# CHAPTER: II

# ISOLATION & ESTIMATION OF AZADIRACHTIN-A

## II.1 ABSTRACT

There has been an upsurge of activity in the application of bio-pesticides in recent years, due to the increasing awareness for safe and green environment.

As already known, azadirachtin-A (Aza-A, $C_{35}H_{44}O_{16}$) **10** (Figure:I.2), a naturally occurring bioactive compound isolated from seed kernels of neem (*Azadirachta indica* A.Juss) is shown to be potent insect antifeedant, growth inhibitor and exhibits many other biological activities.

The present chapter describes, a simplified convenient and economical procedure for the extraction of azadirachtin from neem seed kernels. An improved reverse phase high performance liquid chromatography (HPLC) procedure has also been described to estimate Aza-A content in crude methanolic extracts of seed kernels. It has been shown earlier through several HPLC-UV determinations that Aza-A was always obtained as a distinct peak from other components. In view of this, we have developed a preparative HPLC method for purification of Aza-A up to 91 % from a crude extract containing about 9.14 % Aza-A obtained from seed kernels. The structure of Aza-A was confirmed by PMR, $^{13}$CMR, IR, UV and comparison with other analytical data.

In continuation with this, a short and economical procedure for its preliminary purification by using quick and inexpensive flash column chromatography coupled with preparative HPLC is also reported herein.

Two consecutive flash chromatography steps, thus quickly gave Aza-A residue (65-70 %) from a crude extract obtained from seed kernels. Aza-A pre-purified as above has been further purified by reverse phase preparative HPLC up to 91 % purity.

We also present, herein, our results of Soxhlet extraction of Aza-A from defatted neem seed cake using methanol, as a function of time, which is found to be more efficient than the conventional extraction procedure.

## II.2 INTRODUCTION

### Extraction Technologies

Extraction methods are as important as analytical methods in the plant chemistry. The method to be adopted for the extraction of a compound or a group of compounds should ensure that complete extraction of the desired compound takes place without decomposition, isomerisation or polymerisation.[1] An ideal solvent dissolves only desired compounds, leaving other plant material as such which may contaminate the extract. The basic principle "like dissolves like" is applied, e.g., for extracting lipidic compounds non-polar solvents are used and for extracting polar compounds polar solvents are used.[1]

The extraction technologies for the processing of desired compounds from plant biomass can be broadly classified into two main groups.[2]

**(1) Primary Extraction Technologies**

**(2) Secondary Extraction Technologies**

**Primary Extraction Technologies:** Primary extraction technology is the first step of the extraction of different compounds from the plant biomass.[2]

The primary extraction technologies are of the following types.[2,3]

- Ghani Extraction
- Expeller
- Steam Distillation

- Solvent Extraction
- Microwave-assisted Extraction
- Phytonic Extraction
- Supercritical fluid Extraction

- Ghani extraction

The original animal powered ghani, which is generally used in rural areas, consists of a wooden mortar and pestle. The mortar is fixed to the ground, while the pestle attached to a pair of animals is rotated in the mortar. The generated pressure crushes the seeds, the oil moves out through an opening at the bottom and the cake is scooped out from the top of the mortar.[3]

- Expeller

The expellers are of two types. One is to obtain maximum oil yield from the oil seeds. This is achieved by a single pressing through a screw press at high pressure. The second type is to press the seed to obtain a cake of moderate oil content.[3] The cake is then further processed, in a solvent extraction plant to recover the remaining oil from the cake.

- Steam Distillation

Steam distillation is the major primary technology by which essential oils from aromatic plants are being produced in the world today.[4] The process is carried out by loading the plant raw material in a distillation tank and passing steam at suitable pressure and flow rate through it, which volatilizes the essential oil. The mixture of essential oil vapors and steam is condensed and essential oil is separated from the condensed water by an oil separator.[2]

- Solvent Extraction

Solvent extraction is the primary technique for the production of total extracts of medicinal plants.[2] The choice of solvent is guided by the solubility of the desired constituents present in the plant material. Solvent extraction involves the partition or distribution of a solute between two immiscible liquids in contact with each other.[1] It is divided into cold and hot extraction methods.

Cold extraction: Cold extraction method is used for the thermally labile compounds like volatile oil, alkaloids etc.[1] In this process, the finely powdered material is kept in a solvent with occasional or continuous stirring for long time ranging from 30 minutes to 72 hours. The solvent is then filtered and another aliquot of fresh solvent extracts the residue further. The material may be extracted repeatedly till the desired compounds are extracted. The extracts are pooled and distilled in vacuum.[1]

Hot extraction: Hot extraction is more efficient, where the plant material is boiled in a solvent for a stipulated period. Soxhlet extraction method is the ideal choice for the hot extraction, where minimum solvent is required for complete extraction of the desired compound.[1]

Solvent extraction method is further classified as

(a) Batch extraction (b) Counter current extraction

(c) Extractions of solids (Soxhlet extraction)

(a) Batch extraction

This is the most common type of extraction technique in which organic solvent is added to the solution to be extracted in a separating funnel. After agitation for sufficient length of time, the

two layers are allowed to separate and the extracted layer is taken out.[5]

(b) Counter Current extraction

In a counter current extraction, two immiscible solvents come in contact with each other as they flow through one another in opposite directions. Such extraction procedures have been found to be very efficient because fresh extractant is brought into contact with the solute-depleted phase and the solute-enriched extractant is brought into contact with fresh aqueous solution.[4]

(c) Extraction of Solids

In this procedure, the solid is covered with the liquid extractant and after agitation for a sufficient period, the liquid and solid are separated by decantation or filtration.[5]

Continuous extraction procedures are used commonly for the extraction of solids. In this procedure, small volume of extracting solvent is used. By means of a specialized apparatus, the solution of extracting solvent and solute is continuously separated into a boiling flask from the mixture being extracted. The solution is subjected to continuous distillation and the condensed distillate returned as fresh extracting solvent to the extraction vessel and reused.[6] During the process, the concentration of the material under extraction, gradually increases in the boiling flask.

For separation of the components of a solid mixture by continuous solid-liquid extraction, a Soxhlet extraction apparatus (Figure: II.1) is convenient.

The solid is placed in a porous thimble in the chamber and the extracting solvent in the boiling flask below. The solvent is heated at reflux temperature, and the distillate as it drops from the condenser,

collects in the chamber. By coming in contact with the solid in the thimble the liquid effects the extraction.

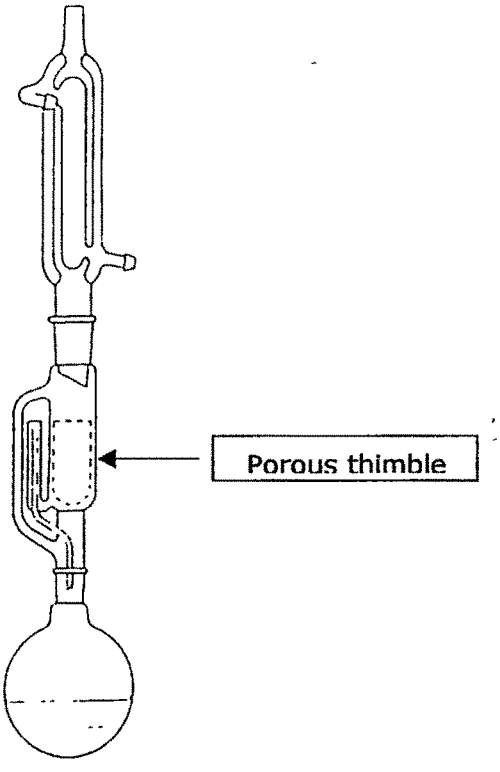



Figure :II.1 Soxhlet extraction apparatus

After the chamber fills to the level of the upper reach of the siphon arm, the solution empties from the chamber into boiling flask by siphoning action.[6] This process may be continued automatically and without attendance for as long as is necessary for effective removal of the desired component, which will then be contained in the solvent in the boiling flask.[6]

- Microwave-assisted Extraction

This is a new concept in extraction of natural products which has been introduced by J. R. Pare *et al.*[7] The principle of this process is

that, when a plant material immersed in a solvent is exposed to microwave heating the organic constituents in it get dispersed and dissolved in the solvent used.[2,7c]

- Phytonic Extraction

This is a patented process from UK, which makes use of the new CFC-free refrigerant 134-A (a hydroflurocarbon) at a high pressure of 5.6 kg/cm$^2$ for the extraction of large range of plant products. It has been used for extracting essential oils, flower absolute and spice oleoresins.[8]

- Supercritical Fluid Extraction

This is one of the most sophisticated and novel extraction method used to isolate the desired compounds from the plant materials.[9] In this process, extraction is carried out by using mineral carbon dioxide ($CO_2$) instead of organic solvents at critical pressure and critical temperature, yielding liquid and gas properties in a single phase.[9]

**Secondary Extraction Technologies**: Secondary extraction technologies are concerned with the processes required to further purify the primary extracts into desired constituents or products. The isolation of the natural products from the plant sources have several problems, such as compounds may be heat-, light- or moisture-sensitive or present in small quantities. For this purpose a proper selection and good knowledge of the different secondary separation technologies is essential.[2]

- Fractional Distillation

Fractional distillation occupies an important place amongst the technologies for separation of the essential oils into their various

constituents. Essential oils are basically complex mixtures of terpenes, hydrocarbons, etc. For isolation of pure compounds from essential oils, packed fractionating columns are employed due to low-pressure drop and higher separation efficiency. The whole process is carried out under high vacuum in order to prevent heat-sensitive products from decomposition by lowering their boiling points. The packing of the column plays an important role in the separation efficiency of the column.[2,10]

- Recent Developments in Chromatography

Chromatography basically involves separations due to differences in the equilibrium distribution of sample components between two different phases. Separation of complex mixtures arising from natural products can be done by passing it through a column packed with an adsorbent like silica gel, alumina etc. Recently more advanced chromatographic techniques, for instance flash chromatography, low and medium pressure liquid chromatography, counter current chromatography and HPLC have been developed, which are rapid and do not lead to decomposition or material loss.[11]

Flash Chromatography:

Flash chromatography is a preparative air-pressure driven liquid chromatographic technique. It is an improved version of open column chromatography, which results in much faster column operation by creating a positive pressure on the top of column.[12] The apparatus required for this technique consists of a set of chromatography columns and a flow controller valve. The column is a flattened bottom tube fitted with a Teflon stopcock. The flow controller valve is a simple variable bleed device for precise regulation of the elution

rate and is constructed from a glass/Teflon needle valve.[12] An improved method for the rapid partitioning of natural product extracts known as reverse phase flash chromatography has also been developed.[13] It has been used for the isolation of alkaloids, terpenoids, phenolic acids etc., from plant source.

Low Pressure Liquid Chromatography (LPLC)

Low-pressure liquid chromatography is employed for relatively large packings having particle size of 40-60 μm, which enables high flow rates at pressures from 5-10 bar. This type of technique provides a simple and versatile means of isolating substances up to gm scale and is used for the isolation of flavanoids, alkaloids and glycosides from the natural plant materials.[2, 14]

Medium Pressure Liquid Chromatography (MPLC)

This technique makes use of pressure up to 40 bars and easily accommodates much larger sample loads than generally applied for LPLC system. This method uses a finer particle size (15-40 μm) of adsorbent in a closed column and employs a piston pump to create pressures in a the range of 5 to 40 bar to pass the solvents through the adsorbent bed.[15a] MPLC has been used for processing of glycosides from natural plant materials. [15b,c]

Counter Current Chromatography:

Counter current chromatography is a liquid–liquid separation method, which does not require a sorbent. The technique has advantages over liquid chromatography because of the total recovery of introduced sample, no irreversible absorption and low solvent consumption.[11] The HPLC process scale up depend on a solid

adsorbent for achieving separation of natural product. Some chemical constituents like quaternary indole alkaloids and carotenoids have chemical reactions with solid adsorbents and hence HPLC can not be used efficiently as an efficient device for separating these compounds.[2,11,16] Two types of instruments have been developed for the counter current chromatography technique, e.g. droplet counter current chromatography and high speed centrifugal counter current chromatography.[2,17,18]

High Pressure Liquid Chromatography (HPLC):

In a classical column chromatography procedure, the liquid phase passes through the stationary phase under the influence of the gravity resulting in the development of the chromatogram and subsequent separation of the components. This technique is time-consuming and frequently gives poor recovery due to band tailing and sometimes products undergo isomerisation, polymerization or decomposition.[12,19]

The speed and versatility of column chromatography can be greatly improved by pumping the liquid phase through the column at high pressure. This process is called the high pressure column chromatography.[19]

In basic principle of HPLC involves, separations due to differences in the equilibrium distribution of sample components between two different phases one of these phases is a stationary phase column, while the second phase is solvent system or mobile phase. The sample components move through the column, towards the detector, only when they are in the mobile phase. From this it is easy to understand that the velocity of migration of the components is a function of the equilibrium distribution between the mobile phase and

the stationary phase. The components having distribution favoring the stationary phase will move more slowly down the column than those with a distribution favoring the mobile phase. The separation that results is explained on this basis.[20]

This technique has several advantages over other forms of chromatography. Efficiency is much higher than that in TLC. It is used for the non-volatile and high-molecular weight compounds whereas GC is not practically used. Another valuable aspect of HPLC is that, it is normally carried out at room temperature, so that there is no decomposition or molecular rearrangement of the molecules. A major drawback associated with HPLC in residue analysis is detector sensitivity, but this limitation has to a large extent been overcome due to the methods of extraction and cleans up deigned for LC.[19] HPLC is used both analytically as well as preparatively.

➢ Analytical Chromatography

Analytical chromatography can be defined as the separation of a complex mixture on a chromatographic support, in order to determine the composition of the solution. In general, the purpose of analytical chromatography is determining the number of distinct components, the identity of each component and the quantity of each component.[21]

In analytical chromatography, the variables, which affect the separation, can be

- Column packing,
- Column configuration,
- Elution condition

The factors that constitute the elution conditions are the eluent composition, the elution profile (isocratic, linear gradient or step gradient), the flow rate and the temperature. The variables of the

column packing are the separation mode, bonded phase, end capping, pore size, particle size, carbon load and surface area. The variables in the column configuration are the length, internal diameter and material of construction (stainless steel, plastic, polyetherether ketone (PEEK).[21]

Frequently, experimentation is the best method to determine the optimum set of variables. However a theoretical model has been developed, that also helps in developing a separation. In the theoretical model, the factors affecting a separation are divided into chemical factors, known as the selectivity and the capacity as well as physical factors, known as the efficiency or the theoretical plate count.[22-25]

- Preparative Chromatography:

Liquid chromatography (LC) was first developed as a powerful analytical technique, but it is now being increasingly applied on the large scale too. The term preparative LC describes any LC separation, which is used to isolate, enrich or purify one or more compounds of a mixture from the sample material.[26]

Factors Affecting the Preparative Separations:

The factors that govern a preparative separation are very similar to those that influence on analytical separation. Plate count, capacity factor and the selectivity factor will affect their solution. The particle size of the packing material and column length can affect the column efficiency and the speed of the separation.[26]

Altering any one of these parameters can have multiple effects on the separation and knowing which one to alter is important for over all strategy. For example, when isolation needs to be done once only,

relying on a column with a high plate count to drive the resolution can save time. When the separation needs to be done many times, optimizing the selectivity and capacity factors will have a significant impact on the cost, and effectiveness of the separation.

Mobile phases are often manipulated to cause the change in selectivity, however altering the stationary phase can also play a significant role. Using shorter columns with small particle size packings yields the same, column efficiency as longer columns packed with larger particle size packings. The use of shorter, high-resolution columns will typically reduce solvent consumption and result in more concentrated fractions.[27]

Separation Scale up:

Usually a separation that is going to be scale up is first run on analytical column, to optimize the chromatographic conditions. Scale up the separation is then accomplished by adjusting column dimensions, load, flow rate and gradient conditions. Once the separation conditions have been optimized, the load or injection volume on the small column is increased to determine the maximum load that retains adequate resolution or purity.

The equation given below are used to determine the size of the preparative column required for a particular mass or volume of starting material or the amount of a particular size.[28]

$$M_P = M_A \times L_P / L_A \times d_P^2/d_A^2$$

Where M is the maximum mass load, L and d are respective column lengths and diameters. The subscripts P and A refer to preparative and analytical columns, respectively.

Scaling the Flow Rate: To maintain the same retention times when scaling, the flow rates needs to be scaled proportionally. The preparative flow rate can be determined by the following equation.

$$F_P = F_A \times L_P/L_A \times d_P^2/d_A^2$$

Where F is the volumetric flow rate.

Advantages And Disadvantages of Various Extraction Methods:

The quantity and quality of various constituents present in plant materials not only depend on its species, chemo types, soil, climatic conditions, plant part and stage of maturity but also on the conditions like method of extraction, mode of drying, level of crushing, nature of solvent, temperature and pressure of extraction etc. Significant saving in labor cost, energy and more uniform product quality are obtained to be the advantages of stem distillation procedure. However, it is a time consuming technique for extraction, may some times lead to degradation of the thermally labile compounds.[29] Cold solvent extraction is applicable in thermally labile compounds like terpenoids, alkaloids etc, it has simple operation technique and equipments.[30] The main disadvantages of cold solvent extraction is that the residual amount of solvent present in the product, which may be unacceptable for pharmaceutical applications. Additionally, use of high toxic solvent, which can affect the eco-system or increase the pollution and some times low boiling point solvent can cause fire.[30]

The main benefit of extraction of oil by expeller is that it reduce the capital cost, no danger of fire from combustible solvent compared to solvent extraction method, easily operated by less skilled employed. However, the drawbacks are the oil recovery is not complete and high mainted costs.[3]

In phytonic extraction process achieves a more complete and balanced extract composition. Other advantages claimed for the process are environmentally friendly and inert solvents used, less than 20 ppm solvent residue in the extract and almost complete recycle of the solvent in this process, but it is not widely applicable method. [8] The advantages in supercritical fluid technology with $CO_2$ as a solvent for extraction of plant material are no residual toxic solvents involves, no fire and hazard due to solvents, improved selectivity and use of inert and cheaper solvent.[9] It has some intrinsic problems such as process is performed at above room temperature and at tremendous pressure, thus in turn causes the process equipment to be expensive to purchase. Additionally super critical fluid extraction uses mineral $CO_2$ that is discharged into the environment there by causing a net increase in the atmospheric $CO_2$ burden and unbalance the eco-system.[31]

## II.3 BACKGROUND AND OBJECTIVES

Azadirachtin-A was first isolated by Butterworth and Morgan in 1968, from neem seed kernels.[32] Following the discovery of its potent activity as an insect antifeedant, growth regulator in addition to and other biological activities,[33,34] large quantities were needed for structural and biological studies.

