Identification of Nematicidal Fatty Acids and Triglycerides from Seeds of *Jubaea chilensis* by GC-EI-MS and Chemical Transformation Methods

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Nematicidal bioassay-guided fractionation of the *n*-hexane extract of the seeds of *Jubaea chilensis* led to the identification of eight known fatty acids and a mixture of triglycerides, reported for the first time for this species. In addition, their corresponding methyl esters were identified to be artifacts generated during the extraction and isolation procedures by using GC-EI-MS and chemical transformation methods. The fatty acid composition of the triglycerides was analyzed by GC-EI-MS and chemical transformation techniques. Among the 17 compounds, only lauric acid and myristic acid exhibited significant inhibitory effects on the movement of *Caenorhabditis elegans* with minimum inhibitory concentrations (MIC) of 75 μg/ml.

Key words: Jubaea chilensis, *Caenorhabditis elegans*, Fatty Acids GC-EI-MS

Introduction

Jubaea chilensis (Molina) Baill. (Arecaceae) commonly known as "Chilean wine palm", "coquito palm", or "honey palm" is an evergreen palm tree with mighty straight trunk up to ~ 25 m in height (Mabberley, 1997). This species indigenous to the coastal areas of central Chile also grows in areas with Mediterranean climates, such as California, the southern European coast and South Africa. Its sweet fruits, seeds, and sap have been used in sweetmeats, candies and wine. An edible oil can also be obtained from its seeds. Up to the present, no phytochemical and biological studies have been reported for this plant. As part of a collaborative search for novel bioactive principles of plant and microbial organisms from dryland biodiversity of Latin America (Timmermann *et al.*, 1999), the MeOH extract of the seeds of *J. chilensis* exhibited significant inhibitory effects on the growth of *Caenorhabditis elegans* with a minimum inhibitory concentration (MIC) of 600 μg/ml. Nematicidal bioassay-guided fractionation of this extract led to the identification of eight known fatty acids (**1**–**8**) and their corresponding methyl esters (**1a**–**8a**) (Fig. 1), along with a mixture of triglycerides. We report herein the identification of these compounds by GC-EI-MS and chemical transformation techniques, their inhibitory effects on the movement of *C. elegans*, and an investigation into the possible artifactual nature of **1a**–**8a**.

Materials and Methods

General experimental procedures

Linoleic, oleic, palmitic, stearic acids were purchased from Fisher Scientific (Houston, Texas, USA) while capric, lauric, myristic, and octanoic acids were obtained from Sigma-Aldrich (Milwaukee, Wisconsin, USA). GC-EI-MS data were obtained with a Varian Saturn 2100T GC-MS Workstation (Varian, Lake Forest, California, USA) including a data system software (Version 5.2) interfaced to a 3900-GC, a 2000-MS detector, and a 1079-injector. The gas chromatograph was fitted with a WCOT Fused Silica Chrompack capillary column packed with CP Sil 8 CB (30 m × 0.25 mm). Ultra pure helium at 1.2 ml/min was used as the carrier gas, and the injector, transfer line, and trap temperatures were 250 °C, 250 °C,

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and 200 °C, respectively. The temperature of the column oven was programmed at 80 °C for 5 min, and then to 280 °C at 10 °C/min, followed by 280 °C for 20 min. NMR spectra were recorded in $CDCl_3$ at room temperature on a Bruker Avance 300 NMR spectrometer in 5-mm NMR tubes with TMS as the internal standard. Column chromatography (CC) was conducted on silica gel (63–200 μm, Scientific Adsorbents Incorporated, Atlanta, Georgia, USA). A middle pressure LC (MPLC) system was set up with a Büchi 688 chromatography pump (Büchi Labortechnik AG, Flawil, Switzerland), a Büchi Borosilikat 3.3 glass column (2 × 45 cm) packed with C_{18} Packing silica gel (15 g, 5 μm, Aldrich, Milwaukee, Wisconsin, USA), and a Spectra/Chrom® CF-1 fraction collector (Spectrum Chromatography Inc., Houston, Texas, USA). Analytical TLC was performed on Whatman Diamond K6F silica gel 60A (250 μm) and Merck RP-18 WF_{254S} (200 μm) plates. Compounds were visualized on TLC plates by dipping in anisaldehyde or vanillin-sulfuric acid reagents followed by charring at 110 °C for 5–10 min.

