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Original Article

SYNTHESIS AND DOCKING STUDIES OF 2-(NITROOXY) ETHYL-4-(2-(SUBSTITUTEDPHENYL)-4-(SUBSTITUTEDPHENYL)-1H-IMIDAZOL-1-YL) BENZOATE AS ANTI-INFLAMMATORY, ANALGESIC AND NITRIC OXIDE RELEASING AGENTS

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ABSTRACT

Objective: The objective of the present study was to develop potent and non toxic Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) by using heterocyclic nuclei and having Nitric Oxide releasing group.

Methods: The compounds were designed with the help of docking studies. In the synthetic study, the target compounds were obtained by reacting substituted diphenyl imidazole benzoic acid (2a-2x) with nitro-oxy alkyl bromide in the presence of dimethyl formamide and potassium carbonate to give substituted 2,4-diphenyl nitric oxide releasing imidazole derivatives (3a-3x). The synthesized compounds were characterized with the help of different analytical studies and further evaluated for anti-inflammatory, analgesic and nitric oxide releasing activity.

Results: In the docking study compounds 3a, 3b, 3c, 3e, 3r and 3s showed significant G-score. In the anti-inflammatory and analgesic study compounds 3a, 3b, 3c, 3e, 3r and 3s exhibited promising activity. All the synthesized compounds exhibited significant nitric oxide releasing properties both *in-vitro* and *in-vivo*.

Conclusion: Compounds 3a, 3b, 3c, 3e, 3r and 3s exhibited prominent anti-inflammatory and analgesic activity.

Keywords: Imidazole, Docking, Anti-inflammatory, Analgesic, Nitric oxide.

INTRODUCTION

Painkiller is the most commonly taken drug today, it reduces mainly the fever and inflammation. Selective Cyclo-oxygenase-2 (COX-2) inhibitors elicit less or no GI damage and bleeding compared with conventional Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), although the magnitude of this reduction continues to be debated in the literature [1]. As widely reported in the lay press, the selective COX-2 inhibitors also cause significant adverse effects in the renal and cardiovascular systems, possibly more serious than those caused by conventional NSAIDs.

Recent strategies adopted to minimize the side effects of NSAIDs include the use of the dual LOX/COX inhibitors, the use of selective COX-2 inhibitors, and the use of hybrid molecules made up of non-selective or selective COX inhibitors together with a nitric oxide-releasing functional group [2-4].

The imidazole ring forms the core of many pharmacological important molecules having a wide array of activities like anti-inflammatory [5-7], anticancer, antimicrobial and antioxidant [8]. The top selling active pharmaceutical ingredients comprise of an imidazole nucleus are losartan, olmesartan and ondasetron. The imidazole derivatives are also found naturally in the amino acid like histidine, in vitamin B12 and a component of the DNA base structure.

Synthetic approaches based on chemical modification of NSAIDs have been taken with the aim of improving safety profile and in turn therapeutic window of the resultant NSAIDs. Our previous studies had described the synthesis of hybrid molecules with nitric oxide-releasing group that resulted in an increased anti-inflammatory activity with reduced GI-ulcerogenicity [1].

In our attempt to continue to discover new, safer, and potent agents for the treatment of inflammatory diseases, we have synthesized compounds containing pharmacophore of 1,3,5 triaryl imidazole ring, the pharmacophore somewhat similar to coxibs and nitric oxide-releasing group to accentuate potency and reduce GI toxicities associated with the traditional NSAIDs. The compounds designed so were found to possess much significant anti-inflammatory, analgesic with significant nitric oxide releasing activity.

MATERIALS AND METHODS

Synthetic studies

All the compounds were synthesized using the reported literature procedures, and synthetic procedures were set and optimized as and were required. All the chemicals & solvents were purchased from avra chemicals & sigma-aldrich. Melting points were uncorrected & recorded on optimelt digital melting point apparatus. IR spectra were recorded on bruker alpha E FTIR spectrophotometer.^{1}HNMR were recorded on varian 400MHz spectrometer by using TMS as internal standard and $CDCl_3$ as a solvent. Mass spectra were recorded on scinpor Q-TOF.

General procedure for the synthesis of 2-bromo-1-(substituted phenyl)-ethanone(1a-1x)

A solution of 0.42 moles of acetophenone in pure anhydrous ether was placed in two neck round bottom flask fitted with a reflux condenser. The solution was cooled in an ice bath and 0.5 g of anhydrous aluminum chloride was introduced and 0.42 moles of bromine were added gradually with stirring. After the addition of bromine, ether and the dissolved hydrogen bromide were removed under reduced pressure. The product was remained as a solid mass. The crude product was recrystallized from methanol.

General procedure for the synthesis of 4-(2-(substituted phenyl)-4-(substituted phenyl) imidazol-1-yl)-benzoic acid (2a-2x)

A mixture of benzaldehyde (2 mmol), p-amino benzoic acid (2 mmol), substituted bromophenylethanone (1a-1x, 2 mmol) and ammonium acetate (3 mmol) was stirred at 130 °C for 2 h, then the reaction mixture was cooled to room temperature and the product precipitated from a 1:1 mixture of acetone–water and then recrystallized from *n*-hexane–ethyl acetate [9].

Synthesis of nitrooxy ethyl bromide

A bromo alcohol (10 mmol) was added drop wise to a solution of 70% HNO_3 (1.1 ml) and 95% H_2SO_4 (2.4 ml) at 0 °C, and the reaction was allowed to proceed at the same temperature for 1 h with stirring. The resulting suspension was poured into water (50 ml),

extracted with CH_2Cl_2 (3 × 200 ml), and dried $MgSO_4$, and the solvent was removed to give the nitrooxy ethyl bromide [10].

General procedure for the synthesis of 2-(nitrooxy)-ethyl-4-(2-(substitutedphenyl)-4-(substitutedphenyl)-1H-imidazol-1-yl)-benzoate (3a-3x)

A solution of 2-nitooxyethyl bromide (1.2 mmol), substituted diphenyl imidazole benzoic acid (2a-2x, 1 mmol) and K_2CO_3 (1.5 mmol) in dry DMF (5 ml) was stirred at 25 °C for 12 h. Water (15 ml) was added, and the mixture was extracted with EtOAc (3 × 20 ml) and washed with brine (20 ml). The EtOAc fraction was dried over Na_2SO_4, the solvent was removed in vacuo, and the residue obtained was purified by silica gel column chromatography using ethyl acetate–hexane (1:1, v/v) as eluent to furnish compound [11].

Analytical data

2-(nitrooxy)ethyl-4-(2,4-diphenyl-1H-imidazol-1-yl)benzoate (3a)

White solid; IR: 3052, 2880, 1730, 1540, 1680, 1580 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$): δ= 4.6 (t, 2H,CH_2), 4.7 (t,2H, CH_2), 7.01 (m, 4H, CH), 7.22 (m, 3H, CH), 7.25 (d, 3H, CH), 7.40 (m, 2H, CH), 7.45 (d, 2H, CH), 8.40 (s,1H,CH). MS: *m/z* 430 $[M+H]^+$. Elemental analysis: Found C (67.11), H (4.47), N(9.80) Calculated C (67.13), H (4.46), N (9.79).

