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Synthesis and Molecular Drug Validations of 1*H*-Benzo[*d*]imidazol-2-amine Derivatives: Molecular Docking and its Biological Activities

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Molecular adaptation of small molecules that are targeted as therapeutic agents is a most anticipated one in drug designing and development. In the present approach, a family of substituted 1*H*-benzo[*d*]imidazol-2-amine derivatives (**5a-d** and **6a-e**) were effectively synthesised and testified for their molecular adaptations in order to develop them as novel medications against oxidation, inflammation and inflammation associated cancer types by means of *in silico* and *in vitro* assessments. Chronic inflammation, regardless of infectious agents, plays a vital role in various cancer development. Moreover, hypoxia-inflammation-cancer are highly associated together. Hydrogen peroxide free-radical scavenging, HRBC membrane stabilization assay and cell viability test by MTT assay (macrophage) were executed to establish antioxidant, anti-inflammatory and anticancer properties of these compounds. As the prostaglandin-endoperoxide synthase 2 is highly involved in inflammation and cancer development respectively, molecular docking was executed on the corresponding X-ray crystallographic models (PDB structures).

Keywords: 1*H*-Benzo[*d*]imidazol-2-amine, Molecular docking, Prostaglandin-endoperoxide synthase 2 (COX-2), Biological activities.

INTRODUCTION

Small molecule drug designing is one of the important area in medicinal chemistry [1]. Computational tools are non-time consumable effective tools to identify efficacious drugs now a days [2]. As the drug resistance and drug validity are decreasing the effectiveness of an available drug in market, moreover increased side-effects on these drugs induces the need of search of novel and safer medicines. Among a range of different chemical entities, benzoimidazoles/benzimidazoles are notable with higher medicinal values [3]. Benzimidazoles have showed a range of biological activities starting from anti-tuberculosis [4], anticancer [5], antimicrobial [6], intestinal antiseptic [7], antidiabetic [8], *etc*. Their flexibility in electron exchange between various electron donor and acceptors between the receptors making benzimidazoles as a chief inhibitors based therapeutic agents. They are inhibitors of kinases [9,10], cyclo-oxygenase [11], H^+, K^+ ATPase/proton pump [12,13], acetyl-cholinesterase [14] and protein kinase CK2 [15], *etc*.

In this study, newly synthesized 1*H*-benzo[*d*]imidazol-2-amine derivatives (**5a-d** and **6a-e**) (Fig. 1) were assessed for their bioactivity potentials through various bioactivity prediction tools such as Molinspiration (molinspiration.com) and PASSonline. As the results suggested, anti-inflammation potentials of these compounds were executed through HRBC membrane stabilization, macrophage cell viability and determination of proinflammatory and anti-inflammatory interleukin levels through macrophages [16]. As the oxidation plays a major role in inflammation development, antioxidant properties of these compounds also assessed through H_2O_2 radical scavenging analysis. A molecular docking was carried out using X-ray crystallographic structure (PDB ID: 5F1A) of prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2) [17], is an enzyme that in humans is encoded by the PTGS2 gene [18]. In human, it is highly participating in the alteration of arachidonic acid to prostaglandin H2. Prostaglandin H2 an imperative precursor of prostacyclin, which is often expressed in inflammation [19].

EXPERIMENTAL

All organic chemicals and reagents procured from Sigma-Aldrich, Spectrochem and Avra. Solvents and common reagents used as without doing any further purification. For thin-layer

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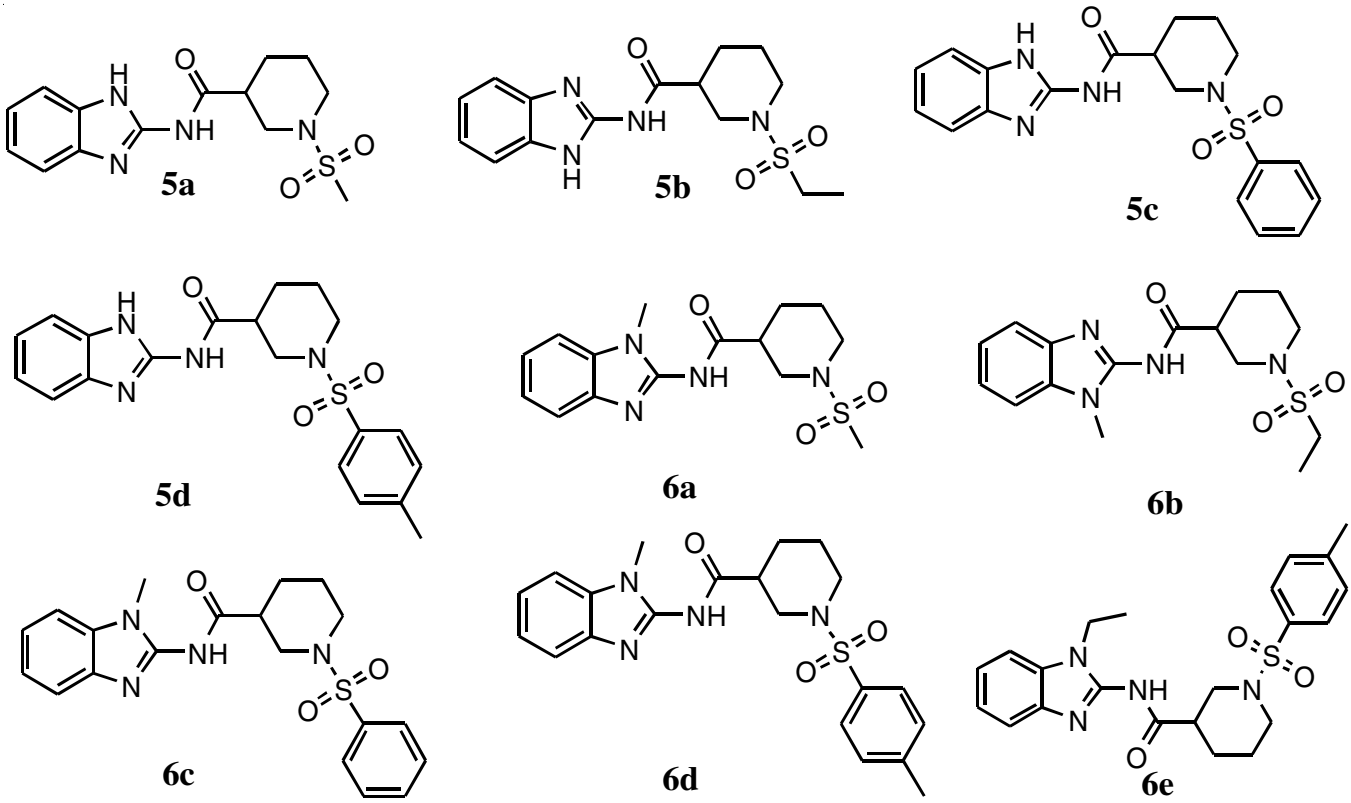


