IMMUNOTOXICOLOGICAL STUDIES OF SUBACUTE PERMETHRIN EXPOSURE AND ITS AMELIORATING POTENTIAL BY *CURCUMA LONGA* IN MICE

A THESIS
SUBMITTED TO THE
ANAND AGRICULTURAL UNIVERSITY
IN THE PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE

OF

Master of Veterinary Science

(Veterinary Pharmacology & Toxicology)

BY

VAIBHAVI VIJAY PAWAR
B.V.Sc. & A.H.
(Reg No: 04-0215-05)

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY
COLLEGE OF VETERINARY SCIENCE & ANIMAL HUSBANDRY
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CERTIFICATE

This is to certify that the thesis entitled **"IMMUNOTOXICOLOGICAL STUDIES OF SUBACUTE PERMETHRIN EXPOSURE AND ITS AMELIORATING POTENTIAL BY *CURCUMA LONGA* IN MICE"** submitted by **VAIBHAVI PAWAR (Reg. No. 04-0215-2005)** in partial fulfillment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE** in the subject of **VETERINARY PHARMACOLOGY & TOXICOLOGY** of the Anand Agricultural University is record of bonafide research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Anand (A.M Thaker)
Date: Major Advisor

CERTIFICATE

This is to certify that I have no objection for supplying to any scientist only one copy of any part of this thesis at a time through reprographic process, if necessary for rendering reference service in a library or documentation center.

Place: Anand

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ABSTRACT

IMMUNOTOXICOLOGICAL STUDIES OF SUBACUTE PERMETHRIN EXPOSURE AND ITS AMELIORATING POTENTIAL BY *CURCUMA LONGA* IN MICE

Name of student
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Present study was planned to know the toxic effect of permethrin on immune system and reversal effect of *Curcuma longa* if any on permethrin induced immunotoxicity in mice. Approximate medium lethal dose (ALD_{50}) of permethrin taken into consideration for the study was 540 mg/kg. Mice were divided into eight different groups each comprising of ten mice. The group C1 was administered corn oil and served as vehicle control. The group C2 was administered ethanolic extract of *Curcuma longa* rhizomes at the dose rate of 10 mg/kg and acted as plant control. Group T1 was given $1/40^{th}$ of LD50 (13.5 mg/kg), group T2 was put on $1/30^{th}$ of LD50 (18 mg/kg) and group T3 received $1/20^{th}$ of LD50 (27 mg/kg) of permethrin suspended in corn oil. Group T4 was given permethrin at dose rate of $1/40^{th}$ of LD50 (13.5 mg/kg) along with ethanolic extract of *Curcuma longa* rhizomes at the dose rate of 10 mg/kg. Group T5 was given permethrin at dose rate of $1/30^{th}$ of LD50 (18 mg/kg) along with ethanolic extract of *Curcuma longa* rhizome at the dose rate of 10 mg/kg. Group T6 was given permethrin at dose rate of $1/20^{th}$ of LD50 (27 mg/kg) along with ethanolic extract of *Curcuma longa* rhizome at the dose rate of 10 mg/kg. Once daily oral dosing was carried out for 28 days.

All the mice were monitored for any observable toxic symptoms throughout the experimental period and they were also weighed weekly to monitor body weight gain. The blood samples were collected before termination of study and were analyzed for hematological, biochemical and immunological parameters. Antibody titre against SRBC were measured by haemagglutination test to see the effect on humoral immunity while DTH was evaluated by footpad thickness against SRBC challenge to monitor development of cell- mediated immunity. Spleen and thymus weight were recorded and collected for histopathological examinations.

Severity and extent of the toxicity signs varied according to dosage of permethrin administered to the mice. The most common signs, which were observed, were reduced feed intake, hyperactivity and hyperexcitability to external stimuli, and rough hair coat, which was prominent in high dose of permethrin, treated animal. Rough hair coat was most commonly observed in females. No clinical signs were observed in mice given *C.longa* extract along with permethrin.

Body weight of mice was not affected in any of the permethrin treated group and administration of *Curcuma longa* does not affect the body weight of mice.

Significant reduction has been recorded in total leukocyte count and lymphocyte count and a nonsignificant effect was noted on granulocyte and monocyte count due to permethrin exposure. There was significant increase in TLC with concurrent administration of permethrin and *C.longa* extract whereas a non significant effect was observed on granulocyte and monocytes. A non significant effect on serum total proteins, serum globulin and serum albumin was observed in permethrin as well as *C.longa* extract along with permethrin treated mice. A dose dependent significant decrease in the

antibody titre was noted in permethrin treated mice. A significant increase in antibody titre against SRBC was observed in plant control group as well as *C.longa* along with the different doses of permethrin as compared to their respective dose of permethrin. Cell mediated immunity was affected nonsignificantly as tested against SRBC in permethrin treated groups of mice. There was significant increase in skin thickness in *C.longa* extract along with permethrin treated mice as compared to only permethrin treated mice.

Decrease in the size of spleen was observed on its gross examination. On microscopic examination depletion of lymphocytes in some spleenic follicles was observed in medium and high dose permetrin treated groups as well as in *C.longa* extract along with high dose permethrin treated groups, whereas no microscopic lesion was observed in control groups. No gross changes were observed in thymus, where as on microscopic examination slight hypocellularity was observed in the cortex region in high dose permethrin treated group.

These findings are suggestive of the fact that the permethrin seems to be toxic to the immune system in mice at the doses administered and considering all the parameters *C.longa* does not possess an observable protecting effect against permethrin induced immunotoxicity.

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Place: Anand

(Vaibhavi Vijay Pawar)

Date: / / 2007

Contents

ABBREVIATIONS

%	Per cent
CMI	Cell mediated immunity
DDT	Dichlorodiphenyltrichloroethane
DNFB	2, 4-dinitrofluorobenzene
LGL	Large granular lymphocytes
MALT	Mucosa associated lymphoid tissues
MCPA	4-chloro-2-methyloxyacetic acid
@	at the rate of
µg	Microgram
µg/g	Microgram per gram
µ l/day	Microliter per day
2,4-D	2,4-dichlorophe-noxyacetic acid
A: G	Albumin: Globulin
Ab	Antibody
AchE	Acetylcholine esterase
ATR	Atrazine
BCG	Bacillus calamite Guanidine
BZLF1	Bam H fragment z left frame 1
C. longa	*Curcuma longa*
CCl4	Carbon tetrachloride
Cd	Cadmium
CEPA	California Environmental Protection Agency

CH	Chlorinated hydrocarbons
CPCSEA	Committee for the purpose of control and supervision of experiments on animals.
CTL	Cytotoxic T lymphocyte
DDD	Dichloro Diphenyl Dichloroethane
DLC	Differential leukocyte count
DMA	2-4-D Dimethylammonium salt of 2-4 dichloro-phenoxy-acetic-
DNCB	1-chloro 2,4-dinitrobenzene
DTH	Delayed Type Hypersensitivity
DTP	Diphtheria, tetanus. Pertussis vaccine
E.D.T.A	Ethylene diamine tetra acetic acid
e.g.	exampli gratia
EAC	Erythrocyte-Antibody-Complement
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
et al.	et alibi
etc.	etcetra
F	Bioavalability
g	Grams
g/dl	Gram per decilitre
g/kg	Gram per kilogram
GABA	Gamma amino benzoic acid
H & E	Haematoxylin and Eosin

HA	Heam agglutination
Hb	Haemoglobin
Hg	Mercury
HI	Heam agglutination Inhibition test
HMI	Humoral Mediated Immunity
i.e	id est
I.P.	Intra Peritoneal
IFN	Interferon
IL	Interleukin
K_3	Potassium
LC_{50}	Median Lethal Concentration
LD50	Median lethal dose
LDH	Lactate Dehydrogenase
mg	Miligram
mg/cm^2	Miligrams per centimeter square
mg/dl	Miligrams per deciliter
mg/kg	Miligram/kilogram
mg/m^3	Miligrams per cubic meter
MHC	Major Histocompatibility Complex
ml	Milliliter
MLR	Mixed lymphocyte response
mm	Millimeter
NDV	New Castle Disease Vaccine

NK	Natural killer
NO	Nitrous oxide
NOEL	No Observed Effect Level
NRCC	National Research Council of Canada
O: BW	Organ: Body Weight
OP	Organophosphate compound
Pb	Lead
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCBs	Polychlorinated biphenyls
PCV	Packed Cell Volume
PFC	Plaque Forming Cells
PHA	Phytohaemagglutinine
ppb	Parts Per Billion
ppm	Parts Per Million
RBC	Red Blood Cells
S: BW	Spleen : Body weight
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SP	Synthetic Pyrethroid
spp.	Species
SRBC	Sheep Red blood Cell
T: BW	Thymus : body weight

t1/2̣	Half life
TEC	Total erythrocyte count
Th 1	T helper Cells
TIG	Total immunoglobulin
TLC	Total leukocyte count
ULV	Ultra-low volume
V	Volume
*V*ss	Steady state volume
WBC	White Blood Cells
WHO	World Health Organization
WLH	White Leghorn
Wt	Weight

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CHAPTER-I

INTRODUCTION

Immunotoxicology is the study of adverse effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals, and, in some instances, biological materials.

Immunotoxicology is also defined as the discipline dealing with the interaction of test substances and the immune system (Banerjee *et al*. 1996b). The objective of the discipline is how to protect humans and animals against the harmful effects of chemical factors present in the environment and introduce/evaluate methods used for determination of interactions between these factors and immune system (Kacmar *et al*. 1999). Immunotoxicity produced by a variety of chemical exposures has been reported for several years, and the number of recognized immunotoxicants is increasing (Luster and Rosenthal, 1993). The types of effects shown to occur are often chemical-specific as well as species-specific and include immunomodulation (i.e., suppression or potentiation), targeting either systemic or local immunity (e.g., lung or skin), hypersensitivity disease, manifested as respiratory tract allergies or contact dermatitis, and in certain instances autoimmunity.

Immunotoxicity is subdivided into three main research areas:(a) studies of altered immunological events associated with exposure of humans and animals to xenobiotics, including altered host resistance to infectious disease; (b) studies of allergy and autoimmunity resulting from xenobiotic exposure; and (c) implementation of analytical immunological methods into toxicology research. Laboratory studies, conducted primarily in rodents, have provided evidence that the immune system is very sensitive to

chemical injury. This sensitivity is probably due as much to the general properties of xenobiotic (e.g. reactivity with macromolecules) as to the complex nature of the immune system, which encompasses antigen recognition and processing. One of the first studies in immunotoxicology was conducted by (Friend and Trainer, 1970), wildlife researchers who demonstrated that exposure to polychlorinated biphenyls (PCBs) increased the susceptibility of young mallards (*Anas platyrhynchos*) to duck hepatitis virus. Immunotoxicity studies with rodents have investigated mechanisms of toxicity, including effects on various immunological functions and resistance to disease challenge. Furthermore, protocols for screening immunotoxic effects during product development have been developed for mice and rats (Luster *et al.*, 1988; ICICIS Group, 1998).

Immunotoxicants of concern have included not only environmental pollutants but also certain therapeutics, consumer products, and biologicals (e.g., the therapeutic use of recombinant materials). Besides, interest has also focused on such diverse materials as silicone implants and pollutants common to the indoor environment. The latter include both chemical agents and bioaerosols such as viruses, bacteria, fungi, algae, and protozoa that have the potential to act as either sensitizing agents or mediators of infectious disease. Immunotoxicology in veterinary medicine deals mostly with the problems of dominant ecological toxicants such as pesticides.

Pesticides are one of the most widely used agrochemicals of toxicological importance. They are used in all aspect of the environment to control undesired pests, such as insects, weeds, fungus, rodents, bacteria and other organisms. The term “pesticide” includes insecticides, herbicides, rodenticides, as well as disinfectants, fumigants and wood preservatives. It has been estimated that 85-90% of the pesticides

applied in agriculture never reach their target organisms, but instead are dispersed in the air, water and soil (Repetto and Baliga, 1996). Based upon such estimates, pesticide exposure is likely for non-target organisms.

Since the World War II, the number of pesticides used has dramatically increased. In the United Kingdom, the number of pesticide active ingredients approved for use increased from 11 to 105 between 1957 and 1995 (Sotherton and Holland, 2003). Similar increase in the use of pesticides occurred in North America. In the USA, herbicide usage increased by 180 % in the period 1971-1987 and in Canada, herbicide usage increased threefold and insecticide usage fivefold during the same period (Sotherton and Holland, 2003). In Sweden currently there are approximately 90 different active pesticide ingredients registered for use within the agricultural sector, and approximately 1700 tons of these are applied each year.

In India about 400 different types of pesticides are registered for use (Tamang *et al.,* 1988). Accidental spills and dumpsites also provide for a part of the environmental pesticide input. In contrast to many other man made chemicals present in the environment, pesticides are deliberately spread into the environment. They are manufactured to be harmful to specific target organisms, or groups of organisms, and their toxic properties are essential to give the pesticides a satisfactory function. Due to pesticide's toxic properties there is an obvious risk that non-target organisms are affected, either at the application site, or due to unintentional spreading, at nearby, or even distant, areas.

Introduction

Application of pesticides has become inevitable in the modern agriculture to augment the farming output. However it is now being realized that the unsystematic and lackadicidal use of pesticides might endanger the disease fighting potentialities of animals, man including poultry. The principal classes of pesticides include organophosphates, organochlorines, carbamates and pyrethroids.

The newest major class of insecticide is the synthetic pyrethriods a group of chemical entering the marketplace in 1980 but by 1982 accounting for approximately 30% of the worldwide insecticide usage (Anon, 1977, Vijverberg and Van den Berken, 1982). The historical development of the synthetic pesticides called pyrethroids is based on the pyrethrins, which are derived from chrysanthemums. Pyrethrins are a "natural" environmental product that is of low toxicity to mammals. They are highly photolabile and degrade quickly in sunlight, and the cost of reapplying them has limited their widespread agricultural use. Pyrethroids have been synthesized to be similar to pyrethrins yet more stable in the environment. They work by quickly paralyzing the nervous systems of insects, producing a quick "knockdown" effect on insect pest populations.

Permethrin (3-phenoxyphenyl methyl (+) cis, trans-3-(2,2-dichloroethenyl)-2,2 dimethylcyclopropane carboxylate) is a synthetic, third generation, type I pyrethroid pesticide that is commonly used in human and veterinary medicine for the prevention and treatment of ectoparasites such as fleas, ticks, lice, and scabies (Schreck *et al*. 1986, Schreck and Kline 1989, Sholdt *et al.* 1989, Asakawa *et al.* 1996, Llewellyn *et al*. 1996, Fuortes 1999). This insecticide has been considered relatively safe. However, despite its presumed relative safety as a topical insecticide, recent reports suggest that low levels of permethrin (34 g/kg/day topically in treated military clothing) may contribute to the

persistent local and systemic immunotoxicity referred to as the "Persian Gulf Syndrome" (Snodgrass, 1992).

Most of the studies of pesticides have been focused on enzyme alterations, gross pathological effects, mutagenic and carcinogenic potential of these agents. In recent years the effects of pesticides on immune response have received attention. It is now clear that important changes in host immunity may occur after pesticide ingestion. Many pesticides like pyrethroids, organophosphorus compounds and organochlorines are known to cause suppression of immune system (Wiltrout *et al.* 1978).

Permethrin and several analogs (cypermethrin, bioallethrin, and deltamethrin) have demonstrated immunomodulatory effects in murine and human models, including diminished natural killer cell cytotoxicity, T cell and antibody-mediated immunomodulation, alterations in class II MHC cells, modified cytokine levels, and variations in thymocyte numbers, distribution and function (Puig *et al*. 1989, Enan *et al.* 1996, Santoni *et al*. 1997, 1998, Diel *et al.* 1998, Murphy *et al*.1999, Zhang *et al.* 1999). These data suggest that this class of insecticides may significantly alter local and systemic immunity. Limited information is available about precise mechanisms of immunomodulation caused by permethrin.

The exposure of animals to residual concentration of pesticides can lead to immunosuppression either directly or participation of stress mechanisms (hunger, thirst, unfavorable microclimate conditions, long distance transport fatigue and others). Immunosuppression leads to change in length of life, increased susceptibility to infectious diseases and decreased immune response to vaccination. Thus there is an

urgent need to obtain more information regarding the manner in which various pesticides alter the immune system.

These adverse effects caused by the pesticides on the immune system can be reversed by the use of herbal immunomodulatory agents. These agents may prevent the damage caused by pesticides to the immune system by their immnostimulant properties and thus can be an area of interest for further research.

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects. Herbal medicine (based on plants), also referred to as alternate medicine/traditional medicine/complementary medicine, has been in use in India since time immemorial. India has a rich history of using plants for medicinal purposes. Nearly 70% of the human population is reported to be dependent on plant-based medicines. The current value of the Indian system of medicine (Ayurveda, Sidha and Unani) and homeopathy is estimated to be around Rs 4000 crores.

In olden times, *vaidyas* used to treat patients on individual basis, and prepare drug according to the requirement of the patient. But the scene has changed now; herbal medicines are being manufactured on a large scale in mechanical units.

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. More than 13,000 plants have been studied during the last 5-year period. The use of medicinal plants for the treatment of many diseases is associated to folk medicine from different parts of the world. Natural products from some plants, fungi, bacteria and other organisms, continue to be used in pharmaceutical

preparations either as pure compounds or as extracts. India has a rich history of using plants for medicinal purposes. There are great varieties of compounds that can be extracted and characterized from plants.

Scientific literature is continuously reporting plant drugs having immunomodulatory activity; most of the leads for this activity are from traditional medicines from different parts of the world. (Mukherjee, 2002,Gauniyal *et al*. 2005) The Indian system of medicine 'Ayurveda' conceptualizes a category of drug activity known as ' Rasayana'. The word Rasayana is composed of two words Rasa meaning elixir and Ayana meaning house. Rasayana therapy prevents diseases and counteracts the ageing process by means of optimization or homeostasis. Many plants have been extensively used as 'Rasayana' drugs in ayurveda for the management of neurodegenerative diseases, as rejuvenators, immunomodulators, aphrodisiac and nutritional supplements. (Tandon, 1992.; Joshi, 2006)

The modulation of immune response with the aid of various medicinal plants in order to alleviate certain diseases is an active area of interest. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of impaired immune response. (Ramasundaram, S. *et al,* 2007)

Immunomodulatory agents of plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. Adaptogenic agents can prevent disease and maintain good health. As adjuvants to other specific treatments, Adaptogens can also help in "altering the course of the disease. These

Introduction

Adaptogens include "Anti-ageing", "Antioxidants", "Immunity Boosters", and "Anti-stress" agents.

About 34 plants have been identified as *rasayanas* in Indian Ayurvedic system of medicine having various pharmacological properties such as immunostimulant, tonic, neurostimulant, antiageing, antibacterial, antiviral, antirheumatic, anticancer, adaptogenic, antistress etc. An entire section of materia medica of Ayurveda is devoted to drugs entitled as 'Rasayana' used for enhancement of body resistance. (Thatte *et al,* 1997)

Many plants with potential immunomodulatory activity are reported, some of these have already been undertaken for evaluation of their activities in animals, and also to some extent in humans. Some glaring examples with promising activity are *Asparagus racemosus, Azadirachta indica, Curcuma longa, Ocimum sanctum, Panax ginseng, Picrorrhiza kurroa, Tinospora cordifolia, Withania somnifera* etc. A lot more are still to be explored and offer scope for further investigation. (Agrawal and Singh, 1999).

Modulation of the immune response through stimulation or suppression may help in maintaining a disease free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy.

Turmeric (*Curcuma longa*) is a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine as home remedy for various diseases (Ammon, 1991, Eigner, 1999). It is much more than the familiar spice that gives curry blends their yellow colour and imparts to them a slightly bitter or astringent taste. Turmeric enhances the flavour of food but also aids digestion, particularly of protein, promotes absorption and regulates metabolism. It is an excellent spice to add to cooking if concerned about weight.

Introduction

Turmeric helps to regulate intestinal flora and is well worth taking during and after a course of antibiotics and by those suffering from Candida or thrush. It has a long history of use for eradicating worms. It has a soothing and bolstering effect on the mucosa of the gut and boosts stomach defences against excess acid, drugs and other irritating substances ingested and from the effects of stress, thereby reducing the risk of gastritis and ulcers. It is said to lower blood sugar in diabetics.

Turmeric has beneficial effects in the liver, which include stimulating the flow of bile, protecting against damage from toxins (Kiso, 1983) and improving the metabolism of fats. By enhancing liver function, turmeric helps to cleanse the blood of toxins and impurities. It has been shown to lower harmful cholesterol levels, to inhibit blood clotting by blocking prostaglandin production and to help prevent as well as remedy atherosclerosis, thus playing a significant role in the prevention of heart and arterial disease. (Srivastava, 1995)

Turmeric extracts have been shown to possess powerful antioxidant, anti-inflammatory, lipid reducing, chemopreventive, immunodulatory, and sedative actions. Looking to this immunomodulatory effect of ethanolic extract of *C.longa* is envisaged in subacute exposure of permethrin in mice and hence the present investigation has been planned with the following objectives.

Objectives

1. To study the immunotoxic effect of Permethrin in mice.
2. To determine the specific immune functions that may be affected by permethrin exposure: organ to body weight ratios, histopathology, total protein, albumin and globulin, total leukocyte count and differential leukocyte count.

3. To study the effect of permethrin on humoral and cell mediated immunity.
4. To study the immunomodulatory effect of *Curcuma longa* if any on Permethrin induced immunotoxicity in mice.

The finding of this study will help in understanding the mechanism as to how this insecticide alters the resistance of animals and thus make them prone to disease, leading to economic losses. It may also help to find the ameliorating potential of *Curcuma longa* against permethrin induced changes on humoral and cell mediated immunity.

CHAPTER- II

REVIEW OF LITERATURE

Immunity by definition is a homeostatic condition in which the body maintains protection from infectious diseases. Immunity is a series of delicately balanced, complex, multicellular, and physiological mechanisms that allow an individual to distinguish foreign material from self and either neutralize or eliminate it (Burns *et al.* 1996). It is characterized by a virtually infinite repertoire of specificities, highly specialized effectors, complex regulatory mechanisms, and an ability to travel throughout the body.

2.1 Immune System:

The immune system is body's defense against invasion by foreign substances. It provides surveillance against pathogens, parasites, foreign proteins, and cancerous cells. All agents that affect the fine balance mechanisms mentioned can cause agent specific immunosuppression (Kacmar *et al*. 1999). Unlike most organ systems the immune system has the unique quality of not being confined to a single site within the body. It is comprised of numerous lymphoid organs and numerous different cellular populations with a variety of functions.

Organs of the Immune System

The organs of the immune system are divided into primary and secondary lymphoid organs. The primary lymphoid organs are the bone marrow and thymus. These organs support the production of mature T and B-lymphocytes and myeloid cells, such as macrophages and polymorphonuclear cells from stem cells. The bone marrow is the site of origination of the pluripotent stem cells, a self-renewing cell from which all other hematopoietic cells are derived. The thymus is the site at which all T-cell precursors

migrate and undergo selection for recognition of self or nonself antigens. The secondary lymphoid organs are the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT), which include the tonsils and Payer's patches (Burns *et al*. 1996; Roitt *et al*.1998). The key events that occur in the secondary lymphoid organs are: (1) specific antigen recognition; (2) clonal expansion of antigen-specific cells; and (3) differentiation of antigen stimulate lymphocytes (Sharma and Reddy 1987; Burns *et al.* 1996).

Cells of the Immune System

Immune responses are mediated by a variety of immune cells. Leukocytes are central to all immune responses, but other cells within tissues also participate by signaling to lymphocytes and responding to cytokines released by T lymphocytes and macrophages. There are three major groups of leukocytes: lymphocytes, phagocytes and auxiliary cells. The lymphocytes consist of T cells, B cells and large granular lymphocytes (LGL). The phagocytes consist of mononuclear phagocytes, neutrophils and eosinophils. The auxiliary cells are basophils, mast cells and platelets.

Lymphocytes are responsible for specific immune recognition of pathogens. T lymphocytes develop in the thymus and B lymphocytes develop in the bone marrow. B cells have a specific surface receptor for a particular antigen. When recognizing that antigen, the B cell differentiates into a plasma cell and produces large amounts of the receptor molecule, or antibody. These antibodies bind to the specific antigen and induce further immune cell response. T lymphocytes consist of two different groups. Helper T cells interact with B cells to aid in division, differentiation and antibody production. The other, cytotoxic T cells, interact with the mononuclear phagocytes and aid in destroying antigens. T cells generate their effect by either releasing soluble proteins called cytokines,

which act as secondary messengers and signal other cells, or by direct cell-cell interactions

The phagocytic cells internalize particles, such as infectious agents or xenobiotics, and destroy them. The mononuclear phagocytes are located in the blood (monocytes) and migrate to tissues where they become macrophages. These cells are antigen-presenting cells to T lymphocytes. Also, neutrophils are important phagocytic cells. They are derived just as macrophages and respond to certain stimuli, however they are much shorter lived.

