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Synthesis of novel benzocoumarin derivatives as lipid lowering agents

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ABSTRACT

A series of novel benzocoumarin derivatives were synthesized and evaluated for their in vivo anti-dyslipidemic and in vitro antioxidant activities. Preliminary screening indicates compound **4** as potential lead with significant lipid lowering and antioxidant activities. The study revealed that such attempts on benzocoumarin-based pharmacophores which is a biologically important scaffold might result in identification of new lead for anti-dyslipidemia.

Numerous compounds with biological and pharmacological activities have been investigated, however, many of them are not suitable for therapeutic use due to their toxicity. A rational approach is to make modifications of active chemical structures, in order to synthesize compounds with improved therapeutic activity and reduced toxicity. Coumarins are an elite class of oxygen heterocycles which occupy a special role in nature. Coumarins and their derivatives have attracted intense interest in recent years because of their diverse pharmacological properties.[1–6] In addition, many coumarin derivatives have the special ability to scavenge reactive oxygen species (ROS) and to influence processes involving free radical injury.[7] Furthermore, 7-hydroxy coumarin and its derivatives are shown to have lipid lowering potential.[8,9] The coumarins are extremely variable in structure, due to the various types of substitutions in their basic structure, which in turn can influence their biological activity. Figure 1 underscores the importance of some representative structures of compounds incorporating coumarin nucleus present in therapeutically utilized products.[10]

Atherosclerosis is the major cause of heart disease, stroke, and death in both developed and developing countries. It is well established that elevated blood lipid levels constitute the primary risk factor for atherosclerosis.[11] Epidemiological studies have indicated that dyslipidemia and coagulation disturbances are among the most significant risk factors of the development of atherosclerotic condition.[11]

Current pharmacological treatment of atherosclerosis includes the use of the statin class HMG-CoA reductase inhibitors and the fibrate class PPARα agonists. Yet they are not effective in lowering triglycerides (TG) and low-density lipoprotein (LDL). Most patients still experience adverse coronary events despite statin therapy, in addition, recent reports of undesirable side effects (myopathy) of some 'super statins' indicate that the idea of improving the potency of this class of drugs may not outweigh the increase in side effects.[12] The fibrate class of drugs, which are mostly used to treat hypertriglyceridemia and low HDL cholesterol, requires high doses to show significant efficacy.[13] In addition, a combination of fibrate and statins has met with serious safety concerns as exemplified by the withdrawal of Cerivastatin in 2001. Therefore, there is a need for a different class of compounds to treat dyslipidemia without severe side effects.

Oxidative stress has recently been implicated in the pathogenesis of various diseases such as diabetes and CAD. The involvement of hydroxyl free radicals has been found to be a major causative factor for the peroxidative damage to lipoproteins present in the blood, which are responsible for the initiation and progression of atherosclerosis in the hyperlipidemic subjects.[14] Therefore, it is envisaged that, if a chemical compound has both cholesterol lowering and hypolipidemic properties it will be able to protect endothelial and myocardial function as well as serve as a better anti-atherosclerotic agent.

Recently, we have reported the synthesis and biological evaluation of benzocoumarin derivatives with potential antioxidant and lipid lowering activities.[15] The result prompted us to use the benzocoumarins as a molecular template and an active pharmacophore for further diversification. Based on our hybrid struc-

