JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

An Efficient Method for the Purification and Characterization of Nematicidal Azadirachtins A, B, and H, Using MPLC and ESIMS

VANDANA SHARMA,† SURESH WALIA,† JITENDRA KUMAR,† MURALEEDHARAN G. NAIR,*,‡ AND BALRAJ S. PARMAR†

Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi-110 012, India and Department of Horticulture and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan 48824

Azadirachtin A enriched concentrate containing 60% active ingredient (a.i.) was prepared from the methanolic extract of the de-fatted neem (*Azadirachta indica* A. Juss) seed kernels. Azadirachtins A, B, and H, the three major bioactive constituents of neem seed kernel, were purified from this methanolic concentrate by employing reverse phase medium-pressure liquid chromatography (MPLC), using methanol−water solvent system as an eluant. The three pure azadirachtin congeners thus obtained were characterized by their unique mass spectral fragmentation, using electrospray probe in positive ion mode (ESI). All three azadirachtins exhibited nematicidal and antifungal activities. Azadirachtin B was the most effective against the reniform nematode *Rotylenchulus reniformis* (EC_{50} 96.6 ppm), followed by Azadirachtin A (119.1 ppm) and H (141.2 ppm). At 200-ppm concentration, the test compounds caused 50−65% mortality of *Caenorhabditis elegans* nematode. Azadirachtin H showed the highest activity against the phytophagous fungi *Rhizoctonia solani* (EC_{50} 63.7 ppm) and *Sclerotium rolfsii* (EC_{50} 43.9 ppm), followed by B and A. The isolation of pure azadirachtins A, B, and H directly by MPLC purification from its concentrate and their characterization by ESIMS are unique and less time-consuming.

KEYWORDS: ***Azadirachta indica*; azadirachtin; *Caenorhabditis elegans*; *Rotylenchulus reniformis*; *Rhizoctonia solani*; *Sclerotium rolfsii*; antifungal; nematicidal; ESIMS**

INTRODUCTION

Azadirachtin-A, the major bioactive secondary metabolite of *Azadirachta indica* A Juss (neem), is well-known for its excellent insecticidal, antifungal, and growth disruptive activity against a variety of insect pests (*1*−*6*). An increased understanding of its molecular structure, as well as that of its natural and synthetic congeners, revealed interesting structure−activity relationships (*7*−*10*). Among the large number of tetranortriterpenoids isolated from various parts of *A. indica*, azadirachtin A and its several congeners have been found to exhibit significant biological activity. Several reports are now available about the extraction and isolation of neem azadirachtinoids (*1*, *11*−*13*). The tedious process of the isolation of these constituents involves extraction, partitioning of extractives between different solvents, column chromatography, preparative thin-layer chromatography, and preparative high performance liquid chromatography. Cost intensive and time-consuming high performance liquid chromatography has been employed in the past for the separation of azadirachtins A, B, D, H, and I from the seed kernel concentrate (*14*−*16*). In these studies, partially purified compounds obtained from the combined peaks for azadirachtins A + D and azadirachtins H + I in the first preparative LC run on one column were further subjected to another preparative LC run on RP-8/RP-18 columns to obtain azadirachtins A and D as well as H and I, respectively. Recently, minor meliacin constituents such as 11-epi-Azadirachtin H and 11-epi-azadirachtin D, the epimers of azadirachtins H and D (*17*−*19*), and three other azadirachtin congeners, namely azadirachtin K (*20*), 13,14-desepoxyazadirachtin A (*21*), and 1-tigloyl-3-acetyl-11-hydroxy-4-methyl meliacarpin (*22*) have been separated and identified from neem seed extracts. While azadirachtin A was present in the extract to the extent of 85%, azadirachtins B and H, the other two major components, were present only at concentrations up to 15% (*23*). Other azadirachtin congeners occurred as minor constituents in neem seed extracts. Despite the considerable progress that has been made in neem research so far, only a few methods are available for the separation of major azadirachtins. To determine activity profile and content of major azadirachtins in azadirachtin-based neem formulations and technical materials, azadirachtin congeners A, B, and H are required both as test materials and as reference standards. Because these materials are not available commercially, a fast and economical method for their separation and characterization from neem extractives in adequate quanti-

* To whom correspondence should be addressed. Telephone: (517) 353-2915. Fax: (517) 432-2310. Email: nairm@msu.edu.
† Indian Agricultural Research Institute.
‡ Michigan State University.

10.1021/jf0342167 CCC: $25.00
Published on Web 05/31/2003

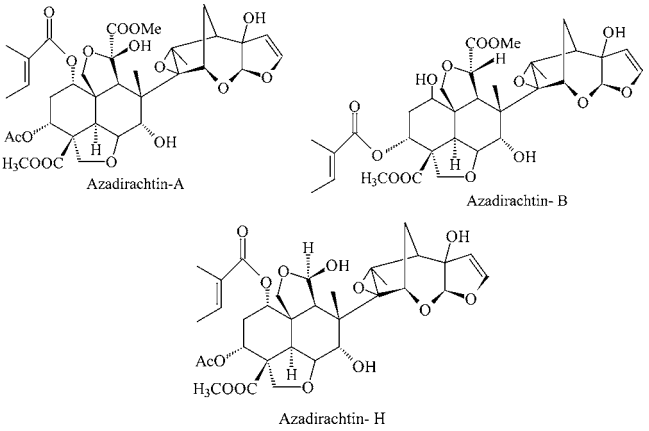

Figure 1. Structures of azadirachtins A, B, and H.

ties is in urgent need. This paper describes a simple method for the isolation of azadirachtin A powder concentrates, as well as separation of three major azadirachtins, A, B, and H (**Figure 1**) from such concentrates by MPLC. In addition to mass spectral evidence described in this paper for the first time to establish their structures, these compounds, along with azadirachtin concentrates, were also evaluated for their nematicidal activity against the reniform nematode, *Rotylenchulus reniformis*, and anti-fungal activity against the two phytopathogenic fungi, *Rhizoctonia solani* and *Sclerotium rolfsii*.

