Synthesis and SAR Study of Antioxidant Potential of Polyhydroxy Coumarin Derivatives
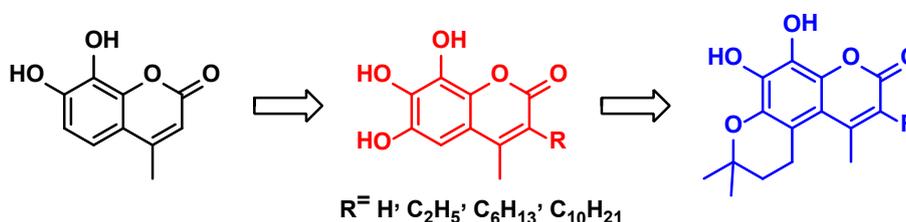
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Abstract

A series of polyhydroxycoumarin derivatives, that are analogs of naturally occurring compounds, have been synthesized and their antioxidant activity (AOA) examined using DPPH, ABTS, and *in vitro* lipid peroxidation inhibition assays. The SAR for differently substituted polyhydroxycoumarins is reported by evaluating the positional effect of hydroxyl groups and the effect of incorporation of the lipophilic group on antioxidant activity. Many of the compounds synthesized have 4-5 fold higher AOA than 'Trolox' taken as standard. In DPPH and ABTS assays, the trihydroxycoumarins were observed to have a potent antioxidant activity. It has been observed that alkylation at C-3/C-4 position as well as the incorporation of pyran ring on coumarin skeleton led to the reduction in AOA in the above two assays. However, in lipid peroxidation inhibition assay an enhancement in AOA was observed for such modifications. The rationale for the observance of variation in AOA in different assays is also studied.

$$R = H, C_2H_5, C_6H_{13}, C_{10}H_{21}$$

Keywords: Antioxidant activity; Polyhydroxy coumarins; Lipid peroxidation inhibitory activity; Structure-activity relationship

Introduction

Antioxidant enzymes are known to control oxidative stress by preventing the Reactive Oxygen/Nitrogen Species (ROS/RNS) induced damage to biomembranes, nucleic acids, proteins, etc. The antioxidant substances mimic such enzymes and are involved in the prevention of cellular damage, the pathway leading to cancer, aging and various other diseases. Besides medicinal applications, the antioxidants are also useful for preventing the deterioration of goods, e.g., oils, foodstuffs, cosmetics, pharmaceuticals, etc. [1,2]. Phenolic compounds obtained from plants, e.g., tocopherols, phenolic acids, anthocyanins, flavonoids, tanins, etc. have established to be safe and effective antioxidants. However, there is an ever increasing demand for more effective antioxidants that are analogues of naturally occurring phenolic compounds.

The method of evaluation of antioxidant capacity of a molecule involve radical scavenging or radical formation inhibition and follow either homolytic hydrogen atom transfer (HAT) or single electron transfer (SET). In HAT, a hydrogen atom from the antioxidant (ArOH) is captured by the free radical and antioxidant itself becomes a radical. Polyphenolics follow such a mechanism due to their ability of phenoxide ion delocalization. The bond dissociation enthalpy (BDE) of the O-H bonds is an important parameter besides the factors such as hydrogen bonds, conjugation, and resonance, in evaluating the antioxidant action. SET on the other hand led to the generation of a radical cation due to the transfer of an electron from the antioxidant to the free radical. The stability of radical cation is an important criterion for the evaluation of antioxidant potential so that it itself does not react further with the substrate molecules. The ionization potential is an important parameter in SET. An antioxidant may follow either HAT and SET mechanisms simultaneously or one mechanism can outweigh the other and this is dictated by the chosen analytical method and conditions [3,4].

The antioxidant potential of a variety of phenolic compounds isolated from natural sources as well their synthetic analogues has been studied in detail, and the number as well as position of hydroxyl substituents plays a key role in attenuating their ability to scavenge free radicals [5,6]. Coumarin (2*H*-1-benzopyran-2-one) which contains an aromatic ring fused with 6-member lactone ring, is a naturally occurring compound whose phenolic derivatives are known to possess potent antioxidant potential [7]. This is mainly due to their extensive conjugated π-electron system that facilitates prompt donation of electrons (SET) or hydrogen atoms (HAT) to free radicals. Among various hydroxycoumarins, the dihydroxy derivatives having *o*-dihydroxy groups in the benzonoid ring are reported to have better radical scavenging activity than their corresponding monohydroxy, *m*-dihydroxy, or *p*-dihydroxy substituted analogues [8,9]. In *o*-dihydroxycoumarins the phenoxy radical formed after the H-atom transfer is either stabilized by resonance or hydrogen bonding. Further, it may also oxidize to a quinone-type moiety through single-electron reduction by the second hydroxyl group (Figure 1) [9].

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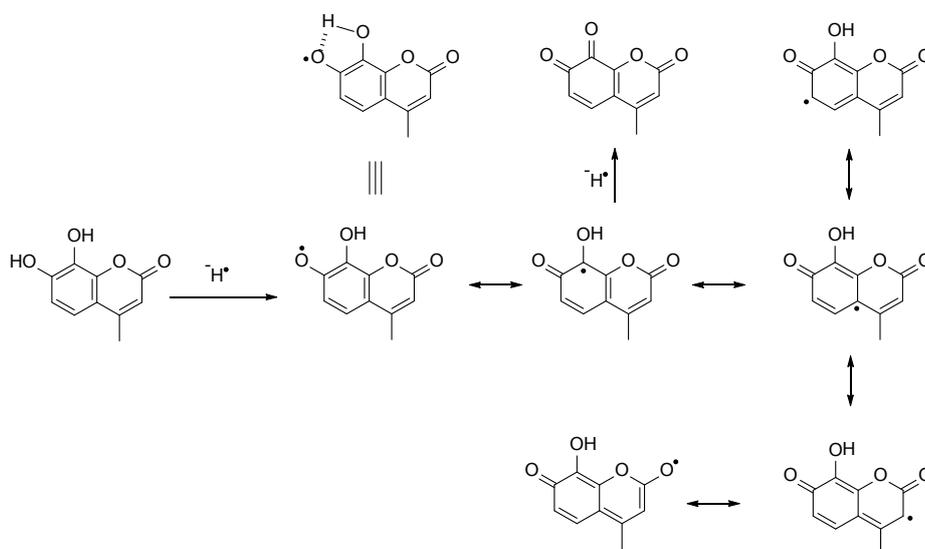
Figure 1: Resonance stabilization of phenoxy radical [9].

