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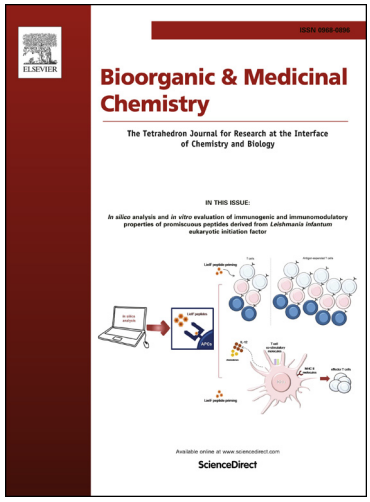

Synthesis, biological evaluation and docking study of a new series of di-substituted benzoxazole derivatives as selective COX-2 inhibitors and anti-inflammatory agents

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Synthesis, biological evaluation and docking study of a new series of di-substituted benzoxazole derivatives as selective COX-2 inhibitors and anti-inflammatory agents.

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Abstract

A new series of substituted-N-(3,4-dimethoxyphenyl)-benzoxazole derivatives **13a-13p** was synthesized and evaluated *in vitro* for their COX (I and II) inhibitory activity, *in vivo* anti-inflammatory and ulcerogenic potential. Compounds **13d**, **13h**, **13k**, **13l** and **13n** exhibited significant COX-2 inhibitory activity and selectivity towards COX-2 over COX-1. These selected compounds were screened for their *in vivo* anti-inflammatory activity by carrageenan induced rat paw edema method. Among these compounds, **13d** was the most promising analogs of the series with percent inhibition of 84.09 and IC_{50} value of 0.04µM and 1.02µM (COX-2 and COX-1) respectively. Furthermore, ulcerogenic study was performed and tested compounds (**13d**, **13h, 13k, 13l**) demonstrated a significant gastric tolerance than ibuprofen. Molecular docking study was also performed with resolved crystal structure of COX-2 to understand the binding mechanisms of newly synthesized inhibitors in the active site of COX-2 enzyme and the results were found to be concordant with the biological evaluation studies of the compounds. These newly synthesized inhibitors also showed acceptable pharmacokinetic profile in the *in silico* ADME/T analyses.

Keywords: Benzoxazole derivatives, selective COX-2 inhibitors, anti-inflammatory activity, ulcerogenic liability.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are generally used for the treatment of inflammation and to relieve pain either associated with surgery or any clinical conditions.[1] Inflammation is a multi-step and complex biological response of the body to any harmful stimulus. It is mediated by the release of pro-inflammatory mediators such as bradykinin and cytokines, which increases the rate of synthesis of prostaglandin.[2] NSAIDs, alters the biosynthesis of prostaglandin, by the inhibition of cyclooxygenase (COX). It occurs in two isoforms: COX-1 and COX-2. COX-1, a constitutive isozyme, performs vital functions of gastro and vascular protection. On the contrary, COX-2 is an inducible isozyme responsible for the prostaglandin synthesis that triggers inflammatory responses.[3] NSAIDs' available in the market such as aspirin (**1**), ibuprofen (**2**) and indomethacin (**3**) shows their anti-inflammatory effect through nonselective inhibition of COX. The adverse effects associated with the chronic use of these drugs are gastric bleeding,[4-7] ulceration[8] and kidney problems.[9-10] The selective COX-2 inhibitors (coxibs) [celecoxib (**4**), valdecoxib (**5**) and rofecoxib (**6**) (Figure 1)] are developed for the treatment of inflammation have shown to produce lower gastrointestinal (GI) side effects. However, prolonged use of few coxibs found to possess high incidence of cardiovascular disorders, due to which valdecoxib **(5**) and rofecoxib (**6**) are withdrawn from the market[11] (Figure 1). Therefore, it is imperative to come out with the scaffolds which have the anti-inflammatory effect but reduced side effects and improved gastric safety profile.[12]

Among the family of heterocycles, benzoxazole is known to possess a wide range of biological activities such as anti-inflammatory,[13-17] anti-bacterial,[18-20] antifungal,[21] anti-convulsant[22] and analgesic activity.[23-24] The literature reports reveal that benzoxazole moiety (**7-10**) (Figure 1) can be a good template for COX-2 inhibitory activity, with appreciable GI safety margins.[16, 25]

Prompted by the aforementioned findings, in the present study, we hereby report the synthesis and biological evaluation of novel series of di-substituted benzoxazole incorporating 3,4-dimethoxyphenyl ring as less ulcerogenic bioisostere. Besides, an *in silico* studies was also conducted to understand the binding mechanism of newly synthesized compounds on the crystal structure of COX-2 and ADME/T analyses was performed to evaluate their suitability as an active drug molecule. Computer aided drug design assist in designing selective and potent

inhibitors as well as vaccines. Among others, molecular docking approach is one of the most rational and authentic approaches in the drug design and discovery for studying the molecular interaction of small molecules.[26-28]

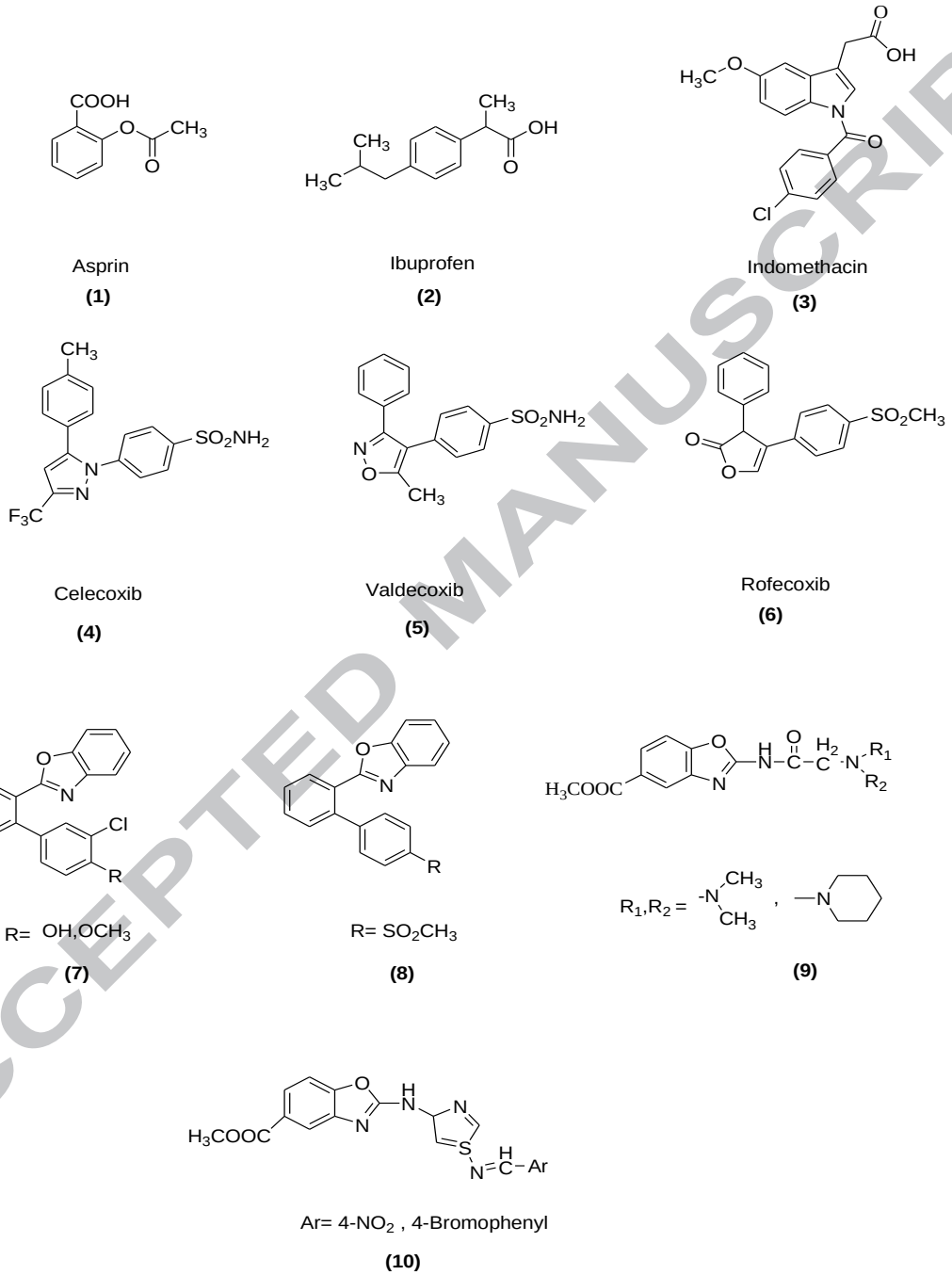


Figure 1. Chemical structures of non-selective NSAIDs (**1-3**), COX-2 selective drugs (**4-6**), and the reported benzoxazole derivatives (**7-10**) with COX-2 activity.