MATERIALS AND METHODS

Chemicals and Solvents. Laboratory grade reagents and solvents were locally procured. The solvents for extraction were distilled, and wherever required, dried before use. HPLC grade methanol and "SQ" methanol procured from Qualigen Fine Chemicals, a Division of Glaxo Smith Kline Pharmaceuticals, Mumbai, India, were used for the analytical HPLC and MPLC separation of azadirachtins. Water for chromatographic analyses was purified using MilliQ water purification system. Premixed solvents for LC analyses were filtered through a Millipore filter (0.45 μm). All other chemicals used in the study were of reagent or analytical grade and used as received.

Neem Materials, Fungal and Nematode Cultures. Neem seed kernels were procured from Neem Mission Pune, India. Standard reference azadirachtin (95% purity) was obtained from Trifolio-M GmbH, Germany, through the courtesy of Neem Mission Pune, India. Ready-made potato-dextrose-agar (PDA) medium was obtained from Hi-Media Lab, Mumbai, India. The culture of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, was obtained from the Division of Nematology, Indian Agricultural research Institute, New Delhi. The nematode *Caenorhabdities elegans* was maintained at Bioactive Natural Product Laboratory, Michigan State University. Phytopathogenic fungi, *Rhizoctonia solani* (Kuhn) and *Sclerotium rolfsii* Sacc, were obtained from the Division of Mycology and Plant Pathology of Indian Agricultural Research Institute, New Delhi.

General Procedures. Thin-layer chromatography (TLC) was performed on silica gel coated plastic plates (Analtech) using dichloromethane/methanol (96:4) as solvent system. The medium-pressure liquid chromatography (MPLC) was performed on a column (50 × 10 cm) packed with C-18 silica (60 micron). The spots were visualized under a UV-lamp at 254 nm and developed by spraying with dilute sulfuric acid (10% v/v), followed by heating. The ^{1}H NMR spectra were recorded on a Varian INOVA 300 MHz spectrophotometer in $CDCl_3$, using tetramethylsilane (TMS) as an internal standard. Electrospray mass spectroscopy was carried out on a VG platform quadrupole mass spectrometer (Fisons Instrument), by flow injection analysis via a Rheodyne injection loop (10 μL) into a stream of acetonitrile-water (1:1, v/v) flowing at 5 μL min^{-1} into the electrospray probe. The spectrometer was scanned in positive ion mode from 100 to 800 amu.

High Performance Liquid Chromatography (*HPLC*). Analytical reverse phase HPLC was performed on a Waters HPLC system fitted with LichroCart^R 250−4 mm, LiChrosphere^R 100 RP-18e column (5 μm), procured from Merck KgaA, Darmstadt, Germany. The mobile phase, methanol/water (50:50, v/v), was used under isocratic conditions at a flow rate of 0.75 mL min^{-1}. The samples were filtered through a 0.25-μm Millipore filter before injection (10 μL) by the Waters 717 auto-sampler. A Waters 919 photodiode array detector (PDA, Millipore Corporation)) was used. Peaks were detected at 217 nm, and the retention time (R_t) for each compound was measured. The calibration and quantification were carried out using Waters Millennium 2010 Chromatography Manager GPC software version 2.0 (Millipore Corporation, Waters Chromatography Division).

Isolation of Azadirachtin A Concentrates from Neem Seed Kernel. Ground neem seed kernels (500 g) were extracted with *n*-hexane (4 × 500 mL) at room temperature (25 °C) to separate neem oil. The de-fatted need seed cake was then extracted with methanol (4 × 500 mL), and the combined extract was concentrated to one-fourth of the volume. It was partitioned with *n*-hexane (250 mL) and 95% aqueous methanol (250 mL) to remove the residual oil and other nonpolar constituents. The methanol extract after concentration under vacuum was again partitioned with water (250 mL) and ethyl acetate (250 mL), and the organic phase was decolorized with activated charcoal (5 g), filtered, and evaporated under vacuum to obtain a viscous concentrate (6 g). The concentrate was dissolved in ethyl acetate (10 mL) and precipitated in excess of hexane to obtain a powder enriched in azadirachtin A (20%, 5.0 g). The process was repeated again twice to further enrich azadirachtin A and yielded a powdered concentrate containing 60% azadirachtin A (2.6 g).

MPLC Purification of Azadirachtins A, B, and H. Azadirachtin concentrate (60% purity) was purified by MPLC. The glass column (tayperling type, 600 × 40 mm, Dychrome, Sunnyvale, CA) pre-fitted with glass guard column (15 × 25 mm) and an automatic fraction collector. The column was fitted with an MPLC pump (Sanki Engineering Ltd, Model LBP-V). Both of the columns were packed with RP-18 material (40−63 μm). The pump was operated at 10−15 psi pressure, and the column was preconditioned with methanol/water (65:35) for 2 h at a flow rate of 2-ml min^{-1} to ensure that no other contaminants were present on the column. To develop an efficient method of separation to yield pure azadirachtins, crude azadirachtin concentrate (750 mg, 60% purity) was dissolved in methanol/water (65:35, v/v, 5 mL) and loaded onto the guard column. The column was then eluted with a series of (1) methanol/water (65:35, 55:45, 50:50, and 45:55, v/v), (2) acetonitrile/water (60:40, v/v), and (3) methanol/acetonitrile/water (35:25:40, v/v) solvent systems to determine the best elution system for the separation of aszadirachtins A, B, and H. Among the various solvent systems tried as eluants, methanol/water (50:50) at a flow rate of 2 mL min^{-1} provided the optimum separation. Also, loading of the MPLC column with 750 mg of azadirachtin concentrate per injection facilitated better separation. Therefore, the final purification was accomplished by injecting 750 mg of crude azadirachtin mixture and eluted with methanol/water (50:50) at a flow rate of 2 mL min^{-1}. Fractions (350) of 10 mL each were collected, and fractions with similar TLC profiles were combined. The fractions containing azadirachtins A, B, and H, detected as single spot on the TLC plates, were pooled and evaporated under reduced pressure at 45 °C. Pure azadirachtins H (10 mg), A (256 mg), and B (15 mg) were isolated as white powders from the pooled fractions 45−51, 90−104, and 170−175, respectively. Azadirachtin H, mp 247−252 °C, Rf 0.65 (dichloromethane/methanol 96:4, v/v), R_t (HPLC) 8.785 min; Azadirachtin A, mp 155−156 °C, Rf 0.72 (dichloromethane/methanol 96:4, v/v), R_t (HPLC) 13.842 min; Azadirachtin B, mp 202−204 °C, Rf 0.82 (dichloromethane/methanol 96:4, v/v), R_t (HPLC) 16.770 min. The HPLC chromatograms of azadirachtin concentrate (60%) along with pure azadirachtins A, B, and H are shown in **Figure 2**.

