SOME TRITERPENIC SAPONINS, SAPOGENINS AND PHYTOALKANOATES AS AZADIRACHTIN ADJUVANTS

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SOME TRITERPENIC SAPONINS, SAPOGENINS AND PHYTOALKANOATES AS AZADIRACHTIN ADJUVANTS

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This is to certify that the thesis entitled **"Some triterpenic saponins, sapogenins and phytoalkanoates as azadirachtin adjuvants"** submitted in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY IN AGRICULTURAL CHEMICALS,** to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, embodies the results of ***bona fide*** research work carried out by **Mr. Supradip Saha,** in the laboratories of the Division of Agricultural Chemicals, IARI, New Delhi under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received by him during the course of investigation and the source of literature drawn are duly acknowledged.

Place: New Delhi
Date: September 11, 2003

(Suresh Walia)
Chairman
Advisory Committee

...This thesis

is

solely dedicated

to

my beloved parents,

to whom

my success is their success

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introduction

"From the Earth came herbs and from the herbs came seed that gave life to humans"

-Taittiriya Upanishad (11.1)

Conventional pesticides in use today are inherently toxic and known to harm non-target organisms. Their excessive use over a period of time has led to several problems such as rapid development of resistance, pest resurgence, contamination of food chain and environmental hazards. Non-target organisms like domestic animals, wild life, aquatic zooplankton and above all pollinators, parasitoids, predators and microorganisms are not exempted from the curse of synthetic pesticides. Having taken cognizance of the hazards associated with these pesticides, scientists throughout the world are engaged in developing environmentally benign pesticides that can provide safe and efficient crop protection option without harming people, livestock and the environment.

Plant secondary metabolites or allelochemicals, serve no apparent function in the fundamental physiological or biochemical processes of the plant but provide natural defense to plants against insect pests and pathogens. In the past, several useful plants have shown wide range of pest control properties including phagodeterrent, growth regulatory, oviposition deterrent, fecundity, nematicidal and antifungal properties. Since the advent of synthetic pesticides, no botanical insecticide apart from pyrethrum has generated as much as interest among the scientific community and industry as has neem extractives. The neem tree *Azadirachta indica* A Juss is native to the Indian subcontinent. Parts of this tree have been used for medicine, shade, building material, fuel, lubricants and above all as pesticides that now draws interest from industrialized countries. The discovery of antifeedant activity of neem seed kernel extract for the first time (Pradhan *et al.*, 1962) gave the momentum and increased focus on this plant as potential source of crop protection chemicals. It is non-perceived as environmentally safe alternative to synthetic pesticides. Many biologically active compounds can be extracted from neem including triterpenoids, phenolics, carotenoids and ketones. Among these, the tetranortriterpenoid azadirachtin has received the most attention as

pesticide because of its relative abundance and biological activity on a wide range of insects. Azadirachtin, virtually a mixture of several congeners is known for its potent insect antifeedant and growth disruptive properties. It is isolated as a major compound from the seeds of *Azadirachta indica* (Butterworth and Morgan, 1968). Its strong antifeedant, insect growth regulatory and reproductive effects are well documented. In spite of its excellent pest control properties under laboratory conditions, its field performance is generally poor as they are slow in action, possesses short residual life and show quick degradation etc. It is thus essential to overcome such undesired traits to develop cost-effective products with improved stability and enhanced bioactivity.

Besides azadirachtinoids, saponins have been reported to possess wide spectrum of biological activities. *Madhuca indica (latifolia)* and *Sapindus mukorossi* are the two plants, which contain significant amounts of triterpenic saponins in seed kernel and pericarp of the fruit respectively. Neem oil, mahua oil and karanj oil are also known for their mild insecticidal properties. These compounds can be utilized to boost up the activity and stability of neem-based phytoproducts. Although a plant product based on the combined crude extract of neem, karanj and mahua known as RD-9 (Repelin 100E), has been developed and commercialized, such products however, remained unstanderdized due to the lack of information about the exact chemical nature of the active ingredients.

In light of these observations, studies have been conducted to develop standard azadirachtin based neem products and investigate the potential of saponins, sapogenins, and other additives such as fatty acids and their salts and esters in improving the stability and enhancing bioactivity of azadirachtin and neem oil based biopesticides.

The proposed research work has been carried out with the following objectives:

i. Isolation and identification of principal constituents such as triterpenic saponins, sapogenins from *Madhuca indica*, *Sapindus mukorossi* and phytoalkanoic acid and alkanoates from *Azadirachta indica* and *Madhuca* indica.

ii. Effect of the isolated phytochemicals on the stability and activity profile of azadirachtin-A.

review of literature

2.1 THE NEEM TREE (*Azadirachta indica* A. Juss)

The neem tree, *Azadirachta indica* A. Juss (syn. *Melia indica* Brandis, *Melia azadirachta* Linn. and *Melia parviflora* Moon) belongs to the family Meliaceae and is native to India and Burma. Neem is known for its bitterness and is recorded growing wild in Shivalik hills and in the dry forests of Andhra Pradesh, Tamil Nadu and Karnataka and has now spread to several Asian and African countries. This undemanding tree survives varied climatic and edaphic conditions and is found growing in an altitude of 15 m to 1500 m. It can tolerate long dry season and rainfall as low as 130 mm and can withstand temperature up to 49^0C. It is now planted widely in India and several other countries. Neem has high species diversity and it shows genetic variation in its widely distributed populations in India and other countries. Interestingly *A. indica* var siamensis is found wild in Myanmar, Thailand, Cambodia and Laos, wjhile other species of the genus besides neem viz., *Azadirachta excelsea* (Jack) Jacob (syn. *A. integrifolia Merr. Melia excelcea* Jack) is found in Phillipines and Indonesia etc. Ecologically, *Azadirachta excelcea* is more adapted to moist tropical conditions.

Neem has emerged as a single most important renewable source of phytoinsecticides. While all parts of the tree such as leaf, flower, fruit, seed kernel, bark, wood, twig etc. are biologically active, the maximum activity resides in seed kernel extractives and its pure constituents. These activities include insect feeding and oviposition deterrent, repellent, insect growth regulatory (IGR) activity, impairment of egg hatchability and sterilant effects. The utility of neem and its derivatives in phytonematode and plant pathogenic fungi control has also been established. Realising the importance of neem as the most versatile multipurpose tree in national, regional, and international perspective, there is an urgent need for value addition, and to develop and promote the uses of neem based botanical pesticides in pest management programmes.

Neem plant elaborates a vast array of chemically diverse and structurally complex bioactive compounds of which tetranortriterpenoids group of compounds commonly referred to as C-secomeliacins or more specifically called limonoids, are found in abundance in neem seed extracts. Some of the potential compounds of this group include salannin, desacetyl salannin (salannin group), nimbin, desacetyl nimbin, 4-epinimbin (nimbin group), and azadirachtinoids (azadirachtin group).

2.2 Azadirachtins

Azadirachtin, a tetranortriterpenoid (C_{26} - terpenoid) has received the utmost attention as bioactive molecule because of its relative abundance in neem seed kernel. Its presence in neem seed kernel varies from 0.02% - 0.66% (Bally *et al.*, 1996). It exhibits powerful biological activity against a wide range of insects (Schmutterer, 1990a). Besides antifeedant, repellent and insect growth regulatory activity, azadirachtin has properties like a partial reduction or complete inhibition of fecundity and/or egg hatchability, reduction of the life span of adults, insect sterilization, oviposition deterrence, ovicidal, formation of deformed larvae, moults between larval or nymphal instars giving rise to larval-pupal, nymphal-pupal, nymphal-adult and pupal-adult intermediates (Ascher, 1993). Most research that has been done on neem plant has focused intensively on azadirachtin because this unique key active ingredient exhibits peculiar bioactivity, as it does not immediately kill the insect like most pesticides do. Instead it actively attacks the insect's reproductive cycle, it's feeding pattern, its bodily development, as well as acting as a direct toxin. As a result of azadirachtin spray, the insect is no longer able to reproduce, eat or grow.

2.2.1 Extraction and isolation of azadirachtins

The azadirachtins are a mixture of twelve isomeric compounds, which significantly reduces the chances of tolerance or resistance development in any of the target organisms (Rembold, 1994). Kraus (1986) classified azadirachtins in three groups, namely azadirachtins, azadirachtols, and meliacarpins. According to this system of classification, azadirachtin B, G, and F belongs to azadirachtol group. Other having

the same skeletal structure with minor variations in the periphery (Rembold, 1989), such as A, E, H and L belong to azadirachtin group, and azadirachtin D and I belong to meliacarpin group. The compound, described as azadirachtin B by Rembold has also been described as 3 – tigloylazadirachtol by Kraus and co-workers (Klenk *et al.,* 1986). Furthermore, the structures of azadirachtins C, E, G and M are poorly illustrated. Several procedures have been described for the extraction and isolation of various azadirachtins and azadirachtin concentrates.

In 1940, Siddique succeeded in isolating the first major bitter principle, nimbin in a crystalline form followed by nimbinin and an amorphous bitter principle, nimbidin. Although nimbin, nimbinin and nimbidin could be isolated in pure form, their complex nature did not permit the structure elucidation for almost twenty years till the advent of new spectroscopic and chromatographic techniques. Subsequently, desacetylnimbin and two related tetranortriterpenoids namely nimbin and vepinin were characterized from the neem oil. Later a nimbin epimer, 4-epinimbin was reported from the seeds of *A. indica* by Devakumar and Mukherjee (1985).

Butterworth and Morgan (1968) were the first to isolate azadirachtin from neem seed kernel. They reported the compounds as a feeding deterrent par excellence against the desert locust *Schistocerca gregaria* at a concentration of 40 µg/litre. After that many researchers refined the procedure for its isolation. Uebel et al (1979) obtained 8.7g from 48.2 Kg of neem seed kernel and Yamasaki *et. al.* (1986) obtained 56 mg from 1 Kg of kernel. For better yields, plant material must be shade dried at ambient temperature to avoid decomposition of the active principles. Neem meliacins such as azadirachtins can be isolated by defatting the kernels employing hexane prior to methanol extraction. Based on earlier chromatographic techniques, ethanol (95%) and methanol (Schroeder and Nakanishi, 1987; Warthen *et. al.,* 1984) proved more useful. Among various solvents tested for their suitability of extracting azadirachtin, it was found that methanol, water, methyl ethyl ketone, azotropic mixture of methyl *tert* - butyl ether and methanol were most suitable (Feruhake, 1984).

Rembold (1984) described a process for the isolation of azadirachtin from neem seed kernel. The ground neem kernel obtained from neem seeds, was extracted with hexane to remove oil and the defatted powder was then extracted with acetone that after evaporation yielded brown, viscous residue containing the entire amount of azadirachtin. The compounds present in the acetone extract were then separated by column chromatography. The azadirachtin containing second fraction was further purified by reverse – phase column chromatography on a RP-8 column (54 cm length × 54 mm i.d., 100 g weight of adsorbent) with methanol – water (7: 3) as an isocratic eluant. Each time, 5 g of azadirachtin was loaded to the column and 15 ml each fractions were collected and checked by TLC for azadirachtin content.

Another method for enrichment of azadirachtin from neem seed kernel extract employed a vacuum column chromatographic technique after two successive solvent – solvent partitioning of the ethanolic neem seed extract (Schroeder and Nakanishi, 1987). From 52 g of ethanolic extract, 13 g of azadirachtin enriched (70 – 80% aza A) fraction was obtained after elution with hexane – ethyl acetate mixture (1: 3). In another method, Govindachari *et al.* (1991) extracted azadirachtin from powdered neem seed using 95% ethanol in Soxhlet apparatus. The viscous residue obtained was dissolved in 90% methanol and partitioned with hexane to remove traces of oil. The methanolic extract was processed to remove the proteins and carbohydrates, to obtain an enriched azadirachtin-A concentrate (25% aza A by HPLC). Several methods for extraction and isolation of azadirachtin from neem seed kernel were later reported including column chromatography, preparative thin layer chromatography and vacuum liquid chromatography on silica gel (Ara *et al.*, 1990; Bokel *et al.*, 1990; Rojatkar and Nagasampagi, 1993; Kraus *et al.*, 1991; Thejavathi *et al.*, 1996).

A process has been reported for the isolation of azadirachtin-A from defatted neem seed kernel following extraction with 95% aqueous methanol and then partitioned with hexane, after adding 5% brine solution (Sundaram, 1996). The resultant methanol extract was concentrated and partitioned with dichloromethane: water.

The dichloromethane layer was dried over anhydrous sodium sulphate, concentrated in a flash evaporator and the residue enriched with azadirachtin-A was subjected to column chromatography over Florisil column. Azadirachtin-A was isolated by sequential elution with ethyl acetate – hexane solvent systems. An efficient column chromatographic method for separation of azadirachtin-A from neem seed kernel extract using Florex RVM has also been reported (Johnson and Morgan, 1997).

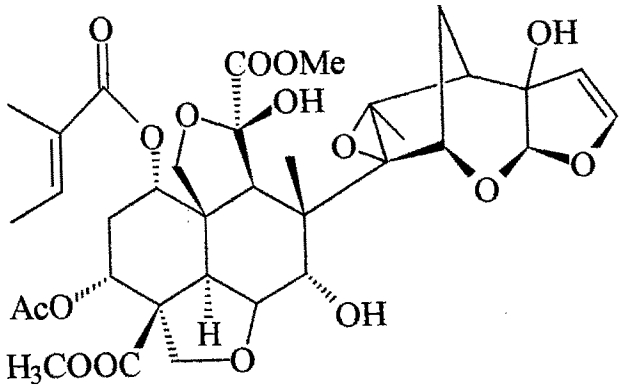
In yet another method preparative liquid chromatography was used for enrichment of azadirachtin-A from the column separated azadiractin-A rich crude product (Govindachari *et al.* 1991). Sharma *et al.,* 2003 reported an efficient method for the purification of nematicidal azadirachtins A, B and H, using reverse phase medium pressure liquid chromatography (MPLC). Unlike earlier reported methods, the reported MPLC method is comparatively simpler, more convenient, most cost-effective and less- time consuming. Among various solvent system tried, methanol: water (50:50) at a flow rate of 2 ml min^{-1} provided optimum separation. The best separation was achieved when the column was loaded with 750 mg of the test sample.

In yet another method, methanolic extract (500 mg) of neem seed kernel was injected into the preparative HPLC column (Shimpack ODS column, 25 cm × 20 mm i.d.) and the column eluted with methanol – water (60: 40) @ 15 ml/minute was run for 40 minutes, and afterwards methanol – water (70: 30) at the flow rate of 20 ml/minute (Govindachari *et al.*, 1994). The combined azadirachtin-A enriched was submitted to a second preparative HPLC run on a reverse phase column, using acetonitrile – water (28: 72) as mobile phase. Azadirachtin-A was eluted at R_t of 53.65 minutes and was recrystallized from ethyl acetate – hexane.

2.2.2 Physico-chemical properties of azadirachtin-A

Azadirachtin-A (molecular weight 720.7) is a tetranortriterpenoid meliacin having a garlic sulfur odour possibly due to sulphur impurities present in technical material. It

is light yellow to amber in colour and has melting point of 159 – 160^{0}C and a relative density of 1.4 g ml^{-1} at 24^{0}C. Johnson *et al.*, 2000 reported its melting point as 149-150°C, while Govindachari *et al.*, 1994 reported its melting point as 174°C. The highly pure sample is white in colour. The active ingredient has no real vapour pressure due to high melting point. It is stable in solvents like methanol, dichloromethane, acetone, ethylacetate, and in fixed oils. It is insoluble in hydrocarbon solvents and is completely hydrolyzed in sterile distilled water and decomposed in water after 100 h at 50^{0}C. Azadirachtin concentrate is stable at cool temperature, in the absence of water and light.



Azadirachtin-A

2.2.3 Structure elucidation of azadirachtins

Butterworth *et al.*, 1972 first reported a partial structure of azadirachtin based on IR, UV and ^{1}H NMR and mass spectroscopy. Nakanishi, 1974 and Zanno *et al.*, 1975 proposed the first complete structure of azadirachtin by applying ^{13}C - nuclear magnetic resonance spectroscopy. Since the ^{1}H and ^{13}C NMR spectra of azadirachtins were very complex, the structure based upon these spectra was tentative. Klenk, 1985 and Bilton *et al.*, 1985, later postulated provisional structures for azadirachtin. It was not until 1983, when Rembold discovered that 'azadirachtins' are mixture of several isomers, the major ones being azadirachtins A and B (Rembold, 1984). Till date, a total of twelve azadirachtin congeners have been isolated (Rembold, 1994). The confirmatory structure of azadirachtin-A was later given by Kraus *et al.*, (1985, 1986, 1987b), Broughton *et al.* (1986), Bilton *et*

al. (1987), and Turner *et al.* (1987). Govindachari and Gopalkrishnan , 1997 reported a 600 MHz PMR and 150 MHz ^{13}C NMR data of azadirachtin-A. Govindachari *et al.,* 1994 crystallized azadirachtin-A from ethyl acetate – hexane and obtained needle shaped crystals. Kabaleeswaran *et al.* (1994) first elucidated the crystal structure of azadirachtin-A by X – ray crystallography.

Very recently the structures of three azadirachtin congeners A, B and H have been characterized by their unique mass fragmentation pattern using electro spray (ESI) probe in positive ion mode. Besides their respective molecular ion peaks $[MH^+]$ at m/z 721, 663 and 663, the major fragment ion peaks for aza-A, B and H originated as a result of the sequential elimination of one neutral molecule each of water (18 amu), acetic acid (60 amu) and tiglic acid (100 amu) indicating the presence of hydroxyl, acetyl, and tigloyl functions in the molecule. Besides two major sodium and potassium adduct ion peaks [M+Na], $[M+K]^+$ with the respective molecular ions of aza A, B and H were also detected (Sharma *et al.,* 2003)

2.2.4 Structure- Activity Relationships (SAR) studies

Structure-activity relationships studies have so far mainly been carried out on insects only as very little information is available with respect to nematodes, mollusks, bacteria, fungi and other organisms. The available information cannot be correlated or compared because of the different set ups of bioassays, different test organisms and different structure types and extractives.

Morgan (1981) was the first to study the structure – bioactivity relationship of azadirachtin. Deacetylated and dihydro (22, 23 double bond) derivatives of azadirachtin-A were found to retain all its antifeedant activity against the desert locust (*Schistocerca gregaria*), but modification of hydroxyl groups by acetylation / trimethylsilylation significantly reduced the activity. Later, Rembold and coworkers (1984) reported that hydrogenation of the olefinic bond in the dihydrofuran moiety of azadirachtin had little or no effect. For activity, both lipophilic (ester function) and hydrophilic sites (hydroxyl groups) are required. Olefinic groups are not crucial for

bioactivity as 2′, 3′, 22, 23 tetrahydroazadirachtin-A was as active as azadirachtin-A (Sharma, 2002).

Blaney and coworkers (1990) bioassayed a large number of analogues of azadirachtins and gave a comprehensive description of structural requirement of azadirachtins for biological activity. Hydrogenation of C – 22/ C – 23 of the furan ring was not found to increase the antifeedant activity, but alkoxy substitution at that position increased the bioactivity. Substituents at C – 1, C – 3, and C – 11 are important in determining the antifeedant activity, but substituents at C – 1, C – 3 and presence of a D ring epoxide are critical for IGR activity. On the basis of the *Epilachna* bioassay, Rembold (1989) drew some important conclusions. For example, creation of a free hydroxy group by deacetylation of azadirachtin-A increased the activity four folds, while hydrolysis of 3-tigloyl moiety in azadirachtin B resulted in a 16 folds increase in the growth inhibitory activity. No significant difference in bioactivity was observed between azadirachtins A and D. While deducing SARs, Rembold *et al.*, (1987) concluded that C – 13/ C – 14 oxirane ring was essential for growth regulatory activity as removal of this moiety or blocking it through steric hindrance reduced the bioactivity. Hydrogenation of C – 22 / C – 23 double bond or formation of an alcohol adduct across it slightly increased the biological activity. Further, the substitution at C – 1 and C – 3 positions is another decisive factor for bioactivity. While hydroxyl function at both the positions showed the strongest growth regulating activity, the presence of only one free hydroxyl group results in decreased biological activity. In another study, Yamasaki and Klocke (1987) prepared eight azadirachtin-A derivatives, namely, 3 – deacetylazadirachtin-A, 11–O–acetylazadirachtin-A, 11–O – methylazadirachtin-A, 22, 23 – dihydroazadirachtin-A, 2′, 3′, 22,23 tetrahydroazadirachtin-A, 1 – detigloyl - 22, 23 – dihydroazadirachtin-A, 2′, 3′ - dihydroxy - 2′, 3′, 22,23 tetrahydroazadirachtin-A, 11, 20 –_ *O, O* dicarbmethoxy 22, 23 – dihydroazadirachtin-A, and compared their IGR activity against *Heliothis virescens*. It was observed that neither C-3 deacetylation nor C_{22}-C_{23} hydrogenation at the dihydrofuran moiety had any significant effect on the increase or decrease of

growth regulatory bioactivity as compared with azadirachtin-A. While acetylation at the 11 position caused a slight decrease in the bioactivity (EC_{50} = 0.18 ppm), replacement of hydroxyl group with a methoxy group resulted in a drastic reduction in activity (EC_{50} = 5.10 ppm). Ley *et al.,* (1993) compared the antifeedant efficacy of azadirachtin-A with 30 other structurally similar synthetic derivatives against African leafworm *Spodoptera littoralis.* Some of the compounds were as active as azadirachtin-A. It was reported that stereochemistry at C – 7 is crucial for bioactivity. Besides, the bridging oxygen substituent at C – 6 plays some role in antifeedant activity.

Investigations directed toward the total synthesis of azadirachtin, resulted in various intermediate compounds and containing decalin or dihydrofuran segments (Anderson and Ley, 1990; Kolb and Ley, 1991; Ley *et al.*, 1987, 1991, 1993). Compounds related to the general structure of azadirachtin were used to establish the absolute configuration of azadirachtin (Ley *et al.,* 1992). Antifeedant activity towards *Spodoptera littoralis* was found for furan moiety and its dihydro product (65.8% and 54.8%, respectively, at 1 ppm; Ley *et al.,* 1987), but much lower activity was observed with *S. frugiperda*, *Heliothes virescens,* and *Helicoverpa armigera* (Blaney et al., 1990). While the bridged epoxy dihydrofuran acetal moiety in azadirachtin is important for the antifeedant activity (Ley et al., 1989), this part of the molecule must also be important also for the IGR activity, which is markedly greater in 22, 23-dihydro-23β- methoxyazadirachtin (Baumann, 1985; Kraus *et al.,* 1991, 1993; Schwinger, 1984).

2.2.5 Biological activity of natural and stabilized azadirachtins

Azadirachtin is a potent botanical pesticide with a wide spectrum of pest controlling properties. Neem oil, crude neem extractives and pure constituents such as azadirachtins manifested acute biological activity as reflected by their multiple acute and chronic effects such as insect growth disruption, feeding, and oviposition detterence, repellant and mortality inflicted upon a variety of treated pests. These products also exhibited nematicidal, antifungal and herbicidal properties. An

excellent summary of the biological activity of the neem constituents on insects has been reported by Schmutterer (1987) and Mordue and Blackwell (1993). Most of these studies have been carried out with neem seed kernel extracts (NSKE) as well as with pure compounds like azadirachtins. There are obvious limitations in the studies using neem extractives, in which there are bound to be variations in the active principles and their relative proportions. Laboratory testing and small-scale field trials indicate that neem insecticides are effective against more than 400 pest species (Isman, 1994)

2.2.5.1 Insect growth regulatory activity

The most of the reported insect regulatory effects of neem oil and azadirachtin based products include i) delay and/ or inhibition of molt into the successive instar, ii) disturbance of molting process, iii) inoculation of malformation, iv) highest mortality between molts, v) reduced uptake of food delaying post embryonic development, vi) treated larvae may become permanent larvae which are unable to molt, vii) delay, disturbance or inhibition of ovarian development, and viii) decreased fecundity and egg sterility. These effects have now been described throughout a wide variety of insect taxa including Lepidoptera (Anarson *et al.,* 1985; Schluter *et al.,* 1985; Barnby and Klocke, 1987; Koul *et al.,* 1987); Diptera (Miller and Chamberlain, 1989); Orthoptera (Seiber and Rembold, 1983; Mordue (Luntz) *et al.,* 1985; Rao and Subrahmanyam, 1986; Ascher *et al.,* 1989; Champagne *et al.,* 1989); Hemiptera (Redfern *et al.,* 1981; Koul, 1984; Garcia and Rembold, 1984; Dorn *et al.,* 1986), and Hymenoptera (Rembold *et al.,* 1982, 1984). The developmental effects of azadirachtin-A were attributed to a disruption of endocrine events and molt inhibition due either to a total blockage of haemolymph ecdysteroid or to a delay in the appearance of the last ecdysteroid peak. In the former case, the molt would not be initiated due to the lack of haemolymph ecdysteroids leading to inhibition of molting and cuticle synthesis, whereas in the latter case, molt inhibition may occur due to prevention of the release of the neurosecretory peptide, eclosion hormone from brain, corpus cardiacum complex.

Table 1. IGR Activity of azadirachtin-A against various agriculturally important insect pests

Test insect	IGR	Reference
Peridroma plorans	0.4 ppm	Champagne *et al.*, (1989)
Peridroma saucia	2.4 ppm	Isman *et al.*, (1990)
Pieris brassicae	30.0 ppm	Arpaia & Van Loon (1993)
Spodoptera littoralis	0.06	Pflieger & Muckensturm (1987)
Rhophalosiphum padi	250 ppm	West & Mordue (1992)
Sitibion avanae	250 ppm	West & Mordue (1992)
Locusta migratoria	3.0 ppm	Mordue *et al.*, (1998)
Schistocerca gregaria	0.001 ppm	Mordue *et al.* (1998)
Schistocerca gregaria	1.0 ppm	Nasiruddin & Mordue (1994)
Halitosis zea	0.7 ppm	Kubo & Klocke (1986)
Spodoptera frugiperda	0.4 ppm	Kubo & Klocke (1986)
Pectinophora gossypiella	0.4 ppm	Kubo & Klocke (1986)
Peridroma saucia	0.26 ppm	Koul *et al.* (1990)
Oncopeltus fasciatus	3.5 ng /nymph	Warthen, J.D. Jr. (1989)
Spilosoma obliqua	2.128 mg/ml	Agarwal *et al.*, (2001)
Achoea janata	MI_{50} 4.1µg g^{-1}body wt. through injection	Rao & Subrahmanyam (1987)
Spodoptera frugiperda	1.9µg g^{-1} body wt. of insect, applied orally.	Simmonds *et al.*, (1990)
Spodoptera litura	0.29 µg g^{-1} body wt. of insect through oral application	Kumar & Parmar (1996)
Oncopeltus fasciatus	0.14 ppm	Isman *et al.*, (1990)
Locusta migratoria	2.0 ppm	Mordue *et al.*, (1985)
Locusta migratoria	1.3 ppm	Sieber & Rembold (1983)
Melanoplus sanguinipes	3.2 ppm, injection and 11.3 ppm oral	Champagne *et al.*, (1989)

These results are not only dose dependent, but also the response increases with earlier larval stages. A few insects show no mortality or metamorphic abnormalities until the final moult to an adult insect, at which time very high rates of death are observed. Moulting inhibition can be seen at very low topical and ingestion rates of one ppm. Even though this observation was made in a laboratory, under field conditions these are effective at rates much lower than those required to elicit other responses (Isman *et al.*, 1990; Schmutterer, 1990b; Stark *et al.*, 1990; Wood 1990). Studies conducted by Rembold and his group revealed that in the pharmacological range of 1-5 ppm concentration, they do not deter feeding but act as potent growth inhibitors.

The physiological effects in insects are more predictable than behavioral ones. For example, azadirachtin was equipotent as a larval growth inhibitor of six noctuid species, but as an antifeedant, bioactivity differed by more than 30 fold between the species (Isman, 1993). Bioactivity among aphid species was very variable. In one study, azadirachtin LC_{50} ranged from 2.4 ppm for the peach aphid, *Myzus persicae* to 635 ppm for the strawberry aphid, *Chaetosiphon fragaefolii.* Such differences in susceptibility among the species may be a consequence of the relative translaminar movement of azadirachtin in different host plants, rather than intrinsic differences among the different species. For example, when azadirachtin is applied to the foliage, peach aphid nymphs are more than 20 times less susceptible on corn than on mustard.

The highest IGR activity is associated with 3- detigloylazadirachtin-B (LC_{50}, 0.08 ppm) in which both hydroxyl groups are free followed by hydrogenated products namely dihydroazadirachtin-B, dihydroazadirachtin-A and the ethanol adducts 23 ethoxy 22,23- dihydroazadirachtin-A which were equally effective in exhibiting LC_{50} of 0.74 and 0.52 ppm respectively (Yamasaki and Klocke, 1987). Azadirachtins B and F were significantly more active than azadirachtin-A. The most critical structural element seems to be the 13, 14-epoxy group as any compound devoid of this function such as salannin are significantly less active.

2.2.5.2 Antifeedant effects

The antifeedant effects of azadirachtin-Are well known (Jacobson, 1989; Schmutterer, 1990; Ascher, 1993; Mordue and Blackwell, 1993). The discovery of antiffedant activity of neem seed kernel extract gave the momentum to investigate the plant thoroughly (Pradhan *et al.*, 1962). Azadirachtin affect feeding, primarily through chemoreception (primary antifeedancy) and also through gut mobility and reduction in the food intake due to toxic effects if consumed (secondary antifeedancy) (Schmutterer, 1990; Ascher, 1993). Studies have revealed that Lepidopteran insects are extremely sensitive to azadirachtin-A showing antifeedancies ranging from < 1-50 ppm. While coleoptera, Hemiptera and Homoptera are less sensitive to azadirachtin behaviourally with upto 100% antifeedancy at 100-600 ppm, orthoptera show an enormous range in sensitivity from the most sensitive *S. gregaria* (EC_{50} 0.05 ppm), through the moderate sensitivity of *Locusta migratoria* (EC_{50} 100.0 ppm) to the extreme insensitivity of *Melanopus sanguinipes* (EC_{50} > 1000 ppm). Antifeedant effects have received much attention especially in crops that suffer from excessive insect damage. The desert locust is believed to be the most sensitive insect to antifeedant effects of azadirachtin, but the migratory grasshopper feeds undeterred on the cabbage treated with 500 ppm, a rate that would deter many other insect species. Growth reduction as a preliminary indication of food refusal can be seen at 0.1 ppm azadirachtin but antifeedant activity often requires higher concentrations, usually over 200ppm.

In *Rhodinus prolixus*, antifeedant activity is observed at 600 times the amount needed to disrupt development. Gustatory and non-gustatory sensilla as well as reduced guttural motility may contribute to deterrent responses (Koul et al, 1991; Schmutterer, 1990b; Wood, 1990; Zehnder et al., 1990). Inhibition of feeding behaviour of azadirachtin results from blockage of input receptors for phagostimulants or by the stimulation of deterrent receptor cells or both (Mordue and Blackwell, 1993).

Table 2. Antifeedant Activity of azadirachtin-A against various agriculturally important insect pests

Test insect	Antifeedancy	Reference
Epilachna varivestis	FI_{50} = 0.0014%	Schwinger *et al.* (1984)
Spodoptera litura	Per cent Feeding Index (PFI) = 11.5 at 50µg/cm application	Govindachari *et al.*, (1995)
Spilosoma obliqua	EC_{50} = 1.1mg/ml	Agarwal *et al.*, (2001)
Achoea janata	54% antifeedency at 1.0 ppm conc.	Ramchandran *et al.*, (1989)
Earius insulana	100 % antifeedency at 50.0 ppm conc.	Meisner *et al.*, (1981)
Spodoptera frugiperda	43% antifeedency at 50.0 ppm conc.	Raffa (1987)
Spodoptera litura	37% antifeedency at 50.0 ppm conc.	Ramachandran *et al.*, (1989)
Acalymma vittatum	98% antifeedency at 100.0 ppm conc.	Reed *et al.*, (1982)
Leptinotersa decemlineata	No antifeedency even at 600 ppm conc.	Klocke & Branby (1989)
Myzus persicae	80% antifeedency at 100.0 ppm conc.	Nisbet *et al.*, (1982)
Eyprepocnemies plorans	85% antifeedency at 0.01 ppm conc.	Ascher *et al.*, (1989)

The relative consumption rate of *Helicoverpa virescens* larvae treated with azadirachtin was 25% of the control, attributing to the lowest assimilation efficiency of all natural insecticides tested. In another study, *Helicoverpa virescens* larvae consumed less food, gained less weight and were less efficient at converting ingested and digested food into biomass (Barnby and Klocke, 1987).

Often Lepidoptera appear most sensitive to azadirachtin's antifeedant effects with Coleoptera, Hemiptera and Homoptera being less sensitive (Mordue and Blackwell, 1993). Antifeedant activity of azadirachtin-A and parthenin against *Spodoptera litura* was compared by short-term dual choice and no choice tests. Azadirachtin-A was considerably more active than parthenin in causing antifeedancy.

Azadirachtin has been reported to reduce food intake and utilization efficiency in the penultimate instar larvae of the African armyworm (*Spodoptera exempta*) when applied either topically or mixed with the diet of the insect. At 1μg larva^{-1}, azadirachtin inhibited feeding by 50 percent and at 10 μg larva^{-1}, food intake was reduced by 80 per cent at 72 hours after treatment (Tanzubil, 1995). The reduced uptake of food has been found to delay postembryonic developments leading to abnormal insect cycle. The overlapping of antifeedant effects with growth regulatory activities accounts for disturbance of metamorphosis and reduction of insects employing pure azadirachtins. It is understood that the release of neuropeptides (BHs) disrupts the control of insect metamorphosis and behaviour on the level of molting, leading to inhibition of ecdysis. Another view is that azadirachtin blocks receptors located in the central nervous system for ecdysteroids, which are needed for larval development.

2.2.5.3 Reproduction

Interruption of insect reproduction is an important and potent effect leading to disruption of ovarian development, fecundity and fertility and prevention of oviposition. Female *S. exempta* when emerged from larvae topically treated with 0.01 and 0.1 μg azadirachtin exhibited reduced fecundity due to failure of many oocytes to mature (Tanzubil and Mc Caffrey, 1990). Hatchability of *O. fasciatus* eggs and *S. exempta* eggs finally treated with 1.25 -640 μg /100μl /100eggs, or 1 μg azadirachtin was not affected (Dorn, 1986; Tanzubil and Mc Caffery, 1990), although in the latter case, the emerging larvae were considerably less viable, probably as a result of consuming azadirachtin with the egg shall, eaten after hatching (Tanzubil and Mc Caffrey, 1990). Reproductive behaviour of females may

also be affected by azadirachtin treatment of the brown planthopper, Nilaparvata lugens (Saxena, 1989), and a prolapse of the anal brush in *Spodoptera litura* (Rao and Subrahmanyam, 1987). While a dose of 3 ppm azadirachtin caused spermatocysts to degenerate in Mamestra brassicae (Shimizu, 1988), an injection of male *O. fasciatus* with 0.125 µg/insect severely reduced male potency as seen by 80% reduction in the fecundity of normal females (Dorn, 1986).

2.2.5.4 Fungicidal activity

Effects of azadirachtin and neem extracts on fungal pathogens, including the inhibition of spore germination and mycelial growth of *Helminthosporium nedulosum* and *Pyricularia grisae* on finger millet, revealed acetone extracts of neem to be more active than the water extracts (Jagannathan and Narsimhan, 1988). Paddy (*Oryza sativa*) also suffers from fungal attack and a neem seed extract significantly reduced the infection of *Oryza sativa* seeds by *Trichoniella padwickii* (Shetty *et al.,* 1989). In addition the infection of foot rot pathogen, *Sclerotium rolfsii* has been reduced by 8 per cent by neem oil (Singh and Dwivedi, 1990). Arya, (1988) found the neem oil extract to be effective in controlling fruit rots in a laboratory experiments. Neem however is not universally effective against fungi, as 14 common pathogenic fungi (dermatophytes, yeasts and moulds) could not be affected by dry neem materials (Khan *et al.,* 1988). Muthuswamy (1988) reported that the seed extract and oil of neem seeds were highly effective in inhibiting spore germination by 96.2 and 96.0 per cent, respectively. Few spores germinated in neem oil and neem seed extract treatments showed no elongation of germ tube, thus revealing the fungicidal effects of the products. Neem seeds treated with neem seed kernel powder, neem leaf powder and neem oil inhibit the growth of *Aspergillus flavus*, *Penicillium* sp. and *Mucor* sp. (Srivastava *et al.,* 1997). Neem extract gave satisfactory rose powdery mildew control caused by *Sphaerotheca pannosa var.* rosae (Pasini *et al.,* 1997). Recently, Sharma, 2003 reported in details the fungitoxic activity of azadirachtin-A, B and H against two phytopathogenic fungi *Rhizoctonia solani* and *Sclerotium rolfsii.* It was found that azadirachtin B and H was considerably more fungitoxic than the azadirachtin-A. Fungicidal activity

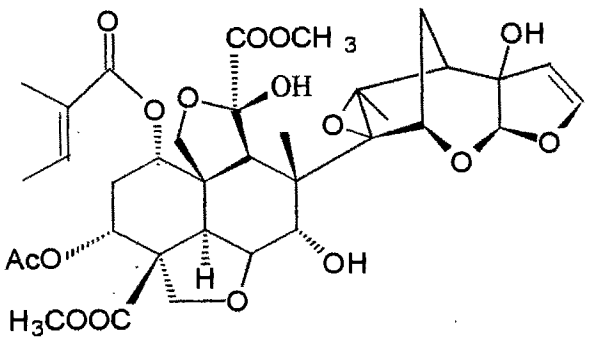
against both the fungi was more in case of azadirachtin H as compared to azadirachtin B. Of the three pure azadirachtins, azadirachtin-H was found to be the most active against *R. solani* (ED_{50}, 63.7) and *S. rolfsii* (ED_{50}, 43.9 ppm) followed by azadirachtin-B, which showed ED_{50} of 85.9 and 76.2 ppm respectively against the two fungi.

2.2.6 Photolability: Light-induced degradation of azadirachtin

Azadirachtins either break down or isomerizes rapidly in the presence of light due to the presence of photolabile unsaturated moieties such as vinyl ether and α, β – unsaturated ester [(E)-2- methyl-*b*ut-2-enoate] and other functions such as acetyl, epoxide, methoxy carboxyls (Barnby et al., 1989). Being a photolabile molecule, the degradation of azadirachtin in the field takes place at a much faster rate than in the laboratory conditions due to the influence of environmental factors (Schmutterer, 1988). Under field conditions, the residual life of azadirachtin lasts for 4 – 8 days. The rate of degradation of aza-A depends upon light intens*ity*, duration of exposure, and pathway of light, aza-A concentration, and effective wavelength of incoming radiation (Sundaram, 1996). Johnson et al., (1994) reported that in the photoreaction of azadirachtin-A in benzene solution, (E)–2– methylbut–2––enoate ester group of aza-A was converted into (Z)–2-methylbut–2–enoate. Johnson 2000) also reported the same photoproduct by exposing aza-A thin film on the glass surface to UV light (254 nm). Furthermore, when aza-A was irradiated with UV light (300 – 360 nm), the isomerized product did not form. Yakkundi et al., (1996) reported that alcohol adduct across the C-2′ – C-3′ double bond in the tigloyl moiety was obtained when aza-A was exposed to UV light in alcohol. On exposure to sunlight for 7 days, antifeedant activity of azadirachtin was reportedly reduced by more than half, as compared with the non–exposed azadirachtin against the first instar larvae of *Spodoptera frugiperda*. After 16 days of the exposure there was total loss of activity (Stokes and Redfern, 1982).

Photoproducts of azadirachtin-A, obtained through UV irradiation, however, retain some of the biological activity (Barnby et al., 1989). A significant decrease in activity

was observed in case of exposed aza-A as compared to unexposed condition (Johnson *et al.*, 2003). It was concluded that photolysis, thermolysis, volatilization and enzymatic reactions were responsible for such a short duration of persistence. Studies conducted on the persistence of aza-A on glasss slides, with or without foliar wax coating under sunlight, revealed that the loss was the highest on glass slides and the half – life was found to be 10 – 14 hours. Foliar wax coating did not stabilize azadirachtin against photolysis.



Azadirachtin-A

Unsaturation (double bond): **Oxidation, cleavage**
Ester functions (O-acetyl and O-tigloyl): **Hydrolysis or photolysis**
Others: Phototransformation, isomerisation, polymerisation, rearrangement

Chowdhuri (1996) studied the impact of UV and sunlight on the degradation of aza-A in methanolic solution as well as a thin film on glass surface. In methanolic solution, it has a half - life of 0.53 hours, but as a thin film, its half - life was 3.30 hours. In methanolic solution, aza-A was photodecomposed to a mixture of more polar transformed products. However, under sunlight irradiation, reverse result was obtained. The half - life of azadirachtin-A in methanolic solution was 1.83 days, as compared to 1.6 days as a thin film. The photodegradation of azadirachtin under sunlight on leaf surface was also studied. Here, the degradation was faster with a half-life of 0.73 days. The faster degradation of azadirachtin-A could be attributed to the moisture content of the leaf surface, absorption or translocation of azadirachtin by plant leaf, losses due to wind current, or possibly chlorophyll sensitized

phototransformation. In another experiment, the half-life of aza-A was reported as 48 minutes and 3.98 days, when the experiment was conducted as thin film on glass surface under UV and Sunlight respectively (Johnson *et al.*, 2003).

2.2.6.1 Stabilization and bioactivity enhancement of azadirachtins with stabilizers and other adjuvants

2.2.6.1.1 Photostabilization

Azadirachtin is vulnerable to various natural environmental factors (Stokes and Redfern, 1982). It has been a matter of concern, to stabilize azadirachtin with various photostabilizers including antioxidants and UV screens to achieve significant persistence. Under field conditions, degradation of azadirachtin takes place at a faster rate than in the laboratory. The residual effect of azadirachtin-based pesticides under tropical conditions usually lasts for about 4 – 5 days. However, in some plants like potato, spruce etc., where it shows systemic action, it lasts for longer duration. Therefore, utilization of azadirachtins as an insect control agent requires choice of proper stabilizers that protect it against various natural forces such as humidity, sunlight and warm conditions that are responsible for its degradation.

2.2.6.1.2 Synthetic antioxidants UV screens

Oxidation and photodecomposition are two major changes that occurs during preparation, storage and application of pesticides. Antioxidants and UV-screens therefore play a significant role in preventing undue changes and prolonging shelf life of pesticides. An antioxidant is any substance that, when present at low concentrations compared to those of an oxidisable pesticidal substrate significantly delays or prevents the oxidation of substrates. UV-screens on the other hand filler out harmful UV-rays responsible for photodeterioration or photooxidation of pesticidal substrates.

Stokes and Redfern, 1982 studied the effect of sunlight on the degradation of azadirachtin solution in acetone alone or in combination with some potential

stabilizers (1% level). In acetonic solution without any antioxidant, complete loss of azadirachtin was observed within 16 days, where as with p-amino benzoic acid (PABA), about 15 – 20% of initial azadirachtin was left even after 14 days of exposure. Antioxidants, namely citric acid, *trioctanoin* and butylated hydroxy toluene (BHT) could not, however, protect azadirachtin from photodegradation. Sundaram and Curry (1996) tested several stabilizers including lecithin as an alternative to the conventional synthetic surfactants in azadirachtin *for*mulation for the purpose of photostabilization as well as prolonging its shelf life. A noticeable photostabilization was observed both under UV and sunlight, when lecithin was *mixed* with azadirachtin in 2: 1 ratio. Three different UV absorbers viz. fluorescent brightener – 28 (Calcofluor white M2R, sodium salt of bis – triazinyl – 4,4′ - diamino–stilbene – 2,2′- disulfonic acid), Uvinul – 400® (2, 4 – dihydroxybenzophenone) and para – aminobenzoic acid (PABA) were also used to photostabilize azadirachtin-A. When mixed with azadirachtin-A in 1: 1 (w/w) ratio and exposed to sunlight, uvinul – 400 gave the highest protection as half – life of azadirachtin-A was 20 days. It was attributed to the similar absorption maxima (λ_{max}) of Uvinul – 400 and azadirachtin-A. Half–lives of unstabilized azadirachtin-A and azadirachtin-A + PABA was 5.62 and 8.68 days, respectively. The fluorescent brightener, Calcofluor white M2R on the other hand destabilized azadirachtin, with a half – life of 1.55 days, due to the energy transfer from the fluorescent bightner to azadirachtin-A. The effective stabilization of azadircht*i*n-A was achieved with 8-hydroxy quinoline and tertiarybutyl hydroquinone under both UV and sunlight out of 57 stabilizers tested, whereas *tert* butyl-p-cresol was found effective only under sunlight (Johnson *et al.*, 2003). 8-hydroxy quinoline was found to be the best stabilizer tested with the half-life of 56.8 hour and 44.42 days under UV and sunlight as compared to 48 minutes and 3.98 days in case of unstabilized azadirachtin-A. Azadirachtin-A + *tert* butyl hydroquinone or *tert* butyl-*p*-cresol showed stabilization of the molecule with the half-life of 49.50 and 2.16 hour under UV light and 35.90 and 38.50 days under sunlight respectively. Khan *et al.,* 2003 reported that adjuvants like neem oil, butylated hydroxy anisole (BHA), propyl gallate, bisphenol-

A, salanin and nimbin gave photostability of aza-A. Neem oil and BHA induce maximum stability to the compound.

2.2.6.1.3 Plant Oils and natural stabilizers

Use of plant oils particularly non-edible oils like neem (*A. indica*), karanja (*P. glabra*), mahua (*M. indica*) and undi (*Calophyllum inophyllum*) oil are well known for protecting stored grain and other commodities against pest infestation. Some of the plant oils containing other bioactive constituents have been used as stabilizers to prolong the shelf life of phytochemical pesticides.

Strokes and Edfern, 1982 evaluated a number of plant oils namely soyabean, tangerine, neem, angelica, sesame, castor, corn, calamus and olive oil to stablize azadirchtin from sunlight. 1000-ppm azadirachtin solution was mixed with these oils in the ratio of 5:1 and the mixture was thinly applied over a glass slide and exposed to sunlight for a fortnight. Neem oil and castor oil gave the greatest protection from photodegradation, followed by soybean and sesame oils. The rest of the test oils were not effective. Neem oil, turmeric oil, curcumin I and their different mixtures were used for photostabilization of aza-A under sunlight as a thin film on glass and leaf surface (Chowdhury, 1996). On glass surface, azadirachtin-A with turmeric or neem oil plus curcumin gave the highest stabilization, as about 25% of initial aza-A was left, even after six days of irradiation. Next best effect was obtained when aza-A was mixed with these two plant oils without curcumin. However, neem oil alone did not stabilize significantly. On leaf surface, similar trend was obtained, though extent of azadirachtin-A stabilization was somewhat lower. Of the various curcumins and their derivatives, Curcumin III in azadirachtin: curcumin (1:1) mixture provided the highest stability under UV light, as even after 16 hours, 48.6 % azadirachtin was still detectable. Johnson *et al.,* (2000) studied the suitability of four different phyto-oils to stabilize azadirachtin-A under UV photolytic conditions. While neem oil showed some effectiveness, the other plant oils, namely linseed, castor and olive oil had an antagonistic effect and promoted degradation. While saturated fatty acids, namely palmitic and stearic enhanced the rate of photodecomposition,

myristic, arachideic, oleic, elaidic and linoleic acids reduced it. As compared to azadirachtin ($t_{1/2}$,48 minutes), incorporation of linoleic acid and arachideic acid improved the half-life of azadirachtin to 70 and 67 minutes, respectively, under the impact of UV light. Unsaturation in the fatty acid thus promoted photostabilization of azadirachtin-A.

2.2.7 Thermal stabilization

The effect of heat on azadirachtin-A along with nimbin, and salannin was evaluated by Jarvis *et al.,* 1998). Twelve mg each of these compounds, in their solid form was taken and heated in oven at 55°C and 100°C for 24 hours. While at 55°C, there was no noticeable decline of all the 3 limonoids, about 8% of aza-A and 29% salannin were lost at 100°C. Sixteen different technical concentrates of varying azadirachtin-A content (4.06 – 33.0%) under ambient conditions (12 – 33.5°C, 41 – 78% RH) were evaluated for a period of 19 months and recorded the loss of 59.52 – 81.27% azadirachtin content during storage was observed (Kumar and Parmar, 2001). Interestingly, the loss was more in concentrates of higher azadirachtin content as compared to those with lower azadirachtin content. Niwas and Parmar (2001) reported 23.74% loss of azadirachtin-A within 14 days of incubation of azadirachtin-A in its technical powder under accelerated storage (54±1°C) condition. No loss of azadirachtin-A was, however, observed when such powder was stored at 5 ± 1° C. Kumar *et al.,* (2003) tested the ability of different stabilizers, namely thiourea, n-propyl gallate, phenothiazines, ascorbic acid, BHA, methyl paraben, propyl paraben and anthraquinone to stabilize azadirachtin concentrate with 1, 2 and 3% level of stabilizer. Under conditions of accelerated storage condition, the maximum stabilization was noticed with anthraquinone, followed by thiourea and propyl paraben.

2.2.8 Stability in aqueous solution

Butterworth *et al.* (1972) observed that azadirachtin was very sensitive to extremely acidic and alkaline conditions. Sundaram *et al.* (1995) exhaustively studied the stability of azadirachtin in buffered and natural water at different pH. At 20 °C and at

pH 4.0, 7.0 and 10.0 half – life of azadirachtin-A was 19, 12.9 days and 2 hours respectively. Further aza-A decomposition was rapid in sterilized water (pH 8.08) than in unsterilized pond water (pH 7.36). Another study conducted on the hydrolytic stability of azadirachtin-A at different pH at 35° C revealed its half – life of 11.6 days at pH 4.0 and 8.6 days at pH 6.0 (Szeto and Wan, 1996). Azadirachtin is apparently more susceptible to hydrolysis at alkaline pH, than at acidic pH. Stability of azadirachtin was the greatest between pH 4.0 to 5.0, it began to fall below pH 4 and declined rapidly above pH 7.0. Extreme alkaline and acidic conditions are detrimental for azadirachtin, where maximum degradation occurred in alkaline conditions (Jarvis et al, 1998). The temperature also played a significant role in hydrolysis of azadirachtin-A as at pH 7.0, the half – life of azadirachtin-A was 24 days at 20° C, 11.75 days at 25° C, and only 20.5 hours at 45° C.

The degradation of azadirachtin was of a lower magnitude at pH 2.3, 4.0 and 7.0 as compared to that at pH 9.2 and 0.6 (Sinha, 2001, Dureja *et al.,* 1999). Amongst the three azadirachtins, azadirachtin H was the most stable at almost all pH (except at pH 2.3). Azadirachtin A, the principal and the currently employed reference meliacin showed the poorest stability at all the pH. Azadirachtin B ranged between H and A. The pH range of 4-7 is apparently more suitable for the stability of all the azadirachtins. It appears that the structural differences among the three azadirachtins govern the extent of their stability in water.

2.2.9 Stability in organic solvents

Azadirachtin has been reported to be less stable in chloroform and deuterochloroform solution (Zanno *et al.,* 1975), owing to its slight acidic nature. This observation was later confirmed by Jarvis *et al.,* (1998), who observed no loss of azadirachtin content when dissolved in methanol, acetone and toluene and stored at 25°C under dark conditions for upto nine months. Larson (1989) found that azadirachtin-A in ethanol was more stable at lower pH than at higher pH. In another study, methanolic solution of azadirachtin (1000 ppm) stored at – 20° C remained stable for at least six months (Hull *et al.,* 1993). Jarvis *et al.,* (1998) observed that

while nimbin was comparatively stable to refluxing in methanol or methanol-water mixture, azadirachtin-A and salannin were unstable and degraded faster. The half – life of aza-A in methanol was 6.96 days at 50°C and 11.7 hours at 90°C. In aqueous solution, degradation was much faster, as the corresponding half – life was 14.9 hours and 18 minutes at 50°C and 90°C respectively, whereas these values for phosphate buffer were 9.9 hours and 8.9 minutes. Dureja *et al.* (1999) reported that aza-A was most stable in acetone and methanol than in chloroform, methyl chloride, carbon tetrachloride, ethanol and water at 29±1°C up to 25 days of incubation. Similarly azadirachtin-A degraded faster in methanol, followed by aromax, ceenine and acetone. A similar trend of loss, but of a much lower magnitude, was observed when these solutions were kept under refrigerated storage (5±1 ° C) for 14 days (Niwas and Parmar, 2001).

2.2.10 Stability in formulations

The stability of azadirachtin was studied in its emulsifiable concentrate (EC) formulation containing 0.3 – 1.0 % azadirachtin with 18 % neem oil (Schiffers *et al.*, 1997). While the nature of the solvent was crucial for the stability of azadirachtin, neem oil improved its stability of azadirachtin in neem oil based formulations. Immaraju, 1998 evaluated that only 3–5 % of azadirachtin-A was lost during one-year storage at ambient temperature and low humidity. An emulsion concentrate containing 20 per cent (v/v) neem oil and 40 per cent (w/v) aqueous extract of *Tetrapleura tetraptera* was found to be most stable when subjected to viscosity measurement, creaming rate determination, droplet size analysis and accelerated storage tests (Olaifa *et al.,* 1993). Kumar and Parmar (2000) tested the stability of azadirachtin-A in 16 different EC formulations under accelerated storage (at 54 ± 1 ° C for 14 days) and observed that nearly 96 – 99% of the initial aza-A concentration was lost within 14 days. Most of the degradation occurred by the third day of incubation. The half – life of azadirachtin-A in various formulations varied from 1.84 – 4.53 days. Further the half – life of dihydroaza-A was nearly double in EC formulations with 25% neem oil and 1.4 times in EC formulations without neem oil during 14 days of heat storage as compared to azadirachtin-A (Niwas and

Parmar, 2001). Kumar *et al.,* (2001) observed that formulation based on epoxygeneted neem oil gave the highest protection to aza-A (67.3 % degradation), followed by oleic acid epoxide (70.7 % loss). Neem oil based formulations could not stabilize azadirachtin to a considerable extent (83.7 % degradation). Among the three synthetic additives, namely phenothiazine, *n*–propyl gallate (NPG) and thiourea, NPG gave the highest protection, followed by thiourea.

In different solid carriers such as attapulgite, kaolinite, fuller's earth, hydrated calcium silicate and fly ash, azadirachtin degradation was observed to the extent of 70 – 95 % as compared to 56 % in neem oil, during the 14 days heat storage studies (Kumar and Parmar, 1999). Employing either anthraquinone or epichlorohydrin as stabilizers considerably reduced the degradation.

2.3 Enhancing bioactivity of azadirachtin through use of adjuvants, activators and synergists

Azadirachtin, a potent insecticide with exceptional environmental characteristics is needed in modern IPM programmes because it is selectively toxic, do not bioaccumulate and exhibit relatively short persistence in the environment. While being exceptionally effective at low concentrations (20g $acre^{-1}$), azadirachtin is very expensive with retail price greater than US$ 3000 per kg. Such high cost has limited its use applications and therefore efforts must be made to make its use cost effective by reducing the cost of manufacture and application. One way to significantly reduce the application cost is through the use of additives such as adjuvants, activators and synergists, which enhance both stability and activity profile of azadirachtin.

