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ORIGINAL PAPER

In vitro, in vivo and in silico-driven identification of novel benzimidazole derivatives as anticancer and anti-inflammatory agents

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Abstract

The synthesis of novel benzimidazole derivatives with varied carbon chain length was achieved via "one-pot" nitro reductive cyclization (**6a–o**). In each case, compounds were determined by the elemental analyses, FT-IR, mass, ^{1}H and ^{13}C NMR spectroscopy. Further, these derivatives were screened for their in vitro anticancer, in vitro and in vivo anti-inflammatory activities. The results revealed that the length of the carbon chain greatly affects the activity. Among the 15 derivatives, compound **6d** induced maximum cell death in HeLa and A549 cell lines and compound **6a** emerged as a potent anti-inflammatory agent. Also, the physicochemical properties of potent compounds were studied.

Keywords Benzimidazole · Anticancer · Anti-inflammatory · ADMET

Introduction

Benzimidazole and its derivatives are important nitrogen heterocycles, having significant applications in both pharmaceutical and technological fields. From a medicinal chemistry point of view, benzimidazole has been used as privileged scaffolds in numerous therapeutic areas such as antimalarial [1], pancreatic lipase inhibitor [2], antihypertensive [3], antiviral [4], antioxidant [5], antimicrobial [6], anticancer [7] and antiparasitic [8]. Omeprazole, glasdegib, crenolanib, and rabeprazole (Fig. 1) are some of the marketed drugs which contain benzimidazole nucleus [9]. Benzimidazole derivatives can easily interact with the biomolecules in living organisms because of their structural isosteres of naturally occurring nucleotides [10].

Cancer which is the second leading root of death is caused due to mutation in normal cells [11]. The widespread malignant disease affecting women is cervical cancer [12]. Also, lung cancer is a well-known form of cancer caused due to smoking and the consumption of tobacco [13]. Deaths due to cancer are predictable to augment over 13.1 million in 2030 [14]. The existence of chemotherapy and radiotherapy poses excruciating side effects. For this reason, there is still an inflated demand for novel anticancer drugs with a diminution of side effects and high tumor selectivity. Thus, the development of potent and effective antineoplastic drugs is one of the most persuaded goals. The benzimidazole-based approved anticancer drugs are Bendamustine [15] and Veliparib [16] (Fig. 1). Henceforth, novel benzimidazole derivatives were designed and synthesized to check for better anticancer activity.

Inflammation can be differentiated macroscopically as tumor (swelling), dolor (pain), rubor (redness), loss of function and calor (heat). Meticulously, it is experienced in expanding vascular permeability, inflammatory cell infiltration and vasodilation [17]. The key role in the production of inflammatory response is prostaglandins (PGs). The generation of PGs depends on PGG/H synthases, which are popularly known as COXs. COXs exist as COX-1 and COX-2 isomers [18]. 5-Lipoxygenase (5-LO) and cyclooxygenase (COX) are the

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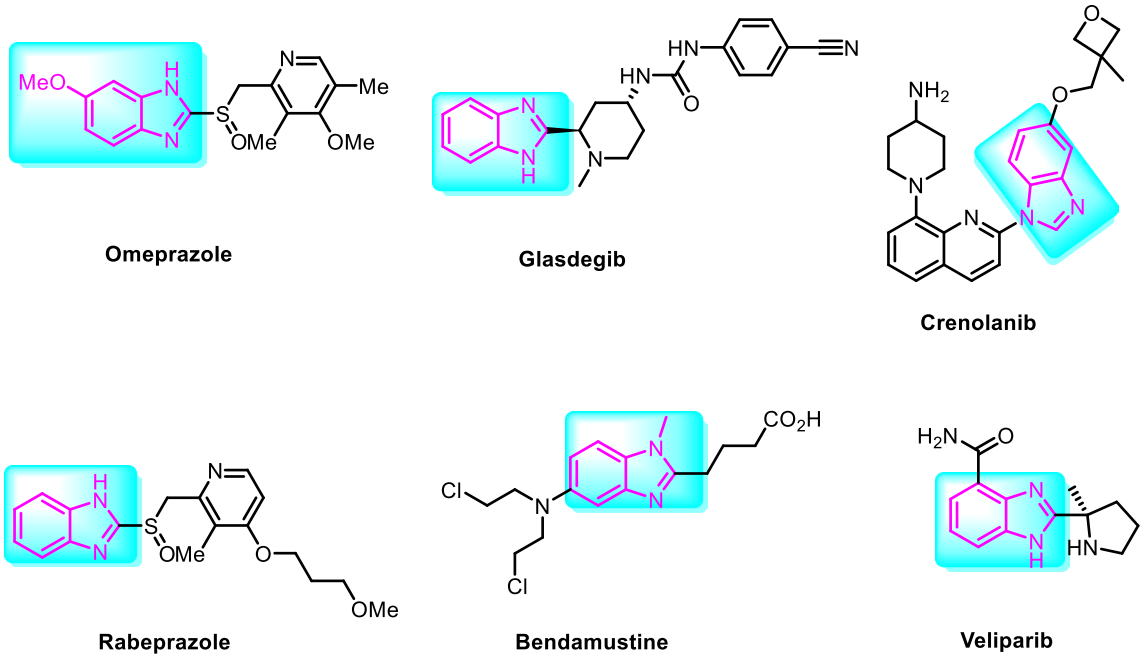


Fig. 1 Marketed drugs having benzimidazole moiety

lead enzymes that enact in the metabolism of arachidonic acid (AA) to pro-inflammatory prostaglandins (PGs) and leukotrienes (LTs), respectively. The main function of nonsteroidal anti-inflammatory drugs (NSAIDS) is to inhibit the COX enzyme which lessens the cytoprotective PGs and improves the transformation of AA to LTs by the 5-LO enzyme [19]. Over 30 million people practice NSAIDs which are utmost used drugs all over the world. NSAIDs are believed to have good anti-inflammatory, analgesic and anti-pyretic therapeutic assets. But it includes a wide variety of side effects such as renal toxicity, weakening of congestive heart failure and increased blood pressure [20]. Drugs with twofold inhibition of 5-LO and COX would be of great interest. Although the huge number of anti-inflammatory drug exist in the market, it is still a challenging task for scientists to find out new and effective drugs against anti-inflammatory with fewer side effects.

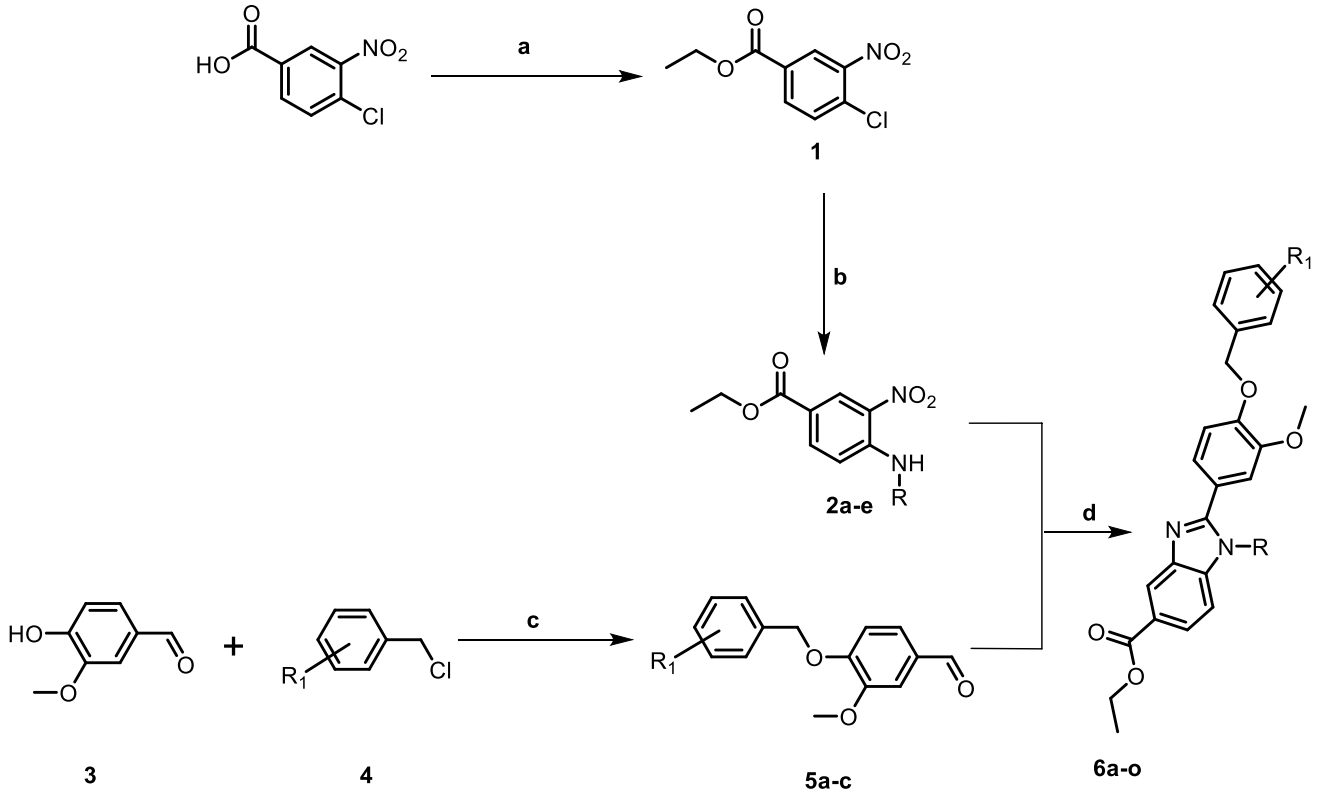
Benzimidazole represents a class of compounds that has awakened an interest in our research group, its synthesis and applications [21, 22]. This article describes the synthesis of novel benzimidazole derivatives as promising anticancer and anti-inflammatory agents. Also, in silico study of potent compounds has been determined.

Results and discussion

Chemistry and spectral analysis

The synthetic pathway for compounds **6a–o** is presented in Scheme 1. According to the reported procedure [21], esterification was carried out under reflux condition using a catalytic amount of conc. H_2SO_4 to afford intermediate **1,** followed by nucleophilic substitution reaction by primary amines bearing varied alkyl chain length in presence of triethylamine as a base in dry THF to afford intermediate **2**. Further, substituted 4-(benzyloxy)-3-methoxybenzaldehyde (**5**) was prepared by refluxing vanillin with substituted benzyl chlorides using potassium carbonate as a base in DMF media for 3 h. The subsequent one-pot reaction between intermediate **2** and **5** in dry DMSO using sodium dithionite as a reducing agent produced the title benzimidazole derivatives (**6a–o**). The physical properties are compiled in Table S1 (ESI).

The structures of the intermediate and the target compounds were confirmed by ^{1}H NMR, ^{13}C NMR, FT-IR, mass spectrum (MS) and elemental analyses. The formation of **2e** was supported by FT-IR spectrum which showed



2a: R = CH_3; 2b: R = C_3H_7; 2c: R = C_4H_9; 2d: R = C_5H_{11}, 2e: R = C_6H_{13}; 5a: R_1 = 4-Cl; 5b = 2,4-Cl_2; 5c = 3-F

6a: R = CH_3, R_1 = 4-Cl; 6b: R = C_3H_7, R_1 = 4-Cl; 6c: R = C_4H_9, R_1 = 4-Cl; 6d: R = C_5H_{11}, R_1 = 4-Cl; 6e: R = C_6H_{13}, R_1 = 4-Cl; 6f: R = CH_3, R_1 = 2,4-Cl_2; 6g: R = C_3H_7, R_1 = 2,4-Cl_2; 6h: R = C_4H_9, R_1 = 2,4-Cl_2; 6i: R = C_5H_{11}, R_1 = 2,4-Cl_2; 6j: R = C_6H_{13}, R_1 = 2,4-Cl_2; 6k: R = CH_3, R_1 = 3-F; 6l: R = C_3H_7, R_1 = 3-F; 6m: R = C_4H_9, R_1 = 3-F; 6n: R = C_5H_{11}, R_1 = 3-F 6o: R = C_6H_{13}, R_1 = 3-F.

