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7-Hydroxy-(*E*)-3-phenylmethylene-chroman-4-one analogues as efflux pump inhibitors against *Mycobacterium smegmatis* mc^2 155

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ABSTRACT

Efflux pump (EP) induces resistance in mycobacteria and hence could be explored as a new target for the discovery of anti-TB agents. In search for efflux pump inhibitors from natural products, bonducellin, a homoisoflavonoid was isolated from *Caesalpinia digyna* roots and evaluated for modulation and EP inhibitory activity. Bonducellin showed modulation in the MIC of EtBr by eight fold at a concentration of 62.5 mg/L and also showed significant EP inhibitory activity. A synthetic scheme was designed to prepare analogues of 7-hydroxy-(*E*)-3-phenylmethylene-chroman-4-one by modification at the phenylmethylene-ring and the synthesized compounds were evaluated in accumulation and efflux assays. Analogues **1**, **7**–**11**, **13**–**15**, **17** and **19** were found to be good modulators and decreased the MIC of EtBr by ≥4 fold at sub-inhibitory concentration. The compounds **8**, **13** and **17** were the most potent inhibitors of ethidium bromide efflux in *Mycobacterium smegmatis* mc^2 155.

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1. Introduction

There is an emergence of multi-drug resistance (MDR) and extensively drug-resistance (XDR) to the first line and second line anti-TB drugs because of mutation of the target protein, enzymatic inactivation of the antibiotic or inhibition of accumulation of antibiotics by over expression of Efflux Pump (EP) within the cell of bacteria. Hence, understanding drug resistance in *Mycobacterium tuberculosis* could serve as one of the measures for the disease control strategy. Numerous putative efflux pumps have been reported in *M. tuberculosis* [1]. The active multidrug efflux pumps (EPs) have been described as one of the mechanisms responsible for resistance in Mycobacteria [2]. Drug efflux pumps have been identified in several mycobacteria including *Mycobacterium smegmatis*. *M smegmatis* LfrA, a major facilitator superfamily transporter, was the first multidrug efflux pump reported for mycobacteria. The *M smegmatis* genome is reported to contain several genes encoding putative efflux pumps but with the exception of LfrA, it is not clear whether other genes contribute to the intrinsic drug resistance to this bacterium. However, Li X-Z et al showed that the homologues of *M. tuberculosis* Rv1145, Rv1146, Rv1877, Rv2846c (*efpA*) and Rv3065 (*mmr and emrE*) were detectable in the *M. smegmatis* mc^2 155, by reverse transcription-PCR technique [3]. It is also reported that deletion of *lfrA* (strain XZL1675) and *mmr* homologue rendered the strains more susceptible to several antimicrobials, such as ethidium bromide (EtBr), acriflavine and fluoroquinolones (Two to eight fold decrease in MICs) [3,4]. In recent years, it is observed that efflux pumps have important functions in other cellular functions, such as physiological homeostasis, resistance to stress conditions, lipid transport, and virulence in *M. tuberculosis* [5]. Bioinformatics as well as direct and indirect evidence have established efflux as a mechanism of drug resistance in *M. tuberculosis* [6]. *M. smegmatis* mc^2 155 is being used in recent years as the model system for expressing putative EP genes and to study the efflux mechanism, because it is rapidly growing strain and non-pathogenic to humans [4,7]. In search for bacterial efflux pump inhibitors (EPIs), natural products have been explored in the past and provided several small molecule natural product compounds as potential EPIs [8].

Considering the ethnomedicinal use of *Caesalpinia digyna* for the treatment of TB [9], homoisoflavonoids were isolated from *C. digyna* [10] and evaluated for modulation and efflux pump inhibitory (EPI)

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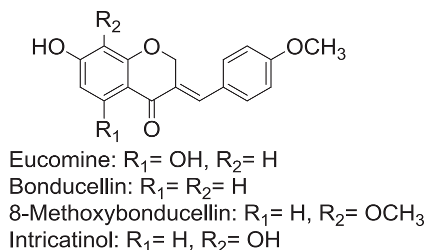


Fig. 1. Structure of isolated homoisoflavonoids studied for modulation effect.

activity in *M. smegmatis* mc^2 155. Standardized homoisoflavonoid-enriched fraction [11] prepared from methanol extract of *C. digyna* roots and four isolated homoisoflavonoids viz. bonducellin, 8-methoxybonducellin, intricatinol, eucomin (Fig. 1) were studied for modulation activity. The homoisoflavonoid enriched fraction and bonducellin showed significant modulation of MIC of ethidium bromide (EtBr) against *M. smegmatis* mc^2 155. Further bonducellin with a basic structural skeleton, 7-hydroxy-(*E*)-3-phenylmet hylene-chroman-4-one, exhibited significant accumulation and efflux effects. Based on these observations, a synthetic scheme was designed and analogues of 7-hydroxy-(*E*)-3-phenylmethylene-chroman-4-one were synthesized by modification in phenyl methylene-ring using various aldehydes [12–14]. The synthesized analogues were evaluated for MIC and modulation of EtBr, isoniazid (INH) and ofloxacin against *M. smegmatis* mc^2 155. Those compounds showing modulation were further tested for accumulation and EPI activity. The dose dependent effect was determined for the most active compounds.

2. Results and discussion

2.1. Chemistry

Bonducellin, 8-methoxybonducellin, intricatinol, and eucomin were isolated from the methanolic extract of *C. digyna* as reported previously by us [10]. The methoxy group at the *para* position in phenylmethylene-ring of bonducellin was replaced by several other functional groups as shown in Scheme 1 by condensation of 7-hydroxy-chroman-4-one (**II**) with aromatic aldehydes to give the compound **1**–**19** [15]. 7-Hydroxy-chroman-4-one (**II**) was synthesized by acylation of resorcinol with 3-chloropropionic acid in the presence of trifluoromethanesulphonic acid to yield an intermediate **I** which was then cyclized using sodium hydroxide (Scheme 1). The *E*-configuration of the synthesized homoisoflavonoids (**1**–**19**) was confirmed by a characteristic signal (a singlet) of vinylic proton of the *trans* (*E*) double bond at C_3–C_9, appearing downfield at δ_H 7.58–7.79 ppm as compared to δ_H 6.78–7.07 ppm for the *Z*-isomer [14,16].

2.2. Biology

2.2.1. Antimicrobial activity and modulating activity

Isolated and synthetic homoisoflavonoids were evaluated for MIC and modulation of EtBr's MIC in *M. smegmatis* mc^2 155. For a test compound to qualify as EPI, it should not possess an intrinsic antibacterial activity. Therefore, the MIC for all the tested compounds was determined to exclude intrinsic antimicrobial activity for screening modulation effect in MIC of EtBr. Bonducellin (**13**) and compound **15** showed the best modulating activity at 62.5 mg/L and reduced MIC of EtBr by 8-fold against *M. smegmatis*. The compounds **1** and **17** also modulated EtBr's MIC by 8-fold but at 125 mg/L concentration (Table 1). Modulating activity of the isolated and 19 synthetic homoisoflavonoids was further evaluated with clinically used antibiotics (isoniazid and ofloxacin) using the same protocol but none of these compounds modulated the MIC of isoniazid and ofloxacin. The compounds that showed at least 4 fold or more reduction in MIC of EtBr with modulation factor (MF) ≥4 (**1**, **7**–**11**, **13**–**15**, **17** and **19**) were further studied for the accumulation and efflux effect. This is based on the literature information that the compounds with MF less than 4, generally do not show significant accumulation and EPI activity in *M. smegmatis* mc^2 155 [17,18].

