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Resorcinolic Lipids from Yucatecan Propolis

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Propolis is a material produced by bees from a combination of plant exudates and wax, used to fill out cracks in the beehive and to defend against intruders and pathogenic microorganisms; it is recognized for its many biological activities and its chemical composition depends on the botanical sources close to the beehive. The objective of this investigation was to isolate and identify metabolites with antioxidant activity present in a propolis sample collected in Yucatan, Mexico. Purification of the bioactive metabolites was carried out using argentation chromatography, while the combination of ¹H nuclear magnetic resonance (NMR), laser desorption ionization (LDI), gas chromatography-mass spectrometry (GC-MS) and biosynthetic origin data allowed their identification as resorcinolic lipids. Finally, the resin of *Mangifera indica* was identified as the botanical source of these metabolites.

Keywords: argentation, antioxidants, *Apis mellifera*, structure elucidation, laser desorption ionization

Introduction

Propolis is a material that bees produce by collecting exudates of plants and mixing it with wax and other materials; it is used by the bees to condition their nests, to fill out cracks in the beehive and to defend the beehive against intruders and pathogenic microorganisms.¹⁻³ The odor, color and chemical composition of propolis depends on the botanical sources close to the beehive, as well as the geographic and climatic conditions of the region.^{4,5} Recently, five types of propolis have been identified according to their chemogeographic patterns:⁶ propolis type I, commonly found in temperate zones of West Asia, Europe and North America, where its main source is the resinous exudate of the poplar tree (*Populus nigra*, Salicaceae) and its main components are flavonoids without substituents in ring B and phenylpropanoid acids and their esters; propolis type II, also called Brazilian green propolis, whose main source of resin is *Baccharis dracunculifolia* and contains prenylated

phenylpropanoids and caffeoylquinic acids; propolis type III, found in Cuba and Venezuela, which contains mainly prenylated benzophenones and its main source of resin are the flowers of *Clusia* spp; propolis type IV, also known as Pacific type, is found in Taiwan and the Okinawa region where is derived from exudates of the fruits of *Macaranga tanarius* and its main components are geranyl flavanones; finally, propolis type V, found in Greece, Crete and Turkey, contains mainly diterpenes and anthraquinones, and its main sources are plants of the Cupressaceae family. Propolis is also recognized for its many biological activities including antimicrobial, antitumor, antiinflammatory, antioxidant and hepatoprotective.^{7,8} Temperate poplar propolis (type I) and Brazilian green propolis (type II) are the two best known and most investigated types of propolis, particularly in terms of their biological activity and the polyphenolic metabolites they contain; these include artepilin C, the phenethyl ester derivative of caffeic acid, caffeic acid, chrysin, galangin, quercetin, apigenin, kaempferol and pinocembrin.^{5,6} Recently, propolis has been recognized as an important source of plant bioactive

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metabolites since bees, independently of the geographical zone, are always able to find and use appropriate plant sources for propolis production;^{5,9} not surprisingly, a wide variety of biologically active secondary metabolites have been reported from propolis samples collected in lesser known regions of the world, including cytotoxic cycloartane triterpenoids from Myanmar propolis,¹⁰ cytotoxic galangin derivatives from Mexican propolis,¹¹ and phenolic lipids with antioxidant activity from Asia^{1,9,12} and Africa.¹³ Here we wish to describe the isolation and identification of resorcinolic lipids with antioxidant activity from a sample of propolis collected in Yucatan, Mexico.

Experimental

General

Analytical thin layer chromatography (TLC) was carried out using aluminum-backed silica gel plates (Merck, Darmstadt, Germany, 0.2 mm); chromatograms were visualized using a solution of phosphomolybdic acid (20 g) and ceric sulfate (2.5 g) in 500 mL of 5% sulfuric acid followed by drying and heating. Preparative TLC (prep TLC) was carried out using glass-coated 20 × 20 cm silica gel plates (Merck, Darmstadt, Germany, 0.25 mm) impregnated with AgNO₃. Flash column chromatography purifications were carried out using silica gel (Merck, Darmstadt, Germany) 200-400 mesh, and open column chromatography (CC) purifications were carried out using AgNO₃-impregnated silica gel (Sigma-Aldrich, St. Louis, USA) 70-230 mesh. Nuclear magnetic resonance (NMR) analyses were carried out in a Bruker Avance III (Billerica, USA, 400 MHz) or an Agilent-AR premium COMPACT (Santa Clara, USA, 600 MHz); samples were dissolved in CDCl₃ and the residual solvent signal was used as reference.