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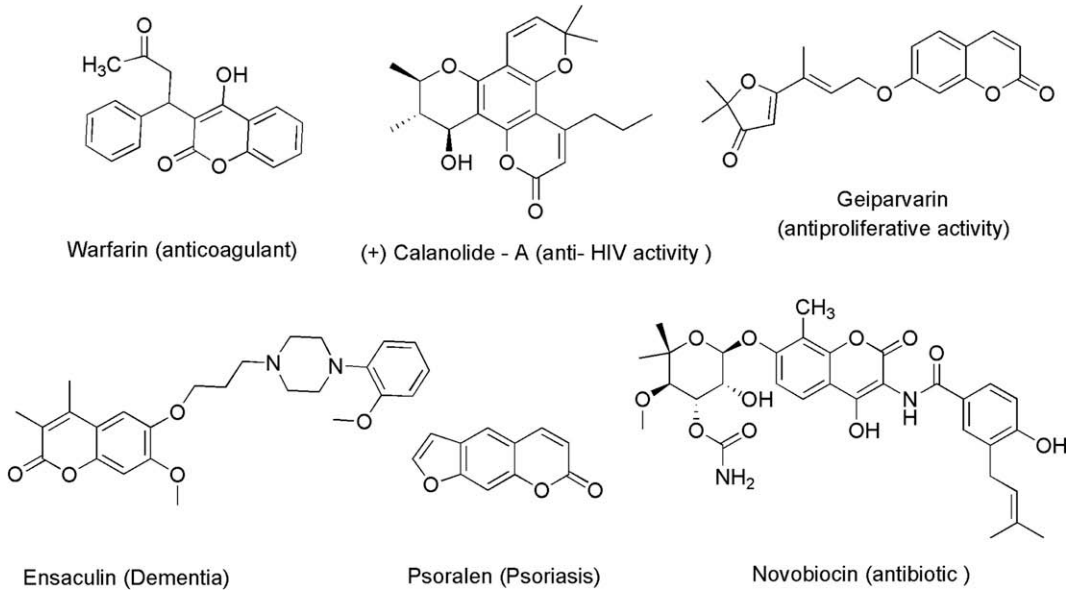
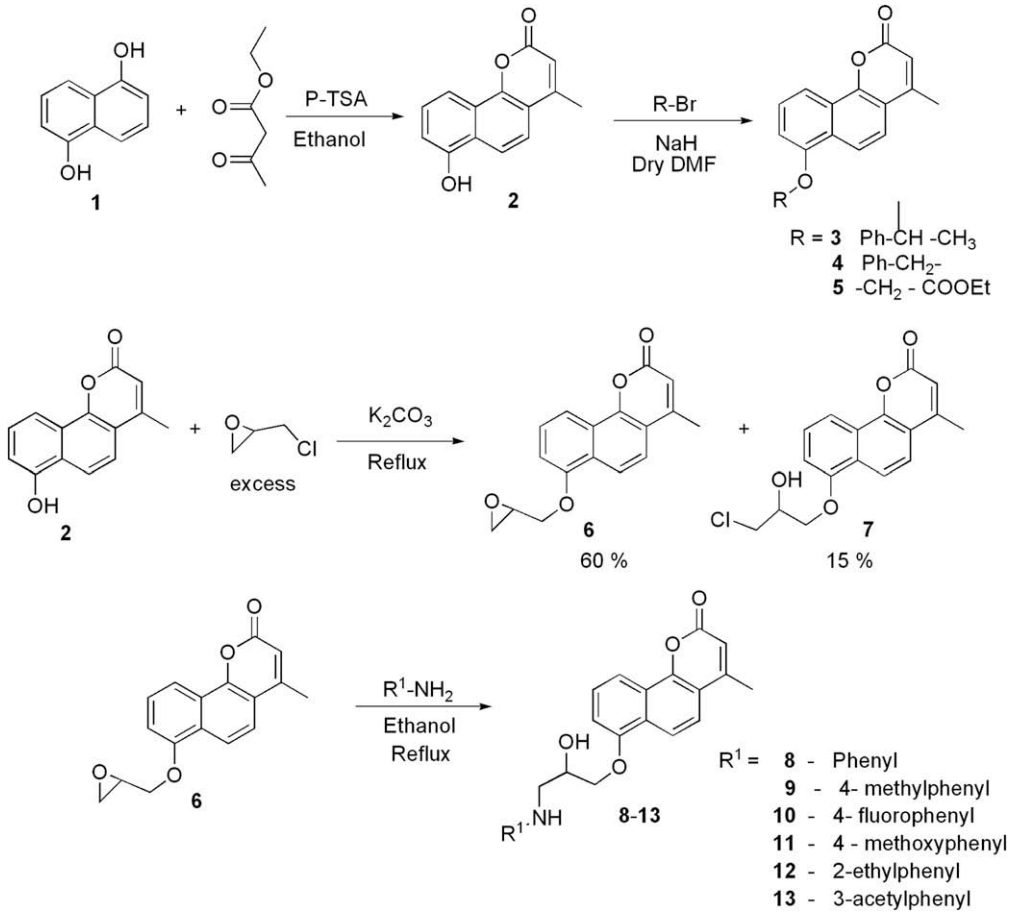


Figure 1. Coumarin-containing biologically potent molecules.

ture of novel benzocoumarins, we set out to conduct an SAR study towards the identification of a new anti-dyslipidemic agent with antioxidant activity. Thus, a series of amino alcohol derivatives of benzocoumarins were synthesized from nucleophilic opening of oxirane with different amines, furnishing a variety of 2-hydroxy amino derivatives (**8**–**13**) is presented in Scheme 1. In addition, we also synthesized simple alkyl substituted benzocoumarins (**3**–**5**) and all the derivatives were evaluated for potential lipid lowering activity, in vivo, and antioxidant activity, in vitro.



