

THE ACUTE TOXICITY STUDIES OF PESTICIDES OBTAINED FROM
INDIGENOUS PLANTS-VIZ ASADIRACHTA INDICA (NEEM),
PONGAMIA GLABRA (KARANI) AND ANNONA SQUAMOSA
(CUSTARD APPLE) AND THE NEUROTOXIC POTENTIAL OF NEEM

THESIS SUBMITTED TO
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHARMACEUTICAL SCIENCES
IN
PHARMACOLOGY

SUBMITTED BY
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UNDER THE GUIDANCE OF
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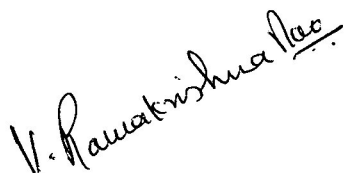
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Introduction

Pesticides are widely used in agricultural management for protection of crops, in public health programmes, against the control of vectors, transmitting diseases.

Pest problems and the measures to alleviate them are nearly as old as the dawn of civilization. The earliest record of pest control was the use of sulfur by ancient Sumarians around 2500 B.C. Around 1000 B.C., Homer spoke of the use of sulfur for fumigation. In 900 A.D., Chinese were using arsenic against garden insects and in 1101 A.D., they discovered soap to control pests and in 1300 A.D. Marco Polo referred to the use of oils against the control of camel mange. Then by 1600 A.D. tobacco infusions and arsenic were commonly used^{1,2}. The use of plants and herbs as food ingredients or for medical purposes or for pest control are recorded^{1,3}. Chemical pesticides were first introduced in 1940⁴. Since then various types of pesticides such as chemical pesticides - chlorinated hydrocarbons, organophosphorus pesticides and carbamate pesticides and phenyl mercurials; pesticides from plant origin - pyrethrum, nicotine, neem, karanj; biocides or microbial pesticides and even natural predators have helped in pest control.

Pesticides have become indispensable for protecting crops from insects, diseases, weeds and are playing a significant role in public health by eradicating vector-borne diseases.

Despite the immense benefits these pesticides have in increasing the food production and eradicating certain vector-borne diseases, pesticides are not only toxic to domestic animals, humans and other beneficiary insects but also affects other members of the environment. The pollution of water, soil, air and the residues in food have become a global concern. Added to their possible toxicity, continuous use of pesticides has also led to development of resistant strains of insect pests.

Pesticides enter the environment through spillage or drift at the time of pesticide application or because of their volatility, or through wastes of human,

animal, plant and industrial processes⁵. Wide spread use of pesticides has resulted in the fear of adverse effects to humans and domestic animals. Wildlife, birds and fish may be exposed to health hazards when pesticides are used for the control of unwanted plants, or via contaminated air, food and water⁶. Pesticides may enter the body through oral ingestion as residues in food and drinking water or from accidental or intentional ingestion or through the respiratory system (inhalation) or through skin. The basic chemical and/or their degradation products may persist in the soil or plant or animal tissues in wildlife in quantities that may affect the preservation of endangered species and the ecosystem.

Pesticides may find their way into human system through the food chain as residues. Residues exceeding extraneous residue limits of DDT and HCH were found in 11 out of 62 milk samples obtained from rural areas of Ahmednagar district, Western Maharashtra⁷. Residue of endosulfan and monocrotophos were found in grains from green and dried pods of pigeon pea⁸. Major environmental pollution affecting wildlife, including birds and fish is through deposits resulting from application of chemicals to control agricultural pests or insects and animals responsible for vector-borne diseases.

It is estimated that the total production of technical grade chemical pesticide for 1989-90 was 60,000 tonnes⁹. Amongst them organophosphorus pesticides are the major ones used. Largest pesticide used in India is BHC followed by DDT, endrin, parathion and malathion¹⁰.

In spite of sufficient control and precaution during the manufacture, distribution and sale of pesticides incidents of death and morbidity are known to occur. The well-known Bhopal tragedy of 1984 is an incidence that occurred at Union Carbide Pesticide plant¹¹. The toxic effects of pesticides can result in acute or chronic toxicity. They may also cause organ damage or carcinogenicity, mutagenicity or teratogenicity. Alachlor was found to cause nasal tumors on chronic administration¹². Organophosphorus pesticides are known to disrupt the

nervous system function by inhibiting the enzyme cholinesterase¹³. A study showed that exposure of pesticide sprayers to multiple pesticides affected the normal functioning of different organ systems¹⁴. The fear of delayed or cumulative effects always persists.

As a result of consumer safety awareness, regulatory bodies are laying down stricter guidelines to ensure that consumer products are evaluated for safety before use. Variety of toxicity studies such as acute, subchronic and specialised studies such as carcinogenicity, mutagenicity, teratogenicity, neurotoxicity in different species of animals are required by the regulatory agencies around the world to assess and prevent the possible hazards of pesticides. Since evaluation can not be done directly in humans due to ethical reasons, the first step required is to predict the possible hazards to humans through other models and then follow-up with controlled field trials or consumer surveys.

Most regulatory decisions are based on the results of animal studies using mammalian species. Extrapolation from animals to man has been the most successful for certain studies such as carcinogenicity, acute toxicity and metabolic interference as well as for deafness, behavioural changes and influence on immune system¹⁵. As all the types of toxicity that occur can not be predicted by conducting studies in one species due to species variation, different species of animals are used for assessing and predicting a possible or more realistic toxic effects in human beings or animals or the ecosystem.

Efforts are being constantly made to manufacture chemical pesticides selective to target species but safe or least toxic to other living systems. The problems of resistance to pesticides and the contamination of the biosphere with pesticides has dictated the need for effective, biodegradable pesticides with greater selectivity and which are also less expensive. This awareness has created a worldwide interest in the use of traditional botanical pesticides. Accordingly,

attempts have been made to manufacture “economically friendly” botanical pesticides.

It is in this context that the discovery and reports on azadirachtin, karanj, annonaine and several other plant extracts assume relevance¹⁶. For these botanical products to be marketed as pesticides, they have to be thoroughly evaluated for their toxicity and the subsequent submission of the toxicity data to the Pesticide Regulatory Body for necessary registration.

To assess the possible hazards, Pesticides Regulatory Authorities require various toxicity studies to be conducted on pesticides. Huge quantities of pesticides are used in agriculture and in health programmes which warrants evaluation of their potential for adverse or toxic effects on agricultural worker, public and environment. The concern is specially for their acute, chronic and cancer effects. The effect of pesticides during occupational exposure has been linked to acute health effects such as rashes, skin, eye and respiratory illness and death¹⁷.

Acute toxicity occurs mainly due to carelessness or lack of understanding on the part of the user or by deliberate misuse or accidental or intentional poisoning. Acute toxicity (single dose) data forms a mandatory requirement for the evaluation of safety of pesticides. This data is required to support the registration of the product. Acute toxicity study studies are most commonly used to predict the human response to short-term, high-level exposure as may occur following an accident. They can also provide a measure of toxic potential of different compounds. It is usually an initial step and provides information on the health hazards likely to arise due to single exposure by oral or dermal or inhalation route. The study is to evaluate the median lethal dose (LD_{50} : dose required to produce toxicity symptoms and kill 50% of the animals), its statistical limits using a single exposure and a 7 or 14 days post-exposure observation.

LD₅₀ serves as a basis for classification, labelling and packaging and also as a guide for selection of doses or dosage regimen for subchronic and chronic studies (long-term, low-level exposure) and may also provide information on the mode of toxic action of the pesticide and comparative toxicity about other pesticides.

The present study was undertaken to study the different types of acute toxicity of extracts of *Azadirachta indica* (neem), *Pongamia glabra* (karanj) and *Annona squamosa* (custard apple) in different animal species viz. mice, rats, chickens, pigeons, fish and rabbits and compared with quinalphos, an organophosphorus pesticide.

The following acute toxicity studies were carried out :

- * Acute oral toxicity in mice and rats (LD₅₀)
- * Acute oral toxicity in chickens and pigeons (MLD)
- * Acute toxicity in fish (LC₅₀)
- * Acute dermal toxicity in rabbits (LD₅₀)
- * Primary skin irritation in rabbits
- * Mucous membrane irritation in rabbits.

Also, the neurotoxic potential of neem in chickens was studied.

Review of Literature

Self sufficiency in food production and control of vector-borne diseases are two of the major priorities with planning in our country. Apart from food production, its storage facilities have also become important. Pests are responsible for incalculable damage to crops in agriculture, during storage of food and in spread of vector-borne diseases. Pesticides play an important role in controlling pests.

Crops are affected by several different pests and competition from weeds. Several insects arthropods, fungi, molluscs and bacteria attack crops and result in considerable losses. The losses of crops caused by pests are great in developed as well as developing countries. It is estimated that about 10% of the crops are annually lost due to insect pests. On an average, our country loses Rs. 6000/- to 7000/- crores worth of crops annually either in the field or during storage due to pests^{18,19}. Losses caused by pests after the crops are harvested and during storage, are usually greater and more significant. In India, according to an expert committee 9.33% of the total food grains produced are lost during post-harvest. There are more than 100 insect species which are found to be damaging stored grains²⁰.

The most important group of pests of economic importance are the rodents which multiply fast and breed throughout the year. They contaminate the grains with their excreta, hair, urine and transmit a number of diseases in human beings²⁰.

The data available in various regions show a correlation between pesticide use and crop yield. The average production of pesticides in India in 1989-90 was 60,000 tonnes⁹ and presently the annual demand is estimated at 1.25 lakh tonnes and by 2000 A.D. it is estimated to reach 2.00 lakh tonnes²¹.

In the year 1993-94, the crop production reached 180 million tonnes and it is estimated that by 2000 A.D., the food requirement would be 225-250 million tonnes. To meet this challenge, 45-70 million tonnes of additional food grains

must be produced, which is possible by the use of good seeds, fertilisers, irrigation and suitable plant protection methods²².

Apart from the use of pesticide for plant protection, pesticides are also used to control rodents, insects, molluscs responsible for transmission of vector-borne diseases^{23,24,25}. Insecticides have made significant contributions in improving public health by reducing vector-borne diseases like malaria and filaria²⁶.

The diversity of species and harmful forms of insects and mites, the features of their structure, biology and ecology has forced mankind to develop hundreds of types of pesticides as a way to control them. Modern pesticides belong to different classes and have different modes of action^{13,27,28,29,31}. Depending on their specific properties and a single mechanism of action, pesticides fall in one of the following groups : organochlorine compounds, organophosphorus compounds, carbamates, nitrophenols and synthetic pyrethroids.

The insecticidal property of DDT was first discovered in 1939 by Paul Muller and which became the most widely used insecticide in the world²⁹. The use of organochlorine compounds began with DDT, which was employed first in Switzerland. Factor that limits the practical use of organochlorine insecticides is the hazard associated with their use. Some of the insecticides possess a long residual action which may be of great advantage in controlling certain pests but which may persist in food plants consumed by man and animals. Some of these pesticides are stored in fat or are excreted in the milk of animals as residues.

The organophosphorus compounds form one of the most important groups of pesticide. The development of organophosphorus compounds stemmed from the wartime research on highly toxic nerve gases for use in chemical warfare. Subsequent research was directed to development of selective and less toxic insecticides. Malathion in 1950 was the first wide spectrum insecticide with a very low mammalian toxicity^{13,29,30,31}. The organophosphorus compounds have a

high potency as insecticides as well as ascaricides and miticides. Many of these compounds are also extremely toxic to man and other warm blooded animals because of their action as an irreversible inhibitors of cholinesterases^{19,31}.

Organophosphorus compounds control a wide range of pests and disease carriers. Certain of these compounds possess systemic action, a characteristic that offers great promise for controlling important insect pests of crops as well as livestock²⁷. They are used extensively, in place of the chlorinated hydrocarbons and other insecticides such as rotenone. Several of the organophosphorus compounds do not bio-accumulate in the meat and milk as readily as do certain chlorinated hydrocarbon pesticides³².

Carbamates include highly active pesticides. They are used as insecticides, herbicides and fungicides. Carbamates like organophosphates, inhibit insect cholinesterase, however, their mode of action is reversible^{19,31}.

Nitrophenols are highly toxic to insects, bacteria and fungi in all stages of their development and to green plants and warm-blooded animals. They can separate the process of respiratory phosphorylation and stimulate the hydrolysis of ATP in the course of reaction of respiratory chain at the time of penetrating into an organism and as these processes are common to all living beings including man, the hazard of nitrophenol predominates in all living beings¹⁹.

All the above pesticides vary in their mode of action, uptake by the body, metabolism and toxicity. Pesticides enter the body mainly through the skin and eyes or by inhalation or by ingestion³³.

Environmental pollution has increasingly become a concern of not only the ecologists but also the governments around the world. It is believed that one of the many contributing factors of pollution is the indiscriminate use of the highly toxic pesticides in agriculture and public health programmes. This has resulted in not only destruction of crops, but also has become a potential health hazards to humans, animals, and also affects the ecological balance. Additionally

it is also believed to upset the beneficial and helpful insects and resulting in pests attaining virulent form³⁶.

Problems like appearance of resistant strains of pests, resurgence of treated population, sudden outbreak of pests previously considered minor, detrimental effects on non-target organisms which are otherwise beneficial to man (predators and pollinators) and most important, the widespread pollution have resulted due to extensive use of pesticides. Pesticides are also known to alter the crop physiology as well^{34,35}.

Pesticide resistance in India was first noticed in insect pests of public health importance. Mosquitoes transmitting malaria and other vector-borne diseases were first to become resistant to pesticides. Pesticide resistance in an agricultural pest was first noticed in 1963, when Singhara beetle, *Galerucella birmanica*, from Delhi was reported to be resistant to DDT and HCH³⁵. *Musca domestica* and *Culex molestus* (1946), head louse and *Anopheles sacharovi* (1951) became resistant to DDT³⁶.

Human exposure to pesticides can be described in several ways e.g. acute or chronic, occupational or non-occupational, accidental or intentional. Exposure can be by oral (by mouth), respiratory (by inhalation), or dermal (through skin) routes.

Acute exposures may be due to accidental, occupational or intentional and may lead to either systemic effect or local effects. Many pesticides, such as parathion, mevinphos, endrin^{28,32,37}, known to be highly hazardous are either banned or severely restricted in industrialised countries.

The continuous use of pesticides may also affect the environment. Air can become contaminated with pesticides during operations. In Pakistan (1976), during the malaria eradication programme with parathion, 2800 were poisoned and five died out of 7500 spraymen exposed to Parathion³⁹.

Treatment with pesticides to prevent losses of food due to pests during storage may result in high concentrations of pesticides as residues. There is one

report from Wales, where about 150 people were reported ill and approximately 30 had epileptic fits as a result of eating bread prepared with endrin contaminated flour⁴⁰.

Over hundred deaths in India during the spring 1958, resulted from food accidentally contaminated with parathion during shipment⁴¹. In United States during 1957, out of total 252 cases of human poisoning reported in California, 189 were due to organophosphorus compounds⁴².

In India, ethyl parathion and endrin have been shown to cause poisoning and death of 102 people in Kerala, due to careless handling and storage of wheat with these two pesticides⁴¹. The tragic accident at Bhopal, where people were exposed continuously to the gas, methyl isocyanate, an intermediate for manufacturing of pesticide, resulted in irritation of skin and eyes, blindness, tightness of chest. Death was reported in 2500 cases out of five lakh people exposed^{11,38,104}.

Blindness, cancer, deformities, diseases of liver and nervous system resulting from pesticide poisoning have been identified in cotton districts of Maharashtra and Andhra Pradesh. Also poisoning due to children consuming pesticides by mistake, families using pesticide containers for storing food or water, the contamination of food or clothing during transport or storage have been reported³⁸.

Water may be contaminated due to accidental spillage of pesticide formulation or discharge of surplus pesticide after spraying operations or by application of pesticides to rivers or ponds to kill fish, which are then removed and eaten. Chlorinated pesticide residue in marine sediments have been analysed and the concentrations of Aldrin, BHC, Dieldrin and DDT in marine sediments of the east coast of India were found to be 0.02-0.53, 0.01-0.02, 0.05-0.51 and 0.02-0.78 $\mu\text{g/g}$ wet sediment respectively⁴. Yamuna river water in Delhi contain an average of 0.24 mg/l of DDT. The level of BHC in water taken

from the Caverry (Karnataka) was over 1000 ppb and methyl parathion 1000 ppb³⁸.

Acute poisoning, including suicide attempts, mass poisonings from contaminated food, chemical accidents in the industry and occupational exposure in agriculture, is the cause of most of the serious health effects associated with chemical pesticides.

If the use of chemical pesticides doubles in the next ten years in developing countries like India and if agricultural practices continue to develop, it is likely that toxicity due to intentional and accidental acute poisoning may increase accordingly. To reduce the use of toxic chemical pesticides as methods of pest control other types of pesticides have been tried including pesticides obtained from plants, biological pesticides and even natural predators.

Before the advent of synthetic pesticides, people were using plants and plant derived substances to try to repel and kill mosquitoes and other domestic pests. The best known examples of botanical pesticides are *Derris*, *Tephrosia*, *Chrysanthemum* and *Nicotiana* from which compounds rotenone, pyrethrum and nicotine were derived^{43,44,45}. These compounds were once the basis for a major part of pesticides. But they never found wide application against pests, largely because of their toxicity to non-target organisms.

The presence of the poisonous alkaloid, nicotine in tobacco, discovered in 1828 by Posselt and Reiman, is well known to be toxic to human beings. Rotenone, an active principle from tuba-root of *Derris elliptica* was used as a fish and arrow poison for the control of insect pests of nutmeg in 1848 by Oxley. Rotenoids are proved to cause local effects such as conjunctivitis, dermatitis, pharyngitis and rhinitis. Pyrethrum, obtained from *Chrysanthemum cinerarifolium* was manufactured in 1828 by Juntikoff to be used against insects. The pyrethrins affect the cardiovascular system, liver and kidney functions. Contact dermatitis is the most common form of pyrethrum poisoning¹³.

Considering the disadvantages of the synthetic and natural insecticides currently in use, attention has been focused to develop a new breed of natural products as potential pesticides. However the presently available insecticides are not very selective in their action and are generally toxic to many non-target species including man and other desirable forms of life that co-exist in the environment. Attempts have been continued to produce new and safer insecticides.

India has a rich and varied plant flora, which are known for medicinal properties^{46,47}. There are many plants which are known for pesticidal activity. Ahmed et al (1984) have compiled a list of 2121 plant species reported to possess pest control properties. Plants namely *Pongamia glabra*, *Anethum sowa*, *Acorus calamus*, *Milletia ovalifolia*, *Mesua ferrea*, *Azadirachta indica*, *Lantana camara*, *Parthenium hysterophorus*, *Annona squamosa*, *Eucalyptus globulus* have been investigated^{48,101}.

However, detailed toxicological evaluation of indigenous plants with proven bio-efficacy are yet to be undertaken for regulatory purposes.

Azadirachta indica

The neem tree (*Azadirachta indica* A.Juss., family Meliaceae) has attained world-wide prominence in recent years.

Insect repellent property of neem leaves has been shown in rural India since ages. The first recorded indication on neem acting as an insect repellent was by Mann and Burns in 1927, who quoted that the adult locusts did not feed on neem leaves during the locust cycle of 1926-27⁴⁹. Astrakhantzev et al. (1936) has reported the efficacy of water and alcohol extracts of neem as aphicide against *Brevicoryne brassicae* L. Pruthi (1937) also found that neem leaves mixed with grain or kept in 5 to 7 cm layer over the stored grain, protected the commodity from damage by storage pests⁴⁹. Volkonsky (1937) reported that extracts of leaves of *M. azedarach* when sprayed on other plants gave protection against locust⁴⁹.

Extracts of neem have been tested and found effective against number of other insect pests specially lepidopterous larvae viz. *Heliothis zea*, *H. virescens*, *Spodoptera frugiperda*, *Plutella maculipennis*⁵⁰.

The tests with neem products have been conducted not only against leaf eating and tissue boring insects but also against sucking insects like aphids, jassids, coccids and other sap sucking bugs⁵⁰.

