumarins are known to act as antioxidants in biological systems, but it is difficult to distinguish their

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antioxidant activity from the many other effects they produce in cells. Recently, it has been published [5,6] that as expected, the *o*-dihydroxy substituted coumarins are excellent radical scavengers. Surprisingly, the corresponding *o*-diacetoxy derivatives also turned out to be good radical scavengers [6], further it has been reported [7] that dihydroxy and diacetoxy derivatives of thionocoumarin are more potent in comparison to the corresponding coumarins.

The aim of this study has been to test a set of *o*- and *m*-dihydroxy-4-methylcoumarins and of their derivatives having substituents at the C-3 position as chain-breaking antioxidants using the kinetic model of bulk phase lipid autoxidation. Data is available only for some compounds concerning their radical scavenging activities towards model radicals, but this activity differs significantly from the chain-breaking antioxidant activity, which is the capacity of the phenolic compounds to shorten the oxidation chain cascade. Furthermore, the importance of phenolic compounds and of coumarins, in particular as potential bio-antioxidants, *i.e.*, compounds with antioxidant and biological activity, has been demonstrated recently [2,3,6,8]. Therefore, in searching new effective bio-antioxidants, we chose in this study various dihydroxycoumarins (having OH groups at the positions: C-7 & C-8; C-6 & C-7 and C-5 & C-7) and their derivatives having substituents at the C-3 position. Comparison with standard and known phenolic antioxidants (DL-α-tocopherol and caffeic & *p*-coumaric acids) as well as with 7-hydroxy-4-methylcoumarin has been made. Thin layer chromatography (TLC)-1,1-diphenylpicrilhydrazyl (DPPH) radical scavenging test has also been applied to all the compounds. A lot of mechanistic studies have been carried out on phenolic antioxidants, which have indicated that the chain reaction is controlled mainly through free radical scavenging by phenolic hydroxyl of the antioxidants. So, if a proper theoretical parameter to characterize the ability of antioxidants to scavenge free radicals can be found, it will be possible to predict their antioxidant activity, which will undoubtedly improve the selection of new antioxidants. Correlation between radical scavenging & antioxidant activities and theoretical calculations has been made in the present study and used for explaining their structure–activity relationship.

2. Materials and methods

2.1. Phenolic antioxidants

α-Tocopherol (TOH) and caffeic & *p*-coumaric acids were procured from E. Merck, Germany. 7-Hydroxy-4-methylcoumarin (**1**), 5,7-dihydroxy-4-methylcoumarin (**2**) and 7,8-dihydroxy-4-methylcoumarin (**5**) were synthesized and characterized by the method of Parmar, Tomar and Malhotra [9–11]. 6,7-Dihydroxy-4-methylcoumarin (**3**), 6,7-dihydroxycoumarin (**4**) and 7,8-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin (**6**) were synthesized and characterized by Parmar et al. [12] according to the procedure of Chakravarti [13]. The compounds **7** and **8** were prepared by the acetylation of compounds **5** and **6**, respectively using acetic anhydride/pyridine and catalytic amount of 4-dimethylaminopyridine by the method used by Parmar et al. [14,15] for the acetylation of similar coumarin derivatives.

2.2. Screening for free radical scavengers by TLC–DPPH rapid test

The compounds were dissolved in acetone and spotted onto silica gel 60 F_{254} plates (E. Merck, Germany). The plates were air-dried and sprayed with 0.03% DPPH radical solution in methanol for detecting the compounds with rapid scavenging properties [16]. The compounds that showed white or yellow spots on a purple background were considered as active radical scavengers. Taking into account that the stability of DPPH radical is much higher in acetone solution than in methanol [17], the same concentration of DPPH radical in acetone was prepared and used to test the activity of the studied compounds. The effect of initial concentration (1 mM and 10 mM) of all the samples studied on their radical scavenging activity was also determined.

2.3. Lipid samples

Triacylglycerols of commercially available sunflower oil (triacyl glycerols of sunflower oil, TGSO) were cleaned from pro- and antioxidants by adsorption chromatography [18] and stored under nitrogen at minus 20 °C. Fatty acid composition of the lipid substrate was determined by GC analysis of the methyl esters of the total fatty acids (six different fatty acids were detected), obtained according to the method of Christie [19] with GC-FID Hewlett–Packard 5890 equipment (Hewlett–Packard GmbH, Austria) and a capillary column HP INNOWAX (polyethylene glycol mobile phase, Agilent Technologies, USA) 30 m × 0.25 mm × 0.25 mm. The temperature gradient started from 165 °C, increased to 230 °C with increment of 4 °C/min and held at this temperature for 15 min; injection volume was 1 μl. Injector and detector temperatures were 260 °C and 280 °C, respectively. Nitrogen was the carrier gas at flow rate of 0.8 ml/min. The analyses were performed in triplicate. Lipid samples (LH) containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone were added to the lipid sample. Solvents were removed by blowing nitrogen gas through the solutions.

2.4. Lipid autoxidation

It was carried out at 80 °C (±0.2) by blowing air through the samples (2.0 ml, at a rate of 100 ml min^{-1}) and at 37 °C (±1) in a petri dish in the dark. The process was monitored by withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary products (lipid hydroperoxides, LOOH) concentration, *i.e.*, the peroxide value (PV) [20]. All kinetic data were calculated as the mean result of two independent experiments and were processed using the computer programs Origin Pro 8 and Microsoft Office Excel 2003.

2.5. Determination of the main kinetic parameters of the studied compounds [8,21–23]

2.5.1. Antioxidant efficiency

The antioxidant potency (or antioxidant efficiency) refers to the increase in the oxidation stability of the lipid sample by blocking the radical chain process and can be expressed by the following kinetic parameters.

- *Induction period* (IP), *i.e.*, the time, in which the concentration of antioxidant is fully consumed, and can be determined as a cross point for the tangents to the two parts of the kinetic curves of lipid autoxidation; IP_A – in the presence of antioxidant and IP_C for the control lipid sample without antioxidant.
- *Protection factor* (PF) means as to how many times the antioxidant increases the oxidation stability of the lipid sample and can be determined as a ratio between the induction periods in the presence (IP_A) and in the absence (IP_C) of the antioxidant, *i.e.*, $PF = IP_A/IP_C$.

2.5.2. Antioxidant reactivity

Antioxidant reactivity expresses the possibility of the antioxidant to take part in side reactions of the oxidation process, *i.e.*, to

change the initial oxidation rate and can be represented by the following kinetic parameters.

➢*Initial rate of lipid autoxidation* (R_C in the absence and R_A in the presence of antioxidant) and can be found from the tangent at the initial phase of the kinetic curves of hydroperoxides accumulation.
➢*Inhibition degree* (ID) is a measure of the antioxidant reactivity, *i.e.*, as to how many times the antioxidant shortens the oxidation chain length ($ID = R_C/R_A$).

2.5.3. Antioxidant capacity

Antioxidant capacity can be presented with the following kinetic parameters.

➢*Main rate of antioxidant consumption* (R_m), which means the main rate of inhibitor consumption during the induction period of the inhibited lipid autoxidation, *i.e.*, $R_m = [AH]/IP_A$, AH stands for a monophenolic antioxidant, *viz*. coumarin **1**.
➢*Relative main rate of antioxidant consumption* (RR_m) means as to how many times R_m differs from R_A, *i.e.*, $RR_m = R_m/R_A$.

2.6. Synergism, additivism, antagonism [8,21]

If two or more antioxidants are added to the oxidizing substrate, their combined inhibitory effect can be either additive, or antagonistic, or synergistic.

