1.0. Introduction

The present market of the anti-inflammatory drugs is encumbered with NSAIDs associated with serious adverse effects like ulceration and gastric haemorrhage [1]. A prolonged consumption of these drugs may cause gastric injuries [2]. The pro-inflammatory mediators like TNF-α, NO and IL-1β play an imperative role in the inflammatory reactions like tissue destruction, shock and organ failure [3-5]. The aim of this work is to develop new molecules with a potential to inhibit the target specific overexpression of the proinflammatory mediators such as TNF-α, NO and IL-1β without inducing any gastric ulceration. 2-mercaptobenzoxazoles are the thiol derivatives (Figure 1) of simple benzoxazole nucleus. They exist in two tautomeric forms i.e. thiol and thione [6].

![Tautomers of 2-mercaptobenzoxazole](image)

**Figure 1.** Tautomers of 2-mercaptobenzoxazole

2-mercaptobenzoxazoles have emerged as a potent medicinal scaffold and have remained the focus of drug discovery due to their important therapeutic values [7]. Benzoxazoles and their derivatives are known to exhibit antibacterial, antifungal, antitumor, anti-tubercular, anti-inflammatory and HIV-1 reverse transcriptase inhibitory activities [8-12]. 1,2,3-triazole nucleus is present in many drugs [13] and has been found to be associated with potent antimicrobial [14,15], anti-inflammatory [16], local anaesthetic [17], anticonvulsant [18], anti-neoplastic [19], antimalarial [20] and antiviral activities [21]. In continuation of our efforts to develop novel molecules for the treatment of inflammatory disorders like osteoarthritis and crohn’s disease we have conjugated 2-mercaptobenzoxazoles with 1,2,3-triazoles under one construct through a methylene linkage. *In silico* molecular docking studies have been done with respect to COX-2 and TNF-α target in order to get an insight into the binding modes of the novel synthesized ligands with their active sites. The compounds showing significant *in vivo* anti-inflammatory activity have been further screened to study their inhibitory effects on the *in vivo* levels of the COX-2 and proinflammatory mediators like TNF-α, IL-1β and NO. The potent anti-inflammatory molecules thus obtained
were subjected to *in vivo* antinociceptive activity by writhing test method followed by their gastric ulceration study. Since the free radical production occurs simultaneously during the inflammation therefore we have also screened our compounds for their antioxidant activity using reduced glutathione and lipid peroxidation assays. All the synthesized compounds have been found to possess antioxidant potential.

2.0. **Results and discussion**

2.1. **Analytical**

A focused library of eighteen novel 2-mercaptobenzoxazole based 1,2,3-triazoles (1-18) has been synthesized (**Table 1**) starting from 2-mercaptobenzoxazole. The conjugation of the 2-mercaptobenzoxazole with the 1,2,3-triazoles nucleus was supported by the presence of a singlet for two methylene protons (-CH$_2$) in a range of δ 4.76-4.81 in the $^1$H-NMR spectra of the synthesized compounds. The formation of 1,2,3-triazoles was confirmed by the resonance of the triazollyl proton as singlet in a range of δ 8.29-8.91 for different derivatives. The final compounds varied from each other on the basis of nature and position of the substituents on aryl ring attached to the 1,2,3-triazole ring. The structure of all the compounds was confirmed by the IR, $^1$H-NMR, $^{13}$C-NMR spectra and ESI-MS mass spectral analysis.
Table 1. Novel 2-mercaptobenzoxazole based 1,2,3-triazoles.

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2.2. **In silico molecular docking studies**

2.2.1. **Molecular docking studies on COX-2**

In order to get the insights of binding mode of ligands with COX-2, these ligands were docked against COX-2 (PBD NO: 3LN1). Before docking these ligands, the docking methodology of Schrodinger was validated by predicting the binding mode of celecoxib itself against the developed grid. Hydrogen bonding between LEU-338 residue & celecoxib (**Figure 2a**) is highlighted in red color. **Figure 2a** clearly shows that adapted Schrodinger methodology successfully predicted the binding mode of crystallographic celecoxib with Root mean square deviation of 1˚A. All the ligands were docked against COX-2 target protein separately. **Figures 2b and 2c** show that the compounds 4 and 9 have purely hydrophobic interactions with the COX-2 protein. The binding energies of the eighteen new ligands was found to be in the range of -23.07 to -48.4 kcal/mol. Ligands 4 (G score = -8.6) and 9 (G score = -8.14) were found to have the closest binding efficiency with respect to celecoxib (G score = -11.29).

![Figure 2a](image)

**Figure 2a.** Superimposed binding orientation of the crystallographic celecoxib (green) and docked celecoxib (maroon) as predicted by Schrodinger glide software. The amino acid residues are shown in stick model (blue).
Figure 2b. Compound 4 shows no H-bond formation indicating that the interactions are mainly hydrophobic and shape driven.
Figure 2c. Compound 9 shows no H-bond formation indicating that the interactions are mainly hydrophobic and shape driven.
The predicted binding energies are summarized in Table 2. The QikProp program was used to predict the ADME (absorption, distribution, metabolism and excretion) properties of the ligands. Normal mode was applied in the program to predict partition coefficient (log P o/w), van der Waals surface area of polar nitrogen and oxygen atoms (PSA), aqueous solubility (log S) properties. The results obtained are listed in Table 3 respectively. As seen from the table the values of calculated properties are within acceptable ranges.

Table 2. *In silico* docking scores of celecoxib and 18 novel ligands with COX protein.

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<tr>
<th>Ligand</th>
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Table 3. *In silico* ADME properties of the ligands with respect to COX-2 protein.

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<th>PSA</th>
<th>LogP o/w</th>
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2.2.2. Molecular docking studies on TNF-α

The binding pocket of 2AZ5 is large and without defined cervices. The binding site is mainly hydrophobic consisting of glycine, leucine, and tyrosine residues. In order to bind to this hydrophobic large pocket, the ligands also need to be hydrophobic and of large size. Before docking the new ligands against the generated grid, the reference ligand was separately docked against the generated grid to validate the grid and docking methodology. Figure 3a shows comparison between the original binding modes of reference ligand against docked binding mode as predicted by Schrodinger Glide software. Figure 3a clearly shows that adapted Schrodinger methodology
successfully predicted the binding mode of crystallographic mode with root mean square deviation of 0.003 Å. All the ligands were docked against TNF-α target protein separately.

**Figure 3a.** Superimposed binding orientation of the reference ligand (green) and docked reference ligand (maroon) as predicted by Schrodinger glide software.

