CHAPTER II

SECTION I

GENERAL EXPERIMENTAL PROCEDURE
The total synthesis as mentioned in the present thesis is comprised of two parts - I and II respectively.

**PART - I**

**SYNTHESIS OF VARIOUS N-[2-(PHENOXY AND SUBSTITUTED PHENOXY) ACETYL/PROPIONYL] BENZIMIDAZOLES, BENZOTRIAZOLES AND IMIDAZOLES**

This part consists of four steps.

**STEP-FIRST**

**SYNTHESIS OF VARIOUS CHLORO, BROMO, NITRO AND METHYL PHENOLS**

Bromo and nitro substituted phenols were synthesised in the laboratory whereas chloro and methyl phenols were used commercially available. The synthesised phenols were confirmed by their sharp melting points and microanalyses.

**STEP-SECOND**

**SYNTHESIS OF VARIOUS [2-(PHENOXY AND SUBSTITUTED PHENOXY) ACETIC/PROPIONIC] ACIDS**

Equimolecular quantities (0.07 - 0.1 mole) of various phenols (dissolved in 33-81 ml of 33% aqueous sodium hydroxide solution) and monochloro acetic acid and α-monochloro propionic acids were refluxed separately in a 250 ml round bottomed jointed flask fitted
with a water condenser for 30 minutes. The reaction mixture was then transferred into a 250 ml beaker and it was then evaporated almost to dryness. The residue was then dissolved in 100 ml of water. The solution was cooled and acidified with dilute hydrochloric acid to yield the free acid. The mixture was then extracted with ether, after washing with water, the ethereal layer was evaporated to yield the various phenoxy and substituted phenoxy acetic/propionic acids.

\[
\begin{align*}
\text{X} & \quad \text{ONa} & \quad \text{Cl-CH(R)CO}_2\text{H} \\
\quad & \quad \text{excess alkali} \\
\text{X} & \quad \text{OCH(R)CO}_2\text{Na} & \quad \text{dil. HCl} & \quad \text{X} & \quad \text{OCH(R)CO}_2\text{H} + \text{NaCl}
\end{align*}
\]

where, \( X = \) substituents; \( R = H \) or \( CH_3 \)

The structures of the various phenoxy and substituted phenoxy acetic/propionic acids were confirmed by their sharp melting points and microanalyses (yield 58 - 78%).

**STEP-THIRD**

**SYNTHESIS OF VARIOUS [2-(PHENOXY AND SUBSTITUTED PHENOXY) ACETYL/PROPYNYL] CHLORIDES**

In the various phenoxy and substituted phenoxy acetic/propionic acids (0.046 mole dissolved in the
appropriate organic solvent) were added excess of thionyl chloride (0.056 mole) in a round bottomed jointed flask fitted with a water condenser and the mixture was refluxed on a water bath separately for about 7 hours. Excess of thionyl chloride were distilled off under reduced pressure to give the corresponding phenoxy and substituted phenoxy acetyl/propionyl chlorides.

\[
\begin{align*}
X - \frac{A}{OCH(R)CO_2H} + SOCl_2 & \rightarrow X - \frac{A}{OCH(R)COCl} + SO_2 + HCl
\end{align*}
\]

The reason for using excess of thionyl chloride is to avoid the anhydride formation.

\[
\begin{align*}
X - \frac{A}{OCH(R)CO_2H} & \rightarrow X - \frac{A}{O-CH(R)COCl} \\
\end{align*}
\]

The structures of all the phenoxy and substituted phenoxy acetyl/propionyl chlorides were confirmed by their sharp melting points/boiling points and microanalyses (yield 78-85%).
STEP-FOUR

(a) SYNTHESIS OF N-[2-(PHENOXY AND SUBSTITUTED PHENOXY ACETYL/PROPIONYL] BENZIMIDAZOLES

To an ice-cooled solution of benzimidazole (0.036 mole in methanol) in a 500 ml beaker was added 5 ml 4N sodium hydroxide solution. The dropwise addition of phenoxy and substituted phenoxy acetyl[propionyl] chlorides (in appropriate organic solvent) in the above solution separately with constant stirring for about 40 to 90 minutes afforded various phenoxy and substituted phenoxy acetyl[propionyl] benzimidazoles, which were separated by ether. The ethereal solution was washed with sodium bicarbonate solution followed by distilled water (4x30 ml) and on concentration yielded solid products which were purified over the column of neutral alumina/silica gel using the appropriate organic solvent(s) as eluant. Finally, the eluate was concentrated and the products were crystallised from appropriate solvent(s).
(b) SYNTHESIS OF VARIOUS N-[2-(PHENOXY AND SUBSTITUTED PHENOXY) ACETYL/PROPIONYL] BENZOTRIAZOLES

To an ice-cooled alkaline solution of benzotriazole (0.036 mole in ethanol) were reacted with phenoxy and substituted acetyl/propionyl chlorides separately and worked up as usual (page 38) to yield the final products.

\[
\begin{align*}
\text{Phenoxy acetyl/propionyl chloride} \\
\end{align*}
\]

