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MULTI-INSECTICIDE EXTRACTIVE TECHNOLOGY OF NEEM SEEDS FOR SMALL GROWERS

[TECNOLOGÍA EXTRACTIVA MULTI-INSECTICIDA DE SEMILLA DE NIM PARA PEQUEÑOS PRODUCTORES]

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SUMMARY

The neem tree (*Azadirachta indica* A. Juss.) is known for its insecticidal properties. In this study an extraction method for neem seeds based on cold extrusion with methanol was applied. On the resulting extract (azadirex), limonoids azadirachtin A and B (AZA and AZB), salannin, and nimbin were quantified by HPLC. A 10 years old neem orchard was used (19° 11.65' N, 96° 20.07' W). The extrusion of 1.0 kg of dry endocarp, ground seeds previously immersed in 150 mL of methanol during 20 min, was performed in a manual hydraulic press, at 20 kg cm^{-2} and room temperature. Concentration of limonoids underwent an analysis of variance and means separation (Tukey, $P < 0.05$). The seed with endocarp showed a salannin concentration of 4500 mg kg^{-1}, 3450 mg kg^{-1} of nimbin and 2784 mg kg^{-1} of the azadirachtins A and B mixture. Azadirex had a typical composition of AZA, AZB, nimbin and salannin, however the latter (3866 mg kg^{-1}) was found in a significantly higher proportion compared to the others (Tukey, $P < 0.05$). No significant differences were found between the mixture of AZA + AZB (1818 mg kg^{-1}) and nimbin (1280 mg kg^{-1}).

Key words: Neem; *Azadirachta indica*; nimbin; salannin; azadirachtin A; azadirachtin B.

RESUMEN

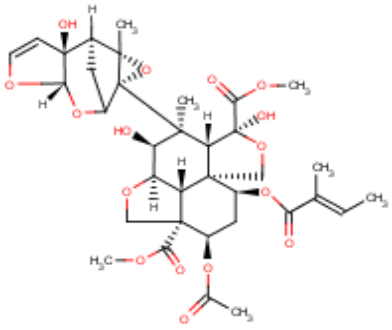
El árbol de nim (*Azadirachta indica* A. Juss.) se conoce por sus propiedades insecticidas. En este estudio se aplicó la extracción por extrusión en frío con metanol a la semilla de nim y en el extracto resultante (azadirex) se cuantificaron por HPLC, los limonoides azadiractina A y B (AZA y AZB), salanina y nimbina. Se utilizó semilla de una plantación de 10 años de edad, (19° 11.65' LN, 96° 20.07' LO). Se realizó por triplicado la extrusión de 1.0 kg de semilla con endocarpio seca, molida y previamente inmersa en 150 mL de metanol por 20 min, en una prensa hidráulica manual a 20 kg cm^{-2} y temperatura ambiente. Las concentraciones de los limonoides se sometieron a un análisis de varianza y comparación de medias (Tukey, $p < 0.05$). La semilla con endocarpio mostró una concentración de 4500 mg kg^{-1} de salanina, 3450 mg kg^{-1} de nimbina y 2784 mg kg^{-1} de la mezcla de azadiractinas A y B. El azadirex tuvo una composición típica de AZA, AZB, nimbina y salanina, aunque esta última (3866 mg kg^{-1}) se encuentra significativamente en mayor proporción que las anteriores (Tukey, $p < 0.05$). No hubo diferencias significativas entre la mezcla de AZA + AZB (1818.7 mg kg^{-1}) con la nimbina (1280 mg kg^{-1}).

Palabras clave: Neem; *Azadirachta indica*; nimbina; salanina; azadiractina A; azadiractina B.