**Figures 3b and 3c** show that the compound 8 is embedded in the pocket of the TNF-α protein and compound 10 is involved in H-bond formation with GLY 121 residue respectively. The binding energies of the eighteen novel ligands were found to be in the range of -35.09 to -41.4 kcal/mol. Ligands 8 (G score = -6.38) and 10 (G score = -6.77) exhibited the closest binding efficiency with respect to reference ligand 2AZ5 Ligand (G score = -7.1). The predicted binding energies are summarized in Table 4. The QikProp program was used to predict the ADME (absorption, distribution, metabolism and excretion) properties of the ligands. Normal mode was applied in the program to predict partition coefficient (log P o/w), van der Waals surface area of polar nitrogen and oxygen atoms (PSA), aqueous solubility (log S) properties. The results obtained are listed in Table 5 respectively. As seen from the table the values of calculated properties are within acceptable ranges.
Figure 3b. Compound 8 is deeply buried in the pocket.
Figure 3c. Compound 10 shows H-bond formation with GLY 121 residue.
Table 4. *In silico* docking scores of reference ligand and 18 new molecules with TNF-α PDB No: 2AZ5).

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### Table 5. *In silico* ADME properties of the ligands with respect to TNF-α protein.

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3.0. Biological activity

A focused library of eighteen compounds has been synthesized and all the compounds have been screened for their anti-inflammatory activity. These compounds have also been screened for their antioxidant activity. The compounds 4, 5, 7, 9 and 16 showing significant in vivo anti-inflammatory activity were further screened for their in vivo COX-2, TNF-α, NO and IL-1β inhibitory potential. The compounds showing potent anti-inflammatory activity have further been screened for their analgesic activity by tail immersion and acetic acid induced writhing test. Finally the ulcerogenic study of the active compounds 4, 5, 7, 9 and 16 has been done to see their tolerance towards gastric mucosa.

3.1. In vivo anti-inflammatory activity

All the synthesized compounds have been screened for their in vivo anti-inflammatory activity by carrageenan-induced hind paw edema model. All the compounds showed a time-dependent decrease in the inhibition of inflammation after 3 h and 5 h. The results thus obtained exhibited that the compound 4 showed potent anti-inflammatory activity with 66.66% and 61.11% inhibition after 3 h and 5 h as compared to celecoxib which showed 72.22% and 65.55% inhibition after 3 h and 5 h respectively (Figure 4 and Table 6). The compound 9 exhibited 68.88% inhibition at 3 h post-carrageenan and 55.55% inhibition 5 h post-carrageenan administration as compared to celecoxib. It was observed that the compounds 5, 7 and 16 exhibited moderate anti-inflammatory activity in comparison to the standard drug celecoxib.

Figure 4. In vivo anti-inflammatory activity of the 2-mercaptobenzoxazole based 1,2,3-triazoles by carrageenan induced hind paw edema method.
Table 6. *In vivo* anti-inflammatory activity of the 2-mercaptobenzoxazole based 1,2,3-triazoles.

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<td></td>
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<td>0.90 ± 0.06</td>
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<td>7.</td>
<td>-</td>
<td>0.43 ± 0.04***</td>
<td>0.50 ± 0.06**</td>
</tr>
<tr>
<td>8.</td>
<td>-</td>
<td>0.50 ± 0.06***</td>
<td>0.55 ± 0.07*</td>
</tr>
<tr>
<td>9.</td>
<td>-</td>
<td>0.28 ± 0.03***</td>
<td>0.40 ± 0.06***</td>
</tr>
<tr>
<td>10.</td>
<td>-</td>
<td>0.48 ± 0.08**</td>
<td>0.53 ± 0.07*</td>
</tr>
<tr>
<td>11.</td>
<td>-</td>
<td>0.41 ± 0.07***</td>
<td>0.48 ± 0.06**</td>
</tr>
<tr>
<td>12.</td>
<td>-</td>
<td>0.51 ± 0.08*</td>
<td>0.55 ± 0.05*</td>
</tr>
<tr>
<td>13.</td>
<td>-</td>
<td>0.58 ± 0.07</td>
<td>0.61 ± 0.08</td>
</tr>
<tr>
<td>14.</td>
<td>-</td>
<td>0.53 ± 0.07*</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>15.</td>
<td>-</td>
<td>0.45 ± 0.05**</td>
<td>0.50 ± 0.03**</td>
</tr>
<tr>
<td>16.</td>
<td>-</td>
<td>0.36 ± 0.06***</td>
<td>0.43 ± 0.04***</td>
</tr>
<tr>
<td>17.</td>
<td>-</td>
<td>0.56 ± 0.07</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>18.</td>
<td>-</td>
<td>0.60 ± 0.07</td>
<td>0.66 ± 0.07</td>
</tr>
</tbody>
</table>

Data is analyzed by one way ANOVA followed by Dunnett’s ‘t’ test and expressed as mean ± SEM from six observations; ***p < 0.001, ** p < 0.01 & *p < 0.05.
The structure activity relationship of the synthesized compounds has been analysed with respect to the nature and the position of the substituents attached with the aromatic ring connected to the 1,2,3-triazolyl ring. The compounds having substituted aromatic ring on the 1,2,3-triazolyl system exhibited more potent anti-inflammatory activity as compared to unsubstituted derivative of the 1,2,3-triazolyl ring i.e. the compound 3 exhibited lesser anti-inflammatory activity. The compounds containing electron donating groups on the aromatic ring exhibited more potent anti-inflammatory activity in comparison to the compounds containing electron withdrawing groups. The compounds 4, 5 and 16 containing the C\textsubscript{2}H\textsubscript{5}, OCH\textsubscript{3} and OC\textsubscript{2}H\textsubscript{5} groups respectively at the para position displayed potent anti-inflammatory activity. The presence of the strong electron withdrawing NO\textsubscript{2} group at the meta 1 and para 2 positions resulted in a significant loss in the anti-inflammatory activity. Amongst the halogens the anti-inflammatory activity was found to decrease with a decrease in the size of halogen (Br>Cl>F) i.e activity was found to decrease in the order 9>7>10. The para substituted halogen containing compounds 7, 9, and 10 exhibited more potent anti-inflammatory activity as compared to their corresponding ortho substituted halogen containing compounds i.e. 8, 12 and 14.

3.2. *In vivo* IL-1β, TNF-α and COX-2 assay

During the inflammatory reactions, large amounts of the pro inflammatory mediators are generated which affect the immune system by suppressing the proliferation of T and B cells, as well as cytokine synthesis [22]. Blockade of these molecules results in a reduction of disease severity and bone resorption [23-26]. Pro inflammatory mediators IL-1β and TNF-α have an important role in the perpetuation of chronic inflammation and tissue damage during progression of inflammatory disorder. There is a significant increase in the level of TNF-α, IL-1β and COX-2 in carrageenan induced edema rats as compared to the control (Figure 5). Administration of the selected active compounds 4, 5, 7, 9 and 16 suppressed the increase in the level of IL-1β, TNF-α and COX-2 significantly when compared with the edema group. The compound 16 exhibited a reduction to 2.66 ± 0.13 pg/ml in the level of IL-1β in comparison to celecoxib which showed a reduction to 2.9 ± 0.16 pg/ml. The compound 5 suppressed the level of TNF-α to 3.10 ± 0.17 pg/ml in comparison to celecoxib which showed a reduction to 3.35 ± 0.16 pg/ml. A significant decrease in
the amount of COX-2 was observed for the compound 5 which reduced the COX-2 level to $6.54 \pm 0.26$ mol/min/ml as compared to celecoxib which showed a reduction of $7.90 \pm 0.25$ mol/min/ml.

