5.1 Antibacterial Activity

Introduction

Since the beginning of life on the earth, bacteria play very important role to the ecosystem. In fact first life on the earth was started by bacteria called cyanobacteria. Later to it, eukaryotic life started and evolved biological life like animals and humans as well. And one most important thing with bacteria is its inclusion into the eukaryotic system was nothing but the most important organelle of cell called mitochondria.

Antibiotics discovery was the beginning of great revolution in Second World War. Alexander Fleming is the first scientist who discovered first antibiotic called penicillin in his small lab and accidentally. When he was working with some fungus, he found that there is zone of inhibition in the plate. Then he isolated the active ingredient and studied it found that it is having potential to kill micro-organism called bacteria.

There is great importance to bacteria in recent world to produce industrial enzymes as well as biotechnologically derived human therapeutic proteins. It has become possible by genetic engineering. Human genes are isolated and recombined into the bacterial genetic system and genes are expressed inside the bacteria and therapeutic proteins are isolated and purified and marketed. For example human insulin expression in \textit{E. coli}. Human intestinal microflora play important role to the host by producing vitamins and enzymes. They also induce some substances which keeps live the immunity of the host. For example Lactobacillus species converts sugar into lactic acid

BACTERIAL CELL STRUCTURE

The bacterial cell differs dramatically in structure and function compared to mammalian cells. The bacterial cytoplasm is separated from the external environment by a cytoplasmic membrane as shown in Fig. 5.1A. The bacterial cell wall is chemically distinct from mammalian cell walls and so is constructed by enzymes that often have no direct counterpart in mammalian cell construction. In addition, bacteria possess a crucial structure surrounding the entire cell, the Peptidoglycan (PG), which forms a saccus around the bacterial
cell, is an essential cell wall polymer since interference with its synthesis or structure leads to loss of cell shape and integrity followed by bacterial death.

The peptidoglycan\(^2\) layer as shown in Fig. 5.1.B consists of a matrix of polysaccharide chains composed of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) sugar moieties cross-linked through pentapeptide side chains.

**Fig. 5.1.A: Cell Structure**
CLASSIFICATION OF ANTIBACTERIAL AGENTS

Based on the severity of damage to the bacteria they are divided into:

1] Bactericidal agents act primarily by killing bacteria with an efficiency of >99.9%

2] Bacteriostatic agents act primarily by inhibiting the growth of the bacteria.

Based on the mode of action they can be further divided into following four main categories.

1] Cell wall synthesis inhibitors
2] Cell Membrane Agents
3] Protein synthesis inhibitors
   i] Impairing 50S subunit
   ii] Impairing 30S subunit
4] Nucleic acid synthesis inhibitors
   i] DNA replication and repair
ii] Transcription

A representative listing of the antibacterial compounds currently in clinical practice or in development along with a schematic overview of their targets \(^4\) is shown in Fig. 5.1.C More recently it has been shown that the bactericidal antibiotics, having distinct drug-target interactions stimulate the production of highly harmful hydroxyl radical in Gram-Positive and Gram negative bacteria which finally contribute to the cell death. In contrast, Bacteriostatic drugs tested doesn’t lead to production of hydroxyl radical.\(^5\)

Fig. 5.1.C Schematic view of a bacterial cell with sites of action of various antibiotics
5.1.1 Antibacterial Screening

Some of the synthesized derivatives from chapter two, chapter three and chapter four were then assayed for their \textit{in-vitro} antibacterial activity against a panel of pathogenic as well as standard bacterial strains such as \textit{Staphylococcus aureus}, \textit{Klebsiella pneumoniae}, \textit{Salmonella typhimurium}, \textit{Pseudomonas aeruginosa}, \textit{Bacillus subtilis}, \textit{Proteus vulgaris}, \textit{Xanthomonascampestriv.Pv.Citri}, \textit{Xanthomonascampestriv.Malvacerum}, \textit{Bacillus thurengensis} and \textit{Escherichia coli}. Based on previous literature & scope of the bacterial species were selected under the different scheme.

Gentamicin, Kanamycin, Streptomycin and cefotaxime sodium were procured from commercial sources. The purities and potencies of the agents recovered from commercial preparations were documented by showing that the MICs of antibacterials were within acceptable limits against the known strains.