➢*Synergism* – the inhibiting effect of the binary mixture (IP_{1+2}) is higher than the sum of the induction periods of the individual phenolic antioxidants ($IP_1 + IP_2$), *i.e.*, $IP_{1+2} > IP_1 + IP_2$. The percent of synergism is expressed by the formula [24]:

$$\%\ \text{Synergism} = \{[IP_{1+2} - (IP_1 + IP_2)]/(IP_1 + IP_2)\} \times 100$$

➢*Additivism* – the inhibiting effect of the binary mixture (IP_{1+2}) is equal to the sum of the induction periods of the individual phenolic antioxidants ($IP_1 + IP_2$), *i.e.*, $IP_{1+2} = IP_1 + IP_2$
➢*Antagonism* – the inhibiting effect of the binary mixture (IP_{1+2}) is lower than the sum of the induction periods of the individual phenolic antioxidants ($IP_1 + IP_2$), *i.e.*, $IP_{1+2} < IP_1 + IP_2$

2.7. Statistical analysis of IP determination

The standard deviation (SD) for different mean values of IP (determined by carrying out ten independent experiments) was (in h): IP = 2.0, SD = 0.2; IP = 5.0, SD = 0.3; IP = 15.0, SD = 1.0; IP = 25, SD = 1.5; IP = 50.0, SD = 3.0. The SD of PV determination (in meq kg^{-1}), according to the modified iodometric method [25] for different mean values of PV, was: PV = 12.0, SD = 1.0; PV = 30.0, SD = 2.0; PV = 70.0, SD = 5.0; PV = 150.0, SD = 10; PV = 250.0, SD = 20. The R_A and R_C were quite constant (variation by less than 2%).

2.8. Quantum chemistry calculations [26–28]

Molecular mechanic method MM2 was used to optimize the molecular structures, and then Austin Model 1 (AM1), Modified Neglect of Diatomic Overlap (MNDO) and Parametric Method 3 (PM3) were applied. The main molecular descriptors appropriate to characterize the free radical scavenging activities of the compounds under study were calculated and compared.

2.9. Lipinski's Rule of Five [29–31]

Lipinski's rule says that, in general, an orally active drug has no more than one violation of the following criteria:

✓No more than 5 hydrogen bond donators (nitrogen or oxygen atoms with one or more hydrogen atoms)
✓No more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms)
✓A molecular weight under 500 Da
✓An octanol–water partition coefficient, log P of less than 5

To evaluate the drug-likeness better, the rules have spawned many extensions, for example one from a study by Ghose et al. in the year 1999 [29]:

✓Partition coefficient, log *P* in −0.4 to +5.6 range
✓Molar refractivity from 40 to 130
✓Molecular weight from 160 to 480 Da
✓Number of atoms from 20 to 70

3. Results

Fig. 1 shows the structures of the studied compounds in 2D and after optimization in 3D.

3.1. Fatty acid composition of the lipid substrate (in wt %)

The composition of six different fatty acids present in TGSO under the present study as determined by us, was: 16:0–6.7%; 18:0–3.6%; 18:1–25.1%; 18:2–63.7%; 20:0–0.2%; 22:0–0.7%, the numbers "*x*:*y*" indicate the number of carbon atoms and double bonds, respectively in the fatty acid.

3.2. Radical scavenging activity using TLC–DPPH rapid test

The yellowish- white spots on the purple background of DPPH radical solution in methanol and in acetone on the plates were observed (Fig. 2). Compounds **3**–**6** (1 mM concentration) showed yellowish-white spots immediately after spraying with DPPH radical in both of its solutions: in methanol (Fig. 2a) and in acetone (Fig. 2c), evidently, they are active as radical scavengers towards DPPH radical. Compounds **1**, **7** and **8** (1 mM concentration) did not show any yellowish-white spots immediately after spraying in any of the DPPH solutions, *i.e.*, they are not active as radical scavengers towards DPPH radical. Compound **2**, however showed a spot of light intensity only with the DPPH in methanol solution (indicating some weak activity). Surprisingly, after 10 min, the compounds **2**, **7** and **8** (1 mM concentration) showed yellowish-white spots with only the methanolic DPPH solution (Fig. 2b), but not in the acetone solution (Fig. 2d). We also checked the effect of the concentrations of dihydroxycoumarins and tested all the compounds at much higher concentration (10 mM). The results obtained demonstrate that at higher concentration, all the compounds **2**–**8**, except the compound **1** showed yellowish-white spots in both the DPPH radical solutions.

3.3. Comparable kinetic analysis

3.3.1. Different terms used in the equations below stand for

$LO_2^{\bullet}$ – Lipid peroxide radicals; P, P_1, P_2, P_3, P_A – Products; QH_2 – Biphenolic antioxidant;
QH• – Semiquinone radical; Q – Quinone; QH–QH – Dimer; R_{IN} – Rate of initiation;

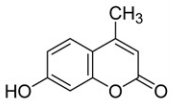
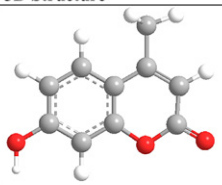
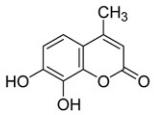
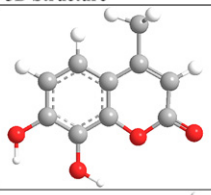
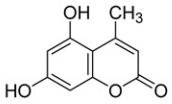
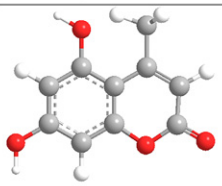
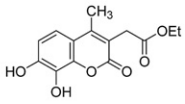
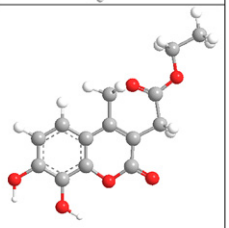
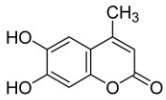
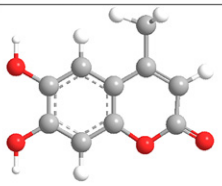
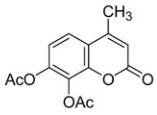
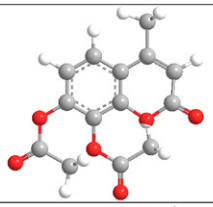
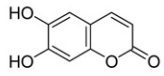
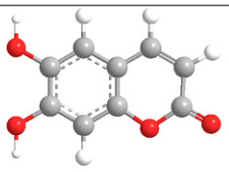
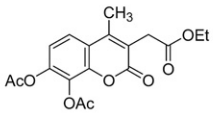
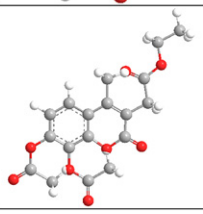
No	2D Structure	3D Structure	No	2D Structure	3D Structure
1			5		
2			6		
3			7		
4			8		

Fig. 1. 2D and 3D structures of coumarins: 7-hydroxy-4-methylcoumarin (**1**), 5,7-dihydroxy-4-methylcoumarin (**2**), 6,7-dihydroxy-4-methylcoumarin (**3**), 6,7-dihydroxy-coumarin (**4**), 7,8-dihydroxy-4-methylcoumarin (**5**), ethyl 2-(7,8-dihydroxy-4-methylcoumaryl-3-) acetate (**6**), 7,8-diacetoxy-4-methylcoumarin (**7**) and ethyl 2-(7,8-diacetoxy-4-methyl-coumaryl-3-) acetate (**8**).

