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ARTICLE

# Synthesis, Characterization and Biological Evaluation of Novel Coumarins as Promising Anti-Inflammatory Agents

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## ABSTRACT

In the present study, coumarins were synthesized by reaction of substituted phenols and ethyl acetoacetate in the presence of concentrated sulphuric acid (Pechmann reaction)and substituted with different electropositive and electronegative groups. The binding affinity of compounds was evaluated by molecular docking in the binding site of phosphodiestrase 4 (PDE4) protein using AutoDockVina and dock scores were calculated. The compounds with good binding affinity were screened for the anti-inflammatory activity using carrageenan induced rat paw edema method. Docking studies showed that compounds **3(d-k)** have good binding affinity with PDE4. Further, pharmacological investigation revealed that compound **3(d-e)** exhibited highest antiinflammatory activity.

## KEYWORDS

Coumarin, Anti-Inflammatory, Phosphodiestrase inhibitors, PDE4B, Docking studies.

**Asian Journal of Organic & Medicinal Chemistry**

Volume: 3 Year: 2018
Issue: 4 Month: October–December
pp: 150–154
DOI: https://doi.org/10.14233/ajomc.2018.AJOMC-P141

Received: 25 August 2018
Accepted: 20 November 2018
Published: 31 December 2018

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## INTRODUCTION

Inflammation is a natural protective phenomenon of the body expressing the response to infections, environmental issues or damage of its cells and vascularized tissue which involves movement of white blood cells from blood vessels to the inflamed site. However, inadequate or excessive activation of this system can lead to several diseases including cancer [1,2]. Therefore, research for novel, more potent compounds as anti-inflammatory agents is continuously growing from several years.

Coumarin ring system present in many natural and synthetic products has grabbed the interest of researchers in past few years owing to their potential to reduce edema and inflammation and also the formation of reactive oxygen species by scavenging the free radicals [3]. Coumarin belongs to benzo-α-pyrone (2*H*-1-benzopiron-2-one) family in which benzene ring is fused to pyrone ring [4]. It is an effective inhibitor of a number of protein and enzyme functions, and substitution of the coumarin ring with different moieties results in therapeutic activity [5]. Novel compounds based on coumarin ring system have been synthesized in recent years to explore their applicability as drugs. Coumarins have been reported to exhibit diverse

pharmacological actions like anti-inflammatory, antioxidant, antitumor, antibacterial, neuroprotective and anticoagulant [6-16]. Recently, novel benzoxazole-coumarin derivatives have been synthesized by coupling benzoxazole and coumarin rings as safe and potent anti-inflammatory agents [17].

The role of phosphodiestrase (PDE), enzyme catalyzing the hydrolysis of cyclic adenosine monophosphate (cAMP) to adenosine monophosphate, is well known in inflammation. In past few decades, great attention has been focused on PDE4 due to its role in pathological process associated with inflammation, angiogenesis and various vascular and neurological disorders [18]. Selective inhibition of PDE4 prevents the release of inflammatory mediators from different cells and is a great therapeutic approach for various inflammation related disorders. Coumarins as PDE4 inhibitors were found to have potential to reduce the symptoms and masked the inflammation associated with dry eye [19]. Various novel PDE4 inhibitors have the therapeutic potential in asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, Alzheimer's disease, encephalomyelitis and depression [20-24]. Roflumilast is selective PDE4 inhibitor approved by FDA in 2011. After roflumilast, apremilast [25] was approved by FDA for psoriatic arthritis in 2014. Crisaborole [26], a selective PDE4B inhibitor has been recently approved by FDA for eczema. Several PDE4 inhibitors have shown promising results in clinical trials, however many have been discontinued from development due to their undesired side effects specifically nausea, emesis, abdominal pain and dyspepsia. It has been ascertained that the side effects of PDE4 inhibitors are a result of their non-selectivity to all four PDE4 subtypes, and thus synthesis of new PDE4 inhibitors with subtype selectivity may provide clinical benefits by maintaining therapeutic efficacy and decreasing the side effects [27].

Based on the available X-ray co-crystallized structures of PDE4 inhibitors in PDB database, their interactions with PDE enzyme were observed and the pharmacophoric features required for PDE4 inhibitory activity were identified as shown in Fig. 1. Attributed to the pharmacophoric features required for PDE4 inhibition and consideration of coumarins as selective PDE4 inhibitors, the novel coumarins were designed and synthesized as potential PDE4B inhibitors and evaluated as anti-inflammatory agents.