Besides the wealth of data available on the antioxidant potential of flavonoids, coumarins, etc. the correlation between AOA and chemical structure is far from clear. This is primarily due to the fact that no single AOA evaluation method is sufficient to estimate a sample accurately and quantitatively. Each method has its own advantages and limitations, even the specificity and sensitivity are different [3,4]. Furthermore, the analytical conditions followed in a particular assay influence the mechanism which in turn affects the kinetics and hence the AOA [10,11]. Lipophilicity is also known to play an important role in the evaluation of AOA of a molecule. Thus, a significant inconsistency in results has been observed for a number of antioxidants and the use of several different methods has been emphasized. Considering the above and encouraged by our recent study on the antioxidant activity of coumarin derivatives [12], herein, we have synthesized newer analogs by incorporating additional hydroxyl groups on the benzenoid ring of coumarin and studied their effect on AOA using three assays i.e., DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), and LPO (lipid peroxidation) inhibition. The DPPH and ABTS methods involves photometric measurements, but the former method is the most frequently used for *in vitro* AOA evaluation [3,4]. On the other hand the lipid peroxidation method is mostly used for *in vivo* AOA measurement as it has relevance to biological systems.

Based on the general agreement that the presence of a catechol moiety in the benzenoid ring of coumarin favors AOA, we have synthesized di- and trihydroxycoumarins and studied the effect of hydroxyl groups on the antioxidant potential. The AOA of compounds has been compared with 'Trolox' taken as a standard and the results have been expressed as Trolox Equivalence Antioxidant Capacity (TEAC). Trolox, tocopherol, and tocotrienol possess same chroman moiety that represents the active antioxidant component of vitamin E, the function of such a group along with long hydrocarbon chain is to enhance generally the solubility of the substrate in lipids with no adverse effects on its antioxidant action [13].

Considering the above, we have incorporated the pyrano moiety in the benzenoid ring (**19-22**) and alkyl chain at C-3 position of the coumarin skeleton (**14-16**), in order to enhance their lipophilicity and consequently the AOA of the corresponding coumarins. This study

is also inspired by the fact that a number of synthetic and naturally occurring polyhydroxy flavanoids have been studied for their AO potential [6,14,15], however, the AOA of polyhydroxy coumarins has not been studied. This is the first report of the SAR between differently substituted di- and trihydroxycoumarins.

Experimental Section

Materials and methods

Materials: All of the chemicals and reagents were procured from Sigma-Aldrich Chemicals and Spectrochem Pvt. Ltd., India. The organic solvents used were dried and distilled prior to their use. Reactions were monitored by precoated TLC plates (Merck silica gel 60F$_{254}$); the spots were visualized either by UV light, or by spraying with 5% methanolic FeCl$_3$ solution. Silica gel (100-200 mesh) was used for column chromatography.

Instruments: Melting points were measured on a Buchi M-560 instrument and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer FT-IR model 9 spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Jeol-400 (400 MHz, 100.5 MHz) NMR spectrometer using tetramethylsilane as the internal standard. The chemical shift values are on a δ scale and the coupling constant values (*J*) are in hertz. The HRMS data were recorded on Agilent-6530, Q-TOF LCMS. UV-Visible absorption spectra were recorded using a Cary-300 UV-Vis spectrophotometer, from Agilent-Technologies. A quartz cuvette of 1 cm path-length was used to record absorption and emission spectra. Purity of the compounds was determined by HPLC (high performance liquid chromatography) on Shimadzu LC-2010HT instrument using Qualisil BDS C8 column. Methanol was used as the mobile phase with a flow rate of 0.3 mL/min and injection volume was 2 μL for all samples.

Different assays for antioxidant activity

DPPH radical scavenging assay: The DPPH assay based antioxidant activity was measured by modifying the method of Blois et al. using the DPPH [16]. Ethanolic solution of DPPH (2 mL) was added to the compound solution in ethanol (1 mL), or 1 mL ethanol for the blank. The final concentration of radical was 0.09 mmol/L.

After 30 min of incubation, the absorbance was measured at 517 nm. All the measurements were done in three replicates. Percentage inhibition of DPPH radical by a 1 μM test compound or standard "Trolox" was calculated by the formula: % Inhibition=[(Blank OD-Sample OD)/Blank OD] × 100. Trolox equivalent antioxidant capacity was calculated by the formula: TEAC=% inhibition by sample / % inhibition by "Trolox".

ABTS radical scavenging assay: Antioxidant activity by ABTS assay was done following the method of Re et al. with slight modification [17]. The fresh ABTS radical cation was synthesized by mixing 7 mM ABTS stock solution with freshly prepared 2.45 mM potassium persulfate (1:1) and incubating for 12-16 h at room temperature in the dark until the absorbance become stable and the reaction gets complete. The UV-vis absorbance of the ABTS solution was equilibrated to 0.70 (± 0.02) by diluting with required amount of water, then 1 mL of this solution was mixed with 1 mL of the test sample and again the absorbance was measured at 734 nm after 6 min. Percentage inhibition of ABTS radical cation caused by a 1 μM test compound or standard "Trolox" was calculated by using the same formula as used for DPPH assay.

Lipid peroxidation inhibitory activity assay: The method standardized by Bishayee and Balasubramaniyam was used for *in vitro* lipid peroxidation inhibition measurement [18]. 100 μL of rat liver homogenate (25%, w/v in 20 mM Tris-HCl buffer, pH 7.0) was incubated with Mohr's salt (0.16 mM), ascorbic acid (0.06 mM) and varying concentrations (0, 1, 2, 5, 10, 20, 50, 75 and 100 μM) of test compounds or Trolox in a final volume of 500 μL for 1 h at 37°C. After incubating for 1 h, 400 μL of the reaction mixture was mixed with 200 μL sodium dodecyl sulphate (SDS) (8.1%), 1.5 mL CH_3COOH (20%, pH 3.5), 1.5 mL thiobarbituric acid (TBA) (0.8%), and 400 μL distilled water and kept in boiling water bath for 1 h. After cooling the reaction mixture 1 mL distilled water and 4 mL n-butanol:pyridine (15:1) mixture were added and the contents were shaken vigorously and then centrifuged at 3000 rpm for 10-15 min. The n-butanol - pyridine layer was separated and UV absorption was recorded at 532 nm. Percentage inhibition of lipid peroxidation caused by different concentrations of test samples and Trolox and was calculated by the same formula as used for DPPH assay. The concentration of Trolox or test compound that caused 50% inhibition was termed IC_{50}. TEAC was calculated by the formula TEAC=IC_{50} of Trolox/IC_{50} of the sample.

Chemistry

General procedure A (synthesis of dihydroxy formylcoumarins, 7-12): To the dihydroxycoumarin (10 mmol) dissolved in 50 mL of TFA, hexamine (14 mmol) was then added and the reaction mixture was refluxed for 1 h. The progress of the reaction was monitored by TLC (5% methanol in chloroform), on completion, the reaction mixture was poured slowly over ice with continuous stirring. The precipitate thus formed was filtered, dried and purified by silica-gel column chromatography using 3% methanol in chloroform as an eluent to afford dihydroxy formylcoumarins (7-**12**).