$A^{\bullet}$ – Phenoxyl radical; A–A – Dimer
k_p, k_t, k_{b1-b3} – Rate constants of the non-inhibition processes of propagation, termination and branching
k_A, $k_{A'}$, k_R, k_D – Rate constants of inhibition reactions (1) and (2), recombination and disproportionation
ν_0 and ν_A – Oxidation chain lengths of non-inhibited and inhibited oxidation

3.3.2. *Basic kinetic scheme of lipid autoxidation [8]*

Non-inhibited lipid (LH) autoxidation (in absence of an antioxidant)

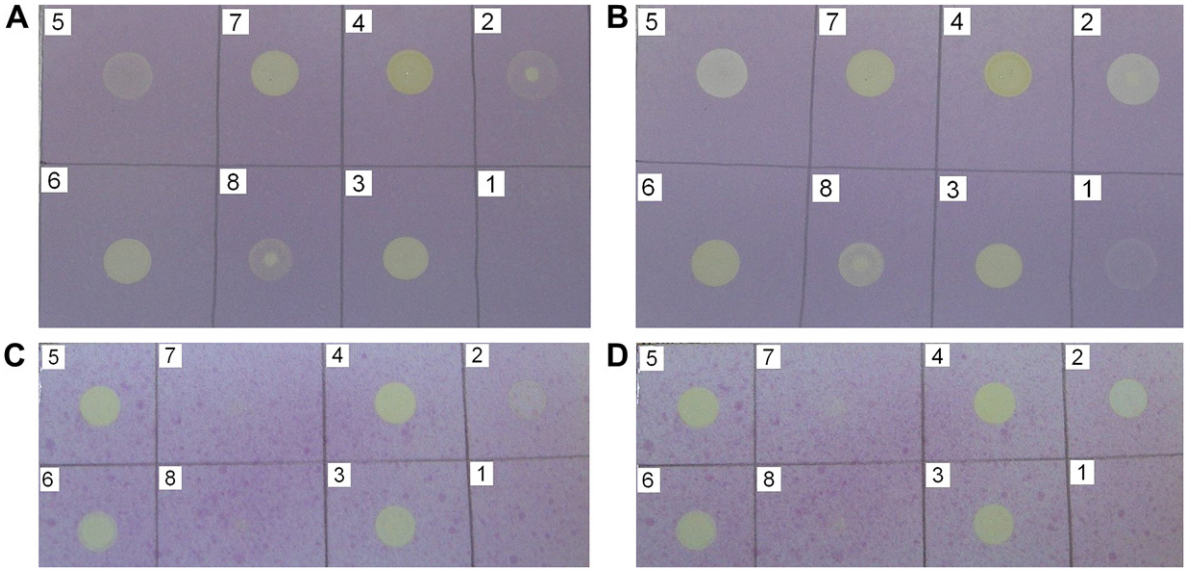


Fig. 2. TLC–DPPH rapid test: immediately after spraying the DPPH radical solution in methanol (A) & in acetone (C), and 10 min after spraying the DPPH radical solution in methanol (B) & in acetone (D).

Chain generation $LH + O_2$ (Y) $\rightarrow LO_2^{\bullet}$ (R_{IN})

Chain propagation $LO_2^{\bullet} + LH (+O_2) \rightarrow LOOH + LO_2^{\bullet}$ (k_p)

Chain termination $LO_2^{\bullet} + LO_2^{\bullet} \rightarrow P$ (k_t)

Chain branching 1 $LOOH (+O_2) \rightarrow \delta_1 LO_2^{\bullet} + P1$ (k_{b1})

Chain branching 2 $LOOH + LH (+O_2) \rightarrow \delta_2 LO_2^{\bullet} + P2$ (k_{b2})

Chain branching 3 $LOOH + LOOH (+O_2) \rightarrow \delta_3 LO_2^{\bullet} + P3$ (k_{b3})

Inhibited autoxidation (in presence of a biphenolic antioxidant QH_2)

Inhibition 1 $LO_2^{\bullet} + QH_2 \rightarrow LOOH + QH^{\bullet}$ (k_A)

Inhibition 2 $LO_2^{\bullet} + QH^{\bullet} \rightarrow P_A$ ($k_{A'}$)

Inhibition 3 (Recombination) $2QH^{\bullet} \rightarrow QH{-}QH$ (k_R)

Inhibition 4 (Disproportionation) $2QH^{\bullet} \rightarrow QH_2 + Q$ (k_D)

Rate of non-inhibited oxidation (R_0) $R_0 = k_p [LH](R_{IN}/k_t)^{0.5}$

Rate of inhibited oxidation (R_A) $R_A = k_p[LH]R_{IN}/nk_A[QH_2]_0$

Inhibited autoxidation (in presence of a monophenolic antioxidant AH)

Inhibition 1 $LO_2^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$ (k_A)

Inhibition 2 $LO_2^{\bullet} + A^{\bullet} \rightarrow P_A$ ($k_{A'}$)

Inhibition 3 (Recombination) $2A^{\bullet} \rightarrow A{-}A$ (k_R)

Rate of non-inhibited oxidation (R_0) $R_0 = k_p [LH](R_{IN}/k_t)^{0.5}$

Rate of inhibited oxidation (R_A) $R_A = k_p[LH]R_{IN}/nk_A[AH]_0$

Oxidation chain length (ν_0) and (ν_A)

Chain length of non-inhibited oxidation (ν_0) $\nu_0 = R_0/R_{IN}$

Chain length of inhibited oxidation (ν_A) $\nu_A = R_A/R_{IN}$

Inhibition degree (ID) $ID = R_0/R_A = \nu_0/\nu_A$

3.3.3. Effect of concentrations of coumarins

This effect of the concentrations of coumarins is shown in Fig. 3a (0.1 mM) and Fig. 3b (1.0 mM), and the main kinetic parameters have been calculated (Table 1) from the kinetic curves. It could be seen that the antioxidant efficiency (PF) is growing when the concentration increases, PF decreases in the following sequences:

0.1 mM: **3** (4.7) > **4** (3.7) > **6** (1.5) > **5** (1.3) > **2** (1.2) = **7** (1.0) = **1** (1.0) = **8** (1.0)

1.0 mM: **3** (16.9) > **4** (12.8) > **6** (5.4) > **5** (4.9) > **2** (3.4) = **7** (1.3) >**1** (1.0) = **8** (1.0)

The antioxidant reactivity, *i.e.*, inhibition degree (ID) decreases in the following sequences:

0.1 mM: **3** (7.0) = **4** (7.0) > **6** (5.6) > **5** (1.3) > **2** (1.1) = **1** (1.0) = **7** (1.1) = **8** (1.0)

1.0 mM: **3** (56.0) > **4** (28.0) > **5** (7.0) > **6** (6.2) > **2** (4.3) > **7** (1.6) > **1** (1.0) = **8** (1.0)

It has been found that the compounds **3**, **4** and **6** showed almost the same ID at 0.1 mM concentration, thus enabling us to separate the studied compounds into two main groups – with high ID (ID = 5.6–7.0) and with a low ID (ID = 1.0–1.3).

However, at higher concentration (1.0 mM), the differences between compounds **3**, **4** and **6** could be seen. Compound **3** showed the highest ID (56.0), which is 2-fold higher value than that of compound **4** (28.0) and 8-fold higher value than that of compound **5** (7.0). Compounds **5** and **6** showed similar ID, so did the compounds **1** and **8**. At higher concentration (1.0 mM) of tested compounds, we could separate them into three main groups: compounds with a strong ID (**3** and **4**), with a moderate ID (**2**, **5** and **6**) and with a low ID (**1**, **7** and **8**).

