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Effects of azadirachtin on insect and mammalian cultured cells

By

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GLASGOW

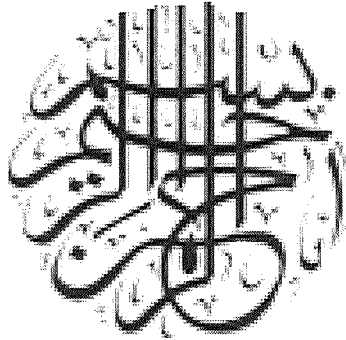
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In the name of

ALLAH

Most gracious Most merciful



Thanking Him

With a full heart and devoted tongue

To My parents

Acknowledgement

I would like to express my sincerest thanks to everyone who assisted me during the course of my work especially:

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DECLARATION

I hereby declare that the thesis which follows is my own composition, that is a record of work done by myself and that has not been presented in any previous application for a Higher Degree.



Areaf Salehzadeh

Summary:

Azadirachtin, a tetranortriterpenoid from the Neem Tree (*Azadirachta indica*), has been shown to affect insects in many ways. Antifeedancy, repellency, and profound effects on growth, development and reproduction, are the most important pesticidal properties of this phytochemical. Many experiments with insects have shown that azadirachtin interferes with the functions of the neurosecretory system. In the last ten years, it has been shown that the compound is cytotoxic to cultured insect cells, but there is still uncertainty as to its exact mode of action at the cellular level.

The aim of this project was to try to find the mode of action of azadirachtin in cultured cells, and to compare its cytotoxicity with some well-known phytochemical pesticides. The results presented here showed that azadirachtin was toxic to the cultured insect cells used (Sf9 and C6/36, derived respectively from *Spodoptera frugiperda* and *Aedes albopictus*) even in very low concentrations with an EC_{50} for the *Spodoptera* cells estimated at 5×10^{-9} M, but that the mammalian fibroblast cell line L929 was little affected except at concentrations greater than 10^{-4} M.

The other major neem terpenoids, nimbin and salannin, showed low toxicity towards the cultured cells. The neurotoxic pyrethrum showed little effect against the cultures, except for some slight stimulatory effect on growth at 10^{-8} M. Rotenone, known to inhibit the electron transport chain, effectively inhibited the growth of both insect and mammalian cells. Nicotine, another neurotoxic phytochemical, had little effect on the growth of the cultured cells. It was concluded that while cell growth assessment is not appropriate for all phytochemical pesticides, it is useful for those, such as azadirachtin and rotenone, whose effect is on the essential mechanisms of insect cells in general.

Rotenone was used as a positive control to investigate if azadirachtin had its effect on respiration of the cells. Only at the highest feasible concentration of azadirachtin, was there a slight but significant (15%) reduction of respiration which was the same in both insect and mammalian cells. As expected, rotenone inhibited both insect and mammalian cells even at concentrations as low as 10^{-11} M.

When the effects of azadirachtin on the cell cycle were examined by means of cell cytometry, it was shown that the compound arrested the cell-cycle in G2/M phase, and that the effect was related to the concentration. Microscopy confirmed that there was a three-fold increase in the mitotic index after 2 hours of exposure to 2×10^{-6} M azadirachtin. The similarity of the of the nuclear profiles and cell-cycle distribution to Sf9 cells treated with colchicine, a well-known antimitotic phytochemical, suggested there was a similarity of action between the two compounds. This was confirmed by the fact that both were apparently able to antagonise the effects of taxol, and by displacement experiments with a fluorescent derivative of colchicine. Azadirachtin was shown to inhibit *in vitro* the polymerisation of purified pig-brain tubulin, suggesting that this was how it prevented cell division.

Although azadirachtin only affected L929 cells at 10^{-4} M, at this concentration the cell cytometry showed an increase in the proportion of cells in S-phase, which could be due to an effect on DNA synthesis. An adverse effect on structure or functions of DNA might also account for the rapid decrease in mitotic index in Sf9 cells after 2 hours in 10^{-6} M azadirachtin.

The cumulative results from this and previous work suggest that azadirachtin in low (nanomolar) concentrations has its effect on insect cells by interfering with the polymerisation

of tubulin, which in turn prevents many cellular functions, including cell division. There may, however, be other sites of action found in mammalian as well as insect cells.

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List of abbreviations

Ach	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
Bub2	Protein required for cell cycle arrest in response to loss of microtubule function
CDK	Cyclin-dependent kinase
CSIs	Chitin Synthesis Inhibitors
CNS	Central nervous system
CoQ	Coenzyme Q or Ubiquinone
CSIs	Chitin Synthesis Inhibitors
D1	One of mammalian G1 cyclins
Da	Daltons
DDT	1,1,1-trichloro,2,2-bis (p-chlorophenyl) ethane
DEET	N,N-diethyl-m-toluamide
DCVJ	9-(dicyanovinyl)-julolidine
DMSO	Dimethyl sulfoxide
EC₅₀	Concentration giving half-maximal response
ED₅₀	Median effective dose, that produces a response in 50% of individuals
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene Glycol bis-(β-aminoethyl ether), N,N,N,N—Tetraacetic Acid
FBS	Foetal bovine serum
FAScan	Cell sorting flow cytometry

FMN	Flavin mononucleotide
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GABA	γ - amino Butyric Acid
γ-BHC	γ -benzylhexachloride
HPLC	High performance liquid chromatography
IC₅₀	Concentration of an inhibitor at which 50% inhibition of the response is seen
IGR	Insect growth regulator
JH	Juvenile hormone
MDR	Multiple drug resistance
MEM	Minimum essential medium
MEN	Mitotic exit network. This indicates a network of genes, now known as the controlling mitotic exit at some level
MOPS	4-[Morpholino]butanesulfonic acid
MTs	Microtubules
MAP	Microtubule associated protein
MTT	3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of Nicotinamide adenine dinucleotide
P16	One of cell cycle inhibitor genes, also referred to as cyclin-dependent kinase inhibitors (CDKi)
P21	Human cyclin-dependent kinase inhibitor
P53	The protein p53 which is critical in maintaining ordered proliferation, growth, and differentiation of normal cells.
PBS	phosphate-buffered saline
PMS	Phenazine methosulfate

PSMOs	Polysubstrate monooxygenases enzyme. The earlier name for these enzymes was mixed-function oxidases (MFOs)
PNS	Peripheral nervous system
Rb protein	Retinoblastoma tumour suppressor a nuclear protein
RNA	Ribonucleic acid
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TCA cycle	Tricarboxylic acid cycle
TEPP	Tetraethyl pyrophosphate
TEMED	N,N,N,'N'-tetramethylethylenediamine
TP53	Tumour Protein 53
UV	Ultraviolet
Vimentin	Intermediate filament protein (58 kD) found in mesodermally-derived cells (including muscle).
v/v	Volume to volume
XTT	sodium (2,3-bis[2-Methyl-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide)

Chapter 1

Introduction

1.1 The phylum Arthropoda and its impact on man

The largest phylum in the animal kingdom is the Arthropoda, which contains about 80% of the known species of animals (Kettle, 1995). In addition to insects, this phylum contains a tremendous variety of forms, such as crabs, sowbugs, spiders, mites, trilobites, centipedes, and many others. As insects gradually evolved morphological, physiological, and behavioural adaptations to meet new ecological conditions they, perhaps more than any other terrestrial group of animals, successfully subdivided their habitats into very small ecological niches. They currently occupy a wide range of habitats, ranging from living on other insects or plants to living in or on higher animals. Although only small proportion of arthropoda interfere directly with human life, their effects on different aspects of human communities are quite obvious.

1.1.1. Arthropod pests of plants

Damage to plants by arthropods occurs in myriad ways. All the major field crops grown either as food or as a cash crop suffer losses from insect attack. In developing countries one of the major reasons for low agricultural yields is crop pest attack affecting both the quantity and quality of the harvest. Also, insect pests cause heavy food grain loss in storage, particularly at the farm level in tropical countries (Iqbal, 1999). Approximately one third of the world food crop is damaged or destroyed by insect pests during growth, harvest, and storage. In 1961, it was estimated that there were 9000 species of insect and mites that infest crops and most of these are insects that have moved from native vegetation onto the introduced crop (Copping *et al*, 1998). Losses are considerably higher in many countries of Asia and Africa. The monetary loss due to feeding by larvae and adults of pest insects amounts to billions of dollars each year (Jacobson *et al*, 1998).

Because they feed on all the natural foods eaten by humans, arthropods have been considered our most serious competitor for the food and fibre products (Ross, 1982).

1.1.2. Arthropod Pests of animals and humans

Parasitic arthropods continue to be a major factor influencing human and animal health in many parts of the world. Damage to humans and domestic animals is caused chiefly by “bites” inflicted by species with sucking mouth parts, such as mosquitoes, fleas, lice or ticks. They can act directly on their animal hosts, causing reduced weight gain, anaemia due to loss of blood, behavioural modification, immune suppression and damage to hides. They can also act indirectly as vectors of various viral, rickettsial, bacterial, and protozoan diseases and the site of their bite may provide a route for other infection. A number of these insects transmit organisms causing some of the worlds worst diseases (Table 1.1). For example there are half to one million malaria-related deaths per year in African children under 5 years (Alles *et al*, 1998) and the number of African trypanosomiasis cases reported by WHO was more than 25,000 cases per year (Hide *et al*, 1997). Attempts to control arthropod-borne disease can, however, act at a variety of levels. The disease itself can be treated, or vaccination can be used to protect against the pathogen. Attempts to control arthropod parasites and vectors have taken various forms, ranging from species-specific, as in the case of the sterile male technique to combat the screw-worm fly in North and Central America, to large scale modification of the environment, to control tsetse flies in Africa (Dransfield *et al*, 1991). Historically, the greatest emphasis has been placed on vector control, but there are still many major arthropod-borne diseases (Petney, 1997).

Table1.1. The major arthropod borne diseases

Arthropod Vector	Disease	Disease Agent	Method of Human Exposure
Arachnida			
Mite: <i>Leptotrombidium sp.</i> (red mites)	Scrub Typhus (Tsutsugamushi disease)	<i>Rickettsia tsutsugamushi</i> (bacteria, intracellular)	Bite of larval mite
Mite: <i>Liponyssoides sanguineus</i> (mouse mite)	Rickettsial pox	<i>Rickettsia akari</i> (bacteria)	Bite of mite
Tick: <i>Dermacentor sp.</i>	Tularemia	<i>Francisella tularensis</i> (Gram negative bacteria)	Bite of tick
Tick: <i>Dermacentor sp.</i> And other Ixodid ticks	Rocky Mountain Spotted Fever	<i>Rickettsia rickettsia</i> (bacteria)	Bite of tick
Tick: <i>Ornithodoros sp.</i>	Endemic Relapsing Fever	<i>Borrelia sp.</i> (bacteria, spiral shaped)	Bite of tick
Tick: <i>Ixodes sp.</i>	Babesiosis	<i>Babesia microti</i> (parasite, protozoan)	Bite of tick
Tick: <i>Ixodes sp.</i>	Lyme disease	<i>Borrelia burgdorferi</i> (bacteria, spiral shape)	Bite of tick
Tick: <i>Dermacentor variabilis</i> , <i>Amblyomma americanum</i>	Ehrlichiosis, Sennetsu fever	<i>Ehrlichia canis</i> <i>E. sennetsu</i> <i>E. chaffeensis</i> <i>E. equi</i> <i>E. phagocytophilia</i> (bacteria, intracellular)	Bite of tick
Tick: <i>Dermacentor sp.</i>	Colorado Tick Fever	CTF virus, Eyach virus, or strain S6-14-03 (Reoviridae)	Bite of tick
Tick:	Russian Spring-Summer Encephalitis, Louping Ill Encephalitis, Langat Encephalitis, Powassan Encephalitis, Omsk hemorrhagic fever	Russian Spring-Summer Encephalitis, Louping Ill Encephalitis, Langat virus, Powassan virus, Omsk hemorrhagic fever virus (Flaviviridae)	Bite of tick
Tick:	Nairobi Sheep fever, Crimean hemorrhagic fever	Nairobi sheep disease virus, Crimean-Congo hemorrhagic fever virus (Bunyaviridae)	Bite of tick
Crustacea			
Copepod: <i>Cyclops sp.</i>	Diphyllobothriasis, fish tapeworm	<i>Diphyllobothrium latum</i> (parasite, cestode, tapeworm)	Arthropod is 1st intermediate host then man swallows infected fish
Copepod: <i>Cyclops sp.</i>	Sparganosis	<i>Diphyllobothrium spiroметра</i> (parasite, cestode, tapeworm)	Man swallows infected <i>Cyclops</i> .

Tble 1.1 Continued

Copepod: <i>Cyclops</i> sp.	Dracunculosis	<i>Dracunculus medinensis</i>	Man swallows infected <i>Cyclops</i> .
Crabs, crayfish: various freshwater species	Paragonimiasis	<i>Paragonimus westermani</i>	Man eats infected crustacean.
Insecta			
Lice: <i>Pediculus Humanus</i>	Epidemic typhus	<i>Rickettsia prowazekii</i> (bacteria)	"Bite," contaminated by louse feces or crushing louse on skin
Lice: <i>Pediculus humanus</i>	Trench fever, bacillary angiomatosis, bacillary peliosis	<i>Bartonella quintana</i> (Gram negative bacteria)	"Bite," contaminated by louse feces or crushing louse on skin
Lice: <i>Pediculus humanus</i>	Louse-borne relapsing fever or epidemic relapsing fever	<i>Borrelia recurrentis</i> (bacteria; spiral shape)	"Bite," contaminated by louse feces or crushing louse on skin
Flea: <i>Xenopsylla cheopis</i>, and various other rodent fleas	Plague	<i>Yersinia pestis</i> (Gram negative rod shaped bacteria)	"Bite" and feces of flea
Flea: <i>Xenopsylla cheopis</i>	Murine typhus	<i>Rickettsia typhi</i> (bacteria)	"Bite" and feces of flea
Flea: <i>Xenopsylla cheopis</i>, and various other rodent fleas	Rat tapeworm infection	<i>Hymenolepsis diminuta</i> (parasite; cestode; tapeworm)	Swallowing infected flea
Flea: various species	Dog tapeworm infection, Dipylidiasis	<i>Dipylidium caninum</i> (parasite; cestode; tapeworm)	Swallowing infected flea
Bug: <i>Triatoma</i> species, <i>Panstrongylus</i> sps (Kissing assassin bug, Reduvid bug)	Chaga's disease	<i>Trypanosoma cruzi</i> (parasite; protozoan)	Rubbing infected feces on mucous membranes or skin
Beetles: Flour beetle	Hymenolepsis	<i>Hymenolepsis nana</i> (parasite; tapeworm; cestode)	Swallowing infected beetle
Fly, gnat: <i>Glossina</i> sp. (tsetse fly)	African trypanosomiasis, African sleeping sickness	<i>Trypanosoma brucei rhodesiense</i> and <i>T.b. gambiense</i>	Bite of infected fly
Fly, gnat: <i>Simulium</i> sp. (black fly)	Onchocerciasis, River blindness	<i>Onchocerca volvulus</i> (parasite; round worm; nematode)	Bite of infected fly

Table1.1. Continued

Fly, gnat: <i>Chrysops</i> sp.	Tularemia	<i>Francisella tularensis</i> (Gram negative rod shaped bacteria)	Bite of infected fly
Fly, gnat: <i>Phlebotomus</i> sp., <i>Lutzomyia</i> sp. (sandflies)	Leishmaniasis	<i>Leishmania donovani</i> (Visceral, dum dum fever, kala-azar), <i>L. tropica</i> (cutaneous; oriental sore, Delphi boil), <i>L. braziliensis</i> (mucocutaneous; espundia, american leishmaniasis, chiclero ulcer) (parasite; protozoan)	Bite of infected fly
Fly, gnat: <i>Phlebotomus</i> sp. (sandfly in Peru, Ecuador and Columbia)	Bartonellosis, Oroya fever, Carrion's disease	<i>Bartonella bacilliformis</i> (Gram negative bacteria)	Bite of infected fly
Fly, gnat: <i>Chrysops</i> sp. (mango flies)	Loiasis, Eye worm	<i>Loa loa</i> (parasite; nematode; roundworm)	Bite of infected fly
Fly, gnat: sandfly	Sandfly fever, Rift Valley fever	Sandfly fever Naples virus, Sandfly fever Sicilian virus, Rift valley fever virus (Bunyaviridae)	Bite of infected fly
Mosquito: <i>Anopheles</i> sp.	Malaria	<i>Plasmodium falciparum</i> , <i>P. malariae</i> , <i>P. vivax</i> , <i>P. ovale</i> (parasite; protozoan)	Bite of infected mosquito
Mosquito: various species	Bancroftian filariasis, filarial Elephantiasis	<i>Wuchereria bancrofti</i> (parasite; nematode; roundworm)	Bite of infected mosquito
Mosquito: various species	Malayan filariasis, filarial Elephantiasis	<i>Brugia malayi</i> (parasite; nematode; roundworm)	Bite of infected mosquito
Mosquito: various species	Dirofilariasis	<i>Dirofilaria immitis</i> (parasite; nematode; roundworm)	Bite of infected mosquito
Mosquito: <i>Aedes aegypti</i>	Yellow fever	Yellow fever virus (Flaviviridae)	Bite of infected mosquito
Mosquito: <i>Aedes</i> sp.	Dengue fever, Break Bone fever	Dengue fever virus (Flaviviridae)	Bite of infected mosquito
Mosquito: <i>Culiseta melanura</i> , <i>Coquillettidia perturbans</i> , <i>Aedes vexans</i>	Eastern Equine encephalitis	Eastern Equine Encephalitis virus (Togaviridae)	Bite of infected mosquito
Mosquito: <i>Aedes triseriatus</i>	La Crosse encephalitis	La Crosse Encephalitis virus (Bunyaviridae)	Bite of infected mosquito
Mosquito: <i>Culex</i> sp.	St. Louis encephalitis	St. Louis Encephalitis virus (Flaviviridae)	Bite of infected mosquito

Table1.1 continued

Mosquito: <i>Culex</i> sp., <i>Culex tarsalis</i>	Venezualan equine encephalitis, Western equine encephalitis	Venezualan Equine Encephalitis virus, Western Equine Encephalitis virus (Togaviridae)	Bite of infected mosquito
Mosquito	Chikungunya forest fever	Chikungunya virus, Mayaro fever, Mucambo fever, O'Nyong-Nyong fever, Pixuna fever, Ross River fever (Togaviridae)	"
Mosquito	Fevers and encephalitis	Nile fever, Japanese encephalitis virus, West Nile fever, Zika fever, Wesselsbron fever, Kyasanur forest disease virus (Flaviviridae)	"
Mosquito	Fevers and encephalitis	Oropouche virus, Bunyamwera, Bwamba fever, Guama fever, Oropouche fever, California Encephalitis virus (Bunyaviridae)	"
Mosquito	Fevers	Chandipura fever, Piry fever (Rhabdoviridae)	"

Table was taken from “ <http://www.kcom.edu/faculty/chamberlain/arthro.htm> ”

1.2. Chemical control of pests

The use of chemicals to control insects possibly dates back to 2500 BC, when Sumerians were using sulphur compounds for insect control. Later, in China, seeds were treated with various natural organic substances to protect against insects, mice and birds, while inorganic mercury and arsenic compounds were applied for the control of body lice and other pest (Conway *et al*, 1991). Homer mentioned the fumigant value of burning sulphur, which was used by Greeks and Romans.

By the sixteenth century moderate amounts of arsenicals were employed by Chinese and not long afterwards nicotine, in the form of tobacco extracts, were used to control the plum curculio (*Conotrachelus nenuphar* (Herbst)). The middle of the nineteenth century, marked the beginning of the first systematic scientific studies into the use of crop protection chemicals.

Work on arsenic compounds led to the introduction in 1867 of “Paris Green” an impure copper arsenate. It was used in the USA to check the spread of the colorado beetle (*Leptinotarsa decemlineata* Say) and by 1900 its use was so widespread that it led to the introduction of what was probably the first pesticide legislation in the world. In the years between the two world wars both the number and the complexity of crop protection chemicals increased. The cosmopolitan nature of contributions to chemical pest control was even more evident after 1939. During the Second World War, the insecticidal potential of DDT was discovered in Switzerland and insecticidal organophosphorus compounds were developed in Germany. Shortly afterwards, the insecticidal carbamates were developed in Switzerland (Hassall, 1982).

In the 1960s, organophosphorus compounds were developed very fast and some companies started to make synthetic pyrethroids to offset the failures of natural compounds.

The discovery of the insecticidal properties of the synthetic organic compounds made a dramatic change in the pattern and in the consequences of pesticide use. In a remarkably short time, they were being used in almost every crop and most countries of the world.

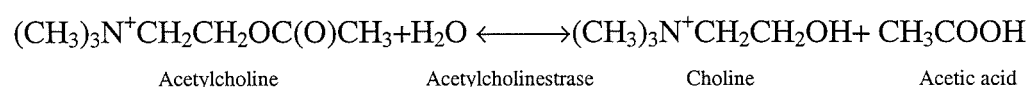
1.3. Insect nervous system

The importance of the nervous system to the overall function of animals has made it an extremely sensitive target for action of pesticides. A discrete lesion in metabolism at one site may be lethal to an animal through the consequence of general destruction of nervous integrity (Jabbar, 1982).

The target sites within the nervous system of insects known at present are very restricted. In most cases, they consist of: sodium channels; the components of the nicotinic cholinergic synapse and the γ -aminobutyric acid (GABA) and octopamine receptors (Copping *et al*, 1998). Nerve function is a transfer of electrical pulses through nerve cells and across the gaps between nerve cells, the synapse, so that a message is transmitted from the brain to muscles or other responsible tissue or from sensory tissue to the brain. It is possible to interfere with nerve function in a number of ways. Nerve impulses pass down the axons of nerve cells as a result of changes in

the permeability of the axon membrane to sodium and potassium ions. When at rest, the electrical potential within the membrane is negative in comparison to the outside. The concentration of sodium inside the cell is low and the concentration of potassium is high (Mathews *et al*, 1990). Potassium and sodium ions enter the cell using two mechanisms: sodium and potassium channels (or gates) allow a rapid passive movement when opened, whilst a slower, active movement occurs through ion pumps. A nerve impulse passing down an axon is a wave of changing polarity. It is caused by the sodium gate opening so that sodium passes in and then the potassium gate opening so that potassium can move out, thereby restoring the electrical polarity. The resting condition is restored by the operation of the ion pump taking up potassium at the expense of ejected sodium. Ion channels have been extensively studied in the fruit fly *Drosophyla melanogaster* by Wicher *et al* (2001). At the end of an axon, where it meets another nerve cell or an effector cell (a cell such as a muscle or a gland cell), there is a gap or junction that is usually about 10 to 20 nm wide and this is known as a synapse. The passage of nerve impulse across this synapse is chemical rather than electrical. When the nerve impulse reaches a synapse it initiates the release of a neurotransmitter that is usually acetylcholine. Other transmitters have been identified and these include L-glutamate and γ -aminobutyric acid (GABA). The released acetylcholine interacts with a receptor on the adjoining cell and the binding of the acetylcholine with this receptor causes this post-synaptic cell to pass on the impulse (if it is another axon) or do work (if it is a muscle or gland cell).

After that the neurotransmitter is rapidly hydrolyzed by an enzyme called acetylcholinesterase.



As choline, the product of hydrolysis, is a very poor neurotransmitter, degradation of acetylcholine restores the resting potential in the post-synaptic membrane. In order to prepare the synapse for another impulse, the empty synaptic vesicles, which are returned to the axonal terminal bulb by endocytosis, must be refilled with acetylcholine. This is accomplished by an acetylcholine transporter protein, which brings newly synthesised acetylcholine into the vesicles by exchanging it for protons (Mathews *et al*, 1990). On the other hand if this enzyme does not work properly, acetylcholine will remain unchanged and the message will continue to be transmitted

without the electrical stimulation from the axon. (Copping *et al*, 1998). Acetylcholinesterase is the main target of carbamate and organophosphate pesticides, which the latter are used extensively worldwide and is the most important group of insecticides (Kwong, 2002).

1.4. Synthetc pesticides

1.4.1. Organochlorines

Organochlorine compounds contain carbon, hydrogen and and chlorine in their structure. The other names for these pesticides are chlorinated hydrocarbons, chlorinated organics, chlorinated insecticides, and chlorinated synthetics, which most of them (e.g. DDT, eldrin and dieldrin) are not used longer.

1.4.1.1. Dichlorodiphenylene derivatives

This group is the oldest group of the organochlorines, which includes DDT, DDD, dicofol, ethylan, chlorobenzilate, and methoxychlor. DDT (Figure 1.1), the most important member of this group, was first prepared by Zildel, but its insecticidal properties were not discovered until 1939 by Muller (Cremlyn, 1978). It is probably the most notorious (see section 1.11.4 and 1.11.5) chemical of the 20th century. DDT is also fascinating, and remains to be acknowledged as the most useful insecticide developed. The application of DTT in public health was so useful that Muller was awarded a Nobel prize in 1948. Indeed, before the introduction of this pesticide 200 million people suffered of malaria, with an average of 2.5 million deaths per year (Turusov *et al*, 2002). DDT also was effective in halting epidemics of typhus, plague and urban yellow fever. It was used extensively in second the World War for the control of lice, by direct application to human body.

More than 4 billion pounds of DDT were used throughout the world, beginning in 1940, and ending essentially in 1973, when the U.S. Environmental Protection Agency cancelled all uses (Korrick *et al*, 2001). The remaining First World countries rapidly followed suit.

DDT is a crystalline non-polar solid that is soluble in most organic solvents. The intoxication of DDT is characterised by motor unrest, spontaneous movement, hyperirritability, tremors, ataxia, convulsions and death.

In insects, the primary action of DDT is on peripheral nerve fibers, whereas in vertebrates the action is both the central and peripheral components of the nervous system. DDT is not well absorbed via the skin, but, inhalation of its dust produces lung irritation and pathological changes in the liver, including cell and mitochondrial enlargement. The neurotoxic action of DDT is on sensory and motor nerve fibers, as well as on the motor cortex. It alters sodium and potassium ion transport across membranes of the nerve axon, actually blocking the potassium efflux across the membrane. It is chemically stable, and only a small amount of the environmental level of DDT is broken down in the ecosystem via dechlorination, resulting in accumulation in bio-organisms and in the environment.

Other chlorinated hydrocarbon insecticides introduced between 1939 and 1945 include aldrin, dieldrin, endrin and chlordane.

The mode of action of DDT

It seems that DDT destroys the delicate balance of sodium and potassium ions within the axons of the neurone in a way that prevents normal transmission of nerve impulses, both in insects and mammals. It apparently acts on the sodium channel to cause "leakage" of sodium ions (Yan *et al*, 1998). Also there is reports of structural activity of DDT and inhibition of calcium-independent ATPases(Beeman, R.W. 1982) Eventually the neurones fire impulses spontaneously, causing the muscles to twitch—"DDT jitters", followed by convulsions and death. DDT has a negative temperature correlation--the lower the surrounding temperature the more toxic it becomes to insects.

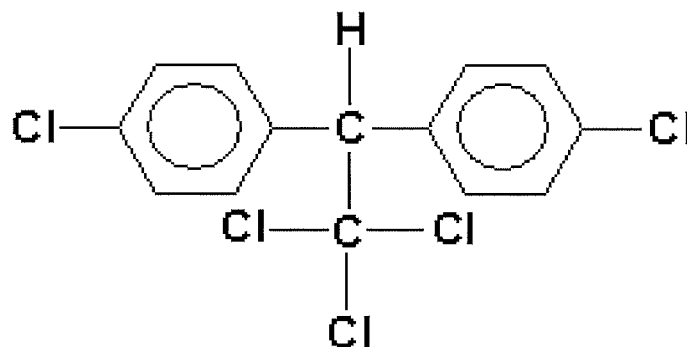


Figure 1.1 DDT

1.4.1.2. Lindane

The modern era of synthetic insecticides actually began with the discovery of hexachlorocyclohexane (HCH) which is also known as benzenehexachloride (Fig.1.2). This chemical was synthesised in 1825 by Michael Faraday (Cremllyn, 1978). In its technical grade, there are five isomers, *alpha*, *beta*, *gamma*, *delta* and *epsilon*. Surprisingly, only the *gamma* isomer (γ -1,2,3,4,5,6-hexachlorocyclohexane), or lindane has insecticidal properties. Consequently, the *gamma* isomer was isolated in manufacture and sold as the odourless insecticide. HCH has a strong musty odour and flavour, which can be imparted to treated crops and animal products. It easily evaporates from the surface of plants, especially the surface of leaves (Gryniewicz *et al.*, 2001). Lindane is a crystalline solid which is soluble in organic solvents. It is considered to be more acutely toxic than DDT, with a rat oral LD₅₀ of 125 mg/kg (Lehman, 1951) and dermal LD₅₀ of 500 mg/kg, while its human LD₅₀ was estimated at 150 mg/kg. The rat oral and dermal LD₅₀ values vary for DDT, depending on formulation (113-118 mg/kg, oral; and 2510 mg/kg, acute percutaneous (Worthing, 1987). The estimated oral fatal dose of DDT in man is 500 mg/kg (Salem *et al.*, 1988). Because of its very low cost, HCH is still used in many developing countries.

The mode of action of lindane

Like DDT, lindane probably kills insect by bringing about sodium-potassium imbalance in nerve membranes but it occurs much more rapidly. The *gamma* isomer is a neurotoxicant whose effects are normally seen within hours as increased activity, tremors, and convulsions leading to prostration which is related to binding to GABA receptor (Zisterer *et al.*, 1995). Like DDT it exhibits a negative temperature correlation, but not as pronounced as that of DDT.

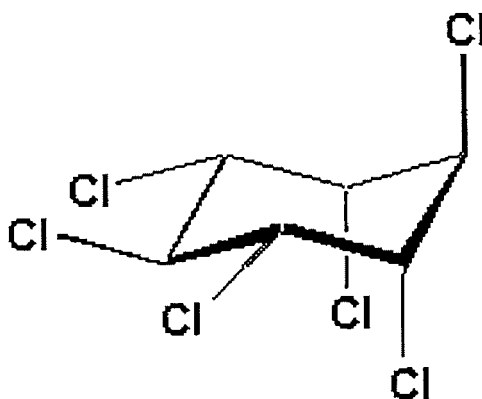


Figure 1.2: γ benzene hexachloride (Lindane)

1.4.1.3. Cyclodienes

After World War II some other chlorinated insecticides were discovered: chlordane, 1945; aldrin and dieldrin (Fig.1.3), 1948; heptachlor, 1949; endrin, 1951; mirex, 1954; endosulfan, 1956; and chlordane (Kepone(r)), 1958 (Cremllyn, 1978). There were other cyclodienes of minor importance developed in the U.S.A and Germany. Most of the cyclodienes are persistent insecticides and are stable in soil and relatively stable to

the ultraviolet of sunlight. As a result, they were used in greatest quantity as soil insecticides (especially chlordane, heptachlor, aldrin, and dieldrin) for the control of termites and soil-borne insects whose larval stages feed on the roots of plants.

The cyclodienes were the most effective, long-lasting and economical termiticides ever developed. Because of their persistence in the environment, and *biomagnification* (tendency of pollutants to concentrate as they move from one trophic level to the next) in wildlife food chains, (Van Wyk *et al*, 2001), most agricultural use of cyclodienes has been banned in the majority of countries.

The mode of action of cyclodienes

Unlike DDT and HCH, the cyclodienes have a positive temperature correlation. Their toxicity increases with increases in the surrounding temperature. Their modes of action are also not clearly understood. However, it is known that this group acts on the inhibitory mechanism called the GABA (gamma-aminobutyric acid) receptor (Purkerson-Parker *et al.*, 2001). This receptor operates by increasing chloride ion permeability of neurons. Cyclodienes prevent chloride ions from entering the neurons, and thereby antagonize the "calming" effects of GABA. Cyclodienes appear to affect all animals in generally the same way, first with the nervous activity followed by tremors, convulsions and prostration.

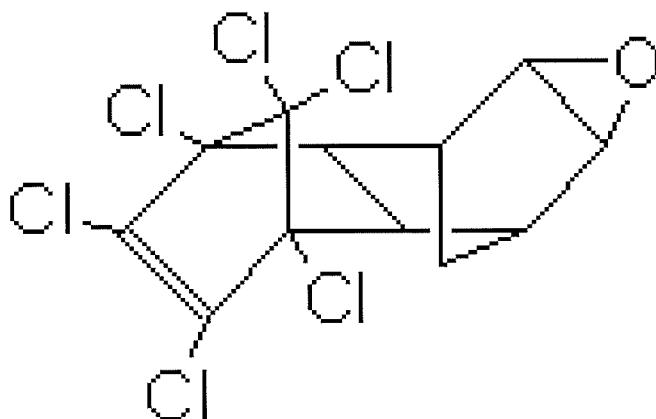


Figure 1.3 Dieldrin

1.4.1.4. Polychloroterpenes

Toxaphene (Fig.1.4) is the most important compound of this group and had by far the greatest use of any single insecticide in agriculture (Hopper *et al.*, 1979), while strobane was relatively insignificant. Toxaphene was used on cotton, first in combination with DDT, for alone it had minimal insecticidal qualities. Then, in 1965, after several major cotton insects became resistant to DDT, toxaphene was formulated with methyl parathion, an organophosphate.

Toxaphene is a mixture of more than 177 C₁₀ polychlorinated derivatives (Budavari, 1989). These materials persist in the soil, though not as long as the cyclodienes, and disappear from the surfaces of plants in 3-4 weeks. This disappearance is attributed more to volatility than to photolysis or plant metabolism. However traces of toxaphen have been found in sediments of lake even 3-9 years after treatment (Brown, 1978). Toxaphene is rather easily metabolized by mammals and birds, and is not stored in body fat nearly to the extent of DDT, HCH and the cyclodienes. Despite its low toxicity to insects, mammals and birds, fish are highly susceptible to toxaphene poisoning, in the same order of magnitude as to the cyclodienes.

Mode of action of toxaphene

Toxaphene acts on the neurones, causing an imbalance in sodium and potassium ions, similar to that of the cyclodiene insecticides. Because of their persistence in the environment, ability to concentrate up the food chain, continued detection in the food supply and breast milk, and ability to be stored in the adipose tissue of animals and humans, organochlorine pesticides have received the most attention (Snedeker, 2001) and dairy intake is the main route of human exposure to this chemicals (Jan *et al*, 2001)

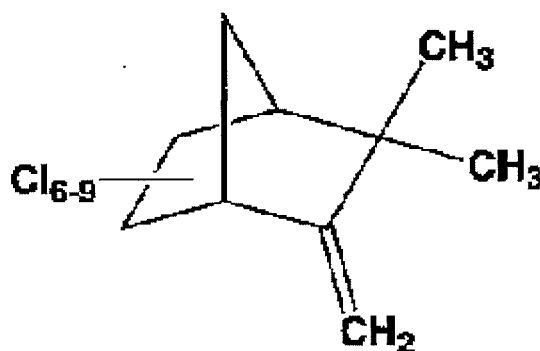


Figure 1.4 Toxaphene

1.4.2. Organophosphorus esters

Organophosphorus pesticides (OPs) are the largest and the most diverse group of insecticides and account for about 40% of registered pesticides.

They have been known since early 1800s and contain diverse compounds which all have phosphorus in their structure (Khurana *et al*, 2000).

These compounds have unlimited structural arrangement, potency and physiological properties. This group are also called *organic phosphates*, *phosphorus insecticides*, *nerve gas relatives*, and *phosphoric acid esters*. All organophosphates are derived from one of the phosphoric acids, and as a class are generally the most toxic of all pesticides to vertebrates. OPs are widely used to control insects and other harmful arthropods in a variety of diverse applications. They are highly electrophilic and thus may interact by covalent binding, with a large number of molecules such as proteins, RNA and DNA (Rodriguez *et al.*, 1999).

Because of the similarity of chemical structures of OPs to the "nerve gases", their modes of action are also similar. Their insecticidal qualities were observed in Germany during World War II in the study of the extremely toxic OP nerve gases *sarin*, *soman*, and *tabun*. Initially, the discovery was made in search of substitutes for

nicotine, which was heavily used as an insecticide but in short supply in Germany. The OPs have two distinctive features: they are generally highly toxic (Videria *et al.*, 2001) classes of insecticides, and most are chemically unstable or nonpersistent. It is this latter characteristic that brought them into agricultural use as substitutes for the persistent *organochlorines*.

There are several different substitution patterns within the group. The inhibition of acetylcholinesterase by OPs is through an attack on the relatively positive phosphorus atom by the hydroxyl group of a serine residue at the enzyme's site of action. Electron withdrawing substitutions within the OP tend to make the phosphorus more positive and, therefore, more reactive. Also this type of substitution makes the compound less stable hydrolytically (Corbett *et al.*, 1984). Although all of these compounds inhibit the action of several ester splitting enzymes in living organisms, AChE is particularly sensitive (Hartley *et al.*, 1969). The binding of OPs to acetylcholinesterase is often irreversible.

Mode of action

As it mentioned in the OPs work by inhibiting *acetyl cholinesterase* (AChE) (Storm *et al* 2000). The enzyme is said to be *phosphorylated* when it becomes attached to the phosphorous moiety of the insecticide. This inhibition results in the accumulation of acetylcholine (ACh) at the neuron/neuron and neuron/muscle (neuromuscular) junctions or synapses, causing rapid twitching of voluntary muscles and finally paralysis.

The “muscarinic” cholinergic effects following OP exposure can be alleviated by atropine. The oxymes, 2-pyridine aldoxime methiodide (2-PAM) and diacetyl monoxime (DAM), can reverse AChE inhibition only if administered soon after OP poisoning, since aged phosphorylated enzyme is not reversible by oximes. Pralidoxime is now standard in the treatment of OP intoxication. The effectiveness of oxymes is somewhat limited since they do not readily cross the blood-brain barrier (BBB) but work primarily on the PNS. Effective therapy for OP poisoning requires a combination of atropine and oxymes (Salem *et al* 1988).

1.4.2.1. Classification of OPs

All OPs are esters of phosphoric acid having varying combinations of oxygen, carbon, sulfur and nitrogen attached, resulting in six different subclasses: phosphates, phospho-nates, phosphorothioates, phosphorodithioates (Lai., *et al* 1995), phosphorothiolates and phosphoramidates. These subclasses are easily identified by their chemical names. The OPs are generally divided into three groups--*aliphatic*, *phenyl*, and *heterocyclic* derivatives.

1.4.2.2. Aliphatics

The aliphatic OPs are carbon chain-like in structure. Tetraethylpyrophosphate (TEPP) was the first organophosphorus pesticide. It was synthesized by DeClermont and was developed in Germany during World War II and it is very toxic (Nicolas *et al.*, 1994). Although it was synthesized in 1854, its toxicity was not studied until 1942, when Schrader discovered that it was toxic to both insects and mammals.

Other examples are malathion (Fig. 1.5), trichlorfon (Dylox), monocrotophos (Azodrin), dimethoate (Cygon), oxydemetonmethyl (Meta Systox), dicrotophos (Bidrin), disulfoton (Di-Syston), dichlorvos (Vapona), mevinphos (Phosdrin), methamidophos (Monitor), and acephate (Orthene) (Worthing & Walker 1987).

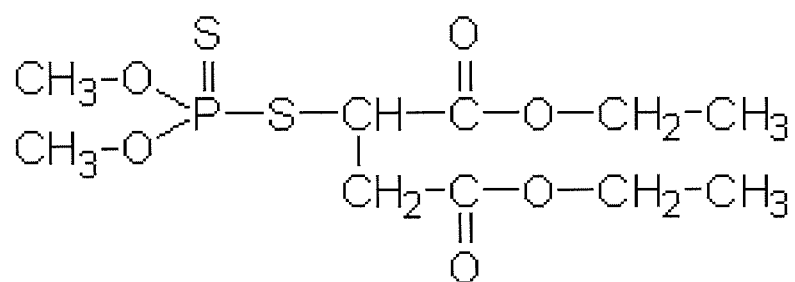


Figure 1.5 Malathion

1.4.2.3. Phenyl derivatives

The phenyl OPs contain a phenyl ring with one of the ring hydrogens displaced by attachment to the phosphorus moiety and other hydrogens frequently displaced by Cl, NO₂, CH₃, CN, or S. The phenyl OPs are generally more stable than the aliphatics, thus their residues are longer lasting. The first phenyl OP brought into agriculture was parathion (ethyl parathion), figure (1.6) in 1947. Examples of other phenyl OPs are methyl parathion, profenofos (Curacron) (Kumar *et al*, 2001).

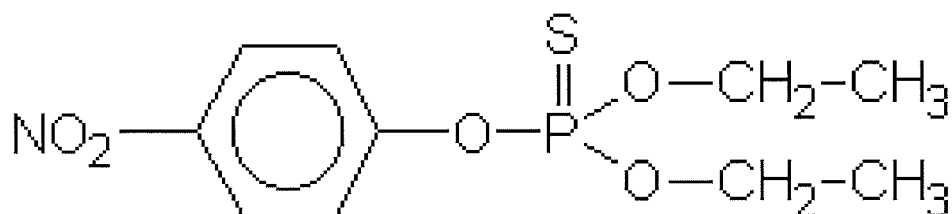


Figure 1.6 Ethyl parathion

1.4.2.4. Heterocyclic derivatives

Heterocyclic OPs contain different or unlike atoms, e.g. oxygen, nitrogen or sulfur in their ring structure. The first of this group was diazinon (Fig. 1.7) introduced in 1952. Other examples in this group are azinphos-methyl (Guthion), azinphos-ethyl (Acifon), Gusathion), chlorpyrifos (Dursban), Lorsban, Lock-On, methidathion (Supracide), phosmet (Imidan), isazophos (Chapman *et al.*, 1982).

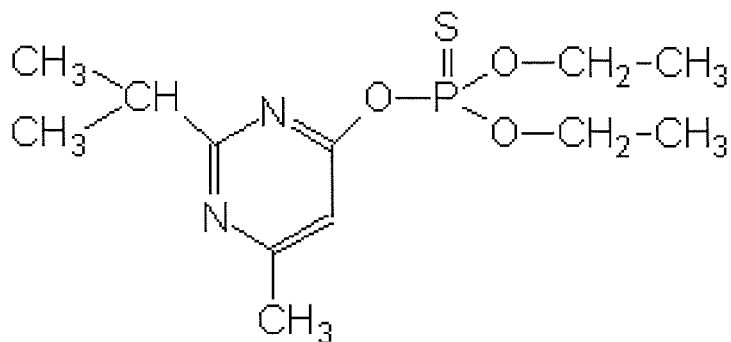


Figure 1.7 Diazinon

1.4.3. Organosulfurs

This group of insecticides are used only as acaricides (miticides). They contain two phenyl rings, resembling DDT, with sulfur in place of carbon as the central atom. These include tetradifon (Tedion(r)), propargite (Omite, Comite), and ovex (Ovotran) (Villarroel *et al.*, 2000).

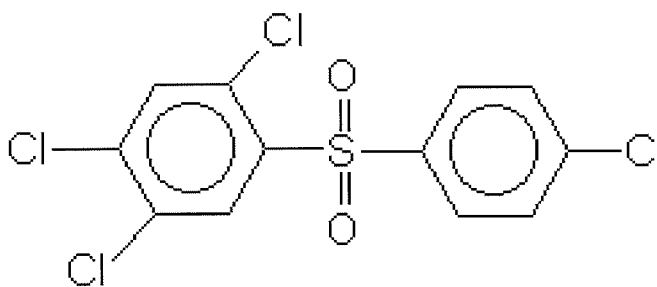


Figure 1.8 Tetradifon

1.4.4. Carbamates

Derivatives of carbamic acid or carbamates also inhibit acetylcholinesterase (Vyas *et al*, 1998, Beauvais *et al*, 2001). They are synthetic derivatives of physostigmine or eserine which is the principal alkaloid of calabar beans. In spite of the similarity of intoxication by carbamates and OP compounds, carbamates are not broad-spectrum insecticides (e.g., bees are extremely sensitive to the carbamates, whereas the housefly and German cockroach are relatively resistant). Also in this case atropine is an effective treatment, but 2-PAM is not recommended since there is little or no evidence that oxime therapy is effective in carbamate poisoning (Salem *et al*, 1988).

The first successful carbamate insecticide, carbaryl (Sevin(r)) (Fig.1.9), was introduced in 1956. More of it has been used worldwide than all the remaining carbamates combined. Two distinct qualities have made it the most popular carbamate: its very low mammalian oral and dermal toxicity and an exceptionally broad spectrum of insect control. Other carbamates are methomyl (Lannate), carbofuran (Furadan), aldicarb (Temik), oxamyl (Vydate), thiodicarb (Larvin), methiocarb (Mesurol), propoxur (Baygon), bendiocarb (Ficam), carbosulfan (Advantage), aldoxycarb (Standak), promecarb (Carbamult), and fenoxycarb (Logic, Torus) (Worthing *et al*, 1987).

Mode of action

As with OP pesticides, carbamates inhibit AChE, and they behave in an almost identical manner in biological systems, but with two main differences. Some carbamates are potent inhibitors of aliesterase (an enzyme, which hydrolyzing low molecular weight aliphatic esters whose exact functions are not known), and their selectivity is sometimes more pronounced against the AChE of different species. AChE inhibition by carbmates is reversible (Lui, 2000). When AChE is inhibited by a carbamate, it is said to be *carbamylated*, as when an OP results in the enzyme being *phosphorylated*. In insects, the effects of OPs and carbamates are primarily those of poisoning of the central nervous system, since the insect neuromuscular junction is not cholinergic, as in mammals. The only cholinergic synapses known in insects are in the central nervous system. (The chemical neuromuscular junction transmitter in insects is thought to be glutamic acid, but that has not been proved.)

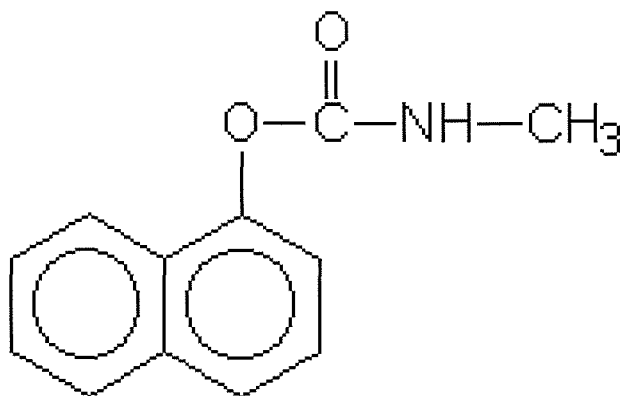


Fig. 1.9 Carbaryl

1.4.5. Formamidines

Formamidines are a small group of insecticides. Three examples are chlordimeform, Fundal(r), and amitraz (Fig.1.10) (Hiripi *et al*, 1999). Their current value lies in the control of OP- and carbamate-resistant pests.

Mode of action

The mode of action of these pesticides are distinctly different from other insecticides. Their probable action is the inhibition of the enzyme monoamine oxidase (Alemany *et al*, 1995), which is responsible for metabolic inactivation of the neurotransmitters norepinephrine and serotonin. This results in the accumulation of these compounds, which are known as *biogenic amines*. Affected insects become quiescent and eventually die.

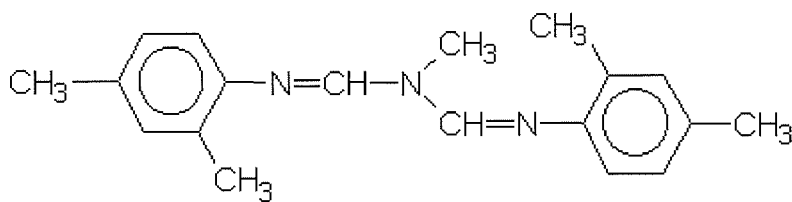


Figure 1.10 Amitraz

1.4.6. Dinitrophenols

The basic dinitrophenol molecule (Fig.1.11) has a broad range of toxicities (Popovic *et al*, 1989). Of the insecticides, binapacryl (Morocide(r)) and dinocap (Karathane(r)) were the most recently used (Worthing & Walker., 1987). Dinocap is an effective miticide and was very heavily used as a fungicide for the control of powdery mildew fungi. Because of the inherent toxicity of the dinitrophenols, they have all been withdrawn.

Mode of action

Dinitrophenols act by uncoupling (or inhibiting) oxidative phosphorylation, which basically prevents the formation of the high-energy phosphate molecule, adenosine triphosphate (ATP)

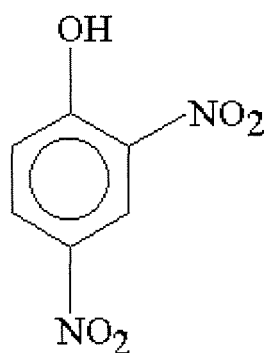


Figure 1.11 2,4-Dinitrophenol

1.4.7. Organotins

The organotins are a group of herbicides that double as acaricides (Dacasto *et al.*, 1994). Of particular interest is cyhexatin (Plictran(r), one of the most selective acaricides known, introduced in 1967. Fenbutatin-oxide (Vendex) has been used extensively against mites (Hoy *et al.*, 1998).

Mode of action

These tin compounds inhibit oxidative phosphorylation at the site of dinitrophenol uncoupling, preventing the formation of the high-energy phosphate molecule adenosine triphosphate (ATP) (Maier *et al.*, 1990). These trialkyl tins also inhibit photophosphorylation in chloroplasts, (the chlorophyll-bearing subcellular units) and could therefore serve as algicides.

1.4.8. Pyrethroids

The first synthetic forms of pyrethroids were developed during the early 1960's.

They were originally referred to as *synthetic pyrethroids* (Shono, 1984). These are very stable in sunlight and are generally effective against most insect pests when used at the very low rates .

Due to their evolution, the pyrethroids can be divided into four generations. Allethrin is the only sample of first generation. Its synthesis was very complex, involving 22 chemical reactions to reach the final product.

The second generation includes tetramethrin (Neo-Pynamin), and resmethrin (Synthrin). The third generation includes compounds like permethrin (Ambush) (Fig. 1.12), (Haustain, 1991). These became the first agricultural pyrethroids because of their exceptional insecticidal activity and their photostability. They were virtually unaffected by ultraviolet in sunlight, lasting 4-7 days as efficacious residues on crop foliage.

The current, fourth generation is effective in the range of 0.01 to 0.05 lb ai/A (active ingredient in acre). It includes insecticides such as bifenthrin (Capture), (Wrothing and Walker ., 1987), lambda-cyhalothrin (Demand), (Soderlund *et al*, 2002). All of these are photostable, that is, they do not undergo photolysis (splitting) in sunlight. And because they have minimal volatility they provide extended residual effectiveness, up to 10 days under optimum conditions.

Recent additions to the fourth generation pyrethroids are acrinathrin, which is a contact and systemic insecticide and is used on a variety of fruit and vegetables (Malato *et al*, 2000).

Mode of action

The mode of action of pyrethroids resembles that of DDT, and they are considered axonic poisons (Vais *et al*, 2001). The primary target of the pyrethroids is the sodium channel and they work by keeping open the sodium channels in neuronal membranes but, in high molecular concentrations, they also may affect other channels. Also the effect of pyrethroids on the immune system may be attributed to their affect on calcineurin which is a particularly important protein phosphatase in this system (Fakata *et al*, 1998). There are two types of pyrethroids. Type I have a negative

temperature coefficient, resembling that of DDT, but type II, in contrast, have a positive temperature coefficient, showing increased kill with increase in ambient temperature. Pyrethroids affect both the peripheral and central nervous system of the insect. They initially stimulate nerve cells to produce repetitive discharges and eventually cause paralysis. Such effects are caused by their action on the sodium channel, a voltage-controlled pore through which sodium ions are permitted to enter the axon to cause excitation. The stimulating effect of pyrethroids is much more pronounced than that of DDT.

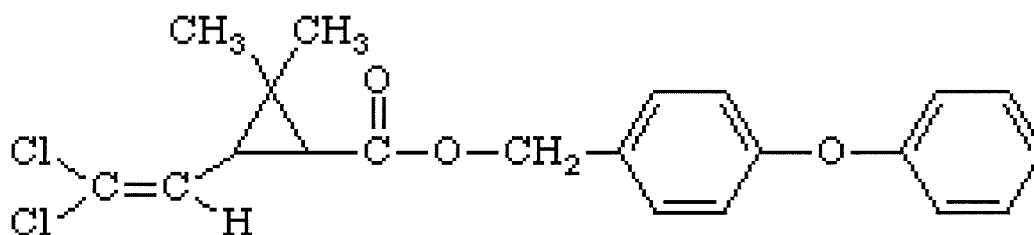


Figure 1.12 Permethrin

1.4.9. Nicotinoids

They are a new class of insecticides with a new mode of action. They have been previously referred to as nitro-quanidines, neonicotinyls, neonicotinoids, chloronicotines, and more recently as the chloronicotinyls. Like pyrethrum and pyrethroids the nicotinoids are modeled after the natural nicotine (Tomizava *et al.*, 2001). Imidacloprid was introduced in Europe and Japan in 1990 and first registered in the U.S. in 1992. It is currently marketed as several proprietary products worldwide, e.g., Admire, Confidor, Gaucho, and Provado (Matsuda *et al.*, 2001). Very possibly it is used in the greatest volume globally of all insecticides.

Imidacloprid is a systemic insecticide, having good root-systemic characteristics and notable contact and stomach action. It is used as a soil, seed or foliar treatment in cotton, rice cereals, peanuts, potatoes, vegetables, pome fruits, pecans and turf, for the control of sucking insects, soil insects, whiteflies, termites, turf insects and the

Colorado potato beetle, with long residual control. Imidacloprid has no effect on mites or nematodes.

Mode of action

The nicotinoids act on the central nervous system of insects, causing irreversible blockage of postsynaptic nicotinic acetylcholine receptors.

1.4.10. Spinosyns

Probably spinosyns are the newest class of insecticides, represented by spinosad (Creemer *et al*, 2000). They are a novel family of insecticidal macrocyclic lactones which, are active on a wide variety of insect pests, especially lepidopterans and dipterans. Also they exhibit a very favorable environmental and toxicological profile, and possess a mode of action that appears unique. Spinosad (Fig.1.13) is a fermentation metabolite of the actinomycete *Saccharopolyspora spinosa*, a soil-inhabiting microorganism. It is a mixture of spinosyns A and D (thus its name, spinosAD). Spinosad has both contact and stomach activity against lepidopteran larvae, leaf miners, thrips, and termites, with long residual activity. Crops registered include cotton, vegetables, tree fruits, ornamentals and others.

Mode of action

This pesticide interferes with binding of acetylcholine in nicotinic acetylcholine receptors at the postsynaptic cell. Also some studies suggest that both nicotinic and gamma-aminobutyric acid receptor functions are altered (Sparks *et al*, 2001)

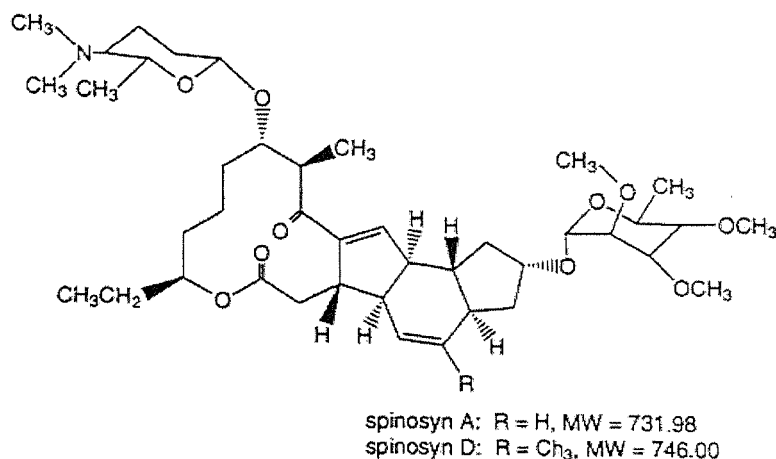


Figure 1.13

1.4.11. Phenylpyrazoles

Fipronyl ((Fig. 1.14), an N-phenylpyrazole with a trifluoromethylsulfinyl substituent, represents a new chemical class of insecticide (Hainzl *et al*, 1998).

It has been shown to have good insecticidal activity against a number of veterinary and agricultural pest species and to be an efficient larvicide against mosquitos and chironomid midges (Kolaczinski *et al.*, 2001). Other sensitive species are the house fly and the fruit fly. Fipronil (Regent, Icon, Frontline) is the only insecticide in this new class (Schlenk *et al*, 2001).

Fipronil has a more favorable selective toxicity for insects relative to mammals than most of the first generation of insecticidal chloride channel blockers, i.e., the chlorinated cyclodienes (Hainzl *et al*, 1998).

Mode of action

Fipronil acts at the γ -aminobutyric acid receptor as a noncompetitive blocker of the GABA-gated chloride channel (Ikeda *et al.*, 2001). Recently some studies have shown cross resistance between fipronil and cyclodiens (Kolaczinski *et al*, 2001).

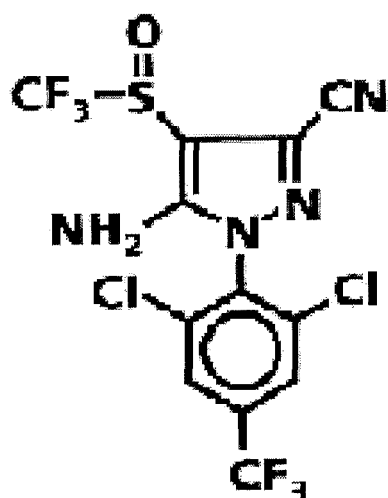


Figure 1.14 Fipronil

1.4.12. Pyrroles

Chlorfenapyr (Alert, Pirate), (Fig.1.15) is the only pesticide of this group (Guglielmone *et al*, 2000). It works as both a contact and stomach insecticide-miticide. Chlorfenapyr is used on cotton and experimentally on corn, soybeans, vegetables, tree and vine crops, and ornamentals to control whitefly, thrips, caterpillars, mites, leafminers, aphids, and Colorado potato beetle. It has been reported as an effective pesticide on lice of cattle (Kaufman *et al*, 2001).

Mode of action

Chlorfenapyr is an "uncoupler" or inhibitor of oxidative phosphorylation, preventing the formation of the crucial energy molecule adenosine triphosphate (ATP)

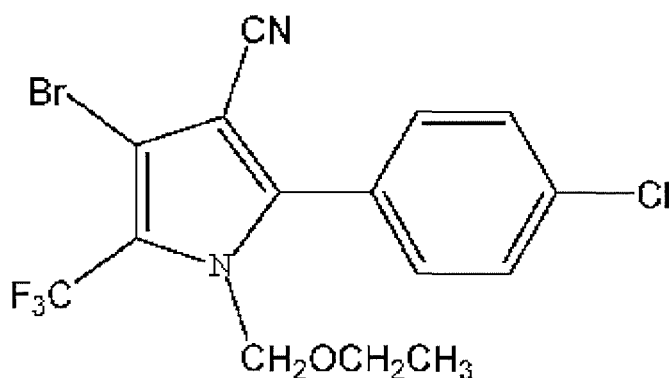


Figure 1.15 Chlorpenapyr

1.4.13. Pyrazoles

In recent years, some compounds of this group have been developed as acaricides and released commercially in close succession. The pyrazolecarboxamide, tebufenpyrad was discovered in 1987 and by 1993 was being used in France, Belgium, Switzerland and Japan. Fenpyroximate, pyridaben and fenazaquin are, respectively, a pyrazole, pyridazinone and quinazoline, and were reported during the same period. One other, pyrimidifen, is a very recent addition, and is registered only in Japan. Owing to their efficacy against many mite species, all four major compounds are now in widespread use globally, but as yet have limited registration in the UK.

Tebufenpyrad (Fig.1.16) is currently the most extensively used acaricide in hops (*Humulus humuli* L) in the UK, where it has been approved since 1993. It accounted for 76% of the hop area treated with acaricides in 1996. Since its introduction, the average annual number of acaricide applications made per treated area has declined from four in 1992 to two in 1996 (Devin *et al*, 2001).

Mode of action

Pyrazoles act on complex I of electron transport chain in mitochondria at the NADH-CoQ reductase site, leading to the disruption of adenosine triphosphate (ATP) formation.

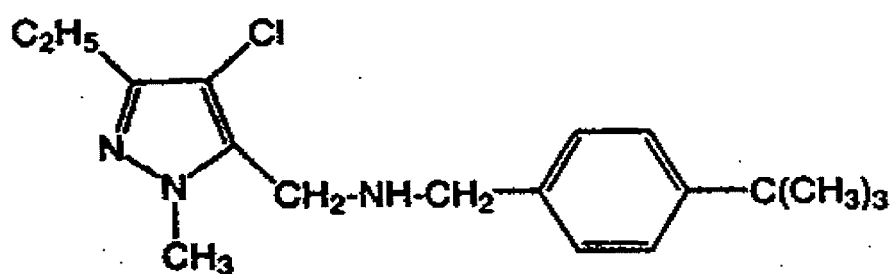


Figure 1.16 Tebufenpyrad

1.4.14. Quinazolines

There is only one pesticide, fenazaquin (Matador) in this group. Fenazaquin (Fig.1.17) is a contact and stomach miticide (Latli *et al*, 1996). It has ovicidal activity, gives rapid knockdown, and controls all stages of mites. It is used on cotton, stone and pome fruits, citrus, grapes and ornamentals.