7,8-Dihydroxy-4-methyl-2-oxo-2H-chromene-6-carbaldehyde (7): The title compound **7** was synthesized from dihydroxycoumarin **1** using the general procedure A as a white solid in 35% yield. mp: 270-271°C (literature mp=268°C [19]); IR (KBr, cm⁻¹): 3402, 1718, 1658, 1622; UV (acetonitrile, λ_{max}): 278 nm; ¹H NMR (400 MHz, DMSO-d_6, δ): 10.23 (s, 1H, CHO), 7.59 (s, 1H, H-5), 6.26 (s, 1H, H-3), 2.40 (s, 3H, C-4 CH₃); ¹³C NMR (100.5 MHz, DMSO-d_6, δ): 191.2, 159.2, 153.7, 152.4, 146.6, 132.6, 119.3, 116.7, 112.9, 111.7, 18.1; HRMS: Calculated for $C_{11}H_8O_5$ [M+H]⁺ 221.0444, found 221.0418.

3-Ethyl-7,8-dihydroxy-4-methyl-2-oxo-2H-chromene-6-carbaldehyde (8): The title compound **8** was synthesized from dihydroxycoumarin **2** using the general procedure A as a white solid in 40% yield. mp: 230-231°C; IR (KBr, cm⁻¹): 3447, 3143, 2968, 1709, 1642; UV (acetonitrile, λ_{max}): 280 nm ;¹H NMR (400 MHz, DMSO-d_6, δ): 10.12 (s, 1H, CHO), 7.51 (s, 1H, H-5), 2.46 (q, 2H, J = 7.32, H-1′), 2.28 (s, 3H, C-4 CH₃), 0.94 (t, 3H, J = 7.32 Hz, H-2′); ¹³C NMR (100.5 MHz, DMSO-d_6, δ): 191.5, 159.8, 151.3, 146.6, 145.4, 132.3, 124.3, 119.2, 116.8, 113.5, 20.2, 14.3, 12.9; HRMS: Calculated for $C_{13}H_{12}O_5$ [M+H]⁺ 249.0757, found 249.0763.

3-Hexyl-7,8-dihydroxy-4-methyl-2-oxo-2H-chromene-6-carbaldehyde (9): The title compound **9** was synthesized from dihydroxycoumarin **3** using the general procedure A as a yellowish white solid in 42% yield. mp: 196-197°C; IR (KBr, cm⁻¹): 3448, 3196, 2954, 2926, 1710, 1646; UV (acetonitrile, λ_{max}): 281 nm; ¹H NMR (400 MHz, DMSO-d_6, δ): 10.73 (brs, OH), 10.16 (s, 1H, CHO), 9.98 (brs, OH), 7.53 (s, 1H, H-5), 2.49 (m, 2H, H-1′), 2.31 (s, 3H, C-4 CH₃), 1.38-1.33 (m, 2H, H-5′), 1.28-1.21 (m, 6H, H-2′-H-4′), 0.80 (t, 3H, J = 6.22 Hz, H-6′); ¹³C NMR (100.5 MHz, DMSO-d_6, δ): 191.5, 160.0, 151.4, 146.8, 145.4, 132.3, 123.0, 119.1, 116.7, 113.4, 31.0, 28.6, 28.1, 26.8, 22.0, 14.6, 13.9; HRMS: Calculated for $C_{17}H_{20}O_5$ [M+H]⁺ 305.1384, found 305.1386.

3-Decyl-7,8-dihydroxy-4-methyl-2-oxo-2H-chromene-6-carbaldehyde (10): The title compound **10** was synthesized from dihydroxycoumarin **4** using the general procedure A as off white solid in 40% yield. mp: 191-192°C; IR (KBr, cm⁻¹): 3530, 2956, 2922, 2852, 1698, 1674; UV (acetonitrile, λ_{max}): 282 nm; ¹H NMR (400 MHz, DMSO-d_6, δ): 10.16 (s, 1H, CHO), 7.54 (s, 1H, H-5), 2.47 (m, 2H, H-1′), 2.31 (s, 3H, C-4 CH₃), 1.38-1.33 (m, 2H, H-9′), 1.23-1.16 (m, 14H, H-2′-H-8′), 0.77 (t, 3H, J = 6.59 Hz, H-10′); ¹³C NMR (100.5 MHz, DMSO-d_6, δ): 191.4, 160.1, 151.4, 146.9, 145.4, 132.3, 123.0, 119.2, 116.7, 113.5, 31.3, 29.0, 28.9, 28.9, 28.7, 28.2, 26.9, 22.1, 14.7, 13.9; HRMS: Calculated for $C_{21}H_{28}O_5$ [M+H]⁺ 361.2010, found 361.2022.

7,8-Dihydroxy-2-oxo-4-phenyl-2H-chromene-6-carbaldehyde (11): The title compound **11** was synthesized from dihydroxycoumarin **5** using the general procedure A as a white solid in 40% yield. mp: 250-251°C; IR (KBr, cm⁻¹): 3216, 3060, 1700, 1652, 1586; UV (acetonitrile, λ_{max}): 280 nm; ¹H NMR (400 MHz, DMSO-d_6, δ): 10.18 (s, 1H, CHO), 7.57-7.52 (m, 5H, H-2′-H-6′), 7.27 (s, 1H, H-5), 6.27 (s, 1H, H-3); ¹³C NMR (100.5 MHz, DMSO-d_6, δ): 190.7, 159.1, 155.6, 152.7, 147.2, 134.8, 133.1, 129.8, 128.9, 128.4, 119.5, 118.0, 111.9, 111.8; HRMS: Calculated for $C_{16}H_{10}O_5$ [M+H]⁺ 283.0601, found 283.0610.

4-(Chloromethyl)-7,8-dihydroxy-2-oxo-2H-chromene-6-carbaldehyde (12): The title compound **12** was synthesized from dihydroxycoumarin **6** using the general procedure A as a yellowish white solid in 35% yield. mp: 245-246 °C; IR (KBr, cm⁻¹): 3482, 1720, 1654; UV (acetonitrile, λ_{max}): 279 nm; ¹H NMR (400 MHz, DMSO-d_6, δ): 10.25 (s, 1H, CHO), 7.68 (s, 1H, H-5), 6.54 (s, 1H, H-3), 4.99 (s, 2H, CH₂Cl); ¹³C NMR (100.5 MHz, DMSO-d_6, δ): 191.3, 159.3, 152.8, 151.3, 147.1, 133.2, 119.7, 116.9, 112.9, 110.5, 41.4; HRMS: Calculated for $C_{11}H_7ClO_5$ [M+H]⁺ 255.0055, found 255.0064.

General procedure B (synthesis of 6,7,8-trihydroxycoumarins, 13-18): To the mixture of 4.5 mmol of dihydroxy formylcoumarin in 25 mL of 2% NaOH cooled at 0°C, 6 mL of 6% H_2O_2 was added drop wise with continuous stirring for 1 h. In case of compounds **15** and **16**, 10 mol% of TBAHS, a phase transfer catalyst was added simultaneously. The progress of the reaction was monitored using TLC (5% methanol in chloroform). On completion of the reaction, the reaction mixture was acidified with dil. HCl and allowed to stand for 10 min. The solid

precipitate thus obtained was filtered, washed with cold water and dried. The resulting crude product was purified by silica-gel column chromatography using 5% methanol in chloroform as eluent.

