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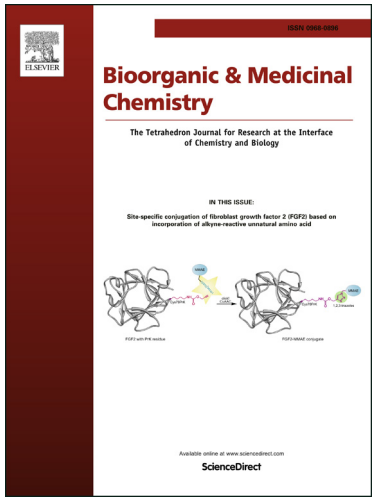

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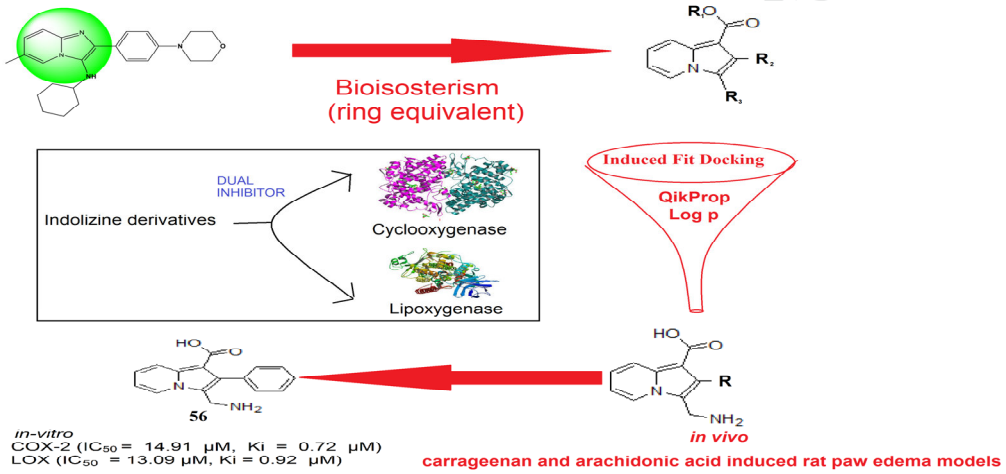
Sushant K Shrivastava[a]*, Pavan Srivastava[a], Robin Bendresh[a], Prabhash Nath Tripathi[a], and Avanish Tripathi[a]

[a] Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi-221005, U.P., India



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[a] *Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi-221005, U.P., India.*

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ABSTRACT

Some novel indolizine derivatives were synthesized by bioisosteric modification of imidazo[1,2-a]pyridine for anti-inflammatory activity. The physicochemical characterization and structure of compounds were elucidated by state of the art spectroscopic technique. Induced fit docking was performed for initial screening to elucidate the interactions with corresponding amino acids of cyclooxygenase (COX-1, COX-2) and lipoxygenase (LOX) enzymes. The target compounds **53**–**60** were then evaluated against *in vivo* carrageenan and arachidonic acid induced rat paw edema models for anti-inflammatory activity. Amongst all the synthesized derivatives, compound **56** showed the significant anti-inflammatory activity in both rat paw edema models with very less ulcerogenic liability in comparison to standard diclofenac, celecoxib, and zileuton. The compounds **56** was further assessed to observe *in vitro* enzyme inhibition assay on both cyclooxygenase and lipoxygenase enzyme where it showed a preferential and selective non-competitive enzyme inhibition towards the COX-2 (IC_{50} = 14.91 µM, Ki = 0.72 µM) over COX-1 (IC_{50} > 50 µM) and a significant non-competitive inhibition of soybean lipoxygenase enzyme (IC_{50} = 13.09 µM, Ki = 0.92 µM). Thus, *in silico, in vivo*, and *in vitro* findings suggested that the synthesized indolizine compound **56** has a dual COX-2 and LOX inhibition characteristic and parallel *in vivo* anti-inflammatory activity in comparison to the standard drugs.

* Corresponding author. Tel.: +919452156527; fax: +91-542-2368428; e-mail: skshrivastava.phe@itbhu.ac.in

1. Introduction

The prostaglandins and leukotrienes are naturally occurring twenty carbon fatty acid derivatives produced through biochemical oxidation of arachidonic acid (AA), which play an essential modulatory role in many normal and disease-related cellular processes.[1] In fact, much of the inflammation, pain, fever, nausea, asthmatic and allergic reaction occurs due to excessive production of prostaglandins and leukotrienes.[2]

The primary enzyme involved in the first step of the AA cascade is cyclooxygenase (COX), which exists in three isoforms as COX-1, COX-2, and COX-3. COX-1 and COX-2 are structurally 63% identical and 77% similar at the amino acid level.[3] The COX-1 is ubiquitous form typically produced in normal, quiescent condition and remains as a constitutive protein of normal cell. It is also important in the production of prostaglandins that regulates cellular homeostasis, such as renal blood flow, and in circumstances where prostaglandins have a protective function such as gastric mucous production.[4] COX-2 is the inducible form of the enzyme, expressed in the endothelial cell, chondrocytes, and osteoblast of traumatic tissue after tissue trauma and therefore plays a major role in inflammation. COX-3 is an enzyme mostly present in the brain, expressed under the influence of COX-1 gene, but not functional in the human being.[5]

There are some other important AA metabolites like leukotriene produced by Lipoxygenase (LOX) enzyme activity. LOXs are the member of non-iron containing dioxygenases family and be available for animals, plants, and fungi. In humans, three functional isoforms of LOX exist as 5-, 12-, and 15-LOXs, whereas two isoforms 9- and 13-LOX exist in plants.[6]

The crystal structures of two COXs suggested that the active site has a narrow hydrophobic channel extending from the membrane-binding region to the protein core. Initially, the binding of the substrate at COX enzyme occurs at the channel opening pocket lined with Arg120 and Ile345 in both the enzymes.[7] The key difference between the COX-1 and COX-2 isozyme active site is the exchange of isoleucine in COX-1 for valine in COX-2 at positions 434 and 523.[8] The differences in the amino acid sequence make the COX-2 substrate-binding site more flexible and somewhat larger by creating a secondary pocket. The COX-2 selective inhibitors explicitly bind to this secondary binding pocket (lined by His90, Arg513, and Val523) resulting in specific inhibition of COX-2 activity. Apart from this secondary pocket, another critical region in the COX-2 active site lined by Trp387, Tyr385, Phe518, Phe381, Met522, and Leu352 is known as the hydrophobic pocket. The selective COX-2 inhibitors acquire a pharmacophore which can selectively bind to the secondary pocket and bring enough steric bulk to block the hydrophobic channel of COX-2.[8] The active site of 5-LOX is an elongated cavity, with no clear access to bulk solvent, lined with both invariant (Leucines 368, 373, 414, and 607 and Ile406) and 5-LOX-specific amino acids (Tyr181, Ala603, Ala606, His600, and Thr364).[9] Further, alignment studies of five isoforms of LOX and two isoforms of COX suggested that pharmacophoric interaction with amino acid Tyr181, Phe359, Phe421, and Trp599 at 5-LOX binding site may increase specificity towards COX-2 and 5-LOX.[10]

Non-steroidal anti-inflammatory drugs (NSAIDs) as COX inhibitors are the leading prescription medicine worldwide for the cure of inflammation, but their long-term use is restricted due to gastrointestinal, bronchoconstriction and hepatotoxic side effects.

