RESEARCH ARTICLE

Studies on extraction and HPLC Analysis of Azadirachtin from Kernels of Neem Seeds

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ABSTRACT

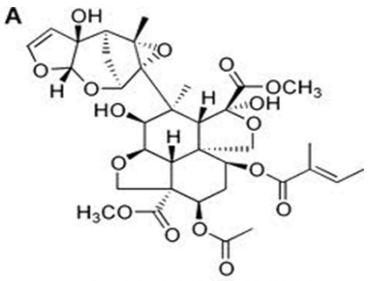
The neem tree has been known for its unique properties in improving the human health and it also acts as an antiseptic agent. Among the various limonoid components of the neem, Azadirachtin is the most important component which has many anti infective and anti microbial properties. Azadirachtin is a tri terpenoid limonoid obtained from various parts of the neem. Azadirachtin is extracted from the dried neem kernel powder using Di-Chloro methane as solvent. The UV absorbance of Azadirachtin was found to be 220nm. The qualitative analysis of Azadirachtin was carried out by HPLC, on a C-18 column at a flow rate of 1ml/min, using Aceto nitryl: Methanol: 1% Triethyl amine p^H 4 (60:40:1) as mobile phase at 210nm. The TLC, UV and HPLC reports indicate the isolation of Azadirachtin from the seed kernel powder of the neem.

Keywords: Azadirachtin, Neem seed kernels, Di-Chloro methane, Soxhlet extractor, UV Spectrophotometer, HPLC.

Introduction

The botanical name of the neem is 'Azadirachta indica' and it belongs to the family 'Meliaceae'. Neem is an ever green tree which grows well in all types of soils.[1] About 14 million neem trees are present in India. It has become focus of attention all over the world due to its medicinal and pest control activities. It grows about 40 to 60 feet high. It is used in treating many kinds of diseases in Ayurveda.[2] Various components of neem consists of varied amount of Azadirachtin. It is shown in the Table no.1

- Neem compounds belong to a general class of natural products called 'limonoid'.
- Almost each and every part of the neem tree have medicinal properties. About 60% of the neem fruit goes uncollected.[3]
- Fruits and seeds are used for oil extraction whereas neem oil is widely used in soap industry. Neem extracts are used as technical material for formulations; these formulations are used in crop protection.
- Seeds contain kernel, which in turn contains 'Azadirachtin' and other bitter compounds
- **Azadirachtin** is the most important and active component. The other components present are Nimbin, Nimbinin, Nimbidin, oleic stearic and Palmitic acids, Quercitin and other limnoids.
- Azadirachtin is currently considered as neem's main agent for controlling insects.[4]
- Molecular weight of Azadirachtin is 720; and its melting point is 160ºC



Structure of Azadirachtin

Materials and Methods

Chemicals:

The chemicals used like Di-Chloro methane, Ethyl acetate, Hexane. Acetonitrile (HPLC grade), methanol (HPLC grade) were obtained from Merck specialties private limited, Mumbai, India. Triethylamine (GR grade) was obtained from Merck specialties private limited, Mumbai. Ortho-phosphoric acid (GR grade) was obtained from S.D. Fine Chemicals Ltd, Mumbai, India.

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Materials:

Ripe neem fruits were obtained from the local vegetable market, Hyderabad.

Instruments:

Soxhlet set up, Shimadzu UV-visible Spectrophotometer (model UV-1800), HPLC

Method

Preparation of neem extract:

The whole neem tree contains bitterness in varied extent, but higher concentration of it is found in the neem kernel. Neem kernel is a valuable source of major limnoid. It is therefore essential to understand the scientific method of fruit collection and depulping to get kernels.

Collection of Neem Fruits:[3]

The neem yields fruits during May to August every year. Being rich in carbohydrates neem fruits gets attacked by fungi when come in contact with soil. Such fruits may damage the quality of the final products prepared from these fuits. Hence it is strongly recommended to avoid contact of neem fruits with soil. As the fruit ripes they must be depulped as early as possible.

Depulping of Neem Fruits:

Depulping is a process to remove seed coat and pulp from the neem seed. It is done by hand and using mechanical depulper. Rub the ripe neem fruits between palms in the bucket of water and wash the seed. Use clean water for depulping. Neem Research and Technology Development Centre (NRTDC) developed a mechnical depulper to handle large quantity of neem fruits. After depulping and cleaning, dry the neem seeds in the shade in a thin layer. Keep the neem seeds in a cool and dry place. If processed properly these neem seeds can be stored for about 6-12 months.

Fig. 1 Dried neem seeds

Fig. 2 Dried neem kernels

Separation of the kernels:

The dried neem seeds are ground slightly by hand and the outer shell of the seed is removed. Kernels are present inside the shell which are separated and then made into powder using a grinder. It should be pounded such that no oil comes out of it. This coarse powder is used for further studies for the extraction of Azadirachtin.

Extraction of Azadirachtin from neem kernel powder:

For extraction of Azadirachtin by solvent process, weigh about 500g of fine tea powder sized clean neem kernel powder. It should be pounded in such a way that no oil comes out of it. Make a thimble and fill it with kernel powder, place it in a Soxhlet apparatus and add about 600ml of Di-Chloro methane. Keep it on a heating mantle and heat to reflux for about 12 hours. When the powdered kernel is extracted with solvents like Di-Chloro methane or ethyl acetate, liminoids and other constituents get dissolved in it, leaving the seed cake without any active components. The solvent from this mixture is recovered by distillation. The distilled or concentrated solution is kept aside for cooling. Hexane is added to the concentrate and then filtered using Vaccum pump. The above residue is dried which gives a pale greenish colored powder. This powder consists of Azadirachtin and a very small quantity of Nimbin. 100gm of neem kernel powder on extraction gives about 1gm of Azadirachtin.

