Azadirachtin Extraction Using Cold Press and Soxhlet Methods

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ABSTRACT Cold press and soxhlet methods were compared for the extraction of azadirachtin from neem seeds (*Azadirachta indica* A. Juss.) using methanol, hexane, water, and terpene as solvents. Cold press method using methanol yielded significantly higher azadirachtin concentrations (2478 ppm) than the other three solvents ($P < 0.0001$). Soxhlet with methanol produced significantly more azadirachtin (1470 ppm).

KEY WORDS : *Azadirachta indica*, bioinsecticide, methanol extraction, neem

INTRODUCTION

Extraction of bioinsecticide components from neem (*Azadirachta indica* A. Juss.) seeds is considered a critical process to obtain quality products. *A. indica* seeds have 45% oil content, with a number of compounds from the tretranortriterpenoid chemical group. In spite of the diversity of components present in the seed, azadirachtin (AZA) has shown the most relevant insecticide activity in several types of neem seed extracts (Johnson *et al.*, 1996), being AZA A and AZA B the two important structural forms (Sharma *et al.*, 2003). Due to the importance of this metabolite, quality of extracts obtained from neem seed kernels might be granted if AZA content is monitored during the extraction process.

Development of better extraction methods have focused on azadirachtin solubility in different solvents, including hexane, chloroform, acetone, ethyl acetate and methanol (Feuerhake, 1985). Most processes are based on seed oil pre-extraction with ether, hexane or heptane, followed by the use of soxhlet with polar solvents as acetone, methanol or ethanol; however, these methods are complicated and require repeated extraction cycles. To obtain AZA isolated from other limonoids, pretreated seeds are then treated with chloroform and finally ethanol is added for AZA precipitation (Puri, 1999).

Water extraction has been used by peasants under field conditions, but is not considered an adequate extraction method due to the low solubility of other non-polar metabolites imbibed in the oil matrix (Puri, 1999). Aqueous extract by immersion of neem seed in water contains AZA, nimbin and salannin (Coventry and Allan, 2001); however, methanolic neem seed oil extraction contains some other limonoides, including epoxyazadion, gedunin, nimbin and nimolicinol (Luo *et al.*, 1999, 2000; Hallur *et al.*, 2002).

Others have used several combinations of solvents and extraction methods to obtain pure AZA or included in a mixture of limonoids. In general, crude seed polar extracts remove some oil fraction, as well as tannins, carboxylic acids, carbohydrates, proteins,

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pigments, organic and inorganic salts (Sankaram *et al.,* 1999). Besides, seed organic extractions yields a complex compound mixture, and also needs standardization in relation to AZA content and its biological effect. Soxhlet has been used for the comparative study with some other processes, however, it is highly recommended to avoid higher temperatures due to soxhlet pre-extraction with polar solvents (Sankaram *et al.,* 1999). Kovo (2006) compared soxhlet hexane and soxhlet ethanol extraction, but reported only differences due to impurities in the seed kernel and the solvent. Romero and Vargas (2005) compared soxhlet extraction with hexane, ether and ethanol, where oil from soxhlet with hexane was 10% higher than the cold press extract.

Cold press is commonly considered an extraction method with no solvents. A cold extraction process was developed by the Central Oil Technology Research in India to facilitate the acquisition of a crude extract rich in AZA (2000 ppm) (Ramakrishna *et al.,* 1996). Sanguanpong (2003) also studied a pilot equipment to obtain a crude extract rich in AZA with methanol. Jenkins *et al.* (2003), and Ramakrishna *et al.,* (1996) used a manual equipment to produce a crude extract of neem seed kernels. Romero and Vargas (2005) found a linear effect between cold pressure on seeds and the percentage of extraction.

Therefore, AZA extraction processes from neem seed kernels are generally done in two steps, first neem oil separation and second obtaining AZA concentration. However, this criterion eliminates other active limonoids to be present in the final extract used as insecticide, losing some of the inbuilt potential of neem as insecticides (Stark and Walter, 1995). Here, a single step extraction process is proposed, with the extraction of oil, AZA and possibly some other bioactives present in the seed. In processes where the main goal is the production and application of *A. indica* extract as a bioinsecticide, insufficient research has been performed to achieve the maximum quantity of AZA in the extract. It is well known that different solvents and extraction methods might be selecting some other terpenoids with different modes of action in addition to the effects caused by AZA in the final extract.