2-(nitrooxy)ethyl-4-(4-phenyl-2-(pyridin-2-yl)-1H-imidazol-1-yl)benzoate (3b)

Yellow solid; IR: 3070, 2851, 1745, 1555, 1590, 1627 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.56 (t, 2H,CH_2), 4.69 (t, 2H, CH_2), 7.21 (m, 4H, CH), 7.30 (m, 3H, CH), 7.41 (d, 2H, CH), 7.5 (m, 2H, CH), 7.6 (d, 2H, CH), 8.63 (s, 1H, CH). MS: *m/z* 431 $[M+H]^+$. Elemental analysis: Found C (64.19), H (4.22), N (13.01) Calculated C (64.18), H (4.22), N (13.02).

2-(nitrooxy)ethyl-4-(4-phenyl-2-(thiophen-2-yl)-1H-imidazol-1-yl)benzoate (3c)

Off white solid; IR: 3060, 2857, 1755, 1528, 1657, 1587 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.18 (t, 2H,CH_2), 4.35 (t, 2H, CH_2), 7.22 (m, 4H, CH), 7.32 (m, 3H, CH), 7.45 (d, 3H, CH), 7.48 (m, 2H, CH), 8.6 (s, 1H, CH). MS: *m/z* 436 $[M+H]^+$. Elemental analysis: Found C (60.69), H (3.94), N (9.67) Calculated C (60.68), H (3.93), N (9.65).

2-(nitrooxy)ethyl-4-(2-(4-chlorophenyl)-4-phenyl-1H-imidazol-1-yl)benzoate (3d)

Buff solid; IR: 3090, 2940, 1695, 1535, 1680, 1535 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.48 (t, 2H,CH_2), 4.66 (t, 2H, CH_2), 7.18 (m, 4H, CH), 7.31 (m, 3H, CH), 7.44 (d, 3H, CH), 7.52 (m, 3H, CH), 8.33 (s, 1H, CH). MS: *m/z* 464 $[M+H]^+$. Elemental analysis: Found C (62.17), h (3.89), N (9.08) Calculated C (62.14), H (3.91) N (9.06).

2-(nitrooxy)ethyl-4-(2-(4-fluorophenyl)-4-phenyl-1H-imidazol-1-yl)benzoate (3e)

White solid; IR: 3011, 2955, 1709, 1522, 1626, 1563 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.42 (t, 2H,-CH_2), 4.7 (t,2H,-CH_2), 7.20 (m, 4H, CH), 7.30 (m, 3H, CH), 7.40 (d, 3H, CH), 7.55 (m, 3H, CH), 8.69 (s, 1H, CH). MS: *m/z* 448 $[M+H]^+$. Elemental analysis: Found C (64.45), H (4.08), N (9.37) Calculated C (64.43), H (4.06), N (9.39).

2-(nitrooxy)ethyl-4-(2-(4-cyanophenyl)-4-phenyl-1H-imidazol-1-yl)benzoate (3f)

Pale yellow solid; IR: 3034, 2848, 1696, 1535, 1598, 1636, 2242 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.5 (t, 2H,CH_2), 4.68 (t, 2H, CH_2), 7.22 (m, 4H, CH), 7.32 (m, 3H, CH), 7.5 (d, 3H, CH), 7.52 (m, 3H, CH), 8.65 (s, 1H, CH). MS: *m/z* 455 $[M+H]^+$. Elemental analysis: Found C (66.09), H (4.01), N (12.34) Calculated C (66.08), H (3.99), N (12.33).

2-(nitrooxy)ethyl-4-(2-(4-methoxyphenyl)-4-phenyl-1H-imidazol-1-yl)benzoate (3g)

White solid; IR: 3047, 2822, 1681, 1511, 1551, 1511, 1190 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.9 (s, 3H, CH_3), 4.7 (t, 2H,CH_2), 4.65 (t, 2H, CH_2), 7.11 (m, 4H, CH), 7.34 (m, 3H, CH), 7.44 (d, 3H, CH), 7.5 (m, 3H, CH), 8.59 (s, 1H, CH). MS: *m/z* 460 $[M+H]^+$. Elemental analysis: Found C (65.37), H (4.62),N(9.17) Calculated C(65.35),H(4.61),N (9.15).

2-(nitrooxy)ethyl-4-(2-(4-hydroxyphenyl)-4-phenyl-1H-imidazol-1-yl)benzoate (3h)

White solid; IR: 3044, 2888, 1787, 1565, 1520, 1627, 3370 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.5 (t, 2H,CH_2), 4.6 (t,2H, CH_2), 5.0 (s, 1H, OH), 7.22 (m, 4H, CH), 7.30 (m, 3H, CH), 7.40 (d, 3H, CH), 7.55 (m, 3H, CH), 8.46 (s, 1H, CH). MS: *m/z* 446 $[M+H]^+$. Elemental analysis: Found C (64.73), H (4.31),N(9.45) Calculated C(64.72),H(4.30),N (9.43).

2-(nitrooxy)ethyl-4-(4-(4-methoxyphenyl)-2-phenyl-1H-imidazol-1-yl)benzoate(3i)

Yellow solid; IR: 3087, 2817, 1781, 1533, 1532, 1172, 1607,3353 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.90 (s, 3H, CH_3), 4.5 (t, 2H,CH_2), 4.6 (t,2H, CH_2), 7.21 (m, 4H, CH), 7.35 (m, 3H, CH), 7.42 (d, 3H, CH), 7.55 (m, 3H, CH), 8.62 (s, 1H, CH). MS: *m/z* 460 $[M+H]^+$. Elemental analysis: Found C(65.36),H(4.63), N(9.17) Calculated C(65.35), H(4.61), N (9.15).

2-(nitrooxy)ethyl-4-(4-(4-methoxyphenyl)-2-(pyridin-2-yl)-1H-imidazol-1-yl)benzoate (3j)

White solid; IR: 3096, 2829, 1795, 1555, 1518, 1148, 1632 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.92 (s, 3H, CH_3), 4.44 (t, 2H,CH_2), 4.5 (t, 2H,CH_2), 7.1 (m, 4H, CH), 7.35 (m, 3H, CH), 7.44 (d, 3H, CH), 7.52 (m, 2H, CH), 8.61 (s, 1H, CH). MS: *m/z* 461 $[M+H]^+$. Elemental analysis: Found C(62.61),H(4.40),N(12.18) Calculated C(62.60),H(4.38),N (12.17).

