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SYNTHESIS AND BIOLOGICAL ACTIVITY OF SUBSTITUTED 4-METHYL-7- [(5-PHENYL-1, 3, 4-OXADIAZOLIDIN-2-YL) METHOXY]-2*H*-CHROMEN-2-ONES

AM. Anushree[1], HD. Bandhavya[1], S. Ramachandra Shetty[2], TV. Namratha[1] and KC. Chaluvaraju[1*]

[1]Department of Pharmaceutical Chemistry, Government College of Pharmacy, No.2, P. Kalingrao Road, Subbaiah Circle, Bengaluru-560 027, Karnataka, India.
[2]Department of Pharmacology, Govenment College of Pharmacy, No.2, P. Kalingrao Road, Subbaiah Circle, Bengaluru-560 027, Karnataka, India.

ABSTRACT

In the current study, a series of five novel substituted 4-methyl-7-[(5phenyl)-1, 3, 4-oxadiazole-2-yl) methoxy]-2H-chromen-2-one (5a-5f) were synthesized by a synthetic protocol in 4 steps (scheme 1). The intermediate involved are acetate derivative of coumarin (3) synthesizedfrom 7-hydroxy 4-methyl coumarin in the presence of K_2CO_3, acetohydrazide (4) by reacting (3) with hydrazine hydrate. The final derivatives were obtained from aceto hydrazide in the presence of $POCl_3$ by reacting it with substituted benzoic acids. All the reactions were carried out in our laboratory by conventional methods. The newly synthesized compounds were confirmed by TLC, melting point, IR and NMR studies. All the synthesized compounds were evaluated for their Anticoagulant and Anti-inflammatory activity by subaqueous tail bleeding model and HRBC method respectively. The results of the study revealed that compound NMPC (5d) having strong electron withdrawing group showed Anticoagulant activity and compounds BMPC and CMPC with bromo and chloro substitution showed Anti-inflammatory activity comparable to that of standard warfarin and diclofenac respectively. While the compound MPOC (5a), showed moderate activities. However, the compound AMPC (5e) having electron donating groups has negative impact on the potency and showed less Anticoagulant and Anti-inflammatory activity as compared to other derivatives.

Keywords: 1, 3, 4-oxadiazole, coumarin, Anticoagulant, Anti-inflammatory and HRBC.

INTRODUCTION

The physiological systems that control blood fluidity are both complex and elegant. Blood must remain fluid within the vasculature and yet clot quickly when exposed to endothelial surfaces at sites of vascular injury. When intravascular thrombi do occur, a system of fibrinolysis is activated to restore fluidity. A delicate balance prevents both thrombosis and haemorrhage and allows physiological fibrinolysis without excess pathological fibrinogenlysis. The mostly used drugs have different mechanisms of action, but all alter the balance between procoagulant and Anticoagulant reactions. With these drugs, efficacy and toxicity are necessarily inter twined Coumarins are competitive inhibitors of Vitamin-K in the biosynthesis of prothrombin. The coagulation cascade relies on the conversion of prothrombin to thrombin in a very important step under the coagulation condition.[1]

The inflammatory process is the response to an injurious stimulus. It can be evoked by a wide variety of noxious agents (e.g., infections, Antibodies, or physical injuries). The ability to mount an inflammatory response is essential for survival in the face of environmental

pathogens and injury; in some situations, and diseases. The initiating stimulus, the classic inflammatory response includes warm, pain, redness, swelling. It has been proposed that some, but not all, NSAIDs may interfere with adhesion by inhibiting expression or activity of certain of these cell-adhesion molecules. Novel classes of Anti-inflammatory drugs directed against cell-adhesion molecules are under active development but have not yet entered the clinical area. Coumarin and their derivatives are highly effective against inflammatory response.[1] From the above facts it can be concluded that coagulation and inflammation are interrelated and hence drug with Anti-inflammatory and Anti-coagulant activity will be the choice for treatment.