Mode of action

Like pyridazinone pesticides fenazaquin affect electron transport at mitochondria.

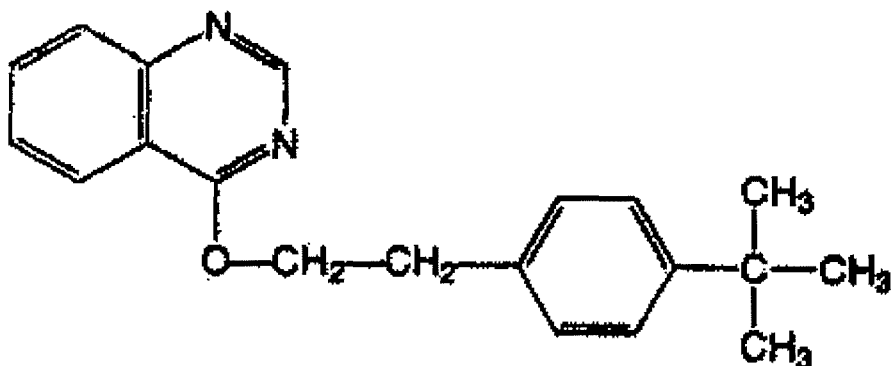


Figure 1.17 Fenazaquin

1.4.15. Synergists or activators

In toxicology, synergism is defined as the case where the toxicity of two compounds applied together is greater than would be expected from the sum of their individual effects. The use of synergists originally arose from the observation that sesame oil would potentiate the action of natural pyrethrins (Corbett *et al*, 1984)

The first synergist was introduced in 1940. Since then many materials have appeared, but only a few are still marketed. Synergists are found in most household, livestock and pet aerosols to enhance the action of the fast knockdown insecticides pyrethrum, allethrin, and resmethrin, against flying insects. Current synergists, such as piperonyl butoxide (Fig. 1.18), contain the methylenedioxyphenyl moiety, a molecule found in sesame oil and later named *sesamin* (Pap *et al*, 2001).

Mode of action

The mode of action of most synergists is that they inhibit cytochrome P-450 dependent polysubstrate monooxygenases (PSMOs). This enzymes produced by endoplasmic reticulum of cells epecially in liver of mammals and in some insect

tissues (e.g., fat bodies) (Becker *et al*, 1984; Casida, 1970). The earlier name for these enzymes was mixed-function oxidases (MFOs). These PSMOs bind the enzymes that degrade selected foreign substances, such as pyrethrum, allethrin, resmethrin or any other synergized compound. Synergists simply bind the oxidative enzymes and prevent them from degrading the toxicant (Bernard *et al*, 1993).

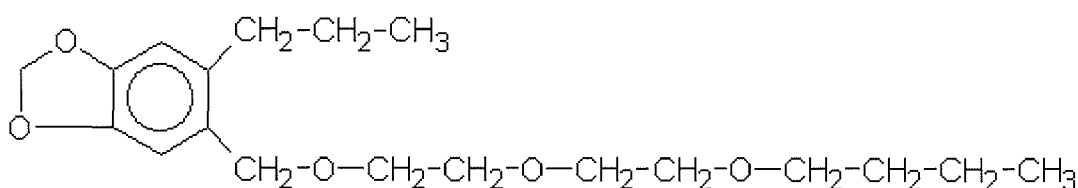


Figure 1.18 pyperonyl butoxide

1.5. Insect growth regulator (IGRs)

The IGRs include various chemical classes with different modes of action, which represent a relatively new category of insect control agents. They act principally on embryonic, larval and nymphal development by interfering with metamorphosis and production. Indeed this property may limit their application in practice, necessitating more specific and sophisticated control strategies with a sufficient knowledge of the biology of the target pests (Graft, 1993).

With the growing concern over insecticide residues in food and water (Konda *et al*, 2001), and widespread resistance to most of the neurotoxins on the market (Pittendrigh *et al*, 2001; Chen *et al*, 2001), there has been a considerable effort to develop new insecticides with unique mode of action and selectivity toward insects. Since insects possess unique biochemical and physiological mechanisms essential to growth and reproduction, disruption of these processes should result in toxicity to insect pests

while minimizing adverse effects to man and the environment (Kostyukovsky *et al*, 2000).

The growth and development of insects is governed by hormones, which, among other things, control moulting and excretion (Takeuchi *et al* 2001). Because insect hormones are vital in controlling the development of insects, they are of interest as a tool for insect pest control.

Insect growth and metamorphosis are controlled mainly by the lipophilic molting hormone, 20-hydroxyecdysone, and juvenile hormone (JH), (Fig.1.20) and proteinaceous hormones like prothoracicotropic hormones (Nakagawa *et al*, 2000).

These hormones coordinately control the development of insects. Ecdysone is a steroid responsible for initiating each molt as the insect progresses, discontinuously, from egg to larva, to pupa, and adult. It exerts its primary effect by serving as a ligand for a nuclear receptor.

Ecdysone secretion promotes the expression of early genes - *Broad-Complex (BR-C)*, which eventually cause production of transcription factors (Dubrovsky *et al*, 2001, Restifo *et al.*, 1998).

Juvenile hormones are sesquiterpenoid molecules in the larval insect and are produced by two small paired glands near of the brain known as the *corpora allata* ("white bodies") (Williamson *et al*, 2001). They are under the control of further hormones known as allatotropins and allatostatins (Engelman *et al*, 2000) which, stimulate and suppress JH synthesis respectively. In most insects juvenile hormone also regulates reproductive maturation in adults (Palli *et al*, 1999)

The presence of JH must be precisely timed in order to exert juvenilizing activity at the appropriate stage in development.

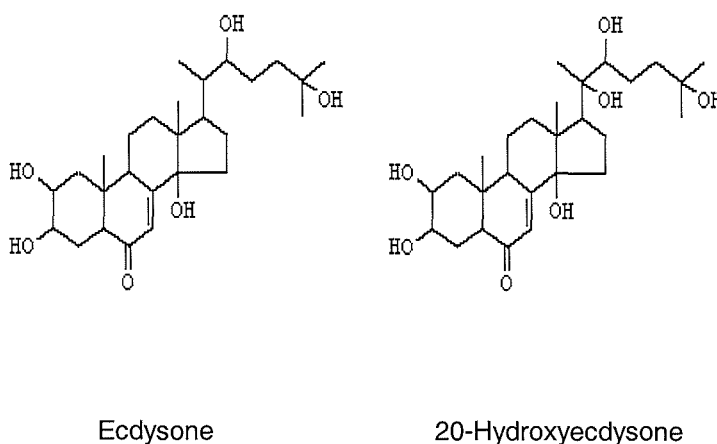
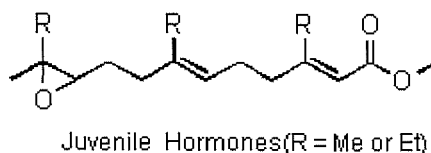


Figure 1.20

1.5.1. Juvenile hormone agonists

Most of the currently registered IGRs mimic the juvenile hormone. Juvenile hormone is important in controlling the moulting cycle of insects and the presence and absence of the hormone in the haemolymph (blood) of the insect at critical times during a larval stage (instar) determines whether the insect will moult and to what stage the insect will moult to (Truman *et al*, 2002). These insecticides are specific for insects, have very low mammalian toxicity, are nonpersistent in the environment, and cause death slowly.

The use of the natural juvenile hormone as a selective insecticide is not feasible because of its environmental instability and difficulties of synthesis. The major breakthrough came when it was observed that synthesized aromatic terpenoid ethers

were several hundred-fold more active than the natural hormone. Juvenile hormone analogues functionally resemble JH, and may or may not be similar in structure. The lipophilic nature of JH allows use of structural analogues as pesticides since they can penetrate the insect cuticle.

1.5.2. Anti-juvenile hormones

The high titre of juvenile hormone in the early larval stages is necessary for retaining the larval morphology. If the action of juvenile hormone can be negated in the early stages then the larva can be forced to moult into a miniature pupa or adult which will be sterile.

Precocenes are the most important compounds of this group which are derived from the bedding plant (*Ageratum houstonianum*) and observed to have anti JH activity. Because these compounds induced precocious development they were named precocenes (Bowers, 1984, De Azambuja *et al*, 1991).

The mechanism of effect of precocene is unique since it is a selective cytotoxin for the active corpus allatum. Precocene treatment results in progressive degeneration of the corpus allatum. The epoxidase enzyme necessary for the synthesis of juvenile hormone is also responsible for the metabolism of precocene in the corpus allatum. This enzyme reacts with precocene and forms a transient but reactive epoxide which alkylates the proteins in the corpus allatum causing cellular death (Foder *et al*, 1989; Schoneved, 1979). In addition to inducing precocious development in young nymphs, precocene has been shown to prevent ovarian development, induce atrophy of corpora allata, and inhibit *in vitro* biosynthesis of juvenile hormone by corpora allata.

Unfortunately, precocene is generally active on some Heteroptera and some grasshoppers only at extremely high doses. The lack of activity against most holometabola, and the high dosage required for its effect on others, have limited the marketability of these compounds.

1.5.3. Ecdysteroid agonists

Wing *et al*, (1988) reported that some dibenzoyl hydrazines like RH 5849 can mimic the action of ecdysteroid. They showed that *Musca domestica* sexta was sensitive to

this compound. Other researchers showed effectiveness of it to pests such as *Plodia interpunctella*. They found that in concentration of 5 ppm larval growth was inhibited and at 50 ppm the mortality rose to 100%. Recently another member of this class of insecticides, RH5992, has shown great potential for control of agricultural pests (Oberlander *et al*, 1997)

1.6. Insecticides that inhibit cuticle production

These chemicals are known as “Chitin Synthesis Inhibitors” or CSIs. They are often grouped with the IGRs. The most notable chemical being used as a CSI is the benzoylphenyl ureas. This class of insecticides includes lufenuron (Program) (Binnington, *et al* 1985) which is a systemic insecticide used for flea control (Bean *et al*, 1999). Diflubenzuron (Dimilin), (Fig.1.21) used against fly larvae in manure, and hexaflumuron (Sentricon) used in a termite bait (Rojas *et al*, 2001). Chitin is a major component of the insect exoskeleton. Insects poisoned with CSIs are unable to synthesize new cuticle, thereby preventing them from molting successfully to the next stage.

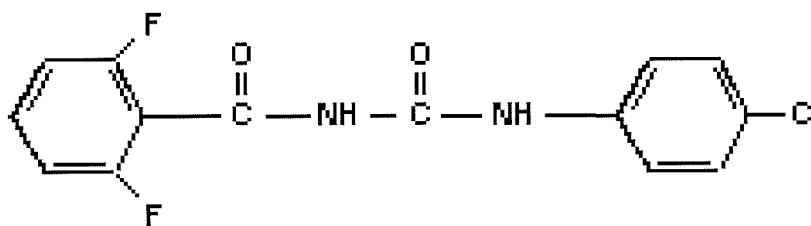


Figure 1.21 Diflubenzuron

1.7. Insecticides affecting water balance

Insecticides with this mode of action include boric acid (Hinkle *et al*, 1995), diatomaceous earth (Smith, 1995), and sorptive dusts (Ebeling, 1975). Insects have a thin covering of wax on their body that helps to prevent water loss from the cuticular

surface. Silica aerogels (sorptive dusts) and diatomaceous earth are very effective at absorbing oils. Therefore, when an insect contacts one of these chemicals it absorbs the protective waxy covering on the insect resulting in rapid water loss from the cuticle and eventually death from dessication. Unfortunately, insects that live in environments with high relative humidities, or that have ready access to a water source, show an increased tolerance to silica aerogels and diatomaceous earth. This is because water loss can be minimized by either of these conditions and the insect may survive despite the absence of a wax layer.

Borate-containing insecticides also disrupt water balance in insects. The exact mode of action (more specifically the target site) of borate containing insecticides is not currently known.

1.8. Fumigants

The fumigants are small, volatile, organic molecules that become gases at temperatures above 40°C (Berk, 1966). They are usually heavier than air and commonly contain one or more of the halogens (Cl, Br, or F). Most are highly penetrating, reaching through large masses of material. They are used to kill insects, insect eggs, nematodes, and certain microorganisms in buildings, warehouses, grain elevators, soils, and greenhouses and in packaged products such as dried fruits, beans, grain, and breakfast cereals.

Methyl bromide is the most heavily used of the fumigants (Yagi *et al*, 1993). The dominant use is for preplanting soil treatments, which accounted for 70% of that global total. Quarantine uses account for 5-8%, while 8% is used to treat perishable products, such as flowers and fruits, and 12% for nonperishable products, like nuts and timber. Phosphine gas (PH_3) has also replaced methyl bromide in a few applications, primarily for insect pests of grain and food commodities (Field *et al* ., 2002). Treatment requires the use of aluminum or magnesium phosphide pellets, which react with atmospheric moisture to produce the gas. Phosphine, however, is very damaging to fresh commodities and is highly adsorbed into oil, thus does not perform as a soil fumigant. It seems that the toxicity of phosphine is due to its effect on cytochrome oxidase (Corbett *et al*, 1984).

1.9. Insect repellents

Chemical repellents can be either the vapour or contact type, and both must induce the insect pest to move away from them and they must be acceptable to the host, particularly if the host is a Man (Cremlyn, 1978). Repellents are chemical products designed to ward off insects and are effective against a wide range of arthropods including mosquitoes and flies. Historically, they have included smoke, plants hung in dwellings or rubbed on the skin as the fresh plant or its brews, oils, pitches, tars, and various earths applied to the body. Before a more edified approach to insect olfaction and behavior was developed, it was assumed that if a substance was repugnant to humans it would likewise be repellent to annoying insects.

The advent of World War II caused the United State of America to embark on a major programme of screening chemicals for repellent properties. The discovery of new repellents was vital for the success of military operation against the Japanese in the Far East. Since DEET (N,N-diethyl-m-toluamide (Fig.1.22) was not effective against all species, during war other repellent like indalone and 2-ethylhexane-1,3-diol which were widely employed together with dimethyl phthalate to give a broader spectrum of repellent activity (Cremlyn, 1978).

Of these, only Deet has survived which have been in use for more than 50 years and most of the others have lost their registrations and are no longer available.

(Holzer, 2001). It is used worldwide for biting flies and mosquitos but great care must be taken and to avoid exposure by children and pregnant women (Carnavel, 1998).

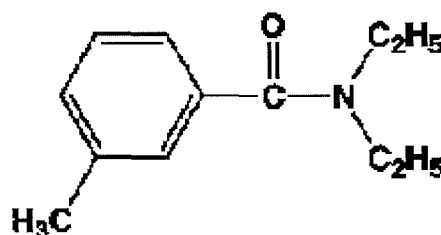


Figure 1.22 N,N-diethyl-m-toluamide (DEET)

1.10. Inorganics

This group of pesticides are those that most of them do not contain carbon. Usually they are white crystals in their natural state, resembling the salts. Usually they are water soluble, do not evaporate and are stable chemicals.

Probably sulfur is the oldest known, effective insecticide. Sulfur and sulfur candles were burned by our greatgrandparents for every conceivable purpose, from bedbug fumigation to the cleansing of a house just removed from quarantine of smallpox. Today, sulfur is a highly useful material in integrated pest management programs where target pests specificity is important. Sulfur dusts are especially toxic to mites of every variety, such as chiggers and spider mites, and to thrips and newly-hatched scale insects.

Several other inorganic compounds have been used as insecticides. The inorganic fluorides were sodium fluoride, barium fluorosilicate, sodium silicofluoride, and cryolite (Kryocide(r)). Cryolite has returned in recent years as a relatively safe fruit and vegetable insecticide, used in integrated pest management programs. This compound can be used as an alternative to synthetic pesticides (Field *et al*, 2002; Vijayan *et al*, 1995). Also sulphur in combination with lime sulphur have been used to control sheep ectoparasite (O'Neill *et al*, 1966). The fluoride ion inhibits many enzymes that contain iron, calcium, and magnesium. Several of these enzymes are involved in energy production in cells, as in the case of phosphatases and phosphorylases.

Boric acid, used against cockroaches and other crawling household pests in the 1930's and, 40's, has also returned (Cochran, 1995). As a salt, it is non-volatile and will

it has the longest residual activity of any insecticide used for crawling household insects, and is quite useful in the control of all cockroach species when placed in wall voids and other protected, difficult-to-reach sites. It acts as a stomach poison and insect cuticle wax absorber.

Sodium borate (disodium octaborate tetrahydrate) (Tim-Bor, Bora-Care) has been shown at concentration of 0.5% to prevent oviposition in *Anastrepha suspensa* for 20 d after treatment and a feeding concentration of 0.2% and 0.5% for 168h caused death in almost all treated cases within 7-day of treatment (Yang *et al*, 2000).

Silica gel is an inert material that is very effective in absorbing moisture. It absorbs the waxy coating on the insect's body and causes death by dehydration.

Silicate dust is very useful for household insect control and protection of food packages (Watters, 1966; Rust *et al*, 1991). The silica aerogels kill insects by absorbing waxes from the insect cuticle, permitting the continuous loss of water from the insect body, causing the insects to become desiccated and die from dehydration. These include Dri-Die, Drianone and Silikil Microcel. Drianone is fortified with pyrethrum and synergists to enhance its effectiveness.

1.11. Problem with synthetic neurotoxic pesticides

It gradually became apparent that these new compounds had severe drawbacks. They were affecting wild life and people in ways which had not been anticipated.

Ideally, a pesticide should be applied to, and only affect the target insect or other unwanted organism and, after killing or damaging it, the pesticide should then immediately break down into benign constituents that have no harmful effect on any other part of environment. In their nature or in the manner in which they are applied, all pesticides should thus be selective and non-persistent. Not surprisingly, this ideal is far from being achieved and, hence, pollution occurs.

1.11.1. Resistance to pesticides

One of the consequences of excessive use of pesticides is the appearance of resistance in pests, which is an increasingly urgent world-wide problem. With each pesticide application, the majority of insects die off, but a few survive, because of unusual

application, the majority of insects die off, but a few survive, because of unusual genetic or behavioural characteristics. For example, a few insects may have the capacity to detoxify the effects of chemicals or have an unusual integument that prevented the penetration of toxic molecules.

The survivors breed, and more and more of their progeny show similar resistant characteristics. Since some pest populations go through a number of generations in a short period of time, the evolution of this kind of pest occurs at a relatively rapid rate. This process begins to accelerate; pests that have evolved resistance to a particular chemical over a number of years will become resistant to that chemical's replacement in a shorter period of time. Some pesticides lead to the development of resistance more quickly than others; the organochlorine insecticides being notoriously bad in this respect (Hemingway *et al*, 1998). Cross-resistance has developed to the point that some pests become resistant to all the chemicals in a given family of pesticides, again at an accelerating rate. In some instances pests even have developed resistance across different families of chemicals. Methyl parathion, for example, has been shown to induce resistance to synthetic pyrethroids among pests in Central American cotton (Murray, 1994). Resistance to one or more pesticides has been documented in more than 440 (Wei *et al*, 2001) species of insects and mites. In vectors of human disease, particularly malaria-transmitting mosquitoes, resistance is a serious threat to public health in many nations. Agricultural productivity is jeopardized because of widespread resistance in crop and livestock pests. Serious resistance problems are also evident in pests of the urban environment, most notably cockroaches.

Resistance management can help to reduce the harmful effects of pesticides by decreasing rates of pesticides use and prolonging the efficacy of environmentally safe pesticides.

1.11.2. The problem of selectivity

Pesticides commonly act by interfering with basic biological processes that are common to a wide range of organisms. The major target organ in insects is the nervous system and the majority of pesticides are neurotoxic compounds. One of the important problems with these compounds is that they act on a very limited number of primary targets: acetylcholinesterases for organophosphates and carbamates, the axonal sodium channel for DDT and pyrethroids, octopamine receptors for

formamidines, and GABA receptors for cyclodienes and avermectins. Such heavy reliance on a few targets is worrying.

A direct and often dramatic consequence of this narrowly focused approach is the rapid development and spread of resistance and cross-resistance, which promotes the need for new substance and which can lead to serious control problem (Graf, 1993).

1.11.3. Pesticides and the environment

After application of a pesticide most of it either is taken in by animals or plants or is degraded by physical and biological factors (e.g. UV light and microorganisms). But a proportion is widely dispersed: some is vaporised to be eventually deposited in rainfall, some remains in the soil, while some reacts with surface water and groundwater, via run-off or leaching.

The presence of pesticides in rainfall has been detected in the USA and in Japan. Since most pesticides are usually applied in Spring of temperate countries, that is when they enter the atmosphere. The retention, though, is short and is soon washed out in rain.

Heavy rain results in rapid removal from the atmosphere – approximately 25 mm of rain in two days in May was enough to halve the concentration of herbicide in the atmosphere. Recent measurements suggest there is little long distance movement, perhaps because pesticides used today in the industrialised countries are not highly persistent (Conway, 1991).

1.11.4. Pesticides in soils

Pesticide that is not lost by volatilization or run-off enters the soil, and there it is degraded or it is lost down the soil profile to groundwater. The time required for degradation varies greatly according to the type of pesticide. Some products break down readily in the soil; others, particularly the organochlorines, can resist degradation for decades. The intermediate products of the degradation process may also be as toxic or more toxic than the original pesticide. One such is DDE the principal metabolite of DDT. It is so stable that, in a controlled 11 year study, the investigators were unable to calculate a half-life; it remained at about 0.4 ppm in the

soil, and 7 ppm in earthworms, over the whole period. In other studies, half-lives have been variously calculated at between 12 years in cultivated soils and 57 years in some uncultivated soils. One authority considers that even though the use of DDT has stopped, soil organisms will remain a source of residue to predators for decades to come (Conway, 1991; Saleh, 1991; Tapp *et al*, 1997).

1.11.5. The effect of pesticides on non target animals

Pesticides, by design, are biocides; their value lies in their ability to kill noxious or unwanted organisms. As mentioned before, they are rarely selective. Most act by interfering with fundamental biochemical and physiological processes that are common to a wide range of organisms; not only pests, but ourselves as well. Some of them have oncogenic properties. For example, the increased incidence of cancer in farmers and agricultural workers has shown a relationship with their occupational background and exposure to pesticides (Safi., 2002).

In developing countries more than 20 poisonings are recorded per 100,000 population. Accumulation of long-lasting pesticides in mammals and other animals is another problem which concerns human health. Soon after their widespread use, persistent synthetic insecticides encountered trouble on an entirely different front. These insecticides accumulate on and in human food and in the environment, where they enter the bodies of small plants and animals such as planktons in ponds and lakes. They concentrate the insecticides in their tissue and are eaten by larger animals such as small fish. At each step the concentration of the insecticide within the organism increases until it eventually becomes dangerous to the larger animals higher in the food chain. For example, DDT and some of its derivative compounds are found in almost all people throughout the world (Conway *et al*, 1991). Also a study from Southern Ontario Canada, showed that 78% of the milk samples analyzed contained 1-10 ppb endosulfan even though application of the insecticide had been stopped for more than 10 years. It has been hypothesized that early and long exposure to low concentrations of estrogenic-like substances in young women might account for the increasing frequency of infertility and associated reproductive disorders in late life and endosulfan is one of this kind of pesticide (Greenman *et al*, 1997)

In recent years, however, public opinion has begun to develop against excessive use of synthetic pesticides. For example, due to an increase in the incidence of cancer incidence in Gaza Governorates (Palestine), the use of more than twenty pesticides has been banned or heavily restricted (Safi *et al*, 2002). A great deal of research is currently being directed towards finding alternatives to current insecticides. Improving some of the botanical pesticides is one approach.

1.11.6. Genotoxic effect of pesticides

1.11.6.1. Carbamates

A growing body of epidemiological studies suggests that long-term exposure to relatively safe pesticides, such as carbofuran (CF), may be associated with increased risk of gastrointestinal, neurological and cardiac dysfunction as well as retinal degeneration. This carbamic pesticide has been reported to be detected repeatedly in drinking water supplies with a typical concentration range between 1.0 and 5.0 µg/l. Therefore, the contamination of the human environment with CF can easily occur and there is an increasing risk of long-term low dose exposure to this pesticide. Moreover, it has been reported that CF alone or in combination with other carbamate insecticides influences the level of reproductive and metabolic hormones, resulting in impairment of endocrine, immune and behavioural functions (Rawlings *et al*, 1998).

Carbamate pesticides are readily converted to N-nitroso metabolites in the presence of nitrites or nitrogen oxides in the stomach under acidic conditions or in the colon by intestinal bacteria. Also they have been synthesized under acid conditions that prevail in the human stomach (Rickard *et al*, 1984; Wang *et al*, 1998). It is reported that 0.5-2% of the carbamate dose could be isolated as the nitroso derivatives from the guinea pig stomach. The nitro derivatives of carbamate pesticides lose the cholinesterase-inhibiting properties of the parent compounds and are less toxic to mammals. However, they induce sister chromatid exchange, chromosomal aberrations, micronucleus formation and aneuploidy *in vitro* and *in vivo*. Like most of the N-nitrosoamide compounds, N-nitrosocarbamates are potent direct acting mutagens and carcinogens.

Apoptosis is a highly regulated process by which an organism eliminates unwanted cells without eliciting an inflammatory response. Some abnormal physiological and chemical injuries, therefore, induce apoptosis. Apoptosis is also triggered in response to various forms of stress and DNA damage. Apoptosis induced by mutagenic or genotoxic carcinogens is vital because it eliminates cells harbouring mutagenic DNA damage from the body. In fact, human cancers contain cells that have been potentially compromised in their ability to undergo apoptosis. N-nitrosocarbofuran (NOCF), but not CF, cause progressive generation of cells with hyploid DNA content that is characteristic of apoptosis. Also this derivative of carbofuran affects the cell cycle of CHL cells. Concentration of 30 μ M caused an increase in the percentage of cells in G2/M phase and also significant decrease in the percentage of G2/M phase (Ji-Yong *et al*, 2001).

Propoxur is another example of a carbamate insecticide. Gonzales *et al* (1990) showed that both propoxur and NO-propoxur were genotoxic for human lymphocytes *in vitro*. NO-propoxur has been shown to be genotoxic in *Salmonella typhimurium* and produce mitotic gene conversion in *S. crevisiae*.

Using human skin cells, Regan *et al*, (1976), showed that carbaryl or its N-nitroso derivative can bind to DNA of these cells. They sedimented the DNA of cells in alkaline sucrose gradients at several times after treatments and demonstrated that NO-carbaryl was apparently split into aromatic and aliphatic fragments, the latter of which binds irreversibly to human DNA, forming many alkali-labile bonds.

1.11.6.2. Organophosphates

Organophosphate insecticides can affect chromosomes. Using the micronucleus test in bone marrow cells of the rat of Wistar strain (*Rattus norvegicus*), Grover *et al*, (1985) investigated the genotoxic effect of some organophosphorous pesticides. Their experiments showed that methylparathion and phorate to be mutagenic, but they could not find any significant effect of fenitrothion. Also methylparathion and phosphamidon have shown a significant increase in the chromosome aberrations of fish gill tissue against control values (Das and John, 1999). Recently, Sobarzo *et al*, (2000) studied the effect of a single injection of parathion on spermatogenesis in mice. They found that this pesticide decreases the number of sperm and increases the

thermal denaturation of DNA. Their experiment showed that, sperm parameter changes were greater in younger animals, so they concluded that parathion had a higher effect at the beginning of spermatogenesis.

1.11.6.3. Pyrethroides

Deltamethrin is an alpha-cyano pyrethroid with marked insecticidal activity and impressive margin of safety to mammalian organisms. Genetic toxicity/mutagenicity studies on deltamethrin have produced mixed results. It has been reported to be positive for chromosomal damage in *Allium cepa* root meristem but negative in *Salmonella typhimurium* and V79 chinese hamster ovary cell assays and bone marrow cytogenetic assay. On the other hand, the genetic toxicity potential of other synthetic pyrethroids, cypermethrin and fenvalerate, has been shown in human peripheral blood lymphocyte cultures, rodent bone marrow cells and *Drosophila*. The experiments of Agarwal et al, (1994) revealed that the exposure of rat bone marrow to this pesticide caused reduction in the mitotic index. The aberration of chromosomes increased with increasing concentrations of deltamethrin. The frequency of micronucleated erythrocytes in the rat bone marrow was also increased 30 h after injection of 11.2 mg/kg of deltamethrine.

1.12. Bacterial Pesticides

There are a large number (>90 species) of insect-specific bacteria which have been isolated from insects, plants, and the soil, but only a few have been developed for biological control. The *avermectins*, (Fig. 1.23) with insecticidal, acaricidal, and antihelminthic activities are well-known agents of this group which, have been isolated from the fermentation products of *Streptomyces avermitilis*, a member of the actinomycete family (Sutherland *et al*, 1990). *Abamectin* is the common name assigned to the avermectins, a mixture of containing 80% avermectin B1a and 20% B1b, homologues that have about equal biological activity. Abamectin has certain local systemic qualities, permitting it to kill mites on a leaf's underside when only the upper surface is treated. The most promising uses for these materials are the control of

spider mites, leafminers and other difficult-to-control greenhouse pests, and internal parasites of domestic animals.

Another compound of this group is emamectin benzoate (Proclaim(r), Denim(r)) which, is an analogue of abamectin, produced by the same fermentation system as abamectin (Kim-Kang *et al*, 2001). It is both a stomach and contact insecticide used primarily for control of caterpillars at the rate of 0.0075 to 0.015 lb (3.5 to 7.0 grams) a.i. per acre. Shortly after exposure, larvae stop feeding and become irreversibly paralyzed, dying in 3-4 days. Rapid photodegradation of both abamectin and emamectin occurs on the leaf surface.

Mode of action

Avermectins block the neurotransmitter gamma aminobutyric acid (GABA) at the neuromuscular junction in insects and mites (Chalmers *et al*, 1986). Visible activity, such as feeding and egg laying, stops shortly after exposure, though death may not occur for several days.

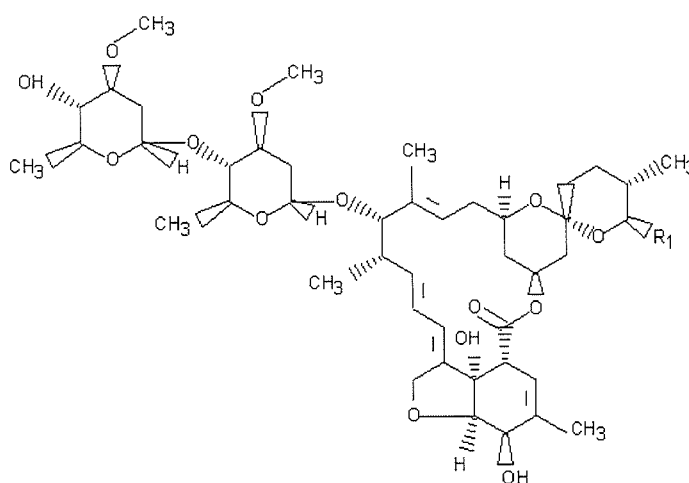


Fig. 1.23 Ivermectin (an example of avermectines)

1.12.1. *Bacillus thuringiensis*

B. thuringiensis (Bt) is an insecticidal bacterium and indeed the key insect-specific bacterium (Copping *et al*, 1998) which, marketed worldwide for control of many important plant pests - mainly caterpillars of the Lepidoptera (butterflies and moths). Bt plays an increasingly important role in mosquito control (Wirth *et al*, 2001). It is a

valuable source of insecticidal proteins for use in conventional sprayable formulations, and it is one of the promising alternative to synthetic insecticides (Ferre *et al*, 2002).

Bt products represent about 1% of the total 'agrochemical' market (fungicides, herbicides and insecticides) across the world. The commercial Bt products are powders containing a mixture of dried spores and toxin crystals. They are applied to leaves or other environments where the insect larvae feed. The toxin genes have also been genetically engineered into several crop plants.

Mode of action

When Bt sporulates it produces a large protein toxin as a crystal. In the gut of an insect larva the toxin protein is cleaved by proteases to produce a smaller active toxin. One part of the toxin protein binds to receptor proteins on the epithelial surface of the gut, then in concert with other toxin molecules, forms a pore in the cell membrane. This destroys the ability of the gut to regulate ion-exchange. The gut epithelium then lyses, feeding stops, and death occurs from 30 minutes to 3 days post infection.

1.13. Botanical pesticides

The animal Kingdom, in size and shape just as diverse as the plant world, includes many herbivores, ranging from plant lice to elephants. Among the herbivores, insects occupy a paramount position, as exemplified by their huge biomass. Total body weight of social insects alone in tropical rainforests is seven times that of vertebrates. Actually, in view of the fact that herbivorous insects generally possess short generation cycles, have high reproductive rates, and are usually winged, they can easily exploit new food sources. It is surprising that, despite the omnipresence of green plants, insects under natural conditions rarely become a threat to the survival of a given plant species. That is, resistant plants are the rule rather than the exception, and very susceptible plants are actually rare. For a long time the relationships between insects and plants were thought to fit a relatively simple pattern: host-plants contain one or a few related secondary plant substances that stimulate feeding and/or oviposition behaviour in insects specialised on them. Insects refrain from feeding on non-host-plants because of the presence of some deterrent substances, which activate

specialised deterrent receptors. As is often the case in nature, reality is far more intricate and subtle than was first thought. It now appears that the plant stimuli not only govern the behavioural responses of herbivorous insects but may also affect physiological processes, such as their morphogenesis, retardation or acceleration of reproduction, or the induction of diapause (Beek, 1993).

It is well-known that some insecticides of plant origin have been in use for a long time, for example the useful property of pyrethrum obtained from the flowers of *Chrysanthemum cinerariifolium* (cinerariaefolium) was known during the time of the Iranian King, Darius the great (521-486 B.C), and Indians have used the extracts of the neem tree for 2000 years (Schmutterer, 1995).

Only a small percentage of plants have been screened for insecticidal activity. In addition, many such studies are not complete and often the bioassay procedures used have been inadequate or inappropriate. Consequently, plants remain a major untapped resource for potential leads to novel insecticides (Beek *et al*, 1993).

1.13.1. Pyrethrins

The pyrethrins are extracted from the flower *Chrysanthemum cinerariaefolium*. The active constituents of the extract are collectively referred to as “pyrethrins”. The pyrethrins consist of the individual compounds cinerin I and II, pyrethrin I and II, and jasmin I and II in varying proportions (Fig. 1.24) (Bruneton, 1995; Brown, 1978) The insecticidal compounds are especially abundant in carpels of young flowers, when at least 3/4 of the disc florets have opened. Crop yield may be as high as 200-1000 kg/ha. The active compounds can be extracted by organic solvents, and since they are not soluble in water, they are applied as aerosol or as dusts which contain 20 percent pyrethrin I and II and 5 percent cinerines. Pyrethrins are quickly inactivated by light and oxygen but few resistant pests have developed as compared to synthetic pesticides.

Although accounts of the discovery of the toxicity to insects vary, it is fairly certain that pyrethrum was in use in Europe as an insecticide more than a century ago and in Persia considerably earlier (Casida, 1973) At present, *Chrysanthemum* species are cultivated throughout the world, (e.g. in Japan, Kenya, Tanzania, Rwanda, Ecuador,

Yugoslavia, California, Mexico, Chile, Brasil, and Russia). The *Chrysanthemum* material produced in developing countries is usually exported to Europe and USA.

Pyrethrins are active as a nervous and contact poison in insects, but also act as a deterrent. Pyrethrins have been found to be effective against many insects (beetles, lepidopteran larvae, aphids, flies, cockroaches, ants, mosquitoes, locusts, thrips, etc.). The insecticidal activity can be enhanced by the use of synergists, like sesamine from sesame oil or piperonylbutoxide (which inhibits mixed function cytochrome oxidases p 450), by antioxidants, such as hydroquinone and tannin, or by activators such as ethyleneglycolether. Pyrethrins are slightly toxic to warm-blooded vertebrates (LD₅₀ in rats, p.o., 1.2g/kg). They can cause severe allergic dermatitis and systemic allergic reactions. Higher doses may cause nausea, vomiting headache, and other CNS disturbances in man: these are common side effects of synthetic insecticides.

Synthetic derivatives, the so called 'pyrethroids', have been synthesized and are much more stable and active (up to factor of 1000) than the natural products (see section 1.4.8). In Germany, it has been discussed whether pyethroids should be banned because of their side effects (according to a note in the German Apothekerzeitung No.14 of March, 1992) (Beek *et al*, 1993)

Mode of action

Pyrethrins (Pyrethrum) are axonic poisons, as are the synthetic pyrethroids and DDT. Axonic poisons are those that in some way affect the electrical impulse transmission along the axons, the elongated extensions of the neuron cell body. Pyrethrum and

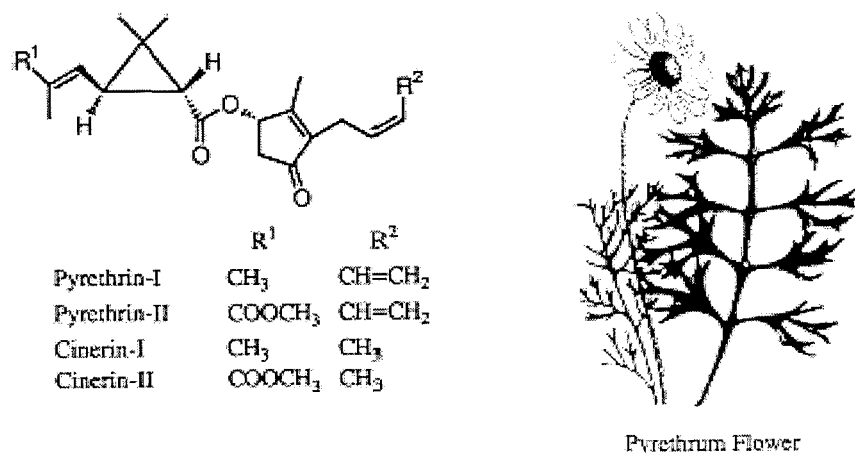


Figure 1.24: Structure of Pyrethrins

some pyrethroids have a greater insecticidal effect when the temperature is lowered (i.e. they have a negative temperature coefficient), as does DDT. They affect both the peripheral and central nervous system of the insect. Pyrethrum initially stimulates nerve cells to produce repetitive discharges, leading eventually to paralysis. Such effects are caused by their action on the sodium channel, a tiny hole through which sodium ions are permitted to enter the axon to cause excitation (Narahashi, 2000). These effects are produced in insect nerve cord, which contains ganglia and synapses, as well as in giant nerve fibre axons.

1.13.2. Nicotine

Nicotine is the main alkaloid of many *Nicotiana* species, such as *N. tabacum*.

In *N. rusticana* leaves it reaches concentrations of 2-8 per cent. Nicotin is slightly lipophilic alkaloid, that can easily penetrate skin and biomembranes by simple diffusion. Thus it can be quickly resorbed by target organisms. Nicotine is a strong agonist of the acetylcholine (ACh) receptor in insects and vertebrates, and acts as a deadly poison even at low concentrations (the LD₅₀ in mice after intravenous application is 0.3mg/kg). It modulates signal transfer in neural synapses and at the neuromuscular plates.

It has been long known, that extracts from *Nicotiana* species can be effectively used to kill insect pests of various classes and first it used as an insecticide in 1763 (Hodgson, 1997) . Nicotine which is produced as a byproduct of the tobacco industry, was used as 40 percent solution of nicotine sulphate in the US ('Black leaf 40'). As a contact poison it is most effective as soap, i.e. as the laurate, oleate, or naphthenate. As a stomach poison a combination with bentonite (a volcanic mineral) has come into use (Merck Index 1989).

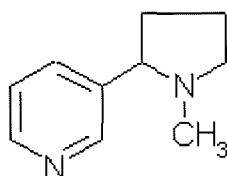
Since nicotine is highly toxic , many cases of intoxication were reported from farm workers, which resulted in nausea, vomiting, evacuation of bowel and bladder, mental confusion, twitching, and convulsions.

Although nicotine was commonly used until the Second World War, it was generally abandoned, because of its toxicity, in the US and Europe , when synthetic insecticides became more available. Derivatives of nicotine, such as nornicotine, anabasine, anatabine, and nicotyrine have also insecticidal properties.

Nicotine is lethal for most insects, except the tobacco hornworm, *Manduca sexta*. *M. sexta* is a specialist and can be fed on daily nicotine which are fatal for man (Heidrich and Winck, unpublished). It is suspected that the insensitivity of this insect is achieved by a mutation of the ACh receptor ; through target site modification nicotine no longer binds to the receptor and thus can not perform its toxic activity. Since nicotine is such a potent insecticide , its use might still be interesting even today provided that safe formulations and applications can be developed. For example, in cases when pests become resistant to synthetic compounds or in third-world countries where nicotine could be produced at relatively low costs (Beek *et al*, 1993).

Mode of action

Nicotine action is one of the first, classic modes of action identified by pharmacologists. Drugs that act similarly to nicotine are said to have a “nicotinic” effect (Suemaru *et al*, 2001). Nicotine mimics acetylcholine (ACh) at the neuromuscular (nerve/muscle) junction in mammals, and results in twitching, convulsions, and death, all in rapid order. In insects the same action is observed, but only in the central nervous system ganglia.



Nicotine

Figure 1.25

1.13.3. Rotenone

The genus *Derris* Lour.(Fabaceae) consists of 80 species of climbing vines found throughout the tropics. Two species, *D. elliptica* (Roxb.) Benth. and *D. malaccensis* (Benth.), have been used as fish poisons throughout the Pacific (Ioset *et al*, 2001; Rickard *et al*, 1986). American *Lonchocarpus* (*L. urucu* Killip and Smith, *L. utilis* A. C Smith) are used for same purposes (Bruneton, 1993). Because of the high rotenone (Fig.2.26) content, both species have been commercially cultivated in Far East as well as the Caribbean and central America as a source of rotenone. The insecticidal properties of *Derris* root were discovered in Malaysia and the East Indies in 1848.

After 1848 until the advent of DDT, rotenone became one of the most widely used insecticides worldwide. The USA imported substantial amounts of *Derris* root from Java, Sumatra, the Philippines, and Malaysia; for example, imports were 3954 t in 1939, 6727 t in 1947, 2356 t in 1948, and 1500 t in 1963. Within Europe (e.g. in Austria and Switzerland), *Derris* extracts were usually combined with pyrethrines.

For its use as an insecticide, root powders of *Derris* are either mixed with neutral soap (1 kg powder + 1.5 kg soap in 100 litres water), paraffin oils or with talcum or clay dust (1 kg *derris* + 5 kg dust). About 30 kg/ha of the dust mixture are needed for an effective control of pests. *Derris* preparations are effective in the control of various aphids, caterpillars, thrips, diptera, beetles, nematodes, and even fungi, and no resistance to this pesticide has been reported.

Rotenone is hardly phytotoxic, but does show some human toxicity (Merk Index 1989): inhalation or ingestion of large dose may cause numbness of oral mucosa, nausea and vomiting, muscle tremors, and tachypnea (Beek *et al*, 1993).

The acute oral LD₅₀ for white rats is 132-1500 mg/kg. It is toxic to pigs and highly toxic to fish (Worthing *et al*, 1987). Lethal effects are caused by respiratory paralysis. Chronic poisoning may produce fatty acid changes in liver and kidney. Direct contact sometimes causes a mild irritation of skin or mucosae (Beek *et al*, 1993).

Mode of action

Rotenone is a respiratory enzyme inhibitor, acting between NAD⁺ (a coenzyme involved in oxidation and reduction in metabolic pathways) and coenzyme Q or ubiquinone oxidoreductase (complex I) (Zhang *et al*, 2001; Lummen, 1998). Since this is a respiratory enzyme responsible for carrying electrons in some electron

transport chains, the result is failure of the respiratory functions. On the other hand recent studies have suggested that there is a relationship between abnormality of complex I in mitochondria and Parkinson's disease (Greenmyre *et al*, 2001)

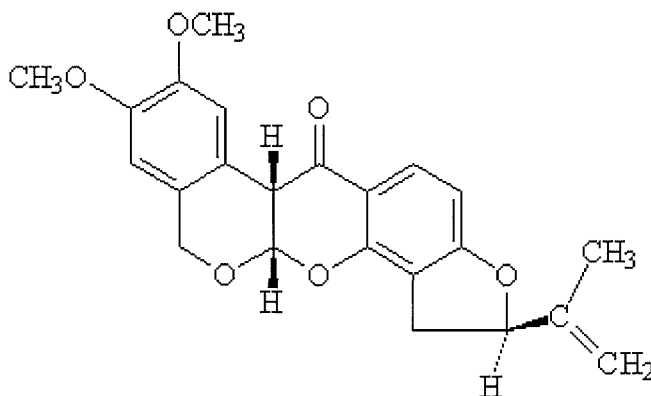


Figure 1.26: Rotenone

1.13.4. Neem

Azadirachta indica, the Neem tree, is native to India and Burma. The word “neem” comes from Sanskrit, the origin of all Indo-European languages, and translates into “the healer and illness reliever.” Around 1500 B.C. this tree was mentioned in this context in religious writings (Norton, 2000).

Indians have long revered the neem tree. They have used different parts of neem for cleaning their teeth, healing skin disorders and keeping away troublesome insects and it plays an important role in Indian healing and cosmetics. Aqueous extracts of powdered neem kernels have been used by these people for control of insect pests for centuries. This practice was given up with the advent of powerful synthetic pesticides, but in the 1960s, when the harmful effects of these pesticides was realised, the quest for more benign pest control agents began (Govindarchari *et al*, 1998). Today the neem tree is widely distributed throughout tropical and subtropical Asia, Africa, Australia and South America (Schmutterer, 1995). Indeed neem products have been obtained from several species of neem trees in the family Meliaceae. Neem tree or *A. indica* A. Juss, is the most important species of this group. Other important species of this group are *Azadirachta excelsa* Jack, *Azadirachta siamensis* Valetton, *Melia azedarach* L., *Melia toosendan* Sieb. and Zucc., and *Melia volkensii* Gurke (Mulla and

Su, 1999). There are many common names for the neem tree, of which some example can be seen in Table (1.2), (Schmutterer, 1995).

1.13.5. Botanical properties of neem

A. indica is a fast growing plant, which usually reaches a height of 15-20 m, and under very favourable conditions up to 40 m (Fig.1.27.a). Despite neem being an evergreen tree, under extreme conditions (such as extended drought) it may shed most of its leaves. The branches spread widely. The fairly dense crown is roundish or oval and may reach a diameter of 15-20 m in old freestanding specimens. The trunk is relatively short, straight and may reach a girth of 1.5-3.5 m. The bark is hard, fissured or scaly, and whitish-grey to reddish-brown. The sap wood is grayish-white and heart wood reddish when first exposed to the air, becoming reddish-brown after exposure. The leaves are unpaired, pinnate with 20-40 cm length. Flowers are white, fragrant and are 5-6 mm long with five petals (Fig.1.27.b). The fruits are olive-like and yellowish-green to yellow when they are mature (Fig.1.27.c). The root system consists of a strong taproot and well developed lateral roots. (Schmutterer *et al*, 1995).

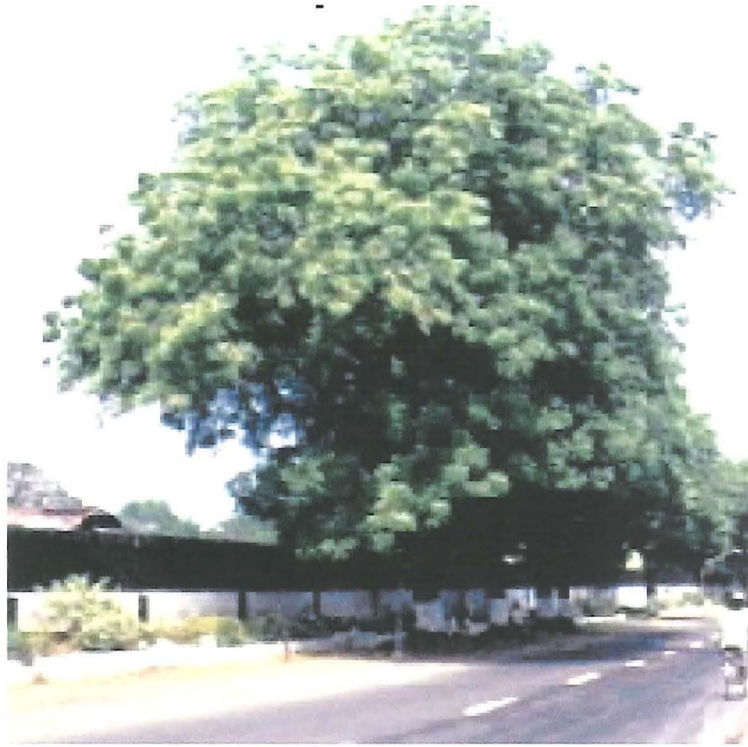


Figure 1.27 (a):Neem tree
The picture was taken from www.prosea.nl/specie_a.html



Figure 1.27 (b):Neem (flowers and immature fruits)
The picture was taken from www.hear.org/pier/azindp.htm



Figure 1.27 (c): Neem tree (Ripe fruits)

The picture was taken from www.keele.ac.uk/depts/ch/groups/ecology/ecology.html

Table 1.2 Selected common names of the neem tree

Asia, Australia, Southpacific	
India	Limba, Limbo, Neem, Nimb, Nimba, Vembu, Vepa, Veppam, etc
Pakistan	Nimmi
Myanmar (Burma)	Tamarkha
Sri Lanka	Kohamba
Thailand	Sadao India, Kwinin, Dao
Indonesia	Imba, Mindi, Mimbo, Intaran
Malaysia (West)	Mambu
Singapore	Nimbagaha
Iran	Azad-darakht-I-hindi, Nib
Yemen	Meraimarah
Australia	Neem
Papua New Guinea	Neem
Fiji	Neem
Africa	
Nigeria	Babo Yaro, Dogon Yaro
Tanzania	Mwarobaini
Cameroon	Ganye, Marrango
Madagascar	Nim
Americas	
U.S.A	Neem
Latin America (Spanish speaking)	Nim
Europe	
Germany	Indischer Zedrach, Großblattriger Zedrach, Indischer Flieder, Niem, Nim, Niembaum
France	Azadira d'Inde, Azadirac, Lilas de Indes, Margousier
Portugal	Margosa
Spain	Nim, Margosa
United Kingdom	Indian Lilac, Neem

The table was taken from Schmutterer, H, (1995)

1.13.6. Insecticidal properties of neem

In the last decades neem has been the subject of many experiments, carried out in at least a dozen countries, and the extracts from different parts of this tree have been studied for their insecticidal activity, repellency and antifeedancy.

Now researchers have found that neem can affect more than 400 species of insects (Iqbal, 1999). Indeed one of most remarkable benefits of neem is the insecticidal activity of its extracts. They attack many pestiferous species while seeming relatively safe for wildlife such as mammals and fishes and, unlike the organochlorines, they are easily biodegradable.

Neem's ability to repel insects was first reported in the scientific literature in 1928 and 1929. Not until 1962, however, was the real significance demonstrated. (National Research Council, 1992).

In Europe, the insecticidal properties of extracts of Meliaceae plants were known as early as 1877, but investigation into the active principles only started in the middle of the twentieth century. Most of the compounds isolated so far from this plant material belong to the class of tetranortriterpenoids and pentanortriterpenoids (Banerjee, 1992).

1.13.7. Medical uses of neem

Neem, known as the "Village Pharmacy", has long been used in both traditional and modern medicine. The bark, the leaves, and the oil from the seeds of neem tree are widely used in folk medicine. The bark has a reputation for being a tonic, the leaves and the oil from the seeds are recommended as antiseptics, and antiparasitic agents (Bruneton, 1995).

In Sanskrit literature, neem is known as *sarva roga nivarini*, "the cure of all ailments." The first indication that it was used medicinally was about 4,500 years ago, in India. Excavations at Harappa and at Mohenjo-Daro, in the north western and western parts of the country, uncovered several therapeutic compounds, some of which included neem leaves (Norten, 2000).

One of properties of neem extract is its effect on arthritis. There is a long history of relieving inflamed joints; it has been used in diabetes (Khosla *et al*, 2000), malaria, rheumatism, and currently there are institutions which search for effects of neem on

AIDS and other viral diseases (Norten, 2000). Also extracts have a contraceptive effect in animals (Garg *et al*, 1994) and have been used as a pain killer (Pardia *et al*, 2002).

1.13.8. Antimicrobial effects of neem extracts

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Investigation on the evaluation of the biological activities of essential oils of some medicinal plant species have revealed that some of them exhibited antimicrobial activities (Cimanga *et al*, 2000).

Virtually all cultures worldwide have relied historically, or continue to rely on medicinal plants for primary health care. Approximately one-third of all traditional medicines are for treatment of wounds or skin disorders, compared to only 1-3% of modern drugs (Mantle *et al*, 2001). For example chewing sticks from *Azadirachta indica* and *Salvadora persica* (Arak), are commonly used as oral hygiene tools in India and some parts of the Middle East, and these have been shown to have good inhibitory effects on *Streptococcus faecalis* (Almas, 1999). Also, one of limonoids from *A. indica*, mahmoodin, has been shown to have significant antibacterial activity against various Gram-positive and Gram-negative organisms (Siddiqui, 1992). There are some differences in antibacterial activity of neem extracts. Species such as neem are widespread in their geographic distribution and consist of many biotypes. It has been documented that the chemical composition can and does vary with biotypes, geographic location, and environmental conditions under which the material is grown and collected. Thus it is difficult to compare the results obtained by numerous researchers over time and geographic distance, evaluating preparations of varying purity and composition. However, it is well established that the neem tree is well endowed with biologically active compounds and that many fungal pathogens can be shown to be sensitive to them at least *in vitro* if not *in vivo*. (Schmutterer, 1995)

There has been some interesting work on antifungal activities of neem extracts and there is evidence that extracts from various parts of the tree have antifungal activity. Purification of antifungal fraction of neem oil suggested that a mixture, rather than the individual compounds are required for maximum activity against fungi.

Coventry *et al*. (2000) showed that despite the antibacterial effect of neem extract, azadirachtin, nimbin and salannin had no activity against *Bacillus mycodies*.

Neem extracts also have been used against viral diseases. Pardia *et al.* (2002) have shown significant inhibitory effect of neem extract but not pure azadirachin on Dengue virus type-2.

1.13.9. Commercial formulations of neem

Many products are obtained from the neem tree, and these are mostly are used as insecticides. Some of these products are; neem seed oil and neem seed cake; aqueous neem seed kernel and leaf extracts; alcoholic seed kernels and leaf extracts; and enriched, formulated seed kernel extracts. The use of large amounts of neem oil for soap-making, dates from after the Second World War. With progress of neem science, there is increasing use of pure and semi-pure extracts. Neem seed oil is pressed from neem seeds in the cold by using an oil presser or prepared by extraction with alcohol or less polar solvents.

Neem seed cake is the residue of neem seeds after extracting the oil. It contains about 2-4% nitrogen and is used as organic fertiliser. (Iqbal, 1999). Table 1.3 Shows some of commercial products of neem.

Table 1.3 Commercially produced neem-based pesticide products

Trade name	Active ingredient or source	Activity claimed
Morgosan-O	Azadirachtin	Insecticide, miticide
Meen	Azadirachtin	Growth reg., repellent, antifeedant
Azatin-EC	Azadirachtin	Insecticide, miticide
Neemazal	Azadirachtin	Insecticide
Godrej Achook	Azadirachtin, nimbocinol, epinimbocinol	Antifeedant, repellent, growth reg.
Field Marshal	Azadirachtin	Antifeedant, repellent
Margocide-CK	Azadirachtin	Antifeedant, growth reg., ovicidal
Moskit	Oil	Mosquito repellent
Neembased EC	Kernel or oil	Pesticide
Neem oil emulsion	Oil	Pesticide
Neemgold	Azadirachtin	Antifeedant
Neemrich	Extracts	Warehouse pests & antifeedant
Nimba	Kernel based	Pesticide
Wellgrow	Kernel powder	Repellent, Antifungal and antiviral, plant nutrition
Align	Azadirachtin	Insecticide
Azatin	Azadirachtin	Insecticide
Turplex	Azadirachtin	Pesticide

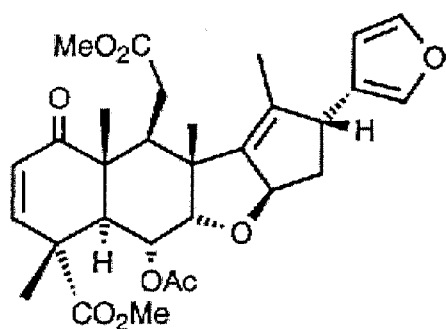
Growth reg: Insect growth regulator (Table was taken from Paranagama, 1994)

1.13.10. Active ingredients of neem

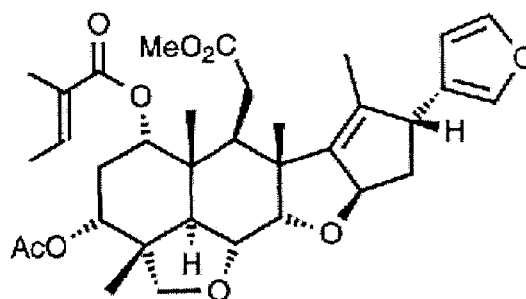
More than 100 compounds have been found in various parts of the neem tree, but the most biologically active ingredients are azadirachtin, salannin, nimbin (Fig.1.28), nimbolide and epoxyazadiradione. These extracts have been shown to possess many useful properties of which, antifeedancy, insecticidal and insect growth disruption are three major properties that are used in control of insect pests (Schmutterer, 1995).

The antifeedant activity of limonoids appears to be due to their effects on chemosensory mouthparts (deterrent activity), and through their postingestive toxic effects on other tissues and organs resulting in an overall loss of "fitness" of the insect and a reduction in food intake. Furthermore, the neem extract appears to disturb the digestive process in insects. For example inhibiting the activity of digestive proteases in larvae of *Spodoptera litura* and *Manduca sexta*. Also ingestion of neem oil

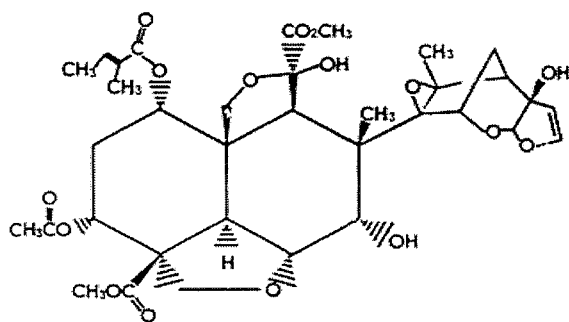
significantly reduced esterase activities in larvae and adult of *Choristoneura rosaceana* (Ortego, 1999).



Nimbin



Salannin



Azadirachtin

Figure1.28 Structure of major terpenoids of neem tree

The growing accumulation of experience demonstrates that neem products work by intervening at several stages of an insect's life (National Research Council, 1992). Dhar, *et al* (1996) have found that by exposure of females of *Anopheles stephensi* and *An. culicifacies* to volatile substances present in neem, the gonotrophic cycle of the female is impaired. Long term exposure to neem odour and some extracts causes vitellogenesis be impaired.

The effects of azadirachtin, salannin, nimbin, and 6-desacetylnimbin on ecdysone 20-monooxygenase (E-20-M) activity were examined in three insect species and it was shown that all four neem tree compounds inhibit, in a dose-dependent fashion, the E-20-M activity in *Drosophila melanogaster*, *Aedes aegypti* and *Manduca sexta* (Mitchell *et al.*, 1997) The cytochrome P-450-dependent hydroxylase is responsible for conversion of ecdysone to 20-hydroxyecdysone in these insects (Ortego, 1999).

A single intrauterine administration of Neem oil fraction caused reversible block in fertility of female wistar rat (Garg *et al*, 1998). This fraction was identified as a mixture of six components which comprising of saturated , mono and di-unsaturated free fatty acids and their methyl esters. However, with the different formulations of neem used and the many varied modes of application, detailed comparisons of efficacy against different pest species are extremely difficult to make (Mordue, 1993).

1.13.11. Azadirachtin as the main biologically active component

Azadirachtin, also termed azadirachtin A (AZA) is a tetranortriterpenoid, which constitutes 15-25% by weight the limonoids in the ripe seeds of neem tree (Morgan *et al*, 2001;Jabbar *et al*, 1998). It is the main biologically active and one of a closely related group of congeners (called AZA A-I) which are present in the seed kernels of the neem tree (Rembold *et al*, 1992; Mordue *et al*, 1998) and appears to cause some 90% of the effect on most pests. (National Research Council, 1992). 3-Tigloyazadirachtol (azadirachtin B) is present at a concentration of 20% that azadirachtin. Other azadirachtins (C-I) occur at much lower concentrations (Mordue, 1993). Azadirachtin A, is a highly oxidized terpenoid with relative molecular mass of 720 (molecular formula $C_{35}H_{44}O_{16}$) and the melting point is 165°C (Iqbal, 1999). It was first isolated by Butterworth and Morgan in 1967 and the most cited paper for the isolation of it is that by Schroeder and Nakanishi (Govindachari *et al*, 1998).

