III. MATERIALS AND METHODS

The present work was taken up to evaluate the Acute oral toxicity, Repeated dose 28-day oral toxicity, Dermal toxicity and Immunotoxicity in Wistar albino rats, Acute eye irritation in New Zealand White rabbits, Skin sensitization in guinea pigs, *in vitro* pharmacological efficacy of pesticide combination were studied against *Rhipicephalus sanguineus* by immersion test and *in vivo* acaricidal efficacy of the pesticide combination was studied in dogs, cattle and sheep. For acute oral toxicity, repeated dose 28 Day toxicity and Immunotoxicity studies cypermethrin and amitraz combination pesticide formulated with odorless kerosene as vehicle was used. For Dermal toxicity, Acute eye irritation, Skin sensitization, *in vitro* and *in vivo* studies Pesticide combination containing cypermethrin (1%) and amitraz (1%) combination formulation was used.

3.1 Materials

3.1.1 Pesticide

Technical grade cypermethrin, amitraz and odorless kerosene as vehicle supplied by M/S Tetragon Chemie Pvt., Ltd., Yelahanka, Bangalore-560 064 was used in the experiment. The structure and chemical formula of cypermethrin and amitraz were as detailed below.

Cypermethrin

![Chemical structure of Cypermethrin](image)

Chemical name (IUPAC):

(RS)-α-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR) -3-(2,2-dichlorovinyl) - 2,2-dimethylcyclopropanecarboxylate

OR

(RS)-α-cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate

Amitraz
Chemical name (IUPAC): N-methylbis(2,4-xylyliminomethyl)amine

3.2 Experimental Animals

Wistar albino rats, New Zealand white rabbits and guinea pigs procured from small Animal House, Veterinary College, Bangalore-24, and Sree Venkateshwara Enterprises, Subramanyanagar, Bangalore- were used in the present work. The animals were kept separately in cages and were allowed to acclimatize to the experimental conditions for one week before the commencement of actual studies under standard hygienic conditions and provided with nutrilab pellet feed supplied by M/s. Tetragon Chemie Ltd, Yelahanka, Bangalore and water *ad libidum*. The animals were maintained as per the protocol outlined in publication of the Committee for the Purpose of Control and Supervision of Experiments on Animals standard guidelines (CPCSEA, 2003) and obtained approval from CPCSEA (Committee for the Purpose of Control and Supervision of Experiments Animals, Animal Welfare Division, Government of India) code number CPCSEA/ CH/ORG/2003/1747 for domestic animals and permission from Institutional Animal Ethics Committee (IAEC) with reference No. LPM/ CPCSEA/04/05 for laboratory animals.

3.3 Design of the experiments
3.3.1 Acute oral toxicity-study

Acute oral toxicity study for the pesticide formulation containing cypermethrin and amitraz combination was conducted in both male and
female Wistar albino rats as per the Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870.1100, Acute oral Toxicity. (Anon., 1998a)

3.3.1.1 Study Procedure
3.3.1.1.1 Animal Preparation

Healthy young Wistar Albino rats aged 8 to 12 weeks weighing 100 to 140 g adult were acclimatized to the laboratory conditions for seven days prior to test before assigning the animals to treatment groups.

3.3.1.1.2 Animal groups and number of animals

The median lethal dose was determined for both male and female rats separately. Six groups of rats consisting 10 males in each group were used as one batch for determining LD$_{50}$ value in male rats. Another six groups consisting 10 female rats in each group were used as another batch for determining LD$_{50}$ value in female rats.

3.3.1.1.3 Dose selection

The doses to be selected were arrived after conducting a preliminary study. Five doses were selected for determining LD$_{50}$ value in both male and female rats.

3.3.1.1.4 Administration of doses

The animals were fasted overnight prior to the administration of the substance. Pesticide combination in graded doses were administered as a single dose to animals by gavage using a stomach tube. The volume of administration was maintained to one ml per animal through proper dilution of pesticide combination. The pesticide combination was diluted such that it contained each of the pesticide in the concentration mentioned below equally.

The group details and dose administered per kg body weight are as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>No of male rats</th>
<th>Dose (mg/kg)</th>
<th>Group</th>
<th>No of female rats</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>10</td>
<td>140+140</td>
<td>Group VII</td>
<td>10</td>
<td>140+140</td>
</tr>
<tr>
<td>Group II</td>
<td>10</td>
<td>160+160</td>
<td>Group VIII</td>
<td>10</td>
<td>160+160</td>
</tr>
<tr>
<td>Group III</td>
<td>10</td>
<td>180+180</td>
<td>Group IX</td>
<td>10</td>
<td>180+180</td>
</tr>
</tbody>
</table>
3.3.1.1.5 Observation of animals

Careful general clinical observations were made every day. All the animals were observed for effects on skin and face, eyes, mucous membranes, respiratory and circulatory systems, autonomic change such as salivation, central nervous system effects including tremors and convulsions, and changes in the level of activity, gait, posture, reactivity to handling or sensory stimuli, and altered strength, health conditions and mortality. The LD\textsubscript{50} was calculated as per the method described by Finney (1971).

3.3.2 Repeated dose 28-day oral toxicity study

Repeated dose 28-day oral toxicity study of the pesticide formulation containing cypermethrin and amitraz combination was conducted in both male and female rats as per the standard guideline of Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870.3050. Repeated dose 28-day oral toxicity study in Rodents. (Anon., 1998b).

3.3.2.1 Study Procedure
3.3.2.1.1 Animal Preparation

Healthy young adult Wistar albino male and female rats aged around 9 weeks weighing 80 to 120 g were acclimatized to the laboratory conditions for seven days prior to the study and before assigning the animals to different groups.