2. Results and Discussion

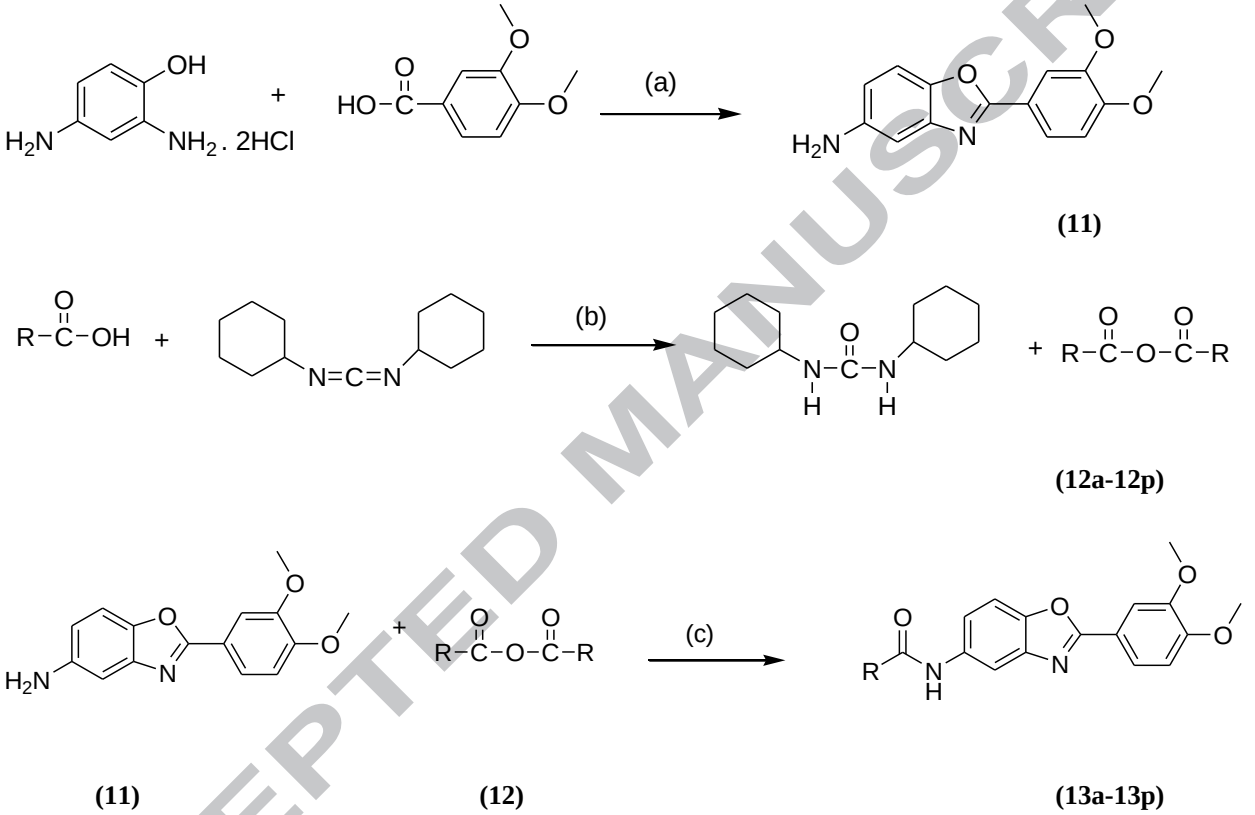
2.1. Chemistry

The title compounds, *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl) substituted benzamide (13a - 13p), were synthesized as outlined in scheme 1. The compound 2-(3,4-dimethoxyphenyl)-benzoxazol-5-amine **(11)** was synthesized by the reaction of 2,4-diaminophenol dihydrochloride with 3,4-dimethoxybenzoic acid in presence of polyphosphoric acid (PPA) followed by the synthesis of the compounds **(12a – 12p)** by the reaction of different substituted acid with dicyclohexylcarodiimide (DCC). Finally, the title compounds **(13a - 13p)** were synthesized by refluxing benzoxazolamine **(11)** with their respective anhydride **(12a – 12p)** in presence of glacial acetic acid (GAA) and zinc (Zn) dust. The poor percentage (%) yield of some of the prepared compounds may be due to the following reasons: (1) The presence of contaminants in the reactants and reagents leads to less efficient reaction; (2) The product loss, due to incomplete extraction or other work-up procedures; (3) The volatilization of products during workup; (4) The incursions of side reactions leading to the formation of by- products.[29]

The progress of the reaction was checked by thin layer chromatography (TLC). Structures of prepared analogs were confirmed by elemental analysis, FTIR, ^{1}H-NMR, ^{13}C-NMR and Mass spectrometry. The IR spectroscopic data showed the presence of –NH-CO- linkage between 1656 – 1684 cm^{-1} and NH stretching in the range of 3282 – 3353 cm^{-1} indicates the synthesis of the compounds. The impression of IR absorption band at 1154 – 1166 cm^{-1} in the synthesized compound **(13b, 13c, 13d, 13l)** displayed the presence of Ar – Cl group substituted at *ortho*, *meta* and *para* position of prepared compounds. The presence of Ar - NO_2 group in derivatives **13k – 13n** displayed symmetric and asymmetric stretches in the range of 1376 – 1394 and 1509 – 1534 cm^{-1}. In case of compound **13o**, the IR absorption band at 580 cm^{-1} corresponds to the C-Br stretching of aromatic-bromo derivative.

The ^{1}H NMR spectra of all prepared compounds are in full agreement with the proposed structures; they showed signals corresponding to aliphatic, aromatic and NH protons. All compounds showed singlet at 3.74 – 4.13 δ ppm due to existence of dimethoxy group. The aromatic benzylic protons (Hb') close to benzoxazole appeared as a singlet in the range of 6.45 – 7.36 δ ppm and other aromatic proton appeared as a multiplet peaks within the range 6.84 – 8.77 δ ppm. Compound **13e – 13g** showed additional singlet within the range of 2.35 – 2.59 δ ppm due

to the presence of methyl group. The compound **13j** showed singlet at 5.09 δ ppm due to existence of hydroxyl group. The NH protons were observed as D_2O exchangeable protons. The elemental analysis data were within ± 0.4% of the theoretical values. Finally, the ^{13}C NMR spectra of the synthesized compound were recorded in $CDCl_3$ and the spectral signals were in accordance to the proposed molecular structure. The physicochemical parameters of the synthesized compounds are presented in Table 1.

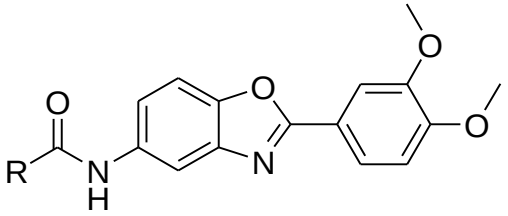


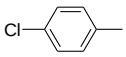
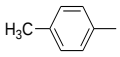
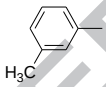
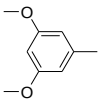
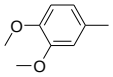
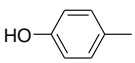
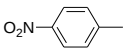
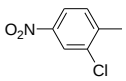
R= **13a** = phenyl; **13b** = 4-chlorophenyl; **13c** = 3-chlorophenyl; **13d** = 2-chlorophenyl; **13e** = 4-methylphenyl; **13f** = 3-methylphenyl; **13g** = 2-methylphenyl; **13h** = 3,5-dimethoxyphenyl; **13i** = 3,4-dimethoxyphenyl; **13j** = 4-hydroxyphenyl; **13k** = 4-nitrophenyl; **13l** = 2-chloro-4-nitrophenyl; **13m** = 2-chloro-5-nitrophenyl; **13n** = 3-nitrophenyl; **13o** = 2-bromophenyl; **13p** = 4-methoxyphenyl.

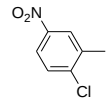
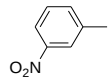
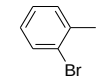
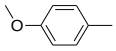
Scheme 1: Synthetic scheme of title compounds (**13a-13p**). *Reagent and conditions*: (a) PPA, 6-7 h 70-80 °C; (b) Dichloromethane; (c) Zn dust, Glacial acetic acid, and Dichloromethane.

