Bis-coumarins; non-cytotoxic selective urease inhibitors and antiglycation agents

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ABSTRACT

The current study is concerned with the identification of lead molecules based on the *bis*-coumarin scaffold having selective urease inhibitory and antiglycation activities. For that purpose, *bis*-coumarins (**1-44**) were synthesized and structurally characterized by different spectroscopic techniques. Eight derivatives **4**, **8-10**, **14**, **17**, **34**, and **40** demonstrated urease inhibition in the range of $IC_{50} = 4.4 \pm 0.21–115.6 \pm 2.13\,\mu M$, as compared to standard thiourea ($IC_{50} = 21.3 \pm 1.3\,\mu M$). Especially, compound **17** ($IC_{50} = 4.4 \pm 0.21\,\mu M$) was found to be five-fold more potent than the standard. Kinetic studies were also performed on compound **17** in order to identify the mechanism of inhibition. Kinetic studies revealed that compound **17** is a competitive inhibitor. Antiglycation activity was evaluated using glycation of bovine serum albumin by methylglyoxal *in vitro*. Compounds **2**, **11-13**, **16**, **17**, **19–22**, **35**, **37**, and **42** showed good to moderate antiglycation activities with IC_{50} values of 333.63–919.72 µM, as compared to the standard rutin ($IC_{50} = 294.46 \pm 1.5\,\mu M$). Results of both assays showed that the compounds with urease inhibitory activity did not show any antiglycation potential, and *vice versa*. Only compound **17** showed dual inhibition potential. All compounds were also evaluated for cytotoxicity. Compounds **17**, **19**, and **37** showed a weak toxicity towards 3 T3 mouse fibroblast cell line. All other compounds were found to be non-cytotoxic. Urease inhibition is an approach to treat infections caused by ureolytic bacteria whereas inhibition of glycation of proteins is a strategy to avoid late diabetic complications. Therefore, these compounds may serve as leads for further research.

1. Introduction

Bis-coumarins are biologically active pharmacophores, initially isolated from natural sources [1,2]. Several biological activities are associated with *bis*-coumarins, such as *α*-glucosidase [3], urease [4], nucleotide pyrophosphatases-1 [5], and DNA polymerase *β*-lyase inhibitory activities [6]. *Bis*-coumarins are also reported to possess anticoagulant, and hemorrhagic properties [7]. However, there is still a need to explore this class for a wide spectrum of pharmacological activities.

Urease (amidohydrolase EC 3.5.1.5) is a metalloenzyme, contains nickel in its active site. It catalyzes the hydrolysis of urea into carbon dioxide and ammonia [8,9]. This enzyme synthesizes by numerous plants, animals, bacteria, and other organisms [10]. Hyperactivity of urease is harmful to human and animal health, as well as for the agricultural sector. Urease is a key virulence in the pathogenesis of urolithiasis, urinary catheter encrustation, pyelonephritis, hepatic coma, and hepatic encephalopathy [11,12]. It also participates in the pathologies caused by ureolytic bacteria *Helicobacter pylori* (HP). It facilitates bacteria to survive in stomach at acidic pH during initial colonization. Therefore, it plays an important role in the pathologies of ulcers (gastric and peptic), and cancer [13-16]. In the agriculture

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sector, hyperactivity of urease leads to considerable economic and environmental damage by liberating aberrantly large quantities of ammonia into the atmosphere, during the process of urea fertilization [17]. Therefore, it is important to develop strategies based on urease inhibition to solve the problems caused by urease producing bacteria.

Glycation is a non-enzymatic reaction in which reducing sugars non-enzymatically bind with the amino terminal of proteins *via* a nucleophilic addition reaction, ultimately giving rise to advanced glycation end products (AGEs). Haemoglobin, serum albumin, collagen, elastin, and crystalline are common proteins that undergo glycation. Changes in their structures and functions lead to different abnormalities such as atherosclerosis, neuropathy, diabetic retinopathy, diabetic nephropathy, *etc*. This process is cumulatively called glycation stress [18,19]. In this process, reactive intermediates such as methylglyoxal (MG) are more prone to bind with amino groups as compared to their carbohydrate precursors. Eighty percent (80%) of blood proteins is serum albumin, which is more likely to be glycated [20]. As a result of complex rearrangements, substitution, and addition reactions of glycated proteins, AGEs are produced in the body which change the functions of proteins, and accumulate with time in different tissues [21-23]. Many late diabetic complications, such as retinopathy, nephropathy, cataracts, atherosclerosis, and osteoporosis are due to the glycation of vital proteins, and accumulation of AGEs [24]. The inhibition of glycation process plays a pivotal role in the prevention of many late diabetic complications. Therefore, it is important to find inhibitors for glycation.

Bis-coumarins have not yet been reported for their antiglycation activity. Fig. 1 showed that chromone ring, a positional isomer of coumarin, is the main scaffold of rutin which encouraged us to evaluate the compounds **1–44** for their antiglycation activity. Furthermore, we have previously reported *bis*-coumarins for urease inhibitory activity [4]. New members of this series were thus evaluated to identify more potent urease inhibitors [Fig. 1]. In brief, forty-four derivatives were synthesized and evaluated for their urease inhibitory, and antiglycation activities. After knowing the selective potential of compounds, cytotoxicity was also checked. To the best of our knowledge, except compounds **6**, **14**, **16**, **17**, **19**, **20**, **23–26**, **28**, and **30–32** [25-29], the rest of the compounds were identified as new.

2. Results and discussion

2.1. Chemistry

Bis-coumarin derivatives **1–44** were synthesized by reacting 6-fluoro-4-hydroxy, 4-hydroxy, and 6-chloro-4-hydroxy coumarins with a variety of benzaldehydes in the presence of tetraethylammonium bromide (TEAB) as a catalyst. Reactions were performed in distilled water (Scheme 1) and checked periodically by TLC analysis. Precipitates of products were obtained in good yields (Table 1). Compounds were structurally identified by various spectroscopic analyses such as ^{1}H- and ^{13}C NMR as well as FAB-, ESI-, and HRESI-MS.

2.2. Characteristic spectral features of representative compound 21

Structure elucidation of a new compound **21** is presented here as an example. ^{1}H- and ^{13}C NMR spectra of compound **21** were recorded in DMSO-d_6. Characteristic signal of methine proton resonated as a singlet at δ_H 5.60, also confirmed the formation of the *bis*-coumarin scaffold. Amongst the protons of coumarin nucleus, H-7 and H-7′ resonated at δ_H 7.53 as triplet, and showed *ortho* coupling (t, $J_{7,6/7',6'} = J_{7,8/7',8'} = 6.9$ Hz) with adjacent protons, H-6/H-6′ and H-8/H-8′. Similarly, H-6 and H-6′ appeared as a triplet at δ_H 7.26, and also showed *ortho* coupling (t, $J_{6,5/6',5'} = J_{6,7/6',7'} = 7.5$ Hz) with neighbouring protons. H-5 and H-5′ appeared as the most downfield signal at δ_H 7.82 with *ortho* coupling (d, $J_{5,6/5',6'} = 6.3$ Hz) with the adjacent H-6 and H-6′. H-8 and H-8′ appeared at δ_H 7.27, *ortho* coupled (d, $J_{8,7/8',7'} = 8.1$ Hz) with H-7 and H-7′. H-2″ and H-6″ of ring R_2 resonated as a broad singlet at δ_H 7.12 (Fig. 2).

Total 16 signals of carbon (eleven methine and fourteen quaternary carbons) appeared in ^{13}C NMR broad-band decoupled spectrum (DMSO-d_6). Most downfield signal of C-2 and C-2′, which are the quaternary carbons of lactone moiety, appeared at δ_C 167.2. Similarly, other quaternary C-10/C-10′, and C-4/C-4′ resonated at δ_C 164.2 and 152.4 as the downfield signals due to the adjacent electronegative oxygen atom. An upfield characteristic signal resonated at δ_C 35.0, corresponding to the methine CH carbon.

The structure of compound **21** was further confirmed with the help

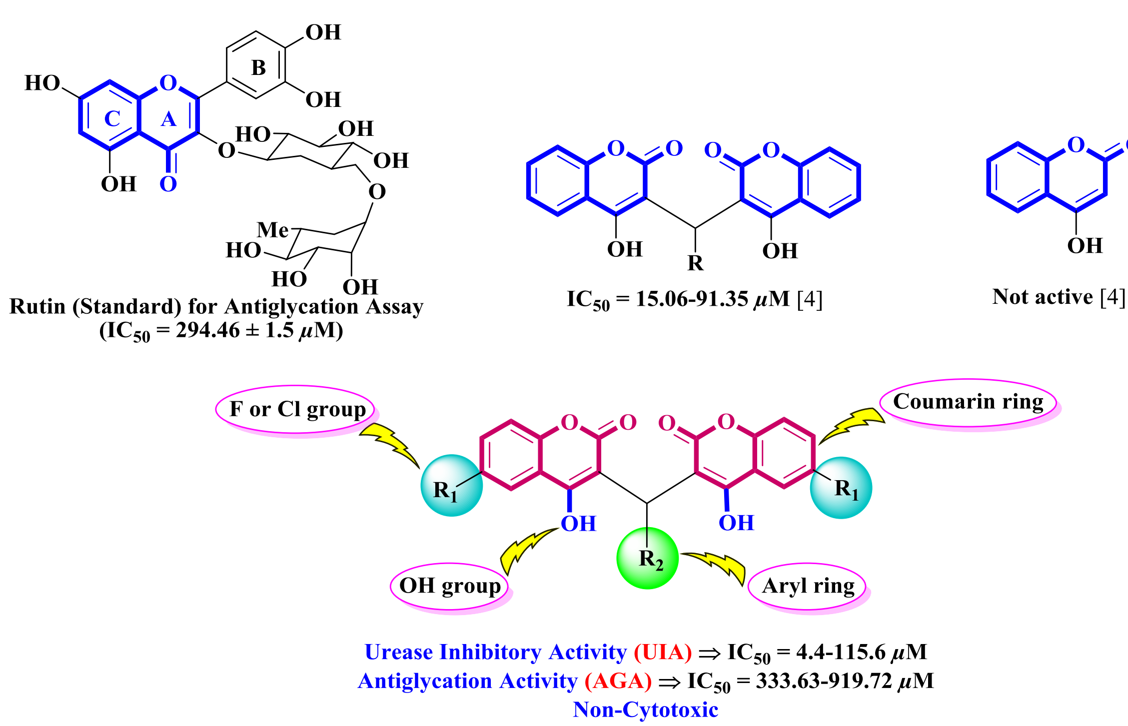
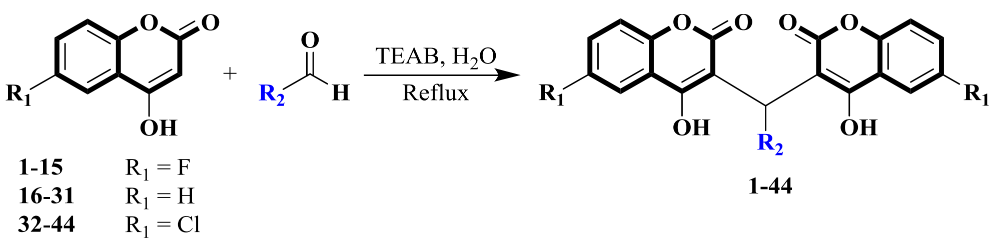


Fig. 1. Structural similarity between rutin and *bis*-coumarins 1–44 as a rationale of the current study.



Scheme 1. Synthesis of *bis*-coumarin derivatives 1–44.

of FAB (Neg.)-MS, ESI-MS, and HRESI-MS. FAB (Neg.)-MS of compound **21** displayed the peaks at m/z = 584 $[M\text{-}H]^{-1}$ 586 $[M+2\text{-}H]^{-1}$ and 588 $[M+4\text{-}H]^{-1}$ which indicated the presence of two bromine atoms. Similarly, ESI-MS of compound **21** displayed the $[M+H]^+$, $[M+2+H]^+$, and $[M+4+H]^+$ at m/z 585, 587, and 589, respectively. HR-(ESI)MS showed the $[M+H]^+$ at m/z 584.9208, corresponding to the formula $C_{25}H_{15}Br_2O_7$ Calcd (584.9184). The UV spectrum of compound **21** showed absorption at 298 nm, characteristic of coumarin moiety. The structure of new compound **21** was thus deduced unambiguously.

2.3. Bioactivities in vitro

Forty-four *bis*-coumarin derivatives were evaluated for urease inhibitory, and antiglycation activities (Table 1). Eight compounds **4**, **8–10**, **14**, **17**, **34**, and **40**, showed a good to moderate urease inhibitory activity in the range of IC_{50} = 4.4 ± 0.21–115.6 ± 2.13 μM, as compared to standard thiourea (IC_{50} = 21.3 ± 1.3 μM). Whereas, compounds **2**, **11–13**, **16**, **17**, **19**, **20–22**, **35**, **37**, and **42** demonstrated good to moderate antiglycation activities in the range of IC_{50} = 333.63–919.72 μM, as compared to standard rutin (IC_{50} = 294.46 ± 1.5 μM). *Bis*-coumarins have never been reported before for its antiglycation activity. It is worth mentioning that the compounds showed selective activity in both assays. *Bis*-coumarins which showed urease inhibitory activity did not give the antiglycation potential, and *vice versa*. All compounds were also checked for their cytotoxicity and largely found to be non-cytotoxic.