Scheme 1 The synthesis of target compounds (**6a–o**). Reagents and conditions: **a** H_2SO_4, dry ethanol, 16 h; **b** n-alkylamine, triethylamine, room temperature; **c** K_2CO_3, DMF, 3 h; **d** substituted benzyloxybenzaldehydes, sodium dithionite, 90 °C, 3 h

N–H stretching vibration at 3371 cm^{-1}, C=O stretching vibration at 1710 cm^{-1} and C–O stretching vibration at 1018 cm^{-1}. In its ^{1}H NMR spectrum (Figs. 2, 3, 4), doublet resonated at δ 8.79 ppm with coupling constant $J = 2.4$ Hz indicates the meta-coupling which corresponds to the 2nd proton of the phenyl ring. A broad singlet that appeared at δ 8.27 ppm indicates the presence of NH proton. A doublet of doublet at δ 7.96–7.99 ppm with coupling constants $J = 2.0$ and 8.8 Hz was due to meta and ortho coupling of 6th proton of the phenyl ring, and a doublet resonated at δ 6.78 ppm with coupling constant $J = 9.2$ Hz was due to ortho coupling of 5th proton of the phenyl ring. The appearance of a quartet at δ 4.26–4.31 ppm indicates the presence of ester CH_2. The hexyl chain length protons resonated in the region δ 0.82–3.25 ppm. The mass spectrum showed the protonated peak at *m/z* 295.10 in agreement with its molecular formula $C_{15}H_{22}N_2O_4$.

Now taking the final compound **6h** to consideration, the =C–H stretching vibration appeared at 3408 cm^{-1}, the sharp absorption peak at 1708 cm^{-1} indicated the presence of the C=O group, and C–Cl stretching vibration appeared at 746 cm^{-1}. Also, ^{1}H NMR (Figs. 5, 6, 7) supports the formation of the final compound by the appearance of singlet at δ 8.49 ppm and a doublet of doublet centered at δ 8.00–8.03 ppm with coupling constants $J = 1.2$ and 8.8 Hz which corresponds to 4th and 6th proton of benzimidazole ring, respectively. The two protons at the 6th and 5th position of 3-methoxyphenyl ring appeared as a doublet and doublet of doublet centered at δ 7.49 and 7.22–7.24 ppm, integrating for one proton each. The two protons at the 5th

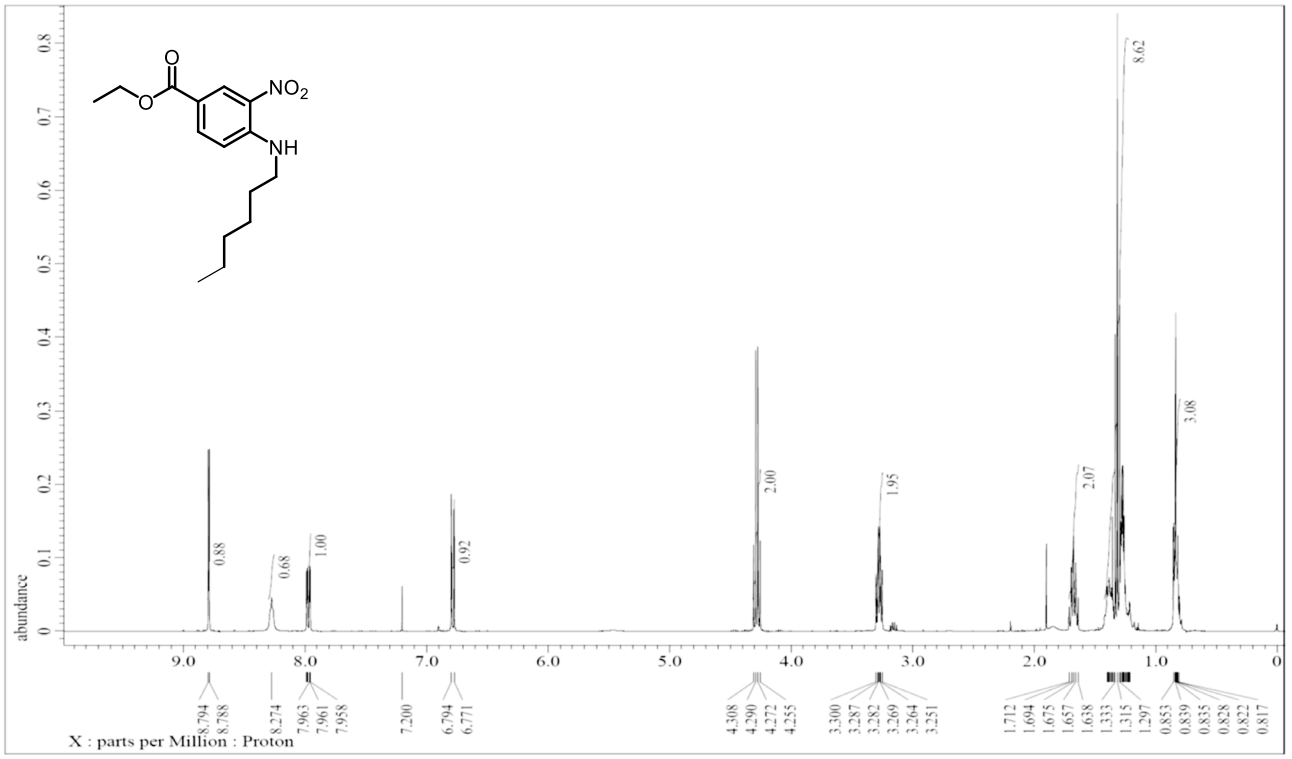


Fig. 2 ^{1}H NMR spectrum of ethyl 4-(hexylamino)-3-nitrobenzoate (**2e**)

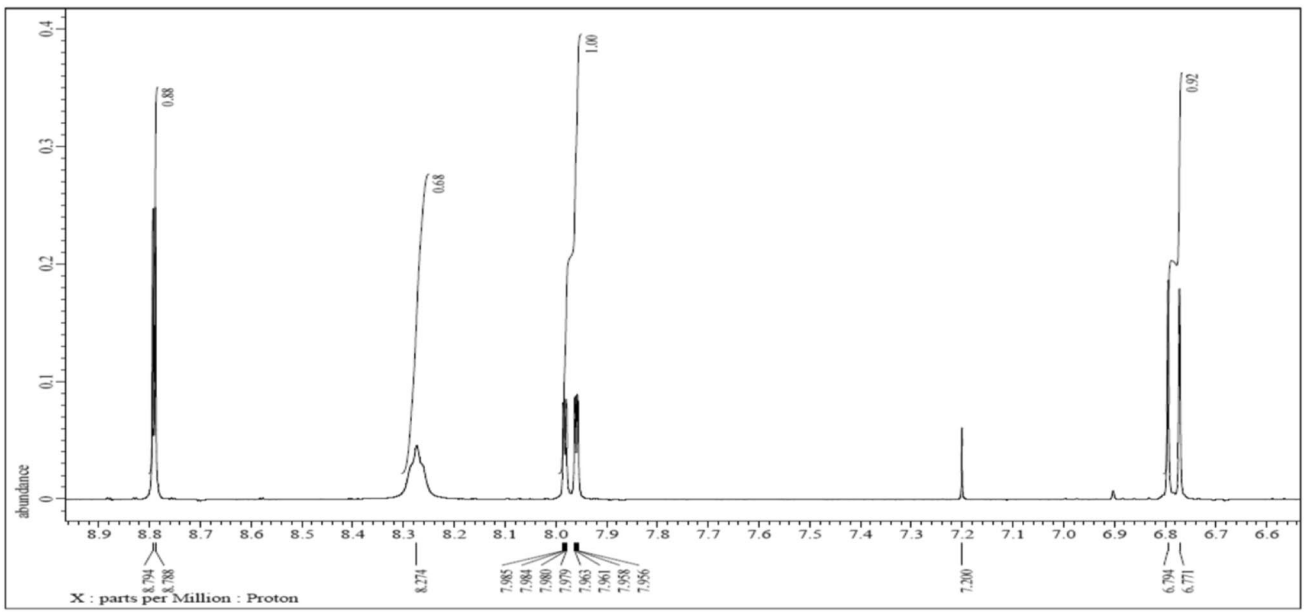


Fig. 3 Expanded ^{1}H NMR spectrum of ethyl 4-(hexylamino)-3-nitrobenzoate (**2e**)

and 6th position of the 2,4-dichlorophenyl ring appeared as a doublet of doublet and doublet resonated at δ 7.12–7.14 and 6.93 ppm, respectively, integrating for one proton each. The remaining three aromatic protons appeared as a multiplet in the region δ 7.33–7.37 ppm. The presence of the ester group was confirmed by the appearance of a quartet and a triplet resonated at δ 4.33–4.93 and 1.36 ppm, respectively. A sharp singlet resonating at δ 5.21 ppm corresponds to

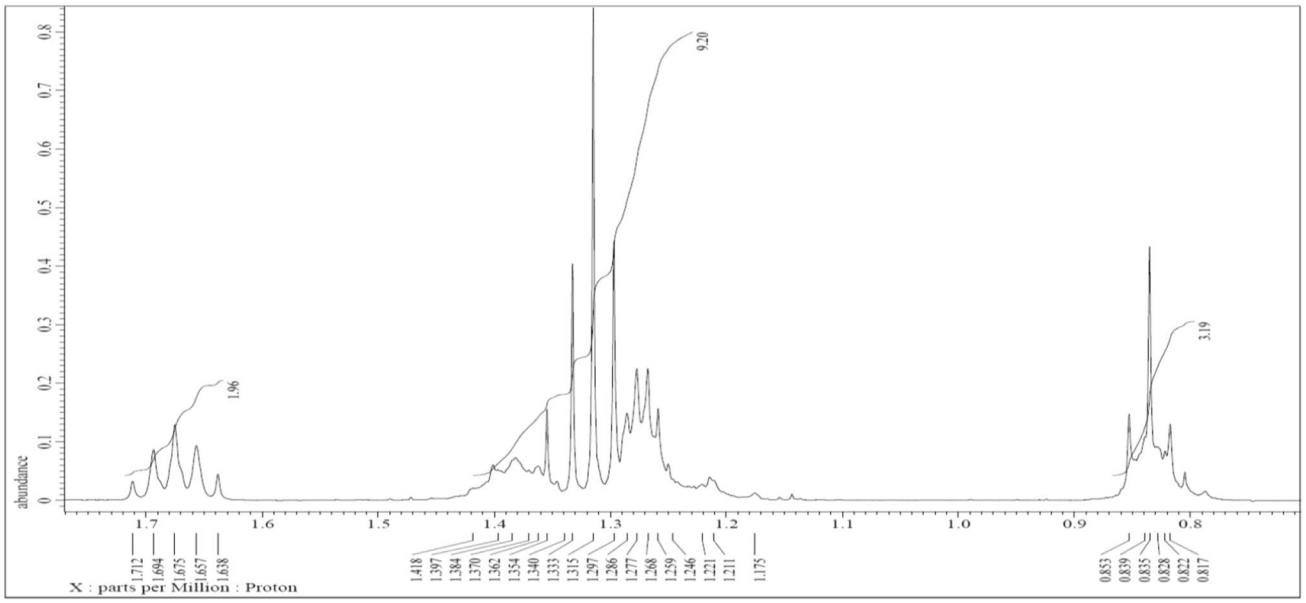


Fig. 4 Expanded ^{1}H NMR spectrum of ethyl 4-(hexylamino)-3-nitrobenzoate (**2e**)

