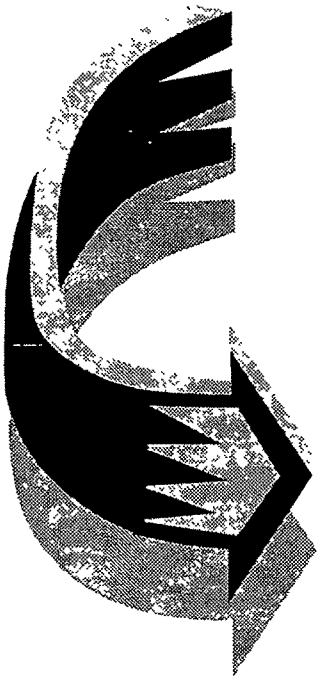
CHAPTER : I

INTRODUCTION



I.1 BIO-PESTICIDES AS AN ALTERNATIVE FOR ENVIROMENTAL PROBLEMS

Insect Resistance Against Synthetic Pesticides

The need for efficient use of diminishing farms and the maximization of crop returns is becoming evermore popular, in view of continuous expansion of the world population. As a result, the concept of insect pest control has gained more importance during the latter half of this century.[1] In developing countries like India, the annual crop losses due to various pests, namely insects, fungi, bacteria, weeds, rodents and nematodes, are estimated to be worth Rs. 6000 to 7000 crores.[2] The warm and humid climate in the tropical and sub-tropical regions of the country is beneficial not only for lush green vegetative growth but also for the multiplication growth of insects, which causes serious damage to the field crops as well as stored grains.[2, 3]

In early days, manual labor was used to solve the weed problem, but the manual control of insect infestations proved in most cases ineffective.[4] Until 1945, the insecticides used against the pests were mainly based on inorganic metal salts (copper, arsenic, etc) and these were efficient in protecting cultivated crops only from some insects.[3,4]

The discovery and use of 2,2-di(p-chlorophenyl)-1,1,1-trichloro-ethane (DDT) in 1940 and then benzenehexacholoride (BHC) and subsequent development of the organochlorines, organophosphates, chlorinated cyclodienes and dinitrophenols have played a major role in the field of crop protection.[1,3,5]

Of course, these synthetic chemical insecticides are important and played a very positive role in controlling at least half of the insect problems in agriculture and public health and kept crop losses under

check for some time. However, their extensive and sometimes indiscriminate use has led to a number of social and environmental problems, such as destruction of natural enemies, turning formerly innocuous species into pests, harm to other non target species and contamination of food.[6, 7]

Pimentel *et al* reported that hardly 0.1 % of the agrochemicals used in crop protection reach the target pest leaving the remaining 99.9 % to enter the environment to cause hazards to non-target beneficial organisms including humans.[8]

The poisoning of live stocks, contamination of food, soil, ground water, fish, wild life and other beneficial organisms has been linked with increased use of synthetic pesticides.[1,3,7] Moreover, chemical pesticides destroy predators, parasites (exercising natural check on pest population) and beneficial insects like honey bee etc.[9] Additionally excessive use of chemical pesticides has developed the resistance to synthetic insecticides in pests, which are responsible for causing increased ecological disturbance as well as pollution of the environment.[1] Varma and Dubey reported that at least 447 species of insects and mites and 200 species of plant pathogens as well as 48 species of weeds are now resistant to chemical pesticides.[3] For these reasons, discovery of target specific methods based on natural resources has been the major goal of research world wide.

It is true that the use of chemical pesticides would continue in foreseeable future. However, there is an urgent need to develop an alternative method for effective pest control to check crop losses, which would be environmentally friendly. Hence a strategy termed as "Integrated Pest Management (IPM)" which means "planned execution of different pest management practices with the objective

of reducing crop losses with minimum adverse ecological implications" was developed.[2]

One of the major components of IPM, which could result in partial replacement of chemical insecticides, is the use of bio-pesticides, especially botanical insecticides. The term botanical insecticide here means chemicals from different plant species or plant parts *per se* having insect control properties.[2]

The development and use of botanical insecticides for protection of crops from insect damage is at a significantly more advanced stage as compared to other alternative methods of pest controls, namely use of living organisms such as bacteria, fungi, viruses, use of pheromones, sterile male techniques etc.[2]

Role of Botanical Products As Insecticides

Plants are the richest source of renewable bioactive organic chemicals, which could be used as pesticides.[10] The total number of plant chemicals may exceed 4,00,000[3]. Of these 10,000 are secondary metabolites whose major role in plants is reportedly defense.[3,10] A number of defensive chemicals belonging to various groups (terpenoids, alkaloids, glycosides, phenols, tannins etc.) which cause behavioural and physiological effects on pests have already been identified.[3]

Grainge *et al* reported that more than 2000 plant species belonging to different families and genera possess the insecticidal properties. However, for effective utilization in pest control only those botanical insecticides would be ideally suited which have the following criteria [11,1]:

- Effective control of broad spectrum of target pests,
- Do minimum damage to non-target organisms including mammals,

- Activity at low concentrations,
- Economic viability, stability and compatibility with existing plant protection methods and
- Environment friendly

Advantages and Disadvantages of Botanical insecticides

There are several advantages of using botanical insecticides in the agriculture. They are generally much safer than conventionally used synthetic pesticides. Pesticidal plants have been in nature as its component for millions of years without any ill or adverse effect on the eco-system.[3] Plant based insecticides would be renewable and cheaper.[3] Some plants have more than one bio active principle, which may exhibit either one or more biological effects. The problem associated with botanical pesticides is their low persistence, hence limiting use in agriculture. Furthermore, lack of standardization processes and less availability of resources also play a major role in their limited use.[12-14]

Various botanical insecticides such as Pyrethroids, Nicotine (tobacco leaves), Absinthin, Withanolides, Bergapten and Neem extracts are investigated for crop protection since last four to five decades.[15] However, the knowledge of the insect repellent property of neem in India is much older.[3,16,17] In 1927, Mann and Burns reported that the neem acts as a repellent and observed that, the adult locusts did not feed on neem leaves.[18] In 1936, Astrakhantzev *et al* reported that the efficacy of water and alcohol extracts of leaves acting as aphicide against *Brevicorgne brassicae* L.[19] This was the first report indicating the insecticidal activity of neem extracts. Pruthi in 1937 also found that neem leaves mixed with grain, protected them from storage pests.[20]

Systematic studies on repellent and insecticidal properties of different parts of the neem and it's products started in the sixties, when Pradhan *et al* reported for the first time, antifeedant properties of neem seed kernel against desert locust.[21] These findings created great interest amongst the research workers throughout the world. They started isolation and identification of the active principles from the various parts of the tree.[16]

During the last five decades, apart from the chemistry of the neem compounds, considerable progress has been made regarding their biological activity and medicinal applications. It is now considered as a valuable source of unique natural product for the development of medicines against various diseases and also for the development of bio-pesticides.[22]

I.2 THE NEEM TREE.

Historical Background

Neem (***Azadirachta indica*** A. Juss) is a versatile Indian tree of great importance, known to man since time immemorial. The history of commercial use of neem tree is shrouded in the mystery and lore of vedic period in India, which began about more than 4000 years B. C.[23]

Various parts of the tree have been and are being used in India for medicinal purpose and Ayurveda has regarded the tree as "sarva roga nivarni". [16,23]

In the 1st century B. C., Charaka, the eminent Indian physicians, gave details of a method for using leaves of neem for contraceptive purposes and for application as paste on the wound.[24]

According to Susruta, in 800 B. C., the decoction of leaves is prescribed in leprosy, urinary disorders, diabetes, eczema of the face and for washing and bathing purposes.[16]

In Ayurvedic medicines, decoction of the bark is prescribed for fever and rheumatism while the oil is used in treatment of tetanus, urticaria, scrofula, skin diseases and in the early stages of leprosy.[16] Neem leaf juice is used for expelling worms and curing jaundice and skin diseases. Neem twigs because of their antipeptic properties are employed in many village communities as a toothbrush.[16]

The name of the genus, ***Azadirachta*** which belongs to Meliaceae family, appears to be derived from the Persian name of the tree i.e. **Azad-darkhat-indica, azad** meaning free, **darkhat** meaning tree and **indica** meaning of Indian origin i.e. the free tree of India. The Sanskrit name of the neem tree is 'Arishtha' meaning 'relieving sickness' and the people of India have long been revered the tree.[22,23]

Azadirachta indica A. Juss and *Melia azedarach* are two closely related species of Meliaceae. The former is popularly known as Indian neem (margosa tree) or Indian lilac, and the latter as the Persian lilac.[22]

The neem tree has been described as ***Azadirachta indica*** A. Juss as early as 1830 by a French botanist Antoine Laurent De Jussieu and its taxonomic position is as follows.[22,23]

Order	Rutales
Family	Meliaceae (mahogany family)
Subfamily	Melioideae
Tribe	Melieae
Genus	*Azadirachta*
Species	*indica*

The genus Azadirachta differs characteristically from its related genera by its leaves being simply pinnate, glabrous innovations, base of the petiole mostly with one pair of orbicular glands and one pair of elongate glands.[22,23]

Occurrence and Geographical Distribution

Azadirachta indica A. Juss an indigenous tree of Indo-Pakistan subcontinent and is widely distributed in Asia, Africa and other tropical parts of the world.[25] It is found in most parts of India in the tropical and sub-tropical, semi-arid to wet tropical regions.[16] It was introduced by an Indian immigrant during the last century into African countries, where it is abundant in the tropical belt from Somalia, in the east to Nigeria, Mauritania, Togo, Ghana etc in the west.[16] It is presently grown in many Asian countries e.g. Pakistan, Nepal, Malaysia, Thailand, Sri lanka, Burma etc.[26] It is now widely planted in many islands in the pacific region and Caribbean nations as well as many countries in central and south America, where it was introduced by Indian migrators.[27] Neem has also successfully been established in Australia, West Africa, and Puerto Rico, Florida and Southern California.[16] Thus, neem tree is distributed in many parts of the world in the tropical and sub-tropical regions.

Neem has adapted to a wide range of climate. It can be established without irrigation in hot and dry regions receiving low annual rainfall of about 500 mm or less. It thrives well in hot weather, nutrient-poor dry soils and tolerant of high temperatures but is susceptible to excessive cold or frost.[27,28] It grows on almost all type of soils including clayey, saline, alkaline soils with pH up to 8.5 but dose not grow well on black cotton soils and deep well drained soil.[29] Although the neem tree grows best in soil at pH 5, its leaf litter gradually brings the surface soil pH to neutrality.[30,31] The native

habitat occurs at altitudes between 50 and 100 m, and 130 mm of rainfall per annum is sufficient for its normal growth. Its deep root system has the ability to extract nutrients and moisture from highly leached soils and it can withstand long periods of drought.[27]

Neem is a large evergreen tree, 15-20 m in height with semi-straight to straight trunk, 30-80 cm in diameter and spreading branches forming a broad crown.[31] It is almost evergreen but the tree becomes near leafless in dry for a short period during February-March before the old ones have all fallen.[31]

In India, white flowers appear from January through April and fruits ripen (mature) from June to August.[31] The fruit is an ellipsoidal drupe, about 1.25 cm long and greenish yellow when ripe, having usually one seed. The tree starts fruiting at the age of five years but economic yield of fruit is obtained at the age of 10-12 years. About 3300–4500 seeds weigh one kg and on an average, a medium sized tree produces 37-55 kg fruits per year.[31,32]

One seed drupe consists of a fleshy pericarp with a moderately soft shell inside, enclosing the oil-rich kernel. The pericarp contains resinous matter and wrinkles as it drys. Fresh fruit yield per tree ranges between 37 to 40 kg per year. Forty kilograms of fresh fruit yields nearly 24 kg of dry fruit (60 %), which in turn gives 11.52 kg of pulp (48 %), 1.1 kg of seed coat (4.5 %), 6.0 kg of husk (25 %) and 5.5 kg of kernel (23 %). The kernel gives about 2.5 kg of neem oil (45 %) and 3.0 kg of neem cake (55 %). The cake and oil contain various bioactive constituents like azadirachtin, nimbin, gedunin, salannin etc.[27]

I.3 CHEMISTRY OF DIFFERENT COMPOUNDS PRESENT IN VARIOUS PARTS OF NEEM

Background

Indians knew medicinal properties of neem, since ancient times. These properties prompted initially the Indian pharmaceutical chemists to study the isolation and identification of active principles from neem oil.

The compounds have been divided into major classes: isoprenoids and others. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, meliacins (azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds) and C-seco meliacins such as nimbin, salannin, and azadirachtin. The non-isoprenoids include proteins and carbohydrates, sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcones, coumarins and tannins, aliphatic compounds etc.[22]

Thus, Warden in 1888 first studied the chemistry of neem oil and found it to contain sulphur.[33] Chatterjee and Sen, isolated an acidic principle from neem oil which they named as margosic acid.[34] However, Dutta and Roy later on proved that margosic acid was nothing but a mixture of known fatty acids contaminated with bitter principles.[35] Watson and co-workers then developed a process for refining the neem oil and isolated steam volatile fraction, which they named as margosopicrin, m.p 221-222°C with the molecular formula $C_{24}H_{32}O_8$.[36] While, Sen and Bennerjee reported a sulphur containing bitter acid from the aqueous extracts of the oil.[37]

Qudrat-i-Khuda *et al* isolated a water soluble bitter compound from the aqueous extract of the oil along with four new fatty acids, later

on identified as known acids.[38] Seshadri and co-workers were the next to examine chemically the neem oil to report two amorphous bitter compounds with grey and dark-brown colour.[39]

All these workers employed drastic conditions such as steam distillation and saponification etc, in their attempts to separate the active principles. However, such drastic conditions may often lead to decomposition of active compounds.

