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Design and synthesis of coumarin–glyoxal hybrids for spermicidal and antimicrobial actions: a dual approach to contraception†

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Today there is an urgent need for safe and effective dual-purpose contraceptive agents, which can simultaneously prevent unintended pregnancies and sexually transmitted infections (STI). There are several naturally occurring antimicrobial and antibiotic drugs (novobiocin, coumermycin and chlorobiocin) reported in the literature, which are based on 4-hydroxy coumarins as the active pharmacophore. Based on these interesting reports, we designed and synthesized a library of new 4-hydroxy coumarin derivatives and evaluated them for spermicidal activity. Among the tested compounds, two compounds (**2a** and **2d**) displayed better activity (greater than 95% sperm immobilization at 0.5 mM concentration) than the positive control nonoxynol-9 (**N-9**). Furthermore, all the compounds were screened for antimicrobial activity against different strains of *Trichomonas vaginalis* and two compounds (**2c** and **2h**) exhibited potent activity as compared to the reference drug metronidazole. The cytotoxicity assay showed that most of these compounds were safer than the N-9 against the human cervical HeLa cell line and normal vaginal flora *Lactobacillus jensenii* strains. The studies have demonstrated that compound (**2a**) is a potential lead to develop a dually active vaginal contraceptive.

1 Introduction

The unabated increase in unintended pregnancies with high rates of STIs (Sexually Transmitted Infections) necessitates the development of novel strategies especially in less developed countries to help individuals in avoiding these risks.[1,2] According to an estimate, ~40% of all pregnancies worldwide are unintended which strongly indicates that the available methods of contraception are insufficient.[3] Human immuno-deficiency virus (HIV) infection and Acquired Immunodeficiency Syndromes (AIDS) have become a major cause of morbidity and mortality in India as well as the world. Now these have assumed epidemic forms. Approximately 2.08 million people of the Indian population were suffering from HIV in 2011 as per the report of NACO (National AIDS Control Organisation). Condoms are used to tackle this problem but their denial, inconsistent or incorrect use and failure results in unintended pregnancies.[4] Herbal contraceptives are cost effective and easily available alternatives with lesser side effects. Many research findings have confirmed about the spermicidal properties in plants of *Achyranthes aspera*,[5] *Staphania hernandifolia*,[5] *Carica papaya*,[6] *Cestrum perqui*, *Hymeno cardiaaceta* and *Lawsonia enermis*[2] *etc.* but most of the plants used as contraceptives either affect the fertility by decreasing spermatogenesis or alter the harmonic level and histoarchitecture of testis.[7]

Sexually Transmitted Infections (STIs), the major cofactor of HIV, are found to increase the risk of HIV infection by 2–9 times.[8] Bacterial vaginosis, Herpes, Chlamydia, Trichomoniasis, Gonorrhea, Hepatitis B virus, HIV and Syphilis are some of

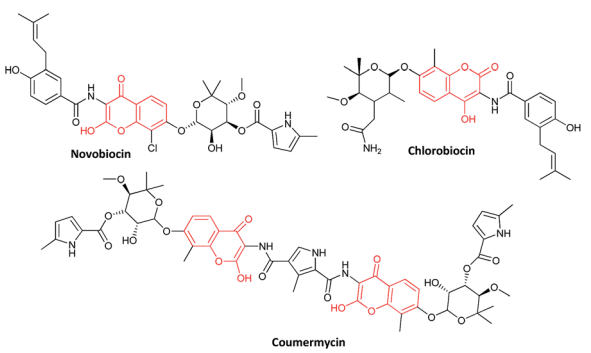

Fig. 1 Structure of naturally occurring antimicrobials with 4-hydroxy coumarins scaffold.

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† Electronic supplementary information (ESI) available: Spectral data of synthesized compounds (^{1}H NMR, ^{13}C NMR and HRMS and HPLC data). See DOI: 10.1039/c6ra12156j

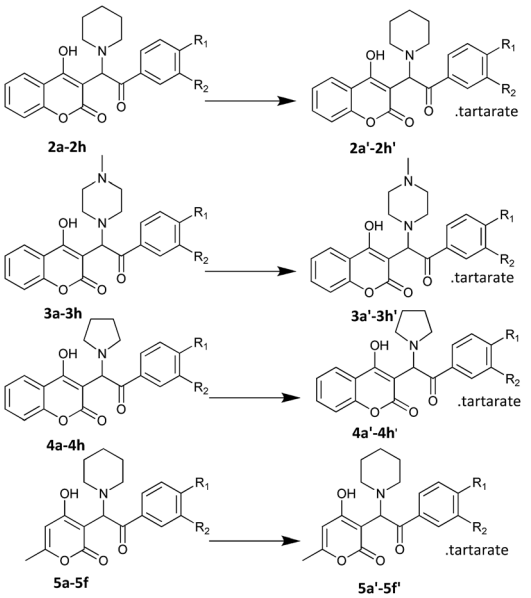


Fig. 2 Structure of synthesized Mannich compounds and their tartrate salts.

the common sexually transmitted diseases.[8] Microbicides can be used to reduce the transmission of STIs including HIV, but microbicides alone without contraceptive activity may not be used with the required compliance during most of the sexual contacts.[9–11] Membrane surfactant nonoxynol-9 (N-9) is used as the main ingredient in most of the currently marketed vaginal contraceptive products.[9] N-9 contain multiple oligomers which have structure affinity with membrane lipids causing cell death in sperm cells by disruption of its membrane integrity.[12] However, consistent use of N-9 or other surfactants causes detergent-type cytotoxicity toward vaginal cells and also inactivates *Lactobacilli*, hence increases the risk of HIV/STI transmission which is the major drawback of using them despite possessing potent microbicidal activity *in vitro*.[13] Therefore, there is a need to develop spermicides with microbicidal activities which are non-toxic to the membrane cells and *Lactobacilli*.

Coumarins, also known as benzo-α-pyrones, belong to flavonoid class of compounds. Compounds containing this pharmacophore act as, *inter alia*, anti-HIV,[14] antimitotic,[15] antifungal,[16] anti-viral,[17] anti-tubulin,[18] vasodilators,[19] anti-coagulants,[20] antioxidant,[21] dyslipidemic,[22] anti-malarial[23] and antihypertensive agents.[24] Some of its derivatives are tyrosine and protein kinase C inhibitors[25] and have been used in phytochemotherapy for treating vitiligo, psoriasis and atopic dermatitis.[26] Coumarins are also active at, benzodiazepine receptors and on lipoxygenase and cyclooxygenase, possessing anticancer and antimutagenic properties and have application in cystic fibrosis treatment.[27] It is also an important pharmacophore in naturally occurring biologically active compounds like Daphnetin, Pachyrrhizine, Pimpinellin, Fraxetin, Aesculetin, Umbelliferone *etc.*[22,28–31]

Anti-microbial drugs or antibiotics such as Novobiocin, Coumermycin and Chloromycin contain 4-hydroxy coumarins as their core structure.[32] Because of this extraordinarily wide range of biological activities, it can be stated that it has "immense" therapeutic potential and these above interesting findings led us to generate a new molecule containing 4-hydroxy coumarin as its core structure with the aim of exploring the effect of such modification on the spermicide as well as antimicrobial profile (Fig. 1–3).

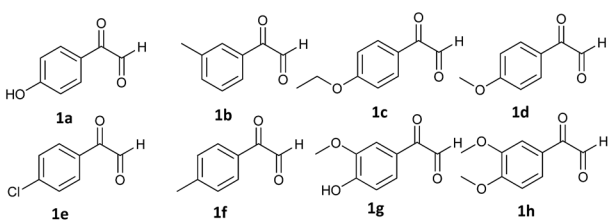
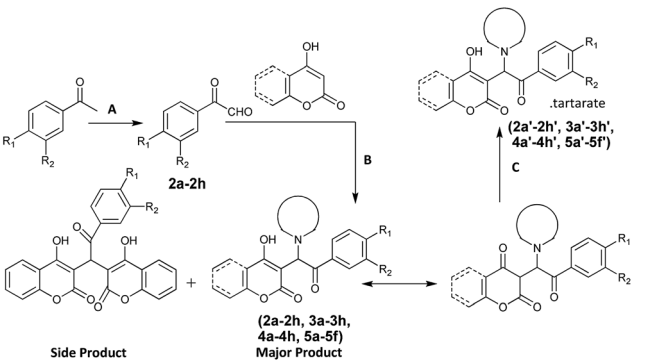


Fig. 3 Different substitutions of phenyl glyoxal used in the reaction.

