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View Article Online
DOI: 10.1039/C8MD00457A

Rationally Synthesized Coumarin Based Pyrazolines ameliorates carrageenan induced inflammation through COX-2/Pro-inflammatory cytokine inhibition

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Abstract

In the present work, coumarin based pyrazolines (**7a-g**) have been synthesized and investigated for their *in-vitro* and *in-vivo* anti-inflammatory potential. Amongst the synthesized compounds, compound **7a**, **7d** and **7f** exhibited significant *in vitro* anti-inflammatory activity as compared to the standard etoricoxib. Keeping this in mind, the *in-vivo* investigations were carried out *via* carrageenan induced inflammation and acetic acid induced writhing models in male Wistar rats and the compound **7a** was found to possess appreciable anti-inflammatory and analgesic potential. The mode of action of compound **7a** was also investigated by using substance P as biomarker, which shows promising results. Further, the selectivity of most active compound **7a** against cyclooxygenase enzyme was supported *via* the molecular docking studies which reveals that compound **7a** has more binding affinity towards COX-2 over the COX-1 and 5-LOX enzymes. *In-silico* ADME analysis of compound **7a** confer the drug like characteristics and *in vivo* acute toxicity study showed the safety of the compound even up to 2000 mg kg^{-1} dose. Thus, compound **7a** was identified as an effective anti-inflammatory agent, and can be explored for further analgesic/anti-inflammatory drug design and development.

Keywords: Coumarin-pyrazolines; COX-2 selective; Anti-inflammatory activity; Cytokine inhibition; Molecular docking analysis.

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1. Introduction

Inflammation is a body's defense response against injury, infectious agents and autoimmune reactions.[1] However, the outcome of chronic inflammation involves the tissue destruction and fibrosis, which may lead to the rheumatoid arthritis,[2] cancer,[3,4] neurodegenerative disorder[5] and cardiovascular diseases.[6,7] Several markers played an important role in inflammation including cytokine receptors, nuclear factor kappa-B (NF-κB), nitric oxide synthase (NOS), tumor necrosis factor alpha (TNF-α), interferons, chemokines and pro-inflammatory enzymes COX-2 and LOX (lipoxygenase).[8-10] Among them, cyclooxygenase and lipoxygenase are the real culprits, and thus, are the primary targets of the anti-inflammatory agents.[11] Cyclooxygenases causes the inflammation *via* arachidonic acid metabolism by catalyzing the formation of prostaglandins H_2, a precursor for the biosynthesis of prostacyclins, prostaglandins, and thromoboxane that virtually affects diverse biological processes such as regulation of immune function, and maintenance of renal blood flow, reproductive biology, and gastrointestinal integrity.[12] Studies have demonstrated that COX exists in multiple isoforms, each with its own physiological expression and function.[13] Two main isoforms are COX-1, designated as 'housekeeping' enzyme present in almost all cells and tissues, and regulates homeostasis and blood clotting, whereas, COX-2 is an inducible enzyme expressed in cells that mediates inflammation such as synoviocytes, macrophages, and monocytes leading to the synthesis of the prostanoids involved in acute and chronic inflammatory conditions. In this context, non-steroidal anti-inflammatory drugs (NSAIDs) are well recognized drugs for the treatment of inflammatory diseases which exerts their therapeutic effects by preventing the metabolism of arachidonic acid *via* inhibition of COX enzymes.[14] However, the conventional NSAIDs, including non-selective COX-1/2 inhibitors such as aspirin, ibuprofen, indomethacin and diclofenac are associated with gastric side effects, whereas, selective COX-2 inhibitors (COXIBs) such as celecoxib **1** prevent these side effect of non-selective NSAIDs.[15-18] The severe side effects of clinically used NSAIDS include gastrointestinal lesions,[19] cardiovascular diseases[20,21] and renal injury,[22] which necessitated the development of new chemical entities having higher efficacy and low/no side effects.

Coumarins have attracted intense interest due to their wide range of applications in pharmacological chemistry such as anticancer,[23] anti HIV,[24] antimicrobial,[25] anticoagulant, antioxidant,[25,26] antiulcer,[27] dyslipidemic,[28] antitumor,[29] and anti-inflammatory.[30] Literature

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reports revealed variedly substituted coumarins, pyrazoles, isoxazoles/isoxazolidines and oxadiazoles etc. as potential anti-inflammatory and analgesic agents *via* inhibition of cycloxygenase/pro-inflammatory cytokines (**Figure 1**).[31-38]

