Extraction and Colorimetric Determination of Azadirachtin-Related Limonoids in Neem Seed Kernel

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A colorimetric method was developed for the determination of total azadirachtin-related limonoids (AZRL) in neem seed kernel extracts. The method employed acidified vanillin solution in methanol for the colorization of the standard azadirachtin or neem seed kernel extracts in dichloromethane. Through the investigation of various factors influencing the sensitivity of detection, such as the concentration of vanillin, acid, and the time required for the formation of color, optimum conditions were selected to perform the assay. Under the optimum conditions, a good linearity was found between the absorbance at 577 nm and the concentration of standard azadirachtin solution in the range of 0.01−0.10 mg/mL. In addition, different extraction procedures were evaluated using the vanillin assay. The HPLC analysis of the extracts indicated that if the extractions were performed in methanol followed by partitioning in dichloromethane, ∼50% of the value determined by the vanillin assay represents azadirachtin content.

Keywords: *Azadirachtin; neem seed; extraction; vanillin assay; total limonoids*

INTRODUCTION

The neem tree, *Azadirca indica* A. Juss, has been increasingly attracting the interest of researchers from various fields. More than 300 compounds have been isolated and characterized from neem seed, one-third of which are tetranortriterpenoids (limonoids) (Kumar et al., 1996). One of these limonoids, azadirachtin (AZ, see Figure 1), is considered to be the most important active principle, due to its various effects on insects (Schmutterer, 1990; Govidachari et al., 1995). AZ content in neem extracts or in commercially available neem-based pesticides can be estimated by HPLC (Sundaram and Curry, 1993; Azam et al., 1995; Yamaski et al., 1986) or by supercritical fluid chromatography (Huang and Morgan, 1990). However, AZ is not the only active component. According to Verkerk and Wright (1993), neem extracts containing equivalent amounts of AZ have 3−4-fold greater activity than the synthetic AZ. This might be due to the presence of other active limonoids in the extract; therefore, knowledge of the total azadirachtin-related limonoids (AZRL) in the extracts can help predict the relative activity of different neem species. We report here a fast colorimetric method for estimating the content of AZRL in neem seed kernel extracts. The reliability of the assay was tested using extracts from neem seed kernel. The content of AZ in the extracts was further investigated by HPLC analysis. The vanillin assay was further used to investigate the efficiency of different extraction procedures.

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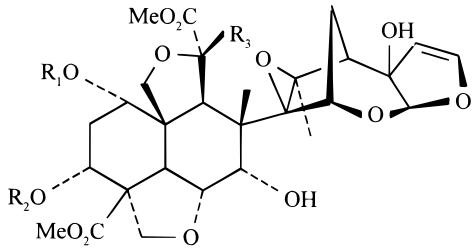

Figure 1. AZ or AZ A: R_1 = tigloyl, R_2 = acetyl, R_3 = OH. AZ B: $R_1 = R_3$ = H, R_2 = tigloyl.

MATERIALS AND METHODS

Materials. Fresh neem seeds were collected from Bangalore, India, during May 1998. The seeds were removed from their shells and blended with a coffee bean blender and stored at below 0 °C. The Synthewave 402 (focused microwave system at atmospheric pressure) was obtained from Prolabo (Fontenay-Sous-Bois Cedex, France). It operates with an emission frequency of 2450 MHz and a 300 W full power. It is equipped with an IR temperature sensor, a 250 mL quartz extraction vessel, and a Graham type condenser. The solvents were evaporated using a Büchi Rotovapor R114 (Fischer Scientific, Montreal, Canada).

Chemicals. Azadirachtin (∼95% purity) was purchased from Sigma Chemical Co. Two stock solutions were prepared: 0.1 mg/mL in dichloromethane and 0.1 mg/mL in methanol, stored below 0 °C in the refrigerator. HPLC grade methanol, dichloromethane, and acetonitrile were purchased from Fisher Scientific; petroleum ether (60−80 °C) was purchased from ACP Chemicals Inc. (Montreal, Canada). Vanillin and concentrated H_2SO_4 (98%) were obtained from Fisher Scientific.

