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Separation of Acid Diterpenes of *Copaifera cearensis* Huber ex Ducke by Flash Chromatography Using Potassium Hydroxide Impregnated Silica Gel

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O uso de cromatografia sob pressão ("flash chromatography") utilizando-se coluna de gel de sílica impregnada com hidróxido de potássio levou ao fracionamento dos constituintes químicos de *Copaifera cearensis* Huber ex Ducke. A fração ácida, após esterificação com diazometano, foi analisada por cromatografia gasosa de alta resolução acoplada à espectrometria de massas ou à espectrometria no infravermelho, possibilitando a identificação de onze diterpenos ácidos. Fracionamentos adicionais da fração ácida por cromatografia líquida de alta eficiência em fase reversa levaram ao isolamento de diversos ácidos diterpênicos e de um sesquiterpênico que foram identificados através de seus dados espectroscópicos. A análise da sílica impregnada com hidróxido de potássio mostrou que, além da deposição do hidróxido na superfície da sílica, ocorreu troca iônica com formação de resíduos Si-OK.

Various acid diterpenes of *Copaifera cearensis* Huber ex Ducke were isolated from the crude extract by flash Silica Gel/Potassium Hydroxide Chromatography. The main components were identified by GC-MS analyses. Further fractionation by reversed phase (RP) semi-preparative HPLC allowed isolation and identification of minor components and provided additional spectral data of those compounds. It was possible to detect the presence of eleven acid diterpenes in addition to a sesquiterpene acid. In addition to potassium hydroxide deposition on the silica surface, the presence of Si-OK residues, resulting from a cation exchange process, was established.

Keywords: *Copaifera cearensis*, acidic diterpene fraction, GC-MS analysis, silica gel/potassium hydroxide chromatography

Introduction

Copaiba oils are exuded from the trunks of trees belonging to the *Copaifera* genus and can be found in many drugstores in Brazil as a phytotherapeutical oil[1,2]. These trees are found in northern and northeastern Brazil, as well as in Venezuela, Colombia, Guyana, French Guiana and Suriname[3,4]. The copaiba oils are known from folk medicine due to their antiseptic, antiinflammatory; and antiblenorrhagic properties[5]. These oils were first described by Gabriel Soares de Souza in 1587[6] and were included in "The British Pharmacopoeia" in 1677 and "The United States Pharmacopoeia" in 1820[7]. Pellegrino *et al.* have studied the use of these oils against infections by *Schistosoma mansoni*[8]. Basile *et al* have shown the analgesic and antiinflammatory properties of these oils[9], confirmed by Fernandes *et al.*[10]. All these studies were carried out with the crude oils, therefore showing the need of a better understanding of which component(s) would be responsible for the pharmacological activities ascribed to the oils. Several *Copaifera spp.* are known, including *Copaifera cearensis* Huber ex Ducke[5], whose acid composition is described in the present work. We also report the characterization of KOH-impregnated silica gel[11,12] by IR spectroscopy.

Experimental

Materials and methods

Crude oil from *Copaifera cearensis* was obtained as an exudate from direct perforation of the trunk of the tree. Melting points were determined on a Kofler block and are uncorrected. Infrared (IR) spectra were recorded as KBr pellets or fluorolube mull on a Perkin Elmer 1760X FT or Nicolet Magma 760 FT (for silica characterization) spectrophotometers from 6000 to 400 cm^{-1}. One- and two-dimensional (1D and 2D) nuclear magnetic resonance (NMR) experiments were carried out on a Bruker 300 AC-P