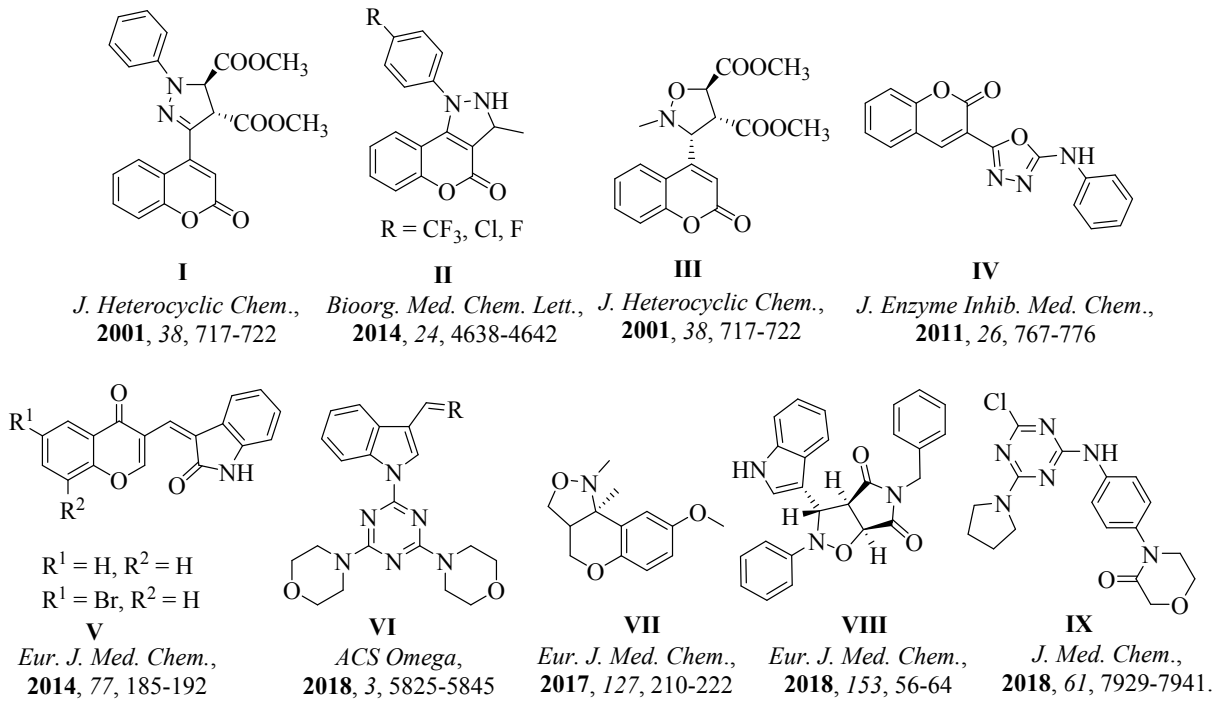

Figure 1: Variedly substituted molecules as potential anti-inflammatory/analgesic agents.

It is well established that a common structural feature of selective COX-2 inhibitors includes the presence of two vicinal aryl rings or 1,3-aryl groups attached with central five or six-membered heterocyclic or carbocyclic motif. On the other hand, various marketed COX-2 inhibitors comprises sulfone moiety which was found to be responsible for their prooxidant activity. This prooxidant effect could be related with the adverse effects observed with rofecoxib and etoricoxib due to which these drugs have not been approved by FDA for the U.S. market.[39a] On the basis of these considerations, the present work describes the rational design and synthesis of coumarin-pyrazoline in which coumarin nucleus was envisaged as a probable replacement for one of the aryl ring in selective COX-2 inhibitor (celecoxib **1)**, and 3-acetyl pyrazoline as central five membered carbocyclic ring with the underlying anticipation that the designed compounds would have a selective COX-2 inhibitory effect with low/no side-effects. Further, the ortho- two aryl groups present in marketed selective COX-2 inhibitors were replaced with meta- two aryl moieties in the newly synthesized compounds (**7a-g**) resulting in more bulky compounds, that

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could maximize the interaction with the hydrophobic residues within COX-2 active site and enhance COX-2 selectivity (**Figure 2**).

Earlier coumarin-pyrazolines have been reported for their anti-tumor activity,[39b] however, to the best of our knowledge, their anti-inflammatory activity has not been reported yet, therefore, keeping in mind their pharmacological potential and their structural resemblance with selective COX-2 inhibitors such as celecoxib, herein, we have re-synthesized coumarin-pyrazoline derivatives **7a** and **7e** along with some novel derivatives **7b**, **7c**, **7d**, **7f** and **7g** and evaluated them for detailed anti-inflammatory and analgesic activity.

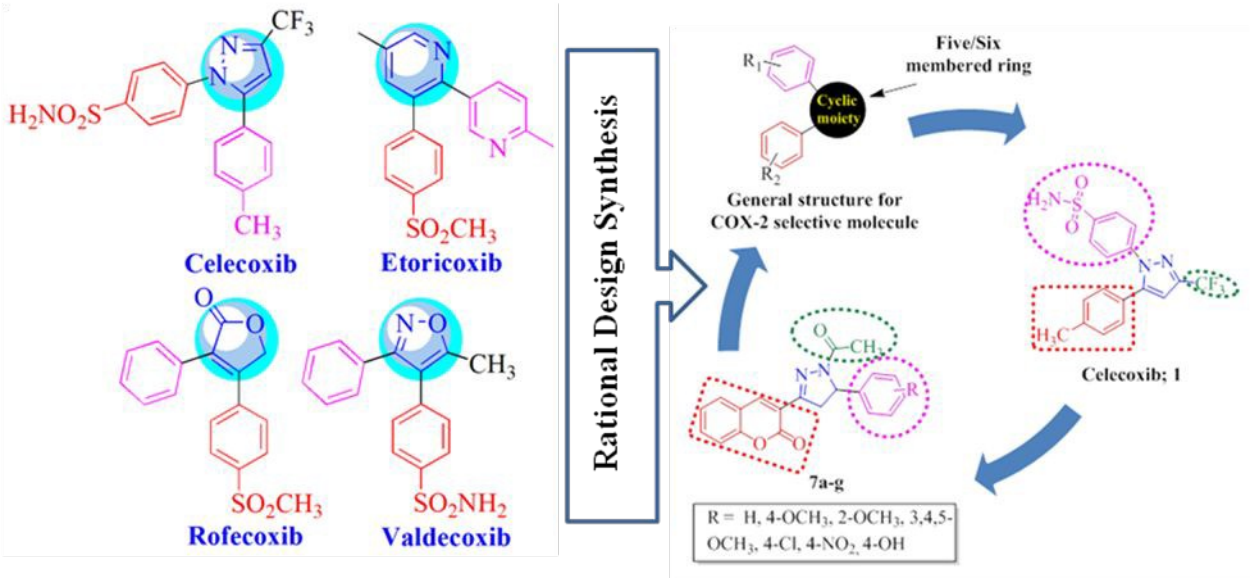

Figure 2: Marketed COX-2 selective inhibitors and design considerations for targeted coumarin-pyrazolines (**7a-g**).