3.3.2.1.2 Selection of doses

The dose is selected on the basis of preliminary study and acute toxicity study where the highest dose was chosen with the aim of inducing toxicity but not death or reverse suffering. Thereafter, a descending sequence of dose was selected with a view to demonstrate any dose related response and No Observable Adverse Effects Level (NOAEL) at the lowest dose. Two to four fold descending dose intervals were selected as per the guidelines.
3.3.2.1.3 Animal groups and number of animals

Male and female rats acclimatized to laboratory conditions were assigned to control and treatment groups. Five groups consisting of 10 male rats and 5 groups consisting of 10 female rats in each group were used for the study.

Satellite group of control and high dose groups were maintained for both male and female rats separately as per the study protocol. These groups were maintained for further two weeks after the 28 Day period without administering the pesticide.

3.3.2.1.4 Administration of doses

The animals were administered with the pesticide formulation containing cypermethrin and amitraz containing each at a concentration and vehicle separately daily for a period of 28 days by oral gavage as a single dose. The volume of administration was maintained at one ml per animal through proper dilutions of pesticide combination.

The group details and doses administered per kg body weight were as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Male</th>
<th>Female</th>
<th>Dose type</th>
<th>Concentration (mg/kg)</th>
<th>Pesticide</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Group I</td>
<td>Group VI</td>
<td>Control</td>
<td>1ml</td>
<td>Saline</td>
</tr>
<tr>
<td>II</td>
<td>Group II</td>
<td>Group VII</td>
<td>Vehicle</td>
<td>1ml</td>
<td>Odorless kerosene</td>
</tr>
<tr>
<td>III</td>
<td>Group III</td>
<td>Group VIII</td>
<td>Low Dose</td>
<td>30+30</td>
<td>Cypermethrin +Amitraz</td>
</tr>
<tr>
<td>IV</td>
<td>Group IV</td>
<td>Group IX</td>
<td>Mid Dose</td>
<td>60+60</td>
<td>Cypermethrin +Amitraz</td>
</tr>
<tr>
<td>V</td>
<td>Group V</td>
<td>Group X</td>
<td>High Dose</td>
<td>120+120</td>
<td>Cypermethrin +Amitraz</td>
</tr>
</tbody>
</table>

3.3.2.1.5 Observations
General clinical observations were made at least once a day throughout the study period of 28 days considering the period of anticipated effects after dosing. All the animals were observed for health condition, morbidity and mortality at least twice daily.

3.3.2.1.6 Body weight and feed consumption

The animals were weighed individually at the beginning of the study and at weekly intervals till the end of study. Feed consumption measurements were made daily and weekly feed consumption was calculated throughout the study period.

3.3.2.1.7 Hematological parameters

Haematological parameters were estimated using blood samples collected from all the animals on Day 7, 14, 21 and 28 by retro-orbital plexus puncture technique using microhaematocrit capillary tubes under ether anesthesia. Disodium EDTA was used as an anticoagulant. The following haematological parameters were estimated following standard methods (Jain, 1990).

1. Total Erythrocyte count (TEC)
2. Haemoglobin concentration (Hb)
3. Haematocrit (Hct)
4. Mean corpuscular volume (MCV)
5. Mean corpuscular haemoglobin (MCH)
6. Mean corpuscular haemoglobin concentration (MCHC)
7. Total leucocyte count (TLC)
8. Differential leucocyte count (DLC)

3.3.2.1.8 Serum biochemical parameters

Serum biochemical parameters were estimated from the serum samples collected from the animals on Day 7, 14, 21 and 28 to investigate toxic effects on organs and tissues specifically on liver and kidney using Euro 5 plus automated clinical analyzer at Vijaya Diagnostic Centre, Bangalore. The commercially available diagnostic kits from M/S swemed Diagnostic,
Jayanagar, Bangalore were employed in the estimation of the following parameters.

1. Aspartate aminotransferase (AST)
2. Alanine aminotransferase (ALT)
3. Alkaline Phosphatase (ALP)
4. Blood urea nitrogen (BUN)
5. Creatinine (Creat)
6. Glucose
7. Total serum protein (TSP)
8. Albumin

3.3.2.1.9 Pathology

At the end of study period on Day 28, all the animals in control and treated groups were humanely sacrificed and subjected to detailed gross necropsy including examination of the external surface of the body all orifices, and cranial, thoracic and abdominal cavities and their contents. The organs were collected for histopathological study.

Collection of organs

After overnight fasting the rats were weighed and sacrificed humanely on Day 28 under ether anesthesia and necropsy was conducted on each carcass to observe any gross pathological changes.

The liver, spleen, kidneys, heart, lungs, stomach, intestine (small and large) and brain were separated from the adhering tissues using saline and collected by placing on the blotting paper and gently pressed to remove excess of saline.

The organs were weighed using analytical balance and the weight of each organ was recorded. Organ to body weight ratio i.e., organ weight/body weight was calculated and expressed in percentage.

Liver, Kidney, lungs, heart, spleen and brain collected were processed for histopathology by cutting sections of five microns thickness and stained with Haematoxylin and Eosin (Luna, 1968).
3.3.3 Dermal toxicity

Dermal toxicity study of the formulation containing cypermethrin (1%) and amitraz (1%) emulsified concentration in odorless kerosene as vehicle was used in both male and female Wistar Albino rats as per the standard guidelines set by Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870. 3200. 21/28 -Day Dermal Toxicity. (Anon., 1998c).