Indeed significant NSAIDs are fluxed in the world market, but the blockade of arachidonate cascade at the COX level diverts the substrate towards increased production of LOX-derived eicosanoids such as leukotrienes (LTs) that cause bronchoconstriction, ulceration, and inflammation, which exists as a big challenge for medicinal chemists.

Considering the pro-inflammatory properties of LTs and prostanoids, the drugs able to block the synthesis of both eicosanoids, should prove itself as a better anti-inflammatory drug molecule with fewer side effects in comparison to established classical NSAIDs and selective COX-2 inhibitors.[11] The COX and LOX drug inhibitors are expected to enhance anti-inflammatory potency without risks of serious side effects. Hence the discovery of dual COX and LOX enzymes inhibitors with reduced toxicity and side effects is the need of pharmacotherapeutics in the modern age.[12]

1.1. Designing considerations

In the field of drug development and therapeutics, bioisosterism is a successful analog designing strategy over the years and translated into the development of drugs like Alloxanthine and Procainamide, etc.[13] Taking a cue from this, we have studied some Imidazo[1,2-a]pyridine derivatives having an aromatic ring at 4th position, and cyclohexanamine or cyclopentanamine at 5th position reported for LOX inhibition (IC_{50} = 0.21 μM) at micromolar concentration.[14] In the designing, the Imidazo[1,2-a]pyridine nucleus was chosen, where bioisosteric replacement of =N- with =CH- (ring equivalent) leads to flanged bicyclic nucleus 'indolizine' that could be considered as a novel class under non-classical bioisostere design.

Compounds with indolizine ring have received attention in recent years like Curindolizine, a chemical generated from Curvularia (species: IFB-Z10) reported for anti-inflammatory activity in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages (IC_{50} = 5.31 μM).[15] Licofelone is another molecule having 3H-pyrrolizine fragment like Curindolizine, reported for 5-LOX (IC_{50} = 0.21 μM), COX-1 (IC_{50} = 0.16 μM) and COX-2 (IC_{50} = 0.37 μM) inhibitory activity and had been passed the safety level in the clinical trial, but the challenge of the ulceration persists in licofelone at doses of 30 and 100 mg/kg.[16] It may be due to the blocking of COX-1 enzyme and the strategy to block COX-2, and LOX enzyme selectively may produce a new dual COX-2 and LOX inhibitor to treat inflammatory disorders.

The primary objective of this study was to design and synthesize some novel 3-(aminomethyl)indolizine-1-carboxylic acid derivatives using the bioisosteric modification of imidazo[1,2-a]pyridine to indolizine (Figure 1) and further evaluated for their particular COX and LOX enzyme inhibition, anti-inflammatory activity, and ulcerogenic liability.

2. Results and discussion

2.1. Chemistry

The targeted compounds were synthesized as per Schemes 1. The known malonate derivatives (**10-17**) were synthesized through the Knoevenagel condensation by reacting substituted aldehydes (**1-8**) with diethyl malonate (**9**) in the presence of catalytic amount of

piperidine.[17] The reported pyridinium bromide salt (**20**) was then prepared by the addition of Bromo acetonitrile (**19**) in pyridine (**18**). Pyridinium bromide salt (**20**) then conjugated with malonate derivatives (**10-17**) through Huisgen [3+2]-cycloaddition reaction in dichloromethane to give unstable intermediate compounds **(21-28)** which than cyclized to five-membered heterocyclic indolizine compounds (**29-36**). Intermediate compounds **(21-28)** are unstable, so we made no attempts to isolate them. Also, indolizine compounds (**29-36**) were carried forward in next step without further purification and reacted with chloranil or chromium trioxide in the presence of the strong base and resulted in the corresponding aromatized indolizine derivatives (**37-44**),[18, 19] which was initially confirmed by positive Dragendorff test on TLC.[20] Further, the nitrile was reduced by nickel boride,