Table 1

Physicochemical parameters of the synthesized compounds **(13a-13p)**



Comp.	R	Molecular formula	Molecular weight	Melting point °C	Percentage yield
13a		$C_{22}H_{18}N_2O_4$	374	122-125	66%
13b		$C_{22}H_{17}ClN_2O_4$	408	110-112	70%
13c		$C_{22}H_{17}ClN_2O_4$	408	100-102	62%
13d		$C_{22}H_{17}ClN_2O_4$	408	94-96	48%
13e		$C_{23}H_{20}N_2O_4$	388	138-140	40%
13f		$C_{23}H_{20}N_2O_4$	388	130-132	42%
13g		$C_{23}H_{20}N_2O_4$	388	140-142	36%
13h		$C_{24}H_{22}N_2O_6$	434	110-112	28%
13i		$C_{24}H_{22}N_2O_6$	434	114-116	34%
13j		$C_{22}H_{18}N_2O_5$	390	141-143	50%
13k		$C_{22}H_{17}N_3O_6$	419	122-126	35%
13l		$C_{22}H_{16}ClN_3O_6$	453	154-156	41%

13m		$C_{22}H_{16}ClN_3O_6$	453	143-145	50%
13n		$C_{22}H_{17}N_3O_6$	419	134-136	48%
13o		$C_{22}H_{17}BrN_2O_4$	453	152-156	36%
13p		$C_{23}H_{20}N_2O_5$	404	121-126	42%

