# CHEMISTRY AND EVALUATION OF NEEM EXTRACT AGAINST Tribolium castaneum (Herbst)

**Thesis** · September 2011

CITATIONS
0

**1 author:**

Ajay Sharma
Sant Longowal Institute of Engineering and Technology
**16** PUBLICATIONS **5** CITATIONS

SEE PROFILE

**Chapter I**

## INTRODUCTION

From the second half of the 20th century, the agricultural modernization has focused on using new techniques which aim to increase the food production for the ever-increasing human population. The increase in the food production is due to the scientific break-throughs and technological innovations which include developing new plant varieties, increase of irrigation facilities and the use of fertilizers and pesticides. This has led to increased crop production but the most important constraint is the post harvest preservation of its quality and quantity. The post-harvest losses and quality deterioration caused by storage pests are major problems throughout the world (Hill 1990). During storage, grains are mostly attacked by pests like *Trogoderma granarium* (Everts), *Tribolium castaneum* (Herbst), *Rhizopertha dominica* (Fab.), *Sitophilus oryzae* (Herbst), and *Alphitobius laevigatus* (Fab.). There is an evidence that several species of storage insect pest attacked granaries and other food structures in ancient times, e.g. in ancient Egypt (Levinson and Levinson 1985). Even today, storage losses remain notoriously high. According to an FAO study, world-wide loss in store approximates 10 per cent of all stored grain, i.e., thirteen million tonnes of grain lost due to insects or hundred million tonnes due to poor storage (Wolpert 1967). In the tropical countries the insect pests cause heavy food grain losses during storage, particularly at the farm level. Food grain losses in India during storage at the farm level approximate 10 per cent of the production (Lal 1988). In Sub-Saharan Africa, food grain losses during storage at farm level can reach as high as 25-40 per cent (Dichter 1976). On the basis of available data the food grain insect pests attributed appreciable amount of loss to the total food grain in India which is up to 12 per cent (Rangi 2001).

Today's agriculture is productivity based and depends heavily on the use of synthetic chemicals to control the pests and diseases. To prevent the loss during storage farmers usually rely on chemical insecticides. These chemical insecticides have been successfully used to protect stored grains from insect infestations but their indiscriminate and massive use have created serious problems which reduce the biodiversity of agroecosystems, provoking their instability and cause harmful effects, such as: environmental pollution, human health issues, insect resistance to pesticides (Denholm *et al* 1998), natural pest predators (Aggarwal and Brar 2006), pollinators (Desneux *et al* 2007) loss and residues in food grains posing health hazards to the consumers as most of the flour mills grind wheat without washing (Fishwick 1988). Moreover in India cereal consumption is quite high as compared to developed countries and therefore even small amount of insecticide residues left on grains result in their large intake**.** Although chemical insecticides play a significant role in reducing storage loss

due to insect pests however their current application for the control of storage insect pests is limited due to resistance developed by the pests and development of resistant strains of many pests (White 1995). Consumer's concern, widespread environmental hazards and increasing cost of application are the reasons for the development of alternative chemicals such as biopesticides.

As a consequence of these environmental security and health issues (Karunamoorthi *et al* 2008), nowadays, new strategies for pest insects control which are less hazardous and more environmentally friendly are searched for; such as the use of plant extracts, one of the most attractive methods for this aim. The practice of using plant extracts in agriculture for pest control is not new; it has been used for at least two millennia, when botanical insecticides were considered important products for pest management in Ancient China (Long *et al* 2006), Egypt, Greece and India (Isman 2006). Even in the United States and some European countries, botanical insecticides were predominantly used, before the discovery of organochlorine and organophosphorus insecticides in the late 1930's and early 1940's (Isman 1997). Plant products have been used for many years by small scale farmers in parts of India, Pakistan, China and many other countries to protect stored grain from insect infestation. Recently, attention has been given to the possible use of plant products or plant derived compounds as promising alternatives to synthetic insecticides in controlling insect pests of stored products (Ohazurike *et al* 2003, Umoetok and Gerard 2003). The effectiveness of many plant derivatives for use against stored grains pests has been reviewed by Jacobson (1989). In last two decades many efforts have been made to screen natural plant products with better biopesticide qualities which can be used as an alternative to synthetic insecticides. Natural plant products that are biodegradable, exhibit structural diversity and complexity constitute one such class of chemicals. These can act as insecticides or may provide lead structure for insecticidal discovery.

Modern agriculture in recent years has focused interest on the use of biopesticides and they are preferred in comparison to synthetic pesticides as they are ecofriendly and biodegradable (Kumar *et al* 2008). They are generally perceived as providing both long lasting insect control and having less potential for causing damage to the environment or to non target organism than synthetic pesticides. In the last 30 years, there has been an increasing research on plant extracts (Clemente *et al* 2003), due to the numerous problems that synthetic insecticides cause; leading to what could be considered a second era of the botanical insecticides (Silva *et al* 2002).

The plants that present biological activity against insects owes this feature to the presence of secondary metabolites, some of which have been widely investigated (Chandra *et*

*al* 2008). The process of knowing and obtaining secondary metabolites against insects is by means of plant extracts, which can have variations; sometimes aqueous extracts can be obtained (Bobadilla *et al* 2005), solvents can be used to obtain different compounds depending on their polarity (Bobadilla *et al* 2002), essential oils can also be obtained or dehydrated plant parts to be used as powders (Silva *et al* 2003).

Natural pesticides are active principles derived from plants for the management of human and animal pest organisms or it can be said to be biologically active ingredients, principally derived from plants, for the management of human and animal pest organisms (Ivbijaro 1990). With the growing global demand for environmentally sound pest management strategies; there is a need to develop alternative pesticides with minimal or non-ecological hazards. Botanical pesticides are easily biodegradable (Devlin and Zettel 1999) and their use in crop protection is a practically sustainable alternative. It maintains biological diversity of predators (Grange and Ahmed 1988) and reduces environmental contamination and human health hazards. The use of plant extracts to control destructive insect pests or disease vectors is not new. Rotenone (*Derris* spp), nicotine and pyrethrins have been used for a considerable time in small-scale subsistence and also in commercial agriculture (Hillock and Bolin 2004). In recent times, considerable effort has been put into the development and promotion of plant based products for the control of pests (Stoll 2000). This has resulted in the development of a science-based approach that uses a formal set of steps to discover and determine their efficacy and attempts to either produce the botanical pesticide on a commercial scale or synthesize it for broader use in commercial agriculture (Philogene and Lambert 1993).

Now there are more than 2000 plant species known to have insecticidal properties, out of which Euphorbiaceae, Asteraceae, Labiatae, Fabaceae, Meliaceae and Solanaceae families stand out. Among the metabolites with biological activities against insects, flavonoids, terpenoids, alkaloids, steroids and phenols are the most prominent (Orozco *et al* 2006). Plant products and their analogues are an important source of agrochemicals used for the control of insect pests (Cardellina 1988). One widely studied plant in this context is the neem tree, *Azadirachta indica* (A) Juss (Meliaceae). *Azadirachta indica* popularly known as Neem, Nim, Margosa or Nimba is native to Indian subcontinent. It grows as a wild tree in India, Bangladesh, Burma, Pakistan, Sri Lanka, Malaysia, Thailand and Indonesia. Presently neem trees can be seen growing successfully in about 72 countries worldwide, in Asia, Africa, Australia, North, Central and South America (Suri and Mehrotra1994).

Neem has been used for centuries in Asia as insecticide, fungicide, anticonceptional in popular medicine and almost every part of this tree i.e. seeds, leaves, roots, bark, trunk and

branches have multiple uses (Chaturvedi *et al* 2003). Neem is being used to manufacture natural bioinsecticides, which are environmentally friendly and do not have any toxic effects on plants and soil. Neem insecticides are used to protect both food as well as cash crops (Anonymous 2008). The insecticidal effect of neem has been proved on several insect groups, including Lepidoptera, Diptera, Coleoptera, Homoptera and Hemiptera species (Sadre *et al* 1983). Along with this neem also shows medicinal properties including antifertility, antifungal (Kher and Chaurasia 1997), immunostimulant (Upadhyay *et al* 1992), antibacterial (Singh and Sastry 1997), antipyretic (Biswas *et al* 2002) acaricidal (Mulla and Su 1999), antioxidant (Sultana *et al* 2007), larvicidal (Miller and Chamberlain 1989) and insect repellent (Palsson and Jaenson 1999).

In addition to its use in afforestation programs, authors from different countries have referred to it as **miracle tree**, **multipurpose crop**, **village dispensary** and **living pharmacy** because of its multiple uses. In fact, in its Asian countries of origin, every part of the neem tree has been extensively used in ayurveda, unani and homeopathic medicines as household remedy against various human ailments from antiquity, leading Biswas *et al* (2002) to conclude that it is a cynosure of modern medicine and this plant is a folk remedy (Biswas *et al* 2002) against various afflictions as illustrated below:

| **Part** | **Medicinal Use** |
|---|---|
| Leaf | Leprosy, eye problem, intestinal worms, anorexia, epistaxis, biliousness and skin ulcers. |
| Bark | Analgesic, alternative and curative of fever. |
| Flower | Bile suppression, elimination of intestinal worms and phlegm. |
| Fruit | Piles, intestinal worms, urinary disorder, epistaxis, phlegm, eye problem, diabetes, wounds and leprosy. |
| Twig | Cough, asthma, piles, phantom tumors, intestinal worms, diabetes, spermatorrhoea and obstinate urinary disorder. |
| Gum | Scabies, wounds, ulcers and skin diseases. |
| Seed | Leprosy and intestinal worms. |
| Oil | Leprosy and intestinal worms |
| Root, bark, leaf, flower and fruit | Blood morbidity, biliary afflictions, itching, skin ulcer, burning sensation and leprosy |

A number of compounds have been isolated from various parts of neem tree. Nimbin was the first bitter compound isolated from neem oil, and thereafter more than 135

compounds have been isolated from different parts of neem viz. azadirachtin, salannin, nimbinin, nimbidinin, gedunin, mahmoodin, meliantriol, gallic acid etc. These compounds are divided into two major classes: isoprenoids (Roy *et al* 2007) and others. The isoprenoids include diterpenoids namely sugiol, nimbiol, margasone and triterpenoids containing protomeliacins, liminoids, azadirone and its derivatives, genudin and its derivatives, vilarin type of compounds and C-secomeliacins such as nimbin, salannin and azadirachtin. The first compound to be studied was nimbin. The non-isoprenoids include proteins and carbohydrates, sulfurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, phenolic acids (Girish and Shankara 2008).

There are several secondary metabolites present in different neem seed kernels extracts which exhibit wide spectrum of biological activities such as insect repellant, larvicidal, antifertility, inhibit feeding and disrupt insect growth, metamorphosis and reproduction. The work on chemistry and potential of polar and non-polar neem extracts have not been reported on *Tribolium castaneum* (Herbst). There is tremendous scope for exploiting activity of neem seed kernels extracts against stored grain insect pest rust red flour beetle *Tribolium castaneum* (Herbst). So the study was planned with the following objectives.

I. Extraction of neem seed kernels extract from neem seed kernels.

II. Isolation of compounds from neem seed kernels extract.

III. Identification of compounds by chemical and spectral techniques.

IV. Bioefficacy studies of neem seed kernels extract against *Tribolium castaneum* (Herbst).

The thesis runs into five chapters, namely, review of literature, materials and methods, results and discussion, which is followed by summary.

Since almost the entire investigation incorporated in this thesis is on isolation and bioefficacy of neem seed kernals extracts against stored products insect pest of wheat i.e. *Tribolium castaneum* (Herbst), a review of literature on the bioefficacy of neem seed kernals extracts against stored product insect pests was thought to be appropriate. This review is given in chapter II of the thesis. In chapter III of the thesis, a brief outline of various methods and techniques employed in the investigation are described. Chapter IV of the thesis is devoted to results and discussion. Chapter V gives the summary of the research work carried out. The references cited in the text are alphabetically arranged at the end of the thesis.

**Chapter II**

## REVIEW OF LITERATURE

Very large and ever growing classes of plant products that represent a rich source of biologically active compounds and are example of molecular diversity, with recognized potential in drug discovery are isoprenoids (diterpenoids and triterpenoids) also known as natural products. Plants produce many compounds that play a useful function in their interaction with the environment (Paiva 2000). These compounds often make plants more successful in terms of competition and reproductive vigour. These compounds hold a major place in traditional medicine practices like homeopathy and ayurveda thus acting as alternatives to pharmaceutical drugs in Western countries and offer an efficient approach towards the discovery of plant extracts as biologically active compounds. Plant extracts are classified chemically as flavonoids, carotenoids, monophenolics, terpenoids, phenolic acids and tannins. The study of natural products involves isolation of these compounds in a pure form and investigation of their structure, formation, use and purpose in the organisms. Plants isoprenoids are known to act as toxins, antifeedant and some of them possess pharmacological activity. Wallach in 1887 (Ruzicka 1932), after carrying out structural investigations of several terpenes, formulated structural relationship among the terpenoids, according to which the terpenoids are made up of two or more isoprene (2-methyl-1,3-butadiene) units joined together in a head to tail manner. Ruzicka (1953) modified Wallach's idea and further formulated biogenetic isoprene rule in the year 1950, which emphasized on the mechanistic considerations of terpenoids synthesis in terms of electrophilic elongations, cyclization and rearrangements.

These plant extracts contain many secondary metabolites. Secondary metabolites feature several properties against insects, like insecticidal, antifeedant and growth regulatory activity. Secondary metabolites considered as that substance or mixture of substances that exert biocide action due to the nature of their chemical structure (Celis *et al* 2008). However, most of the plants used against insects have an insectistatic effect, rather than insecticidal. This refers to the inhibition of the insect's development and behavior, and it is divided into: Repellence, antifeeding activity, growth regulation, feed deterrents (Koul 2004), and oviposition deterrents.

Repellent activity is presented in plants that have compounds with fouling smell or irritating effects, which cause insects to get away from them (Peterson and Coats 2001). Antifeeding activity is exerted by compounds that once ingested by the insect, causes it to stop feeding and eventually die of starvation (Isman 2006). Growth regulating compounds

inhibit metamorphosis or provoke precocious molting. They alter the growth regulating hormones and cause malformations, sterility or death in insects (Celis *et al* 2008).

Secondary metabolites are extracted from many plant families such as Meliaceae, Euphorbiaceae, Asteraceae, Labiatae, Fabaceae and Solanaceae. These families contain many biologically active compounds. The compound extracted from Meliaceae family shows wide range of biological activities mainly insecticidal properties. The Meliaceae family contains about 1400 species, some of which stand out for having insecticidal features; owing to the fact they contain triterpenoid limonoids (Akhtar *et al* 2008). This group of compounds has driven a lot of interest because of their high activity over the behavior and physiology of several phytophagous insect species. Therefore, most of the research on the Meliaceae species has been focused on testing the biological activity of neem extracts on insects (National Research Council 1992).

Neem is one of the general purpose botanical pesticides used in organic agriculture. It is widely used around the world today either as a stand-alone treatment (Kumar and Poehling 2006) or in conjunction with synthetic pesticides or entomopathogens (Mohan *et al* 2007).

**2.1 NEEM (*Azadirachta indica*)**

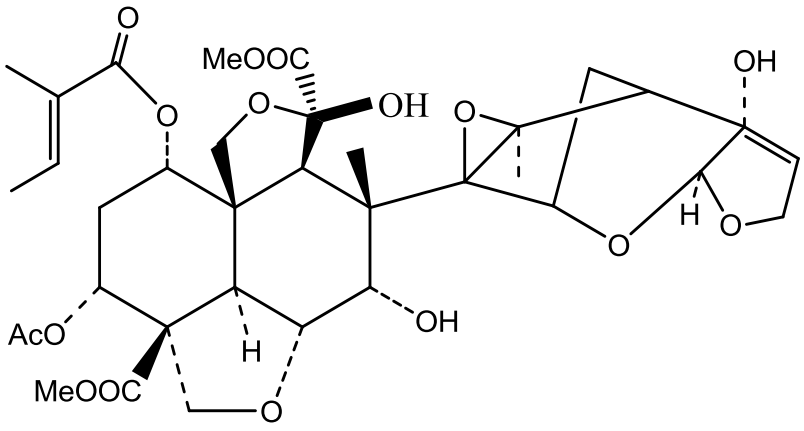
*Azadirachta indica* and *Melia azedarach* are two closely related species of Meliaceae. The former is popularly known as Indian neem (margosa tree) or Indian lilac, and the latter as the Persian lilac. *A. indica* popularly known as Neem, Nim, Margosa or Nimba is native to Indian subcontinent. It grows as a wild tree in India, Bangladesh, Burma, Pakistan, Sri Lanka, Malaysia, Thailand and Indonesia. Presently neem trees can be seen growing successfully in about 72 countries worldwide, in Asia, Africa, Australia, North, Central and South America (Suri and Mehrotra1994). Neem is a member of the Meliaceae family. Taxonomic position of neem is as under:

| | | |
|---|---|---|
| Order | : | Rutales |
| Suborder | : | Rutinae |
| Family | : | Meliaceae |
| Subfamily | : | Melioideae |
| Tribe | : | Melieae |
| Genus | : | *Azadirachta* |
| Species | : | *indica* |

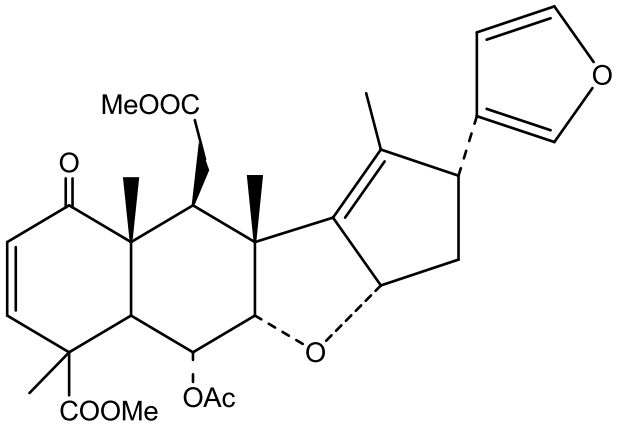
*A. indica* is a small to medium-sized tree, usually evergreen, up to 15 m (30 m max.) tall, with a round, large crown up to 10 m (20 m max.) in diameter, bark moderately thick,

with small, scattered tubercles, deeply fissured and flaking in old trees, dark grey outside and reddish inside, with colorless, sticky foetid sap. Leaves alternate, crowded near the end of branches, simply pinnate, 20-40 cm long, exstipulate, light green, with two pairs of glands at the base, petiole 2-7 cm long, inflorescence axillary, flowers bisexual, actinomorphic, small, pentamerous, white or pale yellow, slightly sweet scented, calyx lobes imbricate, petals free, imbricate, fruit one seeded drupe, ellipsoidal, 1-2 cm long, greenish, greenish yellow to yellow or purple when ripe, exocarp thin, mesocarp pulpy, endocarp cartilaginous, seed ovoid or spherical, apex pointed, testa thin, composed of a shell and a kernel (sometimes 2 or 3 kernels), each about half of the seed's weight.

There have been around 18 secondary compounds identified in neem seed extract, finding azadirachtin (1) in higher concentrations, which can range from 10 to 25 per cent (Govindachari *et al* 2000) due to genetic or environmental causes, fruit developmental stage or even because of seed storage time. Besides azadirachtin, there are other triterpenoids as nimbin (2), meliantrol and salannine. These triterpenoids account for the total bioactivity of the neem seed extract; however, it is considered that 72 to 90 per cent of the biological activity is because of azadirachtin, which is the major, active component (Schmutterer, 1990). Due to this reason it is used for the production of commercial insecticides such as Margosan-O, Neem Gold, Neemark and Azatin (Isman 2006).



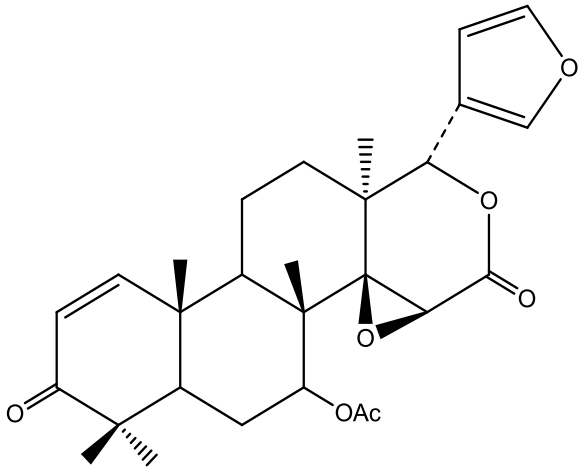

**(1)**




**(2)**

Valladares *et al* (2003) and Isman (2006) explained that the bioactivity of a compound mixture is done by synergism among the different secondary metabolites acting at the same time, when used separately, there is a higher probability of developing insect resistance.

Chemical investigation on the products of the neem was extensively undertaken in the middle of the twentieth century. Nimbin (2) was the first bitter compound isolated from neem oil, and thereafter more than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural diversity of these compounds which are divided into two major classes (Roy *et al* 2007). The isoprenoids include diterpenoids (namely sugiol, nimbiol, margasone) and triterpenoids containing protomeliacins, liminoids, azadirone and its derivatives, genudin (3) and its derivatives, vilarin type of compounds and C-secomeliacins such as azadirachtin (1), nimbin (2) and salannin. The first compound to be studied was nimbin. The non-isoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulfurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds and phenolic acids (Girish and Shankara 2008). Neem extracts also contain significant amount of water soluble electrochemically active compounds, as well as high concentrations of alkaloids, fatty acids, nitrogen and oxygen containing compounds. Neem is bitter in taste and this is due to an array of complex compounds called **triterpenes** or more specifically **limonoids**. Nearly 100 protolimonoids, limonoids or tetranortriterpenoids, pentanortriterpenoids, hexanortriterpenoids and some nonterpenoid constituents have been isolated from various parts of the neem tree (Koul *et al* 1990); still more are being isolated. The most important bioactive principal is azadirachtin; at least 10 other limonoids possess insect growth in regulating activity (Saxena and Kidiavai 1997).




**(3)**

Neem oil extracted from the seeds of *A. indica* has versatile medicinal properties, including antifertility (Upadhyay *et al* 1990), antifungal (Kher and Chaurasia 1997), immunostimulant (Upadhyay *et al* 1992), antibacterial (Singh and Sastry 1997), antipyretic (Biswas *et al* 2002), acaricidal (Mulla and Su 1999), antioxidant (Sultana *et al* 2007), larvicidal (Miller and Chamberlain 1989), insecticidal (Showler *et al* 2004) and insect repellant (Palsson and Jaenson 1999).

### 2.1.1 Extraction of Secondary Metabolites from Neem

Many types of solvents like methanol, water, acetone, chioroform, ethanol and hexane have been used to extract limonoids. However, the composition and proportion of the four main active principles (azadirachtin, salanine, nimbine and meliartenin) depends on the solvent and extraction method used. Most of the investigations use polar solvents, to maintain a rational management of the extracts; besides, using polar solvents, azadirachtin, which is a polar compound, can be obtained in a higher amount and concentration. The extraction methods are varied; however, Romero and Vargas (2005) showed that soxhlet extraction is the best method to obtain the highest yield of neem oil. The extracts obtained from meliaceous plants have been mainly dissolved with water (Garcia *et al* 2006). However, they can be dissolved with other substances such as Tween 20 (Gonzalez *et al* 2006), dichloromethane, dimethyl sulfoxide and acetone as long as they don't affect the biological activity to be tested.

Neem tree is known to be an important source of triterpenoids (Ley *et al* 1993). Most of the research that has been done on neem plant has focused intensely on this compound because it is the principle active ingredient of a unique insecticide which is thought to be the most useful and most fascinating by product of neem. The most important source of the

triterpenoids is the seed kernels and the compound of most biological interest is the highly oxidized and rearranged triterpenoids azadirachtin (Butterworth and Morgan 1968). Azadirachtin and compounds similar to it are structurally related to liomonin, the bitter principle of citrus fruits and are collectively known as limonoids. One of the essential requirements for the commercial trading of neem extracts is an accurate method for the quantitative analysis of their content of different active principle. A number of methods have been used for the separation of neem triterpenoids from seeds including column chromatography on alumina or silica (Harris *et al* 1968, Kraus *et al* 1981), solvent partitions (Schroeder and Nakanishi 1987), semi-preparative high pressure liquid chromatography (Govindachari *et al* 1995) and supercritical fluid chromatography (Johnson and Morgan1997). For long time the preferred method of isolation of these compounds from seed kernels has been by gravity column chromatography on columns of Florex 2, attapulgite clay. This material has lesser tendency then alumina or silica to degrade or retain some of the more reactive and polar compounds. Another improved and quicker method using Biotage 2 flash chromatography column is also preferred over others (Jarvis *et al* 1999).

The supercritical fluid chromatography (SFC) has a number of advantages viz. it is faster and consumes far less organic solvent then high pressure liquid chromatography (HPLC). Analysis of nimbin by HPLC typically uses a reverse phase system that require flushing the column with solvent at the end of each run in order to remove large amounts of non-polar accompanying material (especially in the analysis of neem seed extract) and re-equilibration before the next analysis. Moreover SFC has the ability to perform both temperature and pressure programming that can be giving still higher separation efficiencies. Because the mixture of limonoids in the seed are so complex, they cannot be separated and determined quantitatively directly using a crude terpenoid extract, but some fraction must be carried out first. For larger samples, this can be done quickly with the Biotage system (Jarvis *et al* 1999). For smaller sample a more convenient small scale procedure was required. Jarvis and Morgan (2000) described a system that has been devised for the analysis of limonoids in neem using the principle of SFC for separation of the initial mixture into fractions in which individual compounds can be quantified by SFC or HPLC.

### 2.1.2 Some bioactive compounds of Neem

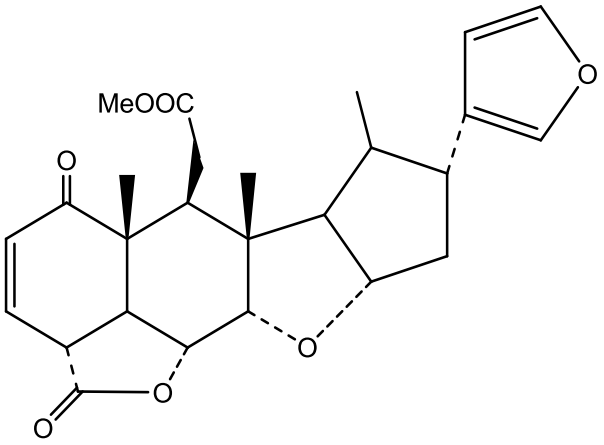
Azadirachtin (1) is a principal active compound of seed extract of *A. indica*. It is a chemical compound belonging to the limonoids. It is a secondary metabolite present in the neem tree seeds. Azadirachtin is a highly oxidized tetranorterpenoid which boasts a plethora of oxygen. It has a complex molecular structure and as a result the first synthesis was not

published for over 22 year after compound's discovery. The first total synthesis was completed by Ley *et al* (2007)

Azadirachtin is the compound that has shown best results, causing mortality, anti-feeding activity and deterrence in important agricultural pests such as: *Spodoptera littoralis* Boisd, *Schistocerca gregaria* Dallas, *S. gregaria* Forskal, among others (Mordue and Nisbet 2000). Azadirachtin is found in the bark, leaves, fruits and mostly in seeds of the neem tree (*A. indica*).

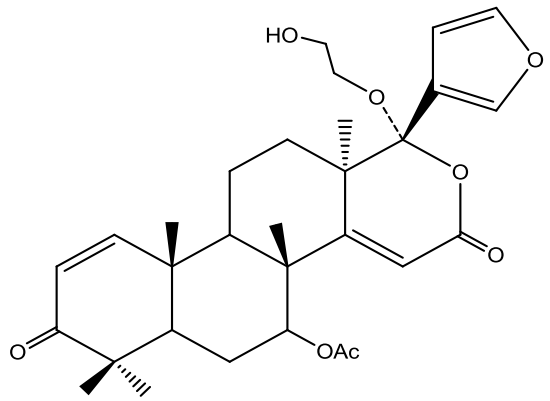
Azadirachtin is biodegradable and shows very low toxicity to mammals. This compound is found in the seeds of the neem tree. Many more compounds related to azadirachtin, are present in the seeds as well as in the leaves and the bark of the neem tree which also show strong biological activities among various pest insects (Senthil-Nathan *et al* 2005). Some laboratory and field studies have found neem extracts to be compatible with biological control as pure neem oil contains other insecticidal and fungicidal compounds in additional to azadirachtin.

