SYNTHESIS AND BIOLOGICAL STUDIES ON
2-SUBSTITUTED BENZIMIDAZOLE
DERIVATIVES

THESIS

Submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

By

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FEBRUARY, 2017
CHITKARA COLLEGE OF PHARMACY
CHITKARA UNIVERSITY

DECLARATION BY THE STUDENT

I hereby certify that the work which is being presented in this thesis entitled “Synthesis and biological studies on 2-substituted benzimidazole derivatives” is for fulfilment of the requirement for the award of Degree of Doctor of Philosophy submitted in the Faculty of Pharmaceutical Sciences, Chitkara College of Pharmacy, Chitkara University, Punjab is an authentic record of my own work carried out under the supervision of Dr. Sandeep Jain, Associate Professor, Deptt. of Pharmaceutical Sciences, GJUS&T, Hisar and Dr. Sandeep Arora, Professor & Director, Chitkara College of Pharmacy, Chitkara University, Punjab.

The work has not formed the basis for the award of any other degree or diploma, in this or any other Institution or University. In keeping with the ethical practice in reporting scientific information, due acknowledgements have been made wherever the findings of others have been cited.

Ritchu Sethi

Date: Regd. No. CUPB/01/Ph.D./11/09
This is to certify that the thesis entitled “Synthesis and biological studies on 2-substituted benzimidazole derivatives” submitted by Ritchu Sethi, CUPB/01/Ph.D./11/09 to the Chitkara University, Punjab in fulfilment for the award of the degree of Doctor of Philosophy in the Faculty of Pharmaceutical Sciences. is a bonafide record of research work carried out by her under our supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institution or University for the award of any degree or diploma.

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(Ritchu Sethi)
OUTCOMES OF DOCTORAL RESEARCH

➢ List of Publications


➢ Conferences Attended/Presented


• Sethi R, Arora S and Jain S (2015) Synthesis and characterization of 1,2-disubstituted benzimidazole derivatives for potential anti-inflammatory, analgesic and gastroprotective activity. 2nd Annual National Conference of Association of Pharmaceutical Teachers of India, Haryana State Branch, Department of Pharmaceutical Sciences, M.D.University, Rohtak. (Poster Presentation)

• Sethi R (2016) A workshop on conceptualization, innovation, commercialization of IPR and pharma innovations and pharma expo, Chitkara College of Pharmacy, Chitkara University, Punjab.
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<table>
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<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>ADT</td>
<td>AutoDock Tools</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl Formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase 4</td>
</tr>
<tr>
<td>DPPH</td>
<td>Diphenyl picryl hydrazide</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalo Virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>IR</td>
<td>Infra red</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>M. audouinii</td>
<td><em>Microsporum audouinii</em></td>
</tr>
<tr>
<td>MAO</td>
<td>Mono amine oxidase</td>
</tr>
<tr>
<td>MES</td>
<td>Maximum electroshock</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MTT 3</td>
<td>(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic Resonance</td>
</tr>
<tr>
<td>NSAIDS</td>
<td>Non steroidal Anti-inflammatory Drugs</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
</tr>
<tr>
<td>OPD</td>
<td>Ortho Phenylenediamine</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton Pump Inhibitors</td>
</tr>
<tr>
<td>PTP IB</td>
<td>Protein tyrosine phosphatase IB</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazole</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>S. epidermis</td>
<td><em>Staphylococcus epidermis</em></td>
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ABSTRACT

Synthesis of \(N-(\text{Benzimidazol-1-ylmethyl})\)-benzamide and \(N-(\text{Benzimidazol-1-ylmethyl})\)-4-chlorobenzamide derivatives was done by Mannich reaction and evaluated for antimicrobial, antioxidant, anti-inflammatory, analgesic and ulcerogenic activity. The compounds were further characterized by spectral and analytical techniques. The synthesized compounds were screened for \textit{in vitro} antimicrobial activity against gram positive, gram negative bacterial and fungal strains. Antioxidant activity of synthesized compounds was also checked by DPPH method using ascorbic acid as standard drug. Molecular docking studies of synthesized compounds were performed for antimicrobial and anti-inflammatory activity to study drug receptor interaction using docking program AutoDock Vina. From the \textit{in silico} study among series-1 and series-2, 26 hit compounds with docking affinity higher than that of internal ligand SC-558 (-7.6 kcal/mol), were evaluated for anti-inflammatory and analgesic activity. On the basis of efficacy and potency of anti-inflammatory (\(p \leq 0.05\)) and analgesic (\(p \leq 0.01\)) potential, five compounds were selected each from series-1 and series-2 respectively for ulcerogenic activity. These compounds also showed significant (\(p \leq 0.05\)) results for ulcerogenic activity as compared to indomethacin as reference drug. Results revealed that molecules 3c, 3o and 3r from series-1 and 3a’, 3e’ and 3h’ from series-2 were most potent among all synthesized derivatives. Compounds were found to have good correlation with \textit{in-silico} study.
CHAPTER 1
INTRODUCTION

A heterocyclic compound contains a ring having more than one kind of atoms. In numerous of the cyclic compounds that we have seen previously, e.g. benzene, naphthalene, cyclohexanol, the rings are composed entirely of carbon molecules. Such compounds are known as homocyclic compounds. But on that point are also rings which contain, additional to carbon, other kinds of atoms, usually nitrogen, oxygen or sulfur e.g.

\[
\begin{array}{cccc}
\text{Pyrrole} & \text{Pyridine} & \text{Furan} & \text{Thiophene} \\
\end{array}
\]

Heterocyclic compounds are present ubiquitously in the biological universe. Carbohydrates are heterocyclic, and thus are the chlorophyll and heme, which makes leaves green and blood red, as well as fetch life to plants and animals. Heterocycles become the primary site of reaction for many enzymes and coenzymes. It too takes on a major function in heredity as DNA is also composed of many heterocyclic rings. Furthermore, heterocyclic compounds are more biologically energetic as compared to others (Padmavati et al., 2007). Benzimidazole is one such compound, which creates a center of attention of synthetic chemists for scheming other effective benzimidazole compounds with miscellaneous biological actions (Walia et al., 2011).

1.1 Benzimidazole

1.1.1 Chemistry

1.1.1.1 Structure

Benzimidazole is a fused heterocycle of benzene and imidazole nucleus (1) (benzene ring is fused with an imidazole ring at the 4 and 5 positions). It is an aromatic heterocyclic organic compound and establish to be a resourceful scaffold for invention of new drugs in pharmaceutical areas.
Introduction

The benzimidazoles (1) can be numbered as given in the structure below. Rarely the 2-position is nominated as the μ-position.

Furthermore, benzimidazoles display fast prototropic tautomerism having unsubstituted NH group (Fig. 1.1) that directs to equilibrium mixtures of asymmetric derivatives. Today, it is a scaffold of preference which exhibit various biological properties. The principal compound containing benzimidazole nucleus, found naturally is N-ribosyl-dimethylbenzimidazole, is an imperative building block of cyanocobalamine (vitamin B₁₂) (Barker et al., 1960).

![Diagram of benzene, imidazole, and benzimidazole tautomerization](image)

**Fig. 1.1:** Fusion of benzene and imidazole ring and tautomerism in benzimidazole

Literature revealed that they were called “anhydrobases” because these compounds were formed by the removal of water. It was also known that “anhydrobases” of this category were created exclusively by means of such reactants where the N-containing
groups were ortho to each other (Wright, 1951). The benzimidazoles can be recognized as benziminazoles or benzoglyoxalines. In the early literature they were named also as derivatives of $o$-phenylenediamine. So, as per to this nomenclature benzimidazole likely to be named as methenyl-$o$-phenylenediamine, as well as 2-methylbenzimidazole possibly be named as ethenyl-$o$-phenylenediamine.

1.1.1.2 Synthesis

Historically, Hoebrecker (Wright, 1951) synthesized the first benzimidazole. He synthesized 2, 5 (or 2,6) dimethylbenzimidazole (3) through the reduction of 2-Nitro-4-methylacetanilide (2) with Sn/HCl.

\[
\text{Sn} \quad \text{HCl} \quad \text{O} \quad \text{N} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{2} \quad \text{H}_3\text{C} \quad \text{CH}_3 \quad \text{C} \quad \text{H}_3 \quad \text{N} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{H}_2\text{O} \quad \text{N} \quad \text{H} \quad \text{N} \quad \text{C} \quad \text{H}_3 \quad \text{3}
\]

After several years, Ladenburg (Wright, 1951) prepared the similar derivative (3) by the reaction of 3, 4-diaminotoluene and acetic acid.

\[
\text{H}_3\text{C} \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{3,4-diamino toluene} \quad \text{+} \quad \text{CH}_3\text{COOH} \quad \text{H}_3\text{C} \quad \text{NH}_2 \quad \text{NHCOCH}_3 \quad \text{acetic acid} \quad \text{+} \quad \text{H}_2\text{O} \quad \text{CH}_3 \quad \text{3}
\]
1.1.1.2.1 Methods for the synthesis of benzimidazole

- **Reaction with carboxylic acids**

  a) **Mono basic acids** - Ortho-phenylenediamine reacts easily with the majority of carboxylic acids to furnish 2-substituted benzimidazoles, generally with very good quality of yields. Regular preparation of benzimidazoles (1) involves the cyclic condensation of ortho-phenylenediamine among carboxylic acids (formic acid). Ortho-phenylenediamine reacts using formic acid to furnish benzimidazole in a 80% yield when the reaction mixture is heated at 100°C (Furniss *et al.*, 1989).

  \[
  \text{NH}_2\text{N}+\text{HCOOH} \xrightarrow{\text{Reflux}} \text{H}
  \]

  

  \[
  \begin{array}{c}
  \text{H} \\
  \end{array} \\
  \text{N} \\
  \text{N} \\
  \text{benzimidazole}
  \]

  \[
  \text{NH}_2 \text{H}_2\text{N}
  \]

  \[
  \text{o-phenylenediamine}
  \]

  \[
  \text{formic acid}
  \]

  \[
  \text{1}
  \]

  \[
  N\text{-monosubstituted o-phenylenediamines give reaction with additional carboxylic acids (other than formic acid) (4) only by adding hydrochloric or phosphoric acid or with orthophenylene diamine dihydrochloride (Wagner *et al.*, 1939; Furniss *et al.*, 1989).}

  \[
  \begin{array}{c}
  \text{NH}_2 \\
  \text{N} \\
  \text{2HCl} \\
  \end{array} \\
  \text{ortho-phenylenediamine dihydrochloride}
  \]

  \[
  \text{R} \\
  \text{RCOOH} \\
  \text{substituted carboxylic acid}
  \]

  \[
  \text{R+} \text{RCOOH} \xrightarrow{\text{2HCl}} \text{R-2HCl} \xrightarrow{\text{substituted carboxylic acid}} \text{2-substituted benzimidazole}
  \]

  \[
  \text{4}
  \]

  b) **Dibasic acids** – Dibasic acids react with ortho-phenylenediamine and the product prepared (5) depend on the molar ratio of reactants and also upon the experimental conditions. Two or more moles of orthophenylene diamine are made to react with one mole of dibasic acid, mostly the product formed are bis benzimidazoles (Shriner *et al.*, 1941).
**Synthesis and biological studies on 2-substituted benzimidazole derivatives**

**Introduction**

```
\[
\begin{align*}
2 \text{ ortho-phenylenediamine} & + (\text{CH}_2)_n (\text{COOH})_2 \\
\rightarrow & \text{ bisbenzimidazole} \\
\end{align*}
\]
```

- **Reaction with acid anhydrides**

a) **Anhydrides of monobasic acids** – The acid anhydrides react with ortho-phenylenediamine give rise to benzimidazole formation or N,N’ diacyl phenylenediamine (Meyer et al., 1902) formation, depending on the conditions given. It was previously under consideration that ortho-phenylenediamine yields benzimidazole by acids and diacyl derivatives by means of acid anhydrides. Virtually the acid anhydride that was used during the synthesis of benzimidazole was acetic anhydride. However mixture of formic- acetic anhydride and benzoic anhydride have also been used fruitfully for example, ortho-phenylene diamine reacts with acetic anhydride under reflux for several hrs and is absolutely transformed to 2- methyl benzimidazole (6) (Wright, 1951).

```
\[
\begin{align*}
\text{ortho-phenylenediamine} & + 2 (\text{CH}_3\text{CO})_2\text{O} \\
\rightarrow & 2-\text{methyl benzimidazole} \\
\end{align*}
\]
```

---

*Synthesis and biological studies on 2-substituted benzimidazole derivatives*
b) **Anhydrides of dibasic acids** – The anhydrides of dibasic acids behaves as monobasic acids, e.g. Succinic anhydride reacts with ortho-phenylenediamine furnishes β-(2-benzimidazole) propionic acid (7) (Wright, 1951).

\[
\text{ortho-phenylenediamine} + \text{succinic anhydride} \rightarrow \text{3-(1H-Benzimidazol-2-yl)-propionic acid 7}
\]

**• Reaction with esters**

Nientkowski (Wright, 1951) was the first to investigate the reaction of esters and ortho-phenylenediamine to provide benzimidazole. 3,4-diaminotoluene dihydrochloride and ethyl formate in equimolar amounts, when heated in a potted tube for a period of 3 hrs at 225°C, gives 84% yield of 5 or 6 methyl benzimidazole hydrochloride (8).

\[
\text{3,4-diamino toluene dihydrochloride} + \text{ethyl formate} \rightarrow \text{5-methyl benzimidazole hydrochloride 8}
\]

**• Reaction with Amides**

Comparatively a small number of amides have been used for the synthesis of benzimidazole. Equimolar amount of 3, 4 diaminotoluene dihydrochloride and benzamide were heated at 240-250°C, furnish an approximately quantitative yield of 2-phenyl-5-methyl benzimidazole (9). In most cases fine yields have been obtained (Wright, 1951).
Introduction

Synthesis and biological studies on 2-substituted benzimidazole derivatives

- Reaction with Lactones

Bistrzycki and Schmutz (Wright, 1951) first investigated the reaction of lactones with ortho-phenylene diamine. Investigation was done on numerous γ-lactones of phenol acids and alcohol acids. Buterolactone on reaction with ortho-phenylenediamine under reflux gives merely a miniature yield of 1,2-(1-methyl trimethylene) benzimidazole (10)

- Reaction with Sodium bisulphite adduct

Equimolar amount of sodium bisulfite adduct of substituted aromatic aldehyde (2mmol) and ortho-phenylenediamine (OPD) (2mmol) were heated in dimethyl formamide (DMF) at 140°C for 4 hrs. The product thus obtained was 2-substituted benzimidazole (11) with substituted aromatic moieties (Devmurari et al., 2010). In the majority of cases excellent yields have been obtained.
• Reaction with urea

\( o\)-phenylenediamine dihydrochloride on reaction with urea at 130ºC yields 2-(3H)-benzimidazolonone (12).

\[
\begin{align*}
\text{NH}_2 & \quad .2\text{HCl} \quad + \quad \text{NH}_2\text{CONH}_2 \\
\text{o-phenylenediamine & dihydrochloride} \quad \text{urea} \quad \text{2-(3H)-benzimidazolonone}
\end{align*}
\]

1.1.1.3 Properties of Benzimidazole

• Solubility

Benzimidazoles with hydrogen at 1-position are generously soluble in polar solvents and solublity in organic solvents to a smaller extent. Benzimidazole solublizes in hot water but insoluble in benzene and only scarcely soluble in ether. The solubility of benzimidazole in nonpolar solvents is increased by introducing other nonpolar substituents in different positions of the benzimidazole ring; e.g. 2-methylbenzimidazole is easily soluble in ether because of the appearance of a methyl moiety at 2 position of benzimidazole. On the other side, the addition of polar functional groups in the molecule enhances solubility in polar solvents; so, 2-aminobenzimidazole is soluble in water. Benzimidazoles are faintly basic, and to some extent less basic than the imidazoles. Therefore, they are mostly soluble in dilute acids. The comparative basicity of the benzimidazole ring was examined by a tortuous qualitative process in which spectrum of a cyanine dye containing the benzimidazole ring as a component was compared with cyanine dyes derived from other basic substances. The procedure requires the determination of the variation in \( \lambda_{\text{max}} \), which was due to the basicity of the heterocyclic moiety. Benzimidazoles are moreover satisfactorily acidic and usually soluble in aqueous alkali and produce N-metallic compounds. Like imidazoles, the acidic characteristics of the benzimidazoles (Remick, 1949), seems to be due to resonance stabilization.
• **Stability**

The benzimidazole ring is extremely stable as it is not borne on by conc. H$_2$SO$_4$ whilst heated underneath pressure at 270°C (Wright, 1951). It remains unaffected by the action of hot HCl and by strong bases. Cleavage of benzimidazole takes place only by oxidation under drastic conditions. Likewise, the benzimidazole scaffold is somewhat defiant to reduction; though, tetrahydro and hexahydro benzimidazoles may be synthesized involving reduction of benzene ring by using appropriate catalyst.

• **Spectral Properties**

Infra red (IR) spectroscopy: The absorption spectra of benzimidazole appears for N-H stretching (near 3107 cm$^{-1}$), C-N stretching (near 1690 cm$^{-1}$), C-H aromatic stretching (near 2850 cm$^{-1}$).

Nuclear magnetic resonance (NMR) spectroscopy: The chemical shift value for benzimidazole aryl ring appears between δ 7-9 ppm when compared with TMS as internal standard.

$^{13}$C NMR: The spectra shows different carbon peaks in the range of δ 115-144 ppm.

• **Chemical Reactions**

A) **Reactions involving benzimidazole ring:**

• Reactions involving 1 and 3 positions: They readily convert into salts when react with acids it forms monohydrochloride, monoacetate, monopicare and mononitrate. They also undergo alkylation with alkyl halides producing 1-alkyl benzimidazoles and produces 1,3-dialkylbenzimidazolium halides (13) under more vigorous conditions. Benzimidazoles also react with grignard reagents and acylating agents.
They also form manich base by reacting with formaldehyde and primary or secondary amine (Selvam et al., 2010).

- **Hydrogenation and dehydrogenation reactions**: As benzimidazole ring is very stable to reduction, 2-phenyl benzimidazole is converted into 2-cyclohexylbenzimidazole. Saturation of only olefinic linkage at 2-position of benzimidazole (14) took place in 2(4-dimethylaminostyryl)-benzimidazole by hydrogenation with nickel at atmospheric pressure.

![Chemical structures](image)

- **Halogenation**: Reaction of 2,5 (or 2,6)-dimethylbenzimidazole with saturated solution of bleaching powder under acidic conditions at 0-5ºC gives 1-chloro-2,5(or 2,6)-dimethylbenzimidazole (15).

- **Nitration**: The nitration of benzimidazole motif takes place readily. Nitration takes place at 5 or 6 position of benzimidazole nucleus. However, if 5 or 6 position of benzimidazole is blocked, it would take place at 4 or 7 position.

### B) Reactions involving substituent groups:

- **Reaction involving 2-benzimidazole carboxylic acids**: 2-benzimidazole carboxylic acid on heating undergo decarboxylation and yields benzimidazole (1).
Introduction

Synthesis and biological studies on 2-substituted benzimidazole derivatives

- **Reaction of 2-(α-haloalkyl)benzimidazoles:** 2-(α-chloroisopropyl)benzimidazole when refluxed with pyridine in dry ethanol gives a high-quality yield of 2-(α-ethoxyisopropyl)benzimidazoles (16).

- **Reaction of 2-(3H)-Benzimidazolones:** 2-chloro derivatives (17) can be synthesized by treating 2-(3H)-benzimidazolone with phosphorous oxychloride or phosphorous pentachloride.

- **Reaction of 2-(3H)-Benzimidazolethiones:** 2-mercapto benzimidazoles are usually stable and alkylation occurs rapidly with substitution of the SH to give S-alkylated analogues (18), and several molecules has been synthesized till now.
• **Reaction of 2-Aminobenzimidazoles:** 2-aminobenzimidazole on reaction with acetic anhydride gives 2-acetyl aminobenzimidazole (19).

\[
\text{NH}_2 + \text{(CH}_3\text{CO})_2\text{O} \rightarrow \text{NHCOCOCH}_3
\]

2-acetylaminobenzimidazole 19

• **Oxidation:** Benzimidazoles are stable to oxidation. Under strong conditions of oxidation like KMNO\(_4\) in the presence of hot basic solution, it is somewhat achievable to oxidize benzimidazoles to achieve a little quantity of imidazole dicarboxylic acid. Since the benzimidazole ring is stable to oxidation, so it is achievable to oxidize substituent group devoid of disturbing the ring. Through the oxidation of the substituent groups several benzimidazole carboxylic acids derivatives have been prepared.

1.1.2 Biological Profile

Benzimidazole is a resourceful heterocyclic scaffold in medicinal compounds, exhibiting a broad spectrum of pharmacological activities. Furthermore, benzimidazoles are structural isosters of nucleotides occurring in nature that allows them to simply work together with the enzymes of the biological system owing to their numerous biological activities.

The literature reveals that benzimidazole-containing compounds show biological activities as anti-allergic agents (Raj et al., 2012), antimicrobial (Deep et al., 2014), antioxidant (Mentese et al., 2015), PARP inhibitors- as anticancer agents (Penning et al., 2009) and as cytomegalovirus (HCMV) inhibitors (Evers et al., 2004), Antiulcer (Bariwal et al., 2008), anti-inflammatory (Mariappan et al., 2015) and as antihistaminic (Wang et al., 2012). Because of their miscellaneous uses, medicinal chemists categorize by calling them as “privileged substructures” for designing of new drug (Evans et al., 1988; Mason et al., 1999).

Approximately all benzimidazole derivatives which differ in their functional substituents, leads to necessary alteration of the physicochemical and pharmacokinetic parameters of these drugs. For last some decades, benzimidazole derived compounds have accomplished a lot of attention owing to their chemotherapeutic standards. Research revealed that 2-substituted benzimidazole hybrids are biologically more
proficient and therefore 2-substituted benzimidazoles serve as the potential areas of drug design and discovery (Preston, 1980; Foks et al., 2006; Ansari et al., 2009).

Large number of benzimidazole derivatives are in market like thiabendazole, flubendazole (Anthelmintic), Enviradine (antiviral), omeprazole, rabeprazole, lansoprazole (antiulcer), telmisartan, candesarten cilexitil (antihypertensives), astimizole (antihistaminic) and several derivatives in a variety of additional curative sections (Gurvinder et al., 2013).

1.2 Mannich bases

1.2.1 Chemistry

Mannich bases are the beta amino ketone hauling compounds and are the last part of mannich reaction (Belinelo et al., 2002). Mannich reaction is a carbon carbon bond building nucleophilic addition reaction that includes condensation of a compound with active hydrogen, with an amine (primary or secondary) and formaldehyde (non enolizable aldehyde). This reaction is valuable for synthesizing N-methyl derivatives and several drug molecules. Mannich reaction has been examined by numerous groups of coworkers in the area of medicinal chemistry, predominantly owing to various pharmacological properties held by Mannich Bases.

Study on the chemistry of mannich bases was first done by Mannich, has been the focus of investigations by an eternally escalating number of researchers (Blicke, 1942; Hellmann and Opitz, 1956; Zaugg and Martin, 1965). Numerous studies that appeared prior to 1960 jointly with books by Reichert (Reichert, 1959) and by Hellman and Opitz (Hellmann and Opitz, 1960) afforded admirable exposure on basically the whole chemistry of mannich bases upto 1960. Furthermore, in last decades various researchers have studied the plentiful applications of mannich reaction, its mechanism and the reactivity of bases which allows the production of several other products (Thompson, 1968; Miocque, 1969). The enormous attention in the chemistry of mannich bases has been fundamentally motivated by two facts:

1. The mannich synthesis introduces a basic function which can make the molecule aqueous soluble when it is transformed into the aminium salt.

2. Mannich bases are very imprudent as they can be changed into various supplementary products. The reactivity of the bases accounts for numerous different biological properties.
1.2.1.1 *Synthesis*

Mannich amino methylation (20) consist of the condensation of substrate having at least one active hydrogen with formaldehyde (or infrequent other aldehydes) and a primary of secondary amine (Blicke, 1942; Smith and March, 1985).

**Mannich Reaction:**

\[
\text{active hydrogen compound} + \text{formaldehyde} + \text{secondary amine} \rightarrow \text{Mannich Base}
\]

- **Reactants and reaction conditions** (Tramontini, 1973)
  - Most commonly used aldehydes are formaldehyde either as paraformaldehyde, trioxymethylene or formalin.
  - The amines can be used as such or as hydrochlorides.
  - Generally ethanol is used as solvent. Other solvents used are- methanol, Isopropanol, water and acetic acid.

It is hard to provide general rules regarding the choice of reagents and reaction conditions (Thompson, 1968), though the most extensively and fruitfully used reaction conditions for diverse substrates are as follows:

- **Alkyl ketones:** Substrate, amine hydrochloride and paraformaldehyde (sometimes 1, 3, 5-trioxan or formalin) are refluxed in alcoholic solvents for a number of hours.

- **Phenols:** Substrate, amine and aqueous formaldehyde are permissible to heat for short time period or permitted to place at room temperature for many days.

- **Heterocyclic Compounds:** Substrate, amine and aqueous formaldehyde are allowed to react in water or alcoholic solvents at room temperature, sometimes with heating.

- **Carboxylic acid derivatives:** Substrate, amine and formalin are made to react at room temperature in water or in alcoholic solvents.
Alkynes: Different reaction conditions as on top are used. The reaction takes place in the presence of Cu$^{2+}$ salts.

The reaction is usually conceded out by addition of substrate, aldehyde and amine in equimolar amount.

An assessment of this system explains that formaldehyde, an electrophile undergo reaction with two nucleophiles: The amine and a carbonic center resulting from the active hydrogen containing compound. At the termination of the reaction, an amino methyl group usually substitutes the active hydrogen atom and products of condensation are recognized as ‘Mannich Bases’.

There has been a large amount of disagreement around the mechanism of the mannich reaction, principally as to whether the aldehyde is primarily reacted with the active hydrogen compound or by the ammonia or amine. The evidence appears to prefer the later path. (Cummings and Shelton, 1960).

1.2.2 Biological Profile

A variety of Mannich Bases have been described to possess analgesic (Datar and Limaye, 2015), anti-inflammatory (Kumar et al., 2015; Koksal et al., 2007), anticancer (Ivanova et al., 2007; Megally Abdo, 2015), anticonvulsant (Vashishtha et al., 2004), antiviral (Edwards et al., 1983) anthelmintic (Bennet-Jenkins et al., 1996) antimalarial (Barlin and Jiravinya, 1990), antibacterial (Ashok et al., 2007), antifungal (Singh et al., 2007; Abrao et al., 2015) as well as several other activities. The most primitive models including Mannich reactions were reported in series by Tollens and Co-workers, Petrenko Kritschenko et al. and by Mannich and Krosche. Furthermore Mannich was the originator to identify the reaction and a detailed investigation began in 1917 (Thompson, 1968). The interest in Mannich reaction and Mannich Bases have been increasing since 1903 owing to the following facts. Firstly, appearance of a basic function which can give the molecule in aqueous solvent. The Mannich Bases being very reactive is without difficulty converted into numerous other compounds. The responsiveness of these bases accounts for their several pharmacological properties. Subjects on the chemistry of Mannich Bases are of interest in various arenas of application. A huge figure of amino alkyl derivatives have been developed in array to show a relationship between their structure and biological activities. Carboxylic acid derivatives containing nitrophenyl moeity, esters, amides, a number of five membered...
Introduction

and six membered heterocyclic compounds, acyclic carboxamides have been reported to condense with ammonia, primary, secondary, cyclic amine or their salts in the presence of formaldehyde or paraformaldehyde.

The examples of medicinaly valuable mannich bases that consists of amino alkyl chain are cocaine, atropine, fluoxetine, trihexiphenidyl, procyclidine, ranitidine, biperiden and many more (Kashiyama et al., 1999; Racane, 2001; Bhusare, 2001).
CHAPTER 2
LITERATURE REVIEW AND OBJECTIVES

2.1 Review of literature

Benzimidazoles are well reputable in literature as imperative biologically active heterocyclic compounds. Derivatives of benzimidazoles are the center of attention of several research studies owing to their prevalent potential biological actions. Literature assessment exposed that 2-substituted benzimidazole derivatives acquire assorted biological activities.

2.1.1 Biological Profile of Benzimidazoles

2.1.1.1 Anti-inflammatory

Managing inflammation is a major concern because it gives rise to several diseases like coronary artery disease, alzheimer’s disease, asthma, gout, multiple sclerosis, degenerative joint disease, rheumatoid arthritis, carcinoma, diabetes mellitus, viral or bacterial infections (Medzhitov, 2010; Grivennikov et al., 2010). Several inflammatory mediators are involved like histamine, prostaglandins, serotonin, plasma proteases, nitric oxide, leukotrienes, tumor necrosis factor-α, interleukins and chemokines (Nathan, 2002). These mediators are formed by a mixture of procedures which involves certain enzymes like cyclooxygenases, cyclin dependent kinases, Janus kinases etc (Bhagwat, 2009; Dinarello, 2010). Research is still going on new anti-inflammatory compounds containing benzimidazole nucleus. Nevertheless, a great number of researchers have enclosed a variety of derivatives of benzimidazole having moderate to outstanding anti-inflammatory action but at present none of the molecule of this category has been in the market. Numerous compounds showing anti-inflammatory activity, considering kinases as target are presently under clinical trials (Sondhi et al., 2002).