2.2. Biological evaluation

2.2.1. *In vitro* cyclooxygenase (COX-1 and COX-2) inhibition assay

The *in vitro* enzyme assay kit used in this study consisted of ovine COX-1 and human COX-2, so we performed the multiple sequence alignment of human and ovine COX-1 sequences using Clustal Omega (Figure 2). The results showed 90% whole sequence identity and 100% conserved catalytic cavity among the two proteins. With this result, we compared the ability of the synthesized compounds to inhibit COX-1 and COX-2 using IC_{50} (µM) values (Table 2). The results of the *in vitro* COX-1 and COX-2 inhibitory studies revealed that the synthesized compounds potentially inhibited COX-2 (IC_{50} = 0.04 - 45.54 µM range) over COX-1 (IC_{50} = 1.02 - 58.24 µM range). Further, the selectivity index (SI) was found to be in the range of 1.27 - 25.5. The results showed that the compounds (**13d, 13h, 13k, 13l and 13n**) were found to be more potent inhibitors of COX-2 (IC_{50} = 0.04 - 0.93 µM range) in comparison to COX-1 (IC_{50} = 1.02 - 6.02 µM range) (Figure 2) among the synthesized compounds. Compound (**13d**) was found to be most potent inhibitor of the series with IC_{50}= 0.04 µM (3.75 fold higher) as compared to celecoxib (IC_{50} = 0.15 µM). The most active COX-2 inhibitory benzoxazole derivatives (**13d, 13h, 13k, 13l and 13n**, IC_{50} < 1 µM), were further evaluated for their *in vivo* anti-inflammatory activity.

```
H-COX1 -MSRSLLLWFLLFLLLLPPLPVLLADPGAPTPVNPCCYYPCQHQGICVRFGLDRYQCDCT 59
O-COX1 MSRQSISLRFPLLLLLLLSPSPVFSADPGAPAPVNPCCYYPCQHQGICVRFGLDRYQCDCT 60
       :*: * * *:**** * **: ******:*****************************
H-COX1 RTGYSGPNCTIPGLWTWLRNSLRPSPSFTHFLLTHGRWFWEFVNATFIREMLMRLVLTVR 119
O-COX1 RTGYSGPNCTIPEIWTWLRTTLRPSPSFIHFLLTHGRWLWDFVNATFIRDTLMRLVLTVR 120
       *********** :*****.:******* *********:*:********: *********
H-COX1 SNLIPSPPTYNSAHDYISWESFSNVSYYTRILPSVPKDCPTPMGTKGKKQLPDAQLLARR 179
O-COX1 SNLIPSPPTYNIAHDYISWESFSNVSYYTRILPSVPRDCPTPMDTKGKKQLPDAEFLSRR 180
       ********** ************************:******.**********::*:**
H-COX1 FLLRRKFIPDPQGTNLMFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLER 239
O-COX1 FLLRRKFIPDPQSTNLMFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLER 240
       ***********.*********************************************
H-COX1 QYQLRLFKDGKLKYQVLDGEMYPPSVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLPGLML 299
O-COX1 QYQLRLFKDGKLKYQMLNGEVYPPSVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLPGLML 300
       ***************:*:**:************************************
H-COX1 YATLWLREHNRVCDLLKAEHPTWGDEQLFQTTRLILIGETIKIVIEEYVQQLSGYFLQLK 359
O-COX1 YATIWLREHNRVCDLLKAEHPTWGDEQLFQTARLILIGETIKIVIEEYVQQLSGYFLQLK 360
       ***:****************************:****************************
H-COX1 FDPELLFGVQFQYRNRIAMEFNHLYHWHPLMPDSFKVGSQEYSYEQFLFNTSMLVDYGVE 419
O-COX1 FDPELLFGAQFQYRNRIAMEFNQLYHWHPLMPDSFRVGPQDYSYEQFLFNTSMLVDYGVE 420
       ********.************:***********:** *:******************
H-COX1 ALVDAFSRQIAGRIGGGRNMDHHILHVAVDVIRESREMRLQPFNEYRKRFGMKPYTSFQE 479
O-COX1 ALVDAFSRQPAGRIGGGRNIDHHILHVAVDVIKESRVLRLQPFNEYRKRFGMKPYTSFQE 480
       ********* *********:***********:*** :**********************
H-COX1 LVGEKEMAAELEELYGDIDALEFYPGLLLEKCHPNSIFGESMIEIGAPFSLKGLLGNPIC 539
O-COX1 LTGEKEMAAELEELYGDIDALEFYPGLLLEKCHPNSIFGESMIEMGAPFSLKGLLGNPIC 540
       *.*****************************************:***************
H-COX1 SPEYWKPSTFGGEVGFNIVKTATLKKLVCLNTKTCPYVSFRVPDASQDDGPAVERPSTEL 599
O-COX1 SPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPDPRQEDRPGVERPPTEL 600
       ******* **********:**********************:*** *:* *.**** ***
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Figure 2. Multiple sequence alignment of Human COX-1(H-COX-1uniport id: P23219) and Ovine COX-1(O-COX-1 uniport id: P05979) using Clustal omega. The alignment shows 90% sequence similarity and 100% conserved catalytic site residues (yellow highlighted).

Table 2

IC_{50} of the synthesized compounds by *in vitro* COX-1 and COX-2 enzymatic assay and COX-2 selectivity index (SI) data.

Compound	IC_{50} (μM)[a]		SI[b]
	COX-1	COX-2	
13a	6.20	1.40	4.42
13b	7.32	1.21	6.04
13c	8.82	1.39	6.34
13d	1.02	0.04	25.50
13e	7.84	1.39	5.64
13f	5.21	1.56	3.33
13g	9.21	4.84	1.90
13h	6.02	0.44	13.68
13i	8.20	2.70	3.03
13j	58.24	45.54	1.27
13k	3.23	0.16	20.18
13l	5.15	0.46	11.19
13m	10.20	7.40	1.37
13n	5.52	0.93	5.93
13o	10.12	2.24	4.51
13p	16.32	10.59	1.54
Celecoxib	6.20	0.15	41.33
Ibuprofen	1.42	1.08	1.31

[a] IC_{50} value is the concentration of the compound required to produce 50% of inhibition of COX-1 and COX-2 respectively using enzyme immunoassay kit (Catalogue no. 560131, Cayman Chemicals, Inc., Ann Arbor, MI, USA).

[b] *In vitro* COX-2 selectivity index(SI): (COX-1 IC_{50}/COX-2 IC_{50}).

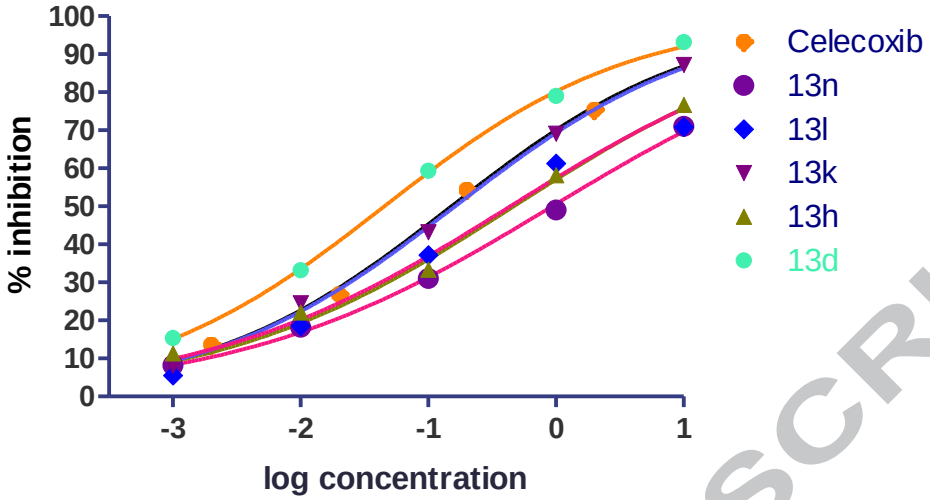

Figure 3. *In vitro* percentage inhibition of COX-2 versus log concentration curve of most potent compounds (**13d, 13h, 13k, 13l, 13n**).

2.2.2. *In vivo* anti-inflammatory activity

Anti-inflammatory activity of the selected compounds (**13d, 13h, 13k, 13l and 13n**) was assessed by the carrageenan induced rat paw edema method. The pharmacological data of selected compounds is shown in Table 3 (Supplementary Figure S1), which clearly implies that the synthesized compounds exhibited significant anti-inflammatory properties ranging from 45% to 84%. Out of the five compounds, four compounds demonstrated better anti-inflammatory activity than standard drug ibuprofen (65.90%). Compound with 2-chlorophenyl (**13d**) emerged as the most promising analog of the series with percentage inhibition of 84.09%. The compounds with 4-nitrophenyl (**13k**), 2-chloro-4-nitrophenyl (**13l**), 3,5-dimethoxyphenyl (**13h**) also showed remarkable efficacy against inflammation with percent inhibition of 79.54%, 72.72%, and 68.18% respectively. On the contrary, compound (**13n**) exhibited weak anti-inflammatory activity of 54.54% than ibuprofen.

Table 3

In vivo anti-inflammatory activity of the most potent compounds using carrageenan-induced rat paw edema method.

Compound	Paw edema volume (ml)		Increase in paw edema (ml)	%Inhibition[b]
	0 h	3 h	(Mean ± SEM)[a]	
13d	0.66	0.74	0.07±0.02	84.09
13h	0.67	0.81	0.14±0.02	68.18
13k	0.71	0.8	0.09±0.02	79.54
13l	0.71	0.77	0.12±0.01	72.72
13n	0.57	0.77	0.20±0.02	54.54
Control	0.7	1.14	0.44±0.05	-
Ibuprofen	0.65	0.8	0.15±0.02	65.90

- = not applicable

[a)] Values are determined after 3 h and are expressed as Mean ± SEM

[b)] $p < 0.05$ (significant difference)

p values were compared with control group (3 h after inducing edema) (Turkey's test). Number of animals (rats) in each group = 5.

2.2.3. Acute ulcerogenic activity

Analogs **13d, 13h, 13k** and **13l** possessing *in vivo* anti-inflammatory activity greater than standard drug ibuprofen were further screened for their ulcerogenic activity according to Cioli method.[30] The results (Table 4) (Supplementary Figure S2) showed that the tested compounds showed better G.I. safety profile with severity index ranging from 0.80 to 2.10, in comparison to standard drug ibuprofen 2.20 ± 0.44. Most potent compound (**13d**) showed severity index of 0.80 ± 0.44 which was 2.75 folds higher in comparison to the standard drug. Hence, these compounds may ascertain to have better safety margin on gastric mucosa than ibuprofen.

Table 4

In vivo ulcerogenic activity of the most active synthesized compounds in rat model.

Compound	Ulcerogenic activity [(a)] (severity index) [(b), (c)] (Mean ± SD)
13d (60 mg/kg)	0.80 ± 0.44
13h (60 mg/kg)	2.10 ± 0.27
13k (60 mg/kg)	1.80 ± 0.44
13l (60 mg/kg)	1.00 ± 0.50
Ibuprofen (60 mg/kg)	2.20 ± 0.44
Control (normal saline)	-

[a)] Number of animals in each group = 5.

[b)] Severity index = Mean score of treated group – Mean score of control group.

[c)] $p < 0.05$ (significant difference).

2.3. *In silico* studies