The purification of azadirachtin is difficult to accomplish, especially on a preparative scale due to the complexity and similarities in structures of compounds found in the neem seeds.[35,36] Although it is present in quantities ranging from 0.1 to 0.6 % in neem seed kernels,[37] its isolation in pure state is tedious and time consuming involving repeated partitioning between various solvents[32,33,37-42] and variety of chromatographic steps including preparative thin layer

chromatography (TLC),[32,41,43] open column reversed-phase chromatography[40] and preparative HPLC.[38,44] Despite these methods, there is still shortage of pure material available for industrial and academic purposes.

Several research papers have been published describing its different isolation techniques from fresh neem seed kernels. The general technique involves extraction of powdered fresh air-dried kernels with polar solvents, followed by partitioning the total extract between hexane and polar solvents. Thereafter, the hexane insoluble part is chromatographed using column and HPLC to give pure azadirachtin.

Butterworth and Morgan isolated 1.5 g azadirachtin from ethanolic extract of 2 kg of seed kernels, followed by partitioning the extract between light petroleum and aqueous methanol (5:95). The methanolic extract gave a dark brown residue, which was chromatographed using ether-acetone (95:5) mixture as eluent and subjecting the active fractions to preparative TLC. Figure II.2 shows the steps involved in the isolation of azadirachtin by Butterworth and Morgan.[32]

In another method, Zanno *et al* isolated 800 mg of azadirachtin from 300 g of seed kernels. The isolation procedure involves the extraction of seeds with ethanol followed by silica gel chromatography and preparative TLC to yield pure amorphous azadirachtin.[41]

Both the procedures described above involve adsorption chromatography followed by lengthy preparative TLC. Further the final purity of the azadirachtin is not reported in both the methods.

Uebel *et al* obtained 8.7 g of azadirachtin (> 90 % purity) from 48.2 kg of neem seed kernels using reversed-phase column chromatography with HPLC-UV monitoring.[40] The flow diagram of

the isolation procedure developed by Uebel *et al* is shown in Figure II. 3. However, this isolation procedure was dependent upon the repetitive use of a phase-bonded $C_{18}$ open column for reversed-phase liquid chromatography.

Yamasaki's group, isolated 56 mg of 99 % pure azadirachtin from 1 kg of seeds via extraction, repeated partitioning, combination of normal and reversed–phase flash chromatography and a combination of normal phase and reversed-phase preparative HPLC runs, as shown in Figure II.4.[38]

Warthen Jr. *et al* in 1984 reported a reversed phase HPLC procedure, whereby azadirachtin content can be estimated in crude extracts of neem and in dust formulations. An estimation of the azadirachtin content is achieved through the use of an external azadirachtin standard and valley-to-valley integration.[45]

The most cited paper for the isolation of azadirachtin is that by Schroeder and Nakanishi, in which about 5 g of azadirachtin was obtained from 2.0 kg of seed kernels after several steps. The method involves grinding of kernels in hexane prior to extraction with 95 % ethanol. The ethanolic extract is then subjected to partitioning between light petroleum and methanol to remove oils and other non-polar material, followed by partitioning between water and ethyl acetate to remove water-soluble proteins and carbohydrates. Subsequent vacuum liquid chromatography of the residue, flash chromatography and crystallization from $CCl_4$ gave 5 g of azadirachtin of unknown purity.[39]

Figure II. 5 represents the flow diagram for the isolation of azadirachtin from seed kernels by Schroeder and Nakanishi method.

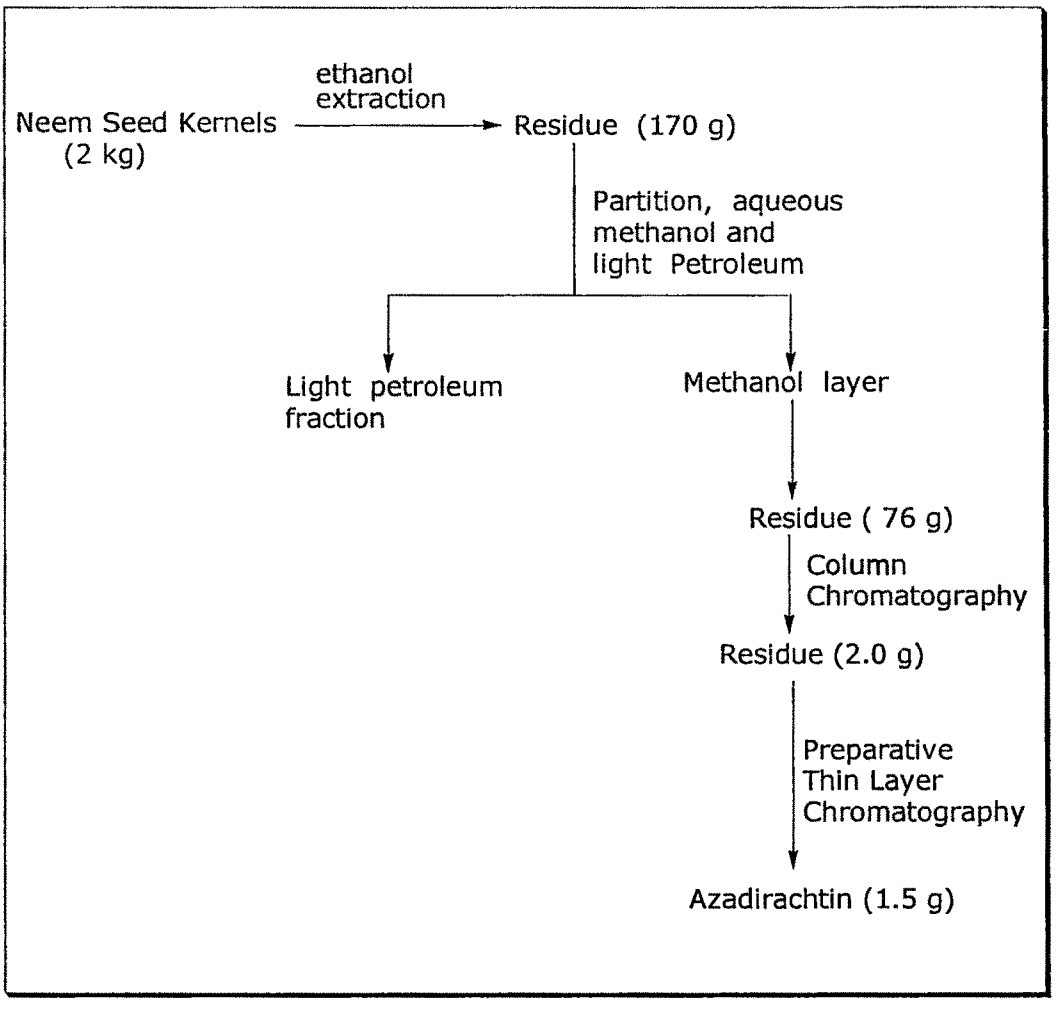



Figure: II.2 Flow diagram of the isolation of azadirachtin from neem seed kernels by Butterworth and Morgan.

A convenient procedure for the isolation of azadirachtin from neem seed kernels extract using preparative HPLC was reported by Govindachari *et al.* The enriched azadirachtin fraction free from water soluble material was subjected to reversed phase preparative HPLC using methanol : water (60: 40) as mobile phase, to resolve

into several peaks, of which those for Aza-H + Aza-I emerged at 16 min, that of Aza-A + Aza-D peak at 25 min and B at 32.8 minutes.

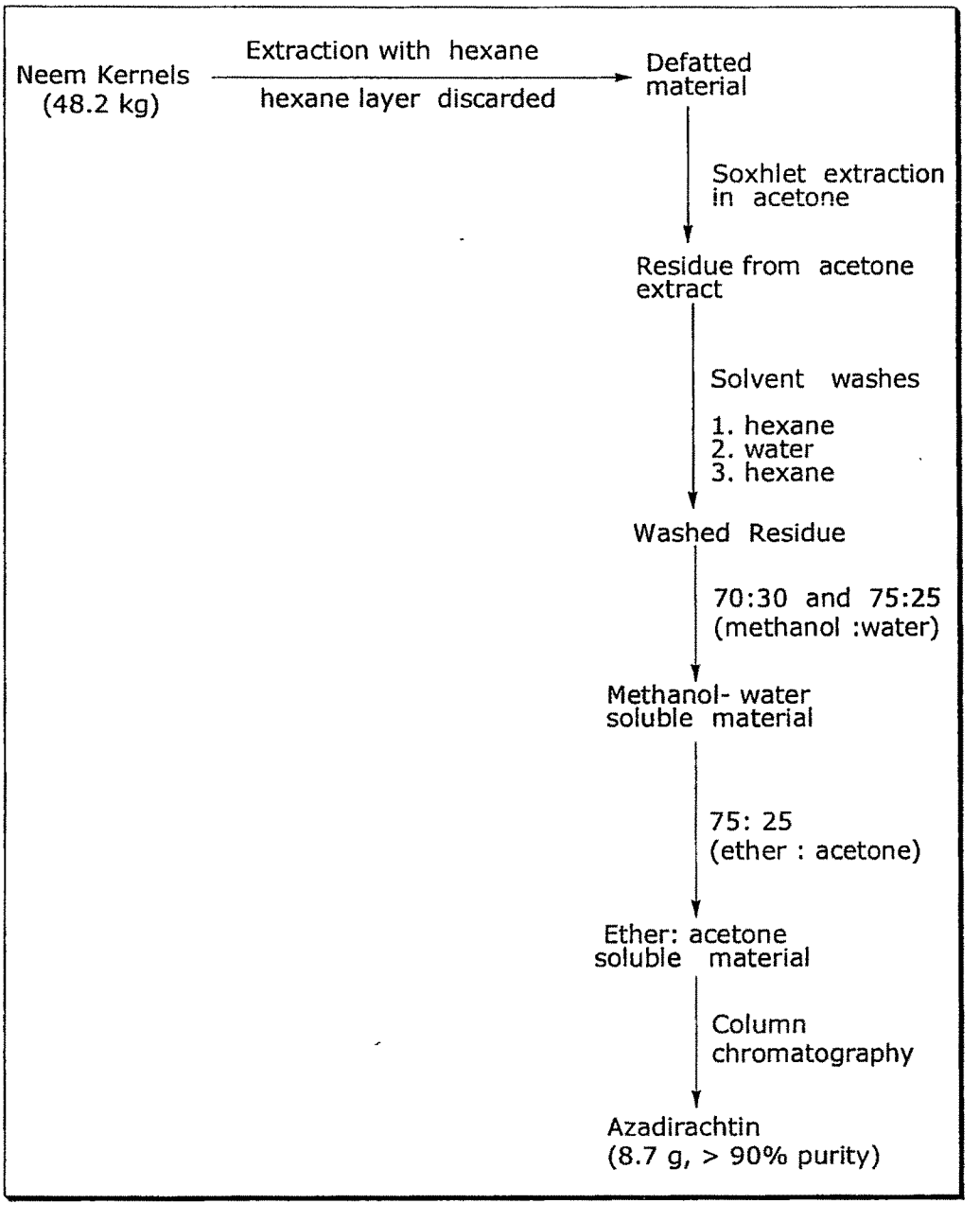



Figure: II.3 Flow diagram of the isolation of azadirachtin from Neem seed kernels by Uebel *et al*

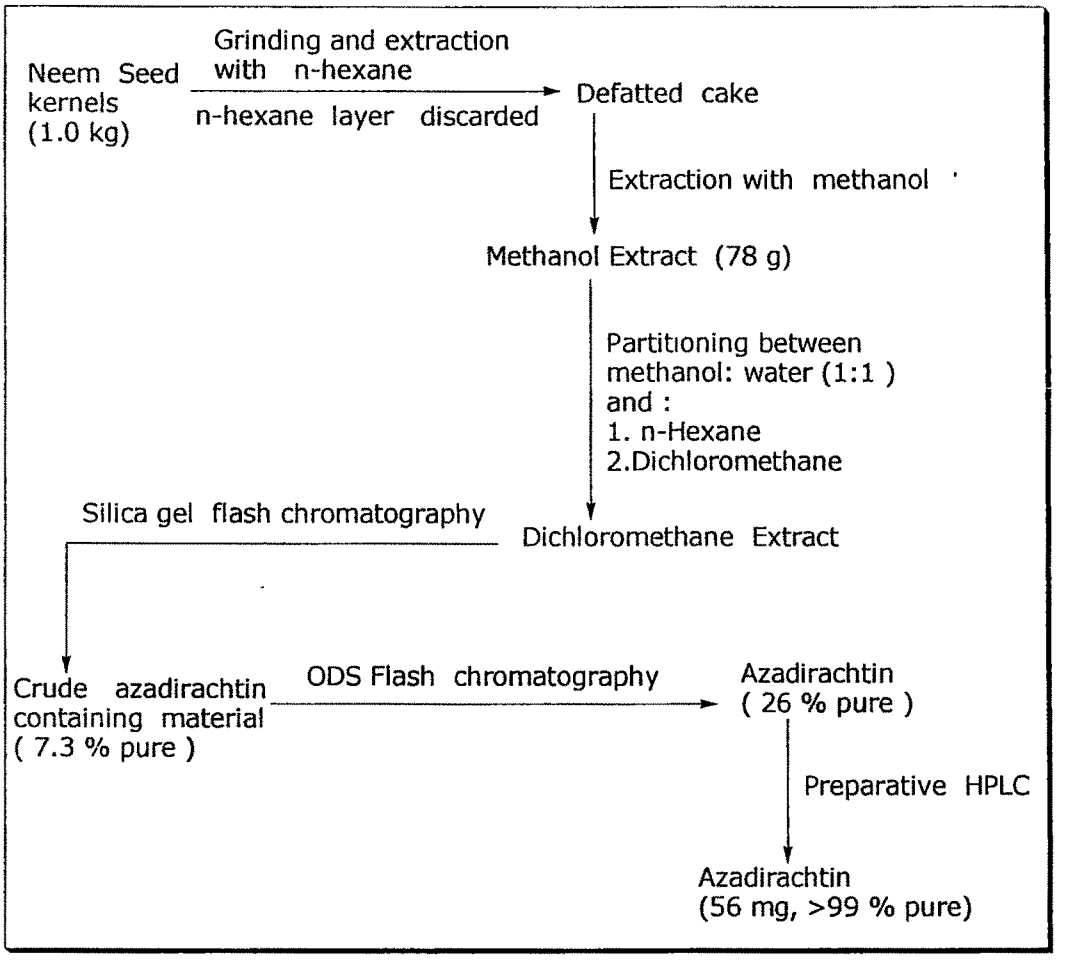



Figure: II.4 Flow diagram of the isolation of azadirachtin from Neem seed kernels by Yamasaki's group

The Aza-A + Aza-D peak was further subjected to second preparative HPLC using acetonitrile : water (28:72) as eluent, whereby 160 mg of Aza-A and 21 mg of Aza-D were isolated.[44a]

In other procedure, the same group has directly injected 500 mg of the ethanolic extract of seed kernels into an ODS reversed phase preparative column and eluted with methanol : water (60: 40) as the mobile phase at a flow rate of 10 mL/min whereby Aza-A was eluted

at 14.4 minute. It is reported that this procedure affords 10-15 mg of pure Aza-A in a single run of about one hour, however, initial and final purity of the azadirachtin samples was not reported.[44b]