Adjuvants are commonly employed to improve the effectiveness or aid the operation of conventional pesticides. These are formulant designed to enhance the activity or other properties of a pesticide mixture (IUPAC glossary, 1996). Such materials include wetting agents, spreader, emulsifiers, dispersing agents and penetrants. While wetting and spreading agents have been shown to improve

coverage and enhance the activity, emulsifier and dispersing agents ensure uniform delivery of the active ingredient on the target site. Walter, 1999 studied in detail the possibility of improving the activity of azadirachtin with adjuvants, activators and synergists. Different formulations change the efficacy of azadirachtin. Wan *et al.,* 1996 reported that the naphthalene carrier in Azatin® increased the toxicity by 10 times to juvenile salmon than Margosan-O®. Stark and Walter, 1995 suggested that presence of other limonoid other than azadirachtin increased the activity of Neemix formulation. These limonoids have no insecticidal activity of their own at this concentration but appeared that they stimulate the activity of azadirachtin. Synergistic secondary metabolites should provide an advantage by producing a greater toxicity at a lower cost (McKey 1979; Berenbaum 1985). Adjuvants containing vegetable or mineral oil gave most satisfactory improvement of Neemix formulation. Neemix alone gave 0.25 dead whitefly per leaf after 18 days after treatment as compared to 1.1 when vegetable oil was used as adjuvants with Neemix. The antiffedant activity of aza-A against *Spodoptera litura* was considerably enhanced with the incorporation of butylated hydroxy anisole and neem oil (Choudhury, 1996; Khan *et al.,* 2003).

Azadirachtin was evaluated for the control of cabbage looper (*Trichoplusia ni*), diamondback moth (*Plutella xylostella*) and silverleaf whitefly (*Bemisia argentifolii*) in cabbage. Azadirachtin was tested at 0, 22, 33 and 44 g a.i./ha and next year azadirachtin was evaluated at 33 g/ha and in combination with commercial formulation based on potassium salts of fatty acids, M-Pede (1%, v/v). Next year two commercial (Align and Neemix) and one experimental hydrogenated (LDF) azadirachtin formulations were evaluated at 11 g/ha. First year azadirachtin alone reduced the number of cabbage looper. Azadirachtin with K salt of fatty acid reduced effectively the number of cabbage looper and diamondback moth larvae. Azadirachtin efficacy against cabbage looper and diamondback moth was enhanced when crop oil (polyol fatty acid esters with polyethoxylated derivatives) was tank-mixed with Align or LDF formulations (Leskovar and Boales, 1996). M-Pede and stylet oil have some insecticidal activity on their own but the combination

with Neemix was found to be more active (Walter, 1999). A tank mix of Neemix at 0.5 gal $acre^{-1}$ plus Xentari (*Bacillus thuringiensis var.* aisawii) at 0.2 lb $acre^{-1}$ gave better marketable yield of pepper than treatment with 1 gal $acre^{-1}$ Neemix or 1 lb $acre^{-1}$ Xentari. Synergism was evidenced when Neemix and Dipel were added in the ratio of 73:25 for the control of *Heliothis virescens*. The observed LC_{50} was 5 ppm for the mixture whereas the expected was 16.9 ppm, proving the synergism. Similar activity was found when another *Bacillus thuringiensis* formulation was used to improve the efficacy of Neemix against Colorado potato beetle, *Leptinotarsa decemlineata*.

2.4 Saponins: potential adjuvants for neem oil/azadirachtin based biopesticides

Saponins are a class of natural products that are surface-active sterol or triterpene glycosides. Chemically saponins are the glycosides which on hydrolysis yield a) one or more sugar units or their oxidation products (pentose, hexose, and/or uronic acid), and b) sugar free aglycone of polycyclic ring systems like cholanes and triterpenes and are commonly known as sapogenins. Saponins are biological detergents because of glycolysation of the hydrophobic aglycone, and when agitated in water produce copious foam. Saponins are widely distributed in both dicotyledonous and monocot plants. Saponins are localized in organelles that have high turnover rate, which implies that they are not only metabolically active but they may be important regulatory substances in the development of an organism. There are a few examples of saponins that have been isolated from animal origin e.g. sea cucumber and starfish (Wang *et al.,* 2003). Saponins have also been characterized by their hemolytic index and formation of precipitates with cholesterol in alcohol.

Saponins have been classified on the basis of their agylcone moiety and are of three types: i. Steroidal saponins, ii. Steroid-alkaloid saponins and iii. Triterpenic saponins. Steroidal saponins, give C_{27} - cholane skeleton on hydrolysis and 3'-methyl-1, 2-cyclopenteno phenanthrene (Diel's hydrocarbon) on selenium dehydrogenation. These group of saponins can be further classified as a) Spirostanolic, where F ring is closed and two oxide bridges characteristic of

spiroketal groupings are present in ring E and F and b) Furostanolic, where F ring open and glycosidation has been found to occur at C_{26} - OH group. The second group of saponins known as steroid-alkaloidal saponins, on hydrolysis produce C_{27} – cholestane type aglycone having nitrogen in a heterocyclic ring or at C_3 as amino group. N atom can be a part of single ring or can bridge two rings. The most commonly occurring third type of saponins known as triterpenoidal saponins, are derived from tetracyclic or pentacyclic triterpenic ring systems and on selenium dehydrogenation give a mixture of naphthalene and phenanthrene hydrocarbon.

Monodesmosidic saponins have a single sugar chain, which is normally attached to carbon atom-3 (C_3) of the triterpene or steroid nucleus. Bisdesmosidic saponins have two sugar chains frequently attached at C_3 through an ether linkage and another one attached either through an ester linkage at C_{28} (triterpene glycosides) or an ether linkage at C_{26} (furastanol saponins). Tridesmosidic saponins have three sugar chains and are rarely found (Waller, 1999). Bidesmosidic appear to be primarily transport form and when plant is damaged they can rapidly converted by enzymes into monodesmosidic which tend to be more active (Bissett, 1991).

2.4.1 Physical and chemical properties of saponins

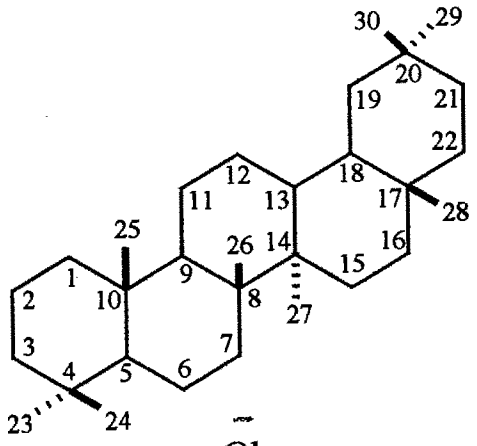
Saponins are generally non-alkaline, amorphous substances with high molecular weights and are characterized by foam formation with water giving colloidal solutions. Foaming is inhibited by the presence of most of organic solvents. Saponins with more than two sugars are rarely crystalline and are generally optically active because of the presence of a number of asymmetric centers.

The saponins on hydrolysis by mineral as well as organic acids or enzyme produce one or more molecules of the same or different sugars or their oxidation products and one or more aglycone, sapogenins. On incomplete hydrolysis saponins yield prosapogenins, saponins attached with usually one or two sugars. Saponins tend to alter the permeability of the cell wall and therefore, exert a general toxicity on all organized tissues. Their haemolytic and antilipenic activities and capacity to lower the serum cholesterol levels can be considered to be their important characteristics.

the serum cholesterol levels can be considered to be their important characteristics. The saponins are characterized by their haemolytic and fish index. While haemolytic index is the measure of the maximum dilution of saponins that is still capable of haemolysing red blood corpuscles at pH 7.48, the fish index determines their toxicity towards fishes.

2.4.2 Triterpenic saponins

Triterpenic saponins are generally the $C_{30}H_{48}$ compounds comprising of six isoprene units, which may vary in the number and positions of carbon and hydrogen atoms and may also poses additional oxygen functions. Triterpenic saponins are generally derived from pentacyclic or tetracyclic sapogenins belonging to oleanane, ursane, lupane, friedelin, dammarane, hopane and lanostane groups. The glycone part of these saponins is generally oligosaccharides, linear or branched, attached to a hydroxyl or a carboxyl group or both. The glycolysation generally observed at 3β - OH groups. However it can occur at C_2, C_6, C_{16}, C_{21} and C_{28} also (Walia, 1981). Based on site of attachment, these saponins can be classified into three groups, monodesmodic (one), bidesmodic (two) and tridesmodic (three).



Oleanane

2.4.2.1 Isolation of saponin

2.4.2.1.1 Extraction of saponin

A review of the literature shows numerous methods for the extraction of saponins. Generally powdered plant material was defatted with petrol and exhaustively extracted with alcohol or methanol at room temperature. The extract was concentrated under vacuo and extracted with ether, acetone or chloroform. Finally the extract was precipitated in acetone or tetrahydrofuran to get saponins mixture. Better yields of saponins were reported by extraction of aqueous plant extract with *n*-butanol in presence of sodium chloride (Wall *et al.*, 1952).

2.4.2.1.2 Liquid Chromatographic separation of saponin

High performance liquid chromatography (HPLC) methods are commonly employed for the separation of saponins. A large number of reports are available in literature for separation of saponins by preparative HPLC and estimation by analytical HPLC (Mahato *et al.*, 1988). In reversed phase HPLC analysis, the peak resolution between isomeric glycosides that differ from each other only in an aldohexosyl or aldopentosyl unit is not always sufficient (Yamaguchi *et al.*, 1986). Three different monodesmosidic saponins from pericarp of *Sapindus mukorossi* were separated by reacting these glycosides into their borate complex with the help of HPLC ion exchange resins. Good separation of each saponin was obtained with the mobile phase of 0.4M boric acid solution in 20% (v/v) aqueous acetonitrile with flow rate of 0.5 ml min^{-1} and column temperature of 80^0C. This method also used to separate 7 ginsenosides isolated from ginsgeng roots and 3 huzhangosides from roots of *Anemone rivularis* (Yamaguchi *et al.*, 1986). Two dammarane saponins were isolated by preparative HPLC on a ODS silica gel column using methanol-water (11:9) as the mobile phase (Zhou *et al.*, 1981). Semipreparative μ-Bondapak C_{18} column and mobile phase acetonitrile-methanol-water (1:1:1) was used for the successful separation of desulfated triterpenoid oligosides from *Actinopyga agassizi* (Kitagawa *et al.*, 1982). Similarly nine triterpene glycosides were isolated from *Medicago sativa* roots by preparative LC employing method developed by Oleszek, 1988. The separation process used C_{18} column (2.5 × 40 cm) and water - Methanol

gradient system. These saponins fractions were further separated by high-resolution chromatography using different columns (Oleszek *et al.,* 1990). In another study saikosaponin c, a and d were successfully separated from *Bupleurum falcatum* by preparative liquid chromatography at the purity level of more than 94%. The purity of the saponins was confirmed by TLC, HPLC and FAB-mass spectrometry (Kim and Park, 2001). The concentration of individual saponins was measured in germinating alfalfa seeds and seedlings of 1 to 16-days old, by HPLC. Total saponin concentration increased from 2.12-µmol/g dry matters at the beginning of germination to 6 µmol/g after 8-16 days of seedling growth. It was concluded that previous reports on saponin concentration in alfalfa seedlings (8-10% in dry matter) as measured by biological tests were overestimated, and that the actual concentration may be several times lower (Oleszek, 1998). Oat kernel saponins were analyzed by reverse phase liquid chromatography using octyl silica column and gradient elution of acetonitrile in water. Two different avenacoside saponins were detected by photodiode array detector from oat meal (Onning and Asp, 1993). Saponin of Shao-yao-tang was analyzed by HPLC at 275 nm with a linear gradient elution system consisting of tetrabutylammonium bromide and acetic acid solution was suitable (Sheu *et al.*, 1998). 13 triterpene glycosides were analyzed by HPLC using 5 µm RP-18 column and water/acetonitrile gradient elution system from different *Astragalus* species within 40 minutes. By using an evaporative light scattering (ELS) detector, the main saponins of *A. membranaceus* could be detected at levels as low as 20.0 µg/ml (Ganzera, *et al.,* 2001).

2.4.2.2 Structure determination

A liquid chromatographic/mass spectrometric method involving electrospray ionization together with up-front collision-induced dissociation, tandem MS or MS^n fragmentation was developed for rapid on-line characterization of triterpene glycosides. Sensitive detection was obtained by post-column addition of alkaline buffer, providing intense deprotonated molecular ions. The experimental method provided information on the sugar sequence and aglycone of each saponin within a crude plant extract. The method was applied for the rapid estimation of saponin

content in methanolic and aqueous extracts of dried berries of the molluscicidal plant, *Phytolacca dodecandra* (Perret *et al.*, 1999). Triterpenoid saponins from *Acanthopanax senticosus* leaves were analyzed by ESI-MS^n. Analysis were performed in positive ion mode with mobile phase of methanol at a flow rate of 3ml min^{-1} with spray voltage of 4.8 kV (Guo *et al.*, 2002). For structure elucidation of saponins of *Quillaja saponaria* ion trap multi-stage tandem mass spectrometry was used as a pre-NMR tool (Setten *et* al., 2000). The technique allowed elucidating the sequence and branching of 6 out of 7 glycosidic linkages.

Five sapogenins and three saponins were identified in bulbs of 14 cytologically defined *Cyclamen* species and their structures were determined by means of mass, ^1H and ^{13}C NMR spectroscopies (Reznicek *et al.,* 1989). Three triterpene glucosides were identified by spectroscopic (^1H NMR, ^{13}C NMR and MS) and chemical methods (hydrolysis and acetylation). Two of the compounds were identified as tormentic acid ester glucoside and 23-hydroxytormentic acid ester glucoside, respectively (Gopalsamy *et al.,* 1988). New triterpene diglucoside was isolated from the methanolic extracts of the fruits. Its structure was established on the basis of hydrolysis and spectral evidence including COSY, NMR and NOE studies (Sotheeswaran *et al.*, 1989). Analysis of the methanol extract of the defatted leaves of *C. montana* yielded a new oleanolic acid-based saponins, characterized by ^1H-NMR and ^{13}C-NMR spectroscopy, FABMS and chemical studies (Jangwan and Bahuguna, 1990). The roots of *G. paniculata* and *G. arrostii* (soap root, Saponariae alba radix) are known as saponin drugs and have been used as detergents and expectorants. Four new triterpenoid saponins were isolated from an aqueous ethanol (1:1) extract of soap roots (*Gypsophila paniculata* + *Gypsophila arrostii*). Their structures were elucidated using a combination of homo- and heteronuclear 2D NMR techniques, without chemical degradation or modification. Two saponins were composed of gypsogenin and another two of quillaic acid as their aglycone part (Frechet *et al.*, 1991).

2.4.2.3 Bioactivity of saponins

2.4.2.3.1 Insect antifeedant activity

Monodesmosidic triterpenoid saponins, 3-O-[O-β-D-glucopyranosyl-(1-4)-β-D-glucopyranosyl]-hederagenin from *Barbarea vulgaris* evidenced antifeedant activity against diamondback moth, *Plutella xylostella* larvae. When the compound was applied to cabbage leaf disk at greater than 0.18 μg mm^{-2} , consumption of the disks by third instar larvae was less than 11% of control. All the instars died on the disks treated with same concentration. The antifeedant activity of saponins was confirmed by evaluating the concentration of saponins present in fresh leaves of *B. vulgaris* was comparable to the effective dose in the cabbage leaf disk tested (Shinoda *et al.*, 2002).

2.4.2.3.2 Antifungal activity

The effect of different concentrations of standard liquorice saponin were evaluated on 18 species of fungi including *Rhizopus oligosporus*, *Fusarium solani*, *Aspergillus niger*, *Penicillium roqueforti* and *Trichoderma viride*. The response of *T. viride* and *F. solani* to saponin isolated from fermented and unfermented liquorice water extracts was also assessed. Results indicated that radial colony growth decreased with increasing concentration of saponin from 0.0002 to 3%, but that this was dependent on the species of fungi. While, saponin completely inhibited growth of *P. roqueforti*, *A. niger* was however more tolerant, demonstrating only 14.14% inhibition at 3% saponin concentration. Radial colony growth of *T. viride* and *F. solani* was 46.64 and 13.45% inhibited, respectively for 8 h (Gamal, 1992). The study conducted on antimycetic activity of triterpenoid saponins from *Solidago virgaurea* L. revealed that the alkaline hydrolysed saponin mixture showed marked inhibitory activity against *Candida albicans* (Bader *et al.*, 1987). Another triterpenoid saponin, identified as Mi-saponin A was isolated from the roots of *Clerodendrum wildii* (Toyota *et al.*, 1990). It was found effective against *Cladosporium cucumerinum* and *Polysticus versicolor*. No fungicidal activity was, however observed against *Candida albicans* (Charrouf, 1991).

2.4.2.3.3 Plant growth promotion and inhibition

Depending upon the chemical structure, some of the triterpenic saponin has been found effective on both plant growth promoter or growth inhibitor. Studies have been revealed that soyasaponin VI stimulates the growth of cells in lettuce roots, *Lactuca sativa* to around 190% of the control (Tsurumi and Tsujino, 1995). The entire molecule of soyasaponin Vi was required for the maximum stimulation of lettuce, whereas, only 10-15% growth stimulation was found for soyasaponin I (Tsurumi and Wada, 1995). The growth stimulation induced by soyasaponin Vi was highest in lettuce, followed by chrysanthemum, leaf mustard, timothy, Italian ryegrass, white clover, salt green, alfalfa, milk vetch and Japanese hornwort.

Soyasaponin I, III and a new saponin inhibited the growth of mungbeans and lettuce growing for 72 hours (Waller *et al.,* 1995; Waller, 1999). Another saponin, helojaposide isolated from *Heloniosis japonica*, which showed a significant inhibition action at 10 ppm for the root growth of rice (ohara *et al.,* 1995). Alfalfa saponins showed high allelopathic potential against plants, fungi and microorganisms (Miller *et al.,* 1992; Oleszek *et al.,* 1992; Oleszek *et al.,* 1996). Though the problem of alfalfa saponins being excreted into the soil and the accumulation in the soil-humus complex was not estimated, 2-24% of saponins were recovered from the humic acid in a range of soils in the laboratory (Okumura *et al.,* 1998).

2.4.2.3.4 Molluscicidal activity

Unlike the bidesmosides the monodesmosidic saponins from unripe berries from *Phytolacca dodecandra* showed increased molluscicidal activity not the (Waller, 1999). The fruits of *Swartzia madagascariensis* have been traditionally used for controlling schistosomiasis-transmitting snails. On investigation it was found that the monodesmosidis glycosides present in the fruits were responsible for the activity (Hostettman *et al.,* 1995). Water extract of the pods exhibited significant activity against *Bulinus globosus* snails at dilution of up to 100 mg ground pods per liter.

Furthermore, half-lives of the saponins were in the range of 12-24 h, thus reducing the risk of human toxicity.

2.4.2.3.5 Spermicidal activity

Yang and Li (1995) studied the activity of polar glycosides, which occur in Chinese medicine such as ginsgeng (*Panax ginsgeng*), south china ginsgeng (*P. notoginsgeg*), and licorice (*Glycrrhizia uralensis*). The saponins of *Oreosolon wattii* showed strong inhibitory activity towards human spermatozoa. At a concentration of 0.01 mg ml^{-1}, mimengoside-A had the same effect as that of nonoxynol, a known spermicidal agent at 0.1 mg ml^{-1}. The triterpenic saponin constituents in *Sapindus mukorossi* have also showed inhibitory activity against both human spermatozoa and ova.

2.4.2.3.6 Nematicidal activity

Triterpenic saponins, albichinin II and sonunin III isolated from *Acacia concinna pods* and steroidal saponins asparanin 1 and asparanin B obtained from *Asparagus adescendens* were found to be posses nematicidal activity affecting larval mobility of root knot nematode, *Meloidogyne incognita* (Meher *et al.,* 1988). Among triterpenic saponins sonunin III showed activity with LD_{50} value of 282 μg ml^{-1}, while asparanin B showed activity with the LD_{50} of 134 μg ml^{-1} after 72 hour of exposure.

2.4.2.4 *Madhuca indica* (Mahua)

Madhuca indica J. F. Gmel. (*syn. Bassia indica, Diploknema indica*) is a medium size to large deciduous tree from the family sapotaceae found in central and northern India and Malaysia. It grows up to an altitude of 1200M. Mahua is valued for its oil-bearing seeds and flowers, which are utilized for alcoholic beverage production. The tree bears ovoid shaped berries up to 5 cm long greenish turning reddish yellow or orange when ripe; seeds 1-4, brown coloured, ovoid shaped, 2.5-3.75 cm long. Under favorable soil and climatic conditions, mahua trees begin to bear fruit at the age of 8-10 years and continue to do so for about 60 years. Mahua fruits ripen in May-June. The seeds are separated from the fruit wall by pressing

and then dried and shelled to get the kernel. Kernels are 70% of the weight of seed and it constitute the mahua seed of commerce. Seed kernel constitutes 51.1% oil, 8.0% protein, 27.9% N-free extract, 10.3 % fiber and 2.7% ash.

2.4.2.4.1 Mahua oil

Fresh mahua oil is yellow in colour and a not unpleasant taste. At temperature prevailing in most part of India during major part of the year, the oil is fluid and in cold weather it solidifies to a buttery consistency.

Table3: Physico-chemical properties and fatty acid composition of mahua oil

Property	Value
Physico-chemical	
Refractive index	1.458-1.462
Specific gravity	0.856-0.870
Saponification value	188-200
Iodine value	53-70
Peroxide value (meq kg^{-1})	0.24
Unsaponifiable matter	1-3%
Fatty acid composition (%)	
Palmitic acid	17.8
Stearic acid	14.0
Oleic acid	46.3
Linoleic acid	17.9
Linolenic acid	1.7
Arachidic acid	0.9
Total unsaturated	65.9
Total saturated	32.7

On the basis of iodine value mahua oil have been classified as non-drying oil. The peroxide value (0.24 meq kg^{-1}) of the oil indicates that it may have low levels of

oxidative and lipolytic activity or contains high level of natural antioxidants. The glyceride structure of the oil is reported to be as dipalmito-stearins (1%), oleo-dipalmitins (1%), oleo-palmitostearins (27%), palmito-dioleins (41%) and stearo-dioleins (30%). Mahua oil contains 65.9% total unsaturated and 32.7% saturated fatty acids and more specifically it constitutes palmitic (17.8%), stearic (14%), oleic (46.3%), linoleic (17.9%), linolenic (1.7) and arachidic (0.9) acid.

2.4.2.4.2 Mahua saponins

In addition to mahua oil, mahua seed kernel contains 2.5% saponins and 0.5% tannins and defatting of seed kernel increased the saponins (9.8%) and tannin (1.0%) contents. These saponins have toxic effect (Birk, 1969; Pederson *et al.*, 1972). The rats fed with mahua meal containing 5-6% saponins at a level of 10-12%, died in a month (Mulky, 1976). Extraction of saponins from *Madhuca indica* seed kernel involves defatting of seeds followed by extraction with ethanol and then concentrated to suryp. It was macerated with ether or acetone and residue was worked up for saponins following extraction with n-butanol. By this method saponin mixture was separated as yellow powder (Hariharan *et al.*, 1972). Another method involves decortication of mahua seeds and defatting of powdered seeds with petrol. The defatted material was then extracted with ethanol. The ethanol extract was concentrated to a syrupy concentrate after drying under vacuo. The concentrated extract was precipitated in large excess of acetone. On repeated precipitation saponins separated as amorphous light brown soild (Misra *et al.*, 1991).

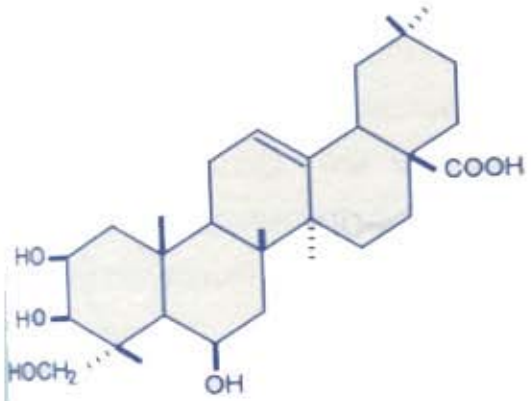
A Method has also been reported for purification of crude saponins by reverse phase HPLC and identified using standardised methods (Massiot and Lavaud, 1995). The method involves a combination of both chemical and spectroscopic techniques. Triterpenoid saponins were isolated as Mi-saponin A and Mi-saponin B. Saponins are bidesmosidic and their aglycone is either protobassic acid (Charrouf et al., 1992) or 16-α-hydroxyprotobassic acid (Charrouf et al., 1992), both belonging to the D-12 oleanane family.

2.4.2.4.3 Isolation and characterization of saponins and protobassic acid

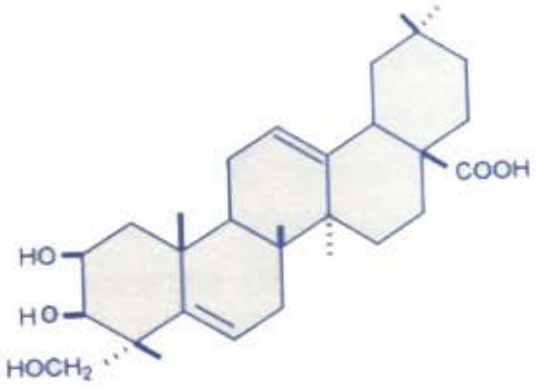
Kitawawa *et* al., 1975 reported two major saponins named Mi-saponin A and Mi-saponin-B from seed kernels of *Madhuca longifolia* and the saponins were elucidated as 3-O-β-D-glucopyranosyl –28-O- [α-L-rhamnopyranosyl (1-3)-β-D-xylopyranosyl (1-4)-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl]-protobassic acid and 3-O-β-D-glucopyranosyl –28-O-{3-O-β-D-apio-D-furanosyl-4-O-[α-L-rhamnopyranosyl(1-3)-β-D-xylopyranosyl]-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl]-protobassic acid.

In another study two different saponins namely butyroside A and B isolated from *Madhuca butyracea*, were chemically characterized as 3-O-β-D-glucopyranosyl protobassic acid –28-O-β-D-apio-D-furanosyl (1-3)-β-D-xylopyranosyl (1-4)-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranoside and 3-O-β-D-glucopyranosyl 16-α-hydroxy protobassic acid –28-O-β-D-apio-D-furanosyl (1-3)-β-D-xylopyranosyl (1-4)-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranoside (Nigam *et al.*, 1992).

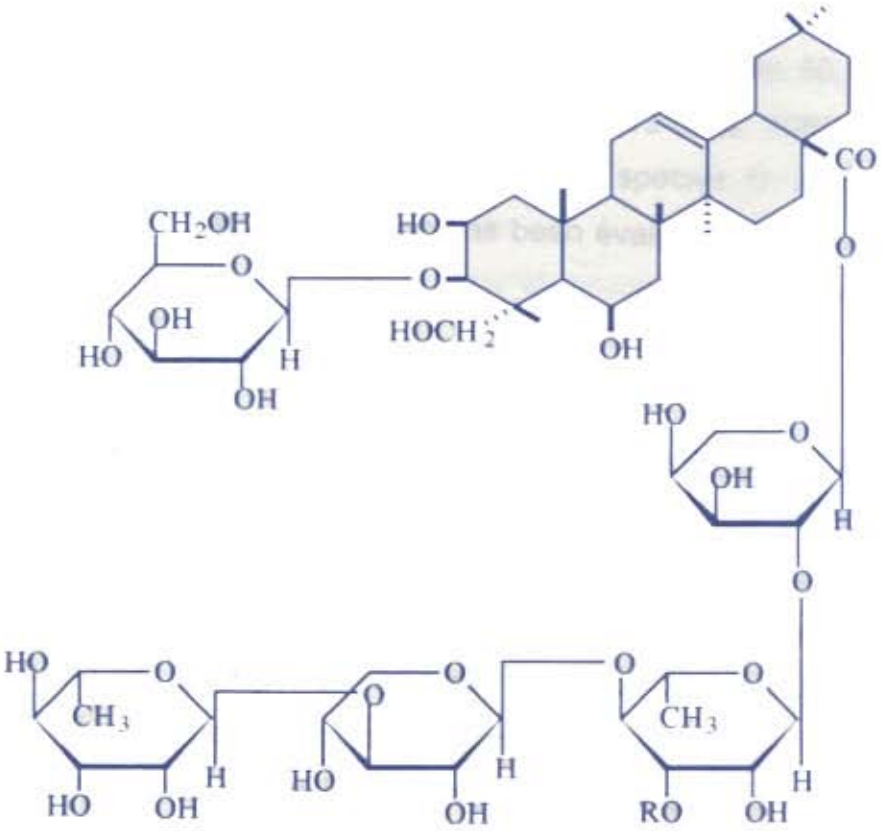
Saponin was converted to its aglycone sapogenin by refluxed with 7% H_2SO_4 in 80% aqueous methanol for 6 hour. After addition of water and removal of solvent the sapogenin, protobassic acid was filtered and chromatographed over silica gel, $CHCl_3$ –MeOH (19:1 v/v) to elute pure acid (Hariharan *et al.*, 1972).



Protobassic acid

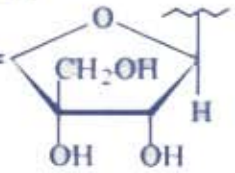


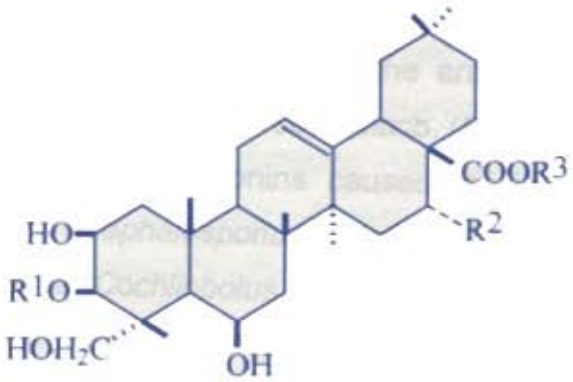
Butyraceol



Mi-saponin A: R = H

Mi-saponin B: R =





Butyroside A: R^1: Glc; R^2: H; R^3: Ara (2 ← 1) Rha (4 ← 1) Xyl (3 ← 1) Api

Butyroside B: R^1: Glc; R^2: OH; R^3: Ara (2 ← 1) Rha (4 ← 1) Xyl (3 ← 1) Api

The structure of protobassic acid was established as 2β, 3β, 6β, 23-tetrahydroxy-olean-12-en-28 oic acid (Yosioka *et al.*, 1974). Misra *et al.*, 1991 reported another sapogenin from the seeds of another *Madhuca* species *M. butyracea*. The new aglycone moiety of madhuca saponin has been evaluated as olean-5, 12-dien-2β, 3β, 23-triol.

2.4.2.5.4 Bioactivity

Combined extracts of *Madhuca indica, Azadirachta indica* and *Pongamia glabra* in the formulated form as RD-9, Repelin® 100E at 1000 and 2000 ml $acre^{-1}$ were evaluated for the control of jassid larvae and white fly adults of cotton. RD-9 controlled both the insects effectively. The activity of RD-9 was comparable to dimethoate (Rogor® 30EC) at 250 ml $acre^{-1}$ (Dhawan *et al.*, 1992). When evaluated against *Heliothis armigera* on cotton and chickpea at 1 and 2% level, it was not as effective as synthetic insecticide quinalphos (Simat *et al*, 1992). RD-9 Repelin (mixture of *Madhuca indica, Sesamum indicum, Ricinus communis* and *Pongamia glabra*) when evaluated against crucifer flea beetle (*Phyllotreta cruciferae*) on canola and found antifeedant activity 1-2 days at 12% and for 6 hour at 1.2 and 6% (Palaniswamy and Wise, 1994). Mahua oil cake showed moderate activity against third instar larvae of white grub (*Leucopholis burmeisteri*) (Padmanavan *et al.*, 1997).

Lalitha and Venkataraman (1991) reported the antifungal activity and mode of action of saponins from *Madhuca butyracea* Macb. (*Diploknema butyracea*) against four different fungi. The test saponins caused maximum growth inhibition of *Penicillium expansum*, *Cephalosporium acremonium* [*Acremonium strictum*], *Helminthosporium oryzae* [*Cochliobolus miyabeanus]* and *Trichoderma viride* (LD_{50} <500 p.p.m.). They also observed growth stimulation for *Botryodiplodia theobromae*, *Pythium* sp. *Penicillium* sp. and *Fusarium* sp. In shake cultures of *T. viride* containing 500, 1000 or 2000 p.p.m. of saponins, *T. viride* showed a sharp growth increase after reaching a min. at 6 h. The saponin concentration decreased steadily over the experimental period, but remained constant in uninoculated

controls. It is suggested that the fungus degraded the saponins. A dose-dependant increase in proteins and nucleic acids in the cell free medium was observed in saponin treated mycelia of *T. viride*, indicating that saponins cause leakage of cellular components into the medium. This was supported by the reduction in the trichloroacetic acid extractable in the saponin treated mycelia. It is concluded that the saponins cause the leakage of small molecular weight compounds. Dev *et al.*, 2002 reported that crude extract of *M. indica* seed showed 10.6 to 36.3 % inhibition at 0.5% concentration and 21.2 to 50.0 % fungal growth inhibition at 1.0% concentration against *Drechslera sorokiniana, Collectotrichum graminicola, Fusarium solani, Phomosis sojae and Macrophomina phaseolina*. A few preliminary biological data have been obtained on Mi saponins (Charrouf, 1991); the crude mixture presents some promising molluscicidal activity against *Biophalaria glabrat*a, (the intermediate guest of *Shistosoma mansoni*).

2.4.2.6 *Sapindus mukorossi* GAERTN

Sapindus mukorossi GAERTN (Ritha) with its large leaves is a handsome deciduous tree of the Indian subcontinent. The species is widely grown in upper reaches of the Indo-Gangetic plains, Shivaliks and sub-Himalayan tracts at altitudes from 200m to 1500m. It belongs to the main plant order Sapindaceae and family Sapindeae. It's known as soap-nut tree, one of the most important trees of tropical and sub-tropical regions of Asia. It is also called ritha, doadni, doda and dodan in Indian dialects. Ritha is a common tree in Shivaliks and the outer Himalayas of Utter Pradesh, Uttranchal, Himachal Pradesh, Haryana and Jammu and Kashmir. In this entire region, starting from Afghanistan in the west to China in the east, it is found growing naturally in suitable tracts. This tree flourishes in deep clayey loam soil and does best in areas experiencing nearly 150 to 200 cm of annual rainfall. Ritha flowers during summer, the fruit appears in July-August and ripens by November-December. These are solitary globose i.e. round nuts of 2 to 2.5 cm diameter, fleshy, saponaceous and yellowish brown in color. The seed is enclosed in a black, smooth and hard globose endocarp. The fruit is collected during winter months for seed and or sale in the market as soap nut.

The dried fruit of Ritha is most valuable part of the plant. Its fleshy portion contains saponin, which is a good substitute for washing soap and is as such used in preparation of quality shampoos, detergents, etc. In fact, the skin of the fruit is highly valued by the rural folks as a naturally produced shampoo for washing their hair. They also use these for washing woolen clothes. This is why some botanists have named the species as *Sapindus detergens*. The fruit is of considerable importance for its medicinal value as well. Ayurvedic, Unani and Tibetan systems of medicine consider it to be useful for treating a number of diseases like common cold, pimples, epilepsy, constipation, nausea, etc. It is also used as expectorant and enthelmintic in small doses. The Central Drug Research Institute, Lucknow, has recently developed a contraceptive cream out of Ritha fruit, under the trade name 'Consap'.

2.4.2.6.1 Sapindus oil

S. mukorossi seed oil contained palmitic, stearic, oleic, linoleic, arachidic, eicosenoic, behenic and docosenoic acid as fatty acid component and a non-glyceridic component, a cyanolipid, 1-cyano-2-hydroxymethyl prop-1-ene-3-ol (Sengupta and Basu, 1982).The seed kernel cake of *Sapindus mukorossi* contained 31.8% crude protein, 7.9% total nitrogen and oil content 36% (Dev *et al.*, 1979a, 1979b, Iqbal *et al.,* 1996). The fatty acid composition of the total lipids was C12:1 (0.3%), C14:0 (traces), C16:0 (4.6%), C18:0 (traces), C20:0 (3.5%), C18:1 (56%), C18:2 (5.8%), C18:3 (1.4%) and C20:1 (28.4%). The fatty acid composition of Sapindus mukorossi seed oil was determined by spectrophotometry, urea complexation, and gas liquid chromatography (GLC). The percentages of individual acids were found to be: palmitic, 4.0; stearic, 0.2; arachidic, 4.4; oleic 62.8; linoleic, 4.6; linolenic, 1.6; and eicosenoic, 22.4 (Sengupta *et al.*, 1975).

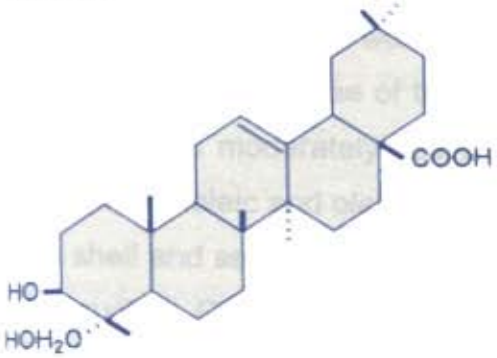
2.4.2.6.2 Sapindus saponins

A review of the literature shows numerous methods for the extraction of saponins. Generally powdered plant material was defatted with petrol and exhaustively extracted with alcohol or methanol at room temperature. The extract was

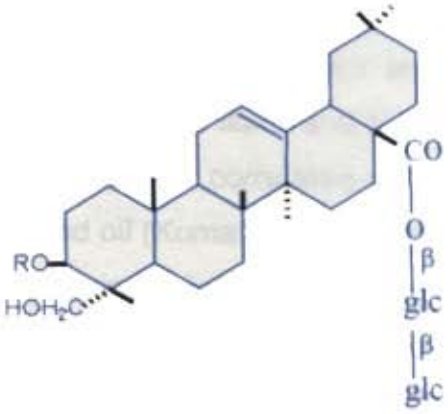
concentrated under vacuo and extracted with ether, acetone or chloroform. Finally the extract was precipitated in acetone or tetrahydrofuran to get saponins mixture (Walia, 1981). Better yields of saponins were reported by extraction of aqueous plant extract with *n*-butanol (Wall *et al.*, 1952). Kimata *et al.*, 1983 reported that *Sapindus* saponin was extracted by defatting the dried material with benzene and then extracted with hot methanol. The resulting methanolic extract was subjected to repeated chromatography on columns of highly porous resin, silica gel and silanized silica gel to isolate saponins.

2.4.2.6.2.1 Chemistry of *Sapindus* saponins

A crystalline saponin, mukorosside was isolated from the pericarp of the *S. mukurossi* fruit. This saponins on hydrolysis produced hederagenin, l-arabinose, d-glucose, l-rhamnose and d-xylose (Gedeon, 1954). In 1969, Chirva *et al.*, isolated 5 glycosides from the same fruit as sapinoside - A, B, C, D and E. Sapinoside - C, D and E contain glucose, arabinose, xylose and rhamnose. Sapinoside-A contains arabinose and rhamnose while sapinoside B possess an O-acyl glycoside bond. Sapinoside–A and B were characterized as 3 (α-L-arabinopyranosyl-2-α-L-rhamnopyranoside), and 3 (α-L-rabinopyranosyl-2-α-L-rhamnopyranosyl-3-β-D-xylopyranoside) of hederagenin respectively (Chirva *et al.*, 1970a; Chirva *et al.*, 1970b).



Hederagenin



Mukurozi-saponin X: R: α ara α rham
Mukurozi-saponin Y_1 : R: α ara α rham β xyl
Mukurozi-saponin Y_2 : R: α ara α rham α ara

From pericarp of *Sapindus mukurossi* a number of monodesmosidic saponins were isolated and three bisdesmosidic saponins were also reported. The structures of bisdesmosidic saponins were elucidated as β-sophorosyl ester of 3-O-α-L-rhamnopyranosyl (1-2) –α-L-arabinopyranosyl hederagenin (Mukuroz-saponin X), 3-O-β-D-xylopyranosyl (1-3)-α -L-rhamnopyranosyl (1-2)-α -L-arabinopyranosyl hederagenin (Mukuroz-saponin Y_1) and 3-O-α-arabinopyranosyl (1-3)-α -L-rhamnopyranosyl (1-2)-α -L-arabinopyranosyl hederagenin (Mukuroz-saponin Y_2) (Kimata *et al.*, 1983).

The basic skeleton of these saponins is of oleanane type and being bisdesmodic, one glycone moiety is attachted with hydroxyl, while another sugar chain with carboxylic acid group. Saponins of *S. mukurossi* have been used as a source of natural surfactants (Nakayama *et al.,* 1986). The saponin isolated from *S. mukorossi* has been shown to have strong anti-inflammatory and haemolytic activity (Park, 1995).

2.4.2.6.3 Insecticidal activity

Sapindus mukorossi and *Murraya koenigii* extracts (0.5%) have been found to effectively control the mustard aphid, *Lipaphis erysimi* when evaluated for their antifeedant/insecticidal properties (Srivastava and Kumar, 1999). Petroleum ether extracts of ritha seeds were tested at up to 1000 p.p.m. for antifeedant activity against 4th-instar larvae of the noctuid *Spodoptera litura* and found that *Sapindus trifoliatus* was moderately active. The activity was correlated with the percentage content of linoleic and oleic acid in the seed oil (Kumar and Thakur, 1988).

The shell and seed powder of soapnut (*Sapindus trifoliatus*) was evaluated against the bruchid, *Callosobruchus chinensis* on stored seeds of cowpea. Mortality was low initially, but increased with time until after 1 week all treatments were significantly superior to the untreated variant. After a period of 5 months, the mean 6.9 per cent damage was found to the seeds with soapnut shell powder as compared with 2.2 for malathion at 15 p.p.m (Yadava, and Bhatnagar, 1987).

A new saponin, named 3-beta-O- [α-L-rhamnopyranosyl (1→3)-beta-D-glucopyranosyl]-hederagenin was isolated from the fruit peel of the medicinal plant, *S. saponaria*, collected from Ceara, Brazil. The compound showed activity against *Pseudomonas aeruginosa, Bacillus subtilis* and *Cryptococcus neoformans* (Lemos *et al.*, 1992). A monodesmodic acetylated saponin and a mixture of 2 monodesmosidic saponins were isolated from the methanol extract of the fruits of *S. saponaria*. The compounds showed molluscicidal activity (LC_{100} values of 5-10 p.p.m.) against *Biomphalaria glabrata*, one of the intermediate hosts of schistosomiasis. The three saponins have been previously reported from other *Sapindus* species (Ribeiro *et al.*, 1995).

The molluscicidal activities of saponins from *Sapindus saponaria* (berries), and gypsogenin of *Saponaria officinalis* were tested against *Biomphalaria alexandrina* and LC_{50}s of 13, and 20 ppm were obtained, respectively. Corresponding LC_{98}s were 80, and 120 ppm. Prolonged exposure to 500 ppm saponin solution from *S. officinalis* gave 100% mortality after 5 days (Motawe, 1994). Monodesmoside, saponin A, B and C and a mixture of bisdesmosides, saponin X, Y1 and Y2 which were isolated also from *Sapindus mukurossi* (Yata *et al.*, 1986).

Sapindin™ is Sabinsa's trademark for Soapnut Saponins, has been prepared from the fruit (nuts) of the Soapnut tree, *Sapindus trifoliatus*. The Soapnut tree is a rich source of saponins that function as a mild cleanser and antimicrobial (Tanaka *et al.*, 1996). Extract of the pericarp mixed with DDT were used as a fungicide and insecticide (Iqbal *et al.*, 1996; Chopra *et al.*,1942; Uppal and Metha, 1952).

2.4.2.6.4. Antifungal activity

Seed extract of *Sapindus trifoliatus* was tested in laboratory against *Sclerotium rolfsii* (*Corticium rolfsii*), causal agent of stem rot of tuberose. At 10% level, the extract showed significant antifungal activity causing 82.7% growth inhibition of the fungi (Das *et al.*, 1997). Methanolic extract of *Sapindus emarginatus* was studied against growth and aflatoxin production of *Aspergillus flavus* and it was found that

the methanolic extract caused 79% inhibition of aflatoxin production (Shankarrao, *et al.*, 1994). *In vitro* and *in vivo* studies were conducted to determine the effect of *Sapindus trifoliatus* on the mycelial growth of *Corticium rolfsii* by Pani and Patra, 1997. Seed extract of *Sapindus trifoliatus* suppressed the growth of the fungus significantly. Water extract of *Sapindus* sp. prepared by macerating smashed pericarp in water with or without prior heating at 60^0C for 24 hours was tested against *Cryptococus neoformans* and *Candida albican* based on modified agar-disc-diffusion method. It was observed that average diameter of inhibition zone was 18 mm in comparison to 36.76 mm diameter resulted from using ketoconazole (Wuthi-udomlert *et al.*, 2000). Soapnut (*Sapindus trifoliata*) inhibited the growth of *Drechslera hawaiiensis* [*Cochliobolus hawaiiensis*], causal agent of papaya leaf blight when the phytoextract was evaluated using a paper disc method (Mistry and Vala, 1998).

2.4.2.6.5 Molluscicidal activity

The molluscicidal activities of saponins from berries of *Sapindus saponaria*, and gypsogenin of *Saponaria officinalis* tested against *Biomphalaria alexandrina* showed LC_{50}s of 15 and 20 ppm, respectively. Corresponding LC_{98}s were 43 and 120. Prolonged exposure to 500 ppm saponin solution from *S. officinalis* gave 100% mortality after 5 days (Motawe, 1994). *S. mukorossi* saponins have also been shown to have strong anti-inflammatory activity. In this study, the acute toxicity, and local irritant and haemolytic activities of the saponin and its sapogenin component, hederagenin, were examined. The acute toxicity of the saponin in mice was very low. Based on LD_{50} values, it showed much weaker toxicity following oral administration than intraperitoneal injection. Hederagenin had a very high LD_{50} value even when administered by intraperitoneal injection. The saponin showed potent local irritation after topical application, whereas hederagenin showed only

weak local irritation. The saponin also showed marked haemolytic activity (Park , 1995).

2.5 Phytoalkanoates

2.5.1 Free fatty acids and esters from *Azadirachta indica, Madhuca indica* and *Pongamia glabra*

Most insects have dietary requirement of fatty acids. However, presences of certain fatty acids in diet have been reported to inhibit insect growth and cause mortality in insects (Mc. Farland, 1978). Free fatty acids have been traditionally used in many Asian and African countries as insecticides to protect grains especially legumes against stored grain pests (Ahmad *et al.,* 1988). Among straight chain fatty acids ($C_9 - C_{11}$) nonanoic (C_9) to undecanoic (C_{11}) acids were found to be most effective as for as insect growth regulatory activity is concerned. These acids were found to be most effective in preventing oviposition of *Callosobruchus maculates* (F.) and causing mortality to *Sitophilus oryzae* adults. Pelargonic (C_9), capric (C_{10}) and undecanoic (C_{11}) acids were the most effective among the lot in terms of oviposition deterrent. The activity was remarkably decreased by the lower (C_5-C_7) and higher acids (C_{17}-C_{18}); the C_{12}-C_{16} acids showed weak activity. At concentration ranging from 0.4-1.6 g kg^{-1}, these compounds exhibited strong insect repellent activity. Among different acids C_{11} acid showed the maximum activity followed by C_9 and C_{10} acids. Interestingly, conversion of fatty acids to corresponding methyl esters resulted in total loss of activity. Fatty acids were also found active against *Sitophilus oryzae*. At 8 g kg^{-1} C_7-C_{11} acids were lethal to adults and acids with higher (C_{12}-C_{19}) or lower (C_5-C_6) number of carbon atoms were weak or not active. Among the active acids C_9 acid was also found to be most active at 2g kg^{-1} application rates. Lalonde *et* al., 1979 reported that capric, lauric and myristic acids exhibited mosquitocidal activity against *Aedes triseriatus* in the range of LC_{50} as 0.0004-0.0014% whereas the higher fatty acids such as palmitoleic, oleic and linoleic acid provided excellent control of *Callosobruchus chinensis* (LC_{50} 435 ppm). These compounds were however, moderately active against *Sitophilus oryza* and *Tribolium castenium.*

The insecticidal activity of linoleic, lauric [dodecanoic] and oleic fatty acids was studied against *Callosobruchus maculatus* on seeds of cowpeas. While, dodecanoic acid had no effect on the development of progeny at conc. between 1.96 and 11.5 g/kg of seed, oleic and linoleic acids on the other hand were active in reducing the development of progeny. Pure oleic acid had an LC_{50} of 1.64 ml/kg seed and was 3 and 8 times more toxic against eggs of *C. maculatus* than groundnut oil and linoleic acid, respectively. The LC_{50}s for the ovicidal activity of acetone-based solutions of dodecanoic, oleic and linoleic acids was 40, 38 and 26 ml $litre^{-1}$, respectively, making them 2-4 times more toxic than acetone-based solutions of groundnut and traditional coconut oils (Don-Pedro, 1990).

Puritch, 1975 reported fatty acids for killing balsam woolly aphid, *Adelges piceae* (Ratz.). They were in two major groups, one centered on capric and caprylic acids within the shorter-chain fatty-acid series, and the other on oleic acid within the unsaturated C_{18} fatty-acid series. Long-chain fatty acid esters of hexadecanoic, arachidonic and octadecanoic acids isolated from *Chenopodium ambrosioides* (fruits), *Conyza dioscordis* (flowers) and *Convolvulus arvensis* (leaves) were highly active against the stored grain pests *Sitophilus granarius* and *Tribolium castaneum.* (Peterson *et al.*, 1989). Ethyl esters of oleic, palmitic, stearic and linoleic acids and the methyl ester of oleic acid were found as assembling scents for the dermestid *Trogoderma granarium* and as repellents to the tenebrionid *Tribolium castaneum.* Methyl oleate at 1.5 and 2.1 µg cm^{-2} and ethyl oleate and ethyl stearate at 1.05µg cm^{-2} had the highest repellency to *T. castaneum.* Methyl oleate and ethyl linoleate had good assembling activity for females and males of *Trogoderma granarium*, respectively, at 100µg. The mixtures of all these esters at the levels of their natural secretion and at the levels found to be active (2.1 and 1.05µg/cm2, respectively) showed maximum repellency to *Tribolium castaneum.* The activity of these esters and their mixtures as both assembling and repelling scents was generally low compared to reported values (Pereira *et al.*, 1987).From the hexane extract of *Dirca palustris* seed, linoleic and oleic acid were isolated by Ramsewak *et al.*, 2001 and

found insecticidal activity against fourth instar *Aedes aegyptii* larvae and exhibited potent feeding deterrent activity against neonate larvae of *Helicoverpa zea*, *Lymantria dispar, Orgyia leucostigma,* and *Malacosoma disstria*.

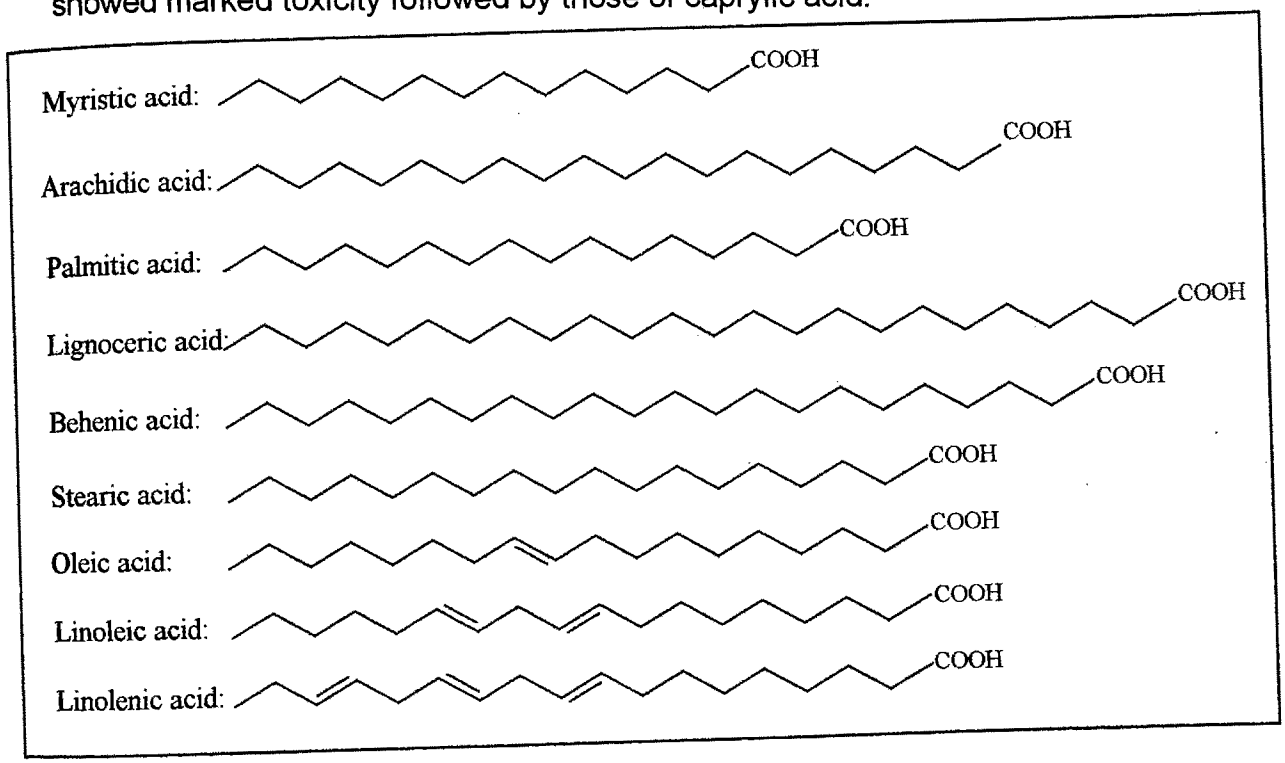
The methyl esters that caused considerable mortality and melanisation on larvae of *Musca domestica* were methyl octanoate and methyl nonanoate, which were approximately equal in toxicity and melanogenic action. Methyl trans-2-octenoate, methyl trans-2-nonenoate and methyl trans-2-decenoate showed marked melanogenic action and, like their saturated analogues, created an imbalance in the series of successive events essential for the formation of normal puparia. Methyl octyne-2-oate and methyl nonyne-2-oate were very toxic, causing complete mortality when applied topically at 0.2 µl larvae^{-1}, but they were not melanogenic. With the exception of ethyl hexanoate and ethyl heptanoate, the ethyl esters were less toxic and less melanogenic than the corresponding methyl analogues (Quraishi, 1972). The effects of ethyl fatty acid ester adjuvant (EOP) on the efficacy of several classes of insecticides were examined both in the laboratory and in the field (Killick and Schulteis, 1998). Vicchem EOP was evaluated for the control of crickets (*Teleogryllus commodus*) and aphids (*Myzus persicae*). In laboratory studies, EOP at 2.0% v/v enhanced the efficacy of beta-cyfluthrin and deltamethrin three-fold against crickets, whereas the activity of dimethoate and carbaryl was not enhanced. In field studies EOP at 1.2% v/v increased the efficacy of methomyl and endosulfan against aphids and worms on maize and imidacloprid (Provado®) against aphids on cotton. It may also soften the cuticle of insects, which would facilitate increased uptake of active ingredients.

The efficacy of 4 selective insecticides against the flatid, *Metcalfa pruinosa* was evaluated in pot and field studies to assess their suitability in biological control programmes. The insecticides tested were a mineral oil (Biolid E®), a fatty acid (Myx 400®), paraffin mineral oil (UltraFine Oil (U.F.O.), and U.F.O. + a natural pyrethrin. All the insecticides resulted in significantly increased mortality of the flatid compared to the control in pot experiments 24 and 48 hours after treatment, and in

the field experiments on pear trees 2 and 5 days after treatment. In general, the mixture of paraffin mineral oil + natural pyrethrin (at a dose corresponding to about 15 + 2.25 litres ha^{-1}) was most effective against the insect among the experimental insecticides tested (Cornale *et. al.*, 1998).

2.5.2 Salts of Fatty acids:

Siegler and Popenoe have reported insecticidal property of fatty acid salts against a number of aphids as early as in the year 1924. No significant toxicity has been encountered with the lower homologues of fatty acid derivatives, the caproic acid showed marked toxicity followed by those of caprylic acid.