2. Results and Discussion

2.1. Chemistry

The synthetic methodology employed for the synthesis of target compounds is illustrated in **Scheme 1**. The reaction of salicylaldehyde **2** with ethyl acetoacetate **3** in the presence of piperidine at RT yielded the 3-acetyl coumarin **4**.[40] Literature reports both acid-base catalyzed methods for the preparation of coumarin-chalcones derivatives, for instance, in the presence of piperidine (at 80°C),[41, 42] 10% NaOH[43] and 40% NaOH.[44] Herein, we have reported the synthesis of coumarin-chalcones by the reaction of 3-acetyl coumarin **4** with substituted benzaldehydes **5** using few drops of conc. H_2SO_4 in glacial acetic acid in excellent yields. Subsequently, chalcone

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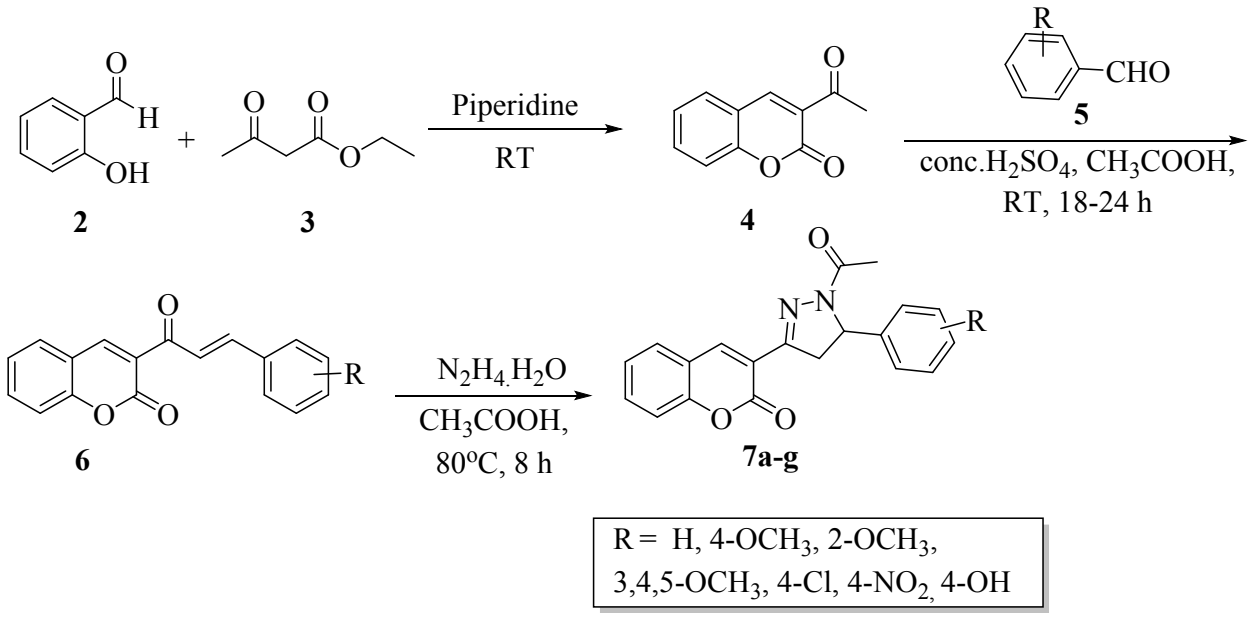
6 was cyclized in the presence of hydrazine hydrate in glacial acetic acid to furnish the desired coumarin-pyrazolines **7a-g** in high yields (**Table 1**).[45]

The synthesized compounds were purified by column chromatography over silica gel and characterized spectroscopically (IR, ^{1}H NMR, ^{13}C NMR and HRMS). For instance, in the ^{1}H-NMR spectrum of compound **7b**, the appearance of a double doublet (dd) of one proton at δ 5.50 ppm, was ascribed to chiral C-5 proton of pyrazoline, whereas, the methylene hydrogens of pyrazoline ring were appeared as two double doublets at 3.88 (1H) and 3.42 (1H) ppm. Two singlets at δ 3.81 ppm and δ 3.77 ppm were ascribed to OCH_3 protons, whereas, a singlet at δ 2.43 ppm correspond to CH_3 protons. The ^{13}C NMR resonance of acetyl carbonyl was appeared at 169.15 and others corroborated well with the structure of compound **7b**. The peak corresponding to the mass of **7b** with m/z 423.1569 (calcd m/z 423.1556 $[M+H]^+$) was observed in the high resolution mass spectra (HRMS), which in agreement with the molecular formula for assigned structure.



Scheme 1. Synthesis of Coumarin-pyrazolines **7a-g**.

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Table 1: Reaction time and (%) yields for compound **7a-g**

Compound	R	Time (h)	(%) yield
7a	4-OCH_3	8	82.1
7b	3,4,5-tri-OCH_3	8	85.5
7c	4-Cl	8	78.1
7d	4-NO_2	8	73.5
7e	H	8	66.1
7f	4-OH	8	65.0
7g	2-OCH_3	8	75.9

2.2. Pharmacological Evaluation

2.2.1. *In vitro* anti-inflammatory activity:

All the prepared compounds (**7a-g**) were evaluated[32] for their potential to inhibit COX-1 and COX-2 using an ovine COX-1/COX-2 assay kit (Catalog No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) and results obtained are presented in **Table 2**. The results of inhibition assay reveal that all the prepared compounds exhibits COX-2 selective inhibition as compare to COX-1. In particular, compound **7a** selectively inhibit COX-2 (IC_{50} = 0.41μM) over COX-1 which is much comparable to Etoricoxib (IC_{50} 0.23μM). In addition, compound **7d** and **7f** were also shows potent inhibitory activity against COX-2 with IC_{50} = 1.79 and 2.39μM, respectively.

Table 2: *In vitro* COX-1 and COX-2 inhibitory activity of coumarin-pyrazolines **7a-g**

Compound Code	COX inhibition (IC_{50}, μM)	
	COX-1	**COX-2**
7a	22.4	0.41
7b	>30	4.35
7c	ND	ND
7d	16.2	1.79
7e	ND	ND
7f	20	2.39
7g	>30	4.37
Etoricoxib	>30	0.23

ND-Not determined

Inhibition of albumin denaturation

Denaturation of proteins is a well-documented cause of inflammation.[46, 47] Thus, the synthesized compounds were investigated for their protective ability against albumin denaturation that could

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indicate their anti-inflammatory capability. The results shown in **Table S1** and **Figure 3a** indicate that synthesized compounds were effective in protecting heat induced albumin denaturation in a concentration dependent manner. Particularly, compound **7a** has shown more significant protection against denaturation. At 100µg/ml, compound **7a** has shown 70% protection as compared to standard (72%).