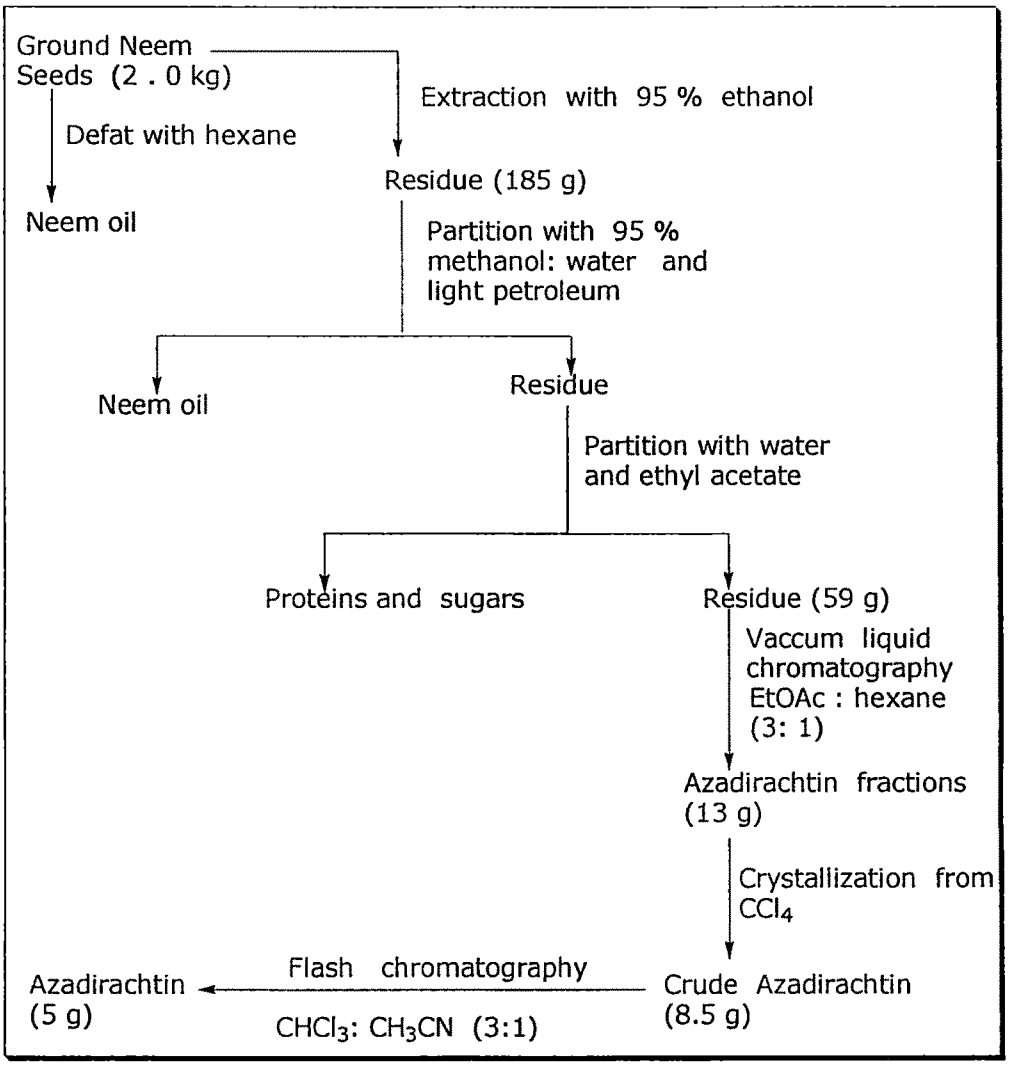



Figure: II.5 Flow diagram for the isolation of azadirachtin from seed kernels of Neem by Schroeder and Nakanishi

The method developed by Sundaram and Curry for isolation of azadirachtin from seed kernels was similar to the method used by Schroeder and Nakanishi, however the partition, column clean up and concentration steps used in the later stages of the procedure were different. In addition, they also reported an improved HPLC procedure to determine azadirachtin content in neem seeds, neem oil and neem based formulations. The steps used in the extraction procedure are given in Figure II. 6. The data showed that 100 g of the kernel from India gave 24.85 mg of azadirachtin whereas the Ethiopian sample gave only 3.10 mg. The percent azadirachtin contents in the two kernel samples were 0.025 % and 0.003 % respectively. The analytical HPLC separation was performed with reversed phase spherisorb $C_{18}$ ODS 5 μm column with acetonitrile: water gradient system at flow rate of 1 mL/min and eluent was monitored with UV detection at 210 nm.[42]

Huang and Morgan reported the quantitative determination of the azadirachtin in the crude extract of neem seeds, conveniently carrying out packed column supercritical fluid chromatography with detection by UV absorption at 210-220 nm and using $CO_2$-methanol as mobile phase.[46] Further Ritieni *et al* applied the supercritical extraction methodology to extract the azadirachtin from neem seed kernels. The neem seed kernels were extracted at 40°C using $CO_2$ in its supercritical fluid state.[47]

Sarojini Sinha *et al* developed a simplified procedure to achieve 50 % enrichment of azadirachtin, using minimum possible steps without resorting to chromatographic techniques.[37] It involves grinding of the seed kernels in hexane and extraction of the defatted material with methanol. The residue from methanolic extract was dissolved in 90 % aqueous methanol and partitioned with hexane, to remove

remaining oil and other non-polar compounds. The methanolic layer was then diluted with water to make it methanol: water (1:1), saturated with sodium chloride and partitioned thrice with different solvents such as ethyl acetate, dichloromethane, toluene, chloroform and carbon tetrachloride. The organic layer was yielded a light yellow to whitish coloured amorphous material containing 25 to 35 % Aza-A. However, this procedure did not mention the exact yield of the material and involved repeated partitioning between different solvent systems. The steps used in extraction procedure are given in Figure II.7.

A number of analytical and preparative methods have been reported in the literature, for the estimation of Aza-A in the neem seeds, oils and its formulations, as well as purification of Aza-A from crude extracts of seed kernels. [37-42,44,45,48-53]

It appeared to us that the analytical methodology for the quantization of azadirachtin was still in developmental stage as compared to established synthetic pesticides. Due to its thermal instability, non-volatility and possible adsorption on a stationary phase, a gas chromatographic method is unsuitable for the analysis of azadirachtin.[42,48] Hence the preferred method for the estimation of azadirachtin is by HPLC using UV detector.

Redfern[50] and Barnby[51] *et al* used HPLC technique to screen the residual amounts of azadirachtin after its exposure to sunlight and UV radiation respectively. Warthen *et al*[45] and Isman *et al*[52] developed HPLC methods for the estimation of azadirachtin in neem extracts and formulations and neem oils respectively. Govindachari *et al*[44] and Yamasaki *et al*[38] have developed the preparative purification method of azadirachtin.

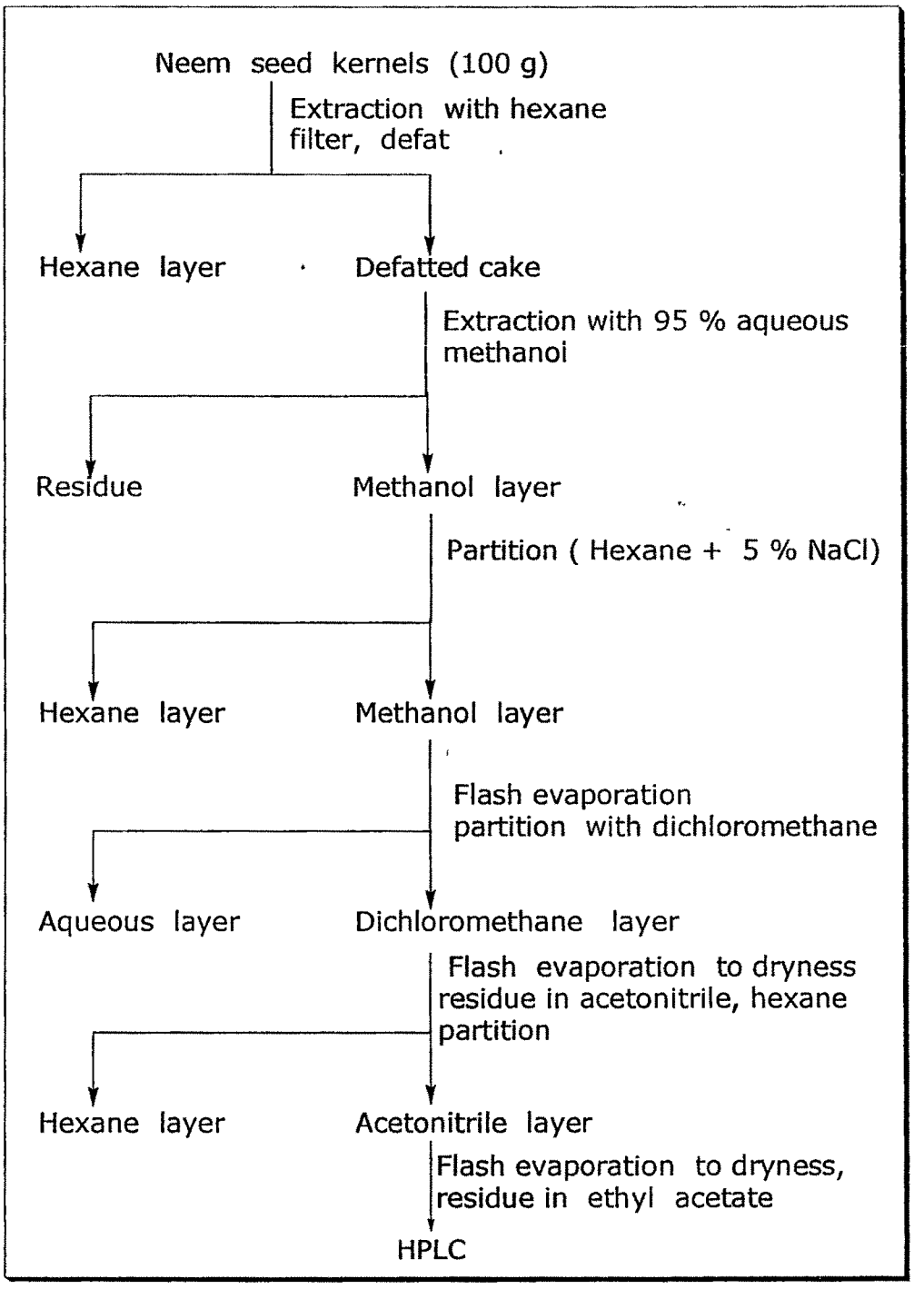



Figure: II.6 Flow diagram for the isolation of azadirachtin from seed kernels of Neem by Sundaram and Curry

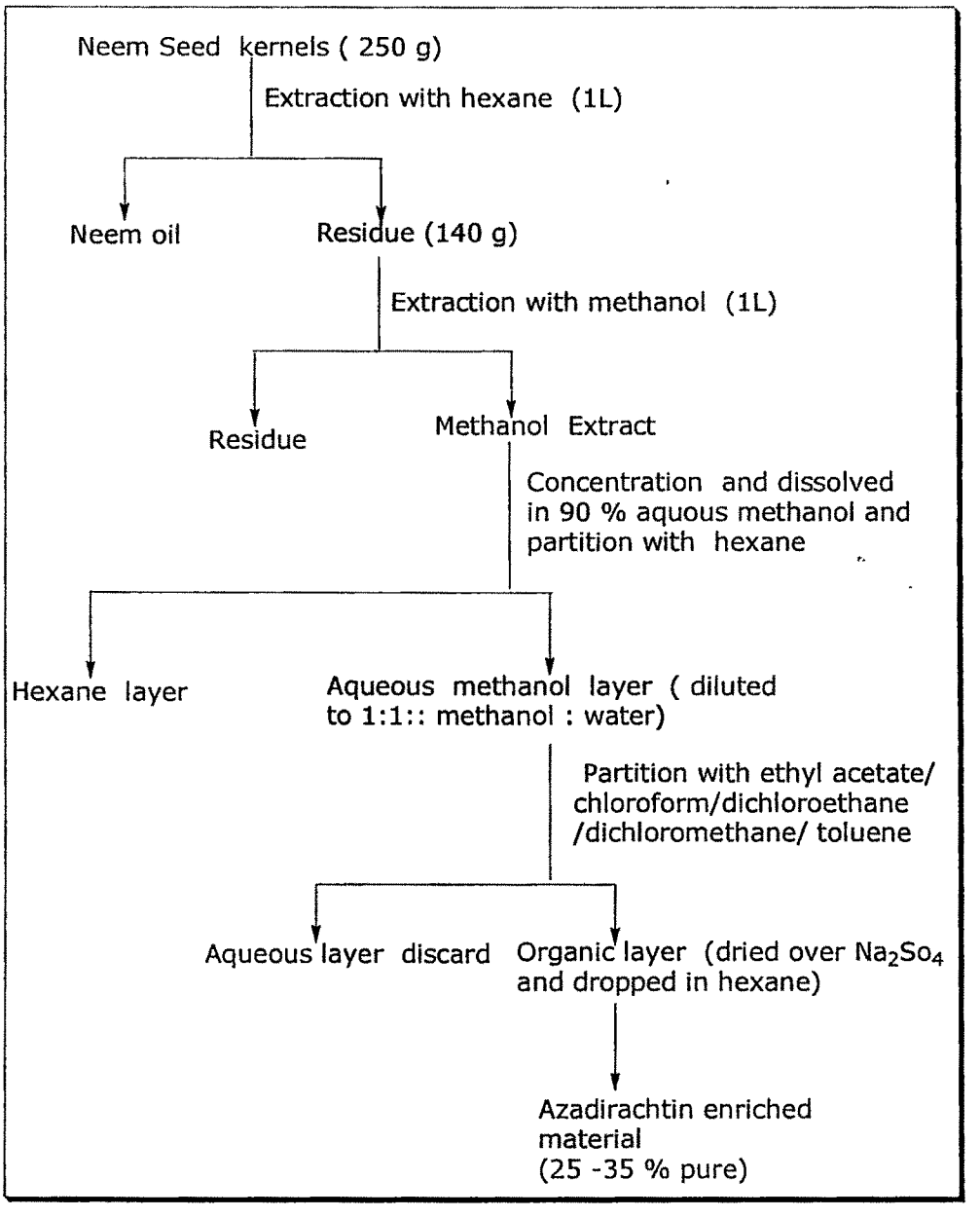



Figure: II.7 Flow diagram for the isolation of azadirachtin from seed kernels of Neem by Sinha *et al*

However, most of the methodologies involved either complex procedures[32,37-39,40-42,44] for isolation or did not report the initial and

final purity of azadirachtin[32,39,41,44,45] or were insensitive.[52] Keeping in view the recent developments in this field, it was thought to develop a short, simplified efficient procedure for the extraction of azadirachtin from seed kernels and a quantitative analytical HPLC-UV method for its estimation.

It has been shown earlier through several HPLC-UV determinations that azadirachtin was always obtained as a distinct peak from those of most other components in methanolic extracts of seed kernels.[44] In view of this, we have developed a preparative HPLC procedure for purification of azadirachtin up to 91% by using reversed phase $C_{18}$ column and methanol: water (60:40) as a eluent, from a crude extract containing about 10 % azadirachtin obtained from plant source.[54]

In addition to that, we have also extended our study to explore the Soxhlet extraction method for extraction of azadirachtin from the defatted neem seed cake.

Open column chromatography is widely used technique for the purification of organic compounds however, this technique is time consuming and gives poor recovery due to band tailing and decomposition, isomerization or polymerization due to longer time for separation.[12]

Therefore, a short and economical procedure for the preliminary purification of azadirachtin from crude extract of seed kernels using the inexpensive technique of flash chromatography coupled with reversed phase preparative HPLC has been developed.

## II.4 RESULTS AND DISCUSSION

The chemistry of the constituents of neem has made rapid progress in recent years, and about hundred triterpenoids are reported to

have been isolated and identified from neem seeds. These include highly active azadirachtin and their isomers, their derivatives, moderately active nimbandiol, salannin and their derivatives and less active gedunin, vilasinin, azadiradione etc. In addition to limonoids, the seeds contain a number of other natural products such as lipids, carbohydrates, terpenoids, fatty acids, hydrocarbon and sulphur compounds.[42,55,56]

Azadirachtin content in the seed kernels was determined by extracting it following the procedure of Schroeder and Nakanishi[39] with some modifications. The changes made were that extraction with ethanol, vacuum liquid chromatography and flash chromatography were omitted to make the process simpler and more efficient. The steps used in the extraction procedure are given in the Figure. II.8. Enrichment by solvent extraction and purification was carried out to prepare neem fractions rich in azadirachtin.

The extraction of Aza-A with methanol was found to be more efficient, when the ground seed kernels were defatted three times with hexane (Table: II.1). The combined hexane extracts were filtered and concentrated under reduced pressure to yield neem oil (40-45 %) having Aza-A content of 0.0235 % (w/w of oil)[57] (Figure:II.9) and defatted cake (B). Estimation of the Aza-A content in the neem oil was carried out by the method of Sundaram and Curry.[42] It is a suitable method, to remove other undesired components present in oil and is found to be highly sensitive to quantify Aza-A in the oil at 6 μg/g levels.[42] The 2.5 g of neem oil was extracted with methanol (50 mL) for 30 minutes at room temperature. The methanolic extract was transfered to the separatory funnel containing equal volumes of water and hexane and 5.0 mL of 5 % sodium chloride solution, shaken well for ten minutes

and the phases were allowed to separate. The aqueous methanolic phase containing Aza-A was separated and partitioned twice more with hexane to remove non-polar compounds. The methanolic layer was concentrated under reduced pressure and the resultant material was further extracted thrice with dichloromethane (60 mL). The pooled organic layer, containing the Aza-A was dried over anhydrous sodium sulphate. The organic layer was concentrated under reduced pressure and the resultant residue was further partitioned with hexane: acetonitrile (1:3). The polar layer was evaporated to dryness and the residue was dissolved in ethyl acetate for analytical HPLC.[42]

For complete recovery of Aza-A, the defatted powder (B) was extracted six times with methanol at room temperature.[38,45,49] Methanol extractions removed the traces of oil left with kernels along with Aza-A, soluble sugars, amino acids and proteins. Our results showed negligible quantities of Aza-A (0.0041 %) left in the defatted neem seed cake after six extractions. The Aza-A content in the six methanolic extracts ranged from 0.2199 to 0.0041% amounting to 0.386 % put together (Table: II.2). The combined methanolic extract was then concentrated under reduced pressure to furnish a brown azadirachtin-enriched residue. It was further extracted with ethyl acetate and partitioned between ethyl acetate: water to remove water-soluble polar compounds such as carbohydrates and proteins (Table: II.3). The ethyl acetate layer was concentrated after drying over anhydrous sodium sulphate and the resultant residue, washed with hexane to get a dry crude powder (G) enriched with Aza-A.