Fig. 1. Achieved final structures of 1*H*-benzo[d]imidazol-2-amine derivatives (**5a-d** and **6a-e**)

chromatography (TLC) analysis, Merck pre-coated plated (Silica gel 60 F254) used and spots visualized under UV light. Redisep silica gel columns used for flash chromatography and mobile phase solvents as indicated in procedures. ^{1}H and ^{13}C NMR spectra were recorded in Bruker-300MHz instrument while LC-MS was recorded on Agilent instruments.

Synthesis of *tert*-butyl 3-((1*H*-benzo[*d*]imidazol-2-yl) carbamoyl)piperidine-1-carboxylate (3) using standard amidation reaction: To a 100 mL single neck round bottom flask, 1-(*tert*-butoxycarbonyl) piperidine-3-carboxylic acid (**2**) (1 equiv. or 1 mol), 1H-benzo[*d*]imidazol-2-amine (**1**) (1.5 equiv. or 1.5 mol), 1-[*bis*(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (1 equiv. or 1 mol) and DMF (8-12 vol.) were added under nitrogen atmosphere. The contents were stirred for 5 min at 20-30 ºC. After clear solution formation *N*,*N*-diisopropylethylamine (DIPEA) (2 equiv. or 2 mol) was added and the reaction mass stirred for 12-16 h at 20-30 ºC. After 12-16 h, the completion of reaction was confirmed by TLC or LC-MS. Ice-cold water (20 vol.) was added slowly to the reaction mixture with stirring. The precipitated solid material filtered and washed with purified water (10 vol.) followed by acetone (5 vol.). The crude material thus obtained further taken for Boc de-protection without purification. The coupling reaction of 1-(*tert*-butoxycarbonyl)piperidine-3-carboxylic acid and 1*H*-benzo[*d*]imidazol-2-amine was optimized by using different bases, solvents and different temperature ranges to get the compound **3** with good yield.

Synthesis of *N*-(1*H*-benzo[d]imidazol-2-yl)piperidine-3- carboxamide (4): To a 100 mL single neck round bottom flask, *tert*-butyl 3-((1*H*-benzo[*d*]imidazol-2-yl)carbamoyl) piperidine-1-carboxylate (**3**) (1 equiv.), distilled water:acetone (1:2, 20 vol.) were added and stirred the reaction mixture for 5 min at 20-30 ºC. 1N HCl (2 vol.) was added and stirred the reaction mass for 2-3 h at 50-55 ºC for reaction completion. Reaction completion was confirmed by TLC. After reaction completion, cooled the reaction mass to 20-25 ºC. The pH was adjusted to 7.5-8 using liquid NH_3 and extracted product using dichloromethane (DCM) (2 × 15 vol.). The DCM layer was separated washed with water (10 vol.) and then DCM layer was dried over anhydrous sodium sulphate and concentrated under vacuum. The obtained products **4** dried thoroughly under vacuum and taken for *N*-substitution reaction without further purification. ^{1}H NMR (DMSO-d_6, 300 MHz): 7.36-7.35 (d, J = 3 Hz, 2H), 7.02-7.01 (d, J = 3 Hz, 2H), 3.64 (s, 1H), 2.96-2.93 (d, J = 9 Hz, 1H), 2.69 (bs, 1H), 2.56-2.41 (m, 2H), 1.79 (bS, 1H), 1.61 (bs, 1H) and 1.42 (bS, 2H).

General procedure for the preparation of target compounds (5-d) by pipiridine-*N*-sulfonylation reaction: To a 100 mL single neck round bottom flask, N-(1*H*-benzo[*d*]imidazol-2-yl)piperidine-3-carboxamide (**4**) (1 equiv. or 1 mol), DCM (20 vol.) were added and stirred the reaction mass for 5 min at 20-30 ºC. Sodium hydroxide powder (3 equiv.) was added to the clear solution and stirred the reaction mass at 0-5 ºC for 10 min followed by the addition of sulfonylating reagents (1.5-2.0 equiv.) and again stirred the reaction mass for reaction completion at 20-30 ºC. Reaction completion confirmed by TLC/LC-MS. After reaction completion filtered the mass through hyflo bed and washed with 2 vol. of DCM. The filtrate dried over anhydrous sodium sulphate and distilled off the volatiles under reduced pressure to get the crude gummy/semi-solid material. These crude materials were purified using column chromatography with silica as stationary phase and DCM:MeOH

as mobile phase to get pure products (**5a-d**). The pure material thus obtained yielded 60-85 % yield of the product. Pipiridine-*N*-sulfonylation reaction using different substituting reagents was optimised by using methyl sulfonyl chloride as substituting reagent with different bases, solvents, catalysts and different temperature ranges to get intermediate compound **4** with good yield.

Synthesis of *N*-(1*H*-benzo[*d*]imidazol-2-yl)-1-(methyl sulfonyl)piperidine-3- carboxamide (5a): Methane sulfonyl chloride used as reagent to get compound **5a** with 76 % yield, m.p.: 249-251 ºC. ^{1}H NMR (DMSO-d_6): 7.36-7.29 (d, J = 21 Hz, 2H, Ar-H), 7.12-7.11 (d, J = 3 Hz, 2H, Ar-H), 3.57 (bs, 1H, -CH), 3.51 (S, 1H, -CH_3), 2.96-2.92 (d, J = 12 Hz, 1H, -CH), 2.73-2.69 (d, J = 9Hz, 1H, -CH), 2.40-2.28 (m, 2H, -CH_2), 1.84 (bs, 1H, -CH), 1.58 (bs, 1H, -CH) and 1.43-1.32 (quat, J = 12 Hz, 2H, -CH2). ^{13}C NMR δ ppm: 151.1 (-HN<u>C</u>OCH), 132.25 (-N<u>C</u>NH); 122.19, 121.90 and 109.26 (arom. C), 51.41 (-N<u>C</u>H_2CH_2), 46.90 (-N<u>C</u>H_2CH), 40.10 (-OC<u>C</u>HCH_2), 31.25 (-S<u>C</u>H_3) 28.88 (HC<u>C</u>H_2CH_2) and 24.85 (-H_2C<u>C</u>H_2CH_2). HRMS for $C_{14}H_{18}N_4O_3S$ calcd. (found): 322.3830 (322.3833).