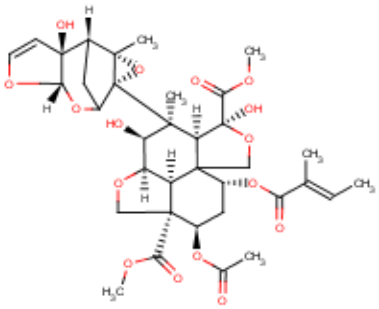
INTRODUCTION

One of the plant species better studied for their pesticide properties is the neem tree (*Azadirachta indica* A. Juss.). It is found in more than 80 countries and there are an estimated 91 million trees around the world. The most important areas where it is distributed are South Asia and the Sahara. In the 20th century, it was introduced to Mexico (Koul, 2004) as a source of raw material for biopesticides. Neem fruit harvest is due when the green epicarp turns yellow. In this stage of maturity the neem fruit has a scarce mesocarp and a hard endocarp surrounding the seed (Puri, 1999a). According to Kaushik *et al*. (2007), the seed contains

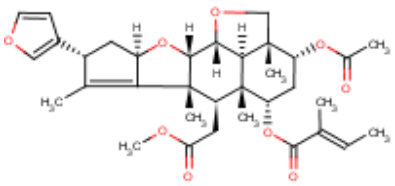
oil in 30 to 50 %, existing a variation in azadirachtin content (AZA) on the seed, due to climatic conditions, in particular temperature and relative humidity. Also, it might vary from tree to tree depending on individual genetic differences (Sidhu *et al.*, 2003). Is in the seed where the highest content of azadirachtin A and other bioinsecticide compounds have been found, isolating there more than 100 triterpenoids (Isman, 2001). Bioinsecticide actives present in greater proportion are the tetranortriterpenoids azadirachtin A (AZA) and azadirachtin B (AZB; Figure 1C). Chemical structures of AZA, AZB, nimbin and salannin are shown in Figure 1. AZA and AZB are produced normally in the seed in a 3:1 ratio (Isman, 2001). Salannin and nimbin (Figures 1B and 1D) are also principal components (Sarais *et al.*, 2008) on the seed at concentrations ranging from 0.1 to 0.9 % (Koul and Wahab, 2004). AZA is detected on the seed right after 50 days of fruit set. The seed reaches its maximum development at 84 days (Puri, 1999b). Yakkundi *et al.* (1995) found that seed color change happens between 63 and 133 days of fruit development. This coloration allows detecting the level of fruit maturity appropriate for seed processing.



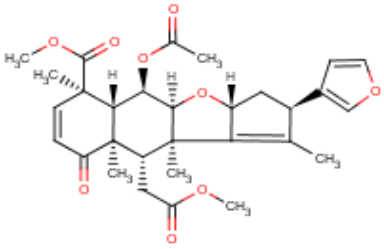
AZA



AZB



Salannin



Nimbin

Figure 1. Chemical structures of azadirachtin A (AZA), azadirachtin B (AZB), salannin and nimbin.

The small scale extraction of neem seed principal actives done by Thailand farmers begins by obtaining crude oil. Then, this oil is processed by solvent bi-partitioning (Sanguanpong, 2003). Two components are obtained here, oil almost free of principal actives and concentrate rich in azadirachtin, free of oil. Nevertheless, it is known that neem oil also contains nimbin and salannin (Stark and Walter, 1995). A neem oil analysis obtained by Bahena-Juárez and García-Chávez (2007) contained 17.1 % of nimbin, 13.7 % of salannin and 12.0 % of AZA.

Several authors use the term "neem extract" in a very imprecise way. Sometimes it is referred to different products derived from fruit, seed or leaf processing, whether containing azadirachtin or not, and without differentiating their insecticidal, bactericidal or medical use. Another inaccuracy is the use of the word "neem" as synonym of "azadirachtin", in relation to the nature of the extract, in spite of being a mixture of substances. It is known that various compounds with insecticide activity are found on neem seed extracts, represented by a group of highly oxidized limonoids. To avoid further confusion, Morgan (2004) suggested

the term azadirex; it is referred to the insecticidal neem seed extract processed without endocarp that contains azadirachtin as the principal ingredient, but containing other active compounds from the triterpenoid group. Azadirex may or may not contain another limonoids, such as nimbin and salannin, and according to this author, salannin was characterized as marginally active. In contrast to the previous definition, here we use the term "azadirex" for extracts based on neem seed with endocarp, because the seed endocarp also contains azadirachtin (Ramos *et al.*, 2004).