## EXPERIMENTAL

The chemicals used in experimental work were purchased from Loba chemie Pvt. Ltd. and Spectrochem Pvt. Ltd. Melting points were determined in an open capillary tube on a melting point apparatus and are uncorrected. IR spectra were recorded

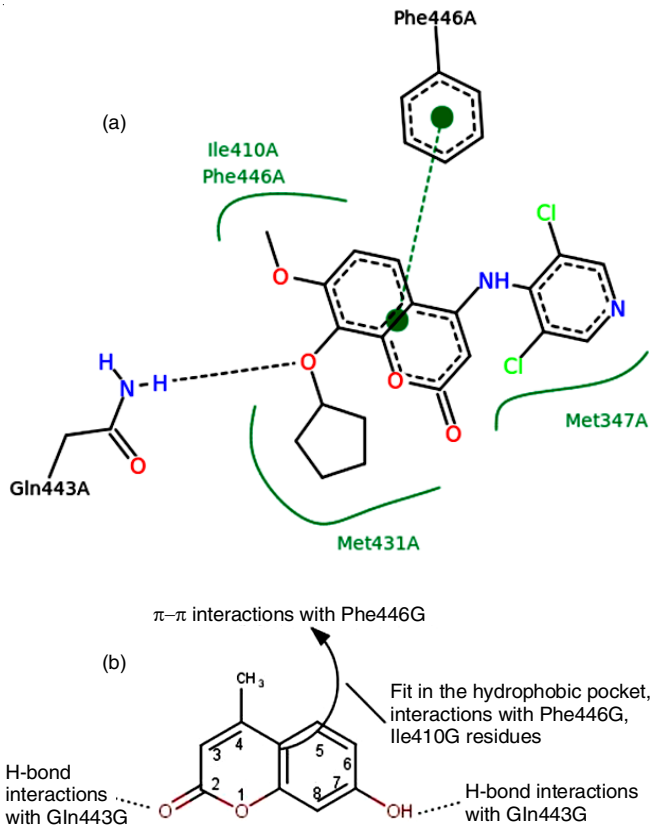
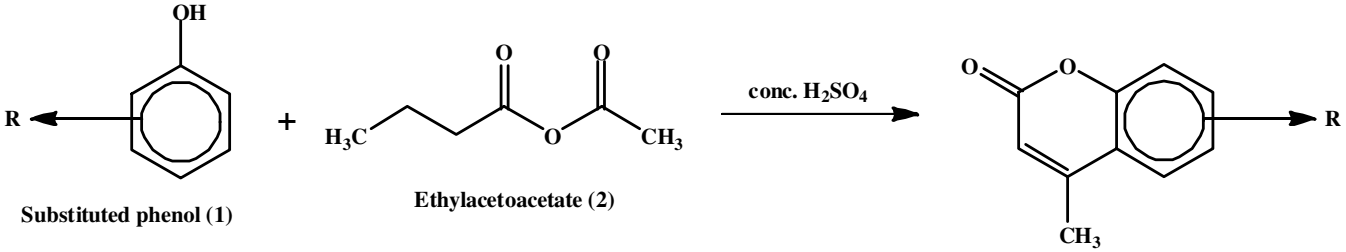



Fig. 1. (a) Binding interactions of co-crystallized PDE4B inhibitor showing H-bonding with the Gln443 residue; hydrophobic and π-π bonding with the Phe446 (b) Structure of coumarin derivative designed as potential PDE4 inhibitors [Ref. 33]

on FTIR spectrophotometer (Shimadzu) by KBr pellet method. $^1$H NMR spectra was recorded on BrukerAvance II at 400 MHz NMR spectrophotometer using DMSO-$d_6$ are expressed in parts per million downfield from tetramethylsilane as an internal standard. $^{13}$C NMR spectra were recorded on the same spectrophotometer at 100 MHz using same solvents.