3.3.3.1 Study procedure
3.3.3.1.1 Animals Preparation

Healthy young adult Wistar albino male and female rats aged between 8 and 9 weeks of age and weighing 80 to 120 gm were selected and acclimatized to laboratory conditions for 7 days prior to the study before assigning the animals to different groups.

3.3.3.2 Preparation of animals skin

Hairs was shaved from not less than ten percent of the body surface area on both right and left flank region of the rats, 24 hrs before testing for application of the test substance. Approximately ten percent of the body surface, the area starting from scapulae (shoulder) to the wing of illum (lip bone) and half way down the flank on each side of the animal was shaved. Repeated shaving or clipping was done at weekly intervals for observations. While shaving or clipping care was taken to avoid abrading the skin.

3.3.3.3 Selection of doses

For repeated dose study three dose levels plus a vehicle level and a control was selected to determine NOAEL. The highest dose level was selected such that it resulted in toxic effects but did not produce severe skin irritation or an incidence of mortality which would prevent a meaningful evaluation. Doses were spaced appropriately to produce test groups with a range to toxic effects to determine a dose-response relationship.

3.3.3.4 Animal groups and number of animals

Hairs clipped/shaved male and female rats acclimatized to laboratory conditions were assigned to control and treatment groups. Five groups of male rats consisting of 10 animals each and 5 groups of female rats consisting of 10 animal each were used for the study.
Satellite group of control and high dose groups were maintained for both male and female rats separately as per the study protocol. These groups were maintained for further two weeks after the 28 day period without administering the pesticide.

3.3.3.1.5 Administration of doses

Three different concentrations of test solution were prepared for different dose levels along with vehicle alone separately. The volume of application was kept constant at 0.3ml for rats through suitable dilutions.

Right flank region was employed for applying the combined pesticide formulation where as left flank region was kept as collateral control.

During the exposure period the test substance was held in contact with the skin with a porous gauze dressing covered with nonirritating tape to retain the gauze for at least 6 hrs day exposure throughout the study period of 28 days.

The group details, concentration, application per group were presented in the following tabular form.

<table>
<thead>
<tr>
<th>Group</th>
<th>Male</th>
<th>Female</th>
<th>Dose type</th>
<th>Concentration (ml/lit of water)</th>
<th>Application per animal (ml)</th>
<th>Pesticide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Group VI</td>
<td>Control</td>
<td>Water</td>
<td>0.3</td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>Group II</td>
<td>Group VII</td>
<td>Vehicle</td>
<td>1</td>
<td>0.3</td>
<td>Odorless kerosene</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>Group VIII</td>
<td>Low Dose</td>
<td>1</td>
<td>0.3</td>
<td>Pesticide combination</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>Group IX</td>
<td>Mid Dose</td>
<td>2</td>
<td>0.3</td>
<td>Pesticide combination</td>
<td></td>
</tr>
<tr>
<td>Group V</td>
<td>Group X</td>
<td>High Dose</td>
<td>4</td>
<td>0.3</td>
<td>Pesticide combination</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3.1.6 Observations

General clinical observations were made at least once a Day throughout the study period of 28 Days considering the period of anticipated
effects after dosing. All the animals were observed for health condition, morbidity and mortality at least twice daily.

3.3.3.1.7 Body weight and feed consumption

The animals were weighed individually at the beginning of the study and at weekly intervals till the end of the study. Feed consumption measurements were made daily and weekly average feed consumption was calculated throughout the study period.

3.3.3.1.8 Hematological parameters

Haematological parameters were estimated using blood samples collected from all the animals on Day 7, 14, 21 and 28 by retro-orbital plexus puncture technique using microhaematocrit capillary tubes under ether anesthesia. Disodium EDTA was used as an anticoagulant. The following haematological parameters were determined following standard methods (Jain, 1990).

1. Total Erythrocyte count (TEC)
2. Haemoglobin concentration (Hb)
3. Haematocrit (Hct)
4. Mean corpuscular volume (MCV)
5. Mean corpuscular haemoglobin (MCH)
6. Mean corpuscular haemoglobin concentration (MCHC)
7. Total leucocyte count (TLC)
8. Differential leucocyte count (DLC)

3.3.3.1.9 Serum biochemical parameters

Serum biochemical parameters were estimated from the serum samples collected from the animals on Day 7, 14, 21 and 28 to investigate toxic effects on organs and tissues specifically on liver and kidney using Euro 5 plus automated clinical analyzer at Vijaya Diagnostics Centre, Bangalore.

The commercially available diagnostic kits form M/S swemed
Diagnostic, Jayanagar, Bangalore- 82 were employed in the estimation of the following parameters.

1. Aspartate aminotransferase (AST)
2. Alanine aminotransferase (ALT)
3. Alkaline Phosphatase (ALP)
4. Blood urea nitrogen (BUN)
5. Creatinine (Creat)
6. Glucose
7. Total serum protein (TSP)
8. Albumin

3.3.3.1.10 Pathology

At the end of study period on Day 28 animals in all groups were humanely sacrificed and subjected to detailed gross necropsy including examination of the external surface of the body, all orifices, and cranial, thoracic and abdominal cavities and their contents. The organs were collected for histopathological study.

Collection of organs

After overnight fasting the rats were weighed and sacrificed on Day 28 under ether anesthesia and necropsy was conducted on each carcass to observe any gross pathological changes.

The liver, spleen, kidneys, heart, lungs, stomach, intestine (small and large) and brain were separated from the adhering tissues using saline and collected by placing on the blotting paper and gently pressed to remove excess of saline.