The oily extract obtained by extrusion is rich in limonoids; but the yield is low from the seed paste holding oil remnants. However, this oil might be enriched with azadirachtin or other compounds obtained by solvent extraction (i.e. ether) (Satyanandam *et al.*, 2011). When the extrusion process is combined with an alcohol such as methanol, the extract might contain AZA, AZB, di-acetyl nimbin, di-acetyl salannin, nimbin and salannin (Hallur *et al.*, 2002), besides other compounds such as 6-deacetilnimbin, azadiradion and epoxiazadiradion (Govindachari *et al.*, 1998). The methanolic extract of neem oil obtained through extrusion might reach an AZA content of 1.9 %, 8.3 % of AZB, 5 % of nimbin and 21.25 % of salannin (Govindachari *et al.*, 2000). This extract has antifeeding activity, but most limonoids are left in seed residues.

The cold extrusion process proposed in this study does not utilize heat or chemical solvents other than ethanol; this could facilitate small growers to obtain their product (Bachman, 2001; Ahmed *et al*., 2009). This method solely requires an alcohol and a manual press, easy to obtain; its advantage is the possibility to obtain products other than azadirachtin with antifeeding effects on pest insects, such as salannin and nimbin (Govindachari *et al*., 2000), from here comes its possible multi-insecticidal effect. However, this method has not been used directly to process endocarp seeds, and it is unknown whether it has the typical composition of azadirex. Thus, the aim of this work was to quantify using HPLC analysis, the amount of main limonoids present in *A. indica* seeds and in the methanolic extract or azadirex, using cold extrusion in small scale.

MATERIALS AND METHODS

A neem seed extract was made with seed from a10 years old *A. indica* orchard, located in the Campus Veracruz, Colegio de Postgraduados, in the municipality of Manlio F. Altamirano, Veracruz, Mexico (19°11.65' N, 96°20.07' W, and a height of 27 m), and a sub-humid warm climate Aw2(x') (García, 1998). The harvest season went from July to August, 2007. Fruit harvest was done when epicarp was in pre-ripening green stage, before switching to yellow, characteristic of fruit maturity. Homogeneous fruit maturity was obtained by placing harvested fruits in a mesh bag for a week. Humid unfleshing of the seed was done with a mechanical berry-coffee pulper. Endocarp seed was dried in the shade and stored in a room at 25±1 °C until extraction. A manual grinder was used, consisting of high resistance iron discs, and a grinding capacity of 0.2 kg min^{-1}. Twenty kg of dry and grinded seed were used, with a particle size of 1.41 mm Ø, 40.33 %; 0.074 mm Ø, 28.63 %; 0.59 mm Ø, 7.83 %; 0.42 mm Ø, 9.84 % and 11.12 % of fines. Solvent used was methanol industrial grade (99.96 %).

Azadirex was obtained by cold extrusion with methanol (Esparza-Díaz *et al*., 2010b). The extruder is portable and manual, with a stainless steel cylindrical chamber that holds in 1.0 kg of seed. At the bottom of the chamber a stainless steel mesh was placed for retention of solids to prevent obstruction. The exit duct had eight rows of holes connected with exit channels. The extraction began with the addition of 150 mL of methanol to 1.0 kg of grinded seed with endocarp. It was mixed until a homogeneous blend was obtained and was left for settlement during 20 min. Then, the seed mixture was introduced into the chamber. Extrusion breaks the seed tissue and releases cellular liquids at room temperature, at 20 kg cm^{-2}, pressure provided by a hydraulic manual system. Methanol dissolves easily some polar components of the neem seed. Azadirex was stored on the dark in 500 mL amber glass bottles, and refrigerated at -4 °C.