2.3.1. Docking studies and ADME/T analysis

The docking study was performed using resolved crystal structure of human COX-2 (PDB ID: 5F19). The docking results showed that among all docked molecules, hydrogen bonds with Arg120 and π-π interaction with Tyr355 is conserved with COX-2 active site (Table 5). Compounds **13d, 13k and 13l** showed the most promising *in vitro, in vivo*, ulcerogenic potential and docking score among all of the newly synthesized compounds. In addition, the hydrophobic cloud was contributed by the Val89, Leu93, Val116, Val349, Leu359, Leu384, Tyr385, Trp387, Phe518, Val523, Ala527 and Leu531 (**Figure 4**). Apart from above mentioned residues, some other residues (Tyr385-**13i**; Ser530-**13k**,**13l**) also found to be involved in interaction and among them compounds **13k** and **13l** found to be amongst most promising candidates of the series.

2.3.1.1 Structure Activity Relationship (SAR)

The benzoxazole moiety plays important role in interacting by H-bond with Arg120 (with N-atom of benzoxazole ring) and π-π interaction (with both aromatic rings of benzoxazole moiety). Docking score showed that the presence of electron withdrawing chloro group at *ortho* position of phenyl ring (**13d**), nitro group at *para* position (**13k**)

and *ortho – para* disubstituted chloro, nitro group on phenyl ring (**13l**) leads to increase in activity. Whereas, compound with electron withdrawing nitro group at *meta* position (**13n**), chloro group at *para, meta* position (**13b, 13c**) produces moderate activity. Unsubstituted phenyl ring (**13a**) also produces significant activity. Substitution of electron donating group at *ortho* **(13g-methyl)**, *meta* **(13f-methyl and 13i-methoxy)** and *para* **(13e-methyl, 13j-hydroxy, 13p-methoxy)** position of phenyl ring produced detrimental effect on the activity profile of compounds. The compound **13h** was found to be exception which possesses electron donating -methyl substituent at *meta* position but showed remarkable *in- vitro* and *in-vivo* activity. The compounds showed comparable docking score to celecoxib.

The celecoxib possessed van der waals interactions with amino acid Val349, Leu384, Trp387, Leu352, Tyr385, Phe518, Val523 and Ala527 and hydrogen bond with Gln192, Ser353, Arg513 and Phe518 (Figure 5f). The non-selective inhibitor Ibuprofen showed no H-bond with COX-2 but salt bridge with Arg120 (Figure 5e). Finally, the Absorption, Distribution, Metabolism, Excretion and Toxicity (ADME/T) parameters for all synthesized ligands were calculated using Qikprop 4.0 (Supplementary Table S1). Assessment of ADME/T property is imperative because they exclude weak or toxic molecule at an early stage of drug discovery and development process. The desirable ADME/T properties of these compounds make them promising candidates as COX-2 inhibitors.

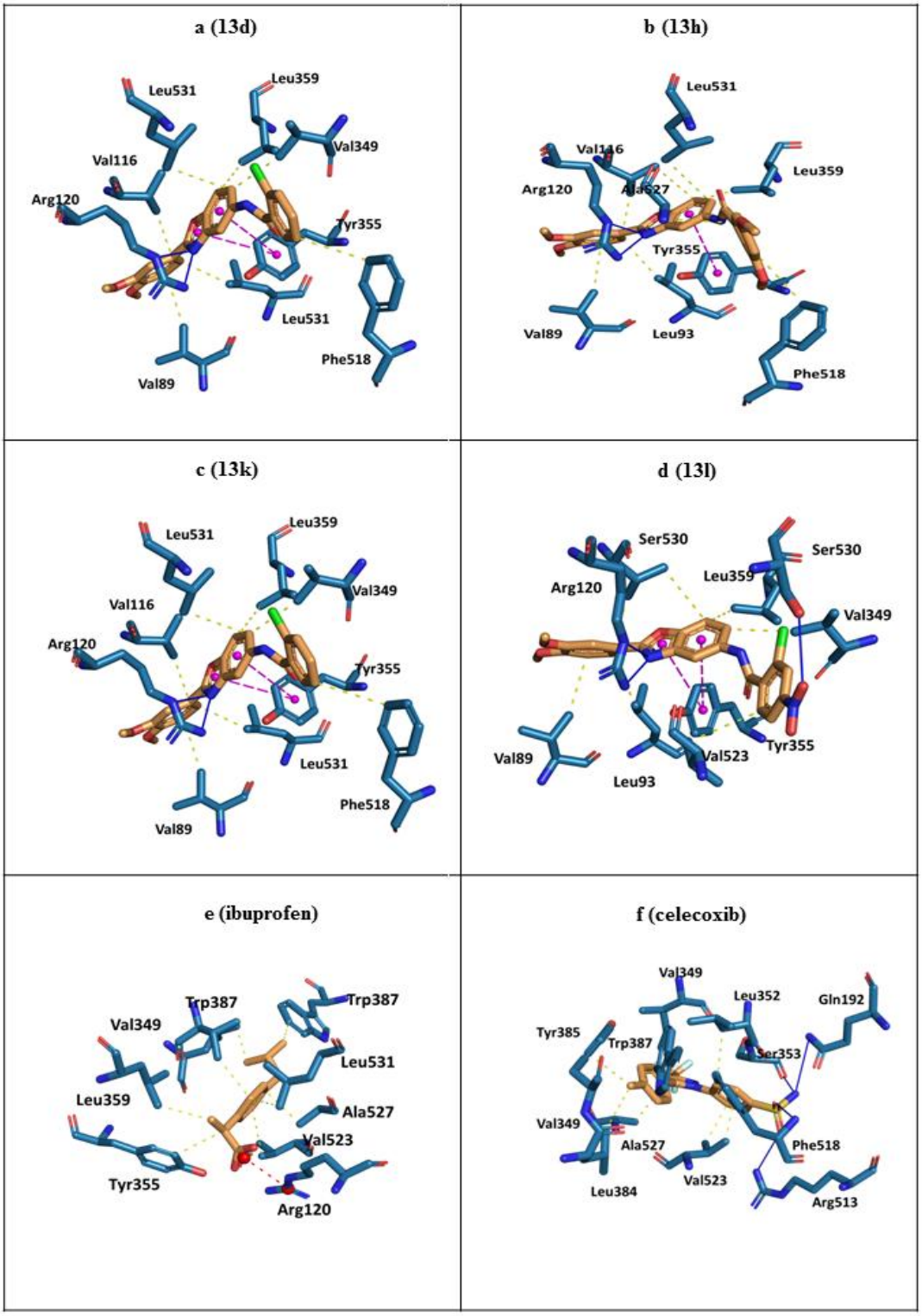

Figure 4. Docked pose of compound (a) **13d**, (b) **13h**, (c) **13k**, (d) **13l** (e) **ibuprofen,** (f) **celecoxib** with COX-2. Blue line indicates hydrogen bond, yellow dashed line indicates hydrophobic interaction, pink dashed line indicates pi-pi stacking and red line indicates salt bridge.

Table 5

Glide score (kcal/mol), type of interactions and interacting residues of the COX-2 protein with synthesized compounds.

Comp	Glide Score (kcal/ mol)	Type of Interactions						
		Hydrogen bonds			π –Interactions			
		Atom of Ligand	Amino acids	Dist (Å)	Type	Ring/Group	Amino acids	Dist (Å)
13a	-9.8	N	Arg120	2.1	π-stacking	Benzoxazole	Tyr355	5.3
		N	Arg120	2.5			Tyr355	5.2
13b	-9.9	N	Arg120	2.3	π-stacking	Benzoxazole	Tyr355	5.3
		N	Arg120	2.7			Tyr355	5.4
13c	-9.8	N	Arg120	2.1	π-stacking	Benzoxazole	Tyr355	5.3
		N	Arg120	2.6				
13d	-10.6	N	Arg120	2.1	π-stacking	Benzoxazole	Tyr355	5
		N	Arg120	1.7			Tyr355	5.1
13e	-9.0	N	Arg120	2.2	π-stacking	Benzoxazole	Tyr355	5.4
		N	Arg120	2.6			Tyr355	5.2
13f	9.1	N	Arg120	2.2	π-stacking	Benzoxazole	Tyr355	5.3
		N	Arg120	2.6			Tyr355	5.2
13g	-8.6	N	Arg120	2.8	π-stacking	Benzoxazole	Tyr355	5.1
		N	Arg120	2.5			Tyr355	5.3
13h	-8.1	N	Arg120	2.3	π-stacking	Benzoxazole	Tyr355	5.3
		N	Arg120	2.7				
13i	-9.2	N	Arg120	2.1	π-stacking	Benzoxazole	Tyr355	5.4
		N	Arg120	2.5			Tyr355	5.3
		OCH_3	Tyr385	3.1				
13j	-8.7	N	Arg120	2.9	π-stacking	Benzoxazole	Tyr355	5.3
		N	Arg120	2.5			Tyr355	5.4
13k	-10.4	N	Arg120	2.2	π-stacking	Benzoxazole	Tyr355	5.2
		N	Arg120	1.6				
		NO_2	Ser530	1.6				
13l	-10.5	N	Arg120	2.1	π-stacking	Benzoxazole	Tyr355	5.1
		N	Arg120	1.7			Tyr355	5.2
		NO_2	Ser530	1.5				
13m	-8.0	N	Arg120	3.1	π-stacking	Benzoxazole	Tyr355	5.4
		N	Arg120	2.8			Tyr355	5.3
13n	-10.0	N	Arg120	2.7	π-stacking	Benzoxazole	Tyr355	5.2
		N	Arg120	2.3			Tyr355	5.5
13o	-9.0	N	Arg120	2.4	π-stacking	Benzoxazole	Tyr355	5.4
		N	Arg120	2.8			Tyr355	5.5
13p	-8.5	N	Arg120	3.0	π-stacking	Benzoxazole	Tyr355	5.5
		N	Arg120	2.6			Tyr355	5.4
Ibuprofen	-7.3	-	-	-	Salt bridge	COOH	Arg120	3.4
Celecoxib	-10.4	NH_2	Gln192	3.5				
		NH_2	Ser353	2.0				
		SO_2	Arg513	2.8				
		SO_2	Phe518	2.6				

3. Conclusion

Summarizing, a series of substituted-N-(3,4-dimethoxyphenyl)-benzoxazole derivatives (**13a - 13p**) were successfully synthesized and evaluated for their selective COX-2 inhibitory potential and the active compounds were further screened for their anti-inflammatory and ulcerogenic activity. The structures of the prepared compounds were confirmed by modern analytical techniques (AT-FTIR, ^{1}H NMR, ^{13}C NMR and mass spectrometry). The docking studies found to be in line with the experimental data and the emerged SAR showed that the benzoxazole ring plays important role in interacting with the COX-2 enzyme. Also, electron withdrawing substitutions have favorable effects on activity of compounds in comparison to the electron donating substitutions. Among the synthesized compounds; **13d**, **13h**, **13k** and **13l** exhibited significant anti-inflammatory and ulcerogenic potentials. The compound **13d** emerged as the most potent compound of the series with improved gastric safety profile. This series of compound can be taken as a lead for development of more safe and effective anti-inflammatory agents.

4. Experimental

4.1. Chemistry

The chemical and solvents used were purchased from commercial vendors and used without purification. The melting points of the prepared analogs were determined on LAB-India MR-VIS visual melting point apparatus and are uncorrected. The Infrared (IR) spectra were recorded on Bruker Optics Spectrophotometer. ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) were recorded in $CDCl_3$ on Bruker, Advance DPX-300 spectrometer. Tetramethylsilane (TMS) was used as internal standard and chemical shifts (δ) were determined in parts per million (ppm). Mass spectral data of analogs were recorded on LCMS/LCQ (Agilent, Advantage-Max) instrument, equipped with electro spray ion (ESI) source. Elemental analyses were carried out on Flash 2000 organic elemental analyzer. The progress of the chemical reaction was monitored using thin layer chromatography (TLC) on pre-coated plates (Merck, Germany) and compounds were purified using column chromatography on silica gel (100 - 200 mesh). Iodine vapors and UV-visualizer were used for detection of spots on TLC plates.

4.2. Synthesis of 2-(3, 4-dimethoxyphenyl)-benzoxazol-5-amine (11)

2-(3,4-Diimethoxyphenyl)benzoxazol-5-amine (**11**) was prepared by heating 2,4-diaminophenol dihydrochloride (0.01 mol) and 3, 4-dimethoxybezoic acid (0.01 mol) in presence

of cyclizing agent PPA (24 gm) at 70 – 80 °C for 6 - 7 h. After the completion of reaction, mixture was poured into ice cold water, neutralized with an excess of 10N NaOH and extracted with toluene, dried over anhydrous sodium sulfate and evaporated under vacuum. The product obtained was boiled with 100 mg of charcoal in ethanol and filtered. After evaporation, the crude product obtained was recrystallized from ethanol.