The objective of present study was to compare cold press and soxhlet systems as two extraction processes to determine the influence on extract production and concentration of AZA produced using methanol, hexane, terpene, and water as solvents.

MATERIALS AND METHODS

A. indica seeds were harvested in August 2004 from a 13 year-old orchard of 225 trees located at Colegio de Postgraduados Campus Veracruz, Municipio Manlio F. Altamirano, Veracruz, Mexico (19°11'658" N, 96°20'069" W, and 27 masl). Neem seeds (20 kg) were dried under shade and ground (Ø 1.41 mm: 40.33%; 0.074 mm: 28.63%; 0.59 mm: 7.83%; 0.42 mm: 9.84% and 11.12% of fines). Seeds were kept at room temperature (approximately 25°C), and processed with a commercial manual grinder of high resistance alloy iron disks with a capacity of 1.0 kg in 5 min. Distilled water (H_2O) and technical grade solvents used were methanol (MeOH; 99.96%), *n*-hexane (C_6H_{14}; Hex; peb @ 55–63°C), and terpene (*d*-limonene; 85%).

Cold Press Extraction

A combination of direct extraction by two methods was used, extrusion and dissolution. Cold press extraction with solvent allows separation of components without thermal degradation of seed content. This is an improved process derived from solvent extraction, eliminating the step of endocarp separation, favoring direct extraction of a rich AZA extract and increasing concentration of other compounds of the neem seed kernels (NIIR Board, 2004). A mechanical pilot expeller with vertical action was used for cold press extraction with solvent. The expeller was equipped with a steel cylinder that holds 1.0 kg of ground seed, a manually operated hydraulic press system (20 kg cm^{-2}), and a cylinder with a reduction in the lower exit and a steel drain used to collect the extract at the bottom (Fig. 1). In addition, prior immersion during 20 min of 1.0 kg of grinded seed in 150 ml of each solvent was required. Three

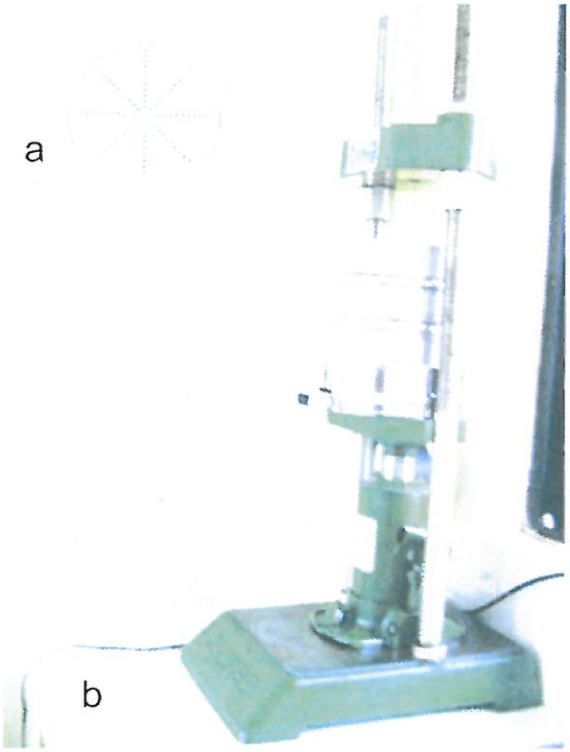

Fig. 1. Colegio de Postgraduados vertical cold press equipment design; a) stainless steel drain, and b) whole equipment view.

extractions were performed at room temperature during 30 min for each solvent.

Soxhlet Extraction

This method was used as a benchmark for cold press extraction. A ground sample of neem seed (64 g) was placed in a "thimble" made of filter paper and placed in the soxhlet extractor. A flask containing 500 ml of the appropriate solvent was attached at the bottom and heated until evaporation, according to the 936.15 AOAC (1990) procedure. This cycle was repeated four times, with a portion of the biopesticide washed off with the help of the solvent. Following completion of all four cycles, the thermostatized evaporation of the solvent was carried out until constant weight on boiling chips at 60°C. Three extractions were obtained for each solvent.