Azadirachtin's structure features a densely packed array of oxygen functionalities of many different types, which make it difficult to determine which portions of the molecule are responsible for its many biological effects. Some studies on structure-activity relationships suggest that the functionalities at the 7 and 11 position of azadirachtin may have an important role to play in its biological effects (Grossman et al, 1994_{a,b})

1.13.11.1. The stability of azadirachtin

The studies on stability of azadirachtin to light and in field conditions, and in the laboratory have shown that its stability can be improved greatly by the addition of UV stabilizers. Temperature is very important in the stability of azadirachtin. It was shown that methanolic solution of azadirachtin was stable at -20 °C for more than six months. On the other hand at higher temperatures the stability is low. For example at pH 7 the half-life was estimated about 11.75 days at 25 °C , decreasing to 20.5 h at 45°C. When the stability of azadirachtin in natural and buffered was investigated the results showed that there was little difference in the rate of decomposition in buffered distilled water between pH 4 (half life 19 days at 20°C) and pH 7 (half life 12.9 days at 20 °C) (Jarvis et al, 1998), but decomposition was rapid at pH 10 with a half life of about 2 h. It has been said that microbial action in the degradation of AZA in the unsterilized pond water is minimal (Sundaram *et al* , 1996). Azadirachtin is a highly oxidized limonoid with many reactive functional groups in close proximity to each other (Mordue, 1993), which may affect its stability in natural conditions.

1.13.11.2. Effect of azadirachtin on pestiferous insects

Azadirachtin-containing pesticides can control many pestiferous insects from different orders. Phytophagous insects especially lepidopterous larvae and orthoptera have been the main target pest of this pesticide. Also some ectoparasites have shown high sensitivity to azadirachtin-based compounds. Methanolic extracts of azadirachtin in concentration 1000 to 2400 ppm reduced the flea, *Ctenocephalides felis*, by 53-93% for 19 days. When azadirachtin was combined with Deet (N, N diethyl-m-toluamide) and citronella (only 500 ppm of azadirachtin), the reduction in fleas was 62-95% for 20 days. This result showed that azadirachtin reduced flea infestation in a dose dependant-manner. It was suggested that Deet with citronella potentiated the

effect of azadirachtin on *Ctenocephalides felis* (Guerrini, 1998). Azadirachtin is potentially useful in the aquatic environment, but further work is required to establish its stability and half-life in water (Mordue, 1993). In *Culex tarsalis* and *C. quinquefasciatus* when the egg rafts were deposited directly in fresh neem suspension and left there for 4 h before transfer to untreated water, 1 ppm of AZA produced almost 100% mortality in eggs (Su *et al*, 1998). Neem has proved to be effective in protecting stored products, particularly grain, whose losses are frequent in developing countries due to inability to apply expensive chemical pesticides. Richter *et al*, (1997) showed that Neem Azal, a commercial neem preparation, with an azadirachtin content of 20%, is a proven growth retardant for the cockroach *Periplaneta americana*. In an investigation carried out on the German cockroach (*Blattella germanica*), it was shown that the effect of azadirachtin was dose-dependent. There was no significant difference in the toxicity of this pesticide to either insecticide-resistance or susceptible strains. Also the roaches injected with 2 and 3 µg of azadirachtin showed a continued time-dependent weight-reduction. It suggested that the toxicity of AZA to the German cockroach may be associated with an altered feeding behaviour and disruption of endocrine events (Prabhakaran, 1996).

1.13.11.3. Antifeedancy

Although the insecticidal properties of extracts of the Meliaceae in Europe have been known as early as 1877 and in some of eastern countries such as India for centuries, world-wide use of azadirachtin goes back only to recent decades (Beek *et al*, 1993). By this time several reports appeared on the ability of neem kernel extract to deter feeding by insect on plants (Mordue, 1993). Using the Mexican bean beetle, *Epilachna varivestis*, Beek *et al*. (1993) investigated the antifeedant activity of tetra- and pentanortriterpenoids and demonstrated an EC₅₀ as low as 13 ppm for azadirachtin.

Azadirachtin affects feeding, primarily through chemoreception and secondarily by reduction in food intake due to a toxic effect if consumed. A large variety of formulations have been applied in many different ways against a great number of insect species. It is clear that Lepidoptera are extremely sensitive to azadirachtin and show effective antifeedancies from < 50 ppm, depending upon species. Lepidopteran larvae are effected through the stimulation of specific deterrent receptors, through the

modification of activity of phagostimulant receptors, or by interaction of the activities of both deterrent and phagostimulant receptors, depending on the individual species (Nisbet *et al*, 1997). Coleoptera, Hemiptera and Homoptera are less sensitive to azadirachtin behaviourally, with up to 100% antifeedancy being achieved at 100-600 ppm, whereas Orthoptera show an enormous range in sensitivity (Table1.4) (Mordue, 1993).

Table 1.4 The antifeedant effects of azadirachtin (AZAD) and Margozan-O (M-O) (0.3% AZAD) on a selected range of insect pest species in laboratory and greenhouse trial.

Species	Treatment	AZAD Content (ppm)	Antifeedancy (%)
Lepidoptera			
(larvae)			
<i>Spodoptera frugiperda</i>	AZAD(fd)	1.0	92
<i>Heliotis virescens</i>	AZAD(fd)	1.0	94
<i>Spodoptera littoralis</i>	AZAD(fd)	1.0	95
<i>Choristoneura fumiferana</i>	M-O (ad)	0.2	50
<i>Choristoneura fumiferana</i>	M-O (ad)	0.3	95
<i>Heliotis virescens</i>	AZAD(ad)	0.07	50
<i>Peridroma plorans</i>	AZAD(ad)	0.4	50
<i>Spodoptera littoralis</i>	AZAD(ad)	10.0	100
<i>Earias insulana</i>	AZAD(ad)	50.0	100
<i>Spodoptera litura</i>	AZAD(t)	50.0	37
<i>Spodoptera frugiperda</i>	AZAD(t)	50.0	43
<i>Peridroma saucia</i>	AZAD(t)	2.4	50
<i>Ostrinia nubilalis</i>	AZAD(t)	24.0	50
<i>Achoea janata</i>	AZAD(t)	1.0	54
<i>Achoea janata</i>	AZAD(t)	10.0	75
<i>Spodoptera littoralis</i>	AZAD(sp)	0.06	50
<i>Macalla thyrsisalis</i>	M-O(sp)	20	41
<i>Spodoptera frugiperda</i>	AZAD(sp)	600.0	100
<i>Pieris brassicae</i>	AZAD(syst)	30.0	56
Coleoptera			
<i>Epilachna varivestis</i>	AZAD(t)	500.0	100
<i>Diabrotica undecimpunctata howardi</i>	AZAD(sp)	100	98
<i>Acalymma vittatum</i>	AZAD(sp)	100	98
<i>Lepitinotarsa decemlineata</i>	AZAD(sp)	600	0
Hemiptera			
<i>Myzus persicae</i>	AZAD (ad)	100	80
<i>Rhopalosiphum padi</i>	AZAD(t)	250.0	50
<i>Sitobion avenae</i>	AZAD(t)	250.0	50
<i>Asymmetrasca decedens</i>	M-O(sp)	60.0	50
<i>Myzus persicae</i>	AZAD(syst)	300.0	30

Table 1.4 –Cont'd

Species	Treatment	AZAD content (ppm)	Antifeedancy (%)
<i>Rhopalosiphum padi</i>	AZAD(syst)	500.0	18
<i>Sitobion avenae</i>	AZAD(syst)	500.0	18
<i>Asymmetrasca decens</i>	M-O (syst)	60.0	100
<i>Rhodnius prolixus</i>	AZAD	25.0	50
Isoptera			
<i>Coptotermes formosans</i>	M-O(fd)	100.0	49
Diptera			
<i>Lirimyza trifoli</i>	M-O(syst)	10.0	31
Orthoptera			
<i>Schistocerca gregaria</i>	AZAD(fd)	0.008	50*
<i>Schistocerca gregaria</i>	AZAD(fd)	0.005	95*
<i>Schistocerca gregaria</i>	AZAD(fd)	0.01	100*
<i>Schistocerca gregaria</i>	AZAD(fd)	0.04	100*
<i>Schistocerca gregaria</i>	AZAD(fd)	0.05	100*
<i>Schistocerca gregaria</i>	AZAD(fd)	0.07	100*
<i>Locusta migratoria</i>	AZAD(fd)	100.0	50*
<i>Locusta migratoria</i>	AZAD(fd)	50.0	100*
<i>Dissostertia carolina</i>	M-O(fd)	150.0	82
<i>Schistocerca gregaria</i>	AZAD(ad)	0.001	100
<i>Melanoplus sanguinipes</i>	AZAD(ad)	1000.0	0
<i>Eyperpocnemis Plorans</i>	AZAD(t)	0.01	44*
<i>Eyperpocnemis Plorans</i>	AZAD(t)	0.1	100*
<i>Eyperpocnemis Plorans</i>	AZAD(t)	0.1	85
<i>Diapheromera femorata</i>	M-O(t)	150.0	75
<i>Diapheromera femorata</i>	M-O(t)	600.0	91
<i>Gryllus pennsylvanicus</i>	M-O(t)	150	100
<i>Schistocerca gregaria</i>	AZAD(sp)	1.0	50

Treatment applied via: fd, filter paper or glass fibre discs; ad, artificial diet; t, topical treatment to leaves, leaf discs; sp, spray application to crop; syst, systemic application. Antifeedancy given in relation to control feeding.

* : records due to different references

(The table was taken from Mordue et al, 1993)

1.13.11.4. Effect of neem azadirachtin on human and other mammals

Azadirachtin, long recognized for its insecticidal activity, has recently received a great deal of attention around the world as a potentially useful and safe biopesticide. Now in some countries it can be used up to, and including, the day of harvest and re-entry is permitted without protective clothing after the spray has dried. The experiments with Margosan-O, a neem extract containing 3000 parts per million azadirachtin, have shown that it is not toxic when fed to mallard ducks, bobwhite quail, and rats. Also this pesticide did not cause an adverse immune response when injected into rats, and did not show any effect in mutagenic assay with bacteria (Stone, 1992). Using albino rats (*Rattus norvegicus*, Druckery strain), Raizada *et al.* (2001) showed that technical azadirachtin 12% at doses of 500-1500 mg/kg/day for 90 days did not produce any sign of toxicity, mortality, changes in tissue weight, serum and blood parameters. In this case the only observed adverse effect was mild aggressiveness in these animals.

The reported adverse effect of neem extracts on animals probably are related to other constituents of these extract rather than azadirachtin. For example Awassthy *et al.* (1999) showed that oral administration of crude ethanol extract of leaves of neem to adult Swiss albino mice in concentrations of 5- 20 mg/10g bw/day after 7 days increased the incidence of structural and mitosis disruptive changes in metaphase chromosomes of bone marrow. Also Mahboobb *et al.* (1998), found that low-level exposure of Vepacide (isolated from neem oil) had a significant effect on xenobiotic detoxification mechanism of different tissues of rats. In their experiments they found that a high dose caused a significant decrease in Cytochrome P-450 (Cyt. P-450) concentration at 45 and 90 days, and the medium dose cause same effect at 90th day of administration in liver and lung.

Structural analyses of azadirachtin carried out by Rosenkranz *et al.*, (1995), indicate that this natural product has the potential for acting as a "genotoxic" carcinogen, but using *Salmonella typhimurium* strains TA98 and TA100, Polasa *et al.* (1987), failed to find mutagenic activity in neem oil. In addition Srivastava *et al.*, (2001), could not find any morphological, visceral and skeletal changes in fetuses of rats which have been administrated azadirachtin from days 6 to 15 of gestation, and mild fetal skeletal variations which reported with high concentration of azadirachtin was not significant.

They have mentioned that chemicals with a molecular weight more than 600 (e.g. azadirachtin) are not readily transigrate through the placenta.

1.13.11.5. Mode of action of azadirachtin

In addition to the antifeedant effect of azadirachtin, it was also found to cause major disorders in some phytophagous insects. The insect growth regulatory (IGR) effects of azadirachtin, manifested in growth and moulting abnormalities, result both from disruption of the endocrine system, by blockage of release of neurosecretory peptides, which regulate synthesis and release of ecdysteroids and juvenile hormone, and from direct effects of azadirachtin on dividing cells (Mordue, 1998).

Azadirachtin A causes ultrastructural changes on the endocrine glands of larvae of *Lucilia cuprina* (Wied) and it was concluded that the degradation of endocrine glands within the ring complex would contribute to a generalized disruption of neuroendocrine function (Meurant, 1994). The developmental effects of azadirachtin are attributed to a disruption of ecdysteroid and juvenile hormone titres through a blockage of morphogenetic peptide hormone release (Ortego, 1999).

The interruption of insect reproduction is also an important and potent effect (Mordue, 1993). The results of studies of Feder *et al.* (1988) demonstrated that azadirachtin causes pronounced sterilization in mature females of *Rhodnius prolixus*. In an investigation carried out on *Labidura riparia*, AZA interrupted the female ovarian development in a dose-dependent manner. Follicles exhibited degenerative changes, separation of follicle cells from the oocyte, and lack of pinocytotic vesicles as of yolk spheres in cortical ooplasm. Gel electrophoresis reveals that vitellogenesis is absent from both the fat body and haemolymph, and that vitellin is not deposited in the ovary. These pathological effects were not linked to an absence of feeding. The effect of AZA on vitellogenesis was rescuable by juvenile hormone treatment. It was supposed that these effects were due to a direct cytotoxic effect as well as to interference by AZA to neuroendocrine system. As in many other insects, vitellogenesis in *L.riparia* depends on precursors of the major yolk proteins which are synthesized in the fat body as vitellogenins, released into the hemolymph, and internalized by the ovaries through receptor-mediated pinocytosis. The sequestration occurs by invagination of oocyte plasma membrane and the proteins are stored inside

yolk spheres; once inside the oocyte these proteins are considered as vitellins (Sayah, 1996).

Aims of the project

The overall aim of the work reported here was to use well-characterised cell lines from insects and other invertebrates, and also mammalian lines, to study at the cellular level the effects of pure neem terpenoids and to compare them to other well-studied phytochemicals with pesticidal actions. The compounds which were to be examined were all to be used in a pure state. They were azadirachtin, nimbin, salannin, nicotine, pyrethrum, and rotenone.

The initial aim was to extend and quantify previous indications that while azadirachtin inhibited the growth of insect cells at low concentrations, it had no effect on mammalian cells except at very high concentrations. By using other invertebrate cell types it was hoped to see if the effects of the neem terpenoids were common to all invertebrates.

Following these studies, the aim was then to use a variety of techniques to attempt to discover the mode (or modes) of action of azadirachtin against insect cells. It was further hoped to be able to discover the reason for any differences between the response of insect and mammalian cells to azadirachtin.

Chapter 2

A comparison of the effects of botanical pesticides on growth of cultured cells

2.1. Introduction

2.1.1. History of animal cell culture

The history of animal cell culture dates from the turn of the twentieth century, when the first experiments published showing frog embryo nerve fibre growth *in vitro*. By the early 1970s, methods were being developed for the growth of specific cell types in chemically defined media (Davis, 1994). Using cultured fish cells for the acute *in vitro* cytotoxicity assays of aquatic pollutants was first suggested by Rachlin and Perlmutter in 1968 (Li *et al.*, 2001).

Originally, tissue culture was regarded as the culture of whole fragments of explanted tissue with the assumption that histological integrity was maintained, at least in part. Now 'tissue culture' has become a generic term and encompasses organ culture, where a small fragment of tissue or whole embryonic organ is explanted to retain tissue architecture, and cell culture where the tissue is dispersed mechanically or enzymatically, or by spontaneous migration from an explant, and may be propagated as a cell suspension or attached monolayer.

The list of different cell types which can now be grown in culture is quite extensive, and includes connective tissue elements such as fibroblasts, skeletal tissue (bone and cartilage), cardiac, epithelial tissues (e.g. liver, lung, breast, skin, bladder, and kidney), neural cells (though neurones do not proliferate), endocrine cells (adrenal, pituitary, pancreatic islet cells), melanocytes, and many different types of tumour (Freshney, 1992).

2.1.2. Drug development and using cultured cells in toxicological assays

Drug development programmes for the identification of new cancer chemotherapeutic agents involves extensive preclinical evaluation of vast numbers of chemicals for identification of anti-neoplastic activity. The safety evaluation of the compounds such as drugs cosmetics, food additives, pesticides, and industrial chemicals necessitates the screening of even greater numbers of chemicals. For example, it has been

estimated that there are currently approximately 100,000 chemicals in commerce, with 700 to 3000 new chemicals produced annually (Li *et al*, 2001).

Animal models have always played an important role in the field of cancer chemotherapy, where the potential value of such systems for cytotoxicity and viability testing is now widely accepted. There is increasing pressure for a more comprehensive adoption of *in vitro* testing in both spheres of application. The impetus for change originates partly from financial considerations, because *in vivo* bioassays are usually costly and time-consuming. There is also an increasing realisation of limitation of animal models in relation to human metabolism, as increasing numbers of metabolic differences between species come to be identified. Finally, there is the moral pressure to reduce animal experimentation. The safety evaluation of chemicals involves an extensive range of studies on mutagenicity, carcinogenicity, and chronic toxicity. Indeed, one of the increasing applications of cultured cells is to test and investigate the mode of action of various products, which may be used as drugs, detergents, cosmetics, insecticides or preservatives, etc. Although results obtained using cells in culture cannot be extrapolated directly to the whole animal, it is fairly certain that if some product produces deleterious effects on several different lines of cells in culture, some ill effect may be expected if the product is applied to whole animals. As well as enabling testing to be performed without the possible suffering of large numbers of animals, the use of human cells allows testing in one animal species not generally available for experimentation, i.e. Man. Moreover, the results of the test are more likely to be reproducible when carried out *in vitro*.

In general, the procedure is to expose cells to a range of concentrations of the chemicals under test for a determined time and then to test for cell viability. Such tests are most easily performed in microtitre plates, which allow rapid quantitation of the results using a micrometer plate reader (Adams, 1990).

Early cytotoxicity studies were largely qualitative, in that explant cultures growing in poorly defined media were used for the study of drug effect, which could be 'quantitated' by assessment either of morphological damage or of inhibition of the zone of outgrowth. The development of better-defined growth media, together with techniques for growing dispersed cells as a monolayer on glass, allowed the screening of identical replicate cell samples in reproducible growth conditions. Using these techniques in conjunction with measurement of protein content of treated and

untreated cells, a correlation between the *in vitro* and *in vivo* activities of neoplastic agents was found, demonstrating the validity of the method. In spite of the diversity of factors that affect these assays the consensus of the majority of reports is that a positive correlation of that more than 90% can be expected between *in vitro* resistance and clinical resistance, and of approximately 60% between *in vitro* sensitivity and clinical response.

Whilst the major application of *in vitro* cultures is currently with analysing acute toxicity, the existence of adequate culture systems would also improve the prospects of chronic toxicity testing. Toxicology and cancer chemotherapy therefore share the aim of determining the acute toxicity of a range of chemicals against a variety of cell type. In both areas there may be several ultimate goals:

- identification of potentially active compounds
- identification of the mechanism by which a compound exerts its toxic effect
- prediction of the effective cytotoxic drug for treatment of cancer patients
- screening to identify the range of activity of a compound
- identification of a potential target cell population
- identification of the toxic concentration range
- relationship of concentration to exposure time (Freshney, 1992).

2.1.3. Effect of pesticides on proliferation of cultured cells

Pesticides can affect growth of cultured cells in several ways. They may for instance inhibit proliferation of cultured cells. It was shown that avermectin C within the concentration of 0.1 to 1 µg/ml inhibited proliferation of human larynx carcinoma Hep-2 (Mosin *et al.*, 2000). Veroseni *et al.* (1993) used the neuroblastoma cell lines, NB41A3 and SH-SY5Y from mouse and human to show the cytotoxic affect of organophosphate on different cell lines. These cell lines have a similar growth curve, and measurable target esterase activities. The cytotoxicity assay was carried out with the help of Neutral Red, which accumulates in the lysosomes of living cells. They exposed the cells to serial dilutions of nine pesticides (aldicarb, parathion, paraoxon, leptophos oxon, DFP, mipafox, fenthion, fenitrothion, and acrylamide) for 72 h. The results showed the concentration of toxicant (IC₅₀) that killed the 50% of cells after

incubation was different in these cell lines. Also the results suggested that the mouse cell line was cytotoxically more sensitive than human cells, to most tested compounds. This difference was less distinct in response to active esterase inhibitors (e.g., paraoxan, mipafox, DFP), than in response to protoxicants (e.g., parathion, fenthion).

In vitro experiments have shown that organotin pesticides are toxic to mouse thymocytes and to rat thymus, bone marrow and red blood cells. Using a [³H]-thymidine incorporation radiometric assay, Dacasto *et al.* (2001) showed that the fungicide; triphenyltin acetate could reduce the proliferation of concanavalin A-stimulated primary cultures of thymocytes. This experiment suggested that triphenyltin acetate decreases the lymphoproliferative response of these cells to T-and B-cell mitogens.

Several xenobiotics such as methoxychlor, dieldrin, and toxaphen have been shown to be estrogenic to animal models (Soto *et al.*, 1994). Preconfluent cultures of normal rat intestinal cells (IEC-6 cell line) and normal human colonic epithelial cells were treated with 0.05-50 μ M doses of atrazine, diazinon, and endosulfan. When the changes in cell proliferation were quantified by cell counting or the MTT growth assay, both intestinal and colonic epithelial cell cultures had increased in cell growth, in concentration of 10^{-6} M of these pesticides (Greenman *et al.*, 1997).

Some chemicals show bimodal effect on proliferation of cultured cells. For example, trivalent arsenicals in concentrations of 0.001 to 0.01 μ M induce an increase in cell proliferation, whereas in high concentration (>0.5 μ M) they inhibit proliferation of normal human epidermal keratinocyte cultures (NHEK) from skin of adult females (Vega *et al.*, 2001). In this case also the experiments have shown that relative toxicities of arsenicals are likely to be a function of their oxidation state and less dependent on their methylation status.

In addition to pesticides sometimes impurity of these chemicals also may affect cell proliferation. For example Hadnagy *et al.* (1999), found that effect of pyrethroids on cell division of V79 line was not directly contribute to the cytotoxic effect of pesticide but, to other factors such as impurity in compounds.

2.1.4. Effect of azadirachtin on cell growth

The many physiological consequences of azadirachtin treatment of insects have made it extremely difficult to separate direct effects on tissues from indirect effects mediated through hormonal disruption (Nisbet *et al.*, 1997). Moreover, assessment of the biological activity of neem terpenoids has generally depended on bioassays using insects, such as the *Epilachna varivestis*: a bioassay developed by Rembold *et al.* (1982). This highly reliable test, however, requires up to three weeks, due to the retardation of larval and pupal development after azadirachtin treatment (Rembold *et al.*, 1993). As an alternative they described the use of the widely available cultured insect cell line, Sf9, derived from ovarian tissue of *Spodoptera frugiperda*. Such methods also can help in understanding the cellular mechanism of action of a compound, identifying specific biochemical lesions at cellular level and showing specific, high affinity binding of the molecule at a site relevant to that lesion (Nisbet *et al.*, 1997). Use of such cell cultures would also pave the way for answering several questions about the *in vivo* molecular targets of azadirachtin. Using the Sf9 cell line, Rembold *et al.* (1993) observed characteristic changes in a monolayer of these cells after treatment with a 1.4×10^{-6} M concentration of azadirachtin. These changes were an increase in the number of floating cells or loosely attached cells in the initial 24 h of treatment, followed by complete cell death so that after 48 h the architecture of the monolayer was completely lost. Jabbar and Strang (1998) compared the effects of azadirachtin on cultured insect and mammalian cells. The insect cells were Sf9 and mammalian cells were L929 (derived from mouse fibroblast); P9 (a liver cell line transformed by means of SV40 virus); and MCF7 (derived from human breast cancer). They observed that the cells of the insect line Sf9 were very sensitive to azadirachtin in a very low concentration, whereas the mammalian cell lines were little affected until the concentrations were very high. The order of potency of effects was very similar to that which had been established for whole, living insects.

Reed *et al.*, (1998) found that azadirachtin in concentrations between 10^{-8} to 10^{-6} M inhibited multiplication of Sf9 cells, whereas even at 10^{-5} M azadirachtin produced no effect on proliferation of mammalian cell line MEL-GM86). On the other hand, using human glioblastoma cells with a different TP53 status, Akuduga *et al.* (2001), found that azadirachtin in a concentration of 28×10^{-6} M inhibited proliferation of G-

28, G-60, and G-44. Also they showed that this terpenoid cause a non-repairable DNA damage in TP53 mutant cells and one wild type (G-62).

Jabbar *et al.* (1995) showed that Sf9 cells grown in the presence of azadirachtin lose their monolayer structure. He proposed that azadirachtin binds with its decalin ring moiety to its specific receptor, but that the whole molecule is important for exerting its effects. Also, if the azadirachtin is fragmented into its decalin and furanoid (Fig.2.1) components, neither inhibits the growth of cells significantly.

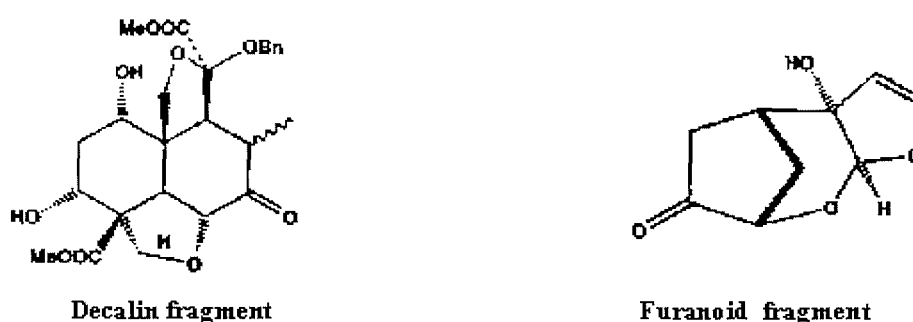


Figure 2.1. The structure of decalin and furanoid fragments of azadirachtin

2.1.5. Effect of azadirachtin on total protein

It is not clear how azadirachtin affects growth of culture cells. An effect on protein synthesis is one possible way, which has been shown in some of experiments. Estimation of the total protein content in treated and normal cells showed considerable differences indicating the reduction in total protein synthesis and turnover in treated cells (Rembold., *et al* 1992). A critical examination of the 2D PAGE gels of the normal Sf9 cells and after the various treatments showed quantifiable differences in the total number of polypeptides. Azadirachtin-treated cells showed a reduction of 77.9% polypeptide spots in comparison with the control. A critical examination of the 2D PAGE gels of the normal Sf9 cells and after the various treatments showed quantifiable differences in the total number of polypeptides.

To verify yolk protein modification in *Labidura riparia* (Insect:Dermaptera)

Sayah *et al.* (1996), injected female insects with azadirachtin. They showed that no vitellogenin, or vitellins were produced in ovaries.

2.1.6. Cytotoxicity of salannin and other limonoids of neem

Neem tree extracts not only contains azadirachtin as the most effective pesticide but also a variety of other limonoids, some of which have cytotoxic properties. For example, in an investigation which Cohen *et al.* (1996) carried out on N1E-115 neuroblastoma (mouse), 143B.Tk osteosarcoma (human) and Sf9 (insect) cultured cell lines, it was shown that the most potent of these limonoids was nimbolide with an IC_{50} ranging $4-10 \times 10^{-6} M$. The average IC_{50} for epoxyazadiradione and salannin were $27 \times 10^{-6} M$ and $112 \times 10^{-6} M$ (respectively), and the cytotoxicity of nimbin, deacetylnimbin and azadirachtin was each $> 200 \times 10^{-6} M$.

2.1.7. The effect of Nicotine and pyrethroids on cell proliferation

Nicotine has been considered in many investigations not only as an insecticide but also as a major component of cigarette smoke. Most studies on the cellular effects of nicotine postulated cytotoxic effects of this alkaloid and indicate that nicotine can be associated with cell loss and desquamation. Villablanca's (1999) studies on vascular endothelial cells, revealed that nicotine in concentration lower than $10^{-6} M$ exhibited cytotoxic properties. However, in concentrations 10^{-14} - $10^{-10} M$ nicotine stimulated initiation of DNA synthesis in this cells.

Some of experiments on estrogenic effect of pyrethroids indicates that these compounds can stimulate growth of cultured cells. Go *et al* (1999) that both Somithrin and Fenvalerate, two synthetic pyrethroids, were able to induce cell proliferation of MCF-7 cells in a dose-response fashion. They showed that permethrin had a noticeable effect on cell proliferation at $100 \times 10^{-6} M$, whereas d-trans allethrin slightly induce MCF-7 proliferation at $10 \times 10^{-6} M$, but was toxic at higher concentrations. Also Garay *et al* (1998) found that fenvalerate and sumithrin caused significant estrogenicity at concentration of $10 \times 10^{-6} M$ on Ishikawa Var-I cells, but Saito *et al.* (2000) could not show any estrogenic activity in fenvalerate.

2.1.8. Estimation of cell number by use of the tetrazolium dyes MTT and XTT

Many biological assays require the measurement of the % of survival and /or the number of proliferating cells. This can be achieved by several methods, e.g., counting cells that include or exclude a dye. Using tetrazolium salts such as MTT (Fig. 2.2) is one of these methods which, when combined with multiwell scanning spectrophotometers (ELIZA readers), can measure large number of samples with a high degree of precision.

The MTT method is a quantitative colorimetric assay for cell survival and proliferation. This is one of the well-known *in vitro* cytotoxic assessment methods (Mosmann., 1983) which measures the metabolic activity of cells. In this assay the dehydrogenases produced in living cells reduce MTT [3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide] and produce a purple insoluble formazan form. Formazan crystals are soluble in DMSO and the absorbance at 492 nm of the dissolved formazan can be immediately recorded. MTT is cleaved by all living, metabolically active cells, but not by dead cells. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population.

MTT is taken into the cell through endocytosis and is reduced. The reduced MTT formazan is exocytosed to form needle-like formazan crystals at the cell surface. The location of the cellular site for tetrazolium salt bio-reduction is controversial. Mitochondria are unlikely to play a significant role in cellular MTT reduction (Liu, 1999).

Although for MTT the exact reduction mechanism is unknown, Berridge and Tan (1993) have shown that NADH and NADPH-dependent mechanisms are involved which are virtually insensitive to inhibitors of the mitochondria respiratory chain (Goodwin *et al.*, 1996). The main advantage of the colorimetric assay is the speed with which samples can be processed.

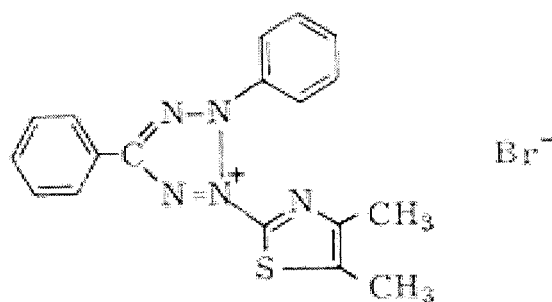


Figure 2.2. The structure of MTT

The aims

The aims of this chapter was to compare the effect of Neem terpenoids on proliferation of different cell lines, as well as evaluation of cytotoxicity of the major phytochemical pesticides on cultured cells and comparison with that of Neem terpenoids.

2.2. Materials and methods

2.2.1. Cell lines

2.2.1.1. Invertebrate cells.

2.2.1.1.1. Insect cell lines

a) Sf9

This cell line, derived from ovarian cells of *Spodoptera frugiperda*, was obtained from stocks in the Division of Biochemistry, IBLS, University of Glasgow. Cells were grown at 27 °C in 75 cm⁻² culture flasks in 10 ml of the insect culture medium TNM-FH (Sigma Aldrich) enriched with 10% v/v foetal bovine serum. The cell doubling time for Sf9 cells under optimum condition ranges between 18-24 h. and they were subcultured every 72 hours.

b) C6/36

C6/36 cells derived from the mosquito *Aedes albopictus* was the kind gift of Dr. A. Bridgen, Division of Virology, IBLS, University of Glasgow. The cells were grown in 162 cm² flasks in 20ml L-15 (LEIBOVITZ) medium (GibcoBRL) containing 10% foetal bovine serum. Cells were grown at the temperature of 27 °C, and were passaged every 6 days. The cell population doubling time was about 30 h.

2.2.1.1.2. Snail

Bge

These cells were derived from embryonic cells of *Biomphalaria glabrata*, and were the kind gift of Dr. C. Coustau, University de Perpignan, France. Cells were maintained in Bge medium supplemented with 10% foetal bovine serum (FBS:Sigma

Aldrich co., poole, Dorset, UK) . Cells were grown in 75 cm² TC flasks under ambient atmospheric conditions at 27 °C cells were passaged every 2 weeks.

2.2.1.2. Vertebrate cells

L929

This culture, derived from mouse fibroblast cells , was obtained from stocks in the Division of Biochemistry, IBLS, University of Glasgow. The culture were grown in 75 cm² flasks in 10 ml of Glasgow MEM (BHK-21) (CibcoBRL) enriched with 10% v/v foetal bovine serum and 4% sodium bicarbonate under a humidified atmosphere of 5% CO₂/95% air. The cells were incubated at 37°C. Under these conditions the cell population doubling time was about 24 h. and they were seeded every 6 days.

All manipulations with cells were done under sterile conditions in a flow hood. For subculturing and experiments the L929 cells were detached from the substrate by trypsinization and the other more loosely attached cell cultures by means of scraper, before disaggregating any clumps by gentle trituration with a sterile pipette.

2.2.2. Estimation of cell numbers

2.2.2.1. Counting

Cells were dislodged and dispersed in medium, and then were counted by light microscopy using a standard haemocytometer. An average of 4 separate counts was done on each suspension. These visual counts were used to check counts made automatically by means of a Coulter counter (Coulter counter Z, Coulter Electronics, Hialeah, FL,USA) Cell suspensions were diluted 1/50 in counting buffer (150 mM NaCl and 15 mM Na citrate pH 4-5). Samples were then counted automatically. Cell numbers obtained by the two methods of counting were plotted against each other to ensure a linear relationship. Thereafter, cell numbers were routinely estimated by means of the Coulter counter.

2.2.2.2. Reduction of tetrazolium dyes

The dyes used were MTT ([3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide] and XTT [sodium (2,3-bis[2-Methyl-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) They, and the intermediary electron acceptors, menadione and phenazine methosulphate (PMS), were purchased from Sigma-Aldrich, Poole, Dorset, UK).

In general, different numbers of various types of cell were incubated in the presence of the dye, which was reduced by cellular dehydrogenases, to form the coloured formazan compound, which was then quantified by spectrophotometry. In the case of MTT, this is a water-insoluble compound, which has to be released from the cells by means of a lipophilic solvent. In the case of XTT, the reduced form of which is hydrophilic, this step was not necessary.

2.2.3. Correlation between numbers of cells and reduced formazan.

In the preliminary experiments, known numbers of cells, estimated by counting, were centrifuged in 15 ml centrifuge tubes (2000 rpm for 5 min), and the supernatant medium removed. The cells were then resuspended in 2 ml of phosphate-buffered saline (PBS contains: NaCl, 10.g; KCl, 0.25 g; Na₂ H PO₄, 1.44 g; KH₂PO₄, 0.25 g, pH 7.2), and 0.5 ml of MTT (15 mM), dissolved in PBS, was added. Blanks contained either cells without MTT or MTT without cells. The cells were incubated in the dark for 45 min at 37°C. The cells were centrifuged as before, the supernatant removed, and replaced with 2 ml of dimethyl sulfoxide (DMSO). The cells were then vortexed until they were fully lysed and the formazan dissolved. The DMSO solution was removed for spectrophotometry, which was done either in a standard 1 cm light-path plastic cuvette, or 200 µl aliquots were placed in the wells of a 96-well plate, to mimic the subsequent cell-growth experiments. Scans were made of the absorbance spectra of both the reduced dyes, to determine their λ max. The absorbance of the reduced formazan was routinely estimated at 492nm, using either a standard spectrophotometer or an ELISA plate reader (SLT. Spectra, Australia). This absorbance was then plotted against the known numbers of cells in the samples.

When XTT was employed, the methods were almost identical except that the step of dissolving in DMSO was omitted. The lower sensitivity of this dye was compensated

for by a much longer time of incubation the dye with the cells: 5 h. In addition, the sensitivity of the XTT method was further increased by the use in the incubation mixture of the intermediary electron acceptors menadione and PMS. They were dissolved in acetone and PBS (respectively) and added in a range of concentrations in order to optimise the reduction of the dye.

In the cytotoxicity experiments, estimation of cells number was done on cultures growing in the wells of 96-well plates. After the determined period of growth, 50 µl of dye solution was added to each well (without removal of medium), and the colour allowed to develop as described above. In the case of MTT, the supernatant was carefully aspirated under suction, before the addition of 200 µl of DMSO. Cell number was then estimated by the absorbance at 492 nm on an ELISA reader. With XTT the absorbance was read without removal of medium.

2.2.4. Botanical pesticides tested

The three terpenoids derived from *Azadirachta indica* were: azadirachtin, salannin and nimbin. The last two were the kind gift of Professor S. Ley, Cambridge University. All were shown by HPLC chromatography to be >95% pure. Nicotine 3-(1-methyl-2-pyrrolidinyl)pyridine was purchased from Sigma-Aldrich, Poole, UK, as the free base (98-100% pure). Pyrethrum and rotenone (95-98% pure) were also purchased from the same source.

All these compounds showed various degrees of hydrophobicity. Consequently, it was necessary to add them to growing cultures in an organic solvent. After preliminary experiments, the solvent used was DMSO that the final concentration of which never exceeded 0.1% .

2.2.5. Preliminary experiments.

2.2.5.1. Determination of the growth curve for the different cells lines.

Cells ($0.7-2.5 \times 10^4$) were seeded into 12 ml of the appropriate cell culture medium. 8-15 flasks (75 cm^2 for Sf9 and L929 and 162 cm^2 for C6/36) were set up simultaneously and incubated under the optimum conditions. Cell counts (in quadruplicate) were made at 24 h intervals, by both haemocytometry and using the Coulter counter. This process continued until the culture ceased to grow after 6-12 days.

2.2.5.2. Effect of organic solvents on cell growth.

As stated above, none of the possible cytotoxic compounds were freely soluble in water. Consequently, it was necessary to use a carrier solvent when adding them to growing cultures. The two solvents tested were ethanol and DMSO. Both insect and mammalian cells were grown in the presence of these solvents in concentrations ranging from 0.1-1%(v/v), and their growth compared to a control without solvent.

2.2.5.3. Determination the most appropriate wavelength for reading MTT and XTT formazan.

2 ml of Sf9 cell suspension were placed into two series 15 ml test tube. 0.5ml of MTT solution was added to the first series of test tubes and 2 ml XTT solution was added to second series test tube. Then, test tubes were gently mixed, wrapped in aluminium foil and incubated in a humidified atmosphere at 37°C for 45 min and five hours (for MTT and XTT respectively). MTT-containing tubes was centrifuged for 5 minutes in 2000-rpm and supernatant discarded. Then, 2 ml DMSO was added to MTT-containing tubes and the absorbion was scanned by spectrophotometer. To determine the λ max for XTT the medium (containing XTT formazan and cell) in a second series of test tube was scanned directly.

2.2.5.4. Investigation of inhibitory effect of rotenone and azadirachtin on activity of dehydrogenase enzymes.

The immediate effect of the pesticide on the dehydrogenase activity in the cells was assayed by means of the reduction of the tetrazolium dye MTT in two series of 1.5 ml Eppendorf tubes. Rotenone and azadirachtin were first dissolved in DMSO at concentration of 10^{-2} M and further diluted to concentrations of 10^{-5} M for rotenone and 10^{-3} M for azadirachtin, so the final concentration of rotenone and azadirachtin were 10^{-6} and 10^{-4} respectively. After adding pesticide and dye to cell suspensions colour was allowed to develop for 45 minutes. The concentration of DMSO in all wells was 1% and control wells contained only DMSO.

2.2.6. Cytotoxicity experiments

Cells were harvested from the maintenance cultures in growth phase, counted, and then appropriately diluted in fresh medium so that 1 ml contained about 10^4 cells. Aliquots (100 μ l) were then pipetted into each of the wells of a 96 well plate.

The compounds to be tested were made up in DMSO in a series of concentrations, each at twice the final desired concentration, and sterilised by filtration.

Volumes (100 μ l) were then added to the wells to give a range of final concentrations from 10^{-10} to 10^{-5} or 10^{-4} M. (The upper limit of the concentrations used was determined by the hydrophobicity of the compound). Control wells contained cells and medium containing DMSO only. Each test concentration was run simultaneously in 8 wells.

Plates were then incubated as previously described for 5-10 days and the cell mass estimated by the use of tetrazolium dyes as described above

2.2.7. Total cellular protein estimation

Total protein assessment carried out using the Folin-Lowry method (Lowry, 1951). In this method tyrosine and tryptophan present in the protein, reduce the phosphomolybdate and intensity of colour is measured at 650 nm.

Reagents:

1. Sodium potassium tartrate $C_4H_4KNaO_6 \cdot 4H_2O$ (2%) purchased from Sigma, UK.
2. Copper sulphate $\% CuSO_4 \cdot 5H_2O$ (1%) Purchased from Riedel-deHaen, Germany.
3. Alkaline sodium carbonate contains 0.1M NaOH, 2% Na_2CO_3 .
4. Folin-Ciocalteu reagent Purchased from Fisher chemicals, UK (commercial reagent diluted with an equal volume of water on the day of use)

Stock solutions of 1-3 were mixed in the ratio of 1:1:100, respectively.

Prior to experiment cells were harvested washed with PBS and resuspended in 10% KOH overnight. Then 1ml of this mixture was added to sample, vortexed and left for 10 minutes. 100 μ l of solution 4 was added and left for further 30 minutes. After that the absorbance was recorded at 650nm. Standard curve was made using 0-50 mg of bovine serum albumin.

2.2.8. Statistical analysis

The statistical significance between control and experimental values was determined by ANOVA (Analysis of variance) test using the statistical package Minitab version 13.

2.3. Results

2.3.1. The relationship between cell number and reduction of MTT

Using Sf9, C6/36 and L929 cell lines in our experiment we showed that there was a linear relationship between the cell number and the amount of MTT formazan generated. The results in figures 2.3 - 2.5 shows that the A_{492} is directly proportional to the number of cells. Therefore using the colorimetric method to assess cell numbers is a good indicator of the number of viable cells and the method is very sensitive.

Standard curve for number of Sf9 cells/ml estimated by MTT method

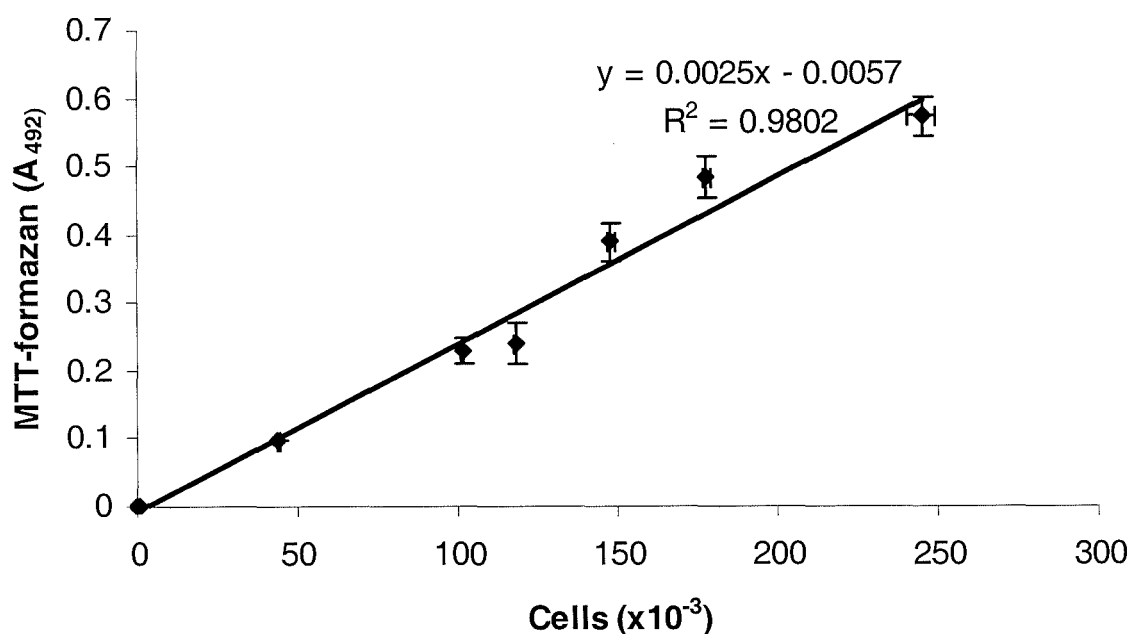


Figure 2.3 Estimation of Sf9 cell number by MTT assay. Means and standard deviation of 3-5 replications per point are shown.

Standard curve for number of C6/36 cells estimated by MTT method

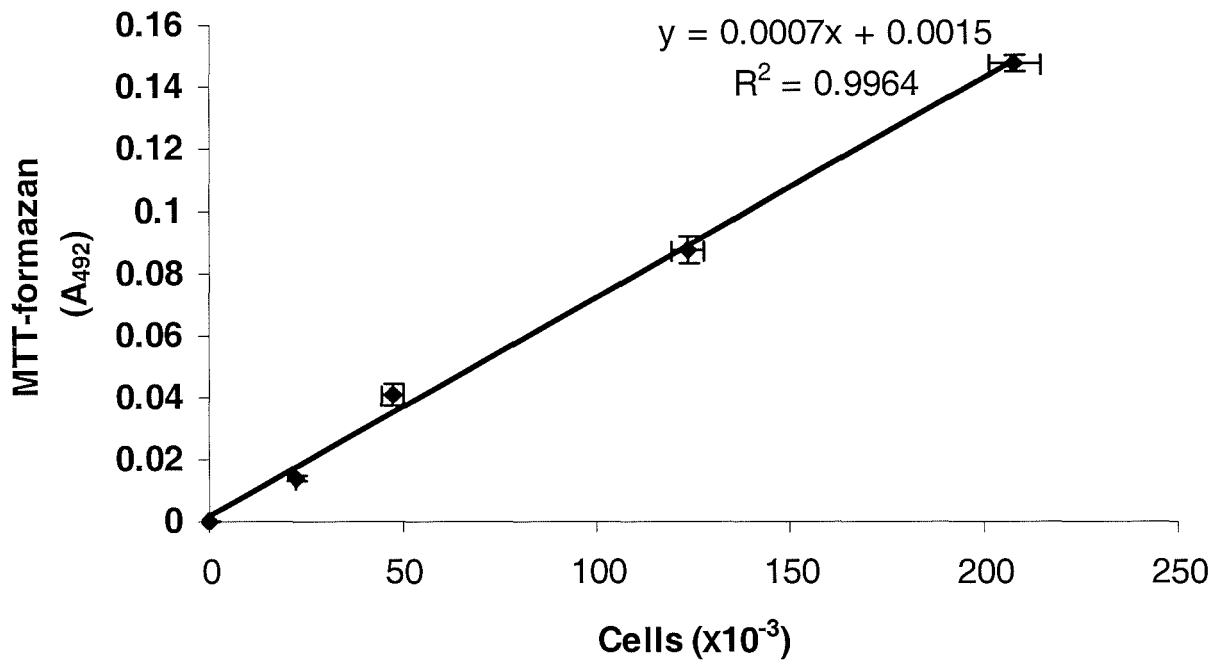


Figure 2.4 Estimation of C6/36 cell number by MTT assay. Means and standard deviations of 3-5 replicate per point are shown.

Standard curve for number of L929 cells estimated by MTT method

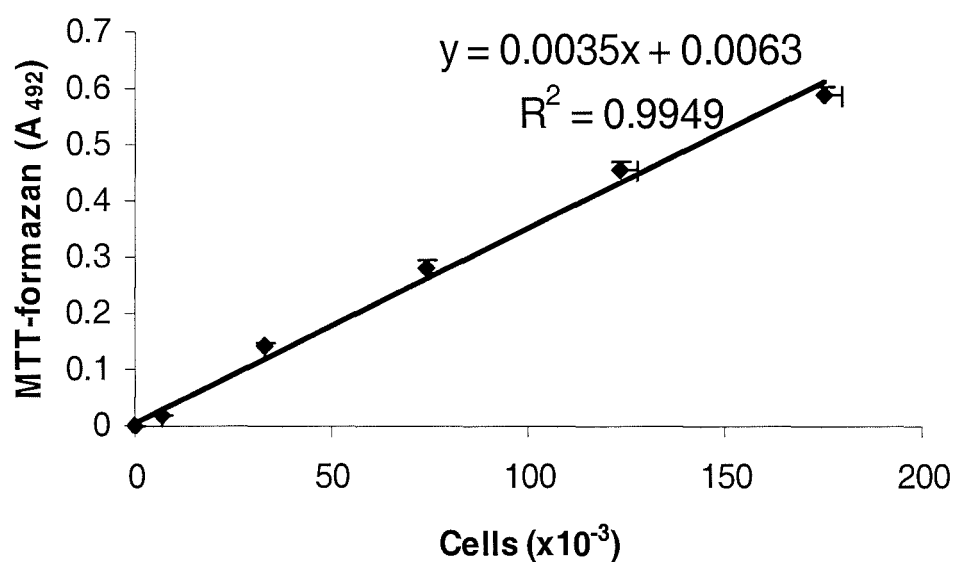


Figure 2.5 Estimation of L929 cell number by MTT assay. Means and standard deviations of 3-5 replicate per point are shown.

In order to find the relationship between the number of cell determined using either haemocytometer or Coulter counter, Sf9 cells were used (Fig. 2.6)

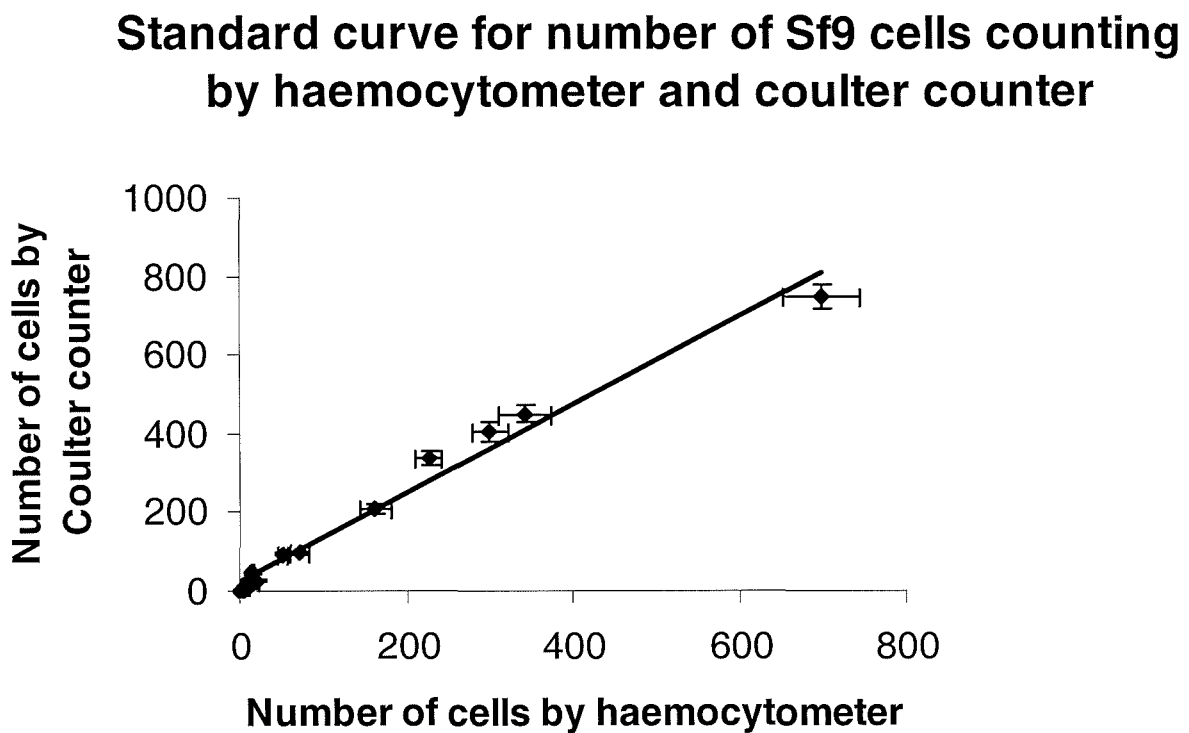


Figure 2.6 The relationship between the number of cell counting by haemocytometer and Coulter counter ($\times 10^{-3}$).

2.3.2. The growth curve of Sf9, C6/36, and L929 cell lines

As the figure 2.7 shows the growth rate of Sf9 cells in first 48 h is low. Then gradually the rate of growth increases and between 72 and 96 h. is very high. Then the growth rate decreases and after 120 h some cells begin to die and the number of cell falls gradually.

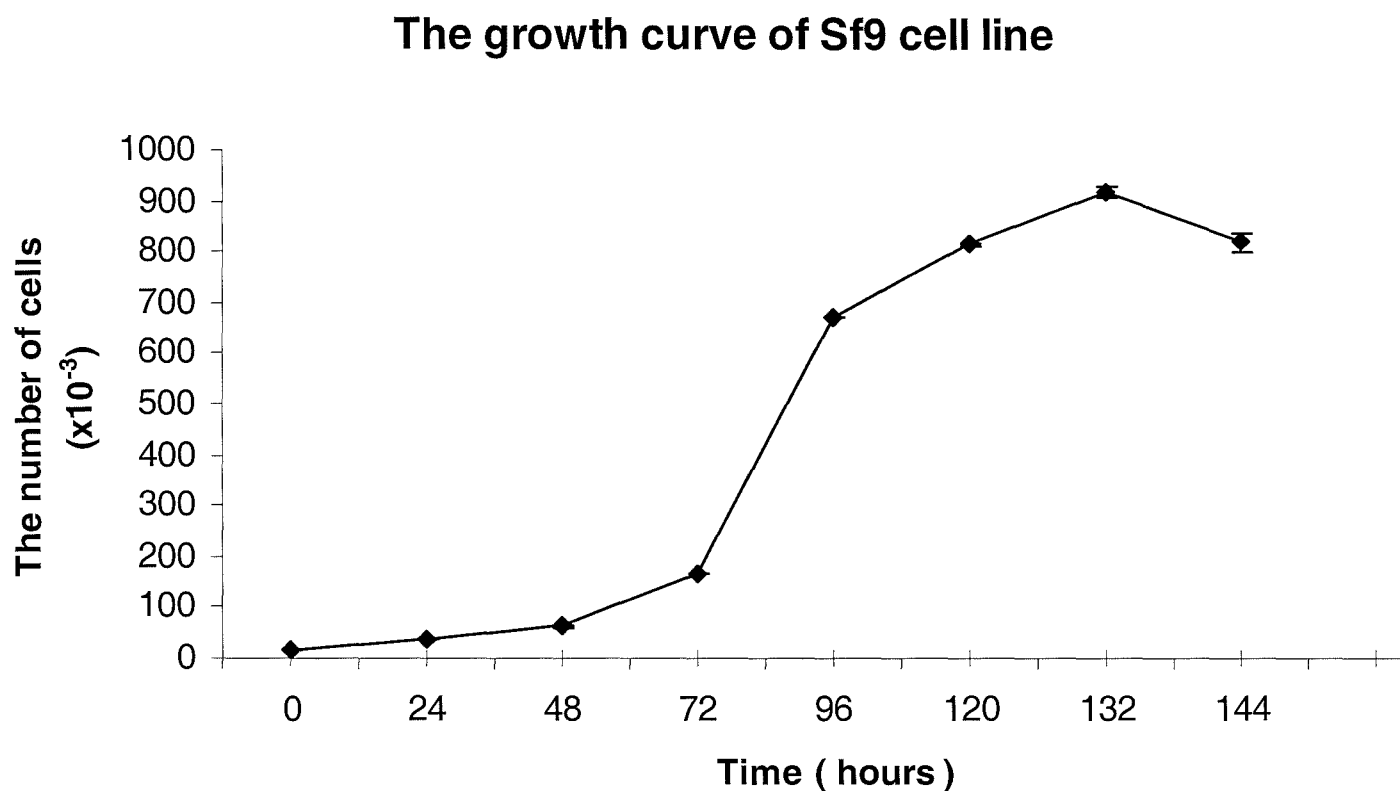


Figure 2.7 The effect of time on proliferation of Sf9 cells. Counting has been done using a Coulter counter. Means and standard deviations of 3 replicates per point are shown.

In the case of the C6/36 cell line the growth rate in the first days is similar to that of Sf9 cells but, the higher rate of growth continues for about 3 days (until 120 h). Then there is a decrease in growth rate (Fig. 2.8).

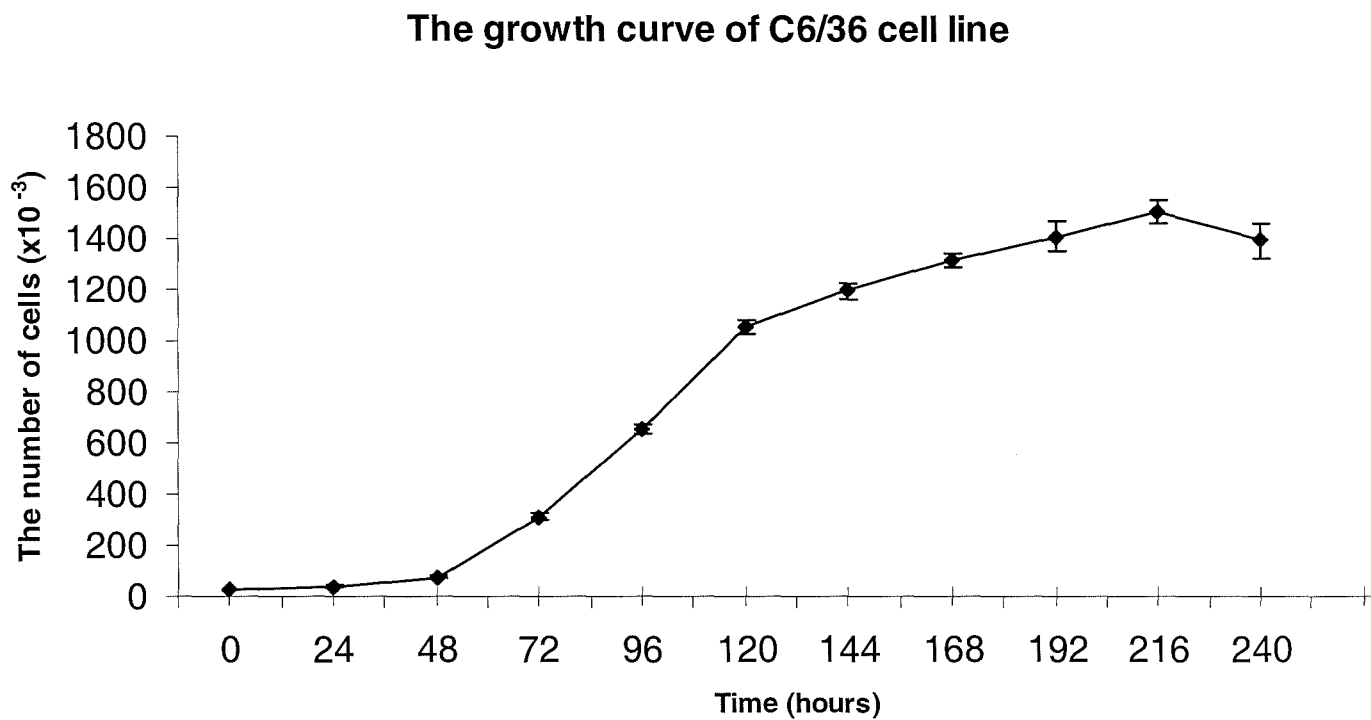


Figure 2.8 The effect of time on proliferation of C6/36 cells. Counting has been done using a Coulter counter. Means and standard deviations are shown (for each point 3 replicates have been used)

The growth curve of L929 cell line

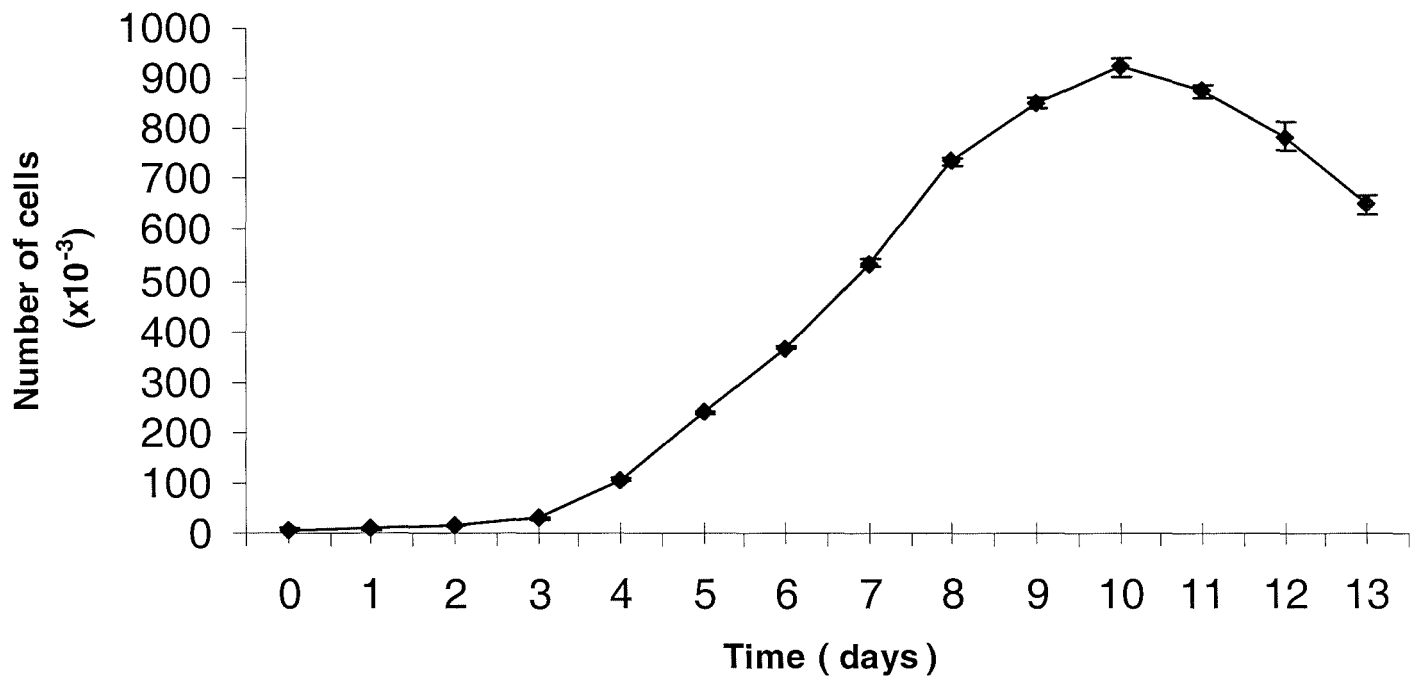


Figure 2.9 The effect of time on proliferation of L929 cells. Counting has been done using a Coulter counter. Means and standard deviations are shown (for each point 3 replicates have been used)

2.3.4. The effect of solvent on growth of Sf9 cells

A quantitative assessment of cell replication (Fig. 2.10 and 2.11) showed that at a concentration of 0.1% of ethanol there is negligible effect on the growth of cells Sf9 and L929. In the same conditions, the 0.1% DMSO has reduced the growth of these cells by 11% and 9% respectively.