Coumarins are heterocyclic ring system containing fused oxygen reported for their biological activities like anti-tumor, anti-HIV, CNS stimulants, Anticoagulant, Antiangiogenesis, Antioxidant[2], Antihyperlipidemic[3], Anti-inflammatory[4], Antimicrobial[5] *etc*.,1, 3, 4-Oxadiazoles are also a class of heterocyclic proved for their Anti-inflammatory, Antioxidant, Inhibition of trypsin, glucuronidase, lipoxygenase and Anticancer activities[6] *etc.,*

No drugs are possessing both Anticoagulant and Anti-inflammatory activity revealed by extensive literature survey. Also a deep literature survey clarifies that coumarins have been reported for their Anticoagulant and 1, 3, 4-oxadiazole for its Anti-inflammatory activity and hence these have been chosen in the present study in order to obtain Anticoagulant and Anti-inflammatory activity by fusing them together chemically.

MATERIALS AND METHODS

Chemicals required for the present study were purchased from CDH, MERK, RANKEM, SIGMA ALDRICH fine Chemicals as synthetic grade and used without purification. Purity of the compounds including the intermediates was checked by TLC using pre coated silica gel plates (604 GF254Merck) with Petroleum Ether: Ethyl Acetate (7:3) as solvent systems. The detection of the spots was done by observing the plates under ARICOUVC abinet at 254 nm. Recovery of solvent from the synthesized compound were carried out by using Buchi rotavapor R-210. Melting points of the synthesized compounds were recorded by using Sigma melting point apparatus. IR spectra were recorded on Shimadzu FTIR IR affinity 1S spectrophotometer. ^{1}H NMR spectra were recorded on Brucker Spectrospin-400 MHz spectrophotometer using MeOD as solvent, TMS as an internal standard. Spectra were obtained from IISc Bengaluru.

Procedure

Synthesis of 7-Hydroxy-4-Methyl-2H-Chromen-2- one (2)

solution of 0.1mol (11g) of resorcinol in 0.1 mol (13 ml) of ethyl acetoacetate was added drop wise with stirring to 0.1 mol (5.3ml) of conc. H_2SO_4 in a RBF surrounded by freezing mixture, so that the temperature of the reaction mixture did not raise above 100 ^oC. The mixture was kept at ambient temperature for 18 h. And then poured with vigorous stirring to the mixture of ice and water. The precipitate resulted was filtered off and washed with cold water and then dried under reduced pressure to afford the crude solid mass. Finally synthesized compound was recrystallized by using ethanol. % Yield:75.2%; M.F:$C_{10}H_8O_3$;m.p.:194.18; m.p:190 ^oC; R_f: 0.51; IR:(KBr, cm^{-1});3100 (C=C aromatic), 1801(C=O), 1597 C=C(aromatic).

Synthesis of Ethyl [4-methyl-2-oxo-2H-chromen-7-yl) oxy] acetate (3)

7-Hydroxy-4-Methyl-2H-Chromen-2- one (0.025 mol, 4.27 g) and ethyl chloroacetate (0.1 mol, 12.25ml) were dissolved separately with, 10 ml of dimethylformamide and then mixed in a single necked RBF. Added 1-2 g of potassium carbonate and refluxed for 48 h. Added reaction mixture with sufficient (400 ml) water to obtain the precipitate. Ethyl acetate (200 ml) was added to dissolve the precipitate, collected the separated ethyl acetate layer and evaporated to get the compound. The obtained compound was collected and recrystallized by ethanol. % Yield:67%; M.F: $C_{14}H_{14}O_5$; M.W:263; m.p.:98-100 ^oC; R_f:0.45;IR(KBr, cm^{-1}): 1795C=O(acetate), 1288(C-O-C), 3142(C-H), 1504(C=C).

Synthesis of 2-[(4-methyl-2-oxo-2*H*-chromen-7-yl) oxy] acetohydrazide (4)

Dissolved Ethyl [(4-methyl-2 oxo-2H-chromen-7-yl) oxy] acetate(0.0038mol,1g) in about triple the quantity of hydrazine hydrate (0.0114mol,0.368g) in two necked RBF fitted with a reflux condenser and dropping funnel and refluxed the mixture for about 24h at 60-80 ^oC and kept at room temperature. The precipitate obtained was filtered, dried and recrystallized from ethanol. % Yield: 55%; M.F:$C_{12}H_{12}O_4$ N_2; M.W:248; m.p:195-196 ^oC; R_f: 0.68; IR(KBr, cm^{-1}):1662(C=O),3205 (N-H stretch),1600(N-H bending).