Extract Analysis of Active Ingredient

Extracts were maintained at -4°C in a hydrothermal chamber, kept away from light inside an amber glass bottle. AZA content on the extract was obtained with methanol at 4°C. AZA content from each extract was determined according to the EPA method developed by Schneider and Ermel (1987), with a modular Perkin Elmer HPLC (ISO-9000 certified) in the "Laboratorio de Alta Tecnología de Orizaba", Universidad Veracruzana, in Orizaba, Mexico. Chromatograph conditions included a column of 125 × 4 mm, a flow rate of 2 ml min^{-1}, a UV-VIS detector (ë 214 nm), a sample volume of 20 µl, a mobile phase using acetonitrile and water, and a relative retention time (tR) of 2.504 min. Quantification was completed by standardization using azadirachtin 95% (Sigma®) ($C_{35}H_{44}O_{16}$; mol. mass 720.72) as a standard. Same conditions were used for AZA analysis on seeds used for extractions.

A completely randomized factorial design, with two extraction processes and four solvents was used. Experimental units were replicated three times, with standardized AZA concentration as the response variable. An analysis of variance was performed, followed by a Duncan's honestly significant difference test for mean separation, with fixed effects for "extraction processes" due to the significance of the main interaction (Statistica® 2003, version 6.1, Start Soft Inc., Tulsa, USA). Pilot equipment comparison with the laboratory method might indicate whether this equipment is useful in low scale extraction processes.

RESULTS

Experimental seeds had an azadirachtin content of 1820 ppm, in accordance with the range of 430 to 3830 ppm reported in India by Pattnaik *et al.* (2006). Thus, neem seeds from Veracruz were as good as seeds from India for use as insecticide raw material.

Analysis of variance on AZA extract contents showed a significant effect due to the process employed ($P = 0.0016$), type of solvent used ($P < 0.0001$), and the interaction of both factors ($P = 0.00028$). Process-solvent interaction of mean azadirachtin concentration can be seen in Fig. 2. Methanol presented lower values in the Soxhlet bar compared to cold press, however, confidence interval for hexane, water and terpene in both extraction