At a concentration of 0.5%, ethanol elicits an inhibitory effect about 8% and 6% in Sf9 and L929 respectively, whereas the effect of DMSO in similar concentration is 20% and 19%.

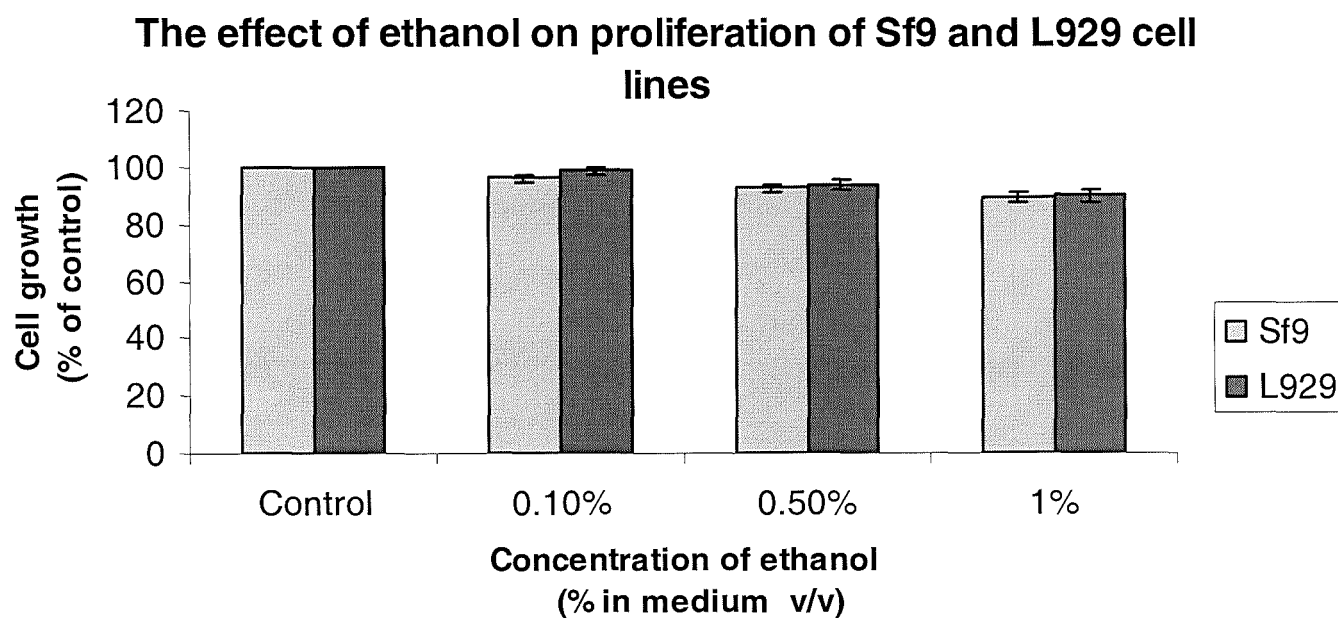


Figure 2.10. The inhibitory effect of ethanol on proliferation of Sf9 and L929 cells. The standard deviations of 8 replicates has been shown.

The effect of DMSO on proliferation of Sf9 and L929 cell lines

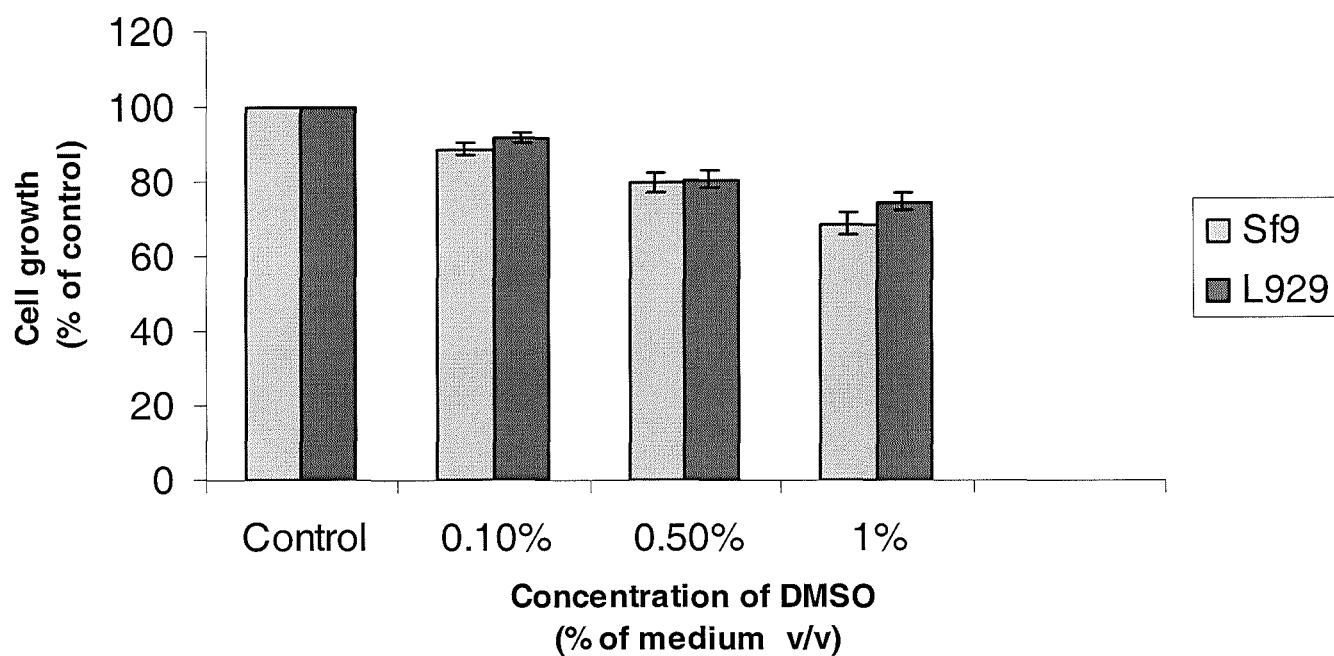


Figure 2.11. The inhibitory effect of DMSO on proliferation of Sf9 and L929 cells. The standard deviations of 8 replicates has been shown.

2.3.5. The potential immediate effect of rotenone and azadirachtin on the reduction of MTT by cellular dehydrogenase enzymes

The method of estimating cell numbers by means of reduced tetrazolium dyes, depends on the uninhibited activity of the cellular dehydrogenase enzymes. It is well established that the main toxic action of rotenone is on the mitochondrial NADH dehydrogenase (Berridge *et al.*, 1996). It was possible therefore that the presence of rotenone (and possibly also azadirachtin) would cause an underestimate of cell numbers by acutely inhibiting a major pathway leading to the reduction of the MTT.

In Sf9 cells it was found that the presence of rotenone at a concentration of 10^{-6} M reduced the rate of formazan production by a maximum of only 15%. Azadirachtin at a concentration of 10^{-4} M had no significant effect on the rate.

Cellular fractionation by differential centrifugation showed that only about 10% of the total cellular ability to reduce MTT resided in the mitochondria. It was concluded that the presence of rotenone and azadirachtin did not result a serious underestimate of the cell numbers.

2.3.6. Determination the most appropriate wavelength for reading MTT and XTT

A spectrophotometric scan of the absorbance spectrum of the solution of MTT formazan in DMSO indicated a λ -max of 511 nm. Previous studies (Jabbar *et al.*, 1998) used a wavelength of 540 nm for estimation of the concentration of the reduced dye. In the present case the wavelength used to estimate the formazan was 492 nm and was imposed by the limitation of plate reader employed, the most appropriate filter for which was 492 nm. All quantitative estimates of the reduced dye whether by plate reader or other spectrophotometric means were made at this wavelength throughout the experiments.

2.3.7. The comparison of sensitivity of XTT and MTT dyes

It is clear from the results presented in Table 2.1 that despite the much longer time allowed for the colour to develop using XTT, in the absence of intermediary electron acceptors, MTT is almost 10 times more sensitive as a means of estimating the cell numbers.

It has been observed (Berridge *et al.*, 1996) that the addition of intermediary electron acceptors greatly increases the rate of reduction of XTT. Consequently, menadione and phenazine methosulphate were added at different concentrations. The results are shown in table 2.1 and it is clear that PMS is the more effective adjuvant. It had the effect of increasing the reduction of the XTT by almost 30-fold when used in concentration of 2 μM .

Table 2.1. The effect of adding the intermediary electron acceptors menadione and phenazine methosulphate to the XTT assay.

Intermediary electron acceptor (IEA)	Final concentration of IEA ($\times 10^{-6}$ M)	A492
None	0	0.015
Menadione	1	0.08
Menadione	2	0.14
PMS	1	0.29
PMS	2	0.44
PMS	3	0.61
PMS	4	0.70
PMS	5	0.74

Each assay was done in three replicate and the results averaged. All assays contained the same number of cells (16×10^4). Reduced XTT were estimated by spectrophotometry at 492 nm. Cell numbers were estimated by means of a Coulter counter.

Also it is clear that at low concentrations PMS greatly increase the efficiency of the reduction of XTT until concentration of 4 μM , but additional PMS causes less effect.

The results of preliminary experiments indicated that both MTT and XTT (in presence of PMS) have high sensitivity, but due to complexity of using XTT and relatively swift response of MTT (45 min in comparison to 5h), throughout the experiments and in all cytotoxicity assay the MTT assay was used.

2.3.8. The effect of azadirachtin on proliferation of Sf9 cell line

Figure 2.12 shows the effect of growth time on the reduction of cell number due to the presence of 10^{-6} M azadirachtin. After 24 h of growth, the reduction of growth in the treated cells was not statistically lower than controls. By 48 h, growth of the control cultures had greatly outstripped the azadirachtin-treated cultures. However after 72 h. the number of cells in azadirachtin treated cells were significantly different from that of control.

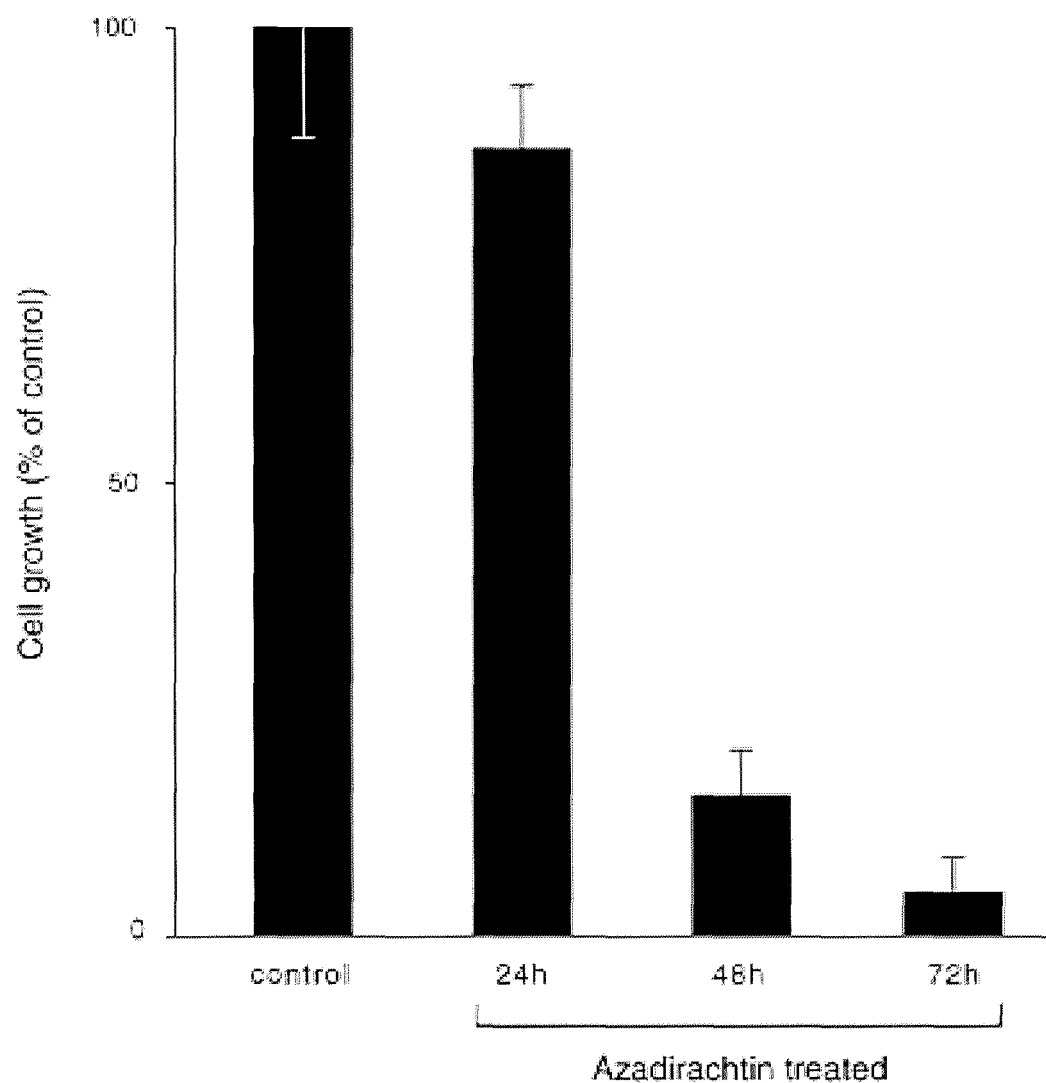


Figure 2.12. Time course of the effect of azadirachtin on the replication of Sf9 cells. The cells were grown in the presence of 10^{-6} M azadirachtin for the times shown in the figure. Cell numbers were estimated by MTT method. Each result is the mean (\pm SD) of eight estimates

Figure 2.13. shows a fluorescence micrograph of Sf9 cells after 72 h incubation of same number of cells, A) in absence (control) or B) in presence of 10^{-8} M azadirachtin. As the figure shows the effect of azadiarchtin on cell number after 3 days is quite obvious.

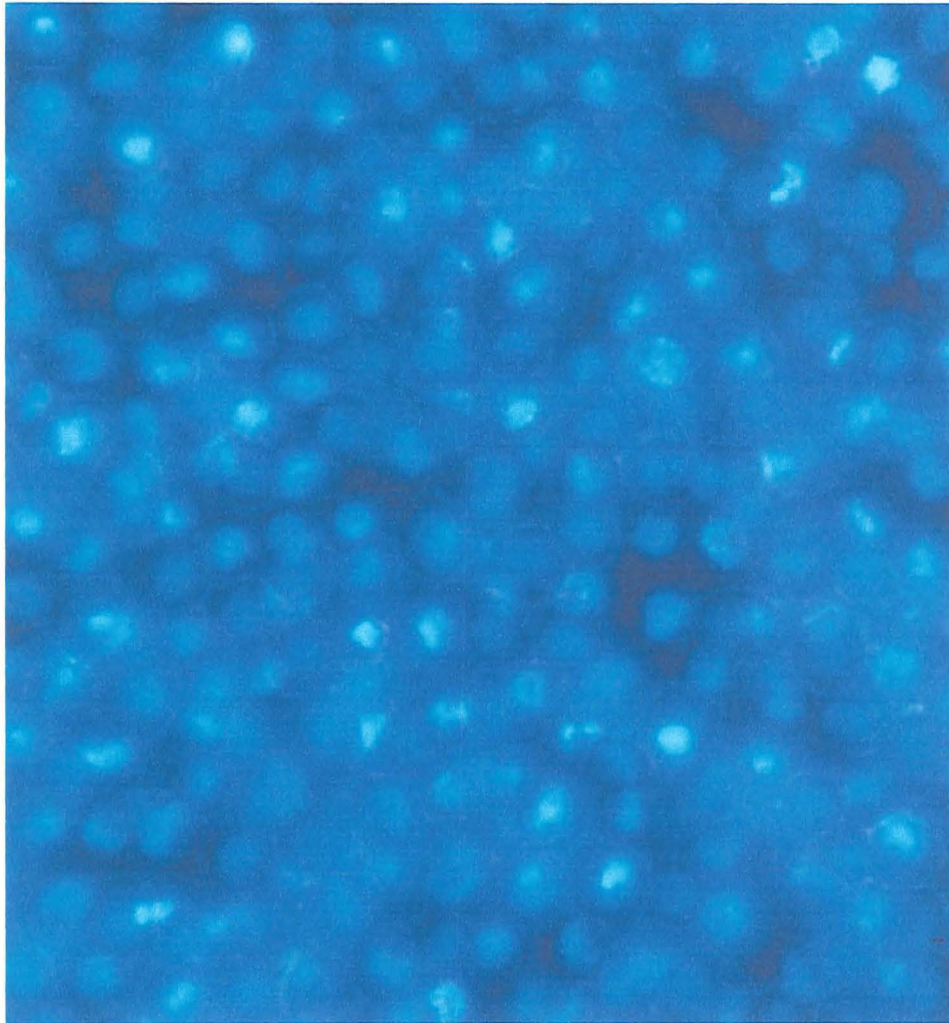


Figure 2.13. A Sf9 cells (control): after 72 h incubation with 0.1% DMSO, cells were washed and stained with Hoechst 33342 dye.

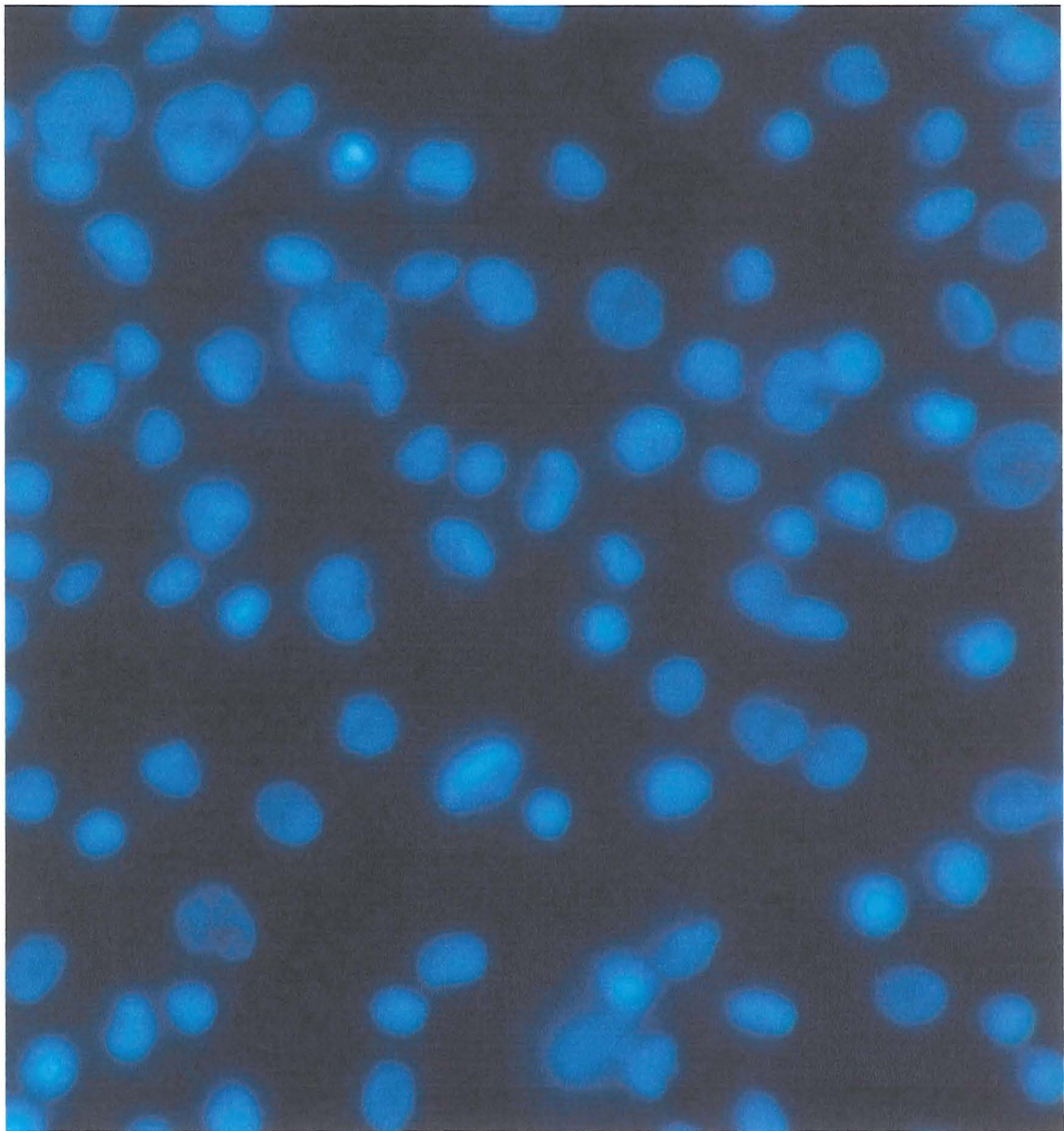


Figure 2.13. B: Sf9 cells after 72 h incubation in presence of 10^{-8} M azadirachtin. After incubation time cells were washed and stained with Hoechst 33342 dye.

Due to longer doubling time of other cell lines, 5 days incubation time was used to assess the effect of the phytochemical on proliferation of cell lines (except Bge cells). When these cells were grown in presence of a series of concentrations of azadirachtin a quantitative assessment of cell replication (Fig 2.14) showed that azadirachtin efficiently inhibited growth in Sf9 cell line after 5 days of incubation. A concentration of 10^{-9} M of azadirachtin caused about 40% reduction in growth rate of these cells. At 10^{-6} M the inhibitory effect was up to 90%. In all concentration the difference between treated and untreated Sf9 cells were statistically significant ($P < 0.001$)

The effect of azadirachtin on proliferation of Sf9 cells

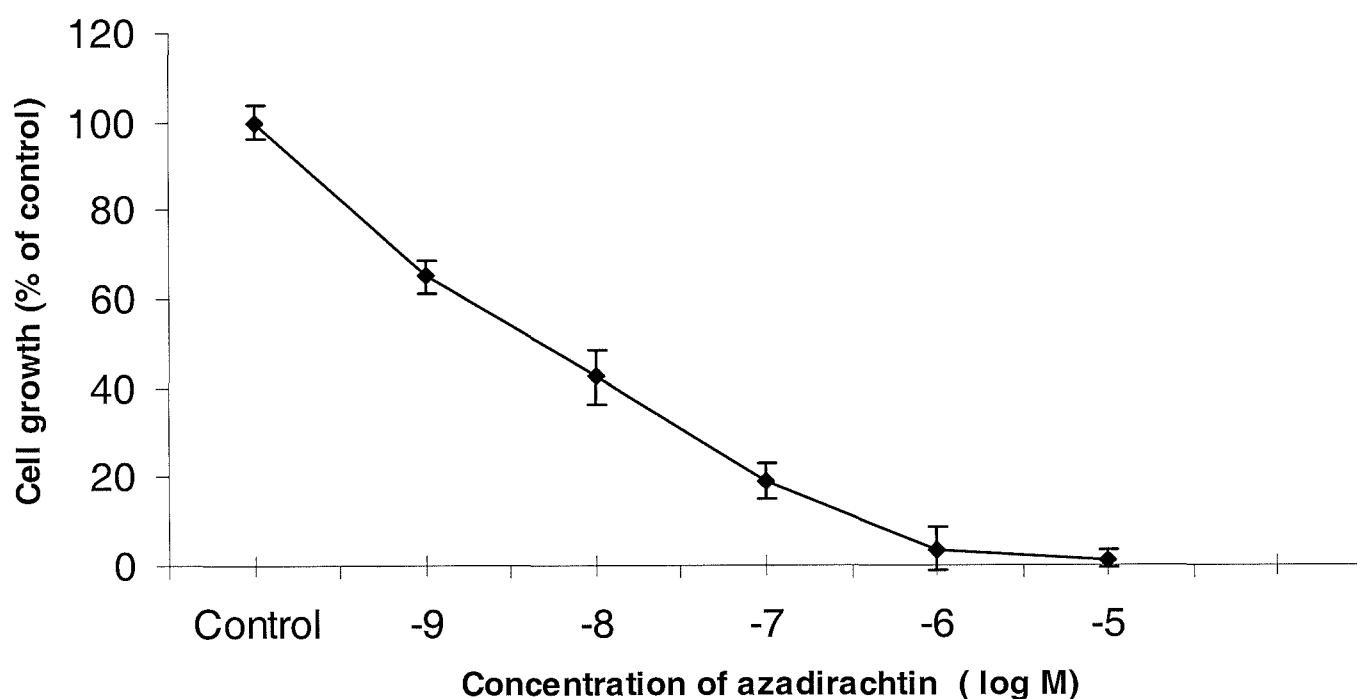


Figure 2.14. The effect of azadirachtin on proliferation of Sf9 cells after 5 days incubation. Each point is the mean of 16 estimates and vertical lines represent \pm SD.

2.3.9. The effect of azadirachtin on total protein in Sf9 cells

Incubation of Sf9 cells for 3-5 days at a concentration of 10^{-8} M azadirachtin did not show any significant difference in total protein/cell compared to that of control cells .

2.3.10. The effect of azadirachtin on proliferation of C6/ 36 cell line

As figure.2.15. shows, although in low concentration (10^{-9} - 10^{-8} M), the effect of azadirachtin on proliferation of C6/36 cells was less than 20%, at higher concentrations azadirachtin caused significant reduction on their growth rate ($P < 0.001$) and at 10^{-6} M, growth was about 50 % of control.

The effect of azadirachtin on proliferation of C6/36 cell line

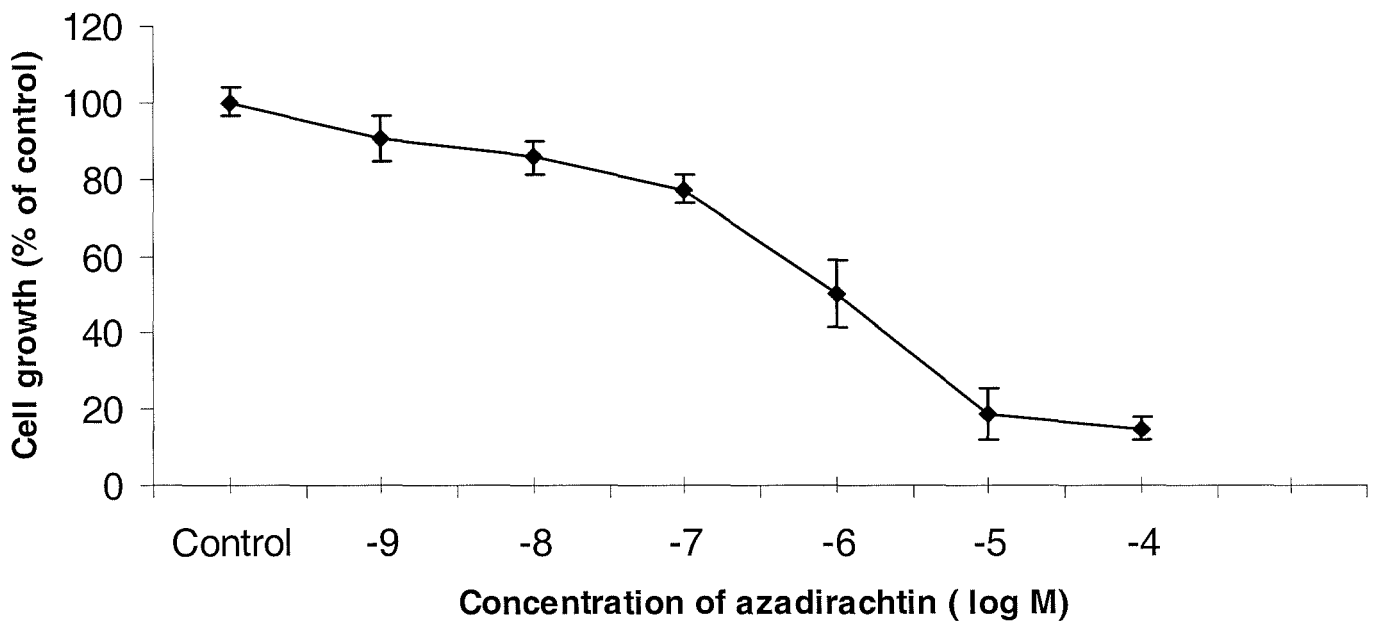


Figure 2.15. The effect of azadirachtin on proliferation of C6/36 cells after 5 days incubation. Each point is the mean of 16 estimates and vertical lines represent \pm SD.

2.3.11. The effect of azadirachtin on proliferation of L929 cell line

Fig. 2.16. represents the effect of azadirachtin on proliferation of L929 cells. As the figure shows, azadirachtin at concentration 10^{-9} to 10^{-7} M slightly reduced the growth of these cells. In this cell line, even in concentration as high as 10^{-4} M, the inhibitory effect was less than 50% of control.

The effect of azadirachtin on proliferation of L929 cell line

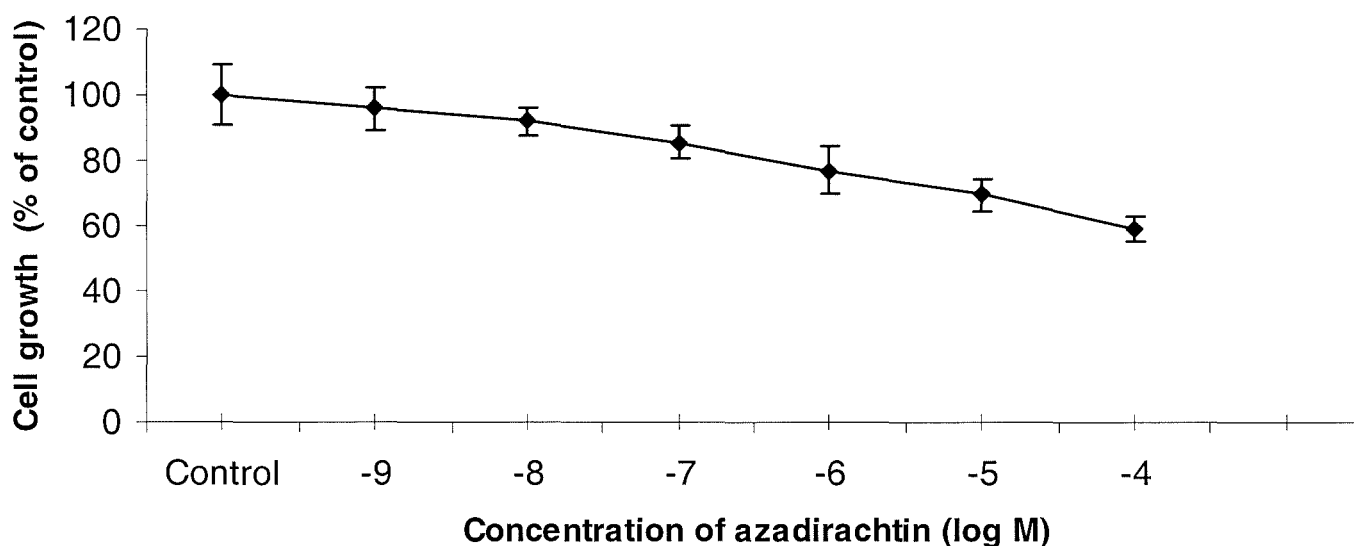


Figure 2.16. The effect of azadirachtin on proliferation of L929 cells after 5 days incubation. Each point is the mean of 16 estimates and vertical lines represent \pm SD.

The comparison of azadirachtin on growth of Sf9, C6/36 and L929 cell lines showed that the most sensitive cell line was Sf9 which even in concentration of 10^{-8} the growth was less than 50% of control. On the other hand the most resistant cell line was L929 which in all concentrations of azadirachtin the inhibitory effect was less than 50% of control.

2.3.12. Comparison of the effect of adding azadirachtin immediately or 24 h after seeding of cells.

To determine the probable interaction of seeding process (such as trypsination and detachment of cells) with inhibitory effect of pesticide on growth of cell lines, the adding of azadirachtin on wells of cultured cells was carried out 24 h after seeding of cells into multiwell plates. Other procedures of cytotoxicity assay were same as previous experiments.

The effect of azadirachtin on proliferation of cell lines 24 hours after incubation of cells

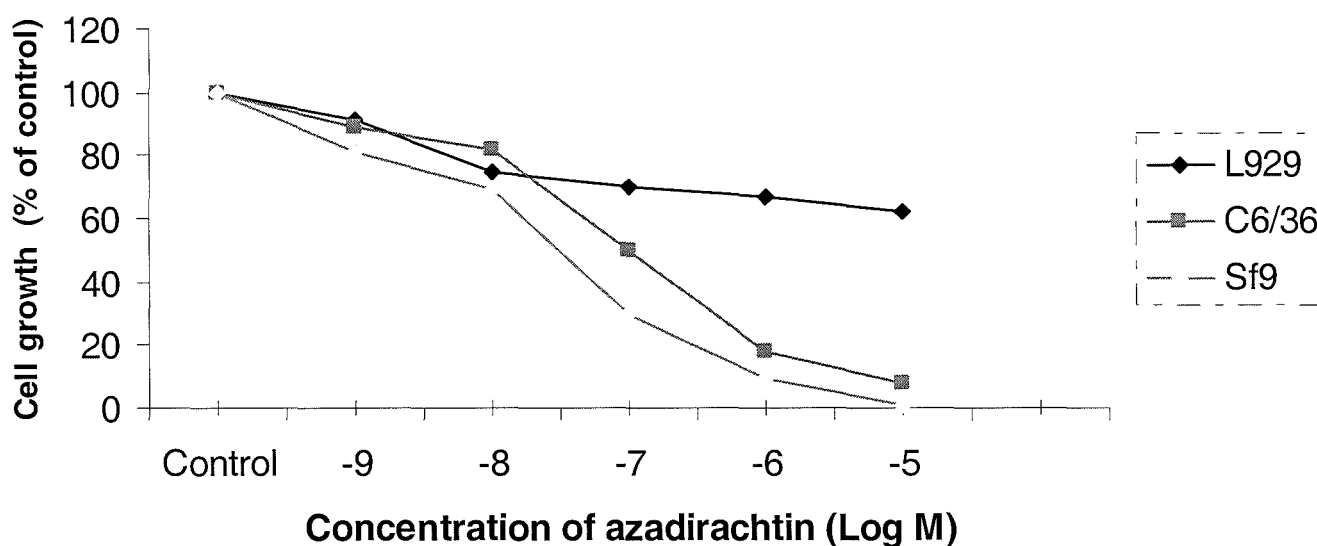


Figure 2.17. The effect of delay on adding azadirachtin to cultured cells, on cell proliferation. (5 days incubation) Each point is the mean of 8 estimates. Pesticide was added at 24 h of seeding.

When there was a 24 h delay in adding azadirachtin to cell culture, there were slight changes in growth of cells. As the results shows (Fig. 2.17) the sensitivity of L929 and C6/36 cells has not changed dramatically. However in case of Sf9 cells, the effect of delay on adding azadirachtin is different and in this case the effect of pesticide was

lower than previous tests (19%, 41%, 70%, 91% and 99%, in comparison to 35%, 55.5% , 81% 96.5% and 99% for concentrations 10^{-9} to 10^{-5} M respectively) .

2.3.13. The effect of azadirachtin on proliferation of Bge cell line

When Bge cells were incubated with azadirachtin, the highest concentration used (10^{-5}) caused a decrease in growth of only 47% (Fig 2.18)

The effect of azadirachtin on proliferation of Bge cell lines

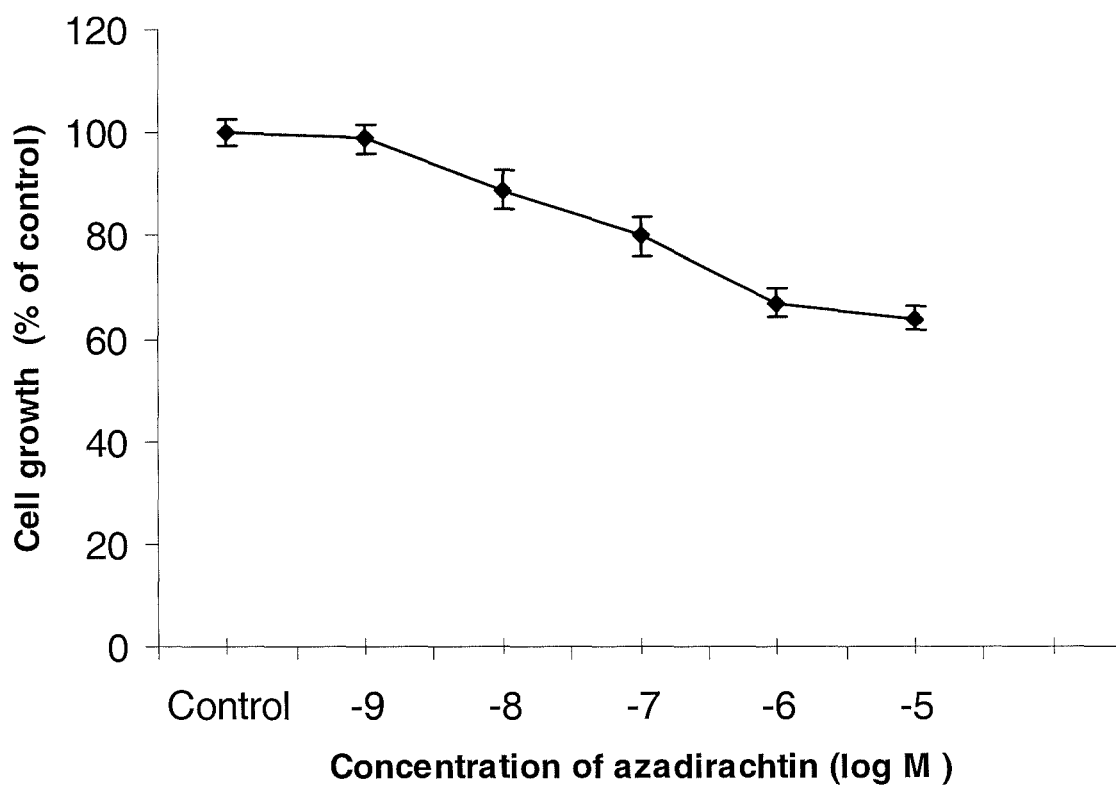


Fig.2.18. The effect of azadirachtin on proliferation of Bge cells after 14 days incubation. Each point is the mean of 3-4 estimates and vertical lines represent \pm SD.

2.3.14. The effect of salannin on proliferation of cell lines

Despite the similarity of Sf9 and C6/36 cell lines in their low sensitivity to salannin (Fig 2.19, 2.21), L929 cells are even more resistant (Fig.2.21). The inhibitory effect of salannin on the proliferation of these cells was many order of magnitude less that of azadirachtin.

The effect of salannin on proliferation of Sf9 cell line

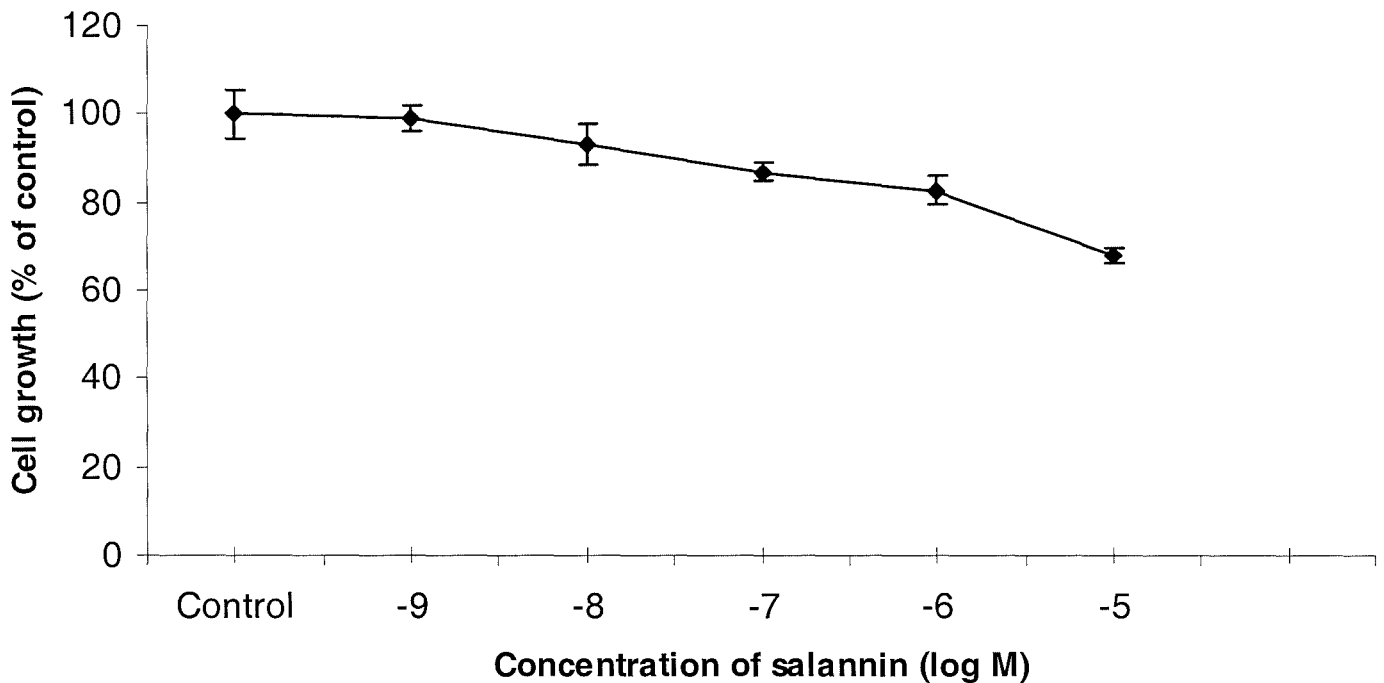


Figure 2.19. The effect of salannin on proliferation of Sf9 cells after 5 days incubation. Each point is the mean of 16 estimates and vertical lines represent \pm SD.

The effect of salannin on proliferation of C6/36 cell line

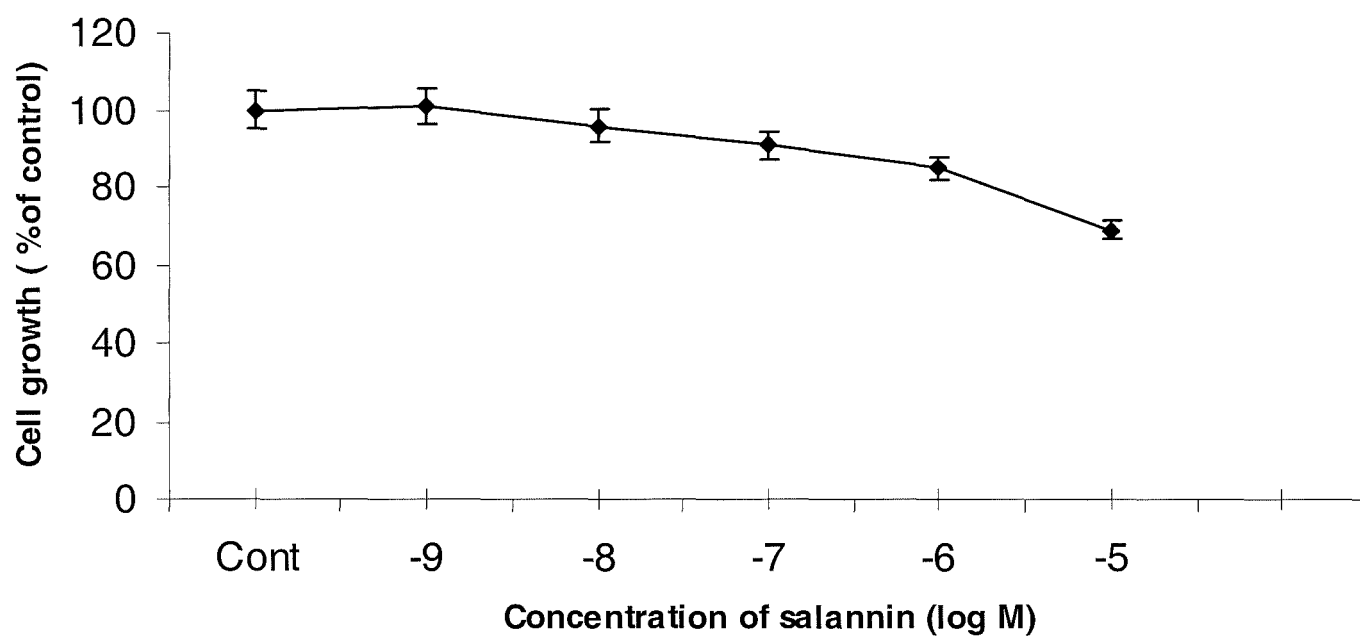


Figure 2.20. The effect of salannin on proliferation of C6/36 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of salannin on proliferation of L929 cell line

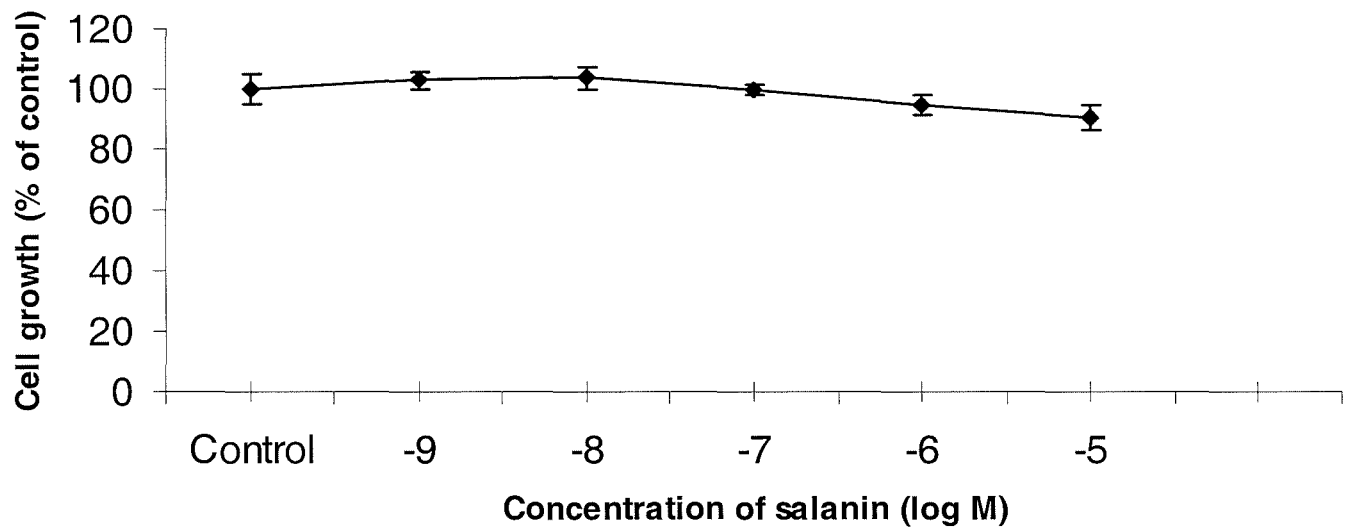


Figure 2.21. The effect of salannin on proliferation of L929 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

2.3.15. The effect of nimbin on proliferation of cell lines

The effect of nimbin on the growth of cultured cell is different from the two other terpenoids. This terpenoid in concentration of 10^{-8} M can significantly ($P < 0.001$) enhance the proliferation of L929 and C6/36 cell lines (Fig. 2.22-2.24)

The effect of nimbin on proliferation of Sf9 cell line

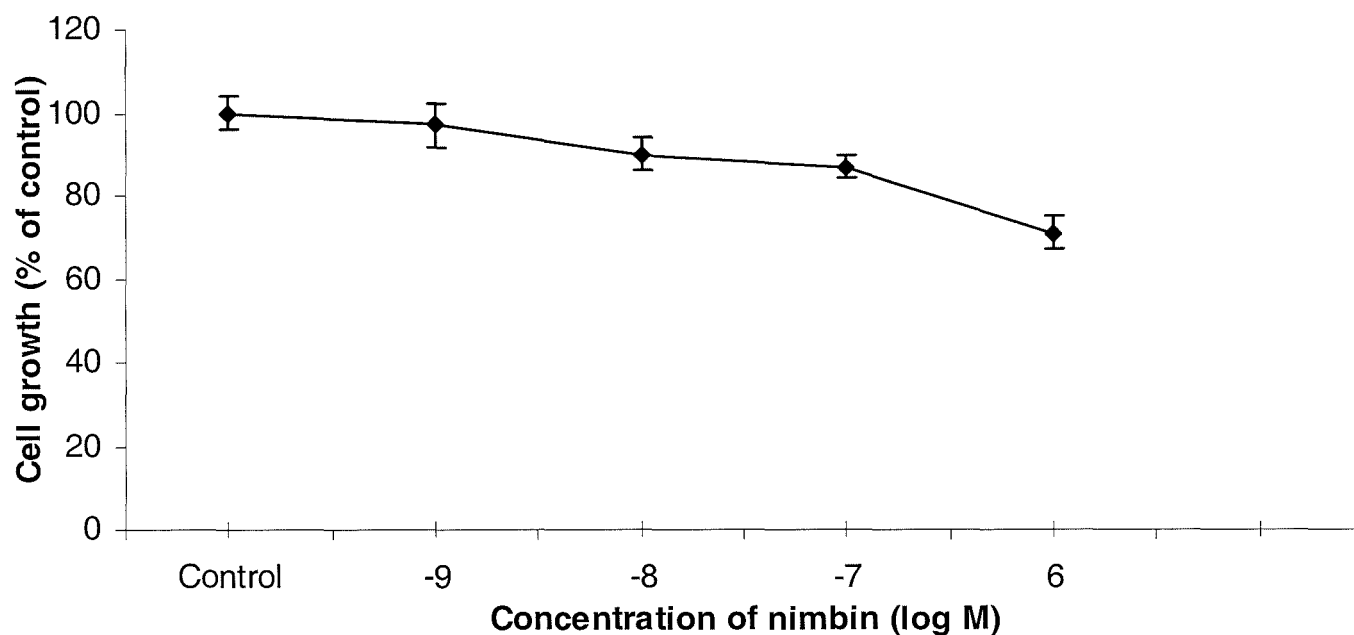


Figure 2.22. The effect of nimbin on proliferation of Sf9 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of nimbin on proliferation of C6/36 cell line

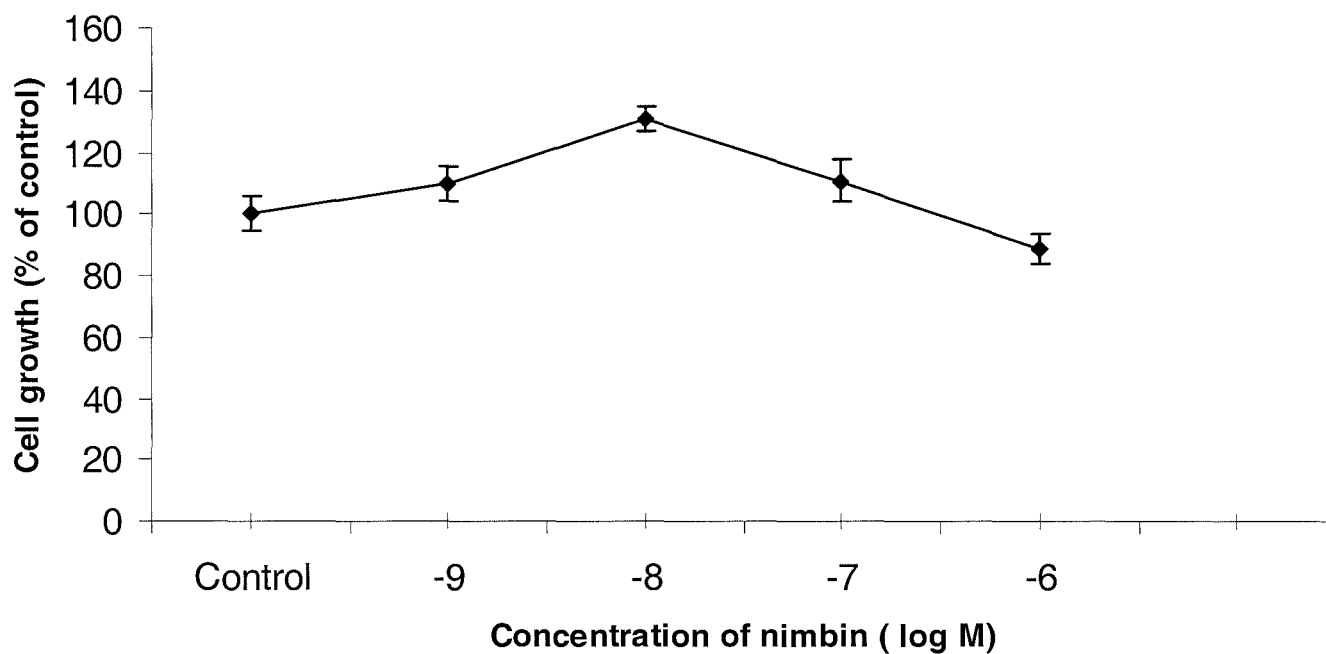


Figure 2.23. The effect of nimbin on proliferation of C6/36 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of nimbin on proliferation of L929 cell line

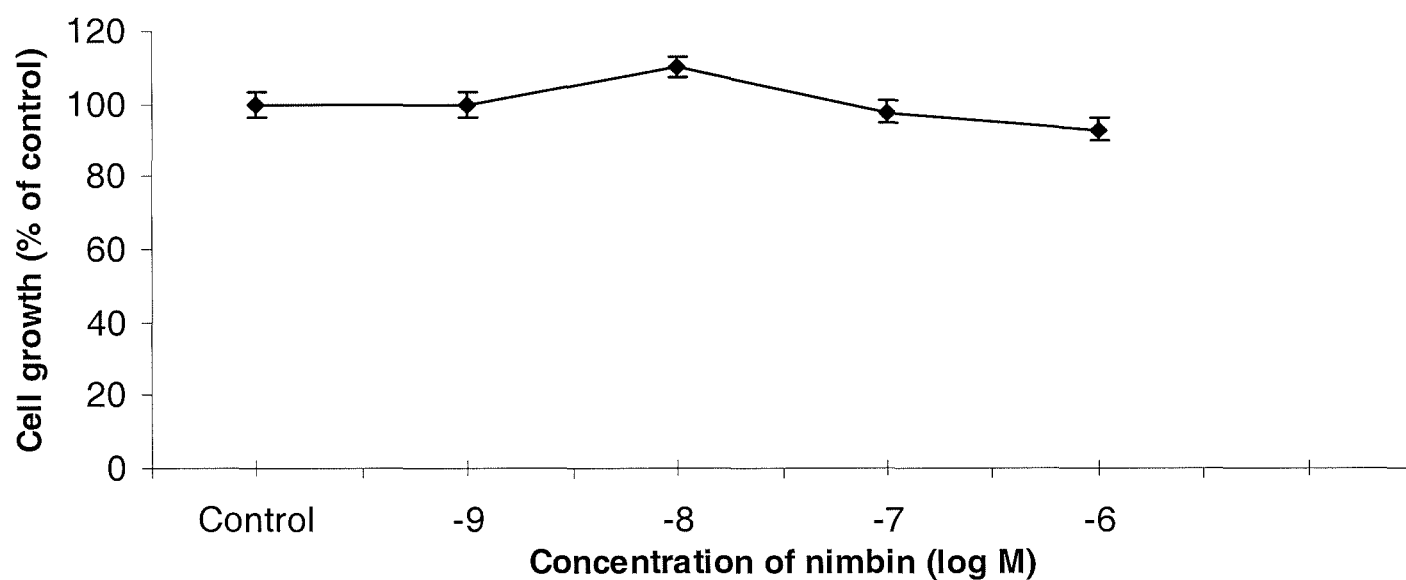


Figure 2.24. The effect of nimbin on proliferation of L929 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

2.3.16. The effect of rotenone on proliferation of cell lines

In all cell cultures, adding rotenone to the medium caused severe reduction in growth rate but only in concentrations of 10^{-7} or greater were the effects statistically significant ($P < 0.001$). Rotenone in concentrations more than 10^{-7} caused all growth of Sf9 and C6/36 to cease, whereas in the case of L929 the cells were more resistant and at a concentration of 10^{-6} M, the inhibitory factor was about 60% (Fig. 2.25-2.27)

The effect of rotenone on proliferation of Sf9 cell line

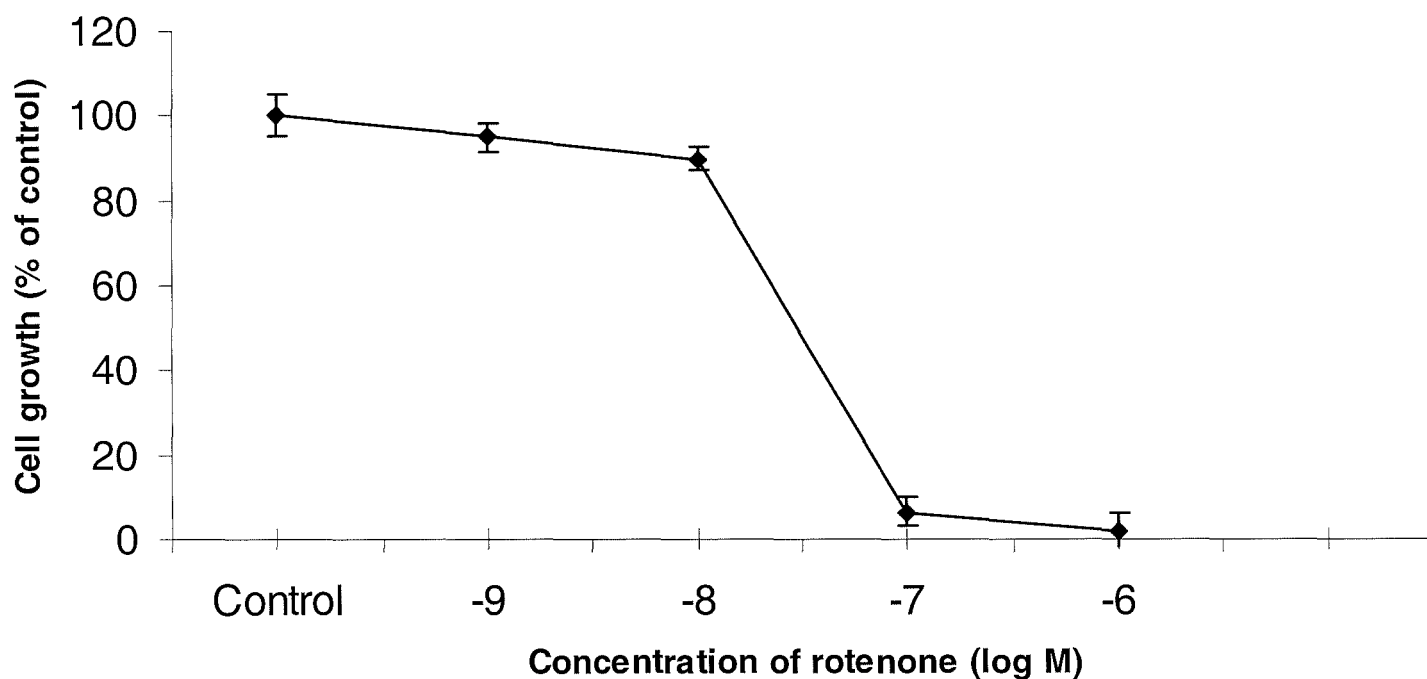


Figure 2.25. The effect of rotenone on proliferation of Sf9 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of rotenone on proliferation of C6/36 cell line

