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Synthesis, biological evaluation and molecular docking studies of stellatin derivatives as cyclooxygenase (COX-1, COX-2) inhibitors and anti-inflammatory agents

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ABSTRACT

Stellatin (**4**), isolated from *Dysophylla stellata* is a cyclooxygenase (COX) inhibitor. The present study reports the synthesis and biological evaluation of new stellatin derivatives for COX-1, COX-2 inhibitory and anti-inflammatory activities. Eight derivatives showed more pronounced COX-2 inhibition than stellatin and, **17** and **21** exhibited the highest COX-2 inhibition. They also exhibited the significant anti-inflammatory activity in TPA-induced mouse ear edema assay and their anti-inflammatory effects were more than that of stellatin and indomethacin at 0.5 mg/ear. The derivatives were further evaluated for antioxidant activity wherein **16** and **17** showed potent free radical scavenging activity against DPPH and ABTS radicals. Molecular docking study revealed the binding orientations of stellatin and its derivatives into the active sites of COX-1 and COX-2 and thereby helps to design the potent inhibitors.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are of huge therapeutic benefit in the treatment of rheumatoid arthritis and various types of inflammatory conditions. The target for these drugs is cyclooxygenase (COX), a rate limiting enzyme which converts arachidonic acid into inflammatory prostaglandins. COX exists in two isoforms, COX-1 and COX-2. COX-1 is known as a housekeeping enzyme and constitutively expressed in all tissues, while COX-2 is constitutively expressed only in kidney, brain and ovaries. COX-2 is increasingly expressed during inflammatory conditions by pro-inflammatory molecules such as IL-1, TNF-α, LPS and TPA.[1–3]

Chromones constitute an important class of natural products and are reported to exhibit anti-inflammatory,[4–6] anti-arthritic,[6] anti-cancer,[7] immune-stimulation,[7] anti-platelet,[8] uricosuric,[6] anti-allergic,[6] anti-bacterial[9] and antioxidant[4] activities. As anti-inflammatory agents, chromones exert their effects by inhibiting various mechanisms such as COX-1 and COX-2 enzymes,[4] NO production,[7] PKC[10] and O_2^- production in PMA- or f-MLF-stimulated human neutrophils.[10] The structures of some naturally occurring anti-inflammatory chromones, aloesin (**1**)[4], norcimicifugin (**2**)[11], petersinone 1 (**3**)[7], stellatin (**4**)[12], eugenin (**5**)[12] and 5,7-dimethoxy-2-methylchromanone (**6**)[12] are shown in Figure 1.

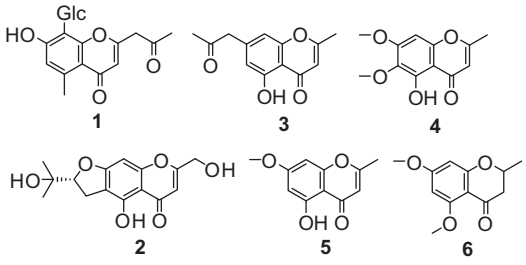

Figure 1. Natural anti-inflammatory chromones of plant origin.

As a part of our continuing program to discover COX-1 and COX-2 inhibitory compounds from Indian medicinal plants,[12–14] the *n*-hexane and EtOAc extracts of *Dysophylla stellata* (Labiatae) were tested in a COX catalyzed prostaglandin biosynthesis assay in vitro and a significant COX-1 and COX-2 inhibitory activity was observed. The chromones **4**–**6** were isolated as the potent COX inhibitory principles and **4** exhibited selectivity towards COX-2 inhibition than COX-1.[12] The HPLC analysis of *D. stellata* revealed **4** as a major constituent[15] and it was concluded that it could be responsible for COX inhibitory activity of *D. stellata*. These findings prompted us to synthesize the derivatives of **4** so as to study the structure–activity relationship (SAR).