Thus, in 1940 Siddiqui employed milder condition like solvent extraction followed by crystallization and isolated first major bitter principle nimbin **1** (Figure: I.1) in a crystalline form, m.p. 205°C, in addition to another crystalline compound, nimbinin m.p. 192°C **2** and an amorphous bitter principle, nimbidin.[40] Subsequently, Siddiqui and Mitra obtained sulphur containing major acid nimbidinic acid and a minor acid, nimbic acid by alkali hydrolysis of neem oil. Nimbidinic acid was found to have pharmacological activity and hence a number of salts namely sodium-, potassium-, zinc-, copper- and quinine nimbidinates were prepared.[41] Both nimbidin and sodium nimbinate were found to stimulate uterine contractions.[41] After removal of bitter principles by alcohol extraction from neem oil, they analyzed it to reveal that it is a good source of stearic and oleic acids. By using low temperature crystallization and UV spectroscopy the following composition of the fatty acids was reported: myristic (2.4 %), stearic (10.2 %), palmitic (6.8 %), oleic acid (59.9 %) and linoleic acid (8.3 %). This is somewhat comparable with the composition reported earlier by Dasa Rao and Seshadri, which are as follows: stearic (18.2 %), palmitic (13.8 %), oleic acid (52.6 %) and linoleic acid (13.6 %).[42]

Around this period, chemical investigations were undertaken extending studies to other parts of the neem tree such as trunk-

bark,[43] root-bark,[44] and flowers. Root-bark and trunk-bark were found to contain nimbin, nimbidin and steronimbosterol. Besides these, trunk-bark was also reported to contain nimbinin. Similarly, neem flowers were yielded nimbosterol, a sterol glycoside nimbosterin and a flavone nimbecetin, which were identified later on by Mitra as keampferol **3**.[45] Seshadri and Pankajamani also isolated three flavonoids and named them as myrcetin **4**, quercetin **5**, and kaempferol.[46] Mukherjee and Shrivastava subjected the neem gum to hydrolysis and one of the product obtained was identified as aldobiuronic acid.[47]

In the sixties, Narashimhan and Narayanan *et al* confirmed the structure of nimbin based on elemental analysis, mass spectroscopy and NMR.[48] Both chemical and spectroscopic evidences led them to assign structure **1** to it. Further extensive studies relating to NMR and Optical Rotatory Dispersion (ORD) of nimbin and its derivatives enabled them to establish the absolute stereochemistry of nimbin. Thus, nimbin the first bitter principle of neem was characterized after a gap of twenty years of its isolation as a growing member of the tetranortriterpenoid, biogenetically derivable from apo-euphol with C-ring oxidatively broken between C_{12} and C_{13} and with appropriate oxidations at other sites.

Around this period Shravankumar and co-workers analyzed the neem seed shell to find that it contained, lignins, protein (8 %) and cellulose (33 %).[49] Choudhary *et al* conducted paper chromato graphy of fatty acids of neem oil and reported the presence of palmitic, stearic, arachidic, myristic, oleic, and palmitoleic acids.[50] Mitra and Mishra estimated the amino acid content in defatted seed meal and found the following amino acid composition, lysine (3.9 %), histidine (3.4 %), arginine (6.3 %), aspatric acid (6.3 %), proline

(5.9 %) and glycine (9.4 %),[51] while Bajpai *et al* standardized the method of hydrolysis of neem gum to obtain the maximum yields of aldobiuronic and aldotriuronic acids.[52]

Naryanan *et al* while continuing their work isolated a derivative of nimbin identified as deacetylnimbin **6** from the neem oil. Its structure was proved by its correlation with nimbin. Further two new tetranortriterpenoids namely nimbinin and vepinin **7** (Figure: I.2) were isolated by them from the same source.[53]

In 1962, Pradhan *et al* discovered the antifeedant activity of neem seeds.[21] This discovery was remarkable because till then neem had only attention of pharmacologists. These workers first time demonstrated the role of neem compounds in the field of plant protection. From then onwards, biologists and chemists started working together and research on neem received worldwide attention.

Then Lavie *et al* isolated one of the antifeedant principles called meliantriol **8**.[54] Henderson *et al* also reported the isolation procedure of salannin, **9** another antifeedant from neem.[55] Prompted by these reports, in 1968 Butterworth and Morgan undertook a chemical examination of neem seed kernel extract and isolated a micro crystalline compound, which was named as azadirachtin (Aza-A) **10** with astonishing activity against insects for the first time.[56]

If the sixties was the period of discovery and identification of biological compounds in neem, the seventies saw the development of isolation techniques aided by diversified biological screenings of pure fractions.

The chemically similar neem constituents were very difficult to separate by the conventional chromatographic techniques. The devel -opment of modern High Performance Liquid Chromatography (HPLC)

by this time was of great help in solving the problem and to resolve the neem compounds to a high degree of purity.

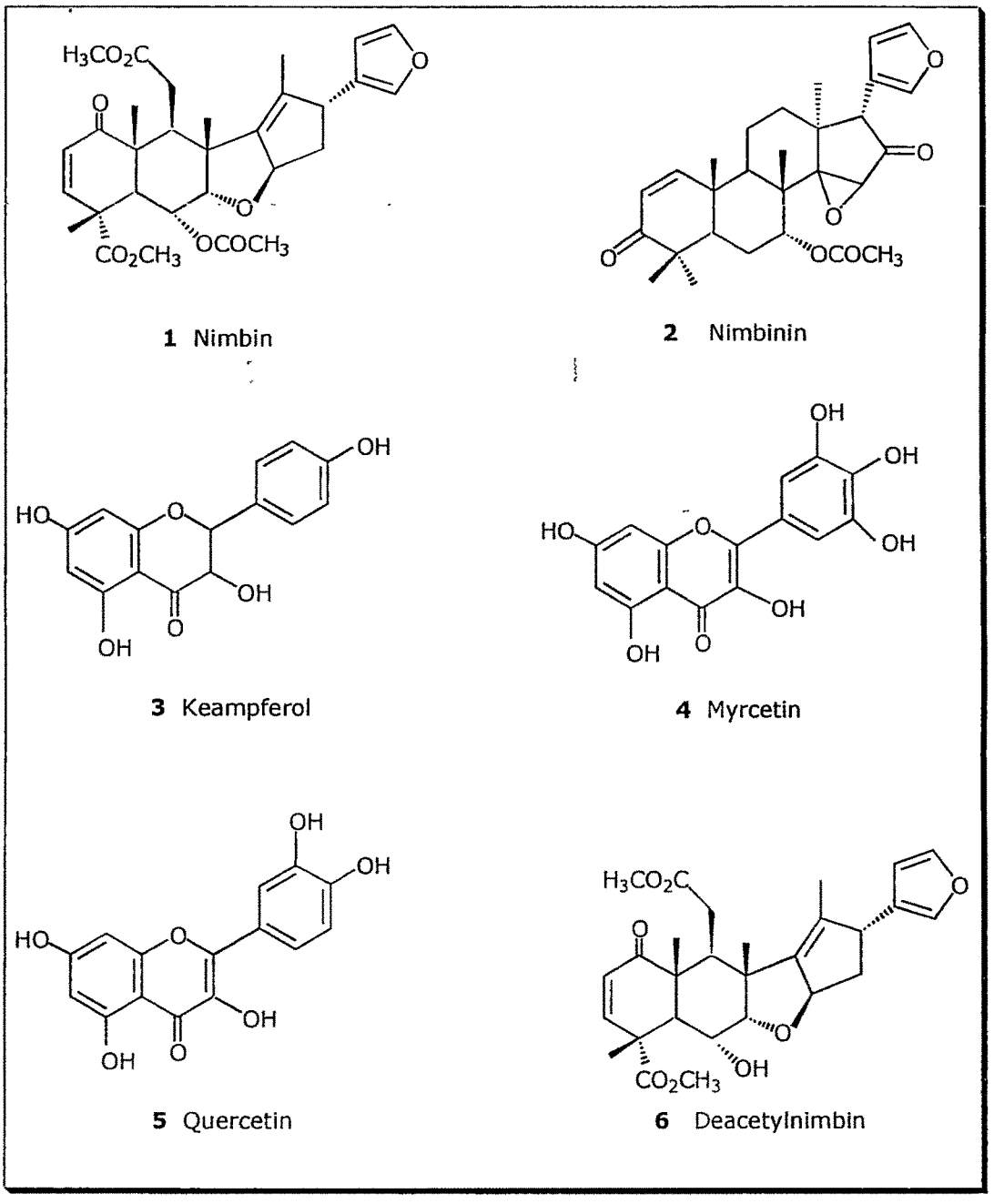


Figure: I.1

By this sophisticated technique, it was easy to purify of the neem fractions in a non-destructive manner. Many of the pure fractions reported by earlier workers turned out to be mixtures as a result of use this instrument. When pure constituents become available, structural chemistry also advanced.

The work carried out by Pradhan *et al* by this time attracted greater attention of biologists. Hence, Gill and Lewis in 1971 and Ruscose independently discovered the growth disrupting activity of azadirachtin.[57] While Warthen Jr *et al* developed a practical method of isolation in large scale of azadirachtin and tested biological activity of the pure substance against nematodes and arthropods.[58] Butterworth and Percy while continuing work in 1972 prepared many derivatives of azadirachtin and Morgan studied the structure activity relationship on feeding inhibition.[59,60] Kraus *et al* reported a few new pentanortriterpenoids which are exceptions to the general chemical nature of neem compounds.[61]

With the ready availability of pure azadirachtin and knowledge of its wide spectrum of biological activities, basic studies such as its mode of action, its different analogous and various isolation techniques become possible in eighties and nineties.

Ley and his group undertook an singal X-ray crystallographic and mass spectroscopic studies together with NMR of the pure azadirachtin and its derivatives.[62] They partially hydrogenated azadirachtin molecule, at the C_{22}-C_{23} position and subsequently treated the material with an excess of sodium periodate and potassium permanganate in the presence of a base to give detigloyldihydroazadirachtin. It was examined by singal X-ray crystallography technique and data revealed the structural fragments and together with detailed NMR and mass spectroscopic studies,

complete structure assignment of the parent azadirachtin molecule was achieved, while Turner *et al* has also reported the detailed results of NMR studies on azadirachtin and its trimethyl ether.[63]

During this period, Schroeder and Nakanishi reported the isolation and purification procedure of azadirachtin from neem seeds.[64] The method involves partition between 95 % aqueous methanol : petroleum ether and ethyl acetate : water, followed by silica gel filtration, vacuum liquid chromatography with ethyl acetate and hexane (3:1), flash chromatography and subsequent crystallization to obtain pure azadirachtin. Uebel *et al*[58], Govindachari *et al*[65], Yamasaki and co-workers[66] and Warthen Jr and co-workers[67] developed the reverse phase HPLC procedure for purification and estimation of azadirachtin in crude neem seed extracts, using different solvent extraction methods and various partition techniques. While Huang and Morgan reported the quantitative determination of the azadirachtin in crude extract of neem seeds by packed column supercritical fluid chromatography.[68] Yamasaki and Klocke have reported the formation of 11,20-dicarbomethoxy-azadirachtin, a result which supports the emerging reactivity pattern for azadirachtin as C_{11} (OH) > C_{20} (OH) > C_7 (OH).[69]

Rembold subjected neem seed kernel extract enriched in azadirachtin to preparative HPLC to obtain, besides Morgan's azadirachtin which he termed as azadirachtin-A and Kraus's 3-tigloylazadirachtol (Aza-B), five other compounds which were named as azadirachtins C to G, and their structures (with the exception of C) were assigned on the basis of their NMR and other spectral techniques.[70] On the other hand, Govindachari and co-workers isolated three other compounds, named azadirachtins H, I and K by preparative HPLC from crude

neem seed kernel extracts and structures were assigned on the basis of mass, PMR and ^{13}CNMR spectroscopy.[71]

Khalid *et al* reported the isolation procedure of Gedunin **11**, an anti malarial agent from neem.[72] Garg and Bhakuni has isolated a new isoprenylated flavanone from the leaves of neem and characterized as 8,3′-di-isoprenyl-5,7-dihydroxy-4′-methoxy flavanone **12** (Figure: I.3) on the basis of physical and spectroscopic evidence. This was the first report of an isoprenyl flavanone from the Meliaceae.[73] While Siddiqui and co-workers have also isolated a new triterpenoid named nimbocinone **13** from fresh, undried winter leaves of neem along with two sterols identified as sitosterol and stigmasterol.[74] They have also isolated two new bitter meliacins, nimocinolide **14** and isonimocinolide **15** from the fresh leaves of neem.[74] The structures of these tetranortriterpenoids were elucidated through chemical and spectral studies. Compounds **14** and **15** acted as insect growth regulators against house flies (*Musca domestica*) and mosquitoes (*Aedes Aegypti*).[74]

Rembold *et al* observed that azadirachtin is not only a feeding deterrent but also an insect growth disruptor and conducted a series of experiments on various arthropods and insects and concluded that neem has both feeding deterrent and insect growth disruptor properties.[75] Ley and co-workers assessed the antifeedant activity of azadirachtin, its derivatives and related limonoids in choice and no-choice bioassays against four species of Lepidoptera. Azadirachtin and dihydroazadirachtin were the most potent of the 40 compounds tested. The results showed that hydrogenation of the C_{22}-C_{23} double bond did not decrease antifeedant activity and the nature of the Substitution at C_1, C_3 and C_{11} were important.[76]

The synthesis of azadirachtin is difficult, because of its complexity. Major effort in this direction was done in nineties by S.V. Ley and his groups.[1,77]

In nineties, Ravindranath *et al* have studied the variation of azadirachtin content during the growth and storage of neem seeds by reverse phase high performance liquid chromatography using anisole as internal standard and concluded that azadirachtin appears only after the 9th week, gradually reaches the maximum around the 17th week and decreases by the 19th week. Thus, the fruits can be profitably harvested in the 17th week of development (when the neem fruit turns from green to yellow) for better yield of azadirachtin.[78] Furthermore, Jitendrakumar and Parmar studied the relationship of various agro-ecological regions and their key factors (ecosystem, growth, period, soil type etc) to the yield of neem oil, its major constituents (azadirachtin, nimbin, salannin etc) and the total as well as the key fatty acids.[79] In addition, they have also examined the physicochemical and chemical variation in neem oils and some bioactivity against *Spodoptera litura*.[79] During this period, Sarojini Sinha *et al* reported the simplified procedure for isolation and enrichment of azadirachtin without resorting to chromatographic techniques.[80] Alberto Ritieni *et al* applied the supercritical fluid extraction technique to extract of azadirachtin from neem seeds.[81] Sundaram and Curry developed a HPLC method for the analysis of azadirachtin in commercial formulations and neem oil.[82]

Govindachari and co-workers reported a direct preparative HPLC method for isolation of the major triterpenoids from neem oil.[83a] They have also obtained azadirachtin-A in a crystalline state for the first time and crystal parameters were measured by X- ray diffraction method. [83b,c]