Synthesis of substituted 4-methyl-7-[(5-phenyl-1,3,4-oxadiazolidin-2-yl) methoxy]-2*H*-chromen-2-ones **(5a-5e)**

2-[(4-methyl-2-oxo-2*H*-chromen-7-yl)oxy] acetohydrazide(0.002 mol,0.5 g) and benzoic acid/substituted benzoic acid (0.004 mol) was

dissolved in ethanol taken in RBF fitted with a reflux condenser and dropping funnel. $POCl_3$ (0.06 mol. 10 ml) was added drop wise as dehydrating agent under constant stirring. The above mixture was then refluxed for 24h to get precipitate. The precipitate formed was filtered and recrystallized.

5a

4-methyl-7-[(5- phenyl-1,3,4- oxadiazolidin-2-yl)methoxy]-2*H*-chromen-2-one (MPOC)

Compound 4 (0.002 mol, 0.5g), benzoic acid (0.002mol, 0.086g) % Yield: 66% ; M.F: $C_{19}H_{14}O_4$ N_2 ;M.W:334 ;m.p:188-190 ^oC ;R_f:0.74 ; IR(KBr, cm^{-1}):1101(C=O), 2372(C-N), 1535N=O; ^1H NMR($CDCl_3$ δppm): 7.2-7.8 (8H-Aromatic Multiplet), 4.1-4.2 (2H, O-CH_2 Singlet), 3.0-3.1(3H,CH_3 Singlet).

5b

Chloro 4-methyl-7- [(5-phenyl-1,3,4-oxadiazolidin-2- yl)methoxy]-2*H*-chromen-2-one(CMPC)

Compound 4 (0.002 mol, 0.5g), p-chloro benzoic acid (0.002mol, 0.182g), % Yield: 68%; M.F: $C_{19}H_{13}O_4$ N_2Cl; M.W:368; m.p:170-175 ^oC; R_f:0.60; IR(KBr, cm^{-1}):742(C-Cl), 1672 (C=C).

5c

Bromo 4-methyl-7- [(5-phenyl-1, 3, 4-oxadiazolidin-2- yl)methoxy]-2*H*-chromen-2-one (BMPC)

Compound 4(0.002 mol, 0.5g), p-bromo benzoic acid (0.002mol, 0.266), % Yield: 67.44% ; M.F: $C_{19}H_{14}O_4$ N_2Br;M.W:412 ;m.p:173-176 ^oC ;R_f:0.51 ; IR(KBr, cm^{-1}):1101(C=O), 2372(C-N), 677(C-Br); ^1H NMR($CDCl_3$ δ ppm): 6.1-7.6 (9H-(Aromatic) Multiplet), 4.2-4.3 (2H, O-CH Singlet), 4.1-4.2 (3H, C-CH_2-C Singlet).

5d

Nitro 4-methyl-7-[(5- phenyl-1,3,4-oxadiazolidin-2- yl) methoxy]-2*H*-chromen-2-one (NMPC)

Compound 4 (0.002 mol, 0.5g), p-nitro benzoic acid (0.002mol, 0.204g), % Yield: 65%; M.F: $C_{19}H_{13}O_6$ N_3; M.W:334; m.p:180-182 ^oC; R_f:0.38; IR (KBr, cm^{-1}):1695(C=O), 1340(C-N), 1111(C-O-C).

5e

p-Amino 4-methyl-7- [(5-phenyl-1,3,4-oxadiazolidin-2- yl)methoxy]-2*H*-chromen-2-one (AMPC)

Compound 4(0.002 mol, 0.5g), p-amino benzoic acid (0.002mol, 0.148), % Yield: 65%; M.F: $C_{19}H_{16}O_4N_3$; M.W:348; m.p:188-190 ^oC;R_f:0.32; IR(KBr, cm^{-1}):1361(C-N), 1604(N-H), 3061(N-H stretch).

Anti-Coagulant Activity[7]

Method: Subaqueous tail bleeding time in rodents

Animals

Swiss albino mice of either sex weighing between 140-200 g were procured from the animal house of the drug technical laboratory, Bengaluru, Karnataka. The animals were kept in well ventilated spacious animal house with 12 ± 1 h day and night schedule in the animal house of Government College of Pharmacy, Bengaluru, India. The animals were lodged in large and hygienically maintained spacious cages during the course of the experimental period. The room temperature was maintained at 27 ± 1 ^oC. The animals were fed with standard mice feed (Lipton India Ltd, Bengaluru) and water adlibitum. The experiments were conducted as per the guidelines of CPCSEA, Chennai, India (185/CPCSEA) and Institutional ethical committee clearance no. DCD/GCP/IAEC/20/E.C/ADM/2015-2016.