Nematicidal Activity. *Rotylenchulus reniformis*. Azadirachtin A concentrates (20, 60, and 90% azadirachtins concentration) and azadirachtin A, B, and H were evaluated for efficacy against nematodes. The nematodes, isolated originally from a single egg mass from castor roots, were cultured, multiplied, and maintained on cowpea plants raised in 30-cm earthen pots containing sterilized soil−sand mixture. The egg

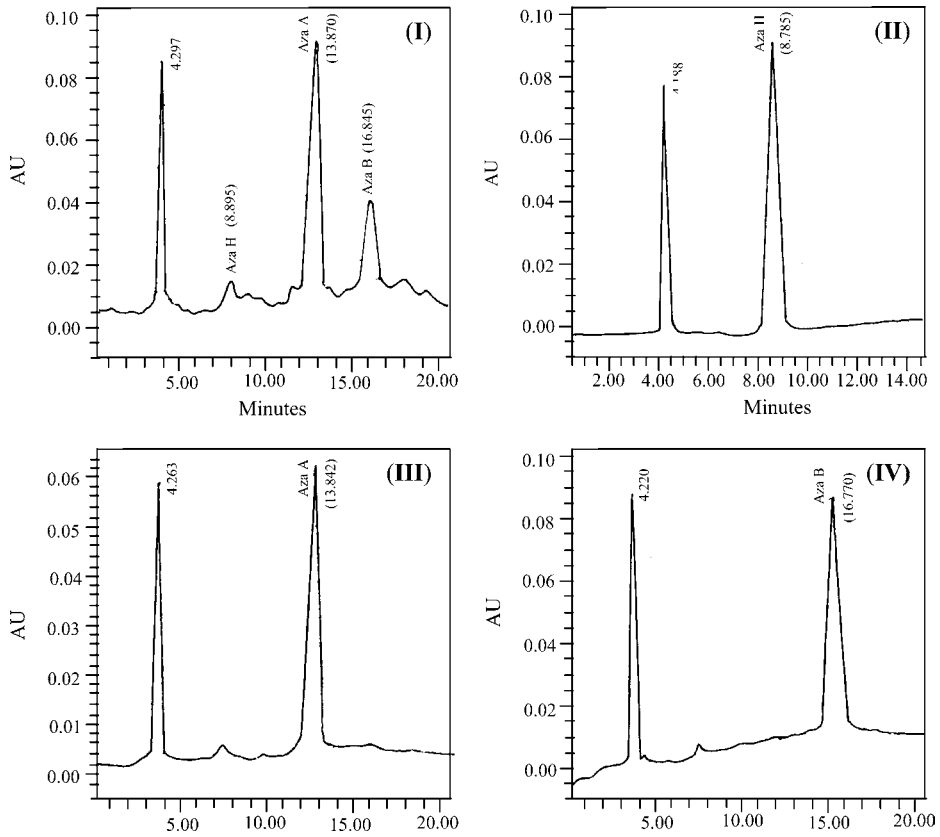

Figure 2. HPLC chromatograms of azadirachtin concentrate 60% (I) and its three major constituents, azadirachtin H (II), azadirachtin A (III), and azadirachtin B (IV), isolated by MPLC. The peak around 5 min in each chromatogram is the solvent front.

masses were collected and kept on two layers of tissue paper supported by aluminum wire gauge (8−10 μ) in 10-cm diameter Petri dishes filled with fresh water. After 4 days, the average population of *R. reniformis* that emerged in the suspension was determined by counting fourth stage juvenile (J 4) of *R. reniformis* in 1 mL aliquots in triplicates.

Azadirachtins (15 mg each) were weighed separately and dissolved in ethanol (0.5 mL), and the volume was made up to 5 mL by 0.5% emulsified water (5 mL of Tween 80 in 1 L of distilled water) to get a stock solution of 3000 ppm. Test solutions of 1500, 750, 500, 250, and 125 ppm were prepared by serial dilutions of the stock solution with 0.5% emulsified water. Suspension of juvenile nematodes was diluted with water to 100 mL to get approximately 50 juveniles ml^{-1}. To one ml of this nematode suspension in Petri dish, an equal volume of test solutions was added separately, to obtain the desired test concentrations of 750, 500, 250, 125, and 62.5 ppm, respectively. Juveniles of the nematode kept in water with ethanol served as the control. After 72 h of exposure, the suspension in three Petri dishes for each treatment was observed under a stereoscopic binocular microscope for determining juvenile immobility. A revival test was performed for each treatment by decanting off the test solution and adding distilled water to the Petri dishes. After 48 h, readings were taken again. The revived juveniles were counted and deducted from the number of immobile juveniles obtained in the previous reading taken after 72 h. Juvenile nematodes found immobile after the revival test were considered as dead. The corrected percent mortality was calculated using the Abbot's formula: Corrected mortality (%) = $((T - C)/(100 - C)) \times 100$, where T = percent mortality in treatment and C = percent mortality in control. LC_{50} values (ppm) were calculated by using a Basic LD_{50} program version 1.1 as described (*24*).

Caenorhabdities elegans: Azadirachtin A concentrates (20, 60, and 90%) and azadirachtins A, B, and H at 200 ppm (0.2 μg ml^{-1} total concentrate basis) were evaluated according to the reported procedure (*23*). A culture of *C. elegans* was grown on NG agar medium containing a strain of *Escherichia coli* in disposable Petri dishes wetted with 2−4 mL of physiological saline solution. The culture was stored at room temperature and subcultured prior to the assay. The assay was conducted in Corning polystyrene 96-well plates. The nematodes were added to 1 mL of the saline solution in a scintillation vial, and the suspension was diluted to a nematode count of 15 to 20, in a 48-μL aliquot. A solution (48 μL) containing nematodes was delivered to each of the three wells per treatment. Two microliters of DMSO (50%) or DMSO (50%) and test compound were added to each well. The plates were then covered, parafilmed, and incubated in a humid chamber. The number of dead nematodes were recorded at 2, 4, 6, and 24 h by observing under a Microscope (*25*).

