5. **GENERAL PHARMACOLOGY**

5.1. **Introduction**

In clinical practice the physician is concerned with the safety margin of a drug. It is well known that most of the drugs produce toxic reactions at higher doses (for a long time). The greater ratio of toxicity against efficacy determines the usefulness of a drug. In common diseases and of minor ailments the drugs used usually have a very large margin of safety, i.e. a better ratio of toxicity upon efficacy, so called as the 'Therapeutic Ratio'. In contrast to this, in severe diseases where cure rates are very low or the disease is of severe nature, the drug which produces toxicity even in recommended therapeutic doses have been used, e.g. anticancer agents. In case of cardiac failure digitalis preparations are used where the margin of safety or therapeutic ratio is very low. A numerical expression is required for expressing the safety of a drug. It is expressed as the ratio of $LD_{50}$ (median lethal dose) to the $ED_{50}$ (median effective dose).

$LD_{50}$ is the dose of drug which causes mortality in 50% of the animals. This is estimated by using different groups of animals of either sexes and of same age and weight and giving different doses so as to kill animals in wide range, ranging from 20% to 80% and then to plot a graph of log dose against mortality from which $LD_{50}$ is calculated.
ED$_{50}$ is the dose of drug which produces the desired effect in the 50% of the animals and in case of antiamoebic drugs it would mean a dose which protects 50% of the animals.

LD$_{50}$ of the acetone extract of the plant O.indicum was studied. To study the side effects of a drug, it is tested pharmacologically using a number of isolated tissue preparations as well as in anaesthetised cat to study the effect of a drug on blood pressure and respiration which is very important.

The acetone extract of O.indicum has been studied pharmacologically on a number of isolated tissue preparations and also on cardio vascular and respiratory systems in cats.

5.2. Materials and Methods

5.2.1. Acute Toxicity Test

For determination of acute toxicity, healthy albino mice (Kasauli strain) of either sex weighing between 13-20 gm. were used. They were fasted for 18 hours prior to the experiment and water was allowed ad libitum. The drugs were suspended in 1% gum acacia, were administered orally with a blunt needle attached to a syringe. The dose given range from 500 mg/kg to 8 gm/kg, single administration. Following the administration of the drug, the animals were observed for the appearance of signs of toxicity for 2 hours and thereafter at intervals upto 8 hours. The mortality was noted over a period of seven days.
Effect on isolated tissue preparations

a) Guinea pig ileum

The guinea pig's isolated ileum is a tissue suitable for bio-assay of histamine. The responses are generally quite consistent and the relation to dose fairly predictable. Discrimination between doses is good enough for purposes of bio-assay. Acetylcholine can also be assayed by this method.

Guinea pigs of either sex weighing between 250-500 gms were used. The guinea pig was killed by stunning and bled. The abdomen was opened. Cecum was lifted forward and the ileocaecal junction was identified. The ileum was cut at this point and transferred to a petri dish containing Tyrode's solution. The lumen was washed thoroughly using Tyrode's solution. A piece of ileum, about 2-3 cm/s in length, was suspended in an organ bath containing Tyrode's solution. One end of the tissue was fixed to the aeration tube and the other to a frontal writing lever. The temperature of the bath was maintained at 37°C and the solution was bubbled with oxygen. The magnified responses were recorded on a smoked drum.

Tyrode's solution of the following composition was used -

(Gm/litre)

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<tbody>
<tr>
<td>Sodium chloride</td>
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<tr>
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<tr>
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</table>
Magnesium chloride - 0.1
Sodium di-hydrogen phosphate - 0.05
Sodium bicarbonate - 1.0
Dextrose - 1.0

When the spontaneous movements of the muscle subsided, the effect of the drug added into the bath was studied.