2 Results and discussion

2.1 Compound design and synthesis

2.1.1 Chemistry. The tartrate salts of Mannich base bearing coumarin derivatives (**2a′–2h′**) (**3a′–3h′**) (**4a′–4h′**) (**5a′–5h′**) have been synthesized during this study. Designed compounds were synthesized in three step procedure. It has been observed that α,β unsaturation is responsible for spermicidal actions. Therefore, we used phenyl glyoxals to increase its unsaturation for spermicidal activity. Substituted phenyl glyoxals were prepared by oxidation of ketones through selenium dioxide as procedure given in reference.[33] These substituted phenyl glyoxals were further reacted with 4-hydroxy coumarin and cyclic secondary amine in the presence of methanol as solvent at refluxing temperature for overnight. 4-Hydroxy coumarin is an important pharmacophore responsible for its antimicrobial property. Cyclic amines have been used to enhance its hydrophilicity, which is an essential condition for compounds used for spermicidal actions as well as for its basic nature. Three different cyclic amines *i.e.* piperidine, pyrrolidine and *N*-methyl-



Scheme 1 Reaction conditions: A = substituted acetophenone (1 mmol), selenium(II) oxide (1.8 eq.), 1,4-dioxane, H_2O, 80 °C, reflux, 5 h, B = 4-hydroxy coumarin (1 mmol), substituted phenyl glyoxal (1.2 eq.), cyclic amine (1 eq.), MeOH, reflux, overnight C = Mannich base product (0.5 mmol), tartaric acid (1 eq), MeOH, rt, 30 minutes.

Table 1 Spermicidal potential in human sperm cells (MEC and percent activity), anti-trichomonas activity in metronidazole (MTZ)-susceptible and MTZ-resistant cell lines (IC_{50}) and cytotoxicity towards vaginal epithelium cells (HeLa cell lines) (IC_{50}) and lactobacilli cell line (IC_{50}) studies of synthesized compounds[a,b]

S. No.	Compound	Spermicidal activity (mM)	Spermicidal activity (%) [mM = % × 10 000/M wt]	Trichomonacidal activity IC_{50} (mM) A (μg ml^{-1} = mM × M wt)	Trichomonacidal activity IC_{50} (mM) B (μg ml^{-1} = mM × M wt)	Hela cell lines IC_{50} (μg ml^{-1})	Lactobacilli cell lines IC_{50} (μg ml^{-1})
1	**2a**	0.5	0.026	0.0294	0.2363	234	>1000
2	**2b**	5	0.263	0.0296	0.4744	212	>1000
3	**2c**	2.5	0.139	0.0140	0.1122	>1000	>1000
4	**2d**	0.5	0.027	0.2302	0.9208	>1000	>1000
5	**2e**	5	0.263	0.1143	0.4570	>1000	>1000
6	**2f**	5	0.262	0.0594	0.2381	>1000	>1000
7	**2g**	5	0.278	0.0280	0.1122	>1000	>1000
8	**2h**	2.5	0.143	0.0136	0.1091	127	>1000
9	**3a**	12.5	0.680	0.0287	0.2298	>1000	>1000
10	**3b**	12.5	0.677	0.1153	0.2306	>1000	>1000
11	**3c**	12.5	0.715	0.1093	0.2185	670	>1000
12	**3d**	12.5	0.697	0.0279	0.2240	>1000	>1000
13	**3e**	25	1.405	0.2224	0.8897	>1000	>1000
14	**3f**	12.5	0.677	0.0575	0.1153	>1000	>1000
15	**3g**	25	1.435	0.0272	0.1089	>1000	>1000
16	**3h**	25	1.470	0.0531	0.2125	>1000	>1000
17	**4a**	25	1.287	0.1213	0.4854	>1000	>1000
18	**4b**	5	0.256	0.0304	0.2437	235	>1000
19	**4c**	12.5	0.678	0.0287	0.1151	>1000	>1000
20	**4d**	25	1.322	0.0590	0.2363	>1000	>1000
21	**4e**	25	1.332	0.0585	0.1173	>1000	>1000
22	**4f**	12.5	0.641	0.1218	0.2437	962	>1000
23	**4g**	25	1.362	0.1147	0.4587	>1000	>1000
24	**4h**	25	1.397	0.0279	0.2236	>1000	>1000
25	**5a**	100	4.950	0.1262	0.2525	>1000	>1000
26	**5b**	50	2.445	0.0638	1.0224	>1000	>1000
27	**5c**	100	5.190	0.1204	0.2408	>1000	>1000
28	**5d**	50	2.535	0.1233	0.4931	778	>1000
29	**5e**	50	2.545	0.1228	0.2456	>1000	>1000
30	**5f**	50	2.435	0.0638	0.5112	>1000	>1000
31	Nonoxynol-9	0.486	0.03	—	—	50.5	35.4
32	Metronidazole	—	—	0.0182	0.3655	—	—

[a] Metronidazole-susp strain with 48 h duration of incubation. [b] Metronidazole-res strain with 48 h duration of incubation, MEC = minimum effective concentration with sperm motility 70–75%.

piperazine were used during the study to check further its role in spermicidal actions. The synthesized Mannich type product were reacted with the tartaric acid in methanol for salt formation. Tartrate salts are formed to improve its hydrophilicity and for better efficacy.[34] Reaction of phenyl glyoxal and 4-hydroxy coumarin gave keto–enol tautomers of Mannich reaction product and dicoumarols (2 molecules of 4-hydroxy coumarin fused with phenyl glyoxal) as a side product. Saeed *et al.* have reported the formation of dicoumarols which was further confirmed by mass and NMR spectra.[35] It was observed that reaction proceeded smoothly and in good yield with electron donating groups while in the case of electron withdrawing groups either reaction does not occur or very low yield is obtained. In case of nitro as a substituent reaction, product was not formed. When simple hydrogen was used as a substituent a dimer of 4-hydroxy coumarin fused with phenyl glyoxal was obtained as major product instead of our desired compound. When methoxy group is attached on 3, 4, 5 position then also reaction didn't proceeded may be due to steric hindrance. To increase the hydrophilicity of these Mannich products, tartrate salts were made using 1 equivalent of tartaric acid in methanol. Reaction scheme is illustrated in figure given below. These compounds were structurally identified through ^{1}H and ^{13}C NMR spectroscopy and HRMS (High Resolution Mass Spectrometry) to confirm their structure (Scheme 1).

2.1.2 Biology. Spermicidal activity was observed in comparison with the compound currently in clinical use, nonoxynol-9 (N-9). In this experiment it was observed that in case of replacement of 4-hydroxy coumarin with 4-hydroxy-6-methyl-2*H*-pyran-2-one, compounds are either inactive or showing very low spermicidal activity. Therefore, it can be said that phenyl ring of 4-hydroxy coumarin is responsible for its spermicidal activity and in the absence of this ring, spermicidal activity decreased. A series of compounds was formed fused with piperidine, pyrrolidine and *N*-methyl piperazine. It was observed that spermicidal activity was highest in case of piperidine chain, moderate in pyrrolidine and lowest in case of *N*-methyl piperazine except few cases. In case of 4-hydroxy and 4-methoxy substitution, compounds with *N*-methyl piperazine was more active in comparison of pyrrolidine. Polar substitution like OH, OCH_3 group on 4-position of phenyl glyoxal induces activity in the compound. When OCH_3 group was added on 3-position of these compounds, then activity decreased and when CH_2 was inserted in methoxy group ($-OCH_2CH_3$) then also activity decreased. Spermicidal activity was observed increasing with increase in polarity of substitution on 4-position of phenyl glyoxal. 16 compounds showed spermicidal activity at concentrations ranging from 0.02 to 1.0%. Out of these, 2 compounds (**2a** and **2d**) showed activity better than nonoxynol-9. In case of anti-trichomonas activity experiment, 18 compounds showed activity at concentrations ranging from 0.0136 mM to 0.0638 mM in comparison of metronidazole susceptible trichomonas cell lines and 21 compounds showed better activity (concentrations ranging from 0.1089 mM to 0.2525 mM) in comparison of metronidazole resistant trichomonas cell lines. 5 compounds were showing excellent anti-trichomonas activity in both metronidazole susceptible and resistant strains. 2 compounds (**2c** and **2h**) showed potent activity at 0.0140 mM and 0.0136 mM respectively in case of metronidazole susceptible strain and 0.1122 mM and 0.1091 mM in case of metronidazole resistant strains which is better than metronidazole. Most of the compounds were found safe in cytotoxicity assay (HeLa cell lines) and safety (*Lactobacillus jenseii* strains) studies (Table 1).

