BioMetals (2005) 18:143–154
DOI 10.1007/s10534-004-4256-3

Studies on structure activity relationship of some dihydroxy-4-methylcoumarin antioxidants based on their interaction with Fe(III) and ADP

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Received 17 May 2004; accepted 5 October 2004; Published online: March 2005

Key words: ADP, antioxidants, chemical speciation, dihydroxy-4-methylcoumarins, formation constants, iron, structure activity relationship, ternary complex

Abstract

Three dihydroxy-4-methylcoumarin (DHMC) derivatives, namely 7,8-DHMC, 6,7-DHMC and 5,7-DHMC alone and complexed with Fe (III) and ADP have been tested for their antioxidative potential. Chemical speciation studies and formation constants reveal the formation of strong DHMC–Fe–ADP (1:1:1) ternary complex. *In vitro* studies were done for their antioxidative property by scavenging the superoxide radicals ($O_2^{\cdot-}$) generated by xanthine + xanthine oxidase (XO) reaction. The IC_{50} values for 7,8-DHMC, 6,7-DHMC and 5,7-DHMC and their ternary complexes with Fe (III)–ADP worked out to be 34.0 μM, 62.0 μM, 8.80 mM and 10.5, 11.5 and 148.5 μM, respectively. The results indicate that $O_2^{\cdot-}$ scavenging potential of all the three DHMCs increased significantly after forming the ternary complex with Fe(III) and ADP. The structure activity relationship studies suggest that the introduction of hydroxyl group at 7th and 8th positions in the coumarins, irrespective of Fe(III)–ADP complexation, increases the antioxidative efficacy. No change in uric acid production in the reactions done for all studies further reveals that the coumarin derivatives and their complexes were the only causative factors for $O_2^{\cdot-}$ scavenging and not the suppression of the enzyme, xanthine oxidase.

Introduction

During the recent years, researchers began to pay attention to coumarin and its derivatives, following their use as effective remedies for various ailments; especially the reactive oxygen species (ROS)-mediated diseases (Chlodius & Pillar 1982; Marshall *et al.* 1987, 1989; Casley-Smith *et al.* 1973, 1993a, b; Levenson 2003). *In vivo* studies on anti-oxidative property of esculetin (6,7-dihydroxycoumarin) on glutathione system has revealed the stimulation in GSH/GSSG ratio and affording the protection of lipid peroxidation (Martin-Aragon *et al.* 1998). Exhibiting the interactions with ROS, a few natural/synthetic coumarin dihydroxy derivatives have been found to be effective lipid peroxidation inhibitors and among them *ortho*-dihydroxy were more effective than *meta*-substituted, where 5,7-dihydroxy-4-methylcoumarin was a non-prooxidant Fe chelator (Paya *et al.* 1992). 5,7-DHMC and 6,7-DHMC have been reported to reduce the duration of ventricular fibrillation in post ischemic reperfused isolated heart caused by mediation of ROS (Hoult & Paya 1996). 7,8-DHMC has been found to protect the peroxidation of linoleic acid in micellar membrane damaged separately, by 2,2′-azobis (2-methylpropionamidine) dihydrochloride and by benzophenone, where 6-hydroxy-4-methylcoumarin was effective against benzophenone only, but 4-methylcoumarin

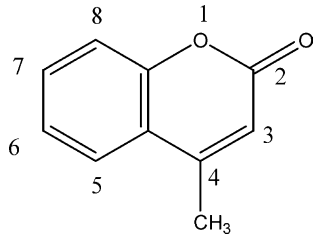

Figure 1. 4-methyl coumarin.

was found to be ineffective against both damaging factors (Yu *et al.* 1999).

The presence of phenolic hydroxyl group and/or carboxylic acid happened to be the key factor for higher activity of coumarin against *Helicobactor pylori* (Kawase *et al.* 2001). Experiments with a series of simple coumarins against eight microorganisms for structure activity relationship assessment revealed that the methoxy group at 7th position, with OH group at 6th or 8th was invariably effective in gram −ve bacteria and gram +ve *Staphylococcus aureus* (Keyser & Kolodzioj 1999). Encouraging data for curing lung cancer are also available for warfarin, which also works as vitamin K antagonist and is used as an anticoagulant (Chahinian *et al.* 1989).

Our group is working on antioxidant properties of coumarins and in this direction we have already proposed a novel mechanism for coumarins' antioxidative behavior (Raj *et al.* 1998). In the present work we report chemical speciation diagrams and formation constants of the ternary complexes of DHMCs with Fe(III)–ADP and their correlation with the antioxidative potential of these DHMCs.

Experimental

Materials

Xanthine oxidase, adenosine-5′- diphosphate disodium salt (ADP-Na_2), 5,7-DHMC and 6,7-DHMC were procured from Sigma-Aldrich Corporation, USA. Xanthine, L-methionine, ferric chloride and nitroblue tetrazolium were purchased from CDH, Mumbai, India. All other chemicals/solvents used were of highest purity available commercially. 7,8-DHMC was synthesized by well-known Pechman condensation method (Parmar *et al.* 1996).