We have also developed an analytical HPLC-UV method to estimate Aza-A content in the crude powder. The final operating parameters to analyze the Aza-A content in the seed kernels were optimized by trial

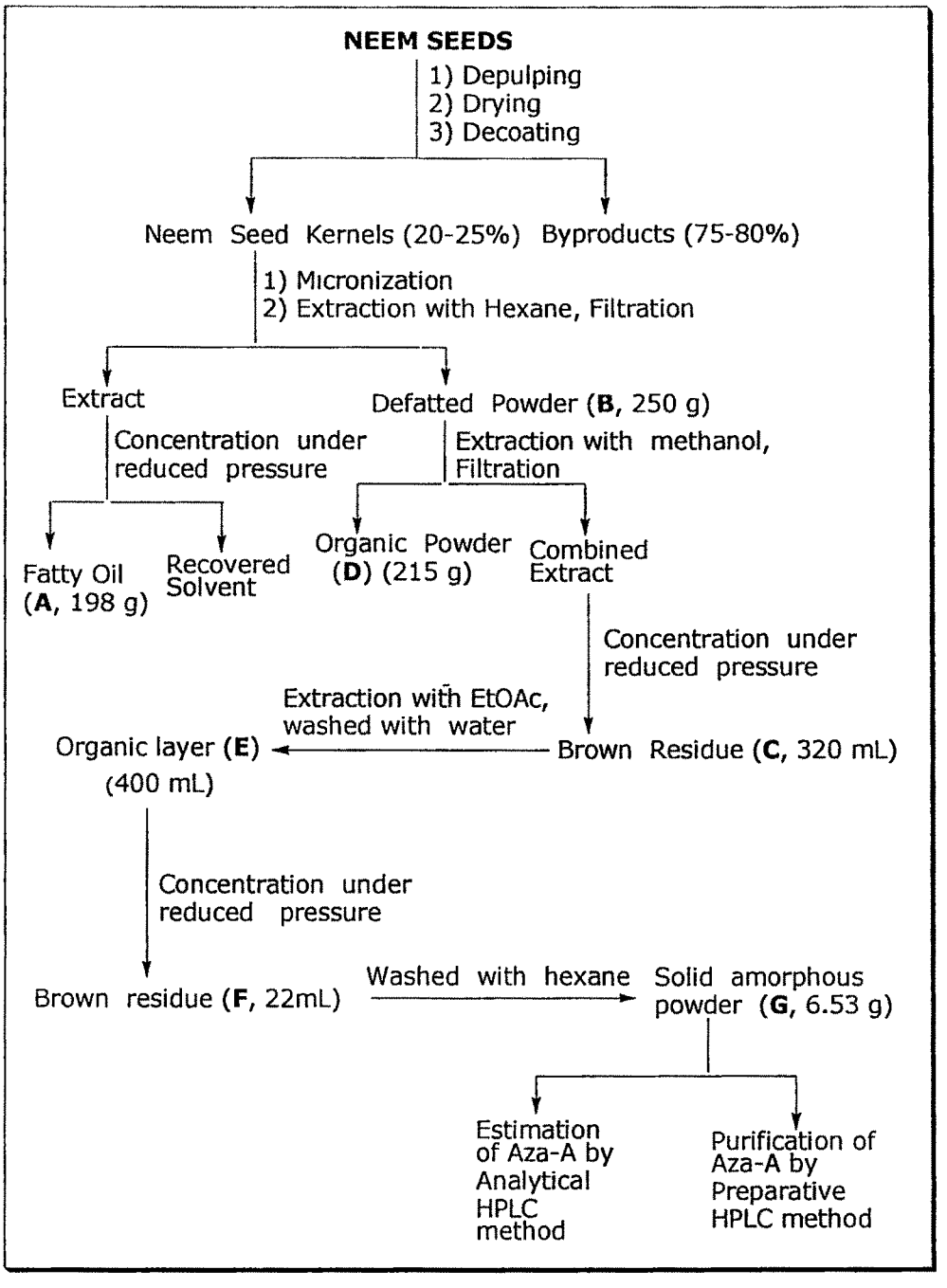



Figure: II.8 Flow diagram for the isolation of azadirachtin from Neem seed kernels

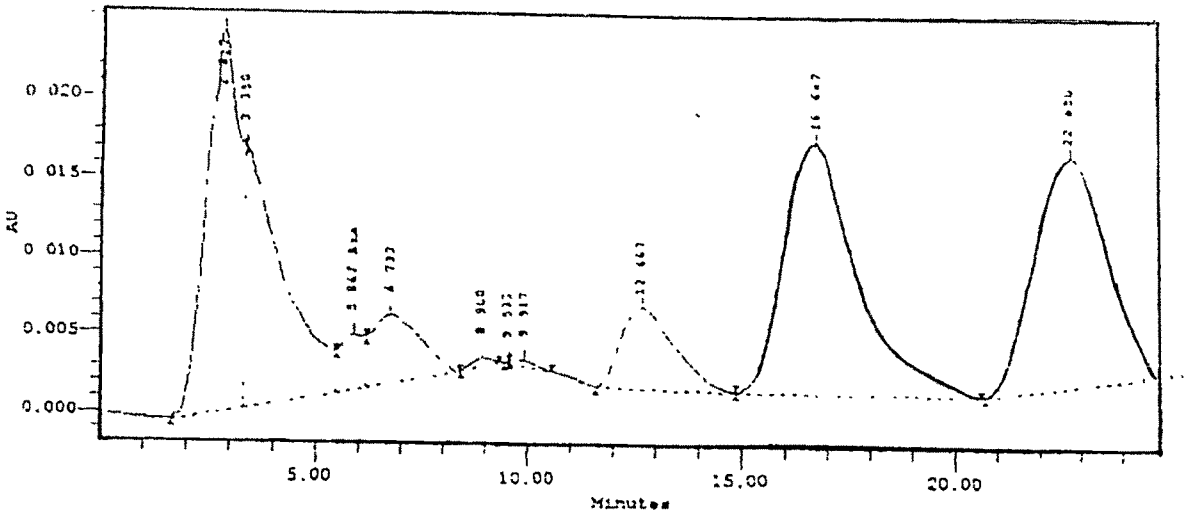
and error. Standard solutions of increasing concentration of Aza-A were injected separately into reverse phase column for a fixed set of instrumental parameters and HPLC response was examined. The parameters were altered in sequence and according to the exploratory runs to obtain the best HPLC response.[42]

For reverse phase analytical HPLC method, $C_{18}$ Bondapak column (3.9 mm I.D. × 300 mm) with 10 μm particle size and 125Å pore size, gave the good resolution of Aza-A, in the methanolic extracts as well as enriched powder with an average retention time ($t_R$) of 6.95 minutes.

The chromatograms were sharp, narrow and symmetrical with maximum separation efficiencies from solvent front and interfering peaks due to endogenous materials present in the matrices. Frequent flashing of the column with mobile phase was necessary to maintain its performance, to minimize band tailing and to remove the late euters.[42]

The aqueous methanol (60:40) as an isocratic solvent system was found to be suitable for the separation of Aza-A giving sharp peaks and good resolution without much base line drift. The 217 nm was chosen as the most suitable wavelength for the analysis of Aza-A in this study.

The flow rate used in this study was 1.0 mL/min. As expected, the higher flow rate lowered the retention time but increased the column backpressure. Therefore, a flow rate of 1.0 mL/min was used in this study and provided optimum band spacing during analysis with good resolution. The 20-μL injection volume was used, as it gave better detection limits and a good separation of the analyte.




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type | % Area | Units | % Height |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 2.817 | 1218915 | 24012 | | BV | 17.77 | | 28.21 |
| 2 | | 3.350 | 1017298 | 16675 | | VV | 14.83 | | 19.59 |
| 3 | Aza | 5.867 | 139032 | 3433 | 231.792 | VV | 2.03 | ppm | 4.03 |
| 4 | | 6.733 | 337276 | 4331 | | VB | 4.92 | | 5.09 |
| 5 | | 8.500 | 15044 | 478 | | BE | 0.22 | | 0.56 |
| 6 | | 9.533 | 511 | 110 | | BV | 0.01 | | 0.13 |
| 7 | | 9.917 | 13468 | 351 | | VB | 0.20 | | 0.41 |
| 8 | | 12.667 | 442364 | 5086 | | BB | 6.45 | | 5.97 |
| 9 | | 16.667 | 1901715 | 15956 | | BB | 27.73 | | 18.74 |
| 10 | | 22.650 | 1772293 | 14690 | | BB | 25.84 | | 17.26 |

Figure: II.9 HPLC Chromatogram of Fatty Oil

The HPLC chromatogram of standard azadirachtin-A is shown in Figure II.10. The retention time ($t_R$) of standard Aza-A in our system was 6.95 minute. The HPLC chromatogram of crude powder containing Aza-A is shown in Figure II.11. In addition to Aza-A peak, a number of less distinct peaks due to endogenous materials in the extracts were also present. These may be due to other azadirachtin isomers and coextracted limonoids, triglycerides and other natural products compounds.

The chromatographic method used was efficient and the resolution of Aza-A was good. However, carryover peaks were observed during the analysis and these were eliminated by flashing the HPLC column periodically with methanol.

The concentration of Aza-A in the crude methanol extracts and in the solid powder (G) was determined from the calibration curve obtained by plotting of peak area versus Aza-A concentration, by injecting 20 µL of standard Aza-A solution in triplet, ranging in concentration from 31.25 to 250 ppm (Figure: II.12). A very good linear relationship ($R^2$= 99.9 %) was observed in the range tested with Y-intercept near zero.

From the HPLC-UV analysis and following calculations, the percentage purity of Aza-A content in crude powder was found to be 9.14 %, while the same in seed kernels was found to be 0.1188 % and that in the fruits to be 0.03029 %.

Peak Area of Sample : 451473

Peak Area of Standard : 3283131

Dilution of Sample : 1000 ppm

Dilution of Standard : 700 ppm

% Purity of Standard : 95 % (by TLC, Sigma Chemical Co.) azadirachtin-A

$$\% \text{ Purity of Sample} = \frac{\text{Peak Area of the Sample x Dilution of the Standard x \% Purity of the Standard}}{\text{Peak Area of the Standard x Dilution of the Sample}}$$

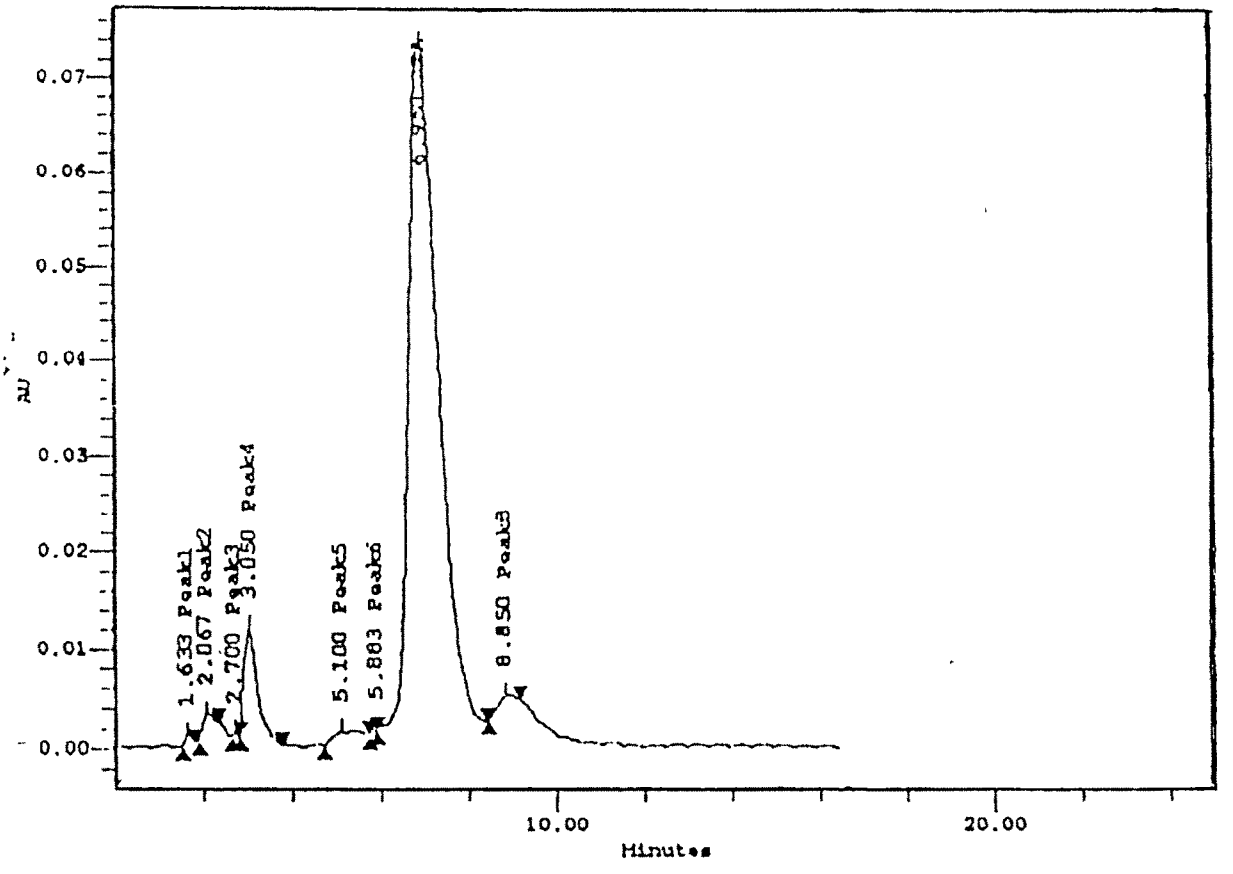
$$= \frac{451473 \times 700 \times 95}{3283131 \times 1000} = 9.14\ \%$$

Thus, 500 g neem seed kernels ≡ 6.5 g 9.14 % crystalline powder
≡ 0.594 g azadirachtin present
100 g neem seed kernels ≡ 0.1188 % azadirachtin present
Now 1 kg neem fruits give 255 g seed kernel on an average
255 g neem seed kernel gives 0.3029 g azadirachtin
0.03029 % azadirachtin present in the given fruits
Neem fruits content the 0.03029 % azadirachtin

Our results are in close agreement with those reported in literature.[57,58] However, variations in the yield and purity of azadirachtin in the enriched crude powder and in neem seed kernels are probably due to the geographical locations of the plant, time of harvest, the method of extraction, exposure to sunlight, processing of seed collection and duration of seed storage, difference in conditions of soil, rainfall and humidity.[37,39,40,53,59] Seeds from arid regions were found to contain higher percentages of Aza-A.[42]

It should be mentioned here that the extraction procedure reported, herein, is much shorter and economical as compared to the earlier reported procedures.[37-39,42]

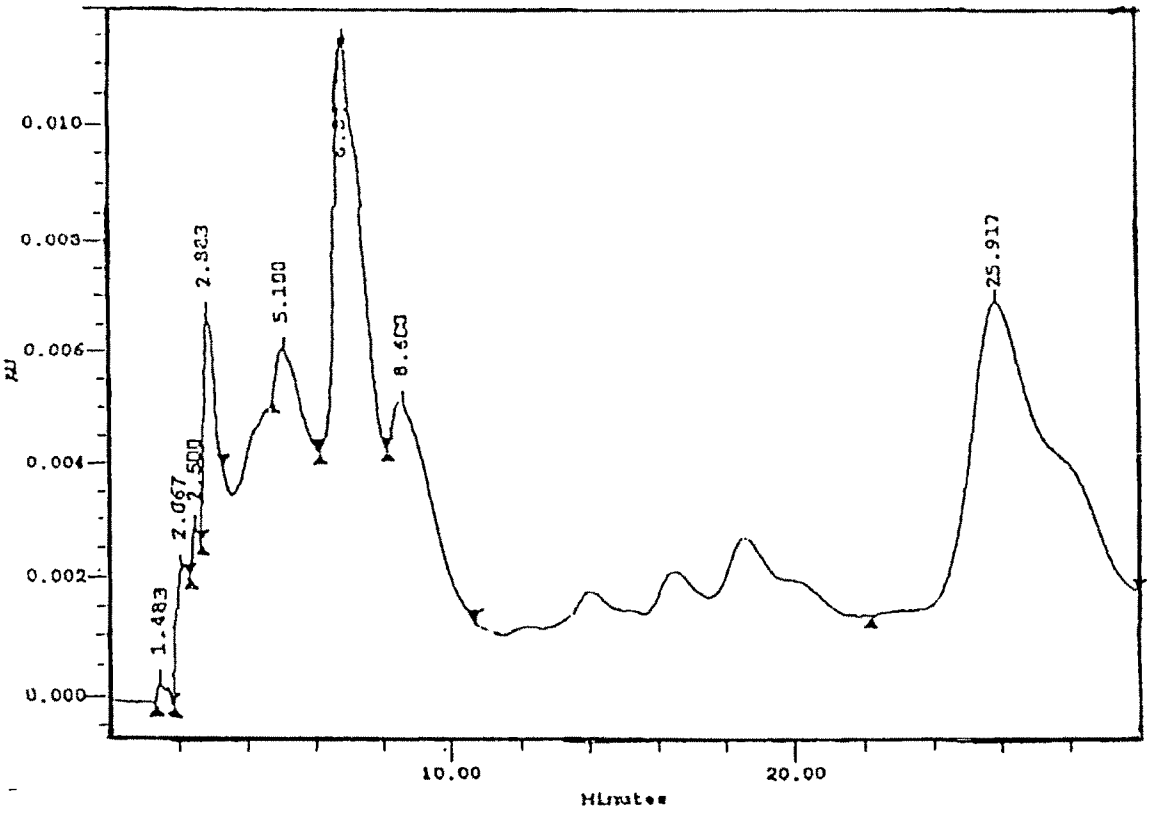
The preparative HPLC-UV method used in this study was chosen on the basis of the results obtained from analytical HPLC-UV. The preparative HPLC chromatogram of crude Aza-A extract is shown in Figure II.13. The retention time ($t_R$) of Aza-A in our system was 9.16 minutes (Figure: II.13). Each run of 1 mL injection of methanolic