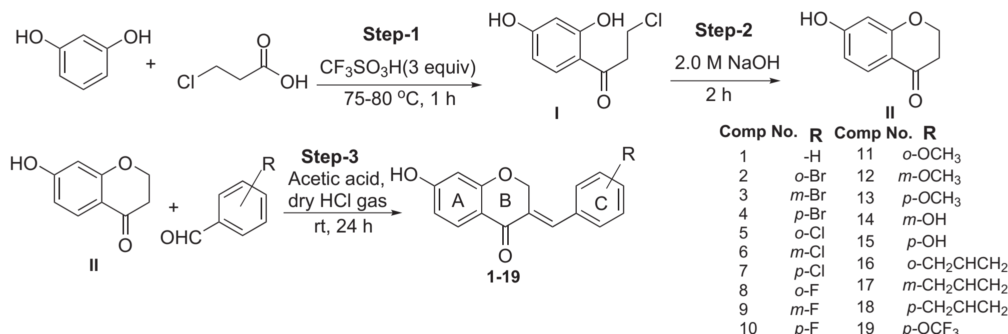
2.2.2. Accumulation assay and efflux pump inhibitory assay

The accumulation and efflux assay were validated using standards verapamil, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and reserpine (Fig. 2). Compounds (**1**, **7**–**11**, **13**–**15**, **17**) which modulated the MIC of EtBr by ≥4 fold, were subjected to accumulation and efflux assays (Fig. 3). Among all tested compounds, **1**, **7**–**11**, bonducellin (**13**) and **17** were found to be a better modulators than the standard verapamil against *M. smegmatis* mc^2 155 (Fig. 3). Compounds **14** and **15** have shown comparable activity as that of verapamil but greater than CCCP.

2.2.3. Dose dependent activity

Dose dependent effect of **1**, **8**–**11**, **13**, and **17** were studied in accumulation and efflux assays at a concentration of 1/2, 1/4 and 1/8 of their MIC. The higher concentration of compounds exhibited stronger accumulation and slower efflux of EtBr in *M. smegmatis* mc^2 155 cells (Fig. 4). The results revealed that the tested compounds enhanced the accumulation and inhibited efflux of EtBr in a dose-dependent manner during the assay period. All the above mentioned compounds exhibited better EPI activity than the standard, verapamil at all the tested concentrations 1/2, 1/4 and 1/8 of their MIC value. These results suggest that bonducellin (**13**) and the synthetic homoisoflavonoid analogues (**1**, **8**–**11**, and **17**) are potential EtBr efflux inhibitors in *M. smegmatis* mc^2 155.

It is reported that the presence of an intrinsic EP system in *M. smegmatis* is responsible for the development of intrinsic resistance to the EtBr and other antibiotics [19]. Rapid occurrence of



Scheme 1. Method used for the synthesis of (*E*)-3-benzylidene-7-hydroxychroman-4-one analogous.

Table 1
MIC and modulation factors of methanol extract, homoisoflavonoid rich fraction, bonducellin from *C. digyna* and compounds **1**–**19** for *M. smegmatis mc*^{2} 155.

Compound/Extract	MIC Value (mg/L)	Concentration as modulator (mg/L)	Modulation factor (MF)	FICI[a]
Methanol extract	250	125	2	1
HRF	500	250	4	0.75
E-Eucomine	500	250	4	0.6
		62.5	2	
Bonducellin[b]	125	62.5	8	0.62
		31.2	4	0.50
E-8-methoxybonducellin	125	62.5	2	**1**
Intricatinol	125	62.5	2	1
1	500	125	8	0.37
		62.5	4	0.37
2	125	62.5	2	1
3	500	125	2	0.75
4	500	125	2	0.75
5	125	62.5	2	1
6	250	125	2	1
7	250	125	4	0.75
8	125	62.5	4	0.75
9	250	125	4	0.75
10	250	125	4	0.75
11	250	125	4	0.75
12	500	125	2	0.75
14	500	125	4	0.50
15	250	125	16	0.56
		62.5	8	0.37
16	500	125	2	0.75
17	250	125	8	0.62
		62.5	4	0.50
18	250	125	2	0.75
19	250	125	4	0.75
Reserpine	256	40	4	0.40
Verapamil	300	40	2	0.63
		80	4	0.52
CCCP	25	16	2	1.14

MIC of EtBr = 16 mg/L.
[a] FICI (fractional inhibitory concentration index) ≤0.5 synergism; ≥4.0 antagonism; 4.0 > FICI > 0.5 no interaction.
[b] Bonducellin = **13** (Synthetic).

bacterial resistance against antibiotics can be achieved in several ways: (i) mutation of target proteins, (ii) active efflux systems and reduced cell-wall permeability, and (iii) enzymatic inactivation of the antibiotic. The characterization of mycobacterial EPs is a relatively new field compared to the characterization of EPs from other Gram-positive and Gram-negative bacteria. Also due to the high incidence levels of MDR-TB cases, there is an urgent need to identify and characterize new anti-TB compounds or enhancer/EPIs for existing TB compounds. The efflux of an antibiotic in bacteria may be inhibited by plant-derived compounds which can be evidenced by an example of 5′-methoxyhydnocarpin (5′-MHC). 5′-MHC did not possess any antibacterial activity but inhibited the MDR-dependent efflux of ethidium bromide and berberine from *S. aureus* [20].

2.2.4. Structure activity relationship (SAR)

In this study we observed that four isolated and nineteen synthetic homoisoflavonoids showed weak amtimycobacterial activity at ≥125 mg/L. The isolated homoisoflavonoids except bonducellin (**13**) showed very weak modulating activity (MF ≤ 2) at sub-inhibitory concentration of 62.5 mg/mL (Table 1). Compounds **1**, **7**–**11**, **13**–**15**, **17** and **19** showed a modulation effect on EtBr's antimycobacterial activity when added at sub-inhibitory concentrations. The identified modulators **1**, **7**–**11**, **13**–**15**, **17** and **19** were further tested for the accumulation and efflux assay. The results revealed that the compounds **1**, **8**–**11**, **13** and **17** enhanced the accumulation and inhibited the efflux of EtBr in the *M. smegmatis* cells more potently than verapamil (Fig. 4). Compounds **8** and **17** found to be the best EPIs since they showed much better accumulation as well as efflux inhibition of EtBr in *M. smegmatis* cells at 31.25 mg/L as compared to verapamil at 150 mg/L concentration.