**Synthesis of coumarin derivatives:** Briefly, substituted phenol (1 M)) in ethyl acetoacetate (1 M) was added in an excess quantity of sulphuric acid. The mixture was heated on a water bath at 75-80 ºC for 20 min. It was poured into excess ice water. The pale yellow solid was collected by suction filtration. The solid was washed with cold water and dried at 60 ºC followed by recrystallization from ethanol or methanol [28] (**Scheme-I**).

**Docking studies:** The catalytic domain of human phosphodiesterase 4B in complex with a coumarin-based inhibitor was obtained from the protein data bank (PDB Code: 3LY2). The chemical structures were drawn using Marvin sketch and mole-




**Substituted phenol (1)** **Ethylacetoacetate (2)** **Coumarin analogs 3(a-o)**

**where R = H, OH, Cl, F, Br, $NH_2$, $NO_2$, COOH, $CH_3$**

**Scheme-I**

cular docking studies were performed using Autodock Vina [29]. The protein 3D structure was cleaned by deleting the water molecules, cofactors and other ligands. This was followed by adding hydrogen atoms in their standard geometry, adjusting the bond orders & formal charges. Mole2 files were created for ligands followed by creating PDBQT files for both protein & ligands respectively. The compounds were finally analyzed in PyMOL to obtain the overlay & nature of bonding between the ligands [30].

**Spectral data of some selected compounds**

**7-Hydroxy-4-methyl-2*H*-chromen-2-one (3a):** Yellowish crystals; m.w. 176, m.p. 185 ºC. Elemental analysis for $C_{10}H_8O_3$ calcd (found) %: C 68.18 (68.18); H 4.56 (4.58); O 27.22 (27.24). IR (KBr, $\nu_{max}$, $cm^{-1}$): 3500.8 (O-H *str.*), 1265.3 (C-O *str.*), 1386.82 (C-H bend.), 1608.63 (Ar C=C *str.*), 1674.21 (C=O *str.*).

**6-Chloro-2*H*-chromen-2-one (3f):** Brownish-black crystals, m.w. 180, m.p. 190 ºC. Elemental analysis for $C_9H_5O_2Cl$ calcd (found) %: C 59.84 (59.86); H 2.75 (2.79); Cl 19.60 (19.63); O 17.70 (17.72). IR (KBr, $\nu_{max}$, $cm^{-1}$): 1265.3 (C-O *str.*), 1720.5 (C=O *str.*), 1606.7 (Ar C=C *str.*), 692.44 (C-Cl *str.*). $^1$H NMR ($CDCl_3$, δ ppm): 2.42 (d, 1H, CH of C3 of coumarin ring), 3.16 (d, 1H, CH, C4 of coumarin ring), 6-7.5 (m, 3H, CH of aromatic ring). $^{13}$C NMR (400MHz, $CDCl_3$, δ ppm): 152.98 (Ar-CH), 132.45 (Ar-CH) 115.80 (C=C), 40.43 (C-O), 39.18 (C-Cl).

**8-Chloro-2*H*-chromen-2-one (3g):** Light-brown crystals, m.w. 180, m.p. 190 ºC. Elemental analysis for $C_9H_5O_2Cl$ calcd (found) %: C 59.84 (59.86), H 2.75 (2.79), Cl 19.60 (19.63%), O 17.70 (17.72%). IR (KBr, $\nu_{max}$, $cm^{-1}$): 1093.94 (C-O *str.*), 615.29 (C-Cl *str.*). $^1$H NMR ($CDCl_3$, δ ppm): 1.62 (d, 1H, CH of C3 of coumarin ring), 2.09 (d, 1H, CH, C4 of coumarin ring), 6.0-7.5 (m, 3H of arom. ring). $^{13}$C NMR (400MHz, $CDCl_3$, δ ppm): 129.41 (C=C), 125.91 (C=C), 118.04 (C=C), 40.60 (C-O), 39.35 (C-Cl).