The main rate of antioxidant consumption during the induction period, R_m increases in the following sequences:

R_m, 10^{-9} M (0.1 mM): **3** (3.9) < **4** (4.9) < **6** (12.6) < **5** (13.8) < **2** (16.3) < **1** (18.5) = **7** (18.5) = **8** (18.5)

R_m, 10^{-9} M (1.0 mM): **3** (10.9) < **4** (14.5) < **6** (34.3) < **5** (37.5) < **2** (54.5) < **7** (138.9) < **8** (173.6) < **1** (185.0)

3.3.4. Effect of equimolar binary mixtures with DL-α-tocopherol

In order to study the possible synergism between two phenolic antioxidants, the antioxidant efficiency and reactivity of three binary mixtures of coumarins and TOH (**5** + TOH, **3** + TOH and **1** + TOH) were tested and compared, Fig. 3c and Table 1 present the results obtained. Higher oxidation stability of the lipid substrate in the presence of all the three binary mixtures was observed as compared to the corresponding values for the individual compounds.

3.3.5. Effect of temperature

Fig. 3d and Table 1 present the results obtained by studying the effect of temperature. The concentration of 1×10^{-5} M of the studied compounds was chosen, because it was used for testing their biological activity [6]. It could be seen that all the chosen compounds have the same antioxidant potential, especially during the first 3 days, the differences between antioxidant properties of these compounds are much lower than at higher temperature.

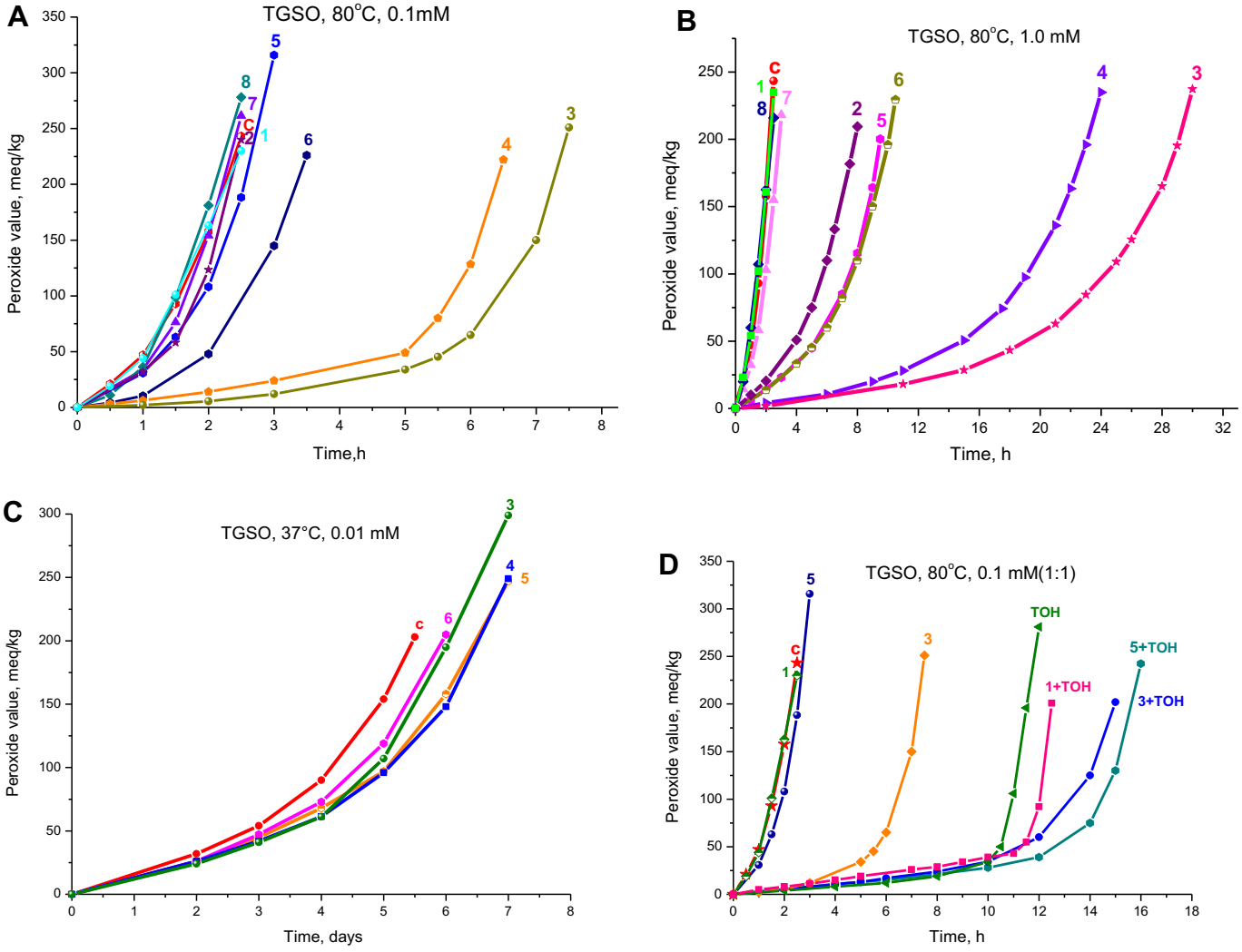


Fig. 3. Kinetic curves of hydroperoxides accumulation during TGSO autoxidation at 80 °C, in absence (control sample, c) and in presence of coumarins **1**–**8** at concentrations of A) 0.1 mM and B) 1.0 mM, C) at 37 °C, control sample (c) and in presence of selected coumarins **3**–**6** at concentration of 0.01 mM; D) at 80 °C, control sample (c) and in presence of 0.1 mM of selected coumarins (**1**, **3**, **5**) and DL-α-tocopherol (TOH) and for their equimolar binary mixtures (1:1) with DL-α-tocopherol (**1** + TOH, **3** + TOH and **5** + TOH).

3.3.6. Comparison with standard (DL-α-tocopherol) and known antioxidants (caffeic and p-coumaric acids)

Structure–activity relationship of coumarins under study was studied by comparing kinetic analysis, all main kinetic parameters, *viz.* antioxidant efficiency (PF, RAE), antioxidant reactivity (R_A, ID) and antioxidant capacity (R_m, RR_m) were compared and analyzed with the same kinetic model (TGSO, 80 °C) during bulk lipid autoxidation (see Table 1). It could be seen that the catecholic structures in the antioxidants' molecule is one of the most important factor that influences the chain-breaking antioxidant activity.

The following orders of PF and ID at the same molar concentration (0.1 mM) was obtained:

PF: TOH (7.0) > CA (6.5) > **3** (4.7) > **4** (3.7) > **6** (1.5) > **5** (1.3) > **2** (1.2) > **1** (1.0) = **7** (1.0) = **8** (1.0) = *p*-CumA (1.0)

ID: TOH (18.7) > CA (9.3) > **3** (7.0) = **4** (7.0) > **6** (5.6) > **5** (1.3) > **2** (1.1) = **7** (1.1) ≥ **1** (1.0) = **8** (1.0) = *p*-CumA (1.0)

It could be seen that the most powerful antioxidants in these experimental conditions are TOH and the *ortho*-biphenolic antioxidants like caffeic acid and the dihydroxycoumarins **3**–**6**. The 5,7-dihydroxycoumarin **2** demonstrated the same antioxidant capacity as the 7-hydroxycoumarin (**1**). These results confirm the data obtained earlier for phenolic antioxidants, that arrangement of two phenolic groups in *meta* position on the phenolic ring leads to significant decrease in its antioxidant activity. It has also been observed that *p*-coumaric acid does not show any antioxidant properties and the 7-hydroxycoumarin (**1**) is more active than *p*-CumA.