They also reported the fatal activity of capric, lauric and myristic acid soaps against same aphids. After experimenting with a number of fatty acid fractions, potassium salt of capric acid (C_{10}) showed maximum activity. Commonly used soaps containing potassium salts and coconut oil are effective in controlling many soft-bodied insects such as aphids, caterpillars, crickets, fleas, flies, and mites. Several commercial products are available on the market under trade names such as M-Pede®, Acco Plant Wash®, Safers Insecticide Concentrate® and several other

Safers-brand products. For the potassium salt soaps, saturated fatty acids containing 10 carbon molecules are most insecticidal. Eight carbon and 12 carbon fatty acids also produce soaps with high insecticidal activity compared to other carbon chain lengths. Safers Insecticidal Soap is derived from oleic acid and a 10-carbon saturated fatty acid. Salts produced by unsaturated fatty acids are most insecticidal around the 18-carbon chain. In another significant study azadirachtin efficacy against cabbage looper and diamondback moth was enhanced when crop oil (polyol fatty acid esters with polyethoxylated derivatives) was tank-mixed with Align or LDF formulations (Leskovar and Boales, 1996). A miticidal composition consisting of an insecticidal soap containing a mixture of monocarboxylic acids and the alkali metal salts having 15 and 18 carbon atoms including oleic acid and one or mixture of ((Phenyl-C $(-CH_3)_2-CH_2-)_3-SN-)_2-O$ has been patented (Gorman and Puritch). An insecticidal composition consisting of Bt originated insecticide and sodium, potassium or ammonium salt of an unsaturated fatty acids having 18 carbon atoms selected from the oleic acid, linoleic acid and a mixture thereof in an amount sufficient to synergistically enhance the efficacy of the bacterial insecticide has been patented (Gaudet and Puritch).

The response of some common forest fungi to certain fatty-acid compounds in the form of water-soluble K salts (soaps) was tested by an agar-plate bioassay method and found effective against *Fomes annosus* and *Ceratocystis ulmi*, *Europhium clavigerum* and two other *Ceratocystis* spp. at 1% concentration. K-oleate was found non-toxic to these fungi. (Puritch and Etheridge, 1975). The K soaps were more effective than the corresponding acids; the soaps of caprylic, capric, oleic and linoleic acids were the most effective. The eggs were less sensitive to the soaps than later stages of the aphid, and there was a large variation in their response to soap treatments. (Puritch, 1975)

Following application of K salts of fatty acids to *Botrytis cinerea* in agar culture, growth inhibition increased with the number of C atoms of the fatty acid salts, reached a peak around C_{10} (caprate), and then declined. Salts of dicarboxylic acids

were nontoxic at the highest levels tested, while the unsaturated C11:1, undecylenate, was as potent as caprate. Caprate at 0.1% inhibited growth of the fungi isolates and prevented the remainder from forming sclerotia. Conidial germination of all isolates was prevented by 0.05% caprate. Fatty acids formulated as potassium salts significantly and consistently reduced powdery mildew, caused by *Sphaerotheca pannosa var.* rosae severity (Pasini *et al.*, 1997). Not only potassium salts, copper salts of fatty acids have also been found effective against a wide range of plant diseases of flowers, fruits, & vegetables. Concern copper soap fungicide™ is a patented, fixed copper fungicide, made by combining a soluble copper fertilizer with a naturally-occurring fatty acid.

2.5.3 Plant Oils

Oils are safe, eco-friendly, and biodegradable with minimal effects on the natural enemies. No species of insect has developed resistance to oils even after many decades of their continuous use. Oils therefore can safely be applied to complement chemical, biological, and cultural methods of pest control to combat insect resistance, environmental pollution, and the emergence of secondary pest problems in a variety of field and garden crops. Oils produce a variety of effects on insects, mites, and fungal plant pathogens. Petroleum oils block respiratory holes (spiracles) through which insects and mites breathe, causing them to die of asphyxiation or suffocation. They may act as poisons by interacting with the amino acids and, eventually, the intercellular structures. Oils may also disrupt insect feeding, which is important in the transmission of some plant viruses by aphids. Ovipositional deterrent effects have also been observed in fruit flies and thrips. Petroleum oils also provide UV shield when used with biopesticides. Oils exhibit fungicidal and fungistate action against fungal pathogens. They increase resistance the of the host plant by changing its physiology. Oils also act as carriers for ultra low volume (ULV) and low volume (LV) sprays. They are used as adjuvants, spreaders, and stickers in various pesticides to facilitate distribution, holding them to the leaf surface, resist weathering, and enhance pesticide absorption by the insects (Khajuria and Gupta, 2003).

Glyceridic oil extracted from the neem plant (*Azadirachta indica* A. Juss) is well known to contain biologically active substances such as azadirachtin and limonoids. Neem oil produce antifeedant, repellent, metabolic-inhibiting, toxicant, chemosterilant, ovipositional deterrent, and ovicidal effects on a variety of insects. They also possess good fungicidal properties. Similarly, chinaberry (*Melia azedarach* L.) oil produces neem like effects and oil from pongam tree (*Pongamia pinnata* (L)) contains karanjin, which produces antifeedant, juvenile hormone analogues (JHA) and toxicant effects on insects. Plant oils such as those derived from neem, mahua and karanj have been found effective against various insect pests including stored grain insect *Callosobruchus chinensis*. Two applications of neem oil (2%) at 30 and 50 days after sowing were found effective against sesame pod borer, *Antigastra catalaunalis* as it resulted in less damage for up to 7 days after each treatment (Muralibaskaran *et al.*, 1993). Neem, karanj and mahua oil (1.0% each) when tested against nymphs of citrus blackfly, *Aleurocanthus woglumi* and results revealed that neem oil 1.0% was as effective as 0.025% phosalone up to 7 days after treatment and superior to karanj and mahua oils (Katole *et al.*, 1996). Ten vegetable oils including neem and karanj oil each at 0.5, 0.75, and 1% concentrations were tested as seed protectants of pigeon pea against *Callosobruchus chinensis.* Their effect on adult mortality and oviposition up to 100 days of storage at the 1% level was studied. Among various treatments, neem and karanj oil showed a significant repellent action for egg laying to adult bruchids (Khatre *et al.*, 1993). In another experiment, oil cakes of karanj, neem and mahua at the rate of 8.5, 19.0, 34.53, g/pot basis were tested against third-instar larvae of white grub, *Leucopholis burmeisteri*. Karanj oil cake was found to be comparatively more effective than the other plant products after 30 days of application (Padmanaban *et al.*, 1997).

Karanj oil gave highest grain yield (12.9 q/ha) with 44% pod damage in 1992-93 as compared to 81.7% damage in control in a field experiment tested against *Helicoverpa armigera* infesting chickpea (Bajpai and Sehgal, 1999). Among the

three pesticidal formulations, the cost: benefit ratio was highest for endosulfan (14.8) followed by Green Mark® (6.3) and karanj oil (5.1) in first year.

Neem oil (1 %), neem seed kernel extract (2%) and neem leaves extract (2%) were the most effective against capsule borer, *Antigastra catalaunalis* and phyllody in sesame and resulted in high yields. Results were compared with 0.07% endosulfan with yield of 372 kg ha^{-1} to 290 kg ha^{-1} for neem oil (Singh and Singh, 1997). Mahua oil (1 and 2 %) was found to be most effective, followed by neem seed kernel extract (2 and 5%), respectively as compared to dimethoate (0.05%) with the lowest aphid population (0.00/plant) against the rose aphid, *Microsiphum rosae* after twenty-four hours of spray. A moderate level of reduction was encountered in 1 and 2% karanj oil (Reddy *et al.*, 2002). The neem seed kernel powder, neem oil and mahua oil proved very repulsive, potent oviposition inhibitor and acted as effective protectant of pigeon pea during storage against *Callosobruchus chinensis*. These treatments protected the seed for 8 months with negligible seed damage. Egg laying was observed only on 0-1% treated seeds compared to 45.3-59.3% on the untreated seeds (Singal, *et* al., 1998).

The 2nd instar rice leaf folder larvae were found more susceptible on 24 hr old films of three concentrations of neem oil (2.00, 3.00 and 4.00%), NSKE and monocrotophos(0.03, 0.04 and 0.05%). The maximum mortality however was observed with monocrotophos (Baitha *et* al., 2000). Neem oil treated beans showed 100% adult mortality of *Callosobruchus chinensis*. Very few eggs were laid after three days of release. Neem oil completely inhibited adult emergence and appeared to be most promising as a seed protectant against *C. chinensis* (Ahmed *et* al., 1999). Neem oil (1.0%) was also found promising against tobacco aphid, *Myzus nicotianae* (Patil *et* al., 1999).

Neem oil, karanja oil and mahua oil at dosages of 0.5 and 1.0% level effectively protected green gram from pulse beetle, *C chinensis*. These oils caused a significant reduction in oviposition and adult emergence. Neem oil at one per cent

level gave the best protection, followed by karanj and mahua oil. These oils also exhibited contact toxicity, and no adults could survive in neem-treated green gram at 5% concentration (Reddy *et* al., 1999). Neem oil 5, 10 and 15 ml kg^{-1} seeds also caused in 76 to 88% adult mortality in green gram at zero days to 38 to 52% at 120 days after treatment (Kalyan and Dadhich, 1999). Neem products such as Achook, Neem Gold and 3% neem oil significantly reduced the thrips population, with resulting in 28-29% lower population, than no treatment against *Scirtothrips dorsalis* on groundnuts (Senguttuvan, 1999). Neem oil (Nimbecidine, 1%) was toxic to the adults of *Sitophilus oryzae, Sitotroga cerealella, Rhyzopertha dominica, Trogoderma granarium* and *Tribolium castaneum*. Neem oil (Nimbecidine, 2%) effectively reduced the emergence of F1 and F2 progeny of all the pests and completely protected maize up to 9 months (Sharma, 1999). Neem oil was found better than neem kernel extract in significantly reducing safflower aphid, *Dactynotus carthami* populations in safflower with either wheat or gram intercropping (Swaminathan *et al.,* 1999).

Neem oil and karanj oil based emulsifiable concentrate (EC) formulations, viz., neem oil 60 EC (acetic acid) [NO 60 EC (Acetone)], neem oil 60 EC (citric acid) [NO 60 EC(C)] and neem oil + karanj oil 60 EC (citric acid) [NO+PO 60 EC(C)], were evaluated for their efficacy against sheath rot (*Sarocladium oryzae*) of rice and found effective in inhibiting mycelial growth of the causal pathogen under *in vitro* conditions. These formulations effectively controlled rice sheath rot and led to increased yield in 5 field trials. Among the various treatments, the formulation NO 60 EC (A) achieved the highest grain yield in 4 out of 5 field trials, with a pooled mean grain yield of 4684 kg/ha *vs* 3882 kg/ha in the control. NO 60 EC(A) achieved the maximum cost-benefit ratio of 1:4.8, followed by NO+PO 60 EC(C), with 1:3.3 (Narasimhan *et* al., 1998). A 2% aqueous emulsion of the clarified neem seed oil was moderately fungicidal to *Botrytis cinerea* and *Glomerella cingulata* in inoculated fruit, but had little activity against *Penicillium expansum*. Neem seed oil was as effective an antifungal $CaCl_2$, but the effects of the two combined extracts were not additive (Moline and Locke, 1993).

In comparison with the control, neem oil showed a significant reduction in the mycelial growth of *Sarocladium oryzae,* the causal agent of sheath rot disease of rice at all the test concentrations (0.225, 0.45 and 0.9%) and storage durations (0, 3, 6 and 9 months) (Rajappan *et* al., 1999). Neem oil inhibited *Magnaporthe grisea*, *Sarocladium oryzae* and *Cochliobolus miyabeanus* but had no effect on *Pseudomonas fluorescens* or *Bacillus subtilis* and appeared to promote growth of *Trichoderma viride* (Rajappan *et* al., 2000). Neem oil was also found effective in reducing mycelial growth of the *Macrophomina phaseolina*, the causal agent of charcoal rot of mungbean (Vigna radiata)(Ilyas *et al.,* 1997). 2% neem oil suspension reduced the primary phase i.e. root hair infection and the secondary phase i.e. club formation of colonization by *Plasmodiophora brassicae* on Chinese cabbages, (Bhattacharya and Pramanik, 1998). Evaluation of the activity of neem oil against *Cochliobolus miyabeanus*, *Fusarium oxysporum* and *Alternaria alternata* showed that the active antifungal fraction was a mixture of tetranortriterpenoids such as 6-deacetylnimbin, azadiradione, nimbin, salannin and epoxyazadiradione. Pure azadiradione, nimbin, salannin and epoxy-azadiradione did not show antifungal activity. However, when these terpenoids were mixed and bioassayed, they were found effective suggesting possible additive/synergistic effects (Govindachari *et* al., 1998).

When tested against the larvae of *Epilachna vigintioctopunctata* neem oil at 1.5% produced the highest mortality of second and third instars (95.23%), and fourth instars (76.19%). In comparison with monocrotophos and quinalphos which resulted in 95.24% larval mortality neem oil at 0.25 and 0.5% concentrations caused 57.1 and 85.7% mortality in second larval instars, 47.6 and 85.7% in third instars and 57.1 and 80.9% in fourth instars respectively (Shanmugapriyan and Kingsly, 2001). Further, neem oil at 0.5% concentration effectively controlled the leaf spot disease of groundnut and significantly increased the yield over the control and was found to be cost effective (Srinivas *et al.*, 2000).

2.6 Relevance of the present investigations

Research into the insecticidal effects of azadirachtins, a limonoid from the Indian neem tree, and development of products for commercial use has been ongoing for at least last four decades. A large amount of data is available in literature detailing isolation, separation, purification, characterization as well as their feeding deterancy and the growth disruptive properties of azadirachtin and neem oil based pesticide formulations against a variety of phytophagous pests and vectors of diseases. Although many of the physiological effects are well understood, much remain to be done in terms of improving stability and performance under field conditions.

Triterpenic saponins and sapogenins of plant origin are well known for their very diverse biological activity. Anti-inflamatory, spermicidal, bactericidal and pest control properties. Are described to saponins and they play important roles in food, animalstufs and pharmaceutical properties. *Madhuca indica* (mahua) and *Sapindus mukorossi* (ritha) are two widely occurring plants known to contain considerable amount of triterpenic saponins. Inspire of their wide ranging botanical properties, such saponins and related products were not been evaluated for their nematicidal, antifungal and insect controlling properties. In addition to triterpenic saponins, their hydrolytic counterparts sapogenins and other additives such as neem, mahua and karanj fatty acids, and their salts and esters have the potential to be used as pesticidal adjuvants. Further more bioactive principles in various combinations are likely to exhibit potentiating or synergistic effect. Keeping in view the huge potential of such widely occurring plants containing pesticidal constituents, it is desirable that these plants are effectively explored to develop environmentaly benign and stable crop protection chemicals.

materials and methods

3.1 Chemicals

Laboratory grade chemicals, reagents and solvents were locally procured. All the solvents were freshly distilled and dried before use. Common laboratory glass apparatus was used for the experiments and chromatography. HPLC grade methanol and acetonitrile (LiChrosolv®) were procured from Merck Ltd., Mumbai and were used for analytical HPLC separation of azadirachtin-A and preparative HPLC separation of saponins. Water for chromatographic analysis was purified using US Filter water purification system (PURELAB™ Classic) with resistance of 18.2 MΩ. cm. Standard Azadirachtin (95%) was procured from Sigma-Aldrich Co., Bangalore. *Tertiary*-butyl hydroquinone was procured from Acros Organics and was used as standard stabilizer.

3.1.1 Plant and seed materials

Neem (*Azadirachta indica*) seed kernels were procured from Neem Mission, Pune, India. Ritha (*Sapindus mukorossi*) seeds were procured from palampur, Himachal Pradesh. Mahua (*Madhuca indica*) seeds were procured from Bihar. Karanj (*Pongamia glabra*) seeds were purchased from local market. Healthy seeds of rice (var. BC) and maize (var. Jaunpur local) were procured from Seed Technology Division and Division of Genetics and Plant Breeding, I.A.R.I.

3.1.2 Formulated insecticides and fungicides

Econeem (0.3%) from Margo Bio-Controls (Recommended dose 600ml ha^{-1}), Mancozeb M-45 (Indofil M-45; 75%WP) and Triazophos were used as standard insect control agent, fungicide and nematicide respectively for comparison.

3.2 Chromatography

3.2.1 Thin-layer chromatography (TLC)

TLC plates were prepared by spreading slurry of silica gel G (with binder; 10% gypsum) in water on 6 cm × 20 cm glass plates uniformly maintaining a thickness of 0.25 mm with the help of TLC applicator. The plates were activated at 110 ^{0}C for 1 hour before use. The sample solutions were spotted on the TLC plates using capillary tubes. Plates were developed in suitable solvent system(s) and air – dried. Different

solvents namely chloroform, methanol and water in different proportions were tested to achieve the best separation of both *Madhuca* and *Sapindus* saponins on the TLC plate. The spots were visualized after spraying with 10% H_2SO_4 solution followed by heating the plate at 120^{0}C for half an hour. For the preparative thin layer chromatography, glass plates (20 cm × 20 cm), coated with silica gel slurry (1.0 mm) containing 10% binder (gypsum) were used. During preparative separation, a glass TLC plate (6 cm × 20 cm) was also run along with preparative TLC plate as reference. Spots were marked on the preparative plates by comparing it with reference plate and the corresponding silica gel was scrapped and subsequently extracted with suitable solvent. Solvent was then evaporated in vacuum rotary evaporator to obtain the compounds.

The sugars present in glycon moiety of saponins were identified by co-TLC or co-paper chromatography (descending) on Whatman no. 1 filter paper by comparison with authentic samples (E-Merck AG, Darmstadt). Solvent system used was $CHCl_3$: MeOH: H_2O (65:35:10,v/v/v) for TLC and butanol: acetic acid: water (4:1:5). Aniline-phosphoric acid and or aniline hydrogen phthalate was used as visualizing agent. After spraying the reagent, the plates were heated at 105°C for 10 minutes.

3.2.1.1 Preparation of spraying reagent (Aniline- phosphoric acid and aniline hydrogen phthalate)

Aniline-phosphoric acid was prepared by mixing 30 ml of 2N aniline solution in water-saturated *n*-butanol with 60ml of solution of 2N *o*-phosphoric acid in *n*-butanol and used for spraying.

Aniline hydrogen phthalate was prepared by dissolving 0.93 g aniline and 1.66 g *o*-pthalic acid in 100 ml water-saturated *n*-butanol.

3.2.2 Column chromatography

Column chromatography was carried out for purification of saponins. Two grams of saponin was loaded on to a 100 cm long glass column (2.5cm dia), containing silica gel (100-200 mesh particle size, pre-activated at 110°C) as adsorbent. It was eluted with varying compositions of chloroform: methanol mixture (95:5 to 70:30 v/v) and fractions of 15 ml each were collected. Each fraction was analyzed for the presence of

saponin(s) by TLC. The fractions containing different saponin of same R_f (TLC) were combined and concentrated under vacuo at 45°C obtain pure saponins.

3.2.3 High performance liquid chromatography (HPLC)

Analytical reverse phase HPLC was performed on Waters HPLC system fitted with LichroCART® 250-4 mm, LiChrosphere R 100 RP-18e column (5μm) procured from Merck KgaA, Darmstadt, Germany or on Novapack phenyl 16R, 4μm particle size, 3.9 x 150 mm ID cartridge column containing dimethylphenyl propyl silyl bonded amorphous silica. Waters HPLC system equipped with 600 series pump, Rheodyne injector, 996 PDA detector at 217nm for azadirachtin-A, and 206 and 213 nm for both types of saponins was used for analysis of these compounds in various samples. Separation of azadirachtin-A was achieved under isocratic conditions at a flow rate of 0.75 ml min^{-1} using a mobile phase of methanol: water (65:35 v/v). A 20μl volume of sample was injected each time via a Rheodyne injector (20μl loop) for a run time of 15 minutes. The samples were filtered through a 0.25 μm Millipore filter before injection. Peaks were detected at the corresponding λ_{max}. The retention time (R_t) for each compound was measured.

The calibration and quantification was carried out using Waters Millennium 2010 Chromatography Manager software version 2.1 (Waters Chromatography Division). Azadirachtin in the samples was quantified by employing standard azadirachtin sample (95 % pure) obtained from Sigma-Aldrich Co., Bangalore.

Azadirachtin content = $(A_1/A_2) \times (m_2/m_1) \times P$

Where, A_1 = peak area of azadirachtin in sample.

A_2 = peak area of azadirachtin in reference standard.

m_1 = mass, in grams, of the test sample.

m_2 = mass, in grams, of the reference standard

P = Purity of the reference standard sample.

3.2.4 Preparative high performance liquid chromatography (Prep-HPLC)

Preparative high performance liquid chromatography (Prep-HPLC) was performed on a Prep-HPLC instrument (MERCK-KNAUER) fitted with Wellchrom preparative HPLC pump K-1800 and RP-18 column (Lichrospher®, 250 x 25 mm; 10μm) and self-packed RP-18 column (250 x 50mm; 15-25μm) and an automatic fraction collector. The

instrument was fitted with UV detector K-2600 and KNAUER preparative dynamic mixing chamber. The pump was operated at 0-40 Mpa pressure. The analysis was performed by EuroChrom® preparative version (Version 3.01) HPLC-software.

3.2.5 Gas chromatography (GC)

Analysis of methyl esters of neem, mahua and karanj fatty acids were performed on a Hewlett-Packard (5890) gas chromatograph fitted with megabore column (10 m, 0.53 i.d.) film thickness 2.65 mm with FID detector. The flow rate of N_2 was 15 ml min^{-1} with injection volume of 3µl. The oven, injector and detector temperature was 250°C, 270°C and 275°C respectively.

3.3 Spectroscopy

3.3.1 Nuclear magnetic resonance spectroscopy (^{1}H NMR)

^{1}H-NMR spectra of compounds were recorded on a Brucker (300 MHz) spectrometer. Deuterio-methanol (CD_3OD) was used as solvent and tetramethyl silane (TMS) was used as standard.

3.3.2 Mass spectroscopy

Electrospray mass spectroscopy was carried out on AB/MDS Sciex-API 2000 triple quadrupole mass spectrometer using TurboSpray source.

3.4 Isolation and purification of azadirachtin concentrates

3.4.1 Isolation of azadirachtin powder concentrate from neem seed kernels

Neem seed kernel powder (1Kg) placed in a conical flask containing hexane (2 litres) was agitated with a mechanical stirrer for 30 minutes. The material was allowed to stand for some time after which the mixture was once again agitated for five minutes. Hexane extract was separated after filtering the material through buchner funnel under vacuo and the resulting neem seed cake was again extracted twice with 1 liter hexane to ensure complete removal of neem oil. The combined hexane extract was concentrated under vacuo to obtain neem oil. The de-oiled seed cake was then extracted in the same manner with methanol (3 × 1 liter) and the combined methanol extract after filtration was concentrated under vacuo at 50 ^{0}C to one fourth of its volume. The alcohol extract was then quickly partitioned between n-hexane and 95%

aqueous methanol to remove the residual oil and other non-polar constituents. Water-soluble proteins and sugars were removed by partitioning the concentrated methanol extract between water and ethyl acetate. The organic phase was concentrated under vacuo to one fourth of its volume and subjected to flash chromatography over a bed of silica-gel-celite to obtain fractions, which were concentrated to yield azadirachtin rich concentrate. The concentrate was dissolved in minimum quantity of water immiscible solvent such as ethyl acetate (25 ml) and precipitated with a non-polar solvent such as hexane/petroleum ether to obtain azadirachtin powder concentrate (≈20 %, 7.0 g). The extraction process was repeated each time with 1 Kg batch of neem seed kernel to obtain sufficient quantity of technical azadirachtin –A powder concentrate.

3.4 Isolation of salanin-nimbin-desacetyl nimbin powder concentrate from neem oil

Neem oil (50g), obtained from the hexane extract of seed kernel of *A. indica* was partitioned between n-hexane (200 ml) and aqueous methanol (1:1; 200ml) and the methanol extract was pooled after repeating the procedure for another two times. The methanol extract was concentrated under vacuo to obtain the material rich in salanin, nimbin and desacetyl nimbin constituents (1.25g). The component in the mixture was analyzed by HPLC using RP-18 column and acetonitrile: water (50:50) as eluant (Fig. 2).

3.6 Isolation of saponins from *Madhuca indica*

Ground mahua seeds (1Kg) placed in a conical flask containing hexane (2 litres) were soaked overnight and agitated next day with a mechanical stirrer for 45 minutes. The material was filtered through buchner funnel under vacuo and the resulting mahua seed cake was again extracted twice with 1 liter hexane to ensure complete removal of mahua oil. The combined hexane extract was concentrated under vacuo to obtain mahua oil. The de-oiled seed cake was then extracted in the same manner with methanol (3 × 1 liter) and the combined methanol extract after filtration was concentrated under vacuo at 45 ^{0}C to viscous syrup. The extract was partitioned between water and n-butanol to remove water-soluble free sugars. The combined butanol extract was then concentrated to viscous liquid at <70^{0} C under vacuo. The viscous concentrate was dissolved in minimum quantity of methanol (25 ml) and

precipitated with large excess of acetone to obtain saponin mixture (≈ 12.5 g). The extraction process was repeated each time with 1 Kg batch of mahua seed to obtain sufficient quantity of saponins.

3.6.1 High performance liquid chromatographic (HPLC) analysis of *M. indica* saponins

A method has been developed for the analysis of mahua saponins by high performance liquid chromatography employing reverse phase HPLC column. The saponins were detected at 213 nm using PDA detector. Separation of saponins was achieved under isocratic conditions at a flow rate of 0.4 ml min^{-1} using methanol: water (60:40 v/v) mobile phase. A 20µl volume of sample was injected each time via a Rheodyne injector (20µl loop) for a run time of 30 minutes. The samples were filtered through a 0.25 µm Millipore filter before injection. The retention time (R_t) for each compound was measured. The calibration and quantification were carried out using Waters Millennium 2010 Chromatography Manager software version 2.1 (Millipore Corporation, Waters Chromatography Division).

3.6.2 Separation of saponins

3.6.2.1 Column chromatography

Mahua saponins mixture (2g) was loaded on to a 100 cm long glass column (2.5cm dia), containing silica gel (100-200 mesh particle size, pre-activated at 110°C) as adsorbent. It was eluted with varying gradient of chloroform: methanol: water (65:35 to 65:35:10 v/v) and fractions of 15 ml each were collected and, each fraction analyzed for the presence of saponin(s) by TLC. The fractions containing saponins of same R_f (TLC) were pooled and concentrated under vacuo at 45°C to obtain pure saponins.

3.6.2.2 Preparative high performance liquid chromatography (Prep-HPLC)

Preparative high performance liquid chromatography (Prep-HPLC) was used for the isolation of pure saponin constituents from saponin mixture concentrate of *M. indica*. The pump was operated at 0.19-0.23 Mpa pressure and the column was run with methanol-water gradient system for 40 minutes at the flow rate of 10 ml min^{-1}. The desired amount (50 mg) of crude saponin mixture was dissolved in minimum quantity

of the eluting solvent (10 ml) and loaded onto the column. The best separation was achieved using following gradient system.

Time	Flow rate	Solvent system	
		%A (Water)	%B (Methanol)
0	10	62	38
10	10	62	38
40	10	60	40
60	10	60	40

Different peaks were collected with the help of direct control system in the operating software and fractions were analyzed and detected as single spot on analytical HPLC. The procedure was repeated sufficient times and same resoluting peak fractions pooled. The compounds were isolated after evaporating off the solvent under *vacuo* at temperature not exceeding 50^0C. Two madhuca saponins abbreviated as MI-I and MI-III was obtained in pure form and were characterized spectroscopically.

3.6.3 Hydrolysis of mahua saponin: Isolation of genin

In two different experiments, solution of mahua saponins (MI-I and III, 2g each) in ethanol was refluxed with 5% H_2SO_4 (1:1) for 4 hours. After cooling the solution was neutralized and partially hydrolyzed saponins was separated out from the solution as off-white colour substance. The partially hydrolysed products was filtered, washed with sufficient quantity of water and transferred back to another round bottom flask. Since this saponins is not hydrolysed under acidic conditions, the partially hydrolyzed saponin so obtained was dissolved in ethanol and refluxed with 10% aqueous KOH (1:1) for 3 hours. The sapogenin (16α hydroxy protobassic acid) was separated out as white powder after cooling and neutralization of the solution with 5% H_2SO_4 solution. The genin was reported to be present in *Madhuca* sp. (Nigam *et al.*, 1992).

3.6.4 Acid hydrolysis of mahua saponin: Identification of sugars

Saponin (MI-I and III, 100 mg) in ethanol was refluxed with 1.5 M HCl-MeOH (100 ml) for 8 hours. After hydrolysis was complete, the reaction mixture was diluted with water and extracted with chloroform or dichloromethane (3 X 50 ml). While prosapogenin was detected in organic phase, sugars were found in aqueous hydrolysate. The water

layer was neutralized with barium carbonate and then deionized with Amberlite (IR-400, H^+), concentrated under vacuo (≈50° C) and then analysed by descending paper chromatography or TLC for its sugar constituents. For TLC analysis, $CHCl_3$-MeOH-H_2O (6:4:1) lower layer, and for PC analysis BuOH-AcOH-H_2O (4:1:5) upper layer was used. Spots were detected after spraying with aniline-hydrogen-pthalate.

3.6.5 Kiliani hydrolysis of MI-I and MI-III

MI-I and MI-III (100 mg each) was dissolved separately with Kiliani mixture (acetic acid: water: 35% HCl (35: 55: 10 v/v/v) (15 ml) and kept first at 50°C for 24 hours, then at 75°C for further 48 hours and for 2 days at 100°C. It was analyzed regularly for the liberated sugars by PC with solvent system of BuOH-AcOH-H_2O (4:1:5) upper layer. The sequence of the sugars detached from MI-I and MI-III were fixed on the basis of colour intensity of the spots in paper chromatography.

3.6.6 Spectral analysis of *Madhuca indica* saponin MI-I and MI-III by ^1H NMR and MS spectroscopy

Madhuca saponin-I (MI-I)

^1H NMR (CD_3OD) δ: 0.901, 0.939, 1.059, 1.13, 1.164 and 1.226 (3H each, s, H-29, H-30, H-27, H-26, H-24 and H-25 respectively); 1.247 (2H, H-5); 1.270, 1.613 (2H, H-15) 1.306 (2-H, H-7), 1.610 (1H, H-9); 1.722, 1.761 (1H, H-11 or H-22); 3.41 (1H, s, H-3), 3.371 (2H, H-23); 3.56 (1H, m, H-18); 4.502 (1H, d, H-2); 5.092 (1H, br s, H-16); 5.127 (1H, br s, H-6); 5.347 (1H, glc anomeric); 5.630 (1H, br s, H-12). Signal pattern of protons attached to glycone moiety at δ 3.4-4.82 was unclear.

ES-MS: m/z 1241 $[M+ H]^+$, m/z 1223 $[M - 18]^+$, m/z 1205.9 $[M - 18\text{-}18]^+$, m/z 1073 $[M - 18\text{-}150]^+$, m/z 1059 $[M - 182]^+$, m/z 927.7 $[M - 182 - 132]^+$, m/z 795.8 $[M - 182\text{-}132\text{-}132]^+$.

Madhuca saponin-III (MI-III)

^1H NMR (CD_3OD) δ: 0.903, 0.940, 1.060, 1.133, 1.224, 1.310 (3H each, s, H-29, H-30, H-27, H-26, H-24 and H-25 respectively); 1.182, 1.760 (2H, H-2); 1.203 (1H, H-5); 1.248, 1.613 (2H, m, H-7), 1.273, 1.294, 1.724 (1H, m, H-9); 1.761 (2H, H-22); 3.34, 3.75 (2H, H-23), 3.564 (1H, m, H-18); 3.713 (1H, m, H-3); 4.524 (1H, d, H-2); 3.487,

3.520, 3.770, 3.826, 3.837, 3.858, 3.915, 4.420, 4.460, 4.540 (anomeric and glycon moiety protons); 5.088 (1H, br s, H-16); 5.150 (1H, br s, H-6); 5.349 (glc H-1); 5.629 (1H, br s, H-12).

ES-MS: m/z 1535.9 $[M]^+$, m/z 1518 $[M - 18]^+$, m/z 1403 $[M - 132]^+$, m/z 1373 $[M - 132-30]^+$, m/z 1373 $[M - 162]^+$, m/z 1355 $[M -162-18]^+$, m/z 1241 $[M - 132-162]^+$, m/z 1241 $[M - 162-132]^+$.

3.6.7 Alkali hydrolysis of mahua saponin: Isolation of prosapogenin

Mahua saponin (MI-III, 2g) was dissolved in ethanol and refluxed with 20% aqueous KOH (1:1) for 4 hours. The solution was cooled at room temperature and diluted with water. After neutralizing the solution with 5% H_2SO_4, partially hydrolyzed saponins were separated out. The prosapogenin (~600 mg) was filtered through buchner funnel under vacuo and repeatedly washed with water to remove the free sugars.

3.6.7.1 Characterization of prosapogenin

^{1}H NMR (CD_3OD) δ: 0.88, 0.937, 1.028, 1.091, 1.238, 1.278 (3H each, s, H-29, H-30, H-27, H-26, H-24, H-25 respectively); 1.67 (1H, H-9); 1.91, 1.92 (2H, H-11); 3.337 (1H, m, H-); 3.650 (1H, m, H-3); 3.660 (1H, m, H-18); 4.041, 4.053 (2H, H-23); 4.82 (1H, H-2); 4.902 (1H, br, s, H-16); 5.328 (2H, br, s, H-6 and glc anomeric); 5.56 (1H, br s, H-12). Proton signals appearing in the region 3.0-4.5 corresponded to anomeric and other protons of the glycone moieties.

ES-MS: m/z 990 $[M^+ - OH]^+$, m/z 974 [M +H - OH], m/z 520 $[C_{30}H_{48}O_7^+]$, m/z 504 $[C_{30}H_{48}O_6^+]$, m/z 504 $[C_{18}H_{16}O_{17}]$, m/z 487.6 $[C_{18}H_{16}O_{16}]$,

3.6.7.2 Acidic hydrolysis of prosapogenin: Identification of sugars

Madhuca prosapogenin (500 mg) in ethanol was refluxed with 5% H_2SO_4 as in section 3.6.4. The genin was filtered out or extracted out with dichloromethane to yield protobassic acid (Co-TLC). Reducing sugars present in aqueous hydrolysate saponins were identified by TLC by comparing the R_f of standard sugars. The solvent system for the TLC was chloroform: methanol: water (65:35:10). Aniline-phosphoric acid was used as visualizing agent.

3.6.8 Saponin acetate

Mahua saponin concentrate (200 mg) in pyridine (5 ml) was mixed with acetic anhydride (5 ml) and kept at room temperature for overnight. The solvent was evaporated under vacuo and then it was precipitated in ice-cold water. The precipitate was filtered and dried as white powder. The absence of saponin spots on TLC in benzene: acetone (4:1) solvent system indicated complete acetylation of the saponin.

3.6.9 Preparation of propionyl, butanoyl and pentanoyl esters of saponin

3.6.9.1 Acid chlorides

Acid chlorides needed for the preparation of saponins esters were prepared according to the literature procedure (Vogel, 5th Edition, Section 5.12.1, Experiment 5.138 and cognate preparations). A general method of preparing acid chloride is described below.

A 50 ml two-necked flask was fitted with a dropping funnel and reflux condenser connected at the top to the gas absorption trap. 22 g (0.25 mol) propionic acid contained in the dropping funnel was added to the redistilled thionyl chloride (36 g, 0.3 mol) kept in the flask, over a period of about 30-40 minutes. Then the mixture was refluxed by heating on a water bath for another 30 minutes, distilled and redistilled through short fractionating column to collect the propanoyl chloride (22g) b.p. 100-101°C. Thionyl chloride was similarly reacted with butanoyl and pentanoic acid to obtain corresponding acid chloride.

3.6.9.2 Saponin esters

Mahua saponin (500 mg), dissolved in pyridine (5 ml) was taken in a conical flask (25 ml) and 3 ml dichloromethane was added into it very slowly. The beaker was cooled in ice bath and pentanoic acid chloride (0.036 mol) was added drop wise into the flask. The mixture was stirred for half an hour and kept overnight at 0^{0}C. Dichrolomethane (40 ml) was added and then washed with 2M H_2SO_4 solution first and then with water thrice. The dichloromethane extract was then washed first with saturated $NaHCO_3$ and then with water thrice to remove excess alkali. The dichloromethane was evaporated, and ester was obtained as viscous liquid and ester was precipitated in MeOH-water as pinkish yellow solid. Propanoic acid chloride and butanoyl chloride was similarly reacted with saponin to obtain corresponding esters.

3.7 Isolation of saponins from *Sapindus mukrossi* fruit pericarp

From *Sapindus mukrossi* (ritha) fruits, pericarp was removed and seeds were separated. Ground pericarp (1Kg) was placed in a conical flask containing hexane (2 litres) was agitated with a mechanical stirrer for 45 minutes. The material was filtered through buchner funnel under vacuo and the resulting material then extracted with methanol (3 × 1 liter). The combined methanol extract after filtration was concentrated under vacuo at 45^{0}C to yield viscous syrup. It was then diluted with water and partitioned thrice with *n*-butanol (3 × 500 ml) to remove water-soluble sugars. The combined butanol extract was concentrated under vacuo to obtain viscous liquid. The concentrate was dissolved in minimum quantity of methanol (25 ml) and precipitated with large excess of acetone to obtain saponins mixture (≈20.5 g). The extraction process was repeated each time with 1 Kg batch of ritha pericarp powder to obtain sufficient quantity of saponins mixture.

3.7.1 Development of high performance liquid chromatographic (HPLC) method for analysis of *S. mukorossi* saponins

A method has been developed for the analysis of *S. mukorossi* saponins by high performance liquid chromatography using reverse phase (RP-18) HPLC column and PDA detector at 206 nm. Separation of saponins was achieved under isocratic conditions at a flow rate of 0.4 ml min^{-1} using a mobile phase of acetonitrile: water (53:47 v/v). A 20µl volume of sample was injected each time via a Rheodyne injector (20µl loop) for a run time of 30 minutes. The samples were filtered through a 0.25 µm Millipore filter before injection. Peaks were detected at 206 nm using photodiode array detector. The retention time (R_t) for each compound was measured. The calibration and quantification were carried out using Waters Millennium 2010 Chromatography Manager software version 2.1.

3.7.2 Separation of *S. mukorossi* saponins

3.7.2.1 Column chromatography

Two grams of saponin was loaded on to a 100 cm long glass column (2.5cm dia), containing silica gel (100-200 mesh particle size, pre-activated at 110°C) as adsorbent. It was eluted with varying gradient of chloroform: methanol (95:5 to 70:30 v/v) and fractions of 15 ml each were collected and, each fraction analyzed for the

presence of saponin(s) by TLC. The fractions containing different saponin of same R_f (TLC) were pooled and concentrated under vacuuo at 45°C and combined to isolate pure saponins.

3.7.2.2 Preparative high performance liquid chromatography

Preparative high performance liquid chromatography (Prep-HPLC) was used for the separation of major saponins from the saponin mixture concentrate of *S. mukorossi*. The pump was operated at 0.19-0.23 Mpa pressure and the column was run with acetonitrile-water (47:53 v/v) isocratic system for 40 minutes at the flow rate of 40 ml min^{-1}. The desired amount (50 mg) of crude saponin mixture was dissolved in minimum quantity of the eluting solvent (10 ml) and loaded onto the column. Different peaks were collected with the help of direct control system in the operating software and fractions were analyzed and detected as single peak on analytical HPLC. The procedure was repeated sufficient times and same resoluting peak fractions pooled. The compounds from the pooled fractions were isolated after evaporating off the solvent under *vacuo* at temperature not exceeding 50 ^{0}C.

3.7.3 Hydrolysis of *S. mukorossi* saponin

Saponin (4g) solution in ethanol was refluxed with 5% H_2SO_4 (1:1) for 4 hours. After cooling the solution was neutralized and partially hydrolyzed saponins was separated out from the solution as white colour substance. The compound was filtered after washing with water and transferred back to round bottom flask. The partially hydrolyzed saponin was again dissolved in ethanol and refluxed with 20%aqueous KOH (1:1) for 3 hours. The sapogenin was separated out after cooling and neutralizating with 5% H_2SO_4.

3.7.4 Acidic hydrolysis of *S. mukorossi* saponin and identification of resulting monosaccharides

Saponin (200 mg) solution in ethanol was refluxed with 1.5 M HCl-MeOH (10 ml) (1:1) for 6 hours. After cooling the solution, it was diluted with water and extracted with dichloromethane. Sapogenin was detected in dichloromethane layer by Co-TLC with authentic samples. The water layer (aqueous hydrolysate) was neutralized with freshly prepared barium carbonate and deionised by passing through Amberlite (IR-400, H^+),

concentrated under vacuo and then subjected to Co-TLC and Co-PC analyisis with authentic samples. For TLC analysis chloroform: methanol: water (6:4:1) lower layer, and for PC analysis butanol: actetic acid: water (4:1:5) upper layer was used. The spots were detected after spraying with aniline hydrogen pthalate solutions.

3.7.5 Alkaline hydrolysis of *S. mukorossi* saponins

Saponin mixture (2g) was dissolved in ethanol and refluxed with 15% aqueous KOH (1:1) for 4 hours. The solution was cooled at room temperature and diluted with water. After neutralizing the solution with 5% H_2SO_4, alkali hydrolyzed saponins were separated out. The compound (~800 mg) was filtered through buchner funnel under vacuo after washed with water to remove traces of acids and free sugars.

The prosapogenin (100 mg) so obtained was subjected to further hydrolysis with mineral acid (MeOH-HCl). The monosaccharides present in the aqueous hydrolysate of the prosapogenin were identified as rhamnose, arabinose and xylose by their comparision (TLC/PC) with the authentic samples.

3.7.6 Characterization of *Sapindus mukorossi* saponin, SM-I

^{1}H NMR (CD_3OD) δ: 0.701, 0.815, 902, 0.936, 0.972 and 1.173 (3H each, s, H-29, H-30, H-26, H-27, H-24 and H-25 respectively); 3.299(1H, m, H-3); 3.520 (2H, m, H-23); 5.215 (1H, d, H-12). Other NMR peaks appearing in the region 3.4-5.0 corresponded to anomeric and other protons of glycone moiety.

ES-MS: m/z 1417 $[M+Na]^+$, m/z 942.7 $[M^+$ - (rha+arab+xyl-Oac, 452 amu)], m/z 925 $[M^+$ -(glu+glu-A+arab, 490 amu] m/z 1374 [1417- $COCH_3$, m/z 793 [925-arab], m/z 782 [942-glu], (m/z 414, 386.7, 369.7) derived from glu-glu-A-arab fragment. M/z 223, 248, 205, and 187 (RetroDiels-Alder fragments)

3.8 Phyto-oils, fatty acids and their salts and esters

3.8.1 Neem oil, mahua oil and karanj oil

Neem seed kernel powder (1Kg) placed in a conical flask was soaked overnight in hexane (2 litres) and agitated next day with a mechanical stirrer for 30 minutes. The material was allowed to stand for some time after which the mixture was once again agitated for five minutes. The material was filtered through buchner funnel under

vacuo and the resulting neem seed cake was again extracted twice with 1 liter hexane to ensure complete removal of neem oil. The combined hexane extract was concentrated under vacuo to obtain neem oil.

Mahua seed powder (1Kg) and Karanj seed powder (1Kg) placed in a conical flask was similarly extracted as above with (1×2 lit. and 2×1 lit.) The combined hexane extract was concentrated under vacuo to obtain respective mahua and karanj oil.

3.8.2 Fatty acids

Neem, mahua and karanj oil (10g each) was separately placed in three round bottom flasks and refluxed with 40 ml 0.5 N ethanolic KOH for one hour. The reaction mixture was cooled and alcohol removed under vacuo. The saponified oil was treated with dil. HCl and fatty acids were extracted after partitioning with ether. The ether was removed under vacuo to obtain mixture of fatty acids.

3.8.3 Salts of fatty acids

Neem, mahua and karanj oil was separately used to prepare respective mixtures of Na, K and NH_4 -salts of fatty acid. The oil (10 g) was taken in a beaker was treated with 20% alcoholic KOH and subsequently stirred on a magnetic stirrer. pH of the oil was checked in interval after every addition of alkali. Stirring and adding of KOH was continued till all the fatty acids were saponified and pH reached to $\cong$7.0 permanently. Same procedure was followed for preparation of Na and NH_4-salts using NaOH and NH_3 solution for saponification.

3.8.4 Preparation of methyl ester of fatty acids

Neem, mahua and karanj fatty acid mixtures were placed in a round bottom flask and dissolved in anhydrous methanol (1:4). To the solution, 1% concentrated H_2SO_4 was added and the mixture refluxed for three hours. After completion of the reaction water was added and the mixture extracted with solvent ether. The un-esterified acids were removed by washing with dil. K_2CO_3 solution and again the ether solution was washed with water to remove the excess alkali present. The organic phase was dried over anhydrous Na_2SO_4 to obtain fatty acid methyl ester. The esters were analyzed by GC for their chemical constituents using megabore column (10 m, 0.53 i.d.) with FID

detector. The flow rate of N_2 was 15 ml min^{-1} with oven, injector and detector temperature was 250°C, 270°C and 275°C respectively.

3.9 Photodegradation of azadirachtin concentrate

3.9.1 Preparation of standard stock solutions

A standard stock solution of azadirachtin concentrate (20 % aza-A) containing 2 mg/ml (2000 ppm) was prepared in a volumetric flask by dissolving 200 mg of the sample in 100ml of methanol. From this stock solution, 1000ppm sub stock was prepared by serial dilution for photodegradation studies. The stock solutions were stored in a refrigerator prior to use and the aza-A content in irradiated and control samples was checked periodically by HPLC.

3.9.2 Effect of light on the degradation of azadirachtin-A (20%) concentrate

3.9.2.1 UV light, methanolic solution

Azadirachtin solution in methanol (10 ml, 1000 ppm) prepared as above was kept in four quartz tubes (20 ml capacity, length 15cm), three of them were irradiated in a water cooled chambers with UV light emanating from a high pressure mercury vapour lamp (125 W; 200-250 V; 6200 Lumens) for 8.5 h. The fourth tube containing 10 ml of the test solution, kept in dark under similar experimental conditions, served as control. The samples from each tube were collected at half-hourly intervals, filtered and kept in the refrigerator prior to HPLC analysis.

3.9.2.2 UV light, thin film on glass surface

Azadirachtin solution in methanol (1 ml, 2000 ppm) was applied uniformly in sufficient number of 5 cm diameter Petriplates using a pipette. The solution was spread as a thin film of uniform thickness onto the glass surface with the help of the pipette tip. Solvent from Petriplates was allowed to evaporate quickly at ambient temperature to form a uniform thin film of azadirachtin. Plates were kept in a chamber maintained at 30 ^{0}C and irradiated upto 14 h under a germicidal lamp (254 nm) kept at a distance of 10 cm from the irradiating source. Sufficient numbers of coated petriplates wrapped in aluminium foil were kept in dark under similar experimental conditions, which served as control. The samples were taken (3 at a time) at 15 min. interval. The azadirachtin

layer from each Petriplate was extracted with methanol (2 × 1ml) to obtain 2 ml of extract for HPLC analysis.

3.9.2.3 Sunlight, methanolic solution

Azadirachtin solution in methanol (10 ml, 1000ppm) was kept in four pyrex tubes (20ml capacity, length 15cm). Three of them were irradiated under sunlight (8-9h / day) upto 15 days. The fourth tube, containing the test solution wrapped with aluminium foil and kept in dark under similar experimental conditions, served as control. The samples from each tube was collected every alternate day, filtered and kept in the refrigerator prior to HPLC analysis.

3.9.2.4 Sun light, thin film on glass surface

Azadirachtin solution in methanol (1ml, 2000 ppm) was applied uniformly in sufficient number of 5 cm diameter Petriplates using a pipette. The solution was spread as a thin film of uniform thickness onto the glass surface with the help of a pipette tip. Solvent from Petriplates was allowed to evaporate quickly at ambient temperature to form a thin film of azadirachtin. Petriplates were kept in a chamber maintained at 30 ^{0}C irradiated under sunlight (8-9 hr/day) upto 15 days. The equal number of coated Petriplates were kept in dark under similar experimental conditions and served as control. The samples were taken (3 at a time) every day. The azadirachtin layer from each Petriplate was extracted with methanol (2 × 1ml) to obtain 2 ml of extract for HPLC analysis.

3.10 Thermal degradation of azadirachtin-A under accelerated storage (54±1^{0}C)

3.10.1 Methanolic solution

A 5000 ppm solution of azadirachtin-A (20%) was prepared by dissolving 50 mg of 20% pure azadirachtin in 10 ml of methanol. This solution was kept in an oven maintained at 54± 1^{0}C at 25g/cm^2 for 14 days. Samples were drawn every alternate day, filtered and kept in the refrigerator prior to HPLC analysis.

3.10.2 Emulsified water solution

A 5000 ppm solution of azadirachtin-A (20%) was prepared as above in 10 ml of 0.5% emulsified water (prepared by dissolving 5ml of Tween 80 in 1000ml of distilled water).

This solution was kept in an oven maintained at 54 ± 1°C at 25g/cm^2 for 14 days. Samples were drawn at equal intervals, filtered and kept in the refrigerator prior to HPLC analysis.

3.10.3 Solid phase

Azadirachtin-A (20%) concentrate powder (10 mg) was taken in a glass vial and kept in a heating chamber maintained previously at 54 ± 1°C for 15 days. The sample (2 mg) was withdrawn on 0, 5, 10 and 15 days and dissolved in 2 ml methanol and injected in HPLC for analysis of azadirachtin content.

3.11 Effect of some botanical adjuvants (saponins, sapogenin and phytoalkanoates) on the photostability of azadirachtin-A

3.11.1 Preparation of stock solution

A 6700 ppm (6.7mg ml^{-1}) and 5000 ppm (5.0 mg ml^{-1}) solution of azadirachtin concentrate (20% aza-A) was prepared by dissolving 670 mg and 500 mg of 20% pure azadirachtin in 100 ml of HPLC grade methanol. A 6700 ppm stock solution of each of the test adjuvants was similarly prepared by dissolving 670 mg of each stabilizer in 100ml of HPLC grade methanol.

3.11.2 Stability of azadirachtin-A in azadirachtin- adjuvant (3:1) mixture

3.11.2.1 UV Light, methanolic solution

To 12 ml of 6700 ppm azadirachtin (20% aza-A) solution in methanol, 12 ml of 6700 ppm of adjuvant solution was added to obtain a mixture (24 ml) containing 3350 ppm each of azadirachtin and stabilizer in 1:1 ratio. Each of the azadirachtin: stabilizer solution (8 ml) was taken in three quartz tubes for irradiation under UV light emanating from a high pressure mercury lamp upto 8.5 hours. Fourth quartz tube containing 8 ml of the test solution and kept in dark served as control. Samples (0.5 ml) from each of the azadirachtin – test stabilizer mixture was withdrawn at hourly intervals from each of the tubes, filtered and kept in the refrigerator prior to HPLC analysis.

3.11.2.2 UV light, thin film on glass surface

Azadirachtin (20% aza-A) (18 ml, 6700 ppm) solution was mixed with stabilizer solution (6 ml, 6700 ppm) to obtain a mixture (24 ml) containing 5000 ppm of

azadirachtin and 1667 ppm of stabilizer in 3:1 ratio. 0.5 ml of this solution was uniformly applied on the Petriplate surface and air-dried to obtain a thin layer on the Petriplate. Sufficient numbers of Petriplates were subsequently irradiated upto 3h under a germicidal lamp (254nm). One set of unexposed petriplates served as control. The samples were taken (3 at a time) at 15 min. intervals. The azadirachtin layer from each Petriplate was extracted with methanol (2 × 1ml) to obtain 2ml of extract, which was analyzed by HPLC for its aza-A content.

3.11.2.3 Sunlight, thin film on glass surface

Azadirachtin: adjuvant (3:1) solutions (0.5 ml) prepared as above containing 5000 ppm of azadirachtin-A was uniformly applied on the Petriplate surface and air dried to obtain a thin layer on the Petriplate. . Petriplates were kept in a chamber maintained at 30^0C irradiated under sunlight (8-9 hr/day) upto 15 days. The samples were collected (3 at a time) at 1 day interval. The azadirachtin layer from each Petriplate was extracted with methanol (2 × 1ml) to obtain 2ml of extract for HPLC analysis.

3.11.2.4 Sun light, methanolic solution

Azadirachtin: adjuvant (3:1) solutions (8 ml) prepared as above were taken in three pyrex tubes, parafilmed and irradiated under sunlight (7-8 hours/day) for 15 days. One set of unexposed served as control. Samples (0.5 ml) from each of the azadirachtin–test adjuvants solutions were withdrawn every day, filtered and stored in the refrigerator prior to HPLC analysis.

3.12 Effect of adjuvants on the thermal stability of azadirachtin under accelerated storage (54+ 1^0C)

3.12.1 Emulsified water

To 18 ml of 6700 ppm azadirachtin (20% aza-A) solution in emulsified water (prepared as in section 3.8.2), 6ml of 6700 ppm of solution of saponins, its different derivatives and phytoalkanoates was added as above to obtain a mixture (24 ml) containing 5000 ppm of azadirachtin and 1667 ppm of adjuvant in 3:1 ratio. All the vials containing azadirachtin: stabilizer (3:1) solutions were kept in an oven maintained at 54± 1^0C at 25 g cm^{-2} for 14 days. Samples were drawn at regular interval, filtered and kept in the refrigerator prior to HPLC analysis.

3.12.2 Methanolic solution

To 18 ml of 6700 ppm azadirachtin (20% aza-A) solution in methanol, 6ml of 6700 ppm of adjuvant solution was added to obtain a mixture (24 ml) containing 5000 ppm of azadirachtin and 1667ppm of stabilizer in 3:1 ratio. All the vials containing azadirachtin: stabilizer (3:1) solutions were kept in an oven maintained at 54± 1^{0}C at 25g/cm^2 for 14 days. Samples were drawn every alternate day, filtered and kept in the refrigerator prior to HPLC analysis.

3.12.3 Solid phase

Azadirachtin-A (20%) concentrate powder (6 mg) was mixed thoroughly with 2 mg each phyto-adjuvants and stored in aproperly stopped glass vials. The samples in triplicate were stored under accelerated temperature of 54 ± 1°C for 15 days. The samples were withdrawn after 0, 5, 10 and 15 days, dissolved in 6 ml methanol and the solutions analysed by HPLC for azadirachtin content.

3.13 Insecticidal, antifeedant and insect growth regulatory activity

3.13.1 Rearing of test insect *Spodoptera litura*

The larvae of *Spodoptera litura* were collected from the fields and reared in the laboratory on castor leaves (*Ricinus communis* Linn.) in glass jars (15 cm × 20 cm) at 27 ± 1^{0}C and 60% relative humidity. Everyday fresh and clean leaves were provided in each jar after removing the remnant leaves and excreta of the insects. Roughly about one hundred larvae were kept in each jar and a disease free culture was maintained avoiding overcrowding and unsanitary conditions. The larvae, up to last instar, were reared in these glass jars. The fully-grown, about to pupate larvae were then transferred to clean jars having thick layer of sterilized soil for pupation. The moths emerging after a week were collected and transferred to the clean jars containing a suspended cotton swab soaked with 10% honey solution and pieces of folded papers at the bottom for oviposition. The freshly hatched larvae of the same batch were removed and maintained separately on fresh tender castor leaves upto fourth instar larvae. These larvae were then exposed separately to the test compounds for studying their insect growth inhibition and antifeedant activity.

3.13.2 Stock and test solutions of test compounds

The test compounds (100 mg) were weighed accurately in 5 ml volumetric flask and dissolved into a little amount of distilled acetone. The volume was then made to 5 ml with acetone to obtain 2% stock solution. From these stock solutions, different concentrations, (1.0, 0.5, 0.25, 0.1, 0.07, 0.05, 0.01, 0.007,and 0.005 %) were prepared separately by serial dilution with 0.5 % emulsified water, which in turn was prepared by dissolving 5 ml of Tween 80 emulsifier in 1 liter of distilled water.