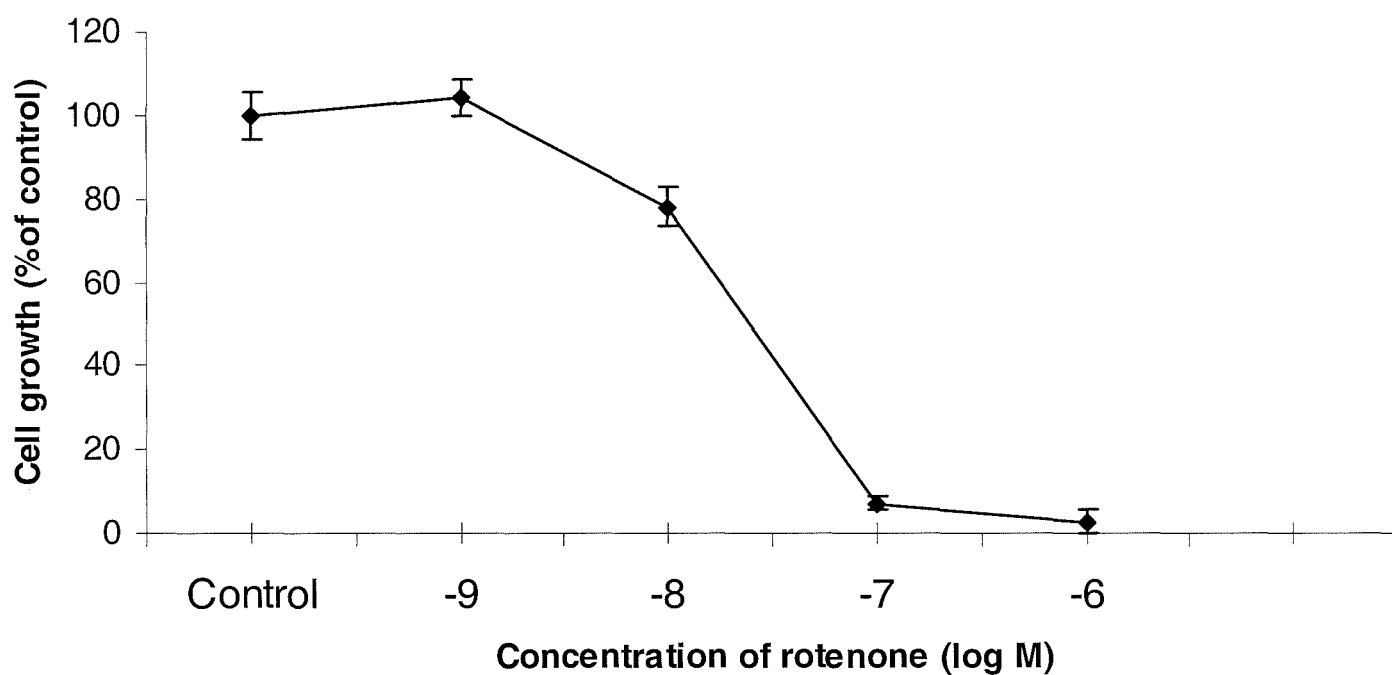


Figure 2.26. The effect of rotenone on proliferation of C6/36 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of rotenone on proliferation of L929 cell line

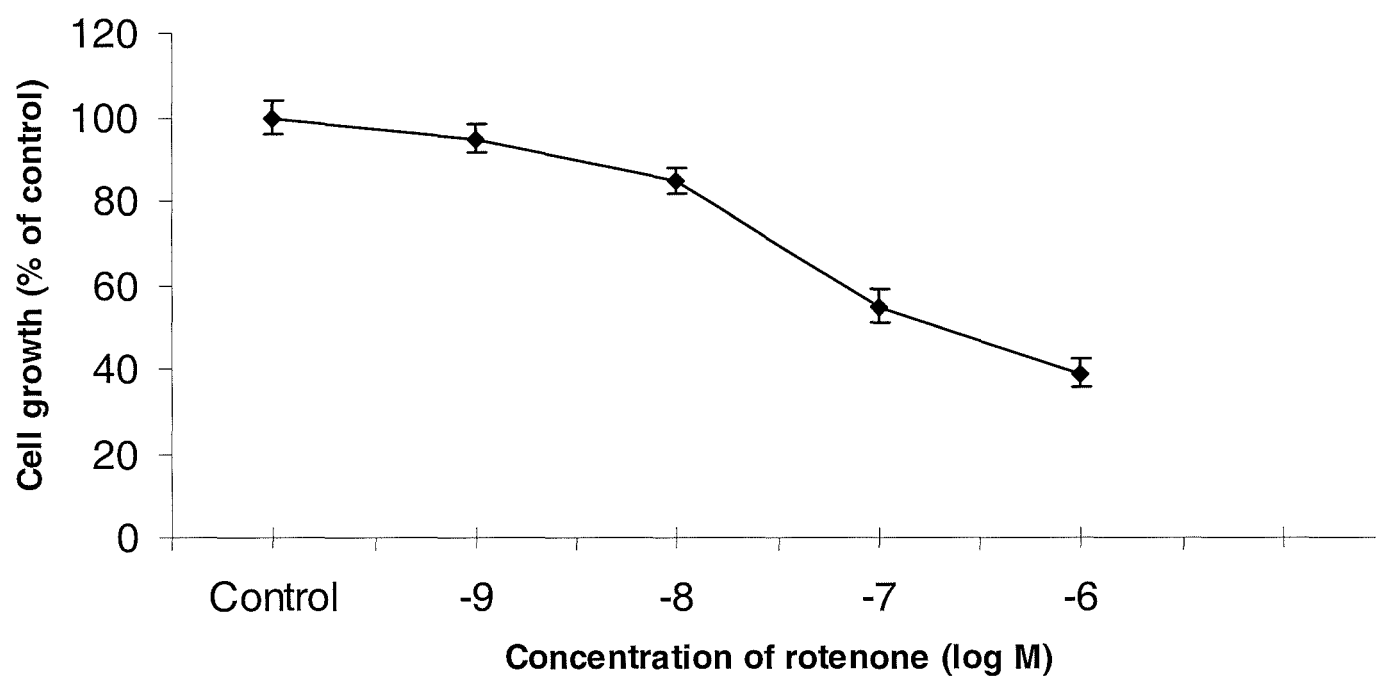


Figure 2.27. The effect of rotenone on proliferation of L929 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

2.3.17. The effect of nicotine on proliferation of cell lines

Nicotine in concentrations less than 10^{-6} M did not affect growth of L929, C6/36 or Sf9 cells and in concentration 10^{-5} M the only cell line which showed significant reduction in growth was the Sf9. Also nicotine in concentrations 10^{-9} to 10^{-7} M showed a weak stimulatory effect on the growth of Sf9 and C6/36 cell lines (Fig. 2.28-2.30)

The effect of nicotine on proliferation of Sf9 cell line

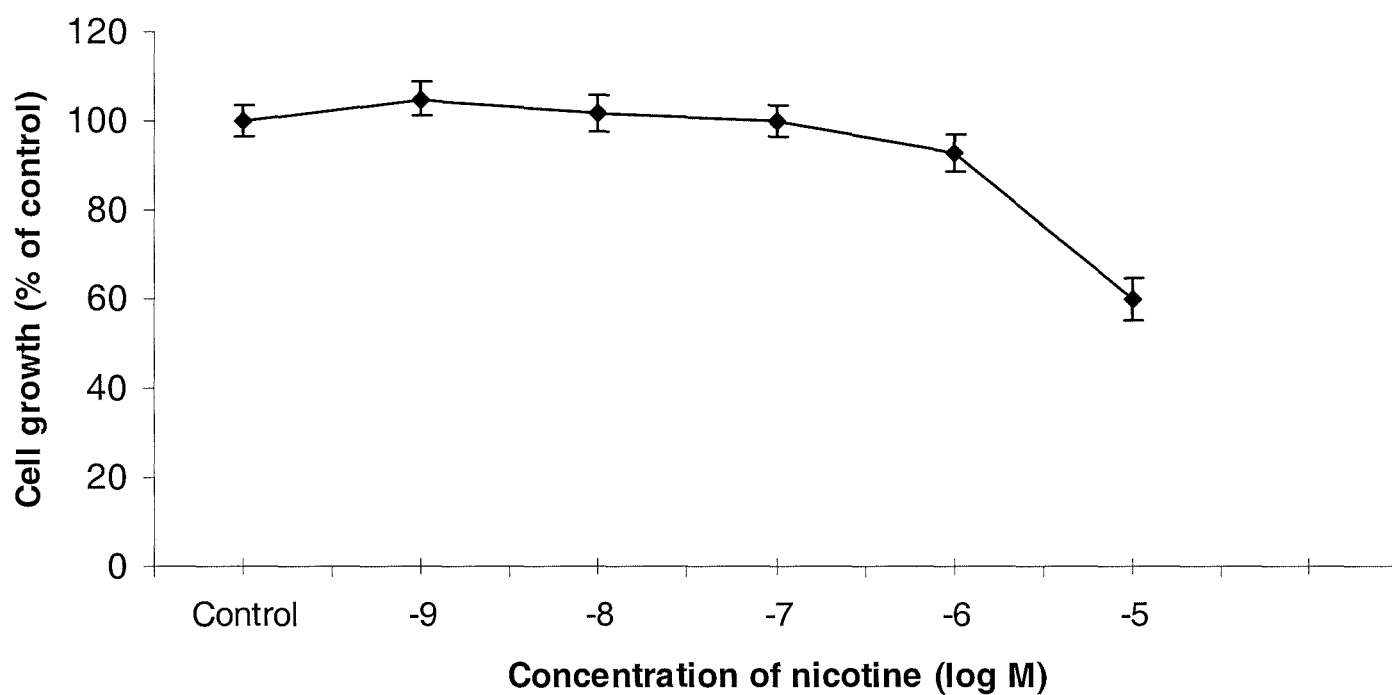


Figure 2.28. The effect of nicotine on proliferation of Sf9 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of nicotine on proliferation of C6/36 cell line

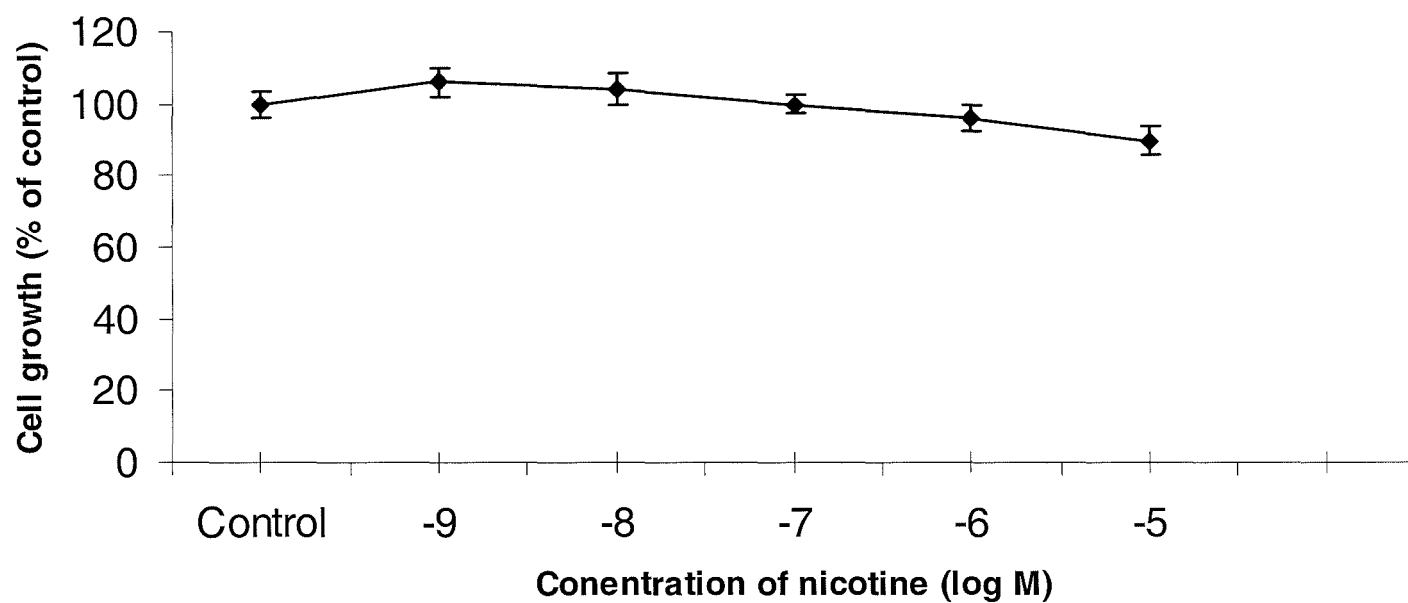


Figure 2.29. The effect of nicotine on proliferation of C6/36 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of nicotine on proliferation of L929 cell line

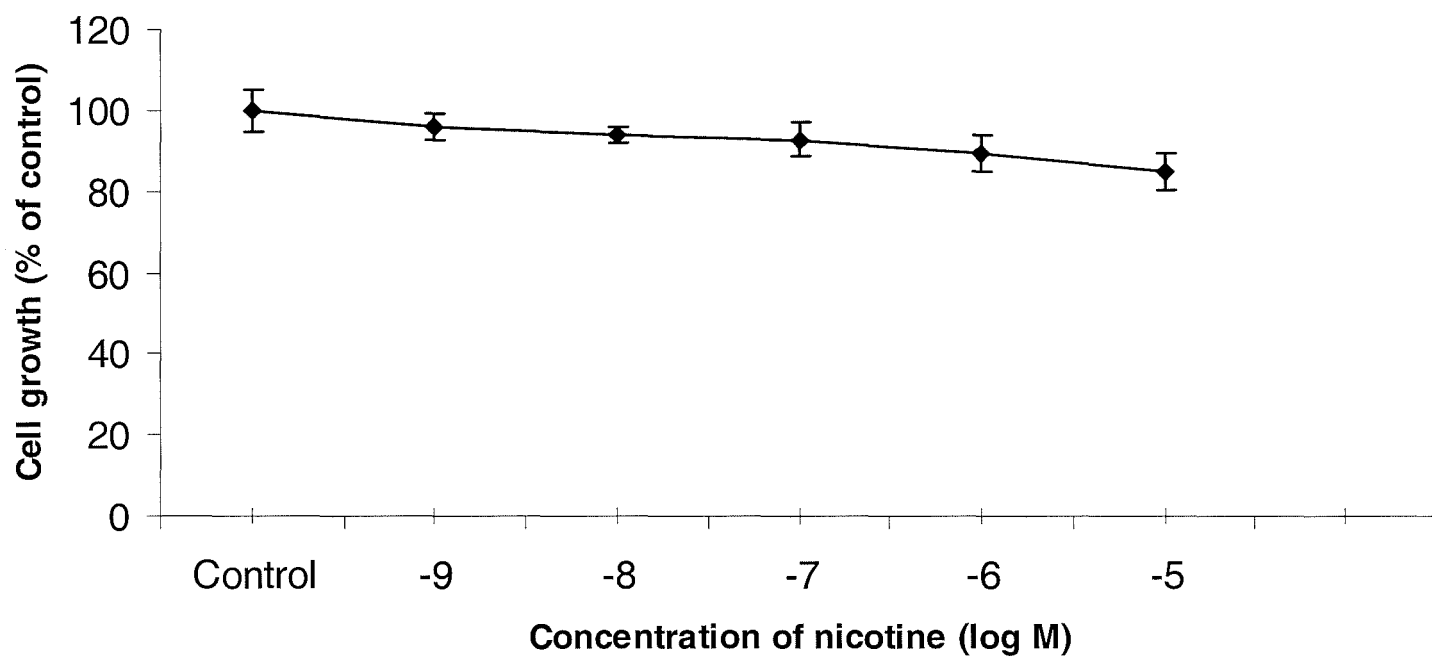


Figure 2.30. The effect of nicotine on proliferation of L929 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

2.3.18. The effect of pyrethrum on proliferation of cell lines

Adding pyrethrum to the cell culture medium did not cause significant changes in the growth of L929 cell line. The only cells that showed reduction in growth was Sf9 which at the highest concentration of pyrethrum (10^{-6} M) there was 20% reduction in cell number after 5 days incubation. The C6/36 cell line showed an increase in growth in concentrations of 10^{-7} – 10^{-10} M. The highest increase of growth was at a concentration of 10^{-8} M which was not highly significant ($P < 0.05$), (Fig.2.31-2.33).

The effect of pyrethrum on proliferation of Sf9 cell line

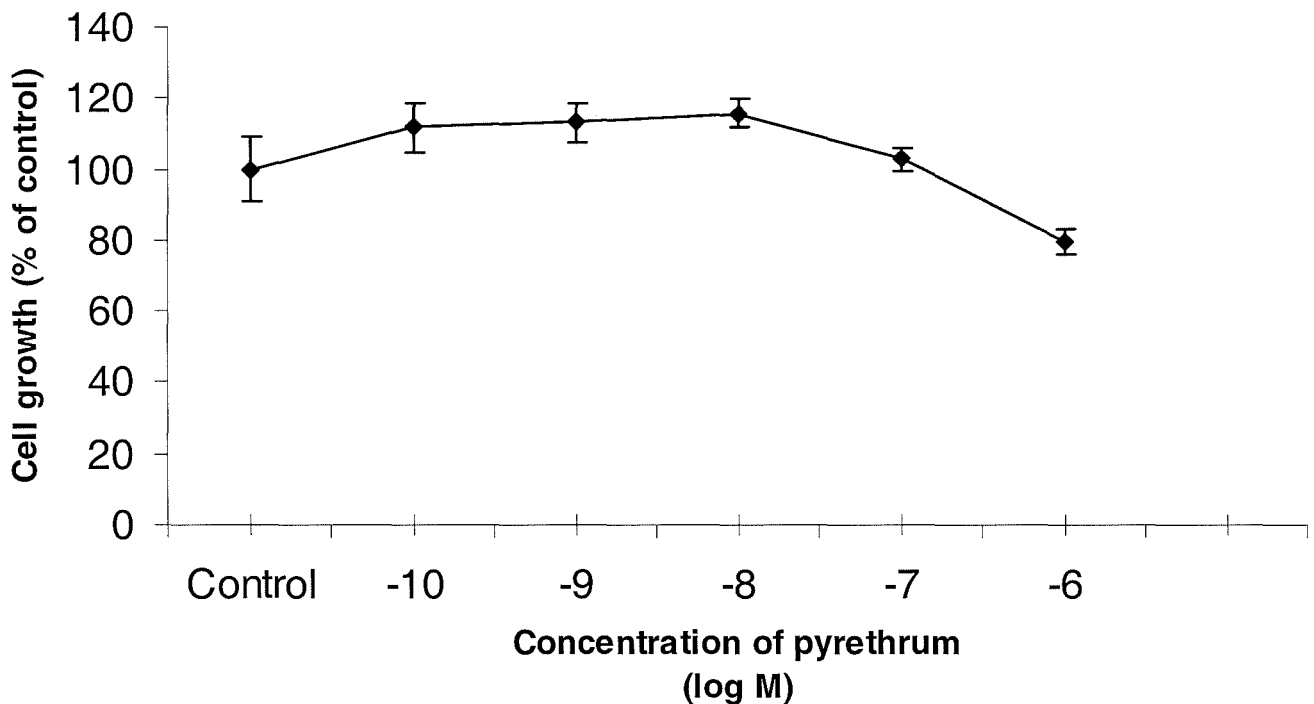


Figure 2.31. The effect of pyrethrum on proliferation of Sf9 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

The effect of pyrethrum on proliferation of C6/36 cell line

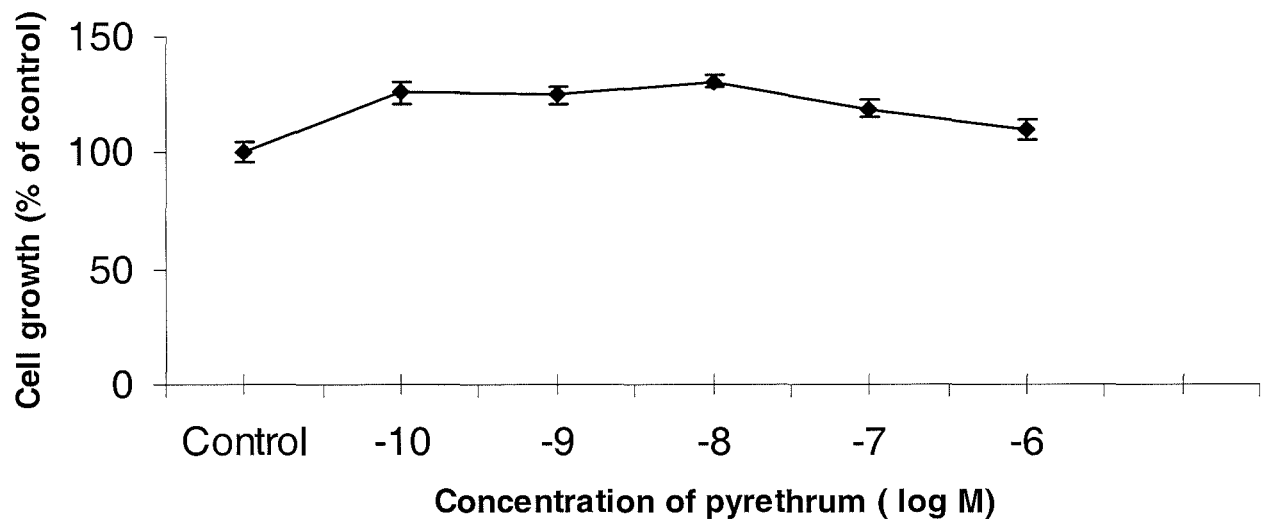


Figure 2.32. The effect of pyrethrum on proliferation of C6/36 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

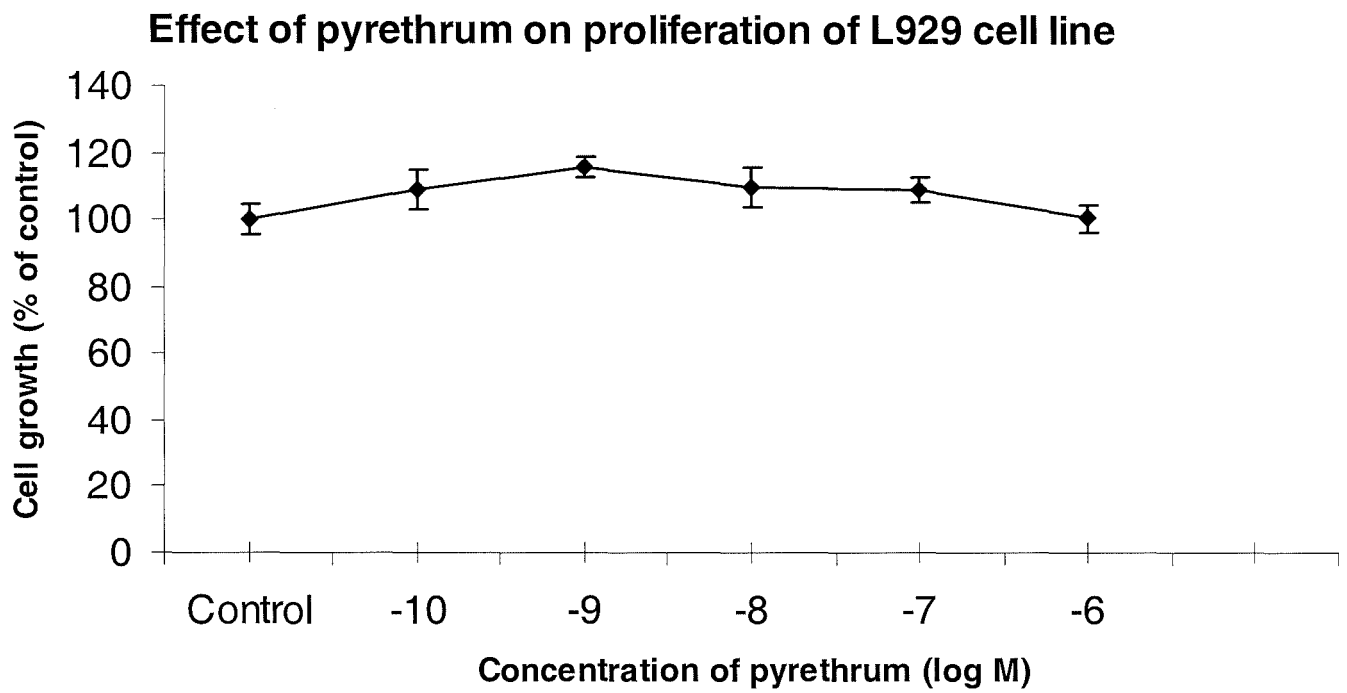


Figure 2.33. The effect of nicotine on proliferation of L929 cells after 5 days incubation. Each point is the mean of 8-16 estimates and vertical lines represent \pm SD.

2.4. Discussion

In vitro cellular models have proved to be extremely useful in some areas of toxicological research. The mechanism of action of a toxic chemical and its metabolism can be studied in *in vitro* systems under strictly controlled conditions (Jover *et al.*, 1994).

Despite numerous studies which have been done on pesticides, there are only a few examples in the literature of studies in which the effect of pesticide on proliferation of cells is compared. Our experiments showed that azadirachtin is toxic to insect cells (Sf9 and C6/36) even in very low concentrations. This is in agreement with results of Rembold (1998) and Jabbar *et al.* , (1998). Also the results of these experiments demonstrated that the mammalian cell line L929, was relatively resistant and only in high concentration can azadirachtin affect proliferation of these cells. In their work, Reed *et al.* (1998) reported that there was no effect on mammalian cells.

On the other hand Akuduga *et al.* (2001) has reported azadirachtin effectively reduced cell survival of glioblastoma cell line. The difference between cytotoxicity of azadirachtin on different mammalian cells perhaps arises partly from difference in cell lines and also the methods that researchers have used. For example, the range of concentration which Akudugu *et al* have used is between $1-6 \times 10^{-5}$ M and they have used different cell lines to those of Reed *et al.* Paraganama *et al.* (1993) have shown that some cell such as nervous tissues shows more affinity to azadirachtin, which suggests selectivity of azadirachtin in affecting (binding) different tissue.

Such differences in sensitivity of cells also can be seen in cell lines derived from insects. For example in present study there was difference in toxicity of azadirachtin in Sf9 and C6/36 cell line.

The present study revealed that the snail cell line, Bge, was not significantly affected by azadirachtin, whereas Singh *et al.* (1996) found that pure azadirachtin was even more toxic to snails than synthetic molluscicides. This difference may be explained by difference in genus of snails, effect of azadirachtin on special tissue in snail or difference in concentration of used pesticides. Because in their experiment Singnh., *et al* have used the Genus *Lymaea* whereas Bge cell line has derived from Genus *Biomphalaria* and concentration of azadirachtin was much more higher than present experiment (almost 70 times).

Difference in sensitivity of animals also can be seen in the case of other properties of azadirachtin. For example in class Insecta; *Orthoptera* are more sensitive to antifeedant effect of azadirachtin than *Isoptera* or *Coleoptera* (see Chapter 1)

The combination of results of this study with previous studies suggests that there is a wide range of diversity in sensitivity of different cell lines to azadirachtin. The most obvious point is that the cells, which have derived from closer source show more similarity. Therefore the most resistant cells seems to be among mammalian cells and the least sensitive cells are cells derived from insects.

The toxicity of salannin and nimbin are lower than azadirachtin and in concentration of 10^{-8} M of nimbin there was even some stimulatory effect on growth of cells. This property also was seen in case of pyrethrum. Because of widespread uses of pyrethroid and presence of salannin and nimbin in neem based pesticides evaluation of this property in further experiments is important. On the other hand, the experiments with neurotoxic phytochemicals (Pyrethrum and nicotine), and rotenone (which does not affect nervous system specifically), showed that cytotoxicity assays which depends on growth of cells may have some limitation. In this case, the toxicity of pesticides which specifically affect nervous system are much more significant in whole animal than cultured cell. Indeed using this method is more likely to show the side effects of neurotoxic pesticides rather than their main toxic property upon target organism (i.e. insect). This can be conclude from cytotoxicity assay of nicotine and pyrethrum which despite their well-known toxicity on insects (and in case of nicotine also mammals), they showed little if any inhibitory effect on growth of culture cells.

Although rotenone is toxic for both insect and mammalian cell lines, it should be considered that in Sf9 cell line, this chemical in its highest used concentration reduced the activity of dehydrogenase by only 15%. However, rotenone did not cause significant immediate effect on reduction of MTT by dehydrogenase enzymes of C6/36 and L929 cell lines. Moreover Berridge. (1996) demonstrated that cellular reduction of MTT was more related to glycolytic rate and thus to NADH and NADPH produced in cytoplasm than to respiration which was in agreement with our experiment with cell fractions (see Chapter 3).

The effect of pesticides on growth of cells may look variable or even sometimes controversial. For example some organophosphorus compound such as diazinone has

been shown to enhance the growth of intestinal epithelial cells (Greenman *et al.*, 1997), whereas other organophosphorus insecticides (i.e. parathion and paraoxon reduce the DNA synthesis in mouse semiferous tubules (Rodriguez *et al.*, 2000).

Also in the case of organochlorine pesticides, Kannan *et al.* (2000) have shown that endosulfan lowered cell viability and growth in a dose and time dependant manner, but Soto *et al.* (1994) demonstrated that endosulphan had estrogenic effects on human cultured cells. In some investigations nicotine has suppressed DNA synthesis (Opanashuk *et al.*, 1991) but in other investigations this alkaloid has shown a stimulatory effect on cell growth.

In our studies we found that salannin is not as toxic as azadirachtin whereas Cohen *et al.* (1996) reported that salannin was more toxic.

It seems that most of this diversity is due to differences in factors which affect the cytotoxicity test . These include differences in cell line clones, culture conditions, and serum lots. Also it looks like the dose of pesticide is very important in quality of effect. For example, present experiments showed that there was dramatic difference in cytotoxicity of concentration of 10^{-8} and 10^{-7} M of rotenone on insect cells.

Our experiments showed that nicotine in lower concentrations slightly stimulate Sf9 and C6/36 cell growth. However its stimulatory effect was not as high as the results which Villablanca has reported (Villablanca, 1999) and was not seen in L929 cells. He has shown that the response of endothelial cells is bimodal. In lower concentrations ($< 10^{-8}$) it stimulates DNA synthesis, but in higher concentrations (10^{-6}) nicotine exhibits cytotoxic effect. Villablanca has demonstrated that the stimulatory effect of nicotine is enhanced by serum and blocked by nicotinic-receptor antagonist hexamethonium.

Chapter 3

The effect of azadirachtin and rotenone on oxygen consumption by mammalian and insect cultured cells

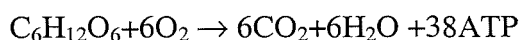
3.1. Introduction

The inhibitory effect of azadirachtin and rotenone on the growth of cells reported in Chapter 2 shows that both azadirachtin and rotenone reduce the growth of insect cultured cells in a dose and time-dependent manner. Although azadirachtin, even in high concentration, only slightly reduced the growth of mammalian cell line L929, both mammalian and insect cells were quite sensitive to the effect of rotenone.

Interference with the respiration process and an effect on cell division (see Chapter 4) are the ways which rotenone affects cultured cells. Because of effect of rotenone on the respiratory chain, it interferes with energy metabolism which eventually causes reduction in growth of cultured cells. Also effect of rotenone on polymerization of tubulin causes arrest of cell cycle which decreases cell growth. In Chapter 3 the effect of rotenone on total oxygen consumption of cultured cells has been compared to that of azadirachtin. Then, using rat liver mitochondria, the effect of these phytochemicals on mitochondrial respiration has been studied.

3.1.1. Respiration

All organisms need energy for their survival. Indeed, nothing in cells happens without the support of their respiration, from the division that brings them into existence through the maintenance of their maturity and their decline into senescence (James., 1971). In higher organisms, energy metabolism depends on respiration, which takes place through oxidation of organic molecules. Organic food-stuffs contain carbohydrates, fats or proteins, and these are first broken down into their constituent monomers which are, respectively, sugars, acetyl groups and amino acids. The carbon skeletons of the sugar monomers are subsequently oxidised through glycolysis and /or the tricarboxylic (or Krebs) cycle in which the two-carbon units of acetyl CoA are degraded to CO₂, which can be summarised as following equation:



The amount of yielding energy produced depends on the tissue and the kind of reactions (Delvin, 1997). Although the overall energy yield is 38 ATP (4 ATP, 10

NADH and 2 FADH₂), due to consumption some of ATP in the respiratory reactions such as transporting pyruvate, usually the actual acquired ATP is lower.

3.1.1.1. Glycolysis

Glycolysis can occur in either the absence or presence of oxygen and is a universal energy pathway. During glycolysis, glucose is broken down to pyruvic acid, yielding 2 ATP and 2 NADH⁺. The end of glycolysis or substrate level under anaerobic conditions can be lactic acid derived from pyruvic acid (Bryant, 1971). Glycolysis occurs in the cytoplasm of cells, not in organelles, and occurs in all kinds of living organisms. Some cells of eukaryotic organisms such as red blood cells and those of the retina lack mitochondria and use glycolysis as their only mechanism for ATP production (Delvin., 1997). Prokaryotic cells use glycolysis and the first living cells most likely used glycolysis.

3.1.1.2. Fermentation

During fermentation, which occurs in the absence of oxygen, the pyruvic acid from glycolysis is converted to either ethanol (alcoholic fermentation, occurring for instance in yeast), or lactate (e.g. in mammals), (Van Dijken *et al.*, 2002). This continued utilization of pyruvic acid during fermentation permits glycolysis to continue with its associated production of ATP (Albert *et al.*, 1983).

3.1.1.3. Formation of acetyl CoA

Most organisms live under aerobic conditions surrounded by an environment, which contains oxygen. Under this condition, the pyruvate, which has been produced in the cytoplasm is further metabolised to acetyl CoA. The conversion of pyruvate to acetyl CoA takes place in mitochondria through a oxidative decarboxylation and then the bond energy is completely metabolized to H₂O and CO₂ (Corbett *et al.*, 1984).

3.1.1.4. Mitochondria and oxidative phosphorylation

Mitochondria (Fig. 3.1) are the main power centre of energy production of animal and plants. These are organelles in animal and plant cells in which oxidative phosphorylation takes place. There are many mitochondria in animal tissues (2000 to 3000 in some of cells); for example, in heart and skeletal muscle, which require large

amounts of energy for mechanical work; in the pancreas where there is biosynthesis; and in the kidney where the process of excretion begins. Mitochondria have an outer membrane, which allows the passage of most small molecules and ions because of many copies of transmembrane protein (porin), and a highly folded inner membrane (cristae), which does not even allow the passage of small ions and so maintains almost a closed space within the cell. The outer membrane of bacteria, like that of mitochondria, is permeable to most small metabolites. The electron-transferring molecules of the respiratory chain and the enzymes responsible for ATP synthesis are located in and on this inner membrane, while the space inside (matrix) contains the enzymes of the TCA cycle. A large family of transporters shuttles metabolites such as ATP and citrate across the inner mitochondrial membrane. The enzyme systems primarily responsible for the release and subsequent oxidation of reducing equivalents are thus closely related so that the reduced coenzymes formed during catabolism (NADH and FADH) are available as substrates for respiration (Stryer, L, 1995) .

Mitochondria have their own DNA. Mutation in mitochondrial DNA has been found to be causes of a wide variety of neuromuscular and other disorders such as cardiomyopathy, deafness, diabetes, and epilepsy. Unlike most inherited conditions, mutations of mitochondrial DNA are inherited solely from maternal DNA (Schon, 2000).

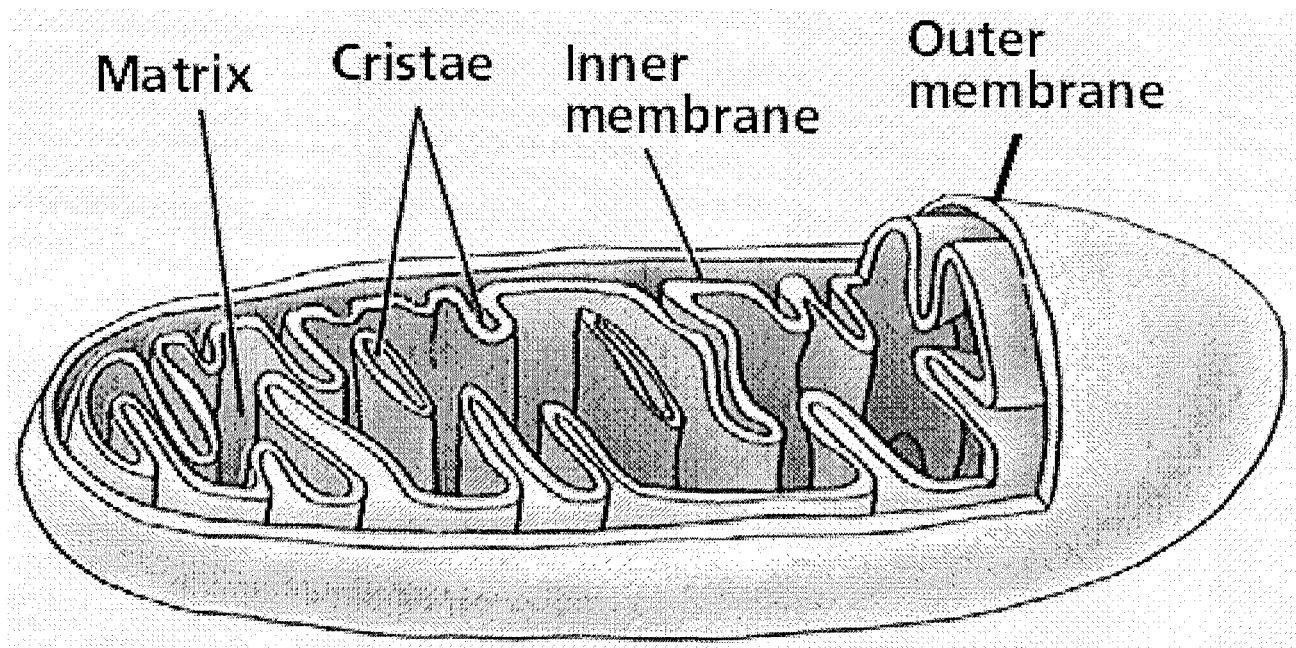


Figure 3.1 A mitochondrion

The figure was taken from: <http://ntri.tamuk.edu/cell/mitochondria4.gif>

3.1.1.5. Electron transport chain

The electron carriers that participate in the flow of electron to O_2 are a structurally diverse group. Occupying a central position are a series of heme-containing proteins, the cytochromes.

Haemes are porphyrins with iron at the centre (Fig. 3.2). Four types of haeme are known in eukaryotes (Atamna *et al.*, 2002). Also three main types of cytochromes, a, b, and c, which were first discovered by their distinctive absorption spectra (Albert *et al.*, 1983) and are structurally distinguished by different subunits on the periphery of the porphyrin ring and different mode of attachment of the porphyrin to protein. Cytochrome c is a small, water-soluble protein associated loosely with the inner mitochondrial membrane. Another cytochrome (c_1), two b cytochromes (b_L and b_H), and two a cytochromes (a and a_3) are embedded in the membrane as parts of large complexes. The Fe atoms of the cytochromes undergo oxidation and reduction during respiration, cycling between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states (Berg

et al., 2002). The absorption spectra of the oxidized and reduced forms differ. This property can be used to measure the oxidation-reduction states of cytochromes in living cells. Under anaerobic conditions the cytochromes rapidly become reduced: in the presence of O₂ they become oxidized. Certain molecules that inhibit respiration (CO, N₃⁻, or CN⁻) block the oxidation ; other inhibitors (amytal, rotenone, and malonate) block the reduction.

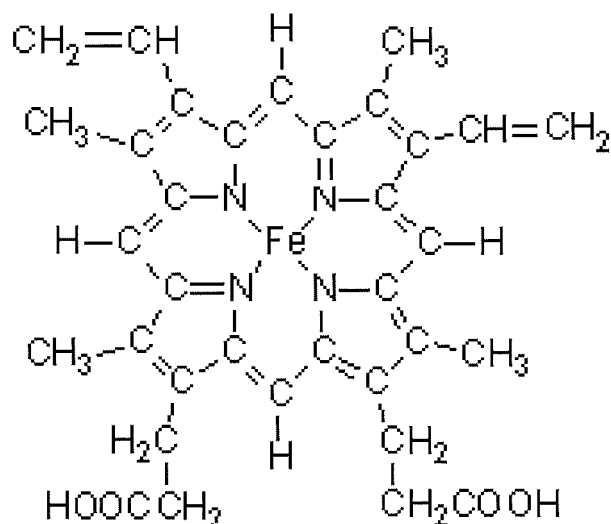


Figure 3.2 Structure of heme

Three other types of electron carriers that participate in the electron-transport chain are flavoproteins, iron-sulfur proteins, and ubiquinone.

The concentration of UQ in the mitochondrial inner membrane far exceeds that of the cytochromes. In heart mitochondria, for example, the concentration of UQ is about seven times that of cytochrome a_3 . Because of its hydrophobic character, the UQ is able to move freely in the phospholipid bilayer of membrane.

The reduction of UQ can be measured by the disappearance of an absorption band at 275 nm. By using this technique, it was shown that adding a substrate such as succinate caused a rapid reduction of essentially all the UQ present in the inner

membrane; the resulting UQH₂ could be reoxidized by the cytochrome system in the presence of O₂. To determine whether UQ is a necessary participant in electron transport from succinate to O₂, the quinone was removed from mitochondria by selective extraction with an organic solvent. The depleted mitochondria were incapable of respiration but recovered this activity when UQ was added back (Zubay, 1998).

3.1.1.6. The translocation of protons in mitochondria

The proton translocation between matrix and intermembrane space of mitochondria takes place with help of electron transport complexes, which are residing in the inner mitochondrial membrane.

These complexes are: Complexes I (NADH dehydrogenase), Complexes II (succinate dehydrogenase), Complexes III (cytochrome bc₁ complex), complexes IV cytochrome oxidase, and complexes V (F₁F₀-ATP synthase) (Schagger., 2001; Van Coster *et al.*, 2001).

Complex I contains noncovalent bound FMN and several iron sulfur clusters as prosthetic groups. It is a multimetric enzyme composed of at least 43 different subunits, 7 encoded by the mitochondrial DNA and the others by the nuclear genome (Velazquez *et al.*, 2001). The main function of these electron transport complexes is to create a proton gradient across the inner membrane of mitochondria (Fig. 3.3).

Complexes I and II transfer electrons from NADH and succinate to UQ. Complexes III transfer electrons from UQH₂ to cytochrome c, Complex IV transfers electrons from cytochrome c to O₂, and complexes V, which is composed of two main sectors a catalytic core (F₁) and a membrane proton channel (F₀) is the terminal enzyme of respiratory chain and responsible for ATP synthesis from ADP and inorganic phosphate (Kim *et al.*, 2000). This complex works as a tiny rotary machine to couple proton gradient generated by respiratory chain to ATP synthesis. As protons pass through a cylindrical rotor (F₀) portion of the complex embedded in the inner membrane, ADP is converted to ATP in the spherical stator (F₁ portion) protruding into the matrix (Schon., 2000).

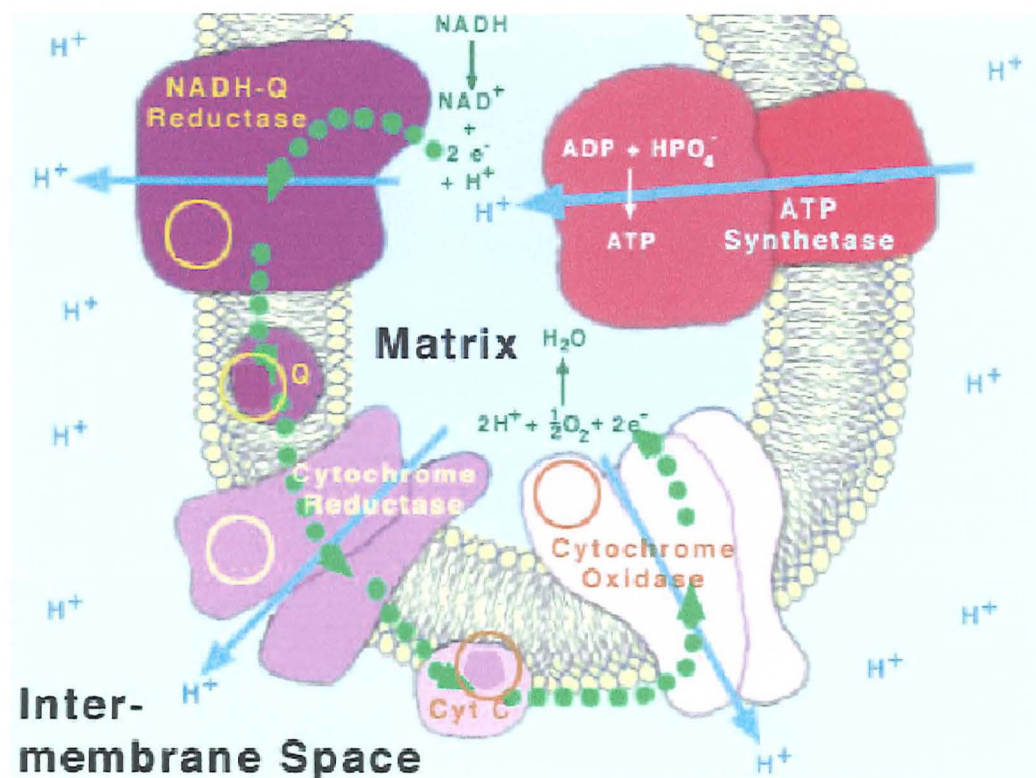


Figure 3.3 The electron transport complexes in the inner mitochondrial membrane.

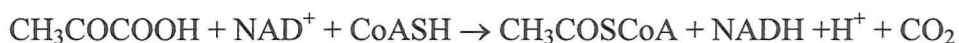
The figure was taken from:

<http://wunmr.wustl.edu/EduDev/LabTutorials/Cytochromes/cytochromes.html>

3.1.2. Pesticides and respiration

3.1.2.1. Effect of toxins on conversion of pyruvate to acetyl CoA

The conversion of pyruvate to acetyl CoA is accomplished by pyruvate dehydrogenase complex. The complex is huge with a molecular mass of over 7 million daltons in mammals. The overall reaction of this conversion is:



There are two known commercial pesticides which interact with these reactions; Copper compounds, which are used as antifungal agents and arsenic containing pesticides (e.g. arsenous oxide (rodenticide), Paris green (insecticide), Sodium arsenite (insecticide and herbicide). Although their main effect is on pyruvate

dehydrogenase (Petrick., *et al* 2001), it is likely that α -oxoglutarate dehydrogenase is also affected (Simpson *et al.*, 1979).

The arsenic-containing pesticides have been in use for many years.

Some of these compounds produce arsenite (ion of arsenious acid $\text{As}(\text{OH})_3$ or AsO_3^{-3}) and some others arsenate (ions of arsenic acid $\text{O}=\text{As}(\text{OH})_3$ or AsO_4^{-3}), which are both toxic to insects and may be interconverted in living systems. Insects poisoned with arsenite show symptoms which resemble those caused by the respiratory inhibitor rotenone but are unlike those caused by neuroactive insecticides. So death is caused by progressive inactivity, and does not involve convulsions.

3.1.2.2. Action of pesticides on electron transport

A number of toxins (including some pesticides) can affect electron transport in mitochondria. Rotenone is one of well known of this group, which has been described in previous pages (see chapter 1).

Two other examples are cyanide and phosphine.

3.1.2.2.1. Cyanide

Hydrogen cyanide (HCN) and calcium cyanide $\text{Ca}(\text{CN})_2$, which decomposes to HCN in the presence of water, has been used to control insect in enclosed areas by fumigation.

Cytochrome oxidases catalyse the terminal step in the electron chain, the oxidation of reduced cytochrome c by oxygen (Petersen., 1977). Cyanide in a concentration of 10^{-8} M reversibly inhibits this reaction by 50% (Corbett *et al.*, 1984).

3.1.2.2.2. Phosphine (PH_3)

In the presence of moisture, aluminium phosphide produces PH_3 (phosphine). Although phosphine is toxic to mammals, its toxicity to insect and its volatility make it suitable for use as a fumigant for stored grain. Phosphine is a non-competitive inhibitor of cytochrome oxidase (Bolter *et al.* , 1990).

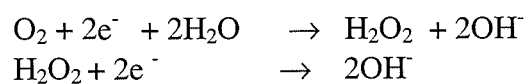
3.1.3. Measurement of respiratory chain electron transport with the oxygen electrode.

Electron transport and oxygen uptake can be measured using the oxygen electrode which continuously determines the concentration of oxygen in solution. The output of the electrode is transferred to a chart recorder. Intact coupled mitochondria, in the absence of ADP, take up oxygen only slowly with suitable substrate. Adding ADP can cause a significant increase in consumption of oxygen until all the ADP has been converted to ATP. Mitochondria, responding in this way, are said to have 'respiratory control' or to be 'coupled', since electron transport only occurs when ADP is present (the slow background oxygen consumption is due to damaged mitochondria and can be ignored).

Now if an inhibitor of electron transport is added, electrons cannot be passed down the respiratory chain to oxygen, and oxygen consumption ceases. On the other hand if an uncoupler of oxidative phosphorylation is added, the effect is to remove the respiratory control and allow maximum oxygen consumption whether or not ADP is added. Finally an inhibitor of oxidative phosphorylation (but not electron transport) will prevent oxygen uptake by coupled mitochondria, but once they are uncoupled, electron transport, and hence oxygen uptake, can proceed unimpeded (Corbett et al, 1984).

3.1.3. 1. The oxygen electrode.

The oxygen electrode (Fig 3.4) comprises a platinum anode and an silver cathode linked by KCl bridge. A teflon membrane, which is permeable to oxygen, separates the electrodes from reaction medium. With application of suitable potential difference between anode and cathode, oxygen is reduced at the cathode.



At the anode



This electrical current is directly proportional to the concentration of oxygen in the solution and the current continues until the oxygen supply is exhausted. This current has been converted to a voltage signal.

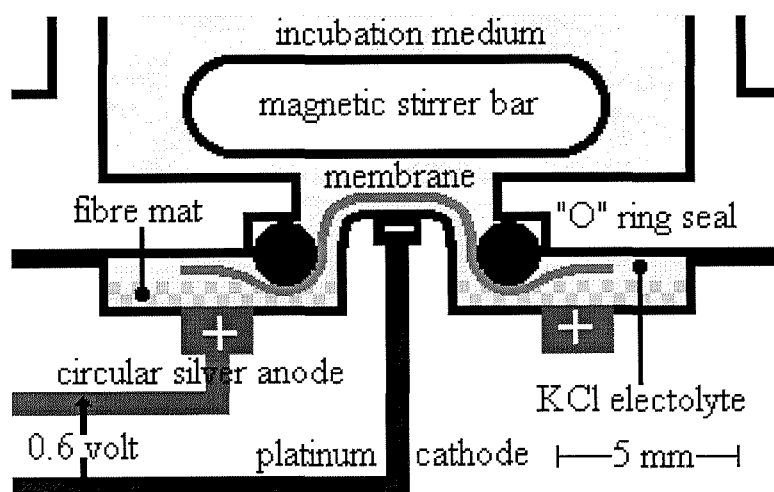
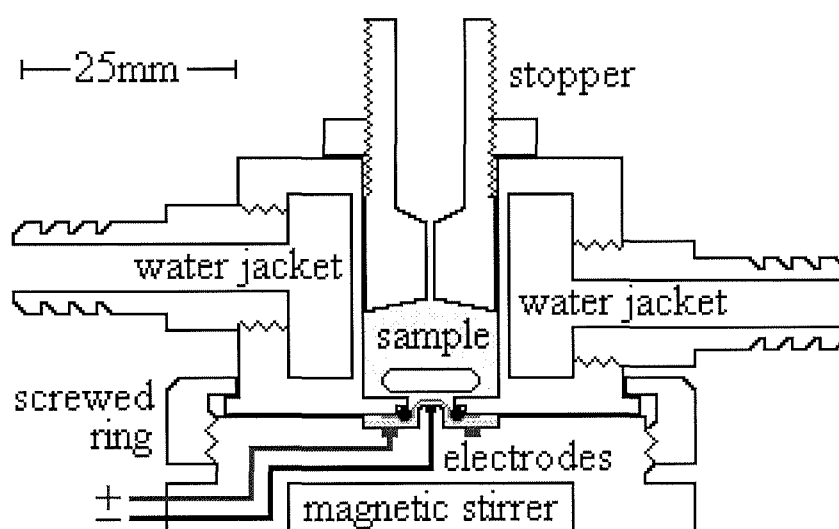


Figure 3.4 The oxygen electrode

The diagram was taken from: <http://www.bmb.leeds.ac.uk/illingworth/oxphos/electrod.htm>

3.2. Materials and methods

3.2.1. Cell lines

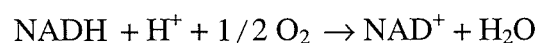
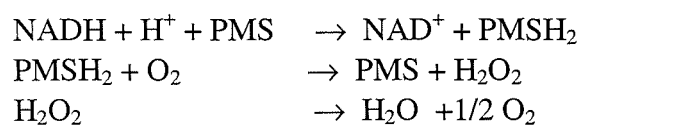
Cell lines and cell counting methods have been described in the previous chapter.

3.2.2. Chemicals

Chemicals (including rotenone , azadirachtin, PMS and MTT), have been described in previous chapter. NADH, KCl, sodium dithionite, malic acid, glutamic acid, succinate, ADP, TMPD, and catalase were purchased from Sigma (Sigma-Aldrich).

3.2.3. Measurement of oxygen consumption

The water-jacketed electrode was maintained at a constant temperature by means of water circulating from a thermostatically-controlled water-bath. Estimates of oxygen uptake for Sf9 and L929 were done at 30 °C and for L929 at 37 °C. Throughout the reported work, the volume of PBS, in which the cells were suspended, was 3 ml. The signal from the electrode was fed into a chart recorder with a 10mV maximum range. Addition of small amounts of sodium dithionite to the medium completely depleted the dissolved oxygen, and allowed the establishment of the base-line, before replacing the oxygen-depleted medium, and determining the dissolved oxygen concentration by NADH, PMS and catalase as shown below:



The concentration of the NADH solution was measured accurately by spectrophotometry at 340 nm based on an extinction coefficient of $6.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

3.2.4. Using mitochondria in the respiration assay

3.2.4.1. Isolation buffer

Isolation buffer contains 0.225 M mannitol, 0.07 M sucrose, 0.4 mM EGTA and 2 mM MOPS (pH 7.2).

3.2.4.2. Preparation of mitochondria from rat liver

After killing the animal, a medial incision was made from groin to sternum of a rat, and 100 ml of ice-cold 0.9% NaCl was poured into peritoneal cavity. Then by cutting it off at the base the liver was removed and dropped into a second beaker of ice-cold saline solution.

Before homogenizing, the liver was washed three times in ice-cold isolation buffer. Then tissues were chopped with a pair of scissors and transferred to a 50 ml glass homogenizing tube in 20 ml isolation buffer. Homogenization was done using first a loose-fitting pestle and then repeated with a tight-fitting pestle to ensure complete breakage of cells. Homogenized cells (including washings) were transferred to a 50 ml centrifuge tubes and after making up the tube, the homogenate with isolation buffer were centrifuged at 2000 rpm for 10 minutes at 4 °C. To bring down the mitochondria pellet the supernatant, carefully poured in to a clean centrifuge tube and without filling the tube, was centrifuged at 11000 rpm (9400 x g) for 10 min to collect the mitochondria. The supernatant was discarded and the white foamy material near the top of the tube was removed by wiping the inside of tube with a lab wiper. Then the mitochondrial sediment was resuspended in isolation buffer and disaggregated by hand homogenizing and centrifuged at 11000 rpm for 7 minute. The supernatant was discarded and a Pasteur pipette used to remove the last bit of liquid. A glass rod was used to gently resuspend the remaining pellet. The paste was transferred to an eppendorf tube and air spaces removed by pipetting with a yellow micropipettor and stored at 4°C.

3.2.5. Effect of azadirachtin on oxygen consumption of cultured cells.

3.2.5.1. Acute effect.

Insect and mammalian cells grown to confluence were harvested, and washed with PBS after centrifugation at 800 rpm for 5 min, and finally resuspended in 3ml PBS at a concentration of $5\text{--}10 \times 10^5$ cells.ml⁻¹. The electrode was then closed and the rate of oxygen consumption followed over a period of 15 min before adding azadirachtin or rotenone to the suspension of cells.

Various concentrations of the two possible inhibitors of respiration were made up in DMSO, before being added to the respiring suspension in the oxygen electrode, in a total of 100 µl to give final concentrations from 10^{-7} – 10^{-4} M for azadirachtin and 10^{-11} – 10^{-6} M for rotenone (the highest concentrations were dictated by the solubility of compounds).

The final concentration of DMSO was 1%, shown in controls to reduce oxygen consumption by $14 \pm 4\%$. The respiration was followed for a 30 min after the addition of the azadirachtin or rotenone. The rate of respiration after the addition of compounds was compared to that before, with each cell suspension acting as its own control.

3.2.5.2 Chronic effect of azadirachtin on consumption of oxygen by Sf9 cells

In order to investigate the long term effect of azadirachtin on respiration of insect cells, azadirachtin dissolved in DMSO was added to culture of Sf9 cells in final concentration of 10^{-8} M. After appropriate time of incubation (30min to 15 h) cells were harvested and resuspended in PBS in the absence of azadirachtin and oxygen consumption was measured as indicated above. The controls contained only DMSO. Samples were taken to estimate oxygen uptake at 30 min intervals.

3.2.6. Effect of serum on activity of azadirachtin and rotenone

Since albumin in serum can bind unspecifically to many compounds such as pesticides (Purcell *et al.*, 2001; Sogorb and Vilanova., 2002), it was important to investigate the possibility of an interaction of serum albumin with phytochemicals

which might reduce the effect of the phytochemicals on cell respiration. To achieve this, concentrations of 10% and 25% of FBS were used in cell suspensions containing and respiration was monitored.

3.3. Results

3.3.1. The effect of foetal bovine serum on inhibition of cell respiration by azadirachtin or rotenone.

Preliminary results indicated that concentration of 10^{-4} M of azadirachtin had the maximum effect on cell respiration. Rotenone in concentration of 10^{-8} M reduced the cell respiration by 50%. However it was shown that bovine serum in concentration of 10% and 25% could effectively reduce the acute inhibitory property of rotenone and azadirachtin in Sf9 cells (Table 3.1).

Table 3.1 The effect of Foetal Bovine Serum on activity of azadirachtin and rotenone on respiration of Sf9 cell line.

Phytochemical	Concentration of F.B.S	Reduction in consumption of oxygen (% of control)
Azadirachtin	0%	16±3.5
	10%	12±1.8
	25%	9.3±0.7
Rotenone	0%	51.8±4
	10%	40.5±4
	25%	29.9±2.44

Concentration of azadirachtin and rotenone were 10^{-4} and 10^{-8} M respectively. Temperature was 30 °C and number of cells were $0.5-1 \times 10^6 \text{ cm}^{-3}$. Results of replicate in each case were compared to the related control contained 1% DMSO and appropriate concentration of serum.

Due to the effect of bovine serum on activity of azadirachtin and rotenone in the following experiment cells were washed with PBS prior to estimation of respiration.

3.3.2. The effect of azadirachtin on oxygen consumption of cell lines

The basal respiratory rates of Sf9, C6/36 and L929 cells were shown to be $8.3 \pm 0.53 \times 10^{-9}$, $1.75 \pm 0.19 \times 10^{-9}$, and $27 \pm 0.22 \times 10^{-9} \mu\text{m O}_2/\text{cell}/\text{min}$ respectively.

