SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF 3, 6 DI-SUBSTITUTED 1-BENZOPYRAN-4-ONE ANALOGS AS ANTI-INFLAMMATORY AGENTS.

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Abstract: A series of 3, 6 di substituted-7-hydroxy-2-methyl-4H-1-benzopyran-4-one derivatives (Chromone Biheterocycles) were synthesized and their structures were established on the basis of modern analytical techniques (^{1}HNMR, ^{13}CNMR and HRMS). All the synthesized compounds were tested for their in vitro anti-inflammatory activity. Among the 21 Synthesized derivatives, Pyrazole linked benzopyran-4-one derivative Compound **7s** (IC50= 23.14), Imidazole linked benzopyran-4-one derivatives Compounds **7g** (IC 50= 17.52) and **7k** (IC50= 19.68) have significant anti-inflammatory activity in comparison with the Diclofenac as control standard (IC50= 16.23). Further demonstrated the mechanism of anti-inflammatory activity for the compounds **7g** (2 IC50= 13.5) and **7k** (2 IC50= 17.1) using TNF-α ELISA based assay in comparison with the Infliximab (IC50= 12.9) as control standard. Animal experiments were carried out to find the in-vivo efficacy of compound **7g** through carrageenan-induced rat paw edema assay method. The results demonstrated that Compound (**7g**) had significant anti-inflammatory activity at doses of 50 and 75 mg/kg, had the best significant reduction and inhibition of edema with 82.28, 88.61% and 84.34, 91.57% at third hour and fourth hour respectively, and similar as compared with standard drug Diclofenac 50 mg/kg body weight ($p < 0.05$). Molecular docking interactions of Compound **7g** with TNF-α (2AZ5) using glide protocol of Schrodinger suite revealed best possible pose with a dock score of 9.6. Altogether, our findings suggest that Compound **7g** is a promising anti-inflammatory drug candidate, in the management of inflammation and pain conditions.

Keywords: Chromones, Imidazole, Pyrazole, Anti-inflamatory activity.

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INTRODUCTION

Inflammation is a complex natural response to harmful stimuli, such as physical trauma, noxious chemicals and pathogens like bacteria, viruses or fungi [1]. The immune and inflammatory reactions serve as the major defense mechanism against an injury, and play a key role in acute as well as chronic diseases [2]. Prolonged inflammation can lead several diseases that collectively represent the leading causes of disability and mortality worldwide, such as cardiovascular disease [3], cancer [4], diabetes mellitus [5], chronic kidney disease [6], non-alcoholic fatty liver disease [7] and autoimmune and neurodegenerative disorders [8]. Tumour Necrosis Factor alpha (TNF alpha), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis [9]. The inappropriate or excessive production of TNF-α can be harmful and may lead to several diseases. Rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriatic arthritis (PsA), psoriasis (PS) and noninfectious uveitis (NIU) are induced by the abnormal secretion of TNF-α. Thus, TNF-α can be classified as a key factor in the pathological development[10].

Drugs used in the treatment of inflammation are non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs. However, both can cause severe adverse side effects. In the past two decades, several anti-TNF therapeutics has revolutionized in the management of autoimmune diseases[11, 12]. Furthermore, this treatment is associated with some adverse effects such as increased risk of infection, and even triggered the de novo development of autoimmune diseases. Hence, the introduction of new, safe anti-inflammatory compounds is necessary.

Chromones (1-Benzopyran-4-one) are naturally occurring flavonoids that are universally present in human health diet [13]. Chromones and its analogs are considered as important pharmacophore, and privileged structures have been featured in a number of clinically used drugs like cromolyn sodium (or cromolyn) and nedocromil [14]. Aloesin, aloeresin A, isoaloeresin D and aloeresin E are the most significant active constituents of Aloe vera, bearing Chromone as the active pharmacophore used for treatment of inflammatory skin disorders[15]. The active chemical constituents khellin and visnagin obtained from the Ammi visnaga (Umbelliferae) seeds bearing Furochromone skeleton , used for wound healing, inflammation conditions, poisonous bites and various kidney related inflammations[16].

Further, Imidazole and Pyrazole are also important class of heterocyclic scaffold possess good anti-inflammatory activity [17, 18]. Some examples of imidazole and pyrazole derivatives such as deracoxib, SC-558, mefobutazone, ramifenazone, famprofazone[19], and Imidazole salicylate[20] have been reported as potent NSAIDs.

The combination of these active heterocyclic moieties may provide synergistic effect to improve anti-inflammatory activity. Therefore, in our endeavor to find new anti-inflammatory agents with better efficacy and less toxicity, we designed, synthesized a series of 1-benzopyran-4-one derivatives (Chromone bi-heterocyclic compounds) coupled with Imidazole or Pyrazole heterocyclic scaffold and screened for their anti-inflammatory efficacy.

EXPERIMENTAL WORK

Synthetic methodology:

Synthesis of 1-(2-hydroxy-4-methoxy-5-nitrophenyl)-2-phenylethan-1-one derivatives (3): Calculated quantities of various substituted phenyl acetic acid molecules (s) (10 mmol) and 3-methoxy-4-nitrophenol (10 mmol) were combined in a round bottom flask and flushed under nitrogen. Boron trifluoride diethyl etherate (5 ml) was added to the solid mixture in the flask and the mixture was stirred under nitrogen and solution temperature was raised to 100 ºC and continued for 3 hours. After cooling to room temperature, mixture(s) were acidified with 1N HCl (7 mL) and diluted with EtOAc. The aqueous phase was extracted with EtOAc (8 mL x 2) and the combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated in vacuum. Purification of the crude product(s) was done by liquid chromatography using EtOAc: Hexane afforded the pure desired 1-(2-hydroxy-4-methoxy-5nitrophenyl)-2-phenylethan-1-one derivatives.

Synthesis of 7-methoxy-2-methyl-6-nitro-3-phenyl-4H-1-benzopyran-4-one derivatives (4): The above purified compounds were taken into a reaction flask and dissolved in dried DMF and a reagent mixture of (1:5 (%v/v)) triethylamine in dried DMF was added dropwise and heated the mixture to 125 ºC for 5h. Then the reaction mixture was cooled to room temperature and poured into cold water containing 5 ml of hydrochloric acid. The precipitate that deposited was filtered off and washed with water and, after drying, was crystallized from ethyl acetate. Purification of the crude product was done by liquid chromatography using EtOAc: Hexane afforded the pure desired 7-methoxy-2-methyl-6-nitro-3-phenyl-4H-1benzopyran-4-one derivatives.

Synthesis of 6-amino-7-methoxy-2-methyl-3-phenyl-4H-1-benzopyran-4-one derivatives (5): To a solution of above synthesized compounds (10 mmol) in EtOAc (75 mL), 5% platinum-carbon (Pt-C) (300 mg) was added, and the reaction contents were agitated for 2.5 hours at room temperature under hydrogen conditions. After removing Pt-C, the extract was concentrated in a vacuum. Purification of the residue using column chromatography (eluent, EtOAc: hexane = 1.5:8.5 to 5:5) yield 6-amino-7-methoxy-2-methyl-3-phenyl-4H-1benzopyran-4-one derivatives in the form of crude precipitate.

Synthesis of 3,6 di-substituted 7-methoxy-2-methyl-4H-1-benzopyran-4-one derivatives (6): The above compounds (10 mmol) and substituted 4-(2-chloroethyl)-4H-imidazole (s) or substituted 4-(2-chloroethyl)-4H-pyrazole (10 mmol) were solubilized in 1-methyl-2pyrrolidone (5.0 mL) and agitated for 3 hours at 100 °C. After bringing the vessel contents to normal conditions, it was diluted with 100 mL EtOAc and partitioned with saturated $NaHCO_3$ solution (50 mL). Washed a layer of the organic phase with saturated NaCl solution (50 mL), dried it over $MgSO_4$, and concentrated it in a vacuum. Purification of the residue using column chromatography (eluent, MeOH: EtOAc = 1:4 to 2:8) yield 3,6 di-substituted 7methoxy-2-methyl-4H-1-benzopyran-4-one derivatives as white crystals.