Laser desorption ionization (LDI) analysis

General laser desorption ionization (LDI) experiments were carried out in linear negative mode on a Biflex III time of flight (TOF) mass spectrometer (Bruker, Billerica, MA, USA), equipped with a 337 nm pulsed nitrogen laser (model VSL-337i, Laser Sciences Inc., Boston, USA). Spectra were acquired within a mass range of 20-2000 *m/z* in automatic mode. An overall of 150 acquisitions were summed for obtaining final spectra. Acceleration voltage was set to 19 kV, pulse ion extraction was 200 ns, and laser frequency was 5 Hz. Applied laser energy was individually adapted to sample requirements and is expressed as percent of maximal laser power as well as calculated theoretical values in μJ based on the instrument's settings. Samples

C3 and C4 were analyzed at a laser power of 90% (112 μJ) while 70% (105 μJ) was used for C5.

Preparation of AgNO₃-impregnated silica gel plates for prep TLC

Silica gel plates were eluted with a 5% AgNO₃ solution in acetonitrile and allowed to dry before use.

Preparation of AgNO₃-impregnated silica gel for column chromatography

Silica gel (10 g) was combined with 50 mL of a 5% AgNO₃ solution in acetonitrile and allowed to stand for 5 min before evaporating the solvent.

Collection and extraction of propolis

Propolis was collected in February 2015 by Mr Lino Mex Mex, a beekeeper of *Apis mellifera* in Hunucmá, Yucatán, Mexico. The propolis sample was ground to a homogeneous powder using liquid nitrogen and 1 g of the propolis powder was extracted three times (2 h) with ethanol (20 mL), at room temperature. The combined filtrates were kept overnight at -18 °C and then filtered to remove waxes.² Finally, the solvent was evaporated under reduced pressure to obtain 738 mg (73.8%) of propolis extract.

Collection and extraction of plant material

The resin sample of *Mangifera indica* was obtained in April 2017 from a tree growing in an area close to the beehive in Hunucmá, Yucatán, Mexico. The resin (0.9 g) was extracted using the procedure previously described for the propolis sample to yield 13.9 mg (1.5%) of the corresponding resin crude extract.

Gas chromatography-mass spectrometry (GC-MS) analysis of propolis and *M. indica* extracts

The sample was analyzed in an Agilent Technologies Gas Chromatograph 6890N connected to an Agilent Technologies Mass Detector 5975B, with an ionization voltage of 70 eV; the separation was carried out using an Agilent HP-5MS column (30 m × 0.25 mm internal diameter with 0.25 mm film thickness), with helium as a carrier gas (1 mL min⁻¹) and a temperature program of 110 °C (2 min), 110-260 °C (15 °C min⁻¹), 260 °C (3 min), 260-280 °C (2 °C min⁻¹), 280 °C (5 min), 280-300 °C (5 °C min⁻¹), 300 °C (30 min). Injector and detector temperatures were set to 280 and 150 °C, respectively.

Preliminary identification of the different components was carried out by comparing their fragmentation patterns with those contained in the NIST 05 data base.

Gas chromatography-mass spectrometry (GC-MS) analysis of propolis purified fractions

The samples were analyzed in an Shimadzu Gas Chromatograph GCMS-QP2010 SE with an ionization voltage of 70 eV; the separation was carried out using a phenomenex Zebron column (30 m \times 0.250 mm internal diameter with 0.25 mm film thickness), with helium as a carrier gas (2 mL min⁻¹) and a temperature program of 110 °C (0.5 min), 110-280 °C (20 °C min⁻¹), 280 °C (20 min), 280-300 °C (5 °C min⁻¹), 300 °C (2 min). Injector and detector temperatures were set to 280 and 150 °C, respectively.

