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Synthesis, Molecular Docking, and Anti-inflammatory Activities of Some Novel Benzimidazole Derivatives as Potential Cyclo-oxygenase-2 Inhibitors

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Abstract

Due to the association of inflammatory, cancerous, and neurological illnesses with COX-2 overexpression, targeting of this enzyme has become a potent tool for therapeutic research. In the current research, synthesis of novel, straightforward benzimidazole derivatives was described that were selective COX-2 inhibitors and exhibit a striking *in vivo* anti-inflammatory activity. The most active candidates were five novel molecules **4a, 4b, 5, 6** and **9** showing promising selectivity and *in vitro* COX-2 inhibition with IC_{50} of 0.23, 0.27, 0.24, 0.13, and 0.15 µM respectively in comparison with indomethacin IC_{50} of 0.41 µM. Additionally, using a carrageenan-induced paw edema method, the anti-inflammatory effectiveness of molecules **4a, 4b, 5, 6,** and **9** was assessed *in vivo*. Results revealed that the synthesized compounds had significant inhibitory potency with action like that of the common drug indomethacin. Finally, to comprehend their way of binding, these substances were docked in the crystal structure of the COX-2 enzyme (PDB ID: 4COX). These discoveries may lead to the creation of novel COX-2 inhibitors with enhanced selectivity.

Key words: Benzimidazole, Indomethacin, COX-2 inhibitors, Anti-inflammatory, NSAIDs

1. Introduction

An essential defense mechanism against all infectious, physical, and chemical aggressions is inflammation. Deregulation of this process leads to pathological conditions in the body, as seen, for instance, in allergies, autoimmune conditions, and organ-rejection [1]. Non-steroidal anti-inflammatory drugs, or NSAIDs, are commonly given medications in modern medicine. NSAIDs are widely used by millions of patients worldwide because they are highly effective at reducing pain, fever, and inflammation [2]. NSAIDs work pharmacologically by preventing COX-1 and COX-2 from doing their action [3] thus lead to stop arachidonic acid's enzymatic biotransformation into the associated pro-inflammatory prostaglandins and thromboxane (TXs) [4]. Two isoenzymes of cyclooxygenase were first introduced by Needleman and Isakson in 1997. COX-1, known as the "House Keeping" enzyme, oversees a fundamental level of PGs for conservation of homeostasis, including gastro-intestinal integrity. COX-2, known as the "Inducible" enzyme, is activated by various stimuli and mediates inflammatory responses. This is why, compared to conventional non-selective NSAIDs, the development of NSAIDs with targeting of COX-2 over COX-1 is significant for the treating inflammatory disturbance with fewer side effects, such as gastrointestinal toxicities [5]. The precise functions of a COX-3 isoform that is only expressed in specific regions of the brain and spinal cord are still unknown [2].

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Numerous pathogenic and pro-inflammatory stimuli, such as cytokines, lipopolysaccharides, and phorbol esters, increase the production of the 2nd COX type, COX-2. Under acute inflammatory conditions, COX-2 is in charge of the manufacture of PGs and the generation of inflammation [6]. COX-2 is implicated in a variety of clinical conditions, including inflammation, cancer, and neurological diseases, according to abundant data. Consequently, inhibitors of COX-2 have been used for cancer therapy beside their intended usage as anti-inflammatory drugs. Therefore, it is a desirable objective for both pharmaceutical and academic research to develop selective COX-2 inhibitors to serve as anti-inflammatory and anti-cancer medicines [7, 8].

Conventional NSAIDs **(Fig.1)** exert their therapeutic effects by non-selectively inhibiting COX-1 and COX-2; which is accompanied with major side effects e.g. stomach pain, ulcers, bleeding, and renal problems. However, selective COX-2 inhibitor drugs like celecoxib, rofecoxib, and valdecoxib [9, 10] were developed to minimize these severe adverse effects. Similar to non-selective COX inhibitors, these drugs have anti-inflammatory and analgesic activities, but with enhanced gastric safety profiles **(Fig.2)** [11]. Unfortunately, both valdecoxib and rofecoxib have been taken off from the market as their substantial side effects in the biochemical COX pathway, with increased occurrence of high blood pressure and myocardial infarction [12]. The negative impacts of rofecoxib and valdecoxib were linked to their individual chemical composition. Therefore, the search for selective anti-inflammatory drugs with better safety profiles than the currently available NSAIDs continues.

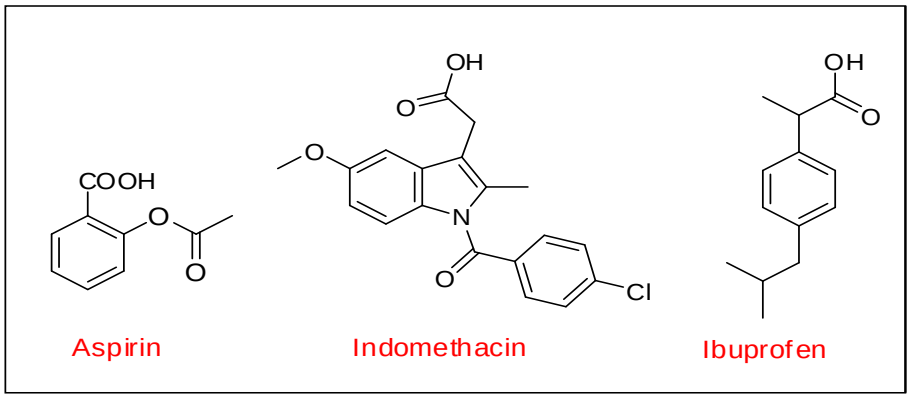


Fig. 1. Traditional NSAIDs (nonselective COX-1, COX-2 inhibitors).

Benzimidazole is an important multi-target scaffold. It has been broadly investigated against both infectious and noninfectious diseases, and various benzimidazole based drugs are in clinical use as antihistaminic (astemizole), antiulcer (omeprazole), antihypertensive (telmisartan) and anthelmintic (flubendazole) agents. [13] Recently, there have been strong efforts to develop benzimidazole based anti-inflammatory therapeutics **(Fig.3)**. [14] In 2003, a group of benzimidazoles/benzothiazoles was created and studied for their capability of inhibiting human COX-2 enzyme by Paramashivappa *et al.* [15]. Compound **I** demonstrated a 384-fold preference for COX-2 over COX-1 with an IC_{50} value of 1 µM.