Synthesis of *N*-(1*H*-benzo[*d*]imidazol-2-yl)-1-(ethyl-sulfonyl)piperidine-3-carboxamide (5b): Ethane sulfonyl chloride used as reagent to get compound **5b** with 79 % yield, m.p.: 242-243 ºC. ^{1}H NMR (MeOD-d_4): 7.55 (bs, 2H, Ar-H), 7.38 (bs, 2H, Ar-H), 3.91 (bs, 1H, -CH), 3.77 (bs, 3H, -CH_2 and -CH), 3.44-3.40 (d, J = 12Hz, 1H, -CH), 2.97-2.95 (m, 1H, -CH_2), 1.79-1.64 (m, 4H, -CH_2 and -CH_2) and 1.30-1.20 (m, 3H, -CH_3). ^{13}C NMR δ ppm: 151.98 (-HN<u>C</u>OCH), 144.39 (-N<u>C</u>NH); 129.65, 128.21, 125.16, 124.91, 112.88 and 110.66 (arom. C), 49.52 (-S<u>C</u>H_2CH_3), 45.91 (-N<u>C</u>H_2CH_2), 43.52 (-N<u>C</u>HCH), 29.31 (-OC<u>C</u>HCH_2) 28.18 (HCCH_2CH_2), 22.08 (H_2C<u>C</u>H_2CH) and 6.71 (-H_2C<u>C</u>H_3) ppm. HRMS for $C_{15}H_{20}N_4O_3S$ calcd. (found): 336.4100 (336.4123).

Synthesis of *N*-(1*H*-benzo[*d*]imidazol-2-yl)-1-(phenyl-sulfonyl)piperidine-3-carboxamide (5c): Benzene sulfonyl chloride used as reagent to get compound **5c** with 81 % yield, m.p.: 228-230 ºC. ^{1}H NMR (DMSO-d_6): 8.17-8.15 (m, 1H, -NH), 7.86-7.51 (m, 6H, Ar-H), 7.37-7.26 (m, 3H, Ar-H), 3.85-3.74 (m, 1H, -CH), 3.46-2.85 (m, 4H, -CH_2) and 1.75-1.44 (m, 4H, -CH_2). ^{13}C NMR δ ppm: 151.66 (-HN<u>C</u>OCH), 144.48 (-N<u>C</u>NH); 135.32, 133.78, 130.96, 130.4, 129.80, 128.51-128.40, 127.26, 125.22, 125.54, 113.48 and 110.30 (arom. C), 50.21 (-N<u>C</u>H_2CH_2), 46.02 (-N<u>C</u>H_2CH), 45.34 (-OC<u>C</u>HCH_2), 30.28 (HC<u>C</u>H_2CH_2) and 28.34 (H_2C<u>C</u>H_2CH). HRMS for $C_9H_{20}N_4O_3S$ calcd. (found): 384.4540 (384.4545).

Synthesis of *N*-(1*H*-benzo[*d*]imidazol-2-yl)-1-tosylpiperidine-3-carboxamide (5d): *p*-Toluenesulfonyl chloride used as reagent to get compound **5d** with 79.5 % yield, m.p.: 212-214 ºC. ^{1}H NMR (MeOD-d_4): 7.82-7.64 (m, 5H, Ar-H), 7.51-7.44 (m, 3H, Ar-H), 4.07 (bs, 1H , -CH), 3.86 (S, 3H, -CH_3), 3.22-3.15 (m, 3H, -CH and -CH_2), 2.87-2.85 (m, 1H, -CH) and 1.88-1.69 (m, 4H, -CH_2). ^{13}C NMR δ ppm: 151.64 (-HN<u>C</u>O-CH), 144.40 (-N<u>C</u>NH); 135.37, 133.91, 130-02, 129.89-129.84, 128.56-128.48, 127.74, 125.82, 125.21-124.96, 113.67 and 111.38 (arom. C), 50.28 (-N<u>C</u>H_2CH_2), 46.52 (-N<u>C</u>H_2CH), 45.65 (-OC<u>C</u>HCH_2), 30.24 (HC<u>C</u>H_2CH_2), 28.12 (H_2C<u>C</u>H_2CH) and 21.83 (-HC<u>C</u>H_3)ppm. HRMS for $C_{20}H_{22}N_4O_3S$ calcd. (found): 398.4810 (398.4816).

General procedure for the preparation of target compounds (6a-e) by benzimidazole-*N*-alkylation of compounds (5a-d): To a 100 mL single neck round bottom flask, compounds **5a-d** (1 equiv. or 1 mol), DCM (20 vol.) were added and stirred the reaction mass for 5 min at 20-30 ºC. Tetrabutyl-ammonium hydrogen sulphate (0.010 equiv.) was added and stirred for 10 min. Potassium hydroxide powder (3.0 equiv.) was added to the clear solution and stirred the reaction mass again at 0-5 ºC for 10 min followed by the addition of methyl iodide/ethyl iodide reagents (1.5-2.0 equiv.) and again stirred the reaction mass for reaction completion at 20-30°C. Reaction completion was confirmed by TLC/LC-MS. After reaction completion filtered, the mass through hyflo-bed was washed with 2 vol. of DCM. The filtrate was dried over anhydrous sodium sulphate and distilled off the volatiles under reduced pressure to get the crude gummy/semi-solid material. These crude materials were purified using column chromatography with silica as stationary phase and DCM:MeOH as mobile phase to get pure products (**6a-e**).