Acute toxicity study

The acute toxicity of synthesized 4-methyl-7-[(5-phenyl-1, 3, 4-oxadiazolidin-2-yl) methoxy]-2*H*-chromen-2-one derivatives were determined by using female albino mice (120-130 g) maintained under standard husbandry conditions. The mice were fasted overnight prior to the experiment. Fixed dose method of OECD guidelines No. 425 given by CPCSEA was adopted for toxicity studies. Four groups of animals each containing three albino mice were administered with 100 mg/kg, 300mg/kg, 2000mg/kg respectively of synthesized derivatives. The animals were observed for mortality as well as morbidity for 48 h with special attention during first 30 min to 4 h. During this study period neither mortality nor any signs of toxicity were seen and animals were found to be normal up to 3 days of study period. Hence as per OECD guidelines number 425 the dose of 200 mg/kg (1/10th of 2000 mg/kg) was selected for evaluation of anticoagulant activity.

Formulation of suspension and administration of drug

The suspension of the synthesized compounds was prepared by using 3 % acacia in distilled water and administered orally to the rats. Doses were selected based on the toxicity profile of the synthesized derivatives.

Procedure

Anaesthetized rats were fixed in supine position on a temperature-controlled (37 ^oC) heating-table. Following catheterization of a carotid artery (for measurement of blood

pressure) and a jugular vein, the test compound was administered. After a defined latency period, the tail of the rat was transected with a razor blade mounted on a self-constructed device at adistance of 4 mm from the tip of the tail. Immediately after transection, the tail was immersed into a bath filled with isotonic saline solution (37 ^{o}C).

Evaluation

The time until bleeding stops was determined within a maximum observation time of 600 s and the results are tabulated (Table1).

Anti-Inflammatory Activity [8]

Method: Human red blood cell stabilization method (HRBC)

In this method, prevention of hypotonicity in HRBC membrane lysis was taken as a measure of Anti-inflammatory activity of novel coumarin derivatives since HRBC membranes are similar to lysosomal membrane components.

Preparation of HRBC Suspension

Blood was collected from healthy mice which has not received any NSAIDS for 2 weeks prior to the experiment. The collected blood was mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05 % citric acid and 0.42 % NaCl in water) and centrifuged at 3000 rpm. The packed cells were washed with isosaline (0.85 %, pH 7.2) and a 10 % (v/v) suspension was made with isosaline.

Preparation and Estimation of Assay Mixtures

Assay mixtures containing 1 ml of different drug solutions (10 µg/ml), 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of HRBC suspension were prepared separately. Diclofenac was used as reference standard. A control was prepared omitting the drug concentration. All the assay mixtures were incubated at 56 for 30 min. Then the mixtures were centrifuged at 3000 rpm for 20 min. The supernatants were isolated and absorbance measured at 560 nm. The percentage of HRBC membrane stabilization or protection was calculated using the formula and the results are tabulated (Table 2).

Formula

$$\% \text{ Stabilization} = 100-\{(OD_1/OD_2) \times 100\}$$

Where, OD_1=Optical density of test samples

OD_2=Optical density of Control

RESULTS

The study was designed to synthesize and evaluate the Anticoagulant and anti-inflammatory activity of the heterocyclic compounds -coumarin bearing 1, 3, 4-oxadiazole motif. The study model used was *in-vivo* subaqueous tail bleeding and *in-vitro* HRBC method respectively. Treatment with study compounds increased the bleeding time and % of hemolysis. This indicates that all the compounds possess anti-inflammatory and anticoagulant activity, however their potency varies.

Anti-coagulant activity

The bleeding time of compound NMPC with -NO_2 group present on1, 3, 4-oxadiazole motif was found to be 80-83 s. Whereas other compounds MPOC, BMPC(4b), CMPC showed its bleeding time of 64-66 sec,72-74 sec, 69-71 s respectively when the tail dipped into isosaline solution. The standard (warfarin) treated animals showed bleeding time of 90-115 s. The results are compiled in Table 1.