**Figure 5.** Level of TNF-α, IL-1β and COX-2 in carrageenan induced edema rats.
Table 7. Inhibitory activity of the 1,2,3-triazole based benzoxazolinones.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀(µM) (COX-1)</th>
<th>IC₅₀(µM) (COX-2)</th>
<th>Selectivity index COX-1/COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>246.20</td>
<td>3.8</td>
<td>64.79</td>
</tr>
<tr>
<td>5</td>
<td>89.37</td>
<td>2.3</td>
<td>38.86</td>
</tr>
<tr>
<td>7</td>
<td>80.82</td>
<td>1.9</td>
<td>42.54</td>
</tr>
<tr>
<td>9</td>
<td>186.11</td>
<td>2.8</td>
<td>66.47</td>
</tr>
<tr>
<td>16</td>
<td>163.24</td>
<td>3.5</td>
<td>46.64</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>20.40</td>
<td>0.27</td>
<td>75.56</td>
</tr>
</tbody>
</table>

Values are the means ± SEM from three independent experiments using COX assay kits (Cayman Chemicals Inc., Ann Arbor, MI, USA).

The compounds 9 (COX-1 IC₅₀ = 186.11µM; COX-2 IC₅₀ = 2.8µM; SI = 66.47) and 4 (COX-1 IC₅₀ = 246.20µM; COX-2 IC₅₀ = 3.8µM; SI = 64.79) exhibited potent selective COX-2 inhibition (Table 7) as compared to celecoxib (COX-1 IC₅₀ = 20.40µM; COX-2 IC₅₀ = 0.27µM; SI = 75.56). The COX-1/COX-2 selective Index (SI value) of the compounds 4 and 9 shows the selective nature of these compounds towards COX-2 inhibition as compared to celecoxib.

3.3. Gene expression study on COX-2

The results of the gene expression study on COX-2 (Figure 6) show that the compounds 4 and 9 suppressed the expression of COX-2 gene by 0.94 and 0.79 fold in comparison to the standard drug celecoxib. Thus these molecules may be used to develop new leads for the treatment of inflammatory disorders like arthritis.

![Figure 6](image_url). Results of the COX-2 gene expression study.
3.4. **Nitric oxide assay**

Nitric oxide (NO) is an important signalling molecule, produced as part of the inflammatory response from activated cells and macrophages [27, 28]. An increase in the NO level has been previously reported in synovial fluids of patients suffering from rheumatoid arthritis [29]. In the present study, increased NO levels have been detected in carrageenan group similar to those previously reported in synovial fluids of patients with rheumatoid arthritis. Analysis of nitrite estimation is summarised in Figure 7. A significant increase in nitrite was observed in carrageenan induced edema group as compared to control. The synthesized compounds suppressed the increase in the nitrite level significantly as compared to the edema group. Compound 4 significantly suppressed the rise in the NO level to 5.10 ± 0.18 μmol/mg in comparison to celecoxib which showed a reduction to 5.50 ± 0.16 μmol/mg.

![Figure 7. Effect of the synthesized compounds on Nitric oxide.](image)

3.5. **Effect on TBARS and GSH**

Lipid peroxidation has been implicated in the pathogenesis of cancer, atherosclerosis, degenerative diseases, and inflammatory arthritis [30]. During lipid peroxidation, lipid peroxyl radicals are produced that can lead to cell membrane damage. Matrix degradation arising from cytokine-stimulated chondrocytes has been shown to be primarily due to lipid peroxidation [31]. The effect of all the synthesized compounds on TBARS (Thiobarbituric acid reactive substances) level was measured to demonstrate the oxidative damage on lipid.
A significant increase in TBARS level (Figure 8) was observed in carrageenan induced edema group when compared to the control group. The level of TBARS was suppressed to 5.10 ± 0.23 moles by the compound 16 whereas celecoxib reduced the TBARS level to 6.30 ± 0.16 moles. Free radical production that occurs during development of arthritis in the articular cartilage leads to decreased GSH (Glutathione) and SOD (Super oxide dismutase) levels as a result of their consumption during oxidative stress and cellular lysis [32-34]. The concentration of GSH was evaluated to estimate endogenous defences against hydrogen peroxide formation. Figure 9 shows the changes in GSH levels evaluated in the joints of the
experimental groups. A marked decrease in GSH was found in the joint of carrageenan induced (0.58 ± 0.02 μGSH/g tissue) edema rats. However the treatment with compounds 4 (0.84 ± 0.025 μGSH/g tissue) and 16 (0.84 ± 0.027 μGSH/g tissue) significantly inhibited the decrease in GSH as compared to celecoxib (0.81± 0.027 μGSH/g tissue).

3.6. **In vivo antinociceptive activity**

The compounds showing significant *in vivo* anti-inflammatory activity have been further evaluated for their *in vivo* antinociceptive potential. It was found that the compounds 5 and 7 exhibited 55.55% and 51.50% inhibition respectively in comparison to celecoxib which showed 70.89% inhibition (Figure 10).

![Figure 10. In vivo antinociceptive activity of the compounds by writhing test.](image)

3.7. **Ulcerogenic study**

The compounds showing potential *in vivo* anti-inflammatory and *in vivo* antinociceptive activities were further tested for their gastric ulceration activity (Figure 11). When compared with celecoxib, compounds 4, 5, 7, 9 and 16 did not induce any gastric ulceration and rupture of the gastric mucosal layer.
Figure 11. Histopathology report of the active compounds.
4.0. **Experimental**

4.1. **Chemistry**

All commercial chemicals used as starting materials and reagents were purchased from Merck (India), Spectrochem and Sigma Aldrich and were of AR grade. All melting points are uncorrected and have been measured using Veego VMP-DS apparatus. IR spectra were recorded as KBr pellets on a Perkin Elmer 1650 spectrophotometer (USA). $^1$H NMR spectra were determined on a Bruker (300 and 400 MHz) spectrometer and chemical shifts are expressed as ppm against TMS as internal reference. Mass spectra were recorded on 70 eV (El Ms-QP 1000EX, Shimadzu, Japan). Column Chromatography was performed on Silica gel (60-120 mesh). Elemental analysis was carried out using Elementar Vario EL III elemental analyzer. Elemental analysis data is reported in % standard.