Synthesis of 3, 6 di-substituted 7-hydroxy-2-methyl-4H-1-benzopyran-4-one derivatives (7a-7u): To a solution of above recrystallized compound dissolved in NaOH (2.0 mmol) were taken into evacuated flask. Anhydrous NMP (2 mL) was added to the reaction mixture followed by 1-dodecanethiol (1.5 mmol). The reaction mixture was stirred at 130 ºC. After cooling to room temperature, mixture was acidified with 1N HCl (7 mL) and diluted with EtOAc. The aqueous phase was extracted with EtOAc (8 mL x 2) and the combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The precipitate that deposited was filtered off and washed with water and, after drying, was crystallized from ethyl acetate. Purification of the crude product was done by liquid chromatography using EtOAc: Hexane afforded the pure desired 3,6 di-substituted 7hydroxy-2-methyl-4H-1-benzopyran-4-one derivatives.

Anti-inflammatory studies:

In-vitro anti-inflammatory activity:

Albumin Denaturation inhibitory activity*:* The assay was carried out by adopting the methods described by Kumari *et al.* in 2008, with some modifications in which the volume of each component in the reaction mixtures was reduced by half. All newly synthesized target compounds 7a to 7k and 7l to 7u were initially screened at 100 µg/ml for their anti-inflammatory activity using the albumin denaturation method. A positive standard (diclofenac) prepared at 100 µg/ml was selected for the experiment. A reaction vessel for each reaction mixture consisted of 200 µl of egg albumin, 1400 µl of phosphate-buffered saline, and 1000 µl of the test solution. DMSO instead of the sample was used as a negative control. Afterward, the mixtures were incubated at 37°C for 15 minutes and then heated at 70°C for 5 minutes. After cooling, their absorbances were measured at 660 nm, and the data were processed by the Spectra Manager system. The inhibition percentage of protein denaturation was calculated using the following formula:

% Denaturation inhibition = (Abs of control-abs of test x 100 / Abs of control)

Measurement of TNF-α using ELISA assay: RAW264.7 cells ($7x10^5$cells/well) were cultured 20 hours in 24-well plate and pre-protected with the selected target compounds 7g, and 7k, for 2 hours, then added 1µg/mL of LPS to stimulate the cells for 24 hours. The production of TNF-α was determined using the mouse ELISA kit (TNF-α: Excell Biotech, EM008) which is operated according to the manufacturer's instructions. The total amount of the inflammatory TNF-α in the medium was normalized to the total protein quantity of the viable cell pellets.

In-vivo anti-inflammatory activity:

Experimental animals: The animal experiments were conducted after getting prior permission from the institutional

animal ethics committee (PGPLS/IEAC/SP/RP-023/2022), PGP life Sciences, Hyderabad. A total of 24 Wister Albino rats (average weight 213.4g) were used for the study. The gender of the rats does not make difference in inflammatory activity as reported in many earlier studies. The animals were randomly distributed into 4 groups (n=6) and housed in well-ventilated sterile polypropylene cages (6 per cage) and maintained at a controlled temperature of 26±2 °C, relative humidity 44-56 %, and provided 12 h light/ dark cycles. All the animals were fed the standard rodent pellet diet and water ad libitum. The experiment was started after acclimatizing the animals for one week.

Carrageenan-Induced paw edema in mice: Anti-inflammatory activity was measured using carrageenan-induced rat paw edema assay. Edema was induced by subplantar injection of 100 µL of 1% freshly prepared solution of carrageenan in distilled water into the right-hind paws of each rat of all the 4 groups. Animals of group A, B, C, and D were treated with the vehicle (PBS), 50 mg/kg.bw of Diclofenac, 7g_50 mg/kg.bw and 7g_75 mg/Kg.bw respectively, 30 minutes prior to carrageenan injection. Paw thickness were measured just before the carrageenan injection, that is, at "0 min" and then at 30, 60, 90, 120, and 180 mints after carrageenan injection. Increase in paw thickness was measured as the difference in paw thickness at "0 min" and paw thickness at respective time points.

Molecular docking study on TNF-α: The docking technique was performed using glide protocol of Schrodinger suite 11. The X-ray crystal structure of TNF-α was downloaded from Protein Data Bank website (PDB ID: 2AZ5) associated with its inhibitor, 6,7-dimethyl-3[(methyl{2-[methyl({1-[3-(trifluoromethyl)phenyl]-1H-indol-3-yl}methyl)amino]ethyl}amino) methyl] -4H-chromen-4-one. The protein was prepared for the docking and the compound **7g** was imposed based on the selected grid alignment and further protocol was followed as per the instructions.

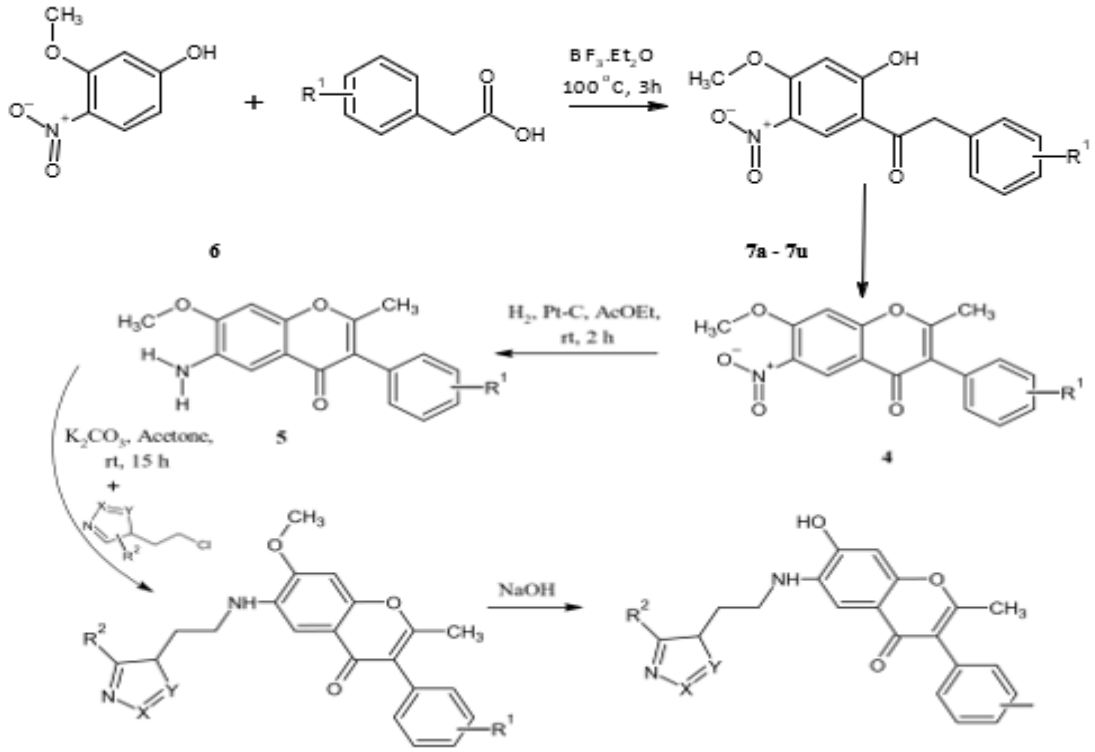
RESULTS AND DISCUSSION

Chemistry:

Total 21 derivatives of title compounds (3, 6-di-substituted 7-hydroxy-2-methyl-4H-1-benzopyran-4-one) were synthesized using multistep synthetic procedure as stated in **Scheme-1**.