3 Conclusions

In Conclusion, we have designed and synthesized a series of novel 4-hydroxy coumarin and glyoxal conjugates using eco-friendly approach with good yields and evaluated them for spermicidal and anti-microbial activity. These compounds were tested about their toxicity towards vaginal epithelial cell lines and safety towards lactobacilli strains. 16 compounds showed activity comparable to N-9 in which compound **2a** and **2d** showed significant spermicidal activity. 18 compounds were equipotential to metronidazole susceptible trichomonas cell lines while 21 compounds displayed prominent activity in comparison of metronidazole resistant trichomonas cell lines. Compound **2c** and **2h** were found most potent in anti-trichomonas assay. These compounds were found highly safe in comparison of N-9 in cytotoxicity and safety assays. 11 compounds were found active in both spermicidal and anti-trichomonas assays. Out of those, compound **2a**, **2c** and **2h** were observed as most potent. Furthermore, compound **2a** was most promising in terms of spermicidal activity and thus can be transformed in a lead molecule towards the development of dually useful contraceptive microbicides.

4 Experimental procedures

All reagents used were commercial and were used without further purification. Compounds were purified on 60–120 mesh and 100–200 mesh silica gels with methanol : chloroform as eluent. All reactions were monitored by silica gel TLC plated with F_{254} fluorescence. The ^{1}H and ^{13}C NMR spectra were determined on 400 MHz and 100 MHz Bruker NMR spectrometers respectively using $CDCl_3$ and DMSO-d_6 as deuterated solvents and TMS as an internal standard. In some cases, increase in the no. of aromatic protons was observed in NMR spectra due to keto–enol tautomerism. Chemical shifts are reported in parts per million. Splitting patterns are described as singlet (s), broad singlet (brs), doublet (d), broad doublet (brd), double doublet (dd), triplet (t), quartet (q), and multiplet (m). Infrared spectra were recorded on a Perkin-Elmer FT-IR RX1 spectrophotometer (4000–450 cm^{-1} range). Melting points were taken in open capillaries on Stuart SME30 melting point apparatus and are presented uncorrected. Electrospray ionization mass spectra (ESI MS) were recorded on Thermo LCQ Advantage Max-IT. HRMS were recorded by Q-TOF mass spectrometer. Purity analysis of compounds were characterized by HPLC analysis (RP-18 Lichrocart ® HPLC system). The HPLC system (Shimadzu, Kyoto, Japan) equipped with Photo Diode Array detector (SPD-M20A), a pump (LC-20AD), controller (CBM-20A), Rheodyne (Cotati, CA, USA) model 7125 injector with a 20 μl

loop. Data was acquired in LC solution software (Shimadzu, Japan). Compounds were monitored at 275 nm with a UV-Vis multiple wavelength detector. Compounds with purity >95% were used for further experiments.

4.1 General procedure of phenyl glyoxal synthesis

SeO_2 (6.4 mmole) was taken in a 50 ml round bottom flask with 1,4-dioxane/water (10 ml/95 : 5) as solvent and heated it at 60 °C for 3 h. Substituted acetophenone (6.5 mmol) was then added and reflux the reaction mixture for 4 h. After completion of the reaction, reaction mixture was filtered and filterate was concentrated. Formation of product (**1a–1h**) was confirmed through TLC and mass and further used without purification.[33]

4.2 General synthetic procedure for 4-hydroxy-3-(2-(4-substitutedphenyl)-2-oxo-1-(piperidin-1-yl) ethyl)-2*H*-chromen-2-one derivatives 2a–2h

Substituted phenyl glyoxal was refluxed with 4-hydroxy coumarin in the presence of piperidine as base and methanol as solvent for 8 h. After completion of the reaction, it was quenched with water and extracted with DCM. The organic layer was separated and dried over sodium sulfate and concentrated *in vacuo*. Crude product was purified by column chromatography using 100–200 mesh silica gels as adsorbent and methanol : chloroform as eluent.

4.2.1 Compound 4-hydroxy-3-(2-(4-hydroxyphenyl)-2-oxo-1-(piperidin-1-yl) ethyl)-2*H*-chromen-2-one (2a). Light orange solid as a mixture of keto/enol tautomers (0.284 g, 75%), mp 184–186 °C, HPLC (purity 95.01%): $t_R = 12.698$ min; FT-IR (KBr, ν_{max}/cm^{-1}): 3396, 3019, 2919, 1630, 1402, 1216, 1155, 1068, 770, 669; δ_H (400 MHz; DMSO-d_6): 15.8–16.0 (1H, brs), 7.77 (1H, dd, J = 7.76 Hz), 7.66 (2H, d, J = 8.72 Hz), 7.45–7.50 (2H, dt, J = 6.96 Hz), 7.18–7.25 (4H, m), 6.67 (2H, d, J = 8.68 Hz), 6.27 (1H, s), 2.98 (4H, m), 1.50–1.70 (6H, m); δ_C (100 MHz; DMSO-d_6): 192.5, 174.2, 162.6, 154.3, 131.7, 130.8, 126.2, 125.4, 122.9, 122.2, 116.4, 115.2, 90.5, 67.3, 54.3, 25.2, 23.5; HR-MS $[M + H]^+$ (m/z) calculated for $C_{22}H_{21}NO_5$ 380.1498 found 380.1481.

4.2.2 Compound 4-hydroxy-3-(2-oxo-1-(piperidin-1-yl)-2-*m*-tolylethyl)-2*H*-chromen-2-one (2b). White solid as a mixture of keto/enol tautomers (0.269 g, 69%) mp 136–138 °C, HPLC (purity 100%): $t_R = 14.271$ min; FT-IR (KBr, ν_{max}/cm^{-1}): 3393, 2922, 1613, 1403, 1218, 1155, 1068, 771; δ_H (400 MHz; $CDCl_3$ + DMSO-d_6): 8.73 (1H, s), 7.98 (2H, d, J = 6.84 Hz), 7.69 (2H, m), 7.37 (2H, brs), 7.17 (2H, brs), 7.05 (3H, s), 6.94 (1H, s), 6.43 (1H, s), 2.18 (4H, m), 1.93 (4H, s), 1.72 (2H, brs), 1.27 (3H, s); δ_C (100 MHz; $CDCl_3$ + DMSO-d_6): 198.4, 170.1, 166.3152.7, 137.4, 136.9, 132.6, 130.9, 128.6, 127.6, 124.9, 124.7, 123.1, 119.8, 115.6, 101.7, 45.0, 43.3, 22.5, 22.2, 21.2; HR-MS $[M + H]^+$ (m/z) calculated for $C_{23}H_{23}NO_4$ 378.1705 found 378.1707.

4.2.3 Compound 3-(2-(4-ethoxyphenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-4-hydroxy-2*H*-chromen-2-one (2c). Whitesolid as a mixture of keto/enol tautomers (0.289 g, 71%) mp 134–136 °C, HPLC (purity 99.36%): $t_R = 12.471$ min; FT-IR (KBr, ν_{max}/cm^{-1}): 3403, 3019, 1632, 1403, 1216, 1155, 1068, 770, 669; δ_H (400 MHz; $CDCl_3$): 8.11 (2H, d, J = 8.48 Hz), 8.00 (1H, d, J = 7.00 Hz), 7.43 (1H, t, J = 7.40 Hz), 7.18 (2H, d, J = 7.96 Hz), 6.84 (2H, d, J = 8.32 Hz), 6.08 (1H, s), 4.03 (2H, s), 1.96 (4H, s), 1.75 (2H, s), 1.37–1.41 (4H, m), 1.27 (3H, s); δ_C (100 MHz; $CDCl_3$): 195.3, 167.4, 162.2, 152.6, 131.7, 130.2, 129.0, 125.0, 123.8, 119.0, 116.5, 115.9, 114.3, 113.7, 102.2, 63.4, 45.4, 43.0, 29.7, 22.8, 14.6; HR-MS $[M + H]^+$ (m/z) calculated for $C_{24}H_{25}NO_5$ 408.1811 found 408.1828.