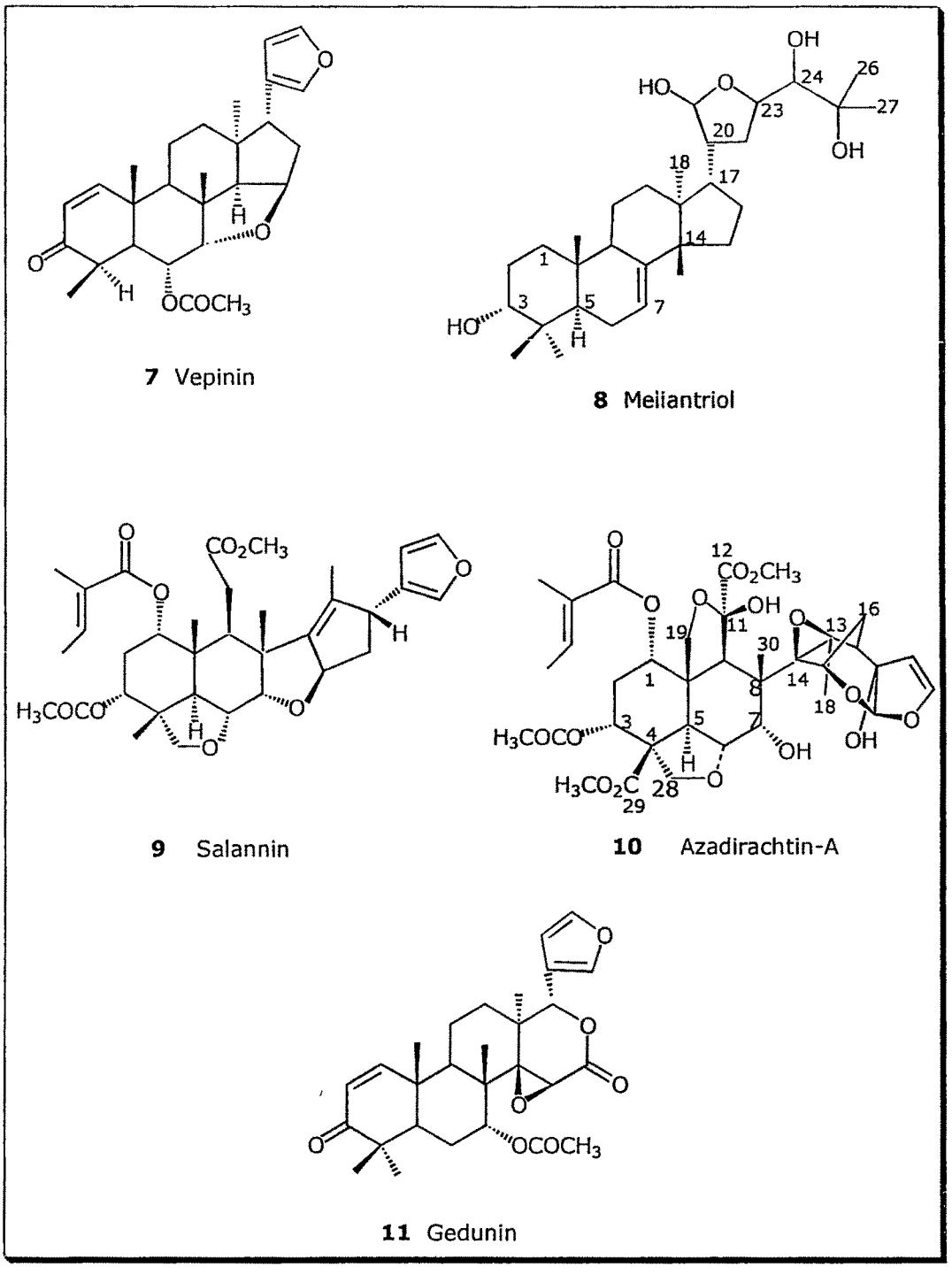
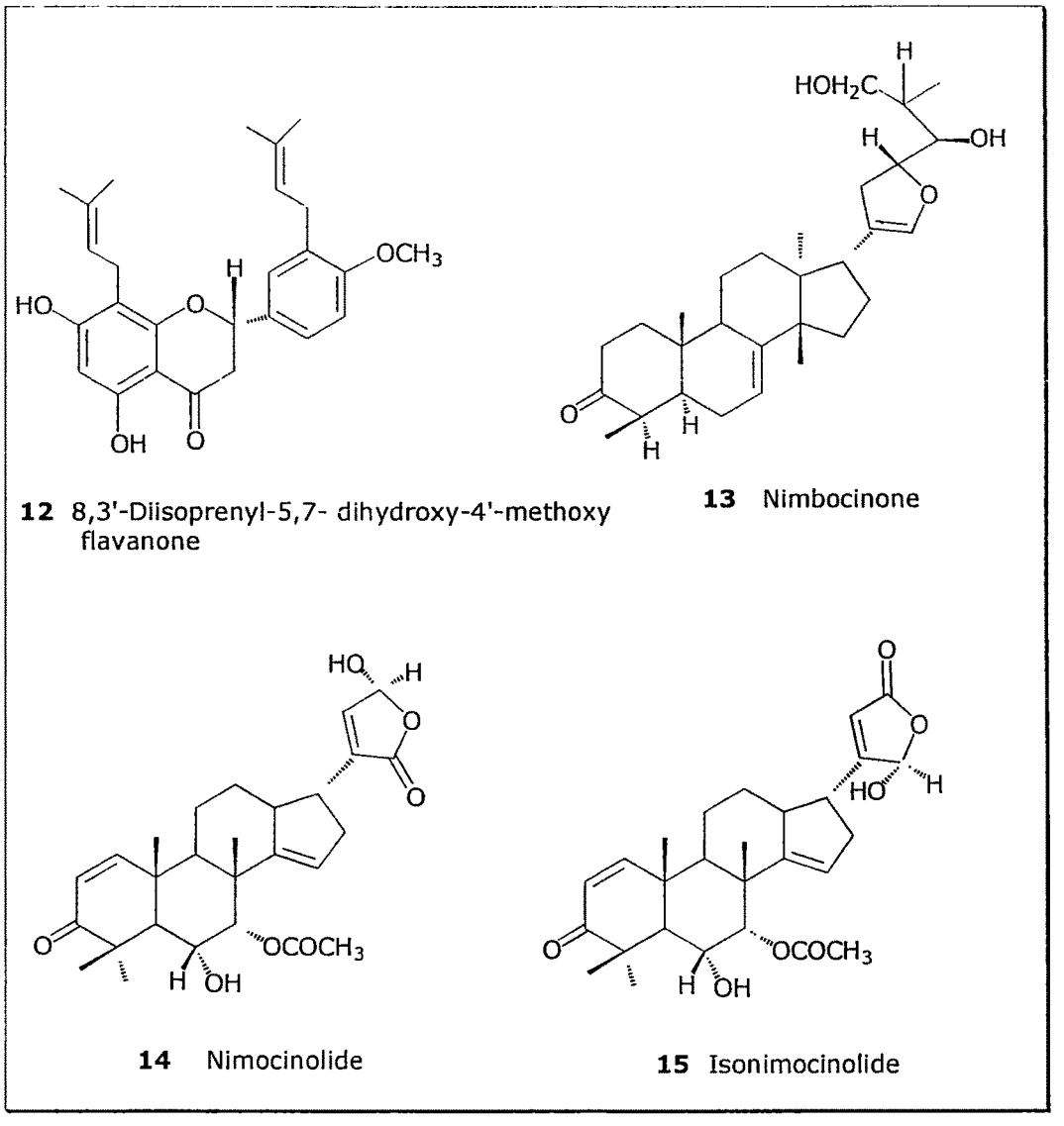

7 Vepinin
8 Meliantriol
9 Salannin
10 Azadirachtin-A
11 Gedunin

Figure: I.2



12 8,3'-Diisoprenyl-5,7- dihydroxy-4'-methoxy flavanone

13 Nimbocinone

14 Nimocinolide

15 Isonimocinolide

Figure: I. 3

Recently, two novel compounds, 29-oxymethylene-11-demethoxy-carbonyl-11α-hydroxyazadirachtin (azadirachtin M) and 22,23-dihydro-23α-hydroxy–3-tigloyl-11-deoxyazadirachtin (azadirachtin-N) together with known compound 11-epi-azadirachtin H were isolated from a methanolic extract of seed kernels of neem by a Xiaodong Luo and his group.[84]

Thus, till today more than hundred terpenoid compounds have been isolated and reported from various parts of the neem by different group of workers.

Distribution of Various Constituents in Different Parts of the Neem Tree

A number of organic compounds have been isolated and investigated for their biological activities from various parts of neem and attempt has been made to group the compounds according to their occurrence in the different parts of tree. These include proto meliacins, meliacins or tetranortriterpenoids, pentanortriterpenoids, hexanortriterpenoids and nontriterpenoidal constituents.

LEAF: The leaf of neem is a storehouse of organic compounds, which are responsible for its various medical and insecticidal properties. Leaves have been shown to contain fibre (11-24 %), carbohydrates (48-51 %), protein (4-18 %), fat (2.3-6.9 %), ash (7.7- 8.5 %) calcium (0.8-2.4 %) and phosphorous (0.13-2.4 %),[85] as well as a number of amino acids including the ten essential ones, e. g., glutamic acid, tyrosine, aspartic acid, alanine, proline and gluta - mine.[86] Fresh matured leaves on steam distillation give an odourous viscous oil. Gas chromatography-mass spectra (GC-MS) analysis of this oil indicated it to be a mixture of tri- and tetracyclic sulfides of C_3, C_5, C_6 and C_9 units.[87]

Siddiqui and his group have isolated a crystalline hydrocarbon fraction from the petroleum ether extract of the fresh, un-crushed leaves. The GC-MS analysis of this fraction indicated the presence of eight saturated hydrocarbons e.g., docosane, pentacosane, heptacosane, octacosane, triacontane, hentriacontane, dotriacontane and nonacosane.[88]

Leaves and twigs were also found to contain different fatty acids such as, dodecanoic acid, tetradecanoic acid, hexadecatrienoic acid, hexadecanoic acid, octadecanoic acid, eicosanoic acid, docosanoic acid and tetracosanoic acid.[88]

There are number of chemically and biologically interesting limonoids present in leaves. Most important among them are: nimbin[40,48] **1**, nimbinene[61]**16**(Figure:I.4), 6-deacetylnimbinene[61]**17**,margocetin deacetylisonimbinolide, nimbandiol[61]**18**,isofraxidin, nimocinol[89]**19**, nimbocinone[74]**13**,nimocinolide[74]**14**,isonimocinolide[76]**15**, nimbocinolide[90] **20** (Figure:I.5), different isocoumarins isonimbocinolide[90]**21**, nimbolide[91]**22**, margosinolide[92], isomargosinolide[92], deacetylisonimbinolide[93], isonimbolide[94] **23**, and (24E)-isopropenyl cholesterol[95] **24**.

Besides these, leaves also contain flavonoids such as hyperoside, quercetin, rutin, kaempferol, â- sitosterol and nimbaflavone.[96] Quercetin (a polyphenolic flavonoid) is known to have antibacterial and antifungal properties.[96]

Tirimanna has also reported that the presence of carotenoids and other compounds in leaves by the using of two-dimensional TLC method.[97]

FLOWERS: The flowers mainly contain flavonoids. Three glycosides namely quercetin-3-galactoside and kaempferol-3-glycoside have been reported to occur in the flowers.[98]

BARK: The chemistry of neem bark is as important as that of other part. The stem and the root bark are reported to possess astringent, tonic, anti-periodic and other medicinal properties. The activity of root bark is more than that of stem bark.[99]

Important compounds isolated from stem bark are: nimbosodione[100a] **25** (Figure:I.6), nimbisonol[100a] **26**,

nimbonone[100b] **27**, nimbonolone[100b] **28**, nimbione[100c,d] **29**, nimbinone [100c,d] **30** and isonimbinolide [100c,d] **31**.

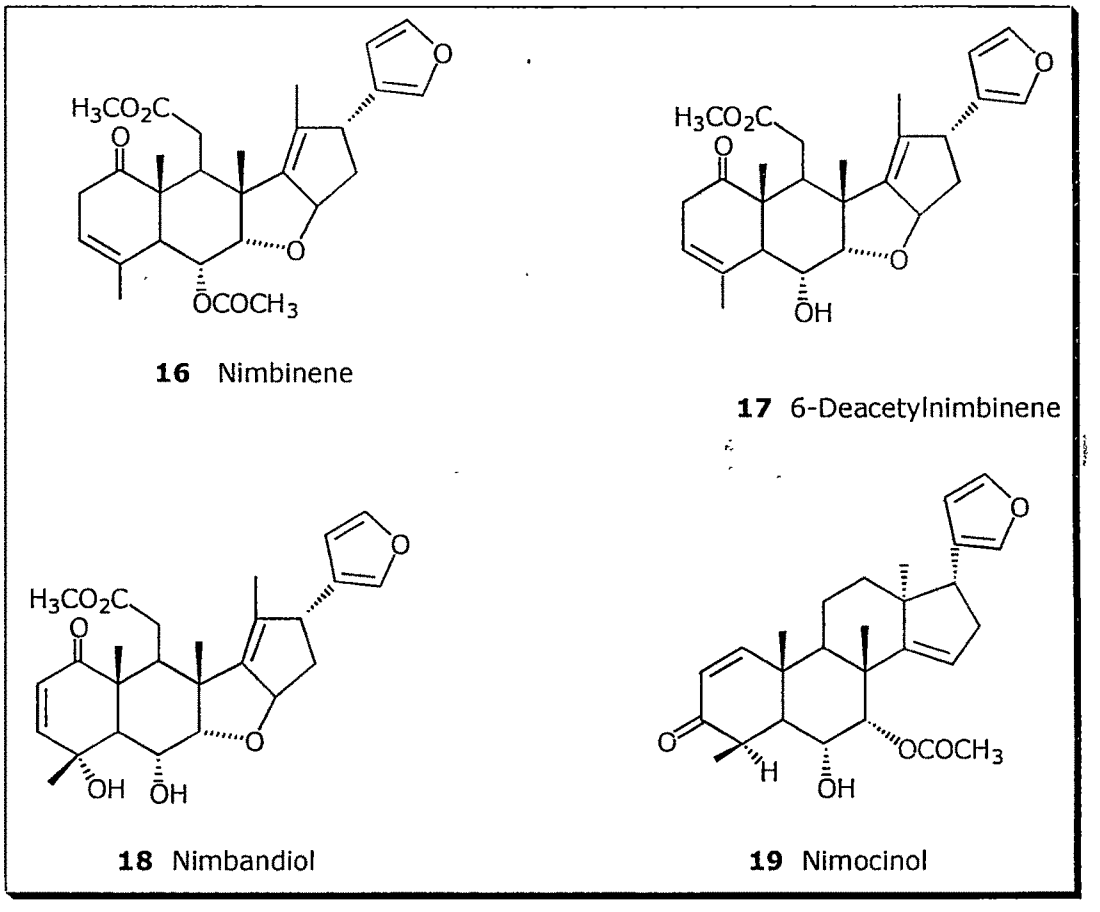
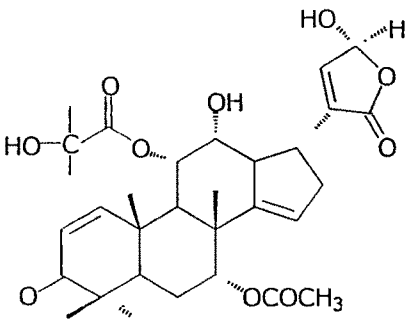


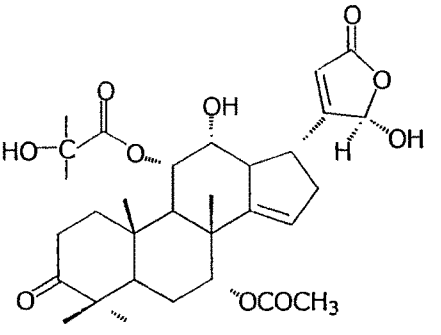
Figure: I.4

Six diterpenoids were isolated from the root bark: margocin [101a] **32** (Figure: I.7), margocinin[101a] **33**, margocilin[101a] **34**, nimbilicin[101b] **35**, nimbocidin[101b] **36** and nimbidiol[102] **37**.