(c) SYNTHESIS OF VARIOUS N-[2-(PHENOXY AND SUBSTITUTED PHENOXY) ACETYL/PROPIONYL] IMIDAZOLES

To an ice-cooled alkaline solution of imidazole (0.036 mole in ethanol) were treated with phenoxy and substituted phenoxy acetyl/propionyl chlorides separately and worked up as usual (page 38) to give the final products.

\[
\begin{align*}
\text{Phenoxy acetyl/propionyl chloride} \\
\end{align*}
\]
The following SCHEME-1 outlines this part of work:

\[ \text{Phenols} \xrightarrow{\text{NaOH}} \text{Sodium salt of phenols} \]

\[ H^+ \cdot \text{CH}(R) \cdot \text{CO}_2 \text{H} \]

Acetic/propionic acids

\[ \text{Hell-Volhard Zelinsky reaction (HVZ)} \]

\[ \text{Cl-CH}(R) \cdot \text{CO}_2 \text{H} \]

\[ \text{Cl-CH}(R) \cdot \text{CO}_2 \text{Na} \]

Sodium salt of \( \alpha \)-monochloro acetic/propionic acids

\[ \text{dil. HCl} \]

\[ \text{Phenoxyacetic/propionic acids} \]

\[ + \text{SOCl}_2 \]

\[ \text{Phenoxy acetyl/propionyl/chlorides} \]
Phenoxy acetyl/propionyl benzimidazoles

Phenoxy acetyl/propionyl benzotriazoles

Phenoxy acetyl/propionyl imidazoles

$X=H, Br, Cl, NO_2$ or $CH_3$ occupied different positions in the ring-$A$.

$R=H$ or $CH_3$

* = Assigned as carbon number-2
PART - II

SYNTHESIS OF N-[2-(1-AND 2-NAPHTHOXY) ACETYL] BENZIMIDAZOLES,
BENZOTRIAZOLES AND IMIDAZOLES

This part consists of four steps.

STEP-FIRST

Both 1- and 2-napthols were used commercially available.

STEP-SECOND

SYNTHESIS OF N-[2-(1/-2-NAPHTHOXY) ACETIC] ACIDS

Equimolecular quantities (0.08 mole) of 1/-2-napthols (dissolved in 30 ml of 33% aqueous sodium hydroxide solution) and monochloroacetic acid have been taken separately and worked up by the same method as described on page 35 to give 1/-2-naphthoxy acetic acids (yield 66%).
STEP-THIRD

SYNTHESIS OF [2-(1-/2-NAPHTHOXY) ACETYL] CHLORIDES

In the solution of 1-/2-naphthoxy acetic acids (0.046 mole in methanol) separately were added excess of thionyl chloride (0.056 mole) and worked up as usual (page 36) to yield 1-/2-naphthoxy acetyl chlorides. The use of excess of thionyl chlorides in this step was to avoid the anhydride formation. The mechanism is same as described on page 37 (yield 79-80%).

\[
\begin{align*}
\text{1-OCH}_2\text{CO}_2\text{H} + \text{SOCl}_2 & \rightarrow \text{1-OCH}_2\text{COCl} \\
\end{align*}
\]

STEP-FOUR

(a) SYNTHESIS OF 1-/2-NAPHTHOXY ACETYL BENZIMIDAZOLES

To an ice-cooled alkaline solution of benzimidazoles (0.036 mole in methanol) were treated with methanolic solution of 1-/2-naphthoxy acetyl chlorides separately and worked up by the same procedure as described on page 38 to afford the final products.

\[
\begin{align*}
\text{NaOH} & \rightarrow \text{Na} \\
\text{1-/2-naphthoxy acetyl chloride} & + \text{NaCl}
\end{align*}
\]
(b) SYNTHESIS OF 1-/2-NAPHTHOXY ACETYL BENZOTRIAZOLES

To an ice-cooled alkaline solution of benzotriazole (0.036 mole in ethanol) were reacted with ethanolic solution of 1-/2-naphthoxy acetyl chlorides separately and worked up as usual (page 38), to yield the final products.

(c) SYNTHESIS OF 1-/2-NAPHTHOXY ACETYL IMIDAZOLES

To an ice-cooled alkaline solution of imidazole (0.036 mole in ethanol) were treated with ethanolic solution of 1-/2-naphthoxy acetyl chlorides separately and worked up by the same procedure as described on page 38 to give the final products.
The purity of the compounds were checked by TLC using silica gel coated plates. Melting points were taken on a Toshniwal melting point apparatus (India). All the compounds were analysed for their C, H and N percentages. Their infra-red spectra were recorded on a Perkin-Elmer-157 (in KBr, cm⁻¹) spectrophotometer.