Based on above observations we propose the SAR as below:

(i) The 7-Hydroxy-(*E*)-3-phenylmethylene-chroman-4-one is required to be present in all the tested compounds for modulation effect (Table 1 and Fig. 5). (ii) The hydroxy group at C-8 and or C-5 or a methoxy group at C-8 reduces the modulation effect. (iii) The compounds with substituents such as fluoro, methoxyl, allyloxy on the phenylmethylene-ring (**8**–**10**, **13**, **17**) enhanced accumulation and efflux pump inhibitory activity as compared to 7-Hydroxy-(*E*)-3-phenylmethylene-chroman-4-one. (iv) The fluoro substituent at *ortho*, *meta* and *para* (**8**, **9**, **10**); methoxyl substituent (**13**) at *para* and allyloxy substituent (**17**) at *meta* position on the phenylmethylene ring potentiated the accumulation and EPI activity. (v) Whereas, a chloro- and trifluromethane substituents (**7**, **19**) at *para*; methoxyl substituent (**11**) at *ortho* and hydroxyl substituent (**14**, **15**) at *meta* and *para* positions on the phenlymethylene ring led to decrease in the accumulation and EPI activity (Fig. 5).

3. Conclusion

In summary, four isolated and nineteen synthetic homoisoflavonoids were screened for their antimycobacterial activity and modulating activities on the MIC of EtBr. An EtBr accumulation and efflux assay were used to determine their potential to inhibit EtBr efflux. The compounds **1**, **8**–**11**, **13** and **17** showed potent accumulation and efflux inhibition of ethidium bromide in *M. smegmatis* mc^2 155, among the tested nineteen homoisoflavonoids as compared to verapamil. We successfully demonstrated that particularly compounds **8** and **17** achieved considerable results as modulator and decreased the EtBr efflux in *M. smegmatis* mc^2 155 at levels either comparable to or lower than the known EPIs. Homoisoflavones have

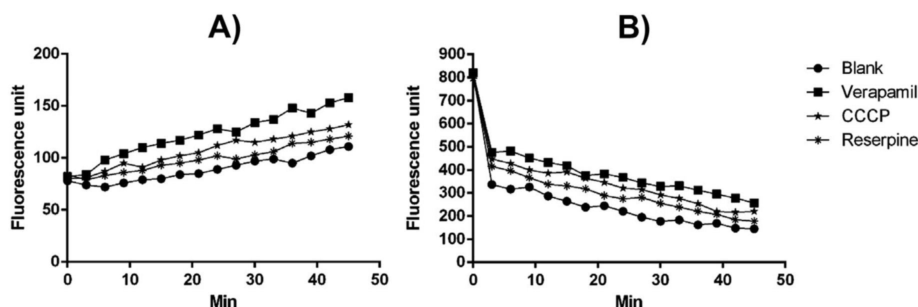


Fig. 2. Effect of verapamil (150 mg/L), CCCP (12.5 mg/L) and reserpine (128 mg/L), on EtBr accumulation (A) and efflux assay (B) in *M. smegmatis* mc^2 155 cells.

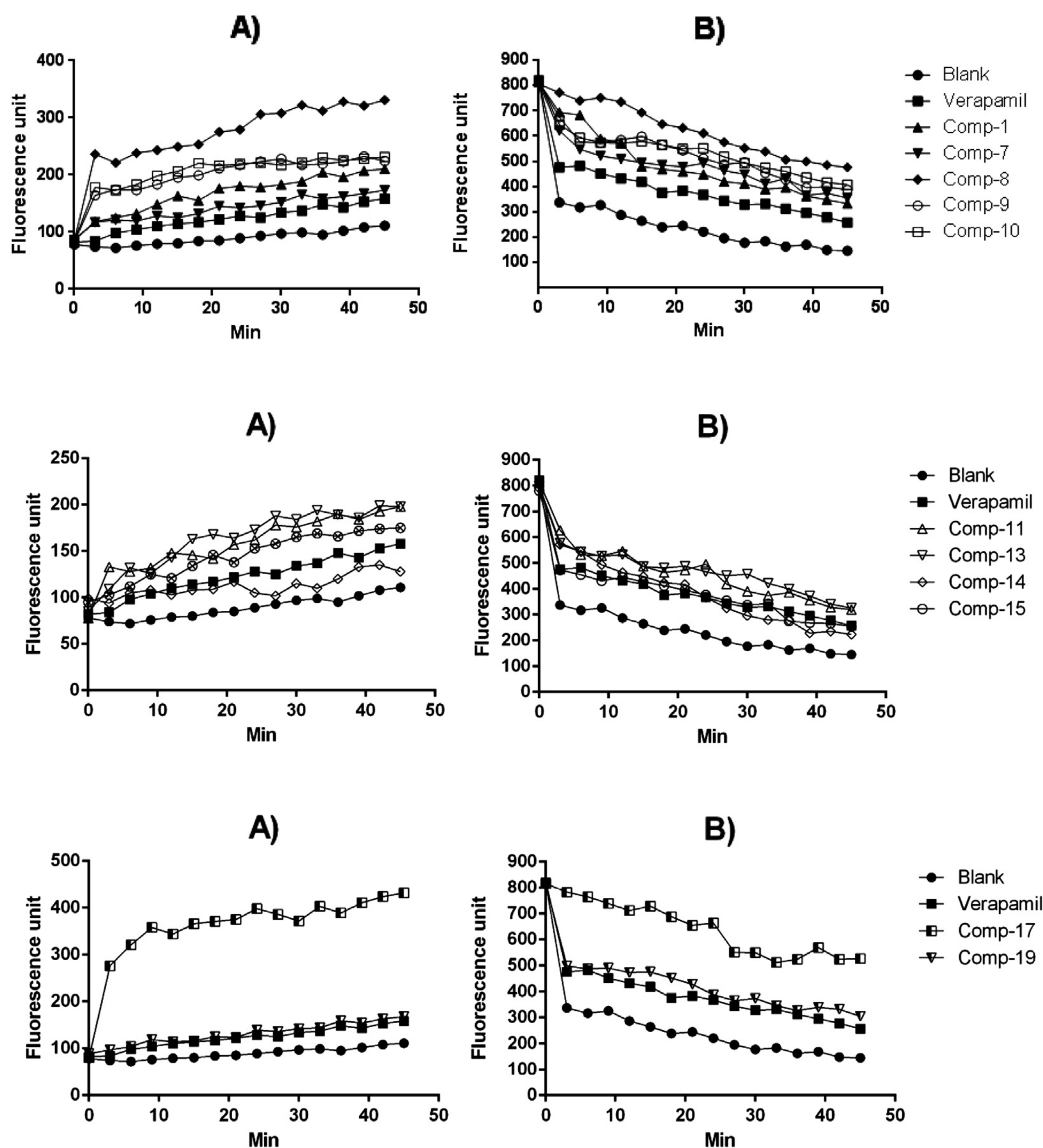


Fig. 3. Effect of test compounds (**1**, **7–11**, **13–15**, **17** and **19**) at half concentration of MIC; on EtBr accumulation (A) and efflux assay (B) in *M. smegmatis* mc^2 155 cells.

not been described as bacterial efflux inhibitors before and thus represent a promising class of compounds to be studied further.