The organs were weighed on an analytical balance and the weight of each organ was recorded. Organ to body weight ratio i.e., organ weight/body weight was calculated and expressed in percentage.

Liver, Kidney, lungs, heart, spleen and brain collected were processed for histopathology by cutting sections of five micron thickness and stained with Haematoxylin and Eosin (Luna, 1968).
3.3.4 Immunotoxicity study

Immunotoxicity study for the formulation containing cypermethrin and amitraz combination pesticide was conducted in both male and female rats to generate information on the possible health effects on immune response which might occur as a result of repeated exposure to Pesticide combination against a known antigen.

Animal preparation, selection of doses, animal groups, number of animals, administration of doses and observations were done as it was carried out in Repeated dose 28-day oral toxicity in rodents.

3.3.4.1 Study Procedure

3.3.4.1.1 Animal preparation

Five groups consisting of 10 male rats in each and another 5 groups consisting of 10 female rats in each were used for the study. The study was conducted for a period of 28 Days and various doses were administered daily orally through gavage for the whole period.

3.3.4.1.2 Selection of doses

The doses employed in the Repeated dose 28-day oral toxicity study in rats were considered for conducting immunotoxicity study in rats against a known antigen.

3.3.4.1.3 Administration of doses

The animals were administered with the amitraz and cypermethrin combination doses and vehicle separately daily for a period of 28 days by oral gavage as a single dose. The volume of administration was maintained one ml per animal through proper dilutions of pesticide combination.

The group details and doses administered per kg body weight are as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose type</th>
<th>Concentration (mg/kg)</th>
<th>Pesticide/Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.4.1.4 Antigen

Sheep red blood cells (SRBC) were used as the antigen. The antigen was administered on Days 14 and 20. Sheep blood was collected in Alsever’s solution and stored at 4°C for one week. SRBC’s were washed three times in pyrogen free sterile normal saline and two per cent SRBC suspension was prepared at the time of administration as per Shah and Gupta (1998).

3.3.4.1.5 Observations

General clinical observations were made at least once a day throughout the study period of 28 days considering the period of anticipated effects after dosing. All the animals were observed for health condition, morbidity and mortality at least twice daily.

3.3.4.1.5.1 Body weight and feed consumption

The animals were weighed individually at the beginning of the study and at weekly intervals till the end of study. Feed consumption measurements were made daily and weekly feed consumption was calculated throughout the study period.

3.3.4.1.5.2 Immunological parameters

The blood was collected on Day 7, 14, 21 and 28 from retro-orbital plexus puncture method using microhaematocrit capillary tubes. Disodium EDTA was used as the anticoagulant. The following immunological parameters were determined following standard methods.

1. Total leucocyte count (TLC)
2. Differential leucocyte count (DLC)
3. Total serum protein concentration (TSP)
4. Albumin
5. Total serum immunoglobulin concentration (TIG)
6. Haemagglutination test (HAT)
7. Hypersensitivity test (DNCB Skin sensitivity test)
8. Phagocytic index (PI)

1. Total leucocyte count (TLC) and Differential leucocyte count (DLC)

   TLC and DLC was done from the blood samples collected on Day 7, 14, 21 and 28 from retro-orbital plexus puncture method using microhaematocrit capillary tubes. Disodium EDTA was used as the anticoagulant. The following immunological parameters were determined following standard methods (Jain, 1990).

2. Total serum protein concentration (TSP) and Albumins

   The total serum protein and albumin concentrations were estimated using Euro 5 Plus automated clinical analyzer at Vijaya Diagnostic Centre, Bangalore. The commercially available diagnostic kits were employed in the estimation.

3. Total serum immunoglobulin concentration (TIG)

   Total immunoglobulin concentration (TIG) in serum was estimated using serum according to the procedure described by Mullen (1975).

   **Solutions used:**

   a) **Zinc sulphate solution**: Freshly prepared solution was used everyday. The solution was prepared by dissolving 208 mg Zinc sulphate (ZnSO₄·7H₂O) in one litre of carbon-di-oxide free distilled water and the distilled water was boiled for 10 to 15 minutes to remove the dissolved carbon-di-oxide.

   b) **Barium chloride solution**: The solution was prepared by dissolving 1.15g of Barium chloride (BaCl₂·H₂O) in 100 ml distilled water. 3 ml of solution was made upto 100 ml using 0.2N sulphuric acid. This solution gives 20 units turbidity reading under standard laboratory conditions.
**Procedure**

The turbidity measurements were made at a wave length of 498nm using a colorimeter. The colorimeter was initially set to zero using blank (distilled water) and the turbidity developed in the control and test solution was measured using blank tube as control.

To the test solution tube containing 6 ml zinc sulphate solution, 0.1 ml serum was added to each tube and a control tube containing 6 ml of distilled water, 0.1 ml of serum and vortexed. The tubes were incubated for one hour at room temperature for development of turbidity.

The difference in the turbidity readings obtained between the control tube and the test solution tube was multiplied by 10. The reading obtained was equivalent to the turbidity of 20 units given by barium chloride standard solution treated similarly.

**4. Haemagglutination test (HAT)**

The titres of the agglutinating antibody in the serum were measured by haemoglobulin test as per Hudson and Hay (1989).

**Procedure**

To micro haemagglutination plates 50 µl of phosphate buffer saline (PBS) was added to all the wells. 50 µl of serum was added to the first well and the contents were mixed. After mixing 50 µl the first well was serially transferred to the succeeding wells and the last 50 ml was discarded. 50 µl of 0.5 per cent SRBC was added to all the wells. The contents were mixed well and incubated at 37°C for one hour. A control was kept which consisted of 50 µl serum and 50 µl 0.5 per cent SRBC and 50 µl PBS and 50 µl 0.5 per cent SRBC suspension.