\textbf{Determination of MIC in terms of Zone of Inhibition:}

The antibacterial activity was tested by agar disc diffusion method. The killing or growth inhibition properties of the agents was scored as clear zone of inhibition surrounding the disc and is measured in mm scale.

\textbf{Materials \\ & Method:}

- The bacterial strains were inoculated into fresh sterile MHB (Muller Hinton Broth) media tube (4.5 ml) and were incubated for 18-24 hrs at 37\degree C in a B. O. D. incubator
- Standard antibiotic Gentamicin, Kanamycin, Streptomycin and cefotaxime sodium were prepared clear solutions with final (1mg/ml).
- The above antibiotic solutions were poured on sterile disc at a final concentration of 40 mcg/disc for Gentamicin, Kanamycin while 40 mcg/disc for Streptomycin and cefotaxime sodium.
- All discs were dried completely by incubating into hot air oven in sterile petri dishes.
- On MHA (Muller Hinton Agar) plates, the bacterial suspension was poured and spread evenly with the help of glass spreader.
• After drying the plates completely, the antibiotic loaded discs were kept on the plates.
• All plates were incubated at 37 °C in a B. O. D. (Biological Oxygen Demand) incubator for 24 hours.
• Results were recorded and antibiotic activity was quantified by measuring the zone of inhibition surrounded to the disc and it were measured in ‘mm’ scale and presented in the respective tables.
5.1.2 Results and conclusion of Antibacterial Screening

Activity data for the substituted 1-(1-((3-methyl-4-(2,2,2-trifluoroethoxy) pyridine-2-yl)methyl)-1H-indole-3-yl)-3-phenylprop-2-en-1-one (Chalcone) (1a-d) and 1-((3-methyl-4-(2,2,2-trifluorotethoxy)pyridine-2-yl)methyl)-3-(5-phenyl-4,5-dihydro-1H-pyrazol-3-yl)-1H-indole (pyrazoline) (2a-d) are represented in Table 5.1 and 5.2 respectively.

Table 5.1. Antibacterial data of some substituted 1-(1-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridine-2-yl)methyl)-1H-indole-3-yl)-3-phenylprop-2-en-1-one

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R1</th>
<th>R2</th>
<th>K. pneumonia</th>
<th>S. typhi</th>
<th>P. auroginosa</th>
<th>B. Subtilis</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>9</td>
<td>4</td>
<td>2</td>
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<td>1b</td>
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<tr>
<td>1c</td>
<td>H</td>
<td>H</td>
<td>F</td>
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<td>2</td>
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<tr>
<td>1d</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
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</tr>
<tr>
<td>Kanamycin (40 mcg/disc)</td>
<td>18</td>
<td>14</td>
<td>21</td>
<td>15</td>
<td>13</td>
<td>9</td>
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</tbody>
</table>

Conclusion: The selected compounds which have been tested for their antibacterial activity by using agar disc method as shown in above table. The antibacterial activity of 1a and 1c has improved against K. pneumonia, but inferior to the positive control. Activity against B. subtilis of 1c is superior amongst the synthesized derivatives. The overall activity of all the compounds is inferior as compared to Gentamicin and Kanamycin. Therefore the synthesized derivatives don’t show any promising activity against Gentamicin, Kanamycin.
Table 5.2 Antibacterial data of new substituted 1-((3-methyl-4-(2,2,2-trifluorotethoxy)pyridine-2-yl)methyl)-3-(5-phenyl-4,5-dihydro-1\textit{H}-pyrazol-3-yl)-1\textit{H}-indole(pyrazoline)(2a-d)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R\textsubscript{1}</th>
<th>R\textsubscript{2}</th>
<th>\textit{K.pneumonia}</th>
<th>\textit{S.typhi}</th>
<th>\textit{P.auroginosa}</th>
<th>\textit{B.subtilis}</th>
<th>\textit{E.coli}</th>
<th>\textit{S.aureus}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>H</td>
<td>H</td>
<td>H</td>
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<td>2</td>
<td>0</td>
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<td>NO\textsubscript{2}</td>
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<td>Cl</td>
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<td>Gentamicin(40 mcg/disc)</td>
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<td>11</td>
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<td>8</td>
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<tr>
<td>Kanamycin(40 mcg/disc)</td>
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<td></td>
<td></td>
<td>18</td>
<td>14</td>
<td>21</td>
<td>15</td>
<td>13</td>
<td>9</td>
</tr>
</tbody>
</table>