2-(nitrooxy)ethyl-4-(4-(4-methoxyphenyl)-2-(thiophen-2-yl)-1H-imidazol-1-yl)benzoate (3k)

Reddish brown solid; IR: 3105, 2810, 1787, 1540, 1530, 1157, 1642 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.85 (s, 3H, CH_3), 4.56 (t, 2H,CH_2), 4.7 (t, 2H, CH_2), 7.22 (t, 4H, CH), 7.35 (m, 3H, CH), 7.42 (d, 2H, CH), 7.50 (m, 2H, CH), 8.64 (s, 1H, CH) MS: *m/z* 466 $[M+H]^+$. Elemental analysis: Found C(59.37), H(4.12), N(9.05) Calculated C(59.35), H(4.11), N(9.03).

2-(nitrooxy)ethyl-4-(2-(4-chlorophenyl)-4-(4-methoxyphenyl)-1H-imidazol-1-yl)benzoate (3l)

White solid; IR: 3046, 2835, 1771, 1519, 1542, 1181, 1626 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.88 (s, 3H, CH_3), 4.47 (t, 2H,CH_2), 4.61 (t, 2H, CH_2), 7.22 (m, 4H, CH), 7.31 (m, 3H, CH), 7.43 (d, 3H, CH), 7.52 (m, 2H, CH), 8.54 (s, 1H, CH). MS: *m/z* 494 $[M+H]^+$. Elemental analysis: Found C(60.82),H(4.10), N(8.52) Calculated C(60.80), H(4.08), N (8.51).

2-(nitrooxy)ethyl-4-(2-(4-fluorophenyl)-4-(4-methoxyphenyl)-1H-imidazol-1-yl)benzoate (3m)

White solid; IR: 3063, 2815, 1788, 1525, 1540, 1210, 1644 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.84 (s, 3H, CH_3), 4.48 (t, 2H,CH_2), 4.66 (t, 2H, CH_2), 7.18 (m, 4H, CH), 7.28 (m, 3H, CH), 7.3 (d, 3H, CH), 7.54 (m, 2H, CH), 8.62 (s, 1H, CH). MS: *m/z* 478 $[M+H]^+$. Elemental analysis: Found C(62.90), H(4.19), N(8.81) Calculated C(62.89), H(4.22), N(8.80).

2-(nitrooxy)ethyl-4-(2-(4-cyanophenyl)-4-(4-methoxyphenyl)-1H-imidazol-1-yl)benzoate (3n)

Pale yellow solid; IR: 3014, 2818, 1748, 1535, 1549, 1201, 2222, 1595 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.80 (s, 3H, CH_3), 4.45 (t, 2H,CH_2), 4.65 (t, 2H, CH_2), 7.22 (m, 4H, CH), 7.28 (m, 3H, CH), 7.35 (d, 3H, CH), 7.4 (m, 2H, CH), 8.58 (s, 1H, CH). MS: *m/z* 485 $[M+H]^+$. Elemental analysis: Found C (64.47), H (4.14), N (11.55) Calculated C (64.46), H (4.16), N (11.56).

2-(nitrooxy)ethyl-4-(2,4-bis(4-methoxyphenyl)-1H-imidazol-1-yl)benzoate (3o)

White solid; IR: 3015, 2799, 1771, 1509, 1556, 1223, 1660 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.8 (m, 6H, CH_3), 4.45 (t, 2H,CH_2), 4.6 (t, 2H, CH_2), 7.20 (m, 4H, CH), 7.32 (m, 3H, CH), 7.40 (d, 3H, CH), 7.54 (m, 2H, CH), 8.2 (s, 1H, CH). MS: *m/z* 490 $[M+H]^+$. Elemental analysis: Found C(63.82), H(4.75), N(8.60) Calculated C(63.80), H(4.74), N(8.58).

2-(nitrooxy)ethyl-4-(2-(4-hydroxyphenyl)-4-(4-methoxyphenyl)-1H-imidazol-1-yl) benzoate (3p)

White solid; IR: 3080, 2829, 1742, 1511, 1549, 1199, 1660, 3369 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.82 (s, 3H, CH_3), 4.45 (t, 2H,CH_2), 4.6 (t, 2H, CH_2), 5.2 (s, 1H, OH), 7.20 (m, 4H, CH), 7.36 (m, 3H, CH), 7.44 (d, 3H, CH), 7.52 (m, 2H, CH), 8.29 (s, 1H, CH). MS: *m/z* 476 $[M+H]^+$. Elemental analysis: Found C (63.16), H (4.46), N (8.85) Calculated C (63.15), H (4.45), N(8.84).

2-(nitrooxy)ethyl-4-(4-(4-fluorophenyl)-2-phenyl-1H-imidazol-1-yl)benzoate (3q)

Off white solid; IR: 3052, 2839, 1733, 1526, 1556, 1642 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.5 (t, 2H,CH_2), 4.7 (t, 2H, CH_2), 7.22 (m, 4H, CH), 7.30 (m, 3H, CH), 7.4 (d, 3H, CH), 7.55 (m, 3H, CH), 8.7 (s, 1H, CH). MS: *m/z* 448 $[M+H]^+$. Elemental analysis: Found C (64.44), H (4.05), N (9.37) Calculated C (64.43), H (4.06), N (9.39).

2-(nitrooxy)ethyl-4-(4-(4-fluorophenyl)-2-(pyridin-2-yl)-1H-imidazol-1-yl)benzoate (3r)

White solid; IR: 3040, 2841, 1720, 1536, 1550, 1633 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$): δ 4.6 (t, 2H,CH_2), 4.7 (t, 2H, CH_2), 7.2 (m, 4H, CH), 7.3 (m, 3H, CH), 7.43 (d, 3H, CH), 7.5 (m, 2H, CH), 8.0 (s, 1H, CH). MS: *m/z* 449 $[M+H]^+$. Elemental analysis: Found C (61.62), H (3.84), N (12.50) Calculated C (61.61), H (3.82), N (12.49).

2-(nitrooxy)ethyl-4-(4-(4-fluorophenyl)-2-(thiophen-2-yl)-1H-imidazol-1-yl)benzoate (3s)

White solid; IR: 3031, 2819, 1754, 1539, 1563, 1626 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.5 (t, 2H,CH_2), 4.7 (t, 2H, CH_2), 7.25 (m, 4H, CH), 7.31 (m, 3H, CH), 7.45 (d, 3H, CH), 7.8 (m, 1H, CH), 8.0 (s, 1H, CH). MS: *m/z* 454 $[M+H]^+$. Elemental analysis: Found C (58.28) H (3.57), N (9.29) Calculated C (58.27), H (3.56), N (9.27).

2-(nitrooxy)ethyl-4-(2-(4-chlorophenyl)-4-(4-fluorophenyl)-1H-imidazol-1-yl)benzoate (3t)

Yellow solid; IR: 3081, 2840, 1752, 1513, 1540, 1612 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$): δ= 4.8 (t, 2H,CH_2), 4.9 (t, 2H, CH_2), 7.21 (m, 4H, CH), 7.30 (m, 3H, CH), 7.5 (d, 3H, CH), 7.55 (m, 2H, CH), 8.1 (s, 1H, CH). MS: *m/z* 482 $[M+H]^+$. Elemental analysis: Found C (59.84), H (3.55), N (8.70) Calculated C (59.82), H (3.56), N (8.72).