2.4. Structure-activity relationship (SAR)

All structural features of *bis*-coumarin derivatives such as coumarin ring, substitutions on coumarin rings (R_1), and aryl ring (R_2) apparently playing their role in the inhibitory activity, and variation in the activity can be attributed to the different substitution pattern on coumarin, and aryl rings (Fig. 3).

Results presented in Table 1 indicate that almost all compounds selectively exhibited their potential in both assays, and found to be non-toxic as well.

Bis-coumarins **1–44** were divided into three categories in order to understand the structure-activity pattern. In category "A", fluoro groups are present at C-6 and C-6ʹ of *bis*-coumarin i.e. **1–15**. Category "B" compounds **16–31** has no substitution on both coumarin rings, and category "C" has chloro groups at C-6 and C-6ʹ i.e. compounds **32–44**.

In category "A", non-cytotoxic compound **8** (IC_{50} = 30.3 ± 1.01 μM) with 3″ -benzyloxy and 4″-methoxy substitutions showed comparable urease inhibitory activity with the standard thiourea (IC_{50} = 21.3 ± 1.3 μM), and did not show any antiglycation potential. The good urease inhibitory activity of the compound **8** could be due to π-π stacking of the benzyloxy group with the active site of urease enzyme. Activity of compound **8** was compared with the activity of non-cytotoxic compound **14** (IC_{50} = 187.3 ± 1.75 μM) which has a bromo group instead of a benzyloxy at C-3″, showed six times less urease inhibitory activity. This indicated the involvement of benzyloxy in the inhibitory activity. Similarly, compound **14** did not show any antiglycation potential. It is worth mentioning that both compounds **8** and **14** showed selective activity towards the urease, as well as being non-cytotoxic. Other compounds with different positional combination of methoxy and bromo groups such as compounds **3**, **5**, and **15**, did not show any urease inhibitory and antiglycation activities. However, all three were found to be non-cytotoxic. The activity of compound **3** can be compared with compound **2** (IC_{50} = 378.03 ± 0.75 μM) which has a fluoro group, instead of a bromo at C-2″, showed an antiglycation potential comparable to the standard rutin (IC_{50} = 294.46 ± 1.5 μM). It showed that fluoro group on aryl ring plays an important role in the antiglycation activity (Fig. 4).

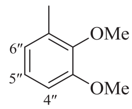
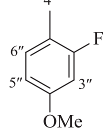
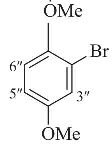
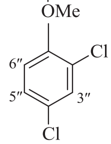
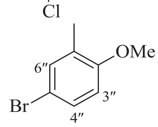
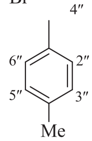
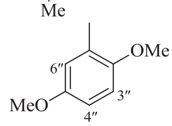
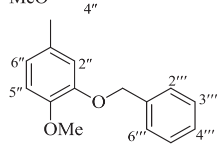
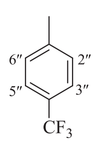
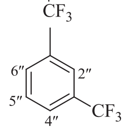
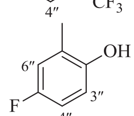
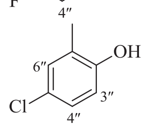
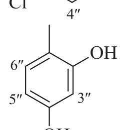
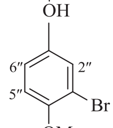
In the same category "A", compounds with the halogen substitutions (**4**, **9**, and **10**), and the compounds with combinations of halogen and hydroxy groups (**11** and **12**), showed a good selectivity and non-cytotoxicity. For example, compound **4** (IC_{50} = 87.0 ± 0.88 μM) with 2″,4″-dichloro substitutions showed a selective urease inhibitory activity, and no antiglycation activity. The two chloro groups on aryl ring might form some polar interactions with the active site of the urease enzyme. Similarly, another compound **9** (IC_{50} = 79.8 ± 1.01 μM) with a 4″-trifluoromethyl group, also showed urease inhibitory activity but no antiglycation activity. This might be that trifluoromethyl group establish polar interaction with the active site of urease enzyme. However, its positional isomer **10** (IC_{50} = 193.7 ± 0.66 μM) with a 4″-trifluoromethyl group, showed two times lower activity. It showed that C-3″ substitution is not favourable for urease inhibition (Fig. 5).

Compounds with the combination of halogen and hydroxy, such as **11** (OH and F *para* to each other) and **12** (OH and Cl *para* to each other) showed no urease inhibition, however, they did show antiglycation activity. Similarly, compound **13** (IC_{50} = 443.64 ± 0.57 μM) with 2″,4″-dihydroxy substitutions also showed antiglycation potential (Fig. 6). Thus hydroxy group is apparently playing an important role in the antiglycation activity. Phenolic OH known to have radical scavenging activity through phenolic OH groups, along with other structural features which may contribute to the inhibition of protein glycation.

Involvement of hydroxy groups in the antiglycation activity was further deduced by examining antiglycation activity pattern of compounds **16** (IC_{50} = 351.85 ± 3.56 μM), **17** (IC_{50} = 374.45 ± 1.21 μM), and **20** (IC_{50} = 434.71 ± 2.73 μM) belong to category "B". Compound **16** (IC_{50} = 351.85 ± 3.56 μM), having a 2,3-dihydroxy substitution, was selectively active against glycation but no urease inhibitory activity. The antiglycation activity of compound **16** can be compared with compound **20** (IC_{50} = 434.71 ± 2.73 μM) which has a 2,4-dihydroxy group, instead of 2,3-dihydroxy, and showed lower antiglycation activity. This shows that the positions of OH substitution also plays a role in the activity. However, incorporation of one or more hydroxy group in compound **17** (IC_{50} = 374.45 ± 1.21 μM) enhanced the antiglycation potential to a considerable level. In addition, compound **17** was found to be a potent urease inhibitor, with five times more activity than the standard thiourea (IC_{50} = 21.3 ± 1.3 μM). Interestingly, this is the only compound which showed dual inhibitory activity against urease and glycation (Fig. 7).

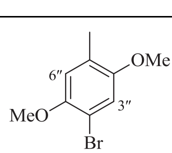
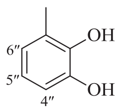
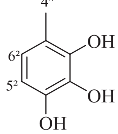
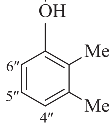
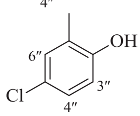
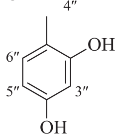
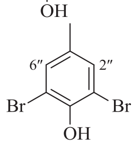
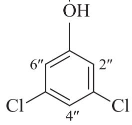
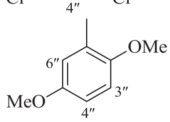
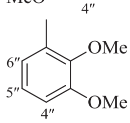
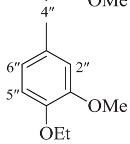
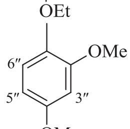
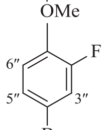
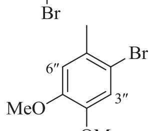
Other compounds with the halogen substitutions such as compound **22** with 3″,5″-dichloro substitutions, showed an antiglycation activity (IC_{50} = 428.84 ± 2.44 μM) but no urease inhibitory activity. Compounds having the combination of halogen (Cl and Br) with the hydroxy group such as **19** and **21**, did not display any urease inhibition, however, both compounds **19** (IC_{50} = 432.58 ± 0.76 μM) and **21** (IC_{50} = 397.07 ± 2.21 μM) showed antiglycation activity (Fig. 8). All

Table 1
In vitro urease inhibitory activity, antiglycation activity, and cytotoxicity of *bis*-coumarins (**1–44**).

Compounds	R_2	Urease Inhibitory Activity IC_{50} ± SEM[a]	Antiglycation Activity IC_{50} ± SEM[a]	Cytotoxicity IC_{50} ± SEM[a]
Category A (R_1 = F)				
1		NA[b]	NA[b]	NT[c]
2		NA[b]	378.03 ± 0.75	> 30
3		NA[b]	NA[b]	NT[c]
4		87.0 ± 0.88	NA[b]	NT[c]
5		NA[b]	NA[b]	NT[c]
6		NA[b]	NA[b]	NT[c]
7		NA[b]	NA[b]	NT[c]
8		30.3 ± 1.01	NA[b]	NT[c]
9		79.8 ± 1.01	NA[b]	NT[c]
10		193.7 ± 0.66	NA[b]	NT[c]
11		NA[b]	399.47 ± 1.88	> 30
12		NA[b]	465.33 ± 1.99	> 30
13		NA[b]	443.64 ± 0.57	> 30
14		187.3 ± 1.75	NA[b]	NT[c]

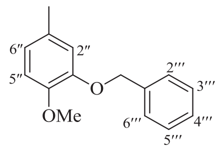
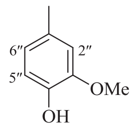
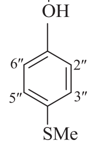
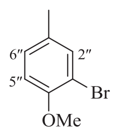
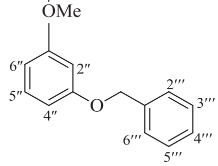
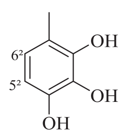
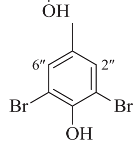
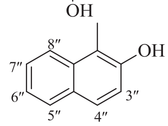
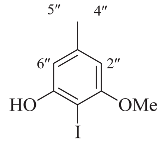
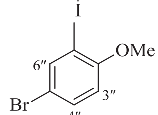
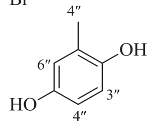
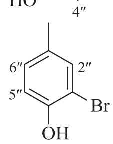
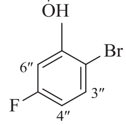
(*continued on next page*)

Table 1 (*continued*)

Compounds	R_2	Urease Inhibitory Activity IC_{50} ± SEM[a]	Antiglycation Activity IC_{50} ± SEM[a]	Cytotoxicity IC_{50} ± SEM[a]
15		NA[b]	NA[b]	NT[c]
Category B (R_1 = H)				
16		NA[b]	351.85 ± 3.56	> 30
17		4.4 ± 0.21	374.45 ± 1.21	18.70 ± 0.57
18		NA[b]	NA[b]	NT[c]
19		NA[b]	397.07 ± 2.21	> 30
20		NA[b]	434.71 ± 2.73	19.42 ± 0.79
21		NA[b]	432.58 ± 0.76	> 30
22		NA[b]	428.84 ± 2.44	> 30
23		NA[b]	NA[b]	NT[c]
24		NA[b]	NA[b]	NT[c]
25		NA[b]	NA[b]	NT[c]
26		NA[b]	NA[b]	NT[c]
27		NA[b]	NA[b]	NT[c]
28		NA[b]	NA[b]	NT[c]

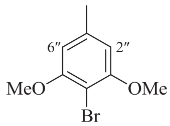
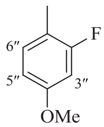
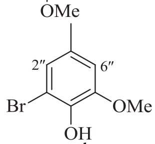
(*continued on next page*)

Table 1 (*continued*)

Compounds	R_2	Urease Inhibitory Activity IC_{50} ± SEM[a]	Antiglycation Activity IC_{50} ± SEM[a]	Cytotoxicity IC_{50} ± SEM[a]
29		NA[b]	NA[b]	NT[c]
30		NA[b]	NA[b]	NT[c]
31		NA[b]	NA[b]	NT[c]
Category C (R_1 = Cl)				
32		NA[b]	NA[b]	NT[c]
33		NA[b]	NA[b]	NT[c]
34		115.6 ± 2.13	NA[b]	NT[c]
35		NA[b]	744.82 ± 5.64	> 30
36		NA[b]	NA[b]	NT[c]
37		NA[b]	919.72 ± 1.98	21.11 ± 1.27
38		NA[b]	NA[b]	NT[c]
39		NA[b]	NA[b]	NT[c]
40		36.5 ± 1.37	NA[b]	NT[c]
41		NA[b]	NA[b]	NT[c]

(*continued on next page*)

Table 1 (*continued*)

Compounds	R_2	Urease Inhibitory Activity IC_{50} ± SEM[a]	Antiglycation Activity IC_{50} ± SEM[a]	Cytotoxicity IC_{50} ± SEM[a]
42		NA[b]	333.63 ± 1.98	> 30
43		NA[b]	NA[b]	NT[c]
44		NA[b]	NA[b]	NT[c]
Standards	**Thiourea**[d]	21.3 ± 1.3		
	Rutin[e]		294 ± 1.5	–
	Cycloheximide[f]		–	0.26 ± 0.1

SEM[a] (Standard error of mean); NA[b] (Not Active); NT[c] (Non Toxic); Thiourea[d] (Standard inhibitor of urease inhibitory activity); Rutin[e] (Standard inhibitor of antiglycation activity); Cycloheximide[f] (Standard inhibitor for cytotoxicity).

three compounds were found to be non-cytotoxic.