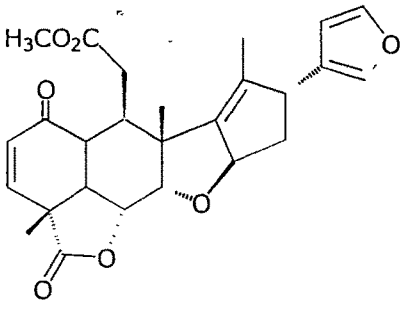
Compounds reported from wood oil are cycloeucalenol and 24-methylenecycloartenol.[103] Heartwood of the neem contains â-sitosterol, thiomyl alcohol 24-methylenecycloartanol[104], nimatone and triterpenes.[105,106] While D-glucosamine[107], aldobioronic and aldotriouronic acids are reported from gum resin of neem.[107]



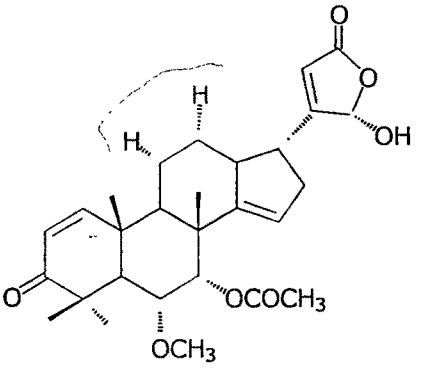
20 Nimbocinolide



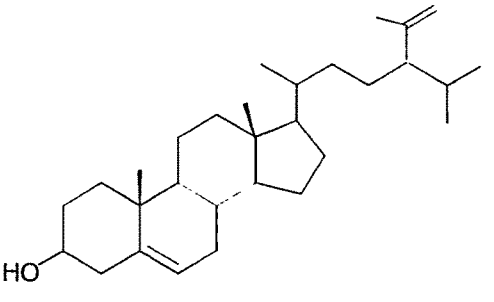
21 Isonimbocinolide



22 Nimbolide

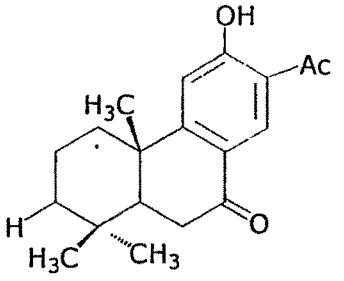


23 Isonimbolide

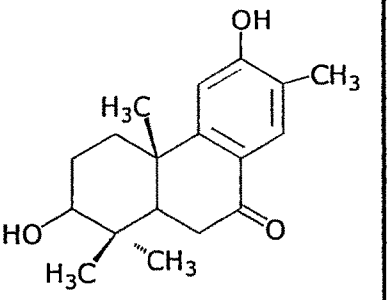


24 (24ξ)-Isopropenyl cholesterol

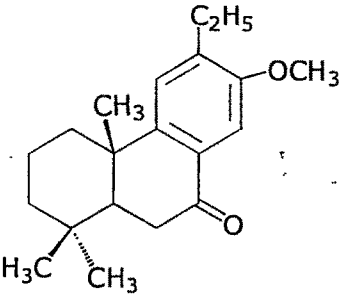
Figure: I. 5



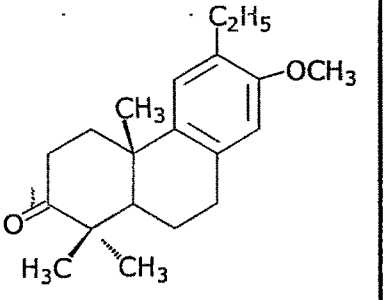
25 Nimbosodione



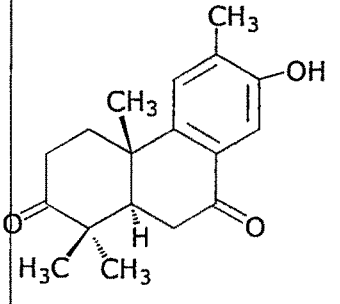
26 Nimbisonol



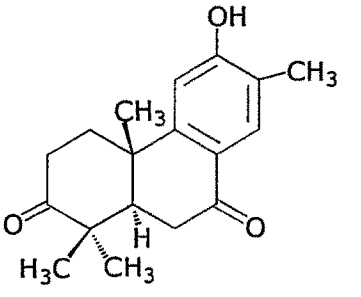
27 Nimbonone



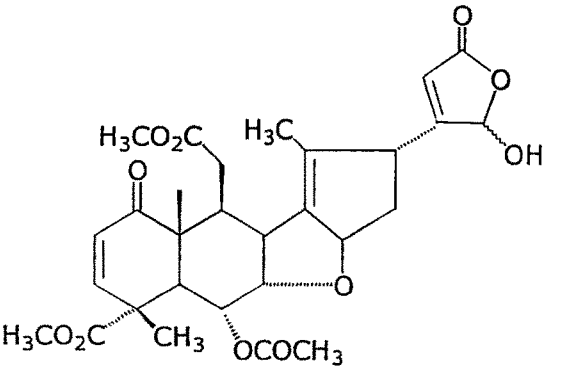
28 Nimbonolone



29 Nimbione



30 Nimbinone



31 Isonimbinolide

Figure: I.6

SEEDS: Seeds of the neem tree are commercially important. They consist of about 45 % kernel and 55 % shell. The volatile compounds from freshly crushed seeds were analyzed by capillary GC-MS and a total of 25 compounds were identified. Di-η-propyldisulphide was the major constituent amounting to 75 % and was shown to be larvicidal to mosquitoes and tobacco budworm etc.[108] These compounds may be responsible for some of the insect repellent properties of neem.[109]

The neem seed kernels are rich in oil, yielding about 40 to 50 % of the oil, with a bitter taste and disagreeable odour.[109] The oil can be extracted either by crushing the kernels in an expeller or by a solvent extraction method. The purified neem oil has following physico-chemical properties. [109]

- Sp. gravity: 0.9087-0.9189 (30°C)
- Refractive index (M_D) : 1.4612-1.4627 (40°C)
- Iodine value (Wijs) : 68-75,
- Saponification value: 193-204 and unsaponifiable matter 0.8 %

The fatty acid composition of the oil is as follows: myristic (0.2 %), palmitic (16.2 %), stearic (14.6 %), arachidic (3.4 %), oleic (56.6 %) and linoleic (9.0 %).[109] The glycerides compounds are palmitodistearin (0.2 %), oleopalmitostearin (20.3 %), oleodistearin (1.6 %), palmitooleolinolein (6.6%) and linoleodiolein (16.5 %)[109]

The biologically active compounds isolated from the neem oil are: nimbin[40], nimbinin[40], nimbidin[40], meliantriol[54] **8**, azadirone[110] **38** (Figure:I.8), azadiradione[110] **39**, epoxyazadiradione[110] **40**, meldenin[111] **41**, vepinin[50] **7**, gedunin[72] **11**, nimbinene[61] **16**, 6-deacetylnimbinene[61] **17**, salannin[55] **9**, tiglic acid [(E)-2-methyl-2-butenoic acid][112] **42** (Figure: I.9), 1,3-diacetylvilasinin[113a] **43**, 3-deacetylsalannin[113a] **44** and salannol[113a] **45**.

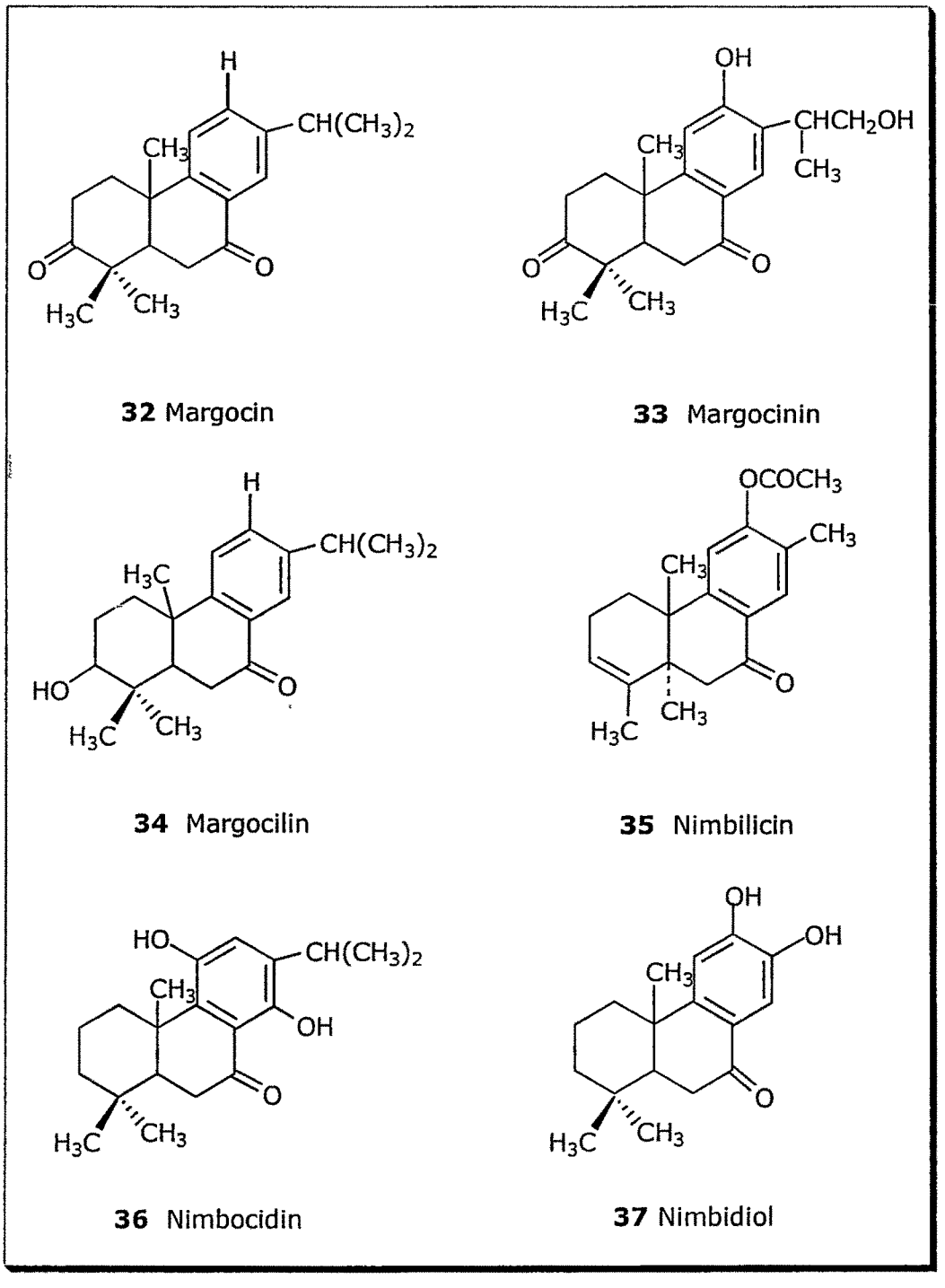

H
CH(CH3)2
CH3
O
O
H3C
CH3
32 Margocin
OH
CHCH2OH
CH3
CH3
O
O
H3C
CH3
33 Margocinin
H
CH(CH3)2
H3C
HO
O
H3C
CH3
34 Margocilin
OCOCH3
CH3
CH3
O
CH3
H3C
35 Nimbilicin
HO
CH(CH3)2
CH3
OH
O
H3C
CH3
36 Nimbocidin
OH
OH
CH3
O
H3C
CH3
37 Nimbidiol

Figure: I.7

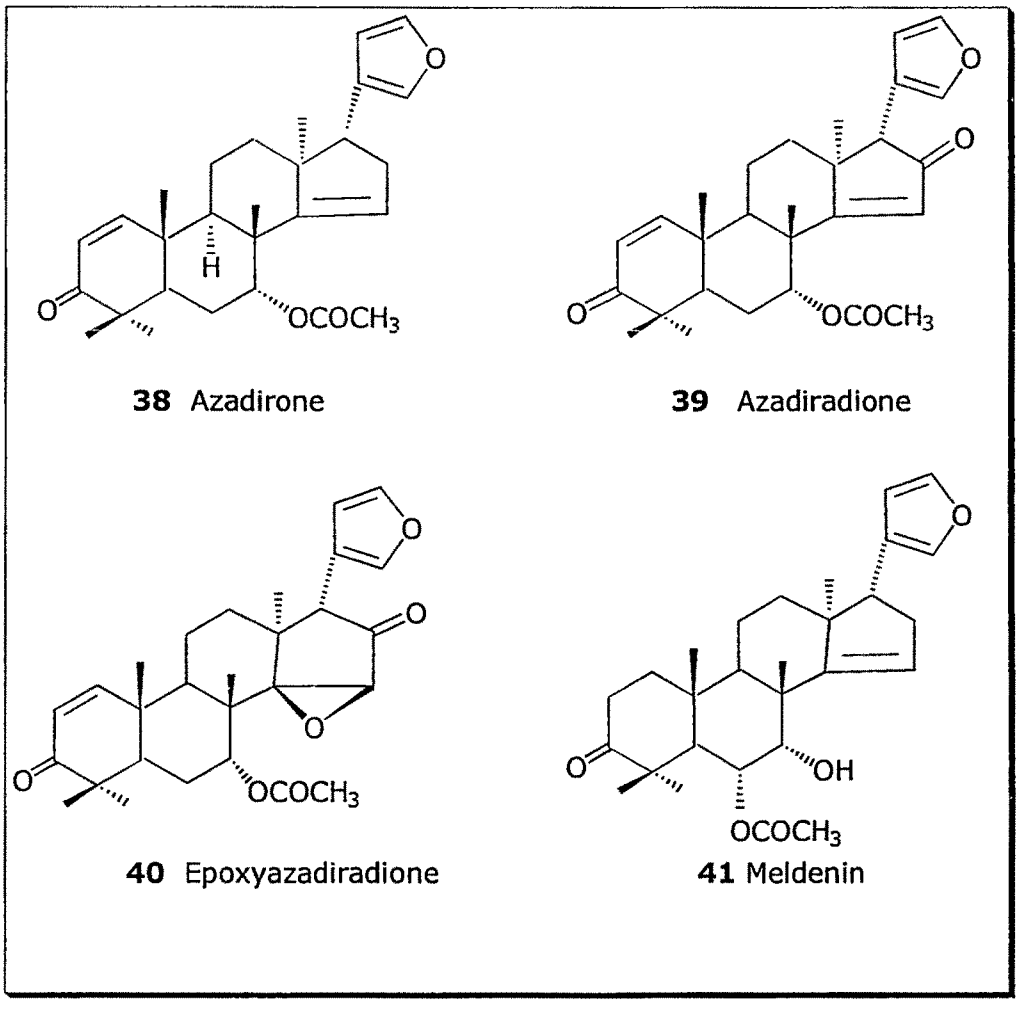


Figure: I.8

NEEM SEED CAKE: The cake left after extraction of the oil is known to contain a large number of triterpenoids, proteins, carbohydrates, crude protein fiber and minerals etc. Important terpenoidal compounds isolated from neem seeds are: azadirachtin[56] **10**, deacetylnimbin[53], 17-epiazadiradione[113b,114] **46** (Figure: I.10),17β-hydroxyazadiradione[113b] **47**, 7-deacetyl-17β-dihydroxyazadiradione **48**,meldenin, 7-desacetyl-7-benzoylazadiradione[61] **49**, nimocin[115a]

50, nimbocinol[115b,c] **19**, azadirachtol[115d], azadira chnol[115d], 4-epinimbin[116], azadirachtin-B[70], azadirachtin-C to G, azadirachtin-H and I[71], azadirachtin-K[71], azadirachtin M and N[84], marrangin **51**[117].