Derivatives of 2-phenyl substituted benzimidazole (1) were made by Leonard et al. and demonstrated its anti-inflammatory potential. Synthesized compounds showed significant protection at 50 mg/kg of dose with p< 0.05 level. Out of four compounds, one compound displayed greatest (54.6%) inhibition of paw edema (Leonard et al., 2006).
Synthesis of novel derivatives of 2-substituted benzimidazoles (2) was done by Mariappan et al. by reacting substituted aromatic primary amines along with 2-chloromethyl benzimidazole. The furnished target derivatives were further tested for anti-inflammatory and analgesic potential. The majority of them showed significant effects in a 100mg/kg p.o. dose at p< 0.05 level (Mariappan et al., 2015).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R_1</th>
<th>R_2</th>
<th>R_3</th>
<th>R_4</th>
</tr>
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<tbody>
<tr>
<td>A</td>
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<td>Cl</td>
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<td>H</td>
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<tr>
<td>B</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>C</td>
<td>NO_2</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>D</td>
<td>H</td>
<td>H</td>
<td>NO_2</td>
<td>H</td>
</tr>
<tr>
<td>E</td>
<td>H</td>
<td>NO_2</td>
<td>H</td>
<td>H</td>
</tr>
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<td>H</td>
<td>Cl</td>
<td>H</td>
<td>NO_2</td>
<td>H</td>
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<tr>
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<td>H</td>
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<td>H</td>
</tr>
<tr>
<td>J</td>
<td>H</td>
<td>H</td>
<td>I</td>
<td>H</td>
</tr>
</tbody>
</table>

2.1.1.2 Diuretic

Srinivasan et al. synthesized benzimidazole containing substituted quinazolinone derivatives (3). Out of the synthesized derivatives, significant diuretic action was shown by compound a and b at dose levels of 50mg/kg and 100mg/kg of body weight. Hydrochlorothiazide was used as reference drug for respective study (Srinivasan et al., 2008).
Antimicrobial agents comprise an assorted collection of chemical substances performing against several forms of germs including bacteria, fungi, helminths, protozoa and viruses. The bulk of the research in improvement of antimicrobials containing benzimidazole moiety has been carried out over the year 2000. Suppression of bacterial nucleic acid and protein synthesis is the likely mechanism by which benzimidazole shows their antibacterial action. This capability of benzimidazole inhibiting microbes is because of their structure is comparable with the purine present in bacterial DNA (Arjmand et al., 2005; Spasov et al., 1999).

Synthesis of some newer benzimidazole derivatives (4) was reported in a research. Three analogues were evaluated for their antibacterial action in opposition to three bacterial strains: S. aureus, B. pumillus and P. Aeurugenosa. Out of three compounds, one showed greater antibacterial activity with MIC (6.25) at 100 µM/ml (Leonard et al., 2006).

Derivatives of 2,3,4-trisubstituted-1,2-dihydropyrimido[1,2-a]-benzimidazoles (5) was synthesized by Deshmukh et al. The prepared derivatives were demonstrated for antifungal action against A. niger and P. chrysogenum by the use of Greiseofulvin as a control (Deshmukh et al., 2009).
2.1.1.4 Antiviral

The current work has discovered that a number of benzimidazole hybrids might show effective anti-HBV (Hepatitis B virus) activity along with extremely little cytotoxicity. The possible antiviral activity is due to their occurrence as 1,3-dideazapurine derivatives and possibly substitute the actual base during the formation of nucleosides in viral replication.

A novel class of 2-aryl benzimidazole derivatives (6) were prepared by Vitale et al., which showed inhibition against pestivirus, flevivirus, picoviridae, reteroviridae, reoviridae, herpesviridae and Poxviridae (Vitale et al., 2008).

Synthesis of 2-(alkylthio) and 2-(benzylthio) derivatives of benzimidazole (7) has been done by Devivar et al.
The synthesized derivatives were screened for antiviral action against HSV-1 and HCMV. Few compounds showed significant results as compared to acyclovir, gancyclovir and foscarnet as standard drugs employed in the respective study (Devivar et al., 1994).

2.1.1.5 Antitumour

Malignant neoplastic disease is the chief health danger which is distressing an extensive preponderance of the planet's inhabitants. A variety of antitumor agents (also referred as anticancer, antineoplastics, antiproliferative) having different mechanisms has been covered for treatment of various forms of neoplasms. However, the most significant side issue related to these agents are toxic towards normal cells owing to no selectivity towards cancerous cells. Hence the seek for anticancer agents is a continuous process for respective years. Benzimidazole is an isoster of the purine which is present in nucleic acid and an imperative pharmacophore in a range of biologically active substances. It is extensively explored for advancement of novel anticancer agents.

Synthesis of some new benzimidazole-4,7-diones (8) derivatives was done by Gellis et al. Amongst synthesized derivatives, three compounds showed tremendous cytotoxic action in opposition to lung (A549), breast (T47D) and colon (HT29) cancer cell lines and least IC₅₀ values of 3µM were described when compared to mitomycin C (Gellis et al., 2008).

\[
\begin{align*}
R_1 &= -\text{CH}_2\text{Cl}, -\text{CH}_2\text{SO}_2\text{C}_6\text{H}_5 \\
8
\end{align*}
\]

2.1.1.6 Antiulcer

The peptic ulcer along with associated diseases include an extended range of clinical defects starting from strong smoldering ache to rigorous impediments like profound ulcers. The stomach is a special organ of gastrointestinal tract that stores and digests the food. The substantial studies (Wolfe et al., 1988) concerning acid secretion showed that proton pump, an imperative mediator of acid secretion, is located in parietal cells in the gastric mucous membrane. On activation, proton pump undergoes a variety of
morphological changes, including oxygen utilization, which brings out acid secretion. The initial therapeutic target was the H₂ receptor and the subsequent therapeutic target accepted was the gastric (H⁺/K⁺)-ATPase known as proton pump. As proton transfer through the (H⁺/K⁺)-ATPase is the last stride in release of acid, it was assumed that drugs depleting this target might become efficient antagonist of acid discharge (Sachs et al., 1995). These compounds are known as proton pump inhibitors (PPI), furthermore they work by forming irreversible covalent disulphide (-S-S-) bonds among enzyme and cysteine of H⁺ /K⁺ -ATPase pump of parietal cell of stomach. As inhibition is irreversible, consequently a fresh enzyme have to be produced to recommence release of acid in stomach. In 1989, Omeprazole was introduced as the foremost therapeutically valuable drug of this category (Lindberg et al., 1990), which contains a benzimidazole nucleus. The additional frequently utilized PPIs are lansoprazole, rabeprazole, pantoprazole and timoprazole. One common thing between them is that all have a benzimidazole core.

Bariwal et al. prepared novel series of pyrimidyl-thio-methyl (9) and pyrimidyl-sulfinyl-methyl (10) derivatives of benzimidazole. The synthesized series was checked for the non-ulcerogenic potential. Synthesized compounds (9) and (10) at two doses (10 and 30 mg/kg) decreased the creation of ulcer appreciably as compared to reference (Omeprazole). From the study it was established that the target compound (10) (sulfinyl derivative) was extra efficient than compound (9) with thio group (Bariwal et al., 2008).
2.1.1.7 Antioxidant

Free radicals e.g. hydroxyl (OH·), nitrous oxide (NO·), superoxide (O₂·) and peroxy (RO₂·) radicals significantly participate in human ailments together with coronary artery disease, rheumatoid arthritis and carcinogenesis (Rice-Evans et al., 1991). Attack of free radical triggered antioxidant guard system, simultaneously superoxide dismutase, glutathione-GSH, catalase, ascorbate and Vitamin E are also activated. So there is an asymmetry among the antioxidant and free radical creation that furnish a variety of diseases together with autoimmune ailment (Aruoma and Haliwell, 1998). The compounds having antioxidant potential are believed to be applied for the avoidance or management of ailments.

Synthesis of 6-flouro-5-substituted benzimidazole (11) derivatives was reported and evaluated for antioxidant activity by Alagoz and Coban. Many compounds were found to be active antioxidants having consequence on superoxide anion at 10⁻³ M conc. (Alagoz and Coban, 2004).

\[ \text{R}=\text{4-CH}_3\text{C}_7\text{H}_{10}\text{N, 4-C}_6\text{H}_5\text{C}_4\text{H}_9\text{N}_2; \text{R}_1=\text{H, Br, }\text{-OCH}_3 \]

2.1.1.8 Anti-diabetic

Diabetes mellitus is a disarray of metabolism which is indicated by elevated blood strength per unit area owing to insulin confrontation and virtual insulin insufficiency. The most predominant type of diabetes mellitus is NIDDM and the chief imperative management of NIDDM is dealing with the levels of blood glucose.

Kumar and Rao synthesized a sequence of new benzimidazole derivatives (12). Compounds have exposed to anti-diabetic activity in opposition to DPP-IV and PTP-IB. Two compounds showed inhibitory action against PTP-IB at 30µM doses and one showed inhibitory action in opposition to DPP-IV at 0.3 µM doses (Kumar and Rao, 2006).
2.1.1.9 Analgesic

Sondhi et al. synthesized novel series of pyrimidobenzimidazoles derivatives (13) by the reaction of \( o \)-phenylenediamines and 3-isothiocyanatobutanal. A compound containing \( R_1 = \text{NO}_2, R_2 = \text{H} \) showed momentous analgesic and anti-inflammatory action when administered at 50mg/kg p.o. Ibuprofen has been considered as reference drug. The rest of the target synthesized compounds showed less analgesic and anti-inflammatory actions (Sondhi et al, 2002).

\[
\text{\begin{figure}[H]
  \centering
  \includegraphics[width=0.5\textwidth]{figure13.png}
  \caption{Compounds 13.}
\end{figure}}
\]

2.1.1.10 Anthelmintic

Solominova et al. synthesized 2-substituted benzimidazole carbamic acid methyl ester derivatives (14). Out of synthesized derivatives, two compounds showed promising anthelmintic activity in opposition to \textit{Nippostrongilus}, \textit{Ankilostoma} and \textit{Haemonhus} larvae with 2.5-50 mg/kg dose (Solominova et al., 2004).

\[
\text{\begin{figure}[H]
  \centering
  \includegraphics[width=0.5\textwidth]{figure14.png}
  \caption{Compounds 14.}
\end{figure}}
\]
2.1.1.11 Anticonvulsant

New pyrrolobenzimidazole derivatives (15) was synthesized and reported by Chimrri et al. Maximum electroshock method was used to determine anticonvulsant activity. One compound showed greatest anticonvulsant activity (Chimrri et al., 2001).

\[
\begin{align*}
R_1 &= \text{Cl, F, H}, R_2 = \text{C}_6\text{H}_5, \text{CH}_3, \\
R_3 &= \text{H}
\end{align*}
\]

15

Shukla et al. synthesized amino/iminomethyl derivatives of benzimidazole (16) and has been demonstrated for monoamine-oxidase inhibitory and neuropharmacological actions. Many derivatives of this category exhibited anticonvulsant, CNS stimulant and MAO (monoamine-oxidase) inhibitory activities (Shukla et al., 1982).

\[
\begin{align*}
R &= \text{H, Cl, F}, R_1 = \text{H, CH}_3, \text{C}_2\text{H}_5 \\
R_2 &= \text{H, C}_6\text{H}_5
\end{align*}
\]

16

A series of benzimidazole containing thiourea scaffolds (17) were prepared by Siddiqui and Alam.

\[
R = \text{H, Cl} \\
R' = \text{C}_6\text{H}_5, 2\text{-CH}_3\text{C}_6\text{H}_5, 3\text{-CH}_3\text{C}_6\text{H}_5, 4\text{-CH}_3\text{C}_6\text{H}_5, \\
2\text{-OCH}_3\text{C}_6\text{H}_5, 3\text{-OCH}_3\text{C}_6\text{H}_5, 4\text{-OCH}_3\text{C}_6\text{H}_5
\]

17
All the prepared derivatives were tested for anticonvulsant activity in zip Maximum electroshock method and sc PTZ model. Further phenytoin was used as a reference drug. Many derivatives explored considerable activity in opposition to both the animal models (Siddiqui and Alam, 2010).

2.1.2 Biological Profile of Mannich Bases

The interest in Mannich reaction and Mannich Bases have been increasing since 1903 owing to the following facts. Firstly, appearance of a basic function which can give the molecule in aqueous solvent. The Mannich Bases being very reactive is without difficulty converted into numerous other compounds. The most primitive models including Mannich reactions were reported in series by Tollens and Co-workers, Petrenko Kritschenko et al. and by Mannich and Krosche. Furthermore Mannich was the originator to identify the reaction and a detailed investigation began in 1917 (Thompson, 1968).

The responsiveness of these bases accounts for their several pharmacological properties. Subjects on the chemistry of Mannich Bases are of interest in various arenas of application. A huge figure of amino alkyl derivatives have been developed in array to show a relationship between their structure and biological activities. Carboxylic acid derivatives containing nitrophenyl moeity, esters, amides, a number of five membered and six membered heterocyclic compounds, acyclic carboxamides have been reported to condense with ammonia, primary, secondary, cyclic amine or their salts in the presence of formaldehyde or paraformaldehyde.

A variety of Mannich Bases have been described to possess analgesic (Datar and Limaye, 2015), anti-inflammatory (Kumar et al., 2015; Koksal et al., 2007), anticancer (Ivanova et al., 2007; Megally Abdo and Kamel, 2015), antiviral (Edwards et al., 1983), anticonvulsant (Vashishtha et al., 2004), antifungal (Abrao et al., 2015; Singh et al., 2007), anthelmintic (Bannet and Bryant, 1996), antimalarial (Barlin and Jiravinya, 1990), antibacterial (Ashok et al., 2007) and several other activities.

The examples of medicinally valuable mannich bases that consists of amino alkyl chain are cocaine, atropine, fluoxetine, trihexphenidyl, procyclidine, ranitidine, biperiden and many more (Racane et al., 2001; Kashiyama et al., 1999; Bhusare et al., 2001).
2.1.2.1 Analgesic and Anti-inflammatory agents

A report has revealed the synthesis of derivatives of 5-nitro-3-substituted piperizinomethyl-2-benzoxazolinones was done by Koksal et al. Mannich bases, (18-a-d) were evaluated for the analgesic action by p-benzoquinone induced writhing test. Among all the prepared derivatives, compounds containing electron withdrawing groups at para position showed excellent analgesic activity (Koksal et al., 2007).

Mannich base derivatives with indole scaffold were synthesized by reacting indole derivatives with a variety of aromatic amines in the presence of formaldehyde and dimethylformamide (19). The entire prepared target compounds were demonstrated by Lohitha et al. for anti-inflammatory activity. It has been reported that synthesized derivatives c, f and g possessed greater anti-inflammatory activity (Lohitha et al., 2011).

A sequence of 4-[(4-aryl methyledine] amino-2-(substituted-yl-methyl)-5-[1-[4-(2-methylpropyl)-phenyl]-ethyl]triazole-3-thiones (20) has been synthesized by Sujith et al. via mannich reaction. Further, the synthesized derivatives were screened for anti-inflammatory action. Among the prepared derivatives, molecules b, f, k and l containing morpholino and methyl piperizino moieties were established to be most active agents (Sujith et al., 2009).
2.1.2.2 Antimicrobial agents

Shivananda and Prakash synthesized mannich bases of 3-substituted mercaptotriazoles (21) and the series was demonstrated for antifungal action using *C. albicans* by disc diffusion method taking fluconazole and nitrofurazone as reference drugs. The outcomes revealed that compounds c, d, f, h, j and n were found to be more active than fluconazole (Shivananda and Prakash, 2011).

A fresh sequence of mannich bases of benzamide (22) were prepared and evaluated by Bala *et al.* for antibacterial activity in opposition to *S. aureus, E. coli, E. faecalis* and *P. aeruginosa*. Serial two fold dilution technique was used for determination of MIC of synthesized target compounds. Cefixime and amoxicillin were used as standard drugs. From the study, it was found that compounds e, f and g were highly active among all the synthesized compounds when compared with amoxicillin and cifixime (Bala *et al.*, 2014).
A new series of 2-(phenyl)-2-(morpholine-4-yl)-N-phenylacetamide (2a-g) \((23)\) mannich bases were prepared and evaluated by Idhayadullah et al. for antimicrobial activity against bacterial strains namely \textit{S. epidermis}, \textit{K. pneumonia} and fungal strains namely \textit{M. audouinii}, \textit{C. albicans}. Clotrimazole and ciprofloxacin were used as reference drugs for antifungal and antibacterial actions respectively. Results revealed that compounds 2c, 2e and 2f had shown good antibacterial activity against \textit{S. epidermis} and \textit{K. pneumonia} as compared to ciprofloxacin. Compounds 2d and 2e had shown equipotent antifungal action against both the fungal strains when compared with clotrimazole as reference drug. However compound 2c has not shown any antifungal effect (Idhayadhulla et al., 2011).

A fresh series of mannich bases of thiomethylpyrimidyl triazoles \((24)\) was prepared by Lingappa et al. and then checked for antibacterial action in opposition to various bacterial strains \textit{E. coli}, \textit{P. aeruginosa}, \textit{S. aureus}, and \textit{S. marcescens}. All the prepared derivatives showed good antibacterial action in opposition to \textit{P. aeruginosa} and found to be less potent against rest of the strains. Among all the synthesized molecules, the compounds substituted with nitro group were established to be highly active (Lingappa et al., 2008).
2.1.2.3 Anticonvulsants

Two series of mannich bases of 1,5 benzodiazepines (25) were synthesized and demonstrated by Pandeya and Rajput for anticonvulsant action. Out of all the prepared derivatives, compounds 1a and 2a were established to be most effective when compared with isoniazid as standard drug (Pandeya and Rajput, 2012).
A new sequence of mannich bases of lamotrigine (26) were prepared and evaluated by Kulkarni et al. for anticonvulsant activity by maximal electroshock (MES) convulsion technique. Lamotrigine and phenobarbitone sodium were used as standard drugs. Results revealed that among the prepared molecules, compounds d and f showed good anticonvulsant activity when compared with reference drug. (Kulkarni et al., 2017).

2.1.2.4 Anticancer agents

New sequence of mannich bases of 2-propoxybenzylidene isonicotinohydrazide (27) were prepared and were evaluated by Malhotra [a] et al. for antitumour activity in opposition to A549 human lung cancer cell lines. MTT assay was used to evaluate anticancer activity and gemcitabine was used as reference drug. Amongst all the synthesized derivatives, compounds c and k exhibited maximum cytotoxic activity as compared to reference drug (Malhotra [a] et al., 2012).
2.1.2.5 Antioxidant

Synthesis of ten novel manich bases (28) was done by Malhotra [b] et al. using (E)-2-{-2-(2,4-dinitrophenyl)hydrazono[methyl]phenol, as an intermediate as well as checked for antioxidant activity. Among all the synthesized derivatives, compounds e, h, i were found to be most potent compounds. (Malhotra [b] et al., 2012).

![Image of Compound 28]

Jagadish et al. synthesized a fresh sequence of manich bases of pyrazolines (29-a-e) and tested for antioxidant potential by NO radical and DPPH radical scavenging techniques. Ascorbic acid and rutin were employed as reference drugs. The results suggested that compounds d and e showed maximum antioxidant activity in comparison to standard drugs (Jagadish et al., 2013).

![Image of Compound 29]

Synthesis of two Mannich bases 1-(1H-benzimidazo-1-yl) methyl urea (BIUF) (30) and 1-(3-Hydroxynaphthlen-2-yl) methyl thiourea (TNTUF) (31) was done by Chakkarvarthi et al. and screened for antioxidant and antimicrobial activity. Hydrogen peroxide radical scavenging, DPPH radical scavenging, and reducing power assays were performed for antioxidant activity evaluation. Ascorbic acid was taken as
reference drug. Both the mannich bases were found to be effective antioxidant and antimicrobial agents because of the presence of electron releasing amide group in them. Also BIUF was found to be more dynamic than TNTUF owing to the presence of N-atoms in benzimidazole adjoining amide group (Chakkarvarthi et al., 2013).

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Synthesis of some new mannich bases of oxadiazole containing benzodioxan moiety (32) was done by Ma et al. Further, all the synthesized derivatives were demonstrated for in vitro antioxidant potential by DPPH, ABTS and FRAP scavenging methods. BHT and trolox were taken as reference drugs. All the synthesized compounds showed good antioxidant results. Amongst all the synthesized derivatives, compound e and f with floroo substitution on phenyl ring showed potent antioxidant action in all the three scavenging techniques (Ma et al., 2013).

2.1.2.6 Anthelmintic agents

Raju et al. synthesized new eight acetohydrazide mannich bases (33) taking different secondary amines like morpholine, piperidine, N-methyl piperazine. All the prepared derivatives were evaluated for anthelmintic action in opposition to P. posthuma. Reference drug used was piperazine citrate. Among all the derivatives, compound 1h was found to be most active (Raju et al., 2014).
2.1.2.7 Antimycobacterial agents

Hybrids of oxadiazole and manich bases (34) were prepared by Ali and Shaharyar and checked for their antimycobacterial action using *M. tuberculosis* H37Rv and isoniazid defiant *M. tuberculosis* by agar dilution methodology. Amongst all the prepared molecules, derivative d was established to be most effective compound in opposition to *M. tuberculosis* H37Rv and isoniazid resistant *M. tuberculosis* having MIC of 0.1µM and 1.10µM respectively (Ali and Shaharyar, 2007).

A fresh series of pyrazoline containing manich bases (35-a-j) were prepared and demonstrated by Taj et al. for antimicobacterial action against *M. tuberculosis* H37Rv.
Reference drugs used were streptomycin and pyrazinamide. The results revealed that compounds c, d, e, g and i showed potent activity. It may be due to the presence of long alkyl chains and electron releasing groups attached to pyrazoline nucleus (Taj et al., 2011).

\[
\text{\text{compound \quad R \quad R'}}
\]
\[
c \quad \text{t-butyl} \quad \text{H}
d \quad \text{n-butyl} \quad \text{H}
e \quad \text{NH} \quad \text{NH} \\
g \quad \text{n-pentyl} \quad \text{H}
i \quad \text{N-O} \quad \text{H}
\]

2.1.3 Mannich base derivatives of benzimidazoles

2.1.3.1 Analgesic and anti-inflammatory

Mohan Rao et al. prepared novel derivatives of [1-(N,N-disubstituted)amino methyl-2-(2,4-dinitrophenyl) sulphanyl]-6-substituted-1H-benzimidazoles (36) by manich reaction of substituted-1H-benzimidazoles with paraformaldehyde and appropriate secondary amines and in the presence of conc. hydrochloric acid and ethanol was used as solvent. The prepared series was further examined for anti-inflammatory and analgesic potential (Mohan Rao et al., 2013).

Mariappan et al. prepared a novel series of 2-ethyl benzimidazole derivatives (37) by the reacting benzimidazole, formaldehyde and primary or secondary amine. The prepared derivatives were assessed for anti-inflammatory as well as analgesic action and several compounds showed effective anti-inflammatory along with analgesic potential (Mariappan et al., 2011).
Novel series of N-mannich bases of benzimidazole (38) were prepared by mannich base reaction by Jesudason et al. All the prepared scaffolds were examined for both analgesic as well as anti-inflammatory activities. Some synthesized derivatives were even more effective than diclofenac and paracetamol. Furthermore, all the synthesized compounds exhibited high-quality of corneal incursion (Jesudason et al., 2009).

Reddy prepared Mannich bases of 2-substituted benzimidazoles (39) by reacting amino phenyl acetic acid and orthophenylene diamine, they were further reacted with formaldehyde and secondary amine (dimethyl amine) and demonstrated for anti-inflammatory activity (Reddy, 2010).
Srivastava et al. reported a novel sequence of 2-phenylhydrazinomethyl (40) and 2-(2-hydroxyphenyl)-benzimidazole (41) derivatives and substitution was done at N1-position of benzimidazole motif. In addition to synthesis, synthesized molecules were also evaluated for analgesic potential. A few of the compounds exhibited potential analgesic activity in comparison to reference drug diclofenac sodium. The inclusion of a phenylhydrazinomethyl moiety at 2-position of benzimidazole compound increases biological activity (Srivastava et al., 2013).

\[
\begin{align*}
R &= \text{Diethylamine, piperazine, piperidine, morpholine, dimethylamine} \\
40 & \\
41
\end{align*}
\]

A series of mannich bases of benzimidazole with substituted phenylpyrazolidindione (42) were prepared by Tiwari and Singh and screened as antinociceptive agents using Eddy’s hot plate and acetic acid-induced writhing models. A number of compounds exhibited potential analgesic action in comparison to standard drug Diclofenac sodium (Tiwari and Singh, 2014).

\[
\begin{align*}
R_1 &= \text{dimethylamine, piperidine, diphenylamine, diethylamine, morpholine} \\
R_2 &= \text{H, 2-methyl} \\
42
\end{align*}
\]
N-Mannich base derivatives of benzimidazole (43) were prepared by Madhavi et al. Synthesized analogues were checked for antibacterial & antifungal potential by cup plate method and also assessed for anti-inflammatory action (Madhavi et al., 2013).

A novel series of mannich bases 1-(N-substituted amino)methyl]-2-substituted benzimidazole derivatives (44) were prepared by Datar and Limaye and were evaluated for analgesic activity. Several compounds exhibited potential analgesic action in comparison to diclofenac sodium which was taken as reference drug (Datar and Limaye, 2015).

2.1.3.2 Antimicrobial

Mannich base of benzimidazole and salicylic acid (45) was synthesized by Patel and Singh by reacting benzimidazole, 4-amino salicylic acid and formaldehyde in ethanol.
Further complexes of mannich bases by means of transition metals were also synthesized. Synthesized compounds were screened for antifungal activity (Patel and Singh, 2009).

Synthesis of 1,2-disubstituted benzimidazole derivatives (46) was completed by Anil, 2010 by means of mannich base reaction. The product was first prepared by reacting orthophenylenediamine and phenylglycine in acidified ethanol then substituted benzimidazole was reacted with secondary amine along with formaldehyde. The target compounds were screened for antimicrobial activity (Anil, 2010).

New N-mannich base derivatives of 3,4 dihydropyrimidine-2-(1H)-one (47) was prepared by reacting active hydrogen compound, heterocyclic amines and formaldehyde by Shah et al. Results revealed that all the synthesized scaffolds exhibited significant antimicrobial activities against two fungal (A. niger, C. albicans) and two bacterial strains (E. coli, B. subtilis) (Shah et al., 2009).
Kumar et al synthesized a new sequence of manich bases of 2-substituted benzimidazole derivatives (48) and all the structures were analysed by various spectral techniques. The preface in vitro antibacterial and antifungal screening outcomes of novel benzimidazole derivatives [(A-G) a,b] exhibited superior to reasonable antimicrobial activity. The compound E (a) and (b) was having wide range of antibacterial activity and antifungal activity. Highly efficient compounds were established to be non-toxic (Kumar et al., 2013).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (a)</td>
<td>-H</td>
<td>-CH3</td>
<td>-CH3</td>
</tr>
<tr>
<td>A (b)</td>
<td>-H</td>
<td>-C2H5</td>
<td>-C2H5</td>
</tr>
<tr>
<td>B (a)</td>
<td>-CH3</td>
<td>-CH3</td>
<td>-C2H5</td>
</tr>
<tr>
<td>B (b)</td>
<td>-CH3</td>
<td>-C2H5</td>
<td>-C2H5</td>
</tr>
<tr>
<td>C (a)</td>
<td>-C2H5</td>
<td>-CH3</td>
<td>-CH3</td>
</tr>
<tr>
<td>C (b)</td>
<td>-C2H5</td>
<td>-C2H5</td>
<td>-C2H5</td>
</tr>
</tbody>
</table>
New mannich Schiff bases of 2-phenylbenzimidazole (49) were prepared by Misra et al. Anthranilic acid was made to react with alkyl amide by means of refluxing to form 2-alkyl-4-(3H)-quinazolinone which further endure mannich reaction. All the entire prepared derivatives showed superior effectiveness as antimicrobial agents (Misra et al., 2010).

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>D (a)</td>
<td>-C₆H₄(2-OH)</td>
<td>-CH₃</td>
</tr>
<tr>
<td>D (b)</td>
<td>-C₆H₄(2-OH)</td>
<td>-C₂H₅</td>
</tr>
<tr>
<td>E (a)</td>
<td>-C₆H₄(2-OH)(5-SO₂OH)</td>
<td>-CH₃</td>
</tr>
<tr>
<td>E (b)</td>
<td>-C₆H₄(2-OH)(5-SO₂OH)</td>
<td>-C₂H₅</td>
</tr>
<tr>
<td>F (a)</td>
<td>-COOH</td>
<td>-CH₃</td>
</tr>
<tr>
<td>F (b)</td>
<td>-COOH</td>
<td>-C₂H₅</td>
</tr>
<tr>
<td>G (a)</td>
<td>-C₆H₄(2-COOH)</td>
<td>-CH₃</td>
</tr>
<tr>
<td>G (b)</td>
<td>-C₆H₄(2-COOH)</td>
<td>-C₂H₅</td>
</tr>
</tbody>
</table>

Cyclization reaction was done to synthesize mannich base derivatives of benzimidazoles (50) by Saraswathi et al. All the prepared scaffolds were screened for antimicrobial activities and a few of them showed reasonable antibacterial and none of the compound was found to be active in opposition to fungal strains (Saraswathi et al., 2010).
Mannich bases of benzimidazoles (51) were prepared by taking some secondary amines and formaldehyde by Afaf et al. Compounds were evaluated for gram positive (B. cereus) and gram negative (E. coli) bacterial strains, yeast (S. cerevisae) and fungus (A. niger). Few compounds showed good actions against bacterial strains but no one was effective under antifungal action (Afaf et al., 2000).