Anti-inflammatory activity

Among the compounds screened for anti-inflammatory activity, the compounds NMPC, CMPC, BMPC with electron withdrawing groups (-NO_2, Cl, Br) showed comparable activity to standard diclofenac. The study revealed that compound having –NO_2 group showed a total % of hemolysis of 30-32%, Chloro group showed 42-44% and bromo showed 40-42%. However, the standard Diclofenac showed 62-63% of Hemolysis. The results are compiled in Table 2.

DISCUSSION

Chemistry

Substituted 4-methyl-7- [(5-phenyl-1, 3, 4-oxadiazole-2-yl) methoxy]-2H-chromen-2-one were synthesized by sequence of reaction from resorcinol. Resorcinol was converted into 7-Hydroxy-4-Methyl-2H-Chromen-2- one with catalytic amount of Conc.H_2SO_4 following pechmann reaction. The structure of this was confirmed by disappearance of OH stretching of resorcinol (3200-3600 cm-1) peak in IR spectra, also supported by appearance of C=O stretching of carbonyl group at 1670-1820 cm-1. The acetate derivative of 7-Hydroxy-4-Methyl-2H-Chromen-2- one was converted into acid hydrazide using hydrazine hydrate in ethanol. This was confirmed by appearance of –NH stretching of acid hydrazide at 3331 cm-1 to 3188 cm-1 in IR spectra. This hydrazide was made to react with substituted benzoic acids such as p-chlorobenzoic acid, p-bromobenzoic acid, p-nitrobenzoic acid, p-aminobenzoic acid, in the presence catalytic

amount of $POCl_3$ to give 1, 3, 4-oxadiazolederivatives of respective benzoic acid. These were confirmed by disappearance of –NH stretch at 3331 cm-1 to 3188 cm-1 and appearance of intence peaks at 1400-1600 cm-1 and CN peak at 2210 to 2260 cm-1 and N-O stretch from 1515 to 1560 cm-1.In proton NMR (δ ppm) the δ ppm values from 6.5-7.2 indicates aromatic hydrogens. δ ppm 4.1-4.2 indicates a singlet of O-CH_2. And a singlet at δ ppm 3.0-3.1 indicates -CH_3 for compound 5a. Similarly, for 5e δppm at 6.1-7.6 shows the presence of aromatic H and 4.2-4.3 indicates O-CH_2. The δ ppm at 4.1-4.2 singlet shows the presence of - CH_2. All the above facts confirm the formation of compound 5a and 5e respectively.

Anticoagulant activity

All of the compounds (MPOC, CMPC, BMPC, NMPC, and AMPC) were screened for their possible anticoagulant activity by subaqueous tail bleeding model. The results of anticoagulanet activity are depicted in Fig.1 Among the tested compounds NMPC having NO_2 (strong electron withdrawing group) present on oxadiazole motif at para position of phenyl ring, at 200 mg/Kg dose demonstrated the Anticoagulant activity comparable to that of standard warfarin at 200 mg/Kg where it was interesting that amine group which is an electron withdrawing group have negative impact on anticoagulant activity. From the result it can be concluded that the presence of electron withdrawing and releasing groups will have impact on Anticoagulant activity.

Anti-inflammatory activity

The compounds MPOC, CMPC, BMPC, NMPC and AMPC were screened for their possible Anti-inflammatory activity by HRBC method. The results of anti-inflammatory activity are depicted in Fig.2 Among the tested compounds CMPC, NMPC and BMPC having Cl, NO_2, Br (electron withdrawing groups) present at para position of phenyl ring attached to 1, 3, 4-oxadiazole motif at 10 µg/kg dose showed better Anti-inflammatory activity comparable with Diclofenac 10 µg/kg. Where the electron donating group showed less Anti-inflammatory activity. By the result we can conclude that electron withdrawing and electron releasing groups have possible effect on Anti-inflammatory activity.

CONCLUSION

A series of coumarins compounds (5a-5e) bearing 1, 3, 4-oxadiazole have been synthesized by conventional methods in our laboratory. The synthetic protocol involves a scheme as depicted in methodology section.