Nimbidin, another crude bitter principle extracted from the oil of seed kernels of *A. indica* showed several biological activities. From this crude product some tetranortriterpenes, including nimbin (2), nimbinin, nimbidinin, nimbolide (4) and nimbidic acid have been isolated (Siddiqui 1942). Nimbidin and sodium nimbidate possess significant dose dependent anti-inflammatory activity against carrageenin induced acute paw oedema in rats and formalin-induced arthritis (Pillai and Santhakumari 1981). Oral administration of nimbidin demonstrated significant hypoglycaemic effect in fasting rabbits (Pillai and Santhakumari 1981). A significant anti-ulcer effect was observed with nimbidin in preventing acetylsalicylic acid, indomethacin, stress or serotonin-induced gastric lesions as well as histamine or cysteamine-induced duodenal ulcers (Pillai and Santhakumari 1984). Nimbidin can also suppress basal as well as histamine and carbachol-stimulated gastric acid output and may act as an antihistamine by blocking $H_2$ receptors, thereby helping as an antiulcer agent (Pillai and Santhakumari 1985). Nimbidin also revealed antifungal activity by inhibiting the growth of *Tinea rubrum*. *In vitro*, it can completely inhibit the growth of *Mycobacterium tuberculosis* and was also found to be bactericidal (Murthy and Sirsi 1958). Nimbolide (4) has been shown to exert antimalarial activity by inhibiting the growth of *Plasmodium falciparum* (Khalid *et al* 1989). Nimbolide was found to show antibacterial activity against *S. aureus* and *S. coagulase* (Rojanapo *et al* 1985).

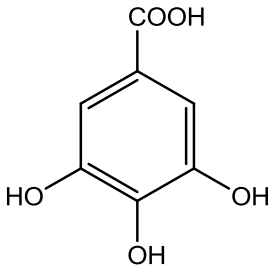



**(4)**

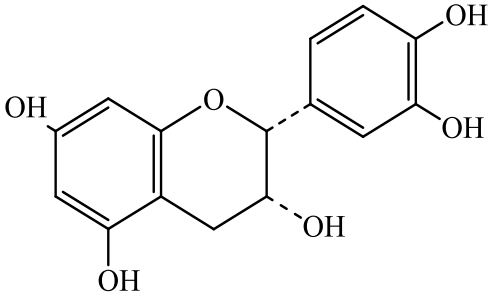
Gedunin (3), isolated from neem seed oil has been reported to possess both antifungal and antimalarial activities (Khalid *et al* 1989). Mahmoodin (5), a deoxygedunin isolated from seed oil, has been shown to possess moderate antibacterial action against some strains of human pathogenic bacteria (Devakumar and Dev 1996). Condensed tannins from the bark contain gallic acid (6), (+) gallocatechin, (–) epicatechin (7), (+) catechin (8) and epigallocatechin, of which gallic acid, (–) epicatechin and catechin are primarily responsible for inhibiting the generation of chemiluminescence by activated human polymorphonuclear neutrophil (PMN) (Vander-Nat *et al* 1991), indicating that these compounds inhibit oxidative burst of PMN during inflammation. Three tricyclic diterpenoids, margolone (9), margolonone (10) and isomargolonone (11) isolated from neem stem bark were found to be active against *Klebsiella*, *Staphylococcus* and *Serratia* species (Ara *et al* 1989). Sulfur containing compounds such as cyclic trisulfide (12) and tetrasulfide (13) isolated from the steam distillate of fresh, matured neem leaves showed antifungal activity against *Trichophyton mentagrophytes* (Pant *et al* 1986). Several polysaccharides from neem exhibited various biological effects. Two water soluble polysaccharides GIa (14) and GIb isolated from the bark of *Melia azadirachta*, demonstrated strong antitumour effect with complete regression of the tumours, when administered in mice at a daily dose of 50 mg / kg for four days after subcutaneous inoculation of Sarcoma-180 cells (Fujiwara *et al* 1982). Two more polysaccharides, GIIa (15) and GIIIa (16) isolated from *M. azadirachta* bark also showed significant anti-inflammatory effect on carrageenin induced oedema in mice (Fujiwara *et al* 1984). Two polymers isolated from an aqueous extract of neem bark possess anticomplement activity, amongst which the compound NB-II, a peptidoglycan of lower molecular weight was found to be more potent (Vander-Nat *et al* 1989). Some active ingredients (phytosterol fraction) isolated from the lipid part of neem fruits, exhibit antiulcer activity in stressinduced gastric lesions (Moursi and Al-Khatib 1984).


HO
O
O
O
O
O
OAc

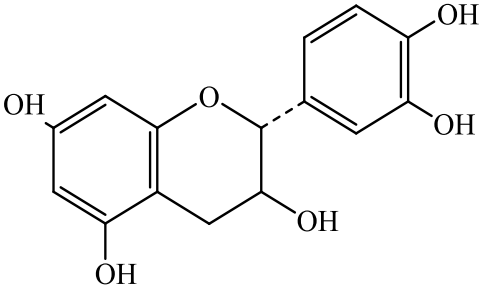
**(5)**


COOH
HO
OH
OH

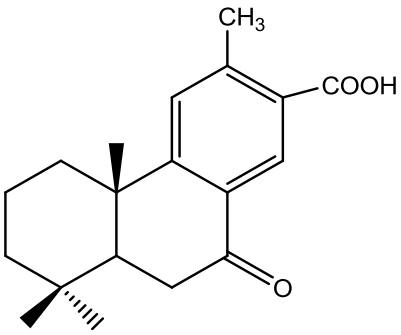
**(6)**


OH
OH
O
OH
OH
OH

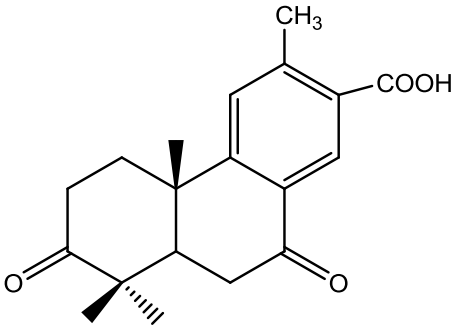
**(7)**


OH
OH
O
OH
OH
OH

**(8)**


CH3
COOH
O

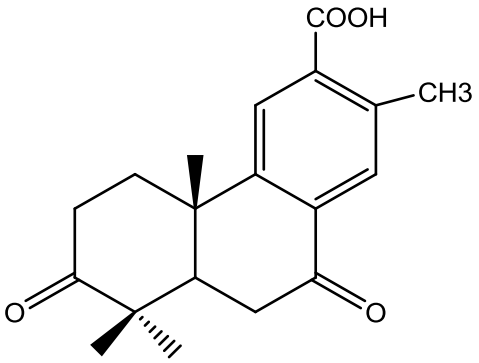
**(9)**


CH3
COOH
O
O

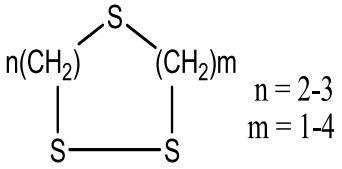
**(10)**

**Some Bioactive compounds from Neem (Biswas *et al* 2002; Grish and Shankara 2008):**

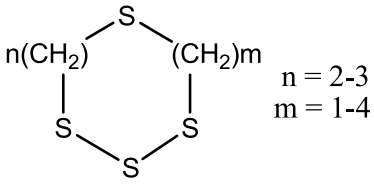
| Neem compound | Source | Biological activity |
|---|---|---|
| Nimbidin | Seed oil | Anti-inflammatory, Antiarthritic, Antipyretic, Antigastric ulcer, Antifungal, Antibacterial, Diuretic, Hypoglycaemic and Spermicidal |
| Sodium nimbidate | Seed oil | Anti-inflammatory |
| Azadirachtin | Seed | Insecticidal and Antimalarial |
| Nimbin | Seed oil | Spermicidal and Insecticidal |
| Gedunin | Seed oil | Antifungal and Antimalarial |
| Nimbolide | Seed oil | Antibacterial and Antimalarial |
| Mahmoodin | Seed oil | Antibacterial |
| Propyldisulphide, Meliacin and Meliacinol | Seed | Insecticidal |
| Meliatetraolenone, Sesquiterpene and Benzopyranoids | Fruit coat | Insecticidal |
| Azadirone | Fruit coat | Antitumor and Antibacterial |
| Salannin and Cardenolide | Seed oil | Insecticidal |
| Nimocinol | Leaves | Insecticidal |
| Fatty acid esters | Fruit coat and Seed | Insecticidal and Immunocontraceptive |
| 2,6-Bis-(1,1)-dimethylethyl-4-methyl phenol | Fruit coat | Antioxidant |
| Gallic acid and (–) epicatechin | Bark | Anti-inflammatory and Iimmunomodulatory |
| Margolone, margolonone and isomargolonone | Bark | Antibacterial |
| Cyclic trisulphide and cyclic tetrasulphide | Leaf | Antifungal |
| Polysaccharides GIa and GIb | Bark | Antitumor |
| Polysaccharides GIIa and GIIIa | Bark | Anti-inflammatory |


COOH
CH3
O
O

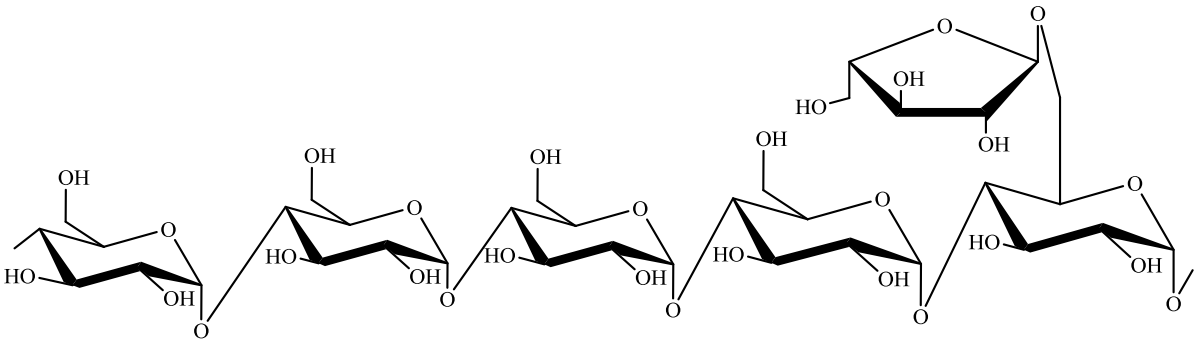
**(11)**


S
n(CH2)
(CH2)m
S
S
n = 2-3
m = 1-4

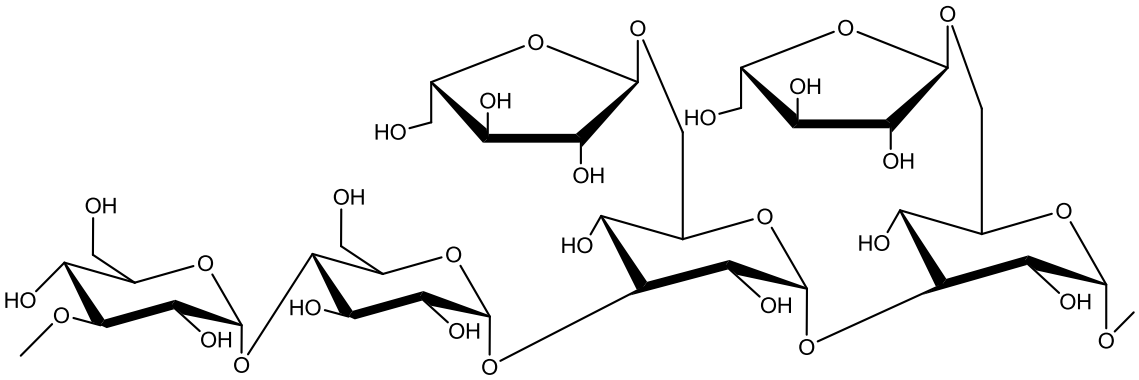
**(12)**


S
n(CH2)
(CH2)m
S
S
S
n = 2-3
m = 1-4

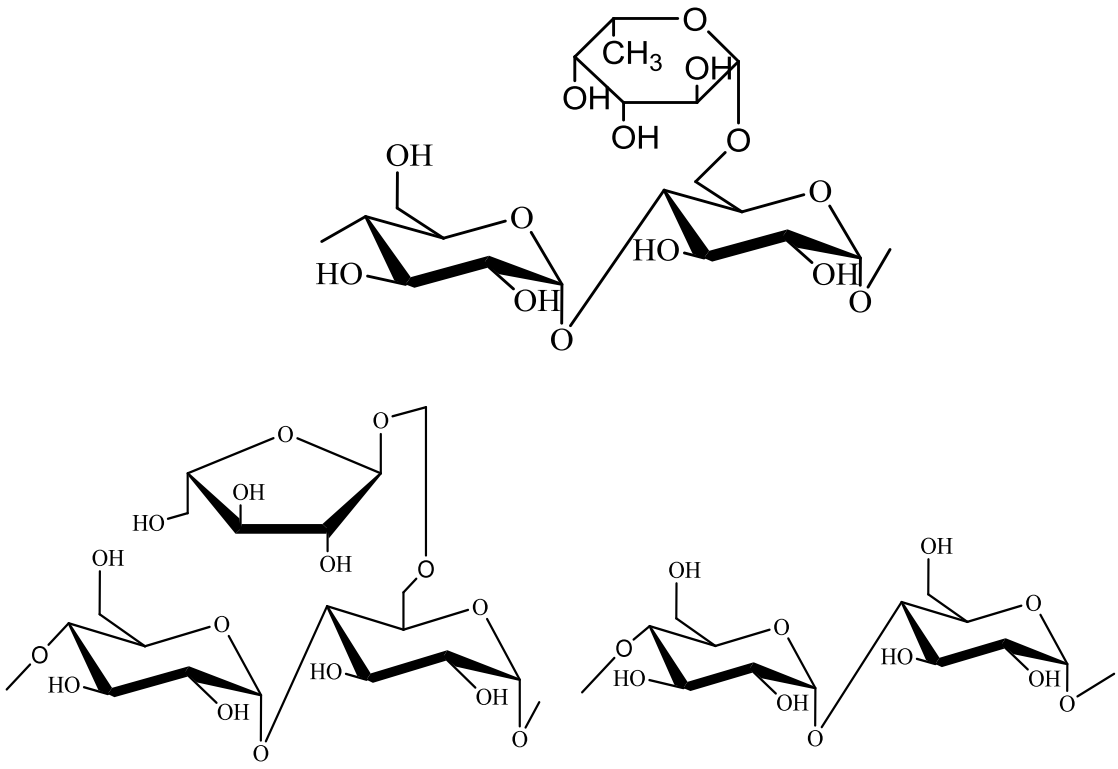
**(13)**


OH
HO
O
OH
OH
HO
O
OH
OH
HO
O
OH
OH
HO
O
OH
O
HO
OH
OH
O
HO
OH
O

**(14)**

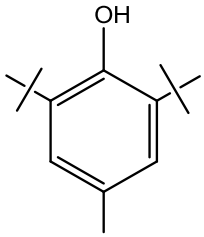

O
O
HO
OH
OH
O
O
HO
OH
OH
OH
HO
O
OH
O
HO
OH
OH
O
HO
OH
O

**(15)**

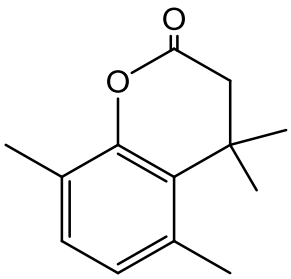


**(16)**

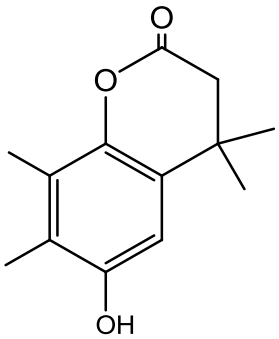
Phytochemical and bioassay-guided fractionation suggests that the insecticidal properties of neem extracts and neem products are mediated by groups of compounds such as the tetranortriterpenoid, meliatetraolenone (Siddiqui *et al* 2000). Insecticidal aromatics, benzopyranoids, sesquiterpene and monoterpene from fractions of the fruit coatings of *A. indica* (2,6-bis-(1,1)-dimethylethyl-4-methyl phenol (17); 3,4-dihydro-4,4,5,8-tetramethylcoumarin (18); 3,4-dihydro,4,4,7,8 tetramethyl-coumarin-6-ol (19); a-hexylcinnamaldehyde (20); methyl (2E,6E)-farnesoate (21); galoxolide (22); methyl 14-methylpentadecanoate (23); ethyl palmitate (24); ethyl oleate (25); dihydromyrcenol (26); asarone (27) (Siddiqui *et al* 2004).



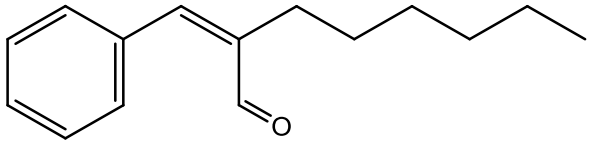
**(17)**



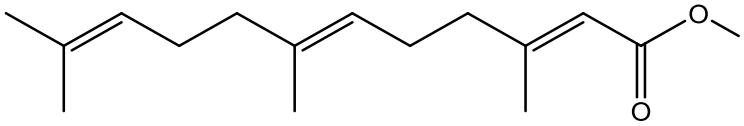
**(18)**



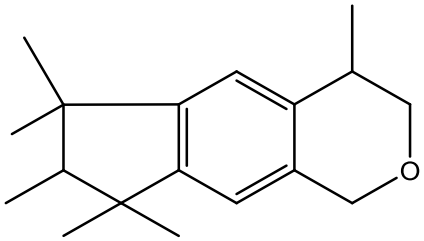
**(19)**



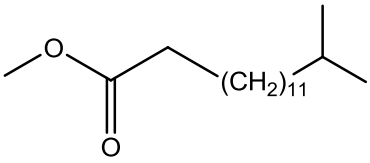

**(20)**



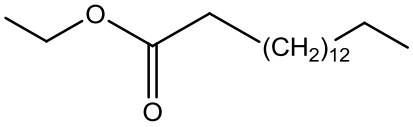

**(21)**



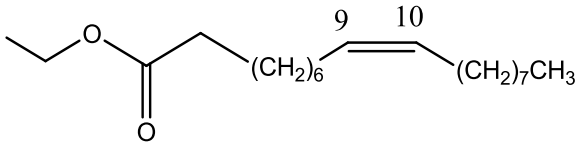

**(22)**



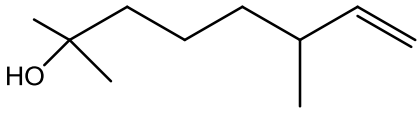

**(23)**



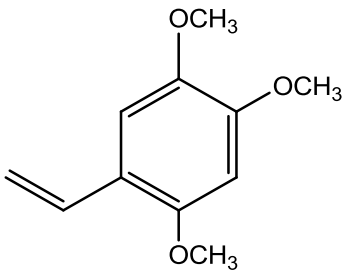

**(24)**




**(25)**

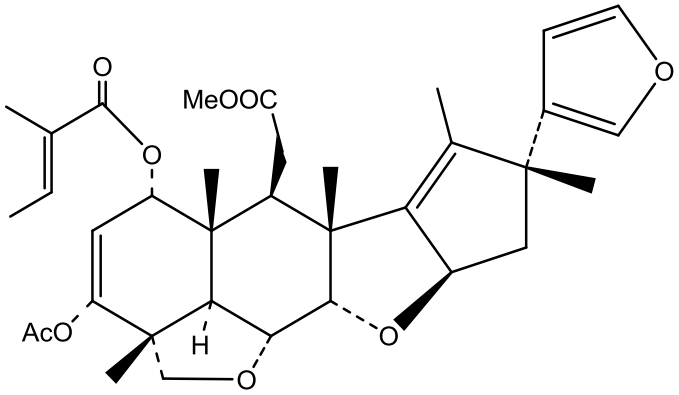



**(26)**

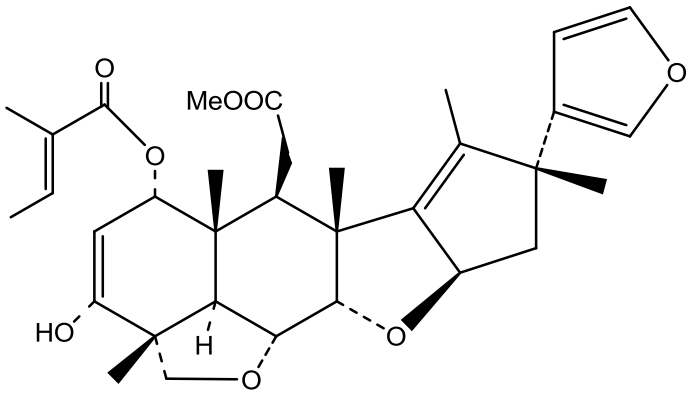



**(27)**

Other insecticidal components of neem include volatile di-n-propyl disulfide from neem seeds (Koul 2004), 6-b-hydroxygedunin (Koul *et al* 2003), Salannin (28) 3-deacetyl salannin (29 (Simmonds *et al* 2004) triterpenoids (22,23-dihydronimocinol and desfurano-6a-hydroxyazadiradione), meliacin (7a-senecioyl-(7-deacetyl)- 23-O-methyl-nimocinolide) and meliacinol [24,25,26,27-tetranorapotirucalla-(apoeupha)-1a-trimethylacryloxy-21,23-6a,28-diepoxy-16-oxo-17-oxa-14,20,22-trien-3a,7a-diol; Siddiqui *et al* 2000, 2002].



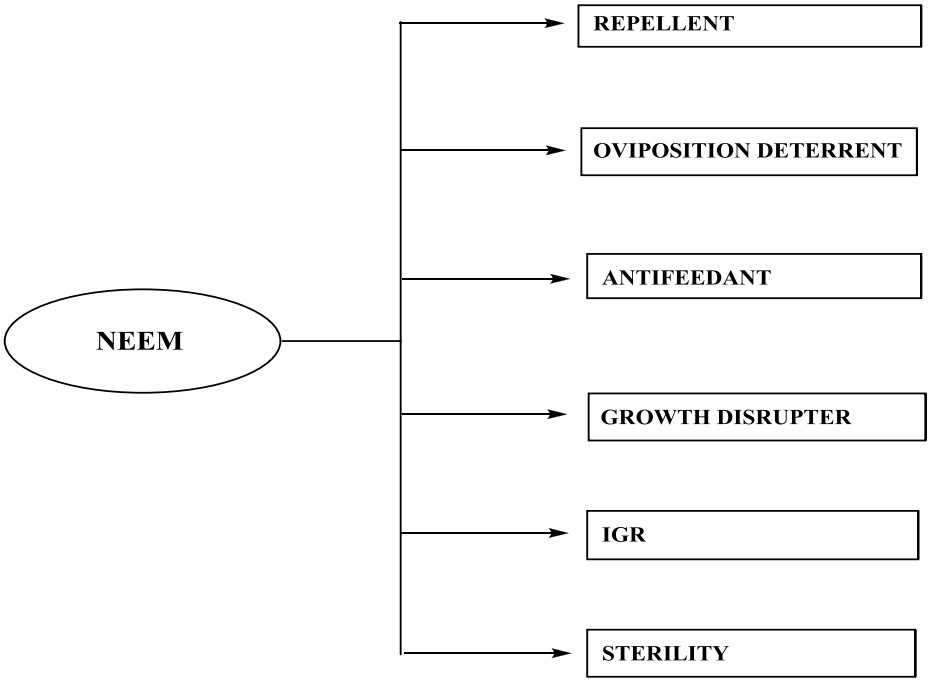

**(28)**




**(29)**

### 2.1.3 Scientific Basis for Effectiveness of Neem Materials

Neem materials, whether raw, enriched, or purified, including biactive compounds, such as azadirachtin, affect behavior, growth and development, survival and reproduction of stored product insects has been reviewed (Mordue and Blackwell 1993, Pascual *et al* 1990, Singh 1993). Neem extract is a potent repellent, antifeedant, growth regulator and oviposition deterrent affecting more than 200 species of pests (Martinez-Villar *et al* 2005). Although the sensitivity of stored product insect pests to neem materials varies, almost all the species were sensitive to neem. There are a few exceptions, such as *Oryzaephilus surinamensis* (Sarup and Srivastava 1971) and *O. acuminatus* (Thomas and Woodruff 1983), which infest old neem seed kernels.




**Biological effect of neem on insect**

Although seed damage is not always reduced by neem materials at par with synthetic insecticides (Sehgal and Ujagar 1990), the advantage of neem treatment is that it does not impair the germination of stored seed (Gupta *et al* 1989). In fact, rice seedlings raised from seed treated with 2.5 per cent neem seed kernel extract or with 2 per cent neem cake were more vigorous and had higher root and shoot growth indices and dry weights than those germinated from untreated seed (Kareem *et al* 1989).

Azadirachtin stimulates specific deterrent cells in mouth part chemoreceptors and also blocks the firing of sugar receptor cells which stimulates feeding thereby resulting in starvation, and death of insect species (Mordue *et al* 1998). The growing accumulation of literature also reported that *A. indica* products worked by intervening at several stages of insect's life.

Oil content in seeds of *A. indica* varies with geographical location. In seed from India, maximum oil content was obtained in seed from Hisar region (Kaura *et al* 1998). The high insecticidal activity of neem tree extract is due to the major tetranortriterpenoid isomer **azadirachtin A**. Azadirachtin is well known as a potent phytogenic insect growth inhibitor (Pavela 2007). It inhibits feeding and growth of insects belonging to several orders, like Lepidoptera (Koul *et al* 1987, Pavela and Holy 2003), Diptera (Champagne *et al* 1989), Orthoptera (Ascher *et al* 1989), Hemiptera (Dorn *et al* 1986) and Coleoptera (Ladd *et al* 1984) etc. through intervention in endocrine events (Sieber and Rembold 1983).

### 2.1.4 Traditional Uses of Neem

It has been an age-old practice in India to mix dried neem leaves with grains meant for storage. The practice of mixing neem materials with stored products became rooted as part of traditional wisdom and culture. Pruthi and Singh (1944) recorded that neem leaves were spread in 5-7 inches thick layers in grains and neem fruits were crushed on the inner surfaces of grain containers. Mixing of neem leaves (2-5 per cent) with wheat, rice, or other grains is even now practised in many villages in India and Pakistan. Other common practices include mixing of neem leaf paste with the mud that is used for making earthen bins and overnight soaking of gunny bags in boiled neem leaf extract (2-10 per cent), which are then used for storing grain.

The traditional uses of neem may differ in different regions or with farmers of different cultural backgrounds. For example in southern Sind, Pakistan, farmers mix dried neem leaves with grains stored in jute sacks, or they apply crushed neem leaves on the inner surfaces of mud bins before filling them with grains (Jilani and Amir 1987). In central Sind, where ***palli*** (a giant basket) made of plant materials is a common storage structure, crushed neem leaves mixed with mud are used as plaster for its inner sidewalls and top. In southern Punjab, Pakistan, neem leaf extract is sprinkled on wheat straw packed at the bottom of ***palli*** 2 to 3 days before filling with grain. A survey of various types of on-farm storage practices revealed that a combination of two or three control measures, including the use of neem leaves, was used by 29 per cent of the farmers in Punjab and 47 per cent of the farmers in Sind (Borsdorf *et al* 1983). In Sri Lanka, farmers burn neem leaves to generate smoke for fumigation against insect pests that attack stored paddy and pulses (Ranasinghe 1984). Also, chopped green leaves are kept over the heap of paddy in a container; as leaves dry up, they are replaced periodically.

Ahmed and Koppel (1987) conducted a survey of post-harvest control practices of 145 farmers in 11 districts of six provinces in India. They found that 30-60 per cent of the farmers who stored wheat, rice, sorghum, and millet, used 4-10 per cent neem leaves (wt/wt) for protection. The grain was stored in large, open straw baskets or in jute bags.

In Ghana, cacao beans mixed with 8 per cent neem leaves remained free from attack by *Ephestia cautella* up to 9 months in storage (Fry 1938). In Nigeria, the traditional use of neem for protecting stored grains is well-documented (Bugundu 1970).

The traditional use of neem materials simply emerged from experience and understanding that relatively less damage occurred in the treated stored commodity than when stored without neem. Little consideration was given to the large quantities of neem material

needed for affording protection because of the ubiquity of neem tree in villages and on homesteads. The characteristic garlic like odour of neem materials permeating the closed storage environment presumably repelled insects and bitter compounds in neem materials mixed with the stored grain discouraged insect feeding. Probably, the oil present in neem seed or kernel also discouraged egg deposition on grains, particularly on leguminous seeds. There could also be other less visible but significant effects of neem on behavior and physiology of stored product pests.

## 2.2 BIOLOGICAL ACTIVITY

Use of synthetic chemicals, no doubt improved crop yields quantitatively but has created undesirable side effects in form of health hazards, deterioration of environment quality and development of resistance and cross resistance. Due to these problems, efforts are being made to find alternatives to these chemicals. Among the various alternatives, use of natural plant products, called allelochemicals, offers a new approach for the management of noxious weeds and pests in suitable manner (Macias *et al* 2001). These are biodegradable, and rarely contain halogenated atoms. They possess novel target sites that are different from synthetic chemicals, and can thus be explored as lead chemicals for synthesis of new herbicides. They also exhibit a large degree of novelty and structural diversity and are highly sought after for the discovery of new agrochemicals (Duke *et al* 2000). A plant growth regulator is an organic substance other than nutrients, which in low concentration promotes or inhibits or quantitatively modifies growth and development of the plant. The agricultural scientists of the world now feel strongly than before, that the role of agricultural chemicals including plant growth regulators rank high to increase the food productivity for the increasing population in the world.