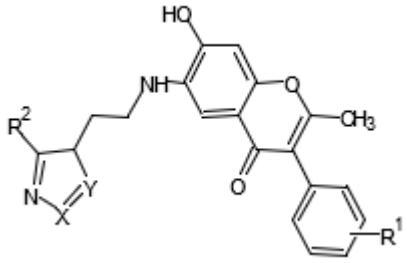
The synthetic strategy was selected in which 3-methoxy-4-nitrophenol (1) and substituted phenyl acetic acid molecules (2) were used as starting material and the reaction was catalyzed by $BF_3 \cdot Et_2O$, which was found to be a very efficient catalyst for the synthesis of functionalized benzylic ketone. Initially phenol was esterified and the intermediate ester readily rearranged it to the formation of Compound (3) in presence of high Lewis acid character of $BF_3 \cdot Et_2O$. The ^1H NMR spectra of Compound (3) showed signal at δ 11.88 due to phenolic OH, singlet at δ 4.29 due to CH2C=O (benzylic keto group). Further intermolecular cyclization of Compound (3) using triethylamine in dried DMF under reflux condition has been employed to afford the synthesis of 7-methoxy-2-methyl-6-nitro-3-phenyl-4H-1-benzopyran-4-one derivatives (Compound 4). The ^1H NMR spectra of Compound (4) showed signal at δ 8.51 due to aromatic hydrogen adjacent to nitro group, singlet at δ 3.79 and δ 3.91 due to methoxy groups.

Scheme-1



Further coupling of various Imidazoles or Pyrazole heterocycles to the benzopyan-4-one have been employed, starting with reduction of nitro group at position-6 of Compound (4) to form 6-amino-7-methoxy-2-methyl-3-phenyl-4H-1-benzopyran-4-one (Compound 5). ^{1}H NMR spectra of Compound (5) showed signal at δ 4.79 due to NH2 protons. Compound (5) was then treated with various 4-(2-chloroethyl)-4H-imidazoles or substituted 4-(2-chloroethyl)-4H-pyrazoles in presence of 1-methyl-2- pyrrolidone under reflux conditions to afford 3,6 di-substituted 7-methoxy-2-methyl-4H-1-benzopyran-4-one derivatives (Compound 6). At last, de-methylation of methoxy group at position-7 of Compound (6) was employed to afford formation of 3, 6-di-substituted 7-hydroxy-2-methyl-4H-1-benzopyran-4-one derivatives (7a-7u). All the synthesized 21 derivatives were characterized by ^{1}H NMR, which contains signal at δ 10.14 due to OH at position-7, signal at δ 4.79 due to NH protons at 6 th position, signals at δ 3.79 and δ 3.89 due to methoxy groups, signal at δ 2.10 due to methyl group, signals at δ 2.29 and δ 3.46 due to ethylene protons of CH2CH2N, signals at δ 8.14 and δ 8.36 due to hetero aromatic protons. The 13C NMR, were also confirms the formation of desired products (7a-7u). HRMS study of all the synthesized compounds showed prominent abundance at $[M+H]^+$ peak.

Target compounds:



7a to 7u
X or Y = N

Table 1. Series of 2,3,6,7 tetra-substituted 4H-1-benzopyran-4-one derivatives

S.No	Compound No	X	Y	R^1	R^2
1	7a	-CH	-NH	3-OCH_3	5-OCH_3
2	7b	-CH	-NH	3-Cl	5-CH_3
3	7c	-CH	-NH	2-Br	5-CH_3
4	7d	-CH	-NH	4-CH_3	5-OCH_3
5	7e	-CH	-NH	3-CF_3	5-CH_3
6	7f	-CH	-NH	4-OAc	5-OCH_3
7	7g	-CH	-NH	3-Br, 4-Cl	5-CH_3
8	7h	-CH	-NH	2-Br, 3-F	5-CH_3
9	7i	-CH	-NH	3-CH_3	5-OCH_3
10	7j	-CH	-NH	4-F	5-CH_3
11	7k	-CH	-NH	3-CF_3	5-OCH_3
12	7l	-NH	-CH	3-CH_3	5-OCH_3
13	7m	-NH	-CH	4-F	5-CH_3
14	7n	-NH	-CH	3-CF_3	5-OCH_3
15	7o	-NH	-CH	2-Br	5-CH_3
16	7p	-NH	-CH	4-CH_3	5-OCH_3
17	7q	-NH	-CH	3-CF_3	5-CH_3
18	7r	-NH	-CH	4-OAc	5-OCH_3
19	7s	-NH	-CH	3-Br, 4-Cl	5-CH_3
20	7t	-NH	-CH	2-Br, 3-F	5-CH_3
21	7u	-NH	-CH	3- OAc	5-CH_3

Characterization of Target compounds:

7-hydroxy-6-{[2-(5-methoxy-4H-imidazol-4-yl)ethyl]amino}-3-(3-methoxyphenyl)-2-methyl4H-1-benzopyran-4-one (7a):

Yield: 74.1%; Colorless crystals; mp: 289.9 ^{o}C; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.36 (t, J = 4.2 Hz, 1H), 8.14 (s, 1H), 7.55 (d, J = 1.0 Hz, 1H), 7.40 – 7.33 (m, 2H), 7.20 (s, 1H), 6.97 (d, J = 1.5 Hz, 1H), 6.87 (s, 1H), 4.79 (t, J = 1.8 Hz, 1H), 3.89 (s, 3H), 3.79 (s, 3H), 3.46 (dt, 2H), 2.29 (s, 3H), 2.10 (dt, J = 6.4, 4.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.85, 175.26, 166.97, 165.90, 160.03, 155.70, 149.56, 132.96, 132.29, 129.05, 123.01, 121.38, 115.84, 113.81, 112.77, 110.04, 100.61, 67.52, 55.16, 54.77, 40.99, 26.36, 18.78; HRMS (ESI) (m/z), $[M+H]^+$ calculated for C23H23N3O5: 422.1710, found: 422.1711.

7-hydroxy-6-{[2-(5-methyl-4H-imidazol-4-yl)ethyl]amino}-3-(3-chlorophenyl)-2-methyl-4H1-benzopyran-4-one (7b):

Yield: 78.3%; Colorless crystals; mp: 283.5 ^{o}C; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.43 (s, 1H), 8.37 (t, J = 4.2 Hz, 1H), 7.68 (s, 1H), 7.61 (d, 1H), 7.45 – 7.37 (m, 2H), 7.35 (s, 1H), 6.87 (s, 1H), 4.45 (t, J = 1.6 Hz, 1H), 3.40 (dt, J = 4.8, 4.7 Hz, 2H), 2.34 (s, 3H), 2.29 (s, 3H), 2.21 (dt, J = 6.6, 4.7 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.57, 177.45, 166.73, 156.24, 155.70, 149.56, 133.92, 132.98, 131.92, 129.94, 129.33, 128.78, 128.26, 121.72, 115.84, 110.04, 100.61, 70.92, 41.22, 28.84, 18.78, 16.27; HRMS

(ESI) (m/z), [M+H]+ calculated for C22H20ClN3O3: 410.1265, found: 410.1254.

7-hydroxy-6-{[2-(5-methyl-4H-imidazol-4-yl)ethyl]amino}-3-(2-bromophenyl)-2-methyl-4H1-benzopyran-4-one (7c):

Yield: 71.7%; Colorless crystals; mp: 276.4 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.42 (s, 1H), 8.37 (t, J = 4.2 Hz, 1H), 7.84 (d, J = 1.4 Hz, 1H), 7.73 (d, J = 1.7 Hz, 1H), 7.50 (dd, J = 7.7, 1.4 Hz, 1H), 7.40 (s, 1H), 7.34 (dd, 1H), 6.88 (s, 1H), 4.45 (t, J = 1.6 Hz, 1H), 3.40 (dt, J = 4.8, 4.7 Hz, 2H), 2.35 (s, 3H), 2.30 (s, 3H), 2.02 (dt, J = 6.9, 4.7 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.57, 177.45, 166.12, 156.24, 155.73, 149.56, 132.98, 132.37, 131.88, 131.51, 130.03, 128.29, 124.42, 118.35, 115.90, 110.02, 100.61, 70.92, 41.22, 28.84, 18.72, 16.27; HRMS (ESI) (m/z), [M+H]+ calculated for C22H20BrN3O3: 454.0706, found: 454.0751.