4.2.4 Compound 4-hydroxy-3-(2-(4-methoxyphenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-2*H*-chromen-2-one (2d). Light brown solid as a mixture of keto/enol tautomers (0.291 g, 74%), mp 116–118 °C, HPLC (purity 95.71%): $t_R = 14.166$ min; FT-IR (KBr, ν_{max}/cm^{-1}): 3393, 3015, 1665, 1398, 1256, 1218, 1173, 765; δ_H (400 MHz; $CDCl_3$): 8.11 (2H, d, J = 8.76 Hz), 7.99 (1H, d, J = 7.24 Hz), 7.43 (1H, t, J = 7.16 Hz), 7.18 (2H, d, J = 8.08 Hz), 6.86 (2H, d, J = 8.68 Hz), 6.11 (1H, s), 3.80 (3H, s), 2.13 (2H, s), 1.96 (4H, s), 1.40–1.80 (2H, brs); δ_C (100 MHz; $CDCl_3$): 192.7, 165.3, 162.5, 160.2, 152.5, 131.6, 130.1, 124.9, 123.7, 122.8, 115.8, 113.1, 102.2, 55.0, 45.5, 30.9, 29.6, 22.7, 22.3; HR-MS $[M + H]^+$ (m/z) calculated for $C_{23}H_{23}NO_5$ 394.1654 found 394.1646.

4.2.5 Compound 3-(2-(4-chlorophenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-4-hydroxy-2*H*-chromen-2-one (2e). Light brown solid as a mixture of keto/enol tautomers (0.286 g, 72%), mp 120–122 °C, HPLC (purity 98.69%): $t_R = 14.587$ min; FT-IR (KBr, ν_{max}/cm^{-1}): 3388, 3018, 1603, 770, 668; δ_H (400 MHz; $CDCl_3$): 7.9–8.12 (2H, m), 7.87 (1H, d, J = 8.4 Hz), 7.39 (1H, t, J = 7.8 Hz), 7.24–7.32 (1H, m), 7.0–7.2 (3H, m), 6.17 (1H, s), 3.47 (1H, s), 3.18 (4H, t, J = 5.44 Hz), 1.81 (4H, d, J = 4.88 Hz), 1.65 (2H, d, J = 4.8 Hz); δ_C (100 MHz; $CDCl_3$): 198.9, 175.1, 166.9, 153.9, 152.8, 139.4, 138.0, 133.3, 131.2, 129.6, 129.3, 128.6, 128.2, 125.0, 123.4, 122.9, 122.2, 116.2, 115.7, 101.3, 95.6, 79.2, 56.3, 45.0, 22.8, 22.4; HR-MS $[M + H]^+$ (m/z) calculated for $C_{22}H_{20}NO_4Cl$ 398.1159 found 398.1152.

4.2.6 Compound 4-hydroxy-3-(2-oxo-1-(piperidin-1-yl)-2-*p*-tolylethyl)-2*H*-chromen-2-one (2f). Light orange solid as a mixture of keto/enol tautomers (0.271 g, 72%), mp 189–191 °C, HPLC (purity 95.65%): $t_R = 14.961$ min; IR (KBr) (cm^{-1}): 3388, 1606, 1542, 1403, 1217, 1154, 1068, 771, 668; δ_H (400 MHz; $CDCl_3$): 7.98–8.02 (3H, m), 7.40–7.44 (1H, m), 7.16 (4H, d, J = 6.72 Hz), 6.14 (1H, s), 2.90–3.90 (4H, br), 2.33 (3H, s), 1.97 (4H, s), 1.72 (2H, s); δ_C (100 MHz; $CDCl_3$): 197.7, 170.8, 166.6, 153.1, 142.6, 131.1, 129.1, 128.6, 128.1, 125.2, 122.9, 120.1, 115.6, 102.2, 63.3, 46.3, 31.1, 29.7, 21.3; HR-MS $[M + H]^+$ (m/z) calculated for $C_{23}H_{23}NO_4$378.1705 found 378.1690.

4.2.7 4-Hydroxy-3-(2-(4-hydroxy-3-methoxyphenyl)-2-oxo-1-(piperidin-1-yl) ethyl)-2*H*-chromen-2-one (2g). White solid as a mixture of keto/enol tautomers (0.286 g, 70%), mp 213–215 °C, HPLC (purity 98.32%): $t_R = 12.995$ min; FT-IR (KBr, ν_{max}/cm^{-1}): 3396, 2927, 1645, 1513, 1404, 1216, 1069, 768; δ_H (400 MHz; DMSO-d_6): 16.00 (1H, s), 8.20–8.80 (1H, br), 7.78 (2H, d, J = 7.16 Hz), 7.49 (2H, t, J = 7.92 Hz), 7.40 (1H, s), 7.31 (1H, d, J = 7.84 Hz), 7.14–7.28 (4H, m), 6.69 (1H, d, J = 8.24 Hz), 6.32 (1H, s), 3.72 (3H, s), 3.01 (4H, s), 1.64 (4H, s), 1.56 (2H, s); δ_C (100 MHz; DMSO-d_6): 196.2, 168.7, 164.5, 152.9, 150.8, 147.0, 131.5, 128.5, 124.5, 123.4, 122.3, 120.0, 115.9, 115.0, 112.1, 101.8, 55.8, 44.2, 43.2, 22.6, 22.0; HR-MS $[M + H]^+$ (m/z) calculated for $C_{23}H_{23}NO_6$ 410.1603 found 410.1627.

4.2.8 Compound 3-(2-(3,4-dimethoxyphenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-4-hydroxy-2*H*-chromen-2-one (2h). Light orange solid as a mixture of keto/enol tautomers (0.313 g, 74%)

mp 223–225 °C, HPLC (purity 97.47%): t_R = 13.230 min; FT-IR (KBr, ν_{max}/cm^{-1}): 3408, 3019, 1654, 1403, 1268, 1216, 1154, 770; δ_H (400 MHz; $CDCl_3$): 11.34 (1H, s), 8.10 (2H, dd, J = 7.96 Hz), 7.63 (2H, t, J = 8.52 Hz), 7.50 (1H, s), 7.37–7.45 (5H, m), 6.76 (1H, d, J = 8.44 Hz), 6.13 (1H, s), 3.87 (6H, d, J = 5.56 Hz), 1.58 (10H, s); δ_C (100 MHz; $CDCl_3$): 195.1, 168.7, 165.1, 154.0, 152.4, 150.6, 148.9, 133.0, 124.9, 124.5, 122.2, 116.6, 110.9, 110.0, 102.0, 56.0, 55.8, 42.6, 29.6, 27.3; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{24}H_{25}NO_6$ 424.1760 found 424.1746.

4.3 General synthetic procedure for 4-hydroxy-3-(2-(4-substitutedphenyl)-2-oxo-1-(piperazin-1-yl)ethyl)-2*H*-chromen-2-one derivatives 3a–3h

Substituted phenyl glyoxal was refluxed with 4-hydroxy coumarin in the presence of piperazine as base and methanol as solvent for 8 h. After completion of the reaction, it was quenched with water and extracted with DCM. Organic layer was separated and dried over sodium sulfate and concentrated *in vacuo*. Crude product was purified by column chromatography using 100–200 mesh silica gel as adsorbent and methanol : chloroform as eluent.

4.3.1 Compound 4-hydroxy-3-(2-(4-hydroxyphenyl)-1-(4-methylpiperazin-1-yl)-2-oxoethyl)-2*H*-chromen-2-one (3a). pale yellow solid as a mixture of keto/enol tautomers (0.307 g, 78%) mp 204–206 °C, HPLC (purity 98.89%): t_R = 11.339 min; IR (KBr) (cm^{-1}): 3432, 1638, 1401, 1385, 1219, 1069, 771; δ_H (400 MHz; $CDCl_3$ + DMSO-d_6): 7.88 (1H, d, J = 7.76 Hz), 7.78 (2H, d, J = 8.72 Hz), 7.52 (1H, s), 7.34 (1H, t, J = 8.0 Hz), 7.06 (2H, m), 6.65 (2H, d, J = 8.76 Hz), 5.91 (1H, s), 2.80–3.20 (4H, brs), 2.50–2.70 (4H, brs), 2.24 (3H, s); δ_C (100 MHz; $CDCl_3$ + DMSO-d_6): 196.7, 180.1, 169.6, 167.5, 158.9, 136.2, 135.4, 130.6, 130.0, 127.4, 126.5, 121.0, 120.1, 93.6, 72.6, 56.5, 54.4, 50.1; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{22}H_{22}N_2O_5$ 395.1607 found 395.1602.