Membrane stabilization assays

The human red blood cell (HRBC) membrane stabilization has been used as an approach to examine the *in-vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the compounds may well stabilize the lysosomal membranes.[48,49] Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extracellular release. Therefore, to check the effect of synthesized compounds on lysosomal membrane, membrane stabilization assays (heat and hypotonicity induced hemolysis) have been performed.

Heat induced hemolysis:

The synthesized coumarin-pyrazolines (**7a-g**) inhibited the heat induced hemolysis in concentration dependent manner as shown in **Table S2** and **Figure 3b**. Among all the synthesized compounds, compound **7a** has shown more significant protection against the damaging effect of the heat compared to the control. At 10, 25, 50 and 100 µg/ml, the compound **7a** has exhibited protection against heat induced hemolysis by 60, 63, 67 and 69%, respectively as compared to standard Etoricoxib 65, 73, 78 and 76%, respectively.

Hypo-tonicity induced hemolysis:

The results showed that synthesized coumarin-pyrazolines at concentration range (10-100 µg/ml) protect the erythrocyte membrane against lysis induced by hypotonic solution (**Table S3** and **Figure 3c**). Among all the synthesized compounds, compound **7a** has shown more significant protection against the damaging effect of the hypotonic solution compared to control. At 10, 25, 50 and 100 µg/ml, the compound **7a** has exhibited protection against hypotonicity induced hemolysis by 62, 63, 67 and 68%, respectively as compared to standard Etoricoxib 52, 62, 66 and 69%, respectively.

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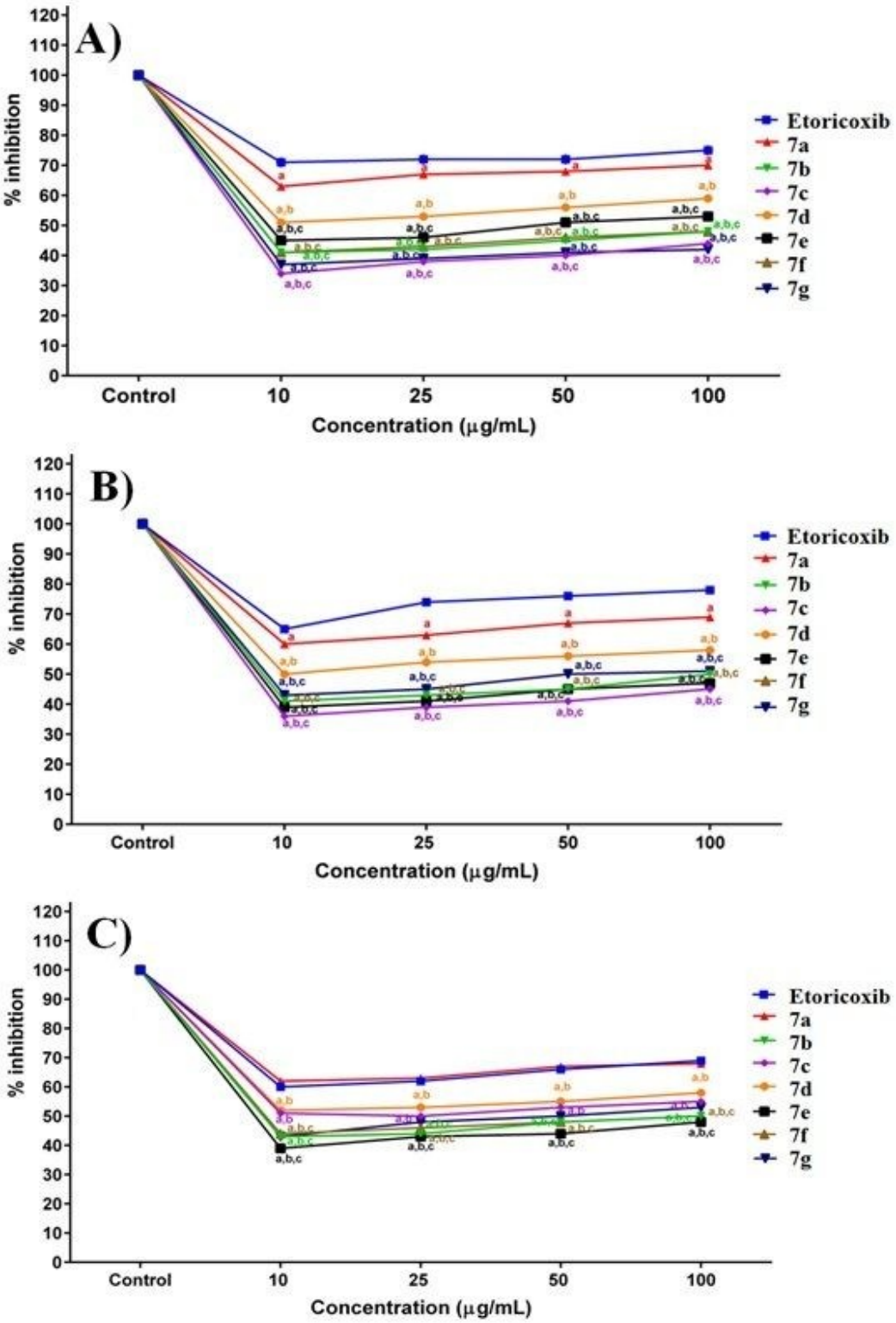

Figure 3: *In vitro* anti-inflammatory activity **a)** inhibition of albumin denaturation **b)** inhibition of heat induced hemolysis **c)** inhibition of hypotonicity induced hemolysis. Data is expressed as mean ± S.D. Significantly different $^{a}p< 0.05$ as compared to Etoricoxib, $^{b}p< 0.05$ as compared to **7a**, $^{c}p<0.05$ as compared to **7b** using ANOVA.