### 2.2.1 Insecticidal Properties

Dunkel *et al* (1995) reported that ethanol extract of seeds of *A. indica* mixed with beans protected the grains against pest infestation during storage without affecting instrumental hardness and consumer acceptability. Insecticidal activity of neem extracts has also been reported against *Clavigralla scutellaris*, *Gryon fulviventre* (Mitchell *et al* 2004), *Cowpea bruchid* and *Callosobruchus maculates* (Lale and Mustapha 2000). Siddiqui *et al* (2000) reported that the insecticidal properties of neem extracts and neem products are mediated by groups of compounds such as the tetranortriterpenoid, meliatetraolenone. Azadirachtin formulation (1 per cent), commercially called **Neemazal** has been found effective against roots sucking (*Aphis fabae*) and free-feeding pest insects (*Heliothis armigera*) in a concentration and time dependent manner (Hummel and Kleeberg 2002).

Pavela *et al* (2009) reported the efficiency of the botanical insecticide (BI) Neemazal T/S (containing 1 per cent Azadirachtin A), on the basis of azadirachtin applied in a dose of 20 g against Brassica pod midge (*Dasineura brassicae*), monitored for four years. The biological efficiency of BI was compared with the efficiency of some synthetic insecticides. It was ascertained that BI was very efficient in decreasing the number of damaged oilseed rape pods (ranging from 56.5 to 85.9 per cent compared to untreated plants) and its efficiency was comparable with synthetic insecticides based on Chloronicotinyl (Thiacloprid) and Neonicotinoid (Acetamiprid). BI's efficiency was, in some years, even significantly higher compared to pyrethroid (cyhalothrin). The high biological efficiency of azadirachtin was significantly resulted in increasing potential crop yields. The increased yield of azadirachtin ranged between 9.3 to 19.4 per cent compared to the control sample. Azadirachtin showed the highest yield for the whole time of experimentation, and in some years the increased yield was significantly higher compared to some synthetic agents.

Singh *et al* (1988) reported the insecticidal activity of an aqueous, an ethanolic and a hexane extract obtained from neem *(A. indica*) seed kernels against the mustard aphid, *Lipaphis erysimi*. The hexane extract, which exhibited a much higher activity than the two other extracts, had an $LC_{50}$ of 0.674 per cent. When the hexane extract was partitioned with ethanol, the ethanol-soluble fraction had an $LC_{50}$ of 0.328 per cent, whereas the ethanol-insoluble part showed no activity even at one per cent.

#### 2.2.1.1 Effectiveness of Different Neem Products against Insect Pests of Major Stored Food Grain

**Legumes:** In India, green gram, chick pea, cowpea, and pea could be protected from damage by the pulse beetles, *Callosobruchus* spp., for 8-11 months by mixing powdered neem kernel with grains at 1 or 2 to 100 parts (Jotwani and Sircar 1967). Neem kernel protected the legumes against *C. chinensis* and *C. maculatus* and stopped the development of progeny even 12 months after *C. chinensis* was released on treated lentil seed (Yadav 1973). Likewise, chick pea and pigeon pea seeds remained undamaged up to 12 months after treatment with 2 g neem kernel powder per 100 g seed. Application of 1 to 3 parts of neem oil per 100 parts of Bengal gram rendered complete protection against *C. chinensis* for at least 135 days, without impairing seed germination.

Ketkar (1976) tested the efficacy of neem kernel and oil for protecting bagged leguminous seeds (peas, Bengal gram, *Phaseolus,* and *Vigna* spp.) from pulse beetles during eight months storage in warehouse trials conducted in Pune, India. Neem oil treatment at 8 ml / 1 kg of grains reduced the infestation to almost zero in Bengal gram and *Phaseolus* (*vs*. 14 per cent in untreated Bengal gram and 26 per cent in untreated *Phaseolus*), and by 50 to 70

per cent in treated peas and vigna; treatment with kernel was less effective. Neem oil did not affect seed viability and unused seeds were fit for animal and even human consumption. Ali *et al* (1983) reported that neem oil at 1 ml/100 g seed killed all the pulse beetle grubs and adults, and no eggs were laid on treated seed. On cowpea and bambara groundnut, neem oil at 8 ml/kg seed not only reduced oviposition, but also killed larvae; the activity persisted more than 90 days on cowpea and for 180 days on bambara groundnuts (Pereira 1983). Green gram was completely protected against *Callosobruchus* spp. when soaked for 20 minutes in a 1 per cent solution of neem oil extract (Attri and Prasad 1980).

In a warehouse trial conducted in Togo, white cowpea treated with 0.5 per cent neem oil was protected from *C. maculatus* for up to 6 months of storage and even after 10 months of storage only 18 per cent of the initial weight was lost (Zehrer 1984). The neem oil-treated cowpea had good texture, but tasted slightly bitter. However, the influence of taste was mattered little compared with the overall nutrient value and acceptability. Probably, eating quality could have been improved further by thorough rinsing or by removing the seed coat prior to cooking. Neem oil was found to be highly suitable for cowpea preservation and well adapted to the life styles of subsistence farmers, as it was inexpensive and its application did not need any tools.

In Ghana, Tanzubil (1987) demonstrated that cowpea treated with neem oil at 0.5 per cent, or mixed with powdered fruit at 10 per cent remained undamaged by *C. maculatus* over a 16 week storage period; mixing neem leaf dust in the grain was less effective, while untreated cowpea had 90 per cent grain damage.

**Sorghum and corn:** In India, sorghum seed mixed with powdered neem kernel in a proportion of 100 to $\geq$ 1.5 (wt/wt) remained protected from damage by *Sitophilus oryzae* (Deshpande 1967). Corn seed soaked for 20 minutes in a 1 per cent solution of neem oil extractive was resistant to attack by *S. oryzae* (Attri and Prasad 1980). In Togo, Adhikary (1981) found that neem treatment of corn stored in sacks or unpeeled corn cobs held in bins was quite simple and effective against *S. zeamais, Rhyzopertha dominica*, and *Cathartus* spp.

**Wheat:** Jotwani and Sircar (1965) in India were the first to demonstrate that powdered neem kernel when mixed with wheat seed at a proportion of 1-2 to 100 (wt/wt) parts satisfactorily protected against *S. oryzae, R. Dominica* and *Trogoderma granarium* for 270, 320, and 380 days, respectively. Rahim (1997) found that an ethanolic neem kernel extract, containing azadirachtin, at 75mg / kg protected stored wheat against *R. dominica* for up to 48 weeks. In warehouse trials, wheat grain treated with neem oil at a proportion of 8 ml / 1 kg grain, prior to storing for 8 months in gunny bags, had 50 to 70 per cent less infestation by *S. oryzae*, *R. Dominica* and *Cryptolestes* spp. (Ketkar 1976). Application of neem oil at a

low concentration of 0.1 per cent (wt/wt) to wheat grain reduced egg laying by *Sitotroga cerealella* as effectively as a 5 per cent malathion dust treatment (Verma *et al* 1985).

In commercial trials conducted in Pakistan, it was demonstrated that paper or cloth grain storage bags treated with water extract of neem leaves at 20 per cent (wt/vol) or water extract of neem seed at 5 per cent (wt/vol) checked the penetration of stored grain pests into the bags for 6 months during storage (Jilani 1981). In an on-farm trial conducted in Sind, Pakistan for 13 months, the application of ethanolic neem seed extract (600 µg /$cm^2$) to storage bags or directly to wheat grain controlled more than 80 per cent of the population of *R. dominica*, *S. oryzae*, and *S. cerealella* and prevented grain damage up to 6 months (Jilani and Amir 1987). The treatments remained effective up to 13 months, providing more than 70 per cent protection; insect infestation and the per centage of weevil attacked grains was much lower than in the untreated control.

**Rice and paddy:** In Malaysia, mixing neem leaves with paddy grain in a proportion of 2 to 100 parts (wt/wt), bag treatment with 2 per cent neem leaf water extract (wt/wt), or placing barriers of neem leaves between bags and storage floor, significantly reduced the infestation by *S. oryzae* and *R. dominica* and damage to paddy grain stored in 40 kg jute bags for 3 months (Muda 1984). Although it was not clear which treatment was superior, but all treatments had potential for adoption in rural areas.

In a warehouse trial conducted in the Philippines, Jilani and Saxena (1988) evaluated the effectiveness of neem oil alone or in combination with fumigation against five species of major stored grain pests infesting rice and paddy grains. Rice grain treated with 0.05 to 0.1 per cent neem oil or treated with neem oil after fumigation with **Phostoxin**, and stored for 8 months contained significantly less *T. castaneum* adults than in the untreated control.

**Potato tubers:** Neem can also be applied in reducing damage due to the potato tuber moth, *Phthorimaea operculella,* during storage. In India, in simulated storage trials as well as in actual storage trials conducted in a warehouse, a 4 month protection was achieved against the pest when harvested potato and the covering material were sprayed with 5 and 10 per cent enriched neem seed extract (Sharma *et al.* 1984). Serious potato tuberworm damage to stored potato was averted also in the Sudan by spraying potato with a 2.5 per cent neem leaf or seed extract prior to bagging (Siddig 1987).

### 2.2.2 Antifungal Properties

A number of in vitro and in vivo studies appear to confirm the fungistatic and fungicidal activity of neem extracts. The extract of *A. indica* has fungicidal effect against

pineapple fruit-rotting fungus, *Ceratocystis paradoxa* (Damayanti *et al* 1996), fungistatic and fungicidal activities against *Aspergillus spp*. and *Candida spp*. (Fabry *et al* 1996), *Basidiobolus haptosporus* and *Basidiobolus ranarum* (Nwosu and Okafor 1995) and *Pestalotiopsis mangiferae*, the causative agent for the serious leaf-spot disease of *Magnifera indica* (Rai 1996). Neem extract has also been found to possess fungistatic and fungicidal effects against plant pathogenic fungi like *Fusarium oxysporum*, *Alternaria solani*, *Curvularia lunata*, *Helminthosporium spp.* and *Schlerotium rolfsii* (Bhonde *et al* 1999) and against dermatophytes such as *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton violaceum*, *Microsporum nanum*, and *Epidermophyton floccosum* (Natarajan *et al* 2002). Other workers have suggested that neem oil and leaf extracts significantly inhibit production of fungal toxic secondary metabolites, e.g. patulin produced by *Penicillium expansum* (Mossini *et al* 2004). Sairam *et al* (2000) reported that neem products may be useful in the management and treatment of dermatophytic and fungal infections of animals and plants as well as in the biological control of biodeterioration of stored agricultural products. Some extracts from neem plant have been shown to be toxic to fungal pathogens, such as *Poria monticolad* infecting wood (Dhyani *et al* 2004), *Aspergillus flavus* from soybean seeds (Krishnamurthy *et al* 2008), *Pyricularia oryzae* infecting rice plant in field and the harvested rice (Amadioha 2000).

Paul and Sharma (2002) showed that neem extract induced plant defense reactions and was useful in management of leaf stripe disease of barley. Cao *et al* (2006) proved that disease resistance in harvested Yali fruit can be affected by salicylic acid treatment on the trees in field during fruit growth and development. The neem extract has the ability of inhibiting the growth of fungi through inducing plant defense reactions and postponing formation of the spores in fruit of Yali pear and plum.

Mondall *et al* (2009) have reported the efficacy of different extracts of neem leaf on seed borne fungi *Aspergillus*, *Rhizopus*. They also studied chemical characterization of the neem leaf extracts in vitro on the culture medium. The growth of both the fungal species of all ages were inhibited significantly and controlled with both alcoholic and water extract at all concentrations used. The alcoholic extracts of neem leaf were most effective in comparison to aqueous extract for retarding the growth of *Rhizopus* and *Aspergillus*. The crude aqueous and alcoholic leaf extracts of neem were more effective in inhibiting of growth of the fungi *Aspergillus* in comparison to inhibitory effects on *Rhizopus* growth in the artificial culture medium. Leaf extracts of neem which are cheap, environmentally safe and promising extracts for protecting crop species against the fungal infestation and leading towards improvement of the crop in terms of yield and productivity.

Neem products inhibit the production of aflatoxins, which can grow in some food. Zeringue and Bhatnagar (1996) reported that nonvolatile, somewhat heat labile constituents in neem leaf extract, when added to fungal growth medium before inoculation, blocked aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus*. Practical applications of this finding may eliminate pre-harvest contamination of crops with aflatoxins.

### 2.2.3 Repellent Properties

Liu and Stansly (1995) reported that steroid-like triterpenoid derived from the neem tree can have many effects on insects including repellency, moulting disruption, growth reduction, interference with development and oviposition and high mortality particularly in immature insects as documented for a wide group of phytophagous insects including sweet potato whitefly. *A. indica* was found to repel mosquito up to 70 per cent (Palsson and Jaenson 1999) while a number of reports have also indicated that neem oil may also possess significant insect repellant activity. Sharma and Ansari (1994) burnt kerosene containing 0.01–1.0 per cent neem oil and found insignificant reduction in the biting of human volunteers and catches of mosquitoes resting on room walls in two Indian villages near Delhi.

Zaidi *et al* (2003) reported that extracts of neem, turmeric and sweet flag were evaluated as repellents against *Sitotroga cerealella* in vitro and found that the acetone extract of neem was the most effective botanical insecticide. Deterrent effects of powders made from leaves, seeds and bark of *A. indica* and *Nerium oleander* were tested against *Rhizopertha dominica* and got its repellency upto 96 per cent from neem leaves and seeds by Khan and Marwat (2003). In laboratory studies, neem seed oil obtained from different localities was appraised against Red Flour Beetle for its repellence and achieved it upto 52.25 per cent from oil obtained from Hyderabad (Nazli *et al* 2003). Fields *et al* (2001) found that neem was repellent to many stored product-insects when tested by exposure on filter paper or in preference chambers.

Anwar *et al* (2005) reported the insect repellent properties of neem oil with 5, 10, 15 and 20 per cent concentrations at intervals of 30, 60 and 90 days on packaging materials of two different densities used for storage of wheat in warehouse under natural conditions. The warehouse had *Rhizopertha dominica, Sitophilus granarius, Tribolium castaneum and Trogoderma granarium* in abundance. As per statistical analysis, 10 per cent oil treatment reduced the insect penetration more than those of 5 per cent significantly but it was not significantly different from that of 15 and 20 per cent. Moreover deterrence to penetration decreased with the passage of time. Per cent mortality could be achieved only upto 62.21 to 82.99 per cent for different insects. With all concentrations, per cent mortality was more at first interval than at second and third interval. Mortality was directly proportional to the

concentration of the spray material. Population build up in the packages and mortality showed no significant relation to the changing temperature and relative humidity in the warehouse but it showed significant change due to type of packaging materials. No concentrations of oil imparted any taint to the wheat stored or alter its viability.

### 2.2.4 Larvicidal, Acaricidal and Nematicidal Properties

Larvicidal, acaricidal and nematicidal properties are the underlying bases for the use of neem products for control of agricultural pests. These effects have been reported for methanolic extracts of defatted neem seed kernels. In eggs, immature and adult stages of *Hyalomma anatolicum excavatum* at concentrations of 1.6, 3.2, 6.4 and 12.8 per cent, Abdel-Shafy and Zayed (2002) observed a significant increase in the hatching rate during the first 7 days post treatment, giving incompletely developed and dead larvae, and after 15 days, it resulted in hatching failure, induced a significant increase in mortality rates of newly hatched larvae, unfed larvae and unfed adults.

Unal and Akkuzu (2009) investigated the effects of azadirachtin, a natural extract of the neem tree (*A. Indica*), on larval growth and feeding activity of pine processionary moth (*Thaumetopoea pityocampa* (Schiff.)) in laboratory experiments conducted in 2007-2008. The organic insecticide Neemazal-T/S (a commercial neem preparation) was applied as suspension in distilled water at concentration levels of 0.3, 0.5 and 1 per cent. Comparing with the untreated leaves, the effect of the insecticide Neemazal-T/S was tested on the $10^{th}$ day after treatment. The results obtained in conducted experiments indicated that Neemazal-T/S was effective against *T. pityocampa* larvae, significantly inhibiting larval growth and reducing feeding activity.

Rahman *et al* (2005) reported that the toxicity of Azadiractin (commercial product Nimbicidin) to different larval instars and adults of *Cryptolestes pusillus* (Schon.) after 24 and 48 h of exposure. The $LC_{50}$ values were 7.829, 19.402, 404.815, 29.374 and 5.659, 13.962, 12.297, 10.203 gm $cm^2$ for first to fourth instars larvae of 24 and 48 h respectively. For adults the $LC_{50}$ were 410.722 and 416.895gm $cm^2$ of 24 and 48 hours respectively.

Miller and Chamberlain (1989) reported that azadirachtin extracted from neem seed and ethanolic extract of grounded seed blended into cow manure or administered orally to cattle, exhibited effects similar to those of insect growth regulators as they were found to be larvicidal against Horn fly (*Haemotobia irritans*), Stable fly (*Stomoxys calcitrans*) and House fly (*Musca domestica*). The larvicidal effect of *A. indica* and other plants on *Aedes aegyptica* and *Culex quinequefaciatus* (Wandscheer *et al* 2004) third instar larvae of *Drosophila melanogaster* and fifth instar larvae of *Manduca sexta* (Mitchell *et al* 1997) have been

demonstrated. There are also reports that aqueous extract of de-oiled neem seed kernel caused complete mortality in the fourth instar larvae and pupae at the concentration of 100 ppm with no significant effect on the development period of *Culex quinquefasciatus* (Sagar and Sehgal 1996) and anopheline pupae (Rao *et al* 1995).

Bioassay-guided fractionation and phytochemical studies revealed azadirachtins A, B and H (Sharma *et al* 2003) as well as salannin, nimbin and 6-desacetylnimbin as the larvicidal component of neem extracts. Furthermore, studies by Koul *et al* (2003) showed 6b-hydroxygedunin isolated from *A. indica* to be the active agent against the gram pod borer, *Helicoverpa armigera* (Hubner), and Asian armyworm, *Spodoptera litura* (Fabricius; Lepidoptera: Noctuidae), alone and in combination with other limonoids, gedunin, salannin, nimbinene, and azadirachtin. However, work of Siddiqui *et al* (2002) implicated two triterpenoids, 22, 23- dihydronimocinol and desfurano-6a-hydroxyazadiradione isolated from methanolic extract of the fresh leaves of *A. indica* as well as meliacin, 7a-senecioyl- (7-deacetyl)-23-O-methyl nimocinolide (which showed mortality for fourth instar larvae of the mosquito *Anopheles stephensi*, with $LC_{50}$ values of 60 and 43 ppm, respectively), as other larvicidal agents in neem plant.

### 2.2.5 Antibacterial and antiviral properties

Several reports in literature suggested that different extracts of *A. indica* possess significant antibacterial and antiviral properties. Using semiquantitative quadrant streaking method, Pai *et al* (2004) demonstrated that mucoadhesive dental gel containing alcoholic neem extract significantly reduced (P\0.05) the plague index and bacterial count in the oral cavity, while Wolinsky *et al* (1996) showed that incubation of oral Streptococci with the neem stick extract resulted in a microscopically observable bacterial aggregation, indicating that neem stick extract can reduce the ability of some Streptococci to colonize tooth surfaces. Utilizing other techniques such as Ditch plate method, tube dilution technique and agar dilution methods, a strong antibacterial effect of neem extract has also been reported against *Bacillus cereus*, *Escherichia coli*, *Salmonella infantis* (Alzoreky and Nakahara 2003). Das *et al* (1999) and Biswas *et al* (2002) indicated that different neem extracts may also possess antibacterial activity against fish pathogenic bacteria such as *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *E. coli* and Myxobacteria, as well as other organisms like *Vibrio cholera*, *Mycobacterium tuberculosis* and *Mycobacterium pyogenes*, which are of public health importance.

Although limited in number, some studies have suggested that neem extract may possess significant capacity for antiviral activity. Parida *et al* (2002) have demonstrated that azadirachtin obtained from neem inhibited Dengue virus type-2 replication as confirmed by

the absence of Dengue-related clinical symptoms in sucking mice and absence of virus specific 511 bp amplicon in Reverse Transcriptase– Polymerase Chain Reaction (RT–PCR). Similarly, the antiviral activity of neem leaf extract against group B Coxsackie viruses (Badam *et al* 1999) has been reported. Biswas et al. (2002) suggested that the methanolic extract of neem leaves (NCL-11) is most effective in Coxsackie B-4 viruses as a virucidal agent, in addition to its influence at the early events of its replication.

Phytochemical studies have implicated limonoids such as mahmoodin and tetranortriterpenoids like azadirone, epoxyazadiradione, nimbin, gedunin, azadiradione, deacetylnimbin and 17-hydroxyazadiradione, as well as the protolimonoid, naheedin as phytochemicals responsible for the reported antibacterial and other antimicrobial activities (Biswas *et al* 2002; Subapriya and Nagini 2005; Bhattacharyya *et al* 2007; Girish and Shankara 2008).

### 2.2.6 Antifertility effects of Neem

Khillare and Shrivastav (2003) demonstrated that aqueous extracts of old and tender *A. indica* leaves immobilized and killed human spermatozoa within 20 s with minimum effective spermicidal concentrations of 2.91 ± 0.669 and 2.75 ± 0.754 mg/ml sperm, respectively. Studies on the mechanism by which *A. indica* may influence male fertility reveal that many processes may be at play. For example, ultrastructural studies by Ghodesawar *et al* (2004) on Cauda epididymidal cell types revealed hypoandrogenic effect as characterized by enlarged nuclei of principal cells, reduced number of coated micropinocytotic vessels of the apical cytoplasm, missing microvilli and mitochondrial cristae, golgi complex disruption, abounding lysosomal bodies in the cytoplasm, decreased chromatin content of nuclei, vacuolized cytoplasm and bulging nuclear membrane. These results appear to collaborate that of Kasturi *et al* (2002), who observed changes such as intracellular spaces and vacuolization in Sertoli cells, diminished Lydig cells and cytoplasmic inclusions of testis, hence concluding that *A. indica* leaves extract might affect spermatogenesis through antispermatogenetic and antiandrogenic properties.

Upadhyay *et al* (1990) reported that different parts of *A. indica* exert significant modulatory effects on female fertility. A single intrauterine application of 100 µl *A. indica* oil has been found to induce a pre-implantation block in fertility, which was reversible after 5–6 months, suggesting its possible use as a long-term female contraceptive. Earlier workers (Prakash *et al* 1988) had demonstrated that neem seed oil administered as a sub-cutaneous dose of 0.3 ml/rat exerted post-coital contraceptive effect, but with less possibility of eliciting less side effects than the steroidal contraceptives, since it does not possess estrogenic, antiestrogenic or progesteronal activity.

### 2.2.7 Anti-inflammatory properties

Kaur *et al* (2004) evaluated the anti-inflammatory effects of nimbin and nimbidin, which is a mixture of tetranortriterpenes that forms the major active principle of neem seed oil. Their results revealed that nimbin significantly inhibited some of the functions of macrophages and neutrophils relevant to the inflammatory response following both in vivo and in vitro exposure. They reported that oral administration of nimbidin at 5–25 mg / kg body weight to rats for three consecutive days significantly inhibited the migration of macrophages to their peritoneal cavities in response to inflammatory stimuli and also inhibited phagocytosis and phorbol-12-myristate-13-acetate (PMA)-stimulated respiratory burst in these cells. Also, in-vitro exposure of rat peritoneal macrophages to nimbidin also inhibited phagocytosis and PMA-stimulated respiratory burst in these cells as well as nitric oxide (NO) and prostaglandin E2 (PGE2) production in lipopolysaccharide (LPS)-stimulated macrophages, while interleukin 1 (IL-1) was only weakly inhibited. Furthermore, Kaur *et al* (2004) observed that nimbidin ameliorated NO inhibition via the induction of inducible NO synthase (iNOS) without any inhibition in its catalytic activity, and also attenuated degranulation in neutrophils were assessed in terms of release of beta-glucuronidase, myeloperoxidase and lysozyme. This demonstrated ability of nimbin and nimbidin to suppress the functions of macrophages and neutrophils relevant to inflammation, made these authors to conclude that they can be valuable in treating inflammation and inflammatory diseases.

### 2.2.8 Antioxidant Activity

Ghimeray *et al* (2009) reported that the leaf and bark fraction extracts of *A. indica* grown in the foothills (subtropical region) of Nepal were evaluated for their antioxidant activity, total phenolic (TP) and total flavonid (TF) contents. HPLC method was employed to quantify the amount of azadirachtin and nimbin present in the seed, leaf and the bark extracts of neem. The result showed that the highest azadirachtin content was found in the methanolic extract of the seed (3300 μg/g dw). Similarly, the hexane fraction of bark showed the highest nimbin content (271 μg/g dw) followed by the methanolic extract (260 μg/g dw). Antioxidant activity was determined by measuring 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity, hydroxyl radical scavenging activity, DNA protection assay, metal chelating and the inhibition of peroxidation using linoleic acid system and their results were found at different magnitudes of potency. The results of TP content expressed in tannic acid equivalents ranged from 66.63 to 629.04 μg/mg in the bark extracts and 23.85 to 237.00 μg/mg in the leaf extracts. Likewise, the content of TF expressed in quercetine equivalents ranged from 12.87 to 17.07 μg/mg in the bark and 13.72 to 93.17 μg/mg in the leaf extracts.

### 2.2.9 Antitrypanosomal activity

Yanes *et al* (2004) have demonstrated that fractions of chloroformic extracts of *A. indica* leaves markedly inhibited growth of *Trypanosoma cruzi epimastogotes* with ultrastructural changes such as vacuolization, organelle degeneration and cell division disruptions. Similarly, De-Azambuja and Garcia (1992) have shown that a major phytochemical in neem, azadirachtin a tetranortriterpenoid, when administered a single dose through blood meal, blocked the development of *T. cruzi* and induced a permanent resistance of the vector against reinfection with *T. cruzi*.

### 2.2.10 Antiulcerogenic effect

The antiulcerogenic effect of *A. indica* has indeed been demonstrated in clinical trials involving humans (Bandyopadhyay *et al* 2004). In that trial, which involved administration of lyophilized aqueous extracts to Indian patients suffering from acid-related problems and gastroduodenal ulcers for 10 days at daily twice dose of 30 mg, it was observed that neem bark extract not only has therapeutic potential for controlling gastric hypersecretion and gastroesophageal and gastroduodenal ulcers, but can also heal the duodenal ulcers as monitored by Barium Meal X-ray and endoscopy. Using important blood parameters for organ toxicity, these investigators further established the relative safety of the extracts when administered at 30–60 mg/kg bw for 10 weeks.

Chattopadhyay *et al* (2004) concluded that neem leaf extracts offer antiulcer activity by blocking acid secretion through inhibition of enzyme ATPase and by preventing oxidative damage and apoptosis. The involvement of enzyme ATPase inhibition and scavenging of hydroxyl radical as mechanisms for the gastroprotective effect of aqueous neem bark extract have also been demonstrated by Bandyopadhyay *et al* (2004).

### 2.2.11 Antidiabetic action

The hypoglycemic and anti-hyperglycemic properties of *A. indica* plant parts have been evaluated using different animal models. In normal and streptozotocininduced diabetic animals, aqueous leaf extracts of *A. indica* displayed anti-hyperglycemic effect, but showed little or no action on peripheral utilization of glucose and hepatic glycogen metabolism (Chattopadhyay 1996). The blood lowering effect of *A. indica* leaf extract, which was found to be either through significant blockage of the inhibitory effect of serotonin on insulin secretion mediated by glucose or peripheral utilization of glucose and glycogenolic effect due to epinephrine action blockage, was observed to be statistically higher than that of *Catharanthus roseus*, *Gymnema sylvestre* and *Ocimum sanctum*. Because *A. indica* extract

exhibited antiperoxidative, hypoglycaemic and cortisol lowering activities, Gholap and Kar (2004) suggested that it may potentially regulate corticosteroid-induced diabetes mellitus. Also, in polyherbal preparation as an ayurvedic herbomineral formulation called hyponidd, oral administration of *A. indica* at 100 and 200 mg/kg bw for 45 days did not only cause decreased levels of glycosylated hemoglobin, plasma thiobarbituric acid reactive substances, hydroperoxides, but also elevated the levels of endogenous antioxidants, especially, plasma reduced glutathione and vitamin C (Babu and Prince 2004) in streptozotocin-induced diabetic animals.