**Conclusion:** The antibacterial activity with series of 2 was performed by agar disc method by measuring the zone of inhibition in mm. In this series there has been marked antibacterial activity was seen in 2c against \textit{B. subtilis} and \textit{E.coli}. The other derivatives tested were almost inactive or mild active against the above bacterial species. The overall activity of all the compounds is inferior as compared to Gentamicin and Kanamycin. Therefore the synthesized derivatives don’t show any promising activity against Gentamicin, Kanamycin.
Activity data for the substituted N-((1-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridine-2-yl)methyl)-1H-indole-3yl) methylene) aniline(5a,c,d and e) and substituted N-benzylidene-1-methyl-1H-indazole-3-carboxhydrazide (7a-d) is represented in Table 5.3 and 5.4 respectively.

**Table 5.3** Antibacterial activities data of some substituted N-((1-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridine-2-yl)methyl)-1H-indole-3yl) methylene) aniline (5a,c,d and e)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>K.pneumoniae</th>
<th>S.typhi</th>
<th>P.aeruginosa</th>
<th>B.Subtilis</th>
<th>E.coli</th>
<th>S.aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>7</td>
<td>3</td>
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<td>4</td>
</tr>
<tr>
<td>5d</td>
<td>H</td>
<td>H</td>
<td>NO2</td>
<td>H</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>5e</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>H</td>
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<td>14</td>
<td>21</td>
<td>15</td>
<td>13</td>
<td>14</td>
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</tbody>
</table>

**Conclusion:** Amongst the existing derivatives, the series, the series of 5 has shown some promising activity against the *K. pneumoniae* except 5d. 5d and 5e derivative has improved mild activity against *P. aeruginosa*. 5c has given some remarked activity against the some bacterial species *k. pneumonia and S.typhi*. The overall activity of all the compounds is inferior as compared to Gentamicin and Kanamycin. Therefore the synthesized derivatives don’t show any promising activity when compare with Gentamicin and Kanamycin.
Table 5.4 Antibacterial data of substituted N-benzylidene-1-methyl-1H-indazole-3-carbohydrazide(7a-d)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>K.pneumonia</th>
<th>S.typhus</th>
<th>P.aeruginosa</th>
<th>B.aureginiosa</th>
<th>E.coli</th>
<th>S.aureus</th>
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</thead>
<tbody>
<tr>
<td>7a</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7b</td>
<td>H</td>
<td>H</td>
<td>CF₃</td>
<td>H</td>
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<td>5</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>7c</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>8</td>
<td>4</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>7d</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>H</td>
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<td>3</td>
<td>4</td>
<td>7</td>
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<td>1</td>
</tr>
<tr>
<td>Gentamicin(40 mcg/disc)</td>
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<td>11</td>
<td>17</td>
<td>15</td>
<td>11</td>
<td>7</td>
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<td></td>
</tr>
<tr>
<td>Kanamycin(40 mcg/disc)</td>
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<td>14</td>
<td>21</td>
<td>15</td>
<td>13</td>
<td>9</td>
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<td></td>
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</tr>
</tbody>
</table>

**Conclusion:** The above series tested for their antibacterial activity with respect to agar disc method. The fluoro groups 7b and 7c has improved the antibacterial property as compared with the other derivatives of series 7. The derivatives 7b and 7c has good killing properties for K. pneumoniae in the group. The bactericidal properties of 7b and 7c against the species of P. aeruginosa have improved over other derivatives of the same group. But the overall killing or inhibiting the growth of bacteria is inferior as compared with the standard Gentamicin and Kanamycin drugs.
Table 5.5 The Antibacterial data of some synthesized substituted 3-methyl-7-(1-methyl-1H-indazol-3-yl carboxamido)-8-oxo-5-thia-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid. (1.1A-1.1C)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Bacteria</th>
<th>Streptomycin 100 µg/ml</th>
<th>Comp 1.1A</th>
<th>Comp 1.1B</th>
<th>Comp 1.1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Klebsiella pneumonia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td><em>Salmonella typhi</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td><em>Bacillus Subtilis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td><em>E.coli</em></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td><em>Proteus vulgaris</em></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>7.</td>
<td><em>Xanthomonas campestris</em> P v.Citri</td>
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<td></td>
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<tr>
<td>8.</td>
<td><em>Xanthomonas campestris</em> P v.Malvacerum</td>
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<td></td>
</tr>
<tr>
<td>9.</td>
<td><em>Bacillus thurengensis</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Zone of inhibition in mm**