Methods

Chemical speciation and formation constant studies. pH-metric technique is a robust and versatile way of measuring the ionization and distribution of drugs in biological fluids and assessing their interaction with metal ions. pH-metric titrations for DHMC–Fe(2:1) and DHMC–Fe–ADP(1:1:1) have been carried out using digital pH-meter (ELICO LI 120) with a combined glass electrode. Glass electrode was calibrated before the titrations (Martell & Motekaitis 1992). To ensure constant ionic strength (0.1 M) during the titrations, an electrolyte, sodium nitrate ($NaNO_3$) was added in requisite amount. A solution of tetramethylammonium hydroxide (TMAH) in *N*,*N*-dimethylformamide(DMF)/water was used as the titrant. Metal ion solution, prepared with ferric chloride, was standardized by the standard method (Schwarzenback *et al.* 1969; Vogel 1978). The titrations were performed in a covered glass jacketed titration cell under a stream of presaturated nitrogen. All measurements were made in 50% DMF/water medium at 25 ± 0.5 °C maintained constant by using Julabo VC type thermostat. Solution concentrations of coumarins in the presence and absence of metal ions were of the order 10^{-2} M. Stepwise dissociation constants within the range of the potentiometric titrations (pH 2.0–11.0) were calculated by fitting the pH-data with the help of the program PKAS (Martell & Motekaitis 1992). Formation constants and speciation of the complexes were determined by direct potentiometric titrations using the program BEST (Martell & Motekaitis 1992). Correction factor was applied for the glass electrode in aqueous DMF solution (Gonzalez *et al.* 1986).

Assay for superoxide anion ($O_2^{\cdot-}$) generation. The superoxide generation assay (Athar *et al.* 1996) was performed with a little modification in the method adopted elsewhere (Darr *et al.* 1987). The typical reaction mixture in a total volume of 1.0 ml contained, phosphate buffer (0.1 M, pH 7.4), L-methionine 10 mM, nitroblue tetrazolium (NBT) 57 mM, xanthine 1.0 mM. The reaction mixture was incubated for 5 min at room temperature (25 ± 2 °C). The reaction was initiated by the addition of 50 milliunits of xanthine

oxidase. The linear rate of reaction was recorded as an increase in optical density at 550 nm on a spectrophotometer (SL 159, ELICO Inc., India) monitoring the production of formazan, a reduction product of NBT.

Assay for Superoxide anion scavenging by dihydroxy-4-methylcoumarins and their Fe(III)-ADP ternary complexes. Various concentrations of DHMCs (1.0 μM–50.0 mM for coumarins alone and 1.0 μM–500 μM for ternary complexes) were added in the reaction mixture for the assay of $O_2^{\cdot-}$ generation described above while testing the antioxidative efficacy. 0.15 mM ferric chloride and 3.0 mM ADP were added for the formation of ternary complexes. The concentrations of constituents (Fe(III) and ADP) of adduct moiety were used as in our previous studies (Raj *et al.* 1998). The kinetics of the reaction was followed in three sets i.e. controls, by adding coumarin analogs and by adding the latter with Fe(III) and ADP. The percent inhibition in formazan formation comparing the controls was recorded due to scavenging of $O_2^{\cdot-}$ in later two sets.

In the above reaction, the product is uric acid (trihydroxy purine) formed as a catabolism of xanthine (dihydroxy purine), reacted upon by xanthine oxidase. The production of uric acid was also measured spectrophotometrically at 290 nm (Pasternack & Halliwell 1979) in controls as well as by adding the DHMCs, $FeCl_3$, $FeSO_4$ and ADP individually or in required combinations. These experiments were conducted to workout if there were some alterations in $O_2^{\cdot-}$ generation due to the enzyme inhibition by the adducts/DHMCs.

Results and discussion

The results of formation constants, chemical speciation and antioxidative potential studies for the coumarin derivatives with and without Fe(III)–ADP are presented in Tables 1–3 and Figures 2–9.

Table 1. Protonation constants of DHMCs.

Sl. no.	Compounds	pKa_1	pKa_2
1.	7,8-dihydroxy-4-methylcoumarin	10.35	8.00
2.	6,7-dihydroxy-4-methylcoumarin	10.28	8.52
3.	5,7-dihydroxy-4-methylcoumarin	10.16	8.90

Table 2. Equilibrium constants of DHMC–Fe(III) (2:1) complex and DHMC–Fe(III)–ADP (1:1:1) complex.

Sl. no.	Compounds	logK_{ML2} (1:2)	σ_{fit}	logK_{MLA} (1:1:1)	σ_{fit}
1.	7,8-dihydroxy-4-methylcoumarin	6.60	0.0303	7.30	0.0409
2.	6,7-dihydroxy-4-methylcoumarin	6.87	0.0625	7.64	0.0184
3.	5,7-dihydroxy-4-methylcoumarin	5.43	0.0859	6.63	0.0539

M, Ferric ion (Fe^{3+}); L, DHMC; A, ADP.