2-(nitrooxy)ethyl-4-(2,4-bis(4-fluorophenyl)-1H-imidazol-1-yl)benzoate (3u)

White solid; IR: 3045, 2856, 1740, 1548, 1565, 1629 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.7 (t, 2H,CH_2), 4.8 (t, 2H, CH_2), 7.24 (m, 4H, CH), 7.38 (m, 3H, CH), 7.46 (d, 3H, CH), 7.54 (m, 2H, CH), 8.5 (s, 1H, CH). MS: *m/z* 466 $[M+H]^+$. Elemental analysis: Found C (61.95), H (3.70), N (9.05) Calculated C (61.94), H (3.68), N (9.03).

2-(nitrooxy)ethyl-4-(2-(4-cyanophenyl)-4-(4-fluorophenyl)-1H-imidazol-1-yl)benzoate (3v)

Off white solid; IR: 3080, 2822, 1710, 1517, 1535, 2210, 1626 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.52 (t, 2H,CH_2), 4.64 (t, 2H, CH_2), 7.24 (m, 4H,-CH), 7.3 (m, 3H,-CH), 7.5 (d, 3H, CH), 7.55 (m, 2H, CH), 8.39 (s, 1H, CH). MS: *m/z* 473 $[M+H]^+$. Elemental analysis: Found C (63.57), H (3.64), N (11.86), Calculated C (63.56), H (3.63), N (11.86).

2-(nitrooxy)ethyl-4-(4-(4-fluorophenyl)-2-(4-methoxyphenyl)-1H-imidazol-1-yl)benzoate (3w)

White solid; IR: 3022, 2839, 1725, 1529, 1549, 2231, 1175, 1660 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 3.8 (s, 3H,-CH_3), 4.52 (t, 2H,-CH_2), 4.6 (t, 2H,-CH_2), 7.28 (m, 4H,-CH), 7.32 (m, 3H,-CH), 7.51 (d, 3H, CH), 7.6 (m, 2H, CH), 8.19 (s, 1H, CH). MS: *m/z* 478 $[M+H]^+$. Elemental analysis: Found C(62.90), H(4.24), N(8.81) Calculated C(62.89), H(4.22), N(8.80).

2-(nitrooxy)ethyl-4-(4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-1H-imidazol-1-yl)benzoate (3x)

Buff solid; IR: 2989, 2830, 1719, 1510, 1556, 2230, 3310, 1636 cm^{-1} ^{1}HNMR (400 MHz, $CDCl_3$):δ= 4.55 (t, 2H,-CH_2), 4.6 (t, 2H,-CH_2), 5.2 (s, 1H,-OH), 7.22 (m, 4H,-CH), 7.30 (m, 3H,-CH), 7.52 (d, 3H, CH), 7.65 (m, 2H, CH), 8.1 (s, 1H, CH). MS: *m/z* 464 $[M+H]^+$. Elemental analysis: Found C(62.21), H(3.93), N(9.09) Calculated C(62.20), H(3.92), N(9.07).

Pharmacology

Animals

Swiss albino mice of either sex weighing 20–25 g and wistar rats weighing in the range 140–160 g were used. All the animals were housed under standard ambient conditions of temperature (25±2 °C) and relative humidity of 50±5%. A 12/12-h light/dark cycle was maintained. All the animals were allowed to have free access to water and standard palletized laboratory animal diet 24 h before pharmacological studies. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of the Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad constituted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

Preparation of test compounds

After suspending the test compounds in 1.0% aqueous solution of sodium carboxy methyl cellulose (CMC), the test samples were administered to the test animals orally. The positive and negative control group animals received the same experimental handling as those of the test groups except that the drug-treated control group animals received only appropriate volumes of the vehicle and of the reference drug, celecoxib.

Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the well known carrageenan-induced rat paw edema model [12], using twenty six groups of six animals each. A freshly prepared aqueous suspension of carrageenan (1.0% w/v, 0.1 ml) was injected in the sub planter region of the right hind paw of each rat. One group was kept as control, and the animals of the other group were pre-treated with the test compounds, 1 h before the carrageenan treatment. The volume was measured before and after carrageenan treatment at 60 min intervals with the help of digital plethysmometer.

Analgesic activity

The analgesic activity was evaluated using the acetic acid induced writhing method [13], using groups of six animals each. A solution of acetic acid (1% v/v) in distilled water was prepared and injected intraperitoneally in a volume of 0.1 ml. One group was kept as control, and the animals of the other group were pre-treated with the test compounds, 1 h before the acetic acid (1% v/v) treatment. The writhing episodes were recorded for 15 min, stretching movements consisting of arching of the back, elongation of the body, and extension of hind limbs was counted.

***In vitro* and *in vivo* screening of nitric oxide**

Vasorelaxing activity [14]

In order to determine a possible vasodilator mechanism of action, the compounds were tested on isolated aortae of male normotensive wistar rats (250–350 g). The rats were sacrificed by cervical dislocation under light ether anesthesia and bled. The aortae was immediately excised, freed of extraneous tissues and prepared as multiple-ring preparations. Then the vessels were suspended, under a preload of 2g in 10 ml organ baths, containing tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 7; H_2O 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously bubbled with a mixture of O_2 (95%) and CO_2(5%). Changes in tension were recorded by means of an isometric transducer (BIOPAC System, Inc., MP 35, USA). After an equilibration period of 60 min, the endothelial integrity was confirmed by acetylcholine (ACh) (55 µM)-induced relaxation of norepinephrine (NE 20 µg/ml) pre contracted tissues. A relaxation ≥ 70% of the NE induced contraction was considered representative of an acceptable presence of the endothelial layer, while the organs, showing a relaxation<70%, were not used in the experimental

procedures. At 30–40 min after the confirmation of the endothelial integrity, the aortic preparations were contracted by treatment with a single concentration of NE (20 µg/ml) or KCl (30 mM), and when the contraction reached a stable plateau, the test compounds in concentration (0.1 mg/ml) were added cumulatively.

Detection of nitrite [15]

A solution of the appropriate compound (20 µl) in dimethyl sulfoxide was added to 2 ml of 1:1 v/v mixture either of 50 mM phosphate buffer (pH 7.4) or of HCl solution (pH 1) with methanol, containing 5 × 10^{-4}M L-cysteine. The final concentration of the test compound was 10^{-4} M. After 1 h at 37 °C, 1 ml of the reaction mixture was treated with 250 µl of Griess reagent [sulphanilamide (4 g), N-naphthyl ethylene diamine dihydrochloride (0.2 g), and 85% phosphoric acid (10 ml) in distilled water (final volume: 100 ml)]. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 µmol/ml) were used to construct the calibration curve. The results were expressed as the percentage of NO released (n-2) relative to a theoretical maximum release of 1 mol NO/mol of test compound.