In category "C", compound **40** (IC_{50} = 36.5 ± 1.37 µM) having 3″-bromo-4″-hydroxy substitutions, showed a urease inhibitory activity comparable to standard thiourea (IC_{50} = 21.3 ± 1.3 µM) and found non-cytotoxic. The hydroxy group is apparently making the hydrogen bonding interaction as well as the bromo group involved in some polar interaction with the active site of the enzyme. Activity of compound **40** can be compared with analogs **44** and **35** which have additional methoxy and bromo group, respectively, at 5″-position of R_2, a complete loss of activity was observed. The inactivity of the compounds might be due to the additional groups which create the steric hindrance for the hydroxy group to bind with the enzyme active site. However, compound **35** (IC_{50} = 744.82 ± 5.64 µM) showed selective antiglycation activity and also found to be non-cytotoxic. Furthermore, non-cytotoxic compounds **42** (IC_{50} = 333.63 ± 1.98 µM) and **37** (IC_{50} = 919.72 ± 1.98 µM) showed a selective good to moderate antiglycation activity. Compound **42** has a 3″,5″-dibromo-4″-hydroxy substitution while compound **37** is with 4″-bromo-3″,5″-dimethoxy substitutions at phenyl ring R_2. It is worthy to note that compounds **35**, **37**, and **42**, are 3″,4″,5″-trisubstituted analogs which demonstrated the selective antiglycation potential. Another compound **34** (IC_{50} = 115.6 ± 2.13 µM) having a 3″,4″,5″-trihydroxy group, showed selective but weak urease inhibitory activity. Its activity compared with the structurally similar and most potent compound **17** (IC_{50} = 4.4 ± 0.21 µM). Compound **34** might have a conformation which does not fit well into the active site of enzyme. Similarly, additional chloro groups at C-6 and C-6′ positions are not playing their role in the activity (Fig. 9).

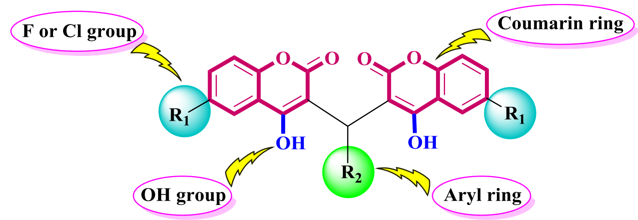


Fig. 3. General structural features of *bis*-coumarin.

In brief, it was observed that all derivatives, except **17**, were found to be selective and non-cytotoxic. Our next goal is to place heterocycles on *bis*-coumarin skeleton in order to see their effects on selectivity and cytotoxicity.

3. Conclusion

Synthetic *bis*-coumarins **1–44** were evaluated for their urease inhibitory and antiglycation activities. Seven derivatives **4**, **8–10**, **14**, **34**, and **40** showed selective urease inhibition, whereas, twelve analogs **2**, **11–13**, **16**, **17**, **19–22**, **35**, **37**, and **42** demonstrated antiglycation potential. Only compound **17** showed dual inhibition. All compounds were largely found to be non-cytotoxic. Newly identified compounds, based on *bis*-coumarin scaffold, may serve as leads for future research for more powerful, non-cytotoxic, and selective agents against urease and protein glycation.

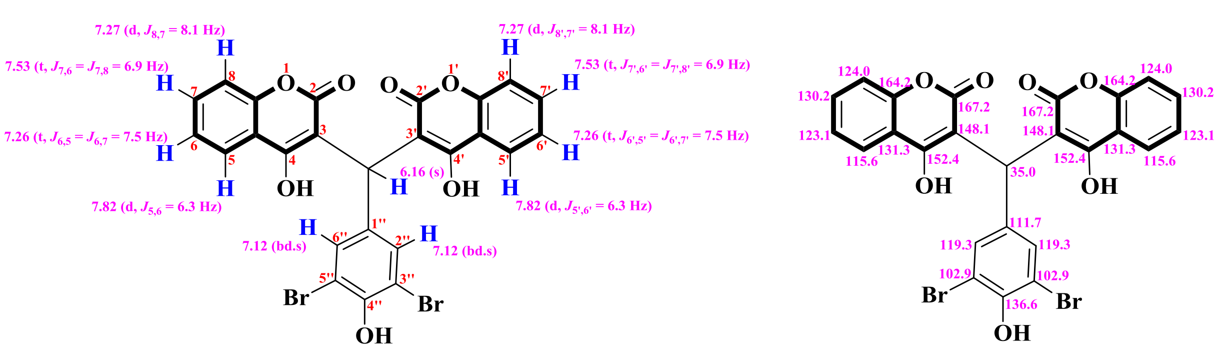


Fig. 2. ¹H- and ¹³C NMR chemical shifts of compound 21.

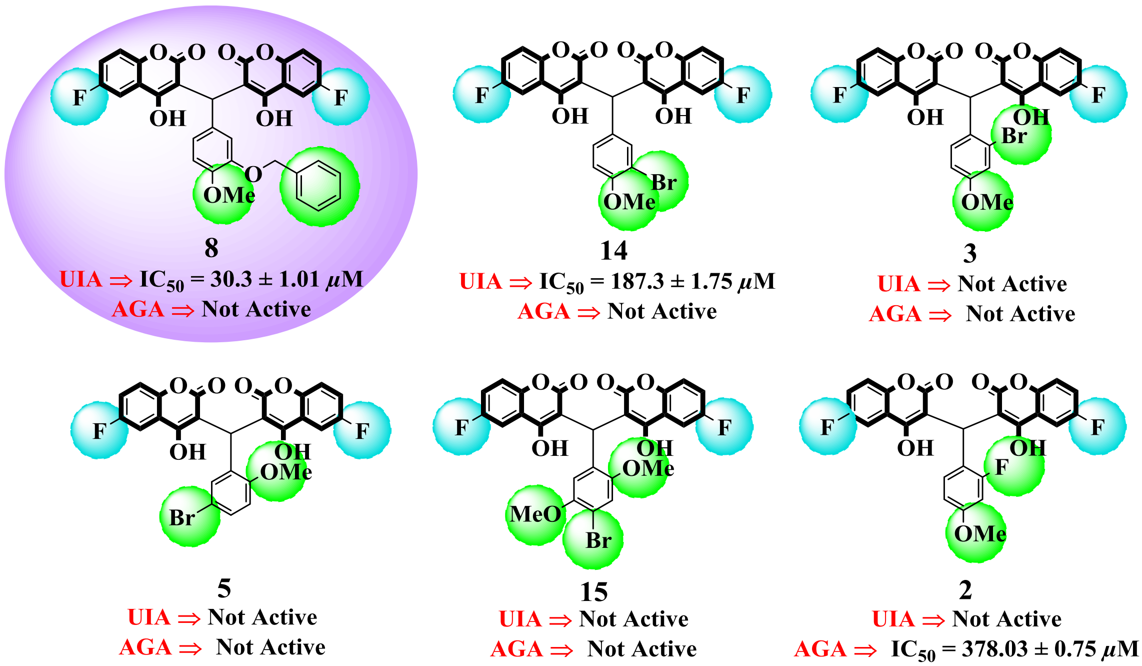


Fig. 4. Structure-activity relationship of compounds 2, 3, 5, 8, 14, and 15.

4. Experimental

4.1. Materials and methods

Thin layer chromatography (TLC) was performed on pre-coated silica gel aluminum plates (Kieselgel 60 F-254, 0.20 mm, Merck, Darmstadt, Germany). Chromatograms were visualized by using a handhold UV lamp at 254, and 365 nm or placing in iodine vapors. Fast atom bombardement mass spectra (FAB MS) were recorded on a Finnigan MAT-311A (Germany) (70 eV) spectrometers, electrospray ionization mass spectra (ESI-MS, HRESI-MS) were recorded on a QSTAR XL LCMS-MS, and ABSciex (Germany) (50 kV) mass spectrometers, and the data are tabulated as *m/z*. ^{1}H - and ^{13}C NMR spectroscopic analysis was performed on an Avance Bruker (Germany) AM spectrometer 300 MHz machine. Splitting patterns for ^{1}H NMR spectra were as follows, s (singlet); d (doublet); t (triplet); m (multiplet). Chemical shifts are reported in δ (ppm) and coupling constants are given in Hz. All solvents and reagents were of reagent grade, and used directly without purification. Melting points of the compounds were determined on Büchi-M560 melting point apparatus.

4.2. General procedure for the syntheses of bis-coumarin derivatives 1–44

6-Fluoro-4-hydroxy /6-chloro-4-hydroxy /4-hydroxy coumarin (1 mmol), and a variety of aromatic aldehydes (0.5 mmol), as well as 10 mol% of tetraethylammonium bromide (TEAB) were dissolved in distilled water (15 mL) in a 100 mL round-bottommed flask. The reaction mixture was refluxed for 2 h. Periodic TLC was taken to check the progress of reaction. Resulting precipitates were filtered, and washed with distilled water. This afforded products **1–44** in high yields.

4.3. 3,3′-((2′′,3′′-Dimethoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (1)

White Solid; Yield: 82%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.25 (s, 2H, 2OH), 7.67 (d, $J_{5,6/5',6'}$ = 8.7 Hz, 2H, H-5, H-5′), 7.50 (d, $J_{7,8/7',8'/8,7/8',7'}$ = 5.4 Hz, 4H, H-7, H-8, H-7′, H-8′), 7.01 (m, 3H, H-4′′, H-5″, H-6′′), 6.22 (s, 1H, −CH−), 3.81 (s, 3H, OCH_{3a}), 3.49 (s, 3H, OCH_{3b}); ^{13}C NMR (75 MHz, DMSO-d_6): δ165.8, 165.8, 163.8, 168.3, 159.3, 159.3, 156.1, 152.3, 148.5, 146.4, 136.0, 124.4, 122.3, 121.1, 121.0, 120.9, 118.1, 117.8, 117.5, 117.3, 110.5, 109.3, 108.9, 104.5, 59.3, 55.4, 32.7; FAB (Pos.)-MS *m/z* = 509 $[M+H]^+$; ESI-MS *m/z* = 509 $[M+H]^+$; HRESI-MS Calcd for $C_{27}H_{19}F_2O_8$ $[M+H]^+$: *m/z* = 509.1047, found 509.1090.

4.4. 3,3′-((2′′-Fluoro-4′′-methoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (2)

White Solid; Yield: 87%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.31 (bd.s, 2H, 2OH), 7.68 (d, $J_{5,6F/5',6F}$ = 8.7 Hz, 2H, H-

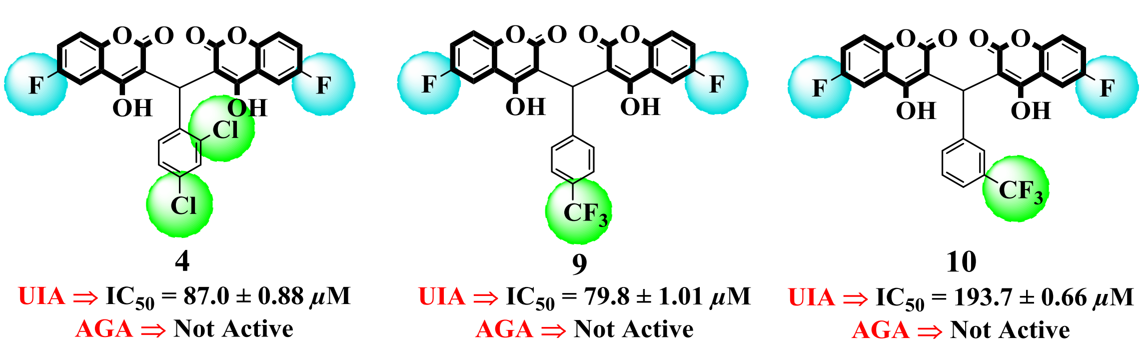


Fig. 5. Structure-activity relationship of compounds 4, 9, and 10.

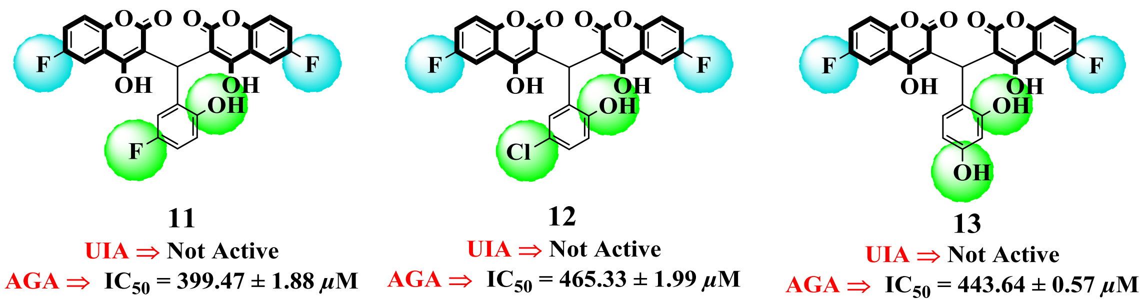


Fig. 6. Structure-activity relationship of compounds 11–13.

5, H-5′), 7.52 (d, $J_{8,7/8',7'/6'',5''}$ = 5.4 Hz, 3H, H-8, H-8′, H-6″), 7.32 (m, 1H, H-3″), 6.75 (m, 3H, H-7, H-7′, H-5″), 6.19 (bd.s, 1H, −CH−), 3.80 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.2, 159.9, 159.9, 159.2, 159.2, 156.7, 149.7, 148.5, 129.1, 120.0, 119.7, 119.3, 118.7, 118.4, 117.4, 117.3, 114.9, 114.8, 113.6, 112.1, 109.5, 109.2, 108.5, 101.5, 55.5, 28.3; FAB (Neg.)-MS *m/z* = 495 $[M\text{-}H]^{-1}$; ESI-MS *m/z* = 477 $[M+H\text{-}HF]^+$; HRESI-MS Calcd for $C_{26}H_{15}F_2O_7$ $[M+H\text{-}HF]^+$: *m/z* = 477.0785, Found 477.0787.