A focused library of eighteen novel compounds has been synthesized. As shown in **Scheme 1**, 2-mercaptobenzoxazole on being refluxed with propargyl bromide for 5-7 h in the presence of potassium carbonate in dry acetone yielded the propargyl derivative **A**. The 1,3-diploar cycloaddition of the propargylated 2-mercaptobenzoxazole derivative **A** with different substituted aromatic azides under click chemistry conditions resulted in the formation of novel 2-mercaptobenzoxazole based 1,2,3-triazoles (1-18) in quantitative yields. Compound **A** obtained from the propargylation of 2-mercaptobenzoxazole was dissolved in 20 mL of $^1$Butanol: water (1:1) solvent at ambient temperature. CuSO$_4$·5H$_2$O was charged into it and the reaction mixture was stirred for 5 min. Reaction mixture became light blue in colour. Then sodium ascorbate was added to the reaction mixture and stirred for 15 min. The colour of the reaction mixture changed to dark yellow. After 15 min azides were added. The reaction mixture was allowed to stir for further 5-7 h at ambient temperature. After the completion of the reaction, monitored by TLC, reaction mixture was quenched with water and extracted with ethyl acetate. Combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to obtain the final product.
Scheme 1. Synthesis of novel 2-mercaptobenzoxazole linked 1,2,3-triazoles

4.1.1. 2-[1-(3-Nitro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfonyl]-benzoxazole

Yield : 85%

Physical appearance : Yellowish brown crystals

m. p. : 210-211 °C

Rf : 0.45 (toluene: ethyl acetate: formic acid 5:4:1)

IR (KBr) cm⁻¹ : 3145, 3130, 1580, 1525, 1390, 924

¹H NMR (DMSO-d₆, 400 MHz) : δ 4.80 (s, 2H), 7.31-7.37 (m, 2H), 7.66 (d, 1H, J=1.6 Hz), 7.68 (d, 1H, J=2.0 Hz), 7.88 (t, 1H, J=8.0 Hz), 8.31 (dd, 1H, J=1.2 and 8.0 Hz), 8.38 (dd, 1H, J=1.2 and 8.0 Hz), 8.71 (t, 1H, J=2.0 Hz), 9.08 (s, 1H)

¹³C NMR (DMSO-d₆, 100 MHz) : δ 26.57, 110.06, 115.32, 118.45, 121.14, 123.23, 124.21, 124.46, 125.95, 130.93, 137.56, 141.66, 145.20, 148.85, 152.09, 163.96

MS (ESI) m/z : 354 (M+1)⁺

Elemental Analysis : Molecular formula C₁₆H₁₁N₅O₃S

Calculated : C, 54.38; H, 3.14; N, 19.82; S, 9.07%

Found : C, 54.39; H, 3.13; N, 19.83; S, 9.06%
4.1.2. 2-[1-(4-Nitro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

![Chemical Structure]

Yield : 88%
Physical appearance : Yellowish brown crystals
m. p. : 205-206 °C
R<sub>f</sub> : 0.47 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm<sup>-1</sup> : 3155, 3127, 1598, 1533, 1376, 855
<sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz) : δ 4.80 (s, 2H), 7.32-7.37 (m, 2H), 7.68 (dd, 2H, J=1.6 and 7.2 Hz), 8.22 (d, 2H, J=9.2 Hz), 8.43 (d, 2H, J=6.9 Hz), 9.06 (s, 1H)
<sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz) : δ 26.42, 109.91, 118.33, 120.45, 121.32, 124.09, 124.34, 125.30, 140.93, 141.53, 144.99, 147.01, 151.89, 163.69
MS (ESI) m/z : 354 (M+1)<sup>+</sup>
Elemental Analysis : Molecular formula C<sub>16</sub>H<sub>11</sub>N<sub>5</sub>O<sub>3</sub>S
Calculated : C, 54.38; H, 3.14; N, 19.82; S, 9.07%
Found : C, 54.36; H, 3.12; N, 19.81; S, 9.05%

4.1.3. 2-(1-Phenyl-1H-[1,2,3]triazol-4-ylmethylsulfanyl)-benzoxazole

![Chemical Structure]

Yield : 90%
Physical appearance : White powder
m. p. : 178-179 °C
R<sub>f</sub> : 0.70 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm<sup>-1</sup> : 3155, 3127, 1598, 1533, 1376, 855
4.1.4. 2-[(4-Ethyl-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

\[
\text{H NMR (DMSO-d}_6, 400 \text{ MHz)}: \delta 1.20 (t, 3H), 2.67 (q, 2H, J=6.8 Hz), 4.77 (s, 2H), 7.31-7.36 (m, 2H), 7.41 (d, 2H, J=8.0 Hz), 7.67-7.69 (m, 2H), 7.77 (d, 2H, J=8.4 Hz), 8.79 (s, 1H)
\]

\[
\text{C NMR (DMSO-d}_6, 100 \text{ MHz)}: \delta 16.86, 26.30, 109.41, 117.13, 121.25, 122.22, 124.09, 124.34, 125.30, 140.93, 141.53, 142.24, 144.99, 147.01, 152.65, 163.75
\]

\[
\text{MS (ESI) m/z: 337 (M+1)^+}
\]

Elemental Analysis: Molecular formula C\textsubscript{18}H\textsubscript{16}N\textsubscript{4}O\textsubscript{3}

Calculated: C, 64.26; H, 4.79; N, 16.65; S, 9.53%

Found: C, 64.27; H, 4.78; N, 16.64; S, 9.52%
4.1.5. 2-[1-(4-Methoxy-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

\[
\text{Yield} : 92\%
\]

Physical appearance : White powder

m. p. : 178-179 °C

R_f : 0.62 (toluene: ethyl acetate: formic acid 5:4:1)

IR (KBr) cm\(^{-1}\) : 3165, 3116, 1468, 1433, 1356

\(^1\)H NMR (DMSO-d\(_6\), 400 MHz) : \(\delta\) 3.82 (s, 3H), 4.76 (s, 2H), 7.11 (d, 2H, \(J=9.2\) Hz), 7.31-7.37 (m, 2H), 7.67-7.69 (m, 2H), 7.77 (d, 2H, \(J=8.8\) Hz), 8.73 (s, 1H)

\(^{13}\)C NMR (DMSO-d\(_6\), 100 MHz) : \(\delta\) 25.62, 52.34, 110.61, 117.23, 118.43, 120.34, 121.35, 125.14, 126.20, 141.93, 142.13, 143.39, 146.31, 152.89, 162.79

MS (ESI) m/z : 339 (M+1)^+

Elemental Analysis : Molecular formula C\(_{17}\)H\(_{14}\)N\(_4\)O\(_2\)S

Calculated : C, 60.34; H, 4.17; N, 16.56; S, 9.48%

Found : C, 60.32; H, 4.18; N, 16.54; S, 9.49%

4.1.6. 2-[1-(3-Chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

\[
\text{Yield} : 94\%
\]

Physical appearance : Brown crystal

m. p. : 203-204 °C

R_f : 0.62 (toluene: ethyl acetate: formic acid 5:4:1)

IR (KBr) cm\(^{-1}\) : 3205, 3150, 1460, 1510, 1260
1H NMR (DMSO-d$_6$, 300 MHz) : δ 4.77 (s, 2H), 7.31-7.36 (m, 2H), 7.51-7.67 (m, 4H), 7.88 (d, 1H, J=7.8 Hz), 8.00 (s, 1H), 8.91 (s, 1H)

13C NMR (DMSO-d$_6$, 75 MHz) : δ 26.72, 110.04, 118.47, 120.75, 121.06, 124.13, 124.41, 128.84, 130.78, 135.51, 137.70, 141.76, 144.64, 152.10, 164.08