The chemical modifications of **4** were carried out at 5-OH to introduce the different functionality. The 5-O-alkylation was

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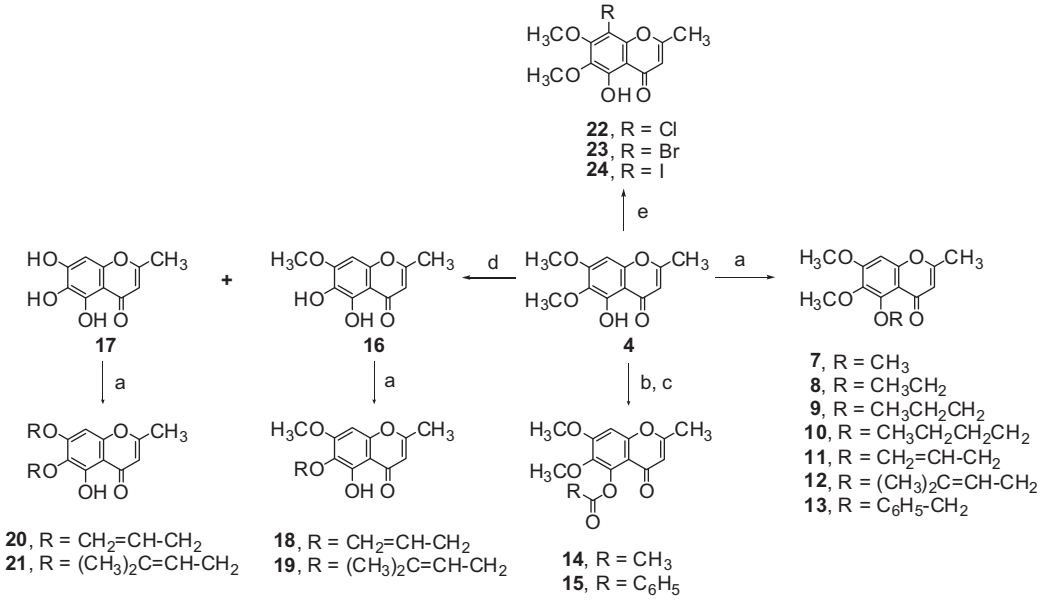

Scheme 1. Reagent and conditions: (a) $K_2CO_3/(CH_3)_2CO$, RX, reflux, 12–24 h, 35–85%; (b) $(CH_3CO)_2O/CH_3COONa$, reflux, 2 h, 75%; (c) $K_2CO_3/(CH_3)_2CO$, PhCOBr, reflux, 24 h, 64%; (d) $AlCl_3/C_7H_8$, reflux, 12 h, 30–70%; (e) NCS (**22**), NBS (**23**), NIS (**24**)/ACN, rt, 4–24 h, 20–75%.

carried out with alkylating agents using anhydrous K_2CO_3 in acetone[16] to furnish compounds **7**–**13** (Scheme 1) in good yields (65–85%), except **10** (35%). The reaction of **4** with $(CH_3CO)_2O$ and anhydrous CH_3COONa afforded **14** in a fairly good yield (75%)[17] while **15** was synthesized in the similar way as *O*-alkyl derivatives. To introduce the free OH, demethylation was performed with anhydrous $AlCl_3$ in toluene.[18] The reaction of **4** with $AlCl_3$ for 2 h resulted in the formation of **16** in a high yield (80%), but, when the reaction time was extended to 12 h, **16** (70%) and **17** (20%) both were formed. Ethers **18**–**21** were synthesized from **16** and **17** respectively, using K_2CO_3 and alkyl bromides in high yields (80–90%). The reaction of **16** with alkyl bromide resulted in the regioselective monoalkylation at 6-position (**18**–**19**). Similarly, the regioselective dialkylation at 6-and 7-positions was observed in **20**, **21**. The reaction of **4** with NCS/NBS/NIS in acetonitrile gave **22**–**24** wherein regioselective halogenation was observed at 8-position. Of the synthetic derivatives, **8**–**13**, **15**, **18** and **20**–**24** were found as new compounds when they were searched on Scifinder and Reaxys databases (The spectral, MS and elemental data of new compounds are submitted as Supplementary data).