Aanadhi et al. developed a novel sequence of mannich bases of benzimidazoles (52-54) from o-phenylenediamine in two steps through benzimidazole intermediates. The prepared derivatives were screened for antifungal activity in opposition to fungal strains specifically, Aspergillus niger, and Candida albicans and antibacterial activity in opposition to B. subtilis, S. aureus, E. Coli, and S. Typhi by serial two fold dilution process. Among all the synthesized derivatives, molecules 52, 53 and 54 showed excellent antibacterial action and compound 52 exhibited good antifungal activity in comparison to others (Aanadhi et al., 2013).
Synthesis of novel Mannich base cyclization derivatives of benzimidazole was done by Ur Rehman et al. in the course of three-element condensation reaction of 2-aminobenzimidazole, formaldehyde and primary amines. The transition metal complexes of resulting mannich bases were also prepared. Furthermore mannich bases (55) along with their metal complexes were assayed in opposition to different pathogens by means of MIC method. All the synthesized derivatives and their metal complexes exhibited good activity in opposition to various bacterial strains (Ur Rehman et al., 2014).

Mannich bases of 2-phenyl substituted benzimidazoles (43) were prepared by Madhavi et al. All the synthesized hybrids were checked for antibacterial & antifungal actions by cup plate method and also demonstrated for anti-inflammatory action (Madhavi et al., 2013).
Synthesis of novel β-naphthol Mannich bases with benzimidazole and \( p \)-toluidine was done by Chakkarvarthi et al. through condensation reaction with suitable aldehydes. The compounds (56) and (57) were prepared and were further evaluated for antibacterial and antifungal action in opposition to various bacterial and fungal strains. Amongst the prepared target motifs, TNBIF exhibited enhanced antimicrobial and antioxidant activity in comparison to TNPTB (Chakkarvarthi et al., 2014).

New mannich base derivatives of benzimidazole (58) were prepared by Rehman et al. by the condensation of benzimidazole derivative with formaldehyde and primary or secondary amine. Complexes of Mannich bases with copper, cobalt, nickel and Zinc were also synthesized. The synthesized library was checked for \emph{in-vitro} antimicrobial action in opposition to a variety of bacterial and fungal strains. Nearly all the derivatives exhibited potent activity in opposition to microorganisms. Furthermore the synthesized molecules were also screened for their cytotoxicity studies and results revealed that only Ni complexes showed some cytotoxicity while all other compounds were almost inactive (Rehman et al., 2013)
2.1.3.3 Antiviral

Selvam et al. synthesized some N-Mannich bases of benzimidazole (59) derivatives by reacting formaldehyde, 2-substituted benzimidazole and active hydrogen compounds (sulphadimidine, sulphamethoxazole, sulphanilamide, 2-amino pyrimidine, pthalimide, benzamide, anthranilic acid and 2-mercaptobenzimidazole). They were evaluated for anti HIV and antiviral activity and all the compounds showed significant activity (Selvam et al., 2010).

2.1.3.4 Antioxidant

Chakkaravarthi et al synthesized two Mannich bases 1-(1H-benzimidazolyl)methyl urea (BIUF) (30) and 1-(3-Hydroxynaphthlen-2-yl)methyl thiourea (TNTUF) (31) were prepared and screened for antioxidant and antimicrobial activity. Hydrogen peroxide radical scavenging, DPPH radical scavenging and reducing power assays were performed for antioxidant activity evaluation. Ascorbic acid was taken as reference drug. Both the mannich bases were found to be effective antioxidant and antimicrobial agents because of the presence of electron releasing amide group in them. Also BIUF was found to be more dynamic than TNTUF owing to the presence of N-atoms in benzimidazole adjoining amide group (Chakkaravarthi et al., 2013).
Synthesis of novel β-naphthol Mannich bases with benzimidazole and p-toluidine was done by Chakkarvarthi et al. through condensation reaction with suitable aldehydes. The compounds 3-((phenyl-(p-tolylamino)methyl) naphthalene-2-ol (TNPTB) (56) and 3-((1H-benzo[d]imidazole-1-yl)methyl)-naphthalene-2-ol (TNBIF) (57) were prepared and were further evaluated for antibacterial and antifungal action in opposition to various bacterial and fungal strains. The prepared derivatives were also checked for antioxidant activity. Amongst the synthesized scaffolds, TNBIF exhibited enhanced antimicrobial and antioxidant activity in comparison to TNPTB (Chakkarvarthi et al., 2014).

2.1.3.5 Anthelmintic

Rita and Shrivastava synthesized N-Mannich bases of benzimidazolyl substituted 1H-isoindole-1-(2H)-dione (60). All the prepared target scaffolds were demonstrated for anthelmintic action by Watkins method, in opposition to common Indian earthworm *P. posthuma*. Piperazine hydrochloride was taken as reference drug. All the title compounds showed noteworthy anthelmintic action whereas the compounds substituted with piperazine, morpholine, diphenylamine, chloro, nitro and dinitro groups exhibited superior activity than rest of the derivatives (Rita and Shrivastava, 2012).
2.1.3.6 Anticancer

New two sequences of manich bases of benzimidazole (61, 62) were prepared by Kaur et al. The structures were recognized by IR spectral analysis and $^1$HNMR spectrum. Sulfordamine B (SRB) assay was performed in vitro for screening of anticancer activity of synthesized compounds. Cytotoxicity of compounds was demonstrated for lung, prostrate, colon and breast tissue. Some of the synthesized compounds showed significant results (Kaur and Wakode, 2015).

2.1.3.7 Antitubercular

Two diverse sequence of benzimidazolyl trizole (63 and 64) derivatives were prepared by Maste et al. Prepared scaffolds were demonstrated for anti-tubercular and antimicrobial actions. It has been revealed that majority of the synthesized molecules explored potential anti tubercular activity as compared to reference drug streptomycin, as well as also showed good antimicrobial activities (Maste et al., 2011).
2.2 Research Envisaged and Objectives

Looking into the above findings together with diverse biological activities exhibited by 2- substituted benzimidazoles and increased activity by the formation of mannnich bases showed the path to commence the synthesis of derivatives of 2-substituted benzimidazoles via mannnich reaction, represented by following general structures (3a-3x) and (3a’-3m’), with the objectives that these compounds might serve as better anti-inflammatory, analgesic, non-ulcerogenic, antioxidant and antimicrobial agents. The novel scaffolds would also be screened by in silico method (Molecular Docking Studies) to study drug receptor interaction between novel compounds and target protein.
2.3 Plan of Work

This project was undertaken to synthesize some new derivatives of 2-substituted benzimidazole. The work was planned in the following steps:

- Synthesis of new derivatives of 2-substituted benzimidazole via mannich reaction.
- Characterization of synthesized compounds by determining the physico-chemical properties (melting point, solubility, Rf value) and by spectral analysis (IR, NMR and Mass spectra).
- Screening of synthesized scaffolds by computational methods (Molecular Docking studies).
- Biological evaluation of synthesized compounds.
  - Antimicrobial activity
  - Antioxidant activity
  - Anti-inflammatory, Analgesic and Ulcerogenic activity
CHAPTER 3
SYNTHESIS AND CHARACTERIZATION

3.1 General schemes for the synthesis of compounds

SERIES-1 Synthesis of N-(Benzimidazol-1-ylmethyl)-benzamide derivatives

STEP-1 Synthesis of 2-substituted benzimidazoles

[Scheme-(1a-1e)]

\[
\text{o-phenylenediamine} + \text{HCOOH} \xrightarrow{\text{Reflux}} \text{benzimidazole} 2a
\]

Scheme 1a: Synthesis of benzimidazole using o-phenylenediamine

\[
\text{o-phenylenediamine dihydrochloride} + \text{RCOOH} \xrightarrow{} \text{2-substituted benzimidazole}
\]

<table>
<thead>
<tr>
<th>Compound code</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>2b</td>
<td>CH₃</td>
</tr>
<tr>
<td>2c</td>
<td>CH₂Cl</td>
</tr>
<tr>
<td>2d</td>
<td>C₃H₅</td>
</tr>
<tr>
<td>2e</td>
<td>C₄H₇</td>
</tr>
</tbody>
</table>

Scheme 1b: Synthesis of 2-substituted benzimidazoles using o-phenylenediamine dihydrochloride

\[
\text{o-phenylenediamine} \xrightarrow{\text{CNBr}} \text{2-amino benzimidazole} 2g
\]

Scheme 1c: Synthesis of 2-amino benzimidazole using o-phenylenediamine

<table>
<thead>
<tr>
<th>Compound code</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>2f</td>
<td>C₄H₉</td>
</tr>
<tr>
<td>2h</td>
<td>CH(OH)CH₃</td>
</tr>
<tr>
<td>2j</td>
<td>CH₂SH</td>
</tr>
<tr>
<td>2v</td>
<td>CH₂C₆H₅</td>
</tr>
</tbody>
</table>
Synthesis and Characterization

Scheme 1d: Synthesis of 2-mercapto benzimidazole using o-phenylenediamine

Scheme 1e: Synthesis of 2-substituted benzimidazole using o-phenylenediamine

STEP-2 Synthesis of N-(Benzimidazol-1-ylmethyl)-benzamide derivatives

[Scheme-2]
Synthesis and Characterization

<table>
<thead>
<tr>
<th>R</th>
<th>R</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>H</td>
<td>3i</td>
</tr>
<tr>
<td>3b</td>
<td>CH₃</td>
<td>3j</td>
</tr>
<tr>
<td>3c</td>
<td>CH₂Cl</td>
<td>3k</td>
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<td>3d</td>
<td>C₂H₅</td>
<td>3l</td>
</tr>
<tr>
<td>3e</td>
<td>C₃H₇</td>
<td>3m</td>
</tr>
<tr>
<td>3f</td>
<td>C₄H₉</td>
<td>3n</td>
</tr>
<tr>
<td>3g</td>
<td>SH</td>
<td>3o</td>
</tr>
<tr>
<td>3h</td>
<td>CH(OH)CH₃</td>
<td>3p</td>
</tr>
</tbody>
</table>

Scheme 2: Synthesis of mannich bases from 2-substituted benzimidazoles

SERIES-2 Synthesis of N-(Benzimidazol-1-ylmethyl)-4-chlorobenamide derivatives

[Scheme-3]

2-substituted benzimidazole + HCHO + formaldehyde $p$-chloro benzamide

\[
\text{Reflux} \quad \text{C}_2\text{H}_5\text{OH},\text{HCl} \quad \text{Mannich Base} \quad 3a'-m'
\]

<table>
<thead>
<tr>
<th>R</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a'</td>
<td>CH₂Cl</td>
</tr>
<tr>
<td>3b'</td>
<td>C₆H₅</td>
</tr>
<tr>
<td>3c'</td>
<td>3-NC₃H₄</td>
</tr>
<tr>
<td>3d'</td>
<td>2-OH-C₆H₄</td>
</tr>
<tr>
<td>3e'</td>
<td>2-Cl-C₆H₄</td>
</tr>
<tr>
<td>3f'</td>
<td>3-Cl-C₆H₄</td>
</tr>
<tr>
<td>3g'</td>
<td>4-Cl-C₆H₄</td>
</tr>
<tr>
<td>3h'</td>
<td>2-Br-C₆H₄</td>
</tr>
<tr>
<td>3i'</td>
<td>3-Br-C₆H₄</td>
</tr>
<tr>
<td>3j'</td>
<td>4-Br-C₆H₄</td>
</tr>
<tr>
<td>3k'</td>
<td>4-NO₂-C₆H₄</td>
</tr>
<tr>
<td>3l'</td>
<td>4-F-C₆H₄</td>
</tr>
<tr>
<td>3m'</td>
<td>2-NH₂-C₆H₄</td>
</tr>
</tbody>
</table>

Scheme 3: Synthesis of mannich bases from 2-substituted benzimidazoles
3.1.1 Synthesis

Chemicals used in synthesis were of AR/LR grade, procured from Qualigens, LobaChemie, S.D. Fine Chemicals and Sigma Aldrich and were purified whenever necessary, employing usual literature methods. Melting points were determined using the melting point apparatus (PERFIT) in open capillary tubes and were uncorrected.

TLC helped in analyzing the purity of synthesized novel compounds on precoated silica gel G F\textsubscript{254} plates with visualization by iodine vapor/UV chamber. The molecular structure of the newly synthesized compounds was characterized by FTIR, \textsuperscript{1}H NMR, \textsuperscript{13}C NMR and Mass spectrometric techniques. The FTIR spectra were obtained from a Perkin Elmer Spectrum Version 10.03.08 using KBr disc method for the preparation of the sample. The NMR spectra were obtained using a Bruker Avance II 400 MHz spectrometer in deuterated dimethylsulfoxide as the solvent, and the chemical shifts, δ, were recorded in parts per million (ppm) downfield from tetramethylsilane (Me\textsubscript{4}Si) as the internal standard. The coupling constant (J) was expressed in Hz. The mass spectra were obtained on an Applied Biosystems API 2000TM mass spectrometer with ESI positive mode. The molecular ion peaks and the base peaks were identified by mass to charge (m/z) vs percentages of peak abundances.

All the spectras were recorded at Panjab University, Chandigarh.

3.1.1.1 General method for the synthesis of N-(Benzimidazol-1-ylmethyl)-benzamide derivatives (SERIES-1)

The title compounds were prepared by the following steps.

STEP 1

a) Synthesis of benzimidazole [2a]

Unsubstituted benzimidazole was synthesized by the reaction of \textit{o}-phenylenediamine with formic acid by the method described in the literature (Furniss et al., 1989).

\textit{o}-phenylenediamine (0.025 mole, 2.7gm) was added to 90% formic acid (0.034 mole, 1.6 ml). The reaction mixture was heated on the water bath at 100°C for 2 hours. NaOH solution (10%) was gradually added to the cooled reaction mixture, until the mixture became distinctly basic. The product was filtered off at the pump, washed thoroughly with water and recrystallized it from 10% aqueous ethanol.
b) Synthesis of 2-substituted benzimidazoles from \textit{o}-phenylenediamine dihydrochloride [2(b-f), 2h, 2j, 2v]

2-substituted benzimidazoles were synthesized by the reaction of \textit{o}-phenylene diamine dihydrochloride with substituted carboxylic acid by the method described in literature (Furniss \textit{et al.}, 1989).

\textit{o}-phenylenediamine dihydrochloride (0.01 mole, 1.81gm), substituted carboxylic acid (0.03 mole), 20 ml of water was taken and refluxed for 4-6 hrs. Concentrated ammonia solution was added slowly, until the reaction mixture is basic to litmus. Precipitates were collected and re-crystallized it from 10 percent ethanol. Quantities of substituted carboxylic acid are given in \textbf{Table 3.1}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Compound.} & \textbf{R} & \textbf{Substituted carboxylic acid} & \textbf{Quantity Taken} \\
\hline
2b & CH$_3$ & Acetic acid & 1.7 ml \\
\hline
2c & ClCH$_2$ & Chloroacetic acid & 2.8 gm \\
\hline
2d & C$_2$H$_5$ & Propionic acid & 2.2 ml \\
\hline
2e & C$_3$H$_8$ & Butyric acid & 2.8 ml \\
\hline
2f & C$_4$H$_{10}$ & Pentanoic acid & 3.3 ml \\
\hline
2h & CH(OH)CH$_3$ & Lactic acid & 2.2 ml \\
\hline
2j & SHCH$_2$ & Thioglycolic acid & 2.1 ml \\
\hline
2v & CH$_2$C$_6$H$_5$ & Phenylacetic acid & 4.1 gm \\
\hline
\end{tabular}
\caption{Quantity of substituted carboxylic acid taken:}
\end{table}

c) Synthesis of 2-amino benzimidazole [2g]

Solution of cyanogen bromide (0.034 mole, 3.60 gm) was added in miniature amount to a solution of \textit{o}-phenylenediamine (0.034 mole, 3.67gm) in water (40 ml). The reaction mixture was then cooled at room temperature and stirred for 36 hrs. The solution was set aside overnight. The reaction mixture was made basic by gradual addition of sodium hydroxide (0.034 mole, 1.36 gm) and the resulting solution was evaporated. The crude product was filtered and recrystallized from boiling water (Simonov \textit{et al.}, 1979).
d) **Synthesis of 2-mercapto benzimidazole [2i]**

A mixture of of o-phenylenediamine (0.1 mole, 10.8 gm), KOH (0.1 mole, 5.61gm) and CS$_2$ (0.1 mole, 6.04 ml) and 15 ml of water was refluxed for 3 hrs in 100 ml of 95% ethanol. Then 1-1.5 gm of charcoal was added and the reaction mixture was refluxed for additional 10 minutes. Charcoal was removed by filtration and the filtrate was heated to 60-70º C. Then 100ml of water was added and acidified with dilute acetic acid with stirring. Product separated as white crystals (Wang *et al*., 2007). The product was recrystallized with ethanol.

Physical data of synthesized compounds is given in **Table 3.2**.

**Table 3.2: Physical data of synthesized compounds (2-substituted benzimidazoles-aliphatic series)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Melting Point(ºC)</th>
<th>Yield (%)</th>
<th>R$_f$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>H</td>
<td>C$_7$H$_6$N$_2$</td>
<td>118.14</td>
<td>171-172</td>
<td>65</td>
<td>0.43</td>
</tr>
<tr>
<td>2b</td>
<td>CH$_3$</td>
<td>C$_8$H$_8$N$_2$</td>
<td>132.16</td>
<td>176-177</td>
<td>54.5</td>
<td>0.29</td>
</tr>
<tr>
<td>2c</td>
<td>ClCH$_2$</td>
<td>C$_8$H$_7$ClN$_2$</td>
<td>166.61</td>
<td>146-148</td>
<td>59.3</td>
<td>0.53</td>
</tr>
<tr>
<td>2d</td>
<td>C$_2$H$_5$</td>
<td>C$<em>9$H$</em>{10}$N$_2$</td>
<td>146.19</td>
<td>175-176</td>
<td>53.4</td>
<td>0.44</td>
</tr>
<tr>
<td>2e</td>
<td>C$_3$H$_8$</td>
<td>C$<em>{10}$H$</em>{12}$N$_2$</td>
<td>160.22</td>
<td>150-153</td>
<td>62.5</td>
<td>0.63</td>
</tr>
<tr>
<td>2f</td>
<td>C$<em>9$H$</em>{11}$</td>
<td>C$<em>{11}$H$</em>{14}$N$_2$</td>
<td>174.24</td>
<td>146-147</td>
<td>42.5</td>
<td>0.49</td>
</tr>
<tr>
<td>2g</td>
<td>NH$_2$</td>
<td>C$_7$H$_7$N$_3$</td>
<td>133.15</td>
<td>224-226</td>
<td>75</td>
<td>0.23</td>
</tr>
<tr>
<td>2h</td>
<td>CH(OH)CH$_3$</td>
<td>C$<em>9$H$</em>{10}$N$_2$O</td>
<td>162.16</td>
<td>178-179</td>
<td>39.5</td>
<td>0.33</td>
</tr>
<tr>
<td>2i</td>
<td>SH</td>
<td>C$_7$H$_8$N$_2$S</td>
<td>150.20</td>
<td>298-300</td>
<td>63</td>
<td>0.52</td>
</tr>
<tr>
<td>2j</td>
<td>SHCH$_2$</td>
<td>C$_8$H$_8$N$_2$S</td>
<td>164.23</td>
<td>265-266</td>
<td>30.4</td>
<td>0.36</td>
</tr>
<tr>
<td>2v</td>
<td>CH$_2$C$_6$H$_5$</td>
<td>C$<em>{14}$H$</em>{12}$N$_2$</td>
<td>208.26</td>
<td>191-192</td>
<td>69.5</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Stationary phase: Silica gel G, Mobile phase for TLC : chloroform: methanol (9.5:0.5)

**e) Synthesis of 2-substituted benzimidazoles (aromatic series) [2(k-u), 2w, 2x]**

2-substituted benzimidazole derivatives were synthesized by the reaction of o-phenylenediamine (OPD) and substituted benzaldehyde by the procedure reported in literature (Devmurari *et al*., 2010).
**Synthesis and Characterization**

**Step-1 Preparation of Sodium bisulfite adduct**

Aromatic aldehyde (0.015 mole) was dissolved in 50 ml of ethanol. The solution of sodium bisulfite (1.6gm) in water (10ml) was added gradually to a cooled solution of aromatic aldehyde in ethanol. Stirred vigorously, then more ethanol was added and cooled again. The precipitates were filtered off and dried. Quantity of substituted aromatic aldehyde is given in **Table 3.3**

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Aromatic aldehyde</th>
<th>Quantity Taken (gm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2k</td>
<td>Benzaldehyde</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>2l</td>
<td>2-pyridine carboxaldehyde</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>2m</td>
<td>3-pyridine carboxaldehyde</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>2n</td>
<td>Salicyldehyde</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>2o</td>
<td>2-chloro benzaldehyde</td>
<td>2.10 gm</td>
</tr>
<tr>
<td>2p</td>
<td>3-chloro benzaldehyde</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>2q</td>
<td>4-chloro benzaldehyde</td>
<td>2.10 gm</td>
</tr>
<tr>
<td>2r</td>
<td>2-bromo benzaldehyde</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>2s</td>
<td>3-bromo benzaldehyde</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>2t</td>
<td>4-bromo benzaldehyde</td>
<td>2.77 gm</td>
</tr>
<tr>
<td>2u</td>
<td>4-nitro benzaldehyde</td>
<td>2.26 gm</td>
</tr>
<tr>
<td>2w</td>
<td>4-fluoro benzaldehyde</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>2x</td>
<td>2-amino benzaldehyde</td>
<td>1.81 gm</td>
</tr>
</tbody>
</table>

**Table 3.3: Quantity of aromatic aldehyde taken**

**Step-2 Preparation of 2-substituted benzimidazole from Sodium bisulfite adduct and ortho-phenylenediamine**

Mixture of Sodium bisulfite adduct of aromatic aldehyde (0.002 mol) and orthophenylene diamine (OPD) (0.002 mol, 0.216 gm) was heated in dimethyl formamide (DMF) (10 ml) at 140ºC for 4 hrs. The reaction blend was cooled and poured into ice cold water. The product obtained was recrystallized with ethanol. Quantity of sodium bisulfite adduct is given in **Table 3.4** and physical data of synthesized compounds is given in **Table 3.5**.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantity of Sodium bisulfite adduct (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2k</td>
<td>0.42</td>
</tr>
<tr>
<td>2l</td>
<td>0.42</td>
</tr>
<tr>
<td>2m</td>
<td>0.42</td>
</tr>
<tr>
<td>2n</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**Table 3.4: Quantity of Sodium bisulphite adduct taken**
Synthesis and Characterization

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Melting point</th>
<th>Yield (%)</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2k</td>
<td>C₆H₅</td>
<td>C₁₃H₁₀N₂</td>
<td>194.23</td>
<td>292-293</td>
<td>68.4</td>
<td>0.78</td>
</tr>
<tr>
<td>2l</td>
<td>2-NC₃H₄</td>
<td>C₁₂H₉N₃</td>
<td>195.22</td>
<td>216-218</td>
<td>57.0</td>
<td>0.56</td>
</tr>
<tr>
<td>2m</td>
<td>3-NC₃H₄</td>
<td>C₁₂H₉N₃</td>
<td>195.22</td>
<td>252-253</td>
<td>59.3</td>
<td>0.40</td>
</tr>
<tr>
<td>2n</td>
<td>2-OH-C₆H₄</td>
<td>C₁₃H₁₀N₂O</td>
<td>210.23</td>
<td>238-240</td>
<td>66.0</td>
<td>0.90</td>
</tr>
<tr>
<td>2o</td>
<td>2-Cl-C₆H₄</td>
<td>C₁₃H₉ClN₂</td>
<td>228.68</td>
<td>230-232</td>
<td>68.4</td>
<td>0.83</td>
</tr>
<tr>
<td>2p</td>
<td>3-Cl-C₆H₄</td>
<td>C₁₃H₉ClN₂</td>
<td>228.68</td>
<td>231-234</td>
<td>67.9</td>
<td>0.91</td>
</tr>
<tr>
<td>2q</td>
<td>4-Cl-C₆H₄</td>
<td>C₁₃H₉ClN₂</td>
<td>228.68</td>
<td>297-298</td>
<td>69.2</td>
<td>0.81</td>
</tr>
<tr>
<td>2r</td>
<td>2-Br-C₆H₄</td>
<td>C₁₃H₉BrN₂</td>
<td>273.13</td>
<td>231-234</td>
<td>82.7</td>
<td>0.89</td>
</tr>
<tr>
<td>2s</td>
<td>3-Br-C₆H₄</td>
<td>C₁₃H₉BrN₂</td>
<td>273.13</td>
<td>235-236</td>
<td>89.2</td>
<td>0.77</td>
</tr>
<tr>
<td>2t</td>
<td>4-Br-C₆H₄</td>
<td>C₁₃H₉BrN₂</td>
<td>273.13</td>
<td>296-297</td>
<td>91.5</td>
<td>0.85</td>
</tr>
<tr>
<td>2u</td>
<td>4-NO₂-C₆H₄</td>
<td>C₁₃H₉N₃O₂</td>
<td>239.23</td>
<td>235-237</td>
<td>41.8</td>
<td>0.67</td>
</tr>
<tr>
<td>2w</td>
<td>4-F-C₆H₄</td>
<td>C₁₃H₉FN₂</td>
<td>212.22</td>
<td>240-241</td>
<td>70.2</td>
<td>0.80</td>
</tr>
<tr>
<td>2x</td>
<td>2-NH₂C₆H₄</td>
<td>C₁₃H₁₁N₃</td>
<td>209.25</td>
<td>210-212</td>
<td>67.8</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Table 3.5: Physical data of synthesized compounds (2-substituted benzimidazoles-aromatic series)

Stationary phase: Silica gel G. Mobile phase for TLC: chloroform: methanol (9.5:0.5)

STEP 2

General method for the synthesis of Mannich bases of 2-substituted benzimidazole [N-(Benzimidazol-1-ylmethyl)-benzamide] derivatives [3(a-x)] (Series 1)

2-substituted benzimidazole (0.01 mole) was added to the ethanolic solution of benzamide (0.01 mole, 1.21gm). Formaldehyde (37-41%) (0.01 mole, 0.3 ml) was added dropwise and the reaction blend was then adjusted to the pH of 3.5 with conc. HCl. The reaction mixture was kept in efficient ice cooling for half an hour and then it was refluxed with stirring at 80°C for 9-12 hrs. Additional amount of formaldehyde was added in portions to the reaction mixture for completion of the reaction. Final stage
of the reaction was monitored by thin layer chromatography (TLC). The reaction mixture was evaporated under reduced pressure and the resulting solution was put in the refrigerator overnight. The product was collected, washed with water and recrystallized from ethyl alcohol (Srivastava et al., 2013; Manikpuri et al., 2010).

Similar procedure was followed and 24 novel compounds were synthesized. Quantity of 2-substituted benzimidazole is provided in Table 3.6 and Physical data of synthesized compounds is provided in Table 3.7.