The highest dilution showing complete agglutination of erythrocytes was taken as an antibody titre of the serum samples and the results were expressed in log2.

**5. Hypersensitivity Study (DNCB Skin sensitivity test)**
The delayed type of hypersensitivity was measured by using dinitrochlorobenzene (DNCB) skin sensitivity. The test was conducted to assess the cell mediated immunity according to the procedure described by Brummerstedt and Basse (1973).

**Procedure**

An area of 3cm in diameter was marked on the left flank of the animal and the hair around the site was clipped close to the skin. 0.4 ml of 2 per cent solution of 2, 4-dinitrochlorobenzene in acetone was applied drop by drop on the marked area for primary sensitization on Day 14. The solution was allowed to evaporate quickly by blowing-gently. The sensitizing dose was applied only once. After 14 Days of the primary sensitization i.e., on Day 28 a challenge dose of 0.25 ml was applied at the same site.

The skin thickness was measured using slide calipers before challenge dosing at zero hour and at 24 and 48 hours intervals after the challenge dose.

6. Phagocytic Index (PI)

The phagocytic index was assessed by the procedure described by Vanfurth *et al.* (1979) using Staphylococcus organism.

**Procedure**

Staphylococcus killed organisms of 0.1 ml cell suspension was added to 1 ml of heparinized blood samples which were incubated at 37°C for 1hr. Then smears were made and stained with Giemsa stain. The mean number of bacteria ingested per 100 phagocytes were calculated. The phagocytic index was calculated using the following formula.

\[
\text{PI} = \frac{\text{Number of bacteria ingested by phagocytes}}{\text{Number of phagocytes involved}}
\]

3.3.5 Acute Eye Irritation Study

Acute eye irritation study in New Zealand white rabbits was conducted to evaluate the effects of the formulation containing cypermethrin (1%) and amitraz (1%) combination pesticide on mammalian eyes according to
standard environmental protection agency. (EPA), Health Effects Test Guidelines, OPPTS 870. 2400, Acute eye irritation. (Anon., 1998d)

3.3.5.1 Study Procedure

3.3.5.1.1 Animals preparation

Health adult New Zealand white rabbits aged between four and six months of age were selected and allowed to acclimatize to laboratory conditions for a period of 7 days prior to the start of study.

3.3.5.1.2 Selection of doses

The doses selected for the present study were 1, 2 and 4 ml per liter of water to 3 different animal test groups.

3.3.5.1.3 Animal groups and number of animals

Four groups consisting of 4 New Zealand white rabbits in each were maintained for the present study. The details of the groups, number of animals and doses are presented in the following table:

<table>
<thead>
<tr>
<th>Groups</th>
<th>No of animals</th>
<th>Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups I</td>
<td>4</td>
<td>control</td>
</tr>
<tr>
<td>Groups II</td>
<td>4</td>
<td>1ml/ltr</td>
</tr>
<tr>
<td>Groups III</td>
<td>4</td>
<td>2ml/ltr</td>
</tr>
<tr>
<td>Groups IV</td>
<td>4</td>
<td>4ml/ltr</td>
</tr>
</tbody>
</table>

3.3.5.1.4 Administration of test substance

0.1ml of the test substance pesticide combination doses selected was placed in the conjunctiva sac of one eye of each animal by gently pulling the lower lid away from the eyeball. The lids were then held gently together for about one second in order to limit doses of the material.

3.3.5.1.5 Observations

Clinical examination of both eyes of all rabbits was made prior to instillation of pesticide combination and at 1, 24, 48, and 72 hours after instillation. Eyes were examined for ocular irritation or corrosiveness, their reversibility or irreversibility and observations on cornea, iris and conjunctivae was made.
The grades of ocular reaction using the following table was done at ‘0’, 1, 24, 48 and 72 hours.

<table>
<thead>
<tr>
<th>Cornea</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Opacity: degree of density (area most dense taken for reading). No ulceration or opacity</td>
<td>0</td>
</tr>
<tr>
<td>Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible</td>
<td>*1</td>
</tr>
<tr>
<td>Easily discernible translucent area, details of iris slightly obscured</td>
<td>*2</td>
</tr>
<tr>
<td>Nacrous area, no details or iris visible, size of pupil barely discernible</td>
<td>*3</td>
</tr>
<tr>
<td>Opaque cornea iris not discernible through the opacity</td>
<td>*4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Iris</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Markedly deepened rugae, congestion, swelling moderate circumcorneal hyperemia, or injection any of these or combination of any thereof, iris still reacting to light (Sluggish reaction is positive)</td>
<td>*1</td>
</tr>
<tr>
<td>No reaction to light, hemorrhage, gross destruction (any or all of these)</td>
<td>*2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conjunctivae</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Redness</strong> (refere to palpebal and bulbar conjunctivae, excluding cornea and iris)</td>
<td>0</td>
</tr>
<tr>
<td>Some blood vessels definitely hyperemic (injected)</td>
<td>1</td>
</tr>
<tr>
<td>Diffuse, crimson color, individual vessels not easily discernible</td>
<td>*2</td>
</tr>
<tr>
<td>Diffuse beefy red</td>
<td>*3</td>
</tr>
<tr>
<td><strong>Chemosis</strong> (refers to lids and /or nictitating membranes)</td>
<td></td>
</tr>
<tr>
<td>No swelling</td>
<td>0</td>
</tr>
<tr>
<td>Any swelling above normal (includes nictitating membranes)</td>
<td>1</td>
</tr>
<tr>
<td>Obvious swelling with partial eversion of lids</td>
<td>*2</td>
</tr>
<tr>
<td>Swelling with lids about half closed</td>
<td>*3</td>
</tr>
<tr>
<td>Swelling with lids more than half-closed</td>
<td>*4</td>
</tr>
</tbody>
</table>

* Starred figures indicate positive grades.
3.3.6 Skin sensitization study

The skin sensitization study was carried out in guinea pigs to evaluate the effects of the formulation containing cypermethrin (1%) and amitraz (1%) combination pesticide on skin sensitization (allergic contact dermatitis) which is an immunologically mediated cutaneous reaction which includes erythema and edema as per the standard protocol of environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870, 2600, Skin Sensitization (Anon., 1998e).