The synthesized chromones (**7**–**24**) were evaluated for in vitro COX-1 and COX-2 inhibitory activity in a COX catalyzed prostaglandin biosynthesis assay as described by us previously.[12,13] Initially, the compounds were tested at 30 μM and their effects on COX-1 and COX-2 inhibition are shown in Table 1. The study was further extended to examine the concentration-activity responses at different concentrations to determine the IC_{50} values for COX-1 and COX-2. Five compounds (**16**, **17** and **19**–**21**) showed better COX-1 inhibitory activity than stellatin with IC_{50} values in the range of 14–22 μM; whereas seven compounds (**12** and **16**–**21**) exhibited more COX-2 inhibitory activity than that of stellatin (IC_{50s}, 8–18 μM). Compound **17** (5,6,7-trihydroxy-2-methylchromone) exhibited the highest COX-2 inhibition (IC_{50} 8.87 μM) followed by **21** (IC_{50} 10.35 μM). The COX-2 inhibitory effect of **17** was >2-fold as compared to stellatin (IC_{50} 19.7 μM). The results of the present study suggests that chromones acts as non-selective inhibitors of COX enzymes and showed anti-inflammatory effects.

In case of ether derivatives, the introduction of a chain length up to four carbons (**7**–**11**) and a benzyl group (**13**) resulted in a decreased COX-1 and COX-2 inhibitory activity; however, an isoprenyl group at 5-position (as in **12**) did not alter the COX-1 inhibition whereas it showed improved COX-2 inhibition. The conversion of OH into an ester (**14** and **15**) also decreased the COX-1 and COX-2 inhibitory activity. The conversion of methoxyl groups of **4** into the hydroxyl groups improved both COX-1 and COX-2 inhibitory activity (**16** and **17**) with more pronounced effect towards COX-2 inhibition. Eugenin (**5**), an isolated chromone with one methoxyl group less than stellatin, showed better COX-1 inhibition than stellatin without having much effect on COX-2. The replacement of free OH groups at 6- and 7-position by an allyl or isoprenyl groups (**18**–**21**) improved the COX-1 and COX-2 inhibitory activity (except **18**, in case of COX-1). The halogenated derivatives (**22**–**24**) did not show 'noticeable' effect on COX inhibition as their inhibition was even less than that of stellatin.

The reports on SAR of flavonoids (2-phenylchromones) suggested that 4-oxo functional group of the C-ring is essential for COX inhibitory activity; the C_2–C_3 double bond enhances the activity and reduction of this double bond results in decreased or loss of inhibitory activity.[19,20] The similar trend was also observed in the case of the studied chromones and inhibition was seemed to depend on number and position of hydroxyl residues. The presence of 5-OH is essential for COX inhibitory activity, however, isoprenyl group seems to be ideal replacement. The presence of 6-OH further increased the COX-1 and COX-2 inhibitory activity. Therefore, the SAR study helped us to establish a basic pharmacophore (Fig. 2) responsible for the activity. A chromone with C_2–C_3 double bond, a carbonyl group at 4-position of A-ring and a 5-OH on B-ring are essential features for the COX inhibitory activity.

Ten compounds (**11**, **12** and **16**–**23**) were evaluated further for anti-inflammatory in a TPA-induced mouse ear edema assay as described earlier by us.[12,13] All the compounds showed significant anti-inflammatory activity. Compound **17** exhibited the highest anti-inflammatory activity with 90.7% reduction in the ear edema followed by **21** (85.1% reduction). The anti-inflammatory effects of **17** and **21** were more than that of stellatin (69.4%) and indomethacin (81.4%) at the same dose levels. Compounds **12**, **16**, **19** and **20** showed anti-inflammatory activity comparable to indomethacin (Table 1) and their anti-inflammatory effects were in order: **20** > **16** > **19** > **12**.