6,7,8-Trihydroxy-4-methyl-2H-chromen-2-one (13): The title compound **13** was synthesized from 6-formyl-7,8-dihydroxy-4-methylcoumarin (**7**) as a white solid in 40% yield by following the general procedure B. mp: 277-278°C (literature mp=274-275°C [19]); IR (KBr, cm^{-1}): 3411, 3151, 1651, 1604; UV (acetonitrile, λ_{max}): 319 nm; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.34 (brs, 3×OH), 6.58 (s, 1H, H-5), 6.10 (s, 1H, H-3), 2.29 (s, 3H, C-4 CH$_3$); ^{13}C NMR (100.5 MHz, DMSO-d_6, δ): 160.5, 153.6, 142.8, 138.6, 137.6, 133.0, 111.2, 110.7, 100.1, 18.4; HRMS: Calculated for C$_{10}$H$_8$O$_5$ [M+H]$^+$ 209.0444, found 209.0447.

3-Ethyl-6,7,8-trihydroxy-4-methyl-2H-chromen-2-one (14): The title compound **14** was synthesized from 3-ethyl-6-formyl-7,8-dihydroxy-4-methylcoumarin (**8**) as a white solid in 60% yield by following the general procedure B. mp: 220-221°C; IR (KBr, cm^{-1}): 3436, 1648, 1620, 1542; UV (acetonitrile, λ_{max}): 317 nm; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.22 (brs, 3×OH), 6.59 (s, 1H, H-5), 2.53 (q, 2H, J = 7.33 Hz, H-1′), 2.27 (s, 3H, C-4 CH$_3$), 1.01 (t, 3H, J = 7.33 Hz, H-2′); ^{13}C NMR (100.5 MHz, DMSO-d_6, δ): 160.9, 146.6, 142.7, 137.4, 136.0, 132.7, 123.0, 111.7, 100.1, 20.2, 14.4, 13.1; HRMS: Calculated for C$_{12}$H$_{12}$O$_5$ [M+H]$^+$ 237.0757, found 237.0762.

3-Hexyl-6,7,8-trihydroxy-4-methyl-2H-chromen-2-one (15): The title compound **15** was synthesized from 3-hexyl-6-formyl-7,8-dihydroxy-4-methylcoumarin (**9**) as a white solid in 35% yield by following the general procedure B. mp: 189-190°C; IR (KBr, cm^{-1}): 3454, 3152, 2958, 2852, 1642, 1610; UV (acetonitrile, λ_{max}): 323 nm; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.25 (brs, 3 × OH), 6.58 (s, 1H, H-5), 2.49 (m, 2H, H-1′), 2.26 (s, 3H, C-4 CH$_3$), 1.40-1.36 (m, 2H, H-5′), 1.30-1.20 (m, 6H, H-2′-H-4′), 0.83 (t, 3H, J = 6.59 Hz, H-6′); ^{13}C NMR (100.5 MHz, DMSO-d_6, δ): 161.2, 146.9, 142.7, 137.4, 136.1, 132.7, 121.8, 111.7, 100.1, 31.1, 28.7, 28.3, 26.9, 22.1, 14.7, 13.9; HRMS: Calculated for C$_{16}$H$_{20}$O$_5$ [M+H]$^+$ 293.1384, found 293.1394.

3-Decyl-6,7,8-trihydroxy-4-methyl-2H-chromen-2-one (16): The title compound **16** was synthesized from 3-decyl-6-formyl-7,8-dihydroxy-4-methylcoumarin (**10**) as a white solid in 35% yield by following the general procedure B. mp: 192-193°C; IR (KBr, cm^{-1}): 3456, 3112, 2920, 2850, 1648, 1576; UV (acetonitrile, λ_{max}): 323 nm; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.22 (brs, 3×OH), 6.58 (s, 1H, H-5), 2.49 (m, 2H, H-1′), 2.26 (s, 3H, C-4 CH$_3$), 1.41-1.35 (m, 2H, H-9′), 1.29-1.22 (m, 14H, H-2′-H-8′), 0.83 (t, 3H, H-10′); ^{13}C NMR (100.5 MHz, CDCl$_3$/DMSO-d_6, δ): 161.2, 146.9, 142.7, 137.4, 136.1, 132.7, 121.8, 111.7, 100.1, 40.4, 31.3, 29.0, 28.9, 28.7, 28.4, 26.9, 22.1, 14.8, 14.0; HRMS: Calculated for C$_{20}$H$_{28}$O$_5$ [M+H]$^+$ 349.2010, found 349.2003.

6,7,8-Trihydroxy-4-phenyl-2H-chromen-2-one (17): The title compound **17** was synthesized from 6-formyl-7,8-dihydroxy-4-phenylcoumarin (**11**) as yellowish white solid in 40% yield by following the general procedure B. mp: 239-240°C; IR (KBr, cm^{-1}): 3404, 3214, 1664, 1448; UV (acetonitrile, λ_{max}): 332 nm; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.53-7.48 (m, 5H, H-2′-H-6′), 6.35 (s, 1H, H-5), 6.08 (s, 1H, H-3); ^{13}C NMR (100.5 MHz, DMSO-d_6, δ): 160.4, 155.8, 142.7, 138.9, 138.2, 135.7, 133.4, 129.4, 128.7, 128.3, 110.6, 109.9, 101.8; HRMS: Calculated for C$_{15}$H$_{10}$O$_5$ [M+H]$^+$ 271.0601, found 271.0609.

4-(Chloromethyl)-6,7,8-trihydroxy-2H-chromen-2-one (18): The title compound **18** was synthesized from 6-formyl-7,8-dihydroxy-4-(chloromethyl)coumarin (**12**) as a yellowish white solid in 35% yield by following the general procedure B. mp: 251-252°C; IR (KBr, cm^{-1}):

3452, 3094, 1654, 1432; UV (acetonitrile, λ_{max}): 334 nm; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.54 (brs, OH), 9.44 (brs, 2×OH), 6.69 (s, 1H, H-5), 6.39 (s, 1H, H-3), 4.86 (s, 2H, CH$_2$Cl); ^{13}C NMR (100.5 MHz, DMSO-d_6, δ): 160.3, 151.0, 142.9, 139.1, 138.1, 133.2, 111.4, 108.6, 100.2, 41.7; HRMS: Calculated for C$_{10}$H$_7$ClO$_5$ [M+H]$^+$ 243.0055, found 243.0055.

General procedure C (synthesis of dihydroxy pyranocoumarins, 19-22): To 3.6 mmol of trihydroxycoumarin dissolved in 20 mL of toluene taken in RB flask, p-toluenesulfonic acid (4.3 mmol) was added at 25°C. After 10 min 2-methyl-3-buten-2-ol (5.4 mmol) dissolved in 10 mL of xylene was added slowly with continuous stirring. The reaction mixture was refluxed for 24 h using dean stark apparatus and the progress of the reaction was monitored by TLC (5% methanol in chloroform). On completion of reaction, toluene was evaporated under reduced pressure and ethyl acetate was added to the reaction mixture. The organic layer was washed with water (3 × 30 mL) and then with brine solution. The ethyl acetate layer was dried over anhydrous Na$_2$SO$_4$ and the solvent was evaporated under reduced pressure to get crude product. The crude product, thus obtained was purified by silica-gel column chromatography using 3% methanol in chloroform as eluent.