The cytotoxic cells, which have the capacity to kill other cells, are either cytotoxic T cells, LGLs or eosinophils. LGL's, or natural killer cells, recognize surface antigens on tumor or infected cells. Eosinophils are a special group of phagocytic leukocytes that can damage large extracellular parasites.

Lastly, two auxiliary cells that mediate inflammatory responses are basophils and mast cells. These cells contain mediators, which initiate inflammation in tissues as well as other immune reactions. Mast cells act on the blood vessel walls. Basophils are similar to mast cells but circulate in the blood.

2.1.1 Classification of immunity

Immunity is classified as innate immunity, a non-specific immune response resulting during the genetic constitution of an organism, and acquired immunity, a specific response resulting from direct exposure to a foreign substance (Herbert and Wilkinson 1971; Sharma and Reddy 1987). Innate immunity acts as a first line of defense against infectious agents eliminating most potential pathogens before significant infection occurs. It includes physical and biochemical barriers both inside and outside of the body as well as immune cells designed for specific responses. Acquired immunity can be

divided into two subclasses, humoral and cell mediated immunity (CMI). Humoral immunity is mediated by antibodies produced by B cells and CMI is mediated by macrophages, T cells and other phagocytic immune cells. CMI response is often related to delayed hypersensitivity or graft rejection (Herbert and Wilkinson 1971).

Previous research has reported that various chemical agents cause immune dysfunction in both humoral and CMI response (Casale *et al*. 1983; Johnson *et al* 1987; Barnett and Rodgers 1994; Banerjee *et al*. 1996b; Banerjee *et al*. 1998; Koner *et al*. 1998).

The immune system provides the means to initiate rapid and highly specific responses against a myriad of potentially pathogenic organisms. In light of the central role that the immune system plays in the maintenance of the health of the individual, the interaction of xenobiotics (pharmacological agents, environmental contaminants, and other chemicals) with various components of the immune system has become an area of profound interest. Indeed, in some instances the immune system has been shown to be compromised (decreased lymphoid cellularity, alterations in lymphocyte subpopulations, decreased host resistance, altered specific immune function response) in the absence of observed toxicity in other organ systems. Decreased immunocompetence (immunosuppression) may result in repeated, more severe, or prolonged infections as well as the development of cancer. Immunoenhancement may lead to immune mediated diseases such as hypersensitivity responses or autoimmune disease. Because of the potentially profound effects resulting from disruption of the delicately balanced immune system, there is a need to understand the cellular, biochemical, and molecular mechanisms of xenobiotic induced immunomodulation.

Immunosuppression by pesticides is a complex phenomenon as several sets of pesticide interactions with the immune response are possible such as antigen processing and presentation, cellular co-operation for the activation of antibody producing cells and synthesis of antibody (Casale *et al*. 1984).

2.1.2 Chemical Induced Immunotoxicity

Immunotoxicology is relatively new interdisciplinary scientific field focused on identification and analysis of chemical and, in a broader sense, also physical and biological factors of the environment which can cause unwanted and usually incidental immunomodulations (Dietert *et al*. 1996).

Interactions of various xenobiotics with live organisms pose diverse immunological problems. According to reliable literary sources almost none of the active ingredients present in pesticides that are used in our country were subject to immunotoxicological testing. The initial studies of the susceptibility of the immune system and its possible use for detection of subclinical toxic states were published in the seventies and early eighties (Vos and van Genderen 1973; Vos 1977; Loose *et al.* 1978; Faith *et al.* 1980) when the attention began to focus on the immune system as an important object of toxic action, particularly in connexion with the experiments on rodents. The susceptibility of the immune system to toxic damage can result from several factors. Host resistance to infectious agents and spontaneous neoplasms depends on immunocompetent cells, which are subject to continuous proliferation and differentiation and because of that they become excessively susceptible to various agents. The immune system is known for highly organized cooperation and regulation of various cells, which is ensured on the one hand by soluble mediators (immunoglobulins, immunohormones,

cytokines) and on the other by intercellular interactions on the level of membrane receptors and antireceptors. All agents that affect the fine balance (homeostasis) mechanisms mentioned could cause agent-specific or species specific immunity damage, which, in majority of cases, results in immunosuppression (e.g. decreased resistance to infectious agents and development of tumours) (Kackmur *et al*., 1999). Manifestations of immunosuppression were observed either on systemic or on the local level (e.g. in lungs or skin, Luster *et al*. 1996).)

Industrial development increased considerably the risk of different xenobiotics in the outer environment to which they have been introduced incidentally (emissions, accidents) or intentionally (the use of various potentially toxic chemicals such as pesticides). It is the latter group of substances that includes chemicals, which do not always have acute toxic effects. However, they exhibit considerable potential for chronic toxicity, which is most frequently accompanied by impairment of the immune system (Saunders and Harper 1994). Individual immune status indices allow us to reveal the toxic action of a number of substances (Zavazal and Richter 1985) even in those cases in which the conventional toxicological examinations give frequently only negative results (Desi *et al*. 1986).

Toxicological assessment for approval of environmental chemicals and pharmaceutical products typically does not include evaluation of the immune system. The need to incorporate immunotoxicity testing has only been realized in recent years. Risk assessment models for immunotoxicity evaluation have been studied as researchers recognize it is temporally and economically impractical to complete a full evaluation of all arms of the immune system for each compound in question (Luster *et al.* 1992, 1993).

Therefore, a risk assessment model was developed such that individual and pair-wise predictive values were established for the various immunological assays in an attempt to expediently quantitate the possibility of decreased host resistance to disease following chemical challenge using a few selected immune tests. Earlier studies had outlined a tiered approach to full evaluation of the immune system that included immunopathology, cell-mediated and antibody-mediated immune evaluation, nonspecific immunity, and host resistance challenge models (Luster *et al.* 1988), which supplied the background information necessary for the development of these risk assessment models.

Over the centuries, humans have developed many indigenous methods in their attempts to control the invertebrates, vertebrates, and microorganisms that constantly threatened the supply of food, as well as posing a threat to health. The historical literature is replete with descriptions of plant diseases and insect plagues and the measures taken to control them.

By the beginning of World War II, there were number of pesticides including dichlorodiphenyltrichloroethane (DDT), dinitrocresol, 4-chloro-2-methyloxyacetic acid (MCPA), and 2,4-dichlorophe-noxyacetic acid (2,4-D) under experimental investigation, much of this activity being kept under wraps during the war (Kirby, 1980).

After IInd war era, there was rapid development in the agrochemical field, with a plethora of insecticides, fungicides, herbicides, and other chemical agents being introduced. In no other field of synthetic organic chemistry has there been such a diversity of structures arising from the application of the principles of chemistry to the mechanism of action in pests to develop selectivity and specificity in agents toward certain species and

same time efforts were made to reduce the toxicity of these agrochemical to human being and animals.

Causes of pesticide toxicity:

- Agricultural exposure is the most common epidemiological site of pesticide poisoning, and any worker in the industry can be affected. This may include pesticide manufacturers, field workers, truckers who transport pesticides or produce, and crop dusters.
- Pest control workers, custodial workers, veterinarians, and pet groomers are the people at the risk of poisoning.
- Wind shifts near sprayed fields can result in accidental exposure to the unsuspecting public.
- Intentional ingestion in a suicide attempt.
- Occurrence of accidental ingestion of household pesticides by children or pets.
- Contamination of public foodstuffs and public places.
- Military use of nerve gas has caused mass fatalities in warfare and accidental poisoning of military personnel who handle these weapons.
- Pesticides impregnated into military clothing as an insect repellent in order to protect the soldiers from insect-borne disease.

2.2 Classification of Pesticides

First generation insecticides

The 1950s were dominated by the organochlorine insecticides. The organochlorines were regarded as very safe for human use as well as very effective at killing insects and were cheap to make. The organochlorines includes a broad range of chlorinated hydrocarbons viz. DDT, DDD, Perthane, Kelthane, Methoxychlor Chlorobenzilate, Chlorobenside and several others. The organochlorines had three properties:

- Persistence
- Fat solubility (that caused biomagnification)
- Chlorine atoms that made the molecules easy to find even at extremely low concentrations.

Second generation insecticides

Organophosphate or carbamate insecticides were developed to have particular properties, which made them less hazardous to the users, consumers or other organisms.

- More toxic to vertebrates than other classes of insecticides.
- Chemically unstable
- Non-persistent

Third generation insecticides

To overcome the problem of residues in food and in the environment, there was a drive towards finding insecticides that are more potent. Several new groups of insecticide have great potency:

- Synthetic pyrethroids.
- New acetylcholine mimics such as the synthetic "nicotine analogue" imidacloprid.
- New GABA antagonists like fipronil.
- Insect growth inhibitors such as the chitin synthesis inhibitors diflfubenzuron.
- Insect juvenile hormone mimics like fenoxycarb.

Major classes of pesticides

- ***Insecticides & acaracides***

  Organophosphorus compounds

  Carbamates

  Chlorinated hydrocarbons

- ***Natural Products***

  Nicotinoids

  Pyrethroids

  Rotenoids

- ***Miscellaneous compounds***

Thiocyanates	Sulfides
Nitrophenols	Sulfones
Organofluorine compounds	Fumigants
Sulfonates	Fluorides
Mercurials.	Arsenicals

- ***Activators or synergists***

  Piperonyl butoxide (commonly used with pyrethrins)

- ***Carrier / Bulk***

  Natural organic, e.g., petroleum products

  Natural inorganic, e.g., dusts

- ***Attractants***

  Food attractants and Sex attractants (Pheromones)

- ***Repellents***

- ***Growth Regulators***

- ***Chemosterilants***

2.2.1 Pyrethroids:

Taking into account the serious adverse effect of conventional insecticides, a relatively new group derived from flower head of pyrethrum (*Chrysanthemum cinerariafolium*) has taken over their place. Now the synthetic pyrethroids are being used extensively as contact insecticides. They have very low mammalian toxicity and are

devoid of any persistent effect. Pyrethroid use has grown to represent approximately 25% of the insecticide market worldwide

Pyrethrins were developed as pesticides from extracts of dried and powdered flower heads of *Chrysanthemum cinerariaefolium*. The active principles of these are esters of chrysanthemic acid (R1 = CH3) or pyrethric acid (R1 = CH3O2C) (both cyclopropane carboxylic acids), with one of three cyclopentanone alcohols (cinerolone, R2 = CH3; jasomolone, R2=CH2CH3; or pyrethrolone, R2 = CHCH2), giving six possible structures. These natural pyrethrins have the disadvantage that they are rapidly decomposed on exposure to light and quite expensive to be used as insecticides. Therfore modifications of the chemical structures of the natural pyrethrins was attempted to develop a series of photostable synthetic pyrethroids with improved physical and chemical properties and greater biological activity. Synthetic pyrethroids with the basic cyclopropane carboxylic ester structure (and no cyano group substitution) are known as type I pyrethroids while the synthetic pyrethriods produced by addition of a cyano group at the benzylic carbon atom are called as type II pyrethriods.

The first commercial synthetic pyrethroid, allethrin, was produced in 1949, followed by others including dimethrin, tetramethrin and resmethrin. In 1960s 3-Phenoxybenzyl esters were also found to be active as pesticides (e.g. phenothrin, permethrin).

2.2.2 Mechanism of Action

Pyrethroids modify the gating characteristics of voltage-sensitive sodium channels in mammalian and invertebrate neuronal membranes (Eells *et al*., 1992; Narahashi, 1989) to delay their closure. They are dissolved in the lipid phase of the

membrane (Narahashi, 1996) and bind to a receptor site on the alpha sub-unit of the sodium channel (Trainer *et al.,* 1997). The interaction of pyrethroids with sodium channels is highly stereospecific (Soderlund and Bloomquist, 1989), with the 1R and 1S cis isomers binding competitively to one site and the 1R and 1S trans isomers binding non-competitively to another. The 1S forms do not modify channel function but do block the effect of the 1R isomers (Ray, 1991). The prolonged opening of sodium channels by the neurotoxic isomers of pyrethroids produces a protracted sodium influx, which is referred to as sodium "tail current" (Miyamoto *et al.,* 1995; Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1982). This lowers the threshold of sensory nerve fibres for the activation of further action potentials, leading to repetitive firing of sensory nerve endings (Vijverberg and van den Bercken, 1990), which may progress to hyperexcitation of the entire nervous system (Narahashi *et al*, 1995). At high pyrethroid concentrations, the sodium "tail current" may be sufficiently greater to depolarize the nerve membrane completely, generating more open sodium channels (Eells *et al*, 1992) and eventually causing conduction block. The depolarizing activity is specific for the neurotoxic isomers (Eells *et al*., 1992), and parallels mammalian toxicity.

Deltamethrin > cypermethrin > fenvalerate >> permethrin (Clark and Marion, 1989; Eells *et al.,* 1992).

Although both type I and type II pyrethroids primarily affect sodium channels, experimental studies have identified some specific differences in their effects. These are summarized below and may, in part, account for the differences in clinical manifestations observed following experimental intoxication with type I and type II pyrethroids. Type I pyrethroids (without the alpha-cyano group) keep sodium channels open (Narahashi,

1989), produce repetitive firing of sensory nerve endings (Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1982), modify sodium channels in the resting or closed state so that they subsequently open more slowly (Dorman and Beasley, 1991).

Type II pyrethroids (mainly alpha-cyano-3-phenoxybenzyl esters) cause depolarization of myelinated nerve membranes without repetitive discharges (Dorman and Beasley, 1991; Vijverberg and van den Bercken, 1982), are associated with a decrease in action potential amplitude (Dorman and Beasley, 1991), stabilize a variety of sodium channel states by reducing transition rates between them (Dorman and Beasley, 1991; Eells *et al.*, 1992; Narahashi, 1989), causing a greatly prolonged open time (Vijverberg and van den Bercken, 1982), and producing stimulus-dependent nerve depolarization and block (Soderlund and Bloomquist, 1989), may act post-synaptically by interacting with nicotinic acetylcholine and GABA receptors (Dorman and Beasley, 1991; Eells *et al.,* 1992).

Type I and type II pyrethroids:

Type I	**Type II**
Allethrin	Cyfluthrin
Bioallethrin	Lambdacyhalothrin
Permethrin	Alphacypermethrin
Prallethrin	Fenpropathrin
Bioresmethrin	Esfenvalerate
Tetramethrin	Flumethrin
Bifenthrin	Deltamethrin
d-Phenothrin	Cypermethrin
Resmethrin	Fenvalerate

Tefluthrin	Flucythrinate, Cyhalothrin

2.3 PERMETHRIN

Permethrin (3-phenoxyphenyl methyl (+) cis, trans-3- (2,2-dichloroethenyl)-2,2 dimethylcyclopropane carboxylate) is a synthetic, third generation, type I pyrethroid insecticide that is commonly used in shampoos and topical creams in human and veterinary medicine to eliminate ectoparasites such as fleas, ticks, lice, and mites (Asakawa *et al.* 1996, Llewellyn *et al.* 1996, Fuortes 1999). Permethrin is also used in the prevention of ectoparasite infestation and possible resultant insect-borne disease via direct topical application or permeation of military and hunting clothing (Schreck *et al*.1986, Schreck and Kline 1989, Sholdt *et al*. 1989). The degree of topical absorption is species-dependent, and ranged from 2% in human beings and rabbits, 10% in mice, 15% in rhesus monkeys (variation according to isomer and site of application), to as much as 44% in rats. It is also used against a variety of pests, on nut, fruit, vegetable, cotton, ornamental, mushroom, potato, and cereal crops. Permethrin is available in dusts, emulsifiable concentrates, smokes, ULV (ultra-low volume), and wettable powder formulations.

2.3.1 Structure of Permethrin

Permethrin insecticide is a mixture of four chiral isomers, a consequence of two chiral centers in the cyclopropane ring of the molecule (Figure 1).

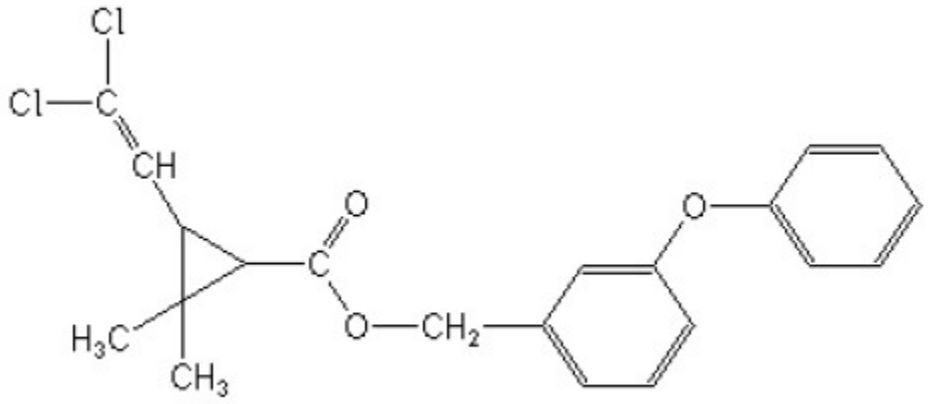


Figure: 1 Chemical Structure of Permethrin, a synthetic pyrethroid insecticide

The insecticidal activity of the two (cis & trans) isomers differs depending on the target insect (Elliot *et al*. 1978). Trans permethrin is metabolized more rapidly than cis in mammals, that is why trans isomers dominate the commercial product, comprising 60-75% of permethrin used (Gaughan *et al.* 1977 and Casida and Ruzo 1980). Although between the years 1982 and 1988 there were 573 cases of acute pyrethroid poisonings, permethrin is classified as moderately to practically non-toxic, of a toxicity class 2 or 3 depending on formulation (Extoxnet, 2000).

2.3.2 Physical and Chemical Properties of Permethrin

The physical and chemical properties of permethrin are shown in the following list (CEPA, 1992).

Common name:	Permethrin
Chemical name:	3-(phenoxyphenyl) methyl (±)-*cis, trans*-3- (2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate
Trade names:	Permanone, Ambush, Pounce, Ectiban, FMC 33297, PP557, BW-21-Z, NRDC 143

CAS registry no.:	52645-53-1
Molecular weight:	391.3
Empirical formula:	$C_{21}H_{20}Cl_2O_3$
Physical state:	Clear viscous liquid
Color:	Medium to dark amber
Odor:	Moderate aromatic
Melting point:	55.7-56.3°C (cis) 45.7-46.3°C (trans)
Boiling point:	220°C at 0.05 mm Hg
Density:	1.0138 at 25°C
Solubility:	0.07 mg/L at 25°C in water Mixable with most organic solvents
Vapor pressure:	2.15×10^{-8} mm Hg at 25°C (cis) 0.69×10^{-8} mm Hg at 25°C (trans)
Hydrolysis:	Stable under acidic or slightly acidic conditions (pH 3-6) at 25-45 °C, but hydrolyzes slowly at pH 9, increasing with temperature ($t_{1/2}$ = 3 days at 45°C). The cis isomer is more stable.
Photolysis:	Degrades slowly in sterile water (pH 5) and soil with exposure to xenon arc lamp at 25°C (60-86% remained intact after 32-35 days)

The amount of permethrin that is lethal to one-half (50%) of experimental animals exposed to it is referred to as the lethal dose fifty, or LD50, of this insecticide. The median lethal oral dose in rats is 4 g/kg, and this level varies slightly according to the age, nutrition, gender, and strain of rat. However, the median lethal oral dose of permethrin in corn oil vehicle is 380 mg/kg. This finding raises concern about the extent of systemic absorption and resulting toxicity with topically applied insecticides, since most commercially available insecticides are only available as a mixture of active ingredients and inert solvents or vehicles (Metker *et al.* 1977, McCain *et al.* 1997).

Permethrin exhibited extremely low mammalian toxicity. Based on oral LD50 values in rats, permethrin is about 3 times less toxic on a milligram per kilogram basis than the organophosphate malathion, 15 times less than the carbamate, carbaryl, and about 40 times less than the organochlorines, lindane or DDT (Taplin and Meinking, 1990). The oral LD50 in rats is 430 to 4,000 mg/kg. Aqueous suspensions usually produced the least toxic results, LD50 values ranging from 3,000 to >4,000 mg/kg. However, corn oil is the more standard vehicle for pyrethroids and yielded LD50 values of about 500 mg/kg for oral administration in rats and mice. Permethrin is more toxic when formulated with corn oil, dimethyl sulfoxide, and propylene glycol than when in an aqueous suspension (perhaps because of greater solubility of permethrin in organic solvents than in water) Death in animals occurs within 3 days of exposure to permethrin. The LD50 is over 270 mg/kg when injected into the veins.

Permethrin appears to be less toxic than other synthetic pyrethroids, such as cypermethrin and fenvalerate (NRCC, 1986). The cis/trans isomeric ratio also appears to affect toxicity, the cis isomer being more toxic than the trans isomer in animals. The *cis*

isomer has a greater potential for mammalian toxicity than the *trans* isomer, which is more rapidly metabolized and excreted (WHO, 1990).

2.3.3 Oral LD_{50} values for technical Permethrin:

Species	Sex	Route	Vehicle	LD_{50} (mg/kg body weight)	Reference
Rat	M	Oral	DMSO	1,500	Clark, 1978
	F	Oral	DMSO	1,000	Clark, 1978
	M	Oral	Corn oil	500	Jaggers and Parkinson, 1979
	M	Oral	Corn oil	430	Kohda *et al*., 1979
	F	Oral	Com oil	470	Kohda *et al*., 1979
	M, F	Oral	Corn oil	1,200	Braun and Killeen, 1975
Mouse	F	Oral	Water	>4,000	Parkinson *et al*., 1976
	M, F	Oral	DMSO	250-500	Clark, 1978
	M	Oral	Corn oil	650	Kohda *et al*., 1979
	F	Oral	Corn oil	540	Kohda *et al.,* 1979

	M	sc	Corn oil	>10,000	Kohda *et al*., 1979
	F	sc	Corn oil	10,000	Kohda *et al*., 1979
Rabbit	F	Oral	Water	>4,000	Parkinson *et al*., 1976
Guinea pig	M	Oral	Water	>4,000	Parkinson *et al*., 1976
Hen	—	Oral	—	>1,500	Millner and Butterworth, 1977

2.3.4 Toxicokinetics

The toxicokinetics of permethrin have been studied in the rat by (Anadon *et al.* (1991). A single dose of permethrin was administered by the oral or intravenous route. The kinetics of permethrin after IV administration in rats was best described by a two compartment open model, with a relatively rapid distribution phase ($t_{1/2}$= 0.46 hr) and a more prolonged elimination phase ($t_{1/2}$= 8.67 hr). A similar kinetic profile was observed in rats orally given permethrin. The apparent volumes of distribution during the elimination phase (V = 0.72 liter) and at steady state (V_{ss} = 0.65 liter) were relatively large. These values, and the high lipid solubility of permethrin, suggest a penetration and distribution of the pyrethroid in body fluids including intracellular water. After a single oral dose, permethrin was both absorbed (Tmax = 3.52 hr) and eliminated slowly ($t_{1/2}$= 12.37 hr). The observed low total plasma clearance (CL = 0.058 liter/hr) also explains the slow elimination of permethrin in the rat. In the study by Anadon *et al*., (1991), the bioavailability of permethrin was relatively low (F = 60.69%) following oral

administration due to permethrin degradation at the site of absorption and a first pass effect.

The (1R,trans)- and (1R,cis)-esters, the active isomers of permethrin, are readily metabolized by ester cleavage, by hydroxylation of the terminal dimethyl group in the acid, or the phenoxy group of the alcohol, and by conjugation of the resulting carboxylic acids and phenols, with *cis*-permethrin being more stable than *trans*-permethrin. The metabolites are quickly excreted and do not persist significantly in tissues (Elliott *et al.,* 1976). In the study by Anadon *et al.* (1991), the metabolism of permethrin was rapid and both metabolites, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, were detected in plasma and tissues. Percutaneous absorption of permethrin has been investigated in rat, rabbit, dog, and man, and the degree of absorption is highly species dependent. When applied in an alcoholic vehicle, 60% is absorbed in the rat, 30% in the rabbit, and 12% in the beagle dog, but less than 2% is absorbed percutaneously in human studies (Taplin and Meinking, 1990).

Permethrin has also been shown to inhibit acetylcholinesterase (AchE) activity in rat brain cortex by 97-98% within 45 minutes of *in-vitro* (5 ppm) exposure or by 50% within 30 minutes following *in-vivo* (250 mg/kg body weight) administration, which would exacerbate permethrin's neurotoxic effects (Bandyopadhyay, 1982). The maximum plasma concentrations are achieved at 3.52 hours (Anadon *et al.* 1991), peak neurotoxic effect of permethrin is at 5 hours post oral administration in rodents. Low doses of permethrin cause mild neurological signs and immunomodulation. Higher doses of permethrin result in more severe peripheral and central nervous system clinical signs including hyperactivity, convulsions, paralysis, and even death (Hansen *et al*.1994).