The summarized syntheses is given in SCHEME-2.
The following SCHEME-2 outlines this part of work -

\[
\begin{align*}
&\text{H-CH}_2\text{-CO}_2\text{H} \\
&\text{Acetic acid} \\
&\text{Hell-Volhard Zelinsky reaction (HVZ)} \\
&\text{Cl-CH}_2\text{-CO}_2\text{H} \\
&\text{monochloro acetic acid} \\
&\text{NaOH} \\
&\text{Cl-CH}_2\text{-CO}_2\text{Na} \\
&\text{Sodium salt of monochloro acetic acid} \\
\end{align*}
\]

\[
\begin{align*}
&\text{1-/2-naphthol} \\
&\text{NaOH} \\
&\text{Sodium salt of 1-/2-naphthol} \\
\end{align*}
\]

\[
\begin{align*}
&\text{Sodium salt of 1-/2-naphthoxy acetic acid} \\
&\text{dil. HCl} \\
&\text{1-/2-naphthoxy acetic acid} \\
&\text{SOCl}_2 \\
&\text{1-/2-naphthoxy acetyl chloride}
\end{align*}
\]
(a) Benzimidazole

(b) Benzotriazole

(c) Imidazole

$\text{* = Assigned as carbon number - 2}$
SECTION II

GENERAL DESCRIPTION OF THE ACTIVITY PERFORMED, TECHNIQUES OF THE EVALUATION OF ANTIMICROBIAL, ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES
GENERAL INTRODUCTION TO PHARMACOLOGY

Pharmacy in some guise has been inseparable from mankind's history since it fulfills one of our most basic needs. As man made his way through remote times or places, he shielded himself against disease as best he could, reaching out, often blindly, toward the resources of nature but in the process gradually elaborating pharmaceutical theories, techniques, and implements. The person supplying this essential service may not be recognizable always as a pharmacist in sense of the term.

One of the main functions of the pharmaceutical industry is to create products. Products are defined as drugs, devices, or services that have a perceived import on the health care systems. Many important examples of the impact of drugs on health and longevity may be cited. Much of the credit can go to research, along with the development, production and distribution facilities of the pharmaceutical industry. Chemist and other Physical scientist have been predominantly responsible for synthesis, isolation and characterization of medicinal agents. However, biological scientists have played an equally essential role in originating meaningful screening and testing models and in the overall evaluation of new agents. Organic chemists synthesize
new drug as well as isolate and characterise natural products. In each case there is interest in the complex relationship between chemical structure and pharmacological action.

The pharmacological activity of a compound is involved function of the structure, and very small changes may profoundly modify the pharmacological effect. These structural modifications may involve replacing one group with another at a specific point in the molecule, shifting the same group from place to place in the parent molecule, saturating valence bonds or modifying the acidity or basicity.

The use of any drug in the treatment of disease may be discussed under two heads. The first of these include the drugs that are used in the treatment and cure of specific diseases and the second category is the one which has the characteristic effect upon the animals and organisms but are not the specific remedies for a particular disease for example morphine, cocaine etc.

The development of new drugs depends upon clinical trials and its use in medicine. A successful drug would be one which is:

(a) readily absorbed and slowly excreted and
(b) having low toxicity to invading organisms.
Their relationship is frequently gained by the ratio:

$$\frac{\text{Maximum tolerated dose (MTD)}}{\text{Minimum curative dose (MCD)}}$$

which is called chemotherapeutic index and larger the ratio and safer would be the drug.

These two factors depend upon mainly on the relationship between biological activity and chemical constitution of a particular drug. These relationships are identical to serve as a guiding factor in mapping the structural features of the compounds with analogous activities; hopefully more potent, more specific and less toxic. The biological activity of the drug is not the sum of the activities of groups or atoms present in it but due to the molecule as a whole. The individual activity of all the groups or atoms associated in the molecule is changed during the synthesis of the drug.

The idea about the structure activity relationship underwent gradual changes with the advancement in the knowledge of chemical and physical properties of the molecule. Even the most advanced and carefully considered theories have not revealed regularities in the relation of chemical structures to physiological action which could be used indiscriminately in one
series of compounds after providing their value in the other. According to W.A. Sexton physical properties and reactivity of a molecule after the structural variations may cause changes in distribution in the cells and tissues and access the active sites of enzymes and receptors in reaction rates at such sites and in excretion pattern.

This attractive hypothesis suggested a new approach in chemotherapeutic research with comprised of the trials of compounds closely related to an essential metabolite of a micro-organism. The slightest change in structure often does produce considerable change in biological properties. Therefore, in evaluating structure activity relationship, the total picture of steric factor, electron density, localisation and the resultant physical and chemical reactivities of a given compound need be considered.

Chemotherapeutic value of a compound is usually determined in different stages. First the preliminary in vitro tests are performed and if the compounds are found active in such tests, these are subjected in vivo tests along with the tests to determine their toxicity in order to find their possible practical usefulness as a drug.
GENERAL INTRODUCTION TO ANTIMICROBIAL

The antimicrobial drugs occupy a unique niche in the history of medicine. The germ theory of disease was the vehicle of a dramatic revolution in medicine. During the entire preceding history of medicine, fewer than a handful of drugs had a known locus of action, and even fewer had been submitted to systematic laboratory investigation. The first systemic antimicrobial drugs revolutionized in the treatment of certain protozoal infections, especially syphilis, but the second major revolution in medicine in which the antimicrobial drugs played a major role awaited the appearance of sulphanilamide and penicillin; the exponential development in the antibiotic and systemic antibacterial field is the inevitable result of the momentum created by those two agents.