spectrometer (300.13 MHz for ^{1}H NMR and 75.25 MHz for ^{13}C NMR) using CDCl$_3$ as solvent. ^{13}C NMR were obtained under Composite Pulse Decoupling (2 μs; 90º 4.4 ms). Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C NMR spectra were obtained considering *J* (CH) 145 Hz. Shift Correlated Spectroscopy (COSY) ^{1}H NMR spectra were obtained using standard Bruker software COSY.AUR program (512 points F2; 256 points F1). Heteronuclear Shift Correlation (HETCOR) ^{13}C NMR spectra were obtained using XHCOORD.AUR program (2048 points F2 ^{13}C; 256 points F1 ^{1}H; *J* (CH) 10 Hz for pulse intervals). A Hewlett-Packard HP 5890A Gas Chromatograph equipped with capillary columns, on-column or split/splitless injectors and a flame ionization detector (FID), was used. A Hewlett-Packard HP 5890A Gas Chromatograph equipped with a capillary column (BP5-SGE, 12 m, 0.33 mm, 1.0 mm, He as the carrier gas:10 psi) and on-column injector (SGE 021) coupled to a FTIR HP 5965A (4000 - 750 cm^{-1}), was used. Transfer line and IR cell were kept at 250ºC. GC conditions: 40 to 160ºC at 50ºC min^{-1}, 160ºC (1 min) to 270ºC at 5ºC min^{-1}. A Hewlett-Packard GC-MS system HP 5988, electron impact (EI), at 70 eV, 200ºC ion source temperature, or chemical ionization (CI), 230 eV, CH$_4$ as the reagent gas, 100ºC ion source temperature, equipped with a capillary column (He as carrier gas), on-column (SGE 021) or split/splitless injectors, was used. Flash chromatography using potassium hydroxide modified silica was carried out with the material produced as follows: 200 g of silica gel 60 (230-400 mesh) in *i*PrOH (1 L) were stirred with 400 mL of a saturated solution of KOH (50 g) for a period of 10 min[11,12]. This mixture was then transferred into a glass column and washed with 800 mL of hexane, under air pressure (less than 1 atm)[13]. Crude oil (5.0 g) was then applied to the top of this column and eluted successively, under air pressure, with 400 mL of hexane, CH$_2$Cl$_2$ and MeOH respectively. The MeOH fraction was concentrated to a quarter of the initial volume, acidified to pH 4 and extracted with CH$_2$Cl$_2$ which was then evaporated. The CH$_2$N$_2$ solution used to produce methyl esters was obtained as follows: a solution of 45 g of Diazald® in 450 mL of Et$_2$O was slowly added to a mixture of 45 g of KOH, 90 mL of H$_2$O and 72 mL of EtOH, so producing a very gentle distillation of CH$_2$N$_2$ / Et$_2$O, which was collected on an ice bath and used whenever needed.

Spectral data

Eperuic acid methyl ester (**1**)[14]: (GC) EIMS *m/z* 320 (M$^+$, 35%), 305 (90), 289 (7), 192 (10), 191 (20), 177 (60), 137 (100); (GC) CIMS *m/z* 321 (M$^+$+1, 100 %); (GC) IR ν$_{max}$ /cm^{-1} 3087, 2940, 2881, 1757, 1642, 1162, 892.

Cativic acid methyl ester (**2**)[15]: (GC) EIMS *m/z* 320 (M$^+$, 20%), 305 (40), 289 (14), 264 (10), 196 (27), 191 (80), 124 (20), 122 (100); (GC) CIMS *m/z* 321 (M$^+$+1, 100%); (GC) IR ν$_{max}$ /cm^{-1} 2992, 2858, 1757, 1160, 833.

Copalic acid methyl ester (**3**)[16]: (GC) EIMS *m/z* 318 (M$^+$, 18%), 303 (70), 286 (3), 271 (10), 244 (20), 229 (13), 205 (18), 192 (2), 177 (20); (GC) CIMS *m/z* 319 (M$^+$+1, 100%); (GC) ν$_{max}$ /cm^{-1} 3087, 2940, 2854, 1738, 1648, 1221, 1190, 893.

Kolavenic acid methyl ester (**4**)[17]: (GC) EIMS *m/z* 318 (M$^+$, 14%), 303 (18), 275 (10), 271 (8), 243 (20), 191 (32), 189 (100), 175 (20); (GC) CIMS *m/z* 319 (M$^+$+1, 100%); (GC) ν$_{max}$ /cm^{-1} 2953, 1738, 1648, 1221, 1150, 862.