**6-Amino-2*H*-chromen-2-one (3l):** Black crystals, m.w. 161, m.p. 175 ºC. Elemental analysis for $C_9H_7NO_2$ calcd (found) %: C 67.04 (67.08); H 4.35 (4.38); N 8.65 (8.69); O 19.80 (19.85). IR (KBr, $\nu_{max}$, $cm^{-1}$): 3261.63 (O-H *str.*), 1541.12 (Ar C=C *str.*), 1211.3 (C-N *str.*). $^1$H NMR ($CDCl_3$, δ ppm): 1.23 d, 1H, CH of C3 of coumarin ring), 2.49 (d, 1H, CH C4 of coumarin ring), 3.38 (s, 2H, $NH_2$), 6.0-7.5 (m, 3H, CH of aromatic ring), $^{13}$C NMR (400MHz, $CDCl_3$, δ ppm): 115.70 (C=C), 118.18 (C=C), 122.36 (C=C), 127.35 (C=C), 139.92 (C=C), 151.55 (C-$NH_2$).

**8-Amino-2*H*-chromen-2-one (3m):** Grey crystals, m.w. 161, m.p. 175 ºC. Elemental analysis for $C_9H_7NO_2$ calcd (found) %: C 67.04 (67.08); H 4.35 (4.38), N 8.65 (8.69), O 19.80 (19.85). $^1$H NMR ($CDCl_3$, δ ppm): 2.07 (d, 1H, CH C3 of coumarin ring), 2.49 (d, 1H, CH, C4 of coumarin ring), 3.41 (s, 2H, $NH_2$), 6.0-7.5 (m, 3H, CH of aromatic ring). $^{13}$C NMR (400 MHz, $CDCl_3$, δ ppm): 125.80 (C=C), 128.28 (C=C), 132.46 (C=C), 137.45 (C=C), 149.99 (C=C), 161.65 (C-$NH_2$).

**Animals:** Wistar rats (150-300 g) were procured from the disease-free Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (India). The rats were kept in standard polypropylene cages (two rats/cage) and maintained under controlled room temperature (22 ± 2 ºC) and humidity (55 ± 5 %) with 12:12 h light and dark cycle. All the rats were provided with commercially available rat normal pellet diet (Ashirwad Feeds) and water *ad libitum*. The experimental protocol was approved by Institutional Animals Ethics Committee (Approval No. JCDMCOP/IAEC/06/16/35) and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

**Acute toxicity studies:** Acute toxicity studies were carried out to find the safe dose of synthesized derivatives. The nine animals were divided into three groups of three animals each. Each group of animals were administered different doses (10, 100, 1000 and 2000 mg/kg) of test compound. The animals were placed under observation for 24 h to monitor their behaviour as well as mortality. The acute toxicity study showed that dose given orally at 2,000 mg/kg caused no mortality after 24 h [31].

**Carrageenan induced paw edema in rats:** The anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan induced paw edema in rats. The animals were starved over night and only water was given *ad libitium*. The initial paw volume of animal was measured by digital plethysmometer. The animals were divided into different groups (control, standard and test groups), each consisting of six rats. Rolipram was used as standard anti-inflammatory drug. The control group was treated with Tween 80 (1 % w/v) suspension. The standard group was treated with standard drug rolipram (25 mg/kg) and the test group were treated with the suspension of test compounds (100 mg/kg, orally). After 30 min, the animals were injected with 0.1 mL of carrageenan (1 % w/v) in sub-planter region of left hind paw of rats. The final paw volume was measured immediately after injection and at 2 h intervals till 4 h [32].

## RESULTS AND DISCUSSION

In the present study, novel coumarin derivatives were synthesized by reaction of substituted phenols with ethyl acetoacetate in the presence of conc. sulphuric acid. The physico-chemical properties of the synthesized compounds were determined and are presented in Table-1. The purity of novel compounds was confirmed by their IR and NMR. The IR spectra of the novel compounds revealed absorption band in the regions 3500 $cm^{-1}$ corresponding to the -OH group in coumarin ring. The IR spectra of the novel compounds also revealed the absorption bands in the region 1700-1600 $cm^{-1}$, which shows the presence of aromatic C=C and cyclic ketone. The in-plane C-H bending vibrations were observed at 1300-1000 $cm^{-1}$. Another peaks near 700 and 1350-1000 $cm^{-1}$ revealed the presence of -Cl and -$NH_2$ groups, respectively. The $^1$H NMR spectra of synthesized coumarin derivatives had doublet signal for ring carbons of coumarin ring. The spectra also revealed the singlet signal for two protons of -$NH_2$ group around δ 3 ppm.