During the early part of this century, tremendous studies were made in the systemic treatment of certain microzoal infections. Nevertheless, these advances did not greatly affect directly the overall practice of medicine. The advent of sulphanilamide in 1935 marked the beginning of a major revolution in the practice of medicine. The subsequent profusion of antibacterial agents overwhelmed the physician with golden tools. The realization that certain
microorganisms are successfully resisting the "wonder drugs" not only impels a ceaseless search for new systemic antibacterial agents but also forces a sober return to certain ancillary art of the medical and surgical management of infectious disease.

The sulphonamides originally possessed a wide antimicrobial spectrum which included all gram-positive cocci, except enterococcus, all gram-positive bacilli, nearly all Enterobacteriaceae and gram-negative cocci, H.influenzae, B.pertussis, Pasteurella and some Pseudomonas chlamydia.

The mechanism of the antimicrobial action of the sulphonamides has been analysed extensively. The sulphonamides compete with p-aminobenzoic acid and prevent its normal cellular utilization, particularly its incorporation into folic acid (pteroylglutamic acid, PGA). Thus, sulphonamide sensitive organisms are primarily those which synthesise their own folic acid. Organisms able to utilise preformed folic or tetrahydrofolic acid or the tetrahydrofolate-dependent pyrimidines and thymidine are not generally affected by these agents. This mechanism is of importance as an example of the general concepts of biological antagonism and antimetabolites. The efficacy of sulphonamides is generally enhanced when the drugs are used in
combination with the folic acid antagonist trimethoprim. Sulphonamides are the drugs of choice only for the treatment of chancroid, nocardiosis and acute uncomplicated urinary tract infections caused by E.coli and P.mirabilis.

Fungi are plantlike, nonphotosynthetic eukaryotes; they grow either in colonies of single cell (yeast) or in filamentous multicellular aggregates (molds). Most fungi live as saprophytes in soil or dead plant material and are very important in the mineralisation of organic matter. Unfortunately, some species are parasites of terrestrial plants and cause serious damage to crops. A smaller number produce disease in humans and animals. The etiologic agents and methods of prevention and treatment differ for each group. The organisms that cause contagious skin infections are specialised saprophytes that digest keratin in soil as well as skin. The fungi that cause noncontagous air-borne infections include yeasts and molds. Most are soil saprophytes and accidentally invade human hosts through inhalation of soil debris. An exception is Candida albicans which is part of the normal flora of the human gastrointestinal tract and vagina. This yeast can cause minor lesions of the skin and mucous membranes, and in immunologically weakened individuals, serious systemic disease.
Clinical manifestations of disease caused by these fungi include chronic granulomas with necrosis and abscess formation. Clinical symptoms in humans following ingestion or other contact with the toxins include blood dyscrasia, hepatic cirrhosis and carcinoma, hallucinations and dermatitis.
EXPERIMENTAL

This part has been divided into two heads:

(a) Evaluation of antibacterial activity; and
(b) Evaluation of antifungal activity.

(a) EVALUATION OF ANTIBACTERIAL ACTIVITY

Various methods\textsuperscript{142-149} are available for the evaluations of antibacterial activity. However, the most widely used method consists in determining the antibacterial activity of the drug by adding it in varying concentrations to the cultures of the test organisms. In the present work, activities of the synthesised compounds were evaluated by standardized single disk method\textsuperscript{150}. The main aim of these investigations was to study the changes in the activity with the variation in the structure of the molecule and thereby establishing a correlation between the structure of the compounds and their antibacterial properties.

All the synthesised compounds (in ethanol) have been screened \textit{in vitro} against the following bacteria using streptomycin as a standard.

(A) \textit{Escherichia coli}, gram (-)
(B) \textit{Proteus vulgaris}, gram (-)
(C) \textit{Staphylococcus aureus}, gram (+)
(D) \textit{Bacillus anthracis}, gram (+)
The few compounds of benzotriazoles were only tested against *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae*, *Klebsiella pneumoniae* with same standard, all are gram (-) bacteria.

This method consists of the following steps:

(1) Preparation of the medium, its sterilization and tubing;

(2) Treatment of the glass apparatus and its sterilization;

(3) Pouring of the needed medium into sterilized petridishes;

(4) Preparation of the required concentration of the compounds and dipping the sterilized Whatman filter paper disc (6 mm) into it;

(5) Incubation at particular temperature and

(6) Measurement of the zones of inhibition.

Out of the different steps in the above method, the most important is the selection of the suitable medium and its preparation because it is the composition of the medium which exerts greatest influence upon the activity of a compound. The other factors which influence in vitro tests are:

(1) The kind and condition of the test organisms.
(2) The concentration of the drug solution and the dilution of the drug at the site of action (incubation period).

(3) Environment factors which may augment or counteract the interaction of the drug and the parasite.

(4) Temperature of the incubation because for each bacteria there is an optimum temperature and for most of the pathogenic bacteria this temperature is $37^\circ$.

(5) pH of the medium which is usually in the range of 7.2 to 7.6.

In the present work, the medium which is employed, has the following compositions:

- Meat extract - 100 ml
- Peptone - 1 gm
- Sodium chloride - 0.5 gm

For the preparation of meat extract, 500 gm fat free minced meat was placed in 1000 ml of distilled water for 24 hours in cold. The mixture was strained through a muslin cloth. The residue was discarded and the filtrate obtained having red in colour. The surface fat was removed by skimming with filter paper and boil for 15 minutes. The insoluble proteins coagulated. The
fluid was filtered through cheesecloth and make up the
original volume by the addition of distilled water.
This gave the extract clear yellow in colour.