TLC confirmation

Take a small amount of Azadirachtin and dissolve it in methanol. Spot the sample on a TLC plate and place it in a beaker consisting Iso-Propanol : Hexane(2.5:17.5) as mobile phase. Run till 3/4th of the plate, then place it in Iodine chamber. It gives two pale yellow colored

spots. The upper spot indicates Azadirachtin and the below spot indicates nimbin.

UV spectrophotometer [5]

Different concentrations of Azadirachtin were prepared with different solvents and scanned in the UV region. The wavelength 220nm was selected for Azadirachtin, where it show maximum absorbance. Lower concentrations does not show any absorbance, hence concentrations (above 10mcg) were taken for calibration. The calibration curves were linear over the concentration range of 30-70 µg/ml of Azadirachtin. Absorbances were plotted versus respective concentrations.

High performance liquid chromatography [6]

The retention time of Azadirachtin is determined using HPLC. The column used for chromatographic separations was reverse phase phenomenex® Luna 5µ C18 (2) 100A (250 × 4.60 mm i.d). A wave length of 217nm was tried but it gave many peaks of impurities hence, the analytical wave length of 210nm was set and the sample was injected. The chromatographic separations were accomplished using mobile phase consisting of Aceto nitryl: methanol: 1% tri ethyl amine adjusted to pH 4(60:40:1) , filtered through a filter using Value stage vacuum pump. Mobile phase was pumped at a flow rate of 1 ml/min at room temperature.

a) Preparation of sample solutions

Weigh about 10mg of Azadirachtin, transfer into 10ml volumetric flask make up the volume with methanol. The volumetric flask was sonicated for 2-3 minutes to affect the complete dissolution of the compound . Suitable aliquots of compound solutions were prepared and injected to HPLC to obtain concentration in the linearity range. A typical chromatogram obtained from the analysis of Azadirachtin, using the optimized chromatographic conditions at 210nm is shown in Figure 4 which shows a single sharp peak representing Azadirachtin.

The literature review shows that there is no much work done on extraction of Azadirachtin from kernels of neem seeds using Di-Chloro methane as a solvent and HPLC analysis at 210nm.[7-10]

Results and Discussion

TLC studies:

Azadirachtin gave pale yellow colored spot when placed in an Iodine chamber with mobile phase Isopropanol: Hexane in the ratio 2.5:17.5

UV-studies:

Azadirachtin showed absorbance at 220nm in the UV spectrophotometer. Correlation coefficient of Azadirachtin was found to be 0.998. The calibration curve of Azadirachtin is shown in Fig. 3. The calibration data is given in Table no. 2.

HPLC studies:

Using the optimized chromatographic conditions baseline was recorded. The Azadirachtin compound solutions were injected and chromatograms were recorded. Retention time of Azadirachtin was found to be 3.8min. The chromatogram of Azadirachtin at 210nm in Aceto nitryl: methanol: 1% tri ethyl amine adjusted to pH 4(60:40:1) mobile phase is shown in Fig. 4

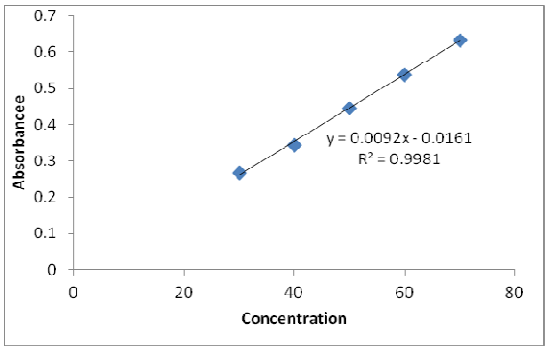

Fig.3 Calibration curve of Azadirachtin

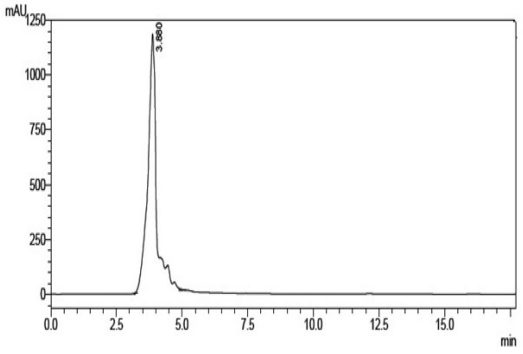

Fig.4 Chromatogram of Azadirachtin

Conclusion

The process adopted for extraction and separation of Azadirachtin from dried neem kernel powder is simple and feasible. The product thus obtained is 40-50% pure and is reproducible. From the TLC, UV and HPLC studies, it is confirmed that the method adopted have resulted in the extraction of Azadirachtin from the powdered neem kernel.

Acknowledgement

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Table no.1 Content of Azadirachtin

Neem part and products	Aza content (%)
Neem oil	0.01-0.1
Aza-enriched oil	0.1-1.0
Seed Cake	0.005-1.2
Seed Kernels	0.35-0.89
Aqueous extract	0.001-0.02

Table 2: Calibration data of Azadirachtin

CONCENTRATION	ABSORBANCE
10mg	0.122
20mg	0.202
30mg	0.268
40mg	0.343
50mg	0.446
60mg	0.538
70mg	0.632
80mg	0.147
90mg	0.813
100mg	1.198

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