Scheme 1. Synthesis of novel benzocoumarin derivatives **3**–**13**.

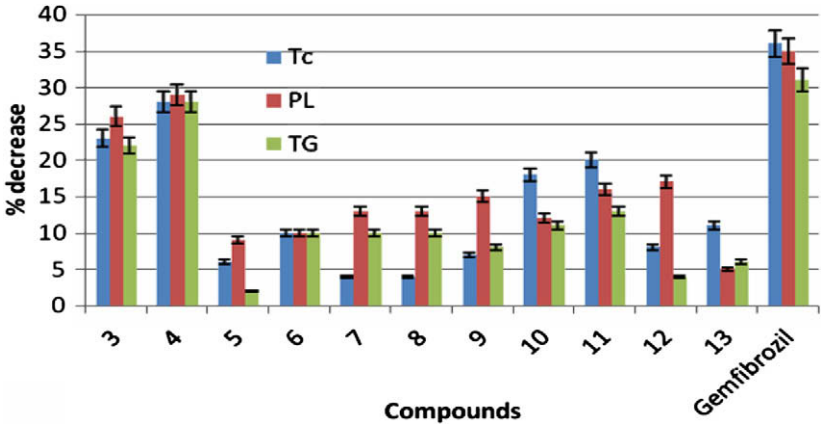


Figure 2. The lipid lowering activity of novel benzocoumarin derivatives (100 mg/kg) in Triton treated hyperlipidemic rats. Triton treated group is compared with control and drug treated group is compared with triton group (units-mg/dL). Values are mean ± SD of six animals.

Using the Pechmann reaction the benzocoumarin **2** was prepared as previously reported.[15] The alkyl substituted compounds (**3**–**5**) were prepared by reacting the phenoxide of **2** with different bromoalkanes in DMF at room temperature for 1–2 h. Alternatively, the synthesis of amino alcohol derivatives (**8**–**13**) was accomplished from the nucleophilic opening of oxirane **6** with different anilines, which, in turn was synthesized by reacting **2** with excess of epichlorohydrin in presence of K_2CO_3 under reflux conditions (Scheme 1). Compound **7** was obtained as a side product in low yield in the preparation of compound **6**. All compounds were characterized using ^{1}H NMR, ^{13}C NMR, mass spectrometry and IR spectroscopy.[16] The purity of these compounds was ascertained by TLC and spectral analysis.

The anti-dyslipidemic and post heparin lipolytic activity of benzocoumarin derivatives (**3**–**13**) were evaluated in an in vivo Triton model.[14,17,18] Administration of Triton WR–1339 in rats induced marked hyperlipidemia as evidenced by increase in the plasma level of total cholesterol TC (2.74-fold), phospholipids PL (2.99-fold) and triglyceride TG (3.36-fold). Triton induced rats caused inhibition of post heparin lipolytic activity plasma PHLA (−24%) as compared to control.[19] Treatment of hyperlipidemic rats with benzocoumarin derivatives at the dose of 100 mg/kg po reversed the plasma levels of lipid to varying extents.[20] Compound **3** significantly lowered the TC, PL, and TG by 23%, 26%, 22%. However, compound **4** was the most potent in the series and showed 28%, 29%, and 28% lowering in TC, PL, and TG, respectively, while compounds **5**–**13** showed mild activity (Fig. 2). These data are comparable with standard drug Gemfibrozil at the dose of 100 mg/kg which decreased level of TC, PL, and TG in plasma by 36%, 35%, and 31%, respectively. In PHLA enzyme activity, only compound **4** showed significant reversal of PHLA in plasma of hyperlipidemic rats by 18%, however, Gemfibrozil causes 24% reversal of activity of this enzyme as compared to the control group.