Various concentrations of acetylcholine solution were added to the bath with the help of a tuberculin syringe to see whether the tissue shows a graded response. A particular dose was chosen giving a medium response. The dose was repeated to see whether it produces the same response, taking care to fill the bath to the same level each time. When the reproducible responses were obtained, acetone extract of O.indicum (suspended in Tween 80) was added and the standard doses of acetylcholine was repeated. The same cycle was repeated for histamine. A constant 3 minutes interval was kept between two doses throughout the experiments.

5.2.2b **The isolated rat uterus**

Isolated rat uterus is utilized for bio-assay of 5-Hydroxytryptamine. It produces graded responses to nanogram doses. The method is very sensitive and discriminating. The disadvantage of spontaneity is overcome by using DeJalon's solution which is poor in calcium.
A young female rat was injected with stilboesterol (0.1 mg/kg) in arachis oil intramuscularly 24 hours prior to the experiment. It was killed by stunning and the abdomen was quickly opened. The bicornuate uterus was identified behind the coils of small intestine, one horn lying in each flank, with large amounts of fat along its inferior border. It was carefully removed and placed in a shallow dish containing DeJalon's solution and dissected free of the fat, ovaries etc. Care was taken not to stretch the preparation. One horn was then mounted in an isolated organ bath in DeJalon's solution at 37°C aerated with oxygen.

A period of about half an hour was allowed for stabilization and the experiment then begun. Responses to 5-Hydroxytryptamine were recorded by means of an isotonic lever loaded for a tension of about 0.5 gm. A contact period of 30 seconds and a recovery period of 3-5 minutes in between successive doses was given. Graded dose of 5-Hydroxytryptamine in nanograms (10, 15, 20, 25) were used.

**DeJalon's solution**

**Composition**

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<th>Gram/litre</th>
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<tr>
<td>Sodium bicarbonate</td>
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</table>
The isolated frog rectus abdominis

A frog was pithed and laid out on the frog dissection board. The two recti were removed and placed in frog Ringer solution in a shallow dish. They were carefully cleaned and one of them was trimmed to the desired size and mounted on organ bath in frog Ringer's solution, at room temperature, aerated with oxygen. A very small bath of 5 ml capacity was sufficient.

For recording purposes, an isotonic lever with sideways writing point was used tangential to the smoked drug, balanced for a tenction of 2.5 gram with an extrac load of 1 gm on long arm.

Responses with 2.0 ug acetylcholine were elicited and the 0.5 ug physostigmin was added and allowed to act for 3 minutes. In the presence of physostigmine, a response was elicited to the same dose of acetylcholine. The fluid was washed and bath was refilled.

Physostigmine potentiated the action of acetylcholine and this potentiation was reversible.

Similar responses were seen with the acetone extract of oroxyium indicum.

Composition of Frog Ringer solution

<table>
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<th>Component</th>
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<tr>
<td>Sodium chloride</td>
<td>6.5</td>
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</table>
Potassium chloride - 0.14
Calcium chloride - 0.12
Sodium dihydrogen phosphate - 0.005
Sodium bicarbonate - 0.4

5.2.2d. The isolated rat phrenic nerve

Diaphragm

At the neuromuscular junction, a nerve impulse liberates acetylcholine from the nerve ending into the cleft between muscle and nerve fibre. This acetylcholine causes a depolarization of the muscle fibre which in turn sets off a muscle action potential and contraction of the muscle fibre. The peculiarity of mammalian muscle is that each muscle fibre has a single end-plate and single nerve fibre innervating it. Hence the contraction obtained by nerve stimulation is a quick, brisk twitch. This is seen in the response of rat phrenic nerve hemidiaphragm preparation and in the contraction of cat's Tibialis muscle nerve preparation. The muscle fibres of lower species like frog are multiply innervated and hence nerve stimulation causes persistent depolarization and a prolonged, slow contracture of the muscle.

On the basis of this concept of muscle function, the actions of various drugs may be considered.