Table 3.6: Quantity of 2- substituted benzimidazole taken

<table>
<thead>
<tr>
<th>Comp</th>
<th>R</th>
<th>Quantity of 2-sub. benzimidazole (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>H</td>
<td>1.18</td>
</tr>
<tr>
<td>3b</td>
<td>CH₃</td>
<td>1.32</td>
</tr>
<tr>
<td>3c</td>
<td>CICH₂</td>
<td>1.66</td>
</tr>
<tr>
<td>3d</td>
<td>C₂H₅</td>
<td>1.46</td>
</tr>
<tr>
<td>3e</td>
<td>C₃H₈</td>
<td>1.60</td>
</tr>
<tr>
<td>3f</td>
<td>C₄H₁₀</td>
<td>1.74</td>
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<tr>
<td>3g</td>
<td>NH₂</td>
<td>1.33</td>
</tr>
<tr>
<td>3h</td>
<td>CH(OH)CH₃</td>
<td>1.62</td>
</tr>
<tr>
<td>3i</td>
<td>SH</td>
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</tr>
<tr>
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<td>SHCH₂</td>
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<td>C₆H₅</td>
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<tr>
<td>3m</td>
<td>3-NC₅H₄</td>
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</tr>
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<tr>
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<tr>
<td>3s</td>
<td>3-Br-C₆H₄</td>
<td>2.73</td>
</tr>
<tr>
<td>3t</td>
<td>4-Br-C₆H₄</td>
<td>2.73</td>
</tr>
<tr>
<td>3u</td>
<td>4-NO₂-C₆H₄</td>
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<td>3x</td>
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Table 3.7: Physical data of synthesized compounds [3a-3x]

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<th>Compound</th>
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<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Melting Point (°C)</th>
<th>Yield (%)</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
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<td>251.28</td>
<td>198-199</td>
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<tr>
<td>3b</td>
<td>CH₃</td>
<td>C₁₆H₁₅N₃O</td>
<td>265.31</td>
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<td>62.4</td>
<td>0.69</td>
</tr>
<tr>
<td>3c</td>
<td>ClCH₂</td>
<td>C₁₆H₁₄ClN₃O</td>
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<td>190-191</td>
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</tr>
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<td>3d</td>
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<td>279.34</td>
<td>210-212</td>
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<td>0.64</td>
</tr>
<tr>
<td>3e</td>
<td>C₃H₈</td>
<td>C₁₈H₁₉N₃O</td>
<td>293.36</td>
<td>208-210</td>
<td>62.5</td>
<td>0.77</td>
</tr>
<tr>
<td>3f</td>
<td>C₄H₁₀</td>
<td>C₁₀H₂₁N₃O</td>
<td>307.39</td>
<td>212-214</td>
<td>60.6</td>
<td>0.91</td>
</tr>
<tr>
<td>3g</td>
<td>NH₂</td>
<td>C₁₅H₁₄N₄O</td>
<td>266.30</td>
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</tr>
<tr>
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<td>CH(OH)CH₃</td>
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<td>190-192</td>
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<td>0.65</td>
</tr>
<tr>
<td>3i</td>
<td>SH</td>
<td>C₁₅H₁₃N₃OS</td>
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<td>170-172</td>
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<td>0.91</td>
</tr>
<tr>
<td>3j</td>
<td>SHCH₂</td>
<td>C₁₆H₁₅N₃OS</td>
<td>297.38</td>
<td>212-214</td>
<td>51.8</td>
<td>0.84</td>
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<tr>
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<td>C₂₁H₁₇N₃O</td>
<td>327.38</td>
<td>195-197</td>
<td>80.4</td>
<td>0.95</td>
</tr>
<tr>
<td>3l</td>
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<td>C₂₀H₁₆N₄O</td>
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<td>218-220</td>
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<tr>
<td>3m</td>
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<td>C₂₀H₁₆N₄O</td>
<td>328.37</td>
<td>205-206</td>
<td>79.7</td>
<td>0.85</td>
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<tr>
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<td>C₂₁H₁₇N₃O₂</td>
<td>343.38</td>
<td>180-182</td>
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<td>C₂₁H₁₆ClN₃O</td>
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<td>190-191</td>
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<td>3-Cl-C₆H₄</td>
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<td>200-202</td>
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<tr>
<td>3q</td>
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<td>C₂₁H₁₆ClN₃O</td>
<td>361.82</td>
<td>195-196</td>
<td>73.6</td>
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</tr>
<tr>
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<td>C₂₁H₁₆BrN₃O</td>
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<td>190-192</td>
<td>75.4</td>
<td>0.81</td>
</tr>
<tr>
<td>3s</td>
<td>3-Br-C₆H₄</td>
<td>C₂₁H₁₆BrN₃O</td>
<td>406.28</td>
<td>192-194</td>
<td>76.7</td>
<td>0.80</td>
</tr>
<tr>
<td>3t</td>
<td>4-Br-C₆H₄</td>
<td>C₂₁H₁₆BrN₃O</td>
<td>406.28</td>
<td>195-196</td>
<td>78.6</td>
<td>0.77</td>
</tr>
<tr>
<td>3u</td>
<td>4-NO₂-C₆H₄</td>
<td>C₂₁H₁₆N₄O₃</td>
<td>372.38</td>
<td>180-183</td>
<td>79.2</td>
<td>0.64</td>
</tr>
<tr>
<td>3v</td>
<td>CH₂C₆H₅</td>
<td>C₂₂H₁₉N₃O</td>
<td>341.41</td>
<td>170-172</td>
<td>64.6</td>
<td>0.70</td>
</tr>
<tr>
<td>3w</td>
<td>4-F-C₆H₄</td>
<td>C₂₁H₁₆F₃N₃O</td>
<td>345.37</td>
<td>230-231</td>
<td>72.7</td>
<td>0.76</td>
</tr>
<tr>
<td>3x</td>
<td>2-NH₂-C₆H₄</td>
<td>C₂₁H₁₈N₄O</td>
<td>342.39</td>
<td>206-208</td>
<td>78.5</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Stationary phase: Silica gel G, Mobile phase for TLC: chloroform: methanol (9:5:0.5)

3.1.1.2 General method for the synthesis of Mannich bases of 2-substituted benzimidazole [N-(Benzimidazol-1-ylmethyl)-4-chlorobenzamide] derivatives [3(a- m‘)] (SERIES-2)

2-substituted benzimidazole (0.01 mole) was added to the ethanolic solution of p-chlorobenzamide (0.01 mole, 1.55gm). Formaldehyde (37- 41%) (0.01 mole, 0.3ml) was added dropwise and the reaction mixture was then adjusted to the pH of 3.5 with conc. HCl. The reaction mixture was kept in efficient ice cooling for half an hour and then it was refluxed with stirring at 80°C for 9-12 hrs. Additional amount of formaldehyde
was added to the reaction mixture in portions to get finished product. Final stage of the reaction was monitored by thin layer chromatography (TLC). The solvent was evaporated under reduced pressure and the reaction mixture was put in the refrigerator overnight. The product was collected, washed with water and recrystallized from ethyl alcohol (Srivastava et al., 2013; Manikpuri et al., 2010).

Similar procedure was followed and 13 novel derivatives were prepared. Quantity of 2-substituted benzimidazole is provided in Table 3.8 and Physical data of synthesized compounds is provided in Table 3.9.

Table 3.8: Quantity of 2-substituted benzimidazole taken

<table>
<thead>
<tr>
<th>Comp</th>
<th>R</th>
<th>2-sub. benzimidazole Quantity Taken (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a’</td>
<td>ClCH₂</td>
<td>1.66</td>
</tr>
<tr>
<td>3b’</td>
<td>C₆H₅</td>
<td>1.94</td>
</tr>
<tr>
<td>3c’</td>
<td>3-NC₅H₄</td>
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</tr>
<tr>
<td>3d’</td>
<td>2-OH-C₆H₄</td>
<td>2.10</td>
</tr>
<tr>
<td>3e’</td>
<td>2-Cl-C₆H₄</td>
<td>2.28</td>
</tr>
<tr>
<td>3f’</td>
<td>3-Cl-C₆H₄</td>
<td>2.28</td>
</tr>
<tr>
<td>3g’</td>
<td>4-Cl-C₆H₄</td>
<td>2.28</td>
</tr>
<tr>
<td>3h’</td>
<td>2-Br-C₆H₄</td>
<td>2.73</td>
</tr>
<tr>
<td>3i’</td>
<td>3-Br-C₆H₄</td>
<td>2.73</td>
</tr>
<tr>
<td>3j’</td>
<td>4-Br-C₆H₄</td>
<td>2.73</td>
</tr>
<tr>
<td>3k’</td>
<td>4-NO₂-C₆H₄</td>
<td>2.39</td>
</tr>
<tr>
<td>3l’</td>
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<td>2.12</td>
</tr>
<tr>
<td>3m’</td>
<td>2-NH₂-C₆H₄</td>
<td>2.09</td>
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</table>

Table 3.9: Physical data of synthesized compounds [3a’-3m’]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Molecular formula</th>
<th>Moleculer weight</th>
<th>Melting Point (°C)</th>
<th>Yield (%)</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a’</td>
<td>ClCH₂</td>
<td>C₁₆H₁₃Cl₂N₃O</td>
<td>334.20</td>
<td>165-167</td>
<td>71.2</td>
<td>0.84</td>
</tr>
<tr>
<td>3b’</td>
<td>C₆H₅</td>
<td>C₂₁H₁₆Cl₃N₃O</td>
<td>361.82</td>
<td>162-164</td>
<td>72.6</td>
<td>0.89</td>
</tr>
<tr>
<td>3c’</td>
<td>3-NC₅H₄</td>
<td>C₂₀H₁₅Cl₃N₄O</td>
<td>362.81</td>
<td>180-182</td>
<td>69.4</td>
<td>0.89</td>
</tr>
<tr>
<td>3d’</td>
<td>2-OH-C₆H₄</td>
<td>C₂₁H₁₆Cl₃N₂O₂</td>
<td>377.82</td>
<td>180-183</td>
<td>65.2</td>
<td>0.88</td>
</tr>
<tr>
<td>3e’</td>
<td>2-Cl-C₆H₄</td>
<td>C₂₁H₁₅Cl₂N₃O</td>
<td>396.27</td>
<td>150-152</td>
<td>65.0</td>
<td>0.67</td>
</tr>
</tbody>
</table>
### Synthesis and Characterization

| 3f' | 3-Cl-C₆H₄ | C₂₁H₁₅Cl₂N₂O | 396.27 | 163-165 | 68.3 | 0.66 |
| 3g' | 4-Cl-C₆H₄ | C₂₁H₁₅Cl₂N₂O | 396.27 | 165-166 | 63.7 | 0.64 |
| 3h' | 2-Br-C₆H₄ | C₂₁H₁₅BrClN₂O | 440.72 | 175-177 | 65.6 | 0.70 |
| 3i' | 3-Br-C₆H₄ | C₂₁H₁₅BrClN₂O | 440.72 | 181-183 | 66.0 | 0.73 |
| 3j' | 4-Br-C₆H₄ | C₂₁H₁₅BrClN₂O | 440.72 | 185-187 | 68.2 | 0.76 |
| 3k' | 4-NO₂-C₆H₄ | C₂₁H₁₅ClN₃O₃ | 406.82 | 175-177 | 59.3 | 0.69 |
| 3l' | 4-F-C₆H₄ | C₂₁H₁₅ClF₃N₂O | 379.81 | 210-213 | 62.6 | 0.78 |
| 3m' | 2-NH₂-C₆H₄ | C₂₁H₁₇ClN₂O | 376.84 | 240-242 | 68.8 | 0.81 |

Stationary phase: Silica gel G, Mobile phase for TLC: chloroform: methanol (9.5:0.5)

### 3.2 Spectral characterization of synthesized compounds

#### 3.2.1 Spectral characterization of N-(Benzimidazol-1-ylmethyl)-benzamide derivatives (3a-3x) [Series 1] (Silverstein and Webster, 1998)

**N-(Benzimidazol-1-ylmethyl)-benzamide (3a):** IR (KBr, cm⁻¹) 3307 (N-H), 3053 (C-H, aromatic str), 2965 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1486 (C=N), 870-675 (CH bend aromatic). ^1H NMR (400 MHz, DMSO-d₆, δ ppm) 7.91-7.39 (m, 10H, ArH), 4.98 (s, 2H, NCH₂N), 8.70 (s, 1H, NH).

**N-(2-methyl-benzimidazol-1-ylmethyl)-benzamide (3b):** IR (KBr, cm⁻¹) 3308 (N-H), 3055 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1487 (C=N), 870-675 (CH bend aromatic). ^1H NMR (400 MHz, DMSO-d₆, δ ppm) 7.92-7.33 (m, 9H, ArH), 4.89 (s, 2H, NCH₂N), 8.88 (s, 1H, NH), 2.80 (s, 3H, CH₃), MS: m/z = 266.2 (M+1).
Synthesis and Characterization

*N-(2-chloromethyl-benzimidazol-1-ylmethyl)-benzamide (3c):* IR (KBr, cm\(^{-1}\)) 3308 (N-H), 3055 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1487 (C=N), 789 (C-Cl), 870-675 (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-\(d_6\), δppm), 8.14-7.40 (m, 9H, ArH), 4.92 (s, 2H, NCH\(_2\)N), 9.0 (s, 1H, NH), 5.19 (s, 2H, CH\(_2\)), MS: m/z = 300.2 (M+1).

*N-(2-ethyl-benzimidazol-1-ylmethyl)-benzamide (3d):* IR (KBr, cm\(^{-1}\)) 3308 (N-H), 3019 (C-H, aromatic str), 2967 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1486 (C=N), (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-\(d_6\), δppm) 7.92-7.39 (m, 9H, ArH), 4.99 (s, 2H, NCH\(_2\)N), 8.80 (s, 1H, NH), 3.27-3.21 (q, 2H, CH\(_2\)), J=7.6 Hz), 1.56-1.52 (t, 3H, CH\(_3\), J=7.6 Hz), MS: m/z = 279.2 (M+1).

*N-(2-propyl-benzimidazol-1-ylmethyl)-benzamide (3e):* IR (KBr, cm\(^{-1}\)) 3308 (N-H), 3056 (C-H, aromatic str), 2967 (C-H, aliphatic str), 1634 (C=O), 1527 (C=C), 1487 (C=N), 870-675 (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-\(d_6\), δppm) 8.12-7.40 (m, 9H, ArH), 4.93 (s, 2H, NCH\(_2\)N), 8.97 (s, 1H, NH), 3.10-3.06 (t, 2H, CH\(_2\)), J=7.6 Hz), 2.11-1.92 (m, 2H, CH\(_2\)), 1.04-1.00 (t, 3H, CH\(_3\), J=7.36 Hz).

*N-(2-butyl-benzimidazol-1-ylmethyl)-benzamide (3f):* IR (KBr, cm\(^{-1}\)) 3307 (N-H), 3055 (C-H aromatic str), 2966 (C-H, aliphatic str), 1635 (C=O) 1527 (C=C), 1486 (C=N), 870-675 (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-\(d_6\), δppm) 8.08-7.18 (m, 9H, ArH), 4.97 (s, 2H, NCH\(_2\)N), 8.84 (s, 1H, NH), 1.00-0.97 (t, 3H, CH\(_3\), J=7.2 Hz), 1.47-1.41 (m, 2H, CH\(_2\)), 1.93-1.90 (m, 2H, CH\(_2\)), 3.19-3.15 (t, 2H, CH\(_2\), J=7.6 Hz).
N-(2-amino-benzimidazol-1-ylmethyl)-benzamide (3g): IR (KBr, cm⁻¹) 3305, 3220 (NH₂ str), 3060 (C-H, aromatic str), 2964 (C-H, aliphatic str), 1638 (C=O), 1529 (C=C), 1484 (C=N), 870-675 (CH bend aromatic). <i>H NMR (400 MHz, DMSO-d₆, δppm) 7.96-7.21 (m, 9H, ArH), 7.97 (s, 1H, NH₂), 4.90 (s, 2H, NCH₂N), 9.03 (s, 1H, NH), 9.68 (s, 1H, NH₂). <sup>13</sup>C NMR (100 MHz, DMSO-d₆, δppm) 166.47 (C=O amide), 150.08 (CH), 133.88 (ArC), 132.97 (ArC), 131.76 (ArC), 131.19 (ArC), 130.16 (ArC), 128.29 (ArC), 128.03 (2×ArC), 127.36 (2×ArC), 122.80 (ArC), 111.44 (ArC), 45.12 (NCH₂N).

N-2-[(1-hydroxy-ethyl)-benzimidazol-1-ylmethyl]-benzamide (3h): IR (KBr, cm⁻¹) 3415 (OH str, br), 3308 (N-H), 1487 (C=N), 3056 (C-H, aromatic str), 2967 (C-H, aliphatic str), 1634 (C=O), 1527 (C=C), 870-675 (CH bend aromatic). <i>H NMR (400 MHz, DMSO-d₆, δppm) 7.92-7.39 (m, 9H, ArH), 4.90 (s, 2H, NCH₂N), 8.97 (s, 1H, NH), 5.70 (br, s, 1H, OH), 1.60-1.59 (d, 3H CH₃, J= 6.4 Hz), 5.05-5.00 (1H, q, CH, J= 6.4 Hz).

N-(2-mercapto-benzimidazol-1-ylmethyl)-benzamide (3i): IR (KBr, cm⁻¹) 3305 (N-H), 1378 (C=N), 3078 (C-H, aromatic str), 2956 (C-H, aliphatic str), 1635 (C=O), 1526 (C=C), 870-675 (CH bend aromatic). <i>H NMR (400 MHz, DMSO-d₆, δppm) 7.95-7.26 (m, 9H, ArH), 4.95 (s, 2H, NCH₂N), 8.86 (s, 1H, NH), 3.4 (s, 1H, SH).

N-(2-mercaptomethyl-benzimidazol-1-ylmethyl)-benzamide (3j): IR (KBr, cm⁻¹) 3308 (N-H), 3056 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1487 (C=N), 870-675 (CH bend aromatic). <i>H NMR (400 MHz, DMSO-d₆, δppm) 8.12-7.40 (m, 9H, ArH), 4.92 (s, 2H, NCH₂N), 8.98 (s, 1H, NH), 3.39 (s, 1H, SH), 4.18 (s, 2H, CH₂), MS: m/z = 298.2 (M+1).
Synthesis and biological studies on 2-substituted benzimidazole derivatives

*N-(2-phenyl-benzimidazol-1-ylmethyl)-benzamide*

(3k): IR (KBr, cm⁻¹) 3308 (N-H), 3056 (C-H, aromatic str), 2965 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1488 (C=N), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.32-7.40 (m, 14H, ArH), 4.93 (s, 2H, NCH₂N), 9.01 (s, 1H, NH). ¹³C NMR (100 MHz, DMSO-d₆, δppm) 166.44 (C=O amide), 149.29 (CH), 133.86 (ArC), 133.67 (ArC), 132.35 (ArC), 131.23 (ArC), 129.36 (ArC), 128.92 (2×ArC), 128.07 (2×ArC), 127.57 (2×ArC), 127.36 (2×ArC), 126.03 (ArC), 124.89 (ArC), 124.79 (ArC), 114.25 (ArC), 111.95 (ArC), 45.15 (NCH₂N), MS: m/z = 329.2 (M+1).

*N-(2-pyridin-2-yl-benzimidazol-1-ylmethyl)-benzamide*

(3l): IR (KBr, cm⁻¹) 3308 (N-H), 3056 (C-H, aromatic str), 2967 (C-H, aliphatic str), 1634 (C=O) 1527 (C=C), 1447 (C=N), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 7.91-7.20 (m, 13H, ArH), 4.96 (s, 2H, NCH₂N), 8.82 (s, 1H, NH).

*N-(2-pyridin-3-yl-benzimidazol-1-ylmethyl)-benzamide*

(3m): IR (KBr, cm⁻¹) 3308 (N-H), 3053 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1635 (C=O), 1527 (C=C), 1486 (C=N), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.96-7.20 (m, 13H, ArH), 4.91 (s, 2H, NCH₂N), 9.53 (s, 1H, NH), MS: m/z = 329.3 (M+1).
Synthesis and Characterization

*Synthesis and biological studies on 2-substituted benzimidazole derivatives*

**N-[2-(2-hydroxy-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3n):** IR (KBr, cm⁻¹) 3450 (OH str, br), 3310 (N-H), 3057 (C-H, aromatic str), 2964 (C-H, aliphatic str), 1633 (C=O), 1527 (C=C), 1491 (C=N), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.18-7.04 (m, 13H, ArH), 4.91 (s, 2H, NCH₂N), 9.03 (s, 1H, NH), 13.6 (br, s, 1H, OH), MS: m/z = 344.8 (M+1).

**N-[2-(2-chloro-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3o):** IR (KBr, cm⁻¹) 3307 (N-H), 3048 (C-H, aromatic str), 2963 (C-H, aliphatic str), 1634 (C=O), 1526 (C=C), 1485 (C=N), 770 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.21-7.41 (m, 13H, ArH), 4.91 (s, 2H, NCH₂N), 9.01 (s, 1H, NH), MS: m/z = 362.2 (M+1).

**N-[2-(3-chloro-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3p):** IR (KBr, cm⁻¹) 3309 (N-H), 3034 (C-H, Aromatic), 2953 (C-H, Aliphatic), 1633 (C=O), 1527 (C=C), 1485 (C=N), 765 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.42-7.46 (m, 13H, ArH), 4.98 (s, 2H, NCH₂N), 8.91 (s, 1H, NH).

**N-[2-(4-chloro-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3q):** IR (KBr, cm⁻¹) 3309 (N-H), 3052 (C-H, aromatic str) 2953 (C-H, aliphatic str), 1632 (C=O), 1566 (C=C), 1489 (C=N), 764 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.61-7.35 (m, 13H, ArH), 4.96 (s, 2H, NCH₂N), 8.90 (s, 1H, NH).
**Synthesis and Characterization**

**N-[2-(2-bromo-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3r):** IR (KBr, cm⁻¹) 3309 (N-H), 3056 (C-H, aromatic str), 2967 (C-H, aliphatic str), 1635 (C=O), 1527 (C=C), 1488 (C=N), 869 (C-Br), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.15-7.40 (m, 13H, ArH), 4.91 (s, 2H, NCH₂N), 8.98 (s, 1H, NH). MS: m/z = 406.2 (M+1).

**N-[2-(3-bromo-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3s):** IR (KBr, cm⁻¹) 3307 (N-H), 3034 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1634 (C=O), 1525 (C=C), 1487 (C=N), 865 (C-Br), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.70-7.19 (m, 13H, ArH), 4.98 (s, 2H, NCH₂N), 9.0 (s, 1H, NH).

**N-[2-(4-bromo-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3t):** IR (KBr, cm⁻¹) 3308 (N-H), 3056 (C-H, aromatic str), 2962 (C-H, aliphatic str), 1635 (C=O), 1527 (C=C), 1486 (C=N), 865 (C-Br), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.62-7.26 (m, 13H, ArH), 4.97 (s, 2H, NCH₂N), 8.89 (s, 1H, NH).

**N-[2-(4-nitro-phenyl)-benzimidazol-1-ylmethyl]-benzamide (3u):** IR (KBr, cm⁻¹) 3308 (N-H), 3058 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1634 (C=O), 1524, 1344 (NO₂ str.), 1463 (C=N), 747 (C-NO₂), 870-675 (CH bend str). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.46-7.24 (m, 13H, ArH), 4.94 (s, 2H, NCH₂N), 8.90 (s, 1H, NH). MS: m/z = 373.7 (M+1).
Synthesis and Characterization

N-(2-benzyl-benzimidazol-1-ylmethyl)-benzamide (3v):
IR (KBr, cm⁻¹) 3308 (N-H), 3054 (C-H, aromatic str),
2966 (C-H, aliphatic str), 1634 (C=O), 1525 (C=C),
1487 (C=N), 675-870 (CH bend aromatic). ¹H NMR
(400 MHz, DMSO-d₆, δppm) 7.93-7.24 (m, 14H, ArH),
4.93 (s, 2H, NCH₂N), 8.62 (s, 1H, NH), 4.53 (s, 2H,
CH₂).

N-[2-(4-flouro-phenyl)-benzimidazol-1-ylmethyl]-
benzamide (3w): IR (KBr, cm⁻¹) 3310 (N-H), 3055 (C-
H, aromatic str), 2927 (C-H, aliphatic str), 1634 (C=O),
1525 (C=C), 1458 (C=N), 848 (C-F), 870-675 (CH
bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm)
8.48-7.41 (m, 13H, ArH), 4.90 (s, 2H, NCH₂N), 9.01 (s,
1H, NH). ¹³C NMR (100 MHz, DMSO-d₆, δppm)
166.41 (C=O amide), 163.51 (CF), 147.78 (CH), 133.87
(ArC), 131.94 (ArC), 131.32 (ArC), 131.02 (ArC),
130.92 (2×ArC), 128.17 (2×ArC), 127.38 (2×ArC),
125.79 (2×ArC), 120.03 (ArC), 120.00 (ArC), 117.02
(ArC), 116.79 (ArC), 113.95 (ArC), 45.15 (NCH₂N).
MS: m/z = 346.3 (M+1).

N-[2-(2-amino-phenyl)-benzimidazol-1-ylmethyl]-
benzamide (3x): IR (KBr, cm⁻¹) 3310, 3201 (NH₂ str.),
3086 (C-H, aromatic str), 2959(C-H, aliphatic str), 1634
(C=O), 1525 (C=C), 1486 (C=N), 870-675 (CH bend
aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 7.91-
7.02 (m, 13H, ArH), 4.96 (s, 2H, NCH₂N), 7.80 (s, 2H,
NH₂), 8.04 (s, 1H, NH).
3.2.2 Spectral characterization of N-(Benzimidazol-1-ylmethyl)-4-
chlorobenzamide derivatives (3a’-3m’) [Series 2]

N-(2-chloromethyl-benzimidazol-1-ylmethyl)-4-
chlorobenzamide (3a’): IR (KBr, cm⁻¹) 3319 (N-H),
3052 (C-H, aromatic str), 2959 (C-H, aliphatic str)
1683 (C=O), 1534 (C=C), 1487 (C=N), 762 (C-Cl),
870-675 (CH bend. Ar). ¹H NMR (400 MHz, DMSO-
d₆, δppm) 7.97-7.51 (m, 8H, ArH), 4.86 (s, 2H,
NCH₂N), 9.20 (s, 1H, NH), 4.31 (s, 2H, CH₂).

N-(2-phenyl-benzimidazol-1-ylmethyl)-4-chlorobenzamide
(3b’): IR (KBr, cm⁻¹) 3316 (N-H), 3054 (C-H, aromatic
str), 2925 (C-H, aliphatic str), 1642 (C=O), 1535
(C=C), 1485 (C=N), 762 (C-Cl), 870-675 (CH
bend. Ar). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.21-
7.25 (m, 13H, ArH), 4.88 (s, 2H, NCH₂N), 9.11 (s, 1H,
NH). ¹³C NMR (100 MHz, DMSO-d₆, δppm) 166.40
(C=O amide) 165.47 (C-Cl), 150.67 (CH), 137.82
(ArC), 135.77 (ArC), 136.33 (ArC), 132.53 (ArC),
130.90 (ArC), 130.36 (ArC), 129.56 (2×ArC), 128.88
(2×ArC), 128.65 (2×ArC), 128.49 (2×Ar),
126.70 (ArC) 122.74 (ArC) 114.74 (ArC), 45.18
(NCH₂N).

N-(2-pyridin-3-yl-benzimidazol-1-ylmethyl)-4-
chlorobenzamide (3c’): IR (KBr, cm⁻¹) 3317 (N-H),
3050 (C-H, aromatic str), 2967 (C-H, aliphatic str),
1642 (C=O), 1531 (C=C), 1487 (C=N), 763 (C-Cl),
870-675 (CH bend aromatic). ¹H NMR (400 MHz,
DMSO-d₆, δppm) 8.20-7.07 (m, 12H, ArH), 4.88 (s,
2H, NCH₂N), 9.13 (s, 1H, NH). MS: m/z = 363.0
(M+1).
Synthesis and Characterization

N-[2-(2-hydroxy-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3d') : IR (KBr, cm⁻¹) 3420 (OH str., br), 3319 (N-H), 3053 (C-H, aromatic str), 2968 (C-H, aliphatic str), 1643 (C=O), 1529 (C=C), 1497 (C=N), 762 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.04-7.13 (m, 12H, ArH), 4.90 (s, 2H, NCH₂N), 8.91 (s, 1H, NH), 13.52 (s, 1H, OH).

N-[2-(2-chloro-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3e') : IR (KBr, cm⁻¹) 3354 (N-H), 3059 (C-H aromatic str), 2974 (C-H, aliphatic str), 1650 (C=O), 1539 (C=C), 1485 (C=N), 760 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.02-7.55 (m, 12H, ArH), 4.91 (s, 2H, NCH₂N), 9.25 (s, 1H, NH).

N-[2-(3-chloro-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3f') : IR (KBr, cm⁻¹) 3357 (N-H), 3068 (C-H aromatic str), 2983 (C-H, aliphatic str), 1656 (C=O), 1527 (C=C), 1487 (C=N), 765 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.12-7.26 (m, 12H, ArH), 4.92 (s, 2H, NCH₂N), 9.12 (s, 1H, NH).

N-[2-(4-chloro-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3g') : IR (KBr, cm⁻¹) 3357 (N-H), 3055 (C-H, aromatic str), 2982 (C-H, aliphatic str), 1653 (C=O), 1525 (C=C), 1480 (C=N), 764 (C-Cl), 870-675 (CH bend aromatic). ¹H NMR (400 MHz, DMSO-d₆, δppm) 8.19-7.25 (m, 12H, ArH), 4.96 (s, 2H, NCH₂N), 8.98 (s, 1H, NH).
Synthesis and Characterization

*N-[2-(2-bromo-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3h'):* IR (KBr, cm\(^{-1}\)) 3349 (N-H), 3076 (C-H, aromatic str), 2957 (C-H, aliphatic str), 1654 (C=O), 1539 (C=C), 1485 (C=N), 760 (C-Cl), 864 (C-Br), 870-675 (CH bend, aromatic). \(^1\)H NMR (400 MHz, DMSO-d\(_6\), δppm) 8.26-7.47 (m, 12H, ArH), 4.87 (s, 2H, NCH\(_2\)N), 9.18 (s, 1H, NH).

*N-[2-(3-bromo-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3i'):* IR (KBr, cm\(^{-1}\)) 3353 (N-H), 3052 (C-H, aromatic str), 2953 (C-H, aliphatic str), 1650 (C=O), 1534 (C=C), 1487 (C=N), 763 (C-Cl), 865 (C-Br), 870-675 (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-d\(_6\), δppm) 7.89-7.21 (m, 12H, ArH), 4.88 (s, 2H, NCH\(_2\)N), 9.23 (s, 1H, NH).

*N-[2-(4-bromo-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3j'):* IR (KBr, cm\(^{-1}\)) 3358 (N-H), 3047 (C-H, aromatic str), 2945 (C-H, aliphatic str), 1647 (C=O), 1537(C=C), 1486 (C=N), 765 (C-Cl), 864 (C-Br), 870-675 (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-d\(_6\), δppm) 8.16-7.26 (m, 12H, ArH), 4.90 (s, 2H, NCH\(_2\)N), 9.29 (s, 1H, NH).

*N-[2-(4-nitro-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3k'):* IR (KBr, cm\(^{-1}\)) 3318 (N-H), 3056 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1634 (C=O), 1534 (C=C), 1532, 1347 (NO\(_2\) str.), 1483 (C=N), 745 (C-NO\(_2\)), 870-675 (CH bend aromatic). \(^1\)H NMR (400 MHz, DMSO-d\(_6\), δppm) 8.43-7.28 (m, 12H, ArH), 4.99 (s, 2H, NCH\(_2\)N), 8.90 (s, 1H, NH).