Crolechinic acid methyl ester (**5**)[18]: (GC) EIMS *m/z* 332 (M$^+$, 4%), 317 (3), 273 (6), 237 (75), 95 (36), 81 (100); (GC) CIMS *m/z* 333 (M$^+$+1, 100%); (GC) ν$_{max}$ /cm^{-1} 2943, 2881, 1747, 1559, 1166, 875; ^{13}C NMR δ 14.8 (C-19), 16.0 (C-17), 18.0 (C-20), 18.1 (C-12), 21.0 (C-1), 24.9 (C-3), 26.2 (C-2), 27.2 (C-7), 36.6 (C-8), 37.3 (C-5), 38.4 (C-11), 38.9 (C-9), 39.9 (C-6), 49.3 (C-10), 50.9 (MeO), 57.6 (C-4), 111.0 (C-14), 125.6 (C-13), 138.4 (C-16), 142.7 (C-15), 174.9 (C-18).

Hardwickiic acid methyl ester (**6**)[18,19]: (GC) EIMS *m/z* 330 (M$^+$, 12%), 315 (5), 299 (10), 283 (12), 235 (45), 203 (65), 175 (20), 139 (100), 96 (60), 95 (42), 81 (80); (GC) CIMS *m/z* 331 (M$^+$+1, 100%); (GC) ν$_{max}$ /cm^{-1} 2962, 2884, 1733, 1638, 1560, 1232, 1166, 936; ^{13}C NMR δ 15.9 (C-17), 17.5 (C-1), 18.2 (C-20), 18.6 (C-12), 20.7 (C-19), 27.1 (C-7), 27.3 (C-2), 35.9 (C-6), 36.3 (C-8), 37.3 (C-5), 38.6 (C-11), 38.8 (C-9), 46.6 (C-10), 51.1 (MeO), 111.0 (C-14), 125.6 (C-13), 136.8 (C-3), 138.4 (C-16), 142.5 (C-4), 142.7 (C-15), 167.9 (C-18).

3-Methyl-5(2',2',6'-trimethyl-6'-hydroxycyclohexyl)-pentanoic acid methyl ester (**7a**)[20] (GC) EIMS *m/z* 270 (M$^+$, 2%), 252 (7), 238 (5), 196 (8), 195 (10), 177 (10), 153 (20), 112 (75), 109 (75), 43 (100); (GC) CIMS *m/z* 253 (M$^+$+1-18, 100%), 271 (M$^+$+1, 2); (GC) ν$_{max}$ /cm^{-1} 2941, 2880, 1757, 1165.

3-Methyl-5(2',2',6'-trimethyl-6'-hydroxycyclohexyl)-pentanoic acid (**7b**)[20]: ^{1}H NMR δ 0.77 (s, 3H), 0.89 (s, 3H), 0.95 (d, *J* 6.5 Hz, 3H), 1.07 (t, *J* 4.5 Hz, 1H), 1.14 (s, 3H), 1.2-1.7 (m, 10H), 1.74 (m, 1H), 1.87-2.00 (m, 1H), 2.10 (dd, *J* 15 and 7.5 Hz, 1H), 2.34 (dd, *J* 15 and 6.5 Hz, 1H); ^{13}C NMR δ 19.8 (C-6), 20.4 (C-4'), 21.3 (C-8), 23.0 (C-9), 23.1 (C-5), 30.8 (C-3), 32.8 (C-7), 35.5 (C-6'), 39.6 (C-4), 41.3 (C-2), 41.4 (C-5'), 43.1 (C-2'), 57.0 (C-1'), 74.9 (C-2'), 178.4 (C-1).

