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Research Article

Anti-Inflammatory Potentials of β-Ketoester Derivatives of N-Ary Succinimides: *In Vitro*, *In Vivo*, and Molecular Docking Studies

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Inflammation, being a well-known and complex pathological condition, is always a challenge to the human health. This research work was designed for a rationale-based anti-inflammatory study on β-ketoester derivatives of N-ary succinimides. The compounds (**A**–**D**) were synthesized by organocatalytic Michael addition. The compounds were initially screened for *in vitro* 5-lipoxygenase (5-LOX) and cyclooxygenase (COX-2) assays. For the *in vivo* activity, carrageenan-induced paw edema and arachidonic acid-induced ear edema tests were used. Furthermore, different *in vivo* pathways such as prostaglandins E_2, histamine, leukotriene, and bradykinin were studied. The results were supported with molecular docking studies. Among the compounds, **D** (ethyl 1-(1-benzyl-2,5-dioxopyrrolidin-3-yl)-2-oxocyclohexane-1-carboxylate) at a concentration of 1000 μg/ml showed significant inhibitory effects of 83.67% and 78.12% against COX-2 and 5-LOX in comparison to celecoxib and zileuton, respectively. Similarly, compound **D** also showed excellent *in vivo* anti-inflammatory potential. Amongst all the compounds, **D** demonstrated excellent (55.92 ± 2.95%) anti-inflammatory potential at maximum tested dose (100 mg/kg) which accomplished the highest significance at 4 h following the carrageenan insertion and stayed considerable ($^{***}P < 0.001$) till the 5th hour of test sample injection. Compound **D** also exhibited excellent percent inhibition (63.81 ± 2.24%) at the highest dose in arachidonic acid-induced ear inflammation. On the basis of *in vivo* and *in vitro* results, compound **D** was subjected to various inflammation-causing agents such as histamine, prostaglandins E_2, bradykinin, and leukotriene via the mouse paw edema test. Compound **D** revealed moderate effect (28.10 ± 1.64%) against histamine-induced paw edema while nonsignificant result (9.72 ± 3.125%) was marked for the bradykinin pathway. Compound **D** showed significance against edematogenic consequence of prostaglandin E_2 (56.28–72.03%) and leukotriene (55.13 ± 2.25%) induced inflammation. In summary, our findings recommended that compound **D** possesses double acting anti-inflammatory properties inhibiting both COX and LOX pathways. Binding orientations and energy values computed via docking simulations support the results of the experimental *in vitro* evaluation.

1. Introduction

Inflammation, a body's protective response to tissue injury, has been known from the era of ancient civilizations. In the early Egypt and Greek era, barks of trees have been used for the management of inflammation and its associated pain [1]. In the early anti-inflammatory drug research, salicin was identified as a major active drug [2]. In the later discovery of anti-inflammatory drugs, the pharmacologically active form of salicin was synthesized from phenol [3] which was further

modified to the more potent and palatable derivative acetylsalicylic acid [4], a well-known anti-inflammatory drug of the era. After this initial commercialization of aspirin (acetylsalicylic acid) by Bayer, several nonsteroidal anti-inflammatory drugs (NSAIDs) were discovered and marketed [5–7].

Arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, is present within the body cells [8]. This is attached covalently as an ester in the body cell membrane. During the inflammation, arachidonic acid is released to the site and is further metabolized by vital enzymes cyclooxygenase (COX) and lipoxygenase (LOX) which form eicosanoids (signaling mediators) [9]. The prostaglandins are produced through the cyclooxygenase pathway while leukotrienes via the lipoxygenase pathway [10, 11]. Both of these are responsible for inflammatory properties. Therefore, the inhibitions of cyclooxygenase and lipoxygenase pathways are the major biochemical targets for the anti-inflammatory properties to evaluate a new drug molecule [12]. Many drugs with potent inhibition properties of COX/LOX pathways are commercially available. However, because of the accompanied unwanted effects with current drugs, the medicinal chemists are in constant search of new COX and LOX inhibitors. In addition, the research of novel COX and LOX inhibitors is more attractive in this period also because besides their use as anti-inflammatory agents, they could be explored in many other diseases such as neuroinflammation and cancer [13–16].

The organic and medicinal chemists are in constant search for new molecules from natural and synthetic sources for analgesia and inflammation [17–19]. Synthetic nitrogenous and other heterocyclic compounds have vital importance in drug design and discovery [20–22]. These compounds are either potential for different pharmacological targets or contribute as vital building blocks in the drug design [23]. Succinimides, compounds with 5-membered nitrogenous ring, possess a well-known pharmacological history. We have previously synthesized various aldehyde [24], ketone [25], ketoesters [26], and cyanoacetate [27] analogues of succinimides for different chemical and biological purposes. In our recent publications, we tested the potential effectiveness of the synthesized succinimide derivatives in the treatment of inflammation. In this study, we have employed β-ketoester derivatives of N-ary succinimide for mechanistic anti-inflammatory studies.

The basic rationale for our designed work is based on the commercially available drugs, literature, and molecular docking approaches (Figure 1). By comparing the structures of marketed drugs for COX-2 (e.g., celecoxib or rofecoxib), we found our designed compounds are more suitable to be evaluated as inhibitors of the inflammatory pathway. Both marketed drugs in Figure 1 contain five-membered ring in their structures, like our compound (D, ethyl 1-(1-benzyl-2,5-dioxopyrrolidin-3-yl)-2-oxocyclohexane-1-carboxylate). Rofecoxib contains carbonyl group at the five-membered ring in a similar position as in our compound. Similarly, in case of celecoxib, the position of CF_3 moiety is on the same side as the benzyl group in our selected compound. Moreover, both these drugs contain vicinal substituents/aromatic moieties attached to the five-membered ring. Our designed compounds also have the 3D positioning for binding with the specific protein for the onset of the pharmacological action.

2. Materials and Methods

2.1. Synthesis of Compounds. For the synthesis of β-ketoester derivatives of N-ary succinimide, 1 M dichloromethane was added in a reaction vial. Two (2.0) mmol of β-ketoesters, such as ethyl 2-oxocyclohexanecarboxylate or ethyl 2-oxocyclopentanecarboxylate, was added to the vial. Afterwards, 8-hydroxyquinoline (20 mol %) or creatinine and potassium hydroxide (20 mol %) were added as organocatalyst. Stir the reaction for 3 or 4 minutes, and then add 1 mmol of N-phenyl or N-benzyl maleimide to it. The reaction progress was monitored via thin-layer chromatography (TLC). After completion of the reaction, the reaction mixture was diluted with 15 ml of distilled water and then added in a separated funnel to separate the DCM layer. The same was repeated by addition of dichloromethane (3×15 ml). The organic layers were combined and dried by using anhydrous sodium sulphate (Na_2SO_4), filtered, and concentrated with the help of rotary evaporator at reduced pressure. By using column chromatography, the crude mixture was purified [26].

3. Evaluation of Anti-Inflammatory Activity

3.1. In Vitro Enzymatic Anti-Inflammatory Activity

3.1.1. 5-Lipoxygenase Inhibitory Activity (5-LOX). The 5-lipoxygenase inhibitory assay was determined using the following reported procedure [28]. In short, synthesized compounds were prepared in different concentrations ranging from 1000 to 62.5 μg/ml. After that, enzyme solution was primed having a concentration of 10,000 U/ml. A solution of linoleic acid 80 mM (substrate) was also primed. Likewise, phosphate buffer (50 mM) was primed having pH 6.3. The total mixture of reaction containing a volume of 2 ml was primed having equivalent enzyme volume; substrate volume and buffer were thoroughly mixed. Various dilutions of the tested compounds and standard drug were (0.2 ml) further added to the reaction mixture. The reaction rate was determined for tested compounds and negative control. The enzyme activity (percent) was determined through raise in absorbance at 234 nm by using a UV-visible spectrophotometer. The percent inhibition was considered via assessing rise in the absorbance of the tested compounds by comparing it with negative control. In this assay, zileuton was used as the standard drug, i.e., positive control.

3.1.2. Cyclooxygenase-2 Inhibitory Activity (COX-2). The COX-2 inhibitory activity was carried out as per the reported method [29]. The solution of COX-2 enzyme was prepared (300 U/ml). For activation, 10 μl of enzyme was kept in a refrigerator for 5 min along with cofactor solution (50 μl) having N, N, N, N-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD 0.24 mM) and glutathione (0.9 mM) along with 1 mM hematin in 0.1 M Tris HCl buffer having

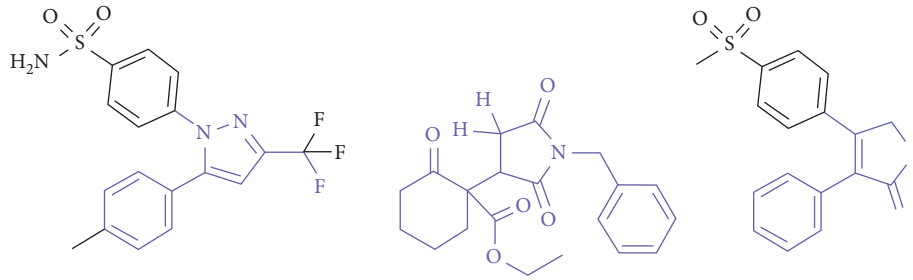

Figure 1: Rationale of the COX-2 inhibitors celecoxib and rofecoxib with our compounds (compound **D**).

pH 8. Then, 60 μl of enzyme solution and 20 μl of the tested compounds having different dilutions be reserved at 25°C for 5-6 minutes. Likewise, the reaction began by the addition of 30 mM arachidonic acid (20 μl). After 5-6 minutes of incubation, absorbance was measured at 570 nm with the help of a UV-visible spectrophotometer. Inhibitory effect of COX-2 was deliberated from that of the absorbance value per unit time. In this assay, celecoxib was used as the standard drug, i.e., positive control.

3.2. In Vivo Anti-Inflammatory Activity

3.2.1. Experimental Animal. Swiss albino mice having an average weight of 25–30 g of both sexes were used. Animals were acquired from Pharmacology Section, NIH (National Institute of Health), Islamabad, Pakistan. The mice will be retained in appropriate cages and maintained at standard laboratory circumstances of 25 ± 2°C plus relative humidity 50 ± 5% with light/dark cycle (12/12 hours), and they were allowed to access food and water ad libitum in the acclimatization stage. The experimental trials were permitted by the Ethical Committee of the Department of Pharmacy, University of Malakand, KPK, Pakistan [12].

3.2.2. Chemical/Drug and Their Solubility. In the current study, we employed COX-2, 5-LOX, carrageenan, histamine, arachidonic acid, bradykinin acetate, leukotriene, prostaglandin, and HOE 140 purchased from Sigma-Aldrich (USA), while chlorpheniramine maleate, acetylsalicylic acid, and celecoxib were from Libra Pharmaceutical (Pvt.) Ltd., Peshawar Industrial Estate Hayatabad, Pakistan. The bradykinins and leukotrienes stock solutions were primed with ethanol (70%) and were extra diluted with ethanol (0.1%). For arachidonic acid dissolution, the carbonated buffer was used having Na_2CO_3, 0.2 M, pH 8.5. The synthesized compounds were primed as 25, 50, and 100 mg in 20 ml of 10% DMSO. All ingredients used were of analytical grade, and normal saline solution (0.9%) was used for its dissolution.

3.2.3. Acute Toxicity. Albino mice were arranged in control and test groups with each group having 8 tested mice. The synthesized compound was given via oral route at different doses ranging from 250 to 2000 mg/kg. Tween-80 solvent was used for dose preparations. After getting the doses, allergic symptoms and abnormal behaviors were observed for 72 hours [12].

3.3. Carrageenan-Induced Anti-Inflammatory Activity. The preliminary anti-inflammatory activity of the compound was evaluated on mice of both sexes weighing 25–30 g. Forty (40) mice were arbitrarily divided into 5 groups (Groups I, II, III, IV, and V) with each group having 8 mice [30]. Group I was taken as the negative control group and was administered 10 ml/kg of 1% DMSO. Group II was taken as the positive control group and given 100 mg/kg acetylsalicylic acid. Groups III, IV, and V received various doses of the tested compound **D** (25, 50, and 100 mg/kg), respectively. After half an hour, freshly primed carrageenan saline suspension (0.05 ml of 1% w/v) was given in the subplantar surface of the right hind paw of the mice. Inflammation was noted instantly via a plethysmometer after the insertion of carrageenan at 60 minutes gap for 5 h. Paw volumes of the animals treated with the standard drug as well as compound **D** were noted time by time and were evaluated with the negative control group. The percent activity was calculated with the following equation:

$$\%\ \text{Inhibition} = \frac{\left(C_g - V_{tg}\right)}{C_g} \times 100, \tag{1}$$

where C_g = average inflammation of the control group and V_{tg} = volume of paws in the tested group.

3.3.1. Arachidonic Acid-Induced Ear Mouse Edema. In this assay, the protocol described by Romay et al. was followed [31]. The synthesized compound in various doses (25, 50, and 100 mg/kg) was applied topically simultaneously with AA (1 mg/ear). Nimesulide (2 mg/ear) was given to the reference group. The animals were killed by cervical dislocation after 1 hour, and then, 6 mm diameter discs were taken from each ear and the weight was determined. Differences in weight between the punches from the right and left ears were taken for measurement of swelling occurred and expressed as an increase in ear thickness.

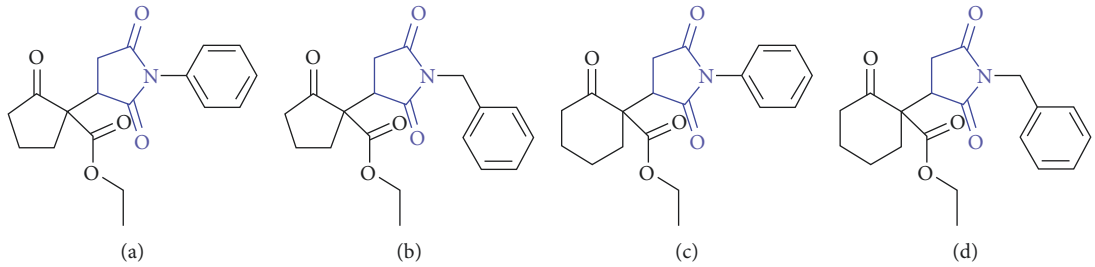

Figure 2: Structures of the synthesized succinimide derivatives.

3.3.2. Anti-Inflammatory Mechanism of the Tested Compound. The anti-inflammatory mechanism was confirmed with various mediators [32]. The experimental mice of both sexes were erratically alienated into different groups. The animals were administered an intraperitoneal injection of 0.9% saline or 1% DMSO or antihistaminic drug such as chlorpheniramine maleate at 25 mg/kg or 10 mg/kg montelukast (leukotriene inhibitor) or 100 mg/kg aspirin or 100 mg/kg of compound **D**. Subsequent to half an hour of the above administration, inflammation was induced in the right hind paw of each mice by subplantar injection of 0.1 ml histamine (1 mg/ml) or leukotriene (10 μg/ml) or prostaglandin E_2 (0.01 μg/ml) or bradykinin (20 μg/ml). Paw volume was calculated prior to and after the subplantar receiving of various irritants (inflammatory mediators) at 1st, 2nd, 3rd, 4th, and 5th hour.

3.3.3. Computational Studies. By using Molecular Operating Environment (MOE 2016.08), the docking studies were carried out [33]. From Protein Data Bank (PDB code 1CX2), the COX-2 structure in complex with SC-558 was downloaded. Preparation of ligands, i.e., 3D protonation, energy minimization, and determination of binding site, was carried out by our previously reported methods. Lowest binding energy ligand enzyme complexes were analyzed by using the MOE ligand interaction module.

4. Results and Discussion

4.1. Succinimide Derivatives/Compounds. The structures of the four compounds (**A**–**D**) are shown in Figure 2. In our previous study, we have synthesized the four compounds and have evaluated them for its anti-Alzheimer studies [26]. Herein, we have resynthesized all the four compounds by using our previous method.

4.2. Cyclooxygenase (COX-2) Inhibitory Assay. In the COX-2 inhibitory assay, all the compounds showed good activity against COX-2, as shown in Table 1. Comparing all the synthesized compounds, the compound **D** showed excellent activity causing 81.70 ± 0.67 μg/ml % inhibition at 1000 μg. The compounds **A**, **B**, and **C** showed 54.43 ± 1.50, 57.39 ± 0.49, and 49.76 ± 0.61 μg/ml inhibitions, respectively, at highest concentration. The IC_{50} values of the compounds **A**, **B,** and **C** were 582, 475, and 1170 μg/ml, respectively. The IC_{50} of compound **D** was 43 μg/ml which is half-active with respect to the standard drug celecoxib IC_{50} value (22 μg/ml).

4.3. 5-LOX Results of the Compounds. In this assay, the inhibitory effect of compound **D** was high as compared to other compounds. Compound **D** caused 72.50 ± 1.04% inhibition at the highest concentration with IC_{50} 120 μg/ml, while the standard drug zileuton exhibited 77.00 ± 0.16 μg/ml at highest concentration with IC_{50} 52 μg/ml. The other compounds **A**, **B**, and **C** exhibited 49.66 ± 0.88, 57.00 ± 1.52, and 44.33 ± 1.76% inhibitions at the maximal concentration (1000 μg/ml). These compounds were less active against the 5-lipooxygenase enzyme. The percent inhibitions of the synthesized compounds against standard drug zileuton with IC_{50} values are shown in Table 2.

4.4. Carrageenan-Induced Paw Edema. The *in vivo* anti-inflammatory activities of all the synthesized compounds were encouraging in carrageenan-induced inflammation. The compounds exhibited dose-dependent significant anti-inflammatory potentials. Compound **D** at doses of 25, 50, and 100 mg/kg showed maximal anti-inflammatory potentials. Compound **D** showed excellent (55.92 ± 2.95%) anti-inflammatory activity at a maximal dose of 100 mg/kg that achieved the upper value at 4 h following carrageenan insertion and remained significant ($^{***}P < 0.001$) up to the 5th hour of tested drug administration, which is shown in Table 3. Aspirin standard drug exhibited a significant effect (57.64 ± 1.54%, $^{***}P < 0.001$) at the dose of 100 mg/kg at 5 h which was almost a comparable effect to that which was produced by compound **D** at the dose of 100 mg/kg. All the other compounds also exhibited moderate activity as shown in Table 3.

4.5. Arachidonic Acid-Induced Ear Inflammation. Synthesized compounds were screened for the arachidonic acid ear anti-inflammatory activity. Compound **D** exhibited marked anti-inflammatory activity. Percent inhibition of inflammation in case of compound **D** was found to be dose dependent. The edema induced by arachidonic acid (AA) at the highest dose (100 mg/kg) displayed excellent activity (63.81 ± 2.24%) and comparable to standard drug nimesulide. The standard drug nimesulide (50 mg/kg) inhibited the edema (67.41 ± 3.04%). Similarly, tested compound B also exhibited good activity and reached the maximal value after 1 h ($^{***}P < 0.001$) after administration of arachidonic acid. Compound **A** showed moderate activity after 15 minutes (39.15 ± 1.16) and then decreased. Tested compound **C** was less active against the arachidonic acid-induced inflammation causing percent inhibition (29.33 ± 3.18) after 30 minutes. The

Table 1: COX-2 inhibitory potentials of the tested compounds (A–D).

Compounds	Concentration (μg/ml)	%Inhibition (mean ± SEM)	IC50 (μg/ml)
A	1000	$54.43 \pm 1.50^{***}$	582
	500	$47.97 \pm 1.25^{***}$	
	250	$41.87 \pm 0.65^{***}$	
	125	$33.17 \pm 0.53^{***}$	
	62.5	$24.17 \pm 0.66^{***}$	
	31.25	$15.60 \pm 2.03^{***}$	
B	1000	$57.39 \pm 0.49^{***}$	475
	500	$51.00 \pm 0.00^{***}$	
	250	$41.33 \pm 0.33^{***}$	
	125	$34.33 \pm 0.66^{***}$	
	62.5	$22.17 \pm 0.53^{***}$	
	31.25	$11.48 \pm 0.23^{***}$	
C	1000	$49.76 \pm 0.61^{***}$	1170
	500	$41.13 \pm 0.80^{***}$	
	250	$34.48 \pm 0.23^{***}$	
	125	$27.67 \pm 0.94^{***}$	
	62.5	$21.37 \pm 0.56^{***}$	
	31.25	$17.05 \pm 0.13^{***}$	
D	1000	$81.70 \pm 0.67^{**}$	43
	500	$74.90 \pm 0.57^{**}$	
	250	$65.73 \pm 0.84^{***}$	
	125	$59.77 \pm 0.82^{***}$	
	62.5	$52.13 \pm 0.70^{***}$	
	31.25	$47.90 \pm 0.78^{***}$	
Celecoxib	1000	85.47 ± 0.59	22
	500	78.60 ± 0.73	
	250	73.32 ± 0.68	
	125	65.42 ± 0.46	
	62.5	59.17 ± 0.66	
	31.25	53.37 ± 0.72	

results of the synthesized compounds and standard drug nimesulide are shown in Table 4.

4.6. Possible Anti-Inflammatory Mechanism. Compound **D**, i.e., ethyl 1-(1-benzyl-2,5-dioxopyrrolidin-3-yl)-2-oxocyclohexane-1-carboxylate, was proved to be the potent among all based on the *in vitro* and *in vivo* experiments. Based on the potency of compound **D**, we further employed this compound for mechanistic studies. We used different mediators of inflammation.

4.7. Compound Effect of Histamine-Induced Inflammation. For the promising anti-inflammatory mechanistic approach, different phlogistic agents were used which include histamine, bradykinin, prostaglandin, and leukotriene. Compound D was the most active compound among the other in both carrageenan-induced paw and arachidonic acid-induced ear inflammation. Different phlogistic agents were used which include histamine, bradykinin, prostaglandin, and leukotriene. Compound **D** exhibited a mild antihistaminic effect (28.10 ± 1.64) that was observed at the highest dose (100 mg/kg) at the 2nd hour of the administration of histamine, which may be due to **D** inhibitory effect on the release of mediators from the mast cells. Furthermore, the standard drug chlorpheniramine maleate, which is an antihistamine drug, has significantly inhibited the edema observed (71.64 ± 2.16) at 1 hr induced by histamine. The antihistaminic effect of the standard drug (chlorpheniramine maleate) and compound **D** is shown in Figure 3.

4.8. The Compound Effect of Bradykinin-Induced Inflammation. After the injection of bradykinin, there was production of edema in mice paw which acquired its maximum limit after 60 min of bradykinin injection. Compound **D** was not active against the edema of mouse paw. At the highest doses (100 mg/kg), compound **D** inhibited paw swelling, 9.72 ± 3.125, 6.94 ± 2.434, 7.85 ± 2.470, 1.16 ± 1.08, and 4.29 ± 2.003 at 1, 2, 3, 4, and 5^{th} hour after the injection of bradykinin, respectively, which is not significant to that of the standard drug (HOE 140). By using Student's *t*-test, the data were analyzed. The result of compound **D** of bradykinin-induced inflammation is shown in Figure 4.

4.9. The Compound Effect of Prostaglandin E_2 (PGE2) Inflammation. The prostaglandin E_2 level increased significantly in the paw tissue after injection of prostaglandin E_2 mediator, as shown in Figure 5. The inflammation induced by phlogistic agent PGE2 was significantly

Table 2: 5-LOX inhibitory potentials of the compounds.

Compounds	Concentration (μg/ml)	%Inhibition (mean ± SEM)	IC50 (μg/ml)
A	1000	$49.66 \pm 0.88^{***}$	930
	500	$45.50 \pm 0.44^{***}$	
	250	$41.00 \pm 2.64^{***}$	
	125	$37.00 \pm 0.00^{***}$	
	62.5	$31.33 \pm 0.33^{***}$	
	31.25	$24.33 \pm 0.66^{***}$	
B	1000	$57.00 \pm 1.52^{***}$	420
	500	$52.66 \pm 1.20^{***}$	
	250	$45.00 \pm 0.16^{***}$	
	125	$39.16 \pm 1.01^{***}$	
	62.5	$32.83 \pm 0.92^{***}$	
	31.25	$25.16 \pm 0.44^{***}$	
C	1000	$44.33 \pm 1.76^{***}$	>1000
	500	$35.16 \pm 1.01^{***}$	
	250	$29.66 \pm 0.88^{***}$	
	125	$22.50 \pm 0.28^{***}$	
	62.5	$11.00 \pm 0.00^{***}$	
	31.25	$05.16 \pm 0.60^{***}$	
D	1000	$72.50 \pm 1.04^{**}$	120
	500	$65.58 \pm 0.69^{***}$	
	250	$56.37 \pm 0.65^{***}$	
	125	$49.44 \pm 0.58^{***}$	
	62.5	$42.16 \pm 0.60^{***}$	
	31.25	$37.37 \pm 0.54^{***}$	
Zileuton	1000	77.00 ± 0.16	52
	500	71.66 ± 1.20	
	250	65.33 ± 0.33	
	125	57.50 ± 0.44	
	62.5	51.00 ± 0.57	
	31.25	44.66 ± 0.88	

Table 3: Percentage inhibitions of compounds in the carrageenan-induced paw edema.

Treatment	Dose (mg/kg)	Percent inhibition of paw edema				
		1 h	2 h	3 h	4 h	5 h
Vehicle	—	7.662 ± 3.75	7.392 ± 2.48	11.41 ± 3.07	14.45 ± 1.57	7.307 ± 3.20
Aspirin	100	$47.54 \pm 4.12^{***}$	$54.63 \pm 1.47^{***}$	$53.26 \pm 1.36^{***}$	$56.92 \pm 2.95^{***}$	$57.64 \pm 1.55^{***}$
A	25	14.70 ± 2.65^{ns}	$22.44 \pm 1.54^{**}$	$24.56 \pm 2.35^{**}$	$28.39 \pm 2.48^{**}$	$26.25 \pm 2.63^{***}$
	50	$20.35 \pm 2.21^{***}$	$24.54 \pm 4.59^{***}$	$26.58 \pm 2.59^{***}$	$29.78 \pm 2.60^{***}$	$27.66 \pm 3.75^{***}$
	100	$31.29 \pm 3.08^{***}$	$31.36 \pm 2.69^{***}$	$28.72 \pm 1.69^{***}$	$37.01 \pm 2.97^{***}$	$33.55 \pm 2.44^{***}$
B	25	$34.68 \pm 4.79^{***}$	$36.35 \pm 3.13^{***}$	$22.55 \pm 2.44^{*}$	$33.84 \pm 2.56^{***}$	$33.55 \pm 2.44^{***}$
	50	$37.45 \pm 2.56^{***}$	$37.68 \pm 3.28^{***}$	$24.70 \pm 1.90^{***}$	$32.32 \pm 2.52^{***}$	$34.57 \pm 2.34^{***}$
	100	41.33 ± 3.27^{ns}	$41.86 \pm 1.19^{**}$	$36.78 \pm 2.60^{***}$	$40.30 \pm 1.18^{***}$	$43.27 \pm 2.57^{***}$
C	25	7.07 ± 2.33^{ns}	17.85 ± 2.23^{ns}	19.19 ± 2.23^{ns}	$28.39 \pm 3.36^{**}$	$26.11 \pm 3.58^{***}$
	50	$20.11 \pm 3.58^{***}$	$21.92 \pm 4.38^{***}$	$24.97 \pm 3.61^{***}$	$31.30 \pm 3.20^{***}$	$27.66 \pm 3.75^{***}$
	100	$29.26 \pm 1.36^{***}$	$36.88 \pm 1.56^{***}$	$34.09 \pm 1.24^{***}$	$35.74 \pm 2.60^{***}$	$33.55 \pm 2.44^{***}$
D	25	$42.85 \pm 3.27^{***}$	$43.83 \pm 2.79^{***}$	$39.87 \pm 3.24^{***}$	$39.04 \pm 3.28^{***}$	$38.80 \pm 1.85^{***}$
	50	$44.11 \pm 3.58^{**}$	$45.28 \pm 2.80^{***}$	$41.75 \pm 3.76^{***}$	$42.84 \pm 2.93^{***}$	$40.07 \pm 2.33^{***}$
	100	$45.77 \pm 2.69^{***}$	$51.05 \pm 3.02^{***}$	$54.36 \pm 2.59^{***}$	$55.92 \pm 2.95^{***}$	$49.25 \pm 2.53^{***}$

Percent inhibition produced by synthesized compounds (25, 50, and 100 mg/kg) of the carrageenan-induced paw edema model in mice. Each percentage point represents the mean ± SEM for a group of 8 mice. Data were analyzed by two-way repeated measures ANOVA followed by Bonferroni's post hoc test. Asterisks show significant values from control (vehicle). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n.s, nonsignificant; $n = 8$ mice per group.

reduced after the injection of compound D which was almost similar to the effect of standard drug celecoxib. The effect of **D** acquired maximal level at the 4th hour (68.03 ± 3.24) which is comparable to the standard drug and remains significant ($^{***}P < 0.001$) till the 5th hour.

4.10. The Compound Effect of Leukotriene Inflammation. Inflammation caused and mouse paws became edematous after the administration of leukotriene and swelling occurred at a maximal level after 30 min of leukotriene administration. Compound **D** became significantly active against the

Table 4: Percentage inhibition of compounds in the arachidonic acid-induced ear edema.

Treatment	Dose (mg/kg)	Percent inhibition of ear edema				
		10 min	15 min	30 min	45 min	60 min
Vehicle	—	8.75 ± 0.56	8.62 ± 1.79	13.33 ± 2.19	16.21 ± 2.86	8.66 ± 3.18
Nimesulide	50	58.66 ± 4.63***	60.33 ± 2.33***	63.67 ± 1.76***	66.00 ± 2.52***	67.41 ± 3.04***
A	25	30.54 ± 2.34***	33.18 ± 3.87***	30.47 ± 3.01***	26.39 ± 2.86*	19.51 ± 2.83*
	50	34.04 ± 4.59***	36.33 ± 2.91***	34.72 ± 2.48***	29.57 ± 2.31**	22.81 ± 2.51**
	100	37.22 ± 1.58***	39.15 ± 1.16***	37.93 ± 1.16***	34.54 ± 1.27***	25.25 ± 3.89***
B	25	33.70 ± 3.35***	34.67 ± 2.27***	37.53 ± 1.56***	28.43 ± 1.48**	26.76 ± 1.86***
	50	39.92 ± 2.87***	38.84 ± 1.98***	41.03 ± 2.88***	0.58 ± 2.54**	29.57 ± 2.31***
	100	43.25 ± 3.09***	45.15 ± 1.67***	48.36 ± 2.87***	36.02 ± 3.54***	34.54 ± 0.64***
C	25	18.57 ± 2.54ns	21.85 ± 4.05**	22.25 ± 2.90 ns	20.88 ± 2.45 ns	14.95 ± 1.95 ns
	50	21.66 ± 3.12**	23.36 ± 2.64**	25.55 ± 1.55**	23.33 ± 1.20 ns	21.73 ± 2.37**
	100	25.69 ± 1.80***	27.40 ± 3.00***	29.33 ± 3.18***	26.02 ± 3.89 ns	22.88 ± 2.24**
D	25	46.44 ± 2.74***	51.07 ± 2.91***	53.14 ± 1.03***	52.33 ± 1.85***	55.69 ± 2.25***
	50	51.63 ± 0.52 ***	54.32 ± 3.61***	57.45 ± 2.56***	55.77 ± 2.88***	61.25 ± 1.78***
	100	54.85 ± 2.18***	57.23 ± 1.75***	60.11 ± 4.79***	58.14 ± 2.92***	63.81 ± 2.24***

Percent inhibition produced by synthesized compounds (25, 50, and 100 mg/kg) of the arachidonic acid-induced ear edema model in mice. Each percentage point embodies the mean ± SEM for a group of 8 mice. Data were analyzed by two-way repeated measures ANOVA pursued by Bonferroni's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n.s, nonsignificant; $n = 8$ mice per group.

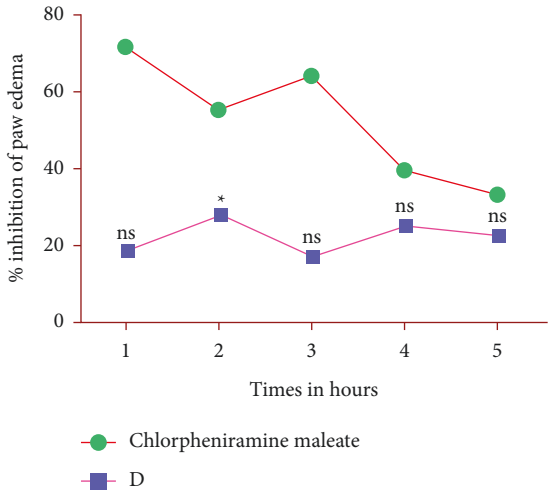

Figure 3: Percent inhibition produced by compound **D** (100 mg/kg) and chlorpheniramine maleate (25 mg/kg) in the histamine-induced paw edema model in mice. Each percent point represents the mean ± SEM for a group of 8 mice. Data were analyzed by Student's *t*-test. Asterisks show significant values. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; n.s, not significant.

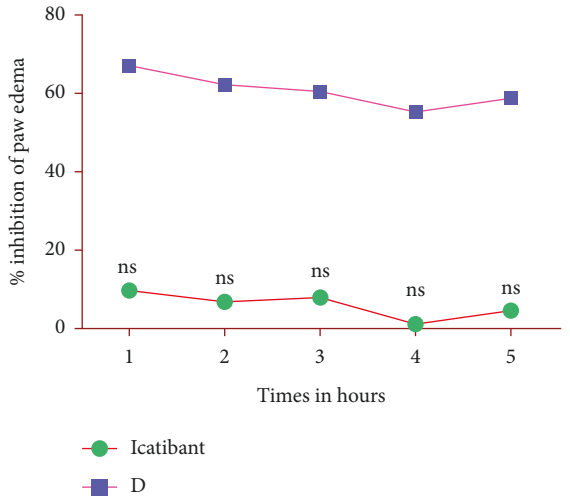

Figure 4: Percent inhibition produced by compound **D** (100 mg/kg) in the bradykinin-induced paw edema model in mice. Each percent point represents the mean ± SEM for a group of 8 mice. Data were analyzed by Student's *t*-test. n.s, nonsignificant values compared to the standard drug (HOE 140).

edema of mouse paw. At the highest doses (100 mg/kg), compound **D** inhibited paw swelling, 46.45 ± 1.40, 47.50 ± 2.54, 55.13 ± 2.25, 44.84 ± 2.20, and 29.43 ± 1.13 at 1, 2, 3, 4, and 5 hours, respectively. The result of compound **D** at the 1st hour was significant ($^{**}P < 0.01$), reached the highest level at the 3rd hour after the administration of leukotriene mediator, and remained significant ($^{***}P < 0.001$) until 5th hour. Montelukast was used as a standard drug which exhibited percent inhibition (67.02 ± 1.74) at the 3rd hour. By using Student's *t*-test, data were analyzed. The result of compound **D** of leukotriene-induced inflammation is shown in Figure 6.

4.11. Docking Studies. We performed docking simulations by using MOE 2016 (Molecular Operating Environment) software package [34]. Three-dimensional COX-2 (code 1CX2) structure in complex with SC-558 was downloaded from PDB (Protein Data Bank). By redocking of the native ligands, the docking procedure was validated. All the compounds were docked into the binding site of COX-2. 2D interaction plots of the compounds are shown in Figures 7 and 8. Compounds **A**, **C**, and **D** exhibited hydrogen bond interactions with Val523 (Figures 7(a), 8(a), and 8(b)), while compound **B** interacts with Ser353 *via* hydrogen bond interactions (Figure 7(b)). The computed binding energy values of the compounds 17–20 are −5.3939 kcal/mol,

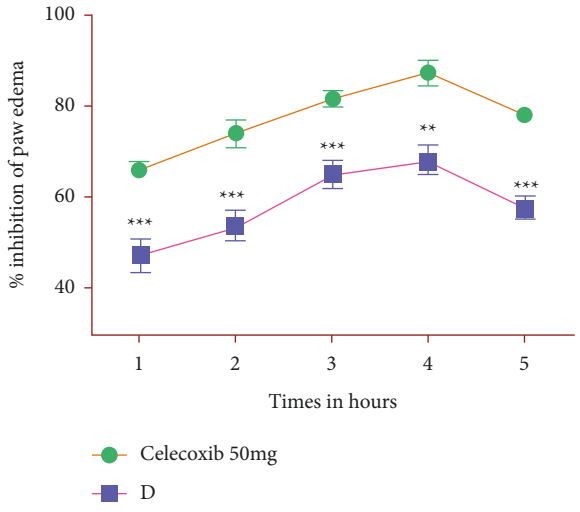

FIGURE 5: Percent inhibition produced by compound **D** (100 mg/kg) in the prostaglandin E_2-induced paw edema model in mice. Each percent point represents the mean ± SEM for a group of 8 mice. Data were analyzed by ANOVA followed by post hoc Dunnett's test. Asterisks show significant values from phlogistic agent prostaglandin E_2. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

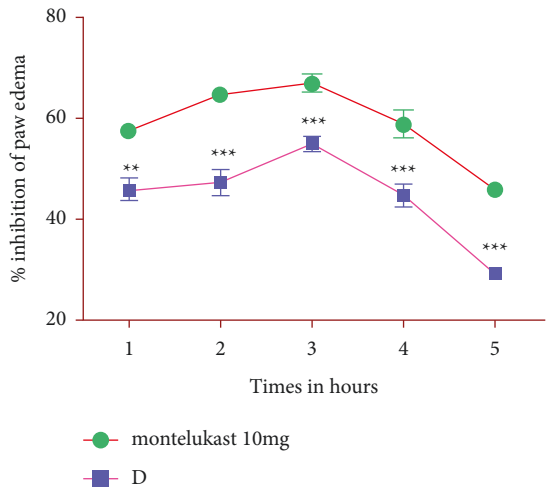

FIGURE 6: Percent inhibition produced by montelukast (10 mg/kg) and compound **D** (100 mg/kg) in the leukotriene-induced paw edema model in mice. Each percent point represents the mean-± SEM for a group of 8 mice. Data were analyzed by ANOVA followed by post hoc Dunnett's test. Asterisks show significant values.$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n.s, nonsignificant value.

−6.8274 kcal/mol, −4.5041 kcal/mol, and −7.6357 kcal/mol, respectively.

The synthetic chemists are constantly in a search of designing new molecules which can be potentially effective as future drugs [35–37]. The COX-2 and 5-LOX are two main enzymes which metabolized arachidonic acid into prostaglandin and leukotriene [38]. The inhibition of both the enzymes produces a wide spectrum of anti-inflammatory effect. The synthesized compounds were studied by using the *in vitro* method using COX-2 and 5-LOX catalyzed prostaglandin and leukotriene biosynthesis assay for COX-2 and 5-LOX inhibition. Among all the compounds studied for *in vitro* COX-2 and 5-LOX inhibitory effect, compound **D** at a concentration of 100 μg/ml was found to show promising COX-2 and 5-LOX inhibitory response (81.70 and 72.50%) in comparison with other compounds. The results for COX-2 and 5-LOX anti-inflammatory activity are shown in Tables 1 and 2.

The most suitable and sensitive model for inflammation is *in vivo* AA mouse ear inflammation, although not specific to test inhibitors of LOX, and rapid onset of edema formation is because of the direct application of AA and is most probably associated to leukotriene release [39]. These are related with transient rise in prostanoid production and obvious cellular influx. This high prostaglandin level is possibly due to COX induction [40]. The anti-inflammatory effect of **D** was higher (54.85 ± 2.18 to 63.81 ± 2.24) in arachidonic acid ear inflammation. Arachidonic acid is a chief constituent of phospholipids of that of the plasma membrane. Phospholipase A2 split up phospholipids. The synthesized compounds could have an inhibitory effect on phospholipase A2 (PLA2). The compound B also exhibited good activity (43.27 ± 2.57) in AA-induced ear edema. It could be considered that the synthesized compound acted on both pathways. For further confirmation of the possible mechanism, the anti-inflammatory potential of **D** (100 mg/kg) was assessed against different mediators. The inflammatory response is adjusted by the release of chemical mediators from the circulatory system, inflammatory system, and injured tissue activity. Chemical mediators include vasoactive amines such as histamine, peptides such as bradykinin, and eicosanoids such as leukotrienes, and prostaglandins.

During inflammatory events, histamine is released in small quantity from basophils [41]. Compound **D** showed 28.10 ± 1.64% antihistaminic effect at the 2nd hour of the histamine administration, which is owing to inhibitory activity of **D** on discharge of various mediators from that of the mast cells. Chlorpheniramine maleate (standard drug) has appreciably inhibited the histamine-induced edema. In the initial stage of inflammation, mediators are released following stimulation, leading to increased vascular permeability and dilation of arterioles and venules. Bradykinin markedly increased microvascular permeability [42]. Compound **D** may not be active against the inhibitory effect of bradykinin-induced inflammation.

Prostaglandins E_2 and leukotriene mediators are the key regulators of inflammation [43, 44]. We used these two mediators in mouse paws. This alternative persuades us to dig out most probable corridor of the anti-inflammatory potential of **D**. In the current study, we established that **D** significantly reserved (53.71–68.03%) the inflammatory prostaglandin *E*2 (PGE2) induced paw edema (Figure 5). The same results were observed in leukotriene-induced inflammation. In leukotriene-induced inflammation, compound **D** significantly inhibited (46.45–55.13%) the teratogenicity of leukotriene-induced paw edema (Figure 6). The literature study demonstrates that the LOX exhibited significant inflammatory activity in carrageenan persuade paw

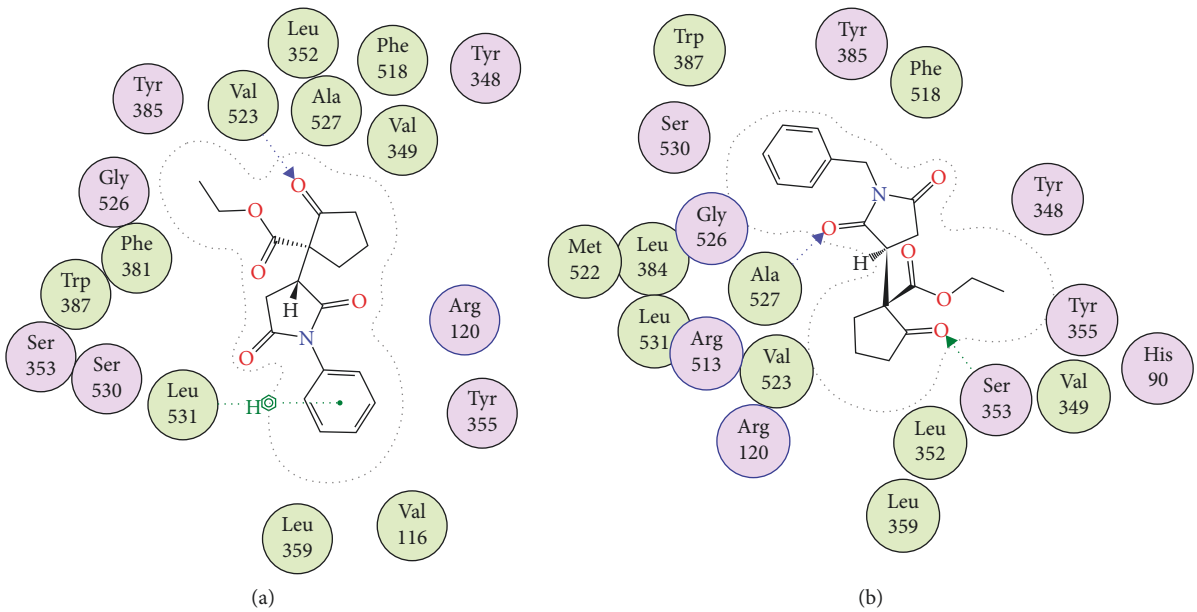

Figure 7: 2D (two-dimensional) binding interaction pattern of compounds (a) **A** and (b) **B** in the binding site of 1CX2.

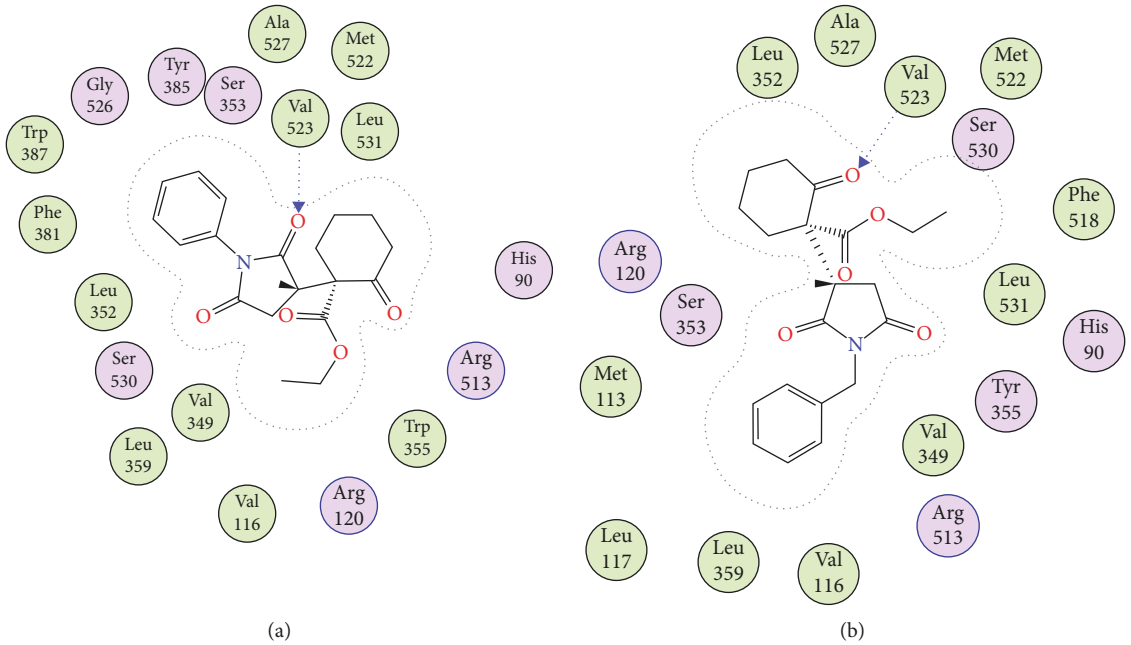

Figure 8: 2D (two-dimensional) binding interaction pattern of compounds (a) **C** and (b) **D** in the binding site of 1CX2.

inflammation [45]. Consecutively, to conclude comparative association of LOX corridor, edema was produced with leukotriene which is tactless to the COX pathway [46]. Compound **D** and montelukast (10 mg/kg) both have appreciably inhibited (55.13% and 67.02% at 3 h) the edema causing effect of leukotriene correspondingly (Figure 6). These findings suggest that compound **D** possesses the double inhibitory assets and has the potential to inhibit both COX and LOX corridors supported by the mild antihistaminic effect of **D**. Analysis of the docking simulations in the binding site of the COX-2 isozyme revealed that all the compounds have shown hydrogen bond interactions with the amino acid residues (Val523 and Ser353) present in the selectivity pocket. The calculated binding energy values also correlate with experimental values.

5. Conclusions

Herein, we have evaluated four β-ketoester derivatives of N-ary succinimides (**A**–**D**) for their anti-inflammatory potentials. Among our compounds, compound **D** (ethyl 1-(1-benzyl-2,5-dioxopyrrolidin-3-yl)-2-oxocyclohexane-1-

carboxylate) exhibited comparatively potent IC_{50} values in COX and LOX assays. Binding orientations and energy values computed via docking simulations support the results of the experimental *in vitro* evaluations. Based on the encouraging *in vitro* results, we used our compounds in experimental animals following carrageenan-induced paw and arachidonic acid-induced ear inflammation tests. In both the *in vivo* experiments, again compound **D** was observed comparatively potent. Compound **D** was further evaluated against various mediators of inflammation. Our results suggest that compound **D** possibly possesses double acting anti-inflammatory properties inhibiting both COX and LOX pathways.

Data Availability

The whole experimental data are available within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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