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type |
|---|---|---|---|---|---|---|
| 1 | Peak1 | 1.633 | 7054 | 816 | | BB |
| 2 | Peak2 | 2.067 | 25351 | 1791 | | BB |
| 3 | Peak3 | 2.700 | 2513 | 380 | | BV |
| 4 | Peak4 | 3.050 | 228757 | 11190 | | VB |
| 5 | Peak5 | 5.100 | 44389 | 1061 | | BV |
| 6 | Peak6 | 5.883 | 7707 | 768 | | VV |
| 7 | AZA | 6.950 | 3283131 | 71835 | 0.070 | VB |
| 8 | Peak8 | 8.850 | 20828 | 1270 | | BB |

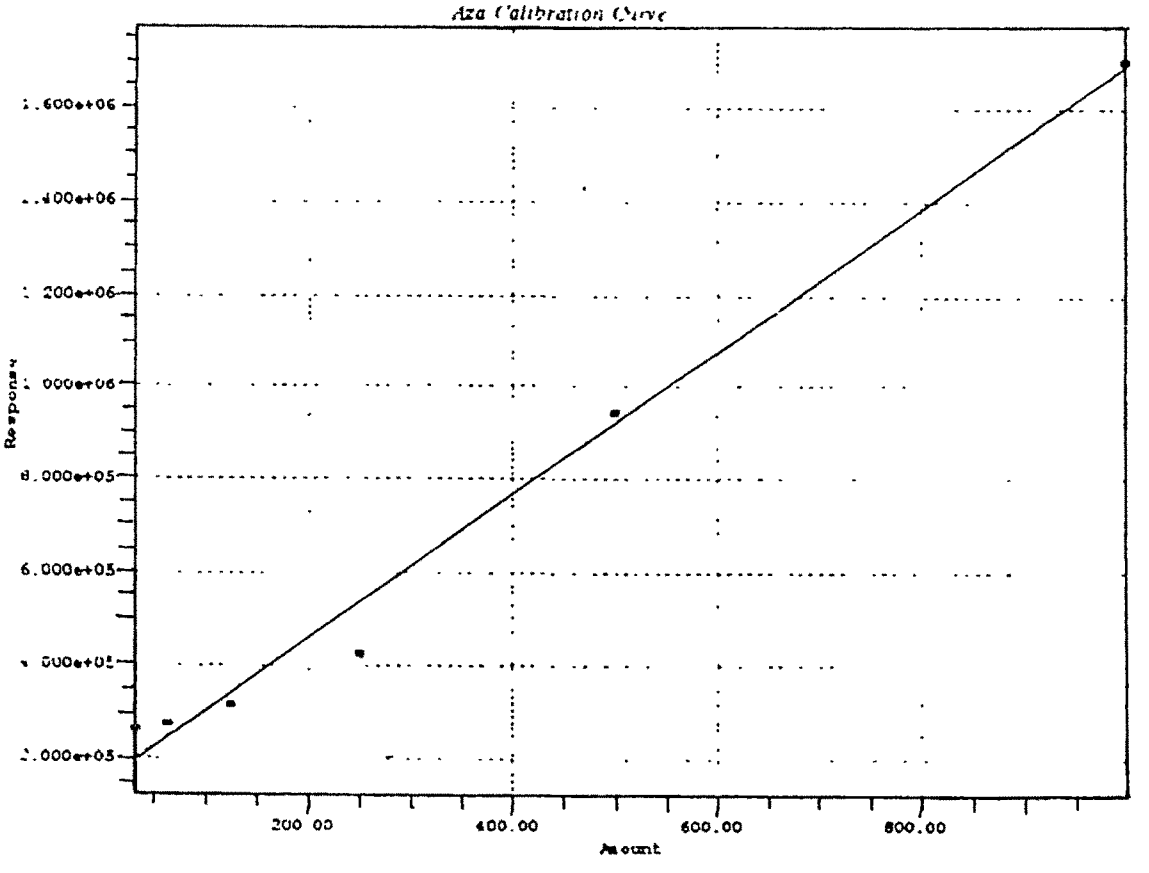
Figure: II.10 HPLC Chromatogram of Standard azadirachtin-A (Sigma)




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type |
|---|---|---|---|---|---|---|
| 1 | | 1.483 | 6054 | 318 | | BB |
| 2 | | 2.067 | 31705 | 1730 | | BV |
| 3 | | 2.500 | 17638 | 1170 | | VV |
| 4 | | 2.883 | 76529 | 3887 | | VB |
| 5 | | 5.100 | 46246 | 1154 | | BB |
| 6 | aza | 6.867 | 451471 | 7692 | 0.193 | BV |
| 7 | | 8.600 | 184262 | 2462 | | VB |
| 8 | | 25.917 | 835314 | 5353 | | BB |

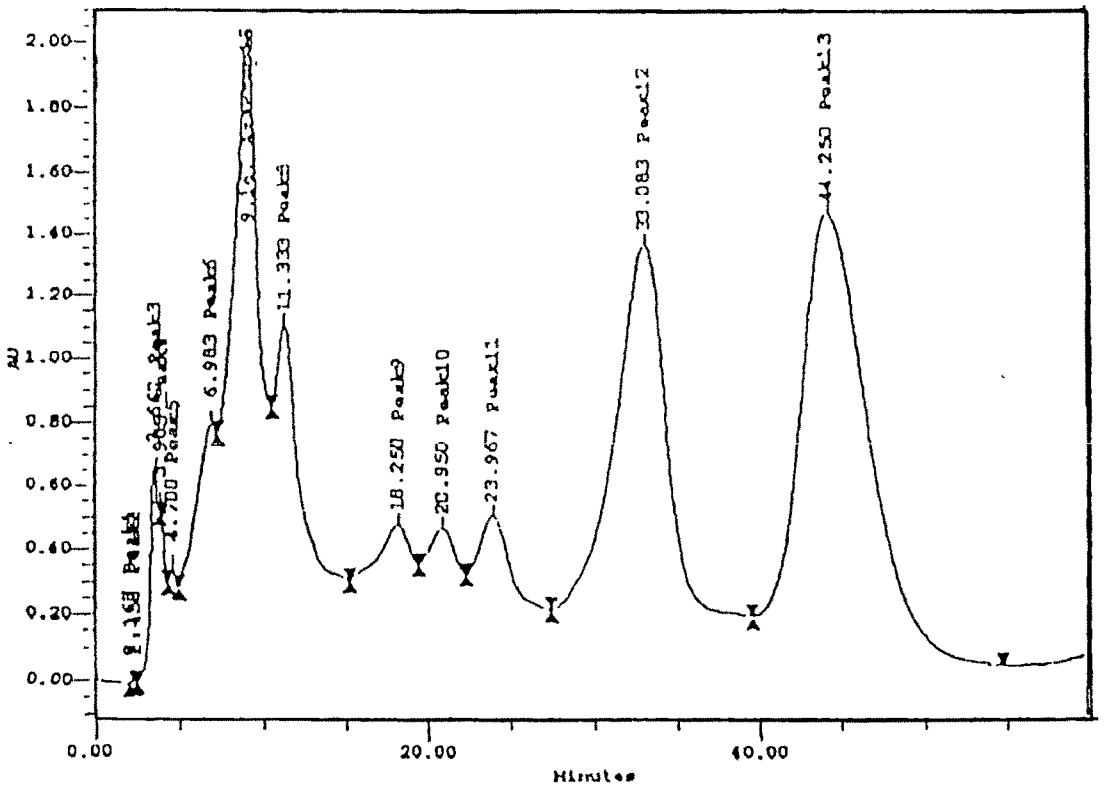
Figure: II.11 HPLC Chromatogram of Crude Powder Containing azadirachtin-A.




Aza Point Table

| # | Amount | Response | Calc. Amount | % Deviation | Manual | Ignore ? |
|---|---|---|---|---|---|---|
| 1. | 31.500000 | 265677.677136 | 40.782309 | 29.468 | No | No |
| 2. | 62.500000 | 277443.541237 | 46.290056 | -25.936 | No | No |
| 3. | 125.000000 | 418903.989104 | 112.509443 | -9.992 | No | No |
| 4. | 250.000000 | 703414.742152 | 245.692448 | -1.723 | No | No |
| 5. | 500.000000 | 1333693.238806 | 540.733616 | 8.147 | No | No |
| 6. | 1000.000000 | 2278463.194154 | 982.992128 | -1.701 | No | No |

Figure: II.12 Calibration Graph of azadirachtin-A.




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type |
|---|---|---|---|---|---|---|
| 1 | Peak1 | 2.167 | 22323 | 3670 | | BB |
| 2 | Peak2 | 2.450 | 39197 | 5105 | | BV |
| 3 | Peak3 | 3.667 | 20592968 | 660229 | | VV |
| 4 | Peak4 | 3.983 | 12866075 | 550047 | | VV |
| 5 | Peak5 | 4.700 | 12996249 | 346200 | | VV |
| 6 | Peak6 | 6.983 | 74651357 | 797508 | | VV |
| 7 | AzaPrep15 | 9.167 | 248371102 | 2004822 | 17.000 | VV |
| 8 | Peak8 | 11.333 | 165842067 | 1101870 | | VV |
| 9 | Peak9 | 18.250 | 96270682 | 467809 | | VV |
| 10 | Peak10 | 20.950 | 67524231 | 453756 | | VV |
| 11 | Peak11 | 23.967 | 99323665 | 491105 | | VV |
| 12 | Peak12 | 33.083 | 369931713 | 1339417 | | VV |
| 13 | Peak13 | 44.250 | 421711954 | 1411340 | | VB |

Figure: II.13 Preparative HPLC Chromatogram of azadirachtin-A

solution containing 9.14 % Aza-A in the residue was completed in 60 min yielding 8 mg of 91 % pure Aza-A. Seed kernels (500 g) thus gave 6.5 g of crude powder containing 9.14 % Aza-A. From 1g of this powder, 90 mg of the 91 % pure Aza-A was isolated (Figure.II.14).

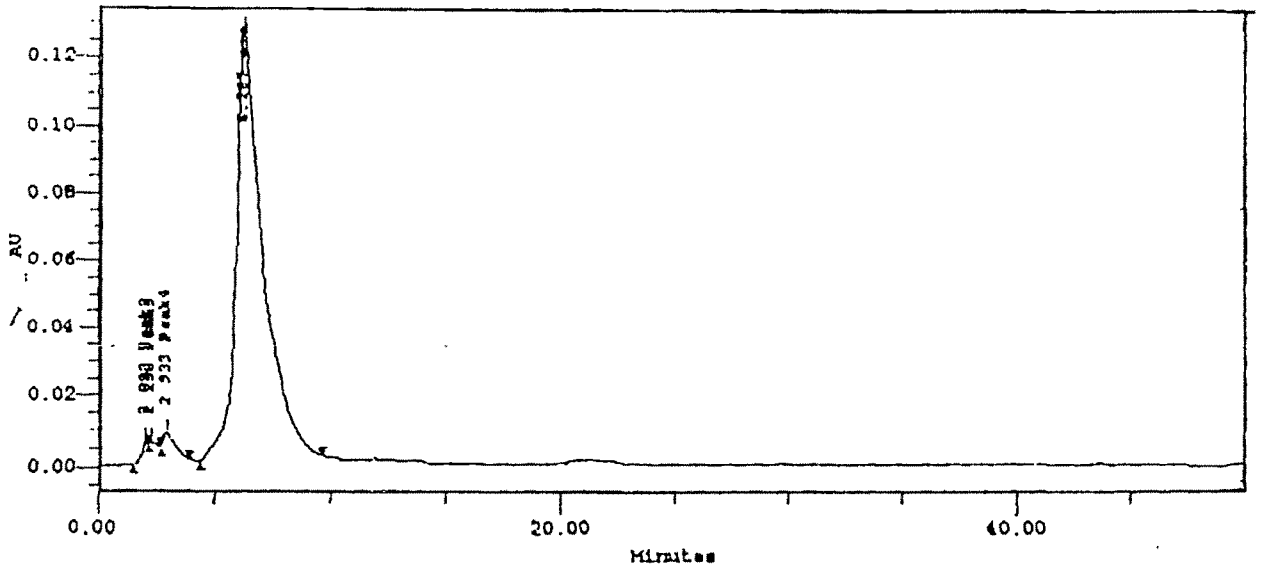
Identification of Azadirachtin-A

IR, UV, PMR, and $^{13}$CMR (Figures: II.15-18) spectra of the sample of Aza-A purified by this method were found to be in good agreement with those reported in the literature.[41,54,60-63] The PMR spectrum of our sample was also found to be identical with that of Aza-A provided by Prof. S. V. Ley, University of Cambridge, Cambridge, U.K.

The identity of our sample of Aza-A was further proved by performing co-chromatography (TLC, HPLC) with an authentic sample (Sigma Chemical Co.).

Soxhlet Extraction Experiments:

The Soxhlet extraction experiments were carried out in a 1 L Soxhlet apparatus. Finely ground powder of neem seed kernels (500 g) was extracted with hexane (1.0 L). The defatted solid cake (B, 230 g) obtained by following the above procedure was taken in an extraction thimble and extracted with methanol (500 mL) for 24 and 72 hours independently, at reflux temperature. Aza-A content was determined by analytical HPLC-UV method by taking samples of extracted solutions at 4-hour interval for total periods of 24 and 72 hours independently. The HPLC-UV analyses showed that the enrichment of Aza-A in the extracted solutions by Soxhlet method (0.449 %) was higher than that in the case of normal extraction method (0.386 %, after six extractions with methanol) thus showing




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type |
|---|---|---|---|---|---|---|
| 1 | Peak1 | 1.633 | | | | Missing |
| 2 | Peak2 | 2.033 | 91828 | 5786 | | BV |
| 3 | Peak3 | 2.250 | 157950 | 5500 | | VV |
| 4 | Peak4 | 2.933 | 291056 | 7714 | | VB |
| 5 | Peak5 | 5.100 | | | | Missing |
| 6 | Peak6 | 5.883 | | | | Missing |
| 7 | aza | 6.267 | 9977308 | 125581 | 0.213 | BB |
| 8 | Peak8 | 8.850 | | | | Missing |

Figure: II.14 HPLC Chromatogram of Pure azadirachtin-A.