Clerodan-15,18-dioic acid dimethyl ester (**8**): (GC) EIMS *m/z* 335 (M$^+$-31, 2%), 307 (3), 251 (4), 237 (100),

205 (55), 177 (70); (GC) CIMS *m/z* 395 (M$^+$+29, 15%), 335 (M$^+$+1-32, 18), 307 (M$^+$+1-60, 100); (GC) ν_{max} /cm^{-1} 2958, 2882, 1749, 1166; ^{1}H NMR δ 0.68 (s, 3H), 0.75 (d, *J* 6.0 Hz, 3H), 0.93 (d, *J* 6.8 Hz, 3H), 0.99 (s, 3H), 1.0-1.5 (m, 16H), 1.5-2.0(m, 3H), 2.1 (dd, *J* 7.0 and 3.0 Hz, 1H), 2.2 (d, *J* 7.0 Hz, 1H), 2.3 (dd, *J* 12 and 6 Hz, 1H), 3.70 (s, 3H), 3.76 (s, 3H); ^{13}C NMR δ 14.8 (C-19), 15.9 (C-17), 18.2 (C-20), 19.8 (C-16), 21.0 (C-1), 24.9 (C-3), 26.2 (C-2), 27.1 (C-7), 29.3 (C-12), 31.0 (C-13), 35.1 (C-11), 36.4 (C-8), 37.3 (C-5), 38.7 (C-9), 39.8 (C-6), 41.5 (C-14), 49.2 (C-10), 50.8 (MeO-C-19), 51.3 (MeO-C-15), 57.5 (C-4), 173.6 (C-15), 175.0 (C-18).

Clerod-3-en-15,18-dioic acid dimethyl ester (**9**)[21]: (GC) EIMS *m/z* 333 (M$^+$-31, 28%), 332 (100), 235 (18), 203 (29), 175 (20), 139 (53); (GC) CIMS *m/z* 393 (M$^+$+29, 10%), 333 (M$^+$+1-32, 100).

Labdanolic acid methyl ester (**10**)[22]: (GC) EIMS *m/z* 338 (M$^+$, 2%), 323 (2), 320 (5), 305 (10), 267 (10), 235 (10), 191 (12), 177 (30), 144 (85), 125 (85), 101 (85), 69 (100); (GC) CIMS *m/z* 339 (M$^+$+1, 5%), 321 (M$^+$+1-18, 100); (GC) ν_{max} /cm^{-1} 3632, 2936, 2880, 1757, 1161, 1085; ^{1}H NMR δ 0.78 (s, 6H), 0.88 (s, 3H), 0.95 (d, *J* 6.50 Hz, 3H), 1.01 (t, *J* 3.10 Hz, 1H), 1.15 (s, 3H), 1.2-1.3 (m, 2H), 1.3-1.5 (m, 6H), 1.5-1.7 (m, 6H), 1.7-2.4 (m, 6H), 2.35 (dd, *J* 15.0 and 6.5 Hz, 1H), 3.68 (s, 3H); ^{13}C NMR δ 15.5 (C-20), 18.5 (C-2), 20.0 (C-16), 20.6 (C-6), 21.5 (C-18), 22.4 (C-11), 24.0 (C-17), 31.1 (C-13), 33.3 (C-4), 33.4 (C-19), 39.2 (C-10), 39.7 (C-12), 40.3 (C-1), 41.3 (C-3), 42.0 (C-14), 44.5 (C-7), 51.4 (MeO-C-15), 56.2 (C-5), 62.1 (C-9), 74.3 (C-8), 174.0 (C-15).

Patagonic acid methyl ester (**11**)[23,24]: (GC) EIMS *m/z* 315 (20%), 314 (100), 299 (7), 271 (10), 203 (8), 175 (30), 139 (18), 105 (25); (GC) ν_{max} /cm^{-1} 2957, 2885, 1787, 1733, 1639, 1232, 1166, 1063, 821; ^{1}H NMR δ 0.76 (s, 3H), 0.83 (d, *J* 6.5 Hz, 3H), 1.29 (s, 3H), 3.74 (s, 3H), 4.85 (dd, *J* 2 Hz, 2H), 6.66 (t, *J* 4 Hz, 1H), 7.14 (t, *J* 3 Hz, 1H); ^{13}C NMR δ 15.4 (C-17), 17.5 (C-1), 18.1 (C-20), 19.0 (C-12), 20.7 (C-19), 27.1 (C-7), 27.2 (C-2), 36.0 (C-11), 36.3 (C-6), 36.3 (C-8), 37.6 (C-5), 38.7 (C-9), 46.6 (C-10), 51.1 (MeO-C-19), 70.1 (C-15), 135.0 (C-13), 136.9 (C-3), 142.3 (C-4), 143.4 (C-14), 167.7 (C-18), 174.2 (C-16).