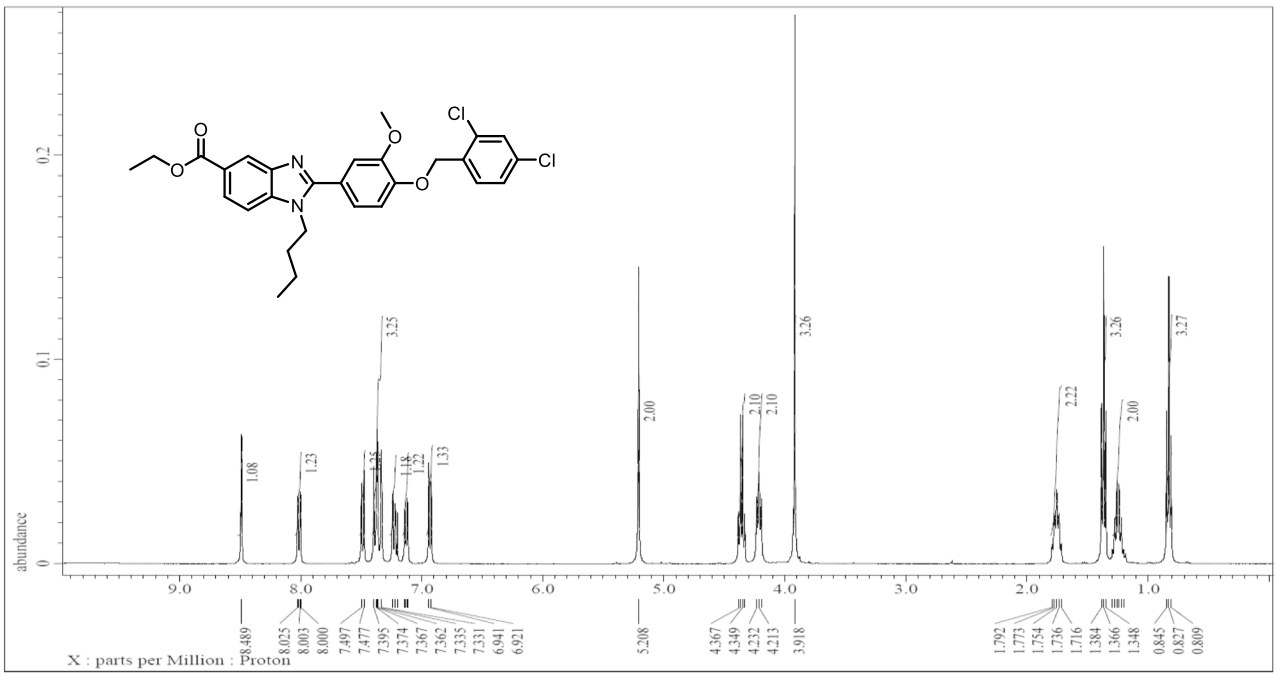


Fig. 5 ^{1}H NMR spectrum of ethyl 1-butyl-2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**6h**)

benzyl protons. The presence of a triplet (δ 4.21 ppm), quintet (δ 1.71–1.79 ppm), sextet (δ 1.20–1.29) and triplet (δ 0.83 ppm) confirmed the presence of butyl chain. The methoxy protons appeared as a singlet at δ 3.91 ppm. Its ^{13}C spectrum (Fig. 8) showed a signal corresponding to carbonyl carbon at δ 170.1 ppm. The aliphatic carbon appeared at δ 67.5, 61.1, 56.3, 45.0, 31.7, 19.9, 14.3 and 13.5 ppm. The aromatic carbon resonated at 166.7, 154.5, 150.0, 149.7, 139.7, 137.6, 134.2, 132.9, 132.8, 129.5, 129.2, 127.4, 125.9, 124.9, 121.8, 121.2, 120.9, 113.4 and 110.1 ppm. The mass spectrum showed the protonated peak at *m*/*z* 527.15 in agreement with its molecular formula $C_{28}H_{28}Cl_2N_2O_4$.

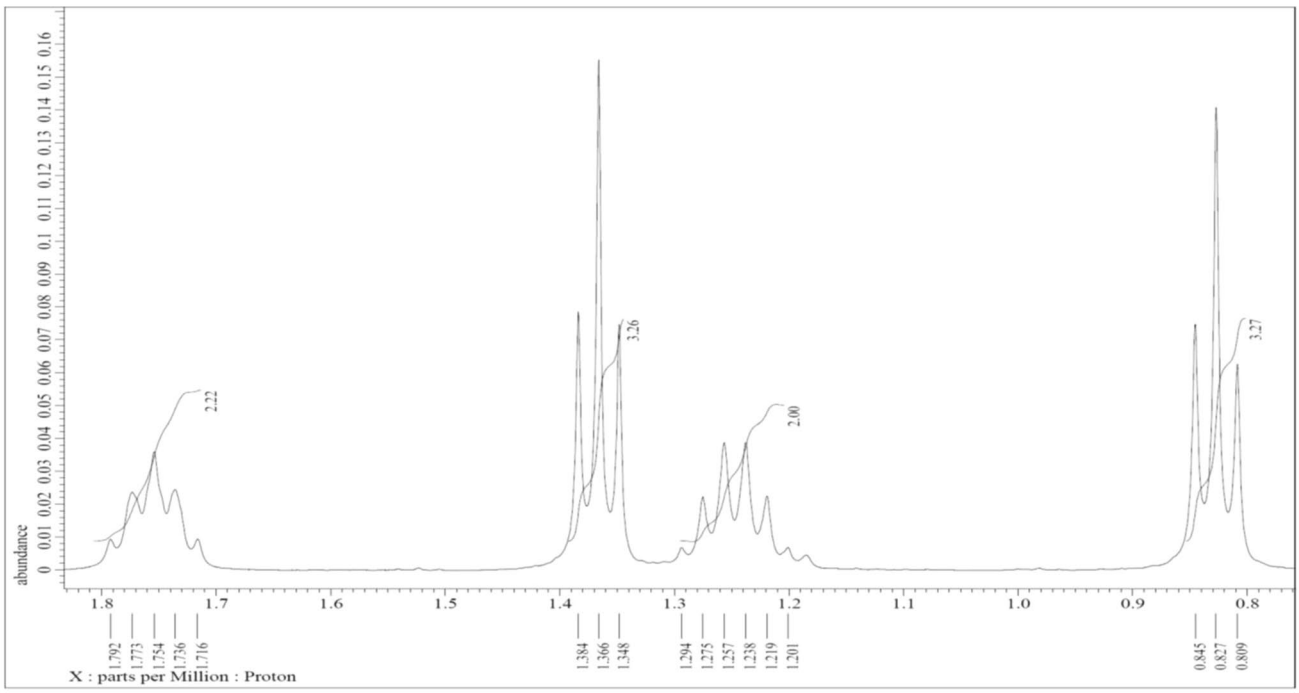


Fig. 6 Expanded ^{1}H NMR spectrum of ethyl 1-butyl-2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**6h**)

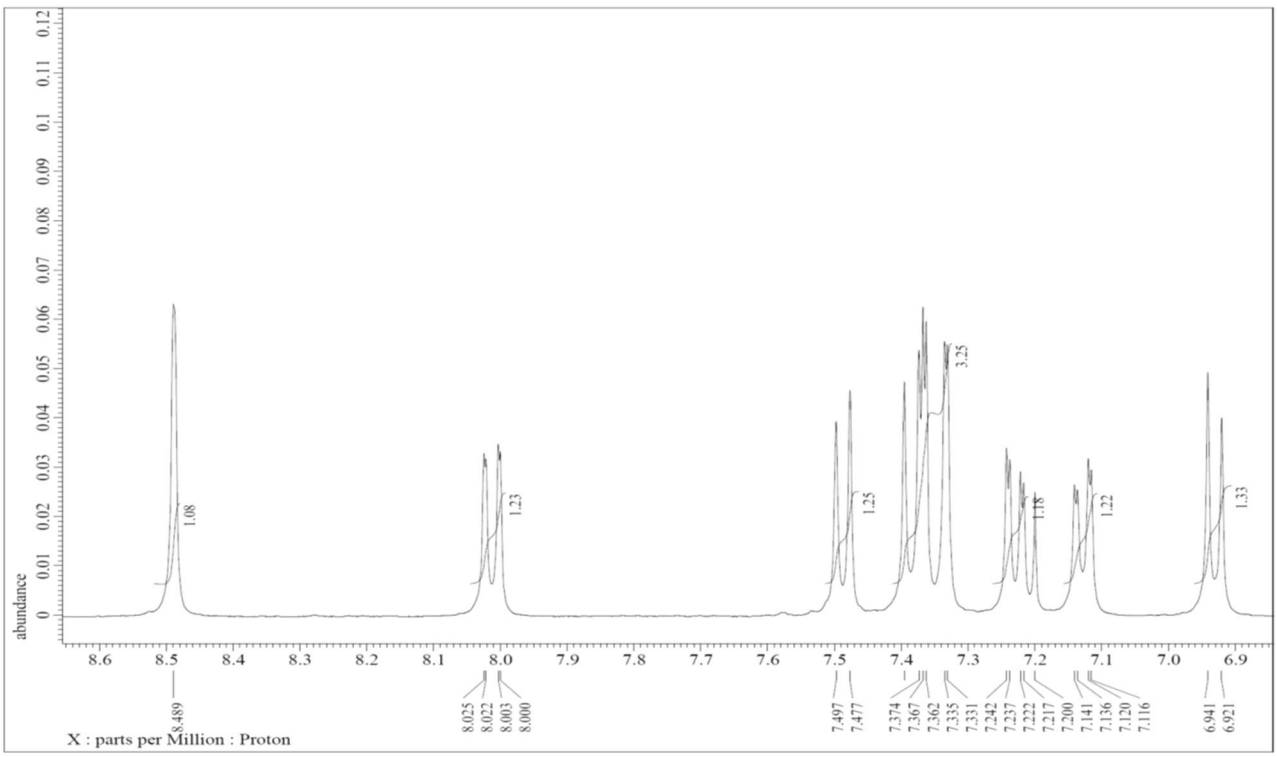


Fig. 7 Expanded ^{1}H NMR spectrum of ethyl 1-butyl-2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**6h**)

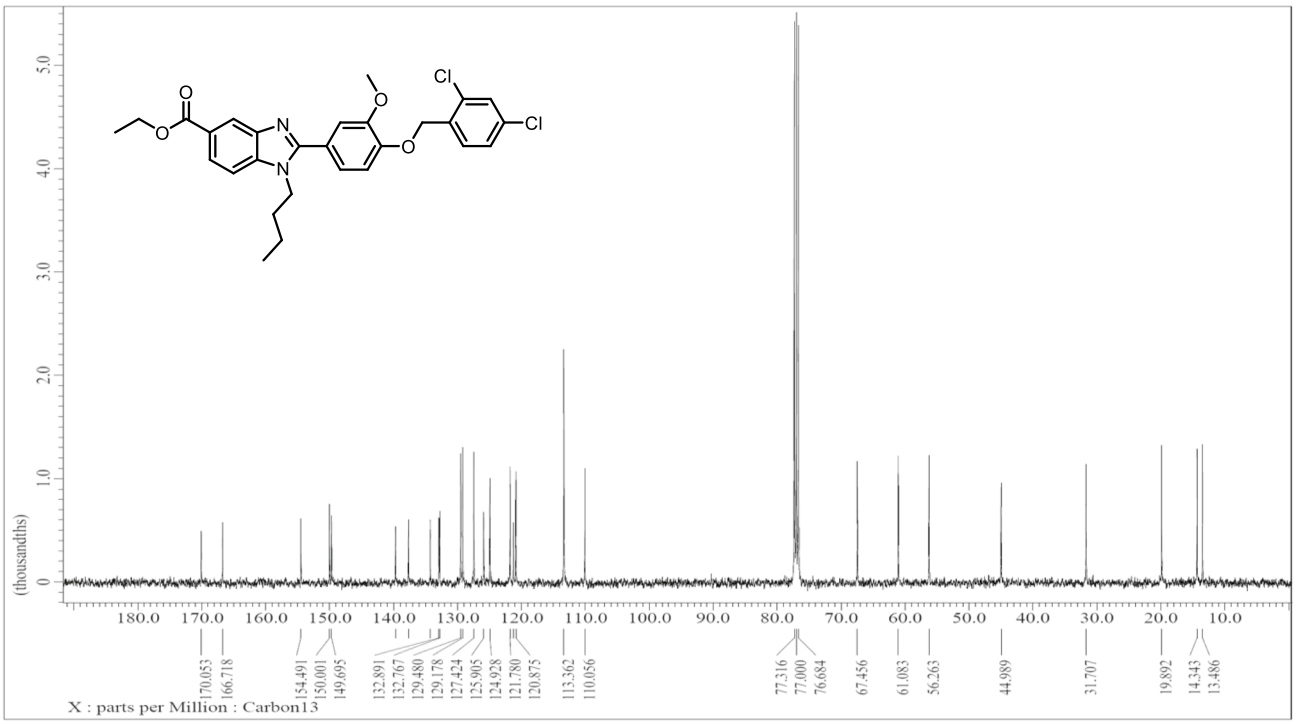


Fig. 8 ^{13}C NMR spectrum of ethyl 1-butyl-2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (**6h**)