Azadirex was analyzed using a modular HPLC system (ISO-9000 certified) Perkin Elmer, in the Laboratorio de Alta Tecnología de Orizaba, Universidad Veracruzana (Orizaba, Mexico). Chromatography running conditions were: Column HS5C_{18}; detector: UV-VIS; wave length: λ 215 nm; sample volume: 20 µL; mobile phase: H_2O: CH_3CN: CH_3OH (15:40:45). Quantification was done standardizing with AZA 90.7 % ($C_{35}H_{44}O_{16}$; m. m. 720.72; CAS#: 11141-17-6), AZB 93.4 % ($C_{33}H_{42}O_{14}$; m. m. 662.69; CAS#: 95507-03-2), salannin 93.3 % ($C_{34}H_{44}O_9$; m. m. 596.71; CAS#: 992-20-1) and nimbin 96 % ($C_{30}H_{36}O_9$; m. m. 545.67; CAS#: 5945-86-8). All standards used were from ChromaDex®. Additionally, amount of seed components were obtained.

Salannin, nimbin, azadirachtin A and azadirachtin B concentration on azadirex was considered as dependent variable. Each neem seed extract was

replicated three times. Actives concentration (mg kg^{-1} of seed) underwent an analysis of variance and means separation (Tukey, $P \leq 0.05$), with the software Statistica v. 6.0.

RESULTS

Limonoids concentration from neem seeds as well as from azadirex, were compared. Seeds with endocarp had an AZA average of 1080 mg kg^{-1}, similar to the interval values from Australia cited by Bally *et al*. (1996) and those cited by Pattnaik *et al*. (2006) for seeds without endocarp from India (430 to 3830 mg kg^{-1} of AZA). According to this, the concentration of AZA found in our studied neem seeds makes viable their use for industrialization in bioinsecticide production. Azadirachtins A and B are isomeric limonoids, much oxidized secondary metabolites with three radicals [-OH], present on neem seeds. AZB showed a mean concentration of 1704 mg kg^{-1}, greater than AZA. Thus, seeds from this agroecological area were rich in AZB. The combined mean value of azadirachtins A and B was 2784 mg kg^{-1} (0.27 %), was compared to the higher values found in the province of Andhra Pradesh, India (Ramesh and Balasubramanian, 1999). Nimbin concentration was 3450 mg kg^{-1} (0.34 %), higher than both azadirachtins in this study and on the interval observed from seed collected in Hyderabad, Andhra Pradesh, and even greater than that from Karnataka (Bangalore) and Tamilnadu (Chennai), India. Also, salannin concentration was 4500 mg kg^{-1} (0.45 %), superior to that found in those localities from India, with 0.06 to 0.07 %, respectively.

Azadirex extract obtained was an amber liquid. Mean concentrations of AZA, AZB, salannin and nimbin are shown in Figure 2. Concentration of actives was significantly different ($P < 0.05$). Mean comparison indicated that salannin is present in significantly higher amounts than the other compounds, which do no present significant differences among them (Figure 2). Although AZA is the main component with insecticidal effect and AZB contributes with this biological activity according to Koul and Wahab (2004), its concentration was lower than that of salannin. On the other hand, the mixture of AZA and AZB had a concentration of 1818 mg kg^{-1} (Std. Error = 348 mg kg^{-1}).

Cold extrusion with methanol process promotes the extraction of metabolites contained in seeds, thanks to the interaction of hydroxyl groups from azadirachtin A and B structure (Figures 1A and 1B), favoring solubility of those molecules in a polar solvent. Seed oil contains salannin and nimbin (Stark and Walter, 1995). Salannin and nimbin from azadirex showed that even the oily seed fraction was extracted. In the oily fraction studied by Stark and Walter (1995), salannin exceeds nimbin, in the same way it did in our azadirex. Although the studied process is a combination of extrusion with solvent, according to Koul and Wahab (2004) azadirex had a typical composition, with azadirachtins A and B, salannin and nimbin as principal components, but having different concentrations. Salannin affects negatively feeding on treated insects and has insecticidal properties, but it has a minor antifeeding effect than azadirachtin (NIIR Board, 2004); if an extract like azadirex contains major concentrations of salannin and nimbin, it could offer a synergic effect, compared with other commercial extracts focusing only on AZA and AZB.