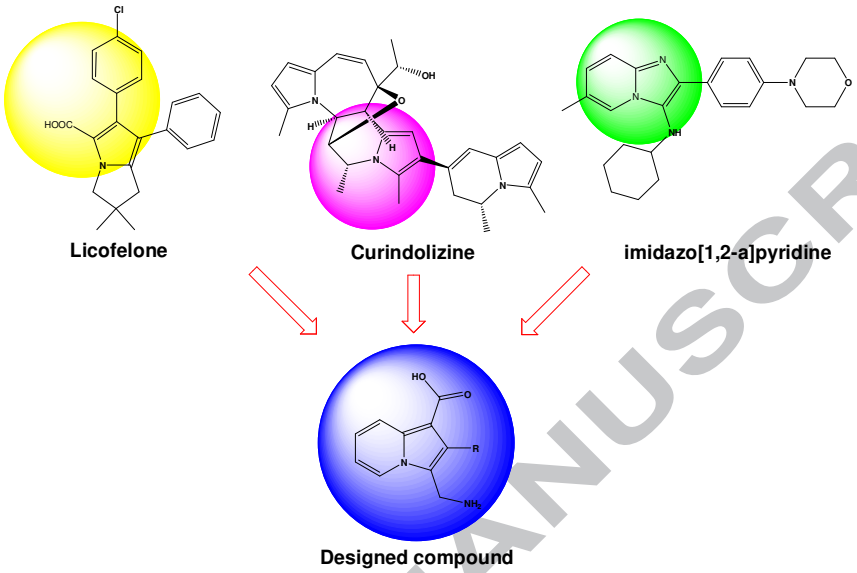
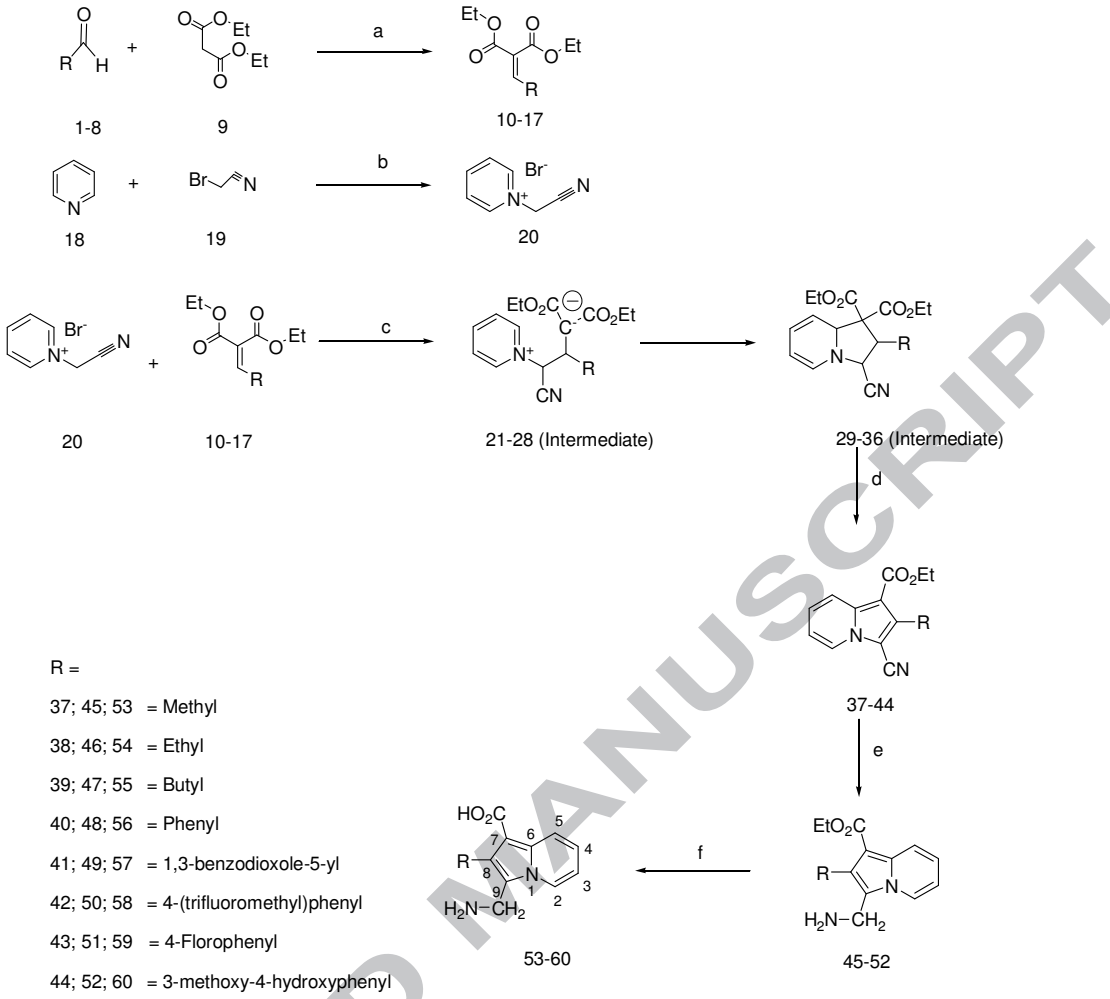


Figure 1: Design of a novel series of compounds as multitarget-directed potential anti-inflammatory agents.



Scheme 1: Reagents and conditions: (**a**) piperidine, EtOH, refluxed, 5- 6 h (**b**) rt, overnight (**c**) NaOH (32%), DCM (**d**) *in situ,* Chromium trioxide/Chloranil, rt, 3- 4 h (**e**) $NiCl_2$, $NaBH_4$, anhydrous EtOH, 0-5°C, 3- 4 h (**f**) NaOH (12%), THF, rt, overnight.

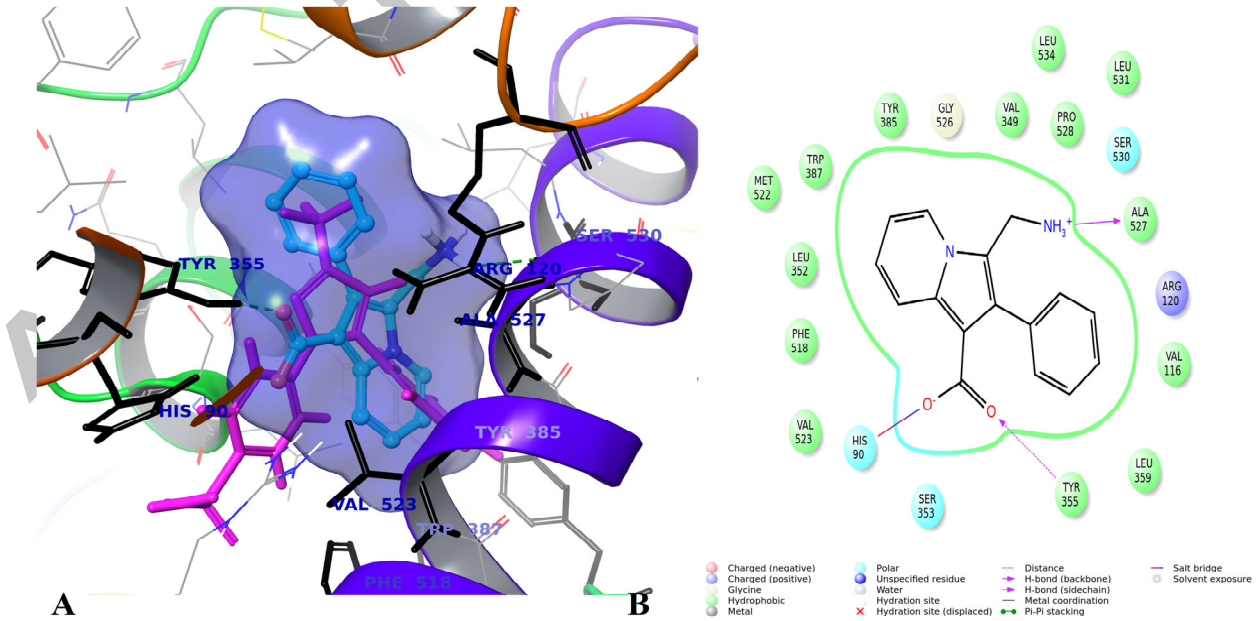


Figure 2. Induced fit docking study of best pose generated on COX-2 (PDB: 1CX2) for **(A)** celecoxib (purple) and compound **56** (sky blue); ligand binding site for docked compound **56** represented in blue, **(B)** 2- Dimensional representation of compound **56** at the active site.

generated from in-situ sodium borohydride and nickel (II) chloride reaction in dry ethanol, resulted in the corresponding aminomethyl carboxylate (**45-52**),[21] which was confirmed by positive Ninhydrin Test on TLC.[22] The novel target 3-(aminomethyl)indolizine-1-carboxylic acid derivatives (**53-60**) were generated by base-catalysed hydrolysis of the ester. Preliminary identification of acid was confirmed by positive Bromo Cresol Green test on TLC.[23]

The FT-IR spectra of nitrile compounds (**37-44**) showed the characteristic medium C-N stretching of nitrile peak in the range of 2300-2350 $cm^{-1}$and C-O stretching of ester peak in the range of 1735-1755 cm^{-1}. Structures of compounds (**37-60**) were also confirmed by NMR spectroscopy. In all compounds, ^{1}H NMR spectra showed a peak of acid at down field (at 11 δ value). The characteristic amine peak was observed at 3-5 δ value with integration value of 2. Another peak of the CH_2 proton with integration value of 2 as triplet was observed at near to 3 δ value. Further, ^{13}C NMR spectra also confirmed the presence of carboxylate group by showing a peak near to 200 δ at down field.