5,6-Dihydroxy-1,8,8-trimethyl-9,10-dihydropyrano[3,2-f]chromen-3(8H)-one (19): The title compound **19** was obtained from the reaction of trihydroxycoumarin **13** with 2-methyl-3-buten-2-ol as an off white solid in 20% yield by following the general procedure C. mp: 208-209°C; IR (KBr, cm^{-1}): 3368, 2926, 1701, 1400; UV (acetonitrile, λ_{max}): 323 nm; ^{1}H NMR (400 MHz, CDCl$_3$, δ): 6.10, 6.04 (m, 3H, 2×OH, H-2), 3.07 (t, 2H, J = 6.59 Hz, H-10), 2.57 (s, 3H, C-4 CH$_3$), 1.84 (t, 2H, J = 6.59 Hz, H-9), 1.36 (s, 6H, C(CH$_3$)$_2$); ^{13}C NMR (100.5 MHz, CDCl$_3$, δ): 160.0, 155.1, 139.0, 138.8, 136.3, 129.6, 113.5, 110.9, 109.2, 74.5, 32.9, 26.2, 25.4, 22.5; HRMS: Calculated for C$_{15}$H$_{16}$O$_5$ [M+H]$^+$ 277.1071, found 277.1074.

2-Ethyl-5,6-dihydroxy-1,8,8-trimethyl-9,10-dihydropyrano[3,2-f]chromen-3(8H)-one (20): The title compound **20** was obtained from the reaction of trihydroxycoumarin **14** with 2-methyl-3-buten-2-ol as an off white solid in 40% yield by following the general procedure C. mp: 212-213°C; IR (KBr, cm^{-1}): 3506, 3400, 1696, 1570; UV (acetonitrile, λ_{max}): 327 nm; ^{1}H NMR (400 MHz, CDCl$_3$, δ): 5.95 (brs, 2×OH), 3.11 (t, 2H, J = 6.22 Hz, H-10), 2.67 (q, 2H, J = 7.32 Hz, H-1′), 2.56 (s, 3H, C-4 CH$_3$), 1.83 (t, 2H, J = 6.22 Hz, H-9), 1.39 (s, 6H, C(CH$_3$)$_2$), 1.13 (t, 3H, J = 7.32 Hz, H-2′); ^{13}C NMR (100.5 MHz, CDCl$_3$, δ): 160.7, 148.9, 138.9, 137.0, 135.0, 129.2, 125.5, 112.0, 108.4, 74.3, 33.1, 26.3, 23.3, 20.9, 20.0, 12.6; HRMS: Calculated for C$_{17}$H$_{20}$O$_5$ [M+H]$^+$ 305.1384, found 305.1390.

2-Hexyl-5,6-dihydroxy-1,8,8-trimethyl-9,10-dihydropyrano[3,2-f]chromen-3(8H)-one (21): The title compound **21** was obtained from the reaction of trihydroxycoumarin **15** with 2-methyl-3-buten-2-ol as a light yellow solid in 35% yield by following the general procedure C. mp: 160-161 °C; IR (KBr, cm^{-1}): 3400, 2924, 1688, 1568; UV (acetonitrile, λ_{max}): 326 nm; ^{1}H NMR (400 MHz, CDCl$_3$, δ): 5.93 (brs, 2×OH), 3.07 (t, 2H, J = 6.59 Hz, H-10), 2.59 (t, 2H, J = 7.32 Hz, H-1′), 2.52 (s, 3H, C-4 CH$_3$), 1.80 (t, 2H, J = 6.59 Hz, H-9), 1.45-1.43 (m, 2H, H-5′), 1.36 (s, 6H, C(CH$_3$)$_2$), 1.29-1.28 (m, 6H, H-2′-H-4′), 0.86 (t, 3H, J = 6.59 Hz, H-6′); ^{13}C NMR (100.5 MHz, CDCl$_3$, δ): 160.8, 148.9, 138.9, 137.0, 135.0, 129.2, 124.5, 112.1, 108.4, 74.3, 33.1, 31.6, 29.3, 28.4, 27.7, 26.3, 23.3, 22.5, 20.3, 14.0; HRMS: Calculated for C$_{21}$H$_{28}$O$_5$ [M+H]$^+$ 361.2010, found 361.2021.

2-Decyl-5,6-dihydroxy-1,8,8-trimethyl-9,10-dihydropyrano[3,2-f]chromen-3(8H)-one (22): The title compound **22** was obtained from the reaction of trihydroxycoumarin **16** with

2-methyl-3-buten-2-ol as a light yellow solid in 30% yield by following the general procedure C. mp: 150-151°C; IR (KBr, cm^{-1}): 3382, 2920, 1684, 1568; UV (acetonitrile, λ_{max}): 325 nm; ^{1}H NMR (400 MHz, CDCl$_3$, δ): 5.90 (brs, OH), 5.73 (brs, OH), 3.10 (t, 2H, J = 6.22 Hz, H-10), 2.62 (t, 2H, J = 7.32 Hz, H-1'), 2.55 (s, 3H, C-4 CH$_3$), 1.83 (t, 2H, J = 6.22 Hz, H-9), 1.51-1.47 (m, 2H, H-9'), 1.39 (s, 6H, C(CH$_3$)$_2$), 1.30-1.26 (m, 14H, H-2'-H-8'), 0.87 (t, 3H, J = 6.96 Hz, H-10'); ^{13}C NMR (100.5 MHz, CDCl$_3$, δ): 160.8, 148.9, 138.9, 137.0, 134.9, 129.2, 124.7, 112.1, 108.4, 74.4, 33.2, 31.8, 29.7, 29.5, 29.5, 29.2, 28.5, 27.7, 26.4, 23.4, 22.6, 20.3, 14.0; HRMS: Calculated for C$_{25}$H$_{36}$O$_5$ [M+H]$^+$ 417.2636, found 417.2633.

5,6,7-Trimethoxy-4-methyl-2H-chromen-2-one (24): To a mixture of 3,4,5-trimethoxyphenol (1.0 g, 5.42 mmol) in ethyl acetoacetate (5.9 mmol) at 0°C, 5 mL of 70% sulfuric acid (in ethanol) was added drop wise. The reaction mixture was stirred at 25°C for 10-12 h. On completion of the reaction as monitored by TLC (20% ethyl acetate in hexane), the reaction mixture was slowly poured over ice cold water with stirring. The crude product so obtained was filtered, dried and purified by column chromatography (10% ethyl acetate in hexane) as a white crystalline solid (1.05 g, 78%). mp: 114-115°C; IR (KBr, cm^{-1}): 1734, 1708, 1604; UV (acetonitrile, λ_{max}): 316 nm; ^{1}H NMR (400 MHz, CDCl$_3$, δ): 6.61 (s, 1H, H-8), 6.01 (s, 1H, H-3), 3.93, 3.88, 3.82 (3s, 9H, 3 × OMe), 2.53 (s, 3H, C-4 CH$_3$); ^{13}C NMR (100.5 MHz, CDCl$_3$, δ): 160.8, 156.2, 153.5, 151.6, 151.3, 139.2, 112.9, 108.0, 96.2, 61.3, 60.9, 56.1, 22.9; HRMS: Calculated for C$_{13}$H$_{14}$O$_5$ [M+H]$^+$ 251.0914, found 251.0934.