MS (ESI) m/z : 343 (M+1)$^+$

Elemental Analysis : Molecular formula C$_{16}$H$_{11}$ClN$_4$OS

Calculated : C, 56.06; H, 3.23; N, 16.34; S, 9.35%

Found : C, 56.04; H, 3.22; N, 16.33; S, 9.34%

4.1.7. 2-{1-(4-Chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl}-benzoxazole

Yield : 90%

Physical appearance : Brown powder

m. p. : 196-197 °C

R$_f$ : 0.68 (toluene: ethyl acetate: formic acid 5:4:1)

IR (KBr) cm$^{-1}$ : 3157, 1886, 1603, 1554, 1398, 924

1H NMR (DMSO-d$_6$, 300 MHz) : δ 4.77 (s, 2H), 7.33-7.36 (m, 2H), 7.64-7.69 (m, 4H), 7.93 (d, 2H, J=9.0 Hz), 8.88 (s, 1H)

13C NMR (DMSO-d$_6$, 75 MHz) : δ 26.65, 110.05, 118.41, 121.07, 121.72, 124.15, 124.41, 129.88, 134.59, 135.35, 141.71, 144.60, 152.07, 164.12

MS (ESI) m/z : 343 (M+1)$^+$

Elemental Analysis : Molecular formula C$_{16}$H$_{11}$ClN$_4$OS

Calculated : C, 56.06; H, 3.23; N, 16.34; S, 9.35%

Found : C, 56.08; H, 3.21; N, 16.35; S, 9.33%
4.1.8. 2-[1-(2-Chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield : 88%
Physical appearance : Yellow powder
m. p. : 190-191 °C
Rf : 0.58 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm\(^{-1}\) : 3139, 3115, 1556, 1500, 1456, 1237, 1137
\(^1\)H NMR (DMSO-\(d_6\), 300 MHz) : δ 4.79 (s, 2H), 7.32-7.35 (m, 2H), 7.53-7.75 (m, 6H), 8.60 (s, 1H)
\(^{13}\)C NMR (DMSO-\(d_6\), 75 MHz) : δ 26.76, 110.01, 118.44, 124.06, 124.35, 125.08, 127.70, 127.89, 128.45, 130.74, 134.74, 141.79, 143.30, 152.06, 164.10
MS (ESI) m/z : 343 (M+1)\(^+\)
Elemental Analysis : Molecular formula C\(_{16}\)H\(_{11}\)ClN\(_4\)OS
Calculated : C, 56.06; H, 3.23; N, 16.34; S, 9.35%
Found : C, 56.04; H, 3.22; N, 16.33; S, 9.31%

4.1.9. 2-[1-(4-Bromo-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield : 86%
Physical appearance : Brown crystals
m. p. : 212-213 °C
Rf : 0.61 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm\(^{-1}\) : 3154, 3116, 1548, 1507, 1454, 1133, 1051
Chapter III

\(^1\)H NMR (DMSO-d\(_6\), 300 MHz) : \(\delta 4.76 (s, 2H), 7.32-7.35 (m, 2H), 7.65-7.68 (m, 2H), 7.78 (d, 2H, \(J=9.0\) Hz), 7.86 (d, 2H, \(J=9.0\) Hz), 8.87 (s, 1H)

\(^{13}\)C NMR (DMSO-d\(_6\), 75 MHz) : \(\delta 26.67, 110.06, 118.43, 120.95, 121.96, 122.49, 124.14, 124.41, 132.87, 135.86, 141.74, 144.69, 152.11, 164.15

MS (ESI) m/z : 387 (M+2)\(^+\), 388 (M+3)\(^+\)

Elemental Analysis: Molecular formula C\(_{16}\)H\(_{11}\)BrN\(_4\)OS

Calculated : C, 49.62; H, 2.86; N, 14.47; S, 8.28%

Found : C, 49.60; H, 2.87; N, 14.48; S, 8.27%

4.1.10. 2-[(4-Fluoro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

![Molecular Structure]

Yield : 92%

Physical appearance : White crystals

m. p. : 194-195 °C

R\(_f\) : 0.66 (toluene: ethyl acetate: formic acid 5:4:1)

IR (KBr) cm\(^{-1}\) : 3154, 3139, 1600, 1549, 1454, 1236, 1131

\(^1\)H NMR (DMSO-d\(_6\), 300 MHz) : \(\delta 4.77 (s, 2H), 7.30-7.36 (m, 2H), 7.40-7.46 (m, 2H), 7.66-7.69 (m, 2H), 7.90-7.94 (m, 2H), 8.81 (s, 1H)

\(^{13}\)C NMR (DMSO-d\(_6\), 75 MHz) : \(\delta 26.74, 110.06, 116.53, 118.45, 121.25, 121.72, 122.55, 122.66, 124.13, 124.40, 141.80, 144.57, 152.15, 164.23

MS (ESI) m/z : 327 (M+1)\(^+\)

Elemental Analysis: Molecular formula C\(_{16}\)H\(_{11}\)BrN\(_4\)OS

Calculated : C, 58.89; H, 3.40; N, 17.17; S, 9.83%

Found : C, 58.87; H, 3.39; N, 17.16; S, 9.84%
4.1.11. 2-(1-o-Tolyl-1H-[1,2,3]triazol-4-ylmethylsulfanyl)-benzoxazole

\[ \text{O} \]
\[ \text{N} \]
\[ \text{S} \]
\[ \text{N} \]
\[ \text{N} \]
\[ \text{N} \]
\[ \text{O} \]

**Yield**: 83%

**Physical appearance**: Yellowish white powder

**m. p.**: 188-189 °C

**R<sub>f</sub>**: 0.66 (toluene: ethyl acetate: formic acid 5:4:1)

**IR (KBr) cm<sup>-1</sup>**: 3144, 3124, 1555, 1505, 1379, 1135, 1100

**<sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)**: δ 2.06 (s, 3H), 4.77 (s, 2H), 7.29-7.34 (m, 2H), 7.38 (d, 2H, J=5.7 Hz), 7.45 (d, 2H, J=5.7 Hz), 7.64-7.67 (m, 2H), 8.48 (s, 1H)

**<sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 75 MHz)**: δ 17.82, 26.83, 110.03, 118.43, 124.09, 124.37, 124.52, 125.95, 126.83, 129.87, 131.47, 133.56, 136.35, 141.79, 143.40, 152.07, 164.28

**MS (ESI) m/z**: 323 (M+1)<sup>+</sup>

**Elemental Analysis**

<table>
<thead>
<tr>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>C, 63.33; H, 4.38; N, 17.38; S, 9.95%</td>
<td>C, 63.32; H, 4.36; N, 17.39; S, 9.93%</td>
</tr>
</tbody>
</table>

4.1.12. 2-[1-(2-Bromo-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

\[ \text{O} \]
\[ \text{N} \]
\[ \text{S} \]
\[ \text{N} \]
\[ \text{N} \]
\[ \text{N} \]
\[ \text{Br} \]

