Materials and Methods
MATERIALS AND METHODS

1. Pesticides chosen

For the present study, four types of pesticides (Technical grade) were chosen. They were viz., Acephate, Quinalphos, Endosulfan and Nimbecidine.

Acephate

Organophosphorus compounds, C₄H₁₀NO₃Ps. It is O, S- dimethyl acetyl phosphoramidothioate. It is a systemic insecticide (Wettable Powder, 95% EC, Tallis India Ltd., Mumbai).

Quinalphos

Organophosphorus compounds, C₁₂H₁₅N₂O₃Ps. It is O, O- diethyl O- quinoxalin-2-yl phosphorothioate. It is a contact and systemic insecticide (96% EC, Sunla Industries, Chennai).

Endosulfan

Organochlorine compounds, C₉H₆Cl₆O₃S. It is (1, 4, 5, 6, 7, 7-hexachloro-, 9, 10-trinorborn-5-en-2, 3-ylenebismethylene) sulfite, 6, 7, 8, 9, 10, 10-exachloro-1, 5, 5a, 6, 9, 9a- hexahydro -6, 9-methano -2, 4, 3-benzodioxathiepine -oxide. It is a mixture of two stereoisomers: alpha-endosulfan, comprises 64 - 67% of the technical grade, beta Endosulfan constitutes 29 - 32% of the technical grade. It is a contact and systemic insecticide (97% EC, Excel Crop Care Ltd., Mumbai).
Nimbecidine

\[ C_{35}H_{44}O_{16} \], Oil extract from the kernals of neem tree *Azadirachta indica*. It is available in emulsifiable concentrate containing 0.30% W/W azadirachtin (Neems India Ltd., Chennai).

3.2. Chemicals for Immunological studies

For immunological assays immunoglobulin and complement assay kits were purchased from BINDARID, Birmingham, UK. For Anti Nuclear Antibody (ANA) assay, Anti Streptolysine ‘O’ (ASO) assay, Catabolic Reactive Protein (CRP) assay and Rheumatoid Arthritis (RA) factor assay kits were purchased from Monozyme India Ltd., Mumbai. Freund's Complete Adjuvants (FCA), Fetal Calf Serum (FCS) and tissue culture medium Rose Perk Memorial Institute 1640 (RPMI-1640) were purchased from Difco laboratories, USA. Lymphoprep, Hangs balanced bile salt solution and all other chemicals were purchased from SISCO Research laboratories (SRL) Pvt. Ltd., Mumbai.

3.3. Animals and Treatment

For the experimental study, Swiss albino mice (BALB/c) were chosen as candidate species. The mice were obtained from Madras Medical College, Chennai and reared in laboratory under standard conditions of light and darkness (12 - 12 hrs) and temperature (22\(^\circ\) C \pm 2\(^\circ\) C). The mice were fed Standard laboratory mice pellet feed (Lipton Ltd., Mumbai) (Consisting protein 15 - 17%, fat 4 - 5%, carbohydrate 45 - 55%, fiber 15%, vitamin A 7000 IU/kg, vitamin E 40 mg/kg, vitamin K 2mg/kg, vitamin B 1g/kg and Hawk-Oser salt 11g/kg) and water ad libitum to all the animals. The access to animal room was limited and kept to minimum.
For the experimental study mice weighing 14.2 ± 0.1 gm (30 days old) were recruited from the acclimatized stock. The mice were grouped into several groups with each group with six individuals. These animals were housed in a specially designed (polyethylene cage) cage with provision for systematic supply of pellets and water. The animals were trained to take water from a feeding bottle kept in a cage.

Test chemicals were given through the drinking water bottle everyday. Using standard toxicological test (Spraque, 1963) LD₅₀ doses for the four pesticides were found out. From the LD₅₀ doses two sublethal concentrations were derived for each pesticide, for further experiments. The sublethal doses were 1/10₀₀₀₀₀ and 1/20₀₀₀₀₀ 0.96 hrs LD₅₀ doses. The sublethal concentrations were viz., Acephate 0.30 and 0.05 ppm; Quinalphos 0.1 and 0.05 ppm; Endosulfan 0.06 and 0.03 ppm and carbendazim 7.50 and 3.75 ppm. The sublethal doses of pesticides were dissolved in water and were given to the animal through feeding bottle. The pesticide doses were renewed everyday. Along with water, standard pellet feed was also given ad libitum.

Pesticide treatment was given to animals for 3 to 5 weeks. During treatment food and water were given in ad libitum. Food consumption, general condition and other symptoms were observed daily and body weights were recorded kly.

Immunization

The test animals were divided into equal groups for stimulation with sheep blood cells (SRBC) and for non-stimulation. For stimulation with antigen, the
nines were immunized (i.p.) with SRBC suspended in normal saline (0.15 M). Approximately 25 x 10^6 cells/ml were administered for the primary and 50 x 10^6 cells/ml for the secondary immunization two weeks after the primary dose. Unstimulated mice were treated similarly except immunization with SRBC antigen.

The stimulated and un-stimulated mice were treated with different sublethal loses of pesticides. For each concentration of pesticide and control groups, replicate experiments were maintained.

5. Experimental system

Blood samples of stimulated and non-stimulated mice were collected on the third, fourth and fifth weeks following pesticide exposure by cardiac puncture, after anesthetizing the mice with chloroform. The serum was separated for each group separately and kept at -20°C, till analysed. Heparin was used in collecting whole blood and leucocyte rich plasma for lymphocyte subset enumeration.

6. Immunological assays

1) Humoral immune response

   - Immunoglobulin titre: Quantitation of serum IgA, IgG and IgM were carried out by radial immunodiffusion (RLD) method.
   - Splenic plaque forming cell assay: To estimate the antibody forming splenic cells.
   - B cell EAC rosette assay: To enumerate B lymphocytes.
   - Serum complement protein factor assay: Quantiation of (C3) by radial immunodiffusion method.
5. **Anti - Streptolysin ‘O’ (ASO)**: To find out of the positivity for Streptococcal nuculation.

6. **Anti nuclear antibody (ANA) screening**: To find IgG and IgM antibodies.

7. **Catabolic reactive protein (CRP) assay**: To study CRP formation against pneumococci bacteria.

8. **Rheumatoid arthritis (RA) factor assay**: To find out the resistance to autoimmune disorders.

b. **Cell - mediated immune response (CMI)**

1. **Delayed type hypersensitivity (DTH)**: DTH studies using dinitrochlorobenzene (DNCB) and tuberculin antigen.

0. **T cell E - rosette assay**: The estimation of T lymphocytes.

**I. Changes in lymphoidal system**

1. **Haematological investigation**: To find out the impact of pesticide on the aemopoietic organs, total and differential count of blood cells mice were studied.

2. **Spleen study**: To find out the changes in spleen, a secondary lymphoidal organ.

3. **Comet assay**: To asses DNA damages in the peripheral lymphocytes.

**II. Gut microbial assay**

4. **Gut microbial study**: To find out the impact of pesticide on the symbiotic microbes inhabiting the gut.

All the experiments (Chart 1) were carried out in triplicates and the mean ± standard deviation were estimated. The data were analysed by two-way analysis of variance (ANOVA) wherein “F” ratios were calculated by comparisons of variance rising within the samples and between the samples.
Chart 1. Immunological assays

Direct immunotoxicological changes

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Indirect immunotoxicological changes