In a separate experiment, antioxidant activities of compounds **3**–**13** at 100 μg/mL were evaluated by generating free radicals [superoxide ions ($O_2^{\cdot}$), hydroxyl radicals ($^{\cdot}$OH), microsomal lipid peroxidation] in vitro in the absence and presence of these compounds.[21–23] The results of this study are shown in Figure 3. The

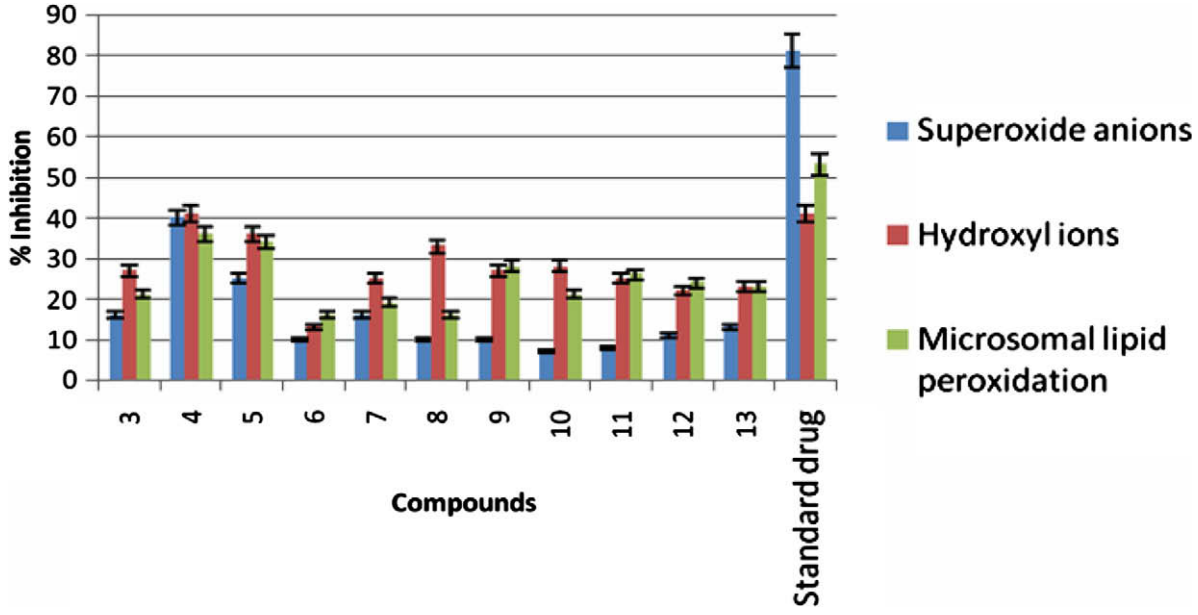


Figure 3. The effect of novel benzocoumarin derivatives (100 μg/mL) on superoxide ion (nmol formazone formed/min), hydroxyl ion (nmol MDA formed/h) and lipid peroxidation in microsomes (nmol MDA formed/mg protein) is shown (standard drugs for superoxide anions-alloperinol (20 μg/mL), hydroxyl ions-mannitol and for microsomal lipid peroxidation-α-tocopherol (100 μg/mL) were used). Values are mean ± SD of six animals.

synthesized compounds **4** and **5** exhibited significant decrease in superoxide ions inhibition by 40% and 28%, hydroxyl radicals inhibition by 41% and 38% and microsomal lipid peroxidation inhibition by 38% and 37%, respectively. The standard drug Alloperinol, at 20 μg/mL, showed 86% inhibition in superoxide ions. Mannitol and α-tocopherol, at the same dose, showed 48% and 45% inhibition of hydroxyl ions and microsomal lipid peroxidation, respectively. The scavenging potential of the other derivatives was modest at best. The wide variation in the free radical scavenging potential may be due to the variation in the proton–electron transfer by the derivatives due to difference in their structures and stability. As propensity of radical formation and stabilization, ability of metal complexation, and lipophilicity are important factors for the antioxidant activity.

In terms of SAR, the benzocoumarin derivatives (**8**–**13**) were found to be inactive maybe due to low lipophilicity, while the simple alkyl substituted benzocoumarins (**3**–**5**) exhibited interesting activity. The most promising was compound **4**, which incorporates an unbranched benzyl group. This compound showed good activity in both lipid lowering and antioxidant experiments. Initial studies indicate compound **4** to be devoid of cytotoxicity in normal cells. Also, when evaluated for dose response activity, at a lower dose of (25 mg/ kg) exhibited 26%, 24%, and 23% lowering in TC, PL, and TG, respectively, and further dose optimization studies are underway. Further studies on **4** are in progress and will be published in the future.