4.5. 3,3′-((2′′-Bromo-4′′-methoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (3)

White Solid; Yield: 79%; M.p.: 230–232 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.47 (bd.s, 2H, 2OH), 7.68 (d, $J_{5,6F/5',6F}$ = 8.1 Hz, 2H, H-5, H-5′), 7.52 (bd.s, 5H, H-7, H-8, H-7′, H-8′, H-3″), 7.32 (d, $J_{6'',5''}$ = 8.1 Hz, 1H, H-6″), 7.02 (d, $J_{5'',6''}$ = 8.1 Hz, 1H, H-5″), 6.15 (bd.s, 1H, −CH−), 3.86 (bd.s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 160.1, 159.5, 159.5, 156.9, 149.9, 148.8, 129.2, 125.2, 120.0, 119.8, 119.4, 118.9, 118.6, 117.6, 117.4, 114.8, 114.6, 113.7, 112.3, 109.4, 109.2, 108.4, 101.6, 55.7, 28.5; FAB (Neg.)-MS *m/z* = 556 $[M\text{-}H]^{-1}$, 558 $[M+2\text{-}H]^{-1}$; ESI-MS *m/z* = 557 $[M+H]^+$, 559 $[M+2+H]^+$; HRESI-MS Calcd for $C_{26}H_{15}BrF_2O_7$ $[M+H]^+$: *m/z* = 557.0047, Found 557.0010.

4.6. 3,3′-((2′′,4′′-Dichlorophenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (4)

White Solid; Yield: 81%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 7.68 (dd, $J_{5,7//5',7'}$ = 2.1 Hz, $J_{5,6F/5',6F}$ = 7.8 Hz, 2H, H-5, H-5′), 7.57 (d, $J_{6'',5''}$ = 8.4 Hz, 1H, H-6″), 7.49 (m, 4H, H-8, H-8′, H-3″, H-5″), 7.39 (dd, $J_{7,5/7',5'}$ = 2.1 Hz, $J_{7,8/7',8'}$ = 6.6 Hz, 2H, H-7, H-7′), 6.24 (s, 1H, −CH−); ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.4, 160.1, 159.3, 157.1, 156.5, 156.1, 148.2, 148.0, 141.5, 135.4, 132.5, 131.7, 130.0, 126.6, 125.0, 124.9, 119.1, 119.0, 115.2, 115.0, 111.4, 111.3, 100.2, 100.0, 28.2; FAB (Neg.)-MS *m/z* = 516 $[M\text{-}H]^{-1}$, 518 $[M+2\text{-}H]^{-1}$; ESI-MS *m/z* = 517 $[M+H]^+$; HRESI-MS Calcd for $C_{25}H_{13}Cl_2F_2O_6$ $[M+H]^+$: *m/z* = 517.0057, Found 517.0071.

4.7. 3,3′-((5′′-Bromo-2′′-methoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (5)

White Solid; Yield: 77%; M.p.: 245–247 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.21 (bd.s, 2H, 2OH), 7.67 (d, $J_{5,6F/5',6F}$ = 8.7 Hz, 2H, H-5, H-5′), 7.51 (d, $J_{8,7/8',7'/6'',4''}$ = 5.7 Hz, 3H, H-8, H-8′, H-6″), 7.43 (bd.s, 2H, H-7, H-7′), 6.96 (d, $J_{3'',4''/4'',3''}$ = 8.7 Hz, 2H, H-3″, H-4″), 6.14 (s, 1H, −CH−), 3.61 (s, 3H, OCH_{3a}); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.8, 165.8, 163.5, 159.3, 157.5, 156.5, 156.2, 148.5, 148.5, 133.1, 131.1, 129.2, 124.2, 120.8, 120.7, 118.3, 117.9, 117.6, 117.4, 113.2, 111.4, 109.3, 109.0, 103.7, 55.8, 32.9; FAB (Neg.)-MS *m/z* = 556 $[M\text{-}H]^{-1}$, 558 $[M+2\text{-}H]^{-1}$; ESI-MS *m/z* = 557 $[M+H]^+$, 559 $[M+2+H]^+$; HRESI-MS Calcd for $C_{26}H_{16}BrF_2O_7$ $[M+H]^+$: *m/z* = 557.0047, Found 557.0010.

4.8. 3,3′-(p-Tolylmethylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (6)

White Solid; Yield: 76%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.43 (s, 2H, 2OH), 7.68 (d, $J_{5,6F/5',6F}$ = 8.7 Hz, 2H, H-5, H-5′), 7.54 (d, $J_{8,7/8',7'/7,8}$ = 5.7 Hz, 3H, H-7, H-8, H-8′), 7.21 (d, $J_{2'',3''/6'',5''/7',8'}$ = 8.1 Hz, 3H, H-7′, H-2″, H-6″), 7.14 (d, $J_{3'',2''/5'',6''}$ = 8.1 Hz, 2H, H-3″, H-5″), 6.10 (bd.s, 1H, −CH−), 2.29 (s, 3H, CH_3); FAB (Neg.)-MS *m/z* = 461 $[M\text{-}H]^{-1}$; ESI-MS *m/z* = 463 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{17}F_2O_6$ $[M+H]^+$: *m/z* = 463.0993, Found 463.1022.

4.9. 3,3′-((2′′,5′′-Dimethoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (7)

White Solid; Yield: 72%; M.p.: 240–242 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.20 (s, 2H, 2OH), 7.67 (d, $J_{5,6F/5',6F}$ = 8.7 Hz, 2H, H-5, H-5′), 7.50 (d, $J_{7,8/7',8'/8,7/8',7'}$ = 5.7 Hz, 4H, H-7, H-8, H-7′, H-8′), 6.91 (m, 3H, H-3″, H-4″, H-6″), 6.10 (s, 1H, −CH−), 3.68 (s, 3H, OCH_{3a}), 3.52 (s, 3H, CH_{3b}); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.1, 165.1, 163.7, 163.7, 159.3, 159.3, 156.2, 152.6, 148.5, 146.3, 131.4, 125.3, 120.5, 120.4, 118.3, 118.0, 117.6, 117.5, 116.4, 111.9, 109.6, 109.3, 108.9, 104.4, 56.2, 55.0, 33.0; FAB (Neg.)-MS *m/z* = 507 $[M\text{-}H]^{-1}$; ESI-MS *m/z* = 509 $[M+H]^+$; HRESI-MS Calcd for $C_{27}H_{19}F_2O_8$ $[M+H]^+$: *m/z* = 509.1047, Found 509.1064.

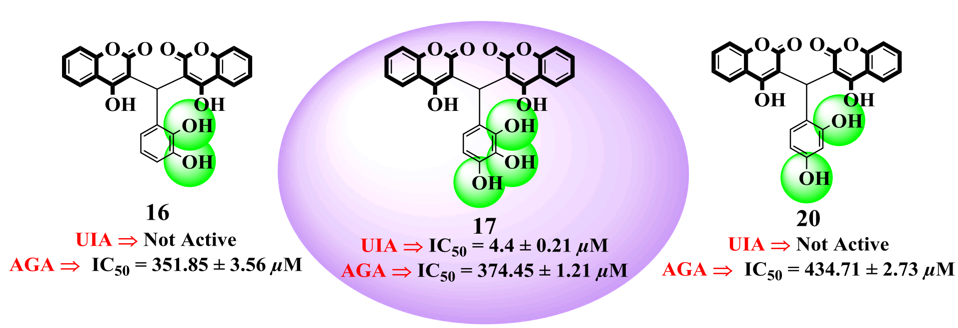


Fig. 7. Structure-activity relationship of compounds 16, 17, and 20.

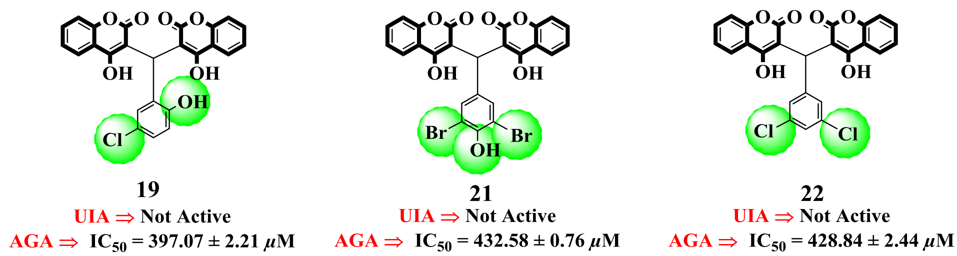


Fig. 8. Structure-activity relationship of compounds 19, 21, and 22.

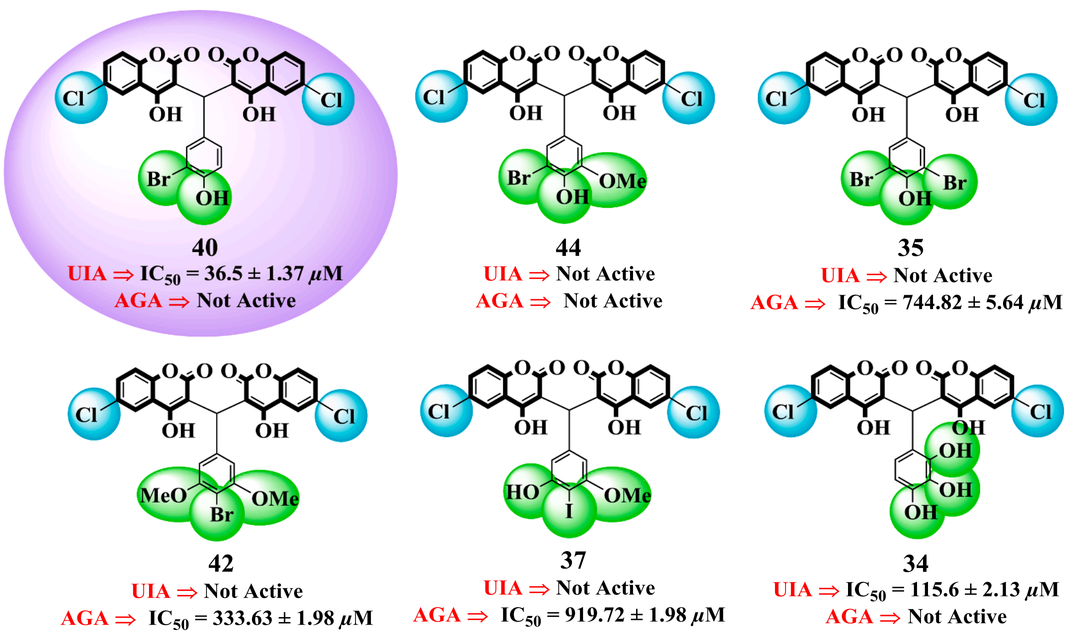


Fig. 9. Structure-activity relationship of compounds 34, 35, 37, 40, 42, and 44.

4.10. 3,3′-((3′′-(Benzyloxy)-4′′-methoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (8)

White Solid; Yield: 80%; M.p.: 200–202 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.41 (s, 2H, 2OH), 7.66 (d, $J_{5,6F/5',6F}$ = 8.4 Hz, 2H, H-5, H-5′), 7.54 (bd.d, $J_{8,7/8',7'/7,8/7',8'}$ = 5.4 Hz, 4H, H-7, H-8, H-7′, H-8′), 7.27 (m, 5H, Ar-H), 6.96 (bd.s, 1H, H-2′′), 6.93 (d, $J_{5'',6''}$ = 7.5 Hz, 1H, H-5′′), 6.87 (d, $J_{6'',5''}$ = 8.0 Hz, 1H, H-6′′), 6.05 (s, 1H, –CH–), 4.97 (s, 2H, –CH_2-), 3.80 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.7, 162.7, 161.6, 161.6, 159.4, 159.4, 149.5, 148.2, 148.2, 146.9, 136.6, 135.4, 128.7, 128.7, 127.5, 127.2, 127.2, 125.3, 125.3, 122.0, 119.2, 119.2, 115.0, 115.0, 114.2, 112.4, 111.5, 111.5, 100.3, 100.3, 71.4, 56.2, 28.4; FAB (Neg.)-MS m/z = 582 [M-H]$^{-1}$; ESI-MS m/z = 585 [M+H]$^+$; HRESI-MS Calcd for $C_{33}H_{23}F_2O_8$ [M+H]$^+$: m/z = 585.1360, Found 585.1332.

4.11. 3,3′-((4′′-(Trifluoromethyl)phenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (9)

White Solid; Yield: 84%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.53 (d, $J_{5,6F/5',6F}$ = 8.4 Hz, 2H, H-5, H-5′), 7.49 (dd, $J_{3'',5''/5'',3''}$ = 3.0 Hz, $J_{3'',2''/5'',6''}$ = 6.0 Hz, 2H, H-3′′, H-5′′), 7.37 (m, 4H, H-8, H-8′, H-2′′, H-6′′), 7.29 (d, $J_{7,8/7',8'}$ = 8.4 Hz, 2H, H-7, H-7′), 6.29 (bd.s, 1H, –CH–); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.8, 162.8, 161.4, 161.4, 159.6, 159.6, 148.0, 148.0, 147.6, 128.9, 128.9, 128.4, 125.3, 125.3, 125.1, 125.1, 124.3, 119.4, 119.4, 115.5, 115.5, 111.6, 111.6, 100.9, 100.9, 28.4; FAB (Neg.)-MS m/z = 515 [M-H]$^{-1}$; ESI-MS m/z = 517 [M+H]$^+$; HRESI-MS Calcd for $C_{26}H_{14}F_5O_6$ [M+H]$^+$: m/z = 517.0710, Found 517.0755.