2.3.5 Clinical signs of toxicity:

Evidence suggests that pyrethriods have a very large margin of safety when used as directed by the label (Aldridge, 1990; Chen *et al.,* 1991; Snodgrass, 1992). In mammals poisoning may cause tremor, ataxia, salivation, nausea, vomition, convulsion, urinary problem, eye irritation, skin irritation and hypersensitivity etc. The systemic symptoms include dizziness, headache, nausea, anorexia and fatigue and, in the most severe cases, fasciculation in large muscles of the extremities. Only paraesthesia could clearly be attributed to exposure to pyrethroids, whereas nausea, headache and dizziness might be induced by organic solvents (He *et al*., 1989).

In all species thus far investigated pyrethroids induce toxic signs that are characteristic of a strong excitatory action on the nervous system. Toxic doses of pyrethroids generally cause hypersensitivity to sensory stimuli, and a number of compounds may induce tingling sensations in the skin. In mammals, two distinct toxic syndromes have been described (Ecobichon, 1991). The T-syndrome is induced by pyrethrins and noncyano pyrethroids, type I pyrethroids, e.g., permethrin, and is characterized by the prominent symptom of whole-body tremors. These compounds initially cause aggressive sparring behavior and increased sensitivity to external stimuli. This is followed by a fine tremor, gradually becoming severe until the animal finally becomes prostate with coarse whole-body tremor.

Chesher and Malone (1974a) administered permethrin by gavage to groups of five female Dutch rabbits in 10 daily doses in corn oil at 0, 200, 400, or 800 mg/kg of body weight. The animals were killed on the 11th day. One rabbit, receiving 400 mg/kg of body weight, exhibited mild hyperactivity and muscular fasciculation, but only on days 6

and 7. Although all animals, including the controls, exhibited some degree of weight loss, it was most marked in the high-dose group.

In another study, groups of six female mice were administered daily oral doses of permethrin (cis/trans ratio, 25:75) in corn oil at 0, 200, 400, 800, or 1,600 mg/kg of body weight for 10 consecutive days. Signs of acute toxicity, such as spasm and convulsion, were seen only in the animals in the highest dose group, half of which died after the initial dose. No significant changes were observed in hematology, clinical chemistry, or body weights after 11 doses. The mice administered permethrin at 800 and 1,600 mg/kg of body weight showed increased liver weights (Wallwork *et al*., 1974a).

Kadota *et al.* (1975) fed Sprague-Dawley rats (16 of each sex per group) permethrin in their diet at 0, 375, 750, 1,500, or 3,000 mg/kg of diet for 6 months. None died, and all animals exhibited normal growth and normal food and water consumption. Urinalysis and hematological and clinical biochemistry values were within normal limits. Signs of hyperexcitability and tremors were observed during the study in animals given 3,000 mg/kg, and their liver weights and liver-to-body-weight ratios were slightly increased.

Butterworth and Hend (1976) fed CD rats (six of each sex per group) permethrin at 0, 30, 100, 300, 1,000, or 3,000 mg/kg of diet for 5 weeks. Persistent tremors were seen in animals fed at 3,000 mg/kg, but none died. Growth was inhibited at that dose in both male and female rats. Relative liver weights were increased in male rats (groups fed 1,000 mg/kg of diet or higher) and female rats (fed 3,000 mg/kg). Histopathological examination of tissues and organs of the animals receiving the two highest doses did not show any adverse effects as a result of permethrin ingestion in the diet.

Parkinson *et al.* (1976) applied undiluted permethrin to the eyes of female rabbits. Application of permethrin only caused minimal pain, redness, or chemosis of the conjunctiva; there was a slight discharge.

In a study by Parkinson *et al.* (1976), guinea pigs were dermally administered permethrin as a 10% solution in dimethylformamide for 3 consecutive days. This was followed 4 days later by challenge doses of 0.1%, 1%, and 10% solutions of permethrin in dimethylformamide. Only very slight erythema was observed. Permethrin was therefore considered to be either nonsensitizing or only mildly so.

Single applications of up to 0.5mL permethrin produced only mild, localized irritation (McCreesh, 1977). The treated area showed focal acanthosis and hyperkeratosis of the epidermis. Those pathological conditions are common skin reactions to nonspecific irritant chemicals.

Metker (1978) evaluated the inhalation toxicity of technical-grade permethrin in guinea pigs, Sprague-Dawley rats, and beagle dogs. The animals were exposed to an aerosol of permethrin at concentrations of 125, 250, or 500 mg/m^3, 6 hr per day, 5 days per week for 13 weeks. At 500 mg/m^3, tremors and convulsions were observed in the rats during the first week of exposure but disappeared in the second week.

Dayan (1980) fed permethrin (cis/trans ratio, 25:75) (94.5% pure) to groups of 10 male and 10 female Sprague-Dawley rats at 4,000, 6,000, or 9,000 mg/kg for 21 days. All animals developed severe trembling and lost weight. Some rats of each sex in the 9,000 mg/kg group died. Histopathological examination of brain, spinal cord, trigeminal and dorsal root ganglia, proximal and distal root trunks, and terminal motor and sensory nerves showed no consistent abnormalities.

When a permethrin formulation was applied to the clipped dorsal surface (0.13 mg/cm^2) of six New Zealand White rabbits (three of each sex) once a day for 16 days, a slight erythema appeared, which correlated with increased cutaneous blood flow. No significant histopathological changes were detected (Flannigan *et al.* 1985).

Pyrethroids can affect behavior patterns. Mice exposed to Ambush (25.6% permethrin) at 0.5, 5.0, or 50 mg/kg orally or 30 or 300 mg/kg dermally displayed an increase in activity (Digiscan optimal animal activity monitor) at the 50- and 300-mg/kg oral and dermal doses, respectively (Mitchel *et al.* 1988).

Clinical signs of toxicity of permethrin, when evident, occur within 2 hr and are associated with central nervous system functions. Permethrin belongs to the Type I group of pyrethroids, and exposure to permethrin is associated with tremors (T syndrome), convulsions, irregular breathing and increased respiratory rates, incoordination, ataxia, hyperactivity, prostration, and paralysis. Other signs that have been reported include hyperexcitability to external stimuli, lacrimation, occasional diarrhea, defecation, and urinary incontinence (Ishmael, 1989). Core body temperature is increased when clinical signs are severe. Signs of toxicity can last up to 3 days after acute exposure. None of the major permethrin metabolites shows greater toxicity than the parent compound.

Neurological effects typical of pyrethroids were observed when rats were exposed to technical-grade permethrin by the inhalation route at concentrations of 2,280 mg/m^3 for 4 hr (internal dose of 365 mg/kg), including paw flicking (probably paresthesias), splayed gait, tail erection, depressed reflexes, and tiptoe gait (Brammer, 1989). Those effects might reflect a higher internal dose rather than a route-specific effect (CEPA,

1992). Death occurred at air concentrations of 2,280 mg/m^3 (internal dose of 365 mg/kg) and higher.

Permethrin is neurotoxic at high doses. It produces a variety of clinical neurotoxic effects in animals. Some of those effects are tremors, salivation, paresthesia, splayed gait, depressed reflexes, and tiptoe gait; reversible axonal injury occurs at high doses (Brammer, 1989; Robinson, 1989a,b). These symptoms appear to be universal for pyrethroids.

In an acute dermal toxicity study, Robinson (1989a) exposed rats to permethrin at 2 g/kg and observed neurotoxic signs such as tiptoe gait, upward curvature of the spine, and urinary incontinence in some of the exposed animals.

Acute toxicity of permethrin from dermal exposure is lower than that from other routes of exposure in several animal species. No deaths were observed when technical-grade permethrin was applied to the skin of rats at 2 g/kg of body weight, but tiptoe gait, upward curvature of the spine, and urinary incontinence were observed in several animals (Robinson, 1989a). The only systemic signs seen when the tick-repellent formulation was applied topically to rabbits at 2 g/kg were weight loss and diarrhea in one animal on days 10-14 (Shapiro, 1989b).

Shapiro (1989a) administered the tick-repellent formulation (0.5% permethrin used on human clothing to repel arthropods) orally to rats at 5 g/kg of body weight in a "limit test." Red nasal discharge, lethargy, and moist rales were observed in a few animals (Shapiro, 1989a). Salivation, lethargy, squinting, and moist rales were seen in rats exposed to an air concentration of the tick-repellent formulation at 4.84 mg/L (774

mg/kg of body weight). Hemorrhaging or white or pale patches in the lungs were observed in 8 of 10 treated rats in this study during gross pathological examination.

Leah (1989b) instilled 0.1 mL of permethrin in the conjunctival sac of rabbits and observed conjunctival erythema, chemosis and discharge. However, no corneal or iridial effects were seen.

Robinson (1989b) showed permethrin to be a moderate skin irritant on the intact and abraded skin of rabbits (Category III). In an acute dermal toxicity test in rats, Robinson (1989a) found an LD50 greater than 200 mg/kg but also observed desquamation, edema, thickening, scab, or skin eruptions in 9 of 10 rats. These skin changes persisted in a few animals up to 10 days (Category III). In the rabbit study, Robinson (1989b) evaluated the skin irritation responses to several concentrations of permethrin. The author observed erythema and edema at a concentration of approximately 80 mg/cm^2.

Some pyrethroids produce tremors and salivation, classified as the intermediate TS-syndrome Following oral administration of permethrin to rats, signs of poisoning became apparent within 2 hr after dosing and persisted for up to 3 days. At lethal levels, these signs included whole body tremors of varying degree from slight to convulsive, which in some cases were accompanied by salivation. Associated signs included hyperactivity and hyper-excitability to external stimuli, urination and defecation, ataxia and lacrimation (WHO, 1990).

Ross and Prentice (1977) orally administered permethrin to 15 hens at 9 g/kg of body weight and again 21 days later. After an additional 21 days, the hens were killed. All

positive control animals (given permethrin at 500 mg/kg) manifested signs of delayed neurotoxicity ranging from slight muscular incoordination to paralysis. No signs of ataxia were seen in any of the hens in the permethrin-treated or negative control animals. Histopathological examination of the nervous tissues of permethrin-treated animals showed none of the degenerative changes observed in the tissues of the animals from positive control groups.

2.4 Effect on Body Weight

Tanaka *et al*., (1967) reported reduced body wt gain in Wister rats maintained for 91 days on a diet containing 5000 mg/kg of tetramethrin. There was no effect on feed and water consumption in rats fed upto 3000 mg/kg tetramethrin for six months .(Wallwork *et al*., 1972) reported slight to moderate decrease in body wt gain in wistar rats which received 500, 1500, 5000 or 10000 mg/kg dietary level of bioallethrin for 90 consecutive days.

Glomot, (1975) reported that the bioresmehrin administered to rats at 2000 mg/kg for three weeks produced slight reduction in body wt. Similar reduction in body wt in rats fed bioresmethrin at 4000 to 8000 mg/kg for 91 days was reported by (Wallwork and Malone, (1971).

Hend and Butterworth, (1976) reported reduction in body wt gain in rats fed 1600 mg/kg cypermethrin for three months. Cypermethrin of 200 mg/kg when applied topically for six hours a day for 13 weeks reduced the feed intake and body wt gain in New Zealand white rabbits (Henderson and Parkinson, 1978).

Goldenthal *et al.*, (1980b) rats fed 50 mg/kg deltamehtrin gained less wt than control but the feed consumption was essentially same in control and treatment groups.

Clark (1982) reported decreased growth when correlated with decreased feed intake in both male and female Wister rats fed with 540 mg/kg alphacypermehrin.

Pickering, (1982) reported decrease in feed intake and body wt gain when alphacypermethrin was fed at a 400 or 800 mg/kg.

Cabral *et al*., (1986) reported slight decrease in body wt when deltamethrin was administered in rats upto 8 mg/kg body wt for 104 weeks.

Bhelonde and Ghosh (2004) investigated the effect of fenpopathrin toxicity on feed consumption and body wt gain in rats. A total number of 72 apparently healthy male albino rats weighing 80-110 gm were divided randomly in to four equal groups. Group I served as control and given distilled water only. The rats of group II and III and IV were given fenpropathrin orally @ 5.916, 2.958 and 1.479 mg/kg b. wt for 90 days. Feed consumption of the rats of group II reduced significantly ($P< 0.05$) from week seven and group III it reduced significantly ($P< 0.05$) only in last two weeks of the study. The body wt gain was affected significantly ($P< 0.05$) in group II rats only, it seems that compound at relatively higher doses affect the feed consumption and body wt gain.

In contrast to decrease in body weight gain observed by most workers, no effect on bodyweight gain by some insecticides is also on record. Feeding of endosulfan for 15 days to rats (Gupta and Chandra, 1977) and mice (Kannan, 1983) and 45 to 90 days feeding of famfur, an organophosphate insecticide to rats (Black *et al.,* 1979) has shown no change in body weight gain.

Many workers observed cumulative toxic effect exerted adversely affecting body weight of poultry upon feeding of cypermethrin (Jabeen, 1984; Quadri *et al.,* 1987; Mohamood and Siddiqui, 1993). Thaker *et al,* (1996) reported decrease in live body

weight gain on feeding of endosulfan at the rate of 1.21 mg/kg and 2.42 mg/kg and malathion at the rate of 9.04 mg/kg and 18.08 mg / kg for 180 days to WLH chicks.

Singh *et al.,* (2001) observed that the fenvalerate significantly decreased the body weight in cockerels following prolong feeding of fenvalerate medicated ration at the rate of 4000 ppm.

Siddiqui, (2004) studied toxicological and immunological effects of sub acute exposure of cockerels to imidacloprid and quinalphos. WLH cockerels of 8 to 10 week vaccinated with F1 vaccine of Ranikhet Disease at day old age were used for study. In different groups of birds, daily oral administration of 50 and 100 µg per kg body weight of quinalphos and 1 and 2 mg per kg body weight of imidacloprid, suspended in 1 ml of groundnut oil, was carried out for 28 days. In first week of treatment, there was no significant change in the mean body weight of quinalphos and imidacloprid treated birds as compared to control. However, in the subsequent week there was a reduction in the body weight of both the pesticides treated birds.

Buckwell and Butterworth, (1977). Reported that feeding of 15000 mg/kg cypermethrin for 13 weeks showed diminished feed intake in beagle hound dogs

Chesterman *et al.,* (1977) reported a reduction in body wt gain in male and female dogs of 25 week age receiving upto 10 mg/kg deltamethrin for 13 weeks.

Griggs *et al.,* (1982) found reduction in body wt gain in male dogs, which received 1000 mg/kg bioallethrin in diet. Further the dietary concentration of 5000 mg/kg of bioallethrin also reduced the body wt gain in both male and female dogs.

Hart, (1975) reported a decrease in body weight and feed consumption in adult beagle dogs fed fenvalerate upto 12.5-mg/kg body wt for 90 days. Parker *et al*., (1986) noticed a decrease in body wt of male rats fed 1000 mg/kg fenvalerate for 13 weeks.

2.5 Effect on Organ Weight and Organ Body Weight ratio

Hend and Butterworth (1976) reported an increase in liver and kidney wts in rats fed cypermethrin upto 1600 mg/kg in feed for three months. Increase kidney wts were noted in the groups receiving 4000 mg/kg cypermethrin in diet. There was an increase in liver, spleen and kidney wts of rats fed 1000 or 3000 mg/kg cypermethrin in diet for five weeks (Hend and Butterworth, 1977b).

An increase in liver weight and decrease in spleen and thymus wt in the rats fed with 4000 mg/kg bioresmethrin for 91 days (Wallwork and Malone, 1971). There was an increase in hepatic organ to body weight ratios in all groups of females and males rats fed 630 to 660 mg/kg resmethrin (Swentzel *et al*., 1977).

Varshneya *et al.* (1992) observed no effect of cypermethrin and carbaryl in rats and chicks respectively on body wt. gain; however, decrease in splenic wt. was recorded in both rats and chicks. In chicks bursal wt. was also decreased. Significant decrease in wt. of Bursa of Fabricius and thymus atrophy has been found in birds given increasing doses of endosulfan in feed for 11 weeks (Kurkure *et al*., 1993).

Intraperitoneal administration of permethrin or deltamethrin results in a calcium/calmodulin-dependent alteration of the protein kinasephosphatase cascade, leading to increased apoptosis of thymocytes and resulting thymic atrophy (Rashatwar and Matsumura 1985, Enan *et al.* 1996).

Immunotoxic Effects of Short-term Atrazine Exposure in Young Male C57BL/6 Mice. Both spleen and thymus weights were dose-dependently decreased by the exposure to ATR The thymus was more sensitive to ATR than the spleen, as the decrease caused by the 125 mg/kg dose of ATR was significant in the thymus but not the spleen. One week after cessation of exposure, the decrease in weight of the thymus caused by ATR exposure was still present, (Nikolay *et al.,* 2005).

Size and wt. of lymphoid organs- spleen, thymus and Bursa of Fabricius was found to be reduced in broiler chicks fed cypermethrin @100 ppm for 8 weeks (Khurana *et al*., 1996a).

Suhash *et al.,* (2004) studied toxicological effect of cypermethrin on male and female Wister rats. Four groups of ten male and female rats were administered cypermethrin orally by gavage at the dose of 0 (control), 3 mg/kg, 9 mg/kg and 30 mg/kg for 28 days. They have reported that cypermethrin didn't have any significant effect on organ wt and organ wt and body wt ratio of any organ in treated group.

Route of exposure to pesticide also affects the organ wt. at necropsy. Following exposure to carbaryl by oral, nasal and dermal routes, there was a dose related decrease in thymus wt. and spleen wt. Significant decrease in liver wt. was found at all oral exposure level. Dermal exposure to carbaryl revealed no significant toxicological effects (Ladies *et al*., 1994).

Mice treated with 100 ppm malathion for 12 weeks showed an increase in liver/body wt. ratio compared to control. The spleen/ body wt. ratio was decreased significantly in mice exposed to 50 ppm malathion for 12 weeks or 100 ppm for 8 weeks and 12 weeks (Bannerjee *et al*., 1998).

Malik *et al*. (2005) studied the biochemical and immunological alteration induced by feeding chlorpyriphos in broiler chickens for a period of 6 weeks. (2 weeks – 8 weeks age). Decreased organ weights particularly of bursa and spleen at different intervals in chlorpyriphos exposed group were observed.

Tripathi (2006) studied subacute exposure Acephate for 28 days to WLH cockerels and found that daily oral administration of acephate resulted in decrease in the spleen: body weight and thymus to body weight ratio.

2.6 Hematological Parameters

There was increase in haematocrit values at the highest dose (LD50/10) tested of supercypermethrin forte in mice (Siroki *et al*., 1994).

Ladies *et al*. (1994) reported that when CD rats were exposed orally to carbaryl (25 mg/kg) for a 2 week period (5 days/ week). There was 33 % increase in RBC counts. At 50 mg/kg dose a 34% decrease in WBC and a 13% increase in RBC counts were observed.

Guilhermino *et al*. (1998) conducted experiments to assess the acute toxic effects of 8.7-30 mg of parathion on the haematology of male Wistar rats. The red cell count and Hb was increased in treated rats.

Choudhary and Joshi (2002) studied the effect of short term endosulfan exposure on haematology of male rat. Endosulfan was administered at the dose of 5, 10, 15 mg/kg body weight per day for 15-30 days. Total erythrocytic count, packed cell volume and hemoglobin content was decreased where as total leukocytic count increased significantly.

Subacute toxicity of imidacloprid was studied in adult male rats following I.P. administration at the rate of 20 and 40 mg/kg daily for 28 days. There was no effect on Hb, PCV and TEC counts at both the dose levels. Imidacloprid caused an inconsistent effect on TLC showing an increase in TLC on seventh day at lower dose and a gradual decrease in TLC upto 28 days at higher dose (Premlata *et al.*, 2006).

An increase in heterophil-lymphocyte ratio and total leukocyte count was observed by various workers in birds with exposure of different insecticide (Mohsin and Ishwar, 1984; Mandal and Lahiri, 1985; Mandal *et al.* 1986; Thaker and Garg, 1993). Whereas a decrease in TLC has been noticed by carbaryl and malathion in chickens (Kakkar *et al*., 1996); lindane in lambs (Khurana *et al*., 1996c); carbofuran in lambs (Khurana *et al*., 1998); permethrin in mice (Shah and Gupta, 1998); fenvalerate in lambs (Khurana and Chauhan, 2000); cypermethrin in chickens (Khurana *et al*., 2000); monocrotphos in sheep (Khurana and Chauhan, 2001) and butachlor in chickens (Kumar *et al*., 2002).

Thaker (1988) studied hematological changes induced in male WLH chicks by long term daily oral administration of endosulfan and malathion. Endosulfan was administered at 1.21 and 2.42-mg/kg body weight and malathion at 9.04 and 18.08 mg/kg body weight. Study did not show any significant alterations in haemoglobin, PCV, DLC, TLC and Total erythrocyte count.

Kakkar *et al*. (1993) reported decrease in total lymphocyte counts in poultry birds when fed low doses of carbaryl 60 ppm for 8 weeks. There was decrease in Hb, PCV and TEC in chickens fed poly-chlorinated biphenyl mixture from 1-42 days of age .

Singh *et al.* (2001c) studied the hematological profiles in cockerels following prolong feeding of fenvalerate-medicated ration at the rate of 4000 ppm. It was found that the fenvalerate significantly decreased TEC, Hb and PCV level.

Administration of traizophos in repeated oral doses of 0.1 and 0.25 mg/kg per day for 21 days in buffalo calves caused a decrease in TEC. Hb was not affected at low dose but higher dose produced a significant decrease in Hb concentration. Insecticide has variable effect on TLC (Sandhu *et al*., 2000).

2.7 Biochemical Parameters:

Serum Total Protein, Total Albumin and Total globulin

Shah and Gupta (2001) conducted subacute toxicity studies of permethrin in young albino male rats. Daily oral administration of permethrin at the rate of 24-120 mg/kg for 30 days showed non-significant changes in the level of total proteins.

Parker *et al*. (1983) reported a decrease in serum albumin concentration in mice fed fenvalerate at the rate of 1250 ppm for 2 years. Triphenyl phosphate as a potential feed contaminant was fed to weanling separation Sprague Dawley rats at the dose level of 0, 0.25, 0.50, 0.75 and 1.00 ppm for 120 days. There was increase in levels of alpha and beta globulins suggestive of increased hepatic activity.

Das and Mukherjee (2000) studied the effect of exposure to sub lethal concentrations of the organophosphate pesticide, quinalphos (1.12, 0.22mg/l) on biochemical parameters of muscle and enzyme activities in brain, liver and kidney of fish (*Labio rohita)*. They found that after 15, 30 and 45 days quinalphos decreased the muscle protein over a period of 45 days.

Subacute toxicity of imidacloprid was studied in adult male rats following I.P. administration at the rate of 20 and 40 mg/kg daily for 28 days. No effect was observed on plasma protein and albumin (Premlata *et al.* 2006).

Khurana *et al*. (1996a) determined serum biochemicals in chickens given cypermethrin at the rate of 100 ppm in feed for 8 weeks. There was a significant depression in serum total proteins, serum globulins and serum gamma-globulins, whereas no effect was observed on serum albumin. The A: G ratio was significantly higher in cypermethrin fed chickens.

A significant depression in serum total proteins, serum globulins and serum gamma globulins was observed when day old broiler chicks were fed endosulfan for 8 weeks (Khurana *et al*. 1996b).

Kakkar *et al*. (1993) reported reduction in serum globulins in poultry birds exposed to low doses of carbaryl (60 ppm) for 8 weeks

Singh *et al.* (2001c) studied the hematobiochemical profiles in cockerels following prolong feeding of fenvalerate-medicated ration at the rate of 4000 ppm. It was found that the fenvalerate significantly decreased serum albumin level.

Garg *et al*. (2002) studied the effect of quinalphos in one week old chicks fed quinalphos at the rate of 8 ppm for 2 months. There was a significant suppression in serum globulins and gamma globulins.

Garg *et al.* (2004) studied haemato-biochemical and immuno-pathophysiological changes following feeding of broiler chicks with 20 ppm fenvalerate (synthetic pyrethroid, SP), 2 ppm monocrotophos (organophosphate, OP) and 2 ppm endosulfan (chlorinated hydrocarbon, CH). Four groups of broiler birds (30 each) were fed poultry

mash without (control) or mixed with pesticides for 8 weeks. Serum globulin levels were decreased in all treated groups compared to control, but not the serum albumin.

Siddiqui (2004) studied toxicological and immunological effects of sub acute exposure of cockerels to imidacloprid and quinalphos in WLH cockerels. Total protein was decreased in all the pesticide treated birds after 14 days of treatment where as total globulin was decreased only in quinalphos treated groups. Large animals.