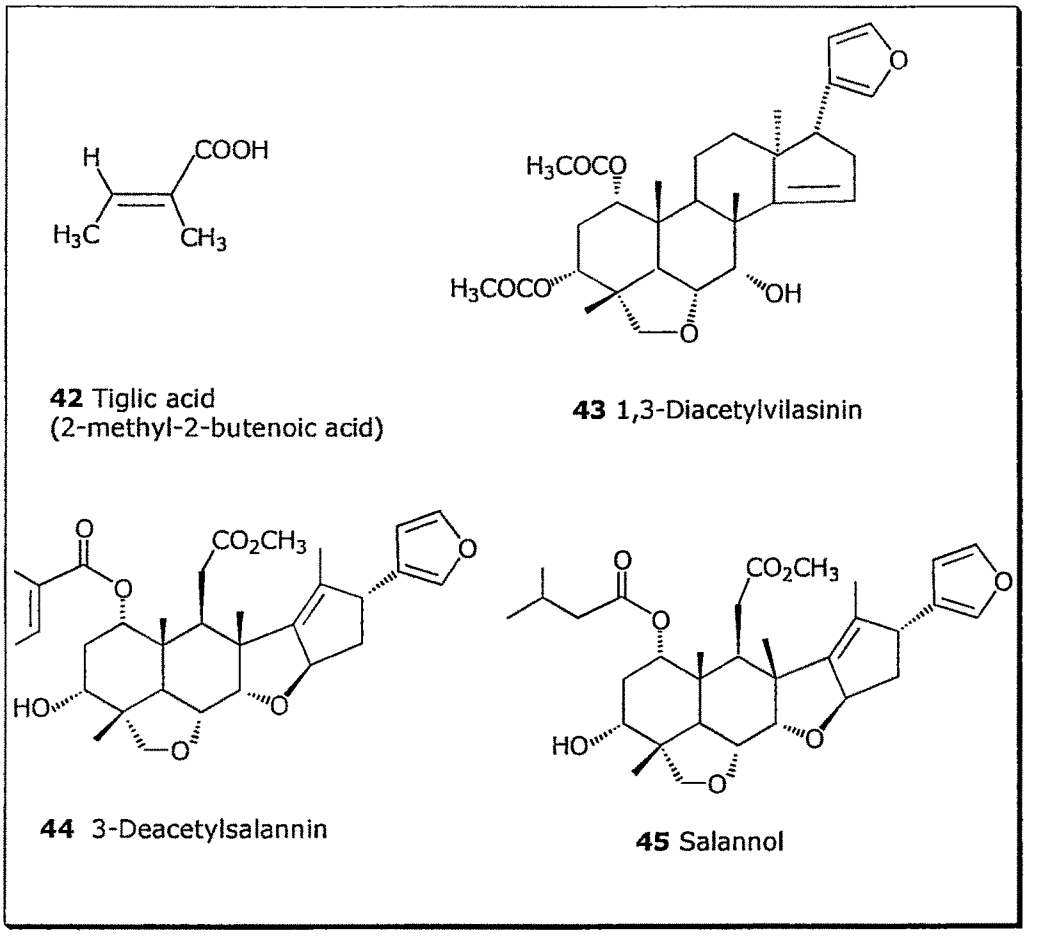
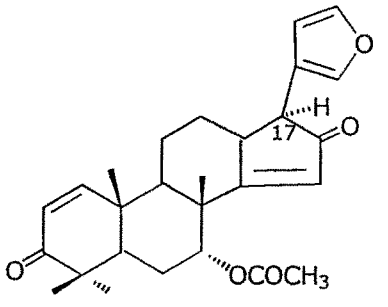
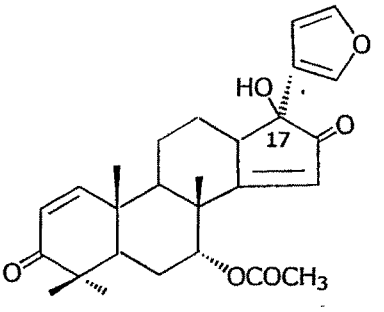


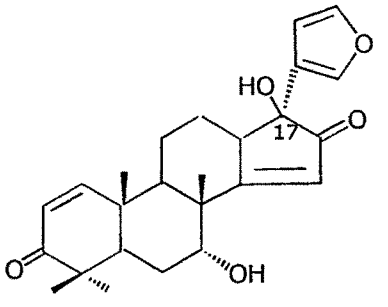
Figure: I.9



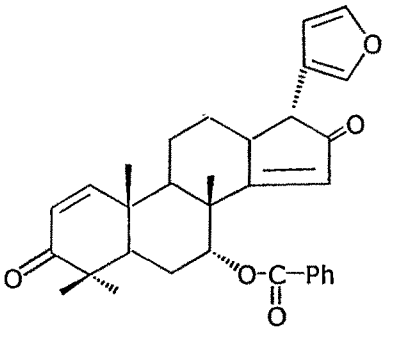
46 17-Epiazadiradione



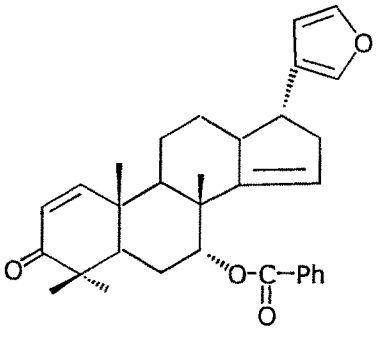
47 17ß-Hydroxyazadiradione



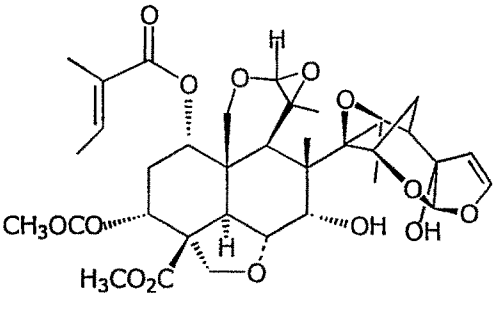
48
7-Deacetyl-17ß-dihyd
roxyazadiradione



49
7-Desacetyl-7-benzoyla
zadiradione



50 Nimocin



51 Marrangin

Figure: I. 10

I.4 APPLICATIONS OF NEEM AND ITS VARIOUS PARTS

The neem tree is a versatile tree and has tremendous potential for human use.[27] Every part of tree has use in toiletries, pharmaceuticals, the manufacture of agricultural implements and furniture, cattle and poultry feeds, nitrification of soils for various crops, pesticides, mosquito repellents, fertilizers, lubricants, gums and even contraceptives.[27] Since neem is a natural resource producing extensive useful biomass, its propagation and economic exploitation will be beneficial for mankind.

Isolation of certain biologically active compounds from neem in recent decades and their effectiveness against insect as well as pharmaceutical applications has been the major focus of activity.

Pharmacological Properties

The neem tree has been used as a traditional remedy in Ayurveda from the vedic times and leaves, bark, seeds, flowers etc have been shown to have medicinal properties. Biological activity of neem is reported with the crude extracts and their different fractions from leaf, bark, root, flower, seed and oil.[16,22]

Although a large number of compounds have been isolated from various parts of the neem, a few of them have been studied for biological activity as shown in Table: I.1.[22]

Pesticidal Properties

It has been an age-old practice in rural India to mix dried neem leaves with stored grains or to place them in warm clothes to repel insects. Farmers used to protect crops with natural repellents found in neem seeds and leaves.[16,22,23,27] However, a real break through

seems to have been made in the early sixties when Pradhan *et al* first reported the extraordinary antifeedant properties of neem seed kernels against desert locust *Schistocerca gregaria*.[21]

TABLE: I 1. Some bioactive compounds from Neem

Neem Compound	Source	Biological activity
Nimbidin	Seed oil	Anti-inflammatory, Antiarthritic, antipyretic, Hypoglycaemic Antigastric ulcer, Spermicidal, Antifungal Antibacterial
Sodium nimbidate		Anti-inflammatory
Nimbin	Seed oil	Spermicidal
Nimbolide	Seed oil	Antibacterial, Anti malarial
Gedunin	Seed oil	Anti fungal, Anti malarial
Azadirachtin	Seed	Anti malarial
Gallic acid, (-) epicatechin and catechin	Bark	Anti-inflammatory, immunomodulatory
Cyclic trisulphide cyclic tetrasulphide	Leaf	Anti fungal
Polysaccharides	Bark	Anti- inflammatory, Anti-tumour

Today, numerous studies are reported describing the insecticidal, antifeedant, growth inhibitory, oviposition deterring, anti-hormonal, anti-fungal, antiviral, nematicidal and anti-fertility activities of neem against a broad spectrum of insects.[27]

These biological activities have been demonstrated for the expelled oil from the seeds, leaves extracts, seed extracts, neem seed cake, fruit extracts, and various isolated compounds e.g., azadirachtin, meliantriol, nimbin, salannol, salannin, azadiranone, nimbinene, nimocinolide and isonimocinolide etc. These materials have been tested against a wide range of pests, insects on vegetables, ornamental crops, stored grains and also against household pest of medicine and hygienic importance.[27]

I.5 AZADIRACHTIN

In the early sixties, several reports appeared on the ability of neem seed kernel to deter feeding by insects on plants and the traditional use of such extracts for insect control. At the same time, Pradhan and his group and Sinha and Gulati reported 100 % antifeedant effect on *Schistocerca gregaria* and *Schistocerca migratoria* by use of neem kernel extract.[21,118,119] and Lavie *et al* isolated one of the antifeedant principles called meliantriol from the fresh fruit of *Melia azadarach* and the oil of *Azadirachta indica*.[54] Prompted by these reports, in 1968 Butterworth and Morgan isolated azadirachtin **10** from neem seed kernel extract, with astonishing activity against insects for the first time.[56]

Several other procedures of isolating azadirachtin have been subsequently reported which are essentially the same, but for the

variations in the adsorbents and solvent systems during the chromatographic operations.

The isolation procedure reported by Uebel *et al*[58] yielded 8.7 g of azadirachtin from 48.2 kg of neem kernels and that of Yamasaki and his group[66], 56 mg from 1 kg of neem seeds. The most cited paper for the isolation of azadirachtin is that by Schroeder and Nakanishi which after many steps, from 2 kg of seed kernels, gives 5 g of azadirachtin of unknown purity.[64] However, Govindachari *et al* reported a convenient procedure for isolation of pure azadirachtin from neem kernel extract using a preparative HPLC and recovered more than 95 % pure azadirachtin.[65]

In addition, Govindachari *et al* also reported the isolation of the major triterpenoids in neem oil by preparative HPLC and quantified their abundance by analytical HPLC. Salannin (1.4 %), nimbin (0.5 %), deacetylnimbin (0.4 %) and epoxyazadiradione (0.13 %) are the major constituents in neem oil, while azadirachtin-A present to the extent of only 0.03 %. Interestingly, neem oil contains other azadirachtins, such as azadirachtins, -B, -D, -H and -I, which together constitute, at least 0.2 % of the oil.[83]

Azadirachtin is a C-*seco* tetranortriterpenoid, present in neem seeds to the extent of 0.1-0.6 %.[78] The azadirachtin content of neem is found to vary depending on the geographical origin of the seeds. It was found to be different in different locations. For example, in seeds from India (0.35 %), Mauritius (0.29 %), Indonesia (0.75 %), Myanmar (0.92 %), Togo (0.4 %), Sudan (0.19 %) and Niger (0.15 %) indicating the involvement of genetic as well as environment factors of different regions and tree variants.[78]

Further azadirachtin content in neem seeds is influenced by temperature, humidity, period of seed collection, processing and

storage conditions.[78] The seeds that fall on the ground are exposed to sunlight, which could lead to a decrease in their azadirachtin content. Moreover, collection of seeds is normally from the ground and the seeds are usually processed much later. Such factors can also affect the content of azadirachtin.[78]

The concentration of azadirachtin content in different parts of the neem tree indicates that the seed kernels contained more azadirachtin than other plant parts. The concentration is found to decrease in the order of seed kernels >>> leaves > bark > roots> stem.[120]

Pure azadirachtin is a microcrystalline solid having molecular formula $C_{35}H_{44}O_{16}$, m.p 154-158°C (dec.).[56,59] Azadirachtin is a complex molecule, having sixteen stereogenic centers, seven of which are quaternary.[77] It has eight condensed rings: three carbocyclic and five heterocyclic. The heterocyclic rings contain oxygen as hetero-atom and include three five membered, one furan and two pyran rings. It contains fourteen of the oxygen atoms deployed in the form of ester groups, secondary and two tertiary hydroxyl groups and a dihydrofuran ring linked to another ether ring. In addition, it has olefinic protons present in tiglate group and dihydrofuran rings.[59]