Synthesis of *N*-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-1-(methylsulfonyl)piperidine-3-carboxamide (6a): Methyl iodide used as reagent to get compound **6a** from compound **5a** as pure compound with 82 % yield, m.p.: 218-221 ºC. ^{1}H NMR (MeOD-d_4): 7.68 (bs, 2H, Ar-H), 7.51-7.50 (m, 2H, Ar-H), 4.14 (bs, 1H, -CH), 3.86 (s, 3H, -NCH_3), 3.47-3.38 (m, 3H, -CH and -CH_2), 3.14-3.12 (m, 1H, -CH), 2.88 (s, 3H, -CH_3) and 1.87-1.79 (m, 4H, -CH_2). ^{13}C NMR δ ppm: 154.06 (-HN<u>C</u>OCH), 148.34 (-N<u>C</u>NH); 121.22 and 113.88 (arom. C), 50.48 (-OC-<u>C</u>H_2CH_2), 45.98 (-N<u>C</u>H_2CH), 45.72 (-OC<u>C</u>HCH_2), 34.68 (-N<u>C</u>H_3 and -S<u>C</u>H_3), 29.14 (HC<u>C</u>H_2CH_2) and 22.75 (-H_2C<u>C</u>H_2CH_2). LC-MS = 338.10 (M+2). HRMS for $C_{15}H_{20}N_4O_3S$ calcd. (found): 336.4100 (336.4115).

Synthesis of 1-(ethylsulfonyl)-*N*-(1-methyl-1*H*-benzo-[*d*]imidazol-2-yl)piperidine-3-carboxamide (6b): Methyl iodide used as reagent to get compound **6b** from compound **5b** as pure compound with 83 % yield, m.p.: 201-202 ºC. ^{1}H NMR ($CDCl_3$) δ ppm: 9.65-9.63 (d, J = 9 Hz, 1H, -NH), 8.51 (s, 1H, Ar-H), 7.69-7.60 (dd, J = 9 Hz and 6Hz, 2H, Ar-H), 7.37-7.32 (t, J = 9 Hz, 1H, Ar-H), 4.13-4.12 (m, 2H, -CH_2), 3.49-3.39 (m, 5H, -CH and -CH_3), 3.22-3.19 (m, 1H, -CH), 3.02-3.00 (m, 2H, -CH_2), 1.89 (bs, 2H, -CH_2) and 1.40-1.32 (m, 5H, -CH_2 and -CH_3). ^{13}C NMR δ ppm: 151.77 (-HN<u>C</u>O-CH), 144.53 (-N<u>C</u>NH); 129.94, 128.66, 125.16, 124.92, 113.66 and 111.34 (arom. C), 49.91 (-S<u>C</u>H_2CH_3), 46.06 (-N<u>C</u>H_2CH_2), 45.66 (-N<u>C</u>HCH), 34.53 (-N<u>C</u>H_3 and -OC<u>C</u>HCH_2), 30.24 (HC-<u>C</u>H_2CH_2), 28.29 (H_2C<u>C</u>H_2CH) and 21.89 (-H_2C<u>C</u>H_3). HRMS for $C_{14}H_{18}N_4O_3S$ calcd. (found): 322.3830 (322.3833).

Synthesis of *N*-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-1-(phenylsulfonyl)piperidine-3-carboxamide (6c): Methyl iodide used as reagent to get compound **6c** from compound **5c** as pure compound with 73 % yield, m.p.: 196-198 ºC. ^{1}H NMR (DMSO-d_6): 7.73-7.19 (m, 9H, Ar-H), 4.02-3.83 (m, 1H, -CH), 3.35 (bs, 2H, -CH_2), 3.06 (bs, 2H, -CH_2), 2.74-2.61 (m, 3H, -CH_3) and 1.72-1.17 (m, 4H, -CH_2). ^{13}C NMR δ ppm: 153.19 (-HN<u>C</u>OCH), 146.80 (-N<u>C</u>NH); 135.84, 133.69, 133.38, 129.94, 129.28, 128.28, 127.85, 125.97, 122.73 and 113.64 (arom. C), 60.23 (-N<u>C</u>H_3), 50.64 (-OC<u>C</u>HCH_2), 46.44 (-N<u>C</u>H_2CH_2), 45.89 (-N<u>C</u>H_2CH), 28.62 (HC<u>C</u>H_2CH_2) and 22.56 (H_2C<u>C</u>H_2CH).

LC-MS = 400.15 (M+2). HRMS for $C_{20}H_{22}N_4O_3S$ calcd. (found): 398.4810 (398.4818).

Synthesis of *N*-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-1-tosylpiperidine-3-carboxamide (6d): Methyl iodide used as reagent to get compound **6d** from compound **5d** as pure compound with 78 % yield, m.p.: 184-186 ºC. ^{1}H NMR (DMSO-*d*6): 8.12 (bs, 1H , -NH), 7.22-7.29 (m, 6H, Ar-H), 7.13-7.11 (bs, 2H, Ar-H), 3.82 (bs, 1H , -CH), 3.72 (s, 3H, -CH_3), 3.10 (bs, 1H, -CH), 2.67 (bs, 1H, -CH), 2.40 (s, 3H, -CH_3), 2.09 (bs, 2H , -CH_2) and 1.76-1.41 (m, 4H, -CH_2). ^{13}C NMR δ ppm: 172.54 (-HN*C*OCH), 154.25 (-N*C*NH); 148.59, 144.00, 132.97, 130.33, 127.92 and 121.01 (arom. C), 50.87 (-OC*C*HCH_2), 46.52(-N*C*H_2CH_2), 45.60 (-N*C*H_2CH), 28.82 (-N*C*H_3), 22.58 (HC*C*H_2CH_2), 21.53 (H_2C*C*H_2CH) and 21.45 (-HC*C*H_3). LC-MS = 414.15 (M+2). HRMS for $C_{21}H_{24}N_4O_3S$ calcd. (found): 412.5080 (412.5089).

Synthesis of *N*-(1-ethyl-1*H*-benzo[*d*]imidazol-2-yl)-1-tosylpiperidine-3-carboxamide (6e): Ethyl iodide used as reagent to get compound **6e** from compound **5d** with 81 % yield, m.p.: 179-181 ºC. ^{1}H NMR (DMSO-d_6): 7.57-7.28 (m, 8H, Ar-H), 3.81 (bs, 1H , -CH), 3.63 (s, 3H, -CH_3), 3.16 (bs, 1H, -CH), 2.92-2.66 (m, 3H, -CH, -CH_2), 2.34 (s, 3H, -CH_3), 2.06 (s, 2H , -CH_2) and 1.69-1.40 (m, 4H, -CH_2). ^{13}C NMR δ ppm: 172.89 (-HN*C*OCH), 152.69 (-N*C*NH), 144.42, 132.59, 130.40, 129.60, 128.84, 127.81, 125.87, 124.71, 124.47, 113.76 and 111.04 (arom. C), 50.36 (-OC*C*HCH$_2$), 46.46 (-N*C*H_2CH_2), 45.73 (-N*C*H_2CH), 31.05 (-N*C*H_2), 28.28 (-CH_2*C*H_3), 22.02 (HC*C*H_2CH_2), 21.36 (H_2C*C*H_2CH) and 21.13 (-HC*C*H_3). LC-MS = 428.15 (M+2). HRMS for $C_{22}H_{26}N_4O_3S$ calcd. (found): 426.5350 (426.5355).