Insecticides like lindane, monocrotophos, carbofuran and fenvalerate when fed for 6 months in lambs at no adverse effect dose cause a significant decrease in serum total proteins, serum globulins and increase in A: G ratio (Khurana *et al*. 1997).

There was significant depression in serum globulins, gamma globulins in lambs fed lindane at the rate of 1.25 mg/kg body wt. for 6 months (Khurana and Chauhan, 1999). Sub-chronic biochemical toxicity (90 days) studies were carried on lambda-cyalothrin 2.5% EC in male Wistar rats. Exposure caused a significant elevation of albumin (Krishnappa *et al*. 2000).

Kaur *et al.* (2000) investigated the toxic effect of chlorpyriphos following repeated oral administration for 28 days on certain blood biochemical analytes and tissue/organs in goats. Blood samples were collected on days 0, 7, 14, 21 and 28 post-chlorpyriphos administration to study the serum total proteins. It produced significant increase in total proteins level.

In lambs fed monocrotophos (0.025 mg/kg) for 6 months significant decrease in serum globulins and gamma globulins was reported (Khurana and Chauhan, 2003).

2.8 EFFECT ON HUMORAL AND CELL MEDIATED IMMUNITY

The potential consequences of immunotoxicity of pesticides can be divided into three groups (Kacmar *et al*. 1999).

- Direct immunotoxicty (connected mainly with immunodepression)
- Hypersensitivity reactions
- Autoimmune reactions

Immunotoxicity of Permethrin

Experiments with laboratory animals indicate that the immune system (used by living things to defend themselves from disease) "appears to be a sensitive target for permethrin activity." Ingestion of permethrin reduces the ability of immune system cells called T-lymphocytes to recognize and respond to foreign proteins. Doses equivalent to 1/100 of the LD_{50}, inhibited T-lymphocytes over 40 percent (Blaylock, 1995). Permethrin ingestion also reduced the activity of a second type of immune system cell, natural killer cells, by about 40 percent. In tests using mouse cell cultures, permethrin had similar effects on the immune system; inhibition of two kinds of lymphocytes (Stelzer and Gordan, (1984). Researchers concluded that "the immune system is exquisitely sensitive at exposure levels that cause no overt toxicity."

Recent studies have demonstrated that low dose sub acute topical exposure (10 days) to permethrin causes diminished antibody-mediated immunity in mice, and oral cypermethrin decreases antibody production in both rats and mice (Desi *et al.* 1985, Tamang *et al*. 1988, Punareewattana *et al*. 2001). A potential mechanism of action has been demonstrated to be inhibited production of cytokines (IFN and IL-4) necessary for antibody production (Diel *et al.* 1998). Further, subacute topical permethrin exposure in

mice depressed splenic macrophage hydrogen peroxide production, and the speculated mechanism included permethrin-induced inhibition of mitochondrial complex I in the electron transport chain, which may interfere with leukocyte respiratory burst development (Gassner *et al*. 1997, Punareewattana *et al.*2001). Other authors have noted that intraperitoneal administration of permethrin or deltamethrin results in a calcium/calmodulin-dependent alteration of the protein kinase phosphatase cascade, leading to increased apoptosis of thymocytes and resulting thymic atrophy (Rashatwar and Matsumura 1985, Enan *et al.* 1996). These reports have demonstrated a few potential mechanisms by which permethrin affects the immune system, but more information is needed in order to fully understand the molecular mechanisms of permethrin's immunotoxicity.

Immunosuppression of the humoral immune response by pesticides has been studied by Casale *et al.,* (1984). It is a complex phenomenon, as several sites of pesticides interaction with the humoral response are possible, such as antigen processing and presentation, cellular cooperation for the activation of antibody producing cell and synthesis of antibody. Secondly, class and chemical formula of the pesticide can be related to some specific interaction with the immune response, such as severe cholinergic stimulation, shown for organophosphate pesticides, subsequently resulting in immunosuppression (Casale *et al.,* 1984).

In an *in vitro* study (Stelzer and Gordon, 1984), permethrin at a concentration of approximately 10^{-5} to 10^{-6} M inhibited the mitogenic response of murine immune lymphocytes. Although permethrin inhibited the mitogenic response of murine

lymphocytes in vitro, mitogenic responses in *invitro* exposures do not represent functional immunity, nor do they simulate in vivo reactions.

Rashatwar and Matsumura 1985, Enan *et al*. (1996) have noted that intraperitoneal administration of permethrin or deltamethrin results in a calcium/calmodulin-dependent alteration of the protein kinasephosphatase cascade, leading to increased apoptosis of thymocytes and resulting thymic atrophy

Cypermethrin has been previously shown to suppress humoral and cell mediated immune response in rats (Desi *et al*., 1986).

Bernier *et al*. (1988) performed comparative studies of sub-lethal exposure to aminocarb and dieldrin in mice. In vivo infection of pesticide exposed mice with *Salmonella typhimurium* and MHV-3 showed that subsequent 1/3 LD 50 doses of aminocarb did not decrease the resistance of animals to the pathogens, whereas exposure to dieldrin resulted in augmented mortality. There was a decrease in anti-SRBC humoral response 10 days after a single oral exposure to1/4-1/6 LD 50 aminocarb. The cellular immune response was unaffected by sublethal exposure to aminocarb.

Tamang *et al.,* (1988) produced cypermethrin toxicity in mice by intra-peritoneal injection of the pesticide at 50 mg/kg body weight per day for 26 days, and in goats by drenching with cypermethrin at 41.6 mg/kg body weight per day for 30 days. The status of cell-mediated immunity (CMI) was assessed by the 2, 4-dinitrofluorobenzene (DNFB) skin sensitivity test. The results indicated significant depression of CMI in the cypermethrin-treated mice and goats. In addition, the humoral immune reaction of the goats intoxicated with cypermethrin was estimated by enumeration of the plaque-forming B-lymphocytes. The rate of plaque formation in the lymphocyte suspension of

cypermethrin-treated goats was significantly reduced and the diameter of the plaques was also significantly lower than that of control animals. The results indicated that cypermethrin suppressed both CMI and the antibody-forming ability of lymphocytes.

Lukowicz-Ratajczak and Krechniak (1992) reported the influence of deltamethrin on the immune system in mice. Female BALB/c mice were given deltamethrin in two daily oral doses; 6 mg/kg for 84 days and 15 mg/kg for 14 days. The humoral immune response in animals immunized with sheep red blood cells was significantly decreased. In addition, the cell-mediated immune response was assessed by alpha-naphthyl acetate esterase activity, by formation of erythrocyte-antibody-complement (EAC) rosettes, and by the footpad reaction test. A decrease in interleukin-1 (Il-1) activity was also ascertained. The obtained results indicated that deltamethrin exhibits an immunosuppressive effect.

Varshneya *et al.* (1992) evaluated the immunotoxic effects of cypermethrin administered orally (in groundnut oil) to male albino rats at dose levels (mg/kg) of 0 (control), 5, 10, 20 and 40 once daily for 90 days. A dose dependent decrease in delayed type hypersensitivity reaction was noticed on day 61 post treatment. Humoral response as evidenced by serum haemagglutinin and haemolysin titres did not show any definite pattern on day 90. Results of the study revealed that low doses (5 and 10 mg/kg) did not have any adverse effects on the immuno-competence of rats.

Siroki *et al.* (1994) reported the immunotoxicological effect of pyrethroid pesticides in mice. A significant decreased in the number of IgM producing plaque forming cells was observed after oral administration of single one half LD_{50} of cypermethrin in mice.

In an *in vivo* study by Blaylock *et al.* (1995), in which mice were orally exposed to permethrin shown to have inhibited cellular immune responses. Permethrin caused decreased responses in immune functions requiring specific antigen recognition and/or effector function, mixed lymphocyte response (MLR) to allogenic lymphocytes, cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activities, while non specific mitogen stimulations and body and organ weights were not affected.

Tulinska *et al.* (1995) investigated the immunotoxic effect of supercypermethrin forte in the Wistar rat. It was found that cypermethrin significantly depressed the cell-mediated immunity in treated rats as compared to control. The results indicated that cypermethrin suppressed both cell mediated immunity and humoral immune responses in Wistar rat.

When cypermethrin was given during gestation (50 mg/kg, 1/20 of the LD50, on days 7–16) to pregnant rats by gavage in corn oil, the pups showed an increase in the blood NK cells and antibody-dependent cytotoxic activity (Santoni *et al.,* 1997). A marked and long-lasting increase was observed in adrenaline and noradrenalin plasma concentrations, concomitant with an increased output of CD5+, CD4+ and CD8+ T-cells from the spleen to the peripheral blood and a consequent lymphocytosis (Santoni *et al*., 1998, 1999).

Shah and Gupta (1998) investigated the effect of permethrin (30-120 mg/kg/day) administered orally for 14 days on the humoral and cell mediated immune response in mice. At lower dose level 30-60 mg/kg/day, permethrin did not alter the primary and secondary humoral response. However, at 120 mg/kg/day, it significantly reduced the

cell-mediated immune response. This observation suggested that permethrin at these doses causes suppression of CMI response in mice.

Immunotoxic effect of a 28 days oral exposure by 55.4, 22.2, and 11.1 mg/kg cypermethrin was investigated in 4 weeks old male Wistar rats. The applied test system involved the determination of general toxicological parameters (body weight gain, organ weight of thymus, heart, lung, liver, spleen, kidneys, adrenals and the popliteal lymph node), hematological parameters (white blood cell count, red blood cell count, haematocrit, mean cell volume of red blood cells, cellularity of the femoral bone marrow), as well as immune function assays (splenic plaque forming cell assay, delayed type hypersensitivity reaction). The highest dose resulted in a significant increase of the relative liver weight, and all three doses resulted in changes in the haematocrit values. The maximum of DTH reaction decreased at all three doses (Institoris *et al.* 1999).

Punareewattana *et al.* (2000) applied Permethrin to the shaved dorsal interscapular region of female C57Bl/6 mice at doses of 0.5 or 1.5 µ l/day in corn oil and neat 5.0 µ l/day. These doses corresponded to approximately 22, 66, and 220 mg/kg/day topical permethrin. Mice were exposed in this manner either daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. Body weight was not affected by any of the treatment regimens. However, thymic weight was decreased and splenic weight was increased by 1.5 or 5.0 µl of permethrin/ day, 2 days after termination of 10 consecutive days of topical chemical exposure. Cell surface antigen expression did not change in any treatment group on thymocytes (CD4, CD8), splenocytes (CD45R, Thy 1.2), or bone marrow cells (CD45, CD45R). A persistent, dose-related inhibition of the

contact hypersensitivity (CH) response occurred in mice at all exposure levels of permethrin tested.

Prater *et al.,* (2002) evaluated immunomodulatory effects of single topical exposure to permethrin in four-to-five-week-old female C57BL/6N mice. Mice exposed to 5-25 mL permethrin (equivalent to 220-1100 g/kg body weight) on the shaved interscapular space were evaluated 48 hours later for: changes in body weight; splenic and thymic organ weight and cellularity; thymocyte cell surface expression, cellular apoptosis, and necrosis; splenic macrophage phagocytosis and H_2O_2 production by chemiluminescence; splenic B cell antibody production and T cell cytolytic activity; and mitogen-induced proliferation of splenocytes and thymocytes after *in-vivo* or i*n-vitro* permethrin exposure. Topical application of permethrin caused significant inhibition of splenic T cell proliferation, but did not appear to affect leukocyte function in the other assays evaluated. A dose-related decrease in thymic cellularity was seen in the permethrin-exposed mice. The CD4+ and CD8+ thymocyte subpopulation was most severely diminished, suggesting possible chemical-induced apoptosis as a mechanism leading to thymic atrophy. Apoptosis was significantly increased in CD4-8- and CD4-8+ thymocytes. Cellularity of the spleen was also reduced by permethrin, an effect that may relate to inhibited proliferation or reduced seeding from the hypocellular thymus.

Single exposure to permethrin resulted in inhibited antibody production by splenic cells in mice. Five day exposure to permethrin caused persistent decreased contact hypersensitivity responses (Prater, 2003).

The immunosuppressive effects of bath exposure to a sublethal concentration of the synthetic pyrethroid alpha -permethrin (3.05x10-4 mg l-1) in the Indian Major carp,

Labeo rohita was studied after 45 days exposure. In some groups, the effects of alpha-permethrin on non-specific defenses and serum enzymes of carp were investigated after challenge with *Aeromonas hydrophila*. Bactericidal activity of rohu serum was reduced significantly in pesticide and bacteria treated fish (Nayak *et al*. 2004).

Suhash *et al.* (2004) studied Immuno-toxicological effect of cypermethrin on male and female Wistar rats. Four groups of ten male and female rats were administered cypermethrin orally by gavage at the dose of 0 (control), 3 mg/kg, 9 mg/kg and 30 mg/kg for 28 days. Blood samples were collected on day 7, 14, 21 and 28 of the experiment period. Total serum protein did not vary significantly. Ab titer in low, medium and high dose treated group did not show any significant variation compared to control group. Total immunoglobulin (TIG) values, Phagocytic index in cypermethrin treated groups in both male and female rats did not differ significantly ($P<0.05$) from that control group. This indicates that cypermethrin doesn't have immunosuppressive effect on T-cells. DNCB skin sensitivity test did not show any significant difference in skin thickness between treated and control group.

Bannerjee *et al*. (1996a) studied the influence of sub chronic exposure to lindane on humoral immunity in mice. Lindane suppressed both primary and secondary antibody responses to SRBC, the effects being more pronounced on the secondary than the primary response.

Kim *et al*. (2003) investigated Simazine, a triazine herbicide, for its in vivo immunomodulatory properties. Male C57Bl/6 mice were treated with vehicle and simazine at the dose of 300 or 600 mg/kg body weight daily orally for 4 weeks. The immune system was evaluated by the antibody response to sheep red blood cells (SRBC);

plaque assay and serum immunoglobulin (IgG). Simazine inhibited the IgM plaque-forming cell numbers and lowered the level of IgG.

Chauhan and Mahipal, (1994) studied the immunotoxicity of cypermethrin in poultry. It was found that when birds were fed with cypermethrin there was a significant reduction in serum globulin and gamma globulin, which is an indicative of non-specific and general reduction in immunity.

Khurana *et al*. (1994) studied the immunotoxic effect of cypermethrin on DTH reaction in chickens fed at a dose rate of 100 ppm and found significant suppression of DTH to DNFB.

Singh *et al*. (1997) investigated the acute toxicity of alphamethrin in chicks. Eighteen, eight weeks old, white leghorn male chicks were randomly and equally divided in three groups and administered single oral doses of alphamethrin at the dose of 0, 100 and 200 mg/kg body wt., respectively, to study humoral and cellular immune responses. Humoral immune response was examined by ELISA, antibody titres were significantly ($P< 0.01$) declined in both the dose groups of alphamethrin as compared to control. Cellular immune response was assessed by DTH against DNFB, macrophage function test and lymphocyte stimulation test. A significant increase in skin sensitivity at low dose (100 mg/kg) was noticed whereas the high dose (200 mg/kg) significantly diminished the skin hypersensitivity of DNFB.

Khurana *et al.* (1998) studied the effect of cypermethrin on humoral immune response in 125 day old broiler chicks. Specific Newcastle disease vaccine induced humoral response was measured by HI and ELISA at weekly interval. There was significant suppression in serum globulin, gamma globulin and specific HI and ELISA

antibodies in cypermethrin fed chicken in comparison to controls indicating the immunosuppressive effect of the insecticide.

Alphamethrin has been found to have immunosuppressive effects at a dose of 5 mg/kg body wt. as indicated by decreased Brucella specific antibodies in calves. This may lead to increased susceptibility to various diseases (Chauhan and Agrawal, 1999). Two groups of six White Leghorn male chickens were administered single oral dose of alphamethrin (alpha-cypermethrin) at 100 or 200 mg/kg body weight at 8 weeks of age. Six chickens were untreated controls. Humoral immune response was examined by evaluating antibody titres against bovine serum albumin using ELISA. Antibody titres significantly declined in all alphamethrin-treated groups compared with untreated controls. Cellular immune response was assayed by delayed type of skin hypersensitivity using dinitrofluorobenzene (DNFB). The skin hypersensitivity in response to DNFB administration significantly decreased after administration of alphamethrin at 200 mg/kg (Singh *et al*. 1999).

To study the effect of cypermethrin on cell- mediated immune response in chickens, 125 day-old boiler chicks were taken and divided into three groups of 40 each. Five birds were used for 0 day observations. The group I birds were kept as unvaccinated control, group II birds were vaccinated with New Castle Disease vaccine at day 0 and 32 and both the groups were given normal broiler ration. The group III birds were fed no adverse effect dose of cypermethrin, 100 ppm in feed for eight weeks. DTH reaction was monitored at 4^{th} and 8^{th} week. A significant suppression in DTH reaction was observed in cypermethrin fed birds as compared to controls (Khurana *et al*. 2000).

Singh *et al*. (2001a) assessed the effect of fenvalerate on cell-mediated immune response in chickens, a total of 60 chickens were used in two groups of 30 each. These birds were vaccinated with New Castle Disease vaccine at day 4 and 12 weeks of age. Group I was kept as control while group II birds were given 200 ppm fenvalerate in feed daily for a period of six months. The reduction in DTH reaction in pesticide fed birds confirmed the suppression of CMI responses in fenvalerate fed birds. Fenvalerate induced stress has been found to decrease humoral immunity. (Singh *et al*., 2001b) and cell-mediated immunity in chicken (Singhal *et al*., 2001). The lowered immunocompetence of birds may enhance the susceptibility, resulting into occurrence of recurrent infection, epidemic of disease and vaccine failures.

Kumar *et al*. (2002) conducted a study to evaluate the effect of butachlor on cell-mediated immunity in chicken. For this study, 80 one-day-old chicks were used and divided into two groups; control and treatment group of 40 birds each. The control group was given normal feed and treatment group was given butachlor in feed at dose rate of 50 ppm, which is considered to be "no observable effect level" (NOEL) dose. Cell-mediated immunity was also assessed by delayed type of hypersensitivity (DTH) reaction to dinitrochloro benzene (DNCB) at the 8th week of experiment. There was significant decrease in reaction to DNCB in butachlor fed group.

Malik *et al*. (2005) mixed chlorpyriphos uniformly in the feed of groups 2, 3 and 4 at 30, 60 and 120 ppm levels, respectively for 6 weeks while group 1 birds were fed normal chick mash with no chlorpyriphos. Humoral immune response was studied by HI test using New Castle Disease Vaccine as antigen. The cell-mediated immune response was studied by skin hypersensitivity test using DNCB as antigen. The chlorpyriphos

exposure caused a decrease in HI titres against NDV. This decrease in HI titre was significant ($P< 0.05$) at 0 day and 4th week of treatment in groups 2, 3 and 4 as compared to control. Cell-mediated immunity as assessed by mean skin thickness at 48 and 72 hour, respectively in group 2, 3 and 4 revealed significantly decrease ($P< 0.05$) when compared to corresponding values in control group.

Chauhan (1998) reported that both the wings of the immune system humoral and cell mediated are adversely affected in calves fed alphamethrin 5 mg/kg body wt. for a period of 4 months. This was indicated by decrease in ELISA values of Brucella specific antibodies IgG, IgM, B and T lymphocyte blastogenesis, CD4+ and CD8+ cells. The effect can be modulated by herbal immmunomodulators.

Khurana and Chauhan (2000) studied the immunopathological effects of fenvalerate on cell-mediated immune response in sheep by oral dosing @ 1.25 mg/kg body wt. for six months. A significant suppression in DTH was observed in fenvalerate fed lambs as compared to control lambs.

Diel *et al*. (1998). Conducted *in vitro* immunotoxicity study of permethrin. The study showed that permethrin inhibited lymphocyte proliferation induced by phytohaemagglutinine (PHA), and decreased the production of IFN and IL-4 by lymphocytes in a concentration-dependent manner.

2.9 Gross Pathology and Histopathology

Poisonous chemicals after entering into the body produce their effects either by producing local injury leading to inflammation and necrosis or by producing gastroenteritis, hepatotoxicity or nephrotoxicity. There are still others, which produce their toxicity by affecting bone marrow or extremities or nervous tissue. The review of

literature of the histopathological changes produced by various pyrethroids, organophosphorus and organochlorine compounds is as under:

The non-target species are often exposed to low doses of pesticides. In practical situation, a single dose is not important, but, slow prolonged exposure is the matter of concern. Such exposure leads to changes in immune system and immune organs and as the first lesion to develop is at the cellular level, the histopathological changes in immune organs are reviewed as under. The effect on liver and kidney has also been reviewed, as liver is the principal site of biotransformation and kidney being the principal organ in excretion. Besides, some pesticides have an effect on nervous system, cardio-vascular system and gastro-intestinal system.

Topical application of permethrin (25 µl, equivalent to 1100 mg/kg b.wt) in mice caused 32% inhibition of splenic T-cell proliferation. Apoptosis was significantly increased in CD4 (-) 8(-) and CD4 (-) 8(+) thymocytes, and the CD4 (+) CD8 (+) thymocyte subpopulation was most severely diminished, suggesting a possible chemical-induced apoptotic mechanism of thymic atrophy. Permethrin also caused splenic hypocellularity by 31% at 15 µl (660 mg/kg bw), and by 50% at 25 µl (1100 mg/kg bw), an effect that may relate to inhibited proliferation or reduced seeding from the hypocellular thymus (Prater, 2002).

Parker *et al*. (1983) fed male mice with dietary concentration s of 10, 50, 250 and 1250 ppm fenvalerate for 2 years. It caused multifactorial granulamata in lymph nodes; liver and spleen in male mice fed 1250 ppm and female mice fed 250 and 1250 ppm.

Histopathological studies in rats and dogs have indicated that hexachlorobenzene has immunotoxic properties. Rats exposed to low doses of hexachlorobenzene showed

lymphoid hyperplasia of the splenic white pulp. In dogs hexachlorobenzene produced hyperplasia of lymphoid tissue in stomach (Vos, 1986).

Thomas and Ratajczak (1988) assessed carbamate pesticide immunotoxicity in rats by feeding 0, 0.1, 1.0, 10, 100 or 1000 ppb of aldicarb daily in water for 34 days. Gross and histopathological examination of tissues relevant to immune system revealed absence of significant effects. Pathological changes have been observed in bursa, thymus and spleen of endosulfan treated birds (Kurkure *et al*. 1993).

Histopatholgically, splenic and thymic atrophy was observed when mice were treated with ethyl carbamate, which was potentiated by the pretreatment with diazinon. In spleen lymphocytes in the periarteriolar lymphoid sheath and the marginal zone appeared to be depleted in the white pulps. In thymus, ethyl carbamate caused a marked depletion of cells in cortex (Cha *et al*., 2000).

Kaioumova *et al*. (2001) investigated the toxicity of the widely used herbicide Dimethylammonium salt of 2-4 dichloro-phenoxy-acetic-acid (DMA 2-4-D) on the lymphoid system of rats after a single dose oral administration. DMA 2-4-D destroyed in a dose- dependent fashion the vascular integrity of the thymus and caused cell depletion in the white pulp of spleen and in the cortex of the thymus.

Khurana *et al*. (1996a) studied the immunopathology of cypermethrin toxicity in broiler chicks when cypermethrin @ 100ppm was given in feed for 8 weeks. There was depletion of lymphocytes, necrosis of lymphoid tissue at the germinal centers and congestion at few places in the bursa, spleen and thymus.

Fenvalerate exposure in birds results in necrosis as well as hyperplasia of lymphoid follicles of spleen and thymus, while adrenal cortex has shown marked degenerative

changes particularly in zona fasciculata (Varshneya *et al.* 1986). Focal necroses in skeletal muscle and enteritis have been observed with oral ingestion of insecticide (Snow, 1973). Retardation in tubular development and excess amount of intertubular tissues in testis four to eight week of age has also been reported (Burlington and Lindeman, 1950).

There was depletion of lymphoid cells in the germinal centers of follicles and increased proliferation of fibroblasts in the bursa, thymus and spleen when birds were fed on NOEL of butachlor for 6 months (Singh *et al*., 2001d).

Garg *et al.* (2004) studied haemato-biochemical and immuno-pathophysiological changes following feeding of broiler chicks with 20 ppm fenvalerate (synthetic pyrethroid, SP), 2 ppm monocrotophos (organophosphate, OP) and 2 ppm endosulfan (chlorinated hydrocarbon, CH). Four groups of broiler birds (30 each) were fed poultry mash without (control) or mixed with pesticides for 8 weeks. Microscopic examination of thymus revealed atrophy/hypoplasia, decrease in the size of follicles with depletion of lymphocytes and haemorrhages. The study revealed that the chronic exposure of chicks to small amount of SP, OP and CH pesticide leads to deleterious effects on immune system of birds.