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\begin{center}
\includegraphics[width=0.8\textwidth]{Chemical_Structure_1.png}
\end{center}

\textit{N-[2-(4-flouro-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3l')}: IR (KBr, cm\(^{-1}\)) 3317 (N-H), 3048 (C-H, aromatic str), 2965 (C-H, aliphatic str), 1635 (C=O), 1525 (C=C), 1486 (C=N), 764 (C-Cl), 822 (C-F), 870-675 (CH bend aromatic). \(1^H\) NMR (400 MHz, DMSO-	extit{d}_6, \(\delta_{ppm}\)) 8.49-7.70 (m, 12H, ArH), 4.89 (s, 2H, NCH\(_2\)N), 9.01 (s, 1H, NH).

\begin{center}
\includegraphics[width=0.8\textwidth]{Chemical_Structure_2.png}
\end{center}

\textit{N-[2-(2-amino-phenyl)-benzimidazol-1-ylmethyl]-4-chlorobenzamide (3m')}: IR (KBr, cm\(^{-1}\)) 3319, 3207 (NH\(_2\) str.), 3056 (C-H, aromatic str), 2966 (C-H, aliphatic str), 1643 (C=O), 1535 (C=C), 1487 (C=N), 766 (C-Cl), 870-675 (CH bend Ar). \(1^H\) NMR (400 MHz, DMSO-	extit{d}_6, \(\delta_{ppm}\)) 7.89-7.27 (m, 12H, ArH), 4.99 (s, 2H, NCH\(_2\)N), 7.82 (s, 2H, NH\(_2\)), 9.16 (s, 1H, NH).

### 3.3 Results and Discussion

2-substituted benzimidazoles (aliphatic series) were synthesized by the reaction of \(\alpha\)-phenylenediamine dihydrochloride with substituted carboxylic acid and aromatic series was prepared by the reaction of \(\alpha\)-phenylenediamine with substituted aromatic aldehyde. Further, Mannich bases were prepared by the reaction of benzamide [series-1] and \(p\)-chlorobenzamide [series-2] (active hydrogen compounds), 2-substituted benzimidazoles (secondary amine), formaldehyde and conc. hydrochloric acid. Purity of all the synthesized compounds was ascertained by TLC. The structures of all the compounds were established by spectral analysis. Melting points were taken by the melting point determination apparatus (PERFIT) in open capillary tubes and were uncorrected.

From IR spectra, the appearance of peaks at 3308-3358 cm\(^{-1}\) and 1400-1500 cm\(^{-1}\) indicated the presence of NH stretching of carboxamide and C=N stretching of benzimidazole respectively. The appearance of peak at 1630-1656 cm\(^{-1}\) indicated the presence of C=O stretching in all the synthesized compounds. Aromatic C-H bending vibrations were observed below 900 cm\(^{-1}\).
From $^1$H NMR spectra, sharp singlet for 2 protons in the range of 4.86-4.99 ppm confirmed the presence of CH$_2$ attached to NH in all the synthesized compounds. The emergence of sharp singlet in the range of 8.62-9.53 ppm indicated the presence of NH of carboxamide in all the synthesized compounds. The appearance of multiplet in the range of 7.18-8.96 ppm confirmed the presence of aromatic and hetero-aromatic protons.

From $^{13}$C NMR spectra, peaks in the range of 166.47-166.40 ppm confirmed the presence of C=O group, peaks in the range of 45.18-45.12 ppm directed the presence of CH$_2$ group, peaks in the range of 137.82-111.44 ppm indicated the presence of ArC in all the synthesized compounds. The calculated molecular weight of the synthesized compounds was comparable with observed m/z value.
CHAPTER 4
ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY

4.1 Microbiology

Microbiology is a type of science, which not only deals with the types, activities and anatomical features of the microbes, but also about their habitats, their role in nature, how they are being distributed amongst themselves and also to the other living creatures. It also studies about their beneficiary and harmful effects on human beings and on the surroundings.

In microbiology various types of microorganisms are being studied out of which unicellular microscopic organisms covers the major part in which only single cell is involved in the whole life process. However, in the advanced forms of life, organisms are made up of many cells, which comprises of greatly specific tissues and organs to execute detailed functions. All living cells are basically similar. They are composed of protoplasm, a colloidal organic complex consisting largely of proteins, lipids and nucleic acids, all are circumscribed by limited membranes or cell walls; and all contain nuclei or an equivalent nuclear substance.

The major categories of life are the plant kingdom, the animal kingdom, and the Protista. The microorganisms classified as protista, may be further divided in two categories: Procaryota and Eucaryota. This classification is based upon differences in their cellular anatomy.

Prokaryotes are the organisms with non-distinct nucleus with absence of cell membranes. Nuclear division is less complex than mitosis; and their gene organization is less sharply defined that occurring in the chromosomes of higher organisms. The blue-green algae and bacteria are prokaryotic organisms. The Eucaryotes possess distinct and well developed nucleus and DNA as a genetic material present as chromosome within the nucleus. Such cells are found among the protozoa, fungi and algae (except for the blue-green algae) (Pelczar et al., 2005).

4.1.1 Bacteria

Bacteria are small (0.01-5000 µm³) prokaryotic, unicellular, filamentous or coenocytic, saprobic, parasitic micro-organisms. They mostly have rigid cell walls containing
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muramic acid and sterols are mostly absent. They are non-motile or motile by simple flagella, having axial filament or gliding motion (Rawlins, 1980). The different forms of bacteria are as:

*Cocccoid*, roughly sphere-shaped cells about diameter of 1µm, in chains, pairs, packets of four or eight or irregular clusters.

*Bacillary*, rods, approximately 0.4 to 1.5 µm broad and 1.5 to 8 µm elongated, exist individually, in chains or uneven clusters.

*Spirillar*, spiral rigid or flexible rods, 0.1 to 1 µm broad and 4 to 500 µm elongated with an axial filament which maintains the spiral structure.

*Mycelia*, with a mass of fragmented or interlacing slender (1.5 µm) threads and bearing external, asexual spores only, resembling microfungi.

*Stalked and sheathed forms*, mostly aquatic in habitat.

*Gliding*, flexible rod-shaped forms 0.5 to 1.5 µm broad and 5 to 10 µm in length moving by a gliding motion and with complex life cycles.

Mainly two types of bacteria are well known i.e. Gram-positive and Gram-negative.

From the staining view point, Gram-positive are the type of microbes which are capable of retaining the dye, crystal violet and hence appearance of deep violet color is observed suggesting the presence of this type; whereas Gram-negative bacteria is able to possess safranin dye and hence appearance of deep red color is observed. The most likely explanations for this occurrence can be correlated with the structure and composition of cell wall. Differences in the thickness of the cell walls among these two groups may also be crucial factor.

Literature has revealed that gram negative bacteria have a thinner cell wall as compared to the gram positive bacteria. Higher concentrations of lipids and low amounts of peptidoglycan in the walls of Gram negative bacteria, due to which they are not thoroughly cross linked and hence has thinner walls than the gram positive bacteria (Pelczar *et al.*, 2005).

Bacteria play an important role in our daily life. Some bacterial species like Azotobacter, Rhizobium etc., fix atmospheric nitrogen and increase the soil fertility.
Unicellular microbes have been cultivated in the food as a direct source since 1900’s.

The thought that microorganisms can be got from the food source and also the construction of single cell protein was discovered at the Massachusetts Institute of Technology (1966). Since then large numbers of bacteria had been extensively studied as an origin of single cell protein. Some of the examples among them have been mentioned: Bacillus spp; Acinetobacter calcoaceticus; Nocardia spp; Methylomonas spp, etc. Butyl alcohol and acetone are manufactured by the fermentation which is brought about by the bacterial activity of Clostridium acetobutylicum on molasses. Some bacteria like Mycoderma aceti and Acetobacter aceti synthesize acetic acid from fermentation of sugary solution and therefore, are used in vinegar production. Bacillus mesentericus is used for commercial production of amylase and protease. Bacillus macerans and B. polymyxa are also used for amylase production. Bacterial protease is employed to desize cotton and degum silk. The commonly observed souring and curding of milk is brought about by the activity of bacteria, Streptococcus lactis, which converts lactose into lactic acid by the action of enzyme produced by them. Similarly, we find that other bacteria like Leuconostoc citrovorum, Streptococcus paracitrovorum are also used in the manufacture of other dairy products like butter, cheese, etc. Beside the innumerable benefits of Bacteria, a few of which are cited above, there are a number of harmful effects also, some examples are: Bacteria like Pseudomonas fragi, Bacillus coagulana, B. stearothermophilus, Clostridium nigrificans and C. thermosaccharolyticum are responsible for the spoilage of meat and other foodstuffs by their enzymatic activities. Psychrophilic bacteria are responsible for spoilage of milk. Spirochete cytophage destroys cotton fibers and other articles made of cotton. A number of bacteria are plant pathogens. They cause various kinds of diseases like rots (Xanthomonas phaseoli, Pseudomonas midicaginis, Erwinia lathyri); spotes (Xanthomonas cucurbitae); galls (Pseudomonas tonelliana); canker (Pseudomonas Citri). These diseases cause great losses to the plants. Bacteria also cause diseases to man some examples are: diphtheria (Corynebacterium diphtheria); tuberculosis (Mycobacterium tuberculosis), leprosy (Mycobacterium laprae), anthrax (Bacillus anthracis), typhoid and paratyphoid (Salmonella typhii and S. paratyphi), tetanus (Clostridium tetani), meningitis (Neisseria meningitides), pneumonia (Streptococcus pneumonia) etc (Purohit, 1994).
4.1.2 Antimicrobial agents

Antimicrobial agents are the agents that inhibit the growth as well as metabolism of microbes and hence finally lead to the death of microorganisms. These antimicrobial agents are commonly known as antifungal agents, antibacterial or bactericidal agents and chemotherapeutic agents depending upon the type of microorganisms they are inhibiting.

4.1.2.1 Methods of testing microbial susceptibility

Degree of susceptibility of various microbes varies with different species and also it is different for each antimicrobial agent. Susceptibility of microbes even get affected due to time, temperature and also can change while being treated with a particular drug. In literature many methods have been quoted which specifies that which antimicrobial agent would be suitable for which species of microorganism. Two general methods are in common use: the tube dilution method and the disk diffusion method (Pommerville, 2004; Tortora, 2004).

4.1.2.1.1 Tube dilution method

The method determines the smallest amount of an antimicrobial agent necessary to destroy a population of a test organism. This amount is known as minimal inhibitory concentration (MIC). To determine the MIC, a series of tubes with variable concentrations of a particular antimicrobial agent is prepared. The tubes, then are inoculated with an identical population of the test organism, incubated, and examined for the growth of bacteria. The minimum concentration of test compound at which the growth of microbe is inhibited is known as its MIC. This method is also known as the broth dilution method or serial dilution method.

4.1.2.1.2 Disk diffusion method

It is the most suitable method having a maximum probability of the positive results also known as Kirby-Bauer test. A standard concentration of a test organism is being inoculated equally over the entire surface of a petri dish containing an agar medium. Lying on the solidified agar surface, filter-paper disks which are embedded with projected concentrations of the antimicrobial agents are placed. During incubation, the test compound gets diffused from the disks into the agar. If the test compound is found to be active, then after a standard incubation time, a zone of inhibition is formed in the
region of the disk. The radius of the zone determines the sensitivity of the microorganism to the test compound under test.

The zone of inhibition and its radius can be measured. In general, larger the zone, the more sensitive the microbe is to the test compound.

4.2 Antibacterial and Antifungal Activity

All the synthesized molecules were further tested for the antibacterial and antifungal activity.

For the present study the following strains were used.

A) Bacterial strains

1. *Escherichia coli* (Gram -ve), (MTCC 40)
2. *Pseudomonas aeruginosa* (Gram –ve), (MTCC 2453)
3. *Staphylococcus aureus* (Gram +ve), (MTCC 96)
4. *Bacillus subtilis* (Gram +ve), (MTCC 121)

B) Fungal strains

1. *Candida albicans* (MTCC 404)
2. *Aspergillus niger* (MTCC 183)

The strains mentioned above were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

*Escherichia coli*

The alimentary tract of nearly all warm-blooded animals usually is populated in a few hours by *Escherichia coli* from ingested food or water or unswervingly from other individuals. *E. coli* strains can attach to the mucous membrane present on the upper layer of the large bowel and distal small bowel. It has been estimated that the *E. coli* gets doubled within the intestine in approximately 40 hours. This species is type of facultative anaerobe which can grow in the absence as well in the presence of oxygen and reside in the large bowel.

Once an *E. coli* strain becomes established within a host, it may perhaps persevere for months or years. Resident strains usually move over a elongated phase, but much more
hastily following enteric illness or antimicrobial treatment. *E. coli* of the normal flora provides protection against colonization by harmful microorganisms.

Even though *E. coli* is a portion of the regular flora of the intestinal tract, some breeds are responsible for causing modest to dreadful diseases e.g. gastroenteritis in human beings and beasts. However, enteropathogenic strains can easily populate in the jejunum and upper ileum portion of small intestine and may produce heightened gastroenteritis in newborns and in infants. The pathogenic strains reside in the epithelial cells of the large intestine and cause diarrhoea in elder kids and adults. Enterotoxigenic (Enterotoxin-producing) strains make either one or both the toxins: a heat-labile toxin and a heat-stable toxin, both toxins produce diarrhoea in adults and infants. Enterotoxigenic strains of *E. coli* are often associated with the Traveler’s diarrhea, a common disease contracted by tourists when visiting developing countries. Other strains of *E. coli* which are usually harmless in their normal habitat can cause disease when they are exposed to the other tissues or the sites. Some of the diseases caused by *E. coli* are urinary tract infections, bacteremia, meningitis, abscesses, septic infections, pulmonary infections, skin and wound infections etc. (Black, 1993; Pelczar *et al.*, 2005).

**Pseudomonas aeruginosa**

The characteristic features of *Pseudomonas aeruginosa* include the straight or slightly curved rods like structure, motile with polar flagella, catalase-positive & usually oxidase-positive. All Pseudomonas can grow aerobically, but some can also grow anaerobically by using nitrate as an electron acceptor.

A water missible blue pigment known as pyocyanin and a water missible fluorescent pigment known to be pyoverdin are produced by *P. aeruginosa*. It is a saprophytic microbe which mainly resides in the soil and water. It is known to be an opportunistic bacteria which can attack the immune compromised patients and can lead to eye, ear, urinary tract infections and also other diseases like skin lesions and septicemia.

**Staphylococcus aureus**

The staphylococci are aerobic, non-motile, glucose fermenting Gram-positive cocci. Most species resides in mammalian skin and mucus membranes and have no other important habitat.

*Staphylococcus aureus* are responsible for causing suppurative (formation of pus) type diseases in humans, which includes, wound infections, superficial and deep abscesses.
and infections of number of internal organs. *S. aureus* is also an important pathogen of domestic animals, being the principal cause of bovine mastitis.

**Bacillus subtilis**

The genus Bacillus is mainly composed of large Gram-positive rods that grow by formation of the spores and aerobic conditions are the best suited conditions for proper growth of the bacteria. Maximum number of species are saprobic in nature and are found on grow best on vegetation and also grows better in soil, water and air. Bacillus species except *B. anthracis* occasionally are responsible for infections in human beings and for pulmonary and disseminated infections in immunologically compromised hosts; bacteremia in patients undergoing hemodialysis; meningitis after ventricular shunting for hydrocephalus; endocarditis in drug addicts; encphtalalimitis associated with intravenous drug abuse or following penetrating eye injuries; wound infections; and crepitant, myonecrosis following trauma, mimicking gas gangrene.

**Candida albicans**

*Candida albicans* is a dimorphic and opportunistic type of yeast or fungus. Growth of candida albicans is either as yeast or as filamentous cells. It causes oral and genital infections in human beings (Ryan and Ray, 2004; D’ Enfert and Hube, 2007). *C. albicans* have come out as important organism which is responsible for morbidity and mortality especially in patients suffering from immuno compromised diseases like AIDS, organ or bone marrow transplantation and cancer chemotherapy. It is a commensal organism which is being benefited from the other microorganism but it is a harmless commensal. It is a part of the regular gut flora containing microbes that resides in the person’s mouth and besides also in the gastrointestinal tract. Its excess growth leads to the fungal diseases named as candidiasis (candidosis). This disease (candidiasis) is usually pragmatic in patients suffering from immunodeficiency disease in which immune system has already been spoiled due to other diseases including HIV-infected patients. Generally, *Candida* infections due to fungi over growth have been seen in the mouth, skin, or vagina for no apparent reason. A common cause for this type of infectious disease may be the utilization of antibiotics that kills the both types of bacteria advantageous as well as unfavorable in the body, authorize *Candida albicans* to grow quicker in that area.
Aspergillus Niger

Aspergillus niger is a one more type of fungus and is a well known species of the genus Aspergillus. It is the contributing agent of the most common ailments known to black mold, especially on fruits, i.e. grapes, apricots and peanuts and on vegetables, i.e. onions, etc. It is also called as the food contaminant. Human beings are very infrequently infected by this type of fungus, except under extreme conditions e.g. In lung infections causing disease Aspergillosis. This type of disease is commonly seen in the people who works in horticulture and inhales the peat dust, which is said to be a good source of Aspergillus spores. It is mentioned in the literature that these types of spores were usually used to be found in mummies present in the ancient Egyptian tombs and should get inhaled by the person who disturb or go nearby that mummy.

A. niger is even responsible for one of the ear infection called as otomycosis, which induces pain, temporary hearing loss, and, in serious cases even can cause impairment to the tympanic membrane and ear canal. A. niger is also grown to extract enzyme glucose oxidase (GO) and Alpha-galactosidase (AGS) which is helpful in designing the glucose biosensors as it has high attraction for β-D-glucose (Staiano et al., 2005; Ghoshdastider et al., 2015).

Standard drugs:

Ciprofloxacin

In view of ciprofloxacin (Williams and Lamke, 2002; Block and Beale, 2004) reported to show remarkable antibacterial effect against the bacteria used in the present study, it was taken as the standard drug. It inhibits the bacterial enzyme DNA gyrase and topoisomerase which lead to the conformation of DNA and also helps in its storage. Conformation of the DNA is altered by cutting the double strand which are being staggered by four base pairs and further by passage of the uncut part of the DNA from the gap and making the DNA molecule again back by the process of resealing. Due to this degree of twist of DNA is changed which further lead to the release of the torsional stress. Now when ciprofloxacin or any antibacterial agent inhibits these bacterial enzymes, which in turn makes the cells of DNA unreachable due to which cell becomes dead. Topoisomerase IV plays important role in some Gram-positive and DNA gyrase in some Gram-negative organisms.
Humans shape their DNA with a topoisomerase II an analogous enzyme that, however, does not bind quinolones at normally achievable doses so the quinolones of commerce do not kill host cells.

**Clotrimazole**

Clotrimazole is an antifungal drug which is typically applied in the management of fungal infections in both man and other animals especially in vaginal yeast infections. It is furthermore first line of treatment in athlete's foot and jock itch. Clotrimazole acts by killing individual *Candida* or fungal cells by making alterations in the permeability of cell walls of the microorganism. It particularly binds to phospholipids which are present in the cell membrane of the microbe and lead to inhibition of the biosynthesis of ergosterol and other sterols which are major requirement for production of cell membrane. Hence it is responsible for the death of the cell due to loss of intracellular elements.

**4.2.1 Experimental**

Serial two fold technique was used to study antibacterial and antifungal activity.

- **Stock solutions**

  Stock solutions of the synthesized title compounds and the standard drug were prepared in dimethyl sulfoxide (DMSO) having the concentration of 100 µg/ml. Further dilutions were prepared from the stock solution.

- **Media**

  a) **Double Strength Nutrient Broth (DSNB)**

     Nutrient Broth was prepared by dissolving 13 gm of nutrient broth in 1000ml of distilled water.

  b) **Sabouraud dextrose broth**

     Sabouraud dextrose broth was prepared by dissolving 39 gm of Sabouraud dextrose broth in 1000ml of distilled water.

- **Sterilization**

  The sterilization of all the apparatus and ingredients was done by using an autoclave at 15 lbs pressure (121º C) for 20 minutes.
**Stock culture and inoculums**

**For Bacteria:**

Bacterial strains (loopful) were transferred aseptically into the already sterilized nutrient broth and was incubated for 24 h at 37 ± 1°C. It was considered as the stock culture. Subculturing of bacteria was done by using aseptic nutrient agar by streaking method. Then the bacterial culture was grown and dilutions were made accordingly with fresh normal saline solution to acquire the microbial count approximately 1 X 10⁶ colony forming unit (CFU) per ml. An aliquot (0.1 ml) of this bacterial culture in sterilized saline solution was taken for inoculation of the culture tubes.

**For Fungus:**

Fungal strains (loopful) were transferred aseptically into the already sterilized sabouraud dextrose broth and was incubated for 48 hrs at 37 ± 1°C for *C. albicans* and for 7 days at 25 ± 1°C for *A. niger*. It was considered as the stock culture. Subculturing of both the fungal strains was done by using aseptic sabouraud dextrose agar by streaking method. Then the fungal culture was grown and dilutions were made accordingly with fresh normal saline solution to acquire the microbial count approximately 1 X 10⁶ colony forming unit (CFU) per ml. An aliquot (0.1 ml) of this fungal culture in sterilized saline solution was taken for inoculation of the culture tubes.

**Determination of the MIC range**

The serial dilution technique was used for evaluation of antimicrobial activity. A stock solution of synthesized compounds was prepared in dimethyl sulphoxide (100µg/ml). Nutrient broth (I.P.) and sabouraud dextrose broth media (I.P.) (Pharmacopoeia, 1996) were used for bacteria and fungi respectively. Ciprofloxacin and clotrimazole were taken as standard drugs for antibacterial and antifungal activity respectively and DMSO was used as control. Sterilized media (Nutrient broth/ Sabouraud dextrose broth) (1ml) were transferred into sterile test tubes. Stock solution (100µg/ml) (1ml) was put in one tube and serially diluted to give concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.56 µg/ml. 0.1ml suspension of bacteria/ fungus in saline was added to all test tubes and were then incubated at 37°C for 24 h for bacterial strains, 48 h for *C. albicans* and 7 days at 25°C for *A. niger*. The test mixture contained 10⁶ organisms/ml (CFU/ml).
Macroscopic examination of inoculated culture tubes was done for turbidity. MIC was the lowest concentration at which microbial growth attenuated and no turbidity was seen in the tube. The similar procedure was followed for reference drugs and the experiment was done in triplicate. (Bala et al., 2014; Cappucino and Sherman, 1999).

Minimum inhibitory concentration of synthesized compounds against bacterial strains of series-1 (3a-3x) and series-2 (3a’-3m’) are given in Table-4.1 and Table-4.3 respectively. The MIC of synthesized compounds against fungal strains of series-1 (3a-3x) and series-2 (3a’-3m’) are given in Table-4.2 and Table-4.4 respectively.

**Table 4.1: Minimum inhibitory concentration (MIC) (µg/ml) of the synthesized compounds (3a-3x) against Gram positive and Gram negative bacteria**

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. coli (MTCC 40)</th>
<th>P. aeruginosa (MTCC 2453)</th>
<th>S. aureus (MTCC 96)</th>
<th>B. subtilis (MTCC121)</th>
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Table 4.2: Minimum inhibitory concentration (MIC) (µg/ml) of the synthesized compounds (3a-3x) against fungal strains

<table>
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Table 4.3: Minimum inhibitory concentration (MIC) (µg/ml) of the synthesized compounds (3a’-3m’) against Gram positive and Gram negative bacteria

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### Table 4.4: Minimum inhibitory concentration (MIC) (µg/ml) of the synthesized compounds (3a'-3m') against fungal strains

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<td>3d'</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>3e'</td>
<td>3.125</td>
<td>3.125</td>
</tr>
<tr>
<td>3f'</td>
<td>6.25</td>
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<tr>
<td>3g'</td>
<td>3.125</td>
<td>6.25</td>
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<tr>
<td>3h'</td>
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<td>3.125</td>
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<tr>
<td>3i'</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3j'</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>3k'</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>3l'</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>3m'</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 4.2.2 Results and Discussion

All the novel synthesized title compounds of series-1, \(N\)-(benzimidazol-1-ylmethyl)-benzamide derivatives (3a-3x) and series-2, \(N\)-(benzimidazol-1-ylmethyl)-4-chlorobenzamide derivatives (3a'-3m') were demonstrated for their antibacterial and antifungal activity and exhibited good to moderate activity in comparison to standard drug. The MIC for series-1 and series-2 was found to be in the range of \(E. coli\) (12.5-3.125 µg/ml), \(P. aeruginosa\) (12.5-3.125 µg/ml), \(S. aureus\) (12.5-3.125 µg/ml) and \(B. subtilis\) (12.5-3.125 µg/ml) in comparison to reference drug ciprofloxacin (MIC 12.5-3.125 µg/ml) and against fungal strains, \(C. albicans\) (MIC 25-3.125 µg/ml) and \(A. niger\) (MIC 25-3.125 µg/ml) when compared with clotrimazole (MIC 6.25) as standard antimicrobial agent. Structure activity relationship of synthesized compounds (3a-3x) revealed that target molecule 3o with its electron withdrawing Cl group at 2 position of phenyl ring which is substituted at 2-position of benzimidazole showed superior activity against \(P. auregenosa\), \(S. aureus\), \(B. subtilis\) and same effect against \(E. coli\) than...
standard ciprofloxacin. Compound 3q with its electron withdrawing Cl group at 4 position of benzene ring substituted at 2 position of benzimidazole scaffold, showed better activity against *P. aeruginosa*, *S. aureus* and similar effect against *E. coli* and *B. subtilis* as compared to reference drug ciprofloxacin. Compound 3r with its electron withdrawing bromo group at 2 position of phenyl ring substituted at 2 position of benzimidazole moiety showed enhanced results against *P. aeruginosa* and similar effect against all the rest of the three strains when compared with ciprofloxacin. Compounds 3t, 3u, 3w with electron withdrawing Br, NO₂ and F groups at 4 position of phenyl ring substituted at 2 position of benzimidazole scaffold respectively showed better activity against *P. aeruginosa* and *B. subtilis* than standard drug ciprofloxacin. Also it was found that amongst all the synthesized compounds, 3o, 3q and 3r were most significantly active in opposition to fungal strains and rest of the compounds showed moderate activity as compared to standard clotrimazole. Also the molecules substituted with electron releasing alkyl groups like methyl, ethyl, propyl, butyl were found to be least active against all the bacterial and fungal strains. Molecules substituted with amino, pyridyl, hydroxyl, mercapto groups showed good results against *E. coli* and moderate to less active against all the bacterial and fungal strains. During analysis of Series-2, it was found that compounds 3e', 3h', 3k' and 3l' were found to be highly active against all the bacterial strains and compound 3e' and 3h' were found to be highly active against both the fungal strains.

Furthermore, It was observed that there was slight significant difference amongst the MIC values of title compounds of Series-1 and Series-2, however the compounds containing chloro, bromo, flouro and nitro group were found to be more active than rest of the title compounds.

### 4.3 Antioxidant Activity

Antioxidant compounds in food participates a vital role in protecting the health of the living beings. It had been proven scientifically that the antioxidants are very important agents which can reduce the prospects of highly persistent diseases like cancer or any disease related to heart (Kamil *et al.*, 2013). Fruits, vegetables and the whole grains are the rich and primary source of the antioxidants. Antioxidants which are mainly obtained from the plants as the source are vitamin C, carotenes obtained mainly from carrots,
Antimicrobial and Antioxidant Activity

phenolic acids and vitamin E are known to be significant agents which can reduce the hazards of or chances of disease (Miller [a] et al., 2000; Miller [b] et al., 2000).

Antioxidant have characteristic feature of trapping the free radicals. The generated free radicals further cause the oxidation of nucleic acids, proteins, DNA or lipids. These antioxidants further find the generated free radicals to scavenge them and then lead to the inhibition of mechanism of oxidation.

A number of antioxidant activity methodologies had been enlisted in order to observe and evaluate the antioxidant activity of compounds. Recently, oxygen radical absorbance capacity assays and improved chemiluminescence assays have been utilized to characterize the antioxidant potential in various types of foods, serums and the biological fluids.

Various methods are available for measuring radical scavenging activity of antioxidants against free radicals for example 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical, the superoxide anion radical, the hydroxyl radical (OH·) or the peroxyl radical (ROO·). The methodologies which are employed to evaluate the antioxidant activity interpret different results as it totally depends on the type of free radical involved as a reactant.

One of the easy, economical and quick methodologies of measuring antioxidant activity is to utilize the DPPH radical i.e. 1,1-diphenyl-2- picrylhydrazyl as the reactant. It has already been employed to test the capacity of the compounds to perform as a free radical scavenger or as a hydrogen donor. This method is suitable for solids as well as liquids. Moreover it is not at all specific for any antioxidant component rather can be utilized for evaluating whole of the antioxidant ability of the compound under test.

4.3.1 Experimental

**DPPH assay (Free radical scavenging activity)**

The *in-vitro* antioxidant potential was characterized by hydrogen donating or free radical scavenging capacity of the prepared compounds by 1,1-biphenyl-2-picrylhydrazyl radical (DPPH) method.