2.2. Log P determination and prediction of drug-like properties

The lipophilicity (log P) of a molecule is considered as a characteristic to establish a relationship between the pharmacodynamic and pharmacokinetic property of drugs.[24] We have determined the partition coefficient of the synthesized compounds and standards by octanol/water system (Supplementary Table 1). Almost, the log P values of compounds were observed under suitable lipophilic range (2-4). The drug-likeness of all the compounds and standard were also calculated through QikProp software tool (Maestro 10.5.014, Schrödinger, LLC, and New York-1), *in silico* ADME/Tox Predictions ensured that all compounds follows Lipinski's rule of five and could be considered as a drug-like molecule. (Table 1)

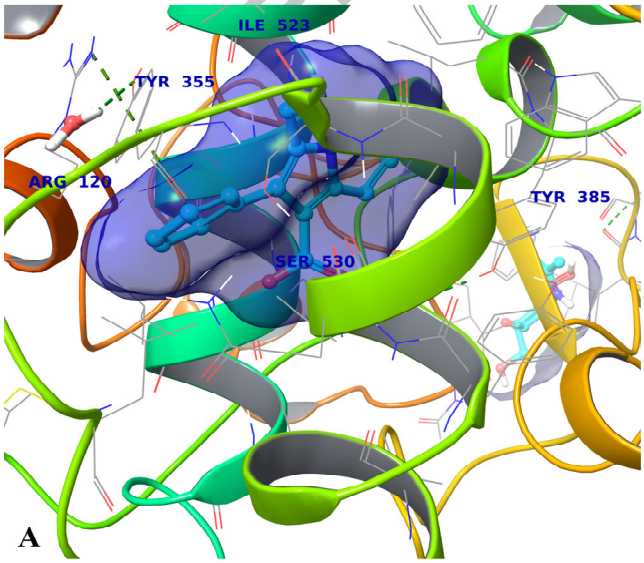
2.3. Docking study

The synthesized compounds (**37-60**) were subject for Rigid Docking on COX-1, COX-2, and LOX enzymes (PDB Code: 1CX2,[25] 3N8Y,[26] and 3V99[27] respectively) using Glide-XP protocol in the Schrödinger software suite. The Glide Score (GScore) and the interactions at the active site were recorded. The compounds **53-60** were observed with top GScore and selected for Induced Fit Docking (IFD) protocol to obtain more accurate ligand placements under fine flexibility treatment of the protein.[28] The GScore and IFD scores of compounds (**53-60**) are depicted in Table 1.

The docking poses of most potential compound **56** on COX-2 enzyme displayed poses similar to that of standard celecoxib (Figure 2A). The indolizine ring of compound **56** was interacting at the hydrophobic channel of COX-2 lined by Leu352, Tyr385, Trp387, Phe518, and Met522 along with contributions from the backbone atoms of Ala527 (via hydrogen bonding). Beyond this hydrophobic pocket, the carboxylic group showed the penetration into secondary pocket lined by His90, Phe518, Trp355, and Val523. One of the oxygen atoms of the carboxylic acid of compound **56** formed a hydrogen bond to Trp355 and linked by a salt bridge with His90 at the secondary pocket (Figure 2B). The interaction of celecoxib (Supplementary Figure 1) and compound **56** at His90 of the secondary pocket of COX-2 enzyme showed the same binding owing to the bioisosteric similarity (SO_2NH_2 vs. COOH).

A similar method was used to compare ligand interactions in COX-1 (PDB code: 3N8Y) enzyme active site. All compounds showed a different conformation in COX-1 with respect to the conformation of the COX-2 active site. (Figure 3)

The docking study of licofelone was performed on 5-LOX enzyme (PDB: 3V99), it interacts with His372 via hydrogen bonding and also showed hydrophobic interaction with Phe359 (Supplementary Figure 2). The binding pose of compound **56** and licofelone was superimposed, and the interactions were compared with respect to the LOX enzyme (Figure 4A). It was found that indolizine rings of compound **56** and pyrrolizine nucleus of licofelone oriented toward the same region and exhibited hydrophobic interaction with surrounding active site amino acids Leu414, Leu607, and Ala603 in the hydrophobic region of LOX. Compound **56** was stabilized in the active site of LOX enzyme by the forming hydrogen bonds with Gln363 and showed π-cation interaction with Phe177. Compound **56** also showed hydrophobic interaction with Phe359 responsible for specificity towards COX-2 and LOX (Figure 4B).



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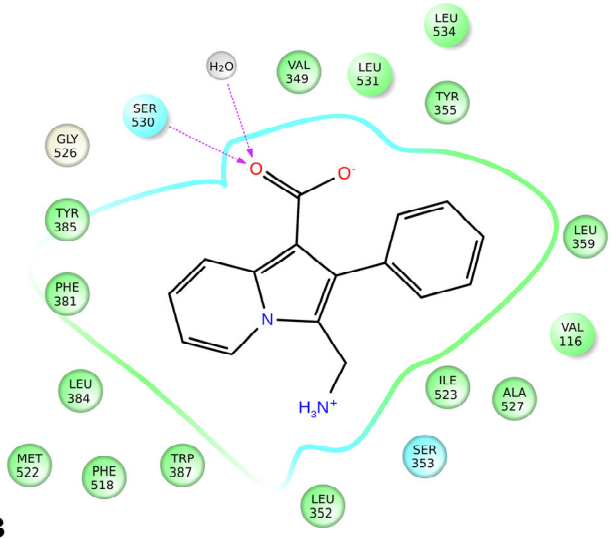

B

Figure 3. Induced fit docking study of best pose generated on COX-1 (PDB: 3N8Y) of **(A)** compound **56**, ligand binding site for docked compound **56** represented in blue, **(B)** 2- Dimensional representation of compound **56** at the active site.