3.13.3 Antifeedant activity of test compounds against *Spodoptera litura* larvae (No choice bioassay)

Leaf disks (6 cm diameter) were cut from castor leaves, washed with water, dried under shade, and then treated with test solutions, dried and transferred to clean rearing bottles, separately. One third-instar larva of *Spodoptera litura* was released in each rearing bottle. The leaf area consumption was measured after 48 hours of treatment by taking observations using graph papers. Consumption data of leaf disks treated with methanol- emulsified water served as control.

Corrected feeding inhibition (%) was calculated by the following formula:

Corrected feeding inhibition (%) = $[(C - T) / (100-C)] \times 100$

Where, T = Consumption of leaf in treatment and C = Consumption of leaf in control.

Antifeedancy index (AI_{50}) values were calculated by using a Basic LD_{50} program version 1.1 as described by Trevors (1986)

The raw data on different parameters was subjected to angular transformation (arc sine percentage) and analyzed by complete randomized design. Analysis of variance was done and means were separated by Critical difference (CD).

3.13.4 Insect growth regulatory activity of test compounds against *Spodoptera litura* larvae

Third instar larvae of *Spodoptera litura* weighing between 30-60 mg were treated with various concentrations of the test emulsions under Potter's direct spray tower at a pressure of 340 g cm^{-2}. The sprayed dishes were dried for five minutes under a fan

after which the larvae were transferred to separate rearing bottles. The larvae, similarly sprayed with emulsified water, served as control. Larval weight was taken at 3 and 7 days after treatment (DAT). The raw data on different parameters was subjected to angular transformation (arc sine percentage)$^{1/2}$ and analyzed statistically by complete randomized design. Analysis of variance was done and means were separated by square difference, i.e., Critical difference (CD). Inhibition concentration (IC_{50}) was determined based on probit analysis. Per cent reduction in larval weight gain over control (% growth inhibition) was calculated as:

[(Weight gain in control - weight gain in treatment)/ Weight gain in control] × 100

IC_{50} values (Inhibition concentration for 50% inhibition of adult emergence) were calculated by using statistical package for social sciences.

3.14 Antifungal activity

Screening for fungitoxicity was carried out by poisoned food technique using potato – dextrose – agar (PDA) medium (Nene and Thapliyal, 1979) against *Rhizoctonia bataticola, Rhizoctonia solani, Sclerotium rolfsii, Aspergillus niger* and *Fusarium udum.*

3.14.1 Test Culture

Cultures of *Rhizoctonia solani, Rhizoctonia bataticola, Alternaria brassicola, Sclerotium rolfsii, Aspergillus niger* and *Fusarium udum* were obtained from the Indian Type Culture Collection, Division of Mycology and Plant Pathology, IARI, New Delhi. The cultures were maintained on PDA slants at 25 ^{0}C and were sub-cultured in Petridishes prior to testing.

3.14.2 Preparation of media

Readymade media procured from Hi-Media Lab contained ingredients such as Potatoes infusion form (200 g/litre), Dextrose (20 g litre^{-1}), Rose bengal (0.0084 g litre^{-1}) and Agar (15.0 g litre^{-1}). This readymade medium (54 gm) was suspended in 1000 ml of distilled water and heated to boiling to completely dissolve the medium. It was then transferred to conical flasks and the flasks were plugged with surgical grade cotton. The medium and the Petridishes were sterilized in an autoclave at 15 psi for 15 minutes.

3.14.3 Stock and test solution

65 mg of test compound was weighed and dissolved in 2 ml of methanol. One ml, 0.5 ml and 0.25 ml of this stock solution when diluted to 65 ml with PDA medium gave 500 ppm, 250 ppm and 125 ppm test solutions, respectively. 100 ppm test solution was prepared by diluting one ml of stock solution to 10 ml with methanol and further diluting one ml from it to 65 ml with PDA medium. To prepare 50 ppm test solution, one ml of 100 ppm test solution was diluted to 2 ml with methanol and one ml of this solution was then diluted to 65 ml of PDA medium. One ml of methanol served as control.

3.14.4 Inoculation and incubation

The medium of each concentration and control was poured into a set of two Petri-dishes (2 replications), suitably labeled, under aseptic conditions in a laminar flow chamber. The dishes were then kept under UV light in the laminar flow chamber till the medium partially solidified. A 5 mm thick disc of fungus (spores and mycelium) cut from earlier sub cultured petridishes was put in the center of the semisolid medium in the test petridishes and the lids of the dishes were replaced. Both treated and control dishes were kept in a BOD incubator at 25 ± 2 ^{0}C till the fungal growth in the control dishes was complete (3–7 days) after which the readings were taken.

The mycelial growth of fungi (mm) in both treated (T) and control (C) Petriplates were measured diametrically in three different directions. From the mean growth of above readings, percentage inhibition of growth (I) was calculated by using the following formula:

$I\ (\%) = [(C - T) / C] \times 100$

3.14.5 Calculation of EC_{50} values

EC_{50} values (effective concentration for 50 % inhibition of mycelial growth) were calculated from the percent inhibition (I) as follows: Percent inhibition (I) was converted to corrected per cent inhibition IC using the following formula.

$IC = [(\%\ I - C.F.) / (100 - C.F.)] \times 100$

C.F. (Correction Factor) = $[(90 - C) / C] \times 100$

where, 90 is the diameter of the Petri-dish in mm and C is the growth of the fungus in mm in control.

EC_{50} value (ppm) was calculated from the concentration (ppm) and corresponding IC data of each compound, with the help of a Basic LD_{50} program version 1.1 using a personal computer (PC) as described by *Trevors (1986).*

3.15 Nematicidal activity against *Meloidogyne incognita* (Kofoid and White) Chitwood and *Rotylenchulus reniformis* Linford and Oliviera

3.15.1 Soil preparation

Soil from IARI farm and river sand was steam-sterilized at 1.05 kg/cm^2 for 4 hrs. The soil and the sand both were exposed to facilitate the aeration for at least 24 hrs before preparation of soil mixture for experimental purposes. Soil-sand mixture in 2:1 ratio and standard dose of NPK were mixed before filling the pots.

3.15.2 Test culture

The culture of root-knot nematode (*M. incognita*) was raised from a single egg mass on tomato grown in 20 cm earthen pots containing sterilized soil-sand mixture. The reniform nematode, *R. reniformis*, originally isolated from a single eggmass from castor, was cultured, multiplied and maintained on cowpea plants raised in 30 cm earthen pots containing sterilized soil-sand mixture. To ensure constant supply of egg masses for experiments, old plants were removed and replaced with new ones by repeated sowing of cowpea seeds at 50 – 60 days intervals.

3.15.3 Collection of J 2 (*M. incognita*) and J 4 (*R. reniformis*) for inoculation

The egg masses were handpicked and kept on two layers of tissue paper supported by aluminum wire gauze (8 – 10 μ size) in 10 cm sized Petri-dishes filled with fresh water. After 24 hours, the population of *M. incognita* and *R. reniformis* emerged in the suspension was determined separately by counting the juveniles in one ml aliquot three times and the average of three counts was taken as pot population in one ml suspension. *M. incognita* culture was straightaway used for bioassay studies whereas *R. reniformis* juveniles were kept for two more days to let them reach to J 4 stage after which they were used.

3.15.4 Preparation of stock and test solutions

The test material (20 mg) was weighed and dissolved in distilled ethanol (0.2 ml) and made up the volume to 10 ml by 0.5 % emulsified water (5.0 ml of Tween 80 in 1 liter of distilled water) to get stock solution of 2000 ppm concentration. Test solutions of 1000, 500, 250, and 125 ppm concentration were prepared by serial dilution of 2000 ppm stock solution with 0.5 % emulsified water.

3.15.5 Treatment

Suspensions of secondary juvenile stage (J2) of *M. incognita* and pre-adult (J4) stage of *R. reniformis* were diluted separately with water to 100 ml to get approximately 25-juveniles/ml. To one ml of these nematode suspensions in Petri-dish (5 x 5 cm size), equal volume of test solutions of 2000, 1000, 500, 250, and 125 ppm were added separately to get the desired test concentrations of 1000, 500, 250, 125, and 62.5 ppm, respectively. Juveniles kept in emulsified water with minimum ethanol served as control. For each count, 3 replicates were taken and were treated for 24, 48 and 72 hours. After the required exposure, the suspensions of three Petri dishes for each treatment were observed under a stereoscopic binocular microscope for determining juvenile mortality in terms of their immobility. Readings were taken after 24, 48 and 72 hours and the corrected % mortality were calculated from the Abbott's formula (Abbot, 1925).

Corrected mortality (%) = [(T – C) / (100 – C)] ×100

Where, T = % mortality in treatment; and C = % mortality in control.

LC_{50} values (ppm) were calculated by using a Basic LD_{50} program version 1.1 as described by *Trevors (1986).*

3.16 Plant growth regulatory activity

3.16.1 Test seed

Plant growth regulatory activities of two saponins were conducted in rice (*Oryza sativa*) (var. BTC) and maize (Zea mays L.) (var. Jaunpur local).

3.16.2 Stock and test solution

The test saponins (10 mg) were weighed accurately in 5 ml volumetric flask, dissolved and made the volume with 0.2 % emulsified water, which in turn was prepared by dissolving 2 ml of Tween 80 emulsifier in 1 liter of distilled water. From the stock

solution, different concentrations, (1000, 500, 250, 125, 50ppm) were prepared separately by serial dilution with 0.2 % emulsified water. Emulsified water was taken as control.

3.16.3 Seed treatment

Seeds were dipped in saponin solution of different concentrations for 24 hours in test tube. The experiment was done in triplicate for each concentration. After that seeds were placed on the bed of tissue paper in petridishes with uniform spacing and kept at room temperature (25-27^{0}C) for ten days. 2-4 ml of water was added in intervals so that seeds remain in moist condition. After 10 days coleoptiles and root length was measured.

3.16.4 Calculation of growth regulatory activity values

Growth regulatory values (per cent increase or decrease in growth of root or coleoptiles length over control) were calculated as follows using the following formula:

Growth promoting activity (%) = $[(T - C) /C] \times 100$

Growth inhibiting activity (%) = $[(C - T) /C] \times 100$

3.17 Statistical analysis

Antifeedant (AI_{50}), insect growth regulatory (IGR) activity and stability study values were calculated by using a Basic LD_{50} program version 1.1 as described by Trevors (1986). The raw data on different parameters was subjected to angular transformation (arc sine percentage) and analyzed by complete randomized design. Analysis of variance was done and means were separated by Critical Difference (CD) with the help of AGRES package (version 7.01).

3.18 Isobolographic/fractional analysis of the mixtures for evaluating synergistic/potentiation/additive/antagonism action

The potency ratio of the mixtures were calculated by isobolographic analysis, using following formula: Potency ratio = $d_A/D_A + d_B/D_B$, where d denotes concentration in combination and D denotes individual activity ($AI_{50}/GI_{50}/ED_{50}/LC_{50}$). If the ratio is less than 1 then synergism/potentiation occurs, likewise it will be joint action and antagonism when the value will be equal to 1 and greater than 1(Nelson and Kursar, 1999).

4.1 Isolation of azadirachtin powder concentrate

Crushed neem seed kernel powder (1 kg) was first extracted with n-hexane (2 lit.) followed by three subsequent extractions with 1 litre each of the solvent. The solvent recovered from the first distillation was reused to further extract the powder. The combined hexane extracts after distilling off the solvent yielded neem oil. The defatted powder was further extracted with methanol to yield extract rich in azadirachtin. This extract was processed to get rid of water-soluble carbohydrates, amino acids and/or proteins. The traces of water present in the concentrate were removed by passing the extract through desiccating materials. The concentrated extract was precipitated with appropriate non-polar solvent to attain a dry-flowable material. Several batches were similarly run to attain sufficient amount of azadirachtin concentrate. The yield of azadirachtin powder concentrate from 1 Kg of neem seed kernels varied from 8.0 to 9.0 g (0.8 to 0.9 % w/w, kernel basis).

4.1.1 Purification and characterization of azadirachtin-A concentrate

Azadirachtin powder concentrate obtained above is a mixture of several structurally related tetranortriterpenoids, among which, azadirachtin congener A (aza-A)($C_{35}H_{44}O_{16}$) is present in large proportion (≈85%). The next most abundant compound in the concentrate is aza-B, which is present to the extent of 13-14 percent. It was possible to detect the presence of major azadirachtins, such as A, B, as well as minors such as D and H in azadirachtin concentrate (20%) by analytical high performance liquid chromatography (HPLC) using acetonitrile: water (50: 50 v/v) solvent system with flow rate of 0.5 ml min^{-1} and RP-18 column (250 x 4 mm) (Govindachari *et al.,* 1995a). Azadirachtin-A, B, D and H were detected at R_t of 16.597, 20.980, 17.663 and 15.597 minutes respectively (Fig. 1). The azadirachtin concentrates analysed for about 13.8 % aza-A, 3.8 % aza-B, 2.0 % aza-D, and about 0.3 % aza-H. This concentrate was further enriched with aza-A by the process developed earlier in our laboratory to obtain aza-A concentrate of upto 60 % purity (HPLC). The material was further purified to obtain analytical sample of pure aza-A (≈90% purity; mp 155-156°C) for use as standard reference. The

identity of the sample of aza-A was established by its ^{1}H-NMR and mass spectral data (Figs. 28 and 29).

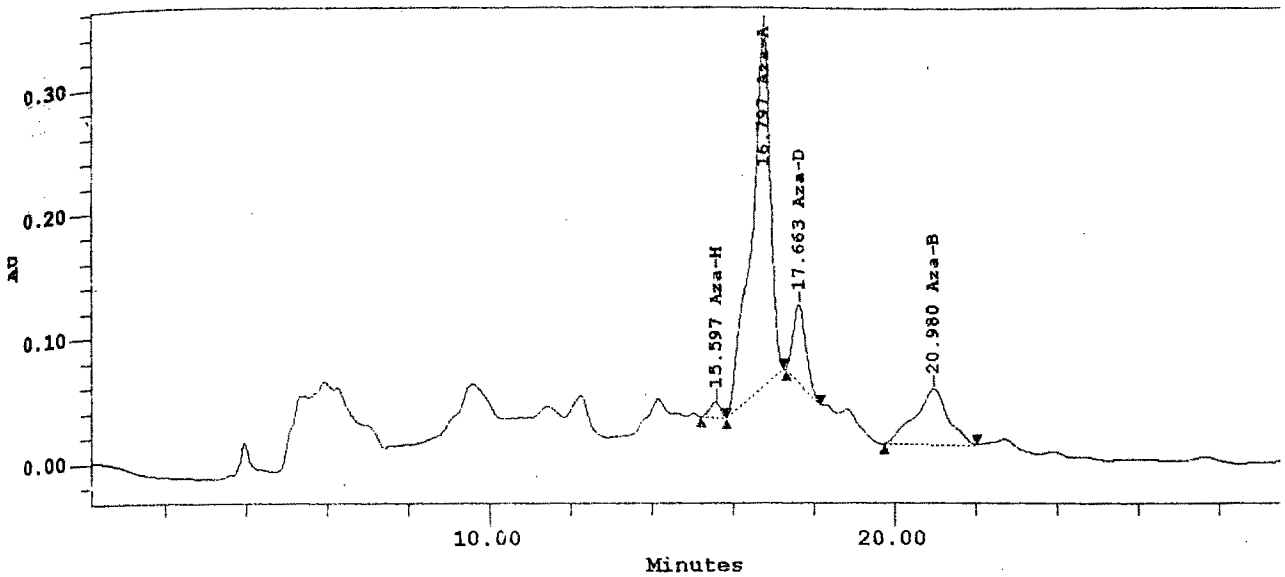
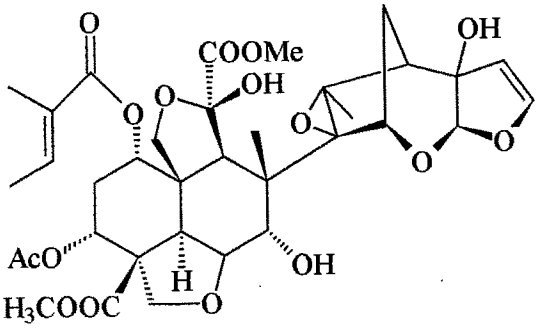


Fig. 1. HPLC chromatogram of azadirachtin concentrate (20 %) containing azadirachtin-A, B, D and H.

The ^{1}H-NMR spectral data and mass fragmentation pattern is listed in Tables 4 and 5. The ^{1}H-NMR spectrum of azadirachtin-A showed, characteristic 12- and 29-carbomethoxy singlets at δ 3.68, and 3.74, 1-H proton multiplet at δ 4.75, 3-OAc singlet at 1.93; 7, 11 and 20-hydroxyl protons at δ 2.89, 5.05 and 2.92, dihydrofuran protons at δ 5.05 (22H) and δ 6.42 (23-H), tigloyl 3'–H proton at δ 6.91, and 4', 5'-methyl protons of tigloyl moiety at δ 1.70 and 1.78 respectively.



Azadirachtin-A

The structure of aza-A was further confirmed by its electrospray mass spectroscopy (ES-MS) which exhibited molecular ion peak at m/z 721 $(M+H)^+$ corresponding to

its molecular formula $C_{35}H_{44}O_{16}$. Other characteristic peaks at m/z 703, 603, 585 and 567 originated as a result of the successive loss of water (18 amu), tigloyl (100 amu), water (18 amu) and water (18 amu) molecules from the parent ion. Other fragment ions at m/z 685, 667, 567 and 507 were formed as a result of the successive loss of two water units, tigloyl and acetic acid (AcOH) moiety from the fragment ion m/z 703.

On the basis of these ^{1}H-NMR data and mass fragmentation peaks the compound was identified as aza-A. The bioassay and stability studies reported in this thesis were carried out with aza-A concentrate (20%).

Table 4. ^{1}H NMR data of azadirachtin-A.

^{1}H atom	δ Value	^{1}H atom	δ Value
1-H	4.74, d, 1H	2-H	2.13, dd, 1H
2-H	2.34, m, 1H	3-H	5.48, dd, 1H
5-H	3.34, d, , 1H	6-H	4.58, dd, 1H
7-H	4.73, d, 1H	9-H	3.32, s, 1H
15-H	4.65, d, 1H	16-H	1.70,dd, 1H
16-H	1.26, d, 1H	17-H	2.36, d, 1H
18-H	1.98, s, 3H	19-H	3.61, dd, 1H
19-H	4.15, dd, 1H	21-H	5.60, s, 1H
22-H	5.05, d, 1-H	23-H	6.42, d, 1-H
28-H	3.72, d, 1H	30-H	1.71, s, 3H
7-OH	2.89, br s	11-OH	5.05, br s
20-OH	2.92, br s	12-CO_2CH_3	3.66, s
29-CO_2CH_3	3.74, s	3-OAc	1.93, s
3'-H	6.91, dq	4'-H	1.70, dq
5'-H	1.78, dq		

Table 5. Positive ion electrospray mass spectral data of azadirachtin-A.

Peak	Atomic mass (m/z)	Relative abundance (%)	Positive ions identified
1	759.3	3.125	$(M+K)^+$
2	743.38	45.0	$(M+Na)^+$
3	721.26	11.25	$(M+H)^+$
4	720	11.25	M^+
5	703	100.0	$(M+H)^+-H_2O$
6	685	36.875	$(M+H)^+-2H_2O$
7	667	4.375	$(M+H)^+-3H_2O$
8	625	1.875	$(M+H)^+-2H_2O$-AA
9	603.34	4.375	$(M+H)^+-H_2O$-TA
10	585.37	11.875	$(M+H)^+-2H_2O$-TA
11	567.32	13.75	$(M+H)^+-3H_2O$-TA
12	507.43	4.375	$(M+H)^+-3H_2O$-TA-AA

4.1.2 Isolation of salannin-nimbin-desacetyl nimbin mixture

Neem oil was partitioned between hexane and aqueous methanol (1:1) to isolate, salannin, nimbin and other bioactive materials. While hexane extract furnished meliacin free neem oil, the aqueous methanol portion of the extract contained all the meliacins particularly salannin and nimbin present in neem oil. Extraction was repeated several times to isolate sufficient quantity of the material rich in salannin and nimbin. As evident from Fig. 2. salannin, nimbin and desacetyl nimbin were present to the extent of 39.62, 18.45 and 18.12%, while meliacins related to azadirachtin group in the concentrate are present to the extent of 18 percent. The mixture was used as such for evaluation as adjuvant for azadirachtin.

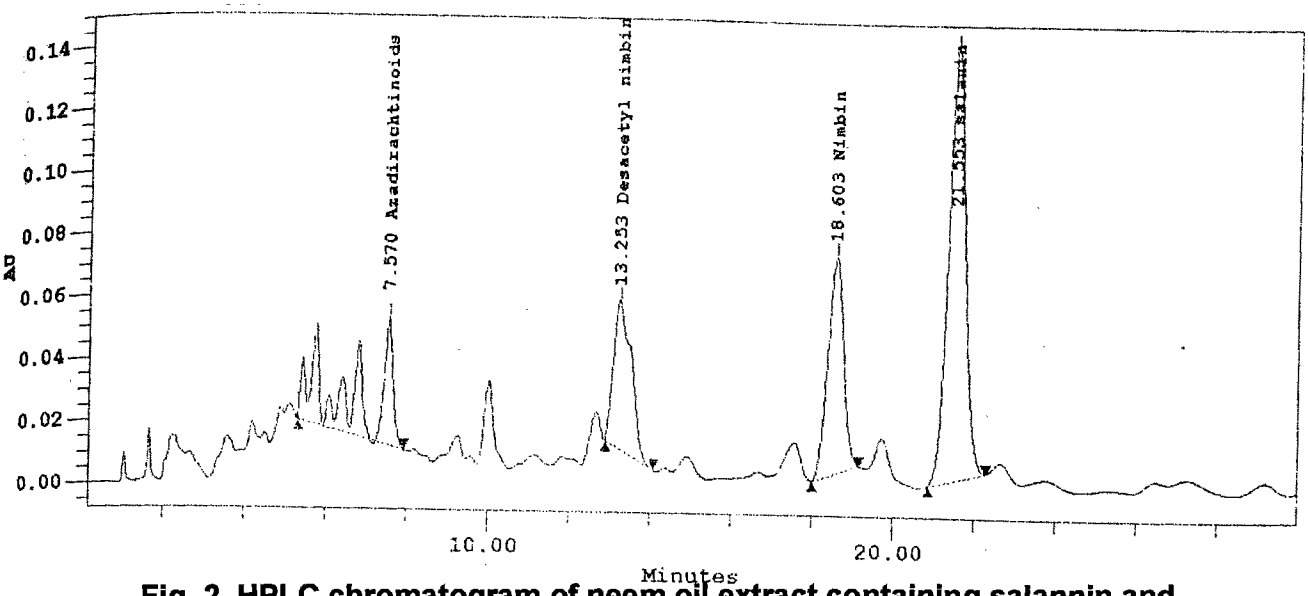


Fig. 2. HPLC chromatogram of neem oil extract containing salannin and nimbin

4.2 Isolation and purification of triterpenic saponins from *Madhuca indica* (mahua) seed kernel

Madhuca indica (Mahua) (syn. *Madhuca latifolia, Bassia latifolia, Diploknema indica*) belongs to the family Sapotaceae. Its seed yields about 40-50% edible oil. Deoiled seed cake contains high level of saponins (6-8%), which make it unsuitable for incorporation in animal feed formulations. Triterpenic saponins isolated from mahua seed kernel, in general, are characterized by its bitter taste, foaming in aqueous solution, haemolytic activity and piscicidal activity (Mulky and Gandhi, 1977). No detailed report is available in literature about their pest control properties. Studies were, therefore, conducted for their isolation, identification and assessment of possible use as botanical pesticide.

Mahua seed procured from Pusa (Bihar) was air-dried, powdered and repeatedly extracted with n-hexane to remove mahua oil. The deoiled seed cake was extracted with methanol and the solvent removed under reduced pressure at temperature up to 50°C to prevent undue thermal decomposition of saponin constituents. The vacuum dried mass was washed with n-hexane and acetone to remove undesired non-polar substances. The residue was partitioned with water: *n*-butanol and the organic phase concentrated under vacuo and precipitated in large excess of

acetone to yield saponin powder concentrate. The crude saponin concentrate was subjected to silica gel column chromatography, eluting the column successively, with chloroform: methanol (65:35) and chloroform: methanol: water (65: 35: 10) to furnish two pure saponins (MI-I and MI-III). Another saponin (MI-II) could not be isolated in pure form. Yield of the pure saponin(s) isolated by column chromatography was, however, very poor. The melting point of saponin MI-I (R_f 0.62) is 235-238°C and saponin MI-III (R_f 0.54), 250-253°C.

4.2.1 Liquid Chromatographic method for the analysis of mahua saponins

High performance liquid chromatography (HPLC) is assuming growing importance in the separation of structurally complex natural products of botanical origin. An improved LC method has been developed to estimate triterpenic saponin content by reverse phase high performance liquid chromatography using photodiode array detector at 213 nm. The method was chosen because of the ready solubility of triterpenic saponins in polar solvents. Based on an initial screen of various proportions of methanol: water and acetonitrile: water solvent systems, methanol: water (60:40 v/v) at a flow rate of 0.4 ml min^{-1} with pressure 1750 psi was chosen for optimum separation of madhuca saponin constituents.

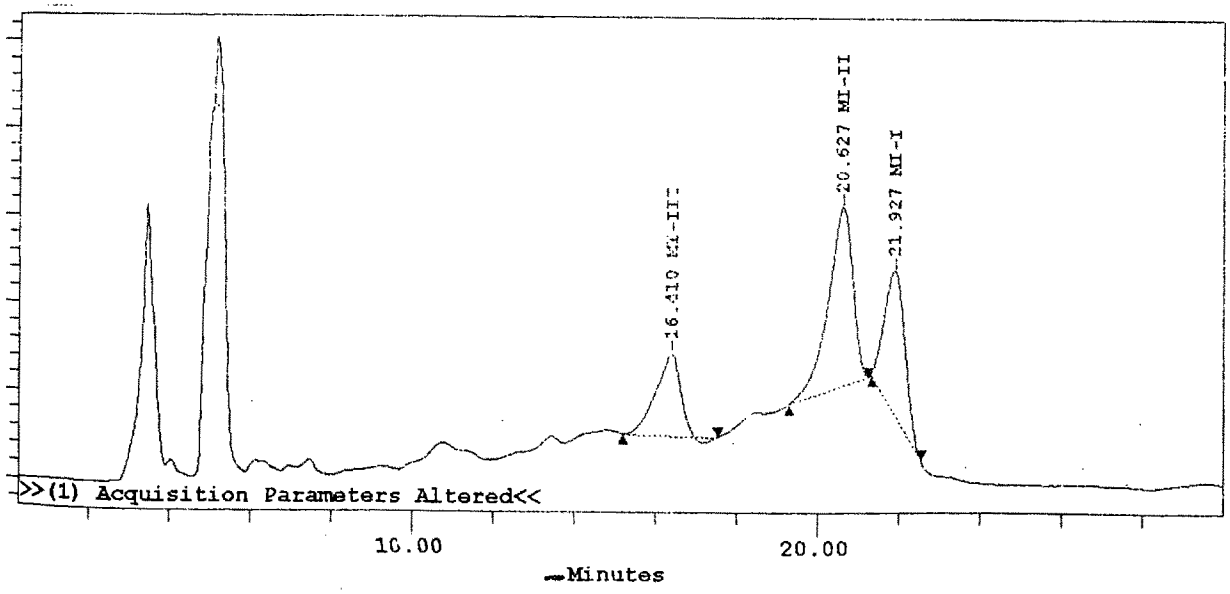


Fig. 3. Analytical HPLC chromatogram of *Madhuca indica* saponins

A chromatogram obtained by injecting 20µl solution of saponin (500 ppm) is depicted in Fig. 3. The retention time of three constituent saponins is shown in Table 3. Resolution of all the three saponins was excellent along the total run time of 30 minutes. In an attempt to determine lower limit of detection, a 20µl injection of 50 ppm solution gave measurable peaks including one of the least sensitive constituent. Figure 3 shows the LC chromatogram at the limit of detection of 50 ppm. The method is simple, specific, and accurate for the analysis and quantification of saponin constituents in madhuca extract.

Table 6. Retention time of Madhuca saponins in HPLC.

No.	Saponin	Retention Time (min.)
1.	MI-III	16.410
2.	MI-II	20.627
3.	MI-I	21.927

4.2.2 Separation of madhuca saponins by Prep-HPLC

Once analytical HPLC method was standardized as above for the analysis of mahua saponins, it was upgraded and fine-tuned for operation on preparative-high performance liquid chromatographic equipment. For each preparative run 50 mg of madhuca saponin concentrate was dissolved in 10 ml of methanol, filtered through a Millipore filter (0.25 µm) and then injected into the preparative column. The prep-HPLC column (RP-18, 250 × 50 mm, 15-25 µm) was run with methanol-water gradient system for 40 minutes with eluant flow rate 40 ml min^{-1} to conveniently separate three saponins constituents. The same resoluting peak fractions were combined and were found to be sufficiently pure when analyzed by analytical HPLC as described above (Fig. 5a and 5b). Peak I (R_t 21.927 min) and peak III (R_t 16.410 min) were found to contain saponin MI-I and MI-III respectively. The middle peak (MI-II) corresponding to R_t 20.627 min could not be isolated in pure form and showed the presence of all the constituents when analysed on HPLC. The identity and purity of two pure saponins was further confirmed by their ^1H NMR and mass spectral data. A representative HPLC chromatogram of one prep-LC run is given in Fig. 4.

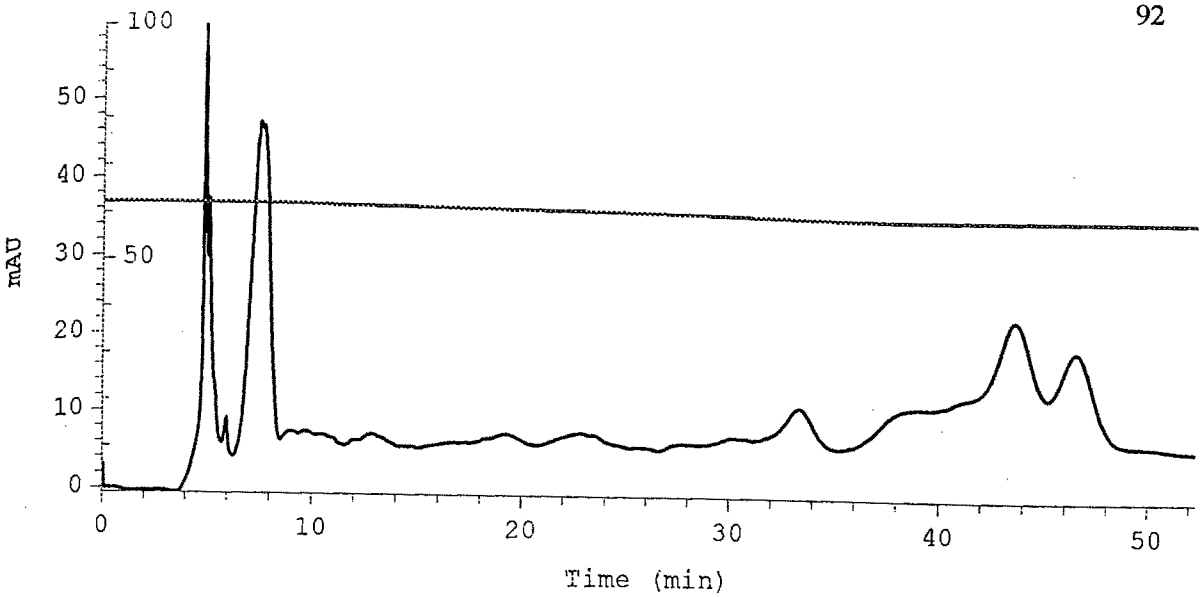


Fig. 4. Preparative HPLC chromatogram of *Madhuca indica* saponins

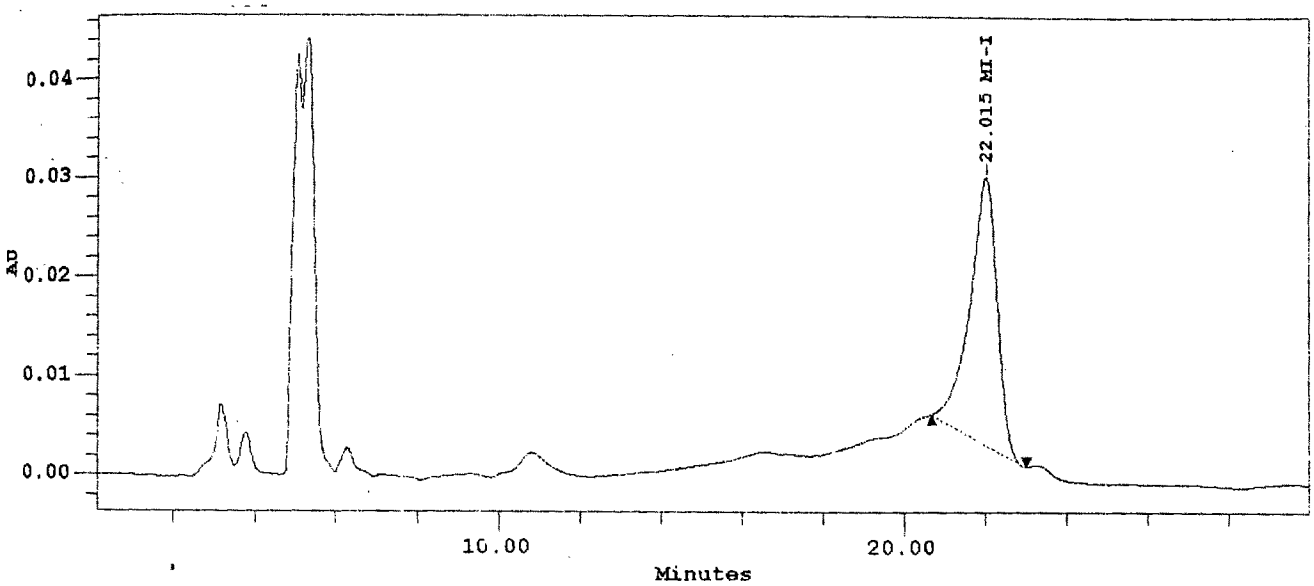


Fig. 5a. Analytical HPLC chromatogram of *Madhuca indica* saponin, MI-I

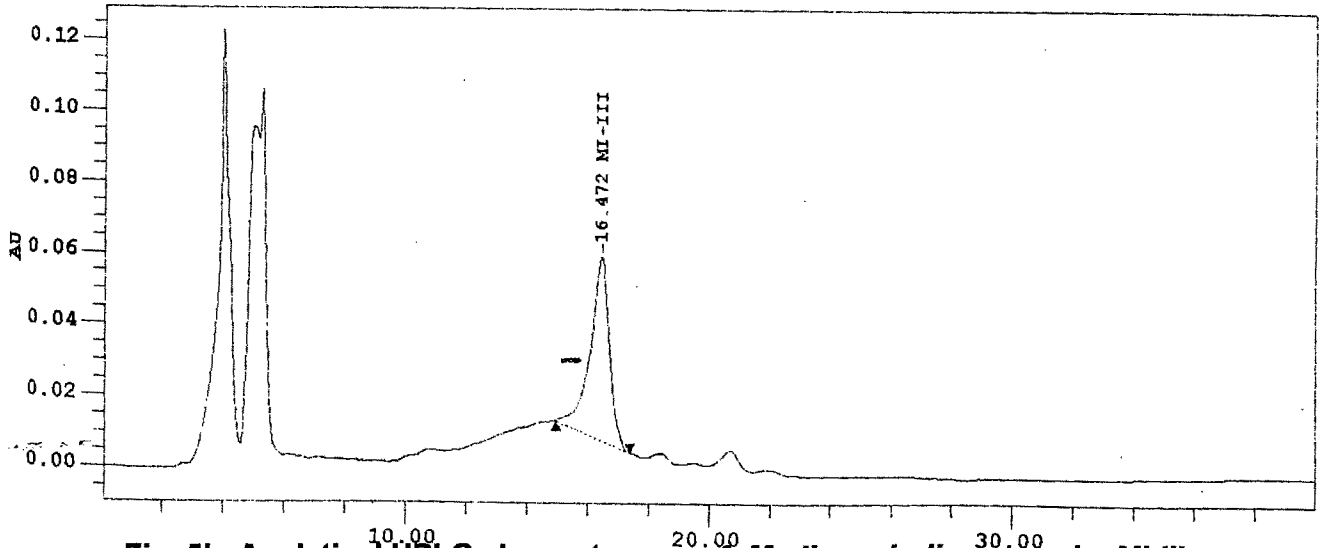


Fig. 5b. Analytical HPLC chromatogram of *Madhuca indica* saponin, MI-III

4.2.3 Acidic hydrolysis of Madhuca saponins MI-I and MI-III: Detection of monosaccharide sugars

Madhuca saponin (MI-I and MI-III) were hydrolyzed with 5 % H_2SO_4 to yield a mixture which when subjected to column chromatography over silica gel furnished prosapogenin as the major product and corresponding genin as the minor product. The prosapogenin(s) mixture was filtered and on further hydrolysis with 7% H_2SO_4 in aqueous methanol yielded the pure genin. Thus for complete conversion of saponin to sapogenin, hydrolysis has to be carried out under strong acidic conditions and for longer duration of time. However, monosaccharide units comprising the sugar moieties get decomposed or altered under strong acidic hydrolytic (H_2SO_4) conditions. For identification of monosaccharides and genin, hydrolysis is best carried out in methanolic HCl solution. Thus complete hydrolysis of saponin (MI-III) with methanolic HCl furnished a genin identified as 16-hydroxyprotobassic acid.

The aqueous hydrolysate obtained after filtration of the genin was concentrated under vacuo, neutralized with barium carbonate solutions and deionized by passing through Amberlite resin (IR 400, H^+) and subjected to thin layer chromatography or paper chromatography to detect the monosccharides. The sugars detected in the aqueous hydrolysate of MI-III were identified as glucose (R_f 0.27), arabinose (R_f 0.40), apiose (R_f 0.49) and xylose (R_f 0.37) by their comparision on TLC and/or PC with the authentic samples. Similarly madhuca saponin MI-I on acidic hydrolysis furnished monosaccharides identified as glucose, arabinose, apiose and xylose. It was thus evident that these two saponins (MI-I and MI-III) differ from each other by the number of monosaccharide units attached to the C-3 and C-28 positions of the sapogenins molecule. Thus MI-III saponin appears to have two additional monosaccharide units (glucose and apiose) attachted at the outer periphery of the two-glycone moieties of MI-I saponin.

4.2.4 Hydrolysis of Madhuca saponin (MI-I and MI-III): Isolation of 16-α-hydroxyprotobassic acid

Since the major mahua saponin is a bisdesmoside with two glycoside linkages at C-3 and C-28 position of the pentacyclic triterpenoid nucleus, it often gives different

products under acidic and /or alkaline hydrolytic conditions. Thus in order to exclusively obtain sapogenin, the major mahua saponin (MI-III) was first hydrolyzed under alkaline (10% KOH) conditions to cleave preferably C-28 ester linkage yielding mixture comprising mainly of prosapogenins with C-3 ester linkages.

The partially hydrolysed product so obtained was then neutralized with dilute HCl, filtered and then subjected to acidic hydrolysis (5% H_2SO_4) to yield genin which was identified as 16-hydroxyprotobassic acid (mp >310^{0}C), by comparison of the ^{1}H-NMR data with that reported in literature (Nigam *et al.*, 1992). Its mass spectrum showed peak at 520 [M^+], 504 [M-OH]$^+$, 487 [M-OH-OH]$^+$. ^{1}H-NMR spectrum shows six singlet peaks at δ 1.04, 1.18, 1.67, 1.81, 2.00, 2.25 for 3-hydroxyl each of methyl protons at H-29, H-30, H-26, H-27, H-24, H-25 respectively. Peaks at δ 3.99, 4.28, 4.30, 4.58, 5.10 and 5.20 were attributed to proton adjacent to –OH functions at H-23, H-3, H-23', H-2', H-16β, and H-6. The peak at δ 5.75 was assigned to H-12 olefinic protons. In protobassic acid molecule peak at δ 5.10 was missing due to non-presence of 16-OH functions.

4.2.5 Spectral characterization of Madhuca saponins

Madhuca saponin MI-I

The first saponin isolated by repeated silica gel column chromatography of the crude saponin concentrate was obtained as amorphous white powder. It was designated as MI-I (m.p. 235-238°C, R_f 0.65 in $CHCl_3$: MeOH: H_2O (65:35:10).

The ^{1}H-NMR spectrum of MI-I showed the existence of six singlet peaks at δ 0.91, 0.94, 1.06, 1.13, 1.16 and 1.23 corresponding to six methyl groups at H-29, H-30, H-27, H-26, H-24 and H-25 positions of the aglycone moiety. A broad singlet at δ 5.63 corresponded to the olefinic proton located at 12th position of the aglycone moiety. Similarly, proton(s) located at carbons adjacent to the hydroxyl functions such as H-3 and H-23 were located at δ 3.41 (m) and 3.71 (br, s). The signal pattern of protons attached at anomeric and other positions of the glycone moiety, as well as methylene and methine protons of the aglycone moiety could not be discerned due to their complex nature in 300 MHz NMR equipment. The ^{1}H-NMR spectrum of MI-I is annexed as Fig. 28.

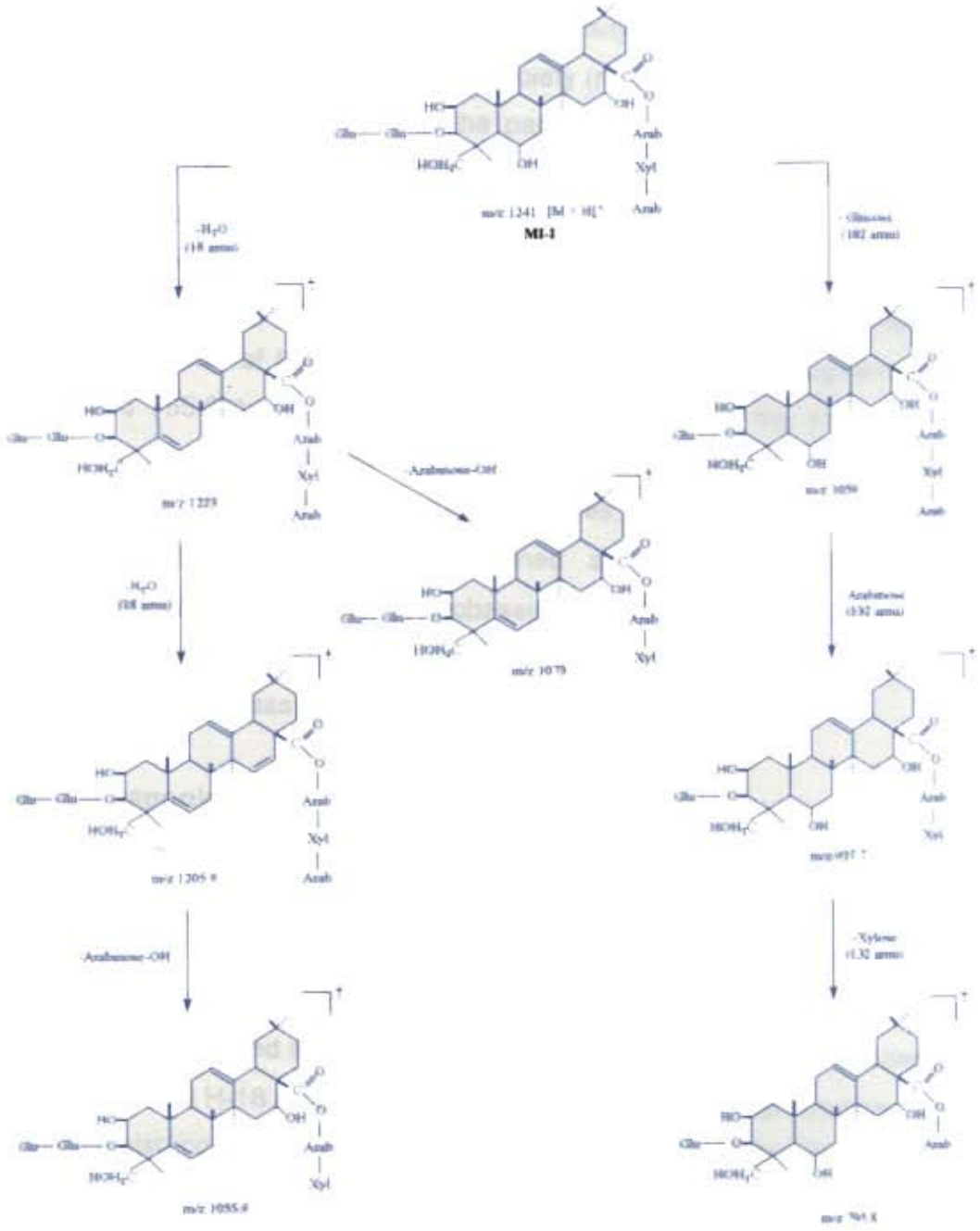


Fig. 6. Mass fragmentation pattern of *Madhuca indica* saponin, MI-I

The mass fragmentation pattern of saponin MI-I is presented in Figure 6. The mass spectrum displayed a quasi-molecular ion peak $[M+H]^+$ at m/z 1241, which corresponded to its molecular formula $C_{57}H_{92}O_{29}$. It further showed two characteristic peaks at m/z 1223 and 1205 emanating as a result of the successive loss of two neutral water (18 amu) molecules from the parent protonated molecular

ion. Further, two fragment ions of lower intensity, detected at m/z 1060 and 1074 were attributed to the loss of glucose moiety (m/z 180 amu) and arabinose moiety (m/z 150 amu) respectively from the parent ion (m/z 1241) and its dehydrated fragment ion (m/z 1223) respectively. The preferential loss of glucose and pentose unit such as arabinose suggest that these monosaccharide units are located at the outer periphery of the glycone moiety of the saponin molecule. Further these monosaccharides (glucose and arabinose) were also detected in the initial phase of Kiliani hydrolysis of MI-I saponin. In addition, characteristic fragment ions at m/z 927.7 and 795.8 were postulated to arise as a result of the successive loss of second and third pentose moiety (150 amu), which may consist of either arabinose and/or xylose. On the basis of spectral studies, the structure of *Madhuca indica* saponin (MI-I) was tentatively assigned as 3-O- [β-D-glucopyarnosyl-β-D-glucopyranosyl] – 16-α-hydroxyprotobassic acid-28-O- [arab-glu-xyl]-arab (Fig. 6). In this tentative structure the linkages between the sugar units have not been established. The mass spectrum is annexed as Fig. 29.

Madhca saponin MI-III

On the basis of ^{1}H-NMR spectral data, the more polar madhuca saponin (MI-III) was identified as bidesmoside of 16-hydroxyprotobassic acid. It showed the presence of six methyl signals at δ 0.90, 0.94, 1.06, 1.13, 1.224 and 1.31 ascribable to H-29, H-30, H-27, H-26, H-24 and H-25 protons respectively. The ^{1}H-NMR spectrum also showed signals at δ 4.52, 3.71, 5.088 and 5.15 corresponding to H-2, H-3, H-6, and H-16 protons on the carbons bearing hydroxyl functions. The NMR spectrum also confirmed the existence of olefinic proton attributable to H-12 proton at δ 5.63 and two hydroxymethyl protons at δ 3.34 and 3.75 located at C-23 position of the aglycone moiety. The remaining anomeric and other protons at δ 3.49, 3.52, 3.77, 3.83, 3.84, 3.86, 3.92, 4.42, 4.46 and 4.54 located on the two glycone moieties attached to C-3 or C-28 positions of the aglycone nucleus could not be assigned to their respective positions.

The mass spectrum of more polar madhuca saponin (MI-III) showed molecular ion peak at m/z 1535.9 corresponding to its molecular formula $C_{68}H_{111}O_{38}$ (Fig. 7). The characteristic fragment ions detected at m/z 1403 and 1373 were attributed to the

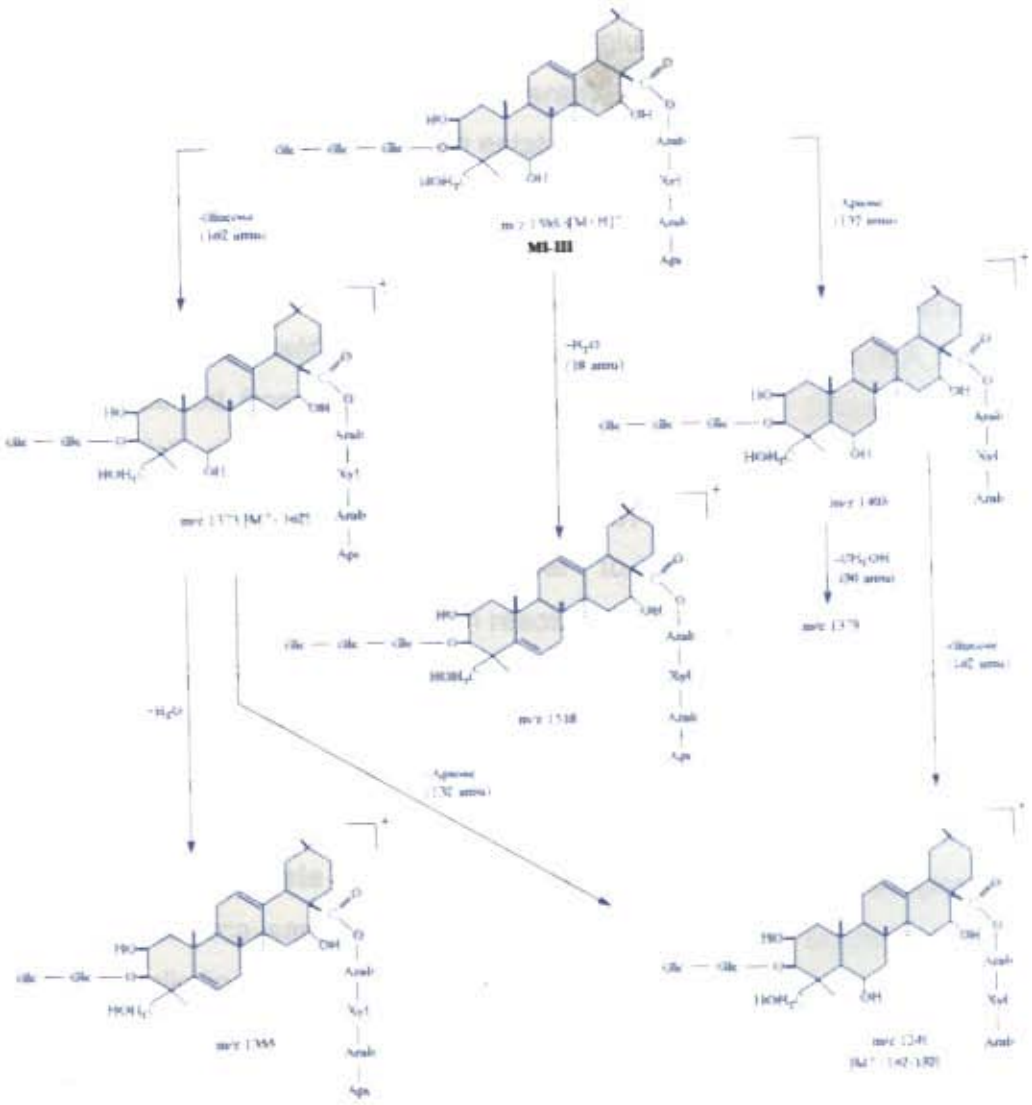


Fig. 7. Mass fragmentation pattern of *Madhuca indica* saponin, MI-III

respective cleavage of pentose such as xylose, arabinose, apiose (132 amu) and hexose such as glucose (162 amu) from the parent ion indicating that one molecule each of glucose and apiose were present in the outer periphery of the two sugar chains attached to the saponin nucleus. Another mass ion peak located at m/z 1241 probably originated as a result of the loss of pentose unit from the parent ion m/z 1373, or hexose unit (glucose) from the fragment ion 1373. Two other fragment ions detected at m/z 1518 and 1355 were caused by the loss of one water molecule (18 amu) from the molecular ion m/z 1535.9 and the fragment ion m/z 1373 respectively. On the basis of spectral studies, the structure of MI-III was tentatively

assigned as 3-O-β-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-α-hydroxyprotobassic acid-28-O-[arab-xyl-arab]- apiose (Fig. 7). Linkages between the monosaccharides have not been established.

As evident from their different molecular mass (MS), two saponins reported herein, appear to be different from those namely Mi-saponin A and Mi-saponin B reported earlier from *Madhuca latifolia* (Kitagawa *et al.*, 1975), and butyroside A, B and other saponins reported from the seeds of *Madhuca butyracea* (Nigam *et al.*, 1992). While *M. latifolia* saponins are derived from the genin protobassic acid, those from *M. butyracea* are glycosides of 16-α-hydroxyprotobassic acid. Two *M. indica saponins* MI-I and MI-III also appear to be the glycosides of 16-α-hydoxyprotobassic acid. Further, high resolution spectral studies would be required to arive at the exact structure of these two highly complex saponins and is beyond the scope of present study. The ^{1}H-NMR and mass spectrum of MI-III are annexed as Fig. 30 and 31 respectively.

4.2.6 Alkaline hydrolysis of Madhuca saponins: Isolation of prosapogenin

The major madhuca saponin (MI-III) separated by column chromatography was subjected to alkaline hydrolysis using 5 % alcoholic KOH to yield partial hydrolytic product (prosapogenin) in which glycon moiety attached to C_3-OH functions remains intact while the one attached to C_{28}-COOH functions gets hydrolyzed. The product separated during hydrolysis was filtered and subjected to column chromatography over silica gel to yield prosapogenin as amorphous cream coloured product (R_f 0.73).

The proton assignments based on its ^{1}H-NMR spectrum made it clear that the prosapogenin is the glycoside of 16-hydroxyprotobassic acid whose hydroxyl group at C_3 is glycosylated. The ^{1}H-NMR spectrum exhibited methyl proton resonances at δ 0.88, 0.94, 1.03, 1.09, 1.24, 1.28 corresponding to H-29, H-30, H-27, H-26, H-24 and H-25 respectively. Other protons chracteristic of the molecule such as olefinic proton at C-12 position, and protons at positions adjacent to carbons having hydroxyl function were also conspicuous by their presence. Thus while a broad singlet at δ 5.56 was attributed to H-12 olefinic proton, four multiplets located at δ

3.65, 4.82, 4.90 and 5.33 were ascribed to H-2, H-3, H-16 and H-6 protons respectively. Other proton resonances corresponding to anomeric and other protons in the glycone moiety, due to their complex pattern, however, could not be assigned to their respective positions.