Toxicity was produced in crossbred calves by oral administration of cypermethrin @ 60 mg/kg body wt. per day for 30 days. At necropsy, the white pulp of the spleen of the crossbred cow calves showed a washed out appearance and the Malphigian corpuscles of the treated cow calves were smaller than normal (Patel *et al*. 1996).

2.10 CURCUMA LONGA (TURMERIC)

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and non-specific immunity (Wagner and Proksh, 1985). Many plants used in traditional medicine are reported to have immunomodulating activities. Some of these stimulate both humoral and cell mediated immunity while others activate only the cellular components of the immune system, i.e. phagocytic function without affecting the humoral or cell mediated immunity. Some of these plants also suppress both humoral and cell mediated immunity (Sharma *et al.* 1994).

Turmeric (*Curcuma longa*) is a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine as home remedy for various diseases. In India it is popularly known as "Haldi". In Malaysia, Indonesia and India it has been well studied due to its economic importance. The coloring principle of turmeric was isolated in the 19th century and was named curcumin, which was extracted from the rhizomes of *C. longa* L with yellow color and is the major component of this plant, being responsible for the anti-inflammatory effects. In old Hindu medicine, it is extensively used for the treatment of sprains and swellings caused by injury (Ammon & Wahl, 1991).

Ethnologically, turmeric, the therapeutic goldmine of the East, occupies an important position in the Hindu psyche. It forms an integral part of many sacred Hindu rituals highlighting its importance for mankind. A systematic scientific study on turmeric in this country was taken up as late as 1970s and that too initially was restricted mostly to its anti-inflammatory characteristics. Lately, this herb has attracted almost globally frantic scientific evaluation on many hitherto unknown and unexplored aspects.

***Curcuma longa* (haldi)**

. In Indian folklore, turmeric is considered highly effective in the management of internal hemorrhages and skeletal injuries people suffer accidentally. Current traditional Indian medicine (Ayurveda) recommends turmeric against bilary disorders, anorexia, coryza, cough, hepatic disorders rheumatism and sinusitis (Ammon & Wahl 1991). Curcumin is a good antioxidant and inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy & Lokesh, 1994).

Allopathy has now affirmed many, if not all, of the above said usages so far laid buried in the ancient Indian texts. Turmeric now is experimentally proved to be antibacterial, antifungal, anti-inflammatory, immunomodulatory, hypolipedemic and hepato-operative. It also shows protease inhibitory effects besides being active oxygen-species free radical scavenger and lipid peroxidase inhibitor (Ammon and Wahl, 1991).

Curcumin the active principle of turmeric is a yellow, crystalline substance that is a potent phenolic antioxidant, the scavenger of free radicals, which account for many diseases including cancer. Turmeric also contains bioactive peptide Turmerin which also has a strong antioxidant and hence protective.

Turmeric was described as *C. longa* by Linnaeus and its taxonomic position is as follows:

Class	Liliopsida
Subclass	Commelinids
Order	Zingiberales
Family	Zingiberaceae
Genus	*Curcuma*
Species	*Curcuma longa*

The wild turmeric is called *C. aromatica* and the domestic species is called *C. longa*.

Habitat

It is distributed throughout tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China.

Physical Characteristics

It is a perennial plant that measures up to 1 m high, having a short stem with large oblong leaves and bears ovate, pyriform or oblong rhizomes, which are often branched and brownish-yellow in colour. Its rhizomes are oblong, ovate, pyriform, often short-branched.

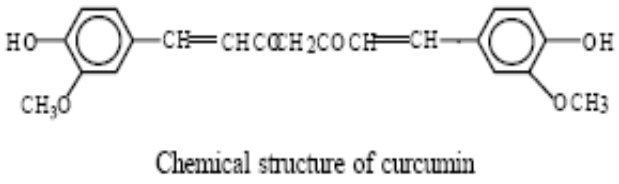
Active constituents:

The active constituent is known as curcumin

Chemical composition of turmeric

Turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The essential oil (5.8%) obtained by steam distillation of rhizomes has *a*-phellandrene (1%), sabinene (0.6%), cineol (1%), borneol (0.5%), zingiberene (25%) and sesquiterpines (53%)5. Curcumin (diferuloylmethane) (3–4%) is responsible for the yellow colour, and comprises curcumin I (94%), curcumin II (6%) and curcumin III (0.3%). Demethoxy and bisdemethoxy derivatives of curcumin have also been isolated. Curcumin is soluble in ethanol, alkalis, ketone, acetic acid and chloroform; and is insoluble in water.

2.10.1 Chemical structure:



Chemical structure of curcumin

Fig 2: Chemical structure of curcumin

Curcumin was first isolated in 1815. The major constituent, curcumin (diferuloylmethane) is in the most important fraction of *C. longa* L. and its chemical structure (Figure and Table), was determined by Roughley and Whiting (1973). It melts at 176-177°C and forms red-brown salts with alkalis. In the molecule of curcumin, the main chain is aliphatic, unsaturated and the aryl group can be substituted or not.

2.10.2 Pharmacological action of turmeric and its extract:

Several pharmacological activities and medicinal applications of turmeric are known. Although curcumin has been isolated in the 19th century, extracts of the rhizomes of *C. longa* have been in use from the Vedic ages.

Effect on gastrointestinal system

***Stomach*:** Turmeric powder has beneficial effect on the stomach. It increases mucin secretion in rabbits and may thus act as gastroprotectant against irritants.

***Intestine*:** Curcumin has some good effects on the intestine also. Antispasmodic activity of sodium curcuminate was observed in isolated guinea pig ileum.

***Liver**:* Curcumin and its analogues have protective activity in cultured rat hepatocytes against carbon tetrachloride, D-galactosamine, peroxide and ionophore-induced toxicity.

Effect on cardiovascular system

Curcumin decreases the severity of pathological changes and thus protects from damage caused by myocardial infarction.

Effect on nervous system

Curcumin and manganese complex of curcumin offer protective action against vascular dementia by exerting antioxidant activity.

Effect on lipid metabolism

Curcumin reduces low density lipoprotein and very low density lipoprotein significantly in plasma and total cholesterol level in liver along with an increase of *a*-tocopherol level in rat plasma, suggesting *in vivo* interaction between curcumin and *a*-tocopherol that may increase the bioavailability of vitamin E and decrease cholesterol levels.

Anti-inflammatory activity

Curcumin is effective against carrageenin-induced edema in rats and mice. The volatile oil and also the petroleum ether, alcohol and water extracts of *C. longa* show anti-inflammatory effects.

Antioxidant effect

The antioxidant activity of curcumin was reported as early as 1975. It acts as a scavenger of oxygen free radicals. It can protect haemoglobin from oxidation.

Anticarcinogenic effect

Curcumin acts as a potent anticarcinogenic compound. Among various mechanisms, induction of apoptosis plays an important role in its anticarcinogenic effect. It induces apoptosis and inhibits cell-cycle progression, both of which are instrumental in preventing cancerous cell growth in rat aortic smooth muscle cells.

Antimutagenic activity

Curcumin exerts both pro- and antimutagenic effects. At 100 and 200 mg/kg body wt doses, curcumin has been shown to reduce the number of aberrant cells in cyclophosphamide- induced chromosomal aberration in Wistar rats.

Anticoagulant activity

Curcumin shows anticoagulant activity by inhibiting collagen and adrenaline-induced platelet aggregation *in vitro* as well as *in vivo* in rat thoracic aorta.

Antifertility activity

Petroleum ether and aqueous extracts of turmeric rhizomes show 100% antifertility effect in rats when fed orally. Implantation is completely inhibited by these extracts.

Antidiabetic effect

Curcumin prevents galactose-induced cataract formation at very low doses. Both turmeric and curcumin decrease blood sugar level in alloxan-induced diabetes in rat.

Antibacterial activity

Both curcumin and the oil fraction suppress growth of several bacteria like *Streptococcus*, *Staphylococcus*, *Lactobacillus*, etc. The aqueous extract of turmeric rhizomes has antibacterial effects. Curcumin also prevents growth of *Helicobacter pylori* CagA+ strains *in vitro*.

Antifungal effect

Ether and chloroform extracts and oil of *C. longa* have antifungal effects. Crude ethanol extract also possesses antifungal activity.

Antiprotozoan activity

The ethanol extract of the rhizomes has anti-*Entamoeba histolytica* activity. Curcumin has anti-*Leishmania* activity *in vitro*.

Antiviral effect

Curcumin has been shown to have antiviral activity. It acts as an efficient inhibitor of Epstein-Barr virus (EBV) key activator Bam H fragment z left frame 1 (BZLF1) protein transcription in cells.

Antifibrotic effect

Curcumin suppresses bleomycin-induced pulmonary fibrosis in rats. Oral administration of curcumin at 300 mg/kg dose inhibits bleomycin-induced increase in total cell counts and biomarkers of inflammatory responses.

Antivenom effect

Ar-turmerone, isolated from *C. longa*, neutralizes both haemorrhagic activity of *Bothrops* venom and 70% lethal effect of *Crotalus* venom in mice. It acts as an enzymatic inhibitor of venom enzymes with proteolytic activities.

Arora *et al*. (1971) investigated the anti-inflammatory activity in different fractions of the petroleum ether extract of the rhizomes of turmeric (two constituents) in animals. They found that the extracts reduced the granuloma growth and no toxic effects were observed.

Sikora *et al.*, (1997) reported that curcumin treatment completely abolished the proliferation of Con A stimulated rat thymocytes and it also suppressed the dexamethasone-induced apoptosis in stimulated as well as nonstimulated rat thymocytes. This inhibition of apoptosis is accompanied by partial or complete oppression of AP-1 activity in nonstimulated or Con-A-stimulated thymocytes, respectively. A similar effect was also observable in rat thymocytes treated with dexamethasone however, curcumin *per se* did not have any adverse effect on AP-1 activity.

Quiles *et al*. (1998) evaluated the antioxidant capacity of a *Curcuma longa* extract on the lipid peroxidation of liver mitochondria and microsome membranes in atherosclerotic rabbits that active compounds in curcuma extract may be protective in preventing lipoperoxidation of liver mitocondria and microsome membranes in a dosage-dependent manner.

Rajakrishnan *et al*. (2002) studied the Effect of curcumin on ethanol-induced stress on mononuclear cells. In order to understand the role of curcumin, an antioxidant principle from *Curcuma longa* on blood mononuclear cells from rabbits given

ethanol for 30 days and ethanol with curcumin, cells were isolated and an attachment assay was carried out. The monocytes from ethanol-treated rabbits showed a lesser attachment to collagen, the major component of the vessel wall subendothelium, and those from curcumin treated animals along with ethanol showed a higher affinity to collagen, causing an alteration in the attachment of monocyte to collagen due to ethanol-induced stress.

Mohanty *et al*. (2004) evaluated cardioprotective potential of *Curcuma longa* on ischemia-reperfusion induced myocardial infarction (MI) and their mechanisms in rats. Wistar rats were divided into three groups and received saline orally (sham, control I/R group) and *Curcuma longa* 100 mg/kg (CL-100 treated group) respectively for one month. Cardioprotective effect of Cl likely results from the suppression of oxidative stress and correlates with the improved ventricular function. Histopathological examination further confirmed the protective effects of Cl on the heart.

IMMUNOMODULATORY EFFECT:

Curcumin has been shown in the last two decades to be a potent immunomodulatory agent that can modulate the activation of T cells, B cells, macrophages, neutrophils, natural killer cells, and dendritic cells.

Curcumin also acts as an immunostimulator, and increased circulating antibody titre, splenic plaque forming cells (PFC), alpha-esterase positive cells, and macrophage phagocytic activity, was reported. Curcumin inhibits Th1 cytokine profile in CD4+ T cells by suppressing IL-12 production in macrophages.

Curcumin not only plays an important role in the Immunomodulation of normal but also transformed T cells, where it adversely affects the cell proliferation of these cells

by suppression of IL-2 gene expression and by inhibiting the activation of NF-κB. These results indicate that the antiproliferative activity of curcumin against T cells may be relevant for T-cell leukemia.

EFFECT OF CURCUMIN ON IMMUNE DISEASES

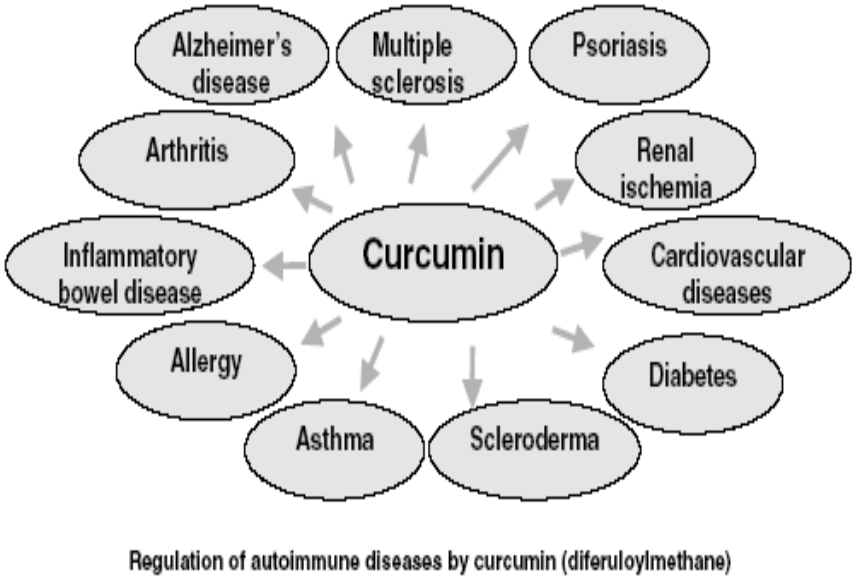


Fig.3 Immune diseases that may be potentially treated with curcumin.

The autoimmune diseases that may be potentially treated with curcumin are:

1) Alzheimer's Disease: Curcumin has potential against Alzheimer's disease. The effect of curcumin in Alzheimer's disease is mediated through the downmodulation of cytokine (i.e.,TNF-α and IL-1β) and chemokine (i.e., MIP-1b, MCP- 1, and IL-8) activity in peripheral blood monocytes and reduces amyloid-β plaque formation. (Giri *et al.,* 2004 , Yang *et al.,* 2005, Zhang *et al.,* 2006)

2) Multiple Sclerosis: Curcumin may have potential against multiple sclerosis. In animal model of this disease, curcumin was found to inhibit IL-12-induced tyrosine

phosphorylation of Janus kinase 2, tyrosine kinase 2, and STAT3 and STAT4 transcription factors. (Natarajan *et al* 2002)

3) Allergy: Based on the experiments *in vivo* (in guinea pigs) and *in vitro* (rat basophilic leukemia cells), curcumin can help to clear constricted airways and increase antioxidant levels. (Suzuki *et al,* 2005, Salh *et al*, 2003)

4) Asthma: It has been reported that curcumin can relieve symptoms of asthma. These effects are linked with reduction of the lymphocytic production of IL-2, IL-5, GM-CSF, and IL-4 that is associated with bronchial asthma. (Gupta *et al* 1999,Suzuki *et al* 2005).

5) Inflammatory Bowel Disease: Curcumin can ameliorate inflammatory bowel disease as shown *in vivo* in humans and rats by reducing inflammatory cytokine levels, blunting NO and O2 production, and suppressing NF-κB activation in colon epithelium. (Salh *et al* 2003,Holt *et al* 2005)

6) Rheumatoid Arthritis: In rheumatoid arthritis, curcumin exerts beneficial effects by inhibiting the expression of collagenase and stromelysin and the proliferation of synoviocytes (Funk *et al* 2006 , Jackson *et al* 2006).

7) Renal Ischemia: In renal ischemia, curcumin can exert beneficial effects that include reducing creatine levels; upregulating Mn-SOD levels; and inhibiting the expression of RANTES, MCP-1, and allograft inflammatory factor (Shoskes *et al* 1998, Shahed *et al* 2001).

8) Psoriasis: Clinical evaluation of topical application of 1% curcumin gel in psoriatic areas reduced the density of CD8+ T cells when compared to untreated areas, where density of CD8+ T cells showed an elevation (Heng *et al* 2000). This and other studies

suggest that curcumin treatment could be an effective paradigm in the treatment of psoriasis as it could also reduce the activity of phosphorylase kinase (Bosman 1994).

9) Scleroderma: Scleroderma is a disease that involves excessive collagen deposition and hyperproliferation of fibroblasts, curcumin provide a therapeutic benefit through its ability to suppress the proliferation of lung fibroblasts in a process involving the inhibition of protein kinase Cε (Tourkina *et al* 2004).

9) Acquired Immunodeficiency Disease (AIDS): There are several reports indicating that curcumin may have potential against AIDS. These effects of curcumin are mediated through suppression of replication of human immunodeficiency virus (HIV) by inhibition of HIV long terminal repeat (Li *et al* 1993, Barthelemy 1998) and HIV protease (Sui *et a.l* 1993), inhibits HIV-1 integrase.

Kuramoto *et al*. (1996) conducted the experiments on rat splenic lymphocytes and showed that curcumin treatment enhances the immune response of the lymphocytes by increasing IgG production.

Ziauddin *et al.,* (1996) shown weight gain by Ashwagandna (*Withania somnifera*) in mice with myelosuppression induced by one or more of the following three compounds: cyclophosphamide, azathioprin, or prednisolone.A significant increase body weight was observed in Ashwagandha-treated mice as compared with untreated (control) mice.

South *et al.,* (1997) studied the dietary effect of curcumin on antibody response in rats. Antibody (IgG) production, delayed-type hypersensitivity and natural killer cell activity were evaluated after 5 weeks of dietary exposure to 1, 20 or 40 mg/kg curcumin and found that the highest dose of curcumin significantly enhanced IgG levels. Rats

receiving lower dietary concentrations (1 or 20 mg/kg) of curcumin were not different in IgG production from rats receiving no curcumin in their diet. Neither delayed-type hypersensitivity nor natural killer cell activity was different from control values at any dietary concentration of curcumin. This shows that curcumin enhances the antibody response in rats.

Deshpande *et al,*. (1998) showed the adaptogenic activity of *Curcuma longa* by demonstratig that pretreatment as well as concurrent treatment of turmeric extract in CCl4 treated rats caused a reduction in cholesterol, bilirubin, SGOT, SGPT and alkaline phosphatase activity; concurrent treatment offering more significant protection.

Agarwal *et al.,* (1999) reported the immunomodulatory activities of extracts from, *Withania somnifera* in mice for immune inflammation, active paw anaphylaxis and delayed type hypersensitivity (DTH). Cyclophosphamide induced immunosuppression was counteracted by treatment with *Withania somnifera*, revealing significant increase in hemagglutinating antibody responses and hemolytic antibody responses towards sheep red blood cells.

Antony *et al.,* (1999) analysed the Curcumin, an active ingredient present in *Curcuma longa,* for the immunomodulatory activity in Balb/c mice. Curcumin administration was found to increase the total WBC count significantly on the 12th day Group of animals treated with vehicle alone showed results similar to that of normal animal (10,130 on 12th day). Curcumin increased the circulating antibody titre against SRBC. Curcumin administration increased the plaque forming cells (PFC) in the spleen and the maximum number of PFC was observed on the 6th day (1,130 PFC/10 spleen cells) after immunization with SRBC. Bone marrow cellularity (16.9x10 cells/femur) and

alpha-esterase positive cells (1,622/4000 cells) were also enhanced by Curcumin administration. A significant increase in macrophage phagocytic activity was also observed in Curcumin treated animals ($P<0.001$). These results indicate the immunostimulatory activity of Curcumin.

Kang *et al.,* (1999) investigated the effects of curcumin, a natural product of plants obtained from *Curcuma longa* (turmeric), on IL-12 production by mouse splenic macrophages and the subsequent ability of these cells to regulate cytokine production by CD4+ T cells and found that pretreatment with Curcumin inhibits Th1 cytokine profile in CD4+ T cells by suppressing interleukin-12 production in macrophages and points to a possible therapeutic use of curcumin in the Th1-mediated immune diseases.

The ability of curcumin to downregulate Th1 cytokine and NO production has been directly correlated to its ability to differentially activate the host macrophages (Bhaumik *et al.*, 2000)

Curcumin exerts a variety of immunomodulatory effects (Churchill *et al*., 2000). Curcuminoids have been shown to be free radical scavengers that suppress the production of superoxide by macrophages, and a significant increase of macrophage phagocytic activity was also observed in curcumin treated animals. He also reported that curcumin treatment stimulates proliferation of B cells in the mucosa of intestine of C57BL/6J-Min/+ (Min/+) mice. These results indicate the immunostimulatory activity of curcumin (Lukita-atmadja *et al*. 2002).

HA titre did not show any significant change with 150 mg/kg/day of *Ashtamangal Ghrita* (polyherbal formulation*)* administration. However, a significant increase was observed at a dose of 300 mg/kg/day with almost a five fold increase as compared to

untreated control animals (P<0.05) .The DTH response, which is a direct correlate of cell mediated immunity (CMI), was found to be increased by 30% at a dose of 300 mg/kg/day of the *Ashtamangal Ghrita* (polyherbal formulation*)*. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavanger cells to the site of reaction (Fulzele *et al.,* 2002).

AL-Sultan (2003) observed the performance in broiler chickens by feeding *Curcuma long* and found that there was increase in body weight and increased spleen to body weight ratio in broiler.

Bin-hafeez *et al.,* (2003) observed that there was increase in thymus to body weight ratio in swiss albino mice due to oral administration of *Trigonella foenum graecum.*

Methanol extract and remaining fraction of methanol extract of *Sphaeranthus indicus Linn* were found potent in protecting cyclophosphamide-induced myelosuppression as evidenced by increasing the levels of total WBC count significantly in swiss albino mice. Petroleum ether and chloroform fraction also raised WBC levels but could not reach up to the normal values. Remaining fraction of methanol extract was the only fraction that increased the total WBC count up to the normal values in a dose dependent manner (Bafna and Mishra, 2004).

Padmaja and Raju (2004) studied the antioxidant effect of curcumin in selenium induced cataract of Wistar rats. Wistar rat pups treated with curcumin, a natural constituent of *Curcuma longa* before being administered with selenium showed no opacities in the lens. The lipid peroxidation, xanthine oxidase enzyme levels in the lenses

of curcumin and selenium co-treated animals were significantly less when compared to selenium treated animals. The superoxidase dismutase and catalase enzyme activities of curcumin and selenium co-treated animal lenses showed an enhancement. Curcumin co-treatment seems to prevent oxidative damage and found to delay the development of cataract.

Rekhate *et al.* (2004) found that supplementation of shatavari root powder in birds, have beneficial effects on body weight and also cause significant increase in ($P<0.01$) serum total protein and globulin. It suggests that aqueous extract of *A. racemosus* having some degree of reversal effect against humoral immunity.

Significant reduction in white blood cell counts was observed in animals treated with cyclophosphamide. Cyclophosphamide induced myelosuppression was counteracted by methanol extract of *Cissampelos pareira* in dose dependent manner with increase in levels of WBC compared to cyclophosphamide group. Higher doses i.e. 400 and 800 mg/kg could bring the levels of WBC to normal (Bafna and Mishra, 2005).

There was significant increase in the total carbohydrate and total carbohydrate/protein ratio in laboratory animals when *Asparagus racemosus* wild and *Withania somnifera* root extract were given with a standard drug (Bhatnagar *et al.,* 2005).

Li X and Liu X (2005) conducted a study on mouse lymphocytes and reported that a low-dose curcumin increased the proliferation of splenic lymphocytes, whereas high-dose curcumin depressed it indicating its ability to differentially regulate the proliferation of splenic lymphocytes.

Yadav *et al.* (2005) studied the immunomodulatory effects of curcumin from *Curcuma longa* on mitogen (phytohaemagglutinin; PHA) stimulated T-cell proliferation, natural killer (NK) cell cytotoxicity, production of cytokines by human peripheral blood mononuclear cells (PBMCs), nitric oxide (NO) production in mouse macrophage cells, RAW-264.7 and found that curcumin inhibits PHA-induced T-cell proliferation, interleukin-2 production, NO generation, and lipopolysachharide -induced nuclear factor-κB (NF-κB) and augments NK cell cytotoxicity. Our results suggest that curcumin most likely inhibits cell proliferation and cytokine production by inhibiting NF-κB target genes involved in the induction of these immune parameters.

Spelman *et al.* (2006) studied the modulation of cytokine expression by the traditional medicines *Acalypha wilkesiana, Acanthopanax gracilistylus, Allium sativum, Ananus comosus, Cissampelos sympodialis, Coriolus versicolor, Curcuma longa, Echinacea purpurea, Grifola frondosa, Harpagophytum procumbens, Panax ginseng, Polygala tenuifolia, Poria cocos, Silybum marianum, Smilax glabra, Tinospora cordifolia, Uncaria tomentosa*, and *Withania somnifera* which are the herbal immunomodulators. The in vitro and in vivo research demonstrates that the reviewed botanical medicines modulate the secretion of multiple cytokines. The reported therapeutic success of these plants by traditional cultures and modern clinicians may be partially due to their effects on cytokines. Phytotherapy offers a potential therapeutic modality for the treatment of many differing conditions involving cytokines.