Determination of antioxidant activity

The assay for the reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was carried out following a modified procedure on a previously reported methodology.¹⁴ In brief, different concentrations of each extract were prepared (1 \times 10⁻¹ to 1 \times 10⁻⁴ g extract mL⁻¹ ethanol) and 200 μ L were added to 1800 μ L of DPPH (0.1 mM ethanol). After 30 min the absorbance was measured at 540 nm. The percentage of remaining DPPH was calculated using the formula % DPPH = [(AbsDPPH 0.1 mM)_{t=0} / (AbsDPPH sample)_{t=30}] \times 100. Methanol was used as a blank and ascorbic acid (1%) as a positive control. The assay was run three times for each extract.

First purification of propolis extract (Figure S1a)

A portion (400 mg) of the propolis extract was dissolved in dichloromethane and adsorbed in 800 mg of silica gel. The sample was placed on top of a chromatographic column and separated by flash column chromatography (5 \times 20 cm) eluting with a mixture of hexane:ethylacetate:methanol 90:8:2. The collected fractions were combined into 17 (A1-A17) semipurified fractions on the basis of their TLC chromatographic profiles. Further purification of active fraction A13 (217 mg) by flash column chromatography (3 \times 20 cm) eluting with hexane:ethylether 6:4 yielded 11 (B1-B11) new semipurified fractions. Finally, prep TLC purification of active fraction B5 (9 mg) using a multiple elution (four times) with dichloromethane:methanol 19:1 on AgNO₃-impregnated silica gel plates produced eight fractions (C1-C8) of which fractions C3 (1.2 mg), C4 (1.0 mg) and C5 (1.3 mg) were active in the DPPH radical reduction assay.

Second purification of propolis extract (Figure S1b)

A portion (420 mg) of the crude propolis extract was dissolved in dichloromethane and adsorbed in 800 mg of silica gel (200-400 mesh). The sample was placed on top of a chromatographic column (5 \times 20 cm) and separated by flash CC using a mixture of hexane-ethyl ether 6:4 + 1 μ L mL⁻¹ of formic acid. The collected fractions were combined into eleven (D1 to D11) semipurified fractions. Open CC (1.5 \times 30 cm) purification of fraction D5 (67.7 mg) using AgNO₃-impregnated silica gel and a mixture of dichloromethane-methanol 19:1 as eluent yielded semipurified fractions E2 (4.2 mg), E3 (19 mg) and E4 (6.2 mg) that were combined and purified by multiple-elution (four times) prep TLC using a mixture of dichloromethane-methanol 19:1 on AgNO₃-impregnated silica gel plates to produce fraction F3 (1.8 mg). Similarly, open CC (1.5 \times 30 cm) purification of fractions D6 (14.8 mg) and D7 (67.7 mg) combined yielded semipurified fractions G3 (8.7 mg) and G4 (10.3 mg) that were combined and purified by multiple-elution (four times) prep TLC using a mixture of dichloromethane-methanol 19:1 on AgNO₃-impregnated silica gel plates to produce fraction H3 (1.8 mg). Finally, open CC (1.5 \times 30 cm) purification of fraction D8 (91.2 mg) yielded the semipurified fractions I5 (11 mg) and I6 (9.9 mg) that were combined and purified by multiple-elution (four times) prep TLC using a mixture of dichloromethane-methanol 19:1 on AgNO₃-impregnated silica gel plates to produce fraction J2 (1.8 mg). Prep TLC purification of fractions I9 (6.1 mg), I10 (11.9 mg) and I11 (4.4 mg) combined using a multiple-elution (four times) with a mixture of dichloromethane-methanol 19:1 on AgNO₃-impregnated silica gel plates yielded fractions K3 (3.1 mg) and K4 (2.8 mg).