Clerod-3-en-15,16-olide-18-oic acid methyl ester (**12**)[25]: (GC) EIMS *m/z* 348 (M$^+$, 3%), 316 (6), 289 (8), 237 (100), 205 (25), 177 (40); (GC) ν_{max} /cm^{-1} 2943, 2884, 1787, 1750, 1146, 1062; ^{1}H NMR δ 0.72 (s, 3H), 0.80 (d, *J* 6.5 Hz, 3H), 1.04 (s, 3H), 3.63 (s, 3H); ^{13}C NMR δ 14.8 (C-19), 15.9 (C-17), 18.0 (C-20), 18.9 (C-12), 21.0 (C-1), 24.9 (C-3), 26.1 (C-2), 27.1 (C-7), 35.6 (C-11), 36.6 (C-8), 37.3 (C-5), 38.8 (C-9), 39.8 (C-6), 49.2 (C-10), 50.9 (MeO-C-19), 57.4 (C-4), 70.1 (C-15), 134.9 (C-13), 142.3 (C-14), 174.3 (C-16), 174.9 (C-18).

Results and Discussion

Characterization of the modified silica

Useful information on the silica surface and SiO$_2$ network can be obtained by IR spectroscopy which is therefore a good method to study silica modifications. Detailed interpretation of the IR of various forms of silica has been published in the literature[26-31].

The IR spectra of silica gel 60 (silica-1) and of the sample treated with a solution of KOH (5%) in *i*PrOH (silica-2) are shown in Table 1. The IR spectra of these samples are very similar in the siloxane bulk region (1500 to 400 cm^{-1}), which suggests no or only minor changes in the bulk of the SiO$_2$ particles. However, the position of the absorption assigned to the Si-OX stretching vibration (a surface mode of Si-OX groups) is shifted from 977 cm^{-1}, in the pure silica to 963 cm^{-1}, which can be attributed to the exchange of H$^+$ by K$^+$ on the surface of the silica particles. In the silanol and water regions (6000 to 1600 cm^{-1}) changes in the bands shapes and positions are observed and the absorption due to the combination of stretching and deformation modes of the Si-OH groups is not observed in the silica-2 spectrum. These results correlate with a large decrease in the number of -SiOH surface groups and indicate a significant modification on the silica surface. When 0.50 g of silica-2 is washed with 50 mL of water, the pH of the resulting solution is equal to 9.5 and after 20 washings (1L of water) the pH stabilizes at 6.0. The washings of silica-1 with 50 mL of water result in a solution of pH 5.5 and this pH is the same after 20 washings. The IR spectrum of silica-3 (prepared by washing silica-2 with 1 L of water) is very similar to that of silica-2, in the 1500 to 400 cm^{-1} region (see Table 1). The absorption assigned to the Si-OX stretching vibration is observed at 966 cm^{-1}, which indicates that the washing procedure removes very few K$^+$ ions from the silica surface. In the 6000 to 1600 cm^{-1} region only minor modifications of bands, shapes and positions could be observed. An additional important observation is the absence of any intense band that could be attributed to silicate stretching modes in the residue from the washings. These results can be explained by the presence of adsorbed KOH on the silica-2 surface that is eliminated by washing. The decrease in the surface area (BET method) from 246 m^{2}g^{-1} (silica-1) to 68 m^{2}g^{-1} (silica-2) is another evidence of KOH deposition on the silica surface.