7-hydroxy-6-{[2-(5-methoxy-4H-imidazol-4-yl) ethyl] amino}-3-(4-methylphenyl)-2-methyl4H-1-benzopyran-4-one (7d):

Yield: 68.5%; Colorless crystals; mp: 283.1 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.36 (t, J = 4.2 Hz, 1H), 8.14 (s, 1H), 7.51 – 7.45 (m, 2H), 7.39 – 7.30 (m, 3H), 6.87 (s, 1H), 4.79 (t, J = 1.8 Hz, 1H), 3.89 (s, 3H), 3.45 (dt, J = 4.8, 3.9 Hz, 2H), 2.41 (s, 3H), 2.29 (s, 3H), 2.10 (dt, J = 6.4, 4.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 179.10, 175.29, 166.99, 165.93, 155.73, 149.59, 137.56, 132.99, 130.02, 129.14, 128.94, 123.83, 116.22, 110.07, 100.64, 67.55, 55.19, 41.02, 26.39, 20.73, 18.75; HRMS (ESI) (m/z), [M+H]+ calculated for C23H23N3O4: 406.1761, found: 406.1739.

7-hydroxy-6-{[2-(5-methyl-4H-imidazol-4-yl) ethyl] amino}-3-(3-trifloromethyl phenyl)-2methyl-4H-1-benzopyran-4-one (7e):

Yield: 72.9%; Colorless crystals; mp: 278.6 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.41 (d, J = 1.8 Hz, 1H), 8.37 (t, J = 4.2 Hz, 1H), 8.00 (s, 1H), 7.73 – 7.67 (m, 2H), 7.61 (d, 1H), 7.39 (s, 1H), 6.87 (s, 1H), 4.45 (t, J = 1.6 Hz, 1H), 3.40 (dt, J = 4.8, 4.7 Hz, 2H), 2.32 (s, 3H), 2.29 (s, 3H), 2.02 (dt, J = 6.9, 4.7 Hz, 2H); 13C NMR (125 MHz, DMSO-d6) δ 178.93, 177.45, 166.88, 156.24, 155.70, 149.56, 132.98, 132.17, 131.91, 131.65, 131.40, 131.09, 131.07, 131.04, 131.02, 129.53, 129.51, 129.48, 129.45, 129.42, 128.20, 128.16, 128.13, 128.09, 126.72, 126.69, 126.66, 126.63, 126.54, 124.37, 122.36, 122.19, 120.02, 115.88, 110.04, 100.61, 70.92, 41.22, 28.84, 18.78, 16.27; HRMS (ESI) (m/z), [M+H]+ calculated for C23H20F3N3O3: 444.1529, found: 444.1536.

7-hydroxy-6-{[2-(5-methoxy-4H-imidazol-4-yl)ethyl]amino}-3-(4-acetoxyphenyl)-2-methyl4H-1-benzopyran-4-one (7f):

Yield: 63.8%; Colorless crystals; mp: 264.2 ºC; ^{1}H NMR(500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.36 (t, J = 4.2 Hz, 1H), 8.14 (s, 1H), 7.65 – 7.59 (m, 2H), 7.37 (s, 1H), 7.35 – 7.29 (m, 2H), 6.87 (s, 1H), 4.79 (t, J = 1.8 Hz, 1H), 3.89 (s, 3H), 3.45 (dt, J = 4.8, 3.9 Hz, 2H), 2.32 (s, 3H), 2.26 (s, 3H), 2.10 (dt, J = 6.4, 4.9 Hz, 2H); **^{13}**C NMR (125 MHz, DMSO-d6) δ 178.97, 175.27, 168.56, 166.89, 165.91, 155.71, 150.44, 149.57, 132.97, 131.43, 125.55, 123.80, 122.08, 115.88, 110.05, 100.62, 67.53, 55.17, 41.00, 26.37, 20.41, 18.77; HRMS (ESI) (m/z), [M+H]+ calculated for C24H23N3O6: 450.1659, found: 450.1632.

7-hydroxy-6-{[2-(5-methyl-4H-imidazol-4-yl) ethyl] amino}-3-(3-bromo-4-chlorophenyl)-2methyl-4H-1-benzopyran-4-one (7g):

Yield: 69.2%; Colorless crystals; mp: 276.9 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.41 (s, 1H), 8.37 (t, J = 4.2 Hz, 1H), 7.92 (s, 1H), 7.64 (d, J = 2.3 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.39 (s, 1H), 6.87 (s, 1H), 4.45 (t, J = 1.6 Hz, 1H), 3.40 (dt, J = 4.8, 4.7 Hz, 2H), 2.30 (s, 3H), 2.27 (s, 3H), 2.02 (dt, J = 6.9, 4.7 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.69, 177.48, 166.78, 156.27, 155.73, 149.59, 135.09, 133.80, 133.01, 131.10, 129.58, 128.77, 122.78, 121.66, 115.87, 110.07, 100.64, 70.95, 41.25, 28.87, 18.81, 16.30; HRMS (ESI) (m/z), [M+H]+ calculated for C22H19BrClN3O3: 488.0370, found: 488.0329.

7-hydroxy-6-{[2-(5-methyl-4H-imidazol-4-yl) ethyl] amino}-3-(2-bromo-3-fluorophenyl)-2methyl-4H-1-benzopyran-4-one (7h):

Yield: 72.3%; Colorless crystals; mp: 258.1 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.41 (s, 1H), 8.37 (t, J = 4.2 Hz, 1H), 7.54 (d, J = 1.5 Hz, 1H), 7.47 (dd, J = 7.8, 5.0 Hz, 1H), 7.39 (s, 1H), 7.23 (d, J = 1.4 Hz, 1H), 6.88 (s, 1H), 4.45 (t, J = 1.6 Hz, 1H), 3.40 (dt, J = 4.8, 4.7 Hz, 2H), 2.35 (s, 3H), 2.30 (s, 3H), 2.02 (dt, J = 6.9, 4.7 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.34, 177.47, 166.52, 161.53, 159.51, 156.26, 155.76, 149.58, 133.94, 133.91, 133.00, 128.71, 128.64, 127.74, 127.71, 122.74, 122.71, 116.60, 116.43, 115.93, 110.04, 107.44, 107.27, 100.63, 70.94, 41.24, 28.86, 18.76, 16.29 HRMS (ESI) (m/z), [M+H]+ calculated for C22H19BrFN3O3: 472.0666, found: 472.1619.

7-hydroxy-6-{[2-(5-methoxy-4H-imidazol-4-yl) ethyl] amino}-3-(3-methylphenyl)-2-methyl4H-1-benzopyran-4-one (7i):

Yield: 70.8%; Colorless crystals; mp: 263.9 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.36 (t, J = 4.2 Hz, 1H), 8.14 (s, 1H), 7.57 (d, J = 1.1 Hz, 1H), 7.45 – 7.35 (m, 3H), 7.23 (d, 1H), 6.87 (s, 1H), 4.79 (t, J = 1.8 Hz, 1H), 3.89 (s, 3H), 3.45 (dt, J = 4.8, 3.9 Hz, 2H), 2.34 (s, 3H), 2.29 (s, 3H), 2.10 (dt, J = 6.4, 4.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.98, 175.20, 166.96, 165.84, 155.64, 149.50, 138.35, 132.90, 131.35, 128.91, 128.45, 128.37, 127.13, 121.72, 116.03, 109.98, 100.55, 67.46, 55.10, 40.93, 26.30, 20.70, 18.72; HRMS (ESI) (m/z), [M+H]+ calculated for C23H23N3O4: 406.1761, found: 406.1722.