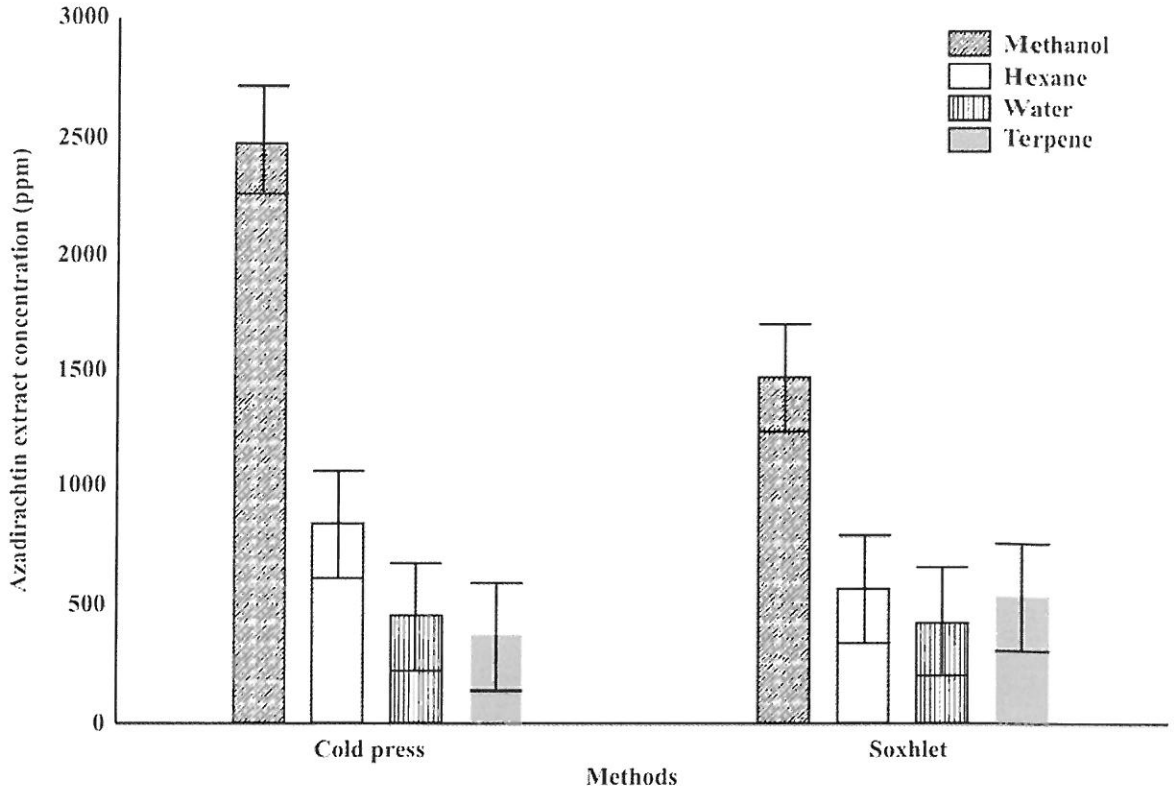

Fig. 2. Azadirachtin concentration in extracts obtained by a combination of cold press and soxhlet extraction methods with methanol, hexane, water and terpene solvents. The bars show the 95% confidence interval.

methods crossed over, indicating similar results.

Methanol was the solvent that extracted the most azadirachtin in both processes, being even significantly greater with cold press. Cold press extraction yielded a significantly greater AZA concentration (2478 ppm; Fig. 3) using methanol or hexane (843 ppm) ($P < 0.0001$), followed by soxhlet and methanol (1470 ppm) ($P = 0.0009$), compared to the other solvents (Fig. 2).

DISCUSSION

In order to obtain maximum extract quantity with maximum AZA content possible, methanol appears to be the best solvent; however, it seem there is room for higher yields with cold press once optimization of extraction conditions are achieved. Soxhlet has the potential to produce more of AZA at higher temperatures, but isomerization and molecule degradation is always a concern. In present study soxhlet extraction yielded less of azadirachtin as compared to cold press extraction product. Govindachari *et al.* (1998), Siddiqui *et al.* (2002) and others have explored the use of hexane, chloroform or benzene to generate hydrophobic extracts with significant azadirachtin concentrations. However, methanol extraction provides a rich AZA crude extract (Sanguanpong, 2003), with the possible presence of other compounds that might combine their mode of actions to improve the biological effect of the product, such as the mortality of insect pests. Liquid-methanol soluble components are easily extracted in cold press extraction and use of methanol as solvent. Because cold press extraction also retains some oil fraction, retention of salannin in the mixture is inevitable, which otherwise will get removed in soxhlet extraction procedure (Stark and Walter, 1995). Water extraction has also been suggested but this cannot be compared to laboratory processes mentioned above. Such extraction is of practical social relevance, allowing impoverished farmers to use homemade aqueous ex-