Pharmacological studies

Anticancer activity (MTT assay)

All the novel benzimidazole derivatives were screened for their in vitro anticancer activity against cervical (HeLa), lung (A549) and kidney (HeK) cancer cells by MTT assay [23]. All the cells were incubated with different concentrations, viz. 1, 10, 25 and 50 µM of compounds for 48 and 72 h, and doxorubicin was used as a reference drug. The anticancer activity of target compounds was expressed as the concentration of the drug inhibiting 50% cell growth (IC_{50}) and summarized in Table 1.

To determine the effect of alkyl chain length and functional group on anticancer activity, compounds **6a–o** with different chain lengths and halogen substitution on benzyloxy-benzylidine were synthesized. Among the series (**6a–o**), only compounds **6b**, **6c** and **6d** were effective in inhibiting the HeLa and A549 cancer cell growth, whereas all compounds were less active in inhibiting HeK cancer cell growth.

From the anticancer activity data, it was observed that only compounds with monochloro substitution on the 4th position of benzyloxy-benzylidine (**6b–6d**) were active. On replacing monochloro with 2,4-Cl_2 and 3-F, the efficacy of the compounds in inhibiting cancer cell growth was reduced against chosen cancer cell line. Also, the

Table 1 IC_{50} values (in µM) of target compounds **6a–o** on selected human cancer cell line[a]

Compound	HeLa[b]	A549[c]	HeK[d]
6a	> 50	> 50	> 50
6b	**47.2**	> 50	> 50
6c	**45.6**	> 50	> 50
6d	**39.7**	**48.2**	> 50
6e	> 50	> 50	> 50
6f	> 50	> 50	> 50
6g	> 50	> 50	> 50
6h	> 50	> 50	> 50
6i	> 50	> 50	> 50
6j	> 50	> 50	> 50
6k	> 50	> 50	> 50
6l	> 50	> 50	> 50
6m	> 50	> 50	> 50
6n	> 50	> 50	> 50
6o	> 50	> 50	> 50
Doxorubicin	13.0	9.5	6.78

[a]50% inhibitory concentration after 72 h of compound treatment

[b]Cervical cancer cells

[c]Lung cancer cells

[d]Kidney cells

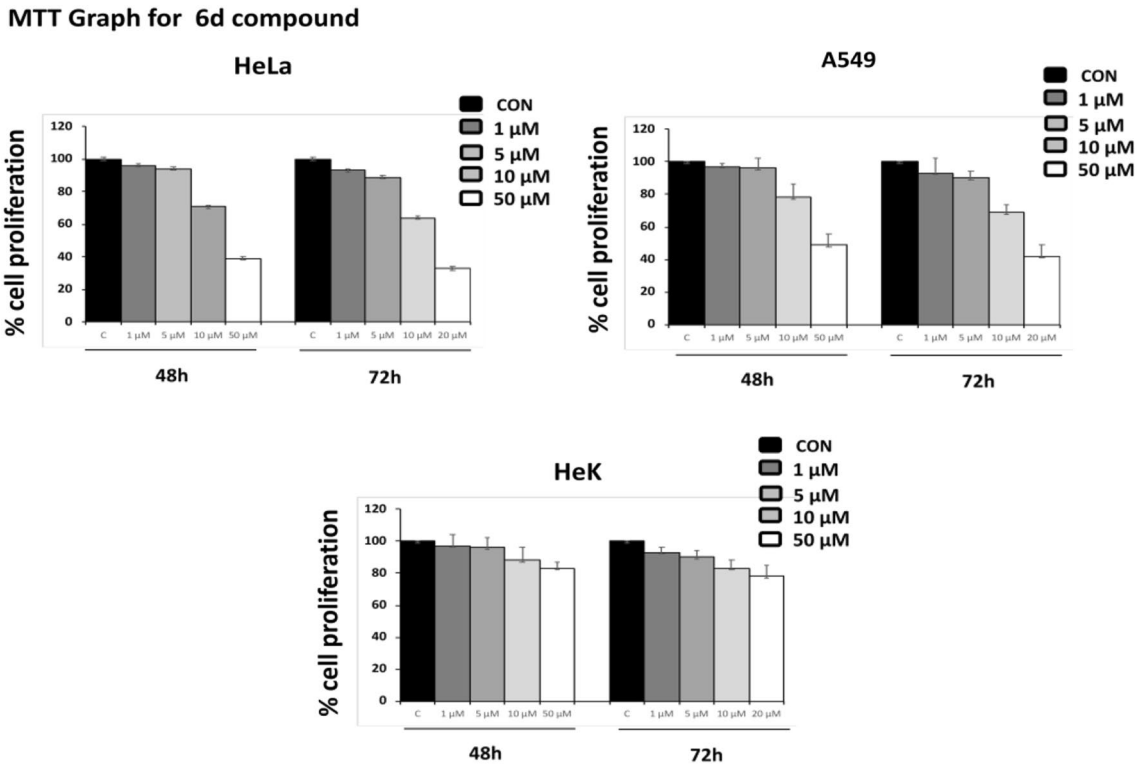


Fig. 9 MTT graph for compound **6d**

length of the carbon chain greatly affected the activity. The compound **6d** with pentyl chain length and 4-Cl substitution on benzyloxy-benzylidine were most effective against HeLa and A549 cancer cell line with IC_{50} value 39.7 and 48.2 µM (Fig. 9). On replacing the pentyl chain with butyl (**6c**) and propyl (**6b**) chain, the activity reduced against the HeLa cancer cell line which showed an IC_{50} value of 45.6 and 47.2 µM, respectively, and displayed the least activity against A549 cancer cell line. Also, compounds with methyl (**6a**) and hexyl (**6e**) chain length exhibited weak activity. Thus, it can be concluded that on increasing chain length the activity increased from methyl up to pentyl group and activity decreased with hexyl chain length. Fascinatingly, compounds with monochloro substitution on benzyloxy-benzylidine were most effective compared to 2,4-Cl_2 and 3-F substitutions.

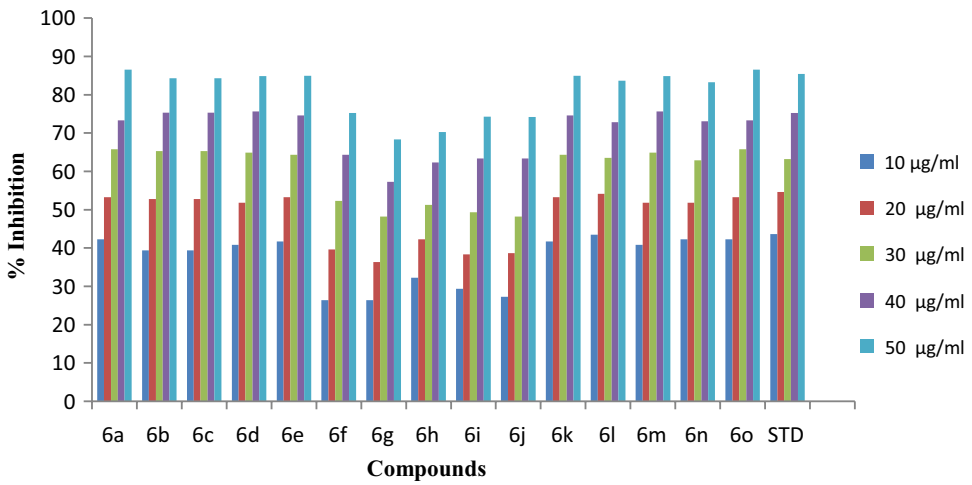


Fig. 10 Graphical representation of percentage inhibition

Table 2 Percentage inhibition of protein denaturation by bovine serum albumin method of target compounds **6a–o**

Compound	Percentage inhibition					
	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	50 µg/ml	IC_{50}
6a	42.25	53.26	65.75	73.29	86.58	22.72
6b	39.36	52.77	65.26	75.29	84.28	23.18
6c	39.36	52.77	65.26	75.29	84.28	23.18
6d	40.85	51.78	64.89	75.66	84.88	23.06
6e	41.69	53.25	64.34	74.58	84.94	22.95
6f	26.38	39.65	52.33	64.36	75.22	29.89
6g	26.38	36.33	48.22	57.26	68.33	33.3
6h	32.22	42.25	51.28	62.35	70.24	30.4
6i	29.36	38.33	49.35	63.33	74.29	30.47
6j	27.25	38.65	48.22	63.36	74.22	30.77
6k	41.69	53.25	64.34	74.58	84.94	22.90
6l	43.44	54.11	63.56	72.87	83.68	23.01
6m	40.85	51.78	64.89	75.66	84.88	23.06
6n	42.26	51.84	62.87	73.04	83.27	23.51
6o	42.25	53.26	65.75	73.29	86.58	22.74
STD (diclofenac sodium)	43.66	54.64	63.22	75.22	85.44	22.57

In vitro anti-inflammatory activity

All the newly synthesized benzimidazole derivatives (**6a–o**) were screened for their anti-inflammatory activity by protein denaturation of bovine serum albumin [24]. The activity of the synthesized compounds was performed with varying concentrations, viz. 10, 20, 30, 40 and 50 µg/mL (Fig. 10) and expressed in IC_{50} value (Table 2). Diclofenac sodium was used as the standard.

From the anti-inflammatory data, it is evident that substitution on benzyloxy-benzylidine and alkyl chain greatly influenced the activity. The compounds with mono substitution (4-Cl and 3-F) on benzyloxy-benzylidine showed excellent activity than compounds with disubstitution (2,4-Cl_2). Among mono-substituted benzyloxy-benzylidine compounds, compounds with methyl (**6a** and **6k**) and hexyl (**6e** and **6o**) chain length emerged as the most active compounds among the series with IC_{50} value 22.72, 22.90, 22.95 and 22.74 µg/mL, whereas compounds with 2,4-Cl_2 substitution on benzyloxy-benzylidine showed moderate activity.

In vivo anti-inflammatory activity

Based on in vitro anti-inflammatory activity, compounds **6a**, **6e**, **6g**, **6k** and **6o** were evaluated for their in vivo anti-inflammatory activity at 50 mg/kg dose level by employing the carrageenan–induced paw edema method [25] and acute toxicity [26]. The standard used was indomethacin. The results are depicted in Table 3.