Results and Discussion

The bioassay-guided purification of the propolis extract, with a strong (half maximal inhibitory concentration (IC₅₀) 0.71 mg mL⁻¹) antioxidant activity when tested in the DPPH reduction assay, produced fraction B5 showing a single component with antioxidant activity in its TLC profile (Figure S2a, Supplementary Information (SI) section). However, the GC-MS analysis of fraction B5 indicated the presence of four major components (Figure S2b, SI section), all of them with a base peak at *m/z* 124 and a fragmentation pattern (Figure 1a) coinciding with that of resorcinolic lipid structures in the database. The fact that the fragment ion of the base peak, which can be explained by a methyl-substituted 1,3-benzenediol structure (Figure 1b), was common to all the components in fraction B5 suggested

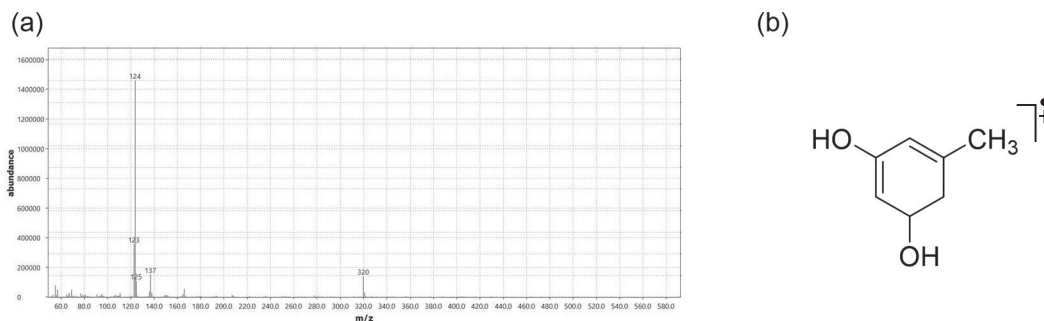


Figure 1. (a) Fragmentation pattern of one of the components (t_R 17.8 min) in fraction B5 showing a molecular ion at m/z 320 and a base peak at m/z 124; (b) structure corresponding to the base peak fragment at m/z 124 ($C_8H_8O_2$).

that they all shared the same resorcinolic basic structure but differed in the length of the lipodic hydrocarbon chain.

Phenolic lipids have often been reported as inseparable mixtures from species of the Anacardiaceae family and from propolis samples collected in different parts of the world;^{1,9,12,13,15} accordingly, various attempts to purify the components in fraction B5 using ordinary chromatographic methods proved to be unsuccessful. However, argentation chromatography, i.e., the impregnation of silica gel and other adsorbents with silver nitrate, has been commonly used to separate structurally-similar metabolites such as triglycerides and lipids, terpenes, essential oils components and resin constituents which differ in the number or geometry of their double bonds,^{16,17} and previous reports have described the use of argentation chromatography as an alternative to separate homologues of phenolic lipids with varying degrees of side-chain unsaturation.¹⁸ In this investigation, argentation of silica gel plates greatly improved the separation of the bioactive components in fraction B5 (Figure S3, SI section); prep TLC purification of fraction B5 using silica gel plates impregnated with silver nitrate produced the purified bioactive fractions C3-C5 (Figure S4, SI section).

While fractions C3 and C5 each showed a single bioactive metabolite on TLC, fraction C4 appeared to be a mixture of the metabolites in fractions C3 and C5. The 1H NMR of C3 (Figure S5, Table S1, SI section) showed the expected signals for a resorcinolic lipid structure,¹⁹⁻²¹ namely two signals corresponding to three aromatic protons at 6.24 (2H) and 6.17 (1H) ppm, one two-proton signal at 4.64 ppm, corresponding to the protons of the two phenolic hydroxyl groups, a two-proton triplet at 2.48 ppm, corresponding to the protons of the benzylic methylene, and a three proton triplet at 0.87 ppm, corresponding to the terminal methyl group of the hydrocarbon chain. However, both the LDI and GC-MS analyses of C3 showed the presence of two components with parent ion peaks at m/z 319/347 and 320/348, respectively (Figure S6, SI section), corresponding to the molecular formulae

$C_{21}H_{36}O_2/C_{23}H_{40}O_2$. To identify the two components in C3 it was necessary to consider the biosynthetic origin of resorcinolic lipids;²²⁻²⁴ taking into account that the hydrocarbon chain of resorcinolic lipids originates from a fatty acyl-coenzyme A (CoA) starter unit (Figure S7, SI section), and the fact that there are only one C-16 (palmitic) and one C-18 (stearic) fully saturated fatty acids, the bioactive resorcinolic lipids in C3 were identified as 1,3-benzenediol-5-pentadecyl (**1**) and 1,3-benzenediol-5-heptadecyl (**2**) (Figure 2). Both bioactive metabolites have previously been reported to co-occur in *Merulius incarnatus*²⁵ and triticale bran,²⁶ and in propolis samples from Cameroon¹³ and Indonesia.¹ Additionally, **2** has been reported from the latex of *Mangifera indica*.²⁷