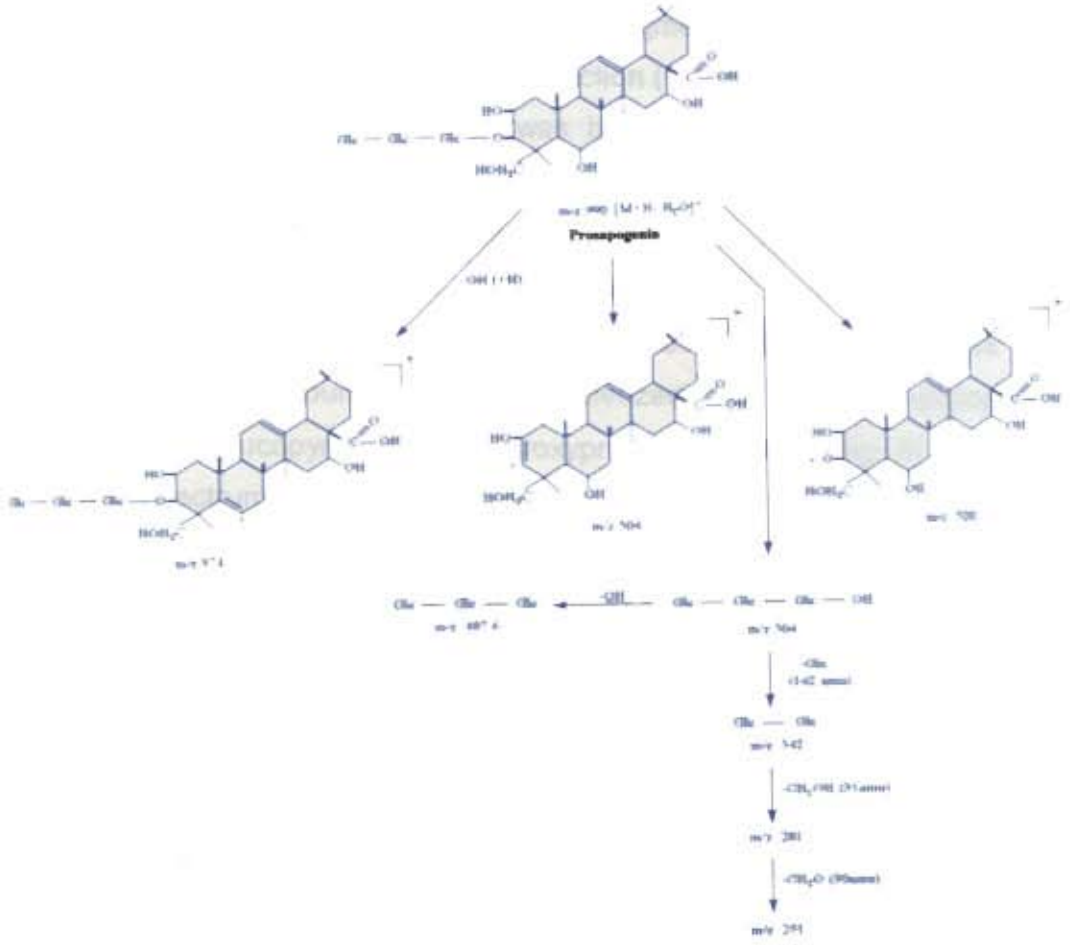


Fig.8. Mass fragmentation pattern of *Madhuca indica* prosapogenin

ESI-MS of the prosapogenin corresponded to the molecular formula $C_{48}H_{79}O_{22}$, the mass spectrum of madhuca prosapogenin displayed characteristic molecular fragment ion peak at m/z 990 $(M+H-H_2O)^+$ and m/z 974 formed as a result of the loss of hydroxyl function from protonated molecular ion respectively. Other characteristic fragment ions at m/z 504 and m/z 487.6 were expected to arise as a result of cleavage of glycoside linkages at C-3 positions. The possibility of any sugar units attached to C-28 carboxylic functions was ruled out as the prosapogenin under discussion was obtained by alkaline hydrolysis of the major madhuca saponin. The first fragment ion at m/z 504 corresponded to the glycone

fragment comprising of three glucose units suggesting that the trisaccharide unit attachted at C-3 hydroxyl position comprised of three glucose units. The other fragment ion corresponding to m/z 342 was assigned to the disaccharide fragment formed as a result of further loss of glucose moiety (162 amu) from the trisaccharide fragment (Fig. 8). The other fragment ions at m/z 487.6 appear to arise as a result of the loss of hydroxyl function (17 amu) from the fragment ion m/z 504. When prosapogenin of MI-III was hydrolysed under acidic conditions, it furnished the genin, which was identified as 16-α-hydroxyprotobassic acid (M^+ 520). The monosaccharide present in the aqueous hydrolysate obtained after neutralization with Amberlite, IR- 400 was identified as glucose (R_f 0.27, Co-TLC and paper chromatography). On the basis of both ^{1}H-NMR and mass spectral analysis the compound was characterized as 3-O-β-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-hydroxyprotobassic acid (Fig. 8). The ^{1}H-NMR and mass spectrum of MI-III are annexed as Fig. 32 and 33 respectively.

4.3 Extraction and isolation of triterpenic saponins from *Sapindus mukorossi* (Ritha) fruit pericarp

Sapindus saponins exhibit the characteristic properties of triterpenic saponins like bitter taste, foaming in aqueous solution. These have been used as a source of natural surfactant or as expectorant rather than for other medicial uses. Studies have been conducted to assess their possible use as botanical pesticide. The pericarp of ritha seed was powdered and extracted with hexane to remove non-polar extractives. The defatted material was then extracted with methanol and solvent removed under reduced pressure. The vacuum dried residue was once again washed with hexane and acetone to remove traces of apolar substances. The remaining residue was partitioned with water: *n*-butanol and the organic phase concentrated under vacuo and precipitated in large excess of acetone to yield saponins mixture. The saponin concentrate was then subjected to column chromatography and the column eluted with different proportions of chloroform: methanol to furnish one major saponin abbreviated as SM-I. Two other minor saponins however, isolated in pure form. The yield of the pure saponin isolated by column chromatography was, however, very poor and therefore separation of the major saponin was attempted on Prep-HPLC.

4.3.1 Liquid Chromatographic method for the analysis of Sapindus saponins

Keeping in view the water solubility of Sapindus saponins a method was developed for their analysis by reverse phase high performance liquid chromatography using photodiode array detector at 213 nm. Several solvent systems were tried to identify the best eluting system to resolve saponin constituents. After considering the various proportions of methanol, acetonitrile and water; acetonitrile: water (47:53 v/v) at a flow rate of 0.4 ml min^{-1} with pressure 1200-1250 psi has been chosen for optimum separation of the saponin constituents.

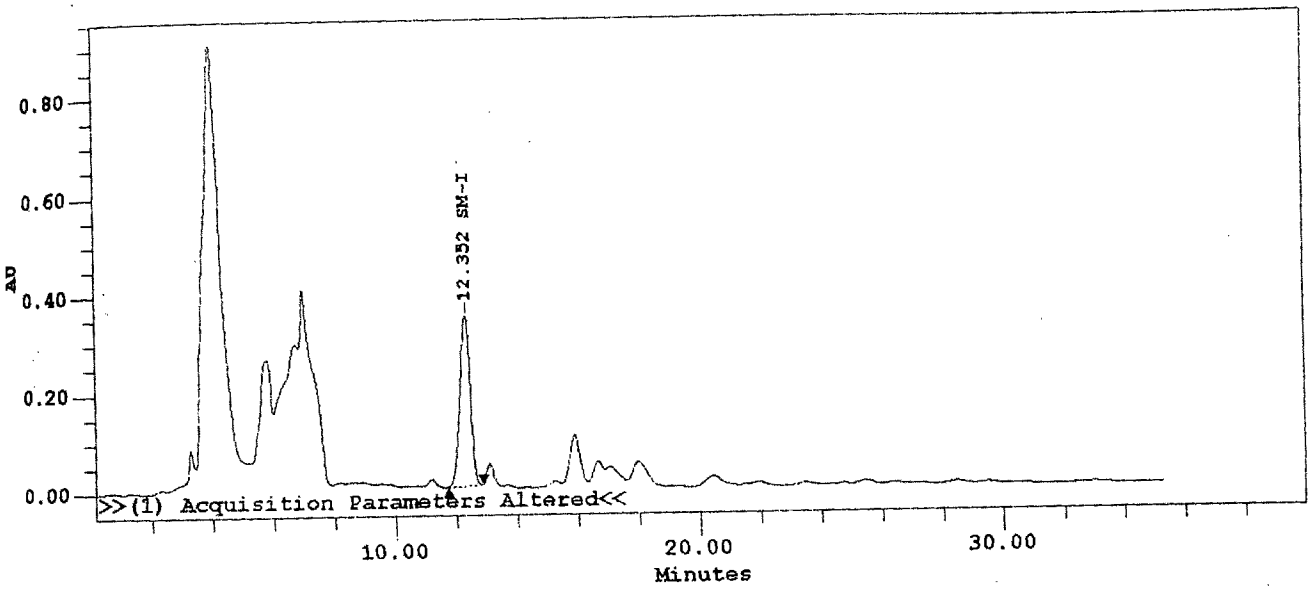


Fig. 9. Analytical HPLC chromatogram of *Sapindus mukorossi* saponins

The saponin concentrate comprised of a single major saponin (>80%) and the rest were detected as minor constituents. A chromatogram obtained by injecting a saponins mixture solution (20µl) containing 500 ppm is depicted in Fig. 9. The retention time of the major Sapindus saponin under these LC conditions is 13.038 minutes. In order to determine the lower limit of detection, 20µl injection of 50ppm solution gave a measurable peak for the least sensitive constituents. The method is simple, specific and accurate for the determination of Sapindus saponins in extractives.

4.3.2 Separation of Sapindus saponin by Prep-HPLC

Analytical HPLC method developed and standardized for the analysis of sapindus saponins was upgraded and fine-tuned for operation on preparative-high performance liquid chromatographic equipment. The prep-HPLC column (RP-18, 250 × 50 mm, 15-25 μm) loaded each time with 50 mg sample was run with acetonitrile-water (47:53 v/v) isocratic solvent system for 40 minutes at the flow rate of 40 ml min^{-1} to separate the saponins (Fig. 10). The same resoluting peak fractions were combined and were found to be sufficiently pure when analyzed on analytical HPLC (Fig. 11).

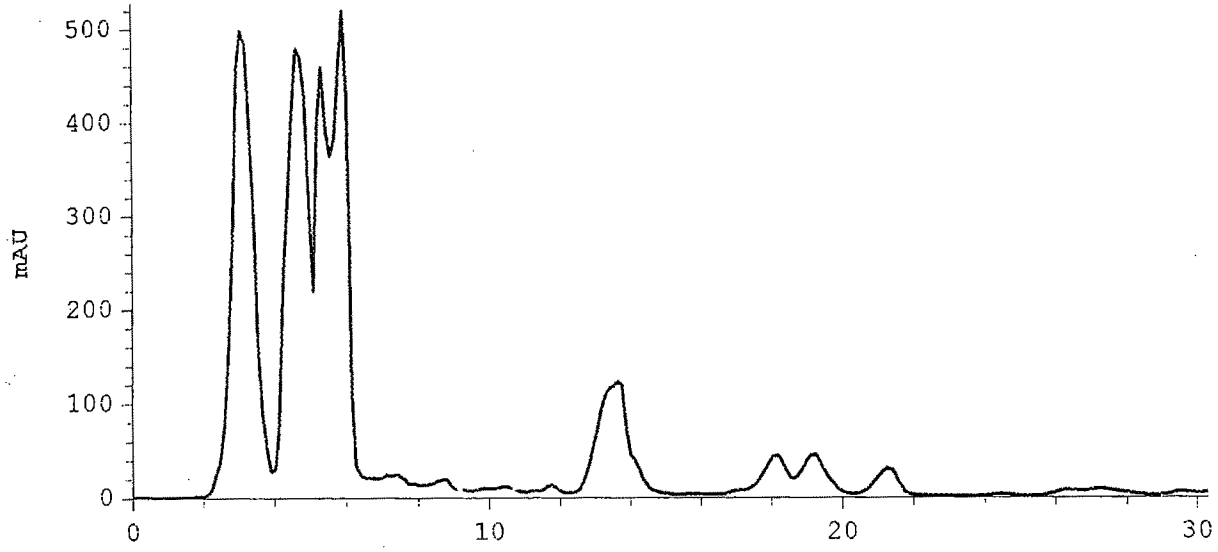


Fig. 10. Preparative HPLC chromatogram of *Sapindus mukorossi* saponins

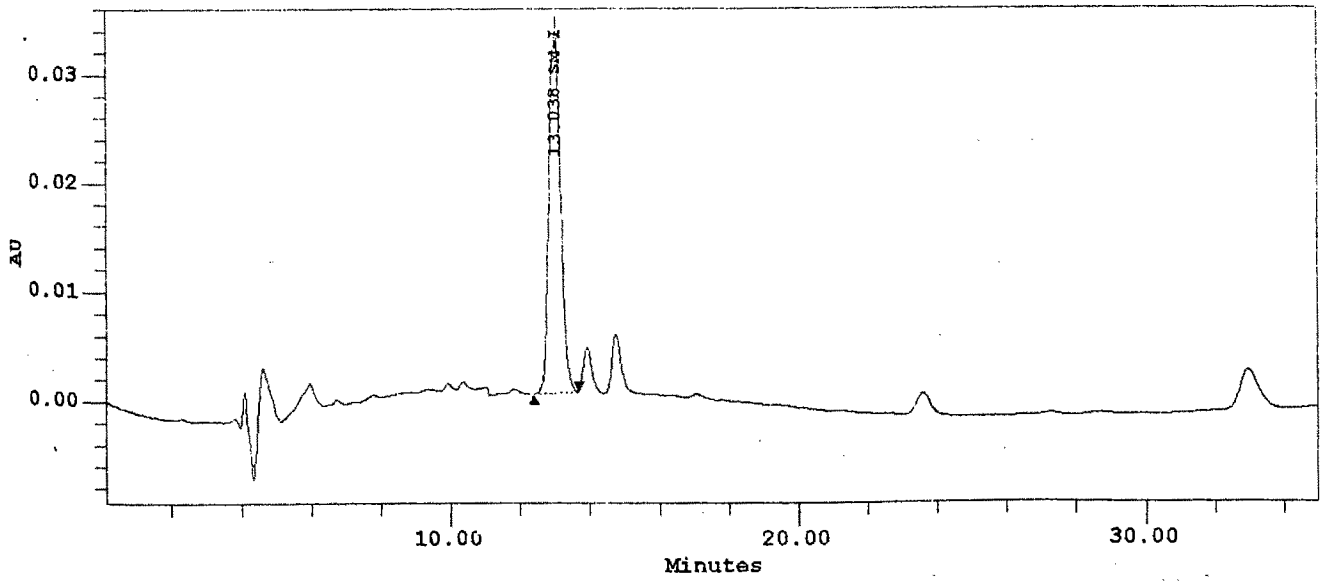


Fig. 11. HPLC chromatogram of *Sapindus* mukoross. SM-I

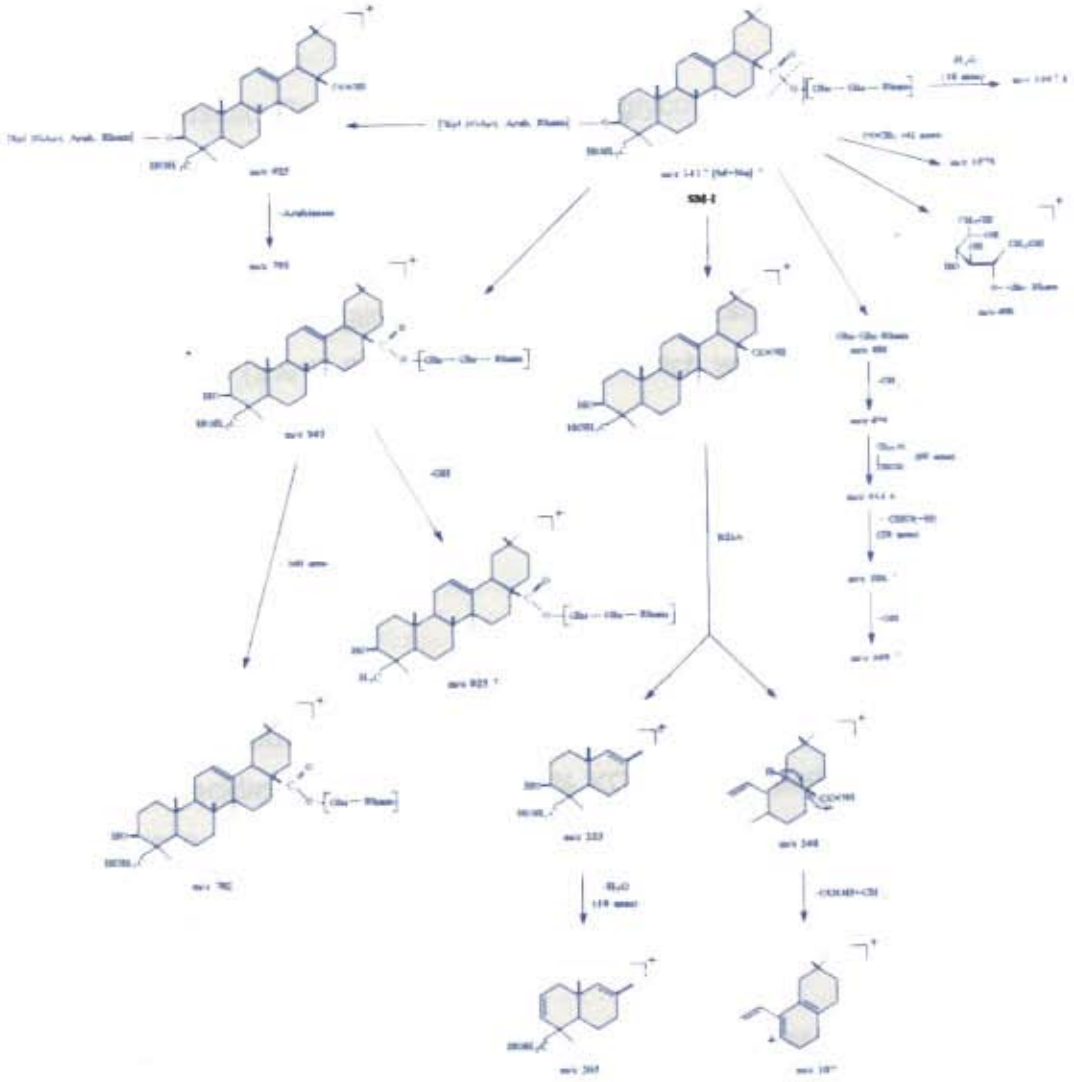


Fig 12. Mass fragmentation pattern of *Sapindus mukorossi* saponin, SM-I.

4.3.3 Hydrolysis of Sapindus saponins: isolation of sapogenin

Saponin was hydrolyzed with mineral acid (methanolic HCl) to yield the sapogenins. Its triterpenic nature was ascertained when it gave positive Liberman Buchard reaction. The sapogenin obtained was identified as hederagenin. The aqueous hydrolysate was neutralized by passing through an ion exchange resin, Amberlite 400 and concentrated under vacuo to obtain product comprising of sugar constituents. The residue comprising of monosaccharide mixture was identified as glucose, arabinose, rhamnose and xylose by comparison of the R_f values on TLC and paper chromatography with the authentic samples.

4.3.4 Complete hydrolysis of Sapindus saponin concentrate: Isolation of hederagenin

The bisdesmosidic Sapindus saponin with glycoside linkage at C-3 and C-28 positions of the pentacyclic triterpenoid nucleus, was first hydrolyzed under alkaline (15% KOH) conditions to cleave preferably C-28 ester linkage yielding a prosapogenin with glycosidic linkage still intact at 3-position of hederagenin. The hydrolytic product so obtained was then filtered, washed, dried and subjected to acidic hydrolysis (5% H_2SO_4) to yield genin, which was identified as hederagenin ($C_{30}H_{48}O_4$), mp 291-293^{0}C, MS 472 $[M]^+$ by comparison of its ^{1}H-NMR and MS data with those reported in literature (Ding *et al.*, 2000). Alternatively complete hydrolysis of saponins concentrate was achieved by reacting with 10% H_2SO_4 in methanol (1:1) for 8 hours.

4.3.5 Alkaline hydrolysis of Sapindus saponins

The major Sapindus saponin (SM-I) separated by column chromatography was subjected to acidic hydrolysis using 10 % KOH to yield partial hydrolytic product in which glycon moiety attached to C-3-OH functions remains intact while that attached to C-28-COOH functions was hydrolyzed. Partially hydrolysed Sapindus saponin was stored in a fridge for evaluation of its pest control properties.

4.3.6 Spectral characterization of *Sapindus mukorossi* saponin, SM-I

Sapindus saponin on acidic and alkaline hydrolysis yielded the sapogenin which was identified as hederagenin by comparison of its ^{1}H-NMR and mass spectrum data with literature values. On hydrolysis with mineral acid it was completely hydrolysed and yielded free monosaccharides identified as glucose, arabinose, xylose and rhamnose, as evident by comparison with authentic samples (TLC, PC). Different partial hydrolysis products inferred that Sapindus saponin is bisdesmosidic with two-sugar moieties attached at C-3 hydroxyl and C-28 carboxyl functions. The constitution of sugar moiety attached at C-3 hydroxyl appears to be the same as reported earlier (Kimata *et al.,* 1983). The sugar moiety located at C-28 position probably comprised of glucose, acid and arabinose. The prosapogenin, resulting from alkaline hydrolysis of the saponin furnished only three sugars namely arabinose, rhamnose and xylose. Besides the characteristic ^{1}H-NMR peaks corresponding to six methyl singlets (0.70, 0.82, 0.90, 0.94, 0.97 and 1.17),

multiplets at δ 3.3 (1H, H-3) and 3.52 (2H, H-23) corresponding to protons adjacent to hydroxyl functions, and an olefinic proton (H-12) at δ 5.22, typical of a hederagenin aglycone moieties, other peaks appearing in the region 3.4-5.0 corresponded to anomeric and other protons of the molecule. Instead of a molecular ion $[M]^+$ peak, exhibited a metal adduct peak at m/z 1416.9 $[M+Na]^+$. Other prominent peaks emerging at m/z 925 are postulated to arise as a result of the C-28 ester cleavage. The fragment ion at m/z 490, 414, 386.7 and 369.7 originated from the C-28 glycone moiety comprising of glucose, and arabinose molecule. Similarly another major fragment ion peak at m/z 942 originated as a result of the cleavage of C-3 glycosidic linkage. Similarly peaks at m/z 793 and 782 were attributed to the loss of glucose and arabinose moieties from fragment ion m/z 925 and 942 repectively. The Sapindus saponin reported here appears to be new. Due to the very complex nature of the major *Sapindus mukorossi* saponin, its exact structure could not be established as it would require special NMR and mass experiments with new generation equipments such as high resolution NMR (600 MHz) or MALDI-TOFF mass spectroscopy. The structure of the saponin was tentatively established as 3-O-[β-D-xyl(OAc). β-D-arabinopyranosyl. β-D-rhamnopyranosyl] hederagenin-28-O[β-D-glu. β-D-glu. β-D-rhamnopyranosyl] ester (Fig. 12).

4.3.7 Saponin acetate and other esters of Madhuca and Sapindus saponins

Pest control properties of naturally occurring polyol esters is well documented in literature. Some of sugar esters reported from *Nicotiana, Solanum,* and other species exhibit remarkable pest control properties. In such sugar esters most active compounds are those in which hydroxyl functions are partially acylated. In view of the several hydroxyl functions in Madhuca and Sapindus saponins, studies were conducted to prepare partial esters following their reaction with acetyl chloride, propionyl chloride, butanoyl chloride and pentanoyl chloride respectively to obtain corresponding esters. Completely acetylated saponins (saponin acetates) were, however, prepared by reacting saponin with acetic anhydride/ acetyl chloride.

4.4 Fatty acids, fatty acid salts and fatty acid methyl esters present in neem, mahua and karanj oil

Neem, mahua and karanj oil were separately saponified with methanolic KOH to obtain corresponding potassium salts of fatty acids. A portion of these salts was stored for bioassay work while the remaining were treated with dilute HCl to obtain the corresponding fatty acids. The fatty acids were refluxed in methanol containing traces of acid to convert them to fatty acid methyl esters. Gas chromatographic analysis of fatty acids derived from neem, mahua and karanj fatty acid methyl esters revealed that all the three oils contain palmitic, stearic, oleic and linoleic acid in varying proportions. Palmitic, stearic, oleic and linoleic acids in the GC chromatogram were detected at their respective retention times (R_t) of 2.74, 4.33, 4.63 and 5.16 minutes for palmitic, stearic, oleic and linoleic acid respectively (Fig. 13). The fatty acids derived from neem oil comprised of palmitic (19.09%), stearic (10.82%), oleic (49.00%) and linoleic acid (16.97%). In mahua and karanj oils 30.11 and 11.80 % palmitic acid, 18.56 and 6.38% stearic acid, 32.74 and 58.37% oleic acid and 14.01 and 17.13% linoleic acids respectively were present. Neem, mahua and karanj oil fatty acids, their potassium salts and methyl esters were subsequently evaluated as potential pesticidal adjuvants.

4.5 Effect of phyto-adjuvants on stability of azadirachtin

The vulnerability of the most active neem constituent, azadirachtin, to various environmental factors such as exposure to air, heat, moisture and sunlight has restricted its use in agricultural applications. Azadirachtin degrades at a faster rate under field conditions specially under abundant sunshine, humidity and temperature. The residual life and toxic effect of azadirachtin-based biopesticides last for 4-5 days. It is therefore necessary to stabilize azadirachtin to improve its shelf-life and bioefficacy by incorporating adjuvants such as stabilizers, antioxidants, UV-screens. Investigations were, therefore, conducted to increase stability and residual toxicity of azadirachtin by incorporating eleven adjuvants derived from neem, mahua and karanj. Two saponin concentrates isolated from *Madhuca indica* and *Sapindus mukorossi*, two corresponding sapogenins namely hydroxyprotobassic acid, and hederagenin, three fatty acid mixtures derived from neem (NOFA), mahua (MOFA) and karanj (KOFA) oil, their corresponding three potassium salts of fatty acids namely NKFA, MKFA and KKFA and one synthetic

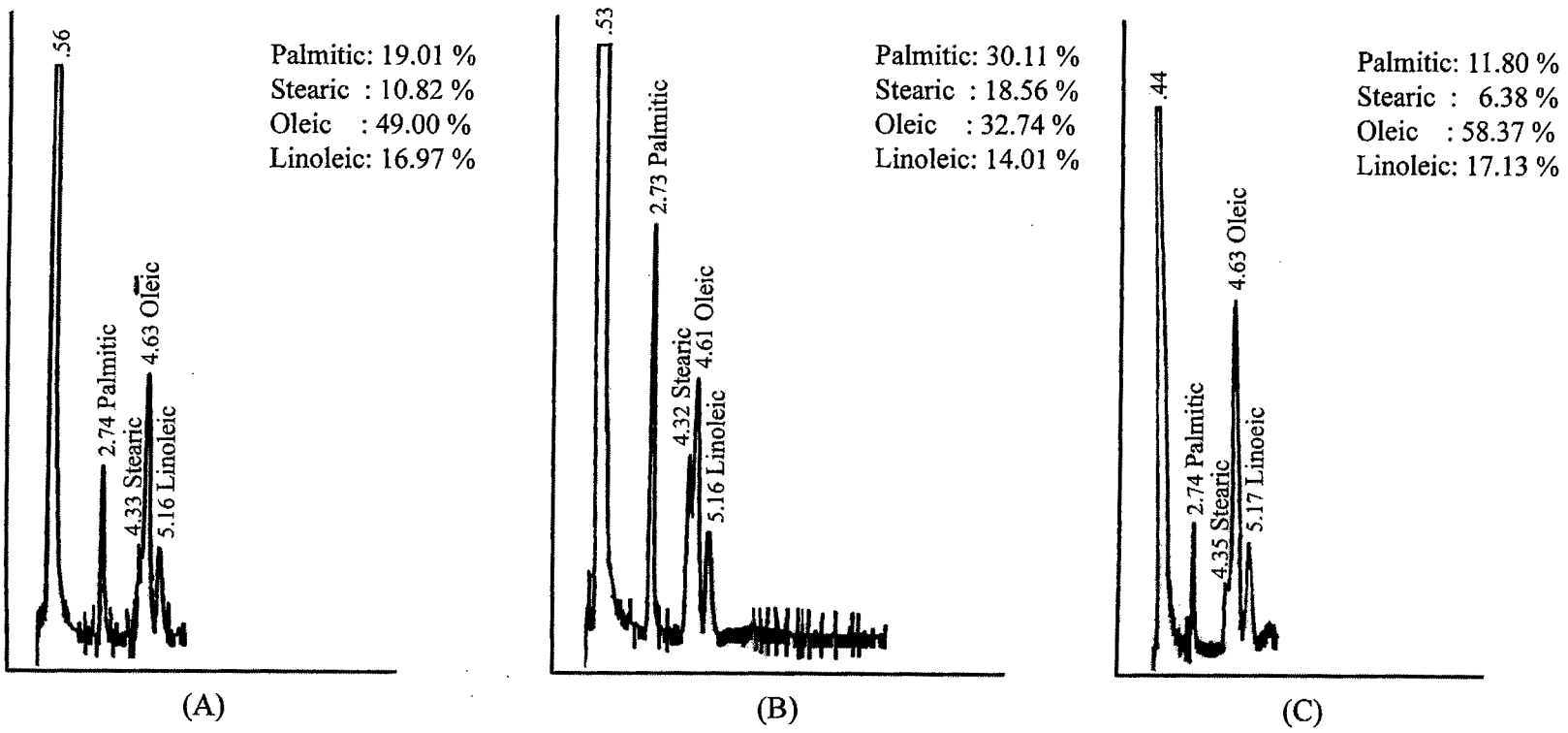


Fig 13. Gas chromatographic analysis of fatty acid methyl esters derived from neem (A), mahua (B) and karanj (C).

stabilizer namely *tert*-butylhydroquinone (TBHQ) were evaluated as potential adjuvants to enhance both efficacy and stability of azadirachtin. Degradation of azadirachtin under exposure to ultraviolet light, sunlight and under accelerated storage conditions has been monitored in the presence of various adjuvants.

4.5.1 UV-photodegradation of aza-A in azadirachtin: adjuvant mixture

4.5.1.1 Thin film on inert glass surface

The concentration (%) of aza-A remaining as thin film on glass surface after different time intervals (min), its half-life ($t_{1/2}$), and stabilization factor (FS) after exposure to UV-light are depicted in Table 7. It is evident from the data that azadirachtin degraded comparatively rapid under UV-photolytic conditions. After 45 and 75 minutes of exposure to UV-light, aza-A was found to the extent of 52.6 and

Table 7 . Aza-A (% remaining) as thin film alone and in combination (3:1) with various phyto-adjuvants under UV light.

Time (min) / Compd.	0	15	30	45	60	75
Aza	100	66.2	55.7	52.6	32.3	18.2
A+ MIS	100	73.4	58.9	54.5	33.9	26.3
A+ SMS	100	71.0	59.8	56.6	32.8	27.3
A+ MISG	100	66.2	56.8	54.2	30.1	18.8
A+ SMSG	100	68.0	56.7	50.4	34.5	20.1
A+FA-NO	100	69.4	56.0	55.1	41.2	21.4
A+FA-MO	100	69.7	57.5	54.9	40.8	22.1
A+FA-PO	100	66.5	55.0	49.1	33.4	15.4
A+KFA-NO	100	68.6	58.1	50.8	38.2	17.6
A+KFA-MO	100	67.0	54.6	50.4	34.3	19.1
A+KFA-PO	100	65.7	60.7	54.7	30.4	20.1
A+TBHQ	100	87.5	77.1	66.8	50.1	30.1

$CD_{5\%}$: Between time: 1.26; between Compound: 1.95; Time x Compound: 4.37
MIS: *M. indica* saponin; SMS: *S. mukorossi* saponin

18.2% respectively. Its half-life ($t_{1/2}$) was calculated as 33.97 min. The photolability of azadirachtin concentrate (20%) was, however, arrested following incorporation of the test adjuvants.

In combination with various test adjuvants, the half-life of azadirachtin was increased to the range of 31.5 to 46.51 min. Among the various test adjuvants, sapogenins (MISG and SMSG) and potassium salts of fatty acids (KFA) were a shade less active than mahua saponin ($t_{1/2}$ 40.06; FS 1.18) and Sapindus saponin ($t_{1/2}$ 41.00 and FS 1.21). Among the fatty acids, those obtained from neem and mahua oil provided better stability (FS 1.15 and 1.17) than the ones derived from karanj oil. The study indicated that *Sapindus mukorossi* saponin concentrate provided the maximum stability (FS 1.21) and was comparable with to the well-known stabilizer *tert*-butylhydroquinone (TBHQ) (Table 8; Fig. 14a).

Table 8. Effect of phyto-adjuvants on the UV light degradation/stability of azadirachtin as thin film on glass surface

Compound	Regression equation	R^2	$t_{1/2}$ (h)	Factor of stabilization (FS)
Aza	y = -0.0204x + 4.6259	0.9295	33.97	1.00
A+MIS	y = -0.0173x + 4.6094	0.9709	40.06	1.18
A+ SMS	y = -0.0169x + 4.5984	0.9529	41.00	1.21
A+MISG	y = -0.0205x + 4.6305	0.9300	33.80	-
A+SMSG	y = -0.0194x + 4.6142	0.9510	35.72	1.05
A+FA NO	y = -0.0177x + 4.6071	0.9052	39.15	1.15
A+FA MO	y = -0.0175x + 4.609	0.9179	39.60	1.17
A+FA PO	y = -0.022x + 4.6482	0.9159	31.50	-
A+KFA NO	y = -0.0201x + 4.6445	0.9066	34.47	1.02
A+KFA MO	y = -0.0197x + 4.6096	0.9412	35.18	1.04
A+KFA PO	y = -0.0199x + 4.6311	0.9295	34.82	1.03
A+TBHQ	y = -0.0149x + 4.7156	0.9075	46.51	1.37

4.5.1.2 Methanolic solution

The concentration (%) of aza-A alone and in aza-A:adjuvant solution in methanol remaining after UV-light exposure of different time intervals is given in Table 9. Its half-life ($t_{1/2}$) and stabilization factor (FS) are reported in Table 10. After 1.0 and 3.5

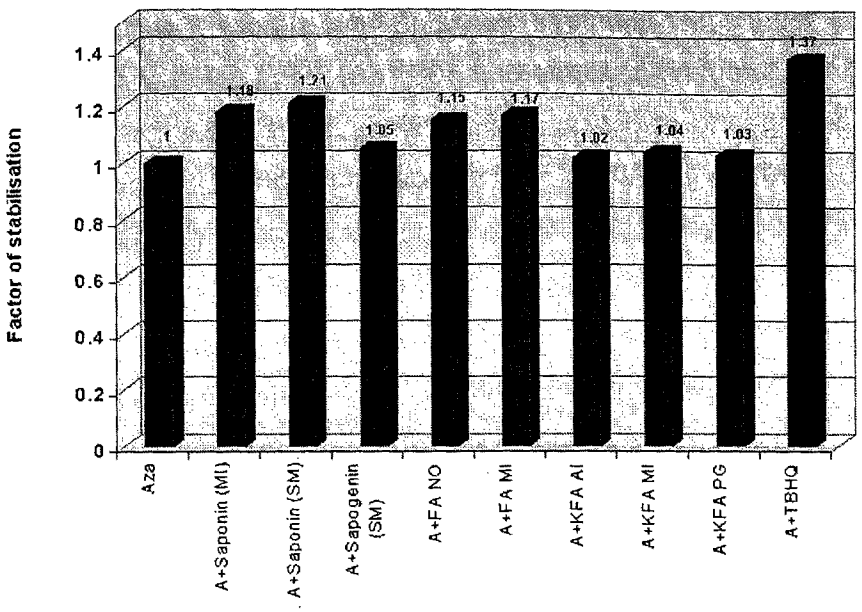


Fig. 14a. Effect of phyto-adjuvants on the UV-light photodegradtion/ stability of azadirachtin as thin film

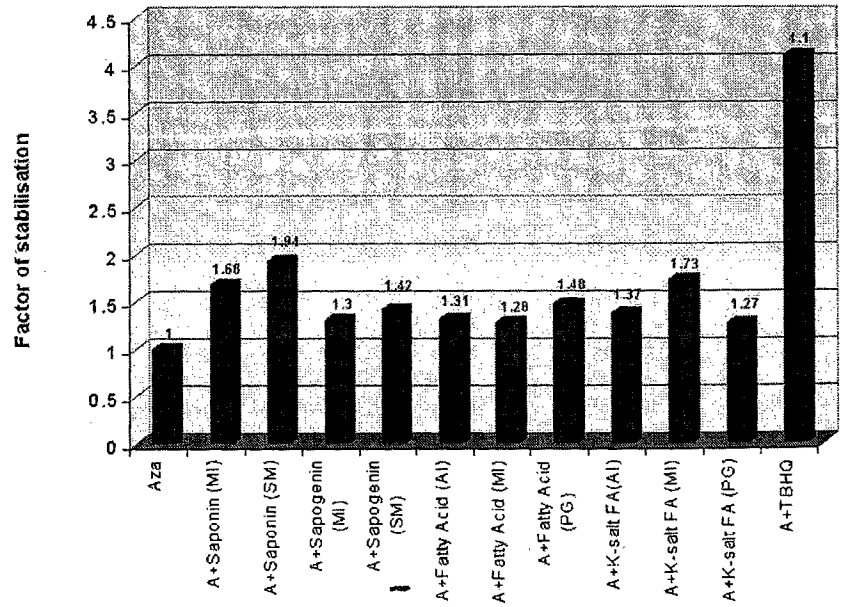


Fig. 14b. Effect of phyto-adjuvants on the UV-light photodegradtion/ stability of azadirachtin in methanolic solution

h of exposure to UV-light, azadirachtin was detected to the extent of 35.6 and 2.3% in methanolic solution. Its half-life in methanolic solution was found to be 0.67 h. Following incorporation of test adjuvants, the half-life and stabilization factor were significantly increased. Mahua and Sapindus saponins were most effective with corresponding half-life ($t_{1/2}$) of 1.13, 1.30h and stabilization factor of 1.68 and 1.94 respectively. Thus the stability of azadirachtin in presence of saponins was almost doubled. Madhuca and Sapindus sapogenins (MISG and SMSG), on the other hand, were moderately effective and could impart stability by the factor of 1.30 and 1.42 only. Among the three fatty acids the one derived from karanj oil increased its

Table 9. Aza-A (% remaining) in methanolic solution alone and in combination (3:1) with various adjuvants under UV light.

Time(h) / Compd.	0	0.5	1.0	1.5	2.5	3.5	4.5	5.5
Aza	100	55.1	39.7	35.6	10.2	2.3	0.0	0.0
A+MIS	100	64.7	54.1	39.8	21.7	15.4	8.6	2.4
A+SMS	100	68.9	57.9	41.2	22.5	16.4	10.4	4.3
A+ MISG	100	57.4	42.8	39.7	18.7	8.7	2.0	0.0
A+ SMSG	100	56.9	45.7	37.4	20.2	7.9	3.1	0.0
A+FA-NO	100	58.9	42.5	36.8	22.1	9.6	1.9	0.0
A+FA-MO	100	61.2	50.7	36.7	14.8	5.6	0.0	0.0
A+FA-PO	100	58.7	52.4	41.0	22.3	11.5	3.2	0.0
A+KFA-NO	100	56.7	40.1	33.8	22.6	8.6	2.4	0.0
A+KFA-MO	100	58.9	42.6	36.4	23.7	11.2	5.6	0.0
A+KFA-PO	100	56.1	41.5	34.8	20.1	8.7	1.6	0.0
A+TBHQ	100	64.8	56.7	54.5	45.8	35.6	26.4	10.2

$CD_{5\%}$: Between time: 0.64; between compound: 0.84; time x compound: 2.22

half-life by a factor of 1.48 ($t_{1/2}$). Similarly among the three fatty acid salts, potassium salt of Madhuca fatty acids could increase the half-life of azadirachtin, to 1.16h with corresponding stabilization factor of 1.73. It was concluded that under UV-light, saponins, particularly Madhuca saponins and Madhuca oil based

potassium salt of fatty acids considerably enhanced the photostability of azadirachtin in methanolic solution (Fig. 14b).

Table 10. Effect of adjuvants on the UV light degradation/stability of azadirachtin in methanolic solution

Compound	Regression equation	R^2	$t_{1/2}$ (h)	Factor of stabilization (FS)
Aza	y = -1.0324x + 4.7213	0.9658	0.67	1.00
A+MIS	y = -0.6099x + 4.6097	0.9703	1.13	1.68
A+SMS	y = -0.5347x + 4.5607	0.9882	1.30	1.94
A+MISG	y = -0.7942x + 4.6572	0.962	0.87	1.30
A+SMSG	y = -0.7328x + 4.5981	0.9841	0.95	1.42
A+FA-NO	y = -0.7868x + 4.6653	0.9448	0.88	1.31
A+FA-MO	y = -0.8053x + 4.6521	0.9879	0.86	1.28
A+FA-PO	y = -0.7009x + 4.646	0.9660	0.99	1.48
A+KFA-NO	y = -0.7507x + 4.5906	0.9612	0.92	1.37
A+KFA-MO	y = -0.5978x + 4.486	0.9862	1.16	1.73
A+KFA-PO	y = -0.8198x + 4.6584	0.946	0.85	1.27
A+TBHQ	y = -0.2521x + 4.4122	0.9361	2.75	4.10

4.5.2 Sunlight photodegradation of azadirachtin in azadirachtin: adjuvants mixture

4.5.2.1 Thin film on inert glass surface

Concentration of aza-A remaining after sunlight irradiation of a thin film of azadirachtin: adjuvant (3:1) mixture on inert glass surface, and their half-lives and factor of stabilization are reported in Tables 11 and 12. As evident from the data, Madhuca saponin ($t_{1/2}$ 2.25 d; FS 1.68) and Sapindus saponin ($t_{1/2}$ 1.76d; FS 1.31) concentrates imparted maximum stability to azadirachtin thin layer followed by potassium salt of Madhuca fatty acids ($t_{1/2}$ 1.64; FS 1.22). The sapogenins (MISG and SMSG) corresponding hydrolytic products of saponin concentrates were least effective, suggesting that glycon moiety in the saponin molecule was essential for imparting stability to azadirachtin molecule. Similarly fatty acids derived from neem,

Table 11. Aza-A (% remaining) as thin film alone and in combination (3:1) with various phyto-adjuvants under sunlight

Time(h) / Compd.	0	1	3	5	7
Aza	100	38.1	18.2	6.5	2.1
A+ MIS	100	48.7	28.9	18.2	9.8
A+SMS	100	45.4	22.7	10.7	5.4
A+MISG	100	38.1	19.7	9.9	2.2
A+SMSG	100	40.8	18.5	6.4	2.1
A+FA-NO	100	39.2	19.2	7.2	2.6
A+FA-MO	100	42.1	21.1	9.8	3.5
A+FA-PO	100	40.1	23.1	10.4	2.3
A+KFA-NO	100	40.7	21.7	8.6	2.5
A+KFA-MO	100	42.7	21.4	10.9	4.2
A+KFA-PO	100	38.9	17.5	9.8	2.0
A+TBHQ	100	68.9	45.2	30.8	25.4

$CD_{5\%}$: Between time: 0.92; between compound: 1.59; Time x compounds: 3.18

Table 12. Effect of phyto-adjuvants on the sunlight degradation/stability of azadirachtin as thin film

Compound	Regression equation	R^2	$t_{1/2}$ (d)	Factor of stabilization (FS)
Aza	y = -0.5187x + 4.4005	0.9845	1.34	1.00
A+MIS	y = -0.3068x + 4.3894	0.9686	2.25	1.68
A+SMS	y = -0.3948x + 4.3716	0.9782	1.76	1.31
A+MISG	y = -0.4945x + 4.4437	0.9683	1.40	1.04
A+SMSG	y = -0.528x + 4.4556	0.9919	1.31	-
A+FA-NO	y = -0.4943x + 4.4136	0.9875	1.40	1.04
A+FA-MO	y = -0.4483x + 4.4206	0.985	1.55	1.16
A+FA-PO	y = -0.491x + 4.4935	0.9675	1.41	1.05
A+KFA-NO	y = -0.4924x + 4.467	0.9838	1.41	1.05
A+KFA-MO	y = -0.4224x + 4.4011	0.982	1.64	1.22
A+KFA-PO	y = -0.5067x + 4.4423	0.9672	1.37	1.02
A+TBHQ	y = -0.1946x + 4.4812	0.9619	3.56	2.66

mahua and karanj oil were ineffective as under sunlight condition they could not improve azadirachtin stability (Fig. 15a). The stabilizing efficiency of saponins, was however, lower than the commercial stabilizer *tert*-butylhydroquinone ($t_{1/2}$ 3.56; FS 2.66).

4.5.2.2 Methanolic solution

Concentration of aza-A (%) remaining after sunlight irradiation of methanolic solutions of azadirachtin: adjuvants (3:1) mixture is given in Table 13. its half-life ($t_{1/2}$) and factor of stabilization alone and in combination with various adjuvants are given in Table 14 and Fig. 15b. As evident from the data, none of the test adjuvants except two saponin concentrates could provide significant stability to azadirachtin molecule. Saponin concentrate isolated from *Madhuca indica* was a shade better than Sapindus saponin in providing stability to azadirachtin molecule. The stabilizing efficiency of mahua saponin concentrate ($t_{1/2}$ 16.73 d) was slightly

Table 13. Aza-A (% remaining) in methanolic solution of azadirachtin alone and in combination (3:1) with various phyto- adjuvants under sunlight

Time(h) / Compd.	0	1	3	5	7	9	11
Aza	100	82.7	76.9	72.4	65.8	56.4	50.7
A+MIS	100	92.7	89.7	84.7	78.4	68.3	61.8
A+SMS	100	92.0	86.7	82.7	75.0	67.8	59.8
A+MISG	100	82.9	78.3	75.4	68.4	59.7	51.7
A+SMSG	100	85.4	79.8	70.4	65.0	59.8	49.8
A+FA-NO	100	83.1	79.4	76.2	66.4	58.0	50.2
A+FA-MO	100	86.1	79.0	75.5	66.4	58.2	51.9
A+FA-PO	100	83.7	79.5	76.3	67.1	56.8	52.6
A+KFA NO	100	82.8	78.6	71.5	68.0	55.7	50.4
A+KFA MO	100	83.0	75.8	72.6	69.4	56.8	51.7
A+KFA PO	100	83.4	76.8	70.5	66.8	57.2	52.3
A+TBHQ	100	89.8	79.8	75.9	70.6	68.9	63.7

CD $_{5\%}$: Between time: 1.02; between compound: 1.44; Time x Compound: 3.52

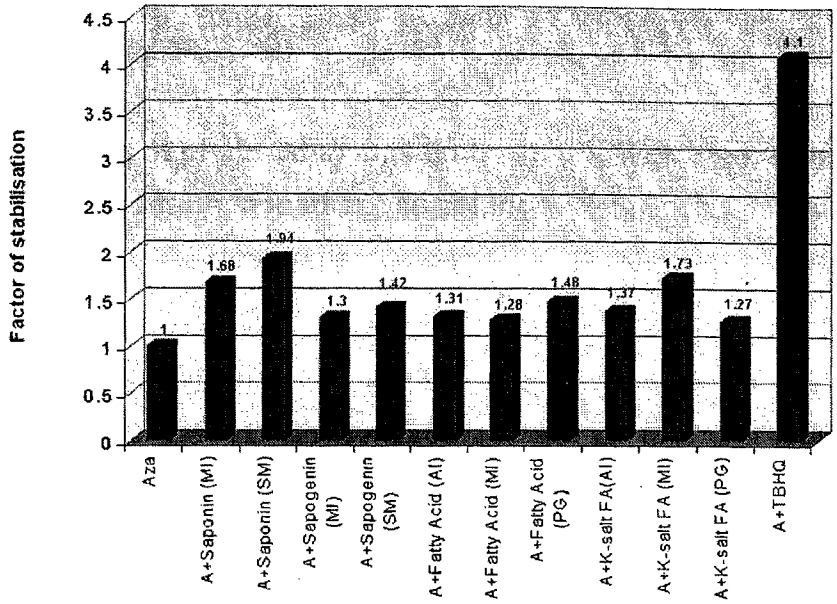


Fig. 15a. Effect of phyto-adjuvants on the sunlight photodegradtion/ stability of azadirachtin as thin film

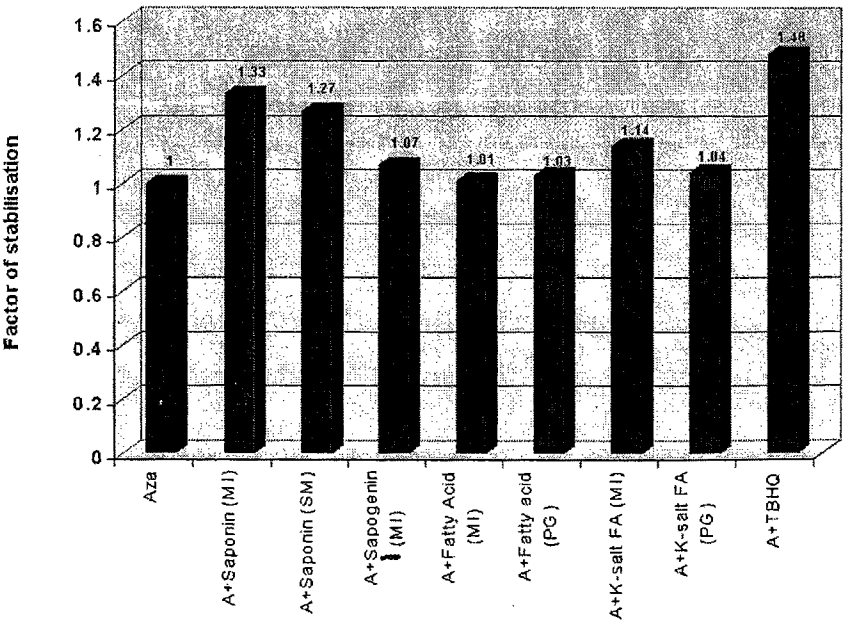


Fig. 15b. Effect of phyto-adjuvants on the sunlight photodegradtion/ stability of azadirachtin in methanolic solution

greater than Sapindus saponin concentrate ($t_{1/2}$ 16.00d). The corresponding factor of stabilization (FS) was calculated to be 1.33 and 1.27 respectively (Table 14). Their stabilizing efficiency was, however, comparable to the commercial stabilizer TBHQ ($t_{1/2}$ 18.63 d, FS 1.48).

Table 14. Effect of phyto-adjuvants on the sunlight degradation/stability of azadirachtin in methanolic solution

Compound	Regression equation	R^2	$t_{1/2}$ (d)	Factor of Stabilization
Aza	y = -0.0552x + 4.5397	0.9634	12.55	1.00
A+MIS	y = -0.0414x + 4.6098	0.9673	16.73	1.33
A+SMS	y = -0.0433x + 4.5983	0.9802	16.00	1.27
A+MISG	y = -0.0517x + 4.547	0.9496	13.40	1.07
A+SMSG	y = -0.0563x + 4.5552	0.9746	12.31	-
A+FA-NO	y = -0.0555x + 4.5576	0.9575	12.49	-
A+FA-MO	y = -0.0548x + 4.5621	0.9772	12.65	1.01
A+FA-PO	y = -0.0537x + 4.555	0.9591	12.91	1.03
A+KFA-NO	y = -0.0559x + 4.5469	0.9576	12.40	-
A+KFA-MO	y = -0.0527x + 4.5369	0.9412	13.15	1.05
A+KFA-PO	y = -0.0529x + 4.5335	0.9614	13.10	1.04
A+TBHQ	y = -0.0372x + 4.5423	0.9393	18.63	1.48

4.5.3 Accelerated storage test

4.5.3.1 Effect of adjuvants on the thermal stability of azadirachtin-A in methanolic solution (Accelertaed storage, 54 ± 1°C)

The vulnerability of azadirachtin to various natural environmental factors is well established (Stokes and Redferm, 1982). Under tropical and warm conditions, the residual effect of azadirachtin-based pesticides usually lasts for 4-5 days. Its residual life, under accelerated storage condition, however, can be increased with the help of suitable adjuvants such as saponins and potassium salt of fatty acids. The residual concentration of aza-A remaining in methanolic solution of aza: adjuvant mixture (3:1) at different time intervals of accelerated storage and their corresponding half-lives and factor of stabilization (FS) are presented in Table 15.

Table 15. Aza-A (% remaining) in methanolic solution of azadirachtin alone and in combination (3:1) with various phyto- adjuvants under accelerated storage (54±1°C)

Time(h) / Compd.	0	21	49	113	202	304	351
Aza	100	73.8	64.2	56.4	45.2	23.0	17.9
A+MIS	100	96.4	94.8	75.9	56.8	43.2	38.9
A+MISG	100	85.8	80.4	76.7	53.4	38.0	30.4
A+SMS	100	88.4	78.3	64.5	54.2	38.4	33.0
A+SMSG	100	89.1	86.6	74.2	56.8	37.3	31.7
A+FA-MO	100	88.5	83.6	76.4	57.7	43.8	39.1
A+KFA-MO	100	80.7	78.6	75.4	49.5	30.4	20.4
A+MFA-MO	100	72.8	62.8	58.1	44.0	25.3	23.4
A+ FA-NO	100	76.9	69.4	58.4	49.2	25.0	18.9
A+ KFA-NO	100	80.6	66.7	59.2	45.3	22.5	18.0
A+ FA-PO	100	78.4	65.1	56.1	46.8	24.6	18.6
A+ KFA-PO	100	80.2	62.1	56.0	40.5	23.4	16.9
A+TBHQ	100	92.8	86.7	59.8	53.1	42.1	32.7

$CD_{5\%}$: Between Time = 1.59, between Compound = 2.16, Time x Compound = 5.72

Perusal of data revealed that with passage of time azadirachtin degraded comparatively rapidly. After 14 days of accelerated storage, azadirachtin content in its methanolic solution was 17.9% only. In combination with various test adjuvants derived from mahua, Sapindus, karanj and neem, the degradation was suppressed and aza-A content in solution phase ranged from 16.9 to 39.1 percent. Among the effective adjuvants, Madhuca and Sapindus saponins as well as mahua fatty acids provided maximum stability to azadirachtin. After 14 days of accelerated storage aza-A content was found to be 38.9, 30.4 and 39.1 % in presence of Madhuca saponin, Madhuca sapogenin and Madhuca fatty acids respectively.

Table 16. Effect of phyto-adjuvants on the degradation/stability of azadirachtin in methanolic solution under accelerated storage condition

Compound	Regression equation	R^2	$t_{1/2}$ (h)	Factor of Stabilization
Aza	y = -0.0044x + 4.4996	0.9643	157.5	1.00
A+MIS	y = -0.0028x + 4.637	0.9945	247.5	1.57
A+MISG	y = -0.0032x + 4.5894	0.9802	216.6	1.38
A+SMS	y = -0.003x + 4.5501	0.9913	231.0	1.47
A+SMSG	y = -0.0032x + 4.6156	0.9892	216.6	1.38
A+FA-MO	y = -0.0026x + 4.5784	0.9928	266.5	1.69
A+KFA-MO	y = -0.0041x + 4.6128	0.9558	169.0	1.07
A+MFA-MO	y = -0.0038x + 4.4577	0.9639	182.4	1.16
A+ FA-NO	y = -0.0043x + 4.5354	0.9628	161.2	1.02
A+ KFA-NO	y = -0.0046x + 4.5465	0.9739	150.7	-
A+ FA-PO	y = -0.0043x + 4.5155	0.9665	161.2	1.02
A+ KFA-PO	y = -0.0046x + 4.512	0.9778	150.7	-
A+TBHQ	y = -0.003x + 4.5726	0.9719	231.0	1.47

As compared to aza-A alone ($t_{1/2}$ 157.5 h), its half-life in combination with three most effective adjuvants namely mahua saponin (MIS), Sapindus saponin (SMS) and madhuca fatty acid (FA-MO) was found to be 247.5, 216.6 and 266.5 h respectively. Mahua saponin was adjudged the best with maximum stabilizing factor of 1.57 followed by mahua fatty acid (FS 1.69) and Sapindus saponin (FS 1.47). Their stabilizing efficiency was superior to the commercial stabilizer *tert* butylhydroquinone (TBHQ) (FS 1.47). Thus under accelerated storage (54 ± 1°C) saponin concentrates imparted considerable stability to azadirachtin in methanolic solution (Fig. 16a).