Molecular docking studies: Docking studies of 1*H*-benzo-[*d*]imidazol-2-amine derivatives (**5a-d** and **6a-e**) into the binding pockets of target protein was carried out using Autodock Tools 1.5.6, Autodock4.2.6, ChemDraw 15.0 and Arguslab 4.0.1. All the parameters were performed according to reported reports [20-22]. 3D crystal structure of prostaglandin-endoperoxide synthase 2 (PDB ID: 5F1A) downloaded from www.rcsb.org/pdb/. Statistical mechanistic values for the target compounds such as lowest binding energy, ligand efficiency and the inhibitory constant (ki) values were extracted. Molecular interaction by means of hydrogen bonding and non-covalent bonding interaction (π-π interaction and π-cation interaction) results were corroborated.

HRBC membrane stabilization examinations: HRBC membrane stabilization study for 1*H*-benzo[*d*]imidazol-2-amine derivatives (**5a-d** and **6a-e**) was completed to confirm the constancy of HRBC membrane, which is supposed to inhibit the inflammatory progression by limiting the discharge of lysosomal enzymes because HRBC are alike lysosomal membrane. Various range of substances that are discharged by a lysosomal enzyme in the progression of inflammation causing multiple disorders. This extracellular activity is known as acute or chronic inflammation. In this study, *in vitro* anti-inflammatory activity was carried out by human red blood cell (HRBC) membrane stabilization method (n = 4) using indomethacin as standard [23]. The percentage hemolysis was calculated by assuming the hemolysis produced in presence of distilled water at 100 %. The percentage of HRBC membrane stabilization was calculated using the following formula:

$$\text{Inhibition of hemolysis (\%)} = \frac{OD_1 - OD_2}{OD_1} \times 100$$

where OD_2 and OD_1 are the optical density of sample and control, respectively.

Hydrogen peroxide radical scavenging assay: The H_2O_2 scavenging prospective of 1*H*-benzo[*d*]imidazol-2-amine derivatives (**5a-d** and **6a-e**) was investigated and the experiment was conducted performed in triplicate [23]. 40 mM H_2O_2 solution prepared using phosphate buffer (pH 7.4). Ascorbic acid was served as the standard. Compounds **5a-d**, **6a-e** and standard were tested at various concentrations (10-250 µg/mL). Test compounds in different concentrations were incubated along with 0.6 mL of freshly prepared H_2O_2 (40 mM) at room temperature for 60 min. Absorbance was registered at 230 nm in UV spectrophotometer in order to decide the relative H_2O_2 percentage decrease activity of compounds **5a-d**, **6a-e** and ascorbic acid. H_2O_2 absorbance in phosphate buffer solution used as control OD and ascorbic acid′s OD as positive control OD. The percentage H_2O_2 scavenging effect of test compounds and ascorbic acid calculated using the following formula:

$$H_2O_2 \text{ reducing activity (\%)} = \frac{OD_0 - OD_1}{OD_0} \times 100$$

where, OD_0 is the optical density (OD) of the control and OD_1 is the OD of compounds **5a-d** and **6a-e**.

Cell viability assay using macrophages: Commercially obtained macrophages (J774A.1) washed with a PBS buffer prior to use it for sub culturing and cytotoxicity assessments [24]. PBS buffer decanted and the cells were seeded in Dulbecco′s modified eagle′s dedium (DMEM) containing 96-well microplates at a density of 8×10^4 cells per well. Further the cells was incubated for 24 h in 5 % CO_2 atmosphere. Subsequently, the compounds (**5a-d** and **6a-e**) was dissolved in DMSO at a concentration of 10^{-1} to 10^{-1} (µg/mL). These concentrations was treated up to 48 h and then 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (10 mg/mL in a PBS buffer) was added and incubated for 6 to 8 h at 37 ºC. Further, formazan crystals that were formed were dissolved in 100 µL of DMSO after removing the DMEM from corresponding plates. The optical density (OD) was resolute at 540 nm in BioRad ELISA plate reader. Four repeated assay was conducted to determine the viability using the following equation:

$$\frac{\text{Mean OD treatment}}{\text{Mean OD control}} \times 100 = _\,\% $$

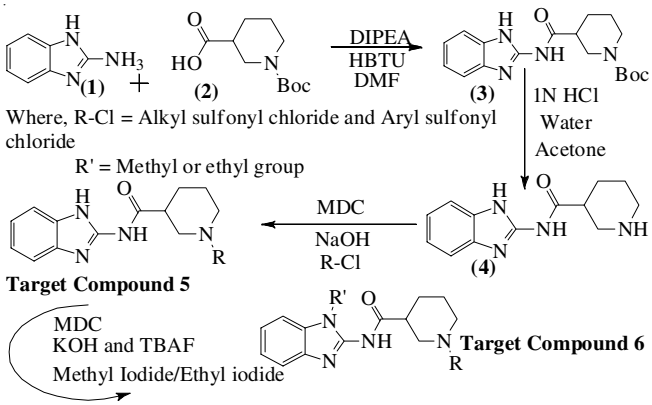
Determination of anti-inflammatory and proinflammatory interleukin levels in LPS induced macrophages: In order to determine proinflammatory and anti-inflammatory interleukin levels in J77A,1 macrophages, the TNF-α, IL-1β, IL-6 and IL-10 serum levels were measured by means of using ELISA method. The OD was measured at 405 nm and the macrophages (J774A.1) were grown with a density of 1×10^6 in DMEM. Further, compounds (**5a-d** and **6a-e**) and indomethacin were treated separately at a concentration range of 1-50 µg/mL and the cells were incubated for 120 min. lipopolysaccharides (LPS) was added to the cells at 1 µg/mL range and incubated for 24 h and further the cell-free supernatants were obtained and analyzed by means of immunoassay and the

cytokines level was quantified. Particularly, the concentrations of TNF-α, IL-1β, IL-6 and IL-10 and in the supernatants of J774A.1 macrophage cell culture determined by means of immunoenzymatic assessments.