Plant material

Seeds of *J. chilensis* were identified and collected in May 2000 in Palmar de Ocoa, V Región, Chile (32° 55′ S; 71° 2′ W) by Gloria Montenegro. A voucher specimen (No. 1009) has been deposited in the herbarium at the Pontificia Universidad Católica de Chile, Santiago, Chile. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between the University of Arizona and Pontificia Universidad Católica de Chile.

Extraction and isolation

The milled and dried seeds (650 g) were extracted by maceration with MeOH (4 × 2.0 l). After filtration and evaporation of the solvent *in vacuo*, the resultant extract was diluted with H_2O to afford a 90% aqueous MeOH solution (0.55 l) and then partitioned with *n*-hexane (3 × 0.55 l) and CH_2Cl_2/H_2O (1:1 v/v, 2 × 1.2 l), sequentially, to afford dried *n*-hexane-soluble (75 g) and CH_2Cl_2-soluble (6.7 g) residues. Among them, the *n*-hexane extract showed the strongest inhibition of the movement of *C. elegans* at a MIC value of 300 μg/ml when compared with MICs of 1200 μg/ml and > 1200 μg/ml for the CH_2Cl_2 and H_2O extracts, respectively. Therefore, the *n*-hexane extract (75 g) was subjected to silica gel CC (750 g, 63–200 μm) by elution with a step gradient of *n*-hexane/acetone (100:1, 75:1, 75:1, 50:1, 10:1, 0:100 v/v, each 16 l) to give six pooled fractions (1–6), respectively. Among the six fractions, fraction 1 exhibited the strongest inhibition at a MIC of 38 μg/ml while the other five fractions all showed inhibition with MICs of > 150 μg/ml. Next, a portion of fraction 1 (2 g) was further fractionated by silica gel CC (130 g, 63–200 μm) to afford four further fractions, fraction 1–1 (50 mg, *n*-hexane/CH_2Cl_2 2.6:1 v/v, 2 l), fraction 1–2 (500 mg, *n*-hexane/CH_2Cl_2 2.2:1 v/v, 3 l), fraction 1–3 (1.0 g, *n*-hexane/CH_2Cl_2 1:1 v/v, 3 l), and fraction 1–4 (235 mg, *n*-hexane/EtOAc 95:5 v/v, 2 l).

^1H and ^{13}C NMR analyses of fraction 1–2 indicated that it was a mixture of triglycerides. A portion of fraction 1–2 (100 mg) was sequentially treated with 6 ml of 5% KOH in MeOH for 15 h and 10 ml of 1 N HCl for neutralization, and then partitioned with $CHCl_3/H_2O$ 1:1 v/v (3 × 25 ml) to give a hydrolysate (60 mg). A portion of the dried hydrolysate (3 mg) was further treated with an excess of diazomethane in a diethyl ether solution prepared using the Diazald® Kit diazomethane generator (Sigma-Aldrich) for 5 min to obtain a product, which was analyzed by GC-EI-MS to evaluate the fatty acid composition in the triglyceride mixture (Fig. 3D).

In turn, fractionation of fraction 1–4 (230 mg) by MPLC (acetonitrile/H_2O 60:40 v/v, 2.5 ml/min, 15 ml/vial, 4 l) sequentially resulted in the isolation of capric acid (**2**, 60 mg, vials 5–12), lauric acid (**3**, 116 mg, vials 20–30), myristic acid (**4**, 10 mg, vials 49–64), and linoleic acid (**6**, 15 mg, vials 70–100).

Capric acid (**2**): Wax, m.p. 29–31 °C. – ^1H NMR, ^{13}C NMR, and GC-EI-MS data (Table I) were identical to those of the commercial standard and the reported values (Pouchert and Behnke, 1993).

Lauric acid (**3**): Wax, m.p. 45–47 °C. – ^1H NMR, ^{13}C NMR, and GC-EI-MS data (Table I) were identical to those of the commercial standard and the reported values (Pouchert and Behnke, 1993).