The antioxidant potential of test sample was measured by estimating the reduction in the absorbance of methanolic solution of DPPH (Albaayit *et al.*, 2014). A stock solution of DPPH (33mg in 1L) was prepared using methanol and 5ml of this stock solution was
Antimicrobial and Antioxidant Activity

Synthesis and biological studies on 2-substituted benzimidazole derivatives

added to 1 ml of test at various concentrations (100, 300, 500, 1000 µg/ml). After 60 min, absorbance was measured at 517nm at different conc. (100, 300, 500, 1000 µg/ml) in comparison to standard drug. Reference compound used was ascorbic acid and experiment was performed in dark. The scavenging activity was calculated in terms of inhibition employing the following formula:

\[
\% \text{ anti-radical activity} = \left[ \frac{(\text{Control absorbance} - \text{Sample absorbance})}{\text{Control absorbance}} \right] \times 100
\]

The antioxidant activity of synthesized compounds (3a-3x) is given in Table 4.5 and Fig. 4.1, synthesized compounds (3a’-3m’) in Table 4.6 and Fig. 4.2.

Table 4.5: Antioxidant activity of synthesized compounds (3a-3x)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>100 µg/ml Avg. ± SD</th>
<th>300 µg/ml Avg. ± SD</th>
<th>500 µg/ml Avg. ± SD</th>
<th>1000 µg/ml Avg. ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a</td>
<td>11.40± 0.85</td>
<td>17.30± 1.35</td>
<td>23.90± 1.40</td>
<td>32.07± 0.31</td>
</tr>
<tr>
<td>2</td>
<td>3b</td>
<td>28.27± 1.00</td>
<td>29.13± 0.49</td>
<td>33.73± 0.80</td>
<td>47.20± 0.53</td>
</tr>
<tr>
<td>3</td>
<td>3c</td>
<td>30.30± 0.95</td>
<td>33.47± 0.46</td>
<td>55.03± 0.50</td>
<td>86.50± 0.66</td>
</tr>
<tr>
<td>4</td>
<td>3d</td>
<td>29.43± 1.00</td>
<td>33.43± 0.78</td>
<td>45.07± 0.55</td>
<td>48.67± 0.55</td>
</tr>
<tr>
<td>5</td>
<td>3e</td>
<td>22.07± 0.67</td>
<td>34.43± 0.65</td>
<td>36.10± 0.92</td>
<td>47.27± 0.42</td>
</tr>
<tr>
<td>6</td>
<td>3f</td>
<td>25.61± 1.04</td>
<td>33.10± 1.81</td>
<td>45.20± 1.00</td>
<td>52.67± 1.03</td>
</tr>
<tr>
<td>7</td>
<td>3g</td>
<td>24.62± 0.95</td>
<td>32.05± 1.54</td>
<td>43.49± 0.99</td>
<td>61.83± 0.60</td>
</tr>
<tr>
<td>8</td>
<td>3h</td>
<td>26.46± 1.00</td>
<td>32.79± 0.60</td>
<td>38.86± 1.25</td>
<td>64.68± 1.16</td>
</tr>
<tr>
<td>9</td>
<td>3i</td>
<td>16.41± 1.02</td>
<td>24.40± 1.02</td>
<td>38.58± 0.81</td>
<td>63.41± 1.06</td>
</tr>
<tr>
<td>10</td>
<td>3j</td>
<td>23.57± 0.61</td>
<td>35.27± 1.37</td>
<td>42.47± 0.59</td>
<td>65.07± 1.68</td>
</tr>
<tr>
<td>11</td>
<td>3k</td>
<td>10.70± 0.66</td>
<td>11.37± 0.51</td>
<td>14.07± 0.31</td>
<td>19.63± 0.81</td>
</tr>
<tr>
<td>12</td>
<td>3l</td>
<td>10.50± 0.75</td>
<td>15.27± 0.86</td>
<td>19.53± 0.87</td>
<td>24.43± 1.00</td>
</tr>
<tr>
<td>13</td>
<td>3m</td>
<td>12.17± 0.25</td>
<td>15.87± 0.80</td>
<td>19.53± 0.70</td>
<td>28.77± 0.55</td>
</tr>
<tr>
<td>14</td>
<td>3n</td>
<td>16.67± 1.19</td>
<td>27.13± 0.49</td>
<td>34.20± 0.66</td>
<td>48.95± 0.07</td>
</tr>
<tr>
<td>15</td>
<td>3o</td>
<td>32.63± 1.06</td>
<td>43.37± 0.96</td>
<td>53.30± 0.46</td>
<td>77.13± 0.35</td>
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<tr>
<td>16</td>
<td>3p</td>
<td>23.48± 1.90</td>
<td>31.13± 1.61</td>
<td>37.63± 0.64</td>
<td>54.45± 0.94</td>
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<tr>
<td>17</td>
<td>3q</td>
<td>33.82± 1.58</td>
<td>46.57± 1.16</td>
<td>62.53± 0.83</td>
<td>74.00± 0.80</td>
</tr>
<tr>
<td>18</td>
<td>3r</td>
<td>21.83± 1.00</td>
<td>30.10± 3.83</td>
<td>49.50± 0.95</td>
<td>72.63± 0.76</td>
</tr>
<tr>
<td>19</td>
<td>3s</td>
<td>22.41± 1.01</td>
<td>33.17± 0.64</td>
<td>47.58± 1.28</td>
<td>52.60± 0.80</td>
</tr>
</tbody>
</table>
Table 4.6: Antioxidant activity of synthesized compounds (3a'-3m')

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>100 µg/ml Avg. ± SD</th>
<th>300 µg/ml Avg. ± SD</th>
<th>500 µg/ml Avg. ± SD</th>
<th>1000 µg/ml Avg. ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a'</td>
<td>35.37 ± 0.57</td>
<td>41.10 ± 1.35</td>
<td>57.57 ± 1.72</td>
<td>81.43 ± 1.66</td>
</tr>
<tr>
<td>2</td>
<td>3b'</td>
<td>12.20 ± 0.53</td>
<td>13.93 ± 1.91</td>
<td>15.27 ± 1.86</td>
<td>21.97 ± 1.50</td>
</tr>
<tr>
<td>3</td>
<td>3c'</td>
<td>13.63 ± 0.32</td>
<td>17.43 ± 1.48</td>
<td>21.00 ± 1.93</td>
<td>28.80 ± 1.32</td>
</tr>
<tr>
<td>4</td>
<td>3d'</td>
<td>19.00 ± 0.46</td>
<td>27.77 ± 0.90</td>
<td>35.30 ± 1.44</td>
<td>49.30 ± 0.61</td>
</tr>
<tr>
<td>5</td>
<td>3e'</td>
<td>36.63 ± 1.06</td>
<td>47.37 ± 0.96</td>
<td>53.30 ± 0.46</td>
<td>73.70 ± 1.72</td>
</tr>
</tbody>
</table>
**Antimicrobial and Antioxidant Activity**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>100 µg/ml</th>
<th>300 µg/ml</th>
<th>500 µg/ml</th>
<th>1000 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>3f'</td>
<td>24.48± 1.90</td>
<td>33.13± 1.61</td>
<td>37.86± 0.64</td>
<td>55.54± 1.94</td>
</tr>
<tr>
<td>7</td>
<td>3g'</td>
<td>35.82± 1.58</td>
<td>48.57± 1.16</td>
<td>54.53± 0.83</td>
<td>70.13 ± 2.26</td>
</tr>
<tr>
<td>8</td>
<td>3h'</td>
<td>25.83± 1.00</td>
<td>33.10± 3.83</td>
<td>52.50± 0.95</td>
<td>67.93 ± 2.05</td>
</tr>
<tr>
<td>9</td>
<td>3i'</td>
<td>24.41± 1.01</td>
<td>33.87± 0.64</td>
<td>49.28± 1.28</td>
<td>52.60± 1.99</td>
</tr>
<tr>
<td>10</td>
<td>3j'</td>
<td>33.14± 1.56</td>
<td>40.42± 1.90</td>
<td>48.53 ± 1.95</td>
<td>64.17 ± 2.08</td>
</tr>
<tr>
<td>11</td>
<td>3k'</td>
<td>23.37 ± 0.45</td>
<td>31.47 ± 0.76</td>
<td>37.63 ± 0.81</td>
<td>40.10 ± 1.32</td>
</tr>
<tr>
<td>12</td>
<td>3l'</td>
<td>26.97 ± 0.31</td>
<td>32.37 ± 1.21</td>
<td>43.97 ± 1.55</td>
<td>58.70 ± 1.67</td>
</tr>
<tr>
<td>13</td>
<td>3m'</td>
<td>28.57 ± 0.40</td>
<td>36.57 ± 1.89</td>
<td>48.60 ± 1.01</td>
<td>56.90 ± 1.50</td>
</tr>
<tr>
<td>Std</td>
<td>Ascorbic acid</td>
<td>75.67 ± 0.76</td>
<td>83.17 ± 0.40</td>
<td>89.30 ± 0.57</td>
<td>92.77 ± 0.38</td>
</tr>
</tbody>
</table>

*n=3*

Fig. 4:2: % age Antioxidant activity of synthesized derivatives and ascorbic acid [3a’-3m’]

**4.3.2 Results and Discussion**

All the novel prepared derivatives (3a-3x) of series-1, and (3a’-3m’) of series-2, were screened for their *in vitro* antioxidant activity and some of them exhibited significant results as compared to standard drug, ascorbic acid.

From Table 4.5, it was found that out of 24 compounds of series-1, eight compounds displayed prominent results when compared with standard ascorbic acid. From all the compounds 3c, 3h, 3j, 3o, 3q, 3r, 3t and 3w showed significantly potent antioxidant activity as compared to standard drug and rest of the compounds showed moderate or less activity.
From Table 4.6, it was established that out of 13 compounds of series-2, five compounds showed momentous results in comparison with standard ascorbic acid. Amongst all the synthesized compounds, 3a’, 3e’, 3g’, 3h’ and 3j’ showed significantly potent antioxidant activity in comparison to standard drug and rest of the compounds showed moderate or less activity. Out of all the compounds, molecules 3c (86.50± 0.66) and 3a’ (81.43 ± 1.66) exhibited highest anti-oxidant activity in comparison to ascorbic acid. Also it was assumed that compounds having good antioxidant activity would be able to exhibit good anti-inflammatory activity. As various free radicals are also responsible for the induction of short term algesia as well as play an important role in the pathogenesis of inflammation. So, the compounds which are good antioxidants might serve as better anti-inflammatory and analgesic agents.
CHAPTER 5
MOLECULAR DOCKING STUDIES

5.1 Computer Aided Drug Design

Computers are an indispensable tool in modern medicinal chemistry and are significant in both drug discovery and drug development. CADD is an exhilarating and distinctive discipline where a number of aspects of applied and basic research merge and stimulate each other. Researchers usually experience small or bigger lack of information regarding structure activity relationship (SAR) at initial stage of discovery (Richards, 1994). Drug design is known to be the rational drug design or rational design. A creative protocol of finding new medicaments established on the information of a biological target. Drug design consists of the design of compounds which are corresponding in shape and charge available to the biological macromolecular target with which they could interact and hence may bind to it (Taft, 2008). Two foremost types of drug designs are described below:

a) Ligand Based Drug design (Indirect Drug Design)

Drug design which is mainly dependent on the knowledge of ligand which binds to the biological target of interest is known as Ligand based drug design. The ligand under test could be utilized for driving of the pharmacophore model that provides the minimum necessary structural characteristics which a molecule should have in order to bind to the target. However, this method in turn could be exploited to design newer chemical entities that may interact with receptor.

b) Structure Based Drug Design (Direct Drug design)

This type of design mainly depends on the knowledge of three dimensional (3D) structure of the target obtained through various methodologies such as X-Ray Crystallography or NMR spectroscopy. So based on the structure of biological target, drugs with higher similarity and specificity could be designed so that they may show proper formation of complex with the receptor and give preferred therapeutic response (Greer, 1994).
5.2 Molecular Docking Studies

In the area of molecular modeling, docking is a technique that determines the favored conformation of one molecule to a second when bound together to form a substantial complex. For the explicit progress of drugs, enzymes participate an important role in the research of the pharmaceutical manufacturing business, as they serve as important target for drug discovery. Inside the span of rational drug design, computational methods become more and more important to plan workflows that are quicker, more proficient and economical. Interaction between biological receptor with ligands is the major principle in drug discovery and development (Silva and Taft, 2005).

Docking of a ligand is characteristically accomplished by creating a number of orientations (or poses) of a ligand inside the active position, and scoring of poses to recognize one or more that closely estimate the bioactive conformation determined by X-ray crystallography. For identification of putative binders from virtual chemical databases and for estimating the binding likeness of protein-ligand complexes docking algorithms are also utilized (Prasanna et al., 2009; La Regina et al., 2008). To establish promising binding modes of a ligand to the active site of a receptor, this computational method is used. It produces a figure of the active site with interaction points called as grid. Further it fits the ligand in the binding position either by search of grid or search of energy. Different types of interaction between receptor and ligand like Vander Waal's interaction, hydrogen bonding, electrostatic and aromatic interactions are considered to calculate the binding energy (Silva and Taft, 2005).

Docking can be classified into two categories on the basis of available software programs. The first one is rigid docking and the second one is flexible docking. In rigid docking both receptor and ligand are rigid. But in real case molecules are flexible even at the lowest energy state. In flexible docking at least the ligand molecule is considered as flexible. Hence calculation of binding energy between receptor and ligand is more accurate by flexible docking (Halperin, 2002). Almost all in modern docking algorithms, such as Molegro Virtual Docker (MVD), AutoDock, DOCK, Flex X, Glide and GOLD, take the ligand with complete flexibility but presume the receptor to be rigid or apply very limited flexibility to side chains. From the above mentioned
Molecular Docking Studies

program, Glide and Molegro Virtual Docker found to be most widely used software’s for docking studies.

In the present study, AutoDock Vina software has been used for docking studies. AutoDock Vina is an integrated program for determining protein–ligand combination. It handles all aspects of the method, starting from preparing the molecules to predicting the possible binding site of the target protein and determining the binding form of the ligand. AutoDock Vina extends better standards of docking which are mainly dependent on a new optimization methodology which could be in combination with a user interface skill focused mainly on usage and efficiency.

5.2.1 Computational Method

Comparative docking of set of ligands with specific proteins involves methodology with easy user interface and their respective scoring function provided by AutoDock Vina.

Steps in methodology:

1) Import a file of protein and ligand followed by formulation of ligands.

2) Second step involves the preparation of the protein and detection of the cavities of the molecules.

3) Execution of a docking set up via docking wizard panel constitutes the third step.

4) Fourth and final step involves procurement of poses of protein-ligand complex after docking has been done with their particular molecular docking scores exhibited in the output file.

5.2.1.1 Ligand preparation

Chemical structures of all the synthesized compounds were drawn using ChemDraw Ultra 8.0 software. Mol2 files of all the derivatives were converted into .pdb files using Marvin Sketch. All the ligand molecules were allowed to be flexible and their torsional roots were detected and chosen. PDB files were further optimised and converted to pdbqt files for molecular docking by using AutoDock Tools 1.5.6.
5.2.1.2 Protein Preparation and detecting cavities of protein molecules

The method of performing docking of ligands to their macromolecular receptors was suitable for present study. In this docking method, semi-flexible docking practice has been followed, in which the receptor protein was kept as rigid while ligands were flexible. The X-ray structure of PDB id: 1C14 and PDB id: 1CX2 was accessible with the help of the protein data bank. Download a target molecule (*.pdb format) and polar hydrogens were added while water molecules were removed by using AutoDock Tools (ADT). Then the *.pdb format of the macromolecule should be converted to *.pdbqt format. Autogrid generation was also performed using AutoDock Tools where values of x, y and z coordinates of active site were determined. Grid based cavity prediction has been attempted for determining binding site. Molecular docking studies of all the derivatives were performed for drug receptor interaction studies for antimicrobial and anti-inflammatory activity.

5.2.1.3 Docking Analysis

Molecular docking analysis was carried out to evaluate the potential of synthetic series against two different targets of antimicrobial and anti-inflammatory activity by using AutoDock Vina keeping all the parameters at default values. Finally, a cnf.txt file was created having receptor and ligand in *.pdbqt format and a grid center with x,y,z coordinates in Angstrom, a grid box size in Å. The Lamarckian Genetic Algorithm (LGA) has been used in order to find best conformers. For each compound, ten different clusters were generated after docking and were ranked based on the free binding energy. The confirmation of the lowest free binding energy has been ranked as the first and considered as best docked conformation. The ligand-receptor interaction images were visualized, which shows the type and distance of interaction and also the atoms of ligand and protein that are involved in interaction. It also helps to find the functional active sites of receptor that were interacting with the ligand. All the results were analyzed on the basis of free binding energy and hydrogen bonding. In docking analysis, less is the binding free energy for a protein, more is the binding capacity of the ligand, was the basic criterion that has been used for evaluating the inhibitory action of the ligands. The resultant receptor-ligand complexes were generated by using PyMol Molecular Graphics System to check whether the synthetic derivatives were bound to the active pocket of the drug targets or not.
5.2.2 Docking studies for antimicrobial activity of synthesized compounds (3a-3x) [Series 1]

Molecular docking studies were performed by means of AutoDock Vina software to get more imminent into the binding interaction of the synthesized compounds. Marvin Sketch application of ChemAxon was used for drawing all the ligands engaged in this study. The 3D structure of enoyl reductase-NAD⁺-triclosan complex (PDB id: 1C14) was procured from Protein Data Bank (www.rcsb.org) (Abo-Salem et al., 2016). Before performing the docking, protein receptor was prepared by merging all the non-polar hydrogens and removing the water of crystallization using the same graphical interface. AutoDock tool uses the hybrid global-local search algorithm which is a big improvement in the genetic algorithm for the best confirmations of legends. Then the protein was defined for the generation of active site, i.e. grid with specific dimensions. The data of docking affinity and interactive amino acids of all the synthesized compounds (3a-3x) is provided in Table 5.1. The parameters of grid box are given in Table 5.2. The secondary structure of the receptor and the interactions of standards with the target protein are displayed in Fig. 5.1 and Fig. 5.2 (a&b) respectively and docking pattern of other synthesized compounds has also been shown in Fig. 5.3-5.10. The AutoDock Vina uses a hybrid scoring function (empirical + knowledge based function) for evaluating binding affinity of ligands with the receptor (Trott and Olson, 2010).

A set of 24 compounds was screened for antimicrobial activity by molecular docking simulations using PDB id: 1C14. The screening results were further compared with the in-vitro studies for their drug receptor interaction.

Table 5.1: Data of docking affinity of synthesized compounds (3a-3x) and interactive amino acids for antimicrobial activity

![Chemical structure of synthesized compounds](image)
## Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

### Table 5.2: x, y, z coordinates of grid box (PDB ID: 1C14)

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<thead>
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<th>Center</th>
<th>X</th>
<th>Size</th>
<th>Y</th>
<th>Size</th>
<th>Z</th>
<th>Size</th>
</tr>
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<tbody>
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### Table 5.3: x, y, z coordinates of grid box (PDB ID: 1C14)

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Fig. 5.1: Secondary structure of protein 1C14

Fig. 5.2: (a) Binding pose for standard ciprofloxacin (docking affinity-6.6 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line

Fig. 5.2: (b) Binding pose for standard clotrimazole (docking affinity-6.4 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line
Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Fig. 5.3: Binding pose for compound 3k (docking affinity -7.7 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line

Fig. 5.4: Binding pose for compound 3l (docking affinity -7.1 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line
Molecular Docking Studies

Fig. 5.5: Binding pose for compound 3m (docking affinity -7.3 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.

Fig. 5.6: Binding pose for compound 3o (docking affinity -8.2 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.
Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Fig. 5.7: Binding pose for compound 3r (docking affinity-7.9 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line

Fig. 5.8: Binding pose for compound 3s (docking affinity-7.8 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line
Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Fig. 5.9: Binding pose for compound 3w (docking affinity-7.8 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line

Fig. 5.10: Binding pose for compound 3x (docking affinity-8.1kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line
5.2.3 Docking studies for antimicrobial activity of synthesized compounds (3a’-3m’) [Series 2]

Molecular docking studies of synthesized compounds (3a’-3m’) were also carried on same microbial protein with PDB id: 1C14, using same docking program AutoDock Vina. All the synthesized compounds were found to have higher docking affinity as compared to standard ciprofloxacin (-6.6 kCal/mol) and clotrimazole (-6.4 kCal/mol). The data of docking affinity and interactive amino acids of all the synthesized compounds (3a’-3m’) is provided in Table 5.3. The parameters of grid box are given in Table 5.2. The secondary structure of the receptor and the interactions of standards with the target protein are displayed in Fig. 5.1 and Fig. 5.2 (a&b) respectively and compounds showing good docking affinity are also shown in Fig. 5.11-5.17. The main amino acid which played a vital role in interaction is Gln1040, Ala43, Glu167 and Val1244. The AutoDock Vina uses a hybrid scoring function (empirical + knowledge based function) for evaluating binding affinity of ligands with the receptor (Trott and Olson, 2010).

A set of 13 compounds was screened for antimicrobial activity by molecular docking simulations using PDB id: 1C14. The screening results were further compared with the in-vitro studies for their drug receptor interaction.

Table 5.3: Data of docking affinity of synthesized compounds (3a’-3m’) and interactive amino acids for antimicrobial activity
### Molecular Docking Studies

**Synthesis and biological studies on 2-substituted benzimidazole derivatives**

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**Fig. 5.11:** Binding pose for compound 3b’ (docking affinity 8.2 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.
Fig. 5.12: Binding pose for compound 3c’ (docking affinity-7.9 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.

Fig. 5.13: Binding pose for compound 3d’ (docking affinity-8.3 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.
Fig. 5.14: Binding pose for compound 3e’ (docking affinity-8.2 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line

Fig. 5.15: Binding pose for compound 3h’ (docking affinity-8.2 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line
Fig. 5.16: Binding pose for compound 3i’ (docking affinity - 8.0 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.

Fig. 5.17: Binding pose for compound 3l’ (docking affinity - 8.1 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line.
5.2.4 Docking studies for anti-inflammatory activity of synthesized compounds (3a-3x) [Series 1]

Before in vivo evaluation, it was thought laudable to study the interaction of synthesized compounds with COX-2 receptor using molecular docking simulations. The principle of the study was to screen the compounds in silico for further in vivo evaluation. Considering 1CX2 (Shah et al., 2010) as target, the series of compounds were docked to get the best in silico confirmations in the domain of selective COX-2 receptor. From the in silico study, 13 hit compounds with docking affinity higher than that of internal ligand SC-558, 4-[4-(4-Bromophenyl)-3-(trifluoromethyl)pyrazol-1-yl]-benzenesulfonamide (-7.6 kCal/mol), were further evaluated for anti-inflammatory, analgesic and ulcerogenic activity. The main amino acids which played a vital role in interaction are Lys194, Asn292, Arg255, Gly99 and Glu193. The data of docking affinity and interactive amino acids of synthesized compounds is provided in Table 5.4. The parameters of grid box are given in Table 5.5. The secondary structure of the receptor and the interactions of internal ligand with the target protein are displayed in Fig. 5.18 and Fig. 5.19 respectively. Compounds showing good docking affinity are also shown in Fig. 5.20-5.25. From the docking study, it has been assumed that the compounds showing good docking affinity as well as interaction with the receptor would acquire better activity.

Table 5.4: Data of docking affinity of synthesized compounds (3a-3x) and interactive amino acids for anti-inflammatory activity
### Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

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Table 5.5: x, y, z coordinates of grid box (PDB ID: 1CX2)

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Molecular Docking Studies

Fig. 5.18: Secondary structure of PDB ID: 1CX2

Fig. 5.19: Binding pose for internal ligand SC-558 (docking affinity-7.6 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Fig. 5.20: Binding pose for compound 3c (docking affinity-9.0 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line
Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Fig. 5.21: Binding pose for compound 3e (docking affinity-6.5 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Fig. 5.22: Binding pose for compound 3h (docking affinity-6.7 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Fig. 5.23: Binding pose for compound 3o (docking affinity-8.9 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line
Molecular Docking Studies

Fig. 5.24: Binding pose for compound 3q (docking affinity -8.6 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Fig. 5.25: Binding pose for compound 3t (docking affinity -8.4 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

5.2.5 Docking studies of synthesized target compounds for anti-inflammatory activity (3a’-3m’) [Series 2]

Molecular docking studies of synthesized target compounds (3a’-3m’) were also carried out in the same manner, on same protein with PDB id: 1CX2 using AutoDock Vina docking program. The main amino acids which played a vital role in interaction are Lys194, Ala98, Ile31 and Gln192. The data of docking affinity and interactive amino acids of synthesized compounds (3a’-3m’) is provided in Table 5.6. The parameters of grid box are given in Table 5.5. The secondary structure of the receptor and the interactions of internal ligand with the target protein are displayed in Fig. 5.18 and Fig. 5.19 respectively and compounds showing good docking affinity are also shown in Fig. 5.26-5.29. From the docking study, it has been assumed that the
compounds showing good docking affinity as well as interaction with the receptor would acquire better activity.

Table 5.6: Data of docking affinity of synthesized compounds (3a’-3m’) and interactive amino acids for anti-inflammatory activity

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<td>3l’</td>
<td>4-F-C₆H₄</td>
<td>-8.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3m’</td>
<td>2-NH₂-C₆H₄</td>
<td>-8.3</td>
<td>1</td>
<td>Lys194</td>
</tr>
<tr>
<td>SC-558</td>
<td>-</td>
<td>-7.6</td>
<td>2</td>
<td>Gly29, Gly32</td>
</tr>
</tbody>
</table>
Fig. 5.26: Binding pose for compound 3a’ (docking affinity-9.0 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Fig. 5.27: Binding pose for compound 3d’ (docking affinity-8.2 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Fig. 5.28: Binding pose for compound 3k’ (docking affinity-8.9 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line
Molecular Docking Studies

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Fig. 5.29: Binding pose for compound 3m’ (docking affinity -8.3 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

5.3 Results and Discussion

5.3.1 Antimicrobial Study

After in vitro antimicrobial evaluation, in silico study was performed using molecular docking to understand the interaction of synthesized compounds with microbial protein. Considering 1C14 as target, all the synthesized compounds were docked to get the best in silico confirmations in the domain of 1C14 protein. Binding affinities of the synthesized compounds were evaluated by using docking program AutoDock Vina, which also optimised the antimicrobial activities of synthesized compounds as possible microbial inhibitors. During analysis, H-bonding and docking affinity were taken as two important parameters for obtaining the hits among the set of compounds. From the in silico study, it was revealed that majority of the synthesized compounds were having higher docking affinity than standards ciprofloxacin (-6.6 kCal/mol) and clotrimazole (6.4 kCal/mol). The main amino acids that have played a vital role in interaction with synthesized compounds are Gln1040, Arg171, Gln40, Arg1171, Ala43, Glu167. Among the synthesized compounds (3a-3x), compound 3o has displayed best docking affinity of -8.2kCal/mol with 1H bond, length of 2.177 Å, shown in Fig. 5.6. In this Fig. it has been shown that 1H bond is formed between ‘N’ of 3o and ‘NH’ group of Arg171. It is a benzamide derivative with 2 Cl phenyl substitution at 2 position of benzimidazole. Substitutions have also been done with aliphatic, aromatic and heteroatoms as well. It has been found that substitutions with methyl, ethyl, butyl,
mercapto, mercaptomethyl, phenyl moieties showed less docking affinity and no interaction with the receptor. Hence, it is assumed that these compounds would also show less antimicrobial activity. Molecules 3r, 3s, 3w and 3x have also displayed good docking affinities of 7.9 kCal/mol with 1H-bond, length of 2.204 Å, 7.8 kCal/mol with 1H-bond, length of 1.905 Å, -7.8 kCal/mol with 1H-bond, length of 2.220 Å, -8.1 kCal/mol with 1H-bond, length of 2.220 Å, respectively and shown in Fig. 5.7, 5.8, 5.9, 5.10. The data of docking affinity and interactive amino acids of all the synthesized compounds (3a-3x) is provided in Table 5.1. The parameters of grid box are given in Table 5.2. The secondary structure of the receptor and interactions of standards with the target protein are displayed in Fig. 5.1 and Fig. 5.2 (a&b) respectively.

Among the synthesized compounds (3a’-3m’), compound 3d’ has displayed best docking affinity of 8.3 kCal/mol with 1H-bond, length of 2.079 Å shown in Fig. 5.13. Molecules 3b’, 3e’, 3h’ and 3i’ has also displayed good docking affinities of 8.2 kCal/mol with 1H-bond, length of 2.075 Å, -8.2 kCal/mol with 1H-bond, length of 1.991 Å, -8.2 kCal/mol with 1H-bond, length of 1.991 Å, -8.0 kCal/mol with 1H-bond, length of 1.851 Å respectively and shown in Fig. 5.11, 5.14, 5.15, 5.16. From Fig. 5.13 and 5.14, it was clearly seen that in both the compounds 3d’ and 3e’, one hydrogen bond was formed between ‘O’ of carbonyl group of 3d’/3e’ and ‘NH’ of Gln1040. The data of docking affinity and interactive amino acids of all the synthesized compounds (3a’-3m’) is provided in Table 5.3.