In conclusion, a series of novel alkyl substituted benzocoumarin derivatives (**3**–**5**) have been synthesized from 7-hydroxy-4-methylbenzo[*h*]chromen-2-one (**2**) and the amino alcohol derivatives of benzocoumarins (**8**–**13**) were synthesized from nucleophilic opening of the oxirane moiety with different anilines. These newly synthesized compounds were evaluated for their anti-dyslipidemic and antioxidant activities. Compound **4** exhibited significant dual lipid lowering and antioxidant activity and lead optimization is underway.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.103.

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16. *General procedure for the synthesis of compounds*: *Synthesis of 4-methyl-7-(1-phenyl-ethoxy)-benzo[h]chromen-2-one* (**3**): To a solution of compound **2** (2 g, 8.85 mmol) in dry DMF (50 mL) was added NaH (0.42 g, 17.6 mmol) at 0–5 °C and the reaction mixture was allowed to stir for 25–30 min. 1-Bromo-ethylbenzene (1.63 g, 8.85 mmol) was then added to the reaction mixture and stirring continued at room temperature for 1.5–2 h. After completion of the reaction, excess DMF was removed under reduced pressure and the residue was diluted with water and extracted with $CHCl_3$ (15 mL × 3). The organic layer was washed with water and dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography over silica (230–400 mesh) and elution with 4% ethyl acetate in hexane furnished the pure compound **3**. This was obtained in 65% yield as white solid. Mp 142–143 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.3 (d, J = 8.8 Hz, 1H), 8.08 (d, J = 8.8 Hz, 1H), 7.61 (d, J = 9.3 Hz, 1H), 7.45–7.31 (m, 6H), 6.85 (d, J = 7.9 Hz, 1H), 6.38 (s, 1H), 5.55 (q, J = 6.1 Hz, 1H), 2.55 (s, 3H), 1.8 (d, J = 6.6 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 159.7, 152.1, 151.9, 149.0, 141.4, 127.5, 126.4, 126.0, 124.1, 123.0, 118.1, 117.3, 114.3, 113.2, 113.1, 108.5, 23.2, 17.9; ESI (m/z) 331 $[M+H]^+$.
Following the above procedure, compounds **4**–**5** have been synthesized.
7-Benzyloxy-4-methyl-benzo[h]chromen-2-one (**4**): This was obtained in 68% yield as white solid. Mp 176–177 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.24–8.17 (m, 2H), 7.61–7.39 (m, 7H), 7.09 (d, J = 7.8 Hz, 1H), 6.4 (s, 1H), 5.28 (s, 2H), 2.55 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 159.6, 152.9, 152.1, 149.0, 135.2, 127.4, 126.8, 126.1, 126.0, 125.7, 123.0, 118.2, 117.2, 114.4, 113.6, 113.3, 106.8, 69.2, 17.9; ESI (m/z) 316 $[M]^+$.
(4-Methyl-2-oxo-2H-benzo[h]chromen-7-yloxy)-acetic acid ethyl ester (**5**): This was obtained in 62% yield as white solid. Mp 148–149 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.27–8.2 (m, 2H), 7.63 (d, J = 8.7 Hz, 1H), 7.53 (t, J = 7.8 Hz, 1H), 6.9 (d, J = 7.8 Hz, 1H), 6.41 (s, 1H), 4.84 (s, 2H), 4.33 (q, J = 7.4 Hz, 2H), 2.57 (s, 3H), 1.34 (t, J = 7.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 168.9, 161.3, 153.9, 153.7, 127.5, 127.3, 124.8, 120.2, 118.9, 116.2, 115.2, 115.1, 108.3, 66.1, 62.1, 19.8, 14.5; ESI (m/z) 313 $[M+H]^+$.