Rathore et al. (2014) investigated the synthesis of 18 benzimidazole-based compounds and looked into the COX-2 inhibitors' potential anti-inflammatory effects [16]. Compound II demonstrated the highest level of inhibitory action with an IC50 value of 8.2 µM. In addition, in 2016, Morsy et.al reported synthesis of some benzimidazole derivatives with good anti-inflammatory activity through COX-2 inhibition, of them compound III showed the best inhibitory action (IC50 value of 0.28 µM). [17] Moreover, in 2017, Ayyad et.al synthesized a series of benzimidazole derivatives which exerted significant COX-2 inhibitory activity, where compounds VI and V showed best activity. [18]

Putting in Consideration the importance of the

benzimidazoles in broad range of anti-inflammatory investigations and treatments in addition to its impressing activity profile, we here described the synthesis of a novel benzimidazole derivatives in the hopes of creating new anti-inflammatory drugs that are both efficacious and selective. The synthetic derivatives' COX-1/COX-2 inhibitory activity was assessed. The cytotoxicity research and carrageenan-induced rat paw edema model were also conducted to evaluate the compounds' potential to reduce inflammation in addition to their safety. Finally, for the purpose of elucidating their potential mode of action, the synthesized substances were docked into the COX-2 active site.

2. Experimental

2.1. Chemistry

In **Schemes 1**, the required compounds **2–9**'s synthesis pathway was displayed. Synthesis of the intermediates **1** and **3** was done in accordance with the reported procedures. [14,15] Procedures and characterization data are present in the supplementary file.

2.1.1 Chemicals and reagents

1,2 benzene diamine, 2-mercaptosuccinic acid, lactic-acid, thioglycolic-acid, thiolactic-acid, thiomalic-acid and ethyl-2-bromoacetate were purchased from Alpha Aesar (England). The use of solvents and other reagents was required only that to be of the highest purity.

2,3-Dihydro-3-mercapto-1*H*-pyrrolo[1,2-a] benzimidazol-1-one 2

A mixture of 3-(1H-benzo[d]imidazol-2-yl)-3-mercaptopropanoic acid **1** (0.01 mol, 3 gm) in acetic anhydride (0.24 mol, 25 mL) and fused sodium acetate (0.018 mol, 1.5 gm) was heated at 90 °C for 24 hrs. Under low pressure, the reaction mixture evaporated. The mixture was placed into ice water after chilling. The precipitate formed was filtered, water washed, dried, and recrystallized from ethanol. [16].

Off white powder. M.p.> 300 ˚C, (yield 52%). IR (cm^{-1}): 740 (C-S), 1620 (C=N), 1701 (C=O), 2638 (SH). Mass spectrum: m/z (%): 204 (M^+, 2.16%), 193 (4.91%), 170 (22.90%), 143 (88.29%), 130 (base peak, 100%), 118 (72.87%), 102 (50.27%), 90 (50.01%), 631 (59.74%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.25-7.19 (m, 2H, Ar-H), 6.97- 6.91(m, 2H, Ar-H), 3.62 (t, 1H, CH-SH, *J*=4 Hz), 3.26 (s,1H, SH), 3.16-3.10 (m, 1H, CH_2), 2.59-2.57 (m, 1H, CH_2). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.48, 152.19, 129.46 (2C), 127.46, 120.35, 117.00, 115.06, 29.45, 27.97. Anal. Calcd. for $C_{10}H_8N_2OS$: C, 58.80; H, 3.95; N, 13.72; S, 15.70. Found: C, 58.48; H, 3.86; N, 13.45; S, 15.59.

Typical method for synthesis of compounds 4a-b, 5, 6

A mixture of lactic acid or thioglycolic- acid or thiolactic- acid or thiomalic- acid (0.0189 mol) in 30 mL of methanol (HPLC) and few drops of triethylamine was stirred for 30 min, and then a solution of compound **3** (0.0189 mol, 3.98 g) in methanol (HPLC) (10 mL) was added. The reaction mixture was heated to 90 °C for 12–16 hrs., agitated for 3 hrs at ambient temperature, evaporated under reduced pressure, and then washed with water. The solid that resulted from this process was recrystallized from acetone [16]

2-((1*H*-benzo[d]imidazol-2-yl) methoxy) propanoic acid 4a

Gray powder. M.p.= 225- 227 ˚C, (yield 71%). IR (cm^{-1}): 1273 (C-O-C), 1597 (C=N), 1643 (C=O), 3093 (NH), 3340 (OH). Mass spectrum: m/z (%): 220 (M^+, 1.47%), 131 (base peak, 100%), 118 (52.44%), 90 (39.34%), 77 (65.17%).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (s, 1H, COOH), 8.50 (s, 1H, NH),7.39- 7.36 (m, 2H, Ar-H), 7.29-7.21 (m, 2H, Ar-H), 4.90 (s, 2H, CH_2), 4.23- 4.17 (q, 1H, CH, *J*=8 Hz), 1.24 (d, 3H, CH_3, *J*=8 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.96, 151.28, 129.46 (2C), 119.80 (2C), 115.86 (2C), 60.99, 52.91, 41.58. Anal. Calcd. for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N, 12.72. Found: C, 59.88; H, 5.16; N, 12.47.

2-((1*H*-benzo[d]imidazol-2-yl)methylthio)propanoic acid 4b

Yellowish white powder. M.p.= 214- 216 ˚C, (yield 77%). IR (cm^{-1}): 732 (C-S), 1597 (C=N), 1630 (C=O), 3051 (NH), 3174 (OH). Mass spectrum: m/z (%): 236 (M^+, 4.36%), 223 (2.54%), 178 (2.58%), 162 (2.49%), 131 (base peak, 100%), 104 (34.98%), 43 (80.81%).^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, IH, COOH), 10.00 (s, 1H, NH), 7.59- 7.54 (m, 2H, Ar-H), 7.42-7.39 (m, 2H, Ar-H), 3.18 (s, 2H, CH_2), 3.01- 2.95 (q, 1H, CH, *J*=8 Hz), 1.26 (d, 3H, CH_3,

J=4 Hz).[13]C NMR (101 MHz, DMSO-d_6) δ 167.75, 151.72, 129.76 (2C), 119.14 (2C), 115.37 (2C), 44.81, 30.08, 22.61. Anal. Calcd. for $C_{11}H_{12}N_2O_2S$: C, 55.91; H, 5.12; N, 11.86; S, 13.57. Found: C, 55.69; H, 4.91; N, 11.67; S, 13.42.