Protonation constants for the coumarin derivatives are listed in Table 1. From the three DHMC–Fe titration curves, it was concluded that the average ligand number does not exceed two in all the three cases, thereby ruling out the tris catecholate coordination to Fe i.e. FeL_3 complexes and indicating the formation of FeL_2 complex. In the DHMC–Fe–ADP titration, one possibility that may be considered, is the formation of a mixture of two binary complexes rather than a mixed ligand complex, containing ADP and DHMC bound to Fe i.e.

$$2M + iA + jL \rightleftharpoons MA_i + ML_j$$

If such were the case, three inflections would be obtained in the mixed ligand titration curve, the first corresponding to the formation of 1:1 primary ligand complex, the second due to the disproportionation of the initially formed complex into 1:2 metal primary ligand complex and third due to

Table 3. IC_{50} values of dihydroxy-4-methylcoumarins and their ternary complexes.

Sl. no.	Coumarin analogs	IC_{50} (μM)	
		Alone	With Fe (III)–ADP
1.	7,8-dihydroxy-4-methylcoumarin	34.0	10.5
2.	6,7-dihydroxy-4-methylcoumarin	62.0	11.5
3.	5,7-dihydroxy-4-methylcoumarin	8800.0	148.5

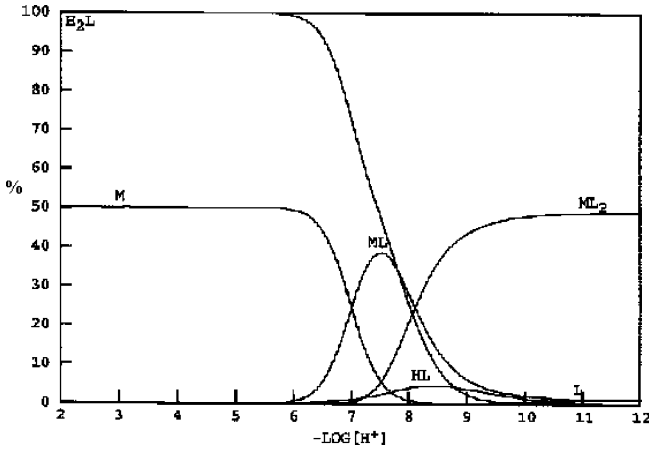

Figure 2. Species distribution curve of 7,8-DHMC–Fe (III). Total ligand concentration, T_L(7,8-DHMC) = 1.022×10^{-3} M, total metal concentration, T_M (Fe(III)) = 0.511×10^{-3} M, ionic strength, $\mu = 0.1$ M ($NaNO_3$), temperature, $T = 25$ °C (± 0.5 °C).

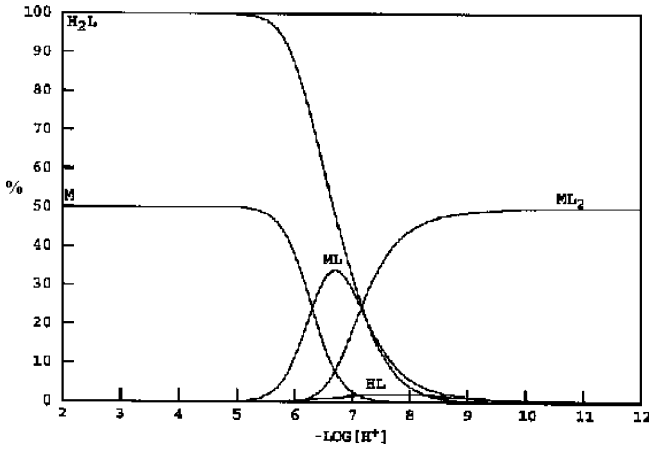

Figure 3. Species distribution curve of 6,7-DHMC–Fe(III). Total ligand concentration, T_L(6,7-DHMC) = 1.022×10^{-3} M, total metal concentration, T_M (Fe(III)) = 0.511×10^{-3} M, ionic strength, $\mu = 0.1$ M ($NaNO_3$), temperature, $T = 25$ °C (± 0.5 °C).

half of the metal forming 1:1 metal secondary ligand complex. However, in the DHMC–Fe–ADP titration curves, only two inflections were observed in all the three cases thereby ruling out this possibility. Formation constants of the DHMC–Fe (2:1) and DHMC–Fe–ADP (1:1:1) complexes were obtained with the program BEST (Martell & Motekaitis 1992).

