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DESIGN, SYNTHESIS, AND EVALUATION OF 2-PHENYL AMINO BENZIMIDAZOLE HYBRIDS AS POTENT ANTI-INFLAMMATORY AGENTS

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ABSTRACT
In present study, a series of 3-[N-(2-hydrazine-acetyl)-2-phenyl amino methyl]-1H benzimidazole derivatives (6a-6u) were designed. Molecular docking (MD) studies were conducted to select compounds with high binding affinity against the COX-2 enzyme (PDB ID: 3LN1). IR, 1H NMR, and mass spectral analysis characterised all test compounds. The synthesised compounds were screened for *in vivo* anti-inflammatory activities by the carrageenan-induced paw edema method and analgesic activities by the acetic acid-induced writhing method. Compounds **6c** and **6r** showed the highest anti-inflammatory activities (% inhibition of 95.71% and 100% at 20 mg/kg b.w.) and analgesic activities (86.66% and 89.33% at 100 mg/kg b.w.). All compounds were non-carcinogenic, non-toxic, and possessed favourable drug-like properties.
Keywords: Benzimidazole, Inflammation, COX-II, MD, Indomethacin
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INTRODUCTION

Inflammation is a localised response of the vascular components of tissue to injury, resulting in the formation of protein-rich exudates.[1,2] This process serves as a protective mechanism, aiming to localize[3], neutralise, or destroy harmful agents during healing. The characteristics of acute inflammation include the exudation of fluid and plasma proteins[3], as well as leukocyte migration. Chronic inflammatory conditions can create a vicious cycle in which inflammation and related pathological states[4], such as obesity, can lead to further inflammation. Benzimidazole, a notable nitrogen-containing heterocyclic system[5], consists of a benzene ring fused to an imidazole ring (Fig.-1), conferring diverse biological activities, including antimicrobial[6], antitumor[7,8], anti-inflammatory[9], antidiabetic[10], antioxidant[11], antiviral[12], and antiseizure effects[13]. Prolonged use of selective cyclooxygenase (COX) II inhibitors such as indomethacin, ibuprofen, and aspirin produces harmful effects on the gastric mucosa and kidneys. Therefore, it is crucial to develop newer COX-II inhibitors that do not cause gastrointestinal adverse effects. It has been reported that the nature of the substituent at the 2-position of the benzimidazole ring plays a crucial role in anti-inflammatory activity. In the present study, 3-[N-(2-hydrazine-acetyl)-2-phenyl amino methyl]-1H benzimidazole derivatives were designed, synthesised, and screened for *in vivo* anti-inflammatory activity.

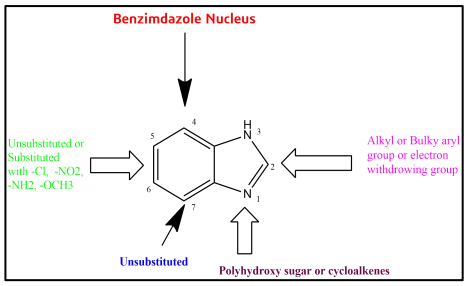

Fig.-1: Structural Requirement for the Benzimidazole Nucleus for Anti-Inflammatory Activity

EXPERIMENTAL

Chemicals and Reagents

All chemicals and solvents were obtained from Merck Lab and S.D. Fine Chemicals, Delhi.