Myristic acid (**4**): Wax, m.p. 53–55 °C. – ^1H NMR, ^{13}C NMR, and GC-EI-MS data (Table I) were identical to those of the commercial standard and the reported values (Pouchert and Behnke, 1993).

Linoleic acid (**6**): Colorless oil. – ^{1}H NMR, ^{13}C NMR, and GC-EI-MS data (Table I) were identical to those of the commercial standard and the reported values (Pouchert and Behnke, 1993).

Identification of components of fraction 1 by GC-MS and chemical transformation methods

A 2 μl aliquot of fraction 1 at 10 mg/ml in CH_2Cl_2 was injected into the capillary column and a representative gas chromatogram is shown in Fig. 2A. Compounds **1**–**5** in fraction 1 were identified by comparison of their GC-MS data (Table I) with their individual commercial standards (1 μg for each standard, Fig. 2B). Furthermore, the identification of **1**–**5** in fraction 1 was confirmed by the observed homogenous GC when fraction 1 was mixed with their individual standards (Fig. 2C).

In order to identify the overlapping GC peaks of fraction 1 around 21 min (Fig. 2A) and confirm the identification of **1**–**5**, fraction 1 (10 mg) was treated with an excess of diazomethane in an ethyl ether solution for 5 min to afford a product, which was analyzed by GC-EI-MS (Fig. 3A). Individual commercial standards **1**–**8** were also treated with CH_2N_2 to afford **1a**–**8a**, which were analyzed by GC-EI-MS (Fig. 3B). In addition, the identification of **1a**–**8a** in the methyl esters of fraction 1 was also confirmed by the observed homogeneous GC when the methyl esters of fraction 1 were mixed with **1a**–**8a** prepared from their individual commercial standards **1**–**8** (Fig. 3C).

*Verification of the origin of **1a**–**8a** in fraction 1*

In order to verify the origin of the corresponding methyl esters (**1a**–**8a**) of **1**–**8** in fraction 1 (Fig. 2A), *n*-hexane instead of MeOH was used to extract the plant material. In summary, the milled and dried seeds (2 g) of *J. chilensis* were sonicated in 30 ml of HPLC *n*-hexane at 30 °C for 60 min. After cooling, the mixture was filtered through Whatman (Clifton, New Jersey, USA) No. 1 filter paper into a 250 ml round bottom flask. The marc was extracted two more times in the same manner. The combined extractions were evaporated *in vacuo* to afford an oil (280 mg). Duplicate sample solutions in *n*-hexane (12 mg/ml) were prepared for GC-EI-MS analysis. A 2 μl aliquot of the *n*-hexane extract was injected into the capillary column and a representative gas chromatogram is shown in Fig. 2D.

Nematicidal assay against Caenorhabditis elegans

A mixed population of larval and adult *C. elegans* (L1–L4 larvae and adults; size range from ~ 0.1–1.0 mm), grown on agar plates with feeder layers of *Escherichia coli*, was prepared as described by Wood (1988). For assays, the worms were transferred into liquid medium with *E. coli* to provide nutrients and stimulate worm motility. Test materials were assayed at concentrations ranging from 600 μg/ml (crude extracts) down to 9.4 μg/ml (pure compounds), with 2 × dilutions between steps (600, 300, 150, etc.). Test materials were solubilized in DMSO/methanol (1:1) and added to individual wells of a 96-well polystyrene microtiter plate (DMSO/methanol also added to control wells). Methanol was allowed to evaporate, and finally 100–200 worms in liquid medium were added to each well (final DMSO content of 1%). At 4 and 24 h of incubation at 20 °C, motilities of the worms in each test well were assessed under a microscope.

Results and Discussion

Bioassay-guided fractionation of the *n*-hexane extract of the seeds of *J. chilensis* led to the isolation of four known fatty acids including capric acid (**2**), lauric acid (**3**), myristic acid (**4**), linoleic acid (**6**, Fig. 1) along with a mixture of triglycerides for the first time as described in Materials and Methods. Compounds **2**–**4**, and **6** were identified by