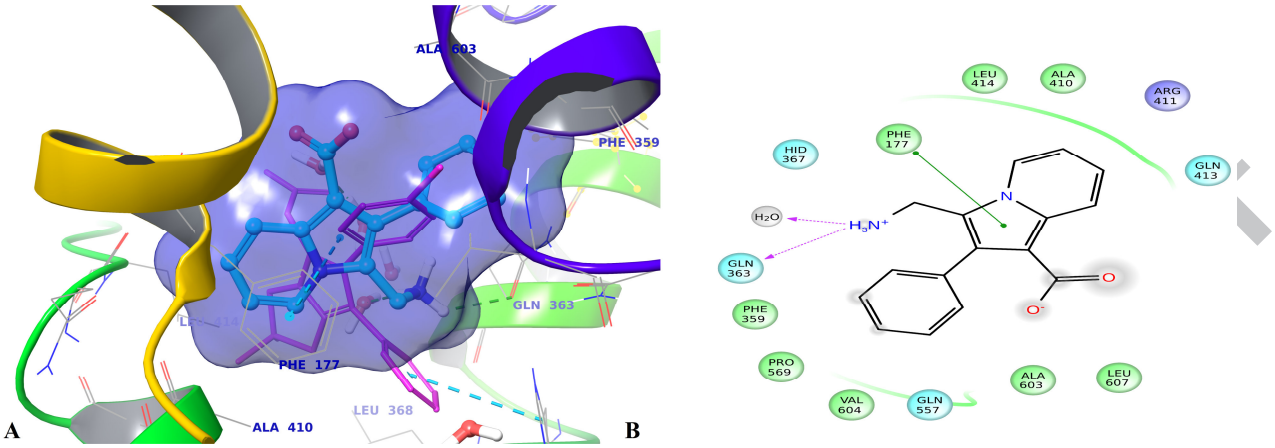

Figure 4. Induced fit docking study of best pose generated on LOX (PDB: 3V99) of **(A)** licofelone (purple) and compound **56** (sky blue), ligand binding site for docked compound **56** represented in blue, **(B)** 2- Dimensional representation of compound **56** at the active site.

2.4. Pharmacology

2.4.1. Carrageenan-induced paw edema

Carrageenan-induced rat paw edema model is considered as standard screening experiment to observe acute inflammation. The paw edema was induced by the subplantar injection of carrageenan and showed a biphasic response. It is reported that early phase releases of pro-inflammatory agents such as serotonin, histamine, whereas kinins and, the late phase (3 h post treatment) mediated by prostaglandins.[29] The outcomes of the anti-inflammatory activity against carrageenan-induced hind paw edema of the target compounds (**53-60**) is summarized in Table 2. Mean changes in the paw edema thickness at two hours intervals from the induction of inflammation up to six hours along with inhibition percentage of edema were calculated and recorded. The compound **56** exhibited parallel inhibition compared to the standard diclofenac which exhibited an inhibitory activity of 90.9 percent at six hours. In all eight evaluated derivatives, compounds **56, 57, 58, 59** and **60** were found to possess good anti-inflammatory potential in the range of 88.6, 78.1, 67.7, 86.4, and 59.4 percent respectively. Compound **56**, bearing a phenyl group on indolizine moiety, exhibited activity (88.6 percent) parallel to diclofenac (90.9 percent). The aliphatic nucleus bearing Methyl (**53**), Ethyl (**54**) and Butyl (**55**), exhibited a decreasing order of anti-inflammatory activity (38.5, 34.3, and 23.9 percent). Structure-activity relationship inferred that substitution pattern at position 8 of indolizine ring is supposed to be an active site for determining the anti-inflammatory activity. The result indicated that substitution of the aromatic ring system at position 8th (**56-60**) resulted in more active compound than the aliphatic chain substituted compounds (**53-55**). Compounds at position 8 with the electronegative groups like CF_3, fluorine and hydroxyl group (**57-60**) showed less anti-inflammatory protection than the other compound (**53-55**). Overall unsubstituted phenyl ring at 8th position showed best anti-inflammatory activity among synthesized compounds. Based on this observation and the biphasic nature of carrageenan-induced paw edema, it is possible to propose that the significant activity

Table 1. Docking and QikProp Analysis

Code	COX-2 GScore	COX-2 IFD score	LOX GScore	LOX IFD score	SASA[b]	HB[c]	HB[d]	QPlogPC16[e]	QPlogPoct[f]	QPlogPo/w[g]	QplogS[h]
53	-8.75	-1100.6	-8.60	-1510.6	428.7	3	3	7.6	13.2	-0.9	-1.4
54	-9.19	-1110.9	-8.53	-1511.2	435.4	3	3	7.8	13.3	-0.6	-1.4
55	-9.11	-1118.5	-6.55	-1508.8	502.8	3	3	8.9	14.4	0.3	-2.4
56	-9.80	-1122.7	-8.26	-1512.3	500	3	3	10.1	16.1	0.5	-2.9
57	-10.26	-1125.9	-8.02	-1509.9	503.7	3	4.5	10.1	17.3	0.1	-2.7
58	-11.12	-1124.1	-8.06	-1511.3	551.8	3	3	9.8	18.4	1.5	-4.3
59	-10.31	-1123.2	-8.41	-1512.3	509.7	3	3	9.7	16.8	0.7	-3.2
60	-10.70	-1124.7	-8.66	-1513.0	546.2	4	4.5	10.9	18.9	0.05	-2.8
celecoxib	-12.69	-1125.1	nd[a]	nd[a]	661.6	2	5.5	11.4	21.2	3.4	-6.4
licofelone	-10.98	-1120.7	-8.07	-1496.1	650.4	1	2	12.5	17.7	6.4	-7.3
zileuton	nd[a]	nd[a]	-7.66	-1500.7	447.7	3	3.7	8.4	14.6	0.9	-1.6

[a] Not determined.
[b] SASA, total solvent accessible surface area (range, 300-1000 Å2).
[c] H-bond donors.
[d] H-bond acceptors.
[e] QPlogPC16, predicted log of hexadecane/gas partition coefficient (range, 4-18).
[f] QPlogPoct, predicted log of octanol/gas partition coefficient (range, 8-43).
[g] QPlogPo/w predicted log of octanol/water partition coefficient (range 2 to 6).
[h] QplogS, predicted log of aqueous solubility (range, 6 to 0.5 M).

observed in the late phase of inflammation is attributed to the ability of the compounds to inhibit the release of late mediators. Thus, it is proposed that the evaluated compounds mitigate the inflammatory effects of carrageenan by inhibiting the release of prostaglandins

2.4.2. Arachidonic acid induced paw edema

AA-induced paw edema is used to distinguish between COX and lipoxygenase (LOX) inhibitors, as the model is known to be more sensitive to LOX inhibitors.[30] A subplantar injection of AA produced significant edema within 30 to 60 minute. The paw edema induced by AA is perceptibly reduced by LOX inhibitors. The results illustrated in Table 3 indicating that zileuton (a selective LOX inhibitor) exhibited an inhibition (72.3 percent) against AA-induced paw edema. Compounds (**56, 58, 59** and **60**) were effective in inhibiting the inflammation induced by AA, with their mean percentage protection as 66.2, 40, 61.5, and 36.9 percent respectively. The outcome of this study proposes that the most active compound **56** mediate their effects by inhibiting the COX pathway as well as LOX pathway.