Azadirachtin is sensitive to acid and alkali. Due to the presence of acid sensitive groups like 3° hydroxyls, a ketal group and a dihydrofuran ring, it is highly unstable under acidic conditions, while the presence of four ester groups makes it unstable under alkaline conditions.[1]

Azadirachtin is very labile when exposed to sunlight, probably because of the presence of π bonds, strained epoxide and furan rings and ester groups in the molecule.[82] For example, the antifeedant activity of azadirachtin exposed to sunlight for 7 days was reduced

by more than half as compared with non-exposed azadirachtin against first instar larvae of *S. frugiperda*, with no activity remaining after 16 days as shown by Stokes and Redfern.[121]

Biosynthesis of azadirachtin

All of the well-characterized components in neem belong to the triterpenoid group. The biosynthetic products arise from successive oxidation and rearrangements reactions of the plant terpenes and may be grouped in to different categories. These include proto-meliacins, meliacins (tetranortriterpenoids, ring C-*seco* tetranortriter penoids), penta- and hexa-nortriterpenoids and nontriterpenoidal compounds.[1,27]

The *in vitro* experiments suggested that proto-meliacins (euphane or tirucallane derivatives) are the biosynthetic precursors of meliacins (tetranortetracyclic triterpenoids).[122,123] Thus, in the biosynthesis of meliacins (Figure: I.11), the euphane or tirucallane derivative undergoes double bond isomerization, to form a Δ^7-isomer of euphane (Δ^7, H-20β, 20R) or tirucallane (Δ^7, H-20α, 20S) derivative i.e butyrospermol. These Δ^7-euphane or Δ^7-tirucallane derivatives (butyrospermol) then undergo epoxidation at C_7-C_8 position to form corresponding epoxy derivatives, which further undergo an euphol-apo-euphol (or tirucallol-apo-tirucallol) rearrangement induced by the opening of the 7α, 8α-epoxide ring, with the migratioin of the methyl group at C_{14}β to C_8 position in the β- face, to form a apo-euphol or apo-tirucallol derivative having a C_7 hydroxyl group, with α -orientation and a double bond at C_{14}-C_{15} position.[124]

The apo-euphol (or apo-tirucallol) derivative is subsequently converted in to the tetranortriterpenoid nucleus via oxidative degradation of the side chain. These are either simple tetra-

nortriterpenes e.g azadirachtol, azadirachnol, nimbocenone etc., or ring C-seco tetranortriterpenoids e.g azadirachtin, nimbin, salannin etc.

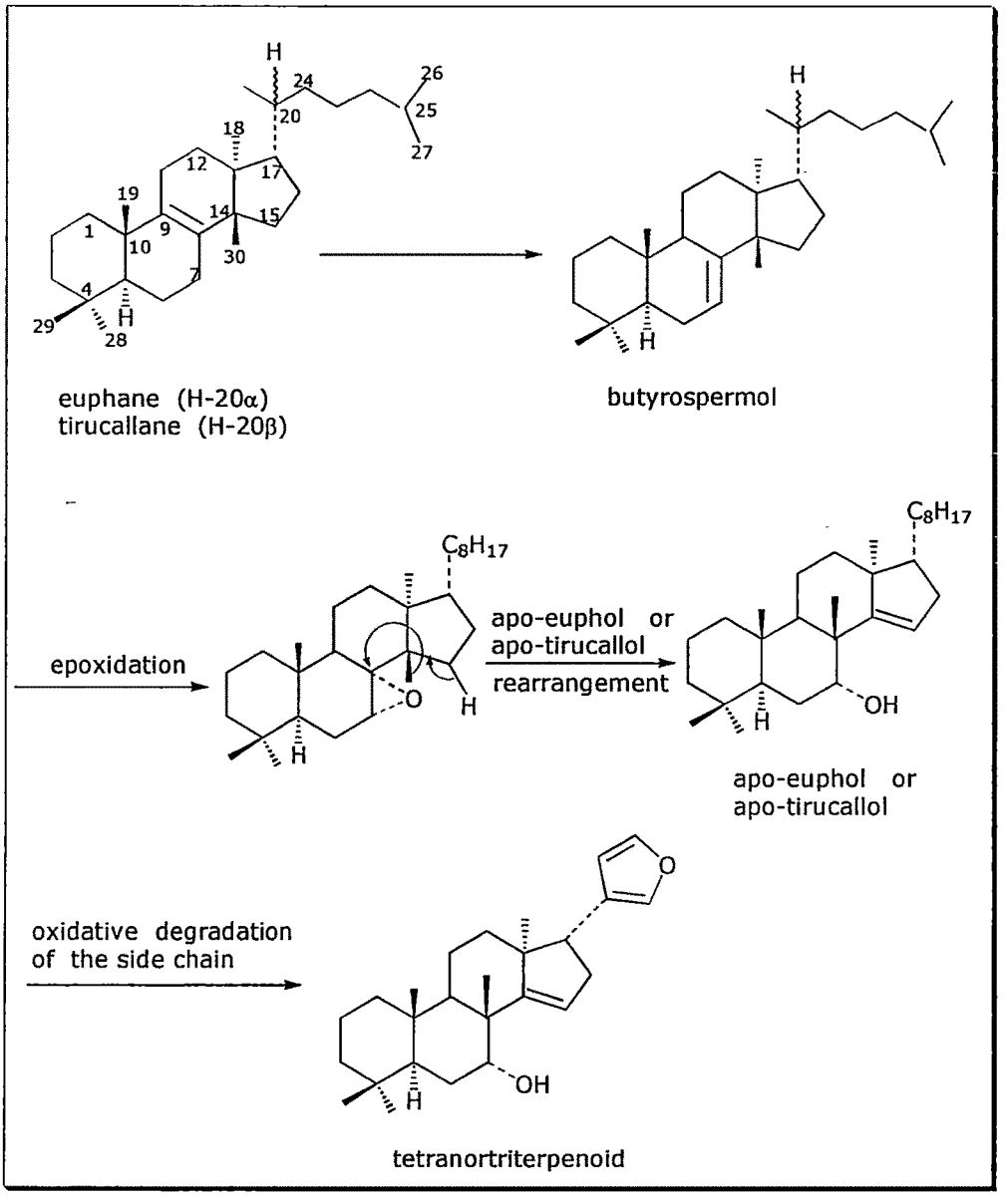


Figure: I.11

It has been further demonstrated by *in vitro* experiments that the formation of the furan ring of meliacin proceeds through a cyclic hemiacetal[122]. The biosynthetic pathway for the formation of the ring C-*seco* compounds is shown in Figure I.12. The euphane or tirucallane derivative might undergo epoxidation at C_8 and C_9 position to form an 8α, 9α-epoxide, which is then converted in to $\Delta^{7,9,(11)}$-diene system. The $\Delta^{7,9,(11)}$-diene undergoes epoxidation at C_7, C_8 position to form the corresponding epoxy derivative, which is further transformed into the apo-euphol or apo-tirucallol derivative. The Δ^9-function would activate the allylic C_{12} position to form a corresponding keto derivative containing a double bond at C_{14}-C_{15}, affording a Δ^{14}, 12-ketone derivative, which upon subsequent oxidation, would lead to ring C-seco tetranortriterpenoid.[124,125]

Isomers of Azadirachtin

Rembold reported that, by submitting neem kernel extract to preparative HPLC, besides Morgan's azadirachtin, which they named as Azadirachtin-A and Kraus's 3-tigloylazadirachtin (Aza-B), other analogus which were named as azadirachtins, C, D, E, F and G were obtained.[70] No structure could be assigned to C, but structures were assigned to the others on the basis of their NMR spectra. Moreover, Govindachari *et al* have isolated three other compounds, azadirachtins H, I, and K by preparative HPLC and structures assigned on the basis of mass, ^{1}H and ^{13}CNMR spectroscopy.[71]

The preparative HPLC of the crude neem seed kernel extract showed Aza-A **10** as the major compound, averaging about 79 % in most of the azadirachtin fraction[126], while the remaining fraction consisted of about 20 % of azadirachtin-B and 1 % of the so called positional isomers (i.e. azadirachtins, -C, -E, -F, -G, -H and –I, etc) as reported by Wan *et al*.[127]

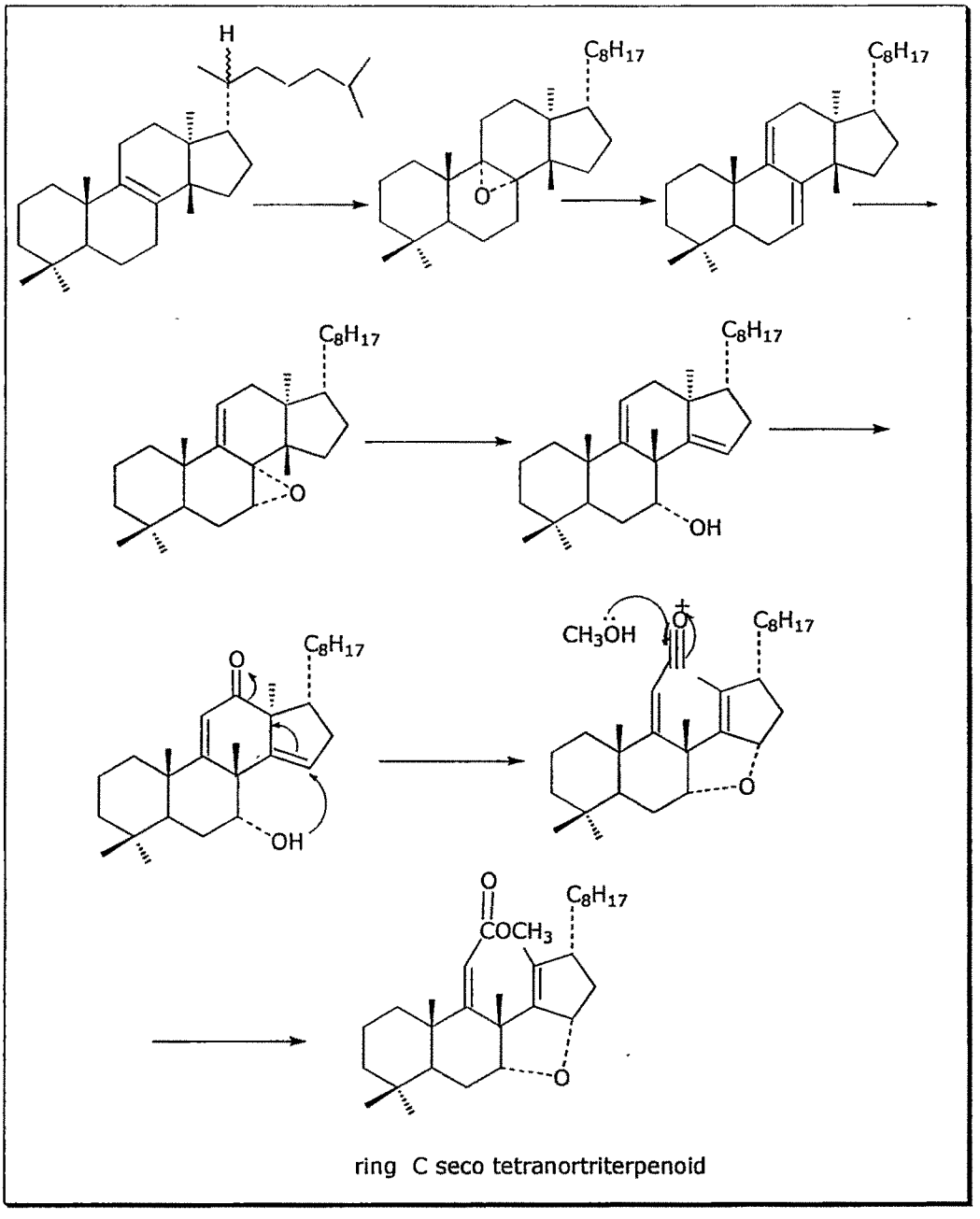


Figure: I.12

3-Tigloylazadirachtol (Aza-B) **52** (Figure: I.13) was first isolated by Klenk *et al* by conventional column chromatography. Then Rembold

et al by preparative HPLC.[70,128] It was also isolated later by Govindachari *et al* by the same technique.[129] Azadirachtin-B differs from azadirachtin-A in which tigloyl group is placed at C_3 instead of C_1. However, the placement of the tigloyl group at C_3 was based entirely on nuclear Overhauser effect (NOE) studies carried out by Govindachari *et al*.[119,129]

Surprisingly, only a partial structure comprising a *trans*-decalin fragment is available for azadirachtin-C.[1] Azadirachtin-D **53** (1-tigloyl-3-acetyl-11-hydroxymeliacarpin) was first isolated by Rembold[70] without furnishing any experimental details of isolation and spectral data and later by Kraus *et al*,[119,130] Rojatkar and Nagasampagi.[131] Azadirachtin-D differs from azadirachtin-A in that C_{29} is not oxidized and is present as an angular methyl group.[1]

Azadirachtin-E **54** corresponds to detigloylazadirachtin, while azadirachtin-F **55** and G **56** are structurally related to Aza-B. For azadirachtin-F, the unoxidized C_{19} angular methyl group is accompanied by a pendent methyl glycolate side chain at C_9, while for azadirachtin-G, the C_{13}-C_{14} oxirane is absent and replaced by a double bond and a C_{17} hydroxyl group.[1]

Azadirachtins H (11-demethoxycarbonylazadirachtin) **57** and I (1-tigloyl-3-acetyl-11-hydroxy-11-demethoxycarbonyl meliacarpin) **58** (Figure: I.14) have been isolated by Govindachari *et al* from neem seed kernels using preparative HPLC and found to lack C_{12} carbomethoxy group.[71a] Azadirachtin-H differs from azadirachtin-A by substitution of a hydrogen in place of the carbomethoxy group at C_{11}. While azadirachtin-I differs from azadirachtin-H having a methyl group in place of the carbomethoxy group at C_4.