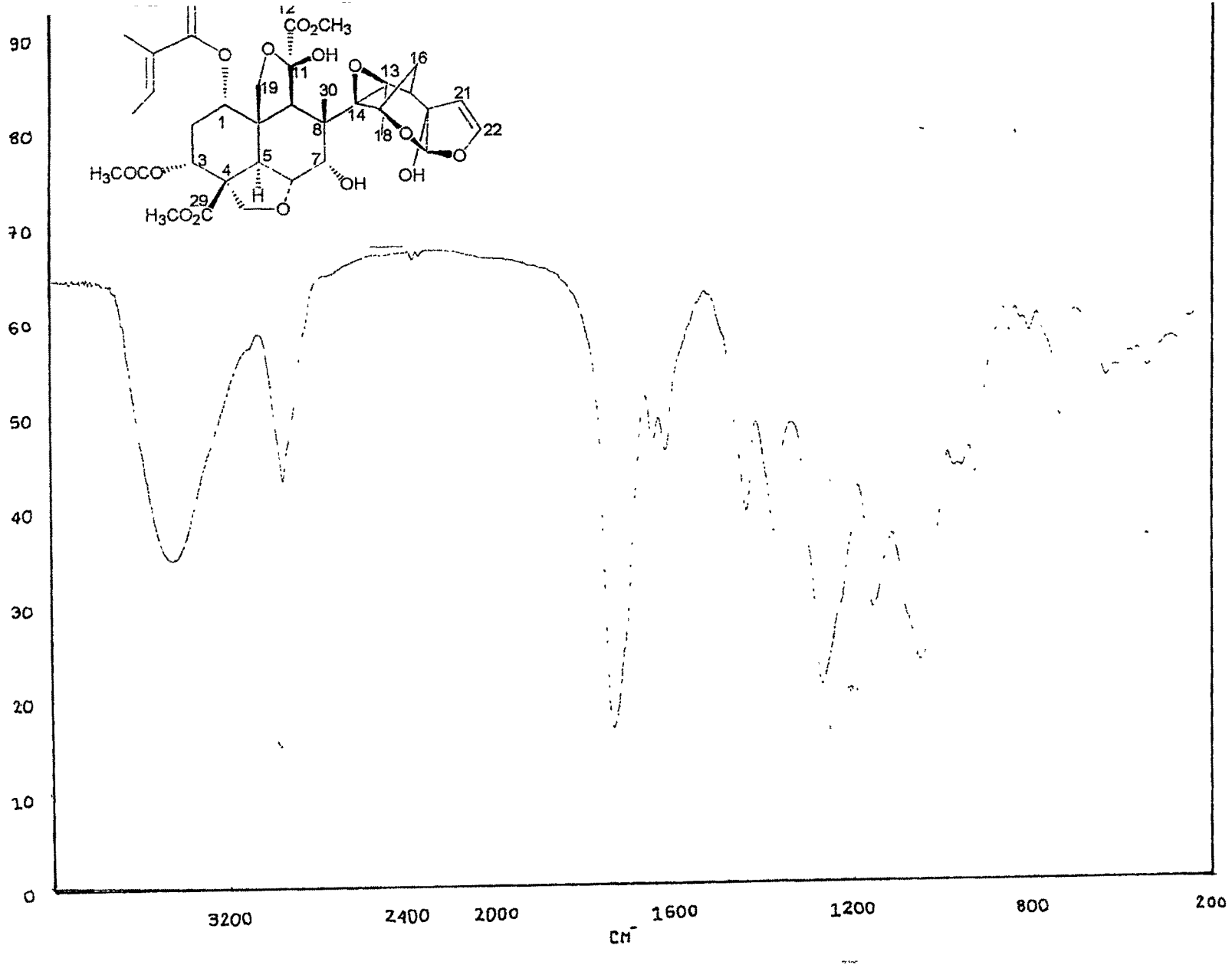



Figure II.15 : IR Spectrum of the Azadirachtin-A

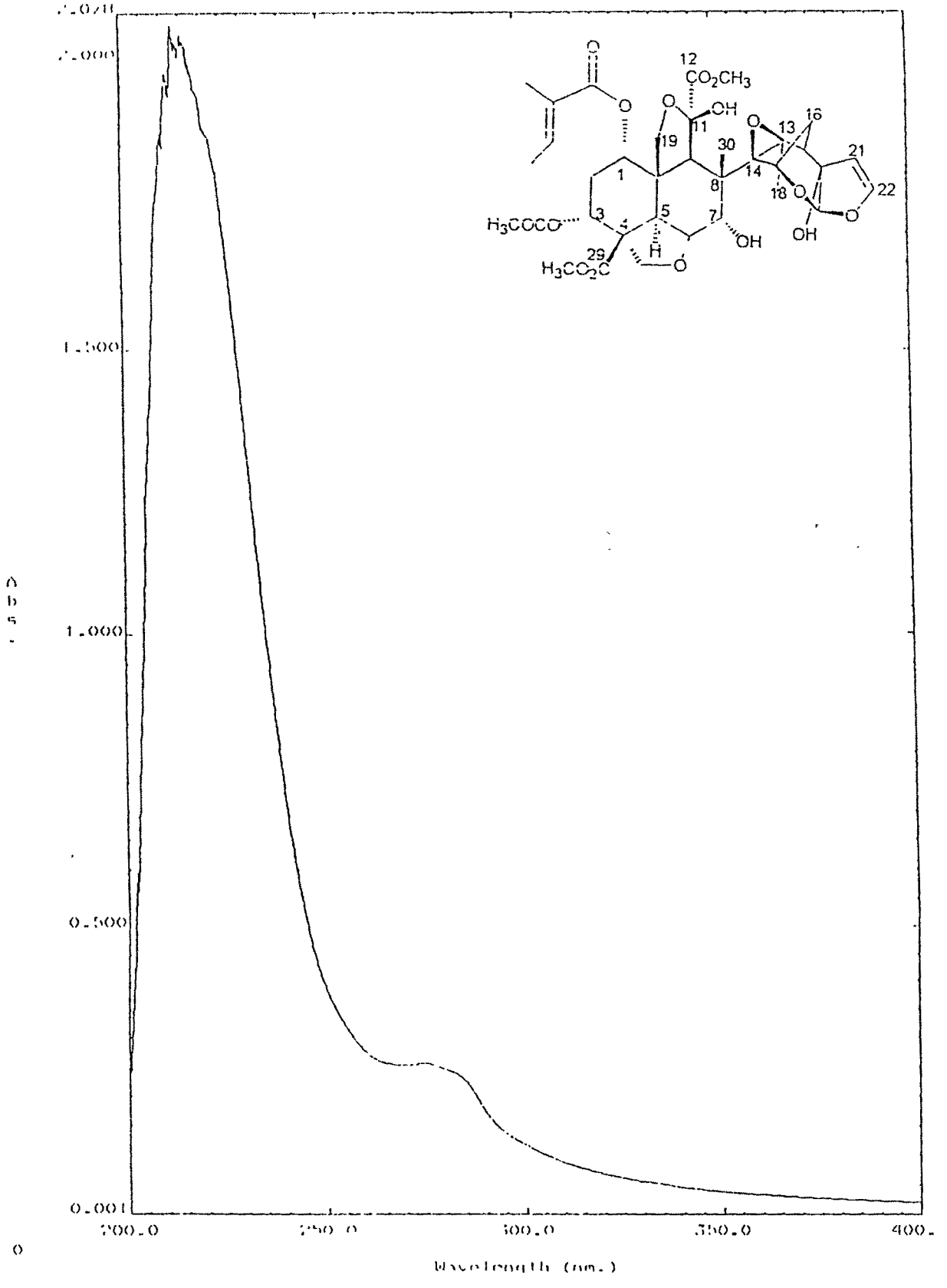



Figure II.16 : UV Spectrum of the Azadirachtin-A

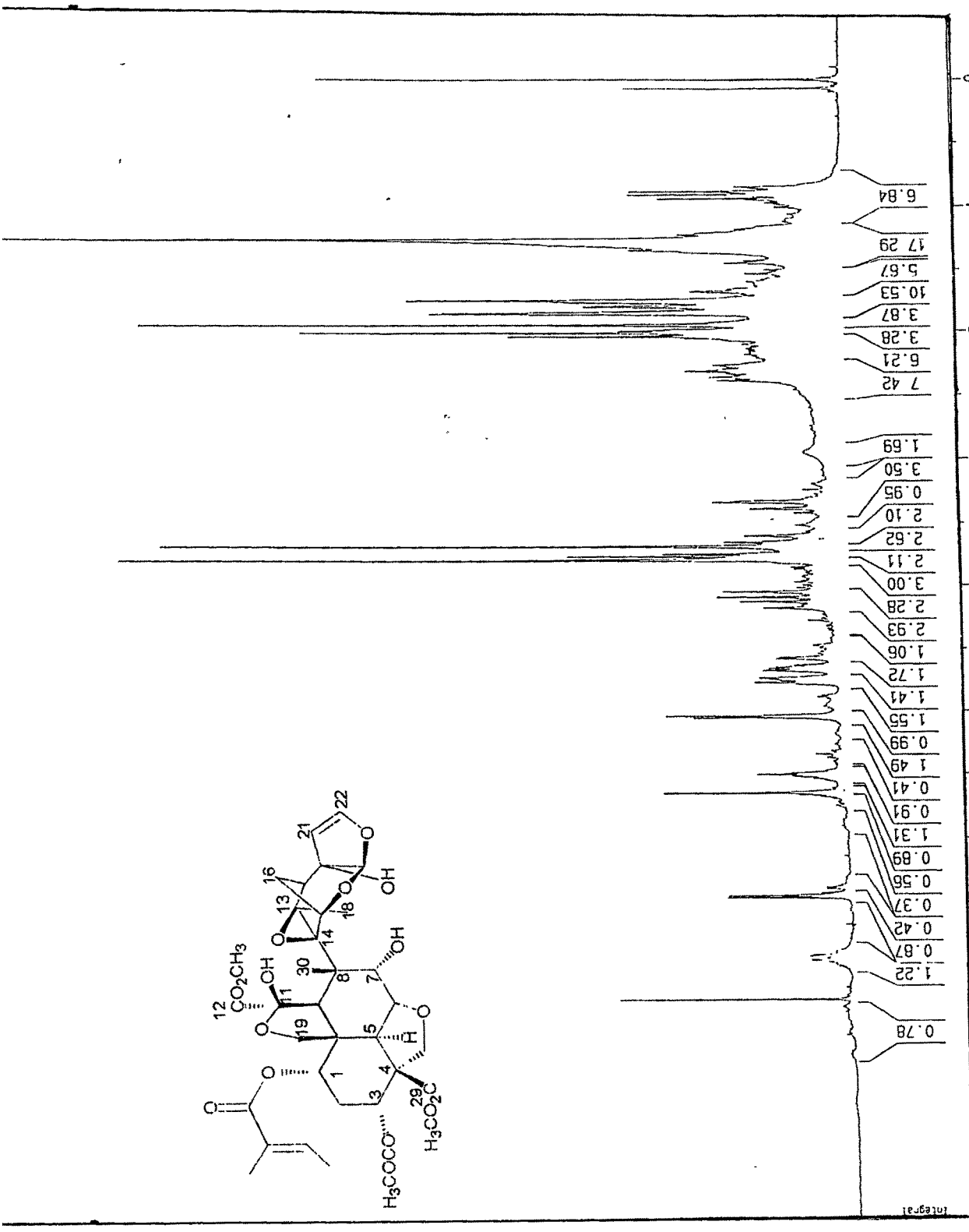



Figure II.17 : PMR Spectrum (200 MHZ) of the Azadirachtin-A

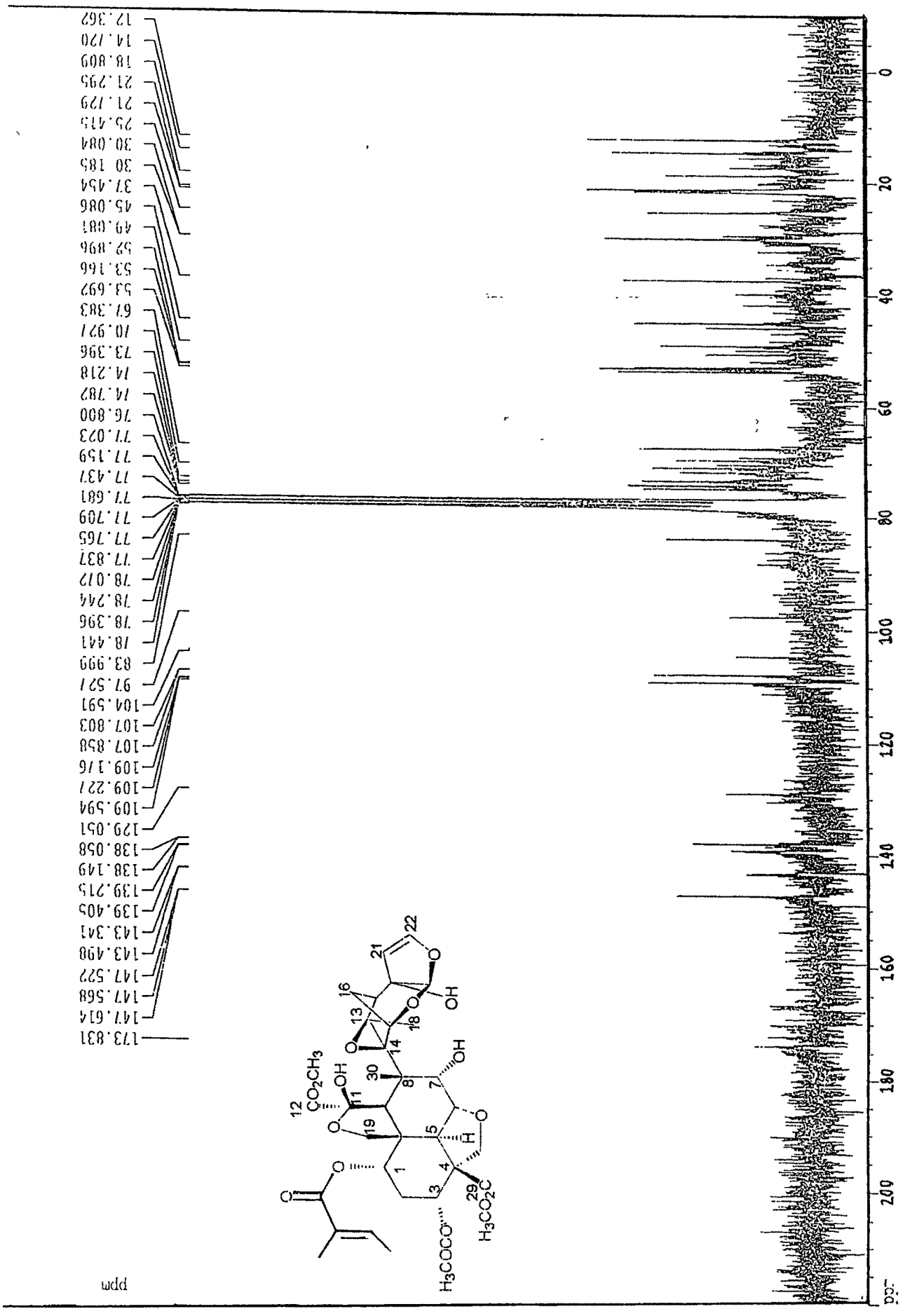



Figure II.18 : $^{13}$CMR Spectrum (50 MHZ) of the Azadirachtin-A

the former method to be more efficient. The plots of the % of Aza-A in extracted solutions versus time in hours for the experiments carried out for 24 and 72 hours are shown in Figures: II. 19 and 20 respectively. The HPLC-UV results and Figures: II. 19 and 20 showed that in Soxhlet extraction, the amount of Aza-A in the extracted solutions increased up to 24 hour initially and decreased gradually thereafter perhaps due to the decomposition of Aza-A.

It is worthwhile to mention at this stage, that prior to our own reports,[64] the Soxhlet method for the extraction of Aza-A from defatted neem cake was not reported earlier.

Thin Layer Chromatography of Azadirachtin-A:

TLC analyses were done on plates of 0.25 mm thick pre-coated layer of silica gel 60 F-254 (Merck). The plates were developed in solvent system, as presented in Table: II. 4. Five analytical solvent systems were used to choose appropriate one for flash chromatography and to monitor the eluted fractions. A vanillin spray reagent was used to visualize azadirachtin on TLC plates, since it gave characteristic colour change from red brown to green.[37-39]

Table: II.4 $R_F$ Values of azadirachtin in various Solvent System

| Sr. No | Solvent system (v/v) | $R_F$ values |
|---|---|---|
| 1 | Ethyl acetate | 0.68 |
| 2 | Ethyl acetate: n-hexane (1:1) | 0.48 |
| 3 | Chloroform: Acetonitrile (3:1) | 0.25 |
| 4 | Isopropyl alcohol: n-hexane (11:9) | 0.56 |
| 5 | Ethyl acetate: n-hexane (3:1) | 0.54 |

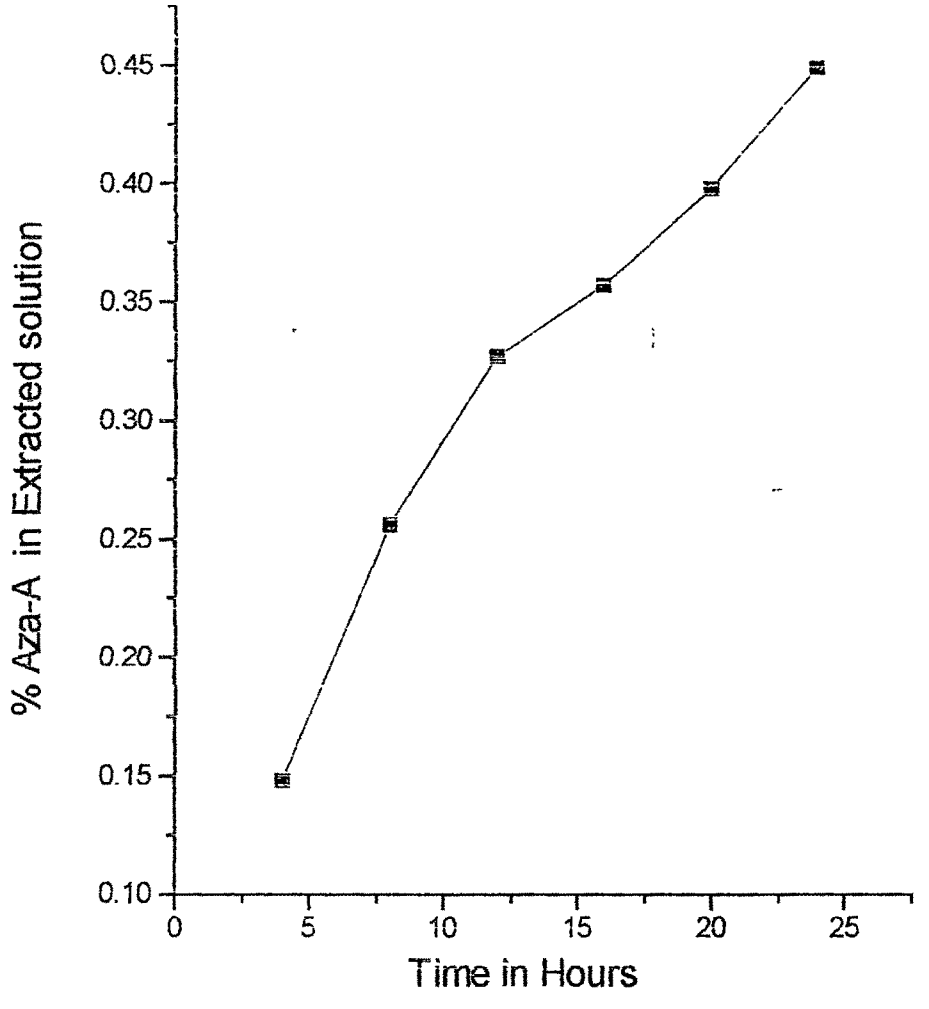



Figure: II.19 Plot of % Aza-A extracted versus time (24 hrs) by Soxhlet method

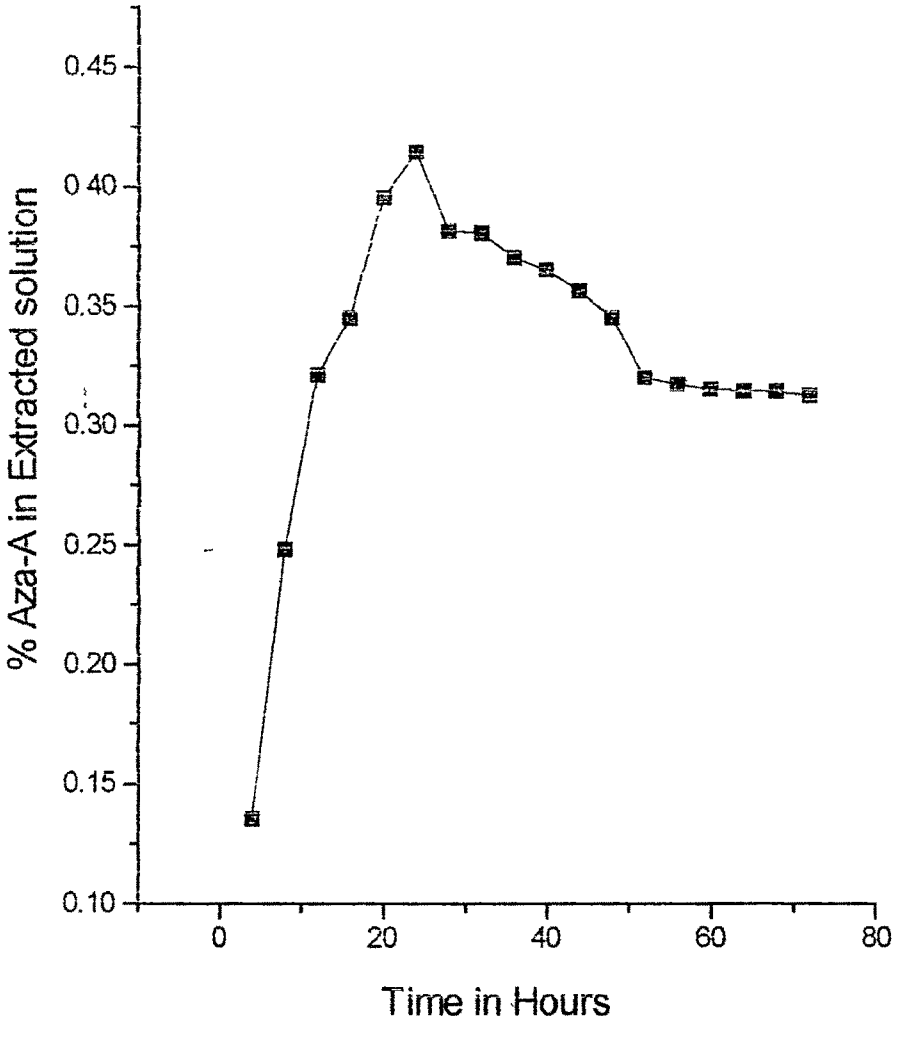



Figure: II.20 Plot of % Aza-A extracted versus time (72 hrs) by Soxhlet method

Flash Column Chromatography of Crude Material Containing 9.14 % Azadirachtin-A:

A long glass column (90 cm × 32 mm I.D.) was selected and a small plug of glass wool was placed in the tube connecting the stopcock to the column body. Column was packed with flash grade silica gel (230-400 mesh size, 75 g) suspended in hexane.

The crude material (5.08 g, 9.14 % Aza-A) dissolved in minimum quantity of methanol was adsorbed on silica gel (8.0 g, 230-400 mesh size) by stirring and evaporating the solvent under reduced pressure on a Buchi rotavapor. The dry powder thus obtained was then placed on the top of the adsorbent column. The flow controller was briefly placed on the top of the column initially and was finally tightly fitted on the top of the column, while applying air pressure of 400-500 Hg from the top of the column through it. Figure II.21 shows the simple flash chromatographic steps involved in the purification of Aza-A from a very complex mixture.

The column was initially eluted with hexane, followed by hexane containing increasing proportions of ethyl acetate and finally with ethyl acetate as flow rate of 8-10 mL/min in to fractions of 15-20 mL.

The column was eluted with a mixture of hexane: ethyl acetate (95:5) in 250 mL portions to remove large amounts of non-polar material in the first seven fractions. Thereafter, column was eluted with increasing proportions of ethyl acetate. In hexane: ethyl acetate (25: 75) the first seven fractions consisted of a complex mixture of the least polar compounds, presumably other tetranortriterpenoids (Figure: II.22) After a small gap, azadirachtin first appeared in next eight fractions as monitored by TLC (Table: II.4). These fractions were combined, concentrated under reduced pressure to furnish a

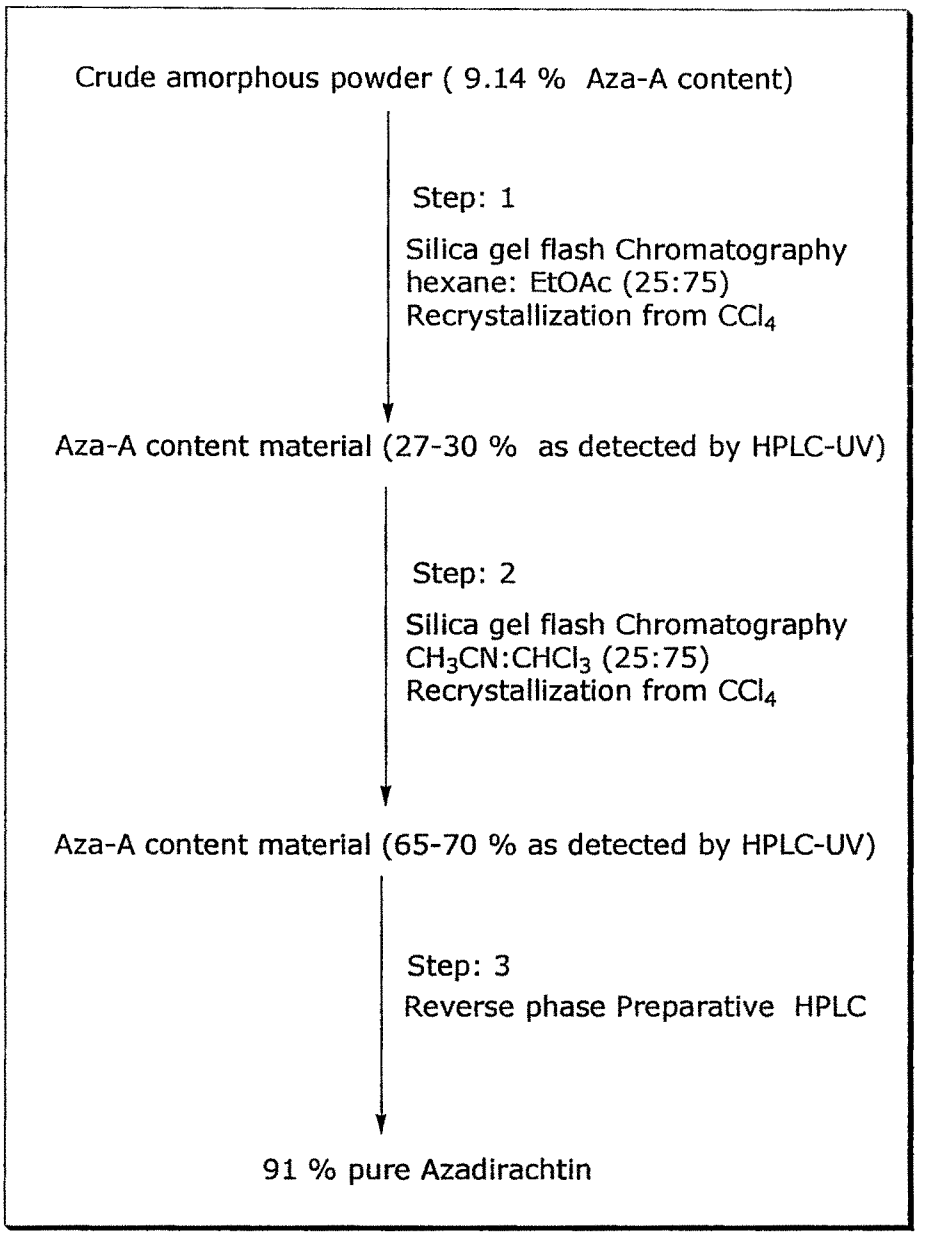
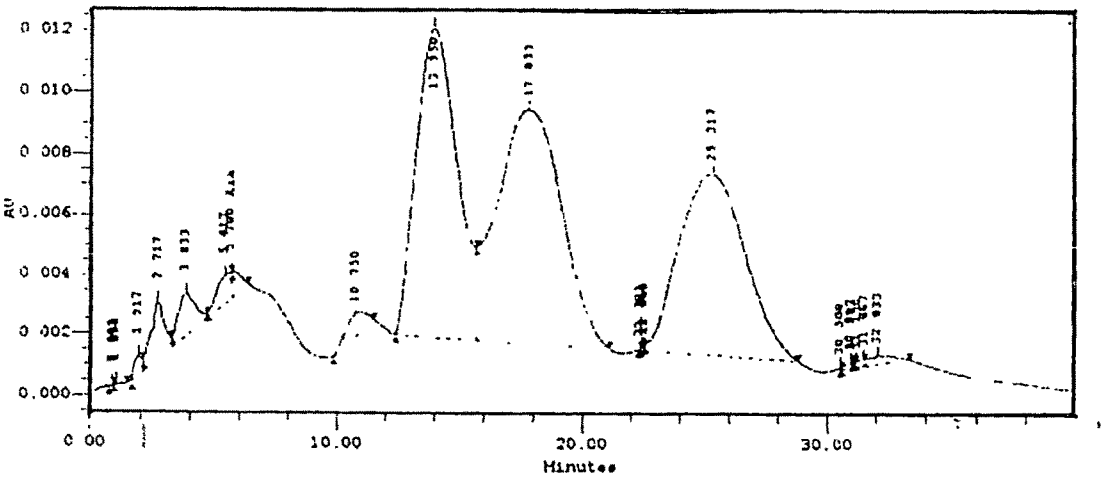



Figure: II.21 Flow diagram of Purification of Azadirachtin by Flash chromatography




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type | % Area | Units | % Height |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 0.883 | 192 | 33 | | BV | 0.00 | | 0.10 |
| 2 | | 1.000 | 660 | 40 | | VB | 0.02 | | 0.11 |
| 3 | | 1.917 | 12461 | 625 | | BV | 0.31 | | 1.98 |
| 4 | | 2.717 | 70930 | 1925 | | VV | 1.78 | | 6.10 |
| 5 | | 3.833 | 66116 | 1391 | | VV | 1.67 | | 4.41 |
| 6 | | 5.417 | 35841 | 893 | | VV | 0.90 | | 2.83 |
| 7 | Aza | 5.700 | 19949 | 821 | | VB | 0.50 | ppm | 2.60 |
| 8 | | 10.750 | 39818 | 713 | | BB | 1.00 | | 2.26 |
| 9 | | 13.950 | 1164159 | 10169 | | BV | 29.23 | | 32.30 |
| 10 | | 17.817 | 1425910 | 7725 | | VB | 35.80 | | 24.49 |
| 11 | | 22.183 | 192 | 46 | | BV | 0.00 | | 0.15 |
| 12 | | 27.900 | 538 | 95 | | VV | 0.01 | | 0.30 |
| 13 | | 32.583 | 665 | 144 | | VV | 0.02 | | 0.46 |

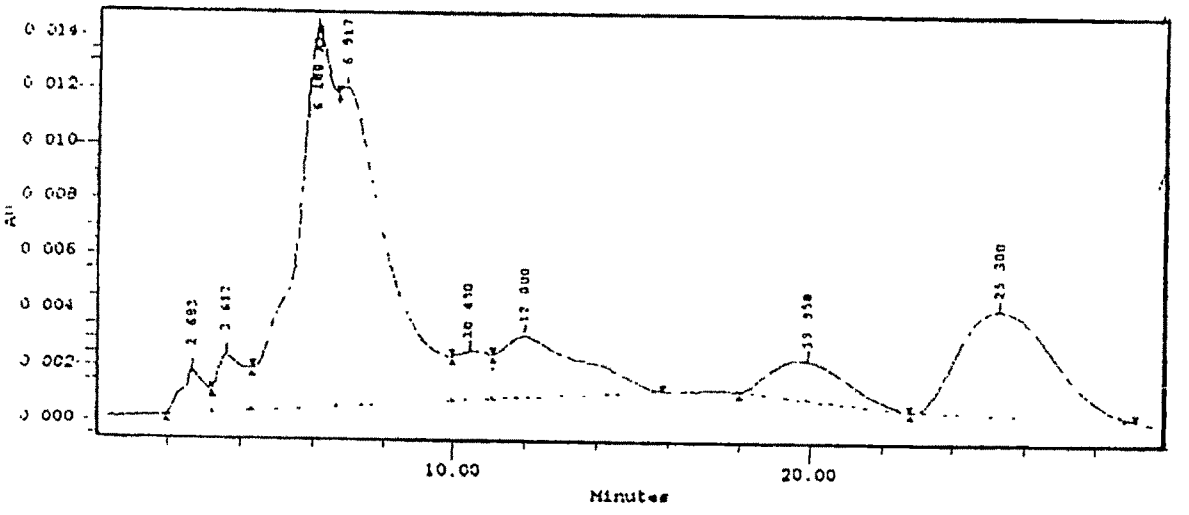
Figure: II.22

solid residue (2.48 g), which on dissolving in boiling carbon tetrachloride and cooling yielded a material (1.8 g) containing 27 to 30 % Aza-A (Figure: II.23). This key crystallization step was repeated again before subjecting the solid to second flash chromatography as shown below. In the second flash column chromatography, the column was eluted with a mixture of chloroform: acetonitrile (75:25) and azadirachtin-containing fractions were again monitored by TLC. These fractions were combined and concentrated under reduced pressure to yield a solid material (925 mg), which upon further recrystallization from carbon tetrachloride gave (750 mg) of the material containing about 65 to 70 % azadirachtin (Figure: II.24). The azadirachtin, thus pre-purified by flash chromatography, was further purified up to 91 % by using reverse phase preparative HPLC (Figure: II.25).

Each run on the flash column was capable of chromatographing gram quantities of the material. Thus, two flash chromatographic steps quickly gave from a crude extract of about 10 % Aza-A to a material containing 65-70 % pure azadirachtin, ready for easy purification by HPLC.

Flash column chromatography is an inexpensive and easily performed technique, with a large sample capacity. The fact that only single runs were necessary in each preparative HPLC step was made possible due to the pre-purification of the neem seed crude extracts by flash chromatography [12,38]

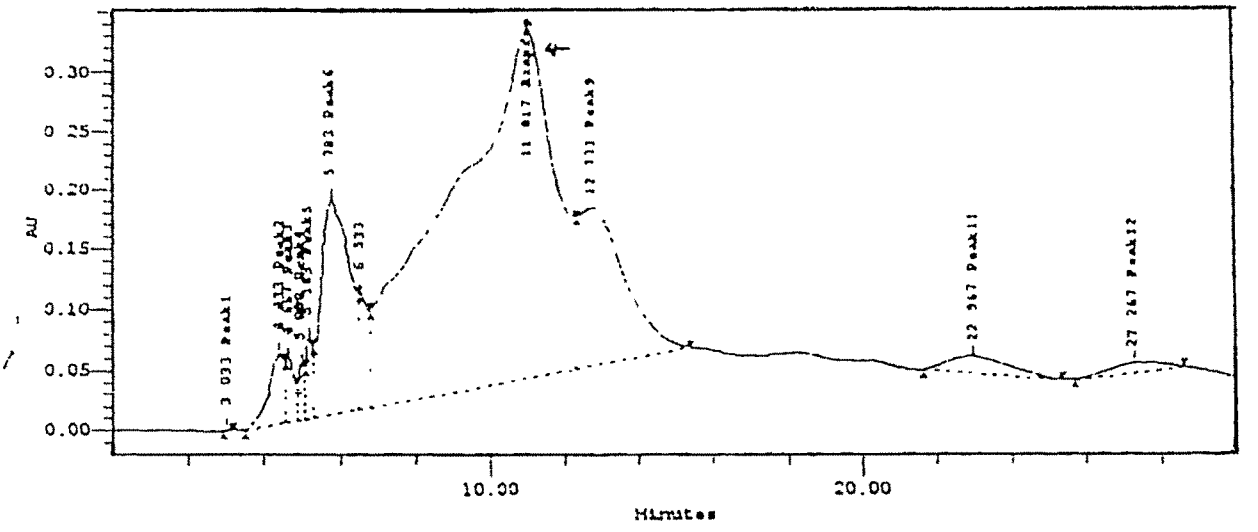
Purification of Aza-A up to 91 % was achieved through the preparative HPLC using crude extracts, while the present procedure affords enrichment up to 65–70 % by two flash chromatographic steps. The isolation of pure azadirachtin by preparative HPLC is much more easier and economical using a material containing 65–70 %




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type | % Area | Units | % Height |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 2.683 | 66531 | 1518 | | BV | 1.84 | | 4.04 |
| 2 | | 3.617 | 106440 | 1996 | | VV | 2.95 | | 5.31 |
| 3 | Aza | 6.100 | 991718 | 13646 | 568.46o | VV | 27.49 | ppm | 36.24 |
| 4 | | 6.917 | 1098707 | 11470 | | VV | 30.46 | | 30.50 |
| 5 | | 10.450 | 115576 | 1702 | | VV | 3.20 | | 4.53 |
| 6 | | 12.000 | 342465 | 2156 | | VB | 9.49 | | 5.73 |
| 7 | | 19.950 | 201522 | 1361 | | BB | 5.59 | | 3.62 |
| 8 | | 25.300 | 683942 | 3761 | | BB | 18.96 | | 10.0[illegible] |

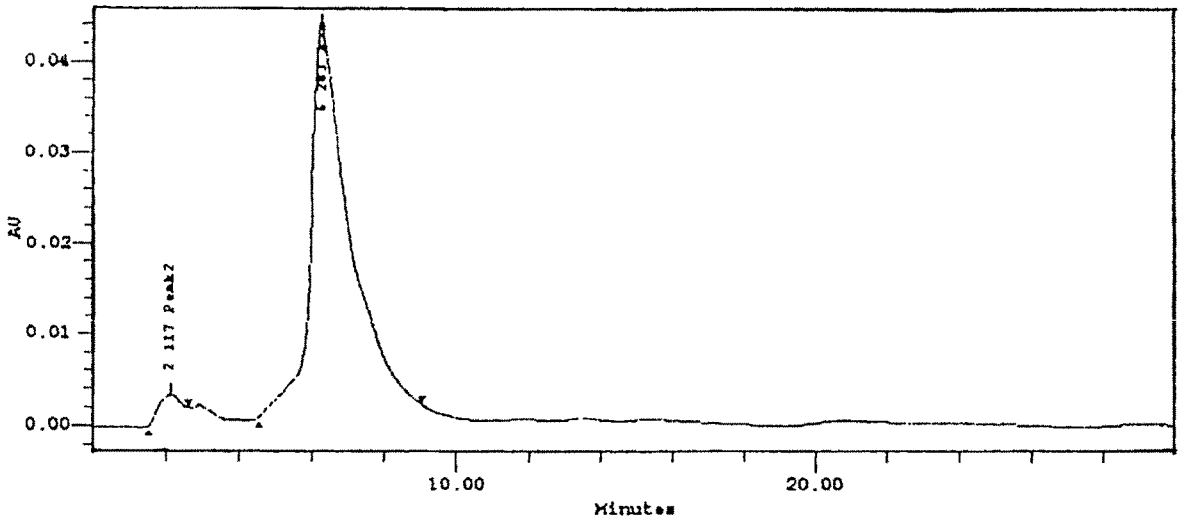
Figure: II.23 HPLC Chromatogram of Crude Material Containing azadirachtin-A.