4.3.2 Compound 4-hydroxy-3-(1-(4-methylpiperazin-1-yl)-2-oxo-2-*m*-tolylethyl)-2*H*-chromen-2-one (3b). Orangish-yellow solid as a mixture of keto/enol tautomers (0.298 g, 76%), mp 119–121 °C, HPLC (purity 100%): t_R = 13.062 min; IR (KBr) (cm^{-1}): 3412, 3019, 1617, 1467, 1404, 1385, 1215, 1069, 758, 669; δ_H (400 MHz; $CDCl_3$): 8.00 (1H, d, J = 7.68 Hz), 7.75 (1H, s), 7.68 (1H, d, J = 6.76 Hz), 7.55 (1H, brs), 6.80–7.20 (5H, m), 6.48 (1H, s), 3.23 (3H, q, J = 7.2 Hz), 2.18 (3H, s), 1.2–1.34 (8H, s); δ_C (100 MHz; $CDCl_3$): 167.3, 152.6, 137.7, 136.7, 134.4, 132.9, 131.6, 128.8, 127.7, 125.0, 124.9, 123.7, 119.2, 115.9, 102.3, 46.7, 43.2, 31.4, 29.4, 22.6; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{23}H_{24}N_2O_4$ 393.1814 found 393.1805.

4.3.3 Compound 3-(2-(4-ethoxyphenyl)-1-(4-methylpiperazin-1-yl)-2-oxoethyl)-4-hydroxy-2*H*-chromen-2-one (3c). Pale yellow solid as a mixture of keto/enol tautomers (0.317 g, 75%), mp 185–187 °C, HPLC (purity 98.89%): t_R = 11.339 min; IR (KBr) (cm^{-1}): 3431, 2069, 1638, 1402, 1385, 1219, 1068, 771; δ_H (400 MHz; $CDCl_3$): 8.00 (1H, d, J = 7.68 Hz), 7.68 (1H, d, J = 6.76 Hz), 7.41 (1H, s), 7.06–7.20 (5H, m), 6.48 (1H, s), 3.48 (2H, s), 3.24 (3H, s), 1.29 (8H, s), 1.20 (3H, s); δ_C (100 MHz; $CDCl_3$): 197.7, 167.3, 152.6, 137.7, 136.7, 132.9, 131.6, 128.8, 127.7, 125.0, 124.9, 123.7, 115.9, 102.3, 64.5, 46.7, 43.2, 30.9, 29.6, 14.1; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{24}H_{26}N_2O_5$ 423.1920 found 423.1432.

4.3.4 Compound 4-hydroxy-3-(2-(4-methoxyphenyl)-1-(4-methylpiperazin-1-yl)-2-oxoethyl)-2*H*-chromen-2-one (3d). Light brown solid as a mixture of keto/enol tautomers (0.314 g, 77%) mp 138–140 °C, HPLC (purity 97.62%): t_R = 11.689 min; IR (KBr) (cm^{-1}): 3415, 1646, 1601, 1528, 1395, 1259, 1218, 1174, 1051, 765; δ_H (400 MHz; $CDCl_3$): 8.14 (1H, d, J = 4.8 Hz), 8.01 (1H, s), 7.42 (1H, t, J = 7.76 Hz), 7.17 (2H, d, J = 7.6 Hz), 7.00 (1H, t, J = 8.8 Hz), 6.85 (2H, d, J = 6.68 Hz), 5.90–6.10 (1H, brs), 3.79 (3H, s), 3.00–3.50 (2H, brs), 2.75 (2H, s), 2.30–2.50 (4H, br), 1.28 (3H, s); δ_C (100 MHz; $CDCl_3$): 192.5, 175.5, 165.1, 164.9, 154.3, 131.5, 130.9, 130.2, 126.9, 125.2, 123.0, 121.7, 116.4, 115.5, 113.8, 113.0, 90.9, 69.4, 55.4, 53.7, 46.1; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{23}H_{24}N_2O_5$ 409.1763 found 409.1761.

4.3.5 Compound 3-(2-(4-chlorophenyl)-1-(4-methylpiperazin-1-yl)-2-oxoethyl)-4-hydroxy-2*H*-chromen-2-one (3e). Dark orange solid as a mixture of keto/enol tautomers (0.309 g, 75%) mp 133–135 °C, HPLC (purity 98.36%): t_R = 14.095 min; IR (KBr) (cm^{-1}): 3399, 1610, 1400, 1385, 1218, 1069, 771, 670; δ_H (400 MHz; $CDCl_3$): 7.99 (1H, d, J = 7.80 Hz), 7.87 (1H, d, J = 8.36 Hz), 7.41 (1H, t, J = 7.24 Hz), 7.12–7.25 (4H, m), 6.48 (1H, s), 3.29 (4H, q, J = 7.28 Hz), 2.70–3.10 (2H, brs), 1.39 (1H, s), 1.37 (3H, s), 1.35 (1H, s); δ_C (100 MHz; $CDCl_3$): 196.9, 166.9, 152.8, 137.8, 131.3, 130.1, 129.4, 128.9, 125.1, 123.5, 119.8, 115.7, 101.7, 68.5, 46.4, 43.4, 29.6; HR-MS $[M + 2H]^+$, (*m*/*z*) calculated for $C_{22}H_{21}N_2O_4Cl$. 414.1346 found 414.1234.

4.3.6 Compound 4-hydroxy-3-(1-(4-methylpiperazin-1-yl)-2-oxo-2-*p*-tolylethyl)-2*H*-chromen-2-one (3f). Dark yellow solid as a mixture of keto/enol tautomers (0.306 g, 78%), mp 76–78 °C, HPLC (purity 95.18%): t_R = 13.888 min; IR (KBr) (cm^{-1}); 3412, 3019, 1617, 1467, 1404, 1385, 1215, 1069, 758, 669; δ_H (400 MHz; $CDCl_3$): 7.94 (1H, d, J = 7.56 Hz), 7.77 (1H, d, J = 7.96 Hz), 7.33 (1H, t, J = 7.56 Hz), 7.08–7.14 (4H, m), 6.97 (1H, d, J = 7.68 Hz), 6.38 (1H, s), 3.24 (4H, q, J = 7.24 Hz), 2.09 (4H, m), 1.31 (3H, t, J = 7.24 Hz), 1.18 (3H, s); δ_C (100 MHz; $CDCl_3$): 197.7, 170.7, 166.6, 152.8, 142.1, 131.0, 128.9, 128.6, 128.1, 125.2, 123.2, 120.1, 115.6, 101.9, 46.5, 43.3, 30.9, 29.7, 21.5; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{23}H_{24}N_2O_4$ 393.1814 found 393.1805.

4.3.7 Compounds 4-hydroxy-3-(2-(4-hydroxy-3-methoxyphenyl)-1-(4-methylpiperazin-1-yl)-2-oxoethyl)-2*H*-chromen-2-one (3g). Light yellow solid as a mixture of keto/enol tautomers (0.331 g, 78%), mp 160–162 °C, HPLC (purity 99.66%): t_R = 11.993 min; IR (KBr) (cm^{-1}): 3410, 3019, 2927, 2400, 1646, 1401, 1385, 1215, 1070, 928, 757, 669; δ_H (400 MHz; $CDCl_3$ + DMSO-d_6): 7.90 (2H, s), 7.49 (1H, d, J = 3.48 Hz), 7.40 (1H, s), 7.32 (2H, d, J = 5.6 Hz), 7.25 (2H, m), 6.58 (1H, s), 6.36 (1H, s), 3.71 (3H, s), 1.29 (3H, s), 1.18 (8H, s); δ_C (100 MHz; $CDCl_3$ + DMSO-d_6): 152.7, 149.9, 146.5, 131.0, 124.9, 123.2, 122.4, 115.6, 113.9, 111.5, 102.2, 55.7, 52.0, 46.5, 43.0, 31.8, 29.5, 29.2, 22.5; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{23}H_{24}N_2O_6$ 425.1712 found 425.1707.