4.12. 3,3′-((3′′-(Trifluoromethyl)phenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (10)

White Solid; Yield: 77%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.52 (bd.s, 2H, 2OH), 7.69 (m, 4H, H-5, H-8, H-5′, H-8′), 7.55 (m, 6H, H-7, H-7′, H-2″, H-4″, H-5″, H-6″), 6.30 (s, 1H, –CH–); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.8, 162.8, 161.4, 161.4, 159.6, 159.6, 148.0, 148.0, 142.7, 131.2, 130.7, 128.7, 127.3, 125.3, 125.3, 124.5, 122.2, 119.4, 119.4, 115.5, 115.5, 111.6, 111.6, 100.9, 100.9, 28.5; FAB (Neg.)-MS m/z = 515 [M-H]$^{-1}$; ESI-MS m/z = 517 [M+H]$^+$; HRESI-MS Calcd for $C_{26}H_{14}F_5O_6$ [M+H]$^+$: m/z = 517.0710, Found 517.0755.

4.13. 3,3′-((5′′-Fluoro-2′′-hydroxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (11)

White Solid; Yield: 73%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.88 (m, 1H, H-5), 7.75 (m, 1H, H-5′), 7.61 (m, 2H, H-8, H-8′), 7.46 (m, 3H, H-7, H-7′, H-6′′), 7.19 (m, 1H, H-4′′), 6.98 (m, 1H, H-3′′), 5.69 (s, 1H, –CH–); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.2, 161.9, 160.5, 160.1, 159.5, 159.0, 155.2, 151.6, 149.5, 148.8, 125.6, 125.1, 122.5, 119.9, 119.5, 117.6, 117.2, 117.0, 114.8, 114.7, 109.7, 109.4, 101.6, 101.4, 28.5; FAB (Neg.)-MS m/z = 462 [M-H–HF]$^{-1}$; ESI-MS m/z = 465 [M+H-H_2O]$^+$; HRESI-MS Calcd for $C_{25}H_{12}F_3O_6$ [M+H-H_2O]$^+$: m/z = 465.0585, Found 465.0549.

4.14. 3,3′-((5′′-Chloro-2′′-hydroxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (12)

White Solid; Yield: 79%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 7.84 (m, 2H, H-5, H-5′), 7.50 (m, 7H, H-7, H-8, H-7′, H-8′, H-3″, H-4″, H-6″), 5.79 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.5, 162.2, 160.4, 160.0, 159.6, 159.3, 151.4, 149.5, 148.9, 126.7, 125.7, 125.2, 122.6, 119.8, 119.4, 117.7, 117.4, 117.1, 114.7, 114.3, 109.8, 109.3, 101.7, 101.5, 28.6; FAB (Neg.)-MS m/z = 497 [M-H]$^{-1}$; 499 [M+2-H]$^{-1}$; ESI-MS m/z = 499 [M+H]$^{+}$; HRESI-MS Calcd for $C_{25}H_{13}ClF_2O_7$ [M+H]$^{+}$: m/z = 499.0318, Found 499.0336.

4.15 . 3,3′-((2′′,4′′-Dihydroxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (13)

White Solid; Yield: 81%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 7.84 (dd, $J_{5,7}$ = 2.1 Hz, $J_{5,6F}$ = 8.7 Hz, 1H, H-5), 7.76 (d, $J_{5',7'}$ = 2.4 Hz, $J_{5',6F}$ = 8.7 Hz, 1H, H-5′), 7.52 (m, 4H, H-7, H-8, H-7′, H-8′), 7.10 (d, $J_{6'',5''}$ = 8.4 Hz, 1H, H-6″), 6.82 (bd.s, 1H, H-3″), 6.65 (m, 1H, H-5″), 5.62 (bd.s, 1H, −CH−); ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.2, 159.9, 159.5, 157.3, 156.7, 156.3, 148.5, 148.3, 129.1, 119.9, 119.6, 119.5, 119.2, 118.7, 118.6, 118.4, 118.3, 115.0, 114.9, 113.1, 111.9, 108.6, 108.3, 102.9, 28.2; FAB (Neg.)-MS m/z = 461 [M-H-H_2O]$^{-1}$; ESI-MS m/z = 463 [M+H-H_2O]$^{+}$; HRESI-MS Calcd for $C_{25}H_{13}F_2O_7$ [M+H-H_2O]$^{+}$: m/z = 463.0629, Found 463.0616.

4.16. 3,3′-((3′′-Bromo-4′′-methoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (14)

White Solid; Yield: 85%; M.p.: 210–212 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.47 (s, 2H, 2OH), 7.69 (d, $J_{5,6F/5',6F}$ = 8.7 Hz, 2H, H-5, H-5′), 7.54 (d, $J_{7,8/8,7/7',8'/8',7'}$ = 6.6 Hz, 4H, H-7, H-8, H-7′, H-8′), 7.52 (bd.s, 1H, H-2″), 7.32 (bd.d, $J_{6'',5''}$ = 8.1 Hz, 1H, H-6″), 7.02 (d, $J_{5'',6''}$ = 8.4 Hz, 1H, H-5″), 6.14 (s, 1H, −CH−), 3.87 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.5, 166.4, 164.1, 159.4, 156.2, 153.1, 148.7, 135.4, 130.8, 127.2, 120.8, 120.7, 118.6, 118.2, 117.7, 117.6, 112.3, 110.0, 109.4, 109.1, 103.7, 56.1, 35.2; FAB (Neg.)-MS m/z = 555 [M-H]$^{-1}$, 557 [M+2-H]$^{-1}$; ESI-MS m/z = 557 [M+H]$^{+}$; HRESI-MS Calcd for $C_{26}H_{16}BrF_2O_7$ [M+H]$^{+}$: m/z = 557.0047, Found 557.0046.

4.17. 3,3′-((4′′-Bromo-2′′,5′′-dimethoxyphenyl)methylene)bis(6-fluoro-4-hydroxy-2H-chromen-2-one) (15)

White Solid; Yield: 83%; M.p.: 205–207 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.30 (s, 2H, 2OH), 7.67 (d, $J_{8,7/8',7'}$ = 8.4 Hz, 2H, H-8, H-8′), 7.49 (d, $J_{7,8/8,7/7',8'/8',7'}$ = 5.4 Hz, 4H, H-7, H-8, H-7′, H-8′), 7.14 (s, 1H, H-3″), 7.10 (s, 1H, H-6″), 6.13 (s, 1H, −CH−), 3.72 (s, 3H, OCH_{3b}), 3.57 (s, 3H, OCH_{3a}); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.7, 165.6, 163.6, 163.5, 159.3, 159.2, 151.8, 149.6, 148.8, 148.5, 131.3, 125.3, 125.2, 120.8, 120.7, 118.3, 117.9, 117.6, 117.4, 116.0, 114.4, 109.3, 109.0, 107.5, 104.0, 56.5, 56.5, 33.0; FAB (Neg.)-MS m/z = 586 [M-H]$^{-1}$, 588 [M+2-H]$^{-1}$; ESI-MS m/z = 587 [M+H]$^{+}$, 589 [M+2+H]$^{+}$; HRESI-MS Calcd for $C_{27}H_{18}BrF_2O_8$ [M+H]$^{+}$: m/z = 587.0153, Found 587.0111.

4.18. 3,3′-((2′′,3′′-Dihydroxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (16)

White Solid; Yield: 82%; M.p.: 110–112 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 8.64 (bd.s, 1H, OH), 8.36 (d, $J_{5,6}$ = 8.1 Hz, 1H, H-5), 8.06 (d, $J_{5',6'}$ = 8.1 Hz, 1H, H-5′), 7.72 (t, $J_{7,6}$ = $J_{7,8}$ = 8.4 Hz, 1H, H-7), 7.61 (t, $J_{7',6'}$ = $J_{7',8'}$ = 7.2 Hz, 1H, H-7′), 7.49 (t, $J_{6,5}$ = $J_{6,7}$ = 7.2 Hz, 1H, H-6), 7.39 (d, $J_{8,7}$ = 8.7 Hz, 1H, H-8), 7.39 (t, $J_{6',5'}$ = $J_{6',7'}$ = 8.7 Hz, 1H, H-6′), 7.24 (d, $J_{8',7'}$ = 8.1 Hz, 1H, H-8′), 6.98 (t, $J_{5'',4''}$ = $J_{5'',6''}$ = 7.8 Hz, 1H, H-5″), 6.88 (d, $J_{6'',5''}$ = 6.6 Hz, 1H, H-6″), 6.76 (d, $J_{4'',5''}$ = 7.5 Hz, 1H, H-4″), 5.67 (s, 1H, −CH−); ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.5, 152.1, 151.9, 151.9, 145.0, 145.0, 132.3, 132.3, 132.0, 132.0, 125.0, 124.3, 124.3, 123.9, 123.9, 123.2, 123.2, 118.2, 116.3, 116.3, 116.1, 116.1, 115.4, 114.0, 28.7; FAB (Neg.)-MS m/z = 425 [M-H-H_2O]$^{-1}$; ESI-MS m/z = 427 [M+H-H_2O]$^{+}$; HRESI-MS Calcd for $C_{25}H_{16}O_8$ [M+H-H_2O]$^{+}$: m/z = 427.0845, Found 427.0868.

4.19. 3,3′-((2′′,3′′,4′′-Trihydroxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (17)

White Solid; Yield: 77%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, Acetone-d_6): δ 8.36 (d, $J_{5,6}$ = 8.1 Hz, 1H, H-5), 8.36 (bd.s, 1H, OH), 8.08 (bd.s, 1H, OH), 8.05 (d, $J_{5',6'}$ = 6.6 Hz, 1H, H-5′), 7.72 (t, $J_{7,6}$ = $J_{7,8}$ = 8.7 Hz, 1H, H-7), 7.60 (t, $J_{7',6'}$ = $J_{7',8'}$ = 7.2 Hz, 1H, H-7′), 7.49 (t, $J_{6,5}$ = $J_{6,7}$ = 7.2 Hz, 1H, H-6), 7.40 (d, $J_{6'',5''}$ = 8.7 Hz, 1H, H-6″), 7.38 (t, $J_{6',5'}$ = $J_{6',7'}$ = 8.1 Hz, 1H, H-6′), 7.24 (d, $J_{5'',6''}$ = 8.1 Hz, 1H, H-5″), 6.66 (m, 2H, H-8, H-8′), 5.60 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.5, 160.5, 156.1, 152.0, 152.0, 151.8, 151.8, 145.5, 145.5, 138.7, 132.9, 132.9, 132.2, 131.9, 124.3, 124.3, 123.8, 123.4, 117.3, 116.2, 116.1, 114.1, 112.2, 28.3; FAB (Neg.)-MS m/z = 441 [M-H-H_2O]$^{-1}$; ESI-MS m/z = 443 [M+H-H_2O]$^{+}$; HRESI-MS Calcd for $C_{25}H_{15}O_8$ [M+H-H_2O]$^{+}$: m/z = 443.0766, Found 443.0744.

4.20. 3,3′-((2′′,3′′-Dimethylphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (18)

White Solid; Yield: 73%; M.p.: 203–205 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.81 (d, $J_{5,6/5',6'}$ = 7.5 Hz, 2H, H-5, H-5′), 7.49 (t, $J_{7,6}$ = $J_{7,8}$ = $J_{7',6'}$ = $J_{7',8'}$ = 6.9 Hz, 2H, H-7, H-7′), 7.24 (d, $J_{8,7/8',7'}$ = 8.1 Hz, 2H, H-8, H-8′), 7.24 (t, $J_{6,5}$ = $J_{6,7}$ = $J_{6',5'}$ = $J_{6',7'}$ = 8.1 Hz, 2H, H-6, H-6′), 7.14 (d, $J_{4'',5''}$ = 8.4 Hz, 1H, H-4″), 6.80 (m, 2H, H-5″, H-6″), 6.02 (s, 1H, −CH−), 2.16 (s, 3H, CH_3), 2.01 (s, 1H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.2, 162.2, 161.4, 161.4, 152.6, 152.6, 136.8, 135.7, 135.4, 128.2, 128.2, 127.5, 125.4, 125.3, 125.3, 123.4, 123.4, 121.4, 116.6, 116.6, 112.2, 112.2, 100.0, 100.0, 28.5, 19.3, 17.2; FAB (Neg.)-MS m/z = 439 [M-H]$^{-1}$; ESI-MS m/z = 441 [M+H]$^{+}$; HRESI-MS Calcd for $C_{27}H_{21}O_6$ [M+H]$^{+}$: m/z = 441.1338, Found 441.1328.