According the earlier workers, neem products were effective against some pests only by, but the perusal of literature shows their efficacy against wide range of insects belonging to different groups. Seventy one species of insects belonging to orders Coleoptera, Diptera, Hemiptera, Isoptera, Lepidoptera and Orthoptera which respond to neem extracts and pure compounds have been listed in the literature⁵³.

The number of insects tested with neem has now increased to more than 300 species. In India, neem has been evaluated against 105 species of insects belonging to 10 orders viz, Orthoptera, Dictyoptera, Lepidoptera, Hemiptera, Diptera, Coleoptera, Hymenoptera, Isoptera, Thysanoptera and Siphonaptera⁵³.

Growth regulatory effect is the most important physiological effect of neem on insects. The seed kernel extracts or azadirachtin when fed or applied at juvenile stages arrest their growth. Depending on the dose, the insects are either killed before reaching the adult stage or produce malformed and miniature adults. The other physiological effects recorded are : prolongation of larval period (up to 2 months), production of larval-pupal and pupal-adult intermediates⁵³.

McMillan et al were the first to report the growth disrupting effect⁵³ of chloroform extract of the leaves of *Melia azadrach*. Sorghum stem when treated with 0.5% concentration of neem seed kernel suspension and fed to *Chilo partellus* larvae resulted in the formation of larval-pupal intermediate or the death of the larvae during the process of development or production of abnormal adults which did not live long enough to continue the next generation. Similar effects were observed when *Heliothis armigera* larvae were fed on tender sorghum grains treated with neem seed kernel suspension.

The effect of azadirachtin on insects is still not fully understood. It is, known that azadirachtin reduces and delays titres of morphogenetic hormones. Azadirachtin interferes with neuroendocrine system which controls ecdysone and juvenile hormones^{52,53}.

Azadirachtin besides being ecdysis inhibitor has also been found to affect vitellogenesis, a process in which maturing adult insects synthesize specific proteins which are incorporated in oocyte leading to their maturation. *Locusta migratoria*, treated with azadirachtin had smaller ovaries and weighed only half that of control. The number of mature oocytes was also less⁵³.

Following azadirachtin application, marked reduction in the fecundity was observed in *Spodoptera litura*. Untreated moths laid 7923 eggs compared to 705 eggs laid by 5 pairs of azadirachtin-treated moths, the reduction being almost 90%. The untreated moths laid eggs up to 7 days while the treated females laid eggs only for 2 days due to delayed oviposition. The egg

hatchability in the treated moths was only 25% as against 75% control⁵³. Neem has an oviposition deterrency on insects which is very useful in protecting crop. *Schistocerca gregaria* treated with 0.1, 0.5 and 1% concentrations of neem seed kernel suspension laid significantly less number of eggs in the sand than untreated control⁵³. Seven oils including neem oil were screened against *Pthorimea operculella* and it was concluded that the neem oil at 0.05% concentration afforded 91 to 100% oviposition deterrency⁵³.

Azadirachta indica preparations have been shown possess various pharmacological effects. Local application of neem derived lotion has been found to be beneficial in many dermatological diseases including eczema, ringworm and scabies. In Haiti, crushed leaves are rubbed on open wounds that have become maggot infested. In India and Bangladesh, villagers apply neem to hair to kill head lice^{54,55}.

A methanolic extract of the leaves has been found to exert antipyretic effect in male rabbits. The plant also possesses analgesic activity which is mediated through opioid receptors in laboratory animals^{54,55}.

Aqueous extracts of leaves administered i.v. in dogs significantly decreased the blood sugar levels and prevented adrenaline as well as glucose hyperglycaemia. Nimbidin (isolated from seed oil) also showed significant antigastric ulcer activity in guinea pigs and rats and also prevented the formation of ulcer and reduced the gastric acidity⁵⁶. Nimbidin gargle and dentrifices have been effective in treating bleeding gums and pyorrhoea⁵⁵.

Neem oil and many of its constituents have been successfully used against a wide range of bacteria, viruses, fungi and parasites^{52,55}. Although in India, a lot of work on pharmacological activity and insecticidal activity of neem extracts have been done, but no detailed toxicological evaluation of neem technical and neem formulations for regulatory purposes have been undertaken. Each indigenously manufactured neem technical and neem formulations, due to secrecy do not specify the solvents used, the nature of emulsifiers or stabilizer

used, percent purity of the technical grade material and hence the magnitude of toxic effect may vary.

It has been reported that leaves of *Azadirachta indica* cause toxic effects on sheep, goats, and guinea pigs⁵⁷. A dose higher than 40 mg/kg of aqueous leaf extract given intravenously caused toxic manifestation leading to death in guinea pigs¹⁰². Liver damage was indicated in chicks fed *A. indica* ripe fruit for 4 weeks, also hepatic excretory dysfunction was suggested. The incorporation of *A. indica* ripe fruit into the basal diet at 2%, 5% and 10% proved toxic to chicks⁵⁸.

Thirteen infants developed vomiting, drowsiness, metabolic acidosis, polymorphonuclear leucocytosis, and encephalopathy within hours of ingestion of margosa oil (obtained from seeds of *A. indica*). Liver biopsy of one infant and necropsy examination of ICR strain mice after experimentally induced margosa-oil poisoning demonstrated pronounced fatty infiltration of the liver and proximal renal tubules as well as cerebral edema. From these findings it was indicated that margosa oil may be involved in the etiology of Reye's syndrome amongst in Malayasian Indians⁵⁹.

When deoiled neem fruit cake was incorporated from 25-50% level with maize in the concentrate mixture of growing sheep no loss in weight was observed but 100% deoiled neem fruit cake, feeding, induced loss in weight⁶⁰. Sodium nimbidinate which is a relatively non-irritating substance can be ingested or injected by any of the conventional routes. The LD₅₀ for i.p. injection in mice was found to be about 700 mg/kg of body weight, whereas dogs tolerated oral, i.m. or i.v. injections up to 200 mg/kg of body weight. A large single dose of 1000 mg/kg per os, or repeated injections led to renal damage⁶¹.

In acute oral toxicity studies of nimbidin upto 2000 mg/kg i.p in albino rats and mice of either sex, no signs of toxicity and mortality was observed during the study period of 7 days. Subacute toxicity studies of nimbidin in albino rats showed increase in liver glycogen and reduction in serum protein in 100

mg/kg group. There were no apparent gross lesions at necropsy, and histological examination of liver, kidney, adrenals, testes and heart muscles did not reveal any pathologic changes suggestive of toxicity of nimbidin whereas in mongrel dogs, no toxic effects were revealed.

Teratogenic studies performed in fertile female rats, with nimbidin at doses 25, 50 and 100 mg/kg did not exhibit any foetal abnormalities in pups born and there were no adverse effects on rearing performance⁵⁶.

Acute oral toxicity studies of ethanolic neem extract containing 3000 ppm azadirachtin ($\pm 10\%$) performed on rats showed no negative effect up to a dose of 5 ml/kg. In another study, methanolic leaf and bark extracts showed an oral LD₅₀ of about 13 gm/kg in acute oral toxicity in mice. General signs of ill-health and discomfort, gastro-intestinal spasm, apathy, refusal of water and feed and hypothermia were observed in the animals⁵⁵.

Acute toxicity study in rats and rabbits using neem seed oil resulted in 24h LD₅₀ of 14 and 24 ml/kg respectively. Target organs for toxic effects were the CNS and lungs⁵⁵.

EPA registered Margosan-O was tested for toxicity to gain registration to be used only on non-food crops. Avian single-dose oral LD₅₀ of Margosan-O to mallard ducks was found to be more than 16.0 ml/kg whereas the avian dietary LC₅₀ with bobwhite quail and mallard ducks was found to be in excess of 7000 ppm⁶².

The LC₅₀ of Margosan-O to rainbow trout was 8.8 ml/l. and the 96-hour no observed-effect concentration was 5 mg/l. The results of acute toxicity to bluegill sunfish showed a 96 hour LC₅₀ of 37 mg/l. and no-observed effect 96 hour level of 20mg/l. In *Daphnia magna*, the water flea the LC₅₀ was 13 mg/l⁶².

Acute oral toxicity in rats dosed once, the LD₅₀ was in excess of 5 mg/kg, the limit of the required test. Acute dermal toxicity (LC₅₀) of Margosan in albino rabbits was in excess of 2 ml/kg. There was low to moderate skin irritation to the shaved area patch and high to moderate irritation to the abraded area^{55,62}.

The acute inhalation (LC_{50}) study for the Margosan-O was more than 43.9 mg/l/hr, the limit of the test⁶².

In the modified eye irritation, Margosan-O was found to show minimal irritation to eyes. Margosan-O did not produce sensitisation on guinea pigs. Results of the mutagenic test indicate that Margosan-O is non-mutagenic⁶².

In the subacute oral toxicity studies with nimbidin in rats fed 25,50, and 100 mg/kg orally did not reveal any toxic symptoms or death⁵⁶. In guinea pigs orally fed with 50 or 200 mg/kg of aqueous suspension of green or dried neem leaves for up to 8 weeks there was a progressive decrease in body weight, pulse and respiratory rates along with diarrhoea⁵⁵.

Subacute toxicity studies in dogs fed with sodium nimbidinate upto 200 mg/kg showed no toxic manifestation even upto level of 2400 mg/kg in one 12 kg dogs but six puppies delivered, died within 10 days. The kidneys of both the dogs showed cloudy degeneration^{55,56,61}.

Subacute dermal toxicity in albino rats was evaluated with Neemrich-100 (Technical grade) a formulation of 30% oil extracted from dry neem seeds which showed no clinicotoxicological symptoms, but the skin had become roughened and thick. There was a 40% increase in size of the liver of the highest dose group. Microscopic examination of skin showed scaling of epidermis and hyperkeratosis of the stratum corneum⁵⁵.

Diarrhoea, nausea and general discomfort have been reported to be caused by neem seed oil intoxication in human. A 4-month-old Indian child died after receiving 12 ml of oil on each day for 2 successive days for cough. Vomiting, drowsiness, respiratory difficulty, seizures after 1-5h of last dose were observed and the liver became enlarged⁵⁵.

Each formulation or preparation needs detailed toxicological evaluation before its registration and commercial use.

Pongamia glabra

The karanj (*Pongamia glabra* Vent, family Leguminosae) has been reported to possess a variety of medicinal and pesticidal properties⁶³. It is reported to be an effective remedy for all skin diseases like scabies, eczema, leprosy and ulcers.

Leaves decoction of *Pongamia glabra* is known for its usefulness in treating diarrhoea and cough. Seed extract of karanj has been reported to possess antibacterial activity. Activity of seed oil on cockroaches and house flies has also been described. The seed extract is also effective against tobacco caterpillars¹⁶.

Pongamia cake has been found effective in controlling ground beetles of tobacco⁶³. It has also been found to have repellent and insecticidal activity against grubs of *H. Vigintioctopunctata*⁶⁴. Seed extracts of *Pongamia glabra* are highly effective against *Spodoptera litura* on groundnut leaves⁶⁵. Kanzi seed (*Pongamia glabra* seed) extract has been found to be effective in controlling Gujarat hairy bollworm, *Amsacta moorei*⁶⁶. *Pongamia* seed extract both at 2% and 4% concentrations were found to be highly effective against citrus leaf miner, *Phyllocnistis citrella* stainton and in par with neem extracts⁶⁷.

Crude oils of karanj has been demonstrated in laboratory and field trials to be effective against rice leaf hopper and plant hopper as well as it affects the survival, oviposition and antifeeding activity of *Chilo partellus*, a serious pest of maize⁶⁸.

Though there are reports on the efficacy of *Pongamia glabra* as stated above the details studies are yet to be undertaken.

Each formulation or preparation needs detailed toxicological evaluation before its registration and commercial use.



Pongamia glabra

Annona squamosa

It belongs to the family Annonaceae. It is an American tree cultivated and naturalised in several parts of India. The seeds contain an oil and a resin which contains an acrid principle and the leaves and seeds contain alkaloid. The seeds, leaves and the immature fruits contain an acrid principle fatal to insects. The dried unripe fruit, powdered and mixed with grain flour is used for killing vermins and the seeds to kill body rice. The powdered seeds and an aqueous infusion of the leaves is believed to possess valuable insecticidal properties. The seeds are insecticidal and are used to remove lice from hair. The seeds are crushed and used to destroy worms in the wound of cattle. The seeds possess insecticidal activity against *Musca nebulo*, *Tribolium castenium* and *Bombyx mori*⁶⁹.

Seeds of *Annona squamosa* are also used to control insects like cabbage aphids, pulse beetles, cotton stainer insects, brown plant hoppers, saw-tooth grain beetles, diamond back moths, white backed plant hoppers and tobacco caterpillars⁶⁹.

Leaves of *Annona squamosa* are useful in reducing grub pollution of *H. vigintioctopunctata*⁶⁴. Seeds, leaves extract of *Annona squamosa* were found to be effective against controlling Gujarat hairy caterpillar, *Amsacta moorei*⁶⁶.

Though there are reports on the efficacy of *Annona squamosa* as stated above the details studies are yet to be undertaken.

Each formulation or preparation needs detailed toxicological evaluation before its registration and commercial use.



Annona Squamosa

Quinalphos

(o,o-diethyl-o-quinoxalin-2-yl-phosphorothioate)

It belongs to the toxicity class WHO II, EPA II^{70,71}.

Quinalphos is an insecticide, and acaricide with contact and stomach action. It is a cholinesterase inhibitor.

It controls many insects pests of the orders Lepidoptera, Coleoptera, Diptera, Hemiptera, etc. It is used against caterpillars on fruit trees, cotton, vegetables, and peanuts; scales on fruit trees; and the pest complex on rice. Also controls aphids, bollworms, borers, leafhoppers, mealybugs, mites, plant hoppers, thrips, etc. on beet, vines, ornamentals, potatoes, soybeans, tea, coffee, cocoa and other crops. It is slightly phytotoxic to certain fruit trees.

It is found to have an acute oral LD₅₀ of 71 mg/kg in male rats, 26 mg/kg in rats, 107 mg/kg in mice and 100 mg/kg in dogs. The acute dermal LD₅₀ in rat is 300 mg/kg. In rats the acute inhalation LC₅₀ is found to be 175 mg/kg.

It has been found to have no teratogenic, mutagenic and carcinogenic potential, though it may cross the placental barrier. On inhalation, it is known to cause nasal hyperaemia and watery discharge, cough, chest discomfort, dyspnea and wheezing due to increased bronchial secretions and bronchoconstriction. Systemic effects include pallor, nausea, vomiting, diarrhoea, abdominal cramps, headache, dizziness, ocular pain, blurred vision, miosis. Reported CNS or neuromuscular effects include ataxia, slurred, speech, areflexia, weakness, fatigue, fasciculations, twitching, tremors possibly of the tongue and eyelids, and eventually paralysis of the extremities and possibly of the respiratory muscles. In severe cases there may also be involuntary defecation and urination, cyanosis, psychosis, hyperglycaemia, acute pancreatitis, cardiac irregularities, pulmonary edema, unconsciousness, convulsions and coma. Death is primarily due to respiratory failure.

Repeated or prolonged exposure results in the effects of acute exposure including the delayed neuropathy. Other effects reported in workers exposed

repeatedly include impaired memory and concentration, acute psychosis, severe depressions, irritability, confusion, apathy, emotional liability, social withdrawal, headache, speech difficulties, delayed reaction times, spatial disorientation, nightmares, sleep walking and drowsiness or weakness, anorexia, and malaise have also been reported^{70,71}.

Materials and Methods

Test Compounds :

Neem technical (*Azadirachta indica* alcoholic kernel extract, purity 100%), Neem formulation (*Azadirachta indica* formulation, purity 12%), Karanj technical (*Pongamia glabra* alcoholic seed extract), *Annona squamosa* technical (methanol and ethyl acetate seed extracts) and quinalphos 70% a.i. (o,o diethyl-o-2-quinoxalinyyl phosphorothioate) were obtained from local manufacturers.

Methods :

Methods followed are as per the guidelines of the International, National and Regulatory Body requirements^{72,73,74,77}.

Acute Oral Toxicity in mice and rats (LD₅₀) :

Acute oral toxicity of neem technical, neem formulation, karanj technical, *Annona squamosa* technical and quinalphos technical were carried out in albino mice (Kasauli strain) weighing 18-22 gms and albino rats (Haffkine strain) weighing 80-120 gms. Ten animals (5 males and 5 females) were taken per dose and for control. All the animals were fasted overnight prior to dosing but water was given *ad libitum*. The test compounds were given as such except for quinalphos which was diluted (1:400) in vegetable oil as it is immiscible with distilled water. Control groups of neem technical, neem formulation, karanj technical and *Annona squamosa* technical received distilled water, whereas control group of quinalphos received vegetable oil.

Preliminary doses were given to one male and one female at 0.6 log interval for selection of dosages for final assay⁷⁵. The final test was terminated one week after dosing where no delayed mortality was observed. When delayed mortality was observed, the observation period was extended to two weeks after dosing. All animals dead during the experimental period and those that were alive at the end of the experimental period were sacrificed and post-mortemed for gross pathological changes. The LD₅₀ values were determined by the method of Litchfield and Wilcoxon⁷⁶.

Acute oral toxicity in chickens and pigeons (MLD) :

Acute oral toxicity of neem technical, neem formulation and quinalphos technical were carried out in chickens (White leghorn, females) weighing 1.5-2.0 kg that were obtained from Central Poultry Farm, Bombay and pigeons (*Columbia livia*, Blue Rock) weighing 300-400 gms (either sex) that were obtained from local supplier. The birds were observed for 7 days before the start of the experiment. Chickens were fed Layer mash while the pigeons were fed millets. Water was given *ad lib*. Three birds were taken per dose group. The birds were fasted overnight before the start of the test but water was given *ad lib*. The test compounds were given as such except for quinalphos which was diluted 1 : 113 times (for chickens) and 1:400 (for pigeons) in vegetable oil as it is immiscible with distilled water. Control groups of neem technical, neem formulation received distilled water, whereas control group of quinalphos received vegetable oil.

Preliminary range finding doses were given to one bird at 0.6 log interval for selection of doses for final assay. The final doses were administered orally to each bird with the help of flexible PVC tubing. The birds were observed for a period of three weeks for clinical symptoms and mortality. The MLD (minimum lethal dose required to kill one out of the three birds) was found^{74,77}.

Acute toxicity in fish (LC₅₀) :

Toxicity study of neem technical, neem formulation and quinalphos were carried out in fresh water fish - *Talapia mossambica*. They were acclimatised at room temperature for ten days before the test. They were regularly fed during the acclimatisation period. Ten healthy fish about 5-7.5 cm in length were used for each experimental concentration of the sample under test and for control. Aged chlorine-free water was used as test water and diluent water was analysed for hardness, acidity, alkalinity, dissolved oxygen, free chlorine, pH and temperature before use.

Preliminary study was carried out on two fish per concentration for the selection of the concentrations for final assay. The test and the control fish were observed at regular intervals for 96 hours for abnormal behaviour, loss of equilibrium and death. The surviving fish were transferred to separate tanks containing diluent water for further period of 24 hours observation. The LC_{50} values were obtained by graphical interpolation⁷⁸.

Acute dermal toxicity in rabbits (LD_{50}) :

Acute dermal toxicity of neem technical, neem formulation and quinalphos were carried out in albino rabbits weighing 1.5-2.0 kg. Four animals (2 males and 2 females) were taken for each dose group and for control. Their backs were depilated by clipping the hair. Known amounts of the samples were applied on the depilated backs. The animals were then placed in an immobilised position in animal holders. The samples were then allowed to remain in contact with the skin for 24 hours. The animals were then observed for any toxic symptoms. At the end of the 24 hours the animals were removed from their respective holders, observed for gross symptoms of toxicity and the subjects were then cleaned thoroughly by wiping. They were then transferred to individual cages for further observation of 15 days for any symptoms of toxicity and mortality. All the animals that were dead during the observation period and those that were alive at the end of the experiment were sacrificed and post-mortemed for gross pathological changes. The LD_{50} was obtained by the method of Litchfield and Wilcoxon⁷⁶.