Statistical analysis: All the results were statistically analyzed by performing one-way ANOVA with Dunnett's post-test. GraphPad Prism version 7.1 for Windows, GraphPad Software, USA (graphpad.com) was used for statistical analysis. The results are expressed as the means ± SEM. A difference was considered statistically significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Synthesis of 1*H*-benzo[*d*]imidazol-2-amine derivatives (5a-d and 6a-e): In the present study, the preparation of title compounds involves four steps. **Scheme-I** explains the route of synthesis of the title compounds **5a-d** and **6a-e**. First step is the acid amine coupling of 1-(*tert*-butoxycarbonyl)piperidine-3-carboxylic acid and 1*H*-benzo[*d*]imidazol-2-amine. Table-1 illustrates the process optimization of synthesis of compound **3** by using different catalysts. In the observations, as mentioned in entry 6 (Table-1), the combination of DIPEA, DMF and HATU with a temperature range of 25-35 ºC reaction for 8 h provided a maximum yield 82 %. Second step is Boc deprotection of amide compounds. This step is the piperidine-*N*-substitution to get compound **5a-d** (Fig. 1) title compounds and the process was optimized (Table-2). Fourth step is the benzimidazole *N*-substitution to get the title compounds **6a-e**



Scheme-I: Synthetic scheme for the preparation of title compounds (**5a-d** and **6a-e**)

(Fig. 1) and the Table-3 illustrates the corresponding process optimization.

Molecular docking: Molecular docking was performed in order to know the binding affinity of 1*H*-benzo[*d*]imidazol-2-amine derivatives (**5a-d** and **6a-e**) to prostaglandin-endoperoxide synthase 2. Based on the binding energy values and ligand efficiency values obtained by the individual compounds, a raking was given for all compounds. We only considered top four compounds for further *in vitro* studies to establish them as drug candidates towards the pre-clinical and other related animal model studies. In the results, all compounds were showed a reasonable binding energy values (-7.24 to -19.81

TABLE-1
REACTION (ACID AMINE COUPLING) CONDITIONS OPTIMIZATION FOR THE PREPARATION OF FINAL COMPOUNDS **3**

Entry	Base	Solvent	Coupling reagents	Temp. (°C)	Time (h)	Yield (%)*
1	TEA	THF	DCC[c]/HOBt[d]	25-35	8.00	NA
2	TEA	THF	DCC/HOBt	Reflux	8.00	NA
3	TEA	THF	EDC.HCl[e]/HOBt	Reflux	8.00	NA
4	TEA	THF	HATU[f]	25-35	8.00	NA
5	DIPEA	THF	HATU	25-35	8.00	NA
6[a]	DIPEA	DMF	HATU	25-35	8.00	82
7	DIPEA	DMA	HATU	25-35	8.00	79
8	DIPEA	DMF	HBTU[g]	25-35	8.00	73
9	DIPEA	DMF	EDC.HCl/HOBt	25-35	8.00	64

6[a] The optimized reaction conditions for preparation of compound **3**. *The yields are mentioned for the isolated products. NA- Incomplete reactions; [c]Dicyclohexylcarbodiimide; [d]Hydroxybenzotriazole; [e]N'-ethylcarbodiimide hydrochloride; [f](1-[*Bis*(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium3-oxid hexafluoro phosphate; [g] 3-[*Bis*(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate

TABLE-2
REACTION CONDITIONS OPTIMIZATION FOR THE PREPARATION OF INTERMEDIATE COMPOUNDS **5(a-d)**

Entry	Base	Catalyst	Solvent	Temp. (°C)	Time (h)	Yield (%)*
1	TEA	–	THF	5-10	4.00	65
2	TEA	–	THF	25-30	4.00	68
3	TEA	TBAF[a]	THF	25-30	4.00	60
4	TEA	TBAB[b]	THF	25-30	4.00	62
5	TEA	TBAF	Methyl THF	25-30	4.00	72
6	TEA	TBAF	DCM	25-30	4.00	80
7	TEA	TBAF	DCM	10-15	4.00	82
8	DIPEA	TBAF	DCM	10-15	4.00	84
9	NaOH	–	DCM	10-15	4.00	90
10[a]	NaOH powder	–	DCM	10-15	4.00	96
11	NaOH powder	TBAF	DCM	10-15	4.00	94
12	NaOH powder	TBAF	DCM	25-30	4.00	90

10[a]Optimized reaction conditions for piperidine N-H substitution reaction; *Yields are mentioned for the isolated products; [a]*t*-Butyl ammonium fluoride. [b]*t*-Butyl ammonium bromide.

TABLE-3
REACTION CONDITIONS OPTIMIZATION FOR THE PREPARATION OF INTERMEDIATE COMPOUNDS **6(a-e)**

Entry	Base	Catalyst	Solvent	Temp. (°C)	Time (h)	Yield (%)*
1	TEA	–	THF	5-10	4.00	NA
2	TEA	–	THF	25-30	4.00	NA
3	TEA	TBAF[(a)]	THF	25-30	4.00	NA
4	TEA	TBAB[(b)]	THF	25-30	4.00	NA
5	TEA	TBAF	Methyl THF	25-30	4.00	NA
6	TEA	TBAF	DCM	25-30	4.00	NA
7	TEA	TBAF	DCM	10-15	4.00	NA
8	DIPEA	TBAF	DCM	10-15	4.00	NA
9	NaOH	-	DCM	10-15	4.00	30
10	NaOH powder	-	DCM	10-15	4.00	35
11	NaOH powder	TBAF	DCM	10-15	4.00	64
12	NaOH powder	TBAF	DCM	25-30	4.00	70
13[a]	KOH powder	TBAF	DCM	25-30	4.00	75

13[a] Optimized reaction conditions for benzimidazole N-methylation reaction; *Yields are mentioned for the isolated products; [(a)]*t*-Butyl ammonium fluoride. [(b)]*t*-Butyl ammonium bromide

kcal/mol) (Fig. 2a). In the individual result assessments, compound **6a** showed an exception values while comparing other compounds. An inhibitory constant values of 0.0085 µM for compound **6a** indicated its strong binding affinity to the target protein. The second most favourable results was found for compound **5a** (binding energy -14.85 kcal/mol and 0.0098 µM). At the same time compound **6b** also showed a nominal value to be considered along with compounds **5a**, **5b** and **6a** for further bioactivity evaluations. Ligand efficiency (LE), another molecular mechanistic value considered in this study, is most the ratio of the affinity of compounds **5a-d** and **6a-e** divided by the number of heavy (non-hydrogen) atoms in the molecule (LE = ΔG/[number of heavy atoms]) [25]. As depicted in Fig. 2b, compounds **5a-b** and **6a-b** were found with the most favourable ligand efficiency.