4.21. 3,3′-((5′′-Chloro-2′′-hydroxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (19)

White Solid; Yield: 78%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.10 (d, $J_{5,6}$ = 7.5 Hz, 1H, H-5), 8.04 (bd.d, 1H, H-5′), 7.73 (t, $J_{7,6}$ = $J_{7,8}$ = 8.7 Hz, 1H, H-7), 7.62 (t, $J_{7',6'}$ = $J_{7',8'}$ = 8.4 Hz, 1H, H-7′), 7.51 (m, 6H, H-6, H-8, H-6′, H-8′, H-3″, H-4″), 7.18 (s, 1H, H-6″), 5.70 (bd.s, 1H, −CH−); FAB (Neg.)-MS m/z = 461 [M-H]$^{-1}$, 463 [M+2-H]$^{-1}$; ESI-MS m/z = 445 [M+H-H_2O]$^{+}$; HRESI-MS Calcd for $C_{25}H_{14}ClO_6$ [M+H-H_2O]$^{+}$: m/z = 445.0478, Found 445.0453.

4.22. 3,3′-((2′′,4′′-Dihydroxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (20)

White Solid; Yield: 82%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.68 (s, 1H, OH), 8.07 (d, $J_{5,6}$ = 6.9 Hz, 1H, H-5), 7.95 (bd.s, 1H, H-5′), 7.69 (t, $J_{7,6}$ = $J_{7,8}$ = 7.2 Hz, 1H, H-7), 7.52 (t, $J_{7',6'}$ = $J_{7',8'}$ = 7.2 Hz, 1H, H-7′), 7.48 (t, $J_{6,5}$ = $J_{6,7}$ = 8.1 Hz, 1H, H-6), 7.43 (d, $J_{8,7}$ = 7.5 Hz, 1H, H-8), 7.25 (m, 2H, H-6′, H-8′), 7.00 (d, $J_{6'',5''}$ = 8.4 Hz, 1H, H-6″), 6.67 (d, $J_{3'',5''}$ = 2.1 Hz, 1H, H-3″), 6.55 (dd, $J_{5'',3''}$ = 2.1 Hz, $J_{5'',6''}$ = 6.0 Hz, 1H, H-5″), 5.57 (s, 1H, −CH−); FAB (Neg.)-MS m/z = 425 [M-H-H_2O]$^{-1}$; ESI-MS m/z = 427 [M+H-H_2O]$^{+}$; HRESI-MS Calcd for $C_{25}H_{15}O_7$ [M+H-H_2O]$^{+}$: m/z = 427.0817, Found 427.0816.

4.23. 3,3′-((3′′,5′′-Dibromo-4-hydroxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (21)

White Solid; Yield: 87%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.82 (d, $J_{5,6/5',6'}$ = 6.3 Hz, 2H, H-5, H-5′), 7.53 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 6.9 Hz, 2H, H-7, H-7′), 7.27 (d, $J_{8,7/8',7'}$ = 8.1 Hz, 2H, H-8, H-8′), 7.26 (t, $J_{6,5} = J_{6,7} = J_{6',5'} = J_{6',7'}$ = 7.5 Hz, 2H, H-6, H-6′), 7.12 (bd.s, 2H, H-2″, H-6″), 6.16 (s, 1H, −CH−); ^{13}C NMR (75 MHz, DMSO-d_6): δ 167.2, 167.2, 164.2, 164.2, 152.4, 152.4, 148.1, 148.1, 136.6, 131.3, 131.3, 130.2, 130.2, 124.0, 124.0, 123.1, 123.1, 119.3, 119.3, 115.6, 115.6, 111.7, 102.9, 102.9, 35.0; FAB (Neg.)-MS m/z = 584 [M-H]$^{-1}$, 586 [M+2-H]$^{-1}$; ESI-MS m/z = 585 [M+H]$^{+}$, 587 [M+2+H]$^{+}$, 589 [M+4+H]$^{+}$; HRESI-MS Calcd for $C_{25}H_{15}Br_2O_7$ [M+H]$^{+}$: m/z = 584.9184, Found 584.9208.

4.24. 3,3′-((3′′,5′′-Dichlorophenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (22)

White Solid; Yield: 82%; M.p.: 232–234 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 8.14 (d, $J_{5,6}$ = 6.3 Hz, 1H, H-5), 8.08 (d, $J_{5',6'}$ = 6.6 Hz, 1H, H-5′), 7.76 (t, $J_{7,6} = J_{7,8}$ = 8.4 Hz, 1H, H-7), 7.62 (t, $J_{7',6'} = J_{7',8'}$ = 7.5 Hz, 1H, H-7′), 7.55 (t, $J_{6,5} = J_{6,7}$ = 7.5 Hz, 1H, H-6), 7.52 (d, $J_{2'',6''/6'',2''}$ = 2.4 Hz, 1H, H-2″, H-6″), 7.43 (t, $J_{6',5'} = J_{6',7'}$ = 8.4 Hz, 1H, H-6′), 7.38 (d, $J_{8,7}$ = 7.2 Hz, 1H, H-8), 7.32 (d, $J_{4'',2''/4'',6''}$ = 2.4 Hz, 1H, H-4″), 7.27 (d, $J_{8',7'}$ = 8.1 Hz, 1H, H-8′), 5.74 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.7, 162.7, 161.6, 161.6, 152.4, 152.4, 145.2, 135.7, 135.7, 128.4, 128.4, 126.8, 126.8, 126.3, 125.3, 125.3, 123.4, 123.4, 116.5, 116.5, 116.3, 116.3, 100.1, 100.1, 28.4; FAB (Neg.)-MS m/z = 476 [M-H]$^{-1}$, 478 [M+2-H]$^{-1}$; ESI-MS m/z = 479 [M+H]$^{+}$, 481 [M+2+H]$^{+}$; HRESI-MS Calcd for $C_{25}H_{15}Cl_2O_6$ [M+H]$^{+}$: m/z = 479.0089, Found 479.0105.

4.25 . 3,3′-((2′′,5′′-Dimethoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (23)

White Solid; Yield: 74%; M.p.: 181–183 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.87 (dd, $J_{5,7/5',7'}$ = 1.2 Hz, $J_{5,6/5',6'}$ = 6.6 Hz, 2H, H-5, H-5′), 7.56 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 8.4 Hz, 2H, H-7, H-7′), 7.31 (d, $J_{8,7/8',7'}$ = 7.8 Hz, 2H, H-8, H-8′), 7.30 (t, $J_{6,7} = J_{6,8} = J_{6',7'} = J_{6',8'}$ = 7.5 Hz, 2H, H-6, H-6′), 6.79 (d, $J_{4'',3''}$ = 8.4 Hz, 1H, H-4″), 6.71 (s, 1H, H-6″), 6.71 (d, $J_{3'',4''}$ = 8.7 Hz, 1H, H-3″), 6.19 (s, 1H, −CH−), 3.60 (s, 3H, OCH_{3a}), 3.48 (s, 3H, OCH_{3b}); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.1, 163.9, 152.7, 152.1, 151.6, 131.1, 131.1, 123.7, 123.7, 123.2, 123.2, 118.5, 116.2, 115.6, 115.6, 111.8, 109.6, 104.2, 56.1, 55.0, 32.9; FAB (Neg.)-MS m/z = 470 [M-H]$^{-1}$; ESI-MS m/z = 473 [M+H]$^{+}$; HRESI-MS Calcd for $C_{27}H_{20}O_8$ [M+H]$^{+}$: m/z = 473.1236, Found 473.1198.

4.26. 3,3′-((2′′,3′′-Dimethoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (24)

White Solid; Yield: 76%; M.p.: 190–192 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.85 (dd, $J_{5,7/5',7'}$ = 1.2 Hz, $J_{5,6/5',6'}$ = 6.9 Hz, 2H, H-5, H-5′),7.53 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 8.4 Hz, 2H, H-7, H-7′), 7.28 (d, $J_{8,7/8',7'}$ = 8.4 Hz, 2H, H-8, H-8′), 7.27 (t, $J_{6,7} = J_{6,8} = J_{6',7'} = J_{6',8'}$ = 7.5 Hz, 2H, H-6, H-6′), 6.89 (m, 3H, H-4″, H-5″, H-6″), 6.29 (s, 1H, −CH−), 3.71 (s, 3H, OCH_{3b}), 3.44 (s, 3H, OCH_{3a}); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.0, 164.0, 152.2, 152.0, 146.5, 135.1, 131.2, 131.2, 123.7, 123.7, 123.3, 123.3, 122.5, 120.8, 118.5, 115.7, 115.7, 110.7, 104.6, 59.2, 55.4, 32.7; FAB (Neg.)-MS m/z = 471 [M-H^{+}]$^{-1}$. FAB (Neg.)-MS m/z = 471 [M-H]$^{-1}$; ESI-MS m/z = 473 [M+H]$^{+}$; HRESI-MS Calcd for $C_{27}H_{21}O_8$ [M+H]$^{+}$: m/z = 473.1236, Found 473.1207.

4.27. 3,3′-((4′′-Ethoxy-3′′-methoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (25)

White Solid; Yield: 78%; M.p.: 195–197 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.81 (dd, $J_{5,7/5',7'}$ = 1.5 Hz, $J_{5,6/5',6'}$ = 6.3 Hz, 2H, H-5, H-5′), 7.50 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 6.9 Hz, 2H, H-7, H-7′), 7.25 (d, $J_{8,7/8',7'}$ = 8.1 Hz, 2H, H-8, H-8′), 7.23 (t, $J_{6,7} = J_{6,8} = J_{6',7'} = J_{6',8'}$ = 7.2 Hz, 2H, H-6, H-6′), 6.73 (d, $J_{5'',6''}$ = 8.7 Hz, 1H, H-5″), 6.64 (s, 1H, H-2″), 6.60 (d, $J_{6'',5''}$ = 8.7 Hz, 1H, H-6″), 6.18 (s, 1H, −CH−), 3.92 (q, 2H, OCH_2−), 3.50 (s, 3H, OCH_3), 1.29 (t, 3H, CH_3); FAB (Neg.)-MS m/z = 485 [M-H]$^{-1}$; ESI-MS m/z = 487 [M+H]$^{+}$; HRESI-MS Calcd for $C_{28}H_{23}O_8$ [M+H]$^{+}$: m/z = 487.1392, Found 487.1385.

4.28. 3,3′-((2′′,4′′-Dimethoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (26)

White Solid; Yield: 72%; M.p.: 197–199 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.82 (d, $J_{5,6/5',6'}$ = 6.9 Hz, 2H, H-5, H-5′), 7.50 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 8.4 Hz, 2H, H-7, H-7′), 7.25 (d, $J_{8,7/8',7'}$ = 8.1 Hz, 2H, H-8, H-8′),7.22 (t, $J_{6,7} = J_{6,8} = J_{6',7'} = J_{6',8'}$ = 7.8 Hz, 2H, H-6, H-6′), 7.06 (d, $J_{6'',5''}$ = 8.1 Hz, 1H, H-6″), 6.39 (m, 2H, H-3″, H-5″), 6.12 (s, 1H, −CH−), 3.68 (s, 3H, OCH_{3b}), 1.29 (t, 3H, OCH_{3a}); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.4, 163.9, 158.8, 158.0, 152.0, 131.2, 131.2, 128.7, 123.6, 123.6, 123.3, 123.3, 120.9, 118.2, 115.7, 115.7, 104.6, 103.8, 98.5, 55.5, 55.0, 32.2; FAB (Neg.)-MS m/z = 470 [M-H]$^{-1}$; ESI-MS m/z = 473 [M+H]$^{+}$; HRESI-MS Calcd for $C_{27}H_{21}O_8$ [M+H]$^{+}$: m/z = 473.1236, Found 473.1230.

4.29. 3,3′-((4′′-Bromo-2′′-fluorophenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (27)

White Solid; Yield: 83%; M.p.: 245–247 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.81 (d, $J_{5,6/5',6'}$ = 6.6 Hz, 2H, H-5, H-5′), 7.52 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 8.4 Hz, 2H, H-7, H-7′), 7.27 (m, 7H, H-6, H-8, H-6′, H-8′, H-3″, H-5″, H-6″), 6.24 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.9, 162.5, 162.2, 160.4, 160.0, 151.4, 150.9, 132.6, 128.4, 127.2, 125.7, 125.2, 121.5, 120.5, 119.8, 119.4, 117.7, 117.4, 114.7, 114.3, 109.8, 109.3, 101.7, 101.5, 28.6; FAB (Neg.)-MS m/z = 507 [M-H]$^{-1}$, 509 [M+2-H]$^{-1}$; ESI-MS m/z = 509 [M+H]$^{+}$, 511 [M+2+H]$^{+}$; HRESI-MS Calcd for $C_{25}H_{15}BrFO_6$ [M+H]$^{+}$: m/z = 509.0036, Found 509.0032.