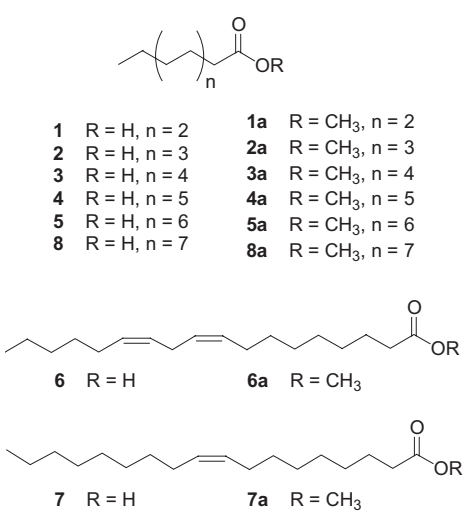

Fig. 1. Structures of fatty acids of *Jubaea chilensis* and their corresponding methyl esters.

comparison of their physical and spectroscopic data (m.p., ^{1}H NMR, ^{13}C NMR, and GC-EI-MS) with those of authentic samples or values reported in the literature. In turn, the mixture of triglycerides was obtained as a colorless oil and identified by its characteristic NMR data [δ_H = 4.16 dd (2H, J = 6.0 and 12 Hz), 4.31 dd (2H, J = 4.3 and 12 Hz), 5.29 m (1H); δ_C = 173.22 (s), 172.80 (s), 68.85 (d), 62.09 (t)] (Jie and Lam, 1995). In addition, the original triglyceride mixture was hydrolyzed with KOH, and further neutralized by the addition of HCl; the resulting mixture of fatty acids was methylated with an excess of CH_2N_2 in diethyl ether. Table I shows the GC-EI-MS data for the fatty acid methyl esters of the triglycerides. The relative compositions of the fatty acids of the triglycerides were estimated to be octanoic (**1**, 3.9%), capric (**2**, 15.6%), lauric (**3**, 100%), myristic (**4**, 20.0%), palmitic (**5**, 12.5%), linoleic (**6**, 4.5%), oleic (**7**, 21.3%), and stearic (**8**, 4.5%) acids if an identical GC response factor was considered for all of their individual methyl esters (**1a**–**8a**).

In order to identify the minor components of fraction 1, its GC-EI-MS data were obtained and are shown in Fig. 2A and Table I. Two additional fatty acids (**1** and **5**) in fraction 1 were sequentially identified as octanoic acid and palmitic acid by comparison of their GC-EI-MS data with their in-

Table I. GC-EI-MS data of **1**–**8** and their corresponding methyl esters **1a**–**8a** identified from *Jubaea chilensis*.