The programs frequently used for the refinement of formation constants based on potentiometric titration include LEAST, MINIQUAD, MINIQUAD 75, SUPERQUAD, HYPERQUAD, LETAGROP and SCOGS. But, in all these programs, refinements are not based on the residuals in the observed quantities i.e volume and pH. This appears to be a motivation behind the program BEST, which minimizes the sum of squared deviations in pH. This program has a number of interesting features. The residuals are assigned weights equal to $(1/\Delta pH)^2$, which, assuming the volume increments are equal, is equivalent to weighting the points in inverse proportion to the slope of the titration curve. The minimization is based on direct incremental variation of the parameters, thus avoiding the use of expense of refinement speed. The program is highly interactive, and also permits the refinement of quantity for use in those cases where the substance cannot be purified. The basic algorithm in BEST can be stated in terms of equation

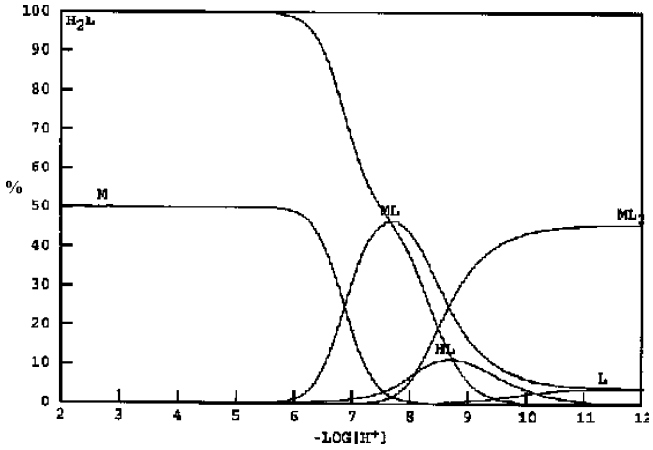

Figure 4. Species distribution curve of 5,7-DHMC–Fe(III). Total ligand concentration, T_L(5,7-DHMC) = 1.022×10^{-3} M, total metal concentration, T_M (Fe(III)) = 0.511×10^{-3} M, ionic strength, $\mu = 0.1$ M ($NaNO_3$), temperature, $T = 25$ °C (±0.5 °C).

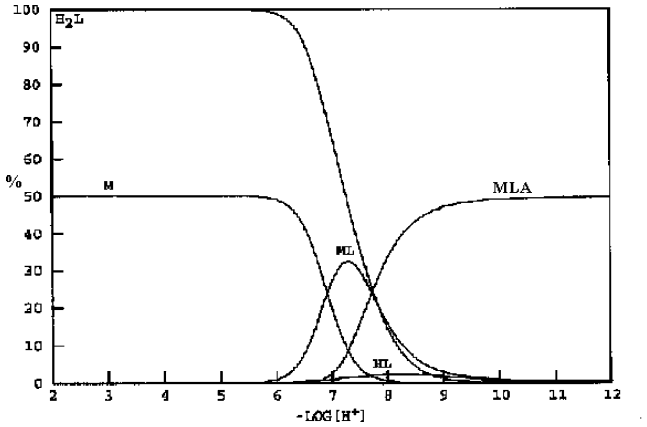

Figure 5. Species distribution curve of 7,8-DHMC–Fe(III)–ADP. Total ligand concentration, T_L (7,8-DHMC) = 0.511×10^{-3} M, total metal concentration, T_M (Fe(III)) = 0.511×10^{-3} M, total ligand concentration, T_A (ADP) = 0.511×10^{-3} M, ionic strength, $\mu = 0.1$ M ($NaNO_3$), temperature, $T = 25$ °C (±0.5 °C).

$$T_i = \sum_{j=1}^{NS} e_{ij}\beta_j \prod_{k=1}^{i} [C_k]^{e_{ij}}$$

This equation indicates the mass balance (at a given titration point) of the *i*-th component in terms of the *j*-th species summed over all species present, NS. Each species concentration consists of a product of the overall stability constant and individual component concentrations $[C_k]$ raised to the power of the stoichiometric coefficient e_{ij}. The standard deviation in pH units is expressed by the use of equation.

$$\sigma_{fit} = (U/N)^{1/2}$$

where $N = \Sigma w$, $U = \Sigma w(pH_{obsd} - pH_{calcd})^2$

So, we used this program for the determination of formation constants and for species distribution. Tables 4 and 5 give the observed and calculated pH values for DHMC–Fe (2:1) and DHMC–Fe–ADP (1:1:1) titrations. The calculated stability constants for the complexes formed, are presented in Table 2. Species distribution curves (Figures 2–7) indicate the formation of DHMC–Fe(III)–ADP ternary complex at different pH values (pH 6.0–12.0).

It is evident from the formation constants of DHMCs that the ternary complex DHMC–Fe(III)–ADP is more stable as compared to ML_2 complex. Results show that in comparison to 7,8-DHMC and 6,7-DHMC, 5,7-DHMC has lower stability constant.

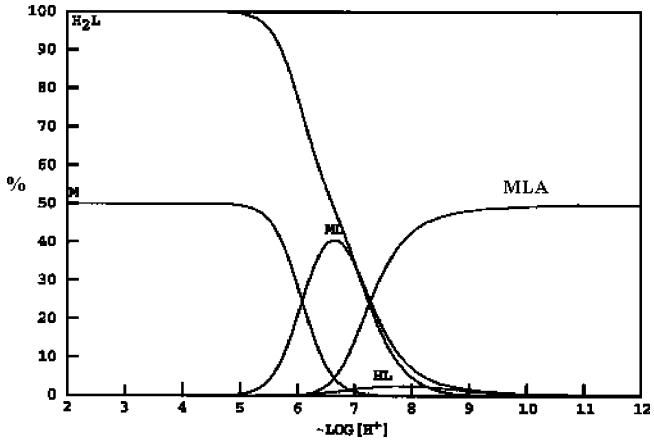

Figure 6. Species distribution curve of 6,7-DHMC–Fe (III)–ADP. Total ligand concentration, T_L (6,7-DHMC) = 0.511×10^{-3} M, total metal concentration, T_M (Fe(III)) = 0.511×10^{-3} M, total ligand concentration, T_A (ADP) = 0.511×10^{-3} M, ionic strength, $\mu = 0.1$ M ($NaNO_3$), temperature, $T = 25$ °C (±0.5 °C).