Synthesis of 4-methyl-7-oxiranylmethoxy-benzo[h]chromen-2-one (**6**): The compound **2** (2.0 g, 8.85 mmol) and anhydrous K_2CO_3 (1.83 g, 13.3 mmol) were taken in epichlorohydrin (75 mL) and refluxed for 1 h. After completion of the reaction, K_2CO_3 was filtered off and epichlorohydrin was removed in vacuo. The residue was diluted with water and extracted with $CHCl_3$ (75 mL × 3). The organic layer was washed with water, brine, and dried over anhydrous Na_2SO_4. Column chromatography over silica gel (100–200 mesh) and elution with 30% ethyl acetate in hexane furnished the epoxide **6**.
Compound **7** was obtained as a side product.
This was obtained in 60% yield as white solid. Mp 142–143 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.12–8.08 (m, 2H), 7.53–7.46 (m, 2H), 6.95 (d, J = 8.1 Hz, 1H), 6.35 (s, 1H), 4.46 (dd, J = 2.9, 11.1 Hz, 1H), 4.11 (dd, J = 5.8, 11.1 Hz, 1H), 3.53–3.48 (m, 1H), 3.0 (t, J = 4.4 Hz, 1H), 2.87–2.85 (m, 1H), 2.5 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 159.6, 152.7, 152.1, 148.9, 125.9, 125.4, 122.9, 118.2, 116.9, 114.3, 113.9, 113.2, 106.4, 68.1, 48.9, 43.4, 17.8; ESI (m/z) 283 $[M+H]^+$.
7-(3-Chloro-2-hydroxy-propoxy)-4-methyl-benzo[h]chromen-2-one (**7**): This was obtained in 15% yield as white solid. Mp 153–154 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.15 (d, J = 8.6 Hz, 1H), 7.94 (d, J = 8.3 Hz, 1H), 7.76 (d, J = 9.1 Hz, 1H), 7.64 (t, J = 8.3 Hz, 1H), 7.24 (d, J = 7.8 Hz, 1H), 6.53 (s, 1H), 5.77 (s, 1H), 4.27 (s, 3H), 3.97–3.85 (m, 2H), 2.56 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.0, 154.4, 154.3, 149.8, 128.3, 126.4, 123.7, 120.9, 118.3, 115.9, 114.5, 114.1, 108.5, 70.0, 69.0, 47.1, 19.0; ESI (m/z) 319 $[M+H]^+$.
7-(2-Hydroxy-3-phenylamino-propoxy)-4-methyl-benzo[h]chromen-2-one (**8**): The compound **6** (0.2 g, 0.70 mmol) and aniline (0.99 g, 1.06 mmol) were dissolved in ethanol (15 mL) and refluxed for 5 h. After completion of the reaction excess ethanol was removed through high vacuo and the residue was diluted with water and extracted with ethyl acetate. The organic layer was washed with water, brine, and dried over anhydrous Na_2SO_4. Column chromatography over silica gel (100–200 mesh) and elution with 50% chloroform in hexane furnished the final product **8**.
This was obtained in 75% yield as white solid. Mp 164–165 °C; IR (KBr, cm^{-1}): 3427, 1714; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.17 (d, J = 9.1 Hz, 1H), 7.91 (d, J = 8.1 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.6 (t, J = 8.4 Hz, 1H), 7.17 (d, J = 7.7 Hz, 1H), 7.06 (t, J = 7.7 Hz, 2H), 6.65 (d, J = 8.4 Hz, 2H), 6.54–6.51 (m, 2H), 5.66 (t, J = 5.2 Hz, 1H), 5.38 (d, J = 4.4 Hz, 1H), 4.23–4.16 (m, 3H), 3.25–3.22 (m, 1H), 2.5 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.1, 160.1, 154.6, 149.8, 149.2, 129.3, 128.3, 126.5, 123.7, 120.7, 118.4, 116.2, 115.9, 114.4, 113.8, 112.6, 108.5, 71.4, 68.0, 46.7, 19.1; ESI (m/z) 376 $[M+H]^+$.
Following the above procedure, compounds **9**–**13** have been synthesized.
7-(2-Hydroxy-3-p-tolylamino-propoxy)-4-methyl-benzo[h]chromen-2-one (**9**): This was obtained in 78% yield as white solid. Mp 177–178 °C; IR (KBr, cm^{-1}): 3420, 1717; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.14 (d, J = 7.1 Hz, 1H), 7.88 (d, J = 6.3 Hz, 1H), 7.74 (d, J = 7.1 Hz, 1H), 7.57 (t, J = 6.3 Hz, 1H), 7.15 (d, J = 5.5 Hz, 1H), 6.87 (d, J = 5.5 Hz, 2H), 6.55 (d, J = 6.3 Hz, 2H), 6.48 (s, 1H), 5.37–5.31 (m, 2H), 4.21–4.14 (m, 3H), 3.18–3.16 (m, 1H), 2.51 (s, 3H), 2.14 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 158.8, 153.3, 153.3, 148.6, 145.7, 128.5, 127.1,