Extraction Procedures. *Procedure 1: Microwave-Assisted Extraction (MAE) with Dichloromethane.* Blended fresh neem seed kernel (2.00 g) was placed in a 250 mL quartz extraction vessel of the Synthewave 402 microwave system. Petroleum ether (15 mL) was then added. The vessel was inserted inside the microwave cavity, fitted with a condenser, and irradiated in the following sequence at 80% power (240 W): 1 min on, 30

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s off, and 1 min on. This process was repeated three times. The petroleum ether extracts were combined and evaporated to yield 0.234 g of fat. The defatted sample was extracted three times with dichloromethane (15 mL) using the following sequence: 30 s on, 30 s off, 30 s on, 30 s off, and 30 s on, at 50% power level (150 W). The dichloromethane extracts were filtered, and the filter paper was washed with dichloromethane (5 mL). The combined dichloromethane extracts were evaporated under vacuum on a rotary evaporator at 40 °C to give a yellow residue (0.047 g) termed MAE-D. The extract was dissolved in dichloromethane (0.40 mg/mL) for the vanillin assay.

Procedure 2: MAE with Methanol Followed by Dichloromethane Partition. The extraction procedure was the same as that of procedure 1 except that the solvent for extraction after the defatting step was replaced with methanol. The petroleum ether extract yielded 0.244 g of fat and the methanol extract 0.214 g of residue. The methanol extract was redissolved in methanol (10 mL) and water (10 mL) followed by the addition of 5% sodium chloride solution (1.0 mL). This mixture was extracted with petroleum ether (6 × 20 mL) to further remove any remaining fat. The residue was then extracted with dichloromethane (3 × 20 mL). The combined dichloromethane extracts were dried over Na_2SO_4, and the solvent was evaporated under vacuum to obtain 0.081 g of an amorphous light yellow solid termed MAE-M. The product was dissolved in dichloromethane (0.33 mg/mL) and in methanol (0.029 mg/mL) for further analysis.

Procedure 3. Room Temperature Extraction (RTE) with Dichloromethane. A suspension of blended neem seed kernel (2.00 g) in petroleum ether (60 mL) was stirred at room temperature for 12 h. The petroleum ether extract yielded 0.406 g of fat. The defatted sample was extracted with dichloromethane (3 × 20 mL) by stirring at room temperature for 12 h. The extract was evaporated under vacuum to give a yellow oil (0.337 g) termed RTE-D. The product was dissolved in dichloromethane (0.80 mg/mL) for the vanillin assay.

Procedure 4. RTE with Methanol Followed by Dichloromethane Partition. The extraction procedure was the same as that of procedure 3 except that the solvent for extraction after the defatting step was replaced with methanol (3 × 20 mL). The petroleum ether extract yielded 0.393 g of fat, and the initial methanol extract yielded 0.249 g of residue. The partition procedure was the same as that in procedure 2. The dichloromethane layer, after vacuum evaporation, gave an amphorous solid (0.101 g) termed RTE-M. The product was dissolved in dichloromethane (0.20 mg/mL) and in methanol (0.024 mg/mL) for further analysis.

Procedure 5. Comparative Study of Different Extraction Methods. Blended neem seed (2.000 g) was stirred overnight in petroleum ether (60 mL) at room temperature. After filteration, the defatted residues were used to study of the efficiency of the different extraction methods.

(a) MAE. The defatted seeds were extracted with methanol (50 mL) using the following irradiation sequence at 150 W: 30 s on and 30 s off for a total of 8.5 min of irradiation time. At the end of the irradiation sequence the solution was left for ~1 min before it was filtered and evaporated in vacuo to yield an orange amorphous solid (0.25 g). The extract was dissolved in dichloromethane for vanillin assay.

(b) RTE. The above procedure was followed except the extraction step was performed with stirring at room temperature for 10 min. Total yield was 0.26 g.

(c) RFX. The same procedure was followed except the extraction was carried out in refluxing methanol for 10 min. Total yield was 0.26 g. All experiments were performed in triplicates.