The modifications of the silica surface can also be analyzed by the changes in the stretching frequency of the surface adsorbed water. The absorptions at 1628 cm^{-1}, 1639 cm^{-1} and 1631 cm^{-1}, ν_2, were assigned to silica-1, silica-2 and silica-3, respectively suggesting that in the

Table 1. Assignments of the observed absorption bands /cm^{-1}

ASSIGNMENT	SILICA-1	SILICA-2	SILICA-3
$(\nu_2 + \nu_3)$ H$_2$O	5274	5250	5263
$(\nu + \delta)$ Si-OH	4532	-	4533^a
	4490	-	4476^a
(ν) OH	3665	3598	3630
	3405	3417	3395
	3248	3272	3296
SiO$_2$ network overtones or combinations	1984	-	1988
	1872	1870	1870
ν_2 H$_2$O	1628	1639	1631
SiO$_4$ asymmetric stretching	1170	1170	1170
	1105	1101	1102
δ Si-OX	977	963	966
SiO$_4$ symmetric stretching	801	800	800
δ Si-O-Si	471	472	472

^{a}Very weak absorpsion.

three samples the water molecules are proton donors[31]. Thus, the basicity of the silica surface can be characterized by an analysis of the shapes and positions of the bands associated with the surface adsorbed water vibrational modes[31,32]. The greater the wavenumber of the ν_2 absorption, the higher the basicity of the silica surface[31,32] and therefore the increasing order of basicity is silica-1 < silica-3 < silica-2. As expected, a decrease in the wavenumber of the band due to the combination $\nu_2 + \nu_3$ of the hydrogen-bonded H$_2$O molecules is observed and follows the trend silica-1 > silica-3 > silica-2. An increase in their width is also noted in the order silica-1 < silica-3 < silica-2. So, the order of basicity also agrees with the variation in the position and shape of this band. These results agree with the hypothesis of deposition of KOH on the surface of silica-2, with the elimination of this KOH by washing in the silica-3, and with the presence of –SiO$^-$K$^+$ groups on the surface of silica-3.

Separation and characterization of the acid diterpenes of Copaifera cearensis Huber ex Ducke

The IR spectrum of the crude copaiba oil showed an absorption at 1740 cm^{-1} ($\nu_{C=O}$) and a broad, medium, band between 3500 and 2600 cm^{-1}, attributed to carboxylic acids. After methylation of the crude oil with CH$_2$N$_2$, the IR spectrum showed a strong absorption at 3300 cm^{-1} (hydroxyl band), and absorptions at 1160 cm^{-1} (ν_{C-O}), and 1740 cm^{-1} ($\nu_{C=O}$), thus showing the presence of carboxylic acids in the crude extract which confirmed previously published results.

GC analysis of the crude oil showed broad tailing peaks between 6 and 20 min, which were related to the presence of free carboxylic acids. GC analysis of the methylated

extract led to a significant improvement of this profile, therefore confirming the presence of carboxylic acids in the extract.

In order to verify the effect of the injection mode, GC analysis of the crude oil was then carried out by using the cold on-column injection mode. The chromatogram obtained showed no difference from the previous one, under split injection mode, *i.e.*, no artifact formation was observed due to the injection mode.

Flash chromatography of 5 g of the crude oil on silica gel/potassium hydroxide was then successively eluted with 400 mL portions of hexane (fraction 1, 2.60 g), CH$_2$Cl$_2$ (fraction 2, 0.50 g) and MeOH (fraction 3, 1.70 g), 96% of the original mass was recovered. The combination of the GC chromatograms of fractions 1, 2 and 3 (after methylation) reproduced qualitatively and quantitatively the chromatogram of the crude oil after methylation.
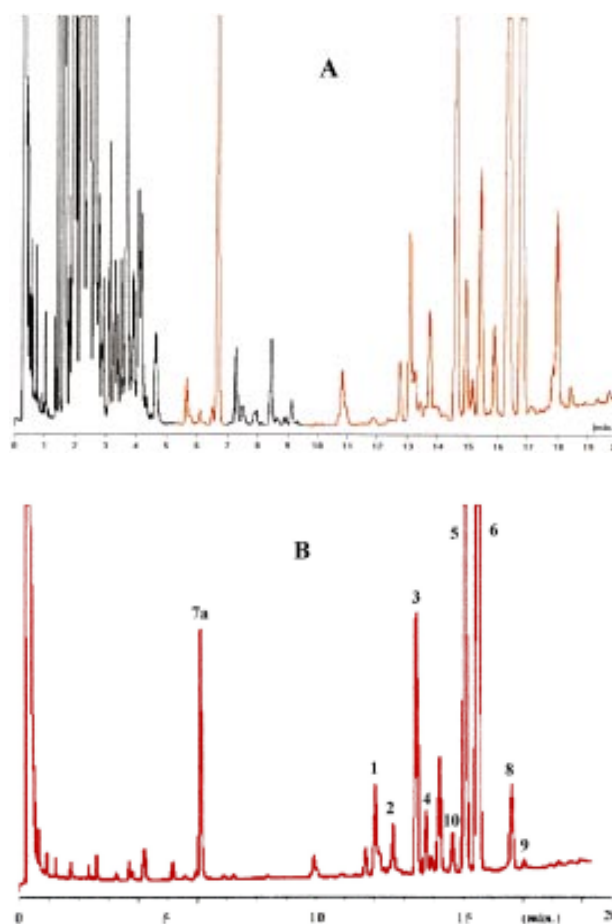