4.5.3.2 Effect of adjuvants on the thermal stability of azadirachtin-A in emulsified water

The residual concentration of azadirachtin-A remaining in solution of aza-A: adjuvant (3:1) mixture in emulsified water at different time intervals of accelerated

storage (54±1^{0}C) is presented in Table 17. The corresponding half-lives ($t_{1/2}$) and factor of stabilization (FS) are presented in Table 18 and Fig. 16b.

Table 17. Aza-A (% remaining) in emulsified water of azadirachtin alone and in combination (3:1) with various phyto- adjuvants under accelerated storage (54±1^{0}C)

Time(h) / Compd.	0	4	7	16	20	25	42	48
Aza	100	83.2	67.0	64.2	58.0	56.8	29.2	28.7
A+SMI	100	94.3	72.6	67.1	64.5	44.2	42.6	42.0
A+MISG	100	93.5	68.2	65.1	43.8	42.8	29.7	29.1
A+SMS	100	95.1	68.4	65.3	56.1	42.6	39.0	37.4
A+SMSG	100	96.5	80.1	72.3	52.8	52.3	45.2	45.1
A+FA-MO	100	91.8	74.3	62.1	58.1	47.7	39.5	36.8
A+KFA-MI	100	93.2	74.3	57.6	52.1	48.7	34.8	30.8
A+MFA-MO	100	92.4	74.0	57.6	52.8	48.9	41.3	41.0
A+ FA-NO	100	90.5	69.0	66.6	59.8	50.7	47.0	44.3
A+ KFA- NO	100	94.8	75.2	73.7	45.6	43.1	37.0	30.5
A+ FA-PO	100	94.0	73.8	64.8	64.3	42.8	41.6	40.8
A+ KFA-PO	100	92.5	72.0	71.0	69.9	50.0	40.7	36.8
A+TBHQ	100	97.6	89.9	85.6	80.7	74.6	66.1	57.4

CD $_{5\%}$: Between Time = 1.62, Between Compound = 2.06, Time x Compound = 5.84

The degradation of azadircahtin in emulsified water alone, and in combination with different adjuvants under accelerated storage conditions was significantly higher than in methanolic solution. Thus while azadirachtin alone in emulsified water degraded to the extent of 28.7% after 48 hours, in its methanolic solution it was comparatively stable as 64.2% of the initial concentration was still detectable after the same time interval. In combination with test adjuvants its degradation was significantly suppressed. After 48 hours of accelerated storage, Madhuca and Sapindus saponins provided maximum stability to aza-A with corresponding half-lives of 37.5 and 39.6 hours, and stabilization factor of 1.36 and 1.44 respectively. Neem, karanj and mahua fatty acids imparted moderate stability with the

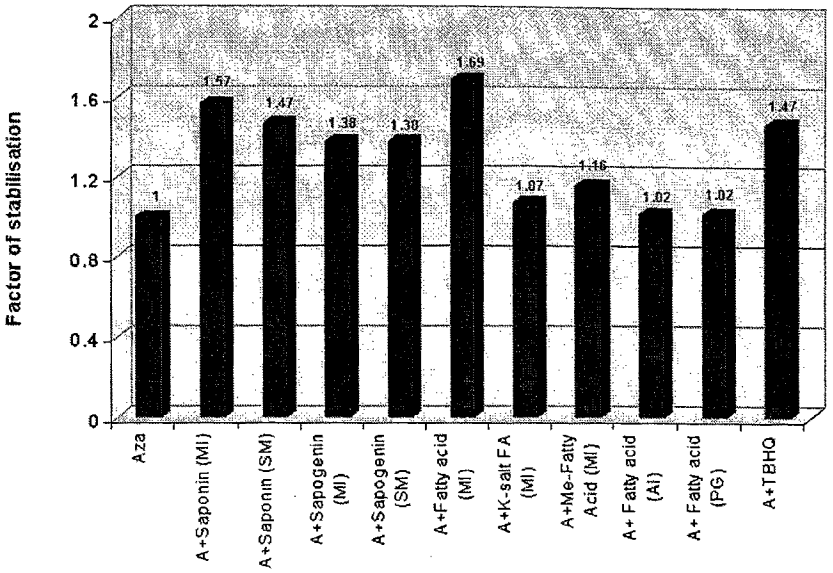


Fig. 16a. Effect of phyto-adjuvants on the degradation/stability of azadirachtin in methanolic solution under accelerated storage condition

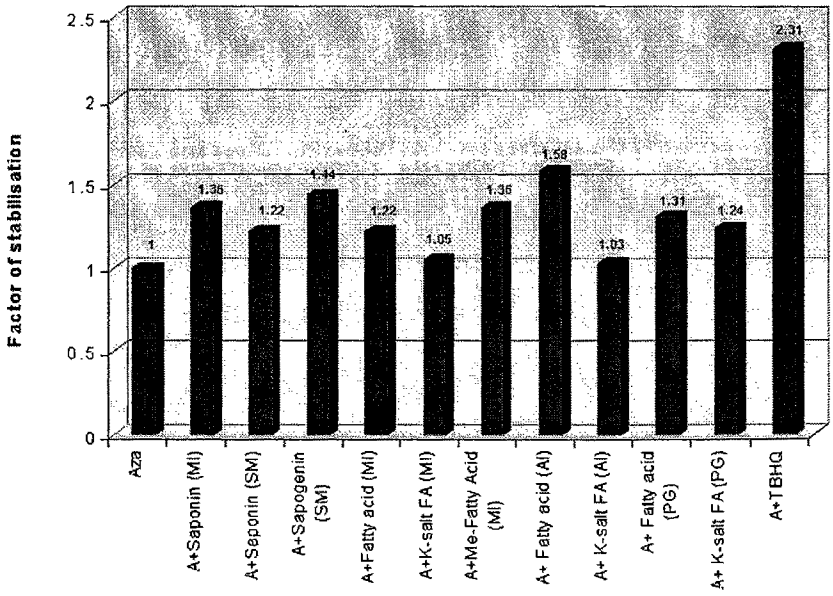


Fig. 16b. Effect of phyto-adjuvants on the degradation/stability of azadirachtin in hydrolytic solution under accelerated storage condition

Table 18. Effect of phyto-adjuvants on the degradation/ stability of azadirachtin in hydrolytic solution under accelerated storage conditions

Compound	Regression equation	R^2	$t_{1/2}$ (h)	Factor of Stabilization
Aza	y = -0.0252x + 4.5385	0.9576	27.5	1.00
A+Saponin (MI)	y = -0.0185x + 4.511	0.8674	37.5	1.36
A+Sapogenin (MI)	y = -0.0265x + 4.5162	0.9308	26.2	-
A+Saponin (SM)	y = -0.0206x + 4.4964	0.8851	33.6	1.22
A+Sapogenin (SM)	y = -0.0175x + 4.5277	0.8705	39.6	1.44
A+Fatty acid (MI)	y = -0.0206x + 4.5137	0.9480	33.6	1.22
A+K-salt FA (MI)	y = -0.0241x + 4.5282	0.9676	28.8	1.05
A+MFA-MO	y = -0.0186x + 4.4733	0.8794	37.3	1.36
A+ Fatty acid (AI)	y = -0.0159x + 4.4714	0.8726	43.4	1.58
A+ K-salt FA (AI)	y = -0.0246x + 4.5463	0.9057	28.2	1.03
A+ Fatty acid (PG)	y = -0.0192x + 4.5114	0.8688	36.1	1.31
A+ K-salt FA (PG)	y = -0.0204x + 4.5549	0.9428	34.0	1.24
A+TBHQ	y = -0.0109x + 4.6058	0.9807	63.6	2.31

respective half-lives of 43.4, 36.1 and 33.6 hour and corresponding stabilizing factor of 1.58, 1.31 and 1.22 respectively. The study confirms our earlier finding that azadirachtin in its aqueous solution is far unstable than in organic solvents. This study has indicated that under accelerated storage conditions (54±1^{0}C), the degradation of azadirachtin was arrested significantly following incorporation of some potential adjuvants like triterpenic saponins and neem fatty acid derivatives.

4.5.3.3 Effect of triterpenic saponins and potassium salt of fatty acids on the thermal stability of azadirachtin-A powder concentrate in solid phase

The amount (%) of azadirachtin-A in powder concentrate remaining after different time intervals under accelerated storage (54 ± 1°C) condition is presented in Table 19. The perusal of data revealed that azadirachtin concentration decreased with time and at the end of 15th day of accelerated storage, 65.5% of azadirachtin-A

could only be detected. Thus 34.5% loss of the intial azadirachtin concentrate occurred. Its half-life was found to be 25 days.

Table 19. Aza-A (% remaining) in solid phase of azadirachtin alone and in combination (3:1) with various phyto- adjuvants under accelerated storage (54±1°C)

Time(d) / Compd.	0	5	10	15
Aza (20%)	100	87.5	79.0	65.5
A+ Saponin (*Madhuca indica*)(3:1)	100	97.6	92.6	84.4
A+Saponin (*S. mukorossi*) (3:1)	100	95.2	84.7	68.0
A+KFA-MO (3:1)	100	79.2	74.6	63.7
A+ KFA- NO (3:1)	100	80.2	73.2	69.9

$CD_{5\%}$: Between Time = 3.65 , between Compound =4.08 , Time x Compound = 8.16

Following incorporation with three selected adjuvants such as triterpenic saponins and fatty acid salts, the half-life of azadirachtin was significantly enhanced. Among the four test adjuvants, saponin isolated from *Madhuca indica* conferred maximum stability to azadirachtin molecule followed by another saponin isolated from Sapindus. Potassium salts of fatty acids derived from mahua and neem oil were next in order and retained between 69.9 and 63.7 percent of the active ingredient. Two triterpenic saponin concentrates after 15 days of accelerated storage resulted in the retention of 68.0 and 84.4% of the initial aza-A content. The most active *M. indica* saponin, showed half-life of 61.9 days with corresponding factor of stabilization of 2.45.

From the forgoing discussion it seems clear that the stability of azadirachtin-A powder concentrate (20%) can be considerably enhanced following incorporation of *M. indica* triterpenic saponins.

Table 20. Effect of stabilizers on the thermal degradation/ stability of azadirachtin in solid phase under accelerated storage conditions

Compound	Regression equation	R^2	$t_{1/2}$ (d)	Factor of Stabilization
Aza	y = -0.0274x + 4.6128	0.9853	25.3	1.00
A+Saponin (MI)	y = -0.0112x + 4.6217	0.9305	61.9	2.45
A+Saponin (SM)	y = -0.0255x + 4.6460	0.9172	27.2	1.08
A+KFA-MO	y = -0.0233x + 4.5573	0.8950	29.7	1.17
A+ KFA-NO	y = -0.0283x + 4.5728	0.9514	24.5	-

4.6 Bioassay

4.6.1 Antifeedant activity of saponins, phyto-oils and their derivatives

Phytochemical insecticides such as those derived from neem and other economically useful plants affect insect feeding primarily through chemoreceptor (primary antifeedancy) as well as through reduction in food intake due to toxic effects of consumed (secondary antifeedancy) food. A variety of crude/refined extractives or pure/partially pure secondary metabolites such as saponins, prosapogenins and sapogenins obtained after partial/complete hydrolysis of both *M. indica* and *S. mukorossi* alone or in combination were evaluated for their insect antifeedant activity against *Spodoptera litura* larvae. Fatty acids, their methyl esters and potassium salts derived from mahua oil, neem oil and karanj oil were also evaluated for their possible additive/ synergistic/potentiating effect on antifeedant activity.

Antifeedant activity of triterpenic saponins and their derivatives from *M. indica* and *S. mukorossi* is depicted in Table 21. A comparison of the antifeedant activities of saponins and their corresponding sapogenins were least active (AI_{50} of 1.40, 1.39, 1.31 and 1.49 %). Their partial hydrolytic products however, showed comparatively higher activity. The partial hydrolytic product(s) obtained as a result of alkaline hydrolysis of Madhuca saponin were far more active (AI_{50}, 0.12 %) than those obtained during their acidic hydrolysis (AI_{50}, 0.38%). Mahua prosapogenins obtained after alkaline hydrolysis comprised of 16-hydroxyprotobassic acid as aglycone with glucose

Table 21. Antifeedancy* (%) of *Madhuca indica, Sapindus mukorossi* saponins and their derivatives against *S. litura*

Conc. / Comp.	1.0	0.5	0.25	0.1	0.05	CD (5%)
M. indica						
Saponin	51.8 (46.04)	44.27 (41.70)	43.25 (41.08)	40.28 (39.40)	39.70 (39.78)	8.54
Alkaline hydrolyzed Saponin	81.82 (64.86)	77.06 (61.43)	56.71 (48.86)	47.62 (43.62)	36.36 (36.99)	10.67
Acid hydrolyzed Saponin	64.51 (53.46)	56.71 (48.86)	38.10 (39.57)	35.50 (36.54)	26.41 (30.89)	8.28
Sapogenin (Protobassic acid)	39.60 (38.99)	27.1 (31.36)	25.95 (30.60)	11.07 (19.40)	10.31 (18.58)	5.86
Saponin acetate	45.90 (42.65)	40.10 (39.28)	36.40 (37.05)	29.40 (32.82)	18.10 (25.15)	8.38
S. mukorossi						
Saponin	48.20 (43.97)	45.60 (42.46)	40.50 (39.54)	38.40 (38.28)	32.82 (34.92)	8.57
Alkaline hydrolyzed Saponin	50.70 (45.41)	41.60 (40.14)	29.55 (32.92)	22.30 (28.08)	20.10 (26.60)	11.53
Acid hydrolyzed Saponin	51.20 (45.69)	47.20 (43.40)	44.12 (41.60)	40.67 (39.60)	38.69 (38.42)	9.89
Sapogenin (Hederagenin)	48.00 (43.86)	45.23 (42.27)	39.25 (38.79)	35.40 (36.51)	33.12 (35.11)	4.15
Saponin acetate	44.80 (41.79)	42.70 (440.80)	32.40 (334.59)	26.00 (30.64)	20.40 (26.83)	11.42

* arc sin transformed data (within parenthesis)

moiety attached at C-3 position, while the other prosapogenin obtained after acidic hydrolysis had partially hydrolyzed glycon moieties at both C-3 OH and C-28 carboxylic acid functions. The study indicated that the antifeedant activity increased

with decrease in number of monosaccharide units in the glycon moiety attached to prosapogenin molecule. Thus monosaccharide with one sugar chain attached at the C-3 hydroxyl function was more active than bidesmoside with two sugar moieties at C-3 hydroxyl and C-28 carboxylic functions. The corresponding products obtained after partial hydrolysis of *S. mukorossi* were just a shade better than corresponding saponins but the increase in the activity was not significant.

Table 22. Antifeedancy* (%) of phyto oils and phytolakanoates derived from mahua, neem and karanj oil

Conc. / Comp.	1.0	0.5	0.25	0.1	0.05	CD (5%)
Mahua oil	40.09 (39.28)	27.97 (31.91)	25.10 (30.03)	20.95 (27.16)	-	7.38
Neem oil	51.56 (45.90)	48.69 (44.25)	37.85 (37.95)	35.62 (36.51)	15.86 (23.41)	9.89
Karanj oil	40.30 (439.40)	30.40 (33.42)	26.90 (31.15)	19.40 (26.11)	12.60 (23.41)	11.53
K-salt of mahua oil	43.57 (41.29)	42.92 (40.93)	37.50 (37.75)	28.33 (32.07)	23.34 (28.89)	7.62
K-salt of Karanj oil	41.19 (39.92)	40.42 (39.47)	34.59 (35.98)	26.67 (30.99)	23.33 (28.88)	7.77
K-salt of Eem oil	50.42 (45.24)	38.33 (38.25)	34.17 (35.76)	30.23 (33.35)	27.91 (31.88)	5.39
Mixture of fatty acids	41.07 (39.79)	32.32 (34.64)	28.14 (31.92)	13.69 (21.67)	6.09 (13.94)	10.86
Mixture of K-salt of mahua and neem oil	67.47 (55.27)	61.05 (51.38)	53.61 (47.29)	46.39 (42.84)	44.58 (41.69)	29.32

* arc sin transformed data (within parenthesis)

Antifeedant activity of another group of potential adjuvants such as phyto-oils (mahua, neem and karanj oils), as well as their fatty acids and potassium salts is depicted in Table 22. Out of three plant glycerides, neem oil was the most active with antifeedant activity of 0.69 percent. The other two namely mahua and karanj oils did not exhibit significant activity. Fatty acids obtained after saponification of the

oil as well as their corresponding fatty acid methyl esters (FAMEs) were also not active. The least activity of fatty acids and their esters is attributed to the fact that invertebrates and vertebrates ingest fatty acids as a significant part of their normal diet. Among the three fatty acid salts evaluated for antifeedant activity, potassium salts of mahua and neem fatty acids were more active (AI_{50} 1.64-1.74%), while salts of karanj fatty acid were least active. The antifeedant activity was, however, significantly enhanced when mahua and neem oil fatty acid salts were combined. As compared to mahua and neem oil fatty acid salts alone (AI_{50} 1.67 and 1.88%), their combination (1:1) was far more active (AI_{50} 0.135%), even much better than neem oil (AI_{50} 0.69%). Under these test conditions antifeedant activity of aza-A concentrate (20%) and Econeem ® was comparable, exhibiting AI_{50} of 0.036 and 0.025 % respectively.

4.6.1.1 Effect of saponins, prosapogenins and potassium salts of fatty acids on the activity of azadirachtins

Synergism or synergy is an adaptive strategy in plant defense may be defined as an interaction among compounds, which produces a greater effect than expected, based on individual activities. Synergistic plant secondary metabolites or their related products may provide an advantage by producing a greater toxicity at a lower cost (McKey 1979; Berenbaum 1985). Instead of having a high concentration of one toxin with a limited range of activity, bioactive phytochemicals may interact to produce a greater activity at a lower total concentration than any single compound alone.

From the foregoing discussion in insect antifeedant bioassay, it appeared that triterpenic saponins and potassium salt of fatty acids were the most active as insect antifeedant. These potential compounds were then tested individually in combination with azadirachtin to assess their possible potentiating /synergistic effect. Unlike aza-A concentrate (20%), triterpenic saponins obtained from *Sapindus mukorossi* and *Madhuca indica* did not exhibit significant insect antifeedant activity (AI_{50} 1.39-1.20%). However, when these were mixed with azadirachtin concentrate in 3:1 proportion, they were able to either retain or enhance the activity of azadirachtin. Thus while aza-A (20%) exhibited antifedant

Table 23. Antifeedancy (%) of azadirachtin alone and with adjuvants against *S. litura*.

Conc. (%) / Comp.	1.0	0.5	0.25	0.1	0.05	CD (5%)
Aza-A concentrate (20%)	82.66 (65.41)	77.13 (61.45)	71.86 (57.97)	67.33 (55.14)	49.24 (44.56)	8.54
Aza + saponin (*M. indica*)	86.40 (68.20)	85.44 (67.57)	78.54 (62.40)	61.68 (51.67)	59.52 (50.49)	2.88
Aza + saponin (*S. mukorossi*)	87.11 (68.71)	77.49 (61.68)	65.45 (54.00)	64.37 (53.35)	51.32 (45.76)	2.36
Aza + prosapogenin (*M. indica*)	83.16 (65.81)	78.79 (63.30)	71.42 (57.45)	54.54 (47.62)	40.06 (39.27)	14.30
Aza + prosapogenin (*S. mukorossi*)	80.80 (63.94)	77.44 (62.28)	70.57 (57.29)	52.52 (46.45)	40.06 (39.27)	13.50
Aza + K-salt of Neem oil	89.63 (71.24)	82.41 (65.32)	79.27 (62.95)	63.35 (52.68)	59.80 (50.65)	7.69
Aza + K-salt of Madhuca oil	90.26 (71.82)	82.73 (65.64)	80.84 (64.07)	65.21 (54.14)	64.89 (53.93)	17.28
Aza + K-salt of karanj oil	88.06 (69.80)	81.15 (64.38)	78.33 (62.79)	62.00 (51.99)	55.40 (48.10)	7.35
Econeem®	58.10* (49.66)	53.24 (46.86)	47.44 (43.53)	42.82 (40.81)	32.02 (34.42)	10.12

* arc sine transformed data within brackets; Concentrations for Econeem®: 0.05, 0.03, 0.02, 0.01, and 0.007%.

activity (AI_{50}) of 0.036%, its combination (Aza-A + Madhuca saponin) performed significantly better with AI_{50} of 0.029 percent. As compared to saponin alone (AI_{50} 1.39-1.20), their partially hydrolytic products of saponins with lesser number of sugar units were not able to enhance the antifeedant activity (Fig. 17).

Table 24. Antifeedancy activity (AI_{50}) of azadirachtin alone and with phyto-adjuvants

Compounds	AI_{50} (%)	χ^2_{exp} (3 d.f)	Fiducial limit
Saponins and their derivatives			
Saponin (*Madhuca indica*)	1.39	0.81	0.14-14.24
Alkaline hydrolyzed prosapogenin	0.12	3.14	0.10-0.18
Acid hydrolyzed prosapogenin	0.34	2.69	0.24-0.483
Sapogenin (Protobassic acid)	1.31	1.68	0.70-2.44
Saponin acetate	1.49	1.76	0.59-3.74
Saponin (*Sapindus mukorossi*)	1.40	0.08	0.27-7.15
Alkaline hydrolyzed prosapogenin	1.18	1.27	0.59-2.37
Acid hydrolyzed prosapogenin	0.91	0.14	0.18-4.61
Sapogenin (Hederagenin)	1.49	0.37	0.29-7.59
Saponin acetate	1.56	0.62	0.60-4.08
Oils, fatty acids and their salts			
Neem oil	0.69	6.01	0.42-1.13
Mahua oil	2.24	1.34	0.72-6.97
Karanj oil	2.54	0.60	0.93-6.89
K-salt of neem oil	1.64	2.03	0.48-5.67
K-salt of mahua oil	1.74	0.87	0.52-5.79
K-salt of karanj oil	2.59	0.60	0.58-11.49
Fatty acid (Mixture of three)	3.20	4.49	0.97-10.54
Methyl ester fatty acids (mix.)	1.38	2.72	0.82-2.34
K-salt of neem and mahua oil	0.14	0.92	0.74-0.25
Azadirachtin and potential adjuvants			
Azadirachtin (20%)	0.036	2.35	0.017-0.079
Aza (20%) + Madhuca saponin	0.029	1.71	0.014-0.061
Aza (20%) + Sapindus saponin	0.040	2.93	0.020-0.081
Aza(20%)+Madhuca prosapogenin	0.081	1.27	0.055-0.118
Aza (20%)+Sapindus prosapogenin	0.084	2.26	0.057-0.126
Aza (20%) + K-salt of neem oil FA	0.029	1.03	0.014-0.060
Aza (20%) + K-salt of mahuaoil FA	0.015	2.21	0.005-0.048
Aza (20%) + K-salt of karanj oil	0.036	0.79	0.019-0.068
Econeem*	0.025	1.10	0.017-0.036

χ^2_{tab}: 7.77; *Concentrations for Econeem®: 0.05, 0.03, 0.02, 0.01, and 0.007%.

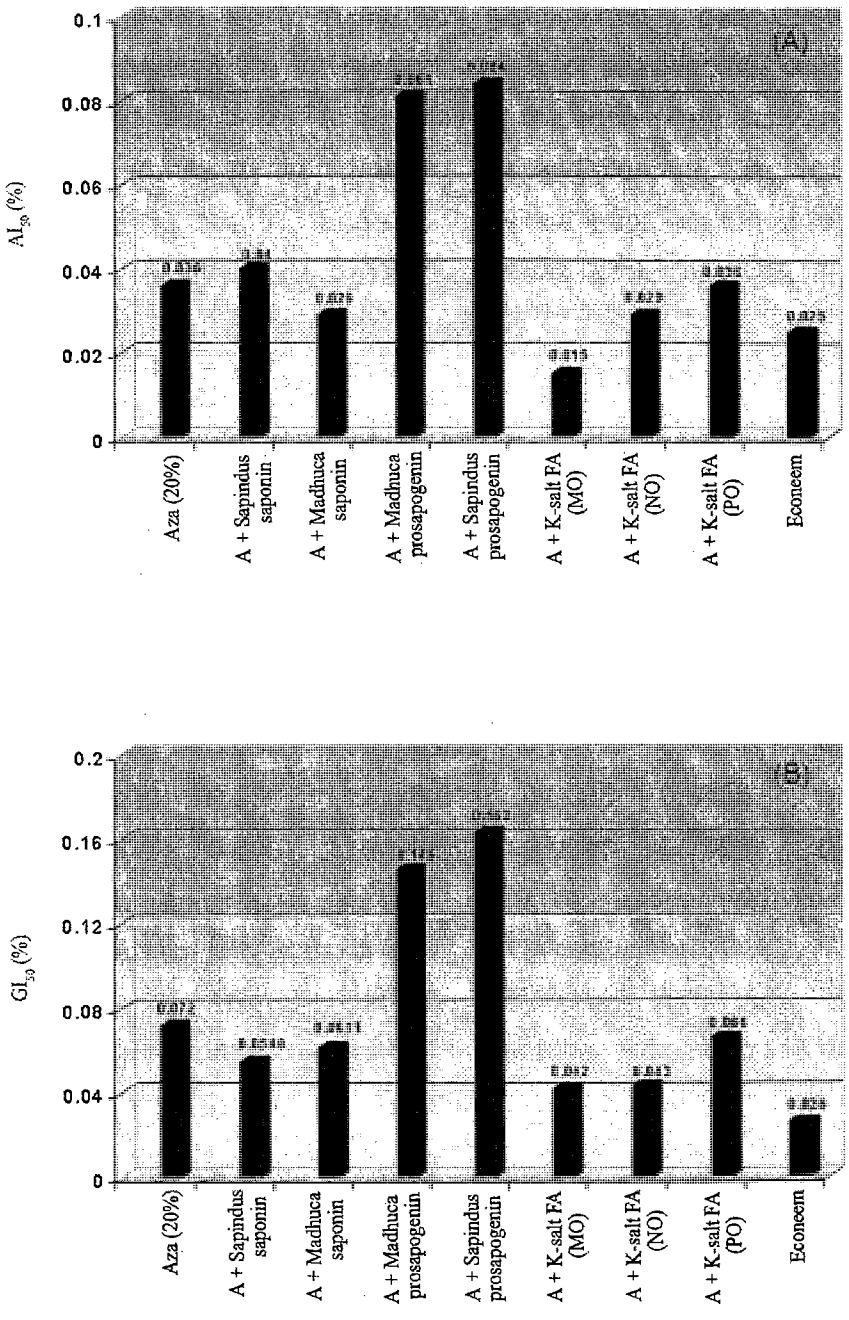


Fig. 17. Antifeedant (A) and insect growth regulatory activity (B) of phytochemicals and related products derived from neem, sapindus, mahua and karanj against *Spodoptera litura* larvae

Like plant saponins, potassium salts of fatty acids derived from three phyto-oils were equally ineffective, exhibiting insignificant antifeedant activity against the test insect. When these were mixed with azadirachtin-A concentrate, they either retained or enhanced the antifeedant activity of azadirachtin concentrate. While karanj oil based fatty acid (FAPS) retained the antifeedant activity of azadirachtin (AI_{50} 0.036%), those derived from mahua and neem oil increased the activity corresponding AI_{50} of 0.015 and 0.029%. Thus incorporation of mahua oil based potassium salts of fatty acids in azadirachtin based formulations can be more useful in developing products with improved activity.

Table 25. Fractional analysis of azadirachtin and two saponins for their synergistic, or joint action in relation to antifeedant activity

Compounds	D_{aza}	D_s	d_{aza}	d_s	d_{aza}/D_{aza} (A)	d_s/D_s (B)	A + B
Aza +SM saponin	0.036	1.40	0.030	0.0100	0.8333	0.0071	**0.84**
Aza + MI saponin	0.036	1.39	0.021	0.0073	0.5830	0.0052	**0.59**
Aza+ KFA (AI)	0.036	1.88	0.022	0.0072	0.6111	0.0038	**0.61**
Aza + KFA (MI)	0.036	1.67	0.011	0.0038	0.3056	0.0023	**0.31**
Aza + KFA (PG)	0.036	2.59	0.027	0.0090	0.7500	0.0035	**0.75**

SM: *Sapindus mukorossi*; MI: *Madhuca indica;* AI: *Azadirachta indica;* PG: *Pongamia glabra;* KFA: K-salt of fatty acids; d: conc. used in combination; D: LD_{50}

From the isobolographic analysis it is evident that potassium salt of mahua oil fatty acids in combination with azadirachtin (20%) exhibited potency ratio of 0.31, denoting high synergistic/potentiating effect of the mixture. Rest of the adjuvants, used in combination with azadirachtin was not that much active to show synergistic effect in that level (Table 25).

4.6.2 Insect growth inhibitory activity of saponins and neem derivatives against *S. litura*

The growth inhibitory effects of triterpenic saponins and neem derivatives are given in Table 26. Unlike aza-A concentrate (20%), which caused significant growth inhibition of *Spodoptera litura* larvae (GI_{50} 0.072%), triterpenic saponins obtained from both mahua (*M. indica*) and ritha (*S. mukorossi*) were far less active with GI_{50}

Table 26. Growth Inhibitory (%) activity of *M. indica* and *S. mukorossi* saponins and their derivatives against *S. litura*

Conc. / Comp.	1.0	0.5	0.25	0.1	0.05	CD (5%)
M. indica						
Saponin	39.57 (38.98)	38.58 (38.40)	25.01 (29.98)	20.30 (26.70)	15.14 (22.82)	5.98
Alkaline hydrolyzed saponin	83.74 (66.43)	66.34 (54.56)	57.21 (52.17)	52.53 (45.46)	26.11 (30.73)	7.65
Acid hydrolyzed saponin	62.18 (52.08)	61.57 (51.70)	56.13 (48.52)	41.02 (39.83)	38.27 (38.20)	5.81
Sapogenin (Protobassic acid)	41.94 (40.35)	38.58 (38.40)	26.08 (30.68)	21.43 (27.57)	15.14 (22.82)	5.01
Saponin acetate	50.56 (45.32)	41.26 (39.95)	34.60 (36.00)	30.18 (33.32)	20.48 (26.74)	9.26
S. mukorossi						
Saponin	42.20 (40.42)	31.28 (38.79)	28.14 (31.83)	20.01 (26.44)	18.65 (25.55)	15.87
Alkaline hydrolyzed saponin	56.40 (48.67)	53.55 (47.05)	37.85 (37.95)	36.90 (37.40)	30.00 (33.09)	9.16
Acid hydrolyzed saponin	69.55 (56.51)	63.54 (52.90)	57.63 (49.39)	54.52 (47.61)	54.48 (47.59)	8.86
Sapogenin (Hederagenin)	44.32 (41.73)	38.54 (38.38)	32.46 (34.71)	24.41 (29.48)	18.50 (25.47)	7.40
Saponin acetate	56.40 (48.68)	53.55 (47.05)	37.85 (37.95)	36.90 (37.40)	30.00 (33.08)	9.16

* arc sine transformed data within parenthesis

of 0.916 and 1.39% respectively. Their acetate derivatives and the hydrolytic products 16-hydroxyprotobassic acid (mahua genin) and hederagenin (ritha genin) were least active (GI_{50} 1.39-1.89%). The hydrolytic products obtained after mild acidic and alkaline hydrolysis comprised of various prosapogenins and were far

more active than the saponins. Unlike saponins, the partial hydrolytic products have less number of sugar units attached to the sapogenin nucleus. While lower level of growth inhibitory effect of saponins containing more number of sugar units can be attributed to comparatively more polar nature of the saponin molecule, the lower level of activity of sapogenins (16-hydroxyprotobassic acid and hederagenin) and saponin acetate is attributed to their non-polar nature. It was thus inferred that for optimum activity, an appropriate hydrophile-lipophile balance (HLB) would be necessary which can be provided by a shorter glycone moiety comprising of lesser number of monosaccharide units.

Table 27. Growth inhibition (%) by phytoalkanoates against *S. litura*

Conc. (%) / Comp.	1.0	0.5	0.25	0.1	0.05	CD (5%)
Mahua oil		34.50 (35.93)	29.00 (32.44)	19.33 (26.02)	-	14.21
Neem oil	52.11 (46.21)	42.22 (40.52)	39.63 (38.59)	29.37 (32.81)	27.91 (31.91)	17.29
Karanj oil	45.61 (42.48)	31.52 (34.09)	25.84 (30.49)	19.56 (26.23)	12.01 (20.17)	8.09
K-salt of mahua oil	44.54 (41.86)	33.49 (35.35)	28.24 (32.07)	19.92 (26.28)	12.72 (20.84)	8.67
K-salt of karanj oil	40.23 (39.36)	36.40 (37.05)	30.45 (33.45)	20.56 (26.87)	12.45 (20.66)	8.65
K-salt of neem oil	77.54 (64.21)	44.04 (41.56)	40.02 (38.23)	33.03 (34.14)	14.03 (21.90)	39.33
Mixture of K-salt of mahua and neem oil	53.71 (47.15)	49.46 (44.69)	47.78 (43.71)	34.24 (35.80)	-	13.20

* arc sine transformed data within parenthesis

Among the three phyto-oils (Table 27), neem oil imparted higher activity (GI_{50} 0.97%) while mahua and karanj oils were least active. Similarly, among the three fatty acid potassium salts (FAPS) one derived from neem oil was more active (GI_{50} 0.36%) followed by mahua (1.64%) and karanj oils (GI_{50} 2.17%). The

corresponding fatty acids (FA) and their methyl esters (FAME) from neem, mahua and karanj oils were the least active. In fact, the mixture of fatty acids methyl esters (FAMEs) obtained from neem, mahua and karanj oil to some extent promoted insect growth. The combination of potassium salts of fatty acids of mahua and neem oil performed better than mahua oil (GI_{50} 0.57%). However, its activity was a shade lower than FAPS of neem oil (GI_{50} 0.36%).

4.6.2.1 Synergistic/potentiating effect of saponins and neem derivatives on the activity of azadirachtin against *S. litura*

The synergistic/ additive or potentiating effect of triterpenic saponins and neem derivatives with azadirachtin concentrate (20%) is given in Tables 28 & 29, and Figure 17. In general azadirachtin: adjuvant mixtures were far more active than either azadirachtin or the test adjuvant(s) alone. As given in Table 29, azadirachtin concentrate (20%) exhibited growth inhibition (GI_{50}) of 0.072% (720 ppm). Following mixing of triterpenic saponins and fatty acid potassium salts (FAPS) with azadirachtin concentrate in 3:1 proportion, insect growth inhibitory effect of azadirachtin was considerably enhanced. Similarly mahua and ritha saponins exhibited potentiating effect and imparted significant increase in the growth inhibitory effect (GI_{50} 0.055-0.061%) of azadirachtin (Fig. 17). Out of the three salts, FAPS derived from mahua in combination with azadirachtin concentrate were most active with GI_{50} of 0.042% followed by neem oil (GI_{50} 0.043%) and karanj oil (GI_{50} 0.066%).

From the foregoing discussions and several reports available in literature, azadirachtin has been found to be largely responsible for insect antifeedant and growth inhibitory effects. The present study revealed that in combination with adjuvants such as saponins and potassium salts of fatty acids particularly those obtained from mahua and neem, the activity of azadirachtin has been considerably enhanced.

Table 28. Growth inhibitory effect (%) of azadirachtin and its combination (3:1) with different adjuvants against *S. litura*

Conc. (%) / Comp.	1.0	0.5	0.25	0.1	0.05	CD (5%)
Aza-A concentrate (20%)	78.50 (62.72)	71.40 (57.75)	68.40 (55.88)	53.20 (46.85)	45.20 (42.25)	13.03
Aza + saponin (*M. indica*)	90.83 (73.80)	76.09 (61.12)	61.22 (51.33)	53.83 (47.20)	57.94 (49.64)	17.73
Aza + saponin (*S. mukorossi*)	83.80 (66.29)	85.42 (68.00)	69.17 (56.36)	51.63 (45.94)	56.62 (48.81)	11.03
Aza + prosapogenin (*M. indica*)	75.23 (57.24)	67.03 (55.18)	55.39 (48.12)	43.43 (41.21)	38.83 (38.52)	12.98
Aza + prosapogenin (*S. mukorossi*)	70.70 (57.24)	65.72 (54.31)	51.52 (45.88)	44.38 (41.77)	38.02 (38.08)	11.37
Aza + K-salt of neem oil	71.57 (58.15)	69.11 (56.23)	68.14 (55.79)	67.26 (55.43)	64.19 (53.64)	22.31
Aza + K-salt of Madhuca oil	77.02 (61.47)	73.48 (59.09)	79.92 (63.38)	67.26 (55.43)	65.76 (54.54)	18.40
Aza + K-salt of karanj oil	69.00 (56.52)	65.80 (54.21)	61.20 (51.47)	64.67 (53.68)	58.16 (49.84)	18.19
Econeem®	58.24* (49.75)	54.26 (47.45)	46.07 (42.74)	39.18 (38.75)	29.08 (32.52)	9.38

*Concentrations for Econeem® (0.3%): 0.05, 0.03, 0.02, 0.01, and 0.007%.

The apparent enhancement of aza-A activity in the presence of both saponins and potassium salts of fatty acids could be attributed to the facilitation of bioavalibility of azadirachtin by these adjuvants at the target site in insect. Since triterpenic saponins and potassium salts of fatty acids are water soluble because of the hydrophilic sugar and salt moieties, their lipophilicity is attributed to the aglycone moiety comprising of polycyclic hydrocarbon skeleton. The bioactive azadirachtin

Table 29. Growth Inhibitory activity (GI_{50}, %) of azadirachtin alone and with adjuvants against *S. litura*

Compounds	GI_{50}	χ^2_{exp} (3 d.f)	Fiducial limit
Saponins and their derivatives			
Saponin (*Madhuca indica*)	0.916	0.898	0.582-1.59
Alkaline hydrolyzed saponin	0.152	6.85	0.117-0.198
Acid hydrolyzed saponin	0.192	1.56	0.118-0.313
Sapogenin (Protobassic acid)	2	1.04	0.790-5.07
Saponin acetate	1.08	1.2	0.506-2.30
Saponin (*Sapindus mukorossi*)	1.39	0.532	0.265-7.23
Alkaline hydrolyzed saponin	0.553	2.52	0.309-0.988
Acid hydrolyzed saponin	0.51	1.65	0.30-0.85
Sapogenin (Hederagenin)	1.69	0.202	0.66-4.34
Saponin acetate	0.882	0.245	0.298-2.60
Oils, fatty acids and their salts			
Neem oil	0.97	0.804	0.42-2.24
Mahua oil	1.89	0.138	0.33-10.7
Karanj oil	1.8	1.02	0.832-3.89
K-salt of neem oil	1.64	0.684	0.785-3.44
K-salt of mahua oil	0.36	12.69	0.284-0.464
K-salt of karanj oil	2.08	1.75	0.84-5.19
K-salt of neem and mahua oil	0.57	1.49	0.28-1.17
Azadirachtin and potential adjuvants			
Azadirachtin (20%)	0.072	0.933	0.042-0.123
Aza (20%) + Madhuca saponin	0.055	2.06	0.033-0.0899
Aza (20%) + Sapindus saponin	0.061	8.72	0.0385-0.0969
Aza(20%)+Madhuca prosapogenin	0.146	0.539	0.102-0.209
Aza (20%)+Sapindus prosapogenin	0.163	0.912	0.109-0.245
Aza (20%) + K-salt of neem oil FA	0.042	0.343	0.0002-0.01
Aza (20%) +K-salt of mahua oil FA	0.043	1.2	0.015-0.121
Aza (20%) + K-salt of karanj oil FA	0.066	0.569	0.027-0.160
Econeem*	0.026	0.51	0.019-0.036

*: conc. 0.05, 0.03, 0.02, 0.01, 0.007 %

molecule with both lipophilic and hydrophilic functions can be transported better to the target receptor sites. Further, in azadirachtin: saponin and azadirachtin: potassium salt of fatty acid combinations, saponins and fatty acid salts probably disrupt the lipophilic matrix of the insect cellular membrane facilitating penetration of azadirachtin to the insect target sites.

Table 30. Fractional/isobolographic analysis of azadirachtin and two saponins for their synergistic, or joint action in relation to IGR activity

Compounds	D_{aza}	D_s	d_{aza}	d_s	d_{aza}/D_{aza} (A)	d_s/D_s (B)	A+B
Aza +SM saponin	0.072	1.39	0.041	0.0137	0.5708	0.0044	**0.58**
Aza + MI saponin	0.072	0.92	0.049	0.0153	0.6805	0.0070	**0.69**
Aza+ KFA (AI)	0.072	0.36	0.032	0.0108	0.4479	0.0300	**0.48**
Aza + KFA (MI)	0.072	1.64	0.032	0.0105	0.4375	0.0064	**0.44**
Aza + KFA (PG)	0.072	2.17	0.050	0.0165	0.6875	0.0076	**0.70**

SM: *Sapindus mukorossi*; MI: *Madhuca indica;* AI: *Azadirachta indica;* PG: *Pongamia glabra;* KFA: K-salt of fatty acids; d: conc. used in combination; D: LD_{50}

The most effective azadirachtin: adjuvant combinations were subjected to fractional/isobolographic anlysis to determine the contribution of each additive towards possible additive of synergistic or potentiating effect. Fractional analysis in Table 30 showed that both potassium salts of mahua and neem fatty acids were more effective synergis/potentiator of azadirachtin with the potency ratio of 0.44 and 0.48 respectively. From these studies it can thus be concluded that triterpenic saponins and potassium salts of fatty acids (potassium-soap) can be incorporated as useful adjuvants for the preparation of more effective azadirachtin based biopesticides.

4.6.3 Antifungal activity

Plant secondary metabolites and their derivatives have been evaluated as viable alternatives to the persistent and less environment friendly synthetic fungicides. Although a large number of phytochemicals are known for their insect control properties, only a few of them are known to impart antifungal activity. For example

Azadirachta indica (neem) constituents such as azadirachtin is well known for its insect control property, but it showed poor antifungal activity. Use of neem oil and crude extractives of neem seeds for the control of plant pathogenic fungi is, however, known (Govindachari *et al.*, 1998). Very recently azadirachtin A, B and H have been found to exhibit considerable antifungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* (Sharma *et al.*, 2003).

In view of the fact that naturally occurring triterpenic saponins isolated from *Madhuca indica* (mahua) and *sapindus mukorossi* (ritha) are known for their surface active, piscicidal and other biological properties, studies have been conducted to evaluate these products for their possible antifungal properties. Studies were also aimed at evaluating some other compounds such as fatty acids, potassium salts of fatty acids derived from neem, mahua and karanj oils for their possible antifungal activity. It is also worthwhile to assess the possible synergistic/potentiating action of neem products and triterpenic saponins when used in combination with azadirachtin.

In earlier studies neem oil has been reportedly used to control phytopathogenic fungi at as high as 2 to 10% concentration under field conditions. Such high concentrations are known to induce phytotoxicity (Govindachari *et al.*, 1998). In the present study, 20 compounds derived from neem (*A. indica*), mahua (*M. indica*), ritha (*S. mukorossi*) and Karanj (*P. glabra*) were initially evaluated for their possible antifungal activity against *Rhizoctonia bataticola*. Most active compounds were subsequently evaluated for their antifungal activity against other test fungi alone or in combination with azadirachtin concentrate. While neem, mahua and pongamia oils did not impart significant antifungal activity; their fatty acids and potassium salts of fatty acids were moderately active (Table 31). Among the three fatty acids, those derived from mahua were more active (ED_{50} 247.4 ppm) while those obtained from karanj oil were the least active (ED_{50} 3404 ppm). Similarly, among the potassium salts, those derived from neem oil (ED_{50} 678.2 ppm) and karanj oil (ED_{50} 868.6 ppm) were moderately active. Unlike fatty acids and their potassium salts, the corresponding methyl ester of neem, mahua and karanj fatty acids did not exhibit significant antifungal activity. Azadirachtin concentrate (20 %) comprising of azadirachtin A, B and H was however most active with ED_{50} of 145.5 ppm.

Table 31. Antifungal activity of saponins and their derivatives, sapogenins and phytoalkanoates against *Rhizoctonia bataticola*

Compound	ED_{50} (ppm)	Compound	ED_{50} (ppm)
Sapindus mukorossi		***Madhuca indica***	
Saponin	181.40	Saponin	229.90
Prosapogenin	397.42	Prosapogenin	402.50
Sapogenin (hydroxyprotobassic acid)	163.58	Sapogenin (Hederagenin)	1143.42
Saponin acetate	1788.72	Saponin acetate	1600.82
Propionic ester of saponin	469.54	Propionic ester of saponin	609.47
Butanoic ester of saponin	303.45	Butanoic ester of saponin	365.48
Pentanoic ester of saponin	203.41	Pentanoic ester of saponin	192.13
Fatty acid of neem oil	203.46	K-salt of neem oil	678.17
Fatty acid of mahua oil	247.39	K-salt of mahua oil	1128.99
Fatty acid of karanj oil	3404.52	K-salt of karanj oil	868.59

Between the two triterpenic saponin concentrates, the one obtained from *Sapindus mukorossi* was slightly more active (ED_{50} 181.4 ppm) than its counterpart from *Madhuca indica* (ED_{50} 229.6 ppm). Their complete hydrolytic products (sapogenin) namely protobassic acid (*Madhuca indica*) and hederagenin (*Sapindus mukorossi*). lose their effectiveness to considerable extent as they were active only in the range of 763.38 – 1143.27 ppm. Similarly, as compared to saponins, their partial hydrolytic products were less active, possibly because they become less hydrophilic and thus lose the required hydrophilic-liphiphilic balance for optimum activity. When Madhuca and Sapindus saponins were converted to their acetyl, butanoyl, propionyl and pentanoyl esters, they lose their activity. Of the two series of esters, Madhuca saponin esters were, however more active than Sapindus saponins and the activity increased with an increase in length of acid moiety. Pentanoic ester of Madhuca saponin was found to be the most active with ED_{50} of 192.13 ppm followed by propionic ester (ED_{50} 609.5 ppm). Comparison of the ED_{50} value of the entire sets of test compounds revealed that saponin concentrates

derived from both mahua (*M. indica*) and ritha (*S. mukorossi*) were the most active. Their corresponding hydrolytic, partially hydrolytic, and esterified products except pentanoic esters of *M. indica* were, however, less active.

The more active saponin concentrates were further evaluated in combination with azadirachtin powder concentrate to assess their possible synergistic or potentiating effect against four phytopathogens namely *Rhizoctonia bataticola, Rhizoctonia solani, Fusarium udum* and *Sclerotium rolfsii.*

4.6.3.1 Synergistic/potentiating effect of triterpenic saponins on the antifungal activity of azadirachtin concentrate

Effect of triterpenic saponins on the antifungal activity of azadirachtin is reported in Table 32. Azadirachtin concentrate (20%) exhibited considerable antifungal activity (ED_{50} 145.5 ppm) against the phytopathogenic fungi *R. bataticola*, responsible for causing root rot and stem wilt. The *M. indica* and *S. mukorossi* saponins on the other hand were comparatively less active with corresponding ED_{50} of 229.9 and 181.4 ppm respectively (Fig. 18). When azadirachtin concentrate was mixed with saponins in 3: 1 proportion the joint action was considerably enhanced. Azadirachtin + Sapindus saponin, and azadirachtin + Madhuca saponin combinations with corresponding ED_{50} of 102.1 and 111.6 ppm were more active than either azadirachtin or saponins alone. Similar trend was observed when azadirachtin and its combinations with two triterpenic saponins were evaluated for their antifungal activity against *R. solani*, a fungus responsible for damping off and root rot of vegetables and other plants (Fig. 19). As compared to azadirachtin (ED_{50} 158.77 ppm), Sapindus and Madhuca saponins were less active with corresponding ED_{50} of 677.74 and 442.28 ppm respectively. The activity of combinations of (azadirachtin + saponins) was comparable to azadirachtin. While azadirachtin: Sapindus saponin (3:1 w/w) showed ED_{50} of 150.3 ppm, azadirachtin: Madhuca saponin (3:1 w/w) exhibited ED_{50} of 163.9 ppm. Similar trend was observed when Sapindus and Madhuca saponins and their combinations with aza-A were evaluated

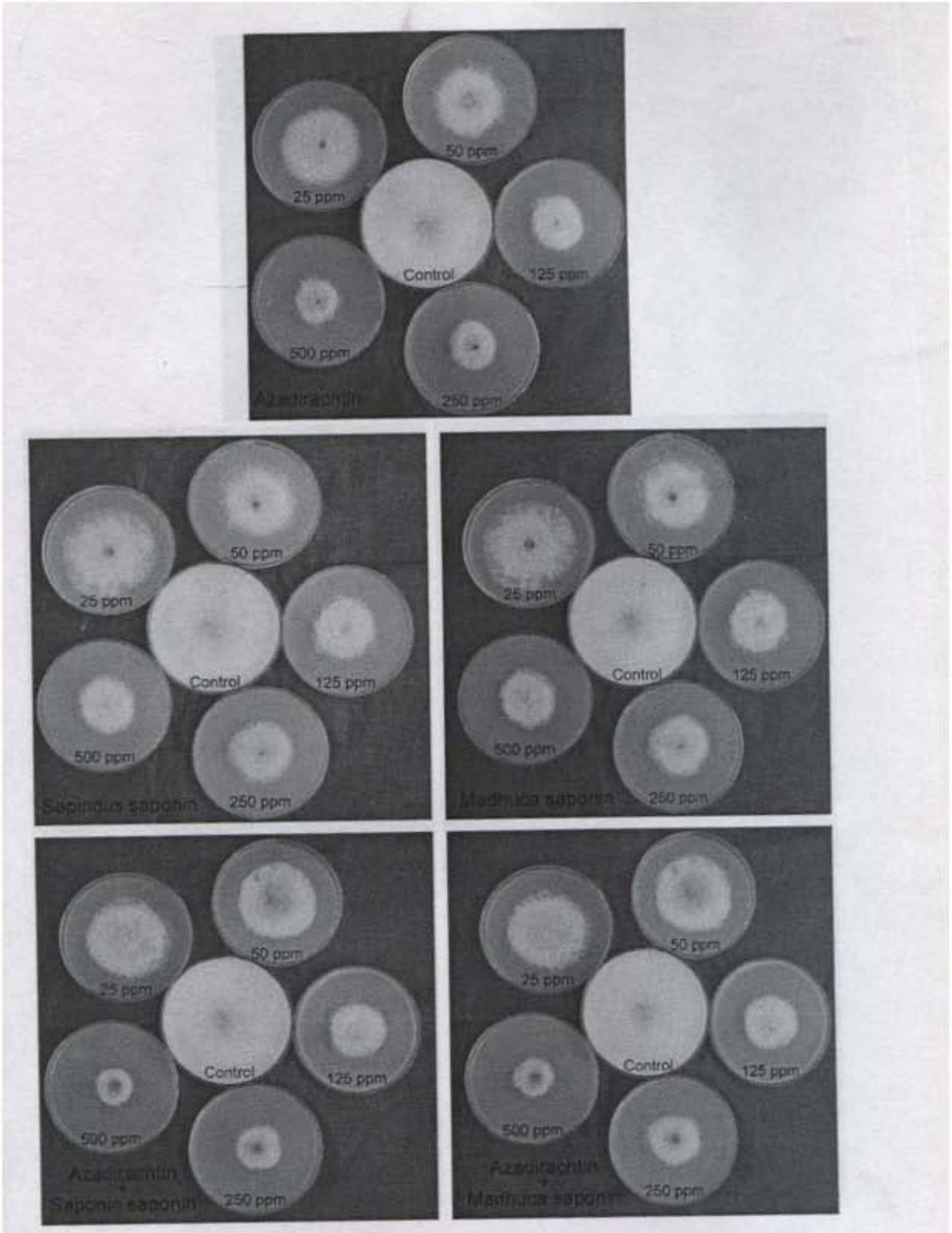


Fig. 18. Antifungal activity of azadirachtin, *Sapindus mukorossi* saponin and *Madhuca indica* saponin alone and in combination against *Rhizoctonia bataticola*

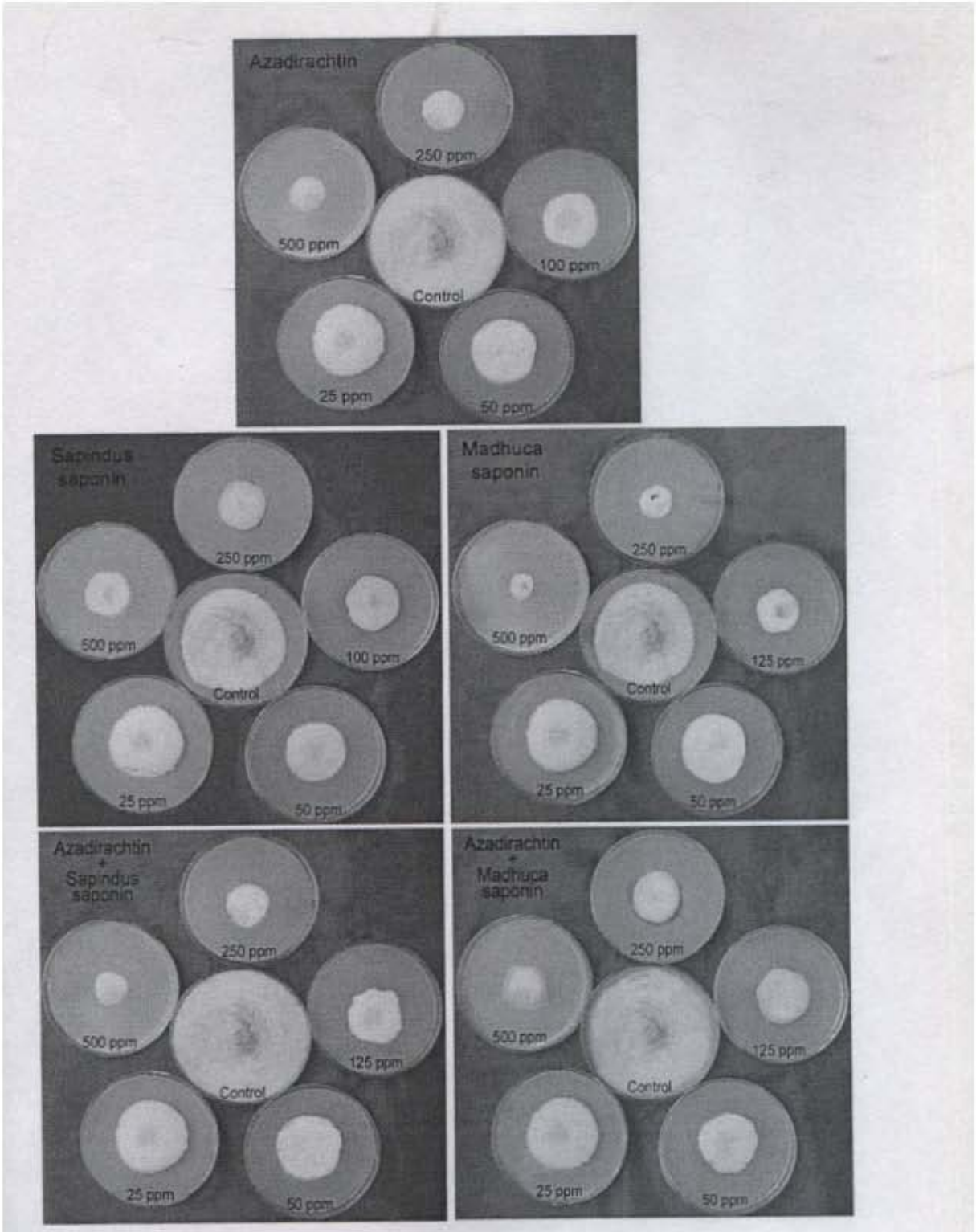


Fig. 19. Antifungal activity of azadirachtin, *Sapindus mukorossi* saponin and *Madhucaindica* saponin alone and in combination against *Rhizoctonia solani*

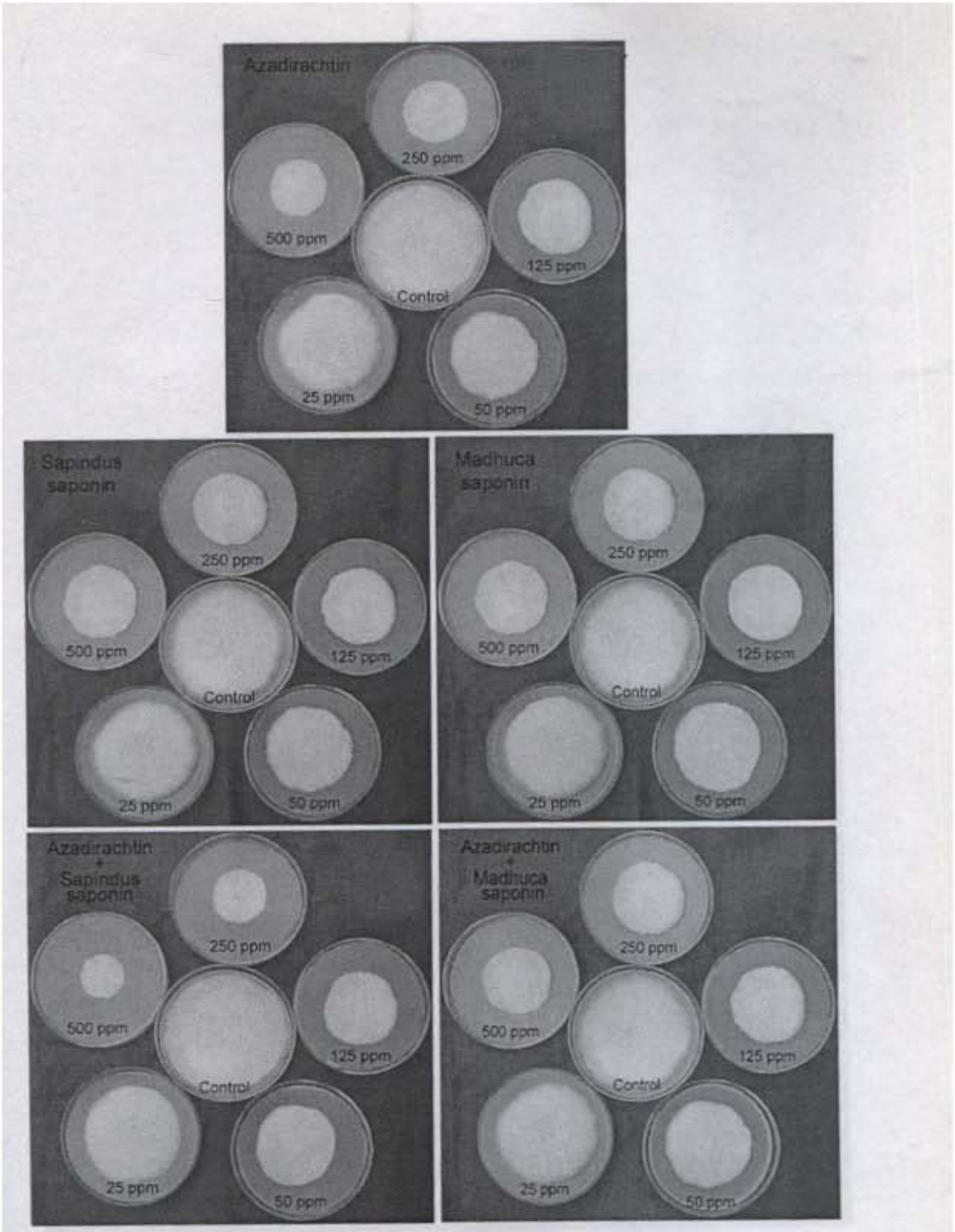


Fig. 20. Antifungal activity of azadirachtin, *Sapindus mukorossi* saponin and *Madhuca indica* saponin alone and in combination against *Fusarium udum*.