### 2.2.12 Immunological properties

There are several reports in literature to suggest that neem products have significant modulating effect on the humoral and cell-mediated immune system. Upadhyay *et al* (1992) studied the immunomodulatory effects of neem oil in mice following a single intraperitoneal dose. They observed that neem oil increased level of leukocytic cells, enhanced phagocytic activity of peritoneal macrophages and expression of MHC class II antigens, as well as induced production of gamma interferon. This led them to conclude that neem oil acts as a non-specific immunostimulants that selectively activates the cell-mediated immune mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge. Other workers have exploited the immune-modulatory properties of neem for immune-contraception in rats and monkeys by using single intrauterine application of neem oil and neem seed hexane extracts (Garg *et al* 1994). This immunocontraceptive activity has been ascribed to a fraction of hexane seed extracts made of six components consisting of saturated, mono- and di-unsaturated free fatty acids and their methylesters (Garg *et al* 1998).

## 2.3 TOXICOLOGICAL AFFECTS OF NEEM (*Azadirachta indica)* PRODUCTS

The neem tree, *Azadirachta indica*, provides many useful compounds that are used as pesticides and could be applied to protect stored seeds against insects. However in addition to possible beneficial health effects, such as blood sugar lowering properties, anti-parasitic, anti-inflammatory, antiulcer and hepatoprotective effects and also toxic effects are described. Boeke *et al* (2004) noted that the nonaqueous extracts appear to be the most toxic neembased products, with an estimated safe dose (ESD) of 0.002 and 12.5 µg/kg bw, while the unprocessed seed oil and the aqueous extracts with ESD of 0.26–0.3 mg/kg bw and 2 µl/1kg bw, respectively, were less toxic. Furthermore, they observed that most of the pure compounds show a relatively low toxicity (ESD azadirachtin 15 mg/kg bw), while for all preparations, reversible effect on reproduction of both male and female mammals seem to be the most important toxic effects upon sub-acute or chronic exposure. Hence, based on review of available data on safety assessments for the various neem derived preparations, they

concluded that, if applied with care, use of neem-derived products as insecticide should not be discouraged.

### 2.3.1 Toxicity in rodents

Acute toxicity studies of neem seed oil established the 24 h $LD_{50}$ to be 14 ml/kg bw in rats and 24 ml/kg bw in rabbits. Prior to death, animals of both species and sexes exhibited comparable pharmacotoxicological symptoms, with lungs and central nervous system as the main target organ of toxicity (Gandhi *et al* 1988). The genotoxicity of neem and neem products has been reported in rodents. Oral administration of a soxhleted crude ethanolic extract of neem leaves to adult male mice for 6 weeks at a rate of 0.5, 1.0 and 2.0 g/kg bw, increased the incidences of structural changes and synaptic disturbances in meiotic chromosomes, and also caused disruption of meiosis, resulting in reduced sperm count, increased frequency of spermatozoa with abnormal head morphology and chromosome strand breakages or spindle disturbances (Awasthy 2001).

Rahman and Siddiqui (2004) studied the biochemical effects of Vapacide a neem based pesticide, administered orally in coconut oil at doses of 80, 160 and 320 mg/kg bw for 45–90 days. They observed that exposure to Vapacide resulted in a significant but reversible increases in acid phosphatase, alkaline phosphatase in serum, kidney, lung and liver tissue (alkaline phosphatase only in liver), which was accompanied with a significant decrease of acid phosphatase in the liver of male and female rats after 45 and 90 days of moderate to high doses, with the lung tissues being the most susceptible and kidney the least susceptible. They attributed the decrease in liver acid phosphatase to the necrosis of cellular tissues, and suggested that these enzyme activities could be useful as biomarkers of exposure to Vepacide. Similar conclusions were reached when aspartate and alanine aminotranferases profiles were established in rats on sub-chronic doses of Vapacide (Rahman *et al* 2001).

### 2.3.2 Toxicity in aquatic animals

In studies on aquatic organisms, 100 per cent mortality among three snail intermediate host species, *Biomphlaria pfeifferi*, *Bulinus truncates* and *Lymnaaecea natalensis* after 24 h exposure to freeze-dried aqueous or ethanolic extract of *A. indica* at 100 mg/l has been reported. Two aquatic crustaceans namely, *Daphnia magna*, *Hyalella azteca* and a dipteran, *Chironomus riparius* have also been found to be susceptible to the toxic effects of Margosan O a neem seed kernel-based insecticide registered in the United States, at concentrations as low as 10 mg/l (Scott and Kaushik 1998). Other workers (El-Shazly and El-Sharnoubi 2000) have also reported varying toxic effects of Neemazal T/S, another neem-based insecticide on various developmental stages of different classes of aquatic organisms,

including *Bufo regularis* (Amphibia), *Aedes caspius* (Insecta), *Gambusia affinis* (Poecilliidae), *Cyclops sp.* and *Daphnia magna* (Crustacea) at concentrations of 10–20 ppm.

### 2.3.3 Toxicity in ruminants

In a comprehensive study involving oral administration of aqueous leaf extract of *A. indica* at doses of 50 and 200 mg/kg bw for 8 weeks to goats and guinea pigs. Ali (1987) observed a progressive decrease in body weight, heart pulse and respiratory rates, weakness, inappetence and diarrhea. It was further observed that goats on higher doses of the plant leaves produced tremors and ataxia during the last few days of treatment, but no statistically significant hematological changes were observed after dosing the animals with *A. Indica* leaves, although there was a tendency toward lowered erythrocyte counts, packed cell volume and haemoglobin concentration. The treatments also caused significant rises in the plasma activity of aspartate transferase, sorbitol dehydrogenase, and concentrations of cholesterol, urea, creatinine and potassium, but no significant changes in the plasma concentration of sodium, chloride or bilirubin were detected. They further reported areas of hemorrhagic erosions, flappy hearts and hydropericardium, while histopathologically; there was evidence of various degrees of hemorrhage, congestion and degeneration in the liver, kidney, lung, duodenum, brain and seminiferous tubules.

### 2.3.4 Toxicity in poultry

After feeding Brown Hisex chicks with 2 and 5 per cent *A. indica* leaf supplemented diet for 28 days, Ibrahim *et al* (1994) reported a depression in body weight gain and efficiency of feed utilization accompanied with clinicopatholological changes such as increase in activities/levels of lactic dehydrogenase, glutamic oxaloacetic transaminase, alkaline phosphatase, uric acid and billirubin concentrations with concomitant decreases in serum total protein. They also observed significant changes in the values of erythrocyte count, hemoglobin concentration, packed cell volume, mean corpuscular volume and mean corpuscular haemoglobin concentration associated with yellow discoloration of the legs and combs and hepatonephropathy.

### 2.3.5 Toxicity in humans

Boral *et al* (2004) have reported the allergizing potential of *A. indica* pollen among an Indian community. Two immunoglobulin reactive proteins have been isolated and characterized from *A. Indica* pollen, and their allergenicity confirmed by skin prick test and Immunoblot Assay (Karmakar and Chatterjee 1994).

**Chapter III**

## MATERIALS AND METHODS

This chapter provides information regarding the experimental procedures employed during the course of investigation. The various chemicals used, methods employed for isolation of neem seed kernel extract using different solvents, rearing of *Tribolium castaneum*, testing of bioefficacy of different extracts against *T. castaneum*, thin layer chromatography of different extracts and column chromatography of hexane extract of neem seed kernels is included in this chapter. All the melting points were determined in open capillaries, on a Büchi B-545 melting point apparatus. IR spectra were measured in $CHCl_3$ solution or nujol on a Perkin Elmer, Model RX-1 FT-IR spectrophotometer. $^1H$ NMR spectra were recorded with Bruker AC (400 MHz) or mentioned otherwise as solutions (in $CDCl_3$) using TMS as an internal reference. The IR and $^1H$ NMR spectroscopic analysis was obtained from Central Instrumentation Laboratories (CIL), Panjab University, Chandigarh. The chemical shifts are expressed in δ (ppm) values and the abbreviations 's', 'brs', 'd', 't' and 'm' stand for singlet, broad singlet, doublet, triplet and multiplet respectively. The molecular masses of the compounds were confirmed by LCMS/MS (WATERS 2489) Department of Chemistry, PAU, Ludhiana.

### 3.1 MATERIALS

**3.1.1 Neem Seed Kernels Extracts -** were obtained from dry powdered neem seed kernels.

The following adsorbents were used for chromatographic separation-

**3.1.2 Silica Gel**

| | | | |
|---|---|---|---|
| i. | Silica gel for column chromatography | : | Qualigens fine chemicals, Mumbai. |
| | Pore size | : | 60-120 mesh |
| | pH (10 per cent aqueous suspention) | : | 7 |
| | Activity according to Brockman and Schodder | : | 2-3 |
| | Chloride max | : | 0.02 per cent |
| | Iron max | : | 0.03 per cent |
| ii. | Silica gel for thin layer chromatography | : | Qualigens fine chemicals, Mumbai. |
| | Calcium sulphate | : | 13 per cent |

### 3.1.3 Various Reagents Used

i. Acetone – Loba Chemie Pvt. Ltd, Mumbai.
ii. Benzene - Glaxo Laboratories Ltd, Mumbai.
iii. Chloroform - Thermo Electron Pvt. Ltd, Mumbai.
iv. Dichloromethane – S.D. Fine Chemicals Ltd, Mumbai.
V. Ethanol – Bengal Chemicals Ltd, Kolkata.
vi. Ethyl acetate – Samir Tech-Chem Pvt. Ltd, Vadodara.
vii. Methanol - Samir Tech-Chem Pvt. Ltd, Vadodara.
viii. Petroleum ether - Thermo Electron Pvt. Ltd, Mumbai.
ix. Sulfuric acid - S.D. Fine Chemicals Ltd, Mumbai.
x. Vanillin - S.D. Fine Chemicals Ltd, Mumbai.

### 3.1.4 Apparatus

Apart from the common laboratories glassware and apparatus, the following specific equipments were used:

1. Glass columns
(1.5 cm id x 40 cm long) with a narrow tip drawn at bottom (locally fabricated)
2. Thin layer chromatographic equipment

i. TLC plates 20 x 20 cm glass plates.
ii. Slurry applicator (Perfit, Ambala, India)
iii. Development tank (Kontes, USA)

3. Soxhlet apparatus.
4. Rotary vacuum pump.
5. Electrical grinder.

## 3.2 EXTRACTION OF NEEM SEED KERNELS EXTRACTS

The extracts were extracted from seed kernels of neem (*A. indica*). The seeds of neem collected from PAU campus were depulped and air dried. The air dried seed kernels were powdered and extraction was done by Soxhlet extraction method. The extracts were extracted from powdered plant material using different solvents such as chloroform, hexane and ethanol for 48 hrs. The extracts so obtained were distilled to yield dense oily liquid. The oil extraction was carried out in number of batches.

## 3.3 ANALYTICAL TECHNIQUES

### 3.3.1 Chromatographic Techniques

Since chromatography is the commonly used method for the isolation and purification of compounds of interest from a mixture. The brief description of various chromatographic techniques used during the work is as follows:

#### 3.3.1.1 Column Chromatography (CC)

Column chromatography involves the separation of compounds from a mixture by eluting the column with solvent of increasing polarity in a step wise manner and the collection of fractions according to the sequence regarding the eluted products being monitored by TLC. Column was packed with silica gel for column chromatography with 60-120 mesh size activated at $110^0$ C for 1 hr. Column clean up was carried out using hexane as the solvent. The material to be chromatographed was dissolved in small quantity of hexane and the solution was allowed to flow down a vertical column. The extraction was carried out by eluting the column with solvent of increasing polarity and the various fractions were collected. For the recovery of the material the solvent was distilled using rotary vacuum pump.

#### 3.3.1.2 Thin Layer Chromatography (TLC)

Chromatography denotes a procedure in which a solution of substance to be separated is passed in a direction determined by the arrangement of the apparatus (bottom to top in case of TLC) over more or less finely divided insoluble organic or inorganic solid resulting in the retention of the individual components to different extent. The underlying mechanism is the partitioning of the moving compounds between the liquid phase and also their being reversibly bound to the surface of the adsorbent. The amount transferred to the solvent will be a function of the distribution of the compound, least strongly adsorbed will be in the highest concentration.

Out of various adsorbents available, the most commonly used is silica gel G. The wide applicability is due to the fact that their adsorbing power towards various classes of compounds can be altered by pre-treatment. The rate of migration of compound on a given adsorbent depends upon the solvent used. The solvent in order of their increasing polarity (increasing eluting powers) are:

Petroleum ether < cyclohexane < carbon tetrachloride < toluene < benzene < dichloromethane < chloroform < ether < acetone < alcohol.

### 3.3.1.3 Preparation of Thin Layer Chromatographic Plates

The silica gel G was dissolved in water to prepare slurry (10 g of silica gel/100 mL of water). Chromatoplates 20 x 20 cm were coated with slurry with the aid of an applicator, giving 0.25 mm thickness. The chromatoplates were air dried for about 4 hrs at room temperature and finally activated at $120^0$ C for 45 min. The chromatoplates were used after cooling for 10-15 min. The spotting of plates was done with the help of capillary tubes. The spot was applied 1 cm upward from the lower end of chromatoplate. After the initial spotting on the stationary phase, the chromatoplate was placed inside the developing chamber and mobile phase is allowed to run up the plate. When the chromatoplate was fully developed that was when the mobile phase reached the upper end of the chromatoplate. The chromatoplate was removed from the developing chamber and was air dried. When the chromatoplate was fully dried, the spots were visualised by spraying reactive spray reagents. The plates after spraying immediately placed in the oven maintained at $110^0$ C for 5-10 min. The advantage of TLC is its ability to detect a wide range of compounds using reactive spray reagents.

### 3.3.1.4 Spray Reagents

The TLC plates were developed in suitable solvents like hexane: ethyl acetate or benzene: ethyl acetate in different ratios and visualization of spots was done by spraying the plates with vanillin or methanol: sulfuric acid as the spray reagents.

**a) Vanillin spray reagent:** Methanol (9 mL), Vanillin (0.5 g), glacial acetic acid (0.5 mL), concentrated sulfuric acid (3 drops).

**b) Methanol**: **sulfuric acid spray reagent:** 95 mL methanol and 5 mL concentrated sulfuric acid.

## 3.4 BIOEFFICACY STUDIES

### 3.4.1 Test Insects

Rust red flour beetle – *Tribolium castaneum*

### 3.4.2 Experimental Grains

The wheat grains (*Triticum aestivum* variety PBW 343, moisture content 11.0 per cent) used throughout these studies were obtained from Department of Plant Breeding and Genetics, PAU, Ludhiana.

### 3.4.3 Rearing and Handling of Test Insects

Adults were collected from the local grain market and were identified by Dr. (Mrs.) B.K. Kang, Associate Professor, Department of Entomology. The adults were released in glass jar (10 × 15 cm) containing wheat flour mixed with 5 per cent yeast powder. Before culturing, the flour was kept at $60\pm1^0$ C in oven for 2 hrs to eliminate contamination of other organisms. The culture jars were placed in incubators maintained at $30\pm1^0$ C and 70±1 per cent relative humidity. After seven days of oviposition period, the adults were removed and the eggs were allowed to develop to the pupae stage. The pupae were shifted from the flour with a 50 mesh sieve and put in small glass jars (5 × 10 cm) containing wheat flour plus yeast. From, these jars, the adults of known age (1-2 weeks) i.e $F_1$ generation were obtained for the experimental purposes. The average weight of 100 freshly emerged adults was 190 mg.

### 3.4.4 Testing the Bioefficacy of different Extracts

The bioefficacy study of different extracts against *T. castaneum* adults was carried out using $F_1$ progeny. For the experiment, wheat grains (20 g) were taken in a bottle. Wheat grains were spiked with different concentrations (viz. 20,000, 15,000, 10,000, 5,000 and 1000 µg $g^{-1}$) of various neem seed kernels extracts. The bottles were put in electric shaker for 5 min to enable thorough mixing of extract with wheat grains. Ten adults of same age were released into each bottle and mouth of bottle was covered with muslin. The observation of mortality of *T. castaneum* was taken after every 24 hrs till complete or constant mortality was obtained. The observation of appearance of larvae and larval growth were also taken out. The corrected per cent mortality was calculated using Abbott's formula.

$$\text{Corrected Per cent mortality} = \frac{\text{Per cent mortality in treated - Per cent mortality in control}}{\text{100 - Per cent mortality in control}} \text{ x } 100$$

### 3.5 TEST PLANT

Neem **(***Azadirachta indica*)

**Classification**

| | | |
|---|---|---|
| Order | : | Rutales |
| Suborder | : | Rutinae |
| Family | : | Meliaceae |
| Subfamily | : | Melioideae |
| Tribe | : | Melieae |
| Genus | : | *Azadirachta* |
| Species | : | *indica* |

*Azadirachta indica* is a small to medium-sized tree, leaves alternate, crowded near the end of branches, simply pinnate, 20-40 cm long, exstipulate, light green, with two pairs of glands at the base, petiole 2-7 cm long, inflorescence an axillary, flowers bisexual, actinomorphic, small, pentamerous, white or pale yellow, slightly sweet scented, calyx lobes imbricate, petals free, imbricate, fruit one seeded drupe, ellipsoidal, 1-2 cm long, greenish, greenishyellow to yellow or purple when ripe, exocarp thin, mesocarp pulpy, endocarp cartilaginous, seed ovoid or spherical, apex pointed, testa thin, composed of a shell and a kernel (sometimes 2 or 3 kernels), each about half of the seed's weight. Seeds of neem collected from PAU campus were depulped and were air dried. The dried kernels were crushed and powered using electric grinder.

### 3.5.1 Extraction of Neem Seed Kernels Extracts

The different extracts were extracted from seed kernels of neem using different solvents viz. ethanol, chloroform and hexane. The air dried seed kernels of neem collected from PAU campus were depulped, powdered and extraction was done using Soxhlet extraction method. 400.0 g of powdered plant material was extracted with 1.0 L of chloroform, hexane and ethanol as solvent for 48 hrs. The extracts so obtained were distilled to yield dense oily liquid. Chloroform, hexane and ethanol extract on distillation yield 125.0, 92.0 and 54.0 g of dense oily liquid respectively. The extracts extraction was further carried out two times using the same amount of plant material and solvents.

### 3.5.2 Partitioning of Hexane Extract

20.0 g of hexane extract of neem seed kernels containing fatty acid was transferred into 250 mL separating funnel. The oil was partioned thrice using, 100, 50 and 50 mL of ethanol. The fatty acid was insoluble in ethanol settled down in the separating funnel and was separated. The yield of ethanol insoluble hexane fraction (Fatty acid) was 8.0 g where as the ethanol soluble part remaining in the separating funnel was collected and distilled. This was yielded 10.0 g of dense oily liquid.

### 3.5.3 Thin Layer Chromatography of different Extracts of Neem Seed Kernels

The different extracts extracted from the neem seed kernels were dissolved in acetone and applied 1 cm from the base on thin layer chromatoplates separately. The chromtoplates were developed for 30 min using benzene: ethyl acetate (95: 5, v: v) as the solvent system in the developing chamber. The chromatoplates were removed from the developing chamber and air dried. The spots were visualised by spraying methanol: sulfuric acid as the spray reagent.

The plates after spraying were placed in the oven maintained at $110^0$ C. Following results were reported for different extracts:

**Chloroform extract:** Six brown coloured spots were observed having $R_f$ values of 0.27, 0.35, 0.42, 0.58, 0.8 and 0.97.

**IR spectral data of chloroform extract**

$\nu_{max}$ ($cm^{-1}$): 3007, 2924, 2853, 2678, 1745, 1712, 1465, 1417, 1378, 1239, 1215, 1165, 1118, 1097, 1056, 1030, 759, 722, 668 and 602.

**Ethanol extract:** Five brown coloured spots were observed having $R_f$ values of 0.27, 0.33, 0.39, 0.83 and 0.93.

**IR spectral data of ethanol extract**

$\nu_{max}$ ($cm^{-1}$): 3418, 3005, 2924, 2854, 2677, 1739, 1713, 1464, 1417, 1378, 1241, 1168, 1118, 1089, 1049, 968, 879, 723, 601 and 461.

**Hexane extract:** Five brown coloured spots having $R_f$ values of 0.37, 0.47, 0.63, 0.83 and 0.95 were observed.

**IR spectral data of hexane extract**

$\nu_{max}$ ($cm^{-1}$): 3006, 2925, 2854, 2677, 1746, 1712, 1465, 1417, 1378, 1240, 1165, 1118, 1098, 1031, 952, 722 and 603.

**Ethanol soluble hexane fraction:** Five brown coloured spots having $R_f$ values of 0.33, 0.43, 0.59, 0.77 and 0.96 were observed.

**IR spectral data of ethanol soluble hexane fraction**

$\nu_{max}$ ($cm^{-1}$): 3430, 3006, 2924, 2854, 2676, 1741, 1713, 1463, 1404, 1378, 1241, 1168, 1119, 1091, 1049, 941, 880, 721, 602 and 461.

**Ethanol insoluble hexane fraction:** one brown coloured sport was observed having $R_f$ values of 0.53.

**IR spectral data of ethanol insoluble hexane fraction**

$\nu_{max}$ ($cm^{-1}$): 3468, 3180, 3063, 2966, 2889, 1899, 1627, 1612, 1577, 1487, 1463, 1446, 1427, 1400, 1386, 1371, 1354, 1309, 1298, 1274, 1236, 1220, 1198, 1129, 1099, 1088, 1001, 955, 931, 896, 822, 751, 723, 677, 598, 514, 454 and 426.

### 3.5.4 Preparation of Standards of different Neem Seed Kernels Extracts

**Standard A (2, 00,000 µg $g^{-1}$)**: 10 g of the test extract was dissolved in acetone and volume was made to 50 mL this was designated as A.

**Standard** B **(20,000 µg $g^{-1}$):** 1 mL of A was taken and dissolved in acetone and the total volume was made to 10 mL this designated as B.

### 3.5.5 Testing the Bioefficacy of different Extracts

The bioefficacy study of different neem seed kernels extracts against *T. castaneum* adults was carried out using $F_1$ progeny. For the experiment, wheat grains (20 g) were taken in a bottle. Wheat was spiked with different concentrations i.e. 20,000, 15,000, 10,000, 5,000 and 1000 µg $g^{-1}$ of different neem seed kernels extracts using standard A and B (Table 1). There were three replications for each treatment and for control treatment, only wheat and acetone was used. The bottles were put in electric shaker for 5 minutes to enable thorough mixing of extract with wheat grains. Ten adults of same age were released into each bottle and mouth of bottle was covered with muslin. The observations of mortality of *T. castaneum* adults were taken after every 24 hrs till complete or constant mortality was obtained. The observation of appearance of larvae and larval growth were also taken out for 45 days. The corrected per cent mortality was calculated using Abbott's formula.

**Table 1 Spiking of wheat at different concentrations using different neem seed kernels extracts using standard A (2, 00,000 µg $g^{-1}$) and B (20,000 µg $g^{-1}$) of the respective test extracts**

| Sr. No. | Spiking level (µg $g^{-1}$) | Weight of wheat grains taken (g) | Volume of standard used (mL) | Volume of acetone used (mL) |
|---|---|---|---|---|
| 1 | 20,000 | 20 | 2.0 (A) | 0.0 |
| 2 | 15,000 | 20 | 1.5 (A) | 0.5 |
| 3 | 10,000 | 20 | 1.0 (A) | 1.0 |
| 4 | 5,000 | 20 | 0.5 (A) | 1.5 |
| 5 | 1,000 | 20 | 1.0 (B) | 1.0 |
| 6 | control | 20 | - | 2.0 |

### 3.5.6 Chromatography of Hexane Extract of Neem Seed Kernels

The separation of hexane extract into various fractions was achieved by extensive column chromatography. Hexane extract (5 g) was dissolved in hexane (5 mL) and was absorbed on the silica gel within the column (180 g). The column was eluted using hexane,

hexane: dichloromethane (1:1, v: v) and dichloromethane as solvents in order of increasing polarity. The various fractions were collected. For the recovery of the material the solvent was distilled using rotary vacuum pump. The result of chromatography is presented in the table 2.

**Table 2 Chromatography of hexane extract of neem seed kernels over silica gel**

| Sr. No. | Eluent (mL) | Weight (g) | TLC based remarks |
|---|---|---|---|
| 1. | Pure hexane (5 x 100) | 2.1 | Mixture |
| 2. | Hexane and dichloromethane 1:1 (7 x 100) | 1.7 | Mixture |
| 3. | Dichloromethane (5 x 100) | 0.9 | Mixture |

### 3.5.7 Thin Layer Chromatography of different Fractions of Hexane Extract of Neem Seed Kernels

The fractions obtained from the hexane extract of neem seed kernels was dissolved in acetone and applied 1 cm from the base on thin layer chromatoplates. The chromtoplates were developed for 30 minutes using benzene: ethyl acetate (95: 5, v: v) as the solvent system in the developing chamber. The chromatoplates were removed from the developing chamber and air dried. The spots were visualised by spraying methanol: sulfuric acid (95: 5, v: v) as the spray reagent. The plates after spraying were placed in the oven maintained at $110^0$C. Hexane fraction I showed three spots on TLC plate, having $R_f$ values of 0.22, 0.83 and 0.92. Fraction II showed three spots on TLC plate, having $R_f$ values of 0.18, 0.66 and 0.84. Fraction III showed only two spots on TLC plate, having $R_f$ values of 0.24 and 0.98.

**Spectral data of fraction 1**

IR $\nu_{max}$ ($cm^{-1}$): 3477, 3006, 2925, 2854, 1747, 1655, 1466, 1418, 1378, 1238, 1164, 1118, 1099 and 588.

$^1$H NMR signals ($CDCl_3$, 400 MHz) of fraction 1 $\delta$ at**:** 0.81 (m), 1.19 (t), 1.54 (q), 1.95 (m), 2.24 (m), 4.07 (q), 4.23 (q) and 5.27 (m).

**Spectral data of fraction 2**

IR $\nu_{max}$ ($cm^{-1}$): 3369, 3006, 2925, 2854, 1746, 1655, 1588, 1465, 1418, 1378, 1239, 1164, 1118, 1098, 799 and 722.

$^{1}$H NMR signals ($CDCl_3$, 400 MHz) of fraction 1 δ at**:** 0.81 (m), 1.21 (q), 1.54 (t), 1.95 (m), 2.24 (m), 4.07 (q), 4.22 (q) and 5.27 (m).

**Spectral data of fraction 3**

IR $\nu_{max}$ ($cm^{-1}$): 2922, 2852, 2678, 1712, 1581, 1462, 1404, 1378, 1342, 1263, 1167, 1120, 1096, 1075, 1039, 1022, 915, 892, 876, 799, 765, 729, 720, 646, 567, 449, 409, and 588.

$^{1}$H NMR signals ($CDCl_3$, 400 MHz) of fraction 1 δ at**:** 0.82 (m), 1.18 (q), 1.34 (m), 1.56 (m), 1.93 (t), 2.25 (m), 4.15 (t), 5.27 (m), 7.46 (q) and 7.64 (q)

**CHAPTER- IV**

## RESULTS AND DISCUSSION

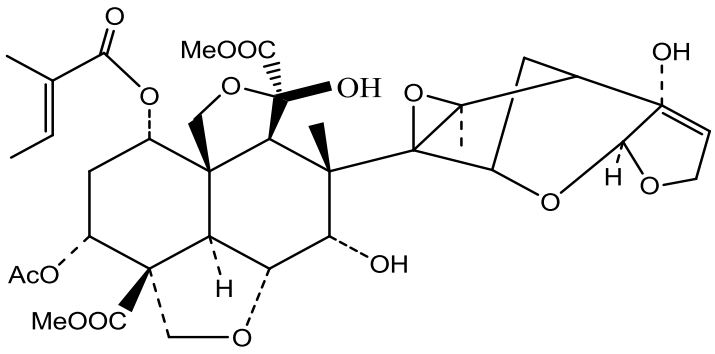
The chemistry of fixed and essential oils is very complex. These oils are consists of various organic constituents and the primary constituents are the terpenoids, oxygenated compounds like alcohols, phenols, aldehydes, ketones, lactones, coumarins, oxides, fatty acid and their derivatives. All naturally occurring terpenoids are made up of isoprene units ($C_5H_8$) linked together head to tail where the branched side of isoprene unit is head and the other end is tail (Ruzicka 1953).

Insects are the major pest of cereal grains or flour during storage. Many pests are the coleopterans and the most destructive tropical species for cereals belong to the genus Sitophilus and Tribolium. Although insecticides or fumigants are used commonly to protect food grains but such practices pose health risks unless the chemicals used are non-hazardous to mammals. Therefore use of essential oils as stored grain protectant is gaining popularity. Fixed or essential oils are often specific and biodegradable having low persistence. They have also been considered as potential pest control agents because they are highly toxic, prevent oviposition and cause egg mortality (Shukla *et al* 2009), possess repellent (Palsson and Jaenson 1999) and antifeedant properties (Tripathi *et al* 2002).