**Yield**: 86%

**Physical appearance**: Yellow flakes

**m. p.**: 202-203 °C

**R<sub>f</sub>**: 0.54 (toluene: ethyl acetate: formic acid 5:4:1)

**IR (KBr) cm<sup>-1</sup>**: 3124, 3125, 1587, 1505, 1457, 1378, 1238, 1155
1H NMR (DMSO-d$_6$, 300 MHz) : \( \delta \) 4.78 (s, 2H), 7.29-7.36 (m, 2H), 7.50-7.58 (m, 2H), 7.61-7.67 (m, 3H), 7.86 (d, 1H, \( J=7.8 \) Hz), 8.54 (s, 1H)

13C NMR (DMSO-d$_6$, 75 MHz) : \( \delta \) 26.80, 110.03, 118.38, 118.47, 124.08, 124.36, 125.15, 128.14, 128.47, 131.15, 133.90, 141.82, 143.25, 152.07, 164.11

MS (ESI) m/z : 387 (M+1)$^+$, 388 (M+3)$^+$

Elemental Analysis : Molecular formula C$_{16}$H$_{11}$BrN$_4$OS

Calculated : C, 49.62; H, 2.86; N, 14.47; S, 8.28%

Found : C, 49.63; H, 2.85; N, 14.46; S, 8.29%

4.1.13. 2-[1-(2-Fluoro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield : 90%

Physical appearance : White powder

m. p. : 187-188 °C

R$_f$ : 0.53 (toluene: ethyl acetate: formic acid 5:4:1)

IR (KBr) cm$^{-1}$ : 3182, 3170, 1595, 1379, 1047

1H NMR (DMSO-d$_6$, 300 MHz) : \( \delta \) 4.81 (s, 2H), 7.31-7.36 (m, 2H), 7.44 (d, 1H, \( J=7.5 \) Hz), 7.52-7.59 (m, 2H), 7.67-7.69 (m, 2H), 7.83 (t, 1H, \( J=7.8 \) Hz), 8.64 (s, 1H)

13C NMR (DMSO-d$_6$, 75 MHz) : \( \delta \) 26.72, 111.02, 116.49, 118.47, 120.36, 121.27, 121.70, 122.54, 122.65, 124.10, 124.42, 141.82, 144.55, 152.18, 164.20

MS (ESI) m/z : 327 (M+1)$^+$

Elemental Analysis : Molecular formula C$_{16}$H$_{11}$FN$_4$OS

Calculated : C, 58.89; H, 3.40; N, 17.17; S, 9.83%

Found : C, 58.87; H, 3.38; N, 17.18; S, 9.82%
4.1.14. 2-[[1-(3,4-Dimethyl-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield: 88%
Physical appearance: Yellow powder
m. p.: 211-212 °C
Rf: 0.71 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm\(^{-1}\): 3145, 3118, 1501, 1454, 1133, 1098, 1045
\(^1\)H NMR (DMSO-\(d_6\), 300 MHz): \(\delta\) 2.27 (s, 3H), 2.30 (s, 3H), 4.76 (s, 2H), 7.30-7.38 (m, 3H), 7.55 (d, 1H, \(J=8.1\) Hz), 7.67 (d, 3H, \(J=6.6\) Hz), 8.72 (s, 1H)
\(^13\)C NMR (DMSO-\(d_6\), 75 MHz): \(\delta\) 19.43, 19.86, 26.85, 110.04, 117.92, 118.45, 121.12, 121.81, 124.07, 124.36, 130.58, 134.84, 137.64, 138.34, 141.83, 144.03, 152.11, 164.33
MS (ESI) m/z: 337 (M+1)\(^+\)
Elemental Analysis: Molecular formula C\(_{18}\)H\(_{16}\)N\(_4\)OS
Calculated: C, 64.26; H, 4.79; N, 16.65; S, 9.53%
Found: C, 64.24; H, 4.78; N, 16.64; S, 9.51%

4.1.15. 2-[[1-(4-tert-Butyl-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield: 88%
Physical appearance: White powder
m. p.: 193-195 °C
Rf: 0.76 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm\(^{-1}\): 3154, 3141, 1555, 1520, 1453, 1378, 1133
$^1$H NMR (DMSO-$d_6$, 300 MHz) : δ 1.31 (s, 9H), 4.77 (s, 2H), 7.32-7.36 (m, 2H), 7.58 (d, 2H, $J$=8.7 Hz), 7.65-7.69 (m, 2H), 7.77 (d, 2H, $J$=8.7 Hz), 8.77 (s, 1H)

$^{13}$C NMR (DMSO-$d_6$, 75 MHz) : δ 26.83, 31.23, 34.76, 110.04, 118.44, 120.33, 121.15, 124.08, 124.37, 126.59, 134.50, 141.81, 144.11, 152.10, 152.22, 164.30

MS (ESI) m/z : 365 (M+1)$^+$

Elemental Analysis : Molecular formula C$_{20}$H$_{20}$N$_4$OS
Calculated : C, 65.91; H, 5.53; N, 15.37; S, 8.80%
Found : C, 65.90; H, 5.54; N, 15.36; S, 8.81%

4.1.16. 2-{[1-(4-Ethoxy-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield : 85%
Physical appearance : White powder
m. p. : 186-187 °C
$R_f$ : 0.80 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm$^{-1}$ : 3155, 3143, 1522, 1498, 1457, 1390, 1251

$^1$H NMR (DMSO-$d_6$, 300 MHz) : δ 1.34 (t, 3H, $J$=6.6 Hz), 4.09 (q, 2H, $J$=6.9 Hz), 4.76 (s, 2H), 7.09 (d, 2H, $J$=9.0 Hz), 7.30-7.38 (m, 2H), 7.66-7.68 (m, 2H), 7.75 (d, 2H, $J$=8.7 Hz), 8.77 (s, 1H)

$^{13}$C NMR (DMSO-$d_6$, 75 MHz) : δ 14.69, 26.84, 63.91, 110.04, 115.25, 118.45, 121.24, 122.24, 124.07, 124.36, 130.22, 132.91, 141.82, 144.04, 152.11, 164.32

MS (ESI) m/z : 353 (M+1)$^+$

Elemental Analysis : Molecular formula C$_{18}$H$_{16}$N$_4$O$_2$S
Calculated : C, 61.35; H, 4.58; N, 15.90; S, 9.10%
Found : C, 61.33; H, 4.57; N, 15.89; S, 9.11%
4.1.17. 2-(1-Pyridin-3-yl-1H-[1,2,3]triazol-4-ylmethylsulfanyl)-benzoxazole

Yield: 84%
Physical appearance: Brown powder
m. p.: 193-194 °C
R<sub>f</sub>: 0.65 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm<sup>-1</sup>: 3155, 3144, 1555, 1505, 1453, 1131, 1095

<sup>1</sup>H NMR (DMSO-<sup>d</sup>6, 300 MHz): δ 4.78 (s, 2H), 7.30-7.33 (m, 2H), 7.59-7.66 (m, 3H), 8.29 (d, 1H, J=7.8 Hz), 8.66 (d, 1H, J=3.9 Hz), 8.29 (s, 1H), 9.10 (s, 1H)