The binding interactions of novel compounds in the active site of PDE4B protein were evaluated by docking studies. Initially, co-crystallized PDB ligand was docked in the active site of PDE4B followed by docking of all the designed compounds. The compounds **3(d-k)** showed good binding interaction as determined by their H-bonding, binding affinity and docking score of best docked poses. These compounds showed similar binding pattern in the active site of enzyme as that of

TABLE-1
PHYSICO-CHEMICAL PROPERTIES AND DOCK SCORE OF THE SYNTHESIZED COUMARIN COMPOUNDS

| Compd. No. | Compound name | m.f. | m.w. | $R_f$ (Toluene:Ether 1:1) | Dock value |
|---|---|---|---|---|---|
| Co-crystallized PDB Ligand | 8-(Cyclopentyloxy)-4-[(3,5-dichloropyridin-4-yl)amino]-7-methoxy-2*H*-chromen-2-one | $C_{20}H_{18}N_2O_4Cl_2$ | 421 | – | -9.3 |
| **3a** | 7-Hydroxy-4-methyl-2*H*-chromen-2-one | $C_{10}H_8O_3$ | 176 | 0.61 | -5.9 |
| **3b** | 4,7-Dimethyl-2*H*-chromen-2-one | $C_{11}H_{10}O_2$ | 173 | 0.64 | -5.3 |
| **3c** | 4-Methyl-2*H*-chromen-2-one | $C_{10}H_7O_2$ | 159 | 0.63 | -5.4 |
| **3d** | 6-Nitro-2*H*-chromen-2-one | $C_9H_5NO_4$ | 205 | 0.65 | -9.0 |
| **3e** | 8-Nitro-2*H*-chromen-2-one | $C_9H_5NO_4$ | 205 | 0.66 | -9.1 |
| **3f** | 6-Chloro-2*H*-chromen-2-one | $C_9H_5O_2Cl$ | 180 | 0.70 | -8.5 |
| **3g** | 8-Chloro-2*H*-chromen-2-one | $C_9H_5O_2Cl$ | 180 | 0.72 | -8.6 |
| **3h** | 6-Bromo-2*H*-chromen-2-one | $C_9H_5O_2Br$ | 230 | 0.73 | -7.9 |
| **3i** | 8-Bromo-2*H*-chromen-2-one | $C_9H_5O_2Br$ | 230 | 0.74 | -8.0 |
| **3j** | 6-Fluoro-2*H*-chromen-2-one | $C_9H_5O_2F$ | 169 | 0.69 | -8.2 |
| **3k** | 8-Fluoro-2*H*-chromen-2-one | $C_9H_5O_2F$ | 169 | 0.71 | -8.3 |
| **3l** | 6-Amino-2*H*-chromen-2-one | $C_9H_7NO_2$ | 161 | 0.75 | -7.1 |
| **3m** | 8-Amino-2*H*-chromen-2-one | $C_9H_7NO_2$ | 161 | 0.77 | -7.2 |
| **3n** | 2-Oxo-2*H*-chromene-6-carboxylic acid | $C_{10}H_6O_4$ | 190 | 0.80 | -7.5 |
| **3o** | 2-Oxo-2*H*-chromene-8-carboxylic acid | $C_{10}H_6O_4$ | 190 | 0.82 | -7.9 |

the co-crystallized ligand. An interesting observation was made that electronegative group at different positions of coumarin ring enhanced the binding affinity much more effectively. In this study, it was found that substitution with electronegative atom or group (*e.g.* chloro, bromo, fluoro, nitro and oxo) showed good binding affinity for the enzyme (Figs. 2 and 3).

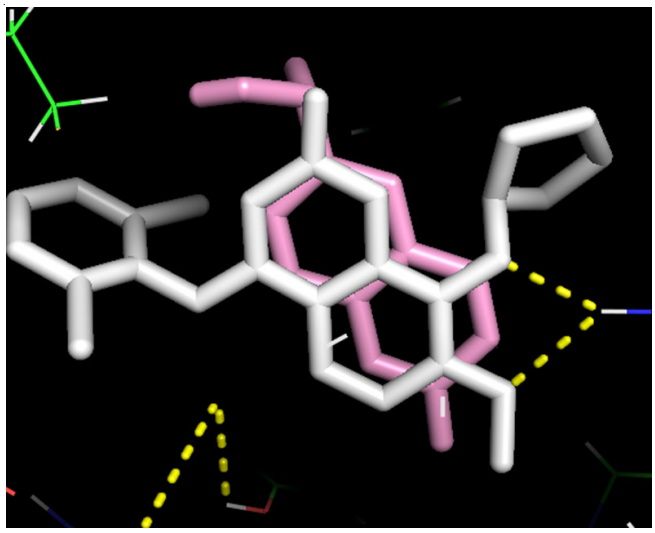


Fig. 2. Overlay of compound **3d** docked in the binding site of PDE 4B in complex (3LY2) with a coumarin-based inhibitor showing H-bond interactions