4.3.8 Compound 3-(2-(3,4-dimethoxyphenyl)-1-(4-methylpiperazin-1-yl)-2-oxoethyl)-4-hydroxy-2*H*-chromen-2-one (3h). Pale yellow solid as a mixture of keto/enol tautomers (0.333 g, 76%) mp 147–150 °C, HPLC (purity 100%): t_R = 13.062 min; IR (KBr) (cm^{-1}): 3407, 3020, 1654, 1601, 1536, 1460, 1408, 1269,

1215, 1025, 762, 669; δ_H (400 MHz; $CDCl_3$): 8.04 (1H, s), 7.80 (1H, d, $J = 6.44$ Hz), 7.67 (1H, s), 7.43 (1H, t, $J = 5.88$ Hz), 7.19 (2H, m), 6.83 (1H, d, $J = 6.52$ Hz), 6.18 (1H, s), 3.86 (6H, s), 3.20–3.60 (4H, br), 2.70–2.90 (4H, br), 2.34 (3H, s); δ_C (100 MHz; $CDCl_3$): 190.4, 175.0, 164.6, 154.2, 148.8, 131.8, 126.8, 124.9, 123.7, 123.1, 121.0, 116.5, 111.0, 110.3, 88.8, 56.0, 52.3, 50.7, 45.2; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{24}H_{26}N_2O_6$ 439.1869 found 439.1863.

4.4 General synthetic procedure for 4-hydroxy-3-(2-(4-substitutedphenyl)-2-oxo-1-(pyrollodin-1-yl) ethyl)-2*H*-chromen-2-one derivatives 4a–4h

Substituted phenyl glyoxal was refluxed with 4-hydroxy coumarin in the presence of pyrollidine as base and methanol as solvent for 8 h. After completion of the reaction, it was quenched with water and extracted with DCM. Organic layer was separated and dried over sodium sulfate and concentrated *in vacuo*. Crude product was purified by column chromatography using 100–200 mesh silica gel as adsorbent and methanol : chloroform as eluent.

4.4.1 Compound 4-hydroxy-3-(2-(4-hydroxyphenyl)-2-oxo-1-(pyrrolidin-1-yl)ethyl)-2*H*-chromen-2-one (4a). Light pink solid as a mixture of keto/enol tautomers (0.274 g, 75%) mp 224–226 °C, HPLC (purity 98.97%): $t_R = 12.027$ min; IR (KBr) (cm^{-1}): 3400, 3021, 1643, 1600, 1514, 1391, 1216, 1072, 767, 670; δ_H (400 MHz; DMSO-d_6): 10.33 (1H, s), 9.30–9.80 (1H, br), 7.83 (1H, d, $J = 7.72$ Hz), 7.77 (2H, d, $J = 8.8$ Hz), 7.44 (1H, dq, $J = 7.08$ Hz), 7.16 (1H, dt, $J = 7.56$ Hz), 7.10 (1H, d, $J = 8.16$ Hz), 6.71 (2H, d, $J = 8.8$ Hz), 6.03 (1H, s), 3.00–3.30 (4H, br), 1.70–2.00 (4H, br); δ_C (100 MHz; DMSO-d_6): 192.5, 174.2, 162.6, 154.3, 131.7, 130.8, 126.2, 125.4, 122.9, 122.2, 116.4, 115.2, 90.4, 67.9, 23.6; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{21}H_{19}NO_5$ 366.1341 found 366.1335.

4.4.2 Compound 4-hydroxy-3-(2-oxo-1-(pyrrolidin-1-yl)-2-*m*-tolylethyl)-2*H*-chromen-2-one (4b). Dark red solid as a mixture of keto/enol tautomers, hygroscopic (0.269 g, 74%), mp 70–72 °C, HPLC (purity 97.82%): $t_R = 13.578$ min; IR (KBr) (cm^{-1}): 3423, 1612, 1396, 1062, 762; δ_H (400 MHz; $CDCl_3$): 8.00 (1H, d, $J = 7.84$ Hz), 7.79 (1H, s), 7.72 (1H, d, $J = 7.36$ Hz), 7.40 (1H, t, $J = 7.96$ Hz), 7.08–7.21 (4H, m), 6.53 (1H, s), 3.31 (4H, m), 2.27 (3H, s), 1.37 (4H, t, $J = 7.28$ Hz); δ_C (100 MHz; $CDCl_3$): 198.3, 170.7, 166.6, 152.8, 132.6, 131.0, 128.7, 127.6, 125.1, 123.2, 120.0, 115.6, 101.8, 46.6, 43.4, 21.3; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{22}H_{21}NO_4$ 364.1549 found 364.1537.

4.4.3 Compound 3-(2-(4-ethoxyphenyl)-2-oxo-1-(pyrrolidin-1-yl)ethyl)-4-hydroxy-2*H*-chromen-2-one (4c). Light orange solid as a mixture of keto/enol tautomers (0.295 g, 75%) mp 163–165 °C, HPLC (purity 100%): $t_R = 12.115$ min; IR (KBr) (cm^{-1}): 3396, 1607, 1392, 1218, 1065, 769; δ_H (400 MHz; $CDCl_3$): 9.01 (1H, s), 7.70–8.00 (3H, m), 7.35 (1H, s), 6.80–7.20 (3H, m), 6.53 (2H, s), 3.71 (2H, d, $J = 7.48$ Hz), 3.49 (3H, s), 2.05 (3H, s), 1.20 1.40 (5H, m); δ_C (100 MHz; $CDCl_3$): 196.4, 167.4, 162.2, 152.5, 131.6, 130.2, 128.8, 124.7, 123.7, 119.1, 115.9, 114.3, 113.6, 102.4, 53.4, 46.2, 42.9, 31.4, 29.7, 24.4, 22.6, 14.5, 14.1; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{23}H_{23}NO_5$ 394.1654 found 394.1640.

4.4.4 Compound 4-hydroxy-3-(2-(4-methoxyphenyl)-2-oxo-1-(pyrrolidin-1-yl)ethyl)-2*H*-chromen-2-one (4d). Dark brown solid as a mixture of keto/enol tautomers (0.288 g, 76%), mp 156–158 °C, HPLC (purity 96.26%): $t_R = 13.299$ min; IR (KBr) (cm^{-1}): 3396, 3018, 1653, 1603, 1541, 1395, 1217, 1175, 1033, 765, 668; δ_H (400 MHz; $CDCl_3$): 8.08 (3H, m), 7.41 (1H, t, $J = 7.12$ Hz), 7.15 (2H, m), 6.82 (2H, d, $J = 7.16$ Hz), 6.16 (1H, s), 3.78 (3H, s), 3.62 (1H, s), 3.47 (1H, s), 2.40–3.00 (2H, br), 1.80–2.20 (4H, br); δ_C (100 MHz; $CDCl_3$): 192.1, 175.5, 165.1, 164.2, 154.2, 131.5, 130.9, 130.2, 126.7, 125.2, 123.0, 121.8, 116.4, 115.5, 113.8, 113.1, 90.9, 69.4, 55.4, 55.0, 46.1, 24.4, 23.7; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{22}H_{21}NO_5$ 380.1498 found 380.1489.

4.4.5 Compound 3-(2-(4-chlorophenyl)-2-oxo-1-(pyrrolidin-1-yl) ethyl)-4-hydroxy-2*H*-chromen-2-one (4e). Light orange solid as a mixture of keto/enol tautomers (0.276 g, 72%) mp 175–179 °C, HPLC (purity 95.21%): $t_R = 13.286$ min; IR (KBR) (cm^{-1}): 3425, 3019, 2958, 2922, 1610, 1546, 1463, 1404, 1270, 1216, 1092, 760, 669; δ_H (400 MHz; $CDCl_3$): 7.95 (2H, d, $J = 6.56$ Hz), 7.87 (1H, d, $J = 8.4$ Hz), 7.39 (1H, t, $J = 7.8$ Hz), 7.28 (1H, d, $J = 8.56$ Hz), 7.00–7.18 (3H, m), 6.17 (1H, s), 3.47 (1H, s), 3.18 (4H, t, $J = 5.44$ Hz), 1.70–1.90 (4H, brs), 1.50–1.70 (2H, brs); δ_C (100 MHz; $CDCl_3$): 199.0, 175.1, 166.9, 153.9, 139.4, 131.3, 131.2, 129.6, 129.3, 128.6, 128.2, 125.0, 123.3, 122.9, 122.2, 116.3, 115.7, 101.3, 79.2, 56.3, 22.8; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{21}H_{18}NO_4Cl$ 384.1002 found 384.0999.