Determination of AZ Content by HPLC. The HPLC Beckman System Gold consisted of a programmable variable-wavelength UV detector Model 166, and a solvent delivery module 110B was used with a Rheodyne injector equipped with a 20 μL loop. The system was controlled by Beckman Gold software. The separation was performed on a Waters Spherisorb ODS-25 column (4.6 mm × 25 cm i.d., 5 μm) equipped with a Waters ODS guard column. The mobile phase was acetonitrile/water (4:6) and the flow rate was 1 mL/min. The detector was set at 214 nm. The concentration of AZ in the extracts was calculated using the peak areas and a calibration curve (R^2 = 0.9993) generated by injecting standard AZ solutions in methanol with concentrations ranging between 2 and 20 μg/mL, taking into account the 5% impurity.

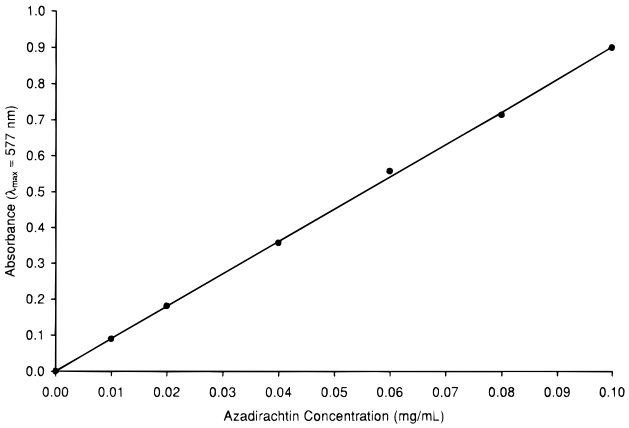

Figure 2. Absorbance vs concentration (milligrams per milliliter) of standard AZ solution subjected to vanillin assay.

Colorimetric Determination of Total AZRL. To a dichloromethane solution (0.7 mL) of standard AZ or neem seed extract was added a methanol solution (0.2 mL) of vanillin (0.02 mg/mL). After shaking manually, the mixture was left at room temperature for 2 min. Concentrated sulfuric acid (0.3 mL, 98%) was then added in three portions (0.1 mL each), and the mixture was stirred for 10 s after each addition. After the addition of sulfuric acid was completed, methanol (0.7 mL) was added to convert the two-layer mixture into a homogeneous solution that instantly developed a blue-green color. The solution was left at room temperature for 5 min before the absorbance was measured at 577 nm, using a Beckman DU-64 spectrophotometer equipped with a 10 mm quartz cell. The blank solution was obtained by substituting the test solution with an equal volume of dichloromethane in the above procedure. Total AZRL in the neem seed extracts (concentrations as stated under Extraction Procedures) were quantified using a calibration curve (R^2 = 0.9995) generated from standard AZ solutions in dichloromethane (0.01−0.10 mg/mL) using the above procedure (see Figure 2).

RESULTS AND DISCUSSION

Although the active component in the neem seed is AZ, the presence of related limonoids in the seed extracts has been shown to enhance the biological activity relative to that of pure AZ (Verkerk and Wright, 1993). Availability of a fast technique to determine total limonoids in the different species of the plant during extractions can be a useful tool to rapidly estimate their relative activity. Acidified methanol solutions of vanillin have been used as spray reagents to visualize terpenes such as limonene (Eweig and Shermer, 1972) and limonoids such as AZ (Yamasaki et al., 1986; Allan et al., 1994) on thin-layer chromatography (TLC) plates. The vanillin reagent could be used as a basis to develop a colorimetric method for the quantification of AZRL in neem seed extracts. Commercially available AZ can be used to develop a calibration curve to estimate the content of compounds structurally related to AZ. Preliminary studies were performed using limonene to reduce unnecessary high costs associated with commercial AZ. These studies have indicated that vanillin and sulfuric acid concentrations and reaction time play a crucial role in determining the sensitivity of the assay. Furthermore, it was demonstrated that limonene could