1. 2
2. 3
3. 4
4. 5
5. 6
6. 7
7. 8
8. 9
9. 10
10. 11

**Summary & Conclusion:** We screened only three compounds 1.1A, 1.1B and 1.1C for antibacterial activity. Concentration of the compounds used in nutrient agar plates were 100 µg/ml each. Linear growths of the test bacteria were measured every day and zone of inhibition of the isolates was recorded. It is clear from Table 5.5 that Compound 1.1C had maximum zone of inhibition for *Bacillus thurengensis*, *Staphylococcus aureus*, *Bacillus Subtilis* and showed least zone of inhibition to *E. coli*. It did not show any zone of inhibition to remaining bacteria. Compound 1.1B showed maximum zone of inhibition to *Bacillus thurengensis* and *E. coli*. Compound 1.1A showed maximum zone of inhibition *Proteus vulgaris*, *Bacillus Subtilis* and *Bacillus thurengensis*. 
Table 5.6. Antibacterial data of substituted 3-(acetoxyethyl)-7-(2-(7-methyl-2-p-tolylimidazo[1,2-a]pyridine-3yl)acetamide-8-oxo-5thia-1-azabicyclo [4.2.0] oct-2-ene-2-carbaylic acid (2.1A-E)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R₁</th>
<th>E. coli</th>
<th>S. typhi</th>
<th>S.aureus</th>
<th>B. megaterium</th>
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<tbody>
<tr>
<td>2.1A</td>
<td>H</td>
<td>CH₂OCOCH₃</td>
<td>20</td>
<td>18</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>2.1B</td>
<td>H</td>
<td>CH₂=CH₂</td>
<td>17</td>
<td>15</td>
<td>-</td>
<td>13</td>
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<tr>
<td>2.1C</td>
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<td>14</td>
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<td>Cefotaxime sodium</td>
<td>30</td>
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</tbody>
</table>

Summary & Conclusion: - We screened all five derivatives of 3-(acetoxyethyl)-7-(2-(7-methyl-2-p-tolylimidazo[1,2-a]pyridine-3yl)acetamide-8-oxo-5thia-1-azabicyclo [4.2.0] oct-2-ene-2-carbaylic acid (2.1A-E). The concentration of derivativs used in disc were 100 µg/ml each. The activity of these derivatives were measured against four different bacterial species by using cefotaxime sodium as standard drugs. Among the tested series 2.1A has shown maximum activity against E.Coli and S.Typhi. 2.1B also showed good activity activity against E.Coli and S.Typhi 2.1E also shown comparatively good activity activity against E.Coli and B.Megaterium. The overall impact of all the derivatives against cefotaxime sodium was inferior against all bacterial species.
5.2. Pharmacological Screening

Inflammation is essentially a local event or any manifestation in the systemic circulation reflects the severity of the inflammatory process. Under normal conditions, the chemical mediators of inflammatory process function to preserve homeostasis in the microenvironment. When the conditions within the microenvironment become hostile as to exceed the normal homeostatic capacity of the chemical mediators, then full inflammatory response occurs. Inflammatory reaction has essentially, in chronological order, four features:

(A) Initial injury causing release of inflammatory mediators (e.g. Histamine, serotonin, leukokinins, SRS-A, lysosomal enzymes, lymphokinins, prostaglandins as shown in the Fig 5.2.(A)

Fig. 5.2.(A): Chemicals that initiate and perpetuate pain signals
(B) Dilation of blood vessels and increased vascular permeability leading to erythema and oedema at the site of noxious stimulus (Tansudative Phase).

(C) Cellular infiltration and general mopping up reaction (Exudative reaction).

(D) Tissue repair or healing (Proliferative Phase).  