3.3.7. Quantum chemical calculations

The optimized structures of all the compounds of the present study and their main molecular descriptors are presented in Fig. 1 and Table 2. The main theoretical parameters, characterizing scavenging activity of antioxidants on free radicals, were obtained by different semiempirical methods (AM1, MNDO and PM3). Fig. 4 exhibits the equilibrium theoretical parameters of compounds **3**, **5** and **6**. Interestingly these different compounds have the same values of C-6 O–H, C-7 O–H and C-8 O–H bond lengths.

3.3.8. Correlation analysis

All compounds have been separated into three main groups: Group A: with high activity (+++), Group B: with moderate activity

Table 1
Kinetic parameters, characterizing the TGSO autoxidation at 80 °C and 37 °C for individual compounds monophenolic (AH) and -biphenolic (QH_2) coumarins and equimolar binary mixtures of selected coumarins with DL-α-tocopherol (TOH). Kinetic parameters for control sample at $t = 80$ °C: $IP_c = (1.5\pm0.5)$ h, $R_c = (5.6\pm0.5)\ 10^{-6}$ M/s and for $t = 37$ °C $IP_c' = (98\pm2)$ h, $R_c' = (0.1\pm0.2)\ 10^{-6}$ M/s.

QH_2 AH	$[QH_2]$ [AH] mM	Antioxidant efficiency		Antioxidant reactivity		Antioxidant capacity		t
		IP_A, h	PF	R_A, 10^{-6}, M/s	ID	R_m, M/s	RR_m, 10^{-3}	°C
1	0.1	1.5 ± 0.5	1.0	5.6 ± 0.5	1.0	18.5 10^{-9}	3.3	80
	1.0	1.5 ± 0.5	1.0	5.6 ± 0.5	1.0	185.0 10^{-9}	3.3	
2	0.1	1.7 ± 0.5	1.2	5.0 ± 0.5	1.1	16.3 10^{-9}	3.3	80
	1.0	5.1 ± 0.9	3.4	1.3 ± 0.3	4.3	54.5 10^{-9}	41.9	
3	0.1	7.1 ± 0.9	4.7	0.8 ± 0.2	7.0	3.9 10^{-9}	4.9	80
	1.0	25.3 ± 1.5	16.9	0.1 ± 0.1	56.0	10.9 10^{-9}	109.0	
4	0.1	5.6 ± 0.9	3.7	0.8 ± 0.2	7.0	4.9 10^{-9}	6.2	80
	1.0	19.2 ± 1.5	12.8	0.2 ± 0.2	28.0	14.5 10^{-9}	72.5	
5	0.1	2.0 ± 0.2	1.3	4.2 ± 0.5	1.3	13.8 10^{-9}	3.3	80
	1.0	7.4 ± 0.9	4.9	0.8 ± 0.2	7.0	37.5 10^{-9}	46.9	
6	0.1	2.2 ± 0.2	1.5	1.0 ± 0.3	5.6	12.6 10^{-9}	12.6	80
	1.0	8.1 ± 0.9	5.4	0.9 ± 0.3	6.2	34.3 10^{-9}	38.1	
7	0.1	1.5 ± 0.2	1.0	5.0 ± 0.5	1.1	18.5 10^{-9}	3.3	80
	1.0	2.0 ± 0.2	1.3	3.6 ± 0.5	1.6	138.8 10^{-9}	38.6	
8	0.1	1.5 ± 0.2	1.0	5.6 ± 0.5	1.0	18.5 10^{-9}	3.3	80
	1.0	1.6 ± 0.2	1.1	5.6 ± 0.5	1.0	173.6 10^{-9}	31.0	
TOH	0.1	10.5 ± 0.9	7.0	0.3 ± 0.2	18.7	2.6 10^{-9}	8.7	80
CA	0.1	9.8 ± 0.8	6.5	0.6 ± 0.2	9.3	2.6 10^{-9}	4.7	80
	1.0	43 ± 5	33.1	0.2 ± 0.2	28.0	6.4 10^{-9}	32.0	
p-CumA	0.1	1.5 ± 0.5	1.0	5.6 ± 0.5	1.0	18.5 10^{-9}	3.3	80
	1.0	1.9 ± 0.2	1.3	2.4 ± 0.3	2.3	76.9 10^{-9}	32.0	
1 + TOH	0.1	11.8 ± 0.9	7.9	0.5 ± 0.2	11.2	2.4 10^{-9}	4.8	80
3 + TOH	0.1	12.7 ± 0.9	8.5	0.4 ± 0.2	14.0	3.0 10^{-9}	5.5	80
5 + TOH	0.1	14.2 ± 0.9	9.5	0.4 ± 0.2	14.0	2.0 10^{-9}	5.0	80
3	0.01	113 ± 2	1.2	0.07 ± 0.01	14	2.5 10^{-5}	3.6	37
4	0.01	125 ± 2	1.3	0.07 ± 0.01	1.4	2.2 10^{-5}	3.2	37
5	0.01	125 ± 2	1.3	0.06 ± 0.01	1.7	2.2 10^{-5}	3.7	37
6	0.01	101 ± 2	1.1	0.08 ± 0.01	1.3	2.8 10^{-5}	3.5	37

Table 2
Main theoretical parameters (molecular descriptors and Lipinski Rule of Five) of all studied coumarins. HF – Heat of formation; TE – Total Energy; HOMO – Highest Occupied Molecular Orbital; LUMO – Lowest Unoccupied Molecular Orbital.

No	Molecular descriptors/methods			Lipinski Rule of Five					
	AM1	MNDO	PM3	miLog*P*	natoms	MW	nON	nOHNH	volume
1	HF: −373.65 Kcal/mol TE: 24.3103 Kcal/mol HOMO: −9.18612 eV LUMO: −0.901814 eV	HF: −373.65 Kcal/mol TE: 24.6576 Kcal/mol HOMO: −9.11481 eV LUMO: −0.891475 eV	HF: −373.65 Kcal/mol TE: 24.6576 Kcal/mol HOMO: −9.11479 eV LUMO: −0.89148 eV	1.887	13	176.171	3	1	153.166
2	HF: −122.98 Kcal/mol TE: 34.90 Kcal/mol HOMO: −9.29098 eV LUMO: −1.03306 eV	HF: −140.71 Kcal/mol TE: 44.57 Kcal/mol HOMO: −9.0709 eV LUMO: −1.01568 eV	HF: −134.69 Kcal/mol TE: 36.08 Kcal/mol HOMO: −9.28478 eV LUMO: −0.93990 eV	1.596	14	192.17	4	2	161.183
3	HF: −117.68 Kcal/mol TE: 31.29 Kcal/mol HOMO: −9.3031 eV LUMO: −1.10957 eV	HF: −145.47 Kcal/mol TE: 35.35 Kcal/mol HOMO: −8.68687 eV LUMO: −0.995849 eV	HF: −133.88 Kcal/mol TE: 28.28 Kcal/mol HOMO: −8.90508 eV LUMO: −0.97444 eV	1.398	14	192.17	4	2	161.183
4	HF: −115.57 Kcal/mol TE: 26.29 Kcal/mol HOMO: −8.9498 eV LUMO: −1.06107 eV	HF: −136.57 Kcal/mol TE: 27.72 Kcal/mol HOMO: −8.89234 eV LUMO: −1.07101 eV	HF: −123.70 Kcal/mol TE: 26.67 Kcal/mol HOMO: −8.86762 eV LUMO: −1.03074 eV	1.021	13	178.143	4	2	144.622
5	HF:−127.44 Kcal/mol TE: 22.31 Kcal/mol HOMO: −9.10201 eV LUMO: −1.03065 eV	HF: −147.73 Kcal/mol TE:32.78Kcal/mol HOMO: −8.86871 eV LUMO: −0.970076 eV	HF: −134.36 Kcal/mol TE: 22.31 Kcal/mol HOMO: −9.10201 eV LUMO: −1.03065 eV	1.627	14	192.17	4	2	161.183
6	HF: −206.57 Kcal/mol TE: 27.99 Kcal/mol HOMO: −9.09451 eV LUMO: −0.919423 eV	HF: −222.00 Kcal/mol TE: 33.78 Kcal/mol HOMO: −8.91901 eV LUMO: −1.00833 eV	HF: −226.52 Kcal/mol TE: 44.09 Kcal/mol HOMO: −9.08004 eV LUMO: −1.07906 eV	1.972	20	278.26	6	2	239.316
7	HF: −184.03 Kcal/mol TE: 66.15 Kcal/mol HOMO: −9.59273 eV LUMO: −1.30985 eV	HF: −207.29 Kcal/mol TE: 87.12 Kcal/mol HOMO: −9.46638 eV LUMO: −1.34331 eV	HF: −205.30 Kcal/mol TE: 67.13 Kcal/mol HOMO: −9.68707 eV LUMO: −1.27986 eV	1.909	22	308.242	8	0	252.176
8	HF: −259.30 Kcal/mol TE: 53.29 Kcal/mol HOMO: −9.33657 eV LUMO: −1.07597 eV	HF: −277.40 Kcal/mol TE: 56.62 Kcal/mol HOMO: −9.20222 eV LUMO: −1.06781 eV	HF: −279.11 Kcal/mol TE: 55.48 Kcal/mol HOMO: −9.25867 eV LUMO: −1.01868 eV	2.255	28	394.332	10	0	330.308