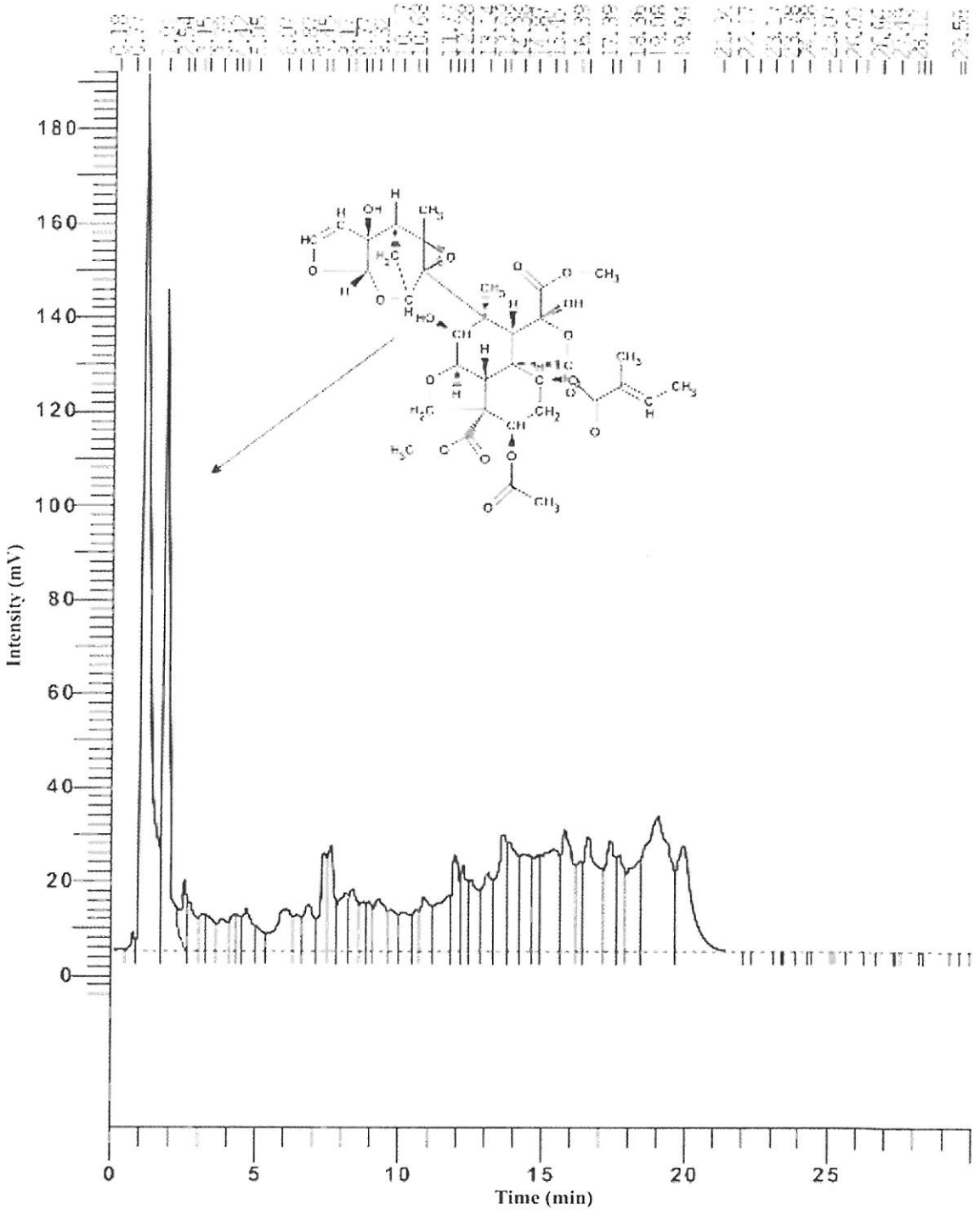

Fig. 3. Neem (*Azadirachta indica*) seed extract chromatogram, obtained by methanol cold press. The HPLC peak represents azadirachtin.

tracts from handpicked seeds on their own crops (Koul, 2004), however, use of such methods further emphasize that cold press methanol extraction technique is a promising and practical tool for produc-

tion of good extracts with high AZA content. Therefore, convenient cold press process allows obtaining a liquid extract rich in AZA, avoiding unnecessary steps in small-scale production. In Tahiland, a small-scale pilot-RIT (Rajamangla Institute of Technology) extractor has been proposed where neem seed kernel is used. The process eliminates the endocarp, followed by the oil extraction and then separates AZA with methanol (Sanguanpong, 2003). In the process described in present study, seed is ground without removing its endocarp. Also, direct extrusion of seed with methanol that helps in AZA separation by dissolution during tissue rupturing and at the same time oil content is released.

Thus, methanol cold press extraction maximizes the quantity of azadirachtin obtained from neem seeds. Optimization of methanol extraction with scaled up and more efficient cold press equipment is required to maximize extract quantities and/or to prevent reduction in AZA concentration.

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