5.3.2 Anti-inflammatory Study

Molecular docking studies of all the synthesized derivatives were also performed for anti-inflammatory action. The basic principle of the study was to screen the compounds in silico for further in vivo evaluation. Considering 1CX2 as target, the series of compounds were docked to get the best in silico confirmations in the domain of COX-2 receptor. From the in silico study among synthesized derivatives (3a-3x), 13 hit compounds with docking affinity higher than that of internal ligand SC-558, 4-[4-(4-Bromophenyl)-3-(trifluoromethyl)pyrazol-1-yl] benzenesulfonamide (-7.6 kCal/mol), were demonstrated for anti-inflammatory, analgesic and ulcerogenic potential by in vivo screening. The main amino acids which played a vital role in interaction with synthesized compounds (3a-3x) are Lys194, Asn292, Arg255, Gly99 and Glu193. Among the synthesized derivatives (3a-3x), 3c has displayed best docking affinity of 9.0 kCal/mol with 2 H bonds, length of 2.216 Å and 2.036 Å shown in Fig. 5.20. In this
Fig., it has clearly been shown that two hydrogen bonds were formed between ‘NH’ group of 3c with ‘O’ of carbonyl group in Glu193 and ‘N’ group of benzimidazole in 3c and ‘NH’ of carbonyl group in Arg255. It is a benzamide derivative with chloromethyl substitution at 2 position of benzimidazole moiety. Substitutions have been done with aliphatic, aromatic and heteroatoms as well and it was found that when sulphydryl group was incorporated in structure that has led to least docking affinity with no interaction shown with the receptor. Even substitutions with aliphatic moieties like methyl, ethyl, propyl, butyl were also not found to possess good anti-inflammatory activity using in silico studies. Furthermore, investigation of whole series led to the conclusion that substitution with sulphydryl, thiomethyl and aliphatic moieties has decreased the activity and might have least potential in vivo as well. The data of docking affinity and interactive amino acids of synthesized compounds is provided in Table 5.4. The parameters of grid box are given in Table 5.5. The secondary structure of the receptor and interactions of standard with the target protein are displayed in Fig. 5.18 and Fig. 5.19 respectively.

Among the synthesized compounds (3a'-3m'), compound 3a' showed best docking affinity of -9.0 kCal/mol with 1 H bond, length of 2.237 Å shown in Fig. 5.21. From the Fig. it has been clearly seen that one hydrogen bond was formed between ‘O’ of carbonyl group of 3a’ and ‘NH’ of Lys 194. It is a p-chloro benzamide derivative with chloromethyl substitution at 2-position of benzimidazole moiety. Substitutions have also been done with phenyl, 3-Cl phenyl, 4-F phenyl moieties as well but no interaction was shown with the receptor. The main amino acids which played a vital role in interaction with synthesized derivatives are Lys194, Ala98, Ile31 and Gln192. The data of docking affinity and interactive amino acids of synthesized compounds (3a'-3m') is provided in Table 5.6. The parameters of grid box are given in Table 5.5.

Hence, from the docking study, it was concluded that the compounds substituted with halogen, hydroxyl, nitro and amino groups has displayed best interactions with the microbial receptor among both the series and the compounds substituted with chloromethyl, pyridyl, halogen, hydroxyl, nitro and amino groups has displayed best interactions with the COX-2 receptor among both the series. Also, it has been assumed that the compounds showing good docking affinity as well as interaction with the receptor would acquire better activity.
CHAPTER 6

PHARMACOLOGICAL EVALUATION

6.1 Anti-Inflammatory Activity

Inflammation is a typical unavoidable response to any type of virulent response, which frightens the subject. Inflammation responses can be varied from a general to a particular one. As such inflammation being a sequence of events which may cause unbearable and hopeless condition when it is severe. These events in general may be summarized as under:-

i. Initial injury to cause release of inflammatory mediators e.g. Histamine, serotonin, prostaglandin.

ii. Vasodilatation

iii. Increase in permeability of the capillary and increase in release of exudates.

iv. Movement of the leucocytes, occurrence of chemotaxis and phagocytic behavior.

v. Expansion of the fibroblasts, macrophages and other connective tissue cells.

Inflammation is the portion of body’s normal guard organization and is a favorable response in reaction to infections and injury. It is a course in which the body’s cells and usual chemicals guard us from corporeal harm and illness caused due to unknown elements for example viruses and bacteria. The majority of infection fighting cells of body are white blood cells or leukocytes. The most important rationale of inflammation is to eliminate unknown substances and to restore the injured tissues (Harper, 2007). In the current years, pain as well as inflammation are renowned as an over powering load to the healthcare grade of our inhabitants and are the fundamental base of a considerable amount of ailments (Edwards, 2005). Inflammation is known to cause various rheumatic diseases such as rheumatoid arthritis, osteoarthritis, gout, neoplasms and various allergic reactions. A normally established stepwise movement towards the treatment of inflammatory disorders involves non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressive agents, anti-rheumatic drugs and corticosteroids (Shaaban et al., 2008). NSAIDs are extensively used as a fore most preference of drug for the management of a variety of inflammatory disorders, in addition to lessen pain and aches (Garavito, 1996). Mechanism of action of NSAIDs includes the inhibition of
prostaglandin biosynthesis, a few of which are pro-inflammatory intermediaries. This is basically due to inhibition of cyclooxygenase (COX) enzyme implicated in the mechanism of inflammation. Another pathway i.e 5-lipoxygenase (5-LOX) is too accountable for production of inflammatory leukotrienes (Fig. 6.1) (Vane, 1971).

Fig. 6.1: Mechanism of Inflammation: Production of arachidonic acid metabolites and their role in inflammation. TXA$_2$- Thromboxane A$_2$, PGE$_2$-Prostaglandin E$_2$, PGH$_2$- Prostaglandin H$_2$, PGD$_2$- Prostaglandin D$_2$, PGG$_2$- Prostaglandin G$_2$, PGI$_2$- Prostacyclin, PGD synthase- Prostaglandin D synthase, PGF synthase- Prostaglandin F synthase, PGE synthase- Prostaglandin E synthase, 5-HPETE-5-hydroperoxyeicosatetraenoic acid

Chronic use of NSAIDs causes ulcerations in gastrointestinal tract owing to inhibition of cyclooxygenase in tissues. The two isoforms of COX are constitutively expressed as COX-1 in the majority of tissues, whereas COX-2 is activated at the site of inflammation (Weberschock et al., 2007). The development of selective COX-2 inhibitors significantly reduces gastric ulceration associated with chronic use of

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NSAIDs. However, because of cardiovascular toxicity, few selective COX-2 inhibitors are withdrawn from the market (Sun et al., 2007). So an actual requirement exists to expand new anti-inflammatory and analgesic agents with enhanced efficacy, decreased toxicity and less gastric ulceration. Furthermore, heterocyclic compounds are famous to take part in drug discovery process, as the greater part of therapeutic drugs includes a heterocyclic component. Beside the enormous collection of heterocycles, benzimidazole established to be a flexible moiety for development of drugs in pharmaceutical arena. Substitution of Benzimidazole at 1, 2, 5 and 6 positions accomplish the structural necessities for anti-inflammatory and analgesic activity (Bansal and Silakari, 2012; Gaba et al., 2010) along with this benzimidazole nucleus substituted with an appropriate group at 2\textsuperscript{nd} position is an essential structural feature for gastric safety of the molecule (Patil et al., 2008). These characteristic features make benzimidazole an important therapeutic lead for drug development. The need for novel and productive investigation paved the requirement of adequate number of the screening tests. However till today no such type of model is available which sufficiently explains the occurrence of arthritic conditions in human. A number of in-vivo animal arrays had been utilized to measure the capacity of anti-inflammatory agents as listed below:

i) Inhibition of edema which has been induced in the rat hind paw by administration of carrageenan to the rats.

ii) Inhibition of the arthritis (adjuvant) in the species of rat which may be due to induction of Mycobacterium butyricum or M. tuberculosis.

iii) Granuloma formation which is due to induction of implanted cotton pellet under the rat abdomen is also being studied for extent of inhibition.

iv) Inhibition of erythema of guinea pig skin is also studied (when it is being exposed under the UV light or radiations).

v) In-vitro techniques include the ability of anti-inflammatory agents in stabilization of the erythrocyte membranes, which in turn is responsible for inhibition of the prostaglandins biosynthesis, especially in human synoviocytes and chondrocytes (cultured) and cultured fluid of monocytes (Vogel, 2008; Youssef et al., 2010).
6.1.1 Experimental

6.1.1.1 Animals

Swiss albino mice (20-25g) and Albino Wistar rat which weighed approximately (150-200g) of either sex were utilized for current animal study. Animals under test were kept in set of 6 per cage at room temperature (25±1 °C) and environment having relative humidity of 45-55%. A dark and light cycle of 12 h was pursued during the whole span of methodology. An open access to food and water *ad libitum* was provided to the animals under study. The *in vivo* experiments were attempted with the approval of Institutional Animal Ethics Committee, Chitkara College of Pharmacy, Punjab, India (Regd No. 1181/PO/Ebi/08/CPCSEA).

6.1.1.2 Acute toxicity studies

The acute toxicity studies were attempted in groups consisting of six swiss albino mice, weighing 25±2g, fasted overnight and further administered orally with test compounds with dosage of 1000 mg/kg body weight for evaluating their toxic effect as per OECD 423 guidelines.

6.1.1.3 Anti-inflammatory activity by Carrageenan- induced rat paw edema method [Series-1]

Anti-inflammatory activity of synthesized *N-(Benzimidazol-1-ylmethyl)-benzamide* derivatives was performed by carrageenan induced paw edema method. Test compounds were selected based on docking studies. A set of 24 compounds were investigated for anti-inflammatory activity by molecular docking simulations using pdbid: 1CX2. The screening resulted in 13 hit compounds which were further characterized for the anti-inflammatory behavior.

Out of 13 compounds, only one compound 3u showed mortality in mice at dosage of 1g/kg *p.o*. Rest all compounds were non-toxic and did not show any mortality in animals.

All the selected prepared derivatives were evaluated for anti-inflammatory activity by orally administering the compounds to the albino rats (either sex) in the form of a suspension in DMSO (10% DMSO in water, 10ml/kg) one hour prior to the carrageenan injection (0.1 ml of 1% w/v suspension) (Winter *et al*., 1962; Fayyaz *et al*., 1992), was given in the right hind paw of the rats under the plantar aponeurosis.
Vehicle treated group was considered as control. The paw edema volume was calculated by plethysmograph using mercury displacement method. According to this method paw volume was measured at 0h and 4h (just after giving injection and 4h after the carrageenan injection had been given). The % age edema which was developed is consecutively tabulated in Table 6.1. Anti-inflammatory activity (% age) has been calculated as per the formula given below:

\[
\text{Percentage inhibition of paw edema} = (1 - \frac{V_t}{V_c}) \times 100
\]

\[
\% \text{ edema} = 100 - [(1 - \frac{V_t}{V_c}) \times 100]
\]

\[
\% \text{ reduction in edema} = (1 - \frac{V_t}{V_c}) \times 100
\]

Where, \(V_t\) and \(V_c\) is the edema volume in drug treated and control groups, respectively.

Final effective dose of compounds was selected by performing pilot studies.

### Table 6.1: Anti-inflammatory activity of test compounds and Diclofenac Sodium [Series-1]

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>Percentage (%) Edema at 4th hr</th>
<th>Percentage (%) Reduction in Edema at 4th hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>(10ml/kg)</td>
<td>100±5.25</td>
<td>0.00</td>
</tr>
<tr>
<td>2.</td>
<td>Diclofenac Sodium</td>
<td>(50 mg/kg)</td>
<td>23.75±1.25 (^a)</td>
<td>76.25±3.75 (^a)</td>
</tr>
<tr>
<td>3.</td>
<td>3c</td>
<td>(100 mg/kg)</td>
<td>29.50±1.55 (^a)</td>
<td>70.50±2.25 (^a)</td>
</tr>
<tr>
<td>4.</td>
<td>3k</td>
<td>(100 mg/kg)</td>
<td>73.85±3.55</td>
<td>26.15±1.20</td>
</tr>
<tr>
<td>5.</td>
<td>3m</td>
<td>(100 mg/kg)</td>
<td>68.35±3.05</td>
<td>31.65±1.29</td>
</tr>
<tr>
<td>6.</td>
<td>3n</td>
<td>(100 mg/kg)</td>
<td>71.25±3.25</td>
<td>28.75±1.34</td>
</tr>
<tr>
<td>7.</td>
<td>3o</td>
<td>(100 mg/kg)</td>
<td>30.34±1.25 (^a)</td>
<td>69.66±2.75 (^a)</td>
</tr>
<tr>
<td>8.</td>
<td>3p</td>
<td>(100 mg/kg)</td>
<td>65.28±3.75</td>
<td>34.72±1.54</td>
</tr>
<tr>
<td>9.</td>
<td>3q</td>
<td>(100 mg/kg)</td>
<td>34.18±1.5 (^a)</td>
<td>65.82±2.15 (^a)</td>
</tr>
<tr>
<td>10.</td>
<td>3r</td>
<td>(100 mg/kg)</td>
<td>33.81±1.2 (^a)</td>
<td>66.19±2.25 (^a)</td>
</tr>
<tr>
<td>11.</td>
<td>3s</td>
<td>(100 mg/kg)</td>
<td>60.23±2.78</td>
<td>39.77±1.46</td>
</tr>
<tr>
<td>12.</td>
<td>3t</td>
<td>(100 mg/kg)</td>
<td>36.59±1.45 (^a)</td>
<td>63.41±2.10 (^a)</td>
</tr>
<tr>
<td>13.</td>
<td>3w</td>
<td>(100 mg/kg)</td>
<td>50.25±2.23</td>
<td>49.75±1.95</td>
</tr>
<tr>
<td>14.</td>
<td>3x</td>
<td>(100 mg/kg)</td>
<td>55.25±2.25</td>
<td>44.75±1.5</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of mean (SEM) (n=6). \(^a\)Statistically significant compared to control group \((p \leq 0.05)\). All the statistical analysis was performed by one-way ANOVA followed by Tukey’s test as a post hoc analysis.
6.1.1.4 Anti-inflammatory activity by Carrageenan-induced rat paw edema method [Series-2]

Anti-inflammatory activity of synthesized N-(Benzimidazol-1-ylmethyl)-4-chlorobenzamide derivatives was performed by carrageenan induced paw edema method. A set of 13 compounds was evaluated for anti-inflammatory activity by the same method as given above. Out of 13 compounds, only one compound 3k' showed toxicity in mice at dosage of 1000 mg/kg p.o. Rest all the compounds were non-toxic and did not show any mortality in animals. The % age reduction in edema is shown in Table 6.2.

Table 6.2: Anti-inflammatory activity of test compounds (3a’-3m’) and Diclofenac Sodium [Series-2]

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>Percentage (%) Edema at 4th hr</th>
<th>Percentage (%) Reduction in Edema at 4th hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>(10ml/kg)</td>
<td>100±4.05</td>
<td>0.00</td>
</tr>
<tr>
<td>2.</td>
<td>Diclofenac Sodium</td>
<td>(50 mg/kg)</td>
<td>23.75±1.15</td>
<td>76.25±2.75a</td>
</tr>
<tr>
<td>3.</td>
<td>3a’</td>
<td>(100 mg/kg)</td>
<td>33.34±1.35</td>
<td>66.66±2.45a</td>
</tr>
<tr>
<td>4.</td>
<td>3b’</td>
<td>(100 mg/kg)</td>
<td>79.17±4.55</td>
<td>20.83±1.15</td>
</tr>
<tr>
<td>5.</td>
<td>3c’</td>
<td>(100 mg/kg)</td>
<td>70.84±3.55</td>
<td>29.16±1.35</td>
</tr>
<tr>
<td>6.</td>
<td>3d’</td>
<td>(100 mg/kg)</td>
<td>75.00±2.9</td>
<td>25.00±1.05</td>
</tr>
<tr>
<td>7.</td>
<td>3e’</td>
<td>(100 mg/kg)</td>
<td>37.50±1.45</td>
<td>62.50±2.55a</td>
</tr>
<tr>
<td>8.</td>
<td>3f’</td>
<td>(100 mg/kg)</td>
<td>66.67±3.55</td>
<td>33.33±1.84</td>
</tr>
<tr>
<td>9.</td>
<td>3g’</td>
<td>(100 mg/kg)</td>
<td>45.84±1.40</td>
<td>54.16±2.1a</td>
</tr>
<tr>
<td>10.</td>
<td>3h’</td>
<td>(100mg/kg)</td>
<td>41.67±1.1a</td>
<td>58.33±2.25a</td>
</tr>
<tr>
<td>11.</td>
<td>3i’</td>
<td>(100 mg/kg)</td>
<td>62.50±2.50</td>
<td>37.50±2.65</td>
</tr>
<tr>
<td>12.</td>
<td>3j’</td>
<td>(100 mg/kg)</td>
<td>50.00±1.35</td>
<td>50.00±2.95a</td>
</tr>
<tr>
<td>13.</td>
<td>3l’</td>
<td>(100 mg/kg)</td>
<td>54.17±2.75</td>
<td>45.83±1.85</td>
</tr>
<tr>
<td>14.</td>
<td>3m’</td>
<td>(100 mg/kg)</td>
<td>58.34±2.85</td>
<td>41.66±1.55</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of mean (SEM) (n=6). *Statistically significant compared to control group (p ≤ 0.05). All the statistical analysis was performed by one-way ANOVA followed by Tukey’s test as a post hoc analysis.

6.1.2 Results and Discussion

After performing in silico studies, compounds showing higher dock score than internal ligand were selected for in vivo studies. Synthesized 26 compounds were screened
Pharmacological Evaluation

Synthesis and biological studies on 2-substituted benzimidazole derivatives pharmacologically for their anti-inflammatory activity and few of them showed appreciable results. Although docking affinity of all the selected compounds were higher than internal ligand, it was assumed that these compounds would show good anti-inflammatory activity. But the molecules substituted with phenyl, 3-pyridyl, 2-OH phenyl, 2-NH$_2$ phenyl, 3-Cl phenyl, 3-Br phenyl and 4-F phenyl groups at 2- position of benzimidazole moiety showed very less anti-inflammatory activity and molecules substituted with chloromethyl, 2-Cl phenyl, 2-Br phenyl, 4-Cl phenyl and 4-Br phenyl groups at 2- position of benzimidazole moiety showed significant results. Compounds substituted with 4-NO$_2$ phenyl moiety at 2- position of benzimidazole displayed good dock score but when studied in vivo, was found to be fatal.

From Table 6.1, it was found that out of 12 compounds of series-1, five compounds exhibited appreciable outcome when compared with standard diclofenac sodium. Out of all the products (3a-3x), molecules 3c, 3o, 3q, 3r and 3t showed statistically significant ($p \leq 0.05$) potent anti-inflammatory activity in comparison to control group and rest of the compounds showed moderate or less activity.

Table 6.2 showed that out of 12 compounds of series-2, five compounds displayed remarkable results when compared with standard diclofenac sodium. Amongst the synthesized compounds (3a’-3m’), molecules 3a’, 3e’, 3g’, 3h’ and 3j’ showed statistically significant ($p \leq 0.05$) potent anti-inflammatory activity in comparison to control group and rest of the compounds showed moderate or less activity. Out of the synthesized derivatives, compounds 3c and 3a’ were found to highly potent and compounds 3k and 3b’ were found to be least active.

All the synthesized compounds that showed better results were substituted with electron withdrawing substituents (Cl, Br) at 2 and 4 positions of phenyl ring substituted at 2 position of benzimidazole.

6.2 Analgesic Activity

6.2.1 Pain

The most pervasive, frightening, and important symptom of injury, and much of disease, is pain. Pain should be considered a syndrome of highly unpleasant sensations rather than a symptom. Everyone knows what pain is, yet it is difficult to define. One concept views pain as a two component phenomenon: the initial sensation, or actual
perception, and the psychological effect or reaction component. Clinically pain is classified into two distinct types: acute and chronic. Acute pain is a package of highly unpleasant bad experiences of emotion often culminating in behavioral responses. Acute pain is, invariably, produced due to any kind of illness, by coming in contact, due to ingesting of some toxic chemicals or any type of injury or affected by physical stimulant (e.g. heat). However chronic pain, due to its persistent and pathological form, does not show any type of interference in the biological functioning. It enforces severe stresses of higher magnitude let it be physical, emotional, or social. The ways in which a patient responds to the both types of pains are entirely different. Another categorization considers pain from its point of origin. Thus visceral pain emanates from non-skeletal parts of the body such as gastric pain, intestinal cramps, and colic. The so-called non-narcotic or milder analgesics are usually ineffective in these instances. Somatic pain emanates from muscle and bone and includes headaches, sprains and arthritic pain (Gringauz, 1997).

6.2.2 Analgesics

An analgesic may be defined as a drug bringing about insensibility to pain without loss of consciousness. Analgesics have been classified in terms of their capability of controlling the severity of pain and the extent to which they are capable of causing dependence and addiction. On this basis, the analgesic drugs have been classified into two main groups.

6.2.2.1 Narcotic Analgesics

These are types of drugs which are responsible for relief of extreme level of pain, but the major disadvantage of these drugs is being moderate or strong addictive in nature. Few examples of this category includes the opiates and related drugs, which bind to opiate receptors, e.g. morphine, codeine, meperidine, methadone etc. (Goodman and Gillman, 2011).

6.2.2.2 Non-narcotic Analgesics

This category of drugs help in reducing as well as relieving soft to moderate levels of pain, and being non addictive is the major advantage which is making these drugs more popular e.g. aspirin, acetaminophen etc. They have no affinity for the opiate receptors and their site of action is peripheral (Goodman and Gillman, 1996).
6.2.3 Study of Analgesic Activity

6.2.3.1 Acetic acid-induced writhing assay

To investigate the analgesic potential of newly selected synthesized compounds (five compounds from each series) showing significant anti-inflammatory activity, acetic acid-induced writhing test was being performed (Koster, 1959). Swiss albino mice were utilized for this type of study. The animals were divided into control, standard (positive control) and different test groups consisting of six mice each. The treatment of control group was done using DMSO (10% DMSO in water, 10 ml/kg, p.o.) however standard (positive control) as well as test compound groups were treated with indomethacin and various selected derivatives respectively at a dose of 30 mg/kg and 100 mg/kg respectively suspended in 10% DMSO p.o, 30 min. prior to the i.p. injection of the acetic acid solution at a dose of 1 ml/kg. The number of writhes per animal was recorded for 15 minutes (Fig. 6.2). The analgesic potential was exhibited in percentage of protection and the results are given in Table 6.3. % age analgesic activity was calculated by the following formula:

\[
\text{% age protection} = \left[ 1 - \frac{\text{No. of writhes of test}}{\text{No. of writhes of control}} \right] \times 100
\]

Final effective dose of compounds was selected by performing pilot studies.

Analgesic activity of test compounds

![Graph showing analgesic activity of tested compounds and Indomethacin](image)

**Fig. 6.2: No. of writhes of tested compounds and Indomethacin**

\(^{a}p<0.001\) vs Vehicle Treated (10 % DMSO in Water, 10 ml/kg), (n=6/group), One-way ANOVA; SEM = Standard error of mean. All the statistical analysis was performed by one-way ANOVA followed by Tukey’s test as a post hoc analysis.
Synthesis and biological studies on 2-substituted benzimidazole derivatives

Table 6.3: Percentage Analgesic Protection of tested compounds

<table>
<thead>
<tr>
<th>S. No</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Percentage Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>(10ml/kg)</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>Indomethacin</td>
<td>(30mg/kg)</td>
<td>66.06\textsuperscript{a}</td>
</tr>
<tr>
<td>3.</td>
<td>3c</td>
<td>(100mg/kg)</td>
<td>63.66\textsuperscript{a}</td>
</tr>
<tr>
<td>4.</td>
<td>3o</td>
<td>(100mg/kg)</td>
<td>61.03\textsuperscript{a}</td>
</tr>
<tr>
<td>5.</td>
<td>3q</td>
<td>(100mg/kg)</td>
<td>53.63\textsuperscript{a}</td>
</tr>
<tr>
<td>6.</td>
<td>3r</td>
<td>(100mg/kg)</td>
<td>56.74\textsuperscript{a}</td>
</tr>
<tr>
<td>7.</td>
<td>3t</td>
<td>(100mg/kg)</td>
<td>52.24\textsuperscript{a}</td>
</tr>
<tr>
<td>8.</td>
<td>3a\textsuperscript{'}</td>
<td>(100mg/kg)</td>
<td>62.63\textsuperscript{a}</td>
</tr>
<tr>
<td>9.</td>
<td>3e\textsuperscript{'}</td>
<td>(100mg/kg)</td>
<td>59.48\textsuperscript{a}</td>
</tr>
<tr>
<td>10.</td>
<td>3g\textsuperscript{'}</td>
<td>(100mg/kg)</td>
<td>53.97\textsuperscript{a}</td>
</tr>
<tr>
<td>11.</td>
<td>3h\textsuperscript{'}</td>
<td>(100mg/kg)</td>
<td>55.34\textsuperscript{a}</td>
</tr>
<tr>
<td>12.</td>
<td>3j\textsuperscript{'}</td>
<td>(100mg/kg)</td>
<td>51.21\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Statistically significant compared to Control group (10 % DMSO in Water, 10 ml/kg ), \((p \leq 0.01)\). All the statistical analysis was performed by one-way ANOVA followed by Tukey’s test as a post hoc analysis.

6.2.4 Results and Discussion

Based on the results of anti-inflammatory activity only ten compounds were selected from series-1 [N-(Benzimidazol-1-ylmethyl)-benzamide] analogues and series-2 [N-(Benzimidazol-1-ylmethyl)-4-chloro-benzamide] derivatives for analgesic activity. Fig. 6.2 and Table 6.3 revealed that all the selected test compounds presented significant \((p \leq 0.01)\) and potent analgesic activity ranging from 51.21\%-63.66\% as compared to control group. Molecules 3c, 3o, 3a\textsuperscript{'} , 3e\textsuperscript{'} showed most significant results.

Although all the selected compounds showed good analgesic effects but molecules substituted with chloromethyl, 2-Cl phenyl, 2-Br phenyl moieties at 2- position of benzimidazole displayed potent analgesic activity when compared to standard indomethacin.

Hence it is concluded that the compounds bearing electron withdrawing substituents in their structure showed better analgesic activity.
6.3 Ulcerogenic Potential

6.3.1 Gastritis

Gastritis is a type of histological diagnosis, although in severe cases it is being diagnosed with the help of endoscopy. One of the type of gastritis is acute gastritis which is usually erosion causing and hemorrhagic also. Neutrophils are mainly accountable for inflammation on the surface of epithelium. In most of the cases, it occurs due to Aspirin or NSAIDs administration. There are no specific symptoms for the acute gastritis, but can be responsible for causing vomiting, anorexia, hematemia or nausea and also dyspepsia. NSAIDs could be one of the reasons for gastric damage proposes two mechanisms of action. First and foremost mechanism is by occurrence of local irritation due to oral administration which leads to back diffusion of the acid increasing the acidic pH of the stomach and finally causing the tissue damage. Other mechanism is due to the parenteral administration of NSAIDS which can even lead to bleeding and damage in the gastric mucosa due to inhibition of the biosynthesis of gastric prostaglandins, specifically PGI$_2$ and PGE$_2$ which are cytoprotective agents in the stomach mucosa.

Literature revealed that benzimidazole nucleus substituted with an appropriate group at 2- position is an essential structural feature for gastric safety of the molecule (Patil et al., 2008).

The track for novel and more creative investigation depends on the accessibility of adequate screening tests. The most commonly used in vivo animal assay measures the gastroprotective agents by the following concepts:

- Inhibition of gastric lesions induced by HCl-Ethanol in rats
- Measurement of gastric acid discharge in pylorus-ligated rats.
- Determination of gastric potential difference (PD) and mucosal blood stream.
- Determination of gastric motility
- Determination of prostaglandin E$_2$ (PGE$_2$)
- Measurement of Nitrite and Nitrate in serum and gastric contents (Vogel, 2008).
6.3.2 Experimental

6.3.2.1 Animals

The test compounds showing significant anti-inflammatory and analgesic activity were tested for their gastric ulcerogenic potential. During the experiments, Wistar rats of either sex weighing 150-200 gm were used. The animals were placed in separate cages and were not given food but permitted to free availability of tap water for 24 h prior to the experiment. Studies were done using twelve groups of animals. Each group contained six animals.

6.3.2.2 Ulcerogenic assay

Wistar rats of either sex weighing 150-200 g were divided into control, standard (Indomethacin) and different test compound groups (n=6). The animals were anaesthetized and the abdomen was opened by mid line incision and pylorus ligated. The test compounds and reference drug were administered in animals orally at a dose of 100 mg/kg and 30 mg/kg, respectively by dissolving in 2% sodium carboxymethyl cellulose (CMC)/suspension in DMSO. Later on after 6 h, the rats were sacrificed for ulcerogenic activity, and their stomach was removed. Formalin (10% v/v) was then injected into the suture ligated stomach for further examination after storage overnight. The stomach was opened next day, washed in normal saline, and examined under magnifier for measuring the length of lesions. The lengths of the longest diameters of the lesions were measured and summated to give a total lesion score (in mm) for each animal, the mean count for each group being calculated (Vogel, 2002) and results are given in Table 6.4.