Statistical analysis

The data obtained for each set of anti-inflammatory model were expressed as the mean of change in paw volume±SD and analyzed by one-way ANOVA followed by Dunnett's test. Data from acetic acid-induced writhing model were expressed as the mean of the number of writhes±SEM and analyzed by one way ANOVA followed by Dunnett's 't' test. Level of significance was set to $P<0.05$. All the statistical calculations were performed using the evaluation version of Graph Pad Prism 3.0 (USA) statistical software.

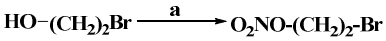
Docking methodology

Molecular docking studies were performed using Glide v6.2 (Schrödinger, LLC). The coordinates for COX-2 enzyme were taken from RCSB Protein Data Bank (PDB Id 1CX2) and prepared for docking using protein preparation wizard. Water molecules in the structure were removed and termini were capped by adding ACE and NMA residue. The bond orders and formal charges were added for hetero groups and hydrogens were added to all atoms in the structure. Side chains that were not close to the binding cavity and do not participate in salt bridges were neutralized. After preparation, the structures were refined to optimize the hydrogen bond network using OPLS_2005 force field. This helps in reorientation of the side chain hydroxyl group. The minimization was terminated when the energy converged or the RMSD reached a maximum cut off of 0.30 Å. Grids were then defined around refined structure by centering on ligand using default box size. The extra precision (XP) docking mode for compounds, optimized by Ligprep, was performed on the generated grid of protein structure [16].

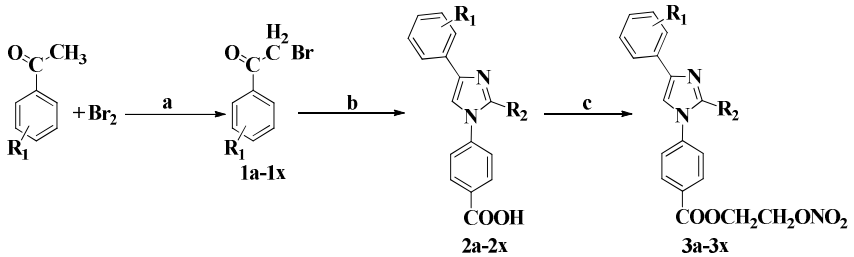
RESULTS AND DISCUSSION

Chemistry

The synthesis of target compounds 3a-3x is shown in scheme 1-2. Substituted acetophenones were brominated in the presence of aluminium chloride and ether to afford substituted bromo phenyl ethanone (1a-1x). The substituted bromo phenyl ethanone were further reacted with 4-amino benzoic acid, substituted benzaldehyde and ammonium acetate to give substituted diphenyl imidazole benzoic acid (2a-2x). Nitro-oxy ethyl bromide was prepared from bromoethanol in the presence of nitric acid and sulfuric acid (scheme 1). The target compounds were obtained by reacting substituted diphenyl imidazole benzoic acid (2a-2x) with nitro-oxy alkyl bromide in the presence of dimethyl formamide and potassium carbonate to give substituted 2,4-diphenyl nitric oxide releasing imidazole derivatives (3a-3x, scheme 2, table1). The structures of various synthesized compounds were assigned on the basis of results of different chromatographic and spectral studies. The physical data, FTIR, ^{1}H-NMR, mass spectral data and elemental analysis data for all the synthesized compounds are given in experimental protocols.



Scheme 1: Synthesis of intermediate nitro-oxy ethyl bromide. Reagents and conditions (a) 70% HNO_3, 95% H_2SO_4, 0 °C, 1h



Scheme 2: Synthesis of compounds 3a-3x. Reagents and conditions (a) $AlCl_3$, Et_2O (b) 4-amino benzoic acid, substituted aldehydes, NH_4OAc, solvent free, 120 °C, 2h (c) O_2NO-CH_2-CH_2-Br, DMF, K_2CO_3, 25 °C, 12 h

Pharmacology

The synthesized compounds were subjected to the evaluation of anti-inflammatory activity, analgesic activity and nitric oxide-releasing properties. Celecoxib was used as reference standard. The experiments were performed using albino rats of wistar strain of either sex, weighing in the range of 140–160 g. The animals were maintained at 25±2 °C, 50±5% relative humidity and 12-h light/dark cycle. All the animals were fasted for 24 h before the experiments and water was provided ad libitum. The test compounds were suspended in 1% aqueous carboxy methyl cellulose (CMC) solution and administered orally to experimental animals for all the studies.

Anti-inflammatory activity

Anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan-induced rat paw edema model and equimolar doses are equivalent to celecoxib. Sub-planter injection of 0.1 ml of 1% carrageenan in rat paw increased the paw volume (edema) in all the animals of various groups. The onset of action was evident during1 h in various test groups. The significant (*P<0.01*) reduction of rat paw edema was observed for most of the test compounds, at 3 h compared to control group and celecoxib (table 2).

Out of the synthesized compounds 3a, 3b, 3c, 3e, 3r and 3s (68.13–72.54%) exhibited very significant anti-inflammatory activity compared to standard drug celecoxib (71.56% at 3 h).

Thus, the compounds having a substitution of hydrogen on R_1 position and phenyl, pyridine, thiophene and fluoro group on the phenyl ring respectively at R_2 position (3a, 3b, 3c, 3e) show equipotent activity with celecoxib. Compound 3r and 3s also shows equipotent activity in which R_1 position is occupied by the fluoro group and R_2 position is occupied with pyridine and thiophene ring. Compound 3k and 3u shows decreased anti-inflammatory activity. (In compound 3k, R_1 position is occupied by methoxy group and R_2 position is occupied by the thiophene ring, whereas in compound 3u, R_1 and R_2 both positions are occupied by fluoro group). Based on the

findings of these preclinical results, further studies need to be carried out to investigate the other specifications, such as *in vitro* assays, toxicological studies and the mechanism by which these drugs exhibit potential analgesic, anti-inflammatory activities.

Table 1: Characterization data for synthesized compounds (3a-3x)