The results revealed that the synthesized compounds exhibited moderate to excellent anti-inflammatory activity ranging from 43.35 to 77.50% inhibition when compared to

Table 3 Anti-inflammatory effect of the selected compounds (50 mg/kg) and indomethacin (10 mg/kg) against carrageenan-induced paw edema in rats

Compound	1 h		2 h		3 h	
	Swelling	Inhibition (%)	Swelling	Inhibition (%)	Swelling	Inhibition (%)
Control	0.283 ± 0.0124	–	0.322 ± 0.0161	–	0.489 ± 0.0116	–
6a	$0.101 \pm 0.0042^{*}$	64.31	$0.113 \pm 0.0013^{**}$	64.90	$0.110 \pm 0.0040^{**}$	77.50
6e	$0.119 \pm 0.0054^{**}$	16.4	$0.116 \pm 0.0059^{*}$	63.97	$0.121 \pm 0.006^{**}$	75.25
6g	0.274 ± 0.01	49.25	0.388 ± 0.01	45.73	0.388 ± 0.02	43.35
6k	$0.188 \pm 0.02^{**}$	65.18	$0.300 \pm 0.02^{**}$	58.04	$0.191 \pm 0.03^{**}$	72.11
6o	$0.135 \pm 0.0043^{***}$	52.3	$0.11 \pm 0.0026^{***}$	64.10	$0.11 \pm 0.0045^{***}$	70.10
Indomethacin	0.170 ± 0.0045	81.29	0.150 ± 0.0065	53.41	0.127 ± 0.0069	74.02

Differences between means at $^{*}p < 0.01$; $^{**}p < 0.005$; and $^{***}p < 0.0001$ were regarded significant

reference drug indomethacin which showed 74.02% inhibition. The in vivo anti-inflammatory results showed a good correlation with in vitro activity. Most of the compounds except **6e** showed tremendous activity in the first one hour of administration, i.e., > 50% inhibition. The compound **6k** having methyl chain length and 3-F substitution on the benzyloxy-benzylidine exhibited a high degree of activity with 65.18% inhibition followed by **6a** having 4-Cl substitution and methyl chain length with 64.31% inhibition. The inhibitory potency of these derivatives increased at the end of the 3rd hour, i.e., up to 77.50% which was in comparable with reference drug indomethacin (74.02%). The same trend was followed by compound **6o** having hexyl chain length and 3-F substitution on benzyloxy-benzylidine with 70.10% inhibition at the end of the 3rd hour. Though compound **6e** with hexyl chain and 4-Cl substitution displayed weak inhibition in 1st hour, it increased gradually at the end of the 3rd hour with 75.25% inhibition. The compound **6g** with 2,4-Cl_2 on benzyloxy-benzylidine substitution displayed weak activity.

The acute toxicity study demonstrated that none of the screened compounds induce any appreciable behavioral change at the administered doses during the observation period.

Drug-like prediction study

In vitro, ADMET assays are now routinely applied during the optimization of drug discovery. To reduce the failure in clinical trials, the physicochemical properties of the compounds typically of interest were studied. Lipinski's rule is used to determine the molecular properties of the potent compounds that are important for drug-likeness [27]. Molecules those obey Lipinski's rule of five are more likely to be a drug candidate.

In the present study, ADMET properties of the potent compounds **6a–e**, **6k** and **6o** were determined using Molinspiration online property calculation toolkit and are summarized in Table 4. The results revealed that most of the compounds (**6a–c**, **6k**) obeyed all the rules except miLogP. The remaining compounds (**6d**, **6e**, **6o**) violate two rules, i.e., miLogP and formula weight. But, these derivatives showed excellent oral absorption (87.40%). The efficiency of the chemical compound depends on the oral bioavailability (absorption, distribution, metabolism, and excretion) in humans. Since these derivatives possess excellent oral bioavailability, violation of any of the properties does not affect the bioavailability of the molecule [28]. The number of hydrogen bond acceptors and hydrogen bond donors of the selected compounds was found to be within Lipinski's limit, i.e., ≤ 10 and ≤ 5, respectively (Table 4).

Conclusions

In conclusion, benzimidazole derivatives with varying alkyl chain lengths were synthesized in good yield and evaluated for their anticancer against three cancer cell lines (HeLa, A549, HeK) and anti-inflammatory activity. Among the series, compound **6d** was more efficient in inhibiting cell growth in HeLa and A549 cancer cells. It was observed the efficacy increased with increasing alkyl chain length up to pentyl chain lengths and decreased in the presence of hexyl chain length. Also, only 4-Cl substitution on benzyloxy-benzylidene showed inhibition. In addition, compound **6a** emerged as a potent anti-inflammatory agent. Thus, these derivatives hold the promise of being effective chemotherapeutic and anti-inflammatory agents.

Experimental section

Materials and methods

All the essential chemicals and solvents were purchased from Spectrochem India, S. D.Fine and Sigma-Aldrich which were used directly without any further purification.

Table 4 Predicted ADME, Lipinski parameters and molecular properties of the synthesized compounds **6a–o**

Compound	% ABS	TPSA	n-ROTB	n-ON acceptors	n-OHNH donors	mi LogP	Formula weight	Volume	n Violations
6a	87.40	62.60	8	6	0	6.05	450.92	394.82	1
6b	87.40	62.60	10	6	0	6.93	478.98	428.43	1
6c	87.40	62.60	11	6	0	7.49	493.00	445.23	1
6d	87.40	62.60	12	6	0	8.00	507.03	462.03	2
6e	87.40	62.60	13	6	0	8.41	521.06	478.83	2
6k	87.40	62.60	8	6	0	5.52	434.47	386.22	1
6o	87.40	62.60	13	6	0	7.96	504.60	470.23	2

ABS: absorption; ADME: absorption, distribution, metabolism and excretion; n-ON acceptors: number of hydrogen bond acceptors; n-OHNH donor: number of hydrogen bond donors; n-ROTB: number of rotatable bonds

The open capillary method was used to determine the melting point of the compound and was uncorrected. Thin layer chromatography plate-aluminium sheet covered with Silicagel $60F_{254}$ was used to determine the progress of the reaction. ^{1}HNMR and ^{13}CNMR spectra were recorded on JOEL, 400 MHz using TMS as an internal standard. The chemical shift (δ) values were expressed in ppm and coupling constant in Hz. Spin multiplicities were stated singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). FT (ATR)-IR absorption spectra were analysed on Shimadzu, in the range 4000–400 cm^{-1} and mass spectra in Schimadzu LCMS-8030. Elemental analyses were recorded in CHNS Elementar Vario EL III.

- Synthesis of ethyl 4-chloro-3-nitrobenzoate (1) [21]
- Synthesis of ethyl 4-(alkylamino)-3-nitrobenzote (**2a–e**)

To a stirred solution of ethyl 4-chloro-3-nitrobenzoate (**1**) (0.01 mol) in dry THF (10 V) were added alkylamine (0.015 mol) and triethylamine (0.03 mol). The reaction mixture was then allowed to stir at room temperature for overnight. The completion of the reaction was monitored by TLC. After completion of the reaction, reaction mass was poured into crushed ice. The precipitate formed was filtered and dried. Ethyl 4-(alkylamino)-3-nitrobenzote with varied alkyl chain length prepared using this method is as follows

- Ethyl 4-(methylamino)-3-nitrobenzoate (**2a**): Mp. 98–100 °C (Lit. Mp. 101 °C) [29]
- Ethyl 4-(propylamino)-3-nitro-benzoate (**2b**): Mp. 74–76 °C (Lit. Mp. 78 °C) [30]
- Ethyl 4-(butylamino)-3-nitro-benzoate (**2c**): [21]
- Ethyl 4-(pentylamino)-3-nitrobenzoate (**2d**):

Yield: 85%; Mp. Low melting; yellow solid; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.81 (d, 1H, $J=2.0$ Hz, Ar–H), 8.29 (bs, 1H, N–H), 7.98–8.01 (dd, 1H, $J=2.0$ and 8.8 Hz), 6.80 (d, 1H, $J=9.2$ Hz, Ar–H), 4.27–4.32 (q, 2H, $J=7.2$ Hz, ester CH_2), 3.26–3.31 (q, 2H, $J=5.2$ Hz, H_1 of pentyl), 1.66–1.73 (quint, 2H, H_2 of pentyl), 1.31–1.38 (m, 7H, ester CH_3, H_3, H_4 of pentyl), 0.86–0.90 (t, 3H, $J=7.2$ Hz, H_5 of pentyl).

Ethyl 4-(hexylamino)-3-nitrobenzoate (2e)

Yield: 87%; Mp. yellow oil; FT-IR (ATR, υ_{max}, cm^{-1}): 3371 (N–H), 2929 (C–H), 1710 (C=O), 1620 (C=C), 1018 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.79 (d, 1H, $J=2.4$ Hz, Ar–H), 8.27 (bs, 1H, N–H), 7.96–7.99 (dd, 1H, $J=2.0$ and 8.8 Hz, Ar–H), 6.78 (d, 1H, $J=9.2$ Hz, Ar–H), 4.26–4.31 (q, 2H, $J=7.2$ Hz, ester CH_2), 3.25–3.30 (q, 2H, $J=5.2$ Hz, H_1 of hexyl), 1.63–1.71 (quint, 2H, H_2 of hexyl), 1.18–1.42 (m, 9H, H_3, H_4, H_5 of hexyl and ester CH_3), 0.82–0.85 (m, 3H, H_6 of hexyl); ESI–MS (*m/z*): 295.10 $[M+H]^+$; anal. calcd. for $C_{15}H_{22}N_2O_4$: C, 61.21; H, 7.53; N, 9.52%. Found: C, 61.23; H, 7.55; N, 9.53%.

Synthesis of 4-(substituted benzyloxy)-3-methoxybenzaldehyde (5a–c)

To a stirred solution of vanillin (0.01 mol) in DMF (10 V) added K_2CO_3 (0.015 mol) at room temperature. To this reaction mixture added substituted benzyl chlorides (0.01 mol) and the reaction was stirred at reflux temperature for 3 h. After the completion of the reaction (monitored by TLC), the reaction mixture was poured into crushed ice, and the precipitate formed was filtered, dried and recrystallized from ethanol.

Procedure for synthesis of target compounds (6a–o)

Sodium dithionite (0.03 mol) was added to the stirred solution of intermediate **2a–e** (0.01 mol) and benzyloxybenzaldehyde (**5a–c**) in DMSO (10 V), and reaction mixture was stirred at 90 °C for 3 h. The reaction mixture was poured into crushed ice after the completion of the reaction. The solid formed was filtered, washed with water, dried and recrystallized from dimethylformamide to obtain **6a–o**.

Ethyl 2-(4-((4-chlorobenzyl) oxy)-3-methoxyphenyl)-1-methyl-1H-benzo[d] imidazole-5-carboxylate (6a)

Yield: 95%; Mp. 140–142 °C; creamish solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3420 (=C–H), 2947 (C–H), 1706 (C=O), 1597 (C=C), 1010 (C–O), 745 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.20 (d, 1H, $J=1.2$ Hz, Ar–H), 7.93–7.95 (dd, 1H, $J=0.4$ AND 2.2 Hz, Ar–H), 7.21–7.30 (m, 6H, Ar–H), 7.08–7.11 (dd, 1H, $J=2.0$ and 8.8 Hz, Ar–H), 6.89 (d, 1H, $J=8.0$ Hz, Ar–H), 5.09 (s, 2H, benzyl), 4.29–4.34 (q, 2H, $J=7.2$ Hz, ester CH_2), 3.95 (s, 3H, CH_3), 3.85 (s, 3H, methoxy), 1.32 (t, 3H, $J=1.8$ Hz, ester CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 165.1, 153.0, 149.7, 149.1, 141.1, 137.6, 134.5, 131.5, 127.7, 127.6, 123.9, 123.1, 122.5, 120.8, 120.6, 112.5, 112.0, 107.6, 67.4, 60.9, 56.3, 32.2, 14.3; ESI–MS (*m/z*): 451.15 $[M+H]^+$; anal. calcd. for $C_{25}H_{23}ClN_2O_4$: C, 66.59; H, 5.14; N, 6.21%. Found: C, 66.60; H, 5.15; N, 6.22%.