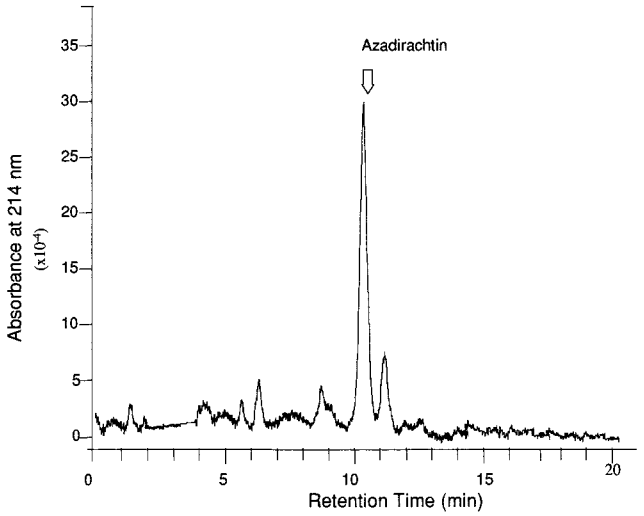

Figure 3. HPLC chromatogram of neem seed kernel extract (MAE-M).

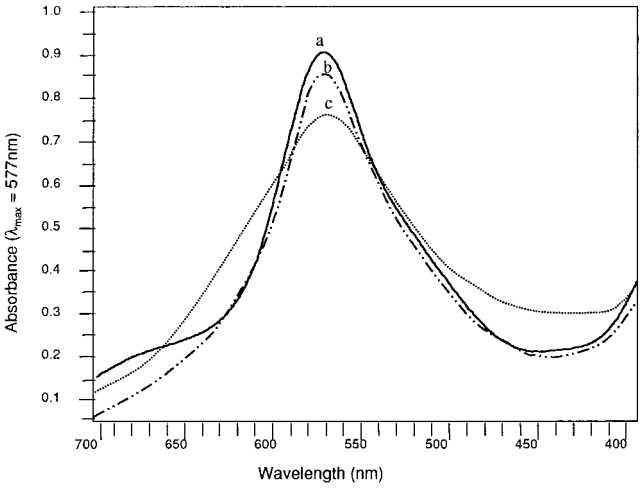

Figure 4. Visible spectrum (700−400 nm) of commercial AZ (—), MAE-M (···), and RTE-M (· · —), subjected to vanillin assay.

be detected in micromolar concentrations when absorbance was measured at 620 nm. A linear response was observed to the limonene concentration in the range of 2−20 μg/mL and with absorbance maxima between 0.09 and 1.0, a suitable range for quantification. In addition, different extraction procedures, including microwave-assisted processes (Paré and Bélanger, 1997), as described under Materials and Methods, were employed to generate extracts from neem seed for the evaluation of the colorimetric method. Furthermore, an HPLC procedure was used to quantify the AZ content of the extracts. According to this procedure, AZ was detected at 214 nm with a retention time of 10.2 min. Figure 3 shows an example of a chromatogram obtained from the neem seed kernel extract termed MAE-M.

Factors Influencing the Vanillin Assay. The initial conditions, to optimize the vanillin assay to determine AZRL, were chosen on the basis of the results of the preliminary investigation with limonene. Figure 4 shows the absorbance, in the visible range, of a commercial AZ solution and neem seed extracts termed MAE-M and RTE-M. Both the extracts and AZ showed similar absorption bands centered at 577 nm (Figure 4). Consequently, this wavelength was chosen to study the effect of time and concentrations of vanillin and

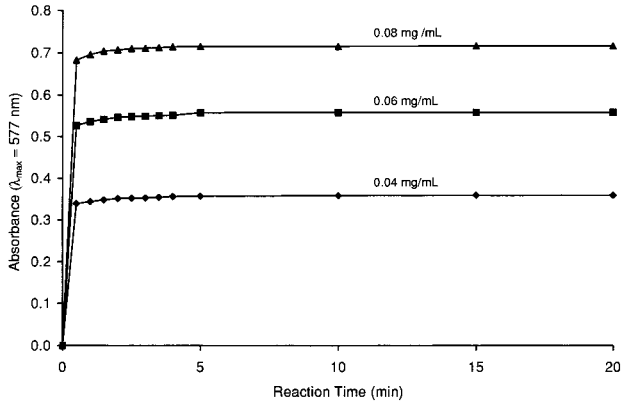

Figure 5. Absorbance vs time (minutes) of AZ solutions at different concentrations subjected to vanillin assay.