4. Experimental

4.1. Materials and equipments

All Chemicals were purchased from Sigma Aldrich. Uncorrected melting points were determined on a digital melting point apparatus of PERFIT, India. ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on Bruker 400 Ultra Shield™ NMR spectrophotometer with TMS as an internal standard. The Mass spectra were recorded on MaXis™ UHR-TOF and Thermo Finnigan LCQ Mass spectrometer. IR spectra were recorded on Nicolet FT-IR (Impact 410, Japan) spectrophotometer.

4.2. General procedure for synthesis of 7-Hydroxy-(E)-3-phenylmethylene-chroman-4-one analogues (*1–19*)

Step A. Acylation of resorcinol: Resorcinol (9.00 g, 82 mM) was acylated by the equi-molar amount of 3-chloropropionic acid in the presence of trifluoromethanesulphonic acid (3 equiv.), stirred at 75–80 °C. After 1 h the solution was cooled to room temperature and poured into $CHCl_3$ (200 mL). This solution was slowly poured into water (200 mL), and the phases were separated. The aqueous phase was partitioned with 2 × 200 mL of $CHCl_3$. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum to give semisolid compound **I** (7.24 g).

Step B. Cyclization of I: Compound **I** (7.24 g) was added to 400 mL of 2 M NaOH at 5 °C and stirred for 20 min. The solution was allowed to reach room temperature for 2 h and again cooled to 5 °C. The pH of the solution was adjusted to 2 with 6 M H_2SO_4. The acidic mixture was extracted with 3 × 200 mL of EtOAc, washed with brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum to give crude solid. Crude solid was subjected to CC over silica gel (#100–200) eluting with petroleum ether and EtOAc (75:25) to give pure **II** (3.50 g).

Step C. Synthesis of homoisoflavonoids by acidic condensation: Compound **II** (100 mg, 0.61 mM) was added in a solution of substituted benzaldehyde (0.62 mM) in acetic acid (20 μL) and dry HCl gas was passed through this mixture for 5 min. The reaction

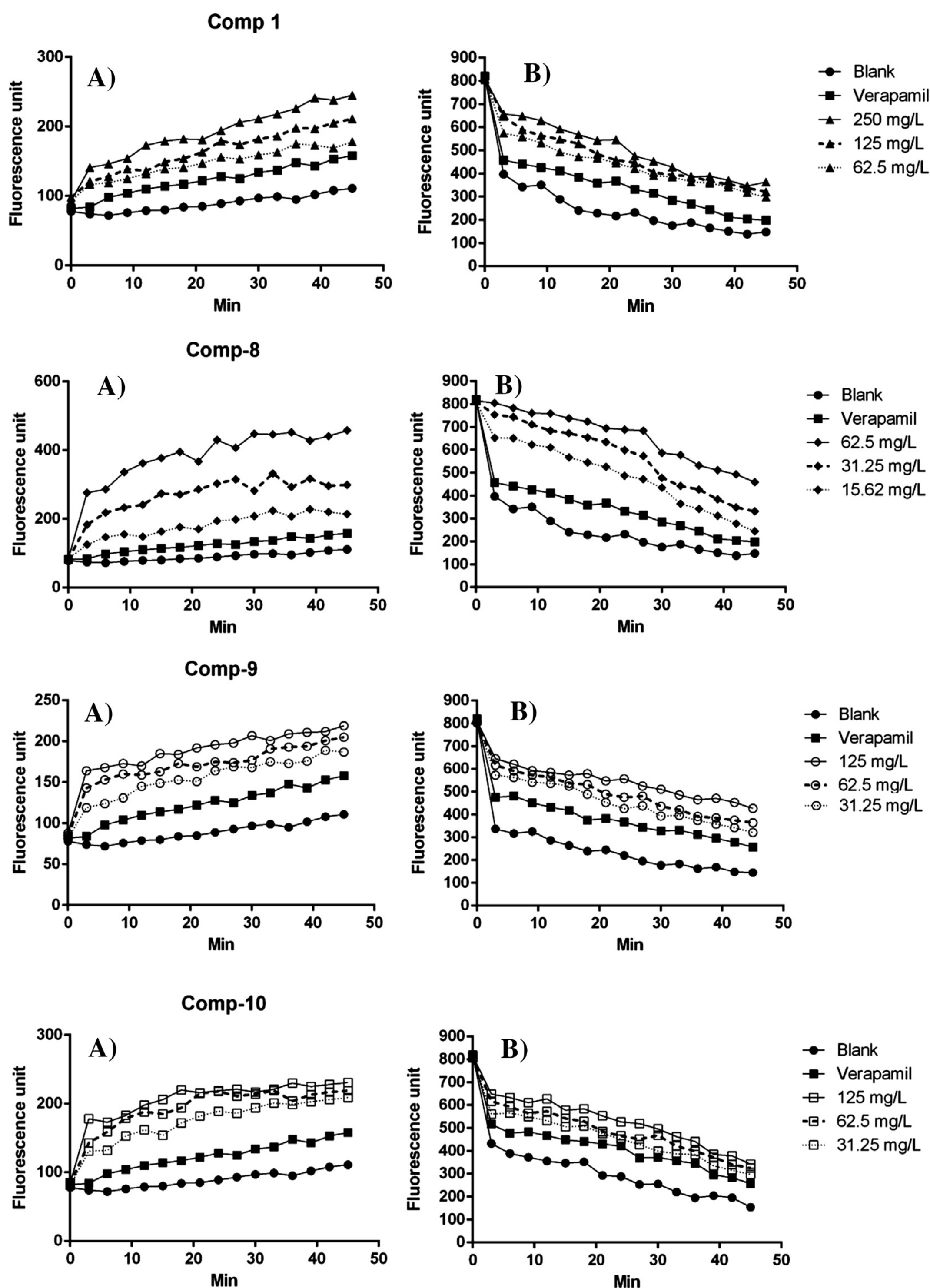


Fig. 4. Dose dependent Effect of Comp (**1**, **8–11**, **13** and **17**) on the accumulation (**A**) and Efflux (**B**) of EtBr from *M. smegmatis* mc^2 155 cells.

mixture was allowed to stand at room temperature for 24 h. The obtained solid mixture washed with water to remove acids and concentrated on a rotary evaporator. Afterwards the mixture was purified by CC on Silica gel (#230–400) eluting with petroleum ether and EtOAc (85:15) to give pure compounds (**1–19**).

4.2.1. 2′,4′-Dihydroxy-3-chloro-propiophenone (*I*)

Yield: 7.24 g, 44.25%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.40 (t, $J = 6.7$ Hz, 2H, H_2-2), 3.90 (t, $J = 6.7$ Hz, 2H, H_2-1), 6.39 (d, $J = 2.4$ Hz, 1H, H-3′), 6.42 (dd, $J = 2.4$, 8.7 Hz, 1H, H-5′), 7.63 (d, $J = 8.7$ Hz, 1H, H-6′), 12.53 (s, 1H, 2′-OH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 38.5, 40.3, 103.6, 108.2, 113.7, 132.1, 163.1, 165.2, 200.6 [12].