Thakur *et al*. (2006) studied the immunomodulatory activity of *Chlorophytum borivilianum* Sant. *F* All the groups were immunized by injecting 20 ml of 5 · 109 SRBC per ml subcutaneously into the right footpad. After 14 days of treatment the thickness of

left footpad was measured. The mice were then challenged by injecting 20 ml of 5 · 109 SRBC per ml intradermally on the left hind footpad (time 0). Foot thickness was measured after 24 and 48 h of challenge. In the control group animals, after 48 and 72 h of challenge the DTH response was either equal or slightly more than the 0 h response; therefore, the peak edema after 24 h of challenge was the evaluating parameter. Ethanolic extract (200 mg/kg-1 per orally) was most effective ($P < 0.05$) compared to sapogenin (100 mg/kg-1 per orally) treatment in increasing the delayed-type hypersensitivity response.

Jagetia and Aggarwal (2007). Curcumin has been shown in the last two decades to be a potent immunomodulatory agent that can modulate the activation of T cells, B cells, macrophages, neutrophils, natural killer cells, and dendritic cells. Curcumin can also downregulate the expression of various proinflammatory cytokines including TNF, IL-1, IL-2, IL-6, IL-8, IL-12, and chemokines, most likely through inactivation of the transcription factor NF kappaB. Interestingly, however, curcumin at low doses can also enhance antibody responses. This suggests that curcumin's reported beneficial effects in arthritis, allergy, asthma, atherosclerosis, heart disease, Alzheimer's disease, diabetes, and cancer might be due in part to its ability to modulate the immune system. Together, these findings warrant further consideration of curcumin as a therapy for immune disorders.

CHAPTER- III

MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

The present study was conducted on 6-8 weeks old male and female ICR strain of mice. The mice were procured from Zydus Cadila Pharmaceuticals, Ahmadabad, Gujarat. All the mice were quarantined for one week prior to initiation of experiment. They were housed in cages at Laboratory Animal House facility of Veterinary College, Anand Agricultural University, Anand and maintained under controlled conditions of temperature (22±1°C), humidity (40-60%), and light (12/12-hour light/dark cycle).

All the protocols as per the CPCSEA Guidelines on the Care and Use of Laboratory Animals were followed and approved by the Institute's Animal Ethics Committee of Veterinary College, Anand. The animals were fed with standard rodent pellet and watered *ad libitum* throughout the course of the experiment. The mice were also examined daily for clinical change.

3.2 EXPERIMENTAL DESIGN

All the mice were randomly divided into eight groups, six treatment groups and two control group (C1, C2, T1, T2, T3, T4, T5 and T6.) each containing ten mice (five males and five females) from either sex. Apparent LD_{50} of permethrin (540 mg/kg) was taken into consideration for calculation of different dose groups (Khoda *et al*. 1979). Mice were treated with permethrin using three dose solutions defined as low-dose (LD50/40), middle dose (LD50/30), and high-dose (LD50/20). Corn oil was used as the control solution. All dosing solutions were kept away from direct exposure to sunlight.

The group C1 was administered Corn oil and served as vehicle control. The group C2 was administered ethanolic extract of *C.longa* rhizomes and served as plant control. Group T1 was given 1/40th of LD50 (13.5 mg/kg in 10 ml corn oil); group T2 was put on 1/30th of LD50 (18 mg/kg in 10 ml corn oil) and group T3 received 1/20th of LD50 (27 mg/kg in 10 ml corn oil) of permethrin suspended in corn oil. Group T4 was given permethrin at the dose rate of 1/40th of LD50 along with ethanolic extract of *C.longa* at the dose rate of 10mg/kg. Group T5 was given permethrin at the dose rate of 1/30th of LD50 along with ethanolic extract of *C.longa* at the dose rate of 10mg/kg. Group T6 was given permethrin at the dose rate of 1/20th of LD50 along with ethanolic extract of *C.longa* at the dose rate of 10mg/kg.

Permethrin in corn oil and ethanolic extract of *C.longa* was (gavaged intragastrically) administered directly in esophagus by using mice oral feeding needle with 1 ml BD syringe. The daily oral administration was continued for 28 days and the live weight was recorded before start of the experiment and thereafter at weekly intervals and mice were observed for any toxicity symptoms during entire period of experiment.

On 29th day of treatment mice were weighed and blood samples were collected from retriorbital plexus before final culling of mice for estimation of Hematological parameters (TLC and DLC), Serum biochemical (Total Protein, serum albumin, serum globulin,) and SRBC antibody titer by Heamagglutination. Mice were sacrificed and the weight of Spleen and Thymus was taken at necropsy for calculation of organ: body wt. ratio. These organs (Spleen and Thymus) were cleared from connective tissue, adipose tissue and then collected in 10% formalin for histopathological examination.

3.3 PREPARATION OF ETHANOLIC EXTRACT OF *CURCUMA LONGA*

The dried rhizomes of *Curcuma longa* were obtained from the Department of Medicinal and Aromatic Plants of Agricultural College of Anand Agricultural University, Anand. The rhizomes were powdered and stored in airtight containers. Ethanolic extract of the *Curcuma longa* was prepared by extracting rhizomes with absolute alcohol in Soxhlet extractor (yield 9.5% w/w). Extract so obtained was decanted in a beaker and then concentrated to $1/6^{th}$ of total volume in water bath. This was preserved in the refrigerator. This aqueous suspension of the extract was administrated to animals by oral gavage at the rate of 10mg/Kg of body weight.

IMMUNOLOGICAL STUDIES:

3.4. HEMATOLOGICAL PARAMETERS:

Blood samples for the hematological evaluation were collected in vials containing K_3 E.D.T.A on 29^{th} day of experiment before sacrifice of the mice. Total leukocyte count (TLC) was estimated as per the method described by Jain (1986). The parameters studied were Total Leukocyte Count (Nos./microliter) and Differential Leukocyte count (lymphocytes, granulocytes and monocytes) by Autoanalyser (Boule Medical Ab, Stockhol, Sweden).

3.5 BIOCHEMICAL STUDIES:

3.5.1 Serum Total Protein

Total Protein level in blood was estimated by standard kit (Merck System) using autoanalyzer (Selectra Junior Clinical Chemistry Analyzer) by Biuret method as described by Doumas (1975). Protein in an alkaline medium binds with cupric ions

present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of proteins present in the sample. Total Protein was expressed as g/dl.

Protein + Cu^{++} _____ Blue Violet Coloured Complex

3.5.2 Serum Total Albumin

Total albumin level in blood was estimated by standard kit from Core System on Photometer BT-224 autoanalyzer (Selectra junior clinical chemistry analyzer) by BCG method as described by Doumas (1975). Albumin present in serum binds with the dye bromocresol green in a buffered medium to form a green coloured complex. The intensity of the colour formed is directly proportional to the amount of albumin present in the sample. Total albumin was expressed as g/dl.

3.5.3 Serum Total Globulin

The total serum globulin was estimated by subtracting total albumin (g/dl) from total serum protein (g/dl). It was expressed as g/dl of serum.

3.6 ORGAN BODY WEIGHT RATIO:

Immune organs: body weight ratio was calculated in sacrificed mice at the completion (29^{th} days) of experimentation to know the effect of Permethrin and plant extract on whole body growth and growth of immune organs. O: BW ratio was calculated by dividing immune organ weight with body weight (gm) and multiplying with 100.

O: BW = Organ weight (g)/ Body weight (g) x 100

Thymus: Body weight (T: BW), Spleen: Body weight (S: BW) ratios were calculated.

3.7 ASSESSMENT OF HUMORAL IMMUNE RESPONSE:

3.7.1 Immunization

Sheep red blood cells (SRBCs) were collected in Alsever's solution, washed in large volumes of sterile 0.9% normal saline thrice and adjusted to a concentration of 5 X 10^9 cells per ml, were used for immunization. Animals were immunized by injecting 0.2 ml SRBC suspension intraperitonially 7^{th} days prior to sacrifice (on 16^{th} day of experiment). Blood was collected from retro orbital plexus under ether anesthesia on 29^{th} day and serum was separated from blood to determine the antibody titer by heamagglutination test.

3.7.2 Antibody titer

Antibody titre was estimated according to protocol of Puri *et al.* (1994). In brief, two fold dilutions of sera were made in 0.15 M Phosphate Buffer Saline (PBS) and aliquoted in "U" bottomed microtiter plates.1% SRBC suspended in PBS was dispended in each well and mixed thoroughly. The plates were incubated for 4 hrs at 37^0c and then observed visually for heamagglutination. The highest dilution of the test serum giving heamagglutination was taken as antibody titer.

3.8 ASSESSMENT OF CELL MEDIATED IMMUNE RESPONSE:

Cell mediated immune response was assessed by the method as described by Lagrange *et al.* (1974). All the animals were immunized by injecting 20 µl of 5 x 10^9 SRBC per ml subcutaneously into the right footpad on 19^{th} day of experiment. Then thickness of left footpad was measured using vernier calipers reading to 0.01 mm on 26^{th} day of treatment. (0 hrs). The mice were then challenged by injecting 20 µl of 5 x 10^9 SRBC per ml subcutaneously in the left hind foot pad (time 0). Foot thickness was

measured after 24 and 48 h of challenge. The difference between the thickness of left foot just before and after challenge in mm was taken as a measure of delayed type hypersensitivity (DTH).

3.9 POSTMORTEM EXAMINATION AND COLLECTION OF TISSUE SAMPLES

On 29th day of study all the mice from each control group C1 and C2 and all the mice from each treatment group were sacrificed. Mice, which died during the experiment, and the mice that were sacrificed 29th day of experiment were subjected to post mortem examination in the confined disinfected laboratory to determine the presence/absence of gross and histopathological conditions.

Post mortem necropsy finding were made by systemic approach (i.e. gross changes in organ size, shape and any visible lesions). Detailed post mortem lesions from all the mice were recorded. For gross (macroscopic) lesions thymus and spleen were examined after opening the body of sacrificed and died experimental mice. For histopathological examinations, tissues from spleen and thymus were collected in 10% formalin and preserved for processing.

3.10 HISTOPATHOLOGY

The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections were cut at 6-8 microns thickness with automatic section cutting machine (SLEE-MAINZ, Germany) and were stained with Haematoxylin and Eosin (H & E) stains (Luna, 1968). The H & E stained slides were observed under microscope and lesions were recorded.

3.11 STATISTICAL ANALYSIS

One-way-analysis of variance (ANOVA) was used to compare the effects of treatment of permethrin and *C.longa* extract along withy permethrin on different body weight, hematological, biochemical, and immunological variables in control and treated mice by using software SPSS (Version 12.1).

CHAPTER IV

RESULTS

The results of oral subacute Permethrin exposure at the dose rates of 13.5 mg/kg (Group T1), 18 mg/kg (Group T2) and 27 mg/kg (Group T3) body weight once daily and administration of *Curcuma longa* extract at dose rate of 10mg/kg along with different dose rate of Permethrin to inbred ICR mice daily for 28 days on clinical signs, body wt., hematology, serum biochemical, humoral immune response, cell mediated immune response, gross and histopathological changes, are given below.

4.1 CLINICAL SYMPTOMS

The mice were regularly and keenly observed throughout the experiment. No signs of toxicity were seen in mice of any groups up to second weak of experiment. After second week, clinical symptoms were observed in extremely toxic dose group i.e. T3 (27 mg/kg mg/kg body weight) of Permethrin. The most common signs, which observed were reduced feed intake, hyperactivity and hyperexcitability to external stimuli, and rough hair coat. Rough hair coat was most commonly observed in females. While in medium and low toxic dose groups i.e. in T2 (18 mg/kg body weight) and T1 (13.5 mg/kg body weight), clinical symptoms were in milder extent. The control groups (C1 and C2) did not show any visible clinical signs.

Mice of plant control group i.e.C2 group expressed increased appetite. Their hair coat was shiny and glossy. While mice of T4 and T5 did not show any observable signs. Although some mice of T6 have shown the decrease in feed and water intake.

Besides there was no mortality at all the dose levels of permethrin administration.

4.2 EFFECT ON BODY WEIGHT

There was insignificant effect on body weight up to end of experiment in permethrin treated groups as well as in those groups in which *C.longa* extract was given along with permethrin.

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract along with Permethrin in different doses on mean live body weight of ICR mice is presented in Table-1 and Figure-4.

TABLE 1: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Body Weight (g) in mice

	BODY WIGHT (MEAN±SE) (n=8)				
	0 DAY	**7 DAY**	**14 DAY**	**21 DAY**	**28 DAY**
C1	30.890±1.486	30.790± 1.461	30.760±1.474	30.710±1.482	30.58±1.146
C2	30.380±0.983	30.360±0.964	29.840±0.966	30.180±0.918	30.16±0.904
T1	31.440±1.547	31.630±1.545	31.550±1.523	31.410±1.502	31.23±1.448
T2	30.470±0.926	30.590±1.004	30.710±0.9930	30.580±0.9616	30.68±0.964
T3	31.360±1.356	31.530±1.372	31.470±1.390	31.390+1.328	31.38±1.305
T4	30.480±1.812	30.360±1.818	30.160±1.837	29.750±1.769	29.50±1.830
T5	31.360±1.404	31.390±1.398	31.440±1.406	31.410±1.411	30.97±1.406
T6	31.570±2.315	31.680±2.201	31.860±2.275	31.710±2.222	31.30±2.225

Values Indicate Mean ± S.E. (figures in the bracket indicate 'n')
Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

	O day		7 day		14 day		21 day		28 day	
Source of variation	DF	MS	DF	MS	DF	MS	DF	MS	DF	MS
Between group	7	2.465 ^{NS}	7	3.300 ^{NS}	7	3.939^{NS}	7	4.851^{NS}	7	4.168^{NS}
Error	72	23.60	72	23.06	72	23.59	72	22.56	72	22.47
Total	80		80		80		80		80	

NS: Non Significant**,***-Significant (P≤0.05)**, ****-Highly Significant (P≤0.01)

4.3 TOTAL LEUKOCYTE COUNT (TLC)

Effect of daily oral dosing of Permethrin in different doses and *C.longa* extract along with different dose of permethrin for 28 days to mice on TLC is presented in Table-2 and also depicted in Figure-5.

There was a dose dependent significant decrease in total leukocyte count in high dose and medium dose permethrin treated groups (T3) and (T2) where as a nonsignificant decrease was observed in low dose (T1) permethrin treated group in comparison to vehicle control group (C1). There was increase in TLC due to combined effect of permethrin and *C.longa* extract in (T5) and (T6) as compared to their respective permethrin treated group (T2) and (T3) and a significant increase in TLC was observed in the low dose permethrin and *C.longa* extract treated group (T4) as compared to its respective permethrin treated group (T1).

4.4 DIFFERENTIAL LEUCOCYTE COUNT

4.4.1 LYMPHOCYTES

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract along with Permethrin in different doses on Lymphocyte Count of ICR mice is presented in Table-3 and Figure-6.

There was dose dependent decrease in lymphocytes count in high dose and medium dose permethrin treated group (T3) and (T2) whereas nonsignificant decrease in low dose group (T1) in comparison to vehicle control group (C1). There was a significant increase in lymphocyte count in *C.longa* along with permethrin treated mice (T5 and T6) as compared to respective permethrin treated mice (T2 and T3), where as there was a nonsignificant increase in lymphocyte count in *C.longa* along with low dose permethrin treated mice (T4) as compared to its respective low dose permethrin treated mice (T1).

TABLE 2: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Total Leukocytes Count in mice.

GROUPS	TLC ($x10^3/\mu l$) (MEAN±SE) (n = 8)
C1	8.510±0.2664 [bc]
C2	9.030±0.4232 [c]
T1	7.240±0.6048 [ab]
T2	6.770 ±0.4782 [a]
T3	6.760±0.3859 [a]
T4	7.860±0.4483 [abc]
T5	6.800±0.6625 [a]
T6	6.770±0.3131 [a]

Values Indicate Mean ± S.E.
Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	8.076**
Error	72	2.164
Total	80	

NS: Non Significant**,** ***-**Significant (P≤0.05)**,** ****-**Highly Significant (P≤0.01)

TABLE 3: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Lymphocytes Count in mice.

GROUPS	LYMPHOCYTES ($x10^3/\mu l$) (MEAN±SE) (n = 8)
C1	4.92±0.194 [ab]
C2	5.24± 0.204 [b]
T1	4.75±0.214 [ab]
T2	4.50±0.221 [a]
T3	4.45±0.187 [a]
T4	4.80±0.175 [ab]
T5	4.52±0.101 [a]
T6	4.46±0.217 [a]

Values Indicate Mean ± S.E.
Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	.778 [NS]
Error	72	.371
Total	80	

NS: Non Significant**,** ***-**Significant (P≤0.05)**,** ****-**Highly Significant (P≤0.01)

4.4.2 GRANULOCYTES

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract along with Permethrin in different doses on granulocytes of ICR mice is presented in Table-4 and Figure-7.

Permethrin treated mice at different doses and *Curcuma longa* extract along with permethrin in different doses did not produce any significant change in the granulocyte count at different period of exposure.

4.4.3 MONOCYTES

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract along with Permethrin in different doses on monocyte count of ICR mice is presented in Table-5 and Figure-8.

Permethrin treated mice at different doses and *Curcuma longa* extract along with permethrin in different doses do not show any significant change in the monocyte count at different period of exposure.

TABLE 4: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Granulocytes in mice.

GROUPS	GRANULOCYTES ($x10^3/\mu l$) (MEAN±SE) (n = 8)
C1	2.690±0.1952 [a]
C2	2.780±0.2154 [a]
T1	2.720±0.2752 [a]
T2	2.340±0.2829 [a]
T3	2.390±0.2142 [a]
T4	2.940±0.2202 [a]
T5	2.470±0.1850 [a]
T6	2.550±0.2172 [a]

Values Indicate Mean ± S.E.

Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	0.430 [NS]
Error	72	0.520
Total	80	

NS: Non Significant**,*-**Significant (P≤0.05)**, ****-**Highly Significant (P≤0.01)

TABLE 5: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Monocytes in mice

GROUPS	MONOCYTES ($x10^3/\mu l$) (MEAN±SE) (n = 8)
C1	1.020±0.1381 [a]
C2	1.260±0.2642 [a]
T1	1.180±0.1340 [a]
T2	1.140±0.1454 [a]
T3	1.070±0.1317 [a]
T4	1.080±0.1083 [a]
T5	1.170±0.1752 [a]
T6	1.160±0.1648 [a]

Values Indicate Mean ± S.E.

Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	0.057 [NS]
Error	72	0.269
Total	80	

NS: Non Significant**, *-**Significant (P≤0.05)**, ****-**Highly Significant (P≤0.01)

4.5 BIOCHEMICAL STUDY

4.5.1 Serum Total Protein

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract along with Permethrin in different doses on serum total protein of ICR mice is presented in Table-6 and Figure-9.

The administration of permethrin exposure at different doses and *Curcuma longa* extract along with permethrin in different doses did not cause any significant change in the levels of serum total protein at different period of exposure.

4.5.2 Serum Albumin

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract (10mg/kg) along with Permethrin in different doses on serum albumin of ICR mice is presented in Table-7 and Figure-10.

The administration of permethrin at different doses and *Curcuma longa* extract along with permethrin in different doses did not cause any significant change in the levels of serum albumin at different period of exposure.

4.5.3 Serum Globulin

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract (10mg/kg) along with Permethrin in different doses on serum globulin of ICR mice is presented in Table-8 and Figure-11.

The administration of permethrin at different doses and *Curcuma longa* extract along with permethrin in different doses did not cause any significant change in the levels of serum globulin at different period of exposure.

TABLE 6: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Serum Total Protein (gm/dl)

GROUPS	SERUM TOTAL PROTEIN (gm/dl) (MEAN ± SE) (n = 8)
C1	6.740±0.2459 [a]
C2	7.030±0.1978 [a]
T1	6.580± 0.2375 [a]
T2	6.760±0.1240 [a]
T3	6.840±0.1809 [a]
T4	6.830±0.1844 [a]
T5	7.410±0.1865 [b]
T6	6.800±0.2033 [a]

Values Indicate Mean ± S.E.

Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	0.310 [NS]
Error	72	0.152
Total	80	

NS: Non Significant**,** ***-**Significant (P≤0.05)**,** ****-**Highly Significant (P≤0.01)

TABLE 7: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Serum Albumin (gm/dl).

GROUPS	ALBUMIN (gm/dl) (MEAN ± SE) (n = 8)
C1	5.48±0.131 [a]
C2	5.63±0.169 [a]
T1	5.44±0.082 [a]
T2	5.34±0.190 [a]
T3	5.34±0.177 [a]
T4	5.47±0.126 [a]
T5	5.62±0.115 [a]
T6	5.53±0.123 [a]

Values Indicate Mean ± S.E.

Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	0.122 [NS]
Error	72	0.205
Total	80	

NS: Non Significant**,** ***-**Significant (P≤0.05)**,** ****-**Highly Significant (P≤0.01)

TABLE 8: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Serum Globulin (gm/dl).

GROUPS	GLOBULIN (gm/dl) (MEAN ± SE) (n = 8)
C1	1.260± 0. 3056 [a]
C2	1.400±0.2333 [a]
T1	1.140± 0.2758 [a]
T2	1.420±0. 2215 [a]
T3	1.500±0.1738 [a]
T4	1.360±0. 2428 [a]
T5	1.790±0. 2558 [a]
T6	1.240±0. 2926 [a]

Values Indicate Mean ± S.E.
Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	0.394 [NS]
Error	72	0.641
Total	80	

NS: Non Significant**, ***-Significant (P≤0.05)**, ****-Highly Significant (P≤0.01)

4.6 Organ: Body Weight Ratio

4.6.1 Spleen: Body Wt. Ratio

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* (10mg/kg) along with Permethrin in different doses on Spleen to Body weight ratio of ICR mice is presented in Table-9 and Figure-12.

There was a dose dependent significant decrease in spleen to b.wt ratio in high dose and medium dose permethrin treated groups (T3) and (T2) in comparison to vehicle control group where as non significant effect was observed in low dose permethrin treated group (T1).

There was significant decrease in spleen to b.wt ratio in *C.longa* extract along with high dose permethrin treated group (T6) in comparison to respective permethrin treated groups where as a nonsignificant decrease in spleen to b.wt ratio was observed in *C.longa* extract along with low and medium dose permethrin treated group (T4) and (T5).

4.6.2 Thymus: Body Wt. Ratio

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract (10mg/kg) along with Permethrin in different doses on Thymus to Body weight ratio of ICR mice is presented in Table-10 and Figure-13.

There was significant decrease in thymus to b.wt ratio in different doses of permethrin treated groups (T1), (T2) and (T3) as well as *C.longa* extract along with different doses of permethrin treated mice in comparison to control mice.

TABLE 9: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Spleen to Body Weight ratio

GROUPS	SPLEEN: B.Wt RATIO (MEAN ± SE) (n = 8)
C1	0.3554±0.01373 [c]
C2	0.3419±0.01064 [c]
T1	0.3170±0.01972 [bc]
T2	0.2657±0.01902 [a]
T3	0.2476±0.01157 [a]
T4	0.3207±0.01123 [bc]
T5	0.2889±0.01699 [ab]
T6	0.2456±0.01198 [a]

Values Indicate Mean ± S.E.

Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source	DF	MS
Between treatment	7	0.018**
Error	72	0.002
Total	80	

NS: Non Significant**,** ***-**Significant (P≤0.05)**,** ****-**Highly Significant (P≤0.01).

TABLE 10: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Thymus to Body Weight ratio

GROUPS	THYMUS: B.Wt RATIO (MEAN ± SE) (n = 8)
C1	0.15340±0.014895 [b]
C2	0.16010±0.008820 [b]
T1	0.11830±0.005663 [a]
T2	0.11800± 0.004851 [a]
T3	0.11680±0.016827 [a]
T4	0.11520±0.010166 [a]
T5	0.11750±0.008423 [a]
T6	0.11600±0.006596 [a]

Values Indicate Mean ± S.E. (figures in the bracket indicate ‘n’)

Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	0.003*
Error	72	0.001
Total	80	

NS: Non Significant**,** ***-**Significant (P≤0.05)**,** ****-**Highly Significant (P≤0.01)

4.7 Effect on Humoral Immune Response

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract (10mg/kg) along with Permethrin in different doses on antibody titer against SRBC of ICR mice is presented in Table-11 and Figure-14.