Mucous membrane irritation study :

The study was carried out on neem technical, neem formulation and quinalphos technical in albino rabbits (females) weighing 1.5-2.0 kg. Six healthy female rabbits were taken for the test and as control. Their vaginal mucous membranes were examined before the test. 0.1 ml of the test compound

was introduced into the upper half of the vaginal vault of the test rabbits. Similarly, 0.1ml of sterile normal saline was introduced in the upper half of the vaginal vault of the control rabbits. Mucous membrane of each animal was examined for reactions at 24, 48 and 72 hours. All the animals were observed for 15 days⁷⁹.

Primary Skin Irritation Study :

The study on neem technical, neem formulation and quinalphos technical was carried out in albino rabbits of either sex weighing 1.5-2.0 kg. Six healthy rabbits were selected for the test and their backs were depilated by clipping the hair. From the depilated backs of each animal four sites of one sq. inch area were selected. Two sites were abraded and two were left intact. 0.5 ml of neem technical and neem formulation and 0.1 ml of quinalphos was introduced under one abraded site and one intact site, while on the remaining two sites 0.5 ml of normal saline was introduced, which served as control. All the four sites were wrapped and held firmly with gauge. The animals were immobilised in animal holders with patches secured in place. After 24 hours exposure, the patches were removed and the resulting skin reaction in terms of erythema and edema were graded from 0-4 after 24 and 72 hours and the Primary Skin Irritation Index was obtained⁷⁹.

Neurotoxicity :

After review of the acute oral toxicity study regarding the lethal dose and variation in animal response, a dose range finding study was performed using one chicken weighing around 1.5 to 2.0 kg to determine the maximum tolerable dose which produces severe symptoms but not mortality within a period of one week (the range finding duration). Additionally, acceptability of diet, body weight, egg laying capacity and other symptoms were observed during the range finding study. Birds were housed individually in cages.



Based on the range finding study, two doses, 2650.0 mg/kg and 1325.0 mg/kg for neem technical and 5100.0 mg/kg and 2550.0 mg/kg for neem formulation were selected to be administered orally.

Group of three chickens (females) were selected for each test group and for control group. The test compounds were given orally to the test chickens for 21 days. Control group received just distilled water. The test birds including control birds were observed for general health, behavioural changes, abnormal symptoms, food intake, body weights, egg laying capacity, egg weights and mortality during the experiment.

Laboratory investigations :

The following tests were performed on all birds before (0 day) and after termination (22 day) of the test. The methods employed for the determinations were as per standard procedures⁸⁰⁻⁸⁷.

Haematology : Haemoglobin, packed cell volume, red blood cell count, white blood cell count, differential count.

Biochemical parameters : Whole blood and plasma cholinesterase, plasma glutamic pyruvic transaminase, plasma alkaline phosphatase, plasma proteins, plasma albumin and blood sugar.

Histopathology : All the birds were sacrificed at the end of the experimental period for recording gross lesions and their tissues were taken for histopathology. The tissues were collected immediately after post-mortem and were fixed in 10% formal saline for histopathology.

Statistical analysis were done as per student 't' test⁸⁸.

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Results

In the present study the acute oral toxicity of neem technical, neem formulation, karanj technical, *Annona squamosa* technical (methanol and ethyl acetate extracts) and quinalphos technical were undertaken in mice and rats. Acute toxicity of neem technical, neem formulation and quinalphos technical were undertaken in chickens, pigeons and fish; acute dermal toxicity study and also primary skin irritation study and mucous membrane irritation study were performed on rabbits.

Results of the acute oral toxicity in mice show that the LD₅₀ of neem technical was found to be 19080.0 mg/kg (Table 1) and of karanj technical (Table 3) to be 8308.0 mg/kg, whereas of neem formulation (Table 2), *Annona squamosa* technical (methanol extract) (Table 4) and *Annona squamosa* technical (ethyl acetate extract) (Table 5) were more than 20400.0 mg/kg, 15135.0 mg/kg and 15285.0 mg/kg respectively, whereas LD₅₀ of quinalphos was found to be 25.10 mg/kg (Table 6).

Results of the acute toxicity study in rats show that the LD₅₀ of neem technical was 16695.0 mg/kg (Table 7) and of karanj technical was 5418.6 mg/kg (Table 9), of neem formulation (Table 8), *Annona squamosa* technical (methanol extract) (Table 10) and *Annona squamosa* technical (ethyl acetate extract) (Table 11) were more than 15300.0 mg/kg, 15135.0 mg/kg and 15285.0 mg/kg respectively, whereas LD₅₀ of quinalphos was found to be 39.55 mg/kg (Table 12).

The pattern of symptoms noted with quinalphos were excitation, respiratory disturbances (gasping), salivation, tremors, convulsions, and death. Symptoms observed with neem technical and karanj technical were excitation, tremors convulsions and death. Animals administered *Annona squamosa* technical (methanol and ethyl acetate extracts) and neem formulation appeared normal. No gross pathological changes were observed in animals on post-mortem studies.

Table 1
Acute Oral Toxicity Study of Neem Technical in Mice (LD₅₀)*

No.	Dose ml/kg mg/kg		Mortality	% Mortality (14 days)
1	control		0/10	0
2	10.0	10600.0	0/10	0
3	15.0	15900.0	2/10	20
4	17.5	18550.0	4/10	40
5	20.0	21200.0	7/10	70

LD₅₀ : 19080.0 mg/kg
 Lower limit : 17066.0 mg/kg
 Higher limit : 21330.0 mg/kg
 (at 95% confidence level)

Table 2
Acute Oral Toxicity Study of Neem Formulation in Mice (LD₅₀)*

No.	Dose ml/kg mg/kg		Mortality	% Mortality (7 days)
1	control		0/0	0
2	5.0	5100.0	0/10	0
3	10.0	10200.0	0/10	0
4	15.0	15300.0	0/10	0
5	20.0	20400.0	0/10	0

LD₅₀ : > 20400.0 mg/kg

Table 3
Acute Oral Toxicity Study of Karanj in Mice (LD₅₀)*

No.	Dose ml/kg mg/kg		Mortality	% Mortality (14 days)
1	control		0/10	0
2	7.5	6773.2	0/10	0
3	8.0	7676.3	2/10	20
4	9.0	8127.9	4/10	40
5	9.5	8579.4	6/10	60
6	10.0	9031.0	8/10	80
7	12.5	11288.7	10/10	100

LD₅₀ : 8308.0 mg/kg
 Lower Limit : 8082.0 mg/kg
 Higher Limit : 8541.0 mg/kg
 (at 95% confidence level)

*LD₅₀ determination by Litchfield and Wilcoxon method⁷⁶

Table 4
Acute Oral Toxicity Study of *Annona squamosa*
(Methanol Extract) in Mice (LD₅₀)*

No.	Dose ml/kg mg/kg		Mortality	% Mortality (7days)
1	control		0/10	0
2	5.0	5045.0	0/10	0
3	10.0	10090.0	0/10	0
4	15.0	15135.0	0/10	0

LD₅₀ : > 15135.0 mg/kg

Table 5
Acute Oral Toxicity Study of *Annona squamosa*
(Ethyl Acetate Extract) in Mice (LD₅₀)*

No.	Doŝe ml/kg mg/kg		Mortality	% Mortality (7 days)
1	control		0/10	0
2	5.0	5095.0	0/10	0
3	10.0	10190.0	0/10	0
4	15.0	15285.0	0/10	0

LD₅₀ : > 15285.0 mg/kg

Table 6
Acute Oral Toxicity Study of Quinalphos in Mice (LD₅₀)*

No.	Dose ml/kg mg/kg		Mortality	% Mortality (14 days)
1	control		0/10	0
2	2.5	7.05	0/10	0
3	5.0	14.1	0/10	0
4	7.5	21.15	3/10	30
5	10.0	28.2	7/10	70
6	12.5	35.25	8/10	80
7	15.0	42.3	10/10	100

LD₅₀ : 25.1mg/kg

Lower limit : 21.1 mg/kg

Higher limit : 29.9 mg/kg

(at 95% confidence level)

*LD₅₀ determined by Litchfield and Wilcoxon Method⁷⁴

Table 7
Acute Oral Toxicity Study of Neem Technical in Rats (LD₅₀)*

No.	Dose		Mortality	% Mortality (14 days)
	ml/kg	mg/kg		
1	control		0/10	0
2	5.0	5300.0	0/10	0
3	10.0	10600.0	1/10	10
4	12.5	13250.0	2/10	20
5	15.0	15900.0	6/10	60
6	17.5	18550.0	6/10	60
7	20.0	21200.0	8/10	80

LD₅₀ : 16695.0 mg/kg
Higher limit : 14774.0 mg/kg
Lower Limit : 18865.0 mg/kg
(at 95% confidence level)

Table 8
Acute Oral Toxicity Study of Neem Formulation in Rats (LD₅₀)*

No.	Dose		Mortality	% Mortality (7 days)
	ml/kg	mg/kg		
1	control		0/10	0
2	5.0	5100.0	0/10	0
3	10.0	10200.0	0/10	0
4	15.0	15300.0	0/10	0

LD₅₀ : > 15300.0 mg/kg

Table 9
Acute Oral Toxicity Study of Karanj in Rats (LD₅₀)*

No.	Dose		Mortality	% Mortality (14 days)
	ml/kg	mg/kg		
1	control		0/10	0
2	1.25	1128.8	0/10	0
3	2.5	2257.7	2/10	20
4	5.0	4515.5	4/10	40
5	7.5	6773.2	6/10	60
6	10.0	9031.0	7/10	70
7	12.5	11288.2	10/10	100

LD₅₀ : 5418.6 mg/kg
Higher limit : 8344.6 mg/kg
Lower limit : 3518.5 mg/kg
(at 95% confidence level)

*LD₅₀ determined by Litchfield and Wilcoxon Method "

Table 10
Acute Oral Toxicity Study of *Annona squamosa*
(Methanol Extract) in Rats (LD₅₀)*

No.	Dose		Mortality	% Mortality (7 days)
	ml/kg	mg/kg		
1	control		0/10	0
2	5.0	5045.0	0/10	0
3	10.0	10090.0	0/10	0
4	15.0	15135.0	0/10	0

LD₅₀ : > 15135.0 mg/kg

Table 11
Acute Oral Toxicity Study of *Annona squamosa*
(Ethyl Acetate Extract) in Rats (LD₅₀)*

No.	Dose		Mortality	% Mortality (7days)
	ml/kg	mg/kg		
1	control		0/10	0
2	5.0	5095.0	0/10	0
3	10.0	10190.0	0/10	0
4	15.0	15285.0	0/10	0

LD₅₀ : > 15285.0 mg/kg

Table 12
Acute Oral Toxicity Study of Quinalphos in Rats (LD₅₀)*

No.	Dose		Mortality	% Mortality (14 days)
	ml/kg	mg/kg		
1	control		0/10	0
2	5.0	14.12	0/10	0
3	10.0	28.25	2/10	20
4	15.0	42.37	6/10	60
5	20.0	56.50	8/10	80
6	25.0	70.62	10/10	100

LD₅₀ : 39.55 mg/kg
Higher limit : 48.726 mg/kg
Lower limit : 32.102 mg/kg
(at 95% confidence level)

*LD₅₀ determined by Litchfield and Wilcoxon method ⁷²

In chickens, MLD (minimum lethal dose) of neem technical and neem formulation were found to be more than 15900.0 mg/kg (Table 13) and 15300.0 mg/kg (Table 14) respectively, whereas of quinalphos it was 10.0 mg/kg (Table 15).

In pigeons, the MLD of neem technical and neem formulation was found to be more than 15900.0 mg/kg (Table 16) and 15300.0 mg/kg (Table 17) respectively, whereas of quinalphos technical was found to be 2.0 mg/kg (Table 18).

The pattern of symptoms observed with quinalphos technical were excitation, respiratory disturbances, salivation, tremors, convulsions, ataxia and death, whereas the birds fed with neem technical and neem formulation appeared normal.

Results of the acute toxicity in fish showed that the LC_{50} of neem technical was 280.0 ppm (Table 19) and of neem formulation (Table 20) was 1300.0 ppm as compared to 1.0 ppm for quinalphos (Table 21). The pattern of symptoms observed were excitation, loss of equilibrium and death.

In the acute dermal toxicity study, the LD_{50} of neem technical and neem formulation were found to be more than 10600.0 mg/kg (Table 22) and 15300.0 mg/kg (Table 23) respectively in rabbits, as compared to 339.0 mg/kg for quinalphos (Table 24). The pattern of symptoms noted with quinalphos were excitation, tremors, convulsions and death, whereas rabbits fed neem technical and neem formulation appeared normal.

In the mucous membrane irritation study and primary skin irritation study of neem technical and neem formulation, conducted on rabbits, they were found to be mild irritants, whereas quinalphos was found to be moderately irritant in comparison (Tables 25-30).

Table 13
Toxicity Study of Neem Technical in Chickens (MLD)

No.	Dose		Mortality
	ml/kg	mg/kg	
1	control		0/3
2	5.0	5300.0	0/3
3	10.0	10600.0	0/3
4	15.0	15900.0	0/3

MLD : > 15900.0 mg/kg

Table 14
Toxicity Study of Neem Formulation in Chickens (MLD)

No.	Dose		Mortality
	ml/kg	mg/kg	
1	control		0/3
2	5.0	5100.0	0/3
3	10.0	10200.0	0/3
4	15.0	15300.0	0/3

MLD : > 15300.0 mg/kg

Table 15
Acute Toxicity Study of Quinalphos in Chickens (MLD)

No.	Dose		Mortality
	ml/kg	mg/kg	
1	control		0/3
2	0.8	8.0	0/3
3	1.0	10.0	1/3
4	1.2	12.0	3/3

MLD : > 10.0 mg/kg

Table 16
Acute Toxicity Study of Neem Technical in Pigeons (MLD)

No.	Dose		Mortality
	ml/kg	mg/kg	
1	control		0/3
2	5.0	5300.0	0/3
3	10.0	10600.0	0/3
4	15.0	15900.0	0/3

MLD : > 15900.0 mg/kg

Table 17
Acute Toxicity Study of Neem Formulation in Pigeons (MLD)

No.	Dose		Mortality
	ml/kg	mg/kg	
1	control		0/3
2	5.0	5100.0	0/3
3	10.0	10200.0	0/3
4	15.0	15300.0	0/3

MLD : > 15300.0 mg/kg

Table 18
Acute Toxicity Study of Quinalphos in Pigeons (MLD)

No.	Dose		Mortality
	ml/kg	mg/kg	
1	control		0/3
2	0.4	1.0	0/3
3	0.8	2.0	1/3
4	2.0	5.0	3/3

MLD : 2.0 mg/kg

Table 19
Acute Toxicity Study of Neem Technical in Fish (LC₅₀)*

No.	Dose ppm	Mortality	% Mortality (96 hrs)
1	control	0/10	0
2	100.0	0/10	0
3	200.0	4/10	40
4	300.0	7/10	70
5	400.0	10/10	100

LC₅₀ : 280 ppm

Table 20
Acute Toxicity Study of Neem Formulation in Fish (LC₅₀)*

No.	Dose ppm	Mortality	% Mortality (96 hrs)
1	control	0/10	0
2	500.0	0/10	0
3	1000.0	3/10	30
4	1500.0	7/10	70
5	2000.0	8/10	80
6	2500.0	10/10	100

LC₅₀ : 1300 ppm

Table 21
Acute Toxicity Study of Quinalphos in Fish (LC₅₀)*

No.	Dose ppm	Mortality	% Mortality (96 hours)
1	control	0/10	0
2	0.5	0/10	0
3	0.8	2/10	20
4	1.0	5/10	50
5	1.2	8/10	80
6	1.5	10/10	100

LC₅₀ : 1.0 ppm

* LC50 determined by graphical interpolation

Table 22
Acute Dermal Toxicity Study of Neem Technical in Rabbits (LD₅₀)*

No.	Dose		Mortality	% Mortality (14 days)
	ml/kg	mg/kg		
1	control		0/4	0
2	2.5	2650.0	0/4	0
3	5.0	5300.0	0/4	0
4	10.0	10600.0	0/4	0

LD₅₀ : > 10600.0 mg/kg

Table 23
Acute Dermal Toxicity Study of Neem Formulation in Rabbits (LD₅₀)*

No.	Dose		Mortality	% Mortality (14 days)
	ml/kg	mg/kg		
1	control		0/4	0
2	5.0	5100.0	0/4	0
3	10.0	10200.0	0/4	0
4	15.0	15300.0	0/4	0

LD₅₀ : > 15300.0 mg/kg

Table 24
Acute Dermal Toxicity Study of Quinalphos in Rabbits (LD₅₀)*

No.	Dose		Mortality	% Mortality (14 days)
	ml/kg	mg/kg		
1	control		0/4	0
2	0.25	113.0	0/4	0
3	0.50	226.0	1/4	25
4	0.75	339.0	2/4	50
5	1.00	452.0	3/4	75
6	1.25	565.0	4/4	100

LD₅₀ : 339.0 mg/kg

Higher limit : 547.49 mg/kg

Lower limit : 209.91 mg/kg
(at 95% confidence level)

*LD₅₀ determined by Litchfield and Wilcoxon method ⁷⁶

Table 25
Primary Skin Irritation Study : Neem Technical
Scores of Irritation

No.	24 hours				72 hours			
	Intact		Abraded		Intact		Abraded	
	Er	Ed	Er	Ed	Er	Ed	Er	Ed
1	0	0	1	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	1	0	0	0	0	0
4	0	0	1	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0

Table 26
Primary Skin Irritation Study : Neem Formulation
Scores of Irritation

No.	24 hours				72 hours			
	Intact		Abraded		Intact		Abraded	
	Er	Ed	Er	Ed	Er	Ed	Er	Ed
1	0	0	0	0	0	0	0	0
2	0	0	1	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	1	0	0	0	0	0
6	0	0	1	0	0	0	0	0

Table 27
Primary Skin Irritation Study : Quinalphos
Scores of Irritation

No	24 hours				72 hours			
	Intact		Abraded		Intact		Abraded	
	Er	Ed	Er	Ed	Er	Ed	Er	Ed
1	0	0	1	0	0	0	1	0
2	0	0	0	0	0	0	0	0
3	1	0	1	0	0	0	0	0
4	1	0	1	0	0	0	0	0
5	0	0	1	0	0	0	0	0
6	1	0	1	1	0	0	0	0

Er : Erythema; Ed : Edema

Note : In control sites (Intact and abraded) the skin irritation index was zero as erythema and edema were not observed.

Table 28
Mucous Membrane Irritation Study : Neem Technical
Scores of Irritation

No.	24 hours			48 hours			72 hours		
	Er	Ed	P.D.	Er	Ed	P.D.	Er	Ed	P.D.
1	0	0	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0	0	0
4	1	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0

Table 29
Mucous Membrane Irritation Study : Neem Formulation
Scores of Irritation

No.	24 hours			48 hours			72 hours		
	Er	Ed	P.D.	Er	Ed	P.D.	Er	Ed	P.D.
1	1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0	0	0
4	1	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0

Table 30
Mucous Membrane Irritation Study : Quinalphos
Scores of Irritation

No.	24 hours			48 hours			72 hours		
	Er	Ed	P.D.	Er	Ed	P.D.	Er	Ed	P.D.
1	2	2	2	1	1	0	0	0	0
2	1	0	0	0	0	0	0	0	0
3	1	1	0	0	0	0	0	0	0
4	2	1	0	1	0	0	0	0	0
5	2	2	1	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0

Er : Erythema;

Ed : Edema;

P.D. : Pus Discharge

Note : In control animals the mucous membrane index was zero as no erythema, edema and pus discharge was observed.

Neurotoxicity Study :

After the review of the acute oral toxicity study in chickens of neem technical and neem formulation, a range finding study was initiated using one chicken per dose group to determine maximum tolerable dose which produces symptoms but not mortality within a period of one week.