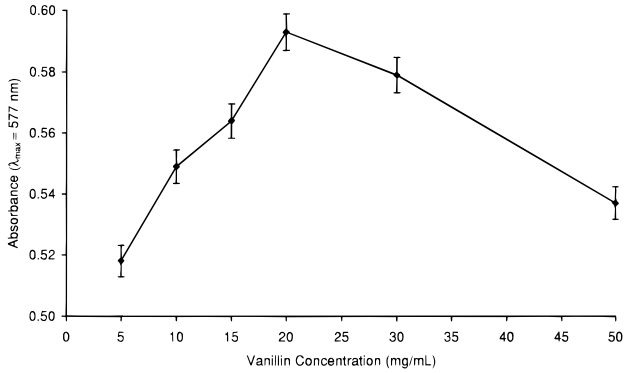

Figure 6. Absorbance vs vanillin concentration.

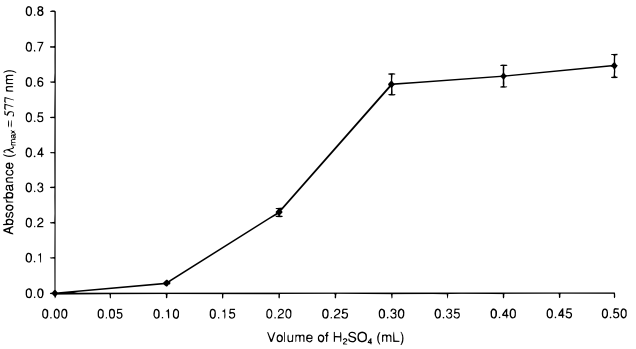

Figure 7. Absorbance vs milliliters of H_2SO_4 (95%).

sulfuric acid on the sensitivity. Color development with time (see Figure 5) was investigated with standard AZ solutions of 0.04, 0.06, and 0.08 mg/mL under the conditions as obtained from the study of limonene. It was found that the color production was stabilized after ∼5 min as shown in Figure 5. On the other hand, the effect of vanillin concentration (see Figure 6) and the amount of concentrated sulfuric acid (see Figure 7) on the intensity of the absorbance at 577 nm was investigated with neem seed kernel extracts termed RTE-M. The results indicated that the absorbance increases with the concentration of vanillin solution up to 0.02 g/mL. At higher concentrations, the blank solution exhibited stronger absorbance than the samples. Accordingly, a concentration of 0.02 g/mL of vanillin was selected as the optimum concentration to study the influence of sulfuric acid. As shown in Figure 7, the absorbance of the sample at 577 nm increases sharply as the volume of concentrated sulfuric acid (98%) increases from 0.1 to 0.3 mL. Similar to vanillin concentration, at volumes

Table 1. Percent AZ and AZRL in Neem Seed Extracts[a]

extraction method[b]	% AZ in the extract	% AZRL in the extract	% AZ in AZRL
MAE-M	15.9 ± 0.04	33.8 ± 0.22	47
MAE-D	nd	19.3 ± 0.28	na
RTE-M	27.4 ± 0.18	49.9 ± 0.69	55
RTE-D	nd	9.4 ± 0.07	na

[a] Values reported are based on five replicate measurements. na, not applicable; nd, not determined. [b] See Materials and Methods, procedures 1−4.