Of the several test methods, skin sensitization was carried out according to Buehler test method (Buehler, 1995).

3.3.6.1 Buehler test procedure
3.3.6.1.1 Preparation of animals

Healthy young adult albino guinea pigs were acclimatized to the laboratory conditions for at least five days prior to the test. Before the test, animals were randomized and assigned to the treatment groups.

3.3.6.1.2 Selection of doses

The doses selected for the present study were 1 ml/lt, 2 ml/lt and 4 ml/lt of water to three different animal test groups.

3.3.6.1.3 Number of groups and number of animals

Four groups consisting of four albino guinea pigs were maintained for the Buehler test. The details of the groups, number of animals and doses selected are presented in the following table:

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose type</th>
<th>Concentration (ml/lt of water)</th>
<th>Application per animal (ml)</th>
<th>Pesticide/Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>Water</td>
<td>0.3</td>
<td>Water</td>
</tr>
<tr>
<td>Group II</td>
<td>Vehicle</td>
<td>1</td>
<td>0.3</td>
<td>Odorless kerosene</td>
</tr>
<tr>
<td>Group III</td>
<td>Low Dose</td>
<td>1</td>
<td>0.3</td>
<td>Pesticide combination</td>
</tr>
<tr>
<td>Group IV</td>
<td>Mid Dose</td>
<td>2</td>
<td>0.3</td>
<td>Pesticide combination</td>
</tr>
<tr>
<td>Group V</td>
<td>High Dose</td>
<td>4</td>
<td>0.3</td>
<td>Pesticide combination</td>
</tr>
</tbody>
</table>
3.3.6.1.4 Procedures

3.3.6.1.4.1 Induction

Day 0

Treated group one flank was cleared of hair by shaving. The test patch was fully loaded with three ml of Pesticide combination diluted concentration to all the animals in treatment groups. The test patch system was applied to the test area of four to six cm² and held in contact with the skin by an occlusive patch and a suitable dressing for six hours.

In Control group one flank was cleared of hair by shaving. The test patch was fully loaded with three ml of distilled water and the test patch system was applied to the shaved area of four to six cm² and held in contact with the skin by an occlusive patch with suitable dressing for six hours.

Vehicle group one flank was cleared of hair by shaving. The test patch was fully loaded with three ml of odourless kerosene and the test patch system was applied to the shaved area of four to six cm² and held in contact with the skin by an occlusive patch with suitable dressing for six hours.

Days 6-8 and 13-15
Vehicle and control group

The same application as on Day ‘0’ was carried out on the same test area cleared of hair of the same flank on Days 6 to 8 and again on Days 13-15.

Challenge dose
Day 27-29 Treated, Vehicle and control group

The untreated flank of treated, vehicle and control animals was cleared of hair. An occlusive patch containing two ml of the diluted concentration of pesticide combination was applied.

3.3.6.1.5 Observations and grading

All the skin and systemic reactions resulting form induction and challenge procedures were observed and recorded according to the Magnusson/ Kligman grading scale as mentioned below:

0- No visible change
1- Discrete or patchy erythema
2- Moderate and confluent erythema.
3- Intense erythema and swelling.

Approximately 21 hours after removing the patch, the challenge area was cleared of hair.

Approximately 3 hours later (approximately 30 hours after application of the challenge patch) the skin reactions were observed and recorded.

Approximately 24 hours after 30 hours observation (approximately 54 hours after application of the challenge patch) skin reactions were observed again and recorded.

3.3.7 In vitro pharmacological efficacy of Pesticide combination against different stages of *Rhipicephalus sanguineus* ticks

Of the several methods generally used for laboratory testing of acaricides of ticks, the immersion test as described by Mira Shah Fischer and Ralph Say (1981) was employed in the present study to evaluate the pharmacological efficacy of the formulation containing cypermethrin (1%) and amitraz (1%) emulsified concentration in odorless kerosene against different stages viz. eggs, larvae, adult male, unengorged female and engorged female ticks of *Rhipilephalus sanguineus*. The pesticide combination concentrations used for the *in vitro* pharmacological efficacy of pesticide combination against different stages of *Rhipicephalus sanguineus* ticks in the present study were control (water), 0.25, 0.50, 1.0 and 2.0 ml per litre of water.

3.3.7.1 Collection, Identification and Rearing of Ticks

The efficacy of pesticide combination was determined on the developmental stages of ticks viz., the egg, larvae, adult male, engorged female and unengorged female *Rhipicephalus sanguineus* ticks. These ticks were collected from dogs from various sources such as the Veterinary College Hospital and City Veterinary Hospitals. Ticks thus
collected were identified based on the characters and keys outlined by Sharif (1928) for *Rhipicephalus* adult stages and Shatas (1956) for larvae of *Rhipicephalus*. Few of adult male and engorged females were utilized in *In vitro* trials. The remaining females were kept in small petridishes with a filter paper at the bottom. The petridishes were then stored in a desiccator containing saturated potassium chloride solution which provides a relative humidity of 80 per cent and 25°C. These conditions were known to be optimum for egg laying. The eggs laid by ticks were collected daily in separate test tubes and kept at room temperature for hatching.