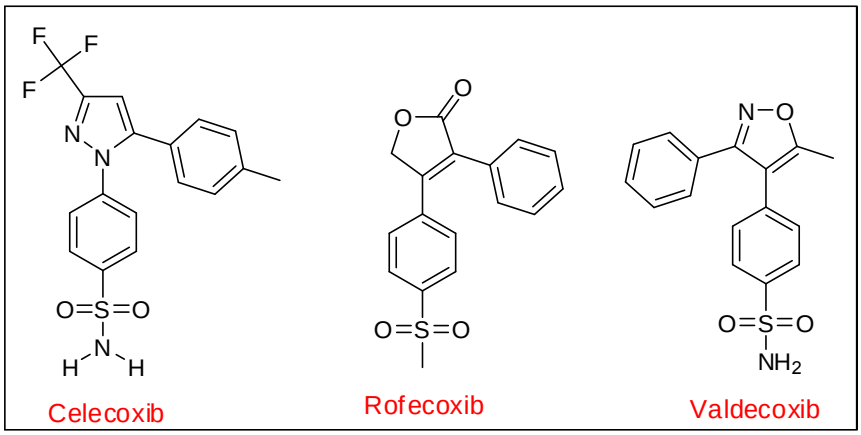


Fig. 2. Some selective COX-2 inhibitors.

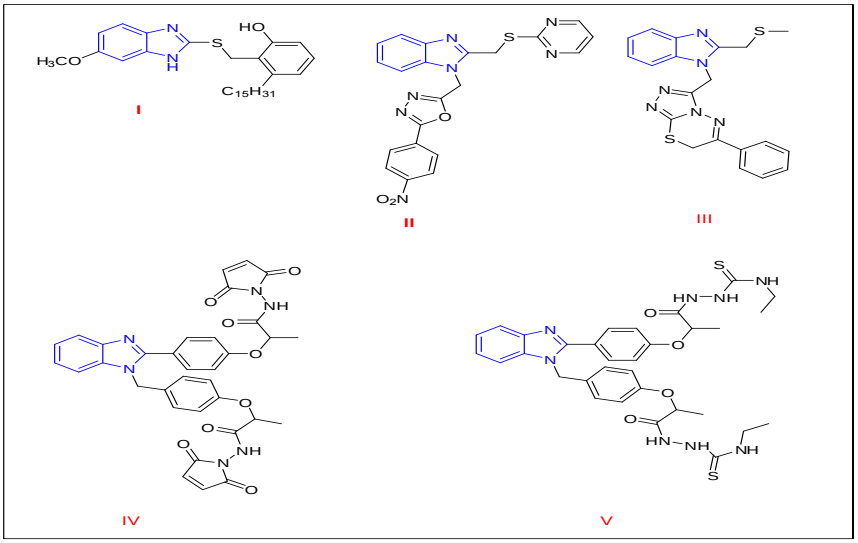


Fig. 3. Some reported benzimidazole based compounds exerting anti-inflammatory activity through COX-2 inhibition.

2-((1*H*-benzo[d]imidazol-2-yl)methylthio)acetic acid 5

M.p.= 185-187 ˚C. (As reported). [17]

2-((1*H*-benzo[d]imidazol-2-yl)methylthio)succinic acid 6

Brown powder. M.p.= 230- 232 ˚C, (yield 49%). IR (cm^{-1}): 744 (C-S), 1597 (C=N), 1674 (C=O), 1678 (C=O), 2762- 3051 (2OH), 3120 (NH). Mass spectrum: m/z (%): 280 (M^+, 0.73%), 263 (base peak, 100%), 220 (2.98%), 159 (8.59%), 131 (96.16%), 104 (14.6%), 77 (12.76%).[1]H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, IH, COOH), 8.58 (s, 1H, NH), 7.58- 7.53 (m, 2H, Ar-H), 7.19- 7.12 (m, 2H, Ar-H), 3.87 (t, 1H, CH, *J*=8 Hz), 3.84 (s, 2H, CH_2-S), 2.90 (t, 1H, CH_2-COOH, *J*=8 Hz), 2.74 (t, 1H, CH_2COOH, *J*=8 Hz). [13]C NMR (101 MHz, DMSO-d_6) δ 175.10, 167.55, 151.76, 129.17 (2C), 119.16 (2C), 115.36 (2C), 49.07, 33.74, 24.26. Anal. Calcd. for $C_{12}H_{12}N_2O_4S$: C, 51.42; H, 4.32; N, 9.99; S, 11.44. Found: C, 51.21; H, 4.15; N, 9.67; S, 11.31.

General procedure for synthesis of compounds 7a-b, 8 and 9

A mixture of compound **4a** or **4b** or **5** or **6** (0.005 mol), acetic anhydride (0.12 mol, 12.24 mL) and fused sodium acetate (0.009 mol, 0.73 g) was heated at 90 °C for 16-24 hrs. Reaction mixture was evaporated under pressure. The mixture was then placed into ice- water after chilling. After being filtered, the precipitate, water washed, dried, and recrystallized from ethanol. [16].

3-Methyl-1*H*-[1,4]oxazino[4,3-a]benzimidazol-4(3*H*)-one 7a

Brown powder. M.p.= 255- 256 ˚C, (yield 45%). IR (cm^{-1}): 1269 (C-O-C), 1508 (C=N), 1666 (C=O). Mass spectrum: m/z (%): 202 (M^+, 0.87%), 187 (1.06%), 174 (1.95%), 158 (9.19%), 143 (39.35%), 131 (base peak, 100%), 118 (34.60%), 102 (25.70%), 77 (38.32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91-7.76 (m, 2H, Ar-H), 7.47- 7.25 (m, 2H, Ar-H), 4.93-4.77 (m, 2H, CH_2), 4.21- 4.15 (q, 1H, CH, *J*=8 Hz), 1.24 (d, 3H, CH_3, *J*=8 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.54, 150.95, 129.15, 127.97, 120.95 (2C), 118.14, 116.18, 78.74, 67.17, 15.15. Anal. Calcd. for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N, 13.85. Found: C, 65.19; H, 5.27; N, 13.66.