4.2.2. 7-Hydroxy-chroman-4-one (*II*)

Yield: 3.50 g, 72.8%; mp: 150–152 °C (lit 145 °C). ^{1}H NMR (400 MHz, CD_3OD): δ 2.70 (t, $J = 5.1$ Hz, 2H, H_2-3), 4.46 (t, $J = 5.1$ Hz, 2H, H_2-2), 6.31 (s, 1H, H-8), 6.47 (d, $J = 8.6$ Hz, 1H, H-6), 7.70 (d, $J = 8.6$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, CD_3OD): δ 36.8,

Fig. 4. (*continued*).

67.0, 102.2, 110.2, 113.9, 128.6, 164.4, 165.1, 191.9. MS (APCI) m/z: 165 $[M + H]^+$ [12].

4.2.3. 7-Hydroxy-(E)-3-phenylmethylene-chroman-4-one (**1**)

Yield: 40.10 mg, 26.1%; mp: 95–96 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 5.32 (s, 2H, H_2-2), 6.32 (s, 1H, H-8), 6.54 (d, J = 8.7 Hz, 1H, H-6), 7.36 (d, J = 7.1 Hz, 2H, H-2′ + H-6′), 7.43–7.50 (m, 3H, H-3′ + H-4′ + H-5′), 7.77 (s, 1H, H-9), 7.82 (d, J = 8.7 Hz, 1H, H-5). ^{13}C NMR (100 MHz, CD_3OD): δ 67.4, 102.3, 111.0, 114.3, 128.4, 129.2, 129.4, 129.7, 131.1, 134.3, 136.4, 163.6, 165.5, 181.5. IR (KBr, cm^{-1}) 3333, 1657, 1612. HRMS (ESI) m/z: Calcd for $C_{16}H_{12}O_3Na$ $[M + Na]^+$ 275.0684; found 275.0680.

4.2.4. 7-Hydroxy-(E)-3-[(2-bromophenyl)methylene]chroman-4-one (**2**)

Yield: 170.21 mg, 84.6%; mp: 209–210 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.20 (s, 2H, H_2-2), 6.33 (s, 1H, H-8), 6.58 (d, J = 8.1 Hz, 1H, H-6), 7.31 (d, J = 7.3 Hz, 1H, H-6′), 7.40 (t, J = 7.3 Hz, 1H, H-5′), 7.50 (t,

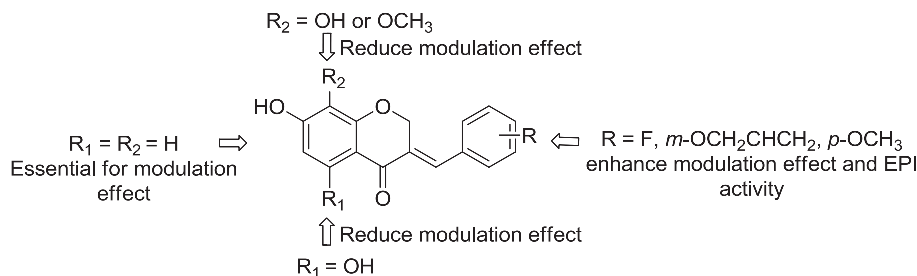


Fig. 5. Structure activity relationship of homoisoflavonoids.

$J = 7.3$ Hz, 1H, H-4′), 7.65 (s, 1H, H-9), 7.78 (d, $J = 8.1$ Hz, 1H, H-5) 7.79 (t, $J = 7.3$ Hz, 1H, H-3′). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.5, 102.9, 111.9, 114.5, 124.6, 128.4, 130.0, 131.4, 131.7, 132.9, 133.4, 134.3, 134.4, 163.3, 165.5, 179.9. IR (KBr, cm^{-1}) 3482, 1655, 1624, 1078. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}BrO_3Na$ [M + Na]$^+$ 352.9789; found 352.9792.

4.2.5. 7-Hydroxy-(E)-3-[(3-bromophenyl)methylene]chroman-4-one (3)

Yield: 182.72 mg, 90.8%; mp: 229–230 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, H_2-2), 6.34 (s, 1H, H-8), 6.58 (d, $J = 8.5$ Hz, 1H, H-6), 7.40–7.46 (m, 3H, H-2′ + H-4′ + H-6′), 7.66 (s, 2H, H-5′ + H-9), 7.76 (d, $J = 8.5$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.7, 102.9, 111.9, 114.5, 122.5, 129.3, 130.0, 131.2, 132.5, 132.7, 132.9, 134.2, 136.9, 163.2, 165.4, 179.8. IR (KBr, cm^{-1}) 3394, 1665, 1623, 1073. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}BrO_3Na$ [M + Na]$^+$ 352.9789; found 352.9793.

4.2.6. 7-Hydroxy-(E)-3-[(4-bromophenyl)methylene]chroman-4-one (4)

Yield: 193.02 mg, 95.9%; mp: 202–203 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.32 (s, 2H, H_2-2), 6.33 (s, 1H, H-8) 6.56 (d, $J = 8.5$ Hz, 1H, H-6), 7.38 (d, $J = 8.4$ Hz, 2H, H-3′ + H-5′), 7.63 (s, 1H, H-9), 7.68 (d, $J = 8.4$ Hz, 2H, H-2′ + H-6′) 7.75 (d, $J = 8.5$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.7, 102.9, 111.8, 114.5, 123.4, 130.0, 132.1, 132.2, 132.6, 133.7, 134.6, 163.1, 165.4, 179.9. IR (KBr, cm^{-1}) 3453, 1666, 1623, 1067. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}BrO_3Na$ [M + Na]$^+$ 352.9789; found 352.9791.

4.2.7. 7-Hydroxy-(E)-3-[(2-chlorophenyl)methylene]chroman-4-one (5)

Yield: 160.95 mg, 92.3%; mp: 225–226 °C.; ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.21 (d, $J = 1.2$ Hz, 2H, H_2-2), 6.33 (d, $J = 2.2$ Hz, 1H, H-8), 6.57 (dd, $J = 2.2$, 8.7 Hz, 1H, H-6), 7.31 (dd, $J = 1.9$, 7.2 Hz, 1H, H-6′), 7.45 (m, 2H, H-4′ + H-5′), 7.59 (dd, $J = 1.9$, 7.2 Hz, 1H, H-3′), 7.70 (s, 1H, H-9), 7.76 (d, $J = 8.72$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.7, 102.9, 111.9, 114.5, 127.8, 130.0, 130.2, 131.4, 131.5, 132.1, 132.5, 133.3, 134.1, 163.3, 165.5, 179.8. IR (KBr, cm^{-1}) 3516, 1658, 1600, 1075. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}ClO_3Na$ [M + Na]$^+$ 309.0294; found 309.0291.