2.4.3. Ulcerogenic risk evaluation

Compound **56** exhibiting promising anti-inflammatory profiles on *in vivo* model was further assessed for its ulcerogenic liability in terms of the ulcer index (UI). The results obtained from the postmortem studies of animals sacrificed on the 21 days of post adjuvant-induced arthritis. The study revealed that compounds **56** were found safer with respect to gastrointestinal (GI) toxicity (UI 10 ± 1.4) in comparison with standard diclofenac, celecoxib, and zileuton (UI 46 ± 4.1, 53 ± 5.5, and 52 ± 4.9 respectively) (Table 4). These findings, corroborated with the outcome of the histological evaluation of the gastric mucosa that support the high gastric tolerability of the compounds relative to standard compounds (Figure 5).

Table 2. Carrageenan-induced paw edema.

Compound	[a]Mean protection (%)		
	2h	4h	6h
control	0	0	0
diclofenac	74.6	91.6	90.9
53	41.3	44.2	38.5
54	30.2	32.5	34.3
55	23.8	24.4	23.9
56	68.2	90.5	88.6
57	61.9	74.4	78.1
58	42.8	62.8	67.7
59	69.8	89.5	86.4

60	50.8	63.9	59.4

Control: 0.3% carboxymethylcellulose sodium (CMC) solution in distilled water (10 ml/kg/p.o.).

Standard: diclofenac (10 mg/kg/p.o) in 0.3% CMC solution.

All other compounds were administered at an equimolar oral dose relative to 10 mg/kg diclofenac

[a]Mean protection (%) was expressed as (%) edema inhibition of the tested compounds relative to (%) edema inhibition of standard.

2.5. COX and LOX inhibition assay

Based on the results of the preliminary *in vivo* screening, the compound **56** showed significant anti-inflammatory activity, and further assessed *in vitro* COX and LOX enzyme inhibition assay. (Table 5)

Table 3. Arachidonic acid induced paw edema.

Compound	%Protection
control	0
zileuton	72.3
53	23.1
54	20.2
55	15.4
56	66.2
57	23.1
58	40.0
59	61.5
60	36.9

Standard: zileuton (10 mg/kg/p.o) in 0.3% CMC solution.

All other compounds were administered at an equimolar oral dose relative to 10 mg/kg standard

[a]Protection (%) was expressed as % edema inhibition of the tested compounds relative to % edema inhibition of standard.

2.5.1. In vitro COX inhibition assay

In vitro COX inhibition of compound **56,** performed on ovine COX-1 and human COX-2 enzymes using colorimetric enzyme immunoassay (EIA).[31] (Supplementary Figure 3) Along with the compound **56**, diclofenac and celecoxib were tested and the data obtained are reported in Table 5. The result obtained infers a specific inhibition of COX-2 over COX-1 of compound **56, (**COX-1; IC_{50} >50 μM, COX-2 (IC_{50} = 14.91 μM) in comparison to standard diclofenac (COX-1; IC_{50} = 0.15 ± 0.009 μM, COX-2; IC_{50} = 0.05 ± 0.001μM) and celecoxib (COX-1; IC_{50} = 6.7 ± 0.029 μM, COX-2; IC_{50} = 0.87 ± 0.041 μM). Compound **56** exhibited a selective COX-2 inhibition very close to the standard celecoxib. It may be due to the proper size, orientation and binding of compound **56** into the secondary pocket of the COX-2 enzyme as observed in IFD studies.

2.5.2. In vitro LOX inhibition assay

The *in vitro* analysis of lipoxygenase inhibitory activity was performed using Soybean lipoxygenase (linoleate 13S- lipoxygenase, linoleate: oxygen oxidoreductase, EC 1.13.11.12).[32, 33] IC_{50} values were determined through Continuous Spectrophotometric Rate Determination method. The LOX enzyme formed the 9- and 13-hydroperoxides by LOX enzyme with substrate linoleic acid and resulted in increased absorbance at 234 nm. Compound **56** showed *in vitro* LOX inhibitory activity (LOX; IC_{50} = 13.09 μM) in comparison with zileuton (LOX; IC_{50} = 3.429 μM) (Table 5). The *in vitro* studies demonstrated that compound **56** inhibits LOX as well as COX-2 activity. The results of *in vitro* activity on both COX and LOX were corroborated with the outcome of earlier *in vivo* results, hence confirmed the dual inhibition nature of compound **56** on both LOX and COX.

2.6. In vitro kinetic study

The mechanism of inhibition of cyclooxygenase and lipoxygenase enzyme by compound **56** was determined by measuring the enzyme activity at fixed concentrations of arachidonic acid in the presence of increasing concentrations of compound **56**. The enzyme kinetic study was observed and determined by Michaelis-Menten equation.[34] The Y-axis on the Lineweaver-Burk Plot was graphed as the $1/V_{max}$ (reciprocal of reaction rate) and the X axis as the reciprocal of substrate concentration (Figure 6). The results obtained from the kinetic inhibition pattern demonstrated a non-competitive inhibition of LOX enzyme (Ki = 0.72 ± 0.060 μM) and COX-2 enzyme (Ki = 0.92 ± 0.056 μM). Thus, the *in vitro* enzyme kinetics and IC_{50} of compound **56** reflected the dual inhibition of COX-2 and 5-LOX enzyme.

Table 4. Ulcer index of the representative compound **56**.

Table 5. IC_{50} values of the representative compound **56**.

Compound Code	COX-1	COX-2	LOX
	IC_{50} (μM) ± SEM	IC_{50} (μM) ± SEM	IC_{50} (μM) ± SEM
56	>50	14.91 ± 0.203	13.09 ± 0.260
celecoxib	6.7 ± 0.029	0.87 ± 0.041	nd[a]
diclofenac	0.15 ± 0.009	0.05 ± 0.001	nd[a]
zileuton	nd[a]	nd[a]	3.429 ± 0.045

[a] Not determined

Compound Code	Ulcer
56	10 ±
celecoxib	53 ±
diclofenac	46 ±
zileuton	52 ±

3. Materials and methods

3.1. Chemistry

Chemicals were purchased from Sigma, Aldrich, SD-Fine, Spectrochem, Avara, and Merck. The synthesized compounds were purified by column chromatography. The reaction was monitored by thin layer chromatography with ethyl acetate: hexane as the mobile

phase and performed on Merck silica gel 60 F254 aluminum sheets (Merck, Germany). FTIR spectra were recorded on Shimadzu 8400S FTIR (Shimadzu Corporation, Japan) by KBr pellets. Elemental analyses for C, H, and N were performed on Exeter CE-440 (Hewlett-Packard, USA) elemental analyzer. ^{1}H NMR spectra were recorded at room temperature on a Bruker 500 MHz using TMS as the internal standard, and the chemical shifts were reported in δ.