A rat weighing 150-200 gm was killed by stunning and cutting the throat. The skin overlying the xiphoid process
of the sternum was picked up and carefully cut upwards, exposing the thoracic cage, and downwards into the abdomen. The thoracic wall was gently separated from the thoracic cage on both sides. Cuts were placed on either side of sternum, proceeding from above downwards, care was taken not to damage the sternal attachment of the diaphragm. From the ends of these cuts, lateral extensions were made so as to expose the contents of the thorax. The diaphragm was identified as the flat pinkish brown structure completely separating the thorax from the abdomen.

The phrenic nerves were seen as thin tough strands embedded in connective tissue, one on either side of the heart; going up into the neck and down to the respective half of the diaphragm which it supplies. The nerve was gently cleared of connective tissue, care was taken to prevent drying by gently dripping Kreb's solution from time to time on the nerve.

A single lobe of the diaphragm was chosen for the experiment, and was cut in the direction of its fibres. This gave a triangular segment, the apex being the tendinous portion and the base of a strip of muscle attached to the ribs (which were cut along with it). The phrenic nerve was cut in the neck and cleared to the point where it enters the muscle substance near the tendinous apex. This preparation now consisted of nerve and hemidiaphragm.

It was tied onto a perspex electrode with three terminals. The strip of ribs was tied onto the horizontal bar of the electrode
while the nerve was gently placed on the sliding terminal. The preparation mounted on the electrode was then set up in a large organ bath (capacity 80 ml) in Kreb's solution, maintained at 37°C and aerated with oxygen.

A suture through the tendinous apex connects this preparation to a Straub's lever with minimal inertia, recording the muscle twitches on a smoked drum. These twitches were elicited either by direct stimulation of the muscle or by indirect stimulation of the muscle through the nerve, each requiring correct connection of appropriate terminal. Stimulat were obtained from a square wave pulse generator, the voltage, pulse width and frequency being adjusted optimally for each preparation. The response elicited by both direct and indirect stimulation was a quick sharp downward twitch, with a rebound flyback upward due to the spring of the lever.

Drugs under consideration were added to the bath fluid and their actions on the muscle twitches elicited directly and indirectly were studied.

5.2.2c. The isolated Frog Heart Perfusion

A frog was killed by stunning and cutting its throat. The thorax was opened quickly. The heart and half inch of aorta were removed and placed in a shallow dish containing warm Ringer Locke solution through which oxygen was bubbled. The heart was gently squeezed to remove blood from cavities of atria and ventricles, and to prevent clotting inside the coronary arteries.
The heart was then cleared of extraneous connective tissue and the aorta was tied in position over the tip of the glass cannula in the Langendorff apparatus. A suture was taken through the apex of the ventricle and the thread was led over the system of pulleys to the bar of the starling lever which records the contractions on a smoked drum.

The coronary flow was measured in terms of cardiac output per minute because in this preparation, the fluid enters the aorta, the coronaries, the right atrium, and right ventricle and leaves by the cut ends of the pulmonary arteries. Drugs to be studied for their action on coronary flow and rate and amplitude of heart beat, are injected into the side limb of the cannula in the aorta and the actions noted.

5.2.3. **Effect on cardiovascular and respiratory system**
(Cat blood pressure and respiration)

Normal healthy cats of either sex weighing approximately between 2-3 kg were used. The cat was fasted overnight, free access to water was allowed. The cat was anaesthetised with sodium pentobarbitone (35 mg/kg intraperitoneally). The cat was taken on operation table and the femoral vein was cannulated using a venous cannula which was connected to a burette containing normal saline. The drugs were injected intravenously through this cannula. The trachea was cannulated to record the respiration through a tambour.
The left carotid artery was cleared, separated from the nerve and cannulated with arterial cannula. The other end of the cannula was connected to a mercury manometer through the 'Y' tube. The space between mercury and artery was filled with 10% sodium citrate solution. The other end of the 'Y' tube was connected to a sodium citrate bottle kept at sufficient height. The blood pressure was recorded on a smoked kymograph paper through a mercury manometer with the help of a free writing pointer.

