PRESSURIZED LIQUID EXTRACTION OF AZADIRACHTIN FROM NEEM (*Azadirachta indica* A. Juss) SEED KERNELS

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Abstract:
Botanical insecticides are in limelight nowadays owing to the increasing sustainable agricultural practices and environmental and health hazards arising out of excessive usage of their synthetic counterparts. The Neem (*Azadirachta indica* A. Juss) tree, belonging to the Meliaceae family, is a storehouse of insecticidal compounds possessing antifeedant, growth disrupting and larvicidal properties against an array of agricultural insect pests. Pressurized Liquid Extraction (PLE) is fast emerging as an efficient means for recovering valuable active ingredients from natural plant matrices at an accelerated rate and with a reduced solvent consumption. The technique employing heated organic solvents at elevated pressures is a potential green substitute for conventional solvent extractions. The present study highlights the applicability of PLE method to obtain azadirachtin rich extracts from defatted neem seed kernels (NSK). Important operating variables like temperature, pressure and extraction time influencing the extraction efficiency were investigated. The azadirachtin content in the extracts was determined by HPLC. Classical solvent extractions were employed for comparative evaluation.

INTRODUCTION

The indiscriminate use of synthetic pesticides has resulted in ecological and health hazards along with development of resistance in insect pests. This has led to the popularity of pest control agents derived from plants as they are biodegradable, environmentally compatible and less toxic to non-target organisms. Neem (*Azadirachta indica* A. Juss), belonging to Meliaceae family, leads the list of plants with the highest potential for this purpose [1]. Neem contains several biologically active chemicals called limonoids such as Azadirachtin A, nimbin, salannin, azadirachtol, nimbidin, gedunin, etc. These compounds are responsible for diverse activities such as insect antifeedant, insect growth disrupting, insecticidal, nematicidal, fungicidal, bactericidal etc. [2]. Azadirachtin is the most potent and the most abundant (0.2 to 0.6 % w/w) chemical found in seed kernels of neem. The bitter extracts from neem kernels obtained by alcoholic extraction contain various bioactive limonoids [3].

Pressurized Liquid Extraction (PLE), also known as Pressurized Hot Solvent Extraction (PHSE), allows the use of solvents or solvent mixtures with different polarities under high pressures (500-3000 psi) which keeps the extraction solvent in the liquid state and temperature ranging from room temperature up to 200 ^{o}C. Increased temperature accelerates the extraction kinetics and enhances diffusivity of the solvent resulting in increased extraction speed. On the other hand, high pressure forces the solvent into the matrix pores and facilitates extraction of analytes [4]. Few recent applications of PLE in natural products field include extraction of several bioactive compounds such as paclitaxel and related compounds from the

bark of *Taxus cuspidata* [5]; naphthodianthrones from St. John's wort, saponins from horse chestnut seeds and terpenes from thyme [6]; berberine from *Coptidis rhizome* [7]; polar steroids from leaves of *Iochroma gesnerioides* [8]; ginsenosides from *Panax ginseng* [9,10]; isoflavones from soybeans [11]; antioxidants from *Spirulina* microalga [12]; rotenone from *Derris elliptica* and *Derris malaccensis* stems and roots [13]; charantin from Momordica charantia fruit [14] and phenolic compounds from parsley flakes [15].
Classical extraction processes like maceration and percolation, which employ large quantities of organic solvents, still remain an important source of obtaining biologically active compounds from neem seed kernels [16]. Abrosino *et al.* [17] reported extraction of azadirachtin A from neem kernels by supercritical CO_2. Johnson and Morgan [18] also reported supercritical fluid extraction of oil and triterpenoids from neem seeds. Microwave assisted extraction (MAE) of azadirachtin related limonoids (AZRL) has also been reported [19]. But, so far, there is no published report on Pressurized Liquid Extraction of neem kernels. The present work aims at the applicability of PLE for the recovery of azadirachtin from defatted neem kernels.