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | % Area | Units | Start Time (min) | End Time (min) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Peak1 | 3.033 | 7034 | 825 | | 0.01 | | 2.900 | 3.183 |
| 2 | Peak2 | 4.433 | 1567462 | 57105 | | 1.84 | | 3.483 | 4.567 |
| 3 | Peak3 | 4.667 | 848652 | 52152 | | 1.00 | | 4.567 | 4.883 |
| 4 | Peak4 | 5.000 | 438487 | 45202 | | 0.52 | | 4.883 | 5.067 |
| 5 | Peak5 | 5.183 | 854629 | 65293 | | 1.00 | | 5.067 | 5.317 |
| 6 | Peak6 | 5.783 | 9223351 | 178334 | | 10.83 | | 5.317 | 6.500 |
| 7 | | 6.533 | 1495475 | 94851 | | 1.76 | | 6.500 | 6.783 |
| 8 | Peak7 | 9.067 | | | | | | | |
| 9 | Azaprep | 11.017 | 57014127 | 292212 | 50.000 | 66.98 | % | 6.783 | 12.300 |
| 10 | Peak9 | 12.733 | 11397191 | 130762 | | 13.39 | | 12.300 | 15.367 |

Figure: II.24 HPLC Chromatogram of azadirachtin-A.




*Peak Results*

| # | Name | Ret Time (min) | Area (uV*sec) | Height (uV) | Amount | Int Type |
|---|---|---|---|---|---|---|
| 1 | Peak1 | 1.633 | | | | Missing |
| 2 | Peak2 | 2.117 | 82168 | 2301 | | BB |
| 3 | Peak3 | 2.700 | | | | Missing |
| 4 | Peak4 | 3.050 | | | | Missing |
| 5 | Peak5 | 5.100 | | | | Missing |
| 6 | Peak6 | 5.883 | | | | Missing |
| 7 | aza | 6.283 | 3189733 | 42236 | 0.068 | BB |
| 8 | Peak8 | 8.850 | | | | Missing |

Figure: II.25 HPLC Chromatogram of Pure azadirachtin-A.

Aza-A, in which each run takes only 30-40 minutes as against 60-90 minutes with crude extracts. This avoids contamination of the column, thereby enhancing its life while maintaining the efficiency, in addition to saving on solvents and time.

## II.5 SUMMARY

The present study describes a short and economical extraction procedure of Aza-A from seed kernel. In addition, an improved reverse phase HPLC method, for the quantification of Aza-A in neem seed kernels and its purification of single peak purity using the preparative HPLC-UV technique is presented.

From the HPLC analysis and calculations shown above, it can be seen that the azadirachtin content in the neem seed kernels is found to be 0.1188 %, while that in the fruits is 0.03029 %.

The HPLC-UV analyses results showed that the Soxhlet extraction of defatted neem cake gives higher percentage of extracted Aza-A, as compared to conventional method of extraction.

It was possible to purify gram quantities of the crude material in each run of flash column chromatography. Two consecutive steps of flash chromatography thus quickly gave material containing 65-70 % Aza-A, from a crude material containing 9.14 % Aza-A obtained from plant source. The product of two flash chromatography steps when subjected to reverse phase preparative HPLC gave up to 91 % pure Aza-A in each single run.

The presented methodology may also serve as a useful analytical tool for future users of this insecticide.

## II.6 EXPERIMENTAL

Melting point is determined by the open capillary method and is uncorrected. Ultra-violet spectrum was recorded on a Perkin-Elmer Lambda-19 spectrophotometer. An IR spectrum was recorded on a Perkin-Elmer PC-16-FTIR spectrophotometer. Proton Magnetic Resonance spectra (200MHz) and $^{13}$CMR (50MHz) were recorded on a Bruker-200-FTNMR spectrometer using $CDCl_3$ as solvent containing tetramethyl silane (TMS) as an internal standard and chemical shift values are reported in $\delta$, ppm downfield with respect to TMS. Microanalyses were performed on a Perkin-Elmer 2400 series II instrument.

Thin layer chromatographic (TLC) analyses were done on plates of 0.25 mm thick pre-coated layer of silica gel 60 F-254 (Merck). The plates were developed in solvent systems, as presented in Table: II.4. The developed plates were air-dried, sprayed with 3 % (w/v) vanillin in absolute alcohol (100 mL) containing 1 % (v/v) conc. sulphuric acid and gentle heating. Azadirachtin was detected by characteristic green colour of the spot.

Flash column chromatography, which refers to the low-pressure system described by Still and co-workers.[12] was performed using Acme's silica gel (230-400 mesh size) unless otherwise stated. The elution was done with hexane : ethyl acetate as well as chloroform : acetonitrile mixtures. The fractions eluted from the flash column were concentrated on a Buchi-RE-111 rotary evaporator.

All the solvents including HPLC grade ones were obtained from Qualigens, Glaxo India Ltd., and filtered through a Millipore filter (0.45 μm). Methanol was dried over magnesium methoxide, prior to distillation. Acetonitrile was dried by treating it successively with activated silica gel anhydrous $CaH_2$ followed by distillation. Water

(18Ω) HPLC grade was obtained by Elga water purification system, Life Science Maxima and filtered as above.

Solvent extractions were carried out at the room temperature using an overhead mechanical stirrer for different time intervals unless otherwise mentioned.

**Plant Material**

The seeds developed over a period of four months from July to October were collected from Kheda region, Gujarat state, India. Seeds were dried and stored at 29 ± 2°C while maintaining relative humidity between 60–65 %, protected from sunlight but with free circulation of air. The soft husk was removed from the seeds using a decoating machine.

**Standard Azadirachtin-A**

Analytical grade standard Aza-A was purchased from Sigma Chemical Co. (95 %, TLC). A 1000 ppm stock solution of the standard Aza-A in methanol (0.5 mg /0.5 mL) was prepared and stored between 0-4°C. Aliquots of the solutions were diluted with mobile phase prior to use, to give working solutions containing Aza-A in the range of 31.25 to 250 ppm, which on injection gave a distinct single peak with retention time of 6.95 minutes (Figure. II.10).

- **Extraction And Enrichment**

Extraction of the kernel powder was carried out by the method of Schroeder and Nakanishi with some modification.[39] The changes made were that extraction with ethanol, vacuum liquid chromatography and flash chromatography were omitted to make the process simpler and more efficient. The steps used in the extraction procedure are given in Figure II.8.

Finely ground powder of neem seed kernel (500 g) was extracted with hexane (1.0 L) for 30 minutes. The hexane extract was filtered and the process repeated with fresh hexane three more times (Table: II.1). The pooled hexane extracts were concentrated under reduced pressure in a rotary evaporator between 45-50°C, which yielded fatty oil (A) (40-45 %) having 0.0235 % Aza-A (w/w of oil) (Figure: II.9) as determined by following the procedure of Sundaram *et al*[42]. The dry defatted cake (B, 250 g) was then extracted with methanol (6 × 500 mL) (Table: II. 2).

The six methanolic extracts were analyzed for Aza-A content by analytical HPLC-UV method (*vide infra)*. These combined extracts were then concentrated under vacuum after filtration to give a brown residue (C, 200 mL) and an organic powder (D). The brown residue containing Aza-A was further extracted with ethyl acetate (300 mL) for 30 minutes to give (E) (Table: II.3). This was then washed with distilled water (150 mL) to remove water-soluble compounds such as carbohydrates, proteins and minerals followed by passing it through a column of anhydrous sodium sulfate (17 mm I.D. × 45 cm). The column was rinsed with ethyl acetate (25 mL), which was added to (E). It was then concentrated under reduced pressure to furnish a brown residue (F), which was washed with hexane (4 × 15 mL) to give a dark brown amorphous solid (G, 6.5 g). A weighed portion of this solid was dissolved in methanol for the estimation of Aza-A content in it using analytical HPLC-UV method (*vide infra*) (Figure:II.11).

- **Soxhlet Extraction Experiments:**

The Soxhlet extraction experiment was carried out in a 1 L Soxhlet apparatus. Finely ground powder of neem seed kernels (500 g) was extracted with hexane (1.0 L). The defatted solid cake (B, 230 g)

obtained by following the above procedure was taken in an extraction thimble and extracted with methanol (500 mL) for 24 and 72 hours independently, at reflux temperature. The temperature was so adjusted to have 250-300 drops of the refluxing solvent per minute. The thimble was taken out, dried and the defatted cake was weighed. The solvent was concentrated using a Buchi rotary evaporator at reduced pressure. These experiments were carried out at 24 hours and 72 hours independently and their Aza-A content were analyzed by HPLC-UV method (*vide infra*) at intervals of four hours.

- **Flash Column Chromatography of Crude Material Containing 9.14 % Azadirachtin-A :**

A method was devised to accomplish a large-scale preliminary purification of azadirachtin from the remaining matrix. Figure: II. 21 shows simple flash chromatographic steps involved in the purification of Aza-A from very complex mixture.

A long glass column (90 cm × 32 mm I.D.) was selected and a small plug of glass wool was placed in the tube connecting the stopcock to the column body. Column was packed with flash grade silica gel (230-400 mesh size, 75 g) suspended in hexane.

The crude material (5.08 g, 9.14 % Aza-A) dissolved in minimum quantity of methanol was adsorbed on silica gel (8.0 g, 230-400 mesh size) by stirring and evaporating the solvent under reduced pressure on Buchi rotavapor. The dry powder thus obtained was then placed on the top of the adsorbent column.

The flow controller was fitted tightly to the top of the column by securing with rubber bands. The needle valve of the flow controller was opened fully. The flow controller was briefly placed on the top of

the column initially and was finally tightly fitted on the top of the column, while applying air pressure of 400-500 Hg from the top of the column through it.

The column was then eluted with hexane, followed by hexane containing increasing proportions of ethyl acetate and finally with ethyl acetate. The flow rate was maintained at 8-10 mL/min and fractions of 15-20 mL were collected.

Fractions containing Aza-A (EtOAc: hexane, 75:25) as monitored by TLC (Table: II.4), were pooled and concentrated under reduced pressure furnished solid residue (2.48 g), which dissolving in boiling $CCl_4$, which upon cooling yielded 27-30 % azadirachtin (1.8 g) containing pale yellow colour microcrystalline material. The HPLC chromatogram of the 27-30 % azadirachtin containing material is shown in Figure: II. 23. This crystallization step is not an exacting process and is reproducible.

This material was dissolved in minimum quantity of methanol, filtered through a Nylon-66 Himedia filter (0.45 removal rating and 0.13 mm dia) applied by pipette to the top of the adsorbent bed of a second flash column (44 cm × 17 mm I.D.) and 300-450 Hg air pressure is applied. The column was then eluted in gradient mode with chloroform-acetonitrile mixture (75:25) at flow rate of 5 mL/min in to fractions of 10- 15 mL each.

Fractions containing azadirachtin ($CHCl_3$: $CH_3CN$, 75:25) were again monitored by TLC, pooled and concentrated under reduced pressure furnished a solid residue, which was recrystallization from carbon tetrachloride yielded 750 mg of 65-70 % pure azadirachtin containing off white material, the HPLC chromatogram of which is represented in Figure: II.24. This material was further pre-purified

up to more than 91 % purity by using reverse phase preparative HPLC (*vide infra*).

- **High Performance Liquid Chromatography**

Analytical as well as preparative HPLC-UV studies were carried out on a Waters LC-4000 system linked with chromatography software Millennium 2010. HPLC system was equipped with a Flow controller (Waters model No: 600), a tunable absorbance UV Detector (190-600 nm, Waters model No: 486), a guard column, Rheodyne injector 7010 and an automatic fraction collector. The instrument employed an automatic degassing system and dual pump heads with common drive flows. The Millennium 2010 provided the chromatograph, calibration curve, peak area, area % and retention time ($t_R$) etc.

Analytical HPLC-UV was carried out on a reverse phase $C_{18}$ Bondapak column (3.9 mm I.D. × 300 mm) with 10 μm particle size and 125Å pore size. The solvent system consisting of methanol: water (60:40) was run isocratically at a flow rate of 1 mL/min and an average pressure of 1500-2000 psi for an injection volume of 20 μL. The peak corresponding to Aza-A was detected at 217 nm. Detected peaks were integrated (valley-to-valley) and retention time was recorded using Millennium 2010 set at peak width of 30.00, retention window 5% and threshold 25. The chromatographic run time was 30 minutes.

The brown amorphous solid (1.0 g, 9.14 % purity) obtained after concentration of organic layer (F), was dissolved in methanol and filtered through a Nylon 66 Himedia filter (0.45 μm removal rating and 0.13 mm diameter). This solution (1 mL) was then injected into a preparative reverse phase $C_{18}$ Bondapak (19 mm I.D. × 300 mm) column, with 15-20 μm particle size and 125 Å pore size, and eluted

isocratically with methanol : water (60:40). The peak was detected at 217 nm under 400-500 psi column pressure at a flow rate of 15 mL/min. Under these conditions Aza–A peak appeared at 9.16 min during the preparative run. When the Aza-A peak ascended from the baseline, the eluent was collected until the peak was at maximum and the peak descended to the baseline again. The presence of the Aza-A in fraction was confirmed by the analytical HPLC-UV (*vide supra*). The less polar compounds could be removed by eluting the column with pure methanol for 10 to 15 min at a flow rate of 20 mL/min. Restabilisation of the column was done with methanol: water (60:40) for another 20-25 min.[44]

Azadirachtin-A

| | | | | |
|---|---|---|---|---|
| M. P. | : | 154-158°C (dec.) | | |
| I.R | : | 3452, 2957, 1738, 1650, 1620, 1438, 1379, 1157, 1050, 738 $cm^{-1}$. | | |
| UV ($\lambda_{max}$) | : | 215 nm ( $CH_3OH$) | | |
| PMR (200 MHz) | : | Figure: II.17 | | |
| $^{13}$CMR(50 MHz) | : | Figure: II.18 | | |
| Analysis | : | Found | C, 58.28 % | H, 6.586 % |
| | | Calculated | C, 58.33 % | H, 6.111 % for $C_{35}H_{44}O_{16}$ |

Table: II. 1 Extraction of Neem seed kernel powder using Hexane

| Exp. No. | Neem seed kernel powder (g) | Temp. | No. of Extractions | Total Volume. of solvent | Stirring time (Min.) | Recovered solvent after Extraction (mL) | Wet cake weight (gm) | Dry cake weight after 3rd extraction (g) | Fatty oil (mL) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 500 | Room Temp. (35°C) | 1 | 1 L | 30 | 800 | 512.00 | 250.00 | 198 |
| | | | 2 | 1 L | 30 | 850 | 473.00 | | |
| | | | 3 | 1 L | 30 | 880 | 450.00 | | |

Table: II. 2 Extraction of Neem seed kernel powder using Methanol

| Exp. No. | Defatted powder (g) | Temp. | No. of Extractions | Total Volume of solvent (mL) | Stirring Time (Min.) | Recovered solvent after Extraction (mL) | Concen-trated Material (mL) | % Azadirachtin |
|---|---|---|---|---|---|---|---|---|
| 1 | 250 | Room Temp. (35°C) | 1 | 500 | 30 | 350 | 320 | 0.2199 |
| | | | 2 | 500 | 30 | 480 | | 0.0959 |
| | | | 3 | 500 | 30 | 485 | | 0.0399 |
| | | | 4 | 500 | 30 | 470 | | 0.0131 |
| | | | 5 | 500 | 30 | 490 | | 0.0127 |
| | | | 6 | 500 | 30 | 490 | | 0.0041 |

Table II. 3 Extraction of the concentrated material (From Table: II. 2) with ethyl acetate

| Exp. No. | Concen-trated material (mL) | Total Volume of solvent | Stirring time (Min.) | Volume of water (mL) | Total Volume of Organic layer (mL) | Concen-trated Brown residue (mL) | Solid powder (g) | % Purity of the powder |
|---|---|---|---|---|---|---|---|---|
| 1 | 320 | 600 | 30 | 150 | 400 | 22 | 6.53 | 9.14 |

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