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Software's

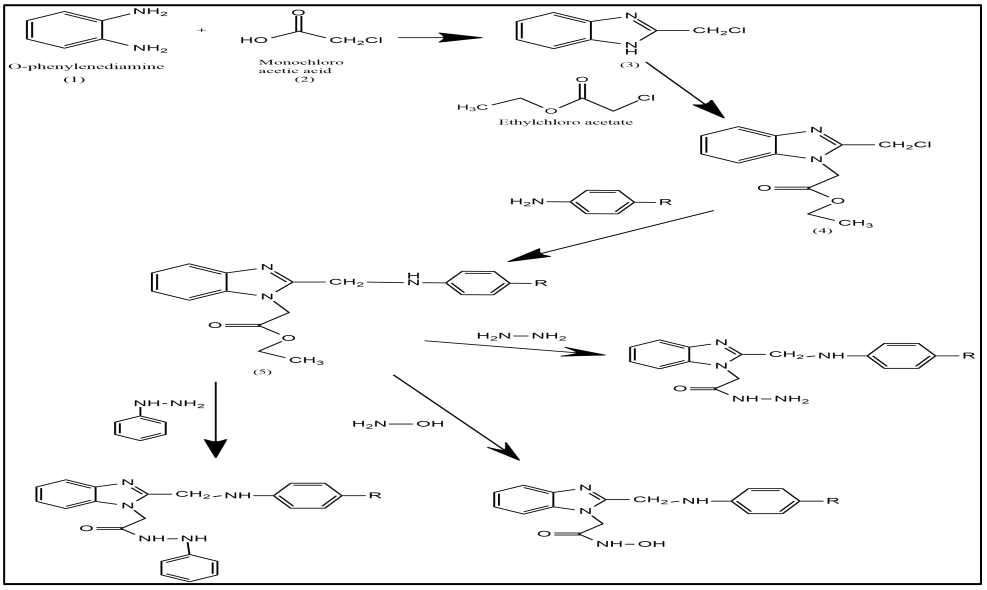
In silico analysis was performed by using PyMOL by Schrödinger LLC, New York, USA, 2020. ChemDraw Ultra 12 Software was used to draw the Chemical Scheme and Structure. Discovery Studio Visualizer (DSV) software 2023 was used to study the interaction of amino acids with the compound.

Preparation of Ligand and Receptor (Protein)

The ChemDraw 12 Ultra Software was used to prepare the ligand. For docking, the Protein Data Bank (PDB) Protein ID: 3LN1 (www.rcsb.org/pdb) was used.[14] After the ligand and receptor production, alterations to the molecules were made, such as the removal of the water molecule, the insertion of polar hydrogen, and the addition of collaman charges. After the simulation was finished, it was converted into a pdbqt file for further analysis.

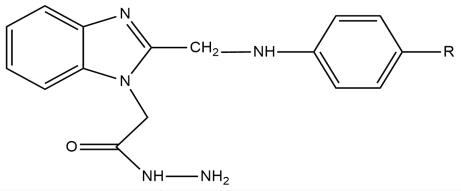
Chemistry

2-chloromethyl 1-H-benzimidazole was obtained by refluxing o-phenylenediamine dihydrochloride (0.01 mol) & chloroacetic acid (0.03 mol) in ethanol for four hours. 2-chloromethyl benzimidazole was prepared. Ethyl chloroacetate (11 mmol) was dissolved in 10 mL of acetone. The solution was mixed with nine mmol of 2-methyl benzimidazole in 10 mL of acetone while stirring continuously. This reaction mixture was treated with 5 mL of acetone & NaHCO3 (12.5 mmol). Ethyl chloroacetate (11 mmol) was dissolved in 10 mL of acetone. The resultant solution was mixed with nine mmol of 2-methyl benzimidazole in 10 mL of acetone. This reaction mixture was treated with 5 mL of acetone and sodium bicarbonate (12.5 mmol) (Scheme-1).

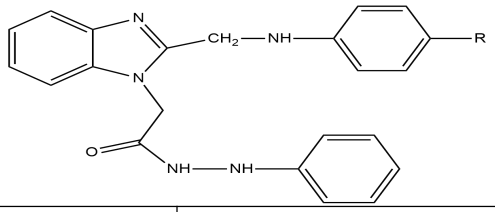


Scheme-1: Synthesis of 3-[N-(2-hydrazine-acetyl)-2-phenyl amino methyl]-1H Benzimidazole Derivatives

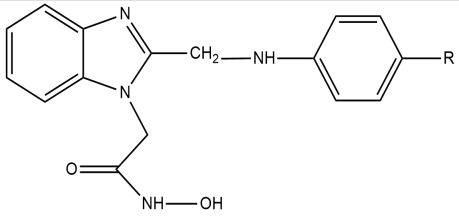
Table-1: Selected Benzimidazole Derivatives