The scheme involves 4 steps, in the first step resorcinol was made to react with ethyl aceto acetate in the presence of concentrated H_2SO_4. The mechanism of preparation involves pechmann reaction. The step 2 describes the synthesis of 4-hydroxy 7-methyl coumarin acetate by using ethyl chloroacetate as reactant and dimethylformamide solvent. The step 3 illustrates the formation of hydrazide from acetate derivative in presence of hydrazine hydrate in ethanol. The step 4 involves formation of substituted 1, 3, 4-oxadiazole derivatives of coumarins from substituted benzoic acid by refluxing with ethanol. The anticoagulant activity was evaluated by considering bleeding time after cutting rats tail about 4mm. From the results it was found that compounds having strong electron withdrawing groups showed Anticoagulant activity comparable to that of standard warfarin. The % of bleeding time is depicted graphically in **Fig.1**. All of the compounds (MPOC, CMPC, BMPC, NMPC, AMPC) were screened for their possible Anti-inflammatory activity by HRBC method by withdrawing blood from wistar albino rats. The different doses of synthesized derivatives were fixed as per OECD guidelines on female albino rats and dose was found to be 100mg. 3% acacia was used as a control and diclofenac as standard and the UV at 560nm was compared with synthesized derivatives. From the results it was found that compounds having strong electron withdrawing groups showed Anti-inflammatory activity comparable to that of standard diclofenac. The % of hemolysis was depicted graphically in **Fig.2.**

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Table 1: Results of mean bleeding time for Anti-coagulant activity

Name of the drug administrated	Bleeding time (Mean ±SEM)
Control	60.3 ± 1.4
Warfarin	115 ± 8.5
5a: MPOC	66 ±0.95
5b :CMPC	71.6 ± 0.66
5c :BMPC	74.33 ± 1.14
5d: NMPC	83±0.57
5e: AMPC	51±1.66

Table 2: Percentage Stabilization/protection of RBC membrane

Name of the drug administrated	% Stabilization / Protection (mean±SEM)
Diclofenac	64±0.577
5a: MPOC	51.6±0.89
5b: CMPC	44±0.8882
5c :BMPC	42±0.577
5d :NMPC	56±0.578
5e :AMPC	32±1.4

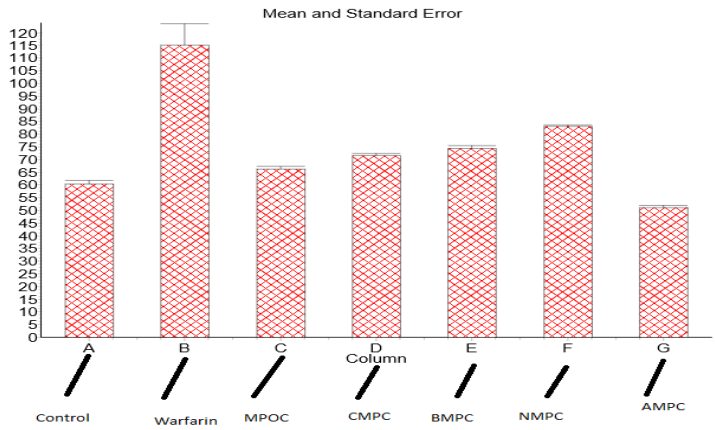


Fig. 1: Graphical representation of Anticoagulant activity of synthesized compound

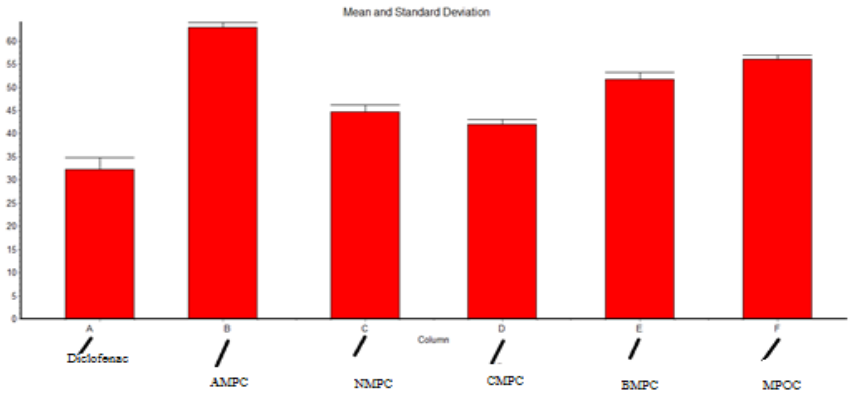
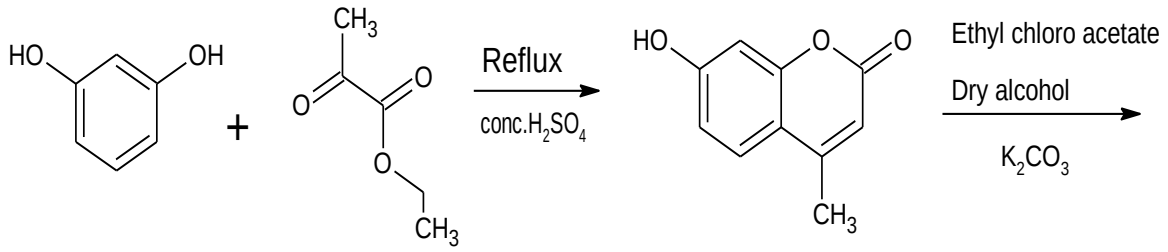