<sup>13</sup>C NMR (DMSO-<sup>d</sup>6, 75 MHz): δ 26.18, 109.61, 118.01, 120.97, 123.75, 123.83, 124.02, 127.63, 133.06, 141.16, 141.24, 144.31, 149.49, 151.59, 163.49

MS (ESI) m/z: 310 (M+1)<sup>+</sup>
Elemental Analysis: Molecular formula C<sub>15</sub>H<sub>11</sub>N<sub>5</sub>OS
Calculated: C, 58.24; H, 3.58; N, 22.64; S, 10.37%
Found: C, 58.22; H, 3.57; N, 22.62; S, 10.36%

4.1.18. 2-[1-(2-Chloro-pyridin-3-yl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole

Yield: 85%
Physical appearance: Yellow powder
m. p.: 183-184 °C
R<sub>f</sub>: 0.54 (toluene: ethyl acetate: formic acid 5:4:1)
IR (KBr) cm<sup>-1</sup>: 3154, 3132, 1571, 1497, 1457, 1135, 1100
Chapter III

\[ ^1 \text{H NMR (DMSO-}d_6, \text{ 300 MHz)} \]:  δ 4.80 (s, 2H), 7.33-7.35 (m, 2H), 7.65-7.71 (m, 3H), 8.21 (d, 1H, J=7.8 Hz), 8.63 (d, 1H, J=3.6 Hz), 8.67 (s, 1H)

\[ ^{13} \text{C NMR (DMSO-}d_6, \text{ 75 MHz)} \]:  δ 26.63, 110.03, 118.47, 123.33, 124.16, 124.42, 124.90, 131.97, 135.84, 141.75, 143.99, 144.81, 150.20, 152.09, 163.94

**MS (ESI) m/z**: 344 (M+1)^+

**Elemental Analysis**

- **Calculated**: C, 52.40; H, 2.93; N, 20.37; S, 9.33%
- **Found**: C, 52.39; H, 2.94; N, 20.35; S, 9.31%

4.2. **Crystallographic study**

Intensity data were collected at 183(2) K on Oxford Xcalibur Sapphire 3 diffractometer (a single wavelength enhance X-ray source with MoK\(\alpha\) radiation, \(\lambda = 0.71073\) Å) [35]. The selected suitable single crystal was mounted using paratone oil on the top of a glass fiber fixed on a goniometer head and immediately transferred to the diffractometer. Pre-experiment, data collection, data reduction and analytical absorption corrections [36] were performed with the Oxford program suite [37] CrysAlisPro. The crystal structures were solved with SHELXS-97 [38] using direct methods. The structure refinements were performed by full-matrix [38] least-squares on \(F^2\) with SHELXL-97. All programs used during the crystal structure determination process are included in the WINGX software [39]. The chemical formula and ring labelling system is shown in Figure 12. Crystal data for compound 6: C\(_{16}\)H\(_{11}\)ClN\(_4\)OS, Mr, 342.80; system, triclinic; space group, P -1; unit cell dimensions, a = 6.7407(3) Å; b = 7.4806(5) Å; c = 15.6537(9) Å; \(\alpha = 78.181(5)^0\); \(\beta = 79.697(4)^0\); \(\gamma = 82.127(5)^0\); \(V = 756.00(7)\)Å\(^3\); \(Z = 2\); T = 298 K; \(R_{int}, 0.0380\); \(R(all), 0.0442\); \(Gof = 1.076; \Delta_{pmax} = 0.23 e Å^3; \Delta_{pmin} = -0.35 e Å^3\). All hydrogen atoms were calculated after each cycle of refinement using a riding model, with C-H = 0.93 Å + \(U_{iso}(H) = 1.2U_{eq}(C)\) for aromatic H atoms, with C-H = 0.97 Å + \(U_{iso}(H) = 1.2U_{eq}(C)\) for methylene H atoms. Crystallographic data for the compound 6 has been deposited with the Cambridge Crystallographic Data Center (CCDC) under the number 950223. Copies of the data can be obtained, free of charge, on application to CCDC 12 Union.
Figure 12. Crystal structure of 2-[1-(3-Chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-benzoxazole(6).
5.0. **Methodology used for the *in silico* molecular docking studies**

5.1. **In silico molecular docking against COX-2**

Crystallized structure of COX-2 protein with reported ligand (Drug celecoxib) was considered for the docking studies. For this purpose we shortlisted Protein 3LN1 from Protein Data Bank as this includes celecoxib drug selectivity bound to binding site of COX. The structure of protein COX-2 along with celecoxib (3LN1) was retrieved from the Protein Data Bank. First protein structure of 3LN1 was imported in Schrodinger using Protein Preparation Wizard. This wizard was used to optimize and minimize the protein structure which involves removing undesirable water molecules and other defects in the target protein. Finally a low energy and structural correct target protein was achieved. This minimized protein was used for further docking analysis. As the target protein had already the site for celecoxib, the grid was generated by selecting the celecoxib ligand as the reference ligand. Finally the grid was validated and was used for further docking with new unknown ligands to predict their docking score. Chemical structures were drawn in maestro and geometrically refined by LigPrep module for Ligand preparation. In this module 2-D structures were converted into 3-D structures, which were further subjected to OPLS-2005 force field to generate single low energy 3-D structure for each input structure. During this step chiralities were maintained. Docking was carried using Glide software. It was carried using Extra precision and write XP descriptor information. This generates favourable ligand poses which are further screened through filters to examine spatial fit of the ligand in the active site. Ligand poses which pass through initial screening are subjected to evaluation and minimization of grid approximation. Scoring is then carried on energy minimized poses to generate Glide score.

5.2. **In silico molecular docking against TNF-α**

Crystallized structure of 2AZ5 was chosen from Protein Data Bank and used as target for molecular docking studies. 2AZ5 structure was reported with the specific ligand celecoxib which inhibits it. 2AZ5 structure was imported in Schrodinger using Protein Preparation Wizard. Missing hydrogen and atoms were added using prime interface. Undesired water molecules were removed. The protein was then optimized and minimized to give low energy and structural correct target protein. As the target
protein had already the site for reference ligand, the grid was generated by selecting the ligand as the reference ligand. Finally the grid was validated and was used for further docking with new unknown ligands to predict their docking score. Chemical structures were drawn in maestro and geometrically refined by Lig Prep module. In this module 2-D structures were converted into 3-D structures, which were further subjected to OPLS-2005 force field to generate single low energy 3-D structure for each input structure. During this step chiralities were maintained. Docking was carried using Glide software. It was carried using Extra precision and write XP descriptor information. This generates favourable ligand poses which are further screened through filters to examine spatial fit of the ligand in the active site. Ligand poses which pass through initial screening are subjected to evaluation and minimization of grid approximation. Scoring is then carried on energy minimized poses to generate Glide score.