Antifungal Activity. The test compounds were assayed for antifungal activity against two fungi, *Rhizoctonia solani* Kuhn. and *Sclerotium rolfsii* Sacc., by the poisoned food technique, using potato-dextrose-agar (PDA) medium. Cultures of the test fungi were maintained on PDA slants at 25 °C and were sub-cultured in Petri dishes prior to testing. The ready-made PDA medium (39 g) was suspended in distilled water (1000 mL) and heated to boiling until it had dissolved completely. The medium and the Petri dishes were autoclaved for 30 min. Stock solutions were prepared by dissolving the test materials (65 mg) in acetone (4 mL), and dilutions of 2, 1, 0.4, 0.1, and 0.02 mL of this solution in 65 mL of PDA medium gave test concentrations of approximately 500, 250, 100, 25, and 5 mg $liter^{-1}$, respectively. Acetone (1 mL) served as the control. The medium was poured into a set of two Petri dishes (two replications) under aseptic conditions in a laminar flow hood. When the medium in the plates was partially solidified, a 5-mm thick disk of fungus (spores and mycelium) cut from earlier subcultured Petri dishes was placed at the center of the semisolid medium, and the lids of the dishes were replaced. The treated and control dishes were kept in an incubator at 26 (± 2) °C until the fungal growth in the control dishes was almost complete (2−3 days). The mycelial growth of fungi (mm) in both treated (T) and control (C) Petri dishes was measured diametrically in three different directions and growth inhibition (I), and corrected inhibition (IC) were calculated using the formula: I (%) = $((C - T) / C) \times 100$, IC = $((I - CF)/(100 - CF)) \times 100$, where CF = $((90 - C_0) / C_0) \times 100$, 90 is the diameter (mm) of the Petri dish, and C_0 is the growth of the fungus (mm) in control. EC_{50} values (effective concentration for 50% inhibition; μg ml^{-1}) were calculated for inhibition of growth using the Basic LD_{50} Program, version 1.1 (*24*).

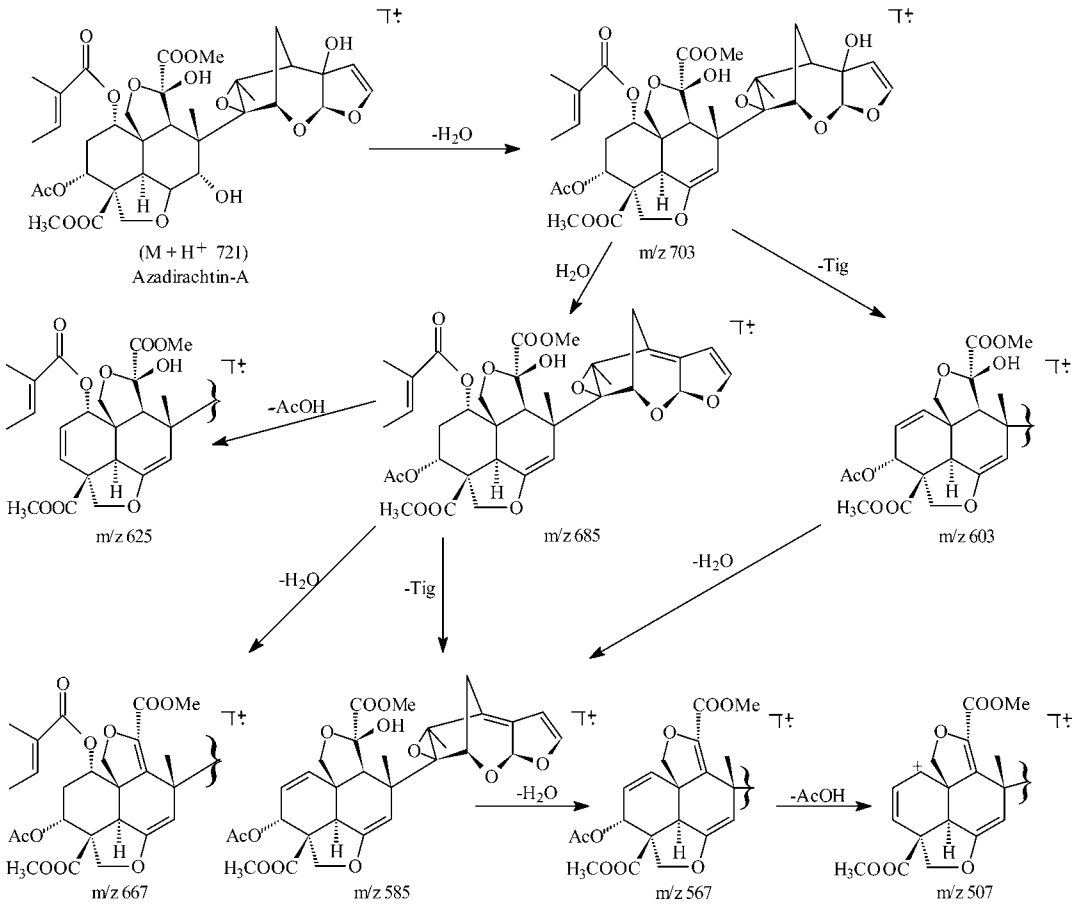

Figure 3. ESI-Mass fragmentation pattern of azadirachtin A.