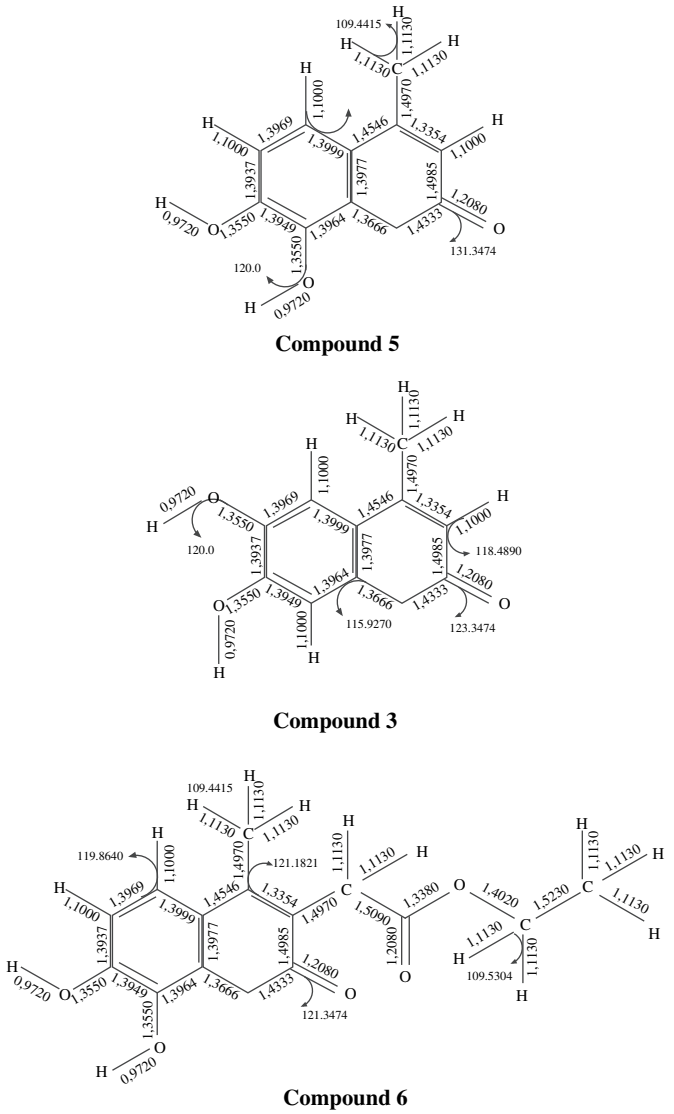


Fig. 4. Equilibrium parameters of coumarins **3**, **5**, and **6**, calculated by semi-empirical quantum chemical calculations (AM1).

(++) and Group C; with weak activity (±) and all parameter values are represented with these symbols (Table 3). It could be seen that a good correlation between experimental kinetic parameters PF and ID and theoretical data has been obtained.

3.3.9. Lipinski's Rule of Five

Table 2 presents the data obtained for all the coumarins of the present study and there is good agreement with *Lipinski's Rule of Five* for all the different coumarins.

4. Discussion

4.1. Rapid TLC–DPPH test

This test separates the studied compounds as radical scavengers into two main groups: group a) *compounds active as radical scavengers towards DPPH radical* (the yellowish-white spots appeared immediately after spraying the DPPH solution); group b) *compounds non-active as radical scavengers* (they do not change the purple background, *i.e.*, they did not react with the DPPH radical). It is important to note that the effect of concentration of studied compounds is of significance for this grouping. For this reason, we have checked the radical scavenging activity against DPPH radical using two concentrations of the studied compounds (1 mM and 10 mM) and also using the DPPH in two different solutions (in methanol and in acetone).

4.1.1. Effect of dihydroxycoumarins concentration

At lower concentrations (1 mM), the *ortho*-dihydroxycoumarins **3**–**6** showed yellowish-white spots immediately after spraying with DPPH radical solutions: in methanol (Fig. 2a) and in acetone (Fig. 2c. On the other hand, the monohydroxycoumarin **1**, the *meta* dihydroxycoumarin **2** and the 7,8-diacetoxycoumarins **7** and **8** did not show any yellowish-white spots immediately after spraying with any of the DPPH radical solutions, this result is in agreement with the expected structure based explanation of the inhibited oxidation that only compounds with free phenolic groups are able to scavenge free radicals, not their acetoxy or methoxy derivatives. However, we have found (by using UV/VIS spectrophotometry study) that the compounds **7** and **8** react with the DPPH radical in methanol solution resulting in the decrease of DPPH radical absorbance with time, $t = 10$ min and $t = 20$ min, respectively. Taking into account this fact, we checked TLC plates 10 min after spraying the DPPH radical in methanol solution (Fig. 2b). Surprisingly, after 10 min, the compounds **7** and **8** without any free OH groups and compound **2** having two hydroxy groups at *meta* positions, also showed the yellowish-white spots with DPPH radical in methanol solution. This result could be explained for compound **2** as it could have a weak activity in comparison to those of the two *ortho*-dihydroxycoumarins **3**–**6**. The explanation of this observation for compounds **7** and **8** is that after 10 min of spraying the DPPH radical in methanol solution, the yellowish-white spots appeared probably as a result of some hydrolysis in methanol of the two acetoxy groups to the corresponding two *ortho* free phenolic OH groups. It may also be noted that the dimers which could form by the reaction between compounds (AH) and DPPH radical could also be active as radical scavengers [8]. It can, thus be concluded that the lower concentration – 1 mM of tested compounds gives more precise separation of active and non-active radical scavengers.