Table 2. Yields of the Different Extraction Procedures

extraction method[a]	wt of extract[b] (g)	wt of extract before partition[b] (g)	extraction conditions
MAE-M	0.081	0.214	4.5 min, 65 °C, 150 W
MAE-D	0.047	na	4.5 min, 40 °C, 150 W
RTE-M	0.101	0.249	36 h, rt
RTE-D	0.337	na	36 h, rt

[a] See Materials and Methods, procedures 1−4. [b] Based on 2.00 g of neem seed. na, not applicable.

Table 3. Percent[a] AZ, AZRL, and Fat in Neem Seed and Comparison with Reported Values in the Literature

extraction method[b]	% AZ	% fat[b]	% AZRL
MAE-D	nd	11.95 ± 0.35	0.39 ± 0.06
MAE-M	0.36 ± 0.06		0.76 ± 0.14
RTE-D	nd	19.98 ± 0.46	1.57 ± 0.12
RTE-M	0.57 ± 0.04		1.03 ± 0.07
Yakkundi (1995)[c]	0.38 ± 0.06		
Azam (1995)[d]	0.29		
Ermel (1986)	0.35 (India), 0.40 (Togo), 0.19 (Sudan), 0.15 (Niger)		
Govindachari (1992)[e]	0.6		

[a] Based on fresh neem seed kernel. nd, not determined. [b] See Materials and Methods, procedures 1−4. [c] Based on dry neem seeds. [d] Based on neem seed kernel from Pune, India. [e] Based on neem seed kernel from suburbs of Madras, India.

>0.3 mL, the absorbance of the blank solution was higher than that of the samples.

AZ and AZRL Content of Neem Seed Extracts. The percentages of AZ and AZRL in the neem seed extracts were determined by HPLC analysis and vanillin assay, respectively. The results are presented in Tables 1 and 2. The neem seeds were extracted according to two different methods (microwave and stirring at room temperature) and using two different solvents (methanol and dichloromethane). In addition, the methanol extracts from both methods were further partitioned between dichloromethane and methanol before analysis for AZ and AZRL contents. The data presented in Table 1 indicate that neem seed extracts could be enriched in AZRL and subsequently in AZ content, if methanol is used for the extraction, followed by partitioning as described under Materials and Methods. Under those conditions, the vanillin assay can predict the relative content of AZ of different extracts. Furthermore, the AZ, fat, and AZRL content of the seed kernel, which constitutes ~45% of the seed (Visvanathan et al., 1996), was also calculated, and the results are shown in Table 3 together with that reported in the literature. The neem seeds from different geographical locations have different contents of AZ due to such variables as temperature, relative humidity, and exposure to sunlight (Ermel et al., 1986). The values reported in Table 3 fall within the range reported in the literature.

Table 4. Comparison of the Different Extraction Efficiencies[a]

extraction method[b]	wt of extract[c] (g)	% AZRL in the extract	% AZRL in the seed
MAE	0.2464 ± 0.0159	15.34 ± 0.85	1.89 ± 0.07
RTE	0.2608 ± 0.0107	15.93 ± 0.45	2.03 ± 0.03
RFX	0.2582 ± 0.0197	16.42 ± 0.25	2.12 ± 0.13

[a] Values reported are based on triplicate measurements. [b] See Materials and Methods, procedure 5. [c] Based on 2.000 g of neem seed.

Evaluation of Different Extraction Methods Using Vanillin Assay. One of the advantages of the vanillin assay is that it allows rapid and convenient estimation of relative AZ content directly from crude extracts without the need to perform lengthy HPLC analysis. Approximately 50% of the value of vanillin assay represents AZ content of the methanol extracts that have been partitioned, as demonstrated above (see Table 1). To evaluate the ability of different procedures to extract limonoids, the neem seeds were defatted under the same condition as described for procedure 5 under Materials and Methods. However, extractions were performed under microwave irradiation (MAE), room temperature (RTE), and reflux (RFX) conditions. All extractions were carried out for 10 min using methanol as the solvent without further partitioning. Vanillin assay performed on the fat extract indicated the absence of AZRL. Table 4 summarizes the results. Single-factor ANOVA analysis of the data from different extraction procedures indicated that there are no significant differences in the total yield and AZRL content of the extracts. In addition, stirring neem seed in methanol at room temperature for 36 h will not increase the yield significantly relative to that obtained at room temperature with a 10 min extraction time (see Tables 2 and 4).