Maximum concentration of azadirachtin can be obtained when precautions are applied to achieve homogeneity in fruit maturity and at the same time taking care of neem seed storage conditions. Azadirachtin content on seeds also may vary based on tree genotype or geographical zone of origin (Sidhu *et al*., 2003), soil type, altitude and environmental conditions such as temperature and relative humidity (Rengasamy and Parmar, 1995; Kaushik *et al*., 2007). Due to this variability, it is important to recognize the value of blends, based on compounds with insecticide or antifeeding activity, such as azadirex, with additional active ingredients, such as salannin, nimbin and AZB. Although AZA has been recognized as the key compound in the bioactivity of this extract, its concentration is not always directly correlated with the insecticidal activity, because other compounds also contribute, such as AZB and salannin (Esparza-Díaz *et al*., 2010a). Besides, azadirachtin is unstable on alkaline extracts. Instead, a methanolic extract (such as azadirex) has the advantage of being stable for at least six months, when stored at 20 °C (Hull *et al*., 1993). The combined process of extrusion with solvent proposed in this study is done with equipment easy to operate; it is potentially useful for small growers who may obtain their own extract from their own seed (Bachman, 2001).

CONCLUSION

Azadirachta indica seed with endocarp harvested from neem trees at the municipality of Manlio F. Altamirano, Veracruz, Mexico, contained important concentrations of nimbin, salannin and azadirachtins A and B (AZA and AZB). Azadirex (an extract of seeds with endocarp) also presented these actives. Salannin was found in greater proportion than the other three.

Azadirachtins A and B were found in similar concentrations than nimbin. Cold extrusion with methanol is a method that promotes extraction of AZA and AZB metabolites contained on seeds. Salannin and nimbin values in azadirex showed that this method extracted even the oily fraction of the seed, where salannin exceeded nimbin. Due to the use of a simple equipment combining extrusion with a solvent, this technology is potentially useful for growers that want to produce the multi-insecticide azadirex.

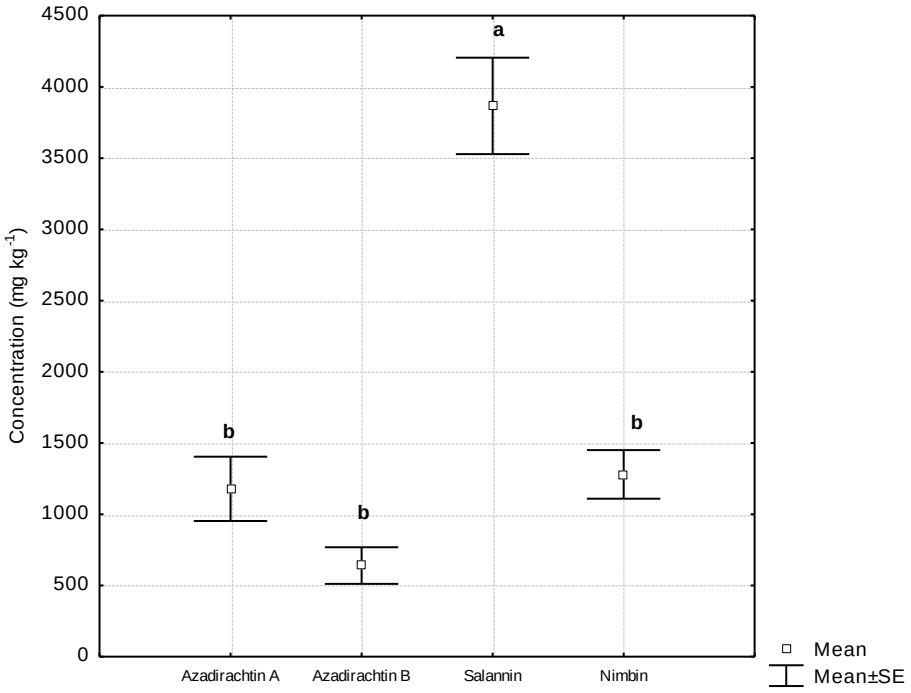


Figure 2. Mean concentration of azadirachtin A, azadirachtin B, salannin and nimbin in azadirex. Means with the same letter do not differ significantly (Tukey, $P < 0.05$).

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