Fig. 1 displayed as the representative to explain the molecular interaction of ligand-receptor complex. A firm bridging between compounds **5a-d** & **6a-e** and prostaglandin-endoperoxide synthase 2 was found to be established by means of π-π interaction (non-covalent) and hydrogen bonding. The hydrogen bonding were established between His207, His214 and Thr212 of prostaglandin-endoperoxide synthase 2 respec-

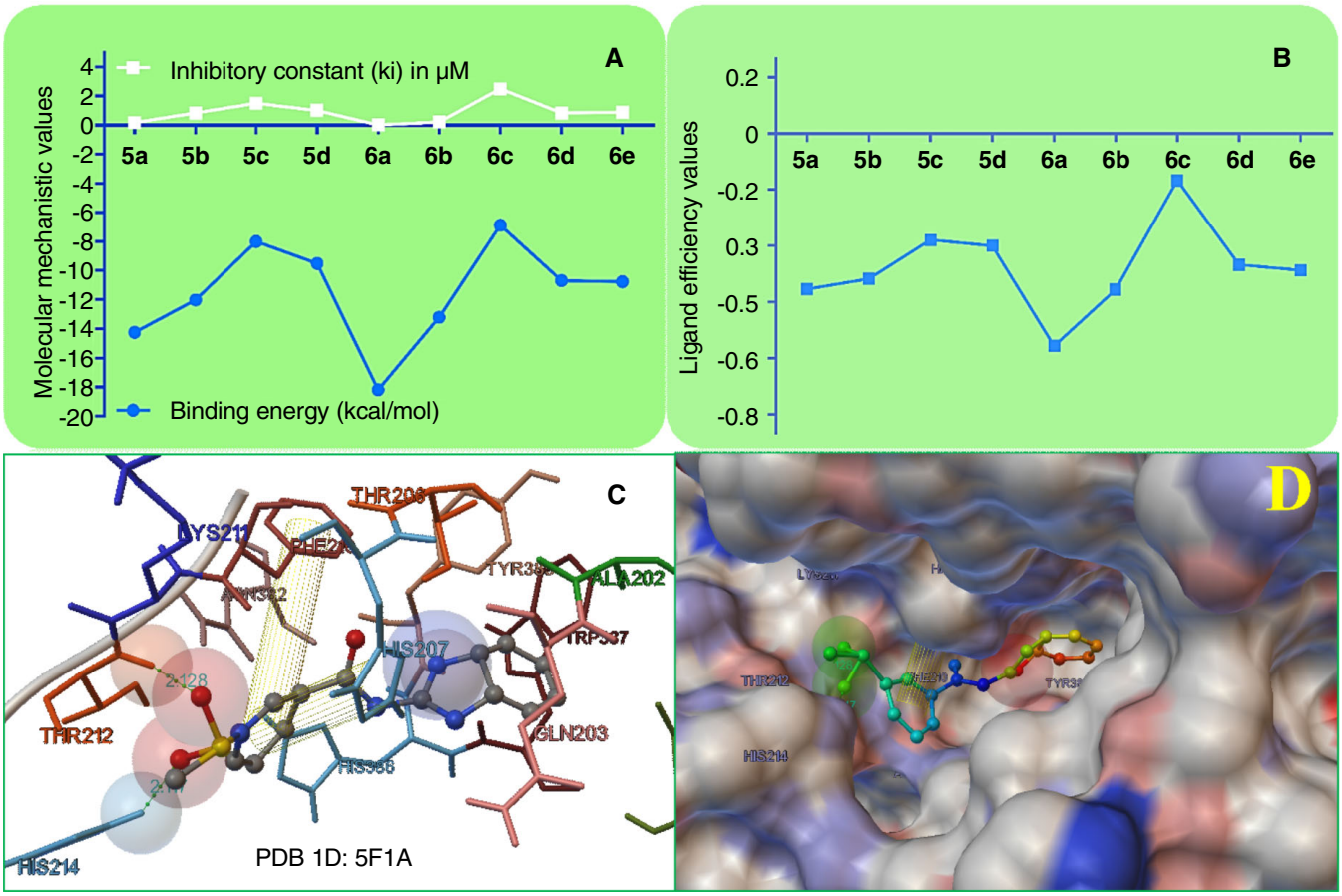


Fig. 2. Molecular mechanistic values (2A & 2B) and molecular interaction (2C & 2D) of compounds **5a-d** and **6a-e**

tively to compound 5a′s sulfoxide and nitro group of imidazole end. Meanwhile, non-covalent π-π interaction was established between the cyclohexane ring to the amino acid residues His207 and Phe210. Higher the hydrogen bonding, higher the bio-availability. Three hydrogen bonding along with the established π-π interaction indicates the possible drug efficacy of this compound.

Anti-inflammatory activity through HRBC membrane stabilization assay: The inhibition ability of discharge of lysosomal enzymes in HRBC was taken as a measure to adjudge the anti-inflammatory effect and determined for compounds **5a-b** and **6a-b**. Relative percentage activity (RPA) and the corresponding IC_{50} values were taken in account to determine the activities among compounds **5a-b** and **6a-b** by comparing the values obtained for standard drug (diclofenac) (RPA = 86.98 ± 2.65 and IC_{50} 0.95 μM). The results were found to be dose depended. An increased lysosomal ingredient discharge from HRBC was found in 0.025 ± 0.002 μM (IC_{50} 0.012 μM) from a concentration range of 1 μM to 25 nM. Serially diluted (initial volume 1 μM in 1 mL) concentrations of the target compounds were assessed in four repeated assays. In the overall results, compounds **5a** and **6a** were showed most valuable results (RPA = 90.12 ± 2.14 & IC_{50} 0.085 & RPA = 96.54 ± 1.86 & IC_{50} 0.025, respectively). Thus, these two compounds were only considered for the further assays.