Ethyl 2-(4-((4-chlorobenzyl) oxy)-3-methoxyphenyl)-1-propyl-1H-benzo[d] imidazole-5-carboxylate (6b)

Yield: 89%; pale yellow oil; FT-IR (ATR, υ_{max}, cm^{-1}): 3418 (=C–H), 2950 (C–H), 1715 (C=O), 1600 (C=C), 1017 (C–O), 750 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm):

8.32 (d, 1H, $J = 1.2$ Hz, Ar–H), 7.94–7.96 (dd, 1H, $J = 0.6$ and 2.4 Hz, Ar–H), 7.23–7.32 (m, 6H, Ar–H), 7.11–7.14 (dd, 1H, $J = 2.0$ and 8.4 Hz, Ar–H), 6.91 (d, 1H, $J = 8.0$ Hz, Ar–H), 5.19 (s, 2H, benzyl), 4.30 − 4.35 (q, 2H, $J = 7.0$ Hz, ester CH_2), 4.33 (t, 2H, $J = 7.5$ Hz, H_1 of propyl), 3.90 (s, 3H, methoxy), 1.69–1.74 (quint, 2H, H_2 of propyl), 1.34 (t, 3H, $J = 1.7$ Hz, ester CH_3), 0.77 (t, 3H, $J = 12.0$ Hz, H_3 of propyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 166.0, 154.1, 149.9, 149.5, 142.1, 139.7, 135.1, 131.9, 128.8, 128.6, 124.6, 123.9, 122.5, 121.6, 121.2, 113.1, 113.0, 106.2, 70.2, 60.8, 56.1, 44.8, 31.8, 14.3, 13.5; ESI–MS (*m/z*): 479.18 $[M + H]^+$; anal. calcd. for $C_{27}H_{27}ClN_2O_4$: C, 67.71; H, 5.68; N, 5.85%. Found: C, 67.72; H, 5.69; N, 5.86%.

Ethyl 1-butyl-2-(4-((4-chlorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (6c)

Yield: 89%; Mp. 82–84 °C; pale yellow solid; FT IR (ATR, υ_{max}, cm^{-1}): 3415 (=C–H), 2953 (C–H), 1708 (C=O), 1020 (C–O), 748 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.45 (d, 1H, $J = 1.2$ Hz, Ar–H), 7.95–7.98 (dd, 1H, $J = 0.4$ and 2.2 Hz, Ar–H), 7.26–7.35 (m, 6H, Ar–H), 7.09–7.11 (dd, 1H, $J = 2.0$ and 8.4 Hz, Ar–H), 6.91 (d, 1H, $J = 8.0$ Hz, Ar–H), 5.11 (s, 2H, benzyl), 4.31–4.36 (q, 2H, $J = 7.2$ Hz, ester CH_2), 4.18 (t, 2H, $J = 7.6$ Hz, H_1 of butyl), 3.89 (s, 3H, methoxy), 1.69–1.77 (quint, 2H, H_2 of butyl), 1.35 (t, 3H, $J = 1.8$ Hz, ester CH_3), 1.18–1.27 (sextet, 2H, H_3 of butyl), 0.81 (t, 3H, $J = 7.2$ Hz, H_4 of butyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 167.1, 155.0, 149.8, 149.4, 142.1, 138.7, 135.0, 133.8, 128.8, 128.6, 125.0, 124.2, 122.9, 121.8, 121.6, 113.5, 113.0, 109.7, 70.2, 60.8, 56.1, 44.8, 31.8, 19.9, 14.3, 13.5; ESI–MS (*m/z*): 493.20 $[M + H]^+$; anal. calcd. for $C_{28}H_{29}ClN_2O_4$: C, 68.22; H, 5.93; N, 5.68%. Found: C, 68.23; H, 5.95; N, 5.69%.

Ethyl 2-(4-((4-chlorobenzyl)oxy)-3-methoxyphenyl)-1-pentyl-1H-benzo[d]imidazole-5-carboxylate (6d)

Yield: 80%; Mp. 80–82 °C; pale yellow solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3422 (=C–H), 2951 (C–H), 1710 (C=O), 1592 (C=C), 1011 (C–O), 743 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.47 (d, 1H, $J = 1.2$ Hz, Ar–H), 7.80–7.83 (dd, 1H, $J = 0.4$ and 1.6 Hz, Ar–H), 7.31–7.40 (m, 6H, Ar–H), 7.11–7.15 (dd, 1H, $J = 2.2$ and 8.4 Hz, Ar–H), 6.97 (d, 1H, $J = 8.0$ Hz, Ar–H), 5.19 (s, 2H, benzyl), 4.34–4.39 (q, 2H, $J = 7.3$ Hz, ester CH_2), 4.18 (t, 2H, $J = 7.6$ Hz, H_1 of pentyl), 3.91 (s, 3H, methoxy), 1.72–1.80 (quint, 2H, H_2 of pentyl), 1.37 (t, 3H, $J = 1.7$ Hz, ester CH_3), 1.20–1.29 (quintet, 4H, H_3 and H_4 of pentyl), 0.81 (t, 3H, $J = 7.0$ Hz, H_5 of pentyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 168.0, 156.1, 149.9, 149.6, 143.1, 139.2, 135.9, 133.9, 129.8, 128.9, 125.9, 124.4, 123.8, 121.9, 121.8, 113.9, 113.5, 109.8, 70.2, 61.8, 57.0, 44.9, 31.9, 30.8, 20.9, 14.6, 13.6; ESI–MS (*m/z*): 507.21 $[M + H]^+$; anal. calcd. for $C_{29}H_{31}ClN_2O_4$: C, 68.70; H, 6.16; N, 5.53%. Found: C, 68.72; H, 6.17; N, 5.56%.

Ethyl 2-(4-((4-chlorobenzyl)oxy)-3-methoxyphenyl)-1-hexyl-1H-benzo[d]imidazole-5-carboxylate (6e)

Yield: 87%; Mp. 70–72 °C; yellow solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3418 (=C–H), 2947 (C–H), 1715 (C=O), 1600 (C=C), 1010 (C–O), 747 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.49 (s, 1H, Ar–H), 7.83–7.86 (dd, 1H, $J = 0.4$ and 2.0 Hz, Ar–H), 7.38–7.47 (m, 6H, Ar–H), 7.13–7.17 (dd, 1H, $J = 2.4$ and 8.0 Hz, Ar–H), 6.97 (d, 1H, $J = 8.0$ Hz, Ar–H), 5.20 (s, 2H, benzyl), 4.35–4.40 (q, 2H, $J = 7.4$ Hz, ester CH_2), 3.25–3.30 (m, 2H, H_1 of hexyl), 3.85 (s, 3H, methoxy), 1.63–1.71 (quint, 2H, H_2 of hexyl), 1.21–1.40 (m, 9H, H_3, H_4, H_5 of hexyl and ester CH_3), 0.82 (t, 3H, $J = 7.0$ Hz, H_6 of hexyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 169.0, 157.1, 150.8, 150.6, 144.0, 140.2, 136.9, 134.8, 130.5, 129.9, 126.6, 125.4, 124.8, 122.6, 122.2, 114.8, 114.2, 109.5, 67.2, 61.8, 55.2, 44.2, 31.1, 29.2, 23.2, 22.5, 14.0, 13.8; ESI–MS (*m/z*): 521.23 $[M + H]^+$; anal. calcd. for $C_{30}H_{33}ClN_2O_4$: C, 69.15; H, 6.38; N, 5.38%. Found: C, 69.16; H, 6.39; N, 5.39%.

Ethyl 2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylate (6f)

Yield: 90%; Mp. 100–102 °C; off-white solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3197 (=C–H), 2980 (C–H), 1707 (C=O), 1597 (C=C), 1024 (C–O), 748 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.34 (s, 1H, Ar–H), 7.90–7.92 (dd, 1H, $J = 1.2$ and 8.4 Hz, Ar–H), 7.39 (d, 1H, $J = 8.0$ Hz, Ar–H), 7.26–7.28 (m, 2H, Ar–H), 7.07–7.13 (m, 3H, Ar–H), 6.82 (d, 1H, $J = 8.4$ Hz, Ar–H), 5.11 (s, 1H, benzyl), 4.21–4.27 (q, 2H, $J = 7.2$ Hz, ester CH_2), 3.84 (s, 3H, methoxy), 3.77 (s, 3H, methyl), 1.26 (t, 3H, $J = 1.8$ Hz, ester CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 166.9, 154.7, 150.0, 149.6, 140.6, 139.0, 134.2, 132.9, 132.8, 129.5, 129.2, 127.4, 125.6, 124.7, 122.1, 121.8, 121.2, 113.3, 113.2, 109.5, 67.5, 61.0, 56.3, 32.2, 14.3; ESI–MS (*m/z*): 485.05 $[M + H]^+$; anal. calcd. for $C_{25}H_{22}Cl_2N_2O_4$: C, 61.87; H, 4.57; N, 5.77%. Found: C, 61.85; H, 4.55; N, 5.76%.

Ethyl 2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1-propyl-1H-benzo[d]imidazole-5-carboxylate (6g)

Yield: 93%; Mp. 98–100 °C; pale yellow solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3190 (=C–H), 2985 (C–H), 1710 (C=O), 1595 (C=C), 1022 (C–O), 750 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.39 (s, 1H, Ar–H), 7.95–7.97 (dd, 1H,

$J = 1.2$ and 8.0 Hz, Ar–H), 7.40 (d, 1H, $J = 8.0$ Hz, Ar–H), 7.31–7.33 (m, 2H, Ar–H), 7.11–7.17 (m, 3H, Ar–H), 6.89 (d, 1H, $J = 8.0$ Hz, Ar–H), 5.15 (s, 1H, benzyl), 4.32–4.36 (q, 2H, $J = 7.1$ Hz, ester CH_2), 4.32 (t, 2H, $J = 7.6$ Hz, H_1 of propyl), 3.89 (s, 3H, methoxy), 1.69–1.74 (quint, 2H, H_2 of propyl), 1.26 (t, 3H, $J = 1.6$ Hz, ester CH_3), 0.75 (t, 3H, $J = 12.0$ Hz, H_3 of propyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 168.7, 155.7, 149.9, 149.6, 140.1, 138.3, 133.5, 133.1, 132.2, 129.7, 129.5, 128.4, 125.7, 123.20, 121.9, 121.0, 120.0, 113.9, 113.1, 109.9, 70.0, 61.5, 56.5, 44.8, 31.7, 14.2, 13.2; ESI–MS (*m/z*): 513.15 $[M + H]^+$; anal. calcd. for $C_{27}H_{26}Cl_2N_2O_4$: C, 63.16; H, 5.10; N, 5.46%. Found: C, 63.15; H, 5.11; N, 5.48%.