Figure 1. A) Chromatogram of the *Copaifera cearensis* oil (esterified). B) Chromatogram of the acid fraction separated by the KOH-modified silica gel column. (after esterification - fraction 3).
The identified methyl esters of the acids are: **1** eperuic; **2** cativic; **3** copalic; **4** kolavenic; **5** clorechinic; **6** hardwickiic; **7a** 3-methyl-5(2',2',6'-trimethyl-6'-hydroxycyclohexyl)-pentanoic; **8** clerodan-15,18-dioic; **9** 3-cleroden-15,18-dioic; **10** labdanolic.

The IR spectrum of fraction 3 showed a carboxylic acid absorption between 3500 and 2500 cm^{-1} which disappeared after methylation, in addition to a $\nu_{C=O}$ at 1750 cm^{-1}.

GC-MS analyses of methylated fraction 3 were undertaken and based on comparisons with the Wiley-HP 59943P database and other published data, it was possible to identify the methyl esters of eperuic acid (**1**), cativic acid (**2**), copalic acid (**3**), kolavenic acid (**4**), crolechinic acid (**5**) and hardwickiic acid (15,16-epoxy-3,13(16),14-clerodatrien-18-oic acid) (**6**) (Scheme 1).
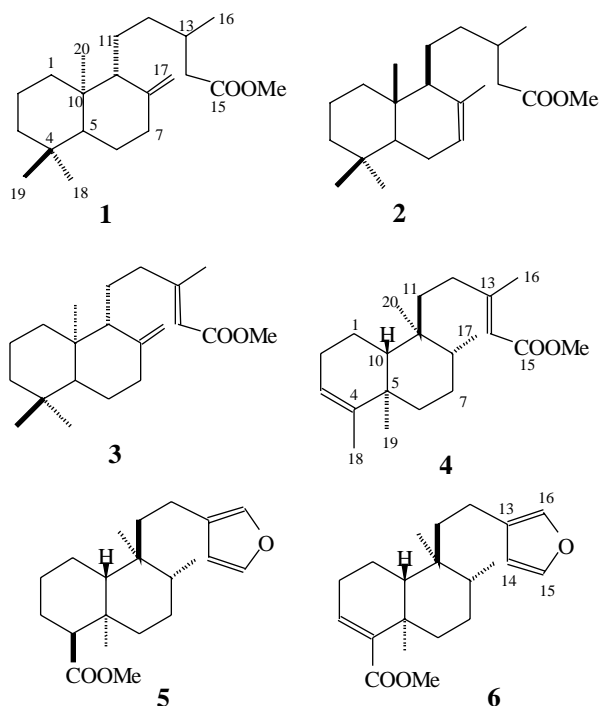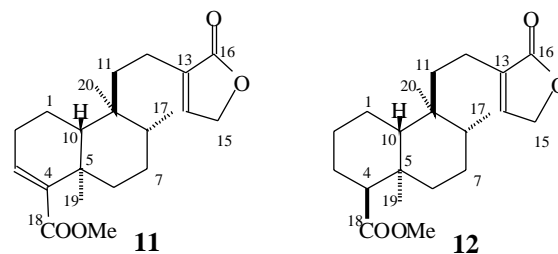

Scheme 1.

Further fractionation of the free carboxylic acids present in fraction 3 was carried out by semipreparative HPLC (300 mm, 7.8 mm ID, 5 mm, μ-bondapak C-18). Elution of fraction 3 with 85% MeOH/H$_2$O (acidified to pH 5 with H$_3$PO$_4$), at 2.0 mL min^{-1} under RI detection, allowed collection of 5 fractions (LC-1 to LC-5). These fractions were then methylated and analyzed by GC-MS. LC-1 was found to be a single compound, a sesquiterpene acid (methyl ester **7a**), LC-2, two diterpene acids (methyl esters **8** and **9**), and LC-3 contained a diterpene acid (methyl ester **10**) as the a main constituent. LC-4 was composed of hardwickiic acid (methyl ester **6**) and crolechinic acid (methyl ester **5**), in addition to two other components. Finally, LC-5 was a complex mixture in which eperuic acid (methyl ester **1**) and copalic acid (methyl ester **3**) were present (Scheme 2).
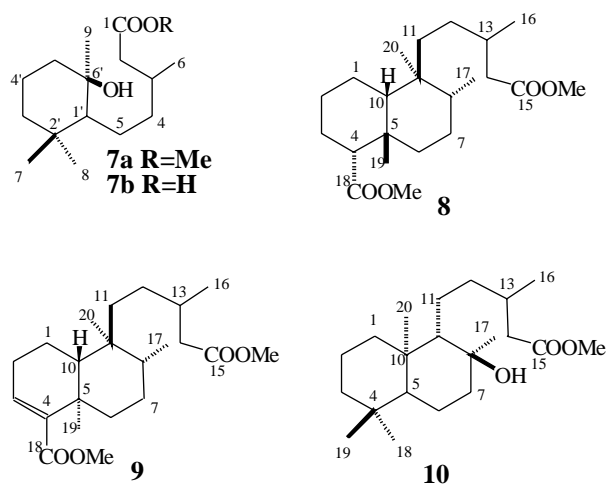
Scheme 2.

In order to investigate the two additional constituents of LC-4, this fraction was submited to further fractionation by HPLC (300 mm, 4.6 mm ID, 5 mm, μ-bondapak C-18) by using 90% CH$_3$CN/ H$_2$O at 1.0 mL min^{-1}. Fraction LC-4a, after methylation, upon GC-MS analysis, contained hardwickiic acid (methyl ester **6**) and crolechinic acid (methyl ester **5**), and LC-4b, after methylation, upon GC-MS analysis, contained patagonic acid (methyl ester **11**) and its Δ^3 saturated form, **c**lerod-13-en-15,16-olide-18-oic acid (methyl ester **12**) (Scheme 3).

Scheme 3.

Fractionation by semipreparative HPLC opened the possibility of obtaining GC-IR data for fractions LC-2, LC-3 and LC-4b and provided amounts of fractions LC-1, LC-2, LC-3, LC-4a and LC-4b large enough to be analyzed by NMR (^{1}H, ^{13}C, COSY, and HETCOR) which confirmed the above findings.

Conclusions

A fraction containing acid terpenes was conveniently isolated from the crude extract of *Copaifera cearensis* by silica gel/potassium hydroxide flash chromatography. The main components of this fraction were identified by

GC-MS. Further fractionation of this extract by reversed phase semipreparative HPLC made it possible, not only to identify some minor constituents, but also to provide additional analytical data on the components of this acidic fraction. The stereochemistry of the compounds showed in the present paper are in agreement with those published in the literature and therefore, must be regarded as relative. The procedure described in the present paper was reproducible qualitatively and quantitatively. The use of this methodology will make possible the necessary quality control of copaiba oils which are commercially available at several drugstores. Based on IR analyses it was shown that the overall effect of washing silica with KOH was to decrease surface area, with deposition of KOH on the silica surface, in addition to formation of Si-OK due to a cation exchange process. In addition, as far as we know, this is the first time that the KOH-modified silica gel, commonly used in chromatography[12,33,34], is characterized by IR spectroscopy.

Acknowledgements

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