5,6,7-Trihydroxy-4-methyl-2H-chromen-2-one (25): 1.0 g (3.99 mmol) of 5,6,7-trimethoxy-4-methylcoumarin (24) in 10 mL of 30% HBr (in acetic acid) was refluxed for 36 h. On completion of the reaction, the reaction mixture was poured over crushed ice. The resulting 5,6,7-trihydroxy-4-methylcoumarin (25) was filtered and washed with water to get pure product. It was obtained as a reddish brown solid (0.416 g, 50%). mp: 279-280°C; IR (KBr, cm^{-1}): 3346, 1666, 1617; UV (acetonitrile, λ_{max}): 314 nm; ^{1}H NMR (400 MHz, DMSO-d_6,
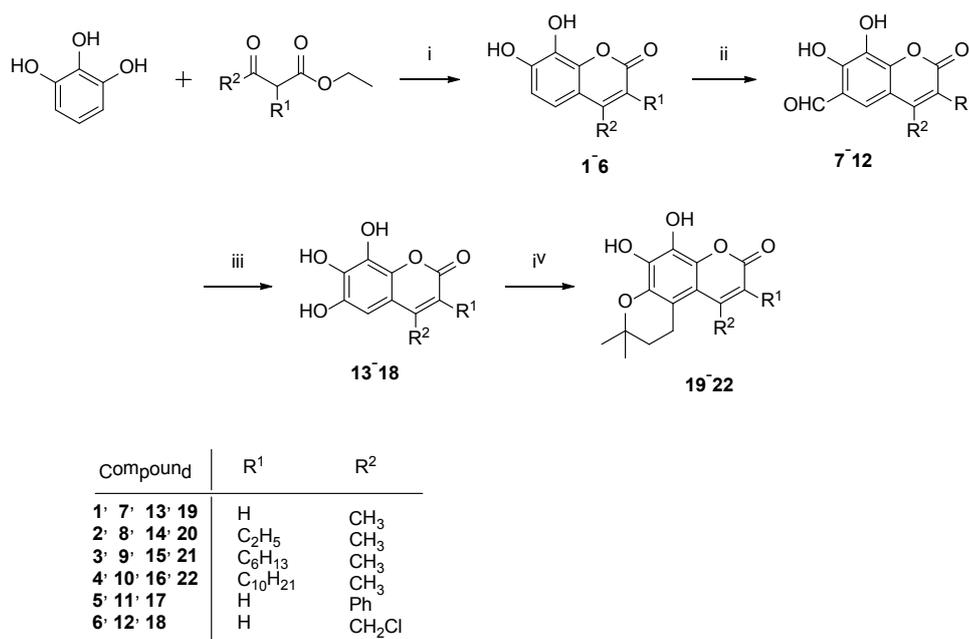
δ): 10.31, 9.30, 8.58 (3s, 3×OH), 6.27 (s, 1H, H-8), 5.84 (s, 1H, H-3), 2.49 (s, 3H, C-4 CH$_3$); ^{13}C NMR (100.5 MHz, DMSO-d_6, δ): 160.3, 154.9, 149.9, 148.2, 145.3, 129.2, 109.3, 102.3, 94.1, 23.3; HRMS: Calculated for C$_{10}$H$_8$O$_5$ [M+H]$^+$ 209.0444, found 209.0442.

Results and Discussion

Chemistry

Trihydroxycoumarins (**13**-**18**) were synthesized from their corresponding dihydroxy precursors **1**-**6**. The dihydroxycoumarins (**1**-**6**) were synthesized from pyrogallol and their corresponding ethyl acetoacetates [20] / ethyl benzoylacetate using Pechmann condensation [21,22] (Scheme 1). Formylation of these compounds (**1**-**6**) using a Duff reaction with hexamine in TFA gave the corresponding formylated product (**7**-**12**) [19]. The reaction was also tried in acetic acid, but the yield of the product (approximately 20%) was found to be very low. The formyl moiety was then converted into a hydroxyl group through Dakin reaction using H$_2$O$_2$/NaOH [19].

The product formation was observed in case of compounds **7** and **8** in the absence of a phase transfer catalyst unlike the compounds **9** and **10**, where, TBAHS (tetrabutylammonium hydrogensulphate) is required due to lower aqueous dispersibility of the reactants having the long alkyl chain at C-3 position. TBAHS was found to be a better phase transfer catalyst with the yield of product 35%, while a lower conversion (approximately 10%) was observed with the other tetrabutylammonium salts (i.e., TBAI (tetrabutylammonium iodide) and TBAB (tetrabutylammonium bromide)). The trihydroxycoumarins (**13**-**16**) were converted into their corresponding pyrano derivatives (**19**-**22**) by treating them with 2-methyl-3-buten-2-ol in the presence of p-TSA (Scheme 1). A poor yield of the product was obtained by using isoprene in place of 2-methyl-3-buten-2-ol as a reactant. 5,6,7-Trihydroxy-4-methylcoumarin (**25**) was synthesized by demethylation of 5,6,7-trimethoxy-4-methylcoumarin (**24**) in HBr-acetic acid. The compound **24** was prepared by Pechmann

Compound	R^1	R^2
1, 7, 13, 19	H	CH$_3$
2, 8, 14, 20	C$_2$H$_5$	CH$_3$
3, 9, 15, 21	C$_6$H$_{13}$	CH$_3$
4, 10, 16, 22	C$_{10}$H$_{21}$	CH$_3$
5, 11, 17	H	Ph
6, 12, 18	H	CH$_2$Cl

Scheme 1: Reagents and conditions: i. Conc. H$_2$SO$_4$, 25°C, 4-5 h; ii. Hexamine, TFA, reflux, 1 h; iii. 6% H$_2$O$_2$/NaOH, 0°C, 1-2 h; iv. 2-methyl-3-buten-2-ol, p-TSA, toluene, reflux, 24 h.

condensation of 3,4,5-trimethoxyphenol (**23**), obtained from 3,4,5-trimethoxybenzaldehyde by the Baeyer-Villiger oxidation using *m*-CPBA in DCM with subsequent hydrolysis using saturated NaHCO$_3$ solution [23] (Scheme 2).