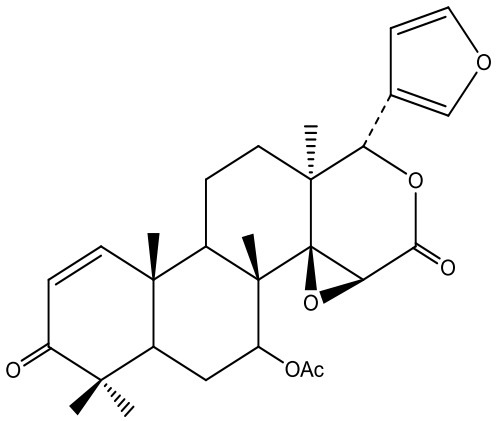
The neem oil was isolated from dried and powdered seed kernels with ethanol, chloroform and hexane using Soxhlet apparatus. The thin layer chromatography of chloroform, ethanol and hexane extracts showed 6, 5 and 5 brown colored spots respectively when visualized by using methanol: sulfuric acid (95: 5, v: v) spray reagent. The ethanol extract showed five brown colored spots having $R_f$ values of 0.27, 0.33, 0.39, 0.83 and 0.93. The chloroform extract showed six brown colored spots having $R_f$ values of 0.27, 0.35, 0.42, 0.58, 0.8 and 0.97 whereas hexane extract showed five brown colored spots having $R_f$ values of 0.37, 0.47, 0.63, 0.83 and 0.95.

The IR data of ethanol and chloroform extract and the study of literature indicated the presence of azadirachtin (1), gedunin (2), mahmoodin (3), nimbin (4) and salannin (5). These compounds form the major part of ethanol and chloroform extract. The biological activity of these extract was attributed to the presence of these active secondary metabolites. The IR data of ethanol extract showed band at 3418 $cm^{-1}$ indicating the presence of hydroxy group, band at 1739 $cm^{-1}$ indicating the presence of carbonyl group and at 1713 $cm^{-1}$ indicating the presence of α, β unsaturated ketone. Bands are also observed at 2924, 2854, 1463 (methlene group) and 1377 $cm^{-1}$ (gem dimethyl). The IR data of chloroform extract showed band at 1744 $cm^{-1}$ indicating the presence of carbonyl group and at 1712 $cm^{-1}$ indicating the presence

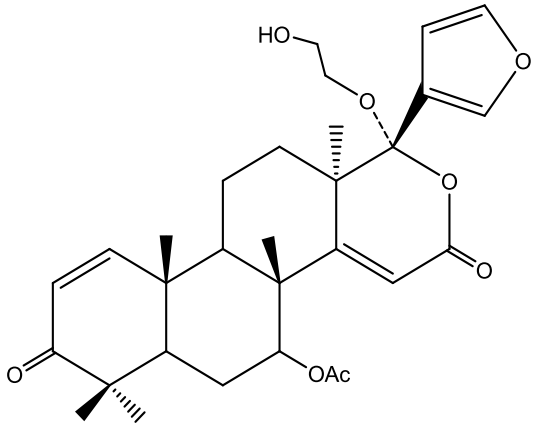
of carbonyl of α, β unsaturated esters. The other bands observed at 2924, 2854, 1464 and 1377 $cm^{-1}$ indicate the presence of methylene and gem- dimethyl group.



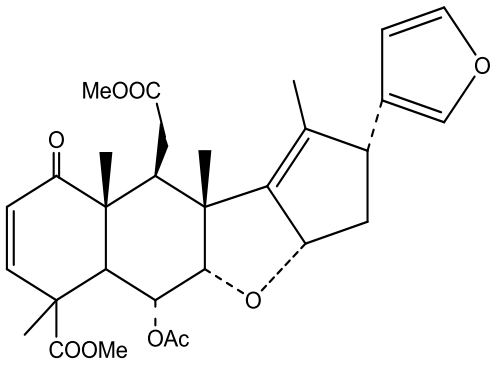

**(1)**



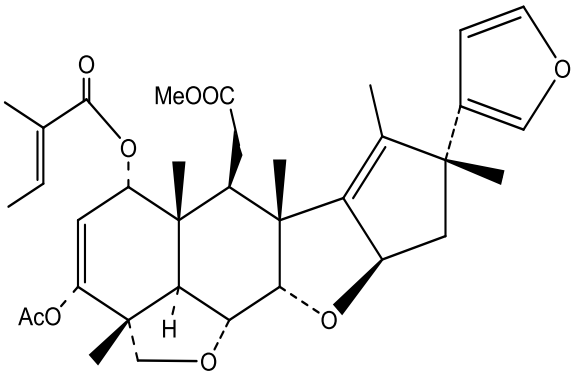

**(2)**




**(3)**



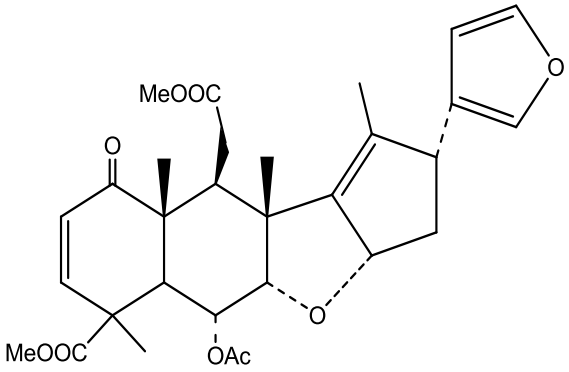

**(4)**



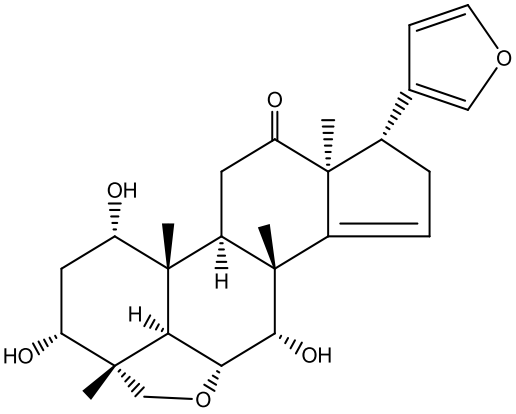

**(5)**

The biological activity of ethanol, chloroform extract and the compounds present in them is known so we became interested in hexane extract for its biological activity. The

ethanol, chaloroform and hexane extracts were tested for their bioefficacy against *Tribolium castaneum* adults. Hexane extract was further partitioned with ethanol to give ethanol soluble and ethanol insoluble fractions. The chemistry and bioefficacy of these two fractions were under taken to know their effect against *T. castaneum*. The IR data of original hexane extract showed band at 1746 $cm^{-1}$ indicating the presence of carbonyl group and at 1712 $cm^{-1}$ indicating the presence of carbonyl of α, β unsaturated esters. The other bands observed at 2925, 2854, 1465 and 1378 $cm^{-1}$ indicate the presence of methylene and gem- dimethyl group. The IR data of ethanol insoluble fraction showed bands at 3468, 3179, 2966, 2889, 1695, 1626,1463, 1274 and 896 $cm^{-1}$. This data shows the presence of hydroxyl group, carbonyl, double bond and methlene and groups in the compounds present in this fraction which are mainly esters of various fatty acids. Study of IR spectra of ethanol soluble and original ethanol fraction revealed that these two fractions are almost identical thereby proving that the polar compounds have been removed from original hexane fraction during its partitioning with ethanol. The study of IR data and literature indicates that nimbin (4), epinimbin (6), nimbidinin (7) and their derivatives are present in ethanol soluble extract. The IR data of ethanol soluble fraction and original ethanol fraction showed prominent band at 3430, 2924, 2854, 1741, 1713 and 1377 $cm^{-1}$ which may be attributed to the presence of compounds (1-7) mentioned above.




**(6)**




**(7)**

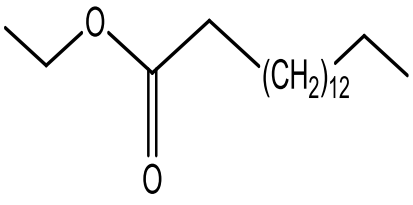
The column chromatography of hexane extract yielded three fractions with increasing polarity solvents such as hexane, hexane: dichloromethane (1: 1, v: v) and dichloromethane. The IR and NMR study of these fractions and their comparison with literature showed the presence of fatty acid, hydroxy fatty acid and their ester. The IR data of fraction 1 showed the hydroxyl band at 3471 $cm^{-1}$, two sharp bands at 2925 and 2854 $cm^{-1}$ which were attributed to the asymmetric $CH_2$ stretch and the symmetric $CH_2$ stretch respectively. The intense band at 1747 $cm^{-1}$ was derived from the existence of the C = O stretch and the band at 1238 $cm^{-1}$ exhibited the presence of the C–O stretch. The O–H in-plane and out-of-plane bands appeared

at 1466 and 975 $cm^{-1}$ respectively. IR data of fraction 2 showed the hydroxyl band at 3369 $cm^{-1}$, two sharp bands at 2925 and 2854 $cm^{-1}$ were attributed to the asymmetric $CH_2$ stretch and the symmetric $CH_2$ stretch respectively. The intense band at 1746 $cm^{-1}$ was derived from the existence of the C = O stretch and the band at 1239 $cm^{-1}$ exhibited the presence of the C–O stretch. The O–H in-plane and out-of-plane bands appeared at 1465 and 965 $cm^{-1}$ respectively. Similarly Fraction 3 showed IR bands at 2922 and 2853 $cm^{-1}$ were attributed to the asymmetric $CH_2$ stretch and the symmetric $CH_2$ stretch respectively. The intense band at 1712 $cm^{-1}$ was drived from the existence of the C = O stretch and the band at 1262 $cm^{-1}$ exhibited the presence of the C–O stretch. A band at 1581 $cm^{-1}$ was due to the presence of double bond. This data showed the probable presence of ethyl palmitate (8), oleic acid (9), ethyl oleate (10), linoleic acid (11), ethyl linoleate (12) and arachidonic acid (13).

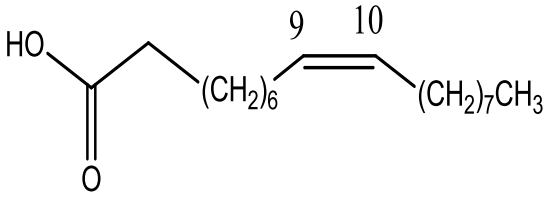
The presence of compounds 8 to 13 as components of neem oil was confirmed by NMR spectral study. The structure 10 was confirmed by NMR. Triplet at δ 0.81 and quadrate at δ 4.07 is due to the presence of $CH_3$-$CH_2$-OCO-. The signal due to olefinic protons appears at δ 5.27. So this supports the presence of ethyl oleate in the hexane fraction as major component. The presence of triglycerides was confirmed by the signals at δ 1.95 and 2.24 which are due to –$CH_2$-COOR.

The position and shape of signal at δ 5.27 may be attributed to the unsaturation present in linoleic acid (11) and hence this may also be present in hexane fraction as such or in its ester form. So it is not possible to distinguished the 11 and 12 in the mixture because ethyl group is also present as ethyl ester of oleic and palmitic acids.

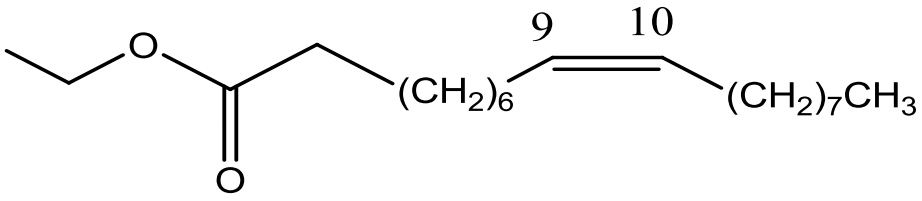
The presence of hydroxy fatty acid ester in the mixture is revealed by a typical signal of ›CH-OH at around δ 4.23. This has been confirmed by the study of NMR spectrum of 3-hydroxy octadecanoate and 17-hydroxy linoleic acid exhibits the signals at δ 4.0 and 4.08 respectively due to ›CH-OH. This showed the presence of various hydroxy esters of fatty acids in hexane fraction.




**(8)**

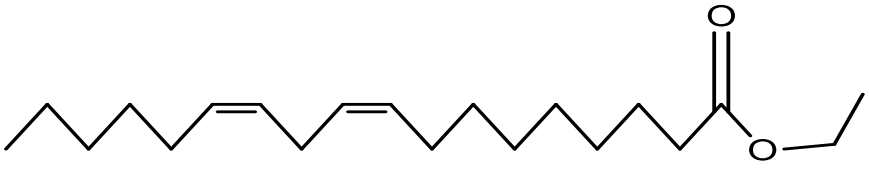



**(9)**

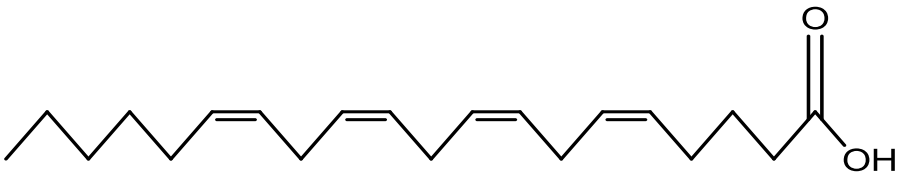



**(10)**


**(11)**




**(12)**




**(13)**

**4.1 BIOEFFICACY STUDIES**

Fixed and essential oils are secondary metabolites abundant in aromatic and medicinal plant families and contain a large number of compounds such as diterpenoids and triterpenoids. Essential oils of these plant families are tested for their potential as protective agents for human and livestock feeds. Many aromatic and medicinal plants are known to possess insecticidal activity, to repel ovipositing insect and reduced the progeny. Essential oils are known to exhibit low toxicity to mammals and the most terpenoids and phenols found in plant essential oils have minimal toxicity and have been approved as flavoring agents in food.

### 4.1.1 Bioefficacy of ethanol extract against *T. castaneum*

The control sample showed no mortality upto 9th day of application. The mortality of 10 per cent was observed on 10th day which was remained constant throughout the experiment. The subsequent corrected per cent mortality of ethanol extract obtained using Abbott's formula against *Tribolium castaneum* is shown in Table 3 and Fig 4.1. On first day of treatment, corrected per cent mortality of 10 was observed at 20,000 μg $g^{-1}$ concentration. Corrected per cent mortality of 60, 40 and 10 was observed at 20,000, 15,000 and 10,000 μg $g^{-1}$ concentrations respectively on 4th day. Corrected per cent mortality of fifty was achieved at 15,000 μg $g^{-1}$ on fifth day of application. On 7th day of treatment, corrected per cent mortality of 100 was achieved at 20,000 μg $g^{-1}$ concentration, whereas corrected per cent mortality of 80 and 20 was achieved at 15,000 and 10,000 μg $g^{-1}$ concentrations respectively. On eighth day of exposure, the corrected per cent mortality of 90, 20 and 10 was achieved at15, 000, 10,000 and 5,000 μg $g^{-1}$ concentration respectively. Complete corrected per cent mortality was achieved at 15,000 μg $g^{-1}$ after 9 days, whereas 10,000 and 5,000 μg $g^{-1}$ concentrations showed corrected per cent mortality of 20 and 10 respectively on same day of application. No mortality was observed in case of 5000 and 1000 μg $g^{-1}$ concentrations till 7 and 12 days of applications. At 10,000 and 5,000 μg $g^{-1}$ concentrations, respectively corrected per cent mortality of 33.3 and 11.1 was found after 12 days. Whereas at 10,000, 5,000 and 1000 μg $g^{-1}$ concentrations, respectively corrected per cent mortality of 55.6, 22.2 and 11.1 was observed after 14 days of treatment. Complete corrected per cent mortality was achieved at 10,000 μg $g^{-1}$ after 19 days whereas 5,000 and 1,000 μg $g^{-1}$ concentrations showed corrected per cent mortality of 66.7 and 11.1 respectively. The corrected per cent mortality was found to be 77.8 and 22.2 per cent at 5,000 and 1000 μg $g^{-1}$ concentrations respectively on 25th day of treatment. However, it was observed that corrected per cent mortality at 5,000 and 1000 μg $g^{-1}$ remained constant after 22 and 25 days of exposure and did not change further. This showed that compounds at these concentrations were not exerting their toxic influence on insects. These data showed that the corrected per cent mortality increased with increase in concentration of the ethanol extract. Satti *et al* (2010) reported that the mortality rates of water extract of neem seeds against *Trogoderma granarium* increased with increase in the concentration of water extract and also with time of application. It was found that at 20,000, 15,000 and 10,000 μg $g^{-1}$ concentrations the corrected per cent mortality increases with increase in time of application till the complete mortality was obtained whereas at 5,000, and 1000 μg $g^{-1}$ concentrations the corrected per cent mortality remain constant or increases slowly in the beginning, increases rapidly in the middle and then became constant as the number of days after application exceed 22 and 25 days. It can be concluded that 20,000,

15,000 and 10,000 μg $g^{-1}$ concentrations were efficient in controlling the infestation in 7, 9 and 19 days respectively. The best concentration was 20,000 μg $g^{-1}$ as complete mortality of *T. castaneum* adults was observed just on $7^{th}$ day of application. Moreover no larval growth was observed in this concentration.

### 4.1.2 Bioefficacy of chloroform extract against *T. castaneum*

The control sample showed no mortality upto $9^{th}$ day of application. The mortality of 10 per cent was observed on $10^{th}$ day which was remained constant throughout the experiment. The subsequent corrected per cent mortality of chloroform extract obtained using Abbott's formula against *Tribolium castaneum* adults is shown in Table 4 and Fig 4.2. On first and fourth day of treatment, corrected per cent mortality of 10 and 50 was observed at 20,000 μg $g^{-1}$ concentration. Corrected per cent mortality of 70, 40 and 10 was observed at 20,000, 15,000 and 10,000 μg $g^{-1}$ concentrations respectively after 5 days. After 7 days of treatment, corrected per cent mortality of 100 was achieved at 20,000 μg $g^{-1}$ concentration, whereas corrected per cent mortality of 70 and 20 was achieved at 15,000 and 10,000 μg $g^{-1}$ concentrations respectively. On ninth day of exposure, the corrected per cent mortality of 90, 30 and 10 was achieved at 15,000, 10,000 and 5,000 μg $g^{-1}$concentration respectively. Complete corrected per cent mortality was achieved at 15,000 μg $g^{-1}$ after 10 days whereas 10,000 and 5,000 μg $g^{-1}$ concentrations showed corrected per cent mortality of 33.3 and 11.1 respectively. At 10,000 and 5,000 μg $g^{-1}$ concentrations, respectively corrected per cent mortality of 55.5 and 22.2 was found after 12 days, whereas that of 88.9 and 44.4 was observed after 15 days of treatment. Complete corrected per cent mortality was achieved at 10,000 μg $g^{-1}$ after 17 days, whereas 5,000 and 1000 μg $g^{-1}$ concentrations showed corrected per cent mortality of 55.5 and 22.2 respectively. The corrected per cent mortality was found to be 66.7 per cent at 5,000 μg $g^{-1}$ concentrations after 19 days of treatment. In case of 1000 μg $g^{-1}$ concentration no mortality was observed till $13^{th}$ day of exposure. A low corrected per cent mortality of 11.1 was observed on $14^{th}$ day of application which increased to 22.2 on $17^{th}$ day respectively. It was observed that corrected per cent mortality at 5,000 and 1000 μg $g^{-1}$ remained constant after 19 and 17 days of exposure and did not change further. This showed that compounds at these concentrations were not exerting their toxic influence on insects. These data showed that the corrected per cent mortality increased with increase in concentration of chloroform extract. It was found that at 20,000, 15,000 and 10,000 μg $g^{-1}$ concentrations the corrected per cent mortality increases with increase in time of application till the complete mortality was obtained whereas at 5,000 and 1000 μg $g^{-1}$ concentrations the corrected per cent mortality remain constant or increases slowly in the beginning, increases rapidly in the middle and then became constant afterward.

**Table 3. Corrected per centage mortality of *T. castaneum* with ethanol extract.**

| Days of application | Concentrations | | | | |
|---|---|---|---|---|---|
| | 20,000 µg $g^{-1}$ | 15,000 µg $g^{-1}$ | 10,000 µg $g^{-1}$ | 5,000 µg $g^{-1}$ | 1,000 µg $g^{-1}$ |
| 1 | 10.0 | 0 | 0 | 0 | 0 |
| 2 | 30.0 | 10.0 | 0 | 0 | 0 |
| 3 | 50.0 | 20.0 | 0 | 0 | 0 |
| 4 | 60.0 | 40.0 | 10.0 | 0 | 0 |
| 5 | 80.0 | 50.0 | 10.0 | 0 | 0 |
| 6 | 90.0 | 60.0 | 10.0 | 0 | 0 |
| 7 | 100.0 | 80.0 | 20.0 | 0 | 0 |
| 8 | - | 90.0 | 20.0 | 10.0 | 0 |
| 9 | - | 100.0 | 20.0 | 10.0 | 0 |
| 10 | - | - | 22.2 | 11.1 | 0 |
| 11 | - | - | 22.2 | 11.1 | 0 |
| 12 | - | - | 33.3 | 11.1 | 0 |
| 14 | - | - | 55.6 | 22.2 | 11.1 |
| 15 | - | - | 66.7 | 33.3 | 11.1 |
| 17 | - | - | 88.9 | 44.5 | 11.1 |
| 19 | - | - | 100 | 66.7 | 11.1 |
| 22 | - | - | - | 77.8 | 11.1 |
| 25 | - | - | - | 77.8 | 22.2 |
| 45 | - | - | - | 77.8 | 22.2 |

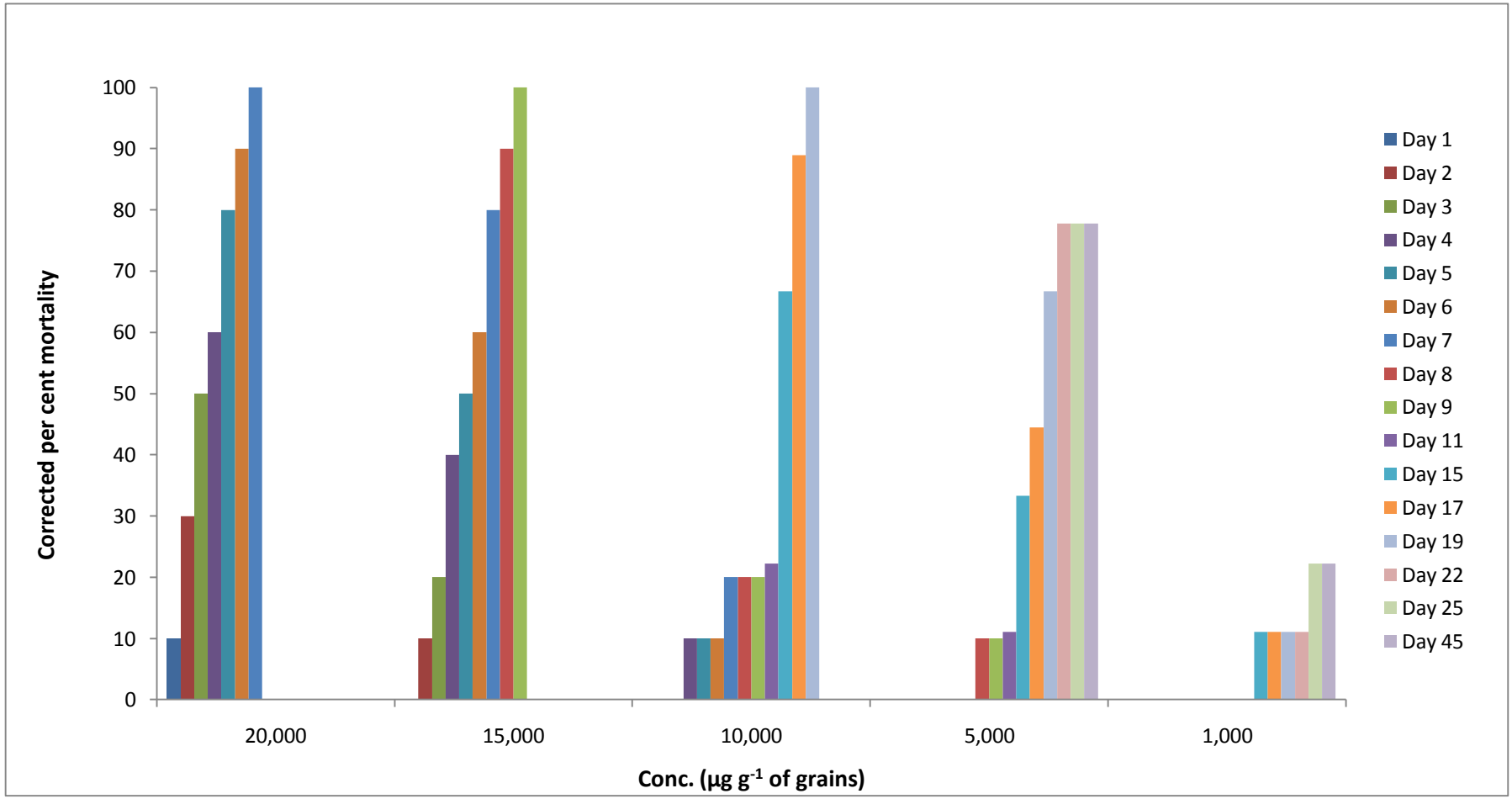



**Fig. 4.1 Corrected per cent mortality of *T. castaneum* with ethanol extract at indicated time interva**

**Table 4. Corrected per centage mortality of *T. castaneum* with chloroform extract.**

| Days of application | Concentrations | | | | |
|---|---|---|---|---|---|
| | 20,000 μg $g^{-1}$ | 15,000 μg $g^{-1}$ | 10,000 μg $g^{-1}$ | 5,000 μg $g^{-1}$ | 1,000 μg $g^{-1}$ |
| 1 | 10.0 | 0 | 0 | 0 | 0 |
| 2 | 20.0 | 10.0 | 0 | 0 | 0 |
| 3 | 30.0 | 10.0 | 0 | 0 | 0 |
| 4 | 50.0 | 30.0 | 10.0 | 0 | 0 |
| 5 | 70.0 | 40.0 | 10.0 | 0 | 0 |
| 6 | 90.0 | 60.0 | 20.0 | 0 | 0 |
| 7 | 100.0 | 70.0 | 20.0 | 0 | 0 |
| 8 | - | 70.0 | 30.0 | 10.0 | 0 |
| 9 | - | 90.0 | 30.0 | 10.0 | 0 |
| 10 | - | 100.0 | 33.3 | 11.1 | 0 |
| 11 | - | - | 44.4 | 11.1 | 0 |
| 12 | - | - | 55.5 | 22.2 | 0 |
| 13 | - | - | 77.8 | 22.2 | 0 |
| 14 | - | - | 77.8 | 33.3 | 11.1 |
| 15 | - | - | 88.9 | 44.4 | 11.1 |
| 17 | - | - | 100.0 | 56.5 | 22.2 |
| 19 | - | - | - | 66.7 | 22.2 |
| 45 | - | - | - | 66.7 | 22.2 |