### 3.3.7.2 Immersion test

#### 3.3.7.2.1 Evaluation of tick sensitivity to acaricides

Acaricidal efficacy of pesticide combination against different stages of *Rhipiaphalus sanguineus* ticks by immersion test by Mira Shah Fischer and Ralph Say (1981)

**3.3.7.2.1.1 Ovicidal efficacy of pesticide combination on eggs**

Five homogenous groups consisting of 100 eggs of *Rhipicephalus sanguineus* ticks each were used in the study. They were immersed directly 3 minutes each in the 100 ml chemical concentration. The chemical concentrations used in the present study were control (water), 0.25, 0.5, 1 and 2 ml / litre of water separately. The ticks were then dried and kept separately in a dark place in petridishes. Mortality was recorded every 24 hours. (Mira Shah Fischer and Ralph Say, 1981).

**3.3.7.2.1.2 Larvicidal efficacy of pesticide combination on larvae**

Five homogenous groups consisting of 50 larvae of *Rhipicephalus sanguineus* ticks in each were used in the study. They were immersed directly 3 minutes each in the 100 ml chemical concentration. The chemical concentrations used in the
present study were control (water), 0.25, 0.50, 1 and 2 ml / litre of water separately. The ticks were then dried and kept separately in a dark place in petridishes. Mortality was recorded after 24 hours. (Mira Shah Fischer and Ralph Say, 1981).

3.3.7.2.1.3 Efficacy of pesticide combination on adult male ticks

Five homogenous groups consisting of 10 adult male *Rhipicephalus sanguineus* ticks in each were used in the study. They were immersed directly 3 minutes each in the 100ml chemical concentration. The chemical concentrations used in the present study were control (water), 0.25, 0.50, 1 and 2 ml / ltr. Of water separately. The ticks were then dried and kept separately in a dark place in petridishes. Mortality was recorded after 24 hours. (Mira Shah Fischer and Ralph Say, 1981).

3.3.7.2.1.4 Efficacy of pesticide combination on unengorged female ticks

Five homogenous groups consisting of 10 unengorged female *Rhipicephalus sanguineus* ticks in each were used in the study. They were immersed directly 3 minutes each in the 100 ml chemical concentration. The chemical concentrations used in the present study were control (water), 0.25, 0.50, 1 and 2 ml / litre of water separately. The ticks were then dried and kept separately in a dark place in petridishes. Mortality was recorded after 24 hours. (Mira Shah Fischer and Ralph Say, 1981).

3.3.7.2.1.5 Efficacy of pesticide combination on engorged female ticks

Five homogenous groups consisting of 10 engorged female *Rhipicephalus sanguineus* ticks in each were used in the study. They were immersed directly 3 minutes each in the 100 ml chemical concentration. The chemical concentrations used in the present study were control (water), 0.25, 0.5, 1 and 2 ml / litre of water separately. The ticks were then dried and kept separately in a dark place in petridishes. Mortality was
recorded after 24 hours. (Mira Shah Fischer and Ralph Say, 1981).

3.3.8 In vivo pharmacological efficacy of pesticide combination against ixodid ticks in different species of animals

The in vivo pharmacological efficacy of the formulation containing cypermethrin (1%) and amitraz (1%) combination pesticide was studied in naturally infested cattle, sheep and dogs against Ixodid ticks.

3.3.8.1 Acaracidal efficacy of pesticide combination against *Rhipicephalus sanguineus* ticks of dogs

Twenty dogs infested with ticks (*Rhipicephalus sanguineus*) were selected for the study. 10 uninfested dogs were kept as control. Some of the twenty dogs had the history of regularly being bathed with organophosphate pesticide once in 15 Days. Their ages ranged between 1 year and 8 years. The different breeds infested with ectoparasites were German shepherd (3), Pommeranian (4), Doberman pinscher (6) and non-descript (7). Similarly, pommeranian (4), German shepherd (2) and non-descript (4) were kept as control.

3.3.8.1.1 Application of ectoparasiticide

The pesticide combination emulsified concentrate solution was used in the recommended concentration of 3 ml per liter of water containing 60ppm of cypermethrin and amitraz concentration equally. The quantity of liquid needed was 5-10 liters depending upon the breed and size of the dog. Dogs were wetted with water and then bathed with the solution after muzzling. After application of the pesticide, dogs were not rinsed with water and were allowed to air dry. Muzzle was removed after 10-15 minutes. The dogs were examined 24hrs after application of the pesticide combination (Yathiraj *et al.*, 1992).

3.3.8.1.2 Haematological parameters

Blood samples were collected from all the dogs 24 hrs after the application of pesticide combination in Disodium EDTA as anticoagulant. The
following haematological parameters were estimated following standard methods (Jain, 1990) at General Bio-Medical Laboratory, Bangalore - 32.

1. Total Erythrocyte count (TEC)
2. Haemoglobin concentration (Hb)
3. Haematocrit (Hct)
4. Total leucocyte count (TLC)
5. Differential leucocyte count (DLC)

3.3.8.1.3 Serum biochemical parameters

Serum biochemical parameters were estimated from the serum samples collected from the animals at approximately 24 hours after the Pesticide combination application using BT 224 automated clinical analyzer at General Bio-Medical Laboratory, Bangalore - 32. The commercially available diagnostic kits from M/S Swemed Diagnostics Pvt. Ltd. Bangalore-82 were employed in the estimation of the following parameters.