There was a significant increase in the antibody titre in the plant control group as compared to the vehicle control group. There was a dose dependent significant decrease in the antibody titre in the high, medium and low dose permethrin treated group (T3), (T2) and (T1) as compared to control (C1). There was an increase in the antibody titre in the *C.longa* along with the high, medium and low dose of permethrin as compared to their respective permethrin treated groups.

TABLE 11: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Antibody Titer

GROUPS	ANTIBODY TITER (MEAN ± SE) (n = 4)
C1	256.00± 57.243[a]
C2	469.33±122.179[b]
T1	213.33± 26.985[a]
T2	202.67± 34.728[a]
T3	181.33± 34.728[a]
T4	416.00± 143.822[ab]
T5	384.00± 57.243[ab]
T6	341.67± 53.970[a]

Values Indicate Mean ± S.E.
Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	DF	MS
Between group	7	71472.762 [NS]
Error	40	36164.267
Total	48	

NS: Non Significant**, ***-Significant (P≤0.05)**, ****-Highly Significant (P≤0.01)

4.8 Effect on Cell- mediated Immune Response

The effect of subacute exposure of Permethrin in different doses and *Curcuma longa* extract (10mg/kg) along with Permethrin in different doses on cell-mediated immune response (Delayed Type Hypersensitivity) of ICR mice measured as increase in skin thickness, is presented in Tables-12 and Figure-15.

There was a nonsignificant increase in paw skin thickness of mice in permethrin treated groups after 24 and 48 hrs of challenge as compared to vehicle control group (C1). A significant increase in skin thickness was observed in plant control group (C2) in comparison to vehicle control group after 24 hrs of challenge A non significant effect was observed on skin thickness after 48 hrs of challenge. Similarly significant increase in skin thickness was observed in animals that received *C.longa* extract along with permethrin (T4), (T5) and (T6) in comparison to control animals. After 48 hrs of challenge there was non significant alteration in skin thickness of paw of all the animal.

TABLE 12: Effect of daily oral dosing of permethrin alone and *C.longa* extract along with permethrin on Cell mediated immunity in mice.

GROUPS	SKIN THICKNESS (mm) (MEAN ± SE) (n = 8)		
	0 hrs	24 hrs	48 hrs
C1	0.2780±0.02816	0.4160±0.02918 [a]	0.3790±0.02387
C2	0.2880±0.01902	0.5350±0.01522 [c]	0.3870±0.02539
T1	0.2660±0.02212	0.4200±0.03109 [ab]	0.3820±0.02220
T2	0.2780±0.02489	0.4390±0.03743 [ab]	0.3910±0.03335
T3	0.3100±0.02256	0.5110±0.01980 [bc]	0.4440±0.02713
T4	0.2840±0.03465	0.5400±0.01612 [c]	0.3900±0.03323
T5	0.2740±0.01784	0.5460±0.05554 [c]	0.3720±0.01843
T6	0.2740±0.01720	0.5550±0.01335 [c]	0.3840±0.01904

Values Indicate Mean ± S.E. (figures in the bracket indicate 'n')
Means having different superscripts at a particular period of treatment differs significantly (P≤0.05)

ONE WAY ANOVA

Source of variation	0 hrs		24 hrs		48 hrs	
	DF	MS	DF	MS	DF	MS
Between group	7	0.002 [NS]	7	0.036 **	7	0.006 [NS]
Error	72	0.006	72	0.009	72	0.007
Total	80		80		80	

NS: Non Significant, *-Significant (P≤0.05), **-Highly Significant (P≤0.01)

4.9 POST MORTEM EXAMINATIONS

4.9.1Gross Lesions

Detailed post-mortem examinations of all the mice of different groups were performed on day 29th of experiment. The common gross pathological lesions observed in spleen and thymuses were as under.

Spleen

Consistency of spleen was normal in all the groups but congestion was observed in mice given only permethrin at different doses. Congestion was more severe in T3 group as compared to T2 and T1 group. There was decrease in the size of spleen in the high dose permethrin treated group (T3). No observable lesions were found in T4 and T5, in T6 group slight congestion was seen and in C2 group spleen was found normal.

Thymus

Consistency of thymus was normal in all the treatment groups. Slight decrease in the size of the thymus has been observed in the high dose permethrin treated group (T3).

4.9.2 HISTOPATHOLOGICAL FINDINGS

Following Histopathological changes in spleen and thymus of mice in different groups were observed.

Group C1 (Normal control)

Group C1 were physiologically normal, so no microscopic changes could be observed in spleen (Figure- 16) and thymus examined (Figure- 24).

Group C2 (Plant control)

As group was of plant control mice so no microscopic changes could be observed in spleen (Figure-17) and thymus (Figure- 25).

Group T1

Very few microscopic changes were observed in spleen. In spleen there were some individual follicles showing depletion of lymphocytes at 29th day but relatively less in comparison to group T3 (Figure-18). No microscopic alterations could be found in thymus

Group T2

Spleen had some individual follicles showing congestion and mild to moderate depletion of lymphocytes but it was less as compared to group T3 (Figure- 19). No microscopic alterations could be found in thymus.

Group T3

Spleen had some focal areas showing moderate lymphoid depletion and congestion (Figure-20) and the thymus was showing slight hypocellularity in cortex region (Figure-26).

Group T4

Spleen had some focal areas showing depletion of lymphocytes (Figure-21). No microscopic alteration could be found in thymus.

Group T5

Mild to moderate depletion of lymphocytes were observed in lymphoid follicles (Figure- 22) No microscopic alterations could be found in thymus.

Group T6

Mild congestion and moderate lymphoid depletion were observed in spleen (Figure- 23) but no changes were seen in thymus of group T6.

CHAPTER V

DISCUSSION

Immune system is an excellent indicator of the overall health of an organism. Functioning of the immune system is delicately balanced to a complex array of cell-to-cell interactions, feedback mechanisms and amplification processes within biological systems. If alterations in the immune system occur, it is likely that there will also be alterations in other systems as well (Sharma and Reddy, 1987). In addition, very low levels of xenobiotic can induce immunotoxicity, often at concentrations much lower than those necessary to achieve target organ toxicity making it a very sensitive indicator of toxicity (Burns *et al.,* 1996). Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles. Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate pathophysiological processes and are hence called immunomodulatory agents. A large number of plant products are being investigated for immune response modifying activity. In light of these points, the effect of subacute permethrin exposure at the rate of 13.5mg/kg (GroupT1), 18 mg/kg (GroupT2), and 27 mg/kg body weight (GroupT3) once daily for 28 days on immune system and immunostimulatory effect of ethanolic extract of *Curcuma longa* if any on Permethrin induced immunotoxicity in ICR mice have been investigated in the present study.

Live body weight at weekly interval was measured to find out the effect of insecticide and *C longa* extract. Organ to body weight ratio of immune organs was measured to monitor the effect on growth rate of immune organs. TLC and DLC were

counted for the evaluation of effect of permethrin and *C.longa* extract on immune cells. Total protein, total globulin, total albumin and antibody titer against SRBC were measured to see the effect of this insecticide at various dose levels on development of humoral immunity and to see the effect of *C. longa* extract on permethrin induced changes in these parameters. Delayed Type Hypersensitivity response was evaluated against SRBC to monitor development of cell- mediated immunity. Histopathological (spleen and thymus) parameters were studied to evaluate the extent of toxicity on immune organs caused by permethrin at the administered doses and effect of *C. longa* extract on permethrin induced microscopic changes in immune organs like spleen and thymus of mice.

An approximate LD_{50} for permethrin utilized in the present study, was 540mg/kg of body weight of mice as reported by Khoda *et al* (1979). The oral LD_{50} of permethrin in male and female rats has been observed as 430 mg/kg and 470 mg/kg, respectively (Kohda *et al.,* 1979). The subacute oral LD_{50} of permetrin for rabbits was >4,000mg/kg (Parkinson *et al*., 1976). Millner and Butterworth, 1977 calculated acute oral LD_{50} of permethrin in Hen as 1500 mg/kg respectively. The variation in LD_{50} or LC_{50} found for permethrin may be due to the species variation or time of determination of LD_{50} or LC_{50}

In present study the body weight of mice was not affected by oral subacute permethrin exposure in any of the doses. This is because permethrin at given doses might not have caused significant effect on body weight. In *C.longa* extract treated groups also did not show any alterations in body weight at any of the doses have been observed which shows that the *C.longa* extract in dose given do not have effect on body weight.

Discussion

Punareewattana (1999) found that the body weight of mice was not affected by topical permethrin treatment.

Similarly feeding of endosulfan for 15 days to rats (Gupta and Chandra, 1977) and mice (Kannan, 1983) and 45 to 90 days feeding of famfur to rats (Black *et al.,* 1979) did not cause much change in body weight.

In contrast to no effect on body weight gain observed, a decrease in bodyweight gain due to some insecticides is also on record as observed by other workers.

Tanaka *et al*., (1967) reported reduced body wt gain in Wister rats maintained for 91 days on a diet containing 5000 mg/kg of tetramethrin. A slight to moderate decrease in body wt gain in wistar rats which received 500, 1500, 5000 or 10000 mg/kg dietary level of bioallethrin for 90 consecutive days has been observed (Wallwork *et al*.,1972). Bioresmehrin administration to rats at 2000 mg/kg for three weeks produced slight reduction in body wt (Glomot, 1975). Reduction in body wt gain in rats fed 1600 mg/kg cypermethrin for three months was reported by Hend and Butterworth, 1976. Cypermethrin at 200 mg/kg when applied topically for six hours a day for 13 weeks reduced body wt gain in New Zealand white rabbits (Henderson and Parkinson, 1978). Rats fed 50 mg/kg deltamethrin gained less wt than control (Goldenthal *et al.*, (1980b). Cabral *et al*., (1986) reported slight decrease in body wt when deltamethrin was administered upto 8 mg/kg.

Pickering, (1982) reported decrease in feed intake and body wt gain when alphacypermethrin was fed at a 400 or 800 mg/kg. Clark (1982) reported decreased growth of rats which was correlated with decreased feed intake in both male and female Wister rats fed with 540 mg/kg alphacypermehrin. A slight decrease in body wt was

reported when deltamethrin was administered in rats upto 8 mg/kg body wt for 104 weeks (Cabral *et al*., 1986). Many workers observed cumulative toxic effect of cypermethrin; it was adversely affecting body weight of poultry (Jabeen, 1984; Quadri *et al.,* 1987; Mohamood and Siddiqui, 1993). Thaker *et al,* (1996) reported decrease in live body weight gain on feeding of endosulfan at the rate of 1.21 mg/kg and 2.42 mg/kg and malathion at the rate of 9.04 mg/kg and 18.08 mg / kg for 180 days to WLH chicks. A significant decrease in the body weight in cockerels was observed following prolong feeding of fenvalerate medicated ration at the rate of 4000 ppm. (Singh *et al.,* 2001). Administration of 15000-mg/kg cypermethrin for 13 weeks showed diminished feed intake in beagle hound dogs (Buckwell and Butterworth, 1977). A reduction in body wt gain was observed in male and female dogs of 25 week age receiving upto 10 mg/kg deltamethrin for 13 weeks (Chesterman *et al.,* 1977). Hart, (1975) reported a decrease in body weight and feed consumption in adult beagle dogs fed fenvalerate upto 12.5-mg/kg body wt for 90 days.

In the present study administration of ethanolic extract of *C .longa* did not show any alteration in body weight in plant control group and also in treatment groups even if increase in appetite has been observed in plant control group. This shows that *C .longa* does not have any effect on the body weight at given rate of 10mg/kg in mice. In contrast to this it was found that supplementation of shatavari root powder in birds, have beneficial effects on body weight (Rekhate *et al.* 2004). Ziauddin *et al.,* (1996) showed weight gain following administration of Ashwagandna (*Withania somnifera*) in mice in which myelosuppression was induced by one or more of the three compounds viz. cyclophosphamide. azathioprin, or prednisolone. AL-Sultan (2003) observed the

performance in broiler chickens following feeding of *Curcuma longa* and found that there was increased body weight.

In the present study there was a dose dependent significant decrease in total leukocyte count in high dose and medium dose permethrin treated groups (T3) and (T2) where as a nonsignificant decrease was observed in low dose (T1) permethrin treated group in comparison to vehicle control group (C1). There was increase in TLC due to combined effect of permethrin and *C.longa* extract in (T5) and (T6) as compared to their respective permethrin treated group (T2) and (T3) and a significant increase in TLC was observed due to combined effect of permethrin and *C.longa* extract in (T4) as compared to its respective permethrin treated group (T1) indicating the immunostimulant effects of plant over the administered doses of permethrin as far this parameter is concerned. Similarly there was dose dependent significant decrease in lymphocytes count in high and medium dose permethrin treated groups (T3) and (T2) where as a nonsignificant decrease was observed in low dose group (T1) in comparison to vehicle control group (C1). There was a significant increase in lymphocyte count in *C.longa* along with permethrin treated mice (T5 and T6) as compared to respective permethrin treated mice (T2 and T3), where as there was a nonsignificant increase in lymphocyte count in *C.longa* along with low dose permethrin treated mice (T4) as compared to its respective low dose permethrin treated mice (T1).

But no significant effect was found on granulocytes and monocytes in present study.

As observed in the present investigation decrease in TLC has also been observed following carbaryl and malathion administration in chickens (Kakkar *et al.*, 1993);

lindane in lambs (Khurana *et al.*, 1996c); carbofuran in lambs (Khurana *et al.*, 1998); permethrin in mice (Shah and Gupta, 1998); fenvalerate in lambs (Khurana and Chauhan, 2000); cypermethrin in chickens (Khurana *et al.*, 2000); monocrotophos in sheep (Khurana and Chauhan, 2001) and butachlor in chickens (Kumar *et al.*, 2002).

Thaker (1988) did not observe any significant alterations in haemoglobin; PCV, DLC, TLC and Total RBC count in male WHL chicks by long term daily oral administration of endosulfan and Malathion. The fenvalerate causes a significant decrease in the TEC, Hb and PCV level as observed by Singh *et al.* (2001c). Subacute exposure of imidacloprid and quinalphos in 8 to 10 week old WLH cockerels did not affect packed cell volume and hemoglobin levels (Siddiqui, 2004). The consequence of insecticide on different haematological parameters has been a matter of controversy. Short term endosulfan exposure on haematology in male rat has shown decreased total erythrocytic count, packed cell volume and hemoglobin content, where as total leukocytic count was found increased significantly (Choudhary and Joshi, 2002).

There is paucity of literature on immunomodulatory studies of *C.longa* extract but various other plants have been reported to cause immunomodulation in animals. Methanol extract and remaining methanol fraction of *Sphaeranthus indicus Linn* were found potent in protecting against cyclophosphamide-induced myelosuppression as evidenced by increasing significantly the levels of total WBC count in swiss albino mice (Bafna and Mishra, 2004).

Similarly, administration of ethanolic extracts and sapogenin extract of *Chlorophytum borivilianum* had beneficial effect on hematological profile wherein WBC counts along with hemoglobin, platelets and RBC were increased in rats. Simultaneous

administration of ethanolic extract and sapogenin extract along with azathioprine resulted in restoration of suppressed values observed after azathioprine treatment alone. Platelet, hemoglobin, RBC and WBC values observed were better than untreated control groups. Among the two test drugs ethanolic extract was pronouncedly more effective on WBC followed by sapogenin treatment (Thakur *et al.,* 2006). Similarly significant reduction in white blood cell counts was observed in animals treated with cyclophosphamide. Cyclophosphamide induced myelosuppression was counteracted by methanol extract of *Cissampelos pareira* in dose dependent manner with increase in levels of WBC compared to cyclophosphamide group. Higher doses i.e. 400 and 800 mg/kg could bring the levels of WBC to normal (Bafna and Mishra, 2005).

In the present study permethrin exposure at different doses and *Curcuma longa* extract along with permethrin in different doses did not cause any significant change in the levels of total protein, globulin and albumin at different period of exposure. The reason for this could be the selection of dose level could be low to produce any change on protein metabolism.

Similar to this, Shah and Gupta (2001) conducted subacute toxicity studies of permethrin in young albino male rats. Daily oral administration of permethrin at the rate of 24-120 mg/kg for 30 days showed non-significant changes in the level of total proteins. This shows that at all dose levels permethrin did not cause any appreciable change in both primary and secondary immune response and total proteins, albumin, and globulin concentrations. Similarly no effect was observed on plasma protein and albumin in adult male rats following I.P. administration of permethrin at the rate of 20 and 40 mg/kg daily for 28 days (Premlata *et al.* 2006), where as a significant depression in serum

total proteins, serum globulins and serum gamma globulins was observed when day old broiler chicks were fed endosulfan for 8 weeks (Khurana *et al*. 1996b). A reduction in serum globulins is also observed in poultry birds exposed to low doses of carbaryl (60 ppm) for 8 weeks (Kakkar *et al*. 1993). It was found that the fenvalerate at the rate of 4000 ppm significantly decreased serum albumin level (Singh *et al.* 2001c). There was a significant suppression in serum globulins and gamma globulins in one week old chicks fed quinalphos at the rate of 8 ppm for 2 months (Garg *et al*. 2002).

Laboratory animals when given *Asparagus racemosus* Wild and *Withania somnifera* Dunal root extract with a standard drug, there was a significant increase in the total carbohydrate and total carbohydrate/protein ratio. (Bhatnagar *et al.* (2005)

Rekhate *et al.* (2004) also reported increase in serum total protein and globulin following supplementation of aqueous extract of shatavari (*Asparagus racemosus*) root powder in broilers.

The result of the present study showed significant reduction in spleen to body weight ratio in the high dose permethrin alone and *C.longa* extract along with high dose permethrin treated group. Similar decrease in splenic weight was recorded in both rats and chicks fed cypermethrin and carbaryl (Varshneya *et al.* 1992 and Bhushan 1993). An increase in liver weight and decrease in spleen and thymus wt was observed in the rats fed with 4000 mg/kg bioresmethrin for 91 days (Wallwork and Malone, 1971). Significant decrease in wt. of bursa of fabricius and thymus atrophy has been found in birds given increasing doses of endosulfan in feed for 11 weeks (Kurkure *et al*., 1993). . Both spleen and thymus weights were dose-dependently decreased by the exposure of short-term atrazine in young male C57BL/6 mice (Nikolay *et al.,* 2005). Size and wt. of

lymphoid organs- spleen, thymus and Bursa of Fabricius were found to be reduced in broiler chicks fed cypermethrin @100 ppm for 8 weeks (Khurana *et al*., 1996a). Following exposure to carbaryl by oral, nasal and dermal routes, there was a dose related decrease in thymus wt. and spleen wt. (Ladies *et al*., 1994).

The spleen to body wt. ratio was decreased significantly in mice exposed to 50 ppm malathion for 12 weeks or 100 ppm for 8 weeks and 12 weeks (Bannerjee *et al*., 1998). Similar reduction in spleen to body wt. ratio was observed following acephate exposure in white leghorn cockrels. (Tripathi, 2006).

A decrease in spleen: body wt. ratio at the highest dose suggests that permethrin might be immunotoxic at higher doses used in the present study. Decrease in spleen: body wt. ratio was supported by histopathological observation indicating depletion of lymphocytes in individual splenic follicles.

The result of the present study showed significant decrease in thymus to b.wt ratio in high dose permethrin treated group (T3) in comparison to vehicle control group where as a non significant effect was observed in medium and low dose group of permethrin treated groups.

Intraperitoneal administration of permethrin or deltamethrin results in a calcium/calmodulin-dependent alteration of the protein kinasephosphatase cascade, leading to increased apoptosis of thymocytes and resulting thymic atrophy (Rashatwar and Matsumura 1985, Enan *et al.* 1996)

Topical application of permethrin in four-to-five-week-old female C57BL/6N mice caused a dose-related decrease in thymic cellularity. The CD4+ and CD8+

thymocyte subpopulation was most severely diminished, suggesting possible chemical-induced apoptosis as a mechanism leading to thymic atrophy. (Prater *et al.,* 2002)

Significant reduction in thymus: body weight ratio was observed extensively in high dose acephate treated group (T3) as compared to control groups due to subacute exposure of acephate in cockerels (Tripathi, 2006). Similarly, decrease in thymus: body wt. ratio has been observed due to polychlorinated biphenyls (PCB) in broiler chickens (Kozutsky and Skrobanek, 1994). The significant reduction in thymus: body wt. ratio in the present study might be due to the thymic hypoplasia caused by exposure of permethrin at higher dose level.

In present study significant decrease in thymus to body weight ratio was observed in animals that were given *C.longa* extract along with high dose permethrin treated group (T6) in comparison to plant control group as well as the respective high dose permethrin treated group, where as a nonsignificant effect was observed in *C.longa* extract along with low and medium dose treated permethrin groups (T4) and (T5). There was significant increase in spleen to body weight ratio and thymus to body weight ratio in plant control group but not in the plant extract and permethrin treated group which shows that *C.longa* has no reversal effect on permethrin induced adverse effect on spleen leading to decrease in spleen to body weight ratio. On contrary Spleen to body ratio was also increased by *Curcuma longa in* broiler chickens (AL-Sultan, 2003) and the thymus: body weight ratio was also increased to the *Trigonella foenum graecum* in swiss albino mice (Bin-hafeez et *al.,* 2004)

In the present study, antibody titres against SRBC were measured by heamagglutination test to monitor humoral immune response. The results indicated that

there was a significant increase in the antibody titre in the plant control group as compared to the vehicle control group. There was a dose dependent significant decrease in the antibody titre in the high, medium and low dose permethrin treated groups (T3), (T2) and (T1) and *C.longa* along with permethrin treated groups (T4, T5 and T6) as compared to control (C1). It definitely indicates that permethrin has produced adverse effects on immunity production in mice even in presence of extract of *C.longa*.

Recent studies have demonstrated that low dose sub acute topical exposure (10 days) to permethrin causes diminished antibody-mediated immunity in mice (Desi *et al.* 1985) as well as oral cypermethrin decreases antibody production in both rats and mice (Desi *et al.* 1985, Tamang *et al*. 1988, Punareewattana *et al*. 2001). By giving deltamethrin in two daily oral doses; 6 mg/kg for 84 days and 15 mg/kg for 14 days significantly decreased the humoral immune response in BALB/c mice immunized with sheep red blood cells (Lukowicz-Ratajczak and Krechniak 1992). Permethrin did not alter the primary and secondary humoral response at doses 30-60 mg/kg/day (Shah and Gupta 1998).

Single exposure to permethrin resulted in inhibited antibody production by splenic cells in mice (Prater, 2003). Cypermethrin at the dose of 0 (control), 3 mg/kg, 9 mg/kg and 30 mg/kg for 28 days did not show any significant variation in the Ab titer in low, medium and high dose treated group as compared to control group. Total immunoglobulin (TIG) values, Phagocytic index in cypermethrin treated groups in both male and female rats did not differ significantly ($P<0.05$) from that control group. (Suhash *et al.* (2004)

Discussion

The various groups of pesticides have been found to cause an immunosuppressive effect in birds and animals. Suppression of humoral immune response was observed by chlorpyriphos in chickens (Malik *et al.,* 2005). There was a decrease in anti-SRBC humoral response 10 days after a single oral exposure to 1/4-1/6 LD_{50} aminocarb in mice. (Bernier *et al*., 1988)

Lindane suppressed both primary and secondary antibody responses to SRBC in mice (Bannerjee *et al*. (1996a). Humoral immune system is adversely affected in calves fed alphamethrin 5 mg/kg body wt. for a period of 4 months which was indicated by decrease in ELISA values of Brucella specific antibodies IgG, IgM (Chauhan, 1998)

In the present study results also indicated that there was a increase in the antibody titre against SRBC in the *C.longa* along with the different doses of permethrin compared to their respective dose of permethrin suggesting that *C.longa* extract has a stimulatory effect on antibody production in mice. Similarly, administration of methanol extract of *Sphaeranthus indicus* and its fractions produced increase in humoral antibody titre as evident by haemagglutination at that dilution. Statistically significant levels were obtained with methanol extract and its petroleum ether, chloroform and remaining methanol fraction at both the dose level (Bafna and Mishra, 2004). HA titre did not show any significant change with 150 mg/kg/day of *Ashtamangal Ghrita* (polyherbal formulation*)* administration. However, a significant increase was observed at a dose of 300 mg/kg/day with almost a five fold increase as compared to untreated control animals. The augumentation of the humoral response as evidenced by an enhancement of antibody responsiveness to SRBC in rats as consequence of both pre and post-immunization drug

treatment indicates the enhanced responsiveness of macrophages and B-lymphocyte subsets involved in antibody synthesis (Fulzele *et al.,* 2002).

In this study DTH response was checked by increased footpad thickness using digital vernier calipers. There was a nonsignificant increase in paw skin thickness of mice in permethrin treated groups after 24 and 48 hrs of challenge as compared to vehicle control group (C1). Significant increase in skin thickness was observed in plant control group (C2) in comparison to vehicle control group after 24 hrs of challenge A non significant effect was observed on skin thickness after 48 hrs of challenge. Similarly significant increase in skin thickness was observed in animals that received *C.longa* extract along with permethrin in comparison to vehicle control group as well as only permethrin treated groups respectively, after 24 and 48 hrs of challenge.