4.2.8. 7-Hydroxy-(E)-3-[(3-chlorophenyl)methylene]chroman-4-one (6)

Yield: 163.4 mg, 93.7%; mp: 232–233 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.31 (s, 2H, H_2-2), 6.32 (s, 1H, H-8), 6.56 (d, $J = 8.5$ Hz, 1H, H-6), 7.35 (s, 1H, H-5′), 7.50 (m, 3H, H-2′ + H-4′ + H-6′), 7.63 (s, 1H, H-9), 7.74 (d, $J = 8.5$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.2, 102.4, 111.4, 114.0, 128.5, 129.1, 129.5, 130.5, 132.2, 133.4, 133.7, 136.1, 162.6, 164.9, 179.3. IR (KBr, cm^{-1}) 3374, 1660, 1620, 1069. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}ClO_3Na$ [M + Na]$^+$ 309.0294; found 309.0289.

4.2.9. 7-Hydroxy-(E)-3-[(4-chlorophenyl)methylene]chroman-4-one (7)

Yield: 168.99 mg, 96.9%; mp: 201–203 °C (lit 196–198 °C) [14]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, H_2-2), 6.34 (d, $J = 1.7$ Hz, 1H, H-8), 6.56 (dd, $J = 1.7$, 8.72 Hz, 1H, H-6), 7.45 (d, $J = 8.4$ Hz, 2H, H-3′ + H-5′), 7.54 (d, $J = 2.1$, 8.4 Hz, 2H, H-2′ + H-6′), 7.65 (s, 1H, H-9), 7.76 (d, $J = 8.5$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.7, 102.9, 111.8, 114.5, 129.2, 130.0, 132.0, 132.3, 133.3, 134.5, 134.6, 163.1, 165.3, 179.9. IR (KBr, cm^{-1}) 3437, 1671, 1601, 1060. APCI (ESI) *m*/*z*: 288.16 [M + H]$^+$.

4.2.10. 7-Hydroxy-(E)-3-[(2-fluorophenyl)methylene]chroman-4-one (8)

Yield: 132.09 mg, 80.2%; mp: 132–133 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.24 (s, 2H, H_2-2), 6.35 (s, 1H, H-8), 6.59 (d, $J = 8.1$ Hz, 1H, H-6), 7.32–7.39 (m, 3H, H-3′ + H-5′ + H-6′), 7.53 (m, 1H, H-4′), 7.67 (s, 1H, H-9), 7.78 (d, $J = 7.2$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.9, 103.0, 111.9, 114.5, 116.2, 122.1, 125.2, 128.0, 130.0, 131.6, 132.3, 133.5, 160.5, 163.3, 165.5, 179.9. IR (KBr, cm^{-1}) 3390, 1664, 1602, 1250. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}FO_3Na$ [M + Na]$^+$ 293.0589; found 293.0591.

4.2.11. 7-Hydroxy-(E)-3-[(3-fluorophenyl)methylene]chroman-4-one (9)

Yield: 137.03 mg, 83.3%; mp: 145–146 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.35 (s, 2H, H_2-2), 6.34 (s, 1H, H-8), 6.59 (d, $J = 8.1$ Hz, 1H, H-6), 7.26–7.32 (m, 3H, H-2′ + H-4′ + H-6′), 7.53 (m, 1H, H-5′), 7.67 (s, 1H, H-9), 7.78 (d, $J = 7.3$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.7, 102.9, 111.8, 114.5, 116.6, 117.1, 126.6, 130.0, 131.1, 132.5, 134.4, 136.7, 162.5, 163.2, 165.4, 179.8. IR (KBr, cm^{-1}) 3405, 1689, 1610, 1244. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}FO_3Na$ [M + Na]$^+$ 293.0589; found 293.0588.

4.2.12. 7-Hydroxy-(E)-3-[(4-fluorophenyl)methylene]chroman-4-one (10)

Yield: 149.05 mg, 90.6%; mp: 195–197 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, H_2-2), 6.33 (d, $J = 2.1$ Hz, 1H, H-8) 6.57 (dd, $J = 2.1$, 8.5 Hz, 1H, H-6), 7.32 (t, $J = 8.8$ Hz, 2H, H-3′ + H-5′), 7.51 (m, $J = 8.8$ Hz, 2H, H-2′ + H-6′) 7.67 (s, 1H, H-9), 7.77 (d, $J = 8.5$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.7, 102.9, 111.8, 114.6, 116.3, 130.0, 131.0, 131.3, 133.0, 134.7, 162.9, 163.0, 165.3, 179.9. IR (KBr, cm^{-1}) 3238, 1675, 1612, 1243. HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{11}FO_3Na$ [M + Na]$^+$ 293.0589; found 293.0590.

4.2.13. 7-Hydroxy-(E)-3-[(2-methoxyphenyl)methylene]chroman-4-one (11)

Yield: 101.66 mg, 59.1%; mp: 166–168 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.85 (s, 3H, 2′-OCH_3), 5.21 (s, 2H, H_2-2), 6.33 (d, $J = 2.1$ Hz, 1H, H-8), 6.57 (dd, $J = 2.1$, 8.7 Hz, 1H, H-6), 7.04 (t, $J = 7.2$ Hz, 1H, H-5′), 7.12 (d, $J = 8.4$ Hz, 1H, H-3′), 7.16 (d, $J = 8.2$ Hz, 1H, H-6′), 7.44 (t, $J = 7.6$ Hz, 1H, H-4′), 7.75 (s, 1H, H-9), 7.77 (d, $J = 8.7$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.0, 68.1, 102.9, 111.7, 111.8, 114.7, 120.7, 122.9, 129.9, 130.8, 131.1, 131.8, 131.9, 158.2, 163.2, 165.2, 180.0. IR (KBr, cm^{-1}) 3414, 1613. HRMS (ESI) *m*/*z*: Calcd for $C_{17}H_{14}O_4Na$ [M + Na]$^+$ 305.0789; found 305.0790.

4.2.14. 7-Hydroxy-(E)-3-[(3-methoxyphenyl)methylene]chroman-4-one (12)

Yield: 144.84 mg, 84.2%; mp: 215–216 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.80 (s, 3H, 3′-OCH_3), 5.35 (s, 2H, H_2-2), 6.33 (s, 1H, H-8), 6.56 (d, $J = 8.5$ Hz, 1H, H-6), 6.97–6.99 (m, 2H, H-2′ + H-4′), 7.02 (d, $J = 8.2$ Hz, 1H, H-6′), 7.40 (t, $J = 7.3$ Hz, 1H, H-5′), 7.66 (s, 1H, H-9), 7.76 (d, $J = 8.5$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.2, 67.4, 102.4, 111.3, 114.1, 115.2, 115.3, 122.2, 129.5, 129.8, 131.1, 135.2, 135.3, 159.3, 162.6, 164.8, 179.5. IR (KBr, cm^{-1}) 3390, 1645, 1614. HRMS (ESI) *m*/*z*: Calcd for $C_{17}H_{14}O_4Na$ [M + Na]$^+$ 305.0789; found 305.0790.