Compound	R
6c	OH
6g	NO_2

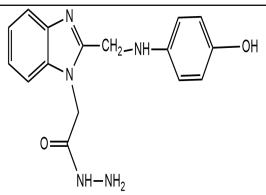
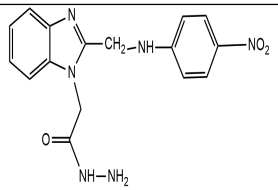


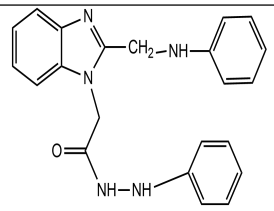
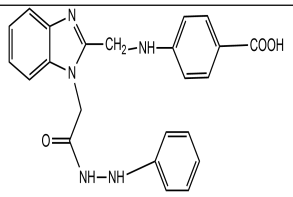
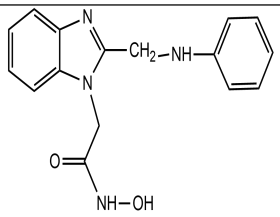
Compound	R
6h	H
6i	COOH

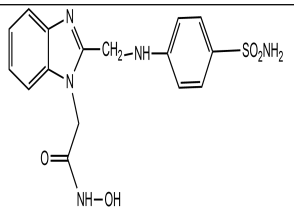


Compound	R
6o	H
6r	SO_2NH_2

Table-2: Spectral Data of Selected Compounds

Comp	Structure	m.p. °C	IR Spectra	NMR value in ppm		Mass At m/z (Mþ 1)
				^1H	^{13}C	
6c		275-285	3440 cm^{-1} – NH stretching, for benzimidazole ring NH 3221 cm^{-1} – NH bending for secondary amide 1642 cm^{-1} C-N bending, for the imidazole ring	3.12 (s, 2H, CH_2) 5.3 (s, 1H, NH, disappeared on D_2O addition) 6.5 (s, 1H, NH, disappeared on D_2O addition) 6.8–7.46 (m, Ar-H)	56, (Methylene carbon) 123–134 (Ar-Carbons) 148, (C]N carbon) 163, (N–C carbon)	254.09
6g		273-283	3458 cm^{-1}– NH bending, for benzimidazole ring NH 3361 cm^{-1} – NH stretching for secondary amide 1636 cm^{-1} CN stretching,	2.39 (s, 2H, CH_3) 2.65 (s, 1H, NH, disappeared on D_2O addition) 6.8 (s, 1H, NH, disappeared on D_2O addition) 5.4–8.6 (m, Ar-H)	51, (Methylene carbon) 54, (Methoxy carbon) 114–142 (Ar-Carbons) 144, (C]N carbon) 153, (N–C carbon)	235.85

			for imidazole ring			
6h		268-278	3659 cm^{-1} – NH bending, for benzimidazole ring NH 3216 cm^{-1} – NH stretching for secondary amide 1642 cm^{-1} C-N stretching, for imidazole ring	2.65 (s, 2H, CH_3) 3.36 (s, 1H, NH, disappeared on D_2O addition) 5.61 (s, 1H, NH, disappeared on D_2O addition) 7.4–7.9(m, Ar-H)	52, (Methylene carbon) 113–146 (Ar-Carbons) 143, (C]N carbon) 147, (N–C carbon)	256.63
6i		287-297	3540 cm^{-1} – NH stretching, for benzimidazole ring NH 3385 cm^{-1} – NH stretching for secondary amide 1627 cm^{-1} C-N bending, for imidazole ring	2.12 (s, 2H, CH_2) 4.39 (s, 1H, NH, disappeared on D_2O addition) 6.46 (s, 1H, NH, disappeared on D_2O addition) 6.8–8.2 (m, Ar-H)	52, (Methylene carbon) 112–146 (Ar-Carbons) 144, (C]N carbon) 148, (N-C carbon)	243.71
6o		275-285	3438 cm^{-1} – NH stretching, for benzimidazole ring NH 3474 cm^{-1} – NH streching for secondary amide 1676 cm^{-1} C-N bending, for imidazole ring	2.18 (s, 2H, CH_2) 4.1 (s, 1H, NH, disappeared on D_2O addition) 6.8 (s, 1H, NH, disappeared on D_2O addition) 6.32–7.87 (m, Ar-H)	52, (Methylene carbon) 56, (Methoxy Carbon) 112–140(Ar-Carbons) 140, (C]N carbon) 149, (N–C carbon)	264.35