3-Methyl-1*H*-[1,4]thiazino[4,3-a]benzimidazol-4(3*H*)-one 7b

Off white powder. M.p.= 270- 273 ˚C, (yield 60%). IR (cm^{-1}): 744 (C-S), 1597 (C=N), 1658 (C=O). Mass spectrum: m/z (%): 219 (M^++1, 2.09%), 204 (1.59%), 192 (1.54%), 161 (6.27%), 131 (17.19%), 118 (50.30%), 90 (30.57%), 43 (base peak, 100%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.48- 7.42 (m, 2H, Ar-H), 7.38- 7.32 (m, 2H, Ar-H), 3.84-3.80 (m, 2H, (CH-C=O), (CH-S)), 3.72-3.70 (m, 1H, CH-S), 1.24 (d, 3H, CH_3, *J*=8 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.80, 155.82, 130.76, 127.98, 119.56 (2C), 115.09, 110.22, 49.05, 30.60, 20.89. Anal. Calcd. for $C_{11}H_{10}N_2OS$: C, 60.53; H, 4.62; N, 12.83; S, 14.69. Found: C, 60.82; H, 4.91; N, 12.54; S, 14.57.

1*H*-[1,4]thiazino[4,3-a]benzimidazol-4(3*H*)-one 8

M.p.= 123-124 ˚C. (As reported) [18]

3,4-Dihydro-4-oxo-1*H*-[1,4]thiazino[4,3-a]benzimidazole-3-acetic acid 9

Black powder. M.p.> 300 ˚C, (yield 35%). IR (cm^{-1}): 744 (C-S), 1620 (C=N), 1654 (C=O), 1755 (C=O), 3417 (OH). Mass spectrum: m/z (%): 262 (M^+, 3.42%), 248 (4.61%), 234 (9.23%), 210 (88.63%), 183 (26.89%), 131 (19.62%), 118 (20.21%), 84 (base peak, 100%), 43 (67.62%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.24- 7.18 (m, 2H, Ar-H), 6.96- 6.91 (m, 2H, Ar-H), 3.73-3.70 (m, 1H, CH-S), 3.62- 3.58 (m, 2H, (CHC=O), (CH-S)), 3.12 (t, 1H, CH_2-COO, *J*=4 Hz), 2.57 (t, 1H, CH_2-COO, *J*=4 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 179.89, 170.48, 152.19, 129.46, 127.46, 120.35 (2C), 117.00, 115.06, 46.27, 35.67, 29.45. Anal. Calcd. for $C_{12}H_{10}N_2O_3S$: C, 54.95; H, 3.84; N, 10.68; S, 12.23. Found: C, 54.72; H, 3.61; N, 10.44; S, 12.02.

2.2 Evaluation of biological activity

***In-vitro* Cyclooxygenase Inhibition Assays**

The enzyme immune assay (EIA) kit (No. 560131, Cayman Chemical, Ann Arbor, MI, USA) was used in accordance with a previously documented method to determine the ability of the compounds under research listed in **(Table 1)** to inhibit bovine COX-1 and human recombinant COX-2 (IC_{50} value, µM) [19].

***In-vivo* anti-inflammatory assay**

Using the carrageenan-induced rat paw edema method, the most selective COX-2 inhibitors (**4a, 4b**, **5, 6** and **9**) were appraised for their anti-inflammatory (AI) effectiveness. (Detailed *in vivo* anti-inflammatory assay procedure was supported in Supplementary file).

Cytotoxicity assay (MTT assay)

The cytotoxicity effects for the most selective compounds were assessed against normal Vero cell line via MTT assay. (Detailed cytotoxicity assay procedure was supported in Supplementary file).

2.3 Molecular docking

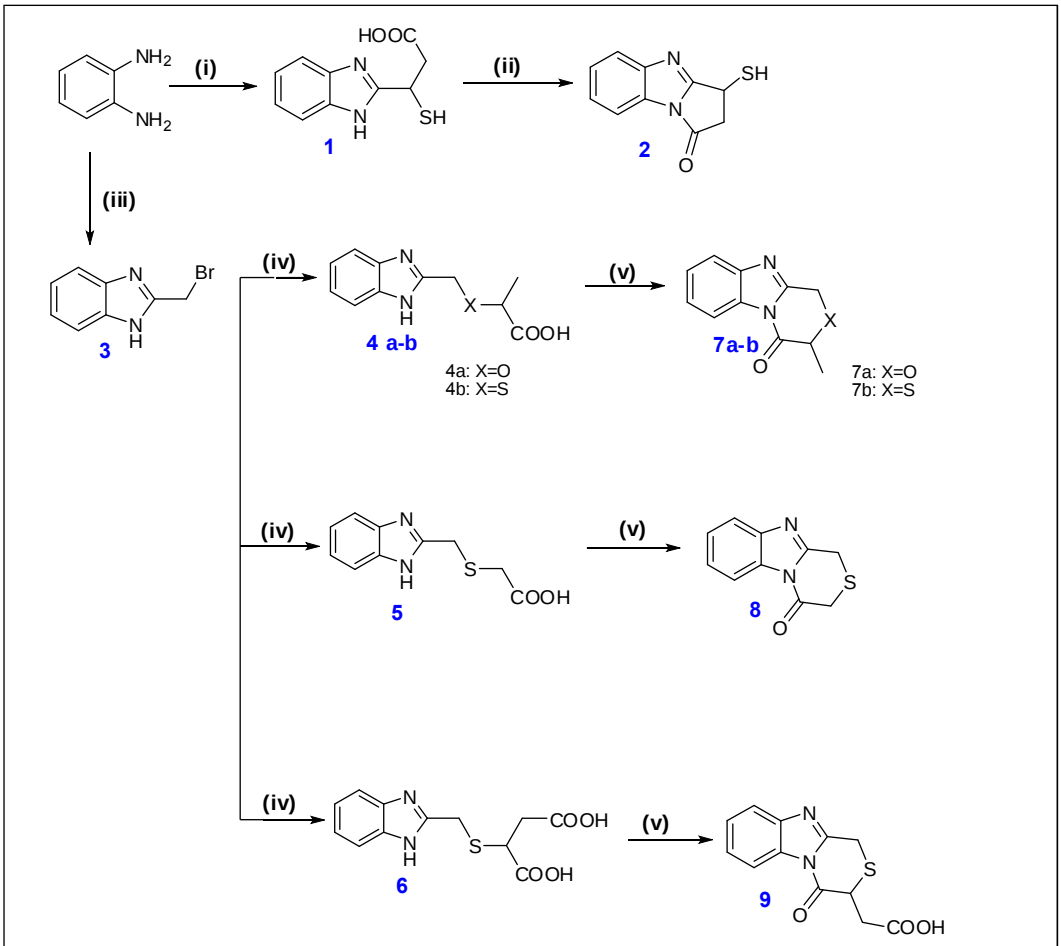
To determine ligand-binding affinity and the preferred orientation of docking pose, the produced compounds and the reference ligand indomethacin were docked with the COX-2 enzyme. (Detailed docking procedure was supported in Supplementary file).