Fig. 2: Graphical representation of anti-inflammatory activity of synthesized compounds



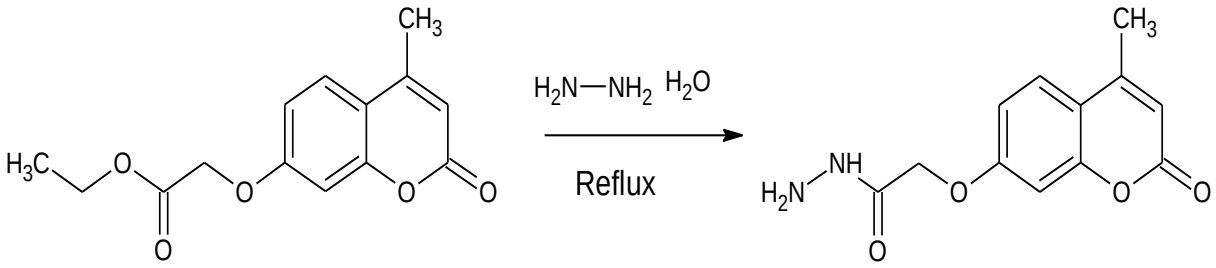
benzene-1,3-diol

ethyl
2-oxopropanoate

7-hydroxy-4-methyl-
2*H*-chromen-2-one

(2)

(1)

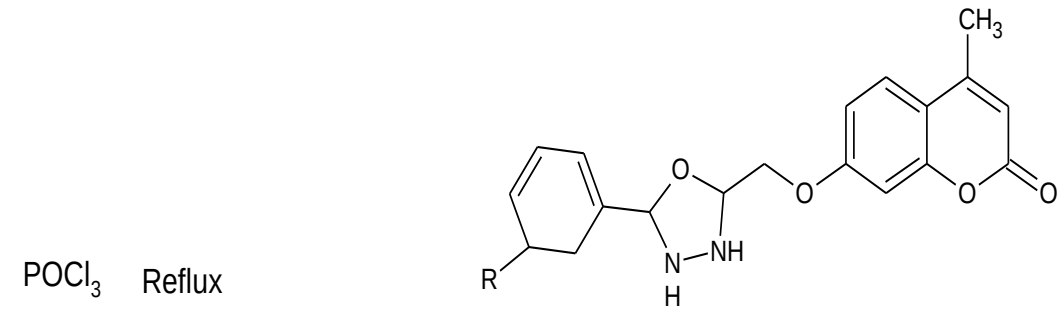


ethyl
[(4-methyl-2-oxo-2*H*-chr
omen-7-yl)oxy]acetate

2-[(4-methyl-2-oxo-2*H*-chromen-7-y
l)oxy]acetohydrazide

(3)

(4)



substituted benzoic acid

substituted
4-methyl-7-[(5-phenyl-1,3,4-oxadiazolidin-2-yl)m
ethoxy]-2*H*-chromen-2-one

(5)

Where, R=H, 4-Chloro, 4-nitro, 4-bromo, 4-amino

Scheme. 1: Synthetic route of substituted 4-methyl-7-[(5-phenyl -1,3,4-oxadiazolidin-2-yl) methoxy]-2*h*-chromen-2-ones

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