6r		274-284	3430 cm^{-1} – NH stretching, for benzimidazole ring NH 3321 cm^{-1}– NH bending for secondary amide 1612 cm^{-1} C-N stretching, for the imidazole ring	2.19 (s, 2H, CH_2) 4.2 (s, 1H, NH, disappeared on D_2O addition) 6.8 (s, 1H, NH, disappeared on D_2O addition) 6.32–8.63 (m, Ar-H)	52, (Methylene carbon) 120–141(Ar-Carbons) 152, (C]N carbon) 154, (N–C carbon)	284.41

In-silico Studies

Molecular Docking

Molecular docking studies were performed using AutoDock Vina software to gain a deeper understanding of the binding interaction of the produced compounds (6a-6u) with the COX-II enzyme (Fig.-3). Twenty-one compounds were evaluated for molecular docking simulations against the COX-II enzyme (Table-3). Compounds 6c, 6g, 6h, 6i, 6o, and 6r have highest docking scores (Fig.-2).

Table-3: Compounds with Docking Score

Compound	Docking Affinity (kCal/mol)	Compound	Docking Affinity (kCal/mol)
6a	-8.2	6l	-8.1
6b	-7.0	6m	-8.1
6c	-8.6	6n	-7.9
6d	-7.3	6o	-8.3
6e	-8.4	6p	-7.4
6f	-7.7	6q	-7.0
6g	-9.2	6r	-8.7
6h	-10.0	6s	-6.4
6i	-8.7	6t	-6.9
6j	-8.1	6u	-7.4
6k	-7.9	Indomethacin	-7.3

Lipinsky Rule of Five Properties

It is a simple molecular descriptor used by Lipinski[14]. The rule states that most drugs like molecules have a log p value less than or equal to five, a molecular weight less than or equal to 500 Dalton[15], several hydrogen bond acceptors less than or equal to ten, and several hydrogen bond donors less than or equal to five.

Table- 4: % Edema Inhibition of Comp and Standard Drug

Compound	Edema Inhibition %				
	0.5h	1h	2h	3h	4h
Control	-	-	-	-	-
6c	34.24	46.51	59.67	78.28	95.71
6g	28.12	39.74	55.26	71.65	84.64
6h	22.24	36.24	49.18	64.87	81.35
6i	26.42	40.78	55.97	74.53	93.47
6o	29.54	41.45	58.76	72.27	89.52
6r	36.87	54.42	68.64	84.74	100
Indomethacin	32.81	51.34	64.28	84.24	100