1. Aspartate aminotransferase (AST)
2. Alanine aminotransferase (ALT)
3. Alkaline Phosphatase (ALP)
4. Blood urea nitrogen (BUN)
5. Creatinine (Creat)
6. Glucose
7. Total serum protein (TSP)
8. Albumin

3.3.8.2 Acaricidal efficacy of pesticide combination against Ixodid ticks of cattle

A preliminary study was conducted by applying pesticide combination at 0.5 ml, 1 ml and 5 ml per liter of water on three naturally tick infested cattle twice of weekly intervals for each concentration before conducting actual study.

The study was conducted at Om Goashram, Rajanukunte, Bangalore. Twenty naturally infested cattle of various ages were selected for assessing the efficacy of the pesticide formulation containing cypermethrin (1%) and amitraz (1%) combination emulsified concentration in odorless kerosene. These animals were fed on green pasture land and reared in shed made of
stones and mud and full of cracks and crevices. The selected animals were equally divided into two groups Group I and Group II.

Group I animals were kept as untreated control group and Group II animals were kept as pesticide combination treated group. The animals in Group II were wetted with water and then bathed with the pesticide formulation after putting muzzle to animal. Group II animals were sprayed with pesticide combination at 3ml per liter of water containing 60 ppm of cypermethrin and amitraz concentration equally. The quantity of liquid needed was 10-15 liters depending on the size of cattle. The cattle were kept under observations for four weeks after spraying.

Before spraying, the ticks on body of each animal were collected for identification. These ticks were identified on the basis of keys given in literature (Sen and Fletcher, 1962; Kaiser and Hogstrall, 1964; Arthur, 1962).

3.3.8.2.1 Haematological parameters

Blood samples were collected from all the cattle 24 hrs after the application of the pesticide combination in Disodium EDTA as an anticoagulant. The following haematological parameters were estimated following standard methods (Jain, 1990) at General Bio-Medical Laboratory, Bangalore-32.

1. Total Erythrocyte count (TEC)
2. Haemoglobin concentration (Hb)
3. Haematocrit (Hct)
4. Total leucocyte count (TLC)
5. Differential leucocyte count (DLC)

3.3.8.2.2 Serum biochemical parameters

Serum biochemical parameters were estimated from the serum samples collected from the animals at approximately 24 hours after the pesticide combination application using BT 224 automated clinical analyzer at General Bio-Medical Laboratory, Bangalore. The commercially available
diagnostic kits from M/S Swemed Diagnostics Pvt. Ltd. Bangalore-82 were employed in the estimation of the following parameters.

1. Aspartate aminotransferase (AST)
2. Alanine aminotransferase (ALT)
3. Alkaline Phosphatase (ALP)
4. Blood urea nitrogen (BUN)
5. Creatinine (Creat)
6. Glucose
7. Total serum protein (TSP)
8. Albumin

3.3.8.3 Efficacy of the pesticide combination against Ixodid ticks of sheep

A preliminary study was conducted by applying pesticide combination at 0.5 ml, 1 ml and 5 ml per liter of water on three naturally tick infested sheep twice of weekly intervals for each concentration before conducting actual study.

The study was conducted at Om Goashram, Rajanukunte, Bangalore. Twenty naturally infested sheep of various ages from local farmers were selected for assessing the efficacy of pesticide combination. These animals were fed on green pasture land and reared in shed made of stones and mud and full of cracks and crevices. The selected animals were equally divided into two groups Group I and Group II.

Group I animals were kept as untreated control group and Group II animals were kept as pesticide combination treated group. Group II animals were sprayed with Pesticide combination at 3 ml per liter of water containing cypermethrin and amitraz concentration equally. The quantity of liquid needed was 7-10 liters depending on the size of animal. The sheep were kept under observations for four weeks after spraying.

Before spraying, the ticks on body of each animal were collected for identification. These ticks were identified on the basis of keys given in literature (Sen and Fletcher, 1962; Kaiser and Hogstrall, 1964; Arthur, 1962).
3.3.8.3.1 Haematological parameters

Blood samples were collected from all the sheep 24 hrs after the application of pesticide combination in Disodium EDTA as an anticoagulant. The following haematological parameters were estimated following standard methods (Jain, 1990) at General Bio-Medical Laboratory, Bangalore.

1. Total Erythrocyte count (TEC)
2. Haemoglobin concentration (Hb)
3. Haematocrit (Hct)
4. Total leucocyte count (TLC)
5. Differential leucocyte count (DLC)

3.3.8.3.2 Serum biochemical parameters

Serum biochemical parameters were estimated from the serum samples collected from the animals at approximately 24 hours after the pesticide combination application using BT 224 automated clinical analyzer at General Bio-Medical Laboratory, Bangalore. The commercially available diagnostic kits from Swemed Diagnostics Pvt. Ltd. Bangalore were employed in the estimation of the following parameters.

1. Aspartate aminotransferase (AST)
2. Alanine aminotransferase (ALT)
3. Alkaline Phosphatase (ALP)
4. Blood urea nitrogen (BUN)
5. Creatinine (Creat)
6. Glucose
7. Total serum protein (TSP)
8. Albumin

3.4 Statistical analysis

The data obtained from the present study were subjected to statistical analysis. The data were analysed by using one-way ANOVA, Kruskal-Wallis test. Mean values and standard error of mean were calculated and all the values are expressed as Mean±SE (GraphPad Prism, 2004).