Over the range of concentrations used, azadirachtin showed only a small inhibitory effect on the total cellular oxygen consumption of either insect and mammalian cells. At the highest feasible concentration, 10^{-4}M , this was $16 \pm 3.5\%$, $10.25 \pm 1.2\%$ and, $10 \pm 2.1\%$, for Sf9, C6/36 and L929 respectively which in all case it was statistically significant ($P < 0.05$), (Fig. 3.5-3.7).

The effect of azadirachtin on consumption of oxygen by Sf9 cells

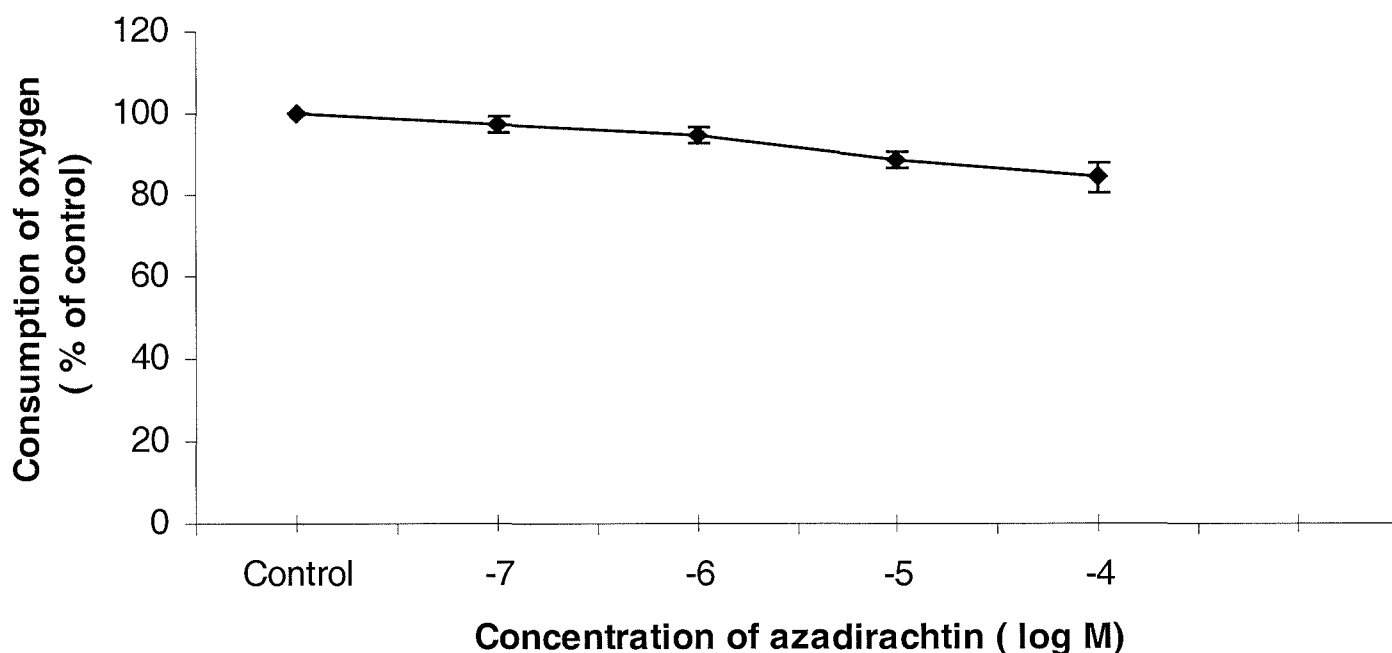


Figure 3.5 Inhibitory effect of azadirachtin on respiration of Sf9 cells. Means and standard deviation of at least 5 replicates per point are shown.

The effect of azadirachtin on consumption of oxygen by C6/36 cells

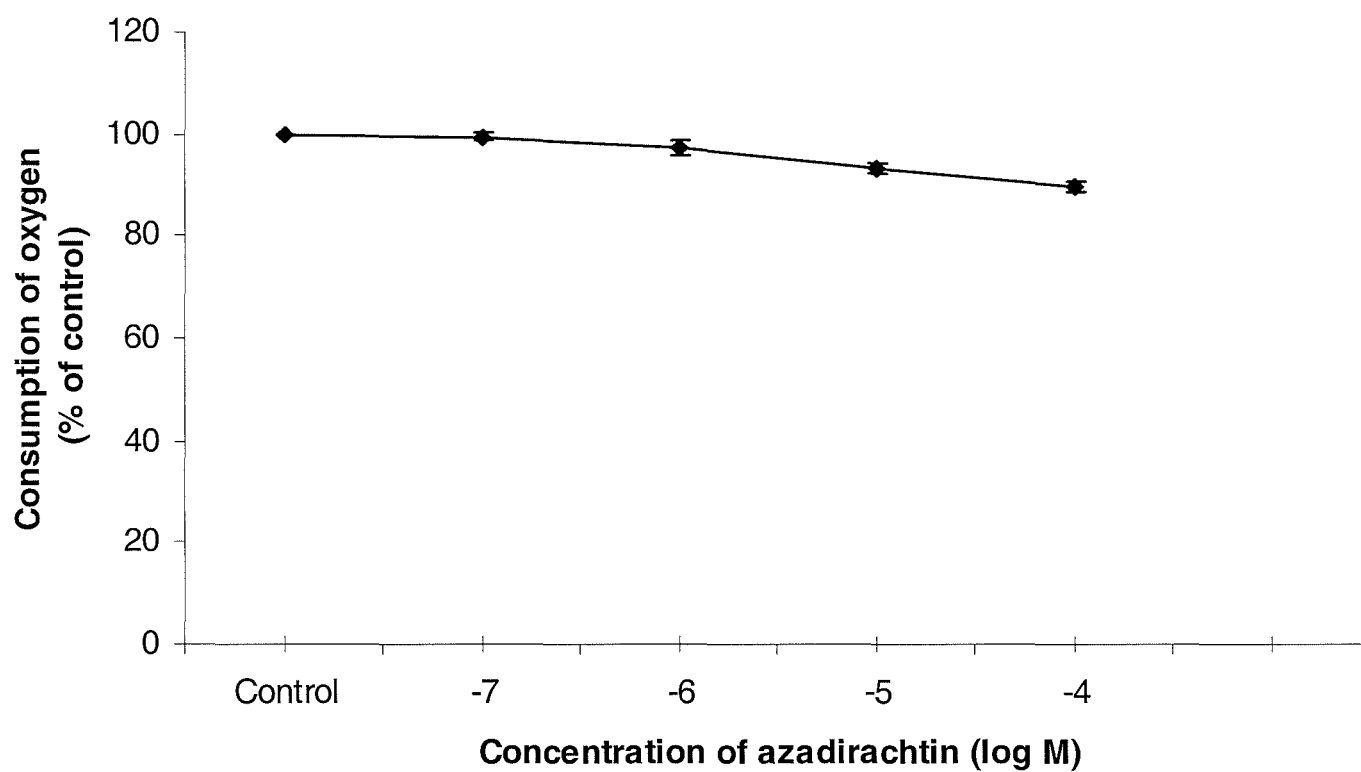


Figure 3.6 Inhibitory effect of azadirachtin on respiration of C6/36 cells. Means and standard deviations of at least 5 replicate per point are shown

The effect of azadirachtin on consumption of oxygen by L929 cells

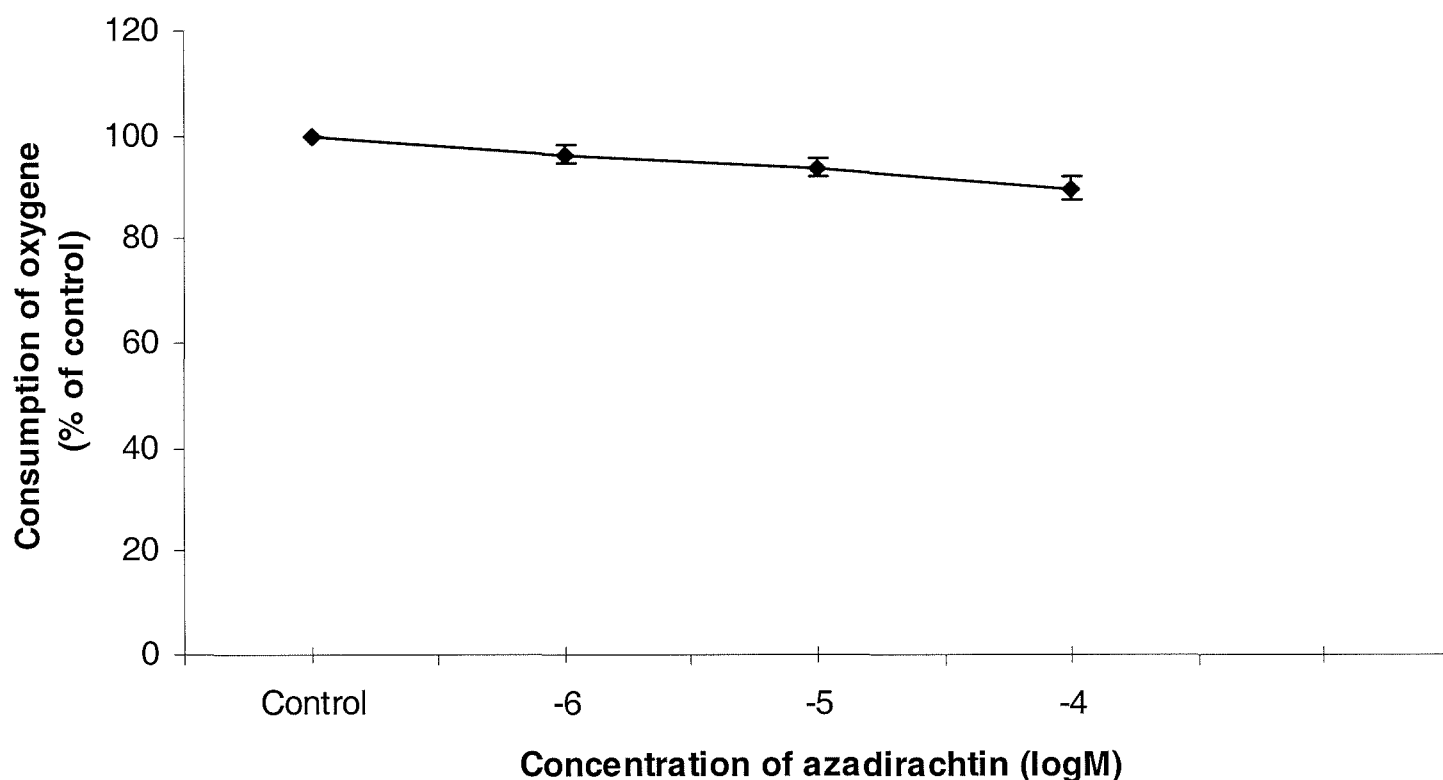


Figure 3.7 Inhibitory effect of azadirachtin on respiration of L929 cells. Means and standard deviations of at least 5 replicate per point are shown.

3.3.3. The chronic effect of azadirachtin on total cellular oxygen consumption of Sf9 cells

When Sf9 cells were incubated with azadirachtin at a concentration of 10^{-8} M, there was no effect on oxygen consumption over 5 h incubation. After 5 h there was slight difference and after 15 h it was 3-5% which was not statistically significant.

3.3.4. Effect of rotenone on acute respiration of cell lines

In contrast to these results with azadirachtin, the results with rotenone showed that it was effective as an inhibitor of respiration at much lower concentrations and to a much greater extent. At the highest concentration (10^{-6} M) it was $78 \pm 7.9\%$, $82.5 \pm 3.5\%$ and $61 \pm 7.5\%$ for Sf9, C6/36 and L929 respectively.

Rotenone even in lower concentration, 10^{-11} M could effectively reduce the respiration between 10- 15% in cell lines (Fig. 3.8-3.10).

The effect of rotenone on oxygen consumption by Sf9 cells

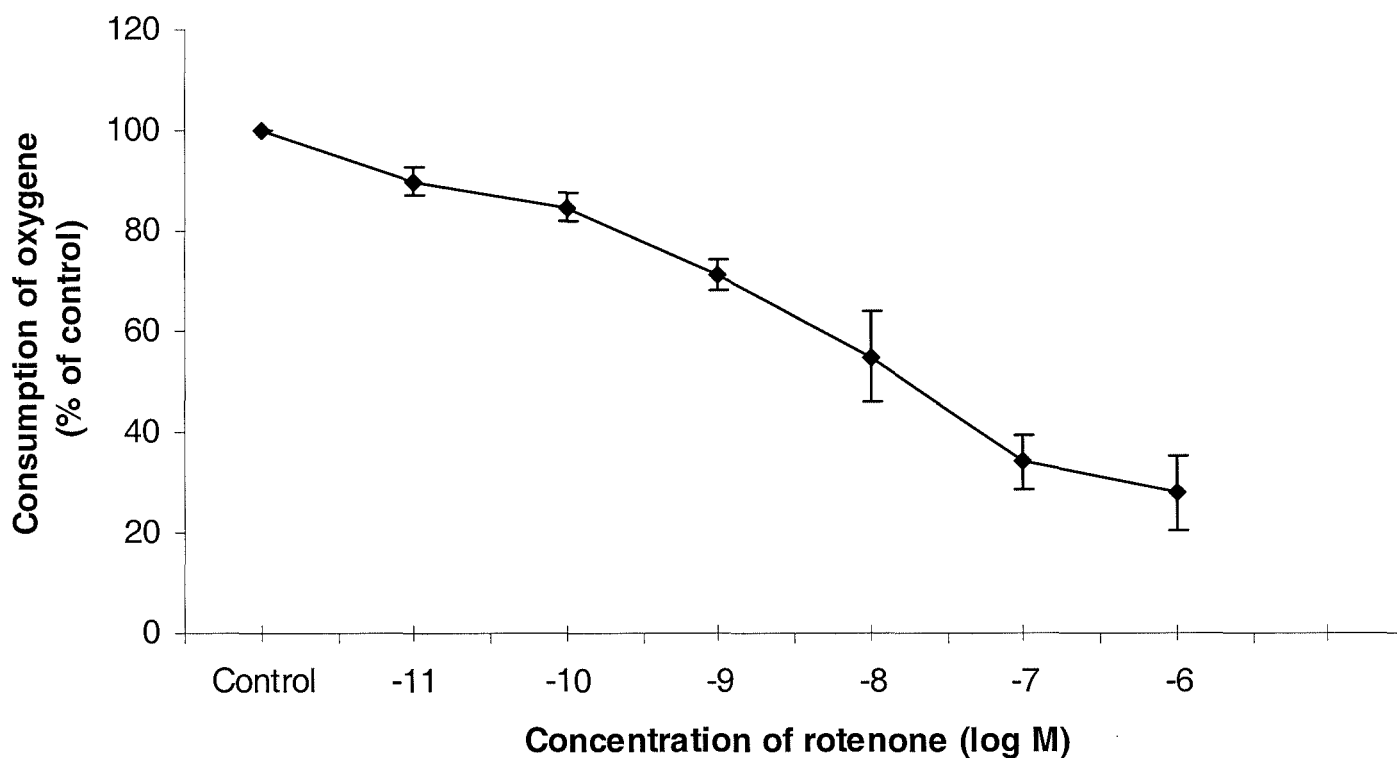


Figure 3.8 Inhibitory effect of rotenone on respiration of Sf9 cells. Means and standard deviations of 4-5 replicate per point are shown.

The effect of rotenone on consumption of oxygen by C6/36 cells

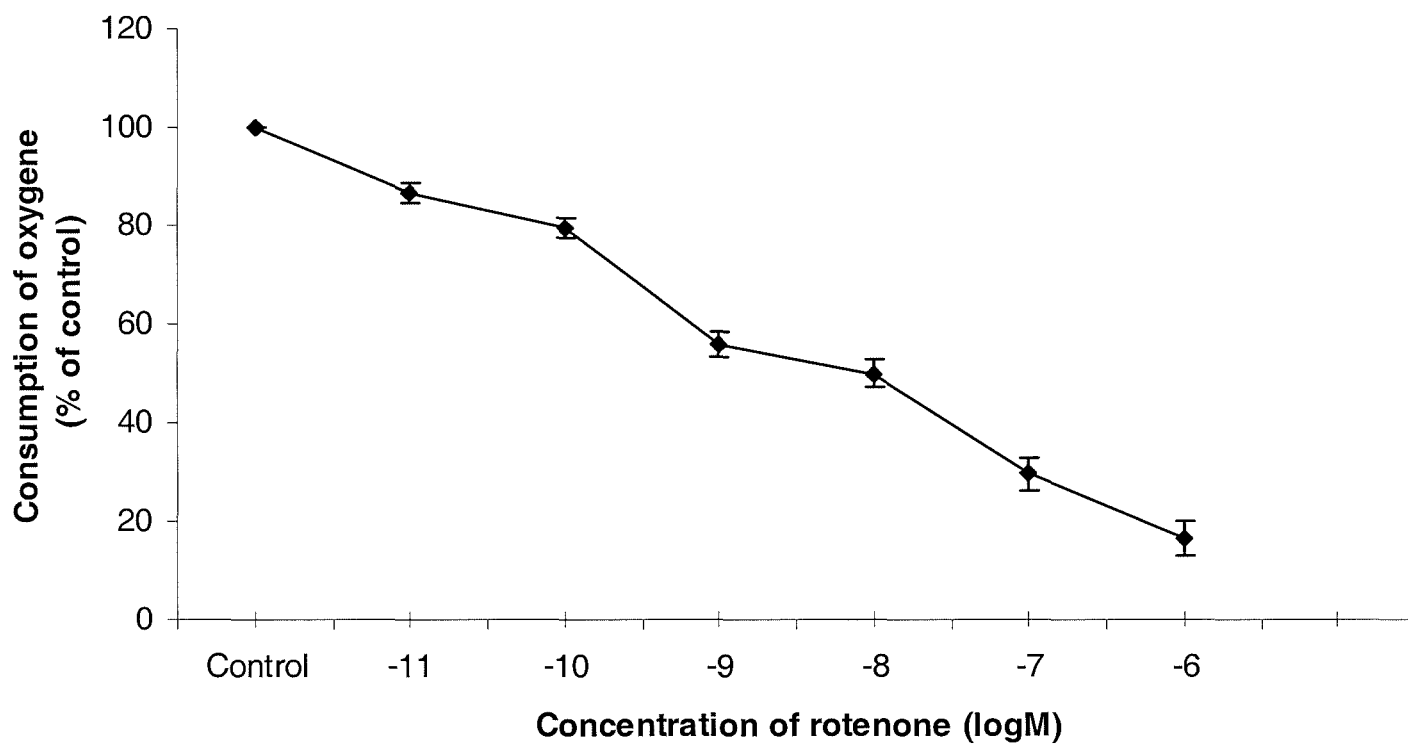


Figure 3.9 Inhibitory effect of rotenone on respiration of C6/36 cells. Means and standard deviations of 4-5 replicate per point are shown

The effect of rotenone on consumption of oxygen by L929 cells

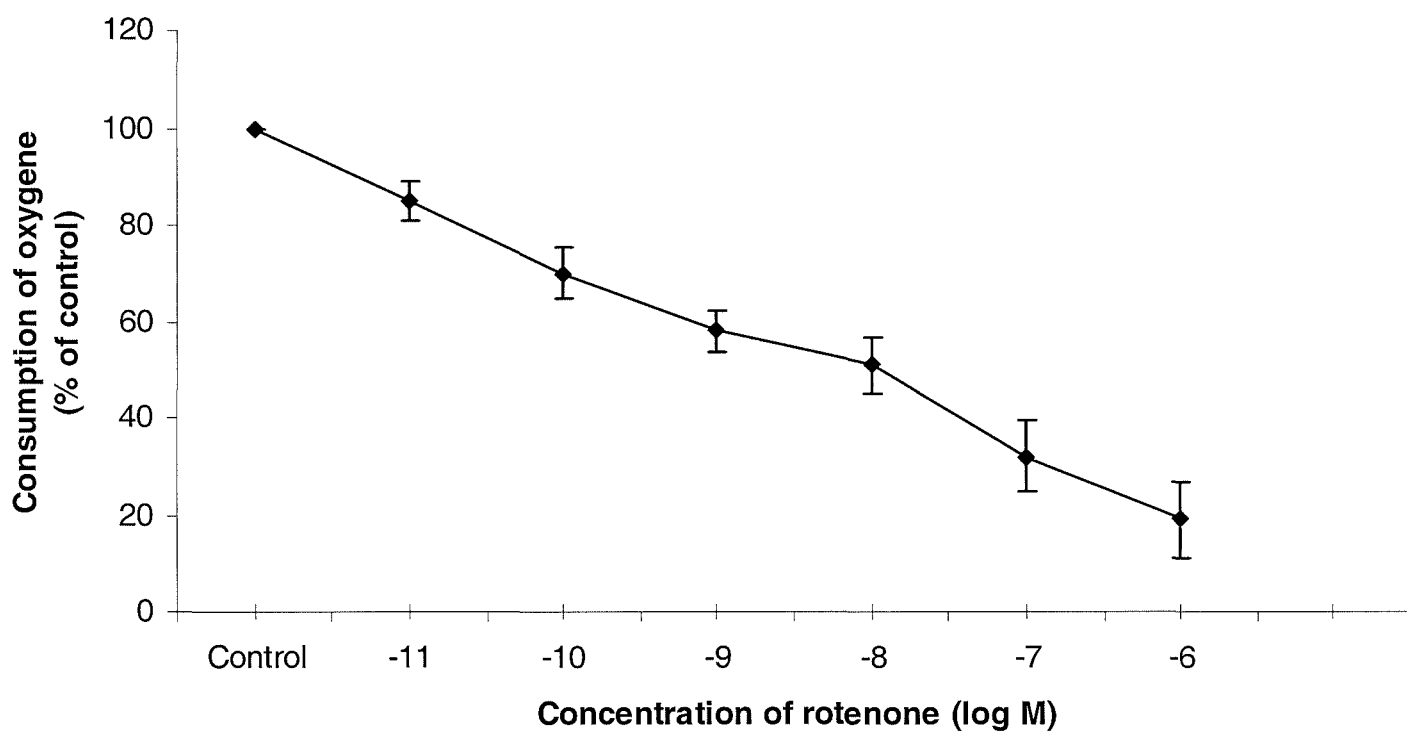


Figure 3.10. Inhibitory effect of rotenone on respiration of L929 cells. Means and standard deviations of 4-5 replicate per point are shown and temperature of measurement chamber was 37 °C.

3.3.5. Effect of azadirachtin on mitochondrial respiration

Rotenone at a concentration of 10^{-6} M, reduced the respiration rate of rat liver mitochondria by up to 90%. In contrast, azadirachtin even in concentrations as high as 10^{-4} M, did not affect respiration in those mitochondria (results not shown).

3.4. Discussion

In previous chapters it was shown that both rotenone and azadirachtin significantly reduced the growth of cell lines. The effect of rotenone on electron transport taking place in the mitochondria of cells is well-known. It was hoped that a comparison of the effect of these two phytochemicals on cell respiration may help to find the possible mode of action of this terpenoid. The results of previous experiment with Sf9 cells and MTT indicated that despite some limited effect of rotenone on dehydrogenase activity, there was not any effect on reduction of MTT by azadirachtin (see Chapter 2). Interestingly in the present test, both azadirachtin and rotenone were shown reduce the total oxygen consumption. Furthermore, using purified mitochondria, the experiments showed that azadirachtin could not reduce the respiration rate significantly. Although the cytotoxicity experiment with azadirachtin showed that Sf9 and C6/36 were more sensitive than L929 cells (see Chapter 2), estimation of total oxygen consumption of cells demonstrated that azadirachtin had only a minor effect on the respiration rate in both mammalian and insect cultured cells and there was only a slight difference between them. Due to low sensitivity of insect cells and also presence of same sensitivity in mammalian cells it is clear that the effect of azadirachtin on cell respiration is different to that of rotenone.

One possibility for this effect of azadirachtin is that at higher concentrations it may affect other binding site. For instance it may affect DNA synthesis or protein synthesis, thus energy requirement. In this case Jabbar *et al.* (1998) have shown that in concentration of 10^{-3} M azadirachtin inhibits the growth of mammalian cells, which is in agree with its general effect on oxygen consumption of insect and mammalian cell lines as shown in the present experiments. The effect of azadirachtin on permeability of cell membrane (see Chapter 4) is one of possibilities that this terpenoid can affect the respiration in different cell lines. Other possibility is that azadirachtin affect cells in a special sector of cell cycle (e.g. M or G2/M phase) as only part of cells are in each phase at the time of measurement of the respiration rate, even a 100% inhibition of respiration would only cause about 33% reduction in overall respiration.

The other interesting point is the difference between the effect of rotenone on the growth of cells and effect on respiration. The results of Chapter 2 showed that rotenone in concentration of 10^{-6} M almost entirely inhibited the growth of insect

cells, whereas the same concentration of rotenone causes about 85% decrease on consumption of oxygen. The difference may be a result of presence of two binding sites within cells. The effect of rotenone on polymerization of microtubules and eventually cell cycle (see Chapter 4) may additionally affect cell growth. These experiments also show some difference in the effect of rotenone on growth and the respiration of L929 cells. In Chapter 2 was it shown that in a concentration of 10^{-6} M, rotenone inhibits the growth to about 39% of controls, whereas the same concentration of rotenone cause about 80% reduction in oxygen consumption of these cells. One possible explanation of the difference between the effect on growth and respiration could be the degradation or metabolism of rotenone during the experiment. In this case Bowman et al. (1978) have shown that the half life of rotenone in the presence of animal chow is between 7 and 8 days. The difference in the temperature of the incubators of insect and mammalian cells may affect this situation. A favourite possibility is that this difference is related to anaerobic metabolism in L929 cell line.

Chapter 4

The effect of azadirachtin on the cell cycle of insect and mammalian cultured cells

4.1. Introduction

The experiments on cell proliferation showed that both azadirachtin and rotenone could effectively inhibit the growth of insect cultured cells. Although insect cells were highly sensitive to the inhibitory effect of the terpenoid, its effect on proliferation of mammalian cells was obvious only at high concentration. The results of the respiration assay showed that despite an effect of azadirachtin on respiration of both mammalian or insect cells the reduction in consumption of oxygen was significant only in a very high concentration and the highest concentration used inhibited respiration in insect cells by 16% . This was much less than the effect of azadirachtin on the growth of insect cells (10-16% reduction in respiration in comparison to more than 90% reduction in growth).

These experiments also indicated that the effect of rotenone on growth of cultured cells is paralleled by its effect on respiration, whereas the inhibitory effect of azadirachtin on proliferation of the insect cell line must be due to its effect on other physiological or biochemical aspects of cell proliferation.

Since cellular proliferation depends on the cell's growth and division cycle (Agami *et al.*, 2002), the experiments described in this chapter were carried out to further investigate the effect of azadirachtin on growth and the cell cycle.

4.1.1. Cell cycle

The cell cycle is the universal process by which cells replicate themselves, and it underlies the growth and development of all living organisms. The most important events of the cell cycle are those concerned with the copying and partitioning of the material of heredity. The cell cycle is composed of a sequence of events, which eventually ends in cell division. The cell cycle conventionally has been divided to G₀, G₁, S, G₂ and M (figure 4.1). Most protein synthesis takes place in G₁, and almost all DNA replication in S phase. In prokaryotes, due to the simultaneous presence of several replication forks in the same chromosome, DNA synthesis overlaps cell division at high growth rates. This mechanism enables microorganisms to grow at a wide range of growth rates. On the other hand animal cells are bound to the cell cycle

where DNA replication occurs only during the S phase (Doverskog *et al.*, 1997). Progression through the cell cycle is mediated by the activation of highly conserved family of protein kinases, the cyclin-dependent kinases (Smith *et al.*, 2001).

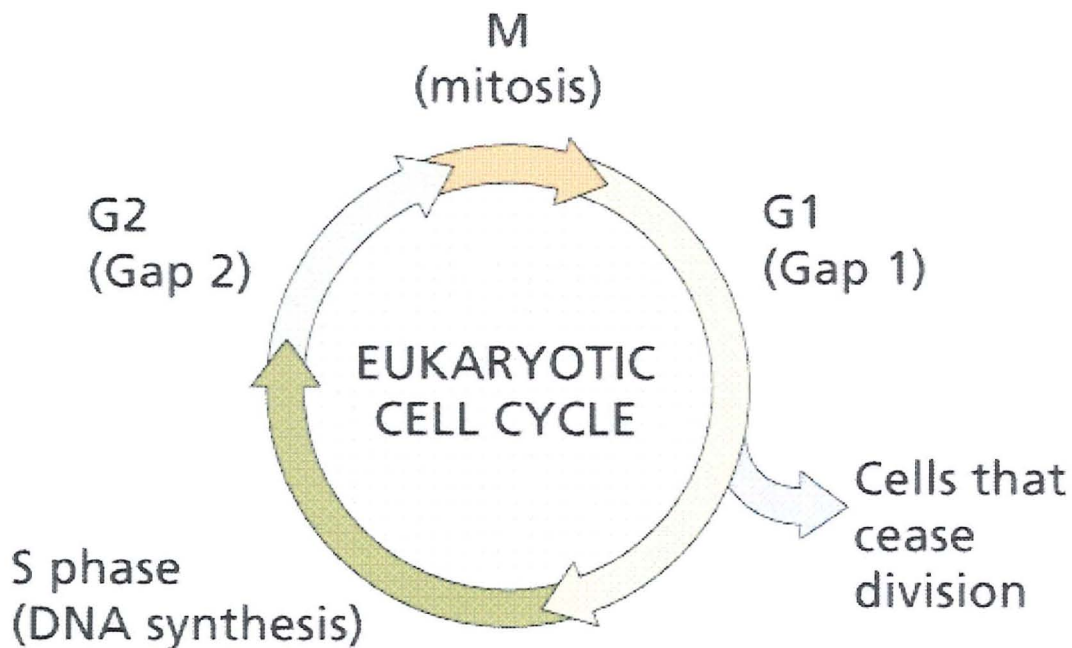


Fig.4.1 Cell cycle in eukaryotic cells

The figure was taken from: gened.emc.maricopa.edu/bio/bio181/BIOBK/BioBookmito.html

4.1.1.1. The G0 /G1 phase

Interphase includes intervals of time both before and after the S phase in which little or no DNA synthesis occurs. These intervals are called the G0, G1 and G2 phases, respectively. The G stands for “gap” because these stages represent gaps or interruptions in DNA synthesis. After undergoing cell division, some cells that have become terminally differentiated, such as neural tissue, enter a quiescent phase, termed G0, where they remain for their entire existence. Cells in other organs, such as liver, for example, are able to re-enter the cell cycle after partial removal of the organ. Other cells such as those from bone marrow progenitors, tumour cells, or cell growing

in tissue culture, enter a growth phase called G1 following cell division (Studzinski, 1999). Indeed most of the regulatory events that affect proliferation occur in the G1 phase (Agami *et al.*, 2002). G1 is the period between the end of the previous division and the onset chromosomal DNA synthesis. The length of G1 is quite variable, depending on the cell type. Even in the same organisms, some cells may spend minutes or hours in G1, whereas others spend weeks, months or even years.

4.1.1.2. The S phase

Since the purpose of mitosis is to generate two daughter nuclei that are identical to the parent nucleus in both amount and kind of information, the parcelling out of chromosomes in the division phase of the cell cycle must be offset by a prior replication of chromosomes. Most cellular contents are synthesized continuously during interphase, so that cell mass increases gradually as the cell approaches division. However, DNA synthesis is restricted to a limited portion of cell cycle, called S phase (Alberts *et al.*, 1994). During the S phase the amount of DNA in the nucleus doubles from what is called the 2C amount present in a diploid cell immediately after division, to the 4C amount, present at the end of the S phase. The doubling of the DNA content of the nucleus reflects the replication of chromosomes during S phase, with each chromosome giving rise to two sister chromatids. Sister chromatids remain attached to each other until they are parcelled out to the two daughter nuclei during mitosis.

The increase in DNA during the S phase can be quantified by determining the total amount of nuclear DNA, using microspectrophotometric methods. DNA replication is a highly ordered process. Each molecule of DNA has one or more specific origins of replication where DNA synthesis begins (Bhavagan, 2002) and the DNA of each chromosome in the nucleus is organised into a series of replicons that replicate independently of each other, but with a definite sequence. Similarly, DNA synthesis does not start simultaneously on all chromosomes within the nucleus, but again there appears to be an order to its onset. Except for the replacement of damaged DNA through what is called repair synthesis, nuclear DNA synthesis is restricted to S phase.

4.1.1.3. The G2 phase

The postsynthetic gap phase, (G2), occurs when the 4C amount of DNA has been reached and ends with the first visible sign that mitosis is beginning. There is as yet little information on the cellular or nuclear process that occur specifically in this stage, nor is clear what determines the length of the G2 stage. Hitomi *et al.* (2001) has suggested that expression of some cyclin, for example cyclin D1, promotes rapid passage through G2 phase and rapidly proliferating cells start to prepare for the next G1/S transition while they are still in G2 phase. In general, G2 is shorter than G1 and is usually much more uniform in duration among the different cell types of an organism. However, G2 arrest is known to occur in some cells, leaving the cell with its DNA fully replicated but without entry to mitosis.

4.1.1.4. Mitosis

Mitosis is nuclear division, involving the separation of duplicated chromosomes into clusters that are genetically identical to each other and to the mother nucleus. Mitosis begins with the progressive condensation (thickening and coiling) of the duplicated interphase chromosomes, accompanied by the migration of structures called centrioles to the opposite ends of the cell. Microtubules then become organised into the fibres of the mitotic spindle, which is responsible for directing chromosome movement. Prior to entry of organized microtubules into nuclei, the nuclear envelope fragments. This involves depolymerization of the lamina, the fragmentation and removal of the nuclear membranes from the chromatin, and the disassembly of the nuclear pore complexes (Aitchison *et al.*, 2002). Then chromosomes are drawn into position at the centre of the cell and sister chromosomes then move toward opposite ends of the cell. Chromosomal movement continues until the two sets of chromosomes are completely separated, by which point cell division is usually already underway. The nuclear envelope then re-forms, delimiting two daughter nuclei of identical genetic makeup. As mitosis is completed, the chromosomes decondense and revert to the extended form characteristic of interphase chromatin.

4.1.1.5. Cytokinesis

In most cases, nuclear division is followed quickly by cell division, so that the two daughter nuclei soon acquire their own cytoplasm. In animal cells and protists, this occurs by formation of a cleavage furrow in the plasma membrane, which gradually deepens until the time when a “decision” is made as to whether and when the cells will divide again. Although nuclear and cytoplasmic division are generally linked, they are separable events, and in some normal circumstances, nuclear division is not followed by cytokinesis. The early *Drosophila* embryo, for example, undergoes 13 rounds of nuclear division without cytoplasmic division, forming a single large cell containing 6000 nuclei arranged in a monolayer near the surface (Alberts *et al.*, 1994).

4.1.1.6. Length of the cell cycle

The cells in the multicellular organism divide at varying rates, but most studies of the cell cycle have been done with cells in culture, where the length of the cell cycle is similar for different cell types. The length of the cell cycle (also called generation time or doubling time) can be estimated for cultured cells either by counting the cells intervals under a microscope or by monitoring the cell mass spectrometrically. For mammalian cells in culture, such as L929 cells, the cycle usually takes about 18-24 hours.

Once the total length of the cycle is known, the length of specific phases can also be determined. The S phase is easiest to measure, which can be determined with using radio-labeled thymidine and autoradiography. The length of M phase can be estimated by multiplying the generation time by the fraction of the cells that are actually in mitosis at any point in time. This fraction is called “mitotic index”. The length of mitosis for cultured cells is often less than 1 hour. G1 and G2 must be determined by less direct methods, because there is no way to label or identify cells in these phases specially (Becker, 1986). The duration of cell cycle phases in Sf9 cell line has been estimated as 6-8, 6, 6-8 and 1h for G0/G1, S, G2 and M respectively. Cells that are not dividing are assumed to be in G0; for example differentiated neurones (Lui *et al.*, 2001) and the transition from G0 to G1 promotes a cell to enter S phase and eventually mitosis. The two key questions are when to enter the S phase (the start

point) and, subsequently, the M phase. Mitosis begins only after DNA synthesis has been completed and requires major changes in cell architecture (Stryer, 1995).

4.1.2. Cell cycle checkpoints

The cell cycle is a highly conserved mechanism by which eukaryotic cells proliferate. The regulation of cell cycle is an important process in that improper cell cycle regulation can lead to cancer (i.e. uncontrolled cellular proliferation). It is also important in biology because organized regulation of proliferative growth is a fundamental requirement for an organism, and absolutely required for the proper development of multicellular structures.

Cell cycle checkpoints are surveillance mechanisms that monitor and coordinate the order and fidelity of cell cycle events. When defects in the division program of a cell are detected, checkpoints prevent the normal cell cycle transition through regulation of the relevant cyclin-CDK (cyclin-dependent protein kinase) complex (es). For example checkpoints that respond to DNA damage have been described for the G1, S and G2 phases of the cell cycle (Koniaras *et al.*, 2001).

A growing body of evidence has indicated that deregulation of the cell cycle can either directly trigger apoptosis or increase sensitivity to apoptotic inducers, which is a mechanism by which eukaryotic cells “commit suicide”. Indeed, successful proliferation requires active suppression of the apoptotic programme.

During both cell cycle and apoptosis processes, cells lose attachment and volume, condense their chromatin, disassemble the nuclear lamina, and display membrane blebbing.

The cell cycle apparatus is composed primarily of three families of proteins: the cyclin-dependent protein kinases (CDKs), the cyclins and the cyclin-dependent kinase inhibitors (CKIs). CDKs allow progression through the different phases of the cell cycle by phosphorylating critical serines and threonines on their target substrates. Their kinase activity is dependent on the presence of activating subunits known as cyclins, whose abundance varies substantially during the cell cycle.

The orderly progression of the cell cycle is driven by sequential activation of CDKs to pass key restriction checkpoints, primarily during the G1/S and G2/M transition of cell

cycle. The checkpoints of the cell cycle ensure that critical events in a particular phase are completed before the next phase can be initiated, thereby preventing the formation of abnormal cells (Liu *et al.*, 2001).

4.1.2.1. G1/S checkpoints

As a result of substantial recent advances in cancer biology, cell cycle regulation in the G1 phase has attracted a great deal of attention as a promising target for the research and treatment of cancer (Owa *et al.*, 2001). Frequently, cells decide to enter or withdraw from the cell cycle at the G1 phase, and thus the G1 checkpoint at the G1/S transition is often the prime target for cell cycle regulation. Cell cycle progression can be blocked at the G1 checkpoint in response to the status of both the intracellular and extracellular environments. For example, growth arrest can be induced when DNA damage is detected. Upon repair of the damage, progression through the cell cycle resumes. An alternative to repairing damaged cells, is simply to eliminate them through the process of apoptosis. Thus it is the cell cycle checkpoints that serve as molecular switches where cells must determine whether to complete cell division, arrest growth to repair cellular damage, or undergo apoptosis if the damage is too severe to be repaired or if the cell is incapable of repairing the DNA.

Also, there are two G0 states called the “senescence G0” state and the “quiescence G0” state. Senescence G0 state can be observed in cell culture in which cells arrest at the end of normal proliferative life span even under optimum culture conditions and enter a state of viable, but permanent growth arrest. This is because of an increase in the level of p16 and p21, which keep the Rb protein in its unphosphorylated state.

In the quiescence G0 state, the CKIs are present in excess of cyclin-CDK complexes and may be caused by growth factor deprivation or high cell density in culture (Liu *et al.*, 2001).

It is apparent that the stress kinase pathways can be activated through events at the cell membrane and in the cytoplasm, whereas, by contrast, the DNA-integrity checkpoints are activated as a result of DNA damage. These findings support the notion that the stress kinase pathways and the DNA-integrity checkpoint pathways are involved in the cellular response to cytotoxic and genotoxic stresses respectively.

Despite these apparently distinct mechanisms of activation, it is clear that a number of parallels can be drawn between these pathways (Pearce *et al.*, 2001).

4.1.2.2. G2 and M phase checkpoints

As cells undergo mitosis, their replicated genetic material must be distributed equally between two daughter cells. In prophase, a bipolar spindle is formed between two microtubule-organizing centres. Condensed sister chromatids are attached to the spindle via their kinetochores in prometaphase and metaphase, and are pulled to opposite poles as they undergo anaphase. As the mis-segregation of sister chromatids leads to aneuploidy, this process must be tightly controlled by checkpoints that monitor the completion of critical steps in the pathway. In human cells, prometaphase is delayed when centrosome separation (a prerequisite for the formation of a bipolar spindle) does not take place.

The metaphase-to-anaphase (M-A) checkpoint inhibits the separation of sister chromatids until all of the kinetochores are attached to a functional spindle. This, so called “spindle checkpoint” is a quality control mechanism that prevents chromosome segregation errors (Hoyt, 2001). The spindle checkpoint allows mitotic delay in response to a spindle defect. This checkpoint delays the onset of anaphase until all the chromosomes are correctly aligned on the mitotic spindle. It means that when unattached kinetochores are present, the metaphase/anaphase transition is not allowed and the time available for chromosome-microtubule capture increases.

Mutational inactivation of spindle checkpoint genes has been implicated in the progression of several types of human cancer.

Finally, exit from mitosis is controlled by a Bub2-dependent checkpoint pathway that inhibits the mitotic exit network (MEN) until the completion of chromosome separation (Wassmann *et al.*, 2001).

Depending on the organism and the dominant control pathways, some cell-cycle delay may be more apparent than others. For example, fission yeast cells spend the majority of their cell cycle in a post-replication, pre-mitotic state such that DNA damage causes arrest before mitosis, which probably centers on preventing the activation of the cyclin-dependent kinase Cdc2. On the other hand, in budding yeast,

DNA damage results in a delay to the metaphase to anaphase transition, which depends on preventing the proteolysis of protein whose degradation sets off a cascade of events leading to chromosome segregation. Also, studies in *Drosophylla* have indicated that in early stages of development, exposure to X-irradiation causes a delay in mitosis but, at later stages, it causes blocking of entry cells into mitosis (Walworth., 2000).

Genomic stability is under constant threat from chemicals, radiation and normal DNA metabolism. Normal cells delay cell-cycle progression in response to agents that damage DNA, which usually cause G2/M arrest in mammalian cells (Luo *et al.*, 2001). Other chemicals, which affect the cytoskeleton may cause arrest in cell cycle progression at G2/M (e.g. arrest of budding yeast due to an effect on polymerization of actin), (Rupes *et al.*, 2001), or other cell cycle phases.

4.1.3. The cytoskeleton

The cytoskeleton is an internal organisation in eukaryotic cells, which co-ordinates of the different parts of its network in the cell and which is of central importance for morphogenesis, organelle transport, and motility (Cau *et al*, 2001). It is transparent in standard light and electron microscope preparations, and is therefore “invisible”. They can be visible after binding to special dyes such as DCVJ or 9-(dicyanovinyl)-julolidine which binds to tubulin and visualise the microtubules (Fig. 4.28). It is usually left out of drawings of the cell, but it is an important, complex, and dynamic cell component. The cytoskeleton consists of 3 protein filament systems: microfilaments, intermediate filaments and microtubules with a large number of associated proteins that regulate each system. Microtubules, together with microfilaments and intermediate filaments, comprise the cytoskeleton network of cells, which play essential roles in diverse cellular functions such as mitosis, cell shape, locomotion, cytokinesis, intracellular transport and translocation of organelles.

4.1.3.1. Microfilaments

Microfilaments consist mainly of actin and are the smallest (about 7 nm in diameter) of the 3 cytoskeletal filaments and are found in all cells. Actin microfilaments are best known for their role in the contractile fibrils of muscles. In cell division, actin microfilaments produce the cleavage furrows that divide the cytoplasm of animal cells after chromosomes have been separated by the spindle (Dustine., 1978).

4.1.3.2. Intermediate filaments

Intermediate filaments are intermediate in size between the microfilaments and microtubules (8-10 nm in diameter). In contrast to microtubules and microflaments, different cell types express different combinations of intermediate filaments. For example, desmin filaments are found in muscle cells, neurofilaments (assembled from a triplet of neurofilament proteins) in neurones, and keratin filaments (assembled from a family of cytokeratin proteins) are found in cells of epithelial origin. Intermediate filaments assembled from vimentin are found in fibroblast cells of connective tissue. They are present singly or in bundles, and appear to play a structural or tension-bearing role in the cell. Intermediate filaments are the most stable and the least soluble constituents of the cytoskeleton (Becker., 1986).

4.1.3.3. Microtubules

Microtubules (MT) are proteinaceous organelles, which are present in nearly all eukaryotic cells. They form a large number of structures such as mitotic spindles, eukaryotic flagella, cilia, cytoplasmic and cytoskeletal elements. Most are single cylinders, but doublet tubules occur in flagellata and triplets in basal bodies and centrioles. Investigation has found that microtubules are principally composed of a highly conserved protein named tubulin.

With a few exceptions, the general morphology of cytoplasmic microtubules appears to be identical in all cell types and in all species studied. It is likely that varying dimensions reported in various ultrastructural studies reflect differences due to

problems with fixation or staining rather than difference in structure (Kenneth *et al.*, 2000).

The walls of microtubules are about 5 nm wide and their total diameter is usually about 25 nm. One or both ends of cellular microtubules are often associated with special structures called “microtubule-organizing centers” (MTOCs), which may be centrosomes, basal bodies, or kinetochores. Microtubules linked to one another and/or to MTOCs form many types of arrays in cells.

At different times in the cell cycle and positions within the organism, microtubules can be very stable or highly dynamic. Stability and dynamics are regulated by interaction with a large number of proteins that themselves may change at specific points in the cell cycle. Exogenous ligands can disrupt the normal processes by either increasing or decreasing microtubule stability and inhibiting their dynamic behaviour. In interphase cells, microtubules span the cytoplasm but are excluded from the nucleus, so using a microtubule binding fluorescence dye this region remains dark (Becher., 1986)

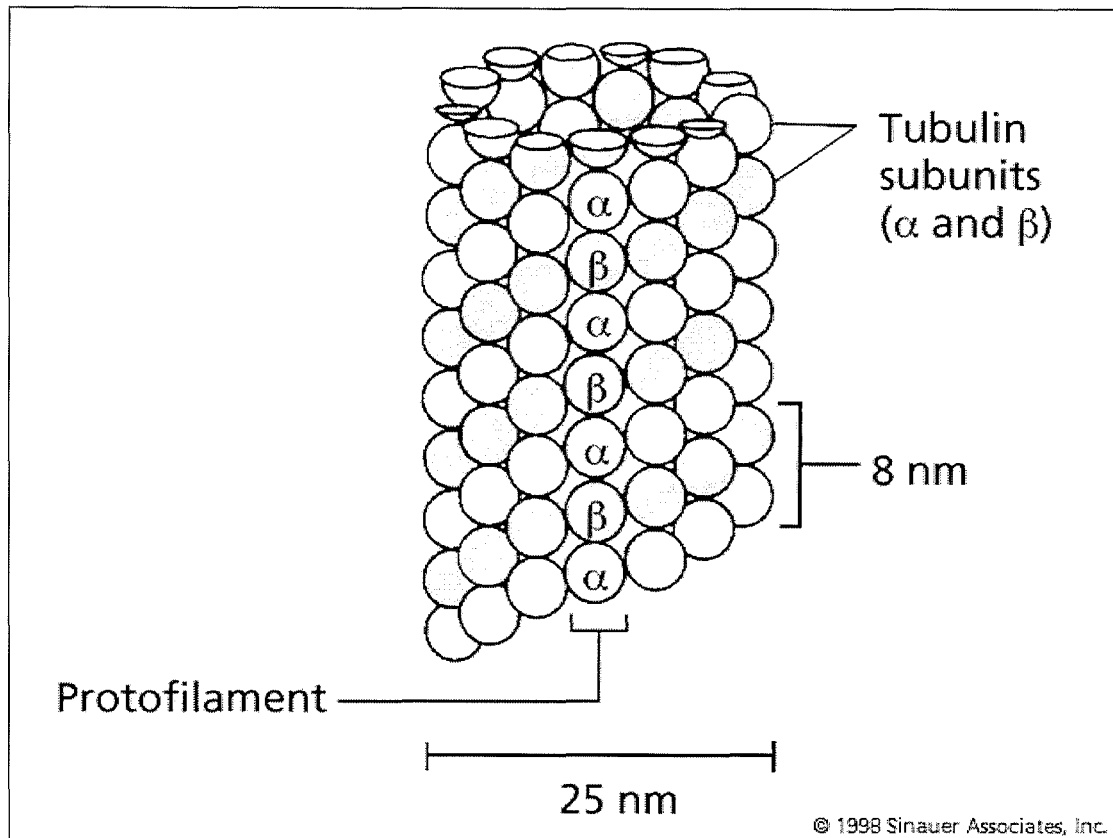


Fig. 4.2 The arrangement of α and β tubulin in the microtubule

4.1.3.4. Protofilament

The wall of microtubules is made of protofilaments. The protofilaments associate in parallel to form the cylindrical wall of the microtubules and themselves are made of linearly arranged alternation of α - and β - tubulin subunits. The protofilament is about 5 nm in width. By varying the conditions of polymerization *in vitro*, one can obtain other morphological structures from tubulin. All these forms of tubulin polymers are composed of protofilaments, put together in different ways. Fragments of a single protofilament can sometimes be seen in the preparation of polymerizing tubulin (Dustine., 1978). The protofilaments remain intact even after they dissociate from each other at the end of the microtubule, forming spirals and rings that appear to break off and then further dissociate to dimmers. The fact that protofilaments can exist after

dissociation from the microtubule suggests that the strength of the lateral interaction is affected more by hydrolysis than by longitudinal interactions (Kenneth *et al.*, 2000).

4.1.3.5. Tubulin

Tubulin is the main building block of all the microtubular systems. Brain tissue has an especially high concentration of tubulin and is used most often for its isolation. On SDS polyacrylamide gels, microtubules show two closely spaced bands, which represent α and β tubulin with approximately 55,000 molecular ratio. In the cells they appear as heterodimers, arranged in longitudinal rows to form protofilaments. α -tubulin is slightly more basic than β -tubulin. The α and β monomers share 36-42% identical sequences (Mitchison., 1993), and their structures are very similar (Kenneth *et al.*, 2000)). Brain α -tubulin contains 451 amino acid residues ; β -tubulin is somewhat shorter, containing 445 residues.

Tubulins have been highly conserved during evolution (Kirschner., 1978). Considerable homologies are found between the amino acid sequences of tubulin from very distant organisms and between the nucleotide sequences of corresponding genes. For example, the chicken tubulin gene hybridizes strongly with the tubulin genes of the alga *Chlamydomonas*. Owing to the conservation of tubulin structure, microtubules of very different organisms can often be stained by the antibody from single origin (Dustine., 1978). However, some exceptional results have also been reported. Thus, only minimal homologies were found between the tubulin gene of the protist *Naegleria* and chicken β -tubulin gene. This finding indicates that the tubulin structure may have undergone considerable changes in certain branches of the evolutionary tree. Also some authors have shown that the same organisms and even the same tissue usually contain multiple forms of α -and β -tubulins distinguishable by isoelectric focusing, peptide mapping, and other methods (Derry *et al.*, 1997). For instance, some experiments have revealed that there are up to six α and six β tubulin isotypes in mammals, γ -tubulin, which is less abundant, appears localized in the centromeres (Dumonent *et al.*, 1999, Field *et al.*, 1984). Some of these isoforms are likely to be coded by different genes. The chick genome has four genes for α - and β -tubulin. Obviously, the number of isoforms in this case

is larger than the number of genes. This suggests that post-translational modifications can generate at least a part of the tubulin heterogeneity. In fact, several types of these modifications have been revealed in various organisms: tyrosination, acetylation, and phosphorylation are most common ways (Mencarelli *et al.*, 2000; Correa *et al.*, 2001). Detyrosination and tyrosination are unique modifications of tubulin; no other protein is known to undergo these modifications. α -tubulin polypeptide is synthesized with a tyrosine at its C-terminus. This tyrosine can be removed by a specific carboxypeptidase. This nontyrosinated α -tubulin is a substrate for addition of tyrosine by another specific enzyme, tubulin tyrosine ligase. Various microtubules of the same cultured cell contain either tyrosinated or nontyrosinated tubulin. These results suggest that tyrosination-detyrosination can be used for the formation of functionally different groups of microtubules, the nature of which is not known. α -tubulin present in the flagella of *Chlamydomonas* appears to be an acetylated form of the α -tubulin present in the body of this unicellular organism. This is the only case in which specific post-translational modification of tubulin seems to be correlated with the special function of microtubules. Because of the relationship between the rate of GTP-tubulin dissociation constant and mechanism of dynamic instability, there is vast range in results reported from different authors and so some controversy over the previously accepted figure which was in the range of $2-10 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$ (Desai *et al.*, 1997). One of the specific properties of the tubulin molecule is the ability to bind colchicine and related organic substances, derived from plants which can affect the functions of microtubules. Tubulin can also bind to calcium and magnesium. In the presence of zinc ions, tubulin molecules are precipitated as sheets, which are used for electron microscopic structural studies. These studies have shown that tubulin consists of elongated, wedge-shaped subunits. Neighbouring subunits in the sheets, which probably correspond to α - and β -chains, have slightly different shapes.

4.1.3.6. Variation in number of protofilaments

Microtubules are one of the varieties of tubulin polymers that have been observed, and the protofilament appears to be a universal component in all of these polymers. Thus

the longitudinal interactions between dimers along the protofilaments is a constant in all of the polymers. The various polymers differ greatly, though, in the lateral interactions between protofilaments. The number of protofilaments in a microtubule can vary from 9 or fewer, to 16, which suggests that there is a high degree of flexibility in the lateral association between protofilaments even within microtubules. (Kenneth *et al.*, 2000). Individual subunits in the wall of microtubules can be seen in preparations pre-treated with tannic acid. The number of these subunits in the cross section of the microtubule corresponds to the number of protofilaments. Examination of protofilaments forming the wall of microtubules, shows that they are parallel to its axis. Tannic acid preparations from different sources have shown that microtubules can have different numbers of protofilaments. Microtubules in brain and other tissue of most animals have 13 protofilaments.

There are, however, certain types of complete microtubules that have not 13, but, 11, 14, 15 or 16 protofilaments. For example, microtubules of most cells of the nematode *Caenorhabditis elegans* have 11 protofilaments; certain neurones of these animals have microtubules with 15 protofilaments. The number of protofilaments in the microtubules in a cell is constant. In contrast, microtubules assembled *in vitro* from brain tubulin or from flagellar tubulin have varying numbers of protofilaments, with 14 predominating. These data suggest that the number of protofilament within the microtubules is not determined by the structure of tubulin, but is controlled by some special cellular mechanism.

4.1.3.7. Structure of tubulin

The tubulin structure can be divided into three domains. The N-terminal half of the sequence, residues 1-206, forms the nucleotide-binding domain.

The middle part of the tubulin sequence, residues 207-384, which shows almost no sequence similarity to other proteins, forms a domain that is involved in contacts between protofilaments. This domain is also involved in hydrolysis, and can thus be considered an essential catalytic subunit of the molecule.

A third domain is formed mainly by two rather long helices, the loop that connects them, and the C-terminal residues. The last 10-18 residues are disordered and thus not

visible in the crystal structure, but they play a significant role in various tubulin activities (Kenneth *et al.*, 2000).

4.1.3.8. Dynamism of microtubules

In eurykaryotic cells there is a state of equilibrium between dimers and polymers of tubulin, so that the network can respond flexibly and quickly to functional requirements (Patocka *et al.*, 1999). Dynamic instability can be characterized by four distinct rate constants: polymerization rate, depolymerization rate, rate of transition from growing to shrinking behaviour, and rate of transition from shrinking back to growing (Belmont *et al.*, 1990). Studies with MTs (microtubules) both *in vitro* and *in vivo*, have demonstrated that MTs also turnover by a process termed “treadmilling” whereby subunit addition is favoured at one end of the polymer and subunit disassembly is favoured at the opposite end of the polymer. (Cassimeris *et al.*, 2001)

The mitotic spindles are composed of dynamic arrays of microtubules (MTs) and associated proteins whose assembly and constant turnover are required for both spindle formation and chromosome movement. As the cells enters mitosis, radial arrays of long interphase MTs are disassembled and converted to bipolar spindles composed of much shorter MTs. This reorganization is accompanied by changes in MT assembly dynamics; entry into mitosis corresponds with a 10-fold increase in the rate of MT turnover.

At the time of nuclear envelope breakdown in mitosis, the total MT polymer level drops precipitously. The remaining MTs show a concurrent dramatic increase in turnover rate. Subsequently, the polymer level returns nearly to interphase level as the spindle assembles. As the cells progress from mitosis to G1, the interphase MT arrays are reformed without the significant loss in polymer observed at the G2/M transition, and the dynamic turnover returns to the slower rate typical of interphase. (Cassimeris, 1999)

Tubulin assembly is very sensitive to many environmental factors. It is inhibited by low temperature and it requires the presence of GTP and magnesium ions (Oxberry *et al.*, 2001). Brain tubulin preparations, containing MAPs (see 4.1.3.10), assemble into microtubules at 37 °C in solutions holding millimolar concentrations

of Mg^{2+} and of GTP, as well as the calcium-chelating agent, EGTA. However, when MAPs are removed from the preparation, assembly of purified tubulin does not take place under these physiological conditions. Purified tubulin without MAPs can be forced to make microtubules only by certain additional alterations of the medium, such as an increase of magnesium concentration up to 10 mM, or addition of 3-4 M glycerol. Conditions of polymerization depend also on the type of tubulin used. There are some exceptional tubulins that are easier to polymerize than brain tubulin. For example, purified tubulin from sea urchin eggs was observed to form microtubules in minimal physiological conditions described earlier. By varying environmental conditions, one can repeatedly polymerize and depolymerize microtubules. This method is used for purification of tubulin from tissue homogenates. Some chemicals such as taxol are often used to stabilize the polymerized microtubules in the course of tubulin and MAP purification. Polymerization of tubulin, like that of actin, can be assessed by measuring certain physical parameters of tubulin solution, principally, light scattering or viscosity.

4.1.3.9. Nucleation of Microtubules

The time course of polymerization of tubulin, like that of actin, is described by a sigmoidal curve: the stage of growth of polymer concentration is preceded by a lag period. Probably, formation of polymerization nuclei takes place during the lag period and microtubule elongation occurs during the growth stage. In fact, when preformed fragments of microtubules are added to the tubulin, rapid increase of polymer concentration begins immediately without the lag period. Nucleation seems to be more sensitive to environmental conditions and to removal of MAPs, than elongation (Karsenti and Vernos., 2001). Elongation of preformed microtubules in the presence of purified brain tubulin can take place in “physiological” conditions without glycerol or high magnesium concentrations. The nature of the nuclei formed during the lag period is not clear. Electron microscopic studies had shown that small fragments of several laterally associated protofilaments are present at the beginning of polymerization. These data suggest that the early stage of microtubule assembly is a two-dimensional process.

4.1.3.10. Microtubule-associated proteins (MAPs)

In gel electrophoresis, tubulin is always accompanied by several associated proteins. These have been found in tubulin preparations from various sources. Their presence, even after a repeated cycle of assembly/disassembly *in vitro* often in stoichiometrical relations with tubulin, suggests that they play a part in the structure of MT. These proteins have been shown to influence both the initiation and elongation processes of microtubule assembly *in vitro* (Kim *et al.*, 1979).

Tubulin interacts with a large number of proteins in carrying out its activities. Some of these proteins modulate the stability of microtubules, while some are active motor proteins that carry “cargo” along microtubules (axonal transport). It is quite clear that the interaction of proteins directly with the C terminus could be involved in some way in the regulation of polymerisation of microtubules (Kenneth *et al.*, 2000).

Microtubule-associated proteins (MAPs) are usually attached to the surface of the wall of the microtubules. They have been known for some times to stabilize or “bundle” microtubules (Jourdain *et al.*, 1997).

Stabilization is a general classification and it could undergo through: slowing dissociation; stimulating addition; blocking catastrophes; stimulating rescue or slowing the rapid shortening phase.

Among the stabilizing MAPs are: mammalian MAP1A and MAP1B, which are large proteins and encoded on two separate genes; MP2, is a heat-stable mammalian MAP A found especially in dendrites of neuronal cells; Tau, increases the amount of MTs assembled mainly by increasing the MT nucleation (Kirshner., 1978); MP4, TOGp, and STOPs stabilize MTs against depolymerization by cold temperature; APC and the EB1 family. Some examples of destabilizing proteins are: MCAK family, Op18/stathmin, Ktannin and MINUS.

The MCAK family are members of the Kin I kinesin subfamily and have the catalytic domain in the center of protein. Overexpression of MACK results in loss of MT polymer, Oncoprotein 18(Op18)/stathmin. This protein was initially identified based on its increased expression in acute leukaemia. Op18/stathmin is a soluble protein that does not show significant binding to MTs. Instead it binds tubulin dimers, primarily through an interaction with a tubulin to form a complex of one Op18/ stathmin/ and two tubulin dimmers. (Cassimeris *et al.* , 2001)

Kinesin and dynein families thought to be major factors in the regulation of MT assembly. The number of known motors of these families continues to grow rapidly (Kenneth *et al.*, 2000). Also dynein is an MAP of special importance because its interaction with tubulin is essential for the motility of ciliae and flagellae. Recently Whealy *et al.* (2001) have reported an essential chromosomal passenger protein INCENP (inner centromeres protein) which directly binds to tubulin and plays a key role in cytoskeletal events during mitosis.