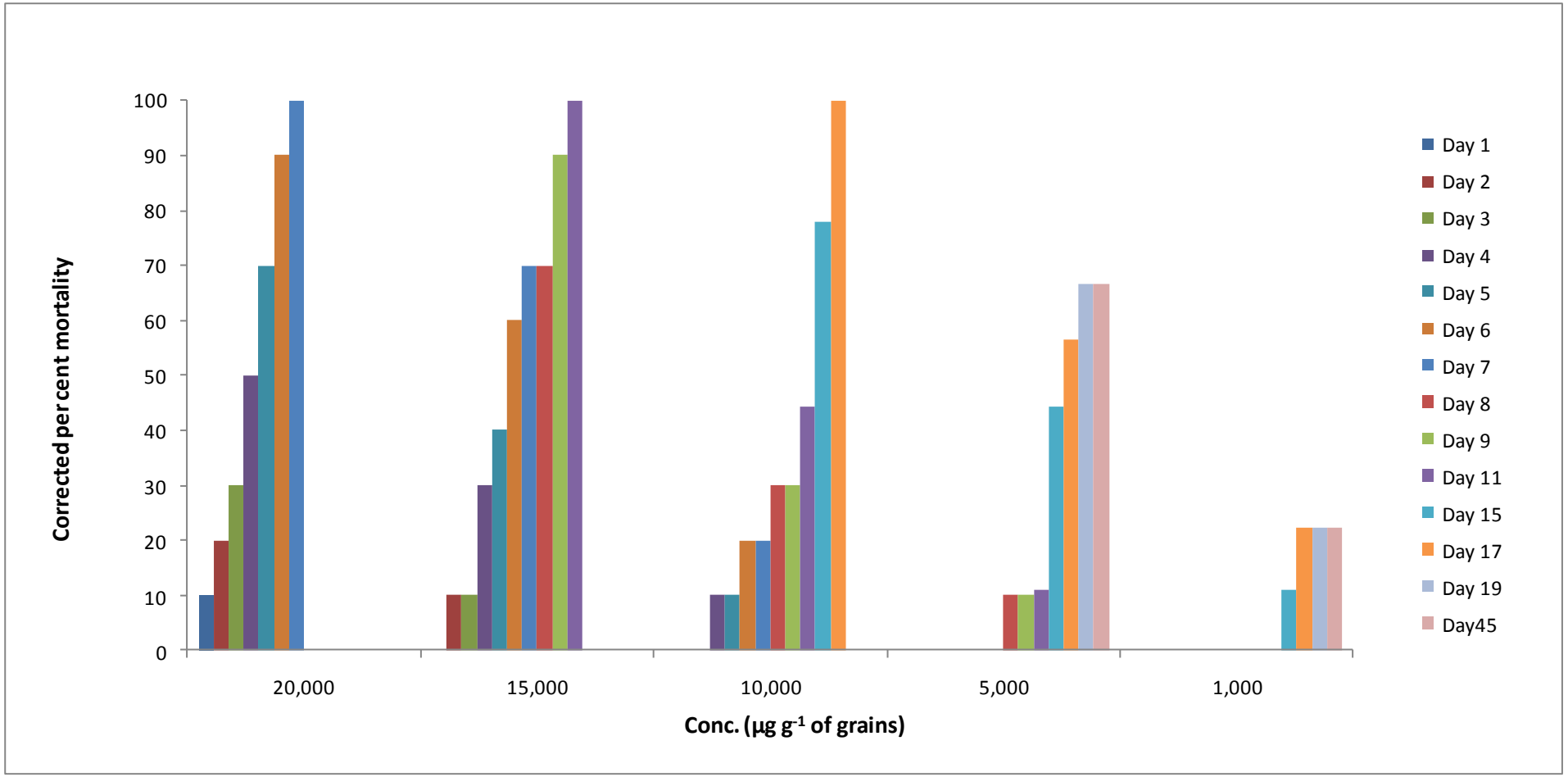



**Fig. 4.2 Corrected per cent mortality of *T. castaneum* with chloroform extract at indicated time interval**

### 4.1.3 Bioefficacy of hexane extract against *T. castaneum*

The control sample showed no mortality upto 9$^{th}$ day of application. The mortality of 10 per cent was observed on 10$^{th}$ day which was remained constant throughout the experiment. The subsequent results of corrected per cent mortality rate obtained using Abbott's formula as a result of the treatment of hexane extract against *Tribolium castaneum* are given in Table 5 and Fig 4.3. The extract showed no mortality on first and second day at all the concentrations tested except 20,000 μg g$^{-1}$ on second day of treatment. However at highest concentrations 20,000 and 15,000 μg g$^{-1}$ corrected per cent mortality of 10 per cent was observed on third day of exposure. Corrected per cent mortality of 10 per cent was observed at concentration of 20,000 μg g$^{-1}$ on second day of treatment whereas it increased to 30 per cent on fourth day at the same concentration. Corrected per cent mortality of fifty was seen at 20,000 and 15,000 μg g$^{-1}$ on fifth and sixth day of application respectively. However, other concentrations tested i.e. 10,000 and 5,000 μg g$^{-1}$ did not reveal any corrected per cent mortality even after 6 days of exposure. On seventh day, the extract started to show corrected per cent mortality of insects at 10,000 μg g$^{-1}$ concentration. After 12 days of treatment, corrected per cent mortality of 100 was achieved at 20,000 μg g$^{-1}$ concentration, whereas corrected per cent mortality of 77.8 and 22.2 was achieved at 15,000 and 10,000 μg g$^{-1}$ concentrations respectively. After this the corrected per cent mortality remained constant at 15,000 μg g$^{-1}$ concentration. On thirteenth day of application, corrected per cent mortality of 22.2 and 11.1 was obtained at 10,000 and 5,000 μg g$^{-1}$ concentrations, whereas corrected per cent mortality of 33.3 and 11.1 was observed at 10,000 and 5,000 μg g$^{-1}$ concentrations respectively after 15 days of treatment. The corrected per cent mortality remained constant at 10,000 and 5,000 μg g$^{-1}$ concentrations after 15 and 13 days of exposure respectively. The extract showed no mortality even after 45 days of exposure at 1000 μg g$^{-1}$ concentration. It was found that the extract was effective only at 20,000 μg g$^{-1}$ concentration where complete corrected per cent mortality was achieved on 12$^{th}$ day of exposure. Concentrations of 10,000 and 5,000 μg g$^{-1}$ showed little toxicity against the insect pest under the investigation, with these concentrations only 33.3 and 11.1 corrected per cent mortality was achieved. Whereas concentration of 1000 μg g$^{-1}$ did not show any toxicity against the insect pest under the investigation even after 45 days of exposure. These data showed that the corrected per cent mortality increased with increase in concentration and time of application of the extract. The concentration of 20,000 μg g$^{-1}$ was most effective whereas 1000 μg g$^{-1}$ was least effective where no mortality was observed even after 45 days of application.

**Table 5. Corrected per centage mortality of *T. castaneum* with hexane extract.**

| Days of application | Concentrations | | | |
|---|---|---|---|---|
| | 20,000 μg $g^{-1}$ | 15,000 μg $g^{-1}$ | 10,000 μg $g^{-1}$ | 5,000 μg $g^{-1}$ |
| 1 | 0 | 0 | 0 | 0 |
| 2 | 10.0 | 0 | 0 | 0 |
| 3 | 10.0 | 10.0 | 0 | 0 |
| 4 | 30.0 | 10.0 | 0 | 0 |
| 5 | 50.0 | 30.0 | 0 | 0 |
| 6 | 70.0 | 50.0 | 0 | 0 |
| 7 | 70.0 | 60.0 | 10.0 | 0 |
| 8 | 80.0 | 70.0 | 10.0 | 0 |
| 9 | 90.0 | 70.0 | 10.0 | 0 |
| 12 | 100.0 | 77.8 | 22.2 | 0 |
| 13 | - | 77.8 | 22.2 | 11.1 |
| 15 | - | 77.8 | 33.3 | 11.1 |
| 22 | - | 77.8 | 33.3 | 11.1 |
| 45 | - | 77.8 | 33.3 | 11.1 |

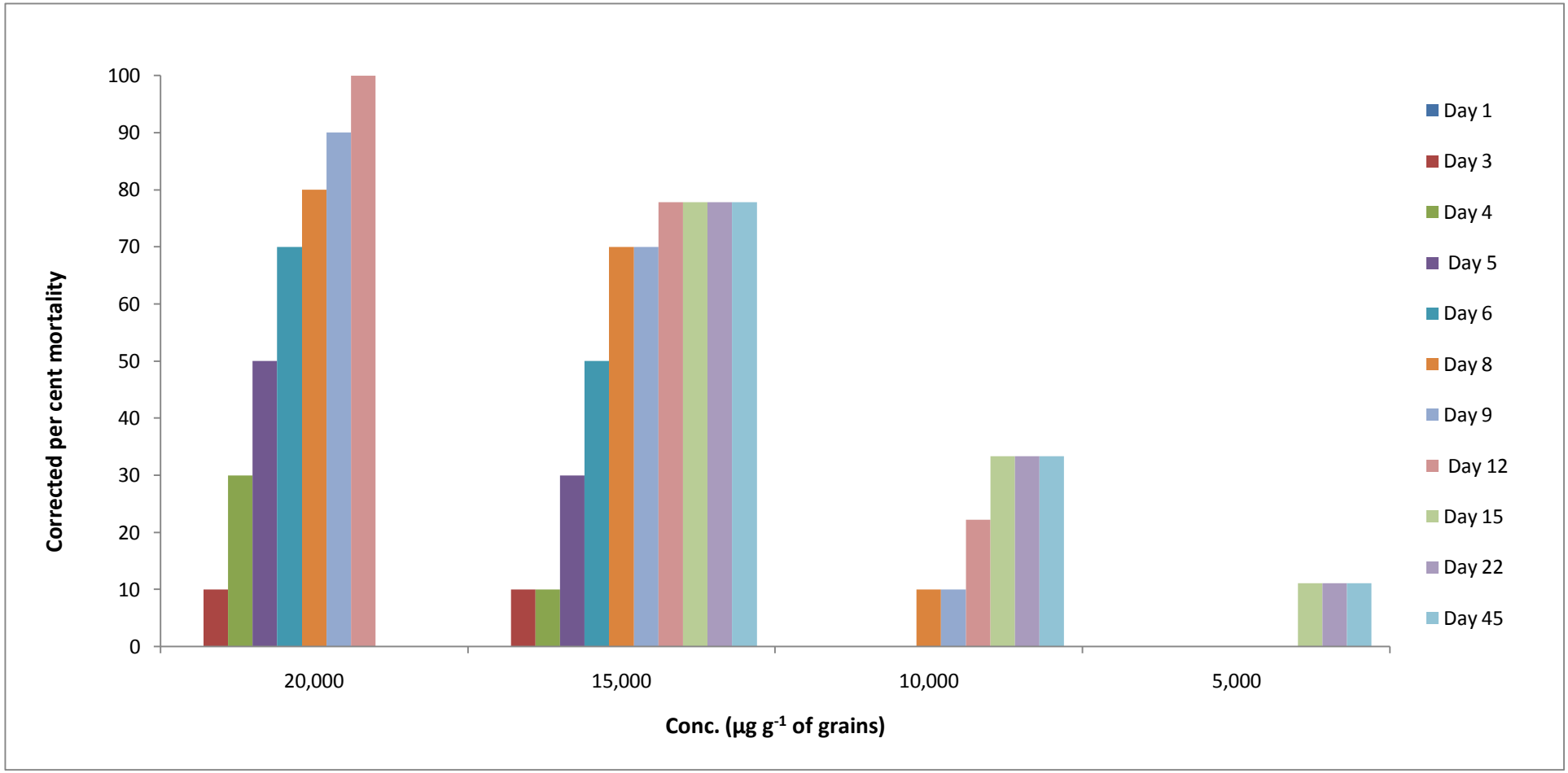



**Fig. 4.3 Corrected per cent mortality of *T. castaneum* with hexane extract at indicated time interval**

### 4.1.4 Bioefficacy of ethanol soluble hexane fraction against *T. castaneum*

As ethanol and chloroform extracts showed almost similar insecticidal activity and the hexane extract showed lower activity as compaired to ethanol and chloroform extracts, so it was decided to carry out bioefficacy studies against *T. castaneum* using ethanol soluble and ethanol insoluble hexane fractions. The control sample showed no mortality upto 4$^{th}$ day of application. The mortality of 10 per cent was observed on 5$^{th}$ day which was remained constant throughout the experiment. The subsequent data on adults corrected per cent mortality obtained using Abbott's formula as a result of the treatment of ethanol soluble fraction against *Tribolium castaneum* is summarized in Table 6 and Fig 4.4. The extract showed no mortality on first and second day at all the concentrations tested except at 20,000 μg g$^{-1}$ on second day. However at highest concentrations 20,000 and 15,000 μg g$^{-1}$ corrected per cent mortality was observed on second and third day of exposure. The table indicates that the ethanol soluble hexane extract at 20,000 and 15,000 μg g$^{-1}$ concentrations caused 40 and 30 per cent corrected mortality of adult of test insect after fourth day of treatment. Corrected per cent mortality of 77.7 and 55.5 was observed at 20,000 and 15,000 μg g$^{-1}$ concentrations respectively on 6$^{th}$ day of application. However no mortality was observed at lower concentrations of 1000, 5,000 and 10,000 μg g$^{-1}$ concentrations respectively. The corrected per cent mortality increased to 88.8, 66.7 and 11.1 at 20,000, 15,000 and 10,000 μg g$^{-1}$ concentrations respectively after 7 days of exposure. The corrected per cent mortality of 100 was achieved at 20,000 μg g$^{-1}$ concentration on the eighth day of treatment, whereas other concentrations 15,000 and 10,000 μg g$^{-1}$ showed corrected per cent mortality of 77.8 and 11.1 respectively after same day of treatment. The corrected per cent mortality of adult increased to 88.9, 22.2 and 11.1 at concentrations of 15,000, 10,000 and 5,000 μg g$^{-1}$ after tenth day of exposure. Complete corrected per cent mortality was achieved at 15,000 μg g$^{-1}$ after 11 days. The corrected per cent mortality of 66.7, 22.2 and 11.1 was achieved at 10,000, 5,000 and 1000 μg g$^{-1}$ concentrations after 19,19 and 22 days of exposure respectively. After 19 days of exposure corrected per cent mortality at10,000 μg g$^{-1}$ became constant showing that the extract was not exerting any further toxic action on the insects. Corrected per cent mortality of 33.3 was achieved at 5,000 μg g$^{-1}$ concentrations after 30 days. However, it was observed that corrected per cent mortality at 5,000 and 1000 μg g$^{-1}$ remained constant after 30 and 22 days of exposure respectively and did not change further. This showed that compounds at these concentrations were not exerting their toxic influence on insects. These data showed that the corrected per cent mortality increased with increase in concentration of ethanol soluble hexane fraction. It was found that at 20,000 and 15,000 μg g$^{-1}$ concentrations the corrected per cent mortality increases with increase in time of application till the complete

mortality was obtained whereas at 10,000, 5,000 and 1000 μg $g^{-1}$ concentrations the corrected per cent mortality remain constant or increases slowly in the beginning, then increases rapidly in the middle only at 10,000 μg $g^{-1}$ and then became constant after 19, 30 and 22 days of exposure respectively. There was no mortality till 19 days at the exposure concentration of 1000 μg $g^{-1}$. Low Corrected per centage mortality of 11.1 was observed on $22^{nd}$ days of application.This may be attributed to the presence of very low concentration of toxic compounds present at this concentration.

### 4.1.5 Bioefficacy of ethanol insoluble hexane fraction against *T. castaneum*

The control sample showed no mortality upto $4^{th}$ day of application. The mortality of 10 per cent was observed on $5^{th}$ day which was remained constant throughout the experiment. The subsequent results of corrected per cent mortality rate obtained using Abbott's formula due to of the treatment of ethanol insoluble hexane fraction against *Tribolium castaneum* are given in Table 7 and Fig 4.5. The extract showed no mortality upto twenty-fourth days at all the concentrations tested. However at highest concentration tested corrected per cent mortality was observed on twenty-fifth day of exposure. Corrected per cent mortality of 11.1 per cent was observed at concentration of 20,000 μg $g^{-1}$ on twenty-fifth day of treatment. However, other concentrations tested i.e. 15,000 and 10,000 μg $g^{-1}$ did not show any corrected per cent mortality even after 25 days of exposure. The extract started to show corrected per cent mortality of 11.1 at 15,000 and 10,000 μg $g^{-1}$ concentration respectively after 30 and 40 days of exposure. Corrected per cent mortality of 22.2 was achieved at 20,000 μg $g^{-1}$ concentrations after 30 days of exposure. After this the corrected per cent mortality remained constant showing that compounds did not show its toxic effect at concentration tested. However no corrected per cent mortality was observed at 5,000 and 1000 μg $g^{-1}$ concentrations even after 45 days of exposure. Very low mortality rates at 20,000, 15,000 and 10,000 μg $g^{-1}$ concentrations tesed may be due to the absence of toxic compound in this fraction as compaired to ethanol soluble fraction where high level of mortality was observed. Comparison of two fraction revealed that the ethanol soluble fraction has more insecticidal activity than ethanol insoluble fraction due to the presence active secondary metabolites. From the above study it was reported that ethanol insoluble hexane fraction showed negligible insecticidal activity against *Tribolium castaneum* even at higher concenterations. Singh *et al* (1988) also reported that ethanol insoluble fraction showed no activity even at 1 per cent agains *Lipaphis erysimi*.

**Table 6. Corrected per centage mortality of *T. castaneum* with ethanol soluble hexane fraction**

| Days of application | Concentration | | | | |
|---|---|---|---|---|---|
| | 20,000 μg $g^{-1}$ | 15,000 μg $g^{-1}$ | 10,000 μg $g^{-1}$ | 5,000 μg $g^{-1}$ | 1,000 μg $g^{-1}$ |
| 1 | 0 | 0 | 0 | 0 | 0 |
| 2 | 10.0 | 0 | 0 | 0 | 0 |
| 3 | 20.0 | 10.0 | 0 | 0 | 0 |
| 4 | 40.0 | 30.0 | 0 | 0 | 0 |
| 5 | 66.7 | 44.4 | 0 | 0 | 0 |
| 6 | 77.8 | 55.5 | 0 | 0 | 0 |
| 7 | 88.8 | 66.7 | 11.1 | 0 | 0 |
| 8 | 100.0 | 77.8 | 11.1 | 0 | 0 |
| 9 | - | 77.8 | 22.2 | 0 | 0 |
| 10 | - | 88.9 | 22.2 | 11.1 | 0 |
| 11 | - | 100.0 | 33.3 | 11.1 | 0 |
| 12 | - | - | 44.4 | 11.1 | 0 |
| 13 | - | - | 55.5 | 11.1 | 0 |
| 15 | - | - | 55.5 | 11.1 | 0 |
| 19 | - | - | 66.7 | 22.2 | 0 |
| 22 | - | - | 66.7 | 22.2 | 11.1 |
| 30 | - | - | 66.7 | 33.3 | 11.1 |
| 45 | - | - | 66.7 | 33.3 | 11.1 |

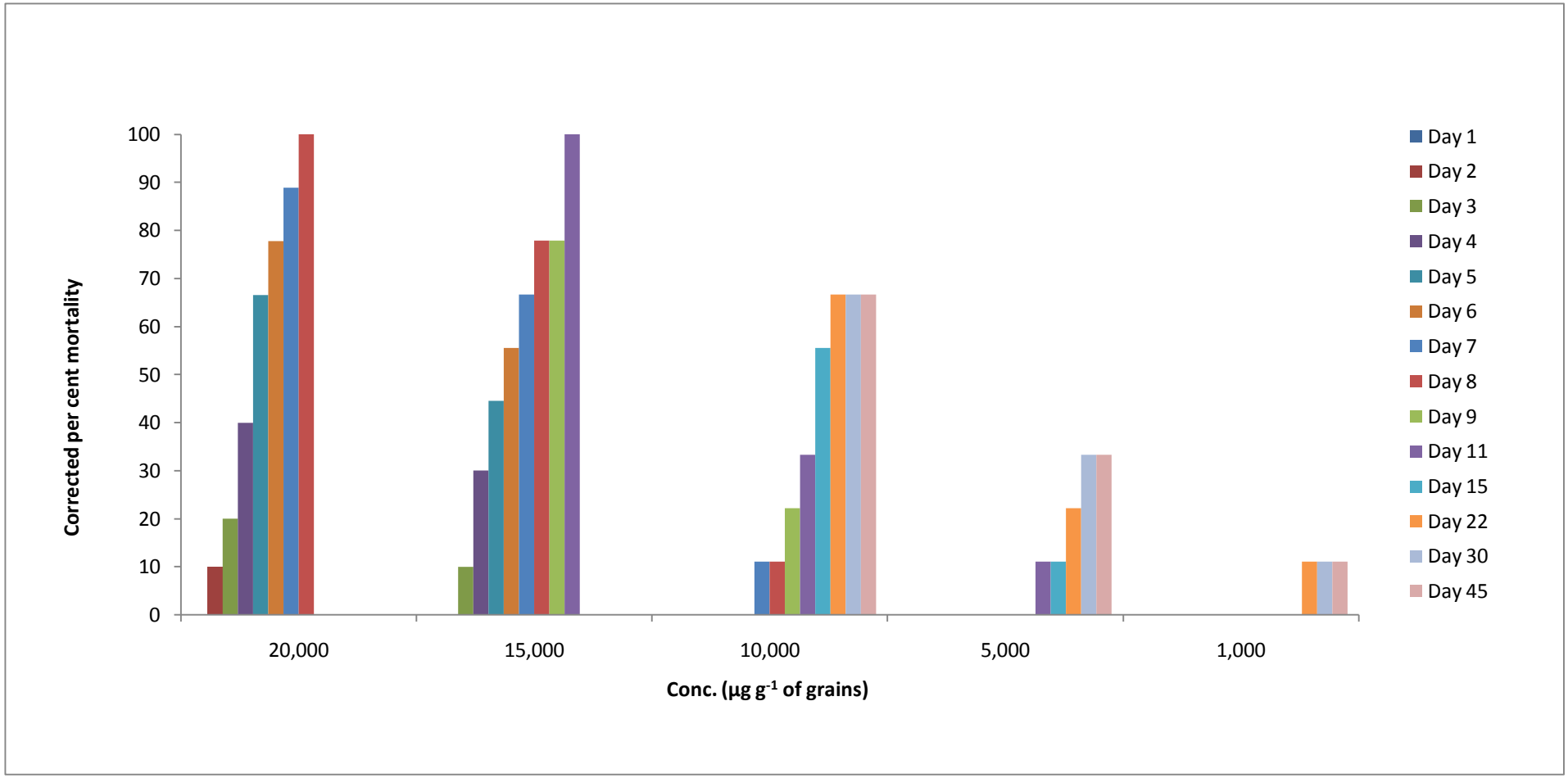



**Fig. 4.4 Corrected per cent mortality of *T. castaneum* with ethanol soluble hexane fraction at indicated time interval**

**Table 7. Corrected per centage mortality of *T. castaneum* with ethanol insoluble hexane fraction**

| Days of application | Concentrations | | |
|---|---|---|---|
| | 20,000 μg $g^{-1}$ | 15,000 μg $g^{-1}$ | 10,000 μg $g^{-1}$ |
| 1 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 |
| 25 | 11.1 | 0 | 0 |
| 30 | 22.2 | 11.1 | 0 |
| 35 | 22.2 | 11.1 | 0 |
| 40 | 22.2 | 11.1 | 11.1 |
| 45 | 22.2 | 11.1 | 11.1 |

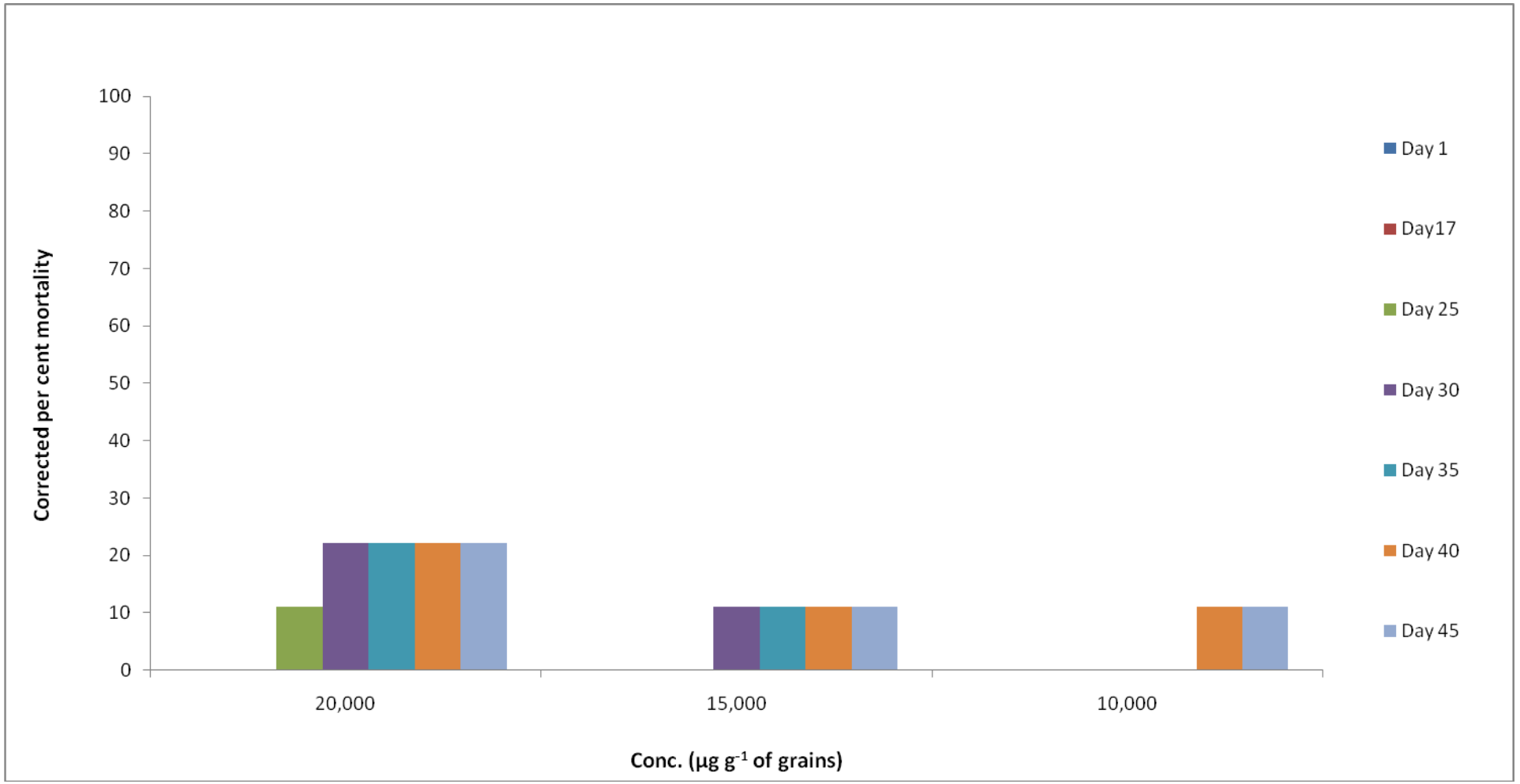



**Fig. 4.5 Corrected per cent mortality of *T. castaneum* with ethanol insoluble hexane fraction at indicated time interval**

### 4.1.6 Comparison of bioefficacy of different neem seed kernel extracts against *T. castaneum* at 10,000 μg $g^{-1}$.

The corrected per cent mortality observed as a result of the treatment of different neem seed kernels extracts against *Tribolium castaneum* at 10,000 μg $g^{-1}$ is shown in Table 8 and Fig 4.6. No corrected per cent mortality was observed in case of different extract tested upto 3 days of exposure whereas corrected per cent mortality of 10 was found in ethanol and chloroform extract after fourth day of exposure. On day 7 of experiment, corrected per cent mortality of 20, 20, 10 and 11.1 was observed in ethanol, chloroform, hexane extract and ethanol soluble hexane fraction respectively and which increased to 33.3, 55.5, 22.2 and 44.4 per cent respectively after 12 days of exposure. Corrected per cent mortality of 66.7, 88.9, 33.3 and 55.5 was achieved in ethanol, chloroform, hexane extract and ethanol soluble hexane fraction respectively after 15 days of treatment. Complete corrected per cent mortality was achieved on day 19 and 17 in ethanol and chloroform extract respectively. No corrected per cent mortality was observed in case of ethanol insoluble hexane fraction upto 40 days of exposure. Corrected per cent mortality was found to be 66.7 and 11.1 per cent in ethanol soluble hexane and ethanol insoluble hexane fraction after 19 and 40 days of treatment. The corrected per cent mortality become constant in hexane extract, ethanol soluble hexane and ethanol insoluble hexane fraction after 14, 19 and 40 days of exposure showing that extracts are no longer toxic towards the insect under investigation. From the above discussion it was found that chloroform extract is more active against *Tribolium castaneum* adults where as the ethanol insoluble hexane fraction was least active at 10,000 μg $g^{-1}$ concentration. The decreasing order of activity of different neem seed kernel extracts against *Tribolium castaneum* adults at 10,000 μg $g^{-1}$ concentration is as follows:

Chloroform extract > Ethanol extract > Ethanol soluble hexane fraction > Hexane extract > Ethanol insoluble hexane fraction.