S. No.	Entry	R_1	R_2	MF	MW	% yield	MP(°C)
1	3a	-H	C_6H_5	$C_{24}H_{19}N_3O_5$	429	88	145-146
2	3b	-H		$C_{23}H_{18}N_4O_5$	430	69	174-176
3	3c	-H		$C_{22}H_{17}N_3O_5S$	435	72	136-137
4	3d	-H	4-ClC_6H_4	$C_{24}H_{18}ClN_3O_5$	463	85	155-157
5	3e	-H	4-FC_6H_4	$C_{24}H_{18}FN_3O_5$	447	88	165-166
6	3f	-H	4-CNC_6H_4	$C_{25}H_{18}N_4O_5$	454	80	188-189
7	3g	-H	4-$MeOC_6H_4$	$C_{25}H_{21}N_3O_6$	459	74	122-123
8	3h	-H	4-OHC_6H_4	$C_{24}H_{19}N_3O_6$	445	86	203-204
9	3i	4-MeO	C_6H_5	$C_{25}H_{21}N_3O_6$	459	80	222-223
10	3j	4-MeO		$C_{24}H_{20}N_4O_6$	460	70	214-215
11	3k	4-MeO		$C_{23}H_{19}N_3O_6S$	465	68	189-190
12	3l	4-MeO	4-ClC_6H_4	$C_{25}H_{20}ClN_3O_6$	493	82	236-237
13	3m	4-MeO	4-FC_6H_4	$C_{25}H_{20}FN_3O_6$	477	64	206-207
14	3n	4-MeO	4-CNC_6H_4	$C_{26}H_{20}N_4O_6$	484	60	109-111
15	3o	4-MeO	4-$MeOC_6H_4$	$C_{26}H_{23}N_3O_7$	489	79	129-130
16	3p	4-MeO	4-OHC_6H_4	$C_{25}H_{21}N_3O_7$	475	83	228-229
17	3q	4-F	C_6H_5	$C_{24}H_{18}FN_3O_5$	447	86	220-221
18	3r	4-F		$C_{23}H_{17}FN_4O_5$	448	67	170-172
19	3s	4-F		$C_{22}H_{16}FN_3O_5S$	453	63	196-197
20	3t	4-F	4-ClC_6H_4	$C_{24}H_{17}ClFN_3O_5$	481	87	175-176
21	3u	4-F	4-FC_6H_4	$C_{24}H_{17}F_2N_3O_5$	465	89	150-151
22	3v	4-F	4-CNC_6H_4	$C_{25}H_{17}FN_4O_5$	472	82	133-134
23	3w	4-F	4-$MeOC_6H_4$	$C_{25}H_{20}FN_3O_6$	477	81	178-179
24	3x	4-F	4-OHC_6H_4	$C_{24}H_{18}FN_3O_6$	463	85	139-140

Table 2: Results of anti-inflammatory activity of synthesized compounds (3a-3x) against carrageenan-induced rat paw edema model in rats

Compound Code	Change in paw volume in (ml) after drug treatment(±SEM)			Anti-inflammatory activity (% Inhibition)		
	1h	2h	3h	1h	2h	3h
Control	1.72±0.038**	1.93±0.05**	2.04±0.017**	-	-	-
Celecoxib	0.67±0.019**	0.63±0.024**	0.58±0.07**	61.04	67.35	71.56
3a	0.69±0.055**	0.67±0.063**	0.63±0.085**	59.88	65.28	69.11
3b	0.65±0.07**	0.62±0.041**	0.56±0.012**	62.20	67.87	72.54
3c	0.66±0.065**	0.64±0.028**	0.58±0.022**	61.62	66.83	71.07
3d	1.11±0.035**	1.13±0.09**	1.16±0.020**	35.46	41.45	43.13
3e	0.72±0.049**	0.68±0.062**	0.65±0.051**	58.13	64.76	68.13
3f	0.79±0.040**	0.76±0.04**	0.74±0.079**	54.06	60.62	63.72
3g	0.93±0.021**	0.91±0.022**	0.88±0.047**	45.93	52.84	56.86
3h	1.01±0.044**	1.06±0.050**	1.09±0.009**	39.53	45.07	46.56
3i	0.83±0.019**	0.81±0.011**	0.78±0.049**	51.74	58.03	61.76
3j	1.03±0.033**	1.06±0.032**	1.08±0.039**	40.11	45.07	47.05
3k	1.13±0.06**	1.15±0.033**	1.17±0.026**	34.30	40.41	42.64
3l	0.71±0.011**	0.68±0.027**	0.66±0.004**	58.72	64.76	67.64
3m	1.01±0.043**	1.05±0.08**	1.07±0.052**	41.27	45.59	47.54
3n	0.73±0.063**	0.71±0.052**	0.68±0.027**	57.55	63.21	66.66
3o	0.90±0.038**	0.88±0.005**	0.85±0.042**	47.67	54.40	58.33
3p	1.06±0.019**	1.08±0.039**	1.11±0.008**	38.37	44.04	45.58
3q	0.91±0.009**	0.88±0.047**	0.86±0.062**	47.09	54.40	57.84
3r	0.68±0.069**	0.66±0.090**	0.64±0.063**	60.46	65.80	68.62
3s	0.66±0.044**	0.63±0.046**	0.60±0.066**	61.62	67.35	70.58
3t	0.88±0.041**	0.84±0.03**	0.81±0.036**	48.83	56.47	60.29
3u	1.12±0.008**	1.15±0.070**	1.17±0.072**	34.88	40.41	42.64
3v	0.81±0.035**	0.79±0.039**	0.76±0.009**	52.90	59.06	62.74
3w	0.99±0.077**	0.97±0.051**	0.94±0.059**	42.44	49.74	53.92
3x	1.03±0.080**	1.05±0.026**	1.08±0.044**	40.11	45.59	47.05

Data analyzed by one way ANOVA followed by Dunnett's 't' test, (n = 6), * P<0.05, ** P<0.01 significant from control *ns* not significant

Analgesic activity

The analgesic activity of the synthesized compounds was studied by using acetic acid-induced writhing test in mice. The analgesic activity was evaluated at equimolar doses equivalent to celecoxib. The important analgesic profile of the compounds was measured by the classical acetic acid-induced writhing model. From the results, it was noticed that all the compounds possess significant analgesic activity (table 3). The analgesic effect of compounds 3a, 3b, 3c, 3e, 3r and 3s (61.68-66.11%) were found to be equipotent compared to standard drug celecoxib (66.56%) similar to anti-inflammatory activity. Compound 3d and 3h shows decreased analgesic activity due to substitution of hydrogen at R_1 position and chloro, hydroxyl group on the phenyl ring respectively at R_2 position.

Table 3: Results of analgesic activity of synthesized compounds (3a-3x) against acetic acid-induced writhing test in mice

Compound Code	No of Writhes in 5-15 min. after treatment (mean±SE)	% Inhibition
Control	26.41±0.57**	-
Celecoxib	8.83±0.19**	66.56
3a	9.21±0.28**	65.12
3b	9.85±0.11**	62.70
3c	8.95±0.13**	66.11
3d	14.36±0.11**	45.62
3e	10.12±0.67**	61.68
3f	13.42±0.38**	49.18
3g	12.35±0.17**	53.23
3h	14.21±0.90**	46.19
3i	10.91±0.82**	58.68
3j	13.98±0.62**	47.06
3k	14.09±0.49**	46.64
3l	12.86±0.44**	51.30
3m	13.03±0.09**	50.60
3n	11.36±0.30**	56.98
3o	12.69±0.35**	61.64
3p	11.94±0.78**	54.78
3q	12.17±0.77**	53.91
3r	9.67±0.33**	63.38
3s	9.03±0.46**	65.80
3t	10.55±0.29**	60.05
3u	13.24±0.29**	49.98
3v	11.99±0.66**	54.60
3w	12.65±0.94**	52.10
3x	13.59±0.39**	48.54