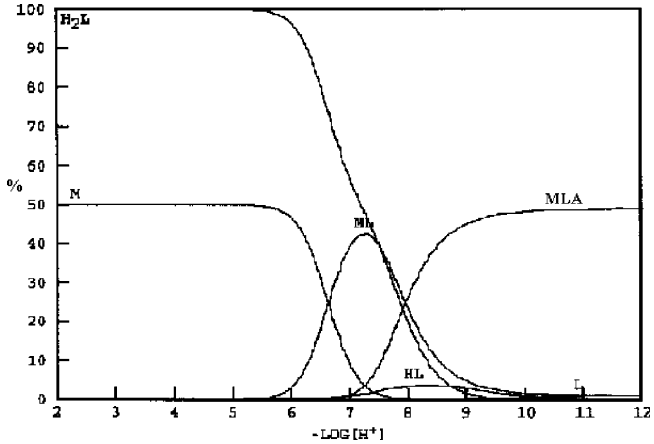

Figure 7. Species distribution curve of 5,7-DHMC–Fe (III)–ADP. Total ligand concentration, T_L (5,7-DHMC) = 0.511×10^{-3} M, total metal concentration, T_M (Fe(III)) = 0.511×10^{-3} M, total ligand concentration, T_A (ADP) = 0.511×10^{-3} M, ionic strength, $\mu = 0.1$ M ($NaNO_3$), temperature, $T = 25$ °C (±0.5 °C).

The antioxidative efficacy of the coumarin derivatives alone and complexed with Fe(III)–ADP are shown in Figures 8 and 9, respectively and in Table 3. In order to know whether there was any alteration in $O_2^{\cdot-}$ generation in the reaction due to enzyme (XO) inhibition following the addition of different components viz. DHMCs, Fe(II) ($FeSO_4$) and Fe(III) ($FeCl_3$) with or without ADP, the production of uric acid was monitored. The observations revealed that there was no marked change in $O_2^{\cdot-}$ generation, as the production of uric acid in all the reactions was almost the same. All the variants in different required combinations were tried and there was variation in the range of ±3.0% only (data not shown here).

The results for antioxidative potential activities show that 7,8-DHMC was very effective at a low concentration for which IC_{50} was worked out to be 34.0 μM, while it was 62.0 μM for 6,7-DHMC. 5,7-DHMC inhibited the formazan formation at a higher concentration exhibiting the IC_{50} 8.80 mM. But when we observed the antioxidant potential after complexing with Fe(III)–ADP, the IC_{50} was recorded 10.5 μM, i.e. more than three times efficient for 7,8-DHMC while for 6,7-DHMC and 5,7-DHMC it was 11.5 and 148.5 μM, respectively. Thus , the IC_{50} values recorded in case of Fe(III)–ADP complexed coumarins were markedly low and indicated the stimulation in antioxidative potential pronouncedly in later two

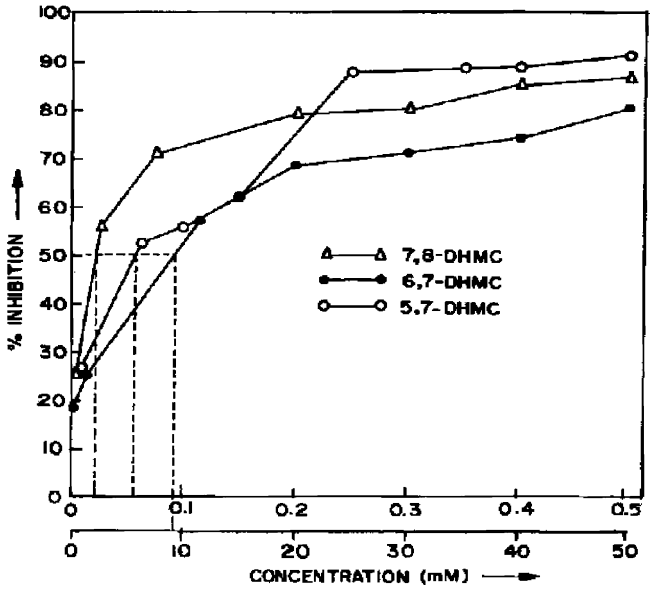

Figure 8. Coumarin derivatives mediated scavenging of $O_2^{\cdot-}$. The typical reaction mixture contained in a total volume of 1.0 ml, phosphate buffer (0.1 M, pH 7.4), L-methionine 10 mM, nitroblue tetrazolium (NBT) 57 mM, xanthine 1.0 mM. The reaction mixture was incubated at room temperature (25 ± 2 °C) for 5 min. The reaction was initiated by the addition of 50 milliunits of xanthine oxidase. The production of blue colored formazan, a reduction product of NBT was monitored at 550 nm as control value. The same reaction was run by adding the various concentrations (1.0 μM to 50 mM) of coumarin derivatives i.e. 7,8-DHMC, 6,7-DHMC and 5,7-DHMC. The decrease in formazan production was recorded and the IC_{50} values for all the three coumarin derivatives were calculated.