Conclusion. The colorimetric method developed, based on the vanillin assay, allows direct and rapid measurement of the total AZRL in the crude extracts of neem seed. Furthermore, relative AZ content of different samples can be estimated from the values of the vanillin assay if extractions are performed with methanol followed by partitioning with dichloromethane. In this assay, the interference from terpenoids and polyphenols is eliminated or minimized due to the differences in the absorption maxima of the color complexes generated by their interaction with vanillin.

LITERATURE CITED

Allan, E. J.; Eeswara, J. P.; Johnson, S.; Mordue, A. J.; Morgan, E. D.; Stuchbury, T. The production of Azadirachtin by in-vitro tissue cultures of neem, *Azadirachta indica*. *Pestic. Sci.* **1994**, *42*, 147−152.

Azam, M.; Rengasamy, S.; Parmar, B. S. Estimation of azadirachtin-A content of emulsifiable and solution concentrates of neem. *J. AOAC Int.* **1995**, *78* (4), 893−896.

Ermel, K.; Pahlich, E.; Schmutterer, H. Azadirachtin content of neem kernels from different geographical locations, and its dependence on temperature, relative humidity, and light. *Proceedings of the Third International Neem Conference*; 1986; pp 171−184.

Eweig, G.; Shermer, J. *CRC Handbook of Chromatography*; CRC: Boca Raton, FL, 1972; pp 561−562.

Govidachari, T. R.; Sandhya, G.; Raj, S. P. G. Azadirachtins H and I: two new tetranoterpenoids from *Azadirachta indica*. *J. Nat. Prod.* **1992**, *55*, 596−601.

Govidachari, T. R.; Narasimhan, N. S.; Suresh, G.; Partho, P. D.; Gopalakriishnan, G.; Kumari, G. N. K. Structure-related

insect antifeedant and growth regulating activities of some limonoids. *J. Chem. Ecol.* **1995**, *21* (10), 1585−1600.

Huang, H.; Morgan, E. D. Analysis of azadirachtin by supercritical-fluid chromatography. *J. Chromatogr.* **1990**, *519*, 137−143.

Kumar, Ch. S. S. R.; Srinivas, M.; Yakkundi, S. Limonoids from the seeds of Azadirachta Indica. *Phytochemistry* **1996**, *43* (2), 451−455.

Paré, J. R. J.; Bélanger, J. M. R. Microwave-assisted process (MAP): Principles and Applications. In *Instrumental Methods in Food Analysis*; Paré, J. R. J., Bélanger, J. M. R., Eds.; Elsevier: Amsterdam, The Netherlands, 1997; pp 395−420.

Schmutterer, H. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica. Annu. Rev. Entomol.* **1990**, *35*, 271−297.

Sundaram, K. M. S.; Curry, J. High performance liquid chromatographic method for the analysis of azadiractin in two commercial formulations and neem oil. *J. Environ. Sci. Health* **1993**, *B28* (2), 221−241.

Verkerk, R. H. J.; Wright, D. J. Biological activity of neem seed kernel extracts and synthetic azadirachtin against larvae of *Plutella xylostella L. Pestic. Sci.* **1993**, *37*, 83−91.

Visvanathan, R.; Palanisamy, P. T.; Gothandapani, L.; Sreenarayanan, V. V. Physical properties of neem nut. *J. Agric. Eng. Res.* **1996**, *63*, 19−26.

Yakkundi, S. R.; Thejavathi, R.; Ravindranath. Variation of azadirachtin content during growth and storage of neem (*Azadirachta indica*) seeds. *J. Agric. Food Chem.* **1995**, *43*, 2517−2519.

Yamasaki, R. B.; Klocke, J.; Lee, S. M.; Stone, G. A.; Darlington, M. V. Isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds using flash chromatography and high-performance liquid chromatography. *J. Chromatogr.* **1986**, *356*, 220−226.

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