RESULTS AND DISCUSSION

Isolation and Characterization of Azadirachtins A, B, and H. An efficient method has been developed for the isolation of azadirachtin A enriched powder concentrates (20 and 60%) from crude azadirachtin A extract obtained by the methanol extraction of the defatted neem seed kernels. In the past, preparative HPLC was used to separate various azadirachtin congeners (*14*−*16*) from azadirachtin concentrates. Most of the preparative HPLC procedures reported (*15*) to separate azadirachtins A, B, D, H, and I from crude azadirachtin concentrates were complicated, time-consuming, and involved the use of numerous preparative HPLC columns. Unlike the earlier reported methods, the MPLC method described for the purification of azadirachtins A, B, and H (**Figure 1**) herein is comparatively simpler, more convenient, more cost-effective, and less time-consuming. We have also optimized the MPLC purification method in terms of MPLC column material, quantity of extract injected, eluting solvent system, flow rate, and fraction sizes. It has been observed that injecting lesser quantity facilitated better separation. The best separation was achieved when the column was loaded with 750 mg of the test sample (azadirachtin A concentrate, 60%). Among the various solvent systems tried in the study, methanol/water (50:50) at a flow rate of 2 mL min^{-1} provided the optimum separation. The azadirachtins thus obtained gave only one peak when profiled by HPLC (**Figure 2**). The MPLC column was flushed with 100% methanol (1 bed volume) prior to each injection. The ^{1}H NMR data of azadirachtins A (*11*), B (*24*), and H (*16*, *17*) were in agreement with the literature values. The unique mass fragmentation pattern of azadirachtins A, B, and H recorded with electrospray probe in positive ion mode (**Figures 3**, **4**, and **5**) provided additional evidence to support their structures.

Mass Spectral Fragmentation Pattern of Azadirachtins A, B, and H. The electrospray mass spectrum (ESIMS) of azadirachtin A in positive ion mode gave the molecular ion $[MH]^+$ peak at *m*/*z* 721 and corresponded to the molecular formula $C_{35}H_{44}O_{16}$. The MS fragmentation pattern of azadirachtin A is presented in **Figure 3**. The major fragment ion peaks originated as a result of the sequential elimination of one neutral molecule each of water (18 amu), acetic acid (60 amu), and tiglic acid (100 amu) from the molecular ion indicated the presence of hydroxyl, acetyl, and tigloyl functions in the molecule. The fragment ion at *m*/*z* 703, formed as a result of the loss of H_2O molecule, underwent further loss of H_2O and tiglic acid units to yield peaks at *m*/*z* 685 and *m*/*z* 603, respectively. The ion at *m*/*z* 661 corresponded to the fragment formed as a result of the loss of an AcOH unit from the MH^+ ion. The fragment ion at *m*/*z* 685 underwent further elimination of H_2O and tiglic acid moieties to form corresponding peaks at *m*/*z* 667 and 585, respectively. Similarly, the fragment ion at *m*/*z* 603 gave fragment ions at *m*/*z* 585 and 567 by successive elimination of H_2O. The MS also exhibited two fragment ions at *m*/*z* 625 and 507, formed as a result of the loss of AcOH molecules from the fragment ion *m*/*z* 685 and 567, respectively. In addition, the ESIMS of azadirachtin A gave two characteristic peaks at *m*/*z* 743 and 759, which were attributed to $[M+ Na]^+$ and $[M+K]^+$, respectively. This report of a detailed mass fragmentation pattern of azadirachtin A is a first. It is interesting to note that azadirachtin A did not show a molecular ion peak in an earlier report; instead, it recorded a fragment ion peak at *m*/*z* 702 due to the loss of H_2O molecule from its molecular ion (*26*).

The ESIMS of azadirachtin B gave an $[MH]^+$ peak at *m*/*z* 663, corresponding to its molecular formula $C_{33}H_{42}O_{14}$. The

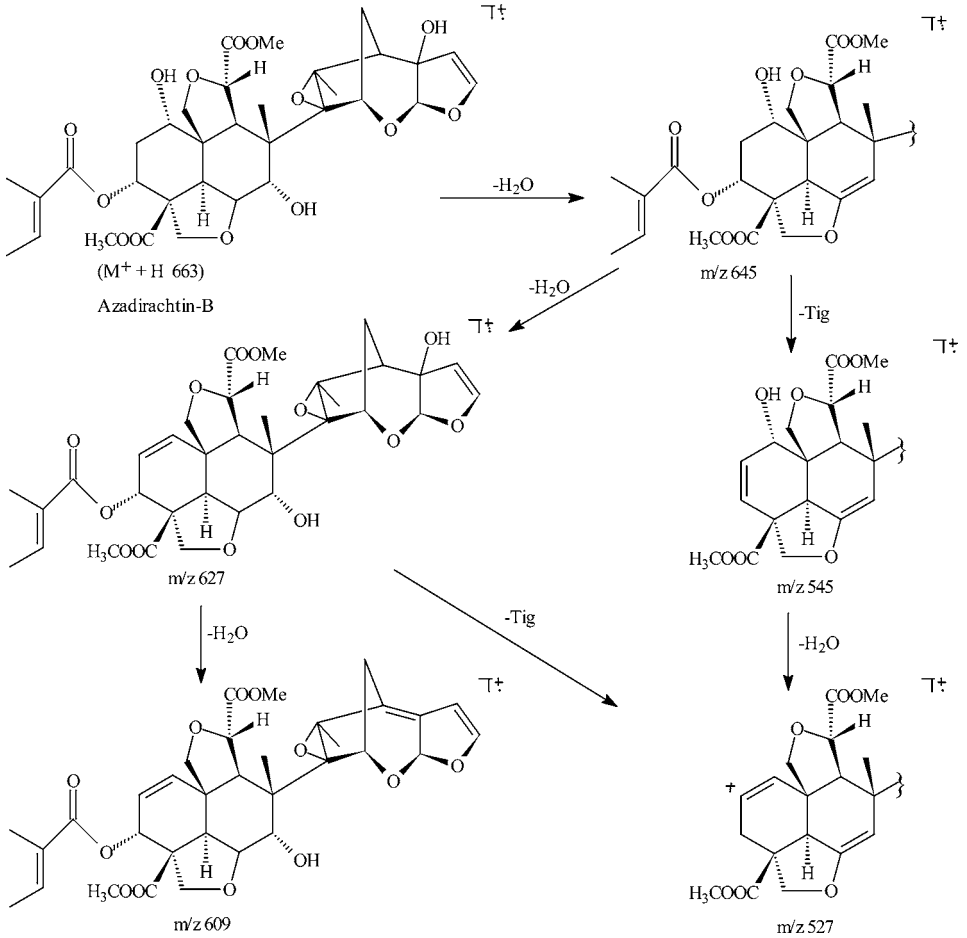

Figure 4. ESI-Mass fragmentation pattern of azadirachtin B.