4.3. General procedure for synthesis of anhydride (12a - 12p)

Mixture of substituted acid derivative (0.02 mol) and dicyclohexylcarbodiimide (0.01 mol) was dissolved in dichloromethane (50 ml) and stirred at room temperature for 3 - 4 h. After then solvent was separated **(12a – 12p)**, the reaction mixture was filtered to remove the precipitated dicyclohexylurea and the filtrate was evaporated to get the oily product **(12a – 12p)**

4.4. General procedure for synthesis of *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl) substituted benzamide (13a - 13p)

A reaction mixture of benzoxazolamine (**1)** (0.012mol), respective anhydride **(12a - 12p)** (0.01 mol), zinc dust (0.010 gm) and glacial acetic acid (0.01 mol) in DCM (15 ml) was refluxed for 4-5 h with constant stirring. After completion of the reaction, mixture was poured into ice cold water and the resultant precipitate was separated and recrystallized with ethanol.

4.4.1. *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5yl)-benzamide (13a)

ATR-FTIR (cm-^{1}): 3284.50 (NH), 2918.20 (-CH), 1680 (-CONH-), 1620.62, 1462 (Aromatic C=C), 1210.20 (C-N), 1100 (C-O), 732.40 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.00 (s, 6H, 2 × -OCH_3), 6.91-7.01 (m, 3H, Hb' merged with He' and Hf'), 7.31-7.33 (d, 1H, J = 6 Hz, H7 of benzoxazole), 7.39-7.45 (t, 3H, J = 6 Hz, Hc, Hd, He), 7.51-7.54 (d, 2H, J = 9 Hz, H4 , H6 of benzoxazole), 7.79-7.82 (d, 2H, J = 9 Hz, Hb , Hf), 8.01 (s, 1H , NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.58, 106.52, 110.52, 112.42, 115.56, 120.72, 127.50, 131.75, 134.99, 135.97, 140.22, 151.41, 162.24, 164.85. MS (m/z): 375 [M +1] $^{+}$. Anal calc for $C_{22}H_{18}N_2O_4$; C, 70.58; H, 4.85; N, 7.48; Found C, 70.56; H, 4.83; N, 7.43.

4.4.2. 4-Chloro-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-benzamide (13b)

ATR-FTIR (cm-^{1}): 3282.60 (NH), 2921.40 (-CH), 1674 (-CONH-), 1630.40, 1466 (Aromatic C=C), 1220.20 (C-N), 1154.32 (Ar-Cl), 1112 (C-O), 734.20 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 4.01 (s, 6H, 2 × -OCH_3), 6.66-6.69 (d, 1H, J = 6 Hz , He'), 6.82 (s, 1H, Hb '), 6.97-7.03 (d, 1H, J

= 9 Hz, Hf ') , 7.43-7.53 (m, 3H, Hc, Hd and H7of benzoxazole), 7.59-7.61 (d, 2H, J = 6 Hz H4, H6 of benzoxazole), 8.00-8.04 (d, 2H, J = 12 Hz , Hf, Hb), 8.13 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.24, 106.88, 110.90, 112.46, 112.58, 115.88, 120.86, 128.98, 130.63, 130.86, 135.40, 137.11, 145.60, 149.84, 150.39, 162.70, 164.80. MS (m/z): 409 [M +1] $^+$. Anal calc for $C_{22}H_{17}ClN_2O_4$; C, 64.63; H, 4.19; N, 6.85; Found C, 64.62; H, 4.22; N, 6.83.

4.4.3. 3-Chloro-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-benzamide (13c)

ATR-FTIR (cm-^{1}): 3302.6 (NH), 2918.4 (-CH), 1662.4 (-CONH-), 1628.26, 1520.26 (Aromatic C=C), 1482.15 (-CH), 1212.2 (C-N), 1160.20 (Ar-Cl), 1050.23 (C-O), 800.91 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 3.88 (s, 3H, -OCH_3), 3.93 (s, 3H, -OCH_3), 6.59 (d/s, 2H, He' merge with Hb'), 6.97-7.11 (m/br, 3H, H7 of benzoxazole),7.43-7.46 (d, 1H, J = 9 Hz, Hd), 7.64-7.68 (dd, 2H, J = 3Hz, H6 of benzoxazole merge with Hb and Hf), 7.77 (s, 1H, H4 of benzoxazole), 7.97 (s,1H,-NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.22, 106.28, 110.90, 112.44, 119.55, 120.86, 127.68, 130.33, 130.63, 134.40, 135.12, 141.72, 145.63, 149.79, 162.82, 164.65. MS (m/z): 409 [M +1] $^+$. $C_{22}H_{17}ClN_2O_4$; C, 64.63; H, 4.19; N, 6.85; Found C, 64.61; H, 4.18; N, 6.83.

4.4.4. 2-Chloro-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-benzamide (13d)

ATR-FTIR (cm-^{1}): 3328.96 (NH), 2910.2 (-CH stretching), 1660.4 (-CONH-), 1625.12, 1524.45 (Aromatic C=C), 1480.19 (-CH bend), 1210.2 (C-N), 1162.50 (Ar-Cl), 1110.62 (C-O), 800.52 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 3.91 (s, 6H, 2 × -OCH_3), 6.62-6.75 (d/s, 2H, Hb', He '), 7.24-7.26 (d, 1H, J = 6 Hz, Hf ' of benzoxazole), 7.31-7.37 (d, 1H, J = 18 Hz, Hd , H7 of benzoxazole), 7.57-7.62 (t, 1H, J = 9Hz, He),7.72-7.74 (d, 2H, J = 6Hz, Hc, Hd), 7.84-7.97 (dd, 3H, H4, H6 of benzoxazole merge with Hf), 8.25 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.40, 106.29, 110.98, 112.36, 112.44, 115.80, 119.68, 120.92, 127.64, 129.12, 132.30, 132.40, 133.68, 135.18, 141.74, 145.92, 149.99, 150.08, 162.79, 165.84. MS (m/z): 409 [M +1] $^+$. $C_{22}H_{17}ClN_2O_4$; C, 64.63; H, 4.19; N, 6.85; Found C, 64.61; H, 4.18; N, 6.83.

4.4.5. *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-4-methylbenzamide (13e)

ATR-FTIR (cm-^{1}): 3326.42 (NH), 2992.64 (-CH stretching), 1680.46 (-CONH-), 1630.12, 1474.20 (C=C), 1458.42 (-CH bend), 1100.14 (C-O), 732.42 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 2.35 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.80-6.82 (d, 1H, J = 4Hz, He'), 6.89 (s, 1H, Hb'), 6.95-6.98 (d, 2H, J = 9Hz, Hf ' merge with H7 of benzoxazole), 7.47 (s, 1H, H4 of benzoxazole), 7.51-7.53 (d, 1H, J = 4 Hz He),7.59-7.62 (d, 1H, J = 9 Hz, Hc) , 7.76-7.78

(d, 1H, J = 6 Hz, H6 of benzoxazole), 7.88-7.92 (d, 2H, J = 12 Hz, Hf, Hb), 8.01 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.60, 56.22, 106.24, 110.92, 112.36, 115.88, 119.68, 127.53, 130.32, 136.67, 147.62, 149.82, 150.36, 162.71, 164.78. MS (m/z): 389 [M +1] $^+$. Anal calc for $C_{23}H_{20}N_2O_4$; C, 71.12; H, 5.19; N, 7.21; Found C, 71.10; H, 5.18; N, 7.20.

4.4.6. *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-3-methylbenzamide (13f)

ATR-FTIR (cm-^{1}): 3326.22 (NH), 2998.78 (-CH stretching), 1684.67 (-CONH-), 1637.24, 1475.40 (C=C), 1460.82 (-CH bend), 1110.28 (C-O) 800.52 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 2.37 (s, 3H, CH_3), 3.95 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 6.87- 6.92 (s/d, 2H, He', Hf '), 6.97-7.00 (d, 1H, J = 9Hz, Hb'), 7.42-7.47 (t, 1H, J = 9Hz, He), 7.50-7.53 (d, 2H, J= 9Hz, Hc and H7 of benzoxazole), 7.59-7.62 (dd, 3H, J_1= 3Hz, J_2= 3Hz, Hf merge with H4 and H6 of benzoxazole), 7.75 (s, 1H, Hb), 8.02 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 23.98, 56.42, 106.95, 110.96, 115.48, 119.52, 120.78, 124.58, 127.44, 127.83, 128.96, 132.55, 149.89, 150.34, 162.91, 164.84. MS (m/z): 389 [M +1] $^+$. Anal calc for $C_{23}H_{20}N_2O_4$; C, 71.12; H, 5.19; N, 7.21; Found C, 71.13; H, 5.20; N, 7.20.

4.4.7. *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-2-methylbenzamide (13g)

ATR-FTIR (cm-^{1}): 3323.42 (NH), 2989.64 (-CH stretching), 1680.48 (-CONH-), 1624.11, 1474.23 (C=C), 1458.28 (-CH bend), 1111.72 (C-O) 780.82 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 2.59 (s, 3H, -CH_3), 3.99 (s, 3H, -OCH_3), 6.47 (s, 1H, Hb'), 6.56-6.57 (d, 2H, J= 3 Hz, Hf', He'), 6.81- 6.88 (t, 2H, J = 12 Hz, He, Hd), 7.41-7.51 (dd, 2H, J_1 = 6Hz, J_2 = 6Hz, Hf and H6 of benzoxazole), 7.69 (s, 1H, H4 of benzoxazole), 7.99 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.18, 56.58, 106.25, 110.92, 112.36, 112.44, 119.56, 125.98, 127.46, 132.15, 135.30, 141.78, 145.69, 149.84, 162.11, 164.69. MS (m/z): 389 [M +1] $^+$. Anal calc for $C_{23}H_{20}N_2O_4$; C, 71.12; H, 5.19; N, 7.21; Found C, 71.11; H, 5.21; N, 7.22.

4.4.8. 3, 5-Dimethoxy-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-benzamide (13h)

ATR-FTIR (cm-^{1}): 3352.64 (NH), 2950.14 (-CH stretching), 1656.04 (-CONH-), 1610.11, 1455.14 (C=C), 1462.48 (-CH bend), 1111.56 (C-O), 810.02 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.81 (s, 6H, 2 × -OCH_3), 3.92 (s, 6H, 2 × -OCH_3), 6.45 (s, 1H, Hd), 6.56-6.57 (d, 2H, J=3Hz, He'), 6.84-6.94 (t, 2H, J= 12Hz, Hb' merged with Hf'), 7.21 (s, 1H, Hb), 7.40-7.51 (dd, 2H,J_1= 12Hz, J_2= 15Hz, H4 mreged with H7 of benzoxazole), 7.67(s, 2H, Hf merged with H6 of