7-hydroxy-6-{[2-(5-methyl-4H-imidazol-4-yl) ethyl] amino}-3-(4-fluorophenyl)-2-methyl-4H1-benzopyran-4-one (7j):

Yield: 65.4%; Colorless crystals; mp: 276.4 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.42 (s, 1H), 8.37 (t, J = 4.2 Hz, 1H), 7.69 – 7.62 (m, 2H), 7.37 (s, 1H), 7.29 – 7.20 (m, 2H), 6.87 (s, 1H), 4.45 (t, J = 1.6 Hz, 1H), 3.40 (dt, J = 4.8, 4.7 Hz, 2H), 2.30 (s, 3H), 2.26 (s, 3H), 2.02 (dt, J = 6.9, 4.7 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.95, 177.42, 166.85, 162.97, 160.99, 156.21, 155.67, 149.53, 132.95, 131.91, 131.84, 127.81, 127.79, 123.71, 115.94, 115.45, 115.27, 110.01, 100.58, 70.89, 41.19, 28.81, 18.73, 16.24; HRMS (ESI) (m/z), [M+H]+ calculated for C22H20FN3O3: 394.1561, found: 394.1528.

7-hydroxy-6-{[2-(5-methoxy-4H-imidazol-4-yl) ethyl] amino}-3-(3-trifluoromethylphenyl)-2methyl-4H-1-benzopyran-4-one (7k):

Yield: 69.3%; Colorless crystals; mp: 268. ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.36 (t, J = 4.2 Hz, 1H), 8.14 (s, 1H), 8.00 (s, 1H), 7.73 – 7.67 (m, 2H), 7.61 (d, 1H), 7.39 (s, 1H), 6.87 (s, 1H), 4.79 (t, J = 1.8 Hz, 1H), 3.89 (s, 3H), 3.45 (dt, J = 4.8, 3.9 Hz, 2H), 2.29 (s, 3H), 2.10 (dt, J = 6.4, 4.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.94, 175.27, 166.89, 165.91, 155.71, 149.57, 132.97, 132.18, 131.92, 131.66, 131.41, 131.10, 131.08, 131.05, 131.03, 129.54, 129.52, 129.49, 129.46, 129.43, 128.21, 128.17, 128.14, 128.10, 126.73, 126.70, 126.67, 126.64, 126.55, 124.38, 122.37, 122.20, 120.03, 115.89, 110.05, 100.62, 67.53, 55.17, 41.00, 26.37, 18.79; HRMS (ESI) (m/z), [M+H]+ calculated for C23H20F3N3O4: 460.1478, found: 460.1452.

7-hydroxy-6-{[2-(5-methoxy-4H-pyrazol-4-yl) ethyl] amino}-3-(3-methylphenyl)-2-methyl4H-1-benzopyran-4-one (7l):

Yield: 63.6%; Colourless crystals; mp: 289.7 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.91 (t, J = 4.9 Hz, 1H), 7.57 (d, J = 1.1 Hz, 1H), 7.45 – 7.35 (m, 3H), 7.23 (d, 1H), 6.92 – 6.85 (m, 2H), 4.47 (t, J = 5.4 Hz, 1H), 3.90 (s, 3H), 3.40 (dt, J = 5.0, 5.0 Hz, 2H), 2.36 (s, 3H), 2.29 (s, 3H), 2.14 (dt, J = 5.0, 0.9 Hz, 2H); **13**C NMR (125 MHz, DMSO-d6) δ 179.06, 167.04, 165.71, 155.72, 154.36, 149.58, 138.43, 133.16, 131.43, 128.99, 128.53, 128.45, 127.21, 121.80, 116.11, 110.03, 100.63, 55.16, 42.20, 39.19, 27.79, 20.78, 18.80; HRMS (ESI) (m/z), [M+H]+ calculated for C23H23N3O4: 406.1761, found: 406.1752.

7-hydroxy-6-{[2-(5-methyl-4H-pyrazol-4-yl) ethyl] amino}-3-(4-fluorophenyl)-2-methyl-4H1-benzopyran-4-one (7m):

Yield: 66.7%; Colorless crystals; mp: 271.9 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.70 (t, J = 5.0 Hz, 1H), 7.68 – 7.62 (m, 2H), 7.37 (s, 1H), 7.29 – 7.20 (m, 2H), 6.87 (s, 1H), 6.75 (d, J = 0.9 Hz, 1H), 4.72 (t, J = 1.6 Hz, 1H), 3.36 (dt, J = 4.7, 4.7 Hz, 2H), 2.29 (s, 3H), 2.16 (s, 3H), 2.04 (dt, J = 4.7, 0.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.98, 174.80, 166.88, 163.00, 161.02, 157.12, 155.70, 149.56, 133.08, 131.94, 131.87, 127.84, 127.82, 123.74, 116.08, 115.48, 115.30, 110.00, 100.61, 45.27, 42.40, 30.24, 18.76, 14.29; HRMS (ESI) (m/z), [M+H]+ calculated for C22H20FN3O3: 394.1561, found: 394.1528.

7-hydroxy-6-{[2-(5-methoxy-4H-pyrazol-4-yl) ethyl] amino}-3-(3-trifluoromethylphenyl)-2methyl-4H-1-benzopyran-4-one (7n):

Yield: 61.5%; Colorless crystals; mp: 282.3 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.00 (s, 1H), 7.91 (t, J = 4.9 Hz, 1H), 7.70 (d, 2H), 7.61 (d, 1H), 7.39 (s, 1H), 6.92 (d, J = 0.9 Hz, 1H), 6.87 (s, 1H), 4.47 (t, J = 5.4 Hz, 1H), 3.90 (s, 3H), 3.40 (dt, J = 5.0, 5.0 Hz, 2H), 2.29 (s, 3H), 2.14 (dt, J = 5.0, 0.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.93, 166.88, 165.69, 155.70, 154.34, 149.56, 133.14, 132.17, 131.91, 131.65, 131.40, 131.09, 131.07, 131.04, 131.02, 129.53, 129.51, 129.48, 129.45, 129.42, 128.20, 128.16, 128.13, 128.09, 126.72, 126.69, 126.66, 126.63, 126.54, 124.37, 122.36, 122.19, 120.02, 115.88, 110.01, 100.61, 55.14, 42.18, 39.56, 39.17, 27.77, 18.78; HRMS (ESI) (m/z), [M+H]+ calculated for C23H20F3N3O4: 460.1478, found: 460.1432.

7-hydroxy-6-{[2-(5-methyl-4H-pyrazol-4-yl) ethyl] amino}-3-(2-bromophenyl)-2-methyl-4H1-benzopyran-4-one (7o):

Yield: 69.7%; Colorless crystals; mp: 275.6 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.84 (d, J = 1.4 Hz, 1H), 7.73 (d, J = 1.7 Hz, 1H), 7.67 (t, J = 4.9 Hz, 1H), 7.50 (dd, J = 7.7, 1.4 Hz, 1H), 7.40 (s, 1H), 7.34 (dd, 1H), 6.88 (s, 1H), 6.75 (d, J = 0.9 Hz, 1H), 4.72 (t, J = 1.6 Hz, 1H), 3.42 (dt, J = 4.8, 4.8 Hz, 2H), 2.35 (s, 3H), 2.16 (s, 3H), 2.04 (dt, J = 4.6, 0.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.52, 174.75, 166.07, 157.07, 155.68, 149.51, 133.04, 132.32, 131.83, 131.46, 129.98, 128.24, 124.37, 118.30, 115.85, 109.97, 100.56, 45.22, 42.35, 30.19, 18.67, 14.23; HRMS (ESI) (m/z), [M+H]+ calculated for C22H20BrN3O3: 454.0760, found: 454.0763.