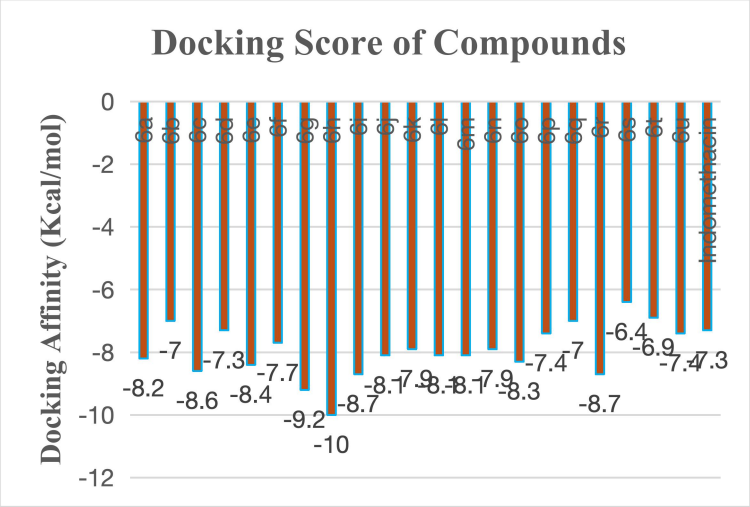


Fig.-2: Docking Score of Selected Compounds

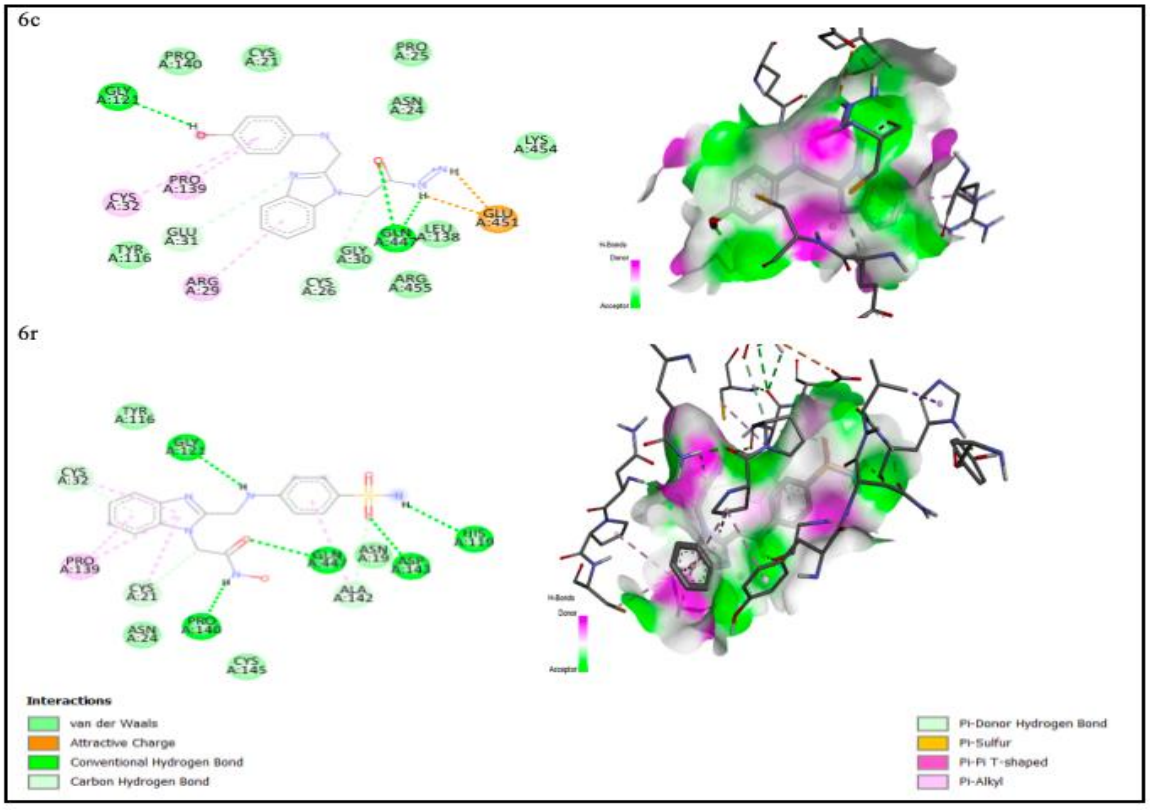


Fig.-3: Three Dimensional and Two-Dimensional Binding Poses of 6c and 6r with COX Enzyme (PDB ID:3LN1)