3. Result and discussion

3.1. Chemistry

It was decided to use **scheme 1** to get the desired chemicals. The target compound **2** was produced through the cyclization of compound **1**, which was produced by reaction with acetic anhydride and fused sodium acetate. The synthesis began with the reaction of benzene 1,2 diamine with 2-mercaptosuccinic acid in presence of 4N HCl. IR spectrum of compound **2** revealed presence of SH group band at 2638 cm^{-1} accompanied by disappearance of broad OH band. ^{1}H-NMR spectrum of the synthesized compound **2** exhibited the downfield multiplet corresponding to four aromatic hydrogen of benzimidazole at δ 7.25-6.91. The spectrum also showed upfield triplet at δ 3.62 for aliphatic proton (CH-S) in addition to upfield

multiplet at δ 3.16-2.57 for aliphatic proton of CH_2. To achieve target compounds **4** to **9**, benzene 1, 2 diamine was alkylated by ethyl bromoacetate to give compounds **3** which was then reacted with either lactic- acid or thiolactic- acid or thioglycolic-acid or thiomalic- acid to provide desired compounds **4a-b,5,6** respectively. The synthesized compounds' IR spectra revealed characteristic broad band of (OH) group at range of 3340- 3051 cm^{-1} and (NH) band at range 3120- 3051 cm^{-1} as well as carbonyl group band at range of 1678- 1630 cm^{-1}. Additionally, IR spectra of compounds of compounds **4b, 5** and **6** showed band matching to (C-S) at range of 744- 732 cm^{-1}. Furthermore, ^{1}H NMR spectra of compounds **4a-b** and **6** exhibited two downfield singlets, one for proton of (OH) at range of δ 10.68- 8.86 and one for proton of (NH) at rang of δ 10.00- 8.50. The spectrum also showed downfield multiplet belonged to the four-aromatic proton of benzimidazole ring at range of δ 7.59- 7.12 and upfield sharp singlet corresponding to two aliphatic protons of (CH_2) at range of δ 4.90- 3.18. proton of (CH_3) in compounds **4a** and **4b** appeared in ^{1}H NMR spectra as upfield doublet at δ 1.24 and 1.26 respectively while proton of (CH) appeared as quartet at range of δ 4.23- 2.95. In ^{1}H NMR of compound **6**, aliphatic proton of (CH) appeared as upfield triplet at δ 3.87 and other aliphatic protons of (CH_2) appeared also as upfield triplets at δ 2.90 and 2.74.



codition and reagents: (i) 2-mercaptosuccinic acid, 4N HCl / reflux 24hrs., (ii) Acetic anhydride, fused sodium acetate, heat at 90°C 24hrs., (iii) Ethyl 2-bromoacetate, 4N HCl, stirr r.t. 4hrs. then reflux 4hrs., (iv) Lactic acid or thioglycolic acid or thiolactic acid or thiomalic acid, MeOH, TEA, stirr r.t. 3hrs. then heat at 90°C 12-16 hrs., (v) Acetic anhydride, fused sodium acetate, heat at 90°C 16-24hrs.

Scheme 1. Target compounds **2-9** synthesis

To obtain target compounds **7a-b**, **8** and **9**, compounds **4a-b, 5** and **6** was cyclized through reaction with acetic anhydride and fused sodium acetate. The success of the reaction was confirmed through disappearance of bands corresponding for (NH) and (OH) from the IR spectra of the synthesized compounds (except for compound **9**

where second (OH) appeared at 3417cm^{-1}). IR spectra also showed sharp carbonyl band within range 1666- 1654 cm^{-1}. In addition, IR spectra of compounds **7b, 8** and **9** exhibited (C-S) band at 744 cm^{-1}. In contrast, ^{1}H NMR spectra of compounds **7a-b** and **9** showed downfield multiplet belonged to four aromatic protons of benzimidazole ring at range of δ 7.91- 6.91 and upfield multiplet belonged to aliphatic protons of (CH_2) at range of δ 4.93- 3.58. Additionally, ^{1}H NMR spectrum of **7a** and **7b** showed upfield doublet for aliphatic protons of (CH_3) at δ 1.24 for both compounds.

3.2 Evaluation of biological activity.

***In vitro* COX-2 inhibitory assay**

The IC_{50} of compound under research was determined after testing them against COX-1 and COX-2. Furthermore, the COX- 2 selectivity indexes (SI) were elaborated (IC_{50} (COX- 1)/IC_{50} (COX- 2)) then compared with the SI of the basic drug indomethacin **(Table 1).** The results showed that all compounds under research inhibited the COX-1 isozyme at larger dosages (IC_{50}= 2.56- 9.35 μM), indicating that the compounds are less effective against the enzyme than the basic drug indomethacin (IC_{50}= 1.56 μM) and may therefore be safer. However, for COX- 2, compounds **4a, 4b, 5, 6** and **9** showed an IC_{50} inhibitory range of 0.13–0.27 μM, that is more powerful than indomethacin (IC_{50} = 0.41 μM). Regarding the SI, the results revealed that compounds **4a, 4b, 5, 6** and **9** exhibited high selectivity (SI range of 22.83- 71.92) compared to indomethacin (SI= 4) where compound **6** was the most selective of them all with SI= 71.92.