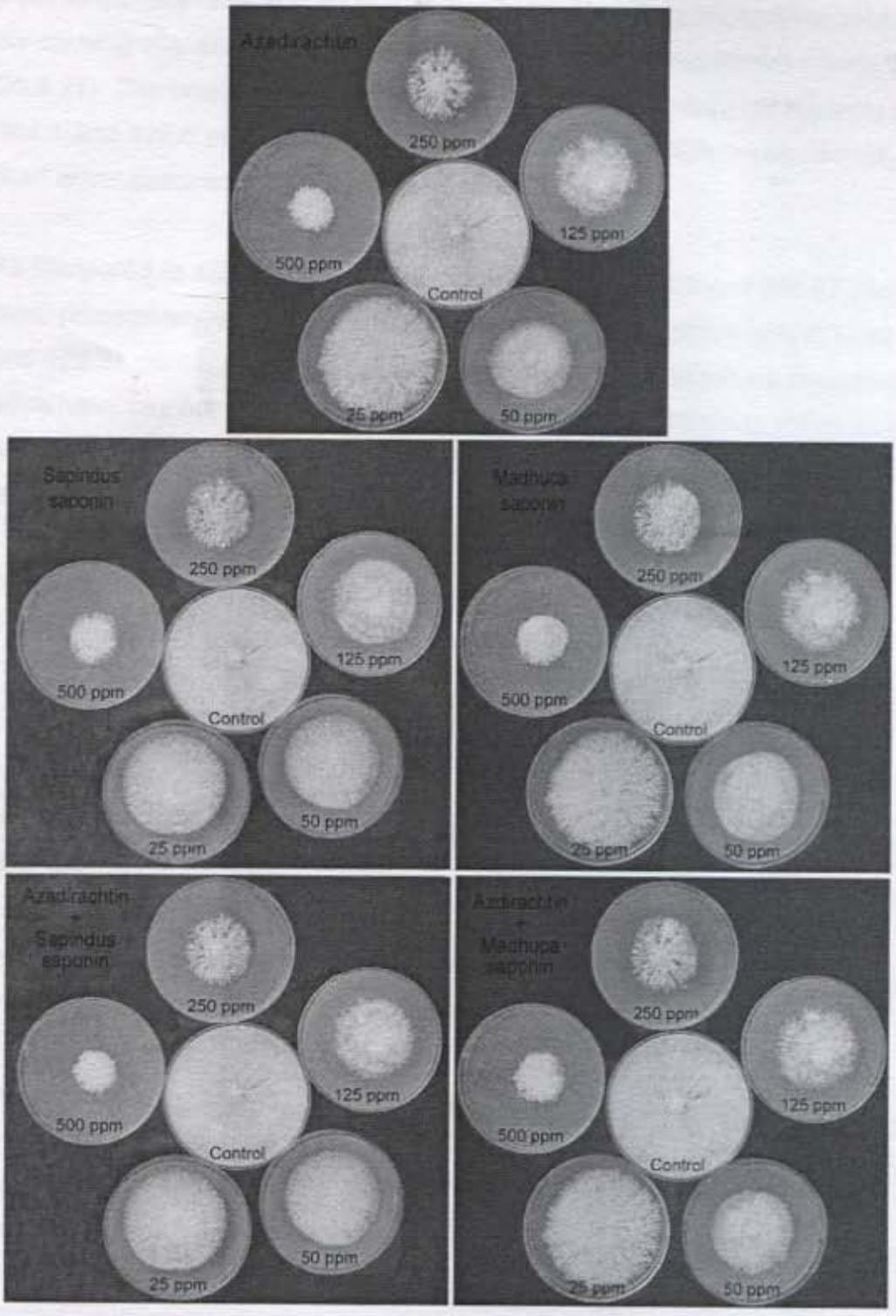


Fig. 21. Antifungal activity of azadirachtin, *Sapindus mukorossi* saponin and *Madhuca indica* saponin alone and in combination against *Sclerotium rolfsii*

against the other two test fungi *Fusarium udum* and *Sclerotium rolfsii*, responsible for causing wilt, seedling blight, stem rot and root rot of vegetables (Table 32; Fig. 20 & 21). The most effective combinations (azadirachtin + sapinus saponin) ED_{50} of 382.1 and 129.6 ppm against *F. udum* and *S. rolfsii*, which is considerably lower than either azadirachtin or saponins alone.

As compared to azadirachtin concentrate which showed ED_{50} of 868.97 and 167.6 ppm, respectively, aza: Sapindus saponin was far more active with ED_{50} of 382.1 and 129.58 ppm against the two test fungi respectively. Madhuca saponin on the other hand was not active against *F. udum* (ED_{50} of 1904.49 ppm). When combined with azadirachtin it showed antagonistic action as activity of azadirachtin (ED_{50} 868.97 ppm) was significantly reduced.

Table 33. Fractional analysis of azadirachtin and two saponins for their synergistic, joint action or antagonistic action

	D_{aza}	D_s	d_{aza}	d_s	d_{aza}/D_{aza} (A)	d_s/D_s (B)	A + B
Rhizoctonia bataticola							
A + SM	145.5	181.4	76.58	25.52	0.526	0.141	**0.67**
A + MI	145.5	229.9	83.7	27.9	0.575	0.121	**0.70**
Fusarium udum							
A + SM	868.97	1982.3	286.65	95.530	0.330	0.048	**0.38**
A + MI	868.97	1904.5	1111.7	370.57	1.28	0.19	**1.47**
Sclerotium rolfsii							
A + SM	167.6	407.41	97.193	32.400	0.58	0.08	**0.64**
A + MI	167.6	454.86	220.58	73.53	1.31	0.16	**1.49**
Rhizoctonia solani							
A + SM	158.77	677.74	112.73	37.58	0.709	0.055	**0.76**
A + MI	158.77	442.28	122.93	40.98	0.770	0.09	**0.86**

A: *Azadirachta indica* SM: *Sapindus mukorossi*; MI: *Madhuca indica;* d: conc. used in combination; D: LD_{50}

The possible synergistic, joint action and/or antagonistic action of the test compounds was established following fractional analysis/isobolographic analysis

proposed by Nelson and Kursar (1999).The potency ratio of the mixtures were calculated by using following formula

The data reported in Table 33 revealed that while Sapindus saponin caused synergistic action with azadirachtin against all the test fungi ($d_A/D_A + d_B/D_B < 1$), Madhuca saponin caused synergistic action against *R.* bataticola and *R. solani; and* antagonistic action against *F.udum* and *S. rolfsii.* Except for azadirachtin + Madhuca saponin combinations against *S. rolfsii* (potency ratio 1.49) and *Fusarium udum* (potency ratio 1.47), all other combinations exhibited potentiating or synergistic action against all the test fungi. Aza + Sapindus saponin combination, with potency ratio of 0.67, 0.38, 0.64 and 0.76 was found to be the most active against *R. bataticola, F. udum, S. rolfsii* and *R. solani* respectively. The reason for their different action remains unclear. This increase in activity (synergism/potentiation) was attributed to the fact that increased permeability of fungal membrane, facilitated penetration of the toxicant inside the fungal hyphae and thus caused increased antifungal activity.

4.6.4 Nematicidal activity of phytochemicals derived from neem, mahua and ritha against *Meloidogyne incognita* Kofoid and White and *Rotylenchulus reniformis*

The root knot nematode, *Meloidogyne incognita* and reniform nematode *Rotylenchulus reniformis* are economically important species and need special attention for control. Unlike more toxic conventional pesticides, plant secondary metabolites may serve as environmentally benign alternative for the control of nematode species that parasitize food and fibre crops. Studies were, therefore, conducted to investigate the nematicidal potential of some phytochemicals derived from *A. indica* (neem), *Madhuca indica* (mahua) and *Sapindus mukorossi* (ritha). The widely used water screening method was used to evaluate the nematicidal activity. The effect of fourteen test chemicals correlating mortality of freshly hatched second juvenile stage of *M. incognita,* and pre-adult or fourth juvenile stage of *R. reniformis* with the concentrations and exposure time, is depicted in Tables 34 and 36.

4.6.4.1 *Meloidogyne incognita*

Perusal of data in Table 34 on mortality of root knot nematode revealed significant nematicidal activity of all the test compounds. At higher dose of 1000 ppm, these exhibited 80-100 percent mortality. The concentration of the chemicals had direct bearing on nematode mortality as the activity decreased with decrease in concentration. Azadirachtin (20%) showed LC_{50} of 215.7, 153.5 and 253.1 ppm after 24, 48 and 72 hours of treatment (Table 35). The increased LC_{50} after 72 hours was probably due to the fact that some of the nematodes turned immobile after 24/48 hours might have recovered and become mobile after 72 hours. Triterpenic saponins obtained from *S. mukorossi* and *M. indica* also exhibited similar level of activity as azadirachtin. Mahua saponin was found to be more active with LC_{50} of 228.7, 159.5 and 220.0 ppm after 24, 48 and 72 hours respectively, than Sapindus saponin with corresponding LC_{50} of 475.5, 164.5 and 245.9 ppm at the respective times. Another neem based concentrate rich in salannin and nimbin was slightly less active than azadirachtin concentrate. Interesting results were observed when saponin concentrates were mixed with azadirachtin concentrate in either 3:1 or 1:1 proportions. In general azadirachtin: saponin mixture was more active than the individual constituents comprising the mixture.

Of the two combinations (3:1 and 1: 1), azadirachtin: Madhuca saponin (3:1) mixture was most active with LD_{50} of 259.5, 114.7 and 120.1 ppm after 24, 48 and 72 hours of exposure. It was followed by azadirachtin: Madhuca saponin (1:1) mixture with corresponding LC_{50} of 133.5, 197.0 and 199.9 ppm after 24, 48, 72 hours of the treatment. Interestingly, azadirachtin in combination with potassium salt of fatty acids derived from both neem and mahua oils were found to be far more active than azadirachtin alone. As compared to azadirachtin alone with LC_{50} of 215.7 and 253.2 ppm after 48 and 72 hours, azadirachtin + potassium salt of neem fatty acids combinations exhibited corresponding LC_{50} of 162.3 and 159.3 ppm after the same time interval. The nematicidal activity of azadirachtin in combination with potassium salt of fatty acids of mahua and neem was however comparable (Table 35). Salannin-nimbin combine isolated from neem oil was moderately active with LC_{50} of 397.9, 339.3 and 228.7 ppm after 24, 48 and 72 hours (Fig. 22).

Table 34 . Nematicidal actyivity of saponins and azadirachtin alone and in combination against root knot nematode, *Meloidogyne incognita*

Conc. (ppm) / Comp.	h	1000	500	250	125	62.5
Aza-A concentrate (20%)	24	100	48.98	34.27	25.51	10.36
	48	94.74	85.47	74.58	44.69	18.73
	72	94.53	83.31	49.04	18.42	5.66
Sapindus saponin	24	73.68	47.53	32.20	18.35	6.65
	48	89.11	77.84	66.76	48.32	20.61
	72	94.34	72.18	46.26	27.05	9.41
Madhuca saponin	24	94.92	82.77	46.44	23.35	12.49
	48	100	89.11	64.29	38.30	10.53
	72	94.34	85.39	55.82	27.02	8.78
Salannin-nimbin-desacetyl nimbin	24	67.80	46.56	46.95	39.66	17.23
	48	74.60	66.79	45.59	27.47	7.29
	72	90.67	80.09	54.55	30.28	10.91
Aza + saponin (*S. mukorossi*) (3:1)	24	89.65	73.68	54.37	23.35	13.33
	48	94.65	81.78	50.05	24.08	5.26
	72	94.44	79.58	50.06	21.58	3.53
Aza + saponin (*S. mukorossi*) (1:1)	24	89.93	55.17	36.21	18.35	10.00
	48	94.65	84.21	61.22	28.05	5.26
	72	100	76.96	63.54	25.73	3.53
Aza + saponin (*M. indica*) (3:1)	24	83.94	66.75	52.79	32.86	14.08
	48	100	93.00	91.06	56.41	20.64
	72	100	98.14	92.74	49.29	15.72
Aza + saponin (*M. indica*) (1:1)	24	100	89.29	77.21	50.12	18.93
	48	89.29	77.41	61.86	33.31	19.27
	72	94.72	82.95	62.35	38.96	9.97

Aza + K-salt of Neem oil (1:1)	24	95.00	78.95	58.49	47.46	22.79
	48	86.17	72.75	45.50	16.45	5.36
	72	95.00	78.95	61.42	47.46	22.79
Aza + K-salt of Madhuca oil (1:1)	24	93.23	84.48	69.50	44.83	18.98
	48	100.0	94.55	74.11	50.95	17.85
	72	93.23	84.48	69.50	44.83	18.98
Salannin-nimbin-desacetyl nimbin + Sapindus saponin (1:1)	24	80.68	59.63	46.56	32.14	25.87
	48	89.12	78.24	59.29	20.22	7.29
	72	94.72	84.90	51.44	27.86	11.88
Salannin-nimbin-desacetyl nimbin + Madhuca saponin (1:1)	24	77.60	66.09	29.32	18.98	-
	48	69.17	58.29	36.54	22.03	5.52
	72	87.29	60.99	43.64	20.48	4.03
Aza + Salannin-nimbin- desacetyl nimbin + Sapindus saponin (1:1:1)	24	68.42	57.89	41.08	22.96	8.63
	48	89.10	73.17	66.73	41.88	16.07
	72	68.42	57.89	41.08	21.65	8.63
Aza + Salannin-nimbin- desacetyl nimbin (1:1)	24	100	100	89.65	75.85	35.11
	48	100	100	91.07	85.22	32.52
	72	100	100	94.64	77.43	44.64

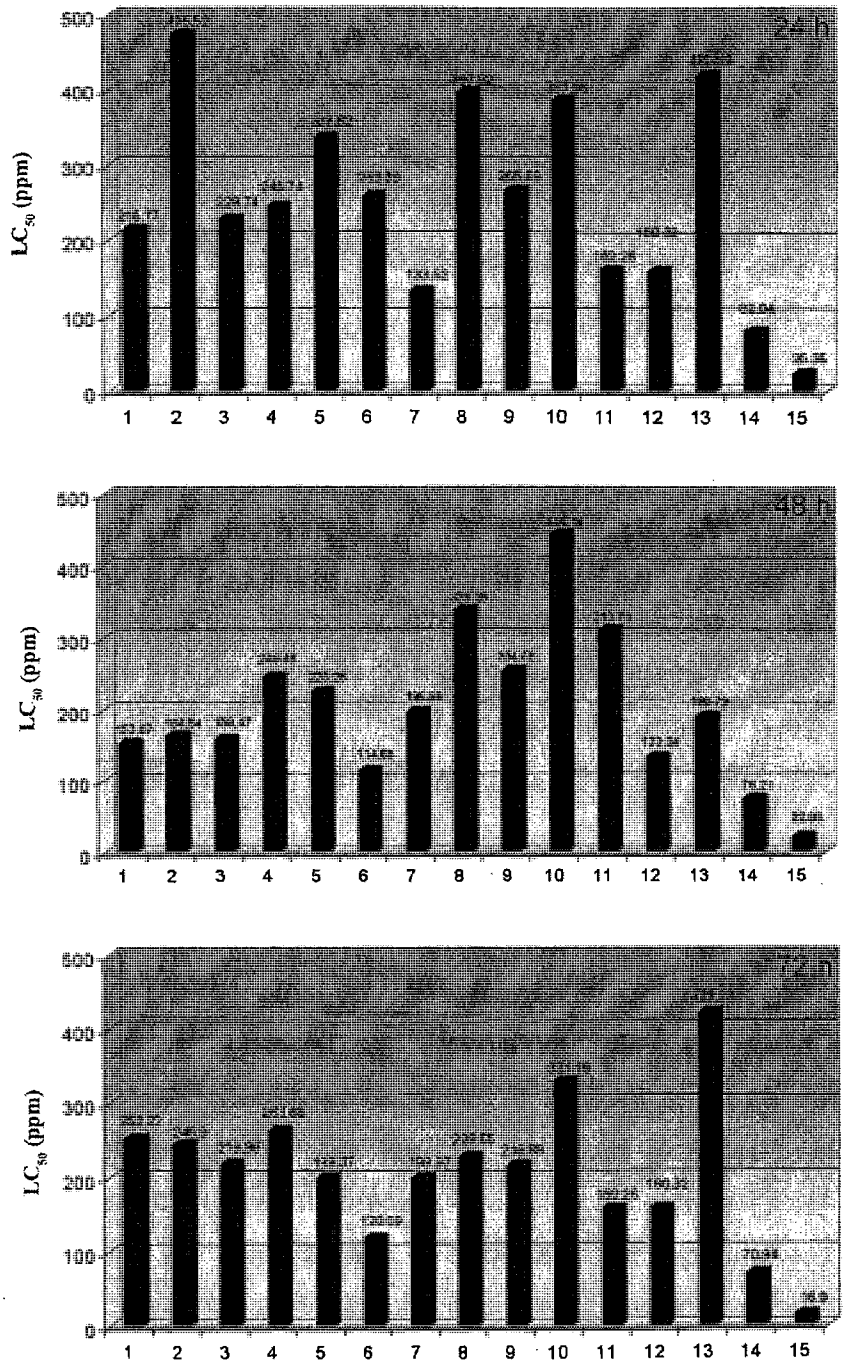
Among all the test combinations, azadirachtin: salannin-nimbin (1:1) combination provided outstanding nematicidal activity with corresponding LD_{50} of 82.04, 76.22 and 70.94 ppm after 24, 48 and 72 hours of the treatment. This was followed by azadirachtin + potassium salt of mahua fatty acids with corresponding LC_{50} of 160.3, 133.3 and 160.3 ppm (Table 35). The study clearly demonstrated that appropriate combinations of neem meliacins, mahua and Sapindus saponin, as well as potassium salt of neem and mahua fatty acids could be employed as effective adjuvants to develop plant based biopesticides with good nematicidal activity.

Table 35. Lethal concentration (LC_{50}) of saponins and azadirachtin alone and in combination against root knot nematode, *Meloidogyne incognita*

Compounds	h	LC_{50} (ppm)	χ^2_{exp} (3 d.f)	Fiducial limit
Azadirachtin (20%)	24	215.7	-	198.14-234.96
	48	153.1	4.72	131.19-178.60
	72	253.2	1.55	225.60-284.21
Sapindus saponin	24	475.5	1.51	395.64-571.52
	48	164.5	3.63	137.25-197.27
	72	245.9	2.83	216.47-279.32
Madhuca saponin	24	228.7	5.34	201.61-259.53
	48	159.5	-	145.86-174.36
	72	220.0	1.30	194.59-148.63
Salannin-nimbin-	24	397.92	7.91	297.88-531.57
desacetyl nimbin	48	339.26	5.82	288.60-398.81
	72	228.65	1.08	199.49-262.06
Aza + saponin	24	245.7	2.02	213.12-283.45
(*S. mukorossi*) (3:1)	48	246.5	0.88	219.45-276.84
	72	263.7	1.30	235.71-294.98
Aza + saponin	24	336.8	8.38	289.98-391.22
(*S. mukorossi*) (1:1)	48	225.3	3.47	200.19-253.47
	72	199.4	-	183.47-216.65
Aza + saponin	24	259.4	1.35	219.52-306.49
(*M. indica*) (3:1)	48	114.7	-	102.75-127.99
	72	120.1	2.67	108.6-132.810
Aza + saponin	24	133.5	-	120.80-147.59
(*M. indica*) (1:1)	48	197.0	1.97	167.97-231.02
	72	199.9	3.58	175.45-227.93

Aza+ K-salt of neem fatty acids (1:1)	24	162.3	4.15	138.67-189.86
	48	310.7	2.14	273.16-353.42
	72	159.3	2.74	135.95-186.57
Aza+ K-salt of mahua fatty acids (1:1)	24	160.3	2.35	137.14-187.41
	48	133.3	-	121.02-146.92
	72	160.3	2.74	137.14-187.41
Salannin-nimbin-desacetyl nimbin + Sapindus saponin (1:1)	24	266.69	3.18	214.99-330.82
	48	254.56	5.79	223.74-289.61
	72	216.89	2.72	191.11-246.14
Salannin-nimbin-desacetyl nimbin + Madhuca saponin (1:1)	24	387.96	6.00	331.64-453.87
	48	444.14	4.74	372.74-529.22
	72	331.16	2.79	290.48-377.55
Aza + Salannin-nimbin- desacetyl nimbin + Sapindus saponin (1:1:1)	24	420.59	2.10	347.94-508.41
	48	190.79	5.31	161.87-224.86
	72	423.23	1.84	350.43-511.16
Aza + Salannin-nimbin- desacetyl nimbin (1:1)	24	82.04	7.67	73.14-92.02
	48	76.21	-	67.12-86.54
	72	70.94	1.46	62.32-80.75
Triazophos	24	25.35	-	22.69-28.33
	48	22.86	7.14	20.82-24.35
	72	16.90	7.22	15.13-18.88

Fractional analysis of the data on nematicidal activity (Table 36) revealed that azadirachtin + saponin mixture and azadirachtin + salannin-nimbin mixture were most active. The potency ratio ($d_A/D_A + d_B/D_B$) of these effective combinations was far less than 1, indicating possible synergistic/potentiating effect of saponins and salannin-nimbin mixture.



1. Aza (20%)
2. Sapindus saponin
3. Madhuca saponin
4. Aza+Sapindus saponin (3:1)
5. Aza+Sapindus saponin (1:1)
6. Aza+Madhuca saponin (3:1)
7. Aza+Madhuca saponin (1:1)
8. Salanin & Nimbin
9. Sala-Nim + Sapindus saponin (1:1)
10. Sala-Nim + Mahua saponin (1:1)
11. Aza + KFA-NO (1:1)
12. Aza + KFA-MO (1:1)
13. Aza+Sal-Nim+Saponin (1:1:1)
14. Aza + Sal-Nim (1:1)
15. Triazophos

Fig. 22. Nematicidal activity of phytochemicals and related products derived from neem, sapindus and mahua against *Meloidogyne incognita*.

Table 36. Fractional analysis of azadirachtin and two saponins for their synergistic, joint action or antagonistic nematicidal action

	D_{aza}	D_b	d_{aza}	D_b	d_{aza}/D_{aza} (A)	d_{ssm}/D_b (B)	A + B
24 h							
Aza+MIS (1:1)	215.7	228.7	66.75	66.75	0.309458	0.291867	**0.60**
Aza + SN* (1:1)	215.7	397.9	41.02	41.02	0.190172	0.103091	**0.29**
48h							
Aza+MIS (3:1)	153.1	159.5	86.025	28.675	0.561888	0.179781	**0.74**
Aza + SN(1:1)	153.1	339.9	38.11	38.11	0.248922	0.112121	**0.36**
72h							
Aza+SMS (1:1)	253.2	245.9	99.7	99.7	0.39376	0.405449	**0.80**
Aza+MIS (3:1)	253.2	220	90.075	30.025	0.355746	0.136477	**0.49**
Aza+MIS (1:1)	253.2	220	99.95	99.95	0.394747	0.454318	**0.85**
Aza + SN (1:1)	253.2	228.7	35.47	35.47	0.140087	0.155094	**0.30**

*SN: salannin-nimbin- desacetyl nimbin combine

4.6.4.2 *Rotylenchulus reniformis*

The nematicidal activity of phytochemicals derived from neem, mahua and Sapindus against the reniform nematode, *Rotylenchulus reniformis* is presented in Table 37 and Figure . In line with the above study, the test compounds were found equally effective against reniform nematode *R. reniformis*. Data depicted in Figure revealed that percent mortality of the test nematode increased with increase in concentration and time of exposure. Azadirachtin concentrate (20%) showed LC_{50} of 236.1 and 197.3 ppm after 24 and 48 hours and 285.2 ppm after 72 hours. This pattern of activity was similar to the one observed against the root knot nematode, *M. incognita*. It appears that some of the immobile/inactivated nematodes taken as dead at 24/48 h of exposure get activated by 72 hour and thus lead to increased LC_{50} value after 72 h. The same, however, does not hold true for saponins, which results in increased activity with increased exposure time. Of the two saponins, mahua saponin was more active. As compared to LC_{50} of 271.6 ppm after 24 hours, Madhuca saponin showed LC_{50} of 170.4 and 168.8 ppm after 48 and 72 hours. Similarly nematicidal activity of Sapindus saponin increased with exposure

time. Thus LC_{50} of 495.5 ppm observed after 24 hours decreased to 208.9 and 181.9 ppm after 48 and 72 hours. The activity of azadirachtin and/or saponin concentrate was further increased when they were used as mixture. Among the various combinations, azadirachtin: Madhuca saponin (3:1) mixture was found to be the best showing LC_{50} of 116.4 and 106.9 ppm after 48 and 72 hours of the treatment. It was followed by azadirachtin: potassium salt of mahua oil fatty acids (1:1) combination with corresponding LC_{50} of 155.9 and 130.7 ppm after 48 and 72 hours of the treatment. Azadirachtin: salannin-nimbin mixture once again showed its superiority exhibiting LC_{50} of 82.1, 97.3 and 91.2 ppm after 24, 48 and 72 hours of the treatment (Fig. 23).

Table 37. Nematicidal actyivity of saponins and azadirachtin alone and in combination against reniform nematode, *R. reniformis*

Conc. / Comp.	h	1000	500	250	125	62.5
Aza-A concentrate (20%)	24	100.0	40.54	31.09	20.00	7.69
	48	91.20	83.17	67.56	36.66	12.30
	72	95.60	78.57	42.75	15.40	2.98
Sapindus saponin	24	74.33	39.99	33.78	21.61	9.58
	48	86.97	76.21	58.36	41.31	14.89
	72	95.76	77.98	49.31	33.03	27.70
Madhuca saponin	24	87.51	75.68	47.21	24.66	8.11
	48	100.0	87.58	62.15	25.97	12.25
	72	100.0	86.02	62.63	35.42	9.53
Salannin-nimbin	24	61.45	40.63	25.76	14.99	7.21
	48	71.78	58.73	52.20	34.44	13.06
	72	81.53	66.61	48.52	31.27	7.67
Aza + saponin (*S. mukorossi*) (3:1)	24	87.50	72.21	37.70	22.68	9.45
	48	91.20	77.95	56.25	42.62	11.86
	72	95.71	80.93	39.20	22.46	15.18

Aza + saponin (*S. mukorossi*) (1:1)	24	86.13	71.23	26.87	16.00	7.90
	48	92.84	83.51	55.58	14.57	1.54
	72	100.0	81.89	67.57	31.94	19.19
Aza + saponin (*M. indica*) (3:1)	24	82.33	64.69	47.02	16.43	1.93
	48	100.0	91.06	86.78	67.93	17.74
	72	100.0	95.38	90.64	75.06	16.90
Aza + saponin (*M. indica*) (1:1)	24	100.0	86.38	72.03	37.94	8.71
	48	86.22	74.68	58.88	27.54	11.68
	72	95.25	84.83	58.28	38.46	15.00
Aza + K-salt of Neem oil (1:1)	24	91.78	81.44	60.57	40.29	33.36
	48	82.10	70.04	52.02	38.48	9.76
	72	92.11	78.18	53.97	38.67	18.46
Aza + K-salt of Madhuca oil (1:1)	24	93.14	83.56	74.33	36.48	15.50
	48	100.0	91.05	70.57	42.81	9.05
	72	99.89	99.89	81.79	54.16	12.92
Salannin-nimbin-desacetyl nimbin: Sapindus saponin (1:1)	24	81.70	59.79	40.64	21.13	5.63
	48	86.82	72.88	59.00	17.33	4.50
	72	90.77	80.28	58.10	17.30	3.49
Salannin-nimbin-desacetyl nimbin: Madhuca saponin (1:1)	24	65.73	56.34	27.61	16.62	36.92
	48	68.81	58.41	32.64	14.50	5.65
	72	78.14	48.61	33.93	20.18	3.05
Azadirachtin: salannin-nimbin-desacetyl nimbin: Sapindus saponin (1:1:1)	24	91.78	81.44	60.57	40.29	33.36
	48	82.10	70.07	52.02	38.48	9.76
	72	92.11	78.18	53.97	38.67	18.46
Azadirachtin: salannin-nimbin-desacetyl nimbin (1:1)	24	67.12	64.04	44.00	18.43	8.11
	48	86.39	72.79	58.79	41.96	10.52
	72	90.71	81.26	69.27	50.10	20.30

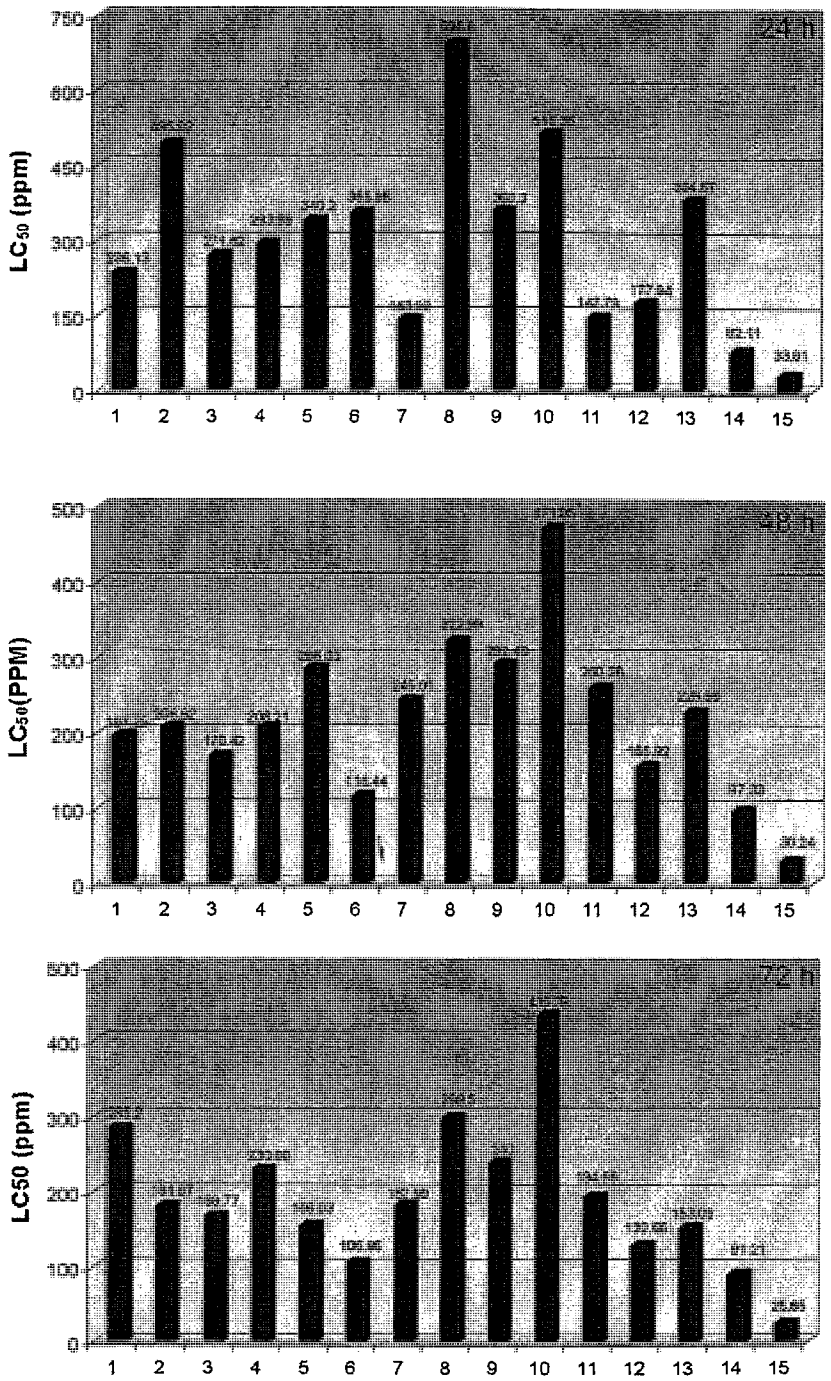
The increase in the nematicidal activity of azadirachtin following incorporation of triterpenic saponins and potassium salts of fatty acids can be once again attributed to the surface tension lowering property of both saponins and potassium salts which facilitates penetration of the active material through cuticle into the insect body both through ingestion, skin contact and/or rupture of cells.

Table 38. Nematicidal activity (LC_{50}) of azadirachtin and various adjuvants alone and in combinations against the reniform nematode, *R. reniformis*

Compounds	h.	LC_{50} (ppm)	χ^2_{exp} (3 d.f)	Fiducial limit
Azadirachtin (20%)	24	236.1	-	217.11-256.81
	48	197.3	5.42	172.22-227.25
	72	285.2	0.92	256.19-317.49
Sapindus saponin	24	495.9	8.68	398.87-616.60
	48	208.9	3.48	177.49-245.92
	72	181.9	-	156.40-211.47
Madhuca saponin	24	271.6	1.16	237.13-311.14
	48	170.4	-	156.45-185.64
	72	168.8	-	154.73-184.08
Salannin-nimbin-desacetyl	24	696.8	0.6	536.52-904.96
nimbin	48	322.9	5.51	262.85-396.89
	72	299.5	5.24	257.43-348.44
Aza + saponin	24	294.0	2.85	255.47-338.30
(*S. mukorossi*) (3:1)	48	208.2	5.29	180.14-240.64
	72	230.9	-	203.12-262.44
Aza + saponin	24	340.2	9.69	295.90-391.93
(*S. mukorossi*) (1:1)	48	286.0	11.14	257.42-317.81
	72	156.0	-	142.38-171.00

Aza + saponin	24	356.0	-	316.65-400.16
(*M. indica*) (3:1)	48	116.4	-	104.01-130.36
	72	106.9	-	94.70-120.58
Aza + saponin	24	143.9	-	130.91-158.24
(*M. indica*) (1:1)	48	245.0	3.58	211.54-283.77
	72	182.9	0.77	159.91-209.40
Aza+ K-salt of neem oil	24	147.8	3.37	122.13-178.81
(1:1)	48	260.4	7.44	222.29-304.98
	72	194.7	0.98	167.31-226.47
Aza+ K-salt of mahua oil	24	177.9	6.57	153.98-205.64
(1:1)	48	155.9	-	142.63-170.45
	72	130.7	2.08	117.20-145.66
Salannin-nimbin- desacetyl	24	360.3	1.64	311.23-416.47
nimbin: Sapindus	48	292.5	11.30	257.84-331.79
Saponin (1:1)	72	240.0	-	210.68-273.39
Salannin-nimbin: Madhuca	24	516.36	2.8	423.26-629.94
Saponin (1:1)	48	471.95	2.84	397.83-559.88
	72	435.38	6.31	373.37-507.67
Azadirachtin: salannin-	24	384.67	7.76	323.20-457.82
nimbin- desacetyl nimbin	48	228.69	8.80	195.93-266.94
: Sapindus saponin (1:1:1)	72	153.06	4.26	127.86-183.23
Azadirachtin: salannin-	24	82.11	-	73.27-92.01
nimbin- desacetyl nimbin	48	97.33	-	87.29-108.53
(1:1)	72	91.21	7.52	81.63-101.92
Triazophos	24	33.81	-	30.86-37.04
	48	30.24	6.85	26.52-35.24
	72	25.65	3.04	23.48-28.01

Fractional analysis (Table 39) was conducted on azadirachtin and its combinations with saponins and potassium salts of fatty acid to determine their possible



1. Aza (20%)
2. Sapindus saponin
3. Madhuca saponin
4. Aza+Sapindus saponin (3:1)
5. Aza+Sapindus saponin (1:1)
6. Aza+Madhuca saponin (3:1)
7. Aza+Madhuca saponin (1:1)
8. Salanin & Nimbin
9. Sala-Nim + Sapindus saponin (1:1)
10. Sala-Nim + Mahua saponin (1:1)
11. Aza + KFA-NO (1:1)
12. Aza + KFA-MO (1:1)
13. Aza+Sal-Nim+Saponin (1:1:1)
14. Aza + Sal-Nim (1:1)
15. Triazophos

Fig. 23. Nematicidal activity of phytochemicals and related products derived from neem, sapindus and mahua against *Rotylenchulus reniformis*.

synergistic/antagonistic or joint action. As evident from the data (Table 39), the test compounds in combination exhibit synergistic action since potency ratio (d_A/D_A + d_B/D_B) value were found to be less than one. It was thus concluded that plant based products like saponins, potassium salt of fatty acids and more particularly neem meliacins such as azadirachtin, salannin and nimbin can be effectively utilized to develop a plant based ecologically sound nematicides. Some of the triterpenic and steroidal saponins have been earlier reported to possess significant nematicidal activity (Meher *et al.*, 1988).

Table 39. Fractional analysis of azadirachtin and two saponins for their synergistic, joint action or antagonistic action against *Rotylenchulus reniformis*

	D_{aza}	D_b	d_{aza}	d_b	d_{aza}/D_{aza} (A)	d_b/D_b (B)	A + B
24h							
Aza+MIS (1:1)	236.1	271.6	71.95	71.95	0.304744	0.264912	**0.57**
Aza + SN (1:1)	236.1	696.8	41.055	41.055	0.173888	0.058919	**0.23**
48h							
Aza+MIS (3:1)	197.3	170.4	87.3	29.1	0.442473	0.170775	**0.61**
Aza + SN (1:1)	197.3	322.9	48.665	48.665	0.246655	0.150712	**0.40**
72h							
Aza+SMS (3:1)	285.2	181.9	173.18	57.725	0.607205	0.317345	**0.92**
Aza+SMS (1:1)	285.2	181.9	78.00	78.00	0.273492	0.428807	**0.70**
Aza+MIS (3:1)	285.2	168.8	80.175	26.725	0.281119	0.158323	**0.44**
Aza+MIS (1:1)	285.2	168.8	91.45	91.45	0.320652	0.541765	**0.86**
Aza + SN (1:1)	285.2	228.7	45.605	45.605	0.159905	0.19941	**0.36**

4.6.5 Germination/growth inhibition/promotion activity of Madhuca and Sapindus saponins

4.6.5.1 Rice

Effect of mahua and Sapindus saponins on the germination and growth of rice and maize seedlings is given in Fig 24-27. Perusal of data revealed that growth (%) of rice coleoptile and root in rice seedlings increased with decrease in Madhuca saponin concentrates. At higher concentrations of 1000 ppm, the increase in rice

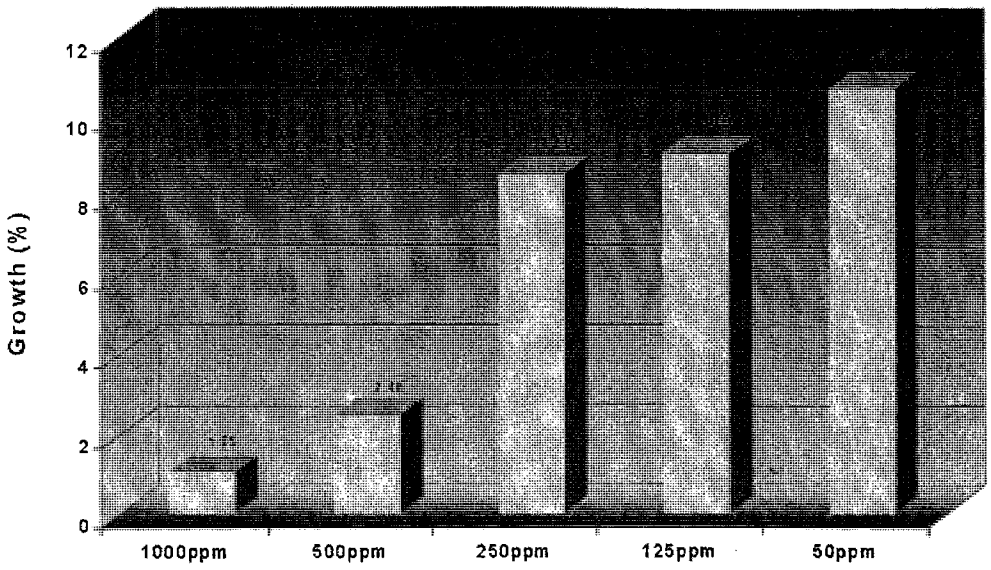


Fig. 24a. Effect of *Madhuca indica* saponin on the coleoptile growth of rice seedling

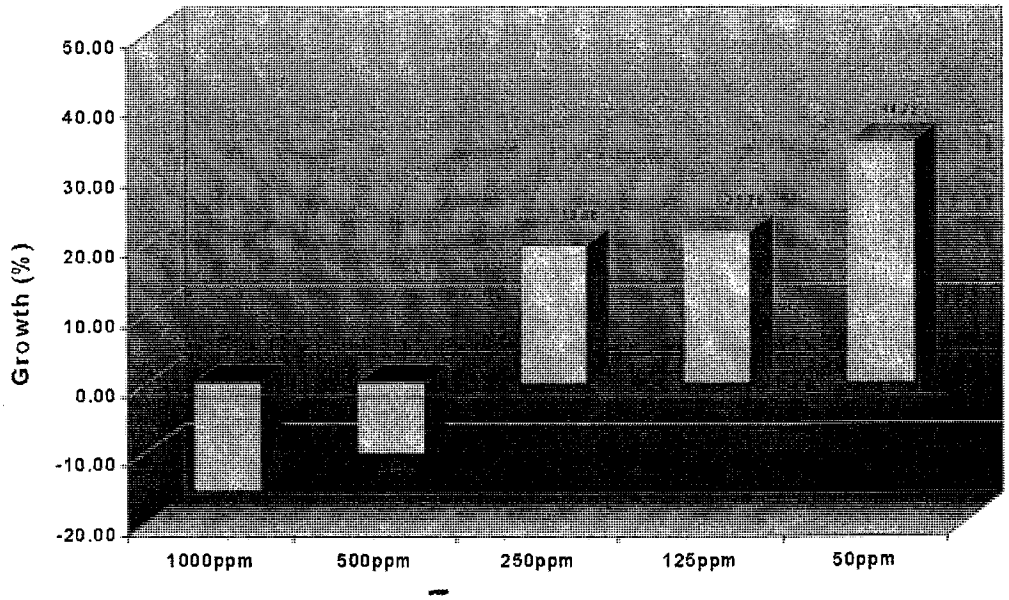


Fig. 24b. Effect of *Madhuca indica* saponin on the root growth of rice seedling

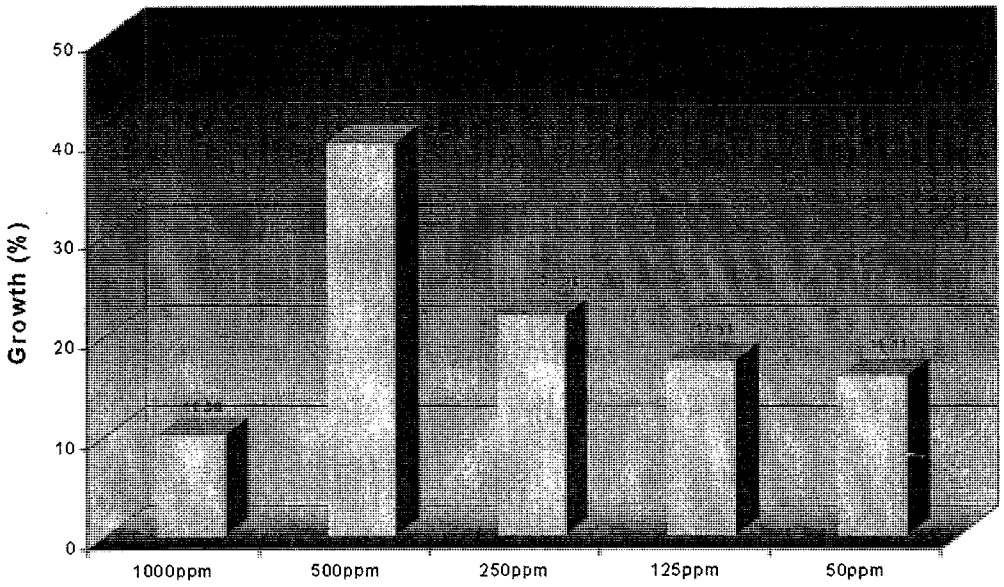


Fig. 25a. Effect of *Sapindus mukorossi* saponin on the coleoptile growth of rice seedling

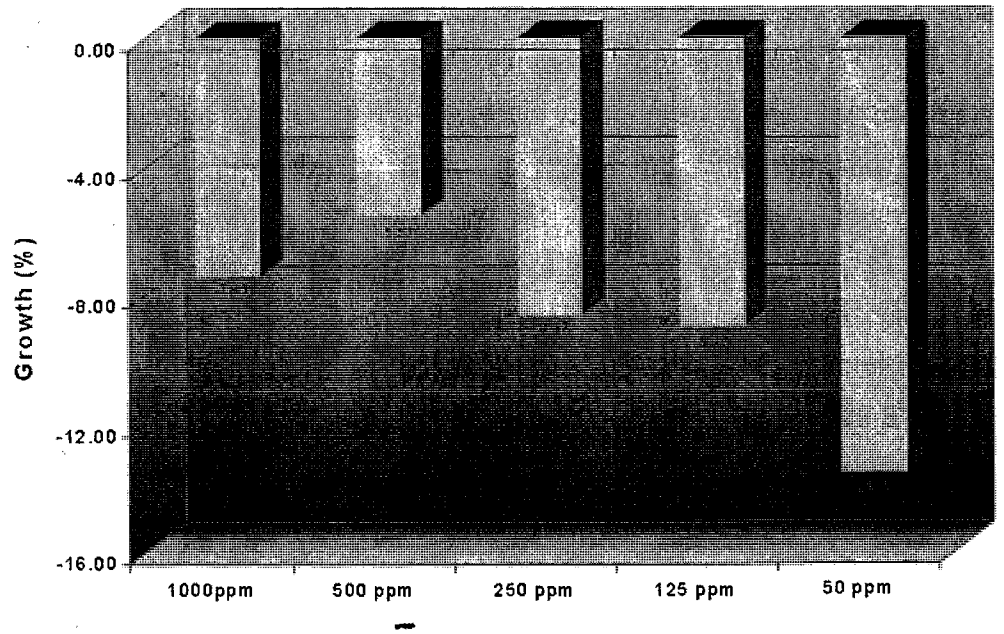


Fig. 25b. Effect of *Sapindus mukorossi* saponin on the root growth of rice seedling

coleoptile and root growth was 1.05 and –15.4% while at lowest concentrations of 50 ppm, coleoptile and root growth was found to be 10 and 34.72 % (Fig. 24). The application of Sapindus saponin on rice seed, however, gave somewhat different results. While at higher concentration of 1000 ppm, it inhibited 90% of the coleptile growth and 7.45% of the root growth, at lower concentrations, it recorded significant increase in coleoptile (shoot) growth (Fig. 25). The highest coleoptile growth (40%) recorded at 500 ppm decreased with further decrease in concentration. At 500 ppm concentration, root growth was stunted by only 5.57%, while at lower concentration of 50 ppm, the root growth was decreased to the extent of 13.8%. Thus at the optimum test concentrations of 500 ppm, mahua saponin recorded 39.2% increase in coleoptile growth, and marginal decrease 5.57% in the root length growth.

4.6.5.2 Maize

Effect of mahua and Sapindus saponins on growth of maize seedlings is given in Fig. 26. Perusal of data revealed that like in rice, the coleoptile length of maize seedlings after germination increased with decrease in application dose of mahua saponin concentrate. At higher concentration of 2000 ppm, it recorded 66.83% increase in shoot length, while at lowest concentration of 50 ppm it showed significantly higher increase 134.3%in the shoot length. Roots on the other hand showed different pattern of growth. While at concentrations of 1000 ppm and higher, it recorded 12.2 to 29.8% decrease in root growth, the root length increased with decrease in concentration of the test compound until it reached 250 ppm when it showed maximum (129.8%) root growth. Decrease in test concentration level beyond 250 ppm led to decrease in the root growth.

Following treatment with Sapindus saponin concentrate, maize coleoptile length increased with decrease in concentration of the test saponin. At optimum concentration of 250 ppm, it recorded maximum shoot growth (122.8%). The shoot growth decreased with further decrease in test concentration. Similarly maize root growth was inhibited (58.2% and 10.4%) at higher concentration (2000 and 1000 ppm), and further decrease in concentration led in increase in the root growth. At lower concentrations, of 500, 250 and 50 ppm; 35, 33 and 18.1% increase in root length has observed (Fig. 27). Thus for optimum growth of maize seedling, 250 ppm concentration of Sapindus saponin was ideal for treating maize seeds before germinations.