Antioxidant activity results

The AOA potential of synthesized polyhydroxycoumarins (**1, 7, 13-22, 25**) was evaluated using three *in vitro* assays i.e., DPPH, ABTS, and lipid peroxidation inhibition assay and expressed as TEAC. Antioxidant potential of 7,8-dihydroxy-4-methylcoumarin (**1**) is well explored in the literature [24-27]. The strong AOA of compound **1** is due to the stability of the transient phenoxy radical and the weakening of an O-H bond (Figure 1) [9].

First the effect of incorporation of electron withdrawing and releasing groups on the antioxidant efficacy of 7,8-dihydroxy-4-methylcoumarin (**1**) was explored by carrying its formylation/ hydroxylation at the C-6 position. The DPPH assay is most widely used *in vitro* antioxidant assay [4]. In DPPH assay, the AOA of dihydroxycoumarin (**1**) decreases on formylation, but increases on hydroxylation at the C-6 position, i.e., 6,7,8-trihydroxycoumarin (**13**) has a higher AOA than the 7,8-dihydroxycoumarin **1**. The AOA order is thus observed to be **13**>**1**>**7**. Moreover, AOA of **13** is approximately four-fold as compared to "Trolox". The observance of higher activity could be explained on the basis of electron donating effect of *ortho* OH group, which by lowering the O-H bond dissociation enthalpy of C-7 hydroxyl group, increases the rate of H-atom transfer leading to enhanced antioxidant activity [28,29]. This fact is supported further by the literature report by Thavasi et al. wherein the O-H bond dissociation enthalpy of phenols, catechol, and pyrogallol has been compared [29]. Pyrogallol's middle OH group having two *ortho* hydroxyls was reported to have lower bond dissociation enthalpy. However, the presence of the electron withdrawing formyl group in compound **7** adversely affects the AOA [30]. To study the positional effect of three hydroxyl groups, we compared the AOA of 6,7,8-trihydroxycoumarin (**13**) with an isomeric compound i.e., 5,6,7-trihydroxycoumarin (**25**). Among the two, the AOA of **25** was found to be lower as compared to **13** and "Trolox". This may be due to the more favourable positioning of electron-donating
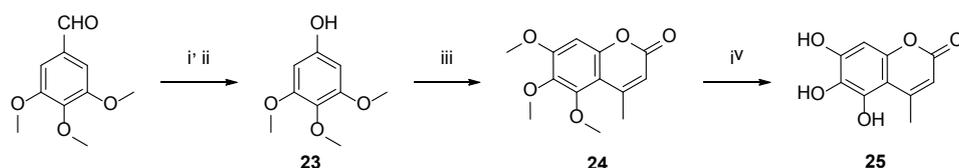
groups for compound **13** as compared to **25**. The presence of hydroxyl groups at C-6 and C-8 position favors the dissociation of C-7 hydroxyl group and the corresponding radical generated is better stabilized due to extended resonance delocalization on benzenoid as well as pyran moiety (Figure 2). While in the case of compound **25** the radical generated by dissociation of C-6 hydroxyl group, due to its middle position, is stabilized only by benzenoid part (Figure 3). To study the effect of hydrophobic group on AOA, 6,7,8-trihydroxycoumarin (**13**) was modified further by incorporating hydrophobic alkyl group at the C-3 position, replacing the C-4 methyl with chloromethyl and phenyl moiety, and by incorporating pyrano ring.

It has been observed that alkyl (ethyl/*n*-hexyl/*n*-decyl) substitution at the C-3 position reduces the AOA in DPPH assays. To evaluate the effect of the C-4 methyl group on AOA of trihydroxycoumarins, the methyl group was replaced with chloromethyl (**18**) and phenyl groups (**17**) and a decrease in AOA was observed in both the cases (Figure 4).

The pyranocoumarins (**19-22**) too were observed to possess lower AOA as compared to the parent trihydroxycoumarins (**13-16**) in DPPH assays and follows the same trend of activity on alkyl substitution i.e., AOA decreases with the increase in size of alkyl chain at C-3 position and following order of activity was observed: **19**≈**20**>**21**>**22** (Figure 4).

In the ABTS assay, an AOA pattern, similar to DPPH assay was observed, i.e., formylation of compound **1** to yield **7** led to a decrease in AOA. Also, hydroxylation at the C-6 position i.e., trihydroxycoumarins (**13-15**) were found to be more active than the dihydroxycoumarin **1** as well as the standard "Trolox". Among the trihydroxycoumarins **13** and **25**, the former was found to have higher antioxidant potential. In this assay too the incorporation of a pyran ring, substitution of alkyl group at the C-3 position, and substitution of CH$_3$ at the C-4 position decreases the AOA efficacy, but still most of the synthesized compounds exhibit higher AOA than the standard (Figure 5).

Lipid is one of the major components of the cell membrane and its peroxidation is directly correlated to peroxidative damage of cells *in vivo*. Hence lipid peroxidation assay (LPO) closely correlates to the true biological assays. In this assay pyrano derivatives of trihydroxycoumarin

Scheme 2: Reagents and conditions: i. *m*-CPBA, DCM, 25°C, 12 h; ii. NaHCO$_3$, H$_2$O; iii. Ethyl acetoacetate, 70% H$_2$SO$_4$ in ethanol, 25°C, 12 h; iv. HBr-acetic acid, reflux, 36 h.
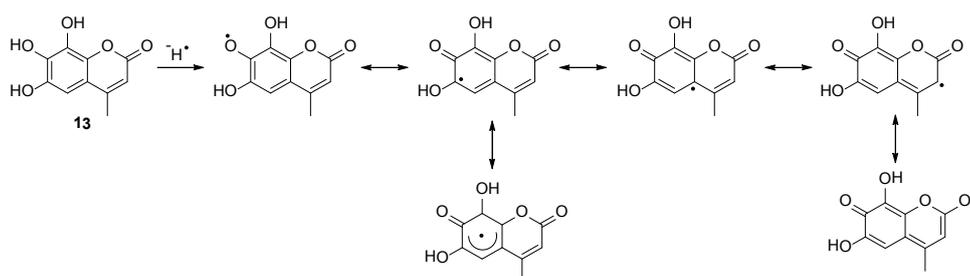
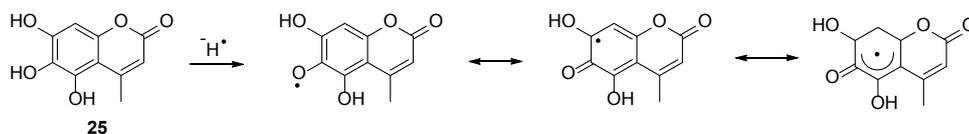**Figure 2:** Resonance stabilization of the C-7 hydroxy radical.