Table-5: ADME Parameters of Selected Compounds

Comp.	Formula	MW	Heavy atoms	Aromatic heavy atoms	Fraction Csp3	MR	TPSA
6c	C16H17N5O2	311.34	23	15	0.12	87.33	105.2
6g	C16H16N6O4	356.34	26	15	0.12	95.09	140.02
6h	C22H20N4O	356.42	27	21	0.09	108.62	58.95
6i	C23H20N4O3	400.43	30	21	0.09	115.58	96.25
6o	C16H16N4O2	296.32	22	15	0.12	83.33	79.18
6r	C16H16N4O3	312.32	23	15	0.12	85.35	99.41

Pharmacology

Animals

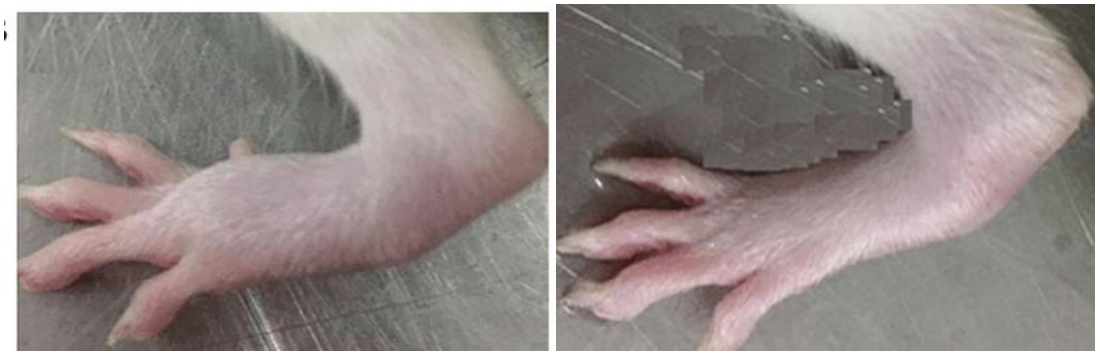
Ten-week-old Wistar rats (150-200 g) were procured from the National Toxicological Research Centre in Pune and maintained in a ventilated room at 25±2°C under a 12 h light/dark cycle. The institutional Animal Ethics Committee accepted the study protocol (ReBi, Reg. No. 316/CPCSEA: Dated 22-12-2024) before the experiment. Animals are divided into the following study groups, each containing six animals.

Carrageenin-Induced Paw Edema Method

The study protocol received approval from the Institutional Animal Ethics Committee (IAEC)[16], as indicated by the registration number 316/CPCSEA. The control group was administered a 0.1% carboxymethyl cellulose (CMC) solution. Subsequently, a 0.1% carrageenan solution in saline was injected subcutaneously into the right hind paw of each rat[19](Fig.-4). The percentage of edema inhibition was calculated based on the mean effect observed in both the control and treated groups, using the following equation:

$$\text{Inhibition of paw edema (\%)} = [(E_c - E_T)/ E_c] \text{ X100}$$

Where E_T represents the mean increase in paw volume in the rat,
Ec represents the mean increase in paw volume in the control group of rats



Rat paw edema after 4 hours of administration

Fig.-4: A comparison of the Rat Paw before and after Edema

Table-6: Edema Inhibition (%) Test of Selected Compound Relative to Indomethacin

Compound	Edema Inhibition %				
	0.5h	1h	2h	3h	4h
Control	-	-	-	-	-
6c	34.24	46.51	59.67	78.28	95.71
6g	28.12	39.74	55.26	71.65	84.64
6h	22.24	36.24	49.18	64.87	81.35
6i	26.42	40.78	55.97	74.53	93.47
6o	29.54	41.45	58.76	72.27	89.52
6r	36.87	54.42	68.64	84.74	100
Indomethacin	32.81	51.34	64.28	84.24	100

Analgesic Activity

The analgesic activity of the synthesised compounds was evaluated using the acetic acid-induced writhing method[3]. Based on the structure-activity relationship (SAR) studies[1], nearly all the compounds demonstrated highly potent analgesic activity[1] compared to the standard indomethacin drug (Fig.-6). Among the tested compounds, 6c and 6r showed significant analgesic activity (86.66% and 89.33% at 100mg/kg b.w) (Table-7).