MTPs show cell cycle dependent localization. For example ZYG9 is cytosolic during interphase but in mitosis it binds to spindle poles (Cassimeris *et al.*, 2001).

4.1.3.11. The microtubule-associated nucleotides and polarity of microtubules

The early work on purified tubulin indicated that the tubulin dimer can bind two molecules of GTP. One of the bound molecules (that bound to β -tubulin) is rapidly exchanged for another GTP or GDP molecule, present in the solution, but the exchange of the second molecule (that bound to α -tubulin) is very slow, so these sites are called exchangeable (E) and nonexchangeable (N) sites. Other nucleotide triphosphates bind weakly, or not at all.

As long as GTP-tubulin is being added to the microtubule, there will always be a cap of GTP-tubulin at the plus end, ensuring stability of the microtubule. However, under conditions where either GDP-tubulin is added or the GTP is lost, the microtubule can enter a phase of depolymerization.

GTP-tubulin is component for assembly, and microtubules containing non-hydrolyzable GTP analogues are highly stable; however, with bound GDP, they are unstable (Kenneth *et al.*, 2000).

Due to their polar structure, microtubules have two unequal ends, called plus and minus ends. These ends have a number of different properties; in particular, the growth of microtubules in tubulin solutions is usually faster at the plus ends than at the minus ends. The demonstration that the nucleotide at the plus end of a microtubule could exchange indicated that the plus end of microtubules was capped by β tubulin. (Mitchison, 1993)

All the protofilaments forming the microtubule wall have the same polarity, so that the whole microtubule is also polar and has unequal plus and minus ends.

4.1.3.12. The role of microtubules in mitosis and other cell functions

Although microtubules are present continuously in many cell types, they also may be formed or broken down as needed by the cell. The most obvious examples of such a cyclic formation and breakdown of microtubules occur during mitosis, but it may also occur during developmental processes involving changes in cell shape (Weisenberg, 1972).

Disassembling the interphase microtubule cytoskeleton and building the array of the spindle requires the rapid but highly regulated turnover of microtubules, so they must be highly dynamic in order to carry out this function (Kenneth, 2000).

The spindle (figure 4.3) itself is a highly dynamic structure as microtubules first explore the cytoplasm to capture all the chromosomes and then move them to the poles by processes that include microtubule shortening as well as motor activity. At the other stages in the cell cycle, or in particular cell types or organelles, stable microtubules are required, such as for transport within axons or for ciliary and flagellar movement. Interfering with microtubule dynamics or stability can inhibit cell division in several ways. Either stabilizing microtubules or inhibiting polymerisation will prevent the cytoskeleton restructuring that is required at several points in the cell cycle and thus stop progression from one stage in the cell cycle to the next. (Wilson *et al.*, 1999). The essential role of microtubules in cell division and the ability of drugs that interact with tubulin to interfere with the cell cycle have made tubulin a successful target for applications that include anticancer drugs, fungicides, and herbicides. It is, of course, of fundamental interest to understand the interactions of tubulin with the proteins that produce motion and that regulate dynamics (Kenneth *et al.*, 2000).

The microtubules are attached to the chromosomes, in most cells, at specialized structures: kinetochores. During mitosis these undergo a progressive differentiation and display their complexity at metaphase. Before separation of the daughter chromosome, the two kinetochores of each chromatid face in opposite direction: the proper orientation of the chromosomes demands that the kinetochore-microtubules extend towards the two poles of the spindle before anaphase. In anaphase, the chromosomes move (or are pulled) toward the poles, and the poles separate from one another.

In metaphase, the spindle shows a variable number of microtubules, which are attached at the vicinity of the centrioles and at the kinetochores. Also microtubules seem to extend from one pole to the other pole. The existence of truly “continuous” microtubule is doubtful, and the polar spindle may be made of long microtubules extending for only part of the spindle length. Most of these microtubules are parallel but it is not always the rule. In many cells the spindles are barrel-shaped, the microtubules curving toward the poles whether the centrioles are present or not. The number of microtubules vary considerably from one type of cell to another. For example, in the endosperm of *Haemanthus catherinae* Back. the spindle contains from 5000 to 10,000 microtubules and from 70 to 150 microtubules are attached to each kinetochore at anaphase and in some yeast there are only a few microtubules in the mitotic spindle (Winey *et al.*, 1995).

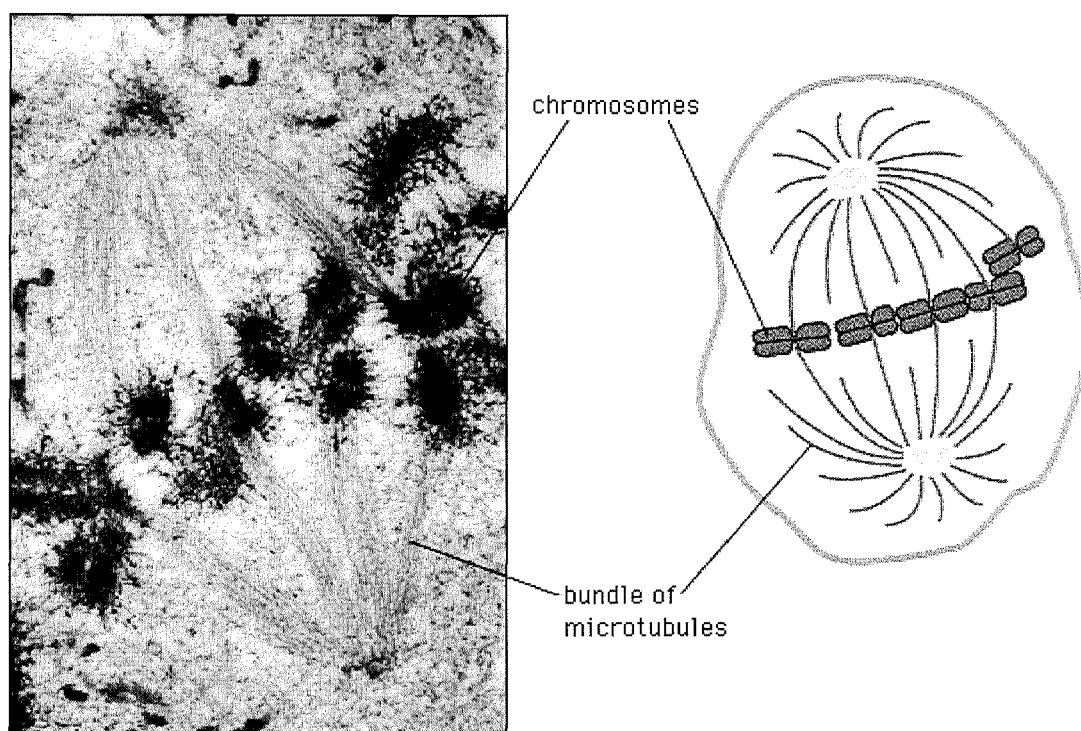


Figure 4.3. Microtubules in mitotic spindle

The figure was taken from bengal.missouri.edu/~forgacsg/Course331/Image1.html

4.1.4. Microtubule poisons

Tubulin is the target of many naturally-occurring toxic compounds that cause cells to arrest in mitosis (Bai *et al.*, 1992). Tubulin-binding agents constitute a large group of compounds which have been used in a wide variety of ways, including as pesticides and antiparasitics and in human therapeutics (Dumonent *et al.*, 1999).

Due to diverse role of MT in cell functions, it is not always easy to find out which effect is mediated via changes in the MT, and some pharmacological effects of these drugs may be unrelated to MT poisoning. One approach to this, which has been followed by several authors, is to study the effect on a definite cell function of a series

of chemically unrelated MT poisons: if they are similar, and can also be imitated by physical means, such as cold or high hydrostatic pressure, it can with some safety be concluded that the effects are mediated through MT poisoning and not by non-specific actions.

The first such agents isolated were obtained from higher plants and include colchicine, podophylotoxin, steganacin, the vinca alkaloids, maytansine and taxol. Subsequently, the ansamitocins, phomopsin, and rhizoxin were obtained from fungal organisms. Later the peptide Dolastatin 10 and the depsipeptide Dolastatin 15 were obtained from the mollusc *Dolabella auricularia*. (Bai *et al.*, 1992). Also recently Haggarty *et al.*, (2000), have reported a number of small molecule agents that interrupt mitosis via either affecting microtubules or in other ways.

For example monastrol which affects Eg5 (a motor protein important in forming and maintaining of mitotic spindle).

4.1.4.1. The classification of antitubulin agents

4.1.4.1.1. Compounds binding to the GTP site

The best known of this group are vinca alkaloids, compounds derived from *Catharanthus roseus* (formerly classified as *Vinca rosea*), a plant from warm climates. The most important compounds of this group are vinblastin and vincristin which composed from a tetracyclic structure of catharantine and a pentacyclic structures of vindoline. The analytical of localization of the vinblastin-binding site on tubulin has indicated that it occurs at the central region of the beta-tubulin subunit (Rai *et al.*, 1996). It has been shown that vinca alkaloids and other related compounds prevent the binding of GTP in this region. Exposure of cells to large concentration of vinblastine leads not only to the disassembly of microtubules, but also to the formation of crystal-like aggregates of tubulin in the cytoplasm (Bershadsky *et al.*, 1988)

4.1.4.1.2. Compounds binding to the colchicine site

Colchicine has not only been an important drug in the reduction the pain of gout (Dustine, 1978) but also it has had a significant role in cell biology and genetics.

Indeed tubulin was originally isolated through its ability to bind colchicine. Colchicine inhibits microtubule formation, and at high enough concentration, disrupts microtubules. Evidence has been presented for interaction of colchicine with both α and β monomers, although most results suggest that the main interaction is with β . Separate experiments have identified crosslinking of colchicine analogues to peptides in β , including residues 1-46 and 216-242 (Uppuluri *et al.*, 1993). However, Bai. *et al* (2000) reported that colchicine binds to cysteine residues 239 and 354 on β tubulin. Binding of colchicine is biphasic and temperature-dependent. The first step is rapid but weak, with the second step slower. The binding of colchicine to tubulin becomes faster when a methyl group replaces the acetyl group present on the amine of β ring, yielding the compound known as colceimide.

Colchicine is capable of inhibiting microtubule polymerization at concentrations that are considerably below the concentration of free tubulin molecules (Bershadsky *et al.*, 1988). Appreciation has grown the normal effect of colchicine in the cell has more to do with stopping the dynamic instability that allows microtubules to explore the cell than with disruption of microtubules *per se* (Wilson *et al.*, 1999; Kenneth *et al.*, 2000)

Nocodazole, as well as Podophyllotoxin which, is a tetracyclic compound obtained from *Podophyllum peltatum*, binds to tubulin at the same sites that colchicine binds.

4.1.4.1.3. Microtubule-stabilizing compounds

Paclitaxel (Taxol) is the active ingredient of one of best-known anticancer drugs. The effect of taxol on the microtubules is in many respects opposite to that of other microtubule- binding drugs. Taxol enhances both the yield and rate of rate of microtubule assembly. It decreases the critical concentration of tubulin required for assembly and promotes polymerization (Bershadsky *et al*, 1988). In the structure of taxol there are two aromatic rings, a tetracyclic structure and an oxetane ring, which is

required for activation of drug. The primary action of this drug is to stabilize microtubules, preventing their depolymerization. The binding site of taxol appears to occur at a different location at the amino terminal of β -tubulin, but binding to the middle region of a α -tubulin has also been reported (Patocka, *et al.*, 1999).

Paclitaxel and related taxanes are among the most important antitumor agents (Snyder *et al.*, 2001; Das *et al.*, 2001) which were first described as members of a MT stabilizing class of compounds, but there is an ever-increasing number of other drugs that also stabilize microtubules and for which there is evidence that they bound in the same region on tubulin (Bershadsky *et al.*, 1988).

4.1.4.1.4. Compounds with disorganizing effect on microtubule network

Some natural marine compounds with anticancer activity have been found to disorganise the microtubule network (Garcia-Rocha *et al.*, 1996). Ecteinascidin 743 and tetrahydro isoquinoline are two examples of this group (Ghielmini *et al.*, 1998).

In addition, there are several distinct classes of drugs that destabilize microtubules for which there is as yet no clear indication of the binding region. These include the benzamidazoles, such as Benomyl, which is used as a fungicide and antihelminth, and dinitroaniline derivatives including Trifluralin, widely use as a herbicide. Based on the lack of competition with other drugs, these appear to bind in sites different from the vinca or colchicine sites (Kenneth *et al.*, 2000).

4.1.4.1.5. Resistance to antitubulin agents

At present the best-described mechanism of resistance to tubulin-binding agents is the MDR (multiple drug resistance) phenotype, mediated by the 170-kDa Pgp efflux pump, encoded by the *mdr1* gene. Both vinca alkaloids and the taxanes are good substrates for this pump. In a number of cases, development of cell lines resistant to vincristine or paclitaxel has been shown to be associated with the expression of *mdr 1*. (Dumontet *et al.*, 1999; Druley *et al.*, 2001; Van der Sandt *et al.*, 2001). The other possibility is that the alterations in the structure and/or function of microtubules represents an important and potentially complex mechanism of resistance to

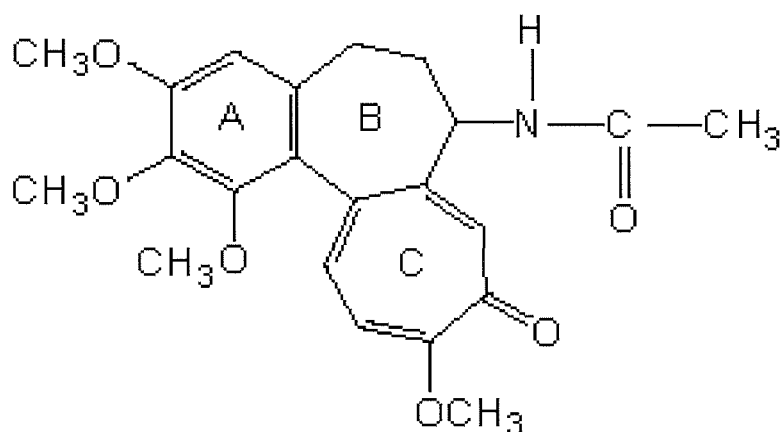
antitubulin agents. For example, a number of cell lines resistant to tubulin-binding agents *in vitro* have been shown to contain tubulin alterations, in term of total tubulin content, tubulin polymerization, or tubulin isotype content (Dumontet *et al.*, 1999).

4.1.4.2. Colchicine

Colchicine consists of pale yellow scales or powder, which darkens on exposure to light. It is an alkaloid soluble in water, freely soluble in alcohol and in chloroform, and slightly soluble in ether. The chemical name for colchicine or Acetyltrimethylcolchicinic acid is: N-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy- 9-oxobenzo [alpha] heptalen-7-yl) acetamide (molecular weight 399.43) (Budavari *et al.*, 1989).

Colchicine was first used over 2000 years ago in the form of preparations of the meadow saffron *Colchicum autumnale*, and is still one of the more effective treatments for the intense pain associated with a gout attack.

As the fig.(4.1) shows, colchicine is a tricyclic alkaloid, the main features of which include a trimethoxyphenyl ring (A ring), a seven membered ring (B ring) with an acetamide at the seven position, and a tropolonic ring (C ring). Colchicine is found in the leaves and seeds of at least 19 species of *Colchicum* and 10 other related genera.



colchicine

Figure 4.4 The structure of colchicine

The goal of most colchicine research is a more thorough understanding of the cause of gout, which is often thought of as a disease of rich living. However, as many victims will testify, the affliction does not limit itself to this lifestyle. Gout, from the Latin *gutta*, meaning drop, was used to describe the symptoms because physicians presumed the disease was caused by the “dropping of phlegm” into the big toe. Hyperuricosia, or elevated blood levels of uric acid, causes the common symptoms of gout (Katzung., 1998). In humans and other primates, uric acid is the final metabolite in the breakdown of purines. When this metabolic pathway becomes overwhelmed, from either an enzymatic deficiency or an increase in dietary purines, uric acid cannot be efficiently eliminated from the body. The poorly-soluble uric acid crystallizes, initiating a response from macrophages and leukocytes. The phagocytosis of urate crystals by the macrophages and leukocytes stimulates the release of cytokines and interleukins, leading to inflammation and the distinctive symptoms. The precise mechanism by which colchicine relieves the intense pain of gout is not known. However, it is believed that the major relief of pain involves colchicine's major pharmacological action: binding to tubulin dimers. Colchicine is a dangerous substance, which needs careful use and disposal. It is very toxic and suspected to be carcinogenic. It is

advisable to wear gloves and a mask when handling either the colchicine or medium containing it so as to avoid hazards should an untimely cough, sneeze or spill occur. One flower of *Colchicum autumnalis* (Fig.4.5) contains about 12 mg colchicine, 20g tuber provides 60mg, single seed provides 3.5mg and less than 2 gram of seeds of this plant is deadly for a child. Autumn Crocus (*Colchicum autumnale*) and Glory Lily (*Gloriosa superba*) contain about 0.1-0.8% colchicine in their bulbs and other parts.

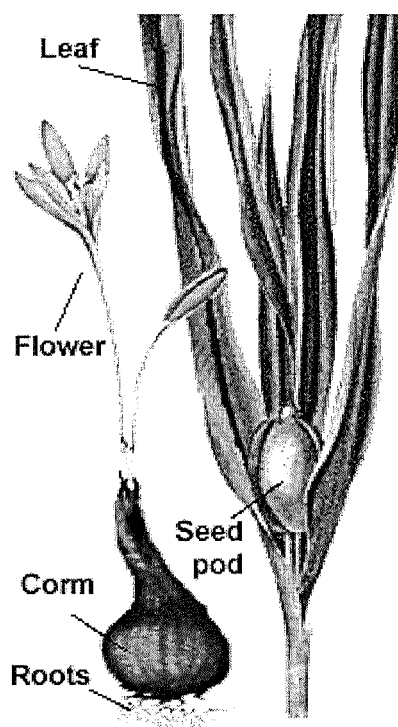


Fig.4.5. *Colchicum autumnalis*

An overdose of colchicine always leads to the delayed onset of multiorgan failure and is frequently fatal. There is no specific treatment and the chances of survival can only be influenced by early and aggressive gastrointestinal decontamination (whether or not the patient is symptomatic).

The toxicity of colchicine for humans is about 7-10 mg, and the ingestion of doses more than 40 mg is always fatal within three to four days of the ingestion of the alkaloid (Bruneton, 1995). At the autopsy of victims of colchicine, haemorrhagic

lung oedema, hypocellular bonemarrow, centrilobular fatty necrosis of the liver and necrosis of the proximal convoluted tubuli of the kidneys have been reported. It is widely accepted that a dose of 0.5 mg/kg can cause gastrointestinal symptoms, while a dose exceeding 0.8 mg/kg usually results in cardiogenic shock. Nevertheless, as little as 7 mg of colchicine have been reported to be fatal (Klintschar *et al.*, 1999).

Cornigerine is a natural product analogue of colchicine produced by *Colchicum cornigerum* and can inhibit tubulin polymerization both with and without microtubule-associated proteins, inhibit the binding of radiolabelled colchicine to tubulin and stimulate tubulin-dependent GTP hydrolysis (Hamel *et al.*, 1988)

4.1.4.3. Taxol

Paclitaxel is one of the metabolites of gymnosperm family or Taxaceae (commonly known as taxads or the yew family). This plant is remarkable in having a single ovule borne terminally on short lateral shoots. In most other conifers the ovules are born axillary on cone scales. Due to the efficacy of taxane compounds in anticancer therapies, since its discovery in 1960s, intensive studies of its chemistry – structural activity relationships have been reported (Gunatilaka *et al.*, 1999; Cheng *et al.*, 2000). The various species of this genus are in the only genus of the the taxaceae family. The leaves are like flat needles, the male flowers have 6-14 anthers shaped like sheilds, and a female apparatus is an ovule surrounded by scales. The most well-known species in Western Europe is *Taxus. baccata* L., a large tree which grows slowly and lives for an exceptionally long time. Since it is highly decorative it often is seen in parks and gardens. The species has been known since ancient times for the toxicity of all of its parts (except for the aril) for human and animals (especially horses) (Bruneton, 1995). There are different categories of metabolites in the leaves and stems, including saccharides, polysaccharides, and cyclitols, fatty acids sterols, bisflavonoids (sciadopittysin and kayaflavone), proanthocyanidins, ligans, and cyanogenic glycosides. Up to now about 300 structurally diverse taxanes have been isolated and identified (Zhang *et al.*, 2000).

The most interesting constituents are diterpenes with a taxane nucleus, including taxusines, taxagifin, baccatin III and derivatives, taxine (a complex mixture of taxine A, B, and of their derivatives), taxol, cephalomannine, taxins and derivatives. Taxol (Fig. 4.6) was initially isolated from the bark of the trunk of the Pacific yew (*T. brevifolia* Nutt.) but it is present only in traces (0.01%). Thus the compound could not be produced without destroying the species. At best, a one hundred year-old tree produces about 3 kg of bark and 300 mg of taxol. Even with optimized extraction methods, 15,000 pounds of dried bark would be required to yield 1 kg of taxol. So in recent years controversial issues surrounded taxol and the Pacific Yew. Most dealt with efforts to obtain sufficient quantities of the chemical compound to get sufficient amount of drug. Systematically screening the taxus genus resulted in the selection and cultivation of *T. x medica* Hicksii. The leaves of this trees are exploitable and renewable source of taxol (Bruneton, 1995). In 1994, the first synthetic analogue of taxol was introduced (Nicolaou *et al.*, 1994). Today several new taxol derivatives and oral preparations of the drug are in clinical trials (He *et al.*, 2001).

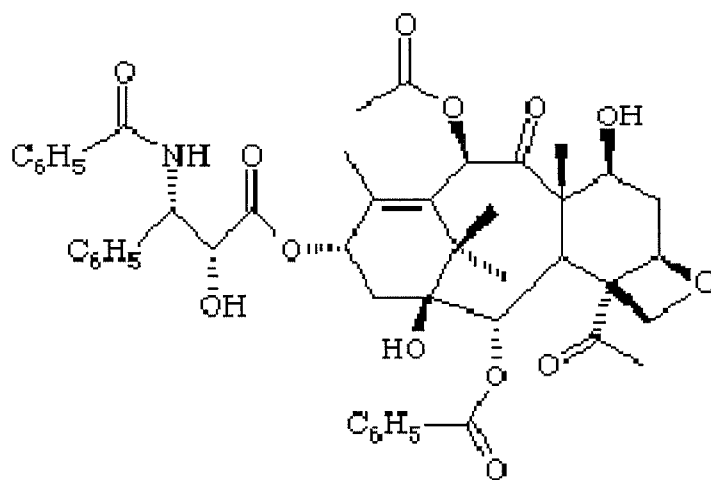


Figure 4. 6 The structure of Taxol

4.2. Methods and Materials

4.2.1. Cell lines

Cell lines and cell counting methods have been described in previous chapters.

4.2.2. Chemicals

Chemicals including taxol (paclitaxel), colchicine, ribonuclease A, propidium iodide, vinblastine, and anti- α -tubulin antibody (clone b-5-1-2) were purchased from Sigma (Sigma-Aldrich, Poole, UK). The dyes “Hoechst 33342”, DCVJ 9-(dicyanovinyl)-julolidine, and fluorescein colchicine (C-662), were purchased from Molecular Probes LTd, The Netherlands.

4.2.3. Fluorescence microscopy

4.2.3.1. Mitotic figures.

Harvested cells were fixed in 50% v/v acetic acid/methanol (first 2 minutes with 50% v/v growth medium and then 15 minutes without medium). Fixed cells were washed with PBS and resuspended in 1 ml phosphate buffered saline (PBS) containing 1 μ g of the DNA-specific “Hoechst 33342” dye. They were incubated for a few minutes at 37 °C prior to observation at 365nm under a fluorescence microscope. All measurements were carried out using a Leitz Laborlux S microscope with Pleomopak fluorescence illuminator under 50 X or 100 X objective lens. Fluorescence was quantified using a Leitz MPV computer with MPV-COMBI control electronics attached to a PC (Silicon Valley) installed with MPV-STAT software for data and statistical analysis (Leica). The microscope was equipped with a standard Hoescht/Dapi optical filter set. Measurement of fluorescence was expressed in arbitrary units (Modha *et al.*, 1997)

4.2.3.2. Cellular binding of azadirachtin and known antimitotic agents.

Sf9 cells were first incubated overnight in the presence of 10^{-8} M taxol in order to stabilise polymerised tubulin. The cells were then incubated for a further period of up to 4 h in the presence of either azadirachtin, colchicine or vinblastine all at a

concentration of 10^{-5} M. Finally the cells were exposed to colchicine-fluorescein at a concentration of 3×10^{-6} M. The effects of the antimitotic chemicals were compared to cells treated only with taxol and the fluorescent derivative of colchicine. The cellular binding of fluorescent colchicine was examined qualitatively and quantitatively by fluorescence microscopy using FITC filter with excitation wavelength of 488 nm.

4.2.4. Flow cytometry analysis (FACS)

Fluorescence-activated cell cytometry was performed on a Becton-Dickinson FACS cytometer. After harvesting, cells were washed twice in phosphate-buffered saline (PBS, pH 7.2). Cells were resuspended by trituration in 2 ml of PBS containing 10 or 20 μ g propidium iodide (for staining mammalian and insect cells respectively), 0.1% saponin and 0.1U/ml RNase and incubated for 30 min at room temperature. At least 1.5×10^4 cells were counted in each assay. The fraction of the total cell population presented in each cell cycle phase (G1, S, and G2/M) was obtained from DNA histograms using Cell Quest and Modfit Software. All cytometry experiments were performed on cells in log phase of growth. This was obtained by seeding cells at $0.5-1 \times 10^5$ cells/ml and allowing the cultures to grow for 48 h.

4.2.4.1. Effect of chemicals on cell cycle distribution of cultured cells.

In this part of experiment a series of concentration of azadirachtin, salannin, nimbin, pyrethrum, rotenone, taxol, colchine was added to medium of cultured cells and incubated for 3-24 h. Also to evaluate the simultaneous effect of some of these chemicals on cell cycle distribution, a series of combinations of taxol-azadirachtin, taxol-colchicine, and colchicine-azadirachtin were used in cell cytometry experiments.

4.2.5. Purification of Microtubules

Tubulin was prepared from pig brain by a method essentially similar to that of Carraway, et al (1992), which is based on the temperature-dependence of polymerisation of tubulin. After homogenization (Potter-Elvehjem homogeniser) in

the cold (4°C) in PM-4M buffer (0.1 M pipes, 2 mM EGTA, 1 mM MgSO₄, 2 mM DTE, 4 M glycerol, adjusted to pH 6.9 with NaOH), the cell debris was spun down at 96,000 x g (35,000 rpm) at 4°C for 45 min. Then GTP was added to supernatant, to bring the concentration to 0.5 mM, and it was incubated for 45 min in 37°C to ensure polymerisation of the tubulin. The polymerised protein was recovered by centrifugation for 40 min at 96,000 x g, at 27°C. The supernatant was removed and the pellet of polymerized microtubules was resuspended in 1/5 volume of the previous stage and chilled at 4°C for 45 min. The assembly and disassembly was repeated and the final pellet of polymerised tubulin stored at -80 °C until required. The purity of the tubulin was assessed by means of electrophoresis and immuno-blotting as described below.

4.2.6. Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

4.2.7. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in stacking (5%) and separating (12.5%) polyacrylamide gels in a Protein II system (BioRad, USA). Samples of purified protein were diluted in an equal volume of 0.125 M Tris-HCL, pH 6.8 with 4% (w/v) SDS, 20% (v/v) Glycerol, 0.002% Bromophenol Blue B and 9% (v/v) β-mercaptoethanol (which was added just before use) and the mixture was immediately boiled for 3-5 min to denature the protein.

Electrophoresis was performed in a 25 mM Tris/0.2 M glycine buffer containing 0.1% (w/v) SDS. Gels were run at 120 mV and 400 mA employing the dye as tracker. Proteins were visualized by staining with 1% Brilliant Blue, and commercial reference proteins (Rainbow, Amersham Pharmaciabiotech Ltd. UK) of known

molecular weights were used to determine the molecular weights of the tubulin monomers.

2.4.8. Immunoblotting

Immunoblotting of purified tubulin was performed as described by Towbin *et al.*, (1979). After electrophoresis in SDS-PAGE, proteins were transferred to nitrocellulose membranes (Sigma, USA) by electrophoretic blotting overnight at 150 mA in 25 mM Tris -HCL, 192 mM glycine with 20% (v/v) methanol. The nitrocellulose membranes were then treated with 3% (w/v) bovine serum albumin solution in Tris-buffered saline (TBS), (50 mM Tris-HCl, 150mM NaCl, pH:7.4) at room temperature for 30 min to eliminate non-specific binding, and incubated at 37°C for 90min with monoclonal anti- α -tubulin antibody (clone b-5-1-2, Sigma Chemicals, Poole, UK) diluted 1:200 in TBS containing 0.005% (v/v) Triton X-100 and 5mM EDTA. After thorough washing with distilled water, membranes were incubated again at 37 °C for 90 min with a 1/16000 dilution of goat antirabbit IgG /horseradish peroxidase conjugate (Sigma. USA). Membranes were then again washed several times with distilled water and binding of the antibody revealed by incubating the membranes with horseradish peroxidase substrate consisting of hydrogen peroxide and 4-chloronaphthol chromogen (BioRad.USA.)

4.2.9. Determination of mitotic index

The mitotic figures of Sf9 cells was examined microscopically in presence of azadirachtin and colchicine. After 48 hours of cell growth , azadirachtin in 10% ethanol was added to 6ml of medium giving final concentrations of 2×10^{-6} - 2×10^{-9} M. Colchicine as a standard anti-mitotic agent was added to cell culture at a final concentration of 2×10^{-7} M. At various times up to 24 hours the cells were fixed and incubated with fluorescence dye “Hoechst 33342” for few minutes at 37°C. Stained cells were examined by fluorescence microscopy and mitotic index estimated based on percentage of mitotic cells in a sample of 100 counted cells in several fields.

4.2.10. Polymerization of Tubulin

This was followed by light scattering according to the method of Oxberry et al (2001). Purified tubulin ($0.5\text{--}1.0\text{mg}\cdot\text{ml}^{-1}$) was dissolved in Pipes buffer (PEM), (0.1M pipes, 0.1 M NaOH, 1mM EGTA, 1 mM MgSO_4 ,) to give a final concentration of 1 mg/ml, and 2ml volumes placed in a standard 1cm light path quartz cuvette. Additional GTP was also added to the cuvette to a final concentration of 1 mM. The contents were mixed and light-scattering was measured continuously at 300 and 390 nm for azadirachtin and colchicine respectively (the preferred wavelength is 300nm as this gives the greatest degree of scattering, but colchicine absorbs strongly in this region) in a Beckman DU-7 spectrophotometer, at 37°C , for 1 h.

4.2.11. Permeability test.

To find the effect of azadirachtin on the permeability of cells, azadirachtin in a concentration of 10^{-4} was added to the cell culture flask of Sf9 cells. Control cells were incubated with solvent only. After 5 hours, cells were harvested and washed twice by PBS. Then cell cytometry was carried out for control and azadirachtin-treated cells: in each case exactly 15 minutes after adding propidium iodide (without saponin or any other permeablizer).

4.2.12. Assessment of uptake of azadirachtin by L929 and Sf9 cell lines

This experiment was based on the effect of cells on concentration of azadirachtin in medium. Two series of Sf9 and L929 cell flasks were prepared and incubated for 4 and 6 days respectively. Also two series of Sf9 cells prepared and incubated for 48 h. Azadirachtin at a concentration of 10^{-6} M in medium was exposed to confluent flasks of first series of cells (either Sf9 or L929 cells) and after 3 h exposure used to grow the second series of cells (Fig.4.27). In the end of experiment the effect of conditioned azadirachtin (azadirachtin after incubation with either Sf9 or L929 cells) on cell cycle of Sf9 cells was estimated and was compared with controls (Table 4.1).

4.3. Results

4.3.1.1. The effect of azadirachtin on cell cycle distribution of Sf9 cells

In the figure 4.7 is shown the cell distribution of Sf9 cell line in their growing phase. As the figure shows most of cells ($61 \pm 6\%$) are in G1 phase. The proportions of cells in S and G2/M, are $11 \pm 6\%$ and $28 \pm 3\%$, respectively.

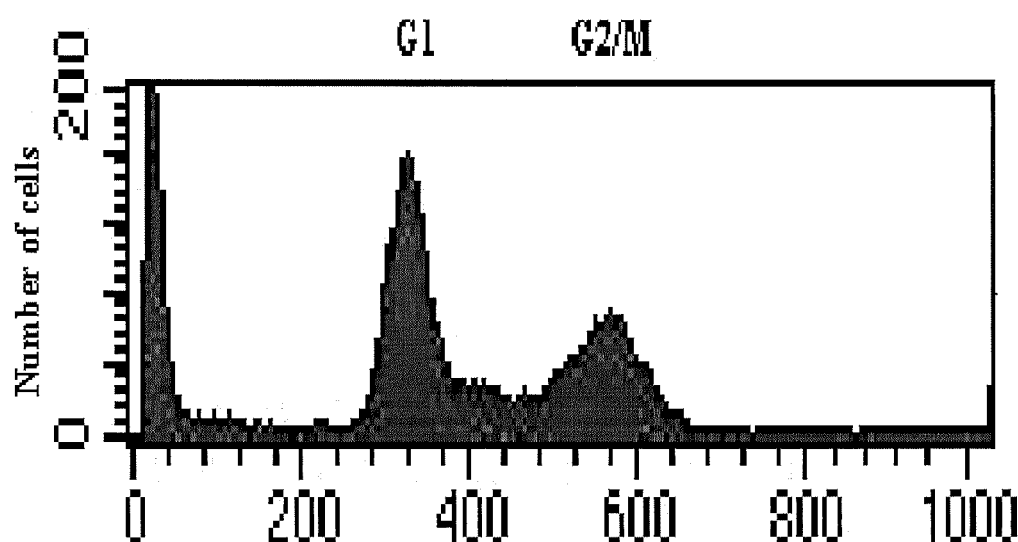


Figure 4.7 Cell cycle distribution of Sf9 cell line. Cells were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 $\mu\text{g}/\text{ml}$ propidium iodide in the presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA.

Figures 4.8-4.12 show the effect of different concentration of azadirachtin on Sf9 cells. As the figure 4.8 shows after 20 h. incubation azadirachtin in concentration of 10^{-8} M has caused an increase to 42% in G2/M phase and those in G1 show decline.

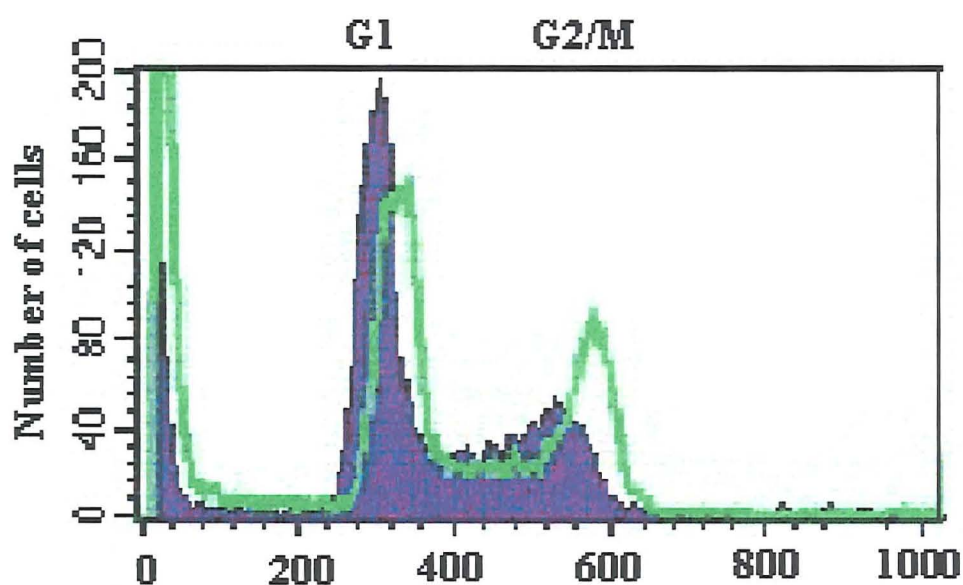


Figure 4.8 The effect of 10^{-8} M azadirachtin on cell cycle distribution of Sf9 cell line. The cells were incubated in presence (green) or absence (blue) of azadirachtin for 20 h, then were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 $\mu\text{g}/\text{ml}$ propidium iodide in presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA.

The effect of higher concentrations of azadirachtin were much more obvious.

At concentration of 10^{-6} M after the same time more than 90% of cells were arrested in G2/M phase (Fig. 4.9).

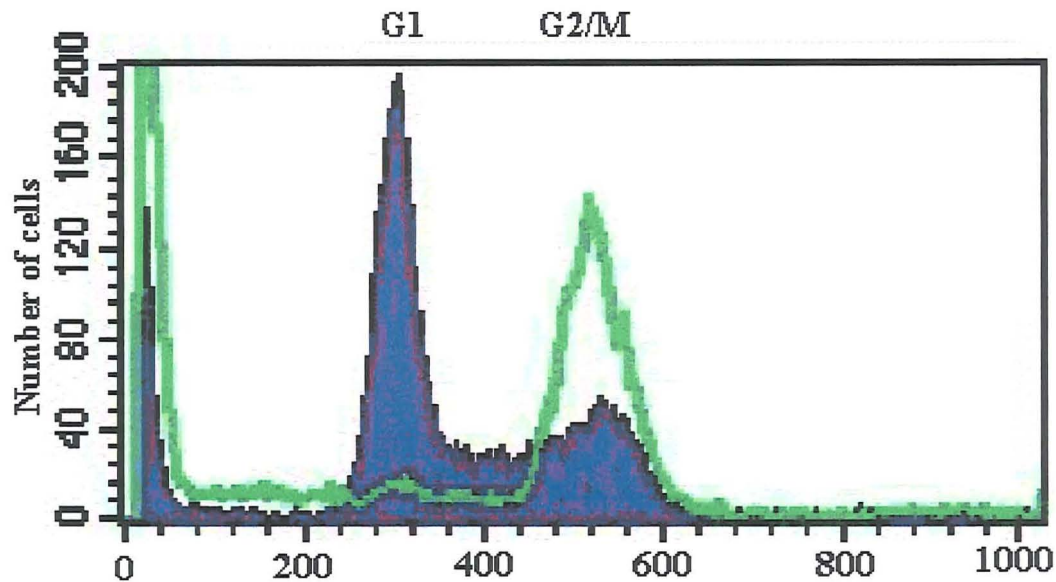


Figure 4.9. The effect of 10^{-6} M azadirachtin on cell cycle distribution of Sf9 cell line. The cells were incubated in presence (green) or absence (blue) of azadirachtin for 20 h. then they were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 μ g/ml propidium iodide in presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA.

Higher concentrations of azadirachtin showed changes in cell cycle in even shorter times. Figures 4.10(A) and 4.10(B) show the effect of concentration of 10^{-5} and 10^{-4} M after 5 and 3 h respectively. As the figure show in both cases there is about 8% change in cell cycle distribution.

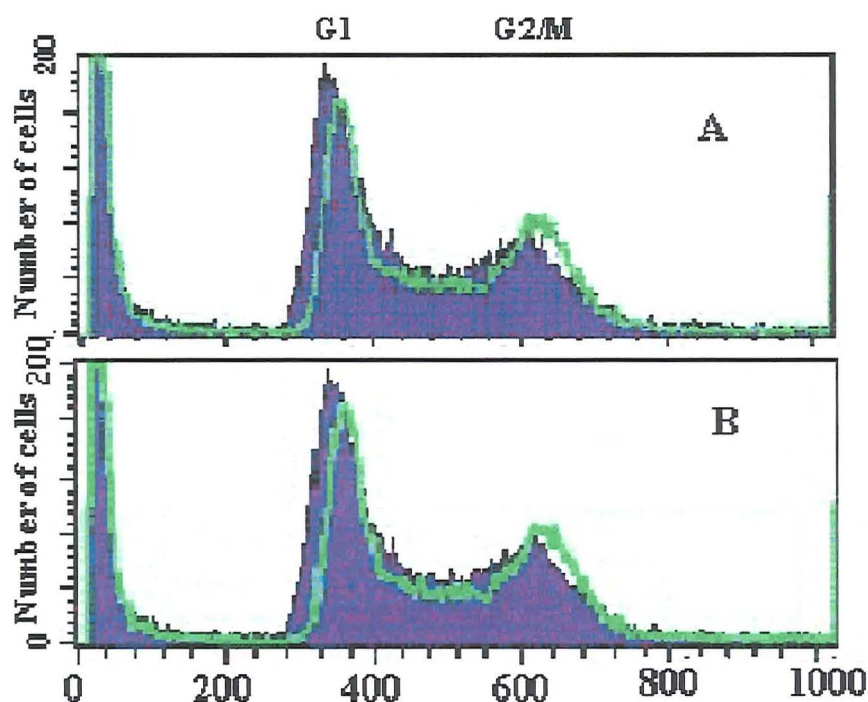


Figure 4.10 The effect of azadirachtin of 10^{-5} M (A) and 10^{-4} M (B) on cell cycle distribution of Sf9 cell line. The cells were incubated in presence (green) or absence (blue) of azadirachtin then were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 μ g/ml propidium iodide in presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA.

4.3.1.2. The effect of azadirachtin on cell cycle distribution of C6/36 cells

The other insect cell line, C6/36, responded in a similar fashion to azadirachtin with an accumulation of cells in G2/M phase and a decline in percentage of cells in G1 phase Fig. 4.11).

In this case the normal cells distribution showed 50% and 32% in G1 and G2/M phase, which after incubation with azadirachtin changed to 7% and 87% respectively.

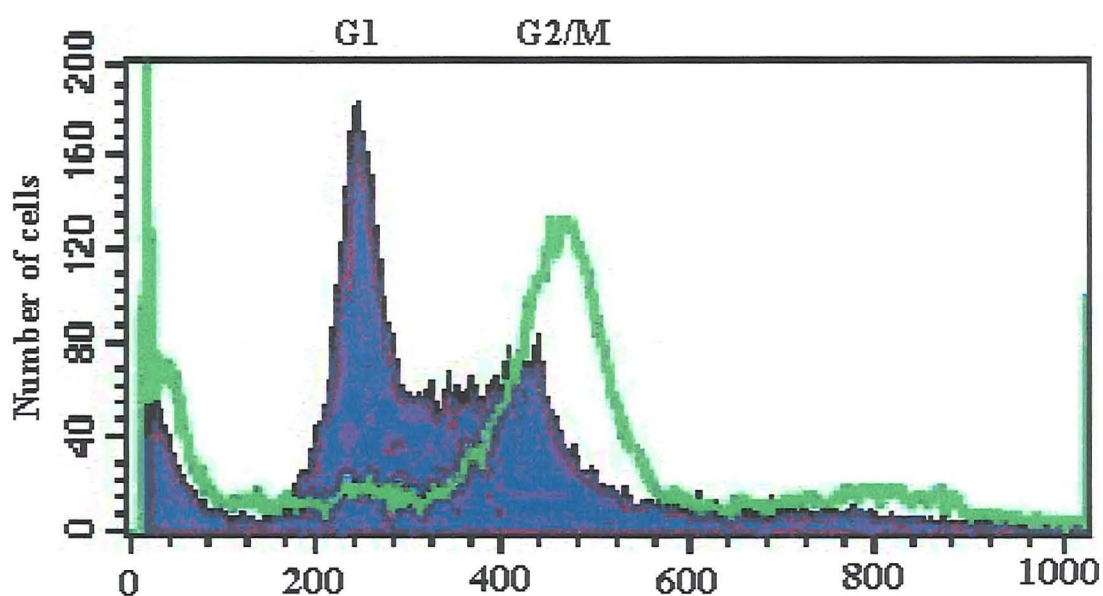


Figure 4.11 The effect of 10^{-6} M azadirachtin on cell cycle distribution of C6/36 cell line. The cells were incubated in presence (green) or absence (blue) of azadirachtin then were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 μ g/ ml propidium iodide in presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

4.3.1.3. The effect of azadirachtin on cell cycle distribution of L929 cells

Unlike insect cell lines, incubation of L929 cells with azadirachtin in concentration of 10^{-6} M did not change cell cycle distribution. The experiment was repeated with a higher concentration and to a period of up 7 days. In this case, for making the constant concentration of pesticide, every day the old medium was changed for medium containing the determined concentrations of azadirachtin, but there was no statistical significant change in cell cycle. Adding concentrations of azadirachtin to 10^{-4} M, caused slight change in S phase of the cell cycle. Interestingly, using a concentration of 5×10^{-4} M, not only caused more obvious change in S phase of cell the cycle but also there was some decrease in G1 and G2/M phase of cell cycle (Fig. 4.12.a and 4.12.b).

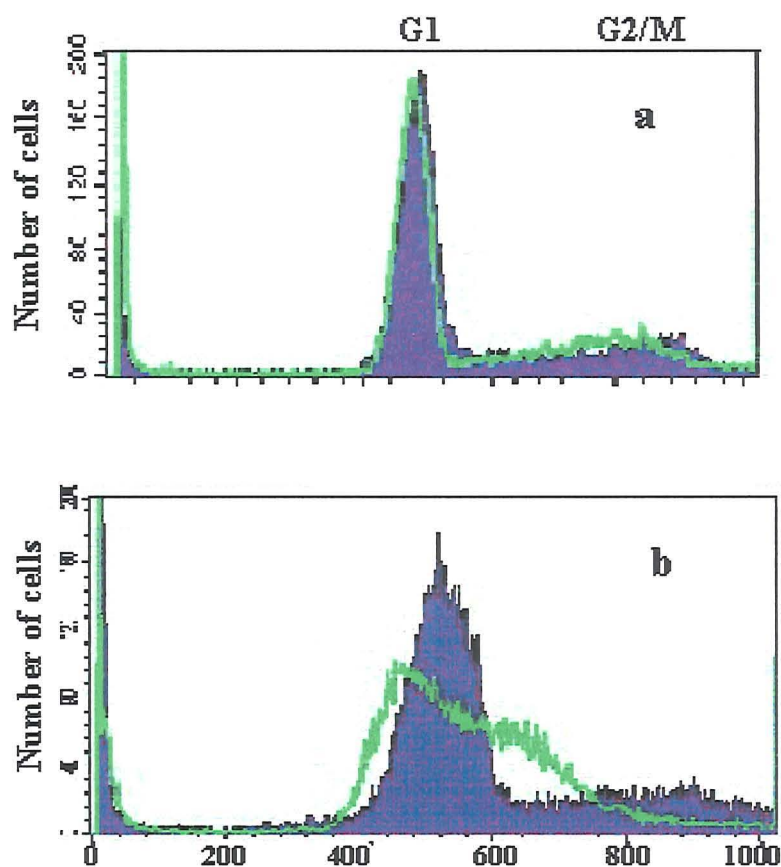


Figure 4.12. The effect of concentrations of a) 10^{-4} and b) 5×10^{-4} M azadirachtin on cell cycle distribution of L929 cell line. The cells were incubated in presence (green) or absence (blue) of azadirachtin for 7 days. After trypsination cells were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with $10 \mu\text{g/ml}$ propidium iodide in presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

The experiment was repeated by adding 1% saponin (as a membrane permeablizer) and concentration of 10^{-6} M verapamil (this calcium channel blocker affects the Pgp efflux pump and in some resistant cells increases the uptake of chemicals like colchicine (McFadzen., *et al.*, 2000)). The effect of saponin was not significant and verapamil even slightly lowered the effectiveness of azadirachtin in this cell line.

4.3.2. The effect of azadirachtin and some other phytochemicals on mitosis in Sf9 cells

The results of cell cytometry demonstrated that azadirachtin arrests cell cycle of insect cells in G2/M. To clarify if the arrest was in G2 or M phase a series of experiment were carried out. In these experiments the effect of azadirachtin and some other phytochemicals on mitosis was compared.

4.3.2.1. The effect of azadirachtin on mitosis in Sf9 cell line

Fluorescence microscopy of Sf9 cells showed that azadirachtin at a concentration of 2×10^{-7} M caused a slow accumulation of mitotic figures of these cells which lasted for more than 24 h. A concentration of 2×10^{-6} M of this chemical caused a sudden increase in mitotic index up to 9% (the value for control cultures was $3.6 \pm 0.8\%$) which contained some abnormal mitotic figures, and was followed by rapid decline in mitotic index (Fig. 4.13 - 4.16).

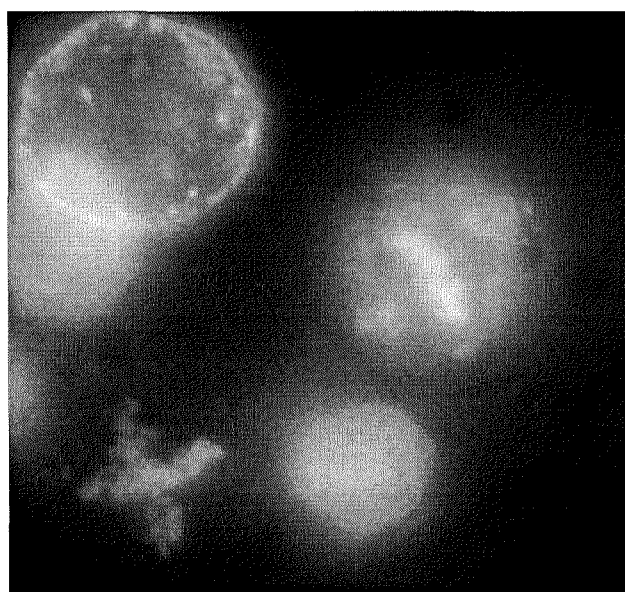


Figure 4.13. The abnormal mitotic figures of Sf9 cells in presence of 2×10^{-7} M azadirachtin. After 20 h incubation, cells were fixed and stained with the DNA-specific dye“ Hoescht 33342 ” and examined microscopically. Cells were in exponential growth phase at time of addition of azadirachtin.

Effect of azadirachtin on mitotic index of Sf9 cell line

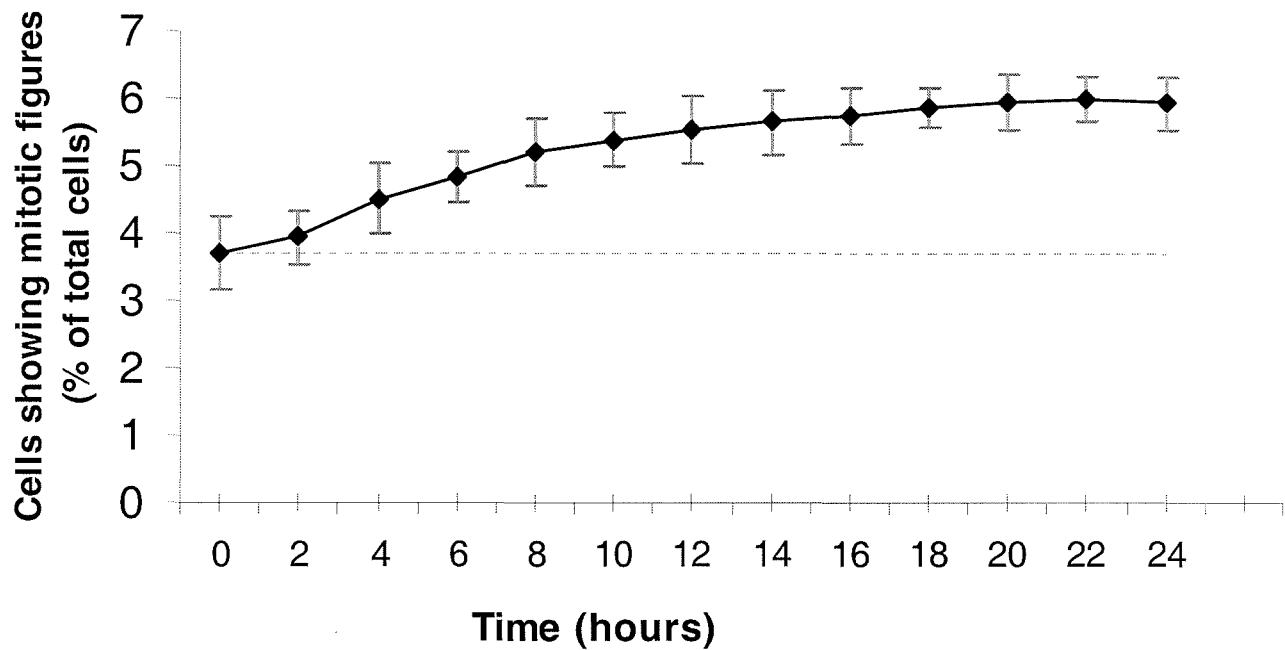


Figure 4.14 Mitotic index of Sf9 cell lines in presence of 2×10^{-7} M azadirachtin. The figure represent the means (\pm S.D.) of 8-9 fields. Horizontal line shows the percentage of mitotic figures in control culture. At time of addition of azadirachtin cells were in exponential growth phase.

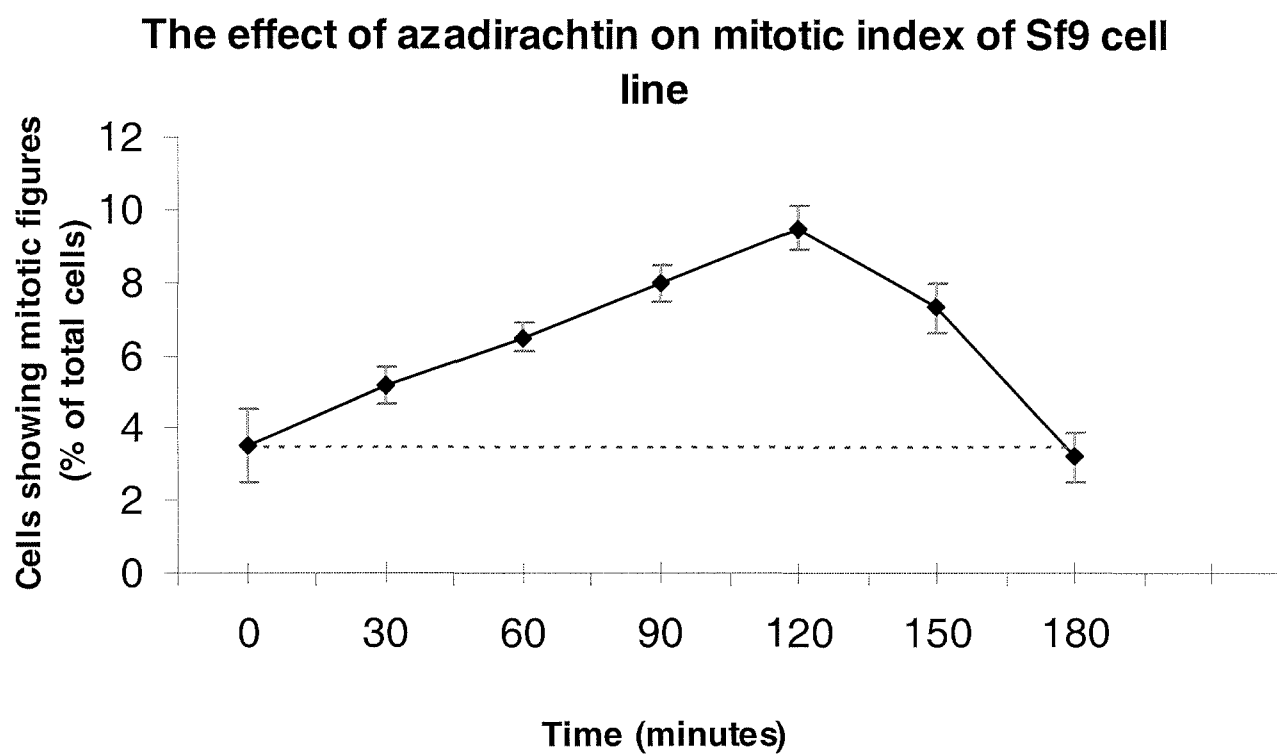


Figure 4.15 Mitotic index of Sf9 cell lines in presence of 2×10^{-6} M azadirachtin. The figure represent the means (\pm S.D.) of 8-9 fields. Horizontal line shows the percentage of mitotic figures in control. At time of addition of azadirachtin cells were in exponential growth phase.

4.3.2.2. The effect of colchicine on mitosis in Sf9 cell line

Colchicine, at a concentration of 2×10^{-7} M, produced almost same proportion of mitotic figures in Sf9 cells. Nevertheless there were some differences in time of accumulation and decline of mitotic index. In comparison to azadirachtin in this case the accumulation of mitotic index appeared later and lasts longer (Fig. 4.16).

The effect of colchicine on mitotic index of Sf9 cells

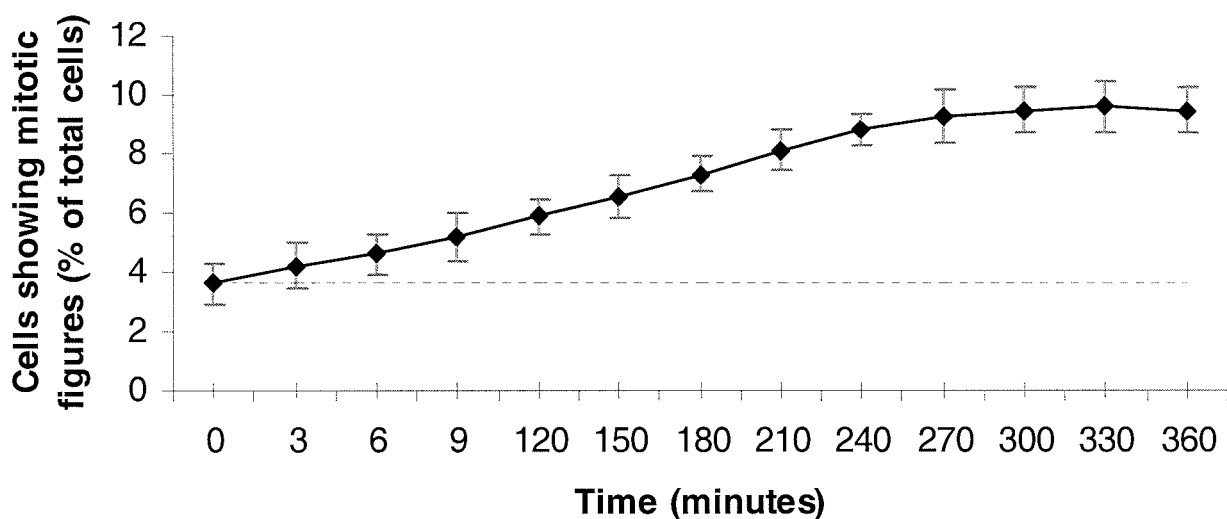


Figure 4.16 Mitotic index of Sf9 cell lines in presence of 2×10^{-7} M colchicine. The figure represent the means (\pm S.D.) of 8-9 fields. Horizontal line shows the percentage of mitotic figures in control. At time of addition of colchicine cells were in exponential growth phase.

Using higher concentration of both azadirachtin ($\geq 10^{-5}$ M) and colchicine ($\geq 10^{-6}$ M) caused sharp decline in mitotic index (results are not shown).

4.3.2.3. The effect of colchicine, taxol and azadirachtin on mitotic figures in Sf9 cells

Since colchicine affects mitosis through inhibition of polymerization of microtubules, in this part of experiments its effect on mitotic figures was compared with effect of taxol (which stabilizes or promotes polymerization of microtubules) and azadirachtin. As the figure 4.17 shows, in comparison to control cells, these compounds have caused some abnormality in mitotic figures of Sf9 cell. Also, anaphase, which can be seen easily in normal cells is absent in cells which treated with either azadirachtin or colchicine.