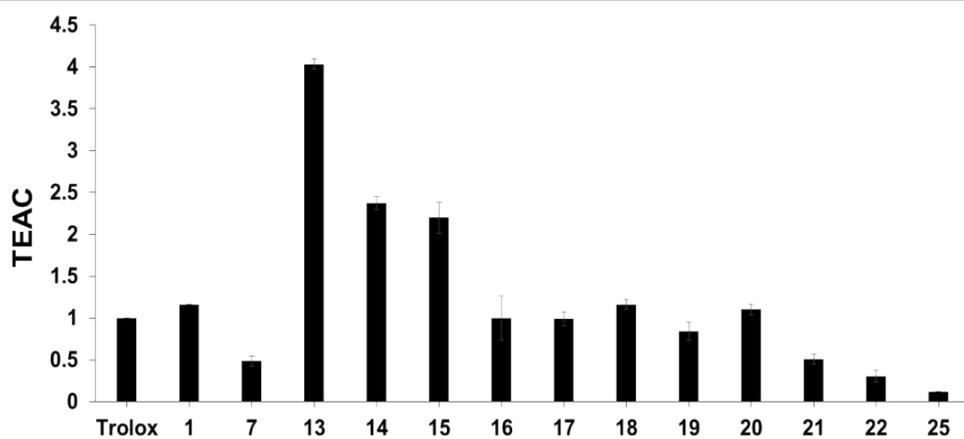**Figure 3:** Resonance stabilization of the C-6 hydroxy radical.

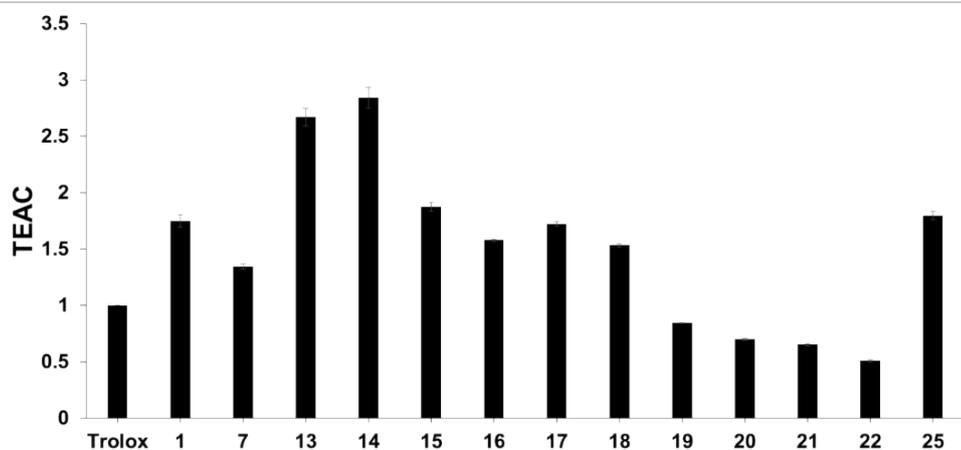**Figure 4:** TEAC of coumarins in DPPH assay.

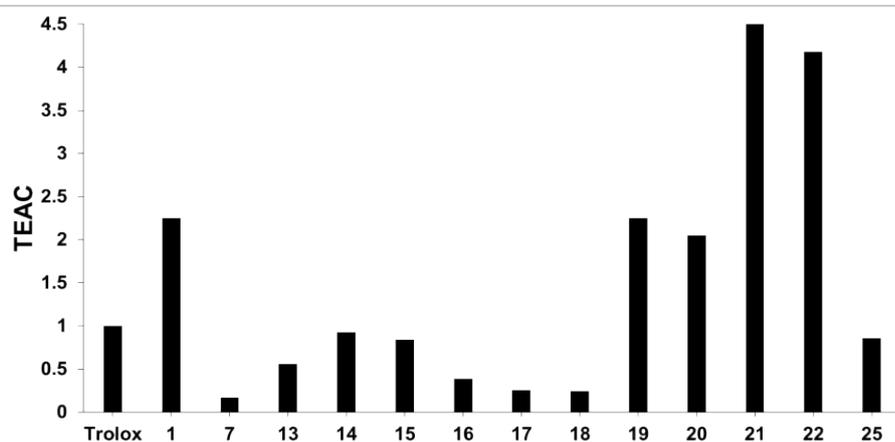**Figure 5:** TEAC of coumarins in ABTS assay.

Figure 6: TEAC of coumarins in LPO assay.

were found to be most active with TEAC value in the range of 2-5, which is higher than their precursors. However, in contrast with the DPPH and ABTS assays the lipophilic groups led to an enhancement in the AOA in the LPO assay. Among the pyranocoumarin derivatives **19-22**, the compound **21** was found to be most active with TEAC approximately 4.5 (Figure 6). The enhancement in AOA with increase in alkyl chain length in LPO assay has been previously observed by our group [12] and Takahashi et al. [31]. These results suggests that the incorporation of the hydrophobic group on the pyranocoumarin enhances the interaction of resulting compound with lipids and thus improves the antioxidant activity.

Although a good correlation is observed between the ABTS and DPPH assays, the corresponding radicals do not exist in biological systems. The ABTS assay is based on the generation of an ABTS radical cation, whereas the DPPH assay uses a radical dissolved in organic media [32]. Oxidizability is a key parameter for AOA evaluation in these two assays. On the other hand, in the LPO assay, in addition to oxidizability, lipophilicity play an additional role and it gives an indication for uptake into the membranes, as the membrane lipids are the target of radical attack. Therefore, the partition coefficients of the coumarins as well as their rates of reaction with the relevant radicals define the antioxidant activities in the lipophilic phase. These facts have been well documented in the literature data, e.g., aglycones are more effective than the corresponding flavonoid glycosides as the sugar moiety is known to mask the antioxidant activity of a flavonoid, probably preventing its access to the lipid membranes [33].

In our experiments, a fundamental requirement for the expression of antioxidant activity appears clearly to be, together with their redox properties, the ability to interact with biomembranes. Though both (HAT and SET) mechanisms are possible for the antioxidant activity, but the electron delocalization stabilization as proposed in this study suggest the HAT mechanism to be a dominant factor for the AOA of coumarin derivatives. In conclusion, the antioxidant activity of coumarins appears to be dictated not only by their structural features, but also by their location in the membrane. This result must be taken into consideration in further developments of these protective antioxidants, which could have important applications in human diseases accompanied by free radical injury.

Conclusions

Thirteen polyphenolic coumarin derivatives were synthesized and characterized from their physical and spectroscopic data. Out of thirteen compounds eight, i.e., **14-16**, **18-22** are reported for the first time. The coumarins synthesized were evaluated for their AOA by using three *in vitro* antioxidant assays (DPPH, ABTS, and LPO). Trihydroxycoumarins were observed to be potent antioxidants. The presence of C-3 alkyl substituent reduces the AOA of trihydroxycoumarins and replacement of C-4 methyl with CH_2Cl/Ph group also exhibits a negative effect on AOA in two assays (DPPH, ABTS). Incorporation of the pyran ring also lowers the AOA in the above two assays. However, an increase in lipophilicity enhances the antioxidant potential in the LPO inhibition assay. A few of these compounds exhibited more than four-fold AOA as compared to standard "Trolox". Since the lipid peroxidation system more closely resembles the biological system than the other two assays, this study may provide useful information for the design and development of antioxidants for a specific purpose.

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