**Table 8. Comparison of corrected per centage mortality of *T. castaneum* with different neem seed kernels extracts at indicated time interval after treatment at 10,000 μg $g^{-1}$.**

| Days of application | Concentrations | | | | |
|---|---|---|---|---|---|
| | Ethanol extract (A) | Chloroform extract (B) | Hexane extract (C) | Ethanol soluble hexane fraction (D) | Ethanol insoluble hexane fraction (E) |
| 1 | 0 | 0 | 0 | 0 | 0 |
| 4 | 10.0 | 10.0 | 0 | 0 | 0 |
| 5 | 10.0 | 10.0 | 0 | 0 | 0 |
| 6 | 10.0 | 20.0 | 0 | 0 | 0 |
| 7 | 20.0 | 20.0 | 10.0 | 11.1 | 0 |
| 9 | 20.0 | 30.0 | 10.0 | 22.2 | 0 |
| 10 | 22.2 | 33.3 | 11.1 | 22.2 | 0 |
| 11 | 22.2 | 44.4 | 11.1 | 33.3 | 0 |
| 12 | 33.3 | 55.5 | 22.2 | 44.4 | 0 |
| 13 | 55.5 | 77.8 | 22.2 | 55.5 | 0 |
| 14 | 55.5 | 77.8 | 33.3 | 55.5 | 0 |
| 15 | 66.7 | 88.9 | 33.3 | 55.5 | 0 |
| 17 | 88.9 | 100.0 | 33.3 | 55.5 | 0 |
| 19 | 100 | - | 33.3 | 66.7 | 0 |
| 30 | - | - | 33.3 | 66.7 | 0 |
| 40 | - | - | 33.3 | 66.7 | 11.1 |
| 45 | - | - | 33.3 | 66.7 | 11.1 |

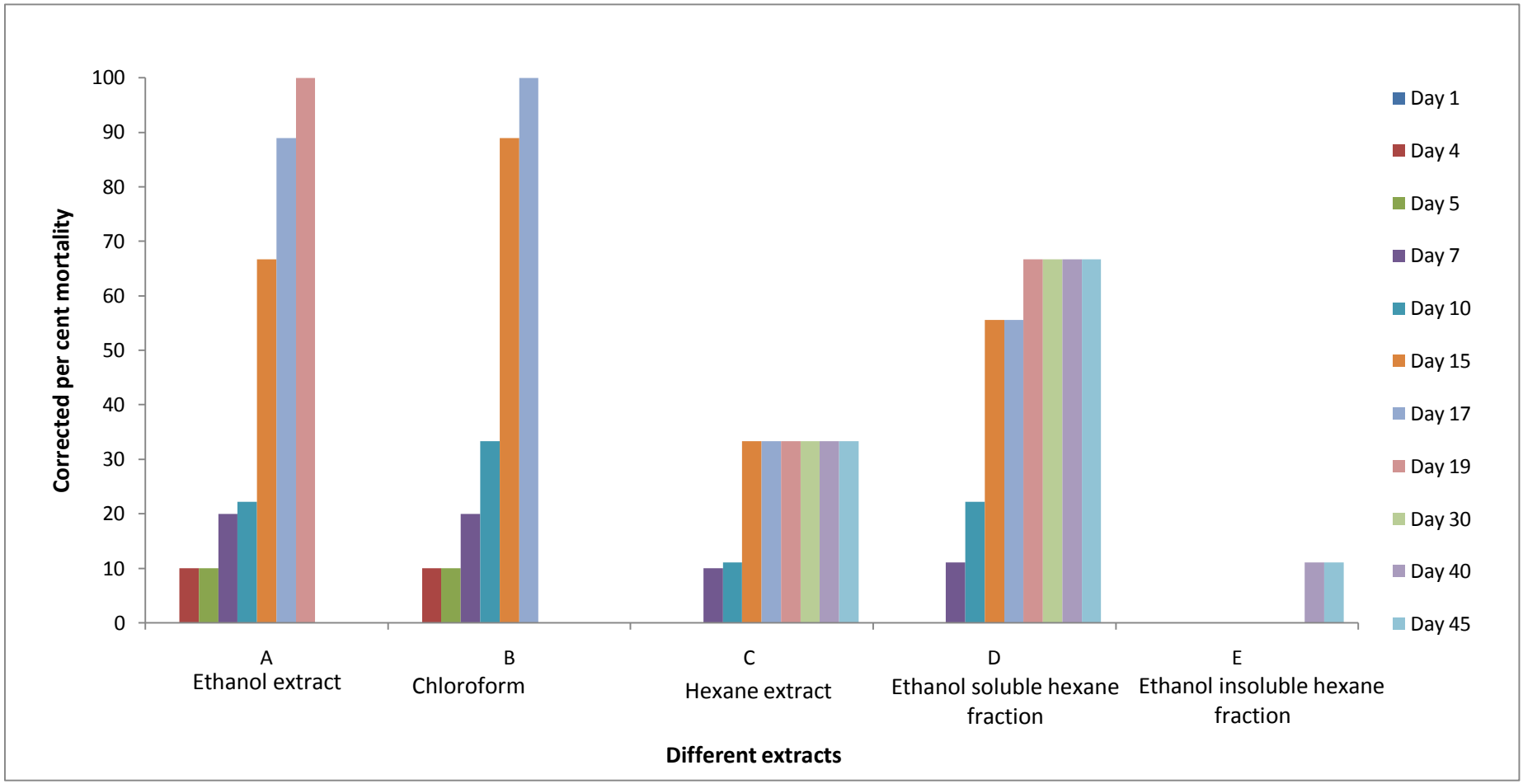



**Fig. 4.6Comparison of corrected percentage mortality of *T. castaneum* with different neem seed kernels extracts at indicated time interval after treatment at 10,000 mg $g^{-1}$**

From the bioefficacy studies of different neem extracts it was found that the polar extract showed more biological activity as compared to non polar ones. This is due to the presence of more polar compounds present in them such as azadirachtin (1), gedunin (2), mahmoodin (3), nimbin (4) and salannin (5) in the polar extracts. So the ethanol and chloroform extract showed almost same insecticidal activity.

It was found that the ethanol soluble fraction showed more biological activity against test insects as compared to the original hexane extract, whereas ethanol insoluble extract showed negligible activity. This is due to the fact that after the partitioning of hexane extract with ethanol the active compounds present in original hexane extract dissolved in ethanol and insoluble fraction contain higher concentration of fatty acid due to this, the activity of the ethanol soluble fraction increases over original hexane extract whereas that of ethanol insoluble fraction decreases. Singh *et al* (1988) also reported that when the hexane extract was partitioned with ethanol, the ethanol soluble fraction had an $LC_{50}$ of 0.328 per cent, whereas the ethanol insoluble part showed no activity even at 1 per cent against *Lipaphis erysimi*.

It was found that the number of larvae, pupae and adults in test sample of ethanol insoluble fraction at 15,000 µg $g^{-1}$ concentration were 44, 12 and 21 respectively, whereas in case of control the number of larvae, pupae and adults were 23, 8 and 20 after 45 days of exposure respectively. This showed that there was an increase in the count of larvae, pupae and adults in test treatment in comparison to the control treatment. This showed that compounds that are fatty acids/esters present in the ethanol insoluble fraction acted as food for the larvae and adults of the test insect, thus regulating their growth.

It was concluded that ethanol and chloroform extracts had almost similar insecticidal activity against *Tribolium castaneum* which was greater than ethanol soluble hexane fraction. The insecticidal activity of original hexane extract was less than ethanol soluble hexane fraction and more than ethanol insoluble hexane fraction. The decreasing order of insecticidal activity of different neem seed kernels extracts against *Tribolium castaneum* adults were ethanol extract = chloroform extract > ethanol soluble hexane fraction > hexane extract > ethanol insoluble hexane fraction.

From the above studies it is recommended that in order to use the neem seed kernels powder or extract as insecticide for storage of wheat, the powder should be thoroughly washed or partitioned with hexane. This will remove the fatty acids and less polar secondary metabolites consequently the relative concentration of more polar and active secondary metabolites will increase thereby the bioefficacy of that powder or extract against the test insect will increase further.

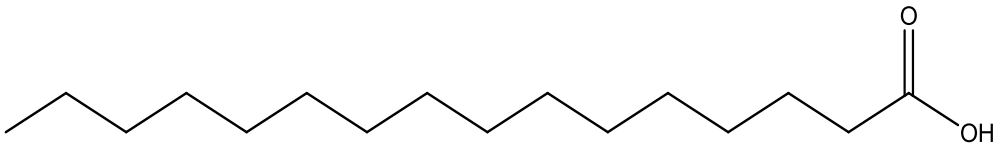
**CHAPTER-V**

## SUMMARY

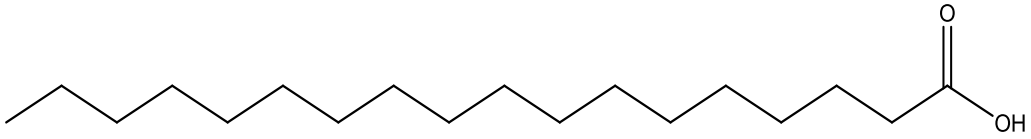
The fixed or essential oils and their component terpenoids are naturally occurring plant products isolated from various aromatic and medicinal plants. Terpenoids are widely used for the fragrance and flavouring ingredients and in formulations of analgesics and antiseptics. The characteristics of these simple compounds that make them attractive for the development as environmentally sound insect-pest control agents include their selectivity, insect toxicity, low mammalian toxicity and biodegradable nature. Hence, attempts were made to extract different extracts having active secondary metabolites that are responsible for toxicity towards adults of insect pest.

The neem seeds were depulped, air dried and powdered. The neem oil was extracted from dried and powdered seed kernels using ethanol, chloroform and hexane using Soxhlet apparatus. The oil was isolated in 13.5, 31.25 and 23 per cent yield respectively. Thin layer chromatography (TLC) of chloroform, ethanol and hexane extracts showed 6, 5 and 5 brown colored spots respectively. The study of IR spectra of polar extract (chloroform and ethanol) and the study of literature indicated the presence of azadirachtin, gedunin, mahmoodin, nimbin and salannin. Hexane extract was partitioned with ethanol to give ethanol soluble and ethanol insoluble hexane fractions. The yield of ethanol insoluble and ethanol soluble hexane fraction was 40 and 50 per cent respectively. TLC of ethanol soluble and ethanol insoluble hexane fractions showed 5 and 1 brown colored spots respectively.

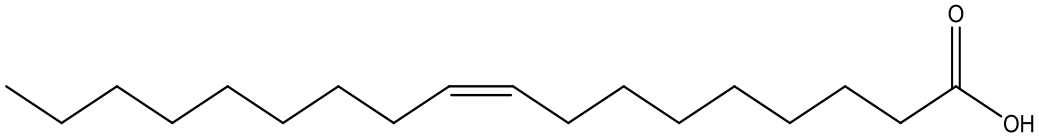
The hexane extract was subjected to column chromatography to have corresponding non-polar and polar fractions. The various fractions were subjected to TLC and similar fractions were pooled together. Three fractions were obtained. Hexane fraction I showed three spots on TLC plate, having $R_f$ values of 0.22, 0.83 and 0.92, fraction II showed three spots having $R_f$ values of 0.18, 0.66 and 0.84 whereas fraction III showed only two spots having $R_f$ values of 0.24 and 0.98 respectively.The IR, NMR and literature studies of these three fractions and their comparison with known data indicates the presence of palmitic (1), stearic (2), oleic (3), linoleic (4), arachidic acid and their esters.



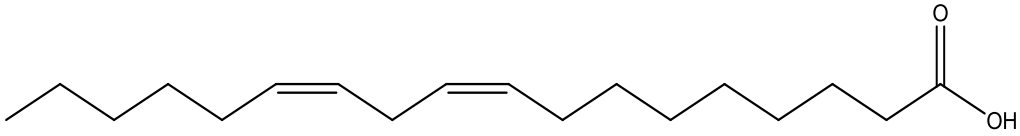

**(1)**




**(2)**




**(3)**




**(4)**

Rearing of insects of *Tribolium castaneum* for raising F1 generation was carried out. The different neem seed kernels extracts were subjected to bioefficacy studies against *Tribolium castaneum* (Herbst) adults. Wheat grains were spiked with various concentrations of test extracts *viz*. 20,000, 15,000, 10,000, 5,000 and 1000 μg $g^{-1}$ of wheat. There were three replications for each treatment and for control treatment, only wheat and acetone was used. The bottles were put in electric shaker for 5 minutes to enable thorough mixing of extract with wheat grains. Ten adults of same age were released into each bottle and mouth of bottle was covered with muslin. The observation of mortality of *T. castaneum* was taken after every 24 hrs till complete or constant mortality was obtained. The observation of appearance of larvae and larval growth were also taken out for 45 days. Corrected per cent mortality was calculated using Abbott's formula.

Results indicate that mortality increases with increase in concentration of the extract applied and also with increase in time of application and then became constant. From the bioefficacy study of different neem extracts it was found that the polar extract showed more biological activity as compared to non polar ones. The best concentration was 20,000 μg $g^{-1}$ in case of ethanol, chloroform extract and ethanol soluble hexane fraction where complete per cent mortality was observed after 7, 7 and 8 days of application respectively. It was observed that no larvae appeared at this concentration in case of ethanol, chloroform extract and ethanol soluble hexane fraction.

It was found that the ethanol soluble hexane fraction showed more insecticidal activity against test insects as compared to the original hexane extract, whereas ethanol insoluble extract showed negligible activity. Hexane extract showed complete mortality at 20,000 μg $g^{-1}$ on $12^{th}$ day of exposure whereas ethanol soluble hexane fraction showed complete mortality on $8^{th}$ day of application. This showed that ethanol soluble hexane fraction has more concentration of active secondary metabolites which have more insecticidal activity as compared to fatty acid and their esters which were the mojor components of ethanol insoluble hexane fraction. In case of ethanol insoluble hexane fraction complete per cent mortality was not observed at any concentration and larvae appeared in all test concentrations and the count of larvae was more in test concentrations as compared to the control. This showed that fatty acids and their esters present in ethanol insoluble fractions acted as food for the larvae and adults of the test insect, thus regulating the growth of larvae and the adults of the test insects.

## REFERENCES


Abdel-Shafy S and Zayed A A (2002) In vitro acaricidal effect of plant extract of neem seed oil (*Azadirachta indica*) on egg, immature, and adult stages of *Hyalomma anatolicum excavatum* (Ixodoidea: Ixodidae). *Vet Parasitol* **106**(1): 89–96.

Adhikary S (1981) Togo experience in moving from neem research to its practical application for plant protection. In: Schmutterer H, Ascher K R S and Rembold H (ed) Natural Pesticides from the Neem Tree (*A. indica*). *Proc 1st Int Neem Conf*. pp 215-22. Rottach-Egern, Germany.

Aggarwal N and Brar D (2006) Effects of different neem preparations in comparison to synthetic insecticides on the whitefly parasitoid *Encarsia sophia* (Hymenoptera: Aphelinidae) and the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae) on cotton under laboratory conditions. *J Pest Sci* **79**: 201-07.

Ahmed S and Koppel B (1987) Use of neem and other botanical materials for pest control by farmers in India: summary of findings. In Schmutterer H and Ascher K R S (ed) Natural Pesticides from the Neem Tree (*A. indica*) and Other Tropical Plants. *Proc 3rd Int Neem Conf*. pp 623-26. Nairobi, Kenya.

Akhtar Y, Yeoung Y R and Isman M B (2008) Comparative bioactivity of selected extracts from Meliaceae and some commercial botanical insecticides against two noctuid caterpillars, *Trichoplusiani* and *Pseudaletia unipuncta*. *Phytochem Rev* **7**: 77-88.

Ali B H (1987) The toxicity of *A. indica* leaves in goats and guinea pigs. *Vet Hum Toxicol* **29**(1): 16-19.

Ali S I, Singh O P, and Misra U S (1983) Effectiveness of plant oils against pulse beetle, *Callosobruchus chinensis* Linn. *Ind J Entomol* **45**: 6-9.

Alzoreky N S and Nakahara K (2003) Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *Int J Food Microbiol* **15; 80**(3): 223–30.

Amadioha A C (2000) Controlling rice blast in vitro and in vivo with extracts of *A. indica*. *Crop Prot* **5**: 287-90.

Anonymous (2008) Green Earth Products (GEP) Neem products. An informative and exhaustive portal covering a wide range of topics on neem including its history, products, applications, usage in different industries. 2pp. Available online@http://www.neemproducts. com/insecticide.html.

Anwar M, Ashfaq M, Ul-Hasan M and Anjum F M (2005) Efficacy of *Azadirachta indica* oil on bagging material against some insect pests of wheat stored in warehouses at Faisalabad. *Pak Entomol* **27**(1): 89-94.

Ara I, Siddiqui B S, Faizi S and Siddiqui S (1989) Structurally novel diterpenoids constituents from the stem bark of *Azadirachta indica. J Chem Soc Perkin Trans* **I**: 343–45.

Ascher K R S, Streloke M, Schmidt G H and Warthen J D (1989) The antifeedant effects of neem seed kernel extract and azadirachtin on nymphs of *Eyprepocnemis plorans*. *Phytoparasitica* **17**: 167–74.

Attri B S and Prasad G R (1980) Studies on the pesticide value of neem oil by-products. *Pestology* **4**: 16-20.

Awasthy K S (2001) Genotoxicity of a crude leaf extract of neem in male germ cells of mice. *Cytobios* **106**(2): 151-64.

Babu P S and Prince P S M (2004) Antihyperglycaemic and antioxidant effect of hyponidd, an ayurvedic herbomineral formulation in streptozotocin-induced diabetic rats. *J Pharm Pharmacol* **56**(11): 1435-42.

Badam L, Joshi S P and Bedekar S S (1999) In vitro antiviral activity of neem (*Azadirachta indica* A. Juss) leaf extract against group *B coxsackieviruses*. *J Commun Dis* **31**(2):79–90.

Bandyopadhyay U, Biswas K, Sengupta A, Moitra P, Dutta P, Sarkar D, Debnath P, Ganguly C K and Banerjee R K (2004) Clinical studies on the effect of Neem (*Azadirachta indica*) bark extract on gastric secretion and gastroduodenal ulcer. *Life Sci* **75**(24): 2867–78.

Bhattacharyya N, Chutia M and Sarma S (2007) Neem (*Azadirachta indica* A. Juss), a potent biopesticide andmedicinal plant: a review. *J Plant Sci* **2**(3): 251–59.

Bhonde S B, Deshpande S G and Sharma R N (1999) In vitro evaluation on inhibitory nature of some neem formulations against plant pathogenic fungi. *Hindustan Antibiot Bull* **41**(1–4): 22–24.

Biswas K I, Chattopadhyay R K, Banerjee U and Bandyopadhyay U (2002) Biological activities and medicinal properties of neem (*Azadirachta indica*). *Cur Sci* **82**: 1336–45.

Bobadilla M, Zavaleta G, Gil F, Pollack L and Sisniegas M (2002) Efecto bioinsecticida del extracto etanólico de las semillas de *Annona cherimolia* Miller (chirimoya) and *A. muricata* Linnaeus (guanábana) sobre larvas del IV estadio de *Anopheles sp. Revista Peruana de Biología* **9**: 64-73.

Bobadilla M, Zavala F, Sisniegas M, Zavaleta G, Mostacero J and Taramona L (2005) (Evaluation of larvicidal properties of aqueous suspensions of the *Annona muricata* Linnaeus against *Aedes aegypti* Linnaeus (Diptera, Culicidae)). *Revista Peruana de Biolog* **12**:15-152.

Boeke S J, Boersma M G, Alink G M, van Loon J J, van Huis A, Dicke M and Rietjens I M (2004) Safety evaluation of neem (*Azadirachta indica*) derived pesticides. *J Ethnopharmacol* **94**(1): 25-41.

Boral D, Chatterjee S and Bhattacharya K (2004) The occurrence and allergising potential of airborne pollen in West Bengal. *Ind Ann Agric Environ Med* **11**(1): 45-52.

Borsdorf R, Foster K, Huyser W, Pedersen J, Pfost H, Stevens H and Wright V (1983) Post-harvest management project design in Pakistan. *Food and Feed Grain Inst Rep*. pp 91. Manhattan.

Bugundu L M (1970) The storage of farm products by farmers in my village. *Samaru Agric Newsl* **12**: 2.

Butterworth J H and Morgan E D (1968) Isolation of a substance that suppresses feeding in locust. *J Chem Soc Chem Commun* **23**.

Cao J, Zeng K and Jiang W (2006) Enhancement of postharvest disease resistance in YaLi pear (*Pyrus bretschneideri*) fruit by salicylic acid sprays on the trees during fruit growth. *Eur J Plant Pathol* **114**: 363-70.

Cardellina J H (1988) Biologically active natural products in the search of for new agrochemicals. In: Gulter H G (ed) *Biologically active natural products: potential use in agriculture*. Pp 305-11. American Chemical Society, Washington DC, USA.

Celis A, Mendoza C, Pachon M, Cardona J, Delgado W and Cuca L (2008) Extractos vegetales utilizados como biocontroladores con énfasis en la familia Piperácea. *Una Revisión. Agronomía Colombiana* **26**: 97-106.

Champagne D E, Isman M B and Towers G H N (1989) Insecticidal activity of phytochemicals and extracts of the Meliaceae. In: Arnason J et al (eds) Insecticides of plant origin, *ACS Symposium Series.*Vol **387**, pp 95-109, New York.

Chandra B, Kumar P and Matiur M (2008) Aphidicidal activity of some indigenous plant extracts against bean aphid *Aphis cracc ivora* Koch (Homoptera: Aphididae). *J Pest Sci* **81**:153-59.

Chattopadhyay I, Nandi B, Chatterjee R, Biswas K, Bandyopadhyay U and Banerjee R K (2004) Mechanism of antiulcer effect of Neem (*Azadirachta indica*) leaf extract: effect on H?-K?- ATPase oxidative damage and apoptosis. *Inflammopharmacol* **12**(2): 153-76.

Chattopadhyay R R (1996) Possible mechanism of antihyperglycemic effect of *Azadirachta indica* leaf extract Part IV. *Gen Pharmacol* **27**(3): 431-34.

Chaturvedi R, Razdan M K and Bhojwani S S (2003) Production of haploids of neem (*Azadirachta indica A. juss.*) by anther culture. *Plant Cell Rep* **21**: 531-37.

Clemente S, Mareggiani G, Broussalis A, Martino V and Ferraro G (2003) Insecticidal effects of Lamiaceae species against stored products insects. *Boletín de Sanidad Vegetal Plagas* **29**: 1-8.

Damayanti M, Susheela K and Sharma G J (1996) Effect of plant extracts and systemic fungicide on the pineapple fruitrotting fungus, Ceratocystis paradoxa. *Cytobios* **86**(346): 155–65.

Das N G, Nath D R, Baruah I, Talukdar P K and Das S C (1999) Field evaluation of herbal mosquito repellents. *J Commun Dis* **31**(4): 241-45.

De-Azambuja P and Garcia E S (1992) Effects of azadirachtin on *Rhodnius prolixus*: immunity and *Trypanosoma cruzi* interaction. *Mem Inst Oswaldo Cruz* **87**(Suppl 5): 69–72.

Denholm I, Cahill M, Dennehy T and Horowitz A (1998) Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly *Bemisia tabaci*. Philosophical Transactions: *Biolog Sci* **353**: 1757-67.

Deshpande A D (1967) *Neem as a protectant against storage pests*. M.Sc. thesis, Indian Agric Res Inst, New Delhi, India.

Desneux N, Decourtye A and Delpuech J M (2007) The sublethal effects of pesticides on beneficial arthropods. *Ann Rev Entomol* **52**: 81-106.

Devakumar C and Dev S (1996) in *Neem* (eds Randhawa and Parmar, B. S.) 2nd edn, pp 77–110.

Devlin J F and Zettel T (1999) *Ecoagriculture: Initiatives in Eastern and Southern Africa*. Weaver Press. Harare.

Dhyani S, Tripathi S and Inder D (2004) Preliminary screening of neem (*Azadirachta indica*) leaf extractives against *Poria monticolad*-a wood destroying fungus. *J Ind Acad Wood Sci* **1-2**: 103-12.

Dichter D (1976) The stealthy thief. *Ceres* (FAO Review) **9**: 51-53, 55.

Dorn A, Rademacher J M and Sehn E (1986) Effects of azadirachtin on the moulting cycle, endocrine system and ovaries in last-instar larvae of the milkweed bug, *Oncopeltus fasciatus*. *J Insect Physiol* **32**: 321–28.

Duke S O, Dayan F E, Romagni J G and Rimando A M (2000) Natural Products as sources of herbicides: current status and future trends. *Weed Res* **40**: 99-111.

Dunkel F V, Serugendo A, Breene W M and Sriharan S (1995) Influence of insecticidal plant materials used during storage on sensory attributes and instrumental hardness of dry edible beans (*Phaseolus vulgaris L.*). *Plant Foods Hum Nutr* **48**(1): 1–16.

El-Shazly M M and El-Sharnoubi E D (2000) Toxicity of a neem (*Azadirachta indica*) insecticide to certain aquatic organisms. *J Egypt Soc Parasitol* **30**(1): 221-31.

Fabry W, Okemo P and Ansorg R (1996) Fungistatic and fungicidal activity of east African medicinal plants. *Mycoses* **39**(1–2): 67–70.

Fields P G, Xie Y S and Hou X (2001) Repellent effect of *Pisum sativum* fractions against storedproduct insects. *J Stored Prod Res* **37**: 359-70.

Fishwick F B (1988) Pesticide residues in grain arising from postharvest treatments. *Aspects Appl Biol* **17:** 37-46.

Fry J S (1938) Neem leaves as an insecticide. *Gold Coast Farmer* **6**: 190.

Fujiwara T, Sugishita E, Takeda T, Ogihara Y, Shimizu M, Nomura T and Tomita Y (1984) *ibid* **32**: 1385–91.

Fujiwara T, Takeda T, Ogihara Y, Shimizu M, Nomura T and Tomita Y (1982) *Chem Pharm Bull* **30**: 4025–30.

Gandhi M, Lal R, Sankaranarayanan A, Banerjee C K and Sharma P L (1988) Acute toxicity study of the oil from *Azadirachta indica* seed (neem oil). *Ethnopharmacol* **23**(1): 39-51.

Garcia J, Grisoto E, Vendramim J and Machado P (2006) Bioactivity of Neem, *Azadirachta indica*, Against Spittlebug *Mahanarva fimbriolata* (Hemiptera: Cercopidae) on Sugarcane. *J Eco Entomol* **99**: 2010-14.

Garg S, Talwar G P and Upadhyay S N (1994) Comparison of extraction procedures on the immunocontraceptive activity of neem seed extracts. *J Ethnopharmacol* **44**(2): 87-92.

Garg S, Talwar G P and Upadhyay S N (1998) Immunocontraceptive activity guided fractionation and characterization of active constituents of neem (*Azadirachta indica*) seed extracts. *J Ethnopharmacol* **60**(3): 235-46.

Ghimeray A K, Jin C W, Ghimire B K and Cho D H (2009) Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta Indica* A. Juss grown in foothills of Nepal. *African J Biot* **8**(13): 3084-91.

Ghodesawar M G, Ahamed R N, Ahmed A W and Aladakatti R H (2004) Ultrastructural changes in cauda epididymidal epithelial cell types of *Azadirachta indica* leaf treated rats. *Ind J Exp Biol* **42**(11): 1091-95

Gholap S and Kar A (2004) Hypoglycaemic effects of some plant extracts are possibly mediated through inhibition in corticosteroid concentration. *Pharmazie* **59**(11): 876-78.

Girish K and Shankara B S (2008) Neem--A Green Treasure. *Elect J of Bio* **4**(3): 102-11.

González R, Otero G, Villanueva J, Pérez J and Soto R (2006) *(Azadirachta indica toxicity and repellence of varroa destructor* (Acari: Varroidae)). *Agrociencia* **40**: 741-51.

Govindachari T R, Suresh G and Gopalakrishnan G (1995) A direct preparative high performance liquid chromatography procedure for the isolation of major triterpenoids and their quantitative determination in neem oil. *J Liquid Chromatogr* **18**: 3465-71.

Govindachari T R, Suresh G, Gopalakrishnan G and Wesley S (2000) Insect antifeedant and growth regulating activities of neem seed oil-the role of major triterpenoids. *J Appl Entomol* **124**: 287-91.

Grange N and Ahmed S (1988) *Handbook of plants with pest control properties*. John Wiles & Sons. New York.

Gupta H C, Verma J P, Bareth S S and Mathur B N (1989) Evaluation of some nonedible oils as grain protectants in wheat and their subsequent effect on germination. *Ind J Entomol* **50**: 147-50.

Harris M, Henderson R, McCrindle R, Overton K H and Turner D W (1968) The constitution and stereochemistry of nimbin. Tetrahedron **24**: 1517-23.

Hill D S (1990) *Pests of stored products and their control*, pp 89-251. Belhaven Press.

Hillock D and Bolin P (2004) Botanical pest controls. Oklahoma Cooperative Extension Service HLA-6433. pp 4. Available online @http://osufacts.okstate.edu.

Hummel E and Kleeberg H (2002) First results of the application of a new Neemazal powder formulation in hydroponicsagainst different pest insects. *Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet* **67**(3):631–39.

Ibrahim I A, Khalid S A, Omer S A and Adam S E (1994) On the toxicology of *Azadirachta indica* leaves. *J Ethnopharmacol* **42**(1): 71-72.

Isman M B (1997) Neem and other botanical insecticides: barriers to commercialization. *Phytoparasitica* **25**: 339-44.

Isman M B (2006) Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Ann Rev Entomol* **51**: 45-66.

Ivbijaro M F (1990) Natural Pesticides: Role and Production Potential in Nigeria. *National workshop on the pesticide Industry*. Sept 24, pp 24-27. Nigeria University of Ibadan, Nigeria.

Jacobson M (1989) Botanical pesticides: Past, present and future. In: Arnason J T, Philogene B J and Morland P (ed) *Insecticides of plant origin.* Pp 1-10. American Chemical Society, Washington DC, USA.

Jarvis A P and Morgan E D (2000) Analysis of small sample of limonoids of neem (*Azadirachta indica*) using Solid Phase Extraction from tissue culture. *Phytochem Anal* **11**: 184-89.