We have checked our hypothesis about possible hydrolysis of the two acetoxy groups to the free *ortho* OH groups by reacting compounds **7** and **8** with the DPPH radical solution in acetone and applying the TLC rapid test, the results obtained with DPPH solution in acetone proved our hypothesis as **7** and **8** did not react with DPPH radical in acetone solution, because the hydrolysis of acetoxy groups in acetone is not possible (it is possible in methanol solution only), consequently free *ortho* OH groups are not formed in the acetone media.

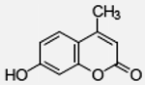
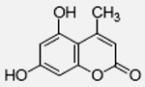
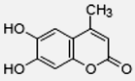
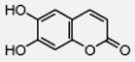
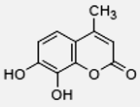
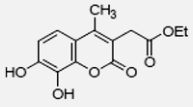
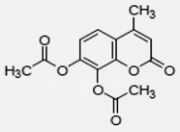
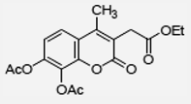
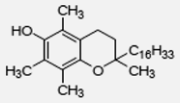
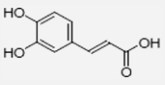
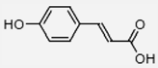
At a higher dihydroxycoumarins concentration (10 mM), the compounds **2**–**8** showed yellowish- white spots immediately after spraying the DPPH radical solutions in both the solvents or 10 min after, only the compound **1** did not show any activity as radical scavenger. These results demonstrated that DPPH solutions in methanol and acetone give some additional reactions with the coumarins **2**–**8**.

4.2. Comparable kinetic analysis and structure–activity relationship

4.2.1. Effect of substitution in ring A

Comparable kinetic analysis showed that the antioxidant efficiency (PF) and reactivity (ID) depend significantly on the relative positions of the two phenolic hydroxyls. It has been found that the antioxidant activity decreases in the following sequence (Fig. 1 and Table 1):

Table 3
Correlation analysis.

No.	Structures of tested compounds	Kinetic parameters				Correlation analysis		
		(AH) nM	PF	ID	R_m 10^{-9} M/s	PF	ID	R_m 10^{-9} M/s
1		0.1	1.0	1.0	18.5	±	±	++
2		0.1	1.2	1.1	16.3	±	±	±
3		0.1	4.7	7.0	3.7	+++	+++	+++
4		0.1	4.7	7.0	4.9	+++	+++	+++
5		0.1	1.3	1.3	13.8	±	±	++
6		0.1	1.5	5.6	12.6	±	+++	+++
7		0.1	1.0	1.1	18.5	±	±	±
8		0.1	1.0	1.0	18.5	±	±	±
TOH		0.1	7.0	18.7	2.6	+++	+++	+++
CA		0.1	8.8	11.0	3.2	++++	++++	++++
p-CumA		0.1	1.0	1.0	23.1	±	±	±

3 > **6** > **5** > **2** ≥ **1** (*i.e.*, 6,7-dihydroxy- > 7,8-dihydroxy- > 5,7—dihydroxy- ≥ 7-hydroxy-).

Replacement of phenolic groups with acetoxy groups imparts lack of activity as antioxidants to the coumarins as the PF and ID values for the coumarins **5**–**8** vary in the order: **6** > **7** and **5** > **8**.

It was recently published that 7,8-diacetoxy compounds have biological and also antioxidant activities during *in vivo* lipid peroxidation [7]. In this paper, we present results proving that the 7,8-diacetoxy-4-methylcoumarins which do not have any free phenolic groups have neither any chain-breaking antioxidant activity *in vitro* nor any radical scavenging activity towards DPPH radical (as seen by TLC—DPPH test).

4.2.2. Effect of substitution in ring B

4.2.2.1. Effect of substitution at the C-4 position. Comparable kinetic analysis demonstrated that the presence of a C-4 methyl substituent leads to a higher antioxidant efficiency (PF) and reactivity (ID):

0.1 mM: (**3**) > (**4**)

It has been seen by us that all the *o*-dihydroxycoumarins having the C-4 methyl substituent are good antioxidants.

4.2.2.2. Effect of substitution at the C-3 position. It was published recently that coumarins carrying a substituent at the C-3 position show antioxidant properties during *in vivo* lipid peroxidation [6,7]. Therefore, we tested and compared the antioxidant properties of compounds carrying the C-3 substituent with those lacking the C-3 subsituent. The results obtained are presented in Fig. 3Aand B, and Table 1. It has been found that both the 7,8-dihydroxycoumarins **5** (lacking the C-3 substituent) and **6** (carrying the ethoxycarbonylmethyl substituent at the C-3 position) are good antioxidants and demonstrated almost the same antioxidant efficiency: IP (2.0 and 2.2, respectively), PF (1.3 and 1.5, respectively); at the same time, it could be seen that they demonstrated some differences in their antioxidant reactivity, ID (1.3 and 5.6, respectively). Thus, it may be concluded that the substituent at the C-3 position does not change significantly the potential of coumarins as antioxidants.

4.2.3. Synergism, antagonism and additivism

4.2.3.1. The synergism was observed only for the binary mixture of 5 + TOH. IP_{1+2} (14.2) > IP_1 (2.0) + IP_2 (10.5), 14% synergism was seen.

4.2.3.2. The binary mixture of 3 + TOH showed antagonism for the two antioxidants. IP_{1+2} (12.7) < IP_1 (7.1) + IP_2 (10.5).

4.2.3.3. In case of 1 + TOH, additivism was observed. IP_{1+2} (11.8) = IP_1 (1.7) + IP_2 (10.5)

4.2.3.4. Explanation for additivism and synergism. The higher oxidation stability of TGSO in the presence of all the binary mixtures with tocopherol may be explained taking into account that both the antioxidants may be regenerated during the oxidation process. It is known that the catecholic system of the coumarin molecules allows the formation of the semiquinone radicals. These semiquinone radicals may regenerate the initial antioxidant molecule during reaction of the bimolecular recombination with homo- and cross-disproportionation of the semiquinone radicals [8,21,32]:

a) *Homo-disproportionation reaction*:

$2QH^{\bullet} \rightarrow QH_2 + Q$ regeneration of QH_2 (coumarin) and

$2TO^{\bullet} \rightarrow TOH + T = O$ regeneration of TOH and

b) *Cross-disproportionation reaction*:

$QH^{\bullet} + TO^{\bullet} \rightarrow QH_2 + T = O$ regeneration of QH_2 and

$TO^{\bullet} + QH^{\bullet} \rightarrow TOH + Q$ regeneration of TOH

These reactions demonstrate that during the oxidation process, the initial molecules of the dihydroxy- coumarins and TOH are regenerated by different mechanisms which make these binary mixtures of the most powerful antioxidant compositions. It could be seen that the positions of the two phenolic groups in the coumarin molecule play an important role in their mechanism of action. In the case of the monohydroxycoumarin **1**, the observed additivism of the binary mixture (**1** + TOH) may be explained with the possible reactions of homo-disproportionation of $TO^{\bullet}$ and cross-disproportionation of phenoxyl radical ($A^{\bullet}$) from **1** and the tocopheryl radical ($TO^{\bullet}$) with regeneration of TOH.

The synergism exhibited by the mixture of α-tocophenol (TOH) and 4-methyl-7,8-dihyroxycoumarin (**5**, DHMC) could be attributed to the increased aqueous solubility and consequently the enhanced bioavailability of TOH due to the presence of DHMC which has two hydroxyl groups and thus is more water soluble and the TOH and DHMC would stick together due to strong intermolecular hydrogen bonding between the two, both in the undissolved (solid state) and in solution. Also strong hydrogen bond can form between the free radical generated from the only OH group present in TOH and the free radical generated from the C-7 OH group of DHMC leaving the C-8 OH in DHMC available for hydrogen bonding with the free radical from TOH [33,34], thus keeping the DHMC–TOH moiety together in the solid, solution and free radical states. The detailed work and data in this direction would be the subject of another publication.