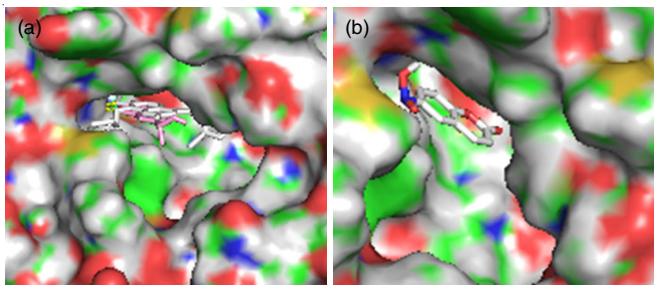



Fig. 3. (a) Compound **3d** docked with catalytic domain of human PDE 4B in complex (3LY2) with a coumarin-based inhibitor (b) without a coumarin-based inhibitor

After carrying out docking studies, the compounds with good binding interactions with PDE4B enzyme were selected for evaluation of anti-inflammatory activity. The anti-inflammatory activity of the selected compounds was evaluated by measuring the volume displaced and calculating percent inhibition of edema.The data was further analyzed by two-way ANOVA. Pharmacological studies indicated that compounds **3 (d-e)** exhibited significant anti-inflammatory activities (Fig. 4). It was found that substitution with electronegative group enhanced the anti-inflammatory activity while substitution with electropositive atom or group (*e.g.* amino, hydroxyl or methyl) showed poor binding affinity for the enzyme as well as poor anti-inflammatory activity.

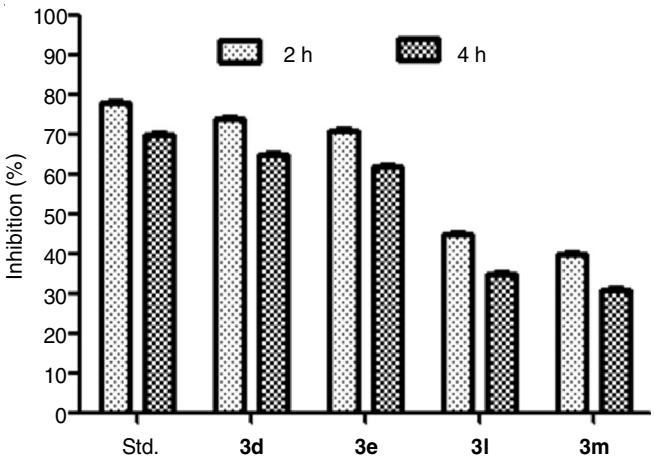



Fig. 4. Anti-inflammatory activity (% inhibition of paw edema) of selected molecules at different time intervals. The values are mean of the six measurements (n = 6). Data are statistically analyzed by two-way ANOVA ($P < 0.05$)

## Conclusion

Novel coumarin derivatives were synthesized as PDE4 inhibitors and evaluated as potent inflammatory agents. Molecular docking was carried out to find the binding affinity and interactions of novel compounds with the enzyme. Based on

good binding affinity in docking studies compounds were selected for further carrying out anti-inflammatory studies. Out of these derivatives, compounds with electronegative atom or group showed good binding affinity for the PDE4B enzyme as well as anti-inflammatory activity and compounds with electropositive atom or group showed poor binding affinity for the PDE4B enzyme as well as anti-inflammatory activity. Compounds **3(d-k)**, showed good binding affinity in docking studies and compounds **3(d-e)** exhibited highest anti-inflammatory activities.

## ACKNOWLEDGEMENTS

The authors are thankful to J.C.D.M. College of Pharmacy and I.K. Gujral Punjab Technical University for providing necessary facilities to complete this research work.

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