4.4.6 Compound 4-hydroxy-3-(2-oxo-1-(pyrrolidin-1-yl)-2-*p*-tolylethyl)-2*H*-chromen-2-one (4f). Light brown solid as a mixture of keto/enol tautomers (0.272 g, 75%), mp 200–202 °C, HPLC (purity 95.53%): $t_R = 14.282$ min; IR (KBr) (cm^{-1}): 3399, 1606, 1385, 1217, 1156, 1069, 771, 668; δ_H (400 MHz; $CDCl_3$): 7.93 (2H, d, $J = 7.76$ Hz), 7.75 (1H, d, $J = 7.32$ Hz), 7.31 (1H, t, $J = 6.72$ Hz), 7.0–7.14 (3H, m), 6.86–7.0 (1H, brd, $J = 3.36$ Hz), 6.39 (1H, s),3.22 (4H, m), 2.09 (3H, s), 1.30 (4H, t, $J = 7.28$ Hz); δ_C (100 MHz; $CDCl_3$): 197.8, 192.6, 170.8, 166.8, 152.8, 142.1, 134.4, 131.0, 128.5, 128.1, 125.1, 123.2, 123.0, 120.0, 115.6, 102.0, 46.5, 43.3, 30.9, 29.7, 21.4; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{22}H_{21}NO_4$ 364.1549 found 364.1541.

4.4.7 Compound 4-hydroxy-3-(2-(4-hydroxy-3-methoxyphenyl)-2-oxo-1-(pyrrolidin-1-yl)ethyl)-2*H*-chromen-2-one (4g). Light greyish solid as a mixture of keto/enol tautomers (0.296 g, 75%) mp 216–218 °C, HPLC (purity 98.97%): $t_R = 12.027$ min; IR (KBr) (cm^{-1}): 3401, 3019, 1646, 1385, 1215, 1069, 758, 669; δ_H (400 MHz; $CDCl_3$ + DMSO-d_6): 7.91 (1H, s), 7.40–7.60 (3H, m), 7.32 (1H, d, $J = 7.48$ Hz), 7.06 (2H, d, 7.8 Hz), 6.73 (1H, d, $J = 8.32$ Hz), 6.01 (1H, s), 3.78 (3H, s), 2.9 (2H, involved in dmso peak), 2.52 (2H, s), 1.97 (4H, s); δ_C (100 MHz; $CDCl_3$ + DMSO-d_6): 191.2, 165.4, 159.0, 156.2, 153.1, 136.0, 127.9, 127.4, 121.0, 119.7, 116.5, 95.5, 74.5, 60.6, 28.3; HR-MS $[M + H]^+$ (*m*/*z*) calculated for $C_{22}H_{21}NO_6$ 396.1447 found 396.1436.

4.4.8 Compound 3-(2-(3,4-dimethoxyphenyl)-2-oxo-1-(pyrrolidin-1-yl) ethyl)-4-hydroxy-2*H*-chromen-2-one (4h). Pale yellow solid as a mixture of keto/enol tautomers (0.301 g, 74%) mp 150–153 °C, HPLC (purity 96.30%): $t_R = 12.631$ min; IR (KBr) (cm^{-1}): 3401, 3019, 1646, 1601, 1546, 1463, 1401, 1385, 1270, 1216, 668, 757, 769; δ_H (400 MHz; $CDCl_3$): 8.04 (1H, d, $J = 5.84$ Hz), 7.85 (1H, d, $J = 8.48$ Hz), 7.70 (1H, s), 7.44 (1H, t, $J = 6.8$ Hz),

7.19 (2H, m), 6.85 (1H, d, $J = 8.48$ Hz), 6.15 (1H, s), 3.83 (6H, d, $J = 6.4$ Hz), 2.30–2.80 (4H, br), 2.14 (4H, s); δ_C (100 MHz; $CDCl_3$): 191.6, 175.5, 165.1, 154.3, 154.0, 148.7, 131.5, 126.7, 125.1, 123.3, 122.0, 116.5, 110.9, 110.4, 91.0, 69.7, 56.0, 56.0, 23.7; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{23}H_{23}NO_6$ 410.1603 found 410.1594.

4.5 General synthetic procedure for 3-(2-(4-substituted phenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-4-hydroxy-6-methyl-2*H*-pyran-2-one 5a–5f

Substituted phenyl glyoxal was refluxed with 4-hydroxy-6-methyl-2*H*-pyran-2-one in the presence of piperidine as base and methanol as solvent for 8 h. After completion of the reaction, it was quenched with water and extracted with DCM. Organic layer was separated and dried over sodium sulfate and concentrated *in vacuo*. Crude product was purified by column chromatography using 100–200 mesh silica gel as adsorbent and methanol : chloroform as eluent.

4.5.1 Compound 4-hydroxy-3-(2-(4-hydroxyphenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-6-methyl-2*H*-pyran-2-one (5a). White solid as a mixture of keto/enol tautomers (0.175 g, 50%) mp 204–206 °C, HPLC (purity 98.64%): $t_R = 11.718$ min; IR (KBr) (cm^{-1}): 3398, 3019, 1666, 1577, 1499, 1403, 1331, 1302, 1217, 1156, 1069, 844, 771, 669, 607; δ_H (400 MHz; DMSO-d_6): 10.00–11.00 (1H, br), 7.68 (2H, d, $J = 8.64$ Hz), 6.73 (2H, d, $J = 8.64$ Hz), 5.68 (1H, s), 5.44 (1H, s), 2.51 (3H, s), 1.93 (3H, s), 1.74 (5H, br), 1.49 (2H, br); δ_C (100 MHz; DMSO-d_6): 192.3, 178.6, 165.3, 162.6, 160.0, 130.6, 126.3, 115.2, 107.6, 88.2, 67.0, 22.7, 22.0, 19.7; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{19}H_{21}NO_5$ 344.1498 found 344.1480.

4.5.2 Compound 4-hydroxy-6-methyl-3-(2-oxo-1-(piperidin-1-yl)-2-*m*-tolylethyl)-2*H*-pyran-2-one (5b). Dark brown solid as a mixture of keto/enol tautomers (0.177 g, 52%) mp 108–110 °C, HPLC (purity 96.73%): $t_R = 12.605$ min; IR (KBr) (cm^{-1}): 3399, 3019, 1678, 1403, 1215, 1157, 1068, 769, 669; δ_H (400 MHz; $CDCl_3$): 7.99 (2H, d, $J = 7.4$ Hz), 7.20 (2H, d, $J = 7.4$ Hz), 5.88 (1H, s), 5.63 (1H, s), 3.24 (4H, br), 2.37 (3H, s), 2.03 (3H, s), 1.90 (4H, s), 1.66 (2H, s); δ_C (100 MHz; $CDCl_3$): 168.7, 161.1, 138.0, 136.2, 134.9, 133.9, 133.3, 129.0, 128.8, 127.8, 124.9, 106.9, 104.0, 101.2, 98.4, 45.0, 41.5, 22.6, 22.3, 21.3, 21.2, 19.5; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{20}H_{23}NO_4$ 342.1705 found 342.1704.

4.5.3 Compound 3-(2-(4-ethoxyphenyl)-2-oxo-1-(piperidin-1-yl) ethyl)-4-hydroxy-6-methyl-2*H*-pyran-2-one (5c). Dark brown solid as a mixture of keto/enol tautomers (0.204 g, 55%) mp 162–164 °C, HPLC (purity 95.05%): $t_R = 13.868$ min; IR (KBr) (cm^{-1}): 3396, 3018, 1653, 1603, 1541, 1395, 1217, 1175, 1033, 765, 668; δ_H (400 MHz; DMSO-d_6): 7.6–7.8 (2H, m), 6.8–7.0 (2H, m), 5.77 (1H, s), 5.66 (1H, s), 4.07 (2H, s), 2.51 (3H, s), 1.9–2.2 (4H, m), 1.77 (2H, s), 1.32 (4H, s); δ_C (100 MHz; DMSO-d_6): 196.2, 165.8, 163.0, 161.8, 159.6, 129.0, 128.1, 125.9, 103.7, 99.8, 64.0, 63.7, 44.1, 22.7, 22.6, 19.5, 14.9; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{21}H_{25}NO_5$ 372.1811 found 372.1795.