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2.2.2. *In vivo* anti-inflammatory activity

The synthesized coumarin-pyrazolines were further tested for *in vivo* anti-inflammatory activity using carrageenan paw edema method.[38] Male Wistar rats (4–6 weeks old), weight between 150 to 200 g were selected for the *in vivo* study. Animals were divided into following five groups: Group I was treated with vehicle and served as control and group II was treated with carrageenan (0.05ml of 1%). In other groups (Group III-X), the compounds were administered at doses of 25 and 50 mg kg^{-1}. After 30 min of the treatment with Etoricoxib or the test compounds, acute inflammation was induced by subplantar injection of 0.05 mL of freshly prepared 1% suspension of carrageenan. The volume displacement was measured by using plethysmography and recorded after 3h, 6 h and finally at 24h. The displacement volume was significantly increased in carrageenan treated group of animals as compared to control group. Among the synthesized compounds, compounds **7a**, **7d** and **7f** showed decrease in displacement volume as compared to carrageenan group in time dependent manner. There was no significant reduction in displacement volume when doses were increased from 25 to 50 mg kg^{-1}. Among all tested compounds, at 0.5, 3, 6 & 24 hrs, compound **7a** has shown more significant reduction in displacement volume as that of standard drug (**Figure 4**). Particularly, compound **7a** at 24 h reduced the displacement volume remarkably by 67.39% which is similar to standard Etoricoxib, whereas, compounds **7d** and **7f** reduced the displacement volume by 52.17 and 55.43%, respectively as compared to carrageenan.

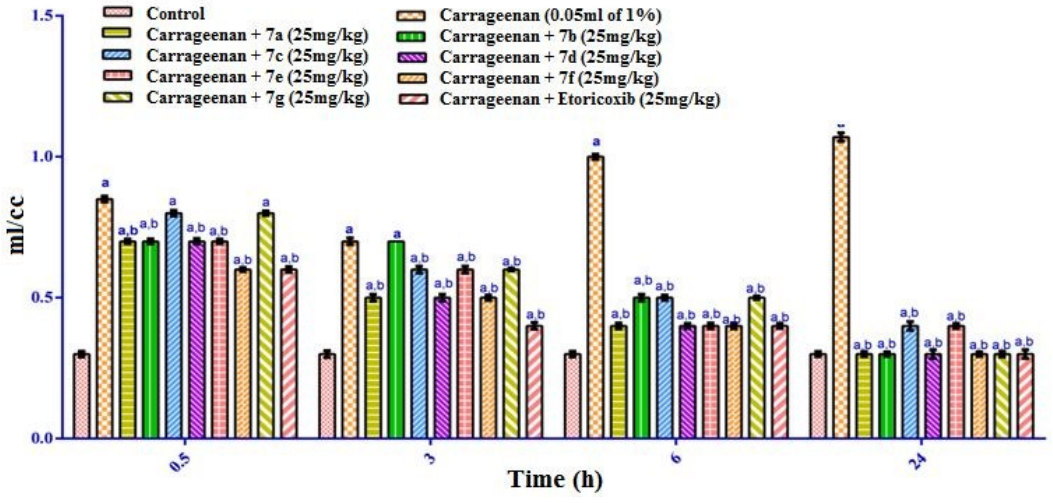

Figure 4: *In-vivo* anti-inflammatory activity of synthesized compounds (**7a-g**). Data is expressed as mean ± S.D. [a]p<0.001*vs* control, [b]p<0.001 *vs* carrageenan.

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2.2.3. Effect on cytokines level

The immune cells modulate the inflammatory responses by release of pro-inflammatory cytokines and contribute to the inflammatory and autoimmune disorders. Thus, the cytokines level were estimated by using rat TNF-α, IL-6, and IL-1β immunoassay kit (KRISHGEN Biosystem, Ashley Ct, Whittier, CA).[38] The tested compounds **7a**, **7d** and **7f** have shown significant decrease in the level of TNF- α, IL-6 and IL-1β which was increased by carrageenan. However, compound **7a** has shown more significant ($p<0.001$) reduction in the level of TNF-α, IL-6, and IL-1β as compared to compound **7d** and **7f** (**Figure 5**).