125.3, 123.3, 122.4, 119.5, 117.1, 114.6, 113.2, 112.6, 111.5, 107.2, 70.2, 66.9, 45.7, 19.2, 17.8; ESI (*m*/*z*) 390 $[M+H]^+$.

7-[3-(4-Fluoro-phenylamino)-2-hydroxy-propoxy]-4-methyl-benzo[h]chromen-2-one (**10**): This was obtained in 72% yield as white solid. Mp 178–179 °C; IR (KBr, cm^{-1}): 3454, 1700; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.17 (d, J = 9.2 Hz, 1H), 7.91 (d, J = 8.9 Hz, 1H), 7.79 (d, J = 9.2 Hz, 1H), 7.6 (t, J = 7.6 Hz, 1H), 7.17 (d, J = 7.9 Hz, 1H), 6.9 (t, J = 9.4 Hz, 2H), 6.66–6.61 (m, 2H), 6.52 (s, 1H), 5.61 (t, J = 6.1 Hz, 1H), 5.39 (d, J = 4.6 Hz, 1H), 4.24–4.15 (m, 3H), 3.21–3.13 (m, 1H), 2.54 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.1, 154.6, 149.8, 146.0, 128.3, 126.5, 123.7, 120.7, 118.4, 115.9, 115.8, 115.5, 114.5, 113.9, 113.4, 113.3, 108.5, 71.3, 67.9, 47.2, 19.0; ESI (*m*/*z*) 394 $[M+H]^+$.

7-[2-Hydroxy-3-(4-methoxy-phenylamino)-propoxy]-4-methyl-benzo[h]chromen-2 one (**11**): This was obtained in 82% yield as white solid. Mp 178–179 °C; IR (KBr, cm^{-1}): 3428, 1720; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.19 (d, J = 9.3 Hz, 1H), 8.04 (d, J = 8.4 Hz, 1H), 7.63–7.49 (m, 2H), 7.04 (d, J = 7.9 Hz, 1H), 6.78–6.69 (m, 4H), 6.38 (s, 1H), 4.36–4.24 (m, 5H), 3.72 (s, 3H), 3.49 (dd, J = 4.7, 13.1 Hz, 1H), 2.54 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.1, 154.6, 154.5, 151.2, 149.9, 143.5, 128.3, 126.6, 123.7, 120.7, 118.4, 115.9, 115.1, 114.5, 113.8, 113.7, 108.5, 71.4, 68.0, 55.8, 47.6, 19.1; ESI (*m*/*z*) 406 $[M+H]^+$.

7-[3-(2-Ethyl-phenylamino)-2-hydroxy-propoxy]-4-methyl-benzo[h]chromen-2-one (**12**): This was obtained in 85% yield as white solid. Mp 176–177 °C; IR (KBr, cm^{-1}): 3427, 1705; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.19 (d, J = 9.5 Hz, 1H), 7.91 (d, J = 8.9 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 7.6 (t, J = 7.5 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H), 7.02–6.96 (m, 2H), 6.64 (d, J = 7.5 Hz, 1H), 6.58–6.52 (m, 2H), 5.46 (d, J = 5.0 Hz, 1H), 4.91 (t, J = 6.0 Hz, 1H), 4.25–4.17 (m, 3H), 3.44–3.28 (m, 1H), 2.54–2.46 (m, 5H), 1.14 (t, J = 7.8 Hz, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.1, 154.6, 154.5, 149.9, 146.1, 128.3, 128.2, 127.9, 127.1, 126.5, 123.7, 120.7, 118.4, 116.6, 115.9, 114.5, 113.9, 110.1, 108.5, 71.6, 67.7, 47.0, 23.8, 19.1, 13.6; ESI (*m*/*z*) 404 $[M+H]^+$.