3.1.1. Synthesis of diethyl 2-benzylidenemalonate ***(10-17)***[17]

The respective aldehyde **(1-8)** (5 mmol) and diethyl malonate **(9)** (5 mmol) were added in the presence of piperidine (4 mmol) under an inert atmosphere and transformed into a viscous solution. It was refluxed for 5- 6 h and quenched with 6N HCl in cold conditions. The product was extracted with ethyl acetate (3×10 mL). The combined organic extracts were dried and evaporated under reduced pressure. Compounds **(10-17)** were recrystallized from 50% aqueous ethanol and carried forward without further purification.

3.1.2.Synthesis of 1-(cyanomethyl)pyridinium Hydrogen Bromide salt ***(20)***[19]

The two-necked round bottom flask fitted with a thermometer, mechanical stirrer, and a dropping funnel was charged with Bromo acetonitrile **(19)** (14.3 mmol) to a solution of pyridine **(18)** (18.6

mmol) in tetrahydrofuran (THF). The reaction mixture was kept with vigorous stirring for overnight at room temperature. The reaction was monitored by TLC and recrystallization was carried out by with 95% ethanol. The pure compound was separated out as the white powder. The pyridinium hydrobromide salt **(20)** was further used in next step of the reaction.

3.1.3. Synthesis of ethyl 3-cyano-2-phenylindolizine-1-carboxylate ***(37-44)***[19]

Malonate derivatives **(10-17)** (500 µmol) and pyridinium hydrobromide salt **(20)** (750 µmol) were dissolved in dichloromethane (15 mL), further 1 mL of NaOH (37%) was added at room temperature. After completion, the reaction was poured into water and then extracted with the dichloromethane. The organic layer was washed with brine solution and dried over anhydrous Na_2SO_4. The reaction mixture was subjected to oxidation by Chromium trioxide/Chloranil (1 equiv.), and the solution was stirred at room temperature for 3- 4 h. The solvent was evaporated, and the residue was subjected to column chromatography (n-hexane/EtOAc). The resulting compounds **(37-44)** were dissolved in chloroform, and the insoluble precipitate was removed by filtration. After evaporation, the product was further purified by recrystallization using diethyl ether.

3.1.4. Synthesis of ethyl 3-(aminomethyl)-2-phenylindolizine-1-carboxylate ***(45-52)***[21]

A mixture of substituted nitrile **(37-44)** (9.8 mmol) and anhydrous nickel (II) chloride (9.8 mmol) was added into pre-cooled dry ethanol (20 ml) at 0-5°C. Sodium borohydride (29.4 mmol) was added slowly to the reaction mixture. The reaction mixture was stirred at 0-5°C and monitored by TLC. Water (6 ml) was added, and inorganic impurities were separated out by filtering the reaction mixture. The resulted compounds **(45-52)** were evaporated under vacuum and purified by column chromatography over silica gel using hexane: ethyl acetate as the eluent.

3.1.5. Synthesis of Substituted 3-(aminomethyl)-2-phenylindolizine-1-carboxylic acid ***(53-60)****.*

NaOH (14mmol) was added to the ethyl 3-(aminomethyl)-2-phenylindolizine-1-carboxylate **(45-52)** in THF and stirred for overnight. After completion of the reaction, THF was removed under high vacuum, diluted with water and then extracted with the ethyl acetate layer. The organic layer was washed with water and 1N HCl. The crude mixture was purified by column chromatography over silica gel (120 mesh) and eluted with 25% ethyl acetate in hexane furnished the target compounds **(53-60)**.

3.2. Molecular docking study

3.2.1 Rigid Docking using Glide-XP

The synthesized compounds and substrate were computationally docked into enzymes using Glide to gain some structural insights into the binding mode of the ligand with COX-1, COX-2, and LOX enzymes. The crystal structure of enzymes in complex with substrate retrieved from the protein data bank and used for molecular docking. Enzymes were subsequently optimized with the "protein preparation wizard" workflow. The ligand was removed, and the ligand binding site was defined. The ligands were built using Maestro 10.5.014 build panel and prepared by Lig Prep (Schrödinger, LLC, USA, 2016-1) application through OPLS 2005 force field. OPLS stands for

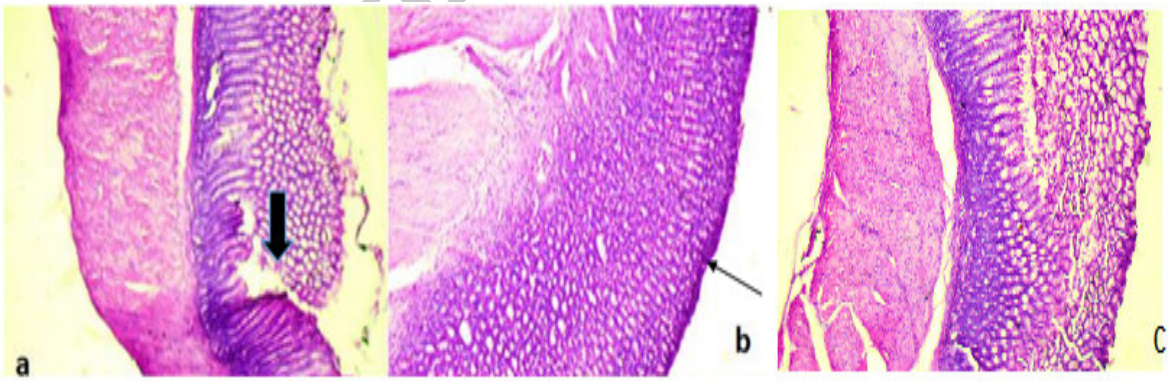

Figure 5. Photomicrographs (10x magnification) of (a) diclofenac (b) control and (c) compound **56** treated groups in rat stomach tissues (hematoxylin and eosin staining). The arrow indicates severe detachment of surface epithelium, resulting in the formation of lesions.

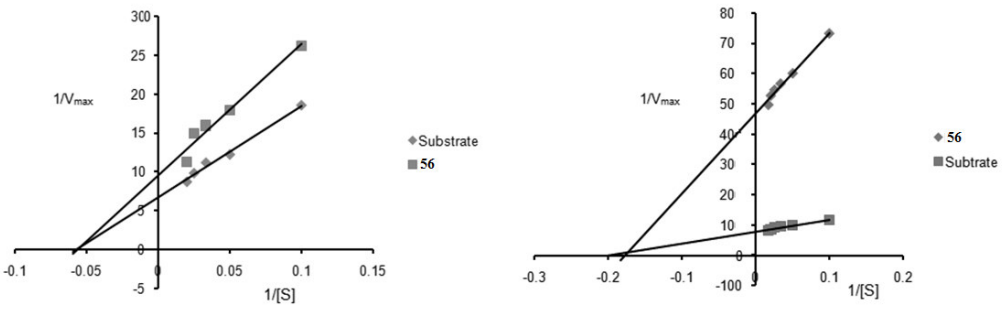

Figure 6. Lineweaver-Burk plot of *in vitro* COX-2 inhibition by compound **56** and *in vitro* LOX inhibition by compound **56**.

optimized potential liquid simulations, and it gave the corresponding energy minima 25 conformers of the ligands. The default settings were used for all other parameters. The extra precision (GLIDE-XP) protocol implemented in GLIDE was used for docking (Glide 5.8, Schrödinger Inc., USA).