Table 6.4: Ulcerogenic activity of test compounds and Indomethacin

<table>
<thead>
<tr>
<th>S. No</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Lesion score (mm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>(10ml/kg)</td>
<td>1.0 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.</td>
<td>Indomethacin</td>
<td>(30mg/kg)</td>
<td>32.50 ± 2.50</td>
</tr>
<tr>
<td>3.</td>
<td>3c</td>
<td>(100mg/kg)</td>
<td>13.90 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.</td>
<td>3o</td>
<td>(100mg/kg)</td>
<td>15.85 ± 1.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.</td>
<td>3q</td>
<td>(100mg/kg)</td>
<td>18.90 ± 1.85</td>
</tr>
<tr>
<td>6.</td>
<td>3r</td>
<td>(100mg/kg)</td>
<td>16.55 ± 1.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.</td>
<td>3t</td>
<td>(100mg/kg)</td>
<td>20.45 ± 2.05</td>
</tr>
</tbody>
</table>
Synthesis and biological studies on 2-substituted benzimidazole derivatives

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>MLS (mm) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.</td>
<td>3a’</td>
<td>100</td>
<td>12.25 ± 0.85</td>
</tr>
<tr>
<td>9.</td>
<td>3e’</td>
<td>100</td>
<td>13.53 ± 0.92</td>
</tr>
<tr>
<td>10.</td>
<td>3g’</td>
<td>100</td>
<td>16.10 ± 1.15</td>
</tr>
<tr>
<td>11.</td>
<td>3h’</td>
<td>100</td>
<td>15.25 ± 1.05</td>
</tr>
<tr>
<td>12.</td>
<td>3j’</td>
<td>100</td>
<td>19.50 ± 1.35</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=6). *Statistically significant compared to Indomethacin (p ≤ 0.05). All the statistical analysis was performed by one-way ANOVA followed by Tukey’s test as post hoc analysis.

6.3.2.3 Statistical Analysis

All the results were expressed as mean ± standard error of mean (S.E.M). Data of the result was analysed by using one-way variance (ANOVA) followed by Tukey’s test. A value of p≤ 0.05 was considered as statistically significant. The SIGMA Plot 13, Systat Software, Inc. 2107 North First Street, Suite 360, San Jose, CA 95131 USA, was used for statistical analysis.

6.3.3 Results and Discussion

The test compounds showing significant anti-inflammatory and analgesic activity were tested for their gastric ulcerogenic potential in terms of mean lesion score (MLS) measured in millimeter (mm). Five compounds were selected from each series 1 and 2. Among ten compounds, six derivatives showed significant (p≤ 0.05) results for the ulcerogenic activity as compared to indomethacin as reference drug (MLS 32.50 ± 2.50). Results revealed that compound 3c, 3o and 3r as indicated by their low lesion score 13.90 ± 0.95, 15.85 ± 1.18 and 16.55 ± 1.25 respectively from series-1 and 3a’, 3e’ and 3h’ as indicated by their low lesion score 12.25 ± 0.85, 13.53 ± 0.92, 15.25 ± 1.05 from series-2 were most potent among ten compounds used for respective study.

Furthermore compounds substituted with chloromethyl, 2-chloro phenyl and 2- bromo phenyl moieties at 2 position of benzimidazole nucleus containing benzamide and p-chlorobenzamide scaffold showed less mean lesion score as compared to the compounds substituted with 4 chloro phenyl and 4- bromo phenyl at 2- position of benzimidazole nucleus. Also it was found that molecules containing p-chloro benzamide moiety were found to have less lesion score as compared to the compounds containing benzamide moiety. The ulcerogenic activity was found in following order: (From lower to higher side):

3a’>3c>3e’>3o=3h’>3g’>3r>3q>3j’>3t>indomethacin
CHAPTER 7
SUMMARY AND CONCLUSION

7.1 Summary

Heterocyclic compounds play a major role in drug discovery, as many of the therapeutic useful drugs contain a heterocyclic moiety. Owing to wide variety of heterocycles, benzimidazole is one such compound, which creates a center of attention of synthetic chemists for scheming other effective benzimidazole compounds with miscellaneous biological actions. Benzimidazole is a fused heterocycle of benzene and imidazole nucleus. It is a heterocyclic aromatic organic compound. Furthermore, it is a resourceful heterocyclic scaffold in medicinal compounds, exhibiting a broad spectrum of pharmacological activities. Also, benzimidazoles are structural isosters of nucleotides occurring in nature that allows them to simply work together with the enzymes of the biological system owing to their numerous biological activities. Benzimidazole containing compounds show biological activities as anti-allergic agents, antimicrobial, antioxidant, PARP inhibitors- as anticancer agents and as cytomegalovirus (HCMV) inhibitors, antiulcer, anti-inflammatory and as antihistaminic.

Furthermore, mannich bases are the beta amino ketone containing compounds and are the last part of mannich reaction. Mannich reaction is a carbon-carbon bond building nucleophilic addition reaction that includes condensation of a compound with active hydrogen, with an amine (primary or secondary) and formaldehyde (non enolizable aldehyde). A variety of mannich bases have been described to possess analgesic, anti-inflammatory, anticancer, anticonvulsant, antiviral, anthelmintic, antimalarial, antibacterial, antifungal and several other activities. These reports provided a platform to carry out the synthesis of some novel benzimidazole compounds via mannich reaction. With the expectations that these compounds might be having enhanced biological activities represented by following general structures (3a-3x) and (3a’-3m’). The novel compounds were also screened by in silico method (Molecular Docking Studies) to study drug receptor interaction between novel compounds and target protein. General structure of title compounds is given below:
7.1.1 Synthesis

7.1.1.1 General method for the synthesis of \( N \)-(Benzimidazol-1-ylmethyl)-benzamide derivatives (SERIES-I)

STEP-1 Synthesis of 2-substituted benzimidazoles [Scheme-(1a-1e)]

Scheme 1a: Synthesis of benzimidazole using o-phenylenediamine

Scheme 1b: Synthesis of 2-substituted benzimidazoles using o-phenylenediamine dihydrochloride

Scheme 1c: Synthesis of 2-amino benzimidazole using o-phenylenediamine
Synthesis and biological studies on 2-substituted benzimidazole derivatives

**Scheme 1d: Synthesis of 2-mercapto benzimidazole using o-phenylenediamine**

**Scheme 1e: Synthesis of 2-substituted benzimidazoles using o-phenylenediamine**

**STEP-2 Synthesis of Mannich bases of 2-substituted benzimidazole derivatives**

[Scheme-2]

**Scheme 2: Synthesis of mannich bases from 2-substituted benzimidazoles**

List of synthesized compounds is provided in Table 7.1 (3a-3x)

**Table 7.1: List of synthesized compounds [Series-1]**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>R</th>
<th>S. No.</th>
<th>Compound</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a</td>
<td>H</td>
<td>13</td>
<td>3m</td>
<td>3-NC₅H₄</td>
</tr>
<tr>
<td>2</td>
<td>3b</td>
<td>CH₃</td>
<td>14</td>
<td>3n</td>
<td>2-OH-C₆H₄</td>
</tr>
<tr>
<td>3</td>
<td>3c</td>
<td>ClCH₂</td>
<td>15</td>
<td>3o</td>
<td>2-Cl-C₆H₄</td>
</tr>
<tr>
<td>4</td>
<td>3d</td>
<td>C₂H₅</td>
<td>16</td>
<td>3p</td>
<td>3-Cl-C₆H₄</td>
</tr>
<tr>
<td>5</td>
<td>3e</td>
<td>C₃H₈</td>
<td>17</td>
<td>3q</td>
<td>4-Cl-C₆H₄</td>
</tr>
</tbody>
</table>
Synthesis and biological studies on 2-substituted benzimidazole derivatives

### Summary and Conclusion

**Synthesis and biological studies on 2-substituted benzimidazole derivatives**

Stationary phase: Silica gel G, Mobile phase for TLC: chloroform: methanol (9.5:0.5)

**7.1.1.2 General method for the synthesis of N-(Benzimidazol-1-ylmethyl)-4-chlorobenzamide derivatives (SERIES-2)**

![Scheme 3: Synthesis of mannich bases from 2-substituted benzimidazoles](image)

List of synthesized compounds is provided in Table 7.2.

**Table 7.2: List of synthesized compounds [Series-2]**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>R</th>
<th>S. No.</th>
<th>Compound</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a′</td>
<td>ClC2H2</td>
<td>8</td>
<td>3h′</td>
<td>2-Br-C6H4</td>
</tr>
<tr>
<td>2</td>
<td>3b′</td>
<td>C6H5</td>
<td>9</td>
<td>3i′</td>
<td>3-Br-C6H4</td>
</tr>
<tr>
<td>3</td>
<td>3c′</td>
<td>3-NClC6H4</td>
<td>10</td>
<td>3j′</td>
<td>4-Br-C6H4</td>
</tr>
<tr>
<td>4</td>
<td>3d′</td>
<td>2-OHC6H4</td>
<td>11</td>
<td>3k′</td>
<td>4-NO2-C6H4</td>
</tr>
<tr>
<td>5</td>
<td>3e′</td>
<td>2-ClC6H4</td>
<td>12</td>
<td>3l′</td>
<td>4-F-C6H4</td>
</tr>
<tr>
<td>6</td>
<td>3f′</td>
<td>3-ClC6H4</td>
<td>13</td>
<td>3m′</td>
<td>2-NH2-C6H4</td>
</tr>
<tr>
<td>7</td>
<td>3g′</td>
<td>4-ClC6H4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Synthesis and biological studies on 2-substituted benzimidazole derivatives
7.1.2 Characterization

Purity of all the synthesized compounds was ascertained by TLC. The structures of all the synthesized title compounds were elucidated by spectral analysis. Melting points were taken by the melting point determination apparatus (PERFIT) in open capillary tubes and were uncorrected.

7.1.3 Antimicrobial and Antioxidant activity

7.1.3.1 Antibacterial and antifungal activity

All the synthesized title compounds were screened for their antibacterial and antifungal activity by serial dilution method. Following strains were used for antimicrobial study.

**A) Bacterial strains**

1. *Escherichia coli* (Gram -ve), (MTCC 40)
2. *Pseudomonas aeruginosa* (Gram –ve), (MTCC 2453)
3. *Staphylococcus aureus* (Gram +ve), (MTCC 96)
4. *Bacillus subtilis* (Gram +ve), (MTCC 121)

**B) Fungal strains**

1. *Candida albicans* (MTCC 404)
2. *Aspergillus niger* (MTCC 183)

All the novel synthesized title compounds of series-1, *N-(benzimidazol-1-ylmethyl)-benzamide* derivatives (3a-3x) and series-2, *N-(benzimidazol-1-ylmethyl)-4-chlorobenzamide* derivatives (3a’-m’) were evaluated for their antibacterial and antifungal activity and showed good to moderate activity with respect to standard drug ciprofloxacin and clotrimazole respectively. The MIC for synthesized compounds (3a-3x) and (3a’-3m’) was found to be in the range of *E. coli* (12.5-3.125µg/ml), *P. aeruginosa* (12.5-3.125µg/ml), *S. aureus* (12.5-3.125µg/ml) and *B.subtilis* (12.5-3.125µg/ml) in comparison to reference drug ciprofloxacin and against fungal strains, *C. albicans* (MIC 25-3.125µg/ml) and *A. niger* (MIC 25-3.125µg/ml) when compared with clotrimazole as standard antimicrobial agent. Results revealed that amongst the synthesized scaffolds (3a-3x), target compound 3o with electron withdrawing Cl group at 2 position of phenyl ring which is substituted at 2-position of benzimidazole and
molecule 3q with electron withdrawing Cl group at 4 position of benzene ring substituted at 2 position of benzimidazole scaffold, were found to be most active against all the bacterial strains when compared with ciprofloxacin. Also it was found that amongst all the synthesized compounds, 3o, 3q and 3r were most significantly active in opposition to fungal strains and rest of the compounds showed moderate activity as compared to standard clotrimazole. However the derivatives substituted with aliphatic moieties were found to be least active. From synthesized title compounds (3a'-3m'), molecules 3e' and 3h', 3k' and 3l' with Cl, Br, NO$_2$, F substitutents were found to be most active against all the bacterial strains as compared to standard ciprofloxacin. Compounds 3a', 3c' and 3i' were found to be least active against most of the bacterial strains. Also molecules 3e' and 3h' were found to be most active against both the fungal strains as compared to clotrimazole. It was observed that there was slight difference amongst the MIC values of title compounds of (3a-3x) series-1 and (3a'-3m') series-2, however the compounds containing chloro, bromo, floro and nitro group were found to be more active than rest of the title compounds.

7.1.3.2 Antioxidant activity

The in-vitro antioxidant activity was evaluated in terms of hydrogen donating or free radical scavenging ability of the synthesized compounds by 1,1-biphenyl-2-picrylhydrazyl radical (DPPH) method. Ascorbic acid was used as reference compound and the experiment was performed in dark. The scavenging activity was calculated in terms of inhibition using the following equation:

\[
\text{\% anti-radical activity} = \left[\frac{\text{Control absorbance-Sample absorbance}}{\text{Control absorbance}}\right] \times 100
\]

All the synthesized compounds were screened for in vitro antioxidant activity and it was found that some of them exhibited significant results as compared to reference drug, ascorbic acid. It was found that out of 24 compounds of series-1 (3a-3x), eight compounds displayed prominent results when compared with standard ascorbic acid. From all the compounds 3c, 3h, 3j, 3o, 3q, 3r, 3t and 3w showed significantly potent antioxidant activity as compared to standard drug and rest of the compounds showed moderate or less activity.

Also, it was established that out of 13 compounds of series-2 (3a'-3m'), five compounds showed momentous results in comparison with standard ascorbic acid.
Amongst all the synthesized compounds \(3a', 3e', 3g', 3h'\) and \(3j'\) showed significantly potent antioxidant activity in comparison to standard drug and rest of the compounds showed moderate or less activity. Out of all the compounds, molecules \(3c\) (86.50±0.66) and \(3a'\) (81.43 ± 1.66) exhibited highest anti-oxidant activity in comparison to ascorbic acid. Also it was assumed that compounds that were showing good anti-oxidant activity would be able to exhibit good anti-inflammatory activity. As various free radicals are also responsible for the induction of short term algesia as well as play an important role in the pathogenesis of inflammation.

### 7.1.4 Molecular Docking Studies

#### 7.1.4.1 Antimicrobial activity

After \textit{in vitro} antimicrobial evaluation, \textit{in silico} study was performed using molecular docking to understand the interaction of synthesized compounds with microbial protein. Considering 1C14 as target, all the synthesized compounds were docked to get the best \textit{in silico} confirmations in the domain of 1C14 protein. Binding affinities of the synthesized compounds were evaluated by using docking program AutoDock Vina, which also optimised the antimicrobial activities of synthesized compounds as possible microbial inhibitors. During analysis, H-bonding and docking affinity were taken as two important parameters for obtaining the hits among the set of compounds. From the \textit{in silico} study, it was revealed that majority of the synthesized compounds were having higher docking affinity than standards ciprofloxacin (\(-6.6\ k\text{Cal/mol}\)) and clotrimazole (\(6.4\ k\text{Cal/mol}\)). The main amino acids that have played a vital role in interaction with synthesized compounds are Gln1040, Arg171, Gln40 and Arg1171. Among the synthesized compounds (3a-3x), compound 3o has displayed best docking affinity of \(-8.2\ k\text{Cal/mol}\) with 1H bond, length of 2.177 Å, shown in Fig. 7.1. In this Fig, it has been shown that 1H bond is formed between ‘N’ of 3o and ‘NH’ group of Arg171. Among the synthesized compounds (3a’-3m’) compound 3d’ and 3e’ has displayed good docking affinity of \(-8.3\ k\text{Cal/mol}\) with 1H bond, length of 2.079 Å, \(-8.2\ k\text{Cal/mol}\) with 1H bond, length of 1.991 Å shown in Fig. 7.2 and 7.3 respectively. From the Fig. it was clearly seen that in both the compounds 3d’ and 3e’, 1H bond was formed between ‘O’ of carbonyl group of 3d’/3e’ and ‘NH’ of Gln1040.

From \textit{in vitro} anti-microbial activity, it has been found that amongst the synthesized compounds (3a-3x), compound 3o was found to be most active, having docking affinity
as well as best MIC against both bacterial and fungal strains. Also, among the synthesized compounds (3a'-3m'), compounds 3d' and 3e' displayed good docking affinity but during *in vitro* studies molecule 3e' was found to be most active against both the bacterial and fungal strains.

![Fig. 7.1](image1.png)

**Fig. 7.1:** Binding pose for compound 3o (docking affinity -8.2 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line

![Fig. 7.2](image2.png)

**Fig. 7.2:** Binding pose for compound 3d' (docking affinity -8.3 kCal/mol) within the domain of microbial receptor showing hydrogen bonding in dashed green line
Synthesis and biological studies on 2-substituted benzimidazole derivatives

7.1.4.2 Anti-inflammatory activity

Molecular docking studies of all the synthesized derivatives were also performed for anti-inflammatory action. The basic principle of the study was to screen the compounds \textit{in silico} for further \textit{in vivo} evaluation. Considering 1CX2 as target, the series of compounds were docked to get the best \textit{in silico} confirmations in the domain of COX-2 receptor. From the \textit{in silico} study among synthesized derivatives (3a-3x), 13 hit compounds with docking affinity higher than that of internal ligand SC-558, 4-[4-(4-Bromophenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide (-7.6 kCal/mol), were demonstrated for anti-inflammatory, analgesic and ulcerogenic potential by \textit{in vivo} screening. The main amino acids which played a vital role in interaction with synthesized compounds (3a-3x) were Lys194, Asn292, Arg255, Gly99 and Glu193. Among the synthesized derivatives (3a-3x), 3c has displayed best docking affinity of -9.0 kCal/mol with 2H bonds, length of 2.216 Å and 2.036 Å shown in Fig. 7.4. In this figure, it has clearly been shown that 2H bonds were formed between ‘NH’ group of 3c with ‘O’ of carbonyl group in Glu193 and ‘N’ group of benzimidazole in 3c and ‘NH’ group in Arg 255. It is a benzamide derivative with chloromethyl substitution at 2 position of benzimidazole moiety. Substitutions have been done with aliphatic,
Summary and Conclusion

Synthesis and biological studies on 2-substituted benzimidazole derivatives

Aromatic and heteroatoms as well and it was found that when sulphhydril group was incorporated in structure that has led to least docking affinity with no interaction shown with the receptor. Even substitutions with aliphatic moieties like methyl, ethyl, propyl, butyl were also not found to possess good anti-inflammatory activity using in silico studies.

Among the synthesized compounds (3a'-3m'), compound 3a' showed best docking affinity of -9.0 kCal/mol with 1 H bond, length of 2.237 Å shown in Fig. 7.5. The main amino acids which played a vital role in interaction with synthesized derivatives are Lys194, Ala98, Ile31 and Gln192. From the Fig. it has been clearly seen that one hydrogen bond was formed between ‘O’ of carbonyl group of 3a’ and ‘NH’ of Lys 194. It is a p-chlorobenzamide derivative with chloromethyl substitution at 2 position of benzimidazole moiety. Substitutions have also been done with phenyl, 3-Cl phenyl, 4-F phenyl moieties as well but no interaction was presented with the receptor.

From the docking study, it was concluded that the compound 3c was found to be most active having best docking affinity among the synthesized derivatives (3a-3x) and 3a’ displayed best docking affinity among the series (3a’-3m’). Both the compounds showed good anti-inflammatory activity as well when studied in vivo.

![Fig. 7.4: Binding pose for compound 3c (docking affinity -9.0 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line](image)
Fig. 7.5: Binding pose for compound 3a’ (docking affinity -9.0 kCal/mol) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

# 7.1.5 Pharmacological Evaluation

All the protocols for animal studies were approved by Institutional Animal Ethics Committee (IAEC) of Chitkara College of Pharmacy, Chitkara University, Rajpura (Punjab).

## 7.1.5.1 Anti-inflammatory activity

Anti-inflammatory activity of all the selected synthesized compounds were evaluated by carrageenan induced right hind paw edema method. The percent anti-inflammatory activity was calculated according to the formula as given below:

\[
\text{Percentage inhibition of paw edema} = (1 - \frac{V_t}{V_c}) \times 100
\]

Where, \( V_t \) and \( V_c \) is the edema volume in drug treated and control groups, respectively. Diclofenac (50mg) was used as reference drug. Results revealed that among the synthesized derivatives (3a-3x), 13 compounds were selected having higher docking affinity than internal ligand. From in vivo studies, five compounds, 3c, 3o, 3q, 3r and 3t showed statistically significant (\( p \leq 0.05 \)) potent anti-inflammatory activity in comparison to control group and rest of the compounds showed moderate or less activity. Out of five derivatives, compound 3c was found to be most active having highest %age inhibition (70.50 ± 2.25) at dose of 100mg/kg as compared to diclofenac sodium (76.25 ± 3.75) at dose of 50 mg/kg. During in silico studies, this compound has also displayed best docking affinity (-9.0 kCal/mol). Furthermore, among the
synthesized derivatives (3a’-3m’), five compounds 3a’, 3e’, 3g’, 3h’ and 3j’ showed statistically significant (p≤ 0.05) potent anti-inflammatory activity in comparison to control group and rest of the compounds showed moderate or less activity. Compound 3a’ was found to be most active showing % age inhibition (66.66 ± 2.45) at a dose of 100mg/kg when compared with diclofenac sodium (76.25 ± 3.75) at dose of 50 mg/kg. Also this compound displayed best interactions (docking affinity -9.0 kCal/mol) with the 1CX2 receptor during in silico studies. Although docking affinty of all the selected compounds were higher than internal ligand, it was assumed that these compounds would show good anti-inflammatory activity. But the molecules substituted with phenyl, 3-pyridyl, 2-OH phenyl, 2-NH₂ phenyl, 3-Cl phenyl, 3- Br phenyl and 4-F phenyl groups at 2- position of benzimidazole moieties showed very less anti-inflammatory activity and molecules substituted with chloromethyl, 2-Cl phenyl, 2-Br phenyl, 4-Cl phenyl and 4-Br phenyl groups at 2- position of benzimidazole moiety showed significant results. Also compounds substituted with 4-NO₂ phenyl moiety at 2-position of benzimidazole displayed good docking affinity but when studied in vivo, caused mortality in rats.

7.1.5.2 Analgesic activity

Acetic acid-induced writhing test was used to evaluate the analgesic potential of most potent compounds showing anti-inflammatory activity. Indomethacin (30mg) was used as standard drug. The number of writhes per animal was recorded for 15 minutes. The analgesic activity was expressed as percentage of protection by the following formula.

\[(1 - \text{No. of writhes of test/No. of writhes of control}) \times 100\]

Results revealed that 10 compounds (5 from each series) showing significant anti-inflammatory activity (p≤ 0.05), were selected for evaluation of analgesic activity. It was found that all the selected compounds showed significant analgesic activity (p≤ 0.01), but compound 3c showed highest percentage protection (63.66) at dose of 100 mg/kg among all the synthesized derivatives (3a-3x) when compared with indomethacin (66.06) at dose of 30 mg/kg. From synthesized derivatives (3a’-3m’), molecule 3a’ showed highest analgesic activity (62.63) at dose of 100 mg/kg as compared to standard at dose of 30 mg/kg.
7.1.5.3 Ulcerogenic Potential

On the basis of efficacy and potency of anti-inflammatory and analgesic activity, five compounds each from series-1 and series-2 were selected for ulcerogenic studies. Among ten compounds, six derivatives showed significant (p ≤ 0.05) results for inhibition of ulcerogenic activity as compared to indomethacin as reference drug (MLS 32.50 ± 2.50). Results revealed that compounds 3c, 3o and 3r as indicated by their low lesion score 13.90 ± 0.95, 15.85 ± 1.18 and 16.55 ± 1.25 respectively from series-1 and molecules 3a’, 3e’ and 3h’ as indicated by their low lesion score 12.25 ± 0.85, 13.53 ± 0.92, 15.25 ± 1.05 from series-2 were most potent among ten compounds used for respective study. Furthermore compounds substituted with chloromethyl, 2-chloro phenyl and 2- bromo phenyl moieties at 2 position of benzimidazole nucleus containing benzamide and p-chlorobenzamide scaffold showed less mean lesion score as compared to the compounds substituted with 4 chloro phenyl and 4 bromo phenyl at 2- position of benzimidazole nucleus. It was also revealed that molecules containing p-chlorobenzamide moiety were found to have less lesion score as compared to the compounds containing benzamide moiety.

7.2 Conclusion

Synthesis of N-(Benzimidazol-1-ylmethyl)-benzamide (3a-3x) and N-(Benzimidazol-1-ylmethyl)-4-chlorobenzamide (3a’-3m’) derivatives was done using classical mannich reaction. Their structures were elucidated by spectral analysis. Synthesized compounds were then screened for in vitro antibacterial and antifungal activity by serial dilution method. Two gram positive (S. aureus and B. subtilis) and two gram negative (E. coli, P. aeruginosa) bacterial strains were used. The synthesized compounds were also tested for antifungal activity on two fungal strains (C. albicans and A. niger). All the novel synthesized compounds showed good to moderate activity with respect to standard drug ciprofloxacin and clotrimazole. Results revealed that amongst the synthesized scaffolds (3a-3x), target compound 3o and 3q were found to be most active against all the bacterial strains when compared with ciprofloxacin. Also it was found that amongst all the synthesized compounds, 3o, 3q and 3r were most significantly active in opposition to fungal strains and rest of the compounds showed moderate activity as compared to standard clotrimazole. However the derivatives substituted with aliphatic moieties were found to be least active. From synthesized title compounds (3a’-3m’), molecules 3e’ and 3h’ were found to be most active against all the bacterial
strains as compared to standard ciprofloxacin. Compounds 3a’, 3c’ and 3i’ were found to be least active against most of the bacterial strains. Hence, it is concluded that the compounds containing chloro, bromo, floro and nitro group were found to be more active than rest of the title compounds. All the novel synthesized compounds of series-1 and series-2 were also evaluated for their in vitro antioxidant activity. Among the synthesized compounds 3a-3x eight compounds displayed prominent results when compared with standard ascorbic acid. From all the compounds 3c, 3h, 3j, 3o, 3q, 3r, 3t and 3w showed significantly potent antioxidant activity as compared to standard drug and rest of the compounds showed moderate or less activity. Amongst synthesized derivatives- 3a’-3m’, molecules 3a’, 3e’, 3g’, 3h’ and 3j’ showed significantly potent antioxidant activity in comparison to standard drug and rest of the compounds showed moderate or less activity. Out of all the compounds, molecules 3c (86.50 ± 0.66) and 3a’ (81.43 ± 1.66) exhibited highest antioxidant activity in comparison to ascorbic acid. Also it was found that compounds showing good antioxidant activity, exhibited good anti-inflammatory activity and exhibited good correlation with in silico studies. Because free radicals are also responsible for the induction of short term algesia as well as play an important role in the pathogenesis of inflammation. So, the compounds which are good antioxidants might serve as better anti-inflammatory and analgesic agents.

Also molecular docking studies were done and their inhibitory activity was tested against PDB ID: 1C14 microbial protein. Both the theory (Docking) and practice (MIC values) demonstrate that N-(Benzimidazol-1-ylmethyl)-benzamide and N-(Benzimidazol-1-ylmethyl)-4-chlorobenzamide derivatives act as potent inhibitor of 1C14 microbial protein. It was observed that there was slight significant difference among the MIC values of title compounds of series-1 and series-2.

Molecular docking studies of synthesized compounds were also performed for anti-inflammatory activity. The principle of the study was to screen the compounds in silico for further in vivo evaluation. Considering 1CX2 as target, the series of compounds were docked to get the best in silico confirmations in the domain of selective COX-2 receptor. From the in silico study among series -1 and series-2, 26 hit compounds with docking affinity higher than that of internal ligand SC-558 (docking affinity -7.6 kcal/mol), were evaluated for anti-inflammatory, analgesic and ulcerogenic activity. Out of selected 26 compounds, 10 derivatives showed significant (p≤ 0.05) anti-
inflammatory activity when compared with diclofenac sodium. Compound 3c showed % age inhibition (70.50 ± 2.25) at dose of 100mg/kg as compared to diclofenac sodium (76.25 ± 3.75) at dose of 50 mg/kg. Compound 3a’ was found to be most active showing % age inhibition (66.66 ± 2.45) at a dose of 100mg/kg when compared with standard among all the synthesized compounds. Simultaneously, molecule 3c (docking affinity-9.0 kCal/mol) and 3a’ (docking affinity -9.0 kCal/mol) possessed highest docking affinity among all the synthesized compounds.

Furthermore, 10 compounds (5 from each series) which were showing significant anti-inflammatory activity (p≤ 0.05), were selected for evaluation of analgesic activity. It was found that all the selected compounds showed significant analgesic activity (p≤ 0.01), but compound 3c showed highest percentage protection (63.66 %) at dose of 100 mg/kg among all the synthesized derivatives (3a-3x) when compared with indomethacin (66.06%) at dose of 30 mg/kg. From synthesized derivatives (3a’-3m’), molecule 3a’ showed highest analgesic activity (62.63%) at dose of 100 mg/kg as compared to standard.

On the basis of efficacy and potency as anti-inflammatory and analgesic activity, five compounds were selected each from series-1 and series-2 respectively. These compounds also showed significant (p≤ 0.05) results for ulcerogenic activity as compared to indomethacin as reference drug. Results revealed that compound 3c, 3o and 3r from series-1 and 3a’, 3e’ and 3h’ from series-2 were most potent among ten compounds used for respective study. The ulcerogenic activity was found in following order:

3a’>3c>3e’>3o>3h’>3g’>3r>3q>3j’>3t>indomethacin

Hence it is concluded that N-(benzimidazol-1-ylmethyl)-benzamide and N-(benzimidazol-1-ylmethyl)-4-chlorobenzamide derivatives with electron withdrawing substituents could be used for designing more potent antimicrobial, antioxidant, anti-inflammatory and analgesic agents with less gastric lesions. Further studies of these derivatives are highly suggested to assess the safety of novel compounds. These agents might be explored for other pharmacological activities.
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