MATERIALS AND METHODS

Plant Material and Chemicals

The neem seeds were collected from Gujarat region (India) in June 2007 and were decorticated in a hand operated Decorticator. The neem seed kernels thus obtained were ground in a kitchen mixer grinder and were defatted with petroleum ether (60-80 ^{o}C). The defatted neem seed kernel powder was used for subsequent extractions employing methanol as solvent. Methanol (AR grade), Petroleum Ether (60-80 ^{o}C), Water and Acetonitrile (HPLC grade) were purchased from Qualigens Fine Chemicals (Mumbai, India). Azadirachtin powder used as reference standard was gifted by M/s Minal Exim (Mumbai, India).

Extraction Procedures

Pressurized Liquid Extraction (PLE)

PLE experiments were performed in a laboratory-assembled system as shown in Figure 1. The extractor made up of stainless steel (Type 316) and having 100 mL internal volume was loaded with a known weight of sample and a plug of glass wool was placed at the top and bottom of the extractor followed by a fine wire mesh cloth to prevent carryover of material in the outlet tubing. The loaded extractor was then connected to the system and hot water from thermostatic water bath set at desired temperature was allowed to flow through the brass jacket surrounding the extractor. The temperature inside the extractor reached the set value (steady state) after about 30 min following which the system was pressurized by pumping the extractant (Methanol) with the help of a high pressure air-driven liquid pump (MSF 72L, Maximator GmbH, Germany). Before entering the extractor, the solvent was preheated to the extraction temperature by passing it through a heating coil which was maintained at the same temperature as the extractor. The outlet pressure control valve V_2 remained closed during this operation. Once the system was pressurized to the desired value, valve V_2 was opened slowly and the dynamic runs started for allowing the extract collection in vials over predefined time. The liquid extracts thus collected were filtered and evaporated to dryness under reduced pressure in a rotary evaporator (Laborota 4000 Efficient, Heidolph Instruments,Germany).

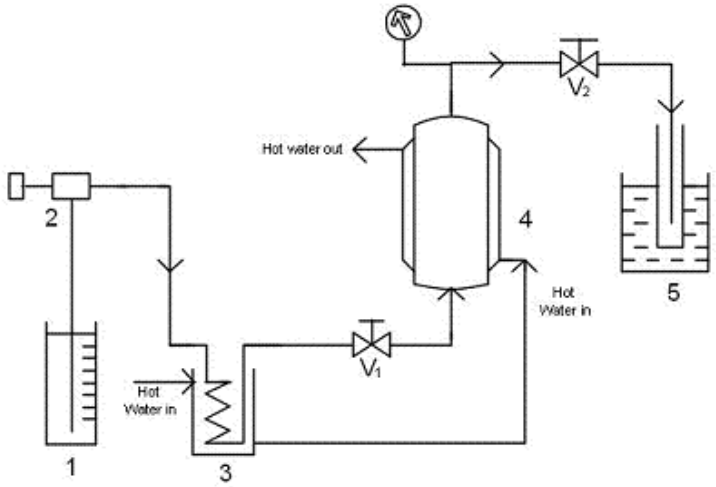

Figure 1: Schematic diagram of PHSE system
1-Extractant reservoir 2-Air-driven high pressure pump 3-Heat exchanger 4-Extractor 5-Extract collector V_1 and V_2-Pressure control valves

Maceration
It is a cold extraction process which was carried out by extracting 30 g defatted kernels with 300 mL (3 x 100 mL) of methanol in a conical flask. The mixture was occasionally stirred and left for 3 days, after which it was filtered and the solvent evaporated under vacuum to obtain the dried crude extract which was weighed. The extraction yield was determined gravimetrically.

Batch Stirring
In batch stirring extraction, 300 mL methanol was added to 30 g of defatted kernel in a conical flask. A magnetic pellet was dropped in the flask which was finally mounted on a magnetic stirrer plate. The mixture was stirred for 8 h. Finally, the solvent was evaporated *in vacuo* to obtain crude extract.