Table 1
COX-1, COX-2 inhibitory and anti-inflammatory activities of the synthesized chromones

Compound	COX inhibition at 30 μM[a]		(IC_{50}, μM)		Anti-inflammatory activity[b]		
	COX-1	COX-2	COX-1	COX-2	Dose (mg/ear)	Increase in ear thickness (mm)	% reduction in ear edema
Control	—	—	—	—	—	0.270 ± 0.008	—
4	71.50 ± 0.32	80.15 ± 0.06	22.3	19.7	0.5	0.082 ± 0.009*	69.44 ± 3.50
5	85.59 ± 0.10	78.50 ± 0.29	17.8	20.1	0.5	0.071 ± 0.004*	73.20 ± 1.78
7	70.35 ± 0.09	47.42 ± 1.12	—	—	—	—	—
8	54.85 ± 1.33	49.75 ± 0.76	—	—	—	—	—
9	40.16 ± 0.13	48.35 ± 0.87	—	—	—	—	—
10	28.45 ± 2.09	52.45 ± 0.33	—	—	—	—	—
11	56.18 ± 0.32	73.87 ± 1.33	38.28	21.25	0.5	0.090 ± 0.012*	66.66 ± 4.69
12	70.09 ± 0.77	82.54 ± 0.19	22.87	17.64	0.5	0.061 ± 0.005*	77.16 ± 2.16
13	63.41 ± 1.66	48.13 ± 0.09	—	—	—	—	—
14	59.02 ± 0.12	46.22 ± 0.56	—	—	—	—	—
15	45.84 ± 2.33	68.14 ± 0.10	—	—	—	—	—
16	78.10 ± 0.87	84.99 ± 0.33	19.75	15.35	0.5	0.058 ± 0.006*	78.40 ± 2.44
17	87.45 ± 1.08	94.12 ± 0.66	14.38	8.87	0.5	0.025 ± 0.006**	90.74 ± 2.28
18	68.33 ± 1.05	81.53 ± 0.45	26.04	18.01	0.5	0.077 ± 0.004*	71.30 ± 2.53
19	80.67 ± 0.34	83.23 ± 2.08	18.67	15.49	0.5	0.060 ± 0.004*	77.77 ± 1.30
20	74.09 ± 0.63	89.54 ± 0.88	21.57	13.54	0.5	0.053 ± 0.008**	80.24 ± 3.09
21	82.33 ± 1.12	91.67 ± 0.34	17.17	10.35	0.5	0.040 ± 0.007**	85.18 ± 2.61
22	66.85 ± 0.66	50.32 ± 1.89	27.18	43.41	0.5	0.105 ± 0.010*	61.11 ± 3.33
23	63.76 ± 0.90	67.03 ± 1.33	29.32	25.42	0.5	0.080 ± 0.007*	70.37 ± 2.26
24	57.21 ± 2.33	37.70 ± 0.76	—	—	—	—	—
Curcumin[c]	59.01 ± 1.76	20.32 ± 2.08	35.09	79.20	—	—	—
Indomethacin[c]	98.23 ± 0.33	50.99 ± 0.34	0.18	—	0.5	0.050 ± 0.005**	81.48 ± 2.15
Celecoxib[c]	13.01 ± 0.63	95.57 ± 0.48	—	0.15	—	—	—

[a] Values are expressed as mean ± S.E.M of three determinations ($n = 3$).
[b] Six animals per group and data is expressed as mean ± S.E.M of six determinations ($n = 6$).
[c] Positive controls used.
* $p < 0.05$ versus control (Dunnett's t-test).
** $p < 0.01$ versus control (Dunnett's t-test).