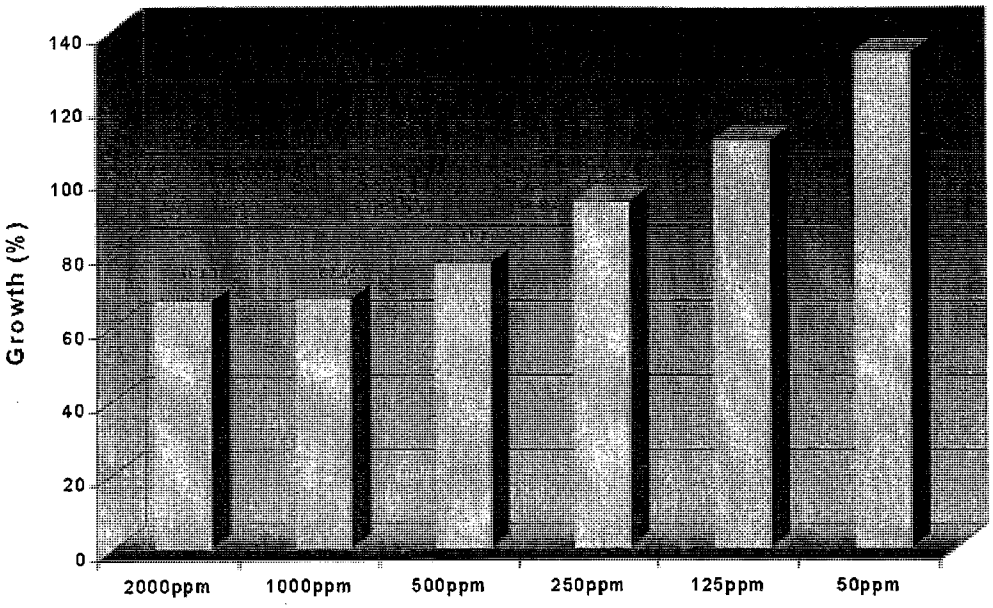


Fig. 26a. Effect of *Madhuca indica* saponin on the coleoptile growth of maize seedling

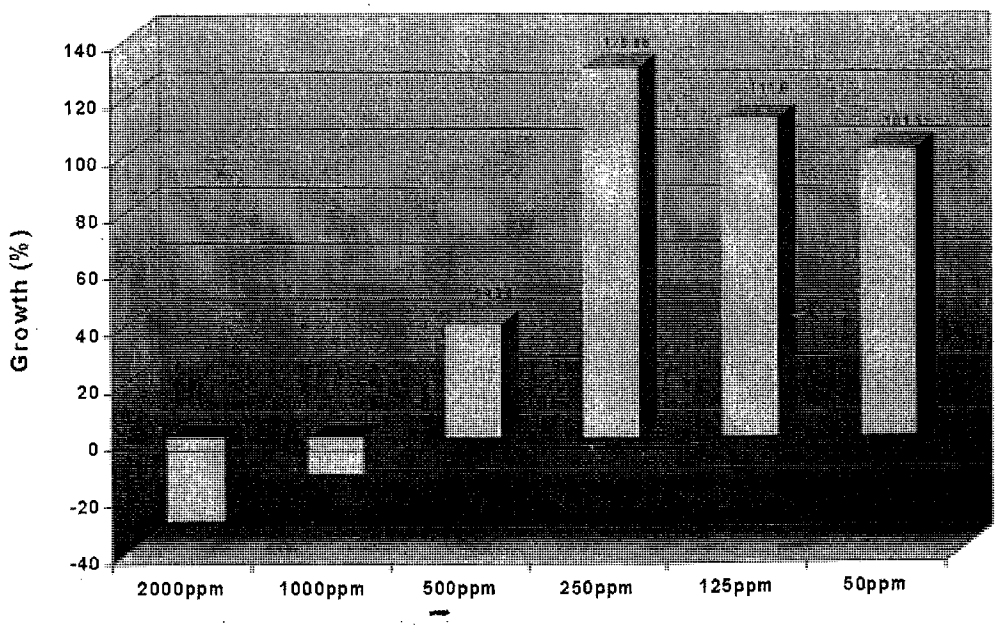


Fig. 26b. Effect of *Madhuca indica* saponin on the root growth of maize seedling

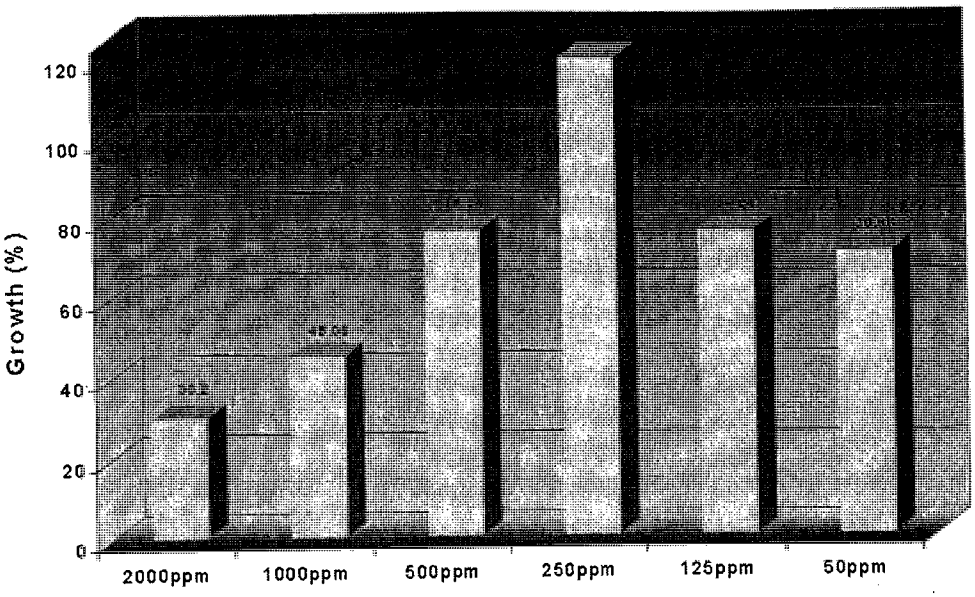


Fig. 27a. Effect of *Sapindus mukorossi* saponin on the coleoptile growth of maize seedling

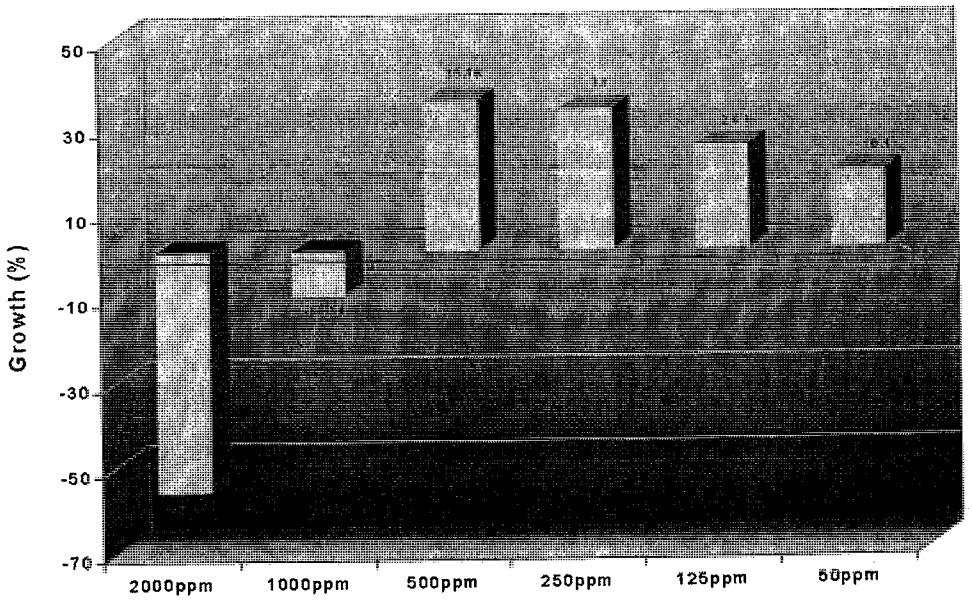


Fig. 27b. Effect of *Sapindus mukorossi* saponin on the root growth of maize seedling

- Azadirachtin powder concentrate (≈ 20 %) was isolated from the methanol extract of defatted neem (*Azadirachta indica*) seed kernel powder by the method developed earlier in our laboratory. The methanol extract after special work-up and enrichment yielded azadirachtin-A enriched concentrate of 40% purity. Azadirachtin concentrate (40%) was further purified yield aza-A of ≈ 90 % purity. The product was characterized by ^{1}H-NMR and mass spectral analysis.

- *Madhuca indica* (mahua) saponins were isolated from the methanol extract of defatted seed kernel cake. *Sapindus mukorossi* (ritha) saponins were similarly extracted from pericarp of the fruit. The methanol extract of both plant materials after removal of the solvent was partitioned with water : n-butanol and the concentrated organic phase precipitated with large excess of acetone to yield saponin powder concentrate.

- Madhuca saponin concentrate comprised of three constituents, of which two (MI-I and III) were separated by column chromatography and preparative high performance liquid chromatography. From sapindus saponin concentrate, the major saponin constituent SM-I was isolated by column chromatography and Prep-HPLC.

- A new reverse phase LC-method has been developed for the analysis of madhuca saponins using PDA detector (213 nm), RP-18 column (250 × 4 mm), and methanol: water (60:40 v/v) solvent system at the flow rate of 0.4 ml min-1. The method is simple, specific and accurate with detection limit of 50 ppm.

- A new LC-method has also been developed for the analysis of sapindus saponins. The method involved the use of RP-18 column (250 × 4 mm), PDA detector at 213 nm, acetonitrile: water (47:53 v/v) at 0.4 ml min-1 flow rate for

optimum separation of saponin peaks. The method is simple, specific and accurate with detection limit 50 ppm.

- LC- analytical method(s) have been upgraded and fine-tuned for operation on preparative high performance liquid chromatography to isolate and purify saponin constituents in large quantities from both plant materials. Solvent system, elution parameters and other parameters have been standardized.

- Madhuca saponin (MI-I) on acidic hydrolysis yielded 16-hydroxyprotobassic acid as genin and glucose, arabinose and xylose as monosaccharides comprising the glycone moiety. The second madhuca saponin MI-III on acidic hydrolysis furnished the same genin and glucose, arabinose, xylose and apiose as monosacchardides comprising the glycone moiety.

- The structures of two madhuca saponins (MI-I and III) were characterized on the basis of their ^{1}H-NMR and mass spectral analysis. Both the saponins exhibited peaks typical of angular methyls, gem dimethyl, olefinic, hydroxymethyl and protons adjacent to hydroxyl functions and others comprising the glycone moiety.

- The mass spectrum of first madhuca saponin (MI-I) exhibited protonated molecular ion peak at 1241 along with other characteristic fragment ions at m/z 1059, 927.7 and 795.8 formed as a result of successive loss of glucose, arabinose and xylose units from the molecular ion. Other fragment ions at m/z 1073 and 1055.9 were postulated to arise from the dehydrated fragment ions at m/z 1223 and 1205.9 respectively.

- The mass spectrum of second madhuca saponin (MI-III) exhibited molecular ion peak at m/z 1535.9 along with three other prominent fragment ions at m/z 1518, 1403 and 1373, arising due to the loss of water (18 amu), glucose (162 amu), and pentose (132 amu) units from the molecular ion. The peaks at m/z

1355 and 1241 originated as a result of the loss of water and glucose moiety from the fragment ions.

- Both the madhuca saponins (MI-I and MI-III) are bisdesmosides of the triterpenic sapogenin 16-hydroxy protobassic acid. On the basis of ^{1}H-NMR and mass spectral analysis, structure of MI-I has been tentatively assigned as 3-O-[β-D-glucopyarnosyl-β-D-glucopyranosyl]–16-α-hydroxyprotobassic acid-28-O-[arab, glu, xyl]-arab. The second saponin MI-III was similarly assigned the tentative structure 3-O-β-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-α-hydroxyprotobassic acid-28-O- [arab, xyl, arab] apiose ester. Linkages between the monosaccharide units have, however, not been established.

- Major madhuca saponin MI-III on alkaline hydrolysis furnished a prosapogenin, which on acidic hydrolysis yielded 16-hydroxyprotobassic acid as the sapogenin and glucose as the only monosaccharide. It showed ^{1}H-NMR peaks typical of the molecule. Its mass spectrum exhibited the major peak at m/z 990 considered to arise as result of the loss of neutral water fragment from protonated molecular ion. The peaks at m/z 520 and m/z 504 were assumed to arise as a result of the cleavage of glycosidic linkage, the former corresponding to sapogenin and later to the glycone moiety. The structure of the prosapogenin was tentatively assigned as 3-O-β-glucopyranosyl-glucopyranosyl-glucopyranosyl- 16-α-hydroxyprotobassic acid. The linkage between the sugar units located at C-3 position have not been established.

- Sapindus saponin (SM-I) was found to be bidesmoside of hederagenin with sugar linkages at both C-3 OH and C-28 carboxylic functions. On prolonged hydrolysis with mineral acid (methanolic HCl), saponin was hydrolyzed to hederagenin (M^+ 472). The monosaccharides in the aqueous hydrolysate were identified as glucose, arabinose, xylose and rhamnose by comparison of R_f values (TLC/PC) with authentic samples. Besides characteristic ^{1}H-NMR peaks of the triterpenic saponins (methyl, hydroxymethyl, olefinic and proton adjacent

to hydroxyl functions), the saponin exhibited a typical molecular-adduct ion $[M + Na]^+$ peak at m/z 1417. Other prominent peaks at m/z 925 and 942 originated as a result of the cleavage of C-28 ester linkage and C-3 glycosidic linkages. Fragment ion at 1374 formed as a result of the loss of 42 ($COCH_3$) suggested that at least one of the hydroxyl function in sugar unit(s) is acetylated.

- Acidic hydrolysis of the sapindus prosapogenin obtained after alkaline hydrolysis of SM-I, furnished three monosaccharide sugars namely arabinose, rhamnose and xylose suggesting that these are located at C-3 position. On the basis of the information generated above, the sapindus saponin (SM-I) was tentatively assigned the structure 3-O-[β-D-xyl(OAc). β-D-arabinopyranosyl. β-D-rhamnopyranosyl] hederagenin 28-O-[β-D-glu. β-D-glu. β-D-arabinopyraosyl) eser.

- Extractions of neem, mahua and karanj seed kernel powder with hexane yielded neem, mahua and karanj oil. Saponificaiton of the oils with methanolic KOH produced corresponding potassium salts of fatty acids. The fatty acid salts on treatment with diluted HCl yielded fatty acids, which on refluxing with methanol under acidic conditions afforded fatty acid methyl esters. The fatty acid methyl esters (FAMEs) were analysed on GC to ascertain the fatty acid composition in the three oils. Fatty acids in neem oil comprised of palmitic acid (19.09%), stearic acid (10.82%), oleic acid (49.0%) and linoleic acid (16.97%). In mahua and karanj oils. Their respective composition has been found to be 31.11, 18.56, 32.74 and 14.01% in mahua oil and 11.80, 6.38, 58.37 and 17.13% in karanj oil respectively.

- Under UV photolytic conditions, fatty acids derived from neem and mahua oil provided better stability to azadirachtin as thin film on neutral glass surface, ($t_{1/2}$ 39.2-39.6 h and FS 1.15-1.17). Similarly *Sapindus mukorossi* saponin provided maximum stability ($t_{1/2}$ 1.21h and FS 1.21) with stabilizing efficiency comparable with TBHQ (FS 1.37). In methanolic solution, karanj oil fatty acids

and their potassium salts were more effective with corresponding stabilizing factor of 1.48 and 1.73. Sapindus saponin with half-life of 1.30h and factor of stabilization of 1.90 was most effective in stabilizing azadirachtin in methanolic solution.

- Under exposure to sunlight, azadirachtin in aza: adjuvant mixures on inert glass surface was best stabilized with madhuca saponin ($t_{1/2}$ 2.25 d and FS 1.68) and with potassium salt of mahua oil fatty acids ($t_{1/2}$ 1.64 d and and FS 1.22). In methanolic solutions, the stabilizing efficiency of both madhuca and sapindus saponin was at par with half-life in the range of 16.0-16.7 d and FS in the range of 1.27-1.33, respectively.

- Under accelerated storage (54±10C) conditions, the stabilizing efficiency of mahua oil fatty acids in stabilizing azadirachtin solution in methanol was maximum ($t_{1/2}$ 266.5 h and FS 1.69) followed by madhuca saponin ($t_{1/2}$ 247.5 h and FS 1.57). In emulsified water azadirachtin degradation was faster, and was controlled to some extent by madhuca saponin and neem fatty acids with stabilizing factor of 1.36 and 1.58 respectively.

- Under accelerated storage conditions (54±1^{0}C) Azadirachtin powder concentrate in solid phase was considerably stabilized with madhuca saponins. It increased the half-life of azadirachtin from 25.3 to 61.9 d with stabilizing factor of 2.45 after 14 days.

- Madhuca and sapindus saponins, their sapogenins and acetylated derivatives did not exhibit considerable antifeedant activity (AI50 1.18-1.57%) against *Spodoptera litura* larvae. The partially hydrolyzed pro-sapogenins, however, were more. Pro-sapogenins obtained after alkaline hydrolysis of madhuca saponin was ten times more active (AI_{50} 0.12%) than the saponins against *Spodoptera litura.* The mixture of potassium salts of both neem and mahua oil

were the most active exhibiting antifeeding index of 0.14% against *Spodoptera litura* larvae.

- Antifeedant activity of azadirachtin (20%) (AI_{50} 0.036%) was considerably enhanced when it was mixed with potassium salts of fatty acids and madhuca saponin concentrate. The mixture exhibited antifeeding index of 0.015 and 0.029%, respectively. Thus aza+potassium salt of mahua oil fatty acid combination (3:1) was 2.4 times more active than azadirachtin concentrate (20%) and 1.4 times more active than Econeem, a commercial product.

- Prosapogenin formed as a result of the partial hydrolysis of sapindus saponin exhibited increased IGR activity *against* S. litura larvae. Madhuca prosapogenin was more active than the one derived from *S. mukorossi*. The most active madhuca prosapogenin exhibited GI_{50} of 0.152%. It was six times more active than the precursor saponin showing GI_{50} of 0.916%. Similarly, potassium salts of mahua fatty acids were moderately active and showed GI_{50} of 0.36 %.

- As evident from the isobolographic analysis, all test saponins and potassium salts of fatty acids potentiated the IGR activity of azadirachtin. They exhibited the potency ratio of less than one which is indicative of their strong potentiating/synergistic effect The increase was more pronounced with potassium salts of fatty acids (GI_{50} 0.042-0.043%) followed by madhuca and sapindus saponin with GI_{50} value of 0.055 and 0.061% respectively. The potentiating effect of madhuca fatty acid salts (potency ratio of 0.44) was comparable with neem fatty acid salts (potency ratio of 0.48).

- Saponins, their derivatives and phytoalkanoates exhibit significant antifungal activity. Among the various test compounds sapindus saponin (ED_{50}, 181.40 ppm) was more active than madhuca saponin (ED_{50} 229.9ppm) against the test fungus *R. bataticola*. The saponin esters were more active than saponins and the antifungal activity increased with increase in acid chain length. Among the

various esters, pentanoic esters of madhuca and sapindus saponins were more active with corresponding ED_{50} of 203.4 and 192.1 ppm. The fatty acids of neem and mahua oil were also active and showed same level of antifungal activity with ED_{50} in the range of 203.5 and 247.5 ppm.

- The antifungal activity of azadirachtin concentrate (20%) against *R. bataticola* (ED_{50} 145.5 ppm) was considerably enhanced when combined with *M. indica* and *S. mukorossi* saponins in 3:1 proportion. The combinations recorded ED_{50} in the range of 102.2-116.6 ppm against *R. bataticola,* 150.3-163.9 ppm against *R. solani,* and 129.6-294.1 ppm *S. rolfsii* respectively. As evidenced from isobolographic/fractional analysis, sapindus saponins potentiated the effect of azadirachtin to a greater extent (potency ratio <1). Enhanced antifungal activity of aza: sapindus saponin combinations can be attributed to the saponin induced increased permeability of fungal membrane facilitating penetration of the toxicant (azadirachtin) inside the fungal hyphae.

- Azadirachtin concentrate (20%) exhibited moderate nematicidal activity against the root knot nematode, *Meloidogyne incognita* (LC_{50} 215.7-253.2 ppm) and reniform nematode, *Rotylenchulus reniformis* (LC_{50} 236.1-285.2 ppm). In combination with the test adjuvants the activity was considerably enhanced. The most active combination comprising of aza + salanin-nimbin-desacetylnimbin (1:1) showed LC_{50} 70.9-82.0 ppm against the root knot nematode and 82.11-97.0 ppm against reniform nematode. Aza + potassium salts of mahua fatty acids combination was next in order with LC_{50} in the range of 133.3-160.3 ppm against *M. incognita* and 130.7-177.9 ppm against *R. reniformis.* As evident from the isobolographic fractional anlysis, the most active saponins, neem meliacins and phyto-oil derivatives potentiated the nematicidal effect.

- The selected test adjuvants were evaluated for their possible plant growth regulatory activity on rice and maize seedlings. At optimum test concentration

of 500 ppm, the most effective madhuca saponins recorded 39.2% increase in coleoptile growth and a marginal 5.57% decrease in root growth of rice seedlings. A 250 ppm dose level of sapindus saponin was found ideal for promoting maize seedling growth in terms of increase in coleoptile and root growth.

- From these studies it is evident that madhuca and sapindus saponin, salanin-nimbin-desacetylnimbin combine, as well as potassium salts of neem and mahua oil fatty acids can be used as potential pesticidal adjuvants for enhancing the stability and potentiating the activity, particularly nematicidal and antifungal activity of azadirachtin-based neem biopesticides.

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APPENDIX

[^{1}H-NMR AND Mass Spectra]

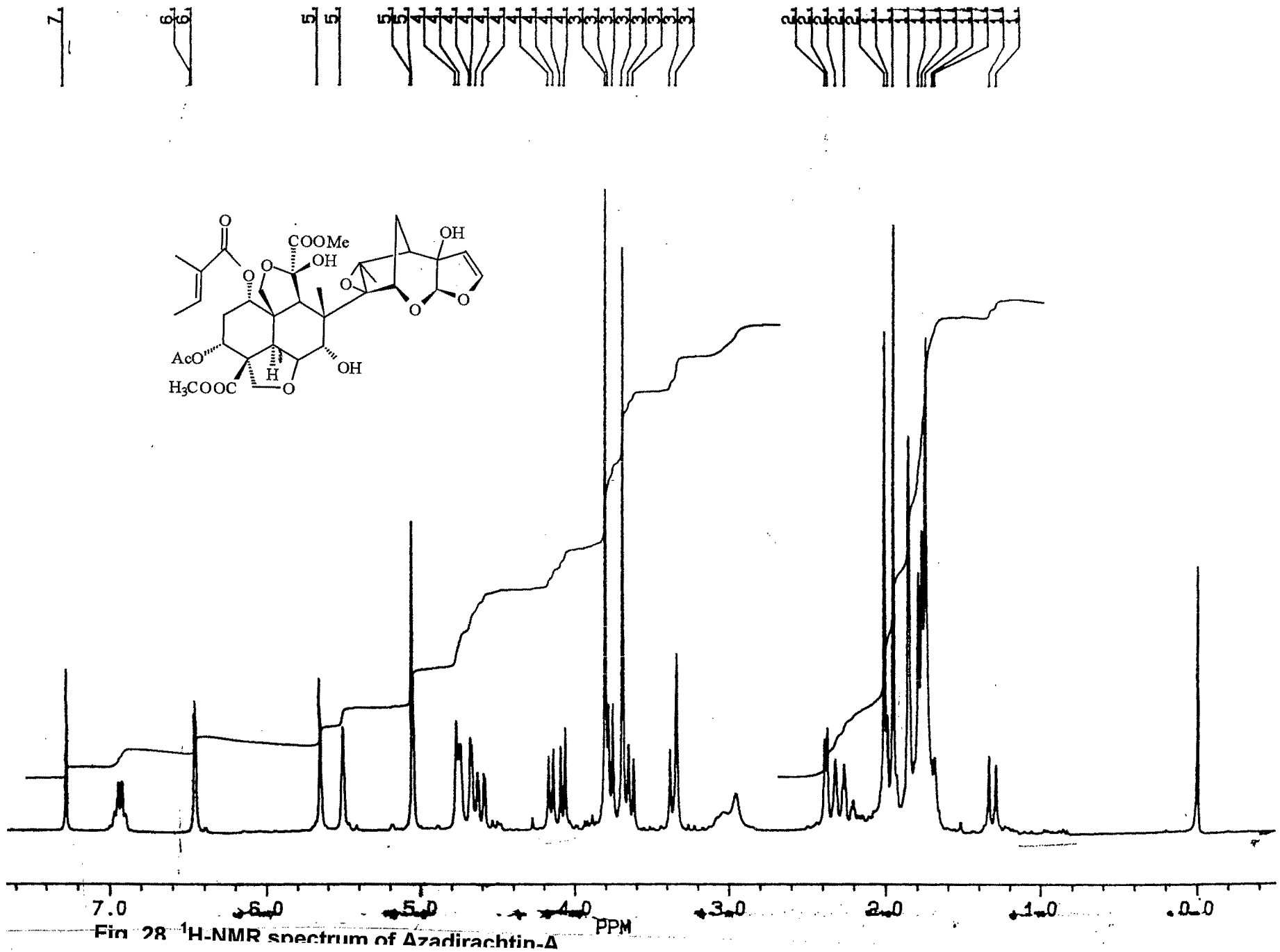

Fig. 28 ^{1}H-NMR spectrum of Azadirachtin-A

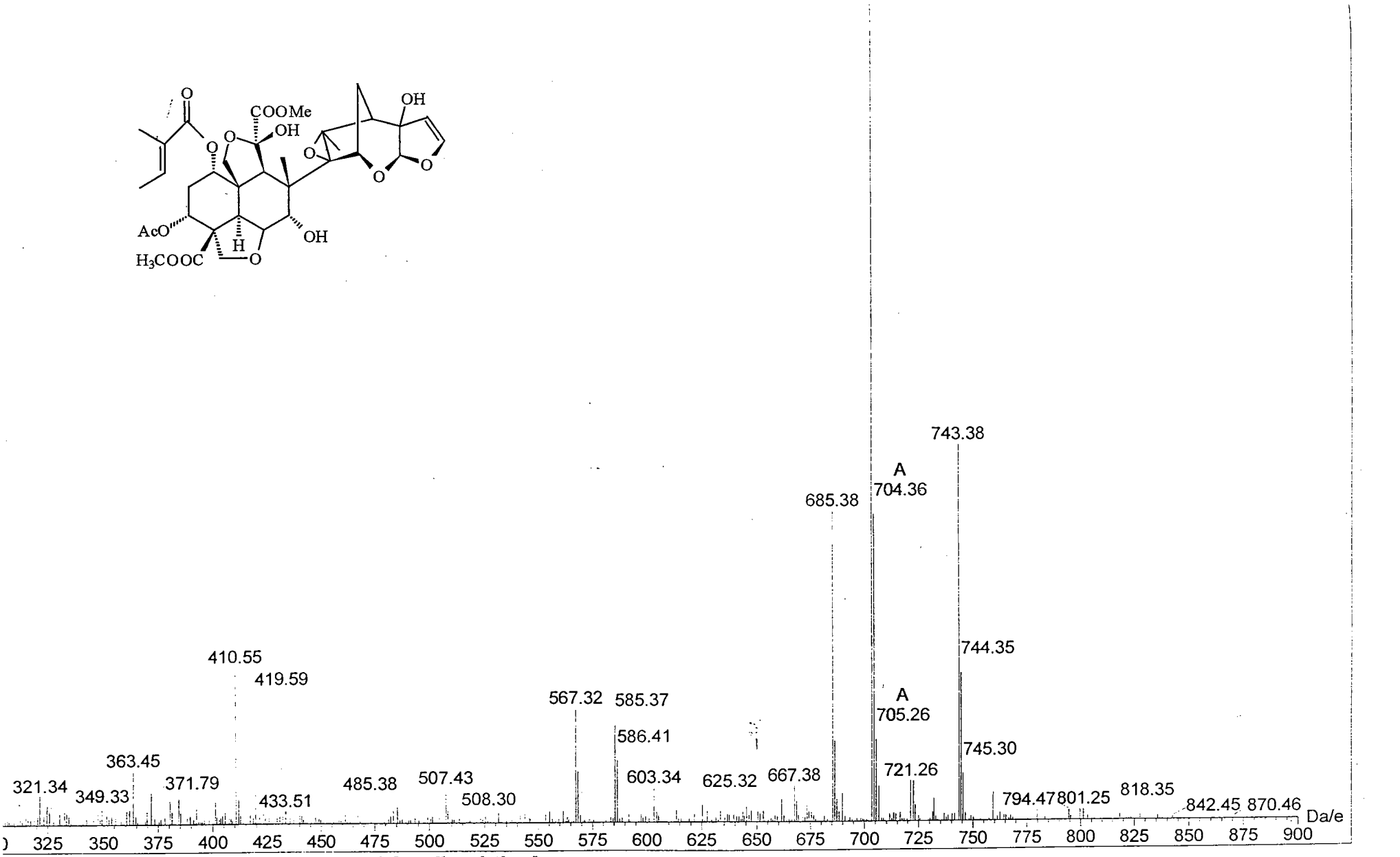

Fig. 29. ES-MS spectrum of Azadirachtin-A

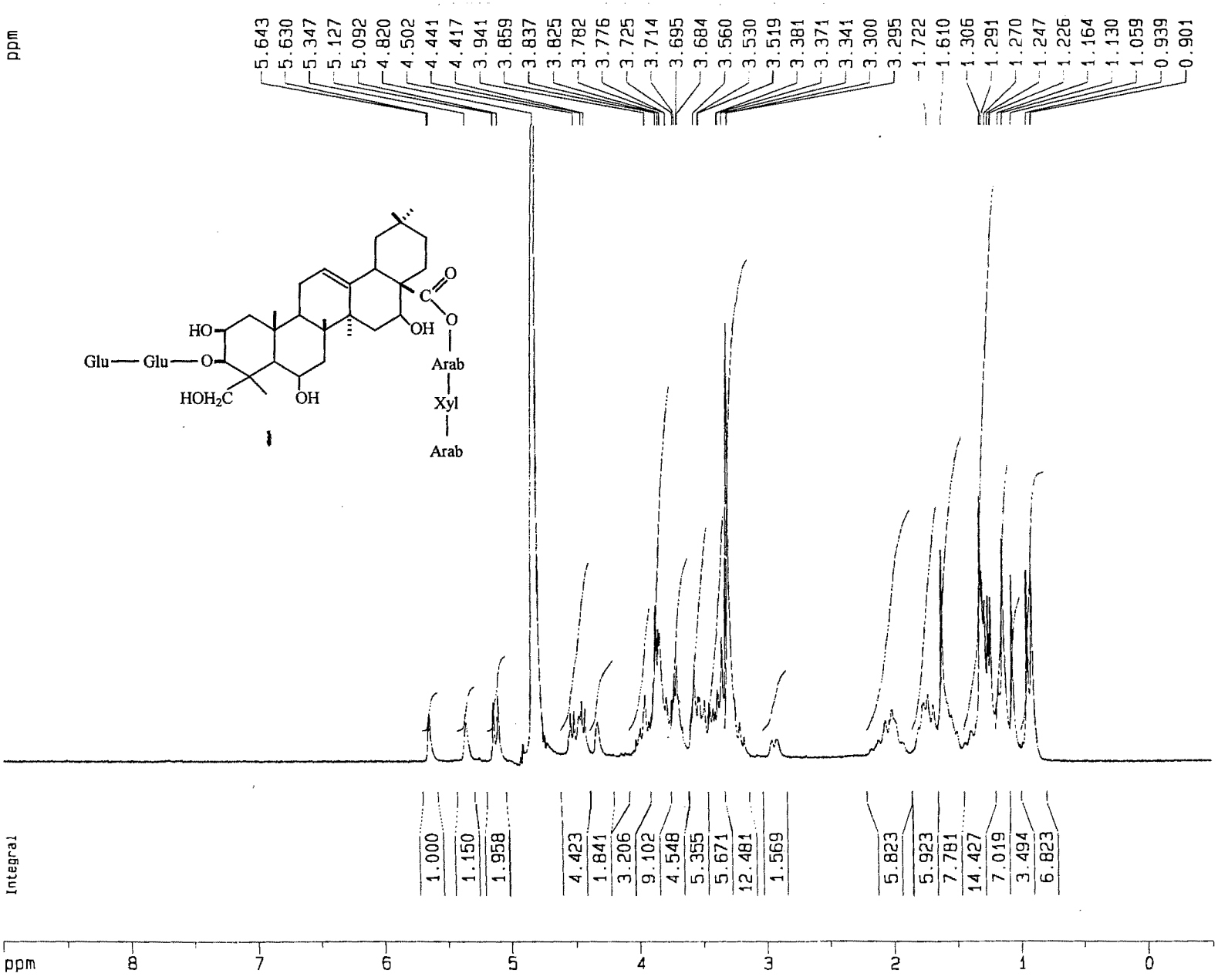

Fig. 30. ^{1}H-NMR spectrum of MI-I

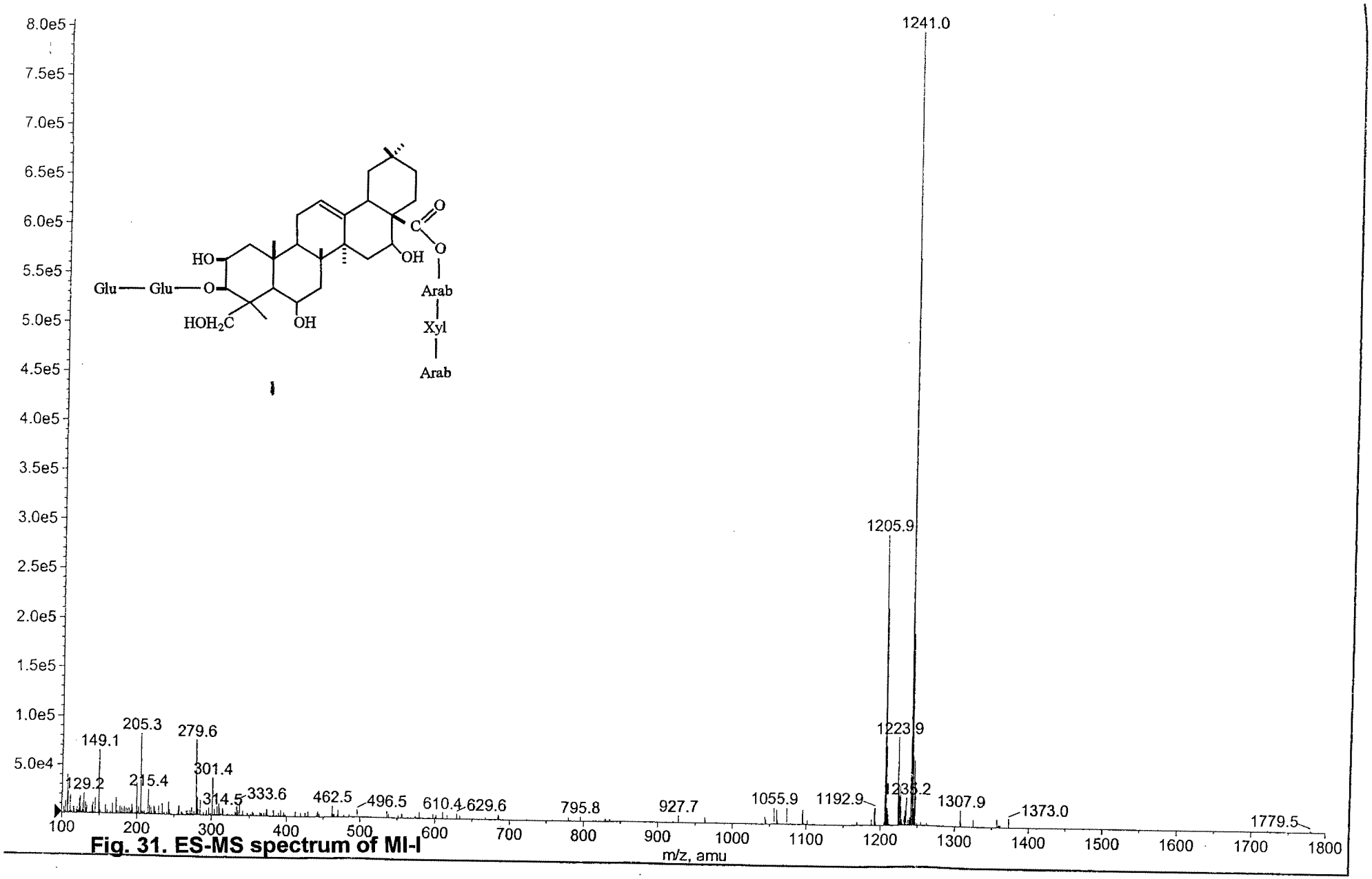

Fig. 31. ES-MS spectrum of MI-I

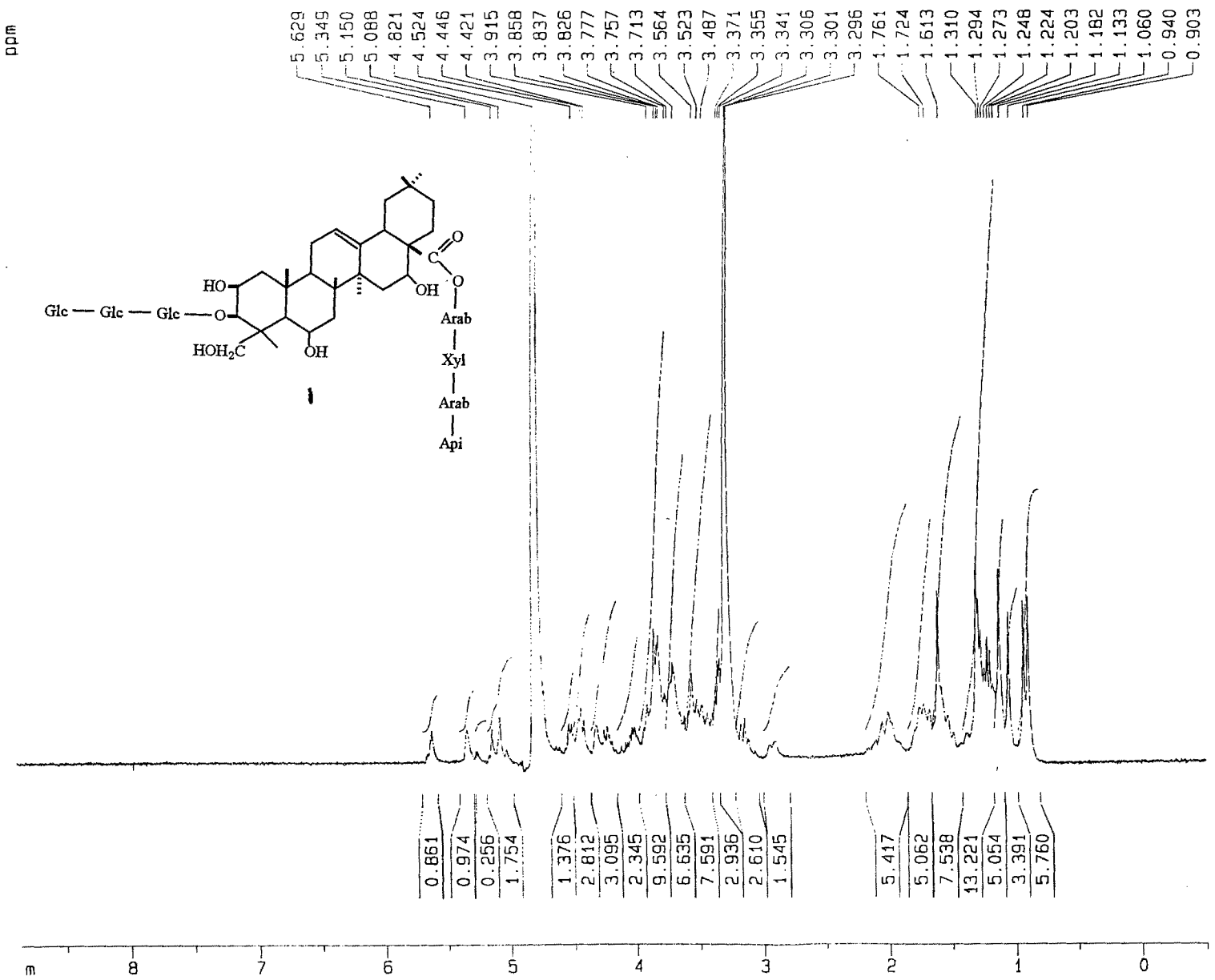

Fig. 32. ^{1}H-NMR spectrum of MI-III

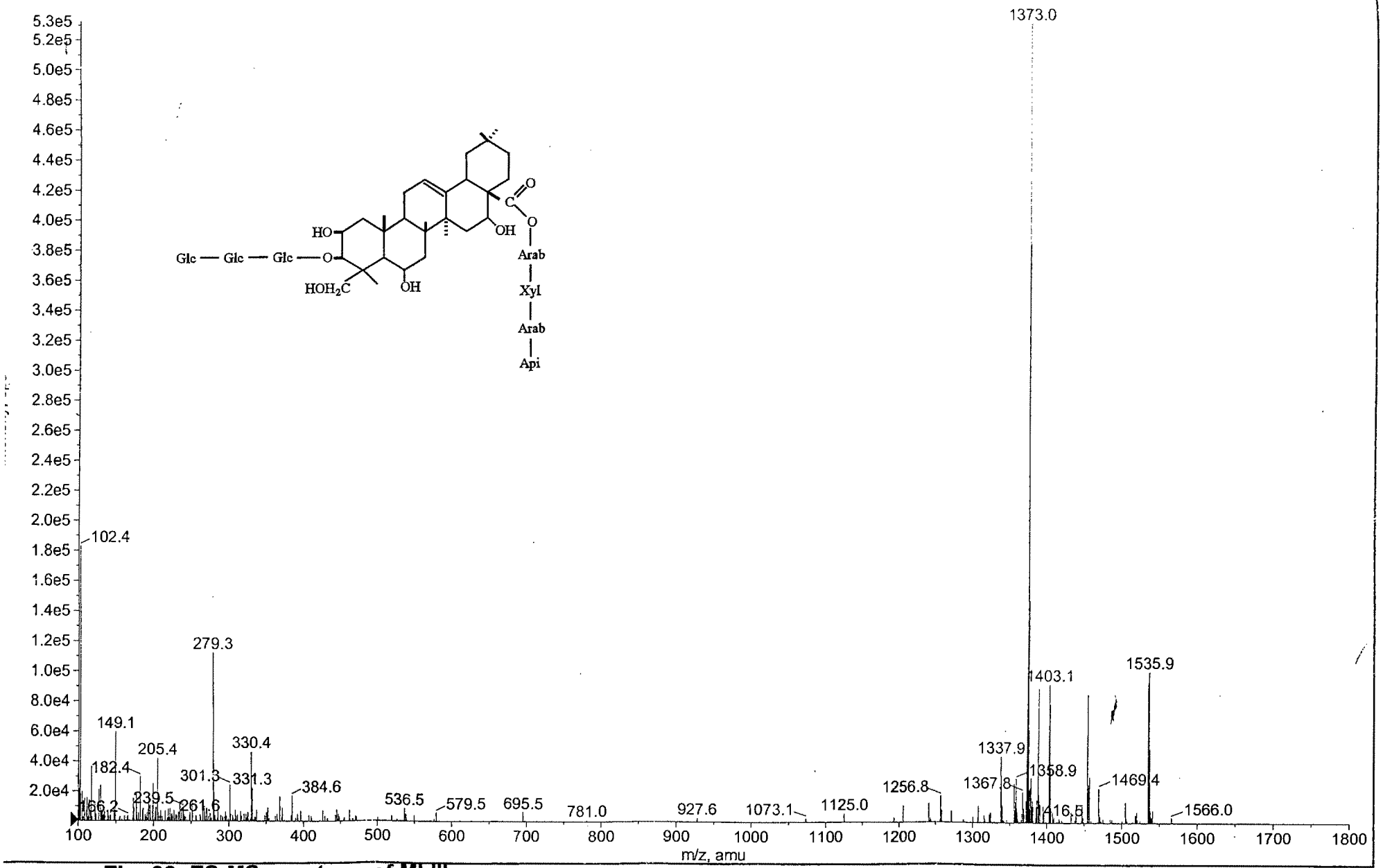

Fig. 33. ES-MS spectrum of MI-III

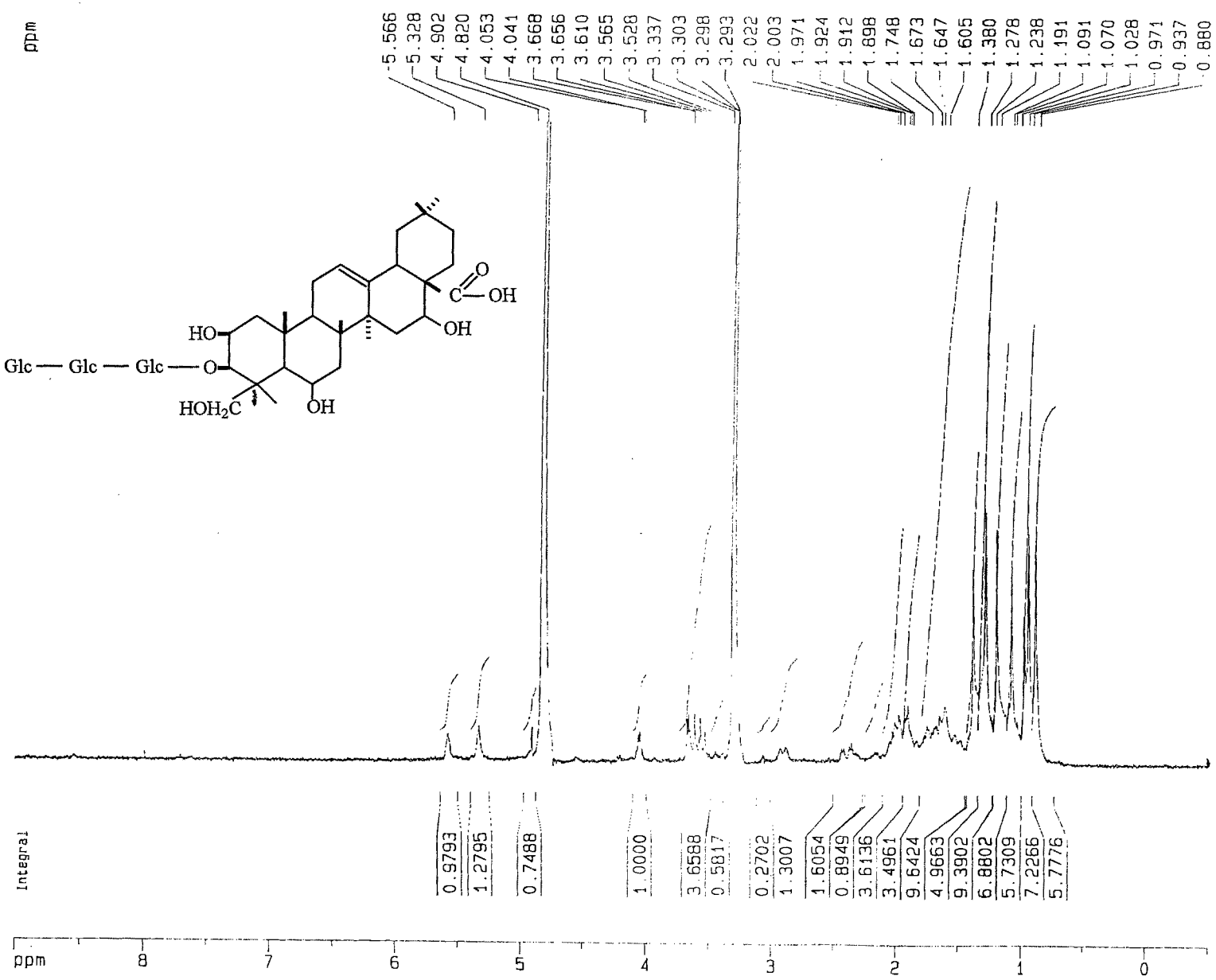

Fig. 34. ^{1}H-NMR spectrum of *M. indica* prosapogenin

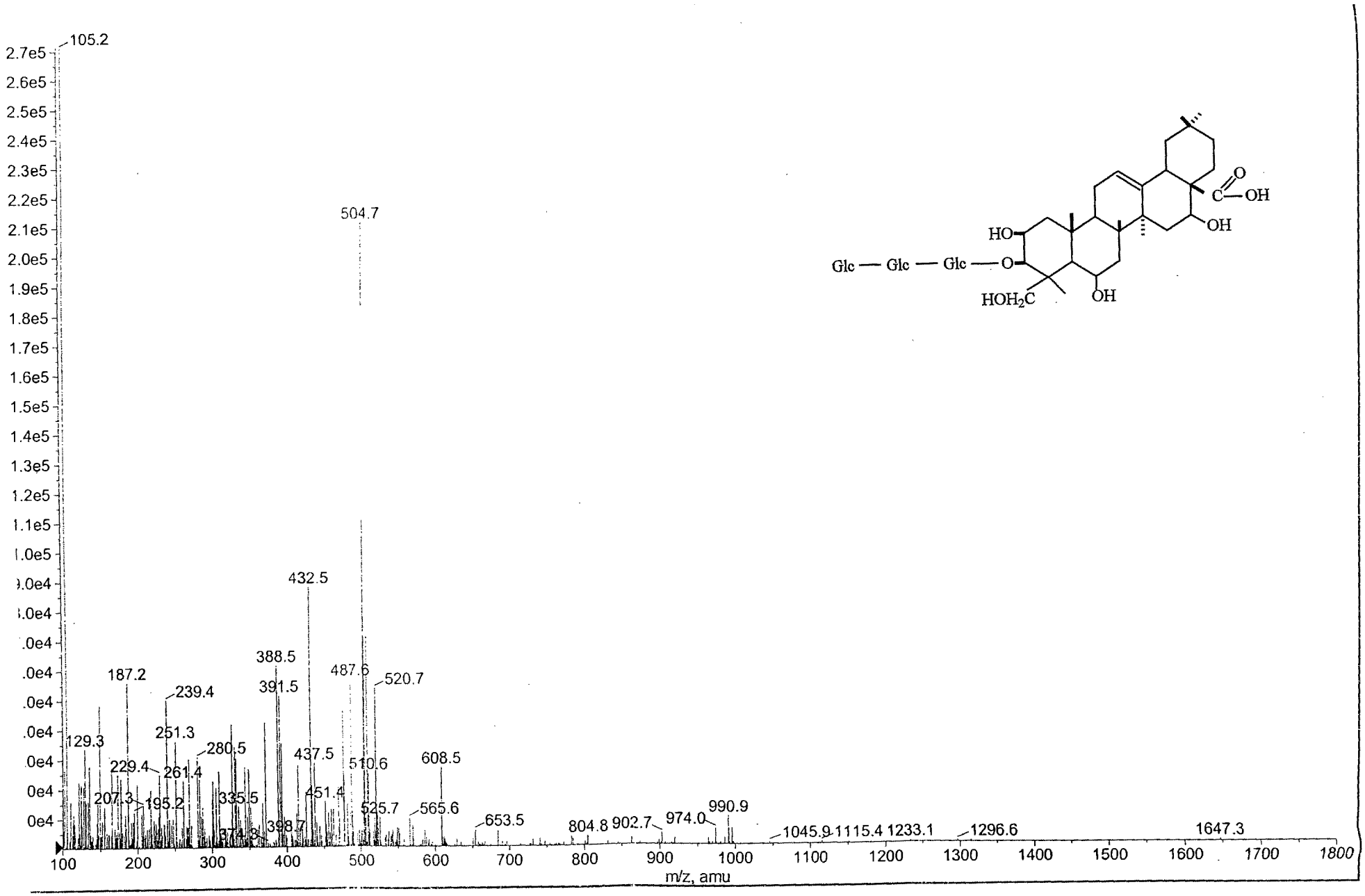

Fig. 35. ES-MS spectrum of *M. indica* prosapogenin

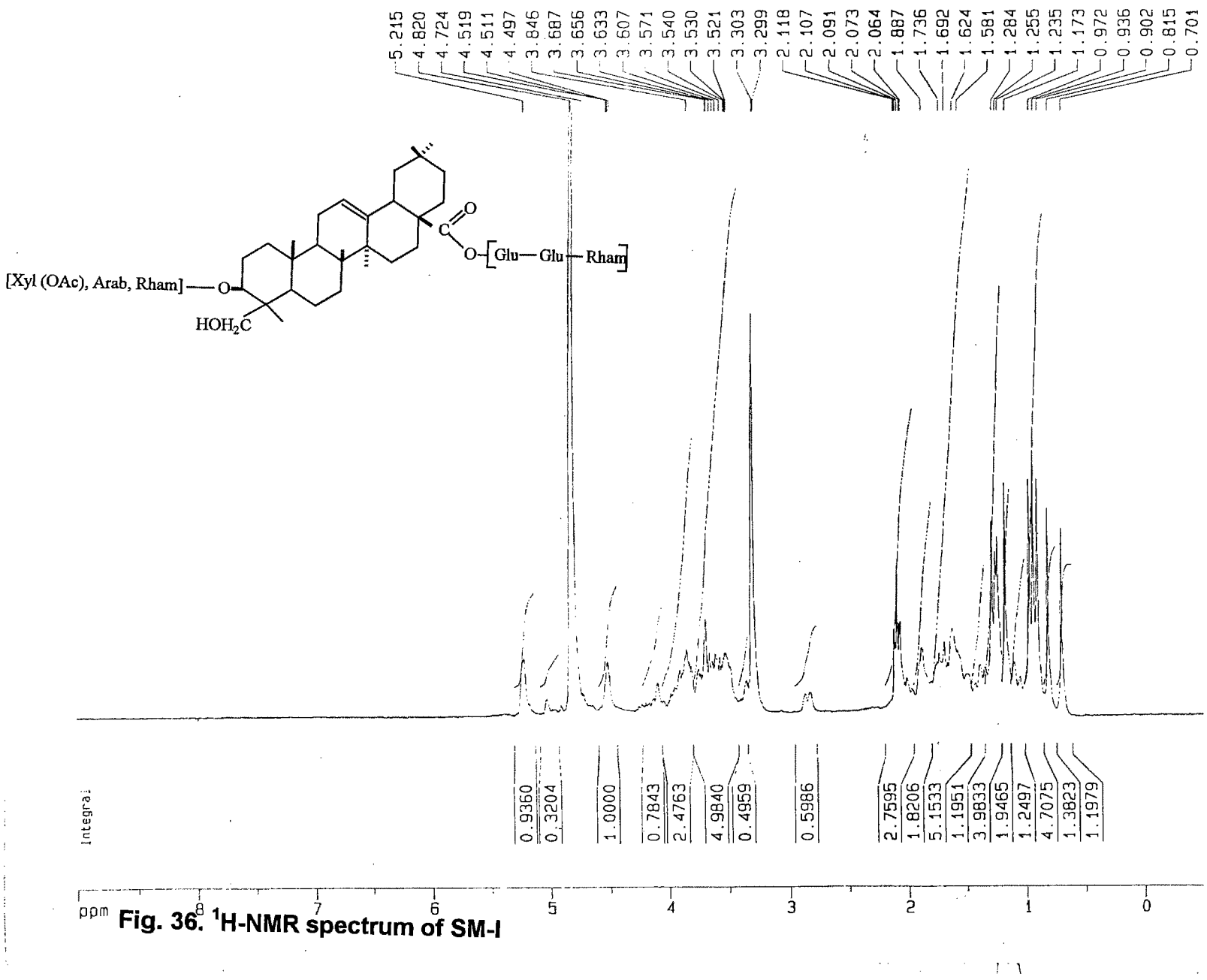

Fig. 36. ^{1}H-NMR spectrum of SM-I

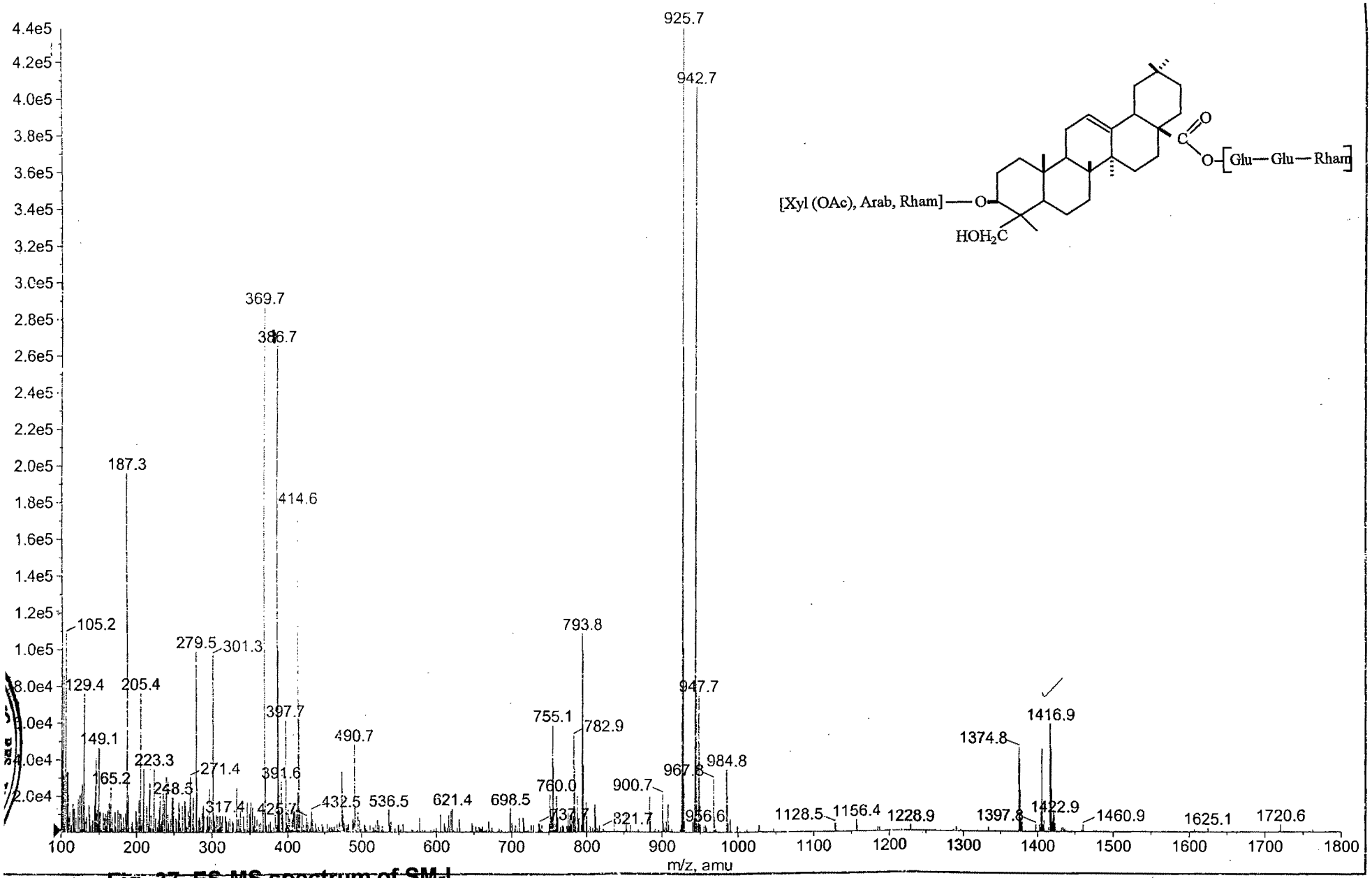

Fig. 37. ES-MS spectrum of SM-I