4.30. 3,3′-((2′′-Bromo-4′′,5′′-dimethoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (28)

White Solid; Yield: 85%; M.p.: 200–202 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.83 (d, $J_{5,6/5',6'}$ = 6.6 Hz, 2H, H-5, H-5′), 7.26 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 7.2 Hz, 2H, H-7, H-7′), 7.26 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 7.25 (t, $J_{6,7} = J_{6,8} = J_{6',7'} = J_{6',8'}$ = 8.1 Hz, 2H, H-6, H-6′), 7.01 (s, 1H, H-3″), 6.97 (s, 1H, H-6″), 5.97 (s, 1H, −CH−), 3.70 (s, 3H, OCH_{3a}), 1.29 (t, 3H, OCH_{3b}); ^{13}C NMR (100 MHz, DMSO-d_6): δ 190.1, 165.3, 163.5, 152.2, 147.6, 147.1, 133.0, 131.2, 131.2, 123.7, 123.7, 123.3, 123.3, 118.4, 116.0, 115.7, 115.7, 114.4, 113.4, 110.5, 104.1, 55.7, 55.6, 37.9; FAB (Neg.)-MS m/z = 549 [M-H]$^{-1}$, 551 [M+2-H]$^{-1}$; ESI-MS m/z = 551 [M+H]$^{+}$, 553 [M+2+H]$^{+}$; HRESI-MS Calcd for $C_{27}H_{20}BrO_8$ [M+H]$^{+}$: m/z = 551.0341, Found 551.0327.

4.31. 3,3′-((3′′-(Benzyloxy)-4′′-methoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (29)

White Solid; Yield: 81%; M.p.: 210–212 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.44 (s, 1H, OH), 8.01 (d, $J_{5,6/5',6'}$ = 6.9 Hz, 2H, H-5, H-5′), 7.74 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 7.2 Hz, 2H, H-7, H-7′), 7.48 (t, $J_{6,7} = J_{6,8} = J_{6',7'} = J_{6',8'}$ = 8.1 Hz, 2H, H-6, H-6′), 7.48 (d, $J_{8,7/8',7'}$ = 7.8 Hz, 2H, H-8, H-8′), 7.26 (m, 2H, H-2‴, H-6‴), 7.20 (m, 3H, H-

3‴, H-4‴, H-6‴), 6.97 (bd.s, 1H, H-2‴), 6.93 (d, $J_{6'',5''}$ = 7.2 Hz, 1H, H-6″), 6.87 (d, $J_{5'',6''}$ = 7.2 Hz, 1H, H-5″), 6.04 (s, 1H, −CH−), 4.96 (s, 2H, −CH−), 3.80 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.9, 162.9, 161.7, 161.7, 152.6, 152.6, 149.8, 146.6, 136.5, 135.7, 128.7, 128.7, 128.4, 128.4, 127.4, 127.2, 127.2, 125.5, 125.5, 123.2, 123.2, 122.4, 116.7, 116.7, 116.2, 116.2, 114.2, 112.4, 100.4, 100.4, 71.2, 28.7, 56.2; FAB (Neg.)-MS m/z = 546 $[M\text{-}H]^{-1}$; ESI-MS m/z = 549 $[M+H]^+$; HRESI-MS Calcd for $C_{33}H_{25}O_8$ $[M+H]^+$: m/z = 549.1549, Found 549.1550.

4.32. 3,3′-((4′′-Hydroxy-3′′-methoxyphenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (30)

White Solid; Yield: 84%; M.p.: 255–257 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.45 (s, 2H, 2OH), 8.02 (d, $J_{5,6/5',6'}$ = 7.2 Hz, 2H, H-5, H-5′), 7.75 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 7.5 Hz, 2H, H-7, H-7′), 7.51 (m, 4H, H-6, H-8, H-6′, H-8′), 6.95 (s, 1H, H-2″), 6.77 (s, 2H, H-5″, H-6″), 6.08 (s, 1H, −CH−), 3.67 (s, 3H, OCH_3); FAB (Neg.)-MS m/z = 457 $[M\text{-}H]^{-1}$; ESI-MS m/z = 459 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{19}O_8$ $[M+H]^+$: m/z = 459.1079, Found 459.1053.

4.33. 3,3′-((4′′-(Methylthio)phenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (31)

White Solid; Yield: 77%; M.p.: 260–262 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 11.50 (s, 2H, 2OH), 8.03 (d, $J_{5,6/5',6'}$ = 8.4 Hz, 2H, H-5, H-5′), 7.76 (t, $J_{7,6} = J_{7,8} = J_{7',6'} = J_{7',8'}$ = 6.9 Hz, 2H, H-7, H-7′), 7.49 (m, 4H, H-6, H-8, H-6′, H-8′), 7.29 (d, $J_{2'',3''/6'',5''}$ = 8.1 Hz, 2H, H-2″, H-6″), 7.23 (d, $J_{3'',2''/5'',6''}$ = 8.7 Hz, 2H, H-3″, H-5″), 6.10 (s, 1H, −CH−), 2.46 (s, 3H, SCH_3); FAB (Neg.)-MS m/z = 457 $[M\text{-}H]^{-1}$; ESI-MS m/z = 459 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{19}O_6S$ $[M+H]^+$: m/z = 459.0902, Found 459.0922.

4.34. 3,3′-((3′′-Bromo-4′′-methoxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (32)

White Solid; Yield: 85%; M.p.: 204–206 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.73 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.55 (dd, $J_{7,5/7',5'}$ = 2.4 Hz, $J_{7,8/7',8'}$ = 9.0 Hz, 2H, H-7, H-7′), 7.32 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 7.15 (bd.s, 1H, H-2″), 7.04 (bd.d, $J_{6'',5''}$ = 8.4 Hz, 1H, H-6″), 6.92 (d, $J_{5'',6''}$ = 8.7 Hz, 1H, H-5″), 6.15 (s, 1H, −CH−), 3.76 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.3, 163.9, 153.1, 151.0, 135.3, 130.8, 130.8, 130.7, 127.2, 127.2, 123.2, 123.2, 121.1, 117.7, 117.7, 112.3, 110.0, 103.8, 56.0, 32.2; FAB (Neg.)-MS m/z = 587 $[M\text{-}H]^{-1}$; 589 $[M+2\text{-}H]^{-1}$; 591 $[M+4\text{-}H]^{-1}$; ESI-MS m/z = 599 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{15}BrCl_2O_7$ $[M+H]^+$: m/z = 588.9456, Found 588.9479.

4.35 . 3,3′-((3′′-(Benzyloxy)phenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (33)

White Solid; Yield: 80%; M.p.: 192–194 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.72 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.54 (dd, $J_{7,5/7',5'}$ = 2.4 Hz, $J_{7,8/7',8'}$ = 6.3 Hz, 2H, H-7, H-7′), 7.31 (m, 4H, H-8, H-8′, H-3‴, H-5‴), 7.24 (m, 3H, H-2‴, H-4‴, H-6‴), 7.09 (t, $J_{5'',4''} = J_{5'',6''}$ = 8.1 Hz, 1H, H-5″), 6.74 (d, $J_{6'',5''}$ = 8.1 Hz, 1H, H-6″), 6.64 (m, 2H, H-2″, H-4″), 6.18 (s, 1H, −CH−), 4.94 (s, 3H, OCH_2); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.1, 166.1, 164.0, 164.0, 158.3, 151.0, 151.0, 143.2, 137.2, 130.8, 130.8, 128.8, 128.2, 128.2, 127.7, 127.6, 127.6, 127.2, 127.2, 123.2, 123.2, 121.1, 119.3, 119.3, 117.7, 117.7, 113.7, 110.9, 104.0, 104.0, 69.1, 36.2; FAB (Neg.)-MS m/z = 585 $[M\text{-}H]^{-1}$, 587 $[M+2\text{-}H]^{-1}$; ESI-MS m/z = 587 $[M+H]^+$; HRESI-MS Calcd for $C_{32}H_{21}Cl_2O_7$ $[M+H]^+$: m/z = 587.0664, Found 587.0666.

4.36. 3,3′-((2′′,3′′,4′′-Trihydroxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (34)

White Solid; Yield: 86%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.26 (bd.s, 1H, OH), 9.06 (bd.s, 1H, OH), 8.56 (d, $J_{5,7}$ = 2.7 Hz, 2H, H-5), 7.95 (bd.s, 1H, H-5′), 7.72 (d, $J_{7,8}$ = 8.7 Hz, 1H, H-7), 7.58 (d, $J_{7',8'}$ = 9.0 Hz, 1H, H-7′), 7.47 (d, $J_{8,7}$ = 9.0 Hz, 1H, H-8), 7.31 (d, $J_{8',7'}$ = 8.4 Hz, 1H, H-8′), 6.55 (d, $J_{6'',5''}$ = 8.4 Hz, 1H, H-6″), 6.46 (d, $J_{5'',6''}$ = 8.4 Hz, 1H, H-5″), 5.56 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.0, 165.7, 164.1, 163.6, 151.4, 151.0, 148.6, 147.4, 133.9, 131.3, 130.9, 129.5, 129.2, 126.7, 126.4, 124.5, 122.9, 122.5, 118.9, 118.6, 116.7, 100.5, 100.2, 108.6, 30.6; FAB (Neg.)-MS m/z = 509 $[M\text{-}H\text{-}H_2O]^{-1}$; ESI-MS m/z = 511 $[M+H\text{-}H_2O]^+$; HRESI-MS Calcd for $C_{25}H_{14}Cl_2O_9$ $[M+H\text{-}H_2O]^+$: m/z = 511.0093, Found 511.0062.

4.37. 3,3′-((3′′,5′′-Dibromo-4′′-hydroxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (35)

White Solid; Yield: 82%; M.p.: > 300 °C dec.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.74 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.55 (dd, $J_{7,5/7',5'}$ = 2.4 Hz, $J_{7,8/7',8'}$ = 6.3 Hz, 2H, H-7, H-7′), 7.32 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 7.13 (s, 2H, H-2″, H-6″), 6.13 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.4, 166.4, 164.3, 164.3, 151.3,150.8, 150.8, 139.0, 134.5, 134.5, 131.0, 131.0, 129.5, 129.5, 126.8, 126.8, 122.9, 122.9, 118.8, 118.8, 110.3, 110.3, 100.2, 100.2, 35.2; FAB (Neg.)-MS m/z = 651 $[M\text{-}H]^{-1}$; 653 $[M+2\text{-}H]^{-1}$; ESI-MS m/z = 653 $[M+H]^+$; HRESI-MS Calcd for $C_{25}H_{12}Br_2Cl_2O_7$ $[M+H]^+$: m/z = 652.8405, Found 652.8436.

4.38. 3,3′-((2′′-Hydroxynaphthalen-1′′-yl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (36)

White Solid; Yield: 86%; M.p.: 252–254 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.83 (s, 2H, 2OH), 9.13 (s, 1H, OH), 8.66 (d, $J_{5'',6''/8'',7''}$ = 8.4 Hz, 2H, H-5″, H-8″), 8.32 (d, $J_{4'',3''}$ = 9.3 Hz, 1H, H-4″), 8.11 (d, $J_{3'',4''}$ = 7.8 Hz, 1H, H-3″), 7.79 (t, $J_{6'',5''} = J_{6'',7''}$ = 7.5 Hz, 1H, H-6″), 7.67 (m, 4H, −CH−, H-5, H-5′, H-7′′), 7.51 (dd, $J_{7,5/7',5'}$ = 2.4 Hz, $J_{7,8/7',8'}$ = 8.7 Hz, 2H, H-7, H-7′), 6.94 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 190.4, 158.1, 156.9, 154.3, 139.6, 139.6, 135.1, 135.1, 134.2, 134.2, 129.9, 129.4, 129.4, 129.2, 129.0, 129.0, 128.8, 128.8, 127.5, 126.4, 125.7, 122.9, 122.5, 122.5, 118.9, 118.9, 116.5, 112.7, 28.6; FAB (Neg.)-MS m/z = 440 $[M-H-2H_2O-2Cl]^{-1}$; ESI-MS m/z = 529 $[M+H\text{-}H_2O]^+$; HRESI-MS Calcd for $C_{29}H_{16}Cl_2O_7$ $[M+H\text{-}H_2O]^+$: m/z = 529.0245, Found 529.0268.

4.39. 3,3′-((4′′-Hydroxy-3′′-iodo-5′′-methoxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (37)

White Solid; Yield: 80%; M.p.: 234–236 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.74 (d, $J_{5,7/5',7'}$ = 2.7 Hz, 2H, H-5, H-5′), 7.54 (dd, $J_{7,5/7',5'}$ = 2.7 Hz, $J_{7,8/7',8'}$ = 6.3 Hz, 2H, H-7, H-7′), 7.31 (d, $J_{8,7/8',7'}$ = 9.0 Hz, 2H, H-8, H-8′), 6.90 (bd.s, 1H, H-2″), 6.64 (bd.s, 1H, H-6″), 6.12 (s, 1H, −CH−), 3.57 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.1, 166.1, 163.8, 163.8, 150.9, 150.9, 146.7, 143.9, 134.4, 130.7, 130.7, 127.8, 127.8, 127.2, 127.2, 123.1, 123.1, 121.0, 117.7, 117.7, 111.2, 104.0, 104.0, 84.1, 55.9, 35.4; FAB (Neg.)-MS m/z = 651 $[M\text{-}H]^{-1}$, 653 $[M+2\text{-}H]^{-1}$; ESI-MS m/z = 653 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{16}Cl_2IO_8$ $[M+H]^+$: m/z = 652.9267, Found 652.9280.