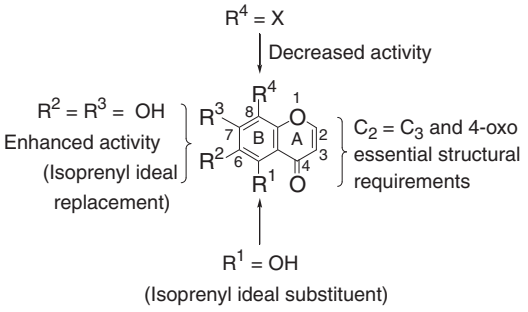

Figure 2. Structure–activity relationship of chromones.

It has been reported that in inflammatory disorders, excessive free radical generation takes place and several NSAIDs and phenolic compounds with anti-inflammatory activity are reported to act as radical scavengers.[21] Therefore, antioxidant property of the synthesized chromones was evaluated using DPPH and ABTS radical scavenging in vitro assays.[12–14] The compounds were tested at 30 μM and it was observed that except **16** and **17**, all other compounds were weakly active and unable to scavenge >10% DPPH radical. No improvement in the radical scavenging property was observed even when their concentration was increased up to 100 μM. Compound **16** (IC_{50}, 13.15 μM) and **17** (IC_{50}, 7.96 μM) strongly scavenged the DPPH radical and the scavenging effect of **17** was about 2-times more than that of trolox (IC_{50}, 15.72 μM) and better than curcumin (IC_{50}, 12.12 μM). The same trend of the antioxidant activity was also observed in the ABTS assay. Compound **16** (IC_{50}, 14.36 μM) and **17** (IC_{50}, 10.60 μM) showed strong ABTS radical scavenging activity and their effects were comparable (**16**) or better (**17**) than those of trolox (IC_{50}, 16.23 μM) and curcumin (IC_{50}, 13.08 μM).

The COX-1, COX-2 inhibitory and significant anti-inflammatory activity of **4** and its derivatives (**11**, **12** and **16**–**23**) prompted us to perform molecular docking studies to understand the ligand-protein interactions, and COX-1/COX-2 selectivity in detail. The docking studies were carried out as per the method described previously by us.[14] The crystal structures of COX-1 (1EQG)[22] and COX-2 (1PXX)[23] complexed with ibuprofen and diclofenac respectively were used for docking. FlexX, an automated docking program, was used to dock these compounds into the active sites of COX-1 and COX-2 enzyme and the most stable conformation based on the best FlexX scoring function was selected. All the calculations were performed using SYBYL7.1[24] software installed on SGI workstation. The active site of the enzyme was defined to include residues within 6.5 Å radius around bound inhibitor. The energy minimization was carried out using Tripos force field and the partial charges were calculated using the same method. The complexes were minimized in a stepwise manner till the gradient convergence 0.05 kcal/mol was reached. The distance dependent dielectric function ($\varepsilon = 4r$) was used. The FlexX successfully docked **4** and its derivatives into the active sites of COX-1 and COX-2 enzyme and the total scores of their complexes are given as a Supplementary data (in Table).

The compound **17** was found to dock into the active site of COX-1 with interaction energy of −14.66 kcal/mol. It formed three hydrogen bonds with Tyr385, Met522 and Ser530 into the active site of COX-1 and bonding distance between 5-OH of **17** and OH of Tyr385 2.62 Å (H···O); 4-C=O of **17** and OH of Ser530 2.58 Å (H···O); 7-OH of **17** and C=O of Met522 1.88 Å (H···O) were observed. The ring A and B of **17** were surrounded by the active site amino acid residues Ser121, Leu123, Val349, Tyr385, Met522 and Ser530 (Fig. 3a). The compound **17** could dock successfully into the COX-2 active site as well. The binding energy of interaction obtained for **17** (−17.74 kcal/mol) was found to be highest. The greater interaction energy of **17** and COX-2 enzyme complex rationalizes the tighter binding of **17** into the COX-2 active site and this binding may be attributed to its three hydrogen bonding interactions with Met522 and Ser530. The bonding distance be-