7-hydroxy-6-{[2-(5-methoxy-4H-pyrazol-4-yl) ethyl] amino}-3-(4-methylphenyl)-2-methyl4H-1-benzopyran-4-one (7p):

Yield: 72.3%; Colorless crystals; mp: 269.5 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.91 (t, J = 4.9 Hz, 1H), 7.51 – 7.45 (m, 2H), 7.39 (s, 1H), 7.36 – 7.30 (m, 2H), 6.91 (d, J = 0.9 Hz, 1H), 6.86 (s, 1H), 4.47 (t, J = 5.4 Hz, 1H), 3.90 (s, 3H), 3.40 (dt, J = 5.0, 5.0 Hz, 2H), 2.41 (s, 3H), 2.29 (s, 3H), 2.14 (dt, J = 5.0, 0.9 Hz, 2H); 13C NMR (125 MHz, DMSO-d6) δ 179.03, 166.92, 165.65, 155.66, 154.30, 149.52, 137.49, 133.10, 129.95, 129.07, 128.87, 123.76, 116.15, 109.97, 100.57, 55.10, 42.14, 39.13, 27.73, 20.66, 18.68; HRMS (ESI) (m/z), [M+H]+ calculated for C23H23N3O4: 406.1761, found: 406.1733.

7-hydroxy-6-{[2-(5-methyl-4H-pyrazol-4-yl) ethyl] amino}-3-(2-bromo3-fluorophenyl)-2methyl-4H-1-benzopyran-4-one (7q):

Yield: 77.5%; Colorless crystals; mp: 281.9 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 8.00 (s, 1H), 7.75 – 7.67 (m, 2H), 7.65 (t, J = 4.9 Hz, 1H), 7.55 (d, 1H), 7.39 (s, 1H), 6.87 (s, 1H), 6.77 (d, J = 0.9 Hz, 1H), 4.72 (t, J = 1.6 Hz, 1H), 3.43 (dt, J = 4.8, 4.8 Hz, 2H), 2.34 (s, 3H), 2.27 (dt, J = 4.8, 0.9 Hz, 2H), 2.16 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.94, 174.81, 166.89, 157.13, 155.71, 149.57, 133.09, 132.18, 131.92, 131.66, 131.41, 131.10, 131.08, 131.05, 131.03, 129.54, 129.52, 129.49, 129.46, 129.43, 128.21, 128.17, 128.14, 128.10, 126.73, 126.70, 126.67, 126.64, 126.55, 124.38, 122.37, 122.20, 120.03, 115.89, 110.01, 100.62, 45.28, 42.41, 30.25, 18.79, 14.30; HRMS (ESI) (m/z), [M+H]+ calculated for C23H20F3N3O3: 444.1529, found: 444.1561.

7-hydroxy-6-{[2-(5-methoxy-4H-pyrazol-4-yl) ethyl] amino}-3-(4-acetoxyphenyl)-2-methyl4H-1-benzopyran-4-one (7r):

Yield: 65.2%; Colorless crystals; mp: 279.8 ºC; ^{1}H NMR(500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.91 (t, J = 4.9 Hz, 1H), 7.62 (d, 2H), 7.37 (s, 1H), 7.32 (d, 2H), 6.92 (d, J = 0.9 Hz, 1H), 6.87 (s, 1H), 4.47 (t, J = 5.4 Hz, 1H), 3.90 (s, 3H), 3.40 (dt, J = 5.0, 5.0 Hz, 2H), 2.39 (dt, J = 5.2, 1.0 Hz, 2H), 2.31 (s, 3H), 2.26 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.93, 168.52, 166.85, 165.66, 155.67, 154.31, 150.40, 149.53, 133.11, 131.39, 125.51, 123.76, 122.04, 115.94, 109.98, 100.58, 55.11, 42.15, 39.14, 27.74, 20.37, 18.73; HRMS (ESI) (m/z), [M+H]+ calculated for C24H23N3O6: 450.1659, found: 450.1633.

7-hydroxy-6-{[2-(5-methyl-4H-pyrazol-4-yl) ethyl] amino}-3-(3-bromo-4-chlorophenyl)-2methyl-4H-1-benzopyran-4-one (7s):

Yield: 69.7%; Colorless crystals; mp: 296.4 ºC; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.92 (d, J = 2.3 Hz, 1H), 7.71 (t, J = 4.9 Hz, 1H), 7.64 (d, J = 2.3 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.39 (s, 1H), 6.87 (s, 1H), 6.75 (d, J = 0.9 Hz,

1H), 4.72 (t, J = 1.6 Hz, 1H), 3.36 (dt, J = 4.7, 4.7 Hz, 2H), 2.29 (s, 3H), 2.16 (s, 3H), 2.04 (dt, J = 4.7, 0.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.66, 174.80, 166.75, 157.12, 155.70, 149.56, 135.06, 133.77, 133.08, 131.07, 129.55, 128.74, 122.75, 121.63, 115.84, 110.00, 100.61, 45.27, 42.40, 30.24, 18.78, 14.29; HRMS (ESI) (m/z), [M+H]+ calculated for C22H19BrClN3O3: 488.0371, found: 488.0324.

7-hydroxy-6-{[2-(5-methyl-4H-pyrazol-4-yl) ethyl] amino}-3-(2-bromo-3-fluorophenyl)-2methyl-4H-1-benzopyran-4-one (7t):

Yield: 72.4%; Colorless crystals; mp: 295.5 °C; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.67 (t, J = 4.9 Hz, 1H), 7.54 (d, J = 1.5 Hz, 1H), 7.47 (dd, J = 7.8, 5.0 Hz, 1H), 7.39 (s, 1H), 7.23 (d, J = 1.4 Hz, 1H), 6.88 (s, 1H), 6.75 (d, J = 0.9 Hz, 1H), 4.72 (t, J = 1.6 Hz, 1H), 3.42 (dt, J = 4.8, 4.8 Hz, 2H), 2.35 (s, 3H), 2.29 (dt, J = 4.6, 0.9 Hz, 2H), 2.16 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d6) δ 178.33, 174.81, 166.51, 161.52, 159.50, 157.13, 155.75, 149.57, 133.93, 133.90, 133.10, 128.70, 128.63, 127.73, 127.70, 122.73, 122.70, 116.59, 116.42, 115.92, 110.03, 107.43, 107.26, 100.62, 45.28, 42.41, 30.25, 18.75, 14.30; HRMS (ESI) (m/z), [M+H]+ calculated for C22H19BrFN3O3: 472.0666, found: 472.0635.

7-hydroxy-6-{[2-(5-methyl-4H-pyrazol-4-yl) ethyl] amino}-3-(3-acetoxyphenyl)-2-methyl4H-1-benzopyran-4-one (7u):

Yield: 75.7%; Colorless crystals; mp: 282.7 °C; ^{1}H NMR (500 MHz, DMSO-d6) δ 10.14 (s, 1H), 7.67 (t, J = 4.9 Hz, 1H), 7.53 (d, J = 1.0 Hz, 1H), 7.40 (dd, J = 8.1, 8.1 Hz, 1H), 7.32 (s, 1H), 7.27 (s, 1H), 7.10 (d, 1H), 6.87 (s, 1H), 6.75 (d, J = 0.9 Hz, 1H), 4.72 (t, J = 1.6 Hz, 1H), 3.36 (dt, J = 4.7, 4.7 Hz, 2H), 2.31 (s, 3H), 2.26 (s, 3H), 2.16 (s, 3H), 2.07 (dt, 2H);^{13}C NMR (125 MHz, DMSO-d6) δ 178.78, 174.76, 168.33, 166.93, 157.08, 155.66, 151.75, 149.52, 133.04, 132.05, 129.31, 123.71, 123.22, 123.09, 121.54, 115.80, 109.97, 100.57, 45.23, 42.36, 30.20, 20.31, 18.74, 14.24; HRMS (ESI) (m/z), [M+H]+ calculated for C24H23N3O5: 434.1710, found: 434.1725.