fragment ion peaks at *m*/*z* 645, 627, and 609 were assigned as a result of the successive loss of three H_2O molecules from its parent ion (**Figure 4**). From the fragment ion at *m*/*z* 645, two peaks emerged at *m*/*z* 545 and 527 as a result of the successive elimination of tiglic acid and water molecules, respectively. The conspicuous absence of the fragment ion corresponding to the elimination of acetic acid (60 amu) from either the molecular ion or subsequent fragment ions indicated the absence of an OAc moiety in the molecule. Like azadirachtin A, the structure of azadirachtin B was further supported by the presence of two characteristic ion peaks at *m*/*z* 685 and 701 and corresponded to the Na (23 amu) and K (39 amu) adducts, respectively (**Figure 4**). The published reports of the field desorption mass spectrum of azadirachtin B showed only two major peaks at *m*/*z* 662 (M^+) and 645 (MH^+-H_2O) (*27*).

The structure of azadirachtin H was corroborated by its ESIMS, which showed $[MH]^+$ at *m*/*z* 663, corresponding to the molecular formula $C_{33}H_{42}O_{14}$. It gave identical molecular mass as azadirachtin B but showed a different MS fragmentation pattern (**Figure 5**). The fragment ions at *m*/*z* 645 and 603 were assigned due to the loss of H_2O and AcOH units from the parent molecule. Similarly, the successive loss of tiglic acid (100 amu) and AcOH (60 amu) from the ion at *m*/*z* 645 yielded corresponding ions at *m*/*z* 545 and 485, respectively. Two major peaks at *m*/*z* 685 and 701, azadirachtin H adducts with Na and K, respectively, were also detected. The sodium adducts peak in all the three azadirachtin congeners appeared as a base peak in their ESIMS spectra. The FABMS of azadirachtin H reported earlier did not show a molecular ion peak; instead, like in azadirachtin, it recorded a fragment ion peak at *m*/*z* 644, due to the loss of H_2O from the molecular ion (*16*).

Nematicidal Activity of Azadirachtins. *R. reniformis*, a reniform nematode, is one of the significant plant pests of tropics, semitropical, and warmer areas of the Temperate Zone. It has been reported to be pathogenic in several vegetables, fruits, legumes, pulses, oilseeds, cereals, and other commercial crops (*28*). Therefore, azadirachtin A concentrates and azadirachtin A, B, and H were evaluated for their potential growth inhibitory activity against this nematode. The EC_{50} values of azadirachtin concentrates and azadirachtins A, B, and H at 72 h are reported in **Table 1**. The nematicidal activity of the azadirachtin concentrates was directly related to the azadirachtin A content. Azadirachtins B and H contributed to the activity of azadirachtin concentrates, as evidenced by the EC_{50} values of 96.6 and 141.2 ppm, respectively. The various meliacins probably contained as minor quantities in these concentrates may also have influenced the activity. Azadirachtins and other neem meliacins such as nimbin and salanin were reported to exhibit significant nematicidal activity against the root knot nematode *Meloidogyne incognita* (*28*). Azadirachtins A, B, H, and the 20% concentrate were also evaluated against the *C. elegans* nematode at 200 ppm concentration. Azadirachtins A, B, and H exhibited 56, 64, and 65% mortality, respectively, after 24 h (**Table 2**). At this concentration, azadirachtin A powder concentrate (20%) exhibited 50% mortality.

Antifungal Activity. *R. solani* and *S. rolfsii* cause severe damage to several crops and agricultural produce. *R. solani* is a deadly soil fungus causing "damping off" disease in several important crops, including peanuts, tomatoes, potatoes, cotton, and radish. The azadirachtin concentrates showed a similar trend in fungicidal activity as that in the case of nematodes tested. The EC_{50} against both *R. solani* and *S. rolfsii* decreased (**Table**