***In-vivo* anti-inflammatory activities**

The carrageenan-induced rat paw edema was applied to examine anti-inflammatory (AI) activity of the most selective compounds with highest SI score (**4a, 4b, 5, 6** and **9**). AI activity was recorded after 1, 2, 3 and 4-hrs post-carrageenan injection as in **(Table.2)**. The findings showed that, except for compounds **4a** and **4b**, all compounds significantly ($p<0.05$) reduced inflammation when compared to carrageenan group at all time periods. They showed average to good anti-inflammatory activity after an hour (AI = 45.5-72.6 %) in compared to indomethacin (AI = 42.3 %) except compound **4b** which showed less AI activity. The target compounds showed extremely increased anti-inflammatory activity after 3 and 4 hrs. The compounds **6** and **9** revealed a highest AI activity during all time intervals (after 1 h = 72.6% and 66.7%, after 2 h= 82.7% and 77.8%, after 3 h = 88.5% and 83.3% and after 4 h = 93.2% and 91.2% respectively).

***In vitro* cytotoxicity assay**

The MTT was performed to appraise the cytotoxicity of the most active compounds against the normal Vero cell line. The compounds were incubated with Vero cells for 24hs, then the percentage of cell viability was measured. All compounds showed a good percentage of cell viability (82.5%-96.9%). Compounds **6** and **9** showed no significant difference ($p>0.05$) compared to control untreated Vero cells with the highest cell viability indicating the least cytotoxicity **(Fig. 4)**.

3.3 Molecular docking

Using the MOE modelling version (2019.0102), docking research was conducted on all synthesized compounds to show potential conformations that bind to the COX-2 receptor. This may have shown the potential way of action for their anti-inflammatory effect toward the COX-2 active site. The docking outcomes are summarized in **(Table.3)** as binding free energy. As can be seen, the binding free energy value is negative, showing that both reference ligands and synthetic molecules bind spontaneously. Then with help of Biovia Discovery Studio visualizer, docking scores of the best-fitted poses were recorded, 2D and 3D images were created.

Human COX-2 is consist of a homodimer of 581 amino acids, it contains four high mannose oligosaccharides, one of them facilitates protein folding and a second regulates its degradation. Each subunit of the dimer comprises of three domains, the epidermal growth factor domain, the membrane binding domain, and the catalytic domain (contains the cyclooxygenase active site).

The COX-2 active site found at the top of an L-shaped channel that originates in the membrane binding domain. The channel starts with a lobby, a wide volume which narrows to a constriction (consist of three residues Arg-120, Tyr-355, and Glu-524) that must open to allow the passing of substrates into the channel [20]. Above the constriction, the channel is bordered by hydrophobic residue and continues into the center of the protein until it reaches Tyr-385, the critical catalytic residue that starts the COX-2 reaction. Additionally, COX-2 is characterized by a side pocket located above the constriction which is bordered by Val-523 and includes a conserved Arg-513 at the base.

Table 1 *In vitro* COX-1/COX-2 enzyme inhibition for compounds under research and Indomethacin.

Compds.	[COX-1 IC_{50} (µM)[a]]	[COX-2 IC_{50} (µM)[a]]	[COX-2 SI[b]]
2	4.89	0.49	9.97
4a	5.25	0.23	22.83
4b	7.23	0.27	26.77
5	7.89	0.24	32.87
6	9.35	0.13	71.92
7a	3.95	0.31	12.74
7b	2.56	0.39	6.56
8	3.59	0.28	12.82
9	7.25	0.15	48.33
Indomethacin	1.56	0.41	4

Compds = Compound. [a]IC_{50}: The concentration caused 50% inhibition of COX, [b] Selectivity index (IC_{50} COX-1/IC_{50} COX-2).

Table 2 Mean ± SD of in-vivo anti- inflammatory action for different compounds and indomethacin against carrageenan- induced rat paw edema

Compds.	% of inhibition			
	at 1h	at 2h	at 3h	at 4h
4a	45.5 ± 3.30	51.2 ± 2.54	66.7 ± 2.41	89.2 ± 2.91
4b	37.2 ± 4.22	48.3 ± 2.43	61.5 ± 4.43	83.7 ± 4.14
5	51.5 ± 3.90*	60.8 ± 3.85*	72.3 ± 4.15*	84.3 ± 4.11
6	72.6 ± 5.06*	82.7 ± 4.89*	88.5 ± 2.22*	93.2 ± 2.54*
9	66.7 ± 3.93*	77.8 ± 4.45*	83.3 ± 3.29*	91.2 ± 2.27*
Indomethacin	42.3 ± 1.97	53.3 ± 3.29	65.8 ± 2.4	88.2 ± 4.52

Compds. = Compound., *Significantly different as compared to indomethacin group using one way ANOVA test ($p<0.05$).

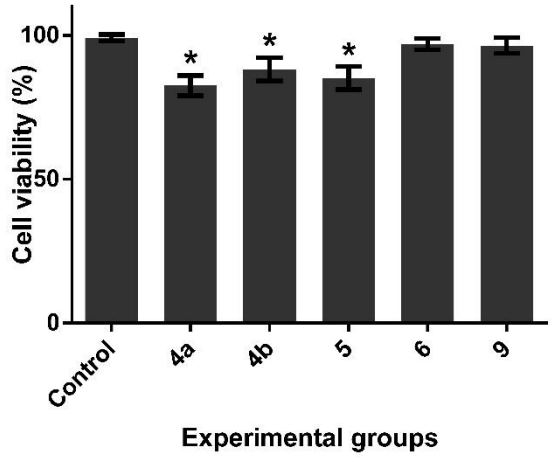


Fig. 4. The cytotoxicity effect of the compounds under research on cell viability in Vero cells. Experimental procedures were performed in triplicate, and findings were expressed as mean±SD. *Significantly different compared to control untreated Vero group using one way ANOVA test, ($p<0.05$).

The solvent accessible surface in the COX-2 active site is large due to the presence of Val-523. On the other hand, the last helix of the membrane binding domain (helix D) shifts the location of Arg-120 present in the constriction site, permitting for a larger solvent accessible surface at the interface between the membrane binding domain and the COX active site. Binding of bioactive substrates (Arachidonic Acid) in the active site occurs through its carboxylate moiety interaction with Arg-120 and/or Tyr-355 at the constriction and the rest of the compound extended upward, looping around Ser-530 and filling the hydrophobic area. Except for interaction with carboxyl group, all interactions are hydrophobic in nature. [21]

There were three different types of interactions for indomethacin in the COX-2 active site [22]: first, hydrophobic contacts between the aromatic rings of indomethacin and the hydrophobic residues Phe381, Leu384, Met522, Tyr385, Trp387, Leu531, Leu352, Ala527, and Val523. Second, a salt bridge was created between the carboxylate group of the inhibitor and Arg120. The inhibitor's hydrogen bonds with Tyr355 (1.60 A˚), Arg120 (1.89 A˚), and Ser530 (1.77 A˚) came in third **(Fig.5)** [23]. Indomethacin's original conformation in crystal structure 4COX served as a guide for analyzing and ranking the produced docking positions. The synthesized compounds' final conformations were examined, and the best poses were chosen based on their capacity to interact similarly to that of reference inhibitor.