Anti-inflammatory activity:

In-vitro anti-inflammatory results

Assessed the in-vitro pharmacological potential of all the synthesized derivatives *(7a-7u)* using Albumin Denaturation inhibitory activity studies. Diclofenac as one of the most effective NSAID was used as a positive control.

From investigation of the obtained results, it was obvious all the synthesized benzopyran-4-one compounds exhibited varying degree of anti-inflammatory activity as shown in **Figure 1**. Among the Imidazole coupled benzopyran-4-one derivatives **7e** and **7h** showed good percentage of protein denaturation inhibition (66.86% and 68.75% respectively), whereas the compounds **7g** (85.80%) and **7k** (83.90%) displayed excellent anti-inflammatory activity. Also among Pyrazole coupled benzopyran-4-one derivatives-**7u** (57.39%)**, 7q** (59.28%), **7t** (61.17%) and **7r** (64.02%) and exhibited moderate to good inhibitory activity. While, the compound **7s (**83.90%) showed excellent anti-inflammatory activity (**Table 2)**.

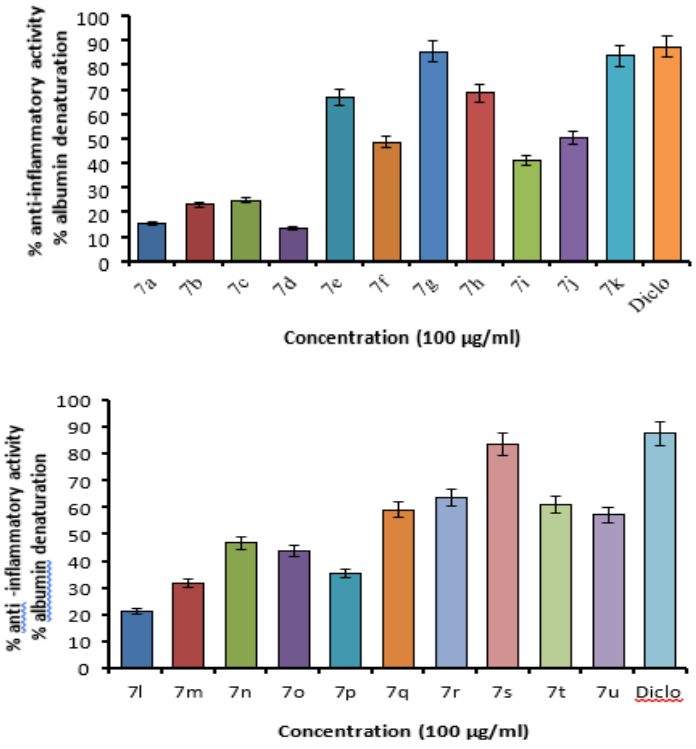


Figure 1: % anti-inflammatory activity of target 4H-1-benzopyran-4-one analogues 7a to 7k and 7l to 7u

Table 2: In-vitro anti-inflammatory results

Compounds	% inhibition	Compounds	% inhibition
7a	15.72 ± 0.81	7l	21.40 ± 1.43
7b	23.30 ± 1.43	7m	31.82 ± 1.89
7c	25.19 ± 1.37	7n	46.97 ± 2.64
7d	13.83 ± 0.95	7o	44.13 ± 2.81
7e	66.86 ± 4.62	7p	35.61 ± 2.76
7f	48.86 ± 2.93	7q	59.28 ± 3.96
7g	85.80 ± 4.18	7r	64.02 ± 3.84
7h	68.75 ± 4.84	7s	83.90 ± 4.73
7i	41.29 ± 2.87	7t	61.17 ± 2.64
7j	50.76 ± 2.69	7u	57.39 ± 3.86
7k	83.90 ± 4.83	**Diclofenac**	87.69 ± 5.07

Further concentration dependent anti-inflammatory activity of 7g, 7k, and 7s as shown in **Figure 2 and Table 3**, revealed that the compound **7s** (IC50= 23.14), **7g** (IC 50= 17.52) and **7k** (IC50= 19.68) have significant anti-inflammatory activity in comparison with the Diclofenac as control standard (IC50= 16.23) **Figure 3**.

Table 3: Concentration dependent anti-inflammatory activity of 7g, 7k, and 7s.

Concentration in µg/ml	7g	7k	7s	Diclofenac
5	23.64 ± 2.8	25.72 ± 2.1	16.59 ± 1.8	22.63 ± 1.9
10	48.11 ± 3.2	45.3 ± 3.6	35.67 ± 2.6	51.37 ± 2.6
20	62.81 ± 3.7	56.23 ± 3.9	49.35 ± 3.5	69.52 ± 2.9
40	71.42 ± 4.5	65.70 ± 4.5	59.86 ± 3.9	79.65 ± 3.5
80	83.53 ± 4.1	80.57 ± 4.8	75.33 ± 4.3	89.34 ± 4.8
160	99.86 ± 5.6	96.2 ± 5.2	92.38 ± 4.7	99.75 ± 5.5

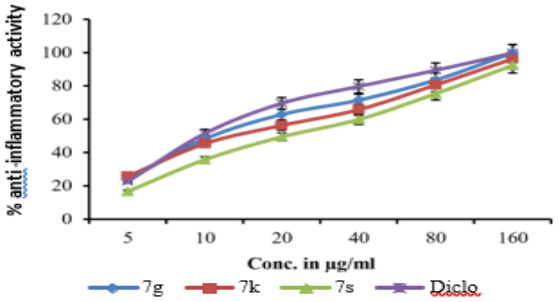


Figure 2. Concentration dependent anti-inflammatory activity of 7g, 7k, and 7s compounds.

Data were presented as means ± SD (n=3).* P < 0.05 compared with control cells

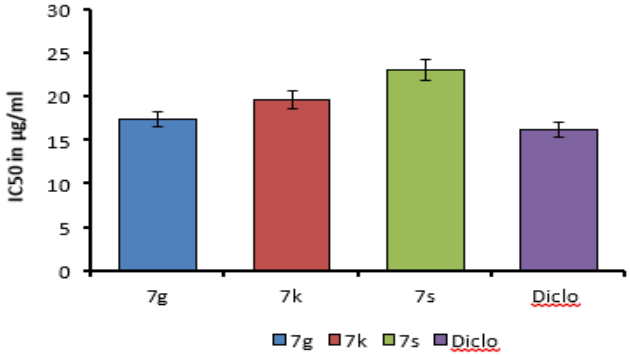


Figure 3. IC50 of selective 4H-1-benzopyran-4-one analogues 7g, 7k, and 7s in µg/ml.

In outlook of above in vitro results, Presence of 3-methoxy phenyl group and 4-methyl phenyl at position 3 of benzopyran-4-one derivatives (Compound **7a** and Compound **7d**) were found to be least anti-inflammatory activity. Whereas the presence of 2-bromo, 3-fluoro phenyl group substitution (compound **7h**), 3-trifluoro methyl phenyl group at position 3 of Chromone bi-heterocyclic compounds **7n, 7e, 7q** and **7k** showed moderate to excellent therapeutic efficacy. The compounds with cc (Compound **7g** and Compound **7s**) have shown excellent therapeutic efficacy.

In order to investigate the mechanism of anti-inflammatory activity exerted by the most active compounds **7g** and **7k**, quantitative measurement of pro inflammatory cytokine, TNF-α using sandwich enzyme immunoassay (ELISA assay) was performed for the detection of TNF-Alpha in the conditioned medium of LPS stimulated RAW-264.7 Cells **Table 4**. Results indicated that the compound **7g** exhibited relatively high activity (2 IC50= 13.5), followed by 7k (2 IC50= 17.1) significantly inhibited LPS-stimulated secretion of TNF-α in comparison with the LPS-stimulated cells with Infliximab (IC50= 12.9) as control standard **Figure 4**.