HPLC Analysis
A known weight of crude extract obtained was re-dissolved in methanol (HPLC grade) and the solution thus obtained was filtered through 0.45 μm membrane filter prior to injection onto the HPLC column. Perkin-Elmer system comprising of Series 200 pump and Series 200 UV-Vis detector set at 217 nm was used. The mobile phase consisted of Acetonitile:Water mixture (35:65, %v/v). The separation was achieved in a Purospher-Star RP 18-e column (250 mm x 4.6 mm id, 5 μm). Injection volume was 20 μL and the mobile phase flow rate was 1 mL/min. External standard calibration method was employed for the quantitative determination of azadirachtin.

RESULTS AND DISCUSSION
Effect of pressure on the extraction yield and the azadirachtin content in the extract:
Four different pressurized liquid extractions were conducted at 50, 100, 150 and 200 bar at a constant temperature of 50^{o}C to investigate the effect of pressure. All the runs were conducted with Methanol as solvent for approx. 30 min, resulting in a collection of 100 mL extract volume. The results are shown in Figure 2. No significant difference was observed

either in the extract yield or the azadirachtin concentration in the extract as the pressure was varied from 50 to 200 bar. It is in good agreement with similar trends reported earlier, proving pressure to be an insignificant parameter in PLE [10,11,13]. The pressure does help in maintaining the solvent in liquid state above its boiling point. Hence, a threshold pressure of 50 bar was chosen for the rest of extractions.

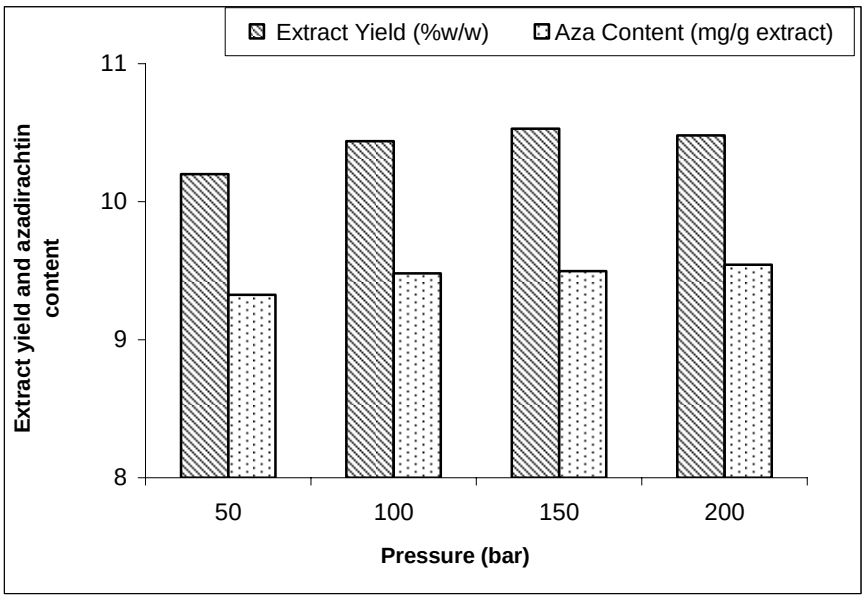

Figure 2. Effect of Pressure on extraction yield and azadirachtin content

Effect of temperature and time on the extraction yield:

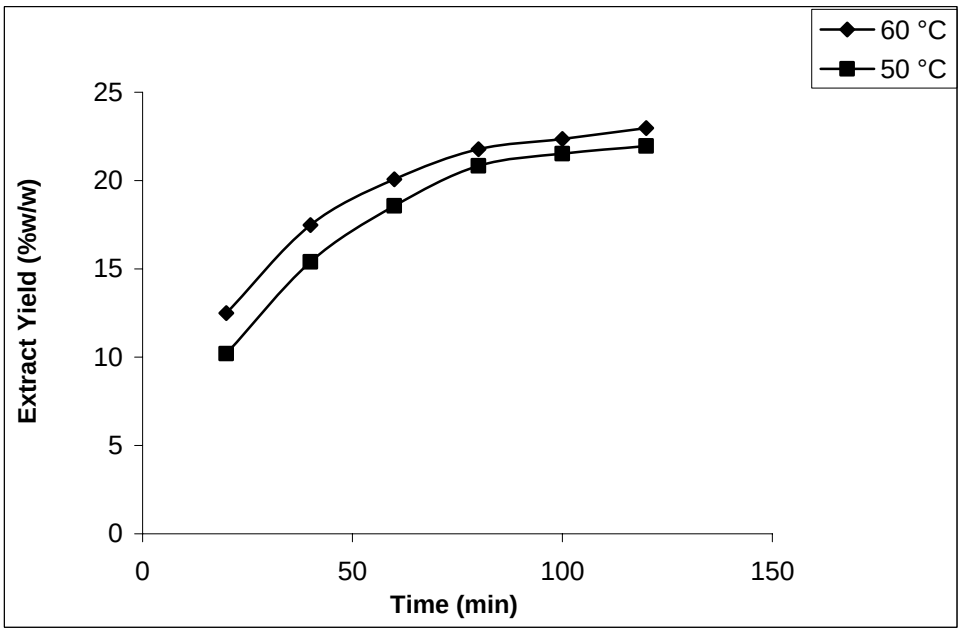

Figure 3. Effect of extraction time on extract yields at different temperatures

The effect of extraction time on the extract yield was tested at two different temperatures (50 and 60 ^{o}C). The pressure maintained was 50 bar and the solvent flow rate through the extractor was 5 mL/min. Figure 3 shows the results obtained. With increase in temperature, an increase in the extraction yield is observed due to the fact that higher temperature disrupts the strong analyte-matrix bonds releasing the solutes. Moreover, enhanced temperature also results in higher solvent diffusivity and higher solubility of analytes in the solvent [4]. However, when analyzing the azadirachtin content of the extracts, a slight decrease was observed at higher temperature due to the thermal degradation of the compound. As far as extraction time is concerned, an increase in the yield of extract was seen till 80 min after which it increased slowly. This indicates that an exhaustion period has reached after 80 min with majority of soluble compounds being leached out of the matrix. A typical HPLC chromatogram of extract obtained at 50 ^{o}C after an extraction time of 80 min is shown in Figure 4. Aza A in the chromatogram is the azadirachtin peak.

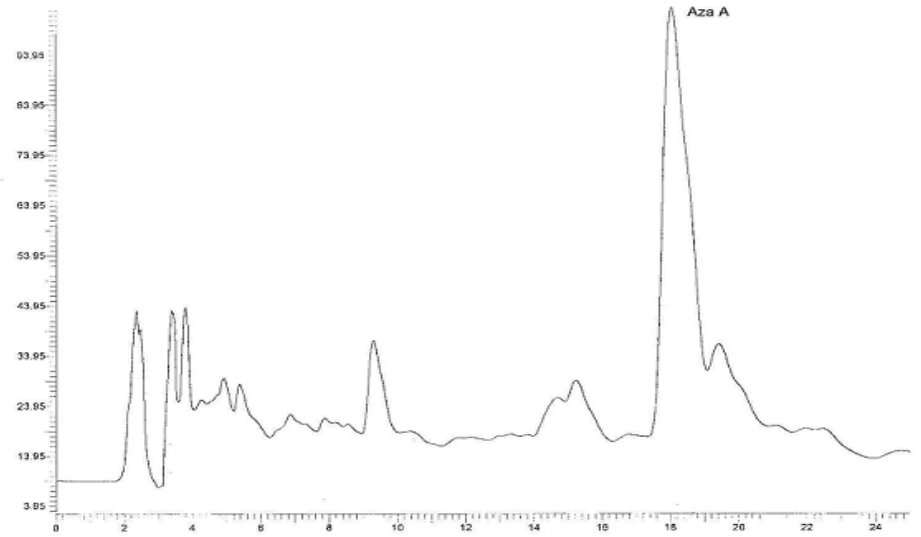

Figure 4. HPLC Chromatogram of PLE extract from defatted neem kernel powder

Comparison of Extraction Methods

To highlight the superiority of PLE on extraction time and solvent usage front, it was compared with conventional methods, viz. Maceration and Batch Stirring. The results obtained from this comparative study are shown in Table 1.