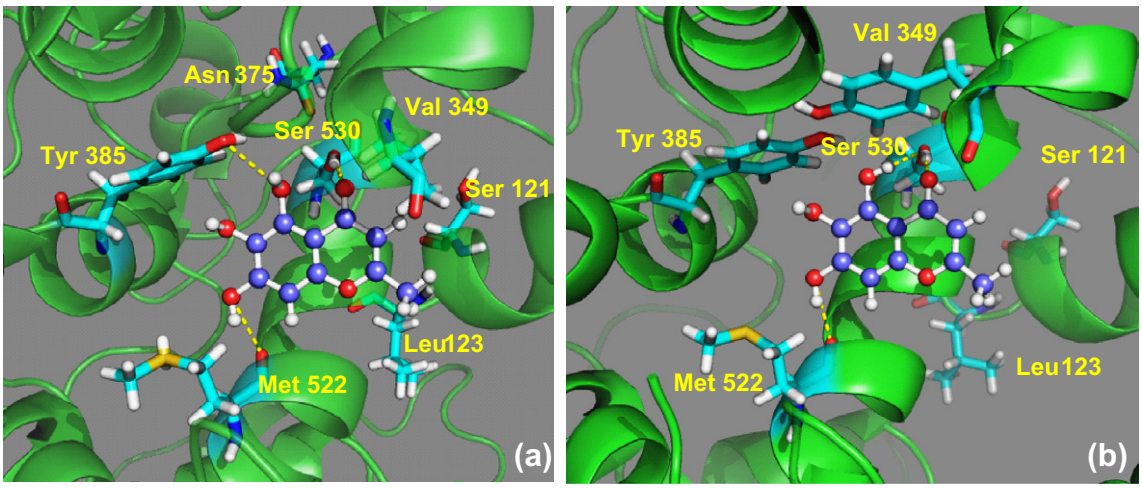

Figure 3. Binding of compound **17** at the active site of COX-1 (a) and COX-2 (b).

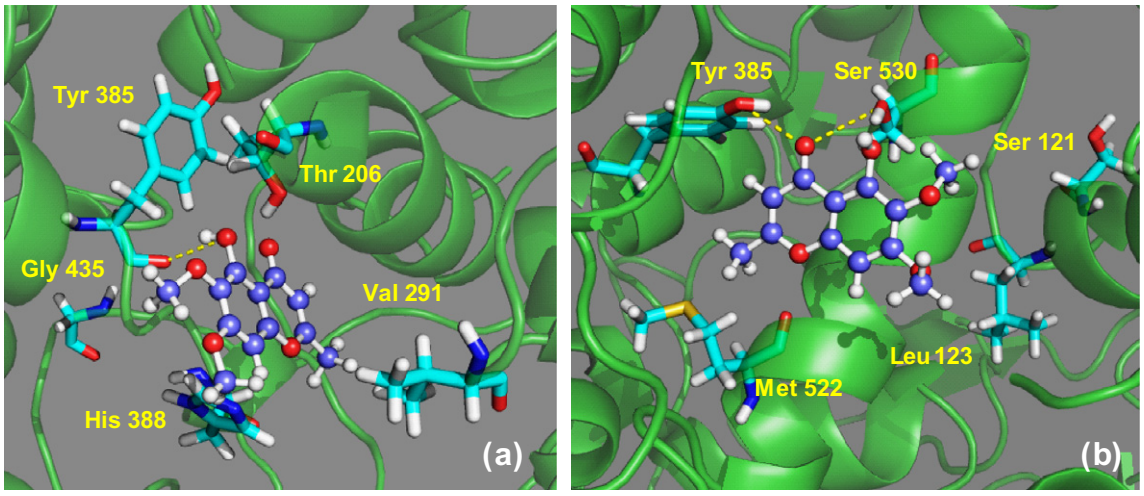

Figure 4. Binding of stellatin (**4**) at the active site of COX-1 (a) and COX-2 (b).

tween 7-OH of **17** with O of Met522 1.99 Å (H...O); 5-OH of **17** and OH of Ser530 1.88 Å (H···O) and 4-C═O of **17** and OH of Ser530 1.98 Å (H···O) of COX-2 were observed. The 6-OH, 5-OH, 4-C═O and 2-Me of **17** were surrounded by the active site amino acid residues Gly526, Ser530, Ser530 and Leu352, respectively (Fig. 3b).