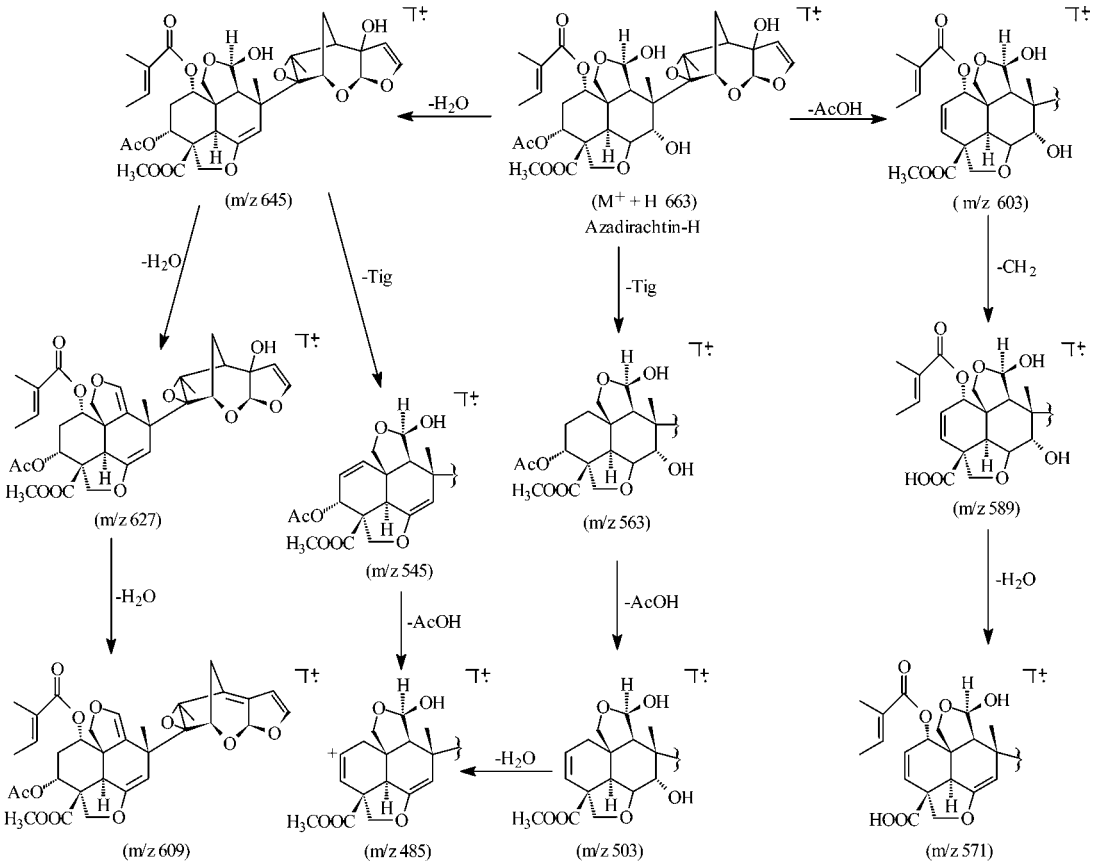

Figure 5. ESI-Mass fragmentation pattern of azadirachtin H.

Table 1. Nematicidal Activity of Azadirachtin A Concentrates and Azadirachtins A, B, and H against *Rotylenchulus reinformis*

treatment	EC_{50} (ppm)	fiducial limits	χ^{2a} (3 d.f; 95%)
azadirachtin A (20%)	193.9	158.3–238.5	1.4
azadirachtin A (60%)	151.9	123.23–187.3	5.9
azadirachtin A (90%)	119.1	93.5–152.7	7.12
azadirachtin B	96.6	77.0–122.3	5.5
azadirachtin H	141.2	114.0–176.1	3.4

[a] All data were homogen.

Table 2. Percent Mortality of *Caenorhabditis elegans* against Azadirachtins at 200 ppm

test compound	mortality (%)
azadirachtin A	56
azadirachtin B	64
azadirachtin H	65
azadirachtin A (20%)	50

Table 3. Antifungal Activity of Azadirachtin A Concentrates and Azadirachtins B and H against *Rhizoctonia solani* and *Sclerotium rolfsii*

test compound	ED_{50} (ppm)	fiducial limits	χ^{2a} (3 d.f.,95%)
	Rhizoctonia solani		
azadirachtin A (20%)	165.2	126.6–215.6	7.2
azadirachtin A (60%)	132.2	101.8–171.7	6.8
azadirachtin A (90%)	104.8	81.9–134.0	7.3
azadirachtin B	85.9	68.0–108.5	5.6
azadirachtin H	63.7	50.4–80.5	7.2
	Sclerotium rolfsii		
azadirachtin A (20%)	140.5	108.3–182.2	6.4
azadirachtin A (60%)	121.9	94.5–157.2	6.3
azadirachtin A	93.6	73.6–118.9	6.5
azadirachtin B	76.2	60.6–95.8	7.6
azadirachtin H	43.9	40.3–64.7	7.2

[a] All data were homogeneous.

3) with an increase in the azadirachtin A content of the concentrate, which indicated that the activity against the two test fungi was directly related to the azadirachtin A content. However, both azadirachtins B and H were considerably more fungitoxic than azadirachtin A and azadirachtin A based concentrates. Unlike the nematicidal activity, the fungicidal activity against both test fungi was higher in the case of azadirachtin H than that in azadirachtin B. Of the three pure azadirachtins, azadirachtin H was found to be the most active against *R. solani* (EC_{50}, 63.7) and *S. rolfsii* (EC_{50}, 43.9 ppm), followed by azadirachtin B, which showed ED_{50} of 85.9 and 76.2 ppm, respectively.

Neem oil and its constituents were reported to possess antifungal activity against some of the phytopathogenic fungi *Drechsiera oryzae*, *Alternaria tenuis*, and *Fusarium oxysporium* (*29*). The neem constituents, such as nimbidin from seed oil (*30*) and isomeldenin and neemonol from neem leaves (*31*), also exhibited antifungal activity. The crude neem seed kernel extracts were found to be effective against several plant pathogenic fungi (*32*). The fungicidal activity was generally attributed to the synergistic presence of tetraterpenoids other than azadirachtins (*29*).

The MPLC results described in this paper are promising, because the paper provides a simple and convenient method for the purification of bioactive azadirachtins A, B, and H. The bioassay results revealed that while azadirachtin B is more effective as a nematicidal agent against *R. reniformis*, aza-

dirachtin H is more potent as a fungicide against the phytopathogenic fungi *R. solani* and *S. rolfsii*. Bioefficacy trials with the azadirachtin A based neem products or azadirachtins A, B, and H must be evaluated under greenhouse and field conditions to determine their potential use as pest managing compounds. Broad conclusions may become possible only after a critical appraisal of a large data. Further research is, however, needed to stabilize azadirachtins A, B, and H or to design analogues with increased shelf life and biological activities.

LITERATURE CITED

(1) Butterworth, J. H.; Morgan, E. D. Isolation of a substance that suppresses feeding in locusts. *J. Chem. Soc. Chem. Commun.* **1968**, 23−24.

(2) Schmutterer, H. *The Neem Tree: Source of Unique Natural Products for Integrate Pest Management, Medicine, Industry and Other Purposes*; VCH: Weinheim, Germany, 1997; p 695.

(3) Randhawa, N. S., Parmar, B. S. *Neem Research and Development*; Society of Pesticides Science, and New Age International Publishers (P) Ltd.: New Delhi, India, 1993; p 332.

(4) Schmutterer, H. Synthesis and Juvenile Hormone Mimetic Insect growth disrupting and fecundity reducing ingredients from the neem and chinaberry trees. In *CRC Handbook of Natural Pesticides*; Morgan, E. D., Mandava, N. B., Eds.; CRC Press: Boca Raton, FL, 1987; Vol III, Part B, pp 119−170.

(5) Schmutterer, H. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annu. Rev. Entomol.* **1990**, *35*, 271−279.

(6) Loke, J. C. In *The Neem Tree*: *Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes*; Schmutterer, H., Ed.; VCH: Weinheim, Germany, 1995; pp 118−125.

(7) Yamasaki, R. B.; Klocke, J. A. Structure-bioactivity relationships of azadirachtin, a potent insect control agent. *J. Agric. Food Chem.* **1987**, *35*, 467−471.