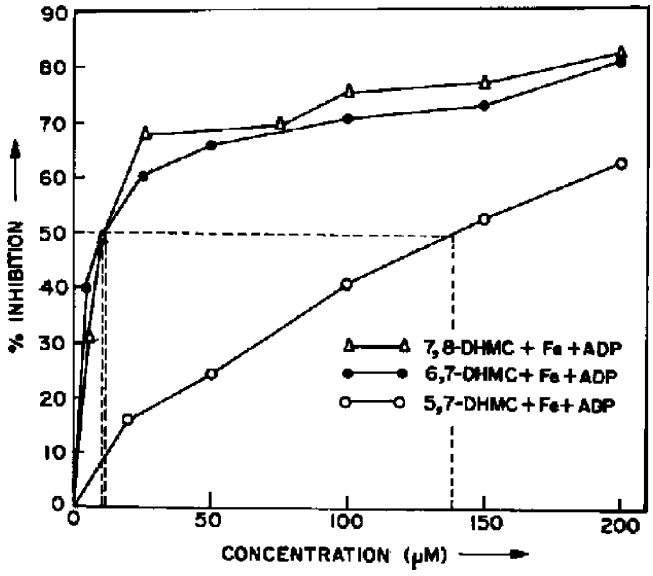

Figure 9. Coumarin derivatives complexed with Fe(III) and ADP mediated scavenging of $O_2^{\cdot-}$. The typical reaction mixture contained in a total volume of 1.0 ml, phosphate buffer (0.1 M, pH 7.4), L-methionine 10 mM, nitroblue tetrazolium (NBT) 57 mM, xanthine 1.0 mM, coumarin derivatives (1.0 to 500 μM), 0.15 mM $FeCl_3$ and 3.0 mM ADP. The reaction mixture was incubated at room temperature (25 ± 2 °C) for 5 min. The reaction was initiated by the addition of 50 milliunits of xanthine oxidase. The complexation of the derivatives further exhibited the stronger antioxidative potential as IC_{50} values recorded were significantly lowered.

Table 4. Results of the DHMC–Fe (III) (2:1) equilibrium computations using program BEST.

TMAH (ml)	7,8-DHMC (Obs. pH)	7,8-DHMC (Calc. pH)	6,7-DHMC (Obs. pH)	6,7-DHMC (Calc. pH)	5,7-DHMC (Obs. pH)	5,7-DHMC (Calc. pH)
0.000	2.220	2.290	2.400	2.347	2.240	2.290
0.100	2.250	2.311	2.420	2.370	2.250	2.311
0.200	2.280	2.332	2.480	2.395	2.270	2.332
0.300	2.320	2.355	2.510	2.420	2.300	2.355
0.400	2.370	2.378	2.540	2.447	2.370	2.378
0.500	2.390	2.403	2.580	2.476	2.410	2.403
0.600	2.430	2.429	2.610	2.506	2.460	2.429
0.700	2.490	2.456	2.730	2.539	2.510	2.456
0.800	2.550	2.484	2.770	2.573	2.550	2.484
0.900	2.590	2.515	2.810	2.610	2.600	2.515
1.000	2.640	2.547	2.850	2.650	2.670	2.547
1.100	2.720	2.582	2.890	2.690	2.740	2.582
1.200	2.820	2.619	2.930	2.742	2.800	2.619
1.300	2.860	2.659	2.980	2.796	2.850	2.659
1.400	2.990	2.703	3.140	2.856	2.920	2.703
1.500	3.100	2.752	3.190	2.926	2.980	2.752
1.600	3.240	2.806	3.290	3.008	3.040	2.806
1.700	3.310	2.867	3.420	3.109	3.100	2.867
1.800	3.430	2.937	3.500	3.238	3.180	2.937
1.900	3.510	3.020	3.600	3.421	3.280	3.020
2.000	3.600	3.122	3.710	3.740	3.360	3.122
2.100	3.680	3.254	5.580	5.393	3.480	3.254
2.200	3.740	3.443	5.670	6.010	3.680	3.443
2.300	3.880	3.780	5.760	6.237	4.080	3.780
2.400	5.920	5.781	5.860	6.424	6.100	6.075
2.500	6.060	6.327	5.970	6.606	6.680	6.613
2.600	6.180	6.559	6.360	6.796	6.990	6.851
2.700	6.310	6.760	6.960	6.999	7.190	7.072
2.800	6.460	6.974	7.320	7.231	7.530	7.363
2.900	6.660	7.214	7.710	7.548	7.830	7.881
3.000	7.040	7.465	8.720	8.304	8.280	8.265
3.100	7.520	7.735	9.980	9.929	8.600	8.565
3.200	8.030	8.102	10.280	10.288	8.990	8.926
3.300	8.840	9.048	10.670	10.481	9.510	9.517
3.400			10.900	10.614	10.010	10.054
3.500			10.120	10.714	10.290	10.332
3.600			10.420	10.796	10.460	10.505
3.700			10.820	10.864	10.590	10.630
3.800			10.980	10.922	10.690	10.726
3.900			11.020	10.973	10.780	10.804
4.000			11.140	11.081	10.870	10.871
4.100					10.930	10.927
4.200					10.990	10.977
4.300					11.050	11.022