benzoxazole), 7.99 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.40, 106.54, 110.90, 112.33, 112.49, 115.42, 119.58, 120.86, 127.56, 135.15, 141.62, 145.67, 149.82, 149.98, 153.24, 162.11, 164.87. MS (m/z): 435 $[M+1]^{+\cdot}$ Anal calc for $C_{24}H_{22}N_2O_6$; C, 66.35; H, 5.10; N, 6.45; Found C, 66.36; H, 5.11; N, 6.46.

4.4.9. 3,4-Dimethoxy-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-benzamide (13i)

ATR-FTIR (cm-^{1}): 3355.42 (NH), 2950.11 (-CH stretching), 1660.04 (-CONH-), 1610.11, 1465.10 (C=C), 1464.52 (-CH bend), 1110.32 (C-O), 800.60 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.94 (s, 6H, 2 × $-OCH_3$), 4.01 (s, 6H, 2 × $-OCH_3$), 6.83-7.03 (br, 3H, He, Hb meged with Hf'), 7.33 (s, 1H, Hb), 7.43-7.45 (d, 1H, J = 6 Hz, H7 of benzoxazole), 7.50-7.53 (d, 1H, J = 9Hz, Hf), 7.59-7.62 (d, 1H, J = 9 Hz, H6 of benzoxazole),7.75 (s, 1H, H4 of benzoxazole), 8.06 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.24, 103.80, 103.82, 106.80, 110.95, 112.34, 115.82, 119.54, 120.86, 135.12, 141.74, 145.68, 149.87, 150.32, 161.85, 164.85. MS (m/z): 435 $[M+1]^{+\cdot}$ Anal calc for $C_{24}H_{22}N_2O_6$; C, 66.35; H, 5.10; N, 6.45; Found C, 66.37; H, 5.11; N, 6.47.

4.4.10. 4-Hydroxy-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-benzamide (13j)

ATR-FTIR (cm-^{1}): 3330.25 (-OH), 3312.42 (NH), 2989.46 (-CH stretching), 1665.82 (-CONH-), 1615.62, 1448.52 (C=C), 1466.20 (-CH bend), 1112.50 (C-O), 820.16 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.87 (s, 3H, $-OCH_3$), 3.91 (s, 3H,$-OCH_3$), 5.09 (s, 1H, -OH), 6.75-6.84 (dd, 2H, Hc, He), 6.90-6.91 (d, 1H, J = 3 Hz,He'), 7.36 (d, 2H, Hb',Hf'), 7.46-7.47 (d, 1H, J= 3Hz, H7 of benzoxazole), 7.52-7.55 (d, 24, H6 of benzoxazole), 7.65-7.68 (d, J = 9Hz, Hb), 7.76-7.78 (d, 1H J = 6 Hz, Hf), 7.94 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.28, 106.28, 110.95, 112.36, 115.84, 116.18, 119.56, 128.95, 128.96, 135.10, 141.70, 145.60, 150.39, 161.20, 164.89. MS (m/z): 391 $[M+1]^{+\cdot}$ Anal calc for $C_{22}H_{18}N_2O_5$; C, 67.69; H, 4.65; N, 7.18; Found C, 67.72; H, 4.67; N, 7.19.

4.4.11. *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-4-nitrobenzamide (13k)

ATR-FTIR (cm-^{1}): 3286.40 (NH), 2928.52 (-CH stretching), 1666.40 (-CONH-), 1626.10, 1528.20 (C=C), 1532.32, 1394.20 (Ar-NO_2), 1488.22 (-CH bend), 1012.32 (C-O) 800.32 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.93 (s, 3H, $-OCH_3$), 3.97 (s, 3H, $-OCH_3$), 6.96-6.98 (d, 1H, J=6Hz, He), 7.31 (s, 1H, Hb'), 7.41-7.48 (dd, 2H, He' merged with H7 of benzoxazole), 7.80-7.81 (t, 1H, J = 3 Hz, H4, H6 of benzoxazole), 8.00-8.09 (t, 3H, Hb, Hf merged with –NH), 8.30-

8.33 (d, 2H, Hc, He). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.26, 106.24, 110.92, 112.36, 112.40, 115.83, 119.69, 121.23, 128.44, 135.16, 140.34, 141.79, 145.60, 149.84, 150.36, 151.96, 162.19, 164.88. MS (m/z): 420 $[M+1]^{+\cdot}$ Anal calc for $C_{22}H_{17}N_3O_6$; C, 63.01; H, 4.09; N, 10.02; Found C, 63.02; H, 4.13; N, 10.03.

4.4.12. 2-Chloro-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-4-nitrobenzamide (13l)

ATR-FTIR (cm-^{1}): 3306.2 (NH), 2908.60 (-CH stretching), 1670.43 (-CONH-), 1628.21, 1528.44 (C=C), 1532.43, 1386.60 (Ar-NO_2), 1474.42 (-CH bend), 1166.40 (Ar-Cl), 1114.80 (C-O), 780.22 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 6H, 2 × -OCH_3), 6.90-6.92 (d, 1H, J = 6 Hz, He), 7.40-7.49 (dd, 2H, Hb', Hf'), 7.53-7.56 (d, 1H, J = 9 Hz, H7 of benzoxazole), 7.65 (s, 1H, H4 of benzoxazole), 7.79 – 7.82 (d, 2H, H6 of benzoxazole merge with Hf), 7.94 – 7.96 (d, 1H,J = 6Hz, He'), 8.11 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.38, 106.54, 110.92, 112.38, 115.82, 119.36, 119.56, 120.81, 124.31, 129.84, 133.26, 135.18, 138.57, 141.75, 145.76, 149.82, 150.31, 162.71, 164.82. MS (m/z): 454 $[M+1]^{+\cdot}$ Anal calc for $C_{22}H_{16}ClN_3O_6$; C, 58.22; H, 3.55; N, 9.26; Found C, 58.23; H, 3.57; N, 9.27.

4.4.13. 2-Chloro-*N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-5-nitrobenzamide (13m)

ATR-FTIR (cm-^{1}): 3308.80 (NH), 2915.80 (-CH stretching), 1672.21 (-CON.H-), 1630.45, 1525.84 (C=C), 1534.44, 1379.80 (Ar-NO_2), 1476.20 (-CH bend), 1162.60 (Ar-Cl), 1110.40 (C-O), 758.44 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.95 (s, 6H, 2 × -OCH_3), 6.89-7.01 (m, 3H, He, Hb, Hf'), 7.60-7.63 (d, 1H, H7 of benzoxazole), 7.74 (s, 1H, H4 of benzoxazole), 7.53-7.56 (d, 1H, J = 9 Hz, H7 of benzoxazole), 7.65 (s, 1H, H4 of benzoxazole), 7.84-7.86 (d, 2H, Hc, H6 of benzoxazole), 8.06 (s, 1H, -NH), 8.23 (d, 1H, Hd). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.42, 106.24, 110.90, 112.53, 115.86, 119.58, 125.87, 129.90, 133.33, 135.17, 138.40, 141.76, 145.68, 150.61, 162.19, 164.66. MS (m/z): 454 $[M+1]^{+\cdot}$ Anal calc for $C_{22}H_{16}ClN_3O_6$; C, 58.22; H, 3.55; N, 9.26; Found C, 58.24; H, 3.56; N, 9.28.

4.4.14. *N*-(2-(3,4-dimethoxyphenyl)-benzoxazol-5-yl)-3-nitrobenzamide (13n)

ATR-FTIR (cm-^{1}): 3289.83 (NH), 2938.40 (-CH stretching), 1670.44 (-CONH-), 1628.80, 1526.40 (C=C), 1509.14, 1392.80 (Ar-NO_2), 1488.54 (-CH bend), 1018.24 (C-O), 810.12 (oop). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.02 (s, 3H,-OCH_3), 4.13 (s, 3H, -OCH_3), 6.90-6.93 (d, 2H, J= 9Hz, He', Hb'), 6.98-7.01 (d, 1H, J= 9Hz, Hf'), 7.42-7.45 (d, 1H, J= 9Hz, H7 of benzoxazole), 7.52-7.56 (t, 1H, J= 6Hz, He), 7.63-7.70 (dd, 2H, Hf, H6 of benzoxazole),7.74 (s, 1H, H4 of

benzoxazole), 8.01 (s, 1H, -NH), 8.28-8.30 (d, 1H, J = 6 Hz, Hd), 8.74 (s, 1H, Hb). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.20, 106.64, 110.90, 112.46, 115.78, 129.81, 135.18, 141.70, 145.59, 148.45, 162.70, 164.80. MS (m/z): 420 [M +1] $^{+\cdot}$ Anal calc for $C_{22}H_{17}N_3O_6$; C, 63.01; H, 4.09; N, 10.02; Found C, 63.02; H, 4.11; N, 10.04.

4.4.15. 2-Bromo-*N*-(2-(3,4-dimethoxyphenyl)benzoxazol-5-yl)benzamide (13o)

ATR-FTIR (cm-^{1}): 3286.2 (NH), 2934.5 (-CH stretching), 1668.6 (-CONH-), 1624.2, 1528.1 (C=C), 1486.2 (-CH bend), 1016.24 (C-O), 758.22 (oop), 580 (C-Br). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.88 (s, 6H, 2 × -OCH_3), 6.77-6.79 (d, 1H, J = 6 Hz, He'), 6.89-6.91 (d, 1H, J = 6Hz, Hf'), 7.18 (s, 1H, Hb'), 7.28-7.29 (d, 1H, H7 of benzoxazole), 7.40-7.43 (t, 1H, He), 7.46-7.66 (br, 3H, H4, H6 of benzoxazole merge with Hd),7.74-7.76 (d, 1H, J = 6 Hz, Hc),7.85-7.87 (d, 1H, Hf), 8.12 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.20, 106.64, 110.90, 112.85, 115.78 , 119.59,129.81,135.18, 141.70, 145.59,162.70,164.68. MS (m/z): 454 [M +1] $^{+\cdot}$ Anal calc for $C_{22}H_{17}BrN_2O_4$; C, 58.29; H, 3.78; N, 6.18; Found C, 58.30; H, 3.79; N, 6.19.

4.4.16. 4-Methoxy-*N*-(2-(3,4-dimethoxyphenyl)benzoxazol-5-yl)benzamide (13p)

ATR-FTIR (cm-^{1}): 3306.22 (NH), 2899.32 (-CH stretching), 1680.72 (-CONH-), 1634.44, 1478.20 (C=C), 1472.12 (-CH bend), 1110.28 (C-O), 810.42 (oop).^{1}H NMR (300 MHz, $CDCl_3$): δ 3.84 (s, 6H, 2 × -OCH_3), 3.89 (s, 3H,-OCH_3), 6.86-6.98 (br, 5H, He', Hb' merge with Hf', Hc and He), 7.53-7.56 (d, 1H, J = 9 Hz, H7 of benzoxazole), 7.61-7.64 (d, 1H, H6 of bezoxazole), 7.73 (s, 1H, H4 of benzoxazole), 7.80-7.84 (dd, 2H, J = 3Hz, Hb, Hf), 8.04 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.29, 106.34, 110.92, 112.48, 114.74, 115.85, 119.48, 120.88, 135.10, 141.79, 145.94, 149.80, 162.72, 164.82. MS (m/z): 419 [M +1] $^+$. Anal calc for $C_{23}H_{20}N_2O_5$; C, 68.31; H, 4.98; N, 6.93; Found C, 68.32; H, 4.99; N, 6.95.

5. Biological evaluation

5.1. *In vitro* cyclooxygenase inhibition assay

The ability of the synthesized compounds to inhibit COX-1 and COX-2 was carried out by enzyme immunoassay (EIA) using *in vitro* enzymatic assay kit (catalog no. 560131, Cayman Chemicals Inc., Ann Arbor, MI, USA). The kit consisted of ovine COX-1 and human recombinant COX-2. To make the results comparable for *in vitro* study which is to be performed on ovine COX-1 and human COX-2, we performed the multiple sequence alignment of Human and Ovine COX-1 sequences. The sequences of both proteins were retrieved from UniProtKB