(8) Ley, S. V.; Denholm, A. A.; Wood, A. The chemistry of azadirachtin. *Nat. Prod. Rep.* **1993**, *10*, 109−157.

(9) Mordue (Luntz), A. J.; Blackwell, A. Azadirachtin: an update. *J. Insect. Physiol.* **1993**, *39*, 903−924.

(10) Rembold, H. Isomeric azadirachtins and their mode of action In *The Neem Tree*; Jacobson, M., Ed.; Focus on Phytochemical Pesticides, CRC Press: Boca Raton, Florida, 1989; Vol. I, pp 47−67.

(11) Yamasaki, R. B.; Klocke, J. A.; 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.

(12) Schroeder, D. R.; Nakanishi, K. A simplified isolation procedure for azadirachtin. *J. Nat. Prod.* **1987**, *50*, 241−244.

(13) Ubel, E. C.; Warthen, O. D.; Jacobson, M. Preparative reversed phase liquid chromatographic isolation of azadirachtin from neem kernels. *J. Liq. Chromatogr.* **1979**, *2*, 875−882.

(14) Govindachari, T. R.; Sandhya, G.; Ganesh Raj, S. P. Simple method for the isolation of azadirachtin by preparative HPLC. *J. Chromatogr.* **1990**, *513*, 389−391.

(15) Govindachari, J. R.; Sandhya, G.; Ganesh Raj, S. P. Isolation of novel azadirachtins H and I by high performance liquid chromatography. *Chromatographia* **1991**, *31*, 303−305.

(16) Govindachari, T. R.; Sandhya, G.; Ganesh Raj, S. P. Azadirachtin H and I: two new tetranortriterpenoids from *Azadirachta indica. J. Nat. Prod.* **1992**, *55*, 596−601.

(17) Govindachari, T. R.; Gopalakrishnan, G.; Malathi, R.; Pradeep Singh, N. D. The 11-a-H and 11-b-H epimers of azadirachtin H. *Nat. Prod. Lett.* **1999**, *13*, 157−162.

(18) Ramji, N.; Venkatakrishnan, K.; Madyastha, K. M. 11-epi-Azadirachtin H from *Azadirachta indica*. *Phytochemistry* **1996**, *42*, 561−562.

(19) Ramji, N.; Venkatakrishnan, K.; Madyastha, K. M. 11-epi-Azadirachtin D: an epimeric azadirachtin analogue from *Azadirachta indica*. *Phytochemistry* **1998**, *49*, 265−267.

(20) Govindachari, T. R.; Sandhya, G.; Ganesh Raj, S. P. Structure of azadirachtin K, a new tetranortriterpenoid from *Azadirachta indica*. *Indian J. Chem.* **1992**, *31B*, 295−298.

(21) Govindachari, T. R.; Gopalkrishanan, G. 13, 14-Desepoxyazadirachtin-A, a tetranortriterpinoid from *Azadirachta indica*. *Phytochemistry.* **1997**, *45*, 397−399.

(22) Rozatkar, S. R.; Nagasampagi, B. 1-Tigloyl-3-acetyl-11-hydroxy-4-methyl meliacarpin from *Azadirachta indica*. *Phytochemistry* **1992**, *32*, 213-214.

(23) Rembold, H.; Azadirachtins In *Insecticides of Plant Origin*; Arnason, J. T. Philogene, B. J. R., Morand, P., Eds.; American Chemical Society Symposium Series 387, American Chemical Society: Washington, DC, 1989; pp 150−163.

(24) Trevors, J. T. A basic programme for estimating LD_{50} values using the IBM-PC. *Bull. Environ. Contam. Toxicol*. **1986**, *37*, 18−26.

(25) Momin, R. A.; Ramsewak, R. S.; Nair, M. G. Bioactive compounds and 1,3-di-[(cis)-9-octadecenoyl]-2-[(cis, cis)-9, 12-octadecadienoyl] glycerol from *Apium graveolens* L. seeds. *J. Agric. Food. Chem*. **2000**, *48*, 3785−3788.

(26) Kubo, I.; Matsumoto, A.; Matsumoto, T. New insect ecdysis inhibitory limonoiddeacetylazadirachtol isolated from *Azadirachta indica* (Meliaceae) oil. *Tetrahedron* **1986**, *42*, 489−496.

(27) Klenk, A.; Bokel, M.; Kraus, W. 3-Tigloylazadirachtol (Tigloyl = 2-Methyl crotonoyl), an insect growth regulating constituent of *Azadirachta indica*. *J. Chem. Soc. Chem. Commun.* **1986**, *7*, 523−524.

(28) Devakumar, C.; Goswami, B. K. Nematicidal principals from neem (*Azadirachta indica* A. Juss) Part III Isolation and bioassay of some neem meliacins. *Pesticide Research J.* **1992**, *4*, 81−86.

(29) Govindachari, T. R.; Suresh. G.; Gopalkrishanan, G.; Banumathy, B.; Masilamani, S. Identification of antifungal compounds from the seed oil of *Azadirachta indica*. *Phytoparasitica* **1998**, *26*, 109−116.

(30) Khan, M. W.; Alam, M. M.; Khan, A. M.; Saxena, S. K. Effect of water-soluble fractions of oil cakes and bitter principles of neem on some fungi and nematodes. *Acta Bot. Indica*. **1974**, *2*, 120−128.

(31) Suresh, G.; Narasimhan, N. S.; Masilamani, S.; Partho, P. D.; GopalaKrishnan, G. Antifugal fractions and compounds from uncrushed green leaves of *Azadirachta indica*. *Phytoparasitica* **1997**, *25*(1), 33−39.

(32) Parveen, G.; Alam, M. M. Bioactivity against plant pathogens. In *Neem Research and Development*; Randhawa, N. S., Parmar, B. S., Eds.; Society of Pesticide Science: New Delhi, India, 1993; pp 144−153.

Received for review March 5, 2003. Revised manuscript received May 7, 2003. Accepted May 8, 2003. Financial support provided by the Indian Council of Agricultural Research (ICAR), New Delhi and the World Bank/ICAR NATP subproject on Environment Friendly Pesticides.

JF0342167