DHMCs, Dihydroxy-4-methylcoumarins.

Table 5. Results of the DHMC–Fe(III)–ADP (1:1:1) equilibrium computations using program BEST.

TMAH (ml)	7,8-DHMC-ADP (Obs. pH)	7,8-DHMC-ADP (Calc. pH)	6,7-DHMC-ADP (Obs. pH)	6,7-DHMC-ADP (Calc. pH)	5,7-DHMC-ADP (Obs. pH)	5,7-DHMC-ADP (Calc. pH)
0.000	2.200	2.244	2.210	2.244	2.250	2.244
0.100	2.220	2.263	2.240	2.263	2.280	2.263
0.200	2.240	2.283	2.260	2.283	2.300	2.283
0.300	2.260	2.303	2.290	2.303	2.320	2.303
0.400	2.290	2.324	2.320	2.324	2.340	2.324
0.500	2.310	2.346	2.350	2.346	2.370	2.346
0.600	2.340	2.368	2.370	2.368	2.400	2.368
0.700	2.360	2.392	2.400	2.392	2.420	2.392
0.800	2.390	2.417	2.430	2.417	2.440	2.417
0.900	2.410	2.443	2.460	2.443	2.470	2.443
1.000	2.440	2.471	2.490	2.471	2.500	2.471
1.100	2.480	2.500	2.510	2.500	2.530	2.500
1.200	2.500	2.531	2.540	2.531	2.560	2.531
1.300	2.520	2.564	2.570	2.564	2.580	2.564
1.400	2.540	2.600	2.620	2.600	2.620	2.600
1.500	2.580	2.638	2.660	2.638	2.650	2.638
1.600	2.610	2.679	2.700	2.679	2.690	2.679
1.700	2.660	2.725	2.750	2.725	2.530	2.725
1.800	2.730	2.775	2.800	2.775	2.580	2.775
1.900	2.780	2.831	2.860	2.831	2.630	2.831
2.000	2.860	2.895	2.910	2.895	2.690	2.895
2.100	2.960	2.970	2.980	2.970	2.770	2.970
2.200	3.070	3.059	3.060	3.059	2.870	3.059
2.300	3.230	3.169	3.160	3.169	2.960	3.169
2.400	3.410	3.317	3.360	3.317	3.140	3.317
2.500	3.610	3.539	3.570	3.539	3.350	3.539
2.600	4.130	4.012	4.060	4.011	3.860	4.012
2.700	6.250	6.207	5.760	5.601	6.010	6.229
2.800	6.410	6.524	5.980	5.923	6.490	6.553
2.900	6.610	6.721	6.210	6.140	6.670	6.772
3.000	6.830	6.895	6.420	6.357	7.040	6.994
3.100	7.120	7.068	6.680	6.620	7.400	7.282
3.200	7.220	7.251	6.980	6.910	7.790	7.618
3.300	7.460	7.456	7.270	7.173	8.070	7.899
3.400	7.710	7.708	7.500	7.451	8.390	8.183
3.500	8.180	8.103	7.920	7.865	8.760	8.590
3.600	9.350	9.370	9.420	9.389	9.600	9.449
3.700	10.030	10.081	10.120	10.109	10.060	10.040
3.800	10.280	10.347	10.380	10.371	10.320	10.315
3.900	10.420	10.511	10.600	10.533	10.460	10.487
4.000	10.630	10.630	10.750	10.650	10.620	10.610
4.100	10.760	10.723			10.700	10.706
4.200	10.870	10.800				

derivatives. But it was interesting to note that the pattern of antioxidative efficacy was unaltered which is: 5,7-DHMC < 6,7-DHMC < 7,8-DHMC, as recorded with the coumarin analogs alone. Fe(II) (ferrous sulphate, freshly prepared aqueous solution) in place of Fe(III) (ferric chloride) was also used in order to verify if Fe(II) has a role in antioxidative mechanism. The experiments revealed