**CYCLOOXYGENASE (COX) PATHWAY**

Arachidonic acid (AA) is the most abundant polyunsaturated fatty acid found in the phospholipid cell membranes. Activation of the phospholipase A2 in response to various stimuli, releases AA, which can be further metabolized by two major enzymatic pathways, cyclooxygenase (COX) and 5-LOX, leading to pro-inflammatory mediators, prostanoids and leukotrienes (LTs) respectively as depicted in [Fig 5.2.B, Cyclooxygenase Pathway].

**COX structure:** The enzyme complex is a dimer of two identical subunits; each subunit has a knob covered with hydrophobic amino acids, pointing downward in this illustration. These knobs anchor the complex to the membrane of the endoplasmic reticulum, shown in light blue at the bottom of the picture in [Fig. 5.2.C]. The cyclooxygenase active site is buried deep within the protein, and is reachable by a tunnel that opens out in the middle of the knob. This acts like a tunnel, guiding arachidonic acid out of the membrane and into the enzyme for processing. In the structure shown above from Protein Drug Bank, a
drug (yellow and green) is blocking the active site in both subunits. The heme groups are also shown above the drug in each subunit.\(^\text{10}\)

COX is an enzyme (EC 1.14.99.1) has two active sites, a cyclooxygenase site and a heme with peroxidase site, with the help of which it catalyzes the formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane) from arachidonic acid. Prostaglandins act (among other things) as messenger molecules in the process of inflammation. This mechanism of action was elucidated by John Vane, who later (1983) received a Nobel Prize for his work.

**COX-2 inhibitors:** e.g. Celecoxib, Rofecoxib, Eterocoxib etc.

Cyclooxygenases is of two types namely COX-1 and COX-2 for different purposes. COX-1 is built to create prostaglandins.\(^\text{11}\) The second enzyme is used for signaling pain and inflammation such as stomach bleeding. But with the discovery of COX-2 in 1991 by Daniel L. Simmons raised the hope of developing an effective NSAID that block just COX-2, leaving COX-1. The inhibition of COX-2 stops the production of prostaglandins and thromboxanes. This, in turn, leads to the following three major effects: -1] Anti-inflammatory 2] Analgesic and 3] Antipyretic effect.

**Dual Inhibitors:** e.g. Licofelone: Blocking the cyclooxygenase (COX) pathway of arachidonic acid (AA) metabolism by conventional NSAIDs not only results in decreased production of gastroprotective prostaglandins (PGs) but also in the increased metabolism of AA via 5-lipoxygenase (5-LOX) route. The 'shunting effect' leads to increased production of leukotrienes (LTS), which contribute to inflammatory processes and further gastrointestinal (GI) damage. Thus, developing dual COX/5-LOX inhibitors may enhance anti-inflammatory effects and replace the undesirable side effects associated with NSAIDs.\(^\text{12}\)

**COX-3 inhibitors:** COX-3 was also discovered by Daniel L. Simmons in 2002 and analyzed this new isozyme's relation to paracetamol (acetaminophen). The author postulated that inhibition of COX-3 could represent a primary central mechanism by which these drugs decrease pain and possibly fever.
NON-STERoidal ANTI-INFLAMMATORY DRUGS (NSAIDs)

Are drugs with analgesic, antipyretic and anti-inflammatory effects they reduce pain, fever and inflammation. The term "non-steroidal" is used to distinguish these drugs from steroids, which (among a broad range of other effects) have a similar eicosanoid-depressing, anti-inflammatory action.

Classification: Based on the chemical structure NSAIDs can be broadly classified in the following manner.

Salicylates: Acetylsalicylic acid (Aspirin), Choline magnesium salicylate, Methyl salicylate, Magnesium Salicylate.

Arylalkanoic acids: Diclofenac, Aceclofenac, Etodolac, Indomethacin, Oxametacin, Proglumetacin, Sulindac, Tolmetin.


N-Arylanthranilic acids (fenamic acids): Mefenamic acid, Flufenamic acid, Meclofenamic acid, Tolfenamic acid.

Pyrazolidine derivatives: Phenylbutazone, Ampyrone, Clofezone, Oxyphenbutazone, Phenazone, Phenylbutazone, Sulfinpyrazone.