Jarvis A P, Morgan E D and Edwards C (1999) Rapid separation of triterpenoids from neem seed extracts. *Phytochem Anal* **10**: 39-43.

Jilani G (1981) Post-harvest protection of food grains with natural insect repellants. *Progr Farming* **2**: 26-29.

Jilani G and Amir P (1987) *Economics of neem in reducing wheat storage losses: policy implications*: Tech Bull 2, Pp 15. Southeast Asian Register Center for Graduation Study and Research in Agriculture, Philippines.

Jilani G and Saxena R C (1988) Evaluation of neem oil, combination of neem oil and fumigation, and Actellic as paddy/ rice protectants against storage insects. *Proc Final Workshop on Botanical Pest Control in Rice-based Cropping Systems.* Pp 28 Int. Rice Res. Inst. Manila.

Johnson S and Morgan E D (1997) Comparison of chromatographic systems for terpenoids from neem (*Azadirachta indica*) seeds. *J Chromatogr A* **761**: 53-63.

Jotwani M G and Sircar P (1965) Neem seed as a protectant against stored grain pests infesting wheat seed. *Ind J Entomol* **27**: 199-202.

Jotwani M G and Sircar P (1967) Neem as a protectant against bruchid *Callosobruchus maculatus* (Fabricius) infesting some leguminous seeds. *Ind J Entomol* **29**: 21-24.

Kareem A, Saxena R C, Boncodin M E M, Krishnasamy V and Seshu D V (1989) Neem as seed treatment for rice before sowing: effects on two homopterous insects and seedling vigor. *J Econ Entomol* **82**: 1219-23.

Karmakar P R and Chatterjee B P (1994) Isolation and characterization of two IgE-reactive proteins from *Azadirachta indica* pollen. *Mol Cell Biochem* **131**(1): 87-96.

Karunamoorthi K, Ramanujam S and Rathinasamy R (2008) Evaluation of leaf extracts of *Vitex negundo* L. (Family: Verbenaceae) against larvae of *Culex tritaeniorhynchus* and repellent activity on adult vector mosquitoes. *Parasitol Res* **103**: 545-50.

Kasturi M, Ahamed R N, Pathan K M, Manivannan B and Aladakatti R H (2002) Ultrastructural changes induced by leaves of *Azadirachta indica* (neem) in the testis of albino rats. *J Basic Clin Physiol Pharmacol* **13**(4): 311-28.

Kaura S K, Gupta S K and Chowdhury J B (1998) Morphological and oil content variation in seeds of *Azadirachta indica A. Juss* (Neem) from northern and western provenances of India. *Plant foods hum Nutr* **52**: 293-98.

Kaur G, Sarwar Alam M and Athar M (2004) Nimbidin suppresses functions of macrophages and neutrophils: relevance to its antiinflammatory mechanisms. *Phytother Res* **18**(5): 419-24.

Ketkar C M (1976) *Utilization of neem (Azadirachta indica Juss) and its bye-products*. Directorate of Non-edible Oils & Soap Industry, Khadi & Village Industries Commission, Bombay, India.

Khalid S A, Duddect H and Gonzalez-Sierra M J (1989) Isolation and characterization of an antimalarial agent from neem (*Azadirachta indica). J Nat Prod* **52**: 922–27.

Khan S M and Marwat A A (2003) Deterrent/ Repellent effect of different plant parts of Neem and Kanair against Lesser Grain Borer (*Rhizopertha dominica* F.). *Pak Entomol* **25**(2): 131-36.

Kher A and Chaurasia S C (1997) Antifungal activity of essential oils of three medical plants. *Ind Drug* **15**: 41-42.

Khillare B and Shrivastav T G (2003) Spermicidal activity of *Azadirachta indica* (neem) leaf extract. *Contraception* **68**(3): 225-59.

Koul O (2004) Biological activity of volatile di-n-propyl disulfide from seeds of neem, *Azadirachta indica* (Meliaceae), to two species of stored grain pests, *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst). *J Econ Entomol* **97**(3): 1142-47.

Koul O, Amanai K and Ohtaki K (1987) Effects of azadirachtin on the endocrine events of *Bombyx mori*. *J Insect Physiol* **33**: 103–08.

Koul O, Isman M B and Ketkar C M (1990) Properties and uses of neem, *Azadirachta indica. Canadian J Bot* **68**: 1-11.

Koul O, Multani J S, Singh G, Daniewski W M and Berlozecki S (2003) 6-beta-hydroxygedunin from *Azadirachta indica*. Its potentiation effects with some non-azadirachtin limonoids in neem against lepidopteran larvae. *J Agric Food Chem* **51**(10): 2937-42.

Kraus W, Cramer R and Sawitzki G (1981) Tetranorterpenoids from seeds of *Azadirachta indica*. *Phytochem* **20**: 117-20.

Krishnamurthy Y L, Shashikala J and Naik B S (2008) Antifungal potential of some natural products against *Aspergillus flavus* in soybean seedsduring storage. *J Stored Prod Res* **44**: 305-09.

Kumar A, Shukla R, Singh P, Prasad C S and Dubey N K (2008) Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. *Innov Food Sci Emerg* **4**: 575-80.

Kumar P and Poehling H M (2006) Persistence of soil and foliar azadirachtin treatments to control sweetpotato whitefly, *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) on tomatoes under controlled (laboratory) and field (netted greenhouse) conditions in the humid tropics. *J Pest Sci* **79**: 189–99.

Ladd T L J, Warthen J D J and Klein M G (1984) Japanese beetle (Coleoptera: Scarabaeidae): the effects of azadirachtin on the growth and development of the immature forms. *J Econ Entomol* **77**: 903-05.

Lale N E and Mustapha A (2000) Potential of combining neem (*Azadirachta indica A. Juss*) seed oil with varietal resistance for the management of the cowpea bruchid, *Callosobruchus maculatus* (F). *J Stored Prod Res* **36**(3): 215-22.

Lal S (1988) Saving grain after harvest. In: Kasturi G (ed) *The Hindu Survey of Indian Agriculture*. Pp 246-48. National Press, Madras, India.

Levinson Z H and Levinson A R (1985) Storage and insect species of stored grains and tombs in ancient Egypt. *Z Angew Entomol* **100**: 321-39.

Ley S V, Denholm A A and Wood A (1993) The chemistry of azadirachtin. *Nat Prod Rep* **10**: 109-57.

Ley S V, Edith Beckmann, Brenda J Burke, Alistair Boyer, Sarah L Maslen and Gemma E Veitch (2007) "Synthesis of Azadirachtin: A Long but Successful Journey". *Angewandte Chemie International Edition* **46**: 7629.

Liu T X and Stansly P A (1995) Deposition and bioassay of insecticides applied by leaf dip and spray tower against *Bemisia argentifolii* nymphs (Homoptera: Aleyrodidae). *Pest Sci* **44**: 317-22.

Long Z, Hock S and Hung S (2006) Screening of Chinese medicinal herbs for bioactivity against *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* (Herbst). *J Stored Prod Res* **43**: 290-96.

Macias F A, Molinillo J M G, Galindo J C G, Varela R M, Sommenet A M and Castellano D (2001) The use of allelopathic studies in the search for natural herbicides. *J Crop Prod* **4**: 237-55.

Martinez-Villar E, Saenz-de-Cabezon F J, Moreno-Grijaba F, Marco V and Perez-Moreno I (2005) Effects of Azadirachtin on the two-spotted spider mite, Tetranychusurticae (Acari: Tetranychidae). *Exp Appl Acarol* **35**: 215-22.

Miller J A and Chamberlain W F (1989) Azadirachtin as a larvicide against the horn fly, stable fly and house fly (Diptera: Muscidae). *J Econ Entomol* **82**(5): 1375-78.

Mitchell M J, Smith S L, Johnson S and Morgan E D (1997) Effects of the neem tree compounds azadirachtin, salannin, nimbin and 6-desacetylnimbin on ecdysone 20-monooxygenase activity. *Arch Insect Biochem Physiol* **35**(1–2): 199-09.

Mitchell P L, Gupta R, Singh A K and Kumar P (2004) Behavioral and developmental effects of neem extracts on *Clavigralla scutellaris* (Hemiptera: Heteroptera: Coreidae) and its egg parasitoid, *Gryon fulviventre* (Hymenoptera: Scelionidae). *J Econ Entomol* **97**(3): 916-23.

Mohan M C, Narasimha P, Reddy N P, Devi U K, Kongara R and Sharma H C (2007) Growth and insect assays of *Beauveria bassiana* with neem to test their compatibility and synergism. *Biocont Sci Technol* **17**: 1059-69.

Mondall N K, Mojumdar A, Chatterje S K, Banerjee A, Datta J K and Gupta S (2009) Antifungal activities and chemical characterization of neem leaf extracts on the growth of some selected fungal species in vitro culture medium. *J Appl Sci Environ Manage* **13**(1): 49 - 53.

Mordue A J and Blackwell A (1993) Azadirachtin: an update. *J Insect Physiol* **39**: 903-24.

Mordue A J and Nisbet A J (2000) Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. *Anais da Soci Entomol de Brasil* **29**: 615-32.

Mordue (Luntz) A J, Simmonds M S J, Ley S V, Blaney W M, Mordue W, Nasiruddin M and Nisbet A J (1998) Actions of Azadirachtin, a plant allelochemical against insects. *Pest Sci* **54**: 277-84.

Mossini S A, de Oliveira K P and Kemmelmeier C (2004) Inhibition of patulin production by *Penicillium expansum* cultured with neem (*Azadirachta indica*) leaf extracts. *J Basic Microbiol* **44**(2): 106-13.

Moursi S A H and Al-Khatib I M (1984) Effect of *Melia azedarach* fruit on gipsing restraint stress induced ulcers in Rat. *Jap J Pharmacol* **36**: 527-33

Muda A R (1984) Utilization of neem as a pest control agent for stored paddy. In: Semple R L and Frio A S (eds) *Health and Ecology in Grain Post-harvest Technology. Proc 7th ASEAN Technical Seminar on Grain Post-harvest Technology.* Pp 117-28. ASEAN Food Handling Bureau, Manila.

Mulla M S and Su T (1999) Activity and biological effects of neem products against arthropods of medical and veterinary importance. *J Am Mosq Control Assoc* **15**: 133-52.

Murthy S P and Sirsi M (1958) *Ind J Physiol Pharmacol* **2**: 387-96.

Natarajan V, Pushkala S, Karuppiah V P and Prasad P V (2002) Anti dermatophytic activity of *Azardirachta indica* (neem) by invitro study. *Ind J Pathol Microbiol* **45**(3): 311–13.

National Research Council (1992) *Neem: A tree for solving global problems*. National Academic Press, Washington D C.

Nazli R, Jilani G, Ibrahim F, Kazmi A R and Solangi A H (2003) Repellency of neem seed oil obtained from different localities of Pakistan against red flour beetle. *Pak Entomol* **25**(2): 201-06.

Nwosu M O and Okafor J I (1995) Preliminary against Basidiobolus and some other pathogenic fungi. *Mycoses* **38**(5–6): 191–95.

Ohazurike N C, Onuh M O and Emeribe E O (2003) The use of seed extracts of the physic nut (*Jatropha curcas* L.) in the control of maize weevil (*Sitophilus zeamaize* M.) in stored maize grains (*Zea mays* L.). *Global J Agric Sci* **2**(2)**:** 86-88.

Orozco J, Soto A and Hipolito A (2006) Efecto de repelencia de *Crotalaria juncea*, *Galactia striata* and *Cymbopogon nardus* para el manejo de *Cyrtomenus bergi* (Hemiptera: Cydnidae). *Revista de Biología e Ciencias Da Tierra* **6**:179-85.

Pai M R, Acharya L D and Udupa N (2004) Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel–a 6-week clinical study. *J Ethnopharmacol* **90**(1): 99-103.

Paiva N L (2000) An introduction to the biosynthesis of chemicals used in plant-microbe communication. *J Plant Growth Regul* **19**: 131–43.

Palsson K and Jaenson T G (1999) Plant products used as mosquito repellents in Guinea Bissau. *West Africa Acta Trop* **72**(1): 39–52.

Pant N, Garg H S, Madhusudanan K P and Bhakuni D S (1986) Sulfurous compounds from *Azadirachta indica* leaves. *Fitoterapia* **57**: 302-04.

Parida M M, Upadhyay C, Pandya G and Jana A M (2002) Inhibitory potential of neem (*Azadirachta indica* Juss) leaves on dengue virus type-2 replication. *J Ethnopharmacol* **79**(2): 273-78.

Pascual N, Marco M P and Belles X (1990) Azadirachtin induced imaginal moult deficiencies in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). *J Stored Prod Res* **26**: 53-57.

Pavela R (2007) Possibilities of botanical insecticide exploitation in plant protection. *Pest Technol* **1**: 47–52.

Pavela P, Kazda J and Herda G (2009) Efectiveness of Neem (*Azadirachta indica*) insecticides against Brassica pod midge (*Dasineura brassicae* Winn.). *J Pest Sci* **82**: 235–40.

Pavela R and Holy K (2003) Effects of azadirachtin on larvae of *Lymantria dispar*, *Spodoptera littoralis* and *Mamestra brassicae*. *Hortic Veg Grow* **22**(3): 434–41.

Paul P K and Sharma P D (2002) *Azadirachta indica* leaf extract induces resistance in barley against leaf stripe disease. *Physiol Mol Plant Pathol* **61**: 3-13.

Pereira J (1983) The effectiveness of six vegetable oils as protectants of cowpeas and bambara groundnut against infestation by *Callosobruchus maculatus* (F.). *J Stored Prod Res* **19**: 57-62.

Peterson C and Coats J (2001) Insect repellents. Past, present and future. *Pesticide Outlook* 154-158.

Philogene B J R and Lambert J D H (1993) Botanical pesticides: optimizing pest control and minimizing environmental impacts. In: Forget G T, Goodman and A de Villiers (ed) *Impact of pesticide use on health in developing countries*. Pp 280-91. IDRC Ottawa.

Pillai N R and Santhakumari G (1981) Anti-arthritic and anti-inflammatory actions of nimbidin. *Planta Med* 43: 59–63.

Pillai N R and Santhakumari G (1981) Hypoglycaemic activity of *Melia azedarach* Linn. (Neem). *Ind J Med Res* **74**: 931-33.

Pillai N R and Santhakumari G (1984) Effects of nimbidin on acute and chronic gastro-duodenal ulcer models in experimental animals. *Planta Med* **50**: 143–46.

Pillai N R and Santhakumari G (1985) Effect of nimbidin on gastric acid secretion. *Ancient Sci Life* **5**: 91-97.

Prakash A O, Tewari R K and Mathur R (1988) Non-hormonal postcoital contraceptive action of neem oil in rats. *J Ethnopharmacol* **23**(1): 53-59.

Pruthi H S and Singh M (1944) Stored grain pests and their control. Imperial Council of Agric. *Res Misc Bull* No. 57.

Rahim M (1997) Biological activity of azadirachtin-enriched neem kernel extracts against *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) in stored wheat. *J Stored Prod Res* **34**: 123-28.

Rahman M F and Siddiqui M K (2004) Biochemical effects of vepacide (from *Azadirachta indica*) on Wistar rats during subchronic exposure Ecotoxicol. *Environ Saf* **59**(3): 332-39.

Rahman M F, Siddiqui M K and Jamil K (2001) Effects of Vepacide (*Azadirachta indica*) on aspartate and alanine aminotransferase profiles in a sub-chronic study with rats. *Hum Exp Toxicol* **20**(5): 243-49.

Rahman M M, Islam W and Sarker P K (2005) effect of azadirachtin on larvae and adults of *cryptolestes pusillus* (schon.) (coleoptera: cucujidae). *Pak Entomol* **27**: 1.

Rai M K (1996) In vitro evaluation of medicinal plant extracts against *Pestalotiopsis mangiferae*. *Hindustan Antibiot Bull* **38**(1–4): 53–56.

Ranasinghe M A S K (1984) Neem and other promising botanical pest control materials from Sri Lanka. *Proc Int Workshop on the Use of Botanical Pesticides.* pp 15. Int Rice Res Inst, Manila.

Rangi P S (2001) Bulk handling and silo storage of wheat. *The Tribune*, Chd. India July 9, 2001.

Rao D R, Reuben R and Nagasampagi B A (1995) Development of combined use of neem (*Azadirachta indica*) and water management for the control of culicine mosquitoes in rice fields. *Med Vet Entomol* **9**(1): 25–33.

Rojanapo W, Suwanno S, Somaree R, Glinsukon T and Thebtaranonth Y (1985). *J Sci Thailand* **11**: 177–88.

Romero C and Vargas M (2005) Extraccion del aceite de la semilla del neem (*Azadirachta indica*). *Ciencia* **13**: 464-74.

Roy M K, Kobori M, Takenaka M, Nakahara K, Shinmoto H, Isobe S and Tsushida T (2007) Antiproliferative effect on human cancer cell lines after treatment with nimbolide

extracted from an edible part of the neem tree (*Azadirachta indica*). *Phytotherapy Res* **21**: 245-50.

Ruzicka L (1932) Third pedler lecture, the life and work of Wallach. *J Chem Soc*: 1582.

Ruzicka L (1953) The isoprene rule and biogenesis of terpenic compounds. *Experentia* **9**: 357-67.

Sadre N L, Deshpande V Y, Mendulkar K N and Nandal D H (1983) Male *Azadirachta indica* in different species. *Proc 2nd Int. Neem Conf.* p 482. Rauischholzhausen, Germany.

Sagar S K and Sehgal S S (1996) Effects of aqueous extract of deoiled neem (*Azadirachta indica A. juss*) seed kernel and karanja (*Pongamia glabra vent*) seed kernel against *Culex quinquefasciatus*. *J Commun Dis* **28**(4): 260–69.

Sairam M, Ilavazhagan G, Sharma S K, Dhanraj S A, Suresh B, Parida M M, Jana A M, Devendra K and Selvamurthy W (2000) Anti-microbial activity of a new vaginal contraceptive NIM-76 from neem oil (*Azadirachta indica*). *J Ethnopharmacol* **71(**3): 377–82.

Sarup P and Srivastava V S (1971) Observations on the damage of neem (*Azadirachta indica* A. Juss) seed kernel in storage by various pest and efficacy of the damaged kernel as an antifeedant against the desert locust, *Schistocerca gregaria* F. *Ind J Entomol* **33**: 228-30.

Satti A A, Ellaithy M E and Mohamed A E (2010) Insecticidal activities of neem (*Azadirachta indica* A. Juss) seeds under laboratory and field conditions as affected by different storage durations. *Agri Biol J N America* **1**(5): 1001-08.

Saxena R C and Kidiavai E L (1997) Neem Seed Extract Spray Applications as Low Cost Inputs for Management of the Flower Thrips in the Cowpea Crop. *Phytoparasitica* **25**: 99-110.

Schmutterer H (1990) Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Ann Rev of Entomol* **35**: 271-97.

Schroeder D R and Nakanishi K (1987) A simplified isolation procedure for azadirachtin. *J Nat Prod* **50**: 241-44.

Scott I M and Kaushik N K (1998) The toxicity of margosan-O, a product of neem seeds, to selected target and non-target aquatic invertebrates. *Arch Environ Contam Toxicol* **35**(3): 426-31.

Sehgal V K and Ujagar R (1990) Effect of synthetic pyrethroid, neem extracts and other insecticides for the control of pod damage by *Helicoverpa armigera* on chick pea and pod damage-yield relationship at Pantnagar in northern India. *Crop Prot* **9**: 29-32.

Senthil-Nathan S, Kalaivani K, Murugan K and Chung G (2005) The toxicity and physiological effect of neem limonoids on *Cnaphalocrocis medinalis* (Guenée) the rice leaffolder. *Pestic Biochem and Physiol* **81**: 113.

Sharma R N, Nagasampagi B A, Bhosle A S, Mulkarni M M and Tungikar V B (1984) **Neemrich** the concept of enriched fractions from neem for behavioral and physiological control of insects. In: Schmutterer H and Ascher K R S (ed) Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss) and Other Tropical Plants. *Proc 2nd Int Neem Conf*. pp 115-28. Rauischholzhausen, Germany.

Sharma V P and Ansari M A (1994) Personal protection from mosquitoes (Diptera: Culicidae) by burning neem oil in kerosene. *J Med Entomol* **31**(3): 505–07.

Sharma V, Walia S, Kumar J, Nair M G and Parmar B S (2003) An efficient method for the purification and characterization of nematicidal azadirachtins A, B, and H, using MPLC and ESIMS. *J Agric Food Chem* **51**(14): 3966-72.

Showler A T, Greenberg S M and Arnason J T (2004) Deterrent effects of four neem-based formulations on gravid female boll weevil (Coleoptera: Curculionidae) feeding and oviposition on cotton squares. *J Econ Entomol* **97**(2): 414–21.

Shukla J, Tripathy S P and Chaubey M K (2009) Toxicity of *Myristica fragrance* and *Illicium verum* essential oils against flour beetles *Tribolium castaneum* Herbst (Coleoptera: Tenebrinoidae). *EJEAF Che* **8**(6): 403-07.

Siddig S A (1987) A proposed pest management program including neem treatments for combating potato pests in Sudan. In: Schmutterer H and Ascher K R S (ed) Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss) and Other Tropical Plants. *Proc 3rd Int Neem Conf*. pp 449-59, Nairobi, Kenya.

Siddiqui B S, Afshan F, Faizi S, Naeem-Ul-Hassan Naqvi S and Tariq R M (2002) Two new triterpenoids from *Azadirachta indica* and their insecticidal activity. *J Nat Prod* **65**(8): 1216-18.

Siddiqui B S, Afshan F, Ghiasuddin Faizi S, Naqvi S N and Tariq R M (2000) Two insecticidal tetranortriterpenoids from Azadirachta indica. *Phytochem* **53**(3): 371–76.

Siddiqui B S, Rasheed M, Ilyas F, Gulzar T, Tariq R M and Naqvi S N (2004) Analysis of insecticidal *Azadirachta indica* A. Juss. fractions. *Z Naturforsch* [C] **59**(1–2): 104-12.

Siddiqui S (1942) A note on the isolation of three new bitter principles from the neem oil. *Curr Sci* **11**: 278-79.

Sieber K P and Rembold H (1983) The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria*. *J Insect Physiol* **29**: 523–27.

Silva G, Lagunes A and Rodríguez J (2003) Control de *Sitophilus zeamais* (COLEOPTERA: CURCULIONIDAE) con polvos vegetales solos and en mezclas con carbonato de calcio en maíz almacenado. *Ciencia e Investigación Agraria* **30**: 153-60.

Silva G, Lagunes A, Rodriguez J and Rodriguez D (2002) Insecticidas vegetales: una vieja and nueva alternative para el manejo de plagas. *Manejo Integrado de Plagas and Agroecologia* **66**: 4-12.

Simmonds M S, Jarvis A P, Johnson S, Jones G R and Morgan E D (2004) Comparison of anti-feedant and insecticidal activity of nimbin and salannin photo-oxidation products with neem (*Azadirachta indica*) limonoids. *Pest Manag Sci* **60**(5): 459-64.

Singh N and Sastry M S (1997) Antimicrobial activity of Neem oil. *Ind J Pharmaciol* **13**: 102-06.

Singh R P (1993) Neem for the management of stored grain insects in developing countries. *World Neem Conf*. pp 69-80, Bangalore, India.

Singh R P, Devakumar C and Dhingra S (1988) Activity of neem *(azadirachta indica* a. juss) seed kernel extracts against the mustard aphid, *Lipaphis erysimii*. *Phytoparasitica* **16**(3): 225-30.

Stoll G (2000) *Natural crop protection in the tropics: Letting information come to life*. 2nd enlarge ededition. pp 376. Margraf Verlag 2000, Weikersheim.

Subapriya K and Nagini S (2005) Medicinal properties of neem leave: a review. *Curr Med Anti-Cancer Agents* **5**: 149-56.

Sultana B, Anwar F and Przybylski R (2007) Antioxidant activity of phenolics components present in barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica* and *Eugenia jambolana* Lam. trees. *J Food Chem* **104**: 1106-14.

Suri Z K and Mehrotra A (1994) *Neem (Azadirachta indica A. Juss.) A Wonder Tree*. Society of Forest and Environmental Managers, Dehra Dun, India.

Tanzubil P B (1987) The use of neem products in controlling the cowpea weevil, *Callosobruchus maculatus*. In: Schmutterer H and K.R.S. Ascher K R S (eds) Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss) and Other Tropical Plants. *Proc 3rd Int Neem Conf*. pp 517-20 Nairobi, Kenya.

Thomas M C and Woodruff R E (1983) First records of a stored product pest, *Oryzaephilus acuminatus* Halstead, from the western hemisphere (Coleoptera: Silvanidae). Entomol. Circular, Div. Plant Industry, Florida Dept. Agric. and Consumer Services. No. 257.

Tripathi A K, Prajapati V, Verma N, Bahl J R, Bansal R P, Khanuja S P and Kumar S (2002) Bioactivity of the leaf essential oil of *Curcumalonga* (*var. ch*-66) on the three species of stored product beetles (Coleoptera). *J Econ Ent* **95**: 183-89.

Unal S and Akkuzu E (2009) Larvaecidal effects of azadirachtin on the pine processionary moth. *Afri J Biotechnol* **8**(19): 5128-31.

Umoetok S B A and Gerard M B (2003) Comparative efficacy of *Acorus calamus* powder and two synthetic insecticides for control of three major insect pests of stored cereal grains. *Global J Agric Sci* **2**(2)**:** 94-97.

Upadhyay S N, Dhawan S, Garg S and Talwar G P (1992) Immunomodulatory effects of neem (*A. indica*) oil. *Int J Immunopharmacol* **14**(7): 1187–93.

Upadhyay S N, Kaushic C and Talwar G P (1990) Antifertility effects of neem (*A. indica*) oil by single intrauterine administration: a novel method for contraception. *Proc R Soc Lond B Biol Sci* **242**(1305): 175–79.

Valladares G, Garbin L, Defago M, Carpinella C and Palacios S (2003) Actividad antialimentaria e insecticida de un extracto de hojas senescentes de *Melia azedarach* (Meliaceae). *Revista de la Sociedad Entomológica Argentina* **62**:53-61.

Vander-Nat J M, Hart L A T, Vander Sluis W G, Van Dijk H, Vander Berg A J J, De Silva K T D and Labadie R P (1989) Characterization of anticomplement compound from *A. indica*. *J Ethnopharmacol* **27**: 15-24.

Vander-Nat J M, Van der Sluis W G, Hart L A, Van Disk H, de Silva K T D and Labadie R P (1991) Activity guided isolation and identification of *A. indica* stem bark extract constituents which specially inhibit Chemiluminescence production by activated polymorphonuclear leukocytes. *Planta Med* **57**: 65-68.

Verma S P, Singh B and Singh Y P (1985) Studies on the comparative efficacy of certain grain protectants against *Sitotroga cerealella* Oliver. *Bull Grain Technol* **24**: 37-42.

Wandscheer C B, Duque J E, da Silva M A, Fukuyama Y, Wohlke J L, Adelmann and Fontana J D (2004) Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito *Aedes aegypti*. *Toxicon* **44**(8): 829–35.

White N D G (1995) Insects, mites and insecticides in stored grain ecosystems. In: Jayas D S, White N D G and Muir W E (ed) *Stored grain ecosystems*, Pp 123-68, Marcel Dekker, New York.

Wolinsky L E, Mania S, Nachnani S and Ling S (1996) The inhibiting effect of aqueous *Azadirachta indica* (Neem) extract upon bacterial properties influencing in vitro plaque formation. *J Dent Res* **75**(2): 816-22.

Wolpert V (1967) Needless losses. *Far Eastern Econ Rev* **55**: 411-12.

Yadav T D (1973) *Studies on the insecticidal treatment against bruchids Callosobruchus maculates (Fab.) and C. chinensis (Linn.) damaging stored leguminous seeds*. Ph.D. thesis, University of Agra, India.

Yanes A, Finol H J and Hasegawa M (2004) Effects of *Azadirachta indica* and *Melia azedarach* (Meliaceae) extracts from leaves on *Trypanosoma cruzi* growth and ultrastructure. *J Submicrosc Cytol Pathol* **36**(2): 149–54

Zaidi S R, Jilani G, Iqbal J, Ashfaque M and Rafique U (2003) Settling response of Angumois moth, *Sitotroga cerealella* (Oliv.) to plant derivatives. *Pak Entomol* **25**(1): 107-12.

Zehrer W (1984) The effect of the traditional preservatives used in northern Togo and of neem oil for control of storage pests. In: Schmutterer H and Ascher K R S (ed) Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss) and Other Tropical Plants. *Proc 2nd Int Neem Conf*. pp 453-60, Rauischholzhausen, Germany.

Zeringue J and Bhatnagar D (1996) Neem and control of aflatoxin contamination. In: Singh R P, Chari M S, Raheja A K and Kraus W (ed) Neem and Environment. *Proc World Neem Conf*. Vol 2, pp 713-27, Bangalore, India 1993. Oxford & IBH Publ Co New Delhi, India,