**NUTRIENT BROTH**

As the meat extract is deficient in nitrogenous materials, the heat resistant protein derivatives in the form of peptone was added. Peptone is the principal source of nitrogen. It may also contain some vitamins and sometimes carbohydrate depending upon the kind of proteinous material digested, sodium chloride is added to increase the salt content.

All the ingredients, peptone (1g), sodium chloride (500 mg) and meat extract (100 ml) were mixed and heated till they dissolved. It was then filtered through a filter paper by adjusting the pH at 7.4 by using normal sodium hydroxide solution. The medium so obtained was poured into the sterile flask and plugged with sterile cotton plug which was then sterilized into an autoclave at 15 lb pressure for 15 minutes.

**NUTRIENT AGAR**

Agar is a complex carbohydrates obtained from certain marine algae and is used as a solidifying agent for media. It is not considered as a source of nutrition for the bacteria. It has the following compositions:
Agar powder - 2 gm
Nutrient broth - 100 ml

The above mixture was boiled in running steam for about half an hour which dissolved completely agar particles. It was then filtered through a glass wool in hot medium by adjusting the pH at 7.4. The medium was then kept in the flasks and heated in an autoclave at 15 lb pressure for 15 minutes and then transferred into the sterilized petri dishes.

INOCULATION OF TEST PLATES

At least four or five well isolated colonies having the same morphological type were selected from an agar plate culture with a wire loop. The top of each colony was touched and transferred the growth to a tube containing 4 to 5 ml of a suitable broth medium. The broth culture was incubated at 37°C for 8 hours.

The sterile cotton swab on wooden application was dipped into the inoculum and the excess of inoculum was removed from the swab by rotating several times firmly inside the wall of the test tube above the final level. The dried surface of a Miller Hinto agar plate was inoculated by streaking the swab over the entire sterile agar surface. The streaking was repeated two or three times so as to ensure an even distribution of the inoculum.
APPLICATION OF DISC: Within about 15 minutes after the plates were inoculated, antimicrobial impregnated disc were applied on the surface of the inoculated plates with sterile forceps. Aseptically each disc was pressed on the medium to ensure complete contact. The spatial arrangements of the discs must be such that they are no closer than 15 mm from the edges of the plate and far enough apart to prevent overlapping of the zones of inhibition that is no more than 24 mm. Since some diffusion of test compound is almost instantaneous, a disc was not moved once it came in the contact with the agar surface.

INCUBATION OF TEST PLATES: The individual plates were evenly dispersed the incubator shelf (at 37°C) so that each plate reached incubator temperature at approximately the same time.

READINGS AND INTERPRETATION: After 24 hours of incubation, the plates were examined and the diameter of the zones of complete inhibition was measured to the nearest whole millimeter with a sliding calipers.

(B) EVALUATION OF ANTIFUNGAL ACTIVITY

There are several methods available for recording the antifungal activity. The one which is in common use in the recent time has been adopted.
All the synthesised compounds (in ethanol) were also screened for their antifungal activity in vitro against the following selected fungi using mycostatin as a standard.

(A) Aspergillus fumigatus
(B) Fusarium oxysporium
(C) Candida albicans

The few compounds of benzotriazoles were only tested against Aspergillus fumigatus, Candida albicans, Microsporum gypseum and Trychophyton mentagrophytes with the same standard.

This method consists of the following steps:

STERILIZATION OF THE APPARATUS: All the glass apparatus were cleaned with chronic acid followed by distilled water and sterilized by heating at 200° in a hot air oven.

PREPARATION OF THE MEDIUM: Sabourauds glucose agar medium was used for antifungal screening which consists of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>40 gm</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.2 gm</td>
</tr>
</tbody>
</table>
Streptomycin was used to check the growth of undesirable bacteria. The above mentioned ingredients were weighted and dissolved in a 500 ml of distilled water. After the ingredients were dissolved completely, more distilled water was added to make the solution up to one litre and the pH of the medium was kept at 7.6 ± 0.1. The medium was heated in an autoclave at 15 lb pressure for half an hour and then transferred into the sterilized petri discs. The broth has prepared in 100 ml by using glucose and peptone.

The spore suspension of each test organism (72 hours culture) was kept in a broth at 35-40°C. The petri dishes were inoculated in the same manner as described on page 60 for antibacterial screening. These petri dishes were incubated at 30°C for 48 hours. The zone of inhibition was considered as an indication for the antifungal activity.
GENERAL INTRODUCTION TO ANTI-INFLAMMATORY

Inflammation is a process by which body fights the energy of any living or non-living foreign body and characterised by redness, swelling, heat and pain. These conditions may be attributed to vasodilation, leakage of plasma into tissues, increase of blood supply and stretching of the tissues respectively.