Compound	R. T. [min][a]	EI-MS (*m/z*, rel. int.%)
Octanoic acid (**1**)	9.38	144 ([M]$^{+\cdot}$, 4), 127 (13), 115 (33), 101 (79), 87 (36), 73 (98), 60 (100), 55 (71)
Capric acid (**2**)	12.50	172 ([M]$^{+\cdot}$, 8), 154 (6), 143 (22), 129 (100), 115 (38), 101 (17), 87 (61), 73 (74), 55 (60)
Lauric acid (**3**)	15.08	200 ([M]$^{+\cdot}$, 19), 171 (27), 157 (59), 143 (39), 129 (100), 115 (47), 87 (89), 73 (92), 55 (79)
Myristic acid (**4**)	17.36	228 ([M]$^{+\cdot}$, 27), 199 (15), 185 (52), 171 (34), 157 (28), 143 (36), 129 (100), 115 (34), 87 (95), 73 (78), 55 (66)
Palmitic acid (**5**)	19.45	256 ([M]$^{+\cdot}$, 33), 227 (15), 213 (30), 199 (19), 185 (38), 171 (37), 157 (39), 143 (32), 129 (81), 115 (34), 97 (35), 87 (100), 73 (69), 55 (64)
Linoleic acid (**6**)	21.09	280 ([M]$^{+\cdot}$, 12), 263 (5), 163 (9), 149 (13), 135 (19), 121 (22), 109 (37), 95 (74), 81 (97), 67 (100)
Oleic acid (**7**)	21.09	282 ([M]$^{+\cdot}$, 3), 264 (45), 246 (9), 235 (11), 221 (16), 207 (11), 193 (11), 179 (14), 165 (15), 151 (22), 137 (26), 123 (33), 111 (42), 97 (90), 83 (100), 69 (97), 55 (91)
Stearic acid (**8**)	21.32	284 ([M]$^{+\cdot}$, 86), 255 (21), 241 (27), 227 (22), 213 (22), 199 (27), 185 (59), 171 (34), 157 (28), 143 (33), 129 (100), 115 (38), 97 (85), 87 (81), 69 (80), 55 (59)
Octanoic acid methyl ester (**1a**)	8.21	158 ([M]$^{+\cdot}$, 6), 127 (33), 115 (44), 101 (34), 87 (62), 74 (100), 55 (51)
Capric acid methyl ester (**2a**)	11.86	186 ([M]$^{+\cdot}$, 12), 157 (21), 143 (93), 129 (27), 115 (10), 101 (39), 87 (74), 74 (100), 55 (53)
Lauric acid methyl ester (**3a**)	14.63	214 ([M]$^{+\cdot}$, 19), 185 (25), 171 (62), 157 (25), 143 (70), 129 (36), 115 (24), 101 (37), 87 (85), 74 (100), 55 (55)
Myristic acid methyl ester (**4a**)	17.00	242 ([M]$^{+\cdot}$, 30), 213 (19), 199 (78), 185 (33), 171 (16), 157 (32), 143 (93), 129 (29), 101 (45), 87 (94), 74 (100), 55 (57)
Palmitic acid methyl ester (**5a**)	19.12	270 ([M]$^{+\cdot}$, 46), 241 (22), 227 (68), 213 (26), 199 (48), 185 (43), 171 (46), 157 (26), 143 (76), 129 (34), 115 (18), 101 (46), 87 (100), 74 (100), 55 (56)
Linoleic acid methyl ester (**6a**)	20.76	294 ([M]$^{+\cdot}$, 6), 262 (14), 191 (5), 177 (7), 164 (13), 149 (25), 135 (27), 121 (26), 109 (30), 95 (66), 81 (95), 67 (100), 55 (26)
Oleic acid methyl ester (**7a**)	20.83	296 ([M]$^{+\cdot}$, 12), 264 (74), 246 (8), 221 (25), 207 (14), 180 (18), 166 (21), 151 (31), 137 (34), 123 (47), 109 (50), 97 (89), 83 (100), 67 (100), 55 (98)
Stearic acid methyl ester (**8a**)	21.05	298 ([M]$^{+\cdot}$, 56), 269 (17), 255 (79), 241 (26), 227 (16), 213 (33), 199 (70), 185 (34), 171 (20), 157 (28), 143 (89), 129 (26), 101 (44), 87 (100), 74 (95)

[a] Retention time.