Data analyzed by one way ANOVA followed by Dunnett's 't' test,(n = 6), ** P<0.01 significant from control

Table 4: EC_{50} values and nitric oxide-releasing properties of the compounds (3a–3x)

S. No.	Compound Code	EC_{50}	% NO release
1	3a	39.85	0.46
2	3b	48.54	0.32
3	3c	37.75	0.49
4	3d	45.55	0.59
5	3e	31.16	0.65
6	3f	50.41	0.78
7	3g	28.56	0.30
8	3h	33.45	0.33
9	3i	55.30	0.44
10	3j	29.65	0.70
11	3k	32.07	0.55
12	3l	36.22	0.61
13	3m	52.06	0.49
14	3n	49.12	0.52
15	3o	42.22	0.77
16	3p	33.10	0.37
17	3q	36.85	0.68
18	3r	46.93	0.66
19	3s	44.68	0.34
20	3t	35.20	0.62
21	3u	34.87	0.55
22	3v	37.73	0.51
23	3w	39.65	0.58
24	3x	46.22	0.43

Nitric oxide-release study

In isolated wistar rat aorta rings, compounds 3a-3x competitively inhibited norepinephrine-induced contraction effects, causing a shift to the right of the norepinephrine concentration response curves. EC_{50} (µg/ml) values were calculated from the cumulative concentration response curves. In order to prove the involvement of nitric oxide in the relaxation process, nitric oxide-releasing properties of synthesized compounds were assessed in phosphate buffer, pH 7.4, in the presence of L-cysteine, relative to nitric oxide

released from standard sodium nitrite solution (table 4). From *in vitro* nitric oxide releasing data, it is observed that compound 3f and 3o shows potent nitric oxide releasing properties, whereas compound 3b and 3h shows less nitric oxide releasing properties.

From nitric oxide releasing activity of rat aortic muscle, it is observed that compound 3g and 3j shows potent EC_{50} values, whereas compound 3i and 3m shows less EC_{50} value.

Docking study

In all series, the docking poses of compounds showing higher docking score (G-score) were compared with that of standard celecoxib in the active site of the COX-2 enzyme. Similarly the docking pose of all other compounds were compared with that of compound showing higher G-score within the series for comparing their pharmacophoric features desired for good binding affinity toward COX-2 enzyme.

The docking study of imidazole derivatives showed that only some of all compounds were docked and few of them showed comparable binding affinity toward COX-2 enzyme (table 5). This may be due to triaryl ring system of imidazole derivatives compared to diaryl rings in celecoxib (fig. 1). The compound 3b showed the good G-score and thus higher binding affinity for the COX-2 enzyme among the imidazole derivatives.

The pyridine ring in compound 3b at R_2 position formed the hydrogen bond with amino acid Tyr 355 which further supports the ligand-enzyme complex (fig. 2). Thus the replacement of pyridine at R_2 position by substituted phenyl ring in other compounds, exhibited the decreased binding affinity of compounds toward COX-2 enzyme, due to lack of H-bond with surrounding amino acids by substituents at R_2 position (fig. 3). However, the compounds substituted by unsubstituted phenyl ring and thiophene ring showed comparably good binding affinity than the compounds containing substituted phenyl ring at R_2 position.

Table 5: Docking score of compounds (3a-3x)

S. No.	Entry	G Score	H Bond	Lipophilic EvdW	Phob En
1	Celecoxib	-10.5	-1.3	-6.1	-1.5
2	3a	-7.32	0	-2.29	-4.86
3	3b	-7.91	-0.66	-2.23	-4.36
4	3c	-7.67	-0.99	-1.87	-4.29
5	3d	-4.25	0	-2.26	-5.27
6	3e	-6.69	0	-2.11	-4.04
7	3f	-4.52	0	-2.61	-4.9
8	3g	ND*	ND*	ND*	ND*
9	3h	ND*	ND*	ND*	ND*
10	3i	ND*	ND*	ND*	ND*
11	3j	ND*	ND*	ND*	ND*
12	3k	-2.1	0	0	-2.8
13	3l	ND*	ND*	ND*	ND*
14	3m	ND*	ND*	ND*	ND*
15	3n	ND*	ND*	ND*	ND*
16	3o	ND*	ND*	ND*	ND*
17	3p	ND*	ND*	ND*	ND*
18	3q	ND*	ND*	ND*	ND*
19	3r	-7.8	-0.66	-2.47	-4.1
20	3s	-6.37	0	-2.25	-3.74
21	3t	ND*	ND*	ND*	ND*
22	3u	-0.78	0	0	-2.11
23	3v	ND*	ND*	ND*	ND*
24	3w	ND*	ND*	ND*	ND*
25	3x	ND*	ND*	ND*	ND*

ND* Not Docked, H Bond: Chem score hydrogen bond pair term, Lipophilic EvdW: Chem score lipophilic pair term and fraction of total protein ligand vanderwall energy, Phob En: Hydrophobic enclosure reward.