The cardiovascular and respiratory responses were standardized. The responses to the intravenous injection of adrenaline, isoprenaline, acetylcholine and histamine were recorded and their optimal doses were selected which could produce either a rise or a fall of about 50 mm of mercury. Sequences of these standard drugs were repeated to determine the reproducibility of the responses. After this, acetone extract suspended in Tween-80 was injected at different doses ranging from 10 mg/kg to 20 mg/kg body weight. The standard drugs were repeated after every dose of the test drug. The blood pressure and respiration was allowed to stabilize before injecting the next dose.

5.3. Results

1. Isolated guinea pig ileum

The extract did not produce per se contraction upto the dose of 800 µg/ml. It caused reversible and dose dependent blockade of acetylcholine induced contractions.
2. **Isolated rat uterus**

   The extract caused reversible blockade of Oxytocin induced contractions.

3. **Frog rectus abdominis**

   The extract caused reversible blockade of acetylcholine induced contractions.

4. **Rat phrenic nerve diaphragm**

   The extract caused slight blockade of indirect muscle stimulation. Direct muscle stimulation was unaffected.

5. **Frog heart perfusion**

   The extract did not produce per se effect and had no effect on adrenaline response.

6. **Cat blood pressure**

   The extract produced dose dependent fall in blood pressure. This was blocked by atropine.

   The extract has non-specific antispasmodic action on smooth as well as skeletal muscle. The fall in blood pressure is cholinergic in nature.
Antibacterial screening of acetone extract of Oroxylum indicum and Fraction (FP) in vitro.

The antibacterial activity of the extract and Fraction (FP) was carried out to find out whether the drug is directly amoebicidal or acting indirectly by killing the associated bacterial flora of the culture and thereby killing the amoebae. For this purpose, the associated bacteria from the culture were isolated, identified and tested along with other bacterial species against acetone extract of O. indicum and compound FP.

The bacteria isolated were identified as E. coli and Streptococcus faecalis. Along with these two isolated bacteria, S. aureus, Salmonella typhosa and Vibrio cholerae were also used in in vitro antibacterial drug action.

The method followed was a tube dilution method (Gould, 1960). Sterile test solutions (drugs) were added in graded concentration to the sterile nutrient broth aseptically. The tubes were then inoculated with 0.1 ml of 1:100 diluted 24 hour old culture of test bacteria. After inoculation, the tubes were incubated at 37°C for 48 hours. The tubes were then examined for the presence or absence of the growth of organism visually. The tubes containing the least concentration showing the absence of growth is considered as the minimum inhibitory concentration (MIC) of the drug. Along with the test drugs of standard drugs were also tested, e.g. Penicillin, chloramphenic, etc.
Antifungal activity of acetone extract of *O. indicum* and Fraction 9 in vitro

The antifungal activity was carried out by tube dilution method of Schraufstaffer (Schraufstaffer et al., 1955). Sterile test solutions of the compounds were added in graded concentration to the sterile Schraufstaffer's liquid medium tubes. These tubes were then inoculated with 0.1 ml of spore suspension of different fungi, e.g. *Candida albicans*, *Microsporum audouinii* and *Trichophytum mentagrophyte*. The opacity of the fungal spore suspension was adjusted to 5 million spores/ml. The tubes were then incubated at room temperature for 14 days. After incubation, the results were read for the presence or absence of the fungal growth. The growth was observed visually and the minimum inhibitory concentration (MIC) was determined. Along with the test drugs, standard drug, viz. Multifungin was kept.

Composition of Schraufstaffer's medium

(Schraufstaffer, E. et al., 1949)

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<th>Ingredient</th>
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<td>Asparagin</td>
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Results

Acetone extract of the plant \textit{Oroxyllum indicum} Vent and the fraction FF did not show any antibacterial activity and antifungal activity upto 1000 \textmu g/ml \textit{in vitro}.