Based on the range study, the following doses were selected to be administered daily orally for 21 days :

Neem concentrate :

Higher dose : 2650.0 mg/kg

Lower dose : 1325.0 mg/kg

Neem formulation :

Higher dose : 5100.0 mg/kg

Lower dose : 2550.0 mg/kg

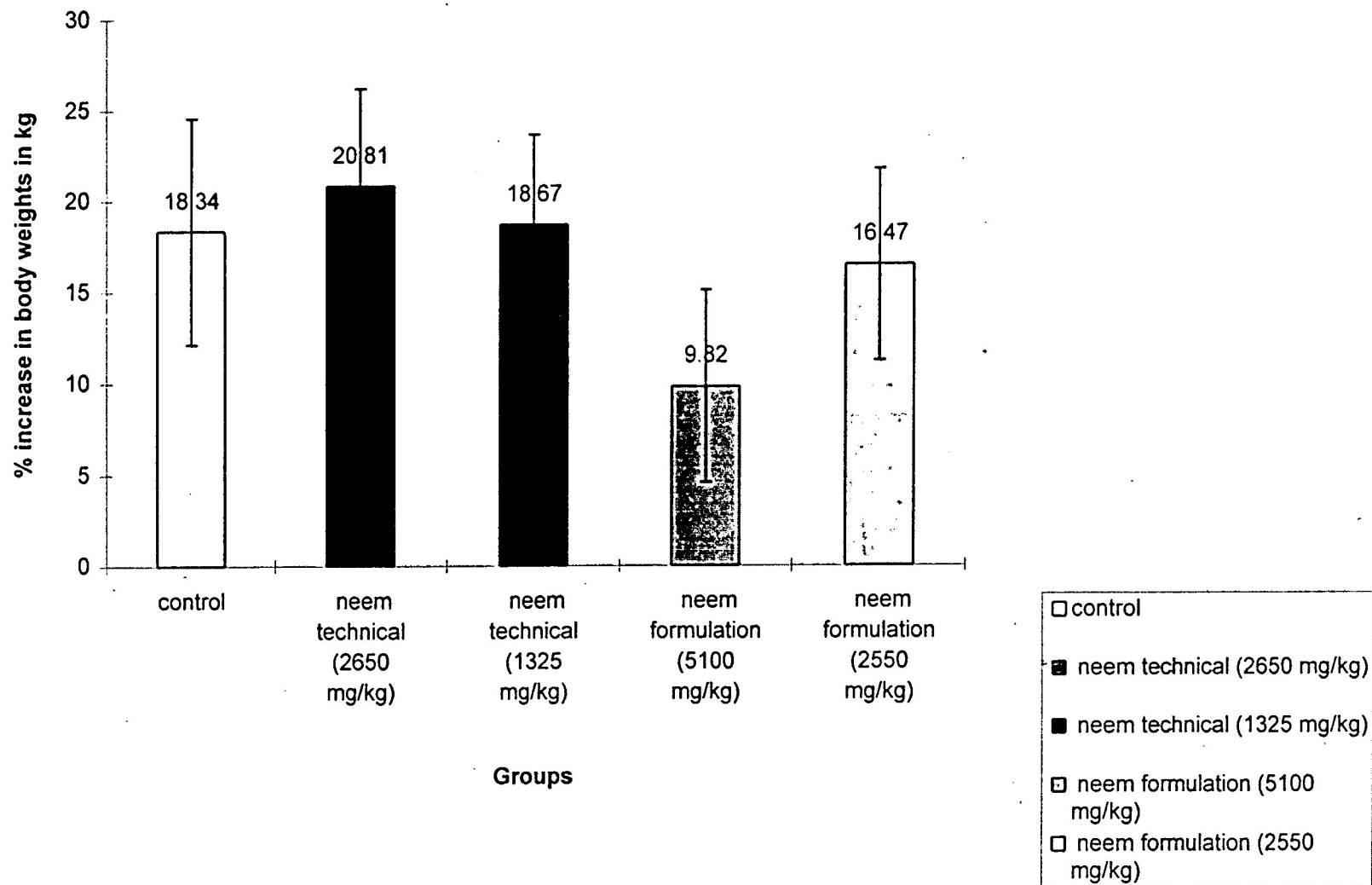
Control group that received distilled water was also included.

In the neurotoxicity studies, no mortality was observed in any of the groups over a period of 21 days. No significant changes in the body weights (Graph 1), food consumption (Graph 2a,2b,2c), egg weights (Graph 3) and number of eggs (Graph 4) laid were observed and ataxia or death were also not observed during the experimental period of feeding the test compounds. The general health was also comparable with the control group.

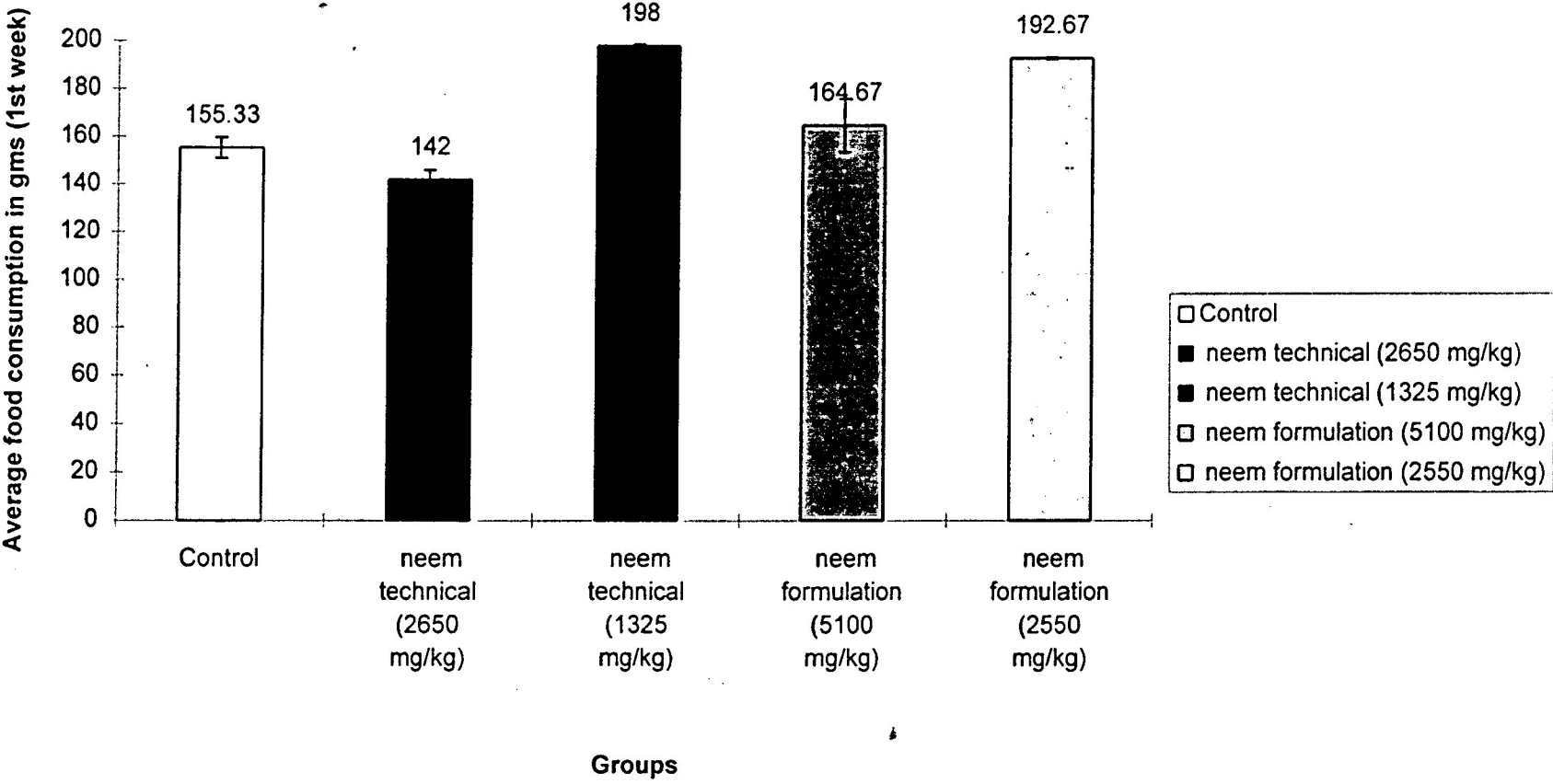
Haematological findings (Table 31 and Table 32) of the test groups did not differ from the control and were found to be within the normal range.

Biochemical parameters, viz. blood glucose, plasma alkaline phosphatase, plasma glutamic pyruvic transaminase, plasma proteins and plasma albumin (Table 33) were comparable with the control and were within the normal range. No reduction in plasma and whole blood cholinesterase (Table 34) was recorded and the levels were found within the normal range.

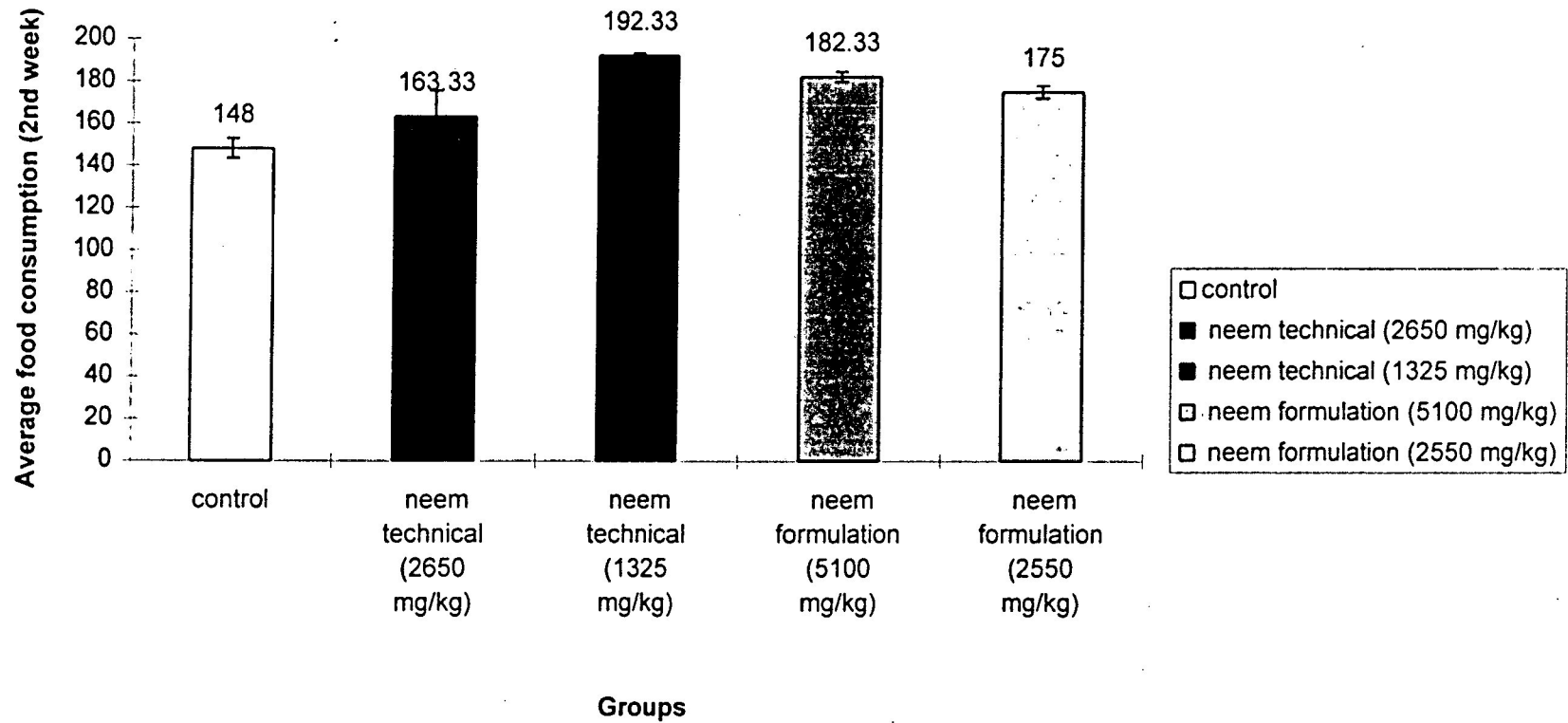
Percent gain in body weights of control, neem technical and neem formulation treated chickens



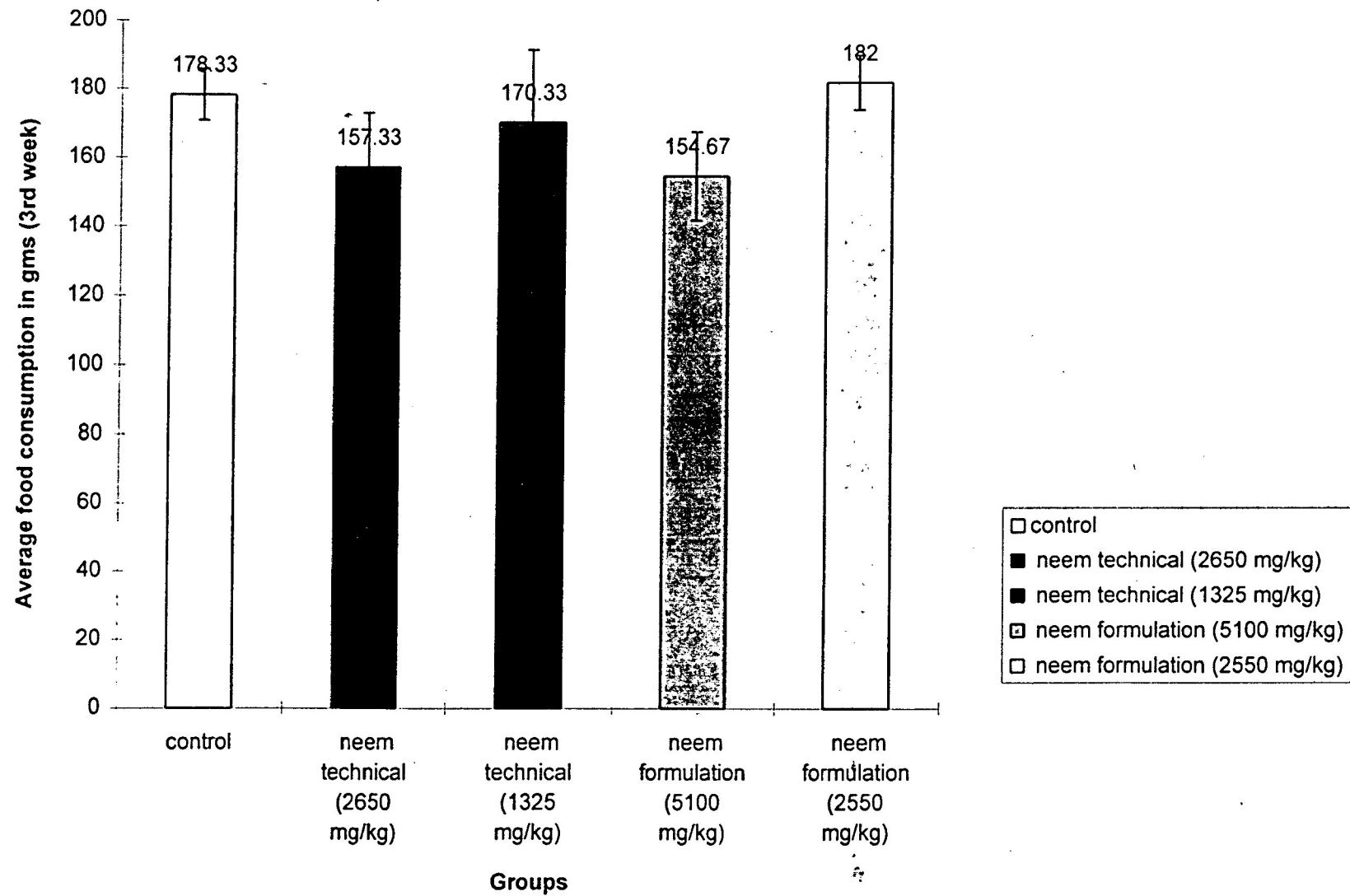
Average weekly food consumption of control, neem technical and neem formulation treated chickens



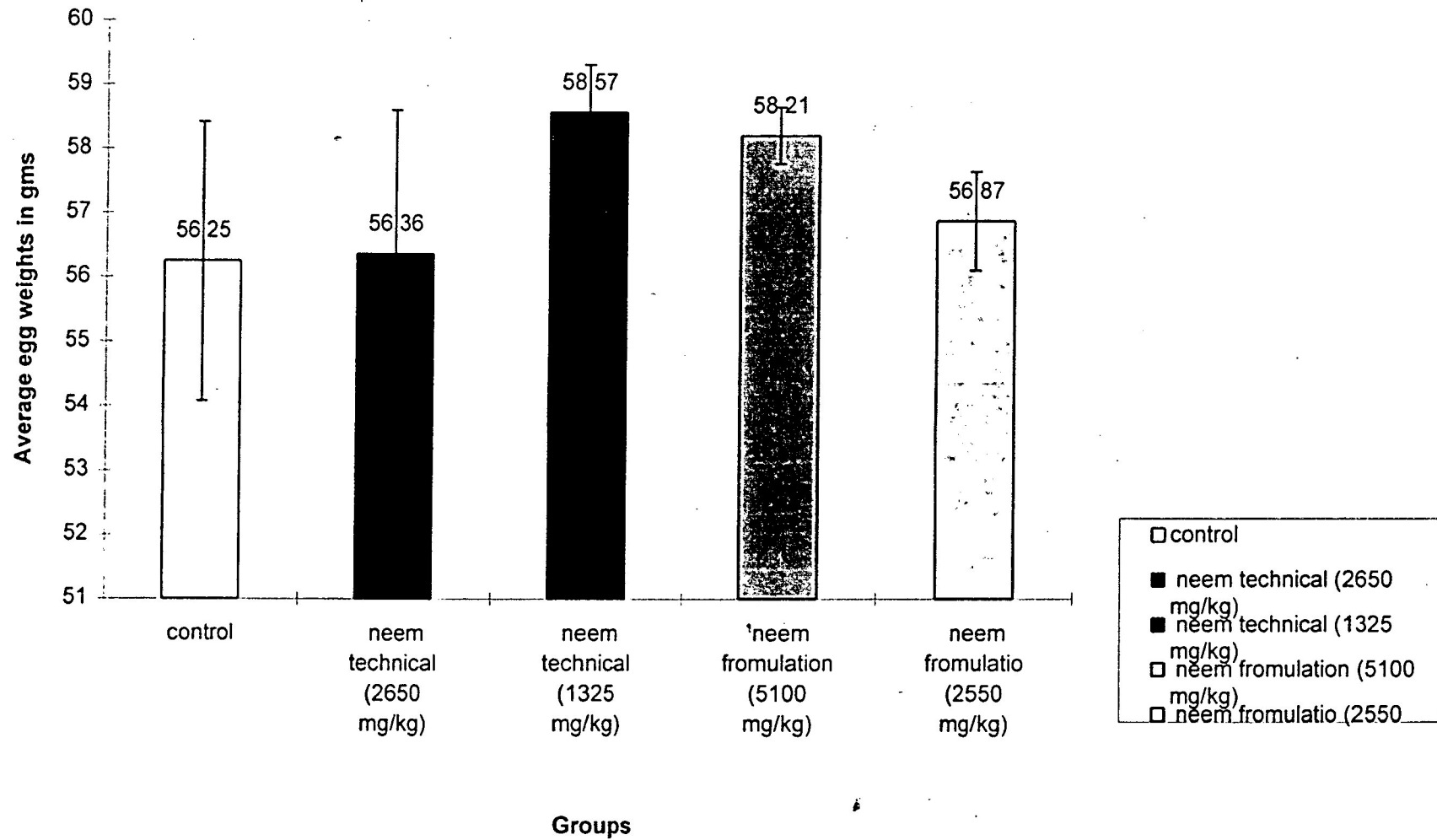
Average weekly food consumption of control, neem technical and neem formulation treated chickens



Average weekly food consumption of control, neem technical and neem formulation treated chickens



Average egg weights of control, neem technical and neem formulation treated chickens