The 1H NMR of C5 (Figure S8, Table S1, SI section) showed the same signals observed in the spectrum of C3 plus one two-proton signal at 5.35 and a four-proton signal at 2.02 ppm corresponding to vinylic and allylic protons, respectively; these signals suggested the presence of a double bond in the lipid part of the molecule. This was confirmed by the LDI and GC-MS analyses of C5 which showed two components with parent ion peaks at m/z 345/373 and 346/374 (Figure S9, SI section), corresponding to molecular formulae $C_{23}H_{38}O_2/C_{25}H_{42}O_2$ and confirming the presence of an extra unsaturation in the resorcinolic lipid structure. The location of the double bond in the lipodic chain of resorcinolic lipids is often established by analyzing the fragmentation pattern of the corresponding dimethyl disulfide-trimethylsilyl derivatives after GC-MS analyses.^{13,16} In this case, the bioactive resorcinolic lipids in C5 were identified by combining the information from their biosynthetic origin and their MS fragmentation pattern after GC-MS analysis. Even though there are five C18-carbon fatty acids with one unsaturation ($\Delta 3, 6, 9, 11$ or 13) that can be used as a starting unit to produce a resorcinolic lipid with a molecular weight of 346, and there are four C20-carbon fatty acids with one unsaturation ($\Delta 9, 11, 13$ or 15) that can be used as a starting unit to produce a resorcinolic lipid with a molecular weight