Ethyl 1-butyl-2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (6h)

Yield: 85%; Mp. 118–120 °C; off-white solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3408 (=C–H), 2931 (C–H), 1708 (C=O), 1612 (C=C), 1020 (C–O), 746 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.49 (s, 1H, Ar–H), 8.00–8.03 (dd, 1H, $J = 1.2$ and 8.8 Hz, Ar–H), 7.49 (d, 1H, $J = 8.0$ Hz, Ar–H), 7.33–7.37 (m, 3H, Ar–H), 7.22–7.24 (dd, 1H, $J = 2.0$ and 8.0 Hz, Ar–H), 7.12–7.14 (dd, 1H, $J = 2.0$ and 8.4 Hz, Ar–H), 6.93 (d, 1H, $J = 8$ Hz, Ar–H), 5.21 (s, 1H, benzyl), 4.33–4.39 (q, 2H, $J = 7.2$ Hz, ester CH_2), 4.21 (t, 2H, $J = 7.6$ Hz, H_1 of butyl), 3.91 (s, 3H, methoxy), 1.71–1.79 (quint, 2H, H_2 of butyl), 1.36 (t, 3H, $J = 7.2$ Hz, ester CH_3), 1.20–1.29 (sextet, 2H, H_3 of butyl), 0.83 (t, 3H, $J = 7.2$ Hz, H_4 of butyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 170.1, 166.7, 154.5, 150.0, 149.7, 139.7, 137.6, 134.2, 132.9, 132.8, 129.5, 129.2, 127.4, 125.9, 124.9, 121.8, 121.2, 120.9, 113.4, 110.1, 67.5, 61.1, 56.3, 45.0, 31.7, 19.9, 14.3, 13.5; ESI–MS (*m/z*): 527.15 $[M + H]^+$; anal. calcd. for $C_{28}H_{28}Cl_2N_2O_4$: C, 63.76; H, 5.35; N, 5.31%. Found: C, 63.77; H, 5.36; N, 5.33%.

Ethyl 2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1-pentyl-1H-benzo[d]imidazole-5-carboxylate (6i)

Yield: 80%; Mp. 96–98 °C; light gray solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3418 (=C–H), 2929 (C–H), 1704 (C=O), 1602 (C=C), 1011 (C–O), 744 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.50 (s, 1H, Ar–H), 8.01–8.04 (dd, 1H, $J = 1.2$ and 8.4 Hz, Ar–H), 7.50 (d, 1H, $J = 8$ Hz, Ar–H), 7.34–7.40 (m, 3H, Ar–H), 7.22–7.25 (dd, 1H, $J = 2.0$ and 8.4 Hz, Ar–H), 7.12–7.15 (dd, 1H, $J = 2.0$ and 8.0 Hz, Ar–H), 6.94 (d, 1H, $J = 8.0$ Hz, Ar–H), 5.21 (s, 2H, benzyl), 4.35–4.40 (q, 2H, $J = 7.4$ Hz, ester CH_2), 4.20 (t, 2H, $J = 7.6$ Hz, H_1 of pentyl), 3.93 (s, 3H, methoxy), 1.74–1.82 (quint, 2H, H_2 of pentyl), 1.40 (t, 3H, $J = 7.0$ Hz, ester CH_3), 1.21–1.30 (quintet, 4H, H_3 and H_4 of pentyl), 0.80 (t, 3H, $J = 7.0$ Hz, H_5 of pentyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 164.9, 153.6, 148.9, 148.2, 141.9, 137.4, 133.6, 131.5, 132.8, 128.4, 128.2, 126.6, 124.4, 123.1, 121.9, 120.7, 120.8, 113.4, 113.1, 108.1, 71.2, 62.8, 58.1, 45.9, 32.9, 31.8, 21.8, 14.6, 13.7; ESI–MS (*m/z*): 541.17 $[M + H]^+$; anal. calcd. for $C_{29}H_{30}Cl_2N_2O_4$: C, 64.33; H, 5.58; N, 5.17%. Found: C, 64.34; H, 5.59; N, 5.19%.

Ethyl 2-(4-((2,4-dichlorobenzyl)oxy)-3-methoxyphenyl)-1-hexyl-1H-benzo[d]imidazole-5-carboxylate (6j)

Yield: 82%; Mp. 79–81 °C; off-white solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3394 (=C–H), 2929 (C–H), 1707 (C=O), 1612 (C=C), 1018 (C–O), 744 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.49 (d, 1H, $J = 0.8$ Hz, Ar–H), 8.00–8.03 (dd, 1H, $J = 1.2$ and 8.0 Hz, Ar–H), 7.51 (d, 1H, $J = 8.0$ Hz, Ar–H), 7.39–7.40 (m, 2H, Ar–H), 7.32 (s, 1H, Ar–H), 7.24 (d, 1H, $J = 7.6$ Hz, Ar–H), 7.16 (d, 1H, $J = 8.0$ Hz, Ar–H), 6.95 (d, 1H, $J = 8.4$ Hz, Ar–H), 5.23 (s, 2H, benzyl), 4.35–4.40 (q, 2H, $J = 6.8$ Hz, ester CH_2), 4.21–4.25 (t, 2H, $J = 7.2$ Hz, H_1 of hexyl), 3.94 (s, 3H, methoxy), 2.58 (bs, 2H, H_2 of hexyl), 1.79 (bs, 2H, H_3 of hexyl), 1.38 (t, 3H, $J = 7.2$ Hz, ester CH_3), 1.20 (bs, 4H, H_4, H_5 of hexyl), 0.80 (bs, 3H, H_6 of hexyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 166.9, 154.7, 149.9, 149.4, 141.2, 138.4, 134.2, 132.9, 132.8, 129.5, 129.2, 127.4, 125.3, 124.5, 122.6, 121.7, 121.5, 113.4, 113.2, 109.8, 67.5, 60.9, 56.2, 45.1, 31.1, 29.7, 26.3, 22.4, 14.3, 13.8; ESI–MS (*m/z*): 555.15 $[M + H]^+$; anal. calcd. for $C_{30}H_{32}Cl_2N_2O_4$: C, 64.87; H, 5.81; N, 5.04%. Found: C, 64.89; H, 5.81; N, 5.05%.

Ethyl 2-(4-((3-fluorobenzyl)oxy)-3-methoxyphenyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylate (6k)

Yield: 85%; Mp. 160–162 °C; off-white solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3385 (=C–H), 2925 (C–H), 1710 (C=O), 1610 (C=C), 1203 (C–F), 1012 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.32 (s, 1H, Ar–H), 7.84–7.86 (dd, 1H, $J = 1.2$ and 8.0 Hz, Ar–H), 7.17–7.26 (m, 3H, Ar–H), 7.01–7.10 (m, 3H, Ar–H), 6.80–6.87 (m, 2H, Ar–H), 5.14 (s, 2H, benzyl), 4.29–4.34 (q, 2H, $J = 7.4$ Hz, ester CH_2), 3.95 (s, 3H, CH_3), 3.85 (s, 3H, methoxy), 1.32 (t, 3H, $J = 7.0$ Hz, ester CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 165.8, 161.3 ($J = 230.5$ Hz), 153.2, 140.3 ($J = 42.5$ Hz), 140.3, 134.0 ($J = 7.2$ Hz), 136.2, 129.9, 129.3, 122.9, 122.1, 121.0, 120.9, 120.0, 119.7, 119.3, 113.2, 112.9 ($J = 22.1$ Hz), 111.8 ($J = 40.0$ Hz), 108.4, 66.2, 59.9, 54.3, 31.3, 14.9; ESI–MS (*m/z*): 435.17 $[M + H]^+$; anal. calcd. for $C_{25}H_{23}FN_2O_4$: C, 69.11; H, 5.34; N, 6.45%. Found: C, 69.12; H, 5.36; N, 6.46%.

Ethyl 2-(4-((3-fluorobenzyl) oxy)-3-methoxyphenyl)-1-propyl-1H-benzo[d] imidazole-5-carboxylate (6l)

Yield: 89%; Mp. 100–101 °C; light brown solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3391 (=C–H), 2932 (C–H), 1712 (C=O), 1612 (C=C), 1214 (C–F), 1010 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.38 (s, 1H, Ar–H), 7.87–7.89 (dd, 1H, $J=1.2$ and 8.4 Hz, Ar–H), 7.20–7.29 (m, 3H, Ar–H), 7.04–7.13 (m, 3H, Ar–H), 6.83–6.89 (m, 2H, Ar–H), 5.15 (s, 2H, benzyl), 4.32–4.36 (q, 2H, $J=7.6$ Hz, ester CH_2), 4.30–4.34 (t, 2H, $J=7.0$ Hz, H_1 of propyl), 3.89 (s, 3H, methoxy), 1.69–1.74 (quint, 2H, H_2 of propyl), 1.26 (t, 3H, $J=7.8$ Hz, ester CH_3), 0.77 (t, 3H, $J=12$ Hz, H_3 of propyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 166.1, 162.2 ($J=233.0$ Hz), 154.7, 140.3 ($J=43.0$ Hz), 141.9, 137.6 ($J=7.2$ Hz), 135.2, 130.6, 130.5, 123.7, 123.9, 122.4, 121.3, 120.9, 120.3, 119.9, 114.5, 113.8 ($J=22.2$ Hz), 112.8 ($J=40.7$ Hz), 109.5, 71.3, 62.3, 56.0, 45.8, 32.9, 13.9, 13.2; ESI–MS (*m/z*): 463.21 $[M+H]^+$; anal. calcd. for $C_{27}H_{27}FN_2O_4$: C, 70.12; H, 5.88; N, 6.06%. Found: C, 70.13; H, 5.89; N, 6.07%.

Ethyl 1-butyl-2-(4-((3-fluorobenzyl)oxy)-3-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (6m)

Yield: 95%; Mp. 82–84 °C; dark yellow solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3389 (=C–H), 2930 (C–H), 1718 (C=O), 1613 (C=C), 1212 (C–F), 1016 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.42 (s, 1H, Ar–H), 7.88–7.90 (dd, 1H, $J=1.2$ and 8.0 Hz, Ar–H), 7.21–7.30 (m, 3H, Ar–H), 7.08–7.17 (m, 3H, Ar–H), 6.86–6.92 (m, 2H, Ar–H), 5.16 (s, 2H, benzyl), 4.31–4.36 (q, 2H, $J=7.6$ Hz, ester CH_2), 4.18 (t, 2H, $J=7.2$ Hz, H_1 of butyl), 3.89 (s, 3H, methoxy), 1.69–1.76 (quint, 2H, H_2 of butyl), 1.34 (t, 3H, $J=6.9$ Hz, ester CH_3), 1.18–1.27 (sextet, 2H, H_3 of butyl), 0.79–0.83 (t, 3H, $J=6.7$ Hz, H_4 of butyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 166.9, 162.7 ($J=244.0$ Hz), 154.9, 145.2 ($J=44.8$ Hz), 141.3, 138.7 ($J=7.3$ Hz), 136.3, 131.9, 131.0, 124.9, 124.0, 123.7, 122.9, 121.8, 121.5, 121.0, 115.7, 114.0 ($J=22.0$ Hz), 113.9 ($J=40.2$ Hz), 110.6, 68.3, 62.0, 57.1, 45.2, 31.6, 20.2, 14.1, 13.3; ESI–MS (*m/z*): 477.22 $[M+H]^+$; anal. calcd. for $C_{28}H_{29}FN_2O_4$: C, 70.57; H, 6.13; N, 5.88%. Found: C, 70.58; H, 6.14; N, 5.89%.

Ethyl 2-(4-((3-fluorobenzyl) oxy)-3-methoxyphenyl)-1-pentyl-1H-benzo[d] imidazole-5-carboxylate (6n)

Yield: 83%; Mp. 70–72 °C; off-brown solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3394 (=C–H), 2931 (C–H), 1707 (C=O), 1612 (C=C), 1209 (C–F), 1018 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.47 (s, 1H, Ar–H), 7.97–8.00 (dd, 1H, $J=1.2$ and 8.4 Hz, Ar–H), 7.27–7.36 (m, 3H, Ar–H), 7.09–7.18 (m, 3H, Ar–H), 6.92–6.98 (m, 2H, Ar–H), 5.17 (s, 2H, benzyl), 4.32–4.38 (q, 2H, $J=7.2$ Hz, ester CH_2), 4.16–4.20 (t, 2H, $J=7.2$ Hz, H_1 of pentyl), 3.91 (s, 3H, methoxy), 1.73–1.80 (quint, 2H, H_2 of pentyl), 1.36 (t, 3H, $J=6.8$ Hz, ester CH_3), 1.17–1.22 (quintet, 4H, H_3 and H_4 of pentyl), 0.79 (t, 3H, $J=6.8$ Hz, H_5 of pentyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 167.1, 163.0 ($J=244.8$ Hz), 155.1, 149.6 ($J=44.5$ Hz), 142.1, 139.1 ($J=7.1$ Hz), 138.7, 130.2, 130.1, 124.9, 124.2, 123.0, 122.6, 121.8, 121.6, 115.0, 114.8, 114.08 ($J=22.1$ Hz), 113.2 ($J=42.1$ Hz), 109.6, 70.1, 60.8, 56.1, 45.0, 29.4, 28.7, 22.1, 14.3, 13.8; ESI–MS (*m/z*): 491.20 $[M+H]^+$; anal. calcd. for $C_{29}H_{31}FN_2O_4$: C, 71.00; H, 6.37; N, 5.71%. Found: C, 71.02; H, 6.38; N, 5.73%.