Table 1: Comparative evaluation of different extraction methods

Parameters	**Maceration**	**Batch Stirring**	**PLE**
Solvent to Solid Ratio (mL/g)	10	10	6
Extraction Time (h)	72	8	1
Extract Yield (% w/w))	10.48	12.14	15.06
Azadirachtin Content in the Extract (ppm)	4330	5140	9510

It is clear that PLE results in a higher crude extract yield along with higher azadirachtin content in the crude extract. Also, a reduction in time of extraction and solvent usage per unit mass of raw material are clearly highlighted in case of PLE. However, due to requirements of high pressure process instruments, the cost of the extract production will be comparatively higher while employing PLE. But the semi-batch processing and possible semi-automation in large scale operation combined with saving of labour and time may help in cushioning the high costs involved in PLE operations in the long run [13].

CONCLUSIONS

The Pressurized Liquid Extraction seems to be a feasible method for recovering azadirachtin rich concentrates from neem seed kernels. The comparison with classical methods shows PLE a more efficient method with higher extract yields at a reduced solvent consumption in a shorter time period. The faster rate of extraction and less solvent use would help in making PLE a potential green method for extracting valuable phytochemicals by reducing ecological footprint of bulk organic solvents utilization.

REFERENCES

[1] Kumar, A.R.V., Current Science, 84, **2003**, 265.
[2] Kavathekar, K.Y., "Neem in India", NISCAIR, New Delhi, **2003**, pp 21.
[3] Govindachari, T.R., Sandhya, G., Ganeshraj, S.P., Journal of Chromatography, 513, **1990**, 389.
[4] Richter, B.E., Jones, B.A., Ezzell, J.L., Porter, N.L., Avdalovic, N., Pohl, C., Analytical Chemistry, 68, **1996**, 1033.
[5] Kawamura, F., Kikuchi, Y., Ohira, T., Yatagai, M., Journal of Natural Products, 62, **1999**, 244.
[6] Benthin, B., Danz, H., Hamburger, M., Journal of Chromatography A, 837, **1999**, 211.
[7] Ong, E.S., Woo, S.O., Yong, Y.L., Journal of Chromatography A, 313, **2000**, 57.
[8] Anne, B., Serge, R., Lidia, M., Phillippe, C., J.L.Veuthey., Journal of Separation Science, 24, **2001**, 865.
[9] Lee, H.K., Koh, H.L., Ong, E.S., Woo, S.O., Journal of Separation Science, 25, **2002**, 160.
[10] Choi, M.P.K., Chan, K.K.C., Leung, H.W., Huie, C.W., Journal of Chromatography A, 983, **2003**, 153.
[11] Rostagno, M.A., Palma, M., Barroso, C.G., Analytica Chimica Acta, 522, **2004**, 169.
[12] Herrero, M., Martin-Alvarez, P.J., Senorans, F.J., Cifuentes, A., Ibanez, E., Food Chemistry, 93, **2005**, 417.
[13] Sar-Yun, A., Ovatlarnporn, C., Itharat, A., Wiwattanapatapee, R., Journal of Chromatography A, 1125, **2006**, 172.
[14] Pitipanapong, J., Chitprasert, S., Goto, M., Jiratchariyakul, W., Sasaki, M., Shotipruk, A., Separation and Purification Technology, 52, **2007**, 416.
[15] Luthria, D.L., Food Chemistry, 107, **2008**, 745.
[16] Schroeder, D., Nakanishi, K.A., Journal of Natural Products, 50, **1987**, 241.
[17] Johnson, S., Morgan E.D., Phytochemical Analysis, 8, **1997**, 228.
[18] Ambrosino, P., Fresa, R., Fogliano, V., Monti, S.M., Ritieni, A., Journal of Agricultural and Food Chemistry, 47, **1999**, 5252.
[19] Dai, J., Yaylayan, V.A., Vijaya Raghavan, G.S., Pare, J.R.J., Liu, Z., Belanger, J.M.R., Journal of Agricultural and Food Chemistry, 49, **2001**, 4584.