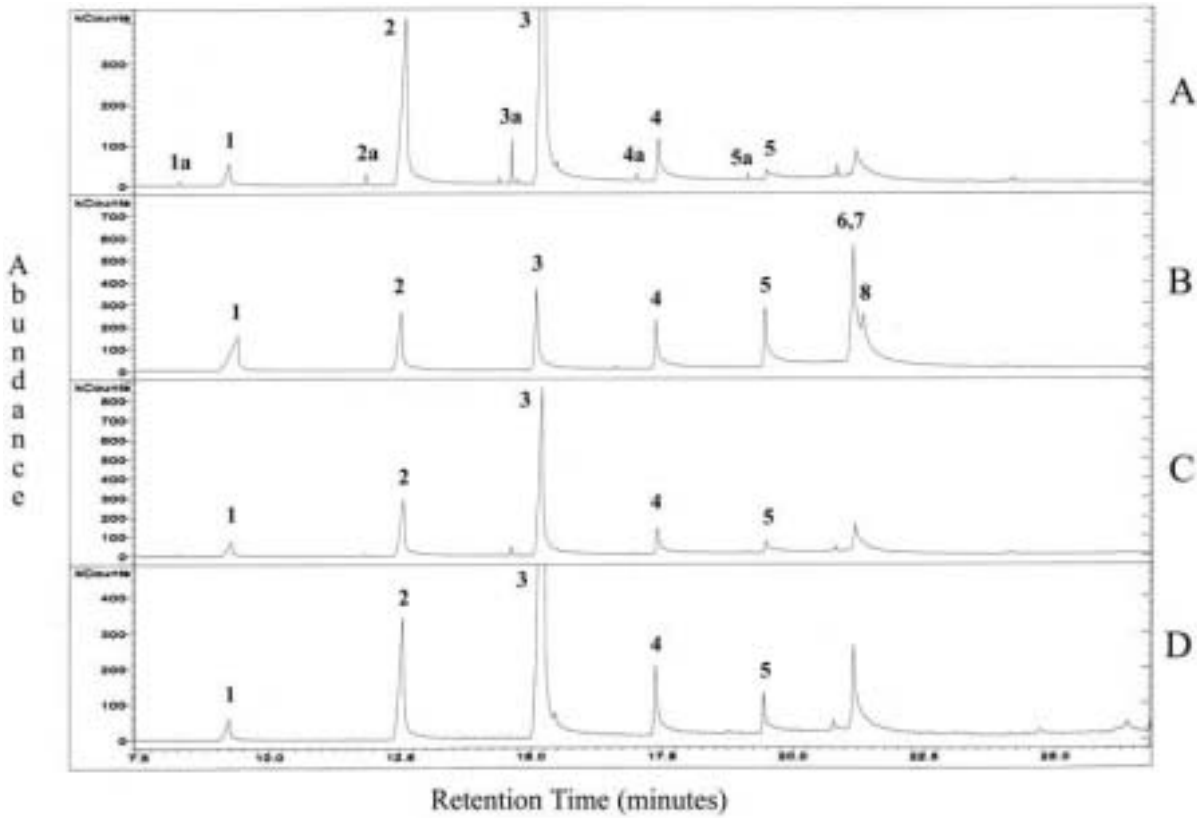

Fig. 2. GC profiles of (A) fraction 1 obtained from *J. chilensis*; (B) commercial standards **1**–**8**; (C) mixture of fraction 1 and commercial standards **1**–**8**; and (D) *n*-hexane extract of *J. chilensis.*

dividual commercial standards (Fig. 2B). The identities of **1**–**5** in fraction 1 (Fig. 2A) were further confirmed by the observed homogenous gas chromatograms after fraction 1 was mixed with their individual commercial standards (Fig. 2C). In addition, the overlapping GC peaks around 21 min (Fig. 2A) were identified as linoleic (**6**), oleic (**7**), and stearic (**8**) acids by comparison of the GC-EI-MS data of the methyl esters of fraction 1 (Fig. 3A) with those of **6a**–**8a** prepared from the individual standards (Fig. 3B) and the observation of their homogenous gas chromatograms (Fig. 3C). Furthermore, all the EI-MS data of **1**–**8** and **1a**–**8a** were confirmed by a mass spectral library (National Institute of Standard and Technology, Washington DC, USA). In turn, the corresponding methyl esters (**1a**–**8a**) of **1**–**8** were also identified from fraction 1 (Fig. 2A) by using the above mentioned techniques. In order to verify the origin of **1a**–**8a**, an *n*-hexane extract was prepared for GC-EI-MS analysis (Fig. 2D). The absence of **1a**–**8a** in Fig. 2D indicated that compounds **1a**–**8a** in fraction 1 were artifacts generated during the extraction and isolation procedures.

All of the 17 compounds (**1**–**8**, **1a**–**8a**, and a mixture of triglycerides) were evaluated for their nematicidal activities against *C. elegans*, as described in Materials and Methods. At 150 μg/ml, lauric acid (**3**) and myristic acid (**4**) were the only purified compounds showing activity. Further characterization of **3** and **4** showed them to be fully active (100% of worms immobile) at concentrations down to 75 μg/ml. Partial activity (reduced movement) was seen at 38 μg/ml. Fraction 1 was also fully active at 75 μg/ml, and sometimes at concentrations as low as 38 μg/ml (2 of 3 assays). Although compound **3** constitutes almost 50% of fraction 1 (Fig. 2A), the activity of fraction 1 may be somehow enhanced by other components in the mixture.