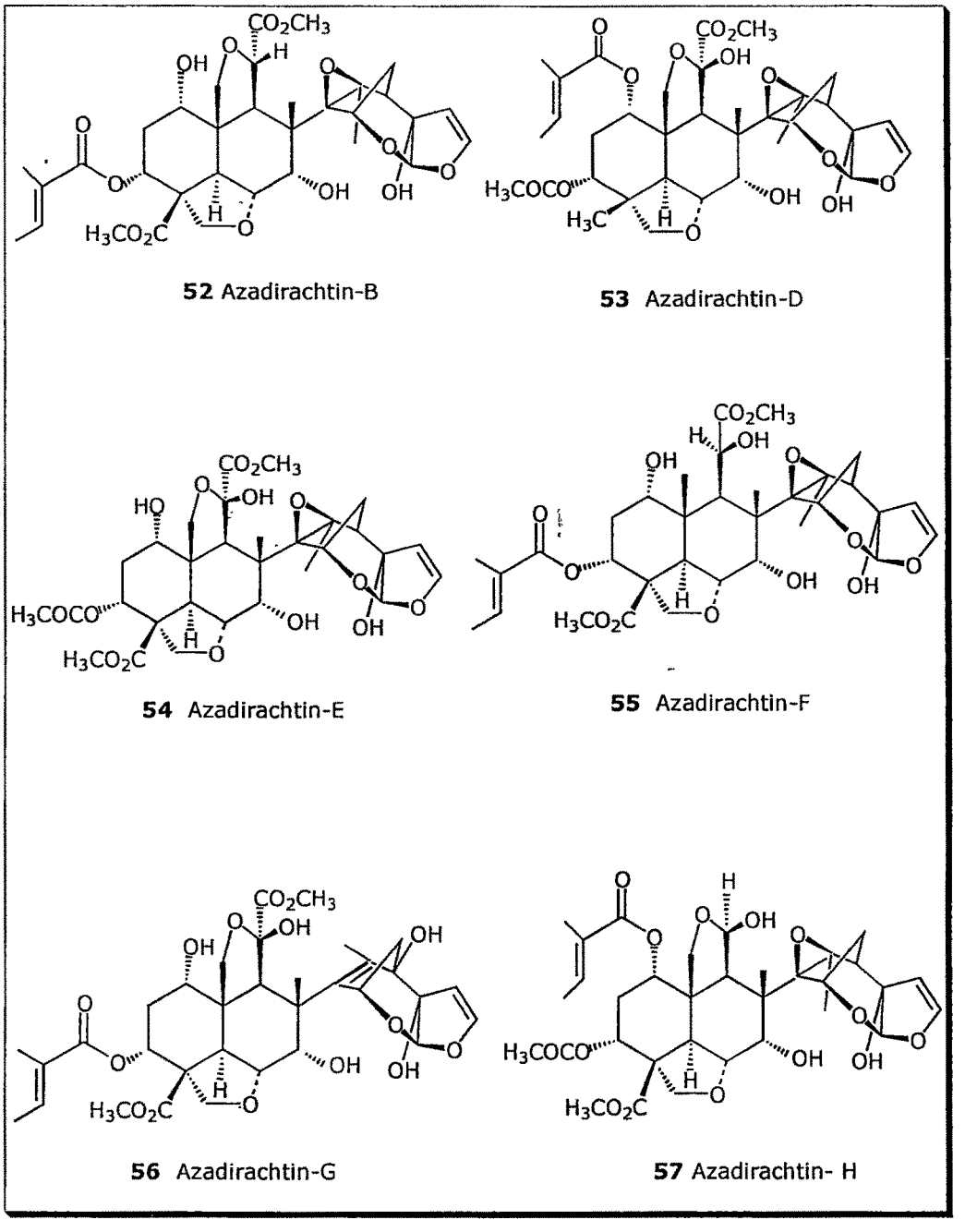

52 Azadirachtin-B
53 Azadirachtin-D
54 Azadirachtin-E
55 Azadirachtin-F
56 Azadirachtin-G
57 Azadirachtin- H

Figure: I.13

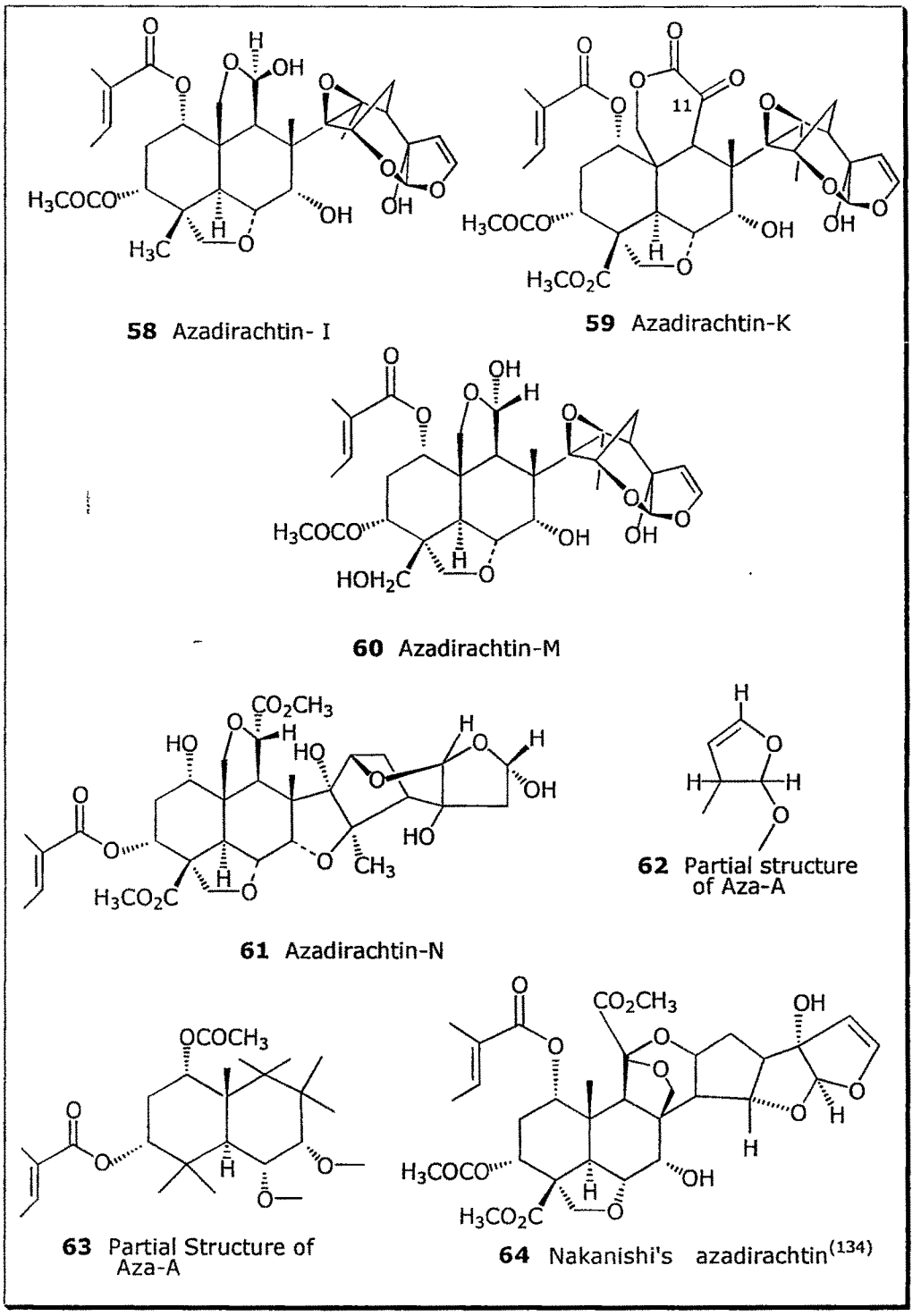

58 Azadirachtin- I
59 Azadirachtin-K
60 Azadirachtin-M
61 Azadirachtin-N
62 Partial structure of Aza-A
63 Partial Structure of Aza-A
64 Nakanishi's azadirachtin(134)

Figure: I.14

Govindachari *et al* have also isolated azadirachtin-K **59** (11-dehydroxy-11-oxoazadirachtin-11,12-lactone) from kernel extract using preparative HPLC.[71b]

In addition azaditachtins, M (29-oxymethylene-11-demethoxy carbonyl-11α-hydroxyazadirachtin) **60** and N (22,23-dihydro-23α-hydroxy-3-tigloyl-11-deoxyazadirachtin) **61** have recently been isolated from methanolic extract of the seed kernels of neem.[84] Besides this, Schmutterer and co-workers have reported the isolation and structure determination of a new azadirachtin congener named marrangin **51**.[117]

Structure Elucidation of Azadirachtin

The elucidation of structure of azadirachtin took nearly eighteen years since its discovery ! Some wrong structures were proposed initially due to the unique properties of this complex molecule, however the final correct structure was reached in 1985. Several laboratories all over the world were engaged in accomplishing this difficult task, which has been admirably summarized in a series of papers. [62,63, 132,133]

In 1968, Morgan and Butterworth described the isolation of azadirachtin.[56] Subsequently the same group identified many of its functional groups and performed the first chemical modification on the natural products.[59] Initially, two partial structures **62** and **63** were offered, a molecular formula ($C_{35}H_{44}O_{26}$) was determined, and it was concluded that the molecule belonged to a new class of hexanortriterpenoids related biogenetically to the C-seco tetranor triterpenoids, salannin and nimbin.[56] However, the authors described the presence of two acetate groups and the absence of either epoxide or hemiacetal functions. These errors serve to

illustrate the often unusual NMR characteristics and chemical reactivity of azadirachtin.[1]

Nakanishi and co-workers reported the first complete structural proposal for azadirachtin **64** in 1975.[134] Their interpretations were based mainly on partially relaxed Fourier transform (PRFT/CWD) ^{13}CNMR spectroscopy and a hypothetical relationship of azadirachtin with the known but less complex compounds, salannin and nimbin, isolated from neem. The NMR evidence used was mainly derived from a study of the PRFT spectrum, the then new technique, in which ^{13}C peaks of different proton coupling have positive or negative signs, thus greatly simplifying the spectrum in crowded regions. Use was also made of proton-decoupling technique, which with assistance from shift reagents, revealed five different spin systems. The carbon skeleton and the substitution in rings A and B were based on analogy to nimbin and salannin. However, some doubt remained about certain parts of the structure. Particularly, the ^{13}CNMR signals of C_{13} (δ 68.69) and C_{14} (δ 68.53) should appear at lower field if they were connected to tertiary hydroxy or alkoxy groups and there is ether link between C_{11} and the right hand segment of the molecule.[134]

Nakanishi's azadirachtin was accepted by the scientific community until 1984, when Kubo *et al* reported the isolation and NMR structure determination of a new azadirachtin congener known as detacetyl-azadirachtinol **65** (Figure: I.15).[135] The proposed structure was the first example of azadirachtin to have a single C_8-C_{14} bond joining the two halves. The implication of this type of behaviour on the structure of a similarly arranged compound prieurianin **66** was known in 1975. This molecule consists of two segaments joined by a single bond, which shows restricted rotation. When this single bond in prieurianin derivatives is reinforced by an ether link, the rotation stops and the

temperature dependence disappears.[136] The only significant possibility for rotation in azadirachtin seems to be about the C_8-C_{14} bond, which implies there is no ether link between C_{11} and the right hand section of the molecule. If this idea had been followed up in 1975, when the facts about prieurianin were already known, the C_{19}, C_{30} uncertainty might have been resolved and the correct structure could have been deduced much earlier.

In 1985, the first reappraised structure of azadirachtin[137] **67** was suggested by Ley and co-workers, through single crystal X-ray analysis.[138] The structure suggested by Ley and co-workers represented a significant advance in showing the presence of a C_8 angular methyl group and a C_9-C_{10} annulated tetrahydrofuranacetal moiety. This revised structure was based on 2D-NOESY and 1D NOE difference spectroscopy, which revealed the presence of H_1-H_9 and H_2-H_9 interactions thus placing the C_{19} methylene unit at C_{10}.

Finally, Ley and co-workers proposed the correct structure for azadirachtin in 1985.[138] This paper contained the details of structure confirmation of azadirachtin through single crystal X-ray crystallographic studies of 22,23-dihydrodetigloylazadirachtin **68** obtained through catalytic hydrogenation of azadirachtin followed by detigloylation and gave the now well-accepted complete structure for azadirachtin **10**.

Simultaneously, Kraus *et al* also reported the correct structure of azadirachtin **10**. Using one-dimensional NOE difference spectroscopy in conjunction with ^{13}C deuterium isotope shift experiments, the authors deduced the presence of C_{11} as a hemiacetal. [139]

Ley *et al* and Kraus *et al* reported the correct structure, which was confirmed by single crystal X-ray crystallographic analysis on detigloyl-22,23-dihydroazadirachtin (DTA) since azadirachtin itself

could not be crystallized. Subsequently, azadirachtin was obtained in crystalline form by Govindachari *et al* from ethyl acetate-hexane solvent system and the structure was determined by X-ray diffraction studies, which fully confirmed the structure arrived at from NMR data.[83b,c, 119]

Biological Activity of Azadirachtin

Azadirachtin and extracts containing it cause various effects in insects. It has been shown to be a potent antifeedant at low concentrations (10-100 ppm). It exhibits ecdysis inhibition activity at much lower concentrations, 1-10 ppm in over 200 species of insects. This prevents the insect larvae from developing in to mature insects, which could further multiply and produce new generations. Besides these it also displays a variety of other biological properties, such as repellency, chitin inhibitor, anti-oviposition, sterilants, fecundity and anti-malarial activity.[16]

Together with its biodegradability, species selectivity and low mammalian toxicity, these factors have resulted in application of azdirachtin in integrated pest management programe and further commercial development.[77a]

The effect upon insect development is most important from the viewpoint of practical insect pest control. The Figure I.16 represents that the all steps involved in the host selection by insects are affected by azadirachtin and azadirachtin-containing neem extracts. Insects escaping one effect for example, repellent have to face other behavioural effects for example antifeedant, antiovipositition etc., If however, they cross these barriers and feed they succumb to physiological effects.[140]