Docking compounds **6** and **9** showed the capability to partially accommodate the equivalent region as the reference inhibitor inside the pocket. Compound **6** demonstrated binding by hydrophobic interactions among Tyr385, Met522, Trp387, and Leu352 that were created by its benzimidazole aromatic rings **(Fig. 6)**, as observed for indomethacin, while compound **9** showed hydrophobic binding with Tyr385, Val 523, Leu352 and Ala527 **(Fig. 8)**.

Table .3: The molecular docking scores and rmsd values of 2, 4a, 4b, 5, 6, 7a, 7b, 8, 9, and the indomethacin (ligand) inner the COX- 2 active site

Compound	Binding score (Kcal/mol)	rmsd
2	-5.85	0.9
4a	-6.51	1.5
4b	-6.40	0.78
5	-6.57	1.09
6	-7.08	1.34
7a	-6.08	0.73
7b	-6.24	0.94
8	-5.80	0.68
9	-6.69	1.64
Indomethacin	8.00	1.10

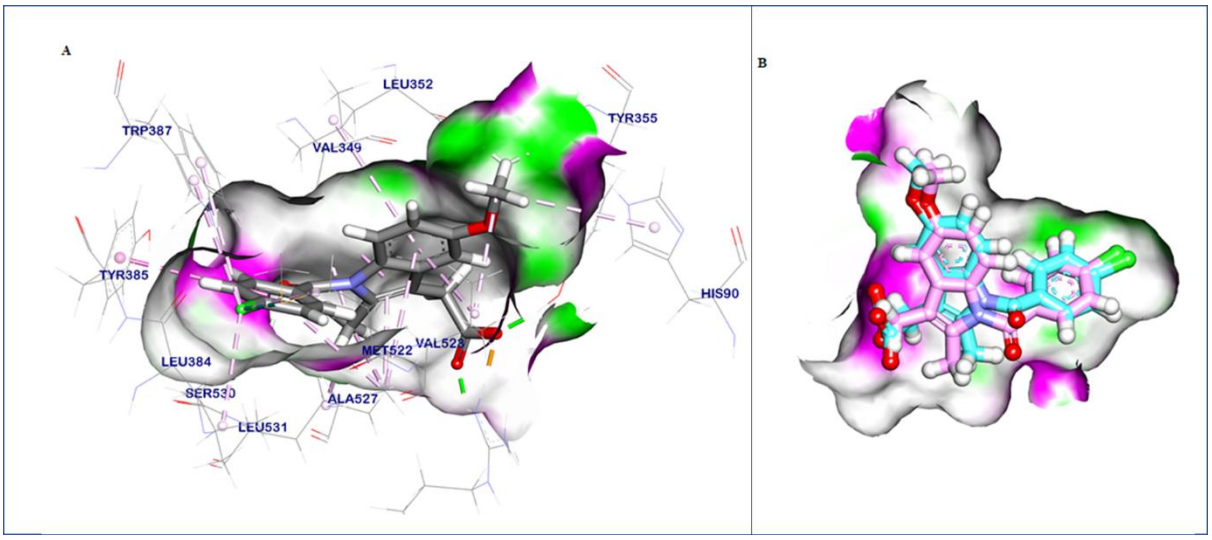


Fig. 5. (A)3D view of docked pose of indomethacin in the COX-2 crystal structure (PDB.4COX). (B) Alignment of the redocked poses of indomethacin (bink) on the crystallized ligand indomethacin (turquoise).

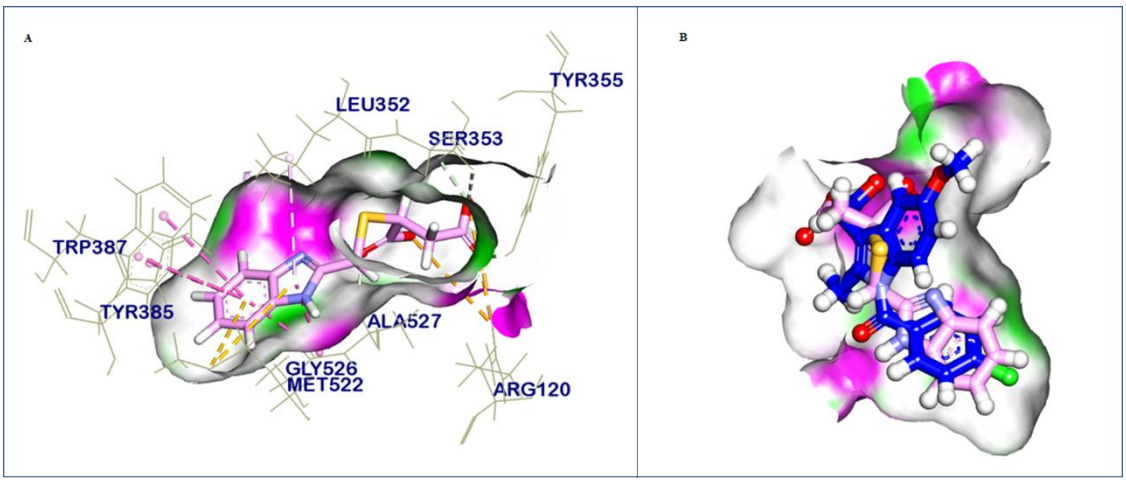


Fig. 6 (A) Shows the proposed binding mechanism of compound **6** in crystal structure of COX-2, where it forms a H bond with Tyr355. (B) The docked postures of the most active compound **6** (bink) on the crystalline ligand indomethacin (blue).

Both compounds **6** and **9** showed additional hydrophobic contacts with Gly526. In addition to salt bridges between carboxylate groups of both compounds **6** and **9** and Arg120, as observed for indomethacin **(Fig. 7)**.