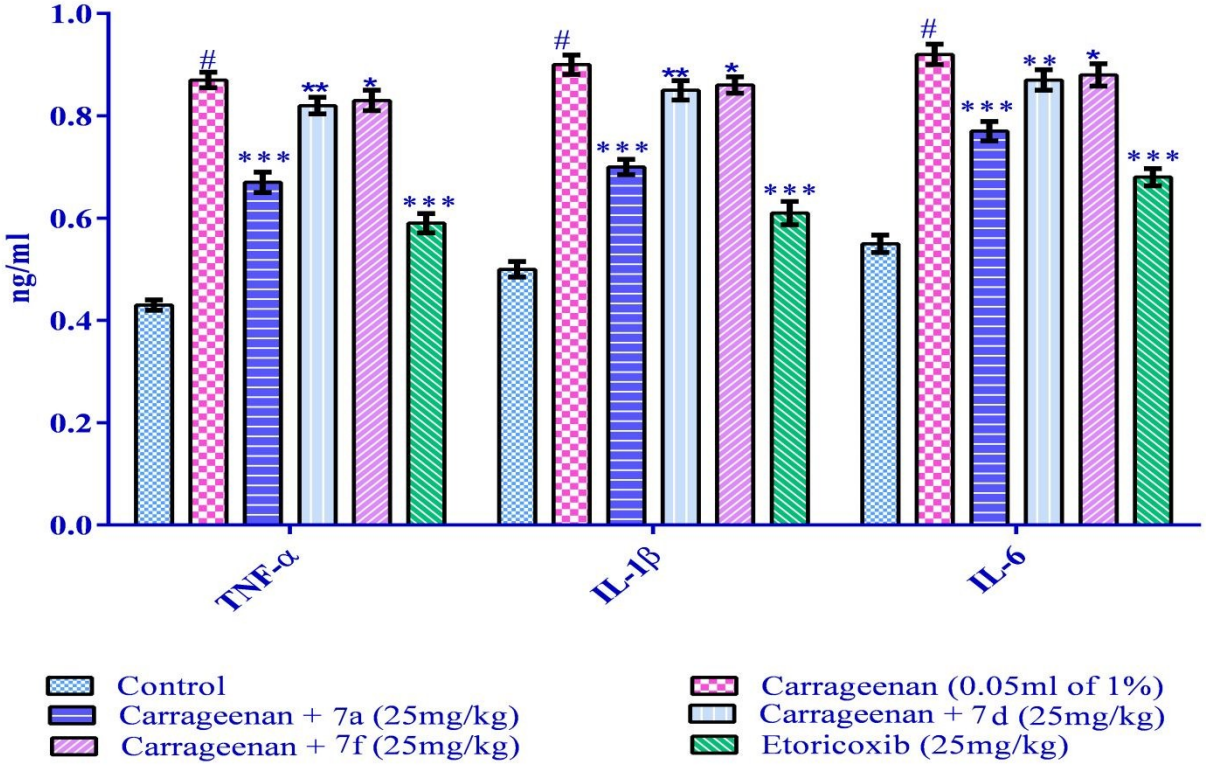

Figure 5: Effect of i.p. administration of compounds **7a, 7d** and **7f** on the serum levels of cytokines. Data is expressed as mean ± S.D. $^{\#}p<0.001$ *vs* control, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* carrageenan.

2.2.4. *In vivo* analgesic activity

The analgesic activity of most potent compound **7a** was assessed by the acetic acid induced abdominal constriction (writhing) test.[38] Animals were divided into three groups of six animals each. Group 1 animals were administered with vehicle and served as vehicle control. Group II

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were administered compound **7a** and Group III animals were administered Etoricoxib. One hour after vehicle and drug administration, each animal was injected with 3% (v/v) acetic acid, 300 mg/kg i.p. The number of stretches or writhes (arching of the back, elongation of the body and extension of the forelimbs) were counted cumulatively over a period of 20 min. The compound **7a** has been observed to significantly reduce the abnormal writhings by 70.5% as compared to control, whereas, Etoricoxib produced 57.6% inhibition of writhing (**Figure 6**).

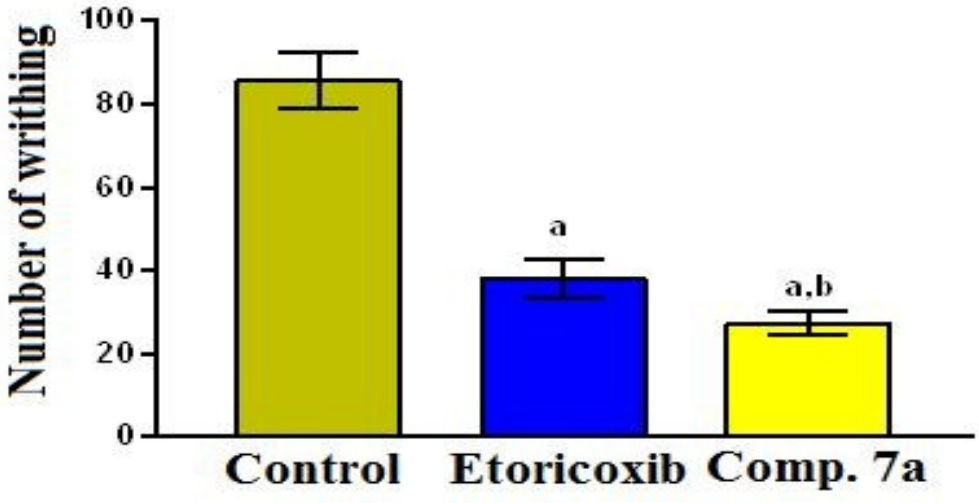

Figure 6: Effect of Compound **7a** on acetic acid induced writhing. All the values are expressed as mean ± S.E.M. [a] $p < 0.05$ *Vs* control, [b] $p < 0.05$ *Vs* Etoricoxib.

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2.2.5. Studies Supporting the Probable Mode of Action of the Compounds

It is well documented that the substance P (SP) is an important neuropeptide involved in neurogenic inflammation and it exerts various pro-inflammatory actions on immune-cells, including macrophages. Prostaglandin E2 (PGE2), a potent lipid mediator produced in nearly every cell type where it is synthesized from arachidonic acid *via* the actions of cyclooxygenase enzymes (COX-1 and COX-2).[50] COX-1 is constitutively expressed and important for normal regulation of vascular activity and gastrointestinal function, whereas, COX-2 is an inducible enzyme stimulated by numerous mitogens, cytokines, oxidants, and microbial products.[51] In particular, COX-2 is primarily responsible for the synthesis of PGE2, and elevates inflammation by increasing vasodilation, vascular permeability, and edema in numerous respiratory diseases, such as acute lung injury, chronic obstructive pulmonary disease, pulmonary fibrosis, and lung cancer. Some studies suggest that COX-2 and PGE2 might be associated with Substance P-

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related inflammatory responses.[52-54] Thus, to study the potential target of this compound, substance P (COX and LOX pathway stimulator) was administered to the animals 30 min before the administration of most potent compound **7a** and then the acetic acid was injected after 30 min.[55] Pretreatment of rat with substance P results in the reversal of analgesic effect of compound **7a** as shown in **Figure 7**. Further, the compound **7a** possesses poor inhibition of 5-LOX (IC_{50} of 12μM) as compared to COX-2 (IC_{50} of 0.41μM) which clearly indicated that COX-2 may be the probable target of **7a**. These results suggest the compound **7a** has analgesic effect in acetic acid induced algesia due to inhibition of cyclooxygenase-2 pathway.