No. of eggs laid by control, neem technical and neem formulation treated chickens

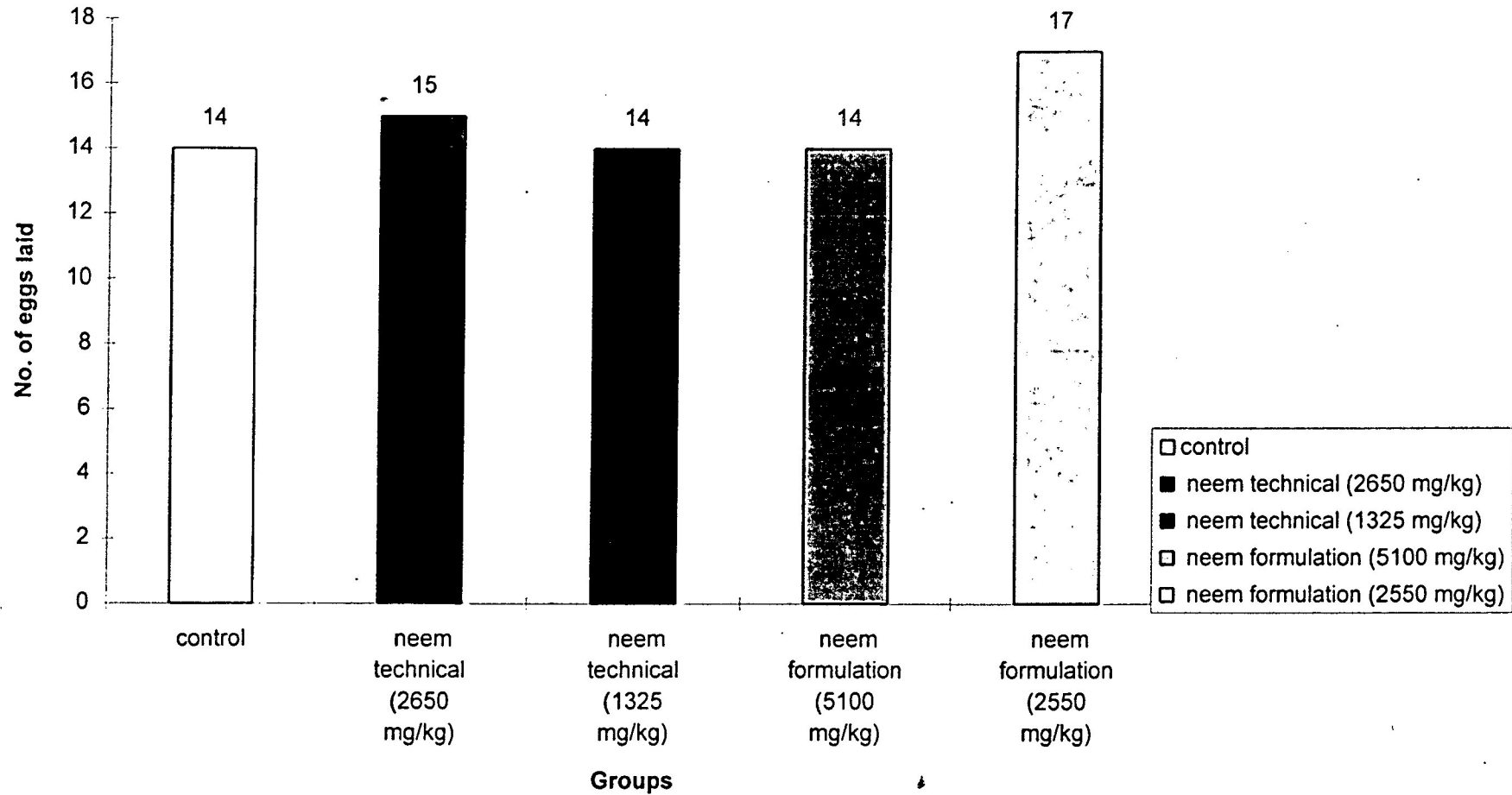


Table 31
Neurotoxicity Study : Haematological Parameters

Pre-Treatment								
Chicken No.	Hb	PCV	R.B.C.	W.B.C.	Differential Count			
					N	E	L	M
Control								
1	8.9	24	2.1	18000	30	0	70	0
2	8.6	26	2.0	19000	28	1	71	0
3	8.9	26	2.0	16000	21	2	75	2
Mean	8.8	25.3	2.03	17666	26.3	1.0	72.0	0.67
±S.D.	0.17	1.15	0.06	1527.5	4.73	1.0	2.65	1.15
Neem Technical 2650.0 mg/kg								
1	8.7	26	2.0	20000	25	1	73	1
2	8.9	24	2.2	18000	23	0	77	0
3	8.8	26	2.1	17000	21	0	78	1
Mean	8.8	25.3	2.1	18333	23.0	0.33	76.0	0.67
±S.D.	0.1	1.15	0.1	1527.5	2.0	0.58	2.65	0.58
Neem Technical 13250.0 mg/kg								
1	9.0	28	2.3	20500	24	1	75	0
2	8.8	26	2.0	20000	20	0	80	0
3	8.9	29	2.1	18000	20	2	77	1
Mean	8.9	27.6	2.13	19500	21.3	1.0	77.3	0.33
±S.D.	0.1	1.52	0.15	1322.8	2.31	1.0	2.52	0.58
Neem Formulation 5100.0 mg/kg								
1	8.9	28	2.0	20000	21	0	79	0
2	9.0	26	2.2	18000	25	2	72	1
3	9.0	29	2.3	20000	23	1	75	1
Mean	8.97	27.6	2.17	19333	23.0	1.0	75.3	0.67
±S.D.	0.06	1.53	0.15	1154.7	2.0	1.0	3.51	0.58
Neem Formulation 2550.0 mg/kg								
1	9.0	30	2.15	20000	25	0	75	0
2	8.8	26	2.0	18000	20	2	71	2
3	8.9	28	2.1	20000	28	1	70	1
Mean	8.9	28.0	2.08	19333	24.3	1.0	72.0	1.0
±S.D.	0.1	2.0	0.08	1154.7	4.04	1.0	2.65	1.0

Hb : Haemoglobin Count (gm%); PCV : Packed Cell Volume(ml);
R.B.C. : Red Blood Cell Count ($10^6/\text{mm}^3$); W.B.C. : White Blood Cell Count (/million);
N : Neutrophils (%); E : Eosinophils (%); L : Lymphocytes(%); M : Monocytes (%).

Table 32
Neurotoxicity Study : Haematological Parameters

Post-Treatment								
Chicken No.	Hb	PCV	R.B.C.	W.B.C.	Differential Count			
					N	E	L	M
Control								
1	9.2	26	2.18	19000	27	1	71	1
2	9.0	28	2.15	17500	22	2	75	1
3	9.1	25	2.25	17000	20	0	80	0
Mean	9.1	26.33	2.19	17833	23.0	1.0	75.3	0.67
±S.D.	0.1	1.52	0.05	1040.8	3.61	1.0	4.51	0.58
Neem Technical : 2650.0 mg/kg								
1	9.0	28	2.2	18500	29	1	70	0
2	9.2	25	2.35	20000	25	2	73	0
3	9.0	25	2.2	19000	20	0	80	0
Mean	9.07	26.0	2.25	19166	24.6	1.0	74.3	0
±S.D.	0.12	1.73	0.09	763.76	4.51	1.0	5.13	
Neem Technical : 1325.0 mg/kg								
1	9.4	29	2.45	18000	22	1	76	1
2	8.9	25	2.25	19500	30	1	69	0
3	8.9	32	2.2	19000	25	1	73	1
Mean	9.07	28.67	2.3	18833	25.6	1.0	72.6	0.67
±S.D.	0.29	3.51	0.13	763.76	4.04	0.0	3.51	0.58
Neem Formulation : 5100.0 mg/kg								
1	9.1	30	2.3	21000	27	2	70	1
2	9.3	27	2.35	19000	20	0	80	0
3	9.2	28	2.40	18000	30	1	68	1
Mean	9.2	28.33	2.35	19333	25.6	1.0	72.6	0.67
±S.D.	0.1	1.25	0.05	1527.5	5.13	1.0	6.40	0.58
Neem Formulation : 2550.0 mg/kg								
1	9.5	30	2.35	21000	20	1	78	1
2	8.9	29	2.3	17500	25	0	80	0
3	9.2	27	2.25	21000	24	1	74	1
Mean	9.2	28.67	2.3	19833	23.0	0.67	77.3	0.67
±S.D.	0.3	1.53	0.05	2020.7	2.65	0.58	3.06	0.58

Hb : Haemoglobin Count (gm%); PCV : Packed Cell Volume(ml);
R.B.C. : Red Blood Cell Count ($10^6/\text{mm}^3$); W.B.C. : White Blood Cell Count (/million);
N : Neutrophils (%); E : Eosinophils (%); L : Lymphocytes(%); M : Monocytes (%).

Table 33
Neurotoxicity Study : Biochemical Parameters

Chicke No.	Control		Neem Technical 2650 mg/kg 1325 mg/kg				Neem Formulation 5100 mg/kg 2550mg/kg			
	I	F	I	F	I	F	I	F	I	F
Total Proteins (gm%)										
1	5.28	7.07	6.20	7.16	5.41	5.89	7.45	7.44	6.95	6.97
2	5.96	6.53	5.89	5.81	5.38	6.53	5.28	6.62	6.07	7.23
3	6.07	7.92	7.99	8.11	6.70	6.74	5.11	6.89	5.97	7.28
Mean	5.77	7.17	6.69	7.03	5.83	6.39	5.95	6.98	6.33	7.16
±S.D.	0.43	0.70	1.13	1.16	0.75	0.44	1.30	0.42	0.54	0.17
Plasma Albumin (gm%)										
1	3.93	4.09	2.72	2.81	2.97	2.90	2.55	3.01	4.55	3.99
2	3.13	3.21	2.75	2.83	3.11	3.24	2.58	3.18	4.08	4.24
3	3.95	4.19	3.12	3.25	3.16	3.44	2.12	3.29	3.29	2.41
Mean	3.67	3.83	2.86	2.96	3.08	3.19	2.42	3.16	3.97	3.55
±S.D.	0.47	0.54	0.22	0.25	0.10	0.27	0.26	0.14	0.64	0.99
Blood Glucose (mg%)										
1	160	152	184	188	182	171	144	177	171	158
2	170	165	161	156	176	163	163	136	173	185
3	135	144	182	190	169	179	144	160	166	164
Mean	155	154	176	178	176	171	150	157	170	169
±S.D.	18.8	11.0	12.9	18.9	6.9	7.8	10.5	20.4	3.5	13.4
Plasma Glutamic Pyruvic Transaminase (K.Units)										
1	20.5	28.7	18.6	19.7	15.6	18.4	28.3	24.2	17.8	22.0
2	22.5	29.3	18.7	17.7	15.9	15.6	14.0	16.2	16.3	18.9
3	26.4	21.5	15.1	29.1	17.8	16.6	12.7	11.2	20.3	16.6
Mean	23.1	26.5	17.5	22.2	16.5	16.9	18.3	17.3	18.2	19.2
±S.D.	3.0	4.4	2.0	6.1	1.2	1.4	8.6	6.6	2.1	2.7
Plasma Alkaline Phosphatase (K.A.Units)										
1	28.7	34.0	37.3	33.6	36.2	37.9	30.7	27.1	26.7	21.4
2	36.3	31.6	32.2	33.2	34.1	32.9	21.9	26.7	25.5	27.2
3	39.7	37.6	35.8	39.3	27.0	22.3	31.1	28.6	29.2	29.6
Mean	34.9	34.9	35.1	35.4	32.4	31.0	27.9	27.4	27.1	26.1
±S.D.	5.6	3.0	2.6	3.4	4.8	7.9	5.3	0.97	1.9	4.19

I : Initial ; F : Final

Table 34
Neurotoxicity Study : Cholinesterase levels

Chicke No.	Control		Neem Technical 2650 mg/kg 1325 mg/kg				Neem Formulation 5100 mg/kg 2550 mg/kg			
	I	F	I	F	I	F	I	F	I	F
Plasma Cholinesterase										
1	4.83	5.23	5.82	6.32	6.63	7.50	4.34	8.61	9.18	8.25
2	6.42	7.51	5.47	5.69	5.77	8.68	5.80	7.73	8.60	9.74
3	5.83	6.25	6.82	7.46	5.30	7.08	6.94	9.99	6.74	7.49
Mean	5.69	6.33	6.03	6.49	5.90	7.7	5.69	8.78	8.16	8.49
±S.D.	0.80	1.14	0.70	0.89	0.67	0.83	1.30	1.14	1.29	1.14
Whole Blood Cholinesterase										
1	30.7	36.8	30.6	33.7	37.8	35.2	33.9	38.9	36.5	35.3
2	32.7	34.2	38.4	39.8	36.4	38.1	31.7	34.9	34.6	38.1
3	34.3	38.4	34.3	40.3	32.4	36.4	44.2	40.6	28.6	33.8
Mean	32.5	36.8	34.4	37.9	35.5	36.5	36.6	38.1	33.2	35.7
±S.D.	1.81	2.11	3.94	3.71	2.79	1.49	6.66	2.90	4.10	2.16

I : Initial ; F : Final

Gross and Histopathological changes :

Gross changes : Macroscopic examination of tissues of birds treated with 2650.0 mg/kg and 1325.0 mg/kg neem technical and 5100.0 mg/kg and 2550.0 mg/kg neem formulation showed no marked changes and were comparable with the control tissues.

Histopathological changes : Observation of the tissues - brain, spinal cord and sciatic nerve from birds treated with neem technical and neem formulation revealed no abnormality and were comparable to the control group. No demyelination or degeneration was observed in any of the above tissues (Table 35).



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Table 35
Neurotoxic potential of Neem Technical and Neem Formulation in Chickens
Histopathological Observations

Chicken No.	Dose mg/kg	Tissue	Histopathological Findings
1	control	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
2	control	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
3	control	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
Neem Technical			
4	2650.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
5	2650.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
6	2650.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
7	1325.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
8	1325.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
9	1325.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
Neem Formulation			
10	5100.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
11	5100.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
12	5100.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
13	2550.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
14	2550.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal
15	2550.0	Brain Spinal cord Sciatic Nerve	Normal Normal Normal

Discussion

Humans, animals, wildlife and all living systems in the environment are constantly exposed daily to a variety of natural and synthetic chemicals. Pesticide chemicals are used extensively in agriculture, against vectors, transmitting diseases and for household purposes.

The use of large amounts of pesticide warrants toxicological evaluation of the potential for their possible adverse effects. Acute, chronic, cancer effects and occupational hazards are of concern.

Hence from the point of view of safety to humans, the regulatory agencies around the world have laid down certain guidelines to assess the potential toxicity of the pesticide. Toxicity studies usually provide the important basic information required for consideration of safe use of pesticides and become extremely important for the regulatory purposes.

Due to ethical reasons, these studies are performed on animal models^{89,99}. Although, animal experiments that are designed to detect toxicity from chemicals do not assure 100% guarantee of total safety of the chemical for man but such tests supply information that will enable the safe use of chemicals through hazard identification and assessment and instills caution in regard to unwarranted exposure of man to many chemicals.

Animal toxicity experiments also prevent distinctly harmful chemicals from being readily available. So even if possible hazard to man cannot be completely eliminated, the inevitable risk observed in animals is sufficient to be socially acceptable as a possible risk.

During their actual usage, pesticides are generally used as their formulations. Formulations are composed of different concentrations of technical grade material along with various solvents, emulsifiers, stabilisers and other additives. Because of the variation in percentage of technical grade of different EC formulations, the impurity levels vary and the possible toxicity. The percentage of varying impurity may be present in the form of solvents, emulsifiers, and other additives. These ingredients may exert their own toxicity

or interfere with the metabolism/kinetics of technical grade material in the formulation. The presence of impurities in the formulations may result in various degrees of toxicity to the biological system on exposure to these pollutants. Therefore the extent of toxic effects that any pesticide may produce depends upon factors like the purity and the percentage of technical grade material, vehicle, nature of the emulsifier and stabilisers used.

There is no single study by which one can judge the total toxicity of a pesticide chemical. Therefore various toxicity studies are laid down by regulatory agencies to assess the potential toxicity of a pesticide chemical. Toxicity studies also provide important information for consideration of the safe use of pesticides and requirements for restriction on use.

Acute toxicity study is one important part of entire toxicological evaluation that is mandatory for regulatory purposes. Accidental, intentional and occupational hazard may occur with any consumer product such as pesticides. Acute toxicity study gives a rapid indication of the potential hazard under above circumstances and indicates the class of toxicity to which the pesticide belongs. Acute toxicity is the effect resulting from the administration of the compound as a single dose over a period of 24 hrs or less⁷⁵. The effect generally reported is mortality and is generally expressed as LD₅₀ (the dose required to kill 50% of the test animals of that dose). The value of LD₅₀ serves a convenient way to classify the pesticide into toxicity classes^{90,105} and also serves as a guide for the selection of doses for long-term studies. The LD₅₀ is expressed in milligrams/kilogram and is a measure of a chemical to cause acute poisoning^{17,91}. Regulatory agencies have issued guidelines for undertaking toxicity studies and types of tests, the type of animal model to be used for safety evaluation^{73,74,75,77}.

Pesticides, technical or their formulations which are used to kill plant and animal pests are not only toxic to insects and other pests, but as well to human beings, animals and environment. The other drawbacks of pesticides include

insect resistance and resurgence and higher farming costs. This has induced scientists to look for alternative methods and sources of pesticides which are pest-specific, nontoxic, safe, biodegradable, less prone to pest resistance and less expensive. Due to lack of detailed toxicological data of such potential pesticides of plant origin, its use inevitably is linked to examination of the potential of such plants or their products to cause risk to health of man and animals and to the ecosystem as a whole. It is invariably believed that medicines or pesticides of plant origin are safe and can be used without precaution. But it may not be true. Sometimes they may have serious side-effects^{55,59,103}. Therefore extracts of plant origin should be used with caution similar to products from synthetic origin. Hence, each preparation needs detailed toxicological evaluation before its commercial use.

In the present study, different types of acute toxicity studies of neem technical, neem formulation, karanj technical, *Annona squamosa* technical (methanol and ethyl acetate extracts) were undertaken in mice, rats, chickens, pigeons, fish and rabbits. For comparison, acute toxicity of quinalphos, an organophosphorus pesticide was also studied.

LD₅₀ values of karanj technical in mice and rats were comparatively less (8308.0 mg/kg and 5418.6 mg/kg respectively) than LD₅₀ of neem technical (19080.0 mg/kg and 16695.0 mg/kg respectively), neem formulation (more than 20400.0 mg/kg in mice and more than 15300.0 mg/kg in rats), *Annona squamosa* technical (methanol extract) and *Annona squamosa* technical (ethyl acetate extract) more than 15135.0 mg/kg and more than 15285.0 mg/kg respectively in mice and rats, whereas LD₅₀ of quinalphos was found to be 39.55 mg/kg in rats and 25.10 mg/kg in mice (Tables 1-12).

Results of the above pesticides in mice and rats suggest that neem technical, neem formulation, karanj technical and *Annona squamosa* technical (both extracts) are practically nontoxic with respect to acute toxicity rating (Appendix 1 and 2) whereas quinalphos is highly toxic.

Birds form a part of the complex ecology of the world and many a time innocent victims of agricultural practice and environmental pollution. The regulatory agencies require toxicity to be carried out on atleast two avian species. Birds may ingest toxic substances and may then be eaten by other animals including man, thus forming part of a food chain. Hence in our study, toxic response in terms of MLD (minimum lethal dose) was found out in chickens and pigeons. MLD is the dose required to kill one out of the three birds of the test group⁷⁷. The results show that quinalphos (MLD : chickens : 10.0 mg/kg and pigeons : 2.0 mg/kg) is very toxic to both species compared to neem technical and neem formulation (MLD more than 15900.0 mg/kg and more than 15300.0 mg/kg respectively in both species) (Tables 13-18).