Oxicams: Piroxicam, Droxicam, Lornoxicam, Meloxicam.
**Mode of action**: It is similar for all the NSAIDs. Aspirin is an effective painkiller; it reduces fever and inflammation when the body gets overzealous in its defenses against infection and damage. It blocks cyclooxygenase, non-selectively, when it attacks the enzyme it acetylates serine amino acid residue, present in the active site thereby permanently inactivating the enzyme.\(^\text{14}\)

The Fig. 5.2.D shows binding of Aspirin (shown in Yellow) to COX enzyme and the discharge from the enzyme with acetyl group (shown in white and red) is attached to the serine amino acid (bright green), and the salicylic acid (large spheres) is bound close by. The backbone of the protein is shown in dark green.

**Fig 5.2.D: Protein Drug Binding with Aspirin**
5.2. Pharmacological Screening

All the newly synthesized series of substituted 3-(acetoxy methyl)-7-(1-methyl-1H-indazol-3-carboxamido)-8-oxo-5-thia-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 1.1(A-E) [Scheme IX] & substituted 3-[(acetyloxymethyl)-7-(2-(7-methyl-2-p-tolylimidazo[1,2-a]pyridine-3-yl) acetamide)-8oxo-5-thai-1-aza bicyclo[4.2.2.]Oct-2-ene-2-carboxylic acid 2.1(A-E) [Scheme X] were subjected for analgesic and anti-inflammatory screening in Rat as animal model by using following protocol.

5.2.1 Anti-inflammatory activity by carrageenan footpad edema:

**Method:** Male Wistar rats (120 - 170 g) kept at the laboratory Animal home. The animals were maintained under standard environmental conditions and had free access to standard diet and water. Antiinflammatory activity was measured using carrageenan induced rat paw oedema assay. Groups of 6 rats were given a dose of 30,60 and 120 mg/kg. Diclofenac sodium was used as the standard drug.(10 mg/kg.p.o). After 1h, 0.1 ml, 1% carrageenan suspension in 0.9% NaCl solution was injected into the sub-plantar tissue of the right hind paw. The linear paw circumference was measured at hourly interval for 2,3 ,4 and 24 hours. Anti-inflammatory activity was measured as the change in paw volume of the test and standard drug treated animals.

A representation of % rise in paw volume after administration of drug and standard in a group of animals is shown in following Fig.5.2.1 a,b,c,d.
**Fig. 5.2.1 (a)** Carrageenan induced rat paw edema.

All data analyzed by Two way ANOVA followed by Bonferroni test (*p<0.05, **p<0.01, ***p<0.001)

**Fig. 5.2.1 (b)** Carrageenan induced rat paw edema.

All data analyzed by Two way ANOVA followed by Bonferroni test (*p<0.05, **p<0.01, ***p<0.001)
Fig. 5.2.1 (c) Carrageenan induced rat paw edema.

All data analyzed by Two way ANOVA followed by Bonferroni test (*p<0.05, **p<0.01, ***p<0.001)

Fig. 5.2.1 (d) Carrageenan induced rat paw edema.

All data analyzed by Two way ANOVA followed by Bonferroni test (*p<0.05, **p<0.01, ***p<0.001)
5.3. Analgesic Activity

Swiss mice (both sexes, 20-30 g) obtained from the serum institute, Pune were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12 hr: 12 hr light–dark cycle. Animals were treated with the extract 1 hr before the experiments. Controls received vehicle at the same volume as the treated groups.

5.3.1 Acetic acid Writhing test in mice:

Purpose and Rational: - Pain is induced by injection of irritants into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior which is called writhing. The test is suitable to detect analgesic activity although some psychoactive agents also show activity. An irritating agent such as Pentazocin or acetic acid is injected intraperitoneally to mice and the stretching reaction is evaluated. The reaction is not specific for the irritant.

Procedure: - Mice of either sex with a weight between 20 and 25 g are used. Pentazocin in a concentration of 0.02% is suspended in a 1% suspension of carboxymethylcellulose. An aliquot of 0.25 ml of this suspension is injected intraperitoneally. Groups of 6 animals are used for controls and treated mice. Preferably, two groups of 6 mice are used as controls. Test animals are administered the drug or the standard at various pre-treatment times prior to Pentazocin administration. The mice are placed individually into glass beakers and five min are allowed to elapse. The mice are then observed for a period of ten min and the number of writhes is recorded for each animal. For scoring purposes, a writhes is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The formula for computing percent inhibition is: average writhes in the control group minus writhes in the drug group divided by writhes in the control group times 100%. The time period with the greatest percent of inhibition is considered the peak time. A dose range is reserved for interesting compounds or those which inhibit writhing more than 70%.