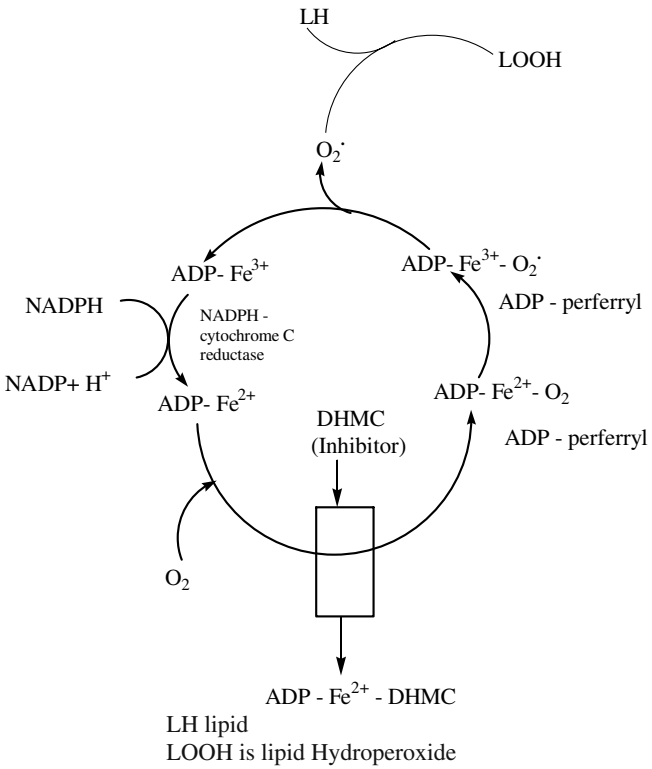

Figure 10. Inhibition of NADPH-dependent microsomal lipid peroxidation.

no effect in antioxidative efficacy when DHMCs were complexed with Fe(II)–ADP.

The pharmacological and biochemical properties and therapeutic usefulness of coumarins have been worked out to be a sequel of the different substitutions in the derivative. It is well established that the antioxidant activity of a compound is more exactly assessed by providing a microenvironment of the reaction medium and nature of the involved ROS rather only by its structure. Hence it is very important to assess the structure activity relationship trying different reactions. Coumarin derivatives have been reported potent inhibitors of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) formation from arachidonic acid and 12-hydroxy-5,8,10-heptadecatrienoic acid in polymorphonuclear leukocytes (PMN). The presence of two adjacent phenolic hydroxyl groups at C-6 and C-7 or C-7 and C-8 positions in coumarin structure is must for its potency for the inhibition of the 5-HETE. Monohydroxy coumarins, umbelliferone and scopoletin also inhibited the formation of 5-HETE, but not very strongly, suggestive of the essentiality of the presence of a phenolic hydroxyl group at C-7 position for this effect (Kimura *et al.* 1985).

In the present study, our results indicate that the pattern of oxidative efficacy consonants the gradual decrease in the position numbers of OH group, being 7th and 8th in 7,8-DHMC, 6th and 7th in 6,7-DHMC and 5th and 7th in 5,7-DHMC depicted the lowering in the activities. The methyl group is on the 4th position in all the three derivatives, which might not be any altering factor.

Recently, we have shown that formation of ADP–Fe-antioxidant (ligand) could assume a crucial role in the prevention of reactive oxygen species (ROS) (Raj *et al.* 1998). The mechanism for inhibition of NADPH-dependent microsomal lipid peroxidation by DHMC is given in Figure 10. As seen in Figure 10, ADP-perferryl ion formation is prevented by DHMC resulting in the production of a stable ternary mixed ligand (ADP–Fe–DHMC), thereby inhibiting the formation of superoxide and other ROS responsible for membrane lipid peroxidation. We have also shown

that ADP–Fe–DHMC is a stable complex devoid of releasing free Fe and can hardly activate the molecular oxygen itself. The sequestration of transition metal ions by antioxidant is one of the most powerful preventive mechanisms *in vivo* (Ernster & Nordenbrand, 1967; Morel *et al*. 1993) and chelation of transition metal by antioxidant ligands can inhibit membrane lipid peroxidation (Bors *et al*. 1994). The polyphenolic structure of DHMC facilitates the inhibition of lipid peroxidation by scavenging free radicals through chelation. It was already reported by us that the chelator 7,8-DHMC is 40 times more potent than vitamin E while performing the lipid peroxidation inhibition experiments (Raj *et al*. 1998). But 6,7-DHMC was found to be a weak antioxidant compared to 7,8-DHMC, which supports the present findings. Studies made with five dihydroxy derivatives of coumarin for antioxidative activity, as inhibitors of 5-lipoxygenase pathway of arachidonic metabolism revealed the high efficacy of these compounds owing to their two fold ability to chelate the iron ions and donating the electron for redox cycling of iron thus rendering the 5-lipoxygenase in catalytically inactive ferrous form (Hoult *et al*. 1994). Studies on chelating behavior of dihydroxycoumarin in Fe(III)-ascorbate–H_2O_2 system revealed *ortho*-dihydroxycoumarin to be a strong metal chelator as compared to metal-substituted compounds (Porter *et al*. 1989) which consonates the results of the present study.

Acknowledgements

Council of Scientific and Industrial Research (CSIR), New Delhi, India, is acknowledged for the financial assistance to carry out this work.

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