This result suggests that permethrin may be toxic in regards to cell mediated immune response at dose rate administered in this study. The administration of *C.longa* extract along with permethrin does not protect the mice from toxic effects of permethrin on the cell mediated immunity.

In contrast to this significant effect of aldicarb on immune system was observed when mice received water containing aldicarb for 34 days, while chlorpyriphos (Malik *et al.,* 2005), cypermethrin (Khurana *et al*., 2000) cause suppression of CMI in chickens. In an *in vitro* study (Stelzer and Gordon, 1984), permethrin at a concentration of approximately 10^{-5} to 10^{-6} M inhibited the mitogenic response of murine immune lymphocytes. In an *in vivo* study by Blaylock *et al.* (1995), in which mice were orally exposed to permethrin shown to have inhibited cellular immune responses Single exposure to permethrin resulted in inhibited antibody production by splenic cells in mice.

Discussion

Five day exposure to permethrin caused persistent decreased contact hypersensitivity responses (Prater, 2003). At 120 mg/kg/day, permethrin significantly reduced the cell-mediated immune response. Cypermethrin significantly depressed the cell-mediated immunity in treated rats as compared to control, which indicated that cypermethrin, suppressed cell mediated immunity. (Tulinska *et al.* 1995).

Administration of methanol extract *Sphaeranthus indicus Linn* and its fractions produced increase in thickness of footpad of mice as a measure of DTH response. Statistically significant increase in DTH response in dose dependent manner by remaining methanol fraction (0.670 ± 0.050 and 0.810 ±0.037) and petroleum ether fraction (0.470 ± 0.050 and 0.510 ± 0.037) was obtained. Benzene fraction slightly reduced the DTH response by reducing thickness of footpad as compared to control group. Methanol extract and chloroform fraction also showed increased DTH response compared to control group (Bafna and Mishra, 2004).

The DTH response, which is a direct correlate of cell mediated immunity (CMI), was found to be increased by 30% at a dose of 300 mg/kg/day of the *Ashtamangal Ghrita* (polyherbal formulation*)*. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavanger cells to the site of reaction (Fulzele *et al.,* 2002). Similarly ethanolic extract of *Chlorophytum borivilianum* (200 mg/ kg per orally) was found to be significantly increasing the delayed-type hypersensitivity response in mice (Thakur *et al.,* 2006). Similarly methanol extract of *Cissampelos pareira* when administered orally showed a linear dose dependent increase in DTH response up to 400 mg/kg, however

statistically significant increase could obtained at 200 and 400 mg/kg doses. At 800 mg/kg DTH response was slightly reduced (Bafna and Mishra, 2005).

DTH is antigen specific and causes erythema at the site of antigen infection in immunized animals. The histology of DTH can be different for different species, but the general characteristics are an influx of immune cells at the site of injection, macrophages and basophils in mice and induction becomes apparent within 24-72 hours. T-cells are required to initiate the reaction (Waksman, 1979; Poulter *et al*., 1982). Increase in the DTH response indicates that plant has a stimulatory effect on lymphocytes and necessary cell types required for the expression reaction.

In the present study no significant gross changes were observed in thymus while spleen showed minor lesions like paleness and reduction in size in some mice of high dose permethrin treated group. Spleen showed depletion of lymphocytes in spleenic follicles while thymus showed the slight hypocellularity in the cortex region. The lesions were principally observed in the highest treatment groups and indicated that higher dose may produce significant signs of toxicity in the immune organs like spleen and thymus.

Topical application of permethrin (25 µl, equivalent to 1100 mg/kg b.wt) in mice caused 32% inhibition of splenic T-cell proliferation. Apoptosis was significantly increased in CD4 (-) 8(-) and CD4 (-) 8(+) thymocytes, and the CD4 (+) CD8 (+) thymocyte subpopulation was most severely diminished, suggesting a possible chemical-induced apoptotic mechanism of thymic atrophy. Permethrin also caused splenic hypocellularity by 31% at 15 µl (660 mg/kg bw), and by 50% at 25 µl (1100 mg/kg bw), an effect that may relate to inhibited proliferation or reduced seeding from the hypocellular thymus (Prater, 2002).

Similarly, depletions in lymphocytes in focal areas in bursa of Fabricius and spleen have been observed by Khurana *et al.* (1996a) in broiler chicks. Cha *et al.* (2000) observed depletion of spleen lymphocytes in the periarteriolar lymphoid sheath and marginal zone in white pulp. However, no changes could be traced in the thymus. Besides, some workers failed to demonstrate any histopathological changes following feeding of famphur (Black *et al.,* 1979) and aldicarb (Thomas and Ratajczak, 1990) in rats. It could be due to low doses or short duration of exposure.

Results indicated that permethrin was found to be immumnotoxic at the dose level tested .The results of present study clearly indicated that following oral administration of permethrin in different doses to mice for 28 days, various physiological parameters of the mice were affected and a state of stress (mild in nature) was produced in the mice. However still more parameters need to be evaluated following the exposure of permethrin to establish animal health hazards and to evolve guideline for acceptable residues in environment. More studies with multiple parameters on dose time relationship in various other species of animal will help in completing the permethrin toxicity on biological systems.

The present investigation therefore also reveals that *C.longa* extract possess immunomodulatory properties but do not protect against the permethrin induced immunotoxicity at different doses. The exact mechanism of action, however, could only be unfolded after detailed characterization of active moieties from different fractions. Further studies are warranted for the understanding the exact mechanisms responsible for immunostimulation by *C.longa* extract in permethrin induced toxicity.

CHAPTER VI

SUMMARY AND CONCLUSIONS

6.1 SUMMARY

The present investigation on permethrin induced immunotoxicity and its reversal by ethanolic extract of *Curcuma longa* was conducted on 6-8 weak old ICR mice. All the mice were randomly divided into eight groups (C1, C2, T1, T2, T3, T4, T5 and T6) each containing ten mice and treatments were given once daily orally for 28 days .The group C1 was administered corn oil and served as vehicle control. The group C2 was administered ethanolic extract of *Curcuma longa* rhizomes at the dose rate of 10 mg/kg and acted as plant control. Group T1 was given $1/40^{th}$ of LD50 (13.5 mg/kg), group T2 was put on $1/30^{th}$ of LD50 (18 mg/kg) and group T3 received $1/20^{th}$ of LD50 (27 mg/kg) of permethrin suspended in corn oil. Group T4 was given permethrin at dose rate of $1/40^{th}$ of LD50 (13.5 mg/kg) along with ethanolic extract of *Curcuma longa* rhizomes at the dose rate of 10 mg/kg. Group T5 was given permethrin at dose rate of $1/30^{th}$ of LD50 (18 mg/kg) along with ethanolic extract of *Curcuma longa* rhizome at the dose rate of 10 mg/kg. Group T6 was given permethrin at dose rate of $1/20^{th}$ of LD50 (27 mg/kg) along with aqueous extract of *Curcuma longa* rhizome at the dose rate of 10 mg/kg.

The mice were regularly and keenly observed throughout the experiment and weekly live body weight was recorded. After 28 days of experiment blood samples were collected from retro orbital plexus of all the mice and various hematological, biochemical and immunological parameters were determined to study the effect of permethrin and *Curcuma longa* on immune system. Spleen to body weight ratio and thymus to body weight ratio was measured to monitor the effect on growth rate of spleen and thymus.

Summary and Conclusions

Live body weight at weekly interval was measured to find out the effect of permethrin and *Curcuma longa* on the growth rate. TLC and DLC were estimated to see the effect of permethrin and *Curcuma longa* on hematological picture. Total protein, total albumin, total globulin and antibody titre against SRBC were measured to see the effect on humoral immunity while DTH was evaluated by footpad thickness against SRBC challenge to monitor development of cell- mediated immunity. Immune organs (spleen and thymus) of sacrificed mice were weighed to calculate organ to body wt. ratios for measuring over all effect of permethrin and *Curcuma longa* on the immune system. Histopathological studies of spleen and thymus were carried out to evaluate the extent of organ toxicity caused by the permethrin at the administered doses and reversal effect of *Curcuma longa* extract.

Severity and extent of the clinical signs varied according to dosage administered to the mice. The most common clinical signs, which observed were reduced feed intake, hyperactivity and hyperexitability to external stimuli, and rough hair coat, while mice of vehicle control group did not show any visible clinical signs.

There was no significant effect on body weight of the permethrin and *C.longa* treated mice at doses administered. TLC and lymphocyte showed significant decrease in permethrin treated mice whereas a non significant effect was observed on, granulocytes, and monocytes,

The total protein, total albumin and total globulin levels were showing nonsignificantly changed. Permethrin caused a significant decrease in the antibody titre of treated mice against SRBC. A nonsignificant increase in paw skin thickness of mice

was observed in permethrin treated groups after 24 and 48 hrs of challenge which shows that cellular immunity was also affected as tested by injecting SRBC in footpad.

Gross and histopathological changes were observed in high dose group. A significant decrease in spleen: body wt. ratio and thymus to body weight ratio was observed in high dose permethrin treated group. Mild to moderate depletion of lymphocytes in some focal areas of splenic parenchyma was also seen and thymus was showing a slight hypocellularity in the cortex region at higher dose of permethrin.

6.2 CONCLUSIONS

Following conclusions could be drawn from the present study.

1. Though permethrin has been reported moderately toxic to the mice, in the present study it seems to be toxic for immune systems in mice at the doses administered.
2. At the dose levels used in this study; the insecticide caused no effect on weight gain as compared to control groups indicating lack of direct toxicity or stressogenic activity of permethrin at given doses.
3. Since permethrin caused significant decrease in total leukocyte count and lymphocyte count it suggest that it has toxic influence on the haemopoetic system at the dose levels employed in present study.
4. No effect on serum total proteins and albumin during experimentation indicate that the insecticide did not affect the protein metabolism at given doses. Globulin was not affected at doses of permethrin administration indicating lack of any effect on immunoglobulin production.

5. Antibody titre against SRBC was dose dependently decreased in the permethrin treated groups indicating significant adverse effect on humoral immunity by permethrin at doses administered.
6. Cell mediated immune response was showing a nonsignificant change in permethrin treated groups when compared to vehicle control group.
7. Mice given *C.longa along* with permethrin at different doses did not show any alterations in body weight in plant control group and also in treatment groups, There was significant increase in TLC due to combined effect of permethrin and *C.longa* extract but non significant effect was seen on differential leukocyte count, total serum protein and total serum globulin.
8. Mice given *C.longa* extract showed a significant increase in antibody titre against SRBC in plant control group and an increase in titre was also observed in *C.longa* along with the different doses of permethrin treated groups as compared to their respective dose of permethrin.
10. Mice given *C.longa* extract showed a significant increase in the skin thickness in plant control group as well as in *C.longa* along with the different doses of permethrin treated groups.
11. Mild microscopic changes have been observed in the immune organs namely spleen and thymus in the high dose of permethrin treated group. Spleen was showing mild depletion of lymphocytes and thymus showed a slight hypocellularity in the cortex region. This indicates that cellular toxicity caused due to permethrin was mild at the selected doses of permethrin.

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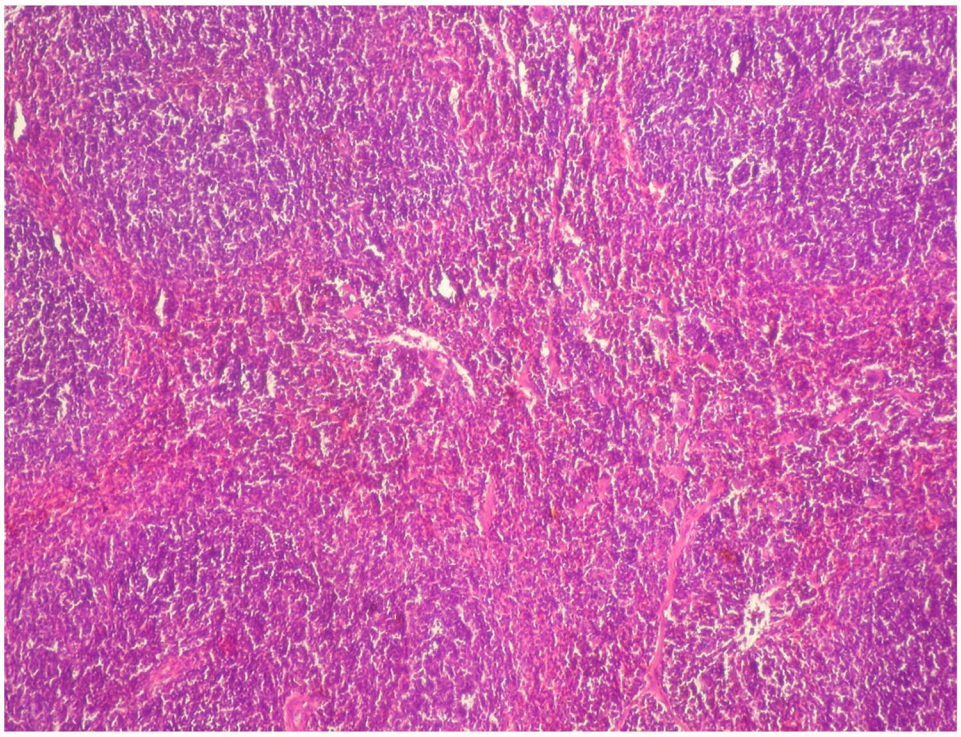

Fig -16: Section showing normal Spleen (Group C1) H & E Stain X 60.

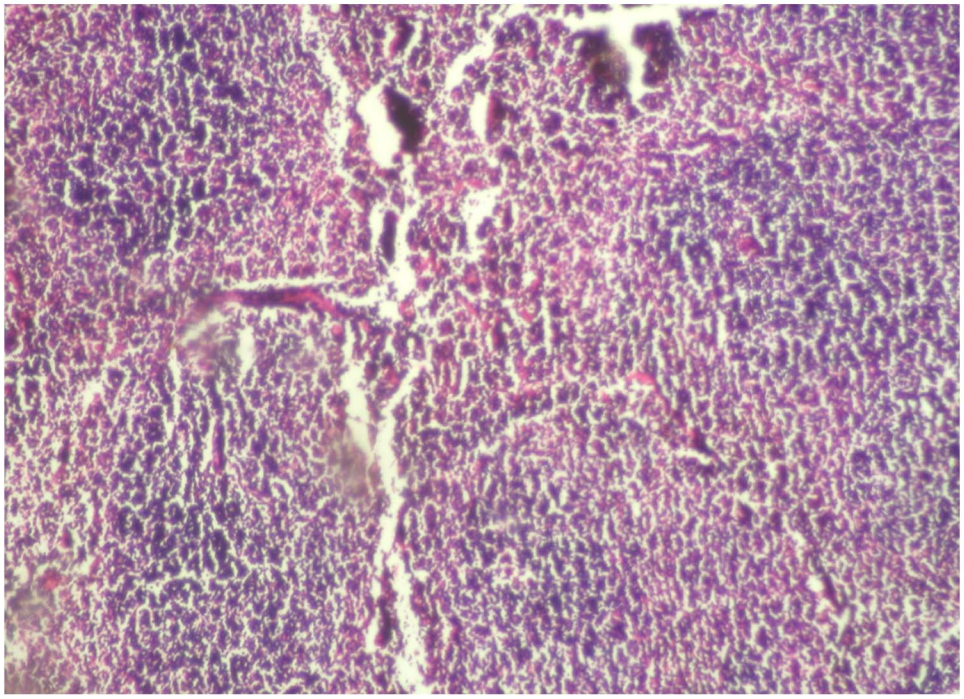

Fig -17: Section showing normal Spleen (Group C2) H & E Stain X 60.

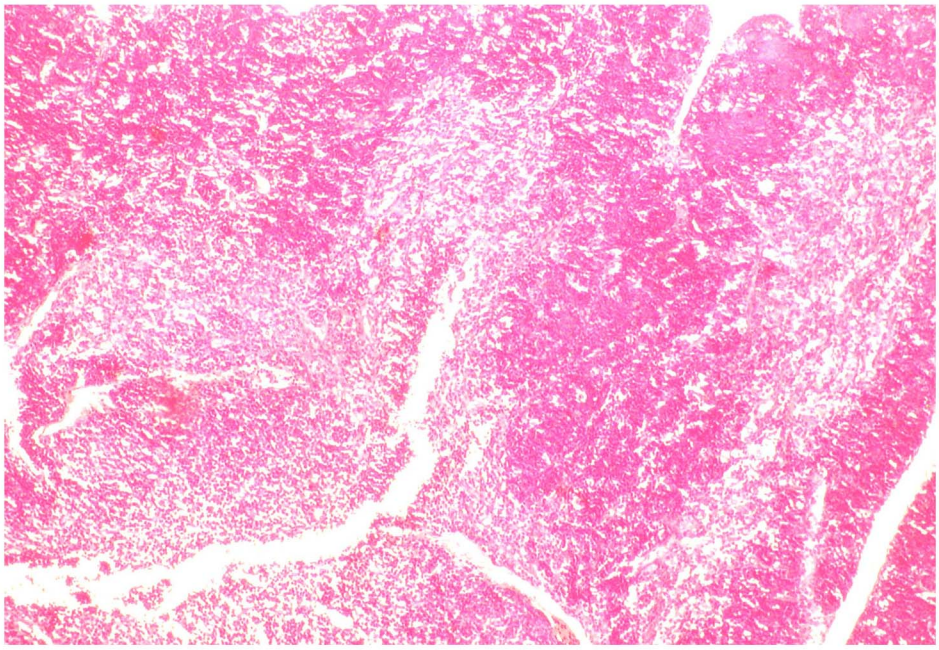

Fig -24: Section showing normal Thymus (Group C1) H & E Stain X 60.

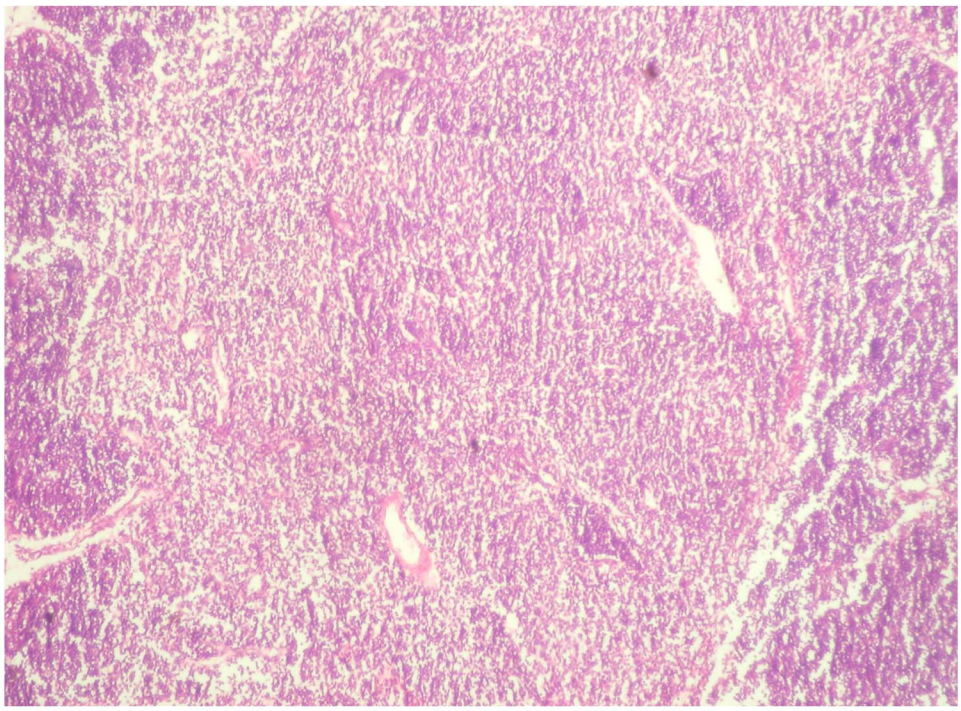

Fig -25: Section showing normal Thymus (Group C2) H & E Stain X 60.

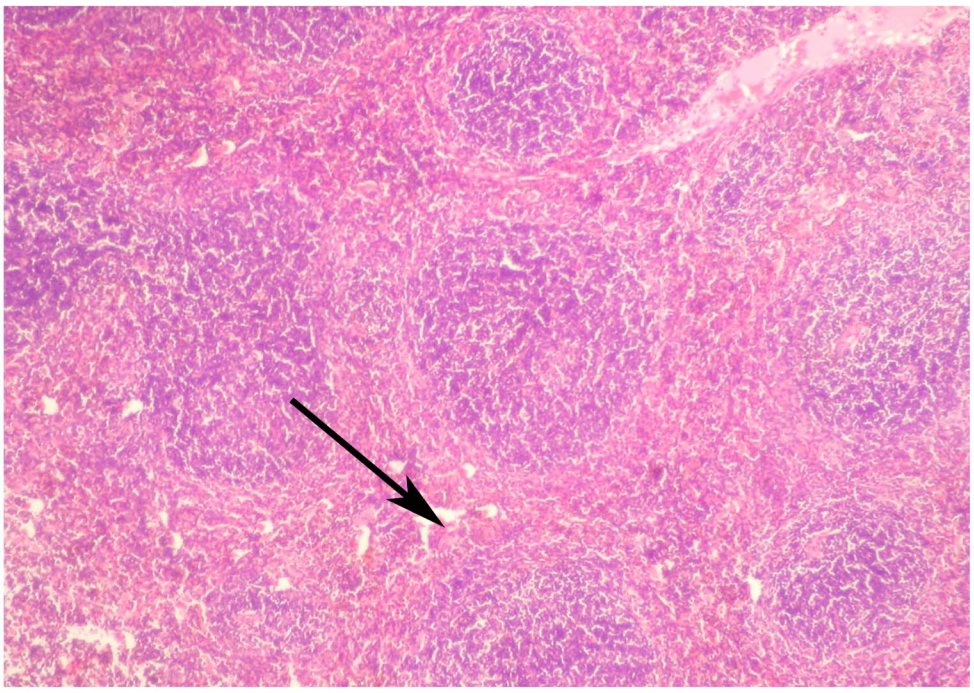

Fig -18: Section of Spleen showing mild depletion of lymphocytes due to permethrin (Group T1) H & E Stain X 60.

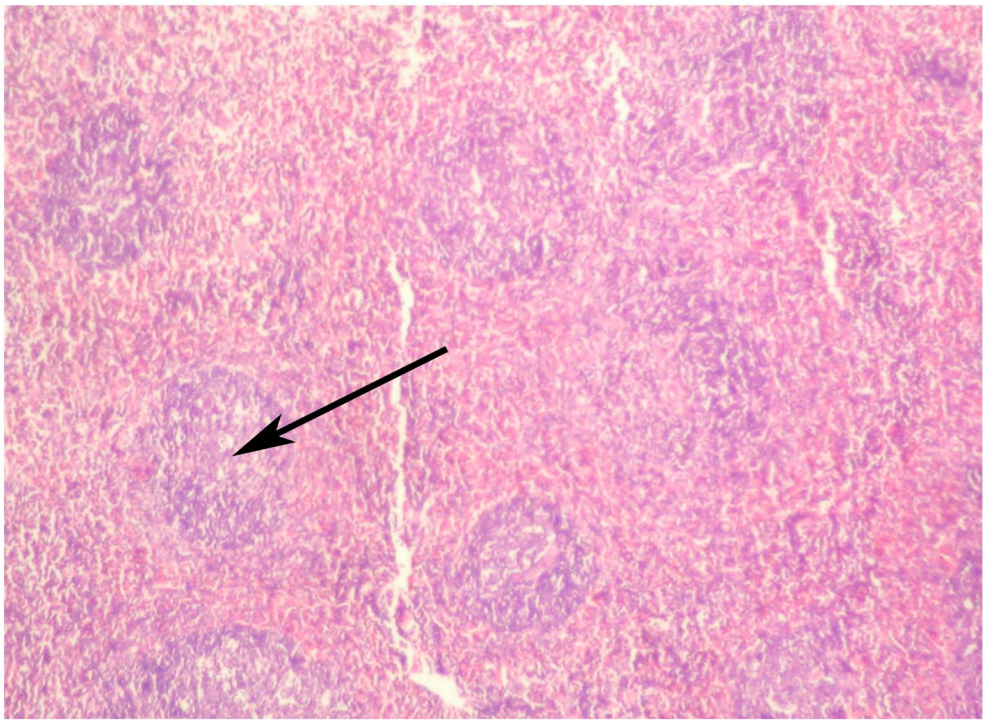

Fig -19: Section of Spleen showing mild to moderate depletion of lymphocytes due to permethrin (Group T2) H & E Stain X 60.