Measurement of TNF-α using ELISA assay:

Table 4: Measurement of TNF-α using ELISA assay

Sample entry	TNF-α amount compared to LPS treatment
Negative Control	1.8
LPS	100
7g_IC_{50}	19.5
7g_2xIC50	13.5
7k_IC50	23.8
7k_2xIC50	17.1
Infliximab_2µg/ml	12.9

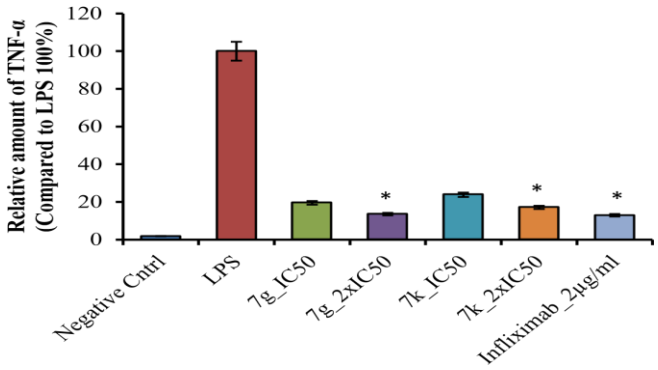


Figure 4: The inhibitory effects of 7g and 7k on LPS-induced TNF-α generation in RAW 264.7 cells.

Data were presented as means ± SD (n=3).* P < 0.05 compared with LPS stimulated cells.

In vivo anti-inflammatory activity:

Further animal experiments were carried out to find the in-vivo efficacy of compound **7g** through carrageenan-induced rat paw edema assay method. The results as shown in **table 4** demonstrated that Compound (**7g**) had significant anti-inflammatory activity at doses of 50 and 75 mg/kg, had the best significant reduction and inhibition of edema with 82.28, 88.61% and 84.34, 91.57% at third hour and fourth hour respectively, and similar as compared with standard drug Diclofenac 50 mg/kg body weight ($p < 0.05$) **Figure 5.**

Table 4: % Edema inhibition over treatments.

Time in minutes	% Edema inhibition		
	Positive control (Diclo)	7g_50 mg.kg.bw	7g_ 75 mg.kg.bw
30	10.34 ±0.41	6.90 ± 0.27	13.79 ± 0.71
60	51.06 ± 0.52	46.81 ± 2.69	53.19 ± 2.53
90	68.85 ± 3.12	63.93 ± 3.17	72.13 ± 3.17
120	82.19 ± 4.19	76.71 ± 4.08	84.93 ± 4.56
180	86.08 ± 4.88	82.28 ± 3.69	88.61 ± 4.82
240	89.16 ± 3.71	84.34 ± 5.27	91.57 ± 5.23

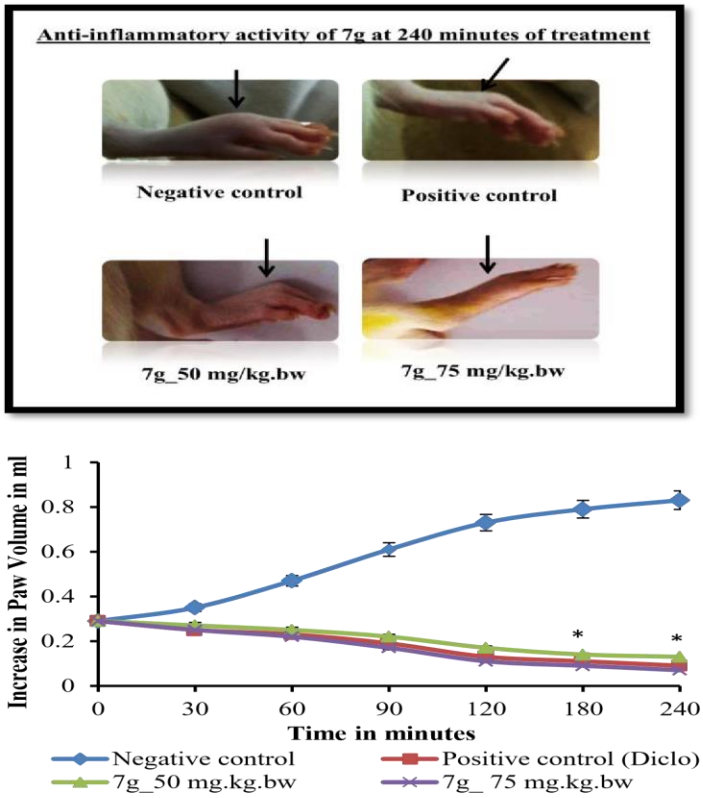


Figure 5: In-vivo anti-inflammatory activity of compound 7g using carrageenan-induced rat paw edema model; (a) observation edema status at 240 minutes over the treatment in various groups. (b) Change of paw volume vs observation time in minutes; Data were presented as means ± SD (n=3).* P < 0.05 compared with LPS stimulated cells.

Molecular docking studies:

The molecular docking studies further help in understanding the various interactions between the ligands and enzyme active sites in detail and thereby helps to design novel potent inhibitor. Molecular docking interactions of Compound **7g** with TNF-α (2AZ5) using glide protocol of Schrodinger suite revealed best possible pose, contains 3 interactions with a dock score of 9.6. The compound **7g** showed hydrogen bond interaction with GLY-121, ILE-155 and Tyr-199 as shown in **Figure 7.**

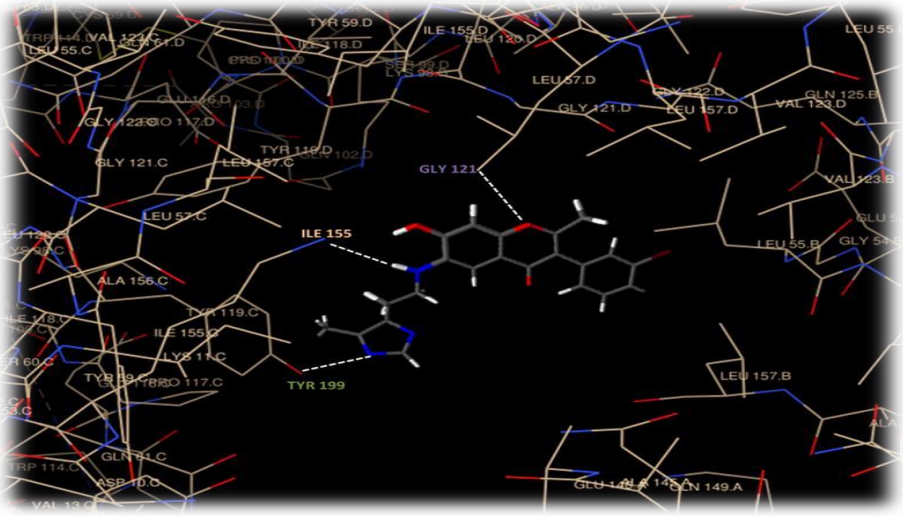


Figure 7: Molecular docking interactions of 7g with TNF-α (2AZ5) using glide protocol of Schrodinger suite.

CONCLUSION

Described herein, a simple and feasible synthesis of the 3, 6-di-substituted 7-hydroxy-2-methyl-4H-1-benzopyran-4-one derivatives via Scheme 1 and this has been supported by ^{1}H

NMR, ^{13}C NMR, and mass spectroscopy. All the synthesized compounds were subjected for the evaluation of in-vitro anti-inflammatory activity and the results showed most of the compounds had good anti-inflammatory activity. Furthermore, based on the results obtained from anti-inflammatory mechanism, it was concluded that compound **7g** could significantly suppress LPS induced expression of pro-inflammatory cytokine- TNF-alpha. A significant correlation was observed between the in-silico and the in-vivo studies of compound **7g.** Thus, this study suggests compound **7g** constitute an interesting template for the evaluation of new anti-inflammatory agents and may be helpful for the design of new therapeutic tools against inflammation.

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Conflicts of Interest - **The authors report no conflicts of interest in this work**

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