Compounds with less than 70% inhibition are considered to have minimal activity. The results of the screening are summarized in Fig. 5.3.1.e, f, g and h.
Fig. 5.3.1(e) Acetic acid induced Wrything.

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)

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Fig. 5.3.1(f) Acetic acid induced Wrything

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
Fig. 5.3.1(g) Acetic acid induced Wrything

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)

Fig. 5.3.1(h) Acetic acid induced Wrything

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
5.3.2. Formalin test in mice:

**Purpose and Rational:** The formalin test in mice has been proposed as a chronic pain model which is sensitive to centrally active analgesic agents by Dubuisson and Dennis (1977).

**Procedure:** Swiss albino mice weighing 25–30 g are administered 0.2 ml of 2% formalin into the dorsal portion of the front paw. The test drug is administered simultaneously either sc. or orally. Each individual rat is placed into a clear plastic cage for observation. Readings are taken at 30 and 60 min and scored according to a pain scale. Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw. Analgesic response or protection is indicated if both paws are resting on the floor with no obvious favoring of the injected paw. The results of the screening are summarized in Fig. 5.3.2 (i, j, k, l, m, n, o and p)

![Formalin Test (Phase 1)](image)

**Fig 5.3.2 (i) Formalin Test (Phase 1).**

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
Fig 5.3.2 (j) Formalin Test (Phase1)

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)

Fig 5.3.2 (k) Formalin Test (Phase1)

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
Fig 5.3.2 (l) Formalin Test (Phase 1)

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)

Fig 5.3.2 (m) Formalin Test (Phase 2)

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
**Fig 5.3.2 (n) Formalin Test (Phase 2)**

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)

**Fig 5.3.2 (o) Formalin Test (Phase 2)**

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
5.4.3. Hot plate method:

**Purpose and Rational:** The paws of mice and mice are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses.

**Procedure:** The method originally described by Woolfe and Mac Donald (1944) has been modified by several investigators. The following modification has been proven to be suitable: Groups of 10 mice of either sex with an initial weight of 18 to 22 g are used for each dose. The hot plate which is commercially available consists of a electrically heated surface. The temperature is controlled for 55° to 56 °C. This can be a copper plate or a heated glass surface. The animals are placed on the hot plate and the time until either licking or jumping occurs is recorded by a stop-watch. The latency is recorded before and after 20, 60 and 90 min following oral or subcutaneous administration of the standard or the test compound. The results of the screening are summarized in Fig.5. 4.3 (q, r, s, and t)
Fig. 5.4.3 (q) Hot Plate Test.

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)

Fig. 5.4.3 (r) Hot Plate Test.

All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
All data analyzed by one way ANOVA followed by Dunnets test (*p<0.05, **p<0.01, ***p<0.001)
5.4 Results and conclusion of Pharmacological Screening

From the present investigation it can be deduced that the series of compounds have analgesic activity in the following order: 1.4D > 1.3C > 1.5E > 1.2B > 1.1 and 2.4D > 2.3C > 2.5E > 2.2B > 2.1A. In the acetic acid induced writhing, the drugs demonstrated a dose dependent activity by inhibiting the number of writhings, showing that the drug has peripheral analgesic activity. However, in the hot plate test, the drugs demonstrated slight inhibition of analgesia as the latency of paw licking was not increased significantly. In the formalin induced hyperalgesia model, the drugs were unable to inhibit the first phase (neurogenic) which shows that the drugs don’t have central analgesic activity. However, the second phase was inhibited dose dependently indicating that the drugs have a peripheral analgesic activity. In the carrageenan induced rat paw edema model, the drugs inhibited the rise in paw volume in a dose dependent manner, depicting the anti inflammatory activity. These observations provide sufficient proof that the series of compounds may act by inhibition of synthesis of prostaglandins by inhibiting the cyclo oxygenase pathway.