3.2.2. Induced Fit Docking

In IFD, softened-potential docking into a rigid receptor was performed to generate groups of poses, and top 20 poses were retained. Further, the ligand was re-docked into low energy induced-fit structures obtained using Prime program in Schrödinger software suite, and then IFD scoring was calculated (IFD score = GScore + 0.05 * Prime_Energy) that accounts for both docking energy (GScore), and receptor strain and solvation terms (Prime_energy).[28]

3.3. Pharmacological screening

3.3.1. Experimental animals

Healthy, adult, Swiss albino mice of either sex having weight 28 to 32 g or Wistar rats of either sex weighing 180-220 g were used for the experimental protocols and procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University. The present biological study was approved by Banaras Hindu University Animal Ethical Committee (BHU; Dean/12-13/CAEC/19). The entire experimental methods were in agreement with CPCSEA guidelines, Ministry of Environment and Forests, Government of India. The animals were adapted to guarded laboratory surroundings (12 h light/dark cycle, 22-24^{o}C at 40-60% R_H) and were allowed to access water and food *ad libitum.*

3.3.2 Carrageenan-induced paw edema (acute inflammatory model)[35]

Anti-inflammatory activity of synthesized compounds was screened using carrageenan-induced rat paw edema model. Paw edema was induced by administration of 0.1 ml of a 1% w/v solution carrageenan into the sub-planter region in the left hind paw of the rats. Briefly, all the animals were randomly divided into various groups and treated with standard diclofenac sodium (10 mg/kg, *p.o.);* test compounds (**53-60**) (dose equimolar relative to 10 mg/kg diclofenac sodium) and the vehicle as a control was administered orally. The paw size was measured in mL using Plethysmometer after two, four and six hour of the intoxication of carrageenan injection. The subjects were pretreated with the selected derivatives and standard drug 1 h before the administration of carrageenan.

3.3.3 Arachidonic acid induced rat paw edema[36]

Paw edema was induced by injecting 0.1 ml of 0.5% w/v AA in 0.2 M carbonate buffer, pH 8.2 via sub-plantar region into right hind paw of rats after 30 min post drug treatment. The 0.3% carboxymethylcellulose sodium (CMC) was used as control (10 ml/kg oral), and the test compounds were given at an equimolar oral dose relative to standard 10 mg/kg zileuton. Changes in thickness (mm) were measured by a digital Vernier caliper 1 h after the AA injection.

3.3.4 Ulcerogenic potential[37]

Ulcerogenic studies were performed on fasting animal via administering test compounds for 7 successive days and was forfeited with cervical dislocation. The incision was done on the peritoneal cavity; stomach was separated and place in a saline plate. A longitudinal incision along with greater curvature was made, and the stomach was cleaned by washing with cooled saline and inspected with a 3X magnifying lens for any evidence of ulcer. Lesions area was counted, and ulcer index was calculated as per Szelenyl and Thiemer.[38] Further, histological studies were carried out (embedded in paraffin blocks, fixed in 10% v/v formalin and stained with hematoxylin and eosin).

3.4. In vitro inhibitory activity

3.4.1. In vitro COX inhibition assays[31]

The COX activity of the tested compounds to inhibit both COX-1 and COX-2 isozymes was evaluated using colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman Chemicals; Item no. 560131). Different dilutions of celecoxib and tested compounds were incubated with the enzymes for 5 min at 25°C. After the incubation, an addition of the colorimetric substrate and AA was done, and the absorbance was measured at 412 nm using plate reader.

3.4.2. Inhibition of LOX

Soybean lipoxygenase (linoleate 13S-lipoxygenase) is frequently used as a dependable screen for LOX inhibition. It has been studied that AA binding sites in soybean lipoxygenases contribute to almost the similar resemblance with animal LOX. It has been reported that amino acid in the binding sites of human LOX shares almost the same similarity with AA binding sites in soybean lipoxygenases. The assay was performed according to Continuous Spectrophotometric Rate Determination method. The stock solution of lipoxygenase was prepared by dissolving 5,000 - 10,000 units/ml of Lipoxidase in 200 mM Borate Buffer, pH 9.0. Six different concentrations of inhibitors were selected and made for determining the enzyme inhibition activity. Increasing concentrations of inhibitors were added to enzyme solution and kept for 5 min followed by substrate addition and further kept at room temperature with stirring for 20 mins. The control contains the only vehicle resulted in 100% enzymatic reaction. Blank contains all the substances except the enzyme solution to account for non-enzymatic reaction. The reaction rates were compared, and the percentage inhibition due to increasing concentration of inhibitors was calculated. The concentration of each test compound was recorded in triplicate, and their IC_{50} values were determined graphically from absorption v/s concentration curve.

3.5. In vitro kinetic study[39]

Continuous Spectrophotometric rate determination method was used to identify the type of inhibition. The substrate (linoleic acid and AA) was used in different concentrations and prepared in tween-20 in 200 mM borate buffer, and pH 9 was maintained. And a fixed concentration of enzyme i.e. 10,000 units/ml of Lipoxidase was prepared in 200 mM borate buffer at pH 9.0 in the presence and absence of most active compound **56** (concluded from IC_{50} determination). The inhibitory kinetics was evaluated by Lineweaver and Burk method.

4. Conclusion

The biological activity with respect to the synthesized indolizine derivative inferred that substitution pattern at position 8 of indolizine ring proved to be an active site for determining the anti-inflammatory activity. The result indicated that substitution of the aromatic ring system at position 8th (**56-60**) resulted in more active compound than substituted aliphatic group (**53-55**). Compounds at position 8 with the electronegative halogen groups like CF_3, fluorine and hydroxyl groups (**57-60**) showed less mean protection to inflammation, in comparison with the other compound (**53-55**).The best compound **56** showed a safer gastric profile than diclofenac, celecoxib, and zileuton as indicated by its low ulcerogenic indices and the histopathological studies. The molecular mechanism of the best compounds **56** was investigated for COX-1/-2 enzymes and LOX enzyme inhibition. The compound **56** was found dual nature of inhibition against both COX-2 and LOX enzymes. The *in vitro* kinetic study of **56** showed a non-competitive type of inhibition on both LOX and COX-2 enzymes. In conclusion, considering the *in vivo*, *in silico*, and *in vitro* results, it can be suggested that the compound **56** is a promising dual COX-2 and LOX inhibitor, deserves further studies which can lead to a discovery of a new lead a potent anti-inflammatory property.

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Supplementary Material

Detailed experimental procedures with analytical data for compounds including FT-IR, ^{1}H NMR, and ^{13}C NMR. (PDF)

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