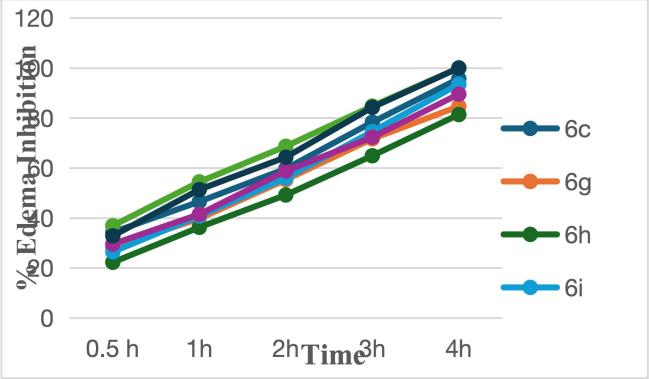


Fig.-5: Graphical Representation of % Inhibition of Selected Compounds and Indomethacin (20mg/kg b. w)

Table-7: Analgesic Activity of Test Compound (100 mg/kg b.w) and Indomethacin (50 mg/kg b.w)

Compound	Mean writhing (X ± SE)	Protection (%)
Control	30.0 ± 1.25**	-
6c	4.52 ± 2.41**	86.66
6g	6.5 ± 1.47**	80.00
6h	6.5 ± 1.95**	80.00
6i	9.14 ± 2.45**	70.66
6o	7.36 ± 2.26**	78.66
6r	3.38 ± 1.12**	89.33
Indomethacin	-	100.00

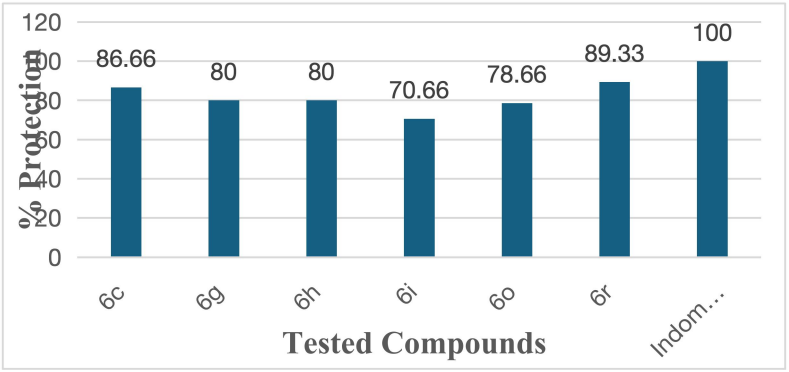


Fig.-6: Analgesic Activity of Test Compound (100 mg/kg b.w) and Indomethacin (50 mg/kg b.w)

Acute Toxicity Study

Acute oral toxicity & estimated lethal dose (LD50) of prospective candidates were assessed in Wistar rats. Each group had six animals, and they were given oral dosages of test chemicals ranging from 200 to 500 mg/kg body weight.[16] The toxic symptoms and fatality rates in each group were evaluated 24 hours following medication delivery.

CONCLUSION

Here, a series of 3-[N-(2-hydrazine-acetyl)-2-phenyl amino methyl]-1H benzimidazole derivatives were synthesized. These compounds possess a key structural feature necessary for COX-II inhibition. Molecular modelling reveals unique binding modes of selected compounds with the target protein. Based on in vivo results, compounds 6c and 6r showed the highest anti-inflammatory activities (% inhibition of 95.71% and 100% at 20 mg/kg b.w.) and analgesic activities (86.66% and 89.33% at 100 mg/kg b.w.). Therefore, compounds 6c and 6r are very promising drugs and warrant further development into safer anti-inflammatory agents.

ACKNOWLEDGMENTS
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ETHICAL APPROVAL
All experimental procedures involving animals were conducted with the approval of the Institutional Animal Ethics Committee (IAEC) ReBi, Reg. No. 316/CPCSEA.

CONFLICT OF INTERESTS
The authors have no competing interests.

AUTHOR CONTRIBUTIONS
The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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