4.3. Quantum chemical calculations and Lipinski's Rule of Five

A simple analysis shows that the strength of the O–H bond in phenolic hydroxyl represents its ability to scavenge free radicals. The weaker the O–H bond, the more active the antioxidant. Therefore, indexes characterizing O–H bond strength may be used as prediction parameters. Theoretically, the bond length and bond order of O–H can measure its strength to a certain extent. For smaller bond orders, the bond is weaker, the hydrogen can be removed more easily, and the phenolic hydroxyl is more active. Bond length also measures bond strength, larger bond length value corresponds to weaker bond, and therefore to smaller bond order. Consequently, the values of bond order and bond length of the antioxidants are opposite. By comparing the antioxidant stability to scavenge free radicals, our results confirm the results available in the literature [21,33,34] that bond orders and bond lengths of different molecules were not able to represent their antioxidant ability (inhibition degree, ID and relative antioxidant efficiency, RAE) to scavenge free radicals. So, these two parameters are not compatible for characterizing antioxidant activity. The highest occupied molecular orbital (HOMO), a parameter representing the molecular electron-donating ability, is also a good index predicting antioxidant activity. HOMO is of great advantage in practice, because the calculation of difference in heat of formation between the antioxidant and its free radical (ΔHOF), the dissociation energy of the O–H bond (D_{O-H} or ΔH_{abs}) is a much more time consuming process as compared with the calculation of HOMO. By comparing the results calculated by different semiempirical methods (AM1, MNDO and PM3), it was found that PM3 was the best for calculating the theoretical parameters, because it demonstrated the middle values of AM1 and MNDO. The highest antioxidant activity of compounds with catecholic moiety may be explained taking into account that the catecholic phenoxyl free radicals can exhibit intramolecular hydrogen bonding, which will increase the stability of the radical [33,34]. Catecholic phenoxyl radical can make the unpaired electron well distributed on atoms and reduce their internal energy. Of course, in the literature all data published recently [35–41] confirmed that the best theoretical parameters for predicting antioxidant activity as free radical scavengers are differences in heat of formation between the antioxidant and its

free radical (ΔHOF), *i.e.*, the dissociation energy of the O–H bond (D_{O-H} or ΔH_{abs}) and O–H bond length & strength, hydrogen bonding, etc. [33,34]. Taking into account that phenoxyl free radicals of antioxidants, may in some conformations form intramolecular hydrogen bonds or not, the conformation analysis is in progress for all the eight coumarins of the present study by Density Functional Theory (DFT) and the solvent effects, and will be the subject of another publication.

It could be seen that all the compounds of the present study are in agreement with the *Lipinski's Rule of Five*, which is of importance for further development of drugs based upon these substances, and their analogs.

5. Toxicity and utility of 4-methylcoumarins

Considerable progress has been made in recent years in relating aging to oxidation in biological cells. The reactive oxygen species (ROS), causing oxidation in biological cells, are mainly involved in detoxification of invading organisms and chemicals, but stray ROS also initiate lipid peroxidation in healthy cells. Lipid peroxidation initiated by oxygen radicals eventually results in membrane degradation and cell death [42], leading to diverse pathologies such as Alzheimer's disease, atherosclerosis, diabetes, Parkinson's disease, etc. [43]. Thus, the rates of reactions of these life-limiting metabolic processes by use of chemicals has been a subject of much interest [44,45]. Even the induction of human cancer involves a multistep process, initiated with DNA damage by endogenous ROS and exogenous activated carcinogens. This is followed by oncogene activation and tumor suppressor gene mutations, which finally lead to the alteration of different signaling pathways. Cell cycle arrest, apoptosis, cell proliferation, and cell differentiation are all mediated through signal transduction processes and appear to be important executive targets for cancer chemoprevention.

Intensive efforts are being made to discover newer antioxidants with greater efficiency to intercept the processes of oxidative stress. In this connection, minor dietary constitutents, especially plant-based foods, have come under serious scrutiny [46]. Phytopolyphenols are widespread in the plant kingdom and are important not only for contributing to the flavor and color of many fruits and vegetables, but are also playing a crucial role in cancer chemoprevention.

Coumarins are widely distributed in the plant kingdom. We have, for the first time, isolated analogs of 4-methylcoumarins, *i.e.*, troupin (4-methyl-6-hydroxy-7,8-dimethoxycoumarin) [47] and trigocoumarin [3-(ethoxycarbonyl)methyl-4-methyl-5,8-dimethoxycoumarin] [48,49] & 3,4,7-trimethylcoumarin [50] from the medicinal plant *Tamarix troupii* and the edible plant *Trigonella foenumgracum,* respectively. The leaves of *T. troupii* are used for the treatment of dysentery, chronic diarrohea, and leucoderma, while the seeds of *Tamarix foenumgraecum* are used for treating dysentery, chronic cough, and diabetes [51]. Unsubstituted coumarins have been found to be toxic since they undergo C-3, C-4 epoxidation by the hepatic CYP2A6 cytochrome P-450 oxidases, followed by oxidative decarboxylation to form *o*-hydroxyphenylacetaldehyde (*o*-HPA), and *o*-hydroxyphenylacetic acid (*o*-HPAA), both of which form very stable complexes with heavy metals inside the body thus causing liver toxicity [52–58]. On the other hand, 4-methylcoumarins are found to be resistant to the C-3,C-4 epoxidation by the hepatic CYP2A6 cytochrome P-450 oxidases due to electronic and steric factors, thus oxidative decarboxylation does not take place and *o*-HPA and *o*-HPAA are not formed [52,57,59–62] and, hence 4-methylcoumarins are non-toxic and possibly safe to use for human consumption. It may be mentioned that all the eight coumarins of the present study (except **4**, which was taken for comparison purpose) are based upon the 4-methylcoumarin skeleton.

6. Conclusions

It has been found that the *o*-dihydroxycoumarins demonstrate excellent properties as chain-breaking antioxidants and free radicals scavengers. Their activity is much higher than that of the *m*-dihydroxycoumarins. The substitution at the C-3 position does not change significantly the antioxidant and antiradical capacities of the studied 7,8-dihydroxy- and 7,8-diacetoxy-4-methylcoumarins. The comparison with standard antioxidants, DL-α-tocopherol (TOH) and caffeic (CA) & *p*-coumaric (*p*-CumA) acids as well as with the 7-hydroxy-4-methylcoumarin shows that the antioxidant efficiency and reactivity decrease in the following sequence:

$$\text{TOH} > \text{CA} > \mathbf{3} > \mathbf{4} > \mathbf{6} > \mathbf{5} > \mathbf{2} > \mathbf{1} = \mathbf{7} = \mathbf{8} = p\text{-CumA}.$$

For the first time, a much higher TGSO oxidation stability has been seen by us in the presence of equimolar binary mixtures of **1** + TOH, **3** + TOH and **5** + TOH in comparison with the individual compounds and 14% synergism has been observed for the (1:1) binary mixture of **5** + TOH. Theoretical calculations were helpful in explaining their structure–activity relationship. Considering that 4-methylcoumarins are not toxic as they are not metabolized to the toxic epoxide intermediates by the human liver oxidases. These results indicate the possible applications of 4-methylcoumarins as powerful antioxidants, and can be developed into neutraceuticals for human use alone or as binary mixtures with another well-known naturally occurring antioxidant, *viz.* α-tocopherol (TOH).

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