Hydrogen peroxide radical scavenging assay: Hydrogen peroxide is widely being used as an investigational source of oxygen-derived free radicals. Radicals are highly responsible for inflammatory disorders and autoimmune diseases, such as rheumatoid arthritis and several types of cancers [26,27]. As they have showed excellent *in vitro* anti-inflammatory activity by means of HRBC membrane stabilization through lysosomal discharge inhibition, compounds **5a** and **6a** were considered for H_2O_2 radical scavenging assay. In the results, compound **6a** showed a dominated result than compound **5a** and ascorbic acid which was served as the standard radical scavenger. The RPA was 98.24 ± 1.66 (IC_{50} 0.00184 μM), 92.84 ± 2.02 (IC_{50} 0.028 μM) and 88.62 ± 1.98 (IC_{50} 0.99 μM) for compounds **6a**, **5a** and ascorbic acid, respectively. The IC_{50} values mentioned here are the average of three repeated assays. The scavenging potential was also a dose depended response showed by all the tested compounds.

Determination of cytotoxicity and pro- & anti-inflammatory interleukin levels in LPS induced macrophages: Lipopolysaccharide can trigger mononuclear phagocytes (macrophages) and other types of cells to discharge TNF-α, IL-6, IL-10 and IL-1β. The excessive and disproportionate discharge of these cytokines may root systemic inflammatory response syndrome (SIRS), severe tissue impairment and septic shock [28,29]. The cell viability of J774A.1 macrophages was evaluated using compounds **5a** and **6a**. Fig. 3 shows that compounds **5a** and **6a** at concentrations of 1 to 20 (nM). Low concentrations (1 to 7 nM) did not affect cell viability, whereas 8 and 20 nM **5a** and **6a** did affect cell viability. Consequently, we used the concentration 5 nM of compounds **5a** and **6a** in subsequent experiments. The IC_{50} was found as 2.05 ± 0.001 nM. The effect of compounds **5a**, **6a** and indomethacin on the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and

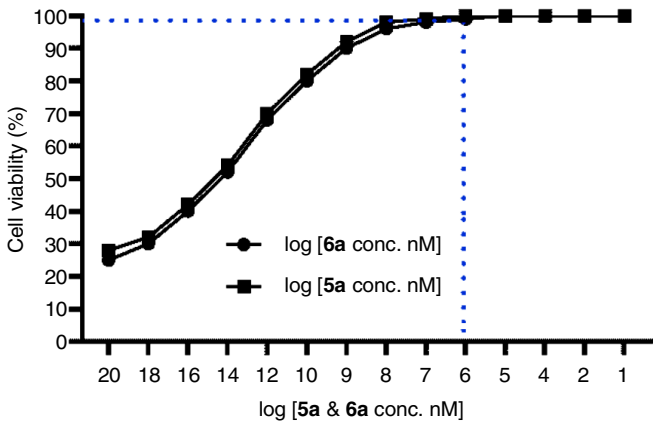


Fig. 3. Cell viability in macrophages treated with **6a** at 1 to 20 (nM), as assessed by the MTT assay. Results are expressed as the % of living cells comparative to control cells. The results are the mean of three repeated assay values

the anti-inflammatory cytokine IL-10 was estimated in the culture medium of macrophages stimulated with 1 μg/mL of LPS alone or in combination with 5 nM of compounds **5a** and **6a** (Fig. 4). Compounds **5a** and **6a** considerably decreased TNF-α, IL-6 and IL-1β, and the properties were comparable to those attained with compounds **5a** and **6a** and higher than indomethacin. Furthermore, compounds **5a** and **6a** showed an augmented IL-10 production at 5 nM concentration matched with the control group, and the increase in IL-10 production was higher than that observed in the group treated with indomethacin.

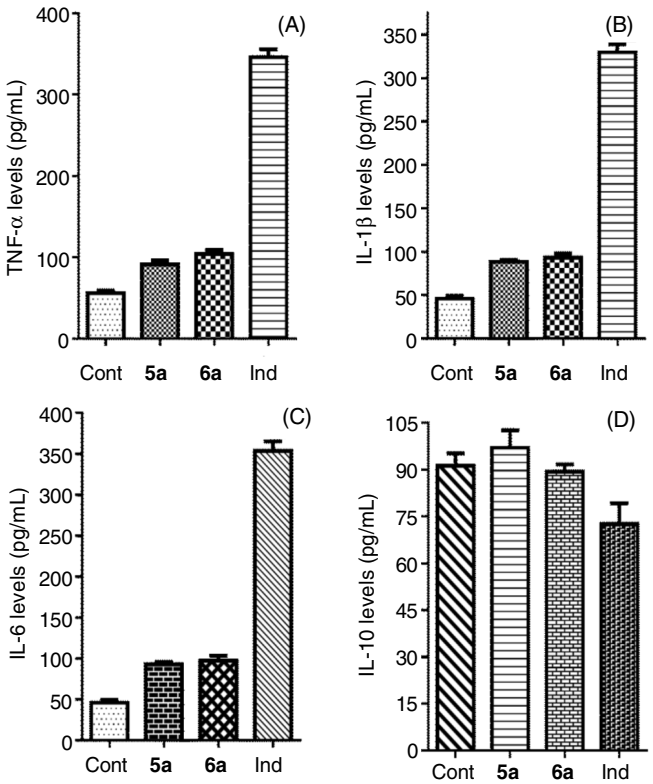


Fig. 4. Effects of compounds **5a**, **6a** and Indomethacin on LPS-stimulated levels of (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-10 levels in macrophages. The concentrations were assessed and finalised by ELISA. The results are the mean of three determinations ± SEM

Conclusion

In this study, compounds **5a-d** and **6a-e** were synthesized and the anti-inflammatory effect was assessed. In all *in vitro* and *in silico* studies, compounds **5a** and **6a** were showed a dominated results in all proposed activity evaluations. Along with indomethacin, compounds **5a** and **6a** were assessed for the levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and the anti-inflammatory cytokine IL-10 in macrophages was determined. Compounds **5a** and **6a** significantly reduced TNF-α, IL-1β and IL-6, and the effects were similar each other and better than with indomethacin. Furthermore, compounds **5a** and **6a** increased IL-10 production at 5 nM concentration compared with the control group, and the rise in IL-10 production was higher than that observed in indomethacin treated group. The need for further animal model and pre-clinical studies for compounds **5a** and **6a** require in order to develop them as future inflammation medications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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