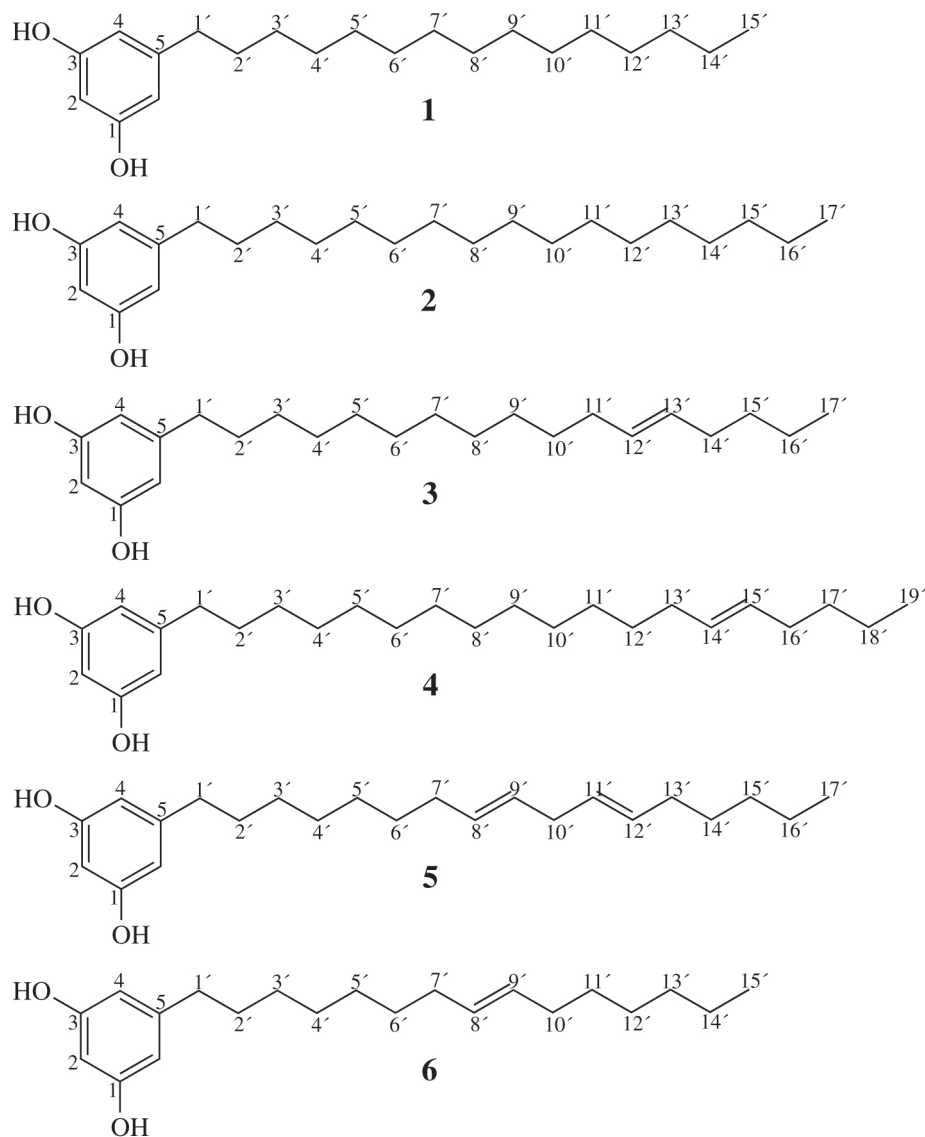


Figure 2. Structures of resorcinolic lipids identified in the propolis sample collected in Yucatan, Mexico.

of 374, the fragmentation pattern of both components in the GC-MS analysis of C5 showed a fragment ion at m/z 97, together with fragments and parent ion peaks at m/z 249/346 and m/z 277/374 in the fragmentation pattern of each component (Figures S10 and S11, SI section); since the m/z 249/97 and m/z 277/97 fragments can only result from an allylic cleavage in an 5- ω C18 and C20 fatty acid-derived resorcinolic lipids, respectively, the bioactive components in C5, one with a Δ 13 18-carbon and the other with a Δ 15 20-carbon fatty acyl-CoA starter units, were identified as 1,3-benzenediol-5-(12-heptadecenyl) (**3**) and 1,3-benzenediol-5-(14-nonadecenyl) (**4**), respectively, (Figure 2). Metabolite 3 has been reported from the peel of the mango (*M. indica*) fruit,²⁸ while **4** has been isolated from the roots of *Ardisia silvestris*²⁹ as well as from wheat bran oil;³⁰ additionally, both metabolites have been reported from

triticale bran²⁶ and a propolis sample from Cameroon.¹³ Finally, and even though C4 appeared to be a mixture of the metabolites in C3 and C5, its LDI and GC-MS analyses (Figure S12, SI section) showed only two components with parent ion peaks at m/z 319/373 and 320/374, respectively, indicating that the purified fraction C4 was in fact a mixture of the bioactive resorcinolic lipids **1** and **4**.

In an attempt to obtain the resorcinolic lipids **1-4** in pure form and/or isolate other resorcinolic lipids, the propolis extract was purified using argentation chromatography throughout the purification process; this resulted in the isolation of bioactive fractions H3, K3 and K4, none of which showed a similar composition to that of fraction B5. However, the GC-MS analysis of H3 showed three major components corresponding to the bioactive resorcinolic lipids **1**, **2** and **4**. A similar analysis of K4 showed the

presence of a single component (Figure S13, SI section) with a parent ion peak at m/z 344, corresponding to a molecular formula of $C_{23}H_{36}O_2$; the number of unsaturation sites calculated from the formula suggested a resorcinolic lipid structure with two double bonds in the hydrocarbon chain. This was confirmed by a four vinylic proton-multiplet at 5.35 ppm in the 1H NMR spectrum of K4 (Figure S14, SI section), together with two signals at 2.77 (2H) and 2.05 (4H) ppm corresponding to the six protons of three allylic methylenes (Figure S14 and Table S1, SI section). Since linoleic acid is the only 18-carbon fatty acid with two double bonds ($\Delta 9$ and $\Delta 12$) that can act as a starter unit, the bioactive resorcinolic lipid in K4 was identified as 1,3-benzenediol-5-(8,11-heptadecadienyl) (**5**) (Figure 2). Metabolite **5** has also been reported from *M. incarnatus*²⁵ and the peel of mango (*M. indica*) fruits,³¹ as well as from propolis samples in Indonesia¹ and Brazil.¹⁵ Finally, the GC-MS analysis of K3 showed the presence of four components (Figure S15, SI section), three of them identified as the bioactive resorcinolic lipids **3**, **4**, and **5**. The unknown component showed a parent ion peak at m/z 318, corresponding to a molecular formula of $C_{21}H_{34}O_2$ which suggested a resorcinolic lipid having a mono-unsaturated 16-carbon fatty acyl-CoA as the starter unit. Since palmitoleic acid is the only known 16-carbon mono-unsaturated fatty acid, the unknown bioactive component in K3 was identified as 1,3-benzenediol-5-(8-pentadecenyl) (**6**) (Figure 2), a metabolite previously reported from *M. incarnatus*.²⁵