As part of environmental effect, fish may also be affected when the pesticides are applied to control pests, reach sources of water following spillage or drift. Thus determination of LC_{50} is important to determine the lethal concentration to fish. In our study, the results suggest that quinalphos (LC_{50} : 1.0 ppm) was very toxic in comparison to neem technical (LC_{50} : 280 ppm) and neem formulation (LC_{50} : 1300.0 ppm) to fish (Tables 19-21).

The skin is a relatively good barrier for lipid- and water-soluble substances. The absorption and distribution pathways of pesticides in the body will depend on factors such as route of exposure, physico-chemical properties of the molecule, species, percent purity of the pesticides solvent used, metabolic conversion of the pesticide, hydration of the skin. In the process of skin protection one barrier that exists lies in the stratum corneum. This barrier layer is said to be very slender in hairy animals and this may be the probable reason why clipped skin of laboratory animals is much more easily penetrated by substances than human skin. Penetration is related to the distribution coefficient between water and non-polar solvent^{92,93,94,95}. Many substances can be absorbed in sufficient quantities to produce acute or chronic systemic effects. Any substance capable of eliciting a reaction when applied topically to the skin, its appendages,

or to mucous membrane demonstrates a capacity for absorption. On absorption by skin or mucous membranes, pesticides may elicit local effects or systemic effects or both. Irritation which is a local effect is an extreme reaction to tissues to an insult or injury. Primary Skin Irritation is measured by a patch-test technique on abraded and intact skin of albino rabbit clipped free of hair (Draize method)⁷⁹. Compounds producing combined average Primary Skin Irritation Indexes of 2 or less are only mildly irritating, whereas those with indexes from 2 to 5 are moderate irritants, and those with scores above 6 are considered severe irritants⁷⁹.

As the rabbit is more susceptible than man to the action of most substances, it is found to be the animal of choice for the acute dermal toxicity^{79,92,93,94}.

The rate of penetration of a compound into the skin and lethal toxicity (local effects) noted following single and multiple exposures, comprise information equally as valuable as the information on internal (systemic) effects^{79,95}.

The acute dermal toxicity study suggests that quinalphos is moderately toxic (LD_{50} : 339.0 mg/kg), whereas neem technical and neem formulation (LD_{50} : more than 10600.0 mg/kg and more than 15300.0 mg/kg respectively) (Tables 22-24) are comparatively nontoxic. The results also suggest that quinalphos even at a very low dose is toxic through the skin.

In the mucous membrane irritation and primary skin irritation study of neem technical and neem formulation conducted on rabbits, they were found to be mild irritants (Tables 25-26,28-29), whereas quinalphos was moderately irritant (Tables 27,30) in comparison.

In recent years there has been an increased interest due to scientific and public awareness to look at effect of chemicals on the nervous system.

Since acute tests are conducted at lethal or near-lethal doses, acute tests are considered to be of limited value for assessing neurotoxic potential of

substances. Signs of convulsions, tremors, ataxia can be associated with acute toxicity studies at systemically toxic or lethal dose levels. Subacute or subchronic studies would however provide more interpretable results due to decreased severity of systemic toxicity when compared with acute study. Therefore in the present study the test sample was administered daily till ataxia develops or for 21 days. Toxicity evaluation was performed on two doses - high dose and low dose levels. Brain, spinal cord and peripheral nerve were examined for histopathology. General behaviour, daily body weight, daily egg collection and egg weight were observed. Haematological and biochemical changes were also undertaken for looking at the long-term effects.

Nervous system has been shown to be particularly vulnerable to chemical insult. The nervous system is influenced by the functioning of other organ systems (e.g. hepatic, cardiovascular and endocrine systems). Thus, toxicant induced alterations in any of these organ systems can be reflected in changes in neurobehavioural output. This fact alone suggests that nervous system function should be among the first to be assessed in cases of exposure to known or potentially hazardous agents like pesticides. Many factors must be taken into consideration with regard to neurotoxicity studies. These include the choice and number of animals, route and duration of administration^{89,100}.

There is no one animal available which will respond to all testing equally well, and also tricresyl phosphate (TCP) does not produce the same neurological symptoms as in man i.e. motor or sensory disturbances. Experiments have failed to induce neurological or other illness in monkeys and baboons even with fairly high doses of TCP daily for over a year. In contrast to this, the adult hen readily develops signs of toxicity at a dose level of TCP comparable to that producing signs in man. The disease process in man and bird appears not earlier than the eighth day in which general gastro-intestinal signs followed by motor paralysis in the leg is observed in both species. Though neurotoxicity studies can be

carried out in different animal species like rats, dogs, cats and adult hen, adult hen is the obvious choice as it is the most sensitive species after man^{96,98}.

Food and water consumption are naturally occurring behaviour that can be measured in the animals' laboratory experiments. Once a stable eating and drinking pattern is established, toxic agents can be introduced and the resulting alteration in behaviour measured⁹⁶.

Since the food intake and the body weight of the experimental birds can be affected due to long term exposure to experimental dose, there might be an undesirable effect on the biochemical parameters of experimental birds. Hence, biochemical studies - blood sugar, plasma proteins, plasma albumin, plasma glutamic pyruvic transaminase, plasma alkaline phosphatase and plasma and whole blood cholinesterase as routine - were studied to get information on its safety on normal use and the probable biochemical disturbances that might occur in accidental oral prolonged exposure.

Pathological alterations in the nervous system lead to changes in behaviour and function. These changes may provide important clues as to the site and even the nature of the underlying structural damage⁹⁷.

Gross pathological examination of any animals that die and of those sacrificed at the termination of each period of the study can provide information concerning affected organs and tissues and the progress of any effect with increasing exposure.

Histopathological examination of the tissues may reveal effects that are not grossly apparent or may indicate the cellular changes involved in a gross effect.

In neurotoxicity studies histopathological examination of brain, spinal cord and sciatic nerve as representative samples of nervous tissue were undertaken.

To look at whether these pesticides of plant origin have potential to cause damage to the nervous system, a preliminary study was undertaken.

Neurotoxicity can occur in many different ways and hence guidelines require test application for signs indicating that a pesticide may affect the nervous system.

To test the neuropathy other than organophosphorus type delayed neuropathy a combined subchronic study which included behavioural, biochemical and haematological parameters along with possible neuropathy were undertaken.

The neurotoxicity studies with two dose groups of neem technical (higher dose : 2650.0 mg/kg and lower dose 1325.0 mg/kg) and neem formulation (higher dose 5100.0 mg/kg and lower dose 2550.0 mg/kg) were conducted in chickens.

Some compounds produce toxic effects following a single exposure; for some compounds, toxic effects follow after prolonged or repeated exposure. Since detection of cumulative toxicity following continued (or intermittent) exposure was one of our objective, a multiple-dosing regimen (21 days) was used in our study.

No mortality was observed in any groups of experimental chickens. Also no significant changes in the body weights, food consumption, number of eggs laid and egg weights were observed during the experimental period of feeding the test compounds. The general health was also observed to be normal and comparable with the control birds (Graph 1,2a,2b,2c,3,4).

Haematological findings in the control and the test groups were comparable with each other and were within the normal range (Tables 31,32).

Blood glucose, plasma proteins, plasma albumin, plasma alkaline phosphatase, plasma glutamic pyruvic transaminase were found to be within the normal range levels and comparable with the control birds (Table 33). No reduction in the plasma and whole blood cholinesterase levels were observed and they were comparable with the control (Table 34).

In our study, Macroscopic examination of all organs of birds treated with 2650.0 mg/kg and 1325.0 mg/kg of neem technical, 5100.0 mg/kg and 2550.0 mg/kg

neem formulation showed no significant changes and were comparable to control tissues.

In the histopathological examination of tissues - brain, spinal cord and sciatic nerve from neem technical and neem formulation treated groups revealed no changes and were normal and comparable to control. No demyelination or degeneration was observed in brain, spinal cord and sciatic nerve.

From our experiment no effect was observed with neem technical and neem formulation.

Conclusion

In the present study, acute toxicity studies of neem technical and neem formulation, karanj technical and *Annona squamosa* technical (methanol and ethyl acetate) were carried out and compared with quinalphos, an organophosphorus pesticide. Neurotoxicity potential of neem technical and neem formulation were also studied. Based on the results, it may be concluded that :

Neem technical (LD_{50} : 19080.0 mg/kg and 16695.0 mg/kg in mice and rats respectively), neem formulation (LD_{50} : >20400.0 mg/kg and >15300.0 mg/kg in mice and rats respectively), karanj technical (LD_{50} : 8308.0 mg/kg and 5418.6 mg/kg in mice and rats respectively), *Annona squamosa* technical (methanol extract) (LD_{50} : >15135 mg/kg in both species) and *Annona squamosa* technical (ethyl acetate extract) (LD_{50} : >15285.0 mg/kg in both species) are practically nontoxic compared to quinalphos technical (LD_{50} : 25.10 mg/kg and 39.55 mg/kg in mice and rats respectively) (Tables 1-12).

The MLD of neem technical and neem formulation (MLD : >15900.0 mg/kg and >15300.0 mg/kg respectively) in chickens and pigeons is very high compared to quinalphos (10.0 mg/kg in chickens and 2.0 mg/kg in pigeons), which suggests that neem products are comparatively non-toxic than quinalphos (Tables 13-18).

The LC_{50} of neem technical (280.0 ppm) and neem formulation (1300.0 ppm) in fish is also very high compared to quinalphos (LC_{50} : 1.0 ppm), which is highly toxic (Tables 19-21).

Results of the acute dermal toxicity study of neem technical and neem formulation (LD_{50} >10600.0 mg/kg and >15900.0 mg/kg) suggest that they comparatively non-toxic when compared to quinalphos (LD_{50} : 339.0 mg/kg) (Tables 22-24).

Neem technical and neem formulation are mild irritants to mucous membrane as well as to skin compared to quinalphos (Tables 25-30).

Results suggest that quinalphos is more than 400 times more toxic than neem in rats, mice by oral route and more than 50 times by dermal route. Also neem products are less irritants than quinalphos.

In neurotoxicity study, no mortality was observed in the control group or any of the test groups treated with neem technical or neem formulation. The general health, food consumption, body weights, number of eggs laid were comparable with the control birds.

The haematological parameters such as Haemoglobin, W.B.C. counts, R.B.C. counts and Differential counts were also comparable with the control (Table 31,32).

The biochemical parameters such as blood glucose, plasma proteins, plasma albumin, plasma glutamic pyruvic transaminase and alkaline phosphatase observed in neem technical and neem formulation treated birds were comparable with the normal (Table 33). No inhibition in the plasma and whole blood cholinesterase were observed with the birds fed neem technical and neem formulation (Table 34).

On the basis of the gross pathological and histopathological studies conducted on neem technical at 2650.0 mg/kg and 1325.0 mg/kg and neem formulation at 5100.0 mg/kg and 2550.0 mg/kg doses, it can be concluded that no changes were observed in the tissues examined, which could be attributed to the administration of neem technical or neem formulation (Table 35).

Based on the results, it can be concluded that neem, karanj, *Annona squamosa* are comparatively non-toxic and seem to be better alternatives for the toxic synthetic pesticides currently in use from acute toxicity point of view.

Bibliography

1. Pedigo P. Larry (1989). Pest Management Theory and Practice. In Entomology and Pest Management. Publ. Macmillan Publishing Co. p. 271-276.
2. Jones L.M. (1957). Insecticides. In Veterinary Pharmacology and Therapeutics, ed. 2, Iowa State University, Iowa, p. 618-641.
3. McCaleb R.S. (1992). Food Ingredients Safety Evaluation. Food and Drug Law Journal. 47 : 657-663.
4. Gaikwad R.W. (1994). Pesticides and its Abatement. Chemical Weekly. 40(8) : 131-134.
5. Gupta P.K. and D.K. Salunkhe (1985). Pesticides. In Modern Toxicology. vol 2, ed 1, p. 44.
6. Patil S.G., Hugar Parmeshwar (1991). Ecological Impacts of Pesticides On Wildlife. Myforest. 27(3) : 244-247.
7. Dethe M.D., Kala V.D., Walunj A.R., Rane S.D. (1991). DDT and HCH in Milk Samples from Rural Areas of Ahmednagar, Western Maharashtra. J. Ecotox. Environ. Monitoring. 1(2) : 95-98.
8. Dethe M.D., Dharme P.K., Kala V.D. (1990). Residues of Endosulfan and Monocrotophos in Pigeon Pea. Growth Development and Natural Resource Conservation. Nature Conservators. p.283-285.
9. Indian Pesticide Directory (1993). Pesticide Industry in India. Ed. Sarathy A.P., Rangarajan S. Publ. Scientia Publications Private Limited.p. 351.
10. Singh S. Amu (1981). Pesticide Pollution : An Analysis of the Problem. Pesticides. 15(6) : 3-12.
11. Bami H.L. (1986). Pesticides - Their Industrial and Health Hazards and Environmental Pollution in India. J. Inst. Chemists (India). 59, part 2 : 83-90.
12. Abby A.Li, Karen J. Asbury, William E. Hopkins, Paul C.C., Feng Alan, G.E. Wilson (1992). Metabolism of Alachlor by Rat and Monkey Liver and Nasal Turbinate Tissue. Drug Metabolism and Disposition. 20(6) : 616-618.
13. Murphy Sheldon D. (1986). Toxic Effects of Pesticides. In Casarett and Doul's Toxicology. ed 3, Ed. Curtis D. Klassen, Mary O. Amdur and John Doul. p.519-581.

14. Srivastava A.K., Gupta B.N., Mathur N., Mahendra P.N., Bharti R.S. (1991). Clinical and Biochemical Study of Pesticide Sprayers. Human and Experimental Toxicology. 10(4) : 279-283.
15. Robert E. Neal (1981). Risk Benefit Analysis : Role of Pesticides in Regulation of Pesticides Registration. In Pesticide Chemistry And Modern Toxicology. p 469-472.
16. Chari M.S., Prabhu S.R., and G. Ramaprasad (1990). Role of Botanicals in Pest Management of Tobacco in India. In Proc. Symp. of Bot. Pesticides in IPM, Rajahmundry. p. 128-133.
17. Woodruff T.J., Kyle A.D. and Bois F.Y. (1994). Evaluating Health Risks from Occupational Exposure to Pesticides and the Regulatory Response. Environ. Health Perspect. 102(12) : 1088-1096.
18. Dubey O.P., Tewari G.C. (1992). Insect Pests of Field Crops and Their Management Practices. In Pest Management and Pesticides. Indian Scenario. Ed. B. Vasantaraj David. p. 28-33.
19. Patel M.K. (1994). Environmental Hazards of Pesticides. Chemical Weekly. 39(39) : 139-145.
20. Sone Lal, R.B. Doharey (1992). Stored Grain Pests of National Importance and Their Management. In Pest Management and Pesticides : Indian Scenario. Ed. B. Vasantaraj David. p. 101-117.
21. Rakila A., N.R. Padmanaban (1995). Knowledge and Factors Influencing Pesticide Use and Frequency of Plant Protection Measures. Pestology. 19(1) : 9-12.
22. Roy N.K. (1995). Evaluation of Agrochemicals in Relation to Food Security and Environmental Protection. Chemical Weekly. 40(30) : 161-171.
23. W.H.O. (1990). Production and Use of Pesticides. In Public Health Impact of Pesticides Used in Agriculture. p. 15-32.
24. Vijagopalan S. (1980). Present Status of Pesticides Industry. Pesticide Annual. p. 8-17.
25. Babu C.J. (1992). Vector Borne Diseases of Human Beings and Their Management in India. In Pesticide Management and Pesticides : Indian Scenario. Ed. B. Vasantaraj David. p. 118-124.

26. Bruce-Chwatt L.J. (1971). Insecticides and Vector Borne Diseases. Bulletin W.H.O. 44 : 419-424.
27. Ellenhorn Mathew J., Barcelown Donald G. (1988). Pesticides. In Medical Toxicology : Diagnosis and Treatment of Human Poisoning. Publ. Elsevier Publishing Co. p. 1069-1108.
28. Patel M.K. (1994). Health Hazards of Organochlorine and Organophosphorus Pesticides. Chemical Weekly. 39(33) : 145-150.
29. Cremllyn R. (1978). Pesticides : Preparation and Mode of Action. Publ. John Wiley and Sons, Chichester, New York, Toronto. p. 1-11.
30. Worthing C.R. (1979). Malathion. In The Pesticide Manual, A World Compendium. ed. 6, Pub. British Crop Protection Council. p. 321.
31. Marderosian Ara H. Des (1990). Pesticides. In Remington's Pharmaceutical Sciences. ed. 18. ED. Alfonso R. Gemario, p. 1249-1271.
32. Bami H.L. (1993). Pesticides Risks - An Overview of Indian Scene. Chemical Weekly. 38(39) : 137-140.
33. Patil Vasant L. (1993). Pesticide Safety : Toxicity, Poisoning, First-Aid/Treatment, Labels and Leaflets. Chemical Weekly. 38(25) : 137-145.
34. Gangwar Santosh K. (1993). Integrated Pest Management - The Concept. Pestology. 17(5) : 28-33.
35. Mehrotra K.N. (1992). Pesticide Resistance in Insect Pests. In Pest Management and Pesticides. Indian Scenario, Ed. B. Vasantaraj David. p. 17-27.
36. Shidravi G.R. (1990). A W.H.O. Global Programme for Monitoring Vector Resistance to Pesticides. W.H.O. Bulletin O.M.S. 68(4) : 403-408.
37. Matthews Graham A. (1994). Pesticides Registration, Formulation and Application in India, Chemical Weekly. 39(37) : 141-144.
38. Kulshethra Niraj (1993). Pesticides and Environmental Pollution : Legislative Control in India. Chemical Weekly. 38(40) : 141-146.
39. Thomson W.A.R. (1981). Making Pesticides Safe For Humans And Animals. Pesticides. 15(11) : 3-4.
40. Hayes W.J. Jr. (1967). Toxicity of Pesticides to Man : Risks from Present Levels. Proc. Roy. Soc. London. 167 : 101-127.

41. Karunakaran C.O. (1958). The Kerala Food Poisoning. J. Ind. Med. Assoc. 31 : 204-207.
42. Kleiman G.D., West I., Augustine M.S. (1960). Occupational Diseases in California Attributed to Pesticides and Agricultural Chemicals. Arch. Environ. and Health. 1 : 118-124.
43. Rao Sheshagiri D. (1972). Insecticides. In A handbook of Plant Protection. Publ. Prasanna Printers and Publishers. p.205-244.
44. Rao Rama M. (1990). Utility of Plant Products in Pest Control of Field and Orchard Crops. Proc. Symp. Bot. Pesticides. p. 125-127.
45. Ayyangar N.R., Nagasampagi B.A.. (1990). Role of Botanicals in Integrated Pest Management. In Proc. Symp. Bot. Pesticides in IPM, Rajahmundry. p. 54-61.
46. Kirtikar K.R., Basu B.D. (1935). Indian Medicinal Plants, vol I-IV, ed 2, Publ. Lalit Mohan Basu.
47. Nadkarni A.K. (1982). Indian Materia Medica vol I-III, Publ. Popular Prakashan Private Limited.
48. Ketkar C.M., Mohan S. Ketkar (1990). Versatile Neem - A Source for Plant Protection. In Proc. Symp. Bot. Pesticides in IPM, Rajahamundry. p. 118-124.
49. Jotwani M.G., K.P. Srivastava (1981). Neem - Insecticide of the Future I - As Protectant Against Stored Grain Pests. Pesticides. 15(10) : 19-23.
50. Jotwani M.G., K.P. Srivastava (1981). Neem - Insecticide of the Future II - Protection Against Field Pests. Pesticides. 15(11) : 40-47.
51. Rajan T.P.S. (1993). Neem - Natures Boon to Humanity. Chemical Weekly. 38(37) : 51-53.
52. Bambarkar Sunil (1990). Neem - A Vast Potential for Agrochemicals. Pesticides. 24(1) : 36-40.