4.40. 3,3′-((5′′-Bromo-2′′-methoxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (38)

White Solid; Yield: 81%; M.p.: 251–253 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.73 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.52 (dd, $J_{7,5/7',5'}$ = 2.7 Hz, $J_{7,8/7',8'}$ = 6.0 Hz, 2H, H-7, H-7′), 7.29 (d, $J_{8,7/}$

$_{8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 7.23 (d, $J_{3'',4''}$ = 6.9 Hz, 1H, H-3″), 7.21 (s, 1H, H-6″), 6.80 (d, $J_{4'',3''}$ = 8.4 Hz, 1H, H-4″), 6.15 (s, 1H, −CH−), 3.53 (s, 1H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.2, 166.0, 163.7, 163.5, 157.7, 150.7, 150.5, 134.6, 131.2, 131.0, 129.8, 129.6, 126.7, 126.5, 129.4, 118.8, 118.6, 115.5, 113.6, 100.3, 100.1, 123.4, 122.8, 122.6, 56.2, 29.7; FAB (Neg.)-MS m/z = 587 $[M-H]^{-1}$, 589 $[M+2-H]^{-1}$, 591 $[M+4-H]^{-1}$; ESI-MS m/z = 599 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{15}BrCl_2O_7$ $[M+H]^+$: m/z = 588.9456, Found 588.9478.

4.41. 3,3′-((2″,5″-Dihydroxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (39)

White Solid; Yield: 76%; M.p.: 274–276 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 9.37 (s, 1H, OH), 8.07 (d, $J_{5,7}$ = 2.1 Hz, 1H, H-5), 8.00 (bd.s, 1H, H-5′), 7.74 (dd, $J_{7,5}$ = 2.4 Hz, $J_{7,8}$ = 6.3 Hz, 1H, H-7), 7.62 (d, $J_{7',8'}$ = 7.5 Hz, 1H, H-7′), 7.49 (d, $J_{8,7}$ = 8.7 Hz, 1H, H-8), 7.35 (d, $J_{8',7'}$ = 8.7 Hz, 1H, H-8′), 7.21 (d, $J_{3'',4''}$ = 8.7 Hz, 1H, H-3″), 6.67 (d, $J_{4'',3''}$ = 6.0 Hz, 1H, H-4″), 6.58 (bd.s, 1H, H-6″), 5.62 (bd.s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.2, 165.9, 164.3, 164.0, 151.3, 151.0, 149.2, 148.7, 131.4, 131.1, 129.3, 129.0, 126.5, 126.2, 124.3, 122.7, 122.5, 118.7, 118.4, 117.2, 116.7, 114.6, 104.6, 104.3, 30.1; FAB (Neg.)-MS m/z = 493 $[M-H-H_2O]^{-1}$; 495 $[M+2-H]^{-1}$; ESI-MS m/z = 495 $[M+H]^+$; HRESI-MS Calcd for $C_{25}H_{14}Cl_2O_8$ $[M+H]^+$: m/z = 495.0038, Found 495.0065.

4.42. 3,3′-((3″-Bromo-4″-hydroxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (40)

White Solid; Yield: 75%; M.p.: 248–250 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 9.84 (bd.s, 2H, 2OH),7.73 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.55 (dd, $J_{7,5/7',5'}$ = 2.7 Hz, $J_{7,8/7',8'}$ = 6.0 Hz, 2H, H-7, H-7′), 7.31 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 7.06 (bd.s, 1H, H-2″), 6.88 (d, $J_{6'',5''}$ = 8.7 Hz, 1H, H-6″), 6.76 (d, $J_{5'',6''}$ = 8.4 Hz, 1H, H-5″), 6.11 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.1, 166.1, 163.9, 163.9, 151.5, 151.5, 151.0, 133.5, 133.5, 130.8, 130.5, 127.2, 127.2, 127.1, 123.2, 123.2, 121.0, 121.0, 117.7, 117.7, 116.0, 108.7, 103.9, 103.9, 35.2; FAB (Neg.)-MS m/z = 573 $[M-H]^{-1}$, 575 $[M+2-H]^{-1}$; ESI-MS m/z = 575 $[M+H]^+$; HRESI-MS Calcd for $C_{25}H_{13}BrCl_2O_7$ $[M+H]^+$: m/z = 574.9300, Found 574.9326.

4.43. 3,3′-((2″-Bromo-5″-fluorophenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (41)

White Solid; Yield: 79%; M.p.: 273–275 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 7.74 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.55 (dd, $J_{7,5/7',5'}$ = 2.7 Hz, $J_{7,8/7',8'}$ = 6.0 Hz, 2H, H-7, H-7′), 7.49 (m, 1H, H-3″), 7.31 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 7.11 (dd, $J_{6'',4'''}$ = 3.0 Hz, $J_{6'',F}$ = 7.8 Hz, 1H, H-6″), 6.99 (m, 1H, H-4″), 5.97 (s, 1H, −CH−); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.0, 166.0, 163.4, 163.4, 161.6, 150.9, 150.9, 142.4, 133.2, 131.4, 131.4, 129.7, 129.7, 126.8, 126.8, 122.7, 122.7, 119.3, 118.7, 118.7, 118.6, 114.5, 104.2, 104.2, 31.8; FAB (Neg.)-MS m/z = 574 $[M-H]^{-1}$, 576 $[M+2-H]^{-1}$, 578 $[M+4-H]^{-1}$; ESI-MS m/z = 579 $[M+H]^+$; HRESI-MS Calcd for $C_{25}H_{13}BrCl_2FO_6$ $[M+H]^+$: m/z = 576.9256, Found 576.9296.

4.44. 3,3′-((4″-Bromo-3″,5″-dimethoxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (42)

White Solid; Yield: 82%; M.p.: 276–278 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 7.74 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.54 (dd, $J_{7,5/7',5'}$ = 2.7 Hz, $J_{7,8/7',8'}$ = 6.3 Hz, 2H, H-7, H-7′), 7.31 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 6.45 (s, 2H, H-2″, H-6″), 6.19 (s, 1H, −CH−), 3.58 (s, 6H, $2OCH_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.0, 160.0, 159.2, 159.2, 155.2, 155.2, 150.6, 150.6, 149.7, 132.2, 132.2, 131.8, 131.8, 129.1, 129.1, 128.7, 128.0, 118.6, 118.6, 117.7, 117.7, 112.2, 112.2, 101.5, 55.5, 55.5, 28.2; FAB (Neg.)-MS m/z = 617 $[M-H]^{-1}$, 619 $[M+2-H]^{-1}$, 621 $[M+4-H]^{-1}$; ESI-MS m/z = 619 $[M+H]^+$; HRESI-MS Calcd for $C_{27}H_{17}BrCl_2O_8$ $[M+H]^+$: m/z = 618.9562, Found 618.9536.

4.45. 3,3′-((2″-Fluoro-4″-methoxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (43)

White Solid; Yield: 75%; M.p.: 243–245 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 8.13 (d, $J_{5,7}$ = 2.4 Hz, 1H, H-5), 7.99 (bd.s, 1H, H-5′), 7.74 (dd, $J_{7,5}$ = 2.8 Hz, $J_{7,8}$ = 6.0 Hz, 1H, H-7), 7.60 (bd.d, $J_{7',8'}$ = 7.2 Hz, 1H, H-7′), 7.50 (d, $J_{8,7}$ = 8.8 Hz, 1H, H-8), 7.33 (d, $J_{8',7'}$ = 9.2 Hz, 1H, H-8′), 7.08 (d, $J_{6'',5''}$ = 8.4 Hz, 1H, H-6″), 7.03 (d, $J_{3'',5''}$ = 2.4 Hz, 1H, H-3″), 6.73 (dd, $J_{5'',3''}$ = 2.4 Hz, $J_{5'',6''}$ = 6.3 Hz, 1H, H-5″), 5.61 (bd.s, 1H, −CH−), 3.77 (s, 1H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.0, 166.0, 163.8, 163.8, 155.9, 151.0, 151.0, 143.0, 131.2, 131.2, 130.8, 130.8, 127.2, 127.2, 123.1, 123.1, 120.8, 118.3, 117.8, 117.8, 104.0, 104.0, 103.9, 96.7, 56.0, 36.7; FAB (Neg.)-MS m/z = 527 $[M-H]^{-1}$, 529 $[M+2-H]^{-1}$; ESI-MS m/z = 529 $[M+H]^+$; HRESI-MS Calcd for $C_{26}H_{15}Cl_2FO_7$ $[M+H]^+$: m/z = 529.0257, Found 529.0257.

4.46. 3,3′-((3″-Bromo-4″-hydroxy-5″-methoxyphenyl)methylene)bis(6-chloro-4-hydroxy-2H-chromen-2-one) (44)

White Solid; Yield: 87%; M.p.: 282–284 °C; ^1H NMR (300 MHz, DMSO-d_6): δ 7.74 (d, $J_{5,7/5',7'}$ = 2.4 Hz, 2H, H-5, H-5′), 7.54 (dd, $J_{7,5/7',5'}$ = 2.4 Hz, $J_{7,8/7',8'}$ = 6.3 Hz, 2H, H-7, H-7′), 7.31 (d, $J_{8,7/8',7'}$ = 8.7 Hz, 2H, H-8, H-8′), 6.70 (bd.s, 1H, H-6″), 6.62 (bd.s, 1H, H-2″), 6.13 (s, 1H, −CH−), 3.58 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.1, 166.1, 163.8, 163.8, 150.9, 150.9, 148.1, 141.4, 133.4, 130.7, 130.7, 127.2, 127.2, 123.1, 123.1, 122.1, 121.0, 121.0, 117.7, 117.7, 110.2, 108.8, 103.9, 103.9, 56.0, 35.6; FAB (Neg.)-MS m/z = 604 $[M-H]^{-1}$, 606 $[M+2-H]^{-1}$; ESI-MS m/z = 604 $[M+H]^+$, 606 $[M+2+H]^+$ 608 $[M+4+H]^+_;$ HRESI-MS Calcd for $C_{26}H_{16}BrCl_2O_8$ $[M+H]^+$: m/z = 604.9405, Found 604.9448.

4.47. Urease inhibitory assay

Urease (EC 3.5.1.5) enzyme solution (25 µL) and 55 µL buffers, containing 100 mM urea, were incubated at 30 °C for 15 min along with 5 µL (1 mM concentration) test compounds in 96-well plates. Ammonia production was measured in order to determine urease activity by utilizing the indophenols method by Weatherburn [25]. Briefly, 45 µL phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. By using a microplate reader (Spectra Max, Molecular Devices, USA), increase in absorbance was recorded at 630 nm after 50 min. All reactions were carried out thrice in a final volume of 200 µL. The change in absorbance per min was processed by serving SoftMax Pro software (Molecular Devices, USA). All the assays were carried out at pH 8.2 (0.01 M $K_2HPO_4{\cdot}3H_2O$, 1 mM EDTA and 0.01 M LiCl). Thiourea was used as the standard inhibitor of urease. Percentage inhibitions were calculated from the following formula:

$$100 - (OD_{testwell}/OD_{control})x100$$

4.48. In vitro BSA-MG Anti-glycation assay

In this assay, BSA (Bovine serum albumin) of concentration (10 mg/mL) and (14 mM) methylglyoxal were prepared in phosphate buffer 0.1 M of pH 7.4, containing 3 mM sodium azide (NaN_3) which was used as antimicrobial agent. 1 mM test compounds were dissolved in the DMSO. Assay was performed in triplicate. Reaction mixtures contained 50 µL BSA, 50 µL methylglyoxal, 20 µL test compound, and 80 µL phosphate buffer of pH 7.4. The reaction mixture was incubated for 9 days at 37 °C under aseptic conditions. Fluorescence was recorded for excitation and emission at 330, and 420 nm on a microtitre plate reader

(Spectra Max M5, Molecular Devices, CA, USA) [30,31]. The percent inhibition of AGE formation was calculated by using the following formula:

$$\% \text{ Inhibition} = (1 - \text{Fluorescence of test sample/Fluorescence of the control})\text{x}100$$

The IC_{50} values were determined at different concentrations (1000–50 μM) of test compounds with the help of EZ-FIT Enzyme Kinetics Program (Perrella Scientific Inc., Amherst, USA). Rutin was used as a standard. In protein model system (BSA-MG glycation model), it showed an IC_{50} of 294 ± 1.5 μM.

4.49. 3T3 cell based (mouse Fibroblast) cytotoxicity assay

Standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-dimethyltetrazolium bromide) colorimetric assay was used to evaluate the cytotoxicity of thirteen *bis*-coumarin derivatives with antiglycation properties in 96-well flat-bottomed microplates. 3 T3 Cells (mouse fibroblasts) in Dulbecco's modified eagle's medium was cultured for this purpose, supplemented with streptomycin, penicillin, and 5% fetal bovine serum (FBS) by using a flask placed in a 5% CO_2 incubator at 37 °C. The growth of cells was harvested exponentially by using hemocytometer, the harvested cells was counted and in a particular medium was diluted. Cell cultures was prepared with a required concentration and plated onto 96-well plates. After overnight incubation, medium was removed and fresh medium was added with different concentrations of the compound. After 72 h, MTT was added to each well, and incubation was continued for 4 h. Subsequently, 100 μ*L* of DMSO was added to each well to solubilize formazan-MTT adduct, formed by the action of enzyme mitochondrial dehydrogenase. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 540 nm by using a microplate ELISA reader [32].

4.50. Statistical analysis

All experiments were conducted using (SpectraMax M5, molecular Devices, CA. USA). Different softweres were used to analyze the results i.e (MS-Excel, SoftMaxPro 4.8, and GraphPad Prism-6.0). In the end IC_{50} values of active compounds were examined by using EZ-FIT, Enzyme kinetics software (Perrella Scientific, Inc., USA)

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103170.

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