A large number of substances the so-called mediators of inflammation are formed or released either concurrently or in successive time sequences at the site of injury from various cell sources in response to an etiologic factor. A variety of cells contain a number of potent mediators and, in some instances, inhibitors of the inflammatory response. These cell sources may include neutrophils (polymorphonuclear neutrophil leukocytes), basophils, mast cells, platelets, macrophages and lymphocytes. The numerous mediators of inflammation implicated in the inflammatory process and elaborated by the foregoing cells include histamine, serotonin leukokininins, slow reacting substance of anaphylaxis, lysosomal enzymes, lymphokines, and prostaglandins. Medicinal effect of bark of Willow (Salix vulgaris) and certain other plants in therapy of inflammation has been known for many centuries. The
presence of salicin in these plants gave an idea to use synthetic salicylates as anti-inflammatory agents such as acetyl salicylic acid and methyl salicylate.

The first non-steroidal anti-inflammatory agent was PARACETAMOL although it has been observed to have a low degree of anti-inflammatory activity but a strong antipyretic activity. During the period between two world wars pyrazolone derivatives gained considerable popularity. Although improved therapy is desperately needed for the rheumatic diseases, especially rheumatoid arthritis and osteoarthritis, beneficial drugs are available. These products relieve pain, swelling, and inflammation and enable many patients with mild to moderate rheumatic disease to lead essentially normal lives. These include drugs such as aspirin and the salicylates, phenylbutazone, indomethacin, ibuprofen, fenoprofen, naproxen, tolfenin, D-penicillamine, mefenamic acid and the gold compounds.

An obstacle to the discovery of new drugs to treat chronic conditions such as rheumatic disease is the difficulty in developing animal models that resemble the disease sufficiently for pharmacologic testing. The most widely used primary test to screen new nonsteroidal anti-inflammatory agents measures the ability of a compound to reduce local edema induced in the rat paw by
injection of the irritant carrageenan, which is a mucopolysaccharide derived from Irish sea moss, chondrus crispus. Most clinically useful anti-inflammatory agents suppress this type of edema. The anti-inflammatory properties of indomethacin, a widely used nonsteroidal anti-inflammatory agent, were initially detected by a carrageenan assay. 'INDOMETHACIN' was discovered at Merck, Sharp and Dohme laboratories in U.S.A. and is highly potent anti-inflammatory drug but exhibited a high degree of gastric toxicity. The advent of propionic acid derivatives in the horizon of anti-inflammatory therapy brought newer hopes in the minds of clinician starting with IBUPROFEN, FENOPROFEN, KETOPROFEN etc. which were found to be extremely safe drugs.

Anti-inflammatory agents act on various systems responsible for inflammation such as plasmin, clotting, arachidonic acid or complement systems. Inflammation can be reduced by inhibition of oxidative phosphorylation, inhibition of protein denaturation and acceleration of sulphhydryl exchange. The agent may also reduce the inflammation by fibrinolysis that is by inhibition of platelets or by mixed lymphocyte reaction or by inhibition of complement. Interruption of arachidonic acid cascade is one of the mechanisms of
anti-inflammatory actions. This can be achieved by inhibiting membrane phospholipids, blocking of cyclo-oxygenase pathway on lipoxygenase pathway. The anti-inflammatory drugs are found to be regulate leukocyte function by inhibition of macrophages, phagocytes and release of lysosomal hydrolases. These drugs also act by inhibiting various enzyme systems like protease, 5 HT decarboxylase, histidin decarboxylase, elastase etc.

Anti-inflammatory agents are mainly used in 'rheumatism'. Rheumatism are connective tissue disease and belongs to the disorder of joints mainly the synovial joint and para or articular joints. Disorder of joints are rheumatoid arthritis, osteo-arthritis, ankylosing spondylitis and gout. Rheumatism arthritis is a chronic disease primarily involving peripheral joints. Osteo-arthritis is basically wear and tear degeneration of synovial joints. This results due to deterioration of articular cartilage and abnormal bony formation in joints. Deposition of crystalline monosodium urate hydrate in joints is known as gout, commonly affect the joints of great toe.

Attempts have been made to differentiate between analgesic and combined analgesic plus anti-inflammatory activities by measuring the edema and pain produced by an irritant. Most compounds with analgesic and
anti-inflammatory actions. This can be achieved by inhibiting membrane phospholipids, blocking of cyclooxygenase pathway on lipoxygenase pathway. The anti-inflammatory drugs are found to be regulate leukocyte function by inhibition of macrophages, phagocytes and release of lysosomal hydrolases. These drugs also act by inhibiting various enzyme systems like protease, 5HT decarboxylase, histidin decarboxylase, elastase etc.

Anti-inflammatory agents are mainly used in 'rheumatism'. Rheumatism are connective tissue disease and belongs to the disorder of joints mainly the synovial joint and para or articular joints. Disorder of joints are rheumatoid arthritis, osteo-arthritis, ankylosing spondylitis and gout. Rheumatism arthritis is a chronic disease primarily involving peripheral joints. Osteo-arthritis is basically wear and tear degeneration of synovial joints. This results due to deterioration of articular cartilage and abnormal bony formation in joints. Deposition of crystalline monosodium urate hydrate in joints is known as gout, commonly affect the joints of great toe.

Attempts have been made to differentiate between analgesic and combined analgesic plus anti-inflammatory activities by measuring the edema and pain produced by an irritant. Most compounds with analgesic and
anti-inflammatory activity do not raise the threshold to pain in normal joints. The term antinociceptive is used to differentiate this combined analgesic and anti-inflammatory activity from simple analgesic activity.