4.2.15. 7-Hydroxy-(E)-3-[(4-methoxyphenyl)methylene]chroman-4-one (13)

Yield: 66.40 mg, 38.6%; mp: 210–211 °C (lit 206–208 °C) [12]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.81 (s, 3H, 4′-OCH_3), 5.35 (d, $J = 1.6$ Hz, 2H, H_2-2), 6.31 (d, $J = 2.2$ Hz, 1H, H-8), 6.54 (dd, $J = 2.2$, 8.7 Hz, 1H, H-6), 7.03 (d, $J = 8.7$ Hz, 2H, H-3′ + H-5′), 7.39 (d, $J = 8.7$ Hz, 2H, H-2′ + H-6′), 7.63 (s, 1H, H-9), 7.73 (d, $J = 8.7$ Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.8, 68.0, 102.9, 111.6, 114.7, 114.8, 127.0, 129.3, 129.9, 132.6, 135.7, 160.7, 162.9, 165.1, 180.0. IR (KBr, cm^{-1}) 3394, 1603. MS (APCI) *m*/*z*: Calcd for $C_{17}H_{14}O_4$ [M + H]$^+$ 283.10; found 283.18.

*4.2.16. 7-Hydroxy-(E)-3-[(3-hydroxyphenyl)methylene]chroman-4-one (**14**)*

Yield: 81.58 mg, 49.9%; mp: >270 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.32 (s, 2H, H_2-2), 6.32 (s, 1H, H-8), 6.55 (d, J = 8.0 Hz, 1H, H-6), 6.84–6.78 (m, 3H, H-6′ + H-4′ + H-2′), 7.29–7.27 (m, 1H, H-5′), 7.58 (s, 1H, H-9), 7.74 (d, J = 8.0 Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.9, 102.9, 111.7, 114.7, 117.0, 117.0, 121.4, 129.9, 130.3, 131.3, 135.7, 136.0, 157.9, 163.1, 165.2, 180.0. IR (KBr, cm^{-1}) 3446, 1654, 1629. HRMS (ESI) m/z: Calcd for $C_{16}H_{12}O_4Na$ $[M + Na]^+$ 291.0633; found 291.0620, Calcd for $C_{16}H_{13}O_4$ $[M + H]^+$ 269.0814; found 269.0805.

*4.2.17. 7-Hydroxy-(E)-3-[(4-hydroxyphenyl)methylene]chroman-4-one (**15**)*

Yield: 52.31 mg, 32.0%; mp: 252–253 °C (lit 248–249 °C) [14,21]. ^{1}H NMR (400 MHz, CD_3OD): δ 5.23 (s, 2H, H_2-2), 6.20 (s, 1H, H-8), 6.42 (d, J = 8.5 Hz, 1H, H-6), 6.78 (d, J = 7.2, 2H, H-3′ + H-5′), 7.13 (d, J = 7.2 Hz, 2H, H-2′ + H-6′), 7.60 (s, 1H, H-9), 7.69 (d, J = 8.5 Hz, 1H, H-5). ^{13}C NMR (100 MHz, CD_3OD): δ 67.6, 102.4, 110.8, 114.4, 115.4, 125.7, 128.1, 129.3, 132.1, 136.8, 159.1, 163.4, 165.2, 181.7. IR (KBr, cm^{-1}) 3451, 1612. MS (APCI) m/z: Calcd for $C_{16}H_{12}O_4$ $[M + H]^+$ 269.07; found 269.10.

*4.2.18. 7-Hydroxy-(E)-3-[(2-allyloxyphenyl)methylene]chroman-4-one (**16**)*

Yield: 64.37 mg, 33.2%; mp: 185–186 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.63 (d, J = 1.9 Hz, 2H, H_2-1″), 5.19 (s, 2H, H_2-2), 5.25 (d, J = 10.5 Hz, 1H, H-3b″), 5.37 (d, J = 17.3 Hz, 1H, H-3a″), 6.04 (m, 1H, H-2″), 6.31 (s, 1H, H-8), 6.55 (d, J = 8.6 Hz, 1H, H-6), 7.02 (t, J = 7.5 Hz, 1H, H-5′), 7.10 (d, J = 8.3 Hz, 1H, H-3′), 7.15 (d, J = 7.4 Hz, 1H, H-6′), 7.41 (t, J = 7.7 Hz, 1H, H-4′), 7.74 (d, J = 8.6 Hz, 1H, H-5), 7.77 (s, 1H, H-9). ^{13}C NMR (100 MHz, DMSO-d_6): δ 68.1, 69.0, 102.9, 111.8, 112.9, 114.7, 118.1, 120.9, 123.2, 130.0, 131.0, 131.1, 131.8, 131.9, 133.8, 157.1, 163.2, 165.2, 180.4. IR (KBr, cm^{-1}) 3442, 1654, 1616. HRMS (ESI) m/z: Calcd for $C_{19}H_{16}O_4Na$ $[M + Na]^+$ 331.0946; found 331.0950.

*4.2.19. 7-Hydroxy-(E)-3-[(3-allyloxyphenyl)methylene]chroman-4-one (**17**)*

Yield: 39.20 mg, 20.21%; mp: 147–148 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.63 (d, J = 5.1 Hz, 2H, H_2-1″), 5.28 (dd, J = 1.4, 10.2 Hz, 1H, H-3b″), 5.34 (s, 2H, H-2), 5.42 (dd, J = 1.4, 17.3 Hz, 1H, H-3a″), 6.06 (m, 1H, H-2″), 6.34 (d, J = 2.1 Hz, 1H, H-8), 6.57 (dd, J = 2.1, 8.7 Hz, 1H, H-6), 6.98 (d, J = 8.0 Hz, 1H, H-6′), 6.99 (s, 1H, H-2′), 7.03 (d, J = 8.2 Hz, 1H, H-4′), 7.39 (t, J = 8.0 Hz, 1H, H-5′), 7.65 (s, 1H, H-9), 7.76 (d, J = 8.7 Hz, 1H, H-5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 67.9, 68.7, 102.9, 111.8, 114.6, 116.4, 116.5, 118.1, 122.9, 129.9, 130.3, 131.7, 132.8, 134.0, 135.8, 135.8, 158.6, 163.1, 165.3, 180.0. IR (KBr, cm^{-1}) 3503, 1660, 1616. HRMS (ESI) m/z: Calcd for $C_{19}H_{16}O_4Na$ $[M + Na]^+$ 331.0946; found 331.0950.