6.0. Biological activity

6.1. Anti-inflammatory activity

The synthesized compounds were tested for their in vivo anti-inflammatory activity using carrageenan-induced hind paw edema method. The rat paw edema was induced by subcutaneous injection of 0.1 ml of 1% freshly prepared saline solution [40] of carrageenan into the right hind paw of rats. The standard drug, celecoxib (0.05 mmoles/kg) was given orally as a positive control. The control group was administered orally with 0.9% of 0.1 ml of saline solution only. The test groups were administered orally with equimolar dosage of the synthesized compounds and the standard drug, 1 h before the administration of carrageenan. The paw volumes were measured using plethysmometer [41] at intervals of 3 h and 5 h.

6.2. In vivo IL-1β, TNF-α and COX-2

Levels of the proinflammatory cytokines (IL-1β and TNF-α) [42] and COX-2 [43] in the serum have been determined by using commercially available ELISA kits (eBioscience and Cayman, USA). Assays have been performed in duplicate in accordance with the manufacturer’s guidelines. Cytokine concentrations were expressed as picograms of antigen per millilitre of protein.
6.3. **COX-2 gene expression study**

6.3.1. **Cell culture experiments**

HeLa cells (ATCC) were seeded in 24 well plate 24 hrs before treatment in DMEM containing 10% calf serum (Invitrogen). After 24 hrs cells were treated with compounds 4 & 9 (10 μM) and standard drug, celecoxib (10 μM) as positive control and DMSO-d₆ as negative control, followed by 24 h of incubation of cells in CO₂ incubator at 37 ºC and 5% CO₂.

6.3.2. **RNA extraction, reverse transcription and gene expression analysis**

After 24 hrs cells were scrapped and collected in 1.5 ml micro centrifuge tubes. The total RNA was isolated by TRI Reagent® (Molecular Research Centre). RNA quantity and quality were determined on a NanoDrop ND-2000c spectrophotometer and integrity was checked on a 1.5% agarose gel. Total RNA (1 μg) was used to generate cDNA using an EZfirst strand cDNA synthesis kit for RT (reverse transcription)–PCR (Biological Industries). Primers for real-time PCR were designed for COX-2 and GAPDH using the Pearl Primer software and are listed in Table 8.

<table>
<thead>
<tr>
<th>Table 8. List of primers used.</th>
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<tr>
<td>Gene</td>
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<tr>
<td>COX-2</td>
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<td></td>
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<tr>
<td>GADPH</td>
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Reactions were run at 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Real-time PCR was performed on an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). PCR was performed in triplicate and was repeated
two times for each gene and each sample. Relative transcript quantities were calculated using the Ct method with GAPDH as the endogenous reference gene.

6.4. *Estimation of thiobarbituric acid reactive substances (TBARS)*

The assay of TBARS was done according to earlier method [44] adapted to microtiter plates by bringing the final volume to 150 µL. In brief, hind paw tissue homogenate was prepared in 0.15 M KCl (5% w/v homogenate) and aliquots of 30 µL were incubated for 0°C and 37°C for 1h. Subsequently, 60 µL of 28% w/v TCA was added and the volume was made up to 150 µL by adding 60 µL of distilled water followed by centrifugation at 3000xg for 10 min. The supernatant (125 µL) was taken and colour was developed by addition of 25 µL of 1% w/v TBA dissolved in 0.05 N NaOH and kept in boiling water bath for 15 min. The absorbance was read at 532 nm in a plate reader (Bio-Rad, U.S.A). The result was expressed in µmoles TBARS formed/hr/g tissue using a molar extinction coefficient of 1.56×10^5 M⁻¹ cm⁻¹.

6.5. *Reduced glutathione (GSH)*

GSH level was measured using the method described earlier [45]. Homogenized hind paw tissue (10% w/v in phosphate buffer pH 7.4) was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4°C for 2 h. The contents were centrifuged at 2000xg for 15 min. 50 µL supernatant was added to 200 µL of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA (pH 8.9) followed by the addition 20 µL of 0.01M DTNB. The absorbance was read in a microplate reader at 412 nm and results are expressed as µg GSH/g tissue using a molar extinction coefficient of 13.6×10^3 M⁻¹ cm⁻¹.

6.6. *Measurement of Nitric oxide (NO) level*

Animals were sacrificed and their hind paw tissues were washed with PBS (pH 7.4) and placed on ice as described earlier [46]. 50 µL of the sample was added to 100 µL of Griess reagent and reaction mixture was incubated for about 5-10 minutes at room temperature and protected from light. The optical density was measured at 540 nm in microplate reader according to the reagent manufacturer’s protocol. Calculations were done after generating a standard curve for sodium nitrite in the same buffer as used for preparation of homogenate.
6.7. **In vivo antinociceptive activity**

6.7.1. **Writhing test**

The writhing test in mice was carried out using the method of Koster [47]. The writhes were induced by intraperitoneal injection of 0.6% acetic acid (v/v) (80 mg/kg). The standard drug i.e. celecoxib was given orally at a dose 0.05 mmoles/kg of body weight. The test compounds were administered orally at an equimolar dosage to groups of six animals each, 30 min before chemical stimulus. The numbers of muscular contractions were counted over a period of 20 min after acetic acid injection. The data represents the total number of writhes observed during 20 min and is expressed as writhing numbers.

6.8. **Ulcerogenic activity**

The test compounds having anti-inflammatory and analgesic activities comparable with the celecoxib were further tested for their ulcerogenic risk evaluation [48]. This was done at three times higher dose in comparison to the dose used for anti-inflammatory activity, i.e. 0.15 mmoles/kg body weight of celecoxib and the test compounds were used. Each group had three animals which were later sacrificed after five hours of oral drug administration. When compared with celecoxib, these compounds did not cause any gastric ulceration and disruption of gastric epithelial cells at the above mentioned oral dose. Hence gastric tolerance towards the test compounds was better than that of celecoxib.

7. **Conclusion**

We have synthesized a focussed library of novel bis-heterocycles encompassing 2-mercaptobenzoxazole and 1,2,3-triazole moieties conjugated through a methylene linkage. The crystallographic study of the compound 6 confirmed the formation of final molecule and supported the spectroscopic data. The compounds 4, 5, 7, 9 and 16 exhibiting potent *in vivo* anti-inflammatory and *in vivo* antinociceptive activities have been further screened against COX-2 selectivity and proinflammatory cytokine mediators like TNF-α, IL-1β and NO. The results of the (COX 1 / COX-2) selective index show that the compounds 4 and 9 exhibit a target specific inhibiton of COX-2, which is comparable with the standard drug celecoxib. The results of the *in silico*
molecular docking studies against COX-2 showed that hydrogen bonding and hydrophobic interactions are responsible for the interactions of the synthesized compounds with their corresponding active sites on the target protein. The *in silico* molecular docking study against TNF-α shows that the molecule 8 interacts with Tyr-59 and Tyr-119 amino acid residues on the TNF-α protein. The molecule 10 was found to show the interactions with Tyr-59 and Gly-121 on TNF-α protein. The compounds 4, 5, 7, 9 and 16 did not induce any gastric ulceration thus showing their effective tolerance towards gastric mucosa. Thus these molecules may be used as leads for the development of novel anti-inflammatory drugs which may help in the treatment of inflammatory disorders like rheumatoid arthritis and crohn’s disease.
References