53. Singh R.P. (1993). Bioactivity Against Insect Pests : Neem Research and Development. Ed. N.S. Randhawa and B.S. Parmar. Soc. Pesticide Science. India. p. 109-122.
54. Dhawan B.N., G.K. Patnaik (1993). Pharmacological Studies for Therapeutic Potential. Neem Research and Development. Eds. N.S. Randhawa and B.S. Parmar, Society of Pesticide Science, India. p. 242-249.
55. Kanungo D. (1993). Pharmacology and Toxicology. Neem Research and Development . Eds. N.S. Randhawa and B.S. Parmar, Soc. Pesticide Science, India. p. 250-252.
56. Pillai N.R., G. Santhakumari (1984). Toxicity Studies on Nimbidin, a Potential Antiulcer Drug. Plant Medica. 50(2) : 146-148.
57. Ali B.H. (1987). The Toxicity of *Azadirachta indica* Leaves In Goats and Guinea Pigs. Vet. Hum. Toxicol. 29(1) : 16-19.
58. Ibrahim I.A., Sawson A. Omer, F.H. Ibrahim, S.A. Khalid, S.E.I. Adava (1992). Experimental *Azadirachta indica* Toxicosis in Chicks. Vet. Hum. Toxicol. 34(3) : 221-223.
59. Sinniah D. and Baskaran G. (1981). Margosa Poisoning as a Cause of Reye's Syndrome. The Lancet. vol. 1, no. 8218 : 487-489.
60. Gupta R.S., Bhaid M.U. (1981). Studies on Agro-Industrial By-Products (deoiled neem fruit cake in sheep feed composition). Ind. Vet. J. 58(4) : 311-315.
61. Bhide N.K., D.J. Mehta, Mrs. W.W. Altekar, R.A. Lewis (1958). Toxicity of Sodium Nimbidinate. Ind. J. Med. Sci. 12 : 146-149.
62. Safety Tests. Appendix A. In Neem - Tree for Solving Global Problems. National Academic Press. Washington D.C.(1992). p.100-103.
63. Dictionary of Indian Medicinal Plants (1992). *Pongamia glabra*. Pub. Director, Central Institute of Medicinal and Aromatic Plants, Lucknow, p. 369.
64. Venkataramireddy P., K.C. Chitra, P. Kameshwara Rao (1990). Efficacy of the Plant Extracts in the Control of Brinjal Spotted Leaf Beetle, *Henosepilachna vigintipunctata* F. In Proc. Symp. Bot. Pesticides in IPM. p. 225-227.

65. Koshiya D.J., A.B. Ghelani (1990). Antifeedant Activity of Different Plant Derivatives Against *Spodoptera litura* (FAB) on Groundnut. In Proc. Symp. Bot. Pesticides in IPM. p. 270-275.
66. Patel J.R., J.I. Patel, D.M.Mehta, B.R. Shah (1990). Integrated Pest Management Of *Amsacta moorei* Butler with Botanical Insecticides. In Proc. Symp. Bot. Pesticides in IPM . p. 327- 331.
67. Jothi Dhara B., P.L.Tandon, Abraham Verghese (1990). Evaluation of Different Plant Oils and Extracts Against Citrus Leaf Miner, *Phyllocnistis citrella*. Stainton (Lepidoptera : Phyllocnistidae). In Proc. Symp. Bot. Pesticides in IPM . p. 340-342.
68. Sharma V.K., Anuj Bhatnagar (1990). Studies on Effects of Non-edible Seed Oils on *Chilo partellus* swinhoe. In Proc. Symp. Bot. Pesticides in IPM . p. 351-359.
69. Dictionary of Indian Medicinal Plants (1992). Publ. Director Central Institute of Medicinal and Aromatic plants, Luchnow, p.36.
70. The Pesticide Manual (1994). 612 : Quinalphos : Insecticide, Acaricide Ed. Clive Tomlin, ed. 10, Publ. The British Crop Protection Council. p. 890.
71. Pestline Vol. II (1991). Publ. Occupational Health Services, Inc. Quinalphos : PST 73112. p. 1736-1738
72. Pesticide Assessement Guidelines. Subdivision F . Hazard Evaluation : Domestic animals. U.S. Environmental Protection Agency, Washington D.C. (1982) : 34-44.
73. Appraisal of Safety of Chemicals in Foods, Drugs, and Cosmetics. Association of Food and Drug Officials in the United States (1975). p.17-25.
74. Report of the Subcommittee on Pesticide, Toxicology, submitted by Dr. B.B. Gaitonde, Central Insecticide Board, Ministry of Agriculture, Government of India, (1978) : 70-74.
75. Balazs T. (1971). Measurement of Acute Toxicity. In Methods in Toxicology. Ed. Paget G.E. Publ. Blackwell scientific publications. P 49-81.
76. Litchfield J.T. Jr., Wilcoxon F. (1949). A Simplified Method of Evaluating Dose Effect Experiments. J. Pharmacol. Exp. Ther. 96 : 99-113

77. WADC Technical Report 55-16.(1955). Handbook of Toxicology, vol.1, Ed. Spector W.S., Prepared by Wright Air Development Center, U.S. Airforce. p.3-4
78. Bioassay Methods For Evaluating Acute Toxicity of Industrial Effluents and Waste Water. IS6582 (1971). Indian Standard Institute, New Delhi.
79. Draize J.H. (1975). Dermal Toxicity. In Safety of Chemicals in Food, Drugs, and Cosmetics. p.46-58.
80. Baker F.J., Silvertown R.E. (1976). Haematology. In Introduction to Medical Laboratory Technology, ed. 5. Publ. Butterworth, London, Boston, p.549-629.
81. Barucha C. Meyer H., Bharucha H., Moody A., Carman R.H. (1974). Haematology. In Handbook of Medical Laboratory Technology. Publ. Christian Medical Association of India. p.53-115.
82. Lucas A.M., Jamroz C. (1974). Atlas of Avian Haematology. Publ. Oxford and IBH Publishing Company, New Delhi. p.222-234
83. Biggs H.G., Shirley Carey, Morrison D.B. (1958). A Simple Colorimetric Method of Measuring Activities of Cellular and Plasma Cholinesterase. J. Clin. Pathol. 7 : 322-326.
84. Richterich R. (1969). Modified Methods for Protein Estimation. In Clinical Chemistry Theory and Practice, Ed. Karger S. Basel., Publ. Academic Press. p.245.
85. Reitman S., Frankel S. (1957). A Colorimetric Method for the Determination of Serum Glutamic Oxalate Transaminase and Serum Glutamic Pyruvic Transaminase. Amer. J. Clin. Pathol. 28 : 56-63.
86. Kind P.R.N., King E.J. (1954). Estimation of Plasma Phosphatases by Determination of Hydrolysed Phenol with Amino Antipyrine. J. Clin. Pathol. 7 : 322-326.
87. Nelson M., Somogyi M. (1951). Colorimetric Method of Estimation of Blood Sugar. In Practical Biochemistry. Ed. Varley H., ed. 4 (Indian ed. 1975). Publ. Arnold Heinemann, Gulab Vazinani. p. 89-90.
88. Snedecor G.W., Cochran W.G. (1971). Statistical Methods, ed. 6, Publ. The Iowa State University Press., Ames, Iowa, U.S.A.

89. WHO (1986). Principles and Methods for Assessment of Neurotoxicity Associated with Exposures to Chemicals. Environmental Health Criteria 60. p. 22
90. Hodge H.C., Sterner J.H. (Dec' 1943). American Industrial Hygiene Association Quarterly. 10(4) : 93.
91. Copplestone J.F.(1988). The Development of the WHO Recommended Classification of Pesticides by Hazard. W.H.O. Bulletin O.M.S. 66(5): 545-551.
92. Barnes J.N. (1968). Percutaneous Toxicity. In Modern Trends in Toxicology 1, London, Butterworth, p. 18-38.
93. Edson E.F., Diana N. Noakes, Sanderson D.M. (1968). Pesticides. In Modern Trends in Toxicology 1, London, Butterworth, p. 275-276.
94. Kimmerle G., Lorke D., (1968). Toxicology of Insecticidal Organophosphates. Pflanzenschutz - Nachrichten Bayer, 21, 111.
95. Hart T.B. (1989) : Pesticide Use - Risk or Safety. Recent Advances in Clinical Pharmacology and Toxicology, London Churchill, Livingstone. p. 197- 218.
96. Glees P. (1967). A Morphological and Neurological Analysis of Neurotoxicity Illustrated by Tricresyl Phosphate Intoxication in the Chicks. In Neurotoxicity of drugs. Proc. Euro. Soc. for the Study of Drug Toxicity, vol. VIII, p.136-148.
97. WHO (1986). Principles And Methods for the Assessment of Neurotoxicity Associated with the Exposure to Chemicals (1986). Environmental Health Criteria 60. p. 71.
98. Sanz P., Emoreno E., Blasco R. and Repetto M. (1990). Study of Delayed Neurotoxicity Caused by Fatty Acid Anilides in Hen. Vet. Hum. Toxicol. 32(5) : 422-427.
99. The Role of Animals in Biomedical Research (1983). An. New. York. Acad. Sci. vol. 406. ED Sechzer J.A.
100. Eisenbrandt D.L., S.L.Allen, P.H. Berry, W.Classen, D. Bury, W. Mellert, R.J. Millischer, W. Schuh, W.J. Boutinck (1994). Evaluation Of The

- Neurotoxic Potential of Chemicals in animals. *Fd. Chem. Toxicol.* 32(7) : 655-669.
101. Chari M.S., G. Ramprasad (1993). Botanical Peasticides in Integrated Pest Management. (Proceedings of the National Symposium).
102. Thompson E.B., Anderson C.C. (1978). Cardiovascular Effects of *Azadirachta indica* Extract. *J. Pharm. Sci.* 67(10) : 1476-1478.
103. Dorman D.C., Beasley V.R. (1991). Neurotoxicology of Pyrethrin and the Pyrethroid Insecticides. *Vet. Hum. Toxicol.* 31(3) : 238-243.
104. Gupta B.N., Rastogi S.K., Chandra H., Mathur A.K., Mathur N., Mahendra P.N., Pangtey B.S., Kumar S., Kumar P., Seth R.K., Dwivedi R.S., Ray P.K. (1988). Effect of Exposure to Toxic Gas on the Population of Bhopal : Part I - Epidemiological, Clinical, Radiological and Behavioral Studies. *Ind. J. Exp. Biol.* 26(3) : 149-160.
105. Gosselin R.E., Hodge H.C., Smith R.P. and Gleason M.N. (1977). Clinical Toxicology of commercial products. Publ. The Willeams and Wilkins. Co. p.2.

Appendix

Appendix 1

COMBINED TABULATION OF TOXICITY CLASSES⁹⁰

Various Routes of Administration

Toxicity Rating	Commonly used term	LD ₅₀ Single Oral dose** Rats	LD ₅₀ Skin Rabbits	Probable Lethal Dose For Man
1	Extremely toxic	1 mg or less/kg	5 mg or less/kg	A taste, 1 grain
2	Highly toxic	1-50 mg	5-43 mg	1 teaspoon, 4 cc
3	Moderately toxic	50-500 mg	44-340 mg	1 ounce, 30 gm
4	Slightly toxic	0.5-5 g	35-2.81 g/kg	1 pint, 250 g
5	Practically non-toxic	5-15 g	2.82-22.59 g/kg	1 quart
6	Relatively non-toxic	15 g and more	22.6 or more g/kg	> 1 quart

Appendix 2

TOXICITY RATING CHART¹⁰⁵

Toxicity Rating	Class	Probable Oral LETHAL Dose Dose For 70 kg Person (150 lbs)	
6	Super toxic	less than 5 mg/kg	A taste (less than 7 drops)
5	Extremely toxic	5-50 mg/kg	Between 7 drops and 1 teaspoonful
4	Super toxic	50-500 mg/kg	Between 1 tsp and 1 ounce
3	Moderately toxic	0.5-5 gm/kg	Between 1 oz and 1 pint (or 1 lb)
2	Slightly toxic	5-15 gm/kg	Between 1 pt and 1 quart
1	Practically nontoxic	above 15 gm/kg	More than 1 quart (2.2 lb)

Appendix : 3

Neurotoxic Potential of Neem Technical and Neem Formulation in Chickens Body Weights (kg)

	Control			Neem Technical						Neem Formulation					
				2650 mg/kg	1325 mg/kg					5100 mg/kg	2550 mg/kg				
days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.27	1.48	1.25	1.43	1.22	1.74	1.71	1.2	1.3	1.47	1.3	1.34	1.37	1.42	1.67
2	1.4	1.43	1.23	1.66	1.4	1.86	1.77	1.29	1.33	1.5	1.42	1.46	1.44	1.55	1.71
3	1.35	1.44	1.31	1.71	1.31	1.87	1.77	1.30	1.36	1.57	1.42	1.46	1.43	1.52	1.76
4	1.27	1.47	1.42	1.7	1.34	1.87	1.81	1.29	1.32	1.53	1.42	1.46	1.48	1.55	1.68
5	1.32	1.5	1.44	1.7	1.35	1.88	1.85	1.27	1.27	1.51	1.42	1.48	1.38	1.59	1.65
6	1.41	1.55	1.44	1.77	1.33	1.88	1.87	1.37	1.25	1.51	1.5	1.47	1.44	1.6	1.62
7	1.4	1.5	1.47	1.8	1.32	1.96	1.85	1.41	1.25	1.57	1.5	1.46	1.36	1.59	1.62
8	1.41	1.59	1.51	1.78	1.33	1.9	1.84	1.35	1.24	1.48	1.47	1.45	1.35	1.58	1.59
9	1.44	1.62	1.47	1.75	1.28	1.91	1.8	1.37	1.24	1.37	1.38	1.47	1.29	1.57	1.63
10	1.48	1.65	1.5	1.85	1.41	1.94	1.85	1.38	1.32	1.43	1.30	1.43	1.32	1.60	1.6
11	1.49	1.72	1.55	1.84	1.32	1.95	1.87	1.38	1.41	1.57	1.32	1.44	1.34	1.63	1.63
12	1.49	1.63	1.58	1.79	1.35	1.99	1.87	1.42	1.56	1.61	1.35	1.5	1.43	1.7	1.62
13	1.46	1.63	1.67	1.85	1.42	1.97	1.83	1.48	1.5	1.55	1.38	1.38	1.5	1.72	1.63
14	1.41	1.6	1.61	1.78	1.43	2.0	1.8	1.49	1.48	1.55	1.35	1.39	1.44	1.68	1.65
15	1.47	1.57	1.57	1.81	1.44	1.92	1.84	1.51	1.51	1.58	1.39	1.49	1.51	1.67	1.71
16	1.49	1.6	1.62	1.85	1.49	1.93	1.86	1.49	1.6	1.69	1.43	1.49	1.5	1.8	1.89
mean	1.41	1.56	1.47	1.75	1.36	1.91	1.82	1.38	1.38	1.75	1.39	1.44	1.41	1.61	1.67
±S.D	0.074	0.083	0.129	0.105	0.07	0.063	0.045	0.09	0.122	0.105	0.063	0.044	0.069	0.089	0.75

Appendix : 4

Neurotoxic Potential of Neem Technical and Neem Formulation in Chickens

Food consumption (gms)

days	Control			Neem Technical						Neem Formulation					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	120	130	120	180	140	160	200	200	200	170	200	140	200	195	195
2	110	140	150	120	160	110	190	185	200	120	180	110	195	200	190
3	190	180	190	190	110	150	200	200	200	150	180	140	200	180	180
4	160	190	150	120	150	130	200	200	195	160	170	150	170	200	200
5	170	180	150	140	130	140	200	200	200	200	200	200	200	190	195
6	170	180	140	140	140	160	190	195	190	190	190	200	180	185	200
7	170	170	140	140	160	170	185	200	200	170	175	180	195	190	190
8	160	190	140	150	170	200	195	195	190	185	195	190	170	170	170
9	120	130	160	160	200	200	190	200	195	190	180	190	165	175	185
10	130	150	170	120	150	190	190	180	190	160	170	175	180	170	190
11	160	170	190	100	140	190	160	190	185	170	190	160	180	190	180
12	170	190	190	160	140	190	120	200	200	150	190	110	200	180	200
13	160	180	185	140	180	190	110	190	180	100	190	200	200	160	190
14	170	180	190	140	140	180	100	190	200	110	100	200	120	160	200
15	160	190	190	140	140	190	150	180	200	130	120	200	150	160	200
16	190	175	180	170	160	180	170	190	185	150	140	190	190	185	190
mean	156.9	170.3	164.7	144.4	150.6	170.6	171.9	194.7	194.4	156.6	173.1	170.9	180.9	180.6	190.9
±S.D	24.14	21.01	23.63	23.66	21.12	26.72	34.15	5.31	6.8	29.48	28.86	31.95	22.3	13.61	8.8

Appendix 5
Normal Range in Chickens
Biochemical Parameters

Parameter	Normal Range	Mean	No. of Animals
Whole Blood Cholinesterase	20 - 59	31.5	40
Plasma Cholinesterase	12 - 47	26.9	40
Plasma Proteins (gm%)	4 - 7	5.47	32
Plasma glutamic pyruvic transaminase (K. Units)	10 - 50	19.71	34
Plasma alkaline Phosphatase (K.A. Units)	8 - 211	56.05	33
Blood Sugar (mg%)	112 - 224	148.44	27

Haematological Parameters

Parameter	Normal Range	Mean	No. of animals
Haemoglobin	7.0 - 10.2	9.09	27
RBC million/mm ³	2.0 - 2.6	2.31	27
WBC /mm ³	18000 - 26000	21622.2	27
Eosinophils (%)	0 - 2	1.4	27
Neutrophils (%)	22 - 87	32.07	27
Lymphocytes (%)	54 - 75	66.85	27
Monocytes(%)	--	--	27

Synopsis

Introduction

Pesticides are widely used for improving agricultural practices, in health programmes and against household pests. Pesticides can be in the form of chemicals or biocides or of plant origin or even natural predators. The use of pesticides has been reported around 1000 B.C., when Homer spoke of the use of sulfur fumigation and in 900 A.D., when the Chinese were using arsenic against garden insects and in 1300 A.D. Marco Polo referred to the use of oils against the control of camel mange¹. The use of plants or herbs as food ingredients or for medicinal purpose or for pest control are recorded ².

An ideal pesticide should be selective in its toxicity to target species but safe to other living systems. However pesticides are not entirely selective in toxicity. They are not only toxic to domestic animals, human beings, and other beneficiary insects but also to the environment. Added to possible toxicity, continuous use of pesticides has lead to development of resistance even in a number of insect pests.

Safety of all types of consumer products, be it chemicals or pesticides has become a priority concern. Wide spread use of pesticide has resulted in the fear of adverse effects in humans and domestic animals. Wildlife, including birds and fish may also be exposed to health hazards when pesticides are used for the control of unwanted plants, and insects thereby contaminating food and water with pesticides³. They may enter the body through oral ingestion as residues in food, drinking water or from accidental or intentional ingestion or through respiratory system (inhalation) or through skin.

The major source of environmental contamination is due to the deposits as a result of the application of these chemicals to control pests. The basic chemicals and/or their degradation products may persist in water,

soil or plant and animal tissues in the wildlife in quantities that may effect the preservation of endangered species and the ecosystem. Residues of DDT and HCH were found in milk samples⁴, endosulfan and monocrotophos in grains from green and dried pods of pigeon pea⁵ and HCH in rice⁶.

The toxic effects of pesticides can result in acute or chronic toxicity. They may also cause organ damage or carcinogenicity, mutagenicity or teratogenicity. Alachlor was found to cause nasal tumors on chronic oral administration⁷. Another study showed that exposure of pesticide sprayers to multiple pesticides affected the normal functioning of different organ systems⁸.

As a result of consumer safety awareness, regulatory agencies are laying down stricter guidelines to ensure that consumer products are evaluated for safety before use. Variety of toxicity studies such as acute, subchronic and specialised studies like carcinogenicity, mutagenicity, teratogenicity, neurotoxicity in different species of animals are required by regulatory agencies around the world to assess and prevent the possible hazards of pesticides. Since evaluation cannot be done directly in humans due to ethical reasons, the first step required is to evaluate indirectly in animal models and predict the possible hazards to humans and then follow up with controlled field trials or consumer surveys.