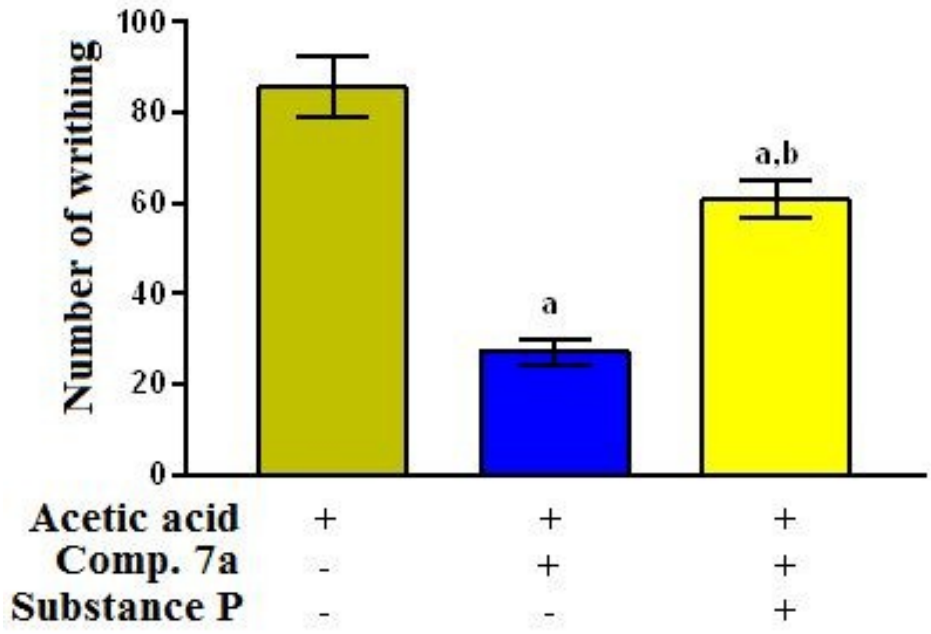

Figure 7: Effect of substance P pretreatment on analgesic effect of compound **7a**. All the values are expressed as mean ± S.E.M. [a] $p < 0.05$ *Vs* control, [b] $p < 0.05$ *Vs* compound **7a**.

2.2.6. Acute toxicity studies

Acute toxicity studies on most active compound were carried out on either sex rats as stated in OECD guidelines.[61] Four groups of animals were included in this study with three animals per group. The first group of animals was served as control and the second, third, and fourth groups were treated with tested compound **7a** at varied doses upto 2000 mg kg^{-1}, respectively, after 4 h of fasting. All the animal groups were continuously observed for the first 4 h and periodically for 24 h. After 14 days, histological studies were performed *via* H and E staining by sacrificing one animal each from the control and the highest dose (2000 mg kg^{-1}). The animals treated with

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under investigation compound **7a** at the dose of 50 mg/kg displayed no behavioral changes, whereas, the animals administered with the dose of 300 and 2000 mg/kg produces some irritation, itching, sneezing occasionally after 1-4 h of treatment. However, it is to be mention here that these symptoms diminish within the next 6 h and no significant changes were observed in the post-mortem analysis of myocardium, liver or kidney tissues of the animals administered with the highest dose of compound **7a** (2000 mg kg^{-1}) as compared to the control group (**Figure 8**).

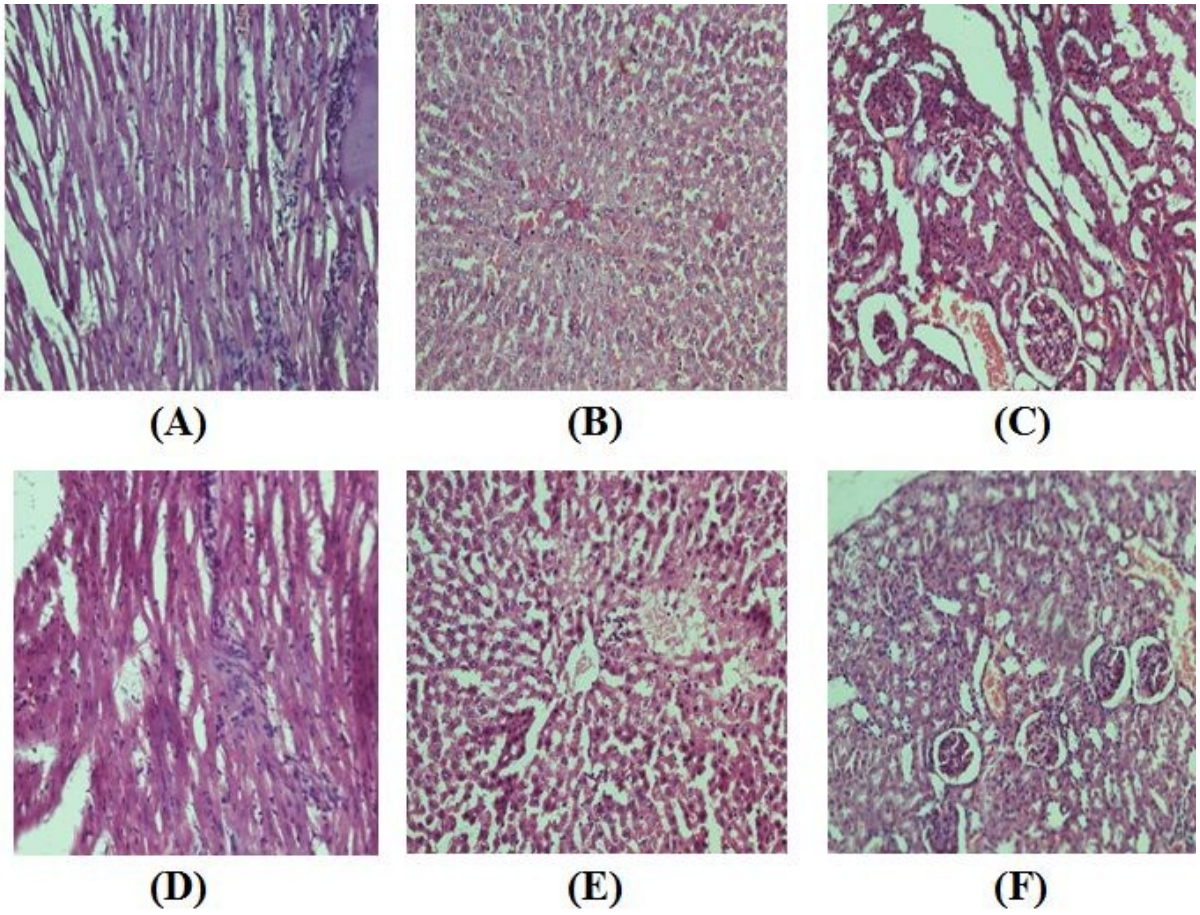

Figure 8: Photomicrographs of hematoxylin-eosin stained sections of control myocardium (**A**), kidney (**B**), and liver (**C**) and compound **7a** (2000 mg kg^{-1})-treated myocardium (**D**), kidney (**E**), liver (**F**). The pictures were taken with light microscope at ×200 magnification