The compound **4** was docked into the active site of COX-1 and the interaction energy of −10.69 kcal/mol was obtained. Only one hydrogen bonding interaction between the 5-OH of **4** and C═O of Tyr385 (H···O, 2.13 Å) of COX-1 was observed (Fig. 4a). Compound **4** was also found to dock into the active site of COX-2 with interaction energy of −12.88 kcal/mol. Two hydrogen bonding interactions of **4** were observed with the bonding distances as follow: 4-C═O of **4** and OH of Ser530 (O···H, 1.88 Å); 4-C═O of **4** and OH of Try385 of COX-2 (O···H, 2.13 Å). The 2-Me and 7-OMe of **4** was surrounded by active site amino acid residues Leu123, Met522 and Tyr385 (Fig. 4b).

The interaction with amino acid Ser530 is important for enzyme inhibitory activity and is well exemplified by the binding interaction of aspirin with COX-1/COX-2.[25] Tyr385 is responsible for the abstraction of 13-pro-*S*-hydrogen from arachidonic acid.[26] In case of complex of **4** with COX-1, the hydrogen bonding interaction with Ser530 was found to be absent, which could be a probable reason for the observed selectivity of **4** towards COX-2 inhibition. Further, the scoring function of **4** and **17** complexes with COX-2 suggests them as the preferred ligand for COX-2 than COX-1 and provides rationale for selectivity of enzyme inhibitory activity. While it was noted that both COX-1 and COX-2 share almost similar active site residues and difference lies in its active site volume (COX2 has larger active site volume- 417 $Å^3$; while COX-1 has smaller active site volume-366 $Å^3$) respectively, determined using Q-SiteFinder program.[27] It further shows that inhibitors of COX-1 can equally inhibit COX-2, thus assuming equipotency, while the larger sized molecule seems to be more selective towards COX-2 due to its increased active site volume. It is also important to note that the mode of binding of compound **17** in COX-1 and COX-2 is slightly different due to its difference in the active site volumes (Fig. 3), and the selectivity issue was further supported by our experimental analysis showing better inhibitory activity of 8.87 μM with COX-2 (Table 1).

In conclusion, eighteen stellatin derivatives including thirteen new compounds were synthesized and seven compounds (**12** and **16**–**21**) showed more pronounced COX-2 inhibition than naturally occurring chromone, stellatin. The SAR study revealed that a chromone skeleton with C_2–C_3 double bond, C═O at 4-position and OH at 5-position are essential features for COX inhibition. Two compounds (**17** and **21**) exhibited greater anti-inflammatory activity than indomethacin and two compounds (**16** and **17**) showed the potent radical scavenging activity as an antioxidant agent. Molec-

ular docking study further helped in supporting the observed COX-2 selectivity. The findings of the study inferred that the dual functioning of **17** as a COX inhibitor and antioxidant agent render it as a lead molecule for further development of new anti-inflammatory agents.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.116.

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16. General procedure for the synthesis of O-alkylated chromones (**7**–**13** and **18**–**21**): To a stirred solution of **4**/**16**/**17** (0.42 mmol) in dry acetone (5–8 mL), K_2CO_3 (0.84 mmol) and an appropriate alkylating agent (0.84 mmol) were added and the resulting mixture was refluxed at 70 °C for 12–24 h. The reaction was stopped by quenching with water and the aqueous portion was extracted with EtOAc (3 × 25 mL). The organic layer was separated, washed with brine solution and dried over anhydrous sodium sulphate. The solvent was removed under vacuum and the crude product was purified by Si gel CC using hexane–EtOAc as an eluent.
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