*4.2.20. 7-Hydroxy-(E)-3-[(4-allyloxyphenyl)methylene]chroman-4-one (**18**)*

Yield: 40.96 mg, 21.1%; mp: 178–179 °C. ^{1}H NMR (400 MHz, $CD_3OD/CDCl_3$, 1:1) δ 4.20 (s, 2H, H_2-1″), 5.32 (d, J = 11.0 Hz, 1H, H-3b″), 5.35 (s, 2H, H_2-2), 5.44 (d, J = 17.2 Hz, 1H, H-3a″), 6.07 (m, 1H, H-2″), 6.35 (s, 1H, H-8), 6.56 (d, J = 8.7 Hz, 1H, H-6), 7.01 (d, J = 7.7 Hz, 2H, H-3′ + H-5′), 7.28 (d, J = 7.8 Hz, 2H, H-2′ + H-6′), 7.76 (s, 1H, H-9), 7.85 (d, J = 8.7 Hz, 1H, H-5). ^{13}C NMR (100 MHz, $CD_3OD/CDCl_3$, 1:1): δ 67.7, 68.8, 102.6, 111.2, 114.7, 114.9, 117.6, 127.1, 129.0, 129.7, 131.9, 132.7, 136.7, 159.6, 163.3, 165.0, 181.8. IR (KBr, cm^{-1}) 3399, 1616. HRMS (ESI) m/z: Calcd for $C_{19}H_{16}O_4Na$ $[M + Na]^+$ 331.0946; found 331.0950.

*4.2.21. 7-Hydroxy-(E)-3-[(4-trifluoromethylphenyl)methylene]chroman-4-one (**19**)*

Yield: 48.21 mg, 24.6%; mp: 198–199 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 5.32 (s, 2H, H_2-2), 6.33 (s, 1H, H-8), 6.56 (d, J = 8.8 Hz, 1H, H-6), 7.57 (d, J = 7.6 Hz, 2H, H-3′ + H-5′), 7.78 (d, J = 7.6 Hz, 2H, H-2′ + H-6′), 7.79 (s, 1H, H-9), 7.84 (d, J = 8.8 Hz, 1H, H-5). ^{13}C NMR (100 MHz, CD_3OD): δ 67.2, 102.3, 111.2, 114.3, 125.2, 125.3, 129.5, 130.1, 133.3, 134.1, 138.1, 138.2, 163.7, 165.6, 180.8. IR (KBr, cm^{-1}) 3445, 1660, 1624. HRMS (ESI) m/z: Calcd for $C_{17}H_{11}F_3O_3Na$ $[M + Na]^+$ 343.0558; found 343.0550.

4.3. Biology

4.3.1. Bacterial strain growing conditions

M. smegmatis mc^2 155 (ATCC 700084) was provided by Dr. S. Majumdar, IMTECH, Chandigarh, India. Mycobacterial strain was grown in 10% oleic acid–albumin–glucose complex (OADC) supplemented Middlebrook 7H11 agar medium.

4.3.2. MIC determination

This assay comprised MIC determination of standards and test compounds as per the reported method [22]. All the compounds are freely soluble in DMSO. Briefly, the compounds was first dissolved in DMSO and then diluted in 7H9/OADC-supplemented medium across a 96 well Microtiter plate in twofold serial dilution. Bacterial inocula equivalent to the 0.5 McFarland standards were prepared in normal saline and diluted to give the final density of 0.5×10^5 cfu/mL. The inoculum (100 μL) was added to all the wells and the microtiter plate was incubated at 37 °C for 72 h. The MIC was recorded as the lowest concentration at which no bacterial growth was observed. This was facilitated by the addition of 20 μL of MTT at the concentration of 10 mg/mL in MeOH to each well and incubated at 37 °C for 20 min where bacterial growth was indicated by purple coloration adhered to cells. Appropriate DMSO, cell and sterile saline controls were carried out in the same set of experiment.

4.3.3. Modulation assay

Test compounds were further screened for their synergistic effects with EtBr prior to efflux assays, according to the modified method described previously [22]. Compounds were dissolved in DMSO and diluted in 7H9/OADC-supplemented medium at various sub-inhibitory concentrations (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/248 of the MIC). Each concentration of the bonducellin and compounds (**1**–**19**) maintained uniformity throughout the experiment, whereas the EtBr was serially diluted (8, 4, 2, 1, 0.5 and 0.25 mg/L) for MIC determination with and without modulator. A modulation factor (MF) was used to express the modulating effects of compounds on MIC of EtBr, where:

$$MF = \frac{MIC(Antibiotic)}{MIC(Antibiotic + Modulator)}$$

Fractional Inhibitory Concentration Index (FICI) was calculated by the equation:

$$FICI = FIC(A) + FIC(B)$$

$$FIC(A) = \frac{MIC(A \text{ in presence of } B)}{MIC(A \text{ alone})}$$

$$FIC(B) = \frac{MIC(B \text{ in presence of } A)}{MIC(B \text{ alone})}$$

Interpretation of FICI was as follows: FICI value of ≤0.5 indicates synergy; ≥4.0 indicates antagonism; and 4.0 > FICI > 0.5 indicates no interaction [17,23].

4.3.4. EtBr accumulation assay by fluorometric method

This assay was performed as per reported methods with some modification [22]. *M. smegmatis* was grown in 10 mL 7H9/OADC-

supplemented medium at 37 °C to an OD_{600} of 0.8. The culture was centrifuged at 13,000 rpm in a microfuge tube for 3 min. The supernatant was discarded and the pellet was washed once and resuspended in phosphate buffer saline (PBS). After adjusting the OD_{600} to 0.4, glucose and EtBr were added to yield final concentrations of 0.4% and 1 mg/L, respectively to one set of microtubes containing 1 mL bacterial suspension.

Aliquots of 95 μL were distributed to replica sets of 0.2 mL microtubes, and 5 μL each of known EPI and test compounds as EPI in case of respective assays, was added. Replica tubes that did not receive any EPI served as a control. In each accumulation assay, the positive controls of the reported EPIs such as verapamil, reserpine and CCCP were dissolved in DMSO and used along with vehicle control (DMSO). The fluorescence was measured by spectroflourimetric microplate reader. The fluorescence at excitation and emission wavelengths of 530 nm (bandwidth 5 nm) and 600 nm (bandwidth 10 nm), respectively, was measured at 3 min intervals for 45 min. Each experiment was repeated three times.

4.3.5. EtBr efflux assay by fluorometric method

The effect of EPIs on EtBr efflux activity was measured according to a modification of recently reported fluorescence techniques [22]. The non-inhibitory concentration of EtBr on working cfu of *M. smegmatis* mc^2 155 was used. The accumulation of EtBr at 25 °C in the absence of glucose, caused a higher accumulation without compromising the cellular viability (4 mg/L, corresponding to one fourth the MIC). The EtBr-loaded cells were centrifuged at 13,000 rpm in a microfuge for 3min and resuspended in EtBr-free PBS containing 0.4% glucose. After adjusting the OD_{600} to 0.4, aliquots of 95 μL were transferred to replicate 0.2 mL microtubes, and 5 μL each of EPI was added. Replica tubes that did not receive any EPI served as a control. In each efflux assay, the positive controls of the reported EPIs such as verapamil, reserpine and CCCP were dissolved in DMSO and used along with vehicle control (DMSO). The EtBr efflux by the cells was monitored in a spectroflourimetric microplate reader. The fluorescence at excitation and emission wavelengths of 530 nm (bandwidth 5 nm) and 600 nm (bandwidth 10 nm), respectively, was measured at 3 min intervals for 45 min. Each experiment was repeated three times.

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