Anti-inflammatory agents are classified as follows:

(1) STEROIDAL ANTI-INFLAMMATORY AGENTS

They exert their action by inhibiting the release of phospholipids in lipoxygenase pathway which inhibited the release of arachidonic acid from membrane, e.g. dexamethasone etc.

(2) NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

They are said to inhibit biosynthesis of prostaglandin at cyclo-oxygenase pathway, e.g. indomethacin, aspirin, mefenamic acid etc.

SCREENING METHODS

The screening methods for anti-inflammatory activity have been classified as follows:

(1) Non-immunological methods;
(2) Immunological methods; and
(3) Miscellaneous method.
(1) **NON-IMMUNOLOGICAL METHODS**

Non-immunological methods have been further divided into the following headings.

(A) For evaluation of acute inflammation: It is of six types:

(i) Carrageenan induced hind paw oedema method\textsuperscript{152}.
(ii) 5-hydroxy tryptamine induced hind paw oedema method\textsuperscript{153}.
(iii) Formalin induced hind paw oedema method\textsuperscript{154}.
(iv) Hyaluronidase hind paw oedema method\textsuperscript{155}.
(v) Histamine induced hind paw oedema method\textsuperscript{155}.
(vi) Turpentine oil induced arthritis in knee joints method\textsuperscript{156}.

(B) For evaluation of subacute inflammation: It is of two types:

(i) Carrageenan granuloma pouch technique\textsuperscript{157}.
(ii) Cotton pellet granuloma technique\textsuperscript{158}.

(C) For evaluation of chronic inflammation: It is of only one type:

(i) Formaldehyde induced arthritis method\textsuperscript{159}.

(2) **IMMUNOLOGICAL METHODS**

Immunological methods are of two types:
(a) Adjuvent arthritis method\textsuperscript{160}.

(b) Tuberculin sensitivity test method\textsuperscript{161}.

(3) MISCELLANEOUS METHODS

It is of two types:

(a) UV erythema method\textsuperscript{161}.

(b) Urate crystal induced synovitis method\textsuperscript{162}.
EXPERIMENTAL

Anti-inflammatory activity of the synthesised compounds were carried out with albino rats weighing (80-120 g) of either sex. The test compound and the standard drug were administered at a dose of 50 or 100 mg/kg body weight i.p. in aqueous suspension. \( \text{ALD}_{50} \) values were determined employing albino rats as test animals. The acute toxicity were determined in rats by intraperitoneal administration of the test compounds at graded doses\(^{163} \) and recording the mortality after 24 hours. All the compounds were found to be relatively less toxic as their \( \text{ALD}_{50} \) values ranged from 250 - 1000 mg/kg i.p.

All the synthesised compounds were tested for anti-inflammatory activity by rat paw oedema test as described by Winter, Risley and Nuss\(^{152} \) utilizing carrageenan suspension as the phlogistic agent. Anti-inflammatory activity was determined by measuring the change in the volume of inflammed foot produced by injection of 0.05 ml of 1% freshly prepared carrageenan suspension.

The volume was measured by 'plethysmograph'. Initial volume of right hind paw of albino rats were measured plethysmographically without administration of test compounds.
Albino rats (80-120 g) were divided into three groups each consisting of four rats. A group of rats was treated intra-peritoneally with 50 or 100 mg/kg body weight of the aqueous suspension (with few drops of Tween-80) of the synthesised compounds. Another group was administered i.p. 100 mg/kg body weight of aqueous suspension of acetyl salicylic acid (ASA) (standard drug) and the third group (control group) was fed with the same volume of distilled water. One hour after the drug administration the animals were injected with 0.05 ml suspension of carrageenan (1.0 percent in 0.9 percent saline) in the right hind paw planter apponeurosis. The measurement of the paw volume were taken using mercury displacement technique with the help of plethysmometer immediately before and 1, 2 and 3 hours after the carrageenan injection. The percent inhibition of inflammation after 3 hours was calculated by the method of Newbould using the following formula -

Percent Inhibition \( I = 100 \left[ 1 - \frac{a-x}{b-y} \right]\)

where, \( x \) = Mean foot volume of rats before the administration of carrageenan injection in the test and the standard groups.

\( a \) = Mean foot volume of rats after the administration of carrageenan and test compounds injection in the test and standard group.
\[ y = \text{Mean foot volume of rats before the administration of carrageenan injection in the control group.} \]

\[ b = \text{Mean foot volume of rats after the administration of carrageenan injection in the control group.} \]

\textit{Aspirin has been included in this study since it is known to have anti-inflammatory action}^{165-167} \textit{and it is the prototype against which the other anti-inflammatory, analgesic and antipyretic drugs are compared}^{168}. \]
GENERAL INTRODUCTION TO ANALGESICS

Analgesics act centrally to increase the capacity to tolerate pain. Analgesic is characterized by altered behavioral response to pain and by diminished ability to perceive pain impulses without loss of consciousness.