Compound **6** could generate a single hydrogen bond with Tyr355 (1.84 A˚) and carbonyl oxygen, but compound **9** created two hydrogen bonds with Arg120 (2.31 A˚) and Tyr355 (1.95 A˚). It is possible for both ligands to occupy the same space in the pocket as shown by the superposition of **6** and indomethacin, which explains why **6** is the most powerful inhibitor in these synthetic molecules **(Fig. 6 B).**

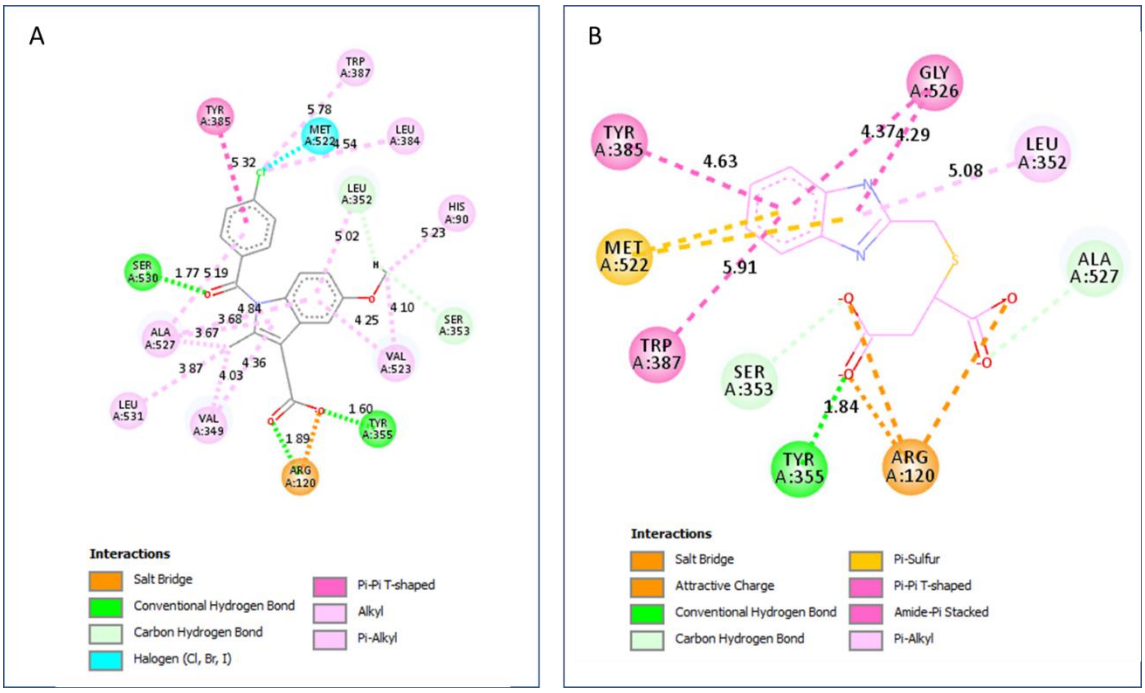


Fig. 7 (A) shows a two-dimensional image of indomethacin docked in the crystal structure of COX-2 (PDB.4COX). (B) A two-dimensional image of compound **6** docked in the COX-2 crystal structure.

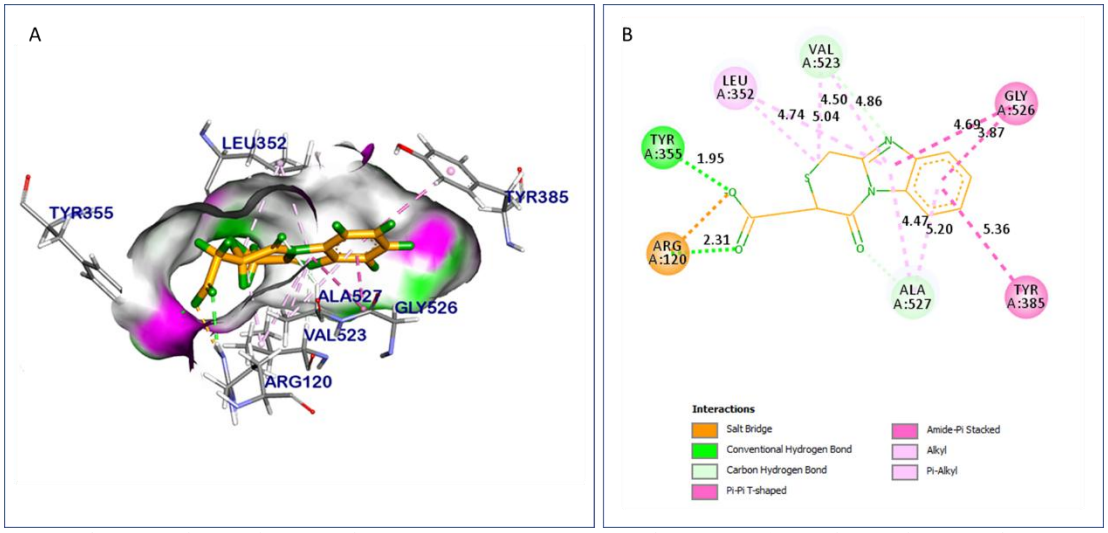


Fig. 8. (A) shows a 3D image of compound **9** docked inside the COX-2 crystal structure. (B) A 2D image of compound **9** docked in the crystal structure of COX-2 reveals established 2-H bonds with Arg120 and Tyr 355.

4. Conclusion

In current work, simple benzimidazole derivatives were synthesized, structurally confirmed and biologically investigated as COX-2 inhibitors. The strongest compounds were compounds **6** and **9**, which can be attributed to their closely binding mode to the reference chemical indomethacin. The relevance of hydrogen bonds formed with Tyr355 and Arg120 and their function in the potency of recently synthesized inhibitor are confirmed by molecular docking analysis of the inhibitor at the COX-2 binding site. The outcomes of current research, in short, offer simple and inexpensive screening procedures for the identification of candidates that are like drugs. Furthermore, these compounds are considered as a start for more investigation of novel anti-inflammatory agents. We are currently extending our research on the synthetic scaffold to improve its lipoxygenase reactivity.

5. Ethics approval

This study was approved by the Committee for Ethics of the Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt (202306RA1).

6. Conflict of interest

The authors claim to have no conflicts of interest.

7. References

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