2.2.7. Molecular docking study

Molecular docking study was performed using Gold score 5.1 software to predict and compare the selectivity of coumarin-pyrazoline derivatives against COX-2 (PDB 3LN1),[56,57] COX-1 (PDB 3KK6)[56,58] and 5-LOX (PDB 3V99) enzymes.[56,59] The affinities of synthesized compounds

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were evaluated in terms of Gold score and it was observed that the synthesized compounds **7a-g** showed good binding interaction with the active site of COX-2 enzyme as compared to COX-1 and 5 LOX, which were also taken in consideration to evaluate the selectivity (**Table S4**). Further, molecular docking results revealed that the most potent compound **7a** was fitted well to the active site of COX-2 with Gold score = 88.25, as compare to celecoxib (Gold Score = 96.42; **Table S4**). The carbonyl of coumarin is involved in hydrogen bonding with Tyr341 and actetyl group of pyrazoline interacts with Ser516. The aromatic ring of coumarin also showed π-H hydrophobic interaction with Val 509 (**Figure 9A**). This may explain the selectivity of the synthesized coumarin pyrazolines against COX-2 enzyme. The complete overlay of standard celecoxib was observed with compound **7a** as shown in **Figure 9B**. The docking results suggest that compound **7a** may potentially and selectively target the COX-2 enzyme. In silico ADME study on synthesized compounds **7a-g** was performed using the Swiss ADME predictor to predict the absorption, distribution, metabolism and elimination (ADME) parameters (**Table S5**). It was observed that all the coumarin pyrazolines **7a-g**, showed the ADME properties in the acceptable range, with no violation according to the Lipinski's rule of five.[60] Therefore, these compounds can be further utilized to be developed as drug candidates.

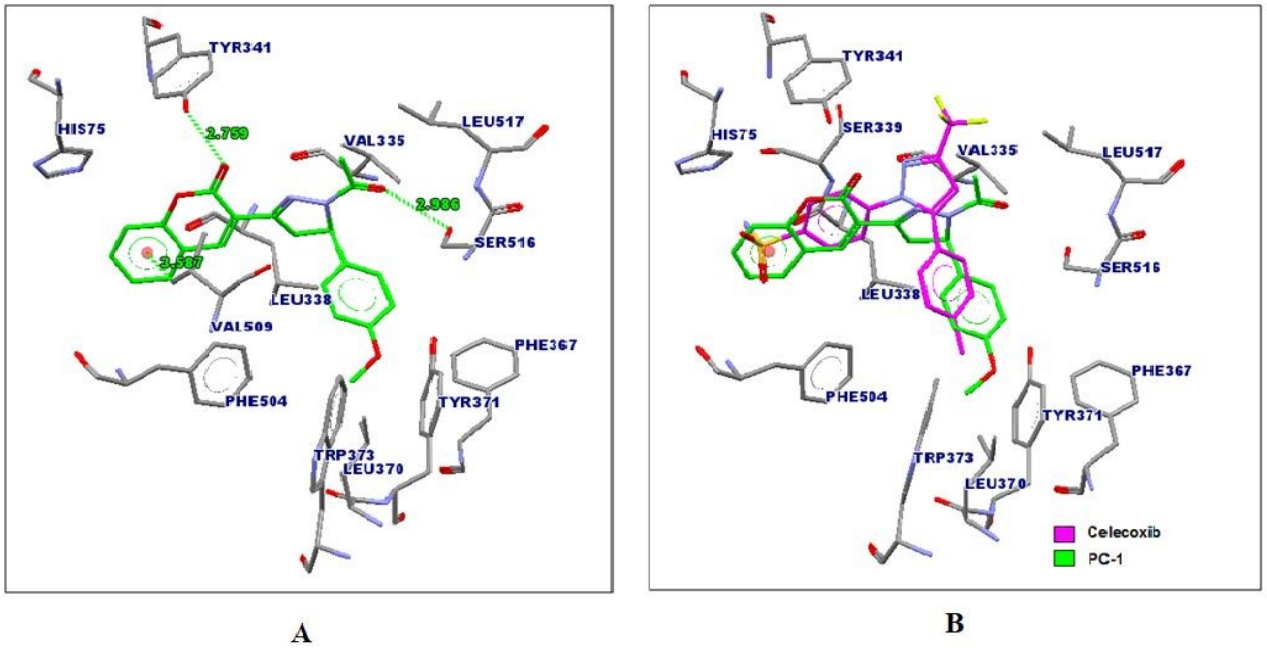

Figure 9: A) Docking of compound **7a** in the active site of COX-2; **B**) Overlay representation of celecoxib with compound **7a**.

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3. Conclusion

Coumarin based pyrazolines **7a-g** were synthesized and evaluated for *in-vitro* and *in-vivo* anti-inflammatory activity. From the *in-vitro* and *in-vivo* experiments, compound **7a** was identified as the highly active anti-inflammatory agent comparable to the standard Etoricoxib. Compound **7a** significantly suppress the cytokines level and showed remarkable analgesic and anti-inflammatory activity with minimum toxicity risk. Testing of this compound on Wistar rat showed reversal of algesia and inflammation with comparable efficacy to the standard drug Etoricoxib by targeting specifically COX-2. Further, molecular docking studies supported that COX-2 is the possible selective target for mode of action of compound **7a,** which was also evident by *in vivo* mechanistic studies using substance P as biomarker. The obtained results suggest that compound **7a** would be used as potential lead for the development of efficient anti-inflammatory agents.

Acknowledgements

Authors are thankful to Mr. Praveen Garg, Chairman, ISF College of Pharmacy, Moga, Punjab, for providing necessary infrastructure and his continuous support and encouragement.

Conflict of interest

The authors declare that they have no conflict of interests.

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GRAPHICAL ABSTRACT

Rationally Synthesized Coumarin Based Pyrazolines ameliorates carrageenan induced inflammation through COX-2/Pro-inflammatory cytokine inhibition

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