Pain is a universal experience of all mankind. Attempts to define this term have not proved satisfactory. However, pain is a highly individualized perception of stimulus as modified by a wide variety of personal, attitudinal and emotional factors. There is also the difference between experimental and pathological pain. Experimental pain is usually brief and is accompanied by a sense of security and is relatively unresponsive to analgesics. Pathological pain may be prolonged. It is associated with anxiety and response to analgesic drugs. Pain receptor organs are distributed through the body. Clinically pain can be considered as:

(a) Superficial or cutaneous pain.
(b) Deep pain from muscles, joints, ligaments and bones.
(c) Visceral pain.
(d) Referred pain.
(e) Psychogenic or functional pain.
Pain from muscles, joints, ligaments and bones usually has a dull character and it may be accompanied by sickening sensation due to an autonomic response. Visceral pain is dull, aching in character and is accompanied by sweating, nausea, fall in blood pressure and even shock in practice. Visceral pain may be due to spasm, ischaemia, myocardial infection, inflammation (appendicitis) or stimulation of sensory nerve ending (peptic ulcer). Deep pain whether visceral or somatic in origin, may sometimes be misinterpreted as it is coming from some part of the body other than the actual site of stimulation. This is called referred pain. Psychogenic or functional pain is usually a vague pain which follows no definite anatomical pattern of distribution. Such pain is usually continuous from day to day and involves more than one part of body. It, however, does not disturb sleep. Pain is mediated by the nerve endings of the non-medulated sensory fibres, which carry it to the spinal cord. The pain fibres are mainly carried to the thalamus. The chemicals may or not be the reason for pain because it is uncertain. Analgesics \(^{170}\) are classified as:

(1) NARCOTIC ANALGESICS

The analgesics under this class not only provide relief from pain but also produce depression of the central nervous system.
(-)-Morphine is the natural product prototype for this class of analgesics. It and its congeners are called opioids, narcotics, narcotic analgesia and centrally acting analgesics. They modify the effects of pain impulses on the central nervous system. The most critical in limiting the usefulness of opioids are centrally mediated tolerance, dependence, and respiratory depression, which is the cause of death from opioid overdose.

Psychologic and physical dependence develops upon extended use of narcotics. Psychologic dependence is characterised by drug seeking behaviour and physical dependence is gradually manifested after stopping narcotics use or is precipitously manifested after administration of a narcotic antagonist. The most common symptoms of the withdrawal syndrome include: anorexia, weight loss, pupillary dilation, abdominal cramps, excessive sweating, nausea, vomiting, muscle spasms, hyperirritability, lachrymation, and increased heart rate. The less severe withdrawal syndromes characteristic of some narcotic analgesics (methadone) have led to their use as substitutes for or morphine and its congeners.
(2) NON-NARCOTIC ANALGESICS

Salicylic acid, acetyl salicylic acid, acetaminilide, and salicin, a constituent of the bark of Salix alba, represent the synthetic and natural product prototypes of this class of drugs.

These analgesics produce relief of pain without hypnosis, or marked impairment of mental activity. They are mainly useful in relieving dull aching pain to low intensity coming from integumental structures such as muscles and joints.

The mechanism of the analgesic effect of salicylates, is still controversial. Some assume that it is to be mainly supraspinal. The thalamus is responsible for the integration of pain sensation and also emotional reactions to pain. They are believed to act by a blockade of the pain centres in the thalamus.

The non-narcotic analgesics appear to have both peripheral (non analgetic - anti-inflammatory) and central (analgesic) mechanisms in relieving pain. However a biochemical explanation for a direct effect on the lateral hypothalamus is lacking.
EXPERIMENTAL

The methods for characterising agents potentially useful for the relief of pain are numerous. There is no single approach that gives complete parallelism between the result in man and animals and clinical practice in man. Part of the difficulty is directly related to the fact that clinical pain is a pathological condition and differs from experimental pain.

Recognizing that the characteristics of clinical pain can only be reproduced in part in the animal models. It is found that battery of tests provide a firmer foundation for the evaluation of analgesic effects of the drugs. The following method has been adopted for the evaluation of analgesic activity:

The experiment was conducted by the modified method of Eddy and Leimbach\textsuperscript{171} using a Techno heated plate analgesic apparatus. Although this method is used mainly in mice. Curzone et al.\textsuperscript{172} used the method in male Sprague Dawley rats in their experiments on analgesic, taking the paw licking response as an index of analgesia.

Albino rats (80-120 g) were divided into three groups each consisting of four rats. A group of rats
were treated intraperitoneally with 50 or 100 mg/kg body weight of the aqueous suspension (with few drops of Tween-80) of the synthesised compounds. Another group was administered i.p. 100 mg/kg body weight of acetyl salicylic acid (ASA) (standard drug) and the third group (control group) was fed with the same volume of distilled water. The rats were placed on the hot plate maintained at the temperature of 55 ± 0.5°C. The time between putting the rat on hot plate and start of licking the paw was considered as reaction time. The reaction time was recorded for 15 minutes interval up to 45 minutes. The percent analgesia score (PAS) recorded by using following formula -

\[
PAS = \frac{T_2}{T_1} \times 100
\]

where,

\(T_1 = \ \text{Reaction time (in seconds) before test compound administration.}\)

\(T_2 = \ \text{Reaction time (in seconds) after test compound administration.}\)