Ethyl 2-(4-((3-fluorobenzyl)oxy)-3-methoxyphenyl)-1-hexyl -1H-benzo[d]imidazole-5-carboxylate (6o)

Yield: 93%; Mp. 74–76 °C; pale yellow solid; FT-IR (ATR, υ_{max}, cm^{-1}): 3389 (=C–H), 2929 (C–H), 1708 (C=O), 1611 (C=C), 1207 (C–F), 1017 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.49 (s, 1H, Ar–H), 7.69–7.93 (dd, 1H, $J=1.2$ and 8.4 Hz, Ar–H), 7.31–7.40 (m, 3H, Ar–H), 7.11–7.22 (m, 3H, Ar–H), 6.93–6.99 (m, 2H, Ar–H), 5.17 (s, 2H, benzyl), 4.37–4.42 (q, 2H, $J=7.0$ Hz, ester CH_2), 3.27–3.32 (m, 2H, H_1 of hexyl), 3.86 (s, 3H, methoxy), 1.65–1.73 (quint, 2H, H_2 of hexyl), 1.23–1.43 (m, 9H, H_3, H_4, H_5 of hexyl and ester CH_3), 0.79 (t, 3H, $J=6.9$ Hz, H_6 of hexyl); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 167.9, 163.7 ($J=245.0$ Hz), 156.2, 150.3 ($J=44.6$ Hz), 142.3, 139.9 ($J=7.1$ Hz), 139.2, 130.9, 130.7, 125.6, 125.0, 123.9, 123.0, 122.0, 121.9, 115.9, 115.0, 114.9 ($J=22.5$ Hz), 114.0 ($J=42.5$ Hz), 110.6, 67.9, 60.2, 55.3, 46.3, 31.9, 30.1, 23.0, 22.9, 14.0, 13.6; ESI–MS (*m/z*): 505.25 $[M+H]^+$; anal. calcd. for $C_{30}H_{33}FN_2O_4$: C, 71.41; H, 6.59; N, 5.55%. Found: C, 71.42; H, 6.60; N, 5.56%.

Biological screening and computational studies

MTT assay

Briefly, cells were seeded at 7500 cells/well into a 96-well plate and incubated overnight. The medium was removed and replaced with fresh media before compound treatment. Cells were treated with different concentrations of **compounds** 1, 10, 25 and 50 µM, respectively, and DMSO was used as negative control. Cells were incubated for 48 and 72 h and subjected to MTT assay with addition of thiazolyl blue tetrazolium bromide (MTT; Sigma; St Louis, MO, USA) and incubated additionally for 4 h. Insoluble formazan crystals was solubilized with 100 µl DMSO. Spectrophotometric absorbance of the plate was recorded at 570 nm using a Tecan Microplate Reader (Tecan Instruments,

Switzerland). Data were collected using Megalan software (Tecan Instruments, Inc) and exported to Microsoft Excel for analysis. Half-Maximal inhibitory concentrations (IC50) will be determined using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) with the 4-parameter logistic function standard curve analysis for dose response. Error bars were calculated based on a minimum of three independent experiments and data represented as histogram.

In vitro anti-inflammatory

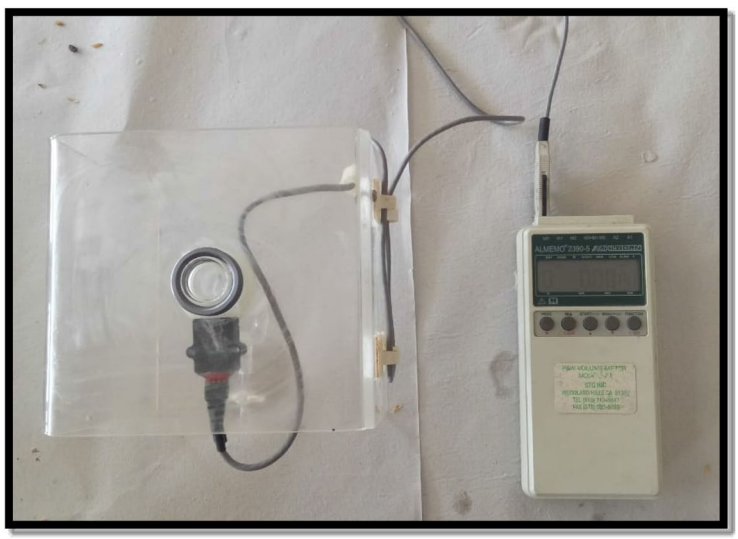
Test solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5%w/v aqueous solution) and 0.05 ml of test solution. The control solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of test solution. Standard solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5%w/v aqueous solution) and 0.05 ml of diclofenac sodium (200 µg/ml). All of the above solutions were adjusted to pH 6.3 using a small amount of 1 N HCl. The samples were incubated at 37 °C for 20 min and heated at 57 °C for 3 min. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance of the solutions was measured using UV-visible spectrophotometer at 416 nm. The percentage inhibition of protein denaturation was calculated using the formula.

$$\% \text{ inhibition} = 100 \times \left[V_t / V_C - 1 \right]$$

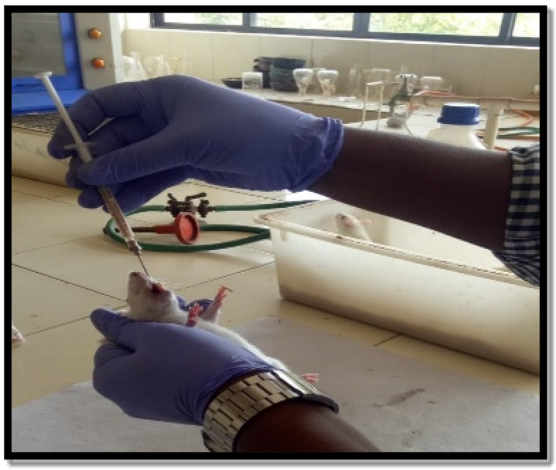
In vivo anti-inflammatory

Animals Wistar rats of either sex and of approximately the same age, weighing about 125–150 g used for the study, were housed in polypropylene cages and fed with standard pellet diet and water ad libitum. The animals were under alternate cycle of 12 h of darkness and light each. Before each test, the animals were fasted for at least 12 h. The experimental protocols according to the CPCSEA guidelines and IAEC clearance were taken prior to the commencement of the study.

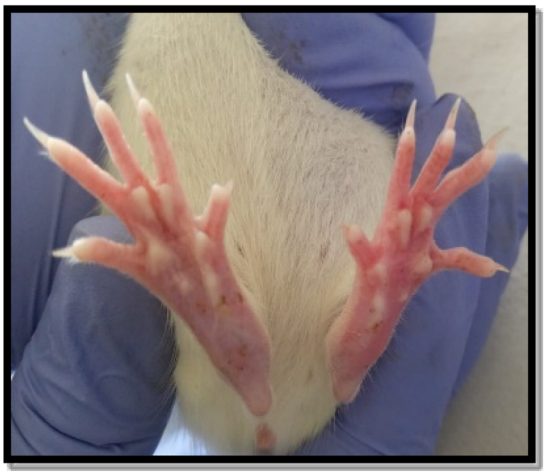
The rats were divided into eight groups of six animals each. Group I (negative control) received 1 ml of normal saline, second Group II (Standard) received 10 mg/kg p.o. indomethacin, and Group III-VIII received synthetic compounds 50 mg/kg, respectively. After 1 h, the rats were challenged with subcutaneous injection of 0.1 ml of 1% w/v solution of carrageenan (Sigma chemical co, St. Louis MO, USA) into the plantar side of the left hind paw. The paw was marked in the cup of the apparatus with water. The digital plethysmograph apparatus used for the measurement of rat paw volume. The paw volume was measured every hour till 3 h after injection of carrageenan to each group. The difference between the initial and subsequent reading gave the actual edema volume.



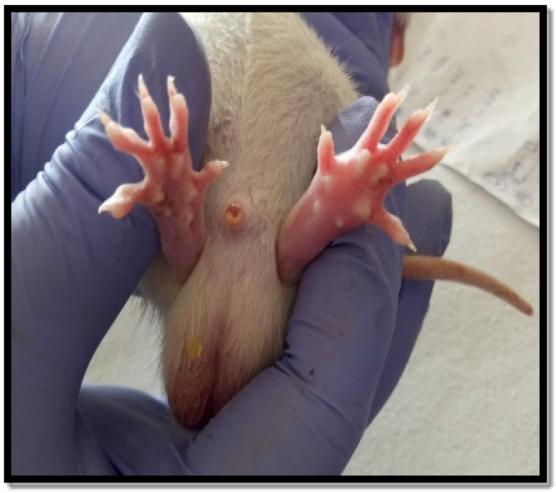
Plethysmograph apparatus used for the measurement of rat paw volume



1



2



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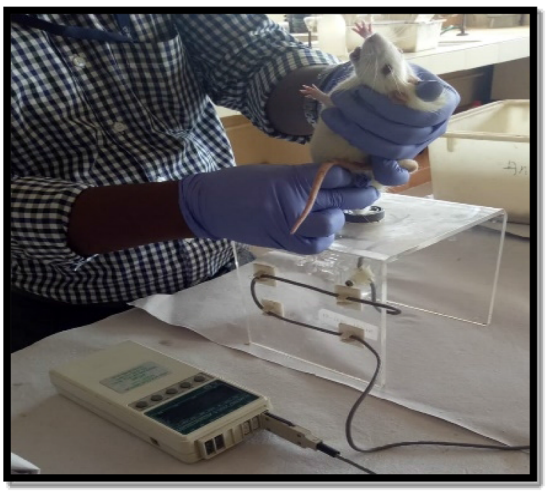

4

Image 1: Introduction of synthesized drugs orally

Image 2: Paw volume of rat after introduction of synthesized drug

Image 3: Paw volume of rat after injecting carrageenan to left hind paw

Image 4: Measuring paw volume using plethysmograph apparatus

Percentage inhibition was calculated using the following formula

$$\left(V_C - V_T / V_C\right) \times 100$$

where V_C = Volume of control, V_T = Volume of test.

Acute toxicity studies

Acute toxicity studies (OECD, 2001) on Wister albino mice were carried out by standard method at oral of 100–1,000 mg/kg body weight as per OECD 425 guidelines. All the animals were continuously observed for 8 h for any signs of acute toxicity such as ataxia, tremors and convulsions. The acute toxicity studies revealed that all the compounds were found to be non-toxic up to 1,000 mg/kg body weight. The control group received 1% Tween 80 suspension. Animals were kept in fasting condition prior to dosing.

Physicochemical properties

The physicochemical properties have been calculated using Molinspiration on-line property calculation toolkit (http://www.molinspiration.com). The formulation of % ABS = 109 − (0.354 * TPSA) was used for estimating percentage of absorption (% ABS) [31].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13738-021-02381-y.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experimental protocols according to the CPCSEA guidelines and IAEC clearance were taken prior to the commencement of the study. The animal treatment protocol was approved by Reg. No. NGSM/IAEC/March-2019/129.

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