4.5.4 Compound 4-hydroxy-3-(2-(4-methoxyphenyl)-2-oxo-1-(piperidin-1-yl)ethyl)-6-methyl-2*H*-pyran-2-one (5d). Dark brown solid as a mixture of keto/enol tautomers (0.186 g, 52%) mp 89–91 °C, HPLC (purity 95.06%): $t_R = 15.444$ min; IR (KBr) (cm^{-1}): 3399, 3019, 1662, 1513, 1403, 1216, 1158, 1068, 771, 669; δ_H (400 MHz; $CDCl_3$): 8.13 (2H, d, $J = 8.52$ Hz), 6.90 (2H, d, $J = 8.32$ Hz), 5.85 (1H, s), 5.63 (1H, s), 3.85 (3H, s), 3.0–3.75 (4H, br), 2.5–3.0 (2H, br), 2.05 (3H, s), 1.92 (3H, s), 1.68 (2H, s); δ_C (100 MHz; $CDCl_3$): 190.1, 179.3, 166.0, 164.2, 161.1, 131.9, 131.0, 127.0, 113.8, 107.6, 88.2, 68.3, 55.4, 23.4, 22.3, 19.7; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{20}H_{23}NO_5$ 358.1654 found 358.1662.

4.5.5 Compound 4-hydroxy-6-methyl-3-(2-oxo-1-(piperidin-1-yl)-2-*m*-tolylethyl)-2*H*-pyran-2-one (5e). Dark brown solid as a mixture of keto/enol tautomers (0.191 g, 53%) mp 150–152 °C, HPLC (purity 96.98%): $t_R = 12.824$ min; IR (KBr) (cm^{-1}): 3399, 3019, 2400, 1660, 1532, 1402, 1215, 1155, 1069, 928, 770, 669; δ_H (400 MHz; DMSO-d_6): 15.01 (1H, s), 7.66 (2H, d, $J = 6.92$ Hz), 7.42 (2H, d, $J = 6.96$ Hz), 5.90 (1H, s), 5.56 (2H, s), 2.02 (7H, s), 1.90 (1H, s), 1.24 (3H, s); δ_C (100 MHz; DMSO-d_6): 191.4, 179.2, 166.0, 161.4, 140.3, 132.6, 129.8, 128.8, 107.5, 88.0, 68.2, 44.7, 29.6, 23.1, 19.7; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{19}H_{20}NO_4Cl$ 362.1159 found 362.1152.

4.5.6 Compound 4-hydroxy-6-methyl-3-(2-oxo-1-(piperidin-1-yl)-2-*p*-tolylethyl)-2*H*-pyran-2-one (5f). Dark brown solid as a mixture of keto/enol tautomers (0.174 g, 51%) mp 120–122 °C, HPLC (purity 95.29%): $t_R = 14.186$ min; IR (KBr) (cm^{-1}): 3681, 3400, 3019, 2399, 1663, 1606, 1529, 1429, 1297, 1215, 1160, 1029, 928, 759, 668, 626; δ_H (400 MHz; $CDCl_3$): 7.99 (2H, d, $J = 7.48$ Hz), 7.20 (2H, d, $J = 7.16$ Hz), 5.88 (1H, s), 5.63 (1H, s), 3.24 (4H, br), 2.37 (3H, s), 2.03 (3H, s), 1.90 (4H, s), 1.66 (2H, s); δ_C (100 MHz; $CDCl_3$): 192.2, 179.2, 166.0, 161.1, 144.8, 131.7, 129.2, 128.6, 107.6, 68.3, 29.6, 23.2, 22.3, 21.7, 19.7; HR-MS $[M + H]^+$ (*m/z*) calculated for $C_{20}H_{23}NO_4$. 342.1705 found 342.1713.

4.6 General synthetic procedure for the formation of tartrate salts of compounds 2–4(a–h) and 5a–5f

Compound was dissolved in methanol and 1 equivalent tartaric acid was added to it. This mixture was stirred for half an hour and then methanol was evaporated *in vacuo*.

5 Biological methodology

5.1 Spermicidal assay

Freshly ejaculated human semen obtained by masturbation from healthy volunteers was collected directly into a sterile plastic tube and transported immediately to the laboratory. Prior informed consent was obtained from the donors. The samples were allowed to liquefy at 37 °C for 45 min. Semen characteristics and analysis were performed according to the normal criteria as per the World Health Organization guidelines. Semen samples with >65 million per ml sperm count, >70% motility and normal sperm morphology were used to determine the spermicidal minimum effective concentration (MEC) of test compounds, *in vitro*. Briefly, the test compounds were dissolved in a minimum volume of DMSO and diluted with physiological saline (0.85% NaCl in distilled water) to make 50 mM of test solution. 0.05 ml of liquefied human semen was added to 0.25 mL of the test solution and vortexed for 10 s at low speed. A drop of the mixture was then placed on a microscope slide, covered with a cover glass and examined under a phase

contrast microscope.[36] The study was approved by the Institutional Ethics Committees of King George's Medical University and CSIR-CDRI, Lucknow, India.

5.2 Trichomonas vaginalis cultures and trichomonacidal assays

Evaluation of antimicrobial properties of compounds was determined by trichomonacidal assay. Trichomonads used for assay were cultured axenically in Trypticase Yeast-Extract Maltose (TYM) (pH 6.8) supplemented with 10% heat-inactivated FBS, 2% vitamin mixture, 100 U of penicillin per ml and 100 μg mL^{-1} streptomycin at 37 °C under partial anaerobic conditions (with ~0.5 ml air trapped above medium). Drug susceptibility assays were conducted as detailed earlier.[37] Briefly, the parasites were incubated under partial anaerobic condition at 37 °C in culture medium containing serially diluted test compounds or MTZ, in 48-well culture plates.0.05% DMSO in culture medium (the highest concentration of DMSO in test-wells) was used as vehicle in the control wells. Cell viability was checked after 48 h by trypan blue exclusion assay and the minimum concentration of the test agent at which all cells were found dead was considered as its MIC. 100% eradication was confirmed by transferring 100 μl of the suspension to a 15 ml tube with fresh medium and recording the growth at 37 °C. MTZ (the most widely used drug against *T. vaginalis*), was used as reference standard. All experiments were repeated three times.[38]

5.3 Cytotoxicity of compounds toward human cervical (HeLa) cells

HeLa cells were procured from National Centre for Cell Sciences, Pune, India, and grown in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with fetal bovine serum (10%) and antibiotics (a penicillin-streptomycin mixture [100 U ml^{-1}]. The cytotoxic effect of test compounds were evaluated in an *in vitro* model of cervicovaginal epithelium (HeLa) cells line, using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Cells seeded at a density of 5×10^4 per well in 96-well plates were incubate in culture medium (DMEM with 10% fetal calf serum) for 24 h at 37 °C in a 5% CO_2–95% air atmosphere. After 24 h, the culture medium was replaced with fresh medium containing dilutions of test compounds (starting with 1.0 mg ml^{-1}) in experimental wells and 0.05% DMSO in culture medium in control wells. After incubation for another 24 h, 5 μl of 5 mg ml^{-1} MTT solution in PBS [pH 7.4] was added to each well. The formazan crystals formed inside the viable cells were solubilized in DMSO, and the optical density at 540 nm (OD_{540}) was recorded in a microplate reader (Microquant; BioTek).[37]

5.4 Compatibility of compounds with commensal vaginal flora – *L. jensenii*

Lactobacillus jensenii (ATCC 25258, strain 62 G) were grown in 6% Rogosa SL broth medium (Hi Media, India) containing 0.132% acetic acid at 37 °C. Briefly, Rogosa SL broth medium was prepared in MilliQ water, boiled for 2 to 3 min, and distributed in 48-well plates (500 μl per well). Serial dilutions of test compounds were added to experimental wells, and vehicle was added to control wells in triplicate. Approximately 1000 CFU of *L. jensenii* were inoculated into each well. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. At the end of the experiment, the cultures were mixed thoroughly, 100 μl from each well was transferred to the corresponding well of a 96-well plate, and the number of lactobacilli were estimated by measuring the turbidity (OD_{610}).[39]

Ethical statement

The *in vitro* studies involving human samples were approved by the Institutional Ethics Committees of King George's Medical University, Lucknow, and the CSIR-CDRI, Lucknow, India. The ICMR guidelines for bio-medical research were followed.

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