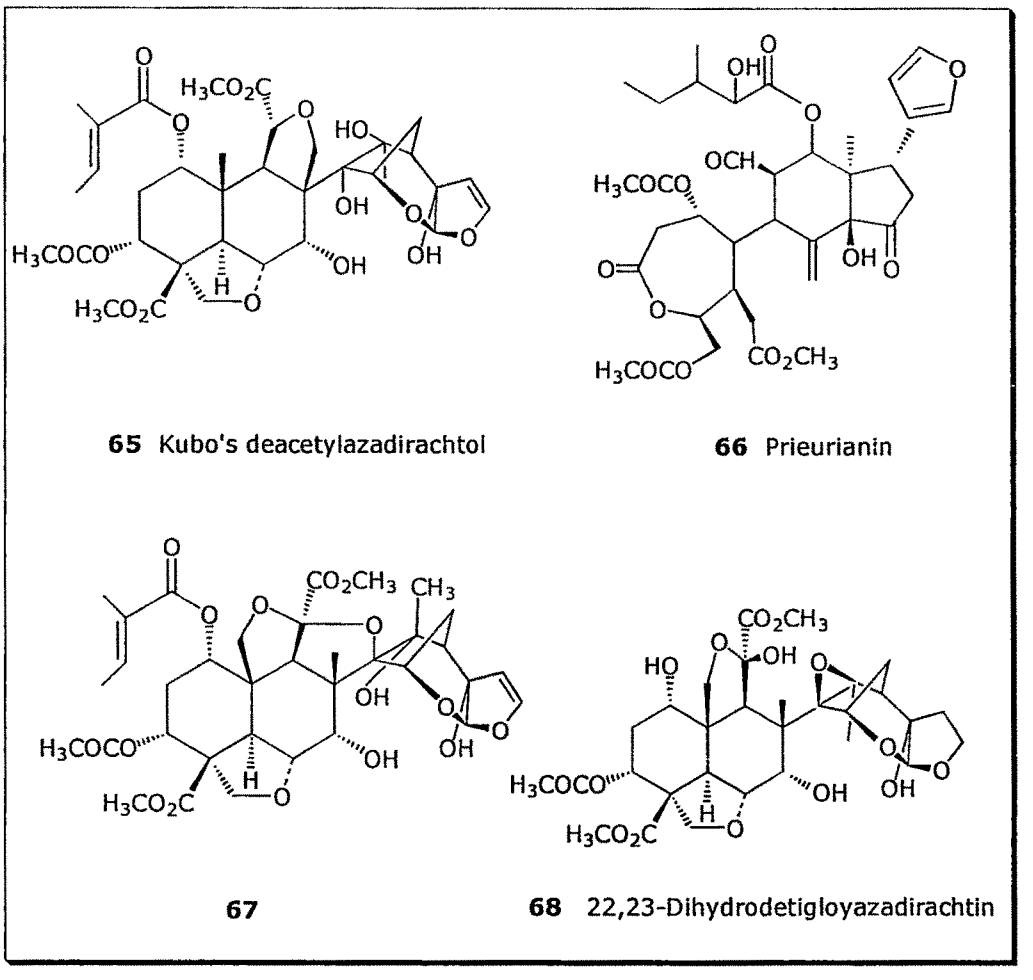


Figure: I.15

Insect Control:

(1) Phytophagous insects

The insecticidal performance of neem products has been assessed in terms of both antifeedancy and insect growth regulatory (IGR) effects. There are numerous examples of laboratory and green house studies of the pest control potential of neem and its major active constituent, azadirachtin. Lepidoptera and other phytophagous

insects remain the main targets.[141] Alternately, modifications of the azadirachtin molecule may significantly increase its stability e.g. hydrogenation of the C_{22}-C_{23} double bond of the dihydrofuran ring yields dihydroazadirachtin, a more stable compound with greater potential for field use. This compound is as active as azadirachtin in feeding trials with four economically important lepidopteran species. It is however, significantly less active than azadirachtin against the locust *Schistocerca gregaria*.[141]

(2) Stored Product Pests

Neem has proved to be effective in protecting stored products, particularly grain whose losses if untreated can be high. Such losses are frequent in developing countries due to the inability to apply expensive chemical pesticides.[142] More diverse effects of neem, other than those on grain, include the preservation of dried fish in Nigeria.[143]

(3) Aquatic Arthropods

Azadirachtin is potentially useful in the aquatic environment. Neem seed kernel extracts, azadirachtin, isonimocinolide and nimocinolide exhibits the toxic effects against mosquitoes larvae and pupae.[141]

(4) Other Organisms

Azadirachtin, apart from its unique mode of action against insects can also affect other organisms including nematodes, fungi, viruses and protozoa. There are numerous instances of the effect of azadirachtin on fungal pathogens, including the inhibition of spore germination and mycelial growth of *Helminthosporium nodulosum*

and *Pyricularia grisea* on finger millet, with acetone extracts of neem being more effective than water extracts.[144]

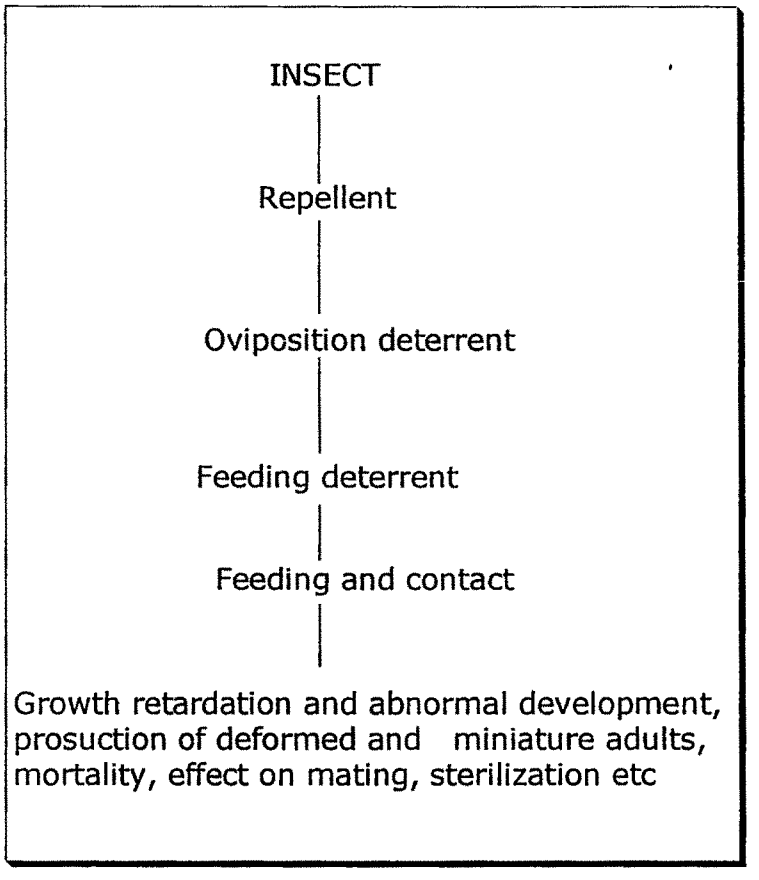


Figure: I. 16 Biological effects of neem on different steps of Host plant selection by insects.

As far as anti-protozoan activity is concerned azadirachtin inhibited microfilarial release from *Brugia pahangi* without affecting the host's motility or viability.[141]

Effects of Azadirachtin

The most important effects of azadirachtin on insects from the applied viewpoint can be described as follows.

First, azadirachtin or azadirachtin containing neem extracts show antifeedant effects. It is a classic example of a natural plant defense chemical affecting feeding, primarily through chemoreception (primary antifeedancy) but also through a reduction in food intake due to toxic effects if consumed (secondary antifeedancy).[141]

A variety of preparations have been applied to assess azadirachtin's antifeedant activity, from crude to refined neem extracts to neem enriched extracts to pure azadirachtin. This large variety of formulations has been applied in many different ways against more than 200 species, e.g., within artificial diets, or on simplified feeding discs, or to the insects as sprays, or by injection so hindering direct comparisons of the susceptibility of different insects species to its antifeedant effects.[141]

There are clear differences, however regarding the strength of these effects, depending on the concentration of the active principle and the species of insects treated. The lepidoptera are extremely sensitive to azadirachtin and show effective antifeedancies from, 1-50 ppm depending upon species. Coleoptera, Hemiptera and Homopetera are less sensitive to azadirachtin, whereas Orthoptera show an enormous range of sensitivity.[141]

A simple and common laboratory bioassay to assess azadirachtin's antifeedant potency is the leaf-, filter paper- or glass fibre- disc feeding test in either a choice or no choice situation.[141] These bioassays provide essential data for more elaborate trials in both the laboratory and field.

When feeding does occur in antifeedant trials with azadirachtin, significant insect mortality is often recorded, particularly in no-choice trials, as secondary toxic effects of azadirachtin begin to predominate.[141]

Second, azadirachtin is a potent disruptor of insect development. Treatment of insects or their food with azadirachtin causes growth inhibition and mortality in a dose-dependent manner. Hence increasing doses of azadirachtin in larval stages result in [141]:

- Adults with reduced longevity and fecundity,
- Wingless adults,
- Nymphs or larvae which die during ecdysis unable to complete the moulting process,
- Pupae with severe deformities to the head and thoracic appendages,
- Insects, which die within hours of treatment.

These phenomena have been observed through out a wide variety of insect species including Lepidoptera, Diptera, Orthoptera, Coleoptera and Hymenoptera. [141, 145]

Third, azadirachtin is an effective sterilant. After uptake of the active material, females of some insect pests species for instance the Colorado potato beetle, *Leptinotarsa decemlineata*, are sterilized to a high degree, sometimes completely.[145] The life span of treated females is prolonged, but their food consumption is very low. However, egg fertility is not influenced by azadirachtin.

Fourth, the fitness of insects is often reduced after application of dosages so low that moulting is not disturbed. Adults resulting from such treatments are for instance, unable to copulate such as males of *Oncopeltus fasciatus*, or cannot recognize the male pheromone, such as females of the fruit fly *Ceratitis capitata.*[145]

Mode of Action

The mode of action of azadirachtin and neem extracts is not understood very well.[146] it is quite possible that different chemicals

or different ratios of chemicals found in neem have diverse effect on insects. Azadirachtin alone probably has several modes and sites of action.[146]

Azadirachtin shows three specific modes of action in insects, as shown by Mordue and Blackwell.[141] First, it has strong antifeedant activity due to its effect on chemoreceptors. Second, it affects ecdysteroid and juvenile hormone titers through a blockage of morphogenetic peptide hormone release resulting in severe growth and molting aberrations. Third, it has direct detrimental and histo-pathological effects on most insect tissues. e.g., muscles, fat body, and gut cuticular epithelial cells.

Blaney and Simmonds have studied the neuro physiological effects of azadirachtin on the chemoreceptors of locusts and lepidopterous larvae and related these effects to feeding responses.[147,148]

Structure activity relationships of azadirachtin have been carried out by several groups of workers using antifeedancy, IGR or both parameters as bioassay.[141]. It is seen that functional groups in both the decalin and dihydrofuran ring contribute to the activity of azadirachtin. The hydroxyfuran acetal moiety is particular important for high levels of antifeedancy. The presence of epoxide ring at C_{13}-C_{14}, as an essential requirement for insect growth inhibitory activity. The hydroxyl groups on C_7 and C_{11} are also important for potency.[1,76]

Toxicity And Resistance

With a large number of organisms being affected by neem extracts, concern was expressed for the welfare of beneficial organisms under management programs using neem extracts. It has been found however, that predator and parasitoid insects are relatively

unaffected when their life cycle involves exposure to neem extracts. Evidently, azadirachtin does not affect these insects in the same way or not enough chemical is taken up in their diet to cause behavioural or metamorphic abnormalities.

Initially, azadirachtin or azadirachtin containing extracts act as oral (stomach) poisons, but in some cases, in the soft-skinned larvae of *Leptinotarsa decemlineata*, the insects also react after dermal contact with active principle.[145] The death of the target insects also dose dependent. It usually occurs a few days after applications of azadirachtin, but in extreme cases the larvae may live up to several weeks when they become unable to moult.

Shapiro *et al* reported that mortality of the gypsy moth was evaluated in the presence of a virus pathogen and also when the moth and virus were subjected to neem treatments. The extracts have no adverse effect on viral activity but, when applied concurrently, moths died sooner.[149] Earth worms actually benefit from soil application of neem by products with increased weight gain.[150] In addition, important natural enemies of pests such as spiders, earwigs ants and some parasitic wasps, butterflies also show no detrimental effects from exposure to azadirachtin or azadirachtin containing neem extracts, in some cases even favoured.[151] This is because of the lack of contact toxicity in most insects, the lack of ovicidal effect. Hence, neem products are quite selective although they have an otherwise broad-spectrum effect.

Limitations:

After discovery of the antifeedant and growth disrupting effects of azadirachtin and other compounds, peoples used azadirachtin and aqueous, alcoholic and other extracts from neem seeds and leaves.

In many cases good to very good pest control was achieved, some times comparable to that of synthetic compounds. However, field trials have also shown a number of limitations connected with compounds like azadirachtin. They are as follows.

As is typical for natural products, one of the main problems of using neem treatments is the durability of azadirachtin in the field conditions. The degradation of azadirachtin in the field takes place faster than in the laboratory, apparently mainly because of the influence of sun light.[145] But leaf pH can also affect detoxification rates and rain can was residue off leaf surfaces.[145] The activity of neem-based products subsides rapidly, lasting four to eight days, meaning that many applications will likely be needed in a season.[121] To overcome the degrading effect of sun light to a certain extent, higher concentrations of the active principle are needed in the field than in the laboratory and uses of the different photostabilizers in the azadirachtin containing preparations, which can enhance their photostability.[152,153]

Temperature seems to play only indirect role higher values may increase the effect, because the target insects are more active under such conditions and an undesirable "primary" antifeedant effect, if there is any is overcome faster than at lower temperatures.[145]

Azadirachtin content in neem kernels and quickness of activity are further considerations in the commercialization of neem extracts. To provide a consistent product, refining kernels with similar levels of compounds are essential. Farmers using synthetic pesticides also are used to quick acting chemicals. They may not be patient enough to wait for the activity of neem-based products to produce results.

I.6 OBJECTIVES

Azadirachtin-A is the major constituent in the neem seed extracts and shows high insecticidal activity. It is shown to be potent insect antifeedant, antimalarial and growth inhibitor amongst many other biological activities.[77] Moreover, azadirachtin has also been reported to be biodegradable, less toxic and non-mutagenic to mammals.[77]

Recently considerable interest has been focused on the isolation and purification of azadirachtin from seed kernels by using various extraction and instrumental techniques. It is not surprising therefore that a variety of procedures have been developed with the aim of isolating pure azadirachtin and to study their biological activities. A literature survey revealed that most of the methodologies involved either complex procedures for isolation or did not report the initial and final purity of azadirachtin or were in sensitive.[56,58,64-68,80]

It was therefore, felt necessary as our first and immediate goal to develop a short, efficient and reliable method for the isolation and purification of azadirachtin from plant material.

Azadirachtin-A degrades in the presence of sunlight, mainly due to the presence of sensitive functionalities.[82] Degradation of the insecticide in the sunlight is the major limitation encountered in its use in agriculture, because the insecticide should remain intact on leaves so as to provide the insect enough time to ingest the material causing the death of the insect. It was therefore proposed that, azadirachtin molecule should be photostabilized by addition of some UV absorbers for its effective use.

Further it is already known that changes in the functional groups present on the decalin fragment of the azadirachtin molecule can change the antifeedant activity of the molecule.[76] Keeping this in

mind, it was proposed to undertake a study of various synthetic transformations on azadirachtin and evaluate the biological activities of the products against insects.

Our efforts directed towards realization of the above objectives are described in the following chapters of the thesis.

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