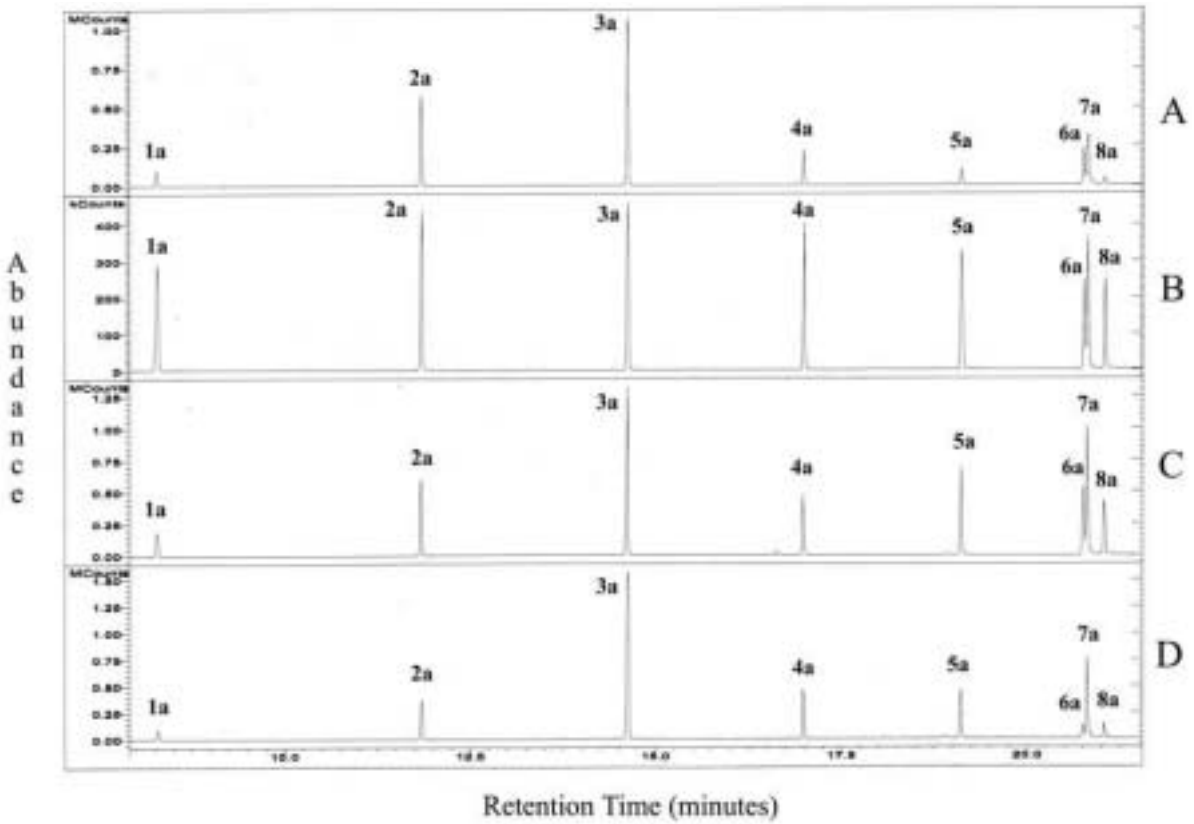

Fig. 3. GC profiles of (A) methyl esters of fraction 1; (B) corresponding methyl esters (**1a**–**8a**) of commercial standards **1**–**8**; (C) mixture of methyl esters of fraction 1 and **1a**–**8a**; and (D) fatty acid methyl esters obtained from the triglycerides of *J. chilensis*.

There were some qualitative differences in the activities of compounds **3** and **4**. The active concentrations of both compounds rendered 100% of the worms immobile at times as early as 1 h, and this was sustained through at least 4 h (standard time for first observation). At the 24 h observation, worms treated with compound **3** remained completely immobile. Worms treated with compound **4** showed recovery at 24 h, varying from only a few worms to as high as 100% (indistinguishable from controls). In one further experiment with a commercial preparation of lauric acid (Sigma-Aldrich product number L-4250), worms initially rendered 100% inactive by a 75 μg/ml dose showed partial recovery by 24 h (~ 50% of worms with slow movement). Although the mechanism for recovery was not determined, degradation of the fatty acids by *C. elegans* or *E. coli* present in the assays is an obvious possibility.

The crude extract of *J. chilensis* was assayed in a nematode-infected rodent model at 2,200 mg/kg of animal weight and was found to be inactive.

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