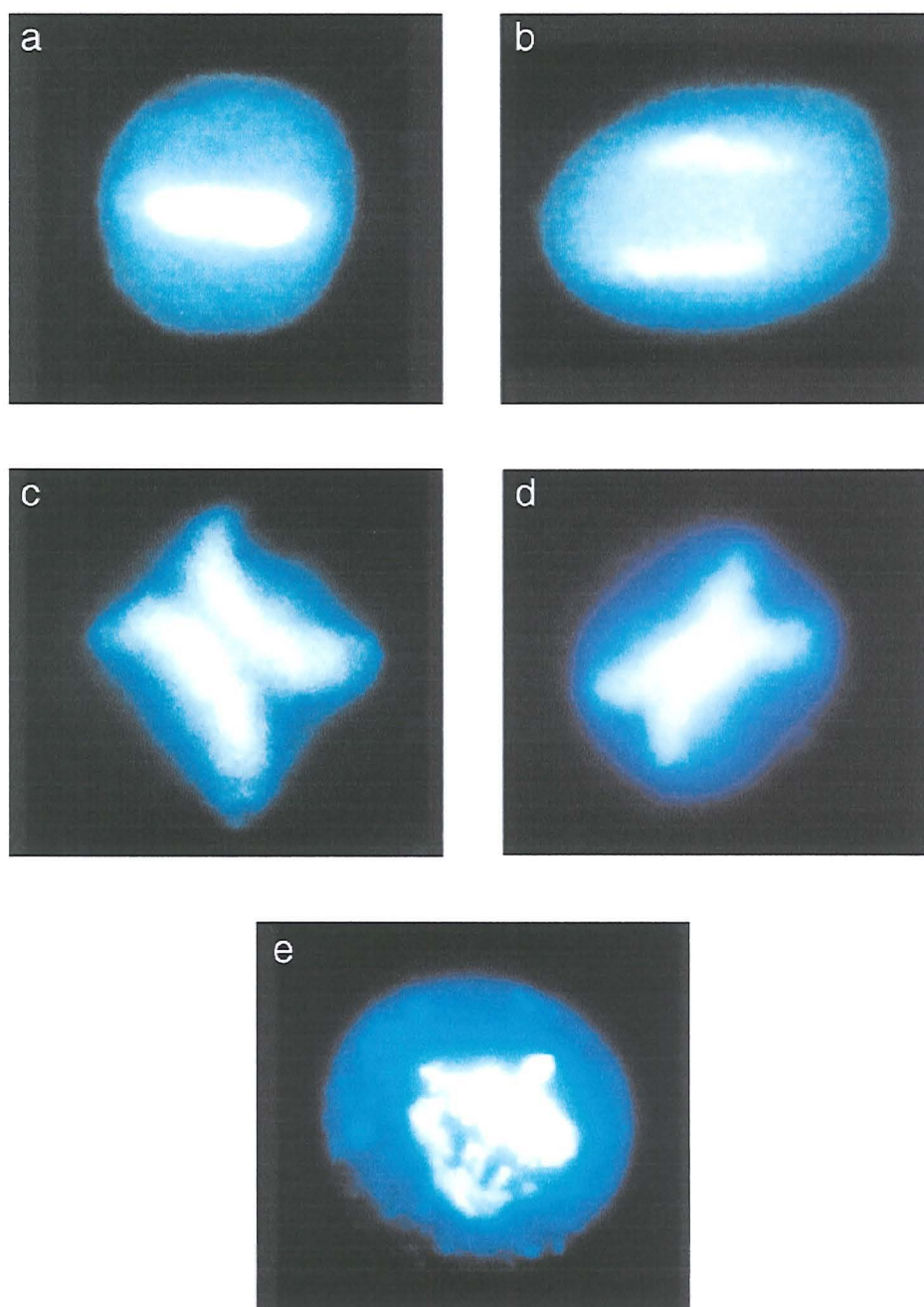


Figure 4.17 Mitotic figures in Sf9 cells. After 3-5 h incubation with phytochemicals cells were fixed and stained with the DNA-specific dye “Hoescht 33342”. a: untreated cell in metaphase; b: untreated cells in anaphase; c: cells treated with azadirachtin at 2×10^{-6} M; d: cells treated with colchicine at 2×10^{-7} M; e: cells treated with taxol at 2×10^{-6} M. Cells were in exponential growth phase at time of addition of phytochemicals.

4.3.3. The effect of colchicine on cell cycle distribution of Sf9 cells

Incubation of Sf9 cells in presence of 10^{-6} M colchicine, after 20 h. incubation caused arrest of almost all Sf9 cells in G2/M phase (Fig. 4.18).

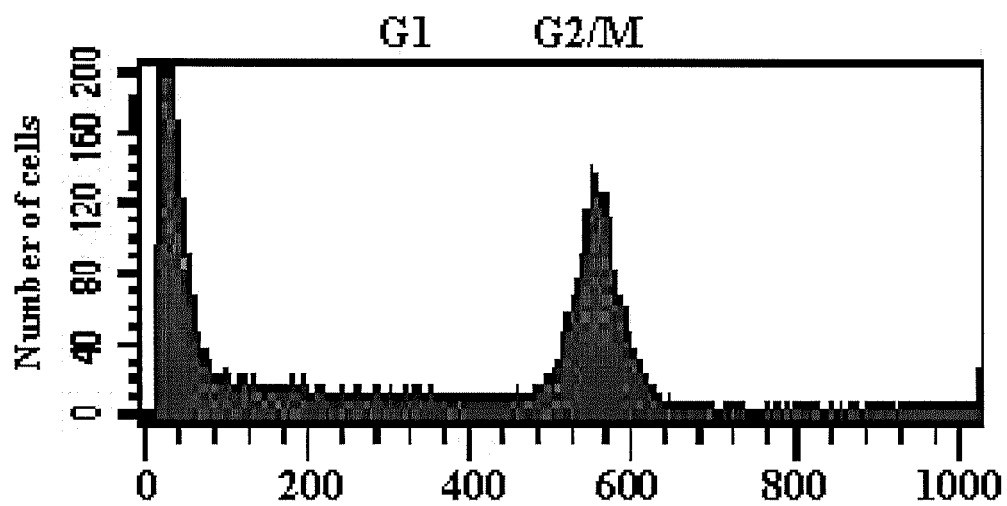


Figure 4.18 The effect of concentration of 10^{-6} M colchicine on cell cycle distribution of Sf9 cell line. After 18-20 h incubation, cells were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 μ g/ ml propidium iodide in presence of RNase and after 30 min incubation in room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

4.3.4. The effect of “Hoescht 33342” on cell cycle distribution of Sf9 cells

To make a comparison with effect of colchicine and azadirachtin on cell cycle distribution, Sf9 cells were incubated with “Hoescht 33342” in concentration of 10^{-6} M for 20 h. The results revealed that this compound (which binds to DNA) not only arrested cells in S and G2/M phases but also it competes with propidium iodide in binding site (Fig 4.19), so treated cells show lower fluorescence in cell cytometry.

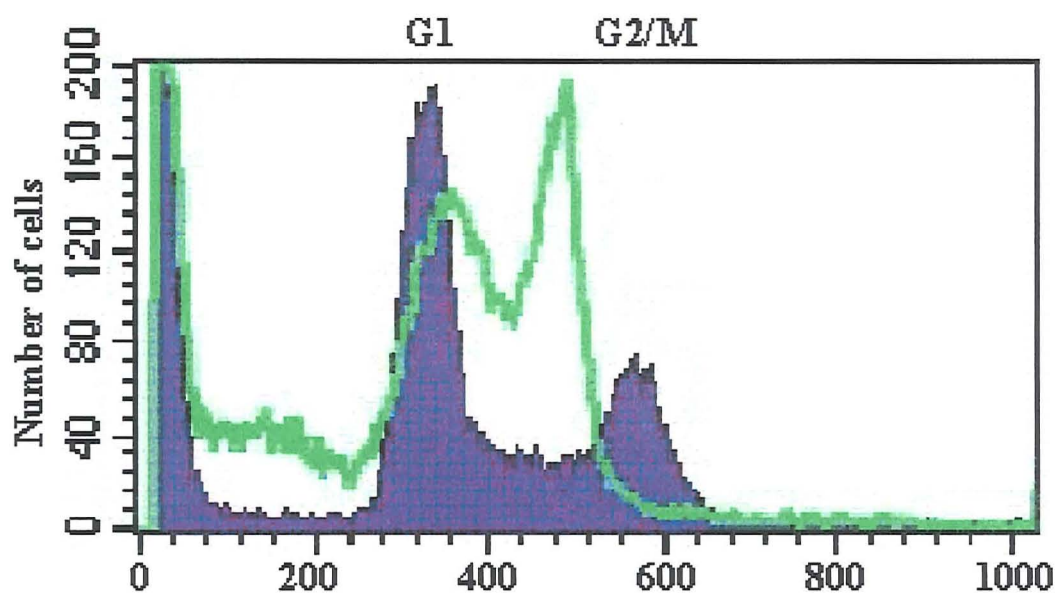


Figure 4.19. The effect of “Hoescht 33342” on cell cycle distribution of Sf9 cell line. After incubation time, cells were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 μ g/ ml propidium iodide in presence of RNase and after 30 min incubation in room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

4.3.5. The effect of pyrethrum and nimbin on cell cycle distribution of C6/36 cell line

In cytotoxicity experiments (see Chapter 2), both pyrethrum and nimbin stimulate the growth of insect cell lines C6/36 at a concentration of 10^{-8} M. When these phytochemicals were added to medium of C6/36 cells, they slightly changed the cell cycle distribution. As figures 4.20.a and 4.20.b show after 20 h. incubation there was slight decrease in G2/M phase of cell cycle. Adding same concentration of pyrethrum and nimbin to medium of Sf9 cell line did not show any change in cell cycle.

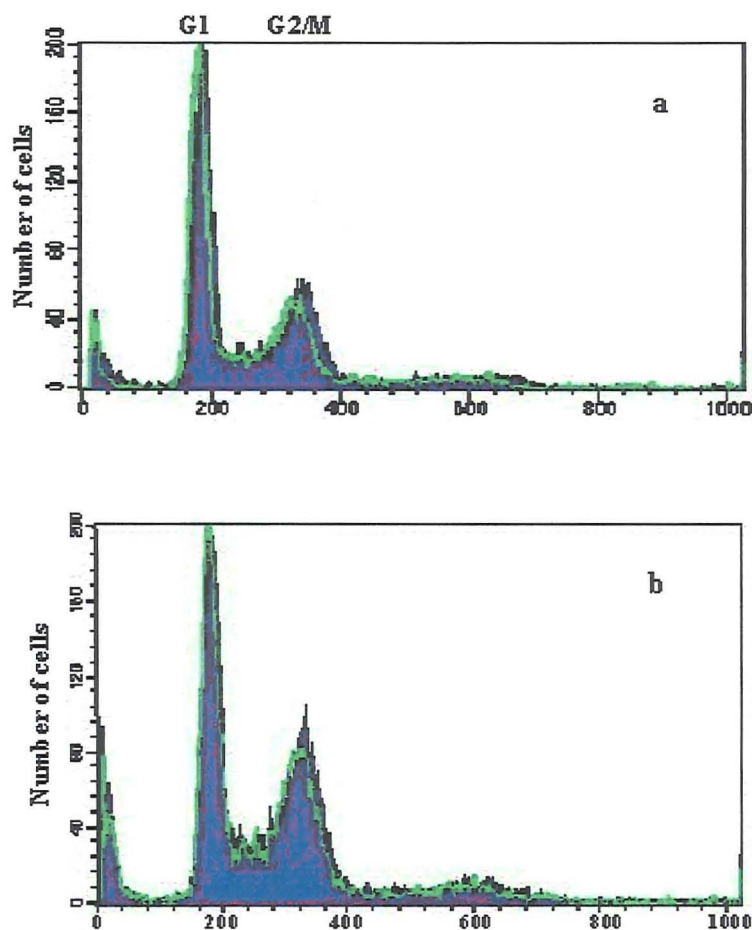


Figure 4.20 The effect of nimbin, (a) and pyrethrum(b) on cell cycle distribution of C6/36 cell line. After 20 h incubation, cells were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 12 $\mu\text{g}/\text{ml}$ propidium iodide in presence of RNase and after 30 min incubation in room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

4.3.6. The effect of combinations of azadirachtin and antimicrotubule compounds on cell cycle distribution of Sf9 cell line

Although both colchicine and taxol arrest Sf9 cells in G2/M phase by affecting their microtubules, the mode of action of these chemicals is different. Colchicine affects the cell cycle by inhibiting the polymerization of microtubules, but taxol in high concentration promotes polymerization of microtubules. Taxol at 10^{-6} M caused an increase in proportion of Sf9 cells in G2/M phase, so that 87% of total cells were in this phase. Incubation of cells in presence of combination of colchicine and azadirachtin showed an increase in percentage of cells in G2/M phase (results are not shown). Interestingly, using a combination of azadirachtin and taxol in concentration of 5×10^{-6} and 1.4×10^{-6} M respectively showed that azadirachtin can partly antagonize the effect of taxol. The combination of taxol and azadirachtin reduced the proportion of cells in G2/M to 74%. Using a combination of 5×10^{-8} M colchicine and 1.4×10^{-6} M taxol showed almost same result (Fig 4.21a – 4.21.d).

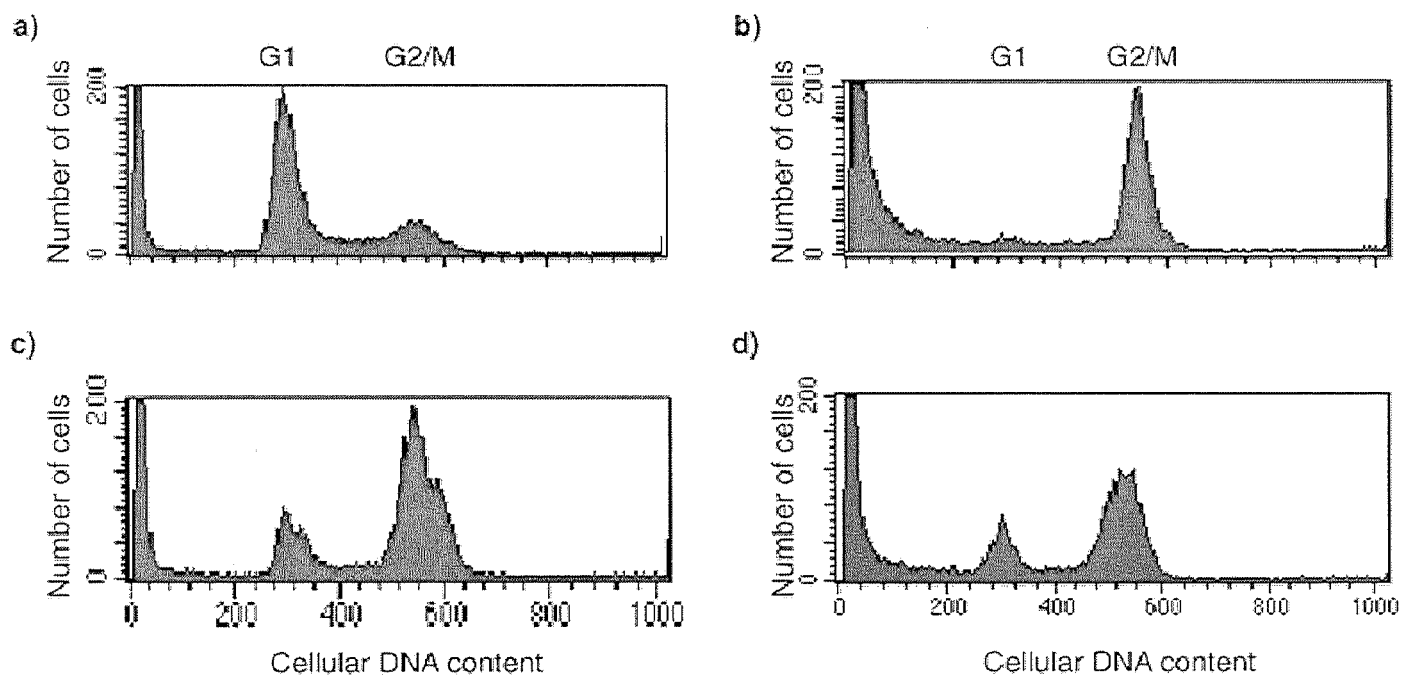


Figure 4.21. The effect of a combinations of phytochemicals on cell cycle distribution of Sf9 cells.

a: Control culture without any phytochemical. **b:** Cells grown in presence of 1.4×10^{-6} M taxol. **c:** Cells grown in the presence of 1.4×10^{-6} M taxol and 5×10^{-6} M azadiachtin. **d:** Cells grown in the presence of 1.4×10^{-6} M taxol and 5×10^{-8} M colchicine. After 18-20 h incubation, cells were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 $\mu\text{g}/\text{ml}$ propidium iodide in presence of RNase and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

4.3.7. Purification of tubulin from pig brain

The experiment with taxol and azadirachtin suggested that azadirachtin may affect the polymerization of microtubules. To evaluate this property of azadirachtin tubulin was extracted and purified from pig brain. The figure 4.22 shows the results of SDS-PAGE electrophoresis at pH 6.8 in a 12.5% gels of the tubulin after purification.

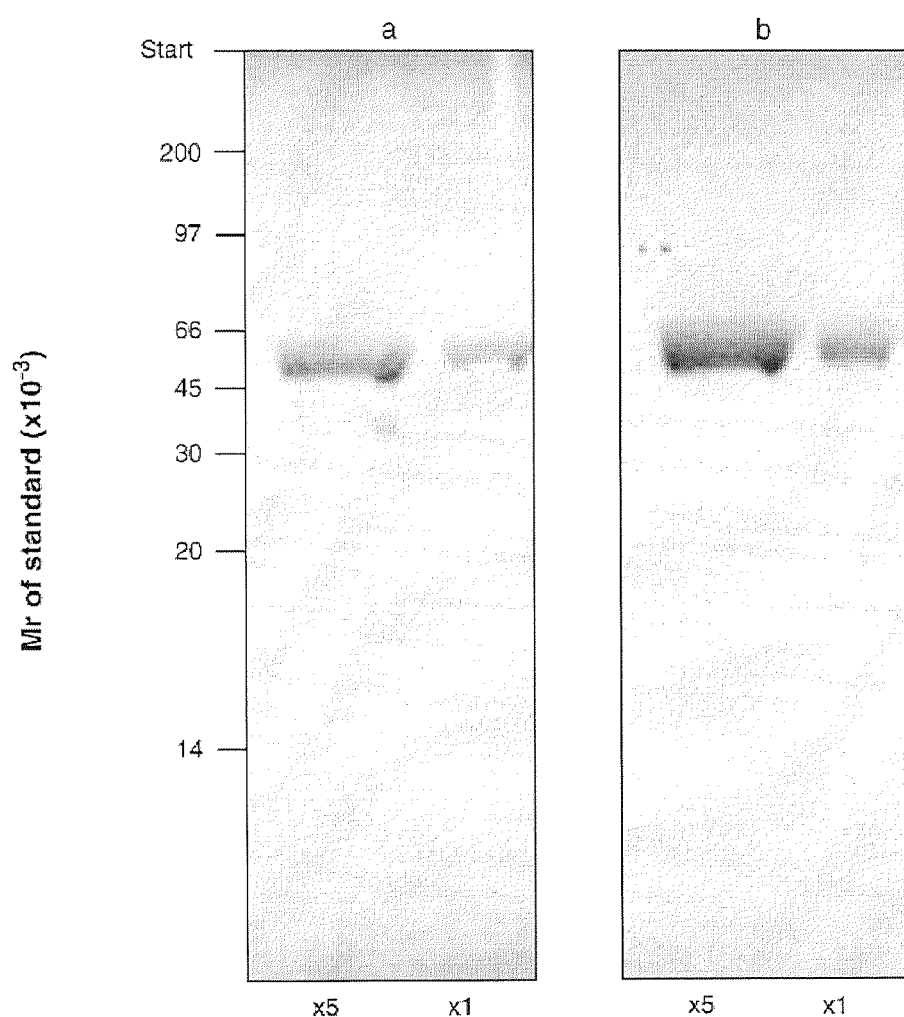


Figure 4.22 The results of SDS-PAGE of purified pig brain tubulin. a; stained for protein with Coomassie Blue; b: immunoblotted with monoclonal antibody (clone b-5-1-2) to the α -tubulin monomer, employing a goat antirabbit IgG/proxidase complex to reveal the IgG. Figures at the bottom represent the relative amount of protein subjected to electrophoresis in each lane. The Mr of the isolated protein was 55,000.

4.3.8. Polymerization of microtubules

The *in vitro* polymerization assay was carried out to compare the effect of azadirachtin with the known effects of colchicine. As expected, untreated solutions of tubulin showed a large increase in scattered light due to polymerization when the temperature was raised from 4 °C to 37°C, which reached a maximum after 40 minutes. The presence in the solution of 10^{-6} M colchicine reduced the polymerization, 60% compared to control. Azadirachtin, at higher concentration of 10^{-4} M, also reduced polymerization, but only by 26% (Fig. 4.23).

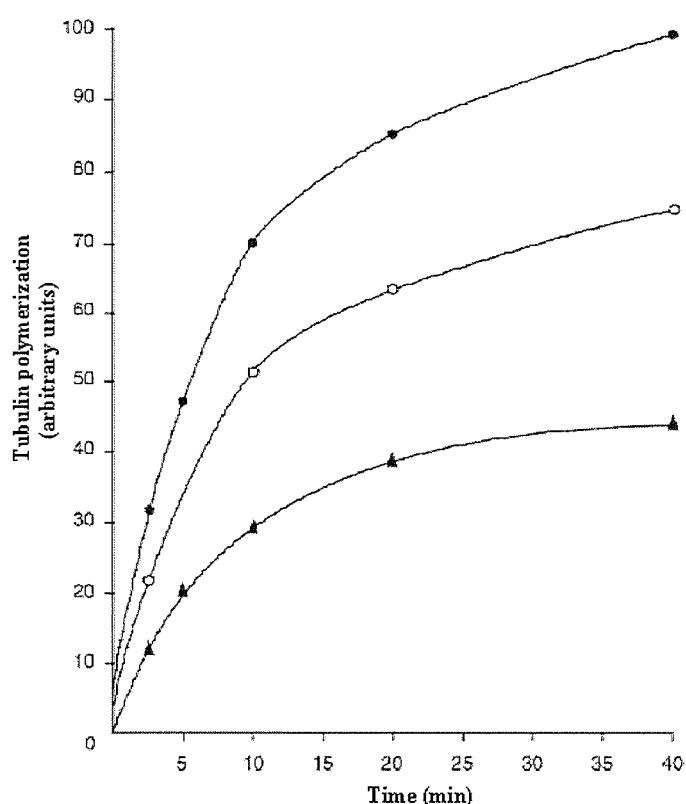


Figure 4.23 Polymerization assay was carried out spectrophotometrically. Colchicine (final concentration 10^{-6} M): ▲; or azadirachtin (final concentration 10^{-4} M): ○; or solvent (ethanol): ●; were added and mixed 30 min prior to the experiment while the preparation was maintained in the ice. The temperature of the assay was then raised to 37 °C and the OD recorded every 60 s for 1 h. To eliminate variation between different batches, the OD of each control preparation after 40 min was taken to 100, and the treated samples run simultaneously expressed as a percentage of that value.

4.3.9. Displacement of colchicine-fluorescence by the antimitotic agents

In figure 4.24 are shown the results of incubating cells with the fluorescent derivative of colchicine, with unlabelled colchicine, azadirachtin, or vinblastine. All three compounds reduced the fluorescence shown in cells exposed to the colchicine-fluorescence only. Reduction of cellular fluorescence of 58%, 45%, and 44% were found due to competition with, respectively, unlabelled colchicine, azadirachtin and vinblastine. Although these results are only semiquantitative, they suggest that most of the fluorescence is specific, and that both azadirachtin and vinblastine can displace the colchicine derivative from its cellular binding sites.

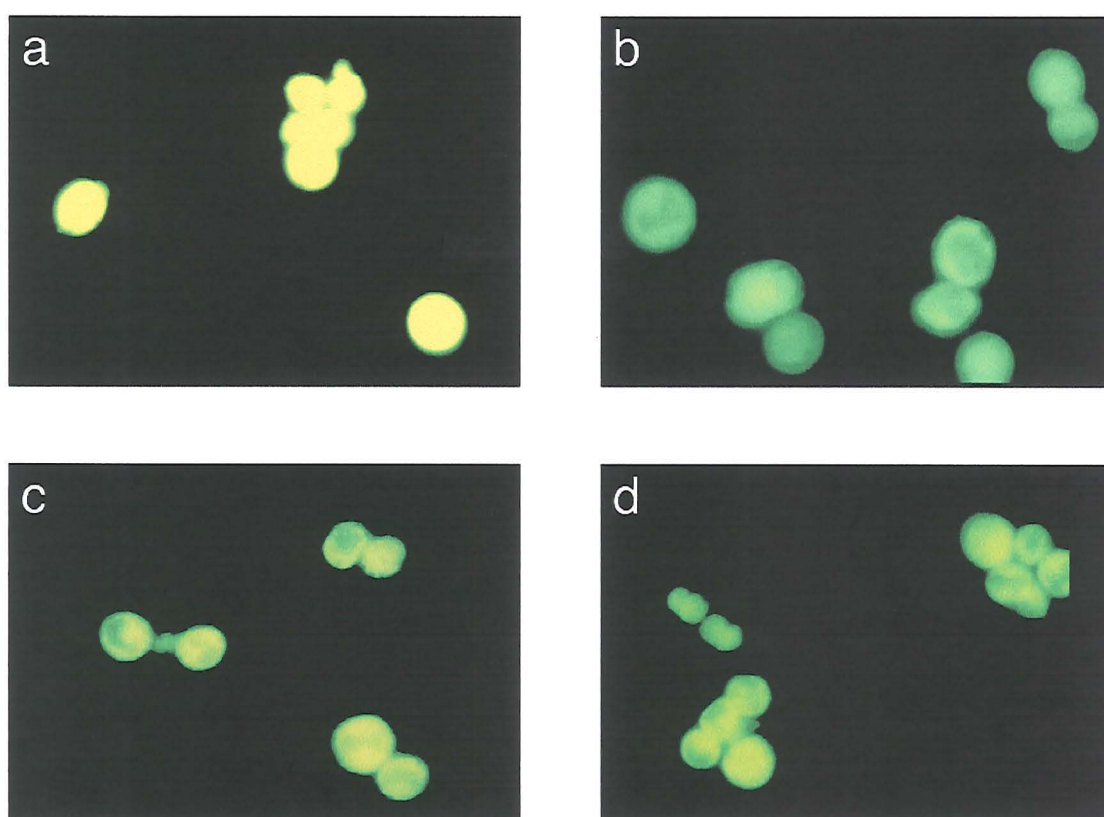


Figure 4.24 Sf9 cells were incubated at a concentration of 10^{-8} M taxol overnight. Then either azadirachtin, vinblastine or colchicine in final concentrations of 10^{-5} , 10^{-5} , and 10^{-6} M (respectively) were added to medium of these cells and incubated for 3-4 h. In the end of experiment cells harvested, fixed, stained with colchicine-fluorescence and photographed through an appropriate filter (maximum absorbance: 490 nm; maximum emission 520 nm). a: control culture, incubated with fluorescein colchicine only; b: incubated with unlabelled colchicine; c: incubated with azadirachtin; d: incubated with vinblastine.

To make a comparison between the results of this experiment based on binding of colchicine to tubulin a similar experiment was carried out. In this experiment the azadirachtin was used to displace the “Hoescht 33342” dye which binds to DNA. As the figure 4.25 shows there was no difference between control and azadirachtin treated cells in fluorescence activity.

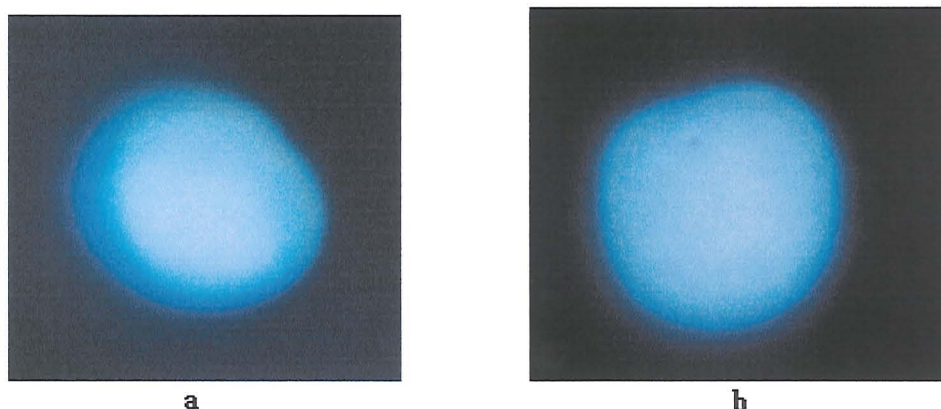


Figure 4.25 Cells were incubated at a concentration of 10^{-8} M taxol overnight. Then azadirachtin at final concentration of 10^{-5} M was added to medium of these cells and incubated for 3-4 h. In the end of experiment cells harvested, fixed, and stained with “Hoescht 33342” and photographed through an appropriate filter. a: control culture (incubated with solvent only); b: incubated with azadirachtin.

4.3.10. The effect of azadirachtin on permeability of cell membrane

The effect of azadirachtin on permeability of membrane was carried out using propidium iodide. The assumption was that cells which have more permeable membrane will take up more dye. The experiment was similar to previous cell cytometry experiments except that in this experiment saponin was not used and timing was much more precise. All samples were assessed at exactly same time after adding propidium dye. As the figure 4. 26 shows azadiarchtin at a concentration of 10^{-4} M after 6 h. incubation not only has changed the cell cycle distribution of Sf9 cells but also the treated cells have taken up even more propidium iodide which can be due to higher permeability of treated cells and so higher uptake of dye.

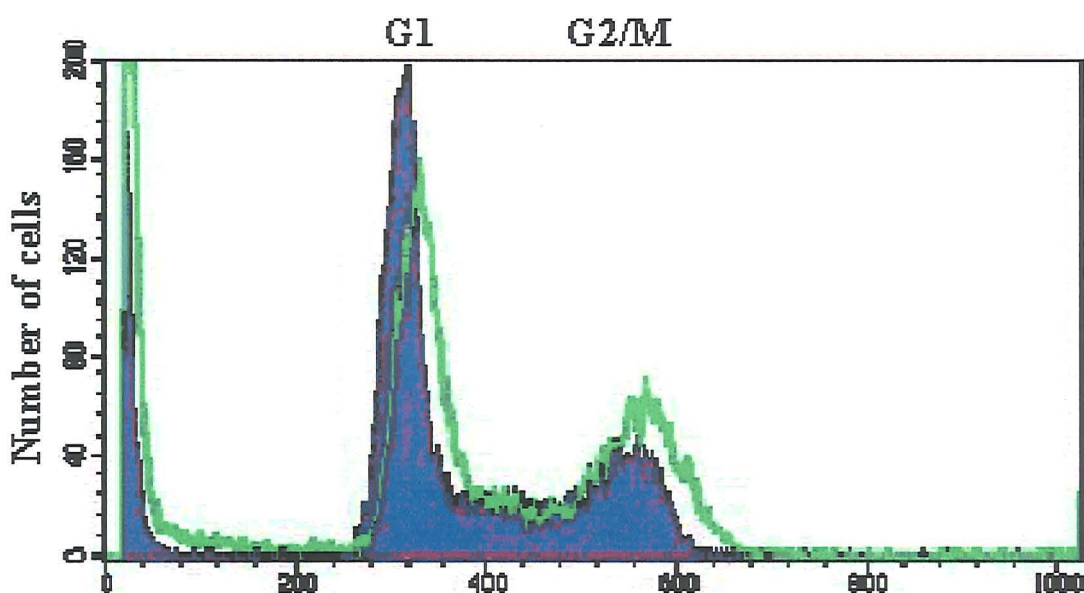


Figure 4.26 The effect of azadirachtin on permeability of Sf9 cells. The cells incubated in absence (blue) or presence (green) of 10^{-4} M, azadirachtin for 6 h. Then cells were harvested and treated with $30 \mu\text{g/ml}$ propidium iodide in presence of RNase and after 45 min incubation in room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA

4.3.11. The effect of Rotenone on cell cycle of Sf9 cell line

In cell cytometry experiments when rotenone applied on Sf9 cell culture, it showed two different effects on the cell cycle. At 10^{-7} M rotenone slightly increased the proportion of cells in G2/M phase, but in concentration of 10^{-6} M and higher despite dramatic effect on cell growth and respiration (see Chapter 2 and 3) there was no significant change in cell cycle of Sf9 cells.

4.3.12. Relative uptake of azadirachtin by Sf9 and L929 cells

Due to a similarity in the structure of tubulin and microtubules in all eukaryotic cells, the expectation is that if some compound affect the microtubules system in insect cells it may affect other cells. Both the cytotoxicity assay and cell cytometry experiments showed that there is big difference in the sensitivity of insect and mammalian cells to effects of azadirachtin.

A difference in the uptake of azadirachtin can be one of possibilities that causes the differences between insect and mammalian cells. In this part of project the cell cytometry assay was used to investigate this difference.

Azadirachtin in concentration of 10^{-6} M was added to the flasks of two series of confluent Sf9 or L929 cells and incubated for 30 min (conditioned medium). Then after 30 min the conditioned medium was added to new flasks containing confluent cells. The process was repeated for 6 times. The medium from last pair of flasks changed with medium of two flasks of Sf9 cells and incubated for 20 h. To evaluate the effect of higher temperature on azadirachtin and the effect of L929 cells on medium also azadirachtin or medium was kept in two separate flasks for 3 h and was added to additional Sf9 flask and incubated for 20 h. The main stages of this experiment has described in following schematic figure (Fig. 4.27)

**Do mammalian cells (L929)
fail to take up azadirachtin?**

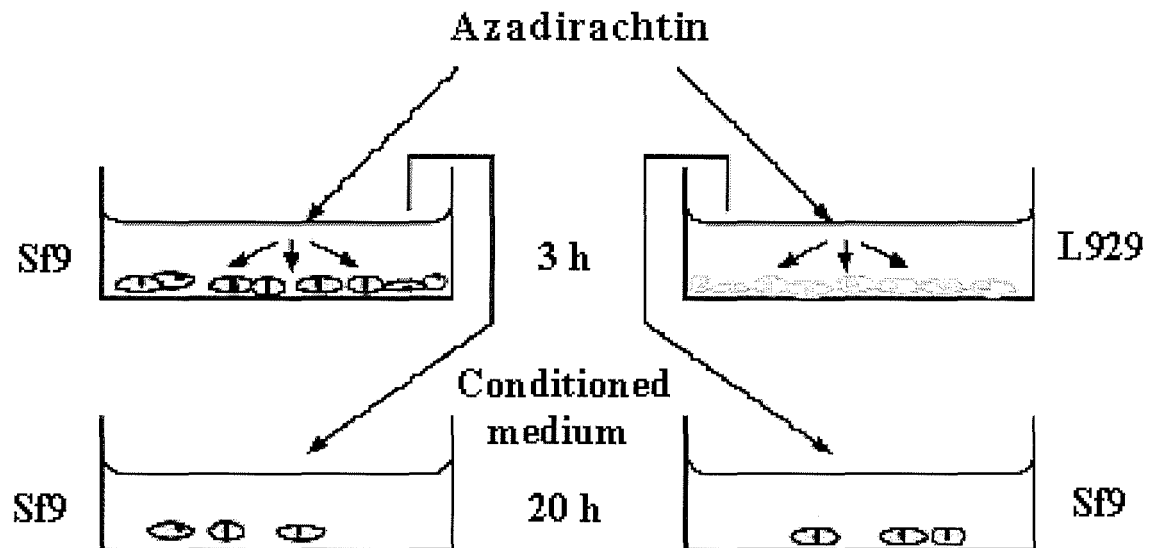


Figure 4.27 Uptake of azadirachtin by insect and mammalian cells

As the table 4. 1 shows incubation of azadirachtin with Sf9 cells has reduced its effect upon the cell cycle of Sf9 cells, whereas incubation with L929 cells, or incubation in 37 °C temperature has not changed the effect of azadiarchtin significantly. Also the effect of L929 cells on medium is negligible.

Table 4.1 Relative uptake of azadirachtin by Sf9 and L929 cells

Test	Azadirachtin	Conditioning of medium		Sf9 cells in G1 (%)
		Cells	Temperature (°C)	
Control 1	-	-	-	61±6
Control 2	+	-	37	8.9±2.3
Control 3	-	L929	37	62±5
Unconditioned	+	-	-	9±2
L929 uptake	+	L929	37	8.2±2.7
Sf9 uptake	+	Sf9	27	19.8±2.6 *

Sf9 cells were grown for 20 h in medium which had been subjected to a variety of conditioning procedures, and then subjected to analysis by FACS by the previously described methods. Control 1 used fresh medium containing solvent only (0.01%DMSO) and without any pre-treatment; Control 2 was to control for the effect of higher temperature at which the L929 cells were grown; Control 3 was eliminate any possible effect due to the L929 cells themselves. Unconditioned medium contained a concentration of 10^{-6} M azadirachtin without any pre-treatment. In all case the conditioning of medium was for a period of about 3 h. * Results significantly different from controls ($P > 0.01$).

4.4. Discussion

The cell cytometry experiments revealed that azadirachtin arrests the insect cell cycle in a time- and doses-dependant fashion. The accumulation of cells with 4N DNA content suggests that azadirachtin may interfere with cell division. When arrested Sf9 cells were examined microscopically, the results indicated that azadirachtin at 2×10^{-6} M increase the mitotic index almost 3-fold within 2 h. The immediate effect of this terpenoid on cell division is in agreement with results which achieved by Schluter (1987), and Linton *et al.* (1997). In this case Schluter found that azadirachtin could affect mitosis in 4th instar larvae of *E.varivestis* and using *S.gregaria* Linton *et al.* showed that azadirachtin arrested spermatogenic meiosis at metaphase I. Figure 4.17 shows a similarity in nuclear profile of Sf9 cells after exposure to azadirachtin and colchicine figure 4.15-4.16 show the mitotic index in same condition. The mitotic index of these cells indicates that both colchicine and azadirachtin cause an increase in mitosis which in case of azadirachtin can be seen shortly after application, but the same percentage of mitotic arrest in colchicine-treated cells can be seen only after 6 h. of exposure. This difference can perhaps be attributed to the relatively fast uptake and irreversible binding of azadirachtin (Nisbet *et al.*, 1995), which in case of colchicine has been said is slow and reversible (Clark *et al.*, 1978).

Despite the effects of colchicine and azadirachtin on increasing the mitotic index of insect cells the mitotic cells hardly raised to more than 10%. In theory, expectation is that arrest in mitosis cause constant increase in number of mitotic cells. It is not clear why the number of mitotic cells remains below 10%. One possibility is that the concentration of the phytochemicals was not enough to affect all cells. Using higher concentration of both azadirachtin and colchicine showed that it was not case. Another possibility is that cells cannot stay in M phase for long so they may escape and inter to G1 after some delay. Alteration in appearance is another possibility, so that they make false G2 cells and can not been distinguished from normal G2 cells andwhich eventually die. The presence of undivided cells is an evidence for such alteration which can be seen in figure 4.17c and 4.17.d.

To make a further comparison between the effect of azadirachtin and some well-known antimitotic compounds, the experiments were repeated with colchicine and taxol. The results showed a similar effect of colchicine and azadirachtin upon the cell cycle of Sf9 line. Using a combination of taxol either with azadirachtin or colchicine, suggested that both colchicine and azadirachtin could partly antagonize the effect of taxol on the cell cycle. The results presented here were achieved with high concentration of taxol, and since taxol has been shown that in high concentration promote the stabilization of microtubules (Jordan, 1993., Snyder, *et al*, 2001), it shows that azadirachtin may interfere with polymerization of microtubules. As the figure 4.21 (b and d) show azadirachtin has rescued some of the arrested Sf9 cells, which not only can be seen in increase of G1 phase but also in decrease of cells debris or dead cells. This result also can be seen in case of colchicine and taxol (Fig. 4.21.c) and is in agreement with finding of Knick *et al.* (1995) who showed using combination of vinorelbine tartrate and paclitaxel (taxol) reduce the cytotoxicity of these agents. Both compounds are anti-microtubule drugs, which are used in treatment of cancer and they showed that the LD₁₀ (dose lethal to 10% of the mice) of vinorelbine tartrate increased approximately 2.5-fold in the presence of paclitaxel.

In the cell cycle when cells progress from interphase to mitosis, large relatively stable microtubule arrays are broken down and reorganized into much more dynamic mitotic spindles. (Belmont, *et al.*, 1990). Colchicine is one of the well known anti-microtubule agents, which at a very low concentration inhibit polymerization of microtubules (Lambeir., *et al* 1980). In present experiment this phytochemical at a concentration of 10^{-6} M reduced the polymerization of microtubules. Conversely, the inhibitory effect of azadirachtin on polymerization of microtubules *in vitro* took place only in high concentrations. In this case the antimitotic property of azadirachtin can be explain by sensitivity of mitotic spindle, because of the absolute requirement of a functional spindle for proper migration of chromosomes during anaphase (Dumontent *et al.*, 1999), which is in agreement with finding of Jordan *et al* (1986) who showed vinblastine and vincristine, even in their lowest effective concentrations, with only subtle changes in the organization of mitotic spindles, can inhibit mitosis. Analyzing the effect of paclitaxel at low concentrations, these authors found the same effect on microtubule dynamics, with no significant alteration in microtubule length. This is in agreement with the finding that Sf9 in presence of 10^{-7} M azadirachtin showed a

higher percentage of mitotic index in comparison to control. It seems that these results are in contrast with finding of Rembold *et al.* (1993) which demonstrated an inhibitory activity of azadirachtin in protein synthesis. If azadirachtin affects protein synthesis at least some of the cells should be arrested in G1 phase (Wintersberger *et al.*, 1983) and the mitotic index should be lower than the mitotic index of control cells. Moreover, the estimation of total cellular protein/cell in present study showed that there was no significant difference in total protein between azadirachtin-treated cells and untreated cells. The reason for this discrepancy may be the difference in concentration of azadirachtin used. In the present study the concentration of azadirachtin for estimation of total protein was lower than of used by Rembold *et al.* (10^{-8} M in comparison to 10^{-6} M). Also the difference in protein content may be related to arrest of cells in G2/M phase. In this case Dversko *et al.* (1997) has suggested that synthesis and uptake of amino acids is regulated in relation to the proliferative status of cells which is in agreement with effect of azadirachtin on the cell cycle.

If the terpenoid inhibits mitosis in insect cells, then it will certainly also affect meiosis. This may largely account for the sterilizing effect of polar neem extracts, which was one of the earliest observed effects in both male and female insects of several species (Steets, 1975; Ascher et al 1984; Bodhade and Borle, 1985; Völlinger, 1987; Schimizu, 1988; Beitzel-Heineke and Hoffman, 1992).

As figure 4.15 shows, after 2 h. incubation of Sf9 cells at concentration of 2×10^{-6} M of azadirachtin, the mitotic index sharply falls which could be related to second binding site which may affect protein or DNA synthesis. Using “Hoescht 33342” dye in present experiments, demonstrated that as it has been reported in previous studies (e.g. Steuer and Breuer., 1990) compounds with high affinity to DNA could cause a dramatic increase in S phase of cell cycle. Azadirachtin at a concentration of 10^{-8} to 10^{-5} M did not cause significant change in S phase of cell cycle in Sf9 cells and only in case of L929 cell there was increase in S phase (Fig. 4.12 a and b). There is a possibility that in highest concentration azadirachtin causes minor damage on DNA, which causes arrest in mitosis or affects G2 checkpoint. In this case the effect of

higher concentration of azadirachtin ($\geq 2 \times 10^{-6}$) should be combination of effect on microtubules which cause arrest in M phase and effect on DNA which causes arrest either in G2 or M phase. So a sharp decrease in mitotic index could be due to the effect of a higher concentration of azadirachtin which after 2 h of incubation of cells at 2×10^{-6} M and eventually causing G2 arrest. In this case the effect of higher concentration of azadirachtin on L929 cells in present experiments could be due to interaction of the terpenoid with DNA synthesis or causing DNA damage in cells as has been shown by Kohn et al., (2002). This is also in agreement with findings of Josephraj Kumar *et al.* (2002) who showed azadirachtin at a concentration of 10^{-3} M inhibited synthesis of DNA in wing discs of last instar *H. armigera*, whereas at lower concentrations (10^{-8} to 10^{-6} M), there was no significant effect on DNA synthesis. In this case the inhibitory effect of azadirachtin on RNA synthesis which was shown by Fritzsche and Cleffman, (1987) and protein synthesis which has been demonstrated by Rembold *et al.* (1993) may be a secondary effect of high concentration of azadirachtin.

Figure 4.24 shows the effect of vinblastine, colchicine and azadirachtin in displacement of fluorescence colchicine. As the figure shows all these compounds can displace colchicine in Sf9 cells. Although both colchicine and the vinca alkaloids disrupt the polymerisation of tubulin, there is uncertainty as to their exact binding sites and possible interactions, with claims made for various sites on both α and β subunits. (Bai et al, 1996; Uppuluri et al 1993; Little and Ludueña, 1985; Williams al 1985; Safa et al, 1987; Wolff et al 1991; Nasioulas et al 1991, Rai and Wolff 1996). Displacement studies are complicated by the fact that colchicine's binding to tubulin is both slow and poorly reversible, while that of azadirachtin appears very fast and almost irreversible. Overall, however, it can be said that the evidence presented here suggests some interaction in binding between the known disrupters of tubulin polymerisation, colchicine and vinblastine, and azadirachtin. It is instructive that previous work has shown that colchicine prevents the development of insect wings (Drozdoskaia and Rapoport, 1988) , as does azadirachtin (Schmutterer and Freres, 1990) and colchicine was used by Schlüter (1987) to suggest an analogy with azadirachtin in its effects on cuticle development by inhibiting mitosis.

Binding of azadirachtin to microtubules also can justify the results of studies of Nisbet *et al.* (1997) who found azadirachtin accumulated in the nuclear fraction of Sf9 cells. In this case using autoradiographs Rembold *et al.* (1993) showed that in malpighian tubules of *L. migratoria* azadirachtin accumulated round the nuclear membrane, where there is network of microtubules. It was found in present study that application of fluorescence dye DCVJ (tubulin specific fluorescence dye); showed accumulation of dye around the nuclei in Sf9 cells (Fig. 4.28.a). In the same experiment when cells were in mitosis the accumulation of DCVJ could be seen around chromosomes (Fig. 28.b) which is related to organization of microtubules in mitotic spindle. This is in agreement with Cassimers *et al.* (2001) and Charrasse *et al.* (1998) who showed localization of MAPs may change during different cell cycle phases. This also can describe the finding of Jabbar *et al.* (1998) who, using radiolabeled dihydro-azadirachtin, demonstrated that in insect cells this terpenoid tends to accumulate in nuclei whereas in mammalian cells azadirachtin can be seen in cytoplasm only.

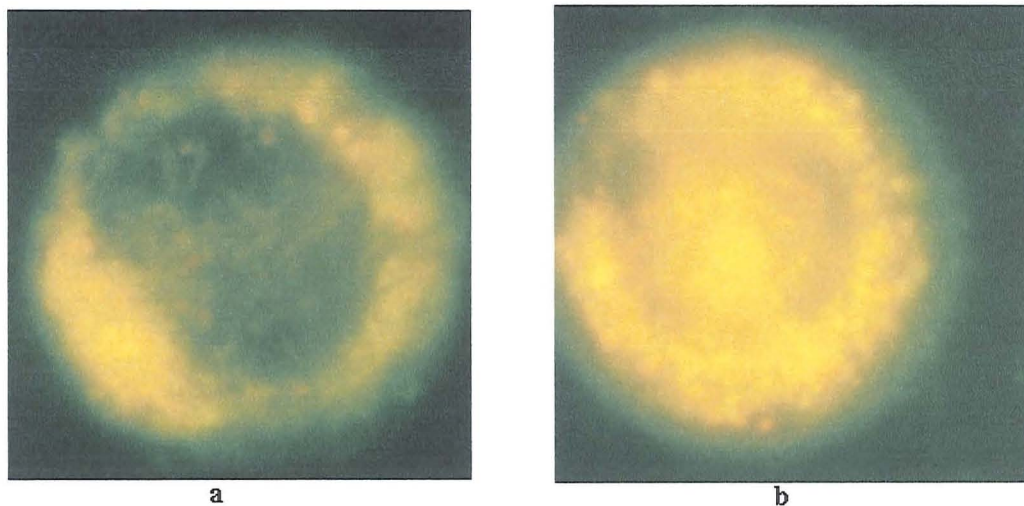


Figure 4.28 localisation of microtubules in interphase and mitotic Sf9 cells

Sf9 cells in interphase (a) and mitosis (b). Cells washed and stained with DVCJ. Figure b represent a mitotic cells and shows accumulation of microtubules around chromosomes.

Because of the effect of microtubules on motility of cells (Ben-Chetri *et al.*, 1993; Hong *et al.*, 1985; Schatten *et al.*, 1982) other experiment also imply the effect of azadirachtin on cytoskeleton. For example, the effect on motility of sperm (Nisbet *et al.*, 1996), antiinflammatory effects (Pardia *et al.*, 2002) and effect on sexual development of malaria (Jones *et al.*, 1994).

Due to the effect of microtubules on cell secretion (Hajnoczky *et al* 1994; Trifaro *et al.*,1992; Hindelang-Gertner *et al.*, 1971) the effect of azadirachtin on the cytoskeleton can also account for its effect on neurosecretory system of insects.

Using *L. migratoria*, Uhl and Rembold (1987), showed a delay and reduction of the haemolymph concentrations of the key developmental hormones ecdysone and juvenile hormone after azadirachtin treatment. Another hormone which is reduced by azadirachtin treatment in the locusts is adipokinetic hormone, which in turn leads to lower circulating concentrations of lipids and thus to reduced capacity for flight (Wilps *et al*, 1992). The key to both these reductions probably lies in events in the neurosecretory cells in the brain and their neurohaemal organs, the corpora cardiaca and allata. Rembold *et al* . (1989) showed that the corpus cardiacum took up the tracer tritiated dihydroazadirachtin, with the cytoplasm of the glandular lobe cells becoming intensely labelled. There was an accumulation in the neurohaemal cells of secretory material as shown by paraldehyde-fuchsin staining.

Indeed the effect of azadirachtin on the cytoskeleton may describe almost all of its effect on the endocrine system such as: the decrease in ecdysteroid titres (Feder *et al.*, 1988), the effect on molting, and even the effect on elevation of serotonin in brain of *S. gregaria* (Mordue *et al.*, 1993). This was also shown by work of Paraganama (1994) who found an interruption in the release of juvenile hormone in tobacco horn worm *Manduca sexta* in the presence of azadirachtin (Linnaeus), which is in agreement with finding of Garcia *et al.*, (1986) who has suggested that the IGR properties of terpenoids is related to their effect on neurosecretory system.

Table 4.1 represents the effect of conditioned azadirachtin on cell cycle distribution of Sf9 cells. As the results show preincubation of azadirachtin by insect cells has reduced the cytotoxic capacity of this medium, which can indicate the relatively fast and

efficient uptake by insect cells. On the other hand incubation of mammalian cells with same concentration of azadirachtin has not changed the cytotoxic capacity. In other words, this experiment suggests that there is difference either in the uptake or binding of azadirachtin to insect and mammalian cells.

Chapter 5

Final discussion

There are two main outcomes of this project:

1- A comprehensive quantitative demonstration of the differential cytotoxicity of azadirachtin towards insect and mammalian cell lines.

2- Evidence for antimitotic activity of azadirachtin possibly through interference with dynamic instability of microtubules.

Cytotoxicity experiments:

Over the past few years a number of cells from mammalian sources, have been qualitatively tested for their response to azadirachtin.

They include: Chinese hamster ovarian cells (Rembold *et al.*, 1993), murine neuroblastoma cells and human osteosarcoma cells (Cohen *et al.*, 1996) and murine erythroleukemia cells (Reed *et al.*, 1998). None of these studies has shown any effect upon growth of cells. The results presented here using cells derived from murine fibroblasts show that azadirachtin even in its highest used concentration (10^{-4} M), inhibits the growth of mammalian cells by less than 50% of control.

In sharp contrast are the effects of azadirachtin on cultured insect cells. Rembold and Annadurai (1993) used a concentration of 10^{-6} M and found profound, although unquantified, effects on the growth of cultures of Sf9 cells. The results of Reed and Majumdar (1998), suggest an EC_{50} for the same type of cells approximately 10^{-8} M which is in agreement with the results of the present work and Jabbar *et al.* (1998) reported even a higher cytotoxicity. The only different result, is that of Cohen *et al.* (1997) who has reported an EC_{50} more than 2×10^{-4} M, which probably is related to difference in cell types or impure material used in their work. The accumulated results indicate that there is a remarkable difference in the effects of azadirachtin on insect and mammalian cells, with the latter at least up to ten thousand times less sensitive than the former. There are differences in response of the two insect cell lines, Sf9 and C6/36, which may indicate higher sensitivity of *Lepidoptera* in comparison to *Diptera* as has been shown by antifeedancy activity of azadirachtin in these orders (see Chapter 1.4). The results in the preliminary experiments confirm that azadirachtin acts through a mechanism which requires cell division over several generations to produce a maximum effect. This is consistent with its normally slow-developing effects *in vivo*.

There are several reports (Muley *et al.*, 1978; Ayoub *et al.*, 1986; Jacobson *et al.*, 1988) that crude aqueous and alcoholic extracts of neem kernels are toxic to a number of species of water-snail. The cells used here were derived from *Biomphalaria glabrata*, a snail reported by Jacobson (1988), as being killed by polar neem-seed extracts. The cultured cells were not much more sensitive to the growth-inhibiting effects of pure azadirachtin than mammalian cells. It is noteworthy, however that the reports cited above indicate a rapid death of snails, sometimes in less than 1 h. This may suggests much more acute effects than shown by insects, which would not be detected by the cell-growth method. As only crude extracts have been used in vivo, it is likely that azadirachtin is not responsible for killing the snails. Since pioneering work of Butterworth and Morgan (Butterworth *et al.*, 1968; Butterworth *et al.*, 1971), attention has rightly focused on azadirachtin as the most potent pesticide of the neem terpenoids, others having a much less significant effect. This is in consistent with the results presented here.

Overall, although there are differences between the responses of whole insects and cultured insects cells towards the neem terpenoids, the results presented here suggest that the toxicity of the various neem terpenoids is decided by pharmacological properties which exist at the cellular level, and that are much more sensitive in cultured insect cells such as Sf9 cell line.

Rotenone also proved to be a highly effective inhibitor of growth of the insect cell lines, with EC₅₀ in the submicromolar range. It was less effective against the L929 line suggesting some difference in affinity for its target.

Control experiments to ensure that rotenone had no direct effect on the rate of reduction of MTT by the cells gave rise to two interesting observations. Rotenone affects the ability of the cells to reduce MTT, but to a very limited extent. At a concentration of 10⁻⁶ M, sufficient for the total suppression of cell growth of Sf9 cells, reduction of MTT was diminished by only 15%. It is unlikely that any residual rotenone in the cells after 5 days would have a direct effect on the rate of reduction of the tetrazolium dye. However, it is surprising that a compound considered to have a high acute oral toxicity to animals should have so little effect on the reduction of MTT by cells. Part of the explanation may lie in the observation of Berridge *et al.* (1996) that MTT was reduced by microsomal rather than mitochondrial dehydrogenases. The

results of the present study have confirmed this finding, and showed less than 10% of the cellular capacity to reduce MTT resides in the mitochondria. Thus it is possible for mitochondrial respiration to be inhibited without much effect on reduction of MTT by whole cells.

Nicotine was found to have little effect on the growth of insect and mammalian cells. It has been reported that nicotine stimulates DNA synthesis in endothelial cells at concentrations lower than 10^{-8} M, and that it is cytotoxic at concentrations $> 10^{-6}$ M. In present experiments only at 10^{-6} M did the alkaloid significantly reduce the replication of Sf9 cells.

Surprisingly, in contrast, the natural pyrethrum did seem to stimulate the growth of both insect and L929 cells. This was most marked with the mosquito-derived cells, which showed an increase of 30% in growth at concentration of 10^{-8} M. The growth in Sf9 cells was stimulated to a smaller extent, but it was still statistically significant. At the moment it is not clear if this effect is related to observation that pyrethrum is tumourigenic (Go *et al.*, 1999).

Antimitotic effect of azadirachtin:

The results of this project and accumulated evidence from previous works suggest that Azadirachtin, at least in insect cells acts in a manner similar to that of well-known antimitotic compound colchicine. The most direct evidence is the effects of these phytochemicals on the polymerization of purified mammalian brain tubulin. The results suggest that azadirachtin is one to two orders of magnitude less effective than colchicine in this particular *in vitro* assay. In this case the sensitivity of dynamism of microtubules in mitosis can justify the effect of azadirachtin on cell division. The observations of mitotic figures in replicating Sf9 cells confirm that azadirachtin is a less effective anti-mitotic agent than colchicine in these insect cells by about a factor of 10. However azadirachtin at concentration of 2×10^{-6} M doubles the number of mitotic figures in the cells within an hour, and at the lower concentration of 2×10^{-7} M cells show a slow linear increase of cells in G2/M over a period of at least 24 hours (i.e. longer than the average doubling time). The results of the cell cytometry studies give quantitative support to the idea that azadirachtin has its effect on insect cell

division, and show that after 20 hours (approximately one cell cycle) in presence of 10^{-6} M azadirachtin, almost all the cells have been arrested in G2/M. At lower concentrations the effects on cell division are the same but take longer to become apparent. These results are consistent with observations of Jordan *et al.* (1993) and Dumontet & Sikic. (1999), which showed antimitotic agents such as colchicine and the *Vinca* alkaloids can prevent replication of cells over several generations. This also explains the the results of cytotoxicity assays which showed the influence of 10^{-6} M azadirachtin on cell numbers was more significant after at least 2 cycles of cell division. The apparently irreversible binding of azadirachtin to its targets, coupled with low levels of metabolism of the terpenoid (Rembold *et al.* ,1988; Paranangama *et al.*, 1993), suggests that the cellular effects could persist over several generations of cell division.

Both azadirachtin and colchicine can apparently “rescue” cell division to some extent from the effect of taxol, allowing more cells to proceed to G1 after 24 hours. This was gained by using each of these phytochemicals in the presence of a high concentration of taxol which has been shown promote polymerization of microtubules (Jordan, 1993., Snyder *et al.*, 2001).

The results with the fluorescent derivative of colchicine, which was shown (Clark and Garland, 1978, Moll *et al.*, 1982) to have the same cellular action as colchicine itself and to displace it from binding to tubulin in cultured cells, suggest that both azadirachtin and the vinca alkaloid vinblastine displace colchicine from binding sites, presumably on tubulin. This could be interpreted as competition for the same site on the protein, but could equally well represent a more indirect effect on colchicine-binding due to conformational changes in tubulin. On the other hand a sharp decrease in mitotic index of Sf9 cells after 2h incubation with concentration of 2×10^{-6} M azadirachtin and its difference with the effect of 2×10^{-7} M of this terpenoid suggest that azadirachtin in high concentration may bind to a second binding site. One possible binding site for higher concentration of azadirachtin could be chromatin which can cause arrest in S, or G2/M Phase (Cliby *et al.*, 2002 ; also Fig. 4.19). In this case the dramatic increase of G2/M phase of insect cells after 20 h incubation with higher concentration of azadirachtin and changes in cell cycle of L929 line in presence of concentration of $1-5 \times 10^{-4}$ M of this pesticides (Fig 4.12 a and b) can be due to combination of its effect on microtubules and chromatin. This explanation is in

agreement with finding of Josephraj Kumar *et al* (2002) who showed azadirachtin in concentration of 10^{-3} M inhibited synthesis of DNA in wing discs of last instar *H. armigera*, whereas in concentration of (10^{-8} to 10^{-6} M) there was no significant effect on DNA synthesis. In this case the effect on RNA, as was shown by Fritzsche and Cleffmann. (1987) seems to be a secondary effect of high concentration of azadirachtin. Also this explanation can justify the inhibitory effect of azadirachtin on protein synthesis (Rembold., *et al* 1992) and the finding of Nisbet *et al.* (2001) who found azadirachtin binds to the nuclear fraction of Sf9 cells.

The studies with cultured cells might suggest that effects in intact insects will be limited to tissues undergoing division, either mitotic or meiotic, but in fact many of the reported effects of azadirachtin may be explained by a general effect on microtubule polymerisation. Any function involving organelle movement within the cytoplasm of the cells will be slowly affected by the terpenoid. Foremost among these will be axoplasmic transport and exocytosis. This in turn will affect the secretion of almost all the hormones which govern the biology of insects.

There is evidence that azadirachtin and colchicine not only affect release of neurohormones but also inhibit the effect of peptide neurohormones in target tissues. Experiments *in vitro* with the production of ecdysterone by the prothoracic glands of *Manduca sexta* (Watson *et al*, 1996) and of polypeptide synthesis by fat body and ovary of *Sch. gregaria* (Paranamgama., 1993) showed that neuropeptides greatly stimulated rate of synthesis, and that this stimulus was eliminated by colchicine in the former case and by azadirachtin in the latter, implying the involvement of microtubules in the response to neurohormonal stimuli in many insect tissues.

Effect on the motility of cells (Nisbet *et al.*, 1996; Sadre *et al.*, 1984) and also effects on the sexual development of malaria (Jones *et al.*, 1994) provide further evidence for an effect of azadirachtin on tubulin which is important in these phenomena (Ben-Chetri *et al.*, 1993; Hong *et al.*, 1985; Schatten *et al.*, 1982).

Although the results presented here offer an explanation for the molecular events, which explain many of the effects of azadirachtin on insect tissues, there is still some ambiguity about the reasons of differential effect of azadirachtin upon different cell lines which especially between insect and mammalian cells. Also, it is not clear yet if

the lower effect of azadirachin on polymerization of pig brain tubulin is related to the difference in tubulin of insects and mammals or not.

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