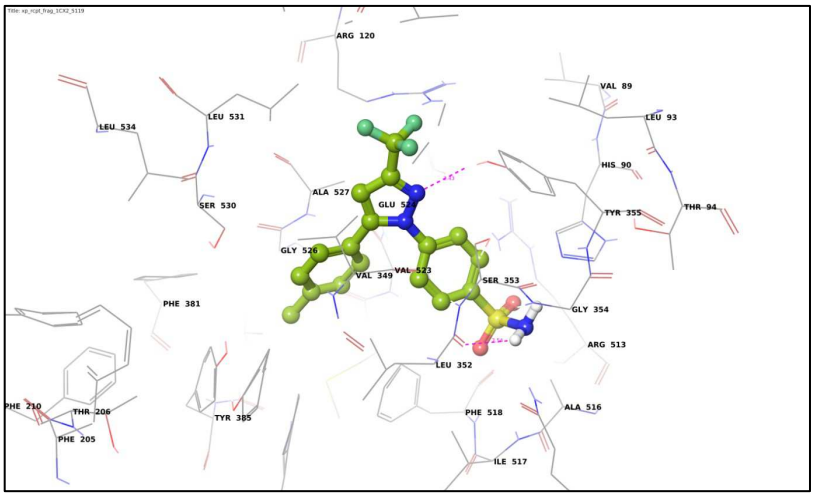


Fig. 1: Docking pose of celecoxib in active site of COX-2 enzyme

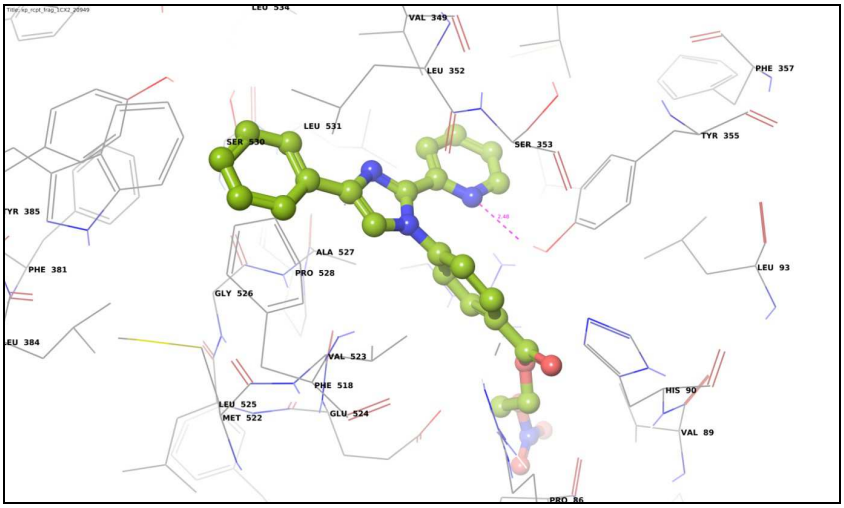


Fig. 2: Docking pose of compound 3b in active site of COX-2 enzyme

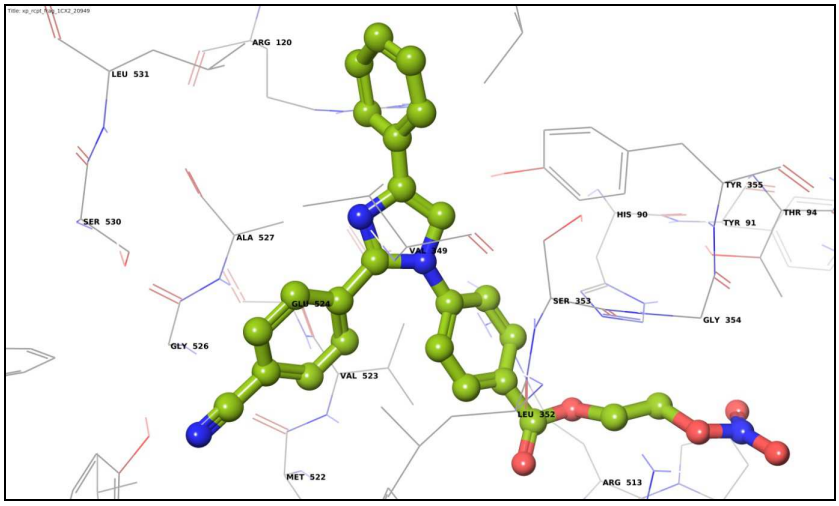


Fig. 3: Docking pose of compound 3f in active site of COX-2 enzyme

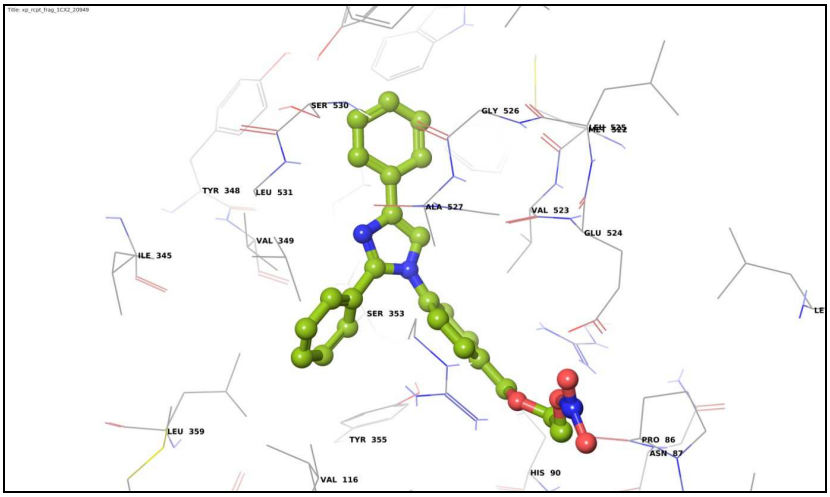


Fig. 4: Docking pose of compound 3a in active site of COX-2 enzyme

Similarly, un substitution and substitution by fluorine atom at R_1 position help the compounds 3a-3c and 3r-3s to fit properly in the surrounding hydrophobic pocket of the COX-2 enzyme (fig. 4).

Thus the replacement of hydrophobic substituents by an electron withdrawing group (OCH_3) at R_1 position decreases the hydrophobic interaction with surrounding amino acids and thus the binding affinity of compounds (fig. 5).

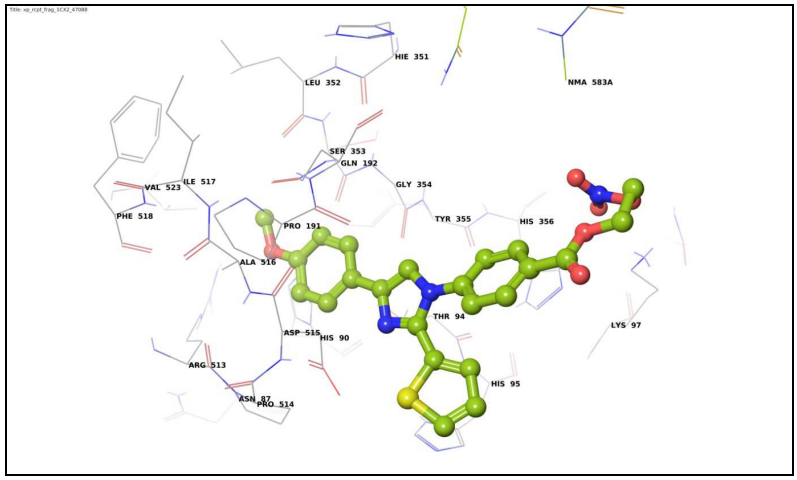


Fig. 5: Docking pose of compound 3k in active site of COX-2 enzyme

CONCLUSION

Twenty four compounds were synthesized and screened for analgesic, anti-inflammatory and nitric oxide-releasing studies. Docking study of these synthesized compounds was also performed. Most of the compounds exhibited significant anti-inflammatory, analgesic and nitric oxide releasing activity. Compounds 3a, 3b, 3c, 3e, 3r and 3s exhibited more prominent and constitute anti-inflammatory activity. Compounds 3a, 3b, 3c, 3e, 3r and 3s showed strong analgesic activity in the acetic acid-induced writhing tests. From the detailed analysis of the results of pharmacological studies, we conclude that the synthesized compounds have not only retained but showed enhanced anti-inflammatory profile. Also, all the synthesized derivatives exhibited significant vasorelaxant activity that is devoid of CVS toxicities associated with coxib families of COX-2 inhibitors. Therefore, it can be concluded that the rational, based on which these NCEs were designed, has been proven to be superior compared to the currently used NSAIDs. The outcome of this research study is very promising. The only concern to be addressed now is the pharmacokinetics profile of the designed NCEs, since molecular weight of all the designed compounds is beyond 500. The most potent derivatives are in the process for chronic toxicity and pharmacokinetic studies, based on those results, a further action plan will be finalized.

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CONFLICT OF INTERESTS

Declared None

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