Most regulatory decisions are based on the results of animal tests using mammalian species. Extrapolation from animals to man has been the most successful for certain effects such as carcinogenicity, acute toxicity and metabolic interference as well as for deafness, behavioural changes and influence on immune system⁹. As all types of toxicity that can occur cannot be predicted by conducting the studies in one species due to species variation, different species of animals are used for assessing and predicting a

possible or more realistic toxic effect in human beings or animals or the ecosystem.

Increasing problems of pest resistance to pesticides and contamination of biosphere with large scale use of synthetic pesticides has dictated the need for effective, biodegradable pesticides with greater selectivity and which are also less expensive. This awareness has created a world-wide interest in the use of traditional botanical pesticides. Accordingly, attempts have been made from time to time to manufacture less polluting and more "economically friendly" botanical pesticides.

It is in this context that the discovery and reports of azaridachtin, karanjin, annonaine and several other plant extracts assume relevance. These extracts have been reported to be equally effective in controlling pests^{10,11}. These extracts have not been studied in detail for their toxicity and also until now, there had been no regulation on pesticides obtained from plant origin. For these products to be marketed, they have to be thoroughly evaluated for their toxicity and the subsequent submission of the toxicity data to the Pesticide Regulatory Body is necessary.

Acute toxicity (single dose) data forms a mandatory requirement for the evaluation of safety of pesticides. This data is required to support the registration of the product. It is usually an initial step and provides information on health hazards likely to arise due to single-exposure by the oral or dermal or inhalation route. The study is to evaluate the median lethal dose (LD_{50}), its statistical limits using a single exposure and a 7 or 14 days post-exposure observation period. LD_{50} serves as a basis for classification, labelling and packaging. LD_{50} is the dose required to produce toxicity symptoms and kill 50% of the animals. It also is an initial step or guide for selection of doses or dosage regimen for subchronic and chronic studies and

may also provide information on the mode of toxic action of pesticide and comparative toxicity about other pesticides.

Aims and Objectives

The present study was undertaken to study the different types of acute toxicity of plant extracts of *Azadirachta indica* (neem), *Pongamia pinnata* (karanj) and *Annona squamosa* (custard apple) in different animal species viz. mice, rats, rabbits, fish, chickens and pigeons to study the species variation and compared with Quinalphos, organophosphorus pesticide. Also, neurotoxic potential of neem in chickens was studied.

Materials and Methods

Test Compounds : Neem technical (*Azadirachta indica* extract), (*Azadirachta indica* formulation), Karanj technical (*Pongamia glabra* extract), *Annona squamosa* technical (methanol and ethyl acetate extracts) and quinalphos (o,o-diethyl-o-2-quinoxalinyI phosphorothioate) were obtained from local manufacturers.

Methods^{12,13,16} :

Guidelines of the methods are as per the International, National and Regulatory Body requirements

Acute oral toxicity in mice and rats (LD₅₀) :

Acute oral toxicity of the above test compounds were carried out in albino mice (Kasauli strain) weighing 18-22 gms and albino rats weighing 80-120 gms. Ten animals per dose group and ten for control were taken for the test. All the animals were fasted overnight prior to dosage but water was given ad libitum. The test compounds were given as such except for

quinalphos which was diluted (1:400) in refined vegetable oil as is it not miscible with distilled water. Control groups of neem technical, neem formulation, karanj technical and *Annona squamosa* technical extracts received distilled water, whereas control group of quinalphos received refined vegetable oil.

Preliminary doses were given to one male and one female animals at 0.6 log interval for the selection of dosages for the final assay¹⁴. The final test was terminated one week after dosing where no delayed mortality was observed. When delayed mortality was observed, the observation period was extended to two weeks after dosing. All animals dead during the observation period and those that were alive at the end of the study period were sacrificed and post-mortemed for gross pathological changes. The LD₅₀ values were determined by the method of Litchfield and Wilcoxon¹⁵.

Acute oral toxicity in chickens and pigeons : (MLD)

Acute oral toxicity of neem concentrate, neem formulation and quinalphos, were carried out in chickens (White leghorn, females) weighing 1.5- 2.0 kg that were obtained from Central Poultry farm, Goregaon, Bombay and pigeons (*Columbia livia*, Blue Rock pigeons) weighing 300-400 gms (either sex) that were obtained from local supplier. The birds were observed for 7 days before the start of the test. Chickens were fed Layer mash while the pigeons were fed milletes. Water was given ad lib. Three birds were taken per dose group and control group. The birds were fasted overnight before the start of the test. Known amounts of test compounds were administered orally to each bird with the help of flexible PVC tubing. The birds were observed for a period of three weeks for clinical symptoms and mortality. The MLD (minimum lethal dose required to kill one out of three animals) was found^{16,17}.

Toxicity in fish : (LC₅₀)

Toxicity of neem concentrate, neem formulation and quinalphos were carried out in fish (*Talapia mossambica*, fresh water fish). They were acclimatised at room temperature for ten days before the test. They were regularly fed during the acclimatisation period. Ten healthy fish 5-7.5 cm in length were used for each experimental concentration of sample under the test. Aged chlorine tap water was used as test water and diluent. The tap water was analysed for hardness, acidity, alkalinity, dissolved O₂ free chlorine, pH and temperature before use. Known amounts of the samples were added to the measured quantity of diluent water in glass aquarium and mixed gently by stirring. Ten fish were also taken as control in diluent water only. Test fish were observed at regular intervals for 96 hours for abnormal behaviour, loss of equilibrium and death. The surviving fish were transferred to separate tanks containing diluent water for further period of 24 hours observation. The LC₅₀ values were obtained by graphical interpolations¹⁸.

Acute dermal toxicity in rabbits : (LD₅₀)

Acute dermal toxicity of neem concentrate, neem formulation and quinalphos were carried out in albino rabbits weighing 1.5-2.5 kg. Four animals per dose group and four for control of either sex were taken for the study. Backs of the rabbits were depilated by clipping the hair. Known amounts of the sample was applied on the depilated backs. The animals were then placed in an immobilised position in animal holders. The samples were then allowed to remain in contact with the skin for 24 hours. The animals were then observed for any toxic symptoms. At the end of 24 hours the animals were removed from their respective holders, observed for gross symptoms of toxicity and the subjects were then cleaned thoroughly by wiping. They were then transferred to individual cages for further

observations for 15 days for any symptoms of toxicity and mortality. All the animals that were dead during the observation period and those that were alive at the end of the experiment were sacrificed and post-mortemed for gross pathological changes. The LD₅₀ was obtained by the method of Litchfield and Wilcoxon¹⁵.

Mucous Membrane Irritation :

The study was carried out on neem concentrate and neem formulation in albino rabbits (females) weighing around 1.5-2.0 kg. Six healthy female rabbits were selected for test and similarly six healthy female rabbits were taken as control. The vaginal mucous membranes were examined before the test. 0.1 ml of the test compound was introduced into the upper half of the vaginal vault of the test rabbits. Similarly, 0.1 ml of sterile normal saline was introduced in the upper half of the vaginal vault of the control rabbits. Mucous membrane of each animal was examined for reactions at 24, 48 and 72 hours. All the animals were observed for 15 days.

Primary Skin Irritation :

The study on neem concentrate and neem formulation was carried out in albino rabbits of either sex weighing 1.5-2.0 kg. Six healthy rabbits were selected for the test. The backs of six healthy rabbits were depilated by clipping the hair. Four sites of one sq.inch area were selected from each animal from the back. Two sites were abraded and two sites were left intact. 0.5 ml of the test compound was introduced under one sq.inch patch on one intact site and 0.5 ml under one sq.inch patch on one abraded site, while on the remaining two sites 0.5 ml of normal saline was introduced, which served as control. All the four sites were wrapped and held firmly with gauge. The animals were immobilised in animal holders with patches

secured in place. After 24 hours exposure, the patches were removed and the resulting skin reaction in terms of erythema and edema were graded from 0-4 after 24 and 72 hours and the Primary Skin Irritation Index was obtained¹⁹.

Neurotoxicity :

After review of the acute toxicity study, regarding the lethal dose and variation in animal response, a dose range finding study was performed using one chicken weighing around 1.5-2.0 kg to determine maximum tolerable dose which produces symptoms but not mortality within a period of one week (range finding duration). Additionally, acceptability of diet, body weight, egg laying capacity and other symptoms were observed during the range finding study. Animals were housed individually in cages.

Based on the range finding study, two doses, 2650.0 mg/kg and 1325.0 mg/kg for neem concentrate and 5100.0 mg/kg and 2550.0 mg/kg for neem formulation were selected to be administered daily orally for 21 days.

Group of 3 chickens (females) were selected for each test group and control group. The compound was given orally to the test chickens for 21 days. Control group received just food and water. All animals were sacrificed at the end of the experimental period. Brain spinal cord and sciatic nerve were collected and studied for histopathology.

Blood samples were collected prior to the experiment and also at the end of the test period.

All the birds including control birds were observed for general health, behavioural changes, abnormal symptoms, food intake, body weights, egg laying capacity and egg weights during the experiment.

Laboratory investigations:

The following tests were performed on all birds before (0 day) and after termination (22 day) of the test. The methods employed for the determinations were as per standard procedures.

Haematology^{20,21,22} : Haemoglobin, packed cell volume, red blood cell count, white blood cell count and differential count.

Biochemical parameters : Whole blood cholinesterase²³, plasma cholinesterase²³, plasma proteins²⁴, plasma albumin, plasma pyruvic glutamic transaminase²⁵, alkaline phosphatase²⁶ and blood sugar level²⁷.

Histopathology : All the animals were sacrificed at the end of the experimental period for recording gross lesions and tissues (brain, spinal cord and sciatic nerve) were taken for histopathology. The tissues were collected immediately after the post-mortem and were fixed in 10% formal saline for histopathology.

Results and Discussion

Large number of chemicals are used as pesticides or newer ones are being discovered for application in agriculture, health programmes and for household purposes. The increasing toxic problems to human beings, environment, and the emergence of pests resistance to chemical pesticides, have induced scientist to look for alternative methods and sources of pesticides. Due to the lack of detailed toxicological data of such potential pesticides of plant origin, its use inevitably is linked to examination of the potential of such plants or their products to cause risk to health of man and animals and to the ecosystem as whole. Biological toxicity experiments are fundamentally incapable of providing 100% guarantee of total safety, as most of the interpretations are based on animal studies, due to ethical

reasons, and also there is no single test or animal model to predict all possible hazards. The risks can be reduced by conducting various planned studies in different species, so that even if possible hazard to man cannot be completely eliminated the inevitable risk observed in animals is sufficient to be socially acceptable as a possible risk.

Acute toxicity study is one important part of entire toxicological evaluation that is mandatory for regulatory purposes. Accidental, intentional and occupational may occur with any consumer product like pesticides. Acute toxicity gives rapid indication of the potential hazard under the above circumstances and indicates the class of toxicity to which the pesticide belongs. Acute toxicity is the effect resulting from the administration of the compound as a single dose or multiple doses over a period of 24 hours or less. The effect generally reported is mortality and is generally expressed as LD₅₀ (The dose required to kill 50% of the test animals of that dose).

It is necessary to evaluate the LD₅₀ so as to classify the ranking order in respect of acute toxicity²⁸ the dose required to produce toxic symptoms and symptoms produced. The LD₅₀ also serves as a guide for selection of doses for long term studies. Regulatory agencies have issued guidelines for undertaking toxicity studies and have specified the number and types of tests, the types of animal model to be used for safety evaluation^{12,13,16}.

In the present study the acute toxicity of neem technical, neem formulation, karanj technical and *Ammona squamosa* technical (methanol and ethyl acetate extracts) were undertaken in mice, rats, chickens, pigeons, fish and rabbits. For comparison acute toxicity of quinalphos, an organophosphorus pesticide was also studied.

Results of the acute oral toxicity in mice show that LD₅₀ of neem technical was found to be 19080.0 mg/kg and of karanj technical to be 8303

technical and neem formulation was found to be 15900.0 mg/kg and 15300.0 mg/kg, whereas MLD of quinalphos was found to be 2.0 mg/kg. In both species quinalphos seems to be very toxic. In pigeons it is 5 times more toxic than in chickens.

Results of the acute toxicity in fish formulation show that the LC_{50} of neem technical 280.0 ppm and of neem formulation was 1300.0 ppm respectively as compared to 1.0 ppm in quinalphos. Quinalphos is very toxic in comparison to neem technical and neem formulation to fish.

In acute dermal toxicity study, the LD_{50} of neem technical and neem formulation was found to be more than 10600.0 mg/kg and 15350.0 mg/kg respectively in rabbits as compared to 339.0 mg/kg of quinalphos, which suggests that quinalphos is moderately toxic whereas neem technical and neem formulation is comparatively non-toxic. It also suggests that quinalphos even at a very low dose is absorbed through the skin.

In the mucous membrane irritation and primary skin irritation study of neem technical and neem formulation conducted on rabbits, they were found to be mild irritants, whereas quinalphos was moderately irritant in comparison.

The neurotoxicity studies with 2 dose groups of neem technical and neem formulation were conducted in chickens. There was no mortality in any groups of experimental chickens fed with 2650.0 mg/kg and 1325.0 mg/kg of neem technical, and 5100.0 mg/kg and 2550.0 mg/kg of neem formulation administered orally for a period of 21 days. No significant changes in the body weight, food consumption, no. of eggs and egg weights were observed and death (if any) during the experimental period of feeding the test compounds. The general health was also compared with the control group. Haematological findings in control and the test groups were comparable with each other and were within the normal range. Biochemical

findings in control and the test groups were comparable with each other and were found to be within the normal range.

Conclusion

The present acute study suggests that Neem technical, Neem formulation, *Annona squamosa* technical and Karanj technical are practically non-toxic by oral and dermal routes. They are mildly irritant to the skin and the mucous membrane. In comparison, quinalphos was highly toxic by oral and dermal routes. It was also moderately irritant to the skin. In the neurotoxicity study in chickens with neem technical and neem formulation there was no effect on general health, body weight, no. of eggs and egg size, food intake in the experimental birds and were similar to the control birds. No ataxia or paralysis or mortality was observed.

Acute Toxicity

Species	Route	Quinalphos	Neem Concentrate	Neem Formulation
Chickens	Oral (MLD)	10.0 mg/kg	>15300.0 mg/kg	>15900.0 mg/kg
Pigeons	Oral (MLD)	2.0 mg/kg	>15300.0 mg/kg	>15900.0 mg/kg
Fish	-- (LC ₅₀)	1.0 ppm	280.0 ppm	1300.0 ppm
Rabbits	Dermal (LD ₅₀)	339.0 mg/kg	> 10600.0 mg/kg	>15900.0 mg/kg

Species	Study/Route	Neem Concentrate	Neem Formulation
Rabbits	Mucous Membrane Irritation	Mild Irritant	Mild Irritant
Rabbits	Primary Skin Irritation	Mild Irritant	Mild Irritant

Bibliography

1. Jones, L.M. (1957). Insecticides. In Veterinary Pharmacology and Therapeutics, ed.2. Iowa State University, Iowa, p.618-641.
2. McCale R.S. (1992). Food ingredients safety evaluation. Food and Drug Law Journal. 47 : 657-663.
3. Patil S.G., Hugar Parmeshwar (1991). Ecological impacts of pesticides on wildlife. Myforest, 27(3) : 244-247.
4. Dethé M.D., Kala V.D. Walunj A.R., Rane S.D. (1991). DDT and HCH residues in milk samples from rural area of Ahmednagar, Western Maharashtra. J. Ecotoxicology and Environmental Monitoring. 1(2) : 95-98.
5. Dethé M.D., Dharme P.K., Kala V.D. (1990). Residues of endosulfan and monocrotophos in pigeon pea. Growth Development and Natural Resource Conservation, Nature conservators. 283-285.
6. Dethé M.D., Kala V.D., Walunj A.R. (1992): Hexachlorocyclohexane (HCH) residues in rice. Ecotoxicology and Environmental Monitoring. 1(2) : 91-94.
7. Abby A. Li., Karen J. Asbury, William E. Hopkins, Paul C.C. Feng, Alan G.E., Wilson (1992). Metabolism of alachlor by rat and monkey liver and nasal turbinate tissue. Drug metabolism and disposition. 20(6) : 616-618.
8. Srivastava A.K., Gupta B.N., Mathur N., Mahendra P.N., Bharti R.S. (1991). Clinical and Biochemical study of pesticide sprayers. : Hum. Expt. Toxicol. 10(4) : 279-283.
9. Bernard A.. Schwetz, Widening concepts of Toxicology. In Pesticide Chemistry and Modern Toxicology p.21-26.
10. Chari M.S., Prabhu S.R. and G. Ramaprasad (1990). Role of botanicals in pest management of tobacco in India. In Proceedings of Symposia of Botanical pesticides in IPM, Rajahmundry, p.128-133.

11. Venkataramireddy P., Chitra K.C. and P. Kameswara Rao (1990) : Efficacy of Brinjal spotted leaf Beetle *Henosepilachna viogintiotopunctata* F. In Proceedings of Symposia of Botanical pesticides in IPM, Rajahmundry, p.255-257.
12. Pesticide Assessment Guidelines. Subdivision F. Hazard Evaluation : Domestic Animals U.S. Environmental Protection Agency, Washington DC 1982. p.34-44.
13. Appraisal of the safety of chemicals in Foods, Drugs and Cosmetics. Association of Food and Drug Officials of the United States 1975. p.17-25.
14. Balazs T. (1971) : Measurement of Acute Toxicity. In Methods in Toxicology, Ed. Paget G.E., Publ. Blackwell Scientific Publications. p.50-51.
15. Litchfield J.T. Jr. and Wilcoxon F. (1949). A simplified method of evaluating dose effect experiments. J. Pharmacol. Exp. Ther. 96:99-113.
16. Report of the Sub-Committee on Pesticide Toxicology by Dr. B.B. Gaitonde to the Registration Committee, Central Insecticide Board, Ministry of Agriculture, Government of India 1978. p.70-74.
17. WADC Technical Report 55-16 (1955). Handbook of Toxicology vol.1 Ed. Spector W.S. Prepared by Wright Air Development Center, United States Air Force, p.3-4.
18. Bioassay Methods for evaluating Acute Toxicity of Industrial Effluents and waste water. IS6582 (1971). Indian Standard Institute, New Delhi.
19. Draize J.H. Dermal Toxicity. In Safety of Chemicals in Food, Drugs and Cosmetics, p.46-58.
20. Baker F.J. and Silverton R.E. (1976). Haematology. In Introduction to Medical Laboratory Technology. 5th ed., Publ. Butterworth, London, Boston, p.549-629.
21. Barucha C., Meyer H., Bharucha H., Moody A. and Carman R.H. (1974). Haematology. In Handbook of Medical Laboratory Technology. Publ. Christian Medical Association of India, p.53-115.

22. Lucas A.M. and Jamoroz C. (1974). Atlas of Avian Haematology. Pub. Oxford and IBH Publishing Company, New Delhi. p.222-234.
23. Biggs H.G., Shirley Carey and Morrison D.B. (1958). A simple colorimetric method of measuring activities of cellular and plasma cholinesterase. J. Clin. Path. 7:322-326.
24. Richterich R. (1969). Modified method for protein estimation for protein estimation. In Clinical Chemistry Theory and Practice Ed. Karger S., Basel. Publ. Acad Press p.245.
25. Reitman S. and Frankel S. (1957). A colorimetric method for the determination of serum glutamic oxalate transaminase and serum glutamic pyruvic transaminase. Am.J. Clin. Path. 28:56-63.
26. Kind P.R.N. and King E.J. (1954). Estimation of plasma phosphatases by determination of hydrolysed phenol with amino antipyrine. J. Clin. Path. 7:322-326.
27. Nelson M. and Somogyi M. (1951). Colorimetric method of estimation of blood sugar. In Practical Biochemistry. ED. Varley H., ed 4. (Indian ed. 1975) Publ. Arnold Heinemann and Gulab Vazinani, p. 89-90.
28. Hodge H.C. and Sterner J.H. (Dec 1943). American Industrial Hygiene Association Quarterly. 10(4):93.