7-[3-(3-Acetyl-phenylamino)-2-hydroxy-propoxy]-4-methyl-benzo[h]chromen-2-one (**13**): This was obtained in 75% yield as white solid. Mp 152–153 °C; IR (KBr, cm^{-1}): 3396, 1702; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.21 (d, J = 8.6 Hz, 1H), 7.92 (d, J = 8.3 Hz, 1H), 7.79 (d, J = 9.2 Hz, 1H), 7.61 (t, J = 7.9 Hz, 1H), 7.2–7.13 (m, 4H), 6.92 (d, J = 8.0 Hz, 1H), 6.52 (s, 1H), 6.02 (t, J = 4.5 Hz, 1H), 5.43 (d, J = 5.4 Hz, 1H), 4.26–4.17 (m, 3H), 3.44 (m, 1H), 2.54–2.46 (m, 6H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 198.8, 160.1, 154.6, 154.5, 149.9, 149.5, 138.2, 129.6, 128.3, 126.6, 123.8, 120.8, 118.5, 117.3, 116.4, 116.0, 114.5, 113.9, 111.3, 108.5, 71.4, 67.9, 46.5, 27.2, 19.1; ESI (*m*/*z*) 418 $[M+H]^+$.

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20. *Lipid lowering activity*: Adult male Charles Foster rats (200–225 g) bred in the animal house of the institute were used for the lipid lowering activity. Rats were divided in control, triton induced, triton plus compounds and Gemfibrozil (100 mg/kg) treated groups containing six rats in each. Hyperlipidemia was developed by administration of Triton WR-1339 (Sigma chemical Co., St. Louis, USA) at a dose of 400 mg/kg body wt. intraperitoneally to animals of all groups except the control. Compounds **3**–**13** were macerated with gum acacia (0.2% w/v), suspended in water and fed simultaneously with triton at a dose of 100 mg/kg po to the animals of treated groups. Animals of the control and triton group without treatment with test compounds were given same amount of gum acacia suspension (vehicle). After 18 h of treatment (50 mg/kg body wt.) 1.0 mL blood was withdrawn from retro-orbital sinus using glass capillary in EDTA coated eppendorf tube (3.0 mg/mL blood). The blood was centrifuged (at 2500*g*) at 4 °C for 10 min and the plasma was separated. Plasma was diluted with normal saline (ratio 1:3) and used for analysis of total cholesterol (TC), phospholipids (PL), triglycerides (Tg) and post heparin lipolytic activity (PHLA) using spectrophotometer, Beckmann auto-analyzer and standard kits purchased from Beckmann Coulter International, USA.
21. *Antioxidant activity (generation of free radicals)*: Super oxide anions were generated enzymatically by xanthine (160 mM), xanthine oxidase (0.04 U), and nitroblue tetrazolium (320 μM) in absence or presence of compounds **3**–**13** (100 μg/mL) in 100 mM phosphate buffer (pH 8.2). Fractions were sonicated well in phosphate buffer before use. The reaction mixtures were incubated at 37 °C and after 30 min the reaction was stopped by adding 0.5 mL glacial acetic acid. The amount of formazone formed was calculated spectrophotometrically. In another set of experiment effect of compounds on the generation of hydroxyl radical was also studied by non-enzymatic reactants. Briefly, OH^- were generated in a non-enzymatic system comprising deoxy ribose (2.8 mM), $FeSO_4{\cdot}7H_2O$ (2 mM), sodium ascorbate (2.0 mM) and H_2O_2 (2.8 mM) in 50 mM KH_2PO_4 buffer (pH 7.4) to a final volume of 2.5 mL. The above reaction mixtures in the absence or presence of test compounds (100 μg/mL and 200 μg/mL) were incubated at 37 °C for 90 min. The test compounds were also studied for their inhibitory action against microsomal lipid peroxidation in vitro by non-enzymatic inducer. Reference tubes and reagents blanks were also run simultaneously. Malondialdehyde (MDA) contents in both experimental and reference tubes were estimated spectrophotometrically by thiobarbituric acid as mentioned above. Allopurinol, mannitol and α-tocopherol were used as standard drugs for superoxide, hydroxylations and microsomal lipid peroxidation. All experimental data were analyzed using Student's *t*-test. Oxidized LDL was compared with the test compounds treated oxidized LDL. The generation of oxygen free radicals was compared in the presence and absence of test compounds. The hyperlipidemic group was compared with control and hyperlipidemic plus drug treated groups P <0.05 was considered to be significant.
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