Since resorcinolic lipids have been reported to be present in the fruits and resin of *Mangifera indica*,^{13,32} we compared the GC-chromatographic profile of the resin of a mango tree (*M. indica*) growing close to the beehive, with the chromatographic profile of the active fractions, and were able to detect the presence of resorcinolic lipids **1**, **3**, **5** and **6** in the resin of the tree (Figure 3). This result confirms the resin of *M. indica* as one of the botanical sources of this type of metabolites in the propolis sample collected in Yucatan.

A recent study of a sample of the propolis extract used in this investigation resulted in the identification of cycloartanes and pentacyclic triterpenes;³³ however, the botanical origin of most of these triterpenoids could not be traced back to the resin of *M. indica* but to the resin of *Bursera simaruba*, a common resiniferous tree found in Yucatan, or to plants belonging to the Anacardiaceae or Clusiaceae families. These results, together with those presented here, confirm the fact that bees use more than one botanical source in their production of propolis and that Yucatecan propolis has a composition which appears to be different from that reported for propolis samples collected in other parts of the world.

Conclusions

The results of this investigation confirm the importance of argentation for the separation of structurally-related resorcinolic lipids and the relevance of LDI, GC-MS and biosynthetic origin data to unequivocally identify structurally-related resorcinolic lipids. Finally, the results obtained allowed the identification of metabolites **1-6** as those responsible for the antioxidant activity originally detected in the propolis crude extract.

Supplementary Information

Supplementary information (chromatographic procedures used for the purification of the propolis extract, TLC chromatographic profile, 1H NMR data, LDI spectrums, GC-MS chromatographic profiles and fragmentation patterns of fractions obtained and the biosynthetic pathway of resorcinol lipids are included) is available free of charge at <http://jbcbs.sbq.org.br> as PDF file.

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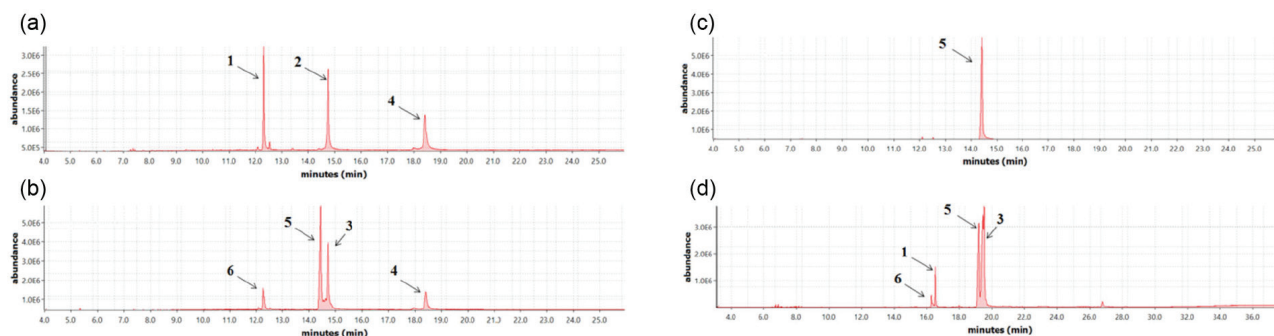


Figure 3. GC-MS chromatographic profiles of purified fractions H3 (a), K3 (b) and K4 (c), and of *M. indica* resin (d).

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