(*Homo sapiens* uniport id: P23219) and ovine COX-1 (*Ovis aries* uniport id: P05979) and multiple sequence analysis was performed using Clustal Omega [31].

The procedure by Consalvi et al.[32] was followed to perform the *in vitro* activity. At the end, the enzymatic reaction produces distinct yellow color which absorbed at 412-415 nm. The intensity of the produced color was determined spectrophotometrically (Bio-Rad ELISA), which is proportional to the amount of PG tracer bound to the well and is inversely proportional to the quantity of free PG's present in the well. The inhibitory efficacy of novel derivatives was calculated by comparison with various control incubations. The efficiencies of the test compounds that causes 50% inhibition of COX-2 was calculated as IC_{50} from the log concentration vs. % inhibition curve.

5.2. *In vivo* activity

The animals (Wistar albino rats) used in the study were procured from Animal House Center and were divided and housed in different cages at 25-28 °C, under well maintained hygienic and environmental conditions with relative humidity of 50–65%, under 12 h light and dark cycles. All animals were acclimatized for a week before use. All experimental work was conducted after receiving the approval from Institutional Animal Ethics Committee (IAEC) via protocol no. IAEC/2015-I/Prot no. 09, 10 and IAEC/2016-I/Prot no. 10, Delhi Institute of Pharmaceutical Sciences and Research, New Delhi. All results are expressed in Mean ± SEM and the *p*- values < 0.01 were statistically significant.

5.2.1. Anti-inflammatory activity

The anti-inflammatory activity of prepared benzoxazole derivatives was evaluated on Wistar albino rat by carrageenan induced rat paw edema as described by Winter et al.[33] The animals were divided into groups consisting of five rats in each group. Prepared compounds were administered orally (20 mg/kg b.wt.) and the volume of paw was determined plethysmographically (Ugo-Basyl, Italy). Control group received equivalent volume of normal saline and ibuprofen (20 mg/kg b.wt.) was administered orally to the reference group. Carrageenan (0.1 ml, 1.0% w/v in 0.9% of normal saline) was injected after half an hour into the sub-plantar tissue of the rat's hind paw. The paw volume was measured at hourly interval for 3 h (0, 1, 2 and 3 h) and the percent inhibition of edema was calculated using formula:

$$\% \text{ inhibition} = (1 - (V_s / V_c)) \times 100$$

Where,

V_s= paw volume i0n sample treated group

V_c= paw volume in control group.

5.2.2. Acute ulcerogenic activity

The ulcerogenic activity of the prepared analogs was performed according to Cioli et al.[30] Each study group consisted of five Wistar albino rats. The animals were fasted for 18 h before the administration of the test compound, while water was given continuously. The dose quantity was made three times (60 mg/kg) of the administered dose for anti-inflammatory studies (20 mg/kg). The control group received only normal saline. After 6 h of the drug administration the rats were sacrificed, stomach was removed and opened around the greater curvature. Inner lining was washed properly with distilled water followed by normal saline. The mucosal damage was examined and number of ulcers and severity index was calculated on a scale of 0 - 3, where: 0 = no lesions; 0.5 = redness; 1.0 = spot ulcers; 1.5 = hemorrhagic streaks; 2.0 = ulcers > 3 but ≤ 5; 3.0 = ulcers > 5.

5.3. *In silico* studies

5.3.1. Software

The *in-silico* experiments were performed on Fujitsu linux workstation (Xeon quad-core E3-1220 processor). Docking and ADME analyses were carried out using LigPrep 3.0, Impact 6.3, Prime 3.6, Glide 6.3 and QikProp 4.0 modules of Maestro 9.8 (Schrödinger, LLC, New York, NY, 2014-2). The ligand and protein interacting behavior was studied using Protein-ligand profiler + server.[34]

5.3.2. Docking Study

The molecular docking study was performed using crystal structure of human COX-2 (PDBID: 5F19). The acetylated Ser530 of this structure was mutated to Ser530, as this structure is reported as aspirin-acetylated human COX-2[35].This protein structure was prepared using Protein Preparation Wizard (Impact 6.3, Schrodinger) as previously described[36-38]. In brief, the structure was processed for addition of hydrogen atoms, formal charges treatment and assignment of correct bond orders. Structures of the molecules were sketched and prepared using LigPrep 3.0 with Epik 2.8 and tautomeric state and protonation states were expanded at 7.0 ± 2.0 pH units. The OPLS 2005 force field was used for both molecules and protein minimization. The glide grid

was generated by specifying the centroid of the residues His90, Thr94, Arg120, Gln192, Tyr348, Val349, Leu352, Ser353, Gly354, Tyr355, Leu359, Phe381, Leu384, Tyr385, Trp387, Arg513, Ala516, Ile517, Phe518, Gly519, Met522, Val523, Gly526, Ala527, Ser530 and Leu531. Prepared and minimized small molecules were docked into the minimized protein structure using Glide 6.3 XP docking. The 3D complex structures of all molecules were analyzed for docking score H-bonding, salt bridge, π-π and π-cation interactions.

5.3.4. Absorption, Distribution, Metabolism, Excretion and Toxicity (ADME/T) study

The ADME/T properties are imperative to decide the role of a new molecule in drug development process and so are considered as crucial for rational drug design. The lack of best fit ADME/T parameters of molecules leads to its denunciation in the progressive stages of drug development process. All the newly synthesized small molecules were analyzed for ADME/T parameters by QikProp 4.0[39-42].

The *in silico* module provides the vision into vital properties like stability (no. of reactive functional groups, no of hydrogen bond donor and acceptors, no of metabolic reactions), druggable behavior and its pharmacokinetics (molecular weight, CNS activity, violation of Lipinski rule of five, octanol/water coefficient, brain/blood partition coefficient, aqueous solubility, skin permeability, binding to human serum albumin, human oral absorption , IC_{50} value for HERG K^+ channel, caco-2 cell permeability, MDCK cell permeability)[43].

The drug's permeability and transporter interactions during drug discovery and development process can be successfully accessed by the human colon adenocarcinoma (Caco-2) and Madin-Darby canine kidney (MDCK) epithelial cell lines which are used as *in vitro* probes. This study has considerable importance in predicting absorption, permeability mechanism, effect of formulation on permeability and probability for transport mediated drug-drug interactions[44].

The HERG channel which is voltage gated potassium channel encoded by Human ether-a-go-go related gene (HERG). These channels are actively involved in cardiac action potential repolarization. The reduced activity of HERG leads to lengthening of ventricular action potentials and extension of the QT interval in an electrocardiogram which in turn increases the risk for fatal ventricular arrhythmias. Therefore, screening of compounds for activity on HERG channels during preclinical safety studies reduces the risk of failure of compounds because of QT prolongation[45].

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Conflict of Interest

The authors have declared no conflict of interest.

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Highlights

- Synthesis of novel series of 3,4-dimethoxyphenyl substituted benzoxazole derivatives
- *In vitro* biological evaluation of compounds by COX-2 enzymatic inhibition assay
- *In vivo* biological evaluation by anti-inflammatory and acute ulcerogenic activity.
- *In silico* study by molecular docking of ligands using crystal